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The Next Generation of Glioma Biomarkers: MGMT Methylation, BRAF Fusions and IDH1 Mutations

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Abstract

For some, glioma biomarkers have been expected to solve common diagnostic problems in routine neuropathology service caused by insufficient material, technical shortcomings or lack of experience. Further, biomarkers should predict patient outcome and direct optimal therapy for the individual patient. Unfortunately, current biomarkers still fall somewhat short of these grand expectations. While there has been some progress, it has generally been slow and in small steps. In this review, the newest set of glioma biomarkers: O^6-methylguanine-DNA methyltransferase (*MGMT*) methylation, *BRAF* fusion and *IDH1* mutation are discussed. *MGMT* methylation is well established as a prognostic/predictive marker for glioblastoma; however, technical questions regarding testing remain, it is not currently utilized widely in guiding patient management, and it has proven to be of no assistance in diagnostics. In contrast, *BRAF* fusion and *IDH1* mutation analyses promise to be very helpful for classifying and grading gliomas, while their potential predictive value has yet to be established.

MGMT PROMOTER HYPERMETHYLATION

Brief history

The gene encoding the O^6-methylguanine-DNA methyltransferase (*MGMT*) has become one of the most, if not the most studied molecular marker in neurooncology since the first description of an association between *MGMT* promoter hypermethylation and response to alkylating drugs a decade ago (23). However, the interest in this gene was particularly enhanced in 2005 by the publication of Hegi *et al* showing in a prospective phase III trial, that glioblastoma (GBM) patients with methylated *MGMT* promoter status demonstrated a significant survival advantage with temozolomide treatment (39). Nonetheless, the dealkylating function of MGMT had already been described in the early 1980s (69, 72), with its cDNA being cloned 10 years later (87). In 1999, promoter hypermethylation was found to be the main inactivating mechanism of this gene in a broad variety of human cancers (22).

Distribution of *MGMT* promoter hypermethylation in tumor entities

MGMT promoter hypermethylation has been identified in a wide range of human cancers, including lung-, head and neck-, pancreatic-, renal- and bladder carcinomas, as well as lymphomas, leukemias and melanoma (22). The reported frequency of *MGMT* promoter hypermethylation in gliomas varies widely. In clinical studies, it has ranged from 35% to 73% in GBM (8–10, 17, 18, 21, 23, 38–40, 74, 90, 96–98, 101). In diffusely infiltrating anaplastic gliomas [World Health Organization (WHO) grade III], it has been found in 50%–84% (7, 91, 99), while 43%–93% of the WHO grade II counterparts are reportedly positive (24, 51, 52). The substantial range of reported *MGMT* promoter hypermethylation frequencies is probably at least partially due to technical challenges (see below).

Mechanisms of action, tumor inactivation and chemosensitivity

The *MGMT* gene located on 10q26 has five exons and a CpG-rich island of 763 bp with 98 CpG sites encompassing the first exon and large parts of the promoter. A minimal promoter and an enhancer region are located within the CpG island (Figure 1) (65). In normal tissue, most CpG sites within the island are unmethylated. In tumors, the cytosine in CpG sites often carries a methyl group, thereby increasing the affinity of proteins like methyl-CpG-binding protein 2 and methyl-CpG-binding domain protein 2 to the DNA. These proteins subsequently alter the chromatin structure and prevent binding of transcription factors, thereby silencing expression of *MGMT* (65).

MGMT is a suicide DNA repair protein that normally catalyzes the transfer of a methyl group from the O^6-position of a guanine DNA nucleotide to a cysteine residue at its own position 145. This

alkylation of MGMT is a one-way process, with alkylated MGMT ultimately targeted for degradation (30). In gliomas, this is relevant because application of alkylating chemotherapeutic drugs like temozolomide cause, among other actions, the binding of an alkyl group to the O^6-position of guanine, thereby inducing DNA-mismatching, DNA-double-stand breakage and ultimately apoptosis in proliferating cells. Thus, MGMT protein counteracts the normally lethal effect of temozolomide by repairing DNA damage. When a tumor has a hypermethylated *MGMT* promoter, the hypothesis is that MGMT expression is reduced and the cytotoxic effects of alkylating drugs are then enhanced.

Yet, this widely accepted and intuitive concept that *MGMT* promoter hypermethylation acts as a chemosensitizer recently became challenged by the observation that patients suffering from anaplastic gliomas with hypermethylated *MGMT* similarly exhibited a survival benefit when treated with radiotherapy alone (90, 99). Therefore, it remains unclear whether MGMT also plays a role in repairing radiotherapy-induced DNA damage, if other DNA repair genes are silenced by promoter hypermethylation in addition to *MGMT*, or if the survival advantages are better explained by other prognostically favorable genetic alterations like the 1p/19q codeletions (6, 63, 90) and *IDH1* mutations (80) that often coexist with *MGMT* promoter hypermethylation.

Clinical relevance

In one of the most important series on therapy of patients with newly diagnosed GBM in the last decade and its follow-up study, it was shown that those patients with a hypermethylated *MGMT* promoter demonstrated survival rates of 49% and 14% at 2 and 5 years respectively, when treated with concomitant and adjuvant temozolomide and radiotherapy. In contrast, estimated 2- and 5-year survival rates were only 24% and 5% respectively, in similar patients that were initially treated with radiotherapy alone. GBM patients whose tumors lacked *MGMT* hypermethylation demonstrated 2- and 5-year survival rates of 15% and 8% when they received combined radiochemotherapy, which dropped to only 2% and 0% when treated with radiotherapy alone (39, 85, 86). As such, temozolomide response was most dramatic in the methylation-positive group, yet there was some advantage even in the non-methylated cohort. Given this data, the fact that temozolomide is a well-tolerated oral drug, and that few highly efficacious alternatives are currently available, most neuro-oncologists still opt to treat their patients with this drug first, regardless of *MGMT* status. Nevertheless, multiple studies have subsequently confirmed the observation that *MGMT* promoter hypermethylation is one of the strongest prognostic factors for patients with newly diagnosed GBM and that it is a potent predictor for response to treatment with alkylating drugs (23, 31, 38, 40, 97), even in elderly patients (9). Furthermore, *MGMT* promoter hypermethylation was identified as the only molecular marker that was enriched in so-called long-term survivors of GBM (OS > 36 months) (54). However, prolonged survival can be seen without *MGMT* promoter hypermethylation, indicating the existence of other factors conferring this favorable prognosis (54, 64). So, while testing currently provides powerful prognostic information, its role in guiding patient management is more tenuous. Presumably however, as additional and perhaps more targeted therapeutic options become available, *MGMT* testing may become all the more important for patient management.

