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Laboratory Testing for Prognostic and Predictive Markers in Gliomas

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Additional information is available at the end of the chapter

<http://dx.doi.org/10.5772/52670>

1. Introduction

Gliomas are the most common tumors of the brain. Normal glia includes astrocytes, oligodendrocytes, and ependyma. Gliomas are analogously designated as astrocytomas, oligodendrogliomas, and ependymomas to reflect the non-neoplastic cell types that they most closely resemble.

Gliomas can also be classified histologically as astrocytomas, oligodendrogliomas, or tumors with morphological features of both astrocytes and oligodendrocytes. The 2007 World Health Organization (WHO) classification recognizes three main histologic types of low grade diffuse glioma grade II: diffuse astrocytoma, oligoastrocytoma, and oligodendroglioma [1]. Diffuse astrocytomas account for approximately 40% of primary intracranial tumors. Their gross, microscopic, and biologic characteristics vary to a considerable degree according to their site. They occur at all ages, although the median age is 30 to 40 for astrocytoma (grade II), 40 to 50 for anaplastic astrocytoma (grade III), and 50 to 60 years for glioblastoma multiforme (grade IV). Diffuse astrocytomas tend to progress to more malignant histologic types, such as anaplastic astrocytoma (WHO grade III) and sometimes secondary glioblastoma (WHO grade IV). Oligodendrogliomas account for approximately 5% of intracranial gliomas. They are most often found in the cerebral hemispheres, where they usually involve the cortex and the white matter. Traditionally the prognosis of oligodendrogliomas has been regarded as relatively favorable, but in practice no valid correlation has been established between the microscopic appearances of these tumors and their clinical evolution. Progression of oligodendrogliomas to anaplastic oligodendrogliomas can be unpredictable. Glioblastomas are malignant, rapidly fatal, astrocytic neoplasms. Glioblastomamultiforme (GBM), account for 30% of primary brain tumors in adults [2, 3]. Patients with glioblastomamultiforme have a mean survival of about 12 months. They may occur in any

region of the central nervous system, however, the cerebral hemispheres, in particular the frontal lobes or temporal lobes, the basal ganglia, and the commissural pathways are sites of predilection. Most GBMs are diagnosed as de novo or primary tumors and are more common in males. A subset of about 5% of GBM tumors, termed secondary GBM, progress from lower-grade tumors (grade II/III), are seen in younger patients, are more evenly distributed among the sexes, and exhibit longer survival times [4]. Depending on the grade and morphologic type of glioma, newly diagnosed patients receive watchful waiting, surgical resection, radiotherapy, or chemotherapy, or some combination of these therapies. Chemotherapy for GBM has very limited efficacy, however it has been shown that certain patients may respond to some treatments [5, 6]. Temozolomide is a novel alkylating agent that has demonstrated activity in recurrent gliomas [7-9]. Regardless of therapy, most patients will progress and have a high risk of mortality and reduced quality of life. For these reasons there has been a great deal of interest in understanding the biology and genetics of gliomas, to provide better diagnostic tools and new therapeutic approaches [10]. Molecular pathology markers are being identified that have been or will soon prove to be clinically useful in treatment of glioma patients.

Molecular genomic-based laboratory assays are often used for detection of prognostic and predictive markers in gliomas. Research efforts have identified a number of cytogenetic and molecular genetic alterations in gliomas [11] that may be exploited to facilitate glioma classification, especially in cases that exhibit inconclusive or borderline histologic features. This chapter will focus on those molecular biomarkers that have been established in glioma diagnostics, namely *MGMT* promoter methylation, 1p/19q Loss of Heterozygosity (LOH), *IDH1* and *IDH2* mutations, and epidermal growth factor variant III (*EGFRvIII*) mutations. Emphasis will be placed on clinical applications, most frequently used methods of detection, as well as issues involved in assay validation, specimen selection, and clinical laboratory oversight.

