IntechOpen

Myeloid Leukemia

Edited by Ahmed Lasfar

MYELOID LEUKEMIA

Edited by **Ahmed Lasfar**

Myeloid Leukemia
http://dx.doi.org/10.5772/intechopen.68969
Edited by Ahmed Lasfar

Contributors

Domenico Mastrangelo, Giuseppe Fioritoni, Lauretta Massai, Nélida Noguera, Francesco Lo Coco, Oxana Klimenko, Seiji Fukuda, Chie Onishi, Mariko Abe, Tomohiro Hirade, Takeshi Taketani, Lei Dong, Katarzyna Derwich, Dorothy Mitkowski, Jolanta Skalska-Sadowska, Tatsuaki Tsuruyama, Zeeshan Ahmed, Imran Ahmed, Sadia Sultan, Masoud Sadeghi, Mehrdad Payandeh, Edris Sadeghi, Mehrnoush Aeinfar, Chenyue Hu, Amina Ann Qutub, Ahmed Lasfar

Notice

First published in London, United Kingdom, 2018 by IntechOpen
eBook (PDF) Published by IntechOpen, 2019
IntechOpen is the global imprint of INTECHOPEN LIMITED, registered in England and Wales, registration number: 11086078, The Shard, 25th floor, 32 London Bridge Street
London, SE19SG – United Kingdom
Printed in Croatia

British Library Cataloguing-in-Publication Data
A catalogue record for this book is available from the British Library

Additional hard and PDF copies can be obtained from orders@intechopen.com

Myeloid Leukemia
Edited by Ahmed Lasfar

p. cm.

Print ISBN 978-1-78923-102-1

Online ISBN 978-1-78923-103-8

eBook (PDF) ISBN 978-1-83881-366-6

Meet the editor

Dr. Ahmed Lasfar is a member of the Cancer Institute of New Jersey, a principal investigator and a faculty member at Ernest Mario School of Pharmacy, Rutgers University, New Jersey. Dr. Lasfar is an innovator and a scientific adviser for pharmaceutical industry. He is an editor, a board member, and a reviewer of several international journals. He is often invited as a speaker for conferences on cancer, immunology and pharmaceuticals. He graduated in France from Paris Rene Descartes University on medical and applied science. He completed his doctoral studies in Immunology at Paris Diderot University and his postdoctoral training in cancer immunology at Robert Wood Johnson Medical School, New Jersey. His research interests involve hematological malignancies, breast cancer, liver cancer and melanoma. His laboratory is more focused on cancer immunology and the elucidation of the mechanisms of cancer metastasis. He published breakthrough articles and holds patents for developing new drugs in oncology and infectious diseases.

Contents

Preface XI

Section 1 Myeloid Leukemia: Multifaceted Disease 1

Chapter 1 **Introductory Chapter: Myeloid Leukemia 3**
Ahmed Lasfar

Section 2 Pathogenicity and Diagnostic 7

Chapter 2 **Cytopathology of MDS/MPN and AML by H&E Staining 9**
Tatsuaki Tsuruyama

Chapter 3 **Acute Myeloid Leukemia in Pediatric Patients: A Review About Current Diagnostic and Treatment Approaches 21**
Katarzyna Derwich, Dorothy Mitkowski and Jolanta Skalska-Sadowska

Chapter 4 **Proteomics in Acute Myeloid Leukemia 43**
Chenyue W. Hu and Amina A. Qutub

Section 3 Genetic and Epigenetic Regulations 63

Chapter 5 **Genomics of Acute Myeloid Leukemia 65**
Zeeshan Ansar Ahmed, Imran Ahmed Siddqui and Sadia Sultan

Chapter 6 **Small Non-Coding RNAs in Regulation of Course and Therapeutic Efficacy in Acute Myeloid Leukemia 85**
Oxana V. Klimenko

Chapter 7 **HIF-Regulated Leukemogenesis Through the Advances on Epigenetic Mechanism 105**
Lei Dong and Yu Zhu

Section 4 Drug Resistance and Potential Treatments 119

Chapter 8 **Molecular Interaction Between the Microenvironment and FLT3/ITD+ AML Cells Leading to the Refractory Phenotype 121**
Seiji Fukuda, Tomohiro Hirade, Mariko Abe, Takeshi Taketani and Chie Onishi

Chapter 9 **Role of Genetic Analysis in New Treatments of Acute Myeloid Leukemia 147**
Mehrdad Payandeh, Masoud Sadeghi, Edris Sadeghi and Mehrnoush Aeinfar

Chapter 10 **High Doses of Vitamin C and Leukemia: In Vitro Update 155**
Domenico Mastrangelo, Lauretta Massai, Giuseppe Fioritoni, Francesco Lo Coco, Nèlida Noguera and Ugo Testa

Preface

Myeloid leukemia is a multifaceted disease, regrouping a variety of myeloid disorders. Recently, important progress has been made in both its diagnosis and treatment. However, some forms are curable and others are treatable but rarely curable. New therapeutic perspectives are now considered for patients.

The aim of our book is to cover key aspects of myeloid leukemia, including its diagnosis and current treatments and the challenges ahead. To apprehend this complex disease, critical molecular and cellular mechanisms including the role of the tumor microenvironment are highlighted. Important molecular targets are described and new therapeutic considerations are discussed:

1. Myeloid Leukemia: Multifaceted Disease
2. Pathogenicity and Diagnostic
3. Genetic and Epigenetic Regulations
4. Drug Resistance and Potential Treatments

The publication of this book was made possible by coordinated efforts and collaborations from many experts in the field. We thank all the contributors for their valuable work and their tremendous efforts.

We are confident that this book will provide valuable insights into myeloid leukemia at different levels. In the meantime, we trust it will bring answers and hope for health care providers, patients, and their families.

Ahmed Lasfar
Member of New Jersey Cancer Institute
Principal investigator and Faculty member
Department of Pharmacology and Toxicology
Ernest Mario School of Pharmacy, Rutgers University
Piscataway, New Jersey, United States

Section 1

Myeloid Leukemia: Multifaceted Disease

Chapter 1

Introductory Chapter: Myeloid Leukemia

Ahmed Lasfar

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.73858

1. Introduction

Myeloid leukemia regroups a variety of myeloid disorders. Some are more frequent and well characterized such as acute myeloid leukemia (AML) or chronic myeloid leukemia (CML). However, other clonal myeloid disorders, designed myelodysplastic/myeloproliferative neoplasms (MDS/MPN), are still subjected to diagnosis and therapeutic challenges. MDS/MPN might possess both dysplastic and proliferative features and cannot simply be listed in myelodysplastic syndrome (MDS) and chronic myeloproliferative disorder (CMPD) categories. Currently, three distinct groups are well classified: chronic myelomonocytic leukemia (CMML), juvenile myelomonocytic leukemia (JMML), and atypical chronic myeloid leukemia (aCML). An additional group called myelodysplastic/myeloproliferative neoplasm-unclassifiable (MDS/MPN-UC) has also been included. Apparently, MDS/MPN-UC shows both MDS and CMPD characteristics but differs at some extent from the other three MDS/MPN groups. MDS/MPN implicates defects in the modulation of myeloid pathways leading to cell survival and proliferation. However, the etiology of the defects remains elusive.

In this introductory chapter, we have succinctly described each of the myeloid disorders and provided some highlights on diagnosis and available therapies.

2. Acute myeloid leukemia (AML)

AML is a complex malignancy characterized by a high heterogeneity of aberrant myeloid precursors. In addition to family history of hematologic disorders and exposure to environmental factors, aging increases the incidence of AML. Although AML arises from transformed hematopoietic stem cells, the etiology of this form of leukemia remains mostly

unidentified. However, we know that consecutive genomic mutations and epigenetic modifications lead to the progression of the disease. Alterations in important genes have been characterized leading to the development of novel targeted therapeutic strategies. However, significant variations in the genetic and the epigenetic of AML are found among patients. Thus, development of efficient treatment of AML remains a significant clinical challenge.

Current AML treatment is generally based on classical chemotherapy, targeted therapy, and stem cell transplantation. The emergence of personalized medicine is still underway. Optimal biological information leading to patient selection and individual therapy are uncommon.

In addition to the age of AML patients, cytogenetics of the tumor and clinical appreciations are fundamental for the prognosis and the success of the treatment. Promyelocytic leukemia is the subset of AML with the highest proportion of cure rate. Patients are benefiting from targeted therapy such as all-transretinoic acid treatment. Currently, several other targeted therapies are available.

The limiting parameters for AML targeted therapy are the tumor heterogeneity and its variability during the course of disease and therapy, resulting in unpredictable response, and constitute the current challenge for establishing successful personalized therapeutic regimens.

3. Chronic myeloid leukemia (CML)

CML is a myeloproliferative disorder characterized by the presence of the BCR-ABL oncogene fusion protein, resulting from reciprocal translocation between chromosomes 9 and 22 [t(9;22)], commonly designed by Philadelphia chromosome (Ph chromosome) (Ref). As a result, Bcr-Abl tyrosine kinase becomes constitutively activated and triggers apoptosis resistance and other biological alterations responsible for CML pathogenesis. With the introduction of tyrosine kinase inhibitors (TKIs), significant benefits for CML patients are currently achieved. Nilotinib, dasatinib, and imatinib are the commonly TKIs small molecules used in the clinic for the treatment of CML. These inhibitors are designed to block the adenosine triphosphate-binding site of the Bcr-Abl tyrosine kinase and inhibit the activation of downstream effector proteins, responsible for CML exacerbations.

The success of TKI therapy is associated with the reduction of BCR-ABL1 transcript levels in the CML patients. Current guidelines refer to major molecular response (MMR) for the BCR-ABL1 level not exceeding 0.1% or deep molecular response (DMR) for the BCR-ABL1 level not exceeding 0.01%. DMR can be achieved in almost 50% of CML patients who stopped TKI treatments. However, important questions remain regarding the mechanisms leading to CML remission and lack of relapse. Addressing such questions will help identify biomarkers that could predict which patient could discontinue TKI therapy without relapse and also determine the long-term success of the treatment.

http://dx.doi.org/10.5772/intechopen.73858

4. Chronic myelomonocytic leukemia (CMML)

CMML displays irregular features, varying from myelodysplastic to myeloproliferative. The majority of CMML patients show persistent somatic mutations. Around 15% of CMML can evolve to AML, and when this transition occurs, a poor prognosis is predicted. Many chemotherapy regimens for CMML have been used with only limited success. Bone marrow transplantation or stem cell transplantation appears to be more effective.

5. Juvenile myelomonocytic leukemia (JMML)

JMML is a rare myeloid disorder of childhood. Although the etiology of JMML is not known, children with neurofibromatosis type 1 (NF1) have high risk for developing JMML. Children diagnostic at 2 years old and up have a poorer prognosis. Thrombopenia and a high HbF level have also been associated with a poor prognosis. Currently, BMT appears to be the best therapy for JMML.

6. Atypical chronic myeloid leukemia (aCML)

aCML is a very rare myeloid leukemia, mainly occurring in elderly people. This disorder displays both MDS and CMPD features. aCML seems to respond poorly to interferon alpha therapy. However, treatment with hydroxyurea may lead to a limited remission.

7. Myelodysplastic/myeloproliferative neoplasm-unclassifiable (MDS/MPN-UC)

MDS/MPN-UC is also called mixed myeloproliferative/myelodysplastic syndrome. Since it shows both MDS/MPN features and not meeting the criteria of the described MDS/MPN categories, this disorder is unclassifiable. A mutation on Jak2 kinase causing its constitutive activation might be involved in the disease at least in some MDS/MPN-UC patients. Adult patients with platelet-derived growth factor receptor gene rearrangements might be treated with imatinib mesylate.

8. Conclusion

Important progress in both the diagnosis and the treatment of myeloid leukemia has been realized. Currently, many treatments are proposed. In CML tremendous success has been achieved with TKI therapy. However, important challenges remain for establishing more focused therapies and determining useful biomarkers associated with the resistance or the success to therapy.

Author details

Ahmed Lasfar

Address all correspondence to: ahmed.lasfar@pharmacy.rutgers.edu

Rutgers-Cancer Institute of New Jersey, Ernest Mario School of Pharmacy, Rutgers University, Piscataway, NJ, USA

Section 2

Pathogenicity and Diagnostic

Chapter 2

Cytopathology of MDS/MPN and AML by H&E Staining

Tatsuaki Tsuruyama

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.71567

Abstract

Bone marrow (BM) clots are routinely sampled in aspiration tests, and their sections are prepared for histological observation by hematoxylin and eosin (H&E) staining. However, H&E-stained sections are considered less informative than those stained by the May-Grünwald Giemsa (M-G) stain; thus, diagnosis using H&E-stained clot samples is challenging for pathologists. In fact, the diagnostic evaluation is limited to the observation of cellular morphology and the myeloid-erythroid cell ratio. Pathologists leave cellular observation to laboratory hematologists, who generally use M-G staining. In this chapter, the utility of bone marrow clot specimens for diagnosis by H&E staining is reviewed. Specifically, the review provides a descriptive and illustrative explanation of the diagnosis of acute myeloid leukemia (AML), myelodysplastic syndrome (MDS), and myelodysplastic syndrome/myelocytic proliferative neoplasm (MDS/MPN) and demonstrates the possibility of diagnosis on the basis of the characteristic features of blast cells. Clot specimens appear to be useful for the diagnosis of hematopoietic dysplasia by pathologists, and this approach can provide more informative findings for hematologists.

Keywords: bone marrow clots, hematoxylin and eosin, acute myeloid leukemia

1. Introduction

Bone marrow (BM) clots and their specimens can be evaluated histopathologically by hematoxylin and eosin (H&E) staining [1, 2]. However, this method is considered to yield limited information regarding cellular morphology, myeloid-erythroid cell ratio, size and morphology of megakaryocytes [3], increase in multiple myeloma [4], involvement of lymphoma, and metastatic tumor such as neuroblastoma [5–7]. In particular, BM clot sections comprising sinusoidal blood are inadequate for morphological interpretation [2]. However, the cellblock technique ensures the capture of cells required for diagnosis, and laboratories routinely apply this sample preparation technique for enhancing the usability of the BM clot specimen [2, 8].

To assist in morphologic diagnosis, immunohistochemistry (IHC) is available for assessment and immunophenotyping. For instance, by IHC, the marker glycophorin can assess the myeloid-erythroid cell ratio, and spectrin can efficiently detect erythroid cellular lineage [9]. P53 can also be used for differential diagnosis between refractory anemia and aplastic anemia [10]. An international Working Party for the Standardization of Bone Marrow IHC was formed by the International Council for Standardization in Hematology to prepare a set of guidelines for the standardization of handling BM specimens and reports [11]. While the histological examination of BM introduces new aspects of blood disease, the diagnosis of hematopoietic disorders using H&E-stained BM clot sections remains a challenge for nonhematopathologists. In fact, hematopoietic disorders have been diagnosed using May-Grünwald Giemsa (M-G) staining by hematologists and pathologists, and the findings obtained from H&E-stained BM clot sections are used as a reference for those obtained from M-G–stained samples.

This review discusses the information obtained from H&E-stained BM clot sections and its advantages and limitations over M-G–stained BM clot sections. The techniques are discussed with respect to examination of erythrocytes and myelocytes and the diagnosis of acute myeloid leukemia (AML), myelodysplastic syndrome (MDS), and myelodysplastic syndrome/myelocytic proliferative neoplasm (MDS/MPN).

2. Overview of various blast cells

2.1. Erythroblasts

In H&E-stained samples, it is difficult to distinguish between erythroblasts, myeloblasts, and megakaryoblasts. Erythroblasts have a large nucleus that occupies the whole cell. In H&E-stained sections, the nucleus is observed to be pale, basophilic, and homogenous, with a fine appearance. Myeloblast lineages have more eosinophilic and rough cytoplasm than the erythroblasts, with distinct nucleoli (**Figure 1A** and **B**). Megakaryoblasts have transparent, lobulated nucleoli, with a higher nuclear-cytoplasmic ratio than that in megakaryocytes. The cytoplasmic border is frequently observed, and cell membranous projections are occasionally observed in some of the megakaryoblasts (**Figure 1C**).

2.2. Immature myelocytic lineage

In general, the morphological characteristics of myelocytic developmental stages have not been sufficiently described using H&E-stained clot samples. This is probably because of the difficulty in recognizing the cytoplasmic granules in the H&E-stained sections. However, H&E-stained sections provide incomplete morphological information regarding the developmental stages. In comparison with erythroblasts, myelocytic lineages are larger and have eosinophilic cytoplasm. Pro-myelocytes are smaller than myeloblasts and have a halo surrounding the nucleus, which is sometimes observed in M-G–stained samples (**Figure 2A**). Myelocytes and meta-myelocytes are easily identifiable owing to their segmented nuclei and smaller size than that of myelocytes (**Figure 2B** and **C**). Eosinophilic and basophilic lineages are identifiable because of the presence of distinct granules (**Figure 2D**).

http://dx.doi.org/10.5772/intechopen.71567

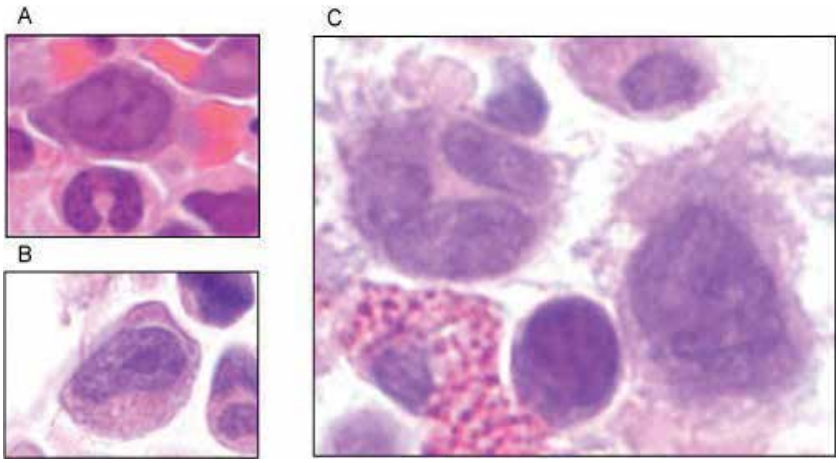

Figure 1. Morphology of erythroblasts and myeloblasts. (A) an erythroblast showing a transparent nucleus with condensed nucleoli (600×). (B) a myeloblast showing a transparent nucleus with one prominent eosinophilic nucleolus. The cytoplasm is eosinophilic, rough, and heterogeneously condensed (600×). (C) Two megakaryoblasts show transparent and lobulated nuclei with prominent eosinophilic nucleoli. The cytoplasm is eosinophilic, rough, and heterogeneously condensed. The edges are irregular, with a number of villi (600×).

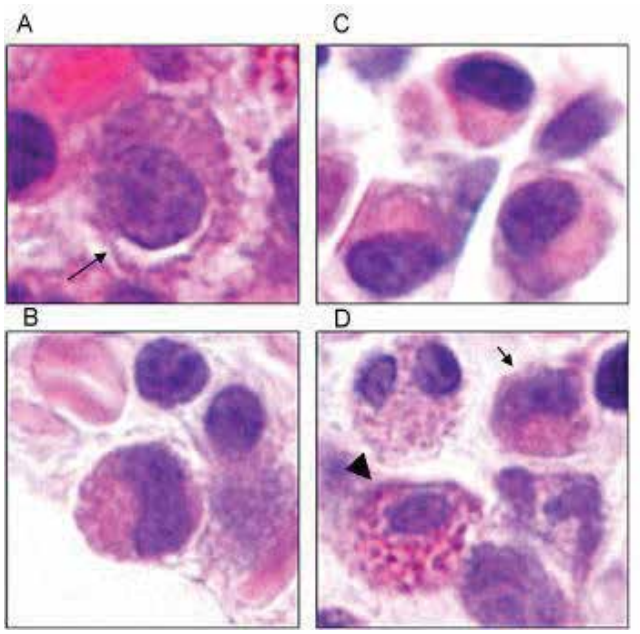

Figure 2. Morphology of myelocyte lineages detected by H&E staining. Immature neutrophil lineages (A–C) and immature eosinophil lineages (D). (A) A pro-myelocyte showing a nucleus surrounded by halo (represented by an arrow). Nucleoli are not prominent relative to myeloblasts. The cytoplasm is eosinophilic, rough, and heterogeneously condensed (600×). (B) A myelocyte showing a regular oval nucleus with a contour and without indentation. The cytoplasm is eosinophilic, rough, and heterogeneously condensed (600×). (C) A meta-myelocyte showing a U-shaped or bent nucleus. The cytoplasm is eosinophilic, rough, and heterogeneously condensed. No nucleoli are visible (600×). (D) Variable immature eosinophil lineages. The arrow indicates a pro-myelocyte, and the arrowhead indicates a myelocyte. A mature eosinophil can be seen in the upper-left corner (600×).

3. H&E-stained AML

3.1. Identifying erythrocytic lineages and AML by H&E-stained BM clots

To determine the utility of H&E-stained BM clots for the diagnosis of megaloblastic leukemia, megaloblastoid cells in MDS, or erythroleukemia, the H&E staining properties of erythrocytic lineages were considered. Pro-erythroblasts are identifiable by their basophilic cytoplasm, transparent nucleus, and multiple deformed nucleoli. A basophilic erythroblast is smaller and has a relatively large nucleus, bearing thick or coarse chromatin (**Figure 3A**). The nucleus occupies a relatively small part of the cell compared to that in basophilic erythroblasts. Importantly, owing to two highly contrasting colors in H&E staining, two distinct types of erythroblasts can be observed: polychromatic and orthochromatic. The polychromatic erythroblast has a lacy nucleus and baso-eosinophilic cytoplasm, while the orthochromatic erythroblast has eosinophilic cytoplasm, in which the nucleus is small, pyknotic, homogeneous, and structure-less, and ultimately becoming blue-black (**Figure 3B**).

3.2. Identifying myeloblasts and AML by H&E-stained BM clots

In this study, the utility of H&E-stained BM clots in diagnosing classic AML subtypes was also examined. H&E-stained clots were prepared for M2, M3, M4, M5, M6, and M7 according to the classic French-American-British classification. Cases of M2, acute leukemia with maturation, as specified in WHO 2008 classification, were the most frequent AML cases

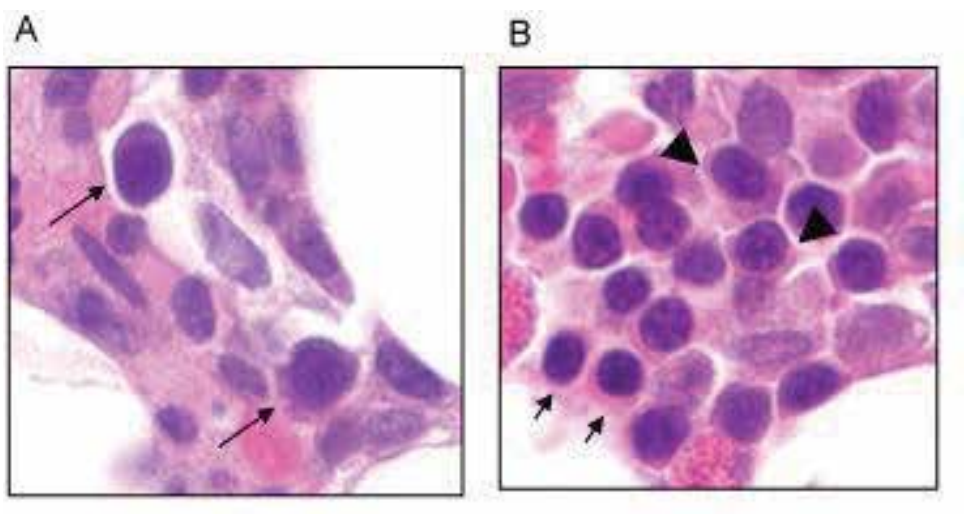

Figure 3. Erythroid lineages detected by H&E staining. (A) Two basophilic erythroblasts represent large nuclei (represented by arrows). Nucleoli are not prominent relative to pro-erythroblasts. The cytoplasm is basophilic, rough, and heterogeneously condensed (600×). (B) Three metachromatic erythroblasts represent lacy and oval nuclei (indicated by two arrowheads). The cytoplasm is baso-eosinophilic, rough, and heterogeneously condensed. Two orthochromatic erythroblasts represent condensed and oval nuclei (indicated by two arrowheads). The cytoplasm is eosinophilic and rough (600×).

encountered; in these cases, myeloblastic leukemic cells were observed. They have similar cytological features as those of normal myeloblasts: (i) eosinophilic, rough cytoplasm and (ii) a prominent nucleolus. **Figure 4A** shows leukemic marrow consisting of myeloblastic leukemic cells. M4, acute myelomonocytic leukemia (**Figure 4C**), and M5, acute monocytic leukemia (**Figure 4D**), have common features of monoblastic cells and are relatively easily identifiable due to a U-shaped nucleus with prominent dual nucleoli. Chromatin condensation was observed in the immature phenotype of monocytic leukemic cells in M4 and M5. The latter is a mix of monoblasts and monocytes and has pyknotic and condensed chromatin. In M6, acute erythroleukemia, atypical erythroblast-like leukemic cells were observed. The rough margin of the nucleus and basophil is a feature of these leukemic cells. In M7, acute megakaryocytic leukemia, the cells are transparent and lobulated without a distinct nucleus, which is observed in MDS. Furthermore, abnormal, deformed, and intensely stained nuclei are observed in MDS/MPN.

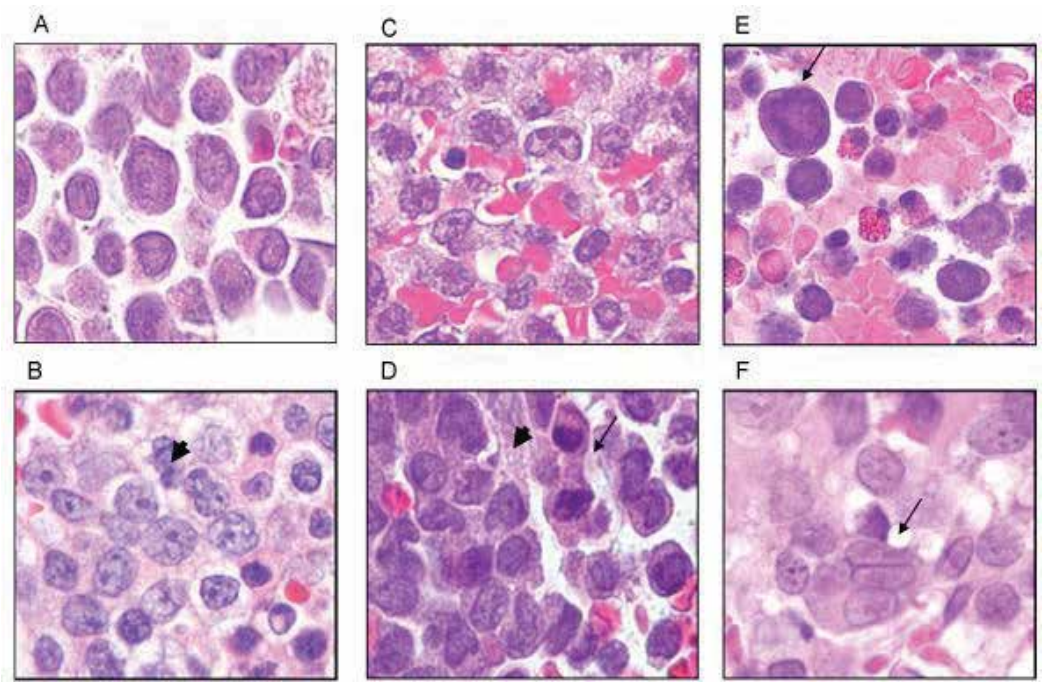

Figure 4. AML diagnosis using H&E staining. (A) Two M2 leukemic cells (AML with maturation) of various sizes mimic myeloblastic morphology and show irregular transparent nuclei with prominent eosinophilic nucleoli (600×). (B) Two M3 leukemic cells mimic pro-myelocytic morphology but present irregular transparent nuclei with prominent eosinophilic nucleoli (600×). (C) Two M4 acute myelomonocytic leukemic (AMMoL) cells, mimicking monocytic and myeloblastic morphology but presenting irregular transparent nuclei with prominent eosinophilic nucleoli (600×). (D) M5 acute monocytic leukemic cells are a mixture of two types of cells: Monoblasts and pro-monocytes. They mimic monoblastic morphology but present an irregular transparent nucleus with prominent eosinophilic nucleoli. Monoblasts have roughly circular and delicate lacy chromatin nuclei with one or two prominent nucleoli. Pro-monocytes have more convoluted nuclei, and the nucleoli are not prominent (600×). (E) M6 leukemic cells mimic erythroblastoid morphology but present irregular transparent nuclei with prominent eosinophilic nucleoli (600×). (F) M7 leukemic cells mimic myeloblastic morphology but present irregular transparent multilobed nuclei with prominent eosinophilic nucleoli (600×).

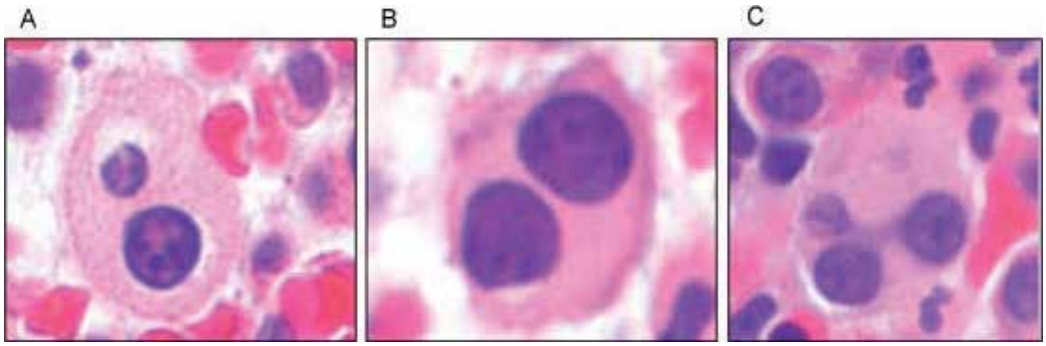

Figure 5. Dysplastic megakaryocytes detected by H&E staining. Megakaryocytes have round, dispersed nuclei (A, B, C). Two or three dispersed nuclei are observed (600×).

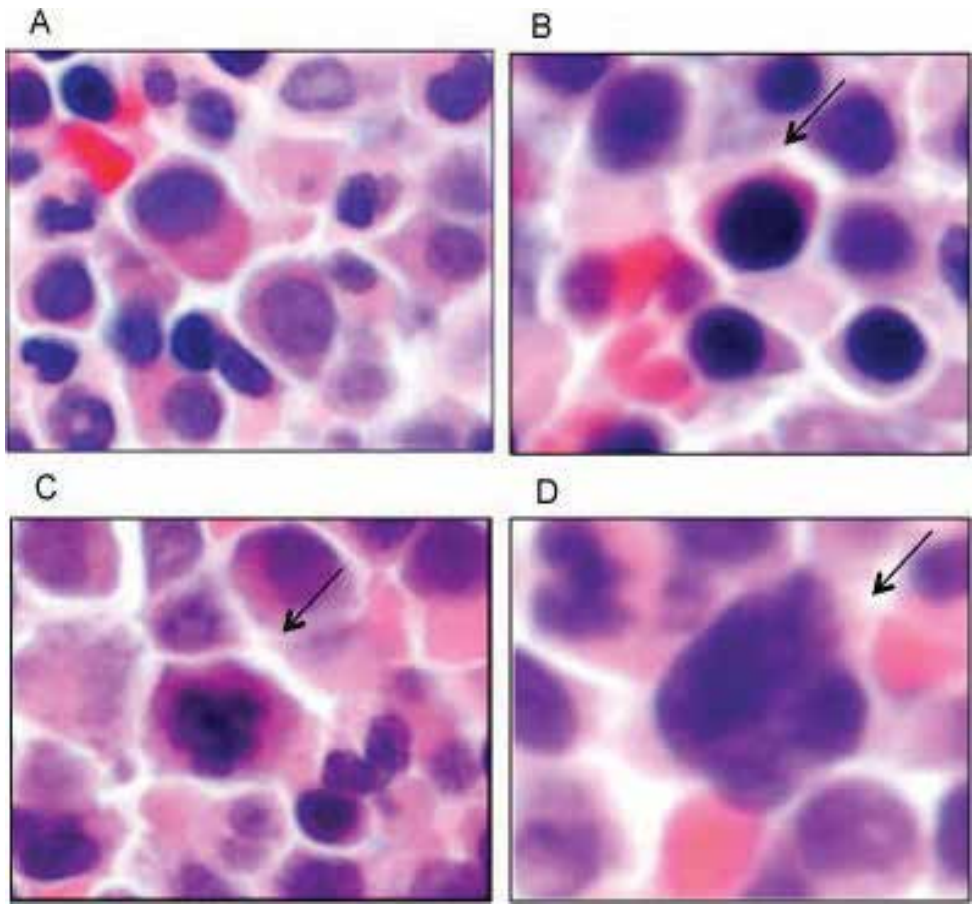

Figure 6. Dysplastic erythrocytes observed by H&E staining. (A, B) nuclear-cytoplasmic dissociation. Arrows represent the dysplastic erythroblasts that have baso-eosinophilic cytoplasm. (C, D) abnormal pro-erythroblasts. Arrows represent the abnormally hyperlobated nuclei.

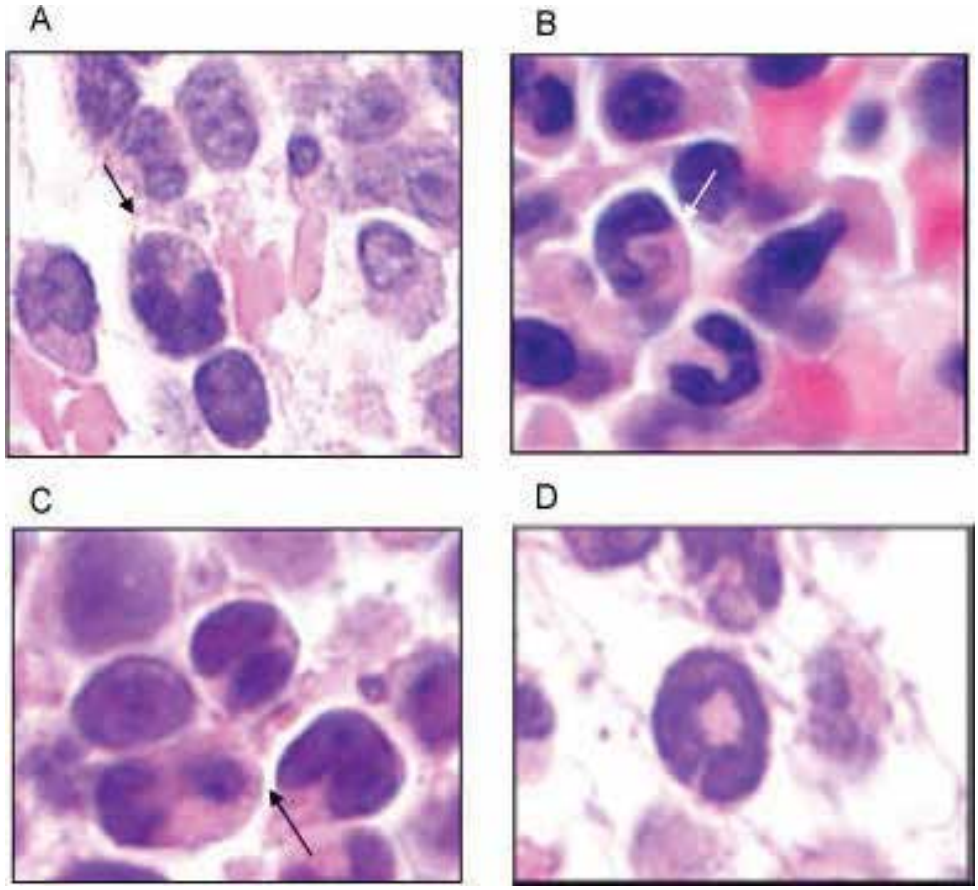

Figure 7. Dysplastic myelocytes observed by H&E staining. (A–D) dysplastic metamyelocytes maturing into neutrophils, which have blots in the cytoplasm (indicated by arrows).

3.3. Dysplastic megakaryocytes in refractory anemia with excess blasts and MDS/MPN

In H&E-stained clot specimens of refractory anemia with excess blasts and MDS, abnormal megakaryocytes, which include micromegakaryocytes and megakaryocytes with large mononuclear form, and dispersed round nucleoli were observed.

Morphological changes and increase in the number of megakaryocytes are informative for diagnosis. Micromononuclear megakaryocytes are detected in MDS, while large, over-mature megakaryocytes have been observed to increase in number in myeloproliferative neoplasms [12]. In anaplastic anemia, the megakaryocytes decrease definitively in number without any evident morphological abnormality [13]. By two-color contrast, megakaryocytic morphological abnormality is more distinct than that by M-G staining. Nuclear-cytoplasmic dissociation was more identifiable in H&E-stained specimens. As shown in **Figure 5**, polynucleated cells and cells with lobulated nuclei were identified in pro-erythroblasts of BM clot samples of MDS patients (**Figure 5A–C**).

In the erythroblast lineage, nuclear-cytoplasmic dissociation (**Figure 6A** and **B**) [2], evident as hemoglobin production in the cytoplasm without enucleation, is identifiable in H&E-stained specimens because of distinct two-color contrast.

Multinuclear abnormal pro-erythroblasts are also the hallmark of dysplasia (**Figure 6C** and **D**) [2]. Furthermore, basophilic heterogeneity in the cytoplasm is observed. Neutrophils exhibited a "blot" or localized heterogeneous staining in the cytoplasm, which is frequently seen in H&E-stained specimens (**Figure 7A–C**) [2]. In addition, a ring-shaped nucleus, which is observed in murine neutrophils, was observed in MDS and MDS/MPN (**Figure 7D**) [2].

4. H&E and M-G staining

Most pathologists are more familiar with H&E-stained samples than with M-G–stained samples in routine diagnostic work. As demonstrated in this study, H&E-stained specimens of BM, as well as M-G–stained samples, were available despite the difficulty in observation of granules in the cytoplasm in the myelocytic lineage. In this chapter, high-power magnification images are provided for understanding features of individual hematopoietic lineages for further clarifying morphological features of the subjects.

As demonstrated, H&E specimens allow the identification of myeloblasts. Therefore, the identification of "blasts" in H&E-stained sections should be clearly defined. In contrast to the M-G-stained sections, H&E-stained sections do show basophilic cytoplasm; rather, the transparency of the nucleus with one or more nucleoli is more identifiable as the feature of blasts in H&E staining. This phenomenon represents another feature of dysplasia with deformity in the shape with or without atypical morphological findings of the nucleus. In dysplasia, hyperlobulated or ring nuclei were observed, which is a diagnostic feature of dysmyelopoiesis. In addition, basophilic heterogeneity, exudation, or a "blot" in the erythroblast cytoplasm is identified in dysplasia. This basophilic exudation was clearer in H&E-stained specimens than in M-G-stained sections. In addition, H&E-stained clot samples are sufficiently useful for the observation of morphological changes and for the assessment of megakaryocytic features.

H&E-stained clot specimens are advantageous for diagnosing AML subtypes because they allow better identification of hematopoietic dysplastic cells than M-G–stained sections do. Pathologists can integrate findings from the three staining methods: H&E, M-G, and IHC: for the decisive diagnosis of AML subtypes. Routine histological observations using M-G or H&E staining will be more useful in combination with flow cytometry analysis for immunohistological assessment using anti-CD33 and anti-CD13, a set of antibodies used to accurately diagnose AML. Myelodysplasia is also identifiable by H&E staining. Using high magnification, such as 1000×, the blot and localized heterogeneous staining or budding of the nucleus is identifiable by H&E staining. In fact, this high-power magnification provides more cytological and histological findings for facilitating the diagnosis of MDS and MDS/MPN.

5. Discussion

5.1. Terminology: dysplastic, atypical, and blastic findings and interrelationship with hematological neoplasm

Dysplastic, atypical and abnormal, and blastic findings, together with hypercellularity, compose the core concepts of hematological neoplastic entities in BM pathology. It is possible to understand the interrelationship of the entities by organizing concepts on the basis of their morphological findings (**Figure 8**). Dysplasia refers to specified morphological features that are distinct from other abnormal or atypical morphologies. As an important example, dysplastic megakaryocytes have circular, dispersed nuclei, which differ from atypical and irregular megakaryocytes in MPN and MDS/MPN. Thus, the terms "abnormal" and "atypical" should be used to describe the morphological changes other than morphological changes in MDS. The cytological spectrum of atypical lymphocytes is extremely broad. For example, small- to large-sized cells with often regular or indented nuclei, moderately condensed chromatin, moderate-to-faint basophilic cytoplasm without azurophilic granules and sometimes with microvacuoles are atypical findings [14].

On the other hand, the term "blast" refers to "immature" cells, which have a transparent nucleus with a prominent clear nucleus. For example, the "centroblast" is a morphological concept that refers to lymphocytes that have a specified morphological feature, such as prominent nucleoli representing an immunologically activated state. As another example, "B-lymphoblasts" refer to the activated B-lymphocytes that undergo hypersomatic mutations and the class switch of immunoglobulin genes. In other words, blastic lymphocytes correspond to the lymphocytes with gene recombination. Myeloblasts and erythroblasts, however, do not necessarily correspond to specific genetic alterations but correspond to the activated state of the first stage of differentiation. Distinguishing these morphological features from hyperplastic marrow will elucidate the mutual relationships among AML, AML arising from MDS, MDS, MDS/MPN, and MPN. MDS, AML, and MPN are specific entities on the basis of dysplastic, blastic (>20% of nucleated cell in BM), and hypercellular findings, respectively.

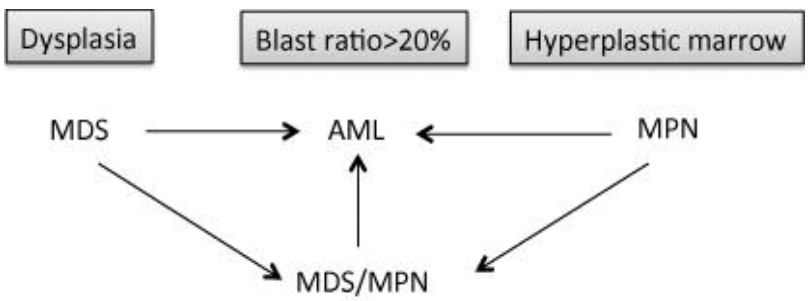

Figure 8. Interrelationships of hematopoietic neoplasms. Dysplastic, blastic findings, together with hypercellularity, compose the core concepts of hematological neoplastic entities.

AML arising from MDS and MDS/MPN are the hybrid entities of dysplasia and blastic findings and dysplastic and hypercellular findings, respectively. On the basis of these three morphological findings, the interrelationships among hematopoietic neoplasms can well be understood (**Figure 8**).

6. Conclusion

Diagnostic evaluation should include H&E-based assessment of morphological dysplastic findings. H&E-stained BM clot specimens have advantage in diagnosis of hematopoietic neoplasms, because they better allow identification of hematopoietic dysplastic and abnormal cells than M-G-stained sections by cases. Pathologists can integrate findings from the two staining methods, H&E, and M-G for decisive diagnosis of hematopoietic neoplasms.

Acknowledgements

We thank hematologist Chiseko Okada for the assessment of AML. This work was supported by a Grant-in-Aid for Scientific Research on Innovative Areas "Fluctuation & Structure," Project Number: 2502, from the Ministry of Education, Culture, Sports, Science, and Technology of Japan. Grant Support: Ministry of Health, Labor, and Welfare of Japan.

Author details

Tatsuaki Tsuruyama

Address all correspondence to: tsuruyam@kuhp.kyoto-u.ac.jp

Department of Pathology, Graduate School of Medicine, Kyoto University, Kyoto, Japan

References

[1] Travlos GS. Histopathology of bone marrow. Toxicologic Pathology. 2006;**34**:566-598

[2] Tsuruyama T, Aini W, Hiratsuka T. Reassessment of H&E stained clot specimens and immunohistochemistry of phosphorylated Stat5 for histological diagnosis of MDS/MPN. Pathology. 2015;**47**(7):673

[3] Sola-Visner MC, Christensen RD, Hutson AD, et al. Megakaryocyte size and concentration in the bone marrow of thrombocytopenic and nonthrombocytopenic neonates. Pediatric Research. 2007;**61**:479-484

[4] Krzyzaniak RL, Buss DH, Cooper MR, et al. Marrow fibrosis and multiple myeloma. American Journal of Clinical Pathology. 1988;**89**:63-68

[5] Cheung NK, Heller G, Kushner BH, et al. Detection of metastatic neuroblastoma in bone marrow: When is routine marrow histology insensitive? Journal of Clinical Oncology. 1997;**15**:2807-2817

[6] Pagani A, Macri L, Faulkner LB, et al. Detection procedures for neuroblastoma cells metastatic to blood and bone marrow: Blinded comparison of chromogranin A heminested reverse transcription polymerase chain reaction to tyrosine hydroxylase nested reverse transcription polymerase chain reaction and to anti-GD2 immunocytology. Diagnostic Molecular Pathology. 2002;**11**:98-106

[7] Parsons LN, Gheorghe G, Yan K, et al. Improving detection of metastatic Neuroblastoma in bone marrow core biopsies: A proposed immunohistochemical approach. Pediatric and Developmental Pathology. 2016;**19**:230-236

[8] Ito M. The diagnosis from the pathological viewpoint of a blood disease. International Journal of Hematology. 2002;**76**(Suppl 2):2-5

[9] Sadahira Y, Kanzaki A, Wada H, et al. Immunohistochemical identification of erythroid precursors in paraffin embedded bone marrow sections: Spectrin is a superior marker to glycophorin. Journal of Clinical Pathology. 1999;**52**:919-921

[10] Iwasaki T, Murakami M, Sugisaki C, et al. Characterization of myelodysplastic syndrome and aplastic anemia by immunostaining of p53 and hemoglobin F and karyotype analysis: Differential diagnosis between refractory anemia and aplastic anemia. Pathology International. 2008;**58**:353-360

[11] Briggs C, Carter J, Lee SH, et al. ICSH guideline for worldwide point-of-care testing in haematology with special reference to the complete blood count. International Journal of Laboratory Hematology. 2008;**30**:105-116

[12] Taubert R, Pischke S, Schlue J, et al. Enrichment of regulatory T cells in acutely rejected human liver allografts. American Journal of Transplantation. 2012;**12**:3425-3436

[13] Ohshima K, Kikuchi M, Takeshita M. A megakaryocyte analysis of the bone marrow in patients with myelodysplastic syndrome, myeloproliferative disorder and allied disorders. The Journal of Pathology. 1995;**177**:181-189

[14] Baseggio L, Berger F, Morel D, et al. Identification of circulating CD10 positive T cells in angioimmunoblastic T-cell lymphoma. Leukemia. 2006;**20**:296-303

Chapter 3

Acute Myeloid Leukemia in Pediatric Patients: A Review About Current Diagnostic and Treatment Approaches

Katarzyna Derwich, Dorothy Mitkowski and Jolanta Skalska-Sadowska

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.70937

Abstract

Acute leukemia is the most common childhood malignancy, accounting for almost 35% of all childhood cancers. Acute myeloid leukemia (AML) represents 15–20% of pediatric acute leukemia. Majority of AML cases appear *de novo*, however a minority of cases can present as a secondary malignancy. AML is a highly heterogeneous disease and its diagnosis involves a combination of diagnostic analyses including morphology, immunophenotyping, cytochemistry, and leukemic blasts derived from peripheral blood or bone marrow demonstrating cytogenic and molecular characteristics. Through the identification of recurrent genetic mutations, it has been made possible to refine individual prognosis and guide therapeutic management. The current survival rate of children with AML is approximately 70%. The standard therapeutic regimen is a combination of cytarabine- and anthracycline-based regimens with allogenic stem cell transplantation in appropriate patients. Relapse in pediatric patients suffering from AML occurs in approximately 30% of cases, whereas death occurs in 5–10% of patients as a result of disease complications or chemotherapeutic side effects. In understanding the genetic basis of AML, targeted therapies will have the ability to reduce treatment-related morbidity and mortality. Here, we provide a comprehensive review of AML, its biology, diagnosis and therapeutic management in pediatric patients.

Keywords: acute myeloid leukemia, pediatrics, diagnosis, immunophenotype, cytogenetics, classification, treatment, hematopoietic stem cell transplantation

1. Introduction

1.1. Epidemiology

Acute leukemia is the most common childhood malignancy, accounting for almost 35% of all childhood cancers. It can be further divided into two main subtypes such as acute lymphoblastic leukemia (ALL), composing 80% of acute leukemia and acute myeloid leukemia (AML) which makes up 15–20% of acute leukemia in pediatric patients [1]. The incidence of AML is greatest in infants at 1.5 per 100,000 individuals per year and decreases to 0.4 per 100,000 individuals aged 5–9 years. After this, the incidence of AML begins to gradually increase reaching its highest point in individuals greater than 65 years of age at 16.2 per 100,000 individuals [1, 2]. A report using data from the surveillance, epidemiology, and end results (SEER) program identified Asian and Pacific Islanders to have the highest rate of childhood AML (0.84 per 100,000) followed by Hispanics (0.81 per 100,000), Caucasians (0.75 per 100,000), and African-Americans (0.66 per 100,000) [3, 4].

1.2. Etiology and pathophysiology

The majority of AML cases appear as a *de novo* malignancy in previously healthy individuals, but there have been cases reported in which AML presents as a secondary malignancy. This has been witnessed in individuals with underlying hematological and genetic disorders such as Fanconi Anemia, Bloom Syndrome, Ataxia Telangiectasia, Shwachman-Diamond syndrome, Noonan syndrome, and Dyskeratosis Congenita. The most common genetic factor for the development of AML is trisomy 21 [3]. Children with Down syndrome have a 500-fold increased risk of developing a unique megakaryoblastic subtype of AML. This classically follows a transient myeloproliferative disorder in the neonatal period, which is characterized by somatic mutations in the GATA1 gene [3, 5]. Recently, a familial predisposition to AML has been suggested, as a number of germ-line mutations, such as GATA2, CEBPA, TP53, and RUNX1 have been found in families with an unexplained high risk of AML [3, 6–10]. In addition, exposure to prior therapy involving topoisomerases II, alkylating agents and radiation therapy have also been associated with an increased risk of developing AML as a secondary malignancy [1, 3].

The pathogenesis of AML involves the abnormal proliferation and differentiation of a clonal population of myeloid stem cells [11]. It is thought to arise from at least two classes of cooperating genetic events, known as a two-hit model of leukemogenesis [12–14]. Type I mutations result in increased and uncontrolled activation of pro-proliferative pathways of the leukemic cell and often involve activating genes that are part of signal transduction pathways, such as FLT3 (28% of cases), K/NRAS, TP53, and c-KIT (12, 8, and 4%, respectively) [15]. Type II mutations occur as a result of genetic aberrations in hematopoietic transcription factors leading to the impairment of normal hematopoietic differentiation. The most common type II cytogenic abnormalities in children, accounting for almost half of all pediatric AML cases are, t(8;21)(q22;q22) in the core-binding factor AML (CBF-AML) and t(15;17)(q;22;q21) in acute promyelocytic leukemia (APL) [16–19]. Other translocations are specific only to children and rarely

found in adults and these include t(1;22)(p13;q13), t(7;12)(q36;p13), and t(11;12)(p15;p13) [20–23]. The NPM1 and CEBPA, type II mutations, are found in approximately 27 and 6% of cases, respectively, and indicate a better prognosis [15]. Moreover, enhanced tyrosine phosphorylation of signal transducer and activator of transcription 3 (STAT3), involved in the stimulation of cellular proliferation and survival, is seen in almost 50% of AML cases and signifies a worse prognosis [24–26]. As stated by the two hit model of leukemogenesis, the pathogenesis of AML is dependent on two classes of cooperating genetic events. A study done by Patel et al. found that the c-KIT mutation has been associated with t(8,21) or inv. (16). Furthermore, they found that NMP1, which is a type II mutation, frequently occurs with FLT3-ITD (a type I mutation) or with mutations in epigenetic genes such as DNMT3A and IDH-1 or IDH-2 [27]. Despite these advancements in the pathogenesis of AML, there still remains much to be discovered on the exact implications that these individual mutations have on the development of AML, particularly in pediatric patients.

2. Therapeutic considerations

2.1. Classification

The first classification system used to distinguish between the different subtypes of AML was the French-American-British (FAB) classification system established in 1976. It identifies eight subtypes of AML (M0-M7) based on the morphological and cytochemical characteristics of the leukemic cells. The FAB classification was replaced by WHO in 2001 which was then revised in 2008 [28]. The WHO classification of AML was once again revised in 2016, this time integrating genetic information such as, karyotypes and molecular aberrations, with morphology, immunophenotype, and clinical presentation. It defines six major disease entities: AML with recurrent genetic abnormalities; AML with myelodysplasia-related features; therapy-related AML; AML not otherwise specified; myeloid sarcoma; and myeloid proliferation related to Down syndrome (**Table 1**) [29].

In **Table 1**, the subtypes of AML with recurrent genetic abnormalities are listed in accordance to their distinct chromosomal translocation. The newly incorporated provisional category of AML with mutated RUNX1 appears to represent a biologically distinct group with a worse prognosis in comparison to other AML subtypes. The category of AML with myelodysplasia-related changes remains to include a history of MDS as an inclusion criteria, however has been re-structured to better include subtypes with features suggesting a poor prognosis. Lastly, the myeloid proliferations of Down syndrome include transient abnormal myelopoiesis and myeloid leukemia associated with Down syndrome. As mentioned previously, both subtypes involve megakaryoblastic proliferations and are characterized by GATA1 mutations and mutations of the JAK-STAT pathway. Transient abnormal myelopoiesis typically occurs at birth or within the first few days of birth and resolves within 1–2 months. Myeloid leukemia associated with Down syndrome occurs later, but within the first 3 years of life, with or without prior transient abnormal myelopoiesis [29].

Types	Genetic abnormalities
AML with recurrent genetic abnormalities	AML with t(8:21)(q22;q22); RUNX1-RUNX1T1
	AML with inv.(16)(p13.1q22) or t(16;16)(p13.1;q22); CBFB-MYH11 APL with PML-RARA
	AML with t(9;11)(p21.3;q23.3); MLLT3-KMT2A
	ML with t(6;9)(p23;q34.1); DEK-NUP214
	AML with inv.(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); GATA2, MECOM AML (megakaryoblastic) with t(1;22)(p13.3;q13.3); RBM15-MKL1 AML with BCR-ABL1 (provisional entity)
	AML with mutated NPM1
	AML with biallelic mutations of CEBPA
	AML with mutated RUNX1 (provisional entity)
AML with myelodysplasia-related changes	AML with minimal differentiation
	AML without maturation
Therapy-related myeloid neoplasms	AML with maturation
	Acute myelomonocytic leukemia
	Acute monoblastic/monocytic leukemia acute erythroid leukemia
	Pure erythroid leukemia
	Acute megakaryoblastic leukemia acute basophilic leukemia
	Acute panmyelosis with myelofibrosis
Myeloid sarcoma	Transient abnormal myelopoiesis
Myeloid proliferations related to Down syndrome	ML associated with Down Syndrome

Abbreviations: WHO, World Health Organization; AML, acute myeloid leukemia; APL, acute promyelocytic leukemia; ML, myeloid leukemia.

Table 1. WHO classification of AML and related neoplasms [29].

In pediatric patients, specifically under the age of 2, it is important to search for translocations that are specific for pediatric AML, as WHO classification does not represent them as new disease categories due to their rarity. These translocations, mentioned above, include t(7;12)(q36;p13) and t(11;12)(p15;p13) [1, 29].

2.2. Diagnostic approach

AML is a highly heterogeneous disease in regards to its morphology, immunophenotyping, and its clinical manifestations [1]. The clinical presentation of AML commonly manifests with leukocytosis, anemia, and thrombocytopenia. Fatigue, anorexia, and weight loss are less commonly seen and symptoms such as lymphadenopathy and organomegaly are not usually present. If a patient is left untreated, death will most likely occur secondary to an infection or bleeding [11]. In order to establish a diagnosis of acute leukemia, 20% or more blasts must be

found in the bone marrow or peripheral blood [30]. To further diagnose AML, a combination of analyses is required, including, morphology, immunophenotyping, cytochemistry, and leukemic blasts derived from peripheral blood or bone marrow demonstrating cytogenic and molecular characteristics [31]. Although there is some overlap between the diagnostic recommendations for AML in children and adults, there are important differences between these two age groups that need to be focused on.

Morphological examination is conducted from blood and bone marrow smears using a May-Grunwald-Giemsa or a Wright-Giemsa stain [30]. The FAB classification is used to define the morphological characteristics of AML, which are based on the lineage of associated phenotypes: undifferentiated, myeloid, monoblastic, erythroblastic, or megakaryoblastic [32]. By looking at the morphology of AML, it is possible to determine the percentage of undifferentiated, atypical, or granulated blasts, intracellular structures such as Auer Rods and the presence of myelodysplasia [31]. Auer Rods are comprised of needle-shaped, azurophilic, cytoplasmic inclusion bodies and are commonly seen in APL, acute myelomonocytic leukemia and in majority of cases of AML with t(8;21) [28]. Once the lineage has been established through morphology, cytochemistry is then used to confirm the affiliation as well as differentiate between myeloid (myeloperoxidase [MPO]-positive) and monoblastic (nonspecific esterase-positive) classification. In certain cases in which the morphology and cytochemistry provide an ambiguous picture, immunophenotyping is required to further support the appropriate diagnosis [31].

Immunophenotyping is necessary to distinguish between AML and ALL. It is also required to classify minimally differentiated AML (FAB M0) and acute megakaryoblastic leukemia (AMKL, FAB M7). In minimally differentiated AML, morphological and cytochemical evaluation do not reveal myeloid differentiation. Although cytochemistry is negative for MPO activity, immunophenotyping is positive for myeloid markers, such as MPO (proenzyme) and/or CD13, CD33, and CD117 [31, 32]. AMKL is a type of leukemia with 20% or more blasts but of this, 50% or more are of megakaryocytic lineage. Immunophenotyping in AMKL is positive for platelet markers, such as CD41 and/or CD61 [30, 31]. The current WHO 2016 classification has not changed significantly from the 2008 classification. Markers required to assign lineages and define otherwise not specified mixed phenotype acute leukemia (MPAL) include MPO, lysozyme, CD11c, CD14, CD64, nonspecific esterase cytochemistry, i(intracellular) CD3, CD19, iCD22, CD79, and CD10 [29]. **Table 2** presents the suggested antigen panel for immunophenotypic analysis required for the diagnosis of AML in pediatric patients. It was modified specifically for pediatric patients by Creutzig et al. from the recommended panel for AML in adults [30, 31].

The use of conventional cytogenetic analysis for the diagnosis of AML is a necessary component of the diagnostic evaluation. It allows for the detection of chromosomal abnormalities in 70–80% of pediatric patients with AML [31]. In the case of which cytogenetic analysis fails, fluorescence *in situ* hybridization (FISH) is an option to detect gene rearrangement. FISH allows for the detection of fusion genes, such as RUNX1-RUNX1T1, CBFB-MYH11, MLL, and EVI1 as well as the loss of chromosome 5q and 7q material [33, 34]. As previously

Diagnosis of AML	Mandatory Markers to fulfill WHO & EGIL Criteria	Other Markers
Precursor stage	**CD34, CD117**	CD133, HLA-DR
Myelomonocytic markers	**CD11b, CD11c, CD13, CD14, CD15**, **CD33**, **CD64, CD65**, iMPO, i-lysozyme	CD4, CD36, CD184 (CXCR4)
Megakaryocytic markers	**CD41**, **CD61**	**CD42**
Erythroid marker		CD235a
Leukemia specific antigen		NG2 homolog
Lineage aberrant antigens		CD2, CD7, CD19, CD56
Pan-leukocyte markers		CD11a, CD45

Table 2. Immunophenotypic markers in Pediatric Patients for the diagnosis of AML.

mentioned, the two most common cytogenetic abnormalities in children are t(8;21)(q22;q22) in CBF-AML and t(15;17)(q;22;q21) in APL, together making up approximately 50% of pediatric AML [16–19].