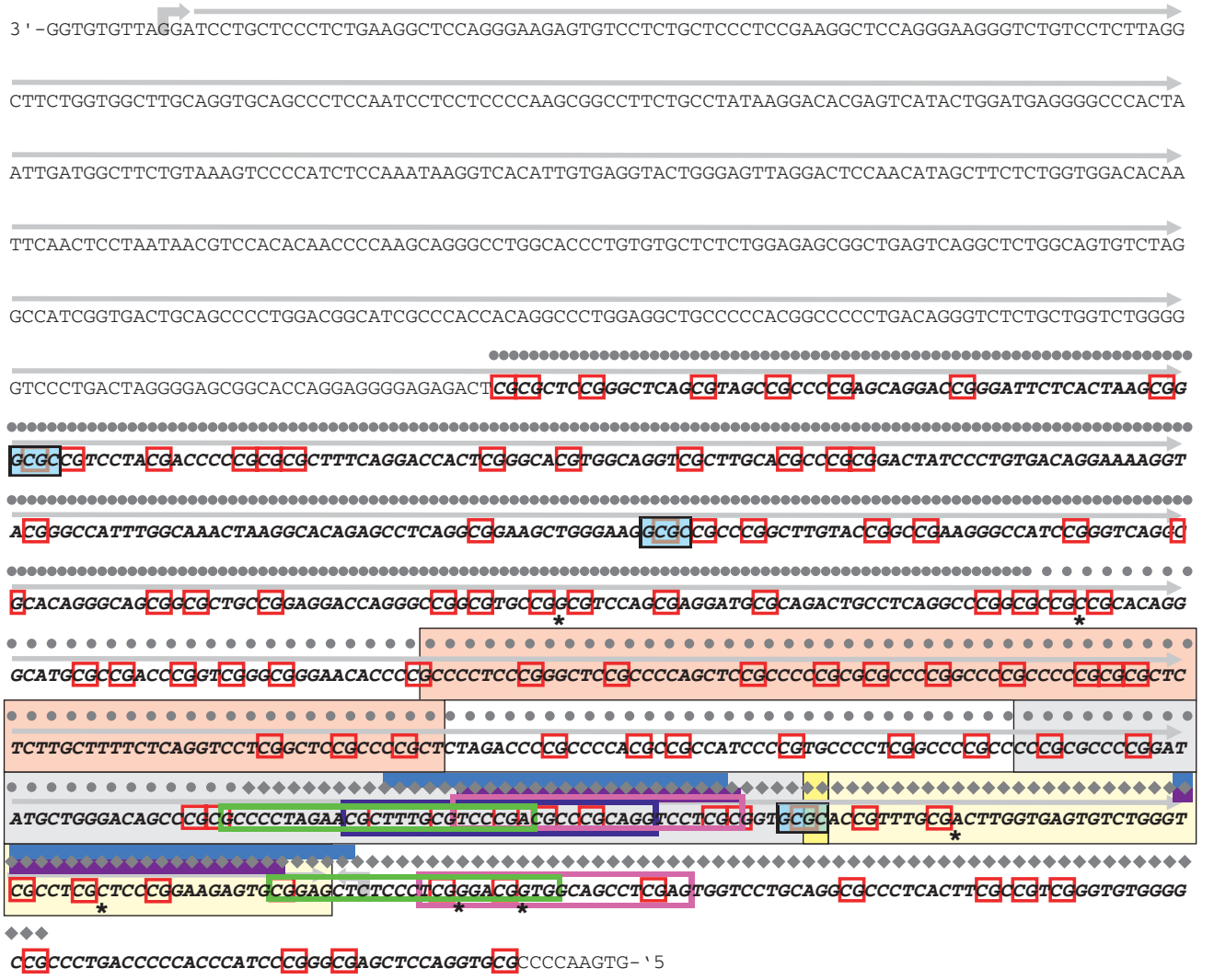
The role of *MGMT* testing in patients with WHO grade II or III diffuse gliomas is even less clear at this time. In anaplastic gliomas, *MGMT* promoter hypermethylation is associated with longer progression free survival for patients treated either by radiochemotherapy or radiation alone (90, 99). A predictive role of *MGMT* promoter hypermethylation for response to temozolomide treatment was documented for grade II gliomas (24, 51). However, a positive prognostic effect of *MGMT* promoter hypermethylation was not found for patients with WHO grade II astrocytomas (A II) (52) and oligodendrogliomas (O II) (94), who received no alkylating chemotherapy.

METHODS FOR MGMT ANALYSIS

MGMT promoter hypermethylation assays

Technical limitations of all available methods used in a routine setting prohibit analysis of all 98 CpG sites. Generally, each method focuses on only a couple of CpG sites based on the assumption that they reflect the methylation status of the whole CpG island, in turn predicting patient response to alkylating drugs. Yet, the methylation pattern is not always homogeneous and methods targeting different CpG sites may yield conflicting results (25). Only a few reports are available that allow conclusions regarding methylation of the majority of the CpG sites in gliomas. By clonal sequencing, Nakagawachi *et al* determined the methylation pattern of all CpG sites in glioma cells that did not express *MGMT*. They identified an upstream highly methylated region (UHMR) and a downstream highly methylated region (DHMR) and a region in between with varying methylation (65). According to their data, all CpG sites of the methylation-specific polymerase chain reaction (MS-PCR) and the commercially available pyrosequencing assay (see below) are located within the DHMR. Everhard *et al* analyzed the methylation profile of 52 CpG sites by pyrosequencing in GBM and compared the results with *MGMT* mRNA expression. In this way, they were able to identify six single CpG sites and two CpG regions that best correlated with overall expression. Only a partial overlap between these CpG sites and the CpG sites tested by the routine assays was observed (25) (Figure 1).

Most clinical studies have used MS-PCR as first introduced by Esteller *et al* (22) to compare *MGMT* status with survival and therapeutic response. As such, MS-PCR has become the standard method for *MGMT* testing to date. Five to nine CpG sites are usually covered by MS-PCR, in which DNA bisulfite treatment converts unmethylated cytosine to uracil. Next, PCR amplification is done with two different primer pairs that align to regions encompassing those specific CpG sites. One primer pair is specific for methylated, "non-uracilized" DNA, while the other is designed for DNA in which cytosine residues have been converted to uracil. In this way, converted and unconverted CpG sites can be distinguished from each other. In addition to the MGMT promoter, a completely unmethylated fragment of DNA (eg, COL2A1 promoter) and a completely methylated sample are often used as negative and positive controls, respectively. Finally, PCR products are visualized on an agarose gel. But while this method does not require expensive equipment and is widely established, the bisulfite reaction requires a great deal of technical expertise and is rather labor-intensive. Also, MS-PCR is frequently used with an increased number of PCR cycles and may result in false positive



