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Abstract: PURPOSE: This review will address the current state of individualized cancer therapy for glioblastoma. Glioblastomas are highly malignant primary brain tumors presumably originating from neuroglial progenitor cells. Median survival is less than 1 year. DESIGN: Recent developments in the morphologic, clinical, and molecular classification of glioblastoma were reviewed, and their impact on clinical decision making was analyzed. RESULTS: Glioblastomas can be classified by morphology, clinical characteristics, complex molecular signatures, single biomarkers, or imaging parameters. Some of these characteristics, including age and Karnofsky Performance Scale score, provide important prognostic information. In contrast, few markers help to choose between various treatment options. Promoter methylation of the O-methylguanine methyltransferase gene seems to predict benefit from alkylating agent chemotherapy. Hence, it is used as an entry criterion for alkylator-free experimental combination therapy with radiotherapy. Screening for a specific type of epidermal growth factor receptor mutation is currently being explored as a biomarker for selecting patients for vaccination. Positron emission tomography for the detection of 3/5 integrins could be used to select patients for treatment with anti-integrin antiangiogenic approaches. DISCUSSION: Despite extensive efforts at defining biological markers as a basis for selecting therapies, most treatment decisions for glioblastoma patients are still based on age and performance status. However, several ongoing clinical trials may enrich the repertoire of criteria for clinical decision making in the very near future. The concept of individualized or personalized targeted cancer therapy has gained significant attention throughout oncology. Yet, data in support of such an approach to glioblastoma, the most malignant subtype of glioma, are limited, and personalized medicine plays a minor role in current clinical neuro-oncology practice. In essence, this concept proposes that tumors that are currently lumped together based on common morphologic features can be subclassified in a way that the resulting subentities are more homogeneous, for example, in molecular signatures and will therefore be amenable to selective therapeutic interventions. At present, the major "biomarkers" used to allocate treatment in glioblastoma are age and Karnofsky Performance Scale score, and these markers have so far survived all efforts at more sophisticated approaches to the management of this disease. Treatment allocation basically means intensity of treatment, especially the use of the standard-of-care or radiotherapy alone beyond age 65 to 70 years or below a Karnofsky Performance Scale score of 60.

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Individualized targeted therapy for glioblastoma: fact or fiction?

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Key words: Glioblastoma, MGMT, IDH, molecular, therapy, angiogenesis

Abstract

Purpose: This review will address the current state of individualized cancer therapy for glioblastoma. Glioblastomas are highly malignant primary brain tumors presumably originating from neuroglial progenitor cells. Median survival is less than one year.

Design: Recent developments in the morphological, clinical and molecular classification of glioblastoma were reviewed and their impact on clinical decision making was analyzed.

Results: Glioblastomas can be classified by morphology, clinical characteristics, complex molecular signatures, single biomarkers or imaging parameters. Some of these characteristics, including age and Karnofsky performance score, provide important *prognostic* information. In contrast, few markers help to choose between various treatment options. Promoter methylation of the O^6-methylguanine methyltransferase gene appears to *predict* benefit from alkylating agent chemotherapy. Hence, it is used as an entry criterion for alkylator-free experimental combination therapy with radiotherapy. Screening for a specific type of epidermal growth factor receptor mutation is currently being explored as a biomarker for selecting patients for vaccination. Positron emission tomography for the detection of $\alpha_v\beta_{3/5}$ integrins could be used to select patients for treatment with anti-integrin anti-angiogenic approaches.

Discussion: Despite extensive efforts at defining biological markers as a basis for selecting therapies, most treatment decisions for glioblastoma patients are still based on age and performance status. However, several ongoing clinical trials may enrich the repertoire of criteria for clinical decision making in the very near future.

The concept of individualized or personalized targeted cancer therapy has gained significant attention throughout oncology. Yet, data in support of such an approach to glioblastoma, the most malignant subtype of glioma, are limited and personalized medicine plays a minor role in current clinical neuro-oncology practice. In essence, this concept proposes that tumors which are currently lumped together based on common morphological features can be subclassified in a way that the resulting subentities are more homogeneous, e.g., in terms of molecular signatures and will therefore be amenable to selective therapeutic interventions. At present, the major "biomarkers" used to allocate treatment in glioblastoma are age and Karnofsky performance score, and these markers have so far survived all efforts at more sophisticated approaches to the management of this disease. Treatment allocation basically means intensity of treatment, especially the use of the standard-of-care or radiotherapy alone beyond age 65-70 years or below a Karnofsky performance score of 60.

Subclassifying glioblastoma

by morphology

The regularly updated WHO classification describes the histological hallmarks of primary brain tumors. Glioblastoma is a distinct glioma entity that can be distinguished from other brain tumors, notably anaplastic gliomas (1). In contrast, primary glioblastoma cannot be morphologically separated from secondary glioblastoma evolving from a prior lower grade tumor. The current WHO classification recognizes two glioblastoma variants, giant cell glioblastoma and gliosarcoma. Both clinical course and treatment are similar to classical glioblastoma, although some authors have proposed that giant cell glioblastoma may have a somewhat less

aggressive course.

by high-throughput analysis

Beyond morphology, an increasing number of publications used high-throughput techniques to derive a subclassification of glioblastomas. One model of molecular classification based on gene expression analyses was proposed by Phillips et al. (2). Selecting a set of genes associated with survival in their patient cohort enriched for long-term survivors (>2 years), they identified three glioblastoma subtypes with distinct molecular signatures which they termed proneural, proliferative and mesenchymal. The proneural signature is associated with oligodendroglial morphology, younger age, lack of *phosphatase and tensin homolog on chromosome ten* (PTEN) or *epidermal growth factor receptor* (EGFR) abnormalities, activation of the Notch pathway, and better outcome. The proliferative and mesenchymal signatures are more common in older patients and are characterized by PTEN loss and Akt pathway activation, and have a less favourable outcome. They are distinguished by a preponderance of either proliferation or angiogenesis. Verhaak et al. (3) took an unsupervised approach, extracting gene expression patterns that yielded four molecular signatures for glioblastoma which they termed proneural, neural, classical and mesenchymal subtypes, in allusion to similarities with signatures of the classification proposed by Phillips and colleagues (2). These subtypes also segregate the characteristic mutations. The proneural subtype comprises most isocitrate dehydrogenase (*IDH)* 1 mutations and is enriched for *p53* mutations, while the classic subtype particularly enriches for *EGFR*-amplified tumors expressing also the *EGFRvIII* variant. The mesenchymal subtype contains most neurofibromatosis (*NF*)-1-mutant tumors. Hence the expression subytypes overlap with major previously identified pathogenetic pathways involved. Of note, O^6-

methylguanylmethyltransferase (*MGMT)* promoter methylation is not particularly enriched in any specific subtype. The authors proposed that patients with classical or mesenchymal glioblastoma derive more benefit from aggressive treatment, but this requires confirmation within a prospective clinical trial.

Other approaches set out to identify gene signatures characterizing cancer-relevant biological features using unsupervised approaches. This, among others, has yielded a stem cell-related gene expression signature dominated by *HOX* genes that was a predictor of failure from the addition of temozolomide to radiotherapy, independent of the *MGMT* status (4). This signature was thereafter independently identified in pediatric glioblastoma, tumors that are otherwise quite different from their adult counterparts. Interestingly, the *HOX* gene signature predicted failure from temozolomide therapy independent of MGMT (5). Another view on the biology of tumors is provided by analysing micro-RNAs that have regulatory functions. microRNA expression profiles yielded biologically meaningful subclassification of glioblastomas, for which 5 five subclasses have been proposed using the TCGA data that relate to developmental patterns. Three of these overlap substantially with 3 of the 4 subclasses based on the Veerhak and colleagues` gene expression classification (2,6).

Yet another approach to characterize tumors is to evaluate aberrant DNA methylation at CpG sites that denotes a major mechanism of epigenetically controlled silencing of genes including non-coding RNAs, such as microRNAs. Noushmer et al. (7) assessed DNA methylation at 27K CpG sites in 272 glioblastomas of The Cancer Genome Atlas (TCGA) and observed several methylation subtypes of which one subgroup exerted a striking pattern of concerted hypermethylation consistent with the delineation of a glioma-CpG island methylator phenotype now commonly referred to

as G-CIMP. The G-CIMP phenotype characterizes a subgroup of tumors with the proneural signature, and is closely associated with *IDH* mutations (see below) and is associated with good prognosis. In glioblastoma G-CIMP is associated with secondary glioblastoma, arising from lower grade lesions (8). Hence not surprising, G-CIMP is also common in grade II and grade III glioma with strong association with IDH mutations. Screening for IDH mutations will identify most G-CIMP-positive gliomas (7). The recently presented molecular data from the Radiation Therapy Oncology Group 0525 / European Organization for Research and Treatment of Cancer 26052 glioblastoma trial support this view. G-CIMP only added an insignificantly different intermediate group to the highly correlating *IDH*1-mutated or G-CIMP-favourable group *versus* the *IDH*1-wild-type / G-CIMP-unfavourable group (9). However, a recent study in anaplastic glioma suggested that G-CIMP outperforms *IDH*1 mutations as a prognostic biomarker although *IDH*2 mutations were not determined (10).

The availability of genome wide methylation analysis provides new opportunities to find new targets for personalized therapy or identify the “Achilles heel” of tumors, as previously described for the silencing of *MGMT* by promoter methylation that sensitizes tumors to alkylating agents (see below) (11,12). Thus, at present, despite promising developments, no specific treatment recommendations can be derived from highthroughput approaches of molecular classification.

Subclassifying glioblastoma by single molecular markers

P53

Mutations of the *p53* gene or its down-stream effector molecules are among the most

common molecular aberrations in human cancers, including gliomas (13,14). Among glioblastomas, p53 mutations are more common in secondary glioblastomas and are thus associated with *IDH* mutation status. However, the p53 pathway in typical primary glioblastomas is also commonly disabled since glioblastoma cells do not readily undergo apoptosis when exposed to ionizing irradiation or DNA-damaging chemotherapy. There is thus no role for the *p53* status in determining treatment decisions in glioblastoma. Promising efforts at exploiting p53 abnormalities are still being evaluated: *p53* mutations may result in protein overexpression and give rise to novel immunogenic targets that might be used for vaccination therapy (15). Moreover, it is likely that tumors with p53 mutations would be susceptible to efforts at reintroducing wild-type p53. This could be accomplished in the form of p53 gene therapy (16) or the development of new experimental agents which restore an active conformation of p53, despite the mutation, and thereby transcriptional activity. Such agents exhibit profound anti-glioma properties *in vitro*, but not of all of their activity could be linked strictly to the predicted effect on mutant p53 variants (17,18).

EGFR

Increased expression of the *EGFR* gene is common in glioblastoma, in particular in primary glioblastoma, and is thus inversely correlated with *p53* and *IDH* mutations. Enhanced EGFR signalling may result from enhanced expression related to amplification or from mutational activation. Loss of exons 2-7 of the EGFR gene affects 801 base pairs and results in a mutant receptor (EGFRvIII) that is constitutively active in the absence of ligand binding (19). Enhanced EGFR signalling activity promotes proliferation, invasiveness and resistance to irradiation and chemotherapy. Extensive efforts at identifying responders to anti-EGFR treatment

have not resulted in a uniform picture: Patients with high EGFR expression and low levels of phosphorylated Akt have been proposed to respond better to erlotinib than patients with tumors with low levels of EGFR expression and high levels of phosphorylated Akt (20). Coexpression of EGFRvIII and PTEN was also reported to be associated with responsiveness to EGFR kinase inhibitors (21). However, these observations were not confirmed in a prospective randomized trial comparing erlotinib with alkylating agent chemotherapy in recurrent glioblastoma (22). A randomized trial of afatinib indicated inferior activity compared with reexposure to temozolomide (23), and addition of erlotinib to combined chemo-radiotherapy in newly diagnosed glioblastoma showed no promising results (24). Several trials aiming at targeting the EGFR in glioblastoma patients have failed to demonstrate meaningful antitumor activity, even though it was shown for gefitinib that high drug levels are reached in the tumor tissue, efficiently dephosporylating the EGFR, however, without measurable effects on downstream targets (25). Similar to p53, there is therefore currently no role for determining the *EGFR* status except that the detection of EGFR amplification or EGFRvIII mutation supports the diagnosis of glioblastoma in cases of doubt.

Nevertheless, targeting the EGFRvIII remains under investigation as a target for immunotherapy. Rindopepimut (CDX-110) is a vaccine product that consists of a 14 amino acid synthetic peptide built from 13 amino acids of EGFRvIII plus a cysteine residue, covalently linked to keyhole limpet hemocyanin as a carrier. This vaccine has been explored in phase II trials in patients with EGFRvIII-positive glioblastoma. Intriguingly, tumors progressing after vaccination therapy had commonly lost EGFRvIII expression, which is probably not the natural course of disease in standard treated EGFRvIII-positive glioblastoma, and responses on immune monitoring defined by antibody reactivity and delayed skin hypersensitivity were associated with

better outcome (26). In this very small trial a progression-free survival of 14.2 months and median overall survival of 26.0 months were observed. This may simply reflect careful patient selection since vaccination was limited to patients who had undergone a gross tumor resection and had completed concomitant chemoradiotherapy without progression. The feasibility of performing a blinded, randomized trial to test the efficacy of this immunotherapeutic approach is currently being explored.

MGMT

MGMT has become the most promising and controversial biomarker in the field of glioblastoma (11,27). MGMT is a DNA repair protein that removes alkyl groups from DNA and is consumed by proteasomal degradation during that process. Its expression by cancer cells confers resistance to alkylating agent chemotherapy and may be a predictive factor for outcome in patients treated with such chemotherapy. Methylation of the *MGMT* promoter was strongly associated with benefit from combined chemo- and radiotherapy compared to radiotherapy alone in the registration trial for temozolomide in newly diagnosed glioblastoma (28). A better outcome of patients with *MGMT* promoter methylation has been confirmed in numerous uncontrolled trials and retrospective analyses of glioblastoma patients treated with alkylating agents. However, a specific prediction of benefit of chemotherapy can only be deduced from data sets which include a chemotherapy-free control arm like the initial temozolomide trial. Surprisingly, two randomized trials in patients with *anaplastic gliomas* containing radiotherapy only control arms reported the same degree of improved outcome in patients with *MGMT* promoter methylation irrespective of whether the patients were treated with radiotherapy alone or chemotherapy alone (29) or radiotherapy alone or radiotherapy plus nitrosourea-

based chemotherapy (30). Whether this is limiting the relevance of MGMT being predictive or just reflecting biological differences between anaplastic glioma and glioblastoma is currently investigated. The missing predictive impact in anaplastic glioma may be due to a retained allele of MGMT on the other arm of chromosome 10q or a strong association with the G-CIMP phenotype at least in anaplastic oligodendroglial tumors (10). So far, these data support the view that anaplastic gliomas and glioblastomas are distinct entities that may be best separated by their *IDH* status (see below) (31).

The *MGMT* status may assume greater relevance in elderly patients with glioblastoma where the efficacy of alkylating agent chemotherapy in addition to radiotherapy has not been demonstrated and where increased toxicity from combined modality treatment remains to be of concern and therefore the use of alkylating agents neither in the first-line or relapsed setting is standard-of-care (32,33). Whether temozolomide alone may be effective as radiotherapy alone in this setting, remains unclear as long as complete data from two large randomized clinical trials are not available. The three-arm Nordic trial that compared standard radiotherapy with hypofractionated radiotherapy and with temozolomide alone in 5-out-of-28 day cycles, reported no difference between the treatment arms (34). A preliminary report of the two-armed, randomized NOA-08 trial that compared standard radiotherapy alone with dose-intensified temozolomide alone (one week on one week off), failed to demonstrate non-inferiority of dose-dense temozolomide (35). A prospective, non-interventional cohort study of the German Glioma Network has identified a strong predictive value of *MGMT* promoter methylation for benefit from temozolomide: there was no evidence for benefit from alkylating agent chemotherapy in glioblastoma patients without *MGMT* promoter methylation whereas, conversely, there was an indication that temozolomide alone might be sufficient for patients with glioblastomas

with *MGMT* promoter methylation (36). Accordingly, the final results from the Nordic trial and NOA-08 need to be awaited and reassessed when data on outcome by *MGMT* status become available.

Much of the current discomfort of using MGMT as a biomarker results from the fact that it has been difficult to establish reliable testing procedures and to establish by consensus which test is best and how to perform it in detail (27). Methylation-specific PCR remains to be the gold standard whereas more expensive, less readily available techniques such as pyrosequencing have not shown to be superior in correlating *MGMT* status with clinical outcome. The failure of MGMT protein assessment to correlate with *MGMT* promoter methylation and outcome has been extensively discussed and reviewed (27,37,38).

The S039 trial analysing enzastaurin and radiotherapy in newly diagnosed non-MGMT methylated patients was the first trial that implemented MGMT status as an entry criterion (39). The most extensive experience with MGMT as a biomarker has been made in the CENTRIC phase III trial that compared standard radiotherapy plus temozolomide with this standard plus the $\alpha_v\beta_{3/5}$ integrin antagonist cilengitide. Based on an uncontrolled phase II trial that indicated preferential benefit from cilengitide in patients with *MGMT* promoter methylation (40), centralized upfront *MGMT* testing was introduced at study entry and enrolment restricted to patients with *MGMT* promoter methylation. While it remains unclear whether such an effort of patient selection was entirely justified for demonstrating efficacy of cilengitide, this trial nevertheless demonstrated the feasibility of molecular testing in large trials for patients with newly diagnosed glioblastoma.

IDH

The identification of somatic mutations of the *IDH* genes in the majority of grade II and III gliomas as well as a minority of glioblastomas (<10%) was an important discovery of molecular neuropathology (41,42). The differential distribution of *IDH* mutations provides a strong rationale to consider grade II/III gliomas and glioblastomas as distinct tumor entities. *IDH* mutations are early events in gliomagenesis and are easy to determine using mutation-specific antibodies. The consistent mutational targeting of specific codons and the heterozygous nature of the mutations strongly suggest that mutant IDH proteins acquire a novel oncogenic activity that is only indirectly related to their physiological function, but results in the accumulation of a candidate oncometabolite, D-2-hydroxyglutarate. Efforts at measuring this metabolite in peripheral blood of patients with *IDH*-mutant gliomas were not successful so far (43), but efforts at detection by magnetic resonance spectroscopy are under evaluation and may provide a non-invasive diagnostic tool to identify and monitor *IDH*-mutant gliomas (44).

The correlation of the neomorphic *IDH* mutants with G-CIMP with has provided interesting mechanistic hint: *IDH*1 or 2 mutations were also correlated with a methylator phenotype in leukemia. Furthermore, *IDH*1 and 2 mutations in leukemia were exclusive with *TET2* mutations. It turned out that D-hydroxyglutarate inhibits TET2 that, in turn, is involved in DNA demethylation (45), suggesting a functional link between *IDH* mutations, the development of a methylator phenotype, and TET2 function: metabolism meets epigenetics. Of note, it has not been demonstrated that this or any other metabolite maintains the neoplastic phenotype of gliomas once the tumors have been established. If this was the case, specific pharmacological targeting of the gain–of-function enzymatic activity of mutant IDH enzymes would be a highly promising targeted therapeutic approach, potentially devoid of side effects. In the absence of such approaches, determining the *IDH* status has diagnostic and

(positive) prognostic impact, but does not help to select among the current treatment options of radiotherapy *versus* chemotherapy *versus* combination thereof.

Angiogenesis

Inhibitors of angiogenesis are currently in the focus of drug development in glioblastoma. Based on uncontrolled phase II data, two agents with differential modes of action, the vascular endothelial growth factor (VEGF) antibody bevacizumab (46) and the RGD-mimetic $\alpha\nu\beta3/5$ integrin antagonist cilengitide (40) are being evaluated in phase III registration trials in patients with newly diagnosed glioblastoma which have completed enrolment in 2011. Similar to traditional approaches to glioblastoma, there is significant heterogeneity in the response of glioblastoma patients to these novel agents, and predictive biomarkers would greatly aid in the selection of specific treatments, both for patient enrichment in clinical trials and in the future with a possible scenario where more than one anti-angiogenic agent might be approved for glioblastoma. So far, efforts at defining either predictive soluble plasma markers or imaging parameters have not been successful, but promising approaches include the labelling of the target integrin of cilengitide by positron emission tomography (47) or the definition of the vascular normalization index consisting of vascular permeability (K^{trans}) and microvessel volume determined by magnetic resonance imaging and circulating collagen IV in plasma (48). Extensive biomarker studies are accompanying most ongoing trials in the angiogenesis field.

Outlook

The perspectives of individualized treatment for glioblastoma depend on the identification and prospective evaluation of biomarkers that allow to predict a

preferential benefit from a specific treatment, depending on the absence *versus* presence of this biomarker. To be clinically useful, the predictive biomarker needs to provide a clear segregation of patients into responders and non-responders, and its evaluation should be based on reproducible, standardized test procedures. The usefulness of the best predictive biomarker we have at present, *MGMT* promoter methylation, is limited for these reasons (27). EGFRvIII is currently a candidate biomarker that might be developed to meet these criteria, pending the demonstration of a test suitable for routine testing and clinical benefit from vaccination against EGFRvIII in a well-controlled clinical trial.

Further, it would be highly desirable if the targeted approach would target the most relevant cell populations within the tumor. While the stem cell hypothesis has its weaknesses, there is nevertheless broad consensus that not all glioma cells within a tumor are alike, and features like spherogenicity, increased clonogenicity, multilineage differentiation potential, and tumorigenicity in rodents at low numbers of injected cells may well characterize a subpopulation of glioma cells that is responsible for resistance to therapy, progression or relapse. However, no reliable stem cell marker has been defined in glioma cells so far, in particular no marker that would define a suitable target for molecular targeted therapy. Candidate pathways include, but are not limited to, the Notch pathway (49) and a *HOX* gene signature (4).

Carefully designed prospective trials are the only way to define a novel scenario where only patients with EGFRvIII mutation are vaccinated, if this vaccination is proven to be of benefit in the future, only patients with *MGMT* promoter methylation receive temozolomide, only integrin-positive patients by positron emission tomography will receive cilengitide, if this concept holds its promises, and a novel biomarker has been established to predict, which patient group benefits from

bevacizumab. This chance should not be missed.

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