Molecular genetics can be performed on bone marrow and/or blood specimens. Both DNA and RNA should be extracted, however if there is a small amount of specimen with a limited number of cells, RNA extraction is preferred. This is because RNA is more suitable for molecular screening for fusion genes and mutations specific to leukemia [30]. The frequency and nonrandom associations of type I and type II mutations (stated previously in Section 1.2) differ between pediatric and adult patients with AML [35]. Mutations in the RAS-RAF-ERK signal transduction pathway (PTPN11, NF-1, N-RAS, K-RAS) occur in 5–21% of pediatric AML cases and even more frequently in cases with CBF-AML and MLL-rearranged AML in young children. C-KIT mutations occur in 4% of all leukemia types, except CBF-AML, in which the incidence raises to approximately 25% in children [15, 30]. Despite the differences between children and adults in the expression of type I and type II mutations routine evaluation as suggested by Creutzig et al. should include FLT2-ITD, WTI, C-KIT, CEBPA, and NPM1 [31].

Gene expression profiling has the ability to contribute to the accurate diagnosis and risk-stratification of pediatric AML patients. However, its use remains limited as large cohorts are required for validation of the true prognostic significance of these genes [36]. Biobanking is strongly recommended for all patients, allowing for confirmation of initial diagnosis, or adding new data in the case of a relapse. Stored material should include AML blasts, DNA, and RNA. Buccal swabs are also recommended to be stored as they allow for discrimination between germ line and somatic genetic aberrations. This material can be used to identify new prognostic markers, monitoring for minimal residual disease (MRD) and contribute to research studies about the biological mechanism, subgroups and leukemogenesis of AML [30, 31].

2.3. Prognostic factors

The most relevant prognostic factors for the survival of pediatric AML are genetic and molecular abnormalities and the initial response to treatment. Both of these prognostic factors are

independent and are both usually essential elements of the risk group classification [31, 37]. Presence of a very high blast count at the time of diagnosis is associated with a higher risk of early death and nonresponse [38]. Other favorable prognostic factors include, t(8;21)(q22;q22)/RUNX1-RUNX1T1, t(15;17)(q22;q21)/PML-RARA, NPM1-mutated AML and CEBPA double mutation - amongst a few [31]. A favorable prognostic group is CBF-AML [19, 39, 40]. MLL-translocations have variable outcomes and depend on the associated translocation. In MLL t(1;11)(q21;q23) has a very favorable outcome in pediatric AML. In comparison, those with t(6;11)(q27;q23) and t(10;11)(p12;q23) translocations have been reported to have poor survival rates [31, 41, 42]. Certain prognostic makers vary between children and adults. For example, deletion of 7q in adults is suggestive of an intermediate prognosis; however in pediatric patients it is associated with a poorer outcome. In these pediatric patients, the outcome has been found to be dependent on other cytogenetic abnormalities in the leukemia cell [19, 43]. Other poor prognostic abnormalities that have been described in adult AML, such as abnormalities of chromosomes 3q and 5q and the monsoonal karyotypes are very rare in children [44–47].

The type I mutations of WT1 and FLT3-itd are indicative of a poor prognosis. FLT3-itd outcome depends on the allele ratio, whereas both of these mutations are described as events in clonal emulation towards relapse [48].

2.4. Pediatric AML treatment

The current survival rate of children with AML has increased to approximately 70%. This increase has been achieved by better risk stratification and intensification of chemotherapeutic regimens. The treatment of childhood AML should be risk adapted according to various biological factors in order to avoid over treatment in patients with a favorable prognosis and provide adequate chemotherapy to improve outcome in those with a less favorable prognosis [31]. This allows for the destruction of leukemia cells with the hope of avoiding side effects or as little late side effects as possible. With regards to infant AML, the most powerful prognostic factor for the outcome has been found to be favorable cytogenetics and a blast count of less than 5% after induction therapy [49].

The standard chemotherapeutic regimen consists of a combination of 4–5 cycles of cytarabine and anthracycline-based regimens with allogenic stem cell transplantation in appropriate patients [1]. Induction therapy typically includes 1 or 2 cycles of chemotherapy in both children and adults. Standard induction therapy involves 3 days of an anthracycline and 7–10 days of cytarabine (“3 + 7” or “3 + 10”). This induction regimen achieves complete remission in greater than 85% of children and adolescence. A third drug, such as etoposide or 6-thioguanine, can be included in induction, but their benefits have not yet been proven [50]. There are a number of various anthracyclines that have been evaluated through randomized controlled pediatric trials. Daunorubicin and mitoxantrone resulted in similar overall survival; however treatments with a mitoxantrone base resulted in lower relapse rates [51]. In comparison, iadrubicin and liposomal daunorubicin had similar survival rates, but liposomal daunorubicin was more effective in cases with a RUNX1/RUNX1T1 translocation and caused less treatment-related mortality [52]. Studies have shown that higher doses of anthracyclines improve the outcome in children and adults [53, 54]. However, at higher doses there is an increased risk

for toxicity, especially acute and late cardiotoxicity. A cumulative dose of >300 mg/m^2 has been associated with significant later cardiac toxicity and should take into consideration factors such as the patients age and sex [55, 56]. To avoid reaching peak serum concentrations, suggestions of splitting the dose, or using prolonged drug infusions have been proposed, however there have been conflicting benefits of dose scheduling and no conclusion on the best regimen has yet been reached [57, 58]. Dexrazozane is another option to reduce cardiotoxicity during anthracycline exposure and has been proven to be beneficial [59]. Failure of induction therapy is seen in 10–15% of pediatric patients. The subsequent outcomes for a patient with induction failure are similar to the patient with AML who relapsed early (<12 months after remission) [60, 61]. These patients, similar to patients that have relapsed, have been shown to have the highest end free survival after stem cell transplantation in comparison to after chemotherapy (31.2 vs. 5%, $p < 0.0001$) [62].

A considerable challenge in the treatment of children with AML is to prolong the initial remission. This is done with additional chemotherapy, similar to that in induction, but with the addition of non-cross-resistant drugs and high dose (HD) cytarabine [63, 64]. The ideal number of post remission cycles of therapy remains unclear, but appears to require at least two courses of intensive therapy, with the addition of the induction course. A study done by the United Kingdom Medical Research Council randomly assigned adult and pediatric patients to four or five courses of intensive therapy. There was no advantage seen in relapse free and overall survival in patients treated with five courses [51, 65].

The benefits of the intensification of cytarabine have been studied by a number of trials. In most studies, intensification of cytarabine from 12 to 36 g/m^2 did not improve survival in adult AML patients [66]. However, a study by the Cancer and Leukemia Group B (CALGB) in adults showed that four courses of HD cytarabine (3 g/m^2 per every 12 h on days 1, 3, and 5) were superior to four courses of lower dose cytarabine (100 mg/m^2 continuous intravenously on days 1–5) but only in patients with CBF-AML and CN-AML [63, 67]. Several pediatric trials (NOPHO AML 93, AML-BFM 2004, AML 10, and AML99 of the JPLSG) showed that the use of intensive chemotherapy courses that include HD cytarabine reduced relapse rates [67–72].

Hematopoietic stem cell transplantation (HSCT) is used as post remission consolidation therapy. Allogenic HSCT (allo-HSCT) has been found to have a greater benefit than autologous HSCT (auto-HSCT). Several trials have found no benefit of auto-HSCT compared with nonmyeloablative chemotherapy during the first complete remission [69, 73–76]. In addition, the degree of toxicity associated with the conditioning regimen greatly out ways the benefits/use of auto-HSCT [77]. In comparison, prospective trials of transplantation in children with AML suggest that 60–70% of children with HLA-matched donors will experience long-term remissions when treat with an allo-HSCT during their first remission [69, 74]. Although there is a significantly lower relapse risk associated with allo-HSCT as compared with post-remission chemotherapy, the improvement in overall survival is controversial [78–80]. The current utilization of allo-HSCT involves incorporation of risk classification to determine whether transplantation should occur during the first remission. There is consensus that favorable-risk patients should not be transplanted in first complete remission but only after the first relapse and achievement of a second complete

remission [73, 78, 79, 81]. Some benefit has been shown in allo-HSCT during first complete remission in intermediate- and high-risk patients. A meta-analysis combining the results of the POG-8821, CCG-2891, COG-2961, and MRC-Leuk-AML-10 showed benefit for allo-HSCT in intermediate-risk patients only [78]. Weakness in this analysis was due to a large percentage of patients not assigned to a risk group resulting in potential selection bias. If transplantation is chosen in pediatric AML, myeloablative chemotherapy is preferred over total body irradiation as the latter is associated with an increased risk of secondary malignancies and more late effects [82–84]. A large prospective CIBMTR cohort study of children and adults with AML, MDS, and CML, showed superior survival of patients in the early stages of the disease and less toxicity with busulfan-based regimens as compared to total body irradiation [85]. Despite these results, the optimal preparative regimen remains undetermined [86–88]. In summary, the role of HSCT in pediatric patients with AML in the first complete remission should continue to be assessed, particularly within specific risk groups. However, there is a consensus that HSCT should be offered to all children with relapsed AML once second remission has been achieved and that favorable-risk patients should not be offered HSCT [79].

The presence of CNS involvement at diagnosis and at relapse is seen in 5–10% of childhood AML cases. An increased risk of CNS involvement is seen in patients with hyperleukocytosis, monocytic leukemia (FAB M4 and M5, especially those with inv(16) or 11q23 chromosomal abnormalities), MLL gene rearrangement, and younger age [89]. All pediatric patients receive CNS treatment, even if no CNS involvement is detected. This is done with the presumption that systemic chemotherapy has little efficacy in penetrating the blood–brain-barrier to eradicate any potential AML blasts in the CNS. The most common regimen used for CNS treatment involves intrathecal chemotherapy-single agent cytarabine or methotrexate, or triple cytarabine, methotrexate and hydrocortisone. Other treatments such as cranial irradiation have also proven to be effective in the treatment of CNS involvement in pediatric AML. According to European protocols, prophylactic cranial radiotherapy is used in pediatric patients with AML. This is based on German pediatric AML studies, BFM-78 and BFM-83 where the use of cranial irradiation suggested to prevent both CNS and systemic relapse [89, 90]. However, due to its high side effect profile (including late toxicities and secondary malignancies), prophylactic cranial irradiation still remains controversial and is used less frequently in other countries [90].

As a result of AML being a highly heterogeneous disease and its ability to present in different ways, management may vary based on age group or the type of AML. One special group is children with DS, as they have a 14–20-fold risk of developing leukemia. In addition to the increased risk of AML during the first 3 years of life, about 10% of neonates with DS also develop a TMD, which usually disappears spontaneously [91]. Despite TMD self-resolving within 4–10 weeks, it can cause severe and life threatening complications such as hydrops fetalis, pleural effusions, liver cirrhosis with hyperbilirubinemia, organomegaly, or hyperleukocytosis [92]. Approximately, 10–20% of these patients will develop myeloid leukemia with megakaryoblastic features (ML-DS) within the first 3 years. Treatment of ML-DS involves intensity reduced chemotherapy and no HSCT in the first complete remission. As children with DS are susceptible to chemotherapy, the event free survival and survival rates are >85% [92–95].

APL is a distinct subtype of AML and is characterized by t(15;17). This translocation involves a breakpoint that includes the retinoic acid receptor and leads to production of the promyelocytic leukemia (PML)-retinoic acid receptor alpha (RARA) fusion protein [96]. Treatment is usually begun immediately with all-trans retinoid acid (ATRA) as APL is associated with an increased risk of a life threatening hemorrhage. In children, a dose of 25 mg/m^2 per day of ATRA should be started and has shown to produce equivalent outcomes to the higher dose of 45 mg/m^2 per day that is commonly used in adults [97–99]. Arsenic trioxide has proven to be an effective agent in combination with ATRA in the treatment of newly diagnosed, refractory, or relapsed APL [77]. In patients with hyperleukocytosis treated with ATRA or arsenic trioxide, approximately 10% of children can develop APL differentiation syndrome. This syndrome is characterized by fever, weight gain, respiratory distress, pleural, and pericardial effusions. The incidence of APL differentiation syndrome can be reduced by combining ATRA with chemotherapy. Pseudotumor cerebri occurs in 11% of children during initial ATRA administration and can be treated with steroids [97, 100].

Although there have been major improvements in treatment outcomes, AML remains a life-threatening malignancy. Approximately, 30% of pediatric patients relapse, with only 30–40% of these relapsed patients surviving, indicating a poor outcome [101, 102]. AML is a highly heterogeneous disease and through gaining knowledge on its molecular and genetic background it will allow new targeted and patient-specific therapies to become available to children.

2.5. Supportive treatment

The estimated incidence in children with high-risk AML of severe bacterial infections is 50–60% and the estimated incidence of invasive fungal infections is 7.0–12.5% [103–105]. The improved outcome in children with AML over the last 10 years may be associated to better supportive care strategies.

Hyperleukocytosis (WBC greater than 100,000/μL) at initial diagnosis is associated with an increased risk of CNS hemorrhage and leukostasis. Patients with monocytic or myelomonocytic (FAB M4 and M5) as well as APL and hyperleukocytosis are at an increased risk of early death [106, 107]. Treatment involves emergency care with intensive monitoring and careful hydration with the addition of rasburicase [108]. In more severe cases involving symptomatic coagulopathy, exchange transfusion or leukapheresis may be required. Controlled and effective reduction in cells with enforced diuresis or hemodialysis, may prevent the occurrence of tumor lysis syndrome [106–108].

Hematopoietic growth factors such as granulocyte-macrophage colony-stimulating factor (GM-CSF) or granulocyte colony-stimulating factor (G-CSF) during AML induction therapy are not recommended in the pediatric population. Although a randomized study in children with AML evaluated G-CSF administered after induction chemotherapy showed a reduction in duration of neutropenia there was no difference in infectious complications or mortality [109]. In addition, a higher relapse rate has been recently demonstrated

for children who over express the differentiation defective G-CSF receptor isoform IV [110]. Therefore, the routine prophylactic use of G-CSF or GM-CSF is not recommended for children with AML but remains an option in shortening neutropenia in critically ill patients.

Bacterial infections occur in up to 70% of children during AML therapy [111]. A retrospective study from St. Jude Children's Research Hospital (SJCRH) in patients with AML found that the use of intravenous cefepime or vancomycin in conjunction with oral ciprofloxacin or a cephalosporin significantly reduced the incidence of bacterial infection and sepsis compared with patients receiving only oral or no antibiotic prophylaxis [112]. Another retrospective study reported a significant reduction in Gram positive, sterile-site infections with antibiotic prophylaxis [113]. While it is suggested that antibiotic prophylaxis is beneficial, prospective randomized trials are required in pediatric patients with AML.

The incidence of invasive fungal infections is up to 15% in children with AML, which is similar to that in adults. They are most commonly caused by *Candida* and *Aspergillus* species [114]. Prophylaxis should be administered to all children with agents such as voriconazole, itraconazole, micafungin, or caspofungin. Due to drug interactions (e.g., itraconazole and voriconazole) and variable pharmacokinetics, voriconazole should be held during courses of chemotherapy and levels should be monitored periodically. Prophylaxis for *Pneumocystis jirovecii* with trimethoprim-sulfamethoxazole should also be administered [114, 115].

3. Conclusion

The diagnosis and treatment of AML has significantly improved over the past decades. Risk stratification has allowed for more targeted and specific therapy while avoiding, over treatment in low-risk patients and allowing for more intensive therapy in others. AML is a highly heterogeneous disease and through gaining knowledge on its molecular and genetic background as well as international collaboration, it will allow new targeted and patient-specific therapies to become available, particularly in pediatric patients.

Author details

Katarzyna Derwich[1]*, Dorothy Mitkowski[2] and Jolanta Skalska-Sadowska[1]

*Address all correspondence to: kderwich@poczta.onet.pl

1 Department of Pediatric Oncology, Hematology and Transplantology, Poznan University of Medical Sciences, Poznan, Poland

2 Poznan University of Medical Sciences, Poznan, Poland

References

[1] de Rooij J, Zwaan C, van den Heuvel-Eibrink M. Pediatric AML: From biology to clinical management. Journal of Clinical Medicine. 2015;**4**(1):127-149

[2] Howlader N, Noone AM, Krapcho M, Garshell J, Miller D, Altekruse SF, Kosary CL, et al. SEER Cancer Statistics Review, 1975-2011. Bethesda, MD: National Cancer Institute; Based on November 2013 SEER data submission, posted to the SEER web site, April 2014. http://seer.cancer.gov/csr/1975_2011/

[3] Puumala S, Ross J, Aplenc R, Spector L. Epidemiology of childhood acute myeloid leukemia. Pediatric Blood & Cancer. 2013;**60**(5):728-733

[4] Linabery A, Ross J. Trends in childhood cancer incidence in the U.S. (1992-2004). Cancer. 2008;**112**(2):416-432

[5] Zwaan C, Reinhardt D, Hitzler J, Vyas P. Acute leukemias in children with down syndrome. Pediatric Clinics of North America. 2008;**55**(1):53-70

[6] Hahn C, Chong C, Carmichael C, Wilkins E, Brautigan P, Li X, et al. Heritable GATA2 mutations associated with familial myelodysplastic syndrome and acute myeloid leukemia. Nature Genetics. 2011;**43**(10):1012-1017

[7] Ganly P, Walker L, Morris C. Familial mutations of the transcription factor RUNX1 (AML1, CBFA2) predispose to acute myeloid leukemia. Leukemia & Lymphoma. 2004;**45**(1):1-10

[8] Owen C, Barnett M, Fitzgibbon J. Familial myelodysplasia and acute myeloid leukaemia—A review. British Journal of Haematology. 2007;**140**(2):123-132

[9] Smith M, Cavenagh J, Lister T, Fitzgibbon J. Mutation of CEBPA in familial acute myeloid leukemia. New England Journal of Medicine. 2004;**351**(23):2403-2407

[10] Link D, Schuettpelz L, Shen D, Wang J, Walter M, Kulkarni S, et al. Identification of a novel TP53 cancer susceptibility mutation through whole-genome sequencing of a patient with therapy-related AML. Journal of the American Medical Association. 2011;**305**:1568-1576

[11] De Kouchkovsky I, Abdul-Hay M. Acute myeloid leukemia: A comprehensive review and 2016 update. Blood Cancer Journal. 2016;**6**(7):e441

[12] Kelly L, Gilliland D. Genetics of myeloid leukemias. Annual Review of Genomics and Human Genetics. 2002;**3**:179-198

[13] Takahashi S. Current findings for recurring mutations in acute myeloid leukemia. Journal of Hematology & Oncology. 2011;**4**(1):36

[14] Kihara R, Nagata Y, Kiyoi H, Kato T, Yamamoto E, Suzuki K, et al. Comprehensive analysis of genetic alterations and their prognostic impacts in adult acute myeloid leukemia patients. Leukemia. 2014;**28**(8):1586-1595

[15] The Cancer Genome Atlas Research Network. Genomic and Epigenomic Landscapes of Adult De Novo Acute Myeloid Leukemia. The New England Journal of Medicine. 2013;**368**(22):2059-2074

[16] Grimwade D. The clinical significance of cytogenetic abnormalities in acute myeloid leukaemia. Best Practice & Research Clinical Haematology. 2001;**14**(3):497-529

[17] Harrison C, Hills R, Moorman A, Grimwade D, Hann I, Webb D, et al. Cytogenetics of childhood acute myeloid leukemia: United Kingdom medical research council treatment trials AML 10 and 12. Journal of Clinical Oncology. 2010;**28**(16):2674-2681

[18] Betts D, Ammann R, Hirt A, Hengartner H, Beck-Popovic M, Kuhne T, et al. The prognostic significance of cytogenetic aberrations in childhood acute myeloid leukaemia. A study of the Swiss Paediatric oncology group (SPOG). European Journal of Haematology. 2007;**78**(6):468-476

[19] von Neuhoff C, Reinhardt D, Sander A, Zimmermann M, Bradtke J, Betts D, et al. Prognostic impact of specific chromosomal aberrations in a large group of pediatric patients with acute myeloid leukemia treated uniformly according to trial AML-BFM 98. Journal of Clinical Oncology. 2010;**28**(16):2682-2689

[20] Mercher T, Busson-Le Coniat M, Khac F, Ballerini P, Mauchauffé M, Bui H, et al. Recurrence of OTT-MAL fusion in t(1;22) of infant AML-M7. Genes, Chromosomes and Cancer. 2001;**33**(1):22-28

[21] von Bergh A, van Drunen E, van Wering E, van Zutven L, Hainmann I, Lönnerholm G, et al. High incidence of t(7;12)(q36;p13) in infant AML but not in infant ALL, with a dismal outcome and ectopic expression ofHLXB9. Genes, Chromosomes and Cancer. 2006;**45**(8):731-739

[22] Slater R, Drunen E, Kroes W, Olde Weghuis D, van den Berg E, Smit E, et al. T(7;12)(q36;p13) and t(7;12)(q32;p13)—Translocations involving ETV6 in children 18 months of age or younger with myeloid disorders. Leukemia. 2001;**15**(6):915-920

[23] Hollink I, von den Heuvel-Eibrink M, Arentsen-Peters S, Pratcorona M, Abbas S, Kuipers J, et al. NUP98/NSD1 characterizes a novel poor prognostic group in acute myeloid leukemia with a distinct HOX gene expression pattern. Blood. 2011;**118**(13):3645-3656

[24] Cook A, Li L, Ho Y, Lin A, Li L, Stein A, et al. Role of altered growth factor receptor-mediated JAK2 signaling in growth and maintenance of human acute myeloid leukemia stem cells. Blood. 2014;**123**:2826-2837

[25] Spiekermann K, Bagrintseva K, Schwab R, Schmieja K, Hiddemann W. Over expression and constitutive activation of FLT3 induces STAT5 activation in primary acute myeloid leukemia blast cells. Clinical Cancer Research. 2003;**9**:2140-2150

[26] Steensma D, McClure R, Karp J, Tefferi A, Lasho T, Powell H, et al. JAK2 V617F is a rare finding in de novo acute myeloid leukemia, but STAT3 activation is common and remains unexplained. Leukemia. 2006;**20**(6):971-978

[27] Patel J, Gonen M, Figueroa M, Fernandez H, Sun Z, Racevskis J, et al. Prognostic relevance of integrated genetic profiling in acute myeloid leukemia. New England Journal of Medicine. 2012;**366**:1079-1089

[28] Vardiman J, Thiele J, Arber D, Brunning R, Borowitz M, Porwit A, et al. The 2008 revision of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: Rationale and important changes. Blood. 2009;**114**(5):937-951

[29] Arber DA, Orazi A, Hasserjian R, et al. The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. Blood. 2016;**127**(20):2391-2405. Blood. 2016;**128**(3):462-463

[30] Dohner H, Estey E, Amadori S, Appelbaum F, Buchner T, Burnett A, et al. Diagnosis and management of acute myeloid leukemia in adults: Recommendations from an international expert panel, on behalf of the European LeukemiaNet. Blood. 2009;**115**(3): 453-474

[31] Creutzig U, van den Heuvel-Eibrink M, Gibson B, Dworzak M, Adachi S, de Bont E, et al. Diagnosis and management of acute myeloid leukemia in children and adolescents: Recommendations from an international expert panel. Blood. 2012;**120**(16): 3187-3205

[32] Bennett J, Catovsky D, Daniel M, Flandrin G, Galton D, Gralnick H, et al. Proposal for the recognition of minimally differentiated acute myeloid leukaemia (AML-MO). British Journal of Haematology. 1991;**78**(3):325-329

[33] Fröhling S, Skelin S, Liebisch C, Scholl C, Schlenk R, Döhner H, et al. Comparison of cytogenetic and molecular cytogenetic detection of chromosome abnormalities in 240 consecutive adult patients with acute myeloid leukemia. Journal of Clinical Oncology. 2002;**20**(10):2480-2485

[34] Lugthart S, van Drunen E, van Norden Y, van Hoven A, Erpelinck C, Valk P, et al. High EVI1 levels predict adverse outcome in acute myeloid leukemia: Prevalence of EVI1 overexpression and chromosome 3q26 abnormalities underestimated. Blood. 2008; **111**(8):4329-4337

[35] Bos J, Toksoz D, Marshall C, Verlaan-de Vries M, Veeneman G, van der Eb A, et al. Amino-acid substitutions at codon 13 of the N-ras oncogene in human acute myeloid leukaemia. Nature. 1985;**315**(6022):726-730

[36] Ross M. Gene expression profiling of pediatric acute myelogenous leukemia. Blood. 2004;**104**(12):3679-3687

[37] Dluzniewska A, Balwierz W, Armata J, Balcerska A, Chybicka A, Kowalczyk J, et al. Twenty years of polish experience with three consecutive protocols for treatment of childhood acute myelogenous leukemia. Leukemia. 2005;**19**(12):2117-2124

[38] Creutzig U, Zimmermann M, Ritter J, Henze G, Graf N, Loffler H, et al. Definition of a standard-risk group in children with AML. British Journal of Haematology. 1999; **104**(3):630-639

[39] Balgobind B, Hollink I, Arentsen-Peters S, Zimmermann M, Harbott J, Beverloo H, et al. Integrative analysis of type-I and type-II aberrations underscores the genetic heterogeneity of pediatric acute myeloid leukemia. Haematologica. 2011;**96**(10):1478-1487

[40] Rubnitz J, Raimondi S, Halbert A, Tong X, Srivastava D, Razzouk B, et al. Characteristics and outcome of t(8;21)-positive childhood acute myeloid leukemia: A single institution's experience. Leukemia. 2002;**16**(10):2072-2077

[41] Balgobind B, Raimondi S, Harbott J, Zimmermann M, Alonzo T, Auvrignon A, et al. Novel prognostic subgroups in childhood 11q23/MLL-rearranged acute myeloid leukemia: Results of an international retrospective study. Blood. 2009;**114**(12):2489-2496

[42] Coenen E, Raimondi S, Harbott J, Zimmermann M, Alonzo T, Auvrignon A, et al. Prognostic significance of additional cytogenetic aberrations in 733 de novo pediatric 11q23/MLL-rearranged AML patients: Results of an international study. Blood. 2011; **117**(26):7102-7111

[43] Hasle H, Alonzo T, Auvrignon A, Behar C, Chang M, Creutzig U, et al. Monosomy 7 and deletion 7q in children and adolescents with acute myeloid leukemia: An international retrospective study. Blood. 2007;**109**(11):4641-4647

[44] Pession A, Masetti R, Rizzari C, Putti M, Casale F, Fagioli F, et al. Results of the AIEOP AML 2002/01 multicenter prospective trial for the treatment of children with acute myeloid leukemia. Blood. 2013;**122**(2):170-178

[45] Kelly M, Horan J, Alonzo T, Eapen M, Gerbing R, He W, et al. Comparable survival for pediatric acute myeloid leukemia with poor-risk cytogenetics following chemotherapy, matched related donor, or unrelated donor transplantation. Pediatric Blood & Cancer. 2013;**61**(2):269-275

[46] Johnston D, Alonzo T, Gerbing R, Hirsch B, Heerema N, Ravindranath Y, et al. Outcome of pediatric patients with acute myeloid leukemia (AML) and −5/5q− abnormalities from five pediatric AML treatment protocols: A report from the Children's oncology group. Pediatric Blood & Cancer. 2013;**60**(12):2073-2078

[47] Manola K, Panitsas F, Polychronopoulou S, Daraki A, Karakosta M, Stavropoulou C, et al. Cytogenetic abnormalities and monosomal karyotypes in children and adolescents with acute myeloid leukemia: Correlations with clinical characteristics and outcome. Cancer Genetics. 2013;**206**(3):63-72

[48] Bachas C, Schuurhuis G, Hollink I, Kwidama Z, Goemans B, Zwaan C, et al. High-frequency type I/II mutational shifts between diagnosis and relapse are associated with outcome in pediatric AML: Implications for personalized medicine. Blood. 2010; **116**(15):2752-2758

[49] Masetti R, Vendemini F, Zama D, Biagi C, Pession A, Locatelli F. Acute myeloid leukemia in infants: Biology and treatment. Frontiers in Pediatrics. 2015;**3**:37. DOI: 10.3389/fped.2015.00037

[50] Kaspers GJ. Pediatric acute myeloid leukemia. Expert Review of Anticancer Therapy. 2012;**12**(3):405-413

[51] Gibson B, Webb D, Howman A, De Graaf S, Harrison C, Wheatley K. Results of a randomized trial in children with acute myeloid leukaemia: Medical Research Council AML12 trial. British Journal of Haematology. 2011;**155**(3):366-376

[52] Creutzig U, Zimmermann M, Bourquin J, Dworzak M, Fleischhack G, Graf N, et al. Randomized trial comparing liposomal daunorubicin with idarubicin as induction for pediatric acute myeloid leukemia: Results from study AML-BFM 2004. Blood. 2013; **122**(1):37-43

[53] Lowenberg B, Ossenkoppele GJ, van Putten W, et al. High-dose daunorubicin in older patients with acute myeloid leukemia. New England Journal of Medicine 2009; **361**(13):1235-1248

[54] Fernandez HF, Sun Z, Yao X, et al. Anthracycline dose intensification in acute myeloid leukemia. New England Journal of Medicine. 2009;**361**(13):1249-1259

[55] Grenier M, Lipshultz S. Epidemiology of anthracycline cardiotoxicity in children and adults. Seminars in Oncology. 1998;**25**(4 Suppl 10):72-85

[56] Kremer L, van der Pal H, Offringa M, van Dalen E, Voute P. Frequency and risk factors of subclinical cardiotoxicity after anthracycline therapy in children: A systematic review. Annals of Oncology. 2002;**13**(6):819-829

[57] Lipshultz S, Giantris A, Lipsitz S, Dalton V, Asselin B, Barr R, et al. Doxorubicin administration by continuous infusion is not cardioprotective: The Dana-Farber 91-01 acute lymphoblastic leukemia protocol. Journal of Clinical Oncology. 2002;**20**(6):1677-1682

[58] Bielack S, Erttmann R, Kempf-Bielack B, Winkler K. Impact of scheduling on toxicity and clinical efficacy of doxorubicin: What do we know in the mid-nineties? European Journal of Cancer. 1996;**32**(10):1652-1660

[59] Lipshultz S, Alvarez J, Scully R. Anthracycline associated cardiotoxicity in survivors of childhood cancer. Heart. 2007;**94**(4):525-533

[60] Wells R, Adams M, Alonzo T, Arceci R, Buckley J, Buxton A, et al. Mitoxantrone and cytarabine induction, high-dose cytarabine, and etoposide intensification for pediatric patients with relapsed or refractory acute myeloid leukemia: Children's cancer group study 2951. Journal of Clinical Oncology. 2003;**21**(15):2940-2947

[61] Aplenc R, Alonzo T, Gerbing R, Lange B, Hurwitz C, Wells R, et al. Safety and efficacy of gemtuzumab ozogamicin in combination with chemotherapy for pediatric acute myeloid leukemia: A report from the Children's oncology group. Journal of Clinical Oncology. 2008;**26**(14):2390-2395

[62] Quarello P, Fagioli F, Basso G, Putti M, Berger M, Luciani M, et al. Outcome of children with acute myeloid leukaemia (AML) experiencing primary induction failure in the AIEOP AML 2002/01 clinical trial. British Journal of Haematology. 2015;**171**(4): 566-573

[63] Mayer R, Davis R, Schiffer C, Berg D, Powell B, Schulman P, et al. Intensive postremission chemotherapy in adults with acute myeloid leukemia. New England Journal of Medicine. 1994;**331**(14):896-903

[64] Cassileth P, Lynch E, Hines J, et al. Varying intensity of postremission therapy in acute myeloid leukemia. Blood. 1992;**79**(8):1924-1930

[65] Burnett A, Hills R, Milligan D, Goldstone A, Prentice A, McMullin M, et al. Attempts to optimize induction and consolidation treatment in acute myeloid leukemia: Results of the MRC AML12 trial. Journal of Clinical Oncology. 2010;**28**(4):586-595

[66] Schaich M, Röllig C, Soucek S, Kramer M, Thiede C, Mohr B, et al. Cytarabine dose of 36 g/m^2 compared with 12 g/m^2 within first consolidation in acute myeloid leukemia: Results of patients enrolled onto the prospective randomized AML96 study. Journal of Clinical Oncology. 2011;**29**(19):2696-2702

[67] Bloomfield CD, Lawrence D, Byrd JC, et al. Frequency of prolonged remission duration after high-dose cytarabine intensification in acute myeloid leukemia varies by cytogenetic subtype. Cancer Research. 1998;**58**(18):4173-4179

[68] Lie S, Abrahamsson J, Clausen N, Forestier E, Hasle H, Hovi L, et al. Treatment stratification based on initial in vivo response in acute myeloid leukaemia in children without Down's syndrome: Results of NOPHO-AML trials. British Journal of Haematology. 2003;**122**(2):217-225

[69] Stevens R, Hann I, Wheatley K, Gray R. Marked improvements in outcome with chemotherapy alone in paediatric acute myeloid leukaemia: Results of the United Kingdom Medical Research Council's 10th AML trial. British Journal of Haematology. 1998;**101**(1):130-140

[70] Creutzig U, Zimmermann M, Reinhardt D, Dworzak M, Stary J, Lehrnbecher T. Early deaths and treatment-related mortality in children undergoing therapy for acute myeloid leukemia: Analysis of the multicenter clinical trials AML-BFM 93 and AML-BFM 98. Journal of Clinical Oncology. 2004;**22**(21):4384-4393

[71] Gibson B, Wheatley K, Hann I, Stevens R, Webb D, Hills R, et al. Treatment strategy and long-term results in paediatric patients treated in consecutive UK AML trials. Leukemia. 2005;**19**(12):2130-2138

[72] Tsukimoto I, Tawa A, Horibe K, Tabuchi K, Kigasawa H, Tsuchida M, et al. Risk-stratified therapy and the intensive use of cytarabine improves the outcome in childhood acute myeloid leukemia: The AML99 trial from the Japanese childhood AML cooperative study group. Journal of Clinical Oncology. 2009;**27**(24):4007-4013

[73] Oliansky D, Rizzo J, Aplan P, Arceci R, Leone L, Ravindranath Y, et al. The role of cytotoxic therapy with hematopoietic stem cell transplantation in the therapy of acute myeloid leukemia in children: An evidence-based review. Biology of Blood and Marrow Transplantation. 2007;**13**(1):1-25

[74] Woods W, Neudorf S, Gold S, et al. A comparison of allogeneic bone marrow transplantation, autologous bone marrow transplantation, and aggressive chemotherapy in

children with acute myeloid leukemia in remission: A report from the Children's cancer group. Blood. 2001;**97**(1):56-62

[75] Ravindranath Y, Yeager A, Chang M, Steuber C, Krischer J, Graham-Pole J, et al. Autologous bone marrow transplantation versus intensive consolidation chemotherapy for acute myeloid leukemia in childhood. New England Journal of Medicine. 1996; **334**(22):1428-1434

[76] Alonzo T, Wells R, Woods W, Lange B, Gerbing R, Buxton A, et al. Postremission therapy for children with acute myeloid leukemia: The children's cancer group experience in the transplant era. Leukemia. 2005;**19**(6):965-970

[77] Sanz M, Grimwade D, Tallman M, Lowenberg B, Fenaux P, Estey E, et al. Management of acute promyelocytic leukemia: Recommendations from an expert panel on behalf of the European LeukemiaNet. Blood. 2008;**113**(9):1875-1891

[78] Horan J, Alonzo T, Lyman G, Gerbing R, Lange B, Ravindranath Y, et al. Impact of disease risk on efficacy of matched related bone marrow transplantation for pediatric acute myeloid leukemia: The Children's oncology group. Journal of Clinical Oncology. 2008;**26**(35):5797-5801

[79] Niewerth D, Creutzig U, Bierings M, Kaspers G. A review on allogeneic stem cell transplantation for newly diagnosed pediatric acute myeloid leukemia. Blood. 2010; **116**(13):2205-2214

[80] Klingebiel T, Reinhardt D, Bader P. Place of HSCT in treatment of childhood AML. Bone Marrow Transplantation. 2008;**42**:S7-S9

[81] Creutzig U, Reinhardt D. Current controversies: Which patients with acute myeloid leukaemia should receive a bone marrow transplantation?—A European view. British Journal of Haematology. 2002;**118**(2):365-377

[82] Bhatia S. Long-term health impacts of hematopoietic stem cell transplantation inform recommendations for follow-up. Expert Review of Hematology. 2011;**4**(4):437-454

[83] Gupta T, Kannan S, Dantkale V, Laskar S. Cyclophosphamide plus total body irradiation compared with busulfan plus cyclophosphamide as a conditioning regimen prior to hematopoietic stem cell transplantation in patients with leukemia: A systematic review and meta-analysis. Hematology/Oncology and Stem Cell Therapy. 2011;**4**(1):17-29

[84] Bresters D, van Gils I, Kollen W, Ball L, Oostdijk W, van der Bom J, et al. High burden of late effects after haematopoietic stem cell transplantation in childhood: A single-centre study. Bone Marrow Transplantation. 2009;**45**(1):79-85

[85] Bredeson C, LeRademacher J, Kato K, DiPersio J, Agura E, Devine S, et al. Prospective cohort study comparing intravenous busulfan to total body irradiation in hematopoietic cell transplantation. Blood. 2013;**122**(24):3871-3878

[86] Koreth J, Schlenk R, Kopecky K, Honda S, Sierra J, Djulbegovic B, et al. Allogeneic stem cell transplantation for acute myeloid leukemia in first complete remission. Journal of the American Medical Association. 2009;**301**(22):2349

[87] Liu D, Xu L, Liu K, Wang Y, Chen H, Han W, et al. Long-term outcomes of unmanipulated haploidentical HSCT for paediatric patients with acute leukaemia. Bone Marrow Transplantation. 2013;**48**(12):1519-1524

[88] Locatelli F, Masetti R, Rondelli R, Zecca M, Fagioli F, Rovelli A, et al. Outcome of children with high-risk acute myeloid leukemia given autologous or allogeneic hematopoietic cell transplantation in the AIEOP AML-2002/01 study. Bone Marrow Transplantation. 2015;**50**(2):320-320

[89] Creutzig U, Zimmermann M, Bourquin J, Dworzasungk M, Fleischhack G, von Neuhoff C, et al. CNS irradiation in pediatric acute myleoid leukemia: Equal results by 12 or 18 Gy in studies AML-BFM98 and 2004. Pediatric Blood & Cancer. 2011;**57**(6):986-992

[90] Dahl G, Simone J, Hustu H, Mason C. Preventive central nervous system irradiation in children with acute nonlymphocytic leukemia. Cancer. 1978;**42**(5):2187-2192

[91] Gamis A, Alonzo T, Gerbing R, Hilden J, Sorrell A, Sharma M, et al. Natural history of transient myeloproliferative disorder clinically diagnosed in down syndrome neonates: A report from the Children's oncology group study A2971. Blood. 2011;**118**(26): 6752-6759

[92] Klusmann J, Creutzig U, Zimmermann M, Dworzak M, Jorch N, Langebrake C, et al. Treatment and prognostic impact of transient leukemia in neonates with down syndrome. Blood. 2008;**111**(6):2991-2998

[93] Creutzig U, Reinhardt D, Diekamp S, Dworzak M, Stary J, Zimmermann M. AML patients with down syndrome have a high cure rate with AML-BFM therapy with reduced dose intensity. Leukemia. 2005;**19**(8):1355-1360

[94] Kudo K, Kojima S, Tabuchi K, Yabe H, Tawa A, Imaizumi M, et al. Prospective study of a pirarubicin, intermediate-dose cytarabine, and etoposide regimen in children with down syndrome and acute myeloid leukemia: The Japanese childhood AML cooperative study group. Journal of Clinical Oncology. 2007;**25**(34):5442-5447

[95] Kudo K, Hama A, Kojima S, Ishii R, Morimoto A, Bessho F, et al. Mosaic down syndrome-associated acute myeloid leukemia does not require high-dose cytarabine treatment for induction and consolidation therapy. International Journal of Hematology. 2010;**91**(4):630-635

[96] Melnick A, Licht J. Deconstructing a disease: RARalpha, its fusion partners, and their roles in the pathogenesis of acute promyelocytic leukemia. Blood. 1999;**93**(10): 3167-3215

[97] Mann G, Reinhardt D, Ritter J, Hermann J, Schmitt K, Gadner H, et al. Treatment with all-trans retinoic acid in acute promyelocytic leukemia reduces early deaths in children. Annals of Hematology. 2001;**80**(7):417-422

[98] de Botton S, Coiteux V, Chevret S, et al. Outcome of childhood acute promyelocytic leukemia with all-trans-retinoic acid and chemotherapy. Journal of Clinical Oncology. 2004;**22**(8):1404-1412

[99] Ortega J, Madero L, Martín G, Verdeguer A, García P, Parody R, et al. Treatment with all-transretinoic acid and anthracycline monochemotherapy for children with acute promyelocytic leukemia: A multicenter study by the PETHEMA group. Journal of Clinical Oncology. 2005;**23**(30):7632-7640

[100] Gregory J, Feusner J. Acute promyelocytic leukemia in childhood. Current Oncology Reports. 2009;**11**(6):439-445

[101] Sander A, Zimmermann M, Dworzak M, Fleischhack G, von Neuhoff C, Reinhardt D, et al. Consequent and intensified relapse therapy improved survival in pediatric AML: Results of relapse treatment in 379 patients of three consecutive AML-BFM trials. Leukemia. 2010;**24**(8):1422-1428

[102] Kaspers G, Zimmermann M, Reinhardt D, Gibson B, Tamminga R, Aleinikova O, et al. Improved outcome in pediatric relapsed acute myeloid leukemia: Results of a randomized trial on liposomal daunorubicin by the international BFM study group. Journal of Clinical Oncology. 2013;**31**(5):599-607

[103] Sung L, Gamis A, Alonzo T, Buxton A, Britton K, DeSwarte-Wallace J, et al. Infections and association with different intensity of chemotherapy in children with acute myeloid leukemia. Cancer. 2009;**115**(5):1100-1108

[104] Kaya Z, Gursel T, Kocak U, Aral Y, Kalkanci A, Albayrak M. Invasive fungal infections in pediatric leukemia patients receiving fluconazole prophylaxis. Pediatric Blood & Cancer. 2009;**52**(4):470-475

[105] Kobayashi R, Kaneda M, Sato T, Ichikawa M, Suzuki D, Ariga T. The clinical feature of invasive fungal infection in pediatric patients with hematologic and malignant diseases. Journal of Pediatric Hematology/Oncology. 2008;**30**(12):886-890

[106] Inaba H, Fan Y, Pounds S, Geiger T, Rubnitz J, Ribeiro R, et al. Clinical and biologic features and treatment outcome of children with newly diagnosed acute myeloid leukemia and hyperleukocytosis. Cancer. 2008;**113**(3):522-529

[107] Creutzig U, Ritter J, Budde M, Sutor A, Schellong G. Early deaths due to hemorrhage and leukostasis in childhood acute myelogenous leukemia. Associations with hyperleukocytosis and acute monocytic leukemia. Cancer. 1987;**60**(12):3071-3079

[108] Malaguarnera G, Giordano M, Malaguarnera M. Rasburicase for the treatment of tumor lysis in hematological malignancies. Expert Review of Hematology. 2012;**5**(1): 27-38

[109] Lehrnbecher T, Zimmermann M, Reinhardt D, Dworzak M, Stary J, Creutzig U. Prophylactic human granulocyte colony-stimulating factor after induction therapy in pediatric acute myeloid leukemia. Blood. 2006;**109**(3):936-943

[110] Ehlers S, Herbst C, Zimmermann M, Scharn N, Germeshausen M, von Neuhoff N, et al. Granulocyte colony-stimulating factor (G-CSF) treatment of childhood acute myeloid leukemias that overexpress the differentiation-DefectiveG-CSFReceptor isoform IV

is associated with a higher incidence of relapse. Journal of Clinical Oncology. 2010; **28**(15):2591-2597

[111] Sung L, Nathan P, Alibhai S, Tomlinson G, Beyene J. Meta-analysis: Effect of prophylactic hematopoietic colony-stimulating factors on mortality and outcomes of infection. Annals of Internal Medicine. 2007;**147**(6):400-411

[112] Kurt B, Flynn P, Shenep J, Pounds S, Lensing S, Ribeiro R, et al. Prophylactic antibiotics reduce morbidity due to septicemia during intensive treatment for pediatric acute myeloid leukemia. Cancer. 2008;**113**(2):376-382

[113] Yeh T, Liu H, Hou J, Chen K, Huang T, Chang C, et al. Severe infections in children with acute leukemia undergoing intensive chemotherapy can successfully be prevented by ciprofloxacin, voriconazole, or micafungin prophylaxis. Cancer. 2014;**120**(8): 1255-1262

[114] Rubnitz J. How I treat pediatric acute myeloid leukemia. Blood. 2012;**119**(25):5980-5988

[115] Lehrnbecher T, Groll A. Invasive fungal infections in the pediatric population. Expert Review of Anti-Infective Therapy. 2011;**9**(3):275-278

Chapter 4

Proteomics in Acute Myeloid Leukemia

Chenyue W. Hu and Amina A. Qutub

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.70929

Abstract

Acute myeloid leukemia (AML) is an extremely heterogeneous and deadly hematological cancer. Cytogenetic abnormalities and genetic mutations, though well recognized and highly prognostic, do not fully capture the degree of heterogeneities manifested in AML clinically. Additionally, current treatment of AML still largely depends on chemotherapy and allogeneic stem cell transplantation, with few options for personalized and molecularly targeted therapies. Proteomics holds promise for unraveling biological heterogeneities in AML beyond the scope of cytogenetics and genomics. In recent years, proteomics has emerged as an important tool for discovering new diagnostic biomarkers, enabling more prognostic patient classifications, and identifying novel therapeutic targets. In this chapter, we review recent advances in proteomic studies of AML, including an overview of AML pathology, popular proteomic techniques, various applications of proteomics in AML from biomarker discovery to target identification, challenges and future directions in this field.

Keywords: AML, proteomics, RPPA, mass spectrometry, leukemia

1. Introduction

Acute myeloid leukemia (AML) is a hematological cancer characterized by rapid proliferation and accumulation of immature, abnormally differentiated clonal hematopoietic cells in the bone marrow and blood [1]. The American Cancer Society estimates about 21,380 new cases of AML and about 10,590 deaths from AML occurring in the United States in 2017. Known as an age-associated disease, the average age of AML patients is 67 years old, and almost all deaths from AML are in adults. Increasing age is also a prognostic factor in AML. The disease has a much lower cure rate in older patients (5–15% over 60 years old) compared to younger patients (35–40% under 60 years old) [2], in part because the elderly are unable to tolerate intensive chemotherapy.

In contrast to breakthroughs made in treating other cancers, the progress of treatment in AML has been slow overall. The main challenge is that the biology of AML is enormously heterogeneous.

This is especially the case in adult AML compared to pediatric AML, as somatic gene mutations accumulate over time and therefore increase the complexity of disease classification and treatment. AML is currently classified into subtypes based on cellular lineage and morphology, cell-surface or cytoplasmic marker expression, chromosome abnormalities, and gene mutations, using the French-American-British (FAB) classification system [3] or the World Health Organization (WHO) classification system [4].

The identification of chromosome abnormalities by cytogenetic tests remains the most effective tool to classify patients into prognostic categories and guide therapy in clinic. The greatest contribution of cytogenetic abnormalities to guide the treatment of AML is in the case of acute promyelocytic leukemia (APL), M3 subtype of AML. APL is characterized by the t(15;17)q (22;12) cytogenetic abnormality, which gives rise to the promyelocytic leukemia-retinoic acid receptor alpha (PML-RARA) fusion protein [5]. The disease was found to respond to all-trans-retinoic acid (ATRA) and arsenic trioxide (ATO) [6, 7]: ATRA enables leukemic promyelocytes to differentiate into mature cells, and ATO accelerates the degradation of PML-RARA. This discovery improved the cure rate in APL from 30% to over 90%, and it marked the first molecularly targeted therapy and one of the greatest breakthroughs in AML therapeutic history [8].

AML patients can be categorized into three risk groups based on cytogenetics: (1) a favorable risk group with relatively good outcomes with chemotherapy; (2) an unfavorable risk group with much worse outcomes, who are usually considered for allogeneic stem cell transplantation; and (3) an intermediate risk group consisting of patients not in the favorable and unfavorable categories. While the favorable and unfavorable risk groups are well defined based on specific cytogenetic alterations, the definition of the intermediate risk group is sometimes unclear and discordant across the medical establishment. The United Kingdom Medical Research Council (MRC-C) prognostic classification system defines the intermediate risk group as a group of patients without identifiable cytogenetic abnormalities of favorable or unfavorable groups [9], whereas the European Leukemia Net (ELN-C) system incorporates common genetic mutation information (NPM1 and FLT3 ITD) to further stratify the intermediate group into intermediate-1 and intermediate-2 groups with higher and lower risks, respectively [2]. The fact that about 50% of AML patients are under the intermediate risk groups indicates the limitations of classifying AML based on cytogenetic alterations alone [10].

It has long been hoped that genetic mutations in AML can provide critical prognostic information to complement cytogenetics and help direct individualized therapy. Indeed, recurrent mutations of genes (e.g. FLT3, NPM1, CEBPA, KIT, DNMT3A, IDH1/2 and TET2) have been identified in AML, some of which were found to associate with patient outcome, and identification of these mutations has already been incorporated into the standard-of-care testing and classification system [11, 12]. Our understanding of the genomic and epigenomic landscape in AML has also been greatly improved in the last decade thanks to the development of next-generation sequencing techniques. In a recent study by the Cancer Genome Atlas Research Network [13], whole-genome (50 cases) and whole-exome sequencing (150 cases), along with RNA and microRNA sequencing and DNA-methylation analysis, were used to analyze the

genomes of 200 adult AML patients. The study revealed that AML genomes on average only have 13 mutations, which is fewer compared to other cancers. Furthermore, 5 of these 13 mutations are recurrent. The limited number of genetic mutations in contrast to the degree of heterogeneity observed clinically indicates the existence and importance of AML heterogeneity beyond genes.

Despite the extensive adoption of genomic approaches in cancer research, it is widely recognized that genomics alone is insufficient to provide an accurate picture of all cellular changes and dynamic states [14]. First, the same mRNA transcript often does not correspond to a single protein but to multiple protein counterparts, thanks to alternative splicing, protein cleavages, and post-translational modifications (PTM). In particular, PTMs (e.g. phosphorylation, acetylation, methylation, glycosylation, ubiquitination) play important roles in cellular processes by affecting the folding, location, and function of proteins. Proteins from the same mRNA transcript can have opposite effects on cellular processes with different PTMs, and it is currently not possible to predict the fate of PTMs from the protein sequence. Second, most cellular processes are executed and regulated by interactions between proteins and interactions between proteins and DNAs. An understanding of these interactions, which is unattainable via genomic approaches, is crucial for predicting cell behavior and discovering new drug targets. Moreover, the discovery of genetic mutations and abnormal gene expressions often does not offer an immediate therapeutic solution, as most drugs target proteins instead of genes.

Though nascent and over-shadowed by genomics in the research community, proteomics can complement the limitations of genomic approaches and advance the discovery of biomarkers and personalized treatments for AML. As the workhorses in cells, proteins can more accurately reflect the real dynamic changes in cellular processes, and offer insights into a heightened level of disease heterogeneity beyond the scope of genomics. In an analogy to screenwriting, genomics is a copy of a script, whereas proteomics is a movie produced from the script. With the same script, different actors, actresses and directors, stage settings and lighting effects will result in different productions. It is also extremely hard to judge whether the show will be a success based on the script alone, because execution matters and one can only be sure after seeing it in action. Therefore, proteomics can capture the real action in cells (e.g. the effects from cellular environment and the response by the cell) that are unforeseen by genomics.

The application potential of proteomics in AML is plenty. First, proteomics can be used to either establish new patient classification systems by itself or improve the current risk stratification system by complementing cytogenetics and genomics. Not all genetic mutations are equally important in driving the disease or in determining a patient's response to therapy. Some genetic mutations might not make a difference at the proteomic level, whereas some proteomic patterns and cell signaling behaviors might not manifest at the genetic level. The combination of proteomic and genomic approaches would be particularly beneficial for subclassifying patients that are currently lumped together in the intermediate risk group. Second, proteomics can be used as biomarkers to guide therapy. Certain protein expression and PTM levels could be effective indicators of whether a patient will develop chemoresistance and hence whether the patient should be referred to allogeneic stem cell transplantation. Moreover,

abnormal expression of proteins can potentially be molecularly targeted, creating more personalized therapy options for AML patients.

The workflow of a typical proteomic project in AML is shown in **Figure 1**. In this chapter, we focus on reviewing the main proteomic techniques and the various applications of proteomics in AML research, the topics of the next two sections. In the last section, we will discuss the main challenges and issues in AML proteomic research by covering topics related to sample collection considerations and proteomic data analysis techniques.

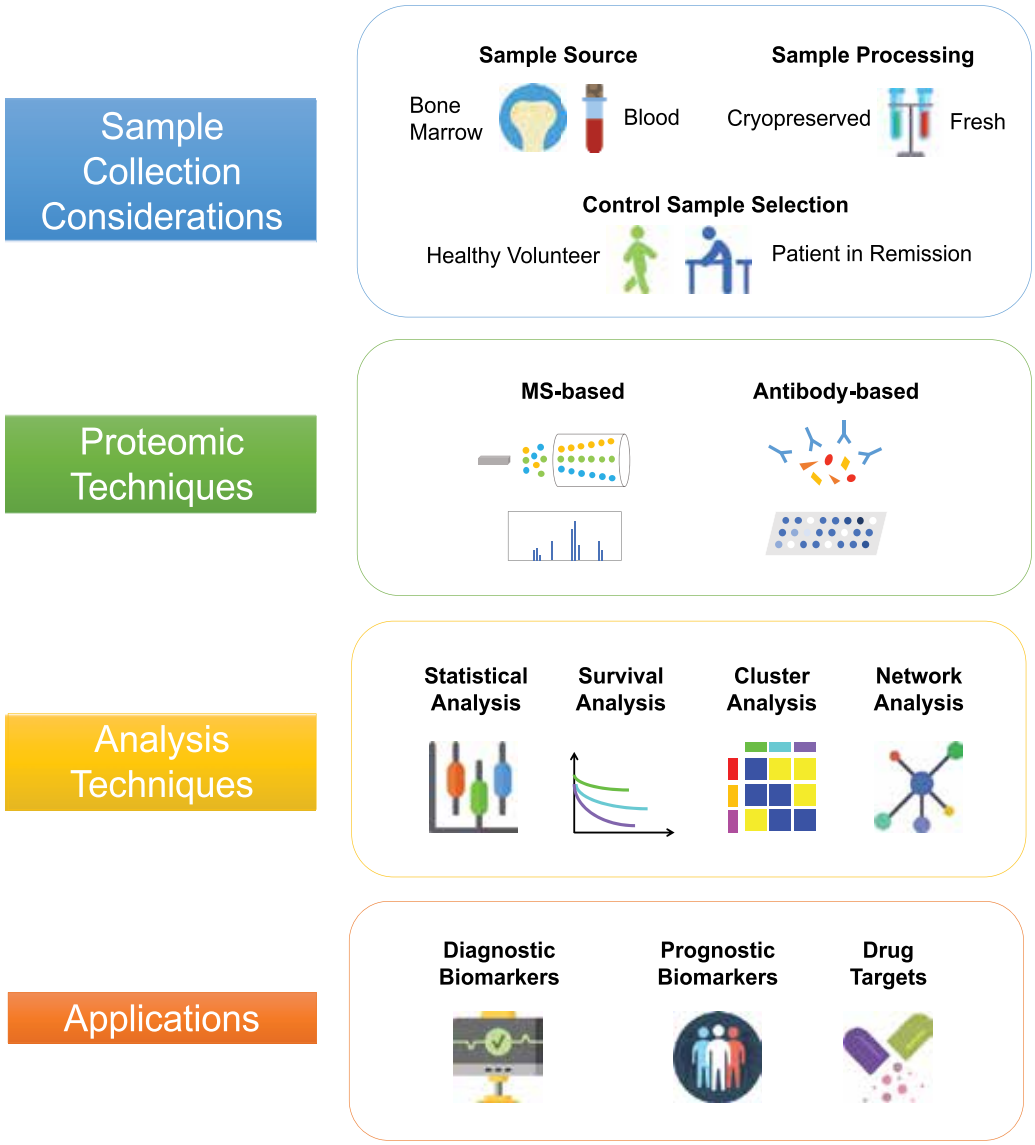

Figure 1. Typical workflow of a proteomic project in AML with methodology choices for each step.

http://dx.doi.org/10.5772/intechopen.70929

2. Overview of proteomic techniques

The development of proteomic techniques in the past 20 years has enabled many research studies to identify the roles of proteins and PTMs in biology and human diseases at a large scale. It has also inspired the Human Proteome Project [15], a global effort that aims to "generate the map of protein based molecular architecture of the human body and become a resource to help elucidate biological and molecular function and advance diagnosis and treatment of diseases". Current proteomic approaches can be divided into two sub-categories: mass spectrometry (MS)-based, and antibody-based. Here, we describe the fundamentals of each technique and their recent applications in AML.

2.1. MS-based methods

One intuitive way to identify a protein is by measuring its mass directly. MS is a widely-used analytical technique that ionizes a sample (solid, liquid, or gas) and measures the mass based on the mass-to-charge ratios of the ions. The ionization causes the molecules to break into charged fragments, which pass through an electric (e.g. time-of-flight (TOF)) or magnetic field that sorts ions by their mass-to-charge ratios. The relative abundance of ions detected as a function of the mass-to-charge ratio is usually presented in a mass spectrum for deciphering the identity of the molecule. MS is often used in tandem with liquid chromatography (termed LC-MS or LC/MS) which separates the liquid compounds chromatographically before passing them through the mass spectrometer.

When applying MS to detect proteins, one can take either a "top-down" or a "bottom-up" approach [16–18]. The "top-down" approach ionizes the intact protein directly, and is usually limited to low-throughput single protein studies. On the other hand, the "bottom-up" approach first digests the protein into peptides using enzymes such as trypsin, and then analyzes the peptides using tandem mass spectrometry. The "bottom-up" approaches using LC-MS are also referred to as "shotgun proteomics" [19]. The "bottom-up" approach is more widely adopted compared to the "top-down" approach in proteomic studies because it is much easier to handle small tryptic peptides and determine their masses with high accuracy than handling intact protein ions. However, the limited protein sequence coverage by peptides, loss of PTM information and redundant peptides of ambiguous origin are some of the disadvantages of "bottom-up" approaches. Notably, an intermediate approach, "middle-down", was proposed to break proteins into proteolytic peptides (size of 2–20 kDa) instead of small tryptic peptides (which is ~8–25 residues long) using proteases such as OmpT [20]. This hybrid approach potentially combines the benefits from the "top-down" and "bottom-up" approaches and overcomes their drawbacks.

Electrospray ionization (ESI) [21] and matrix-assisted laser desorption/ionization (MALDI) [22] are two primary methods for ionizing proteins and peptides. ESI generates ionized molecules by applying a high electric field and dispersing the liquid sample into an aerosol. In contrast, MALDI ionizes the sample by firing laser pulses at the sample mixed with an energy absorbing matrix. Both methods are considered to be "soft" ways of obtaining ions of large molecules with low fragmentation. The main advantage of ESI is that it produces multiply charged ions, extending

the mass detection range of the analyzer. MALDI, on the other hand, is advantageous for its robustness and high speed. ESI is frequently coupled with LC, whereas MALDI is most often used with TOF. A more recent method, Surface-enhanced laser desorption/ionization (SELDI) [23], was proposed as an alternative to MALDI. SELDI is similar to MALDI with the exception that the sample is bound to a surface in SELDI instead of being mixed with a matrix material. The SELDI surface allows for more retention of analytes and therefore is more suitable for detecting proteins in lower concentrations. SELDI is usually coupled with TOF, and it was shown that SELDI-TOF-MS can detect proteins from as little as 1 μL of serum or as few as 25–50 cells [24], which can be very beneficial when studying clinical samples.