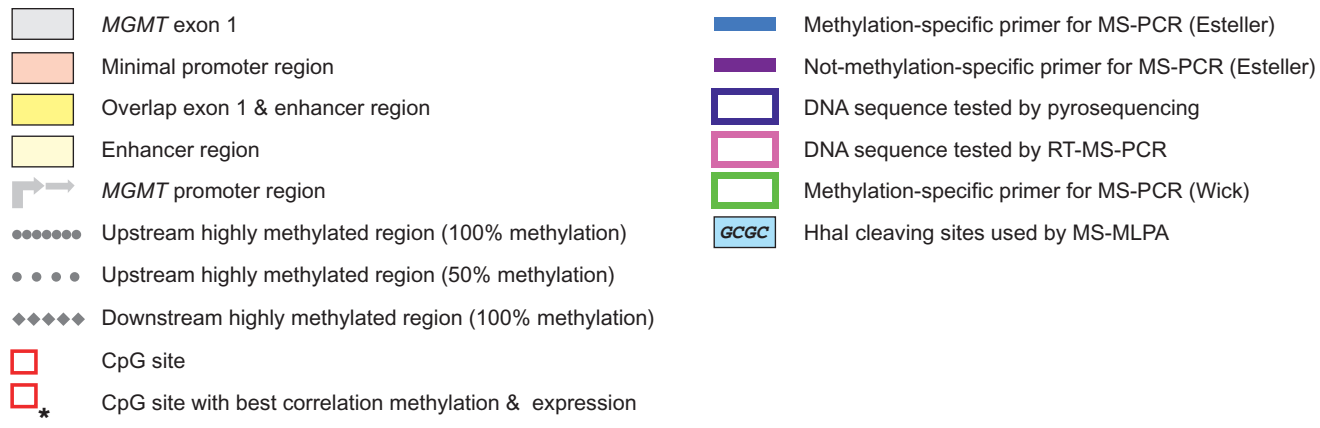


Figure 1. Genomic area covering the promoter, CpG island and exon 1 of O^6-methylguanine-DNA methyltransferase (*MGMT*). Genomic data based on University of California Santa Cruz (UCSC) Genome Browser February 2009 assembly (http://genome.ucsc.edu/), chromosome 10 genomic contig GL000100.1 (GenBank)—a web-based service offered by the NIH, *MGMT* sequence: NM_002412.3 (GenBank), CpG island predicted by UCSC Genome Bioinformatics position chr10: 131 264 949–131 265 710. Whole promoter DNA sequence according to (35). Minimal promoter region, enhancer region, upstream highly methylated region and downstream highly methylated region according to (65). CpG sites with best correlation between methylation status and MGMT expression according to (25). Methylation-specific PCR (MS-PCR) primers according to (23, 39, 99). Pyrosequencing region according to (62). HhaI cleaving sites used for methylation-specific multiplex ligation dependent probe amplification (MS-MLPA) according to (47). Real-time-MS-PC (RT-MS-PCR) region according to (92).

amplification. Depending on numerous factors, PCR efficiency varies, and therefore, the reproducibility is not as high as one would like for a clinical assay.

An improvement on MS-PCR is real-time MS-PCR which allows a higher quantity of standardization, greater throughput and the definition of a cut-off (92). In this variant, a methylation-specific probe has an attached fluorophore and quencher, sitting between primer sites. After the primers hybridize and initiate PCR, the probe is degraded by an exonuclease that acts during the extension phase of PCR. This results in separation of the fluorophore from the quencher, with fluorescence being quantifiable. Results can be expressed as the ratio of methylation-specific amplification of the tumor *MGMT* gene to the *COL2A1* reference gene, that is the methylation index (MI). Based on prior work (90), an MI less than 4 is negative, an MI between 4 and 16 has a low level of methylation with uncertain clinical significance, and an MI greater than 16 is considered methylated.

Another attractive alternative is methylation-specific pyrosequencing (62). This method also requires DNA bisulfite treatment as the initial step, followed by sequencing of a short DNA fragment covering usually five CpG sites using a luciferase-based detection mechanism. Primers can be individually designed, but a commercial kit is available. The method allows analysis of highly fragmented DNA and is therefore robust and reproducible in formalin-fixed, paraffin-embedded clinical tissues. However, methylation-specific pyrosequencing requires expensive equipment and the costs per sample are high if only a few cases are analyzed per run. An unsolved issue is the quantitative character of the results: each of the five tested CpG sites are assigned a value between 0 and 100% which needs to be finally condensed to a qualitative +/− result (Figure 2). No clinical study has yet been produced that allows a valid definition of an algorithm to calculate such a threshold. An example of an ambiguous result is depicted in Figure 2C.

Another method suitable for routine diagnostics that generates quantitative methylation data is methylation-specific multiplex ligation dependent probe amplification (MS-MLPA) (47). MS-MLPA was successfully used to analyze the tumor samples of the large European Organization for Research and Treatment of Cancer (EORTC) 26951 study for *MGMT* promoter hypermethylation (90). MS-MLPA does not require a DNA consuming bisulfite treatment step. After probe annealing and ligation, digestion with the restriction enzyme HhaI only cuts DNA at unmethylated CpG sites. Finally, probes are amplified by PCR and visualized by fragment analysis together with a non-HhaI digested control sample. The short probe annealing sequence should allow analysis of highly fragmented DNA. However, MS-MLPA requires HhaI restriction sites within a CpG island and only one of the three suitable *MGMT* CpG sites are located within the region that is commonly analyzed by MS-PCR or pyrosequencing (Figure 1). Furthermore, similar to pyrosequencing, quantitative MS-MLPA data require an algorithm to calculate a qualitative +/− threshold. In our hands MS-MLPA emerged as a less reliable method when formalin-fixed paraffin-embedded tissue (FFPE) was analyzed.

MGMT protein-based assays

Immunohistochemistry (IHC) would seem to be the most direct and convenient assay to determine amount of actual MGMT protein. Although some studies have reported an association between IHC-based MGMT expression and response to alkylating drugs in gliomas (3, 11, 15, 46, 58), the technique is fraught with problems. In glial tumors, various non-neoplastic cells including lymphocytes, microglia, blood vessel cells and reactive astrocytes all express MGMT, thereby hampering targeted IHC-based evaluation of tumor cells (26). A high interobserver variability has thus been reported and the cut-off levels in the literature for positive staining have ranged from >10% to >50% positive cells (76). Finally, many researchers have failed to identify an association between IHC results and either *MGMT* methylation status based on MS-PCR or patient survival/chemosensitivity (32, 55, 76, 77). Detection of *MGMT* mRNA expression is an alternative to IHC but currently no clinical study is available that has utilized this method. Measurement of enzymatic MGMT activity could potentially provide a more accurate assessment at the mechanistic level, but no study has demonstrated a value to this approach in a clinical setting to date. Both methods suffer from a dependence on fresh frozen tissue and otherwise elaborate, error-prone methodology. Both also suffer the same risk of false-negative "dilution" by non-neoplastic MGMT-producing cells.