1.1. *MGMT* promoter methylation

Chemotherapy for GBM has very limited efficacy, however it has been shown that certain patients may respond to some treatments. Temozolomide is a novel alkylating agent that has demonstrated activity in recurrent gliomas. Alkylating agents cause cell death by forming cross-links between adjacent strands of DNA due to alkylation of the O⁶ position of guanine. The O⁶-methylguanine-DNA methyltransferase (*MGMT*) gene produces the cellular DNA repair protein O⁶-alkylguanine DNA alkyltransferase (AGT) which is a key factor in resistance to alkylating agents. It functions as a DNA repair enzyme that removes the mutagenic alkyl-adducts from the O⁶-position of guanine, and this transfer of alkyl groups to AGT prevents the formation of lethal cross links in DNA. Tumors appear to be heterogeneous with respect to *MGMT* expression, and in a subset of cancer cells, its expression is silenced due to abnormal promoter methylation. Aberrant methylation of CpG islands located in the promoter region of *MGMT* gene is associated with transcriptional inactivation of this gene, and consequent low levels of the *MGMT* DNA repair enzyme. Studies have shown that patients with low levels of this DNA repair enzyme are more likely to experience response to therapy and prolonged overall and disease free survival [12-15].

DNA methylation (for more detailed description and references please see [16]) is a mechanism by which the cell regulates gene expression. Methylation is an enzyme mediated modification that adds a methyl ($-CH_3$) group at a selected site on DNA or RNA. In humans, methylation occurs only at cytosine (C) bases followed by a guanosine (G), also known as CpG dinucleotides. The CpG dinucleotides are prone to spontaneous mutations and have been selectively depleted from mammalian genome. However, some regions of DNA have retained CpG dinucleotides and are referred to as CpG islands. The CpG islands are found primarily in the 5' region of expressed genes, often in association with promoters. When the promoter CpG island is methylated, the corresponding gene is silenced and transcription does not occur. Aberrant CpG island methylation of tumor suppressor genes is frequent in cancer and appears to be an important mechanism of neoplastic transformation.

Quantitative evaluation on methylated *MGMT* in tumors suggests that not all cells in a tumor positive for promoter methylation carry a methylated *MGMT* allele. This raises the question of what level of promoter methylation has clinical significance. A recent study [17] investigated the degree and pattern of *MGMT* promoter methylation in paired samples of glioblastoma tissue and glioblastoma-derived spheres. The degree and density of *MGMT* methylation was then compared with the chromatin structure of *MGMT*, gene dosage, gene expression and enzyme activity, and the tumor cell content of the patient samples. Ten paired samples were evaluated for the extent and density of methylation by clone sequencing 28 of 97 CpGs in the CpG island of the *MGMT* promoter. This region of the promoter encompasses the enhancer element and, according to reporter assays, is associated with complete silencing of the gene when fully methylated [18]. Most assays interrogate CpG methylation in this region. For all glioblastomas with *MGMT* methylation, a band for unmethylated alleles was also detectable, which is expected since benign cells will always be present in the specimen. Sequencing of the original glioblastoma tissue revealed that 10% to 90% of all clones sequenced showed dense methylation, arbitrarily defined as at least 4 consecutive CpGs methylated in a given interrogated clone. The density of *MGMT* promoter methylation, defined as the number of methylated CpGs over 28 interrogated CpGs, ranged from 25% to 90%, never reaching 100%, and showing a characteristic pattern for each tumor.

The study [17] further showed that *MGMT* methylation is associated with no or low *MGMT* expression and closed chromatin structure. *MGMT* activity was below the limit of detection in glioblastoma spheres of completely methylated cases. Moderate activity was measured in the case with one unmethylated allele, and the unmethylated case showed the highest activity. *MGMT* activity was measurable in all respective original tissues likely due to contaminating normal cells. In accordance with lack of *MGMT* expression and *MGMT* activity, the closed chromatin pattern was also observed. Marks of active and inactive chromatin pattern were observed for unmethylated and partially methylated cases.