To quantify the protein levels (or termed "quantitative proteomics"), there are three major groups of labeling methods that can be used in the proteomic workflow: label-free, stable isotope labeling, and multiple reaction monitoring [25]. By its name, label-free methods (e.g. spectral counting and peptide peak intensity measurement) do not use any isotope containing compound to bind to and label proteins [26]. Though easy to perform, inexpensive, high throughput and with a wider dynamic range, label-free methods are in general less accurate [27]. Stable isotope labeling approaches use differential stable isotopes to label and distinguish samples via either metabolic labeling or chemical labeling. One example of metabolic labeling approach is stable isotope labeling by amino acids (SILAC) [28], which feeds cells from different samples with heavy and light forms of arginine or lysine through the growth medium. SILAC generates precise quantitation of proteins, but can only be applied to living or metabolically active samples. An alternative method, "super-SILAC", was developed to extend SILAC to human tissue samples by using a mixture of SILAC-labeled cell lines as the internal standard [29]. A super-SILAC mix based on five AML cell lines (Molm-13, NB4, MV4-11, THP-1, and OCI-AML3) was recently established for quantifying patient AML cells [30].

While most MS-based methods profile proteins from cell lysates, mass cytometry is a fusion technology of MS and flow cytometry that can be used to measure protein levels in single cells [31]. Mass cytometry is also referred to as cytometry by time-of-flight (CyTOF), which is the current commercialized implementation. Mass cytometry overcomes the spectral overlap in flow cytometry by conjugating probes (often antibodies) with heavy-metal isotopes as expression reporters instead of fluorophores. The metal-conjugated antibodies, ionized and detected using the TOF mass spectrometer, greatly increase the number of parameters measureable in single cells due to their little signal overlap. Currently, mass cytometry can be used to detect up to 40 parameters per cell (up to 100 parameters theoretically), including protein levels, PTMs and proteolysis products. Mass cytometry was recently used in pediatric AML to profile both the surface markers and intracellular signaling proteins in single cells [32]. Notably, the study discovered that the surface phenotypes and their regulatory intracellular signaling phenotypes are decoupled in AML, rendering the surface markers unreliable for reporting signaling states. The study also identified a gene signature associated with the primitive signaling phenotype that is predictive of survival.

2.2. Antibody-based methods

The other group of methods for detecting and quantifying proteins is based on the use of antibodies. Antibodies can be engineered to specifically recognize not only proteins but also

their PTMs, which is very favorable for profiling kinases and signaling activities. Commonly used techniques such as western blot and enzyme-linked immunosorbent assay (ELISA) already use antibodies to measure protein expressions. However, these methods are low-throughput, and they are therefore unsuitable to profile a large number of proteins or samples in a timely fashion. Using microarray technologies, multiple types of high-throughput antibody-based methods were developed to enable profiling proteins at a much larger scale, including tissue microarrays (TMA) and protein microarrays. TMA is a proteomic technique in application to tissue samples [33]. TMA assembles up to 1000 tissue samples into one paraffin block to enable simultaneous evaluation of biomarkers. Since tissue samples are of more importance in solid tumors than in leukemia, we will focus the discussion on protein microarrays.

Based on the application purpose, protein microarrays can be divided into two categories: analytical protein arrays and functional protein arrays [34]. Functional protein arrays print a large number of individually purified proteins on an array to investigate their biochemical activities. The use of functional arrays is mostly in basic research, including identifying interactions between protein-protein, protein-DNA, protein-antibody, protein-lipid, protein-RNA, or protein-small molecules, and identifying substrates or enzymes for protein modifications. On the other hand, analytical protein arrays use well-characterized antibodies to measure the amounts of specific proteins in a large scale. These arrays are widely used in clinical research for biomarker discovery and protein expression profiling, and can be applied in disease diagnosis in clinic.

There are two types of analytical protein arrays: forward-phase protein array (FPPA) and reverse-phase protein array (RPPA) [35]. The major difference between FPPA and RPPA is whether antibodies or samples are immobilized. In FPPA, various antibodies are printed on a slide as bait molecules, where each spot on the array is one type of antibody. Each slide is then exposed to a single protein lysate (sample), and multiple protein expression levels are measured. The main advantage of FPPA is that a single slide can provide measurements of many proteins simultaneously. However, FPPA needs two highly specific antibodies (similar to "sandwich ELISA") for assaying each protein, and it also requires a higher amount of the protein lysate sample (which is often a luxury in clinical research). In contrast, RPPA immobilizes protein lysates, where each spot on the slide is a sample from a different source or condition. Each slide is then probed with one type of antibody and provides a read-out of the corresponding protein level across all printed samples, allowing for a direct comparison between samples. To profile multiple proteins, one can prepare a batch of identical slides printed with the same samples (which is straightforward to do), and process them in parallel, each slide with a unique type of antibody. RPPA is known to be highly sensitive and robust, and it is particularly advantageous for clinical applications because it requires lower amounts of samples. In the past decade, RPPA was used in multiple research studies to generate protein profiles and identify biomarkers in AML [36–41].

Compared to MS-based methods, antibody-based methods are less of a de novo discovery approach, and provides less coverage of the proteome. This is mainly because antibody-based methods only profile proteins that are known ahead of the experiment, and the coverage of these methods depend on the availability of specific antibodies. It is still an ongoing effort to generate antibodies that specifically recognize all protein isoforms present in the human

proteome. The Human Protein Atlas project, started in 2003, maps the expression and location of proteins in cells, normal tissues and cancers using an antibody-based approach. Its latest version (16th release) now includes more than 25,000 antibodies that about 86% of all human protein-coding genes [42, 43]. In addition, the quality of antibodies is key to the success of any antibody-based methods. Before printing an array, antibodies need to be validated to ensure that they are highly specific and do not cross-react with other proteins in the lysate. Otherwise, the accuracy of the profiling will be compromised by false signals. Antibodypedia (https://www.antibodypedia.com/), a public database containing validation data of more than one million antibodies, is a useful resource for antibody-based research [44].

3. Applications of proteomics in AML

3.1. Discovery of diagnostic biomarkers

One application of proteomics in AML is diagnostic marker discovery. Comparing the proteomes between AML and healthy samples or between AML and other leukemic subtypes can shed light on the unique disease mechanisms present in AML. Differential protein expression levels can potentially serve as biomarkers for the early detection of the disease and for assisting the current diagnostic system to distinguish AML patients from other leukemic subtypes (for example, acute lymphocytic leukemia (ALL) or myelodysplastic syndromes (MDS)) as well as to classify patients within AML. The identification of these differentially expressed proteins specific to AML or specific to certain AML subtype will provide a deeper understanding of its heterogeneous disease mechanism and facilitate development of personalized therapy.

Multiple studies have compared the proteomes between AML and normal healthy samples to look for AML-specific protein signatures. Using two-dimensional electrophoresis (2-DE) and MS, Kwak et al. identified 8 proteins that were differentially expressed between 12 AML patients and 12 healthy subjects, in which 5 proteins (α-2-HS-glycoprotein, complement-associated protein SP-40, RBP4 gene product, lipoprotein C-III, and an unknown protein) were down-regulated and 3 proteins (immunoglobulin heavy-chain variant, proteosome 26S ATPase subunit 1, and haptoglobin-1) were up-regulated in AML [45]. In another study using 2-DE and MALDI-TOF peptide mass fingerprinting analysis [46], seven proteins (alpha-enolase, RhoGDI2, annexin A10, catalase, peroxiredoxin 2, tromomyosin 3, and lipocortin 1 (annexin 1)) were found to have significantly altered expression in AML blast cells compared to normal mononuclear blood cells. Comparing the proteome of AML against that of normal white blood cells, 31 proteins (including myeloid-related protein 8 and 14, myosin light chain 2 and 3) with significant altered expression were identified [47].

Proteomic comparisons between AML and other leukemia-related diseases may reveal biomarkers to distinguish AML from similar diseases in clinic. Cui et al. identified 27 proteins with differential expression between AML and ALL, including myeloperoxidase [47]. Aiming to characterize the proteomic mechanism underlying MDS progression to AML, Braoudaki et al. identified MOES, ZRI and AIFM1 as potential biomarkers for AML using 2-DE and MALDI-TOF, since these proteins were found to be up-regulated in AML [48]. Foss et al. demonstrated that the use of

alignment-based label-free quantitation approaches in LC-MS/MS to distinguish AML from ALL and CD34+ cells from healthy donors [49]. Based on the same data generated in Foss et al.'s study, Elo et al. used a more advanced statistical method (reproducibility optimized test statistics (ROTS)) to identify biomarkers from the proteomic data and from the transcriptomic data. They found that the alignment-based proteomic method was able to generate novel and significant biomarkers that were not detected by the transcriptomic assay [50]. From the proteomic profiles of 151 AML bone marrow samples generated by SELDI-TOF-MS, Xu et al. developed a proteomic-based decision tree model to classify patients into APL, AML-granulocytic, AML-monocytic, ALL, and control (healthy volunteers) [51].

AML subtypes display unique proteomic patterns, which may present therapeutic opportunities for each of these subtypes. In a study of 38 AML-M1/M2 patients and 17 healthy volunteers [52], Luczak et al. demonstrated the use of 2-DE-MS to distinguish between M1 and M2 patients. They identified five proteins that were differentially accumulated between M1 and M2, in which Annexin III, L-plastin and 6-phosphogluconate dehydrogenase were found exclusively in M2. Comparing the protein expression levels across AML FAB classes, Cui et al. identified 23 proteins differentially expressed between the granulocytic lineage (M1, M2, M3) and monocytic lineage (M5), where they found 7 proteins up-regulated in both M2 and M3, and 15 proteins tightly associated with M3 (e.g. cathepsin G) [47]. In an RPPA study of 256 newly diagnosed AML patients [36], 24 proteins were found to significantly differ in expression between FAB subtypes out of 51 proteins that were tested. The proteins were found to belong to three clusters: (1) total and phosphorylated signal transduction proteins (KCA, PKCA.p, ERK2, AKT.p308, P38.p P70S6K, P70S6K.p, and Src.p527), with lower expression in myeloid subtypes (M0, M1, and M2); (2) PTEN and PTEN.p, with lower expression in M6 and M7; (3) apoptosis, cell cycle or differentiation regulating proteins and activated STAT proteins that have higher expression in myeloid subtypes.

Differences in proteomics (expression patterns, protein interaction pathways, and PTMs) were also found between cytogenetic abnormalities. In a study of 42 AML patients study using 2-DE MALDI-TOF-MS [53], Balkhi et al. showed that there were significant differences of protein expression levels, protein interaction networks and PTMs between cytogenetic groups. PTMs specific to cytogenetic abnormalities were identified, including a b-O-linked N-acetyl glucosamine (O-GlcNAc) of hnRNPH1 in patients with 11q23 translocation, an acetylation of calreticulin in patients with t(8;21), and methylation of hnRNPA2/B1 in patients with t(8;21) and inv(16). In an RPPA study, increased MET phosphorylation levels were found to associate with t(15;17) and t(8;21) cytogenetic subtypes [54].

Proteomic comparisons of relapsed against newly diagnosed patients or patients in remission can reveal biomarkers for early detection of relapse and non-invasive monitoring of minimal residual disease (MRD). Using MALDI-TOF-MS and high performance LC (HPLC)-ESI-MS/MS [55], Bai et al. identified 47 peptides that were differentially expressed between AML and healthy controls. In specific, they built a quality classifier model based on three peptides (ubiquitin-like modifier activating enzyme 1 (UBA1), isoform 1 of fibrinogen alpha chain precursor and platelet factor 4 (PF4)). UBA1 was up-regulated in newly diagnosed AML, decreased to normal level after complete remission, and then elevated again in relapse, whereas the other two peptides had

the opposite response. The three proteins were shown to correlate with patient outcome and can serve as biomarkers for monitoring MRD and detecting relapse. In another study, Pierce et al. performed RPPA on 511 AML patient samples, and found that the expression of protein transglutaminase 2 was higher at relapse compared to diagnosis [41].

Leukemic stem-like cells (LSC) are believed to play critical roles in patient chemoresistance, refractory and relapse. To investigate the biological differences between leukemic stem-like cells (LSC) and common myeloid progenitors (CMP), Kornblau et al. profiled the expression of 121 proteins in Bulk (CD3/CD19 depleted), CD34−, CD34+ (CMP), CD34+CD38+ and CD34 +CD38− (LSC) in AML patients using RPPA [40]. Significant differences in protein expression and protein network patterns were found between LSC and the rest of the cells, indicating unique AML biology existing in LSC. The differentially expressed proteins in LSC (e.g. Mcl1, cIAP, Survivin, and Bcl2) may present as therapeutic targets for selectively targeting LSC.

3.2. Discovery of prognostic factors

Proteomics enables discovery of abnormal expressions of proteins or PTMs that are predictive of patient outcome. Profiling protein expressions in 511 AML patients using RPPA [37], Kornblau et al. found that patients with high levels of FOXO3A phosphorylation have higher rates of primary resistance and shorter remission durations. The prognostic value of highly phosphorylated FOXO3A is independent of cytogenetics, since FOXO3A phosphorylation levels were not found to associate with karyotypes. In another study of the same patient cohort, the overexpression of FLI1 protein was identified as another adverse prognostic factor in AML [38]. In the study by Cui et al. [47], NM23-H1 was identified as a prognostic factor, since it is up-regulated in all FAB subtypes except M3a, a favorable prognosis subtype.

The prognostic protein signatures can potentially complement cytogenetics and genomics to build better classification systems. In a study of 54 AML samples using SELDI-TOF [56], Nicolas et al. showed that proteomic signatures can stratify patient outcome and complement cytogenetic classifications. Based on the proteomic profile, they grouped patients into two clusters and found significant differences in overall and event-free survival between the two clusters. The proteomic-defined clusters were also able to stratify the overall and event-free survival in specific cytogenetic categories: the intermediate risk group was divided into a group of patients with similar outcome to the favorable and a group with similar outcome to the unfavorable; the unfavorable group was divided into a group with similar outcome to the intermediate and a group of similar outcome to the unfavorable. In addition, they isolated a biomarker, S100A8, the expression of which is a predictor of poor survival.

The mutation of p53, resulting in p53 stabilization, is associated with adverse survival, though the mutation is observed in only 5–8% of newly diagnosed AML patients. A recent RPPA study showed that p53 stabilization also occurs in a significant portion of wild-type p53 patients, where the expression of the p53 negative regulator Mdm2 is elevated [39]. Furthermore, patients with overexpressed Mdm2 are subject to poor outcomes similar to patients with p53 mutants. This finding has significant clinical implications as it unveils the p53 dysfunction in wild-type p53 patients who are previously assumed to have intact p53 functions, and it highlights the value of proteomics to complement genetic testing for classifying patients and guiding treatments.

Recently, as part of the Dialog for Reverse Engineering Assessment and Methods (DREAM), a crowdsourcing effort was launched to build, compare and assess prediction algorithms for AML prognosis (DREAM 9 AML Outcome Prediction Challenge) [57]. Based on the data consisting of 40 clinical attributes and 231 RPPA measurements in 191 AML patients (the released training data), participants were asked to build models that predict response to therapy (sub-challenge 1), remission duration (such-challenge 2) and overall survival time (sub-challenge 3) in 100 AML patients (withheld as test data for model evaluation). As one of the conclusions, the study showed that the RPPA data substantially improved the top performing models' performance in predicting response to therapy in AML, illustrating the prognostic and predictive value of proteomics and the potential of combining proteomics with current prognostic factors for more accurate outcome assessment. In addition, the expression of PI3KCA was identified as a highly informative protein biomarker for predicting patient response to therapy.

3.3. Identification of target proteins

Proteomics can provide insights into the effects and mechanisms of genetic mutations and help identify novel drug targets associated with specific mutations. Transcription factor CCAAT enhancer binding protein α (C/EBPα) is an important regulator of the myeloid differentiation. Its mutant form, C/EBPα-p30, is present in about 9% of AML patients. Using 2-DE MALDI-TOF-MS, Geletu et al. identified Ubc9 (an E2-conjugating enzyme) as a target protein for C/EBPα-p30 [58]. The expression of Ubc9 was found to increase when inducing C/EBPα-p30, and the overexpression of Ubc9 was also observed in patients with C/EBPα-p30. In another study using 2-DE-MS proteomic screening, Pulikkan et al. uncovered the association of PIN1 overexpression with C/EBPα-p30 [59]. They then demonstrated that the elevated levels of PIN1 block granulocyte differentiation via c-Jun, and that the inhibition of PIN1 restores myeloid differentiation in primary AML blasts with C/EBPα mutation. This discovery suggests a potential treatment strategy of inhibiting PIN1 for AML patients with C/EBPα mutation.

As another example, RAS mutations occur in 10–25% of AML patients, however the mutation is not known to be prognostic. An RPPA study of 609 patients (11% with RAS mutation) showed that the RAS-Raf-MAP kinase and PI3K signaling pathways are up-regulated in patients with RAS mutation, which indicates RAS and PI3K signaling pathways as potential inhibitory targets for treating patients with RAS mutations [60].

3.4. Proteomics in AML cell lines

Due to the limited availability and difficult culturing conditions of primary patient cells, AML cell lines are often used to study disease mechanisms and biomarker discoveries. In these well-controlled and less heterogeneous experimental environment, one can compare the proteomic profiles between AML cell lines derived from different sources and with different mutations or cytogenetic abnormalities, and then extrapolate findings to the patient category of the cell line's origin. Cell lines are also easier to manipulate (for example, by up or down regulating certain proteins and by introducing mutations), and are therefore a great platform for studying the signaling networks and discovering target proteins.

Recently, Matondo et al. used large-scale quantitative SILAC-MS to identify proteins regulated by proteasome inhibition in two AML cell lines of different maturation stages: KG1a cells

(immature) and U937 cells (mature) [61]. From over 7000 proteins quantified in the two cell lines, the study identified novel regulation targets of the proteasome inhibition, including IL-32, apoptosis inducing factor SIVA, MORF family mortality factors, in addition to known regulation targets such as heat shock and cell cycle proteins. Using 2D-DIGE MALDI-TOF/MS, Hu et al. compared the proteomic profiles between leukemia cell lines, HL-60 (drug-sensitive) and HL-60/ADR (adriamycin-resistant) [62]. Sixteen differentially expressed proteins were identified, among which the up-regulation of nucleophosmin/B23 (NPM B23) and nucleolin C23 were validated in AML patient samples and may be indicators of drug resistance and predictors of prognosis. To investigate how AML exosomes affect the function of hematopoietic stem and progenitor cells (HSPC), Huan et al. compared proteomes of HSPCs treated with exosomes from AML cell line Molm-14 against proteomes of HSPCs treated with media (control) [63]. They identified 282 proteins that were differentially expressed between the two conditions, and the functional annotation of these proteins pinpointed candidate pathways that are involved in the exosome-mediated modulation of HSPC function.

Proteomics were used in multiple studies to investigate the effects and mechanisms of drugs in cell line models. Using SILAC-MS, Weber et al. quantified 10,975 distinct phosphorylation sites to characterize the phosphoproteomic changes in AML cell line KG1 upon pharmacological intervention from erlotinib and gefitinib [64]. They found that the cellular perturbation by the two drugs is rather specific, with fewer than 50 phosphorylation sites significantly changed upon treatment. Most of these phosphorylation changes occur in a network of tyrosine phosphorylated proteins, suggesting that the drugs interfere with leukemic activities by inhibiting signal transduction via Src family kinases and tyrosine kinases Btk and Syk. Proteomics can also be used to compare the mechanisms of two drugs. In a study using 2-DE MALDI-TOF/-TOF-MS, Buchi et al. quantified the protein expression levels in AML1/ETO positive leukemic cells under the treatment of azacitidine and under the treatment of decitabine [65]. The identification of differentially expressed proteins in both conditions as well as differentially expressed proteins exclusive to each condition provides insights into the biological effects of these DNMT inhibitors and the mechanism differences between them.

To develop drugs that specifically target leukemic cells, an understanding of the surface proteomes in cell lines is crucial. Using MALDI-TOF/TOF, Strassberger et al. generated surface proteomes for four AML cell lines (HL60, NB4, PLB985, THP1) [66]. Comparing the AML surface proteomes to that of granulocytes from normal human peripheral blood, they identified multiple proteins that were up-regulated in AML cell lines, including CD33, CD166, integrin alpha-4. An antibody-drug conjugate was then developed using a human monoclonal antibody targeting CD166 and a duocarmycin derivative as the cytotoxic agent, which was shown to be able to kill AML cells in vitro. The study serves as a good example and a basis for developing anti-AML therapeutic strategies using knowledge from cell surface proteomes.

4. Challenges and future directions

Though proteomic technologies are advancing rapidly, a few challenges and issues remain. Therefore, it is worth discussing these challenges as well as the future directions to solve them.

One issue is the choice of control samples. There lacks a consensus as to what samples should serve as the control for AML samples to be compared to. Often, samples from healthy subjects are used as control to represent the normal biology in hematopoiesis, yet samples from patients under complete remission could also make meaningful controls. Another question to ask is what specific samples from healthy subjects should be used: for example, should the samples be derived from healthy bone marrows or peripheral blood; should the samples come from cryopreserved cells or fresh lysates. Though most studies found similar protein expression patterns between bone marrow derived and blood derived samples in AML [36, 48], a comprehensive comparison is yet to be done in a large cohort of healthy subjects. Recently, the influence of freezing on proteomes in AML cells was reported [67], underscoring the importance of establishing more standard sample collection and preservation procedures. In addition, the number of control samples included in the studies is usually small, which makes it hard to deduce statistically valid claims and does not account for the full degree of heterogeneity in healthy individuals.

One challenge facing clinical research is the scarcity of primary patient samples. Most studies profiled proteomes in fewer than a hundred patients, and in some cases fewer than five patients were used. Considering the extreme heterogeneous biology present in AML, the proteomic patterns and biomarkers discovered in a small group of patients may not generalize to the whole AML population. Due to this incomprehensive representation of the AML population, the classification and prognostic power of proteomics will also be limited by drawing conclusions from few clinical samples. Given access to a large cohort of patients, one potential solution is to use peripheral blood samples instead of bone marrow samples. The proteome of blood samples was found to be similar to the proteome of bone marrow samples in multiple studies [36, 48], indicating that blood samples may be a substitute for bone marrow samples in proteomic research. Since obtaining blood samples is less invasive and much more convenient than obtaining bone marrow aspirates, the use of blood samples may grant researchers access to proteomic profiles at more time points (e.g. at diagnosis, through treatment and remission). For this approach to work, more comprehensive comparisons of the proteomes between blood and bone marrow in both AML and healthy subjects need to be carried out. Another potential remedy for the sample availability problem is to openly share data sets generated from quantitative proteomics through common platforms. More statistical power can be achieved when merging findings from multiple datasets of different sources for example using meta-analysis. This approach will greatly benefit from standardizing the choice of control samples and data processing procedures across different studies.

Due to its convenience and almost unlimited supply, cell lines are commonly used as a substitute for primary patient samples for discovering new biomarkers and therapeutic targets, screening for new drugs and investigating therapeutic effects and resistance. However, cell lines may not provide a truthful representation of the biology in AML patients, as cell lines adapt to the culture conditions and selection pressure. The validity of the cell line model is further compromised by the heterogeneity of AML biology. Even if a cell line does preserve the biology of its origin (which is unlikely), a cell line at most represents a tiny fraction of the AML population. To make cell lines more relevant, comprehensive proteomic profiles of cell lines

and primary patients are needed to investigate the degree of biological changes present in cell lines and to match cell lines to specific patient subpopulations. The hope is that cell lines may preserve the biology of their origins in some pathways, and by matching cell lines to specific patient categories we can utilize cell lines to personize treatments for their corresponding patient subpopulations.

To realize the full potential of proteomics in both research and clinic, more advanced computational techniques should be adopted or developed in AML proteomics research. When analyzing proteomic profiles, most studies use standard statistical tests to compile a list of differentially expressed proteins, and some would carry out tests to correlate the protein expression patterns with other clinical attributes and genetic mutations. While these tests are necessary, few studies take the leap to generate pathway level insights by examining the protein expressions in the context of protein interactions. Network-based approaches can be very useful in this regard to organize and visualize protein expressions in protein networks [39], using protein interaction information from public databases (e.g. string [68]) or from graphical reconstruction models. Insights into abnormal pathway regulation beyond the identification of abnormal expression in single proteins can open the door for new drug targets. Another challenge on the computational side is the increasing dimensionality of proteomic data thanks to the improvements in throughput and coverage of proteomic experimental techniques. In this case, more powerful clustering [69, 70] and dimension reduction techniques [71], as well as interactive visualization tools [72], can help researchers to best benefit from this increase in data size and empower then to make data-driven hypotheses and discoveries. Crowdsourcing competitions have also proved to be an effective way to encourage innovative solutions for these challenging computational issues [57, 73].

In summary, proteomics in AML is enabling the identification of new biomarkers and improving the classification of patients. Moreover, new experimental protocols and data analysis methods and tools are emerging to capitalize on the richness of the personalized data from the proteomic screens. Together these technological advances can provide new insight into the heterogeneities and hallmarks of AML.

Author details

Chenyue W. Hu* and Amina A. Qutub

*Address all correspondence to: wendyhu001@gmail.com

Department of Bioengineering, Rice University, Houston, TX, USA

References

[1] Döhner H, Weisdorf DJ, Bloomfield CD. Acute myeloid leukemia. The New England Journal of Medicine. 2015;**373**(12):1136-1152

[2] Döhner H, Estey EH, Amadori S, Appelbaum FR, Büchner T, Burnett AK, et al. Diagnosis and management of acute myeloid leukemia in adults: Recommendations from an international expert panel, on behalf of the European Leukemia Net. Blood. 2010;**115**(3):453-474

[3] Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DA, Gralnick HR, et al. Proposed revised criteria for the classification of acute myeloid leukemia. A report of the French-American-British Cooperative Group. Annals of Internal Medicine. 1985;**103**(4):620-625

[4] Vardiman JW, Thiele J, Arber DA, Brunning RD, Borowitz MJ, Porwit A, et al. The 2008 revision of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: rationale and important changes. Blood. 2009;**114**(5):937-951

[5] Raelson JV, Nervi C, Rosenauer A, Benedetti L, Monczak Y, Pearson M, et al. The PML/RAR alpha oncoprotein is a direct molecular target of retinoic acid in acute promyelocytic leukemia cells. Blood. 1996;**88**(8):2826-2832

[6] Shen Z-X, Chen G-Q, Ni J-H, Li X-S, Xiong S-M, Qiu Q-Y, et al. Use of arsenic trioxide (As_2O_3) in the treatment of acute promyelocytic leukemia (APL): II. Clinical efficacy and pharmacokinetics in relapsed patients. Blood. 1997;**89**(9):3354-3360

[7] Niu C, Yan H, Yu T, Sun H-P, Liu J-X, Li X-S, et al. Studies on treatment of acute promyelocytic leukemia with arsenic trioxide: Remission induction, follow-up, and molecular monitoring in 11 newly diagnosed and 47 relapsed acute promyelocytic leukemia patients. Blood. 1999;**94**(10):3315-3324

[8] Kadia TM, Ravandi F, Cortes J, Kantarjian H. Toward individualized therapy in acute myeloid leukemia: A contemporary review. JAMA Oncology. 2015;**1**(6):820-828

[9] Grimwade D, Hills RK, Moorman AV, Walker H, Chatters S, Goldstone AH, et al. Refinement of cytogenetic classification in acute myeloid leukemia: Determination of prognostic significance of rare recurring chromosomal abnormalities amongst 5,876 younger adult patients treated in the UK Medical Research Council trials. Blood. 2010;**116**:354-365

[10] Grimwade D, Walker H, Oliver F, Wheatley K, Harrison C, Harrison G, et al. The importance of diagnostic cytogenetics on outcome in AML: Analysis of 1612 patients entered into the MRC AML 10 trial. Blood. 1998;**92**(7):2322-2333

[11] Shen Y, Zhu Y-M, Fan X, Shi J-Y, Wang Q-R, Yan X-J, et al. Gene mutation patterns and their prognostic impact in a cohort of 1185 patients with acute myeloid leukemia. Blood. 2011;**118**(20):5593-5603

[12] Patel JP, Gönen M, Figueroa ME, Fernandez H, Sun Z, Racevskis J, et al. Prognostic relevance of integrated genetic profiling in acute myeloid leukemia. The New England Journal of Medicine. 2012;**366**(12):1079-1089

[13] Network CGAR, others. Genomic and epigenomic landscapes of adult de novo acute myeloid leukemia. The New England Journal of Medicine. 2013;**2013**(368):2059-2074

[14] Kanaujiya JK, Lochab S, Pal P, Christopeit M, Singh SM, Sanyal S, et al. Proteomic approaches in myeloid leukemia. Electrophoresis. 2011;**32**(3–4):357-367

[15] Legrain P, Aebersold R, Archakov A, Bairoch A, Bala K, Beretta L, et al. The human proteome project: Current state and future direction. Molecular & Cellular Proteomics. 2011;**10**(7):M111.009993

[16] Chait BT. Mass spectrometry: Bottom-up or top-down? Science. 2006;**314**(5796):65-66

[17] Yates JR, Ruse CI, Nakorchevsky A. Proteomics by mass spectrometry: Approaches, advances, and applications. Annual Review of Biomedical Engineering. 2009;**11**:49-79

[18] Chait BT. Mass spectrometry in the postgenomic era. Annual Review of Biochemistry. 2011;**80**:239-246

[19] Wolters DA, Washburn MP, Yates JR. An automated multidimensional protein identification technology for shotgun proteomics. Analytical Chemistry. 2001;**73**(23):5683-5690

[20] Wu C, Tran JC, Zamdborg L, Durbin KR, Li M, Ahlf DR, et al. A protease for 'middle-down' proteomics. Nature Methods. 2012;**9**(8):822-824

[21] Whitehouse CM, Dreyer RN, Yamashita M, Fenn JB. Electrospray ionization for mass-spectrometry of large biomolecules. Science. 1989;**246**(4926):64-71

[22] Hillenkamp F, Karas M, Beavis RC, Chait BT. Matrix-assisted laser desorption/ionization mass spectrometry of biopolymers. Analytical Chemistry. 1991;**63**(24):1193A-1203A

[23] Issaq HJ, Veenstra TD, Conrads TP, Felschow D. The SELDI-TOF MS approach to proteomics: Protein profiling and biomarker identification. Biochemical and Biophysical Research Communications. 2002;**292**(3):587-592

[24] Issaq HJ, Conrads TP, Prieto DA, Tirumalai R, Veenstra TD. SELDI-TOF MS for diagnostic proteomics. Analytical Chemistry. 2003;**75**(7):148A-155A

[25] Wasinger VC, Zeng M, Yau Y. Current status and advances in quantitative proteomic mass spectrometry. International Journal of Proteomics. 2013;**2013**:180605

[26] Zhu W, Smith JW, Huang C-M. Mass spectrometry-based label-free quantitative proteomics. BioMed Research International. 2009;**2010**:840518

[27] Bantscheff M, Schirle M, Sweetman G, Rick J, Kuster B. Quantitative mass spectrometry in proteomics: A critical review. Analytical and Bioanalytical Chemistry. 2007;**389**(4): 1017-1031

[28] Ong S-E, Blagoev B, Kratchmarova I, Kristensen DB, Steen H, Pandey A, et al. Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. Molecular & Cellular Proteomics. 2002;**1**(5):376-386

[29] Geiger T, Cox J, Ostasiewicz P, Wisniewski JR, Mann M. Super-SILAC mix for quantitative proteomics of human tumor tissue. Nature Methods. 2010;**7**(5):383-385

[30] Aasebø E, Vaudel M, Mjaavatten O, Gausdal G, Burgh A, Gjertsen BT, et al. Performance of super-SILAC based quantitative proteomics for comparison of different acute myeloid leukemia (AML) cell lines. Proteomics. 2014;**14**(17–18):1971-1976

[31] Bandura DR, Baranov VI, Ornatsky OI, Antonov A, Kinach R, Lou X, et al. Mass cytometry: Technique for real time single cell multitarget immunoassay based on inductively coupled plasma time-of-flight mass spectrometry. Analytical Chemistry. 2009;**81**(16):6813-6822

[32] Levine JH, Simonds EF, Bendall SC, Davis KL, El-ad DA, Tadmor MD, et al. Data-driven phenotypic dissection of AML reveals progenitor-like cells that correlate with prognosis. Cell. 2015;**162**(1):184-197

[33] Uhlen M, Ponten F. Antibody-based proteomics for human tissue profiling. Molecular & Cellular Proteomics. 2005;**4**(4):384-393

[34] Hall DA, Ptacek J, Snyder M. Protein microarray technology. Mechanisms of Ageing and Development. 2007;**128**(1):161-167

[35] Boellner S, Becker K-F. Reverse phase protein arrays—Quantitative assessment of multiple biomarkers in biopsies for clinical use. Microarrays. 2015;**4**(2):98-114

[36] Kornblau SM, Tibes R, Qiu YH, Chen W, Kantarjian HM, Andreeff M, et al. Functional proteomic profiling of AML predicts response and survival. Blood. 2009;**113**(1):154-164

[37] Kornblau SM, Singh N, Qiu Y, Chen W, Zhang N, Coombes KR. Highly phosphorylated FOXO3A is an adverse prognostic factor in acute myeloid leukemia. Clinical Cancer Research. 2010;**16**(6):1865-1874

[38] Kornblau SM, Qiu YH, Zhang N, Singh N, Faderl S, Ferrajoli A, et al. Abnormal expression of FLI1 protein is an adverse prognostic factor in acute myeloid leukemia. Blood. 2011;**118**(20):5604-5612

[39] Quintas-Cardama A, Hu C, Qutub A, Qiu YH, Zhang X, Post SM, et al. p53 pathway dysfunction is highly prevalent in acute myeloid leukemia independent of TP53 mutational status. Leukemia. 2017;**31**(6):1296-1305

[40] Kornblau SM, Qutub A, Yao H, York H, Qiu YH, Graber D, et al. Proteomic profiling identifies distinct protein patterns in acute myelogenous leukemia CD34+ CD38-stem-like cells. PLoS One. 2013;**8**(10):e78453

[41] Pierce A, Whetton AD, Meyer S, Ravandi-Kashani F, Borthakur G, Coombes KR, et al. Transglutaminase 2 expression in acute myeloid leukemia: Association with adhesion molecule expression and leukemic blast motility. Proteomics. 2013;**13**(14):2216-2224

[42] Uhlén M, Björling E, Agaton C, Szigyarto CA-K, Amini B, Andersen E, et al. A human protein atlas for normal and cancer tissues based on antibody proteomics. Molecular & Cellular Proteomics. 2005;**4**(12):1920-1932

[43] Uhlén M, Fagerberg L, Hallström BM, Lindskog C, Oksvold P, Mardinoglu A, et al. Tissue-based map of the human proteome. Science. 2015;**347**(6220):1260419

[44] Björling E, Uhlén M. Antibodypedia, a portal for sharing antibody and antigen validation data. Molecular & Cellular Proteomics. 2008;**7**(10):2028-2037

[45] Kwak J-Y, Ma T-Z, Yoo M-J, Choi BH, Kim H-G, Kim S-R, et al. The comparative analysis of serum proteomes for the discovery of biomarkers for acute myeloid leukemia. Experimental Hematology. 2004;**32**(9):836-842

[46] López-Pedrera C, Villalba JM, Siendones E, Barbarroja N, Gómez-Díaz C, Rodríguez-Ariza A, et al. Proteomic analysis of acute myeloid leukemia: Identification of potential early biomarkers and therapeutic targets. Proteomics. 2006;**6**(S1):S293-S299

[47] Cui J-W, Wang J, He K, Jin B-F, Wang H-X, Li W, et al. Proteomic analysis of human acute leukemia cells. Clinical Cancer Research. 2004;**10**(20):6887-6896

[48] Braoudaki M, Tzortzatou-Stathopoulou F, Anagnostopoulos AK, Papathanassiou C, Vougas K, Karamolegou K, et al. Proteomic analysis of childhood de novo acute myeloid leukemia and myelodysplastic syndrome/AML: Correlation to molecular and cytogenetic analyses. Amino Acids. 2011;**40**(3):943-951

[49] Foss EJ, Radulovic D, Stirewalt DL, Radich J, Sala-Torra O, Pogosova-Agadjanyan EL, et al. Proteomic classification of acute leukemias by alignment-based quantitation of LC–MS/MS data sets. Journal of Proteome Research. 2012;**11**(10):5005-5010

[50] Elo LL, Karjalainen R, Öhman T, Hintsanen P, Nyman TA, Heckman CA, et al. Statistical detection of quantitative protein biomarkers provides insights into signaling networks deregulated in acute myeloid leukemia. Proteomics. 2014;**14**(21–22):2443-2453

[51] Xu Y, Zhuo J, Duan Y, Shi B, Chen X, Zhang X, et al. Construction of protein profile classification model and screening of proteomic signature of acute leukemia. International Journal of Clinical and Experimental Pathology. 2014;**7**(9):5569

[52] Luczak M, Kaźmierczak M, Handschuh L, Lewandowski K, Komarnicki M, Figlerowicz M. Comparative proteome analysis of acute myeloid leukemia with and without maturation. Journal of Proteomics. 2012;**75**(18):5734-5748

[53] Balkhi MY, Trivedi AK, Geletu M, Christopeit M, Bohlander SK, Behre HM, et al. Proteomics of acute myeloid leukaemia: Cytogenetic risk groups differ specifically in their proteome, interactome and post-translational protein modifications. Oncogene. 2006;**25**(53):7041

[54] McGee SF, Kornblau SM, Qiu Y, Look AT, Zhang N, Yoo SY, et al. Biological properties of ligand-dependent activation of the MET receptor kinase in acute myeloid leukemia. Leukemia. 2015;**29**(5):1218

[55] Bai J, He A, Zhang W, Huang C, Yang J, Yang Y, et al. Potential biomarkers for adult acute myeloid leukemia minimal residual disease assessment searched by serum peptidome profiling. Proteome Science. 2013;**11**(1):39

[56] Nicolas E, Ramus C, Berthier S, Arlotto M, Bouamrani A, Lefebvre C, et al. Expression of S100A8 in leukemic cells predicts poor survival in de novo AML patients. Leukemia. 2011;**25**(1):57

[57] Noren DP, Long BL, Norel R, Rrhissorrakrai K, Hess K, Hu CW, et al. A crowdsourcing approach to developing and assessing prediction algorithms for AML prognosis. PLoS Computational Biology. 2016;**12**(6):e1004890

[58] Gelutu M, Balkhi MY, Zada AP, Christopeit M, Pulikkan J, Trivedi A, et al. Target proteins of C/EBPαp30 in AML: C/EBPαp30 enhances sumoylation of C/EBPαp42 via up-regulation of Ubc9. Blood. 2007;**110**(9):3301-3309

[59] Pulikkan JA, Dengler V, Zada AAP, Kawasaki A, Geletu M, Pasalic Z, et al. Elevated PIN1 expression by C/EBPα-p30 blocks C/EBPα induced granulocytic differentiation via c-Jun in AML. Leukemia: Official Journal of the Leukemia Society of America, Leukemia Research Fund, UK. 2010;**24**(5):914

[60] Kadia TM, Kantarjian H, Kornblau S, Borthakur G, Faderl S, Freireich EJ, et al. Clinical and proteomic characterization of acute myeloid leukemia with mutated RAS. Cancer. 2012;**118**(22):5550-5559

[61] Matondo M, Marcellin M, Chaoui K, Bousquet-Dubouch M-P, Gonzalez-de-Peredo A, Monsarrat B, et al. Determination of differentially regulated proteins upon proteasome inhibition in AML cell lines by the combination of large-scale and targeted quantitative proteomics. Proteomics. 2017;**17**(7):1600089

[62] Hu J, Lin M, Liu T, Li J, Chen B, Chen Y. DIGE-based proteomic analysis identifies nucleophosmin/B23 and nucleolin C23 as over-expressed proteins in relapsed/refractory acute leukemia. Leukemia Research. 2011;**35**(8):1087-1092

[63] Huan J, Hornick NI, Goloviznina NA, Kamimae-Lanning AN, David LL, Wilmarth PA, et al. Coordinate regulation of residual bone marrow function by paracrine trafficking of AML exosomes. Leukemia. 2015;**29**(12):2285

[64] Weber C, Schreiber TB, Daub H. Dual phosphoproteomics and chemical proteomics analysis of erlotinib and gefitinib interference in acute myeloid leukemia cells. Journal of Proteomics. 2012;**75**(4):1343-1356

[65] Buchi F, Spinelli E, Masala E, Gozzini A, Sanna A, Bosi A, et al. Proteomic analysis identifies differentially expressed proteins in AML1/ETO acute myeloid leukemia cells treated with DNMT inhibitors azacitidine and decitabine. Leukemia Research. 2012;**36**(5):607-618

[66] Strassberger V, Gutbrodt KL, Krall N, Roesli C, Takizawa H, Manz MG, et al. A comprehensive surface proteome analysis of myeloid leukemia cell lines for therapeutic antibody development. Journal of Proteomics. 2014;**99**:138-151

[67] Aasebø E, Mjaavatten O, Vaudel M, Farag Y, Selheim F, Berven F, et al. Freezing effects on the acute myeloid leukemia cell proteome and phosphoproteome revealed using optimal quantitative workflows. Journal of Proteomics. 2016;**145**:214-225

[68] Franceschini A, Szklarczyk D, Frankild S, Kuhn M, Simonovic M, Roth A, et al. STRING v9. 1: Protein-protein interaction networks, with increased coverage and integration. Nucleic Acids Research. 2012;**41**(D1):D808-D815

[69] Hu CW, Kornblau SM, Slater JH, Qutub AA. Progeny clustering: A method to identify biological phenotypes. Scientific Reports. 2015;**5**:12894

[70] Hu CW, Li H, Qutub AA. Shrinkage Clustering: A fast and size-constrained algorithm for biomedical applications. In 17th International Workshop on Algorithms in Bioinformatics. 2017

[71] van der Maaten L, Hinton G. Visualizing data using t-SNE. Journal of Machine Learning Research. 2008;**9**(Nov):2579-2605

[72] Hu C, Bisberg AJ, Qutub AA. Biowheel: Interactive visualization and exploration of biomedical data. bioRxiv. 2017;099739

[73] Hill SM, Heiser LM, Cokelaer T, Unger M, Nesser NK, Carlin DE, et al. Inferring causal molecular networks: Empirical assessment through a community-based effort. Nature Methods. 2016;**13**(4):310

Section 3

Genetic and Epigenetic Regulations

Chapter 5

Genomics of Acute Myeloid Leukemia

Zeeshan Ansar Ahmed, Imran Ahmed Siddqui and Sadia Sultan

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.72757

Abstract

Acute myelogenous leukemia (AML) is a clonal, malignant disease of hematopoietic tissue that is characterized by accumulation of abnormal (leukemic) blast cells, principally in the bone marrow. Representation of these genetic mutations and the involvement patterns seems to follow specific and temporally ordered fluctuating manners. Somatic mutations in these genes are represented as a variety of recurrent chromosomal abnormalities, e.g., t (8;21), t(15;17), etc., or by the presence of prognostic markers, e.g., FLT3, MLL, NPM1 and CEBPA as well as encoding epigenetic modifiers, such as DNMT3A, ASXL1, TET2, IDH1, and IDH2, are commonly acquired early and are present in the founding clone. The same genes are frequently found to be mutated in elderly individuals along with clonal expansion of hematopoiesis that confers an increased risk for the development of hematologic cancers. Furthermore, such genomic changes may persist after therapy, lead to clonal expansion during hematologic remission, and eventually lead to relapsed disease. Majority of genetic data are now being used to classification, risk stratification, and clinical care of patients. The unprecedented molecular characterization provided by advanced and deeply sensitized molecular assays like next-generation sequencing (NGS) offers the potential for an individualized approach to treatment in AML, bringing us one step closer to personalized medicine.

Keywords: acute myeloid leukemia, mutation, Fms-like tyrosine kinase 3 receptor, next-generation sequencing, targeted AML therapy

1. Introduction

The acute myeloid leukemia (AML) is a malignant tumor of hematopoietic precursor cells of non-lymphoid lineage, arising in the bone marrow. It is diagnosed on the basis of clinical features, peripheral and bone marrow morphology, cytochemical stains, immunophenotyping, and cytogenetic analysis [1].

Novel molecular markers of prognostic and more importantly of predictive significance have been identified in different leukemias. The link between the leukemogenic importance of these markers and their role as potential targets for novel drugs has started to contribute to the stepwise replacement of risk adapted by treatment strategies, e.g., imatinib in chronic myeloid leukemia (CML) and all-trans-retinoic acid (ATRA) in acute promyelocytic leukemia (APL).

Over the past few decades, it has become clear that a significant proportion of cases of AML are characterized by at least one of a varieties of recurrent chromosomal abnormalities, e.g., t (8;21), t(15;17), etc., or by the presence of prognostic markers, e.g., FMS-like tyrosine kinase 3 (FLT3), multilineage leukemia (MLL), nucleophosmin 1 (NPM1) and CCAAT/enhancer-binding protein-α (CEBPA). A key challenge for the future is to use information gained from cytogenetic analysis in conjunction with molecular diagnosis and gene expression profiling to achieve greater consensus in the risk group assignment of AML, and risk adapted therapy.

FLT 3 is widely known as FLT3 is a class III tyrosine kinase receptor [2]. Its structure consists of an extracellular ligand-binding domain, a single transmembrane domain, and a cytoplasmic region consisting of juxtamembrane domain and kinase domain interrupted by kinase insert [2, 3]. FLT3 is normally expressed on hematopoietic progenitor stem cells (HPSCs) where it plays an important role in survival and proliferation of stem cells. Its expression is lost with HPSC differentiation [2].

FLT3 mutations have been detected in one-third of AML patients and a small number of ALL patients [2].The mutations most commonly involve internal tandem duplications (ITDs) of the juxtamembrane domain (JM) (detected in 23% of AML patients and <1% of ALL patients) and point mutations in the activation loop of tyrosine kinase domain [2–4]. It has been detected in 7–12% of AML patients and about 3% of ALL patients [4, 5].

Prevalence of FLT3-ITD is according to age, i.e., rare in infants. FLT3 positivity was reported to be 5–10% in patients younger than 10 years, but it rose to 35% in middle-aged patients. Patients with this mutation usually present with increased white blood counts (WBC) and have normal cytogenetics [6]. In literature, there is no marked difference in complete response (CR) between FLT3 positive and FLT 3 negative patients, but relapse rate and overall survival(OS) are lower in FLT3-ITD-positive patients specially in younger than 60 years [7].

Further studies have confirmed that FLT3-ITD is not only inversely correlated with relapse but also associated with decreased overall survival [8–10]. In Kottaridis study, the prevalence of FLT3ITD was 27%. In same study, this mutation was strongly associated with hyperleukocytosis and normal cytogenetics. In literature, on multivariate analysis this mutation has the strongest correlation with decrease DFS [10].

2. Molecular basis and gene expression in acute myeloid leukemia

2.1. Mutations related to pathogenesis

Mutations in AML are different according to the age of patient. Balanced translocations are common in children and adolescents, while in elderly complex karyotypes are common [11]. To date, more than 80 mutations are identified. These rearrangements act as driver mutations to

initiate leukemic phase. Chromosomal derangement leads to disruption of transcription factor that controls directly hematopoiesis. Example is RARα that is formed by realignment of 17q21 [12]. Besides fusion genes other chromosomal abnormalities are also important in pathogenesis of AML. These are divided into three groups. (1) mutations abnormally controlled transcription factors that are helpful in hematopoieses, (2) mutations related to certain receptors, i.e., tyrosine kinase receptors, and (3) mutations involving the gene that encodes nucleophosmin [13].

2.2. Abrasions in gene associated with transcription

Hematopoiesis is regulated by various transcriptions factors which encodes with genes. These genes can get mutation which leads to inactivated or dominated in regulation of hematopoietic functions. Indeed, AML1mutations are detected in up to 25% of M0 cases and are frequently biallelic [14]. The CEBPA, which plays an important role in granulopoiesis, also is a relatively common target in AML, being potentially deregulated by the AML1-ETO and promyelocytic leukemia-retinoic acid receptor alpha (PML-RARα) oncoproteins. Furthermore, mutations involving CEBPA are present in approximately 10% of cases of AML [15].

2.3. Karyotypic abnormalities in AML

Evaluation of karyotypic abnormalities has prognostic and therapeutic implications. Detection of t(15:17) by cytogenetic analysis shows favorable treatment response by ATRA. Similarly, t(9:22) is not responded to conventional chemotherapy but showed good results to imatinib. Approximately 60% of newly diagnosed cases with less than 20% blasts in the bone marrow have abnormal karyotype. Prognostically, t(15:17),inv(16), and t(8:21) showed relatively better prognosis in comparison to monosomies 5 or 7 [16].

2.4. Chromosomal aberration detection by FISH

The absence of fusion genes in good prognostic group necessitates evaluation by FISH to accurately define prognosis. This can be exemplified by complex pattern of losses and loss of chromosome 5 or 7. Other than FISH, southern blot analysis can be used for chromosomal abnormality evaluation. However, it is more laborious than real-time PCR but more informative in certain circumstances, i.e., MLL gene rearrangement [17]. This approach can be used in the future for minimal residual disease assessment in AML.

2.5. Predictive mutations in leukemogenesis

The most notable is FLT3-ITD, which is associated with decreased duration of remission. However, its impact on survival after post bone marrow transplant in the first CR is not clearly documented [18]. The presence of EVI1 in the absence of chromosome 3q abnormalities depicts poor survival. Mutations that show good response to conventional chemotherapy and do not need bone marrow transplant in the first CR are the presence of NPM1 or CEBPA mutation without concomitant FLT3-ITD mutation [19]. In the era of molecular medicine, new tests at molecular levels are being under process. New molecularly targeted agents are underway on the basis of these special tests. Examples of these are FLT3 inhibitors that can modify outcome of poor prognostic group.

2.6. Mutations in AML

In previous few decades, there is a great insight in biology of this disease that leads to risk-adapted treatment approaches. With better understanding of the disease, it is evident that AML is heterogeneous at the molecular level. Around 45% of de novo AML patients belong to normal cytogenetics [20–25]. Recently, molecular dissection of this group identified better prognostication. These molecular alterations include internal tandem duplication of FLT3, partial tandem duplication of MLL gene, and mutations of CEBPA [26–28].

2.7. Prognosis of AML

In AML risk stratification, there are various clinical and biological factors relevant with treatment outcome [29]. These risk factors included are age, performance status, leucocyte counts, platelets counts, lactate dehydrogenase, drug resistance, immunophenotyping, cytogenetics, molecular genetics, epigenetics, micro RNA and so on. In various literature, these factors shows significant role to identify the potential role for treatment outcome in AML; for example, various mutations have targeted agents (e.g., ATRA and arsenic trioxide in PML-RARAα acute promyelocytic leukemia or FLT3 inhibitors in AML with FLT3 mutations), informing decisions on allogeneic transplantation [9, 30].

These clinical and biological analyses classified the AML into three risk stratification groups: favorable, intermediate, and unfavorable. Current updates reclassified into further subgroups after more markers includes with the deep molecular analysis like next-generation sequencing (NGS) of the whole genome of AML patients [5, 31, 32]. There are multiple large cohort done previously and currently as well in normal karyotypes of AML in which significance of mutations like FLT3, NPM1, and CEBPA has been subclassified into subgroups according to their presence and absence for different treatment outcomes and survival rates [33].

When taking into account immunophenotyping, human leukocyte antigen (HLA)-DR and CD14 expression are associated with the elderly, highest WBC count, and unfavorable-risk cytogenetics; CD4, CD7, and CD11b expressions are correlated with the highest WBC count and unfavorable-risk cytogenetics; CD22, CD34, CD123, and terminal deoxynucleotidyl transferase (TdT) expressions are correlated with unfavorable-risk cytogenetics; CD19 is associated with children and favorable-risk cytogenetics; and myeloperoxidase (MPO) and glycophorin A (gly-A) expressions are associated with lower WBC count and favorable-risk cytogenetics [33].

3. Structural and functional characteristics of Fms-like tyrosine kinase 3

3.1. FLT3 receptor

Transmembrane FLT3 receptor is a member of type III receptor tyrosine kinase (RTK) family. Other receptors included in this group are KIT, c-FMS, and platelet-derived growth factor receptor (PDGFR) [34–37]. These receptors keep control in normal maturation and proliferation of hematopoietic precursor cells. The FLT3 is approximately 1000 kilobases in length and consists of 24 exons situated on chromosomes 14 and 15. This gene encodes

a 993-amino-acid protein that is observed as a major 140 kDa band and a minor 160 kDa band because of N-linked glycosylation, and a 130 kDa band when unglycosylated and not membrane bound. The FLT3 receptor has an extracellular domain, one transmembrane region and two cytoplasmic kinase domains. Extracellular domain comprises of five immunoglobulin-like domains, while transmembrane region has a cytoplasmic juxtamembrane (JM) domain and cytoplasmic kinase is linked by an intracellular kinase domain [38].

3.2. FLT3 receptor expression

FLT3 is present in precursors of lymphoid and myeloid cells. These progenitor cells are converted into granulocyte, monocyte, B cell, and T cell, but in comparison to their counterpart, cells are unable to produce erythroid and megakaryocyte cells. FLT3 is also expressed in other tissues like the placenta, gonads, and brain, but its significance in these areas is unknown [27].

3.3. FLT3 ligand(FL)

FLT3 regulates early hematopoiesis by stimulating the FLT3 signal transduction pathway. mRNA of FLT3 is identified in hematopoietic as well as non-hematopoietic tissues. But identification of membrane-bound and soluble isoform is restricted to bone marrow s T lymphocytes and stromal fibroblasts. This protein in non-hematopoietic cells acts similarly as cells expresses FLT3 receptor shows FLT3 has autocrine and paracrine signaling mechanisms. It is identified during resting phase, but it is detected in serum at lower concentration. Under controlled circumstances release of FL is at a lower level to avoid hyperstimulation of progenitor hematopoietic cells. Current research depicts that one pathway for leukemia development is uncontrolled FL secretion [37].

3.4. Synergy of FL with other cytokines

FL needs cytokines for its action and proliferation. Interleukin-3 (IL-3), granulocyte colony-stimulating factor (G-CSF), colony-stimulating factor-1 (CSF-1), and granulocyte macrophage colony-stimulating factor (GM-CSF) are growth factors that help in FL-mediated signal transduction [27]. However, working in conjunction with cytokines, FL induces expansion of hematopoietic progenitor cell [38]. In vivo analysis of FL function further supports its vital role in maintenance and proliferation during early hematopoiesis. Blocking of FL in mice decreased myeloid progenitor cells, whereas stimulation of FL revealed transient HSC proliferation evidenced by bone marrow hyperplasia, splenomegaly, hepatomegaly, and enlarged lymph nodes [39].

3.5. FLT3 receptor signaling

After binding of FL to FLT3 receptor there is a formation of homodimer in plasma membrane. The dimer joins cytoplasmic domains and consequently phosphorylation of tyrosine residues, likely Tyr-589 and Tyr-591, on the JM domain [40]. This combination leads to conformational change at receptor sites to initiate autophosphorylation and leads to downstream signaling cascade which involves activation of cytoplasmic molecules that control pathways of apoptosis, proliferation, and differentiation (**Figure 1**). FLT3 receptor sends signals to the p85 subunit

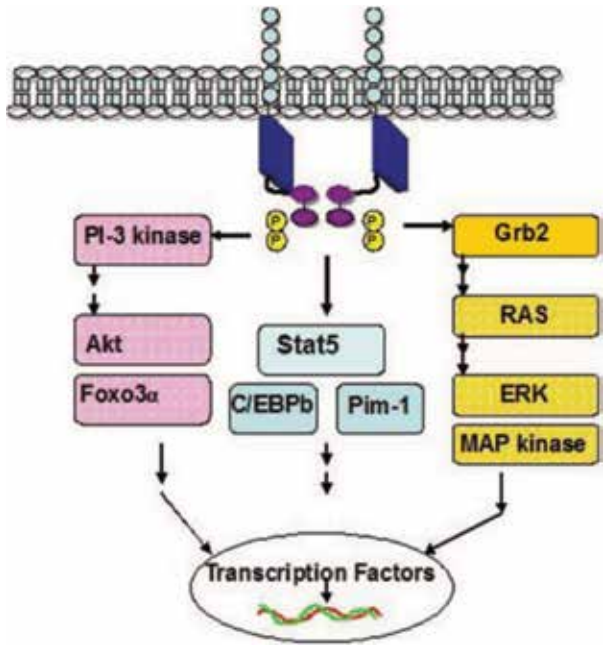

Figure 1. Signal transduction pathways downstream of FMS-like tyrosine kinase-3 receptor activation. Abbreviations: CEBPA, CCAAT/enhancer-binding protein alpha; ERK, extracellular signal-regulated kinase; MAP, mitogen-activated protein; PI3-kinase, phosphatidylinositol 3-kinase. Adapted from STEMCELLS 2006;24:1174–1184. www.StemCells.com

of phosphatidylinositol 3-kinase and to the adaptor protein growth factor receptor-bound protein 2, phospholipase C_1 and a GTPase-activating protein of the Ras proliferation pathway [40, 41]. Normally, FLT3 activates extracellular kinase 1/2 but weaker phosphorylation of STAT5, which is a key target during deregulated signaling [23].

3.6. Significant clinical mutation in FLT3 receptors

FL-FLT3 interaction controls survival, proliferation, and differentiation of stem cells. FLT3 is also expressed on the surface of a high proportion of AML and B-lineage ALL cells [25, 42–45]. In FLT3-expressing leukemia cells, FL-stimulation leads to increased survival of leukemic cells [46]. In 1996, a unique mutation of the FLT3 gene was first identified in AML cells. This mutation (FLT3-ITD) is produced when a fragment portion of the JM domain-coding sequence is multiplied in a direct head-to-tail orientation [47]. There are two types of mutations of FLT3 receptor, i.e., FLT3/ITD and FLT3/KDM that occur in 15–35 and 5–10%, respectively. Mutations of the FLT3 gene are, therefore, the most frequent genetic alterations so far reported as having involvement in AM which should be deleted or written somewhere else [8, 9, 33, 48, 49]. In comparison to AML, FLT3-ITD is far less common in patients with ALL, but FLT3/KDM is recurrently found in patients with ALL, especially in those harboring an MLL gene rearrangement or hyperdiploidy [7]. It is observed that ALL cells, which strongly express FLT3 but do not carry FLT3 mutations, have the same sensitivity to a potent FLT3 inhibitor as leukemia cells with FLT3 mutations [10]. Recently, high concentration of FLT3 transcripts in AML patients without FLT3 mutations is associated with a poor prognosis [6]. These studies indicate that FLT3 is greatly involved in the

pathophysiology of leukemia. Mutations and expression levels of FLT3 distinguish a disease entity of leukemia.

3.7. Clinical prevalence and characteristics of FLT3 mutations

These studies reveal that FLT3 mutations are mainly identified in AML and more infrequent in secondary AML developed from myelodysplastic syndrome (MDS) or therapy-related AML than de novo AML. This mutation is also found in ALL and MDS, but never in chronic myeloid leukemia, chronic lymphoid leukemia, multiple myeloma, malignant lymphoma, or myeloproliferative disorders (MPD) [50]. Frequency of this mutation in MDS is reported to be 3%, but it increased to 15% in advanced stage and after AML conversion [46]. The incidence of FLT3 mutations is directly proportional to the age of patients with AML. In elderly patients, FLT3-ITD has been found in approximately 25% of this population; among them 31.4% were over 55 years of age [51]. In contrast, only 10% pediatric group had this mutation.

3.8. Future perspectives

FLT3 mutations are detected in 25% of AML patients, revealing that the FLT3 gene is the target most frequently mutated. FLT3 is also implicated in the pathogenesis in few patients of ALL, such as those with MLL gene rearrangement or hyperdiploidy. As FLT3 mutation is closely associated with poor prognosis, routine testing of this mutation is recommended to stratify the patients into distinct risk groups. However, optimal treatment strategy according to this mutation is under evaluation because it remains uncertain that high-dose chemotherapy and/or myeloablative therapy followed by hematopoietic stem cell transplantation will change the prognosis of FLT3-mutated patients. Mutated or overexpressed FLT3 is an important molecular target in the treatment of leukemia and several potent inhibitors in clinical phase 1 and 2 trials are underway [52–55]. At present, the clinical efficacy of FLT3 inhibitors seems unimpressive, and problems related to adverse effects and pharmacokinetics are observed. Further studies are required to establish the role of FLT3 inhibitors in the treatment of leukemia.

3.9. Genomic alteration of FLT3 in AML

FLT3 receptor is expressed in the vast majority of human B-lineage acute lymphocytic leukemias (ALL) and most myeloid leukemias in different types of AML (M0–M7) [56–58]. A smaller proportion of T-cell-related leukemias also possess (less than 30%) FLT3 receptor [50, 59–62]. Further analyses revealed that the mean mRNA expression level of FLT3 is higher in leukemic cells than in normal progenitor cells, but percentage is different in various samples of AML patients. Nearly, all primary AML patients and other immature leukemic- cells demonstrate FL and FLT3 co-expression [50, 59–61]. Thus, there is recognized evidence that FL causes autocrine effect in AML, which leads to development of leukemia.

FLT3-activating mutations represent the most frequent identified genetic mutation in de novo AML (−30%) and rarely in myelodysplastic syndromes (−5%). FLT3 genetic aberration is never observed in CML, bi phenotypic ALL, chronic lymphoid leukemia, non-Hodgkin's lymphoma, or multiple myeloma patients.

In 1996, the first type of FLT3 mutation was discovered as a duplication of gene (internal tandem duplication [ITD]) within the JM domain of FLT3 programmed by exons 14 and 15.

The duplicated areas are inconsistent in size (between 3 and 400 bp) and also in location, but the resulting product is always rich in tyrosine residues. It is hypothesized that modification of the length of the JM domain, rather than increase of tyrosine chain, causes enhancement of function of FLT3. Furthermore, an activating point mutation has been identified in the JM domain in immature leukemia cell. Mono Mac 1 and Mono Mac6 causes substitution of valine by alanine at position valine 592 (V592A) [62], but this mutation has not yet been reported in primary AML blasts.

A different group of genetic makeup has been described in the activation loop within the second tyrosine kinase domain of the FLT3 receptor that normally inhibits the binding of adenosine triphosphate and substrate to the kinase domain when the receptor is in a dormant state. Frequently, these involve mutations at aspartic acid 835 (D835) or isoleucine 836 (1836) in exon 20.

3.10. FLT3-ITD

FLT3-ITD mutations are present in 20–25% of adult patients with AML and correlate with higher WBC count at diagnosis, increased relapse risk, and poor prognosis. Moreover, simultaneous loss of the wild-type FLT3 allele is associated with significantly inferior outcome and decreased overall survival in FLT3-ITD patients. In pediatric AML patients, FLT3-ITD is detected in 11–16% of cases and linked to worse prognosis [30, 63–65]. FLT3-ITD mutations more frequently occur in acute prolymphocytic leukemia (30–39%) containing t(15;17), which produces the PML-RARα oncogene. FLT3-ITD receptors are characterized by ligand-independent receptor dimerization and phosphorylation, but the accurate mechanism of this mutation binding and how it causes constitutive kinase activity has not yet been fully elucidated. Both lengthening as well as shortening of the JM domain result in activation of FLT3 receptor [65].

Structural analysis of the EphB2 RTK, it described that JM domain takes up a z-helical conformation, which inhibits the activation of kinase and also self-dimerization. FLT3-ITD would affect the hindrance of kinase domain and, with the help of JM domain, would produce auto phosphorylation. In further studies of HSCT bone marrow cells in mice which done by retro virally transduced with FLT3-ITD, which produced oligo clonal band of MPD due to oncogenic potential of FLT3 ITD mutation [66].

3.11. TKD mutations

Activating TKD mutations are noticed in 7–14% of adults with AML, but D835 has no significant correlation with poor outcome. In pediatric group this mutation was observed 3–8%. Clustering algorithms has identified that childhood acute leukemias carrying rearrangements of MLL gene on chromosome 11q23 show overexpression of wild-type FLT3 mRNA easily distinguishing them from conventional pre-B ALL and AML. Interestingly, FLT3-TKD deletions involving codons D835 and I836 were identified more frequently with MLL gene [67, 68]. Patient with these activating FLT3-TKD alterations expresses higher levels of FLT3 transcripts [69].

Like FLT3-ITD receptors, FLT3-TKD mutations stimulate ligand-independent receptor activation and promote growth factor independence in 32D cells. However, it is under evaluation

whether FLT3-TKD receptors phosphorylate the same receptor and signal similarly to FLT3-ITD receptors. It is well known that FLT3-D835 mutations are not associated with a significant decrease on the overall survival. FLT3-TKD mutations are also less tumorigenic than FLT3-ITD mutations, even hypothesized that FLT3- D835Y receptors show a higher level of tyrosine kinase activity than FLT3-ITD receptors.

4. Co-expression of other mutant genes with FLT 3/ITD & their predictive value

4.1. Prognostic and predictive value of the NPM1, FLT3, and CEBPA genotypes

NPM1 mutation or combined genotype NPM1mut/FLT3-ITDneg is reported as a favorable prognostic marker for attainment of a complete remission after induction therapy [70–73]. Actually, no data is available for specific chemotherapy for NPM1mut [74].

In a more recent study, NPM1 was shown to act as a co-repressor in retinoic acid-associated transcriptional regulation in a manner such that during retinoic acid-induced cellular differentiation, activating protein transcription factor 2 (AP2) recruits NPM1 to the promoter of certain retinoic acid-responsive genes. The German-Austrian AML Study Group (AMLSG) reported favorable effect of ATRA if given with conventional chemotherapy on complete remission rate, event-free survival, and OS in elderly patients with (non- APL) AML1 [50]. This study finding was coinciding with retrospective data in which beneficial effect of ATRA was restricted to NPM1mut/FLT3-ITDneg patients [75]. So, the genotype NPM1mut/FLT3-ITDneg appears as a predictive genotypic marker for the valuable effect of ATRA in non-APL AML.

FLT3-ITD has been reported consistently as an unfavorable prognostic marker for RFS and OS. Whether other molecular markers, in particular NPM1mut, add to prognostication in FLT3-ITDpos, AML is unclear [76, 77]. It is reported in some studies that genotype NPM1mut/FLT3-ITDpos shows more favorable prognosis compared with the genotype NPM1WT/FLT3-ITDpos; however, confirmation by other studies are due now [56]. More recent data give insight that outcome is also related to the concentration of the mutant allele and not just its mere presence [77]. However, if NPM1 mutation status was included to the prognostic model, the mutant wild-type ratio of FLT3-ITD was not an important prognostic factor. Currently in randomized multicenter phase III trial [Cancer and Leukemia Group B (CALGB) 10603; clinicaltrials.gov, NCT00651261] wild-type ratio (high versus low) is applied for midostaurin (PKC412) in young adult AML patients.

CEBPA mutations are another genetic abnormality that consistently associated with good prognosis, either in the subset of patients with intermediate-risk cytogenetics or with normal karyotypes [74]. In the context of other molecular markers, the mutated CEBPA alone retained its prognostic significance for RFS and OS; additional mutations did not affect outcome in the CEBPA mut subgroup. This needs validation. Actually, even in the largest cohort of patients analyzed so far in CN-AML, the sample size in the CEBPA mut subgroup was too

low for meaningful analysis, in particular to compare the different post-remission strategies (chemotherapy versus autologous SCT versus allogeneic SCT) [74]. Therefore, the prognostic marker CEBPA mut cannot actually be used as a predictive marker.

MLL partial tandem duplication (PTD) is exclusively found in normal karyotype (CN)-AML with an incidence reported from 5% to 11%. There are no clinical features differentiating MLL-PTD positive versus MLL wild-type patients [78]. Approximately 30–40% of MLL-PTD-positive patients consist of FLT3-ITD mutations, whereas combined existence of CEBPA with NPM1 mutations is rare. MLL-PTD is linked with shorter complete remission duration or worse RFS; however, in these studies, MLL-PTD did not show any effect on OS [77]. Recently, the CALGB reported relationship of MLL-PTD in young adults who received autologous SCT in the first complete remission. Clinical outcomes between the MLL-PTD-positive and the MLL wild-type groups were equivalent. WT1 mutations were reported in 10–12.6% in CN-AML. However, variable results have been mentioned about the prognostic significance of WT1 mutations. Both CALGB and MRC studies reported the prognostic impact of WT1 mutations in young adults with CN-AML. In both studies, patients with WT1 mutations were an independent adverse prognostic factor with inferior RFS and OS in multivariate analysis. This is in contrast to the findings of Gaidzik et al. who did not observe any decreased RFS and OS in relation with WT1 mutations on RFS and OS neither in univariate nor multivariate analysis. Of note, when performing exploratory subset analysis on FLT3-ITD samples, the WT1mut/FLT3-ITD pos genotype appeared to be associated with worse clinical course. One major difference between the three studies is different doses of cytarabine used. Cumulative dose of cytarabine was significantly higher in the trial reported by Gaidzik et al. (in preparation), suggesting that the negative impact ofWT1 mutations reported by others may be overcome by the use of high-dose cumulative cytarabine. On the basis of the current data, the prognostic impact of WT1 mutation remains unclear and its impact on treatment remains to be elucidated in future studies.

Although the majority of studied related to CN patients contained at least one of the already mentioned genetic alterations. In a study of AML done by one group, almost a quarter of patients did not have FLT3-ITD, FLT3-TKD, MLL-PTD, or mutations in the CEBPA or NPM1 genes [77]. Thus, it is likely that unidentified novel gene mutations and/or abnormal gene expression with prognostic significance will be discovered in the future. Expression of the meningioma 1 (MN1) gene might become such a novel prognostic factor. Same group in study reported also high expression of the MN1 gene related to inferior RFS and OS and a higher risk of relapse in CN aged 60 years or younger with de novo or secondary AML. This observation needs confirmation before implementation.

5. Conventional therapeutic approaches and novel agents in future development

The treatment of AML comprises of two phases, initially induction therapy to achieve complete response and consolidation therapy after achieving CR.

5.1. Induction therapy

The primary objective of induction therapy is attainment of normal bone marrow function. The criteria of CR are a platelet count of >100 × 109/L, neutrophil count of >1 × 109/L and a bone marrow examination with <5% blasts. Patients with persistent >5% blasts in the bone marrow following induction chemotherapy have a poor overall survival (OS) [79, 80]. Despite multiple trials, the standard remission induction therapy consisting of three daily infusions of an anthracycline and cytarabine given as continuous infusion for 7 days (7 + 3 regimen) has not changed much over the years.

5.2. Post-remission consolidation chemotherapy

After achieving complete remission(CR) after induction therapy, disease relapse is a certainly virtual. Median disease-free survival (DFS) in this circumstance is estimated at only 4–8 months. Options for post-remission consolidation therapy include high-dose chemotherapy allogeneic HSCT [81].

5.3. Response assessment

After conventional induction therapy with 3 days of an anthracycline and 7 days of cytarabine ("3 + 7") or other recommended regimens according to guidelines, response assessment is commonly performed between day 21 and 28 after the start of therapy [81].

5.4. Response assessment during follow-up period

Repeat bone marrow examination is recommended every 3 months for the first 2 years; in some cases, it continues every 6 months for the following 2–3 years. Most relapses occur within 1–3 years after the completion of treatment. Standardized schedule is necessary if MRD monitoring is advised. Blood counts should be repeated every 1–3 months for the initial 2 years and then every 3–6 months up to 5 years [77].

5.5. Role of HSCT as a consolidation strategy

Prospective single institution studies comparing allogeneic HSCT as a consolidation treatment in the 1980s and the early 1990s showed lower relapse rates of 181 and improved DFS with allogeneic HSCT in AML patients in CR1, but none conclusively demonstrated a survival advantage [81]. Subsequently, six cooperative group trials prospectively addressed the role of HSCT in AML in CR1 in 1995 [91]. Patients with HLA-identical sibling donors were offered allogeneic transplantation ("Genetic randomization"). Remaining patients were randomized to autologous transplantation, intensive consolidation chemotherapy (ICC) or (depending on individual trial design) no further treatment.

Among these trials, the landmark European Organization for Research and Treatment of Cancer (EORTC)-Gruppo Italiano Malattie Ematologiche Maligne dell'Adulto (GIMEMA) trial showed superior 4-year leukemia-free survival (LFS) with allogeneic (55%) and autologous

(48%) HSCT compared to ICC (30%) [81]. However, despite this improved LFS and higher relapse rate in patients getting ICC, OS was similar in the three groups, since majority of patients relapsing after ICC successfully salvaged with autologous HSCT.

5.6. Novel agents in development

Currently is the era of targeted agents for treatment of malignancies. These targeted therapies for each AML patient are based on unique molecular features. Hypothesis -based study designs can give us proper risk stratification for prognosis and predictive treatment options in AML. In the following section, promising novel agents in development are described:

1. Tyrosine kinase inhibitors.
2. Epigenetic targeting agents
3. New chemotherapies (tipifarnib, cloretazine (VNP40101M), clofarabine)
4. Agents overcoming chemo resistance
5. Antiangiogenic agents

6. Future direction

NGS technologies have made a huge impact in research and clinical diagnostics. It has expanded genes that are causing malignancies and will soon replace routine testing for single-gene mutations with NGS-based gene panel diagnostics. The information will be acquired from NGS assay and will play a role in personalized medicine. This will provide the basis for more comprehensive knowledge data banks that can serve as valuable tools to advance individualized treatment approaches [82]. In addition, we also recorded rapid technical advances that allow for more accurate MRD assessment and started to offer the possibility of capturing leukemia heterogeneity at the single-cell level. NGS will serve as a powerful tool for gaining deeper insights into leukemia stem cell phenotypes, signaling pathways, and function [83]. Finally, population based information will be used in the future to tailor NGS panels, and useful prognostic and predictive markers can be identified. Novel targeted therapeutic approaches hold promise for improving patient outcomes, but it will be important that genomic-based outcome prediction systems stay flexible and adaptable to reflect advances in treatment and changes in disease monitoring.

AML biology is rapidly expanding, and there is a great need to apply knowledge to the clinical context as soon as possible in order to improve the outcomes of our patients. Clinical outcomes will improve to enhance the clinical care of patients with AML, especially older patients for whom clinical outcomes have improved little over the past several decades. Instead of delaying introduction of novel agents to the setting of relapsed/ refractory disease, we propose consideration of frontline treatment with targeted agents either alone or in combination with chemotherapy, in the context of multicenter and/or cooperative group clinical trials, when available.

7. Conclusion

AML is a biologically and clinically heterogeneous disease. Established therapies have given some survival benefit according to risk stratification but overall long-term survival remains poor. Most of the patients on diagnosis are elderly, and they have adverse cytogenetic profile. At the same time, these patients are susceptible for transplant-related complications. The novel targeted therapies may have a good antileukemic activity with reduced toxicity versus available chemotherapeutic options. However, given the molecular diversity of AML, it is unlikely that targeted therapies such as FLT3 tyrosine kinase inhibitors will provide a sole treatment option in FLT3-mutated patients. With improved diagnostic genetic profiling, risk stratification will result in incremental gains in remission and survival.

Furthermore, the identification of cell-specific surface antigens can provide another targetable therapeutic option for recombinant monoclonal antibodies. But now difficulty in selecting to target leukemic myeloid cells while sparing non-malignant myeloid precursors. Lastly, the development of well-tolerated oral therapies, such as clofarabine, will increasingly broaden the range of available treatment for elderly patients at a higher risk of mortality from standard chemotherapy regimens. It is the beginning of a new era in the treatment of AML to make them survive with novel agents with little toxicity, particularly in relapsed or refractory diseases and poor cytogenetic features.

Author details

Zeeshan Ansar Ahmed[1]*, Imran Ahmed Siddqui[2] and Sadia Sultan[3]

Address all correspondence to: zeeshan.ansar@aku.edu

1 Molecular Pathology Section, Department of Pathology and Laboratory Medicine, Aga Khan University Hospital, Karachi, Pakistan

2 Memon Medical Institute, Karachi, Pakistan

3 National Medical Complex, Karachi, Pakistan

References

[1] Vardiman JW, Thiele J, Arber DA, Brunning RD, Borowitz MJ, Porwit A. The 2008 revision of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: Rationale and important changes. Blood. 2009;**114**:937-951

[2] Small D. FLT3 mutations: Biology and treatment. Hematology American Society of Hematology Education Program. 2006:178-184

[3] Knapper S. FLT3 inhibition in acute myeloid leukemia. British Journal of Haematology. 2007;**138**:687-699

[4] Mead AJ, Linch DC, Hills RK, Wheatley K, Burnett AK, Gale RE. FLT3 tyrosine kinase domain mutations are biologically distinct from and have a significantly more favourable prognosis than FLT3 internal tandem duplications in patients with acute myeloid leukemia. Blood. 2007;**110**:1262-1270

[5] Taketani T, Taki T, Sugita K, Furuichi Y, Ishii E, Hanada R, et al. FLT3 mutations in the activation loop of tyrosine kinase domain are frequently found in infant ALL with MLL rearrangements and pediatric ALL with hyperdiploidy. Blood. 2004;**103**:1085-1088

[6] Meshinchi S, Alonzo TA, Stirewalt DL. Clinical implications of FLT3 mutations in pediatric AML. Blood. 2006;**108**:3654-3661

[7] Kiyoi H, Naoe T, Nakano Y. Prognostic implication of FLT3 and N-RAS gene mutations in acute myeloid leukemia. Blood. 1999;**93**:3074-3080

[8] Schnittger S, Schoch C, Dugas M. Analysis of FLT3 length mutations in 1003 patients with acute myeloid leukemia: Correlation to cytogenetics, FAB subtype, and prognosis in the AMLCG study and usefulness as a marker for the detection of minimal residual disease. Blood. 2002;**100**:59-66

[9] Thiede C, Steudel C, Mohr B. Analysis of FLT3-activating mutations in 979 patients with acute myelogenous leukemia: Association with FAB subtypes and identification of subgroups with poor prognosis. Blood. 2002;**99**:4326-4335

[10] Kottaridis PD, Gale RE, Frew ME. The presence of a FLT3 internal tandem duplication in patients with acute myeloid leukemia (AML) adds important prognostic information to cytogenetic risk group and response to the first cycle of chemotherapy: Analysis of 854 patients from the United Kingdom Medical Research Council AML 10 and 12 trials. Blood. 2001;**98**:1752-1759

[11] Grimwade D. The clinical significance of cytogenetic abnormalities in acute myeloid leukaemia. Best Practice & Research Clinical Haematology. 2001;**14**:497-529

[12] Wodnar-Filipowicz A. Flt3 ligand: Role in control of hematopoietic and immune functions of the bone marrow. News in Physiological Sciences. 2003;**18**:247-251

[13] Mathews LS, Vale WW. Expression cloning of an activin receptor, a predicted transmembrane serine kinase. Cell. 1991;**65**:973-982

[14] Rosnet O, Schiff C, Pebusque MJ. Human FLT3/FLK2 gene: CDNA cloning and expression in hematopoietic cells. Blood. 1993;**82**:1110-1119

[15] Small D, Levenstein M, Kim E. STK-1, the human homolog of Flk-2/Flt-3, is selectively expressed in CD34_ human bone marrow cells and is involved in the proliferation of early progenitor/stem cells. Proceedings of the National Academy of Sciences of the United States of America. 1994;**91**:459-463

[16] Agnes F, Shamoon B, Dina C. Genomic structure of the downstream part of the human FLT3 gene: Exon/intron structure conservation among genes encoding receptor tyrosine kinases (RTK) of subclass III. Gene. 1994;**145**:283-288

[17] Abu-Duhier FM, Goodeve AC, Wilson GA. Genomic structure of human FLT3: Implications for mutational analysis. British Journal of Haematology. 2007;**113**:1076-1077

[18] Brasel K, McKenna HJ, Morrissey PJ. Hematologic effects of flt3 ligand in vivo in mice. Blood. 1996;**88**:2004-2012

[19] Dosil M, Wang S, Lemischka IR. Mitogenic signalling and substrate specificity of the Flk2/Flt3 receptor tyrosine kinase in fibroblasts and interleukin 3-dependent hematopoietic cells. Molecular and Cellular Biology. 1993;**13**:6572-6585

[20] Rottapel R, Turck CW, Casteran N. Substrate specificities and identification of a putative binding site for PI3K in the carboxy tail of the murine Flt3 receptor tyrosine kinase. Oncogene. 1994;**9**:1755-1765

[21] Zhang S, Fukuda S, Lee Y. Essential role of signal transducer and activator of transcription (Stat)5a but not Stat5b for Flt3-dependentsignaling. The Journal of Experimental Medicine. 2000;**192**:719-728

[22] Dehmel U, Quentmeier H, Drexler HG. Effects of FLT3 ligand on human leukemia cells. II. Agonistic and antagonistic effects of other cytokines. Leukemia. 1996;**10**:271-278

[23] DaSilva N, Hu ZB, Ma W, Rosnet O, Birnbaum D, Drexler HG. Expression of the FLT3 gene in human leukemia-lymphoma cell lines. Leukemia. 1994;**8**:885-888

[24] Dehmel U, Zaborski M, Meierhoff G. Effects of FLT3 ligand on human leukemia cells. I. Proliferative response of myeloid leukemia cells. Leukemia. 1996;**10**:261-270

[25] Drexler HG. Expression of FLT3 receptor and response to FLT3 ligand by leukemic cells. Leukemia. 1996;**10**:588-599

[26] Drexler HG, Meyer C, Quentmeier H. Effects of FLT3 ligand on proliferation and survival of myeloid leukemia cells. Leukemia & Lymphoma. 1999;**33**:83-91

[27] Rosnet O, Buhring HJ, Marchetto S. Human FLT3/FLK2 receptor tyrosine kinase is expressed at the surface of normal and malignant hematopoietic cells. Leukemia. 1996;**10**:238-248

[28] Zheng R, Levis M, Piloto O. FLT3 ligand causes autocrine signaling in acute myeloid leukemia cells. Blood. 2004;**103**:267-274

[29] Kiyoi H, Towatari M, Yokota S. Internal tandem duplication of the FLT3 gene is a novel modality of elongation mutation which causes constitutive activation of the product. Leukemia. 1998;**12**:1333-1337

[30] Stirewalt DL, Kopecky KJ, Meshinchi S. FLT3, RAS, and TP53 mutations in elderly patients with acute myeloid leukemia. Blood. 2001;**97**:3589-3595

[31] Spiekermann K, Bagrintseva K, Schoch C, Haferlach T, Hiddemann W, Schnittger SA. New and recurrent activating length mutation in exon 20 of the FLT3 gene in acute myeloid leukemia. Blood. 2002;**100**:3423-3425

[32] Kindler T, Breitenbuecher F, Kasper S. Identification of a novel activating mutation (Y842C) within the activation loop of FLT3 in patients with acute myeloid leukemia (AML). Blood. 2005;**105**:335-340

[33] Armstrong SA, Kung AL, Mabon ME. Inhibition of FLT3 in MLL. Validation of a therapeutic target identified by gene expression based classification. Cancer Cell. 2003;**3**:173-183

[34] Ozeki K, Kiyoi H, Hirose Y. Biologic and clinical significance of the FLT3 transcript level in acute myeloid leukemia. Blood. 2004;**103**:1901-1908

[35] Kelly LM, Yu JC, Boulton CL. CT53518, a novel selective FLT3 antagonist for the treatment of acute myelogenous leukemia (AML). Cancer Cell. 2003;**1**:421-432

[36] Stone RM, DeAngelo DJ, Klimek V. Patients with acute myeloid leukemia and an activating mutation in FLT3 respond to a small-molecule FLT3 tyrosine kinase inhibitor, PKC412. Blood. 2005;**105**:54-60

[37] Smith BD, Levis M, Beran M. Single-agent CEP-701, a novel FLT3 inhibitor, shows biologic and clinical activity in patients with relapsed or refractory acute myeloid leukemia. Blood. 2004;**103**:3669-3676

[38] Fiedler W, Serve H, Dohner HA. Phase 1 study of SU11248 in the treatment of patients with refractory or resistant acute myeloid leukemia (AML) or not amenable to conventional therapy for the disease. Blood. 2005;**105**:986-993

[39] Fiedler W, Mesters R, Tinnefeld HA. Phase 2 clinical study of SU5416 in patients with refractory acute myeloid leukemia. Blood. 2003;**102**:2763-2767

[40] Carow CE, Levenstein M, Kanfmann SH. Expression of the hematopoietic growth factor receptor FLT3 (STK-1/Flk2) in human leukemias. Blood. 1996;**87**:1089-1096

[41] Meierhoff G, Dehmel U, Gruss HJ. Expression of FLT3 receptor and FLT3-1igand inhuman leukemia-lymphoma cell lines. Leukemia. 1995;**9**:1368-1372

[42] Brasel K, Escobar S, Anderberg R, de Vries E Gruss HJ, Lyman SD. Expression of the fit3 receptor and its ligand on hematopoietic cells. Leukemia. 1995;**9**:1212-1218

[43] Carow CE, Levenstein M, Kanfmann SH. Expression of the hematopoietic growth factor receptor FLT3 (STK-1/Flk2) in human leukemias. Blood. 1999;**87**:1089-1096

[44] Liang DC, Shih LY, Hung IJ. Clinical relevance of internal tandem duplication of the FLT3 gene in childhood acute myeloid leukemia. Cancer. 2002;**94**:3292-3298

[45] Xu F, Taki T, Yang HW. Tandem duplication of the FLT3 gene is found in acute lymphoblastic leukaemia as well as acute myeloid leukaemia but not in myelodysplastic syndrome or juvenile chronic myelogenous leukaemia in children. British Journal of Haematology. 1999;**105**:155-162

[46] Meshinchi S, Woods WG, Stirewalt DL. Prevalence and prognostic significance of Flt3 internal tandem duplication in pediatric acute myeloid leukemia. Blood. 2001;**97**:89-94

[47] Zwaan CM, Meshinchi S, Radich JR. FLT3 internal tandem duplication in 234 children with acute myeloid leukemia (AML): Prognostic significance and relation to cellular drug resistance. Blood. 2003;**19**:19

[48] Kelly LM, Liu Q, Kutok JL, Williams IR, Boulton CL, Gilliland DG. FLT3 internal tandem duplication mutations associated with human acute myeloid leukemias induce myeloproliferative disease in a murine bone marrow transplant model. Blood. 2002;**99**:310-318

[49] Libura M, Asnafi V, Tu A, et al. FLT3 and MLL intragenic abnormalities in AML reflect a common category of genotoxic stress. Blood. 2003;**102**:2198-2204

[50] Gale RE, Green C, Allen C. The impact of FLT3 internal tandem duplication mutant level, number, size, and interaction with NPM1 mutations in a large cohort of young adult patients with acute myeloid leukemia. Blood. 2008;**111**:2776-2784

[51] Whitman SP, Archer KJ, Feng L. Absence of the wild-type allele predicts poor prognosis in adult de novo acute myeloid leukemia with normal cytogenetics and the internal tandem duplication of FLT3: A cancer and leukemia group B study. Cancer Research. 2001;**61**:7233-7239

[52] Meshinchi S, Pollard JA, Tsuchiya KD, Stirewalt DA, Bernstein ID, Radich J. Gene conversion is a late event in evolution of high risk AML. American Society of Hematology Annual Meeting Abstracts. Am Soc Haemtol. 2007;**110**:207

[53] Raghavan M, Smith LL, Lillington DM. Segmental uniparental disomy is a commonly acquired genetic event in relapsed acute myeloid leukemia. Blood. 2008;**112**:814-821

[54] Raghavan M, Lillington DM, Skoulakis S. Genome-wide single nucleotide polymorphism analysis reveals frequent partial uniparental disomy due to somatic recombination in acute myeloid leukemias. Cancer Research. 2005;**65**:375-378

[55] Gupta M, Raghavan M, Gale RE. Novel regions of acquired uniparental disomy discovered in acute myeloid leukemia. Genes, Chromosomes and Cancer. 2008;**47**:729-739

[56] Gondek LP, Tiu R, O'Keefe CL, Sekeres MA, Theil KS, Maciejewski JP. Chromosomal lesions and uniparental disomy detected by SNP arrays in MDS, MDS/MPD, MDS-derived AML. Blood. 2008;**111**:1534-1542

[57] Fitzgibbon J, Smith LL, Raghavan M. Association between acquired uniparental disomy and homozygous gene mutation in acute myeloid leukemias. Cancer Research. 2005;**65**: 9152-9154

[58] Schlenk RF, Döhner K, Krauter J. Mutations and treatment outcome in cytogenetically normal acute myeloid leukemia. New England Journal of Medicine. 2008;**358**:1909-1918. This study describes the prognostic and predictive impact of gene mutations in a meta-analysis of four prospective clinical trials in intensively treated younger adults; population of the original studies was stated, missing values were addressed, and multivariable analysis was included.

[59] Falini B, Mecucci C, Tiacci E, Gimema Acute Leukemia Working Party. Cytoplasmic nucleophosmin in acute myelogenous leukemia with a normal karyotype. The New England Journal of Medicine. 2005;**352**:254-266

[60] Schlenk RF, Döhner K, Kneba M. Gene mutations and response to treatment with all-trans retinoic acid in elderly patients with acute myeloid leukemia: Results from AMLSG Trial AML HD98B. Haematologica. 2009;**94**:54-60

[61] Liu H, Tan BC, Tseng KH. Nucleophosmin acts as a novel AP2alpha-binding transcriptional corepressor during cell differentiation. EMBO Reports. 2007;**8**:394-400

[62] Schlenk RF, Fröhling S, Hartmann F. Phase III study of all-trans retinoic acid in previously untreated patients 61 years or older with acute myeloid leukemia. Leukemia. 2004;**18**:1798-1803

[63] Döner K, Schlenk RF, Habdank M. Mutant nucleophosmin (NPM1) predicts favorable prognosis in younger adults with acute myeloid leukemia and normal cytogenetics: Interaction with other gene mutations. Blood. 2005;**106**:3740-3746

[64] Heuser M, Beutel G, Krauter J. High meningioma 1 (MN1) expression as a predictor for poor outcome in acute myeloid leukemia with normal cytogenetics. Blood. 2006;**108**:3898-3905

[65] de Greef GE, van Putten WL, Boogaerts M, Huijgens PC, Verdonck LF, Vellenga E, et al. Criteria for defining a complete remission in acute myeloid leukaemia revisited. An analysis of patients treated in HOVON-SAKK co-operative group studies. British Journal of Haematology. 2005;**128**(2):184-191

[66] Cassileth PA, Harrington DP, Appelbaum FR, Lazarus HM, Rowe JM, Paietta E, et al. Chemotherapy compared with autologous or allogeneic bone marrow transplantation in the management of acute myeloid leukemia in first remission. The New England Journal of Medicine. 1998;**339**(23):1649-1656

[67] British Committee for Standards in Haematology, Milligan DW, Grimwade D, Cullis JO, Bond L, Swirsky D, et al. Guidelines on the management of acute myeloid leukaemia in adults. British Journal of Haematology. 2006;**135**(4):450-474

[68] Estey E, Pierce S. Routine bone marrow exam during first remission of acute myeloid leukemia. Blood. 1996;**87**(9):3899-3902

[69] British Committee for Standards in Haematology, Milligan DW, Grimwade D, Cullis JO, Bond L, Swirsky D. Guidelines on the management of acute myeloid leukaemia in adults. British Journal of Haematology. 2008;**135**(4):450-474

[70] Champlin RE, Ho WG, Gale RP, Winston D, Selch M, Mitsuyasu R, et al. Treatment of acute myelogenous leukemia. A prospective controlled trial of bone marrow transplantation versus consolidation chemotherapy. Annals of Internal Medicine. 1985;**102**(3):285-291

[71] Appelbaum FR, Dahlberg S, Thomas ED, Buckner CD, Cheever MA, Clift RA, et al. Bone marrow transplantation or chemotherapy after remission induction for adults with acute nonlymphoblastic leukemia. A prospective comparison. Annals of Internal Medicine. 1984;**101**(5):581-588

[72] Zittoun RA, Mandelli F, Willemze R, de Witte T, Labar B, Resegotti L, et al. Autologous or allogeneic bone marrow transplantation compared with intensive chemotherapy in acute myelogenous leukemia. European Organization for Research and Treatment of Cancer (EORTC) and the Gruppo Italiano Malattie Ematologiche Maligne dell'Adulto (GIMEMA) Leukemia Cooperative Groups. The New England Journal of Medicine. 1995;**332**(4):217-223

[73] Burnett AK, Goldstone AH, Stevens RM, Hann IM, Rees JK, Gray RG, et al. Randomised comparison of addition of autologous bone-marrow transplantation to intensive chemotherapy for acute myeloid leukaemia in first remission: Results of MRC AML 10 trial. UK Medical Research Council Adult and Children's Leukaemia Working Parties. Lancet. 1998;**351**(9104):700-708

[74] Harousseau JL, Cahn JY, Pignon B, Witz F, Milpied N, Delain M, et al. Comparison of autologous bone marrow transplantation and intensive chemotherapy as postremission therapy in adult acute myeloid leukemia. The Groupe Ouest Est Leucemies Aigues Myeloblastiques (GOELAM). Blood. 1997;**90**(8):2978-2986

[75] Suciu S, Mandelli F, de Witte T, Zittoun R, Gallo E, Labar B, et al. Allogeneic compared with autologous stem cell transplantation in the treatment of patients younger than 46 years with acute myeloid leukemia (AML) in first complete remission (CR1): An intention-to-treat analysis of the EORTC/GIMEMA AML- 10 trial. Blood. 2003;**102**(4):1232-1240

[76] Cornelissen JJ, van Putten WL, Verdonck LF, Theobald M, Jacky E, Daenen SM, et al. Myeloablative HLA-identical sibling stem cell transplantation in first remission acute myeloid leukemia in young and middle aged adults: Benefits for whom? Results of a HOVON/SAKK donor versus no donor analysis. Blood. 2007;**109**(9):3658-3666

[77] Keating S, de Witte T, Suciu S, Willemze R, Hayat M, Labar B, et al. The influence of HLA-matched sibling donor availability on treatment outcome for patients with AML: An analysis of the AML 8A study of the EORTC Leukaemia Cooperative Group and GIMEMA. European Organization for Research and Treatment of Cancer. Gruppo Italiano Malattie Ematologiche Maligne dell'Adulto. British Journal of Haematology. 1998;**102**(5):1344-1353

[78] Stone RM et al. Phase IB study of PKC412, an oral FLT3 kinase inhibitor, in sequential and simultaneous combinations with Daunorubicin and Cytarabine (DA) induction and high-dose cytarabine consolidation in newly diagnosed adult patients (pts) with acute myeloid leukemia (AML) under age 61. ASH Annual Meeting Abstracts. 2007;**108**(11):157

[79] Bhalla KN. Epigenetic and chromatin modifiers as targeted therapy of hematologic malignancies. Journal of Clinical Oncology. 2005;**23**(17):3971-3993

[80] Lancet JE, Gojo I, Gotlib J, Feldman EJ, Greer J, Liesveld JL, et al. A phase 2 study of the farnesyltransferase inhibitor tipifarnib in poor-risk and elderly patients with previously untreated acute myelogenous leukemia. Blood. 2007;**109**(4):1387-1394

[81] Baer MR, George SL, Dodge RK, O'Loughlin KL, Minderman H, Caligiuri MA, et al. Phase 3 study of the multidrug resistance modulator PSC-833 in previously untreated patients 60 years of age and older with acute myeloid leukemia: Cancer and Leukemia Group B Study 9720. Blood. 2002;**100**(4):1224-1232

[82] McKerrell T, Moreno T, Ponstingl H, et al. Development and validation of a comprehensive genomic diagnostic tool for myeloid malignancies. Blood. 2016;**128**:e1-e9

[83] Spitzer MH, Nolan GP. Mass cytometry: Single cells, many features. Cell. 2016;**165**:780-791

Chapter 6

Small Non-Coding RNAs in Regulation of Course and Therapeutic Efficacy in Acute Myeloid Leukemia

Oxana V. Klimenko

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.70931

Abstract

Small non-coding RNAs (sncRNAs) are small regulatory molecules, which play key roles in fine-tune of all cell functions. In late 1970s and early 1980s, it was first determined that non-coding RNAs contribute to the cellular regulatory processes. The kingdom of sncRNAs is very numerous and it is clear that functions of different members of this family is different from each other and may be involved in normal and pathologic processes in cell. Recently it was investigated that sncRNAs and long non-coding RNAs play roles in cellular differentiation, proliferation, metabolic processes, bioenergetic regulation, cell death and inter-cellular communications, etc. In embryos, non-coding RNAs control maternal-zygotic transition, the maintenance of pluripotency, the pattering of the body axes, the specification and differentiation of cell types and morphogenesis of organs. Development of hematologic malignancies in humans, their course and regulation of resistance and sensitivity of tumorous cells to therapy are under the control of sncRNAs.

Keywords: small non-coding RNAs, micro-RNAs, antago-miRNA, nano-sized polymer carriers, leukemic cell transformation

1. Introduction

1.1. Small non-coding RNAs

Small non-coding RNAs (sncRNAs) are oligonucleotides with length less than 200 nt. This is numerous family of non-coding genomic regulators. The most investigated sncRNAs are micro-RNAs (miRNAs) and small interfered RNAs (siRNAs). Less studied sncRNAs are piwi-interacting RNAs (piRNAs), small nuclear RNAs (snRNAs) and small nucleolar RNAs (snoRNAs) [1, 2].

1.2. Biogenesis of micro-RNAs and piwi-interacting RNAs

All small non-coding RNAs before they become functionally active undergo different processes of biogenesis from their precursor form. This multistep maturation is necessary for normal functionality of small non-coding RNAs. Imbalance of one or more steps of sncRNAs biogenesis may results in development of different pathologic disorders and carcinogenesis [3].

1.3. micro-RNAs

miRNAs are small, single-stranded RNAs (ssRNAs) that are less than 24 nucleotides in length. They control translation of more than 60% of protein-coding genes. Four different mechanisms of regulation exist: inhibition of translation initiation, suppression of translation elongation, degradation of co-translational protein and initiation of translation termination [4–8].

The biogenesis of miRNAs was previously described [9–13]. Primary miRNAs (pri-miRNAs) are mostly transcribed by RNA Polymerase II (RNA Pol II). After that, pri-miRNAs undergo nuclear processing by the microprocessor complex of RNAse III enzyme Drosha/DGCR8 (DiGeorge syndrome critical region gene 8), followed by export into the cytoplasm as pre-miRNAs complexed with exportin5 and RAN GTP [9–11]. In the cytoplasm, immature miRNAs are duplexes by the cytoplasmic RNase III Dicer together with its catalytic partner Trans-activator RNA (tar)-binding protein (TRBP). Dicer/TRBP cleavage pre-miRNAs in duplex RNA (dsRNA) is loaded onto Argonaute (AGO) proteins to generate the RNA-induced silencing complex (RISC). RISC contains a single-stranded miRNAs and guide them to theirs target mRNAs [12, 13].

1.4. Piwi-interacting RNAs

PiRNAs, 26–32 nt long small non-coding RNAs that are associated with the piwi-class AGO protein family, are generated independently of Dicer from single-stranded precursors [14]. The primary function of these sncRNAs is silencing of transposable elements through de novo DNA methylation [15]. Some piRNAs are silencing target protein-coding genes [16]. Recently two models of piRNA biogenesis were proposed: the primary processing and the secondary amplification (ping-pong amplification loop) pathways. In the germline, piRNA biogenesis involves both pathways. Two putative RNA helicases, Armi and FS(1)Yb (Female Sterile (1) Yb; also known as Yb) and a nuclease, Zuc (Zucchini), as factors of primary piRNA biogenesis. PiRNA precursors or intermediates obtained from uni-strand clusters are cut by Zuc to produce piRNAs that bind to piwi proteins. Amplification of loop pathway is activated by piRNA derived from primary biogenesis pathway. There should be a relative increase in abundance of complementary piRNAs due to the amplification. The primary pool of piRNAs amplifying sequences is silencing active transposons in the secondary ping-pong amplification loops cycles. The ping-pong amplification loop characterized in many animal species requires piwi subfamily proteins: piwi and Aub [17–19]. Sense-strand piRNAs react with Ago3 [20], whereas antisense piRNAs bound to Piwi or Aub [21]. In the ping-pong

amplification loop model of piRNA biogenesis, antisense piRNAs in complex with Aub directly cleaves sense-strand transposon sequences, generating sense piRNAs for Ago3. The Ago3-piRNA complex then directs cleavage of antisense piRNA precursors, generating antisense piRNAs for Aub. The 5′-ends of amplified secondary piRNAs are determined by Aub and Ago3 Slicer [22–25].

1.5. Role of sncRNAs in carcinogenesis

sncRNAs may play double-faced roles in carcinogenesis as oncogenes and tumor suppressors. As oncogenes sncRNAs function in tumor initiation, progression and resistance to therapies; as tumor suppressors, sncRNAs inhibit cell growth, induce apoptosis, block cell cycle and promote cell differentiation [26, 27]. In every pathologic condition, its own biomarker sncRNAs exist which indicate prognosis, course of disease and resistance or sensitivity to ongoing treatment. These biomarkers may be a basis for creation of new drugs for epigenetic therapy of cancers.

2. sncRNAs in AML

In acute myeloid leukemia (AML), some sncRNAs may play roles in oncogenes and other sncRNAs may be tumor suppressors. Oncogenic sncRNAs are miRNA-155, miRNA-17-92 cluster, miRNA-221, miRNA-21, etc. Tumor suppressive functions have miRNA-15a/16 cluster, miRNA-29b, miRNA-181b and members of let-7 cluster [28]. These sncRNAs may be determined as biomarkers and prognostic markers in AML.

2.1. sncRNAs as biomarkers and prognostic markers of AML

Acute myeloid leukemia (AML) is a heterogeneous hematologic disease, which characterizes with disturbances of differentiation and maturation of hematopoietic stem cells or progenitor cells and appearance of immature blasts in periphery blood. Any classifications of AML are existing (WHO, 2008 with corrections in 2016) [29]. Classification of AML in dependence of genetic abnormalities is one of the basic classifications for this disease. All genetic changes in AML are also associated with imbalance of expression of different sncRNAs. These sncRNAs can be determined as biomarkers of particular type of AML. In **Table 1**, presents the most comprehensive analysis of dysregulated sncRNAs in dependence of AML type. All displayed sncRNAs profiles were taken from clinical investigations in AML patients.

2.2. Role of separated miRNAs and piRNAs in AML

miRNA, which most often involved in pathologic processes in AML are let-7 family of miRNAs, miRNA-16, 17–92 cluster, miRNA-29, miRNA-30, miRNA-146, miRNA-150, miRNA-155, miRNA-196 and miRNA-223. The oncogenic markers are miRNA-17-92 cluster, miRNA-155 and miRNA-196; the tumor suppressive are miRNA-15/16, let-7 family, miRNA-29, miRNA-30, miRNA-146, miRNA-150 and miRNA-223 (**Figure 1**).

Type of AML [29, 32]	Downregulated	Upregulated
AML with t(8;21)(q22;q22.1); RUNX1-RUNX1T1	miRNA-9, 18a, 19a–b, 20a, 92, 193a, 196 [34]; miRNA-196b [36]; 21, 26a, 125a, 142-3p, 196a, 494 [37]; 133, 17-3p, 17-5p [38]; let-7b, c [40]	miRNA-126, 130 [33, 36]; let-7a-3, 30a, b, c [34]; 27a, 146a, b, 150, 155, 181a, b, 223 [37]; 17–92, miRNA-24a, b [40]
AML with inv.(16)(p13.1q22) or t(16;16)(p13.1;q22); CBFB-MYH11	miRNA-30b, d, 335 [36]; 196a, b [37]; let-7b, c [40]	miRNA-126 [36]; 126 [40]
AML with t(9;11)(p21.3;q23.3); MLLT3-KMT2A	miRNA-22, 24, 29a, b, 30a, 124, 132, 133a, 133b, 146a, b, 150, 155, 193b, 221, 222, 424, 503, 542 [33]; 23a, 181b, 210, 495 [34, 35]; let-7c, miRNA-26a, 30c, d, 100, 125b, 126-3p, 143, 181a, d [37]; 16, 29s, 34a [38]; let-7b, miRNA-29c [40]	Let-7a-3, miRNA-30a, b, c, 17h, g, 196b, 328 [34, 35]; 21, 196a, b [37]; 17–92, 196a, 219, 326 [38, 40]
APL (M3) with a translocation between chromosomes 15 and 17 (PML-RARA)	Let-7c, miRNA-15b, 107, 143, 210, 223, 342 [33]; miRNA-16 [34]; 23a [35]; 485-5p [36]; let-7b, 18b, 22, 24, 27a, 126-3p, 150, 196a, b, 378 [37]; 17-5p, 20a [39]	Let-7a, let-7d, miRNA-142, 181b [33]; let-7a-3, miRNA-30a, b, c, 337-3p [34]; 125b [35, 37]; 29a, 100, 146a, 146b-5p, 181 a, b [37]; 127, 299, 323, 368, 382 [38, 40]
AML with t(6;9)(p23;q34.1); DEK-NUP214		
AML with inv.(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); GATA2, MECOM	miRNA-449a [35]	
AML (megakaryoblastic) with t(1;22)(p13.3;q13.3); RBM15-MKL1	miRNA-29a [37]	miRNA-335, miRNA-375 [37]
AML with BCR-ABL1	miRNA-203 [41]; 150 [42]; 451 [43]; 120, 151, 155, 31, 564 [44];	miRNA-23a [41]; 125b, 96 [44];
AML with mutated NPM1	miRNA-424, 181a, b [46]; 320, 145, let-7c [47]; 10b, let-7, miRNA-29, 181a, 124, 128, 194, 219, 220a [48]; 204, 126, 130a, 451 [38]	miRNA-10a-5p [45]; 155 [46]; 196a, b [47]
AML with biallelic mutations of CEBPA	miRNA-34a [49]	miRNA-328 [44]; miRNA-181a [38, 48]; 335 [38]
AML with mutated RUNX1	Let-7, miRNA-223, 99a, 100 [48]	miRNA-24, 23, 27, 155, 211, 220, 595 [48]

Table 1. AML with recurrent genetic abnormalities [30, 31].

Let-7. The let-7 is a 13-member family of miRNAs expressed in human tissues. This sncRNAs are induced in embryogenesis and then its high levels are detected in different tissues. Down-expression of let-7 was obtained in early embryogenesis and its suppression was observed in many types of cancers. The main functions of let-7 miRNA are tumor suppression and supporting of cell differentiation. Let-7 targets a human rat sarcoma (RAS) ortholog, a high-mobility group AT-hook 2 (HMGA2) and MYC. These factors are human oncogenes. Let-7 regulates

http://dx.doi.org/10.5772/intechopen.70931

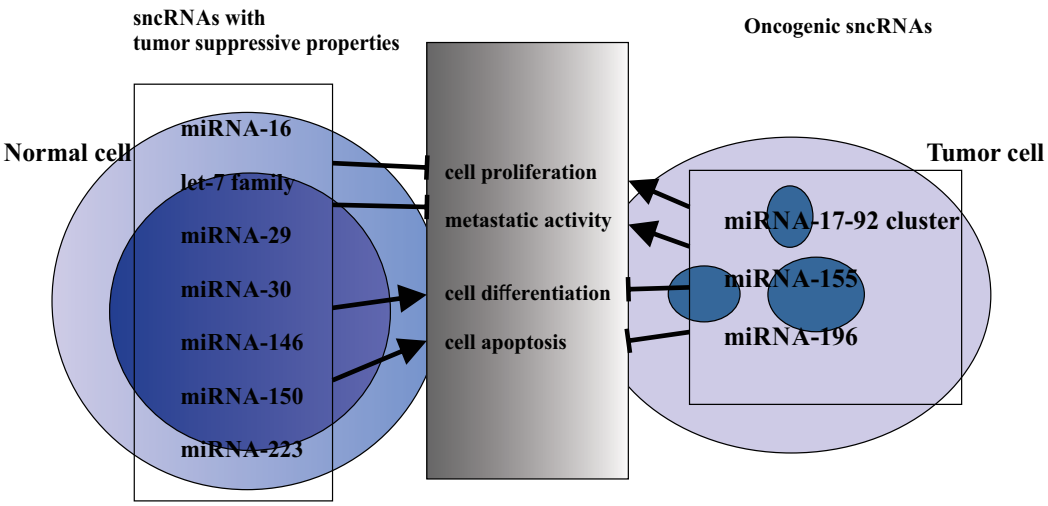

Figure 1. Influence of tumor suppressor sncRNAs and oncogenic sncRNAs on main functions of cells.

cell cycle suppression of any cyclins, such as cyclin A, cyclin D1 and cyclin-dependent kinase 4 (CDK4). It may indirectly suppress LIN28 by the direct inhibition of MYC [23, 24]. Let-7c induces megakaryocytic cell differentiation as the result of targeting of PBX2 in myeloid cells. This factor in cooperation with Meis1 and HoxA9 are required for MLL-dependent leukemogenesis. Acute promyelocytic leukemia (APL) decreases let-7c levels and cooperation of KRAS with PML-RARA results in poor prognosis of disease [25, 26, 50, 51]. One more novel axis was determined in poor prognosis leukemia. This is protein tyrosine phosphatase of regenerating liver 3: PRL-3/LIN28B/let-7 axis. PRL-3 induces activation the PI3K/AKT pathway and Src-ERK1/2 pathways. PRL-3 phosphatase activity upregulates LIN28B, a stem cell reprogramming factor, which in turn represses the let-7 mRNA family, inducing a stem cell-like transcriptional program. These pathways support epithelial-mesenchymal transition and results in metastatic processes. PRL-3 is detected in more than 50% of patients with AML [27, 52].

miRNA-16. miRNA-16 is tumor suppressor sncRNAs. miRNA-16 targets anti-apoptotic gene Bcl-2 (B-cell lymphoma 2) and numerous genes, which are involved in cell cycle regulation. Those genes are cyclin D1, D3, E1 and cyclin-dependent kinase 6 (CDK6). miRNA-16 regulates genes supporting Wnt-signaling pathway, such as WNT3A (wingless-type MMTV integration site family, member 3A). As a result, miRNA-16 can fine-tune cell cycle, support cell apoptosis and inhibit cell proliferation [28, 53]. Erythropoiesis is supported by miRNA-16 and its expression positively correlates with the appearance of erythroid surface antigens (CD36, CD71 and CD235a) [29, 54]. Its level is downregulated in APL. After treatment, ATRA levels of miRNA-16 increase [26, 51].

miRNA-17-92. miRNA-17-92 is one of the most investigated oncogenic miRNA complex in leukemia. Cluster 17–92 is complex of seven co-operating miRNAs. Members of this cluster are miRNA-17, miRNA-17*, miRNA-18a, miRNA-19a, miRNA-20a, miRNA-19b-1 and

miRNA-92a-1. These miRNAs are located at 13q31. The majority of miRNAs from this cluster are overexpressed in MLL-rearranged AML. These miRNAs can support cell proliferation, cell viability, inhibit apoptosis, suppress cell differentiation and induce malignant transformation. In normal hematopoiesis, this complex plays essential roles in monocytopoiesis and megakaryocytopoiesis. Targets for 17–92 complex are factors of cellular proliferation E2F1, E2F2, E2F3, PTEN (tumor suppressor phosphatase and tensin homolog), BIM (pro-apoptotic molecule), AML1, TGFBR2, NCOA3, RBL2, DOCK5, THBS1 and CTGF. RASSF2 and RB1 genes are target genes in leukemia [29–33, 38, 54–57]. In vivo cluster 17–92 expression significantly decreases leukemia latency [34, 40].

miRNA-29. The miRNAs-29 family consists of miRNA-29a, miRNA-29b and miRNA-29c, and is encoded and transcribed in tandem by two genes located on chromosome 7q32.3 (miRNA-29a/b1 locus) or chromosome1q32.2 (miRNA-29a/b2 locus), respectively. As was shown recently, miRNA-29a and miRNA-29b regulate critical anti-apoptotic genes, such as myeloid cell leukemia-1 (MCL-1) and play tumor suppressor role. These results indicated that miRNA-29a and miRNA-29b target Mcl-1 and play roles in regulation of apoptosis in AML. Bcl-2, Mcl-1 and Bcl-Xl are anti-apoptotic proteins and promote cell survival and proliferation. Both miRNA-29a and miRNA-29b not only directly target anti-apoptotic genes but also upregulate pro-apoptotic genes, such as BIM (BCL2L11), p53 and the tumor suppressor programmed cell death-4 (PDCD4) [35, 36, 58, 59]. Overexpression of miRNA-29a, miRNA-29b or miRNA-2c markedly inhibited cell proliferation and promoted cell apoptosis by targeting protein kinase B2 (AKT2) and cyclin D2 mRNAs.

miRNA-29b targets DNA methyltransferases (DNMTs) and downregulates DNA methylation in malignant cells. In AML cells, enhanced expression of miRNA-29b results in the reduction of the expression of DNMT1, DNMT3A and DNMT3B. miRNA-29b downregulates DNMT1 indirectly by targeting Sp1, a trans-activator of the DNMT1 gene due to a decrease in global DNA methylation and re-expression of p15 (INK4b) and ESR1. Moreover, miRNA-29 family members may downregulate the active DNA demethylation pathway members tet-methylcytosine dioxygenase (TET1) and thymine DNA glycosylase (TDG) [37, 60]. miRNA-29 targets CCNT2 that is a component of the positive transcription elongation factor b (P-TEFb). P-TEFb is essential for the elongation of transcription and co-transcriptional processing by RNA polymerase II. Different P-TEFb complexes can regulate subsets of distinct genes that are important for the embryonic development. miRNA-29 family miRNAs downregulate cyclin-dependent kinase 6. It provides a differentiation promoting activity of Runx proteins to be selectively activated in terminally differentiating cells [38, 61].

miRNA-30. miRNA-30 as was shown is tumor suppressor miRNA in AML. miRNA-30 downregulates NOTCH1 gene expression to promote granulocytic differentiation [39, 62].

miRNA-146. miRNA-146a and miRNA-146b are two miRNA, which are highly expressed in primitive bone marrow cells (lineage negative). In mature blood cells, their expressions are different. miRNA-146a is highly expressed in blood cells maturation lineage. The expression of miRNA-146a is regulated by a combination of PU.1 (spleen focus-forming virus proviral integration 1) and c-ETS. It was supposed, that miRNA-146a controls maturation of blood marrow stem cells in adults and plays role in the self-renewal of long-term hematopoietic

stem cells. The decrease or deletion of miRNA-146 levels could result in development of AML in adults [40, 63]. miRNA-146a negatively regulates NF-kB by suppressing two signal transducers, TRAF6 (TNF receptor-associated factor 6) and IRAK1 (IL-1 receptor-associated kinase 1). Absence of miRNA-146a expression is due to NF-kB hyper-expression, which involves miRNA-146a/TRAF6/NF-kB/IL-6 pathway resulting in depletion of hematopoietic stem cells and the development of myeloproliferative diseases [41, 64]. miRNA-146a also downregulates CSF1R. Increased expression of this factor is strongly associated with the development of AML [42, 65]. Furthermore, high expression of miRNA-146a impaired bone marrow reconstitution in mice and reduced survival of hematopoietic stem cells. Treatment of AML patients with ATRA results in downregulation of miRNA-146a, which is associated with upregulation of Smad4 pathway [43, 66]. miRNA-146a is downregulated in MLL rearrangements. MLL-rearranged AML MEIS1 and HOXA9 are key target genes of MLL-fusion proteins. MEIS1/HOXA9 downregulate PU.1 expression, which is essential for miRNA-146a expression. Low levels of miRNA-146a result in high levels of SYK and its activation. SYK expression and activation is switch leukemic cellular transformation [44, 67].

miRNA-150. miRNA-150 is one of the main regulators of hematopoiesis. It is expressed in normal hematopoietic stem cells. The known target for miRNA-150 is Myb, which regulates differentiation of progenitor cells toward megakaryocytes and erythrocytes, supporting c-KIT expression. In majority of cases of patients with AML this miRNA is downregulated [45, 68]. Downregulation of miRNA-150 is associated with misbalance of regulatory factors. MLL-fusion proteins bind to the promoter regions of miRNA-150 and directly promote its expression, however, blocking maturation and biogenesis of this miRNA [46, 47, 69, 70]. Repression of miRNA-150 maturation is associated with expression of Myc protein. Myc is a direct target for MLL-fusion protein, which binds to the promoter region of LIN28 and activates its transcription [48, 71]. miRNA-150 is an important regulator of hematopoietic recovery after treatments with chemotherapeutic drugs. miRNA-150 targets other genes that are necessary for leukemogenesis. These genes are HOXA7, Meis1 and cyclin-dependent kinase 2 [38, 72].

miRNA-155. miRNA-155 expressed in high levels in normal HSC and in low in mature hematopoietic precursors. It controls early maturation of HSC until common myeloid progenitor stage. miRNA-155 supports the leukemogenesis by downregulation of SH2 domain-containing inositol 5′-phosphatase 1 (SHIP1) translation, activating SHIP1-mediated PI3K-Akt pathway. This event is due to myeloproliferation and decrease of erythropoiesis. SHIP1 downregulation results in activation of IL-6 signaling pathway and induces a reactive proliferation of the relatively apoptosis-resistant myeloid precursor cells [49, 73]. Key targets of miRNA-155 are PU.1 and Cebpb in myeloid progenitors. PU.1 transcriptionally activates miRNA-155, forming positive regulatory loop. PU.1 and CCAAT/enhancer-binding protein b (C/EBPb) transcriptionally regulate expression of myeloid-specific miRNA-223, which upregulates myelopoiesis by indirectly regulating C/EBPa [50, 51, 74, 75]. miRNA-155 promotes production of inflammatory cytokines in mature myeloid cells. It negatively regulates anti-inflammatory factors, such as SHIP1 and SOCS1 [52, 76]. miRNA-155 reduces apoptotic activ ity of myeloid cells in vitro and in vivo by the regulation of Akt-signaling pathway [53, 54, 77, 78]. In silico target prediction identified a number of putative miRNA-155 target genes, and the

expression changes transcription factors of myeloid proliferation and apoptosis, such as MEIS1, GF1, cMYC JARID2, cJUN, FOS, CTNNB1 and TRIB2 [55, 79].

miRNA-196. miRNA-196 is a member of homebox regions (HOX) clusters family miRNAs: miRNA-10, 196 and 615. HOX genes encode transcription factors, which control embryonic development. miRNA-196 are highly expressed in AML. MLL-fusion proteins directly upregulate expression of miRNA-196, which is necessary for leukemic cells immortalization. miRNA-196 blocks granulocyte colony-stimulating factor-induced granulopoiesis [56, 80]. Recently it determined double-faced regulation by the miRNA-196 of MLL-associated AML. miRNA-196 may be double-faced Janus in MLL-rearranged AML. On the one hand, it targets MLL-associated HOXA9/MEIS1 oncogenes; on the other hand, it regulates first apoptosis signal (FAS) (also known as Apo-1 or CD95) tumor suppressor gene. High expression of miRNA-196 is associated with the more aggressive leukemic phenotypes and worse prognosis for patients with leukemia [38, 57, 81, 82].

miRNA-223. miRNA-223 is an intergenic miRNA, which is highly expressed in human peripheral blood granulocytes and bone marrow-committed myeloid precursors. It is regulated by two factors PU.1 and C/EBPb [40, 58, 59, 83–85]. miRNA-223 has tumor-suppressive role in AML, and its expression is downregulated in this disease. Targets for miRNA-223 are erythrocyte membrane protein band 4.1 like 3, septin 6, FBXW7, ataxia teleangiectasia mutated, insulin-like growth factor 1, paired box 6 and caprin-1. FBXW7 (F-box protein in the SCF E3 ligase complex) protein leads to ubiquitination of binding proteins and proteasomal degradation. It regulates cell proliferation, differentiation, cell cycle, migration and invasion. miRNA-223 downregulates this factor and results in inhibition of cell proliferation and enhancing apoptosis [60, 86]. Other target genes for miRNA-223 are LMO2, NFI-A, MEF2C (myocyte enhancer factor) and E2F1 (adenovirus E2 promoter-binding factor 1). In normal conditions, increased expression of miRNA-223 promotes differentiation toward granulocytes. Its repression associated with differentiation to erythrocytes and monocytes/macrophages [40, 61, 83, 87]. C/EBPa regulates miRNA-223 that blocks myeloid cell cycle progression by targeting E2F1. Overexpression of E2F1 results in repression of miRNA-223 gene expression by the negative feedback [62, 88].