CONSEQUENCES

Impact on diagnostic aspects

Due to demand by neuro-oncologists for *MGMT* methylation status on their patients' tumors, some neuropathology departments already offer this test. However, in different labs, various methods of testing across diverse CpG sites are applied and even if MS-PCR is chosen, the same primers and conditions are not always used, making it difficult to assess inter-laboratory concordance rates. As a result, divergent results are sometimes generated if the same tumor is analyzed in multiple laboratories. To avoid doubts regarding the quality of *MGMT* testing, a methodological standardization combined with round-robin testing organized by national neuropathology and molecular pathology societies is recommended. Commercially available MGMT test kits may enhance inter-laboratory reproducibility. An alternative and less desirable approach would

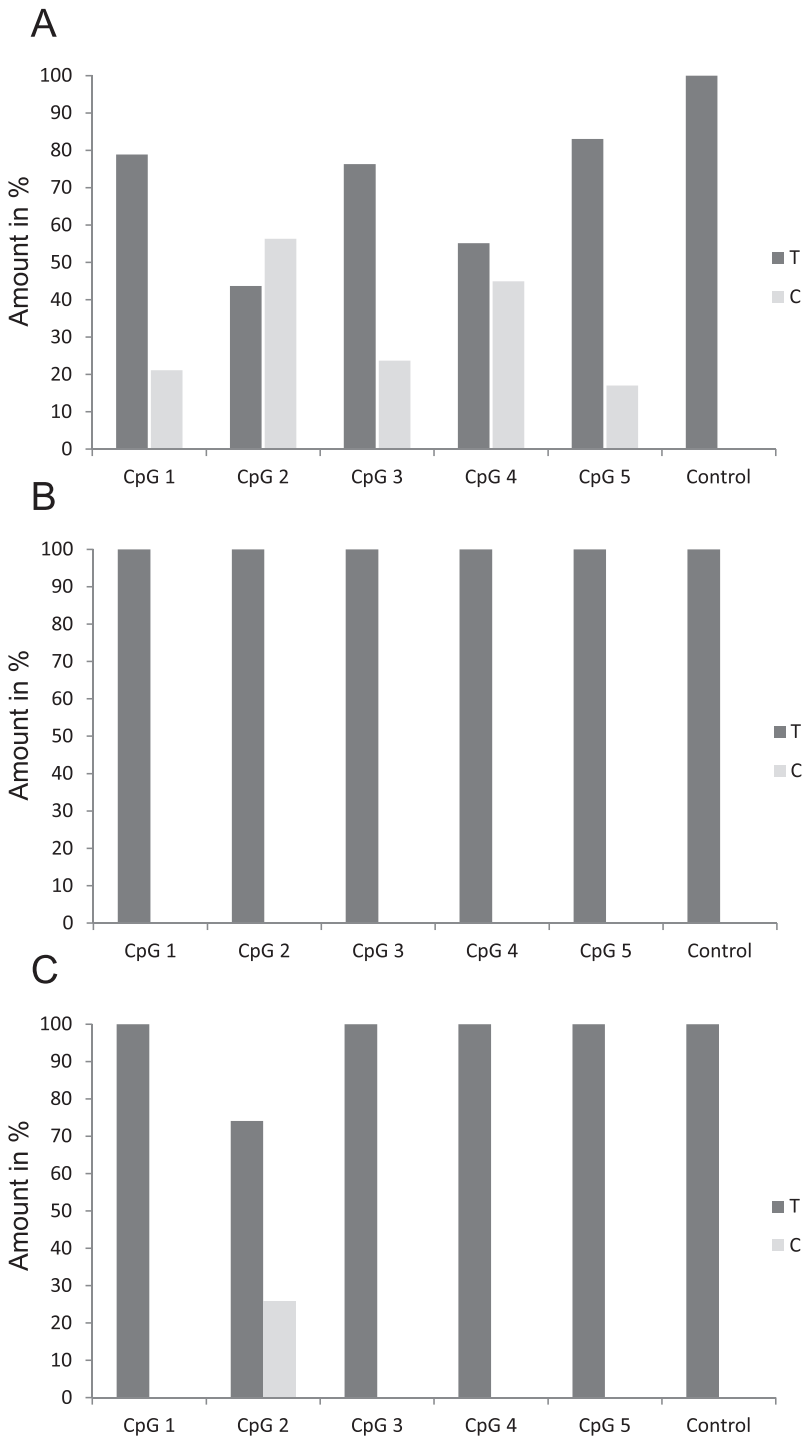


Figure 2. *Bar plot showing different results for* MGMT *promoter hypermethylation analysis by pyrosequencing according to (62). T: amount of thymidine in %. C: amount of cytosine in %. CpG 1—5: the different CpG sites. Control: a cytosine not followed by guanine that always becomes converted to 100% uracil/thymidine when DNA bisulfite treatment was successful.* **A.** Nonambiguous pattern indicating methylation of all five CpG sites between 17% and 53%. **B.** Nonambiguous pattern indicating no methylation of all five CpG sites. **C.** Methylation of only CpG site no. 2 with 26%.

be to delegate *MGMT* promoter hypermethylation analysis to external commercial companies, who are less likely to have skill in glioma tissue evaluation and would not interpret the data in the overall context of other clinicopathological features.

A growing debate in the neuropathology community revolves around whether or not *MGMT* status should be incorporated into the next revision of the WHO classification scheme for central nervous system (CNS) tumors. Multiple studies (see above) clearly indicate that GBM can be separated into two biologically different groups based on *MGMT* status, potentially justifying incorporation of this variable in the next WHO classification. However, molecular assays are still not available worldwide and the WHO classification remains primarily a morphology-based system for tumor stratification, aimed at the broadest audience possible.

Impact on clinical practice

Patients suffering from GBM do appear to benefit from concomitant and adjuvant temozolomide therapy when *MGMT* promoter is hypermethylated (39). However, as already mentioned, the lack of good alternative treatment options for patients with unmethylated GBMs and the small documented number of such patients that still respond to such therapy argues for the commonly utilized approach of trying temozolomide treatment first, independent of the *MGMT* status. Thus, routine *MGMT* analysis does not appear essential for patient management at this time, although *MGMT* testing in new glioma clinical trials has become a *conditio sine qua non* so adequate comparisons with the current standard of care can be made.

Conclusions

Currently, *MGMT* is one of the most requested molecular assays in clinical neuro-oncology. Due to lack of therapeutic alternatives, *MGMT* evaluation is only currently essential for clinical trials, although that is likely to change once effective alternative therapies besides temozolomide are identified. A range of new methodologies have become available for *MGMT* testing that potentially allow higher levels of sensitivity, specificity, robustness, and reproducibility. However, clinical studies that critically compare these new assays to the current "gold standard" of MS-PCR are urgently needed. Furthermore, systematic analyses to identify which CpG sites best reflect treatment outcome and patient survival are still lacking.

BRAF FUSIONS

Brief history

RAF kinases are part of the mitogen-activated protein kinase (MAPK) cascade, a pathway that ultimately leads to the regulation of a wide range of substrates, including transcription factors and other protein kinases that control cell proliferation, differentiation and apoptosis. Oncogenic activation of *BRAF* has been well documented in many tumors (67). However, this usually results from point mutation rather than gene rearrangement, with a hotspot at residue 600 ($BRAF^{V600E}$). An activating rearrangement of *BRAF* in a primary tumor was first reported in four papillary thyroid carcinomas, where an *AKAP9:BRAF* fusion was described (16). Translocations involving *BRAF* have also been reported in large congenital melanocytic nevi (67). Among astrocytic neoplasms, sporadic and hereditary (NF1 associated) pilocytic astrocytomas (PA I) share constitutive activation of the MAPK pathway. A few studies have characterized the proto-oncogene *BRAF* as a particularly important factor in the pathogenesis of PA I in childhood (73, 88). Most notably, duplication of the *BRAF* locus at 7q34 with consecutive up-regulation of *BRAF* expression and MAPK target genes such as *CCND1,* was found in more than 50% of these tumors (73). Several subsequent studies confirmed similar or even higher frequencies of *BRAF* tandem duplications in PA I (2, 28, 42, 45, 48, 49, 56, 83). Moreover, a few studies showed that tandem duplication at 7q34 leads to a fusion between *KIAA1549* and *BRAF* in approximately 70% of these tumors (48, 49, 56, 83).

Distribution of *KIAA1549:BRAF* fusion in tumor entities

The *KIAA1549:BRAF* fusion in PA I has been found across all age groups and in various tumor locations, including cerebellum, cerebral hemispheres, hypothalamus, optic nerve and brain stem, although some studies revealed that *BRAF* duplication/fusion is a more frequent event in cerebellar PA I (28, 48, 49, 83). In contrast, *BRAF* gene fusion is a rare event in diffusely infiltrating gliomas, instead often containing *BRAF* point mutations, whereas, for example, $BRAF^{V600E}$ is found in approximately 25% of grade II–IV pediatric astrocytomas (53, 81). Occurrence and distribution of *KIAA1549:BRAF* fusions in other brain tumor subtypes are still unknown.

Mechanism of action

Genomic sequencing has revealed a few breakpoint variants: *KIAA1549* exon16–*BRAF* exon 9, *KIAA1549* exon 15–*BRAF* exon 9, *KIAA1549* exon19–*BRAF* exon 9, *KIAA1549* exon18–*BRAF* exon 10 and *KIAA1549* exon 16–*BRAF* exon 11 (28, 48, 49, 83, 88). The common thread through all these breakpoint variants is the formation of an oncogenic *BRAF-KIAA1549* fusion incorporating the BRAF kinase domain but lacking the amino-localized autoinhibitory domain. This truncated *BRAF*, is constitutively active (48, 83, 88). While such activity likely is tumorigenic, it is interesting to note that constitutive activation of BRAF can lead to oncogene-induced senescence in slowly growing benign tumors. An alternate mechanism of MAPK pathway activation in PA I constitutes tandem duplication at 3p25 in approximately 2% of cases leading to an in-frame oncogenic fusion between *SRGAP3* and *RAF1*, the latter of which shares high sequence homology with *BRAF* (28, 49, 56). In general, *RAF* gene fusion variants have been found in 80% of PA I and thus constitutes a hallmark aberration in this tumor entity.

Clinical relevance

Prognostic significance of the *KIAA1549:BRAF* fusion, if any, is still unclear, although a few studies have found no differences in survival between tumors with and without *BRAF* duplication/fusion (28, 42, 45, 48). Nevertheless, the frequent *BRAF* alteration in PA I may serve as a novel therapeutic target for pharmacological inhibition of the MAPK pathway, particularly in tumors that are difficult to fully excise surgically (75, 78, 88). Previous *in vitro* studies have revealed that stable silencing of BRAF through lentiviral transduction with inhibition of Map/Erk kinase (MEK)1/2 blocked proliferation and arrested growth of glioma cells (73). In addition, analysis of the *KIAA1549:BRAF* fusion might be very helpful for differential diagnosis between PA I and A II, especially in combination with mutational analyses of the *IDH1/2* genes (53, 81).

METHODS FOR *KIAA1549:BRAF* FUSION ANALYSIS

Fusion detection in genomic DNA

For the mapping of breakpoints on genomic level, long-distance inverse PCR (LDI-PCR) is applied (60). LDI-PCR is based on the

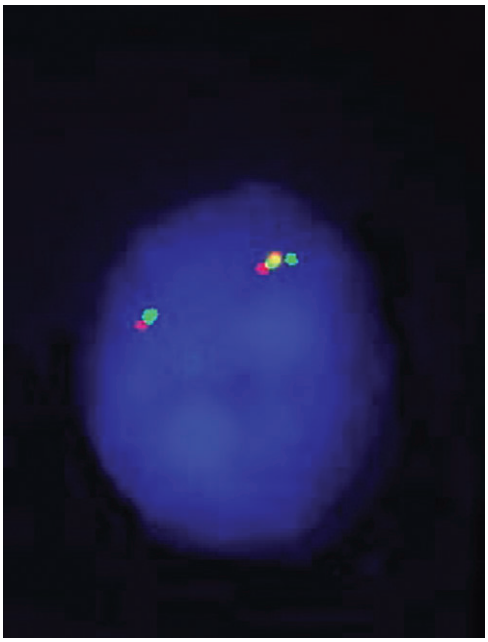


Figure 3. Detection of *KIAA1549/BRAF* fusion by fluorescence *in situ* hybridization (FISH) analysis. Nucleus of pilocytic astrocytoma carrying tandem duplication of *BRAF* (red signals) and *KIAA1549* (green signals) resulting in a fusion of these loci (yellow signal).

hydrolysis of tumor DNA using distinct combinations of restriction enzymes, self-ligation of the resulting DNA fragments and a subsequent PCR reaction using a specific set of oligonucleotides. The analysis is followed by direct-sequencing with corresponding primers, confirming the fusion.

Fusion detection in cDNA

To detect gene fusion, 5′ rapid amplification of cDNA ends (RACE) method is usually applied (48). RACE results in the production of a cDNA copy of the RNA sequence of interest, produced through reverse transcriptase PCR (RT-PCR) assay. The amplified cDNA copies are then sequenced and, if a fusion is present, should map to a unique mRNA that contains exons from both fusion partners (Figure 4).

FISH (fluorescence *in situ* hybridization)

For interphase FISH analysis of *KIAA1549:BRAF* fusion, the simplest strategy is to use two fluorochrome-conjugated probes: a FITC-labeled clone RP11-355D18 corresponding to *KIAA1549* and a digoxigenin-labeled clone 726N20 mapping to *BRAF* (53). The *BRAF-KIAA1549* gene fusion is defined in the cases showing nuclei with a single fusion red-green or yellow signal in addition to the normal pair of split red and green signals (Figure 3). Signals are scored in at least 100 non-overlapping, intact nuclei, and *BRAF* fusion is typically detected in 20%–50% of nuclei in positive cases. Non-neoplastic control tissues do not reveal this pattern.

Conclusions

DNA- and RNA-based methods for detection of *KIAA1549:BRAF* fusion are well-standardized and may be universally applied but snap frozen tissue samples produce the most optimal and reproducible results. Unfortunately, most of these methods require special equipment and are not generally applicable for every day diagnostic practice. In terms of routine application, DNA-based markers for FISH are particularly attractive because of their robustness and applicability to FFPE samples. Nevertheless, some obstacles for universal application of this method exist—such as difficulties in standardization of the optimal cut-off levels for fusion detection, absence of reliable criteria to distinguish real gene fusion from randomly overlapping signals and finally, absence of commercially available probe sets.

IDH1 MUTATIONS

Brief history

IDH1 is no doubt the "new kid in town", receiving considerable attention since the discovery of its relation with human gliomas. Mutations in *IDH1* emerged as a surprising finding from a sequencing project addressing the genome of human GBM with 18/149 of these tumors exhibiting a heterozygous point mutation in codon 132 (70). Interestingly, these *IDH1* mutations were associated with young patient age and the secondary glioblastoma (sGBM) pattern, tumors that evolved from previously confirmed lower grade gliomas. This observation drew attention to A II and anaplastic astrocytoma WHO grade III (A III), both of which were found to carry *IDH1* mutations in the majority of cases (1, 33, 44, 68, 80, 84, 91, 95, 97, 99, 100). Even more provocative was the finding that *IDH1* mutations were just as common in oligodendroglial tumors. Prior to this, much effort had been put in separating astrocytic from oligodendroglial tumors on a molecular level, with the general consensus being that *TP53* mutations are more associated with diffuse astrocytomas while combined 1p/19q deletions predominantly occurred in oligodendroglial tumors. Therefore, the shared trait of *IDH1* mutations in both astrocytic and oligodendroglial gliomas suggests a possible origin of both entities from a common precursor cell type. Apart from A II, A III, O II, anaplastic oligodendroglioma WHO grade III (O III), oligoastrocytoma WHO grade II (OA II), anaplastic oligoastrocytoma WHO grade III (OA III) and sGBM, *IDH1* mutations have been described only in about 10% of acute myeloid leukemias (59, 93). With the exception of rare individual cases, all other neoplasms studied thus far are essentially negative for *IDH1* mutations (4, 50, 79).

Distribution of *IDH1* mutations in tumor entities

Mutations in the cytosolic NADP-dependent isocitrate dehydrogenase (*IDH1*) gene have recently been detected in a fraction ranging between 50% and 80% of astrocytomas, oligodendrogliomas and oligoastrocytomas (1, 41, 44, 84, 95, 100). There is no difference regarding *IDH1* mutation frequency between WHO grade II and WHO grade III tumors, with sGBM also exhibiting a comparable mutation rate. This is contrasted by the distinct rarity of *IDH1* mutations in primary or *de novo* GBMs, arising in the absence of a lower-grade precursor. Interestingly, the rates of *IDH1* mutations in

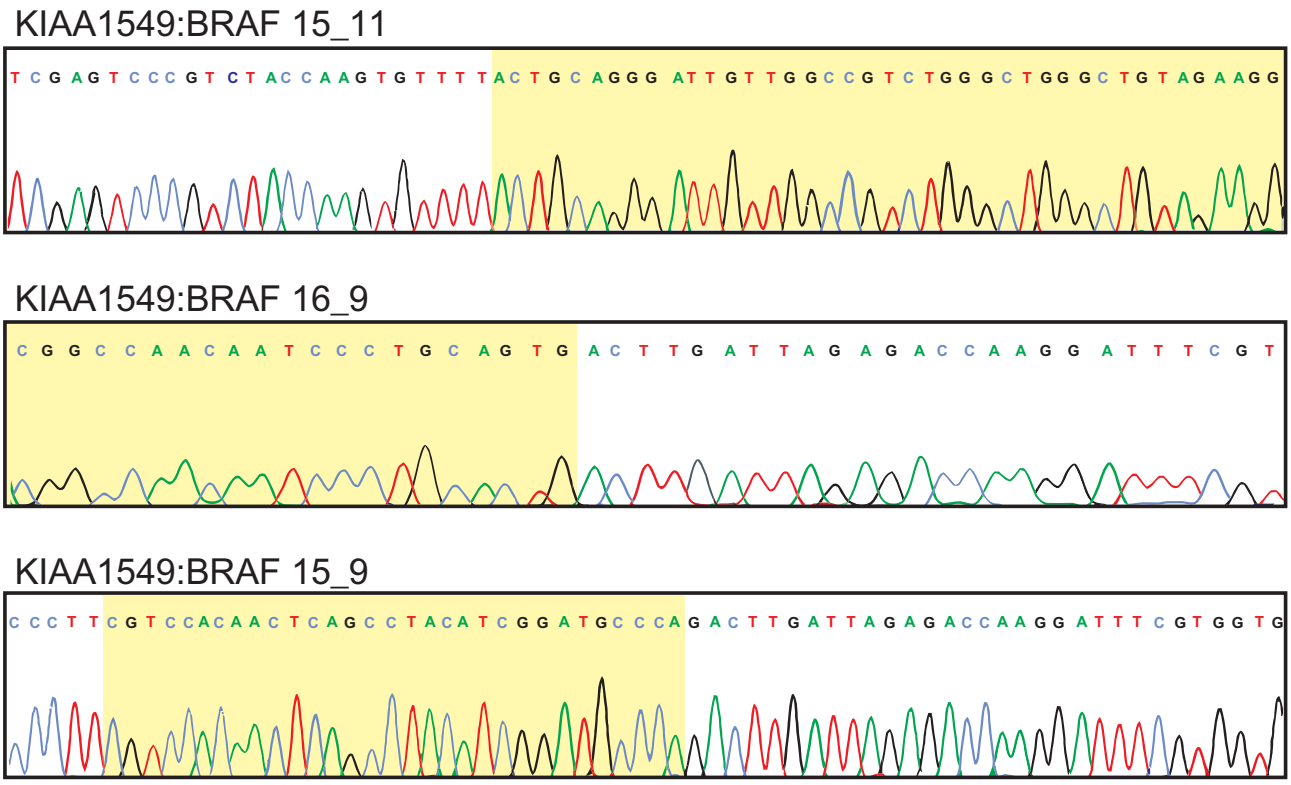


Figure 4. Detection of *KIAA1549/BRAF* fusion by 5′ rapid amplification of cDNA ends (RACE) analysis. Sequence traces consequently demonstrating a fusion between *KIAA1549* exon 15: *BRAF* exon 11, *KIAA1549* exon 16: *BRAF* exon 9 and *KIAA1549* exon 15: *BRAF* exon 9.

PA I and ependymal tumors, as well as in less common glial tumor entities are very low, providing diagnostic utility (1).

IDH1 mutations affect codon 132 in the vast majority of the cases and have been detected in only one of the parental alleles, with the remaining gene copy apparently being wild type. We sequenced the codon 132 region of *IDH1* in more than 3000 primary human brain tumors and detected (as of August 2010) 1212 mutations. Major portions of these data have been reported in prior studies (1, 37, 97, 99), but the distribution of mutation types is summarized in Table 1. In gliomas, approximately 93% of the mutations are of the R132H type, followed by R132C exchanges in 4%, R132S and R132G mutations in approximately 1.5% each, and single R132L mutations.

Table 1. Types of *IDH1* mutations detected in 1212 brain tumors (Heidelberg). Abbreviation: N (%) = number of tumors with mutation and corresponding percentage of mutation type among all mutations.

Nucleotide change	Amino acid change	N (%)
G395A	R132H	1130 (93.2)
C394T	R132C	45 (3.7)
C394A	R132S	16 (1.3)
C394G	R132G	14 (1.2)
G395T	R132L	6 (0.5)
C394G, G395T	R132V	1 (0.1)

This contrasts with the situation in acute myeloid leukemia (AML), the only other tumor entity so far known to carry *IDH1* mutations in a significant portion of tumors, where the distribution of *IDH1* mutations is wider (34, 59, 93). Interestingly, mutations in mitochondrial NADP-dependent isocitrate dehydrogenase (*IDH2*) gene also occur predominantly in astrocytomas, oligodendrogliomas, oligoastrocytomas and AML. *IDH2* mutation are encountered in roughly 3% in gliomas (37), but are more frequent in AML (5, 71, 89).

Mechanism of action

Wild-type *IDH1* decarboxylates isocitrate to α-ketoglutarate (aKG), thereby reducing NADP to NADPH (29). Several concepts for the tumorigenic potential of mutant IDH1 protein have been promulgated. First, the mutation at codon 132 affects the binding site for isocitrate. Hence, it is not surprising that the decarboxylating activity of mutant IDH1 protein is significantly reduced or obliterated (44, 100). Activity drops below 50% because of a dominant effect of the mutation via inhibition of heterodimeric IDH1 complexes (102). Thus, the amount of NADPH equivalents necessary for cellular protection from oxidative stress might be reduced resulting in an increase of reactive oxygen species with subsequent pro-oncogenic DNA damage (57). Another hypothesis suggests that *IDH1* mutations cause reduced production of aKG. Because aKG has prolylhydroxylase activity, it normally promotes degrada-

tion of HIF1-a. Therefore, *IDH1* mutations may promote tumor growth and angiogenesis by reducing inhibition of HIF1-a (102).

However, the hypotheses described above do not entirely explain a peculiarity of *IDH1* mutations, its nearly exclusively heterozygous nature, wherein only one of the two *IDH1* alleles is affected and the other one is perfectly intact, in stark contrast to the two-hit mechanism classically encountered in tumor suppressor genes. Heterozygous *IDH1/2* mutations are more consistent with a gain of function, rather than a loss of function. More recent work has suggested just such a function, in which the mutant enzyme reduces aKG to 2-hydroxyglutarate (2-HG), in the process consuming NADPH. In support of this, gliomas carrying *IDH1/2* mutations have tissue levels of 2-HG that are 1–2 full orders of magnitude higher than comparable wild-type tumors (19). Similarly, the serum of AML patients with *IDH1* or *IDH2* mutations also has markedly elevated 2-HG (33). It bears mentioning, though, that a clear-cut tumorigenic effect of 2-HG has yet to be elucidated.

Clinical relevance

Both the original GBM sequencing work and subsequent studies describing *IDH1* mutations have shown better outcomes in these tumors than grade-matched gliomas lacking this alteration. Analysis of patients enrolled in prospective studies or cohorts have also demonstrated a favorable association of *IDH1* mutation with overall survival in A III (99), O III (91) and GBM (97) patients. From a diagnostic perspective, other studies have established that assays aimed at detecting *IDH1* mutation are fairly sensitive for the presence of a diffuse glioma (vs. reactive gliosis) and are highly specific when other primary CNS tumors are considered in the differential (discussed further below).

METHODS FOR IDH1 ANALYSIS

Current methods of IDH1 analyses usually address either the sequence of the gene or the structure of the protein. However, it also might be possible to focus on analysis of the mutant enzyme product, 2-HG. One such method is gas chromatography-mass spectrometry. Because (so far) all mutations in R132 on *IDH1* and R172 on *IDH2* have been shown to result in the production of 2-HG, this approach might prove to be highly efficient, yet has the obvious limitation of needing a solid sample of unfixed tissue.

Direct sequencing

Nearly all of the published studies rely on sequence analyses from tumor DNA. With development of rapid sequencing facilities, both workload and time required is rapidly decreasing. However, it should be kept in mind that the success of sequencing analysis relies heavily on suitability of the material. Contaminating DNA from adjacent brain tissue, lymphocytic infiltrates, microglial cells and endothelia can dilute mutant DNA below detection thresholds leading to false negative readings. On the other hand, background signals might also reach threshold levels resulting in false positive results. Classic Sanger sequencing requires at least 20% mutant alleles for reliable detection of the mutant. Thus, thorough adjunct histological analysis of tumor tissue prior to DNA extraction is of course mandatory.

Pyrosequencing

Recent studies have detected *IDH1* mutations by pyrosequencing (27, 82). This approach appears as robust as traditional sequencing and allows for rapid analysis and high throughput. A major advantage of pyrosequencing is the quantitative nature of the readings, allowing mutation detection in tissues intermingled with non-tumorous cells. It has been estimated that a concentration of approximately 7% mutant alleles is sufficient for detection. As mentioned above in the context of *MGMT* analysis, though, a key disadvantage of pyrosequencing is the considerable investment in machinery and consumables and the reduced cost efficiency if looking at single samples per run.

dCAPS

An alternative to DNA sequencing is the so-called "derived cleaved amplified polymorphic sequence" (dCAPS) analysis (66). This method relies on the application of mismatched primers, which upon PCR amplification will generate restriction endonuclease sites that will vary depending on whether the mutation is present or not. Consecutive digestion with the appropriate endonucleases will yield DNA fragments of different sizes which can be separated on agarose gels. We have developed a battery of primers that allow the detection of both wild-type *IDH1* and all known R132 mutants, including R132H, R132C, R132G, R132L and R132S (61). While similar DNA quality is needed for this as is needed for sequencing, this approach does not require expensive sequencing facilities. And although only predefined sequence alterations are detectable with this assay, the aforementioned list appears to cover the vast majority of mutant *IDH1*.

Melting curve analysis

Yet another approach to detect *IDH1* and *IDH2* mutations is melting curve analysis performed on real-time PCR products. A suitable protocol has recently been published, utilizing two fluorescent probes, one of which serves as sensor probe either spanning codon 132 of *IDH1* or codon 172 of *IDH2* and a light cycler (43). Having even a single base pair mismatch between the fluorescent probe and the PCR product will result in a lower melting temperature. PCR products from a typical mutated glioma would therefore have two melting peaks, a wild-type sequence showing a higher melting point and a lower one showing a lower melting point. Sequencing can then be targeted to those cases with two peaks to verify the fluorescent melting curve analysis (FMCA). This method, which is already clinically employed in the detection of *KRAS* and *BRAF* point mutations in other tumors, appears to be less vulnerable to non-neoplastic DNA "dilution" such that the detection threshold is roughly 10% mutant alleles.

IHC

The development of an IDH1 R132H mutation-specific antibody (DIANOVA, clone H09) suitable for FFPE tissue immediately made IDH1 analysis in routine specimens accessible and relatively simple even for modestly-sized pathology laboratories (13, 14). A

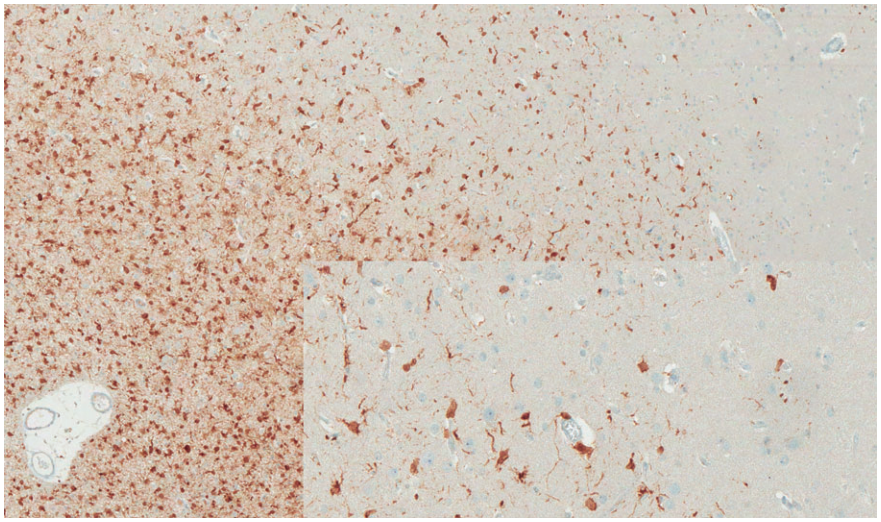


Figure 5. Binding of antibody H09 to tumor cells in infiltrating edge of oligodendroglioma.

caveat is that clone H09 is highly specific to R132H and does not recognize wild-type sequence or the C, S, L or V amino acid substitutions in codon 132. Thus, approximately 93% of brain tumor-associated *IDH1* mutations in codon 132 are readily detectable, while the remaining 7% are missed, as are all *IDH2* mutations. In our hands, however, we found the detection rate of R132H mutations by IHC to be slightly higher than that recovered by sequencing. This is due to the ability to detect single tumor cells (Figure 5), which cannot be accomplished in a practical way by sequencing. Our routine procedure for detection of *IDH1* mutation is therefore an initial IHC screen, followed by DNA sequencing of cases negative or equivocal with the H09 antibody. In order to capture as many possible relevant tumors as possible, those cases are also sequenced for *IDH2* mutations.

CONSEQUENCES

Impact on diagnostic aspects

Knowledge of the *IDH1* status is of both diagnostic and clinical relevance. On diagnostic grounds, *IDH1* status greatly assists classification of gliomas. For example, it clearly separates oligodendroglial tumors from several entities that are sometimes difficult to distinguish, including central (or extraventricular) neurocytoma, tanycytic ependymoma, PA I with oligodendroglial-like cytology and dysembryoplastic neuroepithelial tumor (DNT), all of which are characterized by the absence of *IDH1* mutations (12). Because *IDH1* mutations do not occur in reactive gliosis, conditions producing reactive gliosis can often be separated from

Table 2. *IDH1* mutations in gliomas from the Heidelberg series.

Diagnosis	N	Mut (%)
Astrocytoma WHO grade II (A II)	299	225 (75.3)
Anaplastic astrocytoma WHO grade III (A III)	339	213 (62.8)
Secondary glioblastoma WHO grade IV (secGBM)	16	12 (75)
Oligodendroglioma WHO grade II (O II)	196	160 (81.6)
Anaplastic oligodendroglioma WHO grade III (O III)	227	158 (69.6)
Oligoastrocytoma WHO grade II (OA II)	125	99 (79.2)
Anaplastic oligoastrocytoma WHO grade III (OA III)	260	184 (70.8)
Primary Glioblastoma WHO grade IV (prGBM)	518	42 (8.1)
Giant cell glioblastoma WHO grade IV (gcGBM)	10	2 (20)
Pediatric Glioblastoma WHO grade IV (pedGBM)	13	1 (7.7)
Gliosarcoma WHO grade IV (GS)	10	0
Ependymoma WHO grade II (E II)	17	0
Anaplastic ependymoma WHO grade III (E III)	11	0
Pilocytic astrocytoma WHO grade I (PA I)	114	2 (1.7)
Subependymal giant cell astrocytoma WHO grade I (SEGA I)	12	0
Pleomorphic xanthoastrocytoma WHO grade II (PXA II)	9	0
Ganglioglioma WHO grade I (GG I)	22	0
Dysembryoplastic neuroepithelial tumor WHO grade I (DNT)	21	0
Central neurocytoma WHO grade II	35	0

the frequently mutated diffuse gliomas. The potential of antibody H09 to detect single cells is sometimes of great assistance in detection of tumor in very small samples or in specimens originating from the infiltrating tumor edge. Further, considering the frequency of R132H among *IDH1/2*-mutated gliomas, screening with H09 IHC is expected to reduce the need for sequencing by approximately 90%. An overview of the distribution of *IDH1* mutations in human brain tumors is given in Table 2.

Impact on clinical aspects

Our recent analyses on patients enrolled in the NOA-04 study (99) and the German Glioma Network (97) revealed that *IDH1* status was of higher prognostic relevance than WHO diagnosis within a set of tumors consisting of A III and GBM. In this series, the order of most favorable to least was: A III with mutation; GBM with mutation; A III without mutation; and GBM without mutation (36). We believe that these findings may have imminent importance for the classification of A III and GBM and therefore, also on further management of these patients. There may also be a favorable prognostic effect of *IDH1* mutations in A II, OA II and O II (20, 80).

Conclusions

The recognition of *IDH1* mutations in gliomas has already greatly extended our understanding of these tumors, in particular underscoring the probable role of metabolism in gliomagenesis. The presence of *IDH1* mutations in both diffuse astrocytic and oligodendroglial gliomas highlights the likely common origin of these entities, which have been mostly set apart from each other by molecular markers for the last two decades. Diagnostic challenges may be better met by knowledge of the *IDH1* status. The high relevance of *IDH1* status on clinical outcome of patients with A III and GBM is likely to prompt revisions of the current WHO classification of brain tumors. We expect that on grounds of the strong prognostic effect of *IDH1* status on survival, future clinical studies on diffuse gliomas and GBM will uniformly include this analysis. These implications are of considerable interest to neuropathologists and clinicians, suggesting that determination of the *IDH1* (and *IDH2*) status will be routinely performed in the near future.

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