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Glioma

Contemporary Diagnostic and Therapeutic Approaches

Edited by Ibrahim Omerhodžić and Kenan Arnautović

GLIOMA - CONTEMPORARY DIAGNOSTIC AND THERAPEUTIC APPROACHES

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http://dx.doi.org/10.5772/intechopen.72102
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First published in London, United Kingdom, 2019 by IntechOpen
eBook (PDF) Published by IntechOpen, 2019
IntechOpen is the global imprint of INTECHOPEN LIMITED, registered in England and Wales, registration number: 11086078, The Shard, 25th floor, 32 London Bridge Street
London, SE19SG – United Kingdom
Printed in Croatia

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

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

Glioma - Contemporary Diagnostic and Therapeutic Approaches
Edited by Ibrahim Omerhodžić and Kenan Arnautović

p. cm.

Print ISBN 978-1-78984-953-0

Online ISBN 978-1-78984-954-7

eBook (PDF) ISBN 978-1-83881-655-1

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Foreword 1

It is a tremendous honor for me to have been asked to write an introduction to this book. For a world-class neurosurgeon such as Dr. Omerhodzic, for whom I have tremendous respect, to bestow such an honor upon me is a pleasure.

It was in 1932 that Harvey Cushing wrote: "In these days when science is clearly in the saddle and when our knowledge of disease is advancing at a breathless pace, we are apt to forget that not all can ride and that he also serves who waits and who applies what the horseman discovers…"

Such words are truer today than at any other time in history. It is a credit to Dr. Omerhodzic's sagacity, recognizing that printed books are out of date by the time they hit the bookshelves, that he chose an online format for this compendium such that its information could be updated and disseminated more expeditiously. With that in mind, this book offers the reader the most up-to-date knowledge available on cutting-edge research into novel treatments for patients with gliomas.

But let me be clear, this book is not an entry-level epistle. It contains chapters from cutting-edge glioma research laboratories worldwide—from Boston to Europe, India, China, Mexico City, and beyond. The book covers everything from the extent of surgical resection to molecular genetics and new therapeutic modalities such as laser interstitial thermal therapy, boron neutron capture, and targeting of molecular pathways. This is a book for the serious student with an interest in malignant gliomas. Dr. Omerhodzic has pulled together thought leaders from all continents to offer their most up-to-date research on this deadly disease. I hope you will read with interest.

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Foreword 2

Gliomas are the most common primary brain tumors in neurosurgical practice. The defining characteristics of primary brain tumors have evolved through three periods: classical, histological, and molecular. The classical period began with Rudolf Virchow, who defined tumor cells of glial origin both macroscopically and microscopically in 1863. Dr. Percival Bailey and Dr. Harvey Cushing (1926) noticed that not all glial tumors behave in the same manner. They reviewed more than 400 verified gliomas and concluded that there was more than one tumor type, and prognosis varied according to the pathological findings: the histological period. They made the first classification of glial tumors. Then this classification changed and evolved into four grades defined by the World Health Organization depending on their histopathological features: cellularity, nuclear atypia, mitotic activity, necrosis, and vascular endothelial proliferation, which became accepted worldwide. These histopathological features had been assumed to be intimately related with prognosis. However, longevity had been noticed in patients more than was expected even with higher grade gliomas, so new queries have begun to clarify this black hole. The third period started with advancements in molecular biology and genetics that have yielded a better understanding of gliomas' etiology and prognosis with more delicate knowledge and targeted molecular therapies. Molecular genetic as well as epigenetic regulation was found to be deregulated in gliomas. Some of these changes were proven to be indicators of specific disease processes and have significantly improved our ability to predict clinical behavior by supporting morphological diagnosis. There are many ongoing clinical and laboratory studies whose objective is to develop target therapies for gliomas, aimed at inhibition of tumor proliferation, function, angiogenesis, invasion, apoptosis as well as understanding the tumor microenvironment, all of which increase tumor heterogeneity. All of these advances were made possible by a better scientific understanding of gliomas.

In this book, these research questions have been answered using current knowledge: the receptor tyrosine kinase pathway and its relation with tumor microenvironment, diagnosis and management of diffuse astrocytoma/oligodendroglioma, up-to-date treatment modalities of gliomas, and antioxidant supplementation during treatment.

I believe that this book is a wonderful contribution to neurological sciences and will enhance understanding of tumor pathogenesis and cellular and molecular pathways of gliomas with a glimpse into future treatment modalities that would improve life expectancy and health-related quality of life in glioma patients.

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Preface

Contemporary treatment of gliomas is perhaps one of the best examples of teamwork and collaboration in neurosurgical subspecialties. Along with the neurosurgeon, the neuroradiologist, neuropathologist, neuro-oncologist, radiation oncologist, physiatrist, clinical nurse practitioner coordinator, and others are irreplaceable team members. Furthermore, surgical neuro-oncology has proven to be one of the most resilient neurosurgical subspecialties.

Despite the lack of significant progress in the treatment of high-grade gliomas and the survival of high-grade glioma patients over many years, slow and steady persistence coupled with discoveries in various areas have yielded noticeable improvement in patient survival and quality of life. For example, postoperative survival of more than 2 years for glioblastoma multiforme (GBM) patients has become increasingly frequent. In addition, long-term survivors of the same disease are not necessarily found in rare case reports. Nonetheless, we remain far from meaningful, long-term success in the treatment of those patients.

In the past decade, there has been significant surgical improvement in this field. Improvement of surgical anatomical understanding of the white matter tracts is because of practice by the surgeon and research of brain anatomy acquired during surgical anatomy labs for white matter fiber dissection techniques, as well as the development of more advanced magnetic resonance imaging tractography software. Furthermore, intraoperative frameless navigation systems have become more sophisticated, and intraoperative ultrasound guidance of tumor resection has become more advanced. There have also been further technological improvements of ultrasound tumor aspirators. Every year, surgical microscope manufacturers add more technological features that improve their effectiveness. In addition, 5-aminolevulinic acid fluorescence-guided resection of malignant glioma has recently been gaining popularity.

Molecular diagnostics in gliomas has complemented histopathological analysis, and we are now able to distinguish less aggressive subtypes of GBMs from the more aggressive ones. Treatment protocols matching different molecular characteristics have been established, including protocols for tumor recurrence. Finally, the introduction of portable devices delivering low intensity, intermediate frequency, and alternating electric fields using non-invasive disposable transducer arrays has become the standard of care in the treatment of malignant gliomas.

Given the steady increase in available options in the treatment of malignant gliomas, one should remain cautiously optimistic that significant thresholds in the improvement of patient longevity and quality of life will continue to be achieved.

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Chapter 1

Introductory Chapter: Glioma - Merciless Medical Diagnosis

Ibrahim Omerhodžić

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.82863

1. Introduction

Is not this still the truth? Or, are we today, over 100 years after the first glioma operation, however, nearer to the option that we can treat, even cure, most of gliomas or at least keep the disease under control for very many years? The most frequent primary brain tumor, glioma, is still a nightmare for neurooncologists, neuropathologists, neurosurgeons, and other related professionals, but for patients and their families first of all.

If we look at the results of the papers published in the influential *Medline* database (accessed in December 2018), we will find a paper on glioma published back in 1870 [1]. For the next almost 150 years, in this base alone, we can find more than 87,000 papers on the same topic. On the other hand, in the same base only in the past 5 years, over 22,000 papers have been published having basically a story on gliomas (almost a quarter of all publications on gliomas). Does this mean that in the recent years the glioma field research has been more fruitful than ever before? Does this promise, or at least give hope, that we will find the way to put this serious disease under control?

In the USA, primary brain tumors account for about 2% of all cancers, with an overall annual incidence of 22 per 100,000 population, with nearly 80,000 new cases of which one-third will be malignant [2, 3].

The past three decades have been marked with huge enthusiasm of scientists' and professionals' efforts to bring this serious disease into the context of curable or even cured one. Brain glioma patient treatment has significantly changed over time. Undoubtedly, the architect of this fight, Hurvey Chusing, early in the twentieth century, tried to solve the problem surgically and by tumor removal from the brain. History would very soon show

that there are brain tumors for which surgery is not sufficient; for some of them, it is not the first option or treatment at all. However, gliomas have remained in focus of interest not only of neurosurgeons but of oncologists, pathologists, neuroradiologists, forensics, and other related medical and nonmedical disciplines either. Not unimportant is also the interest of pharmaceutical industry and researchers in the field of biomedical technologies in the glioma field.

2. Where we were

Expansion of new diagnostic modalities and glioma treatment, such as imaging, stereotaxic localization, and standardization of the microsurgical technique, practically started in the 1980s. These methods, however, contributed to a better, safer, and more precise glioma resection, but there was no clear confirmation of better survival. We had to hope for better adjuvant therapy effects.

Until recently, rare were prospective randomized studies confirming that the gross total resection improved the outcome. The use of 5-ALA at resection enabled the doubling of time of 6-month progression free survival (PFS) and overall survival (OS). Significant glioma treatment progress occurred with the introduction of neuronavigational (frameless) biopsy in almost routine practice, followed by analysis of a tumor sample with a series of biomarkers; so, even before entering the operating theater for tumor resection, now it is possible to have a lot of information on its nature; glioma resection can be worked out in much more detail both for low-grade glioma (LGG) and for high-grade glioma (HGG). We should have in mind that today's accepted practice is that, when frozen section shows grade III glioma, we should do the aggressive tumor resection as much as possible. For grade IV (glioblastoma), the extensive resection is also critical for outcome [4]. On the other hand, midline tumors have a poorer prognosis compared to lobar equivalents, probably for the reason that the radical resection is feasible with more difficulties [5].

With time, significant progress has been made in the treatment and strategy of glioma patient treatment. This relates particularly to malignant gliomas. A shift has been made both in treatment and in diagnostic, with an accent on ever more powerful apparatus for neuroradiological scanning, magnetic resonance (MRI) first of all. Introduction of MRI in the late 1980s revolutionized management of intracranial tumors, and advanced neuroimaging today is one of the most important prerequisites for the modern treatment of glioma. This is possible especially because of combined use of contemporary radiological modalities, particularly integration of structural, metabolic, and functional imaging, which provides optimal multifaceted information for detailed characterization of intracranial gliomas [6]. Methods of the definite confirmation of the glioma kind and grade have walked a path from classical macro- and microscopic pathohistological confirmation of tumor, through morphological-histological, to molecular and genetic diagnosis practically accepted today.

http://dx.doi.org/10.5772/intechopen.82863

3. Where we are now

Brain glioma is infrequently also denoted as slowly growing neoplasm that is most often discovered in younger adults, and it is presented with minimal symptoms or no such symptoms at all. The World Health Organization (WHO) classification system for glial tumors offered guidelines, which can predict the disease course; treatment modalities are thus recommended [7]. Nevertheless, these guidelines are based mostly on histological diagnosis. European Association for Neuro-Oncology (EANO) also presented its guidelines [8]. Yet, histologically the same tumors may have different courses, response to therapy and eventually to outcome. Molecular markers that carry both diagnostic and prognostic information add valuable tools by redefining tumor subtypes within each WHO category. That is why these molecular biomarkers have become an integral part of tumor assessment in modern neurooncology [9]. In that sense, markers such as biomarkers (IDH mutations, promoter methylation of MGMT, chromosomal deletion of 1p/19q, mutations of EGFR and ATRX genes, and BRAF fusion) can today guide clinicians to make better decisions in some subtypes of gliomas, including anaplastic oligodendroglioma and GBM in the elderly [9, 10]. The integration of genome-wide data delineated three molecular classes of LGG, and we believe today that those without an IDH mutation were molecularly and clinically similar to GBM [11].

Significant progress has been made generally in glioma treatment with the use of modern radiotherapy ways and new chemotherapeutics. Several studies in the past decade have dealt with very promising temozolomide (TMZ) in glioma treatment. Baumert et al., in recently published randomized study results, have reported that they did not find any significant difference in outcome of the overall patient population treated with either radiotherapy alone or TMZ chemotherapy alone [12]. On the other hand, in the just published EORTC trial, randomized controlled research on phase 2 published in Lancet, van den Bent et al. have found no evidence of improved overall survival with bevacizumab (Avastin) and TMZ combination treatment versus TMZ monotherapy in patients with first recurrence of WHO grades II and III glioma, without 1p/19q codeletion [13].

A new swing in glioma treatment was the defining of a molecular genetics signature, which can predict patient's outcome with the loss of 1p and 19q in anaplastic oligodendroglioma. Specifically, such patients responded very well to procarbazine, cyclohexychloroethylnitrosurea CCNU, and vincristine (PCV) chemotherapy or TMZ. When it comes to glioblastoma multiforme (GBM), hope was set on MGMT gene and its methylation [4].

Surgically, progress was made possible with the development and use of technological aids, first of all of neuronavigation, cortical mapping, electrocorticography, neuromonitoring, functional and intraoperative MRI, magnetoencephalography (MEG). As great hope was placed on extension of tumor resection, brain mapping in particular offered additional safety to the neurosurgeon to provide as good result as possible with maximal and today popular supratotal resection.

The current paradigm shift considers glioma management in a comprehensive perspective that takes into account the intricate connectivity of the cerebral networks. Lesions

previously considered inoperable are today more accessible and safer for resection; the surgeon has greater self-confidence and better preoperative knowledge about the tumor and can expect better result of the glioma resection itself; and patients can expect a better outcome. This is particularly important for those lesions for which neurosurgical resection and the extent of resection (EOR) is still the most important part of treatment and standard of care [14–16].

To achieve as good result as possible, somatosensory evoked potentials (SSEPs), motor evoked potentials (MEPs), visual evoked potentials (VEPs), brainstem auditory evoked potentials (BAEPs), and electrocorticography (ECoG) are used nowadays routinely during glioma surgery. For brainstem gliomas, specific mapping with direct electrical stimulation (DES), corticobulbar tract MEP monitoring, and free-running electromyography (EMG) of the various muscles innervated by the cranial nerves are also required. Awake craniotomy and intraoperative mapping of language and sensorimotor functions with DES have become standard techniques for removal of cerebral neoplasms affecting eloquent cortical areas and subcortical pathways [17].

The present data provide prognostic information for patients with pilocytic astrocytoma and confirm that age and tumor size had a significant effect on OS [18]. For patients with anaplastic astrocytic gliomas, Nayak et al. [18] have found the median overall survival 2.9 years, and 1-year OS rate was 87%. Novel therapeutics is needed in patients with tumors not amenable to resection or radiosurgery [19]. The joint efforts of neuroscientists, researchers, and clinicians have provided an unprecedented ability to localize lesions and to assess the human brain function at the microscopic, mesoscopic, and macroscopic scales [14].

4. Where we are going

Treatments and better outcomes for primary brain tumors have long lagged behind those of other tumors. Rapid advances in neurooncology, cancer and CNS immunology, and progress in genomics have created more therapeutic opportunities than ever before [2, 3]. There is no doubt that significant changes have occurred in management of glioma patients. In the past three decades, we have led to the discovery of hundreds of molecular alterations in grades II, III, and IV gliomas. Among these molecular alterations, three are particularly noteworthy, because they occur early during glioma formation, are prevalent in glioma, or are strongly associated with overall survival. Codeletion of chromosome arms 1p and 19q (1p/19q codeletion), which is associated with the oligodendroglial histologic type and with sensitivity to chemotherapy with alkylating agents. The second was mutation in either IDH1 or IDH2 gene associated with a distinctive tumor cell metabolism. The third was mutation in the promoter of TERT, which is seen in both the most aggressive human glioma (grade IV astrocytoma) and the least aggressive diffuse human glioma (grade II oligodendroglioma) [11, 18].

Pamir and his group (2017) stated that molecular subsets in hemispheric diffuse gliomas result in different tumor biology and clinical behaviors [20]. Eckel-Passow et al. [21] published significant study on a sample of 1081 gliomas, which they divided into five molecular

http://dx.doi.org/10.5772/intechopen.82863

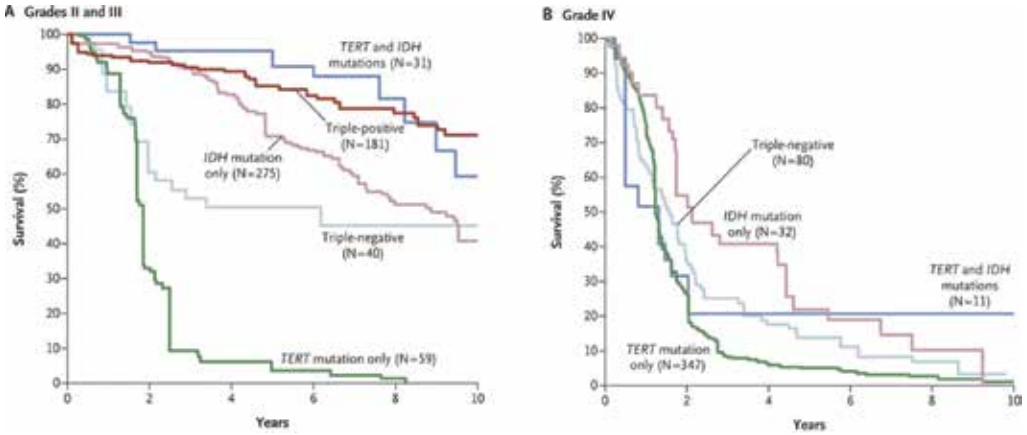

Figure 1. Overall survival in the glioma molecular groups based on 1p/19q, IDH, and TERT promoter mutations in tumors (taken from Eckel-Passow et al. [21]).

groups according to three alterations: mutations in the TERT promoter, mutations in IDH, and codeletion of chromosome arms 1p and 19q (**Figure 1**). They concluded that "the groups had different ages at onset, overall survival, and associations with germline variants, which implies that they are characterized by distinct mechanisms of pathogenesis" [21]. In favor of this, in the future, we should also incorporate alterations in ATRX, TP53, EGFR, or PTEN or other alterations that might be useful to consider in the diagnosis of glioma [11].

WHO grade I tumors and WHO grade II tumors should not be grouped together as low grade, because the two disease processes are markedly different. For patients with grade II glioma who had undergone subtotal tumor resection, Buckner and colleagues suggest combination of chemotherapy in addition to radiation therapy [22]. Opposite to the real low-grade lesions are, for example, dysembryoplastic neuroepithelial tumors are associated with continuous growth and inevitable malignant transformation. This fact supports the concept that grade II gliomas are premalignant and that the use of early aggressive surgical treatment is a very important part of their treatment pathway [18]. Al-Tamimi and Duffau's group [23] suggest that after radical resection, the presence of foci of transformation within a background of grade II tumor does not necessarily require immediate adjuvant therapy. They suggest that a tailored approach should be used, taking into account the extent of resection, the full histopathologic and molecular profile of the tumor, and careful evaluation of the resection margins [15, 17, 23].

Speaking of high-grade glioma, despite the efforts made in research on new therapeutics, the last WHO classification of CNS tumors from year 2016 brought about some changes (**Table 1**) [7].

Demarcation of glioma borders is a subject of comprehensive research, considering that it is difficult to clearly define the line between tumor and healthy brain tissue macroscopically or with today available imaging techniques like functional MRI (fMRI), positron emission tomography (PET), spectroscopy, and diffusion tensor imaging (DTI) [6, 17, 24]. Contemporary neurophysiology plays a very important role in guidance of brain tumor surgery [17]. For tumors located in proximity to critical functional areas, the use of intraoperative electrostimulation

Other astrocytic tumors (often referred as "circumscribed gliomas")

WHO I

- Pilocytic astrocytoma

WHO II

- Pleomorphic xanthoastrocytoma

WHO III

- Anaplastic pleomorphic xanthoastrocytoma

Diffuse astrocytic and oligodendroglial tumors (often referred as "diffuse gliomas")

WHO II

- Diffuse astrocytoma, IDH-mutant
- Diffuse astrocytoma, IDH-wild type
- Diffuse astrocytoma, NOS
- Oligodendroglioma, IDH-mutant and 1p/19q-codeleted†
- Oligodendroglioma, NOS

WHO III (anaplastic)

- Anaplastic astrocytoma, IDH-mutant
- Anaplastic astrocytoma, IDH-wild type
- Anaplastic astrocytoma, NOS
- Anaplastic oligodendroglioma, IDH-mutant and 1p/19q-codeleted
- Anaplastic oligodendroglioma, NOS

WHO IV

- Glioblastoma, IDH-wild type
- Glioblastoma, IDH-mutant
- Glioblastoma, NOS
- Diffuse midline glioma, H3 K27M-mutant

Ependymal tumors

WHO I

- Subependymoma
- Myxopapillary ependymoma
- WHO II
- Ependymoma

WHO II or III

- Ependymoma, RELA fusion-positive
- WHO III
- Anaplastic ependymoma

Neuronal and mixed neuronal-glial tumors

- Diffuse leptomeningeal glioneuronal tumor

WHO I

- Dysembryoplastic neuroepithelial tumor
- Gangliocytoma
- Gangliocytoma
- Dysplastic gangliocytoma of cerebellum (Lhermitte-Duclos)

WHO III

- Anaplastic ganglioglioma

IDH, isocitrate dehydrogenase; NOS, not otherwise specified; RELA, reticuloendotheliosis viral oncogene homolog A [7].
† The last WHO classification of CNS tumors brought about some changes based on molecular findings.

Table 1. Simplified and modified from WHO 2016 classification of neuroepithelial tissue tumors.

mapping (IEM) during awake craniotomy helps to maximize the extent of resection and to minimize the risk of permanent neurological morbidity, allowing a substantial increase in the survival and quality of life of patients [14, 17, 25].

5. Is there hope

Primary brain tumors remain hard and challenging work despite the progress in understanding their genetics and technological progress that enabled safe and extensive tumor resection [3, 15, 17]. As gliomas include a variety of different histological tumor types and malignancy grades, contemporary achievements in terms of molecular imaging have given us a unique

http://dx.doi.org/10.5772/intechopen.82863

chance for a comprehensive interdisciplinary assessment of the glioma pathophysiology, with direct implications in terms of the medical and surgical treatment strategies available for patients [26]. The concept of individualized surgery in brain tumor neurosurgery, that is, specifically in neurooncology of glial tumors is actually based on the goal to provide as radical tumor resection as possible, without causing (additional) neurological deficit (**Figure 2**) [27].

Unfortunately, the prognosis of patients with grade IV malignant glioma particularly recurrent is dismal, and there is currently no effective therapy, but there are some promising agents as vaccine immunotherapy or recombinant nonpathogenic poliorhinovirus chimera (PVSRIPO) [28, 29]. Desjardins et al. have recently reported that overall survival among the patients who received PVSRIPO immunotherapy was higher at 24 and 36 months than the rate among controls [29]. Extension of surgical excision is still an important predictor of outcome. Achieving a gross total resection of the tumor without significant complication requires a thorough understanding of available surgical approaches [15, 17, 30–32]. For majority of those patients, short-course radiotherapy with concurrent and adjuvant TMZ will bring a benefit, while gain from bevacizumab is limited [13]. There have been some ideas that certain antiepileptics also have a favorable effect on the outcome with glioma patients, but these studies have not given affirmative results [33]. To provide a highly personalized medicine, we will also have to make additional effort toward molecular neuropathology [30].

What do we want to see in the future? A patient from the supposed risk group will be scanned with MRI spectroscopy, 7T MRI, or similar MRI prototype. At the level of a robot medical center, needle biopsy of tumor will be performed, which will be followed by oncogenomic characterization of lesion, with gene map reading and defining. Research in the field of stem cells also has an important place and implications in the future. By way of stem cells, a specific

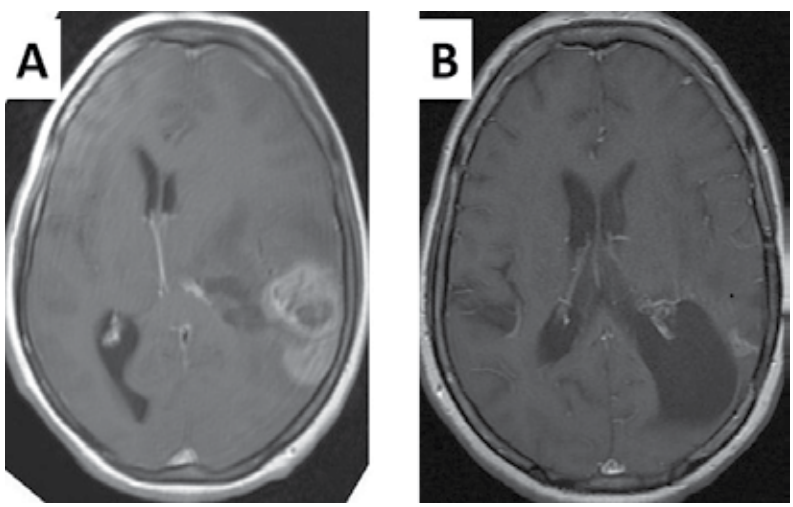

Figure 2. Contrast-enhanced axial T1 MRI scan of 54-year-old female patient with IDH-mutant glioblastoma. (A) Preoperative MRI and (B) 4 years after extensive surgical resection, followed with TMZ and radiotherapy treatment. Small part of recurrent tumor is visible 50 months after initial resection. Patient is still without any neurological deficit.

medicine will be produced, individualized for the particular patient, and in a microcapsule, it will be implanted into the brain zone affected by tumor, by way of robot surgery and injection needle. These are not at all unrealistic expectations in the next decade or two. Perhaps a bit futuristic, but it is also realistic to expect vaccination against glioma [28], that is, specific repair stem cells that will recognize the "glioma-damaged" part of the brain and thus preventively work on it, before the growth of tumor itself.

It is not too much to expect that the current generation of neurooncologists will resolve glioma problem for ever. We should bravely carry on. Time is brain.

Acknowledgements

The book is based on the research, experience, and collaboration of 55 colleagues from 10 countries around the world, from four continents. I would like to give them credit for the effort and for the benefit to the civilization, and would like to thank them very sincerely for the contribution to this small step toward resolving the enigma called brain glioma.

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Chapter 2

Functional and Therapeutic Implications of Mitochondrial Network and Mitochondria-Associated Membranes: The Glioma's Case

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.77224

Abstract

Even today, despite the surgery, radiotherapy, and chemotherapy, gliomas prognosis is still poor. There is a great need to develop new therapies. The understanding of the structural and functional characteristics of mitochondrial network (MN) and mitochondria-associated membranes (MAM) in gliomas is essential for the design of future therapeutic strategies. A huge range of ultrastructural findings is observed in MN and MAM in the human gliomas. These findings imply that a majority of glioma cells are incompetent to produce an adequate amount of energy by means of oxidative phosphorylation and compensatory increases in glycolytic ATP production. Regarding MAM, a "MAM-rich" cell (well-differentiated glioma cells) and "MAM-deficient" cells (glioma like-stem cells) exist. The quantity of MAM could be linked to the functional or metabolic state of the different glioma cells. MAM-resident mTORC2 is a major regulator tumor growth and drug resistance. If sufficient nutrients are present, glioblastoma cells maintain mTORC2 signaling to drive cell proliferation and survival. Consequently, the replacement of fermentable fuels like glucose with non-fermentable fuels like ketone bodies becomes a logical approach. The vision must be targeting the cellular signaling pathways and metabolic reprogramming. Whatever the modality, a holistic and feasible approach must be developed.

Keywords: mitochondria, mitochondria-associated membranes, glioma, glioblastoma, metabolic reprogramming, mTORC2

1. Introduction

In the next lines, we do a brief journey through some aspects of gliomas, included epidemiological, clinical, neuroradiological, neuropathological, ultrastructural, therapeutics, and biologic behavior. An emphasis regarding the functional and therapeutics implications (metabolic therapy approach) of mitochondrial network (MN) and mitochondria-associated membranes (MAM) in astrocytomas is presented.

The MN has been implicated in the process of carcinogenesis, which includes alterations of cellular metabolism and cell death pathways. Defects in mitochondrial function have been suspected to play an important role in the development and progression of cancer [1].

Accumulating evidence indicates that MAMs are a subcellular "hot spot" for the intracellular signaling [2, 3]. Recent research has highlighted and broadened the functional roles of MAM in a variety of cellular processes from lipid synthesis/transport, Ca^{2+} signaling, and ER stress, to mitochondrial shape and autophagy/mitophagy and to inflammation and cell immunity [3, 4]. MAM dysfunction has been associated with several types of cancer [5]. Research from the past decade has identified the MAM as a potentially central regulator of tumor cell metabolism, as exemplified by the presence of critical tumor suppressors and oncoproteins on this structure [6]. The involvement of MAM in cancer has not been thoroughly investigated. Consequently, there is a huge open window for pathophysiological understanding and novel treatment modalities related to MN and MAM functions.

Recently, we provide evidence showing MN and MAM ultrastructural aspects in a range of human astrocytomas, including pilocytic astrocytoma diffuse astrocytoma, anaplastic astrocytoma, and glioblastoma [7–10]. Probably, this represents a contribution to the structural basis of functional roles of MN and MAM in astrocytic tumors as well as therapeutics implications.

2. Epidemiological and clinical aspects

Diffuse astrocytic tumors comprise approximately 60% of primary intracranial tumors. These tumors can arise at any age in children and the very elderly, although incidence increases substantially with advancing age. The median age is 30–40 for diffuse astrocytoma, 40–50 for anaplastic astrocytoma, and 50–60 years for glioblastoma. Older patients are also more likely to have higher grade gliomas, especially glioblastoma. The last one is the most frequent neoplasm in this category, accounting for approximately 80% of the diffusely infiltrative astrocytomas [11, 12].

The clinical presentation of the diffuse astrocytomas varies according to the sites of involvement and the rate of growth. The most common clinical symptoms are new-onset seizures, changes in behavior, motor deficits, and sing/symptoms of increased intracranial pressure (headache, nausea, vomiting, and papilledema). High-grade astrocytoma tend to have a short history with rapid progression, whereas low-grade astrocytoma are more indolent, often with insidious onset and a long, protracted clinical course [11, 12].

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3. Neuroimaging

Astrocytomas are most commonly seen on magnetic resonance imaging MRI as ill-defined, deep-seated, or predominantly subcortical cerebral hemispheric masses. MRI sequences, where signal hyperintensity reflects vasogenic edema generated in response to diffuse infiltration by individual tumor cells. Secondary signs of mass effect include midline shift, ventricular compression, and sulcal effacement. Glioblastoma commonly show a rim-enhancing pattern with a central low-density region of necrosis surrounded by irregular, variable thickness rim of contrast enhancement. This rim-enhancing component is always surrounded by T2- or FLAIR signal hyperintensity that represents an associated diffusely infiltrating neoplasm [11, 12] (**Figure 1**).

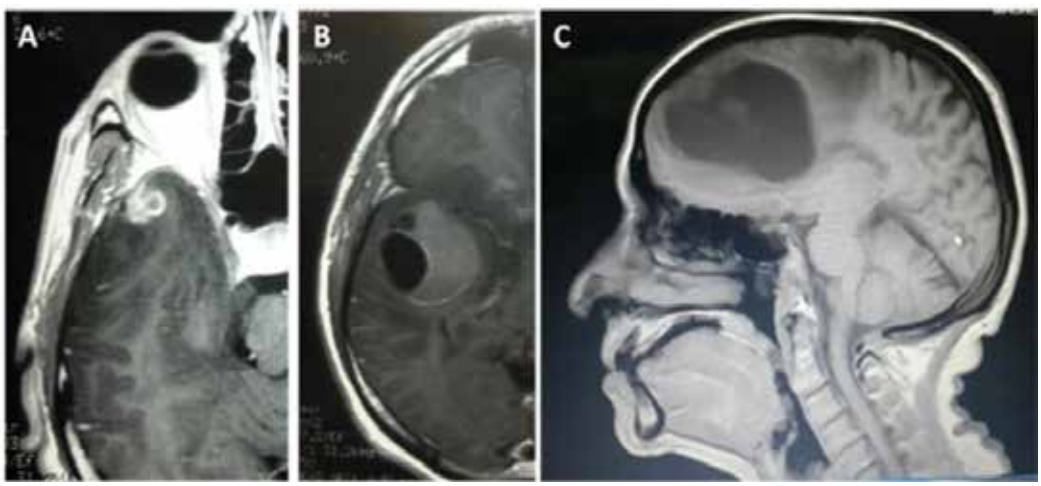

Figure 1. Glioblastoma MRI. (A) Initial MRI from a 50-year-old male patient with seizures and a temporal lobe glioblastoma. (B) The same patient three months later. (C) A huge frontal lobe giant cell glioblastoma from a 65-years-old female patient with changes in behavior.

4. Neuropathological aspects of gliomas

4.1. Gross pathology

Diffuse astrocytomas are ill-defined and subtly discolored, with secondary mass effects. These tumors are most often centered in the subcortical white matter but have a tendency to infiltrate widely and include the cerebral cortex, deep gray structures, and even the contralateral hemisphere. Glioblastoma are classically heterogeneous, with foci of necrosis, and hemorrhage [11, 12].

4.2. Histopathology

Gliomas constitute a heterogeneous group of primary central nervous system tumors. The term astrocytoma includes tumors with astrocytic differentiation. They may have a wide spectrum of

cell types in pure or mixed form. The classical tumor cells may show elongated, irregular hyperchromatic nuclei, often with no discernible cytoplasm, and embedded in a dense fibrillary matrix, mixed with cells that display visible eosinophilic cytoplasmic processes. However,cellular diversity, such as gemistocytic cell, protoplasmic cell, sarcomatous cell, epitheliod cell, granular cell, giant cell, or small cell is eventually observed. Glioblastoma display microvascular hyperplasia and tumor necrosis (pseudopalisading areas or infarct-like areas) [11, 12] (**Figure 2**).

The infiltrative or diffuse forms of astrocytoma are composed of individual tumor cells that infiltrate widely throughout the brain parenchyma with a cellular density and degree of anaplasia that increase with tumor grade. They are characterized by invasive growth such that nonneoplastic cells are often intermixed and may even predominate in some areas. The secondary structures of Scherer include subpial condensation, perineuronal satellitosis, and perivascular aggregation. The extreme end of the infiltrative spectrum, previously assigned as gliomatosis cerebri; it involves multiples lobes of the brain, often bilaterally and frequently extending into the brain stem, cerebellum, and even the spinal cord [11, 12].

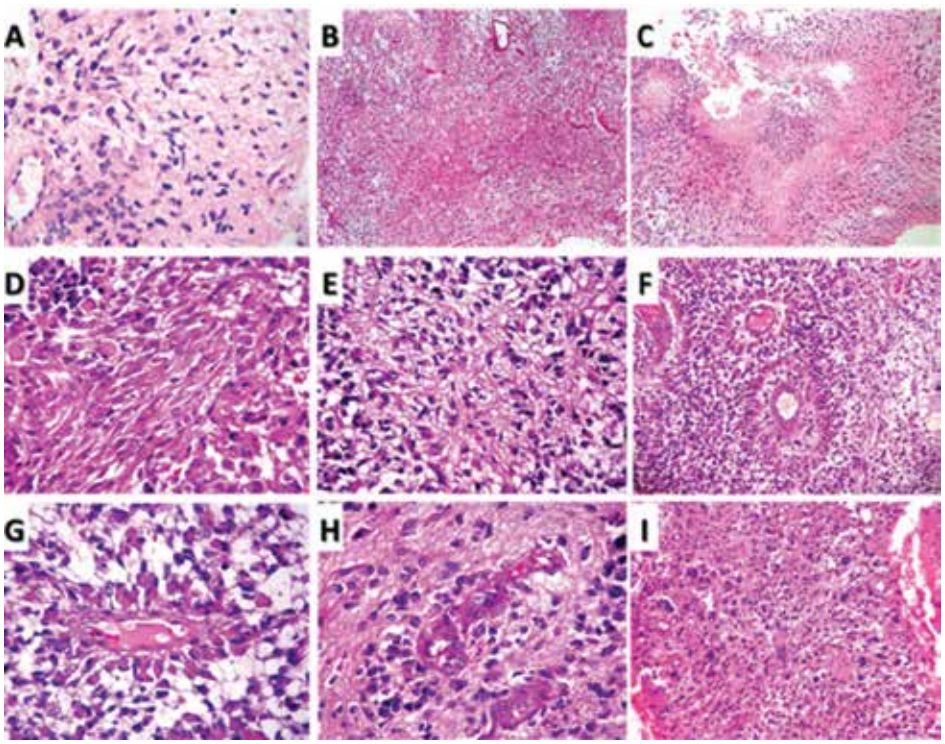

Figure 2. Histopathology. (A) Diffuse astrocytoma. Tumor cells show elongated, irregular hyperchromatic nuclei, with no discernible cytoplasm and embedded in a dense fibrillary matrix, mixed with cells that display visible eosinophilic cytoplasmic processes. (B) Glioblastoma displays a hypercellular-solid neoplasm with fuzzy or ill-defined margins, with diffuse parenchymal infiltration. (C) Glioblastoma: a pseudopalisading necrosis area. (D) Glioblastoma: an epitheliod-like cell area. (E) Glioblastoma: hypercellularity, tumor cells show elongated, irregular hyperchromatic nuclei, with no discernible cytoplasm and embedded in a dense fibrillary matrix, mixed with cells that display visible eosinophilic cytoplasmic processes. (F and G) Glioblastoma: cooption blood vessels surrounded by tumor gemistocytic cells. (H) Glioblastoma displaying microvascular hyperplasia. (I) Giant-cell glioblastoma corresponding to **Figure 1C**.

4.3. The 2016 World Health Organization classification of tumors of the central nervous system

According to the 2016 World Health Organization classification of tumors of the central nervous system [13], the diffuse gliomas include the WHO grade II and grade III astrocytic tumors, the grade II and III oligodendrogliomas, the grade IV glioblastomas, as well as the related diffuse gliomas of childhood. Then, all diffusely infiltrating gliomas (whether astrocytic or oligodendroglial) are grouped together: based not only on their growth pattern and behaviors but also more pointedly on the shared genetic driver mutations in the *IDH1* and *IDH2* genes. This approach leaves those astrocytomas that have a more circumscribed growth pattern, lack IDH gene family alterations, and frequently have *BRAF* alterations (pilocytic astrocytoma, pleomorphic xanthoastrocytoma) or *TSC1*/*TSC2* mutations (subependymal giant cell astrocytoma) distinct from the diffuse gliomas.

The WHO grade II diffuse astrocytomas and WHO grade III anaplastic astrocytomas are now each divided into IDH-mutant, IDH-wildtype, and NOS categories. It is recommended that WHO grading is retained for both IDH-mutant and IDH-wildtype astrocytomas, although the prognosis of the IDH-mutant cases appears more favorable in both grades.

Glioblastomas are divided into: (1) glioblastoma, IDH-wildtype (about 90% of cases), which corresponds most frequently with the clinically defined primary or de novo glioblastoma and predominates in patients over 55 years of age [14]; (2) glioblastoma, IDH-mutant (about 10% of cases), which corresponds closely to so-called secondary glioblastoma with a history of prior lower grade diffuse glioma and preferentially arises in younger patients [14];and (3) glioblastoma, NOS, a diagnosis that is reserved for those tumors for which full IDH evaluation cannot be performed.

5. Biologic behavior

Today, gliomas still represent a serious and discouraging brain tumor; despite the diversity of treatment modalities, generally, the prognosis for patients is still poor (i.e., fatality and sequelae). Even with surgical resection and aggressive treatment with chemotherapy and radiotherapy, the prognosis for patients with astrocytomas remains very poor [15].

6. The mitochondrial network, mitochondria-associated membranes, glioma ultrastructural pathology, and their functional and therapeutic implications

Both the endoplasmic reticulum and mitochondria are highly dynamic organelles, forming networks that may undergo rapid changes in the size, length, and shape, depending on metabolic and Ca^{2+} buffering needs, or in response to different cellular insults [16].

6.1. Mitochondrial network

Ultrastructurally, mitochondrion is an organelle constituted by a peripheral and inner membrane. The peripheral membrane encloses the entire contents of the mitochondrion, and internal membrane forms a series of folds, called cristae, which project inward toward the interior space of the organelle. The area between the peripheral and inner membranes is designated as intermembrane space, and the area enclosed by the internal membrane is labeled as a mitochondrial matrix. Functionally, the outer membrane includes the apoptosis antagonists and agonists and fission/fusion mitochondrial proteins. The inner membrane contains all the respiratory enzyme complexes and the three electron transporters, necessary for oxidative phosphorylation. In major mammalian tissues, 80–90% of ATP is generated by mitochondria in the process of oxidative phosphorylation [17, 18]. The mitochondrial matrix contains the enzymatic system of β-oxidation and tricarboxylic acid cycle. Mitochondria in living human cells display large, elongated and branched structures, actually entitled as mitochondrial network, extending throughout the cytosol and in close contact with the nucleus, the endoplasmic reticulum, the Golgi complex and the cytoskeleton, and is continually remodeled by both fusion and fission events [19].

In some cell types, mitochondria exist as single and randomly dispersed organelles; in other cells, mitochondria may also exit as dynamic networks that often changes shape and subcellular distribution. Depending on the cell type, mitochondria localized in different site-specific regions of a cell may display dissimilar morphology and biochemical properties [20].

6.2. Mitochondria-associated membranes

MAM is a membranous and protein structure (inter-membranous structure) composed by three pieces: (1) endoplasmic reticulum membrane; (2) mitochondrial membrane (outer mitochondrial membrane); and (3) tethers (proteins). Consequently, it displays biological membranous processes such as molecules trafficking and signaling events.

To date, MAM is considered as a fundamental cellular structure tightly regulated and with multifaceted roles that include Ca^{2+} signaling, lipid synthesis and exchange, metabolic control, and others. MAM formation might depend on several factors relating to differences in cell demands or microenvironment stimuli [2, 21].

6.3. Mitochondrial network and mitochondria-associated membranes abnormalities in human astrocytomas

Regards to the mitochondrial network, lucent-swelling mitochondria with disarrangement and distortion of cristae and partial or total cristolysis is predominant in the astrocytoma cells. In a minor proportion of astrocytoma cells, the presence of mitochondria with dense matrix displayed in closed groups exists [7–10].

Considerable variations in MAM ultrastructure is observed in the glioma tissue with respect to density, length, and width of the interfacing ER and mitochondrial membranes (**Figure 3**). In some astrocytoma cells, the MAM displayed a network or “work station”

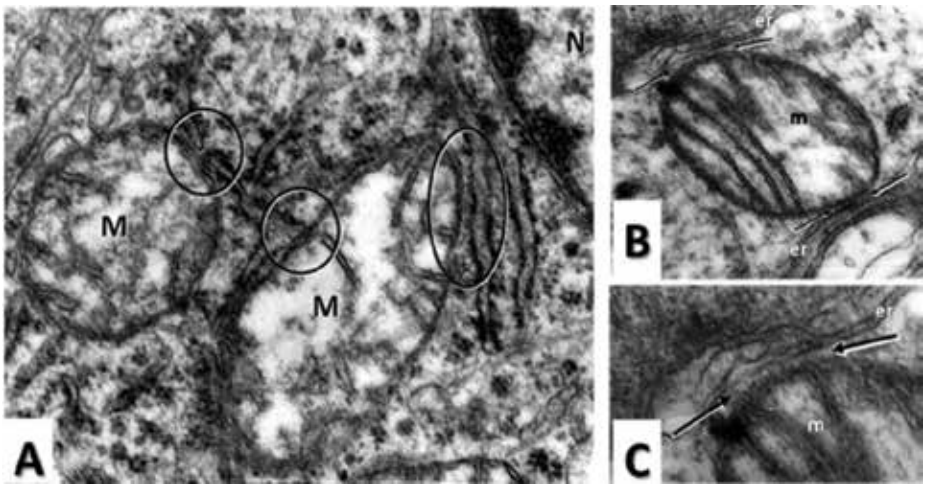

Figure 3. (A–C) Glioblastoma cells displays variable organization of endoplasmic reticulum membrane associated with mitochondria (circles, ellipses, and arrows). M/m denotes mitochondria; er: endoplasmic reticulum profiles. N: cellular nucleus. Lucent-swelling mitochondria with disarrangement and distortion of cristae, and partial or total cristolysis, are seen.

(an area with high density of MAM and predicted the functional activity). Close or direct association (mitochondria-endoplasmic reticulum interface <30 nm) and detached or disrupted (>30 nm) associations is present. The shortest span of MAM was 96 nm, and the longest was 652 nm [10].

In the ultrastructural perspective, we identified two remarkable cell types: (1) poorly differentiated glioma stem cells and (2) well-differentiated glioma cells. The first one exhibits a poorly developed mitochondrial network and scarce MAM (named by us "MAM-deficient cells"). The second contains a well-developed MN and numerous MAM (named by us "MAM-enriched cells"). MAM displayed a network or "work station" in some well-differentiated glioma cells [10] (**Figure 4**).

Previously, we suggest that the MAM could be involved in the invasive properties of glioma cells. Human glioma cell invadopodia show mitochondria with a dense matrix condensed configuration, indicating an active state. The mitochondria were frequently in close contact with an extended smooth endoplasmic reticulum displaying an endoplasmic reticulum subfraction associated with mitochondria MAM. Fluorescent microscopy confirmed that D54 and U251 glioma cells growing in vitro also contained filopodia with mitochondria (**Figure 5**). The U251 glioma cells' filopodia that penetrated through 1.2-μm pores of transwell chambers also contained mitochondria, suggesting that the mitochondria are actively involved in the invasion process [9].

In the vascular microenvironment components of gliomas, the mitochondrial network exhibit similar changes to describe in tumoral cells. The mitochondria display mainly two patterns: (1) swelling associated with disarrangement of cristae and partial or total cristolysis and (2) condensed configuration [8].

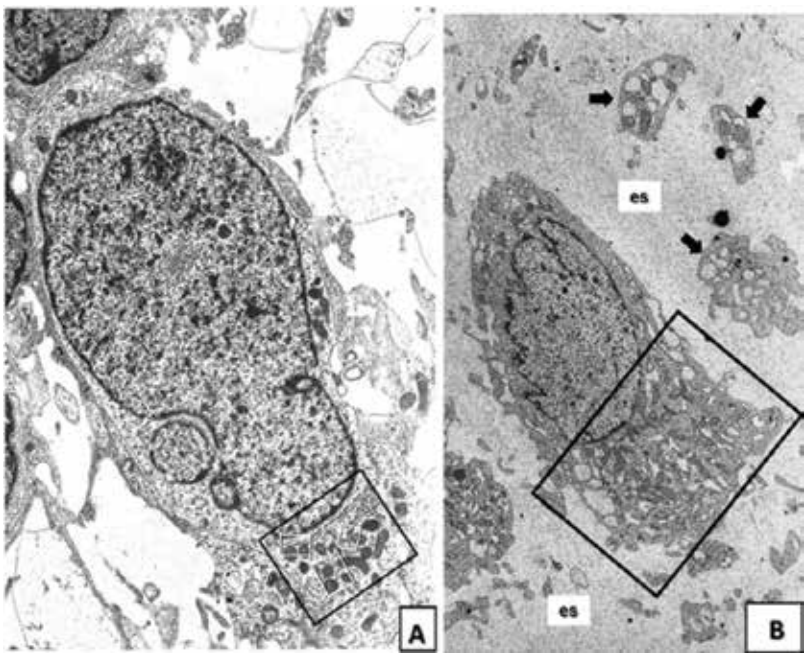

Figure 4. (A) Glioma like-stem cell exhibited, adjacent to nuclei, an endoplasmic reticulum an endoplasmic reticulum profile, and a small amount of electron-dense mitochondrion displayed a "MAM network" (black rectangle) with six direct interorganellar close associations with small span (white rectangles). (B) Well-differentiated tumor cell displays electron-lucent mitochondrion (m) in close association with multiple endoplasmic reticulum profiles establishing multiple MAM (rectangle) conforming a huge "MAM network". Similar fashion is observed in three cellular processes (arrows); es: denotes extracellular space.

6.4. Functional and therapeutics implications

In the case of astrocytomas, the dense mitochondria could be capable of producing energy by oxidative phosphorylation, and lucent-swelling mitochondria with disarrangement and distortion of cristae and partial or total cristolysis are incapable of generating energy by oxidative phosphorylation. Possibly, the astrocytoma cells that hold dense mitochondria are able to generate sufficient ATP concentration by oxidative phosphorylation. In contrast, the astrocytoma cells that contain lucent swelling mitochondria with disarrangement and distortion of cristae and partial or total cristolysis are incompetent to produce an adequate amount of ATP by mitochondrial respiration. These findings suggest that the majority of astrocytoma cells are incompetent to produce an adequate amount of energy by means of oxidative phosphorylation [7–9]. The glycolytic inhibition and inhibition or down-regulation of mitochondrial respiration would be a potential tool for future therapeutic strategies in cases of human astrocytic tumors.

Mitochondria are present at the invadopodia and their apparent function appears linked with the ROS generation and subsequent activation of several pathways essentials for glioma invasiveness. Mitochondria are a major source of ROS, which occurs mainly at complexes I and III of the respiratory chain. In cancer cells, mitochondria can generate ROS and redox signals, specifically via an increase in the NAD^+/NADH ratio [22]. H_2O_2 induces Akt (protein kinase B) activation, and their pathway is redox regulated. Akt activation correlated with the

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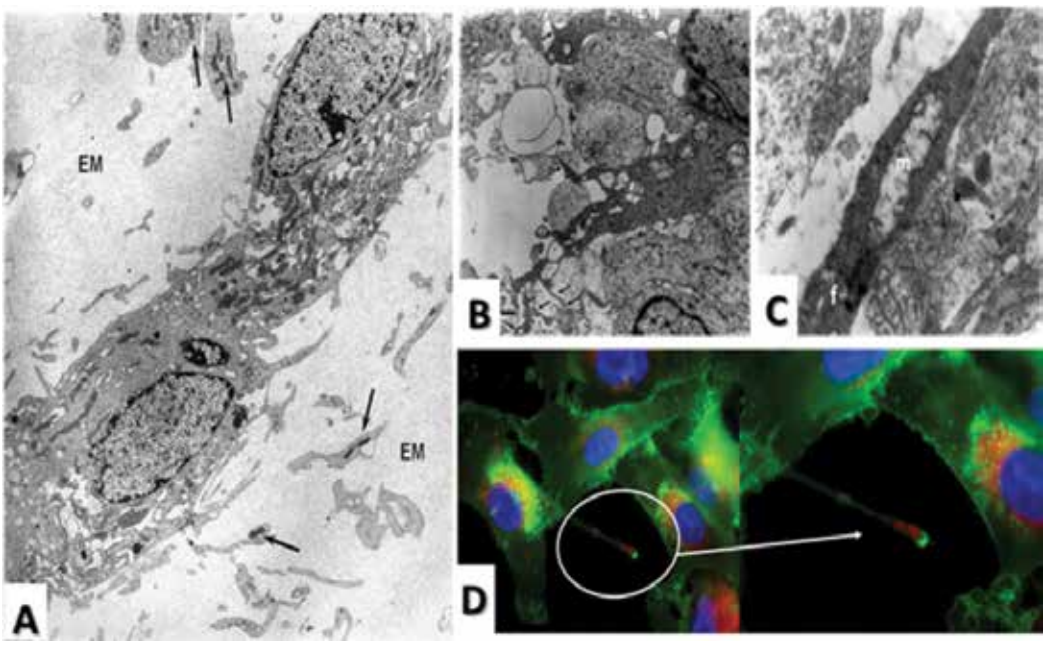

Figure 5. (A) Two glioblastoma multiforme cells exhibit several invadopodia that contain mitochondria with dense matrix condensed configuration (arrows). The cytosol shows multiple mitochondria with similar morphologies and physically adjacent to distended endoplasmic reticulum MAM. EM: denotes extracellular matrix. (B and C) Glioma cells filopodias (f). M denotes: mitochondria; * designates: dilated endoplasmic reticulum cystern; arrows indicate: filiform projections. (D) Under fluorescent microscopy, U251 glioma cells stained with MitoTracker Red (label the mitochondria). The mitochondria are in the filopodia (circle and arrow). Green: actin filaments. Blue: nuclei. (personal communication and courtesy from Martin R. Jadus, Diagnostic & Molecular Health Care Group, Veterans Affairs Medical Center, Long Beach, California, USA, Neuro-Oncology Program, Chao Comprehensive Cancer, University of California–Irvine, Orange, California, and USA; and Pathology and Laboratory Medicine, Med. Sci. I, University of California, Irvine, California, USA).

increased tumorigenicity, stem cell-ness, and invasiveness of invasive glioblastoma cells [23]. Molina et al. [23] reported that the glioma cells with high Akt activation actively invaded the surrounding parenchyma along blood vessels and with matter tracts. In human astrocytomas, the co-option vessel shows invadopodia with mitochondria that display dense matrix condensed configuration [9]. This finding possibly represents the ultrastructural basis of the molecular process expressed above, which permits the invasiveness of glioblastoma cells. On another hand, the PI3K-Rac and PI3K-3/Akt pathways are involved in the production of ROS that accumulates at the membrane ruffles [24], ROS production stimulates cytoskeletal reorganization required for a migratory response. Migrating glioma cells show activation of the PI3K/Akt pathway, and PI3K inhibitors have been tested experimentally, resulting in a decrease in migration [25]. Therefore, Inhibition of mitochondrial ROS generation may represent another important therapeutic target to most gliomas.

The degree of development of MN and quantity of MAM could be linked to the functional or metabolic state of the different tumor cells found in human astrocytic tumors. Then, the well-differentiated glioma cells (or "MAM-enriched cells") could be more active in these processes than the poorly differentiated glioma stem cells (or "MAM deficient cells") [10]. A recent study

showed that glioma stem cells are less glycolytic than differentiated glioma cells, consuming lower levels of glucose, and producing lower amounts of lactate while maintaining higher ATP levels compared with their differentiated progeny [26]. Another study, by means of transmission electron microscopy, analysis revealed that the number of mitochondria with distinct cristae and electron-dense matrices increased significantly in the non-stem differentiated glioma cells when compared to their undifferentiated glioma stem cells. The final conclusion was that glioma stem cells prefer a relatively higher glucose metabolism, which implies that they utilize different mitochondrial biosynthesis and metabolic pathways when compared to differentiated glioma cells [27]. Other research established that glioma stem cells displayed diminished endoplasmic reticulum-mitochondria contacts compared to glioma differentiated cells. Forced endoplasmic reticulum-mitochondria contacts in glioma stem cells increased their cell surface expression of sialylated glycans and reduced their susceptibility to cytotoxic lymphocytes. The final conclusion was that endoplasmic reticulum-mitochondria contacts control surface glycan expression and sensitivity to killer lymphocytes in glioma stem-like cells [28].

The length of the interface is changing under different biochemical conditions [29, 30]. Apparently, the execution of the physiological programs is dependent on the length of the MAM, since the structural plasticity of the MAM cleft accompanies changes in cell metabolism [29]. Changing the thickness of MAM would impact on the activity of several enzymes of the Krebs cycle and on the strength of the IP3R Ca^{2+} signaling pathway [30]. Furthermore, the variability of the ultrastructural aspects observed on astrocytic tumors suggests a dynamic regulation of the interorganellar junction that can be modified by functional requirements needed to adapt to different cell demands. Solid and glycolytic tumor tissue is frequently characterized by a loss of normal MAM architecture and formation [6]. Today, altered Ca^{2+} signaling at the MAM is recognized as a hallmark of cancer cells that shifts their metabolism to glycolysis and increases their resistance to cell death [31]. MAM-resident mTORC2 controls the MAM integrity and mitochondrial functions [4, 32] and is the core of MAM signaling hub that controls growth and metabolism. Recent studies suggest that mTORC2 can promote glioblastoma growth and chemotherapy resistance in cancer cells as well as controlling genome stability and tumor metabolism including glycolysis, glutaminolysis, lipogenesis, and nucleotide and reactive oxygen species metabolism [33]. Glucose is required to activate mTORC2 and promote tumor growth [33] by means an auto-activation loop of mTORC2, rendering glioblastoma resistant to EGFR, PI3K, or AKT-targeted therapies. Then, if sufficient nutrients are present, glioblastoma cells maintain mTORC2 signaling to drive cell proliferation, and survival [33, 34]. mTOCR2 markedly increases glycolysis in glioblastoma [33]. Consequently, replacement of fermentable fuels like glucose and glutamine with non-fermentable fuels like ketone bodies becomes a logical approach to management [35, 36]. The dietary intervention prevents glioma cells accessing their preferred fuel source, i.e., glucose [37–40], and consequently, the signal transduction of mTORC2, cell proliferation and survival are diminished [35]. Therefore, impairments in glucose availability can be devastating for glioma survival [26].

The current standard of care for glioblastoma patients consists of maximal safe resection, followed by radiotherapy, and concurrent chemotherapy with Temozolomide [15, 41, 42]. Despite substantial clinical research efforts over the past decades, therapeutic progress

has been marginal [43]; added benefits from Temozolomide [44] and bevacizumab [45] are modest, and patient overall survival remains poor. Increasing recognition of the metabolic peculiarities of cancer has prompted investigations of nutritional strategies targeting glycemic modulation in cancer treatment, predominantly through the use of high-fat and low-carbohydrate diets (ketogenic diets; KDs), but also caloric restriction (CR), intermittent fasting (IF), and other combinatorial dietary protocols [46, 47]. All of these strategies induce a physiological state of systemic ketosis that metabolically compensates for the therapeutic reduction of carbohydrate intake and a concurrent decrease in blood glucose levels [47]. Both glycemic reduction and systemic ketosis are established key metabolic correlates of these nutritional strategies and are thought to mediate their therapeutic efficacy [35, 47]. The reduced availability of glucose as an energy substrate has been shown to selectively starve glioma cells both in vitro and in vivo [48–54]. Glioma cells are metabolically maladapted to utilize ketone bodies [48, 52, 55]. Unlike highly selective pharmacological blocking agents, KMT might produce a global dampening of insulin-related signaling with potentially more efficacy and less side effects [56]. On a functional level, several preclinical studies could demonstrate that ketogenic metabolic therapy (in particular, KD treatment, and/or CR) induces a metabolic shift in malignant brain tissue toward a proapoptotic, antiangiogenic, anti-invasive, and anti-inflammatory state accompanied by a marked reduction in tumor growth in vivo [57]. According to the current literature, ketogenic metabolic therapy is a safety and feasible alternative for malignant glioma. Cumulative clinical trials suggest that ketogenic metabolic therapy is emerging as a potential therapeutic option and might be combinable with existing anti-neoplastic treatments for malignant glioma [57]. Recently, a press-pulse therapeutic strategy for cancer management was presented [58]. The press-pulse therapeutic strategy for cancer management is illustrated with calorie-restricted ketogenic diets used together with drugs and procedures that create both chronic and intermittent acute stress on tumor cell energy metabolism, while protecting and enhancing the energy metabolism of normal cells. Optimization of dosing, timing, and scheduling of the press-pulse therapeutic strategy will facilitate the eradication of tumor cells with minimal patient toxicity. This therapeutic strategy can be used as a framework for the design of clinical trials for the non-toxic management of most cancers [58].

7. Conclusions

There is a great need to develop new therapies for gliomas. The ultrastructural findings observed in MN and MAM in the human gliomas indicate that: (1) The majority of glioma cells are incompetent to produce adequate amount of energy by means of oxidative phosphorylation and compensatory increases in glycolytic ATP production and (2) The variability of the ultrastructural aspects of MAM observed on astrocytic tumors suggests a dynamic regulation of the interorganellar junction that can be modified by functional requirements needed to adapt to different cell demands. These findings possibly represent the ultrastructural basis of the metabolic processes of glioma cells. MAM-resident mTORC2 controls the MAM integrity and mitochondrial functions, and mTORC2 can promote growth and chemotherapy resistance in cancer cells as well as tumor metabolism including glycolysis, glutaminolysis,

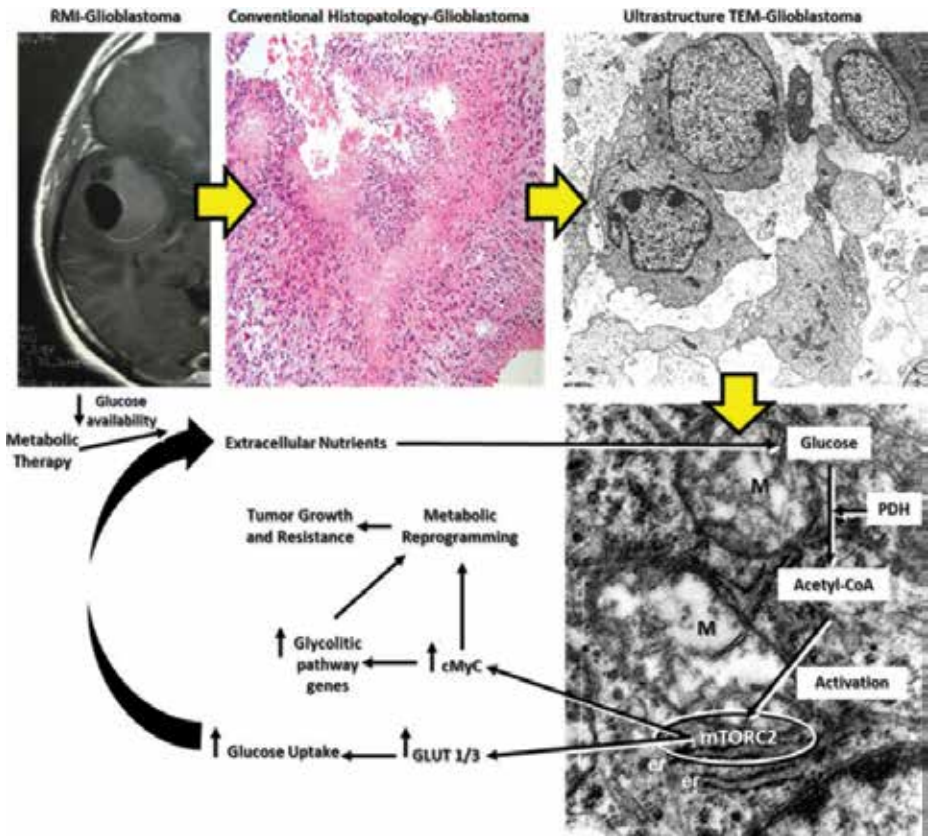

Figure 6. General visualization of the glioma pathology, metabolic aspects and, their metabolic therapy approach. Glucose derived from extracellular nutrients is required to activate mTORC2 and promote tumor growth and resistance. Glucose is converted in acetyl-CoA for the pyruvate deshydrogenase (PDH) action. Acetyl-CoA produces the activation of mTORC2 by acetylation of RICTOR. mTORC2 signaling facilitates the metabolic reprogramming, tumor growth, and resistance. This is a nutrient availability-dependent process, by means an auto-activation loop of mTORC2. The metabolic therapy approach, limit the availability of glucose and consequently, the signal transduction of mTORC2, cell proliferation, and survival are diminished (ellipse denotes MAM).

lipogenesis, and nucleotide and reactive oxygen species metabolism. Considering that therapeutic progress has been marginal, ketogenic metabolic therapy in the context of the press-pulse therapeutic strategy is emerging as a potential therapeutic option (**Figure 6**).

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Chapter 3

Receptor Tyrosine Kinase Interaction with the Tumor Microenvironment in Malignant Progression of Human Glioblastoma

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.76873

Abstract

Glioblastoma (GBM) is the most malignant brain tumor, characterized with a rapid progression and poor prognosis despite modern therapies. Receptor tyrosine kinase (RTK) is a membrane tyrosine kinase that could be activated by binding ligands with the extracellular domain, and communicating signals according to the tyrosine kinase activity of the intracellular domain. Recent studies revealed that RTKs such as EGFR, PDGFR and MET play key roles in cancer progression through regulation of abundant cellular processes. As transmembrane proteins, RTKs work as a mediator between the extracellular environment and intracellular compartments, translating the tumor microenvironment (TME) signals into the tumor cells. TME is also a critical regulator for the malignant process, lately receiving considerable attention. It is composed of extracellular matrix (ECM), the stromal cells (i.e., endothelial cells, microglia and fibroblasts), secreted factors, and hypoxia environment, etc. Among these, the strong invasion and sustained angiogenesis of GBM are closely related to ECM-receptor interaction and -associated signaling events. In this chapter, we consider the interaction and mechanisms of RTKs and TME in GBM progression, especially the role of ECM-receptor mediated signaling in tumor invasion, hypoxia and angiogenesis, glioma stem cells and tumor metabolism. We then summarize and discuss recent improvements on the approaches of targeting RTK and TME as the therapy in the primary GBM.

Keywords: glioblastoma, receptor tyrosine kinase, tumor microenvironment, extracellular matrix, focal adhesion complex, signal transduction, invasion, angiogenesis

1. Introduction

Glioblastoma (GBM) is the most common and aggressive primary brain tumor in adults. Recently, based on mutations in the gene encoding isocitrate dehydrogenase enzyme 1/2(IDH1/2), GBMs were separated into three main groups (2016 WHO classification of CNS tumors): (1) IDH-wild-type GBMs (about 90% of cases); (2) IDH-mutant GBMs (about 10% of cases); and (3) not otherwise specified (NOS) GBMs. Among these, IDH-mutant phenotype is strongly associated with secondary GBM, younger age, and better outcome, while IDH-wild-type with primary GBM. Typical molecular alterations in primary GBM include mutations in genes regulating receptor tyrosine kinase (RTK)/rat sarcoma (RAS)/phosphoinositide 3-kinase (PI3K), p53, and retinoblastoma protein (RB) signaling.

There have been identified approximately 58 mammalian RTKs, which contain an intracellular catalytic protein tyrosine kinase domain and regulatory sequences, transmembrane domain, and an extracellular ligand-binding domain [1]. In response to environmental cues, RTKs are crucial regulators of the growth factor signaling that controls cellular processes including proliferation, metabolism, survival, etc. RTK activation triggers complex signaling network through Ras/Raf/MEK/ERK, PI3K/Akt and other intracellular pathways in both physiological and pathological conditions; RTK dysregulation through mutation and amplification often occurs in a wide range of cancers including GBMs. RTKs such as epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR), c-Met, Tie, Axl, discoidin domain receptor 1 (DDR1), erythropoietin-producing human hepatocellular carcinoma (Eph) and others play a major role in human GBM pathobiology [2]. Therefore, RTK-targeted agents including tyrosine kinase inhibitors and antibodies are currently used in preclinical and clinical settings in cancers including GBM.

The tumor microenvironment (TME) in malignant glioma is a dynamic entity that consists, besides glioma cells [including glioma stem cells (GSCs)], of an intricate network that encompasses various cell types (e.g., endothelial cells, astrocytes, microglia, and pericytes), stromal components, soluble factors, as well as the extracellular matrix (ECM) [3, 4]. Together, these TME elements play an important role in facilitating the integration of tumor cells with their surrounding environment maintaining features of tumor malignancy [3]. Initially, tumor cells actively exploit their stromal environment through the recruitment of nonmalignant cells and elements that may provide physiological resources to facilitate rapid tumor growth. In time, these recruited cells become a major source of secreted factors to mobilize further inflammatory cells into the microenvironment until the entity becomes steady and strong to progression [5]. In the meantime, rapid proliferation of the malignant cells per se has a metabolic effect on the TME, which is rapidly deprived of glucose and oxygen, becoming acidic and hypoxic [6]. Overall, both tumor cells and the TME are adaptive and undergo evolution from time to time during tumor progression. Human brain tumor bears unique TME in that the tumor rarely metastasizes to other parts of the body [7]. Currently, almost in each type of cancer, TME has drawn much attention regarding the mechanisms of cancer biology and novel therapeutic strategies.

In this chapter, we consider the interaction and role of RTKs and TME during GBM progression; especially their close interactions in GBM biology and targeted therapies. We then discuss recent improvements on approaches of targeting RTKs and TME mainly in primary GBM with IDH-wild-type.

2. RTK activation is a hallmark of malignant glioma

2.1. Genetic alterations of RTK in primary GBM

Aberrant RTK activation frequently occurs during glioma initiation and progression and that the associated activation cascades may cooperate through multiple signaling cross-talks in the malignant transformation of cells, tumor growth and progression, treatment resistance, and disease relapse. In 2008, the Cancer Genome Atlas project (TCGA) reported significant alterations in three core signaling pathways, including RTK/RAS/PI3K (88%), p53 (87%), and retinoblastoma protein (78%), in the collected samples from patients with primary GBM, which may represent the majority of human GBM [8]. 60% of the primary GBM harbors RTK amplifications and/or mutations, among them, EGFR amplifications and/or mutations were observed more than 50% of the disease. About half of GBM with EGFR amplification had an in-frame deletion of exons 2–7 from the extracellular ligand-binding domain of EGFR resulting in a mutant protein with ligand-independent receptor activity (designated delta-EGFR or EGFRvIII) [9]. Therefore, EGFRvIII is commonly expressed in a subset of EGFR-amplified cells. Only a small portion (7%) of the tumor showed EGFR genetic alterations in combination with other RTK lesions. Amplification of platelet-derived growth factor receptor alpha polypeptide (PDGFRA) occurs in 13% of GBMs; ErbB2 (HER2/Neu) belongs to the EGFR receptor family that includes the other three members: EGFR, ErbB3, and ErbB4. Activation of ErbB2 depends on the patterns of dimerization within other family members [10]. ErbB2 mutation was observed in 8% GBM tested in a TCGA study [11, 12]. MET amplifications and fibroblast growth factor receptor (FGFR) mutations, including fusion genes, occur in about 2% of the GBMs [1]. Additionally, overexpression of ligand and/or receptor and co-expression of both (autocrine loop formation) are frequent events in cancers, including GBM, and many have been associated with increased malignancy and worse patient outcome.

2.2. Cooperation of RTKs and their downstream signaling pathways

RTK alterations usually coexist with mutations that activate other core regulatory pathways, including intracellular Ras/MAPK and PI3K/Akt pathways, as well as tumor suppressor pathways in certain types of GBM. Furthermore, the frequent co-occurrence of mutations in *PI3K* and deletion of *PTEN*, in addition to the co-occurrence of mutations and/or deletion of cyclin-dependent kinase inhibitor 2A (CDKN2A; encoding both INK4A and ARF) were observed within all of the detectable RTK alterations in primary GBM. This is consistent with the required cooperation of multiple core pathways for tumor formation in genetically engineered mouse models of GBM. Besides, phosphorylated tyrosine kinases of RTK provided PLC-γ1 docking sites for PLCG1 SH2 domains, leading to phosphorylation of tyrosine kinases on PLC-γ1 and signaling activation pathways [13, 14]. JAK/STAT3 signaling was reported associated with EGFR and EGFRvIII signaling [15].

Given that individual tumor cells express multiple RTKs, it is reasonable to speculate that these RTKs are actively interacting with each other. For example, the phosphorylation of c-Met receptor is strongly correlated with functional levels of EGFRvIII, suggesting the presence of cross-talk between these two RTK signaling, although the intermediary molecules were not elucidated [16]. The Axl RTK follows a similar phosphorylation response as a function of EGFRvIII levels [16]. EGFRvIII expressed in glioma cells stimulates upregulation of TGFα and

HB-EGF, which stimulate in turn wild-type EGFR forming an autocrine loop [17]. It was previously reported that EGFR and EphA2 are both expressed in GBM cells and co-localize to the cell surface. EphA2 phosphorylation is dependent on EGFR activity, and EphA2 downregulation inhibits EGFR phosphorylation, downstream signaling, and EGF-induced cell viability [17]. HGF indirectly activates alternative RTKs such as EGFR by upregulating expression of EGFR ligands such as TGF-α and HB-EGF [2]. Previous studies report that EphA4, whose expression is correlated with increasing glioma grade, forms a heteroreceptor complex with fibroblast growth factor receptor 1(FGFR1) in glioma cells and that the EphA4-FGFR1 complex potentiated FGFR-mediated downstream signaling such as Akt/MAPK, Rac1, and Cdc42 pathways, resulting in the promotion of invasion [18]. A few other reports suggest that Tie2 activation regulates angiogenesis in a highly context and tissue-dependent manner and closely collaborates with VEGF and other angiogenesis regulators [19, 20].

2.3. Heterogeneity of RTK expression within the TME

Human GBM is characterized with high degrees of intertumoral and intratumoral heterogeneity. For example, individual GBM tumors display striking histological variations. As a hallmark of GBM development, oncogenic RTK activation is highly responsible for malignant behaviors of multiple cells in the TME other than GBM cells, that is, endothelial cells, epithelial cells, astrocytes, infiltrated immune cells, glioma stem cells (GSC), etc. [2]. The malignant grade in human astrocytoma was associated with an upregulation of the PDGFRβ on vessel endothelial cells indicating the role of paracrine activation in tumor angiogenesis [21, 22]. Besides EGF, five other respective ligands activate EGFR including transforming growth factor alpha (TGF-α), amphiregulin, betacellulin, heparin-binding EGF-like growth factor (HB-EGF), and epiregulin, respectively. These ligands are secreted by glioma cells and received by tumor microenvironmental cells such as microglia and reactive astrocytes [2]. Axl/Gas6 signaling has multiple functions to regulate survival, proliferation, and migration in a variety of cells in vitro including tumor-derived cell lines of epithelial, mesenchymal, and hematopoietic origin [23]. Moreover, the Eph/ephrin system plays a role in many biological processes such as cell adhesion and migration during development, especially in the central nervous system [24]. In glioma, different Eph receptors are overexpressed not only in tumor cells but also in the surrounding tumor-infiltrating cells like tumor-associated macrophages (TAMs) [25], endothelial cells, stromal cells [26], as well as GSCs [27].

Activation of RTK pathways can lead to cellular transformation and result in genetic alteration in GSCs. Fully differentiated neural cells were able to generate malignant glioma upon PDGFA overexpression and showed high expression of stem and progenitor cell markers [28]. Growth factors such as PDGF, bFGF, and EGF were usually added to the serum-free media to maintain properties of cancer stem cells derived from patient tumor biopsies [29]. HGF/c-Met pathway was involved in brain tumorigenesis and malignant progression, and thus, HGF/c-Met signaling may maintain GSC properties [30]. Moreover, RTKs show various regional expression pattern within tumor in situ during tumor progression, for example, histopathological analysis on in vivo human glioma biopsies showed that Ang-2, MMP-2, MT1-MMP, and laminin5γ2 are co-overexpressed in the invasive areas but not in the central regions of the glioma tissues [31]. GBM is characterized with the unique pattern showing that necrotic areas are typically surrounded by “pseudopalisading” glioma cells, which are highly

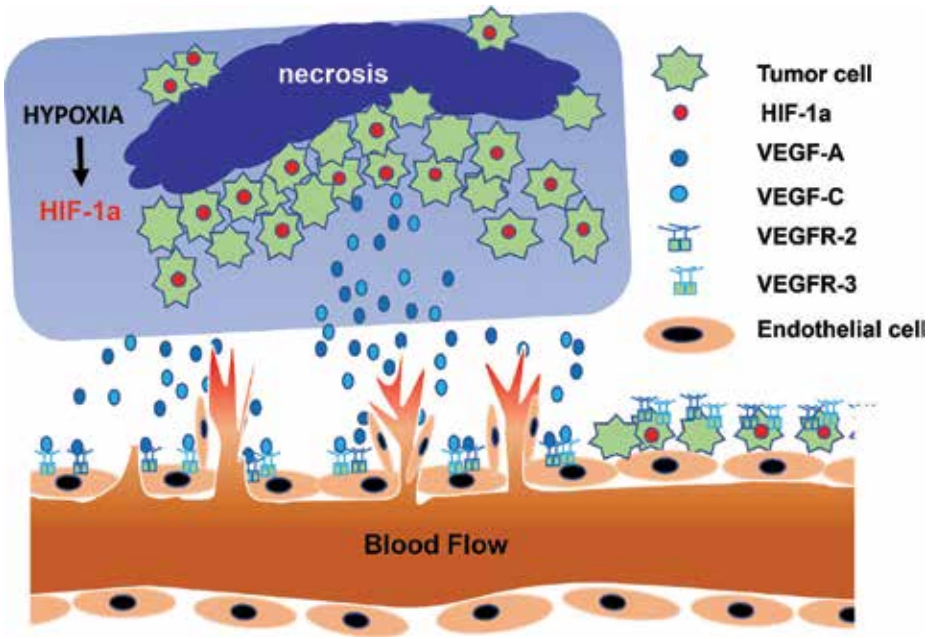

Figure 1. The hypoxic tumor cells stimulate neovascularization in GBM. Under hypoxic conditions, tumor cells secrete enhanced levels of VEGF family members (VEGF-A, VEGF-C). Endothelial cell-specific RTKs (VEGFR-2,VEGFR-3) via ligand (VEGF-A, VEGF-C) binding to stimulate proliferation and migration of endothelial cells. The peri-vascular regions contain glioma stem cells (GSCs).

hypoxic (**Figure 1**). Axl is predominantly expressed in the pseudopalisading cells, along with other markers such as VEGFR, etc. Furthermore, an accumulation of Axl positive tumor cells appeared adjacent to microvascular neoformations, which is a characteristic feature of invading glioma tumor cells spreading along perivascular regions [2].

3. Active interactions between RTK and TME

3.1. RTK, hypoxia and angiogenesis

As one of the most prominent features in human GBM, pseudopalisading necrosis, the area of hypercellularity surrounding necrotic regions, and associated active vascular proliferation and tumor invasion are driven by hypoxia [32, 33]. Tumor cells reside in these regions have a high expression of HIF-1α and release VEGF, which is one of the most important regulators of angiogenesis and neovascularization (**Figure 1**). VEGF family members signal predominantly through the cognate RTKs, VEGFR-1, VEGFR-2, and VEGFR-3, in association with the co-receptors [34] via both hypoxia-dependent and hypoxia-independent mechanisms. Moreover, pseudopalisading necrosis regions protect glioma stem cells (GSC) in the region from therapeutic agents, and this facilitates the GSC niche to expand and contribute to tumor growth [35]. HIF-1α is a transcription factor that regulates the expression of a variety of genes

involved in glycolysis, angiogenesis, invasion and epithelial-mesenchymal transition (EMT), which are critical for tumor growth and progression, and likely cooperate and activate other aberrant RTK signaling pathways [36, 37]. We and other reports showed that, in response to hypoxia condition, significantly increased activity of EGFR, as well as its mutant protein EGFRvIII, which further promoted activation of convergent downstream signaling pathways including Ras/MAPK, PI3K/Akt, JAK2/STAT3, and NF-κB signaling, and enhanced malignant behaviors in GBM cells in vitro, and most likely to act in the same way in vivo [1, 38].

In addition to VEGF, supplementary proangiogenic factors including FGF, PDGF, placenta-like growth factor (PLGF), integrins, HGF/scatter factor, ephrins, angiopoietins (ANGPT), and interleukin-8 (IL-8), matrix-metalloproteinase (MMP)-2, MMP-9, collagen type I α1 (COLIA1), endothelial markers CD34, Tenascin-C, neuron-glial antigen 2 (NG-2) on pericytes, insulin-like growth factor (IGF), and EGF present in GBM [39–42]. Interestingly, many of these factors are RTK ligands and may bind to respective RTK on vascular endothelial cells or GBM cells, act in autocrine or paracrine manner to stimulate the events of neo-angiogenesis. Activation of these proangiogenic factors interacts with a number of signaling pathways include activation of Ras/Raf/MAPK [41, 42], PTEN/PI3K/AKT [40], PLC-γ/protein kinase C (PKC) [40], nitric oxide (NO) [43], PDGFB [44], and Notch1 [45]. GBMs are diagnosed at the advanced stages when they show hypoxia and leaky vasculatures [35]. The critical role that VEGF and these pro-angiogenic factors play in angiogenesis has rendered them appealing targets to exploit in cancer therapeutics [43].

3.2. RTK and ECM/integrin signaling

ECM/ integrins are key components mediating the dialog between cells and the microenvironment. Integrins are composed of two noncovalently associated α and β subunits, which are featured by a large extracellular domain, a short transmembrane domain and a small intracellular noncatalytic cytoplasmic tail [46]. These receptors play a role in the regulation of cell adhesion to ECM proteins or cell surface proteins [47]. Binding of ECM to integrins result in cell adhesion and activation of focal adhesion (FA)-associated signaling pathways [48] and thereafter cascades of intensive activation of downstream signaling that involved in cell proliferation and invasion [49]. Within FA complexes, further auto-phosphorylation of focal adhesion kinase (FAK) leads to its binding to SRC kinase and formation of activated FAK-SRC complexes. Consequently, FAK-SRC complexes activate cascades of downstream pathways including the Ras/Raf/MAPK, RAF/JNK, Rho/Rac/PAK and PI3K/Akt/mTOR [50]. Notably, FAK protein is overexpressed in many tumors including GBM, and its expression level is greatly correlated with poor clinical prognosis [51, 52].

ECM dysregulation is essential for establishing and maintaining a functional tumor microenvironment. ECM in GBM is stiffer and more cross-linked than that in the normal brain tissue, inducing abnormal cell behaviors such as aggressive cell invasion [35]. Dysfunction of ECM and its cognate receptor integrin may lead to aberrant signaling transduction pathways including Ras/Raf/MAPK, Raf/JNK, Rho/Rac/PAK and PI3K/Akt/mTOR, shaping a tumor microenvironment to promote tumor survival, angiogenesis, and invasion [31]. Importantly, many of these cellular signaling pathways are convergent with downstream signaling pathways of RTKs, implicating interaction and cross-talk of RTK- and ECM/integrin-mediated function in GBM

invasiveness and aggressiveness [53]. We demonstrated that hypoxia tumor microenvironment and ECM vitronectin could enhance tumor cell invasion and EGFRvIII activity via EGFRvIII and integrin β3 complex, emphasizing key roles of TME in tumor progression and metastasis [54]. Furthermore, as ECM may act as a reservoir for multiple growth factors such as VEGF, EGF, PDGF and TGF-β, release of these factors and their binding to their cognate receptors may also converge and further strengthen the activation of these signaling cascades, leading to uncontrolled cell behaviors in tumor growth and survival, angiogenesis, and invasion [55].

Knockout studies show the role of integrins in overactive GBM angiogenesis, which highly depends on VEGF and bFGF [56], αvβ3, αvβ5, β1, and αvβ8 notably play an important role during the process. For example, endothelial cells-expressed αvβ3/αvβ5 can provide survival signals and traction for invading cells, which are necessary to angiogenesis [57, 58]. αvβ3/αvβ5-associated neovascularization is respectively dependent on tumor cell-secreted bFGF/TNFα and VEGF and involved in a process leading to active interaction between tumor cells and endothelial cells [59]. Overexpression of αvβ3/αvβ5 in endothelial cells facilitates adhesive interactions with ECM proteins such as vitronectin, fibronectin, fibrinogen, osteopontin, etc. In cooperation with bFGF/VEGF, αvβ3/avβ5 also activates signaling pathways including FAK/ILK, PI3K/Akt, and SDF1-CXCR4 [60] that promote EC proliferation, survival, and migration [61], and initiation of tumor angiogenesis. Collectively, several key integrins such as αvβ3, αvβ5, β1, and αvβ8 appear to be potential targets in GBM to reduce tumor angiogenesis [62].

3.3. RTK-mediated immune suppression

GBM patients show marked intratumoral and systemic immunosuppression. The tumor microenvironment contains multiple immunosuppressive factors including TGFβ2, prostaglandin-E2, IL-10; and receptor molecules B7-H1, Fas-ligand, etc. [63]. The tumor is heavily infiltrated by microglia/macrophages, which can represent up to 30% of viable cells in the tumor mass, but lymphocytes infiltration is not common [64]. These monocytes/macrophages in the tumor environment interact with GBM cells and develop immunosuppressive myeloid-derived suppressor cells (MDSCs). Systemic immunosuppression in GBM patients shows that total T-cell counts are greatly decreased, especially CD4+ T cell counts [65]. Furthermore, T-cell function is markedly abnormal [66]. Besides other factors that may underlie T-cell dysfunction, increases in circulating cell populations such as regulatory T cells (Tregs) and MDSCs may be more important [67, 68].

Because of immunosuppressive features of GBM, in recent years, new therapies such as tumor vaccines and peptides are tested in preclinical and clinical studies [63]. The mutant protein EGFRvIII is a cancer specific antigen bearing a targetable epitope that is almost exclusively present in GBM [69]. Rindopepimut is composed of an EGFRvIII-specific peptide conjugated with an adjuvant protein KLH (keyhole limpet hemocyanin). The vaccination produced active anti-tumor response with significant survival benefit in GBM patients. Furthermore, the underlying immune response is not only effective regarding specific removal of EGFRvIII-positive GBM cells, but also the increase in the titer of anti-EGFRvIII sera in beneficial patients [70]. More importantly, phase II clinical trial with the vaccine confirmed these results [71], and the randomized phase III clinical study is ongoing [63]. EGFRvIII lacks the ligand binding domain

and is persistently activated, promoting tumor formation by activating aberrant signaling pathways, epigenetic mechanisms, and metabolic networks, and thus, is a promising cancer target [72]. Further with rindopepimut efficacy, chimeric antigen receptor (CAR) T cells transduced with humanized scFv against EGFRvIII were produced, and the studies are ongoing [73]. Other immunotherapy approaches that are tested or ongoing in GBM clinical studies include the administration of dendritic cells-based therapies; application of check-point inhibitor drugs, and adoptive cell therapy (ACT), etc.

EGFR also plays a protruding role in GBM cell invasion and aggressiveness [74]. It was previously showed that microglia cells stimulate GBM invasion via the EGFR signaling [75]. Coniglio et al. [76] demonstrated in vitro that microglia secreted EGF, which may activate EGFR and signaling pathways in GBM cells [77]. For example, EGFR or EGFRvIII may activate the STAT3 pathway [38], which is induced in various immune populations, and mediate immunosuppression potentiated by the GSCs [78]. Moreover, recent data implicated VEGF as a potent mediator of immunosuppression, again via GSC-associated mechanisms [79, 80]. A VEGF inhibitor, aflibercept, was applied in combination with an antitumor vaccine. Delayed tumor progression and survival extension were observed, which confirmed the efficacy of combining antiangiogenic and immunotherapy approaches, as well as the value of delineating tumor microenvironment [81]. Antiangiogenic therapy added to immunotherapeutic approaches toward glioma may show clinical benefits, among which the endogenous microenvironment or vaccine-induced inflammatory responses is importantly subsidiary to its effectiveness [82].

3.4. RTK, GSC and tumor metabolism

GSCs or glioma initiating cells (GICs) are preferentially located in perivascular and around necro/hypoxic zones where they closely react with the microenvironment and, thus, escape from apoptotic stimuli and preserve the capacity of self-renewal [83]. These interactions with microenvironment components, such as stromal cells or extracellular matrix (ECM) etc., seem important for GSC maintenance, possibly via metabolic and/or epigenetic modifications [83, 84]. Besides, GSCs may be protected from external factors via specific survival signals that they receive from the niche [85]. For instance, hypoxia induces VEGF expression, which promotes angiogenesis and supports the GSC tumor-initiating capacity [86, 87].

Besides VEGF/VERGFR signaling, HGF/Met signaling involves in regulating cell growth, motility and has a role in embryogenesis, degenerative disease and wound healing [88]. This RTK-mediated signaling also promotes the acquisition of stem-cell like properties in glioma cells and the formation and malignant progression of GBM [89, 90]; overexpressing of Met in vitro in glioma cells was highly clonogenic [88]. Met expression seems to be associated with genetic features with EGFR and the tumor suppressor PTEN inactivation, indicating cooperation among these RTK-mediated signaling in keeping GSC phenotype in glioma [91, 92]. A recent mouse study showed that EGFR inhibition induces increased c-Met expression and associated proliferation of GSCs expressing pluripotency TFs and displaying multi-lineage potential [89]. There is now the debate as to the long-term safety of anti-EGFR treatments, which may possibly induce MET-driven GSC populations [88, 89]. On the other hand, however, it implies the combination of targeting EGFRvIII and GSC as a new therapeutic approach.

Cancer development, progression, and response to treatment are greatly influenced by cancer cells' intracellular metabolism and the exogenous tumor environment [93]. The metabolic reprograming that cancer cells adapted to take up and utilize nutrients to drive tumor growth rigorously often relies on signaling and epigenetic/transcriptional networks induced by activated oncogenes (e.g., EGFR, RAS, MYC) and deactivated tumor suppressor proteins (e.g., TP53) [94, 95]. In primary GBM, the frequent genetic changes of key components of RTK/PI3K/Akt pathways, one of the three core signaling pathways that significantly altered in GBM [96], may result in constitutive activation of mechanistic target of rapamycin (mTOR) signaling [96, 97].

Recent studies showed cooperation between EGFRvIII signaling and c-Myc, the transcription factor and one of the master regulators of cancer metabolism [96], to reprogram cellular metabolism and promote tumor proliferation via activation of mTOR signaling, resulting in changes in intracellular nutrients levels [98–100]. Moreover, RTK- and Myc-dependent metabolic reprogramming maybe also involved in IDH1-mutant glioma malignant progression [101, 102]. Therefore, targeted therapies against Myc-dependent metabolism may be an effective therapy for patients with high-grade gliomas. Recent data indicated that extracellular nutrients such as glucose or acetate were required to maintain EGFRvIII signaling via activating mTORC2 [96], leading to GBM resistance to molecularly targeted therapies [103]. Nonetheless, the intricate interactions between the oncogenic signaling and cancer metabolism have only been recently revealed, and metabolism in primary GBM is dominantly regulated hypoxia and RTK-dependent c-Myc upregulation to modify cancer metabolome and cause resistance to therapies [93]. Future studies are needed to govern regulations on genetic and epigenetic alterations, oncogenic signaling and cancer metabolic reprograming, and translate these insights into more effective treatments for GBM patients.

4. Lessons from RTK-targeted therapies

Since the phase III trials of Temozolomide in 2005, there have been few successes regarding treatment for patients with malignant glioma [82]. RTKs, which are the most commonly amplified and mutated genes in cancers, are the key targets in cancer research including malignant glioma. Up to date, three RTKs and their family members present the major druggable targets, including EGFR and EGFRvIII, VEGFR and PDGFR family.

4.1. Experience with RTK-targeted therapies in GBM

4.1.1. EGFR family and EGFRvIII

Small molecular weight kinase inhibitors include gefitinib (EGFR) and erlotinib (EGFR and EGFRvIII), and these two irreversible inhibitors, unfortunately, have achieved limited success either as a single agent or as combination therapies in numerous Phase I and II trials in patients with newly diagnosed or recurrent GBM [104–107]. The resistance may be driven by a subset of EGFR mutations, activation of alternate signaling pathways and suppression of EGFRvIII on extrachromosomal DNA, etc. [72]. Besides, irreversible inhibitors currently in clinical trials including lapatinib (EGFR, ErbB-2), AEE788 (EGFR, VEGFR), and dacomitinib

(EGFR, HER2, HER4), alone or in combination with other agents, still attained minimal to moderate anti-tumor response in newly diagnosed GBM or recurrent patients [108, 109].

Monoclonal antibodies (mAbs) targeted against both wild-type EGFR and EGFRvIII have also been developed including cetuximab, which showed only minimal anti-tumor effect as a single agent in Phase I/II trials [110], but the drug showed chemosensitizing and radiosensitizing effect and may achieve better effect when combined with TMZ and radiotherapy [111]. Other anti-EGFR antibodies include panitumumab and nimotuzumab. Nimotuzumab, a humanized mAb against EGFR, has shown promising efficacy with significantly higher mean and median survival time in GBM patients in Phase I/II trials via its inhibition on tumor growth and angiogenesis; the antibody drug also shows least cutaneous toxicity [82]. Furthermore, targeting at EGFRvIII which acts as a GBM-specific antigen, rindopepimut is a promising peptide vaccine and has shown its effectiveness and induced strong and specific immune response in Phase II clinical trials [70, 112]. The vaccine is currently investigated in Phase II/III trials in newly diagnosed GBM patients alone, in combination treatment with other agents or standard treatment protocol in recurrent patients [82].

4.1.2. VEGFR family

VEGFR is the most potent stimulator in angiogenesis, mainly including VEGFR1, VEGFR2, and VEGFR3. Several VEGFR inhibitors have been developed and applied in preclinical and clinical studies in GBM.

Cediranib (AZD2171) is a pan-VEGFR RTK inhibitor; in addition, it inhibits activity of RTKs including c-Kit, PDGFRA and PDGFRB. In Phase II trials, cediranib treatment quickly induces tumor vessel normalization and edema reduction which were related with the progression-free survival (PFS) in GBM patients [113]. The treatment with cediranib is associated with improved overall survival (OS) only in newly diagnosed GBM patients [114]. Various other VEGFR inhibitors including aflibercept, BIBF 1120, pazopanib, AMG 386 (trebananib), Vandetanib are tested in combination with other drugs in their Phase I/II trials [115]. Among these, aflibercept inhibits both VEGF and placental growth factor (PGF), and acts as a decoy receptor dubbed VEGF trap, yet shows limited success in Phase II trials for recurrent GBM patients [116]. The mAb against VEGF, bevacizumab (Avastin®) is currently used in patients with GBM, mostly in combination with other treatment or drugs. When used in combination with irinotecan, a cytotoxic topoisomerase I inhibitor, the treatment resulted in objective radiographic responses and improvement in PFS [117]. Since bevacizumab was approved by FDA in 2004, over 60 countries apply it for the treatment of progressive disease including the USA and Japan [118–120]. Two completed Phase III trials indicated an improved PFS but not OS of newly diagnosed GBM patients with combination of bevacizumab with the standard protocol (TMZ and RT), yet showed inconsistent results on patient performance status during the treatment [121, 122].

Research data indicated that resistance to VEGF/VEGFR targeted inhibition in GBM may activate other angiogenic factors, such as FGF and PDGF, and thus, promote alternate signaling for neovascularization [123]. Moreover, the treatment-induced HGF/c-Met activation may contribute to robust invasion in the resistant GBMs [124]. Combinational targeting strategies in a good timing with VEGFR-targeted agents warrant further investigations.

4.1.3. PDGFR family

A number of PDGFR inhibitors with multiple targets are developed and tested, including imatinib mesylate (PDGFR, c-KIT, BCR-ABL), sunitinib (PDGFR, VEGFR, c-KIT), sorafenib (PDGFR, VEGFR, RAF), tandutinib (PDGFR, FLT3, c-KIT), vatalanib (PDGFR, VEGFR, c-KIT), pazopanib (PDGFR, c-KIT, EGFR) or dasatinib (PDGFRB, Src, BCR/Abl, c-KIT, ephrin A2) [125]. Among these, Imatinib (Gleevec®) is already used for the first-line treatment of myeloid malignancies and gastrointestinal stromal tumors; however, as a single agent, it shows minimal efficacy in GBMs. Previously, a combination of imatinib and hydroxyurea, a cytotoxic agent that inhibits DNA synthesis, showed a 20% response rate in progressive chemo- and radio-refractory GBM patients [125]; however, similar combination treatment achieved minimal response in recent studies [126]. Other combinations of imatinib with cytotoxic agents, or other kinase inhibitors, have been tested at the preclinical levels and clinical studies [127, 128]. Thus far, this class of targeted agents only achieved minimal anti-tumor activity either alone or in combination with other therapies [82].

4.2. Mechanisms of resistance to RTK-targeted therapy

RTK targeted therapeutic strategies in cancer came into cancer practice since 2001, when FDA promptly approved imatinib (Gleevec®) as a first-line targeted agent for the treatment of patients with chronic myeloid leukemia (CML). In 2004, FDA approved bevacizumab (Avastin®) as a combination agent with standard chemotherapy to treat progressive disease such as metastatic lung cancer [129]. Thus far, however, RTK-targeted treatment strategies have achieved only moderate anti-tumor activity in patients with GBM [1, 35, 82]. Two RTK family as major targets including EGFR family and EGFRvIII, and anti-angiogenesis therapy against VEGF/VEGFR family are applied in newly diagnosed or recurrent GBM [82]. The experience with EGFR RTK inhibitors in GBM proved that, even if EGFR itself gets efficiently dephosphorylated in tumors, the treatment-induced EGFR-independent regulatory circuits may promote alternate activation of downstream signaling and render clinically ineffective [1]. Similarly, in the case of VEGFR-targeted therapy, alternative activation of other pro-angiogenic factors, such as FGF, PDGF, HGF, ANGPT2 and IL-8 et al. may still activate downstream effectors on converged signaling pathways [117–122]. Moreover, RTK heterogeneity and cooperation between RTKs, as well as secondary activation of downstream signaling pathways, may compensate for the loss of the targeted RTK [2]. For example, EGFRvIII transcriptionally inhibits PDGFRβ in tumor cells. EGFR TKIs reduces such inhibition, enabling tumor cells to switch their dependence to PDGFRβ for growth and survival [1].

The inherent link between RTK and TME greatly contribute to the resistance or even failure with the RTK-targeted therapy and combinational therapies [31, 35, 82]. Treatment with the VEGFR2 inhibitor vatalanib only achieved transient benefits on reduction of tumor vascular volume but induced hypoxia and was related to the increased expression of several pro-angiogenic cytokines and chemokines such as VEGF, SDF-1, HIF-1α, FGF, Ephrin, and their receptors including VEGFR2, VEGFR3, and EGFR, which promoted aggressive tumor invasion [130] and alternative pathway of neovascularization [131, 132]. Other RTK inhibitors such as cediranib and sunitinib have been associated with higher toxicities in clinical trials [133–137]. Recent data suggest that tumors have several distinct mechanisms of

neovascularization including vascular mimicry (VM) [138]. VM is identified as tumor cells, most likely GSCs, transdifferentiate into endothelial cells and form neovascular structures to irrigate the hypoxic tumors for both nutrients and active metabolism [139, 140]. GSCs also transdifferentiate into pericytes to maintain VM [141]. Thus, VM is one of the key tumor-inherent mechanisms to drive the resistance to anti-angiogenesis therapy in GBM [142–144]. Indeed, resistance to RTK-targeted and combination therapies is associated with accumulation of GSC as well as immune suppression. Achyut et al. reported that vatalanib treatment increased the number of CD68+ myeloid cells and the CD133+, CD34+, and Tie2+ endothelial cell signatures in a mouse model of GBM [145]. The enhanced myeloid cell infiltration in the TME following therapeutic resistance was associated with the activation of the CSF1–CSF1R pathway, which results in increased number of tumor-associated macrophages (TAM) within dynamic TME [146, 147].

Collectively, the mechanisms of resistance to RTK-targeted therapy include (1) intratumoral heterogenicity of RTKs, that is, cooperation of various RTKs and their downstream signaling pathways; (2) intertumoral heterogenicity of RTK expression and activity within TME; (3) the treatment-induced shaping and adaption of TME including secondary hypoxia, accumulation of GSC and immune suppression [77]. These mechanisms may cause from ineffectiveness to treatment failure, or even clinical toxicity, leading to GBM recurrence. Moreover, during RTK-targeted treatment, most clinical studies actually lack sufficient information regarding the measurement on intratumoral drug levels, target engagement and the degree of inhibition on the targeted RTK in real time [82]. Nonetheless, design of further combination therapies should consider such information, in addition to monitoring the tumor dynamic profiles, and treat the patients according to the corresponsive patterns in disease progression. Therefore, understanding the biology of CNS tumors and influence of TME on tumor progression is becoming increasingly important for developing new therapeutic strategies for this deadly disease.

5. Conclusion and future perspectives

Not to mention intertumoral heterogeneity of the RTK expression, intratumoral heterogeneity, in particular the heterogeneity of amplified and mutated RTKs, presents a serious challenge to design successful single agent and/or combination therapies for patients with GBM. Thus far, clinical trials with small molecules kinase inhibitors still did not change the clinical practice in human malignant glioma [148]. GBM is one of the most challenging malignancies as featured with its infiltrating nature, recurrent tendencies and poor response to any treatment modalities, besides the intertumoral and intratumoral heterogeneity [31, 82]. The major treatment challenges contain aberrant signaling pathways, hypoxic microenvironment, phenotypic and genetic heterogeneity, GSCs and the blood-brain barrier (BBB) [1, 35, 82]. Nonetheless, aberrant RTK mutation and associated signaling pathways are hallmarks of primary GBM. As we show in this chapter, the functional interaction between RTKs and TME in GBM significantly promotes more aggressive tumor invasion, neovascularization and hypoxia, increases the number of GSCs, and adapts tumor metabolism. Thus, considering the importance of the TME

in modulating cellular, molecular and epigenetic changes in a tumor cell, we propose that immunotherapy, especially vaccine-based treatment, targeting hypoxic cancer cells or HIF, and GSC-based therapies may be among the most promising strategies in GBM, in which reasonable and well-designed RTK-targeted therapy may at least partially contribute to the treatment success [31]. Dynamic treatment data measurement and personalized medicine with new imaging modalities (PET) using hypoxia radiotracers are key to delineating the hypoxic tumor regions, clinical tissue biopsy profile monitoring, and well-adjusted drug delivery systems may be rigorously applied to ensure therapeutic efficacy in GBM.

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Chapter 4

Astrocytomas and miRNAs: Are They Useful?

Mario Perezpeña-Diazconti, Pilar Eguía-Aguilar and Fernando Chico Ponce de León

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.77419

Abstract

Tumours in the central nervous system are a heterogeneous group of neoplasms originating in the neural ectoderm and other layers of the embryo. In the Children's Hospital of Mexico Federico Gómez, in accordance with what has been described in corresponding literature, these tumours occupy the third place, after leukaemia and lymphoma, in cancer cases. MiRNAs are non-codifying RNA molecules, of 18–24 nucleotides which regulate the expression of genes in a post-transcriptional level. Recently, the role of microRNAs (miRNAs) in the development of different types of cancer has been taken into consideration. In the case of astrocytomas, several target molecules of miRNAs have been determined, and their participation in the development of tumours has been proved since they are involved in differentiation, proliferation and apoptosis processes. MiRNAs are less susceptible to chemical modifications and degradement by ribonucleases by comparison with RNAm. The level of expression of miRNAs starting from bodily fluids represents the most promising advance for a non-invasive diagnosis and allows for their use as biomarkers to detect tumours in early stages and correlating them with clinical development.

Keywords: miRNA, CNS tumours, astrocytoma, brain, cancer, biogenesis

1. Introduction

Tumours in the central nervous system are a heterogeneous group of neoplasms originating in the neural ectoderm and other layers of the embryo. In the Children's Hospital of Mexico Federico Gómez, in accordance with what has been described in corresponding literature, these tumours occupy the third place, after leukaemia and lymphoma, in cases of cancer [1]. Fifty-five percent of patients are male. The predominant age was from older nurslings up to school-age children, with over 50% incidence. The tumours were 49% supratentorial and 51% infratentorial.

The most frequent ones were astrocytoma (32%), medulloblastoma (19%), craniopharyngioma (11%) and ependymoma (10%). In fifth place, there are germimoma (with 4%). Mixed glioma, primitive neuroectodermal tumours and ependymoblastomas made up 1–3% [1].

Tumour damage cause into the displacement of encephalic structures, oedema, tissue damage and the symptomatology are according to location, size and time of evolution in the tumour. Cephalea was the most frequent symptom in our hospital, followed by irritability, vomit and papilloedema. The growth of cephalic perimeter is of prognostic value in children less than 2 years of age [1].

Throughout the years, different classifications were postponed and applied for its study, based on histogenesis. Currently, the WHO has published its most recent classification [2] based on morphology and molecular changes. The reduction of costs and the increased ease of access to technology have made several medical centres approach this new era of molecular pathology research [2].

Particularly in the case of astrocytoma, the most frequent tumours in the central nervous system of children, there are several considerations. The diffuse astrocytoma group themselves

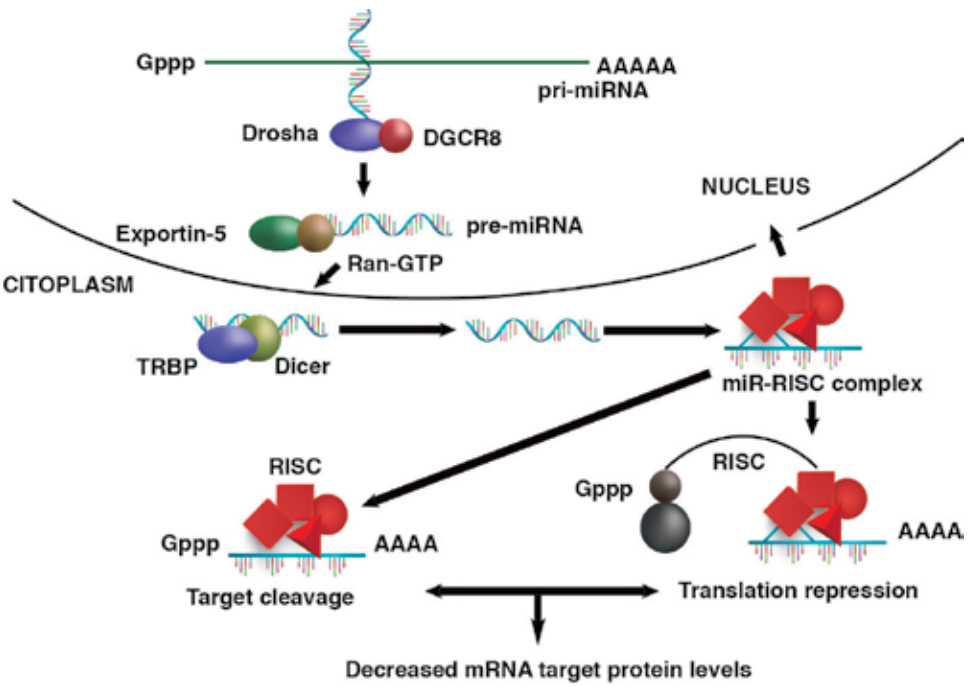

Figure 1. Biogenesis and functions of miRNA. MicroRNA is transcribed by an RNA polymerase II or III so as to generate a transcript of primary RNA within the nucleus. The stem-handle structure of pri-miRNA is recognised and cut by a complex microprocessor composed by Drosha and DGCR8 to produce a precursor microRNA of 60–70 nucleotides in length. Pre-miRNA is then exported to the cytoplasm through the nuclear pores, by means of exportin-5, and it is then processed in the cytoplasm by Dicer-TRBP. The RNA double-chain molecule is separated by a helicase of RNA. One of the strands of the miRNA/miRNA* duplex (the guiding strand or antisense strand) is incorporated, preferentially, in RISC, and it shall guide the miR-RISC complex towards the messenger RNA which shelters a complementary sequence to the miRNA. Once the RNAm is recognised, RISC may regulate the translation by inhibiting the starter or lengthening steps. In some cases, the miR-RISC complex may return to the nucleus [8].

today according to the expression of gene IDH1 or IDH2, which has enabled its correlation with prognosis [2].

Recently, the role of microRNAs (miRNAs) in the development of different types of cancer has been taken into consideration. MiRNAs are small RNA molecules that regulate the expression of genes in a post-transcriptional manner. This regulation is based upon a partial complementarity of microRNA with the target RNAm in such a way that it inhibits the synthesis of proteins (**Figure 1**). In the case of astrocytomas, several target molecules of miRNAs have been determined and their participation in the development of tumours has been proved since they are involved in differentiation, proliferation and apoptosis processes. It is also important to note that tumour cells in high-grade gliomas release microvesicles with miRNAs and proteins which can be detected in patients' serums. This makes miRNAs potential tumour markers. In the case of high-grade astrocytomas, the altered expression of several miRNAs such as miR-15b, miR-21, miR-34, miR-221, miR-10b, miR-124 and miR-181 has been reported, and their participation in the development of the tumour has been proven since they are involved in differentiation, proliferation and apoptosis processes [3–5]. In recent studies undertaken on the serum of patients with GBM, it has been observed that tumour cells release microvesicles, which contain miRNAs among which we can highlight miR-15b, miR-16, miR-21, miR-26a, miR-27a, miR-92, miR-93 and miR-320 [6].

Lages et al. [6] reported six microRNAs which clearly distinguish GBM from oligodendrogliomas. In GBMs, miR-21, -132, -134, -155, -210, and -409-5p were over-expressed. However, miR-128 was more expressed in oligodendrogliomas [7].

2. Importance of microRNAs

MiRNAs are non-codifying RNA molecules, of 18–24 nucleotides which regulate the expression of genes in a post-transcriptional level. They are found in a wide array of organisms, such as animals, plants and viruses, and in each type of cells [8, 9]. It is estimated that the genome of vertebrates codifies over 1000 different miRNAs, which regulate the expression of at least 30% of genes. The low necessary astringency for a functional interaction between miRNA/RNAm gives the capacity to miRNAs to regulate several messengers, besides region 3′UTR of target RNAm frequently harbouring several sites of recognition of microRNAs [10]. Close to 2588 mature sequences of miRNAs have been identified in the human genome [http://microRNA.sanger.ac.uk, version 21]. This number has rapidly increased in the last few years. Nevertheless, little is known about their specific goals and the biological functions that they undertake in the development of cancer and other illnesses [11].

3. Biogenesis

MiRNAs are initially transcribed as a long transcript known as primary miRNA (pri-miRNA) whose length goes between 3 and 4 kb, although some molecules may measure up to 10 kb. Pri-miRNAs are recognised in the nucleus by the complex composed by enzyme RNAse III

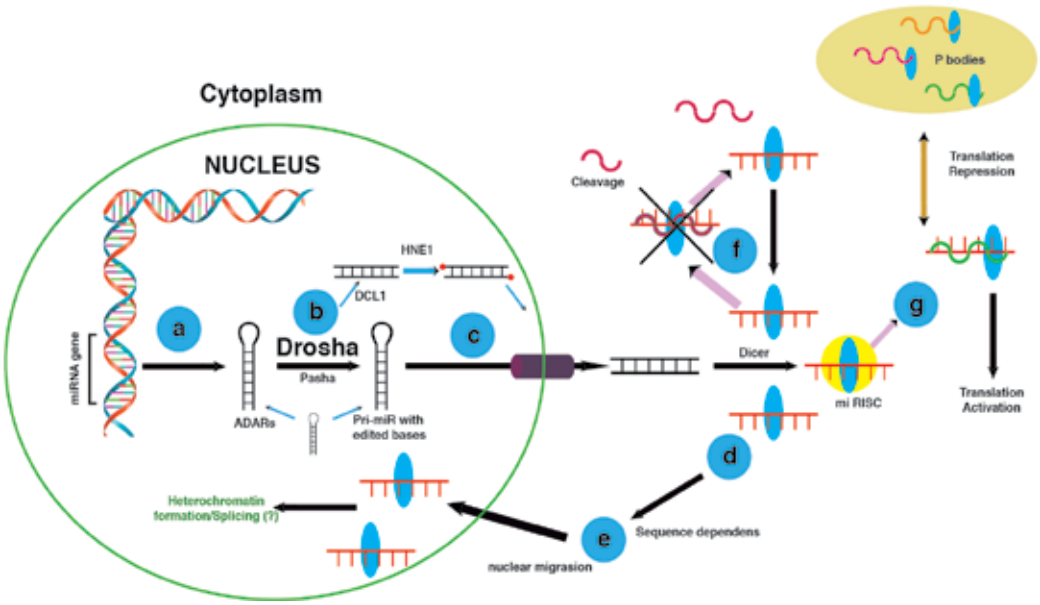

Figure 2. Biogenesis of the microRNA within the nucleus (a–c), maturing of the cytoplasm (d), formation of the microRNA-RISC complex which, depending on the sequence, shall return to the nucleus (e) or shall join its target RNAm to inhibit the translation (f) and finally the microRNA/RISC-RNAm complexes are stored in p-bodies where they are degraded or they return to the translation (g).

Drosha and DGCR8 (protein with a binding domain to double-strand RNA). This complex cuts the structure in a fork, becoming now a precursor miRNA (pre-miRNA) with a length of 60–80 nucleotides. The pre-miRNA is recognisable because of exportin 5 (nuclear exporting factor) and the nuclear protein Ran-GTP. Both transport pre-miRNA towards the cytoplasm. The Dicer and TRBP enzymes (proteins with binding domain to RNA) undertake a second cut in the base of the stem-handle and they generate an RNA molecule, double strand, of 18–24 nucleotides in size [12]. A great protein complex known as silencing complex induced by RNA (RISC) is associated with duplex RNA and separates both chains. RISC is a tetrameric complex made up of Dicer, TRBP (protein with binding domain to RNA), PACT (Activating Protein) and Ago2 (Protein of the Argonaut family). Ago2 identifies the target RNAm based on the complementarity with the associated single-strand microRNA. Recognised sequences of target RNAm are located mainly in region 3, non-translated (3′UTR). Generally speaking, only one strand is incorporated within RISC and the other one is downgraded. This miRNA guides RISC towards the target messenger inhibiting its translation (**Figure 2**) [13, 14].

4. MiR/RISC-RNAm complex

The recognition of the target RNAm takes place because of the complementarity of the sequence known as ‘seed’ (nucleotides 2–8) located in the 5′ extreme of the microRNA with the sequence of the target RNAm. Recognised sequences of target RNAm are in region 3′,

non-translated (3′UTR) (60%), in codifying sequences (25%), in introns, in non-codifying RNA sequences and in 5′UTR. The degree of complementarity between microRNA and RNAm determines the silencing mechanism. When complementarity is 100%, targeted RNAm is downgraded, which mostly happens with plants. In animals, complementarity is 100% in the seed region, but not throughout the microRNA in such a way that a mechanism of inhibition of the target messenger takes place.

The effector complex headed by the Argonaut protein probably interacts with translational systems to inhibit the synthesis of proteins at the beginning or in the elongation step, depending, probably, on the nature of the miRNA and the target transcript [13, 14]. Most microRNAs described in the human body exert their inhibiting effect in the cytoplasm; nevertheless, there exist some microRNAs as miR-29b which has a terminal sequence of hexanucleotides which allows it to return to the nucleus where it possibly undertakes its functions.

The complexes formed by the microRNA/RISC-RNAm do not remain indefinitely in the cytoplasm, but they are rather transported towards structures of cytoplasmic processing, called P-bodies, where the downgrading of RNAm may take place due to deadenylation and decapping, or it is also stored and then separated from the repression complex and the P-body and returns to the translational machinery (**Figure 3**).

MicroRNAs participate in fundamental cell processes such as determining the cell lineage, apoptosis, proliferation, migration and regulation of the cell cycle, in which the translation of specific genes is highly precise and coordinated. MicroRNAs make up complex regulatory networks with its target genes, representing common mechanisms that have evolved

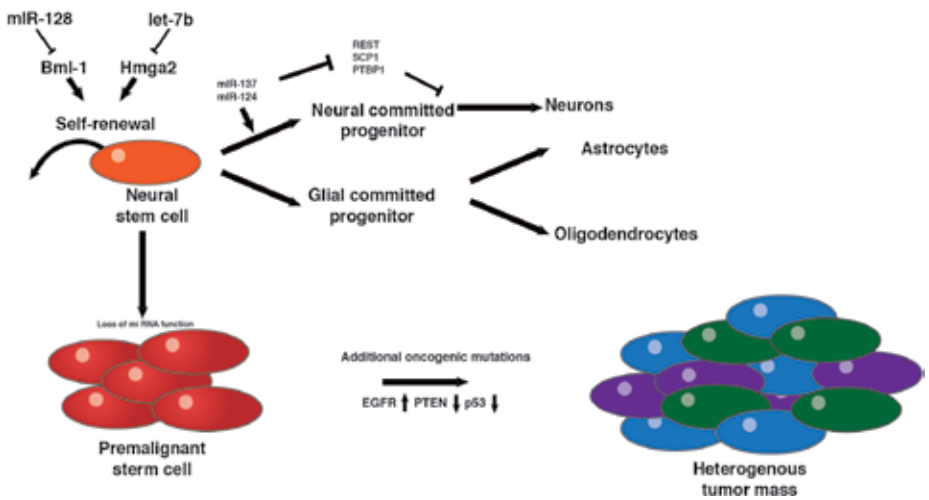

Figure 3. Model which shows the effects of deregulation of miRNAs in the development of the glioma. Under normal physiological conditions, the expression of miRNAs is important for the induction of differentiation in the SNC and abrogation of the self-renewal of the stem cell. The loss of expression of miRNAs results in the creation of pre-malignant stem cells, which are hyperproliferative and non-differentiated, which may progress into a glioma of low to high grade. Additional oncogenic mutations may facilitate the malignant phenotype.

in mammals strengthening genetic regulation. At the same time, microRNAs are regulated by oncogenes, tumour-repressing genes, epigenetic mechanisms, genetic abnormalities and defects in the miRNA biogenesis machinery [15]. Each one of these mechanisms may contribute by themselves or, more likely, together, to alter the expression of miRNAs in cancer [15, 16].

5. Patterns of expression for miRNAs in the brain

The central nervous system of mammals is controlled importantly by genetic regulation mechanisms. MicroRNAs contribute to this regulation; approximately 70% of identified miRNAs until now are expressed in the brain and some of them are specific to the brain [17]. In recent studies, the pattern of expression for microRNAs was determined and it was shown that they regulate both development and functionality of the nervous system [9, 18].

A wide variety of microRNAs are in neuronal subtypes with the highest concentration in the brain cortex and cerebellum [19, 20]. In the central nervous system, there are a large number of genes which originate miRNAs and their expression is different depending on the anatomical region. Specific microRNAs for the brain are miR-9, mir-124, miR-125, miR-128 and miR-129 [21–25]. MiR-124 and miR-128 are expressed mainly in neurons, whereas miR-23, miR-26 and miR-29 can be found enriched in astrocytes [10, 26, 27]. In the same way, the expression profile of miRNAs in the development and differentiation of the nervous system in mammals is fundamental, since changes have been documented in their expression when embryo stem cells develop neurogenesis and gliogenesis, which suggests that they may have an important role in differentiation or determination of the cell lineage [9, 14, 22, 28, 29].

6. MicroRNAs and their relationship with cancer

Calin et al. were the first ones to find evidence regarding the relation between miRNAs and cancer, demonstrating that miR-15 and miR-16 are located in a mutated region, in over half of chronic lymphocytic leukaemias in B-cells [30]. Several following studies have demonstrated that the expression profiles of several miRNAs are altered in different types of tumours such as glioblastoma, pituitary adenoma, prostate cancer, breast carcinoma, hepatocellular carcinoma, lung carcinoma, colorectal carcinoma, ovarian carcinoma, thyroid and cervical carcinoma, lymphoma and chronic lymphocytic leukaemia [31–35]. For this reason, some of them are considered tumour-suppressive genes or oncogenes [36–38]. Genetic events guiding the development of tumours in the brain are yet unknown; nevertheless, there is evidence which suggests that gliomas may surge starting from a subpopulation of cells within the tumoural mass; these cells have been called ‘stem tumour cells’, which maintain their ability for renewal and multi-potentiality. MiRNAs are important regulators of the process of differentiation and proliferation of stem cells (**Figure 4**) [39–41].

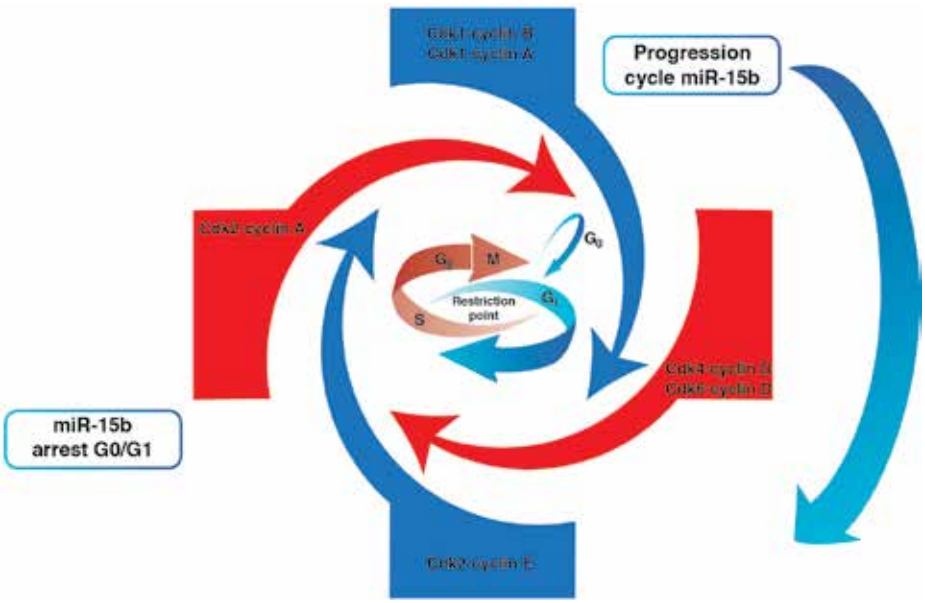

Figure 4. Mir-15b regulates the progression of the cell cycle because it has cyclin E as a target. The over-expression of miR-15b causes an arrest of the cell cycle in the G0/G1 phase, whereas the low expression causes a reduction in the population of cells in G0/G1 and an increase in phase S [51].

7. Expression profile for miRNAs in astrocytomas

Different expression patterns in miRNAs have been described in low- and high-grade astrocytomas including pilocytic, diffuse, anaplastic astrocytomas, and multi-form glioblastoma in adults. In these tumours, miRNAs participate in the cell proliferation, invasion, angiogenesis and differentiation [42, 43]. The first reports are very recent and started with the identification of miRNAs in the GBM in 2005. In this type of tumour, an overexpression of miR-221 was described and proposed as a possible specific marker, whereas miR-128, miR-181a, mir181b and miR-181c were found to be low expression, which probably reflects a loss of expression associated to the lack of differentiation in tumour cells [38]. In that same year, an over-expression of miR-21 in GBM and cell lines was described, comparing it with normal tissue. These effects were related with a reduction of apoptosis and malignant phenotype. On the contrary, the low expression of miR-21 promoted the activation of caspases and apoptosis [44]. Afterwards, in another study, miR-124 and miR-137 were identified, related with the neuronal differentiation in mouse stem cells, derived from a mouse oligodendroglioma and derived of human GBM. Besides, in a cell line of GBM, arrest in the cell cycle after transfecting miR-124 and miR-137 could be observed, which suggests that miR-124 and miR-137 may be target molecules for therapeutic treatments of this illness [44]. These studies suggest that miRNAs participate in multiple biological processes which are characteristic of GBM such as cell differentiation, proliferation, invasion, apoptosis and angiogenesis. Given that miRNAs may promote or limit the development of the tumour, they may be considered as having oncogenic potential or tumour-suppressive activities. MiRNAs

analysed in this study and which are considered in the study as having oncogenic potential are miR-15b, miR-21 and miR-221 and the tumour suppressors are miR-124, miR-128, miR-137 and miR-221. Next, each one of them is described [5, 23, 45–48].

8. MicroRNAs with oncogenic potential: antiapoptotic and proliferative functions

8.1. MiR-9

The gene that codifies miR-9 is located in the genome of three different regions: miR-9-1 is located in the 1q22 chromosome, miR-9-2 in 5q14.3 and miR-9-3 in 15q26.1. This miRNA is expressed almost exclusively in the brain and it is a neurogenetic mediator. In the fetal brain, it is highly expressed, compared with that of an adult [49]. Nass et al. studied the expression of several miRNAs in primary brain tumours and metastatic brain tumours through micro-arrangements and qRT-PCR, and they observed that miR-9/9* were mainly overexpressed in primary brain tumours, by comparison with metastatic brain tumours, and they concluded that it is possible to distinguish between both types of tumours with a high degree of reliability [50]. Up until now, it has only been described as one of its targets for the REST transcription factor.

8.2. MiR15b

Located in chromosome 3q25.33, Xia et al. identified a panel of miRNAs expressed differentially in glioma tissue. One of the significantly deregulated miRNAs was miR-15b. Afterwards, they identified their potential targets being CCNE1 (protein related with the transition of the cell cycle of G1/s) as one of them. The levels of expression of RNAm of CCNE1 in the cell lines after transfection with exogenous miR-15b were analysed, as the anti-senses of miR-15b, and they observed that the levels remained without changes. Nevertheless, protein levels of CCNE1 were significantly reduced after the transfection with exogenous miR-15b and they were increased after transfecting the antisense of miR-15b. These results suggest that CCNE1 is a potential target. The overexpression of this miRNA causes arrest in the cell cycle in its G0/G1 phase, whereas its inhibition results in a reduction of the cell population in G0/G1 and therefore also represents an increase in phase S (**Figure 5**) [40].

8.3. MiR-21

The gene which codifies for the miR-21 is located in chromosome 17q23.1. The overexpression of this miRNA was described for the first time in the GBM and afterwards in other types of solid tumours [31, 44]. Chan et al. studied the expression of miR-21 in patients with GBM and in cell lines of gliomas and observed that, in tissues, the expression of miR-21 was increased five to 100 times in comparison with non-neoplastic brain tissues. There are several important targets which contribute to its anti-apoptotic and proliferative actions, such as some molecules involved in the suppressor tumour routes for p53, TGF-β (β-transforming growth factor) and a mitochondrial apoptotic route [52–54]. In a recent study, developed with 124 samples of astrocytomas of high and low grade, it was found that miR-21 is more sensible to predict the clinical development of

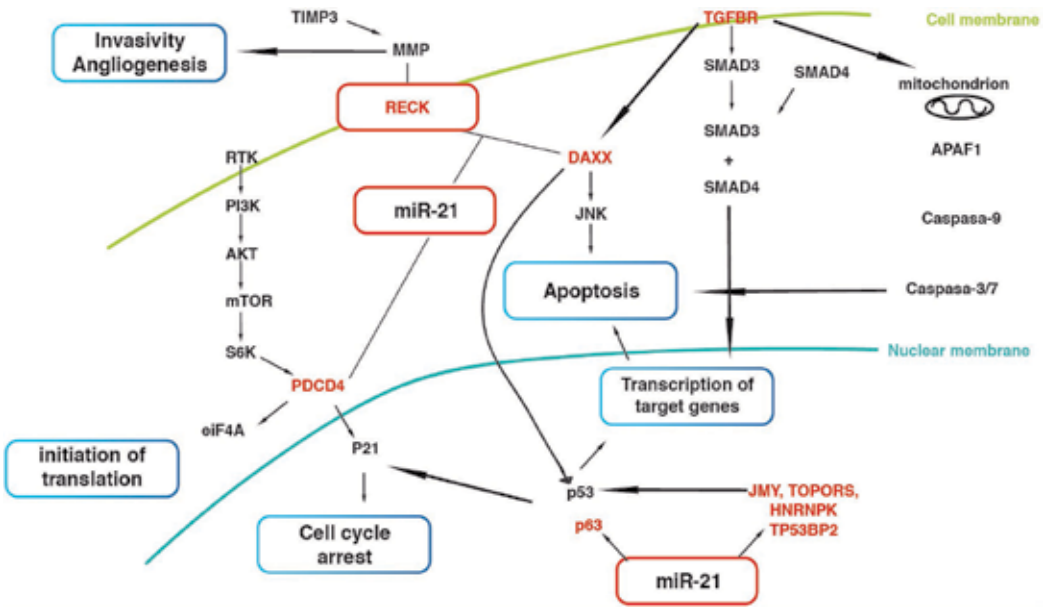

Figure 5. Signalization routes influenced by miR-21 in glioblastoma cells. MiR-21 regulates apoptosis, cell cycle and translation [52].

high-grade astrocytomas, because they observed a greater expression in high-grade tumours and a lower survival rate compared with low-grade astrocytomas [43]. It is evident that the over-expression of miR-21 in astrocytomas results in the activation of multiple oncogenic routes [57]. Many other studies have confirmed the over-expression of this miR in the four grades of astrocytomas and in other tumours of the SNC as oligoastrocytoma, oligodendroglioma and medulloblastomas, having a greater expression in the multi-shaped glioblastoma (**Figure 6**) [26, 38, 58].

8.4. MiR-221/222

MiR-221/222 are located in chromosome Xp11.3 and they are over-expressed in astrocytomas, their expression is co-regulated and they have the same specificity of targets because the region considered as "origin" or "seed" region is the same in both cases [7]. Ciafrè et al., through microarrangements of expression and Northern blot, analysed nine samples of patients with GBM and 10 cell strands of glioma and identified miR-221 as one of the miRNAs with greatest overexpression in comparison with values obtained in normal brains and samples of healthy tissue that were close to the tumour [38]. Gillies et al. 2007 described $p27^{kip1}$ as a direct target of miR-221/miR-222. $P27^{kip1}$ is a protein that regulates the cell cycle, its function is inhibiting the cyclin-depending kinase (CDK) in such a manner that there is an arrest in the cell cycle in the phase G1, avoiding cell proliferation [59] (**Figure 5**). Medina et al. studied the participation of several microRNAs in the regulation of the cell cycle and observed that the expression of miR-221 and miR-222 was increased in human quiescent cells which are stimulated for proliferation. They predicted and proved two targets: p27 and p57; both suppress the cell growth because they inhibit cyclin-dependent kinases. The over-expression of these miRs is closely linked to the control of the cell cycle, which assures

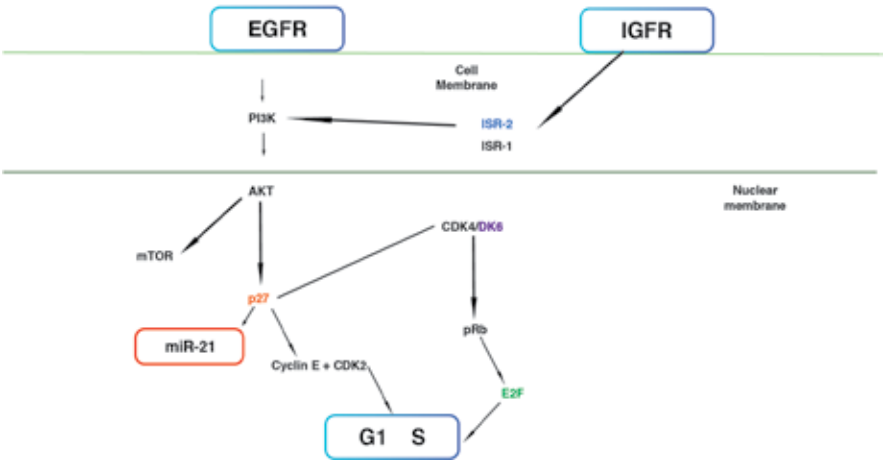

Figure 6. The miR-221 oncogene promotes the progression of the cell cycle because it inhibits the translation of the tumour suppressor $p27^{kip1}$, whose reduction causes the expression of CDK and, with it, the progress of the cell cycle [52].

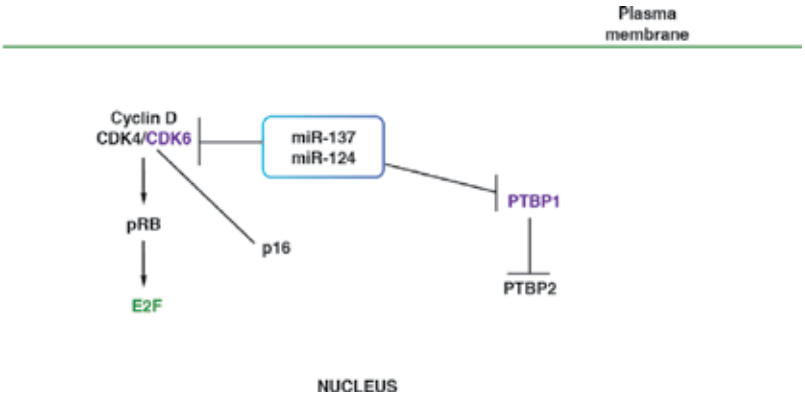

Figure 7. MiR-124 and miR-137 has CDK6 as target, CDK6 which is a regulator of the cell cycle and differentiation. PTBP1 is also one of its targets and is related with alternative 'splicing'.

the survival of the cell by a coordinated competence between the entrance in phase S and signalization routes of the growth factor that stimulates the cell proliferation [55]. The high expression of miR-221 in high-grade astrocytomas and cell strands, and they strongly imply that it is a candidate to becoming a specific tumour marker (**Figure 7**) [59].

9. Tumour-suppressing microRNA: neural differentiation and proliferation

9.1. MiR-124

There are three genes that codify for miR-124 and are located in different regions; thus, we have miR-124-1 located in chromosome 8p23.1, miR-124-2 located in 8q12.3 and miR-124-3 in

20q13.33. It is the most profuse brain-specific miRNA; during neural differentiation, it expresses itself mainly in neurons [49]. It is considered a tumour suppressor weakly expressed in anaplastic astrocytomas and GBM, in relation with the non-neoplastic brain tissue. In this regard, Silber et al. studied the expression of several miRNAs during the differentiation of adult neural stem cells, and it was observed that miR-124 increased its expression eight times, instead of what happens in high-grade tumours, where their expression is less. In this same study, they also determined that miR-124 may induce differentiation and inhibit the proliferation of glioblastoma stem cells when inhibiting CDK6 (cyclin 6, dependent on kinases) which, as a goal, promotes the progress of the cell cycle (**Figure 8**) [45, 54].

9.2. MiR-128

MiR-128-1 is located in chromosome 2q21.3 and miR-128-2 in 3p22.3. It is an miRNA specific to the brain, where it finds itself enriched. On the other hand, in gliomas and glioma cell strands, its expression is lowered [25, 38, 42]. Zhang et al. studied the expression of miR-128 in astrocytomas GII, GIII and GIV and in cell strands, and they observed that it lowers itself progressively as the grade of the tumour increases. Its tumour-suppressing characteristics were evidenced when transfecting miR-128 in glioma cell strands, observing an inhibition in cell proliferation [25]. Godlewski et al. proved the low expression of miR-128 in gliomas and in cell strands and focused in finding a target that was related with cell differentiation and self-renewal. MiR-128 makes up for an important biological target against the 'tumour stem cells' which are characteristic and part of the origin of the glioma (**Figure 9**) [42].

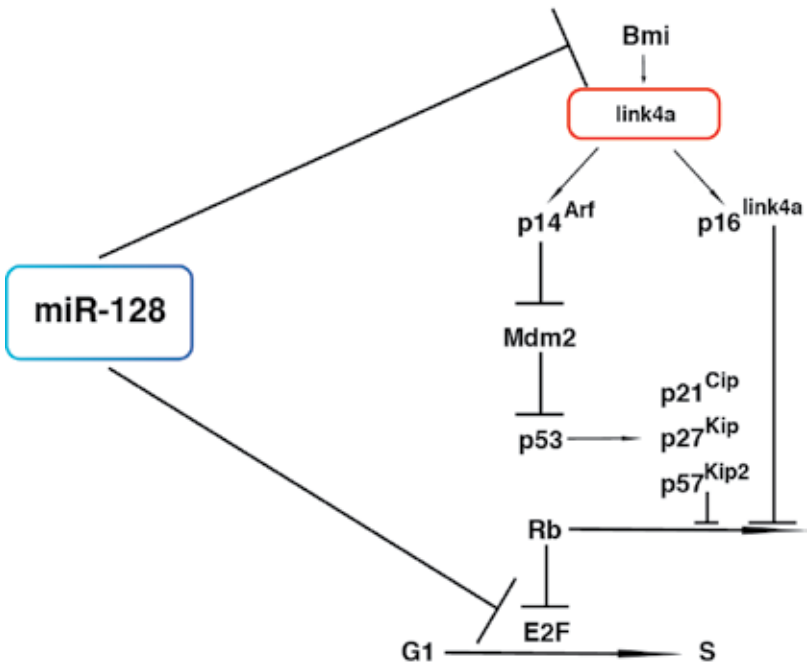

Figure 8. MiR-128 has Bmi and E2F as its main targets.

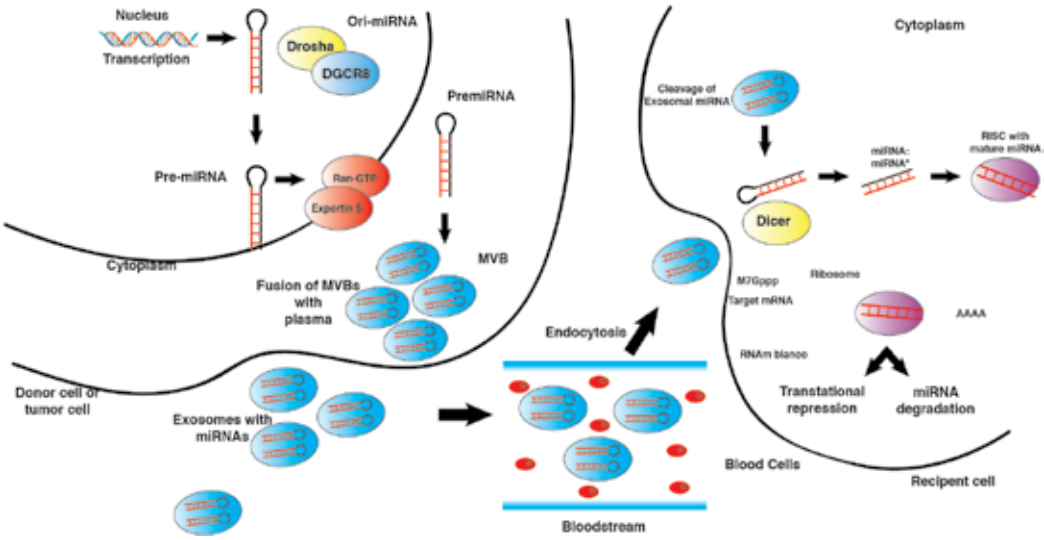

Figure 9. Possible transport route of miRNAs in serum, and their final destination to receptor tissue cells.

9.3. MiR-137

Located in the chromosome 1p21.3, Silber et al. studied the expression of several miRNAs during the differentiation of adult neural stem cells and observed an increase of miR-137 24-fold. This miRNA is considered a strong anti-proliferation factor and a cell pro-differentiator, with tumour-suppressing activity in gliomas, and may be of therapeutic relevance [42]. In high-grade astrocytomas, the expression of miR-137 is lowered. One of its validated targets through the reporting system of luciferase is CDK6, which regulates the progress of the cell cycle and differentiation, suggesting that miR-137 mediates the inhibition of CDK6, which can, in part, cause proliferation and differentiation of CBM cells (**Figure 8**) [54].

9.4. MiR-181

The miR-181 family is made up of miR-181a located in 9q33.3, miR-181b in 1q32.1 and miR-181c located in 19p13.13; miR-181a and miR-181b are enriched in a normal brain. Ciafre et al. studied the expression profile in patients with glioblastoma, finding a low expression of miR-181a, miR-181b and miR-181c in 20–30% of cases. In cell strands, a low expression was also observed, being miR-181a the one with the lowest expression, followed by miR-181b. In this case, low expression was correlated with the lack of differentiation of tumour cells [38]. In the same manner, Shi et al. studied a small series of gliomas in grades II, III and IV and observed a low expression of miR-181a and miR-181b associated with the grade of tumour. They also transfected glioma cell strands with both miRs and they observed an inhibition of the growth, induction of apoptosis and inhibition of the invasion. These effects were more evident with miR-181b [55, 56]. Conti et al. studied the expression of miR-181 in different grades of astrocytoma from a diffuse astrocytoma, grade II up to GBM GIV and observed the low regulation of mir-181b in all grades; nevertheless, the expression levels of miR-181a and miR-181c were similar to those on a normal brain [24]. Zhi et al. studied a total of 124 astrocytomas ranging from GI to GIV and they found low levels of miR181b which were associated with low survival. The authors also mention that miR-181b

is the most sensible way to predict the clinical diagnosis for patients with low-degree astrocytomas. These results suggest that miR-181 may maintain the state of differentiation in normal brain cells for which their diminution would induce the loss of differentiation in tumour cells. The identification of target may provide information regarding the cell differentiation (**Table 1**).

The findings that are registered in the study up to now represent starting studies; nevertheless, it has been established that the deregulated expression of miRNAs participates in the tumourigenesis in several types of tumours such as GBM. Data are scarce regarding the differential expression of miRNAs in low- and high-grade astrocytomas in children. In children, low-degree astrocytomas are the most common; nevertheless, high-grade astrocytomas take place frequently and in advanced clinical studies. In paediatric population, the profile of expression of miRNAs in low- (GI and GII) and high-grade astrocytomas (GIII and GIV) is unknown. With methods of cell and molecular biology, it is possible to generate information regarding the biological behaviour of these molecules and to establish molecular markers which may be used to identify and differentiate the different grades of astrocytomas that have malignity characteristics, despite being low grade. The goal of this work is to determine the

miRNA	**Normal brain**	**Type of glioma and expression**	**Biological function** ***Oncogene/tumour suppressant***	**Target RNAm** **Experimentally validated**	**Number of possible targets**
MiR-9	Abundant	High GIV	Differentiation *Oncogene*	REST	683
MiR-21	Basal	High G II, III, IV	Proliferation and anti-apoptosis *Oncogene*	p63, JMY, TOPORS, TP53BP2, TGFβR2/3, DAXX, HNRPK, PDCD4, RECK, TIMP3, LRRFIP1	210
miR-221	Basal	High G II, III, IV	Proliferation: cell cycle *Oncogene*	CDKN1B/p27 CDKN1C/p57, BIRC1	307
miR-15b	Basal	High (cell strand glioma U118)	Regulates the progression of the cell cycle (arrest in G0/G1) *Oncogene*	CCNE (codifies cyclinE1)	968
MiR-124	Abundant, specific	Low G III, IV	Differentiation, proliferation: cell cycle *Suppressor tumour*	PTBP1 (neural differentiation), CDK6	1299
miR-128	Abundant, specific	Low G II, III, IV	Proliferation: cell cycle *Tumour suppressor*	E2F3a, BMI1	785
MiR-137	Abundant	Degrees III and IV, low	Induces differentiation, inhibits proliferation *Tumour suppressor*	CDK6	468
miR-181a	Abundant	G II, III, IV, low	Induces apoptosis, inhibits invasion and growth *Tumour suppressor*	Not reported	892

Table 1. Expression of microRNAs in normal brain and in astrocytomas, their functions and validated targets.

profile of expression of miRNAs present in low-grade (G I, II) astrocytomas and in high-grade astrocytomas (G III, IV) in paediatric population.

10. Expression of microRNAs in serum

One of the goals within cancer study is to develop non-invasive tests for the diagnosis and follow-up of patients; because of this, there is a great interest in the detection of nucleic acids that are circulating in serum and plasma. Serum and plasma contain a great number of stable miRNAs, despite the high content of ribonucleases in the plasma. This stability may be given by finding itself within the exosomes (organelles derived from endosomes), by chemical modifications or by associating with protein complexes such as RISC [60, 61]. Lawrie et al. [4] reported their first study regarding miRNAs, associated with tumours, in lymphoma patients' serums, and they found that the levels of miR-155, miR-210 and miR-21 were higher than those found in control serums of healthy patients. In this study, they related the high expression of miR-21 with a better prognosis. These results were consistent with previous results in biopsy material from lymphoma patients, in which high levels of miR-21 were associated with a better prognosis [4]. Chen et al. detected and sequenced 100 miRNAs in healthy patients' serums and in patients with lung and colorectal cancers, reporting specific expression patterns of tumour type. In this same study, they distinguished the miRNAs in the serums of other species of small nucleotides such as tRNA or downgraded RNA fragments, concluding that miRNAs are the main fraction present in serum [62, 63]. One of the first undertaken studies in astrocytoma patient serums was the one by Skog et al. in which they report that tumour cells on glioblastomas release microvesicles that contain microRNA, RNAm and angiogenic proteins [64]. These results indicate that patients with cancer present elevated levels of exosomes in plasma, derived from the tumour, in comparison with controls. Although normal cells may contribute to the population of exosomes in the peripheral circulation, the main source of circulating exosomes in cancer patients is originated in the tumour. Nevertheless, little is known about the mechanism by which miRNAs are generated in plasma and the biological impact of these molecules in distant sites of the body [61]. The discovery of miRNAs in serum opens the possibility of using them as biomarkers in different illnesses.

11. Regulator mechanisms of miRNAs

The regulation of miRNAs in cancer is undertaken by multiple mechanisms such as transcriptional regulation, epigenetic alterations, mutations, abnormalities, in the number of copies in DNA and defects in the biogenesis machinery for miRNAs. Each one of these mechanisms may contribute by themselves, or more probably to alter the expression of miRNAs in cancer [11, 15, 65]. Up next, each one of these regulation mechanisms is detailed.

The *transcriptional regulation* contributes to the alteration of expression patterns in miRNAs, an important example is that of miR-34b, a tumour suppressor which is regulated by the transcriptional factor p53. The inactivation of p53 in gliomas reduces the expression of miR-34, which makes it inhibit the cell proliferation, the progression of the cell cycle of G1/s, cell survival,

migration and cell invasion [66], and correction. Another example is miR-451; in this case, it is known that there are two transcription factors, SMAD3 and SMAD4, separated by 157 pb and whose binding sequence is in 1135 pb upstream from the miR-451 sequence. Both factors increase the transcription of miR-451 and induce the inhibition of growth and proliferation [67].

Epigenetic mechanisms may regulate up to a certain degree, the imbalance of miRNAs in tumour cells [68]. The methylation of DNA and modification of histones play a predominant role in the remodelling of chromatin and the general regulation of expression of genes that codify proteins. The hyper-methylation of CpG islands associated with specific miRNAs has been proposed as one of the mechanisms by which a low expression of miRNAs in tumour cells has been observed. The epigenetic silencing of miRNAs that act as tumour suppressors is emerging as an important alteration in cancers. Lujambio et al. studied the expression profile for several miRNAs in cells derived from a metastatic ganglion, and afterwards, the cells were treated with a de-methylating agent, observing that there was some re-expression of some miRNAs such as miR-148a, miR-34b/c and miR-9 [68]. The regulation of miR-124 is given, partly, due to epigenetic mechanisms, which was observed in a cell strand for colon cancer. No expression of miR-124 was observed here, but when cells were treated with a de-methylating agent, their expression was restored and, at the same time, correlated with the inhibition of one of its targets, CDK6. This result is due to miR-124 being located within a great CpG island, which, in a normal colon tissue, would be hypo-methylated, but in colon, tumour finds itself hyper-methylated [69]. In the same manner, the epigenetic silencing of miR-124 was evidenced when treating glioma cell strands with 5-aza-2′-deoxicitidine (a methylation inhibitor) and TSA (histone deacetylase inhibitors), increasing the expression of miR-124 [45]. In gliomas, miR-137 is partially regulated by epigenetic mechanisms, and its expression was increased 12-fold when astrocytoma cell strands were treated with de-methylating agents. This suggests that epigenetic modifications for regulating sequences in CpG islands may contribute to silencing miR-137 in GBM [45] (**Figure 10**).

Somatic mutations and/or in the germinal line, identified in miRNAs, are scarce. Some of the most recent findings have taken place in chronic lymphocytic leukaemia (CLL) [30]. In this illness, 42 genes which codify microRNAs were sequenced and five microRNAs with mutations were found. In the case of solid tumours, 15 miRNAs were evaluated in 91 epithelial-origin tumour cell strands and mutations were found in one case, a variation in the sequence of the precursor miRNA, and 15 variations in the sequence of primary miRNAs [15]. These mutations may be found in pri-, pre- and mature sequences of miRNAs [16].

The *abnormality in the number of DNA copies* is one of the mechanisms which modify the expression and functioning of genes. It is calculated that close to 50% of genes that codify human miRNAs and are registered are located in fragile areas, in regions with minimal loss of heterozygosity (LOH), minimal amplification regions and breaking regions. In chronic lymphocytic leukaemia, region 13q14 is deleted in over 50% of cases, and in this place, there is miR-16-1 and miR-15a. These two miRNAs have Bcl-2 as a target and work as tumour suppressors in this illness. The deletion of these miRNAs has also been identified in pituitary adenomas, ovary adenomas and breast cancer. In patients with lymphoma, the amplification of C13orf25 located in 13q31-32 has been described; in it, seven polycystronic miRNAs have been located. This group of miRNAs work as oncogenes, altering the balance between apoptosis and proliferation through the proto-oncogene c-Myc [15].

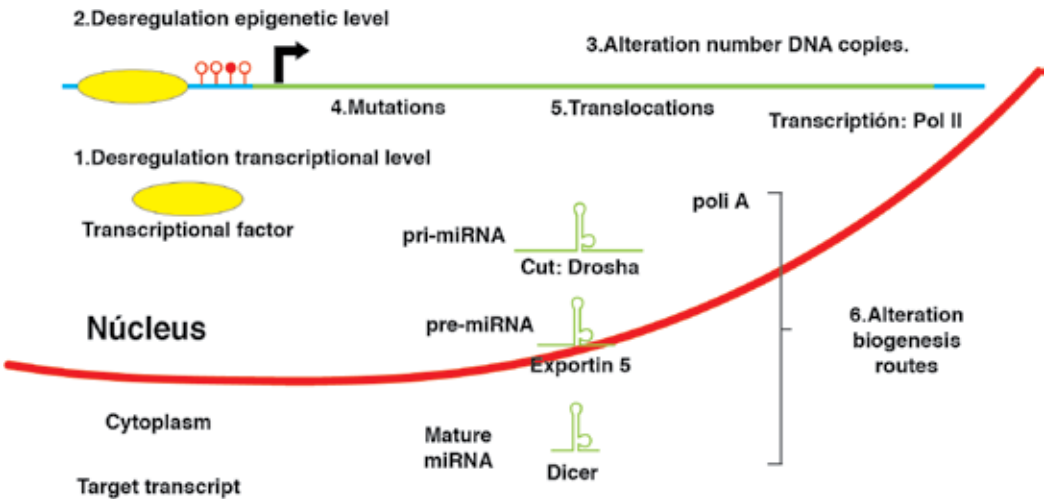

Figure 10. Epigenetic mechanisms regulate the transcription of miRNAs. (A) A CpG island regulates the transcription of an intergenic miRNA. (B) A CpG island regulates the transcription of a gene that harbours an miRNA. (C) An intronic miRNA has its own transcriptional starting point, which is regulated through CpGs. (D) A factor of transcription recruits DNA-modifying enzymes and histones so as to epigenetically regulate a gene that harbours an miRNA which is surrounded by CpGs.

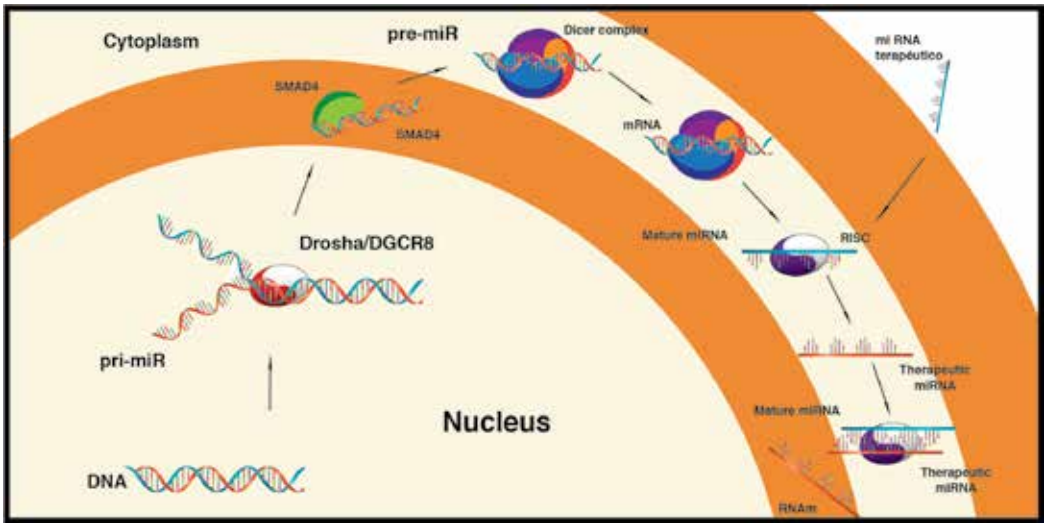

Figure 11. Diverse mechanisms which alter the expression and functionality of miRNAs in human cancer.

Defects in biogenesis of miRNAs. Proteins which participate in the biogenesis of miRNAs may find themselves altered in cancer. In a study that spanned 67 lung cancer patients, a low expression on Dicer1 levels was determined, associated with a poor differentiation of tumour cells and short post-surgery survival [65]. The Argonaut proteins, components of the RISC complex, are in chromosome 1 and are deleted frequently in Wilms' tumours; in

neuroectodermic tumours, an altered expression of these proteins has also been observed. The mechanisms, which alter the expression of miRNAs, are resumed in **Figure 11**.

12. miRNAs as therapeutic targets

Currently, miRNAs are categorised as oncogenes and tumour suppressants in such a manner that a future therapeutic strategy must be headed to inhibiting or activating the altered miRNA, in this sense, in recent years, a therapy of re-expression of microRNAs. The main advantage of miRNA therapy is that its re-expression may influence the expression of hundreds of genes involved in several cell strands and routes. The main obstacle for an effective therapy is the insertion of miRNAs within the cell, because they are molecules that do not freely enter, they are unstable and therefore they may degrade after crossing the membrane of plasma. Another important part is controlling the levels of re-expression of miRNAs to avoid their expression beyond the physiological levels. Another challenge is achieving the antineoplastic agents to cross the haematoma-encephalic barrier. To overcome this inconvenience, different strategies are being developed, such as the intranasal application of oligonucleotides, which is a non-invasive method for the transport of therapeutic agents; unites nucleic acids to cationic lipids, introducing the therapeutic agent by a conjugation with membrane lipids. The *in vitro* studies done with cell strands, antagomiRs, are introduced to cells uniting to their region 5′ a cholesterol molecule; in this way, antagomiR crosses the cell membrane and inhibits the action of the miRNA, sequestering it and uniting by a complementarity of bases, avoiding the inhibition of the target RNAm. Nevertheless, cancer is a complex illness and patients with the same diagnosis may have different genetic and epigenetic alterations and polymorphic variations; therefore, the incorporation of customised medicine is necessary.

In the development of the brain, several microRNAs have been identified with a differential expression profile, for which the differentiation strategy in cancers represents a new approach. There are two focuses on this regard: on one side, there are miRs which favour the growth of the tumour through the inhibition of the cell differentiation, and the maintenance of a small population of tumour stem cells (cells which retain properties of stem cells). In this case, therapies must be directed to these cell under-populations, introducing molecules which block the functions of the miR (antagomiR) [49]. On the contrary, it is known that the overexpression of some miRs such as miR-451 stimulates the CD133+ cells of GBM to differentiate themselves and lose their character of stem cells [67]. MiR-21 regulates several oncogenic routes and strands, for which it participates in the development and progress of gliomas. This makes it a potential therapeutic target in order to treat these tumours. In the same manner, the therapy headed to restore the levels of miR-34a may achieve anti-tumour effects by inducing their differentiation [66]. MiR-124 and 137 inhibit the expression of the RNAm of CDK6, protein CDK6, and they phosphorylate RB in GBM cells, which demonstrate their potential value in treating this illness. Besides, miR-124 and miR-137 have a potent anti-proliferation effect and pro-differentiation effect in GBM CD133+ and CD133− cells [40] (**Figure 12**).

In the following figure, the re-expression of miR-124 is described as a differentiation therapy in GBM.

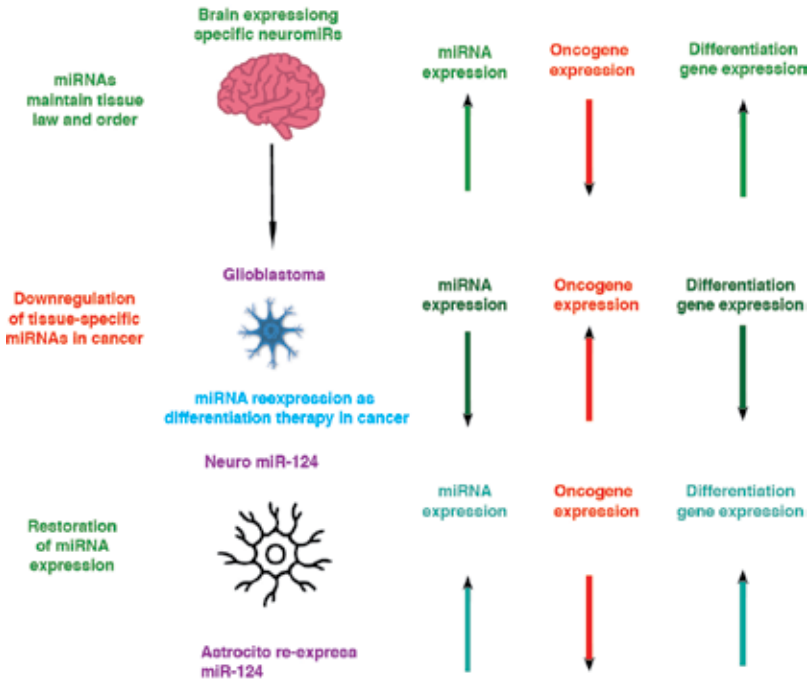

Figure 12. Mature miRNA does not unite to its target RNAm because it is blocked by a complementary therapeutic miRNA.

13. Conclusions and future applications

The expression profile analysis for miRNAs in tumour cells has revealed that the deregulation of these molecules is frequent in a wide array of tumours. MiRNAs may act as tumour suppressants or oncoproteins, which regulate key routes involved in cell growth and apoptosis. Each miRNA may have hundreds of target genes, and several genes are targeted by several miRNAs: this creates a highly complex regulatory network. As we could appreciate in this revision, studies that analyse the expression profile for miRNAs in the different degrees of astrocytomas are scarce; therefore, it is convenient to include a greater number of cases, which helps define the expression profile characteristic for each degree: pilocytic, diffuse, anaplastic and GBM. Within the classing of astrocytic tumours, GBM is the most widely studied tumour, given the fact that it is the most common brain neoplasm in adults and it is quickly disseminated in the adjacent brain tissue, which makes its surgical resection impossible. In GBM, miRNAs participate in several cell processes such as cell proliferation, invasion, angiogenesis and differentiation. Different studies regarding the expression profile of miRNAs in GBM point to overexpressed miRNAs such as miR-10b, miR-21, miR-221 and miR-26 and less expressed miRNAs such as miR-124, miR-128, miR-137, miR-181, miR-7, miR-34 and miR-451. miR-21, miR-221, miR-124, miR-128, miR-181, miR-7 and miR-34 are the best characterised miRNAs with a potential to be used as tumour markers. Nevertheless, it is necessary to correlate the expression profile of miRNAs with clinical and pathological data to answer the therapy or survival of patients.

It is also important to highlight the role that miRNAs undertake in the stem cell, in the differentiation and in cell identity. MiRNAs involved in neural development have also been found deregulated in GBM, which implies that certain miRs allow the growth of the tumour by suppressing the differentiation and maintaining the characteristics of stem cells. Several miRNAs have been identified as having a functional importance in neural development. In particular, miR-7 and miR-124 participate in neural differentiation and are little expressed in GBM. MiR-128 is also altered, but its function in normal cells is unknown. In GBM, the suppression of miR-128 may have severe effects because it may keep the self-renewal of glioma stem cells [42].

The determination and validation of target RNAm will help understand the development of the tumour and will provide potential targets to reduce its growth. In such manner, one of the goals to pursue is to identify a group of miRNAs, whose expression is significantly correlated with clinical parameters and which may be used to classify different degrees of

Little is known about the role of miRNAs as prognosticating indicators. Nevertheless, in astrocytomas, it has been observed that some miRNAs are expressed in a differential manner as miR-221 which is over-expressed in high-grade gliomas, and miR-124 has a lower level of expression in the anaplastic astrocytoma and in the GBM by comparison with low-grade astrocytomas such as the pilocytic and the diffuse astrocytoma. The low expression of miR-137 in astrocytomas is associated with a more advanced clinical phase. The low expression of miR-181b or the high expression of miR-21 was significantly associated with a poor survival of the patient [43].

The miRNAs may have important therapeutic implications, given that they may be functionally antagonised or restored.

MiRNAs are less susceptible to chemical modifications and degradement by ribonucleases by comparison with RNAm. These features of miRNAs allow their detection not only from frozen tissue but also in bodily fluids such as plasma and serum, and even in samples fixed in formol and included in paraffin. This allows for the development of retrospective studies, including a greater number of cases. Particularly speaking, the level of expression starting from bodily fluids represents the most promising advance for a non-invasive diagnosis and allows for their use as biomarkers to detect tumours in early stages and correlating them with clinical development.

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Chapter 5

Mitophagy-Related Cell Death Mediated by Vacquinol-1 and TRPM7 Blockade in Glioblastoma IV

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.77076

Abstract

Glioblastoma IV (GBM) is one of the deadliest malignant diseases in adults and is characterized by a high mutation rate and multiple traits to suppress inborn and acquired immunity. We here approached autophagy-related cell death in newly established GBM cell lines derived from individual tumor isolates. Treatment with a small molecule, termed Vacquinol-1 (Vac) exhibited 100% GBM cell death, which was related to mitochondrial dysfunction, calcium-induced endoplasmic reticulum (ER)-stress, and autophagy. The toxicity of Vac was significantly increased by the inhibition of transient receptor potential cation channel, subfamily M, member 7 (TRPM7). TRPM7 is overexpressed in GBM as well as in many other tumors and thus may be a potential target by the natural compound carvacrol. Of note, at higher concentrations, Vac also induced growth inhibition and cell death in non-transformed cell types. However, in the presence of the TRPM7 inhibitor carvacrol, the tumor-selective effect of Vac was very much increased. Results given in the present study are based on long-term video microscopy using IncuCyteZOOM®, calcium measurements, and 3D ultrastructural analysis using the cryofixed material.

Keywords: glioblastoma IV (GBM), Vacquinol-1, TRPM7, carvacrol, calcium, endoplasmic reticulum (ER)-stress, autophagy, mitochondria, mitophagy, cell death, 3D cryoelectron microscopy

1. Introduction

The search for better tumor treatments in glioblastoma (GBM) and other cancers is ongoing. Recently, a major step forward has been achieved on the basis of immune interventions. Specifically, humanized antibodies directed against immune checkpoint antigens are now available to reconstitute immune recognition of the malignant cell in vivo [1]. In addition to the ongoing search for novel checkpoint interventions, chimeric T cell antigen receptors recognizing clonal neoantigens of a given malignancy are promising candidates for an individualized approach [2]. For a broader range of tumors, however, the identification of novel chemotherapeutic drugs [2], potentially resulting in reduced tumor mass as well as improved stimulation of antigen presentation [3], and remain to be of high interest. In this context, we have been intrigued by the original observations of a small synthetic molecule, named Vacquinol-1, and its capacity to fulminant induce cell death in GBM. Even though the original description has been retracted due to non-reproducibility of the in-vivo data (http://retractionwatch.com/2017/07/20/not-want-create-false-hope-authors-retract-cell-paper-cant-replicate/), we here report novel findings on using Vacquinol-1 (here termed: Vac). The synthetic small molecule Vac appears to impair the hydrostatic balance of a tumor cell and induces vacuolization ending in cell rupture. The small therapeutical concentration window of action by Vac limits its application due to a cascade of side effects to occur in vivo [4, 5]. Interestingly, we recently reported that the discriminative effect by Vac against glioma, as opposed to non-transformed tissues could be increased in the presence of a plant derivative, carvacrol [6], likely acting by inhibiting TRPM7 [7]. We here provide evidence for a mechanism on Vac-induced cell death by deteriorating the mitochondrial membrane potential, increasing high intracellular calcium levels combined with endoplasmic reticulum (ER)-stress [8] and impaired calcium storage, followed by mitophagy and rupture of lysosomes and autophagolysosomes. Lysosomal rupture seems to constitute the final event leading to cell death by hydrostatic pressure-associated rupture of autophagolysosomes and the plasma membrane. The decisive effect of Vac against transformed vs. non-transformed tissues may be explicable by the functional sensitivity of TRPM7 to carvacrol in glioma. Essential aspects proving ER-stress and mitochondrial dysfunction have been unraveled by using 3D cryoelectron microscopy.

2. Results and discussion

We here extended our previous work on Vac sensitivity in 2 glioma cell lines (#12537-GB, U-87), and non-transformed dental pulp stem cells (DPSCs) [6], to a total of 7 other glioma cell lines, two fibroblast cell cultures and bone marrow stroma cell isolates. Using IncuCyteZOOM® long-term video microscopy, we determined Vac concentrations resulting in half-maximal cell death (inhibitory concentration: IC50) after 24 h. Accordingly, IC50 values for Vac to result in 50% cell death after 24 h ranged between 7.7 and 12.2 μM Vac in glioma cell lines, whereas non-transformed cells (fibroblasts, bone marrow stroma, and DPSCs) were less sensitive to Vac, resulting in IC50 values between 9.5 and 22.5 μM Vac. For further studies, we focused on two glioma cell lines with high (#12537-GB, IC50 = 8.2 μM), and low Vac sensitivity, respectively (#12794-GB; IC50 = 10.2 μM), indicating a more resistant phenotype. In the presence of the natural compound

carvacrol, Vac-induced cell death was found to be increased in #12537-GB, as previously reported [6], an observation corresponding to the lower proliferative and less invasive phenotype of GBM when carvacrol was administered [7]. Carvacrol has been demonstrated to inhibit the transient receptor potential cation channel, subfamily M, member 7 (TRPM7 [7, 9], explaining the higher sensitivity of Vac-induced toxicity to the glioma cell line #12537-GB [6]. The gene encoding TRPM7 functions as both: An ion channel and a protein kinase [7]. In our experimental set-up, the addition of carvacrol at concentrations of 100 μM selectively increased the sensitivity to Vac-induced toxicity in our glioma cell lines, but not in non-transformed fibroblasts. To determine cell viability of pre-established cell layers, we applied the live cell imaging system IncuCyteZOOM® equipped with a 20× objective. Propidium-iodide staining was used to identify and count dead cells in a time-dependent manner. As shown in **Figure 1A**, high Vac concentrations >10 μM led to rapid cell death in both GBM cell lines as well as in non-malignant fibroblasts; 7 μM Vac resulted in significant cell death in #12537-GB cells (**Figure 1A**) after 12 h of incubation but not in #12794-GB cells (**Figure 1B**) and non-transformed fibroblasts (**Figure 1C**). The influence of carvacrol on Vac-induced cell death in glioma cell lines is shown in **Figure 1D** (#12537-GB), and **Figure 1E** (#12794-GB), and non-transformed fibroblasts (Hs68-Fi) in **Figure 1F**. After 56 h, carvacrol (100 μM) significantly (Sidak's Post hoc test after two-way ANOVA; p-values between 0.0198 and 0.0001) enhanced Vac-induced cell death in #12794-GB but not in non-transformed fibroblasts (**Figure 1D–F**). Accordingly, only glioma cell lines and not fibroblasts increased their sensitivity against Vac in the presence of carvacrol. To understand the action by carvacrol acting on TRPM7, we performed calcium measurements of glioma cells upon Vac stimulation with and without carvacrol pre-incubation. **Figure 2** demonstrates a rapid loss of cytoplasmic calcium in both GBM cell lines followed by subsequently elevated calcium levels. The addition of ionomycin was included at the end of the experiment to test the residual capacity of the target cell to mount a calcium response. This ionomycin-induced calcium peak was higher in #12537-GB than in #12794-GB. When carvacrol was present, Vac-treatment caused a rapid loss of cytoplasmic calcium again in both cell lines. However, the Vac-induced loss of cytoplasmic calcium was not followed by an increased cytoplasmic calcium level (**Figure 2C** and **D** cf. **Figure 2A** and **B**). The latter might have been due to the inhibition of TRPM7 and loss of control of store-operated calcium channels (SOCEs). Along with this line, Faouzi et al. recently, described the regulation of SOCEs by the kinase activity of TRPM7 [10]. As a consequence, blockade of the TRPM7 would lead to an impaired reconstitution of calcium sequestration to cytoplasmic stores [10].

The molecular events by Vac affecting lipid bilayer integrity and its proton catching properties may contribute to dysfunction of calcium-storage organelles, such as ER and mitochondria. In the end, Vac appears to transiently affect the integrity of lipid bilayers in calcium storage organelles, resulting in transient leakage of bivalent cations such as calcium (**Figure 2**). The partial reconstitution of membrane integrity in the presence of Vac in the range of IC50 values appears to be significantly impaired in the presence of the TRPM7 blocker, carvacrol. As a consequence, calcium influx from external medium would be inhibited [10] and thereby lead to impaired calcium sequestration by ER and mitochondria. In the end, calcium measurements proved, that carvacrol appears to be essential to block endogenous membrane reconstitution. The observations of the combined effects by Vac and carvacrol remind of results obtained in a study performed with curcumin, which was tested on cell death in a melanoma cell line. Bakhshi and colleagues found a dramatic increase of ER-induced cell stress in curcumin-treated melanoma

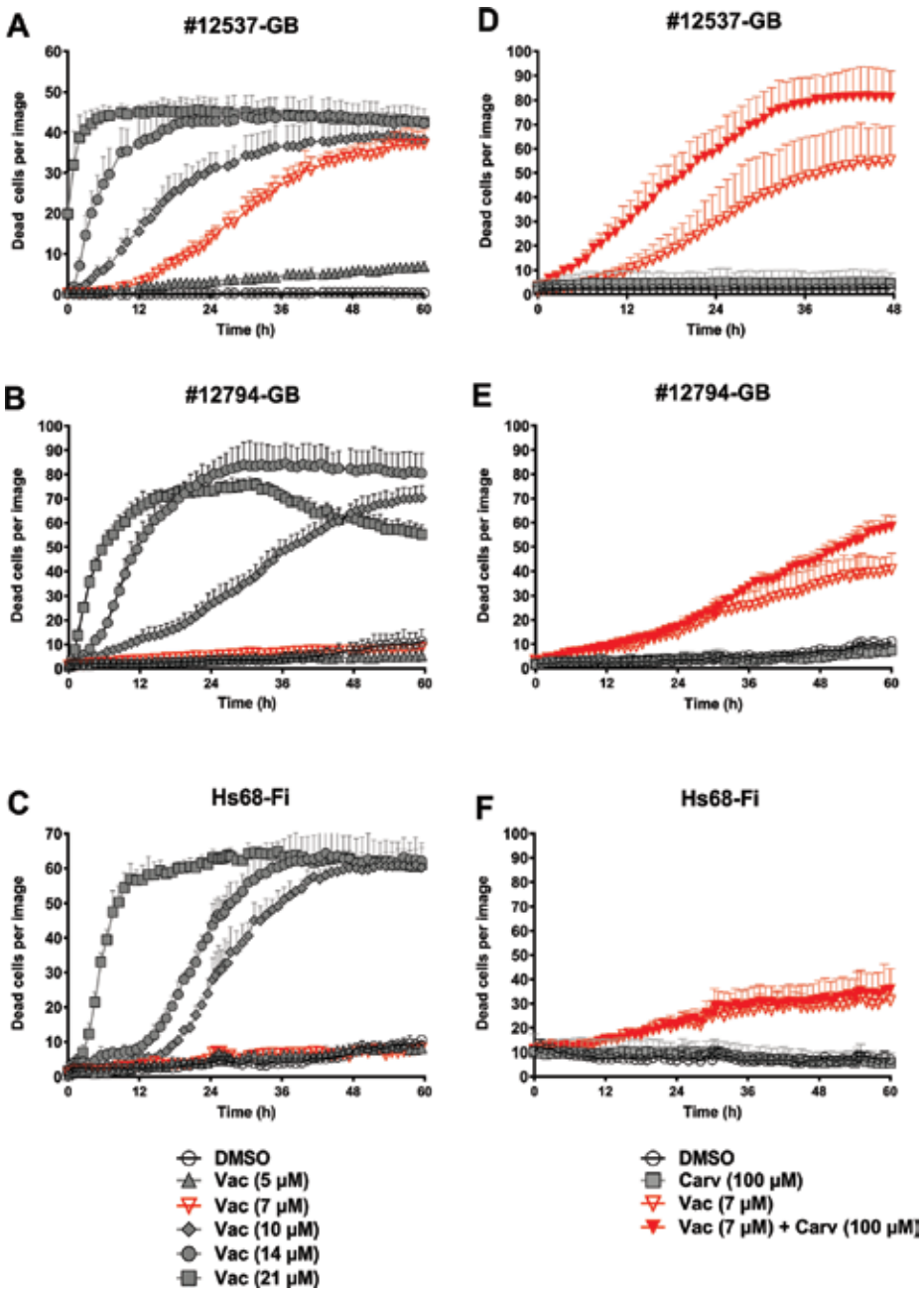

Figure 1. Cell death induced by Vac in the absence and presence of carvacrol. Kinetics of cell death were determined from the PI-positive (dead) cells displayed as red object count/image (y-axis). #12537-GB, #12794-GB, and Hs68 were followed for 60 h (x-axis) (IncuCyteZOOM®). The cells were treated either with DMSO (vehicle control), 5, 7, 10, 14, or 21 µM Vac. All values are means ± SD (biological triplicates) (A–C). Semi-confluent #12537-GB, #12794-GB, or Hs68 were treated with DMSO (vehicle control) or Vac (7 µM) in the presence or absence of 100 µM carvacrol. #12537-GB were followed for 48 h, and #12794-GB, and Hs68 were followed for 60 h (x-axis) (IncuCyteZOOM®). All values are means ± SD (#12537-GB: six biological replicates; #12794-GB and Hs68-Fi: biological triplicates) (D–F). All imaging was performed using IncuCyteZOOM® at 20× objective.

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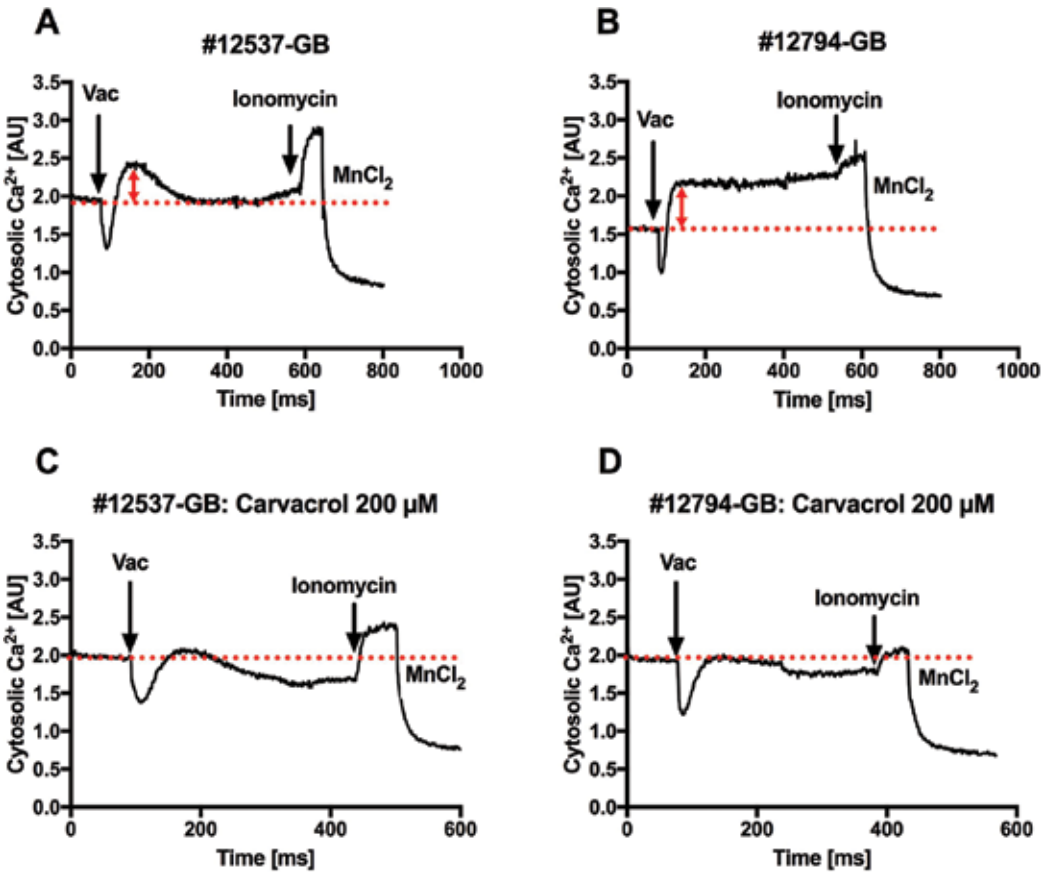

Figure 2. Calcium signaling in GBM cell lines in presence and absence of carvacrol. Calcium responsiveness was tested in Fura-2 (Thermofisher.com)-labeled #12537-GB cells or #12794-GB cells using a LS55 luminescence spectrometer (PerkinElmer.com). After labeling, cells were diluted in HBSS (Hank's balanced salt solution) at a cell concentration of 1×10^6 per ml. The cells were pretreated with DMSO (vehicle control) (A and B) or 200 µM carvacrol for 1 h (C and D). Upon suspension in the cuvette of the spectrometer, suspension cells were stimulated with Vac 7 µM followed by ionomycin (1 µM, Sigma.com). At the end of the experiment, the Fura-2 signal was quenched using $MnCl_2$. Results are given as relative fluorescence ([AU], arbitrary units) and are representative for two independent experiments. The red dashed line emphasizes increased cytoplasmic calcium levels following the calcium depletion induced by Vac (A and B), which is absent after carvacrol pre-incubated cell suspensions (C and D).

cells an effect, which appeared to overrun the chronically active cytoprotection of a malignant, chronically stressed cell type [11]. In contrast to non-transformed tissues, cancer cells are dependent on chronic stress pathway activation [11]. The effector of cell death mediated by curcumin appeared to be due to the massive and acute stress by a curcumin-inducible down-regulation of the protective stress proteins. Curcumin is expected to inhibit the ATPase pump and similarly to carvacrol in GBM, may lead to increased cytoplasmic calcium concentrations. Again, ER-stress appears to contribute to impaired membrane integrity and allows calcium to freely enter the cells. Under conditions of the blocked sequestration of calcium to mitochondria and ER, this effect is expected to be even more dramatic to the target tissue [11]. Pathway analysis proved ER-stress related cell death in this melanoma model. As a consequence, the

authors hypothesized that mechanisms over activating stressors in malignant cells may be crucial for innovative approaches in cancer therapy [11]. In the case of Vac, a mechanism of impaired mitochondrial calcium sequestration is likely due to uncoupling of oxidative phosphorylation by Vac, which has been accurately investigated and described by Feng and colleagues [12]. Uncoupling of mitochondria results in a collapse of the mitochondrial membrane potential (ΔΨm), which may explain the loss of mitochondrial membrane integrity. To assess whether Vac influences ΔΨm in GBM, we used the cyanine dye JC-1 (5, 50, 6, 60-tetrachloro-1, 10, 3, 30 tetraethylbenzimidazolocarbo-cyanine iodide) forming J-aggregates in mitochondria with high ΔΨm spectrally distinguishable from dye monomers at lower ΔΨm [13]. JC-1 monomers emit green fluorescence with a maximum at 530 nm (green), whereas J-aggregates emit orange-red fluorescence with a maximum at 595 nm (orange-red). After 4 h treatment, Vac leads to a significant decrease in the ratio of red/green fluorescence indicating a collapse of the ΔΨm (**Figure 3A**). In summary, the reduction of ΔΨm upon Vac treatment emphasizes the uncoupling properties of Vac as previously described by Feng and colleagues [12].

Moreover, the detrimental process in Vac treated GBM cells occurs in parallel with the upregulation of autophagy, which has been addressed by using an amphiphilic tracer CytoID®. CytoID® stains lysosomes minimally while maximizing the fluorescence of autophagosomes [14]. Using the CytoID® autophagy detection kit (**Figure 3B**), we found increased green fluorescence indicating upregulation of autophagy upon 4 h Vac treatment. These results correspond to upregulation of LC3-II determined by Western-Blot (data not shown). Reactive oxygen species formation (ROS) was also upregulated by Vac treatment as demonstrated in **Figure 3C**. Carboxy-H_2DCFDA is non-fluorescent but in the presence of ROS, when this reagent is oxidized, it becomes green fluorescent [15]. The overall proof of Vac-induced pathology in GBM is here provided by 3D cryoelectron microscopy (**Figure 4**). The screenshot of the 3D tomogram performed from GBM during constitutive culture shows occasional mitophagy, occurring by phagophore formation and subsequent digestion in autophagolysosomes (**Figure 4A**). In addition, relatively high numbers of autophagolysosomes, sparse microtubules, and unorganized actin filaments are present ([16], **Figure 4A**, untreated #12537-GB). By contrast, GBM, treated with Vac for 4 h differ by the following parameters: Identification of (i) massive stress fiber formation, (ii) formation of actin bundles attached to bent mitochondria, and (iii) impressively enlarged cisternae of ER, which indicates ER-stress (**Figure 4B**, 4 h Vac-treated #12537-GB). Moreover, the autophagolysosomes contain electron dense structures, which were structurally compatible with the partially digested mitochondrial material (**Figure 4B**, white arrows). The autophagolysosomes almost never showed a double lipid bilayer, which may indicate that the outer mitochondrial membrane might have been involved in the formation of autophagosomes, as previously described [17]. In the accompanying tomogram (https://mts.intechopen.com/download/index/process/155/authkey/5fc95d7040949214f944031367b18403), a Vac-treated #12537-GB cell has been analyzed from an area in the vicinity of the nucleus. This tomogram shows an elongated, bent mitochondrion, attached to a bundle of active fibers, which appear to be involved in bending (or even traction?) of the organelle. The ER is very close to the mitochondrion with enlarged cisternae, a characteristic of stressed cells as previously demonstrated. This tomogram also shows that the number of autophagolysosomes is dramatically increased when compared to the non-treated #12537-GB (cf. supplementary video I in Gorbunov and Schneider [16]). Finally,

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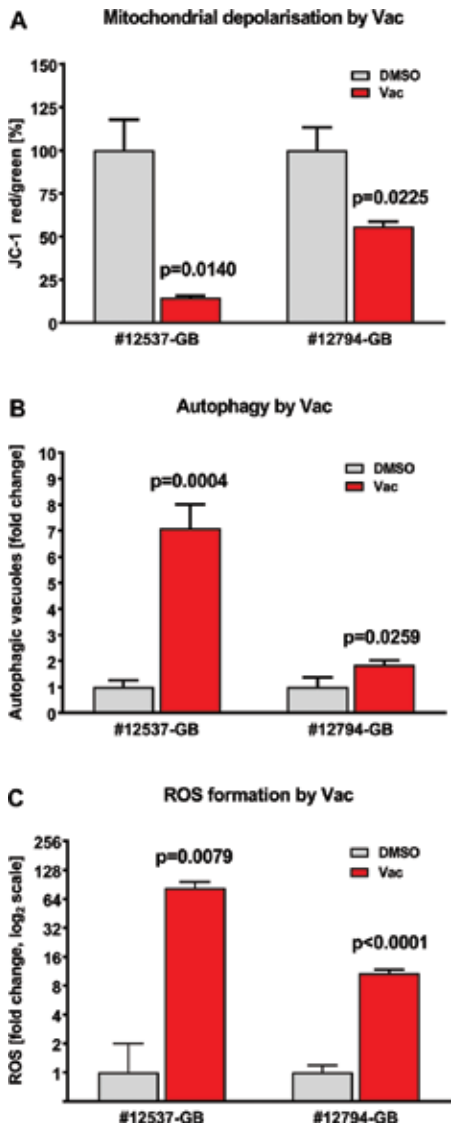

Figure 3. Vac induces autophagy/mitophagy in GBM cells, induces ROS formation and leads to a decrease in ΔΨm. Semi-confluent #12537-GB or #12794-GB cells were stained with JC-1 (2 μg/ml). JC-1 stained cells were treated with DMSO (vehicle control) or 7 μM Vac for 4 h. Quantitative analysis of the ratio red/green fluorescence. Vac leads to a decrease in the ratio red/green in #12537-GB ($p = 0.014$, t-test) as well as #12794-GB ($p = 0.0225$, t-test) indicating a collapse of the ΔΨm. All values are mean values of the total red/green integrated fluorescence intensity per image ± SD (biological triplicates). Data were normalized to the corresponding control (100%) (A). Semi-confluent #12537-GB cells were treated with DMSO (vehicle control) or 7 μM Vac for 4 h. After incubation, cells were stained with the green-fluorescent autophagosome/autophagolysosome dye CytoID®. Quantitative analysis of autophagic vacuoles (fold change compared to DMSO control). Vac leads to an increase in autophagic vacuoles in #12537-GB cells as well as in #12794-GB cells ($p = 0.0004$ and $p = 0.0259$, respectively, t-test). All values are mean values of the green object count ± SD (biological triplicates). Data were normalized to the respective control (B). Semi-confluent #12537-GB or 12794-GB cells were treated with DMSO (vehicle control) or 7 μM Vac for 4 h. After incubation, cells were stained with CM-H_2DCFDA (green fluorescent) to detect ROS. Quantitative analysis of ROS production after 4 h in #12537-GB or #12794-GB. Vac leads to an increased ROS production in #12537-GB ($p = 0.0079$, t-test) as well as #12794-GB ($p < 0.0001$, t-test), (C). All values are mean values of the total green integrated fluorescence intensity per image ± SD (biological triplicates). Data were normalized to the corresponding control. All images were obtained using IncuCyteZOOM® equipped with a 20× objective.

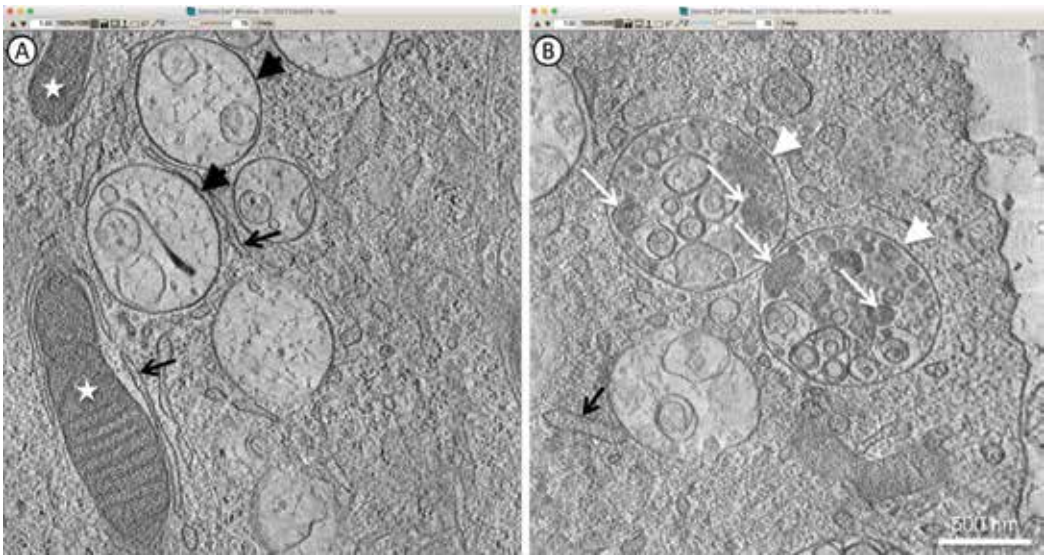

Figure 4. Ultrastructural changes induced by Vac in GBM cells (#12537-GB). Virtual sections of #12537-GB cells investigated by STEM tomography demonstrate active mitophagy in control cells with early phagophore formation around mitochondria (white stars) by smooth ER cisternae (black arrows); in addition, numerous autophagolysosomes (black arrowheads) are shown with fully digested cytoplasmic as well as organelle derived material (A), this video section is part of the video tomogram provided as supplementary file 1 [16]; following Vac-treatment (4 h, 7 μM), phagophore formation is not prominent, ER appears to be swollen (black arrow as an example), and autophagolysosomes (white arrowheads) are more abundant. The material ingested is not fully digested, residual mitochondria can be identified by their morphological remnants (white arrows) (B). https://mts.intechopen.com/download/index/process/155/authkey/5fc95d7040949214f944031367b18403.

the massively upregulated mitophagy in 4 h Vac-treated GBM does not appear to occur with microtubule-assisted mitochondrial fission, as demonstrated in stressed tumor cells [18] since microtubules appear to be randomly distributed (https://mts.intechopen.com/download/index/process/155/authkey/5fc95d7040949214f944031367b18403).

3. Materials and methods

3.1. Cell lines and cell culture

The glioma cell line #12537-GB has been previously described [6]. The #12794-GB cell line has been established from a female patient's tumor material received from the neurosurgical department of the Bezirkskrankenhaus in Günzburg (Universal trial number: U111-1179-3127) with patient-informed consent. The tumor material was minced and cells from the tumor material were taken into the culture by trypsinization of the tumor material (2.5% trypsin), followed by Ficoll separation. Continuous cultures were performed in Iscove's Modified Dulbecco's Medium (IMDM) (Lonza.com, USA) supplemented with 10% fetal calf serum (FCS, endotoxin-free, Batch 0247x, Merck/Biochrom.com, Germany), GlutaMAX (ThermoFisher.com, USA), and antibiotics at 37°C under 5% CO_2. Hs68 fibroblasts (Hs68-Fi) were purchased from ATCC, and cultured in IMDM containing 10% endotoxin-free FCS (Batch No: 0247x, Merck/Biochrom.com).

3.2. Ethical statement

The work with human GBM material/cell lines has been approved by the local Ethics Committee of the University Hospital Ulm (universal trial number: U111-1179-3127) with patient-informed consent.

3.3. Analysis of cell death by IncuCyteZOOM®

Cells were seeded into flat-bottom 96-well microtiter plates (Sarstedt.com, Germany) (density: 17,000 cells per cm^2). Semi-confluent cell layers were treated one day after seeding with DMSO (vehicle control) or Vac (Selleckchem.com, Germany; Stock: 76 mM in DMSO) in the presence of 10 μg/ml PI (Sigma.com, USA). Carvacrol was purchased from Sigma-Aldrich (Sigma.com, USA). Cell death was quantified as the red object count/image by the IncuCyteZOOM® software.

3.4. Detection of autophagy/mitophagy using Cyto-ID® staining

Staining of autophagic vacuoles was performed using the CytoID® autophagy detection kit (Enzolifesciences.com, Germany). GBM cells were seeded into flat-bottom 96-well microtiter plates (Sarstedt.com). One day after seeding, semi-confluent GBM cell layers were treated for 4 h with 0.01% DMSO (vehicle control) or 7 μM Vac to induce autophagy. After treatment, the culture medium was replaced by fresh culture medium containing CytoID® green detection reagent in a 10^{-3} dilution. After 15 min of incubation at 37°C, cells were washed twice in pre-warmed Hank's balanced salt solution (HBSS). Imaging was performed by IncuCyteZOOM®. Green object count reflecting autophagosomes/autophagolysosomes was quantified and collected by IncuCyteZOOM® software.

3.5. Detection of ROS

To detect ROS, carboxy-H_2DCFDA (CM-H_2DCFDA) (Thermofisher.com, USA) was used. In the presence of ROS, the dye gets oxidized and emits green fluorescence. GBM cells were seeded into flat-bottom 96-well microtiter plates (Sarstedt.com). One day after seeding, semi-confluent GBM layers were treated with 0.01% DMSO (vehicle control) or 7 μM Vac. After treatment, CM-H_2DCFDA (stock 5 mM in DMSO) was added in a final concentration of 5 μM to the culture medium (IMDM, 10%FCS). Imaging was performed by IncuCyteZOOM®. Total green fluorescence per image was quantified and collected by IncuCyteZOOM® software.

3.6. Evaluation of mitochondrial membrane potential using JC-1

To evaluate mitochondrial membrane potential, the dye JC-1 (5, 5′, 6, 6′-Tetrachloro-1, 1′, 3, 3′-tetraethyl-imidacarbocyanine iodide) was used (Thermofisher.com). In healthy mitochondria with high membrane potential, JC-1 forms red fluorescent aggregates. In contrast, JC-1 occurs as green fluorescent monomers in mitochondria with depolarized membrane potential. Semi-confluent GBM cell layers seeded into 24-well microtiter plates (Corning.com) (density: 17,000 cells per cm^2) were stained one day after culture with 2 μg/ml JC-1 for 30 min. After incubation, the culture medium was removed and cells were washed twice with pre-warmed HBSS. After

washing, the fresh culture medium was added and cells were treated for 4 h with 0.01% DMSO (vehicle control) or 7 μM Vac. Imaging was performed by IncuCyteZOOM® and red/green total fluorescence per image was quantified and collected using IncuCyteZOOM® software. The ratio between red and green fluorescence was calculated. A shift from red to green fluorescence and therefore, a decline in the red/green ratio indicates mitochondrial depolarization.

3.7. Electron microscopy

Sapphire discs (Engineering Office M. Wohlwend GmbH, Switzerland) were used as support for the adherent glioma cell culture. The 0.170 mm thick sapphire discs were carefully immersed in complete medium and the cells were seeded on top. A 50 μm gold spacer ring (diameter 3.05 mm, central bore 2 mm; Plano GmbH, Germany) was mounted in between two sapphire discs with the cells grown on them, similar to the protocol introduced by Hawes et al. [19]. These sandwiches were high-pressure frozen without aluminum planchettes and without the use of hexadecene. Freeze substitution was performed as described in Villinger et al. [20] with a substitution medium consisting of acetone with 0.2% osmium tetroxide, 0.1% uranyl acetate, and 5% water for 19 h. During this time period, the temperature was exponentially raised from 183 to 273 K. After substitution, the samples were maintained at room temperature for 30 min and then washed twice with acetone. After stepwise embedding of the samples in Epon (Fluka.com, USA) (polymerization at 333 K within 72 h), they were cut using a microtome (Leica Ultracut UCT ultramicrotome) with a diamond knife (Diatome, Switzerland) to semi-thin sections with a nominal thickness of 550 nm.

For STEM tomography, 550 nm semithin sections were coated with 25 nm gold particles serving as fiducial markers for alignment of the images to a tomogram. The tilt series was collected on a JEM-2100F electron microscope operated at an acceleration voltage of 200 kV in the scanning transmission mode. Electron micrographs were recorded with a bright-field detector at a pixel size of 2.74 nm. A tilt-series was recorded from −72° to +72° at increments of 1.5°. The tomogram was created out of 97 images using the IMOD software package. Images were first aligned to an image stack and then computationally reconstructed using a weighted back-projection algorithm to form the tomogram [20].

3.8. Statistical analysis

Statistical analysis was performed using GraphPadPrism v7 (Graphpad.com, USA). To identify significant differences of endpoint analyzes, an unpaired two-tailed t-test was used. P-values are given in the respective graphs (column with the treatment). In case of kinetics, two-way ANOVA followed by Sidak's correction for multiple comparison. Multiplicity adjusted p-values are given. Observations with $p < 0.05$ were considered as significant.

Acknowledgements

We thank the International Graduate School in Molecular Medicine, Ulm University for supporting and evaluating HM, PS, and PSch. We are grateful to IntechOpen publisher for generous support to publish a virtual section of the supplementary file 1 in reference [16], as

Figure 4A (https://www.intechopen.com/books/autophagy-in-current-trends-in-cellular-physiology-and-pathology/introductory-chapter-overview-on-autophagy-in-burden-of-functions).

Conflict of interest

The authors declare no conflicts of interest.

Authors contributions

EMS drafted the manuscript (ms), PW performed STEM microscopy, 3D tomography and reconstruction, PS performed experiments, designed figures and contributed to the ms text; BM performed experiments and drafted the ms, MH, and PSch contributed to chemical property analysis of Vac, HM contributed to trans electron microscopy, AP, CRW, and MG edited the ms. All authors reviewed and approved the final version of the ms.

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Chapter 6

Diffuse Astrocytoma and Oligodendroglioma: An Integrated Diagnosis and Management

Ștefan Ioan Florian and Sergiu Șuşman

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.76205

Abstract

For the first time, the WHO classification of brain tumors has introduced molecular parameters in the diagnosis of brain tumors. Together with embryonal tumors, the diffuse gliomas have suffered significant changes in diagnosis, prognosis, and response to treatment. A new concept of "integrated diagnosis" comes to combine the classical diagnosis with the molecular one. While it is still impossible to disregard the histopathological component, according to the new rule ("molecular beats histology") makes molecular parameters dominant in the final diagnosis. Currently, the diffuse gliomas (oligodendroglial or astrocytic) are nosologically closer than the astrocytomas with a diffuse growth pattern, and the astrocytomas with a more circumscribed growth pattern defined by the presence of the IDH mutation. The family tree was redefined by the presence of the IDH mutation and of the 1p/19q codeletion. The implementation of this new concept in clinical practice will improve patient management, as well as the design of clinical trials and experimental studies. This must also be seen as a model for diagnosis setting in the new molecular era.

Keywords: astrocytoma, oligodendroglioma, glioblastoma, IDH mutation, 1p/19q co-deletion, integrated diagnosis

1. Introduction

The introduction of the new classification represents the first step in the switch of paradigm in brain tumor management toward an individualized-based treatment from the, nowadays, evidence-based management. The classification of diffuse gliomas has undergone significant changes following the introduction of molecular testing, the new WHO 2016 classification introducing a new concept—integrated diagnosis [1]. The current update takes into

account both the phenotype and the genotype [2]. This was the preferred alternative, as it is currently impossible to resort only to the molecular parameters in the definition of tumoral entities [3]. The classification relies both on the morphological character (growth pattern) and on the definition of the genetic status by determining the presence of mutations in the IDH1 and IDH2 genes and of the 1p/19q codeletion [4]. According to WHO 2016, diffuse gliomas are lumped together, regardless of their histopathological aspect (astrocytomas or oligodendrogliomas) [5].

The recourse to an integrated diagnosis makes it possible to formulate a more precise diagnosis of the various entities and acknowledges the existence of new entities [6].

The introduction of molecular parameters comes to improve the clinical management of patients, defining entities which feature a similar prognosis. It also paves the way to the identification of new treatment methods aimed at the biological mechanisms common to this type of tumors [5]. The genetic information supplied by the Cancer Genome Atlas Research indicate that the supratentorial gliomas with a diffuse growth pattern can be categorized separately from the other brain tumors (**Figure 1**) [7]. They are grouped into three categories, according to the genetic profile—the presence or absence of the 1p/19q codeletion and the mutational status of the IDH gene. The first category includes the gliomas with a classical morphology of oligodendrogliomas, having both the IDH gene mutation and the 1p/19q codeletion. The second category is represented by the tumors with an astrocytic histological pattern and IDH mutation, but without the 1p/19q codeletion. In the third category, we find the tumors with an astrocytic phenotype, which display no IDH mutation or 1p/19q codeletion. The latter category usually falls under the classical wild-type glioblastoma diagnosis [8].

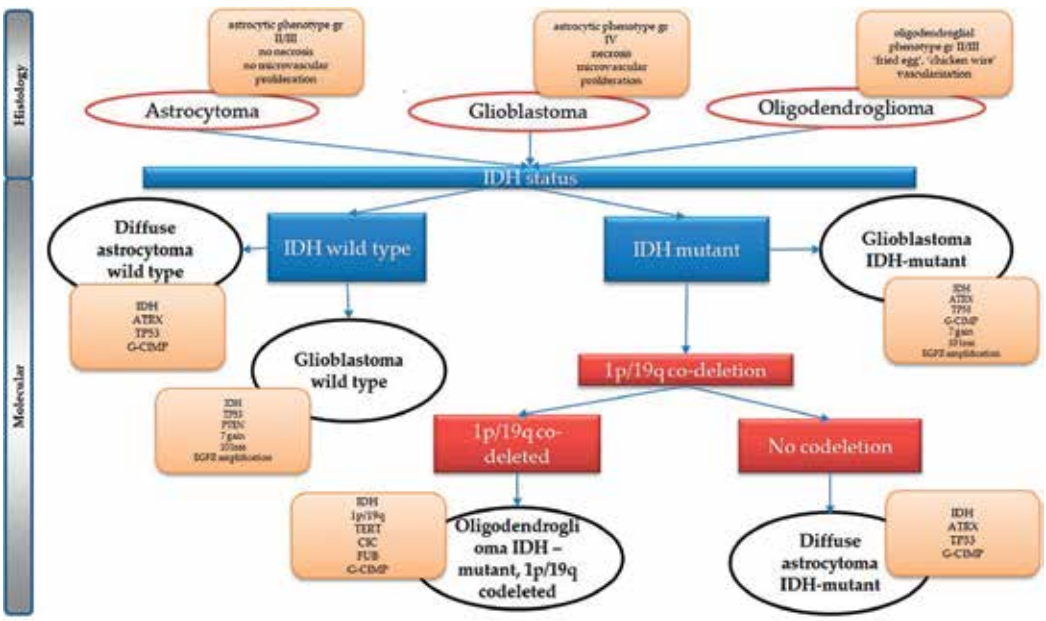

Figure 1. Integrated diagnosis of astrocytic and oligodendroglial tumors.

This approach separates between the astrocytomas with a circumscribed growth pattern, no IDH mutation, and with BRAF mutation (pilocytic astrocytoma, pleomorphic xantoastrocytoma, and subependymal giant cell astrocytoma), on the one hand, and the diffuse astrocytic and oligodendroglial tumors, on the other. According to the new classification, the diffuse astrocytic and oligodendroglial tumors are nosologically closer than the diffuse astrocytoma and the pilocytic astrocytoma [9].

The inclusion in these categories on the basis of genetic determinations also has a role in prognosis [10].

When there is a mismatch between the phenotype and the genotype, genetic tests set the final diagnosis in keeping with the rule "molecular beats histopathology [1]."

Glioblastoma with the IDH mutation show a better evolution than the wild-type ones, generally corresponding to a secondary glioblastoma. They also have a better prognosis than the wild-type anaplastic astrocytoma. The wild-type astrocytomas have the worst prognosis of all astrocytomas, their molecular profile being characteristic for glioblastomas (EGFR amplifications, PTEN mutation, and 10q, 9p loss) [11].

The introduction of molecular parameters in the definition of entities has led to the recognition of a new entity in the group of diffuse pediatric gliomas: the tumors with a midline location, diffuse growth pattern, and the K27 M mutation in the H3 histone gene. This is the first attempt to distinguish between the pediatric brain tumors and their adult counterparts, the difference in behavior between histopathologically identical tumors being long known [12].

As to the histological grading, the WHO 2016 classification keeps the three-tiered system. The shift from low to high malignancy depends upon morphological parameters that reflect the emergence of new biological processes. The *first malignancy* criterion is represented by the variations in the size, shape, and color intensity of the nucleus (*atypia with hyperchromasia*) [13]. The *proliferation* is reflected in the presence of mitoses, which must be unequivocal, with no additional specifications in terms of number and morphology [14]. A significant proliferation typical for high-grade tumors is highlighted by the advent of *necrosis* and of the attempt to compensate for this hypoxia through the emergence of *microvascular proliferation*. According to this classification, the *diffuse astrocytomas* limited to *cytological atypia* are deemed to be *grade II*, while those with *both anaplasia* and *mitotic activity* are deemed to be *anaplastic* (*grade III*). The presence of mitoses must be seen in context as only one mitosis in a large section is not enough for a grade III. If we are dealing with a small biopsy, then the presence of a mitosis may be sufficient. *Grade IV* is reserved to those tumors that display *necrosis and/or vascular proliferation*. Microvascular proliferation is defined as the stratification of the endothelium, or the "glomeruloid" aspect. The necrosis can be of any type [15].

In the group of low-grade gliomas (II and III), the histopathological stratification features a significant interobserver variability, as also demonstrated by the considerable differences in terms of survival rates manifest within this group. The evaluation of molecular parameters can also be useful in the sense of defining groups that correlate better with the prognosis [16].

Gliomatosis cerebri no longer exists as an entity, being considered rather a specific growth pattern. More research is needed in order to identify the biological substrate of this unusual invasive capacity [17].

2. Low-grade gliomas

2.1. Diffuse astrocytoma IDH-mutant (DA IDH-mut)

Definition. Tumoral proliferation which, from a histopathological point of view, shows an astrocytic phenotype with a diffuse growth pattern and IDH1 or IDH2 gene mutations.

As to *grading*, diffuse astrocytoma is deemed to be *grade II* (low-grade diffuse astrocytoma).

Clinically, the emergence of symptoms is usually insidious, as they precede the diagnosis by weeks months or sometimes years. Seizures are the symptom most likely to raise suspicions. There are studies underling the presence of the seizures at the debut in up to 80% of cases, approximately 50% presenting with uncontrollable seizures at the time of surgery. Factors predisposing to a poorer response to antiepileptic drugs are: partial seizure type, temporal location, and a longer seizure duration [18]. The most frequent location of those tumors is in the frontal lobe, followed by temporal and parietal lobe. Other associated symptoms could appear related to the location. Behavioral and personality changes, visual disturbances, aphasia, or agnosia are most frequently mentioned, meanwhile the increased intracranial pressure symptoms install later in the course of disease, related to the tumoral volumes and mass effect.

Imaging. *MRI* is the "golden standard" imagistic tool. For DA IDH-mut, the typical aspect is a homogeneous tumor with low signal intensity on T1-weighted images and a high intensity on T2-weighted sequences. This high T2 signal is rather related to edema, demyelination, or degenerative changes than to cellular atypia. Fluid-attenuated inversion recovery (FLAIR) sequences are the most appropriate for defining the infiltrating tumor margins. Usually, astrocytomas are confined in the white matter, meanwhile the oligodendrogliomas are cortical-based tumor, but this difference is attenuated in the later stages (**Figure 2**). Cystic components are not infrequent and low contrast enhancement could be observed in 20% of cases without malignant transformation [19].

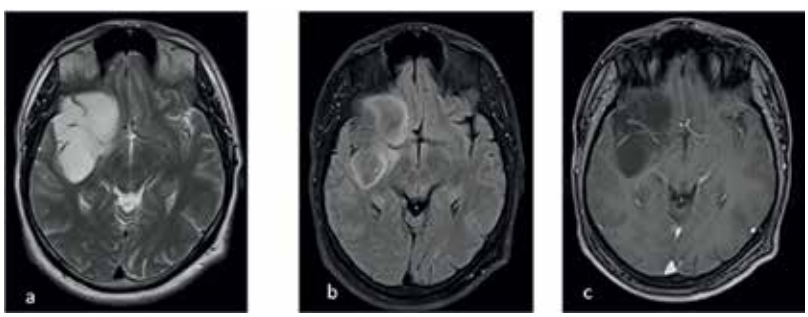

Figure 2. Axial T2W (a), FLAIR (b), and T1W + C (c) MRI examination of a patient with right insular DA IDH-mut (personal archive).

Advanced MRI techniques such as diffusion-weighted imaging (DWI) and MRI spectroscopy will complete the anatomical information, while the functional MRI and diffusion tensor imaging (DTI) will offer important data for surgical planning. On *DWI*, DA IDH-mut presents a decrease cellularity and non-restricted diffusion. *MRI Spectroscopy* will reveal not only decreased N-acetyl-aspartate (NAA) peak, medium choline peaks, absence of lactate peak, and increased myo-inositol [20], but is also able to detect IDH mutation trough oncometabolite 2-hydroxyglutarate (2HG) present in tumor cells [21]. MRI could also serve as a *prognosis tool* if is combined with IDH status. Just recently it was suggested that minimum apparent diffusion coefficient (ADC_{MIN}) threshold of 0.9×10^{-3} mm^2/s or less is associated with a worst prognosis especially when it is combined with IDH wild-type grade II diffuse astrocytomas [22]. *DTI with tractography* usually reveals a displacement rather than an infiltration or destruction of fiber tracts in DA IDH-mut tumors.

CT scan reveals a homogeneous lesion, poorly defined, with no contrast enhancement. This can be associated with cystic changes and calcifications that are more specific for oligodendrogliomas.

Macroscopy. In section, the tumor does not display clearly delineated limits, on account of its infiltrative growth pattern. We can see areas of soft consistency or firmer ones, granular areas, and cystic ones. Cystic changes can include sponge-like areas, consisting of cysts of various sizes that may have a gelatinous aspect. There can be only one large cysts, filled with liquid, and this is associated with the identification of gemistocytes during microscopy. The calcifications can be focal or diffuse, and in this case, the appearance is one of grittiness.

Histological diagnosis. Under the microscope, we see a diffuse tumoral proliferation consisting of atypical fibrillary astrocytes. Hypercellularity is moderately increased, the tumor imperceptibly blending with the surrounding normal structures. Cellular proliferation (star-shaped cells, with extensions) is situated on a fibrillary loose matrix which often forms microcystic structures. The main characteristic is the nuclear atypia, neoplastic astrocytes being based on the aspect of the nucleus. This is enlarged, hyperchromatic, and irregular in shape [23].

The *differential diagnosis* is performed with the help of reactive astrocytosis. The diagnosis can be done only on the basis of morphological criteria, but more often than not, this requires an extremely nuanced approach. The morphological criteria include a numerical increase, but especially the homogeneous aspect of the nuclei, as we are dealing with a clonal neoplastic proliferation. As opposed to neoplastic astrocytes, the reactive ones have a heterogeneous nuclear aspect, with nuclei of various sizes and with cytoplasm in variable quantities. The background of these nuclei is of normal or increased density in the case of tumoral proliferation, and of decreased density in the case of reactive astrocytosis. Immunohistochemistry is extremely useful in distinguishing between reactive and tumoral astrocytes. As the IDH1 mutation falls under the definition of this type of tumor, the antibody identifying the protein altered by the presence of the R132H mutation can be used. Also, the tumoral cells displaying the TP53 mutation can be identified through a recourse to the antibody [24].

Mitotic activity is low to absent, the presence of a mitosis in a large biopsy being compatible with the diagnosis. If a mitosis is present in the context of an important nuclear anaplasia within a small biopsy, then the diagnosis of anaplastic astrocytoma cannot be ruled out. The proliferation index determined by way of Ki-67 is under 4%. If there is a gemistocytic component, the proliferation rate is significantly reduced [25].

As we are dealing with low proliferation rate tumor, the changes induced by hypoxia, such as microvascular proliferation and necrosis, are absent.

Secondary structures (Sherer) such as perineuronal satellitosis, subpial infiltration, and perivascular aggregation can also be present.

Other entities that need to be factored in for the differential diagnosis are: the normal brain, the demyelinating disease, anaplastic astrocytoma, oligodendroglioma, and pilocytic astrocytoma. In what concerns the diffuse pattern, the differential diagnosis must be done with lymphomas and small-cell carcinomas [26].

Gemistocytic astrocytoma is a variant of the grade II diffuse astrocytoma, characterized by the presence of more than 20% angular neoplastic astrocytes, with abundant eosinophilic cytoplasm. The nucleus is pushed toward the periphery, showing nucleoli and a dense chromatin. Electronic microscopy reveals the presence of numerous mitochondria and glial filaments, as also confirmed by the positive GFAP. A characteristic feature is the presence of the perivascular cuffing lymphocyte [27].

The classical morphopathological aspect is astrocytic, but an "oligodendroglioma-like" component can be accepted in the absence of 1p/19q codeletion.

Immunohistochemically, the battery of antibodies that can be used includes: GFAP, vimentin, IDH R132H, p53, ATRX, Olig2, and Ki67. GFAP and vimentin are positive, but of variable expression. The existence of an antibody that makes it possible to indirectly identify the R132H mutation, present in approximately 90% of tumors, is one way of identifying the tumor cells featuring this mutation. Another important antibody is ATRX, and the presence of the mutation leads to the loss of nuclear expression in the tumor cells. P53 can be used, as an intense nuclear expression is consistent with the presence of the TP53 mutation. Olig2 is nearly always present. As already indicated, Ki-67 can be used in assessing the proliferation index [28–31].

Genetic diagnosis. Integrated genomic analysis has made it possible to identify the IDH gene mutation in glioblastomas, leading to the "IDH era" in diffuse gliomas [32]. The sequencing of a large number of brain tumors has revealed the high incidence of the IDH gene mutation in low-grade astrocytomas and oligodendrogliomas, suggesting that it might play a role in the early onset of such tumors [33]. The presence of this mutation is relevant for both diagnosis and prognosis, its absence meaning a less favorable prognosis [34]. A study conducted on a large cohort has indicated a survival rate of 10.9 years for the diffuse astrocytomas with the IDH mutation [35]. The consequence-inducing mechanism involves the excessive production and the accumulation of an oncometabolite—2 hydroxyglutarate [36]. This mutation leads to significant epigenetic changes and to changes in the regulation of the expression of differentiating factors [37]. More particularly, we witness a hypermethylation of the genome, generating tumors with the CpG island methylator phenotype (CIMP) [38]. This group of tumors shows a distinct biological behavior, with epigenetic changes in the whole genome, by remodeling the methylome and reorganizing the transcriptome. This leads to the activation of key genetic programs and to the emergence of a cellular phenotype allowing for better survival rates. Also, the IDH allows for chromosomal aberrant interactions, with the activation of the oncogene expression [39].

ATRX encodes a chromatin-binding protein. The mutations of this gene are associated with epigenetic changes [40]. It also activates the alternative telomere lengthening mechanism, necessary

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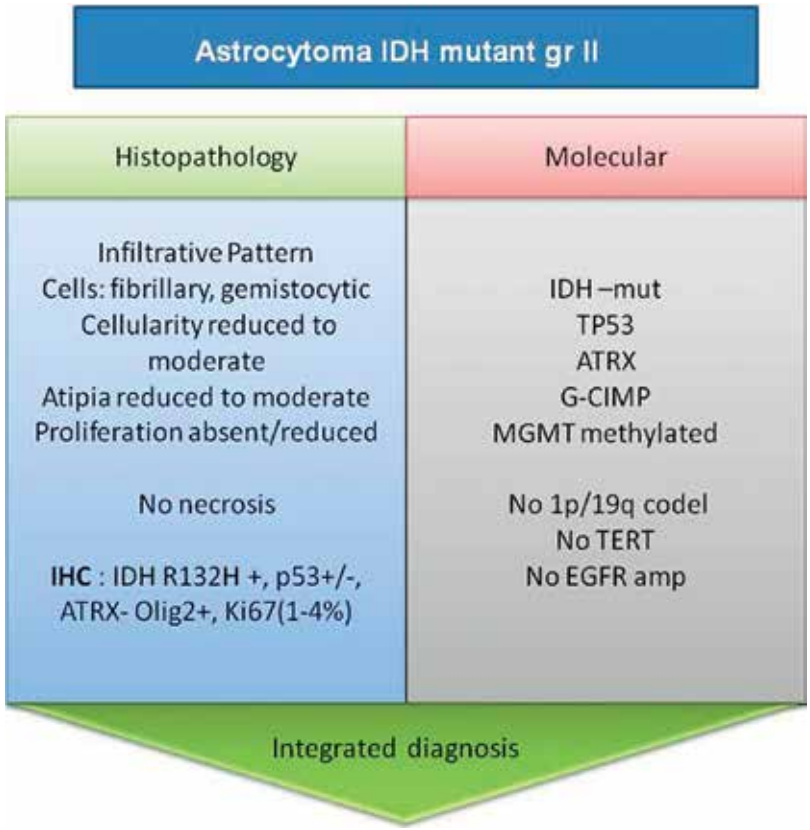

Figure 3. Integrated histological and molecular diagnosis of diffuse astrocytoma IDH-mutant grade II.

in the pathogenesis of diffuse gliomas [41]. Furthermore, ATRX deficiency can create a context of generalized genetic instability which, when P53 is intact, can induce apoptosis. The occurrence of a P53 mutation alongside the ATRX one can allow tumor cells to survive [42]. This instability is reflected in the occurrence of low-level amplifications for other oncogenes, such as MYC and CCND2 [43]. The TP53 mutation is also present in nearly all IDH-mutant gemistocytic astrocytomas, in both gemistocytes and non-gemistocytes, indicating that gemistocytes are oncogenically non-reactive cells [44]. Quite interestingly, the presence of the ATRX mutation is mutually exclusive with the mutation of the gene that encodes the catalytic component of TERT telomerase. The mutations of the TERT gene are characteristic for oligodendrogliomas and wild-type glioblastomas [45].

The methylation of the MGMT gene promoter is present in more than 50% of IDH-mutant astrocytomas, but the presence of this methylation is not correlated with the status of G-CIMP [46]. By definition, 1p/19q codeletion is absent (**Figure 3**).

The genetic profile of diffuse astrocytomas is different in children and in adults; so, we can talk of adult-type and pediatric-type diffuse astrocytomas. The genetic profile of pediatric tumors involves amplifications and rearrangements of the MYB gene, alterations of FGFR1, and mutations of BRAF (V600) and KRAS [47].

There are two entities that increase the susceptibility to diffuse astrocytomas. Low-grade astrocytomas are usually diagnosed in patients with Ollier-type multiple enchondromatosis [48]. Also, those having the Li-Fraumeni syndrome are more likely to develop diffuse gliomas, but these are high-grade anaplastic astrocytomas and high-grade glioblastomas [49].

2.1.1. Multimodal treatment

2.1.1.1. Surgical treatment

In the last decade, a great switch was produced in the therapeutical management of DA. Since it was demonstrated that these tumors have an annual linear grow of 4 mm/year on diameter [50], they are nowadays considered "infiltrating chronic disease that invades the central nervous system, that will ineluctably become malignant" [51]. It is now well established that surgery has a great impact on both the natural history and the malignant transformation. As a consequence, radical surgery becomes the goal in the treatment of diffuse gliomas in order to prevent malignant transformation and to prolong overall survival [52]. Additionally, radical surgery significantly improves seizure control compared with subtotal resection [53].

General principles of surgery, as they were underlined by Hugh Duffau, are generally accepted by the neurosurgical community: early radical surgery, awake surgery in high eloquent areas, cortical mapping, "resection according to cortico-subcortical functional and not oncological boundaries," and "multistage resection in critical regions" [51]. The most difficult part of the operation is at the boundaries of the tumor, where even with adjuvant intraoperative MRI, the distinction between normal brain and infiltrative brain is very difficult. Surgical experience contributes to better surgical results, but even in very experienced hands, there are cases in which a residual tumor could be identified on postoperative MRI (**Figure 4**).

Neuronavigation and intraoperative ultrasound are more and more used in order to improve the surgical resection, since they are able to reveal large residual tumors. Enhanced intraoperative ultrasound is at the beginning of experience, more studies being necessary to establish its usefulness in diffuse astrocytomas resection (**Figure 5**) [54].

2.1.1.2. Adjuvant therapy

Factors to take into consideration for immediate postoperative therapy are those considered risk factors for worse outcome namely age > 40 years, preoperative tumor diameter > 4 cm, incomplete resection, astrocytic histology, and absence of 1p/19q codeletion [55]. In this perspective, cases with DA IDH-mut completely resected, in young patients (<40 year), are candidates for close clinical imagistic observation and no adjuvant therapy is recommended in the immediate postoperative period. However, it is expected that these tumors will recur, so additional surgical and adjuvant therapy will be added at the time of progression. For tumors with subtotal removal, in patients older than 40 years of age, immediate postoperative treatment is recommended. Concerning adjuvant radiotherapy, the recommendation is in favor of lower doses (45–50.4 Gy) which are equivalent in terms of results with the high doses (59.4–64.8 Gy) but with reduced toxicity. Relative to chemotherapeutic regimen, actual data suggest that the PCV (procarbazine, CCNU, and vincristine) formula is superior to temozolomide regimen in terms of overall survival for the treatment of DA IDH-mut, mostly in cases with codeletion of 1p/19q genes [19].

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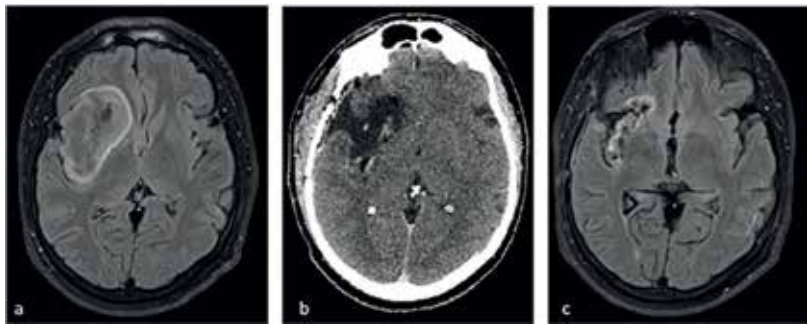

Figure 4. Preoperative FLAIR MR sequence (a) and immediate postoperative axial CT (b) and 3 months postoperative FLAIR examination revealing a complete removal of an insular grade II DA. (c) One year follow-up axial FLAIR MR examination reveal complete removal of tumor with no local signs of recurrence.

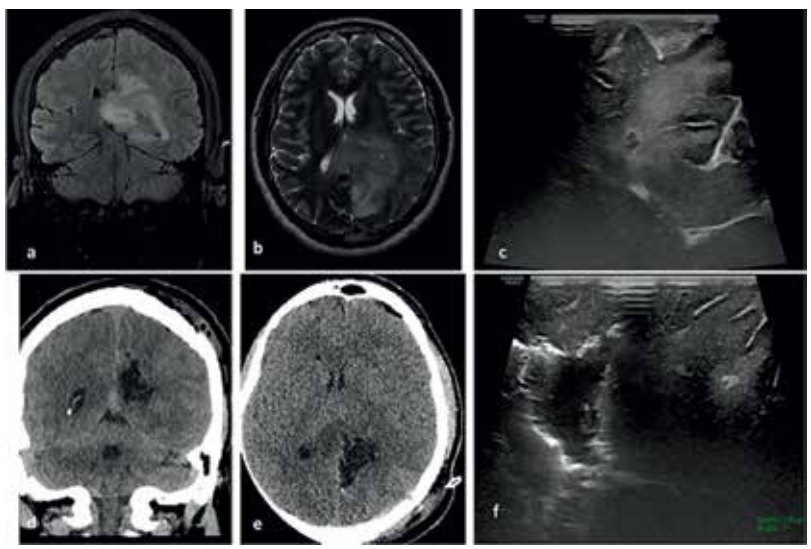

Figure 5. Preoperative coronal FLAIR (a), axial T2W (b) and intraoperative ultrasound examination (c) before resection of a diffuse astrocytoma subtotally removed as it is demonstrated by intraoperative ultrasound (f) and immediate postoperative CT scan (d and e).

2.2. Diffuse astrocytoma IDH wild type

Definition. This astrocytoma is a diffuse growth pattern astrocytoma without the IDH mutation. WHO 2026 lists it as a rare and provisional entity. Most tumors falling under this definition can be classified with the help of genetic testing as a variety of other entities with different clinical evolutions.

Clinically, their behavior is not different from the DA IDH-mut.

The *standard MRI* findings are generally the same, the presence of a more intense enhancement suggesting an increased microvascularization and, as a consequence, a more aggressive evolution. As it was already mentioned, the presence of 2HG peak on MR spectroscopy is suggestive for IDH1 mutation, so the absence of this peak could serve as an indicator for a wild-type tumor. 2HG was also studied as a marker for the response to adjuvant therapy [56].

2.2.1. Multimodal treatment

2.2.1.1. Surgical treatment

Surgical treatment with the goal of early radical surgery is nowadays the first step in the standard of care of low-grade gliomas. Complete tumor removal based on functional borders more than on imagistic ones with the help of neuronavigation, awake surgery, and intraoperative neurophysiological monitoring (IEM) proved to offer a longer progression-free survival (PFS) and overall survival (OS) compared with subtotal removal, with reduced neurological deficits [57, 58].

2.2.1.2. Adjuvant treatment

Knowing that the wild-type astrocytomas have a worse prognosis, a low-dose radiotherapy regimen in the immediate postoperative period is recommended instead of close clinical imagistic observation. There are arguments in favor of radiotherapy in terms of prolonged PFS but not necessarily the OS [59]. Due to the fact that the patients receiving associated radiotherapy and chemotherapy have an improved long-term survival [60], a combined radio-chemotherapy regimen seems to be recommendable. Further studies will have to determine whether temozolomide, which was historically preferred by neuro-oncologists due to its lower toxicity and oral administration, is superior or not to PCV [61].

2.3. Diffuse astrocytoma NOS

Definition. A diffuse astrocytoma for which the presence of the IDH mutation could not be determined.

2.4. Oligodendroglioma, IDH-mutant, and 1p/19q codeleted

Definition. Tumor with a diffuse growth pattern, slow growth, showing IDH mutations and the 1p/19q codeletion. Oligodendrogliomas develop many similarities with DA IDH-mut concerning clinical presentation, imagistic diagnosis, and therapeutical management.

Grading. Grade II WHO.

Clinical presentation. The classical presentation of a patient with oligodendroglioma is with seizures that precede with years and with other neurological signs like mental disturbances, neurological deficits, or signs of increased intracranial pressure (mostly headache). Even in patients with no seizures at the onset, they will develop such clinical manifestations during the course of disease. The brutal debut due to an intratumoral hemorrhage although rare, is more frequent in oligodendrogliomas than in other low-grade gliomas [62].

Imaging. *CT scan* is usually the first imagistic tool in emergency, and for more than 50% of oligodendrogliomas that present intratumoral calcification, it is a useful diagnostic method. On CT scan, oligodendrogliomas are present as well-delineated cortical-based hypodense lesion with intratumoral or peripheral calcification. Cystic component are not rare and hemorrhagic regions may also be encountered (**Figure 6**). Contrast enhancement is absent or marginal.

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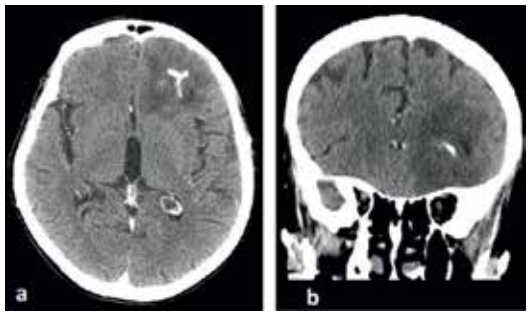

Figure 6. Axial and coronal CT scan examination of a case with an hypodense left frontal lobe lesion with intratumoral calcifications and discrete enhancement (a and b).

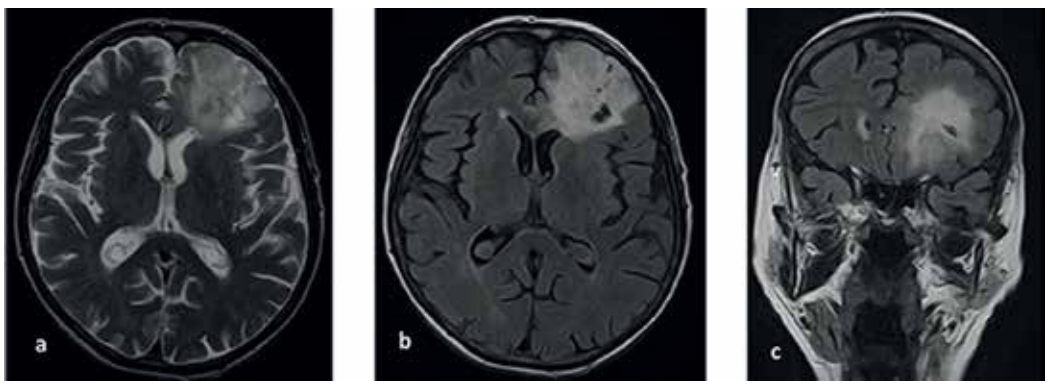

Figure 7. Axial T2W (a), axial (b) and coronal (c) FLAIR sequences of a left frontal oligodendroglioma.

Recently, it was suggested that presence of calcification is highly associated with 1p/19q codeletion and, as a consequence, has a prognosis role [63].

Standard MRI reveals an hyperintense inhomogeneous lesion on T2W and FLAIR sequences, respectively, an inhomogeneous hypointense cortical-based well-delineated lesion on T1W sequences with no or discrete enhancement. Calcifications, cysts, and hemorrhagic lesions increase the heterogeneity of the lesion (**Figure 7**).

Advanced MRI techniques. As for the diffuse astrocytomas, MRI spectroscopy reveals higher NAA (N-acetylaspartate) peak near 2 ppm. The mIns (myoinositol) peak is relatively higher when compared with normal brain; the reduction of peak signaling is a possible malignant transformation of tumor. The NAA/Cr (creatinine) ratio is higher in low-grade gliomas, meanwhile the ratio Cho(Choline)/Cr is higher in high-grade gliomas [64]. As it was already mentioned, 2HG peaks serve as an indicator for IMD 1 mutation. Perfusion MRI, an another physiologic imaging sequence, reveals that relative cerebral blood volume (rCBV) is reduced in low-grade gliomas (LGG) compared with high-grade ones. Increase in rCBV in a LGG is an indicator for rapid progression and possible malignant transformation (**Figure 8**) [65]. Diffusion tensor imaging (DTI) could also add some supplementary information in order to differentiate LGG from high-grade gliomas (HGG) [66].

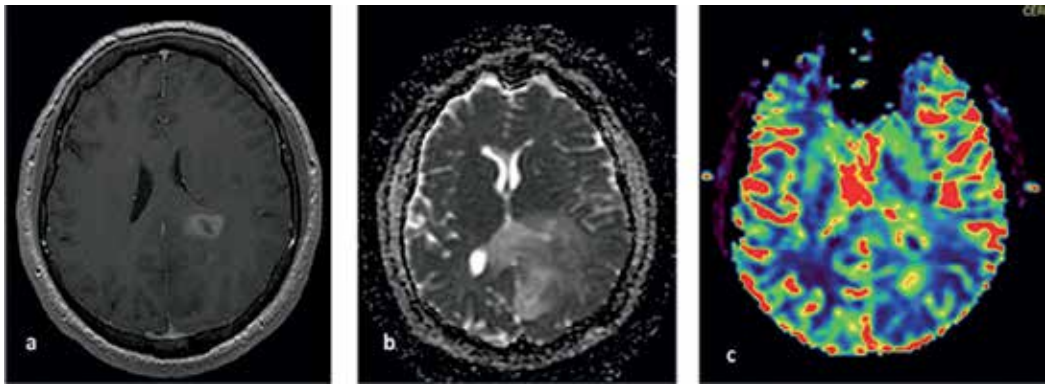

Figure 8. T1W + C sequence (a), DTI (b) and perfusion MR (c) sequences in a patient with a LGG with a nodule of contrast enhancement corresponding to a region of increased rCBV; the patient presented 4 years previously for epileptic fits; MRI examination performed at that time was suggestive for a LGG, but the patient refused any treatment except the antiepileptic one; readmitted for signs of increased intracranial pressure and clear imagistic findings for progression and malignant transformation.

Macroscopy. It is a well-defined lesion at the interface between the white and the gray matter (*with an affinity for the cerebral cortex*). In cross-section, the surface is soft and frequent calcifications gives it a gritty look. Hemorrhagic and cystic degeneration areas can be seen.

Histological diagnosis. The aspect is that of an infiltrative tumor consisting of monomorphic cells. Cellularity is moderately increased, but it can vary considerably. The well-differentiated tumors can feature well-circumscribed nodules of increased cellularity. The nuclei are slightly enlarged, uniform, round (low atypia), and slightly hyperchromatic ("salt and pepper"). In hematoxylin-eosin, they are surrounded by a water clear cytoplasm with sharp borders, which gives them the artefactual aspect of fried egg or honeycomb. Typically, they show a network of branching capillaries in a chicken wire shape. Calcifications, cysts, and areas of mucoid degeneration can also be encountered. Mitoses are rare [67]. Immunohistochemically, the cells are IDH+ and ATRX+, and p53− [3]. GFAP and vimentin are variably expressed. Olig1 and Sox10 are positive but non-specific [68]. The differential diagnosis can be done with macrophage-rich lesions, diffuse astrocytoma, and clear cell ependymoma.

Genetic diagnosis. By definition, these tumors feature mutations in the IDH1 and IDH2 genes, as well as the deletion of the whole arm of the 1p and 19q chromosomes [69]. The incomplete/partial codeletion is present in glioblastomas and anaplastic astrocytomas. TERT mutation is associated with the IDH mutation and codeletion in the early onset of the tumor. *CIC* and *FUB* occur at a later stage [70]. The TP53 mutation is absent. As to the epigenetics, just like in the other cases, the IDH mutation induces a hypermethylated status—G-CIMP [38]. The methylation of the MGMT promoter is associated with a better survival rate, given the response to treatment (**Figure 9**) [71].

2.4.1. Multimodal treatment

2.4.1.1. Surgical treatment

Surgical principles are the same with those of DA IDH-mut, gross total resection (>90% volume reduction on 24–48 h postoperative MRI) being correlated with a longer PFS and OS, compared with subtotal resection [57].

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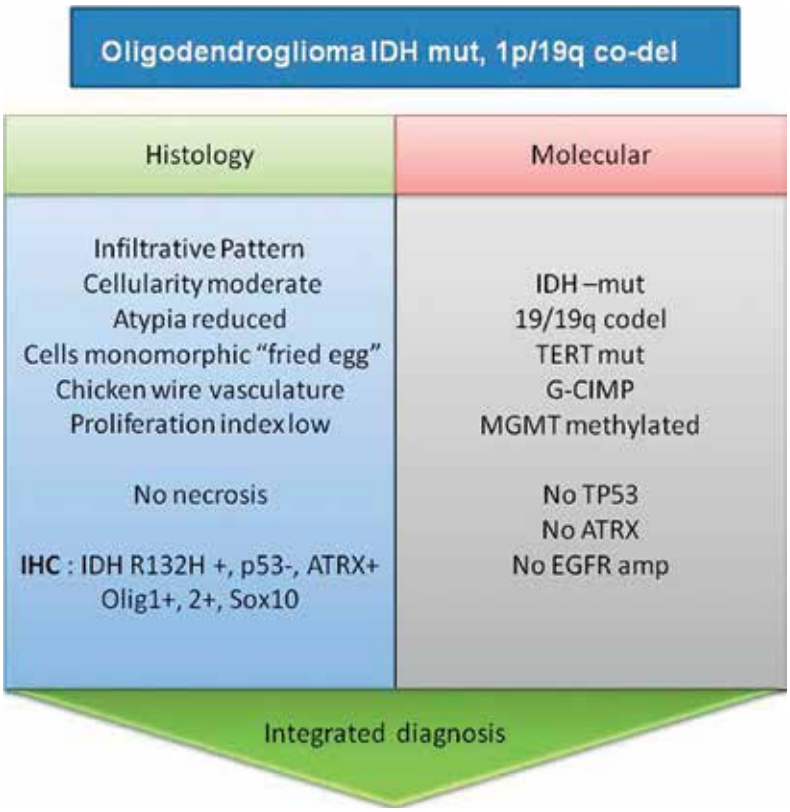

Figure 9. Molecular sand histopathological diagnosis of oligodendrogliomas.

The use of neuronavigation with co-registered preoperative T2W and FLAIR sequences and CT scan data in the presence of intratumoral calcification improved the grade of resection (**Figure 10**). As for other LGG, intraoperative electrostimulation mapping (IEM) on awake patient greatly improved not only functional outcome of the patient but also made possible to extend safely the grade of resection, with a great impact on the prolonged survival rate [72].

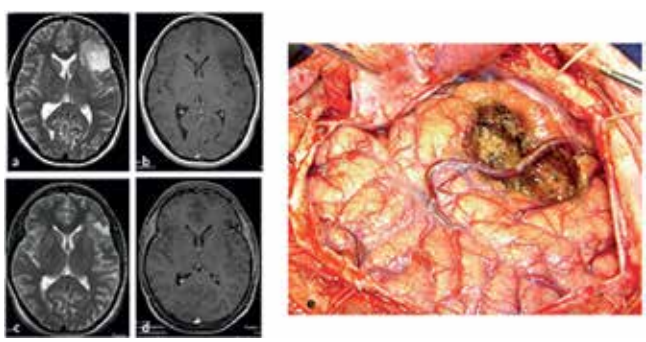

Figure 10. Preoperative T2W and T1W + C (a and b), respectively; 12 months postoperative T2W and T1W + C (c and d) sequences of a case with left frontal oligodendroglioma completely removed without any neurological deficit; (e) intraoperative aspect after completion of radical removal of a right temporal low-grade glioma with the preservation of Labbe vein (personal archive).

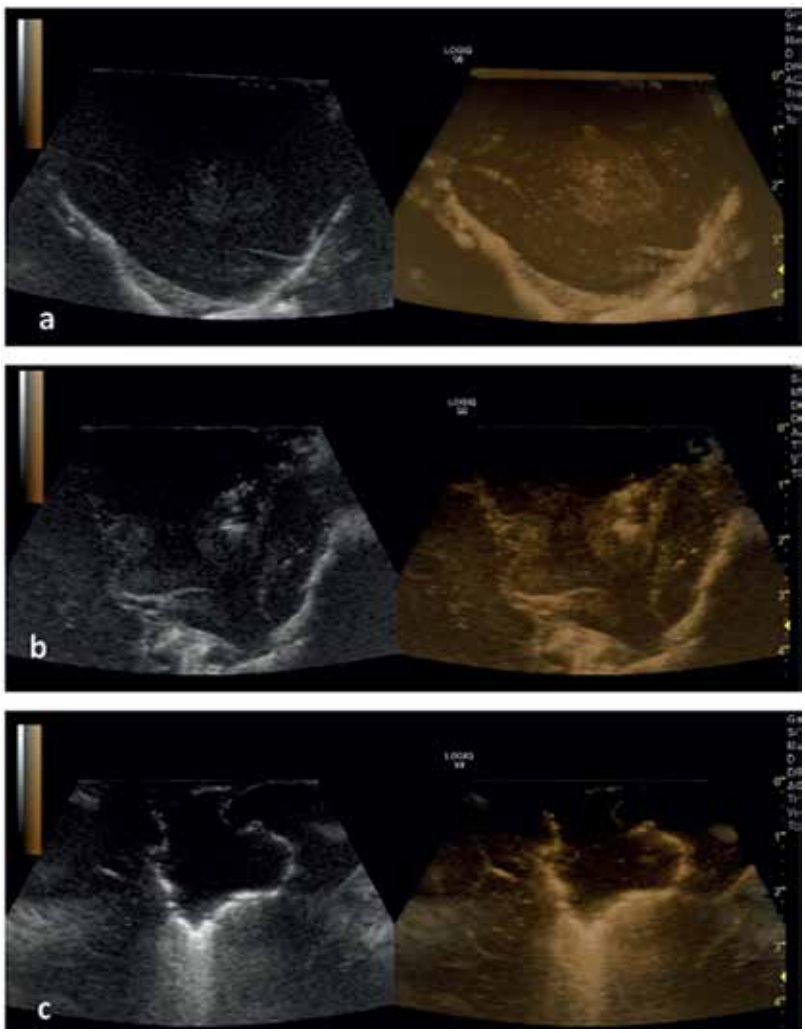

Figure 11. Staged resection of a high-grade gliomas with the aid of intraoperative contrasted ultrasound: after the dural opening (a), partial resection (b) and after complete resection (c) of the tumor.

Despite the fact that there are no randomized trials comparing grade of resection with and without intraoperative MRI, it is intuitive that having real-time data on the progression of removal is at least useful for completion of tumor excision. An alternative is the intraoperative ultrasound which can detect significant remnants in real time without interruption of surgery (**Figure 11**). The introduction of new equipment with 4D and contrast enhancement dramatically improves the quality of images, but their role in detecting fine details in low-grade gliomas is to be established in near future.

2.4.1.2. Adjuvant treatment

Immediate postoperative radiotherapy of completely removed oligodendroglioma IDH-mut 1p/19q codel is still under debate. Based on the results of EORTC 22845 trial which revealed that there is no significant difference in terms of OS between patients receiving immediate

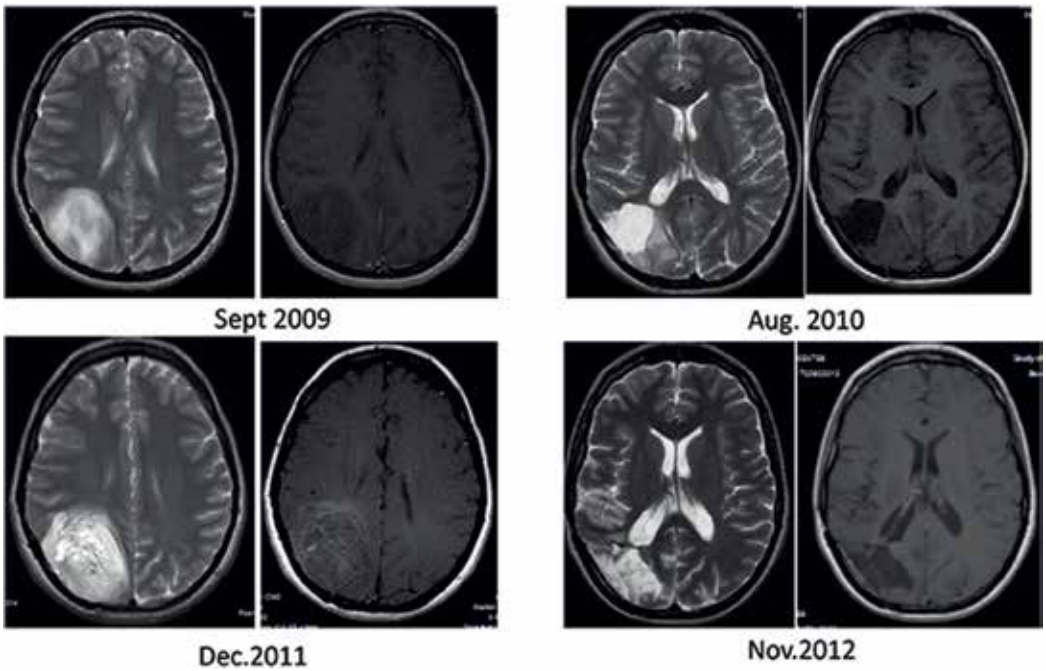

Figure 12. Serial follow-up MRI examination of a patient with oligodendroglioma completely removed in September 2009, presenting with imagistic signs of progression 1 year later, but without any additional clinical manifestation; 15 months later the patient was readmitted for signs of increased intracranial pressure and clear findings of malignant transformation; re-operated in emergency, patient followed the standard radio-chemotherapy regimen.

postoperative radiotherapy and those receiving radiotherapy as a salvage therapy, the actual recommendation is to delay radiotherapy until signs of progression are evident. In cases of incompletely removed tumors or in the presence of any imagistic or clinical signs of progression, combined radiotherapy and PCV chemotherapy is superior to radiotherapy alone (**Figure 12**) [73].

Some authors argued that even in cases with foci of anaplasia imbedded in low-grade glioma, the total resection is sufficient for a long-term PFS, and no adjuvant treatment is needed; but this is not the standard of care as the authors already mentioned at the conclusions [74].

2.5. Oligodendroglioma, NOS

Definition. Infiltrative tumor with the histopathological aspect of oligodendroglioma, for which the genetic determinations relevant for the diagnosis (IDH mutation and 1p/19q codeletion) cannot be determined.

3. High-grade gliomas

3.1. Anaplastic astrocytoma, IDH-mutant

Definition. Tumoral proliferation which, from a histopathological point of view, displays an astrocytic phenotype, a diffuse growth pattern and proliferative activity, and which, genetically speaking, features mutations in the IDH1 or IDH2 genes.

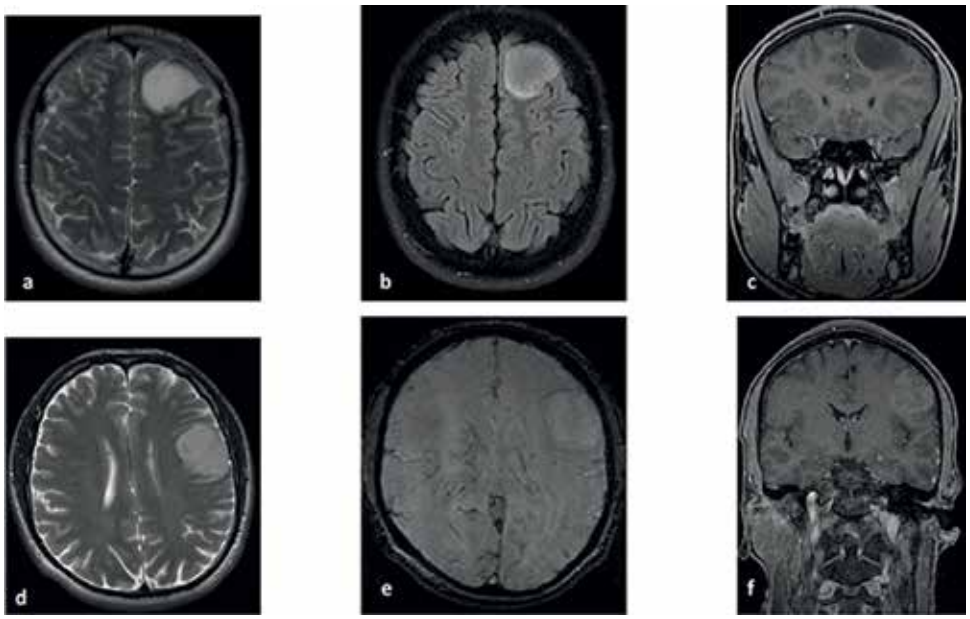

Figure 13. Upper row: left frontal lesion hyperintense in T2W (a) and FLAIR (b) sequences with no contrast and T1W sequence (c) MRI in a 24 years old men diagnosed with grade III astrocytoma; lower row: left frontal lesion having similar characteristics in T2W (d) and FLAIR (e) sequences but with a discrete enhancement in T1W (f) sequences revealed in the young men's father MRI examinations, operated 5 years before with an anaplastic astrocytoma (personal archive) suggesting a hereditary determinism in this particular case.

Grading. Diffuse astrocytoma is deemed grade III WHO.

Clinically, the patients experience seizures, headache, neurocognitive disturbances, and neurological deficits in more advanced stages.

Imaging. MRI features are quite variable, with isointense mixed with hypointense signaling on T2 W sequences and heterogeneous hyperintensity in T2W and FLAIR sequences. The contrast enhancement is subtle or is lacking in majority of cases [75] (**Figures 13** and **14**).

Macroscopy. Anaplastic astrocytoma appears as an expansion of the tissue tending to infiltrate the surrounding nervous structures, but without destroying them. It is difficult to distinguish from the grade II astrocytoma, but the increased cellularity makes it easier to identify the edges of the tumor. Also, in cross section, we see areas of low consistency, granular, or opaque. Cysts are rarely encountered.

Histological diagnosis. The aspect is that of a tumoral proliferation with astrocytic phenotype and a diffuse growth pattern. As compared to grade II astrocytomas, it shows increased cellularity, anaplasia, and mitotic activity. These three parameters can vary between grade II astrocytoma and glioblastoma and must therefore be assessed in context. A heightened mitotic activity is sufficient for a diagnosis, even if the cellularity is low or normal. Also, just one mitosis in a stereotactic biopsy can be sufficient for a diagnosis. There are also atypical mitoses. In a large biopsy,

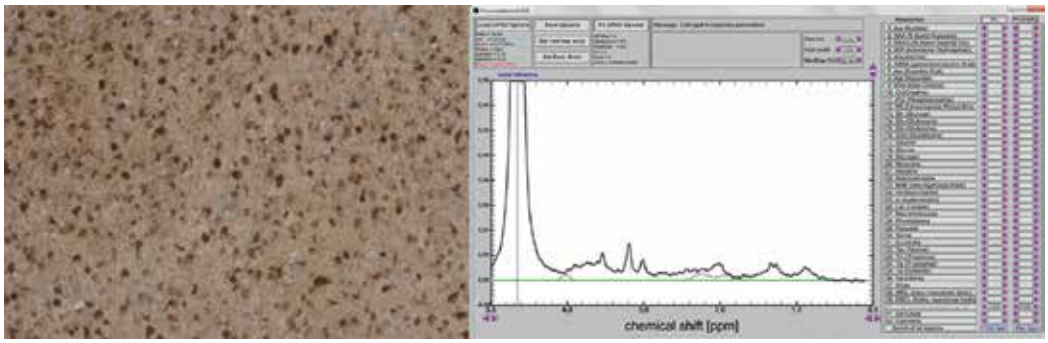

Figure 14. The immunohistochemical analysis (Clone H09) of the IDH1 R132H mutation performed on a patient with anaplastic astrocytoma. Infiltrating tumor cells positives at cytoplasmic level are observed in a background of negative normal astrocytes (left). In vivo single-voxel localized PRESS spectra were performed for the same patient. Black line represents the sum of spectra obtained during MRS. Pink line represent the spectra of 2-hydroxyglutarate identified at TE 35 ms (right).

the presence of several mitoses is not sufficient for a diagnosis. The nuclei are larger, hyperchromatic, and their shapes vary more than in the case of grade II astrocytomas, while the nucleoli are more visible. Multinucleated cells, gemistocytes, small cells, and perivascular lymphocytes can also be seen. There are no calcifications, necrosis, or microvascular proliferation [76].

The immunohistochemical profile is the same as in the grade II IDH-mutant diffuse astrocytoma: Olig2+, IDH1 R132H+, ATRX−, and p53+ in some cases. The Ki-67 proliferation index is generally high (5–10%), but it can vary considerably [77]. The differential diagnosis is performed with reactive gliosis, demyelinating diseases, progressive multifocal leukoencephalopathy, grade II astrocytoma, glioblastoma, and anaplastic oligodendroglioma.

Genetic diagnosis. Generally, it matches that of grade II astrocytomas. The IDH 1/2 mutation is present by default, in association with TP53 and ATRX. Chromosome arm 9p and 19q losses are more frequent in grade III tumors. As with grade II astrocytomas, the presence of the IDH mutation means a better prognosis, with the median survival rate for these patients being 9.3 years. The presence of EGFR, 10q loss, and 7q gain means a less favorable prognosis, and may suggest a molecular diagnosis of glioblastoma, even if there is no indication of it in the histology (**Figure 14**) [35]. By definition, the 1p/19q codeletion is absent.

3.2. Anaplastic astrocytoma, IDH wild-type

Definition. Tumoral proliferation which, from a histopathological point of view, displays an astrocytic phenotype with a diffuse growth pattern, proliferative activity, and increased anaplasia, and which, genetically speaking, does not feature mutations in the IDH gene.

Grading. Grade III tumor.

Genetic diagnosis. One in five anaplastic astrocytomas does not feature the IDH mutation, the genetic profile and the clinical evolution of these tumors being that of wild-type glioblastoma.

3.3. Anaplastic astrocytoma NOS

Definition. Diffuse anaplastic astrocytoma for which the presence of the mutation in the IDH gene could not be determined.

Grading. Grade III tumor.

3.3.1. Multimodal treatment

3.3.1.1. Surgical treatment

Being malignant tumors, early radical surgery represents the first step of the multimodal treatment of anaplastic astrocytomas. Due to the diffuse infiltration of the brain, radical excision is rarely achieved, meaning that if 1% of the initial volume remains in place, a local recurrence is almost certain. Additionally, the location of tumor in high eloquent areas prevents a radical excision in order not to produce severe neurological deficits. The main goals of surgery in high-grade gliomas (HGG) are to reduce the mass effect, to obtain relevant pathological tissue, to reduce the tumor burden, and to prolong survival with improved quality of life. There are many factors influencing OS in high-grade gliomas such as preoperative Karnofsky score, age, general and neurological status at the preoperative and postoperative period, pathology and genetics of the tumor, grade of resection, response and tolerance to the adjuvant therapy. Among all these factors only those related to surgery could be influenced, namely the grade of resection and the neurological status after the operation [78]. There is a very delicate balance between the radical surgery and preservation of neurological function. The more aggressive the surgery, the higher the risk for neurological deficiencies stands (**Figure 15**). After the publication of the trial conducted by Stupp and in 2005 [79], in which the role of surgery was minimized, a large amount of studies were published underlying the importance of gross total resection (GTR) compared not only with biopsy but also with near total resection (NTR) and subtotal resection (STS) as a factor that independently influences OS in HGG. For example, in anaplastic astrocytomas, there is a difference of 12 months in median of survival in favor of GTR compared with STR [80].

A retrospective study performed by us on 266 cases of HGG reveals the fact that gross total removal (GTR) greatly influences survival compared with STR. At the three periods of monitoring (12, 18, and 24 months), the difference regarding survival mean between GTR vs. STR ranged from 2.8 months (at 12 months monitoring) to 4.4 months (at 18 months monitoring) up to 5.1 months (at 24 months monitoring) including all types of malignant gliomas. We also found that the type of surgery and the age are prognostic factors that significantly influenced in all three periods of monitoring, while the histopathology was a prognostic factor for survival only at the 24 months monitoring (**Table 1**) [81].

A more detailed discussion regarding the role of surgery will be presented later in the chapter, along with the types of glioblastomas.

3.4. Anaplastic oligodendroglioma, IDH-mutant, 1p/19q-codeleted

Definition. Infiltrative tumor with the histopathological aspect of oligodendroglioma showing a significant proliferative activity and microvascular proliferations.

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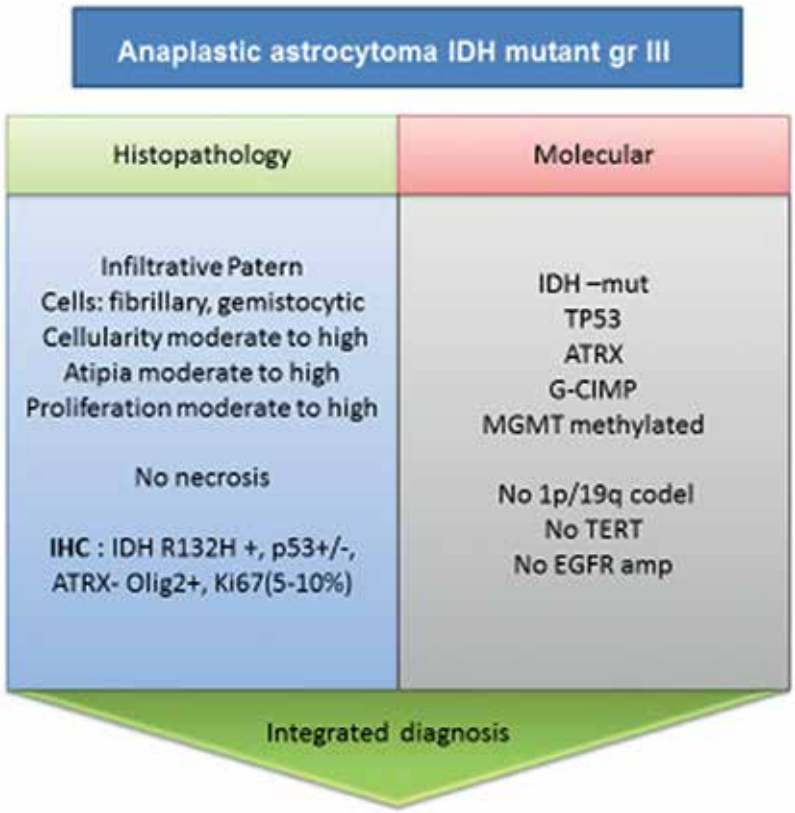

Figure 15. Integrated histological and molecular diagnosis of anaplastic astrocytoma IDH-mutant.

Grading. The tumor is grade III WHO.

Clinically, the most frequent presenting symptom is seizures, less frequent than in the lower-grade tumors, accompanied with signs of increased intracranial pressure and cognitive deficits. Other signs are related to the location of tumor [82].

Imaging. On *MRI* studies, they are typically heterogeneous, predominantly hypointense in T1W, and respectively hyperintense in T2W sequences. The frontal lobe cortical based location, the presence of calcification, cystic degeneration and hemorrhage, and intense enhancement along with minimal perilesional edema are more suggestive for anaplastic oligodendroglioma than for other gliomas. For follow-up imagistic studies, the T2W and FLAIR sequences are more sensitive in identification the local progression [83]. *CT scans* usually performed in emergency may clearly define calcification and hemorrhagic changes (**Figure 16**).

Macroscopy. As compared to grade II oligodendroglial tumors, they show additional necrotic areas.

Microscopic diagnosis. Increased cellularity, in a pattern that is infiltrative or compact with little intervening parenchyma. The cells have the characteristic fried egg aspect, but a significant pleomorphism or giant multinucleated cells can also occur. Significant mitotic activity is

Disease free interval	Log rank (Mantel-Cox) factor: age (<65 years/≥65 years)	Log rank (Mantel-Cox) factor: type of surgery (gross total removal/ subtotal removal)	Log rank (Mantel-Cox) factor: histopathological diagnosis	Log rank (Mantel-Cox) factor: gender
12 months	0.000	0.000	0.090	0.296
18 months	0.000	0.000	0.122	0.836
24 months	0.000	0.000	0.031	0.756

Table 1. Disease free interval regarding the age, type of surgery, and histopathology.

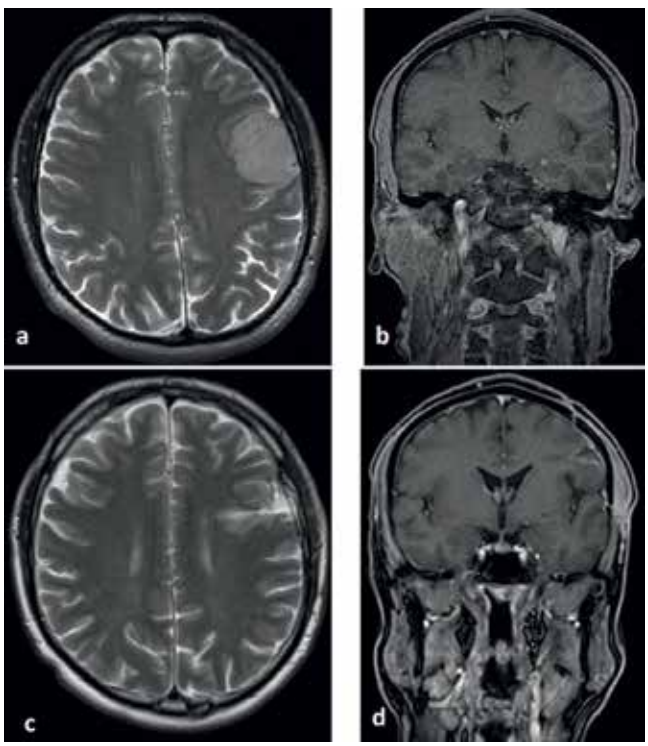

Figure 16. Preoperative T2W and T1W+ C (a and b) sequences of a case with left frontal anaplastic astrocytoma completely removed; follow-up MRI performed at 4 months postoperatively and after concomitant radio- and chemotherapy with temozolomide showed no signs of local recurrence (c and d).

also present [84]. Sarcomatoid areas can be seen. The minigemistocytes are more numerous than in the case of grade II oligodendrogliomas [85]. As a characteristic, the chicken wire aspect is accompanied by microvascular proliferation. As opposed to grade II oligodendrogliomas, they can involve palisading necrosis. Secondary structures can also be encountered. Immunohistochemically, the profile is that of grade II tumors, with a higher proliferation index evinced by way of Ki-67 (generally >5%). The differential diagnosis can be done using clear cell ependymoma, glioblastoma, and anaplastic astrocytoma.

Genetic diagnosis. Apart from the IDH mutation and the codeletion of the whole arm of the 1p and 19q chromosomes, the concurrent polysomy of 1p and 19q is more frequently encountered in anaplastic oligodendrogliomas [86]. Other chromosomal aberrations can occur: gain of 7, 8q, and 15q, and losses of 4q, 18, and 22q. Just like in the case of low-grade tumors, TERT, CIC, and FUBP1 mutations are present. Epigenetically, the IDH mutation induces G-CIMP, while in most cases MGMT is hypermethylated [87].

3.5. Anaplastic oligodendroglioma, NOS

Definition. The diagnosis concerns the anaplastic oligodendrogliomas whose genetic profile (IDH mutation and 1p/19q codeletion) cannot be determined.

The diagnosis of *oligoastrocytoma* has been discontinued following the latest WHO classification.

3.5.1. Multimodal treatment

As for the other types of gliomas, the grade of surgical resection is an independent factor for PFS and OS. As a consequence, GTR is recommendable wherever is possible without neurological damage (**Figure 17**).

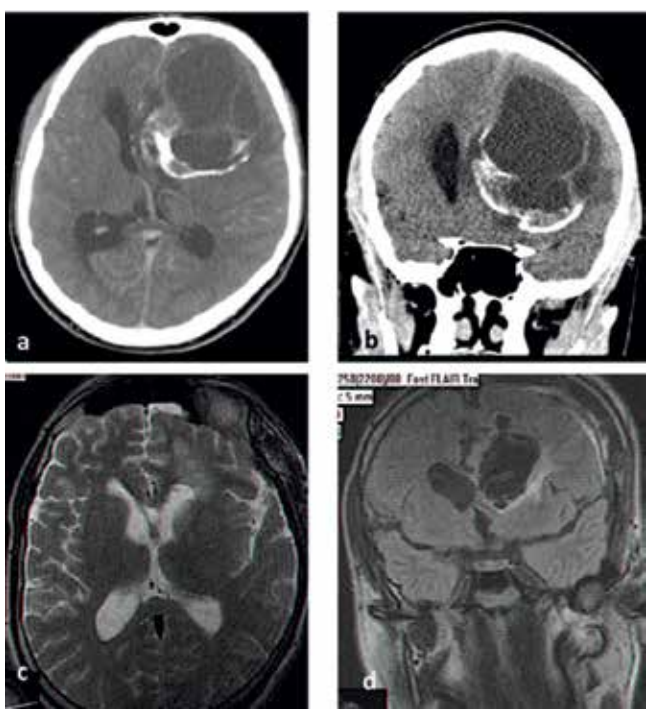

Figure 17. Axial (a) and coronal (b) contrasted CT scan demonstrating the presence of a voluminous expansive lesion with large cystic components and peripheral calcification with a reduced perilesional edema, aspect highly suggestive for an anaplastic oligodendroglioma; after GTR the axial T2W (c) and coronal FLAIR (d) sequences at 2 years follow-up do not indicate a local progression.

Due to the good response of anaplastic oligodendrogliomas to chemotherapy, the subtotal resection with preservation of neurological function is also an acceptable surgical strategy [88].

3.5.1.1. Adjuvant treatment

Adjuvant treatment is recommended in anaplastic oligodendrogliomas on a regular basis. Recent studies failed to demonstrate a benefit of temozolomide over the classical PCV regimen, which remains the main tool for first-line chemotherapy in anaplastic oligodendrogliomas. Radiotherapy is reserved for cases of clear imagistic evidence of tumor progress. Fractions of 1.8–2 Gy for a total dose of 54–60 Gy are the actual recommended radiotherapy regimen based on clinical evidence [89].

3.6. Glioblastoma IDH wild-type

Definition. High-grade tumor with a diffuse growth pattern and a dominantly astrocytic differentiation showing cellular pleomorphism, nuclear atypia, and brisk mitotic activity. Glioblastomas are characterized by the presence of necrosis and microvascular proliferations.

Grading. Glioblastomas are grade IV WHO.

Clinically, the evolution is usually short due to the high growth rate of the tumor [90]. Almost 40% of the patients are admitted for surgical treatment in the first month from the clinical debut marked by progressive headache (almost 80% of cases). Mental disturbances, changes in personality, and neurological deficits are subsequent manifestations of the evolution of the disease. Seizures are less common (18.9% in our study on 276 cases) then in the LGG [81].

Imaging. *Native CT scan* is usually performed in emergency and reveals a heterogeneous expansive process with significant mass effect. Addition of contrast enhancement clearly defines a variable-enhanced lesion with perilesional edema, the classical aspect of a ring enhancement circumscribing an necrotic area which is frequently present (**Figure 18**).

Once the suspicion brain tumor is raised, an *MRI examination* will more accurately define the lesion. T1W and T2W sequences are able to reveal the tumor and also other pathological changes within or around it: edema, necrosis, hemorrhage, and calcifications. In addition, the FLAIR sequences will demonstrate a hyperintense perilesional halo, which is in fact a combination of tumoral infiltration and edema. On enhanced T1W sequences, the classical appearance of "ring enhancement" delineating a central necrosis is the most eloquent aspect of glioblastoma (**Figure 19**).

Nevertheless, *Advanced MRI* techniques will offer functional elements in favor of a malignant lesion. On MR spectroscopy, glioblastoma presents higher Cho (Choline) peaks along with a higher Cho/Cr (creatine) ratio. This ratio is additionally elevated owing to the decrease of Cr compared with the normal level. Lactate and lipid peaks that indicate necrosis and disruption of myelin sheaths could also be detected in glioblastomas [91]. On DWI, high-grade gliomas typically present a restricted diffusion. DTI studies may help in differentiating HGG from LGG trough fractional anisotropy (FA), which is higher in the former [92]. On tractography, HGG will show a disruption of tracts. MRI perfusion studies reveal a heterogeneous relative cerebral volume (rCBV), demonstrating areas of increased cellularity and mitotic activity in glioblastomas (**Figure 20**) [93].

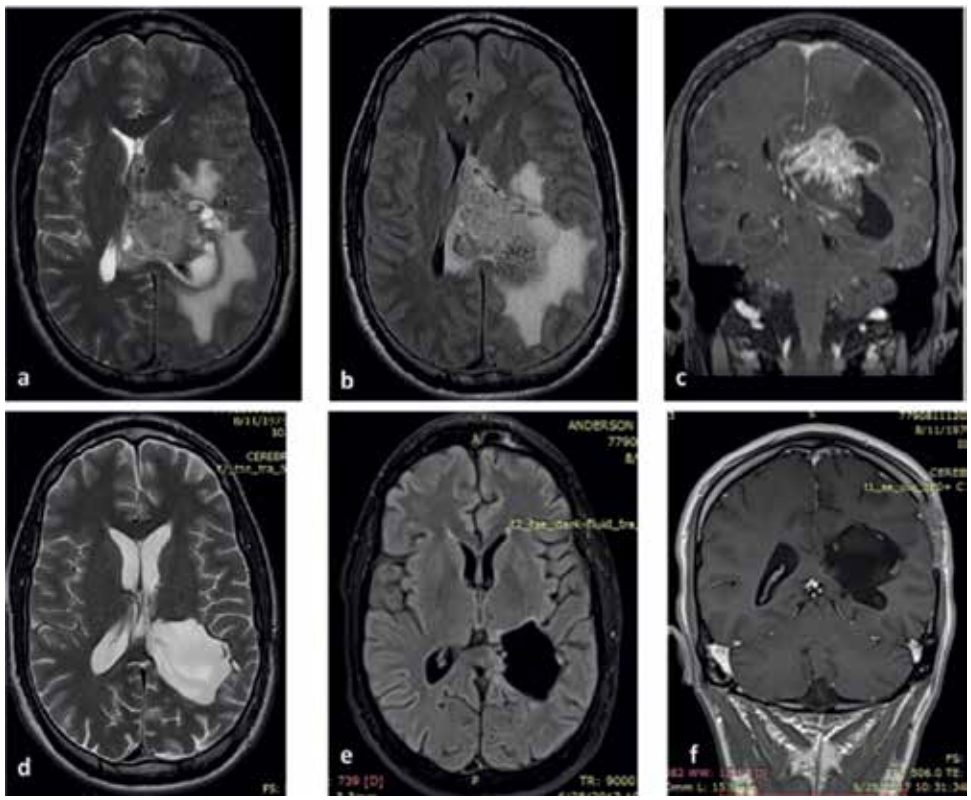

Figure 18. Axial T2W (a), axial FLAIR (b) and coronal T1W + C (c) sequences of an extremely rare intraventricular anaplastic oligodendroglioma completely removed, as it is demonstrated by the 6 months follow-up MRI (d–f).

Advanced MRI techniques are also useful for differentiating between local recurrence and radionecrosis (pseudoprogression). Recently, it was showed that rCBV as a single examination modality is superior to volume transfer constant (Ktrans) or apparent diffusion coefficient (ADC) for the prediction of local recurrence. The combination of rCBV and Ktrans improves the accuracy of the recurrence diagnosis [94].

Macroscopy. Poorly delineated lesions, with yellow, white, or granular areas caused by the necrosis, surrounded by grayish tumoral areas. In glioblastomas, cysts filled with a turbid liquid are formed through the liquefaction of the necrotic tissue. Typically, in cross section, we see purple-red or brown-red areas caused by the hemorrhage occurred during the progression of the tumor. The hemorrhages can be massive. Also in cross section, we can see thrombotic vessels with the aspect of "dark veins."

Microscopic diagnosis. The histology of the tumor is extremely variable and the cellular composition is highly heterogeneous. The astrocytic nature of the tumor can be identified in the well-differentiated tumors. In the poorly-differentiated ones, the astrocytic phenotype is recognized with considerable difficulty [95].

There are a number of cellular morphologies common to glioblastomas: fibrillary astrocytes, gemistocytes, smalls cells (highly infiltrative, bipolar, and mitotically active), epithelioid,

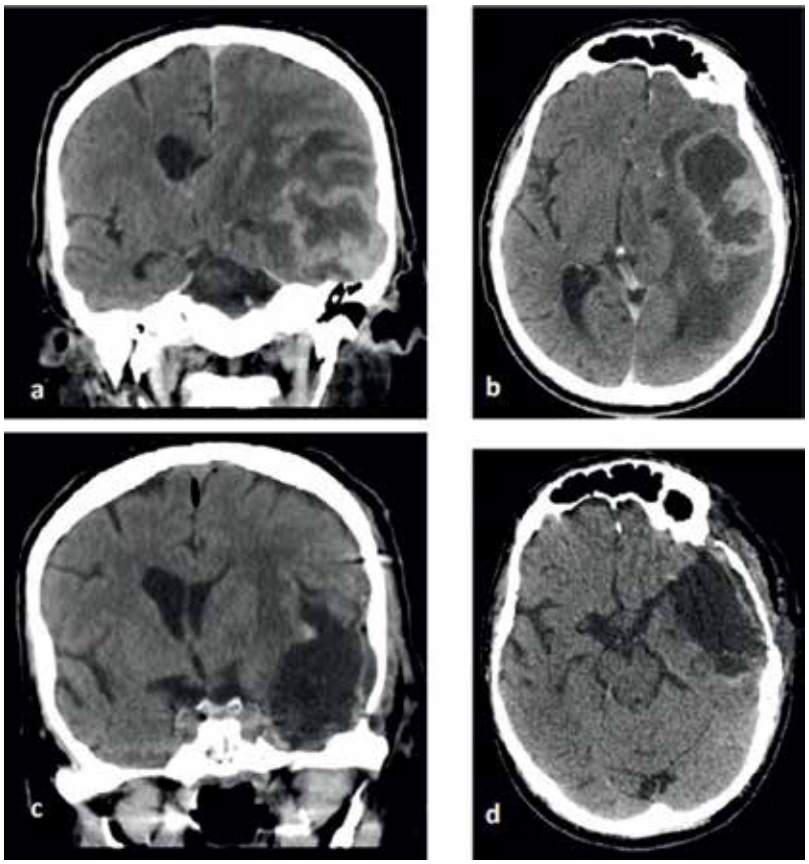

Figure 19. Coronal (a) and axial (b) preoperative contrasted CT scan examination of a case with left temporal inhomogeneous expansive lesion with a "ring enhancement" highly suggestive for a glioblastoma; (c) and (d) immediate postoperative contrasted CT scan examination demonstrating gross total removal of the tumor.

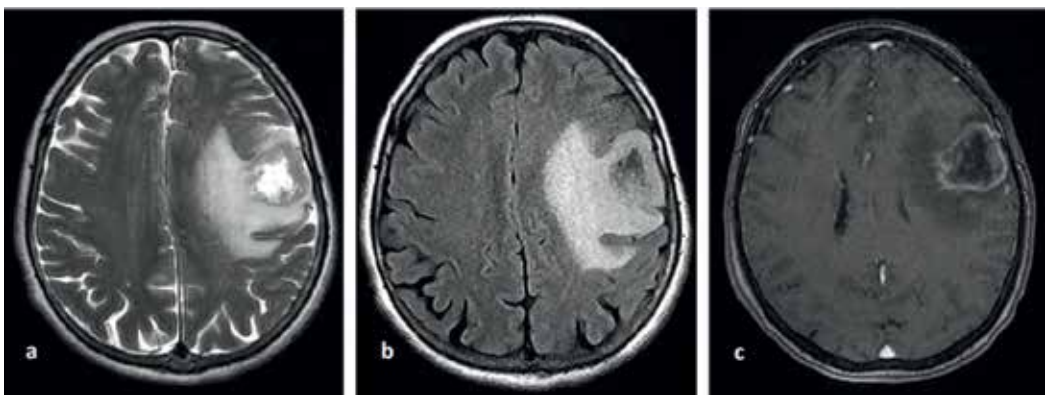

Figure 20. T2W (a), FLAIR (b) and T1W + C MRI (c) sequences of a left frontal expansive lesion, aspects that are highly suggestive for a glioblastoma.

rhabdoid, granular, or lipidized cells. The areas that contain astrocytes whose phenotype is more easily identified can be more or less clearly separated from the areas of high pleomorphism. The cells are poorly differentiated, with pleomorphism, significant atypia, and brisk mitotic activity. The presence of a cellular population having a different phenotype indicates the emergence of a new tumoral clone through new genetic events [96].

The dominance of a specific cell type in the tumor generates a pattern that is useful in the diagnosis of small cells, with a neuroectodermal component, with an oligodendroglial component, with granular cells, with gemistocytes, and with lipidized cells. This is an indicative for the diagnosis but insufficient for a distinct variant.

The small cell glioblastoma pattern consists of small, round, or slightly oblong monomorphic cells, faint atypia, and hyperchomatic nuclei surrounded by a small amount of cytoplasm. One characteristic is the heightened proliferative activity. The GFAP is variably expressed, and when present, it can be seen at the level of delicate processes. Several morphological elements found in this pattern, such as the monomorphic nuclei surrounded by a clear halo, chicken wire vasculature, or calcifications, can cause problems in the sense of a differential diagnosis with oligodendrogliomas [97]. The difference is made here by molecular tests, which evidence a molecular profile typical for glioblastomas: EGFR amplifications, Ch 10 losses, IDH wild-type, no 1p/19q codeletion [98].

The primitive neuronal component glioblastoma is a glioblastoma featuring nodules that show neuronal differentiation. These nodules are clearly distinguishable from the rest of the tumor. Cellularity is increased, and so is the proliferation index in these areas. The cells are similar to those in other CNS embryonal neoplasms or they form Homer-Wright rosettes. The immunohistochemical profile is closer to that of neuronal precursors, being positive for Synaptophysin and with a low GFAP expression [99].

The glioblastoma with an oligodendroglioma component contains foci (often minor) of classic oligodendroglioma, associated with necrosis. The presence of the two components in the same tumor, in association with necrosis, raises the possibility of a grade IV oligoastrocytoma. While the presence and the size of the necrosis means a less favorable prognosis, this type of tumor, nevertheless, has a better prognosis than the standard glioblastomas [100]. From a molecular point of view, this histopathological aspect includes several molecular groups, lacking a distinct molecular signature. The tumor may or may not feature IDH1 mutations or 1p/19q codeletion, and also TP53 mutations, Chr 7 gain, Chr 10 loss, EGFR, and PDGFRA amplifications [101].

Granular cells glioblastomas are tumors with a distinct histological aspect, characterized by an aggressive behavior, despite a morphological character indicative of a grade II or III tumor. The cells are large, similar to macrophages. They are positive for CD163 and CD68 due to the high lysosome content. They are generally negative for GFAP, even if a certain positivity can be occasionally seen in the peripheral cellular areas. They are little understood from a molecular point of view, but a study involving array-based comparative genomic hybridization has indicated a genetic profile very similar to the conventional glioblastomas (gain of CHr 7, loss of Chr. 1p, 8p, 9p, 10p, 13q, and 22q, EGFR amplification, and homozygous deletion of CDKN2A). There are no IDH mutations and no 1p/19q codeletion [102].

The lipidized glioblastoma occurs rarely, a significant number of large cells with a lipidized (foamy) aspect being present against a histological background typical for a standard glioblastoma. In such cases, the diagnosis of pleomorphic xanthoastrocytoma cannot be ruled out. From a genetic point of view, they are little studied [103]. Phenotypically, they are similar to adipocytes, but the fact that they are positive for GFAP comes to confirm their glial origin.

Three histological variants are known: giant cell glioblastoma, gliosarcoma, and epithelioid glioblastoma.

Giant cell glioblastomas are characterized by the occurrence of giant multinucleated cells (up to 20 nuclei), with prominent nucleoli. Also noticeable are the small syncytial fusiform cells. The infiltration patterns typical for the diffuse gliomas are less visible in this variant. It features a significant component of connective tissue in the shape of a reticulin network [104]. Atypical mitoses are frequent. Another characteristic feature of this variant is the perivascular positioning of tumor cells (pseudorosette-like pattern). There is a significant ischemic necrosis. Microvascular proliferation and the pseudopalisading necrosis are rarely encountered. Perivascular lymphocytes may be present [105]. The neuronal immunohistochemical markers are negative. GFAP has variable expression. The nuclear expression of p53 shows a high incidence of TP53 gene mutation [106]. The presence of the connective component, together with their circumscribed nature, makes it more difficult to distinguish macroscopically between these tumors and meningiomas and metastases.

From a molecular point of view, they showed a high incidence of PTEN and TP53 mutations; but, no EGFR amplifications and no homozygous deletion of CDK2A were shown. As a variant of the wild-type glioblastoma, it features no IDH mutations. The prognosis is slightly better than in the case of usual glioblastomas [107].

The epithelioid glioblastoma is characterized by the presence of a significant population of cells with an epithelioid aspect, eosinophilic cytoplasm, peripheral nucleus, and sharp border. Rhabdoid cells with discrete borders and eccentric oval nuclei can be present, as well as necrosis and vascular proliferations. Immunohistochemically, the GFAP has a variable expression [108]. The epithelial markers (EMA and CK AE1/AE5), as well as S100 and vimentin, are positive. The antibody that indicates the BRAF V600 mutation is positive in 50% of cases [109]. From a molecular point of view, the epithelioid glioblastoma occasionally shows genetic events characteristic of glioblastomas (EGFR amplifications, CKD2A deletion, and PTEN mutation). As a variant of the wild-type glioblastoma, it features no IDH mutations. The prognosis is worse than in the case of other glioblastomas [110].

Gliosarcoma is characterized by a monoclonal proliferation with a highly malignant biphasic histological pattern, astrocytic and mesenchymal. The astrocytic component displays the typical aspect of a glioblastoma. The mesenchymal component consists of long bundles of spindle cells, closely packed, showing malignancy (atypia, necrosis, and mitosis), similar to fibrosarcoma or the malignant fibrous histiocytoma [111]. The confirmation of malignancy in the mesenchymal component is required in order to differentiate it from desmoplasia, which can occur in the glioblastomas with meningeal invasion. Elements of differentiation can appear (cartilage, bone, muscle, and osteoclastic giant cells) [112]. The presence of connective tissue

structures in the mesenchymal component (but not in the astrocytic one) can be demonstrated using staining for reticulin and trichrome. As with the giant cell glioblastomas, the mesenchymal component makes the tumor macroscopically similar to a metastasis or meningioma. This entity can also appear in ependymomas and oligodendrogliomas, de novo or following treatment [111]. Immunohistochemically, the astrocytic component is positive for GFAP, while the sarcomatoid one is negative. P53 is positive in both components. IDHR132H is negative. Genetically, this variant shows PTEN and TP53 mutations, as well as CDK2A deletions. There are no EGFR amplifications [113]. The epithelial-mesenchymal transition in the sarcomatoid component is highlighted by the SNAIL and TWIST expression.

Necrosis and microvascular proliferations are required for a diagnosis.

The microvascular proliferations are in direct relation with the necrosis, hypoxia being a significant factor that stimulates the formation of vessels by way of HIF1A and VEGFA [114]. Another characteristic is the thrombosis of small vessels. Although, in glioblastomas, vascularization is very well represented, the vascular structures are immature and largely incapable of compensating for the hypoxia caused by the exuberant proliferation [115].

Necrosis, of the coagulative type, is typical for glioblastoma. It affects both the cells and the vascular structures and is indicative of the extremely high proliferation rate associated with this type of tumor. It is considered to be an aggressive factor in astrocytic tumors, its size being associated with a lower survival rate. In the necrotic areas, we can see "shadows" of the dilated vessels surrounded by tumor cells in various stages of decay [116]. We can also see vessels that still contain oxygenated blood surrounded by viable cells. Undoubtedly, the high proliferation rate of glioblastomas plays an important part in the onset of necrosis, as there is a mismatch between the need for oxygen and the number of functioning vessels. As in the vicinity of the necrotic areas, we notice venal occlusions and vascular thrombosis, and it has been hypothesized that this is the mechanism that could trigger or spread the necrosis. Lesions of the endothelium trigger the release of procoagulants, which generate microscopic or macroscopic vascular thrombosis. Vascular thrombosis is usually accompanied by the so-called pseudopalisading [25]. This is caused by the migration of cells from the central necrotic area to the more oxygenated exterior ones, in a "moving away wave" toward the vessels unaffected by thrombosis and capable of maintaining a level of oxygenation sufficient for their survival. In an attempt to somewhat restore the level of oxygenation, these cells also secrete proangiogenic factors (VEGFs), causing the vascular alterations mentioned above [117].

A perivascular positioning of lymphocytes can occur in the areas with gemistocytes. In glioblastomas, the number of lymphocytes can vary, and they are mainly LT CD8. Quite interestingly, the presence of an extensive LT CD8 infiltration has been identified with the long-term survivors. LT CD4 and LB are also present, but in small numbers. Microglia and histiocytes can also be seen [118].

Immunohistochemically, GFAP has a positive expression, its variability reflecting the heterogeneous nature of the tumor. Sarcomatous and primitive cellular components are negative, while the gemistocytic component is strongly positive. It is also positive in the lipidized variant. S100 and Olig2 are positive in glioblastomas and are quite useful in the diagnosis of poorly differentiated tumors [119]. Nestin is of particular importance in the differential diagnosis in

regard to other high-grade gliomas, as it is positive in glioblastomas [120]. P53 is positive in the glioblastomas with a missense mutation of TP53 [121]. Together with WT1 (which can be positive in low-grade gliomas), it makes the distinction between tumor cells and the reactive post-treatment cells [122]. EGFR indicates the relative amplification of the gene, being expressed in 45–95% of cases. EGFRvIII is present in one third of all cases [123]. The expression of Ki-67 varies. A positive IDH R132H is incompatible with the diagnosis of IDH wild-type glioblastomas.

Genetic diagnosis. Glioblastomas were the first tumors investigated by The Cancer Genome Atlas (TCGA), which highlighted alterations in the signaling pathways of EGFR, PDGFR, PI3K, NF1, TP53, and *Rb* [124]. The genetic profile of wild-type glioblastomas differs from that of IHD mutated glioblastomas, which are considered secondary and which have a genetic profile very similar to that of grade II and III astrocytomas. The characteristic genetic alteration in glioblastomas is 7p gain combined with 10q loss [125].

In what concerns the *tyrosine kinase receptors* and their signaling pathways (PI3K/PTEN/AKT/mTOR and EGFR/RAS/NF1/PTEN/P13K), EGFR is the amplified gene most frequently present in primary glioblastomas, but it is more rarely encountered in the secondary ones [126]. The amplification is accompanied by different truncations in the same tumor. The best known one is EGFRvIII, present in nearly half of the glioblastomas with amplified EGFR. The structure of the receptor is similar to v-erb, and it activates independently of the ligand. Other possible amplifications accompanied by truncation are PDGFRA and MET [127].

The PTEN gene suffers changes almost exclusively in the primary glioblastomas, either by the way of a missense mutation in the area homologous to tensin/auxilin, or following truncation at various sites caused by the loss of the chomosomal region [128].

The *TP53/MDM2/MDM4/p14ARF signaling pathway* is affected in both primary and secondary glioblastomas, especially in the secondary ones, and it is also present in grade II and III astrocytomas. MDM2 amplification is a mechanism whereby the proapoptotic and antiproliferative control of p53 is eluded and encountered in the glioblastomas that do not present TP53 mutations [129].

The CDKN2A locus generates several CDKN2 and p14ARF proteins that act as tumor suppressors. The loss of p14ARF expression is encountered in glioblastomas, being correlated with the methylation of the promoter of the deletion of the CDKN2 gene.

The CDKN2A/CD4/RB1 signaling pathway is altered in most glioblastomas and it occurs in both primary and secondary glioblastomas. The mutations of the RB1 gene are rare, and the methylation of the promoter followed by the loss of protein expression is more frequent in secondary glioblastomas than in the primary ones [7].

TERT can show mutations at the level of the promoter, especially in wild-type glioblastomas, being mutually exclusive with TP53. The occurrence of the mutation (in one of the two hot spots) is followed by the accumulation of the GABP transcription factor at the level of the promoter, leading to the aberrant expression of the gene [130].

The IDH gene is not mutated by definition in wild-type glioblastomas, and the evaluation of the mutational status of this gene can make the distinction between primary and secondary glioblastomas [131].

Epigenetics. A study conducted in cooperation with TCGA and based on the determination of the genomic profile has defined four subtypes of glioblastomas: pro-neural, neural, classic, and mesenchymal. These subtypes are characterized by a different mutational and epigenetic profile, reflected in the response to treatment. These results have paved the way toward the assessment of the epigenetic profile of glioblastomas through a determination of the whole methylation genome profile in the glioblastomas with artificially induced mutations (IDH H3F3A). These experiments have led to the identification of six different subtypes, with a different clinical evolution [132].

MicroRNA and long non-coding RNA have also been studied in glioblastomas. miR10b controls the cycle of stem and tumoral cells in GBM and is associated with a poor prognosis. The role of the interaction between microRNA and the oncogenetic pathways known as drivers in GBM, such as for instance PI3K, has also been demonstrated [133].

The currently defined histological entities have different genetic expression profiles, which are correlated with the grade and are a better predictor of patient outcome [37].

The methylation profile of the MGMT gene promoter is predictive for the response to the treatment with alkylants such as temozolomide or methylants. It is variable, being higher in the IDH-mutant tumors, having a G-CIMP profile [134].

3.7. Glioblastoma IDH-mutant

Definition. High-grade tumor with a dominantly astrocytic differentiation and a diffuse growth pattern, involving the mutation of the IDH gene.

Grading. It is ranked as a grade IV tumor.

Clinically speaking, as it is mostly located in the frontal lobe, it is often accompanied by behavioral and neurocognitive changes. As the growth is more sluggish than in the case of wild-type tumors, the signs indicating an increase in intracranial pressure are also milder.

Imaging. The areas of central necrosis typically seen in wild-type GBM are usually absent here. They are larger in size; the cystic structures are more frequent; and they show no enhancing zones on MRI.

Macroscopy. The aspect is that of a tumor infiltrating the adjacent cerebral parenchyma. The purple hemorrhagic areas and the yellow-white necrotic ones are absent.

Histological diagnosis. The morphological aspect of IDH-mutant glioblastomas is very similar to that of wild-type GBM. The areas of necrosis (ischemic or palisading) are more rarely encountered. On the other hand, the oligodendroglioma-like component is more frequent and has been associated with the presence of the IDH1 mutation [135].

Immunohistochemically, GFAP is positive and shows a certain variability. The presence of genetic events is reflected by the positivity for IDH1 and p53, and the negativity for ATRX. The overexpression of EGFR is unusual, with amplification being a characteristic of the wild-type GBM [136].

Genetic diagnosis. The presence of the IDH mutation falls under the definition of the tumor, and its identification makes it possible to diagnose the two types of glioblastoma. It is an early

	Astrocytoma	Oligodendroglioma	GBM-wt
IDH	+	+	-
ATRX	+	-	-
TERT	-	+	+
p53	+	-	+
1p/19q	-	+	-

Table 2. Synthesis of the mutational status in the main categories of diffuse gliomas.

event in tumorigenesis and remains present during the progression toward glioblastoma. The most frequent mutation of the IDH1 gene (90% of all astrocytic or oligodendroglial tumors) is R132H, when a guanine is replaced by an adenine (CGT->CAT). Other mutations, such as R132C, R132S, or R132L, occur much more rarely [137].

The ATRX gene is also mutated, the same mutation being present in the grade II and III precursor astrocytic lesions. Alongside the ATRX and IDH mutation, TP53 is more frequently mutated in secondary glioblastomas [31]. EGFR amplifications are rare, as opposed to the GBM wild-type, indicating that the genetic onset and progression pathways are different.

The genetic expression of GBM IDH-mutant is relatively homogeneous, most of them falling under the proneural profile [126]. Epigenetically, the occurrence of the IDH mutation induces an extensive hypermethylation of the DNA, all IDH-mutant tumors belonging to a hypermethylated phenotype [138]. The prognosis for GBM IDH-mutant is better than that for GBM wild-type, the survival rate being 2.4 times higher (**Table 2**) [139].

3.8. Glioblastoma, NOS

Definition. High-grade tumor with dominantly astrocytic differentiation and diffuse growth pattern, in which the mutational status of the IDH gene cannot be determined. They are grade IV tumors.

3.8.1. Multimodal treatment

3.8.1.1. Surgical treatment

Sir Rickman Godlee was the first surgeon who reported a resection of a glioma in 1884. More than one century later, the results of the treatment in malignant gliomas remained unsatisfactory. Improvements of surgical techniques and technology developed in this period, including microsurgery, neuronavigation, intraoperative MRI, intraoperative neuromonitoring, and 5-ALA merely improved the grade of resection while increasing the postoperative quality of life of the patient; however, they were unable to change the inexorable course of malignant gliomas to recurrence and death. Perhaps the most important adjuvant of surgery was the introduction of radiotherapy in the middle of the last century, adding a median survival of at least 7 months, as it was highlighted by Ley and coworkers in 1962. In their reported series of

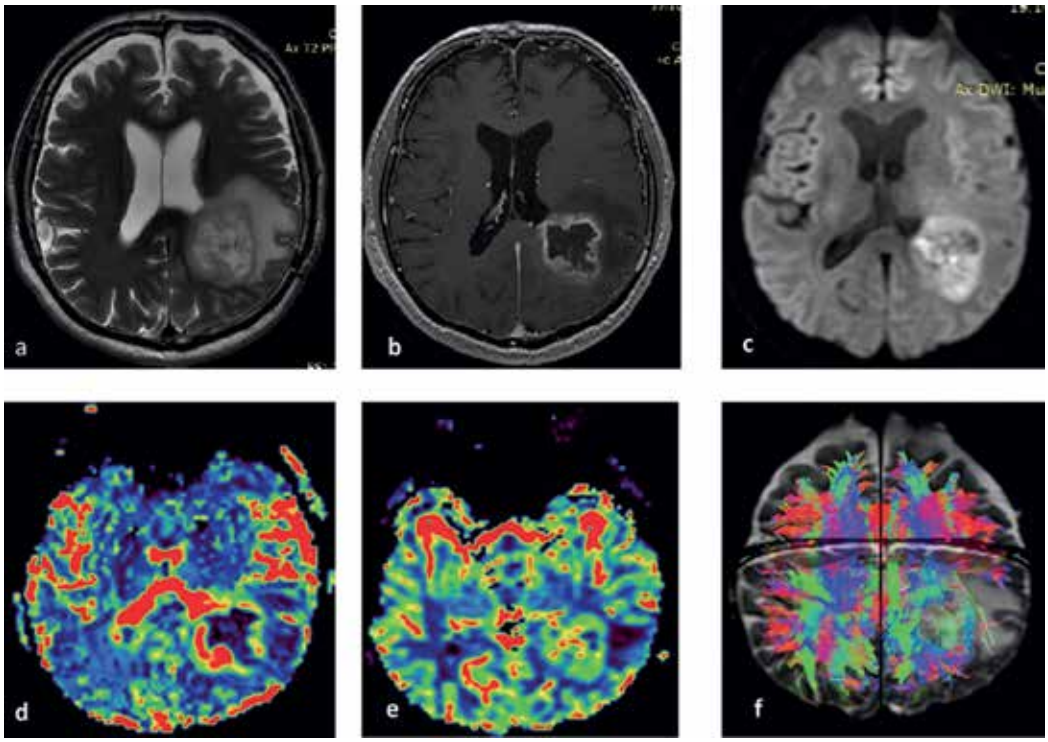

Figure 21. MRI examination of a patient with left paraventricular glioblastoma: (a) T2W sequence reveals an inhomogeneous lesion adjacent to occipital horn of left lateral ventricle surrounded by a hyperintense region of edema; (b) T1W + C sequence expose classical aspect of "ring enhancement"; (c) DWI study demonstrating a restricted diffusion corresponding to the region of tumor; MRI perfusion reveals a heterogeneous rCBV (d) and increased cerebral blood flow (CBF) corresponding to the enhanced lesion (e) ; (f) Tractography shows a disruption of fibers compared to the contralateral side.

glioma, the largest at that time, the median of survival through surgery alone was 7 months, whereas in the group receiving additional radiotherapy the median survival rate was at 15 months [140]. The addition of chemotherapy further improved the survival, but despite the large advancements in research within the last decade, the median survival rate barely increased by no more than 2 months, inexorably close to the data reported 20 years ago [141].

Regarding the role of surgery, there is an increasing body of literature underlining the importance of GTR comparing with STR or biopsy. A meta-analysis covering 41,117 unique patients in 37 retrospective studies revealed a significant improvement of OS in GTR cases compared with STR [142]. Another prospective study comparing grade of resection with the aid of immunofluorescence (5-ALA) versus white light also demonstrated a significant median of survival in favor of those with GTR (16.7 months vs. 11.7 months) [143]. Currently, there is sufficient data favoring GTR to encourage surgeons to increase the grade of resection, while also striving not to cause additional neurological deficits (**Figure 21**).

New tools were introduced in the last two decades in order to facilitate GTR, simultaneously with the permanent improvement of the operative microscope. Contemporary neuronavigation

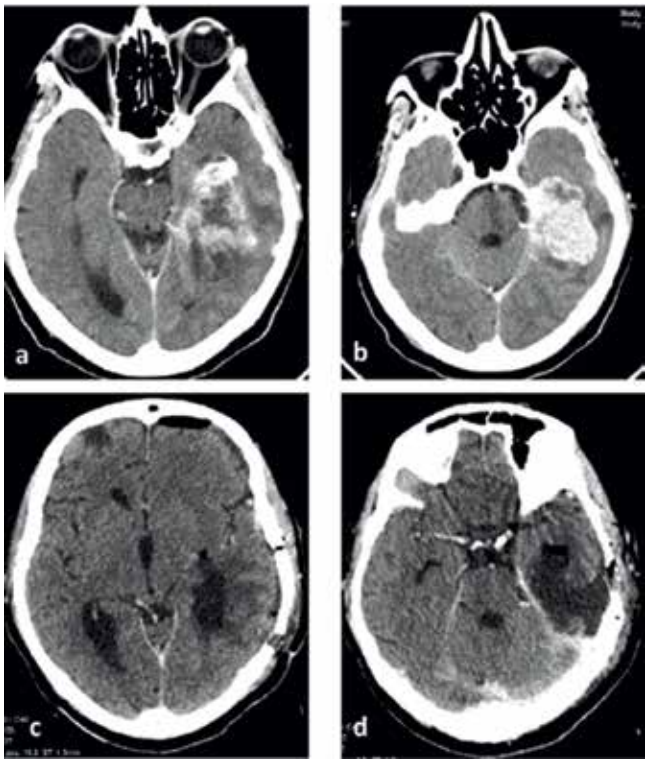

Figure 22. Preoperative (a and b) and postoperative (c and d) contrasted CT scan images demonstrating the GTR of a left gliosarcoma.

equipment offers a more precise localization with real-time correction adapted to the brain shift. Functional and anatomical data are merged in order to facilitate surgical intervention, while also avoiding damage to eloquent areas and tracts. Intraoperative tools, such as intraoperative MRI, are now available in many centers, allowing surgeons to achieve a more complete tumor removal and, as a direct consequence, prolong survival [144]. Intraoperative ultrasonography, having been introduced in practice two decades prior, is at present more accurate in defining intracerebral lesions, especially in cases of HGG, facilitating a real-time control of resection (**Figure 22**) [145].

Contrasted intraoperative ultrasound guidance apparently adds more detailed information concerning the grade of resection [146].

We may conclude that surgery, and radical surgery especially, remains the first and most important step of the multimodal treatment in prolonging the survival of patients with malignant gliomas.

3.8.1.2. Adjuvant treatment

Whole brain radiation therapy stood as the most important adjuvant to surgery up until the randomized trial conducted by Walker el al. in 1980. In this trial, the authors compared the efficacy of radiotherapy alone to the addition of nitrosourea chemotherapy. They demonstrated a

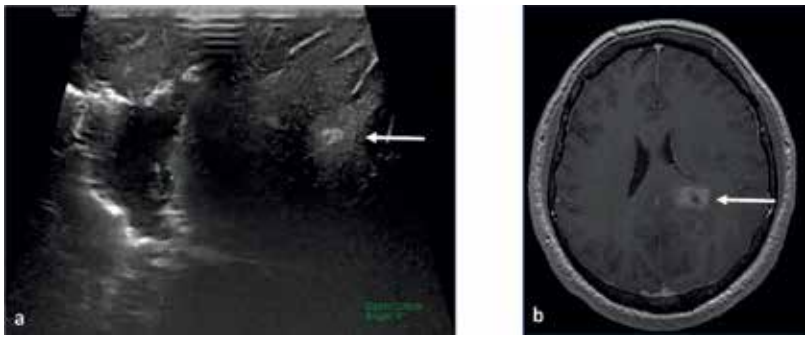

Figure 23. Ultrasonography-guided resection (a) of a diffuse astrocytoma with foci of glioblastoma (arrow) demonstrated on T1W + C image (b).

benefit of 2–3 months in the group treated with combined radio-chemotherapy and this combination has been the standard of care for almost 25 years [147]. In time, whole brain radiotherapy was replaced by a more focal radiotherapy, in order to prevent secondary effects of radionecrosis. The current standard of radiotherapy is a fractionated conformational dose of 2Gy fractions/day, 5 days/week for a period of 6 weeks. The radiotherapy regimen must be initiated in three to no more than 6 weeks after the surgery [148].

Concerning chemotherapy, the actual standard consists of concomitant radiotherapy and temozolomide (TMZ) administration, followed by six cycles of TMZ at every 28 days. This was established in 2005 based on the results of the trial conducted by Stupp et al. (**Figure 23**) [79].

The response of the patients to the TMZ regimen is highly variable, with one of the factors influencing the positive response being the methylation of the MGMT promoter, which is present in less than 50% of glioblastoma cases. It was showed that only 65–70% of new cases of glioblastoma respond to TMZ; although in spite of these evidences, TMZ is still the main agent in multimodal treatment of glioblastoma (**Figure 24**) [149].

A large number of researches were made in order to decrypt the intimate mechanism of apparition and development of glioblastomas, as well as the response of tumor cells to different chemical, physical, or biological agents. Among these agents, only few met the clinical criteria to be introduced in practice. Gliadel (carmustine-impregnated biodegradable wafers) on local application proved to add a median of survival of 2.3 months compared to the standard treatment, yet was accompanied by an increased incidence of local complications (infection and CSF fistula) [150]. We also add our research effort on glioblastoma stem cell cultures in order to improve the response to TMZ by addition of new drugs like arsenic trioxide or metformin as sensitizers [151, 152]. New ways to deliver TMZ have been proposed [153]. Antiangiogenic agents (Bevacizumab) was also tested on glioblastoma stem cells with controversial response (in some cultures, they increased angiogenesis) (**Figure 25**).

The clinical trials testing Bevacizumab in newly diagnosed glioblastoma failed to demonstrate a benefit on survival [154, 155]. Immunotherapy is a highly experimental approach of present days, as are other therapies. A special interest was raised in the use of an electric field delivered by noninvasive transducers placed on a headpiece, the so-called Novocure

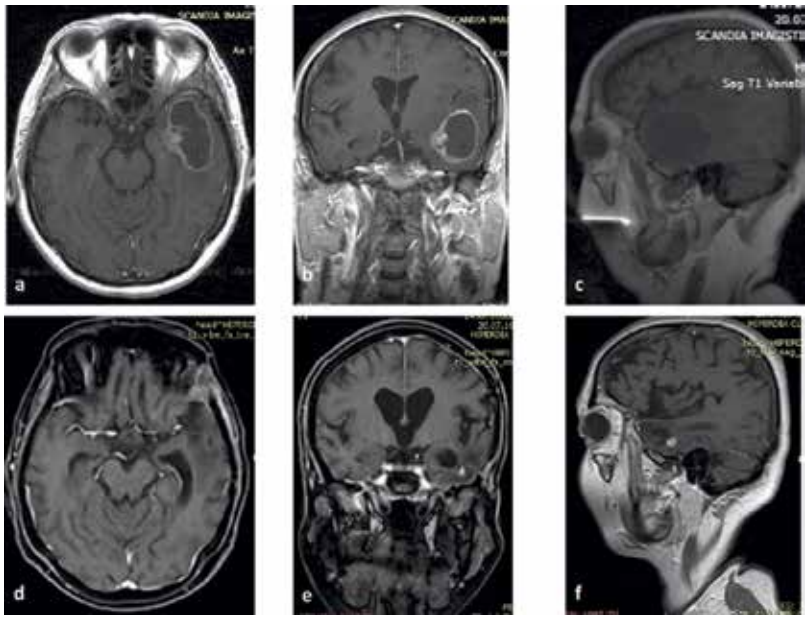

Figure 24. Preoperative axial (a), coronal (b) and sagittal (c) T1W + C sequences of a patient with glioblastoma completely removed and treated with the standard radio-chemotherapy regimen; postoperative enhanced T1W images (d–f) reveal the removal of the tumor with the persistence of a small contrasted nodule, which is stable at 5 years after surgery.

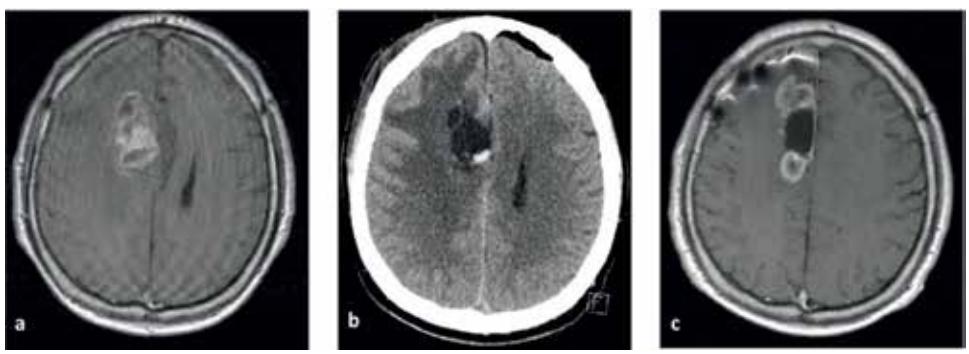

Figure 25. Preoperative T1W + C sequence (a) of a glioblastoma, almost completely removed as it was revealed by immediate postoperative CT scan (b); the patient was not responsive to the standard adjuvant treatment as it is demonstrated by the postoperative enhanced MRI at 4 months (c).

treatment. The addition of this new modality to the standard radio-chemotherapy regimen improved the PFS and OS in glioblastoma-treated patients [156].

We can conclude that despite the huge efforts and investments made in research and therapies, the results still disappoint. This suggests the fact that we are possibly on the correct course, but against the tide. The future multimodality treatment may consist of patient-tailored treatments owing to the multiple individual and tumor-related factors that influence the response to therapy.

http://dx.doi.org/10.5772/intechopen.76205

3.9. Diffuse midline glioma, H3 K27 M-mutant

Definition. High-grade infiltrative astrocytic tumor, with midline location and showing the K27 M mutation in the H3F3A or HIST1H3B/C genes. They are grade IV tumors.

Clinically, it is usually encountered in children and more rarely in adults. The symptoms may be either general (usually produced by the obstructive hydrocephalus) or focal generated by the involvement of different cranial nerves: facial paresis, diplopia, hearing loss, dysphonia, and dysphagia. The extension of the tumor in cerebellar peduncles produces ataxia. The involvement of long tracts determines motor weakness. Particularly in diffuse pontine glioma, behavioral changes such as pathological laughter and anxiety were described before the onset of other more common clinical signs [157].

Imaging. On MRI, these lesions have a diffuse homogenous appearance of hypointense in T1W sequences and hyperintense in T2W and FLAIR ones. The contrast is discrete or absent, but some focal enhancement into an infiltrative hypointense mass on T1W + C sequences could be detected in few cases (**Figure 26**).

MRI spectroscopy will differentiate these tumors from other inflammatory, vascular, or infectious lesions. The Cho/Cr and NAA/Cr ratios are typically increased. An elevation of these ratios after radiotherapy poses the significance of progression [158].

Macroscopy. The infiltrative pattern is reflected in the size and the changes in the shape of the nervous structures affected by the tumor. In cross section, we can see purple hemorrhagic areas or yellowish-white necrotic ones.

Histological diagnosis. The aspect is that of an intensely infiltrative tumor consisting of small-size monomorphic, astrocytic cells. While most are accompanied by necrosis, microvascular proliferations, and an enhanced mitotic index, these elements are absent in a small number of tumors. According to the new rule introduced by the latest WHO classification, whereby "molecular beats histology," even if histologically they are grade II, they shall nevertheless be deemed grade IV. *Immunohistochemistry*. GFAP shows a heterogeneous expression in these tumors. On the other hand, they are positive for S100, OLIG2, and NCAM1. Mutations of ATRX and TP53 can occur, but more rarely. An antibody targeting the protein modified by the presence of the H3.3 K27 M mutation can be used in the diagnosis [159].

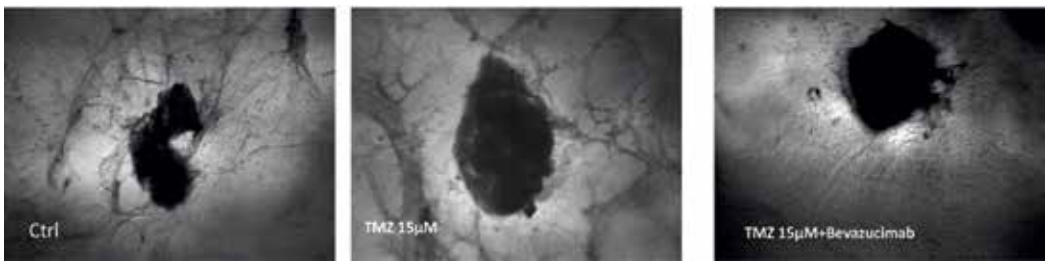

Figure 26. In vitro 3D model of glioblastoma stem cells culture demonstrating an increase angiogenesis in contact with TMZ and resistance to the addition of Bevacizumab compared with the control (ctrl) (personal archive).

Genetic diagnosis. This entity is defined by the presence of the K27 M mutation in the H3F3A (80%), HIST1H3B (20%), and HIST1H3C genes. The genetic profile is completed by mutations in the TP53 and ATRX genes, homozygous CDKN2A deletion, and MYC, CDK4/6, and PDGFRA amplifications [160].

3.9.1. Multimodal treatment

This type of tumor is not amenable for surgical treatment. The single debate is related to the role of biopsy. The actual accepted strategy is that biopsy (open or stereotactic) is indicated only in atypical tumors. The term of atypical is somehow unclear, but generally it is accepted that the unilateral extension of tumor and the presence of cystic components or focal hemorrhages offer a rationale for surgery.

In centers with experience in stereotactic biopsy, this could be the method of choice. Otherwise, open surgery is performed, guided by the most superficial part of lesion or throughout the so-called "safe entry zone." In selected cases, surgical interventions can offer the opportunity of cytoreductive surgery, thus creating proper conditions for radiotherapy (**Figure 27**) [161].

Radiotherapy is the single recommendable therapy for this kind of lesions (**Figure 28**). A total dose between 54 and 60 Gy, administered in fractions of 1.8–2 Gy, is the actual recommendation of oncologists. No chemotherapy regimen was able to demonstrate a benefit in terms of survival. Corticosteroids are also administered during radiotherapy. After a period of symptom amelioration, the tumor will inevitably progress [162].

3.10. Pediatric diffuse astrocytic tumors

Despite having a particular clinical evolution, pediatric tumors have been lumped together with the adult ones, according to the histopathological similarities [163]. We now know that their onset and progression genetic mechanisms are different. A well-defined entity that occurs predominantly in children is the one described above. As opposed to adult tumors,

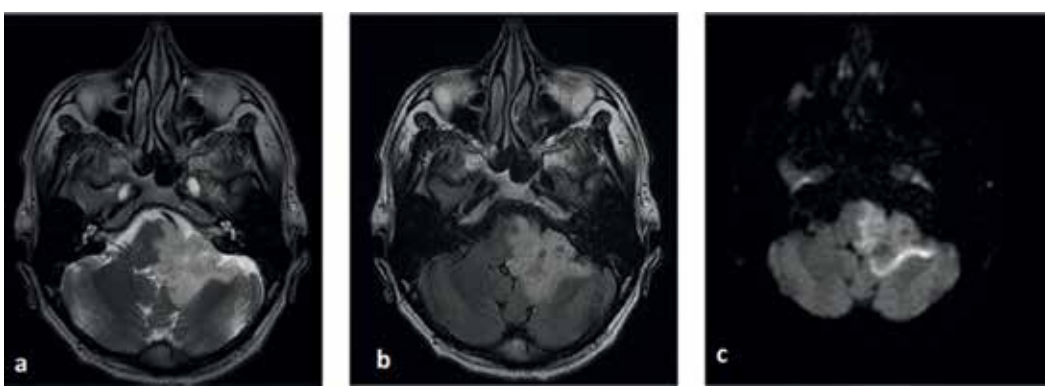

Figure 27. T2W (a), FLAIR (b) and DWI (c) sequences of an infiltrative tumor into the brainstem and left cerebellar peduncles.

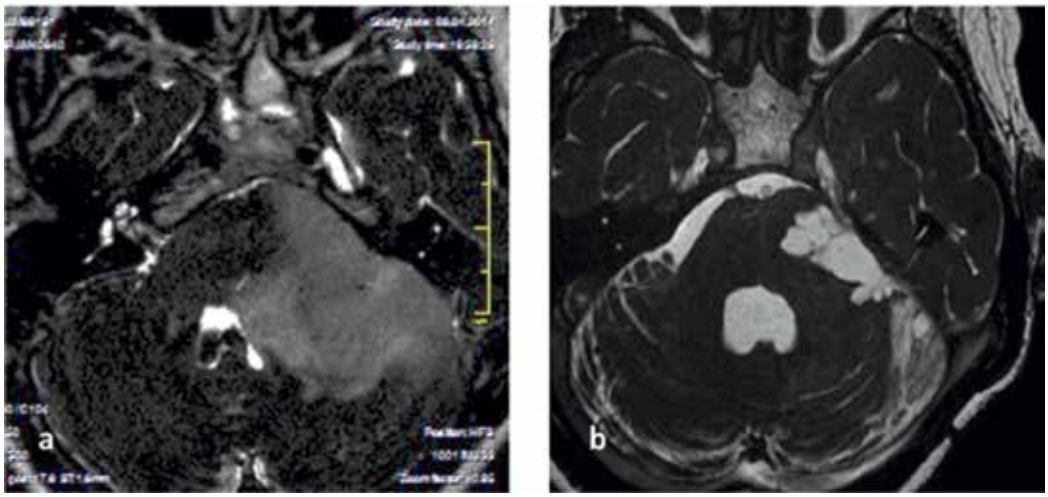

Figure 28. (a) T2W sequence of a diffuse infiltrative brainstem astrocytoma, partially resected; the 18 months follow-up MRI (b) demonstrates a stable lesion after radiotherapy.

the pediatric ones show changes in the genes regulating chromatin structure and the genetic expression profile. Apart from the diffuse midline glioma, H3 K27 M-mutant, another mutation present in the same gene, but in the G34 rather than the K27 position, defines another entity usually encountered in youths. The location differs, as it is no longer situated in the midline area, but in the brain hemispheres. High-grade astrocytic pediatric tumors with a telencephalic location show the TP53, CDK2A, ATRX, and SETD2 mutations [164]. The genetic syndromes that favor the onset of brain tumors during childhood are type 1 neurofibromatosis and Li-Fraumeni syndrome.

4. Conclusions

The 2016 brain tumor classification represents an important step forward in the introduction of molecular diagnosis in the daily current practice. This is also an important step toward a personalized patient-tailored multimodal management of brain tumors. Neurosurgeons, as part of a multidisciplinary team, need to be familiarized with all the aspects regarding specific brain tumors in order to offer the best chance to their patients and to prolong survival with a good quality of life.

Acknowledgements

We thank our colleagues Dr. Reka David for spectroscopy and DTI analysis, Dr. Alexandru Florian and Lecturer Bogdan Aldea for English language improvement and to Dr. Anne-Marie Constantin for manuscript editing.

Conflict of interest

The authors declare that there is no conflict of interest.

Abbreviations

1p/19q-codel	1p/19q-codeleted
ATRX	alpha thalassemia/mental retardation syndrome X-linked
CK	cytokeratin
DA	diffuse astrocytoma
DTI	diffusion tensor images
EGFR	epidermal growth factor receptor
EMA	epithelial membrane antigen
FGFR1	fibroblast growth factor receptor 1
GBM	glioblastoma
GFAP	glial fibrillary acidic protein
HIF1A	hypoxia-inducible factor 1-alpha
IDH	isocitrate dehydrogenase
IDH-mut	IDH-mutant
LT	lymphocyte B
LT	lymphocyte T
MDM2	mouse double minute 2 homolog
MDM4	mouse double minute 4
MGMT	O^6-methylguanine DNA methyltransferase
MRI	magnetic resonance imaging
MRS	magnetic resonance spectroscopy
ms	milliseconds
NCAM1	neural cell adhesion molecule
NF1	neurofibromatosis 1
NOS	not other specified
PI3K	phosphatidylinositol-3 kinase

PRESS spectra	point-resolved spectroscopy
PTEN	phosphatase and tensin homolog
Rb	retinoblastoma
TE	echo time
TERT	telomerase reverse transcriptase
TP53	tumor protein
VEGFA	vascular endothelial growth factor A
wt	wild type

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Chapter 7

Neurosurgical Options for Glioma

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.82603

Abstract

Glioma surgery has been the main component of glioma treatment for decades. The surgical approach changed over time, making it more complex and more challenging. With molecular knowledge and diagnostic improvement, this challenge became maximally safe resection of tumor, which resulted in prolonged overall survival, progression-free period, and a better quality of life. Today, the standard glioma treatment includes maximally safe resection, if feasible, administration of temozolomide, radiotherapy, and chemotherapy. Surgical resection is performed as subtotal resection, gross total resection, and supratotal resection. Subtotal resection is the resection where a part of tumor is left. Gross total resection is a complete removal of the magnetic resonance imaging (MRI) visible tumor tissue. Supratotal resection is performed as gross total resection with excising the MRI visible tumor tissue borders into the unaffected brain tissue. Before we make final decision on which type of resection should be performed, many factors have to be considered. The main question has to be answered: what the actual impact of resection on the progression of glioma is and what the functional risk of resection is.

Keywords: glioma biopsy, subtotal resection, gross total resection, supratotal resection, oncofuntional balance

1. Introduction

Glioma is the most frequent malignant tumor of the brain, with a high mortality as the grade of glioma gets higher [1]. World Health Organization in 2016 classified glioma into four grades, where I and II grades are classified as low grade glioma and III and IV grades as high grade glioma [2]. Glioma surgery is one of the most common and challenging

surgeries for every neurosurgeon. The history of glioma surgery changed during the past century following all technical progresses. One of the biggest technical improvements started with the discovery of nuclear magnetic resonance in the 1950s, now known as magnetic resonance imaging [3]. After MRI introduction, the concept of maximal MRI visible tumor resection started to be the standard approach to glioma surgery. Nevertheless, this concept had a significant rate of morbidity, and it stranded to be valid for decades. During the past 25 years, the concept of surgical removal of gliomas has changed from a maximally aggressive for high grade glioma to minimally invasive but maximally efficient resection. The concept for low grade glioma changed also from "watch and wait" to active surgical treatment [4].

In the course of years, *subtotal resection* (STR) and *gross total resection* (GTR) evolved to *supratotal resection*, which became the surgical option especially for low grade glioma in the eloquent area and younger patients. With supratotal resection, neurosurgeons are trying to utilize minimally invasive surgery for the preservation of life quality as much as possible, but resecting most of tumor tissue using brain monitoring techniques, intraoperative imaging, awake surgery options, etc. The overall survival period with this approach was extended. As the new surgical concept, supratotal resection, which is actually also aggressive but selective, controlled, and monitored approach, over the years confirmed the highest level of general development of glioma surgery. In this respect, supratotal resection is probably becoming the most important part of *state of the art* in glioma management generally, but we are waiting for the results of big clinical trials. When the tumor infiltrates eloquent brain areas the challenge is how much to resect in balance of maximal safe resection and possible neurological deficit or worsening of functional status [4].

In this review, we are going to discuss surgical options for glioma treatment and the impact of it on the patient's life.

2. Different surgical options

Gliomas represent 30% of all brain tumors and 80% of all malignant brain tumors [1]**.** The origin of gliomas are the transformed glioma cells of the central nervous system. Gliomas can be classified by cell type, localization, and grade. The grade classification is performed by World Health Organization (WHO) in 2016 and widely used [2].

There are four grades of gliomas:

Grade I—pilocytic astrocytoma;

Grade II—diffuse astrocytoma, oligodendroglioma;

Grade III—malignant glioma: anaplastic astrocytoma, anaplastic oligodendroglioma;

Grade IV—glioblastoma multiforme (IDH wild type and mutant), diffuse midline glioma, H3 K27M-mutant.

http://dx.doi.org/10.5772/intechopen.82603

Grade I and II gliomas are classified as low grade glioma (LGG) and grade III and IV as high grade glioma (HGG). Surgical treatment options are different for every group of gliomas, LGG and HGG, due the glioma life cycle [2].

2.1. Surgical techniques

In this review, we will present contemporary surgical techniques used in treatment of both glioma groups: LGG and HGG and the impact on patient's life. Surgery remains the core treatment for management of gliomas. Surgical resection of pathological tumor mass, almost nonfunctional brain region, is common, standard, and the oldest neurosurgical approach to contemporary neuro-oncology. Historically, glioma surgery was a controversial topic, but many recent studies have demonstrated the crucial place of glioma resection in the management of low and high grade gliomas [5]. Concerning glioma surgery, there are two big questions: what the actual impact of resection is on progression of glioma and what the functional risk of it is [6]. To improve the outcomes of these two questions in past decades, a huge improvement has been made in intraoperative techniques (neuronavigation, intraoperative MRI, intraoperative ultrasound, stimulation mapping techniques, and fluorescence-guided surgery; **Figure 1**) [7, 8]. These techniques were developed to maximize the resection of glioma and preserve or improve the quality of life [6, 7]. Before glioma surgery, the neurosurgeon must calculate all benefits and possible hazards which could influence on morbidity, mortality, and quality of the rest of life.

There are few surgical options based on glioma type:

- glioma biopsy.
- subtotal resection.
- gross total resection.
- supratotal resection.

Figure 1. Typical intraoperative arrangement of the patient who undergoes tumor resection and the neurosurgeon, in the early stage of glioma surgery, immediately before dural opening. Microscope is positioned close to the surgeon's left arm and neuronavigation tool is in front (picture on the left). After the opening of the dura, characteristic findings of differently colored glioma affecting the brain cortex are presented. Tumor tissue bulks over the dural edge; it is obviously white-grayish and much lighter, with strange pathological vascularization (picture on the right).

2.1.1. Glioma biopsy

Glioma biopsy is the standard procedure for taking tissue samples of tumors with a minimally invasive approach. It consists of taking a sample of tissue through a minimum opening of the skull. Biopsy indications are tumors in difficultly accessible brain zones, multicenter lesions, and pathology where it is advisable to establish a pathohistological diagnosis, which will then determine further surgical or nonsurgical treatment (e.g., whether it is tumor glioma or lymphoma; the former will then undergo resection, while the latter is mainly nonsurgically treated). On the other hand, very advanced gliomas and gliomas in the elderly who are not candidates for a more aggressive approach (open surgery) are also indications for biopsy. Minimally invasive approaches have been favored for a long time in neurosurgery for glioma resection.

2.1.1.1. Frame-based stereotaxis

Today, Leksell G frame is still the most widely used, consisting of two parts—one fixed quadrangular and the other moving, the so-called arc. The fixed part consists of four graduated rods. On the right or left side of the patient, there is a diagonal bar in the form of the letter "N" that connects the corners.

Of the four basic geometric systems used in frame-based navigation systems, the "arc radius" system is the most commonly used, which is the base of the Leksell frame. The principle of the C arc is based on the ability to reach the center of the C arc from all directions by moving the probe along the radius of the frame. The frame itself is fixed on the patient's head, while the C arc is a movable part with the possibility of penetrating with a puncturing needle through any point on the convexity of the skull. The advantages of the Leksell frame design are the ability to reach any intracranial point with great precision and the ability to use it directly under the control of CT or MRI. One of the disadvantages is the bulkiness of the device itself, the obstruction of the operating field, and the "non-real time" procedure if there is no radiological supervision (CT or MRI).

By setting up the frame and the effect of the CT scanning, the center is first marked in anterior–posterior direction. The center is observed on axial CT scans in the form of the letter X by joining the frame corners. On the vertical plane, the center is marked by pulling the corner line through the mid-point of the letter "N." After defining the center, the target point is defined in relation to the geometric center of the frame and it can be accurately calculated, after which the C arc is moved and the probe is placed in the center of the lesion.

2.1.1.2. Frameless stereotaxis

Next evolutionary step in the development of stereotaxis was frameless stereotaxis. Frame-based stereotaxis is a clearly defined relationship between two coordinate systems (preoperative and intraoperative) and a precise position in relation to the planned procedure without further need for determining coordinates. Frameless stereotaxis, unlike frame-based, uses "point pair" registration to establish a relationship between preoperative images and surgical

field or coordinate systems relative to preoperative images and patients during surgery. To connect these two coordinate systems, "registration" is necessary to establish a common relationship between two coordinate systems [9].

The frameless system consists of two parts, an infrared scanner and a dynamic frame, that is fixed to the Mayfield holder. The dynamic scanner transmits the coordination system data to the operating field and integrates them with software with preoperative images. The dynamic framework requires at least three fixed registration points (nasion, lateral cannula, frontal tuber, and tragus). In the further course, an additional multipoint registration on the curvature of the skull is used for the purpose of co-registration and refining the matching of the two coordinating systems. Registration is performed by marker or laser. Once the registration has been made, it is necessary to carry out a check for early identification of errors. The advantages of the frameless system are ease of use, as well as intraoperative possibility, altering the route or plan without the need for additional CT scanning, not taking up a large space, the possibility of absolute freedom of manipulation of the operating field, and no preoperative planning and determination of the coordination point on CT or MRI.

As the leading flaw of the frameless system, there is a decrease in precision compared to frame based, most likely because of less defined registration points in comparison to clearly defined points. However, these deviations from the target point in the brain do not exceed a value of 2–5 mm; they are expected and they are generally accepted when executing the procedure.

2.1.1.3. Navigation-guided glioma biopsy

Most important indications for neuronavigation in glioma surgery are:

a. Neuronavigation driven resections, subtotal resections, and reduction of gliomas (determination of tumor margins, edges of craniotomy, as well as limitations of the form and length of skin incisions)

b. Neuronavigational inducement of glioma biopsy

- early diagnosis with minimal risk
- deep lesions (thalamic gliomas, basal ganglia etc.)
- patients with a high operative risk in case of prolonged anesthesia or those with advanced diseases and poor prognosis compared to co-morbidity
- multiple lesions
- patients who reject the proposed surgery for resection
- patients with "unsafe" radiological diagnosis and suspicion of infectious or demyelinating disease

c. Setting the catheter into tumor cavity

- cyst drainage
- intratumoral chemotherapy
- intratumoral radiotherapy

Unrelated to the use of neuronavigation in oncology or the treatment of gliomas, neuronavigation can be used in ventriculostomy, electrode placement for deep brain stimulation, endoscopic neurosurgery, etc.

2.1.1.4. The present, challenges, and future of biopsy

One of the disadvantages of the established stereotaxy is deviation during the procedure and its execution at the present time in the MRI or CT preoperative images. Currently, efforts are being made to overcome the problem by real-time MRI-induced biopsy with promising results [10, 11]. In certain cases, when we have an extremely small tumor, the precision of biopsy is questionable with disappointing pathohistological results. Today, microelectrodes are used to do the microrecording of the brain electrical potential on the biopsy path and in the target tumor zone with the absence of electrical potentials, performing biopsy by the "real time" method and increasing safety and precision [10].

Since fluorescence 5 ALA has already found its use in tumor surgery and increased radicalism of resection, it is also applied in biopsy; a decrease in negativity of results was observed [11].

Because of the existence of brain blood barriers and inadequate chemotherapeutic penetration of brain tumor, convection-enhanced delivery is developed, which represents a modality of combined surgical and colon tumor treatment. Locally, the medicine (chemotherapeutic) is placed into the brains of rats by using a frameless catheter; it is used in the treatment of diffuse brain stem gliomas [12].

2.1.2. Subtotal resection

Glioma resection in which a portion of tumor can still be seen in postoperative images is called subtotal resection (STR). STR should be performed only when it is not possible to perform gross total resection (GTR). To have better results of STR, tumor mass must be removed as much as possible to preserve functionality of the patient and quality of life. All technical supports should be used to optimize STR. Intraoperative MRI should be used in every STR to optimize glioma safe removal [8].

2.1.3. Gross total resection

Some mathematical modeling studies estimated that at least 78% of preoperative tumor volume must be resected to increase survival and resection brings an incremental benefit of up to 98% [13]. The surgical resection technique that brings this benefit with clear margins is called gross total resection, **Figures 2** and **3**. Since the beginning of wide use of modern operative

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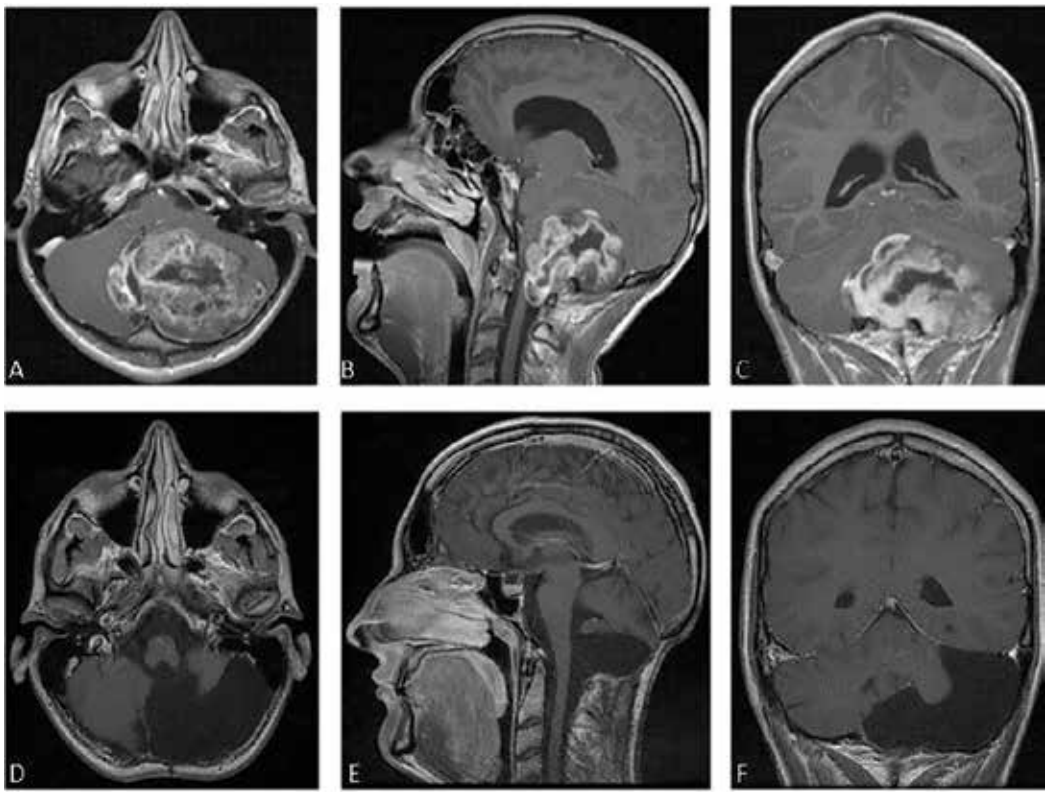

Figure 2. A 19-year-old male patient admitted to our department comatose, with Glasgow Coma Scale score—GCS 4, with respiratory dysfunction (August 2015). Urgent surgery was performed with GTR, pathohistology confirmed LGG. Three weeks later, the patient was discharged from the department in good condition—awake, walking alone with Karnofsky score 80. Preoperative axial, sagittal, and coronal postcontrast T1-weighted MRI (A-C) show a huge tumor in the cerebellum, predominantly on the left side with adherence to the brain stem and propagation down to C2 (Siemens MRI Avanto 1.5 T). Two and a half years postoperative contrast T1-weighted MRI, in March 2018 (D–F), shows complete tumor resection with no signs of tumor rest or recurrence.

techniques, the incidence of gross total resection has increased [8]. On the other hand, without technical development, microsurgical GTR of glioma cannot be always achieved because of the deep location of tumor, located in the eloquent regions, and/or both hemispheres spanned [14]. Aggressive GTR can lead to increased morbidity and complications without improving survival [15, 16]. GTR is shown to be an independent factor of overall survival (OS), but, in respect of STR, evidence is not clear for its benefit [6–8, 15]. Like for STR, in GTR also, intraoperative MRI and all technical supports should be used to have a better resection and to preserve the eloquent brain areas.

2.1.4. Supratotal resection

Surgical resection of pathological tumor mass, almost nonfunctional brain region, is common, standard, and the oldest neurosurgical approach to contemporary neuro-oncology. Historically, glioma surgery was a controversial topic, but many recent studies have demonstrated the crucial

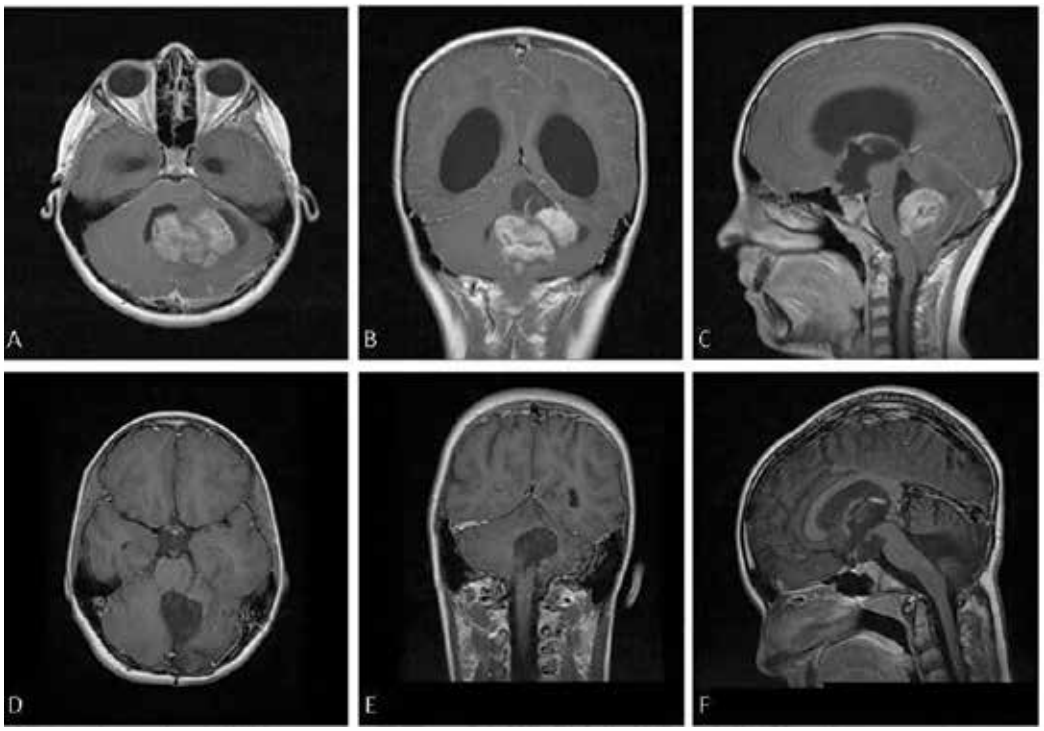

Figure 3. An 11-year old girl with glial tumor in the posterior fossa producing hydrocephalus, admitted to the neurosurgical department with impairment of consciousness, walk and gait disturbance, fully dependent on parents (June 2015). Preoperative T1W axial, coronal, and sagittal contrast brain MRI (A–C) revealed a huge glial tumor, predominantly solid, mostly located in the left cerebellar hemisphere with partial involvement of the fourth ventricular floor and compression to the brain stem (low grade glioma). Two and half years of postoperative contrast T1W MRI shows no recurrence of tumor (D–F, March 2018). The child is in full condition without any neurological deficit.

place of glioma resection in the management of low and high grade gliomas [5]. Over time, the approach to glioma changed from minimally invasive biopsy to maximally possible resection (STR or GTR). Today, total resection changed from temporal lobectomy to very aggressive frontal lesion—paralesional resection of high grade glioma. In the past decade, supratotal resection became a popular approach technique for glioma resection. Supratotal resection includes, like GTR, a total tumor resection with radical resection of perilesional brain tissue, nonglioma region, **Figure 4**. Even after GTR, because of the infiltrative behavior of glioma, probably there are some cells left, which is the biggest challenge to a successful resection and increase in recurrence, OS, and progression-free survival (PFS) [5, 17]. With conventional MRI, it is almost impossible to estimate spatial extent of infiltrative glioma [5]. In low grade glioma, tumor cells are found up to 20 mm beyond MRI seen abnormalities, while in GBM, cells are found diffusely in the hemisphere on the GBM site, even contralaterally [17]. These cells lead to diffuse spread of tumor and its recurrence [5]. Visualization of these cells is beyond any known technique. With supratotal resection of the margins around tumor visible on FLAIR-weighted MRI, there is a limited possibility of leaving residual cells. This resection impacts also on the history of malignant transformation of glioma, which occurs after diagnosis in 4-year

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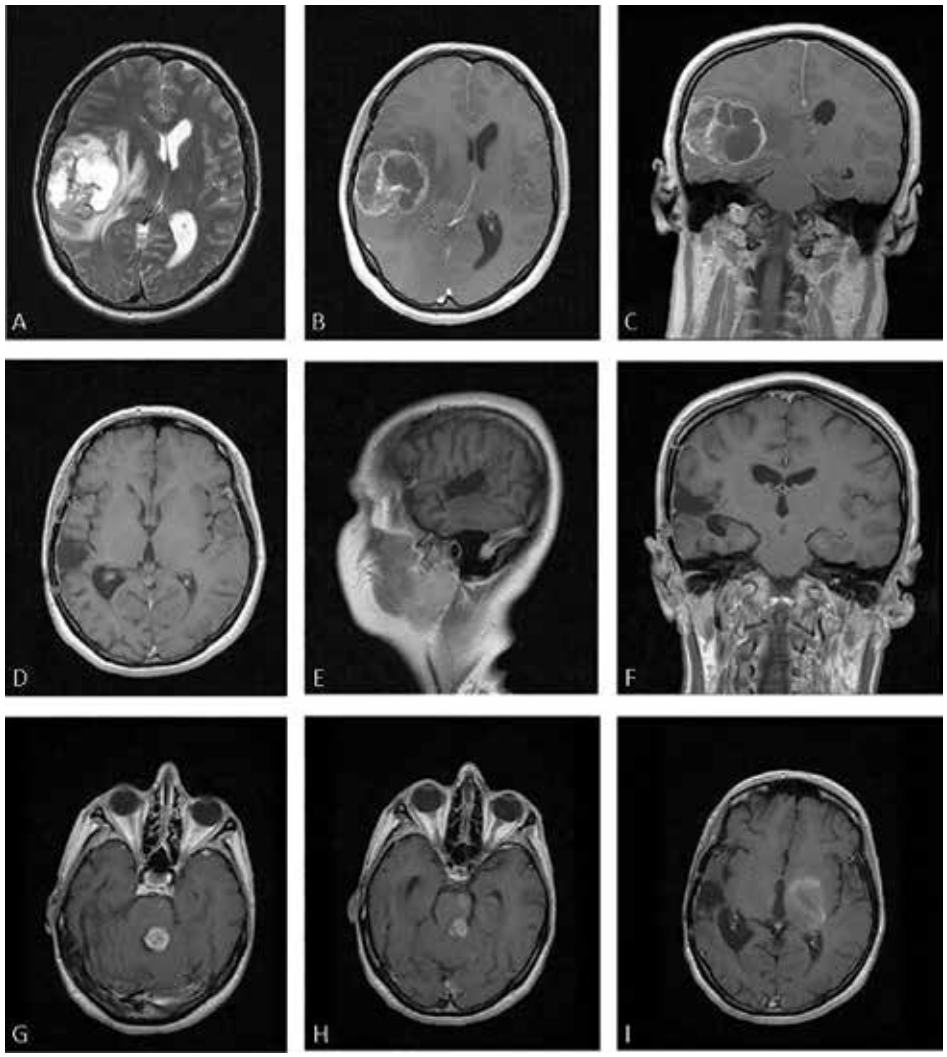

Figure 4. A 38-year old female patient admitted to our neurosurgical department after a large glioma was found in the right temporal region. The patient was with mild left-sided hemiparesis and dizziness (January 2016). Supratotal resection was done and tumor completely resected with 1 cm perilesional tissue. She was discharged from hospital 5 days after the surgery without any neurological deficit. Chemotherapy (temozolomide) and the whole brain irradiation were done immediately. Brain MRI (A–C) was performed prehospitally, T2W axial sequence and T1W axial and coronal postcontrast sequence show extensive, solid cystic tumor located right temporoparietally, surrounded by peripheral edema with compression signs on the right side of the cerebral chamber and signs of mediosagital structure shift to the left. Twelve months after the surgery (March 2017), there was no recurrence on control MRI (middle row, pictures D-F). The same patient 16 months after the surgery (G–I). It was IDH negative glioblastoma multiforme, Grade IV, wild type. Although a complete neurosurgical resection was performed, recurrence was revealed on 16 months follow-up MRI (July 2017), not even on the site of the previous surgery but on the contralateral side as multicentric lesion in the brain stem. MRI T1W axial postcontrast sequence showed multicentric lesions in the left cerebellar pedunculate area, left parietal paraventricular, and left parietal supraventricular areas. The postoperative area on the right did not show any recurrence of the primary tumor. Despite all given therapy, the patient died 17 months after the surgery. This example confirms the thesis that glioblastomas are a diffuse disease, and probably, even after extremely vast surgery, tumor reoccurrence will occur relatively soon. IDH, Isocitrate dehydrogenase.

periods on average [5]. Currently, a randomized controlled clinical trial of supratotal resection for all grade gliomas in noneloquent areas is being conducted with 120 participants; primary results are expected soon (2 years OS, PFS, and Karnofsky Performance Score (KPS)) [18]. The evolution of supratotal surgery from gross total to supratotal was possible due a number of technological advances (light microscope, microneurosurgical tools, magnetic resonance imaging, neuro-navigation, brain mapping, 5-ALA fluoroscency technique, tractography, etc.). Supratotal resection, concerning recently published findings of a few independent authors, resulted in better survival of glioma patients [13, 19].

2.2. Low grade glioma surgical options

For grade II glioma, the recommended treatment is maximally safe resection, with or without radiotherapy followed by procarbazine, lomustine, and vincristine administration or temozolomide plus radiotherapy followed by temozolomide depending of glioma features [20]. When it comes to low grade glioma in the meta-analysis, 5-year OS was markedly increased after resection of low grade glioma, increasing from 50–70% in STR to 80–95% in GTR [7]. GTR has superiority over STR and biopsy with increased OS and PFS [6, 21, 22]. But in another large study, they did not find any difference between STR and GTR in 5-year OS [23]. The impact of STR on OS must be more clearly defined. One study has shown that in younger patients with supratentorial low grade glioma, OS after GTR at 2 and 5 years was 99 and 93%, respectively. The PFS rates at 2 and 5 years were 82 and 48%, respectively [13].

In addition to greater OS GTR for low grade gliomas, it may impact on alerting process from low grade to high grade glioma [7]. In children after GTR, low grade glioma often does not need any further therapy, with 10 years OS rates of 90% or greater, with rare tumor recurrences [24]. In a study including patients with supratotal resection of low grade glioma with 11-year mean follow up, malignant transformation to high grade glioma did not occur. In the control group of total resection, 24% of the patients had malignant transformation from low to high grade glioma ($p = 0.037$) [19]. These promising results come from a pilot study including only 16 patients with performed supratotal resection; a larger study is needed to give a more relevant overview of the behavior of lower grade glioma after supratotal resection.

2.3. High grade glioma surgical options

Recommended treatment for grade III and IV glioma, glioblastoma multiforme (GBM), includes maximally safe resection, if feasible, administration of temozolomide with combination of radiotherapy and chemotherapy depending of favorable and unfavorable prognostic factors and glioma features [20]. The median OS for GMB is 15 months and for grade III tumors between 3.5 and 10 years [14, 25]. In surgical treatment, STR shows better OS compared to biopsy without increasing morbidity [8]. As shown in a meta-analysis of 12,607 high-graded glioma in elderly patients, biopsy OS *vs*. STR was 5.71 months *vs.* 8.68 months, respectively, with lower morbidity rate and longer progression free survival in STR patients [21]. In studies assessing the extent of resection (EOR) in high-grade glioma, in the so-called volumetric studies, STR showed a shorter OS of 2 to 8 months; in nonvolumetric studies, OS was also shorter by 0.9 to 8 months [7]. In their study, Chaichana et al. showed that residual volume and EOR

are independent values for OS and GBM recurrence. They found that the residual volume of <2 cm^3 with 95% resection has the greatest reduction in death in GBM patients [14]. Because of this EOR feature, it is mandatory to perform GTR always when possible, depending on tumor location and quality of life after GTR. GTR has superiority over STR in elderly patients with high grade glioma. GTR resulted in better OS, PFS, and Karnofsky performance score [21]. In children with mean age of 11 years, GTR of high grade glioma resulted in better OS than STR, 3.4 years *vs.* 1.6 years, respectively. Female patients with GTR also had a better OS than male patients, 8.1 years *vs.* 2.4 years, respectively [26]. Concerning higher grade glioma, supratotal resection can have some benefits. In a study of Li et al. of 876 patients who had a GTR (100% EOR), 643 underwent resection of T2 FLAIR abnormality region. Approximately, 18% of them had negative EOR due to postoperative edema, and in positive patients—more than 53.21%—FLAIR resection was associated with improved OS compared with patients with less than 53.21% FLAIR resection (20.7 months *vs.* 15.5 months, $p < 0.001$) [27]. With supratotal resection, the usage of chemotherapy and radiotherapy was reduced after supratotal resection.

3. Surgical technique and oncofunctional balance

As mentioned before, the overall technical development has made supratotal resection possible, with adequate balance between maximal resection of tumor with paralesional region and functional consideration for the eloquent region of the brain. To increase the extent of resection, before surgery, functional MRI, white-matter tractography is performed; during the surgery, intraoperative MRI or ultrasound and 5-ALA-guided resection are used [5]. All these techniques give a rich fund of information, but when the leak of functional information occurs, it signals to us that, while operating, we approached the eloquent brain areas resection [5, 28]. Electrostimulation mapping during supratotal resection is the most important technique used to identify cortical areas and subcortical pathways involved in eloquent functions (especially motor, sensory, language, and cognitive functions) [5, 28, 29]. The usage of electrostimulation in humans started in the 19th century, but the first one in neuro-oncology was used in the 90s of the 20th century by Mitchel Berger. He applied electrostimulation for the mapping of eloquent cortical areas. Hugues Duffau extended and summarized the indication of electrostimulation usage at cortical and subcortical levels intraoperatively [29].

Intraoperative bipolar electrostimulation mapping has become a mandatory tool in neuro-oncology allowing to:

a. study real time individual cortical functional organization;

b. study subcortical connectivity along resection;

c. perform resection according to individual corticosubcortical functional boundaries;

d. make a better neuro-oncological impact, with preservation of the quality of life [28, 30].

With intraoperative electrostimulation, resection is extended into the regions which were considered inoperable. By this extension, a great functional outcome has been documented with

more than 95% of patients recovering to the normal neurological status in 3 months after the surgery, while some patients had improvement in comparison with their preoperative status. In respect of epilepsy, 80% of the patients with preoperative epilepsy did not report it after the surgery [28]. Electrostimulation is a safe, easy, accurate, and reliable technique of individualization of resection for each patient aiming to achieve the "oncofunctional balance."

"Oncofunctional balance" is the term used by Duffau to illustrate the approach to glioma resection through interaction with the patient to determine the best therapeutic sequence according to the patient's needs [31]. Intraoperative electrostimulation is used during the awake glioma surgery, which allows studying interactions between the natural history of tumor and the brain reorganization. With this approach, we are able to preserve the eloquent functions of the brain. Each patient who will undergo supratotal resection should be informed about possible deterioration of the eloquent functions, which of them are most important to him/her to achieve maximal oncofunctional balance. With this approach, glioma surgery becomes an individual surgery, especially designed for each patient.

4. Conclusion

Glioma surgery is one of the most impacting surgical interventions to human health, life, and quality of it. As the molecular knowledge of the life circle of gliomas and techniques improves, it increases our dream that potential curing glioma will come true. Today, the most important treatment option for preserving the quality of life and overall survival is surgical resection. If possible, maximal surgical resection with preservation of neurological functions should be first option in treatment of low and high grade glioma. The resection should be performed with the balance of maximal resection and maximal preservation or improvement of the quality of life. The new promising approach in glioma surgery is supratotal resection with the use of electrostimulation mapping, which makes glioma surgery an individual treatment option for each single patient. But there is a need of more clinical evidence to make supratotal resection widely used. Supratotal resection today is still reserved for individual cases. However, if it is impossible to perform GTR, then STR should be performed. Glioma biopsy is only the last option to confirm the diagnosis for gliomas without possible surgical resection as treatment option.

Acknowledgements

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Conflict of interest

The authors do not declare any conflict of interest.

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Chapter 8

Concurrent Thermochemoradiotherapy in Glioblastoma Treatment: Preliminary Results

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.76264

Abstract

Glioblastoma is the most frequent and aggressive primary brain tumor. The patient can be alive with this pathology using the modern standard of intensive combined treatment less than 2 years. Between December 2013 and August 2017, 30 patients with newly diagnosed supratentorial glioblastoma had received concomitant chemoradiotherapy with transcranial radiofrequency hyperthermia. The gross total or the subtotal resection of the tumor was made previously in all cases. The median follow-up time after operation achieved 12 months (95% confidence interval (CI): 8.5–23 months) in this study. The median disease-free survival time was 9.6 months (95% CI: 7.2–19.0 months). The median overall survival time of patients included in the study was 23.4 months. No increase in the systemic side effects of chemotherapy was found compared with the frequency described in the population. Preliminary results had shown that the usage of concomitant thermochemoradiotherapy with transcranial radiofrequency hyperthermia improves progression-free survival rates. Overall survival rates also tended to increase. Given the absence of severe complications, it is necessary to continue research to achieve statistically significant results.

Keywords: glioblastoma, newly diagnosed glioblastoma, concurrent thermochemoradiotherapy, local hyperthermia, radiofrequency hyperthermia, hyperthermic radiosensitization, hyperthermic chemosensitization

1. Introduction

The median survival time of patients diagnosed with glioblastoma multiforme (GBM) without any treatment is up to 3 months after diagnosis [1]. Modern multimodal treatment including surgery and adjuvant chemoradiotherapy with subsequent chemotherapy results in longer survival. However, the median survival does not exceed 2 years [2–4]. According to population-based studies, the overall 1-, 2-, and 3-year survival rates are approximately 40, 15, and 7–8%, respectively, and the 5-year survival rates range between 0.05 and 5.5% [1, 5–7].

Glioblastoma multiforme is divided into primary and secondary morphological subtypes. Primary GBM accounts for 80–90% of malignant gliomas. They arise de novo and are common in older adults (mean age 55–62 years). Secondary GBMs represent progression from astrocytoma or oligodendroglioma. They manifest in younger adults (mean age 40–45 years) and have a lesser degree of necrosis [8–10]. Various genetic disorders characteristic of the primary and secondary subtypes of glioblastoma have been identified, of which the presence of IDH1/2 mutation is the most reliable molecular marker that is determined in all cases of secondary GBMs, while only about 5% of primary glioblastoma have IDH mutations [1, 11–13]. Several studies have shown that IDH1/2 mutations are positive molecular-genetic prognostic markers. IDH1/2 mutations make the tumor cells more susceptible to genetic rearrangements caused by oxidative stress, thus being a driving force for the development of gliomas. On the other hand, tumor cells containing IDH1 mutations become more susceptible to antitumor therapy, which confers cytotoxicity through the generation of reactive oxygen species [14]. Patients with glioma harboring IDH mutation show a significantly better survival than those with an IDH wild-type glioma (24–36 months vs. 9–15 months). The 5-year survival rate is nearly zero for patients with primary GBM and up to 80% for patients with secondary GBM [12, 15]. In accordance with the updated 2016 edition of the World Health Organization (WHO) Classification of Tumors of the Central Nervous System (CNS), glioblastomas are classified into glioblastoma, IDH wild-type, glioblastoma, IDH-mutant, and glioblastoma NOS. The not-otherwise specified (NOS) is reserved for situations where there is either insufficient material or the facilities for testing for the specific genotype are not available [16].

Promoter methylation of the gene encoding the DNA repair enzyme O(6)-methylguanine DNA methyltransferase (MGMT) is a favorable molecular-genetic prognostic/predictive marker for patients with GBM. Approximately 50% of newly diagnosed GBMs have MGMT gene promoter methylation. MGMT promoter methylation correlates with a low level of MGMT gene expression and may be a predictive marker of sensitivity to alkylating agents, resulting in an almost twofold increase in the median survival after chemoradiotherapy [8, 17]. In addition, the presence of mMGMT is associated with an improved survival of GBM patients regardless of the treatment strategy and reflects a generally more favorable tumor phenotype [15]. The presence of 1p19q co-deletion (loss of heterozygosity on the 1p and 19q chromosome arms) is typical for oligodendroglial tumors and indicates a more favorable prognosis. However, in patients with glioblastoma, the 1p/19q co-deletion may not be associated with survival benefit [18]. The identification of molecular-genetic biomarkers considerably increased our current understanding of glioma genesis. However, further studies are required to identify new biomarkers to define the clinical and biologic subtypes of glioblastoma [1, 8, 13].

Multimodal treatment including surgery and adjuvant chemoradiotherapy with subsequent chemotherapy is the mainstream treatment modality for GBM. Surgery provides material for histological and genetic examinations as well as reduces intracranial pressure in most patients with intracranial hypertension. The gross total or the subtotal tumor resection is an important prognostic factor. Surgery followed by antitumor therapy increases survival rates of these patients [19]. However, aggressive surgery within eloquent areas can worsen the neurological outcome and performance status of patients, thus decreasing overall survival [20, 21]. Moreover, the infiltrative tumor growth beyond the contrast-enhancing areas and the presence of tumor cells in the areas of perifocal edema dictate the need for further antitumor therapy [22]. Survival of patients treated with surgery alone is less than 6 months [23].

Adjuvant radiation therapy at the standard total dose of 60 Gy prolonged overall survival to 10 months. However, tumor recurrence affected about 90% of patients who received radiation therapy [1, 24]. The use of hyperfractionation or dose escalation beyond 60 Gy conferred no survival benefit. Dose escalation up to 90 Gy led to a decrease in 1- and 2-year survival rates [25, 26]. Attempts to escalate the total dose using brachytherapy and radiosurgery also showed no benefit over the standard external beam radiation therapy [27–29].

In the randomized study, concurrent chemoradiation therapy with subsequent adjuvant chemotherapy with temozolomide (TMZ) led to an increase in the overall survival of GBM patients only up to 14.5 months [30]. The use of anti-angiogenic therapy (bevacizumab) in patients with recurrent glioblastomas resulted in survival benefit, with the median overall survival rate from 19.6 to 21.5 months [4, 31, 32]. However, two large randomized phase III trials showed no improvement in overall survival between patients with newly diagnosed GBM receiving and not receiving bevacizumab. The median overall survival of these patients did not exceed 17 months [3, 33].

In recent decades, new approaches to GBM treatment including new drugs, and various biological and physical modifiers have been actively developed. Studies on cell cultures and animal models have shown that low intensity and 200 kHz alternating electric fields have antitumor activity due to mitotic arrest and apoptosis by disrupting mitotic spindle formation during metaphase and causing dielectrophoretic movement of polar molecules during cytokinesis [34, 35]. In a large prospective randomized phase III trial, tumor-treating fields (TTFields) were compared with standard chemotherapy for patients with recurrent GBM. The trial indicated that TTFields had an equivalent efficacy when compared with palliative chemotherapy; however, the quality of life was better in the TTFields group [36]. In patients who underwent surgery and standard chemoradiotherapy, TTFields administered concomitantly with temozolomide significantly improved the median overall survival compared with temozolomide alone (20.5 vs. 15.6 months) [37]. Based on the results obtained, TTFields were included in the standard for the treatment of newly diagnosed GBM in the United States [38].

Experimental studies have shown that high temperatures can directly induce damage to glioma cells and result in radio- or chemosensitization [39–43]. Unlike healthy tissues, tumors have an increased thermal sensitivity, which is caused by biophysical differences between

healthy and tumor cells associated with a low efficiency of ATP production in tumor cells. Under conditions of ATP deficiency, the active transport of ions through the cell membrane is violated and its membrane potential is reduced, thus resulting in higher conductivity and dielectric permittivity in cancer tissue than in normal tissue [44, 45].

As recognized in the early 1970s, the main molecular event underlying the biological effects of local hyperthermia (LHT) in a clinically significant temperature range (39–45°C) is protein damage, including denaturation, exposure to hydrophobic groups, and aggregation with proteins not directly altered by hyperthermia [46–48]. Hyperthermia (temperature above 43°C) causes a large number of macromolecular changes that lead to cell death through extensive protein denaturation and necrosis. Although significantly fewer macromolecular changes occur in the 40.5–42°C range, these changes are still numerous. They occur in different cell components and lead to apoptotic cell death [46, 48–50].

Protein aggregation and denaturation have a significant impact within the cell nucleus. Changes in nuclear proteins, especially those involved in DNA transcription, replication, and repair, cause the inhibition of replication forks and lead to chromosomal aberrations, genomic instability, abnormal chromosome segregation, and cell death [46, 47, 49–52]. Cell membranes are also extremely sensitive to heat stress due to the complex molecular composition of their lipids and proteins. Under the action of LHT, the gel-to-liquid crystal lipid phase transition occurs and the proteins lose their structure, thus resulting in an increased permeability of the cell membrane. The ion balance (Na^+, Mg^{2+}, K^+, Ca^{2+}) in cells and in the extracellular environment is changed, although these changes are not the main mechanism of hyperthermic cell death [49, 52].

Ion imbalance results in significant changes in the mitochondrial membrane potential and disturbance of mitochondrial respiration, causing an increase in the activity of oxygen radicals and a decrease in the level of oxygen consumption in malignant cells [52, 53]. Depolarization of the mitochondrial membrane and the resulting release of oxygen radicals change the oxidation-reduction status of cells and stability of proteins, increasing their sensitivity to LHT. The accumulation of lipid peroxidation changes the distribution of Ca^{2+} and activates the Ca^{2+}-dependent apoptotic pathway. These effects contribute to the protein-unfolding effects of hyperthermia and contribute to effects observed in the nucleus [46, 49].

Irreversible changes in the structure of the protein appear to occur at a temperature of 40°C [54–57]. This temperature threshold also temporarily increases the activity of heat shock genes that encode heat shock proteins (HSPs). The effect of HSPs may depend on their location: intracellularly located HSPs have a protective function, including the correction of misfolded protein molecules, the prevention of aggregation, the transport of proteins, and the restriction of apoptosis. Intracellularly located Hsp70 can act as a cell survival protein by inhibiting the permeability of lysosomal membranes. It also protects tumor cells from monocytic cytotoxicity mediated by tumor necrosis factor [58]. However, HSPs may possess both anti-apoptotic (Hsp27, Hsp70, and Hsp90) and proapoptotic effects (Hsp60 and Hsp10) [59, 60]. Finally, cell death due to apoptosis can occur through various mechanisms and, perhaps, HSPs cannot provide protection against all these mechanisms [61]. In contrast to intracellular HSPs, membrane-bound and extracellular HSPs may have an immunostimulating effect [50]. Heat

has been found to act as the main stimulator of HSP expression and immune stimulation. Hyperthermia treatment at 43.5°C enhances cytotoxicity by antibodies monospecific to specific tumor antigens, suggesting that LHT is capable of enhancing specific immune responses against tumor-associated cell membrane antigens [58, 62].

A simplified view that LHT can lead to immune suppression via induction of thermo-tolerance in tumor cells has been accepted for many years [52, 58]. It is becoming increasingly apparent that in addition to general and limited immune suppression, LHT treatment can lead to specific activation of the immune system by inducing certain modifications of the tumor cell surface and various forms of cell death. Studies have shown that even local exposure to LHT can lead to systemic tumor control by activating the innate immune system [50, 52, 58].

Despite convincing biological justifications, the enthusiasm for the clinical use of LHT as monotherapy has decreased due to the fact that heating of human tumors to cytotoxic temperatures between 42 and 45°C is difficult or almost impossible. In living tissues heated for more than a few minutes, convective heat losses occur near large blood vessels (diameter of ~0.5 mm). Such blood vessels act as a heat sink, and one can expect a temperature drop of up to 50% [63]. Blood flow provides up to 90% of heat removal. A 10-fold increase in blood flow in response to LHT can occur due to the compensatory expansion of arteries and intensive perfusion, especially in normal tissues [64–66].

Intratumoral blood flow varies considerably depending on the tumor type. Moreover, even within the same tumor, the distribution of the vasculature and blood flow is very heterogeneous. Contrary to the general notion that the blood flow in tumors is less than that in normal tissues, blood flow in many tumors, especially in small tumors, is actually greater than that in the surrounding normal tissues under normal conditions. Typically, the bloodstream of the tumor usually decreases as the tumor grows. While studies in small animals suggest that LHT induced a decrease in blood flow at 42–43°C, there is evidence that tumors in large animals and, more importantly, human tumors, are significantly less sensitive to LHT. In general, clinical studies do not suggest a reduction in tumor perfusion at temperatures up to 44°C [67].

An increased blood flow can increase tumor growth, as well as the risk of hematogenous metastases, suppressing the possible therapeutic effect of LHT [48, 52, 68]. However, a high blood flow can have the opposite effect: a high blood flow provides a more intensive exposure to chemotherapy and, through increased oxygenation, sensitizes tumor tissue to radiotherapy [69].

A mild temperature (39–42°C), which is not optimal for the induction of direct cell death or damage to the vascular system of the tumor, is effective in enhancing tumor response to radiation therapy or chemotherapy [70]. Many conditions that contribute to radioresistance, including hypoxia, acid medium, and S-phase of the cell cycle, either increase sensitivity to LHT or do not change it [50, 52, 70, 71]. LHT-induced increase in tumor perfusion leads to an increased tumor tissue oxygenation and an increased tumor radiosensitivity [72].

Under the influence of LHT, both the intrinsic chemical activity of cytostatics and the degree of their penetration into cells increase due to the activation of membrane transport, and the direct effect of LHT is much higher in hypoxic tissues, in which chemoresistance is observed [47, 71].

The mechanism of enhanced cytotoxicity can include the increase in intracellular accumulation of chemotherapeutic drugs, the inhibition of DNA repair, and S-phase cell cycle block, when cells are most sensitive to heat. In addition, LHT increases the production of free radicals and can reverse drug resistance [47, 49, 50].

Based on clinical data demonstrating the synergistic antitumor effect of noninvasive radiofrequency hyperthermia used in combination with chemotherapy and radiotherapy for tumors from various sites and recurrent glioblastomas, the goal of the study was to evaluate the effectiveness and safety of LHT combined with concurrent chemoradiotherapy for newly diagnosed glioblastoma.

2. Methods

2.1. Study population

Between December 2013 and August 2017, 30 patients with newly diagnosed and histologically verified supratentorial GBM were included into the study. All patients underwent gross total or subtotal microsurgical removal of the tumor. Patients with the evidence of recent hemorrhage on baseline magnetic resonance imaging (MRI) of the brain, metal implants, and concurrent severe, intercurrent illness were excluded from the study. The study was approved by the Ethics Committee of Cancer Research Oncology of Tomsk National Research Medical Center. All patients provided informed written consent before being included in the study.

2.2. Study design and treatment

This uncontrolled cohort study aimed to assess the tolerability and efficacy of concomitant transcranial local radiofrequency hyperthermia combined with radiotherapy and chemotherapy with temozolomide to treat newly diagnosed glioblastoma after surgical treatment. The IDH mutation status was determined using immunohistochemical staining with the anti-human IDH1 R132H. Methylation of O6-methylguanine-DNA methyltransferase (MGMT) was evaluated using a quantitative methyl-specific polymerase chain reaction in real time. To assess surgical outcomes, postoperative contrast-enhanced MRI of the brain was used.

External beam radiation therapy (2.0 Gy per fraction, 5 days per week to a total dose of 60 Gy) was delivered using Theratron Equinox device. Chemotherapy with temozolomide was administered at a dose of 200 mg/m^2/day for 5 days for every 28-day cycle. The first course of chemotherapy was administered a week after starting radiation therapy. Patients received local hyperthermia beginning from the second week of administering external beam radiotherapy (**Figure 1**). Local hyperthermia was given two times a week for 60 min. The interval between local hyperthermia session and radiation therapy was 20–40 min.

Local hyperthermia was given using Celsius TCS system, which uses electromagnetic waves with a frequency of 13.56 MHz (radio waves) for energy transfer. The area of heating

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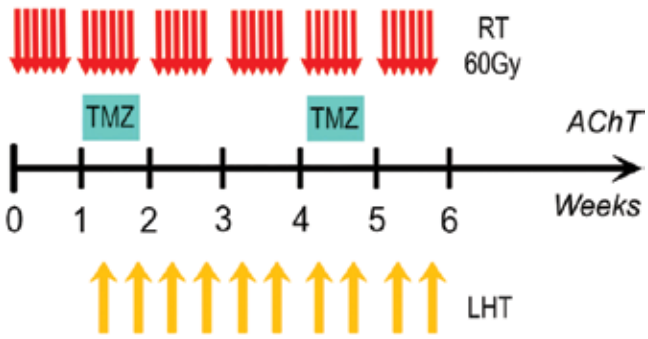

Figure 1. The protocol for transcranial local radiofrequency hyperthermia combined with radiation therapy and chemotherapy with temozolomide.

exceeded the largest tumor diameter by at least 3 cm and increased proportionally to the tumor depth. The heating temperature was gradually increased, focusing on the patient's tolerability of the procedure in accordance with the protocol recommended by the device manufacturer [73].

The applied energy of the electromagnetic field was increased during seven sessions and was dependent on the patients' tolerance. In subsequent treatment sessions, the power did not increase. The duration of the treatment session ranged between 20 and 60 min and the absorbed power during one session was from 42 to over 324 kJ. To prevent thermal burns of the skin and subcutaneous tissue, the surface of the electrodes was cooled by circulating deionized water at a temperature of 12–16°C.

2.3. Patient surveillance and follow-up

Baseline contrast-enhanced magnetic resonance imaging (MRI) of the brain was required before starting concurrent thermochemoradiotherapy (TCRT). All patients underwent a detailed history and physical examination before treatment. Control blood and urine tests were performed every week of thermochemoradiotherapy. The neurological and neuro-ophthalmic evaluations were performed before and after completion of treatment.

Overall survival and time to tumor progression/recurrence are the most important criteria for the assessment of response to adjuvant therapy in patients with GBM. All patients were followed up in the outpatient clinic setting. To assess treatment outcomes, contrast-enhanced MRI was performed a month after completion of treatment, every 3 months in the first 2 years and every 6–12 months thereafter. All MR images were evaluated using Response Assessment in Neuro-oncology criteria, RANO [74]. In case of suspicion of tumor progression, an extraordinary MRI was performed. When a patient did not show up for a scheduled appointment, information on the patient's health status was requested in his family relatives.

Adverse effects of radiation therapy were evaluated using RTOG/EORTC Scoring Criteria (1995), and side effects of chemotherapy were assessed using the NCIC-CTC grading scale. Thermal damage to the skin was classified according to the depth of the lesion.

2.4. Statistical analysis

Progression-free survival and overall survival were assessed using clinical and molecular-genetic prognostic factors.

Statistical analysis was done using statistical software for Microsoft Office Excel 2010 (Microsoft Corporation) and Statistica 10.0 (StatSoft).

2.5. Results

Between December 2013 and August 2017, 30 patients with newly diagnosed supratentorial glioblastoma were included into the study. Eight patients underwent gross total tumor resection, and 22 patients underwent subtotal resection. The status of IDH mutation and MGMT promoter methylation was analyzed in 73% of patients. Mutation of IDH was found in one patient. The frequency of MGMT promoter methylation was 54.5%. There were 19 male and 11 female patients aged from 21 to 71. The highest frequency of glioblastoma occurred in the age range of 50–61 (median age 56 years). Tumor involvement of more than one lobe was the most common. Tumors that infiltrated the parietal, temporal, and frontal lobes occurred less frequently. The medial structures and the occipital lobe were the least frequent sites in which cancer developed (**Figure 2**).

The median Karnofsky Performance Status (KPS) score was 85% (range 40–90%, 95% CI: 60–90%) (**Figure 3**).

The average time from diagnosis to start of radiation therapy was 5.4 weeks. (95% CI: 4.5–6.5 weeks). Three patients who underwent subtotal tumor resection had disease progression at the time of initiation of adjuvant therapy.

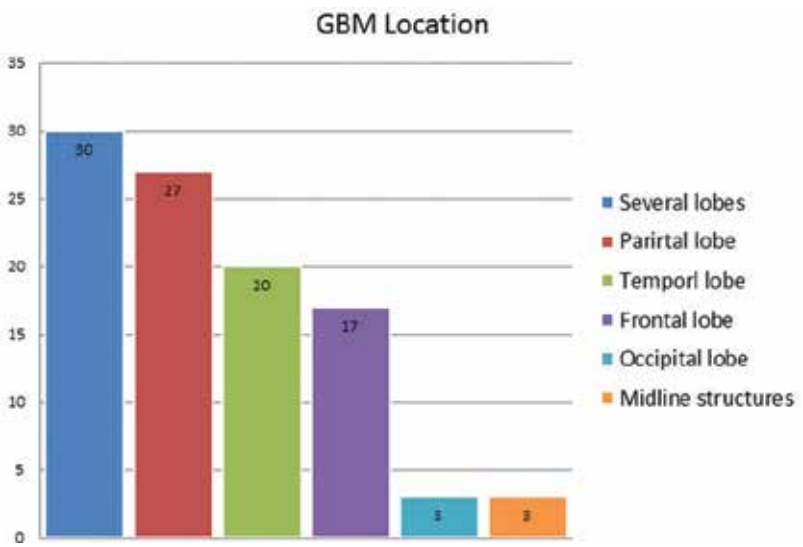

Figure 2. GBM location in patients enrolled into the study.

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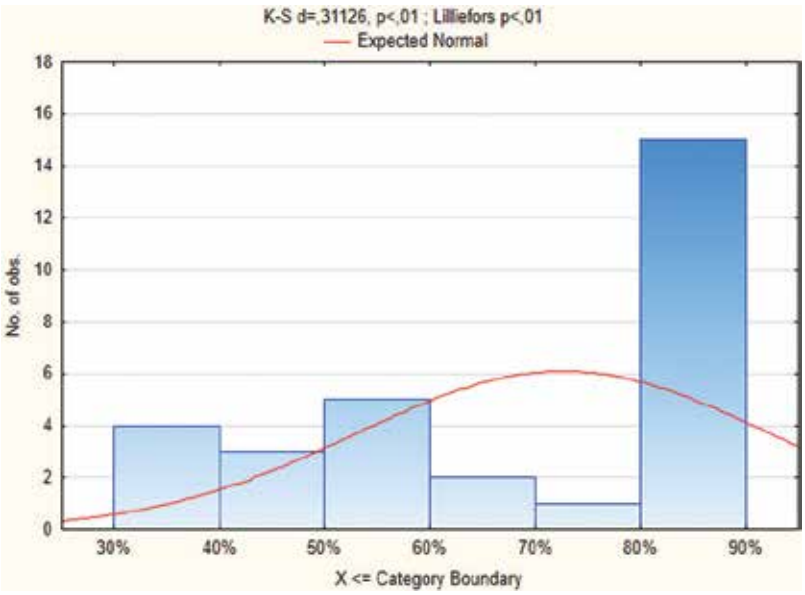

Figure 3. Karnofsky performance status score.

2.6. Treatment

Of the 30 patients included into the study, 29 completed concurrent thermochemoradiotherapy (TCRT). One patient discontinued adjuvant chemotherapy after 42 Gy radiotherapy and eight sessions of local hyperthermia because of tumor progression. In this case, disease progression was diagnosed before starting TCRT. Two patients lost to follow-up after assessing immediate response to TCRT.

Patients who completed TCRT continued to receive chemotherapy with temozolomide at a dose of 200 mg/m^2 per day for five consecutive days in a 28-day cycle. Three patients died within 2 months after completion of treatment with no evidence of disease progression. Among the patients who died, one patient had extensive destructive pneumonia, one patient had severe neutropenia and thrombocytopenia, and another one patient had psycho-organic syndrome occurring during atrophy of the brain.

Patients who had disease progression underwent surgery and second-line chemotherapy. Repeated radiation therapy at a total dose of 40 Gy was administered to patients who developed disease progression 1 year after completion of adjuvant radiation therapy.

2.7. Treatment outcomes

Progression-free survival and overall survival were the primary end points in evaluating treatment response in the study. The median follow-up time was 12 months (range 4–51 months; 95% CI: 8.5–23 months). The median disease-free survival was 9.6 months (95% CI: 7.2–19.0 months), and recurrence most often occurred within 6–12 months after treatment (**Figure 4A**).

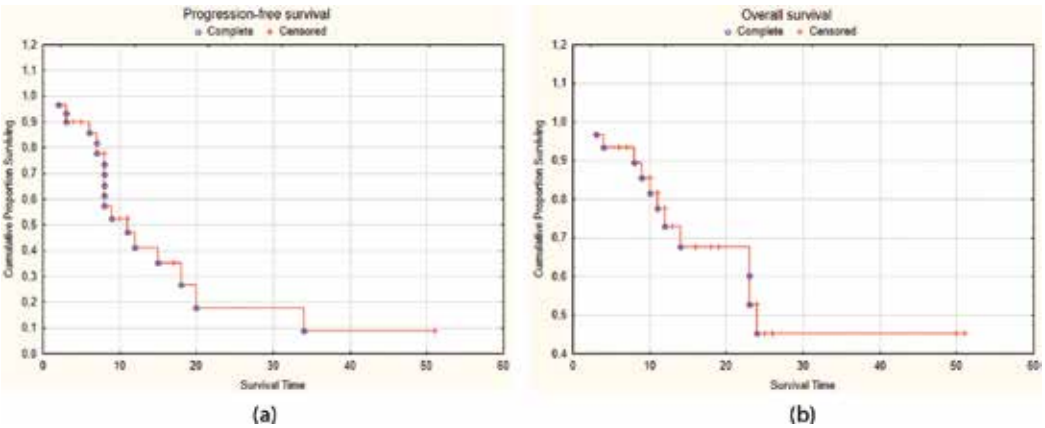

Figure 4. Survival of GBM patients who received concurrent thermochemoradiotherapy. (A) Progression-free survival and (B) overall survival.

The 1-year disease-free survival rate was 41.3 ± 10.6%. Five patients were followed up for more than 24 months. One patient developed recurrent disease 34 months after diagnosis.

During the follow-up period, 11 patients died. Most deaths were registered within 6–12 months (five patients) and 12–24 months (four patients). The median overall survival time was 23.4 months. The 1-year survival rate was 73 ± 8.8%. Four patients had no evidence of recurrence 24 months after completion of treatment (**Figure 4B**).

2.8. Safety and tolerability

Treatment tolerance was assessed by monitoring changes in the functional and neurological status of patients and comparing complications from chemotherapy and radiotherapy.

Most patients had no changes in the functional performance status assessed by the Karnofsky scale before and after TCRT. In three patients with low KPS score (40%) due to neurological deficit, concurrent TCRT led to an increase in KPS score to 60%. One patient had disease progression during TCRT, and his KPS score decreased from 80 to 40%.

Changes in the KPS status before and after TCRT are shown in **Figure 5**. An increased functional activity of patients was observed; however, differences were not statistically significant ($p > 0.05$).

The assessment of neurological symptoms before and after TCRT showed no changes in neurological deficit in most patients. Neurological symptoms were transient in three cases and regressed after vascular therapy in two cases. Worsening of neurological symptoms was observed in three patients. One of these patients discontinued radiotherapy at a total dose of 42 Gy because of disease progression. In two remaining patients, an increase in neurological symptoms was not associated with tumor progression and was characterized by the occurrence of extrapyramidal symptoms in one case and behavioral disorders in another case. Both patients had a history of chronic cerebral ischemia. Regression of focal neurological symptoms manifested as a decrease in the severity of pyramidal symptoms was registered after TCRT

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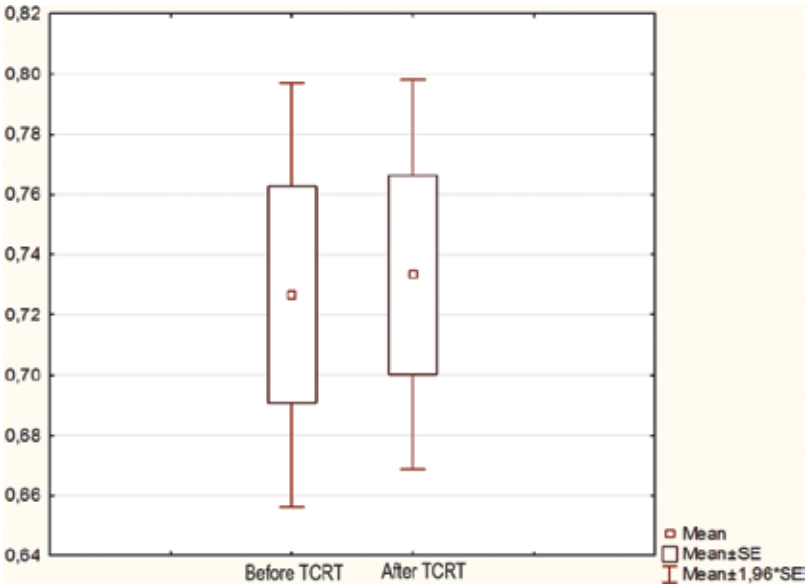

Figure 5. Functional activity of patients before and after TCRT.

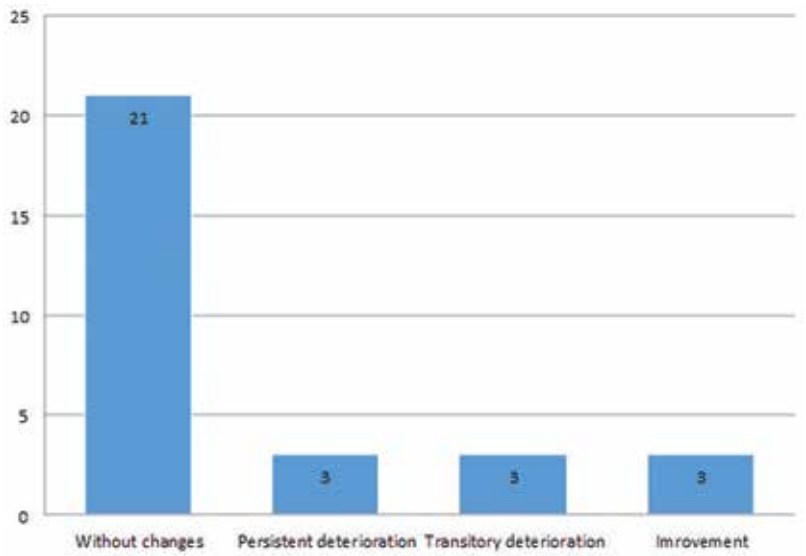

Figure 6. Changes in neurological symptoms after TCRT.

in two patients, and as an improved extremity muscle strength and regression of convulsive syndrome in one patient, thus increasing KPS status from 40 to 60%. The patient characteristics with respect to changes in neurological symptoms are shown in **Figure 6**.

Gastrointestinal toxicity manifested with diarrhea, nausea, or loss of appetite was founded in four (13.3%) patients. Grade 3/4 toxicity was not registered. Hepatotoxicity manifested by a

selective and sustained rise of serum alkaline phosphatase (ALP) activity that was noted in 13 (43.3%) patients. When the level of serum ALP activity was 2.5 times higher than the normal limits, chemotherapy temporarily stopped, and the correction with hepatic protectors was performed.

Hematologic toxicity in the form of grade 1/2 leukopenia, grade 1/2 thrombocytopenia, and grade 1 anemia was observed in 10 (33.3%) patients. Grade 3/4 leukopenia and thrombocytopenia were diagnosed in two (6.7%) patients. Clinical manifestations of grade 3/4 hematological toxicity were characterized by increased hemorrhage, microhematuria, and thrombocytopenic purpura. In one patient, hematoma formation in the tumor bed required surgery. There were no cases of febrile neutropenia. In the cases of grade 3/4 hematologic toxicity, chemotherapy with a reduced dose of temozolomide continued after achieving absolute neutrophil count of >1500 cells/μl and platelet count of >100,000 cells/μl. There were no cases of chemotherapy termination because of hematological toxicity.

Grade 1–2 infectious complications after the completion of chemotherapy were revealed in three (10%) patients. These complications were manifested by chronic pyelonephritis, bronchitis, and oropharyngeal candidiasis and were managed by antibacterial and antifungal therapy. Within a month after completion of TCRT, two patients developed severe infections (pneumonia), requiring hospitalization and prescription of antibiotic therapy. Both patients received dexamethasone at a dose of 16 mg/day intramuscularly.

Acute radiation-induced skin damage was observed in all patients. Allopecia was observed in 29 (96.7%) patients, and a second-degree skin radiation reaction was observed in one case (3.3%). Complications associated with hyperthermia in the form of thermal injury of skin (up to 2 cm in diameter) were diagnosed in three (10%) patients. They did not cause deterioration in the physical status of patients. Treatment was conservative, and interruption or cessation of treatment was not required. One patient developed inconsistency of a postoperative scar with the formation of a cerebrospinal fluid leak. In this case, the excision and suture of the liquor fistula were performed, liquorrhea was stopped, and TCRT was successfully completed.

3. Discussion

Several randomized studies showed a significant increase in the overall and disease-free survival of GBM patients receiving LHT [75, 76]. Seventy-nine patients with newly diagnosed glioblastoma were randomized to receive either interstitial high-frequency hyperthermia in combination with brachytherapy or interstitial brachytherapy alone. The median time to disease progression was longer and the median overall survival was higher in the LHT + brachytherapy group than in the group with brachytherapy alone (35 vs. 57 weeks and 76 vs. 85 weeks, respectively) [75]. Based on the study, interstitial high-frequency hyperthermia for GBM treatment was approved by the Food and Drug Administration (FDA). However, the invasive nature of LHT limited its use by two procedures to prevent complications associated with the installation of antennas and determined the impossibility of combining LHT with external beam radiation therapy.

The effectiveness of magnetic hyperthermia combined with fractionated stereotactic radiotherapy for recurrent GBM was evaluated in a large two-center study [76]. Patients underwent stereotaxic intratumoral injection of a fluid containing magnetic nanoparticles (MNPs), followed by heating in an alternating magnetic field. Side effects were moderate, and no serious complications were observed. The median overall survival time from the diagnosis was 23.2 months. Thus, thermotherapy involving the use of alternating magnetic field in conjunction with MNPs was proven to be an effective method for treating patients with GBM. However, current limitations to the use of magnetic hyperthermia for thermotherapy of GBM patients include the high concentration of MNPs required to generate hyperthermia precluding the use of MRI, as well as the effective delivery of the MNPs [77].

Modern systems for performing deep LHT allow for noninvasive heating of the tumor. In such systems, the electrical parameters of the circuit are automatically measured and individually adjusted to ensure control and high efficiency of the procedure. Temperature monitoring in tumor tissue is provided by calculation based on the measurement of absorbed energy and tissue impedance [66, 68].

There are a number of disadvantages of LHT: the excessive heating of subcutaneous fat, instability in a radiofrequency field and its dependence on the size of electrodes, their location, distance between them, and on the dielectric parameters of tissues, as well as the ease of the formation of the "hot spots," that is, the maximum electrical field in places with a high dielectric contrast [64, 78, 79].

There are published data indicating that the conductivity of the cerebrospinal fluid is at least four to six times higher than that of the gray and white matter. Thus, it is reliable to predict the presence of "hot spots" along the gray matter-cerebrospinal fluid (CSF) boundary, as well as along white matter-CSF boundary. Moreover, the induced electric field distribution is highly nonuniform. The electric field direction plays a significant role: the internal and near-surface electric field is higher in a tissue with low conductivity and lower in a tissue with high conductivity. As a result of tissue heterogeneity, the electric field in the brain does not decrease smoothly with distance from the transducers, as it would in a homogeneous tissue. In addition, electric field "hot spots" can occur far from the arrays, giving rise to a complex spatial distribution [80, 81].

This nonuniformity of the electric field determines high safety requirements for LHT, as it has a number of negative effects on neuronal structures and functions, causing disturbances in electrochemical depolarization, transmembrane ion transport, and destruction of cellular signaling mechanisms and mitochondrial functions. Despite the fact that irreversible changes in the protein structure occur at temperatures above 40°C [55, 56], this temperature threshold also activates heat shock proteins to increase thermal tolerance and enhance cell protection [82]. Since irreversible changes in normal nerve tissue are detected after hyperthermia at 42–42.5°C for 40–60 min [56, 57], the brain temperature should not exceed 42°C.

The attempts to use noninvasive magnetic resonance thermometry during transcranial radiofrequency LHT were unsuccessful, because it was impossible to combine an electromagnetic LHT device with an MRI system. Invasive thermometry for LHT is time-consuming, uncomfortable,

and risky for the patient. Considering these data, we conducted a study simulating radiofrequency deep LHT using a realistic bioequivalent phantom. Results of thermometry showed that the temperature in the normal brain substance and cerebrospinal fluid did not exceed the physiologic parameters. The rise in the tumor temperature enhanced the efficacy of radiotherapy [83].

Several clinical studies on transcranial radiofrequency hyperthermia for relapsed malignant brain tumors showed a low frequency of objective response (from 7 to 25% of cases) and the median overall survival time of 6–9 months after the onset of hyperthermia [84–86]. It was difficult to determine the effectiveness of treatment, since there were no data on the overall survival from the time of surgical treatment.

A preliminary analysis of the results of this uncontrolled cohort clinical trial showed that at a median follow-up of 12 months, the median progression-free survival was 9.6 months in patients who received TCRT (CI 95%: 7.2–19.0 months). These results were better than those described in the randomized study conducted by Stupp [30], who reported that the median disease-free survival time was 6.9 months (95% CI: 5.8–8.2 months) and 7.1 months (95% CI: 5.9–8.2 months) in GBM patients treated with Stupp regimen and tumor-treating fields, respectively [30, 37]. The median overall survival time of patients included in the study was 23.4 months. However, the result was not statistically significant because the median follow-up was up of 12 months.

Given a small number of patients included in the study, the evaluation of molecular-genetic prediction factors (IDH mutations and MGMT methylation) was important to avoid errors associated with a disproportionate number of patients with a favorable prognosis. The molecular-genetic features of tumors in patients enrolled in the study could not be the cause of improved survival. However, the study demonstrated a high frequency of subtotal tumor resections, which was a negative predictor factor [1].

Since radiofrequency hyperthermia was administered locally, an increase in the systemic side effects of chemotherapy compared with the frequency described in the population was not determined. The appearance of neurologic toxicity during chemotherapy with temozolomide not described in the previous studies [30] was more likely to be associated with an increase in edema and ischemic disorders. This was confirmed by the fact that neurological toxicity was mainly observed during the second course of chemotherapy, when external beam radiation dose accumulated. However, none of the patients had evidence of ischemic stroke.

Concurrent hyperthermia and chemoradiotherapy did not result in an increased frequency of local injuries associated with transcranial local radiofrequency hyperthermia [79]. However, it is necessary to pay attention to the fact that during TCRT, one patient developed a fistula in the area of a postoperative scar, which indicated an impairment of reparative processes.

4. Conclusion

Preliminary results of the analysis of 30 patients with supratentorial newly diagnosed glioblastoma who received adjuvant thermochemoradiotherapy using transcranial radiofrequency

hyperthermia showed an increase in progression-free survival rates. Overall survival rates also tended to increase. Given the absence of severe complications, it is necessary to continue research to achieve statistically significant results.

Acknowledgements

The work has been financially supported by the Fundamental Research Program of the Russian Academy of Sciences, project No. 093.

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Chapter 9

Laser Interstitial Thermal Therapy in Glioblastoma

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.77078

Abstract

Laser interstitial thermal therapy is a minimally invasive ablative technique that continues to gain popularity in treatment of a variety of intracranial and spinal disorders. In the field of neuro-oncology it continues to be used for treatment of a variety of intracranial neoplasms, including glioblastoma—the most common malignant primary brain tumor. Maximizing the extent of resection in patients with glioblastoma was shown to prolong patient survival. Many patients present, however, with tumors that are nonresectable due to proximity to eloquent cortical or subcortical areas, or involvement of deep brain structures. LITT procedure, on the other hand, is minimally invasive and involves placing a laser catheter under stereotactic guidance and monitoring the size of the lesion produced as a result of laser ablation using MR thermography in real time. Therefore, a number of studies explored the potential of laser ablation to accomplish significant cytoreduction and thus potentially improve patient's outcomes and prolong survival. The following chapter will review the principles of laser ablation and its current role in treatment of glioblastoma.

Keywords: laser interstitial thermal therapy, laser ablation, glioma, minimally invasive

1. Introduction

Laser interstitial thermal therapy (LITT) is a minimally invasive ablative technique that continues to gain popularity in a variety of domains of neurosurgery including neuro-oncology, epilepsy surgery, spine oncology and degenerative spine surgery, as well as chronic pain syndromes. In neuro-oncology, laser ablation is used for treatment of a variety of intracranial neoplasms but used most frequently for treatment of recurrent brain metastases and radiation necrosis, as well as treatment of recurrent and newly diagnosed, difficult to access gliomas and other deep-seated tumors. The procedure involves placing a laser catheter

under stereotactic guidance, and the size of the lesion produced as a result of laser ablation is monitored using MR thermography in real time. The minimal invasiveness of the procedure makes it a good choice in patients that cannot tolerate a large operation due to either burden of disease or poor performance status.

Glioblastoma is a diffuse primary brain neoplasm with poor prognosis. The invasive nature and malignant potential of this tumor make its treatment a challenge. The current standard of care management paradigm consists of a multidisciplinary approach by combining maximal safe tumor resection with subsequent chemotherapy and radiation [1]. Despite maximal treatment, survival rates of glioblastoma remain poor with median survival of 14–16 months. Recent evidence indicates, however, that the extent of resection of high-grade gliomas correlates with patient survival [2–6]. In a similar sense, laser ablation can provide effective tumor cytoreduction to maximize the effectiveness of adjuvant treatments. Furthermore, there is promising evidence that hyperthermia may have additional synergistic effects with radiation, as well as disrupt blood-brain barrier (BBB) and thus facilitate chemotherapy delivery to target tissues [7, 8].

The following chapter will focus on describing the principles of laser ablation and the equipment used to deliver laser energy to brain tumors, as well as discussing current evidence for the use of laser thermal therapy in management of high-grade gliomas.

2. Background

2.1. The use of hyperthermia

The concept of using heat to destroy cancerous tissue has been attempted multiple times in the past. It was, however, difficult to develop a mechanism of heat delivery to the affected tissues that would allow controlled ablation of tissues in question. One of the earliest references to the efficacy of mild hyperthermia in cancer destruction is found in 1891 in the report by Dr. Coley, an orthopedic surgeon, who made an observation of complete resolution of an inoperable sarcoma in a patient after *Streptococcus pyogenes* infection [9]. He suggested that the high fevers that accompany the illness injured cancer cells sufficiently to destroy them. He followed up that work by describing a series of 10 patients that were successfully treated with "bacterial toxin therapy" [9]. Unfortunately, his results were not reproduced by others.

In the years to come, radiation therapy and chemotherapy have established themselves as mainstream treatments for cancer. It was not until 1967 when Cavaliere et al. demonstrated selective sensitivity to heat of cancer cells, thus suggesting the use of hyperthermia as part of cancer therapy [10]. Follow-up work in animal models corroborated that notion. It was demonstrated that hyperthermia preferentially affects glioma cells compared to surrounding brain tissue. Local hypoxia and more acidic microenvironment within tumor contributes to this selective sensitivity to heat of glioblastoma [11]. Furthermore, hyperthermia potentiates the effects of radiation and chemotherapy observed in vitro [12–14].

Other factors, however, influence the effectiveness of hyperthermia. In vitro experiments showed that only 50% of sarcomas were responsive to hyperthermia resulting in tumor

remission, whereas the other 50% had no response to elevated temperatures [15]. The threshold temperature at which irreversible damage occurs is different across species. While cell cycle arrest and increased cell death in rodent cell lines occurs at 43°C, in human cell lines that threshold is at 41°C [16].

The mechanism by which mild hyperthermia induces damage to cancer cells may involve activation of apoptosis pathways in a temperature dependent fashion, resulting in changes of expression of heat shock proteins such as HSP 27 and 72. The latter display antitumor properties by initiating immunologic response resulting in activation of natural killer cells directed against tumor cells [17].

2.2. Principles of laser thermal therapy

Laser interstitial therapy is an ablative technique that results in tissue destruction as a result of heating. Photons produced by the laser light are absorbed by the surrounding tissues causing excitation and release of excess energy as heat [18]. Once the critical thermal threshold is reached, protein denaturation and irreversible tissue coagulation ensues resulting in permanent tissue damage.

The first lasers that were attempted for tissue ablation were ruby-based [19]. The amount of energy and thus tissue damage produced by these lasers was difficult to control. In 1983, Bown et al. first described the use of the neodymium-doped yttrium aluminum garnet (Nd:YAG) laser for tissue ablation [20]. At present, two commercially available FDA-approved systems are available for use in neurosurgery in the United States: The NeuroBlate System (Monteris Medical, Plymouth, MN) and the Visualase Thermal Therapy System (Medtronic Inc., Minneapolis, MN). Laser ablation uses laser energy in the near-infrared range where the main tissue interactions are heating and coagulation (as opposed to cutting for the CO_2 laser). Both commercial laser ablation systems use diode lasers—one at the Nd:YAG wavelength (1064 nm) and the other at 980 nm. Although there have been claims that one is superior to the other, these are not founded in fact [21]. Lasers in this near-infrared range only penetrate a few millimeters into brain tissue. This heat is then propagated by conduction to allow for ablation radii that may extend up to 15–20 mm.

The wavelength of the laser light is what determines the efficiency of energy transfer to the tissues and, as a result, the volume of the lesion produced. Furthermore, the duration of tissue exposure to the laser light affects the amount of energy transferred and thus the amount of heating produced, with longer duration of exposure resulting in higher temperatures achieved in exposed tissues [22–24]. The design of the optical fiber and the laser catheter further affects the properties of the laser. Initially, lasers had to be used at very low power (1–5 W) to avoid excessive heating that results in tissue charring. Improvement in laser catheter design with the development of cooling mechanisms allowed use of higher power while still protecting nearby tissues. This also provides a non-stick catheter surface that allows the laser probe to glide easily through tissues. Current cooling systems employed in laser probe design use either cooled gas system with CO_2, or a continuous flow of saline through a sheath surrounding the optic fiber.

The laser probe tip is made of either sapphire or quartz to avoid altering the optical properties of the laser light. This design results in a spherical light distribution at the tip of the probe, and as a result, thermal energy is delivered in a symmetrical ellipsoid shape that is centered along the probe axis. The NeuroBlate System, in addition to the spherical probe design, also offers a side-firing probe which allows the surgeon to robotically control the direction of maximal heat distribution and may have an advantage in treating irregularly shaped lesions, or lesions near eloquent areas.

The Visualase system uses a 15 W 980 nm diode laser that is cooled with circulating sterile saline solution [25]. The diameter of the catheter is 1.65 mm. The laser probe tip comes with a light diffusing tip that results in spherical light distribution producing an ellipsoid area of tissue damage. This non-pulsed system produces faster lesions but the application of heat is limited to several minutes. The system is connected to a workstation that displays real-time thermography data as "thermal" and "damage" images [26, 27]. A number of safe points can be set on the pre-treatment MRI, and when the set temperature is reached at that point, the laser is deactivated.

The NeuroBlate System uses a 12 W solid-state Dornier diode laser that operates at Nd:YAG wavelength of 1064 nm [28]. The laser catheter is cooled with CO_2 gas [29]. The probes come in two diameters: 3.2 and 2.2 mm. The light diffusing tip comes in two configurations: spherical, used to produce elliptical lesions along the probe axis, and side-firing probes, that enable treatment of complex and irregularly shaped lesions. The computer interface displays thermal damage as thermal-damage-threshold (TDT) lines. The yellow line represents tissue volume that is exposed to the equivalent of 43°C for 2 min, the blue line is equivalent to exposure to 43°C for 10 min, and the white line surround the volume that received the equivalent of thermal energy of 43°C for 60 min. Based on the Arrhenius equation, the higher the temperature, the less time it takes to generate each TDT-line.

2.3. MR thermography

The use of laser ablation for treatment of tumors was first described by Bown in 1983 [20]. The first report of intracranial use for brain lesion laser ablation came out in 1990 [22]. Despite that, laser interstitial thermal therapy did not gain wide-spread use due to lack of the ability to monitor the extent of ablation and tissue damage. A variety of methods were attempted to measure thermal energy delivered to tissues and included skin thermometers, subcutaneous and interstitial probes, infrared detectors, and thermographic cameras, none of which were accurate enough to predict the size of the resulting thermal lesion [30–32]. Introduction of MR thermography revolutionized the application of laser thermal therapy since for the first time it allowed monitoring of the extent of tissue damage in real time [27]. The principle of MR thermography relies on detecting differential temperature-specific proton resonance frequency in the water molecules. At a given temperature, a proportion of water molecules are interconnected in space via hydrogen bonds between molecules. As the temperature of tissues increase during laser ablation, more water molecules are freed up from the hydrogen bonds between H_2O molecules. During application of the magnetic field, proton nuclei within free water molecules are mobilized more effectively resulting in a different proton resonance

frequency, and this difference is used to interpolate local temperature using well defined relationship [18, 33]. MR thermography does not measure the actual temperature of tissue, rather the change in temperature over time, therefore an accurate core temperature is required at the start of each ablation. The Arrhenius model is then applied to estimate the degree of tissue damage that is produced based on the temperature and the amount of time that the tissues are exposed to a given temperature. Subsequently, computer software is used to visualize the temperature damage produced in real time with accurate temporal and special resolution.

2.4. Biological effect of LITT

Heating tissue results in different types of tissue damage. Several different zones of tissue damage have been described. Heating tissues to up to 40°C typically does not disrupt cellular homeostasis. Once the temperature increases in the range of 42–45°C, the cells display marked susceptibility to cellular damage [34]. This range is typically explored in hyperthermia experiments. Further increase in temperature from 46 to 60°C results in significant cytotoxicity and consequent rapid cell death [35]. At temperatures exceeding 60°C, the damage sustained by mitochondrial enzymes, as well as cellular nucleic acids and proteins is so severe that coagulative necrosis takes place [36]. Finally, heating tissues to near boiling temperatures results in charring, tissue evaporation and carbonization, that may result in life-threatening intracranial pressure increases if not immediately relieved. In addition to temperature thresholds, the length of time that the tissue exposed to a particular temperature determines the extent of tissue damage with longer exposures resulting in equivalent damage that is observed at higher temperatures [18]. For instance, heating tissues to 43°C for 2 min will result in reversible tissue damage. Whereas heating tissues to this temperature for 10 min will result in permanent injury, and for 60 min will result in coagulative necrosis.

As tissue heating occurs, concentric zones of damage can be identified [18, 37–39]. In the area around the fiber, the temperatures can reach high numbers in excess of 60°C resulting in central core area of coagulative necrosis. If the temperature in the area adjacent to the fiber inadvertently reaches 100°C, tissue vaporization occurs and a pseudocavity is formed. Immediately outside the core area lies the intermediate zone of permanently damaged tissue with increased interstitial fluid content. The outermost zone of damage that represents marginal zone consists of edematous but viable brain tissue. Histologically, the marginal zone is defined by lack of evidence of apoptosis and vessel thrombosis, and containing axonal swelling, shrinking neurons, and hypertrophied endothelial cells—markers of reversible tissue injury. Following a laser ablation procedure, tissues typically exhibit an increase in size due to the presence of necrotic tissue and perilesional edema. Over time, however, the necrotic core of the lesion is replaced by granulation tissue resulting in lesion shrinkage and scar formation.

2.5. Radiographic appearance

The typical appearance of high grade glioma is an irregular and heterogeneously enhancing lesion on T1-weighted images. After treatment, there are typical changes that are observed on subsequent imaging studies [40–42]. Immediately after procedure one can appreciate an area of hyperintensity within the lesion on T1-weighted MRI images. This finding corresponds to

coagulated blood products within the ablated area. On the corresponding CT scans this would appear as a hyperdense area typical in appearance of blood products. With administration of contrast, there is typically an area of peripheral rim enhancement. This is thought to represent an area of sublethal tissue damage with disrupted blood-brain barrier and leaky capillaries [7].

3. Application of laser therapy for treatment of intracranial disease

3.1. Laser in glioma treatment

Since the first implementation of laser therapy for intracranial tissue ablation, treatment of a variety of intracranial lesions was attempted, including metastases, radiation necrosis, meningiomas, ependymomas, as well as gliomas. High grade gliomas constitute 14.9% of primary tumors brain tumors and 47.1% of all malignant primary brain and other CNS tumors. Despite maximal therapy, survival remains poor. Survival without any treatment is 9 weeks [43]. With maximal treatment according to the latest guidelines, the survival is prolonged to 14.6 months [1]. The combined use of radiation and temozolomide protocols increase survival rates to 27% at 2 years [1]. Recently, several retrospective studies have demonstrated that increasing the extent of resection of glioblastoma, improves patient survival [2–5]. Furthermore, intraoperative use of surgical adjuncts such as intraoperative MRI or 5-ALA that allow visualization of the partially resected tumor and thus allow for a better extent of resection correlate with prolonged progression free survival [44, 45].

Many newly diagnosed glioblastomas involve deep or eloquent cortical or subcortical areas thus rendering these lesions difficult to resect or unresectable from an open surgery perspective. In these situations, the use of destructive, minimally invasive techniques such as LITT is very attractive as a means to cytoreduce the tumor. At other times patients may be too sick to undergo a lengthy open craniotomy for tumor resection. Finally, treatment of recurrent glioblastoma is challenging as very few effective options exist at present, thus the possibility of using laser ablation for treatment of recurrent tumor ads another tool to the neurosurgeon's armamentarium.

In 2013, the first human phase I study was published that used escalating dose of laser therapy to assess safety of the procedure and its efficacy in controlling tumor growth in patients with recurrent high-grade gliomas [41]. The study recruited 11 patients from two institutions and was completed using the NeuroBlate System. Three thermal damage threshold lines were assessed: yellow line (equivalent to heating of tissues to 43°C for 2 min), blue (43°C for 10 min), and white (43°C for 60 min). Ultimately, ten patients underwent LITT treatments and were followed for a minimum of 6 months or until death. Initially three patients were treated to the yellow TDT line and followed for 14 days to assess for signs of toxicity. If two out of three patients developed signs of toxicity, no further dose escalation was performed. If no toxicity was observed, the dose of treatment was escalated to first the blue line, and then the white line. The mean size of treated tumors was 6.8 cm^3, and an average of 78% of total tumor volume was covered. Median overall survival was 10.5 months after LITT which was increased compared to historic controls of 3–9 months [46, 47]. The median PFS at 6 months

and median overall survival in the study was >30%. One patient had a new permanent postoperative neurological deficit, and one patient had a vascular injury resulting in a pseudoaneurysm. Both patients were in the white TDT line subgroup. This study demonstrated that LITT is a feasible and safe treatment modality for recurrent high-grade gliomas, and that the blue line should be used as the margin of treated area.

The first multicenter study to investigate whether cytoreduction achieved with the use of laser for difficult to access high-grade gliomas could have a similar survival benefit compared to surgery was a retrospective study that looked at outcomes in 34 patients with high grade gliomas that were treated with LITT, 19 of them treated upfront, and 16 patients as salvage therapy [48]. The median overall survival was not reached in the study. One year estimated overall survival was 68%, and median progression free survival was 5.1 months. They also demonstrated that increased coverage by the thermal damage threshold lines correlated with better progression free survival of 9.7 vs. 4.6 months. The latter also relates to tumor volume with smaller tumors being easier to achieve complete coverage with TDT lines. When looking at failure patterns, 5 tumors recurred within the treatment field, 12 patients recurred at the periphery of the treated volume, 5 tumors recurred within 2 cm of the original area of enhancement, and one case had a remote recurrence. Overall, the authors concluded that LITT is an effective treatment modality for newly diagnosed and recurrent high-grade gliomas with improved outcomes correlating with extent of tumor coverage by analogy with extent of resection in surgical series.

Recently, a meta-analysis of the efficacy of LITT treatment of newly diagnosed and recurrent high-grade gliomas was published [49]. Ivan et al. extracted information and analyzed the data pertaining to treatment and outcomes of newly diagnosed high-grade gliomas treated with LITT. They identified four articles that reported treatment of 25 patients with newly identified gliomas. Tumor volume was available for 22 patients and the mean was 16.5 cm^3, whereas the extent of volume treated with laser was available for 9 patients with an average of 82.9% tumor coverage. Complications data was available for 13 patients, and there were no intraoperative mortality or complications. Serious postoperative complications occurred in two patients, one succumbing to postoperative central nervous system infection, and another one requiring hemicraniectomy for malignant post treatment cerebral edema. No permanent new postoperative neurological complications were noticed among these patients. Outcome analysis revealed a mean follow up of 7.6 months, with 12 patients still followed or lost to follow-up. Median overall survival was 14.2 months and the average PFS was 5.1 months. These results are similar to results reported in the literature that vary from 8.5 to 14.5 months [50, 51]. Thus, this systematic review demonstrates that LITT is a safe and effective procedure for newly diagnosed high-grade gliomas achieving outcomes similar to cases with open surgical resection.

Even with the full complement of modern treatments, the survival of glioblastoma patients remains poor in the range of 14–16 months after surgery, chemotherapy and radiation. Recurrence is the rule rather than the exception, at which point the prognosis is quite poor with the 6-month progression free survival rates of 5–15% [52, 53]. Reoperation in the recurrent setting was shown to be of benefit [54]. The risk of complications needs to be weighed against potential survival benefit, which is where the role for the use of LITT in recurrent

high-grade gliomas could be exploited the most. A recent systematic review summarized the outcomes of laser-mediated cytoreduction in high-grade gliomas [55]. Six articles were identified that included outcome analysis for treatment of 64 lesions in 63 patients. The range of pre-treatment tumor volumes was from 0.37 cm^3 to 68.9 cm^3. Postoperatively, serious complications included a permanent neurological deficit in 7 patients (12%), vascular injuries in 3 patients (3%), and wound infection in 1 patient (2%). The authors did not comment on outcome measures due to differences in outcome metrics used in the studies. Thus, they concluded that currently there is insufficient evidence to recommend LITT for treatment of recurrent high-grade gliomas. It is a technique that allows safe and accurate ablation of tumor tissue, though the complication rate associated with this procedure remains around 15% that is similar to open craniotomy procedures [56, 57].

3.2. Laser ablation near eloquent areas

The most common complication reported after laser ablation is a temporary or permanent neurological deficit, such as hemiparesis or aphasia. The reported complication rates range from 0 to 29.4% for transient and 0–10% for permanent postoperative neurological deficits. In many instances it is the damage to subcortical tracts that results in a new deficit. Recently, diffusion weighted imaging (DTI) with fiber-tracking algorithms started to be increasingly used in tumor resection surgery to avoid injury to eloquent white matter tracts. A recent study investigated the role of integration of DTI fiber tracts in laser thermal therapy. Using the NeuroBlate System, Sharma et al. looked that the extent of the overlap of the thermal damage threshold lines with the cortical fibers that would result in a postoperative motor deficit [58]. Retrospective analysis of 80 patients who underwent LITT for tumor near a critical area was performed. Fourteen patients (17.5%) had developed a new postoperative deficit that was temporary in 3 patients and permanent in 11. When looking at the average volume or surface overlap between treated area and the corticospinal fibers, there was a significant difference between the group that developed a postoperative deficit and the group that did not. Therefore, even a minimal overlap between the treated area enclosed within thermal damage treatment lines and the descending motor fibers can cause a postoperative neurological deficit after laser ablation. Addition of DTI tractography to treatment plans of lesions located in proximity to eloquent areas can help avoid fiber damage and thus preserve neurological functioning of the patient and is routinely used in our ablations near critical subcortical fiber tracts.

3.3. Advantages of LITT

There are a number of characteristics of LITT that lead to its recent popularity and investigation for multiple applications in neurosurgery. The main one is the ability to produce a lesion in a location that is difficult to access with open surgery. It is a minimally invasive technique that requires a very small incision and subsequently a very short period of healing. Given the minimally invasive nature of the procedure, the operation can be done under local anesthesia in a cooperative patient. This allows treatment of lesions in patients that cannot otherwise tolerate a large craniotomy.

Furthermore, LITT is a thermal ablating technique, which means that at the time of tumor recurrence the procedure can be repeated. That is not always the case with ionizing radiation, for example, since there is cumulative accumulation of radiation dose that limits the number of treatments that can be safely offered. The procedure also offers the advantage of obtaining a tissue specimen, when combined with needle biopsy, for updated pathology and biomarkers to follow tumor evolution and response to therapy. Finally, the minimally invasive nature of the procedure allows continued use of adjuvant treatments around the time of the surgery, or very shortly thereafter, obviating the need of waiting at least 2–3 weeks for the tissues to heal before restarting chemotherapy of radiation. In fact, there is evidence that LITT may open up the blood-brain barrier in the vicinity of treatment area, thus enhancing delivery of chemotherapeutics in that time range [7].

3.4. LITT and blood-brain barrier

The blood-brain barrier (BBB) is one of the main challenges for chemotherapy delivery to brain tumors. Various methods have been attempted to bypass or disrupt the blood-brain barrier, including convection-enhanced delivery of implanted catheters into the tumor, intra-arterial mannitol injections, or focused ultrasound to temporally disrupt the blood-brain barrier. Recently, laser interstitial thermal therapy was implicated in disrupting the integrity of tumor endothelial cells post-treatment. The core of the lesion that is produced after laser ablation is coagulum that consists of a permanently damaged tissue, whereas at the periphery where the temperature reaches 40°C and is insufficient to result in cell death, however it does lead to physiological temporary disruption in cellular function resulting in transient disruption of the BBB. Imaging of the lesion after laser ablation therapy displays an area of peripheral contrast enhancement that was speculated to represent disruption of the blood-brain barrier. This was demonstrated in a rodent model where there was extravasation of Evans blue dye that was injected intravenously at the periphery of the lesion post ablation [59].

Recently, advanced MRI imaging was used to demonstrate the presence of blood-brain barrier disruption [7]. Dynamic contrast-enhanced MRI was used in 14 patients to determine transfer coefficients (Ktrans) as a measure of permeability at the periphery of the lesion produced by laser ablation. In all patients, Ktrans coefficient peaked after the procedure, and then declined gradually over the next 4 weeks. The authors also used brain specific enolase (BSE) serum levels as a marker of BBB breakdown, and those levels peaked at about 3 weeks, followed by gradual decline and normalization at 6 weeks. This data suggests that there is some breakdown of the blood-brain barrier in the first few weeks following laser ablation of primary brain tumors, and that this may facilitate chemotherapy delivery to residual infiltrated tumor in the immediate post-procedure period.

3.5. Sensitization to radiation

Radiation is one of the non-surgical modalities that has significant impact on survival in glioblastoma patients, yet the control rates remain poor despite maximal therapy. Several studies have demonstrated the synergistic effect of hyperthermia in sensitizing tumor tissues to radiation and improved tumor control [60–62]. A recent study investigated the mechanism by which

thermotherapy affects tumor cells that results in enhanced sensitivity to radiation [8]. Glioma stem cell (GSC) cultures and mice bearing glioma xenographs were first exposed to 42°C for 1 h followed by radiation. When compared to radiation or hyperthermia alone, glioma stem cells are most significantly affected when both modalities are used in combination. Exposure of GSC to heat and radiation reduced stem cell survival, proliferation, and DNA repair, as well as promoted cell death. On the molecular level, there was significantly less AKT phosphorylation in cells exposed to hyperthermia and radiation, and rescuing AKT phosphorylation levels reversed negative effects of heat and radiation maintaining viability of tumor stem cells. Furthermore, exposing the mice bearing glioma xenographs to hyperthermia and radiation consistently reduced tumor size in these animals, and significantly increased their survival compared to animals exposed to either hyperthermia or radiation alone. These results add further evidence that the addition of hyperthermia to the standard radiation treatment may have a significant additive effect with respect to tumor control that is mediated by altering phosphorylation levels of PI3K-AKT pathway. Thus, early radiation therapy after laser ablation procedure may have additive effect on controlling tumor growth and affecting glioma cell viability. Further studies are needed to explore the clinical potential of this combined treatment.

4. Representative cases

4.1. Patient 1

The first patient is a 48-year-old right handed gentleman who presented to hospital after sustaining a fall. A CT head was performed that revealed a 1.9 × 2.0 cm ring-enhancing lesion in the left cingulum with significant amount of surrounding edema (**Figure 1**). On initial evaluation, patient had 4/5 right hand weakness and right-sided pronator drift that improved with initiation of steroids. The lesion was located immediately below the paracentral lobule, and given its proximity to this eloquent area, a needle biopsy with subsequent laser ablation was offered to the patient, who agreed to proceed. The lesion was treated using two trajectories using a side-firing laser (Monteris) to ensure the full tumor volume was included within blue thermal threshold treatment lines. Postoperatively, his neurological exam remained stable with the exception of the transient neglect of right lower extremity. Pathology of the tumor was consistent with glioblastoma. Molecular analysis demonstrated negative staining for IDH-1 and p53, intact 1p/19q, and non-amplified EGFR. Proliferative index was moderate with Ki-67 about 9–10%. The patient underwent subsequent concurrent chemotherapy and radiation according to the Stupp protocol followed by twenty-two cycles of temozolomide. Following that, he was followed with regular MRI brain scans that demonstrated a significant decrease in the size of the lesion over the first 12 months. It remains stable without evidence of recurrence or progression 6 years after the initial procedure (**Figure 1**).

4.2. Patient 2

The second patient is a 66-year-old right handed woman who was evaluated for spells of dizziness and visual disturbance. Brain imaging demonstrated a homogenously enhancing right parahippocampal lesion measuring 1.2 × 0.9 cm with surrounding edema (**Figure 2**). Patient

http://dx.doi.org/10.5772/intechopen.77078

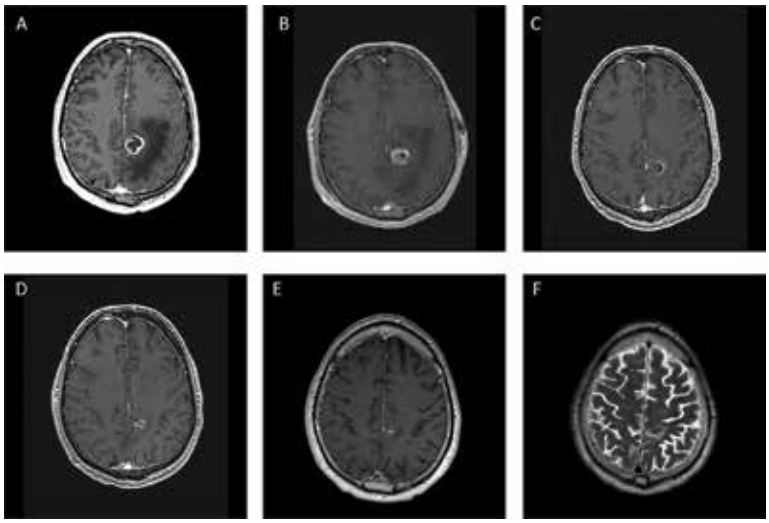

Figure 1. Representative axial T1-weighted MRI images obtained post gadolinium contrast administration. (A) Preoperative; (B) at 24 h post laser ablation; (C) at 3 months; (D) at 1 year; and (E) at 6 years post laser ablation; (F) a corresponding T2 weighted axial slice demonstrating very limited amount of edema surrounding the lesion.

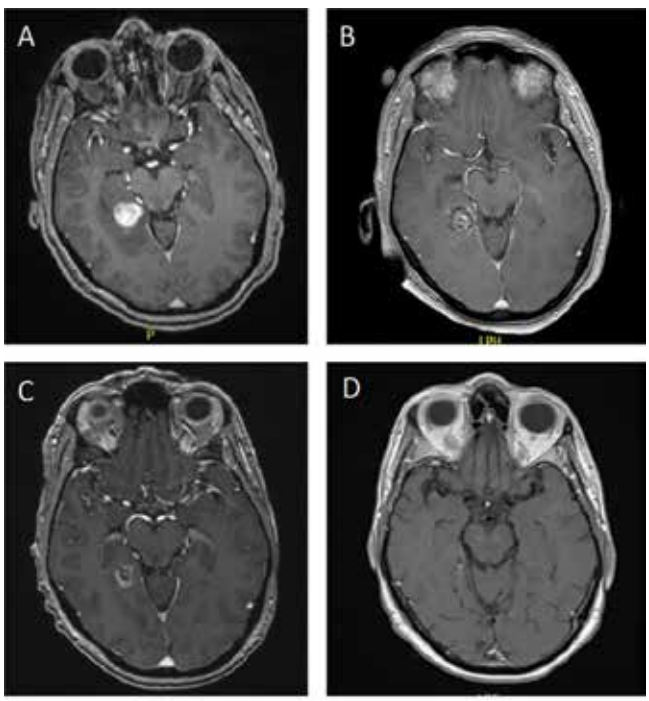

Figure 2. Representative MRI images of the right temporal lesion of patient 2. Axial T1-weighted MR images following contrast administration. The patient originally presented with a right mesial homogenously enhancing lesion (A). Immediately following laser ablation the characteristics of the lesion changed with markedly less contrast uptake (B). In the subsequent months, the lesion has continued to involute and significantly decreased in size at 3 months (C), and almost disappeared completely at 2.5 years (D).

was started on Keppra. Laser ablation of the lesion with a concurrent biopsy was recommended given the deep-seated location of the tumor. A single trajectory was used employing a side firing laser (Monteris). Complete tumor coverage was achieved as indicated by inclusion of the entire tumor volume within the blue thermal damage threshold lines. She had an uneventful postoperative course. No new postoperative deficits were associated with the procedure. Pathology showed a hypercellular glial tumor with marked nuclear atypia, frequent mitoses, and vascular proliferative changes, consistent with the diagnosis of glioblastoma. A Ki-67 labeling index in excess of 30% was focally noted. Greater than 80% of tumor cells stained positively with antibody to p53. 1p/19q chromosomes were intact, and EGFR was non-amplified. Following laser treatment, the patient received chemotherapy and radiation according to Stupp protocol, followed by adjuvant temozolomide for eight cycles that was stopped due to persistent myelosuppression. She was followed with regular MRI scans of brain with great local control with nearly complete resolution of the treated lesion. Unfortunately, at 2.5 years after procedure she developed disease progression at a remote site.

5. Conclusion

Laser thermal therapy is an effective treatment modality for newly diagnosed and recurrent gliomas. It may act in conjunction with radiation thus potentiating the effects of radiation. In addition, it may result in transient disruption of blood-brain barrier in the area of treatment and thus facilitate chemotherapy delivery postoperatively. Further studies examining the outcomes of new and recurrent glioma treatment would help to define the role that laser ablation play in management of this devastating brain tumor.

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Chapter 10

Medicinal Chemistry of Boron-Bearing Compounds for BNCT-Glioma Treatment: Current Challenges and Perspectives

Hugo Cerecetto and Marcos Couto

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.76369

Abstract

Since its first description, boron neutron capture therapy (BNCT) was a special type of radiotherapy for treatment of cancer and with focus mainly on glioma therapeutic. This procedure requires the selective accumulation of boron into the tumoral cells, and due to this requirement, different boron-enriched compounds have been designed and developed. Efforts to circumvent the selectivity-uptake challenge and other problems, such as solubility, stability, and toxicity, have been to driving force behind the medicinal chemistry field in boron-based compounds. In this regard, a wide diversity of medicinal chemistry hypothesis has been used to obtain new and efficient potential BNCT-glioma drugs. In this chapter, these ideas are analyzed focusing on their medicinal chemistry characteristics.

Keywords: glioma, glioblastoma, drug discovery, boron neutron capture therapy, boron-bearing compounds

1. Introduction

1.1. Boron neutron capture therapy: historical account

In 1932 in the UK, Chadwick discovered neutrons, and for his contribution, he was awarded in 1935 with the Nobel Prize in Physics [1]. One year later in the USA, Gordon Locher introduced the concept of boron neutron capture therapy (BNCT) [2]. He hypothesized that if boron could be selectively concentrated in a tumoral tissue and then exposed to a neutrons

beam, a higher radiation dose to the tumor relative to surrounding normal cells would result. A mere 2 years later, Goldhaber, Hall, and Kruger performed the first radiobiological studies using boric acid and slow neutrons in a murine tumoral model [3]. However, the first clinical trials against human brain tumors (glioblastoma multiforme (GBM)) that used BNCT could not be initiated until 1951 at the Brookhaven National Laboratory in collaboration with the Massachusetts General Hospital and the Massachusetts Institute of Technology (MIT) [4, 5]. In this case, ten patients were treated with borax (disodium tetraborate decahydrate, $Na_2B_4O_7{\cdot}10H_2O$; **Figure 1**) and thermal neutrons without much success. While there were no serious side effects of BNCT in the patients, the large doses of borax (^{10}B-enriched) infused 200 mg/kg, inducing slight toxicity symptoms. In order to improve this, a second approach was developed comprising nine glioma patients but now with a less toxic compound, sodium pentaborate (NaB_5O_8; **Figure 1**) in combination with D-glucose. Unlike the first one, a higher dose of ^{10}B was used, and a higher fluency of incident thermal neutron was applied [6]. Unfortunately, again, serious side effects such as radio-dermatoses of the scalp and deep ulcerations were observed [6, 7]. Simultaneously, in 1963, Sweet and co-workers, from the MIT, treated 18 patients using boron-rich disodium decahydrodecaborate ($Na_2B_{10}H_{10}$; **Figure 1**) [8], which was considered to be less toxic and with the ability to deposit more boron atoms per cell. Symptoms of brain necrosis in patients undergoing BNCT were again observed [9]. Due to these disappointing events, the USA halted the progress of research on BNCT in 1961.

On the other hand, in 1968 at Hitachi training reactor, the Japanese neurosurgeon Hiroshi Hatanaka, who in previous years had worked with Sweet in Boston, began treating patients with high-grade malignant gliomas using disodium mercaptoundecahydro-*closo*-dodecaborate ($Na_2B_{12}H_{11}SH$, named as BSH; **Figure 1**) which originally had been synthesized by Soloway in 1967 [10]. The results reported by Hatanaka and co-workers were extraordinary with a 5-year survival rate of 58% [11–13].

From the 1990s to the present day with the development of new boron compounds and the improvement in radiation source, the boron neutron capture therapy has been expanded to several centers worldwide, among them in the USA at Brookhaven and Cambridge, in the Netherlands at high flux reactor in collaboration with the Department of Radiotherapy of the University of Essen in Germany, in Finland at FiR-1 Otaniemi reactor, in Sweden at R2–R0 reactor, in the Czech Republic at LVR-15 reactor, in Italy at TRIGA reactor, in Japan at Kyoto University Research Reactor Institute, in Argentina at RA-6 and RA-3 reactors, and in Taiwan at THOR reactor, just to name a few [14].

1.2. Boron neutron capture therapy: principles and general requirements

BNCT is considered as a rationale and promising binary therapy modality for treatment of several cancers in particular malignant gliomas. The cell-killing effect of BNCT is based on the nuclear reaction $^{10}B(n,\alpha)^7Li$ (**Figure 2**) that occurs when the nuclide ^{10}B, which is a nonradioactive constituent of natural elemental boron (approximately 20% abundance), is irradiated with neutrons of the appropriate energy, thermal neutrons. The nuclear reaction yields excited boron-11 ($^{11}B^*$) that after instantaneous nuclear fission produces two high-linear energy transfer entities, i.e., α-particle ($^4He^{2+}$) and recoiling lithium-7 nucleus ($^7Li^{3+}$). Because of the very short track length of these heavy particles (<10 μm; roughly one cell diameter), radiation damage is confined to those cells loaded with ^{10}B.

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The probability that a nuclide captures a neutron is measured by the neutron capture cross section, σ_{th}, having ^{10}B a value of σ_{th} = 3838 barns [15]. However, other abundant endogenous nuclei present in the healthy tissue, such as ^{1}H and ^{14}N, could also capture neutrons yielding after nuclear reactions of a gamma ray in the first case, $^{1}H(n,\gamma)^{2}H$, and a proton in the second one, $^{14}N(n,p)^{14}C$. However, the σ_{th} of these nuclei is smaller than the value for ^{10}B, i.e., $\sigma_{th,1H}$ = 0.332 and $\sigma_{th,14N}$ = 1.82 barns, and the amount of radiation produced by these nuclear reactions is lesser than the produced by the particle and recoiling nucleus in the case of boron [15].

On the other hand, for brain tumor such as GBM, usually higher energy epithermal neutron beams which have a greater depth penetration being thermal neutrons unable to act on tumors located below the tissue surface because of scattering effects have been used. Epithermal neutrons do not suffer from the disadvantages of H-recoil processes and, consequently, allow capture reactions to occur at some distance within the tissue; then, epithermal neutrons are progressively slowed into thermal neutrons through heat-releasing interactions with the hydrogen atom and constituents of biological system, that do not cause damage to the tissue [16].

In order for BNCT to be successful, the ^{10}B-loaded agent must completely fulfill some overriding conditions, namely, (a) selective uptake by tumor tissue relative to normal tissue (preferably accumulating within specific tumoral cell substructure) with ideal tumor:normal tissues and tumor:blood ratios of 3:1 and 5:1, respectively, and (b) appropriate amount of ^{10}B delivered to the tumor tissue, i.e., at least 20 μg ^{10}B/g tumor, corresponding to about 10^{9} atoms of ^{10}B/cell.

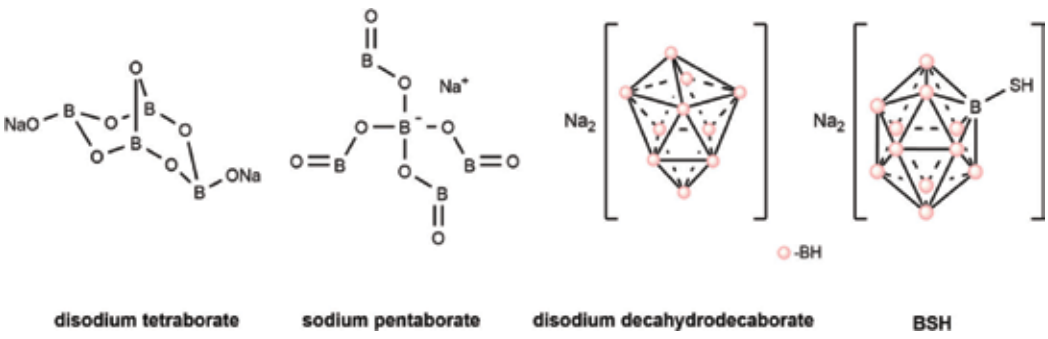

Figure 1. First and current available boron-based drugs for BNCT.

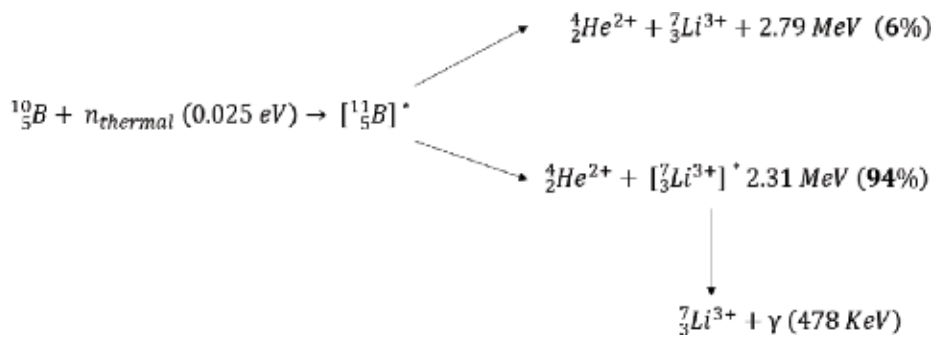

Figure 2. The two parallel nuclear fission reactions that occur upon capture of a slow (thermal) neutron by a ^{10}B nucleus.

However, this amount could be lower if the boron delivery system is concentrated in or near the cell nucleus; (c) retention of ^{10}B in tumor during the BNCT process; (d) rapid clearance from blood and healthy tissues; (e) and adequate lipophilicity especially for glioma treatment where the drug should be able to cross blood-brain barrier (BBB) [17]. Furthermore, like any drug in medicinal chemistry, the ^{10}B-loaded agent must meet the following requirements: (f) absence of systemic toxicity, (g) chemical and metabolic stability, and (h) appropriate water solubility.

1.3. Boron neutron capture therapy: current therapeutic agents

After the first efforts, during the 1940s and 1950s (see Section 1.1.), the lack of selectivity and low boron tumor accumulation observed for the simplest boron salts (**Figure 1**) used until the moment prevented their application in BNCT clinical trials. However, around the 1960s, the first studies of the two compounds currently in clinical began, both ^{10}B-enriched, the polyhedral borane BSH (**Figure 1**) and L-4-dihydroxyborylphenylalanine, known as L-boronophenylalanine (L-BPA; **Figure 3**) [18], which could be accumulated into desired tissues for its structural analogy to some biomolecules.

Due to BSH is a small hydrophilic molecule (**Figure 1**), it does not cross the intact BBB. It only penetrates into the brain passively when the BBB is disrupted [10], as it is observed in the GBM. Although BSH has been applied for the treatment of GBM in infusions with no toxic effects, the efficacy has been limited due to low observed tumor:brain (3:1) and tumor:blood (0.9–2.5:1) ratios [19]. The main structural advantage of BSH compared to L-BPA is that BSH contains 12 times more B per molecule yielding a higher number of events after neutron capture than in L-BPA. BSH has been studied in different therapeutic schedules, combined or not with other small molecules, like L-BPA, or vehicles looking for the improvement of the

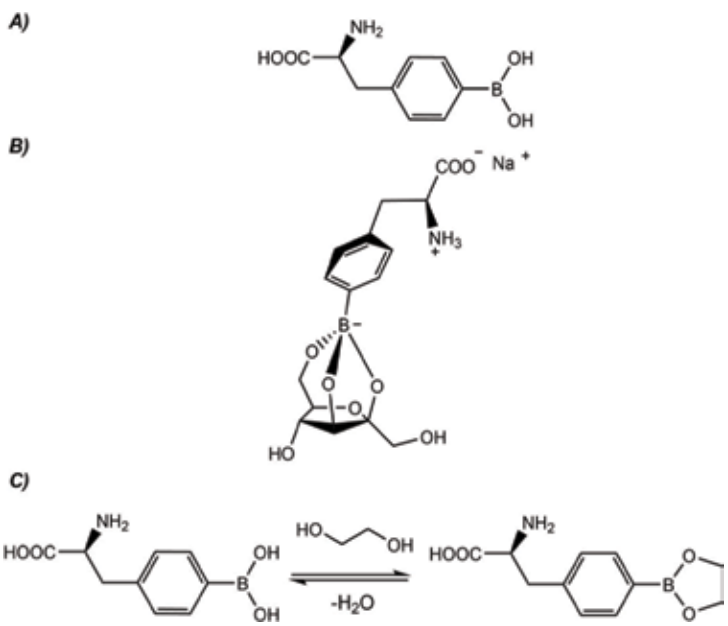

Figure 3. (A) Currently, available boronic acid for treatment of GBM trough BNCT. (B) L-BPA-F complex. (C) Esterification of L-BPA with ethylene glycol.

efficacy and the delivery into the glioma [20]. Medicinal chemistry on BSH, structural modifications, has also been done (see below) seeking better biological behavior.

Nowadays, L-BPA (**Figure 3**) is the standard therapeutic drug used in BNCT [21–23]. Since the L-amino acid transport system is highly expressed in tumor cells compared with normal cells in most organs including the brain, some natural amino acid boron derivatives have been studied [24]. L-BPA has very limited water solubility (1.6 g/L), and searching to circumvent this problem, the standard strategy used for clinical BNCT treatment, it is as a soluble fructose complex, known as L-BPA-F (**Figure 3**), which leads to a pharmaceutical product with favorable biodistribution in human GBM, ratios tumor:blood of 3–4:1 [25], low toxicity, and good capability to cross BBB. Other two strategies include (a) the transformation into the corresponding hydrochloride salt and (b) the esterification of the boronic acid moiety with 1,2- and 1,3-diol producing 1,3,2-dioxaborolanes or 1,3,2-dioxaborinanes, respectively, which is then easily hydrolysable in an aqueous environment (**Figure 3**) [26, 27]. On the other hand, L-BPA is actively transported across the tumor cell membrane, by the L-amino acid transporter system. It is highly expressed in tumor cells, including the brain, compared with normal tissues and can be stimulated by the previous accumulation of the L-DOPA resulting in a substrate-coupled antiport (exchange) mechanism [28]. At this point, L-BPA is considered to be a better B delivery agent than BSH.

2. Medicinal chemistry of boron-bearing compounds for BNCT

2.1. General

From a medicinal chemistry point of view, different strategies have been studied in order to identify new and more selective molecules to glioma cells, with adequate ability to cross the BBB, with higher tumor concentration in the path of the neutron beam and drug-like properties. The third-generation products, which potentially may accumulate into glioma for its structural analogy to some biomolecules, could be classified [15] in (a) macromolecular species and (b) low molecular weight molecules. In reference to the first group, we could mention monoclonal and bispecific antibodies, epidermal growth factor, and encapsulating agents such as boron-containing nanovehicles (liposomes). Here, we will discuss compounds belonging to the second group, like polyhedral boron cluster derivatives, boronic acid derivatives, and other boron-containing small molecules (e.g., oxaborolanes, dioxaborolanes, and azaboro-heterocycles, among others).

2.2. Polyhedral boron clusters

2.2.1. Implications for drug discovery: structural features of closo-carboranes and metallacarboranes. How do they influence on the drug-like properties?

The most known and commonly used class of polyhedral boron compounds in the medicinal chemistry field are the icosahedral dicarba-*closo*-dodecaborane ($C_2B_{10}H_{12}$) commonly referred to as carboranes which exist in three isomeric forms named with respect to the positioning of the two CH vertices (**Figure 4**): 1,2- or *ortho*- (**1**); 1,7- or *meta*- (**2**); and 1,12- or *para*-carborane (**3**); to a lesser extent, their mono-anionic derivatives resulting from the loss of a B vertex, commonly

known as *nido*-carborane (**4** $[C_2B_9H_{12}]^-$); and their metal complexes known as metallacarborane (**5** $[M(C_2B_9H_{11})_2]^-$, where M is a metal) that are generated after removal of the bridge hydrogen from the *nido*-carborane (**Figure 4**) [29, 30].

Among the outstanding and widely explored properties of carboranes and metallacarboranes for medicinal chemistry research are (a) the geometry and the electron-deficient nature of the boron atoms, which generate a strong hydride character in the BH shell, which are some of the main features that determine the intermolecular interactions with the biological targets because they make the B-clusters extremely hydrophobic; (b) both the pharmacokinetics and the bioavailability can be modulated according to the chemotype of boron cluster selected, so the hydrophilicity and lipophilicity, or both, could be tuned; (c) the globular architecture and rigid geometry allow for molecular construction in three dimensions improving the docking, or not, with bio-targets; (d) high boron content per molecule and stability to catabolism are important criteria for the development of agent for BNCT; and (e) the well-established chemistry that makes boron clusters attractive synthons to construct novel pharmaceuticals [31].

Nevertheless, some problems persist today that delay the application of boron clusters in the development of new drugs: (a) the relatively high cost of carboranes and their derivatives even more if they will be used in BNCT because ^{10}B-enriched compound will be needed; (b) the difficulty of in silico drug design and screening of boron cluster drugs, due to the lack of appropriate descriptors for the interaction potentials of boron and the attached hydrogen atoms; and (c) the lack of libraries of boron cluster compounds for high-throughput screening.

2.2.2. Closo-carboranes and metallacarboranes designed and studied for BNCT-glioma treatment

In the past decade, saccharides have been widely studied due to their low toxicities, generally having high water solubility, high expression of specific receptor on the tumor cell surface (specifically in the brain capillary endothelial cells that form the brain barriers, resulting in optimal BBB penetration), and an increased rate of glycolysis in cancer cells. According to this and in order to improve boron uptake, carbohydrates such as ribose, mannose, maltose, and galactose have been chosen to generate carbohydrate-based boron delivery platforms for successful BNCT approach [32, 33]. Since the first description by Hawthorne's group [34], there have been numerous works on the synthesis and biological evaluation of carboranyl-carbohydrate conjugates. Later, Tietze and co-workers developed and evaluated both in vitro and in vivo, against rat glioma cells (C6) and melanoma cell line (B16), a series of carboranyl glycosides including glucoside, lactoside, and maltoside conjugates (**6–8**; **Figure 5**) [35–37]. Although in the in vitro assays significant killing effect was observed because the three derivatives showed an optimum cellular uptake in the C6 glioma cell line, with maltoside **8** being the derivative that exhibited the highest uptake (65.7 ppm at 12 h), the in vivo performance in rat model bearing brain tumor revealed that the concentration of all carboranyl-appended carbohydrate in blood was maintained at levels that were unacceptably high for meaningful use in BNCT. In order to overcome this drawback, Tietze and Yamamoto designed a new type of mixed carboranyl bisglicoside derivatives as prodrugs (**9–12**; **Figure 5**) [38]. Initially, selective uptake of these compounds was not expected due to the hydrophilic sugar moieties at both ends of the carborane preventing cell internalization. The authors demonstrated that the activation of the prodrug could be performed using monoclonal antibodies conjugated

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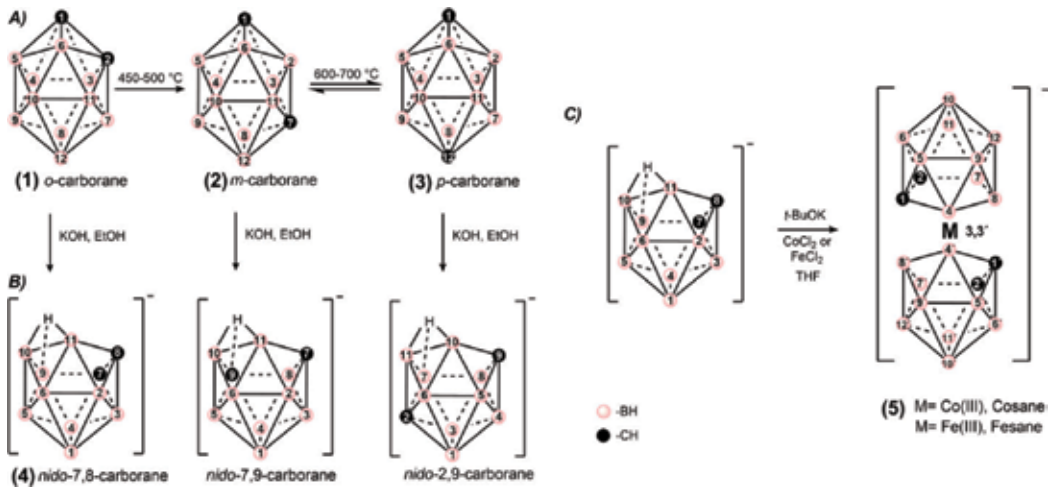

Figure 4. (A) Numbering and nomenclature of the *closo*-carborane systems. Synthesis of *meta* and *para*-carborane derivatives from the *ortho*-carborane isomer through thermal isomerization. (B) Partial degradation of *ortho*-, *meta*-, and *para*-carborane and numbering of nido-carborane system according to IUPAC. (C) Synthesis of metallacarborane from *nido*-carborane.

to glycohydrolases, which bind to tumor-associated antigens, through the glucosidic bond cleavage and concomitant release of carboranyl moiety in the tumor cell surface.

On the other hand, in the last few years, boron-bearing purines, pyrimidines, thymidines, nucleosides, and nucleotides have been widely explored as a novel approach to improve boron uptake in glioma tumor cells. This strategy is based on the fact that tumor cells have higher metabolic activity and an increased requirement for DNA and RNA precursors [39, 40]. Although in recent years several strategies have been addressed, the main focus has been on thymidine analogs substituted with *ortho*-carbonyl cluster at the *N*-3 positions (**13** and **14**, named as 3CTAs; **Figure 6**). Both analogs are potential substrates for human thymidine kinase 1 (hTK1), a cytosolic deoxynucleoside kinase of the DNA synthesis salvage pathway that is predominantly found in proliferating cancer cells. The selective accumulation and retention of 3CTAs in tumor cells via a mechanism known as kinase-mediated trapping (KMT) render these molecules as potential BNCT delivery agents against high-grade brain glioma, such as GBM [41–43]. Another considerable attention has also been to metallacarborane, mainly the bis-(dicarbollyl)-cobalt and bis-(dicarbollyl)-iron derivatives (**15–17**, **Figure 6**). They can also be selectively accumulated in rapidly multiplying neoplastic cells; following their conversion to the corresponding nucleotides, trapped within the cell; or, ideally, incorporated into nuclear DNA of tumors [44]. Despite this, these compounds still have not studied in gliomas.

Barth and co-workers evaluated ^{10}B-enriched derivative **14**, using the RG2 model as in vivo brain tumor model [45]. First, they demonstrated that derivative **14** efficiently delivers boron atoms in cancer cell, which allowed tumor reduction after BNCT in nude mice bearing tumor induced with TK1 positive cell. In addition, based on these favorable results, BNCT studies carried out in the RG2 rat model lead to an increased in life span (ILS) 2.4× in comparison with L-BPA as control therapy. Nevertheless, the greatest percent ILS (122%) was seen in RG2 glioma-bearing rats that received the combination of derivative **14** and

L-BPA, and this correlated with the fact that the tumor in these animals received the highest physical radiation doses. These biological studies clearly revealed the therapeutic potential of derivative **14** although some problems and limitation appeared. Among these, derivative **14** showed clear solubility problems under physiological conditions, possibly due to the presence of the carboranyl hydrophobic core and the absence of any functional groups that can be ionized.

Since the first description by Haushalter and Rudolph in 1978 (**18** and **19**; **Figure 7**) [46, 47], the potential for medical application of several boron cluster-containing fluorescent dyes, including porphyrins and related macrocyclic, have been highly explored and scrutinized for several reasons, among them: (a) well-known ability to selective accumulate into tumor cells in high amounts, (b) subsequent persistence within tumors, (c) general low dark cytotoxicity, (d) capability to bind to DNA due to their plane aromatic structure, and (e) highly fluorescence, thus enabling tumor diagnosis and facilitating treatment planning. Nonetheless, without any doubt, the most exploited feature of these molecules is the ability to act in photodynamic therapy (PDT). PDT combines a photosensitizer (porphyrin), light, and tissue oxygen, to generate reactive oxygen species, including singlet oxygen, triggering subsequently cell death mechanisms by necrosis and/or apoptosis. The combination of this therapy with BNCT has been of particular interest to control local recurrence of high-grade gliomas such as GBM, because they target different mechanisms of tumor cell death and, thus, increase the

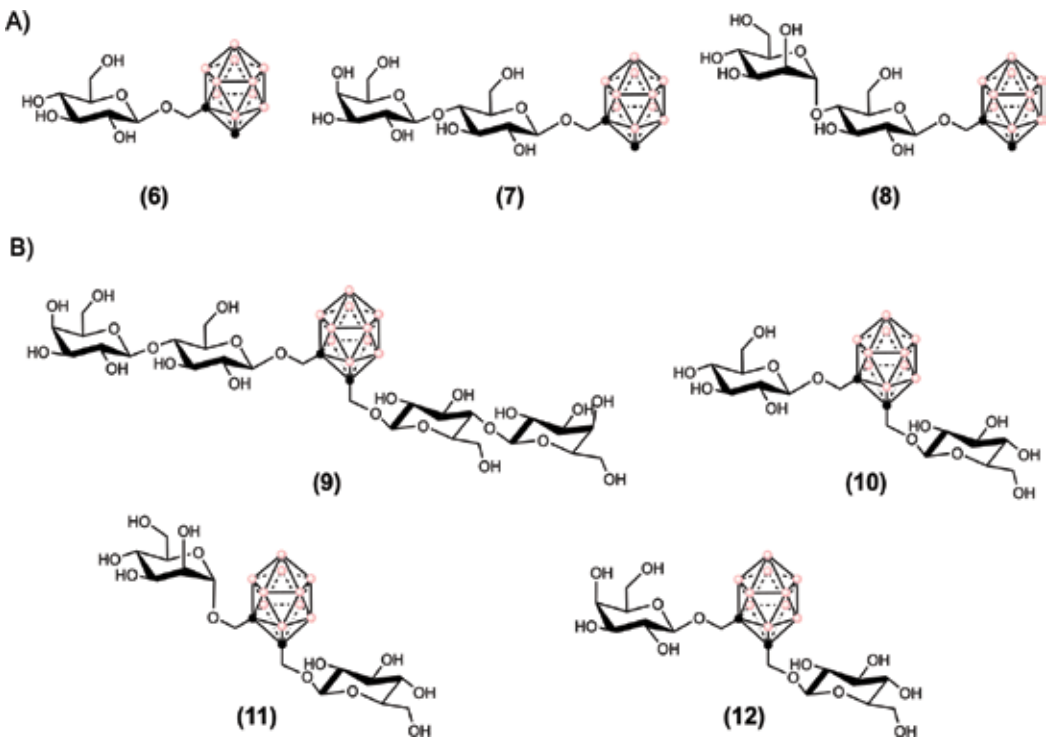

Figure 5. (A) Compound **6** corresponds to carboranyl derivative of D-glucose, and compounds **7** and **8** are derivatives of lactose and maltose, respectively. (B) *Ortho*-carboranyl bisglycosides **9**–**12** containing lactose, glucose, mannose, and galactose in their structures.

therapeutic effect. Both are bimodal therapies, the individual components of which are non-toxic in isolation, but which are tumoricidal in combination.

In the last few years, a large number of boron-containing natural porphyrins have been synthesized and evaluated both in cellular and animal models (**20–23**; **Figure 8**) [48]. To date, the tetrakis-carborane carboxylate ester of 2,4-bis(α,β-dihydroxyethyl)deuteroporphyrin IX (**22**, known as BOPP) was the only compound to be employed in a phase I clinical study against GBM [49, 50]. Pharmacological studies showed its ability to selectively incorporate in tumor cells, in xenograft models of glioma, relative to the surrounding normal brain cells (tumor:brain ratio as high as 400) and situated preferentially in tumor cell mitochondria. Nevertheless, these results could not be replicated in humans.

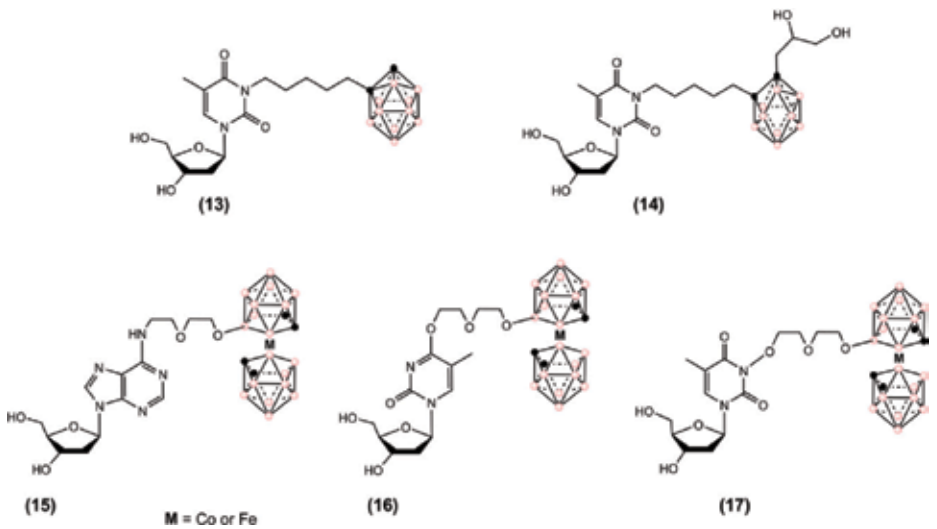

Figure 6. Chemical structure of the main carborane-bearing nucleoside delivery agents.

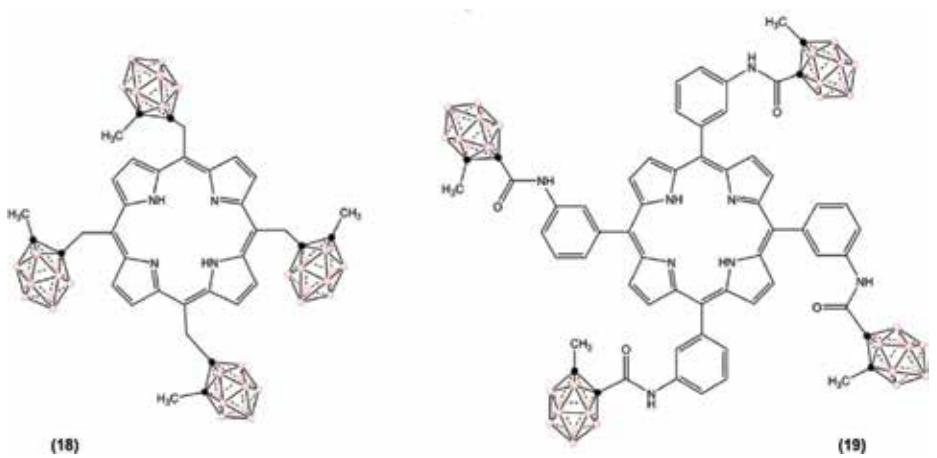

Figure 7. First boron-containing porphyrins developed by Haushalter and Rudolph for catalysis application.

The success of derivative **22** was interrupted in a phase I clinical trial for several reasons: (a) it could not deliver to the tumor a therapeutic amount of boron in patients with GBM; (b) thrombocytopenia was observed in patients due to the direct toxic effect of derivative **22** or its metabolites (probably of carboxylic carborane) on platelets; and (c) the pharmacokinetic behavior of derivative **22** in humans was characterized by a prolonged clearance phase, giving rise to potentially toxic metabolites and cutaneous photosensitivity [51].

In order to overcome this drawback, several researchers designed and synthesized a highly boron water-soluble porphyrins as a possible BNCT agents. In this sense, Vicente and co-workers described *nido*-carborane cluster-linked porphyrins via aromatic linkage (e.g., **24**; **Figure 8**). These amphiphilic derivatives showed very low toxicity, were taken up by 9 L and U-373MG cells in both time- and concentration-dependent manners, and were localized preferentially in cell lysosomes [52]. On the other hand, Deen and co-workers developed the polyboronated ionic porphyrin **25**, known as TABP-1, which was administrated by convection-enhanced delivery (CED) to nude rats bearing intracerebral implant of human glioblastoma cell line U-87MG [53]. In contrast to diffusion, CED uses a pressure gradient established at the tip of an infusion catheter to establish bulk flow and distribute drug and solvent throughout the extracellular space. This demonstrated high tumor and low blood boron concentration through intracerebral administration by CED related to systemic administration.

Carborane-containing nucleosides and analogues as the means to concentrate boron within the tumor cell nucleus were described. However, another class of derivatives, which could interact directly to chromosomal DNA, has been developed and widely explored for boron neutron capture therapy. Among this the following could be mentioned (**Figure 9**): (a) alkylating agents (i.e., **26** and **27**), (b) DNA intercalators (i.e., **28** and **29**), (c) minor groove binders (i.e., **30** and **31**), and (d) cationic polyamines (i.e., **32** and **33**) [54]. Regarding the last group of compounds, it should be stated that natural polyamines such as spermidine, spermine, and putrescine, found in both prokaryotic and eukaryotic cell types, are a class of biologically active compounds known to be essential for every cell to support their function such as growth and differentiation. In addition, because they have a specific transportation system, they have been found in high concentration in rapidly proliferating tumor cells. This characteristic was used by Zhuo and co-workers, synthesizing and evaluating derivatives **32** and **33** [55]. These compounds proved to be highly water-soluble, in vitro they have the ability to interact to calf thymus DNA, and they are rapidly taken up by F98 rat glioma cells at levels which match that of clinically used agents. Unfortunately, in vivo biodistribution studies of these derivatives, performed in mice-bearing intracerebral implants of the GL21 glioma and subcutaneous implants of the B16 melanoma, suggested that they were unable to deliver adequate amounts of boron to tumor.

More recently, Vicente's group proposed other interesting strategies that involved the use of porphyrins bearing *p*-carboranylmethylthiol moiety conjugated to polyamines (**34**–**41**; **Figure 10**) [56]. In vitro, the polyamines displayed low dark cytotoxicity, low phototoxicity, preferential localization in the endoplasmic reticulum, Golgi and the lysosomes, and, except derivative **34**, higher uptake into human glioma T89G cells (up to 12-fold for spermine derivative **39**) than the tri-ethyleneoxy conjugate **41**. All these results suggested that spermine derivative could serve as an effective carrier of boronated porphyrins for the BNCT of tumor.

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2.2.3. BSH structural modifications

As it is indicated above, BSH inadequate drug-like properties, i.e., lack tumor selectivity and poor BBB crossing ability, have conducted to develop BSH hybrid compounds containing other pharmacophoric moieties seeking improvement biological behavior.

In this sense, some approaches were based on the particular tumoral amino acid requirement and the special ability of some peptides to interact with receptors that, via endocytosis, are internalized into tumoral cells. In the first strategy, hybrid **42** (**Figure 11**), carrying a fragment derived from an unnatural amino acid that demonstrated a particular uptake by glioblastoma cells [57], was prepared and in vivo evaluated in F98 glioma-bearing animals [58].

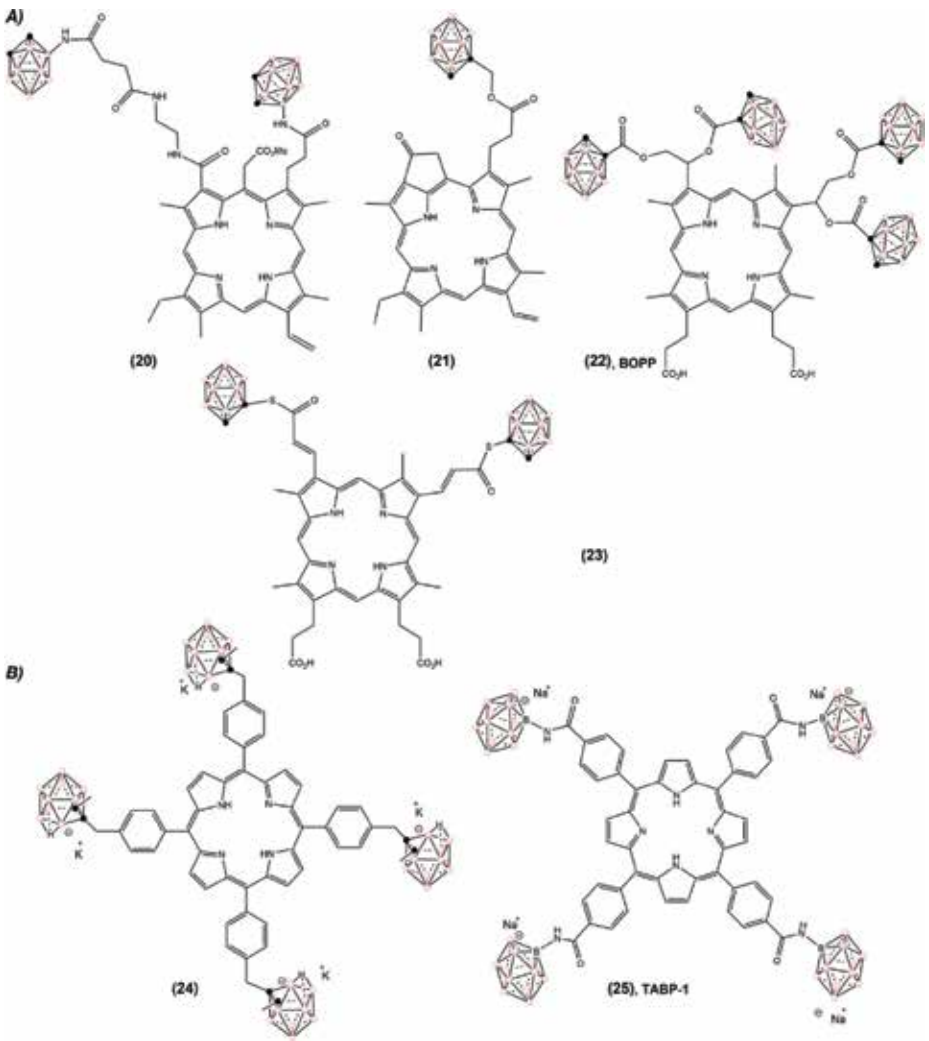

Figure 8. (A) Structures of some relevant boron-containing porphyrins. (B) Some relevant polyhedron boron cluster water-soluble porphyrins.

The biodistribution studies showed higher tumor boron concentrations for derivative **42** than for BSH but lower than for L-BPA-F i.v. treatments. The CED intracerebral administration of derivative **42** was assayed combined with BNCT reaching better animals' survival rates than the treatment with L-BPA-F/i.v. On the other hand, in the use of peptides as carrier strategy, the hybrid **43** (**Figure 11**), derived from Tyr^3-octreotate peptide that interacts to somatostatin receptor, was synthesized [59]. This BSH octapeptide still has not study biologically.

As it was already mentioned, the biochemical peculiarities of the porphyrin system also allowed the delivery of BSH within the tumor. In this sense, hybrid BSH porphyrin **44** (**Figure 11**) was prepared and evaluated in a 9 L glioma-bearing rat model [60]. Compound **44** displayed higher boron tumor:blood ratio in comparison to BSH 24 h after i.v. treatment and in an in vitro BNCT colony-forming assay higher cytotoxicity than BSH. Another series of BSH porphyrin hybrids consists of compounds like **45** and **46** (**Figure 11**) that still has not been biologically studied on gliomas [61].

GBM contains areas with different oxygenation levels, and consequently different metabolic patterns, which make difficult the therapeutic strategies [62]. Highly oxygenated regions are close to blood vessels, present high proliferation rate and oxidative metabolism and are susceptible to metabolism-active drugs and radiotherapy. While hypoxic regions present low proliferation rate, reductive metabolism and are resistant to chemo- and radiotherapy. The hypoxic condition in GBM tumors, which induces metastasis and promotes angiogenesis and resistance, has been attributed to contribute to tumor regrowth and, therefore, in a relapse. The troublesome characteristics of hypoxic regions have been exploited to generate cancer therapeutics known as hypoxia-selective bioreductive prodrugs which are compounds able

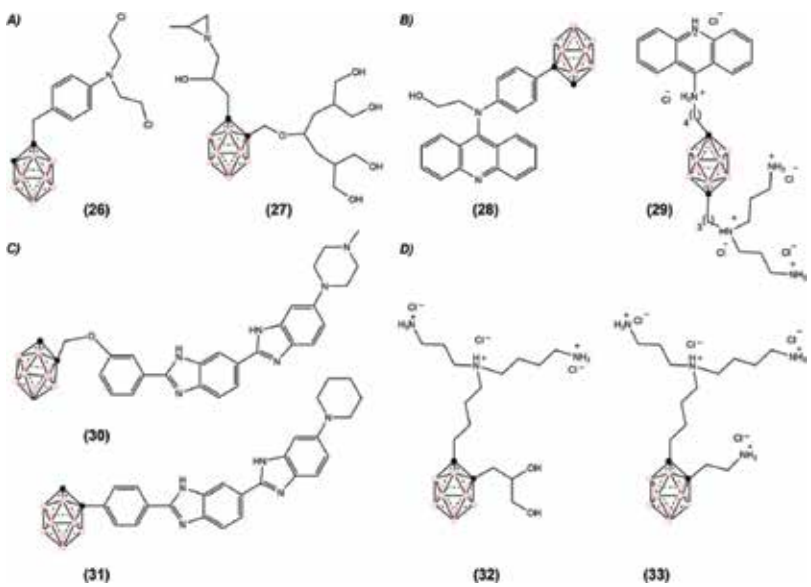

Figure 9. Carborane-containing DNA-interacting agents. (A) Alkylators. (B) Intercalators. (C) Minor-groove binders. (D) Polyamines.

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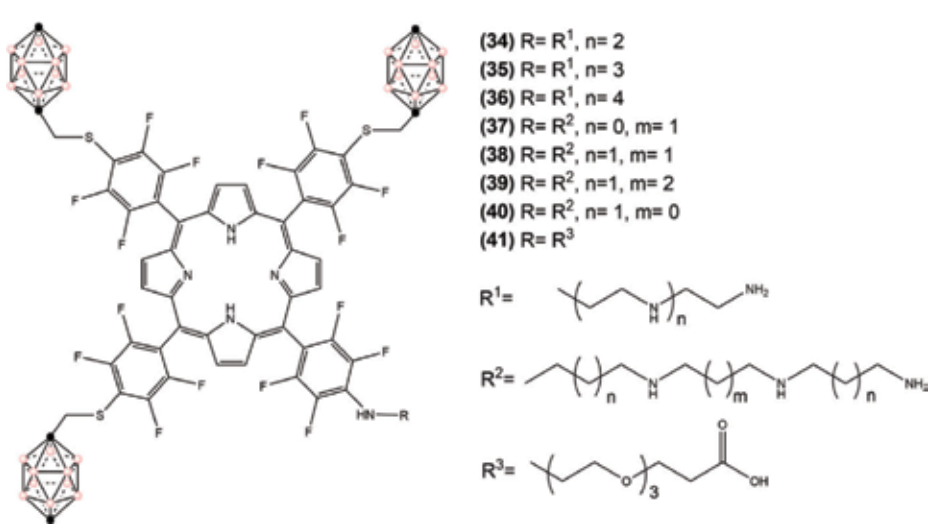

Figure 10. Carborane-containing porphyrins porting polyamine framework.

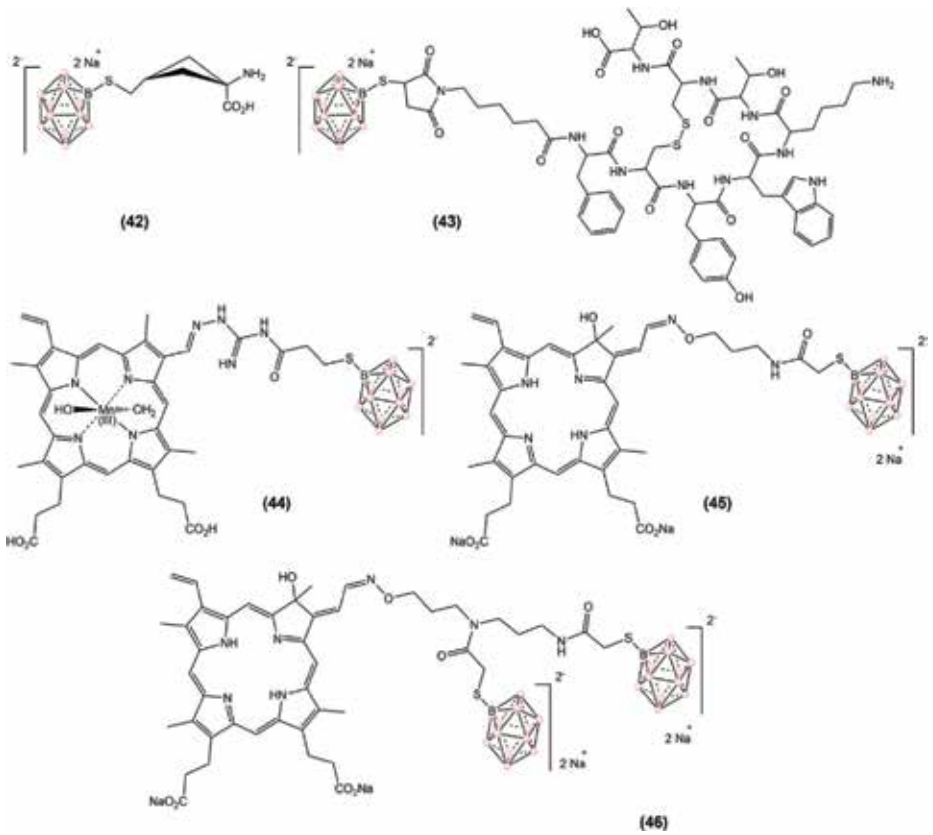

Figure 11. Some BSH hybrids attempting to improve BSH biological behavior.

to undergo reduction producing cytotoxic events. Some of the well-known bioreductive pharmacophores, nitroimidazoles, and *N*-oxide containing heterocycles were used to prepare BSH hybrids for potential use in hypoxia. Thereby, 2-nitroimidazoles **47** and **48** or quinoxaline dioxides **49** and **50** (**Figure 12**) were prepared and evaluated against some tumoral models using BNCT, but unfortunately they have not yet been studied on glioma models [63–65].

2.3. Boronic acids and their esters

Boronic acid (R-$B(OH)_2$) has been utilized as a pharmacophore in the searching of new agents to be employed in BNCT. This functional group has some relevant drug-like properties turning it into a useful moiety for biological applications, for example [66–68], (a) the sp^2-hybridized boron atom possesses a vacant *p*-orbital which, after biomolecule donor atom attacks, allows the interconversion of boron hybridization, from sp^2 to sp^3, to generate a new stable anionic compound; (b) the enhanced ability of $–B(OH)_2$ system to interact with biomolecule via (b1) hydrogen bonds, as acceptor and donor through OH groups, and (b2) strong boron Lewis acidity that allows boron-nitrogen, boron-oxygen, or boron-sulfur bonds; (c) the apparent pK_a of $–B(OH)_2$ ranging 4.5–8.8, for arylboronic, that allows large acid-base behavior finding the protonated and deprotonated form of the acid according to physiological pH conditions; and (d) apart from the reactivity mentioned above (section a) the particular ability to react with diol-containing compounds, like sugars and sugar-containing biomolecules, yielding the corresponding stable cyclic ester 1,3,2-dioxaborolane. Additionally, the $–B(OH)_2$ group has weak electronic effects being electron donor for induction and electron withdrawing for mesomerism. In reference to the lipophilicity of the $–B(OH)_2$ moiety, it is as lipophilic as –CN group, lesser lipophilic than $–CO_2CH_3$ and neutral $–CO_2H$ moieties, and more lipophilic than $–CONH_2$, $–CH_2OH$, and –CHO groups (π Hansch constants which are −0.55, −0.57, −0.01, −0.32, −1.49, −1.03, and −0.65, respectively [69]). In vitro boronic acids degrade leading deboronation via hydrolysis/oxidation yielding boric acid (H_3BO_3). For example, degradation of bortezomib, known as Velcade™ the first boronic acid-containing drug on the market and approved by the US Food and Drug Administration, under acidic and basic conditions seems to be mediated by an initial oxidation producing boric acid and having a plasma half-life of 9–15 h in humans. The end product, boric acid, is not considered especially toxic to humans. For this, it is not expected that boronic acids possess intrinsic toxicity.

The success of the L-BPA in preliminary studies in patients with GBM, followed by PET studies with 1-aminocyclobutane-1-[^{11}C]-carboxylic acid, revealing that cyclic amino acids were located preferentially in this tumor [57], led to the development of series of these cycloalkane-boronic acids (**51**–**54**; **Figure 13**) [70, 71]. They are water-soluble, cross the BBB, and are not metabolized by tumor cells, and while all accumulate into tumor similarly, the *cis*-isomers (**51** and **53**) biodistributed four times higher in tumor than in blood that of the *trans*-ones (**52** and **54**) and that of L-BPA-F [72–74]. By secondary ion mass spectrometry, it was proven that nearly 70% of the boron pool from derivative **51** was in the nucleus and cytoplasm of T98G GBM cells [75]. Until now there have been no studies of BNCT with these compounds, and further studies should be done prior to clinical trials.

As it is mentioned above, polyamines are essential for differentiation and growth of mammalian cell, and their depletion has growth inhibitory effects on tumors. Furthermore, a polyamine-facilitated transport system is present and allows the uptake by malignant cells [76].

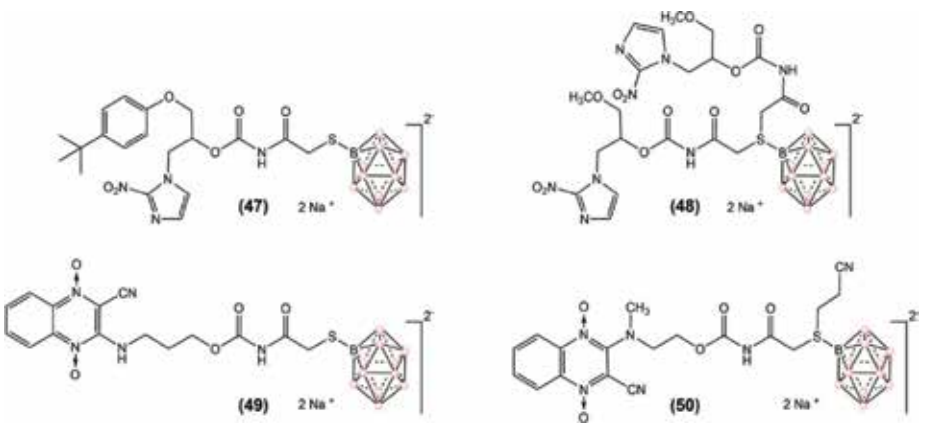

Figure 12. BSH hybrids with potential use in hypoxic glioma.

For that, boronic acid-containing polyamines have been also studied as potential BNCT agents [77]. Compound **55** (**Figure 13**) with higher hydrophilicity that of carboranyls **32** and **33** (**Figure 9**) was designed in order to minimize the carboranyl's nonspecific binding to biolipids that limits the tumor specificity. This boronic acid derivative was able to bind to calf thymus DNA and rapidly was taken up in vitro by F98 rat glioma cells, but the in vivo biodistribution showed unmeasurable levels of boron in tumor, and for this reason, the authors have considered that derivative **55** is not appropriate for BNCT [55].

Thinking about DNA as target to metabolically incorporate boron atoms to the tumoral cell, boronic acid-containing nucleic acids have been studied. One of the first described compounds was the uracil derivative **56** (**Figure 13**) [78] which demonstrated unfavorable tumor:blood and brain:blood biodistribution ratios being in all studied times in favor to blood. After that some boronic acid-containing nucleosides, such as **57** (**Figure 13**), were prepared and studied as potential agents for BNCT; however, no studies with glioma were performed [79]. However, recently nucleoside **58**, a boronic cyclic ester (a dioxaborolane derivative; **Figure 13**), was prepared and evaluated as cytotoxic agent against some tumoral cells [80, 81] including U-118 MG glioblastoma cells [82]. Additionally, the ability of ester **58** to be incorporated into cellular DNA and its selectivity for tumoral cells become its potential usefulness tool in glioma BNCT.

The combination of PDT and BNCT has been also proposed using boronic acid-containing porphyrins, i.e., **59** and **60** (**Figure 14**) [83]. However, it still has not been conducted studies with these compounds in gliomas.

As previously stated the particular hypoxic condition of some GBM regions was used as strategy to produce boronic acid derivatives that could be selectively accumulated in this tissue. For example, the well-known 2-nitroimidazolyl hypoxia pharmacophore was employed as structural guide to boron-enriched tumoral cells generating the boronic acid ester **61** (**Figure 14**) [84]. This dioxaborolane was selectively accumulated in D54 glioma when it was injected intratumorally in a xenograft mouse model reaching 9.5 times the levels of boron into the tumor of L-BPA in the same conditions. However, the pharmacokinetic behavior of derivative **61** should be solved in order to improve the use of this agent in BNCT.

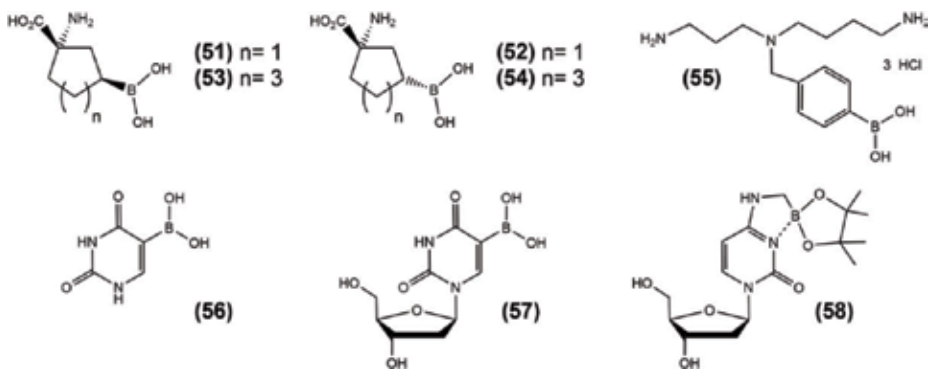

Figure 13. Some boronic acid derivatives developed as potential BNCT agents. Amino acids **51–54** were studied as a mixture of enantiomers.

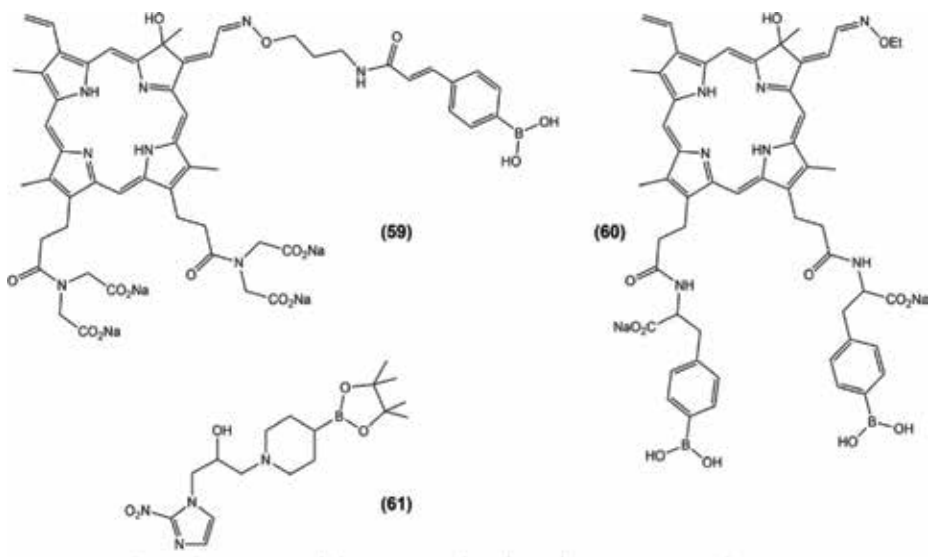

Figure 14. Some boronic acid derivatives developed as two-target drugs.

2.4. Miscellaneous compounds

Other boron-containing moieties have been described for BNCT, for example, in compounds **62–68** (**Figure 15**) [85–90]. However, in spite of their attractive features as pharmacophores, they have not been applied for glioma treatment. Additionally, they contribute with low number of boron atom per molecule which, as mentioned above, which is a disadvantage respect to boron clusters. However, this could be overcome with a high selective accumulation into the tumor cells. For example, the betaine **68**, a boron-containing dipeptide analogue, showed selective accumulation in rat C6 gliosarcomas with ^{10}B tumor:blood and tumor:normal brain ratios of 8.9 and 3.0, respectively [91].

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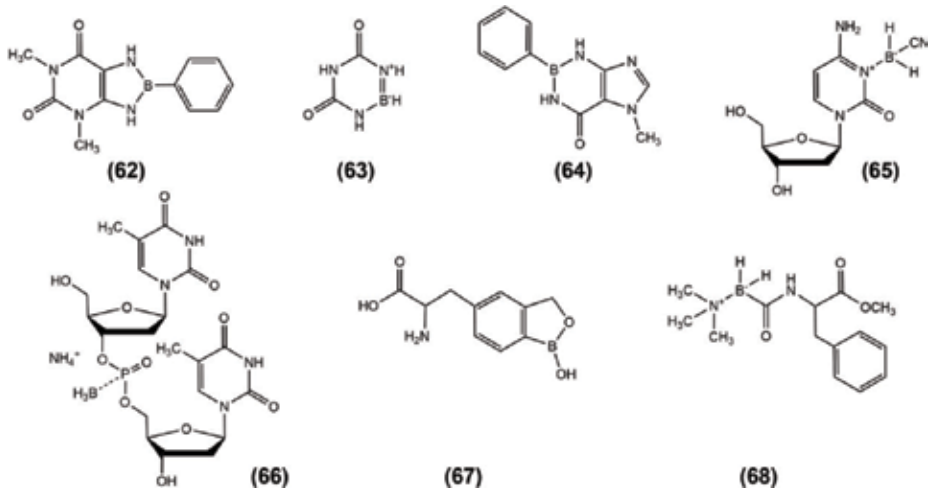

Figure 15. Some other potential boron pharmacophores for glioma BNCT, i.e., boradiazoles, aza-borauracils, 2-boradihydropurines, cyanoboranes, boranophosphates, benzoxaboroles, and trialkylamine-carboxyboranes.

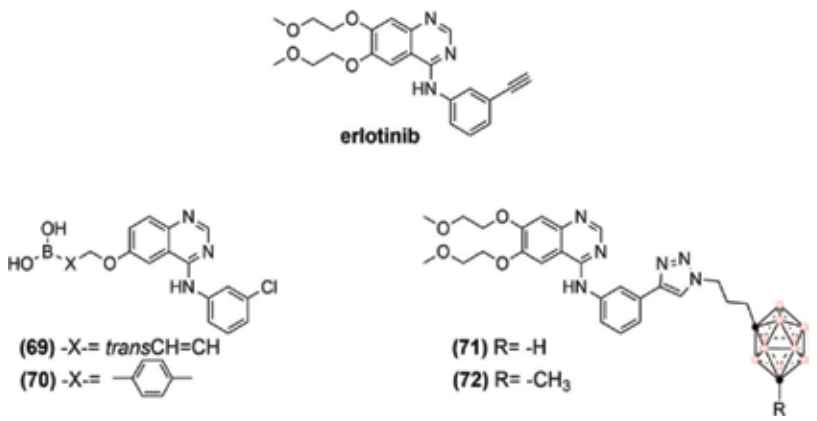

Figure 16. Structure of the well-known PTK inhibitor erlotinib and boron-containing compounds derived from 4-anilinoquinazolinyl pharmacophore.

2.5. Other approaches: site-specific delivery

Beyond the strategies of therapy combination mentioned above, such as phototherapy or hypoxia, another hypothesis has been choosing a bio-system overexpressed in tumoral cells, but not in healthy cells, as a way to selectively accumulate boron drugs in the desired tissue to further BNCT. In this sense, Nakamura and colleagues have described the use of protein tyrosine kinases (PTKs), that its uncontrolled activation is often associated with uncontrolled cell growth and tumor progression, to generate boronic acid and esters hybridized with PTK pharmacophores as new drugs (i.e., **69** and **70**; **Figure 16**) [92]. However, they did not bear

in mind the glioma treatment nor BNCT with this strategy. Nevertheless, we considered the GBM-PTKs [93] as the target to accumulate a boron-enriched drug to further BNCT procedure. Thereby, we hybridized the 4-anilinoquinazolinyl PTKs and the carboranes as boron delivery pharmacophores, i.e., **71** and **72** (**Figure 16**) [94]. Especially, hybrid **71** demonstrated 3.3 times higher activity against C6 glioma cells than the parent drug erlotinib (**Figure 16**), lower cytotoxic effects on normal glia cells, excellent PTK inhibition, capability to accumulate in glioma cells, ability to cross BBB, and stability on simulated biological conditions [94, 95].

3. Conclusion

The development of boron-bearing compound for BNCT has been a vast field of research within medicinal chemistry. New and interesting pharmacophores have been described in order to fulfill the BNCT requirements. Despite this, very few reached the stage of clinical studies, being currently L-BPA and BSH, the only two potential therapeutic agents. However, due to the multidisciplinary approach of the BNCT and the emerging novel structures, we expect with optimism the new developments in the years to come.

Acknowledgements

The authors are SNI-ANII researchers and PEDECIBA members.

Conflict of interest

The authors declare no conflict of interest.

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Chapter 11

Antioxidant Supplementation during Glioma Therapy: Friend or Foe?

Duygu Harmanci

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.77079

Abstract

Gliomas which are one of the most common types of primary brain tumors are originated from glial cells. Type of tumor and tumor location are the most important factors to determine the treatment options. The treatment options might be surgery, radiation therapy, chemotherapy, targeted therapies, and experimental clinical studies. Especially, in course of chemotherapy and radiotherapy, antioxidant levels decrease. Antioxidants fight against the oxidants' negative effects, which include cell damage, oxidative stress, and so on. Recent years, some researchers present that the antioxidant using could be harmful in some cases. A growing body of evidence suggests that antioxidant supplementation might increase the mortality. In this chapter, an overview of antioxidants and their functions has been presented to introduce researchers to the changes and effects of the antioxidants in glioma treatment. The evidence-based studies have been summarized. These experimental studies are important to understand the right option for the patient and transfer the solution from bench to bed.

Keywords: glioma, treatment, antioxidant, oxidative stress, experimental studies

1. Introduction

Central nervous system tumors start in the brain or spinal cord [1]. The common symptoms of these tumors are headache, seizures, weakness, nausea, vomiting, and altered mental status [1, 2]. Gliomas are one of the most common primary brain tumors, which are originated from glial cells. In general, gliomas are classified astrocytoma, oligodendrogliomas, and ependymomas. According to World Health Organization (WHO), the histological classification of gliomas consists of astrocytoma, oligodendroglioma, oligoastrocytoma (low-grade gliomas)

and anaplastic astrocytoma, anaplastic oligodendroglioma, anaplastic oligoastrocytoma, anaplastic ependymoma, and glioblastoma (high-grade gliomas) [1–4]. The histological type of the tumor is the most significant thing to determine the treatment option [2].

Antioxidants are present in plant-based foods, for instance, some types of vegetables and fruits: wine, blueberry, different types of tea, grape, and so on [5, 6]. The main role of antioxidants is prevention of oxidants' harmful effects to human body. Principle regarding oxidant-antioxidant is related to a balance [7]. This balance's side determines the human body reaction. In case of elevated oxidant levels in organism body homeostasis is lost and oxidative stress occurs. Loss of this balance and oxidative stress lead some pathological situations: cancer, neurodegenerative diseases, cardiovascular diseases, immunological diseases, and so on [8–10]. In terms of these diseases with the antioxidant supplementation, cell damage can be fixed.

In this chapter, two different stories will be told and these stories will be turned one story. We will discuss some of the basic concepts of antioxidants, antioxidant systems and antioxidants supplementation and explain how antioxidant supplementation can help with the cancer therapy, especially glioma therapy. Experimental studies are summarized and present evidences are collected under three headings: in vitro studies, animal studies, and clinical trials.

2. What is antioxidant?

To understand the term antioxidant, we have to tell the story from the beginning. The story begins with oxygen. Oxygen is the main source of the life, but in the body oxygen sometimes acts like a foe. Oxygen has two unpaired electrons, which spin in the same direction [9]. For this reason, oxygen is a biradical, so it is a free radical. In general, free radicals are highly reactive compounds, which are called as "reactive oxygen species" (ROS). ROS are intracellular compounds, which consist of oxygen [7, 11]. The most known ROS are listed in **Table 1**.

Oxygen is less dangerous than oxygen-derived free radical species (superoxide, hydroxyl radicals, hydrogen peroxide, etc.), and they react with lipids, proteins, and nucleic acids [12, 13]. Besides ROS, nitrogen-derived molecules are present in human body. These are known as

Name	Molecule formula
Superoxide	O_2^-
Hydroxyl	$OH^\bullet$
Peroxyl	$ROO^\bullet$
Alcoxyl	$RO^\bullet$
Hydroperoxyl	$HO_2^\bullet$
Lipid peroxyl	$LOO^\bullet$
Hydrogen peroxide	H_2O_2

Table 1. Reactive oxygen species (ROS).

Endogenous sources	Exogenous sources
Normal cellular metabolism	UV
• Electron transport chain	Ozone exposure
• Neutrophils, macrophages	Hyperoxia
• Mitochondrial cytochrome oxidase	Burning organic foods
• Smooth muscle cells	Smoking
• Cortisol, catecholamine	Ionizing radiation
• Immune system cells	Air pollutants
	Heavy metal ions

Table 2. Endogenous and exogenous sources of ROS.

reactive-nitrogen species [6, 14]. These molecules can get involved with oxidant molecules, but all oxidants are not free radicals. They produce endogenously or with some exogenous sources' effects [9, 10]. Some endogenous and exogenous sources are shown in **Table 2**.

In human body, antioxidant systems are present to avoid cell damage due to free radicals. These antioxidant systems include a few enzymes for this reason they are called enzymatic antioxidants [9–11, 14]. The definition of antioxidant that it is a molecule reacts with free radicals and neutralizes them [6]. Except enzymatic antioxidants, generally, they occur naturally in foods, especially plant-based foods [15]. For instance, resveratrol is a very popular antioxidant in recent years, and it is found in grape, raspberry, blueberry, wine, and so on [16]. The most known non-enzymatic antioxidants are low-molecular-weight compounds such as vitamin C, vitamin E, beta-carotene, catechins, lycopene, glutathione, and coenzyme Q [5, 12, 17].

In summary, the story starts with oxygen and develops free radicals and stable molecules (DNA, protein, lipids, carbohydrates, etc.). Antioxidants are the good cops and they get involved the free radicals. In normal conditions, this is acceptable as happy ending. In terms of biological perspective, in course of normal metabolism energy production starts with consumption of oxygen and food nutrients. Oxygen and food enter the cell and mitochondria start to produce adenosine triphosphate (ATP). Free radicals form during cell's energy production. These free radicals are neutralized by antioxidant enzyme systems (superoxide dismutase, catalase, glutathione peroxidase, etc.) and non-enzymatic antioxidants [6]. In the presence of any pathological conditions, ROS are highly produced and although antioxidant enzyme systems and antioxidants try to eliminate them to protect the cell, they remain incapable. Redox balance breaks down, oxidative stress increases, and antioxidant levels decrease [14]. In terms of cancer, ROS imbalance is one of the hallmarks of cancer [18].

3. Antioxidants and cancer

Cancer is a malign disease, which is characterized by abnormal cell proliferation [19, 20]. The uncontrolled situation in the cell is a result of endogenous or exogenous effects. According to multistep carcinogenesis theory, cancer originated from one cell, so cancer is a monoclonal

disease and it develops in three stages. These stages are initiation, promotion, and progression [21]. Cells suffer damage with any endogenous or exogenous effects. Defects or mutations accumulate in the cell with these effects. The main effects are listed below [22]:

- Environmental factors,
- Lifestyle,
- Infections,
- Mutations,
- Inherited genetic diseases,
- Viruses
- Reactive oxygen species (ROS).

Aforementioned before ROS cause some pathological situations due to their reactive features [10]. They react with nucleic acids, proteins, lipids, and carbohydrates. As a result of this interaction, it is possible that cancer development may be from one cell. ROS may take a role any stages of carcinogenesis [7]. Proven roles of ROS on cancer progression [23]:

- Some genetic alterations are generated by ROS.
- ROS promote cell migration via invadopodia formation in vitro.
- ROS activate the PI3K/AKT/mTOR and MAPK/ERK mitogenic signaling pathways.

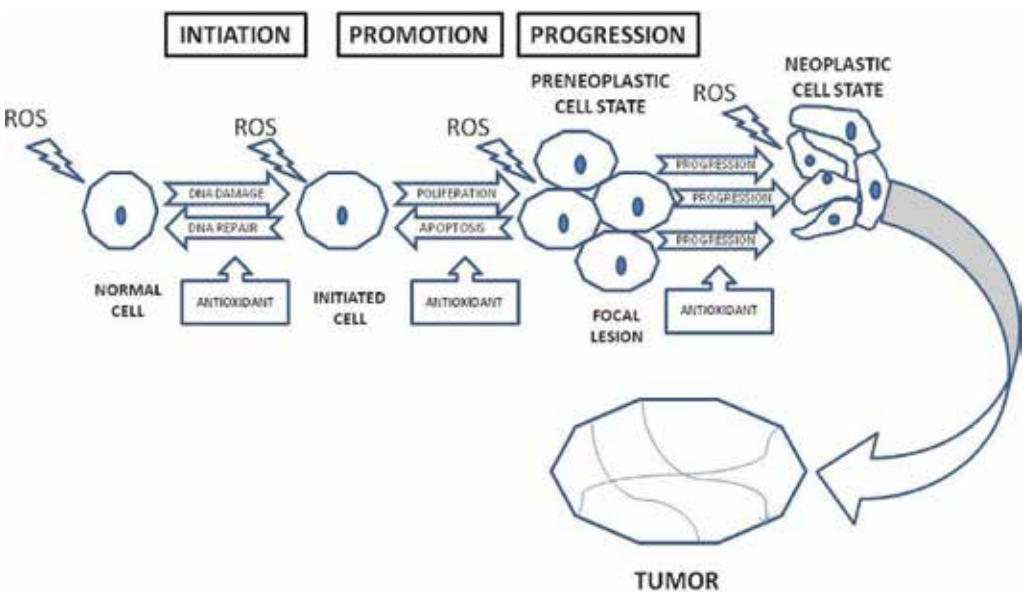

Figure 1. ROS-cancer relationship and antioxidant junction points.

The main objectives of oncological treatment are increasing life-quality and extending survival time. When these objectives are considered, antioxidant supplementation brings to mind some questions. If clinicians add antioxidants to therapy:

- Does the success of therapy increase or decrease?
- Are some of side-effects related to current therapy eliminated by antioxidants?
- Does antioxidant supplementation affect survival rates?

In accordance with these questions, lots of experimental and clinical studies were carried out to prove the role of antioxidants in cancer therapy [16, 24–30]. Results obtained from these studies were variable. This variation is basically related to cancer type and cancer grade. ROS-cancer relationship and antioxidant junction points are described **Figure 1**.

An antioxidant-cancer relationship is deeply discussed next part in terms of glioma.

4. Antioxidants and gliomas relationship

Gliomas are a class of primary central nervous system tumors and they originated from glial cells [1]. Glial progenitor cells have different subtypes: astrocyte, oligodendrocyte, and ependyma. In general, the classification of gliomas is based on these cell types [4]. The most detailed classification belongs to WHO. WHO suggests that four different grades (I–II–III–IV) are described for gliomas according to morphological and histological features [1]. Besides these features, some molecular and genetic features (epidermal growth factor upregulation, isocitrate dehydrogenase 1/2 mutations, p53 mutations, etc.) also alter the grading [2].

Tumor grade and class are major factors to determine the therapy options. Surgery, chemotherapy, and radiotherapy are preferred to treat the gliomas. After surgery, chemotherapy or radiotherapy is applied. For the glioma treatment, the most frequently encountered problems are the blood-brain barrier and drug resistance [31]. The blood-brain barrier is a control mechanism in relation to the transition of ions, molecules, and cells between the blood and brain. If a drug does not pass through the blood-brain barrier, it cannot reach the brain cells [32].

The second problem is drug resistance [33]. Temozolomide is the most common chemotherapeutic agent for gliomas. It is an alkylating agent [34]. In case of elevated levels of O^6-metil guanine DNA methyltransferase expression, temozolomide meets with resistance [35]. On the other hand, increased levels of antioxidant response system SLC7A11 triggered the drug resistance [31].

Over the past decades, antioxidant supplementation becomes a necessity for cancer treatment. Basically, antioxidants use to eliminate the elevated levels of ROS, but cancer in question nothing is understandable. For this reason, researchers have carried out some studies. Understanding the beneficial or harmful roles of antioxidants in cancer treatment is essential. Further to that understanding of ROS effects in terms of cancer progression is really important. ROS is a reason

for cancer progression, but in course of cancer development increased levels of ROS might be a cell-death option. Moreover, increased levels of ROS alter the cell signaling in cancer cell in consequence of acting as secondary messengers [17]. For instance, Akt overexpression is frequently showed in gliomas, and protein kinase C (PKC) activation stimulates some molecules like Akt, MAPK. All these molecules are under the control by cellular redox state [36]. As a result of these features, ROS antioxidants can be provided new approaches in order to treat glioma. It is still an unknown and questionable area for the researchers.

Accumulating data suggest two different approaches regarding antioxidant consumption. One is that antioxidants make tumor cells resistance against chemotherapy or radiotherapy and the survival rates are decreased. On the other hand, the second is that antioxidants protect the normal cells from oxidative damage and they are decreased side effects of therapy and provide better survival [8, 37, 38]. The next part of this chapter is related to evidence regarding these two opinions.

4.1. Evidence-based studies

Gliomagenesis is still an unknown, incurable, and lethal process. New and effective treatment strategies are the necessity and understanding the gliomagenesis is essential in order to develop these options. Experimental evidence indicates that antioxidants are sometimes friend, and in some cases, they are the foe.

4.1.1. In vitro studies

In 1995, Zhu et al. carried out a study to clarify the effects of selenium on rat and human glioblastoma multiforme cell lines. They used sodium selenite and showed that selenium had anti-proliferative effects on both A172 human glioblastoma cells and C6 rat glioblastoma cells, but it was more effective on human glioblastoma cells [39].

In 1997, Vartak et al. showed that some polyunsaturated fatty acids: gamma-linoleic acid (GLA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) supplementations increased the radiosensitivity and also radiation response on 36B10 rat astrocytoma cells [40].

In 1998, Vartak et al. compared the effects of GLA and linoleic acid (LA) on 36B10 malignant rat astrocytoma and normal rat astrocytes. They found that GLA was cytotoxic for astrocytoma cells, but not astrocytes. LA was not effective for both cells. It suggested that GLA might be used for astrocytoma treatment [41].

In 1999, Arora-Kuruganti et al. examined roles of oxidant (H_2O_2) and antioxidant (N-acetylcysteine, NAC) on U373-MG astrocytoma cell line. They observed that tumor cell proliferation was inhibited by NAC. NAC also induced H_2O_2 [25].

In the beginning 2000s, the first study came from Rooprai et al. They checked some anti-invasive and anti-proliferative agents: swainsonine, captopril, tangeretin, and nobiletin on four different glioma cell lines: ependymoma, oligoastrocytoma, anaplastic astrocytoma, and glioblastoma multiforme. Firstly, they observed that each cell line showed difference response

against agents. They found that the most effective agent was nobiletin on four different cell lines and it was decreased the MMP-2 and -9 secretions [42].

In 2001, Naidu et al. studied the effects of ascorbyl stearate (Asc-S) on human glioblastoma multiforme cells. They used different doses of Asc-S on T98G human glioblastoma cell lines for 24 h. They showed that Asc-S inhibited insulin-like growth factor-dependent cell proliferation in a dose-dependent manner. Asc-S modulated IGF-R expression, in consequence of this situation programmed cell death was triggered on T98G cells by Asc-S [43].

In 2007, Rooprai et al. studied on IPSB-18 human astrocytoma cells. They treated cells with selenite and found that selenite was altered the expressions of matrix metalloproteinases and their inhibitors. It was also decreased the epidermal growth factor (EGFR) expression. This was suggested that selenite had anti-metastatic effects [44].

In 2013, Pozsgai et al. studied on quercetin effects on glioblastoma standard treatment. They found that combination treatment provided significant reduction in cell viability in U251 and DBTRG-05MG glioblastoma multiforme cell lines. They also showed that quercetin alone, or in a combination with IR triggered the apoptosis [29]. In 2016, Lou et al. found that quercetin nanoparticles stimulated the autophagy and apoptosis by activating AKT/erk/caspase 3 signaling pathway [45].

In 2017, increasing cell proliferation of glioblastoma multiforme cell lines with low doses of selenomethionine was showed by Harmanci et al. [46].

The combination of berbamine and paclitaxel were decreased the cell proliferation on U87 glioblastoma multiforme cells [47].

Higher levels of ascorbate led the DNA strand breakages by creating genotoxic and metabolic stress on glioma cells, but it also caused the development of radioresistance [48].

4.1.2. Animal studies

In 1981, Newell et al. used a mixture of vitamins C and B12 in high dose on rats with glioma. They observed no difference in survival time between experimental and control groups [49].

In 1989, Wang et al. showed that retinoids (retinal, retinoic acid, retinyl acetate, and retinyl palmitate) and carotenoids (beta-carotene, lycopene, and crocetin) inhibited the tumor growth in C6 glioma cells inoculated rats [50].

A study regarding naringenin using was carried out on rats by Sabarinathan et al. [30]. With supplementation of naringenin in glioma induced rats the status of lipid peroxidation was decreased, on the contrary antioxidant status increased. Besides this, naringenin also modulate the glial-tumor cell proliferation [30].

In 2013, Perez de la Ossa et al. examined that Δ^9-tetrahidrocannabinol (THC) and cannabidiol (CBD) effects on tumor growth in xenograft glioblastoma multiforme model. THC and CBD loaded on microparticles and delivered locally. At the end of the study they found that THC and CBD stimulated apoptosis and induced cell proliferation and angiogenesis [51].

In 2013, Hervouet et al. found that using SUVIMAX-like diet (supplementation en vitamins et minéraux antioxydants), which was enriched with beta carotene, alpha tocopherol, vitamin C, zinc, and sodium selenite, was delayed the clinical signs on ethyl-nitrosourea induced glioma rat model, but gliomagenesis occurred. This diet just decreased the tumor aggressiveness [52].

In 2017, prolonged survival time was showed treatment with coptis chinensis on glioma induced mice model by Li et al. [53].

Combination of berbamine and paxitaxel was delayed the development of tumor U87 xenograft model [47].

4.1.3. Clinical trials

In 1990, Philipov et al. carried out a limited clinical study with 15 patients with malignant brain tumors. There was no significant survival prolongation with selenium addition on patients' diet [28].

In 1996, Lissoni et al. evaluated the effects of melatonin using with radiotherapy on 30 patients with glioblastoma multiforme. They showed that the melatonin addition in normal therapy provided prolonged survival time, decreased side-effects. Based on these results they suggested that concomitant therapy may be more effective for glioblastoma patients [27].

In 2010, Delorenze et al. exhibited that the relationship between daily intake of antioxidants and survival rate was variable depends on tumor grade. They also showed that the supplementation of higher dose vitamin E has increased the survival in grade III gliomas; otherwise, vitamin C and genistein were decreased the survival rate [26].

In 2010, the side-effects welding from radiotherapy were decreased with lycopene supplementation in patients with high-grade glioma [54].

In 2015, Mulpur et al. carried out a study to check complementary therapy options among glioblastoma multiforme patients. They found that multivitamins or omega-3-fatty acids did not affect survival, but for Vitamin D and E further investigations are necessity [55].

5. Conclusions

Cancer is a personal disease, for this reason, it needs special attention. The above-mentioned evidences have shown that antioxidant supplementation cannot safe at times. Researchers advocate two different opinions regarding using antioxidant in course of cancer treatment. The first opinion is traditional approach. It says antioxidants prevent the normal cell from oxidative damage and they induce toxicity and provide better survival rates. The second one shows the dark side of antioxidants. According to the second opinion: antioxidants are decreased the survival rates by triggering drug-resistance. When we consider these two opinions we can easily understand the requirement of this area.

The most urgent thing is clinical trials with larger sample size and long-term following. Evidence obtained from in vitro or in vivo studies cannot representative for the 3D organism. The effects

of antioxidant supplementation can determine appropriate clinical trials. Antioxidants' interference in chemotherapeutic mechanisms is still unknown and clinical fails of therapeutic approaches regarding redox modulation are obvious.

In summary, antioxidant cancer therapy remains incapable. ROS scavengers must give place to antioxidant inhibitors. ROS-related cell death mechanism is a novel approach to provide the selective cell death. Further investigations will need to see the effectiveness of pro-oxidant cancer therapy.

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Edited by Ibrahim Omerhodžić and Kenan Arnautović

The past three decades have been marked with huge enthusiasm from scientists and professionals in an effort to find a cure for glioma disease.

Methods to confirm the kinds and grades of glioma have taken a path from classical macro- to microscopic pathohystological confirmation of tumors, through morphological–histological, molecular, and genetic diagnosis.

Surgically, progress was made possible with the development and use of technological aids, for example neuronavigation, cortical mapping, electrocorticography, neuromonitoring, functional and intraoperative MRI, magnetoencephalography, etc. Great hope was placed on the extension of tumor resection and popular supratotal resection.

Significant progress has been made generally in glioma treatment with the use of modern radiotherapy and new chemotherapeutics.

What do we want to see for the future? By way of stem cells, a specific medicine will be produced, individualized for the particular patient, and by using a microcapsule it will be implanted into the brain zone affected by the tumor by way of robot surgery and injection needle. This is not at all an unrealistic expectation in the next decade or two.

Published in London, UK

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ISBN 978-1-83881-655-1