As combined chemoradiotherapy comprising the alkylating agent temozolomide has become the new standard of care [19], there has been growing interest to use *MGMT* promoter methylation status for individual patient management, and for patient stratification or selection in clinical trials. Knowledge of *MGMT* promoter methylation status is relevant for both prognostic and predictive considerations. Furthermore, *MGMT* promoter methylation status

has been used as a stratifying factor or eligibility criterion in ongoing and accruing clinical trials [20].

Aberrant methylation of CpG islands in the promoter region of many genes has been recognized as an important epigenetic mechanism for gene silencing [21-24]. Inactivation of multiple tumor suppressor genes by aberrant hypermethylation is a fundamental process involved in the development of many malignant tumors [25, 26]. Mapping of methylation patterns in CpG islands has become an important tool in understanding tissue-specific gene expression in both normal and pathologic situations, and several protocols have been published for evaluating methylation status by methylation-specific PCR (MSP). Most of these protocols are based on bisulfite treatment of isolated DNA [27, 28]. Bisulfite treatment chemically changes unmethylated, but not methylated, cytosines to uracil. Methylated DNA can be distinguished from unmethylated DNA using sodium bisulfate treatment of DNA, which converts unmethylated C to uracil (U) but leaves methylated C intact. Detection of methylation or lack of it involves analysis of bisulfite-treated DNA using primer pairs that specifically identify either methylated or unmethylated DNA.

The methylated and unmethylated sequences are detected through the use of methylation-specific primers.



Figure 1. MGMT promoter methylation detection by methylation specific PCR. Methylated bands (M) can be seen in samples S1 and S2 and in methylated control (M). Unmethylated bands (U) are seen in S1, S2, S3, and in negative (unmethylated) control

Methylation Specific PCR (MSP): Methylation is a chemical modification that adds methyl (CH₃) groups at selected sites on protein, DNA and RNA. In humans, DNA methylation only affects the cytosine base (C) when it is followed by a guanine (G). Most CpG islands have been observed in the 5' promoter regions of genes. When promoter CpG islands become methylated, the associated gene is silenced. Small amount of DNA, including those from paraffin embedded tissue, can be used for testing. DNA is first treated with bisulfite which converts unmethylated, but not methylated, cytosine to uracil. This modified DNA is then used as a template for PCR. The sequence differences between methylated and unmethylated DNA after bisulfite treatment allow the designing of PCR primers that are specific for each type. The primers are intended to amplify the identified region of the promoter mentioned above. PCR products are detected by gel electrophoresis or by capillary electrophoresis.

Methylight Protocol: The Methylight assay utilizes the TaqMan PCR principle which requires forward and reverse primers as well as an oligomeric probe which emits fluorescence

only after it is degraded by the 5'-3' exonuclease activity of Taq polymerase. MethyLight protocol is a simple, real-time PCR method to determine the methylation status of CpG islands. Collagen 2A1 (COL2A1) gene is used as the internal reference (amplification control) to assess the quality and quantity of input DNA.

Pyrosequencing: This is a method of DNA synthesis based on sequencing by synthesis principle. The procedure involves taking a single strand of the DNA to be sequenced and then synthesizing its complementary strand enzymatically. The pyrosequencing method is based on detecting the activity of DNA polymerase with another chemiluminescent enzyme [29, 30]. The pyrosequencing reaction occurs in 5 steps. In step 1, a sequencing primer is hybridized to a single-stranded amplicon that serves as a template, and incubated with the enzymes DNA polymerase, ATP sulfurylase, luciferase, and apyrase, as well as substrates adenosine 5' phosphosulfate and luciferin. In subsequent steps triphosphates are added to reaction and each incorporation event is accompanied by release of pyrophosphate (PPi). The PPi is subsequently converted to ATP which then drives conversion of luciferin to oxyluciferin that generates visible light. Addition of dNTPs is performed sequentially. As the process continues, the complementary DNA strand is built up and the nucleotide sequence is determined from the signal peaks in the pyrogram trace.