2.3. Piwi-interacting RNAs

Piwi-interacting RNAs is the most numerous class of sncRNAs, whose action in normal and malignant hematopoiesis is less studied. Piwi-regulatory proteins are Argonaute family proteins, which are essential for germ stem cells self-renewal and maintenance. They are encoded by highly conserved PIWI genes. Human genome has four piwi-coding genes: HIWI, HILI, HIWI3 and HIWI2. Piwi proteins are associated with piRNAs and they act in cooperation with each other. Firstly, piwi proteins were identified in germ cells and then in somatic cells [63, 89]. A growing number of studies have found that piwi proteins in humans and mice, specifically, HIWI, PIWIL2 and PIWIL2-like proteins, are expressed in various types of tumor cells. In addition, piRNAs were also detected in these cells [64, 90].

In AML, piwi proteins and piRNAs are deregulated. Recently, it was shown that high expression of piwi-like protein 4 in leukemic cells in 72% of the patients with different types of AML

compared to healthy controls, and in 90% of the patients with MLL-AF9 rearranged AML [13, 65]. PiRNAs act as sequence-specific guides for piwi proteins, and they support biogenesis and stability of piRNAs. Piwi and piRNAs are involved in the intra-nuclear processes such as heterochromatin formation, mobile transposable elements (TE) silencing and repressive histone modifications (H3K9me3). The piwi protein PIWIL4 is overexpressed in a large proportion of AML leukemia patients. This knockdown results in gross changes in histone methylation and slowed leukemic growth. It suggests a tightly regulated piwi pathway is essential for normal hematopoiesis [66, 91].

Mobile transposable elements, which are under the control of piwi and piRNAs constitute approx. 44% of the human genome. TE could actively contribute to genetic heterogeneity, to alter the behavior during adaptation, and responses to stress. Somatic retrotransposition and its associated insertional mutagenesis have particularly important implications for carcinogenesis and are often associated with different cancers. TE activity can generate a wide spectrum of genomic mutations, ranging from point mutations to gross rearrangements. These events may be due to development of different diseases. Mobile transposable elements such as duplications caused by Alu-Alu (SINE type of TEs) recombination in intron 1 and 6, result in a duplication of exons 2–6 of gene MLL1 [67, 68, 92, 93]. Chromosomal rearrangements in MLL are result of Alu recombination. In partial duplication events, TEs are inserted near the translocation breakpoints. MYB and MLL duplications were also obtained in healthy controls, whereas leukemogenesis is induced by TE insertions during blood cell differentiation [69, 94]. Knockdown of the murine piwi protein MIWI2 leads to abnormal hematopoiesis and ery throid precursors take on characteristics of more differentiated erythroid cells [70, 71, 95, 96]. The roles of piRNAs in hematopoiesis have only just begun to be explored and it seems likely that more will be uncovered in the near future. Certainly, regulation of mobile transposable elements, histone modificatiowns, DNA methylation processes by piwi and piRNAs are important for leukemogenesis and for tumor suppression. Investigation of new piwi/piRNAs biomarkers will be a novel diagnostic tool and possible hopeful epigenetic treatment of patients with AML in future.

2.4. SncRNAs as markers of therapeutic resistance or sensitivity to AML treatment

All sncRNAs from the view of chemoresistance and sensitivity of AML patients to the current treatment can be assigned to the two groups. Group 1 characterized with high level of expression of sncRNAs, which associated with sensitivity to therapy. This group members are miRNA-181a, b; let-7f, miRNA-9*, miRNA-96, miRNA-135a, miRNA-409, miRNA-10, miRNA-29b and miRNA-217 [72, 73, 97–100]. Group 1 members act through activation of cellular differentiation, inhibition of leukemic cells proliferation and survival, regulation of cell cycle, inducing of apoptosis and suppression of migration and invasion. Group 2 characterized with high expression of sncRNAs, which associated with resistance to therapy. Group 2 members are miRNA-155, miRNA-125b, miRNA-126, miRNA-210, miRNA-3151, miRNA-196b, miRNA-199a, miRNA-191, miRNA-644, miRNA-363, miRNA-532-5p and miRNA-342-3p [74, 101].

miRNA-181a is the most investigated miRNA with its chemosensitive action on leukemic cells. Investigations of samples from cohort of patients of different ages, miRNA-181a was

identified as marker of chemosensitivity [75, 102]. The main function of this miRNA is activation of apoptotic activity in cells and inducing sensitivity of leukemic to native immunity action. Direct targets for this miRNA are KRAS, NRAS, transcription factor Prospero homebox protein 1 (Prox1) and HMGB1, a member of the high-mobility group box DNA-binding proteins. The CD4 co-receptor is another target for miRNA-181a. Anti-proliferative effect of miRNA-181a is associated with targeting of FBJ murine osteosarcoma viral oncogene homolog (FOS), which is involved in Moloney murine sarcoma viral oncogene homolog (MOS)/dual specificity mitogen-activated protein kinase (MEK)/mitogen-activated protein kinase (ERK) pathway [76, 103]. miRNA-181a can sensitize leukemic cells to natural killer (NK) cell action [77, 104]. miRNA-181a regulates mechanisms of cell proliferation, differentiation and apoptosis of normal cells. All regulated targets for miRNA-181a are aberrantly expressed and are frequently mutated in acute myeloid leukemia. Promotion of high expression of miRNA-181 in the case of AML may have partial positive effect for chemosensitivity of leukemic cells and for anti-leukemic activity [78, 79, 105, 106].

Other miRNA, which enhances chemosensitivity of leukemic cells is miRNA-217. This sncRNA decreases leukemic cell proliferation via the cell apoptosis pathway. It targets KRAS regulator of signaling links from extracellular space to the nucleus. KRAS connects multiple upstream signals to various downstream signaling pathways [97].

miRNA-155 is oncogenic miRNA, whose high expression is able increase chemoresistant effects of anti-leukemic drugs. Its increased expression is correlated with decreased CR rates and a shorter overall survival (OS) of patients with AML. In our laboratory, primary in vitro leukemic cells were transformed into megakaryocytes after using complexes of polymer carrier with antgo-miRNA-155 [80, 107].

Another miRNA from the second group is miRNA-126. High expression of this miRNA correlated with decreased CR rates and shortened OS. Treatment with nanoparticle-based antagonist of miRNA-126 results in strong anti-leukemic effects in murine leukemia models and chemo-sensitizing effects of cytarabine and idarubicin in AML cell lines.

Recently determined miRNAs predict the prognosis of AML. From the amount of miRNAs were indicated miRNAs, which expression is dysregulated in patients with poor prognosis of AML. miRNA-107, miRNA-155, miRNA-25, miRNA-29b and miRNA-196a are associated with short OS of patients. The worse prognostic marker was miRNA-25 expression dysregulation [76].

3. Conclusion

The normal bone marrow stressor or injury factors act as dysregulators of epigenetic and genetic program of hematopoietic stem cells, which is due to downregulation of separated sncRNAs and upregulation of other sncRNAs. These events result in the development of imbalance of regulating cell program and consequent transformation of normal hematopoietic cells into leukemic. In this case, genotypic and phenotypic markers of pathologic cells

are changing. These cells change its functions. They lose possibility to normal lifecycle: to differentiation, maturation and death. As a consequence, leukemic cells produce abnormal proteins and express particular palette of sncRNAs, which worsens the negative impact into development of AML.

The chemotherapy may change expression map of sncRNAs in the advantageous or in the disadvantageous position. The study of this map may be useful tool for hematologists and hemato-oncologists in the diagnostic of course, efficacy and prognosis of AML in the particular case. In the course of chemotherapy, downregulation of oncogenic sncRNAs and inducing of tumor suppressor sncRNAs associates with favorable prognosis of disease. On the contrary, inhibition of sncRNAs with tumor suppressive properties and high expression of oncogenic sncRNAs due to unfavorable prognosis of AML.

Reversing of changed epigenetic program of cells and its supporting may be the novel tool for the treatment of different hematological malignancies even which have poor prognosis. Treatment with anti-oncomiRNAs and inducing of producing, biogenesis and maturation of miRNAs with tumor suppressive properties may be new therapeutic benefit in complex therapy of AML. However, limitations to this treatment modality include the instability of free-floating anti-miRNAs in the plasma and their vulnerability to breakdown by nucleases, nonspecific tissue uptake and renal clearance. These limitations can be overcome by nanoparticle-based delivery of the anti-miRNAs to target tissues.

Author details

Oxana V. Klimenko

Address all correspondence to: o_klimenko@hotmail.com

SID ALEX GROUP, Ltd. Prague, Czechia

References

[1] Palazzo AF, Lee ES. Non-coding RNA: What is functional and what is junk? Frontiers in Genetics. 2015;**6**:2. He L, Hannon GJ. MicroRNAs: Small RNAs with a big role in gene regulation. Nature Reviews. Genetics 2004 Jul;**5**(7):522-531

[2] Pauli A, Rinn JL, Schier A. Non-coding RNAs as regulators of embryogenesis. Nature Reviews. Genetics. 2011;**12**(2):136-149

[3] Hata A, Lieberman J. Dysregulation of microRNA biogenesis and gene silencing in cancer. Science Signaling. 2015;**8**(368):1-12

[4] Wu N, Yang BB. The biological functions of non-coding RNAs: From a line to a circle. Discover. 2015;**3**(3):e48-e63

[5] Gu S, Kay MA. How do miRNAs mediate translational repression? BMC: Silence. 2010;**1**:11

[6] Li S, Liu L, Zhuang X, Yu Y, Liu X, Cui X, Ji L, Pan Z, Cao X, Mo B, Zhang F, Raikhel N, Jiang L, Chen X. microRNAs inhibit the translation of target mRNAs on the endoplasmic reticulum in Arabidopsis. Cell. 2013;**153**(3):562-574

[7] Dalmay T. Mechanism of miRNA-mediated repression of mRNA translation. Essays in Biochemistry. 2013;**54**:29-38

[8] Hu W, Coller J. What comes first: Translational repression or mRNA degradation? The deepening mystery of microRNA function. Cell Research. 2012;**22**:1322-1324

[9] Bhat SS, Jarmolowski A, Szweykowska-Kulinska Z. MicroRNA biogenesis: Epigenetic modifications as another layer of complexity in the microRNA expression regulation. Acta Biochimica Polonica. 2016;**63**:1-7

[10] Kim VN, Small RNA. Classification, biogenesis, and function. Molecules and Cells. 2005;**19**(1):1-15

[11] Melo CA, Melo SA. Biogenesis and physiology of microRNAs. In: Fabbri M, editor. Non-coding RNAs and Cancer. Vol. VI. Springer; NY. 2014. p. 5-24

[12] Martens-Uzunova ES, Olvedy M, Jenster G. Beyond microRNA—Novel RNAs derived from small non-coding RNA and their implication in cancer. Cancer Letters. 2013;**340**:201-211

[13] Hayashi R, Schnabl J, Handler D, Mohn F, Ameres SL, Brennecke J. Genetic and mechanistic diversity of piRNA 3′-end formation. Nature Letter. 2016;**539**:588-592

[14] Nandi S, Chandramohan D, Fioriti L, Melnick AM, Hebert JM, Mason CE, Rajasethupathy P, Kandel ER. Roles for small noncoding RNAs in silencing of retrotransposons in the mammalian brain. PNAS;**113**(45):12697-12702

[15] Saito K, Inagaki S, Mituyama T, Kawamura Y, Ono Y, Sakota E, Kotani H, Asai K, Siomi H, Siomi MC. A regulatory circuit for piwi by the large Maf gene traffic jam in Drosophila. Nature. 2009;**461**:1296-1301

[16] Sato K, Siomi MC. Piwi-interacting RNAs: Biological functions and biogenesis. Essays in Biochemistry. 2013;**54**:39-52

[17] Thomson T, Lin H. The biogenesis and function PIWI proteins and piRNAs: Progress and prospect. Annual Review of Cell and Developmental Biology. 2009;**25**:355-376

[18] Weick E-M, Miska EA. piRNAs: From biogenesis to function. Development. 2014;**141**: 3458-3471

[19] Costa MC, Leitao AL, Enguita FJ. Biogenesis and mechanism of action of small non-coding RNAs: Insight from the point of view of structural biology. International Journal of Molecular Sciences. 2012;**13**:10268-10295

[20] Katahira J, Yoneda Y. Nucleocytoplasmic transport of microRNAs and related small RNAs. Traffic. 2011;**12**:1468-1474

[21] Iwasaki YW, Siomi MC, Siomi H. PIWI-interacting RNA: Its biogenesis and functions. Annual Review of Biochemistry. 2015;**84**:405-433

[22] Ishizu H, Siomi H, Siomi MC. Biology of PIWI-interacting RNAs: New insights into biogenesis and function inside and outside of germlines. Genes & Development. 2012; **26**:2361-2373

[23] Luteijn MJ, Ketting RF. PIWI-interacting RNAs: From generation to transgenerational epigenetics. Nature Reviews. Genetics. 2013;**14**:523-534

[24] Meister G. Argonaute proteins: Functional insights and emerging roles. Nature Reviews. Genetics. 2013;**14**:447-459

[25] Su J-L, Chen P-S, Johansson G, Kuo M-L. Function and regulation of let-7 family microRNAs. MicroRNA. 2012;**1**:34-39

[26] Huang T, Alvarez A, Hu B, Cheng S-Y. Noncoding RNAs in cancer and cancer stem cells. Chinese Journal of Cancer. 2013;**32**(11):582-593

[27] Calore F, Lovat F, Garofalo M. Non-coding RNAs and cancer. International Journal of Molecular Sciences. 2013;**14**(8):17085-17110

[28] Fabbri M, Garzon R, Andreeff M, Kantarjian HM, Garcia-Manero G, Calin GA. MicroRNAs and noncoding RNAs in haematological malignancies: Molecular, clinical and therapeutic implications. Leukemia. 2008;**22**:1095-1105

[29] Arber DA, Orasi A, Hasserjian R, Thiele J, Borowitz MJ, Beau ML, Bloomfield CD, Cazzola M, Vardiman JW. The 2016 revision to the World Health Organisation (WHO) classification of myeloid neoplasms and acute leukemia. Blood. 2016;**127**:2391-2405

[30] Acute Myeloid Leukemia (AML)–Associated Oncofusion Proteins (Cancer Genome Anatomy Project. In: Mitelman F, Johansson B, Mertens F, editors. Mitelman Database of Chromosome Aberrations and Gene Fusions in Cancer; Lund; Sweden. 2017

[31] Kumar CC. Genetic abnormalities and challenges in the treatment of acute myeloid leukemia. Genes & Cancer. 2011;**2**(2):95-107

[32] How Is Acute Myeloid Leukemia Classified? The American Cancer Society medical and editorial content team; Atlanta, GA. 22 February, 2016

[33] Organista-Nava J, Gomez-Gomez Y, Illades-Aguiar B, Leyva-Vazques MA. Expression of miRNAs associated with the AML 1-ETO, CBFB-MYH1, PML-RARA and AF9-MLL oncoproteins in acute myeloid leukemia. Journal of Human Anatomy & Physiology. 2015;**1**(1):01-05

[34] Shahjahani M, Khodadi E, Seghatoleslami M, Asl JM, Golchin N, Zaieri ZD, Saki N. Rare cytogenetic abnormalities and alteration of microRNAs in acute myeloid leukemia and response to therapy. Oncology Reviews. 2015;**9**(1):261

[35] Gordon JEA, Wong JJ-L, Rasko JEJ. MicroRNAs in myeloid malignancies. British Journal of Haematology. 2013;**162**(2):162-176

[36] Obulkasim A, Katsman-Kuipers JE, Verboon L, Sanders M, Touw I, Jongen-Lavrencic M, Pieters R, Klusmann J-H, Zwaan CM, van den Heuvel-Eibrink MM, Forenrod M. Classification of pediatric acute myeloid leukemia based on miRNA expression profiles. Oncotarget. 2017;**8**(20):33078-33085

[37] Kumar S, Bakhshi S. Diagnostic and prognostic role of microRNAs in paediatric acute myeloid leukaemia. The Indian Journal of Medical Research. 2016;**144**:807-814

[38] Marcucci G, Mrozek K, Radmacher MD, Garzon R, Bloomfield CD. The prognostic and functional role of microRNAs in acute myeloid leukemia. Blood. 2011;**117**(4):1121-1129

[39] Mogilyansky E, Rigoutsos I. The miRNA-17/92 cluster: A comprehensive update on its genomics, genetics, functions and increasingly important and numerous roles in health and disease. Cell Death and Differentiation. 2013;**20**:1603-1614

[40] Schotte D, Pieters R, Den Boer ML. MicroRNAs in acute leukemia: From biological players to clinical contributors. Leukemia. 2012;**26**:1-12

[41] Luan CX, Yan ZX, Chen B. The functional role of microRNA in acute lymphoblastic leukemia: Relevance for diagnosis, differential diagnosis, prognosis, and therapy. OncoTargets and Therapy. 2015;**8**:2903-2914

[42] Kotagama K, Chang Y, Mangone M. miRNAs as biomarkers in chronic myelogenous leukemia. Drug Development Research. 2015;**76**(6):278-285

[43] Lopotova T, Zackova M, Klamova H, Moravcova J. MicroRNA-451 in chronic myeloid leukemia: miRNA-451-BCR-ABL regulatory loop? Leukemia Research. 2011;**35**(7):974-977

[44] Shah NM, Bowles KM, Rushworth SA, MacEwan DJ. Understanding the role for miRNA in human leukemia. RNA Disease. 2015;**2**:e540

[45] Zhi F, Cao X, Xie X, Wang B, Dong W, Gu W, Ling Y, Wang R, Yang Y. Identification of circulating microRNAs as potential biomarkers for detecting acute myeloid leukemia. PLoS One. 2013;**8**(2):e56718

[46] Wang X, Zhu B, Huang Z, Chen L, He Z, Zhang H. MicroRNAs as biomarkers in leukemia. Stem Cell Investigation. 2014;**1**:11

[47] Chiu Y-C, Tsai M-H, Chou W-C, Liu Y-C, Kuo Y-Y, Hou H-A, Lu TP, Lai L-C, Chen Y, Tien H-F, Chuang EY. Prognostic significance of NPM1 mutation-modulated microRNA-mRNA regulation in acute myeloid leukemia. Leukemia. 2016;**30**(2):274-284

[48] Ciccone M, Calin GA. MicroRNA in myeloid haematological malignancies. Current Genomics. 2015;**16**(5):336-348

[49] Pulikkan JA, Peramangalam PS, Dengler V, Ho PA, Preudhomme C, Meshinchi S, Christopeit M, Nibourel O, Muller-Tidow C, Bohlander SK, Tenen DG, Behre G. C/EBPa

regulated microRNA-34a targets E2F3 during granulopoiesis and is down-regulated in AML with CEBPA mutations. Blood. 2010;**116**(25):5638-5649

[50] Zhou J, Bi C, Ching YQ, Chooi J-Y, Lu X, Quah JY, Toh SH-M, Chan Z-L, Tan TZ, Chong PSY, Chng W-J. Inhibition of LIN28B impairs leukemia cell growth and metabolism in acute myeloid leukemia. Journal of Hematology & Oncology. 2017;**10**:138-150

[51] Pelosi A, Careccia S, Lulli V, Romania P, Marziali G, Testa U, Lavorgna S, Lo-Coco F, Petti MC, Calabretta B, Levrero M, Piaggio G, Rizzo MG. miRNA let-7c promotes granulocytic differentiation in acute myeloid leukemia. Oncogene. 2013;**32**:3648-3654

[52] Chen J, Odenike O, Rowley JD. Leukemogenesis: More than mutant genes. Nature Reviews. Cancer. 2010;**10**(1):23-36

[53] Zhou J, Chan Z-L, Bi C, Lu X, Chong PSY, Chooi J-Y, Cheong L-L, Liu S-C, Ching YQ, Zhou Y, Osato M, Tan TZ, Ng CH, Ng S-B, Wang S, Zeng Q, Chng W-J. LIN28B activation by PRL-3 promotes leukemogenesis and a stem cell-like transcriptional program in AML. Molecular Cancer Research. 2017;**15**(3):294-303

[54] Yan X, Liang H, Deng T, Zhu K, Zhang S, Wang N, Jiang X, Wang X, Liu R, Zen K, Zhang C-Y, Ba Y, Chen X. The identification of novel targets of miRNA-16 and characterization of their biological functions in cancer cells. Molecular Cancer. 2013;**12**:92-102

[55] Bissels U, Bosio A, Wagner W. MicroRNAs are the hematopoietic landscape. Haematologica. 2012; **97**(2): 160-167. Hong S-H, Kim K-S, Oh I-H. Concise review: Exploring miRNAs-Toward a better understanding of hematopoiesis. Stem Cells. 2015;**33**:1-7

[56] Mi S, Li Z, Chen P, He C, Cao D, Elkahloun A, Lu J, Pelloso LA, Wunderlich M, Huang H, Luo RT, Sun M, He M, Neily MB, Zelenik-Le NJ, Thriman MJ, Mulloy JC, Liu PP, Rowley JD, Chen J. Aberrant overexpression and function of the miRNA-17-92 cluster in MLL-rearranged acute leukemia. PNAS. 2010;**107**(8):3710-3715

[57] Yan Y, Kasar S, Underbayev C, Prakash S, Raveche E. MicroRNAs in acute myeloid leukemia and other blood disorders. Leukemia Research and Treatment. 2012;**2012**:1-12

[58] Wong P, Iwasaki M, Somervaille TCP, Ficara F, Carico C, Arnold C, Chen C-Z, Cleary ML. The miRNA-17-92 microRNA polycistron regulates MLL leukemia stem cell potential by modulating p21 expression. Cancer Research. 2010; April 20:3833-3842

[59] Garzon P, Heaphy CEA, Havelange V, Fabbri M, Volinia S, Tsao T, Zanesi N, Kornblau SM, Marcucci G, Calin GA, Andreeff M, Croce CM. MicroRNA 29b functions in acute myeloid leukemia. Blood. 2009;**114**(26):5331-5341

[60] Xu L, Xu Y, Jing Z, Wang X, Zha X, Zeng C, Chen S, Yang L, Luo G, Li B, Li Y. Altered expression pattern of miRNA-29a, miRNA-29b and the target genes in myeloid leukemia. BMC: Experimental Hematol Oncol. 2014;**3**:17

[61] Yan B, Guo Q, Fu F-J, Wang Z, Yin Z, Wei Y-B, Yang J-R. The role of miRNA-29b in cancer: Regulation, function and signalling. OncoTargets and Therapy. 2015;**8**:539-548

[62] Wang X-S, Gong J-N, Yu J, Wang F, Zhang X-H, Yin X-L, Tan Z-Q, Luo Z-M, Yang G-H, Shen C, Zhang J-W. MicroRNA-29a and microRNA-142-3p are regulators of myeloid differentiation and acute myeloid leukemia. Blood. 2012;**119**(21):4992-5004

[63] Katzerke C, Madan V, Gerloff D, Brauer-Hartmann D, Hartmann J-U, Wurm AA, Muller-Tidow C, Schnittger S, Tenen DG, Niederweisser D, Behre G. Transcription factor C/EBPa-induced micro-RNA-30c inactivates Notch1 during granulopoiesis and is down-regulated in acute myeloid leukemia. Blood. 2013;**122**(14):2433-2442

[64] Zhao JL, Starczynowski DT. Role of microRNA-146a in normal and malignant hematopoietic stem cell function. Frontiers in Genetics. 2014;**5**:219

[65] Zhao JL, Rao DS, O'Connell RM, Garcia-Flores Y, Baltimore D. MicroRNA-146a acts as a guardian of the quality and longevity of hematopoietic stem cells in mice. eLife. 2013; **2**:e00537

[66] Boldin MP, Taganov KD, Rao DS, Yang L, Zhao JL, Kalwani M, Garcia-Flores Y, Luong M, Devrekanli A, Xu J, Sun G, Tay J, Linsley PS, Baltimore D. miRNA-146a is a significant brake on autoimmunity, myeloproliferation, and cancer in mice. JEM. 2011;**208**(6):1189

[67] Labbaye C, Testa U. The emerging role of MIRNA-146A in the control of hematopoiesis, immune function and cancer. Journal of Hematology & Oncology. 2012;**5**:13

[68] Testa U, Pelosi E, Castelli G, Labbaye C. miRNA-146 and miRNA-155: Two key modulators of immune response and tumor development. Non-Coding RNA. 2017;**3**(3):22

[69] Fayyad-Kazan H, Bitar N, Najar M, Lewalle P, Fayyad-Kazan M, Badran R, Hamade E, Daher A, Hussein N, ELDirani R, Berri F, Vanhamme L, Burny A, Martiat P, Rouas R, Badran B. Circulating miRNA-150 and miRNA-342 in plasma are novel potential biomarkers for acute myeloid leukemia. Journal of Translational Medicine. 2013;**11**:31

[70] He Y, Jaiang X, Chen J. The role of miRNA-150 in normal and malignant hematopoiesis. Oncogene. 2014;**33**:3887-3893

[71] Morris VA, Zhang A, Yang T, Stirewalt DL, Ramamurthy R, Meshinchi S, Oehler VG. Micro-RNA-150 expression induces myeloid differentiation of human acute leukemia cells and normal hematopoietic progenitors. PLoS One. 2013;**8**(9):e75815

[72] Jiang X, Huang H, Li Z, Wang X, Gurbuxani S, Chen P, He C, You D, Zhang S, Wang J, Arnovitz S, Elkahloun A, Price C, Hong G-M, Ren H, Kunjamma RB, Neilly MB, Matthews JM, Xu M, Larson RA, Le Beau MM, Slany RK, Liu PP, Lu J, Zhang J, He C, Chen J. Blockade of miRNA-150 maturation by MLL-fusion/MYC/LIN-28 is required for MLL-associated leukemia. Cancer Cell. 2012;**22**(4):524-535

[73] Bousquet M, Zhuang G, Meng C, Ying W, Cheruku PS, Shie AT, Wang S, Ge G, Wong P, Wang G, Safe S, Zhou B. miRNA-150 blocks MLL-AF9-associated leukemia through oncogene repression. Molecular Cancer Research. 2013;**11**(8):912-922

[74] Ranganath P. MicroRNA-155 and its role in malignant hematopoiesis. Biomarker Insights. 2015;**10**:95-102

[75] O'Connell RM, Rao DS, Chaudhuri AA, Boldin MP, Taganov KD, Nicoll J, Paquette RL, Baltimore D. Sustained expression of microRNA-155 in hematopoietic stem cells causes a myeloproliferative disorder. JEM. 2008;**205**(3):585-594

[76] Bhise NS, Chauhan L, Shin M, Cao X, Pounds S, Lamba V, Lamba JK. Micro-RNA-mRNA pairs associated with outcome in AML: From in vitro cell-based studies to AML patients. Frontiers in Pharmacology. 2015;**6**:324

[77] Joyce CE, Novina CD. miRNA-155 in acute myeloid leukemia: Not merely a prognostic marker? Journal of Clinical Oncology. 2013;**31**(17):2219-2221

[78] Xue H, Hua L-M, Guo M, Luo J-M. SHIP1 is targeted by miRNA-155 in acute myeloid leukemia. Oncology Reports. 2014;**32**(5):2253-2259

[79] Zitzer NC, Ranganathan P, Dickinson BA, Jackson AL, Rodman DM, Croce CM, Marcucci G, Garzon R. Preclinical development of LNA antimiRNA-155 (MRG-106) in acute myeloid leukemia. Blood. 2015;**126**:3802

[80] Palma CA, Sheikha DA, Lim TK, Bryant A, Vu TT, Jayaswal V, Ma DDF. MicroRNA-155 as an inducer of apoptosis and cell differentiation in acute myeloid leukemia. Molecular Cancer. 2014;**13**:79

[81] Chen C, Zhang Y, Zhang L, Weakley SM, Yao Q. MicroRNA-196: Critical roles and clinical applications in development and cancer. Journal of Cellular and Molecular Medicine. 2011;**15**(1):14-23

[82] Li Z, Huang H, Chen P, He M, Li Y, Arnovitz S, Jiang X, He C, Hyjek E, Zhang J, Zhang Z, Elkahloun A, Cao D, Shen C, Wunderlich M, Wang Y, Neilly MB, Jin J, Wei M, Lu J, Valk PJM, Delwel R, Lowenberg B, Le Beau MM, Vardiman J, Mulloy JC, Zeleznik-Le NJ, Liu PP, Zhang J, Chen J. miRNA-196b directly targets both HOXA9/MEIS1 oncogenes and FAS tumour suppressor in MLL-rearranged leukemia. Nature Communications. 2012;**2**(688):1-12

[83] Yalcin S, Chung SS, Park CY. The role of microRNAs in hematopoietic stem cells and leukemia development. In MicroRNA in development and in the progression of cancer. Ed. Singh SR, Rameshwar P Springer. 2014, New York, NY

[84] Vian L, Di Carlo M, Pelosi E, Fazi F, Santoro S, Cerio AM, Boe A, Rotilio V, Billi M, Racanicchi S, Testa U, Grignani F, Nervi C. Transcriptional fine-tuning of microRNA-223 levels directs lineage choice of human hematopoietic progenitors. Cell Death and Differentiation. 2014;**21**:290-301

[85] Eyholzer M, Schmid S, Schardt JA, Haefliger S, Mueller BU, Pabst T. Complexity of miRNA-223 regulation by CEBPA in human AML. Leukemia Research. 2010;**34**:672-676

[86] Kuchenbauer F, Berg T, Mah AM, miRNAkovic-Hosle M, Salmi A, Ruschmann J, Muranyi A, Agiropoulos B, Rouhi A, Starczynowski DT, Heuser M, Hogge DF, Camargo FD, Dohner H, Buske C, Humphries RK. MiRNA-223 is dispensable for the onset of acute myeloid leukemia. Blood. 2010;**116**:501

[87] Xiao Y, Su C, Deng T. miRNA-223 decreases cell proliferation and enhances cell apoptosis in acute myeloid leukemia via targeting FBXW7. Oncology Letters. 2016;**12**(5):3531-3536

[88] Trissal MC, DeMoya RA, Schmidt AP, Link DC. MicroRNA-223 regulates granulopoiesis but is not required for HSC maintenance in mice. PLoS One. 2014;**10**(3):e0119304

[89] Pulikkan JA, Dengler V, Peramangalam PS, Zada AAP, Muller-Tidow C, Bohlander SK, Tenen DG, Behre G. Cell-cycle regulator E2F1 and microRNA-223 comprise an autoregulatory negative feedback loop in acute myeloid leukemia. Blood. 2010;**115**(9):1768-1778

[90] Keam SP, Young PE, McCorkindale AM, Dang THY, Clancy JL, Humphreys DT, Preiss T, Hutvagner G, Martin DIK, Cropley JE, Suter CM. The human piwi protein hiwi2 associates with tRNA-derived piRNAs in somatic cells. Nucleic Acids Research. 2014;**42**(14):8984-8995

[91] Bamezai S, Mulaw MM, Zhou F, Rohde C, Muller-Tidow C, Dohner K, Buske MF, Buske C, Rawat VPS. Knockdown of the piwi-like protein 4 (piwil4) delays leukemic growth and is associated with gross changes in the global histone methylation marks in human MLL-rearranged AML. Blood. 2013;**122**:597

[92] Bamezai S, Rawat VPS, Buske C. Concise review: The piwi-piRNA axis: Pivotal beyond transposon silencing. Stem Cells. 2012;**30**(12):2603-2611

[93] Belancio VP, Deininger PL, Roy-Engel AM. LINE dancing in the human genome: Transposable elements and disease. Genome Medicine. 2009;**1**:97

[94] Schichman SA, Caligiuri MA, Strout MP, Carter SL, Gu Y, Canaani E, Bloomfield CD, Croce CM. ALL-1 tandem duplication in acute myeloid leukemia with normal karyotype involves homologous recombination between Alu elements. Cancer Research. 1994;**54**:4277-4280

[95] Anwar SL, Wulaningsih W, Lehmann U. Transposable elements in human cancer: Causes and consequences of deregulation. International Journal of Molecular Sciences. 2017;**18**:974

[96] Jacobs JE, Wagner M, Dhahbi J, Boffelli D, Martin DI. Deficiency of MIWI2 (Piwil4) induces mouse erythroleukemia cell differentiation, but has no effect on hematopoiesis in vivo. PLoS One. 2013;**8**(12):e82573

[97] Xiao Y, Deng T, Su C, Shang Z. MicroRNA 217 inhibits cell proliferation and enhances chemosensitivity to doxorubicin in acute myeloid leukemia by targeting KRAS. Oncology Letters. 2017;**13**:4986-4994

[98] Wilkes MC, Repellin CE, Sakamoto KM. Beyond mRNA: The role of non-coding RNAs in normal and aberrant hematopoiesis. Molecular Genetics and Metabolism. Published online 28 July 2017. 1-12. DOI: 10.1016/j.ymgme.2017.07.008

[99] Garzon R, Pichiorri F, Marcucci G, Kornblau S, Andreef M, Croce C. MiRNA-29b targets MCL-1 and is down-regulated in chemotherapy-resistant acute myeloid leukemia. Blood. 2007;**110**:717

[100] Lu F, Zhang J, Ji M, Li P, Du Y, Wang H, Zang S, Ma D, Sun X, Ji C. miRNA-181b increases drug sensitivity in acute myeloid leukemia via targeting HMGB1 and Mcl-1. International Journal of Oncology. 2014;**45**(1):383-392

[101] Mosakhani N, Raty R, Tyybakinoja A, Karjalainen-Lindsberg M-L, Elonen E, Knuutila S. MicroRNA profiling in chemoresistant and chemosensitive acute myeloid leukemia. Cytogenetic and Genome Research. 2013;**141**(4):272-276

[102] Zebisch A, Hatzl S, Pichler M, Wolfler A, Sill H. Therapeutic resistance in acute myeloid leukemia. The role of non-coding RNAs. International Journal of Molecular Sciences. 2016;**17**:2080-2096

[103] Dahlhaus M, Schult C, Lange S, Freund M, Junghans C. MicroRNA 181a influences the expression of HMGB1 and CD4 in acute leukemias. Anticancer Research. 2013;**33**:445-452

[104] Nanbakhsh A, Visentin G, Olive D, Janji B, Mussard E, Dessen P, Meurice G, Zhang Y, Louache F, Bourhis J-H, Chouaib S. miRNA-181a modulates acute meloid leukemia susceptibility to natural killer cells. OncoImmunology. 2015;**4**(12):e996475

[105] Huang X, Schwind S, Santhanam R, Eisfeld A-K, Chiang C-l, Lankenau M, Yu B, Hoellerbauer P, Jin Y, Tarighat SS, Khalife J, Walker A, Perrotti D, Bloomfield CD, Wang H, Lee RJ, Lee LJ, Marcucci G. Targeting the RAS/MAPK pathway with miRNA-181a in acute myeloid leukemia. Oncotarget. 2016;**7**(37):59273-59286

[106] Hickey CJ, Schwind S, Radomska HS, Dorrance AM, Santhanam R, Mishra A, Wu Y-Z, Alachkar H, Maharry K, Nicolet D, Mrozek K, Walker A, Eiring AM, Whitman SP, Becker H, Perrotti D, Wu L-C, Zhao X, Fehniger TA, Vij R, Byrd JC, Blum W, Lee LJ, Caligiuri MA, Bloomfield CD, Garzon R, Marcucci G. Lenalidomide-mediated enhanced translation of C/EBPa-p30 protein up-regulates expression of the antileukemic microRNA-181a in acute myeloid leukemia. Blood. 2013;**121**:159-169

[107] Klimenko OV, Shtilman MI. Transfection of Kasumi-1 cells with a new type of polymer carriers loaded with miRNA-155 and antago-miRNA-155. Cancer Gene Therapy. 2013:1-5

Chapter 7

HIF-Regulated Leukemogenesis Through the Advances on Epigenetic Mechanism

Lei Dong and Yu Zhu

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.75769

Abstract

Hypoxia-inducible factor (HIF) is the central master regulator of adaptation to decreased oxygen availability in both physiological and pathological conditions. In leukemia, HIF regulates tumor cell metabolic regulation, metastasis, and other tumor-adaptive survival. However, the regulatory role of HIF in different types of leukemia, including myeloid leukemia, has been unclear. In this chapter, the focus throughout is on the aspects of roles of HIF in the tumor mitochondria metabolic change that are relevant to the assessment and treatment of myeloid leukemia. The connection of HIF with metabolic modification and anaerobic metabolism, along with epigenetic modification, contribute to abnormal biological and clinical behavior of myeloid leukemia, including response to treatment. We have also explored the metabolic requirements of tumor cell proliferation in an attempt to understand why tumor cells escape hypoxia-induced cell growth inhibition. We believe that a better understanding of the mechanistic links between HIF-regulated cellular metabolism, growth control, and epigenetic modifications could be useful for the indication of pharmaceutical agents in myeloid leukemia.

Keywords: myeloid leukemia, HIF, epigenetic modification, metabolism, treatment of myeloid leukemia

1. Introduction

Myeloid leukemia is the most prevalent leukemia in adults, including acute myeloid leukemia (AML) and chronic myeloid leukemia (CML). AML is an aggressive hematologic malignancy that results in the disruption of normal self-renewal, differentiation, and hematopoietic stem and progenitor cell expansion leading to increased proliferation and accumulation of immature nonfunctioning myeloid progenitors. In turn, myeloid progenitors were blocked to

further differentiate into mature myeloid cell to play its role in the hematopoietic system. In comparison, in CML, hematopoietic stem cell is preferred to differentiate myeloid cells (leukemia cells). CML is much milder due to these leukemia cells still partial functions to maintain homeostasis. However, the leukemia cells uncontrollably divide, build up in the bone marrow, and spill over into the blood. Over time, the cells settle in other parts of the body, especially in the spleen (causing splenomegaly), and it can also promote into a fast-growing AML. The American Cancer Society reports that incidence rates have increased over the past few decades, estimating that in 2015 about 20,830 new cases of AML and 14,620 new cases of CML were diagnosed, and 10,460 deaths from AML and 4650 deaths from CML would occur in the USA [1]. Currently, the majority of AML patients still have a poor prognosis, making the development of novel therapies a priority. Prognosis is influenced by a combination of cytogenetic and genetic characteristics of the disease, together with clinical features and the patient's age. In the albeit heterogeneous genetic landscape of myeloid leukemia, many myeloid leukemia patients exhibit recurrent mutations in genes encoding epigenetic regulators [2]. It is thus becoming increasingly clear that epigenetic dysfunction plays a key role in leukemogenesis of AML and CML [3]. More importantly, the epigenetic regulators CREB-binding protein (CBP) and p300 histone acetyltransferases (HATs), as important HIF co-transcriptional factors, facilitate leukemogenesis and represent therapeutic targets in AML [4]. Here, we have focused on the effect of dysregulated epigenetic programs in the development and maintenance of myeloid leukemia. In addition, we have discussed recent advances in therapies specifically targeting these key epigenetic mechanisms.

2. Hypoxia-inducible factor

Oxygen supply and consumption are tightly regulated and dynamically balanced in most normal tissues. However, supply and consumption of oxygen in tumor cells are usually decoupled due to the loss of physiological control and aberrant molecular signaling that provide malignant growth and survival advantages. Hypoxia appears in tumor cells when the metabolic demand for oxygen exceeds its extracellular availability. One of the main early cellular events responded upon hypoxia is activation of hypoxia-inducible factor 1 and 2 (HIF-1 and HIF-2), a critical heterodimeric transcription factor. HIF then in turn binds to hypoxia-responsive elements (HREs), with the minimal core sequence 5′-CGTG-3′, and influences the expression of various genes involved in angiogenesis, metabolism, pH regulation, proliferation, metastasis, and a wide range of other signaling processes.

2.1. Structure of hypoxia-inducible factor and domain structure of α subunits

The structure of HIF was identified as a dimer protein composed of HIF-1β and HIF-1α subunits. HIF-1β, the aryl hydrocarbon receptor nuclear translocator (ARNT), and its highly homologous protein ARNT2 and ARNT3, is constitutively expressed. All the three HIF-β subunits have the potential in forming dimers with various HIF-α subunits [5]. In normoxic conditions, HIF-1α is expressed ubiquitously at low closely balanced levels in all organs and has six different

http://dx.doi.org/10.5772/intechopen.75769

splice variants [6]. HIF-2α is most abundantly expressed in the lung, followed by the heart, brain, liver, and various other organs. Despite their similarities in mediating transcriptional responses to hypoxia, HIF-1α and HIF-2α have distinct, nonredundant functions (reviewed in Semenza [2004] [7]). HIF-3α is the least-studied member of the family and has multiple splice variants [5]. The functional domains of HIF include DNA-binding region basic helix-loop-helix (bHLH), HIF dimerization-binding region PER-ARNT-SIM (PAS), oxygen tension modulated N-terminal transactivation domain (N-TAD), and C-terminal transactivation domain (C-TAD). HIF-α subunit also contains oxygen-dependent degradation domain (ODDD).

2.2. Oxygen-dependent hypoxia-inducible factor regulation

The regulation of HIF by the extent of oxygen pressure is dependent on the intermediator that affects HIF-α protein stability and/or its ability to bind cofactors essential for transcriptional activity. In normoxia, HIF-α is strictly controlled by two types of oxygen sensors. First, 2-oxoglutarate (2-OG)-dependent prolyl hydroxylase domain (PHD) proteins could hydroxylate two prolyl residues (Pro^{402} and/or Pro^{564}) in the N-TAD of HIF-1α ODDD regions (**Figure 1**) [8, 9]. Currently, three functional 2-OG-dependent PHD have been identified—PHD1, PHD2, and PHD3,—and all three require oxygen, Fe^{2+}, and 2-OG as cofactors. This modification of HIF-α promotes its destruction by the proteasomal system through interaction with von Hippel-Lindau (VHL) protein, a component of an E3 ubiquitin ligase complex [10]. A second

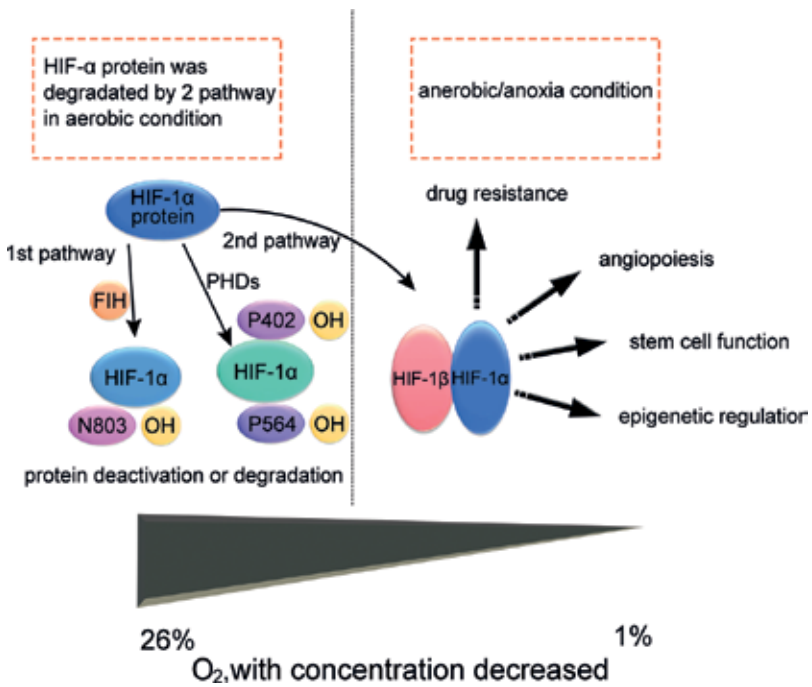

Figure 1. HIF-1α regulation and HIF-1α dependent gene expression under hypoxia.

oxygen sensor called factor-inhibiting HIF-1 (FIH-1) involves hydroxylation of an asparagine residue (Asn^{803}) in the C-TAD of HIF-1α, which also utilizes oxygen as a substrate [11]. The hydroxyl modification of Asn^{803} blocks the binding of the cofactor proteins) CREB-binding protein (CBP) and p300 thus inhibit HIF transcriptional activity. In hypoxia, HIF-α is regulated through "bicephalous" transcriptional nature in an FIH-dependent or FIH-independent manner [12]. In short, PHD has a lower affinity for oxygen than HIF and therefore is more rapidly inhibited. Consequently, genes require only the N-TAD to be induced. As oxygen decreases further, the inhibition of C-TAD is released and HIF-1α retains full transcription activity.

2.3. Metabolic-dependent HIF regulation

The metabolic intermediates are also the key regulators disrupting the hemostasis of HIF activates. As mentioned earlier, PHDs are 2-OG-dependent dioxygenases, catalyzing the conversion of a prolyl residue, molecular oxygen, 2-OG to hydroxyprolyl, carbon dioxide, and succinate using ferrous iron as cofactor. In addition, succinate also intermediates in the tricarboxylic acid (TCA) cycle catalyzed by succinate dehydrogenase (SDH) to fumarate in mitochondria. SDH dysfunction in cells raises the levels of succinate, which accumulates and leaks out to cytosol [13]. The increased level of succinate also inhibits PHDs activity due to accumulation succinate feedback, leading to the stabilization of HIF-α and activation of HIF complex. Similarly, deficiency of fumarate hydratase (FH) leads to accumulation of fumarate in the cytosol. Due to chemical similarity of fumarate to succinate, FH-deficient cells could also inhibit PHDs [14]. Other metabolic changes, such as diseases related to iron homeostasis, also crosstalk with HIF regulation. Hepcidin, a small polypeptide, plays a central role in regulating iron uptake. Iron demand in bone marrow increases when erythropoiesis is stimulated by hypoxia via increased erythropoietin (EPO) synthesis. Iron overload disease like hemochromatosis and iron decrease in anemia, feedback hepcidin production through VHL-HIF regulation [15].

3. HIF regulation in mitochondria metabolic change

HIF is the central master regulator of adaptation to decreased oxygen availability in both physiological and pathological conditions. It is evolutionary pressure to reestablish metabolic balance to allow normal tissue and/or even tumor to survive. Physiologically, in the wound-healing area, damaged tissue leads to hypoxia and facilitates vascular growth. However, pathologically, in the solid tumor region, oxygen demand is in continuous increase due to the uncontrollable growth of the cancer cell. Hypoxia also represents the unifying feature of the microenvironment of solid tumors. The adaptive changes of tumor survival pattern referred to as "hypoxia tumor phenotype" are greatly noticed.

3.1. HIF regulation of metabolic change

HIF upregulation in tumors plays a central role in metabolic switch from aerobic metabolism to anaerobic metabolism. In turn, all the enzymes (e.g., aldolase A and C, enolase 1, hexokinase 1 and 2, pyruvate kinase M (PKM), phosphofructokinase) and glucose transporters (GLUT1, GLUT3) involved in glycolytic pathway are upregulated [16]. Moreover,

conversion of pyruvate to acetyl-CoA, TCA cycle, and mitochondrial biogenesis are inhibited through downregulation of pyruvate dehydrogenase kinase (PDK) 1 and 3 [17, 18]. Even though the glycolysis produces far less energy than TCA cycle per glucose molecule, it has a significant higher throughout. In addition, the accumulated by-products could be used as sources of carbon to produce nucleotides and lipids for proliferating cells [19]. The classic view of metabolism is that of a self-correction of homeostasis responding to microenvironment. In this model, for cancer to arise, tumor hypoxia selects cells depending on anaerobic metabolism [20]. Secondary mutations are needed to give cells the ability to transform the capability to alter existing cell metabolism in a way that supports cell growth. One example is that of mouse embryonic fibroblasts that reduce oxygen consumption when switching from 20% O_2 to 1% O_2, and continued low oxygen consumption when returning to 20% O_2, suggesting HIF stable modified metabolic reprogramming [21].

The direct consequence of glycolysis is the production of lactic acid by hypoxic tumor cells leading to tumor acidosis. Intracellular acidosis poses a threat to cell survival. Readjusting intracellular pH (pHi) is a critical strategy to protect against apoptosis and cell death. HIF upregulated monocarboxylate transporter 4 (MCT4) and Na^+/H^+ exchanger (NHE1) facilitate exportation of H^+ [22, 23]. Moreover, two transmembrane carbonic anhydrases (CAs) catalyze CO_2 to be hydrated to HCO_3^- and H^+, CA IX, and XII overexpressed in tumors also regulated by HIF. This reaction facilitates proton generation in the extracellular space, which contributes to acidification in tumor microenvironment, while preventing acidification of intercellular milieu of cancer cell [24].

4. HIF regulation in epigenetic modification

Wadding first proposed the concept of epigenetics in 1915 and believed that the phenotypes generated from certain genotype within the scope of epigenetics. Meanwhile, he explained the expression of the genetic materials in the entire life process for the first time by using the concept of "Whole View" [25]. Holiday summarized with a more comprehensive explanation that "epigenetics refers to the changes of the gene expression manner with no difference and/or change of heritage DNA sequence" [26]. Epigenetics is involved in individual development and the gene expression regulation in the biological process, however, it could also lead to human diseases when it is interfered [27].

4.1. Histone acetylation

Histone acetylation, a process closely related to transcriptional activation is one of major epigenetic modifications. Histone acetylation occurs in the lysine residue located at the end of the histone N-terminal. Histone acetylation induces relaxed and/or higher-order structure of chromatins through transcriptional regulation [28]. In addition, the acetylated histone produces a signal that binds to the protein, producing intrinsic activities or capped-chromatin remodeling complexes, thereby promoting the transcriptional induction. Histone acetylation is mainly controlled by the inhibitory activities of histone acetyltransferases (HATs) and histone

deacetylases (HDACs), and the substrates of HDAC include non-histone proteins, some transcription factors, and cofactors [29]. As a consequence, histone deacetylases generally inhibit transcription. It is also interesting that they negatively regulate HIF-dependent transcriptions. Previous studies have shown that methylation of Reptin at lysine 67 in hypoxia condition by the methyltransferase G9a negatively regulates hypoxic responses [30]. Consequently, while in hypoxia condition, the chromatin remodeling factor HIF-1α Reptin binds to HDAC1, the target gene of HIF is involved in the supplementation of HDAC1, besides, HDAC4 and HDAC5 complement the expression of HIF target promoter in HIF-dependent transcription to become more active [31]. Moreover, histone deacetylase inhibitors could promote gene expression in the transcription of several HIF target promoters and induce inhibition of HIF-dependent angiogenesis.

The mechanisms of the HDAC-dependent gene activation are still not well defined. However, it becomes increasingly apparent that the HIF C-TAD-p300/CBP interactions are necessary. There are evidences suggesting the targets of deacetylated p300/CBP and HIF. In this concept, HIF, P300 and HDAC4, HDAC5, or HDAC7 have been reported to form multi-polyprotein complexes [31, 32]. This also shows that HDAC4 and HDAC5 could promote the binding between HIF-1 and p300, thereby enhancing the expression of HIF target genes. However, the gene expressions regulated by epigenetic mechanisms that are involved in the hypoxia response are different. It is generally separated into following steps: (1) HIF recruit co-activator enhancing the expression of HIF. (2) The interaction between HIF-p300 and CBP by the inhibition of hypoxia to induce HIF-1 expression. (3) HDAC4 and HDAC5 can promote the interactions between HIF-1α and p300. (4) HDAC4 and HDAC5 promote the interaction between HIF-1α and p300. (5) SWI/SNF complexes alter the chromatin structures in some HIF target promoters and enhancers to benefit their expression [33].

Specifically, hypoxia promotes the changes in the status of the hypoxia-induced gene promoter histone methylation: activation of hypoxia histone demethylase and inhibition of JMJD1A histone demethylase, which can cause H3K9me2 reduction and H3K4me2 increase, respectively, thus enhancing the gene expression [34, 35]. In addition, hypoxia could increase the expression of some HIF target promoters H3K27me3 and H3K4me3. Under hypoxia conditions, the interactions of HIF1-α and Reptin are enhanced, leading to some HDAC1 supplementation of the HIF target genes and negative regulation of transcription; the changes in the status of histone methylation and acetylation promote hypoxia-inhibited gene. Hypoxia could increase the levels of H3K9me2 and H3K4me3, and decrease the levels of H3K27me3 and H3K9ac.

4.2. Histone methylation

Histone methylation, as another main epigenetic modification, is a stringent regulatory process, which relies on the activities of histone methyltransferase and histone demethylase. Histone demethylase induces the dynamic equilibrium of the histone methylation during hypoxia. During hypoxia, histone demethylase can increase the expression of these enzymes, and the decreased enzyme activity can be regulated completely or partly by oxygen deficiency. Some histone lysine methylation can be specifically prevented under hypoxic conditions.

http://dx.doi.org/10.5772/intechopen.75769

Hypoxia-induced histone methylation might be achieved by the partial inhibition of spherical JHDMS and the reduction of histone methylation of some hypoxia response promoters. Interestingly, Jumonji domain-containing protein 1A (JMJD1A) is the HIF target gene itself. Krieg and his colleagues suggested the regulation of feed-forward mechanism in which HIF might represent for the likely HIF-dependent gene expression of JMJD1A [36]. They suggested that JMJD1A maintained the apparent genetic pattern of the activities of the target promoters, thereby minimizing the required energy-supported expression. JMJD1A indicates that more consideration should be given to the induction of differential genes and other JHDMS involved in the activation of hypoxia-responsive genes. Further research is required to determine whether this is true.

Although there is a detailed study of hypoxia-induced conditions on JHDM, this kind of enzyme in hypoxia requires one or more RNA interference-silencing experiment targeting studies of JHDMS or multiple biological effects of JHDMS response. In the apparent regulation of hypoxia, histone modification and chromatin remodeling caused by relative enzymes also play a key role. Hypoxia-induced histone acetylation has become a highly suspected etiology of Alzheimer's disease and attention deficit hyperactivity disorder (ADHD) [37].

Evidence suggests that the increase of H3K9me2 is partly due to hypoxia-induced G9A methyltransferase. During hypoxia, H3K9me2 induces the increase of certain gene promoters as well. Further studies are required to assess the effects of hypoxia-induced epigenetic alterations on the organisms. To activate the gene transcription, a series of specific HIF-targeted genes promoter region is commonly regulated through histone methylation, acetylation, or alteration of chromatin structures. On the other hand, hypoxia could stimulate the inhibition of induced transcription, possibly by supporting the changes of the whole chromatin. Thus, it seems that hypoxia plays a dual role in the studies of epigenetic mechanisms of the genes as well as in controlling the induction and transcriptional downregulation of the HIF target gene.

HIF will be activated when hypoxia occurs in vivo. As a consequence, transcription of more than 100 genes, such as vascular endothelial growth factor (VEGF) and erythropoietin (EPO), can be induced. However, the activation of HIF could be accompanied with significant decline in the activity in many other transcription factors. However, hypoxia-induced gene modulation is not limited to HIF activation. In contrast, epigenetic modification can be involved in this process. The epigenetic mechanisms play dual roles in hypoxia, meaning that they not only upregulate the HIF-controlled target genes but also downregulate the general transcription factors. However, the specific mechanisms remain to be further explored.

In conclusion, hypoxia could induce extensive histone modifications that are usually associated with transcriptional repression or activation. Specifically, however, more research is needed to fully understand its biological functions and to identify the enzymes involved in signal transduction pathways. It provides holistic assessments regarding hypoxia on the epigenetic changes. Under hypoxia conditions, the following epigenetic changes were greatly noticed: (1) p300/CBP histone acetyltransferases interact with HIF and acetylate histones in HIF target promoters. HDAC4, HDAC5, or HDAC7 form a multi-protein complex with HIF-p300 increasing HIF transcriptional activity. HDAC4 and HDAC5 exert their effects by

promoting association between HIF and p300. (2) SWI/SNF are complementary gene promoters of HIF-1α, which is a requirement for the expression of HIF-1α mRNA. The regulation of SWI/SNF could also describe the profound effects of HIF-dependent responses on hypoxia. On the other hand, the SWI/SNF complex alters the chromatin structure in some HIF target promoters or enhancers, thereby favoring their expression. (3) Hypoxia activates JMJD1A which promote a decrease in H3K9me2. In the meantime, oxygen deprivation also inhibits JARID1A histones demethylases which provoke an increase in H3K4me2 levels at their target promoters, thus enhancing gene expression. In addition, hypoxia increases H3K4me3 and H3K27me3 levels in some HIF target promoters, and hypoxia-inductive H3K4me3 seems to depend on the inhibitory effects of histone demethylase [38]. The hypoxia-inducible gene promoter was also observed in EPO, HMOX1, and DAF [39, 40]. (4) The interaction between Reptin and HIF1-α is enhanced in hypoxia, leading to recruitment of HDAC1 to some HIF target genes, negatively regulating their transcription (**Figure 2**). However, more research is still needed to fully understand its biological functions and to identify the enzymes involved in signal transduction pathways. It provides holistic assessment regarding hypoxia on the epigenetic modifications.

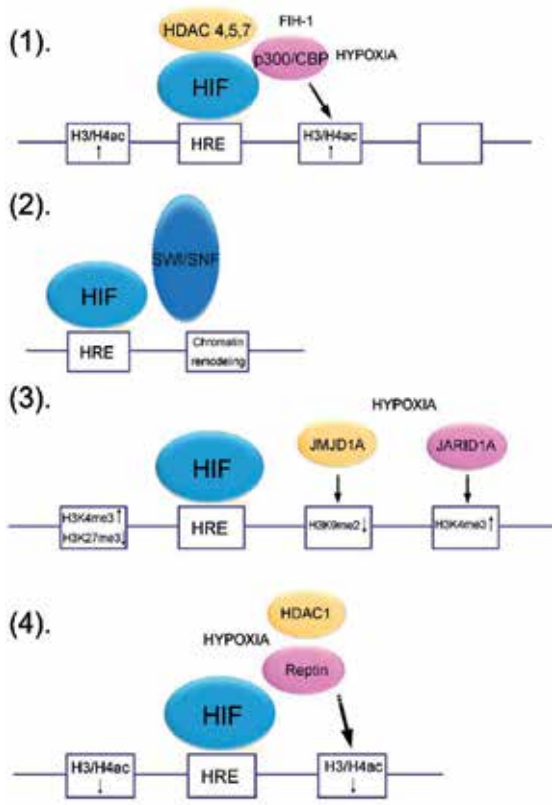

Figure 2. HIF regulated epigenetic changes in response to hypoxia.

http://dx.doi.org/10.5772/intechopen.75769

5. HIF and epigenetic modification in myeloid leukemia

Metabolic flexibility relies on the rewiring of the existing metabolic pathways, which are closely controlled by "pathway switch proteins," to efficient rerouting of metabolites selected by cellular needs. As discussed earlier, HIF controls many aspects of tumor in terms of location, size, cell type, or local invasion. Other aspects, like state of differentiation and hierarchical nature, were also regulated by HIF. Most tumor-initiating mutations occur in stem cell or progenitor populations. The expansion of these mutant cells with a more differentiated phenotype that usually characterizes individual cancers are responsible for the cause of pathogenesis. This was first described in 1997 for acute myeloid leukemia and subsequently extended to solid tumors, including melanoma, Glioblastoma (GBM), and pancreatic cancer [41, 42].

Limited oxygen access is the common feature in solid tumor due to inadequate tissue perfusion, thus, cancer metabolism is heavily influenced by adaptation to highly hypoxic microenvironment. In many cases, HIF is involved as a master regulator. Recently, an increasing number of other proteins, regulated by HIF, are found to influence energy metabolism. In addition, a series of mutations in these proteins—for example, SDH, FH, isocitrate dehydrogenase (IDH), activation-induced cytosine deaminase (AID), or drive altered metabolism. These findings have supported the notion that HIF has a role to play in oncology, and affects diagnostic methods and drug discovery.

In hematological tumors, bone marrow and lymph nodes represent hypoxic environments. The quiescent hematopoietic stem cells (HSCs) in the bone marrow existing in the hypoxic niche utilization of predominantly glycolysis pathway are regulated by HIF-1α stabilization [21]. Hypoxia modulates mitochondrial respiration in an HIF-1α-dependent manner. HIF promoter, the expression of pyruvate dehydrogenase kinase-1 (PDK1), in turn, inhibits pyruvate dehydrogenase (PDH) [43]. In addition, PKD1 activation is also important in inducing pluripotent stem cell, as evidenced by four Yamanaka factors (OCT4, SOX2, KLF4, and c-MYC) sufficient to upregulate PDK1, and initiates a Warburg-like metabolic rewiring which is closely linked with conversion of pluripotency [44]. On the other hand, metabolic reprograming initially triggered by HIF stabilizes HIF expression independently of oxygen to gain tumor survival advantage. As an example, imatinib-resistant cell expresses high levels of HIF-1αs and induces BCR-ABL upregulation [45]. Recent study further suggests that HIF is the potential cause to trigger gene translocation through limiting activation-induced cytosine deaminase (AID) expression [46, 47]. In the therapeutic point of view, the stem cell nature of cancer is also reflected in removing differentiation block therapy. For example, all-*trans* retinoic acid combined with cytotoxic drug was used in the clinical practice for the treatment of acute promyelocytic leukemia (APL) [48]. Other promoting differentiation agent aurora kinase A inhibitors were shown effective in acute megakaryocytic leukemia [49].

Other feature of leukemia cell is attenuated metabolic pathway in glycolysis even in aerobic conditions [50]. Leukemic cells, other than solid tumors, have the advantage to access oxygen; however, levels of HIF-1α, GLUT1, GLUT3, and CA4 are still significantly enhanced compared to normal blood cells. Clinical evidence shows that higher glycolytic rate in leukemic cells

induces resistance to chemotherapeutics. Instead, inhibition of glycolysis using 2-deoxyglucose (2DG) promotes leukemic cell susceptibility to chemotherapeutic treatment, resulting in induction of leukemic cell death in normoxia [51].

Acknowledgements

Fund by Special Program of Talent Development for Excellent Youth Scholars in Tianjin, China (TJTZJH-QNBJRC-2-9).

Author details

Lei Dong[1]* and Yu Zhu[2]*

*Address all correspondence to: ldong@bit.edu.cn and zhuyutj@126.com

1 School of Life Science, Beijing Institute of Technology, Beijing, China

2 Department of Clinical Laboratory, Tianjin Key Laboratory of Cerebral Vessels and Neurodegenerative Disease, Tianjin Huanhu Hospital, Tianjin, China

References

[1] Siegel RL, Miller KD, Jemal A. Cancer statistics, 2015. CA: A Cancer Journal for Clinicians. 2015;**65**:5-29

[2] Kumar CC. Genetic abnormalities and challenges in the treatment of acute myeloid leukemia. Genes & Cancer. 2011;**2**:95-107

[3] Gallipoli P, Giotopoulos G, Huntly BJ. Epigenetic regulators as promising therapeutic targets in acute myeloid leukemia. Therapeutic Advances in Hematology. 2015;**6**:103-119

[4] Giotopoulos G et al. The epigenetic regulators CBP and p300 facilitate leukemogenesis and represent therapeutic targets in acute myeloid leukemia. Oncogene. 2016;**35**:279-289

[5] Maynard MA, Von Ohh M. Hippel-Lindau tumor suppressor protein and hypoxia-inducible factor in kidney cancer. American Journal of Nephrology. 2004;**24**:1-13

[6] Kaur B et al. Hypoxia and the hypoxia-inducible-factor pathway in glioma growth and angiogenesis. Neuro-Oncology. 2005;**7**:134-153

[7] Semenza GL. O2-regulated gene expression: Transcriptional control of cardiorespiratory physiology by HIF-1. Journal of Applied Physiology. 1985;**96**:1173-1177; discussion 1170-1172 (2004)

[8] Bruick RK, McKnight SL. A conserved family of prolyl-4-hydroxylases that modify HIF. Science. 2001;**294**:1337-1340

[9] Jaakkola P et al. Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation. Science. 2001;**292**:468-472

[10] Gossage L, Eisen T, Maher ER. VHL, the story of a tumour suppressor gene. Nature Reviews. Cancer. 2015;**15**:55-64

[11] Lando D, Peet DJ, Whelan DA, Gorman JJ, Whitelaw ML. Asparagine hydroxylation of the HIF transactivation domain a hypoxic switch. Science. 2002;**295**:858-861

[12] Pouyssegur J, Dayan F, Mazure NM. Hypoxia signalling in cancer and approaches to enforce tumour regression. Nature. 2006;**441**:437-443

[13] Selak MA et al. Succinate links TCA cycle dysfunction to oncogenesis by inhibiting HIF-alpha prolyl hydroxylase. Cancer Cell. 2005;**7**:77-85

[14] Isaacs JS et al. HIF overexpression correlates with biallelic loss of fumarate hydratase in renal cancer: Novel role of fumarate in regulation of HIF stability. Cancer Cell. 2005;**8**:143-153

[15] Peyssonnaux C et al. Regulation of iron homeostasis by the hypoxia-inducible transcription factors (HIFs). The Journal of Clinical Investigation. 2007;**117**:1926-1932

[16] Semenza GL, Roth PH, Fang HM, Wang GL. Transcriptional regulation of genes encoding glycolytic enzymes by hypoxia-inducible factor 1. The Journal of Biological Chemistry. 1994;**269**:23757-23763

[17] Brahimi-Horn MC, Chiche J, Pouyssegur J. Hypoxia signalling controls metabolic demand. Current Opinion in Cell Biology. 2007;**19**:223-229

[18] Gillies RJ, Robey I, Gatenby RA. Causes and consequences of increased glucose metabolism of cancers. Journal of Nuclear Medicine. 2008;**49**(Suppl 2):24S-42S

[19] Vander Heiden MG, Cantley LC, Thompson CB. Understanding the Warburg effect: The metabolic requirements of cell proliferation. Science. 2009;**324**:1029-1033

[20] Gatenby RA, Gillies RJ. Why do cancers have high aerobic glycolysis? Nature Reviews. Cancer. 2004;**4**:891-899

[21] Suda T, Takubo K, Semenza GL. Metabolic regulation of hematopoietic stem cells in the hypoxic niche. Cell Stem Cell. 2011;**9**:298-310

[22] Ullah MS, Davies AJ, Halestrap AP. The plasma membrane lactate transporter MCT4, but not MCT1, is up-regulated by hypoxia through a HIF-1alpha-dependent mechanism. The Journal of Biological Chemistry. 2006;**281**:9030-9037

[23] Shimoda LA, Fallon M, Pisarcik S, Wang J, Semenza GL. HIF-1 regulates hypoxic induction of NHE1 expression and alkalinization of intracellular pH in pulmonary arterial

myocytes. American Journal of Physiology. Lung Cellular and Molecular Physiology. 2006;**291**:L941-L949

[24] Pastorekova S, Parkkila S, Zavada J. Tumor-associated carbonic anhydrases and their clinical significance. Advances in Clinical Chemistry. 2006;**42**:167-216

[25] Strizzi L, Hardy KM, Kirschmann DA, Ahrlund-Richter L, Hendrix MJ. Nodal expression and detection in cancer: Experience and challenges. Cancer Research. 2012;**72**:1915-1920

[26] Aslam ML et al. Genome-wide candidate regions for selective sweeps revealed through massive parallel sequencing of DNA across ten Turkey populations. BMC Genetics. 2014;**15**:117

[27] Singhal SK et al. Towards understanding the breast cancer epigenome: A comparison of genome-wide DNA methylation and gene expression data. Oncotarget. 2016;**7**:3002-3017

[28] Zou ZK, Huang YQ, Zou Y, Zheng XK, Ma XD. Silencing of LSD1 gene modulates histone methylation and acetylation and induces the apoptosis of JeKo-1 and MOLT-4 cells. International Journal of Molecular Medicine. 2017

[29] la Rosa AH, Manoharan M, Goolam AS. Current concepts of epigenetics in testicular Cancer. Indian Journal of Surgical Oncology. 2017;**8**:169-174

[30] Lee JS et al. Negative regulation of hypoxic responses via induced Reptin methylation. Molecular Cell. 2010;**39**:71-85

[31] Wu HT et al. K63-polyubiquitinated HAUSP deubiquitinates HIF-1alpha and dictates H3K56 acetylation promoting hypoxia-induced tumour progression. Nature Communications. 2016;**7**:13644

[32] Zhang Y et al. Nucleus accumbens-associated protein-1 promotes glycolysis and survival of hypoxic tumor cells via the HDAC4-HIF-1alpha axis. In: Oncogene. 2017

[33] Wei J et al. Recent advances in the discovery of HIF- 1alpha/p300 inhibitors as anticancer agents. Mini Reviews in Medicinal Chemistry. 2016

[34] Tausendschon M, Dehne N, Brune B. Hypoxia causes epigenetic gene regulation in macrophages by attenuating Jumonji histone demethylase activity. Cytokine. 2011;**53**:256-262

[35] Salminen A, Kaarniranta K, Kauppinen A. Hypoxia-inducible histone lysine Demethylases: Impact on the aging process and age-related diseases. Aging and Disease. 2016;**7**:180-200

[36] Chervona Y, Arita A, Costa M. Carcinogenic metals and the epigenome: Understanding the effect of nickel, arsenic, and chromium. Metallomics. 2012;**4**:619-627

[37] Yagishita S, Hirasawa A. Intermittent hypoxia produces Alzheimer disease? Oncotarget. 2017;**8**:41786-41787

[38] Krzykawska-Serda M et al. Correlation between hypoxia proteins and EPR-detected hypoxia in Tumors. Advances in Experimental Medicine and Biology. 2017;**977**:319-325

[39] Rauner M et al. Increased EPO levels are associated with bone loss in mice lacking PHD2 in EPO-producing cells. Journal of Bone and Mineral Research. 2016;**31**:1877-1887

[40] Cheng CC et al. Blocking heme oxygenase-1 by zinc protoporphyrin reduces tumor hypoxia-mediated VEGF release and inhibits tumor angiogenesis as a potential therapeutic agent against colorectal cancer. Journal of Biomedical Science. 2016;**23**:18

[41] Baccelli I, Trumpp A. The evolving concept of cancer and metastasis stem cells. The Journal of Cell Biology. 2012;**198**:281-293

[42] Bonnet D, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nature Medicine. 1997;**3**:730-737

[43] Kim JW, Tchernyshyov I, Semenza GL, Dang CV. HIF-1-mediated expression of pyruvate dehydrogenase kinase: A metabolic switch required for cellular adaptation to hypoxia. Cell Metabolism. 2006;**3**:177-185

[44] Prigione A et al. HIF1alpha modulates cell fate reprogramming through early glycolytic shift and upregulation of PDK1-3 and PKM2. Stem Cells. 2014;**32**:364-376

[45] Zhao F et al. Imatinib resistance associated with BCR-ABL upregulation is dependent on HIF-1alpha-induced metabolic reprograming. Oncogene. 2010;**29**:2962-2972

[46] Cho SH et al. Germinal Centre hypoxia and regulation of antibody qualities by a hypoxia response system. Nature. 2016;**537**:234-238

[47] Nambiar M, Raghavan SC. How does DNA break during chromosomal translocations? Nucleic Acids Research. 2011;**39**:5813-5825

[48] Abaza Y et al. Long-term outcome of acute promyelocytic leukemia treated with all-trans-retinoic acid, arsenic trioxide, and gemtuzumab. Blood. 2017;**129**:1275-1283

[49] Krause DS, Crispino JD. Molecular pathways: Induction of polyploidy as a novel differentiation therapy for leukemia. Clinical Cancer Research. 2013;**19**:6084-6088

[50] Warburg O. On the origin of cancer cells. Science. 1956;**123**:309-314

[51] Hanahan D, Weinberg RA. Hallmarks of cancer: The next generation. Cell. 2011;**144**: 646-674

Section 4

Drug Resistance and Potential Treatments

Chapter 8

Molecular Interaction Between the Microenvironment and FLT3/ITD$^+$ AML Cells Leading to the Refractory Phenotype

Seiji Fukuda, Tomohiro Hirade, Mariko Abe, Takeshi Taketani and Chie Onishi

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.71676

Abstract

Internal tandem duplication mutations in the FLT3 gene (FLT3/ITD) are detected in 10–15% of children and 30% of adult patients with AML and are associated with an extremely poor prognosis. Although several antagonists against FLT3/ITD have been developed, few of them are effective for the treatment of FLT3/ITD$^+$ AML because of the emergence of drug-resistant cells. The mechanisms responsible for drug resistance include the acquisition of additional mutations in the FLT3 gene and/or activation of other prosurvival pathways such as microenvironment-mediated resistance. Recent studies have strongly suggested that the reciprocal interaction between the microenvironment and AML cells generates specific machinery that leads to chemoresistance. This chapter describes the molecular mechanism responsible for the refractory phenotype of FLT3/ITD$^+$ AML cells resulting from the communication between the microenvironment and FLT3/ITD$^+$ leukemia cells. Understanding this mechanism enables the discovery of novel and innovative therapeutic interventions for resistant FLT3/ITD$^+$ AML.

Keywords: FLT3/ITD, microenvironment, niche, drug resistance, CXCL12/CXCR4

1. Introduction

Mutations in the FLT3 gene represent the most common genetic aberrations among patients with acute myeloid leukemia (AML) [1, 2]. Internal tandem duplication mutations in the FLT3 gene (FLT3/ITD), which are expressed in human acute myeloid leukemia (AML)

stem cells, are found in ~30% of patients with AML [3]. FLT3/ITD$^+$ AML is one of the most intractable hematological malignancies because of the emergence of resistant clones to FLT3/ITD inhibitors or chemotherapies [3, 4]. FLT3/ITD allows ligand-independent activation and phosphorylation of the FLT3 receptor. Ectopic FLT3/ITD expression in IL-3–dependent mouse Ba/F3 or 32D hematopoietic cells results in growth factor–independent proliferation and produces acute leukemia in mice [5, 6]. Studies have indicated that FLT3/ITD transforms mouse hematopoietic cell lines via the activation of the *STAT5*, *RAS-MAPK*, and *PI3-kinase*/*AKT* pathways [5, 7, 8] and blocks differentiation by suppressing *C*/*EBPα*, *PU1*, and *RUNX1* [9–11]. Other studies have reported that *JAK2* and *STAT3* are tyrosine phosphorylated by constitutively active *FLT3* [12]. *ROCK1* [13], *CDKN1a* [14], *SURVIVIN* [15, 16], *RUNX1* [9, 17], *CXCR4* [18, 19], *SOCS1* [20], *PIM1* kinase [21, 22], *FLT3*-ligand [23, 24], *SHP*-2 [25], and micro-RNA-155 [26], and other molecules are reported to be involved in FLT3/ITD signaling. Although FLT3/ITD has been associated with extremely poor patient prognoses, FLT3 inhibitors fail to show significant efficacy in anti-AML therapies. For instance, AC220 (quizartinib), a second-generation class III tyrosine kinase inhibitor (TKI) used in phase II clinical trials, is a very potent and specific inhibitor of FLT3/ITD compared with other TKIs; however, FLT3/ITD$^+$ cells can become refractory to AC220 [9, 27]. The mechanism responsible for the resistance of FLT3/ITD$^+$ AML cells against FLT3/ITD inhibitors can be classified into FLT3/ITD-dependent and FLT3/ITD-independent mechanisms [4, 28]. The former is generally acknowledged as the acquisition of mutations in the FLT3 gene in addition to preexisting FLT3/ITD mutations. The emergence of additional mutations in the kinase domain makes FLT3/ITD no longer sensitive to FLT3/ITD inhibitors by altering the three-dimensional structure of FLT3 kinase, making FLT3 inhibitors difficult to physically interact with FLT3 protein. This mechanism is detailed in the excellent reviews [4, 28]. Although the development of further mutations in the FLT3 gene is associated with being refractory to the FLT3 inhibitor, most patients who became refractory to the FLT 3/ITD inhibitors lacked additional mutation in the FLT3 gene. Therefore, the resistant mechanism of these cases was likely to be attributed to alteration of the activity or levels in the molecules or pathways independent of FLT3/ITD [29], which includes microenvironment-mediated resistance.

Human AML stem cells residing in the endosteal niche of the bone marrow are relatively chemoresistant [30, 31]. This resistance results from survival cues in the form of various cytokines and adhesion molecules provided by niche cells [32]. Studies using the FLT3/ITD inhibitors have demonstrated that FLT3/ITD$^+$ AML blasts circulating in the peripheral circulation were very sensitive to these inhibitors, whereas those residing in the marrow endosteal region remained resistant to the FLT3/ITD inhibitor [33]. Reports have demonstrated that stromal cells protect FLT3/ITD AML cells from apoptosis induced by FLT3/ITD inhibitors [34–36]. These studies suggest that leukemia niches provide survival cues that protect FLT3/ITD$^+$ AML blasts from being eradicated by the FLT3/ITD inhibitors. In agreement with these observations, early study demonstrated that releasing leukemia cells from the marrow niche into the peripheral circulation by blocking the *CXCL12*/*CXCR4* interaction is effective in increasing their sensitivity to cytoreductive treatment [37]. These findings indicate that targeting cells via a cell-autonomous mechanism alone may not be sufficient

http://dx.doi.org/10.5772/intechopen.71676

for treating FLT3/ITD+ AML and that antagonizing these protective interactions between FLT3/ITD+ AML blasts and leukemia niches represents a novel therapeutic strategy to eradicate resistant FLT3/ITD+ AML cells.

2. Microenvironmetal factors inducing the resistance of FLT3/ITD+ AML cells to FLT3 inhibitors

2.1. CXCL12/CXCR4 signaling pathways as a mechanism responsible for the resistance of FLT3/ITD AML cells to the FLT3 inhibitor

One of the machineries that holds AML cells in the bone marrow microenvironment is the interaction between *CXCL12* and *CXCR4* (**Figure 1**). *CXCL12*, a chemokine known as stromal cell–derived factor-1 (*SDF1*) that is expressed by the bone marrow microenvironment, is

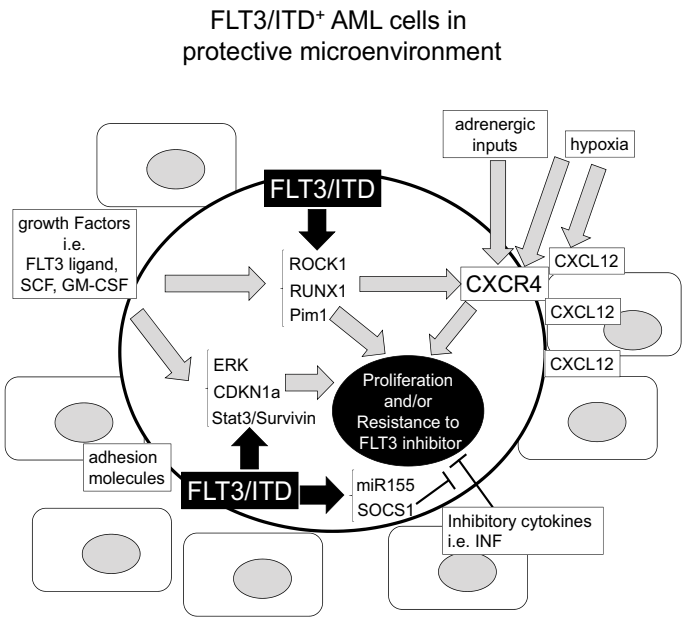

Figure 1. FLT3/ITD+ AML cells in protective microenvironment. Suggested model for the resistance mechanism mediated by the environmental factors is shown. Retention of FLT3/ITD+ cells in the bone marrow microenvironment increases the risk of resistant phenotype of FLT3/ITD+ AML cells. This is mediated by adhesion molecules as well as the interaction between *CXCL12* that is provided by the microenvironment and the *CXCR4* on the AML cells. FLT3/ITD increases cell migration to *CXCL12*, thereby enhancing the interaction between AML cells and the microenvironment. Hypoxia and adrenergic inputs in the marrow environment that can enhance expression of *CXCL12* and/or *CXCR4* likely increase this interaction even further. FLT3/ITD itself activates or modulates several intracellular molecules, such as *ROCK1, RUNX1, PIM1, ERK, STAT3, SURVIVN*, *CDKN1A*, miR-155, and *SOCS1*, through which FLT3/ITD increases cell proliferation. In addition to FLT3/ITD, growth factors, such as *FLT3 ligand*, *stem cell factor* (*SCF*), and *GM-CSF*, can also enhance activity and/or expression of these molecules, events providing survival signaling to the cells independent of FLT3/ITD. Therefore, cells will be able to survive even if FLT3/ITD activity is abrogated by the inhibitors.

responsible for retaining hematopoietic stem cells in the marrow niche through its receptor *CXCR4* that is expressed on HSCs [38–41]. Similar to normal hematopoietic cells, *CXCR*4 is expressed in most AML cells that express *CXCR4* and migrate in response to *CXCL12* [42]. Antagonizing *CXCR4* inhibits the engraftment and development of AML in a human xenograft human AML model, suggesting that *CXCR4* is required for human AML to home to the marrow niche [43]. High expression of *CXCR4* is associated with the poor prognosis of patients with AML [44, 45]. An early study indicated that FLT3/ITD enhanced chemotaxis to *CXCL12* that is expressed in the niche [42]. The data suggest that FLT3/ITD facilitates the interaction between AML cells and the microenvironment via the enhancement of CXCL12/CXCR4 signaling. The expression of *CXCR4* is upregulated by various cytokines, including stem cell factor [46], *VEGF*, *bFGF*, *EGF*, *IL2*, *IL4*, *IL6*, *IL7*, *IL10*, and *IL15* [47]. The induction of *CXCR4* expression by the cytokines derived from the niche suggests that these cytokines promote the migration of AML cells to the microenvironment, thereby increasing the interaction between AML cells and the microenvironment. Indeed, stem cell factor enhances the migration of human AML cells to *CXCL12* [48] and enhances their homing to the bone marrow [49]. By contrast, *FLT3 ligand* [50], *TNFα*, and *INFγ* downregulate *CXCR4* expression [47]. Adrenergic inputs downregulate *CXCL12* in the marrow environment during the daytime [51] but upregulate *CXCR4* on HSCs at night [52]. Hypoxia induces the expression of *CXCL12* [53] and *CXCR4* [54] by inducing *HIF-1α* expression. Hypoxic conditions in the bone marrow niche that induces the expression of *CXCL12* and *CXCR4* can increase the lodging of AML cells in the bone marrow microenvironment. A recent study suggested that the mobilization of FLT3/ITD$^+$AML cells into the peripheral circulation using the *CXCR4* antagonist AMD3465 enhanced the antileukemia effect of chemotherapy and *FLT3* inhibitor sorafenib, resulting in a reduced burden of AML and prolonged survival of mice [19]. A combination of AMD3100 (Plerixafor), Sorafenib, and *G-CSF* in FLT3-mutated patients yielded an overall response rate of 77% [55]. These data indicate that disrupting the interaction between FLT3/ITD$^+$AML cells and the bone marrow microenvironment by antagonizing *CXCR4* is beneficial to overcome the resistance of leukemia cells against the *FLT3* inhibitor or chemotherapy.

Although reports have indicated that *CXCL12*/*CXCR4* signaling can induce apoptosis in human AML cells by regulating BCL-X_L, NOXA, and BAK [56, 57], stromal cells generally protect FLT3/ITD$^+$ AML cells from apoptosis induced by *FLT3*/*ITD* inhibitors [34–36], and *CXCL12* increases the number of FLT3/ITD$^+$ mouse hematopoietic progenitor cells cultured in the absence of hematopoietic growth factors. These data indicate that *CXCL12* can provide a survival effect on the hematopoietic progenitor cells expressing FLT3/ITD [58]. Consistent with *CXCL12* as a survival factor for FLT/ITD$^+$ cells, targeting the microenvironment by the *CXCR4* antagonist overcomes the resistance of FLT3/ITD$^+$ AML cells to the *FLT3*/*ITD* inhibitors [18, 19, 34, 59–61]. Antagonizing *CXCR4* by BL-8040 and FLT3/ITD inhibition demonstrates synergistic effects in inducing the apoptosis of FLT3/ITD$^+$AML cells. The mechanism by which *CXCL12* and *CXCR4* provide resistance to FLT3/ITD$^+$ AML cells includes the expression of *ERK*, *BCL2*, *MCL1*, and *CYCLIN D1* via the downregulation of miR-15a/16-1 expression [18]. Microenvironment-mediated resistance

of FLT3/ITD+ AML cells to *FLT3* inhibitors through *CXCL12* was partially abrogated by activating p53 in the stromal cells using an *HDM2* inhibitor, suggesting that the combination of HDM2 antagonists and the *FLT3* inhibitor may provide therapeutic efficacy [34]. These data demonstrate that, while antagonizing *CXCR4* induces the mobilization of FLT3/ITD+AML cells into the peripheral circulation, which, in turn, sensitizes cells to *FLT3* inhibitors, antagonizing *CXCL12*/*CXCR4* signaling itself can abrogate resistance to FLT3 inhibitors [18, 19, 34, 59–61]. The data clearly indicate that the resistance of FLT3/ITD+ AML cells to FLT3/ITD inhibitors depends on the stromal cells and is at least partially mediated through *CXCL12*/*CXCR4*.

2.2. Cytokine signaling in the microenvironment as salvation factors for FLT3/ITD+ AML

CXCL12 is not the only cytokine that confers the resistance of FLT3/ITD+AML cells to the *FLT3* inhibitor. Stromal cells secrete various cytokines and growth factors, such as angiopoietins, *TNF-α*, *G-CSF*, *GM-CSF*, and *VEGF* [36]. *FLT3* ligand, stem cell factor, *IL-3*, *GM-CSF*, or *G-CSF* existing in the marrow environment can provide a protective effect on the FLT3/ITD+ AML cells against *FLT3*/*ITD* inhibitors [23, 24]. For instance, the culture of FLT3/ITD+ 32D cells with the *FLT3*-inhibitor AC220 in the absence of growth factors induces the rapid decline in the viable cell number, whereas the addition of *IL-3* significantly inhibits the cytotoxic effect of AC220 (Fukuda & Hirade, unpublished observation). Similarly, *FLT3* ligand that is expressed in the marrow microenvironment increases the resistance of FLT3/ITD+ AML cells to the *FLT3* inhibitor [23]. These cytokines subsequently enhance the expression or activity of *SURVIVIN*, *CDKN1a*, *ERK*, *N-RAS*, and *PIM1*, all of which are known to be involved in the resistant phenotype against FLT3/ITD antagonists. The data indicate that cytokines in the marrow environment provide resistant activity to the FLT3/ITD+AML cells against *FLT3* inhibitors (**Figure 1**).

2.3. STAT3/SURVIVIN signaling pathways

SURVIVIN, an antiapoptotic protein that is upregulated by FLT3/ITD, regulates the proliferation of FLT3/ITD+ hematopoietic progenitor cells [16, 62] and mediates the resistance of FLT3/ITD+ AML cells against the FLT/ITD inhibitor ABT-869 [15]. Zhou et al. reported that *SURVIVIN* expression was upregulated by FLT3/ITD, and its expression was even higher in the resistant FLT3/ITD+ AML cells compared with cells sensitive to ABT-869. On the other hand, antagonizing *SURVIVIN* recovered the sensitivity of resistant FLT3/ITD+ AML cells to ABT-869, indicating that *SURVIVIN* expression is one of the mechanisms responsible for the resistance to ABT-869. *SURVIVIN* expression was mediated by the activation of STAT protein, and antagonizing *STAT3* using SRC-*STAT3* inhibitor IDR E804 abrogated the expression of *SURVIVIN*, coincident with a significant reduction of ABT-869–resistant FLT3/ITD+AML cell proliferation *in vivo*. The combination of ABT-869 with IDR E804 further decreased the burden of ABT-869–resistant FLT3/ITD+AML in a xenograft model in mice compared with the administration of ABT-869 or IDR E804 alone [15], suggesting that *STAT3* is also involved in the resistance to ABT-869. Consistent with

this finding, recent data have demonstrated that the stroma-based activation of *STAT3* ^{Y705} confers resistance to AC220 in FLT3/ITD$^+$AML [63]. The culture of FLT3/ITD$^+$ AML cells in direct contact with stromal cells or in the conditioned medium harvested from the stromal cells increased the IC50 of AC220 in FLT3/ITD$^+$AML cells, with a concomitant increase in the phosphorylation of *STAT3*^{Y705} in the AML cells, compared with control medium without stromal cells. Pharmacologic inhibition of *STAT3* using BP-5-087 [64] decreased the IC_{50} of AC220 in the FLT3/ITD$^+$ AML cells cultured in direct contact with stromal cells or in the conditioned medium derived from stromal cells, indicating that *STAT3* confers FLT3/ITD$^+$ AML resistance to AC220 that is induced by stromal cells. This finding is consistent with *SURVIVIN* being a direct transcriptional target of STAT3 in FLT3/ITD$^+$AML and lymphoma cells [15, 65], suggesting that the *STAT3*/ *SURVIVIN* axis protects FLT3/ ITD$^+$AML cells from the antileukemia effect by the *FLT3* inhibitors. *SURVIVIN* expression is also upregulated by exogenous factors such as *FLT3*-ligand [15, 16], which hampers the efficacy of the *FLT3* inhibitor and is involved in the resistant phenotype of FLT3/ITD$^+$ AML cells [23]. Likewise, stem cell factor [66] and GM-CSF [67], all of which are provided by the marrow microenvironment, increase the expression of *SURVIVIN* (**Figure 1**). These data suggest that the marrow niche protects FLT3/ITD$^+$ AML cells from *FLT3*/*ITD* antagonists through the upregulation of *SURVIVIN* by the hematopoietic growth factors secreted by the marrow environmental cells (**Figure 1**). Therefore, antagonizing *SURVIVIN* and/or *STAT3* would overcome the resistance of FLT3/ITD$^+$ AML to *FLT3* inhibitors.

2.4. ERK/MAPK signaling pathways

An additional mechanism responsible for the resistance to the *FLT3* inhibitor by the niche is the activation of *ERK/MAPK* signaling pathways. FLT3 inhibitors induce apoptosis in FLT3/ITD$^+$ AML cells , whereas direct contact and proximity to stromal cells were protective toward FLT3/ ITD+ AML cells against FLT3 inhibition. Coculture of FLT3/ITD$^+$ AML cells with bone marrow stroma cells was associated with cell cycle arrest and persistent activation of ERK, even in the presence of the *FLT3* antagonist [36]. On the other hand, inhibition of MEK significantly abrogated the protective effect of stromal cells or *FLT3* ligand in FLT3/ITD$^+$ AML cells, indicating that *ERK* activation provided by the stromal cells is responsible for the resistance to *FLT3* inhibition in FLT3/ITD$^+$ AML cells. It was also reported that direct cell contact is more essential for the persistent activation of ERK compared with exposure to soluble factors [36]. Consistently, a recent report demonstrated that the treatment of FLT3/ITD$^+$ AML cells with *FLT3* inhibitors for over 48 hours induced rebound in *ERK* phosphorylation [68], suggesting an adaptive feedback mechanism capable of reactivating *ERK* signaling in response to upstream target inhibition in the FLT3/ITD$^+$ AML. These data suggest that antagonizing *ERK/MAPK* signaling pathways can overcome the resistance of FLT3/ITD$^+$AML to the FLT3 inhibitors (**Figure 1**).

2.5. Cyclin-dependent kinase inhibitor 1a/Pbx1 signaling pathways

The report by Yang et al. also noted the cell cycle arrest of FLT3/ITD$^+$ AML cells cocultured by stromal cells [36], indicating that stromal cells provide factors that induce cell cycle quiescence. *CDKN1a* is one of the cyclin-dependent kinase inhibitors that is known

to block G_1/S and G_2/M transition [69–71]. It is reported that cell cycle quiescence of leukemia stem cells is one of the mechanisms that leads to refractoriness to anticancer drugs that normally eliminate cells in S-phase [30]. In human AML cells, *CDKN1a* is upregulated by growth factors, such as stem cell factor, *FLT3*-ligand, and *GM-CSF* [14, 70, 72], all of which are present in the marrow microenvironment. Consistent with *FLT3* ligand–induced upregulation of *CDKN1a*, FLT3/ITD also upregulates CDKN1a via Stat5 [73]. Abe et al. reported that knocking down CDKN1a significantly decreases proliferation and cell cycle progression in FLT3/ITD+ cells concomitant with an increase in Pbx1 mRNA expression [14], indicating that *CDKN1a* that is upregulated by FLT3/ITD negatively regulates proliferation and cell cycle progression of FLT3/ITD+ cells. Knocking down Pbx1 expression using shRNAs abrogated the enhanced proliferation that was induced by CDKN1a deletion. The data demonstrate that FLT3/ITD not only contains stimulating activity but also harbors inhibitory activity on cell proliferation, which is mediated by upregulating CDKN1a and downregulating PBX1 expression. More importantly, FLT3/ITD confers resistance to the *FLT3* inhibitor by inducing the expression of CDKN1a [14]. When FLT3/ITD was antagonized with AC220, a selective inhibitor of FLT3/ITD, CDKN1a expression was decreased coincident with PBX1 mRNA upregulation and a rapid decline in the number of viable FLT3/ITD + Ba/F3 cells; however, the cells eventually became refractory to AC220. Overexpressing CDKN1a in FLT3/ITD + Ba/F3 cells delayed the emergence of cells that were refractory to AC220, whereas silencing CDKN1a accelerated their development. These data indicate that FLT3/ITD can inhibit FLT3/ITD+ cell proliferation through the CDKN1a /PBX1 axis and that antagonizing FLT3/ITD contributes to the subsequent development of cells that are refractory to the FLT3/ITD inhibitor by disrupting CDKN1a expression because of FLT3/ITD inhibition. Similarly, the upregulation of CDKN1a may represent one mechanism responsible for the *FLT3* ligand–induced resistance of FLT3/ITD+ AML cells against the *FLT3* inhibitor [23] because CDKN1a expression is induced by *FLT3* ligand [14]. The data also suggest that *CDKN1a*, which is upregulated by hematopoietic growth factors, such as *SCF* and *GM-CSF*, which are secreted by stromal cells, is also responsible for the refractory phenotype of FLT3/ITD+ AML cells (**Figure 1**).

2.6. RUNX1 in the resistance of FLT3/ITD+ AML

A recent report demonstrated that FLT3/ITD signaling is associated with a common expression signature as well as a common chromatin signature. The study identified that FLT3/ITD induces the chronic activation of *MAPK*-inducible transcriptional factor *AP-1* and that *AP-1* cooperates with *RUNX1* to shape the epigenome of FLT3/ITD+ AML [74]. *RUNX1* is a core-binding transcription factor that plays an important role in hematopoietic homeostasis, particularly in differentiation and proliferation [75, 76]. *RUNX1*-deficient cells showed increased susceptibility to AML development in collaboration with MLL-ENL, N-RAS, and EVI5 [77–79], suggesting that *RUNX1* can function as a tumor suppressor in myeloid malignancies. By contrast, *RUNX1* also promotes the survival of AML cells and lymphoma development and can function as an oncogene [80, 81]. These data suggest that the *RUNX1* has a dual function that promotes and attenuates the proliferation of hematological malignant cells. Hirade et al. identified that *RUNX1*

expression is upregulated by FLT3/ITD and functions as an oncogene in FLT3/ITD$^+$ cells [9]. Another group demonstrated that *RUNX1* cooperates with FLT3/ITD to induce acute leukemia, validating *RUNX1* as an oncogene in FLT3/ITD signaling [17]. With respect to the function of RUNX1 in the resistance to the *FLT3* inhibitor AC220, antagonizing *RUNX1* significantly accentuated the antiproliferative effect of AC220 in FLT3/ITD$^+$ 32D cells. *RUNX1* expression was elevated in the FLT3/ITD$^+$ 32D cells, which became refractory to AC220, whereas knocking down RUNX1 significantly inhibited the emergence and proliferation of FLT3/ITD$^+$ cells refractory to AC220, demonstrating that *RUNX1* mediates the development of FLT3/ITD$^+$ AML cells resistant to AC220 in FLT3/ITD$^+$ cells. RUNX1 upregulation by AC220-resistant cells was not due to the additional mutation in the FLT3 gene because the upregulation of RUNX1 by AC220 was no longer observed when resistant cells were incubated without AC220. The data indicate that the epigenetic mechanism is likely involved in the upregulation of *RUNX1* by AC220 refractory cells [9]. Because *RUNX1* cooperated with *MAPK*-inducible transcription factor *AP1* [74] and *MAPK* is regulated by various growth factors existing in the marrow microenvironment, it is highly likely that *RUNX1* function is indirectly modulated by the microenvironmental factors. On the other hand, *RUNX1* directly binds to the CXCR4 promoter region, and *RUNX1* transactivates CXCR4 in a DNA binding–dependent manner, indicating that *RUNX1* transcriptionally upregulates CXCR4 expression [78]. These findings strongly suggest that the upregulation of *RUNX1* by FLT3/ITD increases the expression of *CXCR4*, which, in turn, enhances the chemotaxis of FLT3/ITD$^+$ AML cells to stromal niche cells, thereby increasing the likelihood of the cells being protected from the insult by the *FLT3* inhibitor in the niche. On the other hand, *RUNX1* downregulates the expression of cell adhesion factors that promote the residency of stem cells and megakaryocytes in their bone marrow niche [82], suggesting that *RUNX1* expression that is induced by FLT3/ITD likely alters the interaction between the FLT3/ITD$^+$ AML cells and niche cells and is involved in the resistance to the *FLT3* inhibitor (**Figure 1**).

2.7. FLT3/ITD evades external inhibitory cytokine control

While it has been unclear how leukemia cells escape from normal cytokine control that is indispensable to maintain normal hematopoiesis, a recent study demonstrated that FLT3/ITD facilitates the development of myeloproliferative disease by inhibiting the interferon response [20, 26]. Interferon exhibits an anti-proliferative effect on primitive hematopoietic cells [83–86], including FLT3/ITD$^+$ cells [20]. In FLT3/ITD$^+$ cells, activated STAT5 up-regulates SOCS1 expression, which inhibits the antiproliferative effect induced by interferon-α or interferon-γ [20]. SOCS1 protects FLT3/ITD$^+$AML cells from external interferon control, thereby promoting myeloproliferative disease. Another report also uncovered a novel mechanism responsible for the escape of FLT3/ITD$^+$ AML cells from interferon signaling. Micro-RNA 155 (miR-155) is significantly overexpressed in FLT3/ITD AML [87–92] and promotes myeloproliferative disease induced by FLT3/ITD. This was coincided with repression of the interferon response compared with that with wild-type FLT3. Inhibition of miR-155 resulted in the elevation of the interferon response and reduction in the proliferation of human FLT3/ITD$^+$ AML cells. The data indicate that

miR-155 promotes FLT3/ITD+ AML cell proliferation by blocking interferon signaling [26]. Taken together, FLT3/ITD stimulates AML cell proliferation by evading external antiproliferative cytokine control that is normally provided by the microenvironment (**Figure 1**). It remains to be determined if these mechanisms are involved in the resistance against *FLT3* inhibitors.

FLT3/ITD+ AML is also found in patients with acute promyelocytic leukemia who harbor the PML-RARα fusion gene resulting from chromosomal translocation. Recent data have demonstrated that the combination of the FLT3/ITD inhibitor and ATRA, which targets PML-RARα, displays a synergistic effect of reducing the burden of FLT3/ITD+ AML both *in vitro* and in a xenotransplantation model [93–95]. This is a promising strategy to facilitate the differentiation of FLT3/ITD + AML in the patients; however, recent data have also indicated the inactivation of retinoids in the marrow niche, thereby inhibiting the differentiation of AML cells [96–98]. In this regard, the effect of ATRA with the FLT3/ITD inhibitor may be more complicated than anticipated because the marrow niche may impede the long-term effect of ATRA.

2.8. Interaction of FLT3/ITD+ AML cells with the microenvironment via adhesion molecules

The interaction between AML cells and the microenvironment is mediated by various factors, such as *CXCL12*, and adhesion molecules. *CXCL12* can activate adhesion molecules, particularly very late antigen-4 (*VLA-4*) and lymphocyte function–associated antigen-1 (*LFA-1*) on hematopoietic stem and progenitor cells, which also regulate the homing process [99]. FLT3/ITD decreases the expression of *VLA4* expression, coincident with a significant reduction in cell adhesion to *VCAM1* [58]. While the data indicate that FLT3/ITD negatively regulates the expression of *VLA4* and adhesion to its ligand *VCAM1*, the inhibition of FLT3/ITD by Fl-700 decreases the affinity of *VLA4* to soluble *VCAM1* [100], indicating that FLT3/ITD modulates the interaction between *VLA4* and *VCAM1*. The interaction of leukemia cells with the microenvironment is also mediated via E-selection [101]. A recent report has demonstrated that a dual inhibitor for *E-selectin* and *CXCR4* (GMI-1359) exerts efficient antileukemia effects in an FLT3/ITD+ AML xenograft model by mobilizing AML cells into the peripheral circulation from the bone marrow [102, 103]. The data suggest that antagonizing adhesion molecules that retain FLT3/ITD+ AML cells in the bone marrow microenvironment is beneficial to abate the resistance of AML cells to the *FLT3* inhibitor by mobilizing AML cells into the blood circulation.

Taken together, these data provide evidence that stromal cells, or other cells comprising the microenvironment, support FLT3/ITD+AML cells via soluble factors and adhesion molecules, which, in turn, activate survival or proliferative signaling in the AML cells (**Figure 1**). However, the machinery provided by the microenvironment is not confined to these factors described above. A recent report has indicated that bone marrow mesenchymal stromal cells transfer their mitochondria to AML cells to support their proliferation [104, 105], possibly representing an additional mechanism that can enhance the resistance to the *FLT3* inhibitor in FLT3/ITD+ AML. Likewise, it is highly possible that microsomes containing micro-RNAs

secreted from the microenvironment modulate the function of FLT3/ITD$^+$ AML cells, although this hypothesis remains yet to be proven.

3. Functional interaction between FLT3/ITD and CXCR4 in the migration and homing of AML cells that are associated with resistance

Because *CXCL12*/*CXCR4* provides a survival signal to FLT3/ITD$^+$ AML cells, it suggests that *CXCL12*/*CXCR4* signaling accentuates FLT3/ITD signaling activity. By contrast, FLT3/ITD regulates cell migration to *CXCL12* [50], indicating that FLT3/ITD modulates *CXCR4* signaling. Therefore, FLT3/ITD and *CXCL12*/*CXCR4* signaling mutually interacts. While an earlier study demonstrated that patients with FLT3/ITD$^+$ AML have higher *CXCR4* expression than those with FLT3 wild-type AML [45], subsequent studies have demonstrated controversial findings. We and other groups have demonstrated that overexpressing FLT3/ITD in mouse Ba/F3 cells or human CD34$^+$ cells significantly downregulated *CXCR4* expression [50, 59]. Incubating human CD34$^+$ cells with *FLT3* ligand also decreased the expression of *CXCR4* [50]. Moreover, the mRNA expression of *CXCR4* was significantly lower in patients with FLT3/ITD$^+$ AML than in those with wild-type FLT3 [9, 106]. These data indicate that FLT3/ITD can reduce the expression of *CXCR4* in contrast to the data of the earlier report. The mechanism responsible for the modulation of CXCR4 expression by FLT3/ITD remains subject to investigation. PIM1, which is activated by FLT3/ITD, upregulates *CXCR4* [107]. Similarly, *RUNX1*, which is elevated in FLT3/ITD$^+$ AML, upregulates CXCR4 transcription [78]. On the other hand, *CEBPα*, a transcriptional factor that increases CXCR4 expression [108], is inactivated by FLT3/ITD [11, 109]. Therefore, the inactivation of *CEBPα* by FLT3/ITD can decrease CXCR4 expression. Because FLT3/ITD inhibits *CEBPα* but enhances *PIM1* and/or *RUNX1* expression, the balance between the inactivation of *CEBPα* and activation of *PIM1* and/or *RUNX1* may determine the expression of *CXCR4* in FLT3/ITD$^+$ AML.

Although the *FLT3* ligand, as well as FLT3/ITD, increases the migration of mouse hematopoietic cells to *CXCL12* [19, 50, 106], *FLT3* signaling can decrease the migration of CD34$^+$ cells and mouse Ba/F3 cells toward *CXCL12* [50, 59]. Enhancing migration and decreasing migration in response to *CXCL12* by FLT3/ITD appear to be controversial, but the reduction of migration toward *CXCL12* is most likely a consequence of a decrease in *CXCR4* expression, which, in turn, induces the quantitative reduction of *CXCR4* signaling. Jacobi et al. reported that the transient expression of FLT3/ITD decreases *CXCR4* expression in human CD34$^+$ cells, coincident with their reduced migration toward *CXCL12* [59]. This is consistent with the reduction in *CXCR4* expression in CD34$^+$ cells or Ba/F3 cells incubated with *FLT3* ligand that is accompanied by a decrease in CXCL12-mediated migration [50]. These data indicate that FLT3/ITD, as well as normal *FLT3* signaling, can inhibit *CXCL12*/*CXCR4* signaling by downregulating *CXCR4* expression. By contrast, the sustained expression of FLT3/ITD enhances migration in response to *CXCL12*, even with a significant downregulation of the *CXCR4* level [50]. Augmentation in cell migration toward *CXCL12* despite the reduction in *CXCR4* expression suggests that the increase in migration was not due to the qualitative increase in *CXCR4* signaling. A subsequent study by Onishi et al. confirmed that enhanced migration by FLT3/ITD was mediated through

http://dx.doi.org/10.5772/intechopen.71676

the qualitative change in *CXCR4* signaling [106]. The data indicated that molecules and/or pathways downstream of *CXCR4* that are regulated in the presence of FLT3/ITD were overlapped but distinct from those regulated in the absence of FLT3/ITD, suggesting that FLT3/ITD regulates *CXCR4* signaling pathways functionally distinct from those of normal cells [106]. This implies that FLT3/ITD functionally alters *CXCR4* signaling. These findings strongly suggest that FLT3/ITD can negatively regulate *CXCR4* signaling by qualitatively decreasing *CXCR4* signaling by downregulating *CXCR4* expression, whereas it also increases *CXCR4* signaling activity by changing the global gene expression downstream of CXCR4 (**Figure 2**). One of the molecules responsible for the activation of *CXCR4* signaling by FLT3/ITD is Rho-associated kinase-1 (*ROCK1*). *ROCK1* promotes the migration of *CXCR4*+ cells to *CXCL12*, whereas antagonizing *ROCK1* displays the opposite effect. *CXCL12* transiently upregulates *ROCK1* expression but subsequently downregulates its expression in the absence of FLT3/ITD. This downregulation is associated with the attenuation in cell migration to CXCL12, suggesting the presence of negative

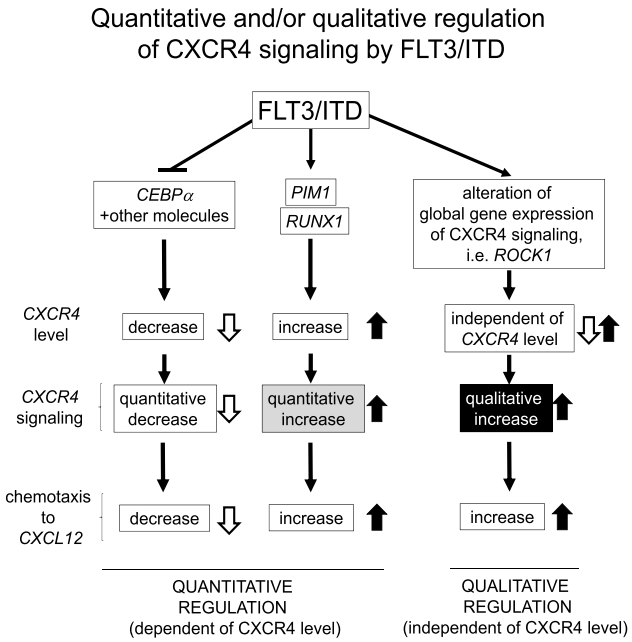

Figure 2. Quantitative and/or qualitative regulation of CXCR4 signaling by FLT3/ITD. *CXCL12*/*CXCR4* signaling augments FLT3/ITD activity, but in contrast, FLT3/ITD modulates *CXCL12*/*CXCR4* signaling, indicating that *CXCL12*/*CXCR4* and FLT3/ITD signaling mutually interacts. Regulation of CXCR4 signaling by FLT3/ITD is classified into two categories: one is quantitative regulation and the other is qualitative mechanism. FLT3/ITD regulates expression of *CXCR4*, depending on the transcriptional mediators or kinases. For instance, inactivation of CEBPα by FLT3/ITD can decrease *CXCR4* expression, whereas activation of *PIM1* and/or *RUNX1* can increase *CXCR4* expression. Downregulation of CXCR4 diminishes cell migration to CXCL12, whereas upregulation of *CXCR4* expression leads to enhancement in cell migration to *CXCL12*. On the other hand, FLT3/ITD modulates global gene expression downstream of *CXCR4*, which leads to the enhancement of cell migration to *CXCL12*. Classification of genes that are regulated by *CXCL12* in FLT3/ITD$^-$ cells and those in FLT3/ITD$^+$ cells based on the molecular pathways or biological process demonstrated that they are functionally overlapped but distinct. The data suggest that FLT3/ITD functionally alters *CXCL12*/*CXCR4* signaling. For instance, downregulation of *ROCK1* expression by *CXCL12* that is normally observed in control cells is abrogated by FLT3/ITD, which is responsible for the enhancement in cell migration to *CXCL12* by FLT3/ITD.

feedback in *CXCL12*/*CXCR4* signaling mediated by modulating *ROCK1* expression to prevent excessive migration in normal cells. By contrast, FLT3/ITD or *FLT3* ligand enhances the expression and prevents the subsequent downregulation of the *ROCK1* level that is normally induced by CXCL12, thereby abrogating the negative feedback generated by *CXCL12* and *ROCK1*. The loss of negative feedback on *ROCK1* expression induced by *FLT3* signaling resulted in the sustained activation of *CXCL12*/*CXCR4* signaling, thereby enhancing the migration of FLT3/ITD$^+$ cells toward *CXCL12*. Enhanced chemotaxis is also mediated through RAS [58].

An additional molecular machinery that specifically mediates the migration of FLT3/ITD$^+$ cells is *PIM1* kinase. The expression and kinase activity of *PIM1* are upregulated in FLT3/ITD$^+$ AML cells [110]. Enhanced *PIM1* activity induced by FLT3/ITD is essential for the migration and homing of AML cells [107]. The effect of *PIM1* on the migration and homing of FLT3/ITD cells is mediated by the increase in *CXCR4* owing to its recycling by the phosphorylation of serine 339 on *CXCR4*. These data indicate that *PIM1* activity is essential for the proper *CXCR4* surface expression and migration of FLT3/ITD$^+$ AML cells toward *CXCL12*. In addition to regulating migration and homing, *PIM1* modulates the resistance of FLT3/ITD$^+$ AML cells to *FLT3* inhibitors [21, 22]. Targeting *PIM1* synergizes with *FLT3* inhibition [111] and restores the sensitivity of *FLT3* inhibitors in FLT3/ITD$^+$ AML cells [21]. A recent study in abstract form indicated that the microenvironment-induced expression of *PIM* kinase supports chronic leukemia (CLL) survival and promotes *CXCR4*-dependent migration [112]. Although this was investigated in CLL, the data suggest that microenvironmental factors increase the expression of *PIM1* kinase, which promotes the resistance of FLT3/ITD$^+$ AML. The upregulated *PIM1* kinase, in turn, would facilitate the migration of FLT3/ITD$^+$ AML toward *CXCL12* by activating *CXCR4* signaling, thereby increasing the interaction between FLT3/ITD$^+$ AML cells and microenvironment cells. In this regard, antagonizing PIM1 represents an additional therapeutic strategy to abrogate the interaction between FLT3/ITD$^+$ AML cells and marrow niches, particularly for those that have become resistant to *FLT3*/*ITD* inhibitors. Similarly, *ROCK1* enhances not only *CXCL12*-induced migration [106] but also the proliferation of FLT3/ITD$^+$ cells [13]. Therefore, antagonizing *ROCK1* is likely to be beneficial to interfere with the communication of FLT3/ITD$^+$ AML cells between the marrow niches and inhibit their proliferation. These data suggest that FLT3/ITD increases the communication with the bone marrow microenvironment by enhancing the chemotaxis toward *CXCL12*. Together with *CXCL12* protecting FLT3/ITD$^+$ AML cells from the insult of FLT3 inhibitors, the findings strongly indicate that reciprocal interaction between FLT3/ITD and *CXCL12*/*CXCR4* signaling exists that accentuates the resistance to FLT3 inhibitors.

4. Effect of FLT3 mutation on the microenvironment

Normal hematopoietic stem cells drive hematopoiesis, but this process requires appropriate factors secreted by adjacent cells, adhesion molecules, neighboring cells such as mesenchymal stromal cells, osteolineage cells, and endothelial cells that exist in the microenvironment [113]. In agreement with the microenvironment mediating the tight control necessary for normal hematopoiesis, earlier studies have demonstrated that malfunction of microenvironmental cells can lead to the development of myeloproliferation, which represents one of the outcomes of aberrant hematopoiesis. Walkley et al. demonstrated

that the loss of retinoic acid receptor gamma (PARγ) resulted in myeloproliferation in mice; however, the transplantation of the marrow cells into PARγ-deficient cells did not cause myeloproliferation in wild-type recipients, whereas the transplantation of wild-type marrow cells caused myeloproliferation in PARγ-deficient recipients, indicating that myeloproliferation caused by the loss of PARγ was microenvironmental [114]. The microenvironmental effect on aberrant myeloproliferation is also supported by experiments using Rb-deficient cells. Knocking out Rb resulted in myeloproliferation in mice; however, the genetic defect in both hematopoietic cells and the microenvironment was necessary for the development of myeloproliferation [115]. Furthermore, deletion of DICER1 in primitive osteolineage cells led to myelodysplastic syndrome and AML [116], indicating that malfunction of *DICER1* in the niche component was sufficient to cause myeloid malignancy. These findings indicate that the genetic alteration and/or malfunction of the microenvironment can induce myeloid malignancies.

Reports have demonstrated that HSCs regulate their own niches by instructing neighboring stromal cells to produce supportive factors or alter the overall microenvironment [117–119]. While the marrow niche supports leukemia cell proliferation or protects cells from chemotherapeutic insult by providing various survival signals, recent evidence has demonstrated that leukemia cells modulate the marrow environment to create a supportive niche favoring survival for AML cells, just as healthy HSCs regulate their niche. Zhang et al. demonstrated that chronic myeloid leukemia (CML) cells modulate the microenvironment in favor of CML cells over healthy HCS by modulating *CXCL12* expression and alter the localization of HSCs. CML cells modulate cytokine expression in the microenvironment, such that they support CML cells [120]. A study by Schepers et al. identified that myeloproliferative neoplasia (MPN) remodels endosteal bone marrow niches by stimulating mesenchymal stem cells to produce functionally altered osteoblastic lineage cells. This results in the creation of a self-reinforcing leukemic niche that impairs normal hematopoiesis and favors leukemic stem cell function [121]. Several cytokines, such as thrombopoietin and CCL3, that direct cell-cell interaction, alteration of TGF-β, and Notch and inflammatory signaling were involved in the expansion and/or remodeling in osteoblastic lineage cells. The osteoblastic lineage cells remodeled by myeloproliferation compromised normal HSCs but effectively support leukemia stem cells [121]. Similarly, the latest study by Mead et al. demonstrated that FLT3/ITD modulates the marrow microenvironment and impaired the number of HSCs. In the marrow of $FLT3^{ITD/ITD}$ mice, FLT3/ITD-induced myeloproliferation was associated with a progressive decline in the HSC compartment. Notably, when $FLT3^{ITD/ITD}$ marrow cells were transplanted with marrow competitor cells from wild-type mice into healthy recipients, the HSCs derived from the competitor cells were significantly reduced, demonstrating the presence of a cell extrinsic mechanism that diminishes the competitor HSC. Loss of competitor cells in the recipient mice that developed FLT3/ITD-induced myeloproliferation was coincided with the disruption of stromal cells in the recipient marrow, an activity that was associated with reduced numbers of endothelial and mesenchymal stromal cells showing increased inflammation-associated gene expression. The study finally discovered that tumor necrosis factor (TNF), a cell-extrinsic negative regulator of HSCs, was overexpressed in the marrow niche cells in $FLT3^{ITD/ITD}$ mice, and anti-TNF treatment partially rescued the loss of HSCs. These data clearly demonstrate that FLT3/ITD compromises HSCs through an extrinsically mediated mechanism of disrupting HSCs that support

bone marrow stromal cells by generating an inflammatory environment [122]. The same study also demonstrated that the expression of FLT3 mRNA and protein is absent in HSCs, strongly suggesting that FLT3/ITD protein is not expressed in most primitive HSCs, even if FLT3/ITD mutation exists in the FLT3 gene in HSCs. Because these HSCs harboring the FLT3/ITD gene but lacking the expression of *FLT3/ITD* protein would not be targeted by the *FLT3* inhibitors, they may represent a reservoir for the development of resistant clones, in which additional mutations can be accumulated. The lack of mutant *FLT3/ITD* protein in HSCs harboring FLT3/ITD mutation on the FLT3 gene implies that current strategies targeting FLT3/ITD protein or activity would be ineffective. In this regard, disrupting the FLT3 gene, for instance, by using a gene-editing strategy, would represent an additional approach to eliminate HSCs containing FLT3/ITD mutation. Moreover, because FLT3/ITD$^+$ AML restructures the marrow environment in favor of AML cells over normal HSCs, factors provided by FLT3/ITD$^+$ AML cells that influence the marrow environment would represent a novel therapeutic target.

5. Summary

FLT3/ITD$^+$ AML can become refractory to *FLT3* inhibitors. Factors derived from the marrow microenvironment represent one such mechanism responsible for the refractory phenotype to *FLT3/ITD* inhibitors. Understanding the molecular mechanism involved in microenvironment-mediated resistance will shed light on the development of innovative therapeutic strategies against FLT3/ITD$^+$ AML, especially for FLT3/ITD$^+$ AML that has become refractory to *FLT3* inhibitors.

Acknowledgements

The authors declare that no potential conflicts of interest associated with this study exist. This work was supported by research support funds from the Grant-in-Aid for Scientific Research (17K10111 to S.F.) and a Grant-in-Aid for Young Investigators (15K19616 to T.H.) from the Japanese Society for the Promotion of Science.

Author details

Seiji Fukuda[1,2]*, Tomohiro Hirade[2], Mariko Abe[2], Takeshi Taketani[2] and Chie Onishi[3]

*Address all correspondence to: sfukuda@med.shimane-u.ac.jp

1 Department of Clinical Nursing, Shimane University School of Medicine, Izumo, Shimane, Japan

2 Department of Pediatrics, Shimane University School of Medicine, Izumo, Shimane, Japan

3 Department of Oncology and Hematology, Shimane University School of Medicine, Izumo, Shimane, Japan

References

[1] Levis M, Small D. FLT3: It does matter in leukemia. Leukemia. 2003;**17**:1738-1752

[2] Ley TJ, Miller C, Ding L, Raphael BJ, Mungall AJ, Robertson A, et al. Genomic and epigenomic landscapes of adult de novo acute myeloid leukemia. The New England Journal of Medicine. 2013;**368**:2059-2074

[3] Hospital MA, Green AS, Maciel TT, Moura IC, Leung AY, Bouscary D, et al. FLT3 inhibitors: Clinical potential in acute myeloid leukemia. OncoTargets and Therapy. 2017;**10**: 607-615

[4] Ghiaur G, Levis M. Mechanisms of resistance to FLT3 inhibitors and the role of the bone marrow microenvironment. Hematology/Oncology Clinics of North America. 2017;**31**: 681-692

[5] Mizuki M, Fenski R, Halfter H, Matsumura I, Schmidt R, Muller C, et al. Flt3 mutations from patients with acute myeloid leukemia induce transformation of 32D cells mediated by the Ras and STAT5 pathways. Blood. 2000;**96**:3907-3914

[6] Levis M, Allebach J, Tse KF, Zheng R, Baldwin BR, Smith BD, et al. A FLT3-targeted tyrosine kinase inhibitor is cytotoxic to leukemia cells in vitro and in vivo. Blood. 2002;**99**: 3885-3891

[7] Hayakawa F, Towatari M, Kiyoi H, Tanimoto M, Kitamura T, Saito H, et al. Tandem-duplicated Flt3 constitutively activates STAT5 and MAP kinase and introduces autonomous cell growth in IL-3-dependent cell lines. Oncogene. 2000;**19**:624-631

[8] Gilliland DG, Griffin JD. The roles of FLT3 in hematopoiesis and leukemia. Blood. 2002;**100**:1532-1542

[9] Hirade T, Abe M, Onishi C, Taketani T, Yamaguchi S, Fukuda S. Internal tandem duplication of FLT3 deregulates proliferation and differentiation and confers resistance to the FLT3 inhibitor AC220 by up-regulating RUNX1 expression in hematopoietic cells. International Journal of Hematology. 2016;**103**:95-106

[10] Zheng R, Friedman AD, Small D. Targeted inhibition of FLT3 overcomes the block to myeloid differentiation in 32Dcl3 cells caused by expression of FLT3/ITD mutations. Blood. 2002;**100**:4154-4161

[11] Zheng R, Friedman AD, Levis M, Li L, Weir EG, Small D. Internal tandem duplication mutation of FLT3 blocks myeloid differentiation through suppression of C/EBPalpha expression. Blood. 2004;**103**:1883-1890

[12] Tse KF, Mukherjee G, Small D. Constitutive activation of FLT3 stimulates multiple intracellular signal transducers and results in transformation. Leukemia. 2000;**14**: 1766-1776

[13] Mali RS, Ramdas B, Ma P, Shi J, Munugalavadla V, Sims E, et al. Rho kinase regulates the survival and transformation of cells bearing oncogenic forms of KIT, FLT3, and BCR-ABL. Cancer Cell. 2011;**20**:357-369

[14] Abe M, Pelus LM, Singh P, Hirade T, Onishi C, Purevsuren J, et al. Internal tandem duplication in FLT3 attenuates proliferation and regulates resistance to the FLT3 inhibitor AC220 by modulating p21Cdkn1a and Pbx1 in hematopoietic cells. PLoS One. 2016;**11**:e0158290

[15] Zhou J, Bi C, Janakakumara JV, Liu SC, Chng WJ, Tay KG, et al. Enhanced activation of STAT pathways and overexpression of survivin confer resistance to FLT3 inhibitors and could be therapeutic targets in AML. Blood. 2009;**113**:4052-4062

[16] Fukuda S, Singh P, Moh A, Abe M, Conway EM, Boswell HS, et al. Survivin mediates aberrant hematopoietic progenitor cell proliferation and acute leukemia in mice induced by internal tandem duplication of Flt3. Blood. 2009;**114**:394-403

[17] Behrens K, Maul K, Tekin N, Kriebitzsch N, Indenbirken D, Prassolov V, et al. RUNX1 cooperates with FLT3-ITD to induce leukemia. The Journal of Experimental Medicine. 2017;**214**:737-752

[18] Abraham M, Klein S, Bulvik B, Wald H, Weiss ID, Olam D, et al. The CXCR4 inhibitor BL-8040 induces the apoptosis of AML blasts by downregulating ERK, BCL-2, MCL-1 and cyclin-D1 via altered miR-15a/16-1 expression. Leukemia. 2017;**31**:2336-2346

[19] Zeng Z, Shi YX, Samudio IJ, Wang RY, Ling X, Frolova O, et al. Targeting the leukemia microenvironment by CXCR4 inhibition overcomes resistance to kinase inhibitors and chemotherapy in AML. Blood. 2009;**113**:6215-6224

[20] Reddy PN, Sargin B, Choudhary C, Stein S, Grez M, Muller-Tidow C, et al. SOCS1 cooperates with FLT3-ITD in the development of myeloproliferative disease by promoting the escape from external cytokine control. Blood. 2012;**120**:1691-1702

[21] Adam M, Pogacic V, Bendit M, Chappuis R, Nawijn MC, Duyster J, et al. Targeting PIM kinases impairs survival of hematopoietic cells transformed by kinase inhibitor-sensitive and kinase inhibitor-resistant forms of Fms-like tyrosine kinase 3 and BCR/ABL. Cancer Research. 2006;**66**:3828-3835

[22] Green AS, Maciel TT, Hospital MA, Yin C, Mazed F, Townsend EC, et al. Pim kinases modulate resistance to FLT3 tyrosine kinase inhibitors in FLT3-ITD acute myeloid leukemia. Science Advances. 2015;**1**:e1500221

[23] Sato T, Yang X, Knapper S, White P, Smith BD, Galkin S, et al. FLT3 ligand impedes the efficacy of FLT3 inhibitors in vitro and in vivo. Blood. 2011;**117**:3286-3293

[24] Zheng R, Levis M, Piloto O, Brown P, Baldwin BR, Gorin NC, et al. FLT3 ligand causes autocrine signaling in acute myeloid leukemia cells. Blood. 2004;**103**:267-274

[25] Nabinger SC, Li XJ, Ramdas B, He Y, Zhang X, Zeng L, et al. The protein tyrosine phosphatase, Shp2, positively contributes to FLT3-ITD-induced hematopoietic progenitor hyperproliferation and malignant disease in vivo. Leukemia. 2013;**27**:398-408

[26] Wallace JA, Kagele DA, Eiring AM, Kim CN, Hu R, Runtsch MC, et al. miR-155 promotes FLT3-ITD-induced myeloproliferative disease through inhibition of the interferon response. Blood. 2017;**129**:3074-3086

[27] Smith CC, Wang Q, Chin CS, Salerno S, Damon LE, Levis MJ, et al. Validation of ITD mutations in FLT3 as a therapeutic target in human acute myeloid leukaemia. Nature. 2012;**485**:260-263

[28] Levis M. Quizartinib for the treatment of FLT3/ITD acute myeloid leukemia. Future Oncology. 2014;**10**:1571-1579

[29] Alvarado Y, Kantarjian HM, Luthra R, Ravandi F, Borthakur G, Garcia-Manero G, et al. Treatment with FLT3 inhibitor in patients with FLT3-mutated acute myeloid leukemia is associated with development of secondary FLT3-tyrosine kinase domain mutations. Cancer. 2014;**120**:2142-2149

[30] Saito Y, Uchida N, Tanaka S, Suzuki N, Tomizawa-Murasawa M, Sone A, et al. Induction of cell cycle entry eliminates human leukemia stem cells in a mouse model of AML. Nature Biotechnology. 2010;**28**:275-280

[31] Ishikawa F, Yoshida S, Saito Y, Hijikata A, Kitamura H, Tanaka S, et al. Chemotherapy-resistant human AML stem cells home to and engraft within the bone-marrow endosteal region. Nature Biotechnology. 2007;**25**:1315-1321

[32] Lane SW, Scadden DT, Gilliland DG. The leukemic stem cell niche: Current concepts and therapeutic opportunities. Blood. 2009;**114**:1150-1157

[33] Smith BD, Levis M, Beran M, Giles F, Kantarjian H, Berg K, et al. Single-agent CEP-701, a novel FLT3 inhibitor, shows biologic and clinical activity in patients with relapsed or refractory acute myeloid leukemia. Blood. 2004;**103**:3669-3676

[34] Kojima K, McQueen T, Chen Y, Jacamo R, Konopleva M, Shinojima N, et al. p53 activation of mesenchymal stromal cells partially abrogates microenvironment-mediated resistance to FLT3 inhibition in AML through HIF-1alpha-mediated down-regulation of CXCL12. Blood. 2011;**118**:4431-4439

[35] Parmar A, Marz S, Rushton S, Holzwarth C, Lind K, Kayser S, et al. Stromal niche cells protect early leukemic FLT3-ITD+ progenitor cells against first-generation FLT3 tyrosine kinase inhibitors. Cancer Research. 2011;**71**:4696-4706

[36] Yang X, Sexauer A, Levis M. Bone marrow stroma-mediated resistance to FLT3 inhibitors in FLT3-ITD AML is mediated by persistent activation of extracellular regulated kinase. British Journal of Haematology. 2014;**164**:61-72

[37] Nervi B, Ramirez P, Rettig MP, Uy GL, Holt MS, Ritchey JK, et al. Chemosensitization of acute myeloid leukemia (AML) following mobilization by the CXCR4 antagonist AMD3100. Blood. 2009;**113**:6206-6214

[38] Greenbaum A, Hsu YM, Day RB, Schuettpelz LG, Christopher MJ, Borgerding JN, et al. CXCL12 in early mesenchymal progenitors is required for haematopoietic stem-cell maintenance. Nature. 2013;**495**:227-230

[39] Mohle R, Bautz F, Rafii S, Moore MA, Brugger W, Kanz L. The chemokine receptor CXCR-4 is expressed on CD34+ hematopoietic progenitors and leukemic cells and mediates transendothelial migration induced by stromal cell-derived factor-1. Blood. 1998;**91**: 4523-4530

[40] Omatsu Y, Sugiyama T, Kohara H, Kondoh G, Fujii N, Kohno K, et al. The essential functions of adipo-osteogenic progenitors as the hematopoietic stem and progenitor cell niche. Immunity. 2010;**33**:387-399

[41] Sugiyama T, Kohara H, Noda M, Nagasawa T. Maintenance of the hematopoietic stem cell pool by CXCL12-CXCR4 chemokine signaling in bone marrow stromal cell niches. Immunity. 2006;**25**:977-988

[42] Voermans C, van Heese WP, de Jong I, Gerritsen WR, van Der Schoot CE: Migratory behavior of leukemic cells from acute myeloid leukemia patients. Leukemia. 2002;**16**: 650-657

[43] Tavor S, Petit I, Porozov S, Avigdor A, Dar AL-TL, Shemtov N, et al. CXCR4 regulates migration and development of human acute myelogenous leukemia stem cells in transplanted NOD/SCID mice. Cancer Research. 2004;**64**:2817-2824

[44] Spoo AC, Lubbert M, Wierd WG, Burger JA. CXCR4 is a prognostic marker in acute myelogenous leukemia. Blood. 2006, prepublished online August 3, 2006; DOI: 10.1182/blood-2006-05-024844

[45] Rombouts EJ, Pavic B, Lowenberg B, Ploemacher RE. Relation between CXCR-4 expression, Flt3 mutations, and unfavorable prognosis of adult acute myeloid leukemia. Blood. 2004;**104**:550-557

[46] Peled A, Petit I, Kollet O, Magid M, Ponomaryov T, Byk T, et al. Dependence of human stem cell engraftment and repopulation of NOD/SCID mice on CXCR4. Science. 1999;**283**: 845-848

[47] Busillo JM, Benovic JL. Regulation of CXCR4 signaling. Biochimica et Biophysica Acta-Biomembranes. 2007;**1768**:952-963

[48] Kim CH, Broxmeyer HE. Vitro behavior of hematopoietic progenitor cells under the influence of chemoattractants: Stromal cell-derived factor-1, steel factor, and the bone marrow environment. Blood. 1998;**91**:100-110

[49] Peled A, Tavor S. Role of CXCR4 in the pathogenesis of acute myeloid leukemia. Theranostics. 2013;**3**:34-39

[50] Fukuda S, Broxmeyer HE, Pelus LM. Flt3 ligand and the Flt3 receptor regulate hematopoietic cell migration by modulating the SDF-1alpha(CXCL12)/CXCR4 axis. Blood. 2005;**105**:3117-3126

[51] Mendez-Ferrer S, Lucas D, Battista M, Frenette PS. Haematopoietic stem cell release is regulated by circadian oscillations. Nature. 2008;**452**:442-4U4

[52] Lucas D, Battista M, Shi PA, Isola L, Frenette PS. Mobilized hematopoietic stem cell yield depends on species-specific circadian timing. Cell Stem Cell. 2008;**3**:364-366

[53] Ceradini DJ, Kulkarni AR, Callaghan MJ, Tepper OM, Bastidas N, Kleinman ME, et al. Progenitor cell trafficking is regulated by hypoxic gradients through HIF-1 induction of SDF-1. Nature Medicine. 2004;**10**:858-864

[54] Staller P, Sulitkova J, Lisztwan J, Moch H, Oakeley EJ, Krek W. Chemokine receptor CXCR4 downregulated by von Hippel-Lindau tumour suppressor pVHL. Nature. 2003;**425**:307-311

[55] Andreeff M, Zeng Z, Kelly MA, Wang R, McQueen T, Duvvuri S, et al. Mobilization and elimination of FLT3-ITD+ acute myelogenous leukemia (AML) stem/progenitor cells by Plerixafor/G-CSF/sorafenib: Results from a phase I trial in relapsed/refractory AML patients. Blood. 2012;**120**:42

[56] Kremer KN, Peterson KL, Schneider PA, Meng XW, Dai H, Hess AD, et al. CXCR4 chemokine receptor signaling induces apoptosis in acute myeloid leukemia cells via regulation of the Bcl-2 family members Bcl-XL, Noxa, and Bak. The Journal of Biological Chemistry. 2013;**288**:22899-22914

[57] Kremer KN, Dudakovic A, McGee-Lawrence ME, Philips RL, Hess AD, Smith BD, et al. Osteoblasts protect AML cells from SDF-1-induced apoptosis. Journal of Cellular Biochemistry. 2014;**115**:1128-1137

[58] Fukuda S, Pelus LM. Internal tandem duplication of Flt3 modulates chemotaxis and survival of hematopoietic cells by SDF1alpha but negatively regulates marrow homing in vivo. Experimental Hematology. 2006;**34**:1041-1051

[59] Jacobi A, Thieme S, Lehmann R, Ugarte F, Malech HL, Koch S, et al. Impact of CXCR4 inhibition on FLT3-ITD-positive human AML blasts. Experimental Hematology. 2010;**38**: 180-190

[60] Weisberg E, Liu Q, Nelson E, Kung AL, Christie AL, Bronson R, et al. Using combination therapy to override stromal-mediated chemoresistance in mutant FLT3-positive AML: Synergism between FLT3 inhibitors, dasatinib/multi-targeted inhibitors and JAK inhibitors. Leukemia. 2012;**26**:2233-2244

[61] Zeng Z, Samudio IJ, Munsell M, An J, Huang Z, Estey E, et al. Inhibition of CXCR4 with the novel RCP168 peptide overcomes stroma-mediated chemoresistance in chronic and acute leukemias. Molecular Cancer Therapeutics. 2006;**5**:3113-3121

[62] Fukuda S, Abe M, Onishi C, Taketani T, Purevsuren J, Yamaguchi S, et al. Survivin selectively modulates genes deregulated in human leukemia stem cells. Journal of Oncology. 2011;**2011**:946936

[63] Patel A, Pomicter AD, Eiring AM, Hein T, Heaton WL, O'Hare T, et al. Stroma-based activation of pSTAT3^{Y705} confers resistance to FLT3 inhibitors in FLT3 ITD-positive AML. Blood. 2016;**128**:34

[64] Eiring AM, Page BDG, Kraft IL, Mason CC, Vellore NA, Resetca D, et al. Combined STAT3 and BCR-ABL1 inhibition induces synthetic lethality in therapy-resistant chronic myeloid leukemia. Leukemia. 2017;**31**:1253-1254

[65] Aoki Y, Feldman GM, Tosato G. Inhibition of STAT3 signaling induces apoptosis and decreases survivin expression in primary effusion lymphoma. Blood. 2003;**101**:1535-1542

[66] Carter BZ, Milella M, Altieri DC, Andreeff M. Cytokine-regulated expression of survivin in myeloid leukemia. Blood. 2001;**97**:2784-2790

[67] Fukuda S, Pelus LM. Regulation of the inhibitor-of-apoptosis family member survivin in normal cord blood and bone marrow CD34(+) cells by hematopoietic growth factors: Implication of survivin expression in normal hematopoiesis. Blood. 2001;**98**: 2091-2100

[68] Bruner JK, Li L, Ma HS, Qin A, Levis MJ, Pratz KW, et al. Signaling adaptation to TKI treatment reactivates ERK signaling in FLT3/ITD leukemia. Blood. 2016;**128**:33

[69] Fukuda S, Mantel CR, Pelus LM. Survivin regulates hematopoietic progenitor cell proliferation through p21WAF1/Cip1-dependent and -independent pathways. Blood. 2004;**103**:120-127

[70] Mantel C, Luo Z, Canfield J, Braun S, Deng C, Broxmeyer HE. Involvement of p21cip-1 and p27kip-1 in the molecular mechanisms of steel factor-induced proliferative synergy in vitro and of p21cip-1 in the maintenance of stem/progenitor cells in vivo. Blood. 1996;**88**:3710-3719

[71] Mantel CR, Gelfano VM, Kim YJ, McDaniel A, Lee Y, Boswell HS, et al. P21waf-1-Chk1 pathway monitors G1 phase microtubule integrity and is crucial for restriction point transition. Cell Cycle. 2002;**1**:327-336

[72] Lee Y, Mantel C, Anzai N, Braun SE, Broxmeyer HE. Transcriptional and ERK1/2-dependent synergistic upregulation of p21(cip1/waf1) associated with steel factor synergy in MO7e. Biochemical and Biophysical Research Communications. 2001;**280**: 675-683

[73] Takahashi S, Harigae H, Kaku M, Sasaki T, Licht JD. Flt3 mutation activates p21WAF1/CIP1 gene expression through the action of STAT5. Biochemical and Biophysical Research Communications. 2004;**316**:85-92

[74] Cauchy P, James SR, Zacarias-Cabeza J, Ptasinska A, Imperato MR, Assi SA, et al. Chronic FLT3-ITD Signaling in acute myeloid leukemia is connected to a specific chromatin signature. Cell Reports. 2015;**12**:821-836

[75] Speck NA, Gilliland DG. Core-binding factors in haematopoiesis and leukaemia. Nature Reviews. Cancer. 2002;**2**:502-513

[76] Mangan JK, Speck NA. RUNX1 mutations in clonal myeloid disorders: From conventional cytogenetics to next generation sequencing, a story 40 years in the making. Critical Reviews in Oncogenesis. 2011;**16**:77-91

[77] Motoda L, Osato M, Yamashita N, Jacob B, Chen LQ, Yanagida M, et al. Runx1 protects hematopoietic stem/progenitor cells from oncogenic insult. Stem Cells. 2007;**25**:2976-2986

[78] Jacob B, Osato M, Yamashita N, Wang CQ, Taniuchi I, Littman DR, et al. Stem cell exhaustion due to Runx1 deficiency is prevented by Evi5 activation in leukemogenesis. Blood. 2010;**115**:1610-1620

[79] Nishimoto N, Arai S, Ichikawa M, Nakagawa M, Goyama S, Kumano K, et al. Loss of AML1/Runx1 accelerates the development of MLL-ENL leukemia through down-regulation of p19ARF. Blood. 2011;**118**:2541-2550

[80] Goyama S, Schibler J, Cunningham L, Zhang Y, Rao Y, Nishimoto N, et al. Transcription factor RUNX1 promotes survival of acute myeloid leukemia cells. The Journal of Clinical Investigation. 2013;**123**:3876-3888

[81] Wilkinson AC, Ballabio E, Geng H, North P, Tapia M, Kerry J, et al. RUNX1 is a key target in t(4;11) leukemias that contributes to gene activation through an AF4-MLL complex interaction. Cell Reports. 2013;**3**:116-127

[82] Behrens K, Triviai I, Schwieger M, Tekin N, Alawi M, Spohn M, et al. Runx1 downregulates stem cell and megakaryocytic transcription programs that support niche interactions. Blood. 2016;**127**:3369-3381

[83] Anguille S, Lion E, Willemen Y, Van Tendeloo VF, Berneman ZN, Smits EL. Interferon-alpha in acute myeloid leukemia: An old drug revisited. Leukemia. 2011;**25**:739-748

[84] Benjamin R, Khwaja A, Singh N, McIntosh J, Meager A, Wadhwa M, et al. Continuous delivery of human type I interferons (alpha/beta) has significant activity against acute myeloid leukemia cells in vitro and in a xenograft model. Blood. 2007;**109**:1244-1247

[85] Ersvaer E, Skavland J, Ulvestad E, Gjertsen BT, Bruserud O. Effects of interferon gamma on native human acute myelogenous leukaemia cells. Cancer Immunology, Immunotherapy. 2007**;56**:13-24

[86] Jiang LJ, Zhang NN, Ding F, Li XY, Chen L, Zhang HX, et al. RA-inducible gene-I induction augments STAT1 activation to inhibit leukemia cell proliferation. Proceedings of the National Academy of Sciences of the United States of America. 2011;**108**:1897-1902

[87] Cammarata G, Augugliaro L, Salemi D, Agueli C, La RM, Dagnino L, et al. Differential expression of specific microRNA and their targets in acute myeloid leukemia. American Journal of Hematology. 2010;**85**:331-339

[88] Garzon R, Volinia S, Liu CG, Fernandez-Cymering C, Palumbo T, Pichiorri F, et al. MicroRNA signatures associated with cytogenetics and prognosis in acute myeloid leukemia. Blood. 2008;**111**:3183-3189

[89] Garzon R, Garofalo M, Martelli MP, Briesewitz R, Wang L, Fernandez-Cymering C, et al. Distinctive microRNA signature of acute myeloid leukemia bearing cytoplasmic mutated nucleophosmin. Proceedings of the National Academy of Sciences of the United States of America. 2008;**105**:3945-3950

[90] Jongen-Lavrencic M, Sun SM, Dijkstra MK, Valk PJ, Lowenberg B. MicroRNA expression profiling in relation to the genetic heterogeneity of acute myeloid leukemia. Blood. 2008;**111**:5078-5085

[91] Marcucci G, Maharry KS, Metzeler KH, Volinia S, Wu YZ, Mrozek K, et al. Clinical role of microRNAs in cytogenetically normal acute myeloid leukemia: miR-155 upregulation independently identifies high-risk patients. Journal of Clinical Oncology. 2013;**31**:2086-2093

[92] Whitman SP, Maharry K, Radmacher MD, Becker H, Mrozek K, Margeson D, et al. FLT3 internal tandem duplication associates with adverse outcome and gene- and microRNA-expression signatures in patients 60 years of age or older with primary cytogenetically normal acute myeloid leukemia: A cancer and leukemia group B study. Blood. 2010;**116**:3622-3626

[93] Chi HT, Ly BT, HA V, Sato Y, Dung PC, Xinh PT. Synergistic effect of alltrans retinoic acid in combination with protein kinase C 412 in FMS-like tyrosine kinase 3-mutated acute myeloid leukemia cells. Molecular Medicine Reports. 2015;**11**:3969-3975

[94] Ma HS, Greenblatt SM, Shirley CM, Duffield AS, Bruner JK, Li L, et al. All-trans retinoic acid synergizes with FLT3 inhibition to eliminate FLT3/ITD+ leukemia stem cells in vitro and in vivo. Blood. 2016;**127**:2867-2878

[95] Scholl S, Muller R, Clement JH, Loncarevic IF, Bohmer FD, Hoffken K. ATRA can enhance apoptosis that is induced by Flt3 tyrosine kinase inhibition in Flt3-ITD positive cells. Leukemia Research. 2006;**30**:633-642

[96] Alonso S, Hernandez D, Chang YT, Gocke CB, McCray M, Varadhan R, et al. Hedgehog and retinoid signaling alters multiple myeloma microenvironment and generates bortezomib resistance. The Journal of Clinical Investigation. 2016;**126**:4460-4468

[97] Ghiaur G, Yegnasubramanian S, Perkins B, Gucwa JL, Gerber JM, Jones RJ. Regulation of human hematopoietic stem cell self-renewal by the microenvironment's control of retinoic acid signaling. Proceedings of the National Academy of Sciences of the United States of America. 2013;**110**:16121-16126

[98] Su M, Alonso S, Jones JW, Yu J, Kane MA, Jones RJ, et al. All-trans retinoic acid activity in acute myeloid Leukemia: Role of cytochrome P450 enzyme expression by the microenvironment. PLoS One. 2015;**10**:e0127790

[99] Peled A, Kollet O, Ponomaryov T, Petit I, Franitza S, Grabovsky V, et al. The chemokine SDF-1 activates the integrins LFA-1, VLA-4, and VLA-5 on immature human CD34(+) cells: Role in transendothelial/stromal migration and engraftment of NOD/SCID mice. Blood. 2000;**95**:3289-3296

[100] Katsumi A, Kiyoi H, Abe A, Tanizaki R, Iwasaki T, Kobayashi M, et al. FLT3/ITD regulates leukaemia cell adhesion through alpha4beta1 integrin and Pyk2 signalling. European Journal of Haematology. 2011;**86**:191-198

[101] Rashidi A, Uy GL. Targeting the microenvironment in acute myeloid leukemia. Current Hematologic Malignancy Reports. 2015;**10**:126-131

[102] Fogler WE, Flanner H, Wolfgang C, Smith JA, Thackray HM, Magnani J. Administration of the dual E-selectin/CXCR4 antagonist, GMI-1359, results in a unique profile of tumor mobilization from the bone marrow and facilitation of chemotherapy in a murine model of FLT3 ITD AML. Blood. 2016;**128**:2826

[103] Zhang W, Ly C, Zhang Q, Mu H, Battula VL, Patel N, et al. Dual E-selectin/CXCR4 antagonist GMI-1359 exerts efficient anti-leukemia effects in a FLT3 ITD mutated acute myeloid leukemia patient-derived xenograft murine model. Blood. 2016;**128**:3519

[104] Marlein C, Zaitseva L, Piddock RE, Robinson R, Edwards DR, Shafat MS, et al. Bone marrow mesenchymal stromal cells transfer their mitochondria to acute myeloid leukaemia blasts to support their proliferation and survival. Blood. 2016;**128**:772

[105] Moschoi R, Imbert V, Nebout M, Chiche J, Mary D, Prebet T, et al. Protective mitochondrial transfer from bone marrow stromal cells to acute myeloid leukemic cells during chemotherapy. Blood. 2016;**128**:253-264

[106] Onish C, Mori-Kimachi S, Hirade T, Abe M, Taketani T, Suzumiya J, et al. Internal tandem duplication mutations in FLT3 gene augment chemotaxis to Cxcl12 protein by blocking the down-regulation of the rho-associated kinase via the Cxcl12/Cxcr4 signaling axis. The Journal of Biological Chemistry. 2014;**289**:31053-31065

[107] Grundler R, Brault L, Gasser C, Bullock AN, Dechow T, Woetzel S, et al. Dissection of PIM serine/threonine kinases in FLT3-ITD-induced leukemogenesis reveals PIM1 as regulator of CXCL12-CXCR4-mediated homing and migration. The Journal of Experimental Medicine. 2009;**206**:1957-1970

[108] Kuo YY, Hou HA, Chen YK, Li LY, Chen PH, Tseng MH, et al. The N-terminal CEBPA mutant in acute myeloid leukemia impairs CXCR4 expression. Haematologica. 2014;**99**: 1799-1807

[109] Radomska HS, Alberich-Jorda M, Will B, Gonzalez D, Delwel R, Tenen DG. Targeting CDK1 promotes FLT3-activated acute myeloid leukemia differentiation through C/EBPalpha. The Journal of Clinical Investigation. 2012;**122**:2955-2966

[110] Kim KT, Baird K, Ahn JY, Meltzer P, Lilly M, Levis M, et al. Pim-1 is up-regulated by constitutively activated FLT3 and plays a role in FLT3-mediated cell survival. Blood. 2005;**105**:1759-1767

[111] Natarajan K, Xie Y, Burcu M, Linn DE, Qiu Y, Baer MR. Pim-1 kinase phosphorylates and stabilizes 130 kDa FLT3 and promotes aberrant STAT5 signaling in acute myeloid leukemia with FLT3 internal tandem duplication. PLoS One. 2013;**8**:e74653

[112] Bialopiotrowicz E, Gorniak P, Pula B, Noyszewska-Kania M, Makuch-Lasica H, Nowak G, et al. Microenvironment-induced expression of PIM kinases supports chronic lymphocytic leukemia cells survival and promotes CXCR4-mTOR pathway dependent migration. Blood. 2017;**128**:3239

[113] Hoggatt J, Kfoury Y, Scadden DT. Hematopoietic stem cell niche in health and disease. Annual Review of Pathology. 2016;**11**:555-581

[114] Walkley CR, Olsen GH, Dworkin S, Fabb SA, Swann J, McArthur GA, et al. A microenvironment-induced myeloproliferative syndrome caused by retinoic acid receptor gamma deficiency. Cell. 2007;**129**:1097-1110

[115] Walkley CR, Shea JM, Sims NA, Purton LE, Orkin SH. Rb regulates interactions between hematopoietic stem cells and their bone marrow microenvironment. Cell. 2007;**129**:1081-1095

[116] Raaijmakers MH, Mukherjee S, Guo S, Zhang S, Kobayashi T, Schoonmaker JA, et al. Bone progenitor dysfunction induces myelodysplasia and secondary leukaemia. Nature. 2010;**464**:852-857

[117] Gillette JM, Larochelle A, Dunbar CE, Lippincott-Schwartz J. Intercellular transfer to signalling endosomes regulates an ex vivo bone marrow niche. Nature Cell Biology. 2009;**11**:303-311

[118] Jung Y, Wang J, Havens A, Sun Y, Wang J, Jin T, et al. Cell-to-cell contact is critical for the survival of hematopoietic progenitor cells on osteoblasts. Cytokine. 2005;**32**:155-162

[119] Zhou BO, Ding L, Morrison SJ. Hematopoietic stem and progenitor cells regulate the regeneration of their niche by secreting Angiopoietin-1. eLife. 2015;**4**:e05521

[120] Zhang B, Ho YW, Huang Q, Maeda T, Lin A, Lee SU, et al. Altered microenvironmental regulation of leukemic and normal stem cells in chronic myelogenous leukemia. Cancer Cell. 2012;**21**:577-592

[121] Schepers K, Pietras EM, Reynaud D, Flach J, Binnewies M, Garg T, et al. Myeloproliferative neoplasia remodels the endosteal bone marrow niche into a self-reinforcing leukemic niche. Cell Stem Cell. 2013;**13**:285-299

[122] Mead AJ, Neo WH, Barkas N, Matsuoka S, Giustacchini A, Facchini R, et al. Niche-mediated depletion of the normal hematopoietic stem cell reservoir by Flt3-ITD-induced myeloproliferation. The Journal of Experimental Medicine. 2017;**214**:2005-2021

Chapter 9

Role of Genetic Analysis in New Treatments of Acute Myeloid Leukemia

Mehrdad Payandeh, Masoud Sadeghi,
Edris Sadeghi and Mehrnoush Aeinfar

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.76050

Abstract

Genetics has an important role in the risk stratification and management of the patients with acute myeloid leukemia (AML). Molecular testing can't take the place of cytogenetic testing results, but has complementary role to help refine prognosis of the disease, especially within specific AML subgroups. Molecular genetic analysis of CEBPA, NPM1, and FLT3 is already the standard of care in AML patients, and mutations in several additional genes are assuming increasing importance. The French-American-British (FAB) classification and the World Health Organization (WHO) classification are the most classifications for AML. The aim of this chapter is a review on the role of genetic analysis in new treatments of AML.

Keywords: acute myeloid leukemia, genetics, mutation, classification, treatment

1. Introduction

Acute myeloid leukemia is the most common acute leukemia in adults and highly heterogeneous disease, characterized by gene mutations, chromosome abnormalities, changes in expression of multiple genes, and microRNAs [1]. In 2008, World Health Organization (WHO) categorized acute myeloid leukemia into four groups; AML with genetic abnormalities, AML with myelodysplasia, treatment-dependent AML, and AML not categorized. In the first group, prognosis depends on cytogenetic findings, so patients with acute myeloid leukemia were divided into different prognostic groups. For example, the translocations t(8;21), t (15;17), and inv(16) indicate a favorable prognosis, whereas 11q23 abnormalities indicate intermediate or worse prognosis. In the last group, 40–50% of AML cases were included, and

Type	Name	Cytogenetics	Percentage of adults with AML (%)
M0	Acute myeloblastic leukemia, minimally differentiated		5 [3]
M1	Acute myeloblastic leukemia, without maturation		15 [3]
M2	Acute myeloblastic leukemia, with granulocytic maturation	t(8;21)(q22;q22), t(6;9)	25 [3]
M3	Promyelocytic, or acute promyelocytic leukemia (APL)	t(15;17)	10 [3]
M4	Acute myelomonocytic leukemia	inv (16)(p13q22), del(16q)	20 [3]
M4eo	Myelomonocytic together with bone marrow eosinophilia	inv (16), t(16;16)	5 [3]
M5	Acute monoblastic leukemia (M5a) or acute monocytic leukemia (M5b)	del (11q), t(9;11), t(11;19)	10 [3]
M6	Acute erythroid leukemia, including erythroleukemia (M6a) and very rare pure erythroid leukemia (M6b)		5 [3]
M7	Acute megakaryoblastic leukemia	t(1;22)	5 [3]

Table 1. French-American-British (FAB) classification of acute myeloid leukemia (AML).

no chromosomal abnormalities were detected by conventional karyotyping. Almost these patients located into the intermediate prognostic group [2]. The French-American-British (FAB) classification system divides AML into eight subtypes, M0 through to M7, based on the morphological and cysto-chemical characteristics of the leukemic cells as shown in **Table 1**. This is done by examining the appearance of the malignant cells with light microscopy and/or by using cytogenetics to characterize any underlying chromosomal abnormalities [4]. In AML, somatic genetic changes are often thought to contribute to leukemogenesis through a "two-hit" process: (1) mutations that activate signal transduction pathways and thereby increase the proliferation or survival, or both, of hematopoietic progenitor cells (class I; Mutations in KIT, FLT3, and N-RAS) and (2) mutations that affect transcription factors or components of the cell-cycle machinery and cause impaired differentiation (class II; RUNX1, CBFβ, CEBPA, NPM1, PU1, MLL, and RARA) [5]. Genes that were significantly mutated in AML were organized into several functional categories (**Figure 1**) [6]. The purpose of AML genetic testing and classification at diagnosis is mainly to risk-stratify patients with AML, and thus determine proper treatment modalities.

2. Class I mutations

2.1. KIT mutations

KIT is a member of the type III RTK family, encodes a receptor tyrosine kinase and ligand-independent activation of KIT. KIT mutations are detected mostly in association with core binding factor (CBF) AML, and correlated with t(8;21), inv (16), and t(16;16) [7, 8].

http://dx.doi.org/10.5772/intechopen.76050

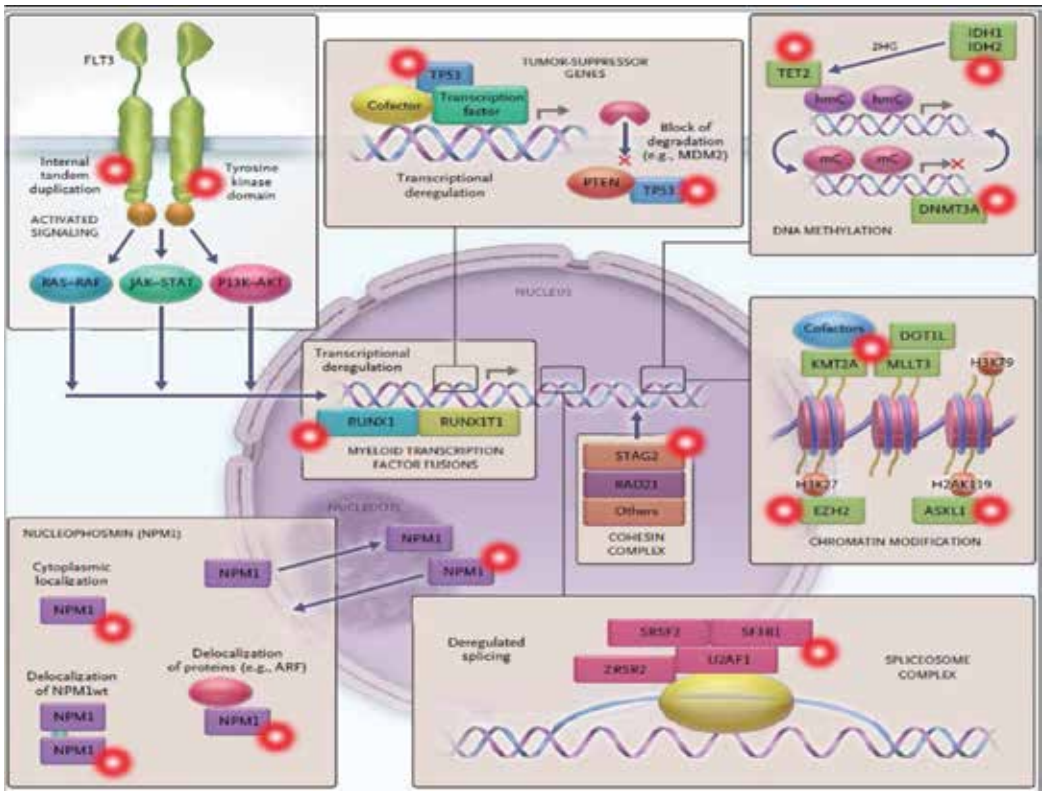

Figure 1. Acute myeloid leukemia and commonly mutated genes. Upper-left box shows mutations in *FLT3* signaling that confer a proliferative through the RAS–RAF, PI3K–AKT, and JAK–STAT signaling pathways. Upper-middle box shows *TP53* mutations, can lead to deregulation of transcription and impaired degradation through the phosphatase and tensin homolog (PTEN) and the mouse double minute 2 (MDM2) homolog. Upper-right box shows *DNMT3A* and *TET2* mutations, also *IDH1* and *IDH2* mutations, can lead to the deregulation of DNA methylation. Center-right box shows mutations of *ASXL1* and *EZH2* genes, cause deregulation of chromatin modification, also *KMT2A-MLLT3* gene fusion, can impair other methyltransferases such as DOT1L. In center-right box shows *RUNX1* and *RUNX1-RUNX1T1* mutations, result in reduced hematopoietic differentiation and transcriptional deregulation. Center-middle box shows mutations in the *RAD21* and *STAG2*, might impair accurate chromosome segregation and transcriptional regulation. Lower-right box shows mutations of *U2AF1*, *ZRSR2*, *SF3B1*, *SRSF2* are involved in deregulated RNA processing. Lower-left box shows *NPM1* gene mutations, resulting in the aberrant cytoplasmic localization of NPM1 and NPM1-interacting proteins.

2.2. FLT3 mutations

Fms-like tyrosine kinase 3 (FLT3) is a receptor tyrosine kinase involved in proliferation, cell survival, and differentiation of hematopoietic progenitor stem cells. FLT3 mutations are one of the most common genetic changes in AML that occur in about 30% of these cases and confer a poor prognosis [9, 10]. Internal tandem duplications (ITD) mutations in the juxtamembrane (JM) domain and missense point mutations in the second tyrosine kinase domain (TKD) of the FLT3 gene are the molecular abnormalities that have been found in 20% of all AML patients [11].

2.3. RAS mutations

Mutations in N-RAS and K-RAS occur in AML as well as solid Tumors in about 10 and 5% of AML patients, respectively [12].

3. Class II mutations

In addition to class I mutations, in AML patients, mutations in brain and acute leukemia gene (BAAL), MLL-MLLT3 gene fusion created by the t(9;11)(p22;q23) translocation is associated with intermediate prognosis in AML, nucleoplasmin 1(NPM1), CCAAT/enhancer-binding protein α (CEBPα), and Wilms tumor gene (WT-1) have also been detected. Lately, mutations in DNMT3A, encode DNA methyltransferases that catalyze the addition of a methyl group to the cytosine residue of CpG dinucleotides, have been recognized in one-third of de novo AML patients with intermediate-risk cytogenetics. Genomes with DNMT3A mutations commonly harbored extra mutations in NPM1, IDH1, and FLT3. The incidence of any DNMT3A mutation, alone or in combination with FLT3 ITD mutation, is related with lower overall survival (OS) [12, 13].

4. Molecule analysis of mutations in AML

The single most important prognostic factor in AML is cytogenetic testing of bone marrow samples. Results are highly predictive of response to induction chemotherapy, relapse risk, and overall survival (OS). Approximately 50–60% of newly diagnosed AML patients can be detected by cytogenetic abnormalities [14]. To recognize cytogenetic abnormalities,

Risk	Cytogenetics	Molecular
Favorable	inv (16) or t(16;16) t(8;21) t(15;17)	Normal cytogenetics with: Isolated biallelic *CEBPA* mutation *NPM1* mutation without *FLT3* ITD
Intermediate	Normal cytogenetics Isolated +8 t(9;11) Other non-good and non-poor changes	*KIT* mutation in core binding factor leukemia: inv (16) or t(16;16) t(8;21)
Poor	Complex (≥3 clonal abnormalities) Monosomal karyotype −5/−5q or − 7/−7q 11q23 rearrangements other than t(9;11) inv (3) or t(3;3) t(6;9) t(9;22)	Normal cytogenetics with: *FLT3* ITD

Table 2. Genetic risk classification of acute myeloid leukemia.

fluorescence in-situ hybridization technique (FISH) and DNA analysis should be done as well routine cytogenetics. Results of these tests are used for patient-risk stratification and to guide patient management. In **Table 2**, most of the prognostic cytogenetic abnormalities are listed, grouped by risk category [15]. AML Patients with the t(8;21)(q22;q22), t(15;17)(q22;q12), and inv (16)(p13.1;q22) are associated with longer survival and remission, whereas alterations of chromosomes 7, 5, complex karyotype, and 11q23 are associated with poor response to therapy and shorter overall survival [16]. The vital chromosomal abnormalities in AML include deletions in chromosomes 5 or 7 or monosomies and trisomy 8 [17]. Furthermore, other abnormalities consist of the balanced translocations between chromosomes 15 and 17 (t (15;17)); long arm of chromosome 11 (11q); chromosomes 8 and 21 (t(8;21)); (q22;q22), (q31; q22), and t(9;11); and inversion for instance inv (16) [18].

5. Oncofusion proteins related with AML

In total, 749 chromosomal aberrations have been recorded in AML [19]. **Table 3** shows the common chromosomal aberrations and their corresponding Oncofusion Proteins in AML [12].

In conclusion, AML is a highly aggressive heterogeneous malignant disease, classified by genetic abnormalities that define subgroups of distinct biological and clinical features. Despite best efforts at targeted therapy, therapeutic approaches have stuck to "one-size fits all" conventional chemotherapy because of lack of targeted therapeutic options.

Translocations	Oncofusion protein	Frequency of occurrence (% of AML)
t(8;21)	AML1-ETO	10
t(15;17)	PML-RARα	10
inv(16)	CBFβ-MYH11	5
der(11q23)	MLL-fusions	4
t(9;22)	BCR-ABL1	2
t(6;9)	DEK-CAN	1
t(1;22)	OTT-MAL	<1
t(8;16)	MOZ-CBP	<1
t(7;11)	NUP98-HOXA9	<1
t(12;22)	MN1-TEL	<1
inv(3)	RPN1-EVI1	<1
t(16;21)	FUS-ERG	<1

Table 3. Acute myeloid leukemia (AML)-associated Oncofusion proteins.

Author details

Mehrdad Payandeh*, Masoud Sadeghi, Edris Sadeghi and Mehrnoush Aeinfar

*Address all correspondence to: md.payandeh@yahoo.com

Medical Biology Research Center, Kermanshah University of Medical Sciences, Kermanshah, Iran

References

[1] Döhner H, Gaidzik VI. Impact of genetic features on treatment decisions in AML. ASH Education Program Book. 2011;**1**:36-42

[2] Chen W, Rassidakis GZ, Medeiros LJ. Nucleophosmin gene mutations in acute myeloid leukemia. Archives of Pathology & Laboratory Medicine. 2006;**130**:1687-1692

[3] Seiter K, Jules EH. Acute myeloid leukemia staging; 20 May 2011. Retrieved August 26, 2011

[4] Bennett JM, Catovsky D, Daniel MT, et al. Proposed revised criteria for the classification of acute myeloid leukemia: A report of the French-American-British cooperative group. Annals of Internal Medicine. 1985;**103**:620-625

[5] Dash A, Gilliland DG. Molecular genetics of acute myeloid leukaemia. Best Practice & Research Clinical Haematology. 2001;**14**:49-64

[6] Döhner H, Weisdorf DJ, Bloomfield CD. Acute myeloid leukemia. New England Journal of Medicine. 2015;**373**:1136-1152

[7] Paschka P et al. Adverse prognostic significance of KIT mutations in adult acute myeloid leukemia with inv (16) and t (8; 21): A cancer and leukemia group B study. Journal of Clinical Oncology. 2006;**24**:3904-3911

[8] Lennartsson J, Jelacic T, Linnekin D, Shivakrupa R. Normal and oncogenic forms of the receptor tyrosine kinase kit. Stem Cells. 2005;**23**:16-43

[9] Small D. Targeting FLT3 for the treatment of leukemia. Seminars in Hematology. 2008;**45**: 17-21

[10] Stirewalt DL, Radich JP. The role of FLT3 in haematopoietic malignancies. Nature Reviews Cancer. 2003;**3**:650-665

[11] Saultz JN, Garzon R. Acute myeloid leukemia: A concise review. Journal of Clinical Medicine. 2015;**5**:33

[12] Kumar C. Genetic abnormalities and challenges in the treatment of acute myeloid leukemia. Genes & Cancer. 2011;**2**:95-107

[13] Ley TJ, Ding L, Walter MJ, McLellan MD, Lamprecht T, Larson DE, Kandoth C, Payton JE, Baty J, Welch J, Harris CC. DNMT3A mutations in acute myeloid leukemia. New England Journal of Medicine. 2010;**363**:2424-2433

[14] Martens JHA, Stunnenberg HG. The molecular signature of oncofusionproteins in acute myeloid leukemia. FEBS Letters. 2010;**584**:2662-2669

[15] Yohe S. Molecular genetic markers in acute myeloid leukemia. Journal of Clinical Medicine. 2015;**4**:460-478

[16] Döhner H, Weisdorf DJ, Bloomfield CD. Acute myeloid leukemia. The New England Journal of Medicine. 2015;**373**:1136-1152

[17] Byrd JC, Mrozek K, Dodge RK, et al. Pretreatment cytogenetic abnormalities are predictive of induction success, cumulative incidence of relapse, and overall survival in adult patients with de novo acute myeloid leukemia: Results from cancer and leukemia group B (CALGB 8461). Blood. 2002;**100**:4325-4336

[18] Mrozek K, Radmacher MD, Bloomfield CD, Marcucci G. Molecular signatures in acute myeloid leukemia. Current Opinion in Hematology. 2009;**16**:64-69

[19] Mitelman F, Johansson B, Mertens F, editors. Mitelman Database of Chromosome Aberrations and Gene Fusions in Cancer 2010. Available from: http://cgap.nci.nih.gov/Chromosomes/Mitelman

Chapter 10

High Doses of Vitamin C and Leukemia: In Vitro Update

Domenico Mastrangelo, Lauretta Massai, Giuseppe Fioritoni, Francesco Lo Coco, Nèlida Noguera and Ugo Testa

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.71484

Abstract

Vitamin C (ascorbic acid) is an essential nutrient with a number of beneficial effects on the human body. Although the majority of mammals can synthesize their own Vitamin C, humans and a few other species, do not produce it and depend on dietary sources for their Vitamin C supply. Among its many effects on cell function and metabolism, Vitamin C has shown, in vitro, a powerful anticancer effect against a number of human tumor cell lines, including myeloid leukemia. There are many different mechanistic explanations for the anticancer/anti-leukemic effects of Vitamin C and the aim of the present review is to illustrate these mechanisms, showing the results of some preliminary in vitro investigations, and outlining their potential clinical relevance.

Keywords: Vitamin C, ascorbate, sodium ascorbate, high doses of ascorbate, intravenous ascorbate, cancer, leukemia, antioxidants, pro-oxidants, free radicals, oxidative stress, redox balance

1. Introduction

Vitamin C is an essential nutrient with a number of beneficial functions, for the organism [1], such as

1. helping the metabolism of tyrosine, folic acid, and tryptophan;
2. increasing the elimination of cholesterol;
3. contributing to the synthesis of catecholamines;
4. helping the body to absorb and breakdown histamine;

5. enhancing the absorption of non-heme iron;
6. promoting the synthesis of collagen (its most widely known physiological function);
7. neutralizing free radicals (it is a reducing agent, "scavenger" of free radicals, and a founder among the natural antioxidants);
8. protecting DNA from damage due to free radicals and mutagens;
9. reducing the risk of premature death;
10. fighting off widespread environmental pollutants; and
11. preventing the development of nitrosamines.

Though ubiquitous, ascorbate is not produced by humans, guinea pigs, some primates, a particular type of fruit eating bat, the majority of fishes and birds [2], who depend on diet for the assumption and use of this fundamental nutrient.

2. Vitamin C and leukemia: historical background

The first mention of the therapeutic potentialities of Vitamin C in leukemia, can be found in the book "*The healing Factor: Vitamin C against disease*," written by the biochemist Irwin Stone, in 1974 [3]. In his book, Stone refers to a study, performed in 1936 by Stephen and Hawley [4], demonstrating, for the first time, that when the blood is separated into plasma, red blood cells, and white blood cells, there is a 20- to 30-fold concentration of Vitamin C in the white blood cells, as compared to plasma.

Following this report, Barkhan and Howard, by studying a few cases of chronic myelogenous and lymphatic leukemia, added the evidence that leukemic patients have substantially lower than normal plasma levels of Vitamin C [5]. As noted by Stone, although this knowledge could suggest the use of Vitamin C as a therapeutic agent, in leukemia, the first clinical trials showed contrasting results, due to the inappropriately *low doses* administered.

Later on, Vogt, in a literature review [6], confirmed that there are high deficits of Vitamin C in leukemic patients, as also confirmed by the reports of Kyhos and Coll. [7] in 1945, and Waldo and Zipf, in 1955 [8].

According to Stone [3], leukemia reduces the body stores of Vitamin C to very low levels, and any residual Vitamin C circulating in the blood is scavenged and locked in the excessive numbers of leukocytes characterizing this disorder. As a direct consequence, the plasma levels of Vitamin C are reduced to zero or close thereto, and tissues are no longer being supplied with this most important metabolite, since it is accumulated in leukocytes.

Stone [3] defined "*biochemical scurvy*" as the condition of insufficient Vitamin C supply to body tissues, and proposed that its correction required the administration of Vitamin C at a rate of 25 g or more per day.

In 2012, 76 years after the first observations on the "concentration" of Vitamin C in leukocytes, an investigation on 131 patients affected by different types of leukemia, definitively confirmed that leukemic patients have significant lower plasma Vitamin C than normal controls. The reduction of plasma Vitamin C levels in leukemia, as predicted by Stone, is due to an increased uptake and utilization by the actively proliferating leukocytes, leading to tissue biochemical scurvy and consequent increased tendency to bleeding and infections, which are the hallmark of this pathological condition [9, 10]. Interestingly, low plasma levels of Vitamin C, have been, very recently, found in around 30% of cases of Non-Hodgkin Lymphoma (NHL), particularly in patients with high bulk disease [11]. With the above data at hand, it is clear that leukemia can be viewed as a condition of functional Vitamin C deficiency, associated with biochemical scurvy, and therefore, all leukemic patients are suitable candidates for the treatment with this nutrient.

3. How much Vitamin C to treat leukemia? The concept of "mega-doses"

In 1949, Frederik Klenner first reported the successful treatment of bulbar poliomyelitis, with high doses of Vitamin C administered by intramuscular, intravenous, and oral route [12]. Klenner had also established clinical protocols using massive doses of Vitamin C to treat a number of different viral conditions, but only more than two decades later, Stone formally defined the concept and rationale for the use of "*mega-doses*" of Vitamin C. In particular, Stone observed that man and only a few other species do not produce their own Vitamin C, while the great majority of mammals do, according to their physiologic requirements [3]. This observation led the author to hypothesize that due to either insufficient intake or increased consumption of the nutrient, or both, man could easily undergo a condition that he defined "*hypoascorbemia*." Hypoascorbemia is a reduced amount of circulating Vitamin C (also called "ascorbic acid"), due to the lack of the enzyme L-gulonolactone oxidase (GLO), as a consequence of an "inborn error of carbohydrate metabolism" [13–15]. This defect, now very well acknowledged and characterized [16], led Stone to hypothesize that to be in good health, man needs mega-doses of Vitamin C (several grams a day) [17, 18], rather than doses in the order of milligrams, as stated by the Recommended Daily Allowances (RDAs) [19].

The rationale behind the use of mega-doses of Vitamin C was further refined by the chemist and twofold Nobel Prize, Linus Pauling. Pauling soon became an enthusiastic supporter of the use of this nutrient, in high doses, not only to prevent disease [20–23], but also to treat a number of pathologic conditions, ranging from common cold [24, 25] to cancer [26] and AIDS [27].

4. Intravenous Vitamin C and cancer

Studies on dose-concentration relationship in humans, performed by Levine and co-workers [28], revealed that at oral doses exceeding 250 mg/day, the plasma levels of Vitamin C reach a

plateau, and any further increase in the amount administered by mouth, does not determine significant increase in plasma concentration. This is due to multiple "control" mechanisms, including, among others, intestinal absorption, tissue accumulation, renal reabsorption and excretion, and utilization. On the contrary, the intravenous administration of high doses of Vitamin C, bypassing the above control mechanisms, allows plasma concentrations that are 100-fold or higher than maximally tolerated oral doses, and the peak could last for hours within the millimolar (mM) range [29].

More importantly, at plasma concentrations easily achievable by intravenous administration (5–10 mM for 1–2 h), Vitamin C induced death in 75% of 48 cancer cell lines tested in vitro [30], but had no toxic effect on human peripheral white blood cells, fibroblasts, or epithelial cells. This selective cytotoxic effect would be achieved since at high doses, parenteral ascorbate is a *peroxide delivery system* for the generation of sustainable ascorbate radical and H_2O_2. H_2O_2 would be produced in the extracellular space, with consequent oxidative damage to cancer cells [31, 32]. Therefore, Vitamin C in high doses would be cytotoxic to cancer cells because of its *pro-oxidant*, rather than anti-oxidant effect, even though some authors remark that the pro-oxidant activity of Vitamin C, may not be relevant, in vivo [33–35].

More recently, Yun and co-workers [36], by investigating the effects of high doses of Vitamin C on KRAS and BRAF mutants cells derived from colorectal cancer (CRC), have further refined the mechanistic explanation of the anticancer properties of Vitamin C. In particular, according to the authors, the death of KRAS and BRAF cell mutants of CRC is not caused by the Vitamin C itself, but rather, by its oxidized form, dehydroascorbic acid (DHAA). While Vitamin C enter cells though specific receptors, called sodium-Vitamin C co-transporters (SVCTs) [37], DHAA competes with glucose, for intracellular uptake by glucose transporters (GLUT), mainly 1 and 4 subtype receptors [38, 39].

Interestingly, both KRAS and BRAF activating mutations are responsible for the upregulation of GLUT1 expression in different types of cancer, including CRC [40, 41].

However, as reported by Yun and Coll. [36], the upregulation of GLUT-1 expression is not always associated with increased sensitivity of tumor cell lines to the cytotoxic effects of DHAA.

Further investigation into the metabolic makeup of KRAS and BRAF mutations CRC-derived cell lines, showed an accumulation of glycolytic intermediates upstream glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) and a contemporary depletion of the metabolites downstream GAPDH. This finding indicates an inhibition or severe reduction of GAPDH activity, which appears to be the key of the cytotoxic effect of DHA.

In summary, the data reported by Yun and Coll. on the effect of DHAA on CRC cell lines, indicate that in glycolysis-addicted KRAS and BRAF mutated cell lines, high amounts of DHAA are transported into the cancer cells, through the overexpressed GLUT-1 receptors. The exceeding amounts of intracellular DHAA are then reduced again to Vitamin C with consequent consumption of glutathione (GSH), redox imbalance, and oxidative stress. Oxidative stress, in turn, causes GAPDH inactivation, with inhibition of glycolysis, and energetic crisis, ultimately leading to cell death [42].

More precisely, beyond being inactivated directly by ROS (including H_2O_2), GAPDH function is also hindered by the depletion of nicotinamide adenine dinucleotide (NAD+), caused by the activation of the DNA repairing enzyme, poly(ADP-ribose) polymerase (PARP), induced by damaged DNA. In fact, the increased production of ROS, in cancer cells, due to the high doses of Vitamin C, produces increased DNA damage and consequent activation of PARP. PARP, in turn, consumes NAD+ with consequent NAD+ depletion, ATP depletion, and cancer cell death [43].

5. High doses of Vitamin C and H_2O_2

The view that Vitamin C in high concentrations, administered by intravenous infusion, acts as a pro-oxidant, leading to the formation of H_2O_2, thus inducing oxidative damage to cancer cells, is not new. In 1969, Benade and co-workers had already demonstrated that Vitamin C could selectively kill cancer cells, without harming normal cells. The authors suggested that the cytotoxic effect of ascorbate could be due ("*in major part*") to the intracellular generation of toxic hydrogen peroxide produced upon oxidation of Vitamin C, by the cells. This view was corroborated by the fact that the toxicity of Vitamin C was greatly enhanced by the concomitant administration of 3-amino-1, 2, 4-triazole (ATA) that inhibits the enzyme catalase, "*thus decreasing or destroying the ability of the cancer cells to detoxify H_2O_2 effectively*" [44]. Further scientific reports confirmed that human cancer cells have low levels of antioxidant enzymes (including, among others, catalase and glutathione peroxidase), and therefore cannot detoxify hydrogen peroxide [45, 46].

According to the pro-oxidant theory, Vitamin C in high concentrations induces the production of H_2O_2 through a Fenton-like reaction. This reaction is the oxidation of organic substrates by iron and hydrogen peroxide, in which trivalent iron (Fe^{3+}) plays a fundamental role. However, since Fenton-like reactions are usually controlled, in vivo, because of iron sequestration by metal binding proteins, the pro-oxidant effect of Vitamin C, in vivo, may be scarcely significant [33–35], and other mechanisms should be hypothesized.

Other authors, using two prostate cancer cell lines (LNCaP and PC-3) have shown that iron at physiological concentrations in cell culture medium and human plasma abrogates the anticancer/cytotoxic effects of Vitamin C. In particular, at physiological concentrations, iron promotes both production and decomposition of H_2O_2, the latter being mediated by a Fenton reaction, which prevents the accumulation of H_2O_2, thus abolishing the cytotoxic effect of Vitamin C. Therefore, for an optimal anticancer effect, Vitamin C should be administered with chelating agents, which remove iron from the medium [47].

On the other hand, Vitamin C readily undergoes pH-dependent autoxidation producing hydrogen peroxide, and catalytic metals only accelerate the oxidation process. Therefore, catalytic iron may not be strictly necessary for the production of H_2O_2. This auto oxidation process (oxidation in the absence of catalytic metals) occurs via the ascorbate di-anion (Asc2−). In particular, at pH 7.0, 99.9% of ascorbate (Vitamin C) is in the form of mono-anion (AscH−). Asc2− increases by a factor ten, with one unit increase in the pH. Therefore, while the production of

H_2O_2 may be scarcely relevant in the absence of catalytic iron (as in the "Fenton chemistry"), it may become considerable when the concentration of ascorbate is in the order of the millimoles, as in the case of the use of Vitamin C as an anticancer compound [48].

Finally, accumulating evidence suggests that cancer cells produce high amounts of hydrogen peroxide [49], and hydrogen peroxide itself is a powerful carcinogen, associated with mutagenic potential [50]. Therefore, the role of Vitamin C as a pro-drug of hydrogen peroxide, to kill cancer cells, is still far from being fully elucidated.

6. Oral vs. intravenous Vitamin C

The pharmacokinetic studies of Levine and Padayatty [28, 29], on Vitamin C, indicate that after oral administration of 200 mg of the nutrient, the maximum plasma concentrations obtained, are not superior to 70–80 μM. This is due to a "tight control," operated by several different mechanisms, including, among others: bioavailability, intestinal absorption, tissue accumulation, renal reabsorption and excretion, and utilization rate as a function of homeostasis. On the contrary, when Vitamin C is administered intravenously, "tight control" is bypassed, until renal excretion restores equilibrium, depending on the dose administered [51].

Therefore, according to these data, the intravenous administration of Vitamin C is the only way to achieve plasma concentrations in the order of millimoles, necessary to kill cancer cells.

However, this view is in disagreement with the following evidences:

a. The results obtained by intravenous administration of Vitamin C, do not show the same large effects reported by Robinson, feeding squamous cell carcinoma implanted mice, with large doses of the nutrient [52];

b. Abram Hoffer [53] used oral high doses of Vitamin C in cancer patients and obtained essentially the same significant results as Cameron and Pauling, Cameron and Campbell [54–58], and Murata [59];

c. Although it is presently believed that only injected Vitamin C delivers the concentrations needed to produce an anti-tumor effect, neither the legendary scientist Linus Pauling nor the consultant surgeon, Ewan Cameron, seemed to know the difference between oral and intravenous administration. In fact, in their clinical trial, the protocol started with a few days of 10 grams of intravenous Vitamin C, followed by 10 grams of oral Vitamin C for the whole life. Interestingly, Cameron and Campbell, who had already reported on the successful treatment of cancer with *oral* Vitamin C, had already observed that "*… with increasing experience, we tend now to believe that the intravenous regime is probably unnecessary as a routine measure, and need only to be employed in situations where vomiting, anorexia, or other complications of malignancy, preclude oral administration*" [58];

d. Plasma concentrations above the 400 μM have been reported, after the administration of a single dose of oral liposomal Vitamin C [60];

e. At times of stress or illnesses (including cancer), the body may absorb extra Vitamin C, as demonstrated by the principle of "bowel tolerance" to the nutrient administered by mouth. According to this principle, when the body is saturated with Vitamin C, slight diarrhea may appear, due to intestinal elimination of the nutrient. However, during stress or disease, the amount of oral Vitamin C a patient can tolerate, before the appearance of diarrhea, increases in proportion with the severity of the condition [61];

f. This means that the "tight control" hypothesized by Levine and Padayatty, over the plasma concentration of Vitamin C, is either inexistent or relative to disease conditions or stress. To achieve the maximum plasma levels, a typical person may need 20 g of oral Vitamin C spread throughout the day (3–4 g every 4 h); but cancer patients may require far more [62]. Such massive intake may result in plasma concentrations that the tumor may absorb, generating hydrogen peroxide that kills cancer cells;

g. More recently, the paradigm according to which antioxidants inhibit tumorigenesis predominantly by decreasing ROS-mediated DNA damage and mutations [63, 64] has been challenged by experimental data. Antioxidants such as N-acetylcysteine (NAC) and Vitamin C exerts their anti-tumorigenic activity by downregulating HIF-1α [65]. Interestingly, these data were obtained not by injecting, but by simply feeding mice with large amounts of NAC or Vitamin C. These findings validate the role of oral administration of Vitamin C (and other antioxidants) in fighting cancer.

7. Vitamin C and leukemia: an in vitro update

As we have previously demonstrated, high ("pharmacologic") concentrations of Vitamin C (in the form of the sodium salt of ascorbic acid) are capable of eliciting a clear-cut pro-apoptotic/cytotoxic effect on human promyelocytic leukemia-derived cell lines (HL60), in vitro [66] (**Figures 1** and **2**). This effect is already evident at concentrations of Vitamin C of 1 mM in the culture medium, and it is proportional to the amount of Vitamin C.

Since clinical investigations using high doses of Vitamin C to treat cancer, have reported plasma levels of more than 30 [67], and up to 49 mM [68], it seems reasonable to conclude that using high amounts of Vitamin C, administered by intravenous injection, is not strictly necessary to kill cancer cells in APL.

Further investigations in leukemia, performed by our research group, have shown that a plasma concentration of 3 mM of Vitamin C in the culture medium, is sufficient to kill more than a half of the cells in culture (LC_{50}) in a number of different human myeloid leukemia cell lines [69] (**Figures 3** and **4**) (**Table 1**). It is of interest to consider that according to our protocol, the leukemic cells are exposed to Vitamin C for no more than 2 h, then accurately "washed," re-suspended in fresh culture medium, without Vitamin C, and further incubated for additional 18–24 h, before the evaluation of cell survival and apoptosis. Given the results obtained, it is reasonable to conclude that the Vitamin C added to the culture medium (in the form of sodium ascorbate) is rapidly internalized by the leukemic cells, and

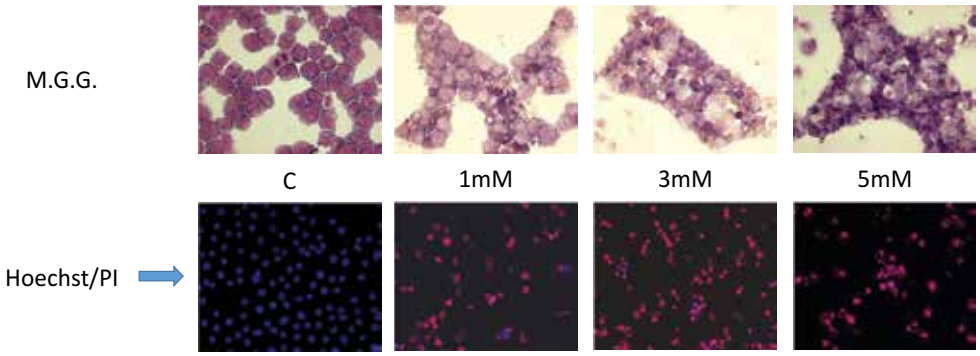

Figure 1. The microphotographs refer to the cytomorphologic modifications of HL60 cell lines (human acute promyelocytic leukemia—APL) exposed for 2 h to increasing concentrations of Vitamin C. It is evident that by increasing the concentration of Vitamin C in the medium (from 1 to 5 mM), APL cells show an increasing degree of morphologic alterations indicating progressive cell death (apoptosis, autophagy, autoschizis). With the Hoechst/PI fluorescent staining, vital cells are colored in blue, while dead/apoptotic cells are stained in red. M.G.G. = May Grunwald Giemsa cell staining; Hoechst33342/Propidium Iodide (PI) = Vital Staining; C = control (untreated) sample; 1 mM, 3 mM, 5 mM = Vitamin C at 1, 3, and 5 mM in the culture medium; original magnification: 400×.

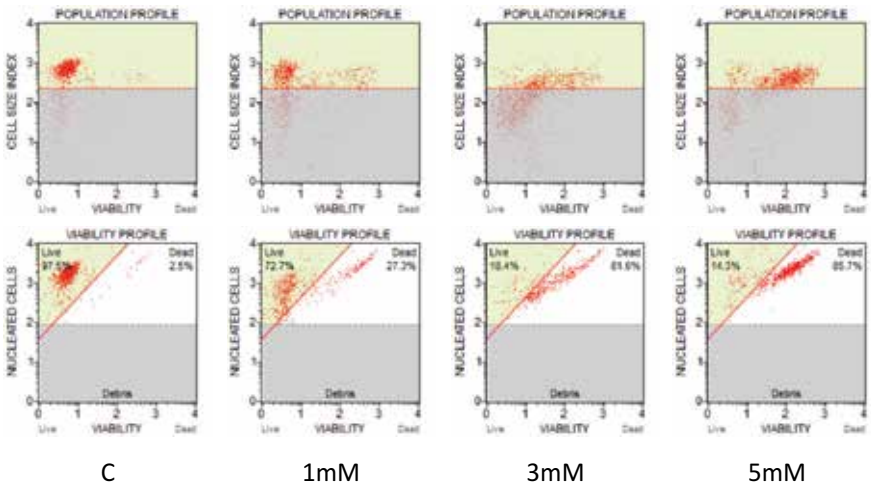

Figure 2. Viability profile of (Human) acute promyelocytic leukemia (APL) cell line (HL60) exposed for 2 h to increasing concentrations of Viability profile of (Human) acute promyelocytic leukemia (APL) cell line (HL60) exposed for 2 h to increasing concentrations of Vitamin C. (Flow Cytometry analysis: see text). The percentage of dead cells in the plates is proportional to the concentration of Vitamin C in the medium. C = control (untreated) sample; 1 mM, 3 mM, 5 mM = Vitamin C at 1, 3, and 5 mM in the culture medium.

its "toxic" effects last for hours (days), even when the nutrient has been removed from the culture medium. This is in agreement with the notion that both normal and leukemic white blood cells tend to concentrate Vitamin C [70–73] to levels that are 10–100 fold higher than plasma [74, 75], and it is in contrast with the view that hydrogen peroxide forms outside the tumor cells [31, 32].

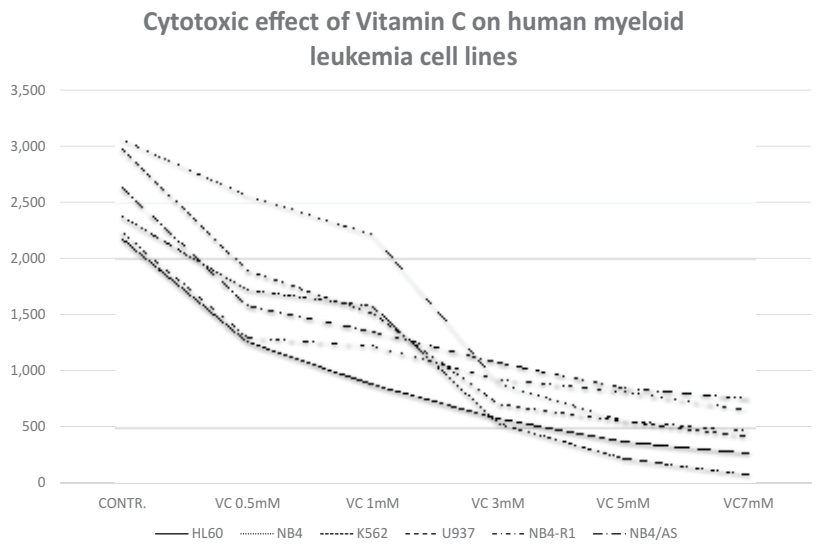

Figure 3. The figure illustrates **Table 1**. The diagram shows the almost uniform decrease in the number of vital leukemic cells in the culture medium, after exposing them to increasing concentrations of Vitamin C, for 2 h.

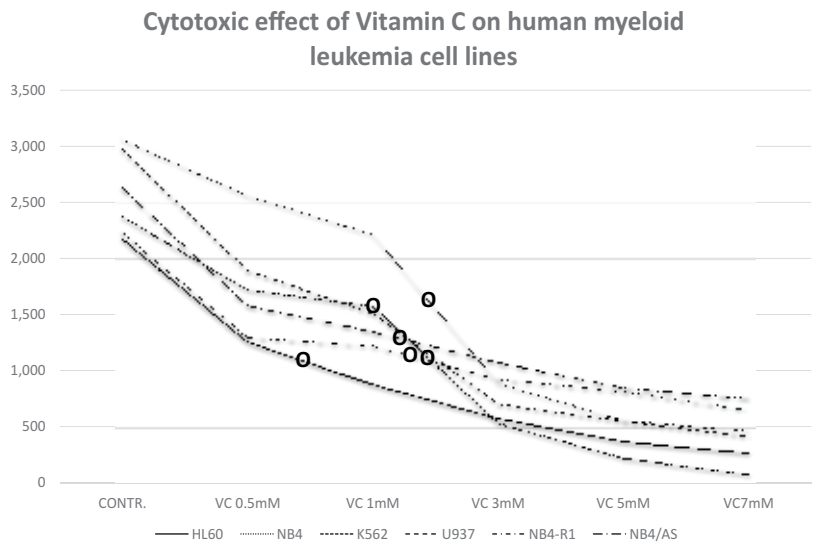

Figure 4. The figure illustrates **Table 1**. Highlighted with colored circles, the LC50 for each human myeloid leukemia cell line tested.

Neutrophils, in particular, accumulate Vitamin C via the sodium-dependent Vitamin C co-transporter 2 (SVCT2) [76], and have intracellular levels of 1–2 mM [77]. Therefore, while there is agreement on the fact that in solid tumors, Vitamin C, initially oxidized to dehydroascorbic acid (DHAA), is internalized by the cell, via GLUT 1 and 4, and finally reduced

		HL60 (2 h)	NB4 (2 h)	K562 (2 h)	U937 (2 h)	NB4-R1 (2 h)	NB4/As (2 h)
Exp. 1	Contr.	471	912	663	1189	337	819
	VC 0.5	296	680	669	479	82.8	42.7
	VC 1	108	494	628	245	39.7	31.9
	VC 3	22.6	163	226	56.4	47	32.2
	VC 5	15.1	143	82	30.2	35	48.4
	VC 7	6.15	85.4	32.2	10.6	24.6	17.6
Exp. 2	Contr.	869	1020	694	829	936	958
	VC 0.5	423	959	399	704	624	823
	VC 1	349	887	445	585	560	674
	VC 3	217	337	200	232	335	581
	VC 5	143	74.6	111	118	344	402
	VC 7	89.5	147	35.2	93.7	255	329
Exp. 3	Contr.	843	1130	1020	969	967	859
	VC 0.5	545	924	660	716	678	722
	VC 1	438	835	507	689	668	649
	VC 3	343	395	113	480	551	499
	VC 5	218	346	35.2	411	443	453
	VC 7	181	241	17.6	320	380	414

The cell lines used in this experiment are variants of human myeloid leukemia cells, and include: HL60, NB4, K562, U937, NB4-R1, NB4/As. It is evident that the total number of cells in culture decreases by increasing the concentration of Vitamin C in the culture medium. C = control (untreated) sample; VC = Vitamin C; VC 0.5 mM, VC 1 mM, VC 3 mM, VC 5 mM = Vitamin C at 0.5, 1, 3, and 5 mM in the culture medium.

Table 1. The number of vital cells after 2 h of exposure to increasing concentrations of Vitamin C in the culture medium.

again to ascorbic acid, with consumption of GSH; this may not be the case in acute myeloid leukemia.

More importantly, the parallel exposure of normal hematopoietic precursors (CD34+), isolated from cord blood, to Vitamin C, at the concentrations that are cytotoxic for leukemic cells did not affect their survival, or impair their capacity to proliferate and differentiate in response to myeloid growth factors. These data confirm that Vitamin C is harmless for normal hematopoietic precursors and therefore highly selective in its anticancer/antileukemic effect.

8. Hypoxia inducible factor (HIF): the forgotten pathway

Hypoxia and induction of hypoxia-inducible factors (HIF) is a hallmark of many tumors [78, 79].

HIF-1 is a heterodimeric transcription factor discovered in 1991 [80], and is composed of two subunits, α and β. The HIF-1α subunit is oxygen sensitive and it is induced by hypoxic conditions, which are very common in cancer. Direct transcriptional targets of HIF-1 include genes regulating, among others, growth and apoptosis, cell migration, energy metabolism, angiogenesis, vasomotor regulation, matrix and barrier functions, and transport of metal ions and glucose [81].

In normoxic conditions, the HIF-1α unit is downregulated by Vitamin C dependent hydroxylases, while in hypoxic conditions (such as those existing in many different types of cancer), HIF-1α hydroxylation is repressed with consequent increase in HIF-dependent gene transcription, neo-angiogenesis, and tumor growth and progression [82].

More importantly, since Vitamin C stimulates HIF-1α prolyl hydroxylases, low levels of Vitamin C promote tumor growth and progression, by reducing HIF-1α hydroxylation [83], thereby stabilizing HIF1-α. On the contrary, high levels of HIF render cancer cells more sensitive to Vitamin C-induced toxicity. To confirm this view, Kuiper and Coll. [84] have recently found an inverse relationship between intra-tumor levels of Vitamin C and HIF activity in both endometrial cancer [85] and colorectal carcinoma (CRC) [86].

In 1925, Otto Warburg observed that cancer cells manifest increased rates of lactate production under aerobic conditions ("Warburg Effect") or, in other words, they preferentially utilize glycolysis, instead of oxidative phosphorylation, for metabolism even in the presence of oxygen [87, 88].

"Hypoxia" (low oxygen concentration) is a hallmark of solid tumors, usually occurring at the center of the tumor mass, where blood vessels are abnormal or insufficient to supply adequate amounts of oxygen [89].

In response to the reduced oxygen tension, the HIF is activated to mediate the primary transcriptional adaption to hypoxic stress in cancer cells [90, 91].

As previously mentioned, HIFs regulate angiogenesis, cell survival, proliferation, apoptosis, adhesion, and metabolism by transcriptionally activating downstream targets such as vascular endothelial growth factor and erythropoietin. Therefore, HIF (HIF1, in particular) plays a major role in tumor growth, and clinical data suggest that the upregulation of HIF, as determined by the low oxygen tension, is usually associated with increased mortality in a number of different cancers [92–94], and may represent a relevant target for new therapeutic approaches to the disease [95–97].

9. The HIF pathway in leukemia

The role of HIF-1α in leukemia, and in particular in acute myeloid leukemia (AML), has only recently emerged and it is still somewhat controversial. One possible explanation for this delayed interest in the role of hypoxia in leukemia could be the fact that leukemia is not considered a "solid" tumor, and therefore, the role of oxygen, in its pathogenesis, has been

considered inconsequential for long time. This erroneous view, has been recently reviewed, as data have emerged, demonstrating that leukemic cells are sensitive to the oxygen tension, and hypoxia influences leukemic cell proliferation, differentiation, and resistance to chemotherapy [98].

Migliavacca and Coll. have recently demonstrated oncogenic function of HIF-1α, in the M5 Fab subtype of AML [99]. In particular, the authors have demonstrated that in M5 AML, HIF-1α mediates the ability of leukemic cells to migrate and invade extramedullary sites. The same group has demonstrated that PML-RARα and other fusion proteins involved in the pathogenesis of acute promyelocytic leukemia (APL) behave as transcriptional coactivators of HIFs, and both HIFs and PML-RARα enhances the progression of APL, by promoting cell migration, homing to bone marrow, and bone marrow neo-angiogenesis [100, 101].

Further investigations [102] have demonstrated that HIF-1α plays critical and pleiotropic roles in the pathogenesis of chronic lymphocytic leukemia (CLL).

Globally, elevated levels of HIF-1α have been reported in AML [103–106], APL [100], acute lymphoblastic leukemia (ALL) [107], and chronic myelogenous leukemia (CML) [108, 109]. Furthermore, HIF-1α overexpression conditions disease severity and outcome in both AML and myelodysplastic syndrome (MDS) [110–112].

Overall, the available data show that hypoxia and HIF-mediated signaling play a crucial role in leukemia, and targeting HIF with specific drugs or natural inhibitors, such as Vitamin C, represents a potentially useful approach to its treatment [113].

10. Vitamin C as a powerful modulator of TET2 activity

Decreased TET expression and loss of 5hmC have been observed in a wide variety of solid tumors, as well as in many hematological malignancies, including acute myeloid leukemias, myelodysplastic syndromes, and clonal hematopoiesis [114].

Recent experimental studies suggest that pharmacological dose of Vitamin C may represent a potentially important strategy in leukemia therapy through a stimulatory effect on TET2 activation and restoration in leukemic cells. Vitamin C is a co-factor of TET2 enzyme and is capable of interacting with the catalytic domain of TET2, enhancing the enzymatic oxidation of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC) [115]. This epigenetic modulation elicited by Vitamin C is able to improve the generation of pluripotent stem cells [116] and to induce a blastocyst-like state in mouse embryonic stem cells [117].

Two recent studies explored the possible epigenetic effects of Vitamin C on leukemia models, mediated by activation and restoration of TET2 activity in leukemic cells. In the first one, authors used a murine model of IDH1-dependent acute myeloid leukemia [118], and 2-phosphate L-ascorbic acid (Asc 2-P). Asc 2-P, unlike native Vitamin C, remains oxidatively stable under standard cell culture conditions [119], and possesses the same modulatory effects of Vitamin C, but, unlike Vitamin C, it does not induce cytotoxic effects of through stimulation of

H_2O_2 production. Asc 2-P added to the cells in culture is stable and releases Vitamin C by plasma membrane alkaline phosphatase hydrolysis [120]. Therefore, Asc 2-P allows a better characterization of the epigenetic activity of Vitamin C, without the "disturbing" H_2O_2-mediated cytotoxic effects of the native molecule. Asc 2-P treatment of the IDH1 AML-mutant mice induced an increase of 5hmC levels, a reduction of leukemic proliferation and an increase in expression of genes involved in leukocyte differentiation [118]. The stimulatory effect of Vitamin C on myeloid differentiation is mediated though the restoration of a normal expression and function of transcription factors, such as PU.1 and RUNX1, required for normal myeloid differentiation.

A second study provided clear evidence that in various leukemia models, Vitamin C treatment induces the restoration of TET2 function, blocking aberrant self-renewal and leukemia progression. Treatment with Vitamin C, mimics TET2 restoration, driving DNA hypomethylation and, by enhancing 5hmC formation, suppresses leukemic colony formation and leukemic progression of primary human leukemia patient-derived xenografts (PDXs). Interestingly, TET2-mediated DNA oxidation induced by Vitamin C-treated leukemic cells, greatly enhances their sensitivity to PARP inhibition and could provide a safe and effective combination strategy to target TET-deficient leukemic cells. These observations suggest that future clinical trials could incorporate high-dose Vitamin C as an adjuvant to standard chemotherapy/demethylating therapy, particularly in TET2-deficient neoplasms [121].

11. What to do next?

The anticancer properties of Vitamin C are known, since at least six decades, even though its use in clinical practice has only recently re-emerged, after the demonstration that in relatively high concentrations, it can selectively kill a number of different human tumor cells, both in vitro and in vivo.

The proof of the anticancer efficacy of Vitamin C in high doses, administered by mouth, has been reported four decades ago, by Linus Pauling [54–57], and further confirmed, very recently, by experimental in vitro and in vivo data [30–32, 66, 69].

Vitamin C is a natural compound, and it is an antioxidant and a life-saving nutrient with multiple beneficial effects on the human body. Man, some primates, and a few other mammals do no longer produce it. Beyond being a natural and essential nutrient, Vitamin C shows, in vitro, an outstanding efficacy in killing a number of different cancer cells, with an efficiency that no other anticancer drug, presently available on the market, has ever shown.

Vitamin C is extremely selective since it kills only cancer cells, by sparing, at the same time, all the other cells of the organism. As a consequence, it is very well tolerated, and devoid of any significant side effects. In fact, the only (relative) contraindication to its use, is the lack of the enzyme glucose-6-phosphate-dehydrogenase (G6PDH), a rare genetic condition also known as "favism." More importantly, within an expensive and often artificially inflated market, such as that of the anticancer drugs [122, 123], Vitamin C, with its low cost, represents an outstanding opportunity for both the patients and the healthcare system.

Unfortunately, in spite of all the above characteristics, Vitamin C has never been easily or favorably accepted as an anticancer drug, by the western Medicine. This also explains why, although the data on its anticancer efficacy are outstanding and straightforward, many scientists still prefer to consider "controversial" the role of Vitamin C in the treatment of cancer.

As we have seen, the idea that the oral administration of Vitamin C, in high doses, is not effective against cancer is a conceptual artefact, originating from questionable interpretations of pharmacokinetics data, after oral and/or intravenous administration. On the other hand, the idea that Vitamin C, administered in high doses by intravenous infusion, behaves as a pro-drug of H_2O_2 beyond being experimentally questionable, has not led to clinically significant results or outcomes [124–128]. More importantly, encouraging results have emerged from unbiased interpretation of the available data [129]. In particular, as it has been shown up to 110 g/m^2/day are very well tolerated by the majority of patients, and even in the absence of any significant clinical remission, intravenous Vitamin C is almost invariably associated with a clear-cut improvement in patient's quality of life.

As a result, History repeat itself! … and just as Vitamin C was dismissed as ineffective, against cancer, more than 30 years ago, on the ground of questionable clinical trials [130, 131], nowadays, it runs again the risk of being definitively discarded, in spite of the large amount of scientific evidence, demonstrating its extraordinary efficacy in fighting cancer!

It is clear that much remains to be understood about the cytotoxic effects of Vitamin C against cancer, and much more can (and must!) be done, to both improve the intravenous therapy and further investigate the oral administration route of the high doses of the nutrient.

Improving the intravenous treatment can (and should!) be achieved, by considering:

a. The type of pharmaceutical preparation, the sodium salt of the ascorbic acid to be preferred, when administered by the intravenous route [132];

b. The time and schedule of administration (slow infusion to be preferred) [133, 134];

c. The level of tissue oxygenation (cell cultures are better oxygenated than tumor tissues, and this may explain the differences in the outcomes between in vitro and in vivo treatment of cancer) [135]. In clinical settings, an improved tumor tissue oxygenation could be obtained with either ozone or hyperbaric oxygen;

d. The level of blood glucose (glucose may interfere with the uptake of ascorbate by cancer cells) [136, 137], and the possibility of associating an adequate dietetic regimen to the treatment with high doses of oral or intravenous Vitamin C.

12. Latest evidence of the role of Vitamin C in leukemia

A recent study provided clear-cut evidence that Vitamin C is a main regulator of hematopoietic stem cell (HSC) function and leukemogenesis. In fact, Agathocleous and co-workers, using a peculiar strategy for isolation of HSCs and hematopoietic progenitor cells (HPCs) from murine bone marrow, showed that HSCs have unusually high levels of Vitamin C,

which decline with differentiation [138]. Importantly, human HSCs and multipotent progenitor cells (MPPs), such as murine HSCs, display high Vitamin C levels.

Using "GULO" mice (deficient in Vitamin C because of the lack of gulonolactone oxidase, the last enzyme in the synthesis of Vitamin C starting from glucose), Agathocleous and colleagues have shown that Vitamin C deficiency induces an increased number of HSCs. A FLT3-internal tandem duplication (ITD) mutation, found in approximately a quarter of patients with de novo AML, imparts a particularly poor prognosis. Using "GULO" mice (deficient in Vitamin C because of the lack of gulonolactone oxidase), Agathocleous and colleagues have shown that Vitamin C deficiency induces an increased number of HSCs. Therefore Vitamin C deficiency, and TET2 mutations, are likely to cooperates with FLT3-ITD to induce leukemia development in murine models of FLT3-ITD-driven leukemia. [138].

Given the above evidence, it will be worth mentioning, once more, that the biochemist Irwin Stone, in his book "The healing Factor: Vitamin C against disease," published in 1972 (45 years ago!), had already warned the scientific community on the role of Vitamin C as a main factor in the prevention and treatment of leukemia. In his words, "*In a leukemic, the biochemical stresses of the disease process has reduced the body stores of ascorbic acid to very low levels … Any ascorbic acid circulating in the blood has been scavenged and locked in the excessive numbers of white blood cells contained in the blood. The plasmas level of ascorbic acid is usually zero or close thereto. A zero level in the blood plasma means that the tissues of the body are not being supplied with this most important metabolite. The ascorbic acid contained in the leukocytes are unavailable for the tissues. The tissues are in a condition of biochemical scurvy and this explains why these depleted tissues are so susceptible to the characteristic hemorrhaging of leukemia and the infections that kill so many of the leukaemics. A leukemic is not only suffering from leukemia but also from a bad case of biochemical scurvy. To correct this condition, ascorbic acid has to be administered in sufficiently large doses not only to saturate the excess of white blood cells but to provide adequate spill over into the blood plasma and tissues so that the seriously ill leukemic will be given a fighting chance to combat the disease. This may require the administration of ascorbic acid at the rate of 25 or more grams per day, as noted in the following case of leukemia treated with megascorbic levels of ascorbic acid.*" [3].

13. Concluding remarks

The rationale behind the use of high doses of Vitamin C in the treatment of acute leukemia is strong and very well grounded. In summary:

a. Leukemic patients, almost invariably show a severe deficiency of this nutrient;

b. While it is currently supposed to kill cancer cells by inducing pro-oxidant damage, Vitamin C is also very effective as an antioxidant by inhibiting the hypoxia inducible factor (HIF);

c. The mechanistic explanation of the pathogenesis of myeloid leukemia, includes the possibility that a Vitamin deficiency may induce the neoplastic transformation of myeloid precursors, through an upregulation of the HIF, and the consequent cascade of HIF-dependent cancer genes;

d. Although administered by intravenous infusion, in the majority of clinical trials performed so far, Vitamin C appears to be effective, in fighting cancer, even when administered by mouth;

e. Vitamin C is very well tolerated, and has no side or undesired effects;

f. Experimental in vitro data unequivocally show the cytotoxic effect of Vitamin C against leukemia.

g. As shown in our study on leukemic and normal cell lines, Vitamin C can kill almost every type of acute and chronic myeloid leukemia-derived cell, without doing any harm to their normal counterpart CD34+ cells;

h. Vitamin C is a natural compound, and it is very cheap.

Do we really need more information or evidence, to start clinical trials on Vitamin C, in the treatment of acute and chronic myeloid leukemia?

Acknowledgements

This chapter has been realized, in part, thanks to the financial support of the "Pescara Cell Factory Foundation".

Conflict of interests

None to declare.

Author details

Domenico Mastrangelo[1]*, Lauretta Massai[1], Giuseppe Fioritoni[2], Francesco Lo Coco[3,4], Nèlida Noguera[3,4] and Ugo Testa[5]

*Address all correspondence to: mastrangelod10@gmail.com

1 Department of Medical, Surgical and Neurological Sciences, University of Siena, Polo Scientifico San Miniato, Siena, Italy

2 Pescara Cell Factory Foundation Onlus, Pescara, Italy

3 Department of Biomedicine and Prevention, University of Rome Tor Vergata, Rome, Italy

4 Santa Lucia Foundation, I.R.C.C.S., Via del Fosso di Fiorano, Rome, Italy

5 Department of Hematology, Oncology and Molecular Medicine, Istituto Superiore di Sanità, Rome, Italy

References

[1] Iqbal K, Khan A, Ali Khan Khattak MM. Biological significance of ascorbic acid (Vitamin C) in human health. A review. Pakistan Journal of Nutrition. 2004;**3**:5-13

[2] Drouin G, Godin JR, Pagé B. The genetics of Vitamin C loss in vertebrates. Current Genomics. 2011;**12**:371-378. DOI: 10.2174/138920211796429736

[3] Stone I. The Healing Factor: Vitamin C Against Disease. New York: Grosset and Dunlap Inc.; 1972

[4] Stephen DJ, Hawley EE. The partition of reduced ascorbic acid in blood. The Journal of Biological Chemistry. 1936;**115**:653-658

[5] Barkhan P, Howard AN. Distribution of ascorbic acid in normal and leukaemic human blood. The Biochemical Journal. 1958;**70**:163-168

[6] Vogt A, Uber den Vitamin C. Verbrauch bei Tumorkranken und bei der Lymphogranulomatose. Strahlentherapie. 1939;**65**:616-623

[7] Kyhos FD, Sevringhaus FL, Hagendorn DR. Large doses of ascorbic acid in treatment of Vitamin C deficiencies. Archives of Internal Medicine. 1945;**75**:407

[8] Waldo AL, Zipf RE. Ascorbic acid level in leukemic patients. Cancer. 1955;**8**:187-190

[9] Pujari KN, Jadkar SP, Mashal SN, Belwalkar GJ, Kulkarni A, Patil CG, et al. Variations in vitamin C levels in leukemias. Biomedical Research. 2012;**23**:307-311

[10] Liua M, Ohtania H, Zhoua W, Ørskovb AD, Charletc J, Zhangd YW, et al. Vitamin C increases viral mimicry induced by 5-aza-2′-deoxycytidine. PNAS. 2016;**113**:10238-10244. DOI: 10.1073/pnas.1612262113

[11] Shenoy N, Bhagat T, Nieves E, Stenson M, Lawson J, Choudhary GS, et al. Upregulation of TET activity with ascorbic acid induces epigenetic modulation of lymphoma cells. Blood Cancer Journal. 2017;**7**:e587. DOI: 10.1038/bcj.2017.65

[12] Klenner FR. The treatment of poliomyelitis and other virus diseases with vitamin C. Southern Medicine and Surgery. 1949;**111**:209-214

[13] Stone I. Hypoascorbemia, the genetic disease causing the human requirement for exogenous ascorbic acid. Perspectives in Biology and Medicine. 1966;**10**:133-134

[14] Stone I. The genetic disease, hypoascorbemia: A fresh approach to an ancient disease and some of its medical implications. Acta Geneticae Medicae et Gemellologiae. 1967; **16**:52-62

[15] Stone I. Hypoascorbemia, our most widespread disease. Bulletin of the National Health Federation 1972;**18**:6-9

[16] http://www.omim.org/entry/240400

[17] Stone I. Megascorbic therapy of the disease called leukemia. Cancer Control. 1974;**2**:1-4

[18] Stone I. My daily megascorbic regime for full health and long life. Better Nutrition; 1977

[19] Panel on Dietary Antioxidants and Related Compounds; Subcommittee on Upper Reference Levels of Nutrients; Subcommittee on Interpretation and Uses of DRIs; Standing Committee on the Scientific Evaluation of Dietary Reference Intakes; Food and Nutrition Board; Institute of Medicine. Dietary Reference Intakes for Vitamin C, Vitamin E, Selenium, and Carotenoids. 2000. 529 p. ISBNs: Paperback: 978-0-309-06935-9 Hardcover: 978-0-309-06949-6

[20] Letter PL. Megavitamin therapy. Journal of the American Medical Association. 1975; **234**:149

[21] Pauling L. Are recommended daily allowances for vitamin C adequate? Review. PNAS USA. 1974;**71**:4442-4446

[22] Pauling L. Vitamin C. Science. 1972;**177**(4055):1152

[23] Pauling L. Evolution and the need for ascorbic acid. PNAS USA. 1970;**7**:1643-1648

[24] Pauling L. Vitamin C and common cold. Journal of the American Medical Association. 1971;**216**:332

[25] Pauling L. The significance of the evidence about ascorbic acid and the common cold. Review. PNAS USA. 1971;**68**:2678-2681

[26] Pauling L. Vitamin C therapy of advanced cancer. The New England Journal of Medicine. 1980;**302**:694-695

[27] Harakeh S, Jariwalla RJ, Pauling L. Suppression of human immunodeficiency virus replication by ascorbate in chronically and acutely infected cells. PNAS USA. 1990;**87**: 7245-7249

[28] Levine M, Conry-Cantilena C, Wang Y, Welch RW, Washko PW, Dhariwal KR, et al. Vitamin C pharmacokinetics in healthy volunteers: Evidence for a recommended dietary allowance. PNAS USA. 1996;**93**:3704-3709

[29] Padayatty SJ, Sun H, Wang Y, Riordan HD, Hewitt SM, Katz A, et al. Vitamin C pharmacokinetics: Implications for oral and intravenous use. Annals of Internal Medicine. 2004;**140**:533-537

[30] Chen Q, Espey MG, Sun AY, Pooput C, Kirk KL, Krishna MC, et al. Pharmacologic doses of ascorbate act as a prooxidant and decrease growth of aggressive tumor xenografts in mice. PNAS USA. 2008;**105**:11105-11109

[31] Chen Q, Espey MG, Sun AY, Lee JH, Krishna MC, Shacter E, et al. Ascorbate in pharmacologic concentrations selectively generates ascorbate radical and hydrogen peroxide in extracellular fluid *in vivo*. PNAS USA. 2007;**104**:8749-8754

[32] Chen Q, Espey MG, Krishna MC, Mitchell JB, Corpe CP, Buettner G, et al. Pharmacologic ascorbic acid concentrations selectively kill cancer cells: Action as a pro-drug to deliver hydrogen peroxide to tissues. PNAS USA. 2005;**102**:13604-13609

[33] Halliwell B. Vitamin C: Poison, prophylactic or panacea? Trends in Biochemical Sciences. 1999;**24**:l255-1259

[34] Carr AC, Frei B. Does vitamin C act as pro-oxidant under physiological conditions? The FASEB Journal. 1999;**13**:1007-1024

[35] Hacisevki A. An overview of ascorbic acid biochemistry. Journal of the Faculty of Pharmacy of Ankara University. 2009;**38**:233-255

[36] Yun J, Mullarky E, Lu C, Bosch KN, Kavalier A, Rivera K, et al. Vitamin C selectively kills KRAS and BRAF mutant colorectal cancer cells by targeting GAPDH. Science. 2015;**350**:1391-1396

[37] Savini I, Rossi A, Pierro C, Avigliano L, Catani MV. SVCT1 and SVCT2: Key proteins for vitamin C uptake. Amino Acids. 2008;**34**:347-355

[38] Vera JC, Rivas CI, Fischbarg J, Golde DW. Mammalian facilitative hexose transporters mediate the transport of dehydroascorbic acid. Nature. 1993;**364**:79-82

[39] Tian W, Wang Y, Xu Y, Guo X, Wang B, Sun L, et al. The hypoxia-inducible factor renders cancer cells more sensitive to Vitamin C-induced toxicity. The Journal of Biological Chemistry. 2014;**289**:3339-3351

[40] Yun J, Rago C, Cheong I, Pagliarini R, Angenendt P, Rajagopalan H, et al. Glucose deprivation contributes to the development of KRAS pathway mutations in tumor cells. Science. 2009;(5947):1555-1559

[41] Sheu JJC, Guan B, Tsai FJ, Hsiao EYT, Chen CM, Seruca R, et al. Mutant BRAF induces DNA strand breaks, activates DNA damage response pathway, and up-regulates glucose transporter-1 in nontransformed epithelial cells. The American Journal of Pathology. 2012;**180**:1179-1188

[42] Van der Reest J, Gottlieb E. Anti-cancer effects of vitamin C revisited. Cell Research. 2016;**26**:269-270

[43] Uetaki M, Tabata S, Nakasuka F, Soga T, Masaru Tomita M. Metabolomic alterations in human cells by vitamin C-induced oxidative stress. Scientific Reports. 2015;**5**:1-9

[44] Benade L, Howard T, Burk D. Synergistic killing of Ehrlich ascites carcinoma cells by ascorbate and 3-amino-1, 2, 4,-triazole. Oncology. 1969;**23**:33-43

[45] Oberley TD, Oberley LW. Antioxidant enzyme levels in cancer. Histology and Histopathology. 1997;**12**:525-535

[46] Cavanagh EMV, Honegger AE, Hofer E, Bordenave RH, Bullorsky EO, Chasseing NA. Higher oxidation and lower antioxidant levels in peripheral blood plasma and bone marrow plasma from advanced cancer patients. Cancer. 2002;**94**:3247-3251

[47] Mojić M, Bogdanović Pristov J, Maksimović-Ivanić D, Jones DR, Stanić M, Mijatović S, et al. Extracellular iron diminishes anticancer effects of vitamin C: An in vitro study. Scientific Reports. 2014;**4**:5955. DOI: 10.1038/srep05955

[48] Du J, Cullen JJ, Buettner GR. Ascorbic acid: Chemistry, biology and the treatment of cancer. Biochimica et Biophysica Acta. 2012;**1826**:443-457

[49] Lòpez-Làzaro M. Dual role of hydrogen peroxide in cancer: Possible relevance to cancer chemoprevention and therapy. Cancer Letters. 2007;**252**:1-8

[50] Lisanti MP, Martinez-Outschoorn UE, Lin Z, Pavlides S, Whitaker-Menezes D, Pestell RG. Hydrogen peroxide fuels aging, inflammation, cancer metabolism and metastasis. Cell Cycle. 2011;**10**:2440-2449

[51] Padayatty SJ, Levine M. Reevaluation of ascorbate in cancer treatment: Emerging evidence, open minds and serendipity. Journal of the American College of Nutrition. 2000;**19**:423-425

[52] Robinson AR, Hunsberger A, Westall FC. Suppression of squamous cell carcinoma in hairless mice by dietary nutrient variation. Mechanisms of Ageing and Development. 1994;**76**:201-214

[53] Hoffer A. The discovery of Vitamin C: Albert Szent-Gyorgi, M.D. Ph.D. 1893-1986. Journal of Orthomolecular Medicine. 1989;**4**:24-26

[54] Cameron E, Pauling L. Supplemental ascorbate in the supportive treatment of cancer: Reevaluation of prolongation of survival times in terminal human cancer. PNAS USA. 1978;**75**:4538-4542

[55] Pauling L. Vitamin homeostasis in the brain and megavitamin therapy. The New England Journal of Medicine. 1977;**297**:790-791

[56] Pauling L. Diet, nutrition, and cancer. The American Journal of Clinical Nutrition. 1977; **30**:661-663

[57] Cameron E, Pauling L. Supplemental ascorbate in the supportive treatment of cancer: Prolongation of survival times in terminal human cancer. PNAS USA. 1976;**73**:3685-3689

[58] Cameron E, Campbell A. The orthomolecular treatment of cancer. Clinical trial of high dose ascorbic acid supplements in advanced human cancer. Chemico-Biological Interactions. 1974;**9**:285-315

[59] Murata A, Morishige F, Yamaguchi H. Prolongation of survival times of terminal cancer patients by administration of large doses of ascorbate. International Journal for Vitamin and Nutrition Research Supplement. 1982;**23**:101-113

[60] Hickey S, Roberts H, Miller NJ. Pharmacokinetics of oral ascorbate liposomes. JNEM. 2008. DOI: 10.1080/13590840802305423

[61] Cathcart RF. The method of determining proper doses of vitamin c for the treatment of disease by titrating to bowel tolerance. Orthomol Psychiatry. 1981;**10**:125-132

[62] Hickey S, Roberts H. The real story of Vitamin C and cancer. Journal of Metals. 2008; **23**:133-138

[63] Poirier MC. Chemical-induced DNA damage and human cancer risk. Nature Reviews. Cancer. 2004;**4**:630-637

[64] Sharma RA, Farmer PB. Biological relevance of adduct detection to the chemoprevention of cancer. Clinical Cancer Research. 2004;**10**:4901-4912

[65] Gao P, Zhang H, Dinavahi R, Li F, Xiang Y, Raman V, et al. HIF-dependent antitumorigenic effect of antioxidants *in vivo*. Cancer Cell. 2007;**12**:230-238

[66] Mastrangelo D, Massai L, Fioritoni G, Iacone A, Di Bartolomeo P, Accorsi P, et al. Megadoses of sodium ascorbate efficiently kill HL60 cells in vitro: Comparison with arsenic trioxide. Journal of Cancer Therapy. 2013;**4**:1366-1372

[67] Monti DA, Mitchell E, Bazzan AJ, Littman S, Zabrecky G, Yeo CJ, et al. Phase I evaluation of intravenous ascorbic acid in combination with gemcitabine and Erlotinib in patients with metastatic pancreatic cancer. PLoS One. 2012;**7**:e29794

[68] Stephenson CM, Levin RD, Spector T, Lis CG. Phase I clinical trial to evaluate the safety, tolerability, and pharmacokinetics of high-dose intravenous ascorbic acid in patients with advanced cancer. Cancer Chemotherapy and Pharmacology. 2013;**72**:139-146

[69] Mastrangelo D, Massai L, Lo Coco F, Noguera NI, Borgia L, Fioritoni G, et al. Cytotoxic effects of high concentrations of sodium ascorbate on human myeloid cell lines. Annals of Hematology. 2015;**94**:1807-1816

[70] Lowry OH, Bessey OA, Brock MJ, Lopez JA. The interrelationship of dietary, serum, white blood cell, and total body ascorbic acid. The Journal of Biological Chemistry. 1946;**166**:111-119

[71] Wilson C. Clinical pharmacological aspects of ascorbic acid. Annals of the New York Academy of Sciences. 1975;**25**:354-376

[72] Lykkesfeldt J. Determination of ascorbic acid and dehydroascorbic acid in biological samples by high-performance liquid chromatography using subtraction methods: Reliable reduction with tris[2-carboxyethyl] phosphine hydrochloride. Analytical Biochemistry. 2000;**282**:89-93

[73] Emadi-Konjin P, Verjeea Z, Levinb AV, Adeli K. Measurement of intracellular vitamin C levels in human lymphocytes by reverse phase high performance liquid chromatography (HPLC). Clinical Biochemistry. 2005;**38**:450-456

[74] Michels AJ, Hagen TM, Frei B. Human genetic variation influences Vitamin C homeostasis by altering Vitamin C transport and antioxidant enzyme function. Annual Review of Nutrition. 2013;**33**:45-70

[75] Bozonet SM, Carr AC, Pullar JM, Vissers MCM. Enhanced human neutrophil Vitamin C status, chemotaxis and oxidant generation following dietary supplementation with Vitamin C-rich sungold kiwifruit. Nutrients. 2015;**7**:2574-2588. DOI: 10.3390/nu7042574

[76] Corpe CP, Lee JH, Kwon O, Eck P, Narayanan J, Kirk KL, et al. 6-Bromo-6-deoxyL-ascorbic acid: An ascorbate analog specific for Na+-dependent vitamin C transporter but not glucose transporter pathways. The Journal of Biological Chemistry. 2005;**280**:5211-5220

[77] Washko PW, Wang Y, Levine M. Ascorbic acid recycling in human neutrophils. The Journal of Biological Chemistry. 1993;**268**:15531-15535

[78] Aprelikova O, Chandramouli GVR, Wood M, Vasselli JR, Riss J, Maranchie JK, et al. Regulation of HIF prolyl hydroxylases by hypoxia-inducible factors. Journal of Cellular Biochemistry. 2004;**92**:491-501

[79] Nagy MA. HIF-1 is the commander of gateways to cancer. Journal of Cancer Science & Therapy. 2011;**3**:035-040. DOI: 10.4172/1948-5956.1000054

[80] Semenza GL, Nejfelt MK, Chi SM, Antonarakis SE. Hypoxiainducible nuclear factors bind to an enhancer element located 3′ to the human erythropoietin gene. PNAS USA. 1991;**88**:5680-5684

[81] Schofield CJ, Ratcliffe PJ. Oxygen sensing by HIF hydroxylases. Nature Reviews. Molecular Cell Biology. 2004;**5**:343-354

[82] Traber MG, Stevens JF. Vitamins C and E: Beneficial effects from a mechanistic perspective. Free Radical Biology and Medicine. 2011;**51**:1000-1013

[83] Knowles HJ, Raval RR, Harris AL, Ratcliffe PJ. Effect of ascorbate on the activity of hypoxia-inducible factor in cancer cells. Cancer Research. 2003;**63**:1764-1768

[84] Kuiper C, Vissers MCM. Ascorbate as a co-factor for Fe- and 2-oxoglutarate dependent dioxygenases: Physiological activity in tumor growth and progression. Frontiers in Oncology. 2014;**4**:1-11

[85] Kuiper C, Molenaar IG, Dachs GU, Currie MJ, Sykes PH, Vissers MC. Low ascorbate levels are associated with increased hypoxia-inducible factor-1 activity and an aggressive tumor phenotype in endometrial cancer. Cancer Research. 2010;**70**:5749-5758

[86] Kuiper C, Dachs GU, Munn D, Currie MJ, Robinson BA, Pearson JF. Increased tumor ascorbate is associated with extended disease-free survival and decreased hypoxia-inducible factor-1 activation in human colorectal cancer. Frontiers in Oncology. 2014;**4**: 1-10. DOI: 10.3389/fonc.2014.00010

[87] Warburg O. Über den stoffwechsel der carcinomzelle. Klinische Wochenschrift. 1925; **4**:534-536

[88] Justus CR, Sanderlin EJ, Yang LV. Molecular connections between cancer cell metabolism and the tumor microenvironment. International Journal of Molecular Sciences. 2015;**16**:11055-11086

[89] Huang D, Li C, Zhang H. Hypoxia and cancer cell metabolism. Acta Biochimica et Biophysica Sinica. 2014;**46**:214-219. DOI: 10.1093/abbs/gmt148

[90] Semenza GL, Wang GL. A nuclear factor induced by hypoxia via *58* protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. Molecular and Cellular Biology. 1992;**12**:5447-5454

[91] Wang GL, Jiang BH, Rue EA, Semenza GL. Hypoxia-inducible factor-1 is a basic-helix-loop-helix-Pas heterodimer regulated by cellular O_2 tension. PNAS USA. 1995;**92**: 5510-5514

[92] Schindl M, Schoppmann SF, Samonigg H, Hausmaninger H, Kwasny W, Gnant M, et al. Overexpression of hypoxia-inducible factor 1 is associated with an unfavorable prognosis in lymph node-positive breast cancer. Clinical Cancer Research. 2002;**8**:1831-1837

[93] Mouriaux F, Sanschagrin F, Diorio C, Landreville S, Comoz F, Petit E, et al. Increased HIF-1a expression correlates with cell proliferation and vascular markers CD31 and VEGF-A in uveal melanoma. IOVS. 2014;**55**:1277-1283. DOI: 10.1167/iovs.13-13345

[94] Kim BW, Cho H, Chung JY, Conway C, Ylaya K, Kim JH, et al. Prognostic assessment of hypoxia and metabolic markers in cervical cancer using automated digital image analysis of immunohistochemistry. Journal of Translational Medicine. 2013;**11**:185. DOI: 10.1186/1479-5876-11-185

[95] Masoud GN, Li W. HIF-1α pathway: Role, regulation and intervention for cancer therapy. Acta Pharmacol Sin B. 2015;**5**:378-389. DOI: 10.1016/j.apsb.2015.05.007

[96] Semenza GL. Targeting HIF-1 for cancer therapy. Nature Reviews. Cancer. 2003;**3**:721-732. DOI: 10.1038/nrc1187

[97] Poon E, Harris AL, Ashcroft M. Targeting the hypoxia-inducible factor (HIF) pathway in cancer. Expert Reviews in Molecular Medicine. 2009;**11**:e26. DOI: 10.1017/S1462399409001173

[98] Muz B, de la Puente P, Azab F, Luderer M, Azab AK. The role of hypoxia and exploitation of the hypoxic environment in hematologic malignancies. Molecular Cancer Research 2014;**12**:1347-1354. DOI: 10.1158/1541-7786

[99] Migliavacca J, Percio S, Valsecchi R, Ferrero E, Spinelli A, Ponzoni M, et al. Hypoxia inducible factor-1a regulates a pro-invasive phenotype in acute monocytic leukemia. Oncotarget. 2016;**7**:53540-53577

[100] Coltella N, Percio S, Valsecchi R, Cuttano R, Guarnerio J, Ponzoni M, et al. HIF factors cooperate with PML-RARalpha to promote acute promyelocytic leukemia progression and relapse. EMBO Molecular Medicine. 2014;**6**:640-650

[101] Percio S, Coltella N, Grisanti S, Bernardi R, Pattini LA. HIF-1 network reveals characteristics of epithelial-mesenchymal transition in acute promyelocytic leukemia. Genome Medicine. 2014;**6**:84. DOI: 10.1186/s13073-014-0084-4

[102] Valsecchi R, Coltella N, Belloni D, Ponente M, Ten Hacken E, Scielzo C, et al. HIF-1a regulates the interaction of chronic lymphocytic leukemia cells with the tumour microenvironment. Blood. 2016;**127**:1987-1997. DOI: 10.1182/blood-2015-07-657056

[103] Gao XN, Yan F, Lin J, Gao L, XL L, Wei SC, et al. AML1/ETO cooperates with HIF1α to promote leukemogenesis through DNMT3a transactivation. Leukemia. 2015;**29**:1730-1740

[104] Forristal CE, Brown AL, Helwani FM, Winkler IG, Nowlan B, Barbier V, et al. Hypoxia inducible factor (HIF)-2α accelerates disease progression in mouse models of leukemia and lymphoma but is not a poor prognosis factor in human AML. Leukemia. 2015;**29**:2075-2085

[105] Kawada H, Kaneko M, Sawanobori M, Uno T, Matsuzawa H, Nakamura Y, et al. High concentrations of l-ascorbic acid specifically inhibit the growth of human leukemic cells via downregulation of HIF-1α transcription. PLoS One. 2013;**8**:e62717

[106] Wang Y, Liu Y, Malek SN, Zheng P, Liu Y. Targeting HIF1α eliminates cancer stem cells in hematological malignancies. Cell Stem Cell. 2011;**8**:399-411. DOI: 10.1016/j.stem.2011.02.006

[107] Frolova O, Samudio I, Benito JM, Jacamo R, Kornblau SM, Markovic A, et al. Regulation of HIF-1α signaling and chemoresistance in acute lymphocytic leukemia under hypoxic conditions of the bone marrow microenvironment. Cancer Biology & Therapy. 2012;**13**:858-870

[108] Chen H, Shen Y, Gong F, Jiang Y, Zhang R. HIF-α promotes chronic myelogenous leukemia cell proliferation by upregulating p21 expression. Cell Biochemistry and Biophysics. 2015;**72**:179-183. DOI: 10.1007/s12013-014-0434-2

[109] Zhang H, Li H, Xi HS, Li S. HIF1α is required for survival maintenance of chronic myeloid leukemia stem cells. Blood. 2012;**119**:2595-2607

[110] Rouault-Pierre K, Lopez-Onieva L, Foster K, Anjos-Afonso F, Lamrissi-Garcia I, Serrano-Sanchez M, et al. HIF-2α protects human hematopoietic stem/progenitors and acute myeloid leukemic cells from apoptosis induced by endoplasmic reticulum stress. Cell Stem Cell. 2013;**13**:549-563

[111] Wellmann S, Guschmann M, Griethe W, Eckert C, Stackelberg A, Lottaz C, et al. Activation of the HIF pathway in childhood ALL, prognostic implications of VEGF. Leukemia. 2004;**18**:926-933

[112] Elias H, Verma A. Activation of hypoxia signaling is marker of poor prognosis in myelodysplasia. Leukemia & Lymphoma. 2012;**53**:2337-2338

[113] Deynoux M, Sunter N, Hérault O, Mazurier F. Hypoxia and hypoxia-inducible factors in leukemias. Frontiers in Oncology. 2016;**6**:41. DOI: 10.3389/fonc.2016.00041

[114] Rasmussen KD, Helin K. Role of TET enzymes in DNA methylation, development, and cancer. Genes & Development. 2016;**30**:733-750

[115] Yin R, Mao SQ, Zhao B, Chong Z, Yang Y, Zhao C, et al. Ascorbic acid enhances Tet-mediated 5-methylcytosine oxidation and promotes DNA demethylation in mammals. Journal of the American Chemical Society. 2013;**135**:10936-10403

[116] Chen J, Guo L, Zhang L, Wu H, Yang J, Liu H, et al. Vitamin C modulates TET1 function during somatic cell reprogramming. Nature Genetics. 2013;**45**:1504-1509

[117] Blaschke K, Ebata KT, Karimi MM, Zepeda-Martínez JA, Goyal P, Mahapatra S, et al. Vitamin C induces Tet-dependent DNA demethylation in ESCS to promote a blastocyst-like state. Nature. 2013;**500**:222-226

[118] Mingay M, Chaturvedi A, Bilenky M, Cao Q, Jackson L, Hui T, et al. Vitamin C-induced epigenomic remodeling in IDH1 mutant acute myeloid leukemia. Leukemia. 2017:1-10. DOI: 10.1038/leu.2017.171

[119] Austria R, Semenzato A, Bettero A. Stability of vitamin C derivatives in solution and topical formulations. Journal of Pharmaceutical and Biomedical Analysis. 1997;**15**:795-801

[120] Takamizawa S, Maehata Y, Imai K, Senoo H, Sato S, Hata RI. Effects of ascorbic acid and ascorbic acid 2-phosphate, a long-acting vitamin C derivative, on the proliferation and differentiation of human osteoblast-like cells. Cell Biology International. 2004;**28**:255-265

[121] Cimmino L, Dolgalev I, Wang Y, Yoshimi A, Martin G, Wang J, et al. Restoration of TET2 function blocks aberrant self-renewal and leukemia progression. Cell. 2017;**170**:1-17

[122] http://www.ascopost.com/issues/february-1-2013/cost-of-cancer-drugs-what-price-for-what-benefit/

[123] http://chronicdisease.uchicago.edu/files/2014/09/Conti-pricing-trends-9-9-2014.pdf

[124] Wilson MK, Baguley BC, Wall C, Jameson MB, Findaly MP. Review of high-dose intravenous vitamin C as an anticancer agent. Asia-Pacific Journal of Clinical Oncology. 2014;**10**:22-37

[125] Unlu A, Kirca O, Ozdogan M, Nayır E. High-dose vitamin C and cancer. Journal of Oncological Science. 2016;**1**:10-12

[126] Nandal S, Ghalaut P, Shekhawat H, Nagar P, Ghalaut R, Meena B. High-dose intravenous vitamin C as an anticancer agent—A literature review. IJARESM. 2014;**6211**:7

[127] Jacobs C, Hutton B, Ng T, Shorr R, Clemons M. Is there a role for oral or intravenous ascorbate (Vitamin C) in treating patients with cancer? A systematic review. The Oncologist. 2015;**20**:210-223

[128] Goodman A. Vitamin C and cancer. AIMS Medical Science. 2013;**3**:41-51

[129] Fritz H, Flower G, Weeks L, Cooley K, Callachan M, McGowan J, et al. Intravenous Vitamin C and cancer: A systematic review. Integrative Cancer Therapies. 2014;**13**:280-300. DOI: 10.1177/1534735414534463

[130] Creagan ET, Moertel CG, O'Fallon JR, Schutt AJ, O'Connell MJ, Rubin J, et al. Failure of high-dose vitamin C (ascorbic acid) therapy to benefit patients with advanced cancer. A controlled trial. The New England Journal of Medicine. 1979;**301**:687-690

[131] Moertel CG, Fleming TR, Creagan ET, Rubin J, O'Connell MJ, Ames MM. High-dose vitamin C versus placebo in the treatment of patients with advanced cancer who have had no prior chemotherapy. A randomized double-blind comparison. The New England Journal of Medicine. 1985;**312**:137-141

[132] http://www.doctoryourself.com/vitciv.html

[133] Duconge J, Miranda-Massari JR, Gonzalez MJ, Jackson JA, Warnock W, Riordan NH. Pharmacokinetics of vitamin C: Insights into the oral and intravenous administration of ascorbate. Puerto Rico Health Sciences Journal. 2008;**27**:7-19

[134] Hickey DS, Roberts HJ, Cathcart RF. Dynamic flow: A new model for ascorbate. Journal of Metals. 2005;**20**:237-244

[135] Carreau A, El Hafny-Rahbi B, Matejuk A, Grillon C, Kieda C. Why is the partial oxygen pressure of human tissues a crucial parameter? Small molecules and hypoxia. Journal of Cellular and Molecular Medicine. 2011;**15**:1239-1253

[136] Malo C, Wilson JX. Glucose modulates Vitamin C transport in adult human small intestinal brush border membrane vesicles. The Journal of Nutrition. 2000;**130**:63-69

[137] Washko P, Levine M. Inhibition of ascorbic acid transport in human neutrophils by glucose. The Journal of Biological Chemistry. 1992;**267**:23568-23574

[138] Agathocleous M, Meacham CE, Burgess RJ, Piskounova E, Zhao Z, Crane GM, Cowin BL, Bruner E, Murphy MM, Chen W, Spangrude GJ, Hu Z, DeBernardinis RJ, Morrison SJ. Ascorbate regulates haematopoietic stem cell function and leukaemogenesis. Nature. Sep 28 2017;**549**(7673):476-481. DOI: 10.1038/nature23876. Epub 2017 Aug 21

Edited by Ahmed Lasfar

This book offers a remarkable coverage of myeloid leukemia from diagnosis to treatment. It provides an updated and new vision of this multifaceted disease, regrouping a variety of myeloid disorders. To ensure the high quality of this book, important insights are included and rigorously discussed in a simple and authentic way. This book is a relevant source of knowledge, very useful for researchers, medical doctors, nurses, students and individuals interested in this complex disease.

Published in London, UK

IntechOpen

ISBN 978-1-83881-366-6