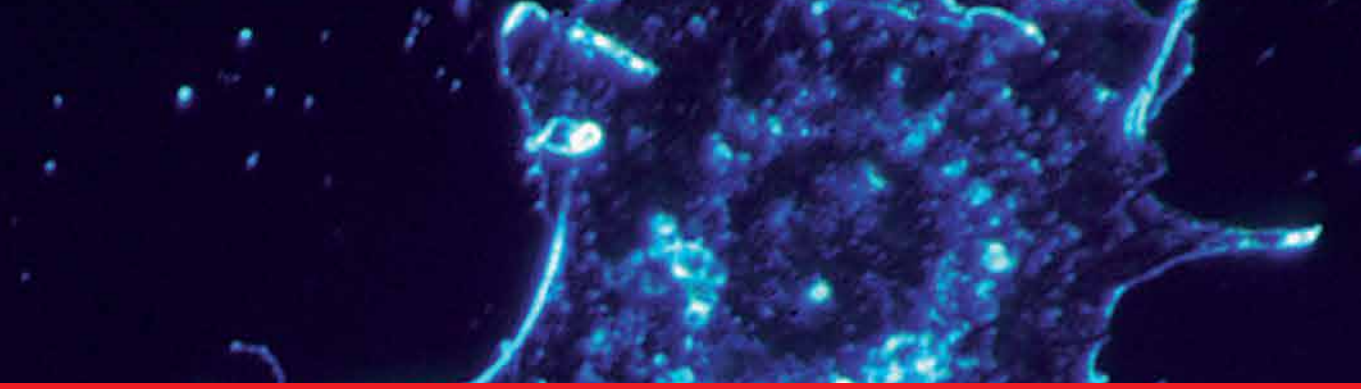

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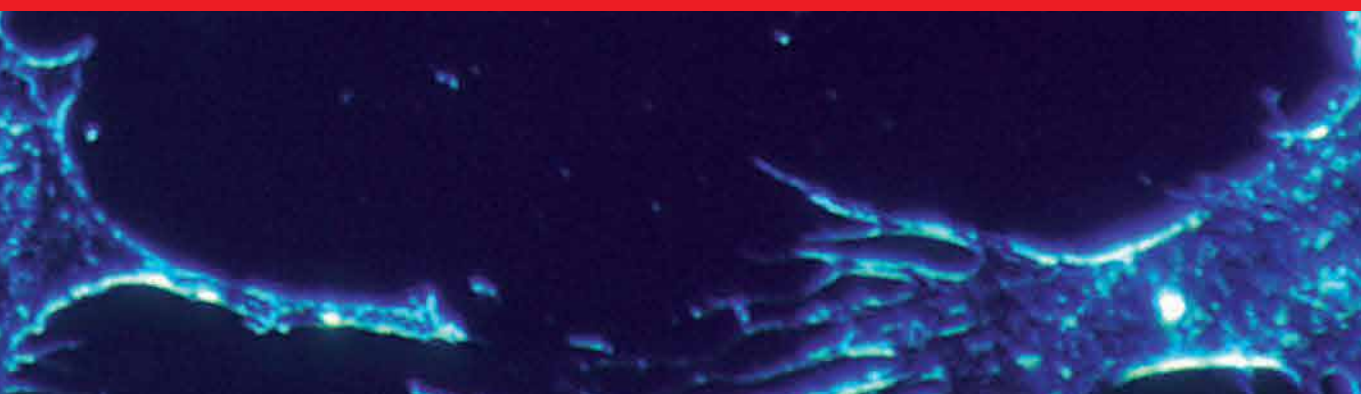

NEOPLASM

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Notice

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Meet the editor

Dr. Hafiz Naveed Shahzad completed his PhD in 2013 from the International Agency for Research on Cancer (IARC), World Health Organization (WHO), France. Currently, he is working as an assistant professor at the School of Biological Sciences, University of the Punjab, Lahore, Pakistan. He is conducting research on multiple aspects of cancer and oncogenic viruses. His research expertise is in microbiology as well as cell and molecular biology. He has published more than a dozen research papers in high-impact factor journals and presented his work at various international conferences across the globe. Moreover, he has been serving as an editorial board member of various journals of international repute.

Contents

Preface

Neoplasm represents the abnormal growth of tissue that arises due to rapid and unregulated proliferation of cells, which lacks coordination with the surrounding tissues. This uncontrolled growth eventually turns out to be a mass or a tumor if it persists in the body. The neoplasm can be noncancerous (benign), precancerous (premalignant), or cancerous (malignant) depending on the severity of disease. Another way of classifying tumors is according to the tissues and cells involved. The development of cancer is a multistep process where normal cells acquire enhanced capacity of proliferation, survival, invasion, and metastasis as a consequence of progressive series of genetic modifications, which accumulate over a longer period of time. The clonal expansion of such transformed cells results in the progression of cancer. In the past several years, tremendous advances have been made in the knowledge of development, diagnosis, and treatment of cancer. This book encompasses the significant advances in the understanding of basic biology, diagnosis, and treatment of some important cancer types. Overall, the book is divided into three sections. The first section summarizes the clinical and pathological features, imaging characteristics, therapeutic approaches, as well as genomic and molecular aberrations associated with colorectal, central nervous system, and uterine neoplasms. The second section includes a comprehensive discussion on cancer metastasis as well as some other novel cancer-related mechanisms, for instance, glucose metabolism and nonalcoholic lipid accumulation. The third section describes various approaches of generalized and targeted cancer therapies. All chapters of this book are clearly written with a considerable detail relating to each of the issues addressed by the authors. Each of the chapters provides a rationale for the topic being covered and some general details about tumors where applicable. The citations used in the chapters are comprehensively referred to, which adds depth to the information being presented. Undoubtedly, this book would be an excellent resource for anyone who wants to learn the basics about multiple aspects of tumor biology and management practices. On the whole, it may prove to be a valuable knowledge resource for target readers.

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Section 1

Various Types of Cancer

Chapter 1

Neoplasms of Central Nervous System: A Diagnostic Approach

Frank Yuan Shan, Dingrong Zhong, Wanming Hu, Nitesh Patel, Ekokobe Fonkem, Dongxia Feng, Yilu Zhang, Jason H. Huang and Arundhati Rao

Additional information is available at the end of the chapter

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Abstract

Neoplasms of central nervous system accounts for approximately 1% of tumors of the human body, and they can be primary or secondary (metastatic), benign or malignant, and intra-axial or extra-axial. This chapter includes some most common brain and spinal cord tumors, like pituitary adenomas, meningiomas and gliomas, with their clinical, imaging, and histological characteristics for the diagnosis purpose, with additional treatment options and prognosis.

Keywords: brain tumors, central nervous system (CNS), World Health Organization (WHO), WHO grades, pituitary adenomas, meningiomas, gliomas, diffuse astrocytoma (WHO grade II), anaplastic astrocytoma (WHO grade III), glioblastoma (WHO grade IV), oligodendroglioma (WHO grade II), anaplastic oligodendroglioma (WHO grade III), magnetic resonance imaging (MRI), computerized tomography (CT), immunohistochemical stain (IHC), isocitrate dehydrogenase-1 (IDH-1), fluorescent in situ hybridization (FISH), chromosome 1p/19q co-deletion, MGMT, molecular diagnosis, neuroimaging study, dural tail

1. Introduction

Human central nervous system (CNS) is composed of brain and spinal cord and their covers. The neoplasms of human CNS account for about 1% of all human body tumors. Location is important information for making diagnosis of brain tumors. Generally speaking, the tumor of the CNS are divided into two large categories: intro-axial and extra-axial tumors; intro-axial

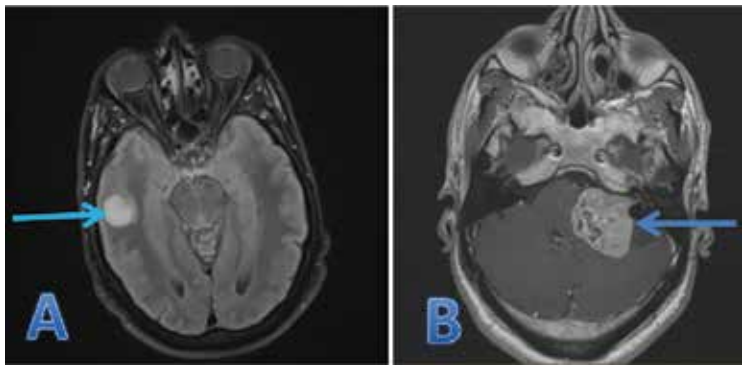

Figure 1. A well-defined right temporal intra-axial hyperintense lesion on FLAIR, see arrow (a); a heterogeneous extra-axial mass at left cerebellopontine angle (CPA) with internal auditory meatus extension on post-contrast T1WI, see arrow (B), is classic for Schwannoma/acoustic neuroma.

tumors mean those tumors that arise from the brain/spinal cord parenchyma, such as astrocytomas and glioblastomas while extra-axial tumors indicate those tumors that originate from the covers or nerve roots of brain/spinal cord, such as meningiomas and schwannomas (**Figure 1**). We include in this chapter only a few most common brain tumors [1].

Tumors of CNS include benign and malignant tumors, based on the World Health Organization (WHO) classification, brain tumors have four grades, from grade I to IV. Grade I tumor is true benign one, like pilocytic astrocytomas in children, with partial or complete surgical resection, the tumor will be cured. Grade II tumors are low-grade malignant potential with both recurrent tendency and progression to higher-grade tumor even after being completely surgically removed. Grade III is high-grade malignant tumor, it not only can be progressing to higher-grade tumor but also has greater recurrent potential, while the grade IV tumor is very high-grade malignant tumor, such as glioblastoma (GBM), even with current combined surgical, radiation and chemotherapies, patients' expected survival time is only approximately 22–24 months [1].

2. Pituitary adenomas

The anterior pituitary gland (adenohypophysis) is an important organ for human development and physiological functions, which comprises several different cell types, responsible for the synthesis and secretion of a specific hormone or group of specific hormone (plurihormonal), such as growth hormone (GH), adrenocorticotropic hormone (ACTH), and prolactin (PRL). Each of these cell types may give rise to a discrete pituitary adenoma subtype that is either hormonal active (functional) or inactive (nonfunctional).

As one of the most common pituitary neuroendocrine tumors, pituitary adenomas constitute the overwhelming majority of tumors arising in the pituitary gland and accounts for 10–15% of intracranial neoplasms. Incidental microadenoma (smaller than 10 mm in diameter) may occur in up to 27% of pituitary glands examined at autopsy and up to one-fifth of the population has

a pituitary abnormality on magnetic resonance imaging (MRI) [10]. The monoclonal nature of the majority of pituitary adenoma subtypes was supported by data from LOH (loss of heterozygosity) analysis and by X-chromosomal inactivation analysis in female patients. Which strongly suggests the view that pituitary adenomas result from a clonal expansion of a single mutated pituitary cell [10] (**Figure 3**).

2.1. Clinical features

The clinical manifestations of pituitary adenomas can be divided into two categories: The first is the endocrine functional changes caused by pituitary adenoma; the second is the local space-occupational effect caused by tumors. They can also invade downwards into the paranasal sinus, laterally into the cavernous sinus (to compress cranial nerves leading to ophthalmoplegias) and upwards into the brain parenchyma (**Figure 2**). Endocrine changes include amenorrhea, lactation, obesity, acromegaly and gigantism, and hypopituitarism, and so on [10]. By testing six types of hormones secreted by the pituitary gland, functional pituitary adenoma can be detected. For example, among adult pituitary adenomas, PRL producing adenomas are the most common hormone-secreting tumors, in which the elevated serum prolactin (prolactinemia) level may be helpful for diagnosis. While nonfunctional pituitary adenomas are often found late and are often found when secondary symptoms of compressing adjacent surrounding tissue becomes apparent, which includes visual field defects, especially the temporal visual field defects, blindness by compressing optic chiasm, and headache, and so on. While the majority of pituitary tumors are nonmetastasizing and slowly growing adenomas, some cases (the proportion is low, but the exact percentage is unknown) will become invasive and a very small portion (approximately 0.1%) will become malignant [9]. Add more growth pattern in different directions.

2.2. Radiologic finding

Pituitary adenomas are characterized by CT low-density occupying lesions on pituitary or MRI (T1WI) low-signal occupying images (**Figure 1**). Contrast enhanced CT and MRI can

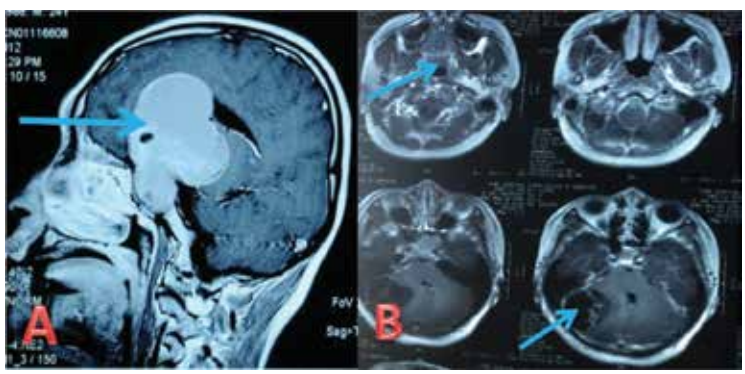

Figure 2. MRI sagittal T1 W1 shows this enhanced mass of giant pituitary adenoma, see arrow (a). MRI shows occupied masses in both sellar region and cerebellum, see arrows, based on the patients' history, the lesions most likely a pituitary carcinoma with metastasis to cerebellum (B).

significantly improve the display and diagnostic rates of pituitary adenomas, especially microadenoma. The pituitary microadenoma with diameter less than 10 mm has little effect on the surrounding bone tissue around the sellar region; however, pituitary adenomas with diameter larger than 10 mm (often referred to as macroadenomas) often result in enlargement of the sellar region and even destruction of the surrounding sphenoid bone (**Figure 2**). Pituitary adenomas with destructive behavior are often diagnosed as invasive pituitary adenomas by radiology experts [10].

2.3. Microscopic finding and diagnosis

The majority of pituitary adenomas are composed of monotonous anterior pituitary gland cells in similar size and shape with abundant small vessels and variant amount of cytoplasm (**Figure 3**). Compared with the normal histology of the pituitary gland, the reticular fibers in pituitary adenomas are significantly reduced, so the reticulin stain can also be helpful to differentiate pituitary adenoma from pituitary hyperplasia, the latter shows only expansion rather than rupture of pituitary acini demonstrated by reticulin stain. The monoclonal nature of the majority of pituitary adenoma subtypes was supported by finding that the tumor cells have the similar morphology (**Figure 3**). The pituitary adenoma subtypes secreting different hormone subtypes are slightly different in morphology and also differ in clinical biological behavior.

2.4. Immunohistochemical findings

A minimal panel of immunostains is helpful for the diagnosis of pituitary adenomas, which includes synaptophysin and low-molecular weight cytokeratin CAM 5.2. Pituitary adenomas, as most neuroendocrine tumors, are uniformly immunoreactive for synaptophysin (**Figure 4**) and variably for chromogranin A (another neuroendocrine marker) and CAM 5.2. In addition, synaptophysin immunohistochemically positive in a finely fibrillary pattern even highlights small fragments of posterior pituitary gland that can be also sometimes included in the specimen, especially in specimens being assessed for Rathke's cleft cyst.

In terms of specific anterior pituitary hormones necessary for subtyping pituitary adenomas, FSH, LH, TSH, ACTH, GH, and PRL (**Figure 4**) as a small group is highly recommended [7, 8].

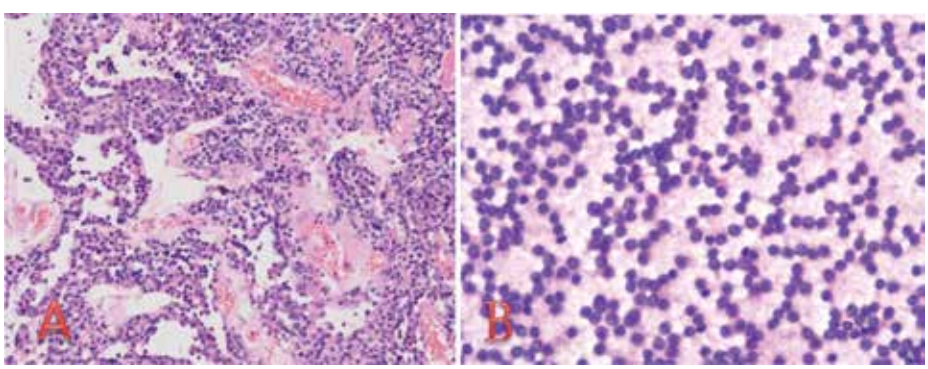

Figure 3. Monotonous tumor cells in size and shape with abundant small vessels in pituitary adenoma on H&E section (a) (H&E stain ×100), and smear (B) (H&E stain ×200).

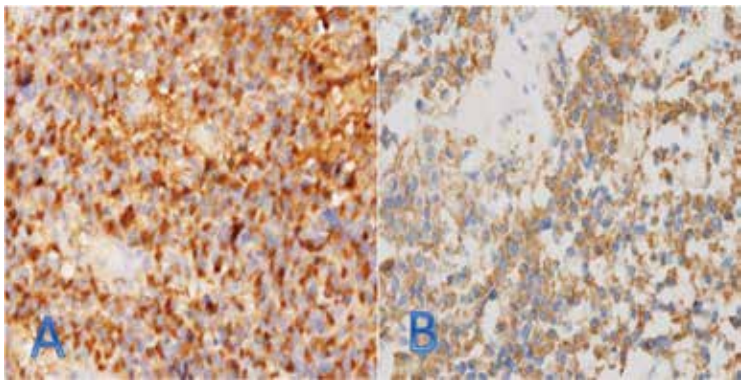

Figure 4. Tumor cells of this pituitary adenoma are immunoreactive for PRL (a), and synaptophysin (B) (immunohistochemical stains ×100 (brown color indicates positive stain)).

In recent 2017 WHO classification, a major technique for tumor classification is the immunohistochemical stain, with the combination of immunostains for the main pituitary hormones (GH, PRL, ACTH, β-TSH, β-LH, β-FSH, and α-subunit of glycoproteins) in order to determine the tumor cell's linage. In some conditions, if the immunohistochemical staining result is inconclusive, immunostains for the pituitary transcription factors (PIT-1, SF-1, T-PIT) may be necessary. In addition, immunohistochemical stains can be used for the subclassification of adenomas variants. For example, low-molecular-weight cytokeratin is very helpful in identifying fibrous bodies in sparsely granulated somatotroph and in acidophilic stem cell adenomas; cytokeratin also highlights corticotroph cell differentiation and Crooke's hyaline changes. With the combination of morphology and immunohistochemical markers, there is minimal need for ultrastructural analysis (electron microscopic) for the classification of the adenomas [9].

According to the 2017 WHO classification of tumors of the pituitary gland, **Table 1** summarizes how these pituitary neuroendocrine tumors are separated according to their clinical behavior. A large proportion of tumors are the adenomas (typical) that show low probability for recurrence. At the other extreme are the pituitary carcinomas, malignant tumors that by definition already have metastasis when diagnosed. Several tumors have higher probability

Low probability for recurrence	High probability for recurrence	Malignant (metastatic) tumor
Pituitary adenoma	Adenomas with elevated proliferative activity	Pituitary carcinoma
	Special subtypes (variants) of adenomas:	
	Sparsely granulated somatotroph adenoma	
	Lactotroph adenoma in men	
	Silent corticotroph adenoma	
	Crooke cell adenoma	
	Plurihormonal PIT-1 positive adenoma	

Table 1. Likelihood of recurrence of pituitary neuroendocrine tumors.

for recurrence than the typical adenomas including adenomas with elevated proliferative activity and the special variants of adenomas previously mentioned [9].

In some pituitary adenomas, prominent cellular atypia can be observed but which is not indicative of malignancy. To date, there is no definitive morphological index and marker for malignant pituitary adenoma, which is consistent with endocrine tumors in other organs. Pituitary carcinoma (**Figure 5**) can be diagnosed only after a definitive metastasis of pituitary adenoma is identified [9]. Atypical pituitary adenomas with biological behavior between pituitary adenomas and pituitary carcinomas were abolished in the recent 2017 WHO classification of pituitary adenomas [9], the reason is that the exact diagnosis of atypical pituitary adenoma is difficult to be unified and quantified (the cutoff of Ki-67 varies in different studies). According to previous studies, the positive rate of Ki-67 and the mutation of P53 tumor suppressor gene are closely related to the invasiveness of pituitary adenomas. Ki-67 and P53 (**Figure 6**) may be an important marker for evaluating the risk of recurrence and invasiveness of pituitary adenomas. Many indicators have been included in the study of pituitary adenomas, such as GSP (G proteins super-family), ras, and PTTG (Pituitary Tumor Transforming Gene), CCND1 (cyclin D1 gene), MEN1, Rb (Retinoblastoma susceptibility gene), p16/CDKN2A (the cyclin-dependent kinase inhibitor p16), *p27/Kip1,* and so on, but now it seems to be of limited clinical significance. Attempts at identifying potential aggressive adenomas should be made on an individual basis by considering the histology, mitotic index, Ki-67 labeling index, and tumor invasiveness [9]. Furthermore, recognition of adenoma variants that have intrinsic substantial risk for recurrence and poor clinical behavior is imperative [9]. Therefore, more research in order to find a reliable and accurate biomarker reflecting biological behavior of pituitary adenomas will be our future goal.

2.5. Treatment for pituitary adenomas

Nonfunctional pituitary macroadenomas are typically managed with transphenoidal surgical resection followed by radiation. With the exception of prolactinomas, functional pituitary macroadenomas require surgical resection via a transphenoidal approach with subsequent radiotherapy, and some patients may also benefit from medical therapy in the postoperative period depending on the specific type of adenoma. After surgical resection and radiation therapy of growth hormone secreting pituitary adenomas, octreotide is often administered to suppress

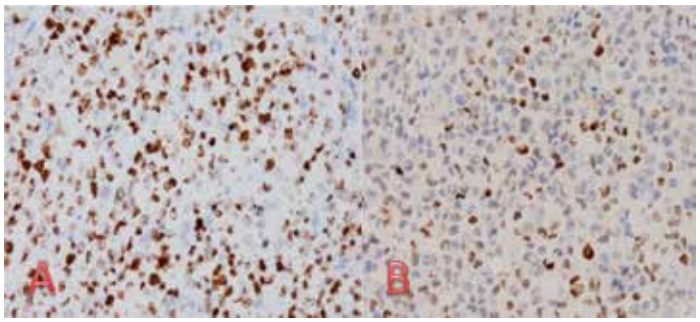

Figure 5. This picture shows the tumor from cerebellar mass of **Figure 1B** as a metastatic pituitary carcinoma, the tumor cells show larger nuclei with prominent nucleoli (arrow), indicating its malignancy (H&E stain ×400).

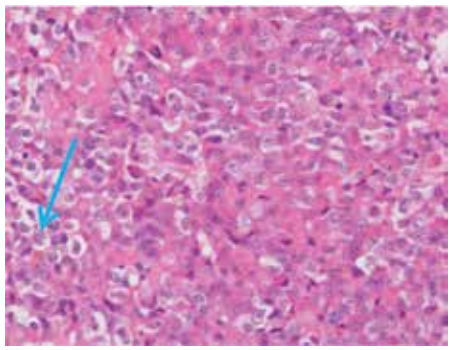

Figure 6. High expression of Ki-67 proliferation index in tumor cells (a) and approximately 30% of tumor cells express mutant P53 protein by nuclear stain (B), both suggesting an aggressive behavior of this tumor (immunohistochemical stains ×200).

growth hormone secretion given that lower postoperative growth hormone levels correlate with a greater probability of remission after radiotherapy. Corticotropin secreting pituitary adenomas undergo surgery and radiotherapy, but medical therapy is only utilized in patients who fail or decline therapy; central or peripherally acting agents are available for use. Gonadotropin secreting macroadenomas are also treated with surgical resection and subsequent radiation, but medical therapy such as bromocriptine or octreotide are only administered to patients who deny surgery or radiation. Thyrotropin secreting macroadenomas respond well to octreotide after surgery and radiation and thus can be a useful adjuvant therapy [13–16].

3. Astrocytic and oligodendroglial neoplasms

Taken together, these neoplasms are also referred to as "gliomas", which are the most common intro-axial tumors of the central nerve system (CNS). The current classification of these tumors is primarily based on the World Health Organization (WHO) classification which is continually updated and internationally accepted [1].

Based on the WHO classification, the gliomas are divided into astrocytomas, oligodendrogliomas, and mixed oligoastrocytomas, and includes the criteria for grading of each tumor from grade II to grade IV, the highest-grade IV tumor as glioblastomas (GBM). The low-grade tumor, although only grade II, due to the diffusely infiltrating nature of the tumor, and their strong tendency toward progression and upgrading, with destruction of the normal brain architecture, the prognosis of those tumors are poor and post a great challenge to the diagnostic pathologists and oncologists.

4. Diffuse astrocytomas (WHO grade II)

4.1. Definition

The WHO grade II diffuse astrocytoma is a well-differentiated, diffusely infiltrative neoplasm [1, 2].

4.2. Clinical features

Diffuse astrocytomas most frequently affect the cerebral hemisphere of young to mid-age adult patients and the brainstem and thalamus of children. Occasionally, the tumors also occur in the cerebellum or spinal cord. Those better circumscribed (non-infiltrating) astrocytomas should be distinguished from diffuse astrocytomas, since the latter carries much worse prognosis [17–24].

Diffuse astrocytomas may produce nonspecific symptoms of mass effect, seizures, and neurologic deficits according to the lesions' size and location, and rate of growth. Seizures are more common than functional deficits due to cerebral parenchymal destruction, which occur more often in highest-grade tumors. Brainstem astrocytomas produce neurologic signs caused by dysfunction of cranial nerve nuclei and compression of the sensorimotor tracts that traverse the pons and medulla [2].

The infiltrating and diffuse forms of astrocytoma occur throughout the CNS, primarily affecting adult patients and mostly involving the cerebral hemispheres. Hypercellularity with nuclear atypia are key points for diagnosis, although mitosis is rarely identified, and necrosis and vascular proliferation should not be observed in this tumor. The tumor can progress to higher grade, like anaplastic astrocytoma (WHO grade III), and even glioblastomas (WHO grade IV). The infiltrating nature of the tumor makes completely surgical resection without damaging the functional cerebral area almost impossible and its resistance to conventional therapy making this type of tumor a medical challenge.

4.3. Radiologic finding

By magnetic resonance imaging (MRI), diffuse astrocytomas (grade II) are present as ill-defined areas of low signal intensity on T1-weighted images. Due to their content of bright edema fluid, they are even more obvious on T2-weighted or fluid-attenuated inversion recovery (FLAIR) images (**Figure 1A**). Contract enhancing should not be observed in grade II diffuse astrocytomas [2].

4.4. Microscopic finding

Hypercellularity and nuclear atypia are the important diagnostic features for grade II diffuse astrocytomas. Those lesions are primarily located in white matter. The tumor cells widely infiltrate the gray matter. The margin of such tumors is indistinct since tumor cells lie individually dispersed within intact parenchyma and often follow fiber pathways for some distance. Such tumor spread is often more apparent in anaplastic astrocytoma (grade III) or glioblastoma (grade IV). Astrocytomas often infiltrate the overlying cerebral cortex or deep hemispheric structures. The so-called "secondary structures of Scherer" indicate the proliferation of tumor cells in subpial, perivascular, perineuronal, and subependymal zones. Such characteristic changes are usually more prominent in oligodendrogliomas, astrocytomas undergoing anaplastic transformation, or in gliomatosis cerebri [1, 2].

In addition to hypercellularity, almost all astrocytomas possess cells with enlarged, cigar-shaped, irregular and hyperchromatic nuclei. Prominent nucleoli are uncommon in grade II

diffuse astrocytomas. As well, mitosis, especially multiple mitoses, should not be observed in most classic grade II diffuse astrocytomas [2].

The cytoplasm of astrocytoma cells varies considerably both in amount and configuration. In some cases, the tumor cells' fibril-free cytoplasm is scant and devoid of processes, making it a feature of "naked nuclei", in some other cases, the tumor cells have varying quantities of fibril-containing cytoplasm and short, often asymmetric processes, which is the basis of the overtly astrocytic nature of this tumor [2]. Gemistocytes (fat astrocytes) occur in varying numbers, and only a few astrocytomas have enough glassy cytoplasm to justify the term gemistocytic astrocytoma. Most tumor cells in gemistocytic astrocytoma show a plump, glassy cell body and an eccentric corona of short, stout-to-delicate processes. Such cells, when scattered and well differentiated, can mimic reactive astrocytes. For reactive changes, the main features are their uniform distribution, minimal increase in number, and symmetric stellate configuration of long-radiating processes. Such cells are best seen in smear preparations or on immunostains for glial fibrillary acidic protein (GFAP).

It is important to know that most gemistocytic tumors fall into the category of anaplastic astrocytoma, or into the highest grade, glioblastoma, when microvascular proliferation and/or necrosis are present (see below).

Most low-grade tumor and reactive changes may contain microcysts and microcalcification, suggesting a long-term process, which are helpful features for making our diagnosis.

4.5. Immunohistochemical findings

Almost all diffuse astrocytomas are both S-100 protein and vimentin protein reactive; the tumor cells cytoplasm often is immunoreactive for GFAP. In gemistocytic tumors, GFAP reactivity is usually more prominent at the periphery of the cells. Staining for p53 protein is variable in diffuse astrocytomas; nuclei are diffusely immunoreactive in approximately one-third of tumors [1, 2].

MIB-1 (Ki67) labeling indices of grade II astrocytoma have generally been less than 2%, often even 1% or less, but there is considerable case-to-case and region-to-region variation.

Immunostains for mutated p53 protein, together with mutated IDH-1 (isocitrate dehydrogenases-1, R132) are used by some people as confirmative markers for the diagnosis of glioma, since both genes and their proteins are involved in the tumorigenesis of gliomas by current understanding (**Figures 7** and **8**).

4.6. Differential diagnosis

The most important differential diagnosis of glioma is reactive gliosis or reactive changes, which may occur in many conditions, such as infarct, infection, and demyelinative disease. Those lesions sometimes can mimic gliomas on imaging study and mistake pathologic diagnosis may result in unnecessary treatment for the patients and even more severe damage to patients may occur. So the diagnosis of glioma should be made cautiously with more supporting evidence ready before making the final diagnosis [2].

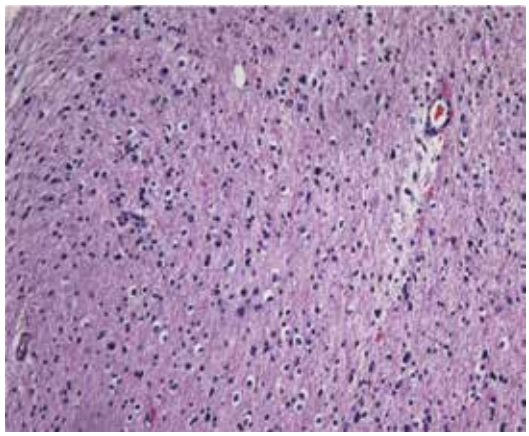

Figure 7. This histologic picture corresponds to the tumor in **Figure 1A**, with increased cellularity and atypia, but no significant mitosis, most likely a low-grade, WHO grade II infiltrating glioma (H&E stain ×400).

4.7. Treatment of gliomas

4.7.1. Low-grade gliomas

Low-grade gliomas (WHO grade I and II) are typically managed with a combination of surgery, radiation therapy, and chemotherapy; however, the precise treatment is often individualized depending on the patient at hand.

The management of WHO grade II tumors, such as diffuse astrocytoma and oligodendroglioma, is more variable. In patients presenting with neurologic impairment, immediate surgical resection is utilized to relieve symptoms and obtain a definitive diagnosis. Asymptomatic patients can elect for surgical resection or choose observation. In patients who opt for careful observation, surgical intervention is indicated if tumor growth accelerates, neurologic impairment develops, or if evidence of transformation to a high-grade glioma is detected. Given that surgery is often not curative, further postoperative therapy with radiation therapy and/or

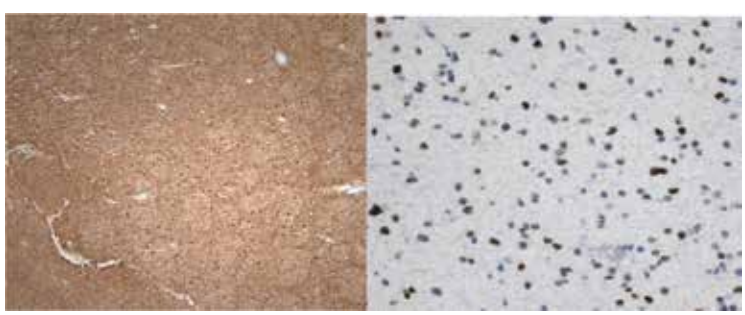

Figure 8. The tumor cells are immunoreactive for GFAP (glial fibrillary acidic protein) (immunohistochemical stain ×100) and p53 (immunohistochemical stain ×400), consistent with WHO grade II diffuse astrocytoma. This tumor is with IDH-1 mutation (no show).

chemotherapy is ultimately needed but the timing will depend on the patient's prognosis. If complete resection is achieved and the molecular characteristics are favorable, postoperative therapy is delayed until recurrence is detected during observation. Conversely, immediate postsurgical chemoradiation is recommended for patients with poor prognostic factors, such as neurological deficits, large tumor size, mass effect, incomplete resection, or advanced age. Conventional radiation therapy at a dose of 50–54 Gy is typically chosen for low-grade gliomas, whereas the chemotherapy regimen is individualized to the patient. PCV (procarbazine, lomustine, and vincristine) and temozolomide are the main regimens, and although no trials have compared these directly, temozolomide is being used more frequently due to improved patient tolerance and ease of administration; despite this, only PCV has proven survival benefit in a randomized control trial [11].

5. Anaplastic astrocytoma (WHO grade III)

5.1. Definition

Anaplastic astrocytoma is a WHO grade III infiltrating astrocytoma, intermediate between grade II diffuse astrocytoma and grade IV glioblastoma [1, 2].

5.2. Clinical features

Generally speaking, anaplastic astrocytomas usually occur one decade later than grade II tumors and a decade earlier than glioblastomas. The tumors in cerebral hemisphere occur most often in the fifth decade, they also appear occasionally in children [1, 2].

5.3. Radiology findings

Some lesions partially enhance on T1-weighted MRI image following administration of contrast agents, but not with the perinecrotic ring (ring enhancing) or "rim" pattern that usually typifies glioblastoma [2]. Still, a significant portion of anaplastic astrocytomas show no contrast enhancing, which makes the pathological diagnosis more informative and important [2].

5.4. Microscopic findings

Anaplastic astrocytoma has more hypercellularity, nuclear pleomorphism, and hyperchromasia; in addition, the presence of mitosis is an important diagnostic feature for grade anaplastic astrocytomas, but lack the necrosis and vascular proliferation, which are the characteristic findings in glioblastomas.

5.5. Immunohistochemical findings

The staining pattern of anaplastic astrocytomas should be the same as grade II diffuse astrocytomas, whose tumor cells are immunoreactive for GFAP and S-100 protein. Not surprisingly, reported ranges of proliferation index of Ki-67 are quite variable, in the range of 5–10% [2].

5.6. Differential diagnosis

The same as for the grade II diffuse astrocytomas, the major differential diagnosis for anaplastic astrocytomas are those of reactive type lesions. The misdiagnosis of demyelinative lesion as a grade III anaplastic astrocytoma may result in unnecessary and fetal radiation therapy to patient, which usually causes a medicolegal issue. In that case, the diagnosis should be made on strong evidence-based practice.

6. Gliobalstoma multiform (GBM) (WHO grade IV)

6.1. Definition and general features

Glioblastoma is a highly malignant astrocytic glioma with WHO grade IV that appears to arise either de novo (primary GBM) or in transition from lower grade gliomas (diffuse astrocytoma or anaplastic astrocytoma, secondary GBM). It typically affects adults and is preferentially located in the cerebral hemispheres. The term of "high grade glioma" is sometimes used to describe both anaplastic astrocytoma and glioblastoma. GBM is the most frequent brain tumor accounting for approximately 12–15% of all intracranial neoplasms and 60–75% of astrocytic tumors. In most European and North American countries, the incidence of GBM is in the range of 3–4 new cases per 100,000 populations per year. GBM may manifest at any age, but preferentially affects adults, with a peak incidence at between 45 to 75 years of age. The male female ratio of GBM patients is 1.26 in USA and 1.28 in Switzerland [1, 2].

6.2. Clinical features

Affecting primarily the cerebral hemispheres of adults and the thalamus and brainstem of children, GBM is the most common malignant glioma. It is less likely to occur in the optic nerve and cerebellum and uncommon in the spinal cord. Most cases are solitary cerebral hemisphere mass lesion, but occasional examples appear radiologically separate and warrant the designation multicentric GBM. True multicentricity is difficult to establish, however, even at autopsy [1, 2].

The clinical presentation of patients with GBM are similar to those of patients with better differentiated diffuse astrocytomas, although accompanying neurologic deficits are more frequent, more abrupt in onset, and more rapid in evolution. Unlike lower grade lesions, GBMs are often expansile and edema generating. As a result, they are more likely to produce frank neurologic deficits and signs of increased intracranial pressure. Acute hemorrhage precipitates symptoms in occasional cases. Rapid growth within months or even weeks can be observed in MRI studies [2].

6.3. Radiologic findings

On MRI, GBM usually have an enhancing ring or rim in post contrast T1-weighted images and a generally broad zone of surrounding edema evident in T2-weighted or FLAIR images. The central, hypodense core of the lesion represents tumor necrosis, while the contrast enhancing ring is highly cellular neoplasm with abnormal vessels that permeable to contrast

agents. The peripheral zone of low attenuation indicates vasogenic edema containing varying numbers of isolated infiltrative tumor cells [2].

6.4. Microscopic findings

GBMs are heterogeneous tumors with multiple histological variations, and characterized by nuclear pleomorphism (different nuclear size and shape), active mitoses, necrosis, especially palisading necrosis and/or vascular proliferation. Most times, GBM have an astrocytic quality with small amount of pink cytoplasm, while others may have oligodendroglioma-like structures with prominent perinuclear halos. Small cell GBM is with monotonous small nuclei with or without halos resembling anaplastic oligodendroglioma, while at the other end is the giant cell GBM, which composed of numerous multinucleated monstrous giant tumor cells with large lipid-rich cytoplasm. The infiltrating tumor cells usually have slightly elongated nuclei [1, 2].

Vascular proliferation is characteristic for GBMs, and takes two forms; the most common is forms of globular masses resembling the glomerular tufts of the kidney. Another form of vascular hyperplasia has endothelial proliferation since it is intraluminal and consists largely of endothelial cells within small to medium-sized vessels. This latter type of endothelial proliferation is less common than glomeruloid proliferation and appears to have a more constant correlation with high-grade gliomas and poor prognosis [1, 2].

Necrosis, the second cardinal feature of GBM, takes the form of either large confluent areas of parenchymal destruction, including of vasculature, or small, often multiple serpiginous foci. On MRI scan, it is the large confluent zone of necrosis that comprises the tumor's hypotense center. Necrotic areas, particularly small foci, often feature peripheral accumulation of somewhat radially oriented cells. Such "pseudopalisading" occurs almost exclusively in high-grade astrocytomas [1, 2]. Sometimes, that necrosis is associated with small vessel thrombosis.

6.5. Immunohistochemical findings

The immunophenotype of GBM includes reactive for GFAP, and S-100 and vimentin. Reactive for vimentin is nonspecific and with no diagnostic significance. In addition, immunoreactive for IDH-1 and p53 is suggestive of a slightly better prognosis [1–3].

6.6. Molecular and cytogenetic findings

Currently, the primary GBM is considered associated with EGFR vIII (Epidermal Growth Factor Receptor variant III) amplification, which carries a poor prognosis, while the secondary GBM is associated with mutations of IDH-1 and p53 genes, which suggests a slightly better prognosis due to more sensitive to modern chemotherapy [3].

6.7. Treatment for high-grade gliomas

High-grade gliomas are also initially managed with surgical resection with the goal of gross total resection. Adjuvant chemoradiation is always required, and temozolomide (TMZ) with concomitant radiotherapy is the current regimen of choice. Patients with high-grade gliomas are also encouraged to participate in the clinical trials.

WHO grade III gliomas, such as anaplastic astrocytoma, will require maximal surgical resection if feasible followed by adjuvant therapy depending on the tumor type. If the tumor is not amenable to resection, open or stereotactic biopsy is indicated to guide subsequent adjuvant therapy. Options for adjuvant treatments include temozolomide, PCV (procarbazine, lomustine and vincristine), and fractionated external beam radiation therapy of 60 Gy. The specific combination of treatment will vary depending on the prognostic factors that were previously discussed for grade II gliomas.

Glioblastoma (WHO grade IV) is initially treated with maximal safe resection for all patients. For well-performing patients ≤70 years old, postoperative fractionated external beam radiation of 60 Gy is indicated, whereas hypofractionated radiation therapy at a lower dose should be considered for patients >70 years old; concomitant temozolomide should be employed in both age groups. In poorly performing patients, monotherapy with adjuvant temozolomide is typically utilized instead of subjecting the patient to radiation therapy, but hypofractionated radiation therapy at a lower dose schedule is a potential option. For any patient on temozolomide, combination treatment with an antiangiogenic agent such as bevacizumab is an option but has not been shown to improve overall survival. Another study demonstrated significant efficacy with combination of temozolomide and TTFields [12].

7. Oligodendroglioma and oligoastrocytomas (WHO grade II)

7.1. Definition

Oligodendroglioma is a WHO grade II infiltrating glioma composed at least in part, of cells resembling oligodendrocytes [1, 2].

The patients with oligodendrogliomas have a significant better prognosis than those with infiltrating astrocytomas of comparable grade. The diagnosis of mixed glioma or oligoastrocytoma is largely due to a favorable outcome that will be attributed to the presence of an oligodendroglioma component whereas overgrowth of the astrocytic component will be used to explain cases that recur rapidly as high-grade astrocytoma [1, 2].

7.2. Clinical features

Olidendrogliomas arise throughout the neuroaxis, but primarily affect adults' cerebral hemispheres. Manifestations of oligodendroglioma include any of the usual consequence of an infiltrating, expanding intracranial neoplasm. Seizures are common at presentation; large lesions may produce signs and symptoms of increased intracranial pressure [1, 2].

7.3. Radiologic finding

A large intracranial lesion on MRI, especially when long segments of the cortical ribbon, are affected. Oligodendroglioma is especially likely when a band of cortical mineralization undulates in a "gyriform" profile. Calcification is not detected in many small oligodendrogliomas, however, grade III lesions are large, and while variably enhancing, generally lack the ring profile so typical of glioblastoma [2].

7.4. Microscopic finding

The tumor cells of olidendroglioma like to infiltrate the cerebral cortex, although not uniformly distributed, the tumor cells are attractive to neurons (perineuronal satellitosis) and to subpia and perivascular regions.

Oligodendroglioma is characteristic for its histology and cytologic monotony, which means the tumor is likely a uniform blueness to the section, and it is confirmed on closer view as sameness of nuclear size and shape. Round nuclei with small but prominent nucleoli, in all grades, the majority of nuclei are round, especially so in grade II lesions, and somewhat less so in intermediate to higher-grade lesions. In contrast to astrocytomas, the chromatin pattern is more open and bland, and there is often a clearly defined, solitary nucleolus. Large hyperchromatic nuclei may also be observed but not the generalized nuclear pleomorphism in many high-grade infiltrating astrocytomas [2].

Perinuclear halos producing a "Fried-egg" artifact (**Figure 9**). Oligodendrocytes, whether normal or neoplastic, are susceptible to the autolytic imbibition of water, resulting in the production of a clear perinuclear halo. Perinuclear halos are not specific findings, since they are present in some other tumors, such as clear cell ependymoma and neurocytoma [2].

Microcysts. Particularly common in the cortex, which are filled with protein-like fluid; again it is not specific for oligodendrogliomas, and can be observed in some other tumors.

Delicate capillaries disposed in a "Chicken-wire" pattern (**Figure 9**). Blood vessels in better differentiated tumors typically consist of short capillary segments arranged geometrically. This arrangement resembles the pattern angulations of "chicken-wire". It is a "soft" finding with little diagnostic use.

Microcalcifications: Many oligodendrogliomas exhibit some degree of calcification within the tumor or in surrounding brain tissue. It is mainly introcortical, and the deposition takes the form of laminated calcospherites. Vascular walls are often affected in heavily mineralized cases.

7.5. Grading

Accounting to the 2007 WHO system, oligodendrogliomas are generally either grade II or III [1].

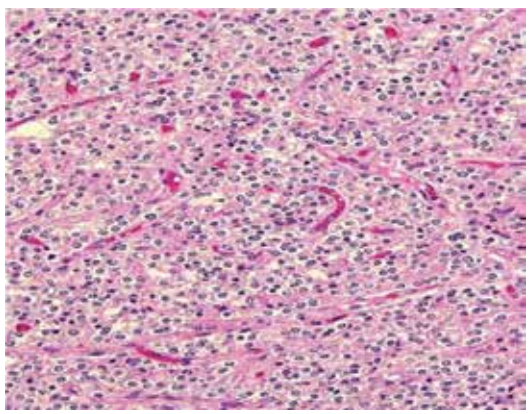

Figure 9. "Fried-eggs and chicken wire" are characteristic histology for oligodendrogliomas (H&E stain ×200).

8. Anaplastic or malignant oligodendrogliomas (WHO grade III)

This cellular tumor is with considerable nuclear hyperchromasia, brisk mitoses, and microvascular proliferation, some with necrosis, but palisading necrosis is usually not seen.

Nuclei of grade III lesions vary with the degree of anaplasia, with those of uniformly round for less anaplasia to those of histologically malignant tumor but still obviously oligodendroglial due to the roundness (more or less) and prominent nucleoli. Truly anaplastic lesions have the nuclear features of high-grade astrocytoma, although they usually retain oligodendroglioma-like round cells, at least in small numbers. Mitosis and microvascular proliferation/hyperplasia are required features in making diagnosis of WHO grade III anaplastic oligodendroglioma [2].

8.1. Immunohistochemical findings

So far, there is no a reliable immunohistochemical marker available that allows the specific and sensitive recognition of human oligodendrogliomas. However, the specific molecular marker is now available for making the diagnosis of oligodendrogliomas (see below).

The proliferation index of MIB-1 (Ki-67) is in the range of 3 to 5% for grade II oligodendroglioma, while the anaplastic oligodendrogliomas have much higher proliferation index by immunostaining for Ki67 [2].

8.2. Molecular marker

The loss of chromosome arms 1p and 19q is an established genetic hallmark of oligodendroglial tumors [3–6]. This combined loss is detected in up to 80% of oligodendrogliomas and up to 60% in anaplastic oligodendrogliomas, with decreasing frequency in mixed oligoastrocytic tumors [6]. The 1p/19q co-deletion has proven its use as both diagnostic and prognostic marker

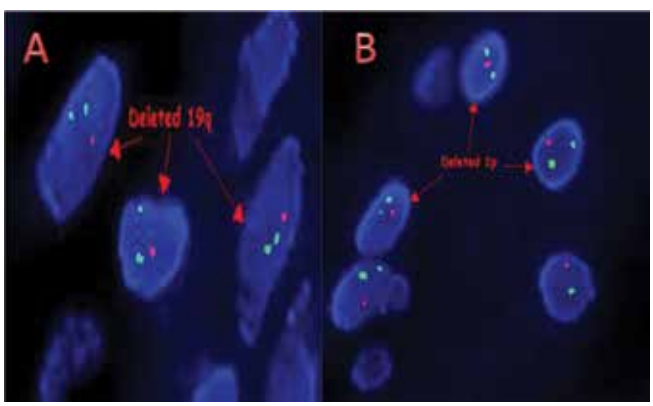

Figure 10. The FISH test shows the 1p (red in a) and 19q (red in B) co-deletion in an oligodendroglioma, the green dots in the pictures as control.

for oligodendrogliomas. The tumor with this genetic mutation has a better response to therapy and a longer survival time, regardless of using chemotherapy, radiotherapy or the combined therapy [4, 5]. Various techniques are available to detect 1p/19q deletion; however, fluorescent in situ hybridization (FISH) is often employed due to its technical ease (**Figure 10**). Another frequently utilized method is loss of heterozygosity (LOH), which is a PCR-based test that compare tumor DNA to the patients "normal" DNA, usually from peripheral blood [5, 6].

9. Meningiomas

9.1. Definition

A group of mostly benign, slow-growing extra-axial neoplasms that most likely derive from the meningothelial cells of the arachnoid layer. People refer to it as "dura-based tumor." They are categorized into three WHO grades (I-III) with more than 15 histologic subtypes [1, 2].

9.2. Clinical features

Meningiomas account for about 36.1% of all primary brain tumors. It is the most frequently reported brain tumor in the USA. They are most likely to be found in adults older than 60; the incidence appears to increase with age. Rarely are meningiomas found in children, but pregnant women carry an increased risk for meningiomas. For brain meningiomas, the incidence between male and female is about the same; but for spinal meningioma, it is female patient dominant, with primarily psammomatous subtype meningioma (**Figure 11**) [1, 2].

Meningiomas usually grow slowly and may reach a large size before interfering with the normal functions of the brain. The resulting symptoms depend on the location of the tumor within the brain. Headache and weakness in an arm or leg are the most common symptoms.

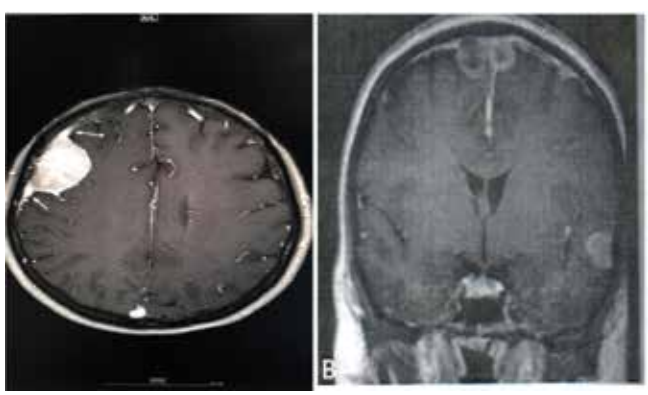

Figure 11. Left temporal intra-axial hyerintense mass with medial uncal herniation compression of the left midbrain on Flair, arrow shows dural tail sign (a). T1WI MRI demonstrates multiple dura-based enhancing mass lesions, most likely NF-2 mutated multiple meningioma (B).

However, seizures, personality changes, and/or visual problems may also occur. Patients often have subtle symptoms for a long period before the meningioma is diagnosed [1, 2].

9.3. Radiologic finding

A contrast enhanced CT (computerized tomography) and/or MRI (magnetic resonance imaging) scan could be used. Typically, it appears as extra-axial masses with a broad enhanced dural base ("dural tail sign"). Some refer to it as "mouse tail sign", which is a radiological diagnostic marker for meningioma (**Figure 12**). They are usually homogeneous and well circumscribed, although many variants are encountered. Peritumoral edema may be seen with grade II or III tumors, secretory or adherent microcystic subtype. While MRIs are in some ways superior, the CT can be helpful in determining if the tumor invades the bone, or if it's becoming hard like bone [2].

9.4. Microscopic finding

Histologically, meningioma cells are relatively uniform, with a tendency to encircle one another, forming meningiothelial whorls and psammoma bodies (laminated calcific concretions). As such, they also have a tendency to calcify and are highly vascularized. There are more than 10 histological subtypes of meningiomas associated with different WHO grading as listed below [2].

- **WHO grade I: 9 subtypes**
  - Meningothelial meningioma
  - Fibrous (fibroblastic) meningioma
  - Transitional (mixed) meningioma
  - Psammomatous meningioma
  - Angiomatous meningioma
  - Microcystic meningioma
  - Secretory meningioma
  - Lymphoplasmacyte-rich meningioma
  - Metaplastic meningioma
- **WHO grade II: 3 subtypes**
  - Chordoid meningioma
  - Clear cell meningioma
  - Atypical meningioma

- **WHO grade III: 3 subtypes**
  - Papillary meningioma
  - Rhabdoid meningioma
  - Anaplastic meningioma

As to molecular pathology, between 40 and 80% of meningiomas contain an abnormal chromosome 22. The cause of this abnormality is not known. Meningiomas also frequently have extra copies of the platelet-derived growth factor (PDGFR) and epidermal growth factor receptors (EGFR), which may contribute to the growth of these tumors. TRAF7 mutations are present in about one-fourth of meningiomas. Mutations in the TRAF7, KLF4, AKT1, and SMO genes are commonly expressed in benign skull-based meningiomas. Mutations in NF2 are commonly expressed in meningiomas located in the cerebral and cerebellar hemispheres. People with neurofibromatosis type 2 (NF2) have a 50% chance of developing one or more meningiomas (**Figure 12B**) [1].

9.5. Immunohistochemical findings

The vast majority of meningiomas are positive for EMA, progesterone receptor and vimentin by immunohistochemical stain. Recently, a new marker somatostatin receptor 2A (SSTR2A) is expressed strongly and diffusely in almost all cases of meningiomas [1]. S100 protein positivity is most common in fibrous meningiomas, but is not usually diffuse, as it is in schwannomas. Other potentially useful immunohistochemical markers in selected cases include Ki-67. Studies have suggested that meningiomas with an index of >4% have an increased risk of recurrence similar to that of atypical meningioma, whereas those with an index of >20% are associated with death rates analogous to those associated with anaplastic meningioma [1, 2].

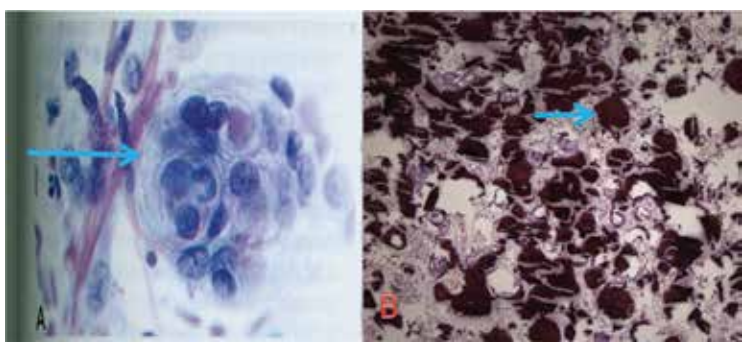

Figure 12. Meningiothelial whorl (see arrow, H&E stain ×400) (a) and psammoma body (brownish blocks, see arrow, H&E stain ×200) (B) are a diagnostic hallmarks for meningioma. (B) Shows a psammomatous type meningioma, it is predominantly in female patient with spinal meningiomas.

9.6. Differential diagnosis

The most important differential diagnosis of meningioma is solitary fibrous tumor/hemangiopericytoma (SFT). It was CD34 and STAT6 immunoreactive, which was not seen in meningiomas. Others were chordoma, schwannoma, superficial glioblastoma/gliosarcoma and metastatic carcinoma, and so on.

Importantly, a so-called collision tumor sometimes occurs between meningioma and metastatic carcinoma, especially metastatic breast cancer is more likely to spread into meningioma, probably due to the presence of progesterone receptor (PR) in meningioma attracts breast cancer cells. It is interesting to know that meningiomas not only occur in the system of CNS but also occur in other organs, such as lung and bone; even skin meningioma has been reported, although the pathogenesis is largely unknown.

9.7. Treatment for meningiomas

The management of meningioma is often guided by tumor size, the presence or absence of symptoms, and the WHO grade; as a result, the specific treatment varies from patient to patient. Management typically revolves around observation versus surgery and/or radiotherapy, and systemic chemotherapy is only considered in cases of malignant tumors, inoperable tumors, or exhaustion of other treatments [25–28].

In asymptomatic patients with WHO grade I meningioma less than 3 cm in size and low potential for neurologic impairment, observation is recommended; however, if symptomatic and amenable to surgery, resection is indicated. Radiotherapy alone is considered for symptomatic patients with meningioma unamenable to surgical resection [25, 26].

Meningiomas greater than 3 cm in size can be observed if asymptomatic, but surgical resection is also indicated. If the patient chooses surgery, radiotherapy is administered if there is subtotal resection of a WHO grade II meningioma or if a grade III meningioma is diagnosed. Symptomatic grade II or III meningiomas greater than 3 cm should undergo surgery followed by radiotherapy, but if inoperable, radiotherapy alone is employed and the patient is counseled on clinical trials for potential systemic therapy [26–28].

10. Conclusion

In summary, the neoplasms of the CNS are uncommon tumors, accounting for approximately 1% of tumors in human body. They can be benign or malignant; primary or secondary (metastatic) by origin; can be extra-axial or intro-axial by locations. Except WHO grade IV glioblastomas, most other CNS tumors can be successfully managed by surgery, chemotherapy and radiation therapies. An accurate diagnosis is vitally important not only for treatment options but also for prognosis. As more and more genetic information about CNS tumors become available, we are optimal to be able to cure those tumors in the near future.

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Chapter 2

Uterine Sarcomas: An Updated Overview. Part 1: Smooth Muscle Tumors

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Additional information is available at the end of the chapter

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Abstract

Uterine sarcomas (USs) account for 3–9% of uterine malignant neoplasia and about 5% of all gynaecologic malignancies. Despite their low prevalence, these tumors stimulate a great interest because of their aggressiveness, poor prognosis and high mortality rate. According to the last World Health Organization (WHO) classification and the International Federation of Gynecology and Obstetrics Committee (FIGO) staging, USs are categorized as pure mesenchymal tumors (endometrial stromal sarcoma, leiomyosarcoma and undifferentiated uterine), and mixed tumors (carcinosarcoma and adenosarcoma). Due to their non-specific signs and symptoms, USs are commonly diagnosed in advanced stage, more often after surgery for a suspected leiomyoma. Although surgery followed by adjuvant therapies represent the common choices for USs, they show poor efficacy due to the early occurrence of metastasis, and the high resistance of tumors to radio-and chemotherapy. Presently, specific expression profiles and new cytotoxic agents are under investigation. In these reviews, we summarized clinical and pathological features, imaging characteristics, therapeutic approaches, genomic and molecular aberration associated with smooth muscle neoplasia (Part 1) and endometrial stromal neoplasia (Part 2); the goal is to understand the biology and the molecular signature of these tumors, in order to focus on their best management.

Keywords: uterine sarcomas, mesenchymal tumors, uterine malignant neoplasia, uterine smooth muscle neoplasia, leiyomiomas, STUMP

1. Introduction: why these chapters?

Cancers of the female reproductive system (cervix uteri, corpus uteri, vulva, vagina, ovaries and fallopian tubes) are important causes of morbidity and mortality worldwide, accounting for almost 15% of all female neoplasia [1].

Uterine cancer is defined as any invasive neoplasm of the uterine corpus and represents the fourth most common malignancies in women, after breast, lung and colorectal cancer [2]. Uterine cancers originating from mesenchymal elements of the uterus are defined as uterine sarcomas (USs). They are very rare, representing about 5% of all gynaecologic malignancies and 3–9% of all uterine cancer [1, 2].

USs usually manifest in postmenopausal women. Obesity, diabetes mellitus, nulliparity and hypertension, considered as certain risk factors for the development of endometrial carcinoma, do not seem to have a crucial role in the genesis of USs [3]. Moreover, most authors reported a three times risk for USs developing in patients undergoing prolonged use of tamoxifen, a synthetic oestrogens (ERs) receptor agonist largely used in the management of women with oestrogens receptor-positive breast cancer [3]. Tamoxifen-related US usually occurs about 5 years following tamoxifen therapy and presents with a higher stage of disease [3].

Because of the rarity of USs, their histopathological heterogeneity and aggressiveness, there is a general lack of consensus regarding risk factors and treatment options [2].

The only certainty is that, if compared with the epithelial counterpart, uterine sarcoma is associated with a poor prognosis, a high rate of local recurrence and distant metastasis [1].

In this chapter, we systematically focus on each single type of USs, starting from epidemiological and etiological factors, going through clinical, morphological and molecular characteristics, differential diagnosis and prognostic features, finally achieving conventional and novel therapeutic approaches.

2. The mesenchyma

Mesenchyma consists of loosely packed and mobile cells embedded in a network of fibers and fluid called intercellular matrix. Mesenchymal cells are spindle-shaped, with oval nucleus and scant cytoplasm.

The loose nature of the mesenchymal cells allows them to easily migrate. Moreover, during embryogenesis and foetal development, their pluripotent nature makes them able to differentiate into a great variety of structures: bones, cartilage, teeth, blood cells, endothelial lining of blood and lymphatic vessels, and smooth muscles.

Mesenchyme derives from mesoderm germ layer and forms early during embryogenesis. Mesenchymal cells also derive from the neural crest, a specialized ectodermal structure. During

gastrulation, mesenchymal cells lose their adhesiveness and separate from the connected sheets of epithelial cells. This process is known as epithelial-mesenchymal transition (EMT). Some important events take place during interactions between mesenchymal and epithelial cells. Epithelial cells are often induced in changing their shape and arrangement in response to signals originating from mesenchymal cells [4].

During EMS, epithelial cells lose their polarity. Cell membranes and desmosomes dissolution, degeneration of cytocheratin filaments, increased cell resistance to apoptosis, and migration of new epithelial cells with mesenchymal phenotype also occur [4]. These changes are induced by the mechanical stimulation of migrating mesenchymal cells or by biochemical mechanisms [4].

EMT has to be considered as a physiological process, playing a role in the development of various embryo tissues, as well as in cells proliferation and tissue repair. EMT is also essential in driving folliculogenesis and ovulation [5]. It has been hypothesized that pathological process such as adenomyosis, endometriosis, malignant neoplasia and metastasis would derive from the dysfunction of EMT within the epithelial cells of the female reproductive system [6, 7].

EMT also participates in other pathological processes, including metastatization [8].

In cancer cell lines, cells gradually change from epithelial to spindle-like shape and acquire fibroblastic morphology [4]. During this process, the expression of epithelial markers decreases and the cells progressively acquire mesenchymal markers [4]. This mesenchymal signature facilitates the detachment of tumor cells, the proteolytic digestion of basement membrane, the vascular invasion, and the migration of circulating cells towards distant sites [8].

Little is still known about the detachment of circulating tumor cells from the cytoskeleton. It seems that EMT produces detyrosinated α-tubulin with the formation of microtubules-based membrane protrusions, which are distinct from the actin-based prolongations known as lamellipodia and filopodia. The new acquired protrusions make circulating tumor cells with mesenchymal phenotype able to attach to endothelial layers and to migrate towards distant sites [9].

Three types of EMT have been described. EMT type 1 plays an important role in the organogenesis; it also generates the primitive mesenchyma during embryogenesis. EMT type 2 is characteristic of pathological, non-neoplastic processes. Through EMT2, fibroblasts are recruited to repair tissue; EMT2 also causes fibrosis as a consequence of chronic inflammations (i.e. renal and hepatic fibrosis, Crohn's disease). It has been demonstrated that about one-third of the fibroblasts causing chronic glomerulonephritis, diabetic nephropathy, lupus nephritis and Alport syndrome occurring during renal fibrosis originates from tubular epithelial cells. They are recruited because of the damage of basement membrane. EMT type 3 allows tumor cells to dissociate, migrate and metastasize [9].

EMT is regulated by transcriptional and post-transcriptional mechanisms, particularly through downregulation of E-cadherin and overexpression of mesenchymal proteins such as vimentin and N-cadherin [9].

If mesenchymal cells would have a crucial role during morphogenesis, they often remain undifferentiated in adults. Undifferentiated mesenchymal cells, known as stem cells, exist in small

quantities in bone marrow, fat, muscles and in dental pulp of baby teeth. They retain the ability to differentiate into different kind of connective tissues for reparative or regenerative reasons [9].

Mesenchymal-epithelial transition (MET) is the reverse process by which mesenchymal cells acquire adhesive properties and arrange themselves into organized sheets. MET would also need to generate the so-called 'secondary epithelium' in various organs (i.e. kidney) [6]. When required, the secondary epithelium can re-differentiate towards mesenchymal tissues by mediation of several genes [6]. The switch EMT-MET would also induce neoplastic cells in acquiring a stem cell pattern. This pattern helps to prevent apoptosis and senescence, and would contribute to both immunosuppression and multidrug resistance [6]. These converted mesenchymal cells are not able to migrate toward the blood flow and cause local recurrence. In metastatic sites, in accordance with local microenvironment, an EMT-MET switch would occur. Restoration of epithelial features allows cells to arrange in clusters contributing to the stability of the metastatic focus.

3. Uterine sarcomas: the history

Sarcomas are malignant neoplasia occurring in any site of the body in which mesenchymal tissues are present. Because of their mesodermal origin, sarcomas are characterized by histological and cytogenetic heterogeneity. The histological classification of sarcomas is made according to tissue differentiation [1].

Homologous USs refer to mesenchymal tissues, which are normally found in the uterus, such as smooth muscle, endometrial stroma, vascular and fibrous tissue. Heterologous USs refer to mesenchymal tissues that are foreign to the uterus, such as cartilage, bone, skeletal muscle and fat.

In the past, sarcomas originating from different organs were grouped together; such classification demonstrated a scant utility from a clinical point of view [1]. Recently, basing on cells differentiation and growth pattern, the World Health Organization (WHO) proposed a separate histological classification for uterine neoplasia. Thus, uterine mesenchymal neoplasias were grouped as smooth muscle tumors and endometrial stromal tumors [10]. Uterine smooth muscle tumors are defined as benign and malignant neoplasms arising in the context of myometrium and composed of cells showing smooth muscle differentiation. Among these, benign leiomyoma, smooth muscle tumor of uncertain malignant potential (STUMP) and leiomyosarcoma (LMS) are listed [10].

Endometrial stromal tumors enclose all the neoplasia originating from the uterine endometrial stroma: endometrial stromal nodule (ESN), endometrial stromal sarcoma (ESS) and undifferentiated uterine sarcoma (UUS) [10] (**Figure 1**).

Nowadays, being carcinosarcoma (CS) considered as a dedifferentiated/metaplastic form of endometrial carcinoma, together with Müllerian adenosarcoma (MA), it is encompassed among the 'mixed epithelial and mesenchymal tumours' [11].

Tumor stage represents the most important prognostic factor for USs. In the past, USs were inadequately staged using the same 1988 staging system utilized for endometrial carcinoma.

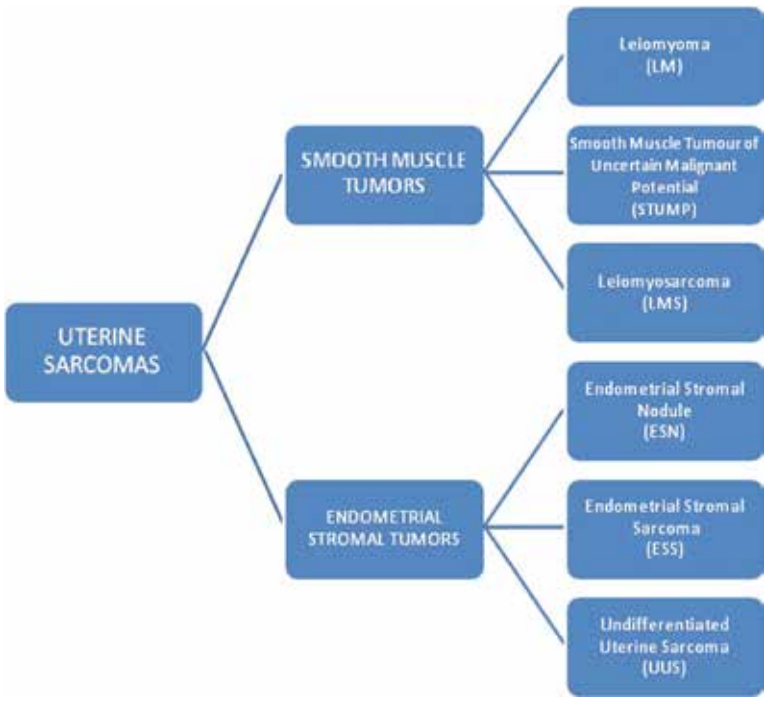

Figure 1. 2014 WHO classification of uterine sarcomas.

In 2009, a new International Federation of Gynecology and Obstetrics Committee (FIGO) staging system was specifically developed [12]. It comprises two sections, one for both leiomyosarcomas and endometrial stromal sarcomas and another for the adenosarcomas. The staging system used for carcinosarcomas is the same used for endometrial carcinomas.

4. The two great excluded

Müllerian adenosarcomas (MAs) account for 5.5–9% of all USs [1]. They are commonly seen in postmenopausal women, even if cases occurring in adolescents and young women are described. MA typically shows benign epithelial cells together with homologous/heterologous mesenchymal sarcomatous components. Neoplasia is often limited to the endometrium, since myometrial invasion is extremely rare. Malignant potential is low as well as histological grade at fist presentation [1]. The 5-year survival rate for stage I is of about 76% [1]. MAs are polypoid in shape and may contain small internal cysts. Tumor cell necrosis, if present, represents the most important prognostic factor [1].

Carcinosarcoma (CS), also known as malignant mixed Müllerian tumor (MMMT), has been recently considered as a metaplastic carcinomas basing on a different derivation from a common monoclonal stem cell [13]. Clinical, pathological and molecular evidences would confirm the monoclonal origin of carcinosarcomas, which would further undergo both epithelial and mesenchymal differentiation during its development [13]. For other authors, a CS would

originate from a carcinoma undergoing sarcomatous metaplasia through a dedifferentiation process [14].

The peculiar molecular features of CSs, as well as their good response to adjuvant therapies, would confirm the epithelial derivation of these tumors which characteristically shows a high aggressiveness and a high frequency of lymph nodal and distant metastases. Thus, the prognosis of CS would depend on the carcinomatous component [15].

Noticeably, patients with MA and CS tend to be much older than patients bearing US [15].

5. Leiomyosarcoma

After the exclusion of CSs, leiomyosarcomas (LMSs) represent the most common USs (30%), being the endometrial stromal sarcomas the second (10–15%). Rhabdomyosarcoma, angiosarcoma and liposarcoma are extremely rare [1].

LMSs develop in the smooth muscle layer of the uterus, called myometrium; thus, malignant cells show smooth muscle differentiation.

5.1. Epidemiology

The worldwide annual occurrence rate of USs is 1.55–1.95 per 100,000 women. The peak of incidence is in the fifth decade (50–55 years), about 10 years later than leiomyoma. In younger women, the incidence of LMS strictly correlates with the use of tamoxifen in adjuvant breast cancer therapy [16].

The percentage of incidental LMS among women undergoing surgery for suspected leiomyoma increases with age, being about 0.2% in patients aging 31–40, 0.9% among those aging 41–50 years of age, about 1.4% in women aging 51–60 and 1.7% in patients ranging from 61 to 81 years of age [15].

LMS is most common in black race. The relative risk and incidence of both leiomyomas and LMS is two- to threefold greater in black women than in white ones [1].

5.2. Aetiology

The risk factors for LMS are still unknown. The role of obesity, nulliparity, hypertension and diabetes mellitus, recognized as influencing the development of other uterine malignancies, are uncertain yet. On the other hand, some evidences demonstrated that the use of tamoxifen for 5 years or more is associated with an increased relative risk of developing an LMS, although the absolute risk remains low [15]. Pelvic irradiation, a history of retinoblastoma in childhood, hereditary leiomyomatosis and renal cell carcinoma are other documented risk factors [15].

Finally, although it is now clear that the vast majority of LMSs arise independently, it is now accepted that a small percentage would derive from the transformation of a pre-existing leiomyomas [17].

5.3. Clinical features

Since pelvic examination cannot distinguish between leiomyoma and LMS, the pre-surgical differential diagnosis is very difficult. In both cases, symptoms are not specific. Patients often present with vaginal bleeding or discharge, lower abdominal mass and pelvic pain. Size, contour and mobility of the uterus along with any other possible findings (i.e. cervical abnormalities or vaginal nodules) should be evaluated during gynecological examination. A fixed mass is commonly suggestive of a malignant neoplasm, even if a malignant neoplasm not infiltrating the uterine serosa is often mobile. A rapidly growing solitary intramural or subserosal uterine mass should be suspected for an LMS, especially in the absence of hormonal stimulation or in non-pregnant women. LMS shows lymph nodal or haematogenous spreads. Lung represents the preferential site for distant metastasis. When local metastases occur, gastrointestinal or urinary symptoms may be associated [1].

5.4. Imaging

Imaging features for LMS are similar to those of leiomyoma.

At transvaginal ultrasound examination, LMS shows echogenic components mixed with anechoic areas due to necrosis. Color Doppler usually demonstrates irregular vessel distribution and low impedance to flow. All of these characteristics may also be found in leiomyomas [18].

Computed tomography (CT) is not able to differentiate between leiomyomas and LMS. A specific characteristic of LMS would be the absence of calcifications, which are usually seen in leiomyomas outgrowing their blood supply [19].

Magnetic resonance imaging (MRI) has been reported to have high sensitivity in LMS diagnosis, although specificity is low [19]. Contrast-enhanced MRI (CE-MRI) demonstrates significantly higher accuracy and specificity if compared with diffusion-weighted MRI (DW-MRI), while sensitivities are comparable [19].

Finally, even if the uptake of fluorodeoxyglucose (FDG) in positron emission tomography/CT (PET-CT) is usually high in LMS and low in leiomyomas, the use of this technique in differential diagnosis is limited, since leiomyomas can uptake FDG too [20].

In conclusion, although most studies demonstrated that pelvic ultrasound followed by MRI represents the most useful strategy in LMS diagnosis, the vast majority of the authors concluded that no pelvic imaging is able to reliably differentiate between LMS and leiomyomas.

5.5. Surgical specimens

LMS is commonly diagnosed after surgery for a suspected leiomyoma [1, 16, 19].

Although fine needle biopsy and curettage samples have been proposed as good diagnostic specimens, their use is limited. In the context of an LMS, areas showing histological features indistinguishable from those of leiomyoma may be seen. For this reason, histological diagnosis requires the evaluation of the entire neoplastic mass, obtained by myomectomy or hysterectomy [21].

In addition, since the distribution of atypia and mitosis is not homogeneous in the context of a malignant mesenchymal mass, an accurate estimation requires extensive sampling.

Intra-operative diagnosis on frozen section demonstrated to be limited too, although this technique remains essential to drive the extension of the surgery [21]. Hysterectomy may be performed by laparotomy or laparoscopy. Using laparotomy, the specimen is not morcellated. By laparoscopy, only the suspected mass is removed; in such cases, the specimen is morcellated and might favor dissemination of malignant cells within the peritoneal cavity [22]. Occasionally, smooth muscle cells have been found in pelvic washing after laparoscopic myomectomy [22].

5.6. Pathological findings

5.6.1. Macroscopic features

About 65% of LMSs are intramural, 20% are submucosal, 10% are subserosal and 5% originate from cervix. Characteristically, LMS presents as a large solitary mass, although the development of an LMS in a uterus harboring multiple leiomyomas is common [1]. Usually, LMS presents as a voluminous mass with a mean diameter of 10 cm. Its margins are often well defined, although focal infiltration of the adjacent myometrium may also be seen. Irregular margins and lacking of a clear line of demarcation separating LMS from normal myometrium usually indicate invasive behaviour. Because of the possible overlap in shape between LMS and ischemic degeneration of leiomyoma, most of smooth muscle neoplasms suspected to be malignant at imaging are found to be benign at microscopic evaluation [23]. Grossly, LMS is very different from leiomyoma, the former revealing a fleshy consistency, a bulging cut surface and a pearly white-to-gray color; necrotic and haemorrhagic foci are often seen. The typical whorled appearance of leiomyoma is always lacking. The presence of hemorrhage and necrosis should always be regarded as suspected for LMS. When cystic changes are present, samples should be mainly taken on the solid areas [1].

5.6.2. Microscopic features

LMS is composed of connected bundle cells showing smooth muscle differentiation. Nuclei are round with one or more prominent nucleoli. Multinucleated giant cells with osteoclast-like shape may be present (**Figure 2A**) [24]. The three cardinal microscopic features characteristics of LMS are tumor cell necrosis (**Figure 2B**), nuclear atypia (**Figure 2C**) and mitotic count >10/10 High Power Fields (HPFs) (**Table 1**). Even if all of these three cardinal features are usually detected in about 80% of typical LMS, the presence of two of three is sufficient to reach the diagnosis [1].

Three types of necrosis have been described in smooth muscle tumors: (1) ulceration with submucosal necrosis; (2) infarct-type necrosis, encountered in both benign and malignant neoplasms and (3) tumor cell necrosis, characterized by distinct and harshly demarcated necrotic zones, suddenly transiting towards non-necrotic zone [1, 26]. Tumor cell necrosis is specific for LMS and should also be distinguished from hyaline or degenerative necrosis, which can be seen in both leiomyomas and other types of sarcomas (**Table 2**) [26].

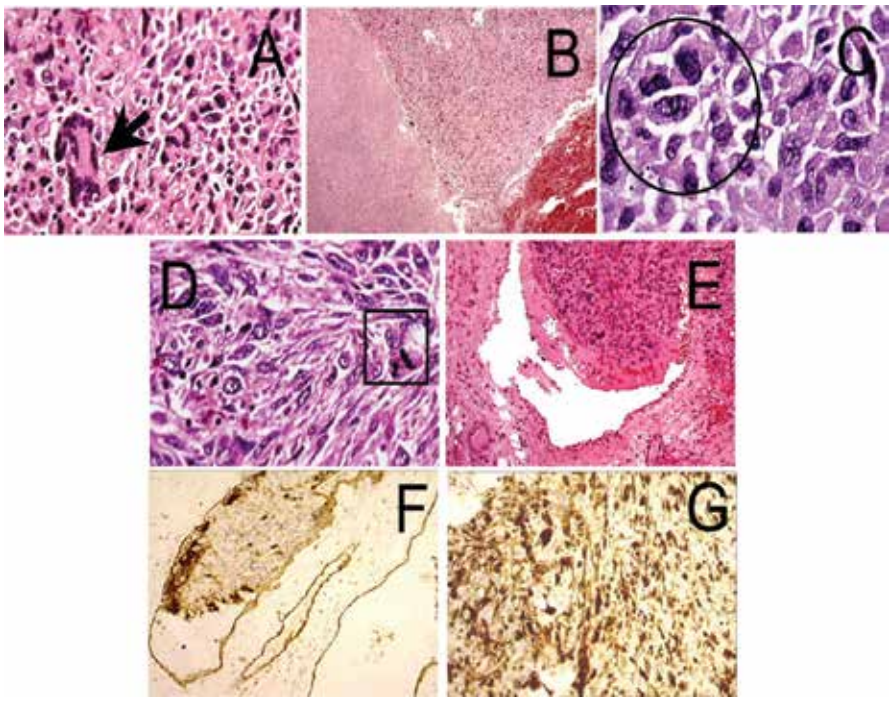

Figure 2. Uterine leiomyosarcoma. (A) Bundles of cells with smooth muscle differentiation, and multinucleated giant cells with osteoclastic shape (arrow), Ematoxilin-eosin (EE), 20x. (B) Tumor cell necrosis: Demarcated necrotic zones abruptly transiting towards non-necrotic zone; EE, 10x. (C) Nuclear atypia (circle), EE, 40x. (D) Mitosis (square), EE, 60x. (E) Vascular invasion with artery embolization, EE, 10x.(F) The wall of the vessel shows CD31 positivity, 10x. (G) Desmin positive stain in neoplastic cells, 10x.

If infarct-type necrosis is present, it should be evaluated in conjunction with nuclear atypia and mitoses (**Figure 2D**), since it is common in both benign and malignant neoplasms [1, 16].

Other features that should be included in the pathological assessment of uterine LMS are tumor size, presence of vascular invasion (**Figure 2E**, **F**), occurring in 10–27% of the cases, and status of surgical margins [26]. Hypercellularity does not discriminate between LMS and leiomyoma [1]. As previously shown, the specimen should be adequate to exactly evaluate the mitotic rate. To obtain adequacy is essential to analyze one section every 1–2 cm of tumor diameter, to count mitotic figures only in mitotically active areas at 60× magnification, and to evaluate five sets of 10 consecutive HPF, excluding degenerating cells [1]. Some drugs and hormones may induce histological changes mimicking necrosis; for example, iatrogenic cell necrosis in a histological background of atypical leiomyoma could lead to a wrong diagnosis of LMS. For this reason, pathologists should be informed of any therapies [1, 26]. A tumor lacking coagulative cell necrosis and nuclear atypia should be diagnosed as mitotically active leiomyoma in the presence of 5–20 mitoses/10 HPF, or as Stromal Tumor of Undetermined Malignant Potential (STUMP) when mitotic count is >20 mitoses/10 HPF. A tumor lacking coagulative necrosis but showing diffuse moderate–severe nuclear atypia should be considered as atypical leiomyoma when mitosis is <2/10 HPF, as STUMP when mitotic count is 2–10/

Tumor cell necrosis	Atypia	Mitosis/10 HPFs	Diagnosis
Present	Diffuse, moderate–severe	Any	Leiomyosarcoma
	None-mild		
		≥10	Leiomyosarcoma
		<10	Leiomyosarcoma
Absent	Diffuse, moderate–severe		
		≥10	Leiomyosarcoma
		<10	Atypical leiomyoma
	None-mild		
		≥10	Leiomyoma
		<10	Mitotically active leiomyoma
	Focal, moderate–severe		
		<10	Leiomyoma with limited experience
		≤10	STUMP

HPF, high power fields; STUMP, smooth muscle tumor with uncertain malignant potential. (Modified from Ref. [25]).

Table 1. Diagnostic criteria for smooth muscle tumors.

Coagulative tumor cell necrosis	Hyalinizing necrosis
Common in malignant smooth muscle tumors	Common in leiomyomas
Sudden transition to vital to necrotic areas	Area of fibrous and granulation tissue between vital and necrotic areas
Necrotic cells look ghostly	No cell shadows are visible
Rare inflammation	Presence of immune complexes
Abrupt borders	Slight borders
Blood vessels are not involved	Blood vessels are involved by fibrin deposits; their walls are thickened
Hyperchromatic and atypical nuclei	Pale nuclei

Table 2. Types and characteristics of necrosis in smooth muscle tumors.

10 HPF, as LMS when mitoses are more than 10/10 HPF. A neoplasia showing coagulative necrosis without a significant nuclear atypia should be classified as STUMP when mitosis is less than 10/10 HPF, as LMS when at least 10 mitoses/10 HPFs are found. Finally, a tumor with coagulative necrosis and significant nuclear atypia, regardless of mitotic count, should be diagnosed as LMS [1].

In the past, Broder's classification was one of the most used systems to grade LMS. It considered four categories: grade 1, mild cytological atypia; grade 2, more nuclear irregularity; grade 3, intermediate between grades 2 and 4; grade 4, the presence of bizarre cells. [27]. Other authors classified LMS basing on the level of differentiation (well, moderately and poorly

differentiated) [28]. A binary categorization into low- and high-grade categories was rarely used, since it has been demonstrated that it is quite impossible to recognize a low-grade uterine LMS at the time of diagnosis [28]. Presently, according to WHO, no grading should be performed for LMS [1].

5.6.3. Immunohistochemistry

Although immunohistochemistry (IHC) does not represent a necessary tool in LMS diagnosis, it may help in distinguishing LMS from other uterine malignancies (**Table 3**).

Desmin, h-caldesmon, smooth muscle actin and histone deacetylase 8 (HDAC8), the so-called smooth muscle markers, are usually expressed in LMS (**Figure 2G**), even if immunoreaction

Antibody markers	**Typical LMS**	**Epithelioid LMS**	**Myxoid LMS**	**STUMP**
Smooth muscle actin	+	±	+	
Desmin	+	±	±	
h-cardesmon	+	±	±	
EMA	+, patchy	+	+, patchy	
CD10	+, patchy	+, patchy	+, patchy	
CD34	—	—	—	
CD44	—	—	—	
Cytokeratins	+, patchy	+	—	
HDAC8	+	+	+	
ER	if +, better prognosis	if +, better prognosis	if +, better prognosis	±
PR	if +, better prognosis	if +, better prognosis	if +, better prognosis	±
p53	±	±	±	±
p21	if +, poor prognosis	if +, poor prognosis	if +, poor prognosis	+
Bcl-2	if +, better prognosis	if +, better prognosis	if +, better prognosis	if +, good prognosis
MIB1	poor prognosis for high percentage	poor prognosis for high percentage	poor prognosis for high percentage	Absent or low percentage
p16	if +, high risk of relapse	if +, high risk of relapse	if +, high risk of relapse	±
Inhibin	—	—	—	
S100	may be +	—	may be +	
c-kit	±	—	±	±
Cyclin D1				

LMS, leiomyosarcoma; STUMP, smooth muscle tumors of uncertain malignant potential.

Table 3. Immunohistochemical features of uterine smooth muscle neoplasia.

for one or more of these markers (particularly for desmin and h-caldesmon) can be lost or may be weak in some LMS variants, such as myxoid and epithelioid ones [29]. LMS is generally negative of focally positive for CD10, but it is still unknown whether CD10-positive foci have to be considered as areas of endometrial stromal differentiation within smooth muscle neoplasms [30].

LMS does not immunoreact with CD44, whereas leiomyoma and normal myometrium express this marker [1]. CD44 demonstrated sensitivity, specificity, and positive- and negative-predictive values near to 100%; thus, it is very useful in problematic cases [29]. Epithelial markers such as cytokeratins (CKs) and epithelial membrane antigen (EMA) may also be expressed in LMS, although their expression is weak and focal [29]. Focal positivity for CAM5.2 may also be seen.

Expression of estrogen (ER) and progesterone (PR) receptors has been reported in 57 and 43% of LMS cases, respectively; the corresponding percentage for leiomyoma is 78 and 88% [31]. In general, LMSs staining positive for ER and PR demonstrated to be less aggressive than the negative counterpart [31]. Positivity for c-kit may also be seen, although a variable proportion of LMS without c-kit mutation has been identified [1]. The percentage of MIB1-positive cells is usually high in LMS, if compared with leiomyomas. p53 positivity is detected in about 50% of LMS but not in leiomyoma [32]; tumors overexpressing p53 are more aggressive than those showing p53 negativity [32].

p16 antibody seems to be useful in distinguishing between benign and malignant uterine smooth muscle neoplasia. In particular, a strong and diffuse p16 positivity associated with p53 positivity would favor an LMS diagnosis. In addition, p16 expression seems to be strictly related to a highest risk of LMS relapse [33].

5.7. Histological variants of LMS

Several histological subtypes of LMS have been recognized, although it is unknown if this classification may have a clinical relevance.

Usual leiomyosarcoma is composed of fascicles of spindle-shaped cells with eosinophilic cytoplasm, resembling the normal myometrial smooth muscle.

A tumor lacking all the three main cardinal histological features seen for LMS should be diagnosed as leiomyoma (**Figure 3**); a tumor showing one of three main features should be categorized as atypical leiomyoma or STUMP [1].

Myxoid leiomyosarcoma is the less common variant. Grossly, it appears as a well-circumscribed, voluminous and gelatinous mass. Commonly, myxoid change is seen in about 30% of the tumor mass. Histologically, it differs from the classic form of LMS due to hypocellularity and myxoid stroma. A significant cytological atypia and a high mitotic activity are usually lacking [34]. Within myxoid areas, no more than 2 mitosis/10 HPFs are often seen, although a higher mitosis number may be present in the context of smooth muscle fascicles. Smooth muscle cells stain positive for smooth muscle markers (**Table 3**). Myxoid LMS usually shows clinically malignancy, since it is highly infiltrative [1].

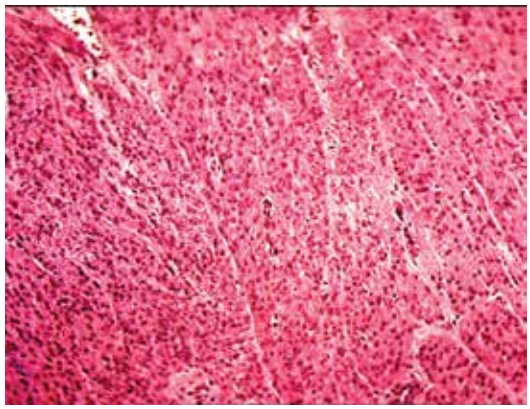

Figure 3. Typical leiomyoma. EE, 10x.

Epithelioid leiomyosarcoma is characterized by the presence of round-polygonal smooth muscle cells with abundant eosinophilic cytoplasm and round nuclei, arranged in nests, cords or plexiform patterns. The clinical behaviour of epithelioid tumors with a moderate mitotic activity (2–4 mitosis/10 HPFs) is not well understood. About 10% of epithelioid LMS larger than 6 cm recurred or metastasized [1]. These cases are classified as STUMP and need a careful follow-up [34].

Leiomyosarcoma with osteoclastic giant cells is a rare but more aggressive variant of LMS [1]. The background is similar to those of typical LMS; however, histiocytic CD68-positive cells may be detected admixed with smooth muscle cells staining positive for smooth muscle cell markers. Overall survival is <2 years after presentation, even with radiation or chemotherapy1 [1].

In **xanthomatous LMS**, smooth muscle cells show microvesicular and foamy cytoplasm.

The three cardinal features used to diagnose typical LMS are often hard to assess in epithelioid and myxoid variants [35]. In patients showing a worse prognosis, two or more of the following features are usually detected: tumor size of 5 cm or more, infiltration of the adjacent tissues, cytological atypia, high mitotic index, necrosis and lymph vascular invasion [35]. Particularly, cut-off values of 10 mitoses/10 HPFs, ≥4 mitoses/10 HPFs and ≥2 mitoses/10 HPFs are used for typical, epithelioid and myxoid LMS, respectively [16].

5.8. The 2009-revised FIGO staging system for LMS

Staging of LMSs is very important to drive treatment. The International Federation of Gynecology and Obstetrics Committee (FIGO) recognized that the old classification was no longer sufficient and that USs require an independent staging [35]. The old staging system looked at how far the cancer spreads; FIGO staging, essentially being a post-surgical staging, relies on histological examination.

Moreover, while FIGO staging is more precise in detecting tumors with a worse prognosis, staging by the American Joint Commission on Cancer (AJCC) demonstrates to be more

accurate in identifying patients with a good prognosis (**Table 4**). In general, neither the former nor the latest are able to provide an exact estimation of the overall survival for LMSs [36].

5.9. Diagnostic problems with LMS

Among uterine sarcomas, LMS represents a source of differential diagnostic problems, particularly with leiomyomas variants, which most often show macroscopic and histological features causing misdiagnosis [1]. This fact would be essentially due to the hormonal uterine milieu that would cause a high mitotic activity. A diagnosis of LMS would also signify a challenge for clinicians, because of problems with its management. As previously shown, among women undergoing hysterectomy or myomectomy for a myometrial mass, the prevalence of LMS is approximately 0.20% [37]. Thus, differential diagnosis between LMS and leiomyoma is the first step to move when a uterine mass is suspected. Since the risk of complications during hysterectomy exceeds the risk of incidental LMS, women with a suspected leiomyoma should be treated with a uterine-sparing surgical option [37]. Some conditions, which may be considered as associated with LMS, but not with leiomyoma, include older age and postmenopausal status. Being leiomyoma responsive to estrogen and progesterone, it frequently arises during reproductive age (below 20 years of age in black women, and between 30 and 40s in white women), while usually stabilizes or decreases in size in postmenopausal patients [37]. Basing on these considerations, a new or growing uterine mass in women above 40 years of age should be suspected for LMS, while the level of suspicion for malignancies may be lower in postmenopausal women undergoing oestrogens therapy [38]. Younger age cannot exclude a

FIGO stage	Definition	TNM Stage
I	**Tumor limited to uterus**	**T1, N0, M0**
IA	<5 cm	T1a, N0, M0
IB	>5 cm	T1b, N0, M0
II	**Tumor extended to the pelvis**	**T2, N0, M0**
IIA	Adnexal involvement	T2a, N0, M0
IIB	Tumor extends to extra -uterine pelvic tissue	T2b, N0, M0
III	**Tumor invades abdominal tissues (not just protruding into the abdomen)**	**Any of the following**
IIIA	One site	T3a, N0, M0
IIIB	More than one site	T3b, N0, M0
IIIC	Metastasis to pelvic and/or para-aortic lymph nodes	T1-T3, N1, M0
IV	**Tumor invades bladder and/or bowel mucosa, and/or distant metastases**	
IVA	Tumor invades bladder and/or bowel mucosa	T4, any N, M0
IVB	Distant metastases, including intra-abdominal metastases and/or inguinal lymph nodes	any T, any N, M1

FIGO, International Federation of Gynecology and Obstetrics Committee; AJCC, American Joint Commission on Cancer.

Table 4. 2009-revised FIGO and AJCC (TNM) staging system for leiomyosarcomas.

diagnosis of LMS. On the other hand, a rapidly growing large uterine mass cannot unequivocally be associated to an LMS [38]. For all these reasons, histological examination represents the milestone to distinguish between leiomyoma and LMS.

Recent data reported an increased risk of undetected LMS among postmenopausal patients who underwent morcellation of uterine tissue [39]. Uterine sarcoma usually spreads via intra-abdominal, lymphatic or haematogenous routes. It is worth noting that some histological variants of leiomyomas may also disseminate. Thus, a careful diagnosis has to be done in the presence of a widespread disease. Failure of medical treatment with gonadotropin-releasing hormone agonist, or unsuccessful non-excisional procedures for a leiomyoma (such as uterine artery embolization), has been reported in some LMS cases [39].

Genetic studies demonstrated that, in a vast majority of cases, an LMS does not originate from a benign leiomyoma. LMS typically shows polyploidy and aneuploidy, while leiomyoma displays genetic rearrangements which are often shared by other benign neoplasms. On the other side, rare cases of leiomyoma progressing to LMS have been described [40]. In the absence of risk factors, the vast majority of the authors agree to manage women for a leiomyoma unless new symptoms develop. Conversely, a suspect of LMS should be put if women failing response to medical therapy or when new symptoms appear.

5.10. Differential diagnosis

5.10.1. LMS versus *intravascular leiomyomatosis, benign metastasizing leiomyoma, disseminated pelvic leiomyomatosis*

Intravascular growth, metastasis and pelvic dissemination are not included among the cardinal features driving LMS diagnosis. Thus, they cannot be used to distinguish an LMS from a leiomyoma. However, intravascular leiomyomatosis, benign metastasizing leiomyoma and disseminated pelvic leiomyomatosis do not show significant cytological atypia, tumor cell necrosis or a high mitotic count [1]. Recent findings demonstrated a distinctive genetic profile in benign metastasizing leiomyomas [41].

5.10.2. LMS versus *endometrial stromal sarcoma with smooth muscle differentiation*

In endometrial stromal sarcoma with smooth muscle differentiation, smooth muscle cells do not show necrosis or a significant mitotic activity. Moreover, these malignancies always contain an endometrial stroma usually lacking in LMS [1].

5.10.3. Epithelioid LMS versus *poorly differentiated carcinoma*

A diagnosis of carcinoma is favored when malignant cells are associated with endometrial hyperplasia. This diagnosis was also supported when neoplastic cells show positivity for keratin and negativity for desmin and h-caldesmon (**Table 3**) [1]. When a distinction is impossible to make, a diagnosis of 'undifferentiated malignant neoplasm' should be put. Electron microscopic examination may sometimes help.

5.10.4. LMS versus *gastrointestinal stromal tumor (GIST)*

Occasionally, GIST extends from the bowel wall simulating a fibroid. In such cases, differential diagnosis between GIST and leiomyoma may be problematic since both tumors show spindled cells without cytological atypia or mitotic activity.

Unlike LMS, GIST frequently shows spindle cells with cytoplasmic vacuoles, while the typical fascicular architecture of muscle cells is lacking. Desmin expression in GIST is rare, while both c-kit and CD34 expression are common. Basing on these evidences, the use of a panel including desmin, c-kit and CD34 may be helpful in differential diagnosis [36].

5.10.5. LMS versus *undifferentiated uterine sarcoma*

Recent data demonstrated that there are no universal histological criteria able to distinguish these two malignancies. In truth, it is also uncertain if there are significant clinical and therapeutic differences between them [36].

5.11. Molecular features

The oncogenic mechanisms leading to LMS remain unknown, even if the accumulation of multiple genetic events has been demonstrated. In general, the number of molecular features characteristic for LMSs is smaller if compared with those of endometrial stromal sarcomas.

Single nucleotide polymorphisms technique, gene expression arrays and DNA methylation analyses show genomic modifications and mosaicisms in LMS; cytogenetic analyses also demonstrated numerical and structural chromosomal abnormalities [42]. On the other hand, no or limited genomic aberrations have been found in leiomyomas [42]. Thus, genomic instability represents the hallmark of uterine smooth muscle malignancies [43]. The most frequent genomic lost found in LMS involves 10q, 11q, 13q and 2p chromosomal arms. Particularly, the loss of genetic material at chromosomal arms 1p, 14q, and 22q seems to be the same for both uterine LMS and gastrointestinal stromal tumors (GISTs) [43]. The most common genomic gains in LMS are Xp, 1q, 5p, 8q and 17p.91 [33]. Loss of heterozygosity (LOH) for long arm of chromosome 10 was found in about 50% of LMSs, but not in leiomyomas [43]. t(12;14)(q15;q23-24) translocation has been detected in a high proportion of leiomyomas but not in LMS [43]. Some LMSs demonstrated some types of X chromosome inactivation differing from those of leiomyomas. This fact would confirm the theory of the independent transformation processes occurring in LMS and leiomyoma [43]. Moreover, convincing evidences regarding the malignant transformation of certain type of leiomyomas, such as the bizarre variant, are still lacking. LMS also shows a significant higher frequency of allelic loss (FAL), if compared with leiomyoma (52 vs. 18%, respectively) [44]. All these findings would support the hypothesis that the pathways for LMS and leiomyoma are different [43]. Genetic instability would be the key to acquire sequential genetic changes and mutations. Although the vast majority of USs are sporadic, some germline mutations (i.e. mutation occurring in fumarate hydratase) are regarded as genetic risk factors for the development of both LMSs and leiomyomas [45]. Most authors put their attention on the mutations occurring in the gene named mediator complex subunit 12 (MED12), located at locus Xq13.1 [46]. MED12 protein complexes with MED13, CDK8 and cyclin D [46]. Mutations of Exon

2 in MED12 gene have been found in 70% of leiomyomas, particularly in the typical and mitotically active variants [46]. For this reason, MED12 mutation cannot be used to determine the behaviour of a smooth muscle neoplasia. The unique role of this marker would rely on the individuation of the smooth muscle differentiation within a mesenchymal neoplasia [46]. Overexpression of high-mobility group AT-hook 2 (HMGA2) protein, frequently mutated in uterine leiomyomas, seems to be inversely related to the presence of MED12 mutations [47].

By FISH analysis, TP53 mutations and PTEN deletions were detected in LMS, atypical leiomyoma and STUMP [34]. A high expression of topoisomerase 2A (TOP2A) has been found in a vast majority of LMSs, while low expression was seen in leiomyoma variants and STUMP [48]. Expression of Stathmin1 activating the phosphoinositide-3-kinase (PI3K) pathway was demonstrated to be significantly higher in LMSs, if compared with other uterine smooth muscle tumors. Thus, the absence of Stathmin1 would not support a diagnosis of LMS [49]. Being the expression of the mRNA-binding protein IMP3 higher in LMS than in benign smooth muscle neoplasia, it must be considered as a useful tool in differential diagnosis [50]. CDC7, CDC20, GTSE1, CCNA2, CCNB1, and CCNB2 are overexpressed in LMS, while K-ras is overexpressed in a small percentage of leiomyomas but not in LMSs [42]. MDM-2 oncogene negatively regulates apoptosis by (1) targeting p53 for ubiquitin-based degradation, (2) blocking p53 transcriptional activation domain and (3) shuttling p53 from the nucleus to the cytoplasm [42]. Amplification of MDM-2 has been reported in 10% of uterine LMS and in extra-uterine LMS, but not in leiomyomas [42]. The block of MDM-2 would enhance p53 function, thus providing a targeted therapeutic strategy. Abnormalities of the retinoblastoma-cyclin D pathway have been found in about 90% of LMSs [42]. All the above mentioned aberrant molecular patterns, the vast majority of which is different for LMSs and leiomyomas, confirm the different nature of these tumors. Cell cycle markers and proliferation proteins (p16, p21, p27, p53, PCNA, Ki-67 and PHH3) are presently under consideration. $p16^{INK4a}$ has been found to be implicated in the genesis of LMS [51]. p16 binds to cyclin D/CDK4 complex regulating cell cycle through G1/S progression. p16-/CDK4A would act as a negative cell cycle regulator, by blocking cell cycle progression of neoplastic cells and accelerating cell senescence. Ki67 antigens identify both normal and neoplastic cells under proliferation. Recently, statistically significant higher level of both PCNA and Ki67 has been found in uterine LMSs in comparison with leiomyomas. The percentage of MIB1-positive cells would help to predict LMS prognosis and neoplastic spread [1].

In conclusion, among the several markers listed above, TOP2A, IMP3, Stathmin1, HMGA2 and MED12 are demonstrated to be promising in distinguishing between LMS and leiomyoma. Most studies recently focused on molecular markers able to predict progression risk and prognosis of a LMS. Slatter et al. correlated the presence of ALT and PML bodies (APBs) to a poor prognosis of LMS [52]. Next-generation sequencing confirmed the presence of ATRX mutations in LMS and their association with a poor survival [53]. RNA sequencing identified three distinct molecular subtypes of LMS; subtype II was demonstrated to have the worse prognosis [54]. Leiomodin (LMOD1) and ADP-ribosylation factor-like 4 C (ARL4 C) are now considered as specific markers for LMS types I and II [54]. The expression of progesterone receptor has been recently included in FIGO staging as an independent prognostic factor for

stage I LMS [2]. On the other hand, overexpression of c-myc proto-oncogene does not correlate with smooth muscle tumor prognosis, since it has been detected in about 50% of both leiomyomas and LMSs [2].

Gene expression profiling individuated 203 probes, which were differentially expressed in primary and metastatic LMSs. Among these, OSTN, NLGN4X, NLGN1, SLITRK4, MASP1, XRN2, ASS1, RORB, HRASLS and TSPAN7 were overexpressed in primary LMSs, while TNNT1, FOLR3, TDO2, CRYM, GJA1, TSPAN10, THBS1, SGK1, SHMT1, EGR2 and AGT were overexpressed in metastatic LMSs [55]. By flow cytometry, about 70% of LMSs showed aneuploidy; thus, DNA ploidy may probably help in identifying cases with adverse prognosis [1]. CGH analysis demonstrated to be useful in distinguishing LMS from STUMP and in predicting the clinical behaviour of the latest [56].

In summary, most molecular markers have been studied in relation to LMSs progression and prognosis. However, large studies are still needed to validate their usefulness as possible therapeutic targets.

5.12. Therapeutic approaches

5.12.1. Surgery

Hysterectomy with tumor debulking may be considered the treatment of choice in patients with uterine LMS [1]. In postmenopausal women, hysterectomy and bilateral salpingo-oophorectomy represent the gold standard. Ovarian preservation may be considered in premenopausal patients with early stage LMS, limited to the uterus [57]. Patients without residual disease after surgical resection would have an improved survival if compared with those undergoing suboptimal surgical resection [57]. The role of lymph node dissection remains controversial, since lymphatic metastases occur only in a small percentage of cases, frequently associated with intra-abdominal disease. The incidence of retroperitoneal lymph node metastases is low in patients harboring a uterine LMS. On the other side, nodal metastasis has been reported in 50% of women with an LMS mass of 6–10 cm. This fact would suggest to also consider tumor size in planning surgical management. Presently, among postmenopausal women harboring an LMS larger than 5 cm its maximum diameter, lymph node dissection should be considered [57], although lymph nodes metastases were identified in 6.6–11% of women undergoing lymphadenectomy [1, 57].

In patients with localized metastases, complete metastasectomy enhances disease-specific survival. Particularly, in patients with pulmonary metastasis, metastasectomy would bring to a 5-year survival rate of 43–46.8%, with an overall 3-year disease-free survival rate of 27.8% [58].

As previously shown, since the vast majority of LMSs are diagnosed after surgery for a suspected benign uterine mass, it would be extremely important to avoid uterine morcellation or intraoperative rupture of the mass into the peritoneal cavity.

5.12.2. Adjuvant therapies

The role of postoperative adjuvant therapies remains controversial, since no study clearly confirmed their benefits in the management of uterine LMSs. Radiation does not show a significant impact on the overall survival, although it seems to have a role in controlling local

disease, local recurrences and in palliation [59]. In general, CT with a single agent did not demonstrate a significant improvement of the LMS outcome, with limited clinical benefits. Moreover, only tumors with ER/PR receptors may respond to hormonal therapy [59]. Adjuvant chemotherapy is not standardly administered in patients who underwent hysterectomy for LMS confined to the uterus (stages I and II) [60]. The management of advanced uterine LMS is now based on a first-line regimen including Doxorubicin/Doxorubicin plus Ifosfamide [61]. The use of Gemcitabine or Gemcitabine plus Docetaxel produced conflicting results [61]. A French randomized study by Pautier et al. demonstrated a better 3- and 5-year disease-free survival in patients with multiagent CT, in comparison with women receiving RT alone [62]. Conversely, the use of multiagent CT or the combination of CT and RT proved to be associated with a significant increase in toxicity [62]. Trabectedin is a tetrahydroisoquinoline alkaloid. Trabectedin interferes with several transcription factors, DNA-binding proteins and DNA repair pathways, thus resulting in G2-M cell-cycle arrest and apoptosis [63, 64]. The two main advantages to use Trabectedin would rely on (1) therapeutic benefits that can be maintained by extending the use beyond six cycles and (2) reliable tolerability. All these findings would underline the possible role of Trabectedin in the management of advanced/persistent/recurrent LMS, although this drug has not been approved by the Food and Drug Administration yet [65, 66]. Some authors reported the cytoreductive surgery with hyperthermic intraperitoneal CT (CRS-HIPEC) as a promising treatment to achieve prolonged survival for peritoneal spreading LMS [67]. In general, chemotherapic protocols do not lead to a clinically significant response in high-grade LMS cases; on the other side, palliative CT is a rationale approach to improve the quality of life in patients with advanced unresectable disease.

A trial by the European Organization for Research and Treatment of Cancer failed to demonstrate some benefits of adjuvant RT in treating patients with LMS in stages I and II after surgery [68, 69]. These data were also confirmed by SEER (Surveillance, Epidemiology, and End Results) database [70]. On the other side, a retrospective study from Sanpath et al. demonstrated an improved outcome in women receiving RT after surgery, in comparison with surgery alone [71]. Finally, a consensus by the Gynecologic Cancer InterGroup (GCIG) established that adjuvant RT does not confer survival benefits to patients undergoing complete resection of uterus-limited LMS. Moreover, in advanced or recurrent LMS, RT may only have a minor role [72].

5.13. Spread and metastases

Although LMS shows metastatic potential and a high rate of recurrence, patients usually present with early stage of disease. If present, the extension of the LMSs outside the uterus occurs into the pelvis. About 3% of LMS at stages I and II show lymph nodes involvement, as a consequence of intraperitoneal spreading. A high proportion of patients without lymph nodes involvement would develop distant metastasis, the favored site being lung, brain, liver and bone. Direct extension to cervix and vagina is commonly observed [1].

5.14. Prognostic factors and survival

LMSs are often associated with a poor prognosis. A 5-year disease-specific survival is about 20–30%; a 5-year survival rate is 50–60% in stage I and 15% in more advanced stages. Death frequently occurs within 2 years from diagnosis, although a long disease-free interval was

described for low-stage LMS confined to the uterus [1]. In stage I, tumor size represents the most important prognostic factors [20]. Age at presentation and mitotic index remain controversial. In a large Norwegian report including 245 uterine LMSs confined to the uterus, tumor size and mitotic rate demonstrated to be useful in stratifying patients in different prognostic groups [73]. In truth, correlation between survival, patients' age, clinical stage, tumor size, the presence/absence of necrosis, mitotic rate, the degree of nuclear pleomorphism and vascular invasion varies among the different studies. Presently, nuclear pleomorphism, high mitotic rate, extensive tumor cell necrosis, vascular invasion, a size greater than 5 cm and non-spindle morphology are considered negative prognostic factors in low-stage LMS [1]. Prognostic significance of DNA ploidy and TP53 expression has been described, although confirmation is still needed [74]. Ancillary parameters such as p53, p16, Ki67 and Bcl-2 have also been explored, but results are still confusing. Recurrences are seen in 53–71% of the cases. All patients with extra pelvic metastasis usually die within 6 years from diagnosis [1].

5.15. Future perspectives

The genetic heterogeneity of the uterine LMSs makes the identification of driver mutations and therapeutic targets more difficult [75]. Recently, recurrent mutations of alpha thalassaemia/mental retardation syndrome X-linked (ATRX) gene have been detected. Although ATRX inhibitors might be considered as new possible therapeutic targets, their benefits are still to be defined [76, 77]. Since MDM2 inhibitors have proven to be efficient in preclinical settings, agents such as AMG232 and RG7112 are currently under investigation in a variety of cancer types [78].

In summary, the standard treatment for both early and advanced uterine LMSs remains the hysterectomy. In postmenopausal women, bilateral salpingo-oophorectomy and complete cytoreduction of the tumor with adherent structures, even if not infiltrated, are recommended. For uterus-limited disease (early stage), neoplastic mass should be removed en bloc. Metastasectomy should be considered in patients with metastatic LMS. Adjuvant RT and CT should not be considered in routine practice, especially in women in which tumor has been completely removed. CT with a single agent (Doxorubicin, Gemcitabine and Trabectedin) or in combination might be promising in patients with advanced, persistent or recurrent LMS. Presently, many efforts are focused to define the molecular etiology of LMS, in order to provide a better care for this highly lethal neoplasia.

5.16. Key points

- Uterine leiomyomas represent the most common gynecological benign neoplasia. Uterine sarcoma is rare. The percentage of incidental LMS among women undergoing surgery for suspected leiomyoma ranges from 0.2 to 1.7% and increases with age.
- Among women in reproductive age, a rapidly enlarging uterine mass should not be suspected for LMS. A new or growing uterine mass in postmenopausal women needs further evaluation.

- Leiomyomas do not appear to progress to sarcoma, with the exception of some histological variants.
- No pelvic imaging is undoubtedly able to distinguish between leiomyoma and LMS.
- It is not recommended to perform hysterectomy to exclude malignant neoplasm. Conversely, hysterectomy is suggested when the presence of LMS is strongly suspected by MRI, in the presence of multiple risk factors or when thoracic imaging demonstrated lung metastases.
- The influence of adjuvant therapy on survival is uncertain. RT may be useful in controlling local recurrences; CT with doxorubicin or docetaxel/gemcitabine should be considered as the first-line choice in advanced or recurrent disease.
- Multidisciplinary evaluation of LMS is essential.

6. Uterine smooth muscle tumors of uncertain malignant potential

Uterine smooth muscle tumors, which cannot be unequivocally diagnosed as benign or malignant, are designated as STUMPs [79]. STUMPs represent a heterogeneous group of neoplasia with a borderline behaviour. Because of their rarity and the evolving knowledge about them, the proper management of patient bearing STUMP represents a dilemma. The lack of uniform diagnostic criteria may often result in STUMP over diagnosis. The term 'STUMP' was first used by Kempson et al., in 1973 [80]. He clustered STUMPs into three groups, basing on cytological atypia, tumor cell necrosis and mitosis (**Figure 4A**, **B**):

1. Atypical leiomyoma with a low risk of recurrence: diffuse moderate–severe atypia, <10 mitosis MFs/10 HPFs and no tumor cell necrosis.
2. Atypical leiomyoma with limited experience: focal moderate-severe atypia, <20 mitosis/10 HPFs and no tumor cell necrosis.
3. Smooth muscle tumor with a low malignant potential: absent-mild nuclear atypia, mitosis less than 10/10 HPFs and the presence of tumor cell necrosis.

Later, Kempson et al. classified STUMPs as those tumor with a mitotic count major than 15 mitosis/10 HPFs [81, 82]. The largest study on uterine STUMP was done by Guntupalli et al. [83], which grouped STUMPs into five categories:

Group 1: the presence of tumor cell necrosis, the absence of atypia, and mitotic count <10/10 HPFs.

Group 2: the absence of tumor cell necrosis, diffuse atypia and mitotic count <10/10 HPFs.

Group 3: the absence of tumor cell necrosis, the absence of atypia and mitotic count >20/10 HPFs.

Group 4: hypercellularity and mitotic count >4/10 HPFs.

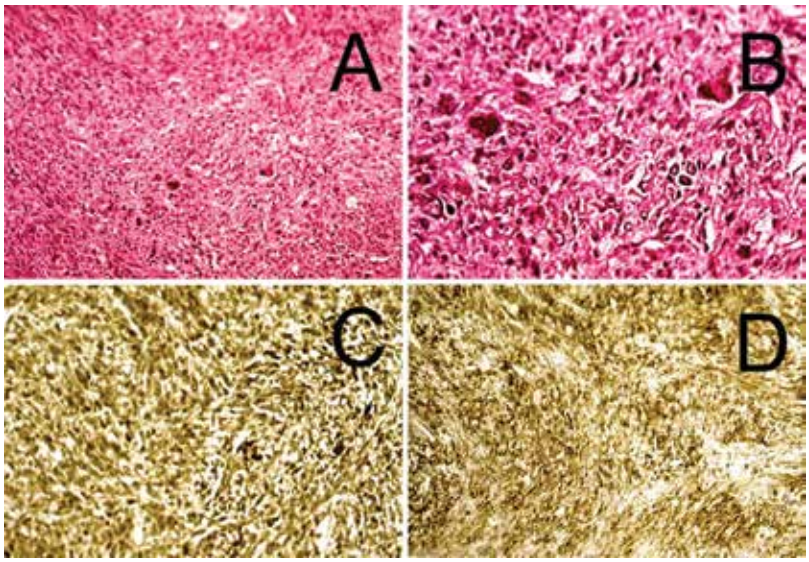

Figure 4. Uterine smooth muscle tumor of uncertain malignant potential (STUMP). (A) Overview, EE, 4x. (B) Nuclear atypia, EE, 20x. (C) p16 positive stain, 10x. (D) Smooth muscle actin positive stain, 10x.

Group 5: irregular margins or vascular invasion at the periphery of the tumor.

Mitotically active leiomyoma, considered as benign variants of leiomyoma, differs from STUMP due to the lacking of recurrences and metastases outside the pelvis [83]. On the opposite side, the difference between LMS and STUMP would rely on the aggressive clinical course, with early recurrence and metastases of the former, and on lower tumor growth and possible delayed recurrence of the latter [83]. The clinical presentation of STUMPs resembles signs and symptoms of uterine leiomyomas: rapidly growing pelvic mass, abnormal uterine bleeding, pelvic pain and vaginal discharge. Risk factors are still unclear, as well as clinical behaviour. The mean age at diagnosis is 45 years and the vast majority of patients are premenopausal women [84].

6.1. Pathological findings

Recent studies characterized the natural history of smooth muscle neoplasms. Physiologically, myometrial stem cells induce cells proliferation and tissue regeneration through strictly regulated processes [85]. Uterine smooth muscle cells undergo multiple cycles of growth and involution induced by oestrogens and progesterone stimulation. These cells also receive paracrine signaling from stem cells, in order to regulate physiologic process [85]. Genetic mutations and chromosomal rearrangements in myometrial stem cells would be induced by repeated endocrine and paracrine stimulation [85]. Mutations and genetic rearrangements would cause unregulated cells proliferation driving smooth muscle tissue towards a spectrum of neoplasia ranging from leiomyomas to LMSs [86]. In particular, the deletion of the short arm of chromosome 1 (1p) has been associated with a possible malignant behaviour of myometrial cells [87].

6.2. Diagnostic imaging

No reliable method is able to pre-operatively distinguish between benign and malignant behaviour of STUMP. Although some MRI features may differentiate tissue intensity, these elements are no specific. Similar to leiomyoma, STUMPs demonstrate homogeneous low signal on T2-weighted images. On the other hand, STUMP and leiomyosarcoma often present with areas of heterogeneous high T2 signal intensity. Recent data would suggest how the combination of hypointense T1 signal, moderate T2 signal intensity and high signal intensity on diffusion-weighted imaging (DWI) might be indicative of a leiomyoma variant or STUMP [88]. The utility of positron emission tomography/computed tomography (PET/CT) is still to be defined.

6.3. Immunohistochemistry

A panel of antibodies such as p16, p53, p21 and Ki-67/MIB1 may be helpful in distinguishing STUMP from leiomyoma and LMS (**Figure 4C**) [1]. Ki-67/MIB1 and p53 expressions are significantly higher in LMS if compared with STUMP. p16 shows a significant increased expression starting from leiomyoma to LMS. Smooth muscle actin is positive in STUMP (**Figure 4D**). A significant difference has been found in PR expression when comparing STUMP and leiomyosarcoma. Bcl-2 is more frequently expressed in leiomyomas with respect to STUMP and LMS. Finally, the expression of Bcl- 2 in STUMP is indicative of a good prognosis [89].

6.4. Therapeutic approaches

6.4.1. Surgery

No standard protocols for the management of patients with suspected STUMP have been defined. Present recommendations are based on guidelines for LMS. Considering the high risk of recurrence, hysterectomy represents the gold standard for women completing their childbearing. Myomectomy followed by hysterectomy after childbearing.is suggested in patients who desired maternity. Since STUMPs may show delayed recurrences, patients with surgically removed STUMP should get CT of chest, abdomen and pelvis at baseline, followed by physical examinations every 6 months for 5 years. When myomectomy is performed for fertility sparing, US evaluation every 6 months, followed by yearly MRI and chest X-ray for 5 years have been proposed [89].

6.4.2. Adjuvant therapies

The usefulness of adjuvant therapy for STUMP is not clear yet, since few studies have been performed. In general, due to the low recurrence rate of these neoplasias, no role has been suggested. If recurrence occurs, surgical excision of the mass is followed by adjuvant therapy, such as pelvic RT. CT (with Doxorubicin and Cisplatin), Medroxyprogesterone or GnRH should be performed [89]. In the presence of metastasis and in premenopausal patients, some authors suggest achieving hormonal suppression to prevent STUMP progression [89].

6.5. Prognosis

STUMP recurrences are observed in about 7% of the cases. The median of survival after recurrence is higher in STUMP than in LMS. In truth, recurred STUMP should be biologically considered as low-grade LMS, even if this diagnosis cannot be achieved until a recurrence develops [90]. In this context, the number of mitosis seems to have the highest value in predicting the clinical behaviour and the prognosis of STUMP [91]. STUMP metastases are rare. They commonly occur in lungs, although the involvement of bones has also been described [1].

In conclusion, the management of STUMPs remains controversial. In general, patients with STUMP should be counseled regarding the potential risk of recurrence. Moreover, because of the risk of metastases even many years after the initial diagnosis, patients with STUMP require a long-term surveillance [92]. These considerations highlight the need of a multidisciplinary approach, which includes gynecologists, gynecological pathologists and oncologists, to early detect disease and to establish the correct management. Finally, the future research should put the attention on the detection of an ideal biomarker, able to predict the outcome of STUMPS and to personalize both surgical and oncological strategies.

6.6. Key points

- STUMPs represent a heterogeneous group of neoplasia and a gray area in diagnostic pathology of uterine sarcomas.
- The vast majority of STUMPs demonstrated a benign behaviour, although follow-up with adjuvant therapy is strongly recommended.
- Immunohistochemistry with Ki67/MIB1 and p53 antibodies may help stratify the prognosis.

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Chapter 3

Uterine Sarcomas: An Updated Overview. Part 2: Endometrial Stromal Tumors

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Abstract

Uterine sarcomas (USs) account for 3–9% of uterine malignant neoplasia and about 5% of all gynaecologic malignancies. Despite their low prevalence, these tumors stimulate a great interest because of their aggressiveness, poor prognosis and high mortality rate. According to the last world health organization (WHO) classification and the International Federation of gynecology and obstetrics committee (FIGO) staging, USs are categorized as pure mesenchymal tumors (endometrial stromal sarcoma, leiomyosarcoma and undifferentiated uterine) and mixed tumors (carcinosarcoma and adenosarcoma). Due to their non-specific signs and symptoms, USs are commonly diagnosed in advanced stage, more often after surgery for a suspected leiomyoma. Although surgery followed by adjuvant therapies represent the common choices for USs, they show poor efficacy due to the early occurrence of metastasis, and the high resistance of tumors to radio-and chemotherapy. Presently, specific expression profiles and new cytotoxic agents are under investigation. In these reviews, we summarized clinical and pathological features, imaging characteristics, therapeutic approaches, genomic and molecular aberration associated with smooth muscle neoplasia (Part 1) and endometrial stromal neoplasia (Part 2); the goal is to understand the biology and the molecular signature of these tumors, in order to focus on their best management.

Keywords: uterine sarcomas, mesenchymal tumors, uterine malignant neoplasia, uterine stromal sarcomas

1. Introduction

Endometrial stromal sarcomas (ESSs) are mesenchymal malignancies mainly occurring in uterine corpus. Alternative origins, such as ovary and peritoneum, have been described. Pathogenesis of ESSs has been widely debated. The rarity of this neoplasia contributed to the difficulty in classifying them into clinically meaningful categories. All started in 1966 with Norris and Taylor, who classified ESS into low-grade and high-grade neoplasia, basing on the degree of mitotic activity [1]. Following studies demonstrated the irrelevant value of mitotic activity as prognostic factor. In 1982, Evans understood the importance of separating tumors with endometrial stromal differentiation from poorly differentiated endometrial sarcoma [2]. Studies from Chang et al. demonstrated that a combined assessment of cytological/nuclear atypia and mitotic index could better provide prognostic information than either feature alone [3].

In 2003, WHO abolished "high-grade" category and adopted a classification based on two categories: low-grade ESS (histological resembling proliferative endometrial stroma) and undifferentiated endometrial sarcoma (UES) [4]. The main perplexity with 2003 WHO classification was related to the heterogeneity of undifferentiated endometrial sarcomas category, which enclosed tumors with different morphology, clinical behavior and outcome. In the following years, cytogenetic and molecular investigations helped to redefine ESSs and, in

FIGO stage	Definition
I	Tumor limited to uterus
IA	Tumor limited to endometrium/endocervix with no myometrial invasion
IB	Less than or equal to half myometrial invasion
IC	More than half myometrial invasion
II	Tumor extended to the pelvis
IIA	Adnexal involvement
IIB	Tumor extends to extrauterine pelvic tissue
III	Tumor invades abdominal tissues (not just protruding into the abdomen)
IIIA	One site
IIIB	More than one site
IIIC	Metastasis to pelvic and/or paraaortic lymph nodes
IV	Tumor invades bladder and/or bowel mucosa, and/or distant metastases
IVA	Tumor invades bladder and/or bowel mucosa
IVB	Distant metastases, including intra-abdominal metastases and/or inguinal lymph nodes

FIGO, International Federation of Gynecology and Obstetrics Committee.

Table 1. 2009-revised FIGO staging system for endomerial stromal sarcomas.

2014, WHO identified four groups of endometrial stromal neoplasia: endometrial stromal nodule-ESN, low-grade endometrial stromal sarcoma-LGESS, high-grade endometrial stromal sarcoma-HGESS, and undifferentiated uterine sarcoma-UUS [5]. Each one demonstrated peculiar molecular signatures, morphological characteristics, and prognosis. FIGO staging system ESSs was revised in 2009 (**Table 1**) [6].

Endometrial stromal sarcoma accounts for approximately 10% of all uterine sarcomas and about 0.2% of all uterine malignant neoplasia. At presentation, the vast majority of patients are in the fifth decade; among these, about 50% are premenopausal women [7]. Although molecular mechanisms involved in the genesis of ESSs are not clear yet, obesity, diabetes, early menarche, and tamoxifen intake have been associated with an increased risk of developing this neoplasia [8]. Abnormal uterine bleeding and pelvic/abdominal pain represent the main symptoms of ESSs, which may be present as uterine mass or endometrial polyp. In the latter cases, endometrial biopsy is more likely to be diagnostic [8]. ESS may most often be an incidental finding in patients undergoing hysterectomy for other reasons; in such cases, pulmonary metastases may be detected at the time of the diagnosis [8].

2. Endometrial stromal nodule (ESN)

ESNs are defined as benign stromal tumors of the uterus. They occur in women ranging from 31 to 86 years, with a mean of about 50 years [9].

2.1. Macroscopic features

ESNs are more common in uterine corpus than in the cervix. They show well defined but expansible margins, absent/minimal myometrial invasion, and no lymph vascular invasion. If myometrial or lymph vascular invasions are present, tumor should be diagnosed as LGESS. ESN may occur as an intramural mass centered in the myometrium, or as a polypoid tumor protruding into the endometrial cavity [8]. On gross examination, ESN size ranges from 1 to 22 cm. The nodule is well demarcated, even if finger-like projections (<3 in number and <3 mm in maximum extension) into adjacent myometrium may occur. In these cases, some pathologists diagnose this neoplasia as "endometrial stromal nodule with limited myometrial infiltration" [8]. The tumor shows a uniform tan-to-yellow soft cut surface; cysts formation, infarct-type necrosis, and hemorrhage are uncommon. If present, cysts may be secondary to necrosis and hemorrhage. Rarely, ESN may exclusively be cystic [8].

2.2. Microscopic features

ESN is composed of cells with uniform round-ovoid nuclei, small nucleoli, and scant-moderate eosinophilic cytoplasms. This diffuse proliferation of monotonous "blue cells" resembles proliferative phase of endometrial stroma (**Figure 1A** and **B**). Cytological atypia is minimal, and although mitotic rate is usually low (up to 5 mitosis/10 HPF), a higher count does not exclude ESN diagnosis [8]. A rich and arborizing network of small arterioles

around which neoplastic cells are concentrically arranged also characterizes ESS [8]. Thick-walled vessels may be present in a minority of cases. The presence of collagen bands or plaques uniformly dispersed in the context of the tumor is often seen (**Figure 1A**). Foamy histiocytes, singly or in clusters are also described, as well as cholesterol clefts. ESNs present some variants including smooth muscle, skeletal muscle, and sex-cord stromal differentiations [8].

2.3. Immunohistochemistry

Vimentin, CD10, actins, WT1, ER, and PR are typically positive in ESN. Rarely, the neoplasia may be CD10 negative. Although, smooth muscle tumors and ESN demonstrate an overlapped immunophenotype, CD10, desmin, h-caldesmon, smooth muscle heavy chain myosin, and HDAC8 facilitate the differential diagnosis. In particular, areas of smooth muscle differentiation stain positive for desmin and h-caldesmon, although areas of stromal differentiation may be desmin positive also; such areas show a typical perinuclear cytoplasmic pattern [8].

2.4. Differential diagnosis

ESN with smooth muscle differentiation can be misdiagnosed as endometrial stromal sarcoma invading the myometrium, due to the presence of interdigitating smooth muscle cells misinterpreted as myometrial invasion [9]. Differential diagnosis would also be needed between ESN, highly cellular leiomyoma, LMS, and LGESS. Highly cellular leiomyoma characteristically shows focal fascicular pattern, margins with a cleft-like zone, and contains thick-walled blood vessels [8]. Uterine LMS does not present as a low-grade neoplasia [8]. Finally, since morphology demonstrates to be not useful in distinguishing between ESN and LGESS, diagnosis relies on the exclusion of myometrial and lymph vascular invasion [8]. A definitive diagnosis of ESN should be provided after a careful examination of tumor borders; thus, specimens from hysterectomy are needed.

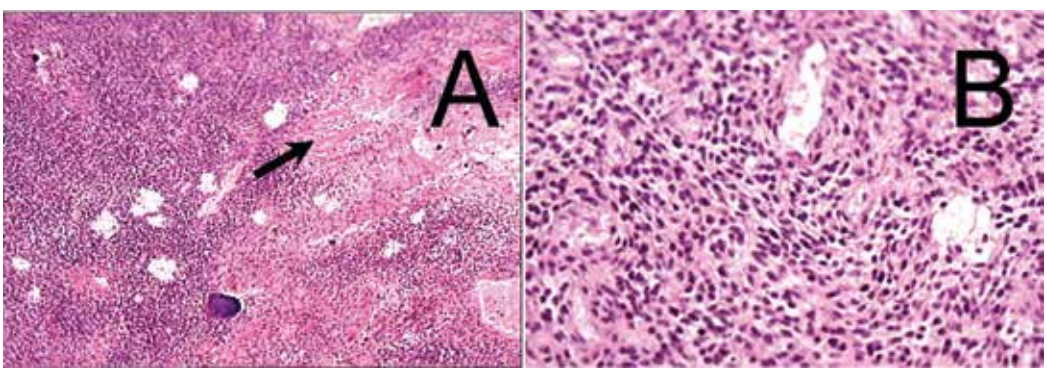

Figure 1. Endometrial stromal nodule. (A) Diffuse proliferation of monotonous blue cells resembling proliferative phase of endometrial stroma. Collagen bands dispersed in the context of the tumor (arrow), EE, 10×. (B) Neoplastic cells show uniform round-ovoid nuclei and scant-moderate eosinophilic cytoplasms, EE, 20×.

On curettage specimens, the distinction between ESN and LGESS is quite impossible. Finally, in ESNs with "limited infiltration," the infiltration is not widespread as showed by LGESS [8].

2.5. Molecular features

ESN is characterized by the chromosomal translocation *t(7,17) (p15;q21)*, resulting in the formation of JAZF1-SUZ12 fusion gene. JAZF1-SUZ12 gene has been found in about 50% of ESNs and in LGESS [10].

2.6. Keypoints

- ESN may demonstrate focal finger-like projections toward myometrium. To confirm ESN diagnosis, the projections should be <3 in number and <3 mm in their maximum extension.
- Both ESN and LGESS share the same genetic aberration; thus, the presence of *t(7;17)* cannot be used to distinguish between the two neoplasia.
- Correlation between immunostain results and morphology may help in distinguishing between ESN with smooth muscle differentiation and pure smooth muscle tumor.

3. Low-grade endometrial stromal sarcoma (LGESS)

This neoplasia is composed of cells resembling those of endometrial stroma during proliferative phase, associated with a broad network of arteriolar-like vessels. The tumor shows infiltrative "tongue-like" growth into the myometrium, with or without lymph vascular invasion.

Accounting for about 0.2–1% of all uterine malignancies, LGESS represents the second most common uterine sarcoma. It is more common than ESN. The age range is similar to that of ESN, more often occurring in perimenopause. The median age is 52 years. No race is favorite [10]. Being a slow-growing tumor with an indolent clinical course, LGESS shown no-specific signs; they include vaginal bleeding and pelvic pain. LGESS most frequently occurs in the uterine corpus, even if extra uterine locations such as ovary pelvis, abdominal cavity, vulva, and vagina are possible. Association with endometriosis has been described [11].

3.1. Macroscopic features

The vast majority of LGESS are diagnosed on hysterectomy that has to be considered as a diagnostic and therapeutic approach. Curettage or myomectomy specimens are not useful, since LGESS diagnosis should substantially rely on the evaluation of the tumor/myometrial interface [8]. Fertility-sparing approaches should be used only in carefully selected cases. Grossly, LGESS is typically poorly defined, but well-circumscribed borders might be found

when myometrial invasion is limited. Neoplastic mass may present as intracavitary or intramyometrial. Similarly to ESN, the cut surface is fleshy with tan-to-yellow or white color. Firmly consistency is reported in the presence of extensive fibrous stroma. Cystic formation, as well as areas of hemorrhage and necrosis might be present [8]. Rarely, LGESS appears as a pure cystic mass.

3.2. Microscopic features

LGESS is characterized by the irregular interface with the myometrium (**Figure 2A**). Myometrial invasion and lymph vascular "tongue-like" patterns represent the cardinal features to put differential diagnosis between LGESS and ESN [8]. Cytological features are identical to those of ESN: monotonous "blue cells," with scant cytoplasm, uniform oval-spindle nuclei, and small nucleoli, typically growing in sheets or storiform patterns (**Figure 2B**). Vessels are thin with hemangiopericytoma-like morphology or, less commonly, thick and placed at the periphery of the neoplasia. Nuclear atypia is not significant and mitotic count is usually less than 5 mitosis/10 HPF ([8], p. 1). Like in ESN, alyne bands, cholesterol clefts in the context of areas of necrosis, and cystic formation may be encountered. Several histological variants of LGESS have been described. In smooth muscle type, smooth muscle component accounts for more than 30% of all uterine mass [8]. On gross examination, this component appears as a firmly area. Microscopically, smooth muscle differentiation is characterized by pink irregular islands of slightly epithelioid cells. Starburst pattern, with a central area of hyalinization and collagen bands radiate toward the periphery, may also be seen [8]. In myxoid and fibroblastic type, neoplastic background is typically hypocellular. Fibroblastic component has been reported in about 50% of ESS with *t(10,17)*. In such cases, a more aggressive behavior has

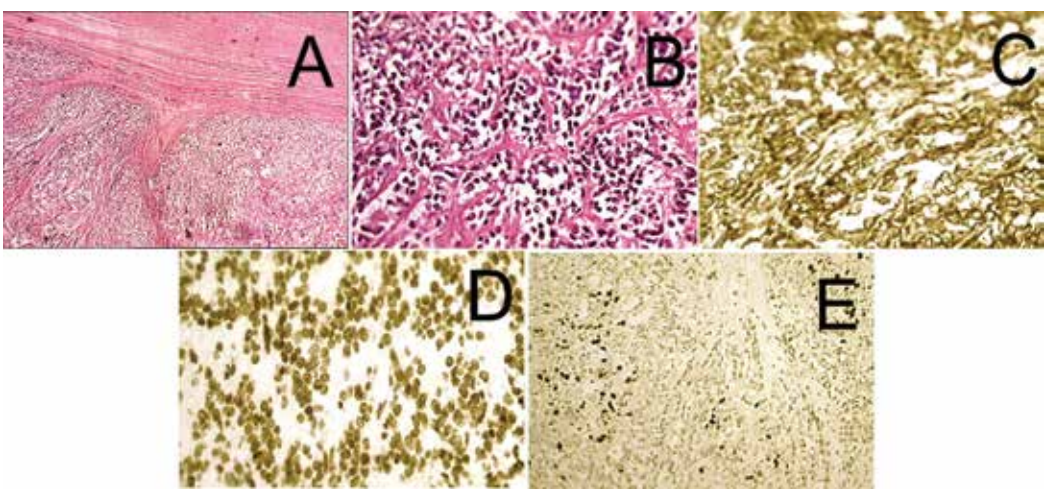

Figure 2. Low-grade endometrial stromal sarcoma. (A) Irregular interface with the myometrium, with 'tongue-like' patterns of invasion, EE, 4×. (B) Monotonous blue cells, with scant cytoplasms and uniform oval-spindle nuclei, EE, 20×. (C) CD10 positive stain, 20×. (D) Estrogens receptor stain, 20×. (E) MIB1 index, 10×.

been demonstrated [8]. Sex cord-like elements, consisting in anastomosing cords, trabeculae, islands, nests, tubules or sheets of cells resembling the pattern typically seen in granulosa and Sertoli ovarian cell tumors, may also be encountered in LGESS. Sex cord-like cells are usually present within the endometrial stroma. Not infrequently, the areas of smooth muscle differentiation may also co-exist [12]. **Glandular elements** showing endometrioid morphology have also been reported in LGESS. Cells have cuboid-columnar shape; with eosinophilic or rarely clear cytoplasm and minimal cytological atypia [1]. **Epithelioid variant** is characterized by cells with oval-polygonal appearance and abundant, often granular, cytoplasm [8]. **Rhabdoid type** shows cells with large and eosinophilic cytoplasmic inclusions, eccentric vesicular nuclei, and prominent nucleoli [13].

Antibody markers	LGESS	HGESS	UUS	UTROSCT
Smooth muscle actin			+, patchy	+
Desmin	+		+, patchy	+
h-cardesmon	+			+
EMA	±		+, patchy	
CD10	++	—	±	
CD34	—			
CD44				
Cytokeratins	±		+, patchy	+
HDAC8				
ER	+	—	- or weakly +	
PR	+	—	- or weakly +	
p53			+	
p21				
Bcl-2				
MIB1			high percentage	
p16			+	
Inhibin				+
S100				
c-kit		+		
Cyclin D1	—	±	—	

LGESS, low-grade endometrial stromal sarcoma; HGESS, high-grade endometrial stromal sarcoma; UUS, undifferentiated uterine sarcoma; and UTROSCT, uterine tumor resembling an ovarian sex-cord tumor.

Table 2. Immunohistochemical features of uterine endometrial stromal tumors.

3.3. Immunohistochemistry

No single marker demonstrated high specificity. Thus, a panel of antibodies should be used (**Table 2**). It is also important to take into account the intensity and the distribution of positive results and to correlate them with histological features and gross findings. CD10 is a very helpful marker, since its expression is generally strong and diffuse in typical LGESS (**Figure 2C**). Positive stain for WT1, ER, and PR is observed in more than 80% of LGESS (**Figure 2D**) [8]. Ki67 mitotic index is high (**Figure 2E**). Cyclin D1 is negative or focally positive [14]. However, in *t(10;17)*, LGESS CD10 may be negative or weakly and focally positive [14–27]. *t(10,17)* LGESSs are frequently negative for ER and PR too [15]. Expression of nuclear β-catenin has been reported in about 40% of *t(10;17)* LGESS, often in association with cyclin D1 positivity [15]. In general, LGESS variants show immunohistochemical pattern concordant with the type of cellular differentiation [15]. Epithelioid LGESS is typically positive for desmin and h-caldesmon, often positive for keratin and EMA, and negative for cyclin D1 [8]. Expression of smooth muscle actin, desmin, and h-caldesmon may be present in typical LGESS [8]. Since CD34 is almost never expressed in uterine LGESS, this marker is helpful to diagnose LGESS of extra uterine sites [15]. p53 is typically absent in LGESS, while it has been reported in HGESS and USS ([8], p. 1). Finally, expressions of PDGFR-α and PDGFR-β were, respectively, found in 50 and 42% of LGESSs [16].

3.4. Differential diagnosis

Differential diagnosis between ESN and LGESS has been previously described. A common problem is to differentiate LGESS from highly cellular leiomyoma. Careful macroscopic and microscopic assessment is needed to put the right diagnosis. Leiomyoma usually forms fascicles of spindle cells with elongated cigar-shaped nuclei; blood vessels are small and do not show the typical arteriolar morphology usually seen in LGESS. In addition, using a panel of antibodies including CD10, desmin, and h-caldesmon, differential diagnosis may be facilitated. It would be always important to correlate immunostain results with morphology [8]. Both LGESS and intravenous leiomyomatosis may show vessel invasion; however, microscopic examination helps to differentiate these entities, since the latter is characterized by a fascicular pattern with large and tick blood vessels [8]. Epithelioid smooth muscle tumor can resemble the smooth muscle variant of LGESS. However, the former lacks of the typical vasculature seen in the endometrial stromal neoplasia. ESS with myxoid differentiation may be confused with myxoid LMS, since both show hypocellularity and a similar pattern of myometrial infiltration. However, LMS usually demonstrates cells with high-grade atypia and brisk mitotic activity [8].

Distinction between LGESS with sex cord-like differentiation and Uterine Tumor Resembling an Ovarian Sex-Cord Tumor (UTROSCT) has to be done. UTROSCT is a rare mesenchymal neoplasm composed of epithelial-like cells showing the typical patterns of the ovary sex-cord stromal tumors; its behavior is benign, without risk of recurrences [17]. Differential diagnosis should rely on the presence of areas of conventional stromal

neoplasia in the LGESSs. Obviously, the distinction cannot be done on biopsy or curettage specimens. Immunohistochemistry may be helpful when using a panel of antibodies including inhibin, calretinin, and Melan A [8]. Stromal component of an adenosarcoma may be morphologically and immunohistochemically identical to that of ESS. However, while the stromal component of adenosarcoma is strictly associated with the epithelial component, in LGESS, glands are few and randomly placed [8]. Finally, GIST comes into differential diagnosis with LGESS; in these cases, a panel of antibodies including c-kit and DOG1 should be used [18].

3.5. Molecular features

The vast majority of LGESSs harbor chromosomal rearrangements.

As previously described, the most common genetic aberration is the *t(7;17) (p15;q21)* translocation that has been found in about 80% of LGESSs and morphological variants. *t(7;17) (p15;q21)* results in JAZF1-SUZ12 gene fusion [19]. Being detected in both ESN and LGESS, *t(7;17)* translocation would represent an early event in the development of ESS. It has been hypothesized that ESS would originate from a benign stromal proliferation, being neoplastic progression the result of additional events [20]. As a counterpart, the lack of JAZF1-SUZ12 gene in most cases of undifferentiated endometrial sarcoma would suggest the existence of a different pathogenesis leading to ESS. Translocation *t(6;17) (p21;p22)* represents the second most common genetic abnormality in LGESS, being the *t(6p;10q,10p)* the third. Other gene fusions, which have been detected in association with ESSs, are JAZF1/PHF1, EPC1/PHF1 MEAF6-PHF1, ZC3H7-BCOR, and MBTD1-CXorf67 ([21], p. 109). JAZF1, SUZ12, PHF1, and EPC1 gene fusions have also been detected in other types of benign and malignant neoplasia [21]. Rearrangement of the X chromosome has also been seen in LGESS, in association with two different transcripts [22]. All of the genes involved in the previously listed chromosomal translocations are implicated in transcriptional regulation. Translocations would lead to oncogenic effects starting from deregulation of transcriptional mechanisms in endometrial stromal stem cells [23]. All of the genetic fusions seem to be equivalent in inducing oncogenic events, since LGESSs having different genotypes show similar clinical behavior. Microscopically, all of the JAZF1 LGESSs demonstrate tongue-like myometrial invasion accompanied by vascular invasion [23]. From a prognostic point of view, both JAZF1-LGESS and LGESS without genetic rearrangements (wild LGESS) usually have a low incidence of recurrences [23]. Finally, although no correlation has been found between morphological variant of LGESS and a specific chromosomal abnormality, PHF1 genetic rearrangement has been frequently observed in LGESS with sex cord differentiation [24].

3.6. Therapeutic approaches

Patients with LGESS are usually treated with hysterectomy plus bilateral salpingo-oophorectomy, the latest to avoid the secondary stimulation of the tumor by ovarian hormones. Younger patients desiring to preserve fertility can be treated with hormonal therapy or aromatase

inhibitors. The adjunct of gonadotropin releasing hormone (GnRH) may reduce ovarian synthesis of estrogens [25]. Since low-grade ESSs show low response rates to conventional CT, there is no evidences supporting its use ex adiuvantibus. On the other hand, the presence of estrogens and progesterone receptors in about 80% of the LGESS would give the opportunity to reduce the recurrence rate and risk of relapse using adjuvant endocrine therapy. Hormone therapy with progestin, aromatase inhibitors, and analogues of the gonadotropin-releasing hormone has become an effective post-surgical treatment in patients with low-grade ESS [26]. Particularly, aromatase inhibitors are becoming the treatment of choice, since progestin is poorly tolerated due to side effects. The duration of hormonal therapy should be protracted for 3–5 years after surgery [26]. Tumors with *t(10;17)* translocation, typically not responding to conventional treatment, should be treated with a more aggressive therapy such as RT and CT combination [23]. Finally, in LGESS showing immunohistochemical positivity for PDGFR-α and PDGFR-β, the PDGF signaling pathway may be considered as a useful therapeutic target for imatinib [26].

3.7. Prognosis

Stage is the most significant prognostic indicator in LGESS. Disease-specific survival is approximately 80–90% at 5 years and 70% at 10 years [26]. Patients with stage I LGESS show a survival rate of about 100% at 5 years and 90% at 10 years; women presenting with high-stage disease have a survival rate of 40%, which is constant at 5 and 10 years [8]. Clearly, tumor grading is also a powerful predictor of disease recurrences, which are observed in about 50% of the patients especially in pelvis, abdomen, and lungs [8]. Recent data demonstrated that stage and mitotic activity correlate with the outcome of the neoplasia, while cytological atypia correlates with increased relapse of LGESSs [8]. A tumor showing both severe cytological atypia and high mitotic rate, with or without the typical endometrial stromal sarcoma growth pattern, should be considered as USS [8]. Cases of USS arising in a background of LGESS have been rarely reported [27]. A tumor with more than 10 mitotic figures/10 HPF without severe cytological atypia should be considered as a pure LGESS. Finally, prognosis of JAZF1-LGESS and wild-LGESS are similar, since no genotype-specific target therapy exists yet [25].

3.8. Keypoints

- Like ESN, LGESS may be well circumscribed on gross examination. Thus, the adequate sampling of the interface neoplastic border/myometrium is imperative to reach a correct diagnosis.
- Biopsy, curettage or myomectomy specimens are not useful.
- Cytological features of LGESS are identical to those of ESN. Nuclear atypia is not significant, mitotic rate is usually less than 5 mitosis/10 HPF, nuclear pleomorphism is absent, and necrosis is rarely present.
- A panel of antibody may help in diagnosis, in tight correlation with gross and microscopical features. In LGESS, cyclin D1 is negative-to-focal, and CD10 and ER/PR are diffusely positive.

- LGESS demonstrate histological variants.
- Since sex cord-like differentiation may occur in LGESS, UTROSCT should be excluded by extensive samples.
- Intravenous leiomyomatosis can be highly cellular and often mimics a LGESS. Intravascular component with cleft-like spaces, fascicular growth pattern, proliferation of spindle cells colonizing the wall of the veins, and thick-walled blood vessels may help to diagnose the former.
- The most common chromosomal translocation in LGESS is *t(7;17) (p15;q21)*, resulting in JAZF1-SUZ12 gene fusion.
- Stage and brisk mitotic activity correlate with the outcome, while cytological atypia correlates with increased relapse.

4. High-grade endometrial stromal sarcoma (HGESS)

The 2014 WHO classification of uterine mesenchymal tumors re-introduced HGESS as a distinct entity [28]. Lee et al. identified a fusion between the tyrosine 3/tryptophan5-monooxygenase gene YWHAE from chromosome 17p13 and the NUT family member gene NUTM (previously known as FAM22) from chromosome 10q22 [29]. The identification of the YWHAENUTM2A/B (also designed as YWHAE-FAM22A/B) gene fusion as a recurrent genetic event in ESS with more aggressive behavior, provided the basis to create a category of ESS intermediate between LGESS and UUS (**Table 3**). Before 2014, HGESS was enclosed in the "undifferentiated endometrial sarcomas" group [30]. Kurihara et al., underlining the heterogeneity of this group, emphasized the importance to distinguish between tumors with nuclear uniformity and YWHAE rearrangement, and tumors with nuclear pleomorphism, more complex karyotypes, and frequent p53 alterations [8]. Separation between LGESSs and HGESSs was also important, due to the peculiar clinical aggressiveness of the latter [31]. The age of women affected by HGESS ranges from thirty to seventy years, with a mean of 50 years. HGESS most commonly presents with abnormal uterine bleeding and symptoms related to extra-uterine spreads [8].

	ESS	**USS**
Age at presentation	Perimenopausal	Postmenopausal
Cytology	Monomorphous	Polymorphic and highly atypical
Growth pattern	Infiltrative	Expansive
Vascular pattern	Intravascular growth	Vascular invasion
Hormonal receptors	Estrogens-related	Not estrogens-related
Prognosis	Good, late recurrence	Poor, early recurrence and distant metastasis

ESS, endometrial stromal sarcoma; USS, undifferentiated uterine sarcoma.

Table 3. Differential diagnosis between endometrial stromal sarcomas and undifferentiated uterine sarcomas.

4.1. Macroscopic features

HGEESs appear as an intracavitary polyp or poorly circumscribed mural plaques-like masses, with a median diameter of 7.5 cm. The cut surface is fleshy and often associated with areas of hemorrhage and necrosis [8]. Being destructive, myometrial invasion of HGESS is different from that of LGESS, which is permeative. Extrauterine extension is frequent [8].

4.2. Microscopic features

At low-power magnification, the morphology of HGESS does not exactly look like the proliferative endometrium. HGESS is characterized by a monomorphic proliferation of round cells arranged in a vaguely nested or pseudo-glandular pattern [8]. The infiltrative growth pattern and the vascularization typical of the LGESSs coexist, together with the features of destructive invasion of the outer half of the myometrium. The tumor often contains both morphologically low- and high-grade areas. Low-grade areas are hypocellular and composed by a uniform population of neoplastic spindle cells, with no apparent nuclear pleomorphism [8]. These areas may also show myxoid appearance. High-grade areas typically show a population of closely packed large (epithelial-like) cells, arranged in nests or cords (**Figure 3A**). The vascular pattern is delicate and arborized (**Figure 3B**) and is different from the spiral arteriolar-like pattern characteristically seen in LGESS. Concentric arterioles may occasionally be present [8]. At high magnification, HGESS cells show eosinophilic cytoplasms; nuclei are large, with irregular contours and prominent nucleoli (**Figure 3A** and **C**). Mitotic activity is brisk. In non-rearranged (wild) HGESS, mitotic count is <5 mitosis/10 HPF, while YWHAE-HGESS shows mitotic rate > 10 mitoses/10 HPF. Coagulative necrosis is absent in wild tumors and present, together with lymph vascular invasion, in rearranged HGESS [8]. Rearranged HGESS always demonstrates uniform high-grade cytomorphology, although high-grade morphology is not always associated with YWHAE genetic rearrangement [32].

4.3. Immunohistochemistry

Low- and high-grade components of HGESS have a different immunohistochemical profile [8]. The low-grade component, similarly to LGESS, stains positive for CD10, ER, and PR. Cyclin D1 is negative-to-focal in rearranged HGESS, while it is diffusely positive in wild neoplasia. CD117

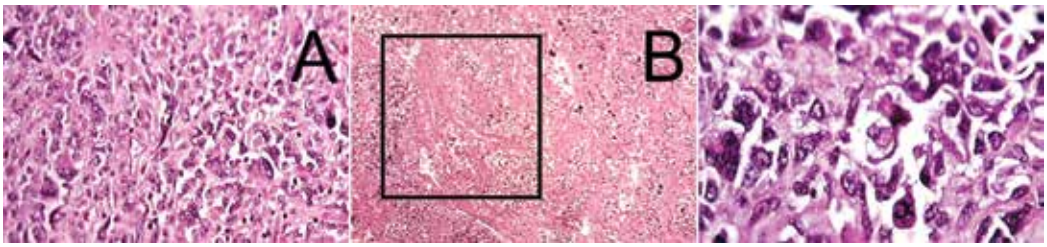

Figure 3. High-grade endometrial stromal sarcoma. (A) High-grade area: proliferation of closely packed large cells with eosinophilic cytoplasms, large nuclei and prominent nucleoli. Cells are arranged in cord pattern, EE, 20×. (B) Delicate and arborized vascular pattern (square), EE, 4×. (C) Pleomorphism and nuclear atypia, 40×.

stains negative [8]. The high-grade component is negative for CD10, ER, and PR in wild tumors and positive in rearranged HGESS [8]. Lack of expression for hormones receptors would have potential treatment implications. CD117 is often positive (**Table 2**) [8]. The different morphological and immunohistochemical features of HGESS may be considered as a surrogate indicator for the underlying genetic rearrangement, even if both fluorescence in situ hybridization (FISH) and reverse transcriptase-PCR (RT-PCR) demonstrated to be more useful [33].

4.4. Differential diagnosis

Adequate sampling is always necessary to diagnose HGESS, since approximately 50% of these tumors contain a low-grade component, which overlaps with LGESS [8]. Low-grade component shows smaller "blue" cells with scanty cytoplasm and smooth nuclear contour, spiral arterioles, infrequent necrosis, positivity for CD10, ER, PR, and negativity for cyclin D1 and c-kit. Moreover, it lacks of biphasic appearance. Differential diagnosis between HGESS and epithelioid LMS should be based on the lacking of a prominent delicate vasculature in the latter. In addition, HGESS does not show a marked atypia. LMS is typically positive for desmin, caldesmon, ER, and PR, but negative (or only focally positive) for cyclin D1 [8]. To differentiate HGESS with intraperitoneal/pelvic locations from GIST, DOG1 antibody is helpful, since it always lack in the former [34].

4.5. Molecular features

As previously shown, genetic fusion YWHAE-FAM22 is characteristic of ESS with high-grade histological features. FISH analysis demonstrated the absolute specificity of YWHAE-FAM22A/B rearrangement in HGESS, since fusion gene has been detected neither in other uterine sarcomas nor in extra-uterine mesenchymal tumors [34]. FISH demonstrated higher sensitivity than RT-PCR in detecting YWHAE-FAM22A/B rearrangement. Croce et al. established that the cut-off of rearranged cells to consider FISH as positive should be 30%; they also recommended to add RT-PCR in borderline cases [35]. Such molecular evidences surely will have diagnostic and therapeutic implications [35].

4.6. Therapeutic approaches

Due to the lack of ER and PR receptors, anti-estrogenic therapy seems to be inappropriate in controlling HGESS growth. Adjuvant CT may provide survival benefit, although experiences are limited [35]. Overexpression of EGFR and Erbb2 has been reported in both HGESS and UUSS. In these cases, treatment with imatinib or trastuzumab may be an option [36]. Recently, overexpression of c-kit has been reported in HGESS carrying the YWHAE/FAM22A/B gene fusion. In these cases, a response to imatinib was also described [36].

4.7. Prognosis

Patient with HGESS typically present with advanced stage disease (stages II–IV). Moreover, in comparison to LGESS, patients with HGESS have earlier (within 1 year after initial surgery) and more frequent recurrences. Particularly, in terms of prognosis, YWHAE-rearranged HGESS is intermediate between LGESS and UUS [37].

4.8. Keypoints

- Myometrial invasion is destructive in HGESS and permeative in LGESS.
- HGESS may morphologically show low- and high-grade areas. Low-grade areas are hypocellular and composed by neoplastic spindle cells, with no nuclear pleomorphism. High-grade areas show round-epithelioid cells.
- Vascular pattern is delicate and arborized.
- It is extremely important to distinguish between rearranged YWHAE HGESS and wild HGESS.
- Mitotic activity in rearranged tumors is <5 mitosis/10 HPF, while it is >10 mitosis/10HPF in wild neoplasia. Coagulative necrosis is absent in the former and present in the latter.
- Mitotic rate should not be used as the unique criterion to distinguish between LGESS and HGESS.
- Distinction between LGESS and HGESS is clinically relevant, since patients with HGESS would have a more aggressive course.
- Cyclin D1 is negative-to-focal in rearranged HGESS and diffusely positive in wild tumors. Immunohistochemistry with CD10 and ER/PR antibodies shows positive results in YWHAE HGESS and negative results in wild HGESS. Due to the lacking of ER/PR receptors, hormonal treatment is not useful in wild neoplasia.
- Because of the frequent positivity for c-kit antibody in both HGESS and GIST, this marker shows low sensitivity. Vice versa, DOG1 is more specific.
- YWHAE-FAM22 fusion gene is characteristic of HGESSs, which show high-grade histological features.

5. Undifferentiated uterine sarcoma (UUS)

In 2014, WHO re-classified gynecological tumors and replaced the old terminology of Undifferentiated Endometrial Sarcoma (UES) with UUS [28]. UUS represents a diagnosis of exclusion, referring to a high-grade sarcoma, which lacks a specific mesenchymal differentiation. UUSs may also enclose poorly differentiated LMS in which smooth muscle cells have been completely replaced by the sarcomatous component [28]. UESs are rare and highly aggressive; they present as intramural mass or intracavitary polyps [8]. Signs and symptoms are non-specific enclosing abnormal uterine bleeding, pelvic mass, or pain due extra-uterine spreads. USS typically occurs in older postmenopausal women [8]. The occurrence of cases with coexisting LGESS component would suggest that USS might also arise from a dedifferentiation of a LGESS [8].

5.1. Macroscopic features

Grossly, cut surface is tan to white, with fleshy consistency. Hemorrhage and necrosis are common [8].

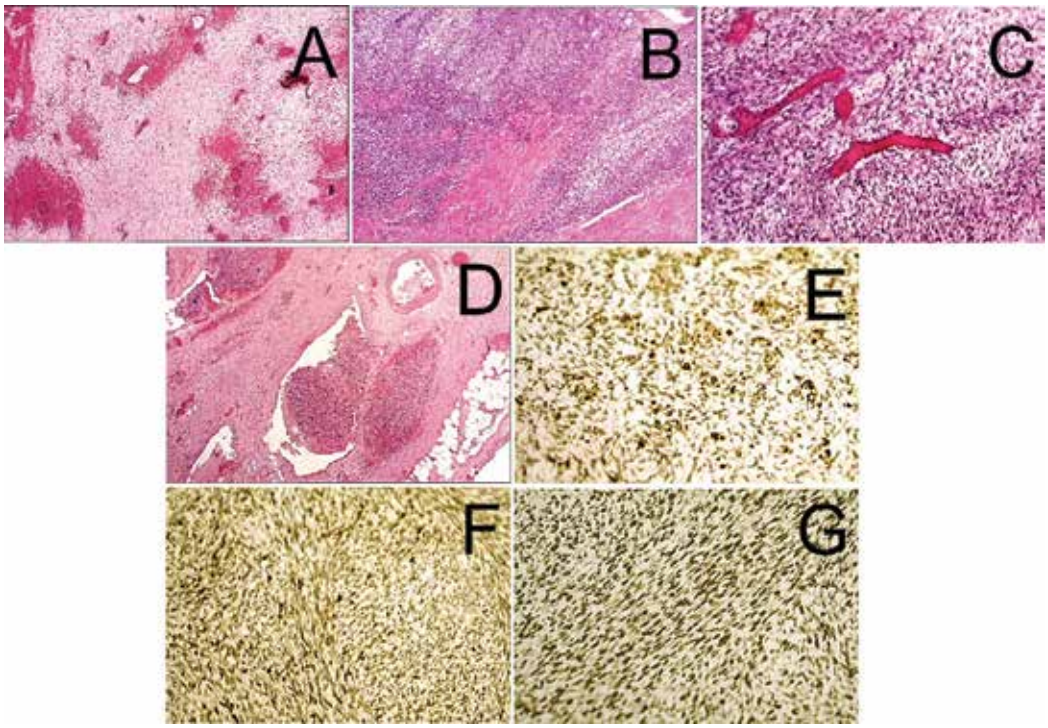

Figure 4. Undifferentiated uterine sarcoma. (A) Tumor-cell necrosis, EE, 4×. (B) Destructive infiltration of the myometrium), EE, 4×. (C) Vascular pattern, EE, 10×. (D) Lymph-vascular invasion with embolism, EE, 20×. (E) CD10 stain, 20×. (F) Desmin stain, 10×. (G) MIB1 index, 10×.

5.2. Microscopic features

UUS shows a combination of severe nuclear atypia, brisk high mitotic rate, and tumor cell necrosis (**Figure 4A**) ([8], p. 1). Histologically, UUS can be distinguished into two histologic variants: uniform UUS (u-UUS) and pleomorphic UUS (p-UUS) [8]. u-UUSs show morphologic and immunophenotypic characteristics of HGESS, and frequently harbor *t(10,17)* rearrangement [38]. p-UUSs show destructive infiltration of the myometrium (**Figure 4B**), highly pleomorphic cells and a fascicular growth pattern not resembling proliferative endometrial stroma [39, 40]. Lymph vascular invasion is common (**Figure 4C** and **D**) [41].

5.3. Immunohistochemistry

Being undifferentiated by definition, the immunohistochemical characterization of UUS it sometimes hard and reflects the heterogenicity of this category of tumors. USSs are typically CD10, p53, and cyclin D1 positive (**Figure 4E**); ER and PR usually stain negative or weakly positive [42]. Desmin, EMA, and keratins may show focal positivity (**Figure 4F**). Smooth muscle actin may be focally positive, although the presence of positive stain for more than one smooth muscle marker should drive to a suspect of LMS [42]. On the other hand, a positive

stain for keratin and EMA should lead to the suspicion of undifferentiated endometrial carcinoma [42]. MIB1 index is usually high (**Figure 4G**).

5.4. Differential diagnosis

Being a diagnosis of exclusion, the suspect of USS should be put after an extensive sampling of the neoplastic mass. Differential diagnosis includes: leiomyosarcoma or rhabdomyosarcoma, which typically show marked cytological atypia and positivity for desmin, h-caldesmon, keratins, and EMA; carcinosarcoma that presents a malignant epithelial component; müllerian adenosarcoma, where benign epithelial cells may be encountered; malignant mixed müllerian tumor, in which the epithelial component is limited [8].

5.5. Molecular features

Genetically, little is known about UUS. In the vast majority of the cases, it has been demonstrated a genetic pathways that is different from those of LGESS and HGESS, although it is not specific (i.e., complex karyotypes, genomic gains, and losses). Particularly, gains of 2q, 4q, 6q, 7p, 9q, 20q and losses of 3q, 10p, and 14q have been detected. A subset of USSs also demonstrated a missense TP53 mutation [15].

5.6. Therapeutic approaches

Patients should be treated with radical hysterectomy and bilateral salpingo-oophorectomy. Adjuvant RT and/or CT are strongly suggested [43].

5.7. Prognosis

Due to their aggressiveness, USSs are associated with a poor prognosis, with an overall survival <2 years [43].

5.8. Keypoints

- USS is a diagnosis of exclusion, lacking of smooth muscle or endometrial stromal differentiation.
- USS diagnosis should be made by extensive sampling of the neoplastic mass, following hysterectomy.
- Histologically, UUS can be distinguished into two histologic variants: uniform UUS and pleomorphic UUS.
- USS is typically CD10, p53, and cyclin D1 positive, while ER and PR are negative or weakly positive. Desmin, EMA, and keratins may show focal positivity.
- USS lacks of a specific molecular pathway, although genetic rearrangements, different from those of LGESSs and HGESSs, have been detected.

6. Imaging in ESS diagnosis

Even if imaging cannot reliably help to diagnose an ESS before surgery, some specific characteristics can be identified. On US, the neoplastic mass is hypoechogenic, with irregular margins. By Doppler, vascularization pattern appears as irregular. On MRI, ESS typically presents as an invasive endometrial mass with wide myometrial involvement and extension along vessels and/or ligaments. ESS also shows intense 18 FDG uptake. The combination of PET and CT demonstrated to be promising for diagnosis [8]. Due to the high incidence of distant metastases at the first presentation, preoperative imaging of the chest and abdomen may be considered when ESS is suspected [8].

7. Therapeutic approaches

7.1. Surgery

Hysterectomy represents the best practice to treat localized ESS. Since ESS typically expresses ER and PR, there would be a higher risk of recurrence if the ovaries are retained; thus, in postmenopausal women, salpingo-oophorectomy should be performed [8]. Tumor morcellation, a widespread technique used for presumed benign disease, demonstrated to have an adverse impact on patient's outcome [8]. The benefit of lymphadenectomy in ESS is still controversial. The incidence of lymph node metastases is generally low, but it is common in higher stages of disease thus resulting in a worse outcome. Overall, systematic lymphadenectomy does not appear to confer a therapeutic benefit. The role of cytoreductive surgery in locally advanced ESS is controversial. Finally, the resection of distant metastases and cytoreductive procedures should be performed in case of recurrent ESS [27].

7.2. Other therapies

7.2.1. Hormonal therapy

The high rate of positive results for hormones receptors has led to interest in using adjuvant hormonal therapy for both early stage and advanced LGESS. Hormonal therapies are generally well tolerated, even if several questions remain controversial, such as doses, regimens (i.e., progestins, gonadotrophin-releasing hormone agonists, and aromatase inhibitors), and duration of therapy. In general, the lack of significant adverse effects would allow the administration for longer periods. Hormonal therapies seem to be effective for metastatic disease also. Conversely, the frequent lack of ER and PR in HGESS and UUS makes the hormonal therapy ineffective in controlling tumor growth [42].

7.2.2. Chemotherapy

Findings regarding the ESS response to CT are scarce, since data from high-grade and low-grade ESS are pooled. In general, the rate of response to CT is low; thus, it should only be used

when hormonal therapies have become ineffective [42]. Anthracycline and/or ifosfamide are presently considered in the first-line therapeutic regimen [41].

7.2.3. Radiotherapy

Although postoperative RT demonstrated some benefits in controlling loco-regional disease, overall survival is rarely improved, since ESSs typically recur distantly. In recurrent or metastatic ESS, palliative radiotherapy is usually used to reduce symptoms [41].

8. Prognostic factors and survival

JAZF1-LGESSs and LGESSs with no demonstrable genetic rearrangements (wild LGESSs) generally show stage 1 at presentation. Their prognosis is excellent with a low risk of recurrence [41]. In comparison, YWHAE–NUTM2 ESSs typically present in advanced stages (stages 2–4); they frequently recur within a few years after the initial surgery [42].

9. Rare sarcomas

9.1. Rhabdomyosarcoma

It is most common in the uterine cervix than in the uterine corpus. Patients typically presents with abnormal postmenopausal bleeding. Uterine mass is typically polypoid, with a fleshy cut surface and areas of hemorrhage and necrosis. Microscopically, sheets of atypical cells with abundant and eosinophilic cytoplasms, large pleomorphic nuclei, and atypical mitosis are seen. Rhabdomyosarcoma stains positive for muscle specific actin and desmin, but it is negative for smooth muscle actin. Expression of WT1, S100, EMA, and keratins is uncommon. The treatment relies on surgery and CT, with or without RT. Adult age, extracervical location, pleomorphism, depth invasion, and distant metastasis are all bad prognostic factors [8].

9.2. Alveolar soft part sarcoma

It frequently arises in uterine cervix of adolescent and young women. Microscopically, alveolar and organoid patterns separated by delicate fibrovascular septa are seen. Cells show abundant and eosinophilic cytoplasms and contain PAS-positive diastase resisting granules or crystals. Nuclei are large and vesicular, with evident nucleoli. Mitosis is rare. The neoplasia typically shows strong TFE3 positivity. Vimentin, smooth muscle markers, CD10, S100, NSE, and HMB45 may also be positive. Surgery represents the elective treatment [8].

9.3. Angiosarcoma

It presents as a hemorrhagic and diffusely infiltrating mass, often simulating a leiomyoma [8]. Neoplastic cells show vascular differentiation; they stain positive for the vascular endothelial markers CD31, CD34, cyclin D1, and vimentin. The lymphatic endothelial marker D2-40 is absent [8].

9.4. Liposarcoma

The fat cell tumors of the uterus are extremely rare, showing an incidence of 0.03–0.2%. The histogenesis of this tumor is not completely clear. The theory of "tumor metaplasia" has been postulated. Uterine liposarcoma shows an aggressive behavior [8].

10. Conclusions

The in-depth investigations of US biology have certainly improved the therapeutic approaches to these malignancies. Presently, a great number of agents are under investigation. TKI, mTOR inhibitors, growth factors/growth factor receptor inhibitors and antiangiogenic agents, represent the most promising drugs. SARC028 and the anti-PD1 antibody pembrolizumab (also called MK.3475) for advanced sarcomas, and nivolumab plus ipilimumab for patients with unresectable sarcoma, are in phase II trials [43]. Pazopanib (a selective multi-targeted inhibitor of tyrosine kinase receptors), trabectedin, and eribulin have been recently approved. On the other side, immunotherapy did not show potential benefits for US. It is our opinion that future genetic research will significantly allow a better identification of the molecular signatures of US, in order to provide the optimal treatment strategy for these malignancies [43–48].

At the end of the present work, which represents the effort to synthesize all of the updated findings on US, we need to emphasize the importance of interdisciplinary approach to achieve the correct diagnosis and the optimal management for this neoplasia.

The availability of clinical and laboratory information, as well as pharmacological anamnesis, would allow pathologist to put the correct diagnosis of US. Moreover, the continuous research of novel therapeutic approaches would guarantee the updated management of these malignancies.

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Section 2

Pathobiology of Cancer

Chapter 4

Novel Mechanism of Nonalcoholic Lipid Accumulation Promoting Malignant Transformation of Hepatocytes

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Zhizhen Dong and Dengfu Yao

Additional information is available at the end of the chapter

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Abstract

The incidence of hepatocellular carcinoma (HCC) is steadily increasing in worldwide, which has been a public concern significantly associated with diabetes and non-alcoholic fatty liver disease (NAFLD) is an emerging risk factor with increasing prevalence nowadays, with gradually instead of HBV and HCV, aflatoxin, or alcohol liver disease as major etiological factors. The deeply worrisome aspects of these high risk factors are their large spread in population. Systemic and genetic mechanisms involved in malignant transformation of liver cells as well as useful biomarkers at early stage of HCC are being investigated. However, the exact mechanisms from NAFLD to HCC still remain to be explored. In this paper, some advances of liver lipid accumulation were summarized on the relationship between NAFLD and hepatocytes malignant transformation.

Keywords: nonalcoholic fatty liver disease, hepatocellular carcinoma, metabolism

1. Introduction

Hepatocellular carcinoma (HCC) is one of the fifth most common malignant tumors, the third most frequent cause of cancer mortality worldwide [1, 2], and ranks the second in China among all malignancies with its mortality almost equal to its morbidity, especially in the inshore area of the Yangtze River [3, 4]. The principal treatment of HCC patient is surgical resection or liver transplantation, depending on whether the patient is a suitable transplant candidate [5, 6]. However, in most HCC patients with diagnosis at early stage is very difficult, thereby excluding the patients from definitive surgical resection. Sorafenib, the most commonly used systemic therapy, has shown to only minimally impact on patient survival

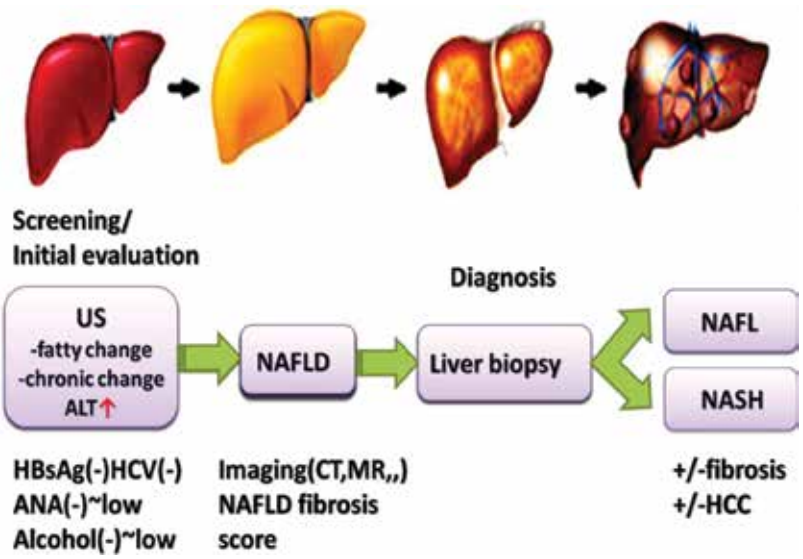

Figure 1. NAFLD progression and clinical diagnosis. ALT: alanine aminotransferase, NAFLD: non-alcoholic fatty liver disease, NAFL: nonalcoholic fatty liver, NASH: non-alcoholic steatohepatitis, HCC: hepatocellular carcinoma, HBsAg: hepatitis B surface antigen, HCV: hepatitis C virus, ANA: antinuclear antibody.

by several months. Besides, neither chemotherapy nor radiotherapy are generally effective. Due to the poor prognosis of HCC patients, the early diagnosis and effective therapy of HCC are needed with several being in development, either in preclinical or clinical studies [7, 8].

The development of HCC is a complex multi-step process involved multiple genes. Major risk factors of HCC include hepatitis B virus (HBV) or hepatitis C virus (HCV) infection, alcoholic or non-alcoholic fatty liver disease, nitrosamines, aflatoxin, and other harmful substances [9–12]. Chronic persistent infection of nonalcoholic is still the main pathological factor of inducing cirrhosis and HCC. However, with the changes of people's dietary structure and lifestyle, the incidence of fatty liver disease (FLD) also rose sharply [13–15]. A median prevalence of alcoholic fatty liver disease (AFLD) and nonalcoholic fatty liver disease (NAFLD) is 4.5 and 15.0%, respectively [16, 17]. It is worrying that if no interference is conducted in the treatment, nonalcoholic steatohepatitis (NASH) or alcoholic hepatitis can also be developed for liver fibrosis, cirrhosis and liver cancer (**Figure 1**), and its exact mechanism is worth exploring. However, the underlying molecular mechanisms that lead to malignant transformation of infected liver cells still remain to be explored. Most of HCC patients died quickly because of the rapid tumor growth, and surgical operation or liver transplantation still is the only effective treatment for HCC [18, 19]. This article summarizes new advances on relationship between NAFLD and hepatocytes malignant transformation.

2. Mitochondria and fatty β-oxidation

2.1. Mitochondria

Liver is one of the most important organs in human for maintaining energy supply and lipid metabolism [20, 21]. The peroxisomal compartment in hepatocytes hosts several essential

metabolic conversions. Upon nutrient deprivation, cells metabolize fatty acids (FAs) in mitochondria to supply energy. FAs mobilization depends on triacylglycerol lipolysis, whereas autophagy feeds the lipid droplet pool for continued fueling of mitochondria. Proteome imbalance of mitochondrial electron transport chain in brown adipocytes leads to metabolic benefits [22]. Lipid metabolism are defective in peroxisomal disorders that are either caused by failure to import the enzymes such as carnitine palmitoyltransferase (CPT) in the organelle or by mutations in the enzymes or in transporters needed to transfer the substrates across the peroxisomal membrane (**Figure 2**). Hepatocytes specific differences have been confirmed in mitochondrial DNA maintenance and expression [23]. Hepatic pathology is one of the cardinal features in disorders of peroxisome biogenesis and peroxisomal β-oxidation, although it rarely determines the clinical fate. Besides of the morphological changes, the impact of peroxisome malfunctions on other cellular compartments includes thermal instability of carnitine palmitoyltransferase II (CPT-II) variants in mitochondria and endoplasmic reticulum (ER) [24–26]. Proteomics analysis revealed numerous enzymes expression involved with the electron transport system, the tricarboxylic acid cycle, as well as lipid and amino acid metabolism in response to anoxia exposure [27].

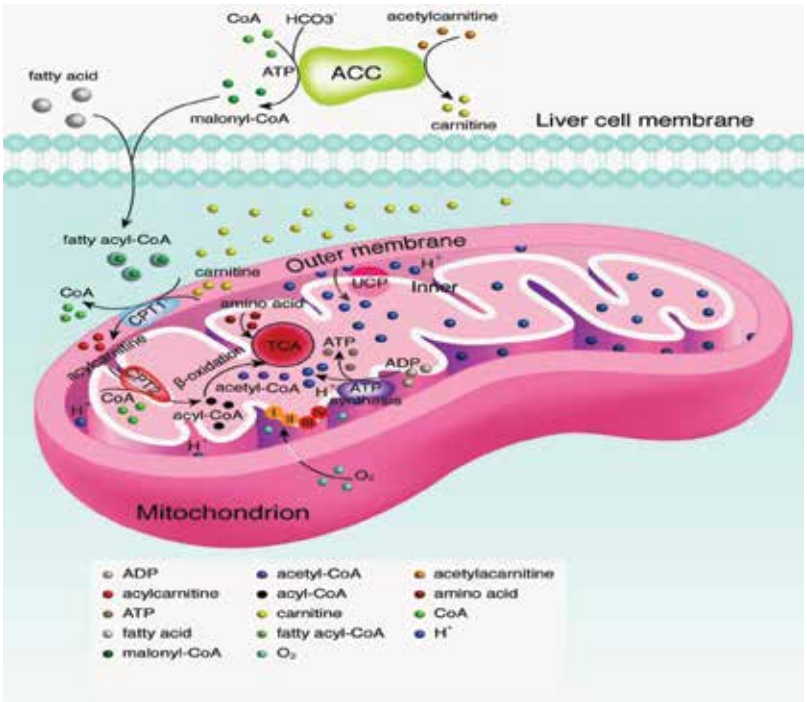

Figure 2. Fatty acid oxidation and ATP production in mitochondria. The distribution of mitochondrial CPT-I or CPT-II with regulation plays important roles in fatty acid metabolism. Fatty acid (FA) β-oxidation requires successive carnitine acyltransferases to translocate acyl-coenzyme As (acyl-CoAs) from the cytoplasm into matrix. As initial and rate-limiting CPT-I generates acylcarnitines that traverse mitochondrial membranes via specific transporters into matrix, CPT-II produces acyl-CoAs from acylcarnitines for FA β-oxidation to acetyl-CoA. Then carnitine crosses the inner membrane, binds with the endogenous or exogenous acyl CoA to prevent acyl CoA accumulation causing poisoning. ACC: acetyl CoA carboxylase, CoA: coenzyme A, TCA: tricarboxylic acid cycle, UCP: uncoupling protein, I II III IV: electron transfer complex [29].

2.2. Carnitine level

Carnitine is a physiological substance that is essential for the proper metabolism of fat and energy production that actually transports both long and medium fatty acid chains. L-carnitine attracts long and medium fatty acid chains, breaks them down, and carries them to the mitochondria of the cells where they are metabolized (burned). The L-carnitine plays important roles in the catabolism of long-chain fatty acids in the mitochondria, not only due to increased mitochondrial fatty acid oxidation reflected by increased mitochondrial biogenesis, but also to changes in plasma clearance and reduced triacylglycerol (TAG) biosynthesis [28]. The ultimate result is that you burn more fats, and in the process give your body more natural energy. In the previous study, the increasing liver weight with lipid accumulation was discovered during the course of the wild-type mice (**Figure 3**) in circulating carnitine analogues [3-(2,2,2-trimethyl hydrazinium) propionate dihydrate, THP] [29, 30].

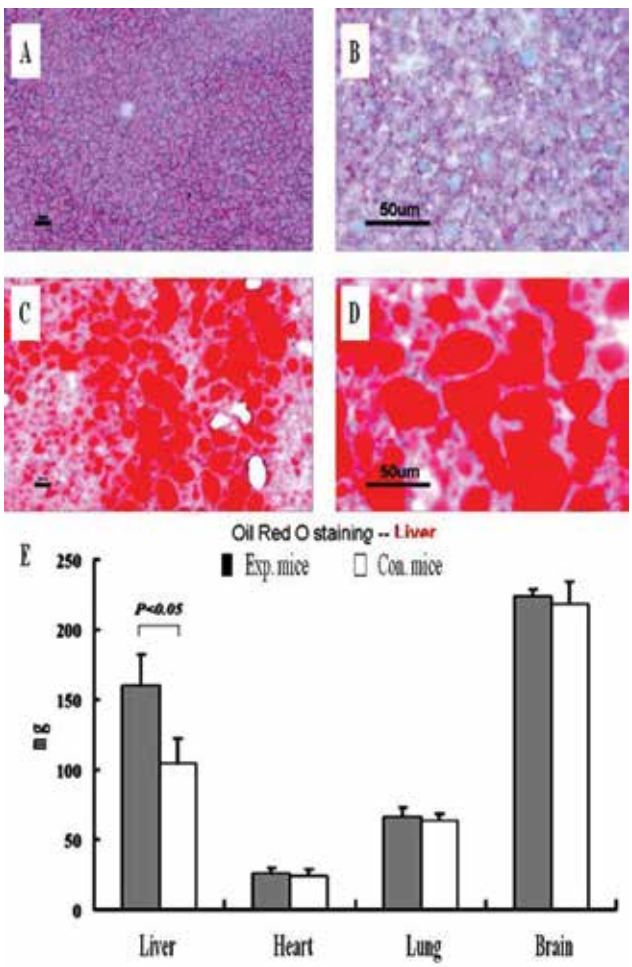

Figure 3. Liver lipid accumulation and liver weight tissues in mice models after carnitine analogues. A–D, the mice liver tissues with Oil red O staining: A & B, the control livers; C & D, the experimental livers; E, the alterations of different tissue weight after the experimental mice with carnitine analogues [29].

2.3. Carnitine palmitoyltransferase (CPT)

Hepatic CPT-II is a mitochondrial protein which is transported to mitochondrial inner membrane. It together with CPT-I oxidizes long-chain fatty acids in mitochondria. Defects or mutation of this gene are associated with mitochondrial long-chain fatty-acid oxidation disorders. Decreasing of its activity is a disorder of mitochondrial fatty acid oxidation with autosomal recessive mode of inheritance. The variants exert a dominant-negative effect on the homotetrameric protein of the enzyme (**Figure 4**), with reduced activities, thermal instability, fatty acid β-oxidation decreased to 30–59%, intracellular ATP to 48–79%, a significantly decreasing of mitochondrial membrane potential with increasing temperature at

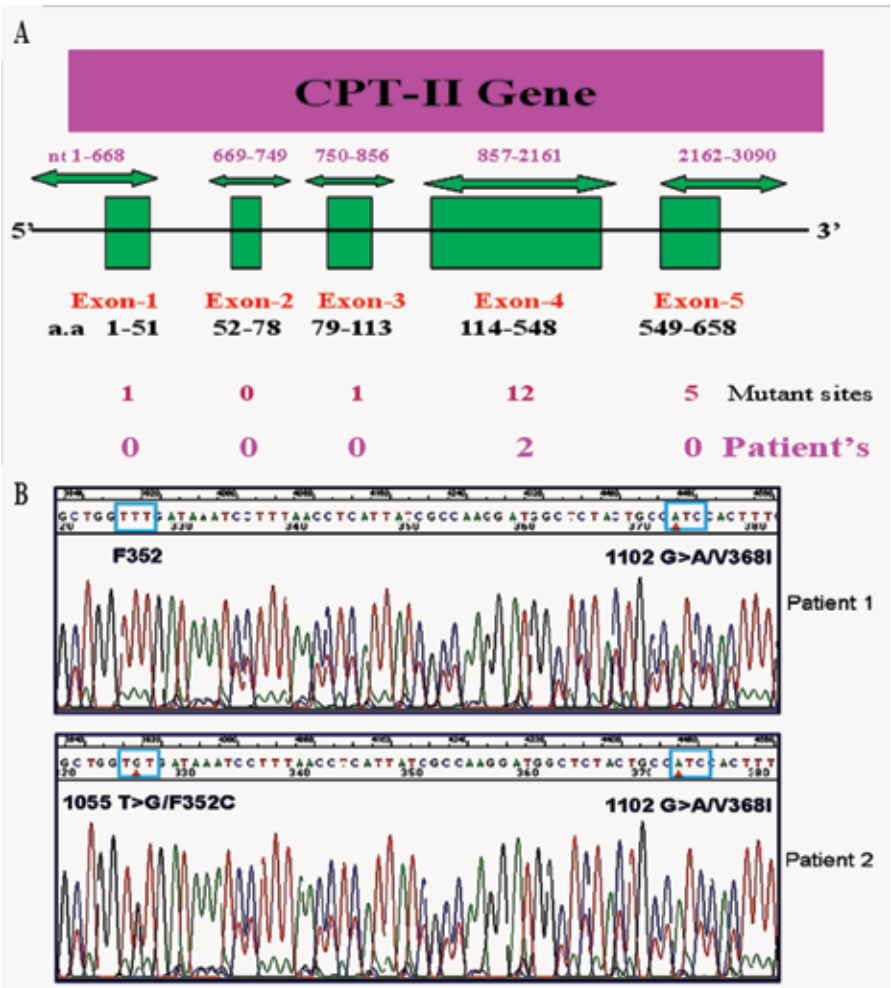

Figure 4. Mutation of CPT-II gene and hepatic lipid accumulation. (A) The CPT-II gene exon 1–5. (B) The sequence fragments of CPT-II gene exon 4 were amplified on mitochondrial inner membrane. The mutation analysis of CPT-II gene exon-4 using the specific primers were designed by sequencing with 1974 nucleotides coded 658 amino acids. Compared with the original sequence from Genbank, the two substitution sites were found at 1618 (G→A) and 1858 (T→C), and code amino acids at V368I and F448L, respectively [29].

41°C, and shortening half-lives of CPT-II, and the enzyme variant proteins were polyubiquitinated and rapidly degraded by a lactacystin-sensitive proteasome pathway [24]. The very unstable CPT II variants with decreased enzymatic activities may bring mitochondrial fuel utilization below the phenotypic threshold during high fever in humans with hepatitis virus infection, and thus might be as novel potential mechanisms for NAFLD formation [31, 32].

The dynamic alterations of hepatic CPT-II expression in the mitochondrial inner membrane were investigated during the malignant transformation of hepatocytes induced by abnormal fatty accumulation. After the male Sprague-Dawley (SD) rats were fed with control, high fat (HF), and HF containing 2-fluorenylaceta-mide (2-FAA) diet, respectively. The rats were divided into control, fatty liver, degeneration, pre-cancerous, and cancerous groups according to the hematoxylin and eosin staining (H&E) of liver pathological examination, hepatic lipids accumulation were confirmed with the Oil Red O staining. Massive lipid accumulation hepatocytes were seen in rats on HF and HF containing 2-FAA diets. The lipid levels in the control group were significantly lower than those in the fatty liver, degeneration, precancerous, and cancerous groups. The serum triglyceride and total cholesterol levels in the degeneration, precancerous, and cancerous groups were 2–3 times higher than those in the control group. The serum aspartate aminotransferase and alanine aminotransferase levels (**Figure 5**) in the degeneration, precancerous, and cancerous groups were significantly higher (4–8 times) than those in the control group. The specific concentration (μg/mg protein) of liver CPT-II expression was significantly reduced during hepatocyte malignant transformation, as confirmed by immunohistochemistry, with the CPT-II levels significantly lower in the cancerous group than in any of other groups, indicated that low hepatic CPT-II expression might lead to abnormal lipid accumulation in hepatocytes, which should promote the malignant transformation of hepatocytes [33, 34].

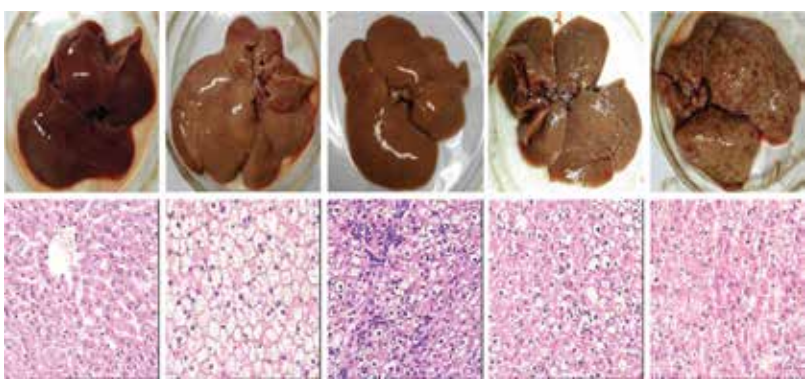

Figure 5. Rat liver tissues and their pathological examination. Liver alterations after the rats (from left to right: upper, A, B, C, D, and E; under A1, B2, C3, D4, and E5) were sacrificed at different time according to the plan schoule. (A) A representative liver from rat with normal diet; (B) a representative liver from the rat with high-fat diet (HFD) without 2-fluorenyl acetamide (2-FAA); (C) a representative liver from the rats with HFD containing 2-FAA at early stage; (D) a representative liver at interim stage; and (E) a representative liver at later stage. The liver sections were examined with hematoxylin and eosin staining and then divided into the control (A1), fatty liver (B1), degeneration (C1), precancerous (D1), and cancerous (E1) groups; A1-E1: The original magnification of the corresponding rat liver sections was ×200 [29].

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3. Abnormal liver lipid accumulation

3.1. Lipid accumulation

Lipid accumulation in liver or HCC will cause tumor-associated molecular signaling alteration including NF-κB (nuclear factor-kappa B), JNK (c-Jun N-terminal kinase)/activation protein-1 activation, and alterations of HCC development-related genes, respectively. For example, liver unsaturated fatty acids (UFA) inhibit the expression of phosphatase and tensin homolog (PTEN) deleted on chromosome 10 (10q23.3) via activating NF-κB/mTOR (mammalian target of rapamycin) complex [35]. As a tumor suppressor gene PTEN regulates the PKB/akt (serine-threonine kinase protein kinase B) pathway, and PTEN deficiency induces the proliferation of hepatocytes by inhibiting cell apoptosis and promoting HCC formation confirmed in mice models with the PTEN deficiency in resembling non-alcoholic steatohepatitis (NASH) features with developing steatosis, and inflammation damages or fibrosis in liver tissues [36].

DNA injury affects hepatic lipid metabolism. Reactive oxygen species (ROS) is an important factor in carcinogenesis. It can be induced in NAFLD patients with contiguous DNA damage by some of hepatic inflammatory cytokines or hepatitis virus infection and react with poly-unsaturated fatty acids derived from hepatocyte membrane phospholipids, and subsequently results in reactive aldehydes production as lipid oxidation (LPO) byproducts, for example, 4-hydroxynonenal (4 HNE) that can react with DNA to form mutagenic exocyclic etheno-DNA adducts. Importantly, they are preferably formed in codon 249 of TP53, resulting in inactivation of tumor suppressor p53 gene, secondary growth advantage, and anti-apoptosis [37].

3.2. Adipokines

Adipokine is a plethora of pro- and anti-inflammatory cytokines that secretes from adipose tissue with low-grade inflammation. Adiponectin and leptin have evolved as crucial signals in many obesity-related pathologies including (NAFLD) [38–40]. Adiponectin regulates the metabolism of blood glucose and hepatic fatty acid, and is decreased in NAFLD that might be critically involved in the pro-inflammatory state associated with obesity and related disorders, overproduction of leptin, a rather pro-inflammatory mediator, is considered of equal relevance [41, 42]. An imbalanced adipokine profile in obesity consecutively contributes to metabolic inflammation in NAFLD, which is also associated with a substantial risk for developing HCC in the non-cirrhotic stage of disease [43, 44]. Both related to liver tumorigenesis especially in preclinical models, especially in hepatic satellite cell activation with stimulating the tissue inhibitor of metalloproteinase 1 production via the JAK/STAT (Janus kinase/signal transducer and activator of transcription) pathway promoting fibrogenesis [45, 46], or angiogenesis or progression from NASH to HCC that has been confirmed in mice models [47, 48].

According to the data from animal models with HCC cell lines, adiponectin could increase JNK activation and induce cell apoptosis with AMPK alteration, which could inhibit mTOR phosphorylation, xenograft growth, tumor growth and metastasis by suppression of tumor angiogenesis in nude mice. However, lower circulating adiponectin favors tumorigenesis in NASH model [49]. Adipose-derived tumor necrosis factor is a potent activator of pro-oncogenic

pathways involving in mTOR, JNK, NF-κB, and extra-cellular signal-regulated kinases; Interleukin-6 (IL-6) combining with its receptors on liver or non-parenchymal cells can promote signal-transmuting receptor (gp130) complex with IL-6R activating JAK1 signaling, and STAT3 activation or phosphorylation promotes the proliferation and anti-apoptosis of cancer cells [50], indicated that adiponectin coefficient action from adipose tissues and related- cytokines affect fatty acid metabolism and hepatocyte malignant transformation via many signal molecules.

4. Inducing roles of related proteins

4.1. Sterol regulatory element-binding proteins (SREBPs)

Lipid reprogramming has been considered as a crucial characteristic in HCC initiation and progression. SREBPs are the key transcription regulators of hepatic lipogenesis, and activate hepatic steatosis at the early stage. Tat-interacting protein 30 (TIP30) is a tumor suppressor protein that has been found to be expressed in a wide variety of tumor tissues that is involved in the control of cell apoptosis, growth, metastasis, angiogenesis, DNA repair, and tumor cell metabolism. TIP30 regulates lipid metabolism in human HCC by regulating SREBP1 (sterol regulatory element-binding protein 1) through the Akt/mTOR signaling pathway [51, 52]. In human HCC tissues, SREBP1 could significantly induce lipogenesis and be associated with a poor prognosis [53].

SREBP1c gene at mRNA level was up-regulated in human HCC tissues and not in their adjacent non-cancerous or non-cancerous liver tissues. The inhibition of SREBP1 expression resulted in growth arrest and apoptosis of cancerous cells, and increased the cell proliferation ability. HBx (HBV protein X) expression induces lipid accumulation in hepatic cells mediated by the induction of SREBP1, a key regulator of lipogenic genes in the liver. HBx interacts with LXRalpha (liver X receptor alpha) and enhances the binding of LXRalpha to LXRE (LXR-response element), thereby resulting in the up-regulation of SREBP1 and fatty acid synthase, suggested that HBV infection can stimulate the SREBP1-mediated control of lipid accumulation [54].

4.2. Loss of tripartite motif 24 (TRIM24)

Aberrantly high expression of TRIM24 occurs in human HCC clinical samples and positively correlated with HCC tumor grade. Its knockdown inhibits proliferation and migration in HCC cells *in vitro*, with impeding of tumor growth *in vivo* [55, 56]. TRIM24 in mice is reportedly a liver-specific tumor suppressor, and appears to promote liver tumor development via AMPK signaling [57]. TRIM24 as an epigenetic co-regulator of some gene transcription that directly or indirectly inhibits mouse hepatic lipid accumulation, liver cell inflammation, liver fibrosis, and hepatocyte damage. Additionally, the global expression analyses of TRIM24$^{-/-}$ livers unveiled signaling pathways that closely associated with some features of NAFLD, inflammatory, cell apoptosis, and hepatocyte damage. The loss of liver TRIM24 expression could lead to the progression from patients with NAFLD to NASH or HCC in a time dependent manner [58].

4.3. Osteopontin (OPN)

According to accumulating data, human liver OPN is a multifunctional protein involved in some pathological alterations including hepatic immunity, hepatocytes inflammation, liver

fibrosis, and the development of HCC. Deficiency of OPN in obese mice fed with a high-fat diet reduced hepatic steatosis and inflammation, and liver cell ballooning, portal leukocyte infiltration and macrophage accumulation were attenuated. It is induced by Hedgehog signaling, may directly promote pro-fibrogenic responses in steatohepatitis, or act as a paracrine factor secreted by bile duct or natural killer T cells (NKT), and also can be as an autocrine factor promoting fibrosis in hepatic satellite cells (HSC) [59].

The silencing OPN gene transcription by specific shRNA could result in increasing Bax, decreasing Bcl-2/Bcl-xL and X-linked inhibitor of apoptosis protein expression, and NF-κB activation, and induction of mitochondria-mediated apoptosis in HCCLM3 cells [19]. There were statistically significant differences in plasma OPN levels between the HCC group and the other groups. Regarding the validity of plasma OPN was a predictor of fatty change, with 50% diagnostic accuracy, 70% sensitivity, 45% specificity, 50% positive predictive value, and 75% negative predictive value at a cutoff value of 134 ng/mL. The data indicated that plasma OPN level could be of diagnostic potential value in NAFLD [60].

5. Promoting role of related cells

5.1. Hepatic satellite cells (HSC)

Human hepatic satellite cells (hHSC) in the perisinusoidal space between sinusoids and hepatocytes are the predominant fibrogenic cells in liver tissues, and activated by liver cell injury to transdifferentiate from a quiescent state to proliferate matrix producing myofibroblasts [61–63]. The excessive production of extra-cellular matrix might result in cirrhosis occurrence. Human amphiregulin could increase the cell proliferation via EGFR, PI3K, and p38 mitogenic signaling pathways, inducing significantly up-regulation of fibrogenic biomarkers, confirmed by the mice NASH model that exhibited rapid progression of advanced fibrosis and HCC, with mimics histological, immunological and transcriptomic features of NASH, and a useful tool for preclinical drug testing [64, 65]. In addition, fatty liver as a pro-metastasis microenvironment with hHSCs could promote HCC migration and proliferation. Fasting and specific microRNAs could inhibit hHSC activation or potentiate anti-cancer activity of Sorafenib in HCC [66, 67].

5.2. Immune cells

Activated immune cells interact with cells in tissues by metabolic stress will migrate to liver and drive the progression from NAFLD to HCC. The dysregulation of lipid metabolism in NAFLD from mice models or human samples causes a selective loss of intrahepatic CD_4^+ but not CD_8^+ T lymphocytes, leading to accelerated hepatocarcinogenesis via cross-talk with liver cells [68, 69]. The NKT cells primarily cause steatosis in liver tissues via secreted a type II trans-membrane protein (a TNF ligand super-family member, TNFSF14), and both of CD_8^+ and NKT cells cooperatively induce liver injury by feeding choline-deficient high-fat diet [70]. CD_4^+ T lymphocytes have greater mitochondrial mass than CD_8^+ T lymphocytes and generate higher levels of mitochondrially derived reactive oxygen species (ROS) [71].

Disruption of mitochondrial function by linoleic acid, a fatty acid accumulated in NAFLD, causes more oxidative damage than other free fatty acids such as palmitic acid, and mediates

selective loss of intrahepatic CD_4^+ T lymphocytes. Hepatic immune cells recognize cell injury or pathogen invasion with intracellular or surface-expressed pattern recognition receptors, subsequently initiating signaling cascades that trigger the release of factors promoting inflammatory response during NAFLD progression, demonstrating that the transition from NASH to HCC through liver cell lymphotoxin-β receptor (LTβR) and NF-κB signaling. *In vivo* blockade of ROS reversed NAFLD-induced hepatic CD_4^+ T lymphocyte decrease and delayed NAFLD-promoted HCC [72, 73].

5.3. Polyploidization

Polyploidization is one of the most dramatic genomic changes with rarely reported. The physiological events occur in liver development or adult life. However, the pathological polyploidization takes place in NAFLD, a widespread metabolic disorder that maybe is a risk factor for HCC. The liver parenchyma in NAFLD models displayed the process alterations with a large ratio of highly polyploid mononuclear cells, but was not observed in normal liver parenchyma. Biopsies from NASH patients revealed the alterations in hepatocyte ploidy compared with tissue from controls. Hepatocytes from NAFLD mice revealed that progression through the S/G2 phases of the cell cycle was inefficient and associated with activation of a G2/M DNA damage checkpoint, which prevented activation of the cyclin B1/CDK1 complex. The oxidative stress promotes the highly polyploid cells, and antioxidant- treated NAFLD hepatocytes resumed normal cell division and returned to normal state of polyploidy, indicated that oxidative stress promote pathological polyploidization in NAFLD that might contribute to HCC [74].

6. Alterations of small molecules

6.1. Oxidative stress

NAFLD is characterized by excess lipids in hepatocytes, due to excessive fatty acid influx from adipose tissue, de novo hepatic lipogenesis, in addition to excessive dietary fat and carbohydrate intake [44, 19]. Serious imbalance was found between limited antioxidant defenses and excessive formation of reactive species produced by liver oxidative stress such as ROS or RNS (reactive nitrogen species). Obese persons could increase free fatty acids uptake, stimulates FA oxidation for compensating excessive liver fat storage, and accelerate β-oxidation leads to increased production of ROS that damage mitochondrial membrane and DNA [75, 37].

Chronic lipid overload in hepatocytes induces mitochondrial oxidative stress or hepatocytes damage leading the NAFLD developing into a more severe liver disease condition, NASH, cirrhosis or HCC. Oxidative stress may induce endoplasmic reticulum (ER) dysfunction for liver malignancy. ER plays an important role in NAFLD pathogenesis, and consecutive increasing oxidative stress, inflammation and activation of NF-κB and JNK signaling pathways lead to the accumulation of intracellular lipids [76]. Extra-cellular signal-regulated protein kinase (ERK) is highly expressed in HCC via PIK13 activation. Among others, copper is one of the main bio-metals required for the preponderance of the enzymes involved in physiological redox reactions, which primarily occurs during mitochondrial respiration. Antioxidant food

agents recognized to improve NAFLD and its complications have been described in the copper-related literatures [77, 78].

6.2. Insulin resistance

NAFLD is associated with insulin resistance (IR) leading to a resistance in the antilipolytic effect of insulin in adipose tissue with an increase of FFAs. The increase of FFAs induces mitochondrial dysfunction and lipotoxicity [79]. Liver steatosis defined as lipid accumulation in hepatocytes is very frequently found in adults and obese adolescents. Etiologically, obesity and IR or excess alcohol intake are the most frequent causes of liver steatosis. Insulin as a key hormone regulates lipogenesis and lipolysis in adipose depots. The adipose tissue becomes resistant to the antilipolytic effect of insulin and FA release is increased with lipolysis or lipid intake, promoting triglyceride synthesis with lipid accumulation occurrence in livers [80, 81].

Liver lipid accumulation causes IR by the activation of NF-κB pathway and leads to hyperinsulinemia to activate phosphatidylinositol-3 kinase (PI3K)/Akt signaling pathway, implicated the malignant transformation of hepatocytes or in hepatocarcinogenesis [82]. Hyperinsulinemia up-regulates insulin-like growth factor-1 that stimulates cell proliferation and inhibits cell apoptosis [83]. Insulin activates insulin receptor substrate-1 (IRS-1) with up-regulating expression in HCC [84]. The IRS-1-mediated related-signaling molecules may act as survival factors, promote liver cell proliferation via mitogen-activated protein kinase and PI3K, and protect against transforming growth factor β1-induced apoptosis in HCC progression [85, 86].

6.3. Iron deposition

Liver is the main storage site for iron in the body because of its rich reticuloendothelial system [87]. Acquired hepatic iron overload is seen in a number of NAFLD patients. The dietary iron supplementation enhances experimental steatohepatitis induced by long-term high-fat diet feeding rats [88]. Excess liver iron may increase NASH risk and its progression to HCC [89, 90]. Abnormal iron deposition in liver is more frequent in NASH patients, in which necroinflammation may be the driving factor. Iron and the coexistence of hyperinsulinemia are risk factors for NASH development and together they may contribute to insulin resistance, disease progression and HCC. Iron reduction has been proposed as treatment for dysmetabolic iron overload syndrome and NAFLD or iron deprivation can suppress HCC growth *in vivo* and *in vitro* experiments [91].

6.4. Alcohol

While tobacco and alcohol are established risk factors for HCC, the most common type of primary liver cancer [92]. Chronic alcohol intake results in the induction of liver cytochrome P_{450} 2E1 leads to generation of ROS with direct or indirect carcinogenic consequences [93]. Many genetic factors regulating alcohol metabolism could predispose in developing alcoholic pancreatitis or cirrhosis. Some studies revealed that alcohol could be metabolized by oxidative and non-oxidative. The main oxidative pathway includes alcohol dehydrogenase, aldehyde dehydrogenase, and cytochrome P_{450} 2E1. In addition, neurocan in neuronal tissue is also expressed in liver and the common polymorphism of its gene rs2228603 is associated with HCC in alcoholic liver disease [94].

6.5. MicroRNA (miR)

Regulating control miRs are highly conserved, small non-coding RNAs (about 18–25 nucleotides in length) regulates transcription or translation of target genes and fatty acid metabolism. Both of miR-197 and miR-99 were associated with liver fibrosis in NASH patients. Altered miRNA expression was associated with activation of major hepatocarcinogenesis-related pathways, including the TGF-β, Wnt/β-catenin, ERK1/2, mTOR, and EGF signaling. The over-expression of the miR-221-3p and miR-222-3p and oncogenic miR-106b~25 cluster was accompanied by the reduced protein levels of their targets, including E2F transcription factor 1, phosphatase and tensin homolog, and cyclin-dependent kinase inhibitor 1. miR-93-5p, miR-221-3p, and miR-222-3p have been confirmed over-expressed in HCC. Aberrant expression of miRNAs may have mechanistic significance in NASH-associated liver carcinogenesis and may serve as an indicator for the development of NASH-derived HCC [94, 95].

Some studies found that miR-122 is a key regulator of glucose and lipid metabolism in livers [96] and significantly higher circulating miR-122, miR-34a, and miR-16 expression were found in NAFLD. During the development of NAFLD patients with simple steatosis to steatohepatitis, the serological levels of miR-122 and miR-34a were positively correlated with disease severity, liver enzyme activities, fibrosis staging, active inflammation, and silencing of microRNA-122 is an early event during hepatocarcinogenesis from NASH [97], suggesting that the alteration of circulating miR-122 could be an early event from NASH to hepatocarcinogenesis.

7. Genetic factors

The development and progression of NAFLD are determined by environmental and genetic factors [10, 98]. The effect of genetic factors has been demonstrated by familial studies, twin studies and several cross-sectional studies. The data from the genome-wide association studies (GWAS) have shown that patatin-like phospholipase domain-containing protein 3 (PNPLA3) involved in metabolism of triglyceride on chromosome 22 is a genetic factor that promotes NASH development, and PNPLA3 gene variant I148M showed a strong relationship with the development and progression of NAFLD, NASH, and NAFLD-related HCC. Single nucleotide polymorphism (SNP, rs738409) is closely related to fatty liver involved in fibrosis progression of NAFLD. The C<G variation in SNP rs738409 also increases HCC risk in NAFLD patients [99, 100].

The whole exome sequencing finds that apolipoprotein B mutations (c.6718A>T, K2240X) represent a paradigm of rare variant influencing liver fat content and HCC risk. Besides, the Patatin-like phospholipase domain-containing 3 [the trans-membrane 6 superfamily member 2 (TM6SF2)] genes variant E167K was associated with NAFLD [101, 102]. Telomerase reverse transcriptase (TERT) mutations have been associated with hepatic steatosis. The deficiency of TERT can reduce the response to liver damage inducing the formation of steatosis and fibrosis. In conclusion, the occurrence of NAFLD-HCC seems to be influenced by common genetic variants as PNPLA3 and by rare genetic variants. Several genes have been proposed as candidate genes to be associated with NAFLD based on case–control studies [103].

8. Microbiota and toxic substances

8.1. Gut microorganisms

NAFLD has become the most common chronic liver disease worldwide and is well-accepted that gut dysbiosis is associated with NAFLD [103]. The gut-liver axis has been proposed as a key player in the pathogenesis of NAFLD, as the passage of bacteria-derived products into the portal circulation could lead to a trigger of innate immunity, which in turn leads to liver inflammation. In intestine, there are trillions of microorganisms including bacteria, archaea, yeasts and viruses collectively called intestinal ecosystem through energy harvesting and fat storage [78, 104]. The relationship between gut microbiota and NAFLD is dependent on levels of choline, bile acid, larger production of endogenous ethanol, higher prevalence of intestinal dysbiosis, higher prevalence of increased intestinal permeability, bacterial translocation, pro-inflammatory molecules, endotoxemia, and cytokines. The hepatic manifestation of the dysregulation of insulin-dependent pathways leads to IR and adipose tissue accumulation in NAFLD patients with liver injury, indicated that the gut liver-axis is the way by which the bacteria and their potential hepatotoxic products (LPS, DNA, RNA, etc.) can easily reach liver [105, 106].

The interaction between the gut epithelia and some commensal bacteria induces the rapid generation of ROS. The main goal of any therapy addressing NASH is to reverse or prevent progression to liver fibrosis/cirrhosis [78]. Recently, a new isoform of human manganese superoxide dismutase (MnSOD) has been shown to be a powerful antioxidant capable of mediating ROS dismutation, penetrating biological barriers via its uncleaved leader peptide, and reducing portal hypertension and fibrosis in rats affected by cirrhosis [107]. Primary bile acids which derived from cholesterol become secondary bile acids under the action of intestinal microbes. If the bile acids bind to G-protein-coupled cell surface receptor (TGR5), it could inhibit inflammation via suppressing NF-κB pathway in macrophages. Many genetic and environmental factors have been suggested to contribute to the development of obesity and NAFLD, but the exact mechanisms might be the issue of further investigations [108, 109].

8.2. Toxic substances

NAFLD has been implicated in some conditions such as IR, obesity, metabolic syndrome, hyperlipemia, hypertension, cardiovascular disease, and diabetes. Dietary or genetic obesity induces alterations of gut microbiota, thereby increasing the levels of deoxycholic acid, a gut bacterial metabolite known to cause DNA damage [78, 110]. Glyceraldehyde-derived advanced glycation end-products (Glycer-AGEs) are the predominant components of toxic AGEs (TAGE). More data suggested that TAGE with its receptor might change intracellular signaling, pro-inflammatory molecules gene expression, and also elicited the oxidative stress generation in liver cells including hHSCs. Circulating TAGE levels were significantly higher in NASH patients than those with simple steatosis or healthy subjects. Moreover, their TAGE levels inversely correlated with adiponectin. Increased lipid availability in livers might provide ATP and structural support for cancerous cell proliferation [111, 112].

9. NAFLD in hepatocarcinogenesis

Recent epidemiological studies have identified NASH, a progressive form of NAFLD, as a major risk factor for HCC. Elucidating the underlying mechanisms associated with the development of NASH-derived HCC is critical for identifying early biomarkers for the progression of the disease and for treatment and prevention [97, 113].

Liver derangements in lipid metabolism, importing FFA and manufacturing, storing, and exporting lipids could lead to NAFLD development [114]. The dysregulation of hormonal axes, mitochondrial carnitine palmitoyltransferase-II inactivity, and cytokines in NAFLD promotes a worse cycle between metabolic and inflammatory stimulus lead to malignant transformation of hepatocytes [33, 71]. The majority of NAFLD patients had steatosis about 20% present as NASH that was defined by microscopic finding, and consists of liver injury, steatosis, parenchymal and portal inflammation, and different fibrosis. Alterations of miRNA in hepatocarcinogenesis were associated with TGF-β, Wnt/β-catenin, ERK1/2, mTOR, and EGF signaling pathways. Importantly, miR-93-5p, miR-221-3p, and miR-222-3p were also significantly overexpressed in human HCC. Aberrant expression of miRNAs might have mechanistic significance in NASH-associated liver carcinogenesis and serve as an indicator for the development of NASH-derived HCC [115, 116].

Hepatic lipid accumulation is accompanied by distinct patterns of perilipin expression, suggested that abnormality of hepatic lipid accumulation might promote hepatocyte malignant transformation [33]. The levels of high leptin and low adiponectin are hallmarks of obesity and involved in NAFLD and carcinogenesis [117]. Obesity-promoted HCC occurrence was dependent on increasing IL-6 and TNF levels, which resulted in liver inflammation and oncogenic STAT3 activation. The long-term chronic inflammatory in obesity plus higher IL-6 and TNF might be the risk factor for HCC [118]. The prospective studies (25,337 patients with HCC) demonstrated that both of excess body weight and obesity in males or females are related to an increased risk factor for HCC occurrence [119]. The prospective studies with longer follow-up periods should screen the malignant transformation of hepatocytes with specific biomarkers among NASH or NAFLD populations [3, 120].

10. Perspectives

In the past decade, the discussion of substantially NAFLD increased by hypernutrition and HCC had become a cocktail party cliché, and its impact on public health cannot be dismissed. With both relationship gradually deepening, more and more evidences have supported that NAFLD might promote the malignant transformation of hepatocytes because of liver lipid accumulation, its toxicity, endoplasmic reticulum dysfunction, IR, and abnormal fat metabolism. Although the exact mechanisms from NAFLD tumor-promoting mechanism triggered by hypernutrition remain to be explored [121], however, the patients with the excessive fat deposition feeds this tumor-promoting inflammatory flame and should be treated in time to avoid the occurrences of hepatocyte malignant transformation [7, 122].

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Abbreviations

CPT	carnitine palmitoyltransferase
HBV	hepatitis B virus
HCV	hepatitis C virus
HSC	hepatic satellite cell
IL-6	interleukin-6
NAFLD	non-alcoholic fatty liver disease
NASH	nonalcoholic fatty hepatitis
NF-κB	nuclear factor kappa B
MiR	microRNA
OPN	osteopontin
HCC	hepatocellular carcinoma
PNPLA	patatin-like phospholipase domain-containing protein
ROS	reactive oxygen species
SREBP	sterol regulatory element-binding protein
TAGE	toxic advanced glycation end-products

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Chapter 5

Glucose Metabolism and Carcinogenesis: The Impact of the Tumor Suppressor p53

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Additional information is available at the end of the chapter

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Abstract

The tumor suppressor protein, p53 responds to cellular stress such as DNA damage, oncogenic activation and hypoxia by transactivating downstream genes that are responsible for apoptosis, DNA repair, senescence, cell cycle arrest and cell cycle progression. However, emerging trends show that p53 also plays multifaceted roles in regulating glucose metabolism. It promotes oxidative phosphorylation, suppresses glycolysis at multiple points as well as controlling glutamine and lipid metabolism. Current findings suggest that p53 actions have potential to influence the Warburg Effect, that is, characteristic of cancer cells. The Warburg phenomenon is characterized by their preference for glycolysis to oxidative phosphorylation for ATP generation, irrespective of adequate oxygen supply. This is often in concomitance with enhanced glucose uptake and leads to increased lactate production and anabolic processes such as lipid synthesis and *de novo* nucleic acid synthesis. The molecular underpinnings of the Warburg Effect are still poorly understood. These important differences between cancer and normal cells have induced interest in glucose metabolism as a drug target. This chapter focuses on the influence p53 exerts on glucose metabolism as well as on the implications of the Warburg phenomenon in carcinogenesis and a review of the ever-increasing number of p53 regulators.

Keywords: p53, glucose metabolism, carcinogenesis, Warburg effect, RBBP6

1. Introduction

Most normal differentiated cells rely primarily on oxidative phosphorylation and, to a lesser extent, on glycolysis for the generation of ATP. Cancer cells prefer glycolysis to oxidative phosphorylation for ATP generation from glucose even in the presence of adequate supply

of oxygen, a phenomenon that was first described by Otto Warburg in 1927 [1]. This often occurs alongside rapacious uptake of glucose and leads to increased lactate production and elevation of the pentose phosphate pathway (PPP). In addition to synthesizing nucleotides, the PPP generates large amounts of nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) conferring anti-oxidant properties to cancer cells and thereby protecting them from potential damage by reactive oxygen species [2].

Previously, Hannan and Weinberg identified and reviewed six hallmarks of cancer [3]. The Warburg effect is now recognized as the seventh [4]. Given the multifaceted interventions by p53 in glucose metabolism, it would not be surprising that this potent tumor suppressor exerts substantive influence on the Warburg effect. p53 is tightly regulated in normal physiology but is activated by post-translational modifications following one or more of several cellular stresses such as DNA damage, oncogenic activation, hypoxia, ribonucleotide depletion and telomere erosion [5]. p53 activation triggers a suit of signaling cascades by either p53 transcription-dependent or -independent pathways. In normal cellular conditions, wild type p53 is kept under tight regulation mainly by the Mouse double minute (MDM2) which uses its E3 ligase activity to ubiquitinate p53 thereby tagging it for proteasomal degradation [6]. This stringent control of p53 is consistent with its well-known role in maintaining genomic and cellular integrity.

Over the years, many negative regulators of p53 have been identified further complicating the functional map of this ubiquitous tumor suppressor. Given recent findings about the importance of p53 in glucose metabolism, it is necessary to reassess the extent to which metabolic reprogramming and absence of functional p53 contributes to carcinogenesis. p53 regulates glucose metabolism at multiple points effecting outcomes such as repression of glucose transport, inhibition of glycolysis, positive influence on oxidative phosphorylation and control of glutamine and lipid metabolism **(Figure 1).**

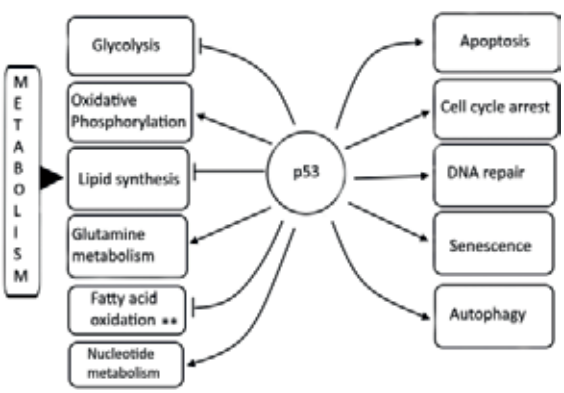

Figure 1. p53, although well-known for inducing apoptosis and cell cycle arrest for tumor suppression, it also employs several strategies via metabolic pathways for tumor suppression such as blocking glucose transport, inhibiting glycolysis, boosting oxidative phosphorylation, promoting glutamine metabolism, enhancing fatty acid oxidation in low glucose conditions and inhibiting lipid synthesis. p53 also promotes nucleotide metabolism. The asterisks indicate that the enhancement of fatty acid oxidation by p53 is only in conditions of low glucose (1 mM).

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2. The impact of p53 on glucose metabolism

p53 is mutated in more that 50% of all cancers. Moreover, the development of many anti-cancer drugs often depends on the status of the *p53* gene in the targeted cancer. Recent findings show p53 intervenes in the glucose metabolic pathways at multiple points **(Figure 2).** Overall, p53 inhibits glycolysis and promotes oxidative phosphorylation **(Figure 2).**

2.1. Control of glucose transport

The first rate-limiting step for glucose metabolism is the uptake of glucose which is facilitated by glucose transporters at the cell membrane such as GLUT1-4. These receptors are often over-expressed in many cancers. Hence, carcinogenesis is associated with increased glucose uptake and glycolysis. This is mediated by glucose transporters. Consequently, glucose uptake is used for diagnosis and monitoring of cancer and metastasis in patients using positron emission tomography (PET) imaging technique that is based on consumption of the glucose analog 2-(^{18}F)-fuoro-2-deoxy-D-glucose by cells [7, 8]. Under physiological conditions, p53 monitors glucose uptake by suppressing the expression of transporters such as GLUT1 and GLUT4 by directly binding to the p53 response elements in their promoters [9]. This indicates that p53 can inhibit energy metabolism by obstructing cellular glucose uptake. p53 can also indirectly repress glucose intake via Glut3 by activating the IKK-NFκB pathway. In p53-deficient cells, there is an increase in aerobic glycolysis and upregulation of Glut3 accompanied by increased

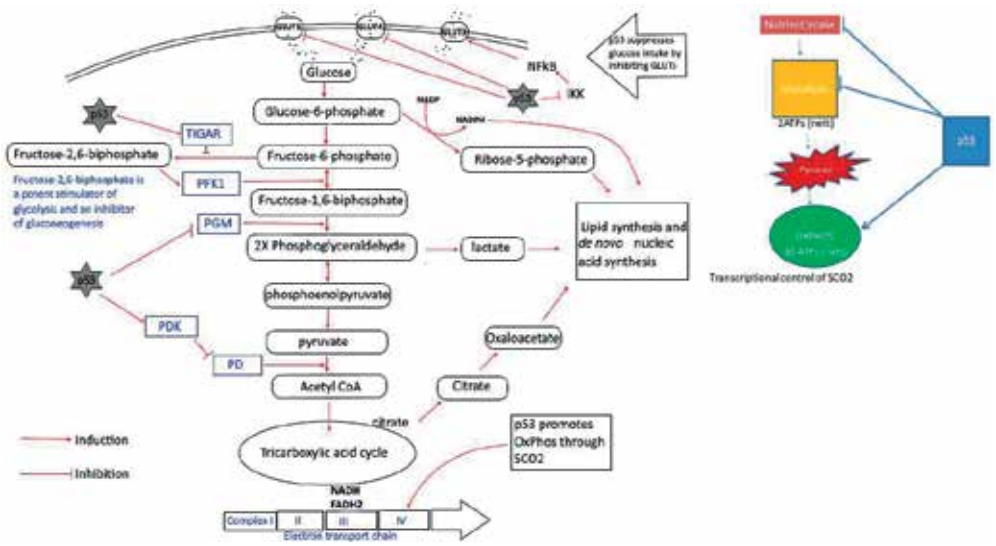

Figure 2. p53 inhibits glucose influx by directly repressing the expression of GLUT1 and GLUT4. However, p53 indirectly inhibits GLUT3 expression by repressing NFkB transcriptional activity on GLUT3 through inhibition of I kappa B kinase (IKK). p53 also represses glycolysis by inhibiting phosphoglycerate mutase expression and activating TP53-induced glycolysis and apoptosis regulator (TIGAR) which suppresses glycolysis. OXPHOS can be promoted by p53 through the activation of SCO2 at Complex IV. p53 can inhibit PDK which can phosphorylate pyruvate dehydrogenase and prevent its catalysis of pyruvate to acetyl-CoA. INSERT: p53 inhibits glycolysis and boosts OXPHOS.

activity of the IKK-NFκB axis. This accelerated glycolysis can be suppressed by elimination of the p65/NFκB complex and restored by expression of Glut3 [10]. These observations indicate that glycolysis may also influence the IKK-NFκB pathway which is activated by inflammatory molecules such as cytokines, reactive oxygen species (ROS) and by radiation.

2.2. Suppression of glycolytic pathway by p53

The p53 target gene, *TP53-induced glycolysis and apoptosis regulator* (*TIGAR*) intervenes at the third step of glycolysis by dephosphorylating fructose,2,6-phosphate, which is a potent stimulator of glycolysis and an inhibitor of gluconeogenesis, to fructose-6-phosphate **(Figure 2)**. p53 also modulates the transcription of TIGAR by binding to p53 response elements in the promoter region, thereby controlling the levels of fructose,2,6-phosphate. Hence, p53 can block glycolysis by activating TIGAR and lowering the levels of fructose-2,6-biphosphate, which is a potent activator of phosphofructokinase-1 that is responsible for converting fructose-6-phosphate to fructose1,6-bisphosphate. Thus, the activation of TIGAR inhibits glycolysis and shunts glucose breakdown through the pentose phosphate pathway [11, 12]. It is expected then that loss of p53, which occurs in most cancers, would lead to increase in fructose,2,6-phosphate and in glycolysis.

Phosphoglycerate mutase (PGM) is a glycolytic isomerase that catalyzes step 8 of glycolysis, converting 3-phosphoglycerate to 2-phosphoglycerate and is repressed by wild type p53 in mouse embryonic fibroblasts (MEFs). When expression of PGM is elevated, high glycolytic flux is observed and the MEFs are immortalized [13]. Furthermore, PGM is upregulated in p53 null cells. Evidence shows that PGM controls a critical step in glycolysis where the process is shunted to the pentose phosphate pathway (PPP) because when it is depleted by shRNA or by an small molecule inhibitor, glycolysis is reduced, PPP flux occurs and cell proliferation and tumor growth are inhibited [14, 15].

p53 also represses the transcription of pyruvate dehydrogenase kinase 2 (PDK2) thereby inhibiting the conversion of pyruvate to acetyl coA and production of lactic acid in breast carcinoma cells (MCF7). This underlines that p53 plays a pivotal role in establishing the Warburg Effect whereby pyruvate is converted to lactate even in abundant oxygen conditions [16].

Taken together, the above observations show that under normal physiological conditions, p53 inhibits glucose metabolism while boosting oxidative phosphorylation (OXPHOS). When p53 is dysfunctional, as it happens in many cancers, this scenario is reversed a situation that is consonant with the Warburg Effect.

2.3. p53 influence on lactate metabolism

Pyruvate, which is the product of glycolysis, is the key branch point at which glucose metabolism can go either to lactate production or to the tricarboxylic acid cycle (TCA). The conversion of pyruvate to acetyl-Coenzyme A, which enters the TCA, is catalyzed by pyruvate dehydrogenase complex (PDC). The enzyme pyruvate dehydrogenase kinase isoenzyme-2 (Pdk2), which inactivates acetyl-CoA production by phosphorylating the PDC, is negatively regulated by p53 [16]. Lactate is no longer regarded as merely a waste product of glycolysis, but

also as a significant regulator of carcinogenesis and metastasis, acting as fuel and as a signal molecule that promotes angiogenesis via VEGF [17, 18]. Moreover, the conversion of pyruvate to lactate is enhanced in neoplastic cells and utilizes NADH and H^+ to yield the NAD^+ that is used in the conversion of glyceraldehyde-3-phosphate to 1,3-biphosphoglycerate which refuels glycolysis. Tumor cells generally export the excessive lactate along with protons via monocarboxylate transporters (MCT) instead of utilizing it as a nutrient. This leads to an acidification of the tumor microenvironment and contributes to immune evasion. Lactate export establishes low pH and induces the production of pro-inflammatory cytokines by inflammatory cells in the tumor microenvironment. The cytokine expression is facilitated by "lactate response elements" in the promoters of the genes encoding these cytokines [19]. Wild type p53 directly interacts with the promoter of the MCT1 gene and represses it. Consequently, cancer cells that are deficient in p53 have elevated levels of MCT1 and proliferate. This phenotype also enables them to modulate import or export of lactic acid depending upon glucose availability [20]. Various isoforms of MCT including MCT1, MCT4 and chaperone CD147, are highly increased in renal cell carcinomas and also indicate poor prognosis. Furthermore, MCT has been suggested as a potential target for anti-cancer drugs, whose strategy is to reverse the Warburg Effect [21].

Lactate dehydrogenase-A (LDH-A), an important prognostic marker in several tumors, catalyzes the conversion of pyruvate to lactate in anaerobic conditions with concomitant oxidation of reduced nicotinamide adenine dinucleotide (NADH) to NAD^+. LDH-A expression is upregulated by several oncogenes, including c-Myc [22], Her2/Neu through heat shock factor 1 [23]) and hypoxia-inducible factor 1α (HIF-1α) [24] all of which are influenced by p53. p53 is also known to regulate lactate metabolism by indirectly suppressing LDH-A activity through miR-34 because p53 transactivates and upregulates them via p53 response elements on their promoters. In turn, miR-34a suppresses LDH-A activity *in vitro* and *in vivo* and has been shown to possess tumor suppressor functions [25–29]. Furthermore, miR-34a activation leads to apoptosis and to upregulation of several genes that are involved in cell cycle regulation, DNA repair and angiogenesis. These findings show that miR-34 is part of the p53 tumor suppression network.

2.4. p53 enhances oxidative phosphorylation (OXPHOS)

Oxidative phosphorylation is a metabolic process that occurs in the mitochondria to generate larger amounts of ATP from glucose as compared to glycolysis. It involves sequential transfer of electrons (electron transport chain (ETC)) facilitated by a series of enzymes located in the inner mitochondrial membrane thereby generating energy that is used to pump protons across the membrane creating an electrochemical gradient. This gradient drives the adenosine 5′-triphosphate (ATP) synthase to "almost mechanically" produce ATP by attaching a phosphate to ADP. By this method, the cell is able to produce 36 ATP molecules from a glucose molecule compared to 2 ATPs produced by glycolysis [30].

p53 displays multifaceted roles in the regulation of energy metabolism via the mitochondria. It assists in the repair or degradation of unhealthy or worn out mitochondria by inducing the expression of mitochondria-eating (mitophagic) protein (MIEAP) through binding to

the MIEAP promoter in response to mitochondrial damage, thereby facilitating good mitochondrial quality [31]. p53 is also involved in preserving mitochondrial genetic integrity by interacting with mtDNA polymerase gamma (mtDNA polymerase γ) thus improving DNA replication and enhancing the mitochondria to respond appropriately to DNA-damaging insults. Loss of p53 increases mitochondrial susceptibility to mutations, a phenotype that is rescued by wild type p53 [32]. This presents another example of p53 subcellular localization and function outside the nucleus. Mitochondrial diseases are characterized by energy depletion probably due to defects in OXPHOS since the primary function of mitochondria is to support aerobic respiration [33]. It is thus relevant to review the role of p53 in maintaining mtDNA integrity and influence in aerobic glucose metabolism.

The mitochondrion is also the key regulator of apoptosis and by extension, critical in cell cycle control and in development of tumors. p53 promotes oxidative phosphorylation by activating synthesis of cytochrome c oxidase 2 (SCO2) gene enzyme in complex IV of the electron transport chain. It transactivates SCO2 by direct DNA binding to the p53 response element at the SCO2 promoter region [34]. SCO2 then regulates cytochrome c oxidase (COX) complex or complex IV, which is the last enzyme in the ETC and regulates the major site of oxygen utilization in the cell.

Matoba et al. showed that even though the total amount of ATP produced by $p53^{-/-}$, $p53^{-/+}$ and $p53^{+/+}$ HCT116 cells was roughly the same, the relative proportion of ATP generated by glycolysis showed an inverse proportion to p53 dosage. This suggests that the Warburg Effect does not reduce the amount of ATP generated but adjusts the contributions made by glycolysis and OXPHOS. Oxidative respiration was, however, rescued in $p53^{-/-}$ cells when the SCO2 protein, which is downstream effector in OXPHOS, was expressed. This shows that the decreased OXPHOS was mediated in part by decreased levels of SCO2. Furthermore, $SCO2^{+/-}$ knockout cells showed the same metabolic profiles and proliferation rate as $p53^{-/-}$ cells [34, 35]. In contrast to glycolysis where p53 plays an inhibitory role, it has a positive effect on OXPHOS (**Figure 2** (insert)). This is consistent with p53 being a driver of the Warburg Effect because when it is mutated one expects that OXPHOS would be downregulated and glycolysis upregulated which is the glycolytic phenotype seen in cancer. However, it appears that this phenotype can occur in the background of wild type p53 suggesting that there are other factors that are crucial for the establishment of the Warburg Effect.

The Apoptosis-inducing factor (AIF) is a mitochondrial protein, encoded by nuclear DNA. It induces apoptosis when apoptotic stimuli cause it to be translocated to the nucleus where, together with cyclophilin, it executes the final events of apoptosis, nuclear chromatin condensation and large scale DNA fragmentation [36]. In the mitochondria, AIF maintains structural integrity especially of the cristae. Consequently, disruption of mitochondrial location of AIF disturbs oxidative phosphorylation [37]. AIF has an important role in the assembly and function of Complex I of the electron transport chain, as demonstrated in isolated cardiac mitochondria obtained from Harlequin and wild type mice [38]. AIF is also a direct transcriptional target of p53 and possesses p53 response elements in its promoter, although only basal levels of p53 are required with higher levels having no impact on AIF dynamics. The induction of AIF expression by p53 is therefore important in supporting cellular OXPHOS activity. AIF and

TIGAR, which are both upregulated by p53, are important examples of genes that regulate both apoptosis and metabolism; showing the diverse approaches of p53 in tumor suppression.

2.5. The role of p53 in glutamine metabolism

Glutamine is a non-essential amino acid that can be synthesized from glucose. When compared to glucose, it is a less celebrated source of energy and biosynthetic molecules. It provides nitrogen for the synthesis of nitrogenous bases and amino acids. Glutamine metabolism also generates reduced NADP which lowers anti-oxidant stress and is required for fatty acid synthesis. Glutaminases catalyze the deamination of glutamine to glutamate, which is further processed to generate α-ketoglutarate (an important metabolite in the TCA cycle) thereby boosting OXPHOS. α-Ketoglutarate can also be converted to citrate, which may be exported out of the cell for amino acid and synthesis of lipid which are important in proliferating cells. Glutamine can also be metabolized to pyruvate, which is further processed to lactate. Two types of glutaminase, encoded by two separate genes have been discovered: the kidney type glutaminase (GLS1) predominantly expressed in the kidneys and the liver type GLS2 which is highly expressed in hepatocytes. GLS1 and GLS2 show contrasting roles in carcinogenesis and seem to depend on the origin of the tumor. For example, increased glutamine metabolism, corresponding to enhanced expression of GLS1 is characteristic of several cancers including colorectal and hepatocellular carcinomas [39, 40] while downregulation in GLS2 is associated with hepatocellular cancers [41]. Moreover, GLS1 is an attractive target in glutamine-addicted tumors. A recent study showed that the small molecule glutaminase inhibitor, CB-839 exhibits anti-proliferative effects in triple negative breast cancer cells [42].

Studies by Suzuki et al. and Hu et al. showed that phosphate-activated glutaminase 2 (GLS2) is a p53 inducible gene which enhances OXPHOS and increases ATP production as well as glutathione levels [41, 43]. Two mechanisms by which GLS2 may inhibit cancer progression have been elucidated. Firstly, GLS2 represses the phosphatidylinositol 3 kinase/protein kinase B (PI3K/PkB) pathway which is upregulated in several tumors [44, 45]. It is noteworthy that the PI3K/PkB pathway, in addition to promoting cell proliferation, also boosts glycolysis [46]. Secondly, GLS2 represses the GTPase protein, Rac1, which is known to promote invasion, migration and metastasis in cancer cells through the regulation of actin dynamics [45]. Moreover, glutamine metabolism is another metabolic signature in carcinogenesis, because cancers consume large amounts of glutamine in culture and *in vivo*. In fact, the term "glutamine-addicted tumors" has been coined for such tumors and may be exploited for anti-cancer drug discovery. In addition to amino acid biosynthesis, glutamine serves other cellular functions including helping to maintain good mitochondrial membrane potential and integrity and modulating P13K-Akt and EGFR signaling which, in turn, stimulate glycolysis [47].

2.6. p53 reduces oxidative stress

p53 generally enhances OXPHOS activities which may inadvertently generate mitochondrial reactive oxygen species (ROS). ROS are key signaling molecules in cell proliferation in both cancer and in normal cells. Nevertheless, ROS can lead to damage of membranes, proteins and DNA, with DNA damage leading to genomic instability [48].

In addition to the incidental ROS production, p53 actively generates ROS by activating a network of genes that are directly involved in ROS generation. Consequently, a deficiency in p53 will results in oxidation of DNA and genomic instability which can be avoided by treatment with anti-oxidants [49]. To circumvent this problem, p53 upregulates handful of genes that protect a cell from oxidative stress. For example, GLS2-mediated glutathione synthesis as a consequence of glutaminolysis is known to protect cells from oxidative stress. A glutathione molecule gets rid of ROS by accepting an electron from it, and is oxidized to glutathione disulfide. Glutathione reductase is an enzyme that uses NADPH to catalyze the conversion of glutathione disulfide back to its reduced form, glutathione. The Tp53-induced glycolysis and apoptosis regulator (TIGAR) was shown to enhance the generation of reduced glutathione thereby protecting cells from ROS-mediated apoptosis [11]. p53 also transactivates glutathione peroxidase 1 (GPX1) [50] and aldehyde dehydrogenase 4 (ALDH4) [51], which are also important in reducing oxidative stress.

2.7. p53 influence in lipid metabolism

More than 90% of lipids required by tumors are produced by *de novo* synthesis. Precursors of these critical elements of the plasma membrane are generated by glucose metabolism. Furthermore, fatty acids play essential roles in signal transduction, phospholipid formation for the synthesis of membranes and energy storage required by rapidly proliferating cells. Metabolism of fatty acids in mitochondria through β-oxidation generates large amounts of energy. The availability of fatty acids has been shown to influence cell proliferation, and their metabolism is deregulated in several cancers. While increased fatty acid oxidation (FAO) may play a role in suppressing tumor growth, increased fatty acid synthesis which correlates with overexpression of fatty acid synthase and ATP citrate lyase connotes poor tumor prognosis [52–54]. It has been demonstrated that p53 influences cellular fatty acid metabolism. A family of transcription factors known as sterol regulatory element-binding proteins (SREBPs) transcriptionally regulates lipogenesis. p53 inhibits SREBP1c which is known to transactivate genes that promote fatty acid synthesis including FASN and ACLY [55]. However, p53 may promote FAO during glucose starvation by transactivating Lpin1a Mg^{2+}-dependent phosphatidate phosphatase enzyme that catalyzes the conversion of phosphatidate (PA) to diacylglycerol.

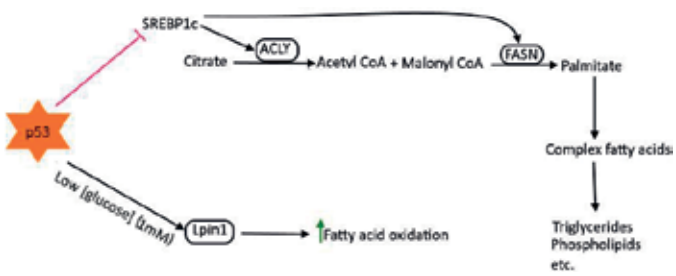

Figure 3. p53 primarily inhibits lipid synthesis by inhibiting SREBP1c which is known to transactivate ACLY and FASN which catalyze fatty acid synthesis. p53 also transactivates Lpin1 during glucose deprivation, thereby increasing fatty acid oxidation for the generation of ATP.

It is a bi-functional protein that represses and promotes FAO in mouse myoblasts at normal (25 Mm) or low (1 Mm) glucose concentrations in a ROS and ATM-dependent manner [56]. p53-mediated upregulation of Lpin1 represents a pro-survival function and confers the ability to cope with stress such as nutrient (glucose) deprivation. Inhibiting fatty acid synthesis by targeting the enzymes and transcription factors that promote lipogenesis is an attractive strategy in the development of anti-cancer drugs. For example, RNAi-mediated knockdown of FASN in prostate carcinoma resulted in decreased levels of triglycerides and phospholipids and a reduction in cell volume as well as cell-to-cell contacts [57]. Furthermore, depleting the lipogenic transcription factor SREBP1 using shRNA slowed down lipogenesis and reduced endometrial tumor growth [58] **(Figure 3).**

3. Gain-of-function mutations in p53 and carcinogenesis

Many cancers are associated with loss of p53 function often due to mutations to its DNA-binding domain. More than half of all human tumors contain mutations or deletions of p53. The remainder involves mutations in genes that regulate the p53 pathway. The hotspots, comprising about 95% of p53 mutations, are missense mutations in the DNA-binding domain. Many of these mutations have been shown to severely restrict p53 function [59]. Since p53 is a major tumor suppressor offering protection against cancers, its loss of function results in vulnerability to cancer-causing agents and aberrant growth of affected cells. Over the years, it has emerged that there is a subset of p53 mutations whereby p53 acquires new properties such as enhanced growth capacity, antiapoptotic activity, invasiveness and anti-cancer drug resistance [60]. These gain-of-function (GOF) mutants have thus become a subject of intense research.

The p53 GOF mutations were formally explored, for the first time, in cell lines whereby mutations introduced in a null p53 background were found to cause increased tumorigenicity in nude mice and enhanced growth support to the cells in soft agar [61]. Critically, these experiments showed that these mutants, in contrast to wild type p53, were able to transform p53 null cells and endow them with new properties. This property was responsible for the erroneous identification of p53 as an oncogene when it was discovered since wild type p53 does not have these characteristics [62]. Later, it was shown that one mechanism by which some of these mutations may affect carcinogenesis is by inhibiting the adenosine monophosphate (AMP)-activated protein kinase (AMPK) a key energy sensor that is activated in conditions of stress [63]. In this study, the GOF mutant p53 inhibited AMPK signaling in head and neck cancers. It was shown that it preferentially binds to the AMPKα subunit thereby increasing anabolic metabolism, causing metabolic reprogramming and inducing an invasive growth phenotype. Normally, p53 and AMPK act via a positive feedback mechanism whereby AMPK establishes a metabolic checkpoint by post-transcriptionally stabilizing p53. In turn, p53 activates AMPK transcriptionally thereby activating the gene that encodes the β subunit of AMPK [64] and sestrin [65, 66]. Among others, key pathways that are upregulated by p53 GOF mutations include the epidermal growth factor receptor (EGFR) [67], the vascular endothelial growth factor expression (VEGFR) [68] and insulin-like growth factor I receptor

(IGF-1R) [69] pathways. These are mostly mitogenic growth factor pathways that play critical roles in carcinogenesis, further underlining the clinical significance of GOF mutants. Many p53 mutations occur in the DNA-binding domain (DBD) and alter the proteins tertiary structure affecting its transcriptional activity and resulting in loss of function. Known GOF mutants include P151S, R175H, G245C and R282W.

There is also evidence showing that the GOF mutants can aggregate with wild type p53 in some cancers and that suppression of this aggregation restores the wild type p53 activity, including that of its paralogs p63 and p73. This introduces a novel mechanism of carcinogenesis involving aggregation [70]. The fact that certain cancers with p53 GOF are more aggressive than others with poor prognosis underlines their significance. Furthermore, some GOFs confer resistance to anti-cancer treatments.

4. p53 negative regulators

p53 is involved in numerous biological activities and new functions continue to be discovered. It is thus not surprising that the list of p53 regulators also continues to grow. **Table 1** shows known negative regulators of p53. It would not be surprising that the list of negative regulators will continue to grow in the near future.

The half-life of p53 is limited only to a few minutes in unstressed cells and its tight regulation in most cells is known to be controlled by Murine double minute 2 (MDM2), its prototypical negative regulator [77]. However, following DNA damage and other cellular stresses, p53 rapidly stabilizes by post-translational modifications such as phosphorylation and acetylation and the half-life extends to hours causing it to accumulate in the cell [5, 78]. The diversity in p53 negative regulators is still not fully understood but some examples show that they can be targets for anti-cancer drug discovery as they provide the mechanism for reactivating p53. This strategy, however, also comes with a critical caveat as it has been shown that in the absence of Mdm2, p53 can be spontaneously activated [79]. The main negative regulators of p53 are E3 ubiquitin ligases which tag p53 for proteasomal degradation and include MDM2, Pirh2 and COP1. These regulators generally bind to the DNA-binding domain of p53, preventing it from transactivating its target genes. Jun-N (amino)-terminal kinase (JNK) also

P53 negative regulators	Mode of action with p53 in brief	Reference
MDM2	E-3 ligase activity. Forms autoregulatory feedback loop with p53	[6]
Pihr2	E-3 ligase activity. Forms autoregulatory feedback loop with p53	[71]
COP1	E-3 ligase activity. Forms autoregulatory feedback loop with p53	[72]
Jun-N (amino)-terminal kinase (JNK) JNK	Binds to and negatively regulates p53 at G0/G1 and S/G2M cell cycle phases	[73, 74]
RBBP6	Binds p53 and E3 ligase activity. Shown to enhance MDM2-mediated ubiquitination.	[75, 76]

Table 1. p53 negative regulators.

influences p53 ubiquitination and stability probably by acting as an adaptor in the formation of the E3 ubiquitin/ligase complex [73]. There is evidence showing that p53 negative regulators can interact with each other independently of op53 and synergistically inhibit it [80].

4.1. Murine double minute 2 (MDM2)

MDM2 is an E3 ubiquitin ligase first discovered in mice. It is the prototypical negative regulator of p53 and an oncogene. It is a p53-inducible gene that binds to the 53 transactivation domain and regulates p53-mediated gene transcription. Using the E3 ligase activity, MDM2 ubiquitinates p53 and tags it for proteasomal degradation. It also transactivates p53 thereby forming an autoregulatory feedback loop [6, 81, 82]. The role of Mdm2 in glucose metabolism in not clearly understood but a Mdm2-p53-pyruvate signaling axis has been demonstrated to be activated in a diabetic situation where it links mitochondrial respiration to glucose homeostasis [83].

4.2. Pirh2

The p53-inducible gene that encodes a RING-H2 (Pirh2) protein interacts with p53 directly and catalyzes its ubiquitination through an intrinsic E3 ubiquitin ligase activity targeting it to proteasomal degradation. p53 also transactivates Pirh2 providing an autoregulatory feedback loop similar to Mdm2-p53 interaction [71]. Pirh2 has been shown to have an impact on lung tumorigenesis as increased expression corresponds to reduced levels of p53 and increased cell proliferation [84]. To our knowledge, the involvement of Pirh2 in glucose metabolism has not been reported.

4.3. Constitutively photomorphogenic 1 (COP1)

COP1 is a plant protein involved in photomorphogenesis. In Arabidopsis seedlings, for example, COP1 controls seedling development by repressing light-mediated gene expression [85]. It contains a RING-finger domain for E3 ubiquitin ligase activity. Recently, the molecular and functional importance of the human homolog of COP1 was characterized in human bone osteosarcoma (U2-OS) cells. Its molecular functions include p53-dependent activities and ubiquitination because it is encoded by p53-inducible gene and has E3 ubiquitin ligase activity. Hence, p53 transactivates COP1 which in turn targets p53 for degradation again forming an autoregulatory feedback loop that is similar to MDM2 and Pirh2. siRNA-mediated knockdown of COP1 stabilizes p53 and causes cells to accumulate in the G_1 cell cycle arrest [72].

4.4. The retinoblastoma binding protein 6 (RBBP6)

The longest RBBP6 isoform is 250 kDa protein. All RBBP6 family proteins contain the N-terminal Domain With No Name (DWNN). The shortest is isoform 3 which is essentially the DWNN. The long isoforms also possess a RING-finger with E3 ligase activity alongside an assortment of other domains and motifs. It is a member of a small class of cell cycle regulators that binds both p53 and pRb [86]. It is a negative regulator of p53 and has been shown to have properties that promote cell proliferation. It is known to act as a scaffold protein associated with nuclear matrix and telomeres of chromosomes suggesting an ability to control cellular functions to do with cell division and replication [87]. In the mouse model, it was demonstrated that RBBP6 enhances ubiquitination catalyzed by MDM2. It was, however, shown

that the phenotype exhibited by *rbbp6*$^{-/-}$ mutants was more severe than the *mdm2*$^{-/-}$ phenotype [75, 76]. Although the *rbbp6*$^{-/-}$ phenotype is strikingly similar to that of *mdm2*$^{-/-}$, these results indicate that RBBP6 also possesses roles that are independent of Mdm2. There are many instances which show a close association of RBBP6 with carcinogenesis making it an attractive candidate for drug targeting. For example, overexpression of RBBP6 alone or in combination with mutant p53 is associated with poor prognosis in patients with colorectal cancer. Moreover, its isoforms are differentially expressed in many cancers [76, 88]. Interest in RBBP6 and glucose metabolism has been activated by an observation that, in normal cells, the various isoforms including isoform 3 which comprises the ubiquitin-like DWNN, become differentially regulated when OXPHOS is boosted in cell culture but in cancer cells only the long isoform is expressed [89]. This raises questions about its involvement in regulating mitochondrial respiration.

4.5. Jun-N (amino)-terminal kinase (JNK)

JNK is a stress activated kinase that was shown to regulate p53 levels in unstressed cells. It is a p53 inducible gene which facilitates p53 ubiquitination tagging it for proteasomal degradation p53 has a JNK binding site which when perturbed either by blocking or by mutation abrogates p53 ubiquitination. Like Mdm2-p53 complexes, JNK-p53 complexes are found at G_0/G_1 and G_2/M checkpoints [73].

5. Anti-cancer drug discovery and development targeting glucose metabolism

Preferential killing of cancer cells while protecting normal cells is the most desired outcome for anti-cancer drug discovery efforts. With this in mind, the Warburg Effect, as a distinct feature of cancer cells, offers many opportunities for drug discovery and development. Nearly a century ago, since the Warburg phenomenon was discovered in cancer cells, it is still unclear whether or not it is a cause or consequence of carcinogenesis [1, 90]. Nonetheless, this metabolic signature is shared by many cancers, making it an attractive target for preferential therapy as it is distinct from most normal cells. Using the Database for Expressed Sequence Tags (dbEST), glycolytic genes have been shown to be upregulated in 24 different types of cancer; representing more than 70% of all cases [91]. Central to the regulation of glycolysis are three transcription factors: p53, c-Myc and HIF-1α [4]. p53 functions primarily as a tumor suppressor that regulates the cell cycle and a network of genes with diverse functions related to cell proliferation and cell survival, but it also plays a pivotal role in the Warburg effect because mutant p53 (mutp35) acquires a gain-of-function phenotype in cultured cells and in knock-in mice in a GLUT1-dependent manner. Inhibition of a downstream effector in this GLUT1 pathway abolishes this GOF phenotype [92]. c-Myc (an oncogene) and HIF-1α act in complex ways to regulate glucose metabolism—sometimes cooperating but at times acting in an opposite manner [93]. A dilemma arises because targeting transcription factors is not an attractive strategy as it may result in non-specific outcomes due to the vast array of genes that they transactivate.

Extensive research efforts are underway worldwide to find strategies by which p53 is targeted for cancer therapy. In many cases, small molecules are designed to reactivate p53 [5]. The intriguing situation is that, the Warburg effect, one of the hallmarks of cancer, can occur in the presence of wild type p53. For example, non-small cell lung cancer cell lines A549 (wild type p53) and H1299 (mutant p53), both exhibit the Warburg effect [94]. Yet it has been shown that perturbation of functional p53 is sufficient to induce the Warburg effect in some cells because a mutant form of p53 (mutp53) acquired this ability [92]. Thus, drug development based on the Warburg Effect should be conducted with some consideration of the p53 status.

In the present study, we update progress made with some drugs and extend the list of drugs that are considered as anti-cancer candidates based on glucose metabolism. Studies show that cancer cells are more sensitive to inhibitors of glycolysis such as 3-bromopyruvate, oxamate and 2-deoxy-D-glucose in hypoxic tumor microenvironments [95–97]. Several inhibitors of glycolysis that target the Warburg effect as a therapeutic strategy in cancer have been reviewed elsewhere [98, 99]. Here, the drugs are divided into categories for convenience:

i. Drugs targeting glucose transport

ii. Drugs targeting glycolysis

iii. Drugs targeting OXPHOS

5.1. Targeting glucose transporters

Enhanced glucose intake is characteristic of several tumors, given their high glycolytic rates, and is facilitated by glucose transporters which are often overexpressed [100]. It has been shown that depletion of Glut1 transporters by shRNA knockdown and blocking the receptor with an anti-Glut1 antibody re-sensitizes cisplatin-resistant cancer cell line [101]. Similarly, a Glut1 inhibitor was used to counter 5-fluorouracil (5-Fu) resistant colon cancer [102]. Targeting the glucose transporter GLUT1 with a GLUT1-specific inhibitor, WZB117, has been shown to inhibit self-renewal and tumor initiation in ovarian, glioblastoma and pancreatic stem cells [103]. Another drug, STF1 which selectively inhibits GLUT1, preferentially killed melanoma cells and synergistically enhanced cell death induced by anti-cancer drugs melphalan, doxorubicin and bortezomib [104]. It has been demonstrated that expression of the GLUIT1 receptor is absent in sarcomas, lymphomas, melanomas and hepatoblastomas and variable in other cancers [105]. This indicates that this drug strategy would require more specific inhibitors to target the remaining glucose transporters to manage diverse cancers.

Indirectly, ritonavir, an HIV protease inhibitor, exhibits an off-target effect on GLUT4 and has been shown to inhibit GLUT4-mediated glucose transport and to induce apoptosis in multiple myeloma cells [100]. Although, we have not found drugs targeting GLUT3 receptors, it appears that inhibition of glucose transport is a feasible strategy for anti-cancer drug development. Imatinib mesylate is essentially a tyrosine kinase inhibitor targeting Bcr-Abl tyrosine kinase but also has an ability to antagonize the translocation of GLUT2 receptors to the plasma membrane thereby reducing glucose uptake leading to cancer cell death [106].

5.2. Drugs targeting glycolysis

5.2.1. Lonidamine and 3-bromopyruvate

Following glucose uptake into the cell, the next step is phosphorylation of glucose by hexokinase II. Both lonidamine and 3-bromopyruvate were identified as drugs that repress glycolysis by inhibiting hexokinase II. It was also found to selectively inhibit the electron transport chain in the mitochondria of cancer cells indicating that it might affect OXPHOS [107, 108]. Furthermore, lonidamine was shown to have an additive rather than a synergistic effect when used together with 2-deoxy-D-glucose (2-DG) [109]. Thus far, the lonidamine mechanism of action is elusive and after many studies, it is still not clear.

The primary mechanism of action for 3-bromopyruvate (3-BP) is via preferential alkylation of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). This results in depletion of ATP selectively in cancer cells as they prefer glycolysis for energy production [110]. Systemic delivery of microencapsulated 3-BP to pancreatic ductal adenocarcinoma xenograft tumors showed promising results with minimal or no tumor progression observed [111]. However, safety concerns in the use of 3-BP only were observed in human trials [112].

5.2.2. Pyruvate esters

Interestingly, some pyruvate esters show anti-cancer properties, albeit via different mechanisms. Methyl pyruvate kills lung cancer cell line-A549 cells alone and in combination with irinotecan but protects the normal lung fibroblast cell line—MRC5 from irinotecan-induced apoptosis probably by inhibiting the p53/p21 axis of the apoptotic pathways during treatment and the mitochondrial pathway as well during recovery. Thus, methyl pyruvate has a potential for use as an adjunctive to chemotherapy [89]. In this case, exogenous pyruvate adds to the endogenous thereby boosting OXPHOS and reversing the Warburg Effect. It is reported that another pyruvate ester, ethyl pyruvate, preferentially killed leukemia cells by concerted mechanisms including cell death by necrosis or apoptosis/ATP depletion and by inhibition of glycolytic and para-glycolytic enzymes. Ethyl pyruvate (EP) was also shown to inhibit glyoxalase 1 (GLO1), an enzyme that detoxifies the glycolytic bi-product, methylglyoxal. It also suppresses glycolytic enzymes, LDH and pyruvate kinase [113]. In another study, ethyl pyruvate-reduced mitochondrial apoptosis and protected the murine myeloid cell line 32D c13 from radiation. It also increased survival of irradiated C57BL/6NHsd mice. In contrast, the inactive analog of EP did not enable such protection [114]. These properties of pyruvate esters are interesting as they point to a potential for this molecule as adjunctive in chemotherapy and in radiotherapy.

5.2.3.-deoxy-D-glucose (2-DG)

2-DG is a synthetic glucose analog in which a hydrogen atom replaces the C2 hydroxyl group such that it cannot be further utilized in the glycolytic pathway. 2-DG competitively inhibits glucose uptake, given that it is imported into the cell by glucose transporters (GLUTs). Following the import of 2DG into the cell, it becomes phosphorylated by hexokinase to 2-DG-6-P (2-deoxy-D-glucose-6-phosphate), rather than glucose 6-phosphate. As such, 2-DG

competitively inhibits the production of glucose-6-phosphate. 2-DG-6-P cannot be further processed, hence it accumulates in the cell and non-competitively inhibits hexokinase and competitively inhibits glucose-6-phosphate isomerase [115]. Both glycolysis and OXPHOS are partially inhibited, given that the first important steps of glycolysis are inhibited by 2-DG [116]. This leads to decreased ATP production which renders cells susceptible to death receptor-induced apoptosis. 2-DG is also known to block the cell cycle.

5.2.4. TEPP-46

Pyruvate kinase M2 (PKM2) is an alternatively spliced isoform of the key glycolytic enzyme PKMI which catalyzes the conversion of phosphoenolpyruvate (PEP) into pyruvate while concurrently producing ATP. PKM2 is expressed during embryonic development and in cancer cells where is promotes the Warburg Effect, but not in normal adult cells making it an attractive target for drug development. Negative regulation of PKM2 in cancer cells results in shunting glycolysis to the anabolic pentose phosphate pathway which benefits cancer cells. TEPP-46 reactivates pyruvate kinase M2 thereby increasing glycolytic flux and reducing lactate production in cancer cells while deleting intermediates required for entry into the pentose phosphate pathway resulting in cancer cell death [117, 118]. It has been demonstrated *in vivo* that reactivation of PKM2 activity and reduction of PPP is a plausible anti-cancer strategy. In a xenograft model for non-small lung cancer, TEPP reduced the size of the tumor [118, 119]. There is some concern about small molecule inhibitors of PKM2 as they may interfere with anabolic activity since they induce constitutive activity. It is noteworthy that PKM2 can act as a transcription factor activating transcription of genes which control other aspects of carcinogenesis such as Oct-4, hypoxia-inducible factor 1-α (HIF-1α), and β-catenin [120–122]. This indicates that small molecule inhibitors of PKM2 may also affect other pathways but this may be a positive factor since these pathways are also implicated in carcinogenesis. It also suggests that studies of these small molecule inhibitors must also include assessment of the potentially affected pathways.

5.2.5. Inhibitors of lactate dehydrogenase-A (LDH-A) and oxamate

LDH-A catalyzes the interconversion of pyruvate, the final product of glycolysis, to lactate and nicotinamide adenine dinucleotide (NAD^+). The NAD^+, thus produced, is reused to fuel further glycolysis. Depletion of endogenous LDH-A by RNAi results in death of cancer cells regardless of p53 status indicating that it supports their proliferation. However, it was shown that the p53 status is required for maintaining the NADH:NAD^+ ratio because acetylated $p53^{+/+}$ is important for modulating the NADH:NAD^+ ratio. This phenotype is dependent upon the NAD^+-dependent deacetylase sirtuin 1 (SIRT1). Hence, the anti-cancer activity of small molecule drug and redox-sensitive anti-cancer drug EO9 (apaziquone) was enhanced only in $p53^{+/+}$ cells. This indicates that LDH-A is a plausible therapeutic target for anti-cancer drug development and that a combinatorial approach that targets LDH-A and the redox status is even more effective [123].

EO9 is a bioreductive alkylating agent that causes DNA damage, creating single strand breaks and crosslinks [124]. Therefore, it does not target glucose metabolism but is regarded as a sensor of the NADH:NAD^+ redox status. These findings indicate that inhibitors of LDH-A

may have potential as anti-cancer drugs. Indeed, a small molecule inhibitor of LDH-A, FX11 (3-dihydroxy-6-methyl-7-(phenylmethyl)-4-propylnaphthalene-1-carboxylic acid) causes ATP depletion, induces oxidative stress and causes cell death which can be partially reversed by anti-oxidants such as *N*-acetylcysteine [125]. FX11 probably acts by upsetting the Warburg Effect because it has been demonstrated that it induces apoptosis in and reduction of patient tumor xenographs in a p53-dependnet manner whereby only mutant Tp53 tumors respond to the drug [126]. This p53-dependence seen in LDH-A-related responses is interesting as it seems to be related to the involvement of p53 in modulating the NADH:NAD^+ ratio. The molecular mechanism by which p53 would influence this phenotype is unclear and certainly requires more investigation. Oxamate is a related drug as it inhibits lactate dehydrogenase (LDH) and has been shown to cause death of a breast cancer cell line [127, 128].

5.2.6. Oxythiamine (OT)

The transketolase-like-1-gene (TKTL1) in urothelial and colorectal cancer is associated with poor prognosis when overexpressed and was identified as a biomarker. It encodes the enzyme transketolase that catalyzes the production of lactate from glucose via the pentose phosphate pathway in cancer cells [129]. Oxythiamine (OT) is a transketolase inhibitor that arrests growth of cancer cells. This discovery introduced the idea that in general, pentose cycle inhibitors provide yet another angle for cancer management [130].

5.3. Drugs targeting OXPHOS or mitochondrial activities

5.3.1. Dichloroacetate

Dichloroacetate (DCA) is an inhibitor of mitochondrial pyruvate dehydrogenase and its effect on metabolism is to redirect glucose metabolism from glycolysis to oxidation which effectively reverses the Warburg effect. Consequently, it inhibits proliferation and induces caspase-mediated apoptosis [131]. DCA has been taken through Phase 1 clinical trials given orally to patients with **World Health Organization (**WHO) grade III–IV gliomas or metastases from a primary cancer outside the central nervous system. It was feasible and well tolerated although genetic factors affecting tolerance were confirmed [132]. In Phase II clinical trials, DCA exhibited synergy when used with cisplatin and docetaxel leading to a suggestion that it might be more effective and preferable for use in combination with other drugs [133]. It is noteworthy that these studies, together with the use of methyl pyruvate mentioned earlier, support a broad principle involving the reversal of the Warburg Effect as a plausible anti-cancer strategy.

5.3.2. Rotenone

Rotenone is a natural insecticide and an inhibitor of NADH dehydrogenase complex or complex 1 of the mitochondrial electron transport chain (ETC). This results in the generation of reactive oxygen species and apoptosis [134, 135]. Because of its activity on ETC complex 1 which is often altered in Parkinson's disease, rotenone is sometimes used to create an animal

Drug	Mechanism of action	Status	Reference
Targeting glucose transporters			
WZB117, STF-31	Inhibits GLUT1, thereby suppressing glucose transport	Preclinical	[140, 141]
STF-31	Selectively inhibits Glut1 expression and completely induces apoptosis sin GLUT1 expressing myeloma cells.	Preclinical	[104]
ritonavir	Has an off-target inhibitory effect on GLUT4.	Approved for HIV	[100]
Imatinib	Inhibits Bcr-Abl tyrosine kinase; suppresses the activity of Hexokinase and G6PD	Approved for clinical use	[142]
Targeting glycolytic pathway			
2-DG	Inhibits HK, thereby suppressing glycolysis	Clinical trials aborted	[143, 144]
3-bromo- pyruvate	An alkylating agent that inhibits HK	Preclinical	[97, 111, 145]
Lonidamine	Suppresses glycolysis by inhibiting hexokinase II and OxPhos by dissociating HK2 from mitochondria	Clinical trials Phase II/III	[107, 108, 146]
FX11	Inhibits LDH-A	Preclinical	[125]
Oxamate	Inhibits LDH-A	Preclinical	[127, 128]
TEPP-46	Activates PKM2 and suppresses the PPP	Preclinical	[118, 147]
Oxythiamine	Inhibits transketolase thereby suppressing the PPP; inhibits pyruvate dehydrogenase	Preclinical	[130]
Targeting OXPHOS			
Dichloroacetate	Inhibits the pyruvate dehydrogenase complex	Phase II	[131–133]
Pyruvate esters	Boost OXPHOS	Experimental	[89, 113, 114]
Rotenone	Inhibits complex 1 in OXPHOS	Preclinical	[135, 148]
Targeting glutamine pathway			
L-γ-glutamyl-*p*-nitroanilide	Inhibits SLC1A5 (also known as (ASCT2)	Preclinical	[138]
BCH	inhibits glutamine/leucine exchange and mTOR activation	Preclinical	[139]
Benzylserine	inhibits glutamine transport by targeting both LAT1 and ASCT2	Preclinical	[139]
BPTES	inhibit glutaminase	Preclinical	[139]
CB-839	unknown	Preclinical	[139]
Compound 968	Probablt tarets by Rho GTPases	Preclinical	[139]
EGCG	inhibits GDH	Preclinical	[139]

Table 2. Potential anti-cancer drugs targeting glucose metabolism.

model of Parkinson's disease [136]. It can thus be neurotoxic. Nevertheless, rotenone, given at subtoxic amounts, was used to reverse tumor necrosis factor-related apoptosis inducing

ligand (TRAIL) resistant non-small cell lung carcinoma (NSCLC) cells in a p53-dependent manner that was also related to generation of reactive oxygen species [137]. Although, rotenone does not target glucose metabolism but rather the integral structure of the mitochondrion, it would affect glucose metabolism indirectly. It falls within a class of drugs that target the structural integrity of the mitochondrion, including resveratrol, Vitamin E analogs, arsenic trioxide, honokiol and betulinic acid [127].

5.4. Drugs targeting the glutamine pathway

Glutamine is a non-essential amino acid that can be produced from glucose. It is known that cancer cells have a propensity to consume large amounts of glutamine and undergo dramatic metabolic programming geared at production of anabolic precursors from glutamine. Glutamine, a mitochondrial substrate, enters the cell via high affinity glutamine transporters and is converted into glutamic acid and then into the tricarboxylic acid (TCA) cycle metabolite, α-ketoglutarate [47]. A window for anti-cancer drug development is therefore open at the level of glucose uptake. The Na(+)-dependent neutral amino acid transporter type 2 (ASCT2) is encoded by the *solute-linked carrier family A1 member 5 (SLC1A5)* gene and inhibited by L-γ-glutamyl-*p*-nitroanilide or GPNA via mTOR signaling [138]. New drugs targeting glutaminolysis have been reviewed by Jin et al. [139] and are listed in **Table 2**.

6. Conclusions

The metabolic reprogramming that occurs during carcinogenesis and also upon infections provides a window of opportunity for anti-cancer drug discovery and development. The molecular mechanisms that underlie this phenotype are being elucidated at a rapid pace further helping the development of therapeutic strategies. However, there are still key questions to be addressed with regards to the Warburg Effect. Firstly, it is still to be ascertained whether this phenomenon is a cause or a consequence of carcinogenesis. Secondly, the role of the tumor suppressor p53 which intervenes at multiple points in the glucose metabolic pathway seems essential as demonstrated by the elegant work of the Feng group at Rutgers University who showed a gain-of-function phenotype in p53 which stimulates the Warburg Effect. However, it seems that this phenomenon can occur in a wild type p53 background. Altogether, glucose metabolism offers exciting opportunities for anti-cancer drug discovery and development.

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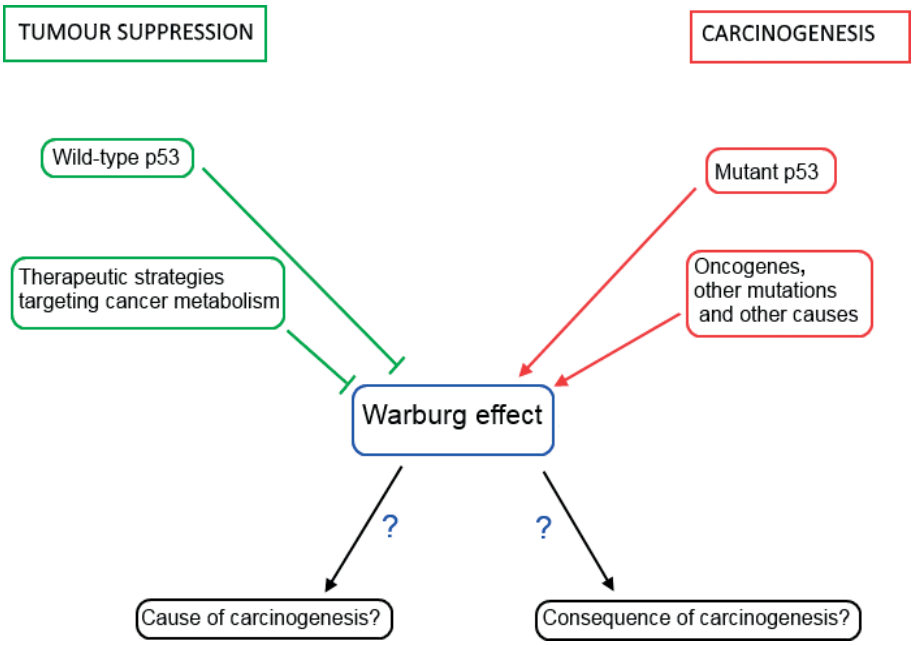

Conflict of interest

The authors declare no conflict of interest.

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Section 3

Cancer Therapies

Chapter 6

An Overview of Cancer Treatment Modalities

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Additional information is available at the end of the chapter

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Abstract

Cancer is a global issue majorly affecting developing countries. According to a survey, 63% of deaths due to cancer are reported from developing countries. There are different conventional treatment modalities that are available to treat and manage cancer. However, new cancer treatment options are being explored continuously as over 60% of all current experimental trials worldwide are focusing on tumor cure. The success of treatment depends upon the type of cancer, locality of tumor, and its stage of progression. Surgery, radiation-based surgical knives, chemotherapy, and radiotherapy are some of the traditional and most widely used treatment options. Some of the modern modalities include hormone-based therapy, anti-angiogenic modalities, stem cell therapies, and dendritic cell-based immunotherapy. This chapter discusses different traditional and novel treatment modalities to combat different types of cancer.

Keywords: cancer, tumor, radiotherapy, surgical knife, chemotherapy, surgery, immunotherapy, stem cell therapy

1. Introduction

Cancer is a major global issue causing more than eight million deaths annually. Recently, the International Agency for Research on Cancer (IARC) reported that 7.6 million deaths worldwide were due to cancer. Likewise, 12.7 million new cases are estimated per year [1]. It has been reported that developing countries are at higher risk of cancer; according to a survey, 63% of cancer-related deaths were reported only from developing countries [1]. Cancer is a multifactorial disorder involving complex modifications in the genome affected by the interactions between host and environment. The hallmarks of cancer include independence from growth signals, irresponsiveness to signals which halt the cell division, uncontrolled replication, evasion of apoptosis, sustained angiogenesis, and finally the capacity to penetrate in other tissues,

known as metastasis [2]. The microenvironment of benign tumor manifests dysregulation of various regulatory proteins and extracellular environment which plays a vital role in origination and development of cancers [3]. Before 1950, only surgery was considered as a preferred treatment option for the cure of cancer. After 1960, radiation therapy was initiated to control local disease. With the passage of time, it was realized that individual treatment of surgery and radiation is not effective as compared to their use in combination to control the cancer. Nowadays, drugs, biological molecules, and immune mediated therapies are being used for treatment. Till today, we have not reached the excepted therapy level that resists the mortality rate and decreases the prolonged survival time for metastatic cancer. Pathways and characteristics of different tumor entities were determined to create new revolution in neoplastic cancer or targeting drugs to tumor. Radiation therapy is based upon the use of physical entities like electrons, protons, and various ions to kill the cancerous cells. The mechanism behind radiation therapy is that high energy radiations halt the cell division and block their ability to proliferate by damaging their genetic material. If it is done before surgery, radiation therapy is given with the intention to shrink the tumor. If done after surgery, radiations will destruct the left behind tumor cells and reduce the cancer relapse [4]. As radiation therapy acts in a localized manner so to treat systemic cancers, chemotherapy is used alone or in combination with radiotherapy. Chemotherapy is considered the most effective and extensively used modality in most types of cancers. Chemotherapy drugs target the tumor cells and mainly produce reactive oxygen species which largely destroy tumor cells by the means of genotoxicity [5]. However, chemotherapy also harms ordinary cells that leads to diverse dose-dependent side consequences such as fatigue, nausea, hair loss, and vomiting or even death in extreme cases [6].

A standout among the best cancer treatment modalities is the gene therapy which is direct in situ insertion of exogenous genes into the tumors which could give a powerful remedial way for the treatment of benign tumors. Similarly, hormonal treatments are also widely used for cancer malignancies and generally considered as cytostatic. Hormonal treatment restricts tumor development by limiting hormonal growth factors. It most likely acts via the down direction of hypothalamic-pituitary-gonadal axis, blockage of hormone receptor, and restraint of adrenal steroid synthesis [6]. Strikingly, the use of stem cell therapy is extended beyond regenerative medicine with increasing knowledge of stem cell behavior. In vitro, stem cells are modified by introducing specifically customized genes with antitumor effects which create tumor-seeking therapeutic vehicles [7]. Among advanced cancer treatment modalities, dendritic cell-based immunotherapy is thought to be the most effective treatment since it manipulates the immune system in a way to destroy tumors without any side effects [8]. This chapter will provide an ample knowledge about the various types of cancer therapies along with a discussion on their new trends.

2. Cancer prevalence in the world

Cancer is the principal cause of death equally in developed and underdeveloped countries but more prevalent in middle-income countries, probably due to prevailing poor socioeconomic conditions. The geographic differences in the prevalence of cancer can be explained by many

contributing factors, like early diagnosis, age factor, occurrence of risk factors, screening tests, and accessibility of quality treatment [9]. According to the report of IARC (International Agency for Research on Cancer), 14.1 million cases of cancers were reported in 2012 globally, of which 8 million were reported from underdeveloped countries that is about 82% of total population of the world [10].

3. Understanding the cancer

Cancer is an abnormal condition in which a group of cells disregard the physiological rules of the cell division and grow in an uncontrolled manner. Cancerous cells do not respond to the signals that activate the normal cell cycle because they have a degree of self-sufficiency which leads to the uncontrolled growth and proliferation of transformed cells [11]. If the proliferation of cancerous cells continues, it can be fatal. In fact, 90% of deaths due to cancers are because of the spread of cancer cells to other tissues which is called metastasis.

During mitosis normal cells grow in an interdependent manner, relying on the availability of external growth factors. So, when the supply of these growth signals is limited or terminates, cells cease to reproduce. In contrast, tumor cells grow independently of any factor or signal [12]. Moreover, normal cells exhibit contact inhibition ability. They cease cell division in response to the presence of enough number of surrounding cells, i.e., after a particular threshold. Conversely, cancer cells lack this contact inhibition ability, leading to the formation of unwanted mass of cells [13]. The life of a normal cell is well-programed; it divides only about 50 times, and then it dies by apoptosis and is replaced by a new cell. This is in accordance with a limited efficiency of DNA replication, as repeated replication leads toward shortening of telomeres. Cancer cells, on the other hand, show high activity of telomerase enzyme that continuously keeps replacing the lost, worn-out ends of telomere, allowing unlimited proliferation of cells [3].

3.1. Tumor biology

Cell division, when grows independent of growth factors, forms tumors, which involve a series of steps. In the very first stage, a large mass of cells known as **hyperplasia** is formed because of uncontrolled cell division. This is followed by **dysplasia** in which cell growth is accompanied with abnormalities. Additional changes occur in the next stage when these atypical cells start to spread over a limited area of the tissue, losing their original function. This phase is coined as **anaplasia**. At this stage, the tumor is not invasive and is considered as benign. In the advanced stage, the tumor cells acquire the ability to metastasize. They begin to invade the surrounding tissues as well as those located away via bloodstream. This stage is considered to be malignant and is very hard to treat. However, not all tumors progress to this level, if identified earlier [14]. Though tumor cells are able to proliferate independent of growth factors, they still require nutrients and oxygen for their growth. All normal tissues are sufficiently supplied with capillaries for the supply of nutrients and oxygen to every cell. Similarly, tumors, as growth progresses, form new blood vessels in a process called as angiogenesis so

that nutrients reach the cells located at the center of the tumor mass which do have access to normal blood vessels [15].

3.2. The types of tumor

3.2.1. On the basis of the type of cell initially altered

Tumors are named depending upon the type of cell from which they originate. These include:

- Carcinomas, which result from altered epithelial cells. They constitute the highest ratio in all types of cancer.
- Sarcomas denote the cancer abnormalities in the bone, muscle, fats, and connective tissue.
- Leukemia, which originate from cancerous white blood cells.
- Lymphoma, which is a malignancy of the lymphatic system or cells which are derived from the bone marrow (BM).
- Myelomas depict the cancers of those particular white blood cells that synthesize antibodies [14].

3.2.2. Classification by grade

This is the abnormality in cells with respect to their surrounding normal tissues. Increase in abnormality increases the grade, from 1 to 4. Well-differentiated cells closely resemble normal cells and belong to low-grade tumors. Improperly differentiated cells are highly abnormal with respect to the surrounding tissues [16]. These are high-grade tumors.

Grade 1: This includes well-differentiated cells having slight abnormality.

Grade 2: These cells are moderately differentiated and a bit more abnormal.

Grade 3: The cells are improperly differentiated and very abnormal in context of having mutated chromosomes and produce some harmful chemicals which affect nearby cells and may enter in the blood.

Grade 4: Cells are immature, primitive, and undifferentiated.

3.3. Causes of cancer

Origin and advancement of cancer depend on many factors inside the cell (mutations, immune conditions, and hormones) as well as external factors from the environment (smoking, chemicals, infectious organism, and radiations). These entire elements act together to cause abnormal cell behavior and uncontrolled proliferation. The resultant unusual cell mass in the body grows and affects normal tissues in their surroundings, and sometimes it also spreads to the other localities in the body (metastasis) [17] (**Figure 1**).

According to the most accepted model for cancer causation, mutations in tumor suppressor and oncogenes is the major factor leading to the cancer development. Another model suggests that some mutation in a master gene that control the division of cells can also shepherd normal

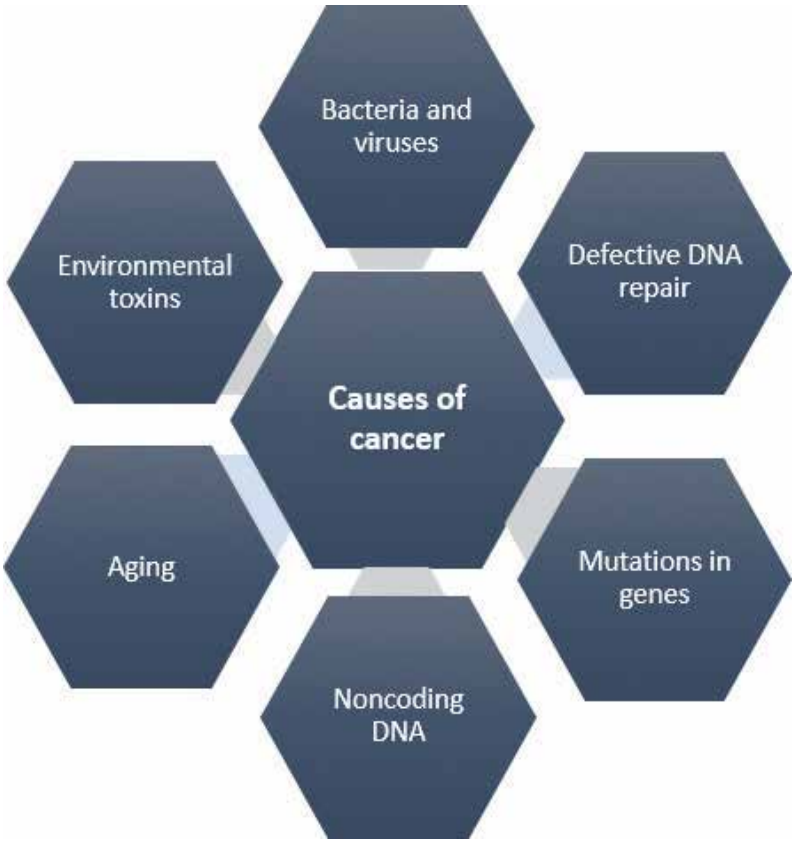

Figure 1. Factors involved in causing cancer.

cells toward abnormal chromosomal replication, which can result in duplication or deletion of the entire sections of chromosomes [18].

This change in genetic content in the cells produces abnormal amount of a specific protein irrespective of the actual need. If any chromosomal aberration affects a protein that plays a crucial role in cell cycle, quantitatively or qualitatively, it may result in cancer. There is also a strong indication that the unnecessary addition (hypermethylation) or deletion (hypomethylation) of methyl groups to genes involved in the regulation of cell cycle, DNA repair, and apoptosis is also associated with some cancers. It is necessary to commemorate that cancers can take months to years for accretion of DNA mutations enough for the resultant cancer mass to be detectable. Thus, there can be several mechanisms which lead to the development of cancer. This further obscures the difficult task of defining the actual cause of cancer [19].

3.3.1. Mutations in the p53 tumor suppressor gene

Considering biochemical pathways the most important component central to human carcinogenesis is the P53 gene whose normal function is associated with gene transcription, DNA synthesis, apoptosis, and DNA repair [20]. Alterations and mutations in p53 elicit the development of primary tumors. The biochemical processes related to the normal function of p53

gene are performed by multiunit protein machines. The functions of these machines are altered by some viral oncoproteins, which bind with the p53 and perturb its interactions with other cellular protein components [21].

3.3.2. Linking tumor viruses to human cancer

Development of human malignancies is strongly associated with viruses. In fact, 15% of the cancer are believed to be caused by oncogenic viruses which include human papillomaviruses (HPVs), Epstein–Barr virus (EBV), Kaposi's sarcoma-associated herpes virus (KSHV, also known as HHV-8), and hepatitis B and C virus (HBV and HCV) [22]. Another virus known as Merkel cell polyomavirus (MCPyV) has been recently described causing Merkel cell carcinoma, a rare but aggressive type of skin cancer [23]. The recent studies on these cancer-causing agents have been very helpful to understand the basic biology of cell and how disturbances in the cellular pathways lead to the initiation and maintenance of cancer.

4. Cancer treatment modalities

Since the recognition of the malignancy, the objective of extraordinary research is to discover novel methods of quality treatment approaches for cancer. Presently, over 60% of all ongoing medical quality treatment trials worldwide are concentrating on cancer [24]. The selection of treatment and its progress depends on the type of cancer, its locality, and stage of progression. Surgery, radiation-based surgical knives, chemotherapy, and radiotherapy are some of the traditional and most widely used treatment methods. Some of the modern modalities include hormone-based therapy, anti-angiogenic modalities, stem cell therapies, immunotherapy, and dendritic cell-based immunotherapy. Side effects associated with traditional methods of cancer treatment highlights the scope of novel cancer treatment methods. Different novel treatment systems utilized for the treatment of malignancy include treatment against angiogenic ability of cancers, oncolytic virotherapy, hereditary control of apoptotic and tumor-attacking pathways, antisense, and RNAi techniques. These treatments are employed against the cancer of the cerebrum, prostate, lung, breast, colorectal, pancreatic, liver, head and neck, bladder, skin, ovarian, and renal malignancy [25]. The coming sections of the chapter will shed light on the abovementioned treatment modalities.

4.1. Surgical removal of tumors

Surgery, resection, or operation is thought as one of the most promising and conventional treatments of many benign and malignant tumors as it assures least damage to the surrounding tissues as compared to chemotherapy and radiotherapy. Another reason of considering surgery as the preferred treatment option is that the tumor can be removed without unnecessary risk of tissue damage. Different kinds of surgeries either **open** or **minimally invasive** can be performed depending upon various factors:

- The reason of the surgery
- The part of the body where surgery is to be performed

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- The mass of tumor to be removed
- Patient's preference

Surgeries also vary depending upon the stage of cancer. Surgery may:

- Remove the entire tumor from a certain part
- Debulk a tumor in case its removal may cause damage to a certain organ
- Ease cancer symptoms in cases when a large tumor is causing pain or intense pressure on any body part

In case of open surgery, one large cut is made, and it usually results in removal of the tumor along with some amount of healthy tissues associated with some closely present lymph nodes. In contrast, for minimally invasive surgery, the surgeon makes a few small cuts instead of one large one and then with the aid of laparoscope which is a thin tube with a camera attached to it views the tumor in detail. The camera shows the image on a screen which helps the surgeon to monitor his activity [26]. The tumor, along with small amount of healthy tissues, is then carefully removed with the assistance of specialized surgery tools.

4.2. Radiation-based surgical knife

4.2.1. Stereotactic radiosurgery (SRS)

Stereotactic radiosurgery (SRS) is a kind of therapeutic radiology in which ionizing radiations are used for the damage and destruction of selected areas within an organ or tissue. This technique exposes a small area of the body to a very high dose of radiations. However, no cutting or blade is used in the entire process, but it is still called a surgery because the results of this treatment are quite similarly an ordinary surgery [27]. As the beam of radiations administered is of very high dose, it is very important that the beam of radiation is highly focused so that the peripheral tissues are left unaffected. It is primarily utilized in cases of brain tumors at locations where conventional surgical techniques are hard or unsafe to use or in other cases when the health status of a patient does not support him to tolerate a surgical procedure [28].

4.2.2. Gamma knife systems

A Gamma Knife technique does not include real surgery, nor is the Gamma Knife actually a blade. It utilizes light emissions, centered gamma beams to treat little to medium-sized sores and tumors. Many radiation beams combine to concentrate on the cell mass under treatment, giving an exceptionally high dose of radiation without a surgical cut or opening [29].

4.2.3. Linear accelerator (LINAC) systems

Linear accelerator (LINAC) systems utilize high-energy X-rays to treat a tumor or other injuries. Some basic kinds of LINAC frameworks include CyberKnife®, X-Knife®, Novalis®, and Peacock®. LINAC frameworks can treat bigger tumors and bigger affected regions than the Gamma Knife. Zones other than the brain can be treated with a LINAC framework [30].

4.2.4. Proton beam therapy or cyclotron

Proton beam therapy is a sort of molecular radiation treatment. As opposed to utilizing beams of radiation, for example, gamma beams or X-beams, molecular radiation treatment utilizes particles, like protons or neutrons [31].

4.3. Radiation therapy

The discovery of X-rays by German physicist Wilhelm Conrad Rontgen in 1895 also marked their clinical importance in the treatment of cancer. After that, almost a hundred years ago, Marie Curie's research in radium makes her a two-time Noble Prize winner and the one to introduce the field of radiotherapy in medicine. The 2011 thus became the Year of Radiation Therapy, announced by the UK, enclosing a century of developments in radiation therapy. Radiation therapy, now a distinguished field of specialization in medicine with branches such as that of radiation oncology, employs professionals from various sectors of health sciences working on the field's advancements [32].

4.3.1. Principles of radiation therapy

Radiation in cancer therapy can be described as a physical entity used to kill the cancer cells. The kind of radiation used in therapy is ionizing radiation. The radiation upon incidence causes particles in biological bodies to charge electrically; thus, the term is "ionizing," and energy is transferred in this way from the rays to the cells of the body through which it passes. This energy can either directly kill cancer cells or genetically alter them so that they accede to apoptosis and cell death.

The mechanism underlying genetic alterations in cells treated with radiation lies in the fact that the damaged DNA is unable to replicate and thus cell division is halted, which in turn causes cells to die. The adverse effect of radiation therapy is that it also hits normal cells lying in the peripheries of the main tumorous mass. However, improved imaging techniques and attempts at accurate targeting of the cancer mass in addition to the normal cells' ability to regain normal function faster than cancer cells as cancer cells lack efficient repair systems minimize the net damage done by radiation [33].

4.3.2. Radiation therapy techniques

4.3.2.1. Fractionation

Fractionated delivery of radiation therapy employs the radiobiological difference of normal and cancer cells, multiplying the survival edge of normal cells over cancer cells, by many folds, since they have an intact repair system triggered by sublethal dosages of radiation.

4.3.2.2. 3D conformal radiotherapy (3DCRT)

The usage of 2D rectangular fields in therapy has become obsolete, making CT scan-based 3D radiation therapy the primary method for detection of cancer masses, avoidance of vital organs, and target selection for radiation therapy [34].

4.3.2.3. Intensity-modulated radiation therapy (IMRT)

This technology uses an inverse planning software, which modulates the intensity of radiation beams used during therapy, resulting in an irregularity of radiation dosages that differentially target tumor as opposed to vital organs [35].

4.3.2.4. Image-guided radiotherapy (IGRT)

Using imaging techniques prior to therapy, such as IGRT, helps position radiation correctly, diverting rays away from critical organs, targeting only tumor masses, and consequently reducing organ damage as a result of errors in aiming [36] (**Figure 2**).

4.4. Chemotherapy

Chemotherapy halts tumor progression by killing off their ability to divide and enforcing apoptosis. Normal biological functioning of the body refreshes cells of the body by removing excess cells or damaged cells and thus signaling new cell formation. In contrast, tumor cells have an increased capacity to divide and the quality of immortality as they are not controlled by apoptosis. Therefore, where in normal bodies the cell proliferation is balanced by cell death and is regulated, in cancerous masses, cell proliferation to cell death ratio is high. Chemotherapy acts here to bring about changes in the tumor cells so that they stop growing or die; thus, the two branches of chemotherapeutic drugs are cytostatic (biological drugs) and cytotoxic, respectively [5].

However, chemotherapeutic drugs also target normal cells, which could result in a variety of side effects depending on the dosage such as hair loss, nausea, fatigue, vomiting, etc. As a result of vigorous chemotherapy treatment, patients become immunocompromised; this can result in complicated infections and consequently death. Out of chemotherapeutic drugs discovered, a total of 132 are FDA approved. These drugs are designed to specifically target

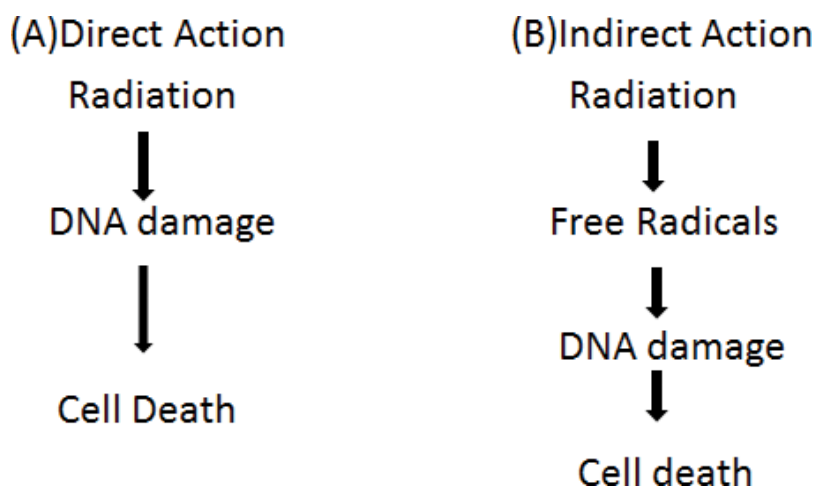

Figure 2. Direct and indirect mechanisms of radiotherapy-based treatments.

tumor cells and kill them by genotoxic effect, i.e., the production of reactive oxygen species. However, to some extent normal cells of the body are also affected by these drugs [37].

The use of chemotherapy as a treatment for cancer started in the beginning of the twentieth century. Effects of drugs studied in four programs conducted in World War II were the leverage over which a national effort to develop drugs was initiated in 1955, known as Cancer Chemotherapy National Service Center. Two diseases, acute childhood leukemia and advanced Hodgkin's disease cured using combination chemotherapy in the 1960s and 1970s, respectively, lead to acceptance of the ability of drugs to cure complicated cancers. This also encouraged studies on adjuvant chemotherapy by the aid of national cancer program. Molecular studies on abnormalities in cancer cells are an important screening process today for checking the effectiveness of new drugs and designing targeted therapies. This has advanced chemotherapy today [5].

Drugs used in chemotherapy are now known to be more than a 100 in number which can be used alone or in combination therapies. Each drug has a different chemical structure and composition. While surgery and radiation are invasive and targeted procedures, chemotherapy is mainly systemic, traveling through the body to reach cancer cells [38].

4.4.1. Different types of chemotherapy drugs

Mode of action, chemical structure, composition, and homology to other drugs are factors that help categorize chemotherapy drugs. Some drugs may fall into more than one category as they may have multiple modes of action. To know the side effects of a particular drug, one must study the mode of action. This information can later be incorporated by oncologists to predict how effective a drug will work. In combination chemotherapies, drug studies help decide the time, order, and dosages of each drug administered in the therapy [39].

4.4.1.1. Alkylating agents

Direct DNA damage by alkylating agents stops division of cancer cells and is efficacious in all stages of the cell cycle. Many cancers are treated with alkylating agents such as lymphoma, leukemia, multiple myeloma, Hodgkin's disease, and sarcomas [40]. Also included are several cancers of the ovary, breast, and lungs. On the downside of alkylating agents, they can cause damage to bone marrow as they damage DNA. Long-term damage can result in acute leukemia, depending on dosages used, although rarely. Leukemia from alkylating agents arises after 5 to 10 years of treatment. Families of alkylating agents are given in **Table 1**.

Based on similar mode of action of alkylating agents and platinum drugs, i.e., cisplatin, carboplatin, and oxaliplatin, they are sometimes grouped together. These drugs have a reduced tendency to cause posttreatment leukemia.

4.4.1.2. Antimetabolites

These drugs are analogs for the units of DNA and RNA, and hence by incorporation, they stop growth of DNA and RNA. Such drugs particularly effect the S phase of the cell and used for

the treatment of leukemia, cancers of ovary, breast, intestinal tract, and various others. Examples of antimetabolites are given in **Table 1**.

4.4.1.3. Anthracyclines

These are antibiotics in nature which target DNA replication enzymes, effecting cells in all phases of the cell cycle. Various cancers lie in the scope of these drug treatments. A big limitation of these drugs is that exceeding a critical limit can permanently damage the heart. Therefore, dose limits for a lifetime are determined for these drugs. Classes of anthracyclines are mentioned in **Table 1**.

4.4.1.4. Other antitumor antibiotics

There are some antitumor antibiotics that do not belong to anthracyclines, including actinomycin D+, bleomycin, and mitomycin C. Another anticancerous antibiotic is mitoxantrone,

Class	Names of drugs
Alkylating agents	• Nitrogen mustards: such as mechlorethamine (nitrogen mustard), chlorambucil, cyclophosphamide (Cytoxan®), ifosfamide, and melphalan • Nitrosoureas: which include streptozocin, carmustine (BCNU), and lomustine • Alkyl sulfonates: busulfan • Triazines: dacarbazine (DTIC) and temozolomide (Temodar®) • Ethylenimines: thiotepa and altretamine (hexamethylmelamine)
Antimetabolites	• 5-Fluorouracil (5-FU) • 6-Mercaptopurine (6-MP) • Capecitabine (Xeloda®) • Cladribine • Clofarabine • Cytarabine (Ara-C®) • Floxuridine • Fludarabine • Gemcitabine (Gemzar®) • Hydroxyurea • Methotrexate • Pemetrexed (Alimta®) • Pentostatin • Thioguanine
Anthracyclines	• Daunorubicin • Doxorubicin (Adriamycin®) • Epirubicin • Idarubicin
Mitotic inhibitors	• Taxanes: paclitaxel (Taxol®) and docetaxel (Taxotere®) • Epothilones: ixabepilone (Ixempra®) • Vinca alkaloids: vinblastine (Velban®), vincristine (Oncovin®), and vinorelbine (Navelbine®) • Estramustine (Emcyt®)
Hormone chemotherapeutic drugs	• Prednisone, methylprednisolone (Solu-Medrol®), and dexamethasone (Decadron®)

Table 1. Various classes of anticancer chemotherapeutic drugs and their examples.

comparable in many ways to doxorubicin, both of which can damage the heart at high dosage. Their mode of action is also the same, i.e., inhibiting the topoisomerase II, and thus can lead to posttreatment acute myelogenous leukemia, after 2–3 years in most cases. Prostate and breast cancers, lymphoma, and leukemia are also treated with mitoxantrone.

4.4.1.5. Topoisomerase inhibitors

Topoisomerase inhibitors deter the unwinding of DNA and hence stop DNA replication. Some leukemia; ovarian, gastrointestinal, and lung cancers; and others are treated with these drugs. Examples of topoisomerase I inhibitors are topotecan and irinotecan (CPT-11), and examples of topoisomerase II inhibitors are etoposide (VP-16) and teniposide. Mitoxantrone also constrains topoisomerase II.

4.4.1.6. Mitotic inhibitors

Mitotic inhibitors are plant alkaloids and other naturally derived products in nature. They inhibit synthesis of proteins necessary for cell division, particularly in the mitotic phase of the cell cycle, subsequently damaging all other phases too. Cancers treated with these drugs include lung, breast, myelomas, leukemia, and lymphoma. Side effects such as peripheral nerve damage can put limits to dosages of these drugs. Examples of mitotic inhibitors are given in **Table 1**.

4.4.1.7. Miscellaneous chemotherapy drugs

Some uncategorized chemo drugs with uncommon modes of action include the enzyme L-asparaginase and an inhibitor of proteasome called bortezomib (Velcade®). Examples include drugs like L-asparaginase; it is an enzyme, and the proteasome inhibitor is bortezomib (Velcade®) [41].

4.5. Hormone therapy

Advancements in the field of molecular biology in recent years clarified the role of hormones in cell growth and in the regulation of malignant cells. Nearly 25% of tumors in men and 40% in women are known to have hormonal basis. Hormonal treatment is effective to treat cancer without any cytotoxicity which is associated with chemotherapy [42]. Steroids are hormone in nature, and such hormone-like drugs are used in treatment of cancers like lymphoma, leukemias, and multiple myeloma. Moreover, corticosteroids are used as antiemetics, which give relief from nausea and vomiting after chemotherapy. Also used before chemotherapy, they mitigate hypersensitivity to the treatment. Only when used in actual chemotherapy procedure, these drugs are called as chemotherapeutic drugs [43]. Examples of such drugs are given in **Table 1**.

4.6. Antiangiogenesis inhibitors

Nutrition to the tumor cells is provided by blood vessels, and the development of these vessels inside tumor tissues is called angiogenesis. Some chemical inhibitors known as “angiogenesis

inhibitors" can cut off the blood supply to the tumor cells. These angiogenic inhibitors like thalidomide, interferon, bevacizumab (Avastin), cilengitide (EMD 121974), and cediranib (Recentin) VB-111 are sometimes administered in combination with the chemotherapeutic drugs in an attempt to increase therapeutic efficiency of both [44] (**Table 2**).

4.7. Role of stem cells in cancer treatment

Stem cells are undifferentiated cells present in the bone marrow with an ability to differentiate into any type of body cells. Stem cell therapeutic strategy is also one of the treatment options for cancer which are considered to be safe and effective. Application of stem cell is yet in experimental clinical trial; for example, their use in the regeneration of damaged tissue like the heart, liver, bones, skin, cornea, etc. is being explored. Mesenchymal stem cells are currently being used in trials which are delivered from the bone marrow, fat tissues, and connective tissues [46].

4.8. Autologous dendritic cell vaccines for cancer immunotherapy

Immunotherapy is a wider term defined as the treatment of diseases by manipulating the immune system. It is of two types: active immunotherapy and passive immunotherapy. Self-limiting infectious diseases are easily controlled by traditional active vaccination strategies. Treatment of chronic infectious diseases or cancer is currently the main objective of immunotherapy, and it requires better understanding of the immune systems in terms of its regulatory mechanisms, identification of appropriate antigen, and optimization of the interaction between antigen-presenting cells (APC) and T cells [47]. Dendritic cells are professional APC. They play a major role in the initiation and control of immune responses by regulating T and B lymphocyte activation. These cells are strategically positioned throughout the body in an immature state, surveying the tissues for invading pathogens, and are unique in antigen capturing, processing, and presentation as compared to other antigen-presenting cells [48].

Class	Mechanism
Selective estrogen receptor modulators (SARM) (tamoxifen and raloxifene)	Block-binding site for estrogen; can slow the growth of estrogen stimulated cancers
Selective androgen receptor modulators (SARM)	Block-binding site for testosterone; can slow the growth of testosterone modulated cancers
Spindle inhibitors	Stops cell replication early in mitosis
Farnesyl transferase inhibitors	Blocks addition of farnesyl group to RAS, preventing its action
Gleevec®	Binds to abnormal proteins in cancer cells, blocking their action
Angiogenesis inhibitors (endostatin, angiostatin)	Prevent angiogenesis by tumor cells
Immunostimulants (interleukin 2, alpha interferon)	Enhance the normal immune response
Herceptin	Antibody that binds to HER2 receptor on tumor cell preventing the binding of growth factors

Table 2. Different drugs involved in the treatment of cancer through different mechanisms [45].

DCs are derived from bone marrow progenitors and circulate in the blood as immature precursors prior to migration into peripheral tissues. Within different tissues, DCs differentiate and become active in the taking-up and processing of the antigen. The location of the DCs inside the body is unique to capture the foreign antigens such as body surfaces like the skin, pharynx, upper esophagus, vagina, ectocervix, and anus and at mucosal surfaces, such as the respiratory and gastrointestinal systems [49]. In steady-state conditions, in most tissues DCs are immature, unable to stimulate the T cells due to the lack of required accessory signals such as CD40, CD54, and CD86, but they are highly equipped with the antigen-capturing Fcγ and Fcε receptors to uptake the antigens [48]. Upon antigen uptake and appropriate stimulation, DCs undergo further maturation and migrate to secondary lymphoid tissues where they present Ag to T cells and induce an immune response [50].

Inaba et al. (1990) first described the role of DCs as adjuvants. In this study, DCs isolated from mouse spleen were primed with the specific antigen overnight. DCs processed and presented the antigen epitopes onto MHC molecules, and Ag-loaded DCs were then injected into mice, which led to Ag-specific T-cell sensitization and development of immunity. The immune response was robust when the mouse was challenged again with the DC pulsed with the same antigen, due to the presence of memory cells [51].

Methods of preparing DCs have changed since they were considered trace cell types of the immune system, when in vitro protocols were employed to grow DCs from their progenitors. Inaba et al. identified and reported clusters of DCs from cultures of mouse blood supplemented with GM-CSF [52]. Bone-marrow being the precursor of DCs, they were soon thereafter identified in the blood culture, and a method was thus described to grow large numbers of DCs from bone marrow (BM) cultures of mice supplemented with GM-CSF [53]. These new methods of DC culture paved the way to further characterize DCs and investigate their clinical application. In order to investigate the capacity of BM-derived DC (BMDC) to be used as an adjuvant to induce immunity against infectious diseases, BMDCs were pulsed with bacillus Calmette-Guerin organism and induced a strong T-cell response when injected in vivo [54].

To investigate the role of DCs as adjuvant in humans, they are prepared from the culture of blood monocytes supplemented with GM-CSF and IL-4 [55]. Later, a method to generate mature DCs from human blood was described in which they used macrophage-conditioned media containing essential maturation factors [56]. DCs generated with this method were clinically more potent as an adjuvant.

Briefly, to produce autologous dendritic cell vaccines for cancer immunotherapy, monocytes are harvested from cancer patients by leukapheresis and cultured in the presence of GM-CSF and IL-4 supplements to generate monocyte-derived DCs. These immature monocyte-derived DCs can subsequently be loaded with tumor-derived antigens using different methods. Firstly, DC can be fed with the autologous tumor lysate prepared from the tumor biopsy of the concerned patients. Secondly, DC can be electroporated with tumor-derived mRNA. However, if the access of autologous tumor is too limiting, then DCs may be loaded with allogeneic tumor proteins or common tumor-associated antigens (TAAs). DCs loaded with the relevant tumor peptides/antigens are activated using Toll-like receptor ligands or activating cytokines. The mature DCs loaded with tumor antigens are then stored and transported in dry ice to be

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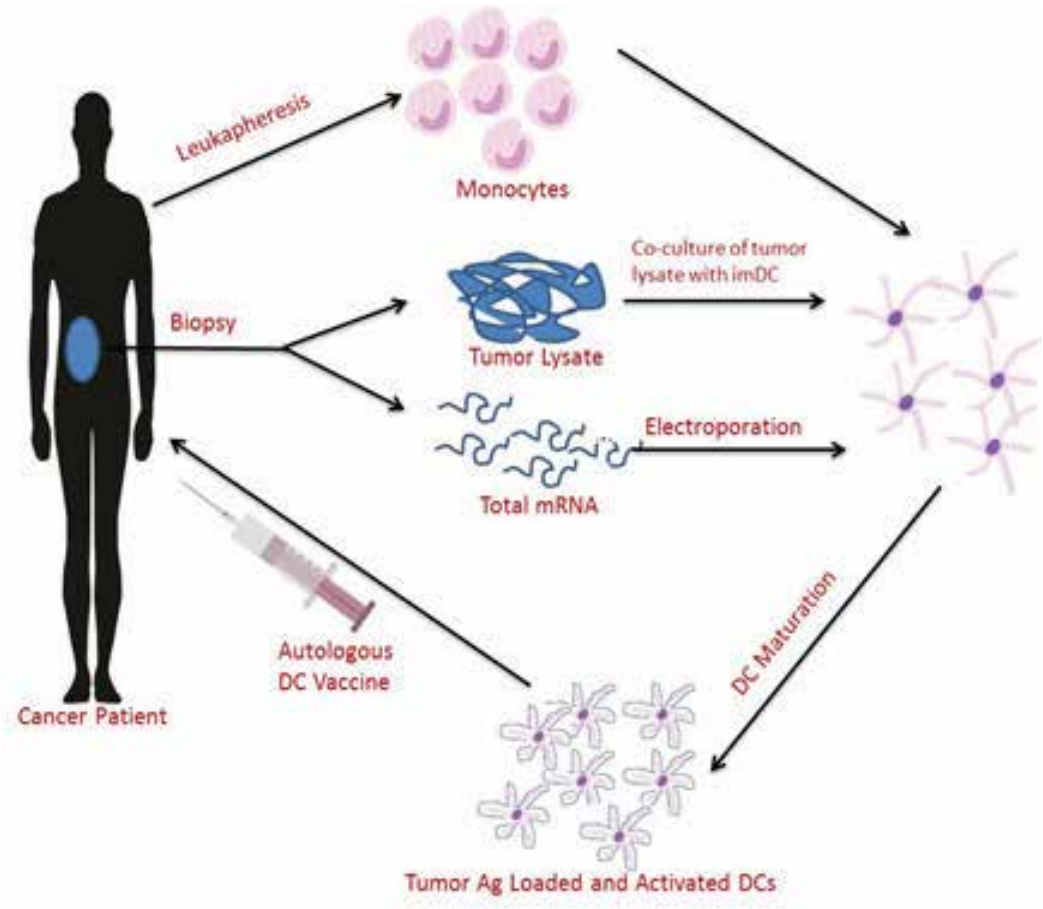

Figure 3. Schematic presentation of autologous dendritic cell vaccine preparation for cancer immunotherapy.

used as autologous DC-based cancer vaccine as shown in **Figure 3**. When injected into cancer patients, tumor antigen loaded DCs are drained into the local lymph nodes and induce tumor-specific T-cell immunity which helps to fight against the cancer cells of the patient [57].

5. Conclusion

Cancer is one of those diseases that are emerging very rapidly throughout the world, and it is affecting about 82% of the world's population. Cancer is a complex disorder involving complex alterations in the physiological conditions of the body. Considering its severe complications, there is a crucial need to search active treatment modalities for cancer. Some of the traditional methods like radiotherapy, chemotherapy, and surgery are still considered effective, but due to certain side effects to the normal body cells, we owe to work for some advancement in cancer treatment modalities. In recent times hormone-based therapy, gene therapy, stem cell

therapy, and dendritic cell-based immunotherapy are introduced which, if used along with traditional therapies, can minimize the chances of relapse in cancer patients.

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Chapter 7

Biomarker-Based Targeted Therapeutics

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Additional information is available at the end of the chapter

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Abstract

Cancer biomarkers are emerging as important tools for disease diagnosis, prediction and prognosis. A significant number of studies have been reported in the field of biomarker discovery due to their potential as personalized targeted therapy. With the converging gap about their utilization as specific targets, studies have focused on identifying disease-specific biomarkers in different cancer types. This chapter provides a comprehensive overview about different cancer-associated biomarkers, their prevalence in different cancer types and their use as targeted therapy. Additionally, we provide an in-sight on the therapeutic and diagnostic potential of different noncoding RNAs as cancer biomarkers.

Keywords: biomarkers, exosomes, chemokines, noncoding RNA, therapeutics

1. Introduction

Cancer is a genetic disease with great molecular diversity and unpredictable nature, which makes it complicated to generate reliable therapeutic interventions for treating cancer. Current treatment strategies include chemotherapy, radiotherapy and surgery. Cancer patients often show primary resistance to directed therapy or often develop adaptive resistance during the course of treatment. Hence it is extremely important to understand the molecular basis of cancer and search reliable biomarkers that can be employed in field of cancer diagnosis and treatment.

Precision medicine, classified as personalized cancer therapy, has increased our knowledge of aberrantly regulated genes and their involvement in tumorigenic pathways towards developing better therapeutic strategies. The advancement in information about the cause and effect of cancer genetics has translated to personalized targeted therapy, one such based on cancer

biomarkers. A biomarker is defined as biological molecule such as a protein, DNA, RNA or circulating extracellular vesicles (EVs) that can be found in blood, biological fluids and tissues and is an indicator of a normal physiologic or a diseased state. Cancer biomarkers are predictive of altered gene signature marks at the transcription level and/or of abnormal proteomic or metabolomic patterns. These biomarkers are used for molecular diagnosis, patient prognosis and to determine the outcome of the targeted therapy. Hence, it is extremely important to understand the evolving landscape of cancer genetics and to combine tumor aberrations with personalized biomarker-based targeted therapy.

Human genome sequencing has identified approximately 30,000–40,000 genes and only 2–3% of the coding sequence of the genome are evolutionary conserved among mammals [1, 2]. About 98% of genome sequence was initially classified as junk DNA, but recent progress in deep sequencing has discovered these to be transcribed as noncoding RNAs (ncRNAs). These ncRNAs are grouped as different types of functional RNAs, such as Piwi-interacting RNA (piRNA), microRNA (miRNA), small nucleolar RNA (snoRNA), circular RNA (circRNA) and long noncoding RNA (lncRNA). ncRNAs have been shown to affect gene expression and disease progression, making them important targets for drug discovery. Clinically, aberrations in ncRNAs show high prognostic and diagnostic importance. With the advancement in technology and enhancement in understanding the nature of ncRNAs, novel therapeutic treatments against cancer can be developed.

This chapter focuses on deciphering a comprehensive approach on the recent biomarkers that are available as therapeutic options, their scope, utility and implementation as prognostic and diagnostic tools for cancer therapy. We will focus on role of ncRNAs and discuss their potential use as a prognostic and diagnostic marker in various cancers. We will also address the challenges and possible solutions in their assessment as biomarker for therapeutic use.

2. Cancer biomarkers

A biomarker is a collection of genetic and proteomic signatures used to distinguish between healthy and diseased individual. These signatures can be in the form of DNA (ssDNA, dsDNA and retrotransposons), RNA (mRNA, miRNA, circRNA and lncRNA) or protein (antibodies and peptides) depending upon the site of secretion and isolation [3]. A biomarker is predictive of disease prognosis and prediction, risk of recurrence or to determine the therapeutic potential of an identified target. The success of biomarker-based therapy can be attributed to the development of new sequencing strategies and characterization of tumor pathways with increasing knowledge about druggable targets and its predictive outcome. One of the earliest biomarkers to reach clinical practice was the identification of mutations in *KRAS* gene in case of metastatic colorectal cancer (CRC), which was predictive of therapeutic response towards anti-epidermal growth factor receptor (EGFR) [4]. However, owing to the heterogeneity of tumor cells, lack of information on specific biomarkers associated with particular disease type (and subtype) and different developmental strategies, biomarker-based therapy has not fully transcended to clinical stages.

Cancer biomarkers are broadly categorized into three divisions based on the specific signature it is associated with: diagnostic, predictive and prognostic biomarkers [5]. As the name

suggests, diagnostics biomarkers predict disease outcome associated with a particular malignancy; predictive biomarkers predict the success of a particular therapeutic strategy applied to treat the disease followed by prognostic biomarkers that predict the risk of disease recurrence in the future. Currently, there are only a handful of FDA approved biomarkers in the market highlighting the present-day challenges, starting from their diagnosis to clinical approval. Some examples of FDA approved biomarkers include: HER2 overexpression as a predictive marker to determine the survival status of breast cancer patients treated with anti-HER2 therapy [6]. Another example of FDA approved diagnostic maker include testing the patients suspected with prostate cancer for the prostate-specific antigen (PSA) to test for malignancy of the associated disease (recent studies have found PSA screening to be inconsistent [7] however, further studies needs to be done to understand the discrepancy). Measurement of 70-gene expression analysis used to predict the recurrence of breast cancer after chemotherapy, is an FDA approved prognostic biomarker-based assay [8]. Apart from the FDA approved biomarkers, there are various new approaches being utilized towards personalized targeted therapy so as to bridge the gap between disease diagnosis and its clinical manifestation.

This section will discuss different types of biomarkers characterized thus far in different cancers; their scope and utility for targeted therapy and will provide an overview of the new biomarkers being identified and their possible translational to clinical levels (**Figure 1**).

2.1. Extracellular vesicles as biomarkers of cancer

Tumor cells are characterized by a neoplastic set of population that continuously divides and evolves into sub-population of cells, each with its own heterogeneity. Among the many reasons for cancer therapeutics failure, one of them is the associated tumor heterogeneity and despite the new sequencing strategies developed, there are major challenges that need to be

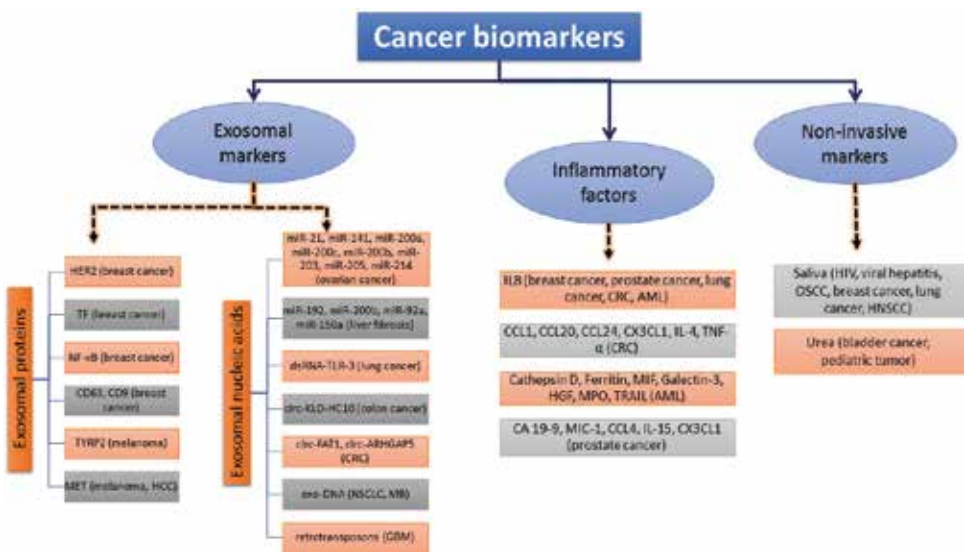

Figure 1. Different cancer-associated biomarkers. An overview of the different biomarkers associated with specific cancer types. Targeting these biomarkers could serve as important therapeutic option for associated malignancies (abbreviations as used in the text).

overcome. Studies have found that tumor cells secrete EVs such as exosomes and macrovesicles into the extracellular environment at a threefold higher rate than normal cells [9, 10]. These vesicles carry important genetic information such as DNA and RNA or protein fragments that act as signatures of the secretory cell type [11]. Identification of EVs from patient's blood stream, urine or plasma provide important insights into the cells they originate from, their genetic constituent and molecular variants. Identification of EVs as potential biomarkers along with the advancement in techniques for their successful isolation has enabled new therapeutic targets for cancer treatment. EVs are isolated from the conditioned media of cells *in vitro* or from biological fluids such as serum and plasma of patients. Some of the important identified secretory signatures in patient-derived vesicles known so far include receptor of a hepatocyte growth factor (HGF) identified in melanoma called MET [12], miRNAs in ovarian cancer [13] and in breast cancer the human epidermal growth factor receptor 2 (HER2/neu) [14] among many. EVs affect the cell milieu by enabling transfer and exchange of important information among different cell types, pre-metastatic niche formation and triggering cell type specific inflammatory immune response [15].

EVs are sub-classified into exosomes, macrovesicles and large oncosomes based on their size. Of these, exosomes (30–120 nm in diameter) are widely considered a valuable source of biomarkers [16] and as important mediators of biological information. Exosomes are bilayered membranous structures comprising of various lipids and proteins, are formed from intracellular vesicles and release their content extracellularly (outside the cell) [17]. Cancer cells secreted exosomes affect the microenvironment not only of the proximally located cells, but also cells of distal origin [18]. Information carried by exosomes not only plays significant role in normal pathological processes, but are also a hallmark of aberrantly regulated pathways in different cell type-associated malignancy. Exosomes have also been characterized as 'liquid biopsy' tools [19] owing to their stability in secreted bodily fluids such as plasma, urine or saliva. Various exosomal markers such as CD63, TSG101 and Alix, among many are known and their detection from conditioned media of cancer cells or from patient-derived tumor samples gives an indication of diseased process [20]. These specific protein markers allow for their characterization as specific liquid biopsy tools in cancers of different origin.

2.1.1. Exosomal proteins as cancer biomarkers

Various studies have reported that secretory information from exosomes could serve as a diagnostic tool in identification of breast cancer types. For example, in secretory exosomes of some breast cancer cell lines, which overexpressed HER2, full-length HER2 protein levels were found to be overexpressed when compared to normal cells [14]. It was found that in EGF (a ligand for HER2)-treated cells, the release of exosomes was higher as measured by the cell-conditioned media and could serve as an important predictive tool in HER2-driven tumors. Monitoring the status of HER2 in blood-derived patient exosomes could therefore serve as a diagnostic tool in breast cancer and to improve the disease outcome. Another study found that a blood clotting specific protein called tissue factor (TF) correlated to increased tumor invasiveness in breast cancer by its incorporation into tumor-derived EVs [21]. TF-derived EVs from highly metastatic triple negative cell line MDA-MB-231 was transferred to less aggressive MCF-7 cell line. It was observed that increased levels of incorporated TF associated with

more aggressive phenotype was responsible for cancer-associated thrombosis. Circulating exosomal vesicles are known to be upregulated during cancer progression and are associated with intercellular communication. A study found that breast cancer-derived exosomes from MDA-MB-231 (MDA-231) and MCF7 cells had elevated levels of transcription factor nuclear factor-κB (NF-κB) and its associated activation of signaling pathway in the macrophages as compared to exosomes from MCF10A cells. Increased NF-κB signaling led to increased production of pro-inflammatory cytokines, which included factors such as interleukin-6 (IL6), granulocyte-colony stimulating factor (GCSF), chemokine ligand 2 (CCL2) and tumor necrosis factor α (TNFα) [22]. The increased inflammatory response in the macrophages contributed to metastatic niche formation and to modulate immune cells activity. Thus, targeting the activity of NF-κB pathway could be used as a therapeutic option to block the secretion of exosomes and consequently the formation of metastatic microenvironment. EVs from brain metastatic cells were found to be within 100 nm diameter size and expressing markers such as CD63 and CD9, characteristic of exosomes [23]. A study found that breast cancer-derived exosomes contribute to the breakdown blood-brain barrier (BBB) and *in vivo* cell metastasis in the brain [23]. Secretion of exosomes was inhibited by targeting the degradation of EV proteins involved in its biogenesis such as neutral sphingomyelinase (nSMase2) and RAB27B. It was found that cells showed reduced migratory potential and that its migratory ability was restored when exosomes derived from breast cancer cells was added. Thus, targeting the cancer-derived exosomes could be an important therapeutic option for the prevention of BBB breakdown and to prevent its associated malignancies.

In another study, exosomes were isolated from the plasma of the patients with tumor grade I–IV [12]. It was found that patients with high exosomal proteins in stage IV had low survival rate as compared to patients in the same stage with low exosomal proteins content. Along with this, increased levels of specific melanoma protein, tyrosinase-related protein-2 (TYRP2) [24] was seen in exosomes isolated from melanoma cell lines. Levels of TYRP2 in exosomes of patients also correlated to their increased metastatic progression of tumors. Given the increasing studies on exosomal horizontal transfer of molecules [25] and intercellular communication, it was hypothesized that an oncogenic protein known to be metastatic could play a role. Among the different known proto-oncogenes such as MET, CD44, Annexin A6 and Hsp70 [26, 27], MET was considered a possible target owing to its role in invasion and metastasis [28]. It was found that in melanoma cell-derived exosomes, secretory vesicles could horizontally transfer MET to bone marrow-derived cells and that the levels of MET and phospho-MET (p-MET) protein was eventually found to increase in exosomes of these cells. Subsequently, MET and p-MET levels was also found to be high in circulating exosomes isolated from patients with stages III and IV grade melanoma. Hence, it was hypothesized that targeting MET protein using specific inhibitors could provide new opportunities to restrict the metastatic progression of cells in tumors. A similar study on exosomes isolated from hepatocellular carcinoma (HCC) cell lines showed MET proto-oncogene isolated from metastatic HCC cell line to increase the migratory potential of non-motile HCC cells [29]. The uptake of exosomes harboring MET protein in the cells triggered PI3K/AKT and MAPK signaling pathways [30] leading to increased metastatic potential of cells. Increased MET and p-MET along with increased p-AKT and MEK1/2 phosphorylation as well as levels of MMP2 and MMP9 were confirmed in the conditioned media of immortalized HCC cells after exosome

treatment from metastatic cells. Data were correlated with that obtained from HCC patients, where aberrant activation of MET/HGF pathway signaling pathway corresponded to poor prognosis and survival [31].

2.1.2. Exosomal nucleic acids as cancer biomarkers

First evidence of exosomal shuttle RNA (esRNA) came from a study which found that exosomes harbor both mRNA and miRNA and that they are involved in intercellular communication [11]. It was found that mRNA and miRNA secreted from mast cells were packed into exosomes and the coding information on mRNA could be translated into protein. The exosomes containing translatable information could be transferred between cell types, thus providing important insights and complexity in which the information is relayed. Targeting the exosomal RNA, that is shuttled between the cells, could therefore allow for targeted therapies in cell type-associated cancer. Subsequently, it was found that in ovarian cancer patients, levels of eight specific miRNAs obtained from exosomes were similar to that obtained cellularly. These circulating exosomal miRNAs isolated from serum samples include miR-21, miR-141, miR-200a, miR-200c, miR-200b, miR-203, miR-205 and miR-214 [13]. This suggests the importance of profiling circulating miRNAs from diseased patients and their use as important diagnostic tools and as liquid biopsy markers. A similar study based on identification of miRNA levels, found different miRNAs in the EVs isolated from the plasma of the patients infected with HBV or HCV infection. Expression profile of miRNAs isolated from circulating vesicles showed reduced miR-192, miR-200b, miR-92a and miR-150a levels [32]. Thus, miRNAs expression levels can be correlated to early stage liver fibrosis identification. Lipid bilayered vesicular contents of exosomes are recognized by multiple pathogen recognition receptors (PRRs), which include retinoic acid-inducible gene I (RIG-I) and toll-like receptors (TLRs) – TLR2, and TLR4 among others [22]. A study found that tumor-derived exosomes stimulated the activation of TLR3 in alveolar epithelial cells followed by the production of pro-inflammatory cytokines and pre-metastatic niche formation [33]. Since TLR3 recognizes dsRNA, RNA isolated from tumor-derived exosomes upregulated the expression of TLR3, thereby leading to cytokine production by activation of downstream NF-κB and MAPK pathways [34]. Activated TLR3 also led to the recruitment of neutrophils in the lungs, which elicited a pro-metastatic inflammatory response. Thus, insights into tumor-derived exosomal RNAs could provide important clues to target tumor metastasis in the lungs.

The first evidence of Exo-circRNA (circular RNA from exosomes) came from a study which showed them to be enriched at a twofold higher rate in liver cancer cells as compared to circRNA present in those cells [35]. Data from RNA-seq showed abundant circRNA in liver cancer cells as compared to normal cells. It was found that levels of these identified circRNA were about sixfold higher than linear RNA in the exosomes of these cells. The researchers further investigated whether serum from cancer affected patients were enriched with circRNA. Serum from patients with colon cancer showed high levels of an exo-circRNA, circKLD-HC10 in the exosomes of patients in comparison to healthy controls. This was the first evidence of exosome-based circular biomarkers as potential therapeutic options. In another study based on circRNA, expression profile of circRNAs present in both cells and exosomes of different

CRC cell lines that differ in the KRAS mutation status were analyzed [36]. KRAS is a proto-oncogene first identified in Kirsten rat sarcoma virus [37] and whose mutation or upregulation is associated with cancer progression based on the different pathways it acts upon. It was found that in cells where KRAS was mutated, a number of circRNA isolated from exosomes were downregulated as compared to those identified in cells having wild-type KRAS allele. These circRNAs include circFAT1 and circARHGAP5. To further investigate the reason for their downregulation, levels of known regulators of circRNA, adenosine deaminases acting on RNA (ADAR, an RNA editing enzyme) [38] and quaking (QK1, an RNA binding protein) [39] were determined. It was found that decreased levels of above-mentioned circRNAs directly correlated to decreased levels for QK1 found in the mutant cells. Levels of circRNAs could therefore serve as important biomarker for disease prediction.

The first report of tumor-derived exosomal DNA (exoDNA) showed that exosomes carried double-stranded DNA (dsDNA). The levels of these exoDNA corresponded to the mutational status of cancer cells and can therefore be used as an important circulating biomarkers in case of cancer-associated metastasis [40]. Mutation status of different genes known to be mutated in cancer cell lines were determined to see if the status of the genes corresponded to that obtained from exoDNA. It was found that BRAF allele was mutated in exoDNA in primary human melanoma cells containing BARF mutation as compared to cells that did not harbor the mutation. Also, status of EGFR, which is known to be mutated in case of non-small cell lung cancer (NSCLC) [41], was determined and same observation as compared to BRAF allele was found, confirming that exoDNA shows the same mutation status as that in parental cells. This shows that, exoDNA reflects the genomic status of the DNA present in cancer cells and because it is stable and feasible to isolate, could serve as an important biomarker. In addition to the dsDNA found in exoDNA, another study found the presence of ssDNA in the microvesicles (another subclass of extracellular vesicles with 30 nm–1 μm in diameter) from the tumor cells in culture in addition to retrotransposon elements [42]. Nucleic acids were identified from microvesicles released by cells *in vitro* and from tumors. It was found that along with RNA, ssDNA in the form of exoDNA as well as transposable elements were present in the microvesicles. Levels of exo-RNA were found to be higher in medulloblastoma (MB) cells, which released more microvesicles as compared to normal cells with intact 18S and 28S ribosomal peaks. Similarly, in MB cells levels of exoDNA was found to be more abundant as that in normal cells and was found to be single stranded. The strand status was confirmed using a detection chip that detected only dsDNA (the isolated single-stranded exoDNA was subjected to second strand synthesis, which converted it into double-stranded form and hence was detected by the chip). Retrotransposons such as human endogenous retroviruses (HERVs), long interspersed nuclear element-1 (LINE-1) and Alu were found to be enriched in microvesicles isolated from glioblastoma (GBM) cells. RNA from these transposable elements were enriched as compared to their cellular levels in these cells. Interestingly, experimental observations also suggested that HERV RNA is elevated in endothelial cells when these were exposed to microvesicles from tumor cells, indicating that these retrotransposons (jumping genes) could lead to genomic instability. Taken together, this study reveals that the uniquely identified retrotransposons RNA could serve as important biomarkers in tumor cells along with DNA from patient-derived body fluids. Additionally, the level of these retrotransposons could be an important indicator of the cell type-specific tumor origin [43].

2.2. Inflammatory cytokines and chemokines as biomarkers of cancer

Levels of invading microbial pathogens during an inflammatory response are regulated by the first line of immune defense called the innate immune system [44]. The pathogen-associated molecular patterns (PAMPs) are bound by the associated PRRs, each recognizing its own unique set of patterns [45]. Upon receptor activation, a pro-inflammatory and antimicrobial response is triggered in the host system, which includes a series of signaling events comprising of small molecules, transcription factors and kinases [46]. Subsequently, the signaling pathways relay the signal activating the associated cytokines or chemokines of the pathway [45], which generate a long-term response, ultimately leading to the activation of adaptive arm of the immune response.

Cytokines and chemokines are key modulators of inflammation and are involved in a variety of diseased processes based on their specific role. These can be pro- or anti-inflammatory, depending upon their class and type. For example, certain pro-inflammatory cytokines include interleukins (IL) such as IL-1, IL-6, TNFα and interferons (IFNs) among many [47]. Examples of anti-inflammatory factors include IL-12 and IL-10 among many [47]. Chemokines are small group of proteins characterized by conserved cysteine residues that recruit leukocytes to the site of inflammation. Common chemokines include any protein with a CCL or CXC motif such as RANTES (CCL5) and IL8 (CXCL8) among many [47]. The level of cytokines and chemokines differ greatly among individuals depending upon normal or diseased outcome. The downstream effects produced by these inflammatory regulators depend on the signaling pathway activated, their targets and the associated patterns. Although these factors are involved in many disease-associated processes, their levels are not consistent across similar diseased processes. The variation is due to the difference in their cut-off levels on what is considered normal versus abnormal [48] as well as the population size of the cohort used in the study (healthy or diseased) [49]. Hence, it is difficult to characterize them as unique biomarkers or as diagnostic tools for any cytokine-associated specific disease. Despite these limitations, considerable progress has been made and recent studies have focused on important cytokines and chemokines, their involvement in cancer-associated diseases and their use as cancer biomarkers.

2.2.1. IL8 as a cancer-associated biomarker

IL8, a 6–8 kDa pro-inflammatory chemokine is the most widely studied for its role in recruiting neutrophils to the site of infection, activation of angiogenesis and metastasis [47]. Many metastatic and tumors of breast, prostate and colon cancer are known to constitutively express IL8 [50]. IL8 ligand is bound and recognized by its G-protein coupled receptors (GPCRs) CXCR1 and CXCR2, with CXCR1 being the more specific receptor for mediating the response [47]. A number of therapeutic strategies therefore utilize the difference in pharmacological properties of these receptors to target and attenuate the effect of IL8 on the tumor microenvironment. High serum levels of IL8 correspond to poor prognosis and increased metastasis as seen in patients with metastatic breast cancer [51]. It was also noted that in cells treated with cytotoxic agents, IL8 levels were high with increased chemo-resistance observed in tumor cells. Another study on breast cancer found the role of a protein of *Drosophila* gene called dachshund (dac) to be important in patient prognosis [52]. *Drosophila dac* gene is involved in differentiation [53] and is a founding member of the human homolog *dac* protein DACH1, a

cell fate determination factor [54]. Levels of DACH1 directly correlated to patient survival and were found to be low in metastatic breast cancer. It was found that DACH1 inhibited cell migration and invasion by decreasing the levels of IL8 in breast cancer cells. DACH1 repressed the IL8 promoter in a dose-dependent manner by occupying the AP-1 and NF-κB sites. Adding neutralizing antibody against IL8 resulted in a decrease in the migratory potential of the cells, showing that targeting IL8 as a biomarker could be a potential mechanism to inhibit cell growth.

In addition to binding to its own receptor, IL8 binds and upregulates the expression of another receptor, CXCR7 in case of prostate cancer leading to cell proliferation and growth [55]. It was observed that CXCR7-mediated growth was dependent on the activation of EGFR and independent of its own ligand, highlighting the importance of targeting CXCR7 as a potential biomarker along with IL8 in case of prostate cancer. In case of lung cancer, a study found that high circulating levels of IL8 were predictive of the risk of lung cancer in patients prior to diagnosis [56]. Along with IL8, IL6 was also found to be predictive of the high risk of lung cancer but only in cases within 2 years of blood collection. However, highest associated risk of lung cancer was determined in patients with several years of smoking habit and showed high IL8 and C-reactive protein (CRP) levels. Thus, plasma levels of IL8 along with CRP could be a more robust predictor of lung cancer (several years before diagnosis) for tumor progression and relapse.

Promoter methylation is often characterized with aberrant transcriptional regulation and/or tumor suppressor genes silencing [57]. It was found that in MDA-231 and MDA-MB-435 (MDA-435), two highly metastatic breast cancer cell lines that produce high levels of IL8, two CpG sites were methylated 1.2 kb upstream of IL8 promoter. The observed methylation pattern showed a positive correlation with IL8 expression, suggesting additional uncharacterized epigenetic control known so far [58]. Similar approach in CRC was taken and it was observed that IL8 promoter was hypomethylated in 64% of tissue samples [59]. Hypomethylation of the promoter led to high IL8 protein levels and associated metastasis, showing that high IL8 levels along with hypomethylated IL8 promoter, could be a useful marker for disease progression. Despite the vast array of information on IL8 and its role in different cancer progression and metastasis, only handful inhibitors against it are used in preclinical studies and even few have reached clinical trials. Neutralizing antibodies against IL8, ABX and HuMax are being used to block the binding to IL8 to its receptor [60]. ABX has shown to reduce tumor growth in mice in case of bladder cancer [61] and reduction in metastasis and angiogenesis along with reduced tumor size in case of melanoma [62]. Reparixin, an inhibitor of IL8 receptor CXCR1/2 is in clinical trials for the treatment of patients affected with HER2-negative breast cancer and patients with TNBC along with paclitaxel (an FDA approved microtubule-stabilizing drug for ovarian, breast and lung cancer treatment [63]).

2.2.2. Other inflammatory factors as cancer biomarkers

In a study on colorectal cancer patients (stages I–IV grade), levels of inflammatory factors were determined in the blood at the time of surgery [64]. It was found that CRC-specific mortality directly correlated to the plasma levels of various inflammatory factors. In particular, levels of IL-4, TNFα, CCL1, CX3CL1, CCL20 and CCL24 were upregulated in patients with CRC-specific mortality. Thus, high levels of these inflammatory cytokines, chemokines

and interleukins found in the plasma of affected individuals could be a diagnostic marker to determine disease outcome and prognosis. A study on acute myeloid leukemia (AML) reported a cohort of seven inflammatory molecules to be upregulated and predictive of AML diagnosis irrespective of the disease heterogeneity [65]. These molecules include Cathepsin D, Ferritin, Macrophage migration inhibitory factor (MIF), Galectin-3, HGF, myeloperoxidase (MPO) and IL8 suggesting that their plasma levels could be predictive of disease diagnosis. In accordance with the TCGA database, levels of two other novel inflammatory molecules TNF-related apoptosis-inducing ligand (TRAIL) and MIF were downregulated and upregulated, respectively, in the plasma of patients. Levels of MIF correlated with that found in other cancer types such as prostate and breast and is known to activate PI3K/Akt pathway, leading to anti-apoptosis and survival [66]. Thus, along with the seven biomarkers for prognosis, MIF could serve as an important therapeutic target in AML.

Carbohydrate antigen (CA) 19-9, is the only FDA approved diagnostic marker for prostate cancer but its diagnosis is limited due to inaccurate sensitivity and specificity in different prostate cancer subtypes [67]. There is thus an ardent need for a personalized biomarker predictive of disease outcome. A study found the levels of macrophage inhibitory cytokine 1 (MIC-1), a novel TGF-β superfamily cytokine [68] in patient serum affected with prostate cancer, to be differentially expressed in comparison to healthy cohorts [69]. The diagnostic sensitivity and specificity percentage of MIC-1 from a total pool of different prostate cancer patients were found to be low but improved significantly when both CA 19-9 and MIC-1 were detected in patient serum [70], showing that these biomarkers together could be used as diagnostic tools in prostate cancer. Another example of the use of multiple biomarkers for patient prognosis and disease outcome in prostate cancer came from a study which identified three inflammatory factors in tissue samples of patients post prostatectomy [71]. Among the 30 cytokines that were measured, the expression levels of CCL4, IL-15 and CX3CL1 were significant and were predictive of recurrence free survival 5, 3 and 1-year post surgery.

2.3. Noninvasive cancer biomarkers

Research on biomarkers and its clinical translation is still in its early phase despite the recent advances in the field. This is due to the complexity in identifying the specific biomarker, its sensitivity of prediction and the isolation method used. There is therefore a need for the development of a noninvasive, inexpensive and accessible biomarker to evaluate disease progression and for better diagnosis. Besides the use of bio-fluids such as saliva and urine for basic health assessment, they are being used to monitor disease progression and possible outcome. The progress in the usage of saliva as a diagnostic biomarker fluid can be attributed to its FDA approval in 2003 for detecting HIV infection. In case of HIV, levels of a microglobin B2M b2, an end product of increased cytokine production and a soluble tumor necrosis factor α-receptor 11 (sTNFαR11) was detected to be higher in saliva of HIV infected patients than in control [72]. Thus, specific kits were designed to measure the levels of these inflammatory molecules from saliva to be predictive of HIV infection. A study showed the comparison between sensitivity and specificity of samples obtained from oral fluid and serum for the evaluation of different viral Hepatitis type. It was found that samples obtained from saliva showed almost 100% sensitivity and specificity for the immunoglobin (Ig) M of Hepatitis A virus (HAV), surface antigen of Hepatitis B virus (HBV) and antibody of Hepatitis C virus (HCV), confirming that oral sampling offers opportunities for efficient prognosis [73]. In

another study based on identification of salivary transcriptome factors in oral squamous cell carcinoma (OSCC), levels of different mRNA signatures were determined. Among the different identified targets, seven genes were found to be significantly upregulated in the following order: high-*IL8*, moderate-*H3 histone family member 3A* (H3F3A), S100 Calcium Binding Protein P (*S100P*), *IL1B*, dual specificity phosphatase 1 (*DUSP1*), low-spermidine/spermine N1-acetyl transferase (*SAT*) and Ornithine decarboxylase antizyme 1 (*OAZ1*) [74]. Each of the identified gene has a role in cancer-specific pathways and is often deregulated leading to diseased progression. Therefore, the combination of identified salivary biomarkers with sensitivity and specificity of around 90% could help in early diagnosis of oral cancer and could be explored for other cancer types as well.

A study analyzed the level of protein c-ErbB-2 or HER2/neu in patient saliva with/without breast cancer [75]. The oncogenic protein is considered a prognostic marker having been identified in the tissue biopsies of patients with malignant tumor. The results showed that c-ErbB-2 level identified from saliva was upregulated and could therefore be used in the diagnosis of patients and to monitor disease recurrence. In another study based on salivary proteomics technology, protein profiles of patients with generalized aggressive periodontitis (GAgP) were compared with that of controls and 11 proteins were found to be altered [76]. Thus, salivary diagnostics identifying peptides and salivary proteins could play a significant role in understanding the cause of associated disease. In case of lung cancer, a study identified the methylation status of promoters of different TSGs in sputum of lung cancer patients. Promoters of genes such as O6-methylguanine DNA methyltransferase (MGMT), RAS association domain-containing protein 1(RASSF1A), death-associated protein kinase (DAPK) and B cell lineage-specific activator protein (PAX5a or BSAP) were highly methylated in patients who had survived lung cancer (these patients had a 6% recurrence risk) [77]. A similar study on head and neck squamous cell carcinoma (HNSCC) found promoters of similar TSGs to be hypermethylated and therefore important for the diagnosis in saliva of patients. These include RASSF1a, p16 INK4A, DAPKI and MGMT, confirming that these biomarkers play an important role and that methylation status of gene promoters is associated with increased cancer risk [78].

Other than saliva, another noninvasive accessible biomarker that can be used for disease diagnosis and prevention is urine. A study identified the methylation status of different TSGs in patients suffering from bladder cancer and found it to correlate with tumor grade. The TSGs identified were cyclin D2 (CCND2), Secretoglobin Family 3A Member 1 (SCGB3A1), BCL2 Interacting Protein 3 (BNIP3), DNA-binding protein inhibitor ID-4 (ID4) and Runt-related transcription factor 3 (RUNX3) [79]. Another study on pediatric tumors identified two biomarkers tissue inhibitor of metalloproteinases-3 (TIMP3) and basic fibroblast growth factor (bFGF) to be involved in detection of juvenile pilocytic astrocytomas (JPAs) in the brain [80]. The expression of biomarkers correlated to tumor grade and their levels decreased after treatment.

3. Overview of noncoding RNAs

Noncoding RNA (ncRNA) is RNA transcript that do not encode for the protein. In term of their sizes ncRNAs on threshold of 200 nucleotides length, are categorized in two types, small noncoding RNAs and long noncoding RNAs (lncRNAs). Small noncoding RNAs are sub categorized into microRNAs (miRNAs), small nucleolar RNAs (snoRNAs) and piwiRNAs

(piRNAs) [81]. On the basis of their location with respect to protein coding genes, lncRNAs are categorized into: intergenic lncRNAs (present between two protein coding genes), intronic lncRNAs (introns of protein coding genes transcribe them), overlapping lncRNAs (a coding gene is located on the intron), antisense lncRNAs (the opposite strand of protein coding gene transcribe them) and processed lncRNAs (lacks an open reading frame ORF) [82]. These ncRNAs play an important role in different biological processes and are often deregulated in cancer. In this section, we will discuss ncRNAs as a potential biomarker, providing rationales for the development of therapeutics targeted against or based on these ncRNAs.

3.1. miRNA

miRNAs are universally present in plants and mammals and are single-stranded RNA of 18–25 nucleotides in length. They regulate gene expression mainly at the posttranscriptional level in a sequence specific manner either by translational repression or by cleavage of their target mRNAs. Lin-4 and let-7 were identified in *C. elegans* as the first miRNAs. They were involved in nematode development. As many as 1881 precursors, 2588 mature; 495 precursors, 765 mature and 1193 precursors, 1915 mature microRNAs have been interpreted in the human, rat and mouse, respectively, till date and this collectively has been cataloged in the miRNA Registry (http://microrna.sanger.ac.uk, V 21 July, 2014) [83]. Approximately one-third of the protein coding genes are believed to be controlled by miRNAs. These are first transcribed as pri-miRNA of more than 150 nucleotide (nt) long and then the stem loop is processed by an exonuclease Drosha in the nucleus, which results in pre-miRNA of 70 nt intermediate. These duplex pre-miRNAs are then exported to cytoplasm by Exportin-5 and Ran-GTP. They are then processed by Dicer to form mature miRNA of 22–29 nucleotide in length. Then they become part of RNA induced silencing complex (RISC), where one strand is cleaved (depending on the stability of 5'end) and the other remaining one functions as mature strand. Then this strand depending on the complementarity of the target mRNAs inhibits the translational initiation. miRNAs are expressed in different cells and at different stages, thereby play a crucial role in the regulation of various biological processes in various stages. They have been involved in several diseases like cancer [84]. Urgency for early cancer diagnosis and differentiating multiple cancer types is guiding the way for identifying miRNA signatures and monitor disease.

3.1.1. Techniques for miRNA quantification

Various methods have been developed by researchers to identify miRNAs in body fluids and tumors. Northern blotting is quantitative technique, which is used to detect RNA, but lacks sensitivity [85]. Researchers have used quantitative polymerase chain reaction (q-PCR) based on stem loop primers, that can differentiate between miRNAs isomers. Like whole genome array, miRNA microarray is used to differentiate deregulated miRNAs. Recently high throughput sequencing techniques have undergone a number of developmental changes and small RNA sequencing has been used to identify the novel miRNAs.

3.1.2. Benefits of using miRNA as biomarkers

miRNAs are deregulated frequently in several diseases and are specific to the tissues but some of them are highly conserved in different species and secreted in body fluids thereby serving as potential candidates for biomarkers. In comparison to large and extensive mRNA

expression signatures and identifying unknown tumor origin, miRNA signatures have shown more predictive power.

3.2. miRNAs as biomarker in different cancers

3.2.1. Lung cancer

Lung cancer is one of the major causes of cancer-related deaths in both men and women. Lung cancer is extremely difficult to detect in its early stages and the most prevalent is NSCLC [86]. The effectiveness of NSCLC treatment is expected to be improved through the implementation of robust and specific biomarkers. Novel targeted therapies are being developed based on molecular characteristics. Junichi et al. provided the first evidence of miRNAs role in lung cancer. Let-7 is reduced in human lung cancer and this alteration may have a prognostic impact in lung cancer patients who are surgically treated [87]. Global profiling studies have been done to identify the relationship between alterations of miRNAs and patient outcome. Deregulation of miR-155 and miR-let-7a-2 correlated with poor survival. Univariate analysis as well as multivariate analysis for hsa-mir-155 predicted poor survival [88]. In another global profiling study, investigators identified a set of five microRNAs to construct a signature by the risk score method. They have shown two microRNAs (hsa-miR-221 and hsa-let-7a) to be protective, and the other group (hsa-miR-137, hsa-miR-372 and hsa-miR-182∗) to be predictive of disease progression [89]. Similar genome-wide miRNA expression in patients with NSCLC (plasma samples) was performed and a signature of 24 circulating miRNAs was identified. This study was done by profiling 754 miRNAs in 100 NSCLC patients, showing a strong and highly predictive miRNA signature [86]. Another study done in TRAIL-resistant NSCLC shows miR-221 and miR-222 expression to be elevated, which is necessary to maintain TRAIL-resistant phenotype, thus making them as potential therapeutics or diagnostic tools [90]. Gasparini et al. profiled NSCLCs and showed that the NSCLCs can be classified into as rearranged ALK, mutated EGFR or mutated KRAS versus wild type based on miR-1253, miR-504 and miR-26a-5p expression levels [91].

3.2.2. Prostate cancer

Prostate cancer accounts for almost 15% of all new cancers in men. Several studies have linked circulating miRNAs expression to serve as accurate biomarkers for prostate cancer diagnosis. Singh et al. profiled expression of miRNAs in serum of prostate cancer patients that underwent radical prostatectomy. They identified a panel of 43 miRNAs that could help in differentiation of disease stages in 14 prostate cell lines and patient samples and correlated the expression of miR-222 and miR-125b as prognostic marker in these patients [92]. Another group identified miR-205 and miR-214, which were downregulated in prostate cancer and predicted it as potential biomarker in prostate cancer [93]. Circulating miRNAs that are most deregulated in men with high risk prostate cancer, metastatic and castrate-resistant prostate cancer (CRPC) includes miR-21, miR-141 and miR-221 [94, 95]. These data suggest the diagnostic importance of less invasive biological fluids as sources of biomarkers than blood or tissue of prostate cancer.

Many miRNAs (miR-155, miR-31, miR-152 and miR-137) host genes promoters that are associated with CpG island, recent studies have shown these to be hypermethylated in prostate cancer [96]. They have shown the upregulation of KDM5B, a lysine-specific demethylase, to be associated with the methylated status of the host gene promoter of miR-137 and miR-155.

These methylated miRNAs host genes are promising diagnostic and/or prognostic biomarkers of prostate cancer. miR-193b has been implicated in prostate cancer with high sensitivity and specificity, whereas high miR-129-2 and miR-34b/c methylation levels are prognostic markers for disease-free survival [97]. Jacob Fredsøe et al. developed a novel urine-based three-miRNA prognostic model (miR-125b-5p*, let-7a-5p/miR-151a-5p) for prediction of biochemical resource after radical prostatectomy in prostate cancer [98]. miR-125b, let-7a and miR-151a inhibit apoptosis, reduce proliferation and promote cell migration and invasion of prostate cancer cells, respectively, suggesting that these miRNAs could play a functional role in prostate cancer progression [98].

3.2.3. Triple negative breast cancer

Triple negative breast cancer (TNBC) treatment is difficult and it accounts for 20% of all breast cancers in women. Researchers are developing markers to detect breast cancer at early stage, which can lead to better disease outcome and prolonged patient survival. miRNAs have been shown to play a regulatory role in cell cycle progression, apoptosis, epithelial-mesenchymal transition, angiogenesis and drug resistance in breast cancer [99]. miR-125b, miR-145, miR-21 and miR-155 were deregulated in a genome-wide miRNA expression profile study. This deregulation correlated with the expression of estrogen and progesterone receptor, stage of tumor and vascular invasion which demonstrate the existence of breast cancer-specific miRNA signatures [100]. In a recent study, researchers identified five miRNA signature (miR-92a-3p, miR-342-3p, miR-16, miR-21 and miR-199a-5p) to investigate the role of plasma miRNAs in TNBC, using a microarray platform. These five miRNA signatures are associated with increased risk of breast cancer [101]. Our group has shown miR-22 to regulate metastasis in breast cancer by downregulating TIP60, an acetyl lysine transferase and miR-22 and TIP60 levels could be used as a prognostic marker for breast cancer [102].

3.2.4. Ovarian cancer

miRNA deregulation is prominent feature in ovarian cancer, thereby playing an important role in regulation of ovarian physiology. miR-125b, miR-29b, miR-29a and let-7 are downregulated in epithelial ovarian cancers (EOC) and are highly expressed in normal ovarian tissues. Researchers in this study have linked this deregulation of miRNA during normal ovarian functioning to EOC pathogenesis. High grade serous EOC with BRCA1/2 mutations or loss have high miR-29a and miR-29b expression [103]. In tumor tissues, 39 miRNAs are significantly deregulated in comparison to normal ovary, of which miR-200a, miR-141, miR-200c and miR200b were most significantly overexpressed whereas miR-199a, miR-140, miR-145 and miR-125b1 were most downregulated [104]. Eight miRNAs (miR-25, miR-506, miR-29c, miR-182, miR-128, miR-101, miR-141 and miR-200a) that were downregulated were predicted to regulate majority of miRNA-associated genes, which suggests the importance of miRNA networks as predictors of EOC survival [105].

3.2.5. Gastric cancer

Gastric cancer (GC) is one of the deadliest cancers in the world. Presence of sensitive and specific biomarkers for early detection and monitoring the progression of GC could lead to the reduction of mortality. Chun et al. used antisense miR-221 and miR-222 in SGC7901 cells and

showed that these miRNAs regulate radio sensitivity, cell growth and invasion by directly modulating PTEN expression in these cells [106]. Their study suggested inhibiting miR-221 and miR-222 might be a novel therapeutic strategy for human GC. Another group showed miR-18a, which is a component of miR-17-92 cluster, to be overexpressed in GC tissue [107]. They found that the cell line overexpressing miR-18a showed increase in cell number and concentration of miR-18a in cultured medium, suggesting that miRNA might be released from cancer cells into the surrounding environment. They then concluded circulating miR-18a to be a useful biomarker for screening and monitoring tumor dynamics in GC [107].

Wang et al. did meta-analysis in 107 studies published in 42 articles and identified circulating miRNAs, miR-203, miR-146b-5p, miR-192 and miR-200c. They used bivariate model to calculate the sensitivity and specificity and used to plot the area under the summary receiver operator characteristic curve (AUC). The AUC is interpreted as the probability to correctly distinguish patients from normal controls. Using these parameters, they showed miR-203, miR-146b-5p, miR-192 and miR-200c has sensitivity of 0.75, a specificity of 0.81 and an AUC of 0.85, showing good diagnostic performance in gastrointestinal cancers [108]. They concluded based on this study that circulating miRNAs specially a cluster of miRNA may present as promising biomarkers for the diagnosis of GC.

3.2.6. Pancreatic cancer

In digestive system, pancreatic cancer is the most aggressive cancer and worldwide it is a serious health problem. There is lack of prognostic and diagnostic marker due to which the overall survival of pancreatic cancer is poor. To search for effective biomarker in pancreatic cancer, miRNAs have been investigated in pancreatic tumor tissue, blood samples, pancreatic juice, stool and urine [109]. The miRNAs, miR-143, miR-223 and miR-30e showed increased levels in urine of stage 1 pancreatic ductal adenocarcinoma in comparison to healthy individuals. The combinational use of miR-143 and miR30e showed a sensitivity of 83.3% and a specificity of 96.2% in PDAC [110]. Another group showed miR-21 and miR-155 upregulation in PDAC and this deregulation is detected early in intraductal papillary mucinous neoplasm (IPMN), which suggests that these miRNAs can be considered as markers of transformation [111].

Li et al. identified another miRNA, miR-1290 in the serum of PDAC patients by q-PCR using TaqMan microRNA arrays [112]. In tumor tissue, it is overexpressed and shows prognostic significance. It also showed a higher diagnostic accuracy than CA19-9 in their cohort (AUC 0.86 vs. 0.77 in the group PDAC vs. healthy controls). Another group showed five miRNAs including miR-10b, miR-155, miR-106b, miR-30c and miR-212 in plasma and bile, had excellent accuracy, sensitivity and specificity for detection of PDAC over the control [113].

3.2.7. Hepatocellular cancer

In HCC, miR-15b,miR-21, miR-130b and miR-183 showed upregulation in HCC tissue as compared to adjacent non-tumor tissue [114]. These miRNAs were detected in serum and cell culture medium but showed decreased levels after surgical resection. They also showed miR-15b and miR-130 could be used to distinguish HCC from healthy samples with high sensitivity and specificity. Kourtidis et al. showed miR-30b overexpression restores the abnormal cell growth in liver cancer cell lines. The abnormal cell growth reversed, on restoring miR-30b levels [115]. The miRNAs have been shown to have a great potential as a biomarker for HCC but

till date there is no consensus on detection or good miRNA sets, for example, there is a better response to interferon therapy on miR-26 downregulation but this is associated with poor survival [116]. A summary of miRNAs involved in different cancers is presented in **Table 1**.

3.3. Long-noncoding RNA

Long noncoding RNAs (lncRNAs) are the RNA transcript that do not encode for proteins and do not have open reading frame. lncRNAs are transcribed by RNA polymerase II and controlled by the transcriptional activators of the SWI/SNF complex. lncRNA transcripts are usually spliced, capped and polyadenylated, similar to mRNAs. lncRNAs represent a heterogeneous group of ncRNAs and they are usually expressed in tissue and cellular context, and are localized in the both nucleus and cytoplasm. The presence of secondary structures in lncRNA such as stem loops and hairpins, help them to interact with proteins and chromatin and are important for various functions of lncRNAs. In general, lncRNAs act as guides to recruit proteins, scaffolds for grouping protein complexes, transcriptional enhancers by chromatin reorganization, decoys to release proteins from chromatin or antagonists for other regulatory ncRNAs, for example, miRNAs.

3.4. lncRNAs as biomarker in cancer

3.4.1. Breast cancer

Expression levels of lncRNAs have been investigated in breast cancer tissues compared to normal tissues indicating that some may be potential biomarkers for breast cancer diagnosis. lncRNA-BC2 and lncRNA-BC5 were upregulated and lncRNA-BC4 and lncRNA-BC8 were downregulated in breast cancer patient samples in a study done by Ding et al. [117]. In grade 3 breast cancer, lncRNA-BC4 expression was significantly lower and lncRNA-BC5 expression was significantly higher. lncRNAs have been demonstrated to be easily detected in bodily fluids by multiple studies, such as lncRNA RP11-445H22.4 was found to be significantly increased in breast cancer patients serum with high sensitivity and specificity [118]. Zhao et al. identified a set of lncRNAs are deregulated in breast cancer patients and distinguish low-risk patients from high risk patients. Breast cancer patients with high expression of LINC00324 and low expression of PTPRG antisense RNA 1 (PTPRG-AS1) and small nucleolar RNA host gene 17 (SNHG17) co-related with longer overall survival and tumor grade [119]. High SPRY4 intronic transcript 1 (SPRY4-IT1) expression levels was increased in 48 breast cancer tissues in comparison to normal tissue and this upregulation was found to be associated with increased tumor size, poorer prognosis and disease-free survival (DFS) [120].

Hox transcript RNA (HOTAIR) is overexpressed in breast cancer tissue and increases the invasion and metastatic capacity. This increased expression is predictive of overall survival and progression-free survival [121, 122]. Metastasis-associated lung adenocarcinoma transcript 1's (MALAT1) is upregulated in 26 pairs of estrogen receptor positive breast cancer patients. Further analysis of a larger group of breast cancer patients comprised of 204 samples and correlated high expression of MALAT1 with ER+ breast cancer patients [123]. In an analysis of 151 breast cancer tissues, which comprised of stages 1–111 invasive ductal carcinoma, lncRNA BC040587 was found to be downregulated and this downregulation was correlated with differentiation of tumor and status of menopause [124]. LINC00472 is highly expressed

in non-metastatic breast cancer tissues and the high expression of LINC00472 was associated with Luminal A type of breast cancer. High expression of LINC00472 showed reduced risk of relapse and death in patients [125, 126]. Lei zhong et al. analyzed 600 breast cancer patients with ER^+ status from the TCGA data and identified six lncRNAs (HAGLR, STK4-AS1, DLEU7-AS1, LINC00957, LINC01614 and ITPR1-AS1) gene signature as prognostic survival biomarkers [127].

3.4.2. Ovarian cancer

Guo et al. performed genome-wide miRNA and lncRNA expression profiles and categorized ovarian cancer patients (BRCA1/2 wild-type) into high and low survival on the basis of LINC01234 and CCDC144NL-AS1 and two miRNAs (miR-637 and miR-129-5p) signatures [128]. Wang et al. identified seven lncRNAs such as XR_948297, XR_947831, XR_938728, XR_938392, NR_103801, NR_073113 and NR_036503, which were deregulated in most ovarian tumor samples and showed significant correlation with a poor chemotherapeutic response of EOC patients [129]. Rong Liu et al. identified signature lncRNAs such as ZFAS1, RP5-1061H20.5, RP11-489O18.1, RP11-136I14.5, RP11-16E12.1, CTD-2555A7.3, LINC01514 and TUG1 to play a mechanistic role in chemotherapeutic resistance in HGS-OvCa tumors and act as diagnostic markers [130].

3.4.3. Leukemia

In AML patients, lncRNAs MEG3 are poorly expressed and overexpression of MEG3 inhibits AML cells proliferation, regulates cell cycle and promotes apoptosis [131]. Diaz-Beya et al. have reported that HOTAIRM1 is highly expressed in 215 intermediate-risk AML patients and this expression is associated with poor prognosis, overall survival and disease recurrence [132]. In AML patients with Nucleophosmin 1 (NPM1) mutation, a higher expression of HOTAIRM1 is associated with poor clinical outcome. Another study by De Calra et al.

Cancer	miRNAs	Expression	Clinical features	Refs.
Breast	miR-21, miR-155	Upregulated	Plasma of patient with TNBC (n = 5) and non-TNBC (n = 5), as well as healthy controls	[100]
	miR-10b, miR-125b, miR-145, miR-21	Downregulated		
	miR-145, miR-451	Downregulated	Novel biomarkers for early detection	[183]
	let7a, miR-21,miR-141, miR-214	Upregulated		
	miR-92a-3p, miR-342-3p	Upregulated	Expression is correlated with tumor stage and subtypes	[101]
	miR-16, miR-21 and miR-199a-5p	Downregulated		
	miR-210	Upregulated	Hypoxic environment in breast cancer	[184]
	miR-451	Upregulated	Multidrug resistance in breast cancer	[185]
	miR-22	Upregulated	Epithelial-mesenchymal transition, metastasis	[102]
	miR-18a	Upregulated	Paclitaxel resistance	[186]

Cancer	miRNAs	Expression	Clinical features	Refs.
Gastric	miR-433, miR-9	Downregulated	Marker for the advanced gastric carcinoma	[187]
	miR-221, miR-222	Upregulated	Regulate radio sensitivity, and cell growth and invasion by directly modulating PTEN expression	[106]
	miR-203, miR-146b-5p, miR-192, miR-200c	Upregulated	Diagnostic marker	[108]
	miR-214, miR-17, miR-20a, miR-200c, miR-107, miR-27a, miR-433, let-7 g, miR-125a-5p, miR-760, miR-206, miR-26a	Upregulated	Poor prognosis in gastrointestinal cancer patients	[188]
	miR-200b, miR-185	Downregulated		
	miR-125a, miR-137, miR-141, miR-146a,	Downregulated	Prognostic marker	[189]
	miR-206, miR-218, miR-486-5p, miR-506			
	miR-451, miR-199a-3p, miR-195	Upregulated	Poor prognosis for recurrence and survival	[190]
	let-7 g, miR-342, miR-16, miR-1, miR-34	Upregulated	Associated with chemosensitivity	[191]
	miR-18a	Upregulated	Increase in cell number and released in cell culture medium	[107]
Hepatocellular carcinoma	miR-15b, miR-21, miR-130b, miR-183	Upregulated	High sensitivity and specificity in HCC patients	[192]
	miR-16, miR-195, miR-199a	Upregulated	HBV-associated HCC samples from healthy controls	[193]
	miR-29a, miR-29c, miR-133a, miR-143, miR-145, miR-192, miR-505	Upregulated	HBV-associated HCC from chronic HBV infection	[192]
	miR-200c, miR-200, miR-21, miR-224, miR-224, miR-10b, miR-222	Upregulated	HBV-related HCC compared to patients with chronic HBV	[194]
	miR-517a, miR-520c	Upregulated	Downregulation of both miR-517a and miR-517c contribute to HCC development through Pyk2 regulation	[195]
	miR-18a, miR-224, miR-199a*, miR-195, miR-199a, miR-200a, miR-125a	Upregulated	Promotes tumor progression	[196]

Cancer	miRNAs	Expression	Clinical features	Refs.
Ovarian	miR-125b, miR-29b, miR-29a, let-7	Downregulated	Epithelial ovarian cancer pathogenesis	[103]
	miR-519a	Upregulated	Poor prognosis of patients with ovarian cancers	[197]
	miR-25, miR- 506, miR-29c, miR-182, miR-128, miR-101, miR-141, miR-200a	Downregulated	miRNA networks as predictors of epithelial ovarian cancer survival	[105]
	let-7e, miR-30c, miR-130a, miR-335	Upregulated	Prognostic tool to monitor the chemotherapy outcome	[198]
	miR- 125b	Downregulated		
	miR-214, miR-199a*, miR-200a	Upregulated	Cell survival and cisplatin resistance	[199]
	miR-100	Downregulated		
	mir-135b, miR-200a, miR-200b, miR-200c, miR-141, miR-429	Upregulated	Prognostic and diagnostic marker	[200]
	miR-205, miR-449, miR-429	Upregulated	Diagnostic marker in endometrioid ovarian cancer	[201]
	miR-204, miR-99b, miR-193b	Downregulated		
Lung	miR-155 and miR-let-7a-2, miR-145, miR-21	Upregulated	Associated with adenocarcinoma patients survival	[88]
	miR-221, miR-222	Upregulated	Induce TRAIL resistance and enhance cellular migration	[90]
	miR-1253, miR-504, miR-26a-5p	Upregulated	Therapeutic target to overcome resistance to ALK inhibitors	[91]
	miR-1343-3p miR-671-3p, miR-103a-3p	Upregulated	Deregulated in cancerous vs. normal lung tissue	[202]
	let-7e, miR-342-3p	Downregulated		
	miR-210, miR-182, miR-486-5p, miR-30a, miR-140-3p	Upregulated	Poor survival in squamous carcinoma	[203]
	miR-31	Downregulated		
	let7g, miR-26	Downregulated	Deregulated in squamous carcinoma vs. adenocarcinoma	[204]
	miR-21	Upregulated	Marker of tumor progression in adenocarcinoma	[205]

Cancer	miRNAs	Expression	Clinical features	Refs.
Pancreatic	miR-143, miR-223, miR-30e	Upregulated	Overexpressed in patients with stage I cancer when compared with age-matched healthy individuals	[110]
	miR-21, miR-155	Upregulated	Expression was significantly correlated with tumor stage and poor prognosis	[111]
	miR-20a, miR-21, miR-24, miR-25, miR-99a, miR-185, miR-191	Upregulated	Prognostic biomarker with high sensitivity and specificity	[206]
	miR-10b, miR-155, miR-106b, miR-30c, miR-212	Upregulated	Excellent accuracy, sensitivity and specificity for detection of PDAC over the control patients	[113]
Prostate	miR-222, miR-125b	Upregulated	Prognostic marker in prostate cancer patients	[92]
			Metastasis	
	miR-21, miR-221	Upregulated	Analysis of miR-21, -141, and -221 in blood of PCa patients reveals different pattern of molecules in clinical subgroups of PCa	[94, 95]
	miR-141	Downregulated		
	miR-155, miR-31, miR-152, miR-137	Upregulated	Hypermethylated	[96]
	miR-1290, miR-375	Upregulated	Decreased overall survival in Castration-resistant prostate cancer (CRPC) patients	[207]
	miR-375, miR-141	Upregulated	Released into incubation medium from androgen-stimulated cells	[208]
	miR-16, miR-92a,miR-103, miR-107,miR-197, miR-34b, miR-328, miR-485-3p, miR-486-5p, miR-92b, miR-574-3p, miR-636, miR-640, miR-766, miR-885-5p	Upregulated	Upregulated in serum from prostate cancer patients compared to normal donor sera	[209]
	miR-125b-5p*let-7a-5p/ miR-151a-5p	Upregulated	Urine-based three-miRNA prognostic model for prediction of BCR	[98]

Table 1. Summary of various miRNAs involved in different cancers, their expression and clinical features.

have recently identified XLOC_109948 long noncoding RNA as a strong prognostic factor in NPM1-mutated patients [133]. An association study performed in a case-control cohort made up of 149 leukemia patients, including Philadelphia positive (Ph(+)) acute lymphoblastic leukemia (ALL) and AML samples, and 183 healthy controls. They found a single nucleotide polymorphism mapping to CDKN2BAS encoding for ANRIL antisense noncoding RNA showed significant correlation with the ALL phenotype [134]. In neoplastic T lymphocytes samples from 21 children with ALL, T-ALL-R-lncR1 was expressed in 11 cases. T-ALL-R-lncR1 might be associated with T-ALL, provide a new entry point for early diagnosis and targeted therapy for T-ALL [135]. In 68 chronic lymphocytic leukemia (CLL) patients, 62 multiple myeloma (MM) patients and 36 healthy controls, 5 lncRNAs (Taurine upregulated gene1 (TUG1), lncRNA-p21, metastasis-associated lung adenocarcinoma transcript-1(MALAT1), HOTAIR and growth arrest-specific 5 (GAS5)) were identified, of which lncRNA-p21 showed low expression in CLL patients [136]. lncRNA-p21 forms a complex with hnRNPK ribonucleo

protein and suppresses cell cycle regulatory genes on stimulation by p53 [137]. lncRNA-p21 could be developed as a biomarker for the disease or drug design. Miller et al. showed translation regulatory long noncoding RNA 1 (TRERNA1) is overexpressed in 144 CLL patients' samples, act as enhancer and regulate expression of-SNAIL in cis-dependent manner [138]. They have also correlated the high expression of TRERNA1 with shorten treatment time. TRERNA1 expression resulted in decreased DNA damage and cell death in B-CLL cell line, suggesting TRERNA1 as a novel biomarker.

3.4.4. Lung cancer

lncRNAs have been successfully isolated from bronchial brushings, biopsies and sputum. Still only, few species of lncRNAs have been identified in biofluids; therefore, more sampling methods are required. Recently, there have been progresses in discovery of lncRNA biomarkers in lung cancer. MALAT-1 emerged as most promising candidate NSCLC in tissue specimens [139]. MALAT-1 is overexpressed and is a prognostic marker for metastasis and poor prognosis in cancer that arises from squamous cell carcinoma. In small cell lung cancer (SCLC) patients, CCAT2 was associated with shorter overall survival. High expression of CCAT2 was an independent unfavorable prognostic factor for SCLC patients as analyzed by univariate and multivariate analyses [140]. Qiu et al. showed CCAT2 is upregulated in NSCLC tissues in comparison with paired adjacent normal lung tissues and this overexpression is significant in lung adenocarcinoma but not in squamous cell carcinoma [141].

Zhang eb et al. have shown that the lncRNA TUG1 was downregulated in lung cancer tissues and this correlates with advanced pathological stage, greater tumor size and shorter survival time in both lung squamous cell carcinoma and lung adenocarcinoma [142]. Sun et al. have demonstrated that the downregulation of SPRY4-IT1 correlated with larger tumor size, advanced pathological stage and lymph node metastasis in NSCLC patients and this reduced expression of SPRY4-IT1 with lymph node metastasis status may serve as a biomarker of late stage and poor survival [143].

3.4.5. Prostate cancer

Recently, a lot of progress has been made in identifying the lncRNAs as biomarker in prostate cancer and the most recent one is the PCA-3 assay, which has been approved by the FDA. In prostate cancer, PCA3 was suggested as a urinary biomarker and detected using q-PCR in a cohort of 108 men [144]. One of the lncRNA extensively studied in prostate cancer pathogenesis is prostate cancer-associated intergenic noncoding RNA transcript 1 (PCAT1). In 102 prostate cancer tissues, PCAT1 was upregulated and correlated with disease progression [145]. Srikantan et al. found prostate cancer gene expression marker 1 (PCGEM1) to be upregulated in African-American men compared to Caucasian-American men and is associated to patients with high prostate cancer risk [146].

Schlap1 showed higher expression in metastatic prostate cancer and the expression of Schlap1 correlated with metastasis and prostate cancer-specific mortality [147–149]. Another study did RNA sequencing and found prostate cancer-associated noncoding RNA transcript 18 (PCAT-18) to be upregulated in prostate cancer in comparison to neoplasm. In prostate cancer cell lines, PCAT-18 inhibits cell invasion, migration and proliferation, which suggest it to be a therapeutic target and biomarker for prostate cancer [150]. Isin et al. analyzed exosomes from

the urinary samples from 30 prostate cancer patients and 49 benign prostatic hyperplasia (BPH) patients and found lncRNA-p21 to be deregulated, whereas another lncRNA GAS5 expression was not changed [151]. This result suggested lncRNA-p21 as a biomarker for the prostate cancer detection. Ren et al. has demonstrated lncRNA FR0348383 to be differentially expressed and its expression levels could differentiate prostate cancer from BPH. Another group has reported same lncRNA as a novel biomarker in prostate cancer detection using post-digital rectal examination (post-DRE) urine of the patients [152].

3.4.6. Liver cancer

The lncRNA urothelial carcinoma-associated 1 (UCA1) is shown as a single lncRNA-based HCC diagnostic approach. In both studies, UCA1 performed better than Alpha-fetoprotein (AFP). In combination with JUN mRNA, UCA1 lncRNA showed 90% sensitivity and 80% specificity and in early stage HCC detection, the same combination showed 100% sensitivity and 80% specificity underlining the importance of RNA-based detection methods for early stage HCC diagnosis [153]. In 86 (35 female, 51 male) HCC patients, Liu et al. showed NEAT1 to be overexpressed in HCC patients and this was an independent risk factor associated with the prognosis of patients [154]. Wang et al. analyzed TCGA data and shown the expression profiles of four lncRNAs (RP11-322E11.5, RP11-150O12.3, AC093609.1 and CTC-297N7.9) for 371 patients with HCC were significantly and independently associated with survival of HCC patients [155]. Several studies suggested circulating ncRNAs and tumor tissue-derived ncRNAs for HCC diagnosis or survival prediction [156, 157]. While tissue-derived ncRNAs might be functionally relevant in the tumor, they are not necessarily good biomarkers for diagnosis. To obtain a tissue sample, a liver biopsy is needed, which is an invasive procedure with potential side effects. Therefore, the detection of circulating ncRNAs in body fluids instead of tumor tissue is advantageous for HCC diagnosis and surveillance. A summary of lncRNAs involved in different cancers is presented in **Table 2**.

3.5. Circular RNA

Circular RNA (circRNA) is a class of RNA that are abundant, evolutionary conserved and stable. They were identified 30 years ago as a result of an error in RNA splicing, but their function in different cellular processes is now being appreciated [158]. In acute lymphoblastic leukemia (ALL) patients and cell lines, Salzman et al. discovered circRNAs by RNA sequencing [159]. After this discovery, many other circRNAs were identified, shown to be endogenously expressed as well as stable. Backsplicing of exons, introns or both results in the formation of exonic or intronic circRNAs [160]. RNA binding proteins act as activators or inhibitors in circRNA formation [39]. Conn et al. have shown that the RNA binding protein QK1 binds to the introns flanking a circRNA and forms a looped structure by dimerization promoting circularization [39]. circRNAs have been classified as noncoding RNA, but recently there have been reports suggesting that they may be translated to protein if there is a presence of internal ribosome entry site (IRES) [161–163]. Recently, circRNAs have been studied extensively in relation to human diseases, especially cancers. Here we will discuss the potential of circRNAs as potential biomarkers and as therapeutic targets. A summary of circRNAs involved in different cancers is presented in **Figure 2**.

3.5.1. Gastric cancer

Lai et al. examined co-expression networks between circRNAs and mRNAs and found three candidate (circRNA0047905, circRNA0138960 and circRNA7690-15) oncogenes in gastric tissue. circRNA0047905 was predicted as biomarker in gastric tissue as it showed highest diagnostic accuracy [164]. Huang et al. analyzed plasma samples from patients with GC and healthy controls and showed hsa_circ_0000745 to be downregulated in GC tissue. Its expression correlated with tumor formation in gastric tissue, whereas in plasma, it correlated with tumor node metastasis (TNM) stage. Expression level of hsa_circ_0000745 in plasma in combination with carcinoembryonic antigen (CEA) level is a promising diagnostic marker for GC [165]. In plasma and GC tissues, hsa_circ_002059 was shown to be upregulated in comparison to adjacent normal tissues and this deregulation was associated with metastasis, TNM, gender and age. This suggests hsa_circ_002059 as a potential stable biomarker for the diagnosis of gastric carcinoma [166]. Another study showed upregulation of circPVT1 in GC tissue due to the amplification of its genomic locus. circPVT1 acts as a sponge for miR-125 family, promotes cell proliferation and also acts as a prognostic marker for disease-free survival and overall survival of GC patient [167].

Cancer	lncRNAs	Expression	Clinical features	Refs.
Breast	lncRNA-BC2 and lncRNA-BC5	Upregulated	Positively correlated with patients' age, clinical stage, progesterone receptor (PR) concentration	[117]
	lncRNA-BC4 and lincRNA-BC8	Downregulated	Negatively correlated with PR concentration	
	LINC00324	Upregulated	Expression pattern is associated with ER+ and ER- subtypes, tumor histology	[119]
	PTPRG-AS1, SNHG17	Downregulated		
	SPRY4-IT1	Upregulated	Prognostic biomarker and therapeutic candidate for breast cancer. Biomarker for overall survival and progression-free survival	[120]
	HOTAIR	Upregulated	Potential tumor marker for breast cancer diagnosis	[121, 127]
	MALAT1	Upregulated	Poor prognosis and correlated with tumor differentiation	[123]
	BC040587, neuroblastoma-associated transcript 1 (NBAT1) and eosinophil granule ontogeny transcript (EGOT)	Downregulated	New marker of prognosis in breast cancer	[124]
	LINC00472	Upregulated	Prognostic and predictive value in the clinical management of breast cancer	[125, 126]
	HAGLR, STK4-AS1, DLEU7-AS1, LINC00957, LINC01614 and ITPR1-AS1	Upregulated	Prognostic biomarker of survival of breast cancer patients	[127]

Cancer	lncRNAs	Expression	Clinical features	Refs.
Ovarian	LINC01234 and CCDC144NL-AS1	Upregulated	Overexpression is correlated with overall shorter survival	[128]
	XR_948297, XR_947831, XR_938728, XR_938392, NR_103801, NR_073113 and NR_036503	Upregulated	Correlated with a poor chemotherapeutic response of EOC patients	[129]
	ZFAS1, RP5-1061H20.5, RP11-489O18.1, RP11-136I14.5, RP11-16E12.1, CTD-2555A7.3, LINC01514 and TUG1	Upregulated	Play a mechanistic role in chemotherapeutic resistance in HGS-OvCa tumors	[130]
Leukemia	MEG3	Downregulated	Regulate cell cycle and promote apoptosis	[131]
	HOTAIRM1	Upregulated	Associated with poor prognosis, shorter overall survival and disease recurrence	[132]
	XLOC_109948	Downregulated	Strong prognostic factor in NPM1 mutated patients	[133]
	ANRIL	Upregulated	Significant correlation with the ALL phenotype	[134]
	T-ALL-R-lncR1	Upregulated	Early diagnosis and targeted therapy of T-ALL suppress cell cycle regulatory genes	[135]
	TRERNA1	Upregulated	Decreased DNA Damage and cell death in B-CLL cell line	[138]
Lung	MALAT-1	Upregulated	Predictive marker for metastasis development and poor prognosis in cancer arising from squamous cell carcinoma	[139]
	CCAT2	Upregulated	Promotes invasion of non-small cell lung cancer	[140, 141]
	TUG1	Downregulated	Correlated with advanced pathological stage, greater tumor size and shorter survival time in both lung squamous cell carcinoma and lung adenocarcinoma	[142]
	SPRY4-IT1	Downregulated	Correlated with larger tumor size, advanced pathological stage and lymph Node metastasis in NSCLC patients	[143]

Cancer	lncRNAs	Expression	Clinical features	Refs.
Prostate	PCA3	Upregulated	Urinary biomarker	[144]
	PCAT1	Upregulated	Implicated in prostate cancer progression	[145]
	PCGEM1	Upregulated	Associated to patients with high prostate cancer risk	[146]
	Schlap1	Upregulated	Prognostic biomarker	[147–149, 210]
	PCAT-18	Upregulated	Inhibits cell invasion, migration and proliferation	[150]
	lncRNA-p21	Downregulated	Biomarker for the prostate cancer detection	[151]
	FR0348383	Upregulated	Novel biomarker in prostate cancer detection using post-DRE urine of the patients	[152]
Liver	UCA1	Upregulated	90% sensitivity and 80% specificity in early stage HCC detection	[153]
	NEAT1	Upregulated	Associated with the prognosis of patients with HCC	[154]
	RP11-322E11.5, RP11-150O12.3, AC093609.1, CTC-297 N7.9	Downregulated	Associated with prognosis of liver cancer, and could provide novel insights into the potential mechanisms of HCC progression	[155]
	HULC and Linc00152	Upregulated	Applied as a potential target for HCC treatment	[157]

Table 2. Summary of various lncRNAs involved in different cancers, their expression and clinical features.

3.5.2. Hepatocellular carcinoma

hsa_circ_0001649 expression is significantly downregulated in 89 HCC samples in comparison to adjacent liver tissue and this correlates with tumor embolus and size, indicating its use as a potential biomarker for HCC [168]. Fu et al. showed lower expression of hsa_circ_0004018 is correlated with serum AFP level, tumor diameters, differentiation, Barcelona Clinic Liver Cancer stage and TNM in HCC [169]. Using a circRNA microarray, Huang et al. identified 226 differentially expressed circRNAs, of which 189 were significantly upregulated and 37 were downregulated. circRNA_100,338, one of the upregulated circRNAs in HCC, is correlated with a low cumulative survival rate and metastatic progression in HCC patients with Hepatitis B [170]. Shang et al. performed circRNA microarray and found circ_0000520, circ_0005075 and circ_0066444 are deregulated in HCC. They found upregulation of only circ_0005075 to be associated with tumor size [171].

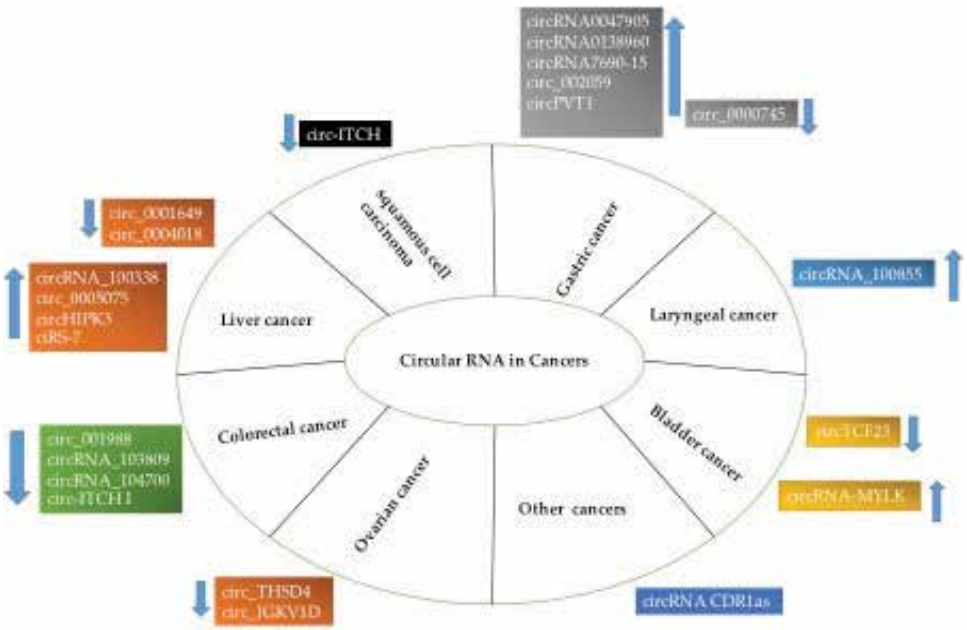

Figure 2. Circular RNAs in cancer. A list of the important circRNAs involved in different cancer types and their associated levels.

3.5.3. Colorectal cancer

In CRC, Wang et al. showed the expression of hsa_circ_001988 was decreased in tumor tissues and the expression was correlated with differentiation and perineural invasion, suggesting hsa_circ_001988 as a novel treatment target and a potential biomarker of CRC [172]. Zhang et al. performed circRNA array in paired tumor and adjacent non-tumorous tissues from six CRC patients and found lower expression of hsa_circRNA_103809 and hsa_circRNA_104700 in CRC tissues. The expression of hsa_circRNA_103809 was correlated with lymph node metastasis and tumor node metastasis stage, whereas expression level of hsa_circRNA_104700 was significantly correlated with distal metastasis. hsa_circRNA_103809 and hsa_circRNA_104700 are involved in the development of colorectal cancer and serve as potential biomarkers for the diagnosis of colorectal cancer. Another circRNA, cir-ITCH was downregulated in CRC and inhibits Wnt/β-catenin pathway by increasing ITCH expression suggesting a mechanistic role for circ-ITCH in CRC by regulating the Wnt/β-catenin pathway [173]. Studies cited above illustrate that circRNAs are promising biomarkers for CRC.

3.5.4. Laryngeal cancer

Till date not much study on circRNA profiling in laryngeal cancer have been done. Microarray analysis in four paired laryngeal squamous cell cancer (LSCC) tissues revealed 698 circRNAs to be altered. hsa_circRNA_100855 was most upregulated in LSCC when compared to adjacent non-neoplastic tissues [174]. This expression of hsa_circRNA_100855 correlated with tumor

grade, tumor stage, neck nodal metastasis and primary location of LSCC. CircRNA_100855 plays an important role in the tumorigenesis of LSCC can be used as a prognostic and diagnostic biomarkers in LSCC.

3.5.5. Bladder cancer

In bladder carcinoma, circRNA expression was done using microarray assay by Zhong et al. and researchers demonstrated that circTCF25 is overexpressed in bladder cancer and this overexpression downregulate miR-103a-3p and miR-107, increase cyclin-dependent kinase 6 (CDK6) expression and promote proliferation and migration *in vitro* and *in vivo* [175]. The data also suggested that circTCF25 might be a new biomarker for bladder cancer. In another study by the same group, they found that circRNA-MYLK and VEGFA were significantly upregulated and co-expressed in bladder cancer [176]. The expression of circRNA-MYLK co-related with the progression of stage and grade of bladder cancer, suggesting that circRNA-MYLK would be a promising target for bladder diagnosis and therapy.

3.5.6. circRNAs in other cancers

In cutaneous squamous cell carcinoma (cSCC), 322 circRNAs were identified to be deregulated and having 1603 miRNA response elements (MREs) [177]. These deregulated circRNA were shown to be involved in tumor formation by acting as a sponge for miRNAs. Another study identified circRNA expression signatures in PDAC by microarray platform [178]. They have shown that initiation and progression of PDAC is controlled by circRNAs. Li et al. found that circ-ITCH expression is downregulated in esophageal squamous cell carcinoma (ESCC) compared to the peritumoral tissue. circ-ITCH act as a sponge for miR-7, miR-17 and miR-214, thereby increasing the level of ITCH and promoting ubiquitination and degradation of phosphorylated Dvl2, thereby inhibiting the Wnt/β-catenin pathway [179]. circRNA CDR1as have 70 selectively conserved target sites of miR-7, and lot of studies have shown that miR-7 can directly downregulate oncogenes [180]. This miRNA regulation by CDR1as has been shown to be involved in cancers such as breast cancer, melanoma, GC, gliocytoma, liver cancer and NSCLC.

3.6. Noncoding therapeutics in clinical trials

To date, the use of miRNA-based therapeutics in malignant disease is poorly explored. Presently, many companies are developing miRNA as therapeutic targets either by overexpressing tumor suppressor miRNA or by inhibiting oncogenic miRNAs. miR-122 has been shown to play an important role in HCC. Currently Santaris pharma has used locked nucleic acid-based antisense oligonucleotide against miR-122, thereby reducing the miR-122 levels and playing a positive role in the regulation of Hepatitis C viral replication [181]. This antisense has already passed Phase II clinical trials and shows promising results in patients infected with HCV infection [182]. Mirna therapeutic has also developed a miR-34a mimic, for miR-34a overexpression and currently is under trials for primary liver cancer. They have also developed anti-miR-155, which has shown a promising result in restoring normal function

and reducing cell proliferation in hematological malignancies. Similarly, miR-34 liposomes are also under stage I clinical trial. Regulus therapeutics has introduced several anti-miR in preclinical trials, for example, in renal fibrosis the expression of miR-21 is high and anti-miR-21 reduces the expression of extracellular matrix proteins. In atheroclosis, anti-miR-33 has been used successfully to regulate cholesterol and fatty acid homeostasis by decreasing LDL triglycerides and increasing HDL. This anti-miR-33 has cleared the preclinical trials. In HCC, anti-miR-221 has been successful in delaying the tumor progression, thereby increasing the survival rate. miRagen therapeutics have also developed anti-miR-92 (for peripheral artery disease) and anti-miR-15 (for myocardial infarction), which are in preclinical trials.

Besides the above examples of successful trials, there are still major obstacles that need to overcome for miRNA-based therapeutics. miRNAs have multiple targets, hence the off target effects need to be examined carefully. Similarly one gene can be regulated by multiple miRNAs that can compromise the effect of miRNA-based treatment. In addition, delivery mechanism for miRNA that show high specificity and efficacy is lacking. Overall, miRNA-based therapeutics hold a promising future for personalized medicine based on miRNA biomarkers but for this, a first step towards better understanding of miRNA biology is required.

4. Conclusion

Recent advancement in techniques and the vast repertoire of information has made it feasible to identify and characterize different biomarkers. However, due to the constant evolving epigenetic landscape of cancer cells along with the heterogeneity in the cell population subtype, their translation into clinical stage offers various limitations. Additionally, population size used in the study as well as tumor samples used to identify the genes and pathways involved needs to be further validated. Samples handling methods need to be specified as variation might occur depending on the site of isolation as well as the method used to identify the specific biomarker type. Therefore, extensive research on various biomarker profiles is important to understand their potential as therapeutic and diagnostic tools in different cancer types. Integration of biomarker discovery with other techniques such as imaging (labeling) of the specified tumor target site can provide information about the disease end point and offer a noninvasive way to monitor dose requirement.

The use of advance sequencing technologies and bioinformatics approaches in studying the transcriptome of cancer has led to the identification of ncRNAs such as miRNAs, lncRNAs and circRNAs. These ncRNAs are deregulated in most of the cancers in comparison to the normal tissue, which suggests that they might play an important role in biological function of these cancers. miRNAs are the most extensively studied ncRNAs, but their potential as effective biomarkers is still in its nascent stage. A number of studies have been done to demonstrate the potential of miRNAs as biomarkers and as diagnostic tools, but challenges still remain. For example, a large dataset has to be used to successfully predict specfic miRNAs as biomarker for prognostic and diagnostics use. To ensure the accuracy of the diagnosis based on miRNAs, one has to identify all the targets of miRNAs, thus removing the false positive targets. New delivery mechanisms also need to be developed for specificity and efficacy. Similarly, the identification of lncRNAs as important regulators of cancers has potentiated their use as

promising tool for biomarkers. lncRNAs are stable in body fluids and their expression is specific to different pathological conditions. However, their development as biomarkers is still in its preliminary stage, proper normalization control and a large cohort has to be used to make the study reliable. Additionally, identification of new mutations, deletions and amplifications in the ncRNAs as well as genomic alterations affect their structure and function. circRNAs are known to be deregulated in cancers and although several studies have documented their role, many questions regarding their biogenesis and function is still unclear.

To summarize, this chapter highlights the different types of biomarkers that have been characterized in different cancer types thus far, their mode of action and their targeting strategies. The therapeutic potential of different biomarkers and their use in clinical trials has also been discussed. Despite the recent advancements, a comprehensive approach on biomarker biogenesis is required to integrate the available information and to translate them as tools of prognostic and diagnostic potential.

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Chapter 8

Targeting the Ubiquitin Proteasome System in Cancer

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Additional information is available at the end of the chapter

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Abstract

The ubiquitin proteasome system is involved in a myriad of biological functions including cell cycle progression, intracellular signaling and protein degradation. As such, it is not surprising to find many components of the system misregulated in cancer. The clinical success of Bortezomib for treatment of multiple myeloma proves that targeting the ubiquitin proteasome system is valid and feasible. Here, a detailed examination of the strategies used to target the ubiquitin proteasome system in cancer is discussed. The inhibitors available, its targets, the cancer type and the developmental stage it is in are discussed.

Keywords: ubiquitin, proteasome, E1, E2, E3, ubiquitin proteasome system, cancer, deubiquitinase, DUBs inhibitors

1. Introduction

The function and activity of most proteins can be partially modulated by posttranslational modifications (PTMs). In particular, ubiquitination has emerged as one of the most versatile PTMs over the past few decades. Ubiquitination is a process that attaches ubiquitin, a short polypeptide of 76 amino acids, for its covalent link to proteins. It is a highly conserved process that mostly targets unwanted proteins for degradation either through proteasome-mediated or by directly sorting proteins to the lysosome and thus helps to maintain cellular homeostasis [1]. However, ubiquitination may also play a crucial role in other non-proteolytic regulatory functions such as protein activation, interaction, and translocation [2].

Ubiquitination is a multistep process and requires the sequential action of three enzymes, the E1 activating enzyme, E2 conjugating enzyme, and E3 ligase [3, 4] (**Figure 1**). The process of ubiquitin attachment commences when E1 recruits free ubiquitin in the cell through its active cysteine residue. The C-terminal glycine residue of ubiquitin is activated through ATP-dependent

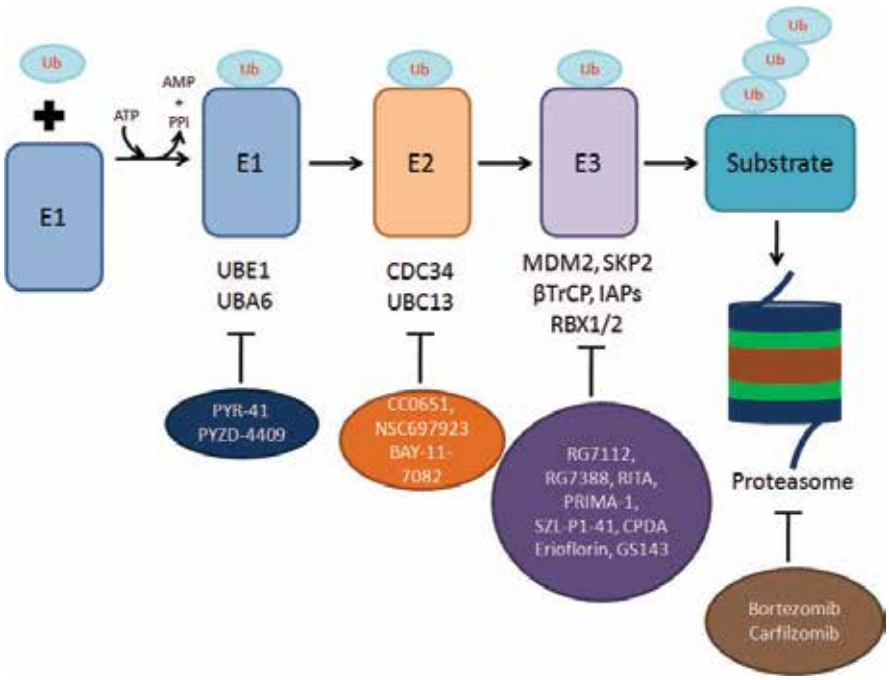

Figure 1. Schematic representation of ubiquitin conjugation cascade and inhibitors targeting specific cascade component.

adenylation and thioester bond formation catalysed by E1, resulting in attachment by non-covalent linkage to the E1 cysteine residue [5–7]. Activated ubiquitin is then transferred from E1 to a cysteine residue of the E2 conjugating enzyme linked through a thioester bond [4, 8].

The E3 ligases are responsible for substrate recognition and facilitates transfer of ubiquitin to the substrates from E2 resulting in covalent attachment of ubiquitin to the substrate's lysine residue [4, 9, 10]. The two major classes of E3s are the RING and HECT domain E3s which transfer ubiquitin through different mechanisms [9, 10]. HECT domain ligases accept ubiquitin from E2 through its catalytic cysteine residue and act as an intermediate entity capable of transferring ubiquitin to its recruited substrate [10]. RING domain ligases, instead of directly transferring ubiquitin, function as scaffolds and allow ubiquitin transfer from the E2 directly to the substrate [9]. In addition, other E3 classes such as ring-between-ring E3s are not discussed here [11].

Moreover, the ubiquitin molecule itself contains seven intrinsic lysine residues (K6, K11, K27, K29, K33, K48, and K63) and Met1 that can be further ubiquitinated allowing for the formation of various types of ubiquitin chains [12]. These come in the form of linear, branched, forked, homotypic, heterotypic kinds of monoubiquitin, multi-monoubiquitin, and polyubiquitin chain types. Each type could be associated with distinct cellular functions. For example, one of the best-known polyubiquitinations is K48-linked ubiquitination which acts as a degradation signal targeting substrate for proteasomal degradation [13].

The degradation of polyubiquitinated proteins is subsequently carried out in the 26S macromolecular proteasome complex which is present in both the cytosol and nucleus of eukaryotic

cells [14]. These complexes keep the proteins under quality checks and help cells to degrade misfolded/unwanted proteins. The proteasome is an approximately 2.5 MDa proteinase complex containing the catalytic active 20S core particle and the regulatory 19S particles [15, 16]. The 20S core particle is a barrel-shaped structure containing four stacked rings with two outer α-rings and two inner β-rings [17]. Each ring is composed of seven distinct α (α1–α7) or β (β1–β7) subunits [17]. The outer α-ring serves as the "gate" for entry of substrates, while the β-rings contain the catalytic activity. Namely, β1, β2, and β5 subunits confer the peptidyl-glutamyl-hydrolysing or caspase-like, the trypsin-like, and the chymotrypsin-like activity, respectively [17].

The 19S subunit can be separated into the "base" and "lid." The base contains ATPase subunits (RPT1–6) and four non-ATPase subunits (Rpn1, 2, 10, 13) [17]. The non-ATPase subunits are ubiquitin receptors that identify ubiquitinated substrates [17]. The lid contains nine subunits (Rpn3, 5–9, 11, 12, 15) and two proteasome-associated deubiquitinating enzymes (UCHL5/Uch37, Ubp6/Usp14) [17, 18]. Together with Rpn11/PSMD14, UCHL5/Uch37 and Ubp6/Usp14 carry out the deubiquitination of substrates before it moves on to the 20S core for degradation [17]. Although it is generally assumed that ubiquitinated proteins end up degraded by the proteasome, a recent review highlighted the strict requirements needed for proteasomal degradation wherein certain ubiquitinated substrates which do not meet these requirements escape from the proteasome and survive degradation [18].

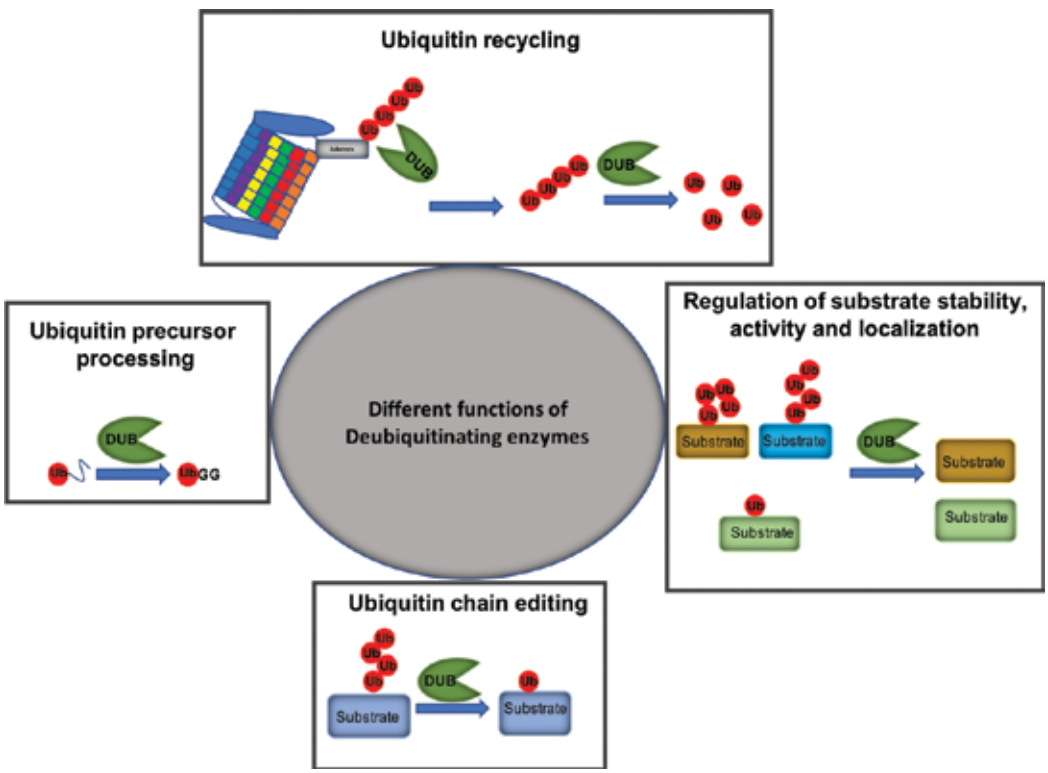

Figure 2. Pictorial representation for involvement of DUBs in different functions.

The ubiquitination process is antagonized by another set of enzymes that specifically removes ubiquitin moieties and counteracts ubiquitin-mediated function of a protein. These specific enzymes are called deubiquitinating enzymes (DUBs). As the name suggests, DUBs are responsible for cleaving the isopeptide bond between protein and ubiquitin. Other than regulating stability and function of its substrates, DUBs are also involved in ubiquitin precursor processing, ubiquitin recycling, and ubiquitin chain editing (**Figure 2**). By conducting the process of removing ubiquitin from its target, DUBs are mostly involved in opposing the effect of ubiquitination on substrates and thus leave a remarkable impact in the field of protein biology.

2. History of the ubiquitin proteasome system

The 2004 Nobel prize for Chemistry was awarded to Avram Hershko, Aaron Ciechanover, and Irwin Rose for the discovery of ubiquitin-mediated protein degradation [19]. Remarkably, the ubiquitin proteasome system (UPS) has been implicated in multiple cellular processes such as cell cycle, stress response, and DNA damage repair [20]. In 1978, Hershko and Ciechanover for the first time showed that ATP-dependent degradation required more than one component [21]. Using reticulocyte lysate and a DEAD cellulose column, they separated 2 fractions that individually do not catalyze ATP-dependent degradation but when combined, restored proteolysis [21]. Shortly after, the 2 fractions were identified. Fraction 1 contained ATP-dependent proteolysis factor 1 (APF-1) which was later identified to be ubiquitin [21–23]. Together with Irwin Rose, Aaron Ciechanover and Avram Hershko identified fraction 2 by further separating it into 2 other fractions containing a 450 kDa protein unknown at that time the proteasome, and the protease system containing E1, E2 and E3 enzymes [24]. It should be noted that prior to this, ubiquitin was first identified by Goldstein in 1975, as a universally present polypeptide, although its function was unknown at that time [25]. Prior to these findings, two reports in 1977 had characterized histone H2A covalently tagged with a single ubiquitin. Although not for degradation, the finding implied that ubiquitin could be used for tagging [26, 27]. Subsequently, a series of papers from the Nobel laureates characterized and defined the multi-step ubiquitin-tagging model for protein degradation through the E1, E2, and E3 enzymatic cascade [4–7, 28, 29]. Additionally, multiple ubiquitin could be tagged to a single molecule of lysozyme showcasing the first polyubiquitin chain [28].

Up till this point, the remaining piece of the puzzle was to identify the downstream protease(s) responsible for degradation of the tagged proteins. In order to characterize the protease(s) responsible, two large multi-subunit proteinase complexes were purified from reticulocytes [15, 16, 30]. One of which requires ATP to degrade the tagged protein (~1500 kDa), while the other is ATP independent (~700 kDa). It was later discovered that these were the 26S proteasome and the 20S core catalytic subunit of the proteasome, respectively [15, 31, 32]. Apart from the ATP-dependent E1 ubiquitin activation step, the process of degradation by the protease was also ATP dependent although the mechanism was unknown [33, 34]. This was resolved when it was found that the assembly of the 26S proteasome from the 20S catalytic core and 19S regulatory subcomplex is ATP dependent, explaining the reliance of energy for substrate degradation by the proteasome [31, 35].

The first papers to show a biological role for the ubiquitin cascade were in 1984 [36, 37]. In a mutant mouse cell line (ts85) that is conditionally lethal and temperature sensitive, monoubiqutinated H2A disappears at high temperatures, suggesting defects in the ubiquitin cascade [38, 39]. As it turns out, the E1 enzyme in ts85 was temperature sensitive, resulting in defects in ubiquitination at high temperatures [36, 37]. Additionally, the cells were arrested at G2 at higher temperature, indicating a role of the UPS in cell cycle regulation. These two papers set the stage for further discovery of biological roles played by the ubiquitin cascade in the coming years [20].

The first observation of deubiquitinating activity was in fact, in the very paper that the first scheme of ATP-dependent degradation was proposed [28]. Specifically, removing ATP from the ^{125}I-labeled ubiquitin-tagged lysozyme in the presence of endogenous proteins reversed the ubiquitin tagging, implying the presence of a DUB which the authors described as an amidase [28]. Subsequently, the first deubiquitination assay was developed and showed the deubiquitinating activity of mammalian ubiquitin C-terminal hydrolase L3 (UCHL3) and its yeast homolog Yuh1, which represented the first DUBs identified and characterized [40, 41]. The work of Varshavsky and colleagues ensued, identifying DUBs in yeast and up till today, there are a total of ~80 known DUBs [42, 43].

3. Ligases in cancer

Since ubiquitination occurs through a multi-step cascade, it can be inferred that multiple proteins along the cascade can be targeted. In particular, inhibitors targeting all three (E1, E2, and E3) classes of enzymes are utilized both in research and clinics. For an overview, the inhibitors that are about to be discussed in this section and the class of enzyme (E1, E2, and E3) which is targeted are summarized in **Figure 1**.

3.1. E1 enzymes

UBE1 and UBA6 are the only two E1 enzymes that are known in humans [44]. Till date, there are only two UBE1 inhibitors, PYR-41 and PYZD-4409 [45, 46]. Among the two, PYR-41 has been shown to inhibit the nuclear factor κB(NF-κB) pathway by regulating the stability of inhibitor of NF-κB (IκB). Additionally, it also prevents the degradation of the tumor suppressor p53 resulting in increased transcriptional activity of p53 [45]. On the other hand, PYZD-4409 was specifically shown to induce ER stress-induced apoptosis in cancer cells and, in a mouse model of leukemia, delayed tumor cell growth [46]. Although these results suggest the potential of targeting E1 in cancer treatment, none of these are currently in clinical trials, perhaps due to off-target effects or poor pharmacokinetic properties.

3.2. E2 enzymes

There are ~38 E2 enzymes in the human genome implying that they serve as more specific targets than E1 [8]. CC0651 is an allosteric inhibitor of CDC34, the common E2 enzyme for

Cullin ligase complexes. Treating cancer cells with CC0651 results in the accumulation of the tumor suppressor p27 and inhibition of proliferation, which suggests that CC0651 could be a potential inhibitor for clinical use [47]. However, development of this compound has met with great difficulties due to pharmacokinetic reasons [48]. Another potential target in cancer is the E2 enzyme UBC13-UEV1A, an important regulator of NF-κB pathway induction through the formation of ubiquitin K63-linked chains. The inhibitor NSC697923 has been shown to inhibit the formation of K63-linked chains by UBC13 *in vitro* and is effective in inhibiting the proliferation and survival of diffuse large B-cell lymphoma (DLBCL) [49]. BAY-11-7082 is a well-known inhibitor of the NF-κB pathway and has been thought to inhibit the IκB kinases [50]. However, it was found to inhibit the E2 UBC13 by preventing ubiquitin conjugation to it, thereby preventing K63-linked chain formation in the same way as NSC697923 [50]. Likewise, it was shown that BAY-11-7082 induces cell death to DLBCL HBL-1 cells [50]. Although E2 inhibitors show immense potential for cancer treatment, so far, E2 inhibitors are present only in preclinical stages.

3.3. E3 enzymes

Amongst enzymes in the ubiquitin conjugation cascade, E3s are the most abundant in number with ~700 ligases identified so far [48]. Due to the large number, targeting E3 will likely increase the specificity and decrease side effects. Due to space limitation, we will be discussing a few of the E3 ligases that are implicated in cancer and refer the readers to the following review about E3 ligases family [51].

3.3.1. Mouse double minute 2 homolog (MDM2)

Termed the guardian of the genome, p53 is frequently upregulated in stress conditions and functions to activate the expression of genes involved in apoptosis and cell cycle arrest to prevent cellular transformation [52]. MDM2 is an E3 ligase of p53 responsible for its degradation and is frequently upregulated in cancer [53, 54]. Thus, targeting MDM2 could be useful for cancer treatment. To this end, several MDM2 inhibitors are available. In particular, the Nutlin family of cis-imidazoline inhibitors shows the greatest potential [55]. One of the latest developed Nutlin inhibitors, RG7112, has been tested in phase I clinical trials and shows activity against relapsed leukemia [56]. Although it showed good clinical outcomes, a high dose was required and it caused gastrointestinal side effects [56, 57]. A more potent pyrrolidine-based MDM2 inhibitor, RG7388 is currently in clinical trial and might be able to overcome these issues [58]. In preclinical setting, RG7388 showed potent tumor inhibition specifically in p53 wild-type xenograft neuroblastoma indicating its possible use in neuroblastoma treatment where majority of tumors are p53 wild-type at diagnosis [59].

A majority of MDM2 inhibitors bind to MDM2 itself to prevent it from binding to p53. RITA (reactivation of p53 and induction of tumor cell apoptosis), however, binds to p53 and prevents MDM2 from interacting [60]. In this case, the mechanism of stabilization might be MDM2 independent and could possibly be used to treat MDM2-independent p53-destabilized cancers. Thus far, the mentioned MDM2 inhibitors aim to restore p53 levels. Given that p53 is known to be mutated in ~50% of all cancers, these therapies are severely limited to a subpopulation of p53 wild-type tumors [48, 61]. An ingenious way to overcome this is through the use

of drugs, which restore mutant p53 function. One example of such an approach is the drug PRIMA-1 which alkylates the thiol groups of mutant p53, correcting protein folding and enabling p53 to carry out its tumor suppressive function [62].

3.3.2. S-phase kinase associated protein 2 (SKP2)

SKP2 is a F-box protein which functions as the substrate recognition subunit of the SCF (SKP1/Cullin/F-box) RING E3 ligase complex [63]. In particular, its role in ubiquitinating and degrading cell cycle regulators, p27 and p21 makes it a potential target in cancer [64, 65]. Additionally, SKP2 is upregulated in several different cancers and serves as a prognostic marker for cancer patient survival [66–68]. Particularly, a structural pocket formed by SKP2 and its neighboring subunit CKS1 within the SCF complex is important for binding and degradation of p27. This outlines a potential vulnerability which could be targeted in cancer therapy. As such, using *in silico* screening to identify inhibitors for this structural pocket, four compounds were shown to increase p27 levels and arrest cells at G1 [69]. Another mechanism that could be utilized to inhibit SKP2 could be by targeting its association with the SCF complex through inhibiting SKP1-SKP2 binding. SZL-P1-41was identified to block SKP1-SKP2 interaction and shows strong antitumor effects against lung and prostate tumor xenograft in mouse models with concomitant increase of p27 [70]. Lastly, CPDA is another compound identified due to its ability to inhibit *in vitro* ubiquitination of p27 by SCF complex [71]. Although its mechanism is unknown, it has been shown to induce cell cycle arrest specifically in leukemic cells but not marrow components [71].

3.3.3. Beta-transducin repeat containing E3 ubiquitin protein ligase (βTrCP)

Like SKP2, βTrCP is a component of the SCF-Cullin E3 ligase complex. It utilizes its N-terminal F-box domain to bind to SKP1 and its C-terminal WD40 domain to bind to substrates including pro-caspase-3, IκB, p53, CDC25, and WEE1. In most cancers, it is upregulated and acts as an oncogene [72]. Erioflorin and GS143 are two βTrCP inhibitors that block the interaction of βTrCP with its targets, PDCD4 and IκB, respectively, leading to their stabilizations [73].

3.3.4. RING box protein 1/RING box protein 2 (RBX1/RBX2)

Both RBX1 and RBX2 are important subunits of the SCF complex and function to physically bring the activated E2 closer to the substrate for ubiquitination [74]. Increased expression of RBX1 is seen in breast, liver, kidney, and lung cancer indicating an oncogenic function [75]. An exception is in melanoma where RBX1 is higher in nevi than in melanomas [76]. Likewise, RBX2 is overexpressed in many human cancers and targets IκB, c-Jun, HIF-1α, and NF1 for degradation [75]. Although its precise mechanism in cancer progression is not well studied, depletion of RBX2 induces apoptosis, decreases tumor growth, and sensitizes cells to DNA damage [77, 78].

3.3.5. Inhibitor-of-apoptosis proteins (IAPs)

The IAPs are RING E3 ligases that inhibit caspases and thereby block apoptosis which makes them putative targets in cancer [79]. During apoptosis, the second mitochondria-derived activator of caspase (SMAC) is released from the mitochondria and binds IAPs which releases

caspases to perform their pro-apoptotic function. IAPs such as c-IAP1 and c-IAP2 are reported to have genomic amplifications in a variety of cancers like hepatocellular carcinoma, cervical, pancreatic and esophageal cancers. SMAC on the other hand gave a better prognosis in breast, colorectal, and bladder carcinomas [80]. Mimicking the SMAC binding region, a few inhibitors were shown to bind IAPs and activate apoptosis in cancer. These are currently in phase I clinical trials [80].

4. Ubiquitin proteasome system in virus-induced cancers

The idea of viral oncoproteins hijacking the cellular degradation system to degrade potential tumor suppressors is exemplified by the early papers showing human papillomavirus (HPV) oncoproteins E6 and E7 utilizing E6-associated protein (E6-AP) and Cullin 2 RING ligase to target p53 and retinoblastoma for degradation, respectively [81–85]. By developing small molecule inhibitors, targeting these ligases in virus-induced cancers holds great potential for cancer therapy. Additionally, depleting E6-AP expression has been shown to increase p53 protein levels and inhibit growth in HPV-positive cells [86]. So far, inhibitors identified that abalte E6-AP and E6 binding using binding assays have been disappointing as they show low efficacy in inducing cell death in culture [86–88]. It is suggested that more structural data are required in order to design better inhibitors [86]. On the other hand, the small molecule RITA mentioned earlier was shown to block the binding of E6 to p53 thereby preventing E6-mediated degradation degradation of p53 [89]. Cervical carcinoma xenografts showed substantial growth suppression when treated with RITA, suggesting its potential use in cervical cancer [89].

Another important tumor suppressor targeted by the HPV E6 protein is Tat-interactive protein 60 kDa (TIP60) [90]. In addition to HPV E6, adenovirus oncoproteins were also reported to target TIP60, implying an important tumor suppressive role played by TIP60 in virus-induced cancer [91]. The mechanism of degradation in HPV-positive cells involves the use of E3 identified by Differential Display (EDD1) to ubiquitinate and target TIP60 to the proteasome [92]. Importantly, overexpression of TIP60 or depletion of EDD1 in cervical cancer mouse xenografts inhibited tumor growth implying that EDD1 could be a novel target in cervical cancer therapy [92]. In addition, EDD1 is also upregulated in ovarian, breast, and pancreatic adenocarcinoma, as such an EDD1 inhibitor could be extended to these cancers [93].

Latent membrane protein 2A (LMP2A) is one of the 9 proteins expressed from Epstein-Barr virus transformed genome and is involved in viral latency and persistence [94]. In order to perform its function, it recruits neural precursor cell-expressed developmentally down-regulated 4-like (NEDD4-like) ligase to facilitate degradation of Lyn, a tyrosine kinase [94, 95]. This in turn blocks signal transduction of B-cell receptor. Although the mechanism is not completely understood, it increases our understanding of how cellular ligases are utilized at different stages of viral-induced cancers [95].

Apart from individual ligases utilized by viral proteins to degrade cellular substrates, there have been many cases reported where viral oncoproteins interact with the proteasome and hijacks it for their own purposes [95]. For example, through binding to the 20S proteasome and the NF-κB precursor p105, Tax, which is the human T-cell leukemia virus (HTLV) oncoprotein,

enhances the proteolytic activation of NF-κB, which sustains T-cell proliferation [95–97]. In particular, this represents a potential susceptibility using proteasome inhibitors in HTLV-infected T-cell leukemia treatment. Indeed, treatment with proteasome inhibitor Bortezomib was investigated in mouse models with mixed results [98, 99]. In HTLV-1-associated xenograft models, Bortezomib inhibited tumor growth and the mice showed prolonged survival [98, 99]. However, heterogeneity in response was seen between tumors treated with vehicle or Bortezomib derived from Tax transgenic mice [98]. More studies need to be conducted before proteasome inhibitors could be used for HTLV infected T-cell leukemia.

Hepatitis B virus X-antigen (HBX) is another oncoprotein known to interact with proteasome subunits PSMA7 and PSMC1 [100]. In the presence of HBX, two well-defined proteasomal substrates had increased half-lives suggesting that HBX can block proteasomal activity [100]. The importance of proteasome inhibition by HBX is shown in its ability to regulate HBV virus replication. Particularly in cells infected with mutated HBV not expressing HBX, proteasomal inhibitors MG132 and Epoxomicin were able to rescue virus replication back to wild-type levels supporting HBX's role in inhibiting proteasome [101, 102].

Apart from the proteasome, HBX also binds to cellular DDB1, a subunit of the Cullin 4 RING ligase (CRL4) complex [101]. Rather than being degraded by the CRL4 complex, it is stabilized and has been suggested to alter CRL4 specificity by displacing DDB1-CUL4-associated factors (DCAFs), which are proteins that confer substrate specificity to the CRL4 complex [103, 104]. Indeed, two recent papers identified structural maintenance of chromosomes 5/6 (SMC5/6) as novel degradation targets of the CRL4-HBX complex [105, 106]. Since SMC5/6 complex is essential for inhibiting the extrachromosomal HBV gene expression, identification of SMC5/6 as CRL4-HBX targets solves the long-standing question of how HBX-DDB1 interaction is important in HBV virus replication [101, 107, 108]. From these data, one plausible strategy would be to design inhibitors to block the interaction between HBX and DDB1 or the proteasome.

5. Families of deubiquitinating enzymes

There are approximately 80 functional DUBs known in humans [109]. These DUBs are mainly divided into six different classes based on their structure and active site homology: ubiquitin specific proteases (USPs), ubiquitin carboxyl-terminal hydrolases (UCHs), ovarian tumor proteases (OTUs), Machado-Joseph disease protein domain proteases (MJDs), JAMM/MPN (JAB1/MPN/MOV34 metalloenzyme) domain associated metallopeptidases (JAMMs), and monocyte chemotactic protein-induced protein (MCPIP) [110] (**Figure 3**). All DUB families belong to cysteine proteases with the exception of JAMMs family of DUBs, which are zinc-dependent metalloproteases. The mechanism of action for cysteine-dependent DUBs is through nucleophilic attack on the isopeptide linkage of an ubiquitinated lysine residue by the catalytic cysteine, which is facilitated by a nearby histidine side chain that helps to decrease the pKa of the cysteine. A third residue, aspartic acid or asparagine, helps in this whole process. This residue aligns and polarizes the catalytic histidine. Some enzymes which do not have this third residue use other means to polarize histidine [111, 112]. On the other hand, the mechanism of action for JAMMs which are metalloproteases is facilitated by two zinc ions which are present

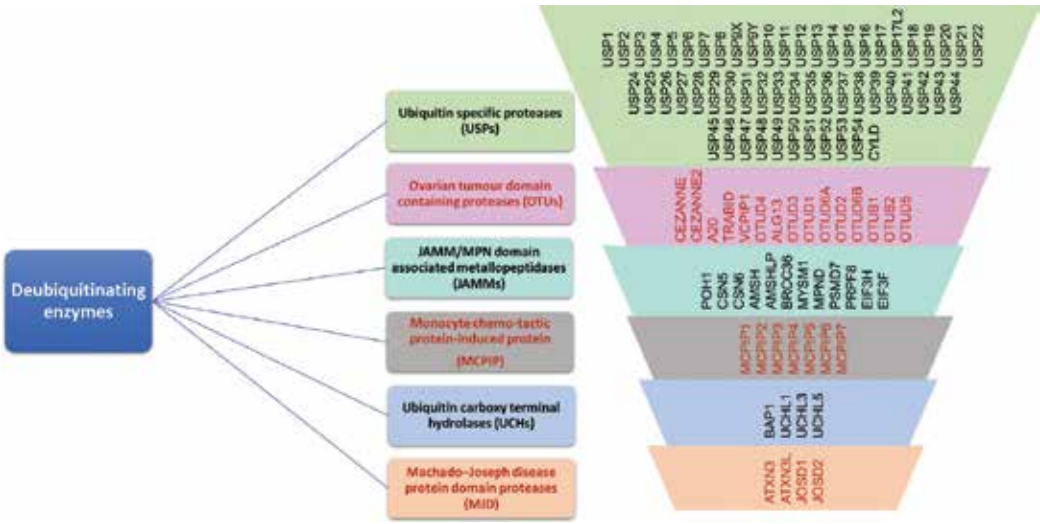

Figure 3. Schematic representation of different families of deubiquitinating enzymes and their members.

within its catalytic site and coordinated by invariant histidine, aspartic acid, and serine side chains [113]. This zinc ion activates a water molecule to form a hydroxide ion which in turn attacks the carboxyl carbon in the isopeptide link [114].

Out of the six classes of DUB families, the USP family is the largest with more than 50 members. These proteins belong to cysteine protease family (clan CA, family C19) [115]. USPs are characterized by the presence of a catalytic core involving histidine and cysteine boxes [116]. DUBs from the USP family contain a highly conserved USP domain characterized by three subdomains which form the palm, thumb, and fingers of a right hand [117]. The active site cysteine is present between the palm and thumb while the finger is used for interaction with ubiquitin. CYLD (cylindromatosis D) is the only USP which does not have the finger domain but possesses an additional domain known as B-box domain [118]. The presence of additional domain and terminal extensions has also been seen in several other USPs, which plays critical roles in conferring specificity to DUBs. For example, USP3, USP5, USP39, USP44, USP45, USP49, and USP51 have zinc finger USP domain, USP25 and USP37 contains ubiquitin-interacting motif, USP5 and USP13 possess ubiquitin-associated domain, USP4, USP11, USP15, USP20, USP33, and USP48 have the domain in USPs (DUSP), and USP52 has the exonuclease III domain. Moreover, several USPs such as USP4, USP7, USP14, USP32, USP47, and USP48 have the ubiquitin-like domain which can be found within and outside of the catalytic domain [115, 119].

UCHs are another family of DUBs, which contain four members in humans, UCHL-1, UCHL-3, UCHL-5, and BAP1. This class of DUBs was the first to be structurally characterized. In particular, UCHs have a short catalytic domain of approximately 200–300 amino acids [109] and can only target short peptide from the C-terminus of ubiquitin because of the presence of a confined loop which prevents polyubiquitin chain recognition and large protein processing. A well-studied member of the UCH class of DUBs is UCHL-1, which is one of the shortest DUBs, having only 223 amino acids [120]. UCHL-1 was initially known to be involved in ubiquitin

maturation by cleaving single amino acids or short peptides from the C-terminus of ubiquitin precursors to generate mono-ubiquitin rather than cleaving ubiquitin from proteins [121].

In UCHL-5 and BAP1, there is the presence of additional C-terminal extension of about 100 and 500 amino acids, respectively. The additional extension at the C-terminus of UCHL-5 directs it to proteasome and helps in trimming polyubiquitin chain from conjugated protein as they are degraded [122]. However, the additional extension of BAP1 contains a nuclear localization signal and helps it to interact with the N-terminal ring finger of BRCA1 (a ubiquitin ligase) [122, 123].

Due to space limitation, we have summarized the targets of different USPs and UCHs in cancer in **Table 1**.

DUBs family	DUBs	Important targets (direct/ indirect)	Mechanism/pathway	Relevance to neoplasm
Ubiquitin specific proteases (USPs)	Cylindromatosis (CYLD)	TNFR-associated factor 2 (TRAF2) and TRAF6 [197]	Promotes apoptosis [198]; negatively regulates NFκB signaling [197]	Downregulated in lung cancer [199], liver cancer [200], colon cancer [200] and multiple myeloma [201]
	USP1	Fanconi anaemia complementation group D2 (FANCD2) [202]; proliferating cell nuclear antigen (PCNA) [203]	Involved in DNA repair and DNA-damage response pathways [202]	Overexpressed in hydatidiform mole [204]
	USP2	MDM2 [205], MDMX [206], Cyclin D1 [207]	Indirect regulation of tumor suppressor p53; increase cell proliferation [208]	Associated with bladder cancer and increase in proliferation, invasion and migration in bladder epithelial cells [209]; overexpressed in prostate cancer [210]
	USP4	TGFβRI [211]	Regulates TGFβ signaling pathway [211]; important player mediating crosstalk between TGFβ and PI3K signaling pathway [211]	Upregulated in human hepatocellular-carcinoma samples and has been suggested to induce aggressive phenotype [212]; downregulated in small cell lung cancer cell lines [213]
	USP5	p53 [214]	Inhibits accumulation of free unanchored polyubiquitin chains	
	USP7	p53 [187], PTEN [188], IRS1/2 [215], Chk1 [216], Claspin [217]	Regulates stability of p53 and MDM2 [218]; reported to induce IGF signaling [215]; modulates ATR-Chk1 pathway [216, 217]	Overexpressed in prostate cancer [188]
	USP8	EGFR [219]; ERBB2, ERBB3 and MET [220]	Regulates endosomal ubiquitin dynamics and required for RTK downregulation following internalization [221, 222]	Gain-of-function mutation in Cushing disease [223]; depletion of USP8 leads to selective death of Gefitinib resistant non-small cell lung carcinoma (NSCLs) cells [220, 224]
	USP9X	SMAD4 [225], β-catenin [226]	Regulates signaling pathway such as TGFβ [225] and MAPK pathway [227]	Overexpressed in breast cancer [228], ERG-positive prostate tumors [229] and osteosarcoma cell line SaOS2 [230] and its

DUBs family	DUBs	Important targets (direct/ indirect)	Mechanism/pathway	Relevance to neoplasm
				increased expression has been correlated with ill prognosis outcomes in multiple myeloma patients [231] and esophageal squamous cell carcinoma [232]
	USP10	p53 [233], T-box transcriptional factor (T-bet) [234]	Regulates ATM-p53 and mismatch repair (MMR) [235, 236]	Overexpressed in breast cancer [228], glioblastoma [237] and in metastatic melanoma [147]
	USP11	TGFβRII [238], p53 [239]	Regulates TGFβ signaling pathway [240] and BRCA2 mediated damage response [241]	High expression of USP11 has been observed in murine lung tissue [238]
	USP12	Androgen receptor (AR) [242]		Suggested as a putative regulator of progression and metastasis in prostate cancer [242]
	USP13	PTEN [243]	Implicated in PI3K signaling	Important for melanoma growth in soft agar assay and nude mice [244]
	USP15	TGFβRI [245], E6 (human papilloma virus (HPV) protein) [246]	Enhances TGFβ signaling pathway [245]	Overexpressed in glioblastoma, ovarian and breast cancer [245, 247]
	USP16	Histone H2A	Regulates progression of cell cycle and gene expression [248]	Regulates stem cell self-renewal and pathologies associated with Down syndrome [249]
	USP17	Ras-converting enzyme 1 (RCE1) [250]	Important for chemotaxis and chemokinesis, and have a crucial role in cell migration [251]	USP17 is amplified in tumors and found to regulate G1/S cell cycle advancement and proliferation [252]
	USP18	EGFR [253]	Involved in interferon signaling [254]	Implicated in regulation of viral disease and malignancies; identified as anticancer target in acute promyelocytic leukemia (APL) [254, 255]
	USP19	KPC (Kip1 ubiquitination-promoting complex) [256]	Regulates cell growth [256]	Putative target for inhibiting proliferation [256]
	USP20	HIF1α [257], Claspin [258]	Promotes transcription of hypoxic response genes [257]	Decreased expression in gastric cancer cells and negative correlation with tumor size and tumor invasion [258]
	USP21	GATA3 [259], Histone H2A [260], EZH2 [261]	Activates transcription [260]	Upregulated in bladder carcinoma [261], breast carcinoma [262, 263] and cancer stem-like cells (CSCs) of renal cell carcinoma cell lines [263] and its expression was correlated with tumorigenic behavior of cells such as tumor size, proliferation, metastasis and invasion
	USP22	Histones H2A and H2B [264]; c-MYC [265]	Regulates epigenetic modulations that support neoplastic change [264, 266]; involved in regulation of various	Elevated expression of USP22 has been reported to be associated with ill prognosis of several cancer like breast [268],

DUBs family	DUBs	Important targets (direct/ indirect)	Mechanism/pathway	Relevance to neoplasm
			tumor associated processes such as cell cycle, proliferation, and apoptosis [267]	colorectal [269] and esophageal squamous cell carcinoma [270]
	USP25	Tankyrases (TNKS1 and TNKS2) [271]	Regulates Wnt signaling pathway [271]	The upregulated mRNA and protein level of USP25 was observed in NSCLC patients which was linked to metastasis [272]
	USP28	c-MYC [273, 274], Chk2 [275], LSD1 (lysine-specificdemethylase1) [276]	Involved in DNA damage response [274, 275]	Somatic mutation has been observed in case of lobular breast cancer [277]; overexpressed in colon [278] and breast cancer [273]
	USP29	p53 [279], Claspin [280]	Involved in regulation of p53 and ATR-Chk1 pathway	
	USP30			
	USP33	Interact with Robol [281]; CP110 (centriolar protein) [169]	Required for Slit signaling [281]; involved in regulation of centrosome duplication and genomic stability [169]	Overexpressed in pediatric acute lymphoblastic leukemia [282]
	USP34	RNF168 [283], AXIN [284]	Regulate genome stability [283]; positively regulates Wnt signaling pathway [284]	
	USP42	p53 [285]	Supports "protect and repair function" of p53 without altering its basal level [285]	
	USP44	Mad2-Cdc20 [286], H2B [287]	Regulates mitotic spindle checkpoint [286]	Overexpression in human T-cell-leukemia [288]; defects in chromatin segregation has been observed with USP44 depletion [286]
	USP47	Polβ [289]	Regulates base excision repair (BER) [289]	USP47 is suggested to be possible therapeutic target as USP47 depletion upregulated level of Cdc25A and decreased cell survival [290]
	USP50	Cdc25B Wee1 [291]	DNA damage response signaling pathway [292]	
Ubiquitin C-terminal hydrolases (UCHs)	UCHL-1	p53 [293]	Involved in ubiquitin maturation [294] and activation of AKT signaling pathway [295]	Linked to several types of cancer including Breast [296], lung [297], colorectal [298] and pancreatic [299]
	BRCA1-associated protein1 (BAP1)	Host cell factor 1 (HCF-1) [300]	Participate in epigenetic regulation in tumor and regulation of histone stability [301]	Mutated in melanoma [302] and implicated in lung and breast cancer [123]
	UCHL-5	Smad2 and Smad3 [303]; NFRKB [304]	Promotes TGFβ signaling [303]; regulates DNA double-strand breaks (DSBs) resection and repair by homologous recombination [304]	Overexpressed in epithelial ovarian cancer [63] and hepatocellular carcinoma [175]

Table 1. USP and UCH family of DUBs, their targets and relevance in cancer.

Ovarian tumor (OTU) represents a superfamily of proteins which are characterized by the presence of an ovarian tumor domain (OTUD) [124]. This domain was first described in the ovarian tumor gene in fruit flies which is involved in the development of ovaries [125]. In 2003, some members of the OTU superfamily were identified to have active cysteine protease site and were described as deubiquitinating enzymes [126]. Based on its characteristics, this class is further subdivided into four groups: Otubains, A20-like OTUs, OTUDs, and OTULIN like OTUs [127]. According to recent studies, the OTU core domain is suggested to consist of five β-strands placed between two αhelical domains. The helical domains vary in sizes among OTU DUBs [128–130]. Like USPs, OTU members also possess additional domains. For example, A20 has A20-type Zn fingers, TRABID has NP14-type Zn fingers, OTUD1 and OTUD5 have ubiquitin-interacting motif, and CEZANNE contains ubiquitin-associated domain [118]. In humans, there are 14 DUBs which belong to the OTU family of DUBs [124]. These DUBs are able to cleave different linkages of ubiquitin chains. For example, OTUB1 and A20 specifically remove K48-linked ubiquitin chains, CEZANNE is specific for K11-linked chains, and TRABID cleaves K29- and K33-linked chains [131].

OTUB1 has a crucial role in DNA damage repair through regulating the RNF8/168 pathway. Recently, OTUB1 is reported to be overexpressed in non-small-cell lung carcinoma (NSCLC) and promotes RAS activation by inhibiting RAS monoubiquitination [132]. Moreover, high expression of OTUD1 is also seen in thyroid carcinoma signifying its oncogenic nature [133]. On the other hand, OTUD5 is linked to apoptosis and is involved in stabilization and activation of p53, suggesting a possible tumor suppressive role [134].

A20 and CEZZANE take part in the negative regulation of NFκB signaling, whereas TRABID positively regulates Wnt signaling pathway [135–137]. A20 is unique and known to have activity of both an E3 ligase and a DUB [138]. A20 cleaves K63-linked ubiquitin chains from RIP1 (receptor interacting protein 1) and negatively regulates the NFκB pathway [137]. A20 genes have been reported to be mutated/deleted in lymphoma suggesting it to be a tumor suppressor [139]. On the other hand, increased A20 is associated with poor outcome in glioma patients [140] and Tamoxifen resistance in breast cancer [141]. Overall, the widespread involvement of these DUBs in a variety of tumorigenic processes makes them potential targets for cancer treatment.

The Josephin family of DUBs is named after a neurodegenerative disease known as Machado-Joseph disease. Particularly, genetic mutations of ATXN3, a member of MJD class of DUBs, are linked to the cause of Machado-Joseph disease [142]. There are four DUBs in humans that form the MJD class: Josephin domain-containing protein 1 (JOSD1), JOSD2, ATXN3-like and ATXN3. ATXN3 can cleave both K48- and K63-linked chains with a higher preference for K63 chains. ATXN3 controls protein folding and stability by editing polyubiquitin chains [143]. The other three members of the Josephin family (JOSD1, JOSD2, and ATXN3L) have highly conserved catalytic triad formed by one cysteine and two histidine residues. An additional domain such as ubiquitin-interacting motif has been identified in ATXN3 and ATXN3L, indicating probable interaction between two distal ubiquitins in a polymer [144]. It has been reported that all Josephin family DUBs especially ATXN3 inhibits PTEN transcription in lung cancer and inhibition of these DUBs induces PTEN expression [145]. In light of these observations, ATXN3 could be a putative target for PTEN repressed tumors. JOSD1 is a membranous DUB and is involved in

regulating membrane dynamics and endocytosis [146]. Moreover, JOSD1 was found to be significantly overexpressed in NSCLCs but its function remains to be elucidated [147].

As mentioned earlier, the JAMM family of DUBs has zinc metalloprotease activity. The crystal structure of AMSH-LP (associated molecule with SH3 domain-like proteases), a DUB from the JAMM family, bound to K63-linked diubiquitin, assisted the understanding of catalytic mechanism of JAMM family [148]. The members of the AMSH family are involved in specific removal of K63-linked polyubiquitin chains and regulate vesicle trafficking and receptor recycling. The domain of AMSH-LP consists of a JAMM core and two conserved insertions. JAMM proteases which do not have AMSH-specific inserts show no specificity for K63-linked polyubiquitin.

There are 12 JAMM proteins along with AMSH-LP that are encoded by human genome. Seven out of the 12 JAMM proteins have isopeptidase activity for ubiquitin or ubiquitin-like proteins while the rest are catalytically inactive. The JAMM proteins with isopeptidase activities are: AMSH-LP, AMSH/STAMBP, BRCC36 (BRCA1/BRCA2-containing complex subunit 36), POH1/PSMD14 (26S proteasome-associated PAD1 homolog 1), MYSM1 (Myb-like with SWIRM and MPN domains 1), MPND (MPN domain-containing protein), and CSN5/JAB1 (COP9 signalosome subunit 5). The high degree of similarity between POH1, AMSH, and AMSH-LP sequences indicates a common mechanism for ubiquitin recognition and catalysis for these JAMMs [148].

BRCC36 belongs to the JAMM class of DUBs whose overexpression has been observed in breast cancer cell lines and tumors [149, 150]. EIF3H and COP6S are other examples of DUBs that belong to JAMM class. COP6S is amplified in breast cancer [151] and EIF3H is amplified in breast and prostate cancer [152].

MCPIP1 proteins possess a domain with deubiquitinating activity which suggests the presence of a sixth family of DUBs in the human genome [153]. This family is suggested to have seven members according to bioinformatics analysis of a recent study [110]. The interaction of MCPIP1, which is the founding member of this family with ubiquitinated proteins, is carried out by ubiquitin-associated domain placed at the N-terminus. However, this domain is not essential for its DUB activity. The other domains of MCPIP1 proteins include N-terminal conserved region, a conserved CCCH-type zinc-finger domain in the middle region of the protein, and a proline-rich domain at its C terminus. The domains that are required for activity of the MCPIP1 proteins are the N-terminal conserved region and zinc finger. In addition, similar to cysteine proteases, the catalytic domain of MCPIP1 also consists of cysteine and aspartic acid boxes but lacks histidine in the catalytic core. However, possibility of histidine outside the core cannot be ruled out [153].

6. Targeting proteasome in cancer

The 26S proteasome is a 2.4 MDa multi-subunit complex responsible for the degradation of intracellular proteins [154]. Currently, there are two FDA-approved proteasome inhibitors

namely Bortezomib (Velcade) and the more potent Carfilzomib (Kyprolis) (**Figure 1**). The FDA initially approved Bortezomib in 2003 for relapsed multiple myeloma (MM) patients [155]. Now, its use has been extended to new MM patients as well as for the treatment of mantle cell lymphoma [156]. Generally, there are three well-accepted models. These are NF-κB inhibition through stabilization of IκB, activation of the unfolded protein response by proteasome inhibition due to high endoplasmic reticulum (ER) stress, and stabilization of pro-apoptotic proteins such as BAX and NOXA [48, 155, 157, 158]. Carfilzomib was approved by FDA in 2012 for relapsed and refractory MM patients, who had previously been treated with Bortezomib [156, 157]. It binds irreversibly to proteasome and inhibits its function by up to 80% resulting in nonfunctional proteasomes as such, it is used for Bortezomib-resistant MM patients [48].

6.1. Proteasome associated DUBs as therapeutic target

Although Bortezomib and Carfilzomib have shown great promise in the clinic [159, 160], it also exhibits side effects [161]. As such, targeting proteasome-associated DUBs might present better specificity by minimizing off-target toxicity attributed to inhibiting the entire proteasome complex. These DUBs play two critical roles in the UPS system. First, by cleaving the attached ubiquitin molecules, it promotes the entry of polyubiquitinated substrate to the 20S catalytic portion of the proteasome. Second, the cleaved ubiquitin would then be available to be recycled as free ubiquitin [162]. Considering the fact that DUBs are intrinsic part of ubiquitin-proteasome system and majority of cancers demonstrate altered expression of DUBs which might drive a number of cancer-associated pathways, targeting DUBs may be considered as a reasonable approach for regulating UPS and is current area of research. There are three DUBs which are associated with the proteasome: PSMD14 (or POH1), USP14, and UCHL5.

6.1.1. PSMD14

PSMD14 is a JAMM metalloprotease. Other than recycling ubiquitin, it is also essential for the structure and function of the 26S proteasome [163]. The importance of PSMD14 in cancer is seen in MM, where its level has been shown to be negatively correlated with the overall patient survival [164]. Depletion of PSMD14 showed decrease in cell viability in multiple myeloma cells. Moreover, upregulation of nuclear PSMD14 is reported in hepatocellular carcinoma and correlates with E2F transcription factor 1 (E2F1) expression and cancer prognosis [165]. PSMD14 is also known to deubiquitinate and modulate the stability of ERBB2 [166]. In addition, PSMD14 has been reported to promote cellular responses to DNA double-strand breaks through homologous recombination. In light of these observations, targeting PSMD14 could lead to better therapeutics in cancer patients.

6.1.2. USP14

Another DUB which is important for ubiquitin recycling is USP14 and has been shown to be involved in delaying protein breakdown by the proteasome and thus, inhibits proteasome activity [167]. USP14 perhaps does so by preventing deubiquitination of proteasome substrate by PSMD14. Although USP14 depletion in mammalian cells has no detectable effect on the accumulation of polyubiquitin [122], it has been shown to inhibit proteasome through its deubiquitinating

activity. USP14 also assists substrate degradation by increasing 20S gate opening [168]. USP14 is overexpressed in NSCLC [169] and in ovarian cancer cells [170]. The expression of USP14 in NSCLC is associated with poor overall survival of patients and tumor cell proliferation, which further strengthens the evidence of USP14 as a tumor-promoting factor in NSCLCs, and a promising therapeutic target. Moreover, USP14 expression in colorectal cancer has been found to be associated with liver and lymph node metastases [171]. It is also implicated in several important signaling pathways [172, 173]. The small molecule inhibitor of USP14, IU1 was shown to stimulate proteasome degradation, further proving its role in proteasome inhibition. This inhibitor specifically binds and inhibits proteasome-bound USP14 [167].

6.1.3. UCHL5

Similar to USP14, UCHL5 is involved in removing ubiquitin from the distal tip of polyubiquitin chains. However, in contrast to USP14, UCHL5 can only release mono-ubiquitin [174]. Clinically, UCHL5 is overexpressed in epithelial ovarian cancer [63] and hepatocellular carcinoma [175]. It has been shown to be associated with poor clinical outcomes in epithelial ovarian cancer [63] and promotes cell migration and invasion in hepatocellular carcinoma [175], implying that it could be a novel predictor of hepatocellular carcinoma reoccurrence. A small molecule compound WP1130 has been shown to inhibit UCHL5 and is expected to functionally block proteasome [176].

Another small molecule compound b-AP15, which was initially identified in cell-based screen, was found to increase the accumulation of polyubiquitin in the cells. Later b-AP15 was identified as an inhibitor of USP14 and UCHL5 [177]. Utilized in solid tumor and MM, b-AP15 showed considerable anti-cancerous effect in animal models. Thus, inhibiting these DUBs by b-AP15, IU1 or related inhibitor may be of therapeutic benefits.

7. DUBs inhibitors

By regulating ubiquitin homeostasis, DUBs have been implicated in tumorigenesis as both its overexpression or loss may drive oncogenesis. Hence, it is not surprising that deregulation of DUBs can lead to severe pathological conditions. To target DUBs, a number of inhibitors either specific for a single DUB or pan-enzyme inhibitors have been identified and are currently explored for its risk-free use in patients. Another approach to target DUBs would be to identify an antagonist that can bind to the DUB's substrate for cancer therapy. DUBs show a great degree of substrate specificity and have a well-defined active site such as the catalytic cysteine which makes DUBs attractive targets for small molecule drug discovery. The active site catalytic cysteine of DUBs is very reactive toward electrophiles. A majority of the DUBs inhibitors are compounds with Michael acceptors such as α, β-unsaturated ketones which are capable of forming covalent adducts with free thiols of nucleophilic cysteine which in turn blocks the DUBs activity [178]. A diverse number of compounds ranging from synthetic small molecules to natural compounds with inhibitory properties for DUBs have been identified and studied. Several strategies can be used to target DUBs and we have summarized them in **Figure 4** and **Table 2**.

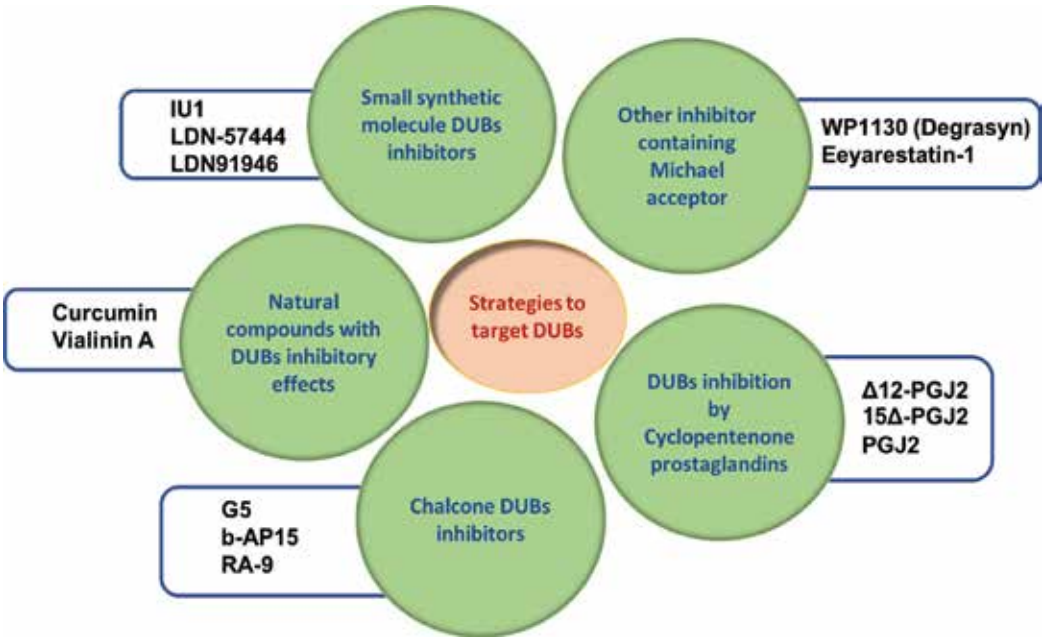

Figure 4. Pictorial representation of different strategies to target DUBs.

7.1. Cyclopentenone prostaglandins

The induction of polyubiquitinated proteins in cells by prostaglandins of the PGJ2 class was first reported by Fitzpatrick and coworkers [179]. DUB activity was shown to be inhibited by prostaglandin PGJ2 which contains α, β-unsaturated ketones. PGJ2 is then further metabolized to Δ12-PGJ2 and 15Δ-PGJ2 [179, 180] which show inhibitory effect toward UCHL3 and UCHL1, respectively [167].

7.2. Chalcone compound with DUB inhibitory effect

A chalcone is an aromatic ketone and an enone that is centrally essential for a broad range of biological compounds. These compounds have cross-conjugated α, β-unsaturated ketones and accessible β-carbons that are important for inhibiting DUBs [181]. These compounds act as either relatively specific or broad-spectrum inhibitors. For example, b-AP15 and its analogue VLX1570 are relatively specific to USP14 and UCH37, whereas another chalcone compound G5 possesses broad inhibitory effect [182, 183].

7.3. Other DUB inhibitors containing Michael acceptors

A small molecule, WP1130, which was derived from a compound with inhibitory activity for Janus-activated kinase 2 (JAK2) kinase was reported to selectively inhibit the activity of USP5 along with USP9X, USP14, and UCH37 [176].

S. no.	Inhibitor	Target (DUB)	Major attributes	Developmental stage
1.	LDN-57444 [195]	UCHL-1	A potent active site directed inhibitor for UCHL-1 [195]; cell permeable inhibitor; decreases proteasome activity	Preclinical
2.	LDN-91946 [196]	UCHL-1	Is able to inhibit UCHL-1 in a noncompetitive manner [196]	Preclinical
3.	15Δ-PGJ2	UCHL-1	Is a metabolite of prostaglandin, PGJ2 that was identified to retain inhibitory effects towards UCHL-1 by affecting overall structure and thus activity [305, 306]	
4.	AM146, RA-9, and RA-14 [307]	UCHL-1	Are chalcones which act as partially selective DUBs inhibitor and can inhibit UCHL-1 activity [307]	
5.	LS1 [308]	UCHL-3	Inhibits UCHL-3, identified in FRET-based screen [309]	
6.	NSC112200 and NSC267309 [310]	TRABID	Inhibited the growth of colorectal tumor cell lines HCT-116 and SW480 [310]	
7.	b-AP15 [177]	UCHL-5 and USP14	Anti-cancerous effect against solid tumor and multiple myeloma *in vivo*	
8.	WP-1130 [176]	USP9X, USP5, USP14, UCH37, UCHL-5	A small molecule, WP1130 serves as a pan DUBs inhibitor which was derived from AG490 (JAK2 inhibitor) and reported to inhibit activity of several DUBs [176]; elicits apoptosis of tumor cells	Preclinical
9.	Pimozide [311] and ML323 [312]	USP1	Works by blocking complex formation between USP1-UAF1, which in turn inhibits USP1activity. ML323 and related N-benzyl1-2-phenylpyrimidine-4-amine derivatives shows higher selectivity and inhibitory potency towards USP1/UAF1 than Pimozide [312]	Preclinical
10.	ML364 [313]	USP2	Is a small molecule inhibitor, which has been identified to enhance Cyclin D1 degradation in colorectal cancer and lymphoma model [313]	
11.	Vialinin A [185]	USP4 and USP5	A natural compound isolated from Chinese mushroom *Thelephoravialis* and has been shown to inhibit enzymatic activity of USP4 and USP5 [185, 186]	Preclinical
12.	P5091 [191]	USP7	Selective inhibitor of USP7, triggers apoptosis in multiple myeloma cells [191]	Preclinical
13.	P22077 [192]	USP7 and USP47	A specific inhibitor of USP7 identified by Progenra [190]	Preclinical
14.	Cpd14 [192]	USP7 and USP47	Resulted in increase in p53 and induction of p21 protein in HCT-116 cells upon treatment [192]	
15.	HBX41, 108 [193]	USP7	HBX 41,108 is an noncompititive reversible inhibitor and it allosterically modulates the catalytic reaction of USP7 [193]	Preclinical
16.	HBX19, 818 [194]	USP7	Binds selectively to the active site of USP7 [194]	Preclinical
17.	HBX28, 258 [194]	USP7	Selective inhibitor for USP7	
18.	HBX90397 [314]	USP8	Specifically target USP8 [116, 314]; inhibited cancer cell growth	
19.	Spautin1 [315]	USP10 and USP13	Induce Vps34 PI3K complex degradation [315]	Preclinical

S. no.	Inhibitor	Target (DUB)	Major attributes	Developmental stage
20.	Mitoxantrone [316]	USP11		Preclinical
21.	IU1 [167]	USP14	Cell permeable; reversible; encourages ubiquitin dependent protein degradation *in vitro*	Preclinical
22.	GSK2643943A [317]	USP20	Identified by GSK from a screen involving compounds targeting USP20/Ub-Rho. It has an IC_{50} of 160 nM [317]	Preclinical
23.	15-oxospiramilacetone [318]	USP30	15-oxospiramilacetone is the only inhibitor for USP30 defined so far which can be used in case of some mitochondrial dysfunctions [318]; natural compound from spiramine A; induce mitochondrial fusion	Preclinical
24.	PR619 [319]	Broad range DUBs inhibitor	Nonselective, noncompetitive, reversible; results in accumulation of ubiquitinated proteins	
25.	1,10-phenanthroline [320, 321]	JAMM type isopeptidase	Chelates active site Zn^{2+}	

Table 2. Different inhibitors and their target DUBs.

7.4. Natural products with DUB inhibitory effect

A number of natural compounds have been identified to have DUB inhibitory effect. One of such is Curcumin, which is a yellow pigment isolated from the *Curcuma longa.* Curcumin possesses two α, β-unsaturated ketones moieties and has been linked with suppression of tumorigenesis and various other diseases. It was reported that Curcumin accelerates polyubiquitinated protein accumulation at concentrations of 40 μM [184]. USP4 has been reported to be targeted by a small natural compound known as Vialinin A. Vialinin A is isolated from the Chinese mushroom *Thelephoravialis* and has been shown to inhibit the enzymatic activity of USP4 and USP5 [185, 186].

7.5. Synthetic small molecule DUB inhibitors

Several inhibitors have been developed to target the multifunctional deubiquitinating enzyme USP7. USP7, also known as HAUSP, is probably the most attractive DUB in the field of cancer biology. USP7 has been reported to regulate the function and stability of at least three important tumor suppressor p53 [187], PTEN [188] and TIP60 [189]. Progenra identified P022077 as a specific inhibitor for USP7 [190]. Other inhibitors of USP7 are P5091 and Cpd14, which triggers apoptosis in MM cells and inhibits tumor growth [191, 192]. Other Hybrigenics compounds which could inhibit USP7 function are HBX41108 [193], HBX19818 [194], and HBX28258 [194]. An isatin O-acyl oxime, LDN-57444 is a most potent active site directed inhibitor for UCHL-1 [195]. LDN-91946 is another compound which was identified as a hit in an *in vitro* screen for identifying blockers of Ub-AMC activity and was able to inhibit UCHL-1 in a noncompetitive manner [196].

Despite the multitude of inhibitors identified to target DUBs, so far, no DUB inhibitors are approved for clinical use. Only a few of these inhibitors, such as VLX1570, are in clinical trial for

cancer therapy. Out of 98 DUBs, only several DUBs have been explored structurally providing a platform for understanding, identifying, and validating various DUB inhibitors for clinical usage.

8. Conclusion

The UPS is implicated in several human diseases such as neurodegenerative disease, inflammation, bacterial and viral infection and most importantly, in cancer. The type of ubiquitin linkages formed/cleaved with the help of a cascade of enzymes (E1, E2, E3/DUBs) intensifies biological complexities. Hence, it is important to discover and identify the targets for therapeutic intervention. One of the strategies that can be used is targeting components of the UPS. Over the past 35 years, our knowledge and understanding of the UPS has significantly increased and it is evident that the UPS plays critical roles in various important cellular functions and can regulate both structural and functional behavior of cells. The success of Bortezomib provides a proof-of-concept to expand the use of other inhibitors targeting different components of UPS system in cancer. However, the results were not satisfying due to challenges in bringing these inhibitors to clinic. This is mostly because E3 ligases and DUBs have multiple substrates which makes it complicated. Therefore, it is critical to find the right target(s) for a specific cancer, to understand how the target functions and eventually find the finest way to effectively manipulate these targets for treatment intervention.

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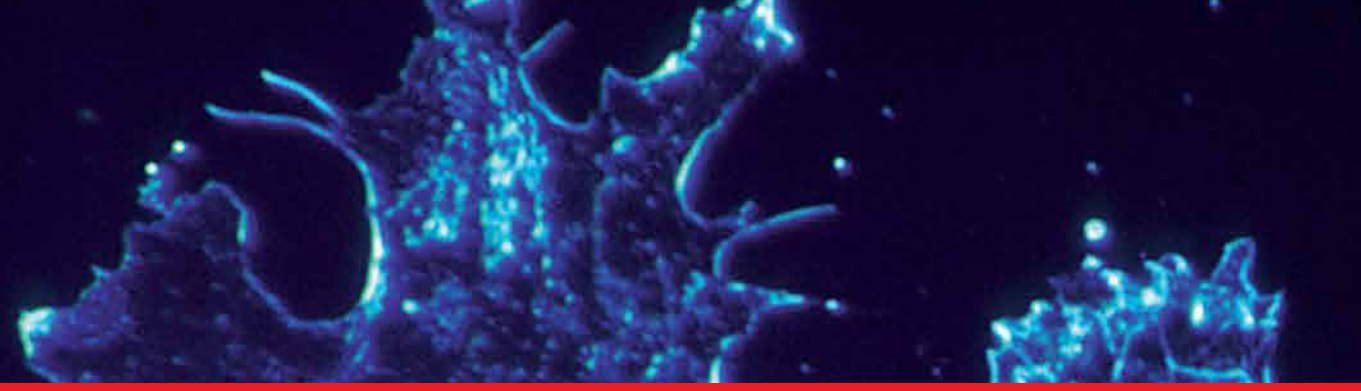

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Neoplasm refers to an abnormal tissue growth that arises as a consequence of rapid cell proliferation and continues to grow abnormally even after terminating the stimuli that had instigated the change. It lacks partial or complete functional coordination with that of the normal tissues. Neoplasms are classified depending on the degree and type of tissues involved. Carcinogenesis is a multistep process where a plethora of endogenous and exogenous factors turns out genetic and epigenetic modifications, which collectively amend some critical cellular pathways controlling the proliferation, apoptosis, and differentiation. The cells having aberrant modifications are transformed into malignant ones of which the clonal expansion results in the development of cancer. This book provides the reader with a comprehensive overview of various cancer types along with their molecular mechanisms of initiation and progression. It also describes the current knowledge about the state-of-the-art measures being employed in cancer diagnosis and therapeutics. Particular attention is paid to make this book equally useful for students, practitioners, and expert scientists.

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