Methylation-Specific MLPA (MS-MLPA): The multiplex ligation-dependent probe amplification (MLPA) method allows for multiplex detection of gene copy number aberrations in a routine laboratory. The methylation-specific MLPA (MS-MLPA) can detect changes in both CpG methylation as well as copy number of up to 40 chromosomal sequences in a simple reaction. In MS-MLPA, the ligation of MLPA probe oligonucleotides is combined with digestion of the genomic DNA-probe hybrid complexes with methylation-sensitive endonucleases [31, 32]. MS-MLPA is not based on bisulfite conversion of unmethylated cytosines, can provide methylation status, is semiquantitative, and can be used to evaluate methylation status of multiple sequences simultaneously. Furthermore, it allows for a combined copy number detection and methylation-specific analysis.

1.2. 1p/19q Loss of heterozygosity (LOH)

Among the major subtypes of gliomas, oligodendrogliomas are distinguished by their remarkable sensitivity to chemotherapy, with approximately 70% of anaplastic (malignant) oligodendrogliomas responding dramatically to treatment with procarbazine, lomustine, and vincristine (termed PCV) [33]. Unfortunately, no clinical or pathologic feature allows accurate prediction of chemotherapeutic response. The prognosis for grade II oligodendrogliomas is significantly better than that for grade II astrocytomas, with average survival times of 10 to 15 years. As with astrocytomas, there is considerable individual variability in time to progression and overall survival. The average survival for anaplastic grade III oligodendrogliomas is 3 to 5 years, although some patients with genetically favorable subset may survive 10 years or longer. The histologic distinction between oligodendroglioma and astrocytoma is often highly subjective, and there has been significant interobserver variation.

Patients with oligodendroglial tumors are often stratified into therapeutic groups according to age, extent of resection, tumor grade, and 1p/19q status. The current national Comprehen-

sive Cancer Network (NCCN) guidelines for central nervous tumor cancers recommend testing for 1p/19q codeletion or unbalanced translocation, for cases of suspected oligodendroglioma. The test is also recommended to distinguish anaplastic oligodendroglioma from anaplastic astrocytomas and glioblastomas. Recent studies [34-36] have shown that: (1) - allelic loss (loss of heterozygosity) of chromosome arm 1p is a statistically significant and currently the best predictor of chemosensitivity; (2) combined loss involving chromosome arms 1p and 19q predicts both chemotherapeutic response and longer survival in patients with oligodendrogliomas; and (3) combined allelic loss of chromosomes 1p and 19q can be considered a molecular signature of oligodendroglioma (present in approximately 70-80% of oligodendroglial tumors and in only 10% of astrocytomas). Anaplastic oligodendrogliomas with loss on 1p, or combined loss on 1p and 19q usually respond favorably to chemotherapy, with about half of such tumors showing complete neuroradiological response. Owing to the major prognostic significance of the 1p/19q status in patients with anaplastic gliomas treated with radio and/or chemotherapy, ongoing prospective trials are no longer stratifying anaplastic glioma patients according to histological types but according to the 1p/19q deletion status [37]. Detection methods for 1p and 19q deletion include polymerase chain reaction (PCR), fluorescent in situ hybridization (FISH), array comparative genomic hybridization, and multiplex ligation-dependent probe amplification (MPLA) [38-40].

| Primary Markers | | Back up markers | |
|----------------------|--------------|----------------------|--------------|
| Microsatellite Locus | Abbreviation | Microsatellite Locus | Abbreviation |
| D1S548 | 1-1 | D1S468 | 1-2 |
| D1S592 | 1-3 | D1S1612 | 1-5 |
| D1S552 | 1-4 | D1S496 | 1-6 |
| D19S219 | 19-1 | | |
| D19S412 | 19-2 | D19S606 | 19-3 |
| PLA2G4C | 19-5 | D19S1182 | 19-4 |

Table 1. Primary and back up microsatellite markers for detection 1p and 19q deletion

1.3. 1p/19q LOH - Microsatellite-based method of detection

Allelic loss is assessed by PCR assay in normal DNA / tumor DNA pairs using markers at both 1p and 19q. For this type of testing, a patient's blood sample is needed to accurately assess patient's genotype and establish the normal DNA baseline. Because there might be partial as well as complete deletions, any reductions in tumor peaks can then accurately be interpreted as deletions. The 6 markers on 1p and the 5 markers on 19q are microsatellites (2 or 4 nucleotide repeats) except PLA2G4C which is a minisatellite (26 nucleotide repeat) polymorphism. The markers were selected based on heterozygosity score, amplicon size, and ease of interpretation. LOH at all informative loci on each chromosomal arm represents the typical finding in oligodendrogliomas with 1p and 19q deletion. To streamline the work

flow process, a set of primary markers is tested first. If at least two markers for 1p and two markers for 19q show either no deletion or clear deletion, the results can be considered valid. If the results with primary markers are not clear, either because the markers are not informative (homozygosity), or they did not amplify, then the back up markers are used in subsequent PCR reaction.

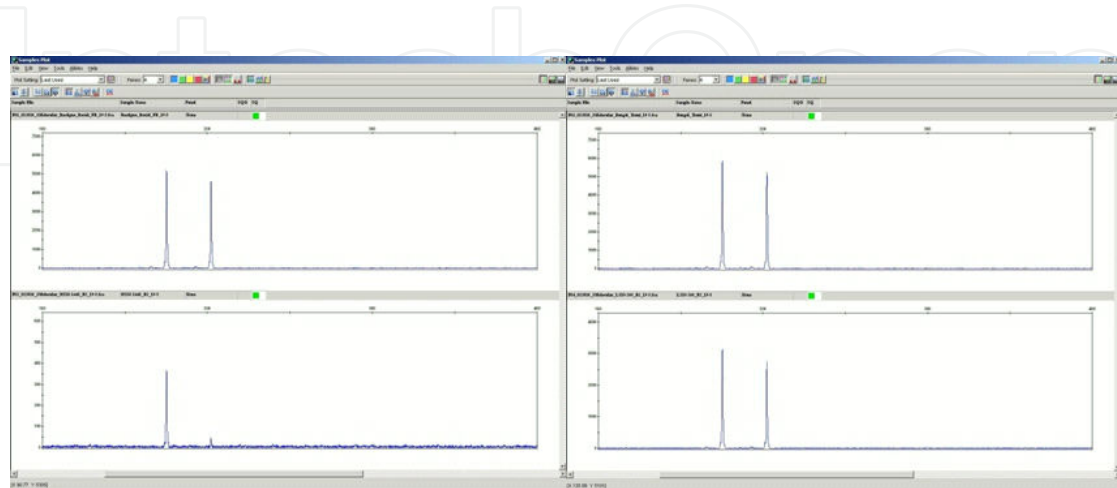


Figure 2. The tumor sample on the left demonstrates 1p deletion. Normal DNA baseline is on the top.

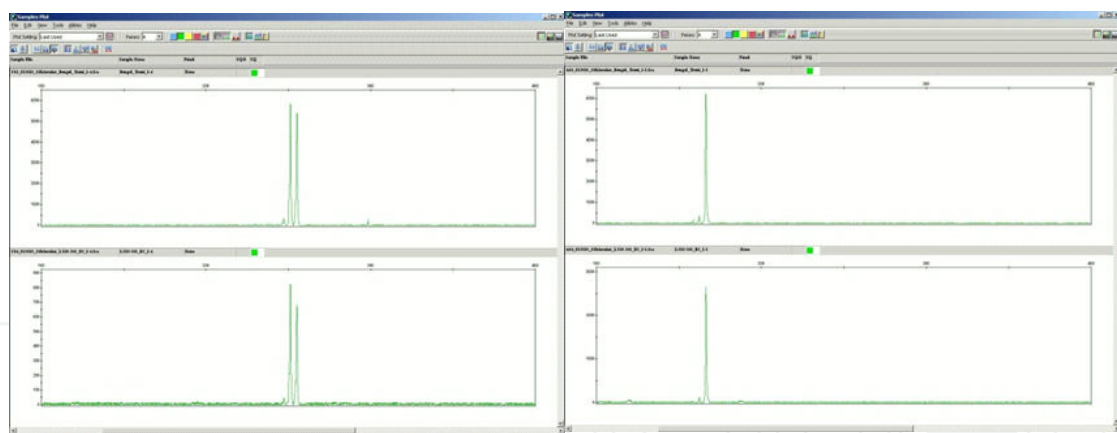


Figure 3. Sample on the left demonstrates two alleles both in base line sample and in tumor sample. The sample on the right has a non-informative microsatellite marker.

Array Comparative Genomic Hybridization (aCGH) - aCGH is a technique to detect genomic copy number variations at a higher resolution level than chromosome-based comparative genomic hybridization (CGH). DNA from a test sample and normal sample are labeled differentially, using different fluorophores, and hybridized to several thousand probes. The probes are derived from known genomic sequences and are printed on glass slides. The fluorescence intensity of the test and of the reference DNA is then measured to calculate the ratio between them and subsequently the copy number changes for a particular location in

the genome. This method allows one to detect microdeletions and chromosomal duplications and is used with increasing frequency for loss of heterozygosity detection [41].

1.4. IDH1 and IDH2 mutations

Point mutations in the cytosolic isocitrate dehydrogenase enzyme gene (*IDH1*) or the mitochondrial version of the same gene (*IDH2*) are frequently detected in low grade astrocytomas, oligodendrogliomas and in secondary glioblastomas. *IDH1* is involved in the metabolic conversion of isocitrate to alpha-ketoglutarate, which reduces NADP to NADPH. In gliomas, mutations in this gene were discovered through large scale DNA sequencing of tumor samples [42]. Among WHO grade II and grade III gliomas, 50% to 80% have mutated *IDH1*, whereas 5% to 10% of WHO grade IV gliomas carry *IDH1* mutation. Studies of clinical trial samples of low grade gliomas showed that mutated *IDH1* may be both prognostic and predictive, because it was associated with longer survival times and better response to temozolomide therapy [43, 44]. Another study with astrocytoma showed an association with improved survival but not with response to temozolomide [45]. Further clarification of the correlation of chemotherapy response and *IDH1* mutational status will be needed for this marker to receive broad clinical use. At present, lack of *IDH1* mutation can not be considered strong enough evidence to alter therapy.

Because the presence of an *IDH* mutation is considered tumor specific, it has a role as a diagnostic marker when morphologic features are inconclusive and a non neoplastic (reactive) condition is possible.

| Nt# | | Nucleotide Change | Amino Acid Change | Frequency (%) (Hartman, 2009) |
|-----|------|--------------------|---------------------|----------------------------------|
| 395 | IDH1 | G395A CGT à CAT | Arg132His, R132H | 92.7 |
| 394 | " | C394T CGT à TGT | Arg132Cys, R132C | 4.2 |
| 394 | " | C394A CGT à AGT | Arg132Ser, R132S | 1.5 |
| 394 | " | C394G CGT à GGT | Arg132 Gly, R132G | 1.4 |
| 395 | " | G395T CGT à CTT | Arg132Leu, R132L | 0.2 |
| 515 | IDH2 | G515A AGG à AAG | Arg172Lys, R172K | 64.5 |
| 515 | " | G515T AGG à ATG | Arg172Meth, R172M | 19.3 |
| 514 | " | A514T AGG à TGG | Arg172Trp, R172W | 16.2 |

Table 2. IDH 1 and IDH2 mutations and resulting amino acid changes

Sanger Sequencing - The ability to sequence DNA has been essential to the field of molecular pathology because sequence information is needed for primer design in PCR-based assays, for determination of target sequence, and for detection of any changes such as mutations, insertions and deletions in DNA sequence. The method for DNA sequencing developed by Sanger [46] is the basis for most DNA sequencing currently performed in clinical molecular laboratories. The Sanger sequencing reaction (for more detailed description see [16]) uses a single DNA primer and DNA polymerase with linear amplification rather than the exponential amplification of PCR. Components essential to the Sanger sequencing reaction include: (1) an electrophoresis technique capable of clearly distinguishing single nucleