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Glioma diagnostics and biomarkers: an ongoing challenge in the field of medicine and science

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Abstract

Glioma is the most common brain tumor. For the more aggressive form, glioblastoma, standard treatment includes surgical resection, irradiation with adjuvant temozolomide and, on recurrence, experimental chemotherapy. However, the survival of patients remains poor. There is a critical need for minimally invasive biomarkers for diagnosis and as measures of response to therapeutic interventions. Glioma shed extracellular vesicles (EVs), which invade the surrounding tissue and circulate within both the cerebrospinal fluid and the systemic circulation. These tumor-derived EVs and their content serve as an attractive source of biomarkers. In this review, we discuss the current state of the art of biomarkers for glioma with emphasis on their EV derivation.

Keywords

Cancer; CNS; glioma; exosomes; microvesicles; extracellular vesicles; biomarker; IDH1/2; EGFRvIII

1. Introduction

The consensus World Health Organization (WHO) classification of tumors of glial cells provides grading from lower to higher levels of aggression [astrocytoma (WHO grade II), anaplastic astrocytoma (WHO grade III), GBM (WHO grade IV)] with similar standards for those of oligodendrocytic origin [oligodendrogliomas (WHO grade II) and anaplastic oligodendroglioma (WHO grade III)]. GBM, the most aggressive, is characterized by cells

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with nuclear atypia and high mitotic rates with contiguous areas of new vessel formation and necrosis. The incidence of GBM is about 3.5 per 100.000 people per year with a mean overall survival of 1.5 years [1]. Patients with a less aggressive glioma have longer survival with concomitant morbidity but cure is uncommon. Neuro-oncologists and neurosurgeons understand primary glial tumors. However, their experience is not available to the molecular biologist, the developer of companion diagnostics, the reference pathologist or the healthcare administrator. Therefore, the aim of the present review is to offer a clinical perspective of glioma for the non-clinician.

The neuropathologic definition of “glioma” based on light microscopic morphology serves as a starting point. However, our review includes changes in classification that are based upon molecular studies of glioma-specific gene mutations and amplifications. Moreover, Extracellular vesicles (EVs), including exosomes, microvesicles, and oncosomes have entered the field of diagnosis. EVs are shed by a variety of cells into biofluids or surrounding tissues as lipid membrane structures with diameter 30–1000 nm [2]. Here, we review in particular the potential of EVs as diagnostic and prognostic biomarkers for glioma.

1.1. Challenges in Glioma Treatment. Epidemiology, Molecular Pathology and Survival

Amongst the 70,000 CNS tumors seen yearly in the United States, there appear those primary in brain and those metastatic to brain. All gliomas afflict 20.59 of 100,000 Americans [3] and represent 80% of tumors starting in the brain. Malignant glioma represent approximately one-third of all brain tumors; with glioblastoma (GBM) more common in males, Caucasians and individuals after the 5^{th} decade (Fig.1). Childhood glioma account for one-quarter of pediatric tumors; second only to leukemia. Risk factors for gliomas include inherited neuro-cutaneous disorders (neurofibromatosis and tuberous sclerosis, basal cell nevus [Gorlin] syndromes); likely familial predisposition [4] and radiation exposure [5]. Malignant glioma in animals has been associated with JC papovapolyomavirus mutation of tumor suppressor genes [6]. Cytomegalovirus (CMV) genomic material has been found in GBM [7,8] and a recent study has proposed that CMV induces glioma by inhibition of tumor suppressor genes [9]. Thus, CMV genomic material has been used as the basis for anti-tumor immunization [10]. GBM are diffusely infiltrative of brain and grow in a microenvironment that favors angiogenesis and peritumoral growth of surrounding glial cells with suppression of the immune response [11–13]. There are 2 forms of GBM, primary and secondary GBM. Primary GBM, developing *de novo*, progress to death usually within less than 2 years. Secondary GBMs, evolving from tumors of lower grades in younger patients, progress over a number of years Although histologically indistinguishable from primary GBMs, these harbor different molecular alterations [14].

As glial tumors are analyzed at increasing molecular resolution, their mutational heterogeneity is becoming clarified, even within the same histo-pathological subtype [15]. Molecular analysis of GBM has revealed alterations in signaling pathways for cellular proliferation, apoptosis, senescence, migration, and cell-to-cell communications. The Cancer Genome Atlas (TCGA) project has identified 3 critical pathways to be affected in most GBM including receptor tyrosine kinase/Ras/phosphatidylinositol-3 kinase, p53, and retinoblastoma signaling [16]. Several molecular subclassifications have been proposed for

GBM such as (TCGA) division into proneural, neural, classical and mesenchymal subtypes based on expression of genes related to epidermal growth factor receptor (EGFR), neurofibromatosis type 1/2 (NF1), platelet-derived growth factor receptor (PDGFR), and isocitrate dehydrogenase 1/2 (IDH1) [16]. Noushmehrin et al [17] further subdivided the proneural subtype based on the expression of IDH1 mutation-associated CPG island methylator phenotype (G-CIMP) [17] occurring in younger patients showing prolonged survival. Multiple alterations in gene and protein expression patterns may be present in a single tumor and may change with time and treatment [15]. Amplification of wild type EGFR is the most common oncogenic alteration in GBM and is highly associated with the classical subtype [16]. Up to 50% of EGFR-amplified tumors also contain a unique EGFR mutation variant (EGFRvIII) resulting in ligandin-dependent constitutive activation of the EGFR pathway [18].

1.2 The Financial Consequence of Providing Care for Glioma Patients

Adult glioma is the most costly cancers in terms of treatment expense as well as the cost to society. Financial costs include those for provision of surgery, radiation therapy, chemotherapeutics, multiple hospitalizations, and supportive medications for brain edema and seizures. A cost utilization study of patients with malignant primary brain tumors in the United States from 1998–2000 (before the introduction of temozolomide) found that the total costs per patient were upwards of $50,000, the majority of which was driven by inpatient hospitalization and surgical fees [19]. To these costs must be added lost productivity, brain tumor-associated thromboembolic complications, seizure activity, infections and corticosteroid-induced muscle weakness, osteoporosis, obesity and diabetes.

2. The Current Care for Glioma

Here, we review diagnostic biomarkers of glioma including those expressed in EVs. We provide definitions of the subtypes of both low grade and high grade glioma. The nonclinician will benefit from an overview of the care of these patients, the paradigm of their care and their clinical outcome.

Patients afflicted by seizures, weakness of the extremities or changes in behavior are provided a neurologic examination to achieve a malignant glioma diagnosis within 3 months [20] rising up to 6 months for those with slowly evolving low grade tumors. As of 2007, there were almost 8000 MRI and over 10,000 CT scanning units in the United States; numbers exceeding the per capita availability of any country [21]. The U.S. MR scans costing $1200 (range, $500 to $3000) are commonly obtained by a general practitioner or by self-referral. MRI depicts and delineates intracerebral tumors, provides for surgical planning, and provides a metric of treatment response in longitudinal followup. GBM appears as a heterogeneous masses of low T1-signal intensity and high T2- signal intensity, with internal cysts and foci of blood products. Gadolinium contrast highlights an intense enhancement pattern, which often encompasses the non-enhancing necrotic central regions of the tumor. Unfortunately, MRI sensitivity for detection of masses is less than 90% and is limited further for the subtypes of glioma [22]. Low grade glioma, lymphoma, metastases, abscesses, and subacute infarcts may mimic the radiographic appearance of GBM. Even

advanced MRI including spectroscopy and perfusion, provides diagnostic specificity ranging from 50 – 80% for distinction of GBM from the above mentioned conditions [23]. Spectroscopic MRI provides sensitivity for tumor diagnosis of 79% and specificity of 77%. The addition of MR perfusion imaging or diffusion studies increases the sensitivity of brain lesion detection to 81% but does not provide molecular information nor identification of tumor subtype [24]. Indeed over standard MRI, dynamic contrast enhanced MR and perfusion MR improved accuracy, sensitivity, and specificity of glioma grading from 64.9%. 78.6%, and 56.5% to 83.8%, 78.6% and 87.0%, respectively (Fig. 2) [25].

Tumor diagnosis is made upon review of tissue obtained at biopsy or surgical resection. Molecular analyses include gene expression testing, DNA copy number, methylation profile, phospho-protein pathway profiling, genetic sequencing, and definition within the Cancer Genome Atlas [26]. Maximal safe resection, the treatment of choice for patients with GBM, enhances the effects of adjuvant therapies, reduces symptoms emerging from brain edema and epileptic seizures, and provides specimens for histologic and genomic studies [27]. In a preliminary review of the SEER database (BC) it was found that the surgical desire to obtain “gross-total resection” of tumor within MRI-delineated regions is achieved in fewer than 30% of patients. This goal is not reached for various reasons including difficulty distinguishing tumor cells from normal brain tissue and peri-tumoral reactive elements; the difference in goals and experience between surgeons in practice and in tertiary facilities and the availability of intraoperative MRI scanning. The Glioma Outcome Project reported a peri-operative complication rate of 24% in patients undergoing first craniotomy for glioma resection, with 8% displaying worsened neurologic status [28]. The incidence of peri-operative complications increased with subsequent operations (33% complications, 18% worsened neurologic status after second craniotomy) [28]. For patients with GBM, surgical therapeutic options include resection, implantation of a nitrosourea polymer wafer, the use of fluorescent guidance systems [29–31], irradiation of the tumor during operation via implanted “brachytherapy” isotopes [32,33] or post-operative radiation [34,35]. Approximately 30% of patients with GBM have tumors that permit only diagnostic biopsy. In an unpublished review (Noorbakhsh, in preparation) 22% of patients were found never provided with an operative diagnosis. The remainder receive biopsy or subtotal resection- a reflection of restrictions imposed by age, comorbidities, multi-focal masses, or tumor location. Pathologic diagnoses based upon biopsy carry with them issues of sampling errors due to tumor regional heterogeneity of architecture, vascularity, cellularity, and necrosis. Thus biopsies have limitations for tumor grading and diagnosis of GBM. Of 81 consecutive patient recipients of stereotactic biopsy [36], subsequent resection resulted in a changed diagnosis in 49%, of whom 26% experienced a change in clinical management. Similarly tumor heterogeneity imposes topographic limitations on mutational analyses [15].

Six weeks after surgery, patients are provided adjuvant therapy using temozolomide and fractionated 60 Gy radiation over 42 sessions, followed by 6 additional monthly cycles of temozolomide [37]. This therapy increased 2-years and 5-years survival rates to 27% and 11% from 11% and 2% respectively, with no significant adverse effects on quality of life [34]. The incremental cost of temozolomide is estimated to be $50,000 per life-year gained [38]. Resistance to temozolomide is described as a function of repair of damaged DNA by the enzyme O^6–methylguanine-DNA methyltransferase (MGMT), by poly(ADP-

ribose)polymerase (PARP) in the base excision repair (BER) pathway, or through tolerance of damaged DNA in mismatch repair-deficient cells [39]. However, other molecular alterations may cause resistance such as the MSH6 mutation [40,41]. Currently, methods for detection of temozolomide resistance other than *de facto* tumor progression do not exist. Thus, many patients undergo long and expensive therapies, which do not provide any benefit for their particular tumor. Biomarkers may provide a measure of response and progression.

At the present time, tumor response to therapy, whether reduction, recurrence or progression, is evaluated by longitudinal serial MRI, an approach with inherent limitations. Recurrence or tumor progression is measured by volumetric or cross-sectional changes in the tumor's T1 enhancement [42]. Treatment of recurrent GBM involves re-resection, [43], hypofractionated re-irradiation and anti-angiogenesis therapy with the use of bevacizumab and/or irinotecan [38, 44]. These therapies increase 6-month survival rates with attendant complications [38] and without curative intent. Salvage chemotherapy with experimental agents do not further improve survival and are provided late in the life of patients now afflicted with morbidities resulting from prior therapies. However, the MRI maximum spatial resolution of millimeters does not provide a measure of tumor cells, only tenths of micrometers in size, which increase in number before radiologic changes are apparent. Concomitant anti-angiogenic therapies make problematic the use of MRI for tumor monitoring [45]. The reduction of vascular permeability resulting from anti-angiogenic drugs reduces passage out of vessels of MRI contrast agents. As a result there is reduction of tumor contrast enhancement, even in the setting of paradoxical tumor growth [46]. Further limiting MRI specificity is the inability to distinguish radiation necrosis (‘pseudo-progression’) from progressive viable glioma [47]. These considerations stimulate the search for biomarkers of diagnosis and as metrics of therapeutic response.

3. EVs as Source of Biomarkers

EVs include exosomes, exosome-like vesicles, microvesicles, and oncosomes which are released by all cell types. The content of EVs and their functions vary with the cells of origin. For example, EVs released from tumor cells contain a wide variety of proteins and lipids, RNA and DNA which support tumor growth by altering multiple hallmarks of cancer. Thus EVs may explain features of oncogenesis including genetic instability, tumor growth, alterations of the microenvironment, cellular invasion, migration and metastasis and immune resistance. Please note that (A) the terminology of EVs is in flux and has yet to come to grips with the diagnostic implications of EV structural features and size differences, the effects of preparative techniques and the differing functional roles of EVs; (B) the number of novel biomarkers will, no doubt, increase in the coming years and (C) future correlative studies will validate the diagnostic value of EV biomarkers for subtypes of glial tumors [48]. EVs emerge from the endosomal compartment and are secreted into the extracellular space but can also detach from the plasma membrane of the cell. The preparative approaches for EV isolation include filtration, ultracentrifugation and column separation followed by electron microscopy and NanoSight analysis [2]. EVs have been isolated from multiple body fluids such as plasma and CSF. They participate in intercellular communication and modulate the microenvironment to alter the immune response. For example, EVs modulate expression of MHC class II molecules on the surface of dendritic cells as well as α-amino-3-

hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) receptor on the surface of cortical neurons. EV-derived mRNA transfer to recipient cells is translated into protein with changes in the recipient cell genotype [2]. EVs from microglia are received by neurons with changes in synaptic function as are EVs from oligodendrocytes. Similar interactions exist between platelet-derived EVs and coagulation and by EVs and recipient cells to facilitate platelet adhesion. EVs contribute to cell invasion and migration by modulation of metalloproteases which alter the extracellular matrix. GBM-derived EVs facilitate angiogenesis. For example, EGFRvIII constitutively signals the initiation of a proangiogenic cascade in GBM. The EV cargo of mRNA, miRNA is transferred into GBM cells [49]. EV DNA and protein appears stable over years, and EV RNA is not likely to be degraded by RNAases and can be isolated at high levels from biofluids. The cargo has been extensively evaluated but the relationship between subsets of EVs and cargo-specificity remains obscure. The literature lacks speciation of EVs that carry specific types of RNA, ncRNA of retroviral origin and miRNA. Many of the latter (miRNA 1, 21, 181D) have been linked to GBM; but their specificity is lacking. For example, sera of GBM patients contain higher levels of miR-21 than sera from 'normal' patients. These single miRNAs may be able to target and regulate over 100 genes. Tumor EVs also contain retrotransposon elements, which can be transferred from tumor cells to normal cells [50]. Thus, genetic information encapsulated in specific fractions of tumor-derived EVs found in biofluids may serve as tumor markers.

4. Potential Glioma Biomarker Candidates

4.1.1. Epidermal growth factor receptor variant III mutation (EGFRvIII)

EGFRvIII mutation is highly specific for GBM. The exon 2-7 deletion from the EGFR gene is found in 20–25% of GBM cases; but reports of frequency vary. Its presence within a tumor is diagnostic and possibly prognostic, but only in patients that live beyond 2 years and in anaplastic astrocytomas [51,52]. Functionally, the presence of EGFRvIII presence is associated with amplification of EGFR *wt*, which subsequently upregulates the PI3K pathway and promotes abnormal cell proliferation and tumor progression [53].

Skog *et al.* detected EGFRvIII from sera EVs from 30 GBM patients [49]. Fourteen of these patients had the EGFRvIII mutation present in matched tissue samples. Using nested RT-PCR, the EGFRvIII mutation was found in 7 of the 30 sera samples. Interestingly, 2 of 7 serum samples in which EGFRvIII was detected had matched tissue samples that had been negative for this mutation, supporting the idea that serum-based biomarkers may provide more sensitive tests of tumor characteristics than tissue specimens. The underlying heterogeneity of GBM cellular distribution may cause EGFRvIII to be undetectable in a small piece of tissue, but a serum sample presumably receives EVs from the entire tumor. After resection, GBM patient serum samples no longer contained EGFRvIII [49]. Analytic techniques have included immunohistochemistry [6], proteomic techniques and flow cytometry [54]. Analyses have been carried out on human tissue specimens [53,55] and non-human CSF [56] in addition to serum/plasma. Ongoing is a multi-center trial to validate the diagnostic utility of EV EGFrvIII mRNA in CSF and plasma. EGFR is the target of first generation inhibitors such as erlotinib and gefitinib, that have not resulted in survival

benefits [57–59]. Forthcoming is a next generation of EGFR inhibitors, including afatinib, dacomitinib, and nimotuzumab [60].

4.1.2 Isocitrate dehydrogenase 1/2 mutations (IDH1/2)

IDH1/2 are $NADP^+$-dependent dehydrogenases catalyzing the oxidative decarboxylation of isocitrate to α-ketoglutarate. Mutation in these genes alters the enzymatic activity of both IDH1 and IDH2. The mutations are found in 80% of low grade glioma and approximately 10% of GBM, but never in normal brain or bodily tissues [14]. *IDH1/2* mutations are associated with young age, secondary GBM, and a longer overall survival [61]. Furthermore, the point mutation is found in cholangiocarcinoma, certain sarcomas, acute myelogenous leukemia and inborn accumulation of 2-hydroxyglutarate (see below). The $IDH1^{R132H}$ mutation represent 93% of glioma-associated *IDH1* hotspot mutations [61]. The mutation in tissue correlates with survival approaching ten years in patients with low grade tumors and possibly improved response to temozolomide therapy in patients with secondary GBM. This response may reflect a correlation with methylation status in patients bearing this mutation. Analyses to detect the mutated versions of IDH1 have been performed on tissue using a monoclonal antibody for immunohistochemical detection followed by DNA sequencing and in EV-derived RNA from tissue and CSF using the highly-sensitive BEAMing qRT-PCR or digital PCR techniques and from DNA obtained from blood [62]. Peripheral blood samples have also been analyzed, and a murine model has been studied for clarification of disease mechanism [63,64]. Identification of the *IDH1/2* mutations may serve not only as a “partial” diagnostic biomarker, but also a prognostic marker for improved survival in GBM. Large scale studies of *IDH1/2* mutations are underway. The molecular mechanisms governing *IDH1/2* mutations are not well understood. It has been stated that for both *IDH1* and *IDH2* mutations, mutant-mediated 2-hydroxyglutarate production inhibits enzymes involved in epigenetic regulation, collagen synthesis, or cell signaling [65]. Although 2-hydroxyglutarate accumulates in tumor tissue and serum, this accumulation alone does not support diagnostic biomarker utility of 2-hydroxyglutarate in addition to *IDH1/2* mutations. The absence of documented cases in which *IDH1/2* mutations co-exist may suggest that each mutation provides a sufficient independent growth advantage [66]. The discovery of mutated *IDH1* within a body fluid sample would likely be tumor specific and diagnostic of glioma. Potentially, targeted agents under development might then be provided. Uncertain is whether these drugs, suppressive of the mutation, will amplify the expression of IDH1 wild type.

4.1.3. Drosophila capicua homolog (CIC)

CIC mutations are associated with 1p/19q deletion and with *IDH1/2* mutations [67, 68]. The reported overall incidence of CIC mutations in oligodendrogliomas is reported at 46–69% [67,69]. The occurrence of the mutation is rare in astrocytomas (approximately 10%) [68,69]. The impact of CIC mutations on molecular pathways is not well understood. It is known to play a role in embryonal development, downstream of the RAS/MAPK pathway [65, 70]. In addition, sequence analysis in tissue has been performed [67–69].

4.1.4. Far upstream element binding protein 1 (FUBP1)

FUBP1 mutations are associated with 1p/19q deletion and with *IDH1/2* mutations. FUBP1 mutations have been reported in 10–24% of oligodendroglioma [68,69] and in 10% of astrocytomas [69]. The mutated FUBP1 product exhibits loss of function and does not bind to its known target site in the Myc oncogene [65,71], resulting in loss of regulation and aberrant cell growth. Sequence analysis in tissue has been performed [68,69,71].

4.1.5. Alpha-thalassemia/mental retardation X-linked gene (ATRX)

ATRX mutations occur in 44% of pediatric GBM [72]; mutations have been reported on 33% of pediatric grade II glioma, 46% of pediatric grade III glioma, 57–80% of pediatric "secondary" GBM, 7% of pediatric "primary" GBM [69, 73], 71% of pediatric grade IIIII astrocytomas, and 68% of pediatric oligoastrocytomas [69]. Reportedly no ATRX mutations are found in pediatric oligodendroglioma [73]. The nonfunctional binding protein encoded by mutant ATRX permits inappropriate recombination, resulting in aberrant telomere lengthening [74]. ATRX mutations have been associated with the *IDH1* mutation [73]. Sequencing studies have been carried out in tissue [69,72,73].

4.1.6. BRAF: $BRAF^{V600E}$

$BRAF^{V600E}$, is a point mutation that is frequently found in ganglioglioma and in about 65% of grade II xanthro-astrocytoma [75]. It is assumed that this alteration constitutively activates the RAS/RAK/MEK/ERK kinase pathway [75]. Incidence is reported to be 18% in brainstem ganglioglioma, 66% in pleomorphic xanthoastrocytoma with anaplasia, and 65% without anaplasia, 9% in pilocytic astrocytoma (PA); 3% in anaplastic astrocytoma [75], and 22.5% in pediatric grade II-IV tumors, but mutations are not found in pediatric grade I tumors [76]. The $BRAF^{V600E}$ mutation in tissues has been detected using *in situ* hybridization. The $BRAF^{V600E}$ mutation in melanoma has been targeted with a small-molecule BRAF kinase inhibitor vemurafenib (PLX4032), which therapy improves progression-free and overall survival [77,78]. When similar treatment effects are validated within low grade glioma, the drug could transform the $BRAF^{V600E}$ mutation from diagnostic marker to a marker which is predictive of response to therapy.

4.1.7. Telomerase reverse transcriptase-encoding gene (TERT)

TERT promoter mutations have been reported in 83% of 78 primary GBM, 10% of 40 astrocytomas, 78% of 45 oligodendroglioma, and 25% of 24 oligo-astrocytomas [74]. Overexpression results in 1.2–1.5 times increased risk of glioma occurrence [79]. TERT promoter mutations upregulate telomerase expression, enabling tumor cells to maintain sufficient telomere length in their genomes, thus removing an obstacle to prolonged cell proliferation. Analyses to detect TERT promoter mutations have been performed on tissue using RT-PCR and sequencing techniques [74].

4.1.8. Histone H3F3A gene

Unique to pediatric high grade glioma are mutations in the genes H3F3A and HIST1H3B which encode histone H3.3 [72]. Alterations in the H3F3A or HIST1H3B genes are present in approximately 80% of diffuse intrinsic pontine glioma and 20% of non-brain stem GBM

in children [80]. There is a potential association of *H3F3A* mutations with ATRX mutations [72]. The effect of *H3F3A* mutations on gene expression is not well understood. *H3F3A* mutations have been proposed to affect epigenetic gene expression regulation, selective gene regulation, or telomere length/stability [80]. The various mutations of this gene all result in the same amino acid substitutions, suggesting a gain-of-function phenotype that could potentially be targeted by drug developers [80]. Like EGFRvIII deletion and *IDH1* mutations, the specificity of the *H3F3A* mutation make it a promising sensitive and specific diagnostic biomarker, though it has yet to be investigated in biofluids.

4.2.Amplifications or mutations not unique to glioma

We have reviewed glioma-specific biomarkers (Table 1). In addition, there have been reports on amplification of receptors and overexpressed normal brain proteins. These are less compelling as diagnostic biomarkers as validation would involve identification of 'cut-points' or threshold values in accessible biofluids, which would separate glioma from both normal and carcinoma-afflicted individuals as well as those suffering nontumor neurologic syndromes.

4.2.1. Epidermal growth factor receptor (EGFR)—Amplification of EGFR has been reported in tissues from 40–50% of all glioma [55,63], 45–60% of primary GBM [54, 81], 10% of secondary GBM [54], and in lung cancer [82]. It plays a fundamental role in normal tissue development. Overexpressed EGFR constitutively activates the PI3-K/AKT/mTOR pathway, resulting in cancer cell proliferation and invasive tumor progression [54, 55, 63, 82]. EGFR amplification in FFPE tissue is detected by genomics and proteomics [63, 82] and in peripheral blood [63].

4.2.2. BRAF: KIAA 1549-BRAF fusion gene—The KIAA 1549-BRAF fusion gene is present in up to 80% of PA [76,83–85]. PA, the most common brain tumor in childhood, is found in cerebellar and non-cerebellar locations [86]. The BRAF fusion gene has been shown to exert its pro-oncogenic activity by activation of the mitogen-activated protein kinase (MAPK) pathway. The prognosis is excellent for surgically resected lesions but this is accomplished in fewer than 50% of patients. Resected PAs contain the Sox-2 stem cell marker, and rarely synaptophysin. However these materials seldom immunostain for BRAF. It is likely that in PAs the RAF/MEK/ERK pathway is activated.

4.2.3. TP53 mutations—TP53 mutations are common in glioma, These mutations have been used as an astrocytic marker to differentiate types of glioma. Mutation of ATRX is found frequently in low grade astrocytomas and secondary GBM, but not in primary GBM. ATRX with TP53 and IDH mutations correlates with improved survival [65]. *CIC* and *FUBP1* are frequently mutated in oligodendroglioma tumors, but needed are insights into their roles in tumor pathogenesis [65].

4.2.4. O-6-Methylguanine-DNA-methyltransferase (MGMT)promoter methylation status—Of marginal utility for diagnosis; but excellent utility for prognosis, are studies of the DNA repair enzyme O-6-methylguanine-DNA-methyltransferase (MGMT). When methylated, the *MGMT* promoter is silenced leading to improved response

to alkylating agents [34, 87]. In a cohort of 301 patients, MGMT promoter methylation in 44% of the patients correlated with improved progression-free and overall survival [63]. The tumor response can be observed as MRI-delineated “pseudo-progression” masses, which are in fact focal, gadolinium-enhanced necrotic lesions. Thus, the MGMT status may serve as a biomarker of pseudo-progression otherwise only identifiable at the time of repeat surgery. MGMT promoter methylation occurs in GBM [55,87,88] including 40% of primary GBM, over 70% of secondary GBM, 50–80% of anaplastic glioma [62] and 50–93% of low-grade glioma [54,55]. Immunohistochemistry of the MGMT protein did not correlate with PCR analysis of methylation. Thus, the ‘gold standard for tissue analyses has yet to be defined and may include methylation-specific PCR pyrosequencing, and/or MPLA. GBM and grade 2–4 glioma tissue along with colon cancer tissue exhibit GCIMP, which correlates with presence of mutation $IDH1^{R132H}$. This biomarker may be useful as a source of patient stratification for clinical trials. MGMT status can be identified in tissue and serum from GBM patients [89,90].

4.2.5. CHI3L1 (YKL-40)—CHI3L1, also known as YKL-40, has been shown to be highly overexpressed in GBM relative to normal brain and other CNS tumors. The overexpression favors the GBM mesenchymal subtype, and older age and is associated with poor prognosis [91]. The gene is not specific as expressed in conditions of extracellular matrix degradation and angiogenesis including severe arthritis, hepatic fibrosis, and other cancers. Elevated YKL-40 levels have been detected in the serum of glioma patients and have been shown to correlate with tumor grade and possibly tumor burden [92].

4.2.6. Phosphatase and tensin homolog gene (PTEN)—PTEN mutations occur in 28–60% of GBM, 7% of anaplastic astrocytomas, and no lower grade glioma [55,81]. Loss of PTEN function likely worsens survival for anaplastic glioma patients. Mutated PTEN gene products result in the loss of inhibition of the PI3K/AKT/mTOR pathway, leading to cell proliferation [55,81]. Analyses have been performed on tissue [55] and at least one GBM cell line [81] using genomics and proteomics [82].

4.2.7. c-Myc—Biofluids contain the c-Myc gene, characteristic of a subtype of childhood medulloblastoma. c-Myc amplification is characteristic of the group C medulloblastomas (Northcott *et al.)* which have significantly poorer progression-free and overall survival than the other three groups of childhood medulloblastomas [93]. Balaj *et al.* successfully measured c-Myc amplification in serum-derived EVs extracted from mice harboring human medulloblastoma xenografts [50].

5. Conclusion

A sensitive and glioma-specific biomarker diagnostic assay would benefit four underserved populations: 1) the 20% of Americans who currently never receive pathologic confirmation of their tumor; 2) the aged, infirm patient whose comorbidities preclude surgical evaluation; 3) patients whose masses are in ‘sensitive’ locations including the brainstem, the posterior fossa, speech and motor areas of cortex and subcortex, as well as those with non-discrete multifocal or diffusely infiltrative lesions; and 4) children for whom surgical morbidities may prove unacceptable. Although a plasma-based assay is preferable, there is consensus

amongst neurosurgeons of the ABC2 Foundation Biomarker Consortium that a biomarker from CSF would be acceptable, less costly and safer than many delicate neurosurgical resections. Minimally invasive diagnostics would change the nature of stratification for clinical anti-cancer trials. The Chief Clinician at Cancer Research UK, and members of the Early Detection Research Network of the US National Cancer Institute recognize that molecular specification of tumors will create a novel clinical trial design by enabling personalized therapy based on a predominant driver mutation or amplification. This would be true for the glioma-specific mutations EGFRvIII and *IDH1/2*. The diagnostic biomarkers would then become the subject of quantitative, longitudinal studies as metrics of therapeutic success. In addition, multiplex assays likely will provide information about downstream pathways as well as resistance mechanisms in glial tumors. MGMT promoter methylation status has been observed to be a predictor of glioma resistance to chemotherapy [94]. The expression of IDH1/2 mutant proteins has also been shown to sensitize GBM cells to ionizing radiation-induced apoptosis improving overall survival of these patients [93, 94].

For many of the biomarkers identified (Table 1) novel therapeutics are in development. For targeting EGFRvIII a monoclonal antibody therapy has been offered as has induction of humoral reactivity against EGFRvIII [95]. Currently, EGFRvIII-targeted vaccination is undergoing evaluation in phase 3 clinical trials [96]. The immunotherapeutic agents which target EGFRvIII include rindopepimut which induces a humoral immune response [97]. Combination therapies also target wild-type EGFR and other growth factor receptors such as insulin-like growth factor receptor or PDGFRI [98]. Murine monoclonal antibodies have been synthesized against IDH1 mutations; in addition, drugs mimicking α-ketoglutarate have been proposed as a potential therapeutic option pertaining to IDH gene mutations [99]. Mutant IDH1 inhibition has been found to release restriction on glioma cell differentiation and deter tumoral growth, thus allowing cancer cells to differentiate within less invasive pathways [100]. Mutant IDH2 inhibition has been observed to have the same effects in leukemia cell lines [101]. The small-molecule agents used in these inhibitions are termed AG-221 and AG-110, respectively, from Agios Pharmaceuticals. An adenovirus vector-based combination PTEN/antisense hTERT therapy has shown benefit in a xenograft murine model [102]. Trastuzumab has been shown to upregulate PTEN activity and thus inhibit the PI3K pathway in metastatic breast cancer with intact PTEN [103]. Therefore, detection of PTEN within EVs could be of significant use to study the levels of PTEN elevated in this type of tumor. Several studies have provided proof-of-principle for $BRAF^{V600E}$ mutant inhibition in the context of malignant melanoma and have identified potent inhibitors, some of which are specific for $BRAF^{V600E}$ (e.g. PLX4720, sorafenib) and targeted inhibition of $BRAF^{V600E}$ in glioma has been proposed [104–109]. MGMT inhibitors are being investigated [110] and methylation-related resistance may correlate with methylation of HOXA7, 9, and 10 genes [111]. A telomerase inhibitor Imetelstat, is currently in phase 2 trials [112], but issues exist involving resistance via molecular pathways that lengthen telomeres [113]. In a broader sense, diagnostic biomarkers could improve the enrollment of glioma patients in phase 2 clinical trials. A 2006 evaluation of NIH-derived patient accrual reported a total of 6 patients accumulated for a phase 1 clinical study investigating glioma (Protocol #: 00-C-0173), and maximum monthly referrals of 2 individuals [114]. However, an estimated 80% of clinical trials fail to recruit subjects in their desired timeframe [115].

Phase 3 trials also carry concerns, as most accrue unselected patients instead of targeted populations [116]. Absent a targeted patient cohort, drug recipients may receive ineffective treatment which logically will bias trial outcomes.

We propose a change in the paradigm of care. On the basis of symptoms, an MRI image will be obtained. A “suspicious” lesion is further evaluated using EV derived plasma or CSF biomarker analytics. From this will emerge the EV-associated molecular pathology of the tumor (Fig. 3). For inoperable patients there will be provided immediate nonsurgical therapy including radiation and/or mutation-specific chemotherapy. Operable candidates will receive pre-operative tumor-specific drug therapy prior to surgery or radiation treatment. For other patients, including children, identification of tumor-specific mutations in blood or CSF precludes biopsy of eloquent brain, or brainstem. This approach reduces cost and surgical morbidity. For example, the identification of an IDH mutant tumor should result in initiation of an anti-IDH1 chemotherapy without biopsy.

Identification of a molecular tumor signature, whose downstream pathways are known, opens the possibility of minimally invasive identification of drug-resistance pathways. These pathways may be modulated in real time, without the need for re-biopsy of tumor. The paradigm of *diagnosis, resection, chemoradiation, imaging, re-biopsy* would be replaced by *clinical symptoms, diagnostic biomarker assay, targeted therapy, resection, chemoradiation, and response assessment by imaging, and biofluid biomarker assay*.

This future paradigm relies on technologies within the grasp of the biomedical community. Readers of this review will likely see a number of these new strategies in place over the next 5–10 years.

6. Expert commentary

5.1. Short discussion of choices of an analysis with EGFRvIII and IDH1/2 mutations as examples

As a detection method, real time PCR assays provide sensitive detection of large gene deletions, mutations or rearrangements. For example, EGFRvIII with deletion of exon 2–7 can easily be detected using real time PCR assays. In the case of rare events and/or point mutations such as in the IDH1/2 genes, there is need for more sensitive assays (digital PCR or targeted sequencing) to increase sensitivity and produce more reliable results. This type of limitation in conventional techniques has stimulated studies to develop a system that can detect single nucleotide alterations in a gene and monitor tumor progression. Novel analytics are based upon evaluations performed of circulating nucleic acids using digital PCR and sequencing [117–119].

5.2. The spike-in cohort analysis and validation processes

It is important to define the detection limits for each assay. Digital PCR and sequencing can detect as low as 1 mutant copy in a background of 100,000 or even millions of wildtype copies. Before any assay can be adopted for clinical application to human samples, it is important to optimize the platform of the specific gene assay. This includes choice of optimal primers for the target sequence, amplification temperatures and times using spike-

ins of ultramers or a plasmid containing the target sequence or synthetic or nonhuman mRNAs. Concerns exist regarding sources of contamination as well as endogenous and exogenous heparin-like molecules [120]. Currently, this paradigm moves from spike-ins in PBS to human normal pooled biofluids and subsequently glioma patient biofluids. These concerns are fundamental for determination of the sensitivity of assays of wild-type and mutant copies of the ‘diagnostic’ biomarker gene.

6.3. Diagnostics and stratification for trials

We have previously shown that blood-derived EVs contain nucleic acid from tumor cells [121]. EV-RNA analysis clearly distinguishes a tumor patient from controls. Upon further analysis of EVs, we can determine glioma-specific mRNAs, miRNAs, ncRNAs in comparison with those of normal controls. Identification of the EV gene expression signature at diagnosis would provide stratification criteria for patients in clinical trials. Thus, blood-derived EVs have been shown to contain a specific gene signature that can clearly distinguish GBM patients from controls [121]. Furthermore, EGFRvIII has been detected in circulating plasma and CSF EVs [49], and mutant $IDH1^{G395A}$ can be detected in CSF derived EVs [62]. These studies together with other biomarker studies [122] offer great hope for fast, specific, and “real-time” minimally-invasive molecular stratification and response evaluation for brain tumor patients.

Blood-based assays are more desirable compared to CSF-based assays as it is less invasive for patients. Ongoing studies have to address which biofluid offers the best detection rate for each molecular target. Detection of RNA or DNA within or removed from EVs may offer different answers. For example, we have been unable to detect mutant *IDH1* mRNA in EVs from serum of patients whose tumor was positive for the *IDH1* mutation [62], whereas this mutation has been readily detected in non-EV DNA from 60% of patients [123].

7. Five-year view

Treatment of patients with glioma evolved slowly in the last 3 decades. A “minimally invasive” diagnostic glioma biomarker will reshape the landscape by providing a rapid confirmation of the molecular subtype of benign and aggressive gliomas. Progression will be made in particular with isolation and characterization of brain tumor-specific EVs. Their mRNA, miRNA and ncRNA cargo will be sequenced to confirm the existence of diagnostic point mutations and amplifications and to identify novel mutations. These analyses, performed without surgical intervention, will create a nosology replacing that which is over 100 years old. New insights will emerge in neurooncology with respect to gliomagenesis, the roles of endogenous brain pleuripotential stem cells and genes that drive pathways of malignant change. These insights may inform whether these tumors stem from environmental, viral and/or genetic risks. The next 5 years can become productive in research neuro-oncological research in the following directions:

1. Classification of glioma will have a fundamental overhaul as a consequence of the mapping of EV-associated gene amplifications and mutations.
2. EV-related IDH1/2 mutations and associated changes in IDH wild type genes and their substrate (2-hydroxyglutarate) will become a scientific ‘hot bed’ as biofluid

diagnostics become available, therapeutic decisions are made on the basis of these data and these become stratifiers for targeted drug trial entry.

3. International collaborations will provide rapid verification of novel biomarkers such as TERT.
4. EV-related studies of methylation status will lead to changes in dose/time schedules of radiation and temozolamide treatment.
5. The role of EV cargoes will be investigated in relation to tumor formation and attention will be focused on the role of peri-ependymal stem cells in both tumor formation and preservation of the ‘healthy brain’.
6. At least two clinically-useful FDA-approved EV-biomarkers of glioma will be available to clinicians, followed shortly thereafter by markers of metastases in brain. Neurosurgical study groups will coalesce to validate these markers.
7. With identification of the above, national priorities will be reset to consider costs of diagnostic approaches of brain tumors, prioritization in clinical trials and the provision of translational research funds.

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Key Issues

- We provide a description of glioma-derived EVs, their detection as well as glioma-specific amplifications and mutations that can serve as diagnostic biomarkers.
- The current sub-typing of glioma neither reflects our understanding of the mechanisms nor molecular pathways of gliomagenesis.
- The treatment of these glioma has only marginally improved without curative intent in the last 30 years. The care of glioma patients remains among the mostly costly in the United States.
- The current paradigm of care seldom takes into account the molecular features of the tumor with few trials driven by identified tumor-specific mutations or amplifications.
- EVs of tumor origin can be isolated from plasma and CSF. Purification strategies are available along with sensitive analytics for amplified mRNA and mutations within glioma-specific genes. These analyses serve as the basis for detection of tumor-specific biomarkers in biofluids obtained with minimal risk for the patient. The most fruitful studies involve the creation of a diagnosis without surgical intervention. Two candidate glioma diagnostic biomarkers are EGFRvIII in plasma and CSF and IDH1/2 in CSF.
- Validation of the sensitivity and specificity of glioma diagnostic biomarkers lead to a novel paradigm of targeted drug therapy.

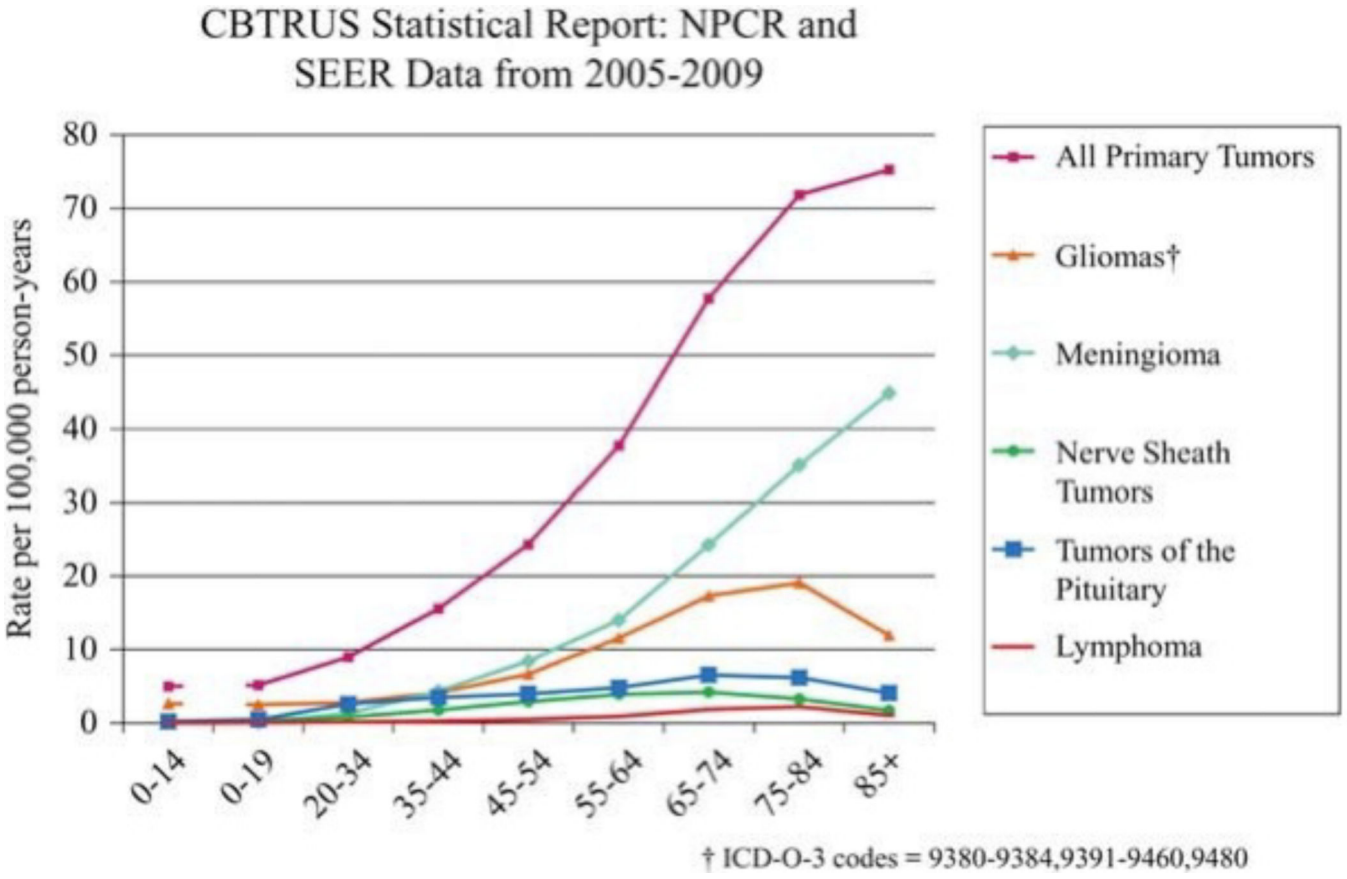

Figure 1.

All CNS tumors and types of brain tumors and their incidence in age categories represented as numbers of individuals versus age. Reprinted with permission from Oxford University Press.

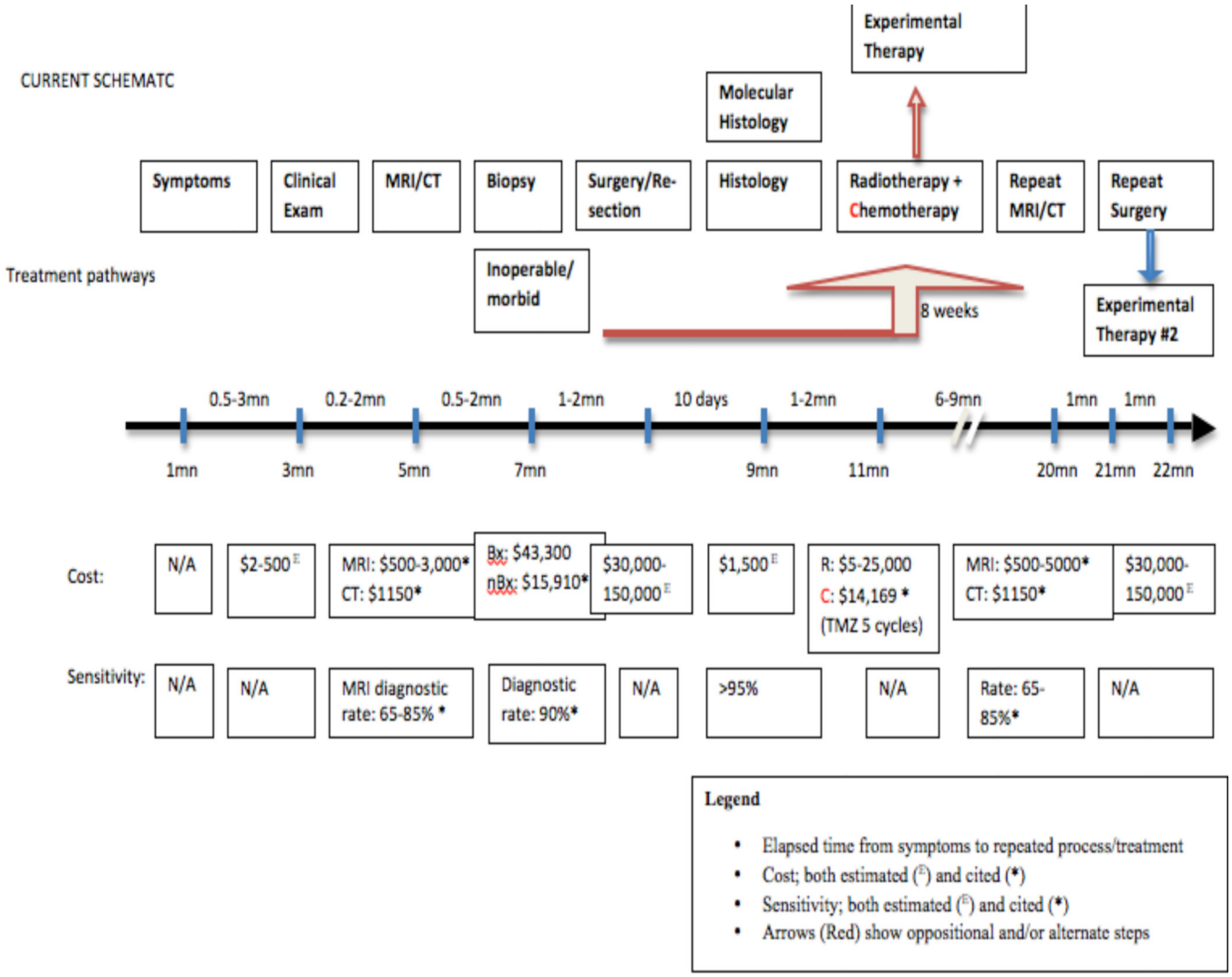

Figure 2.
The current paradigm of care for glioma patients.

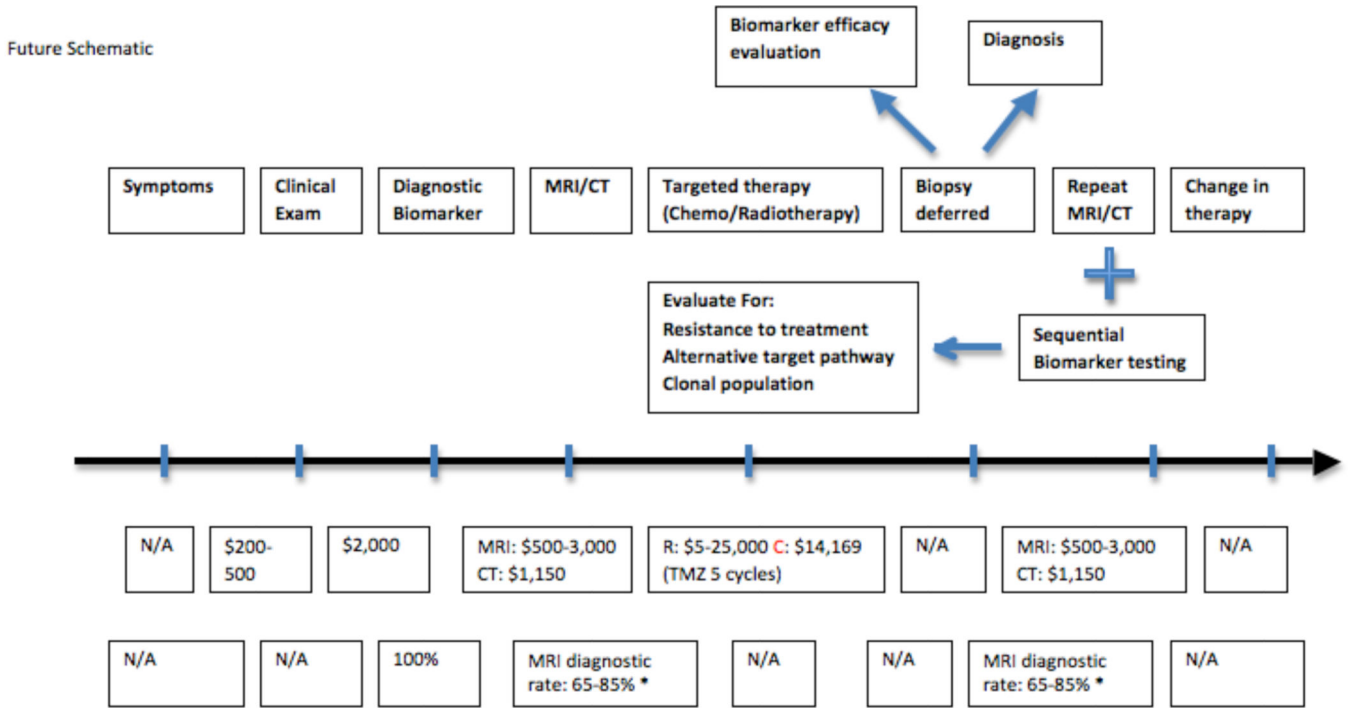

Figure 3.
The future perspective on the paradigm of care for glioma patients.

Table 1

Glioma-specific biomarkers and their relevance in the field of EVs.

Biomarker	Reference Number	Glioma Subtype Correlate	Molecular Correlation	Analytic Technique	Tissue/Biofluid
EGFRvIII	[29, 40, 53, 63, 64, 98]	24–67% of GBM; "primary" GBM; pediatric brainstem glioma	EGFR*wt* amplified; upregulate PI3K pathway	Immunohistochemical analysis, rtPCR, Western blot, flow cytometry	Tissue, CSF, plasma
IDH 1.132	[5, 29, 69, 70, 74]	50%–82% "secondary" GBM; 65–94% oligodendrogliomas/ oligoastrocytomas	2-hydroxyglutarate tissue; MGMT expression	Genomic analysis, gel electrophoresis, rtPCR, knock-in mouse tissue	FFPE tissue, peripheral blood samples
IDH 2	[65, 70, 74]	4.7% grade II oligodendrogliomas, 5.2% grade III anaplastic oligodendrogliomas, 6.2% grade III anaplastic oligoastrocytomas	2 Hydroxyglutarate accumulation. No association with IDH1.132	Knock-in mouse tissue, tissue sequencing	FFPE tissue
CIC (homolog of *Drosophila* Capicua)	[71, 72, 73, 74]	46–69% oligodendrogliomas; app. 10% astrocytoma	FISH 1p/19q deletion; IDH 1/2 mutations. Downstream of RAS/MAPK pathway	Tissue sequencing	FFPE tissue
FUBP1 (Far Upstream Element [FUSE] Binding Protein 1)	[72, 73, 76]	10–24% of oligos, 10% in astrocytomas	FISH tissue 1p/19q deletion; IDH 1/2 mutations; FUBP1 mutated gene does not bind to MYC oncogene	Tissue-based sequencing	FFPE tissue
ATRX (alpha thalassemia/mental retardation syndrome X-linked)	[69, 72, 73, 76]	44% of pediatric GBM; 33%–71% grade II glioma, 68% oligoastroxytomas; 46% grade III glioma, 57–80% "secondary" GBM, 7% "primary" GBM; 0% in oligodendroglioma	Aberrant telomere lengthening. Associated with IDH1 mutation	Tissue sequencing	FFPE tissue
BRAF V600E	[75, 76]	18% brainstem gangliogliomas; 66% pleomorphic xanthoastrocytoma; 9% pilocytic astrocytoma; 3% anaplastic astrocytoma; 22.5% pediatric grade II-IV tumors, 0% in grade I tumors	Activates RAS/RAF/MEK/ERK kinase pathway	DNA sequencing	Cell line
BRAF fusion	[75,80, 81, 84, 85]	Reported 100% in pediatric grade I tumors, 0% in grade II-IV tumors in 10 grade I, 31 grade II-IV gliomas respectively; reported up to 80% incidence in pilocytic astrocytomas; brainstem gangliogliomas; pleomorphic xanthoastrocytoma; pilocytic astrocytoma	KIAA1549-BRAF fusion-mediated upregulation of MAPK pathway	Clinical case report and sequencing	Tissue CSF
TERT	[48, 74]	83% "primary" GBM, 10% astrocytomas, 78% oligodendrogliomas, 25% oligo-astrocytomas; increased glioma risk	Upregulation of telomerase expression	Tissue-based rtPCR and sequencing	Tissue

Biomarker	Reference Number	Glioma Subtype Correlate	Molecular Correlation	Analytic Technique	Tissue/Biofluid
H3F3A/HIST1H3B (Histone H3.3)	[72, 73, 80]	80% pediatric diffuse intrinsic pontine gliomas, 20% pediatric non-brainstem GBM; pediatric high grade glioma, 3.4% adult GBM	ATRX, selective gene regulation/ telomere length/ stability	Whole-genome/targeted sequencing	FFPE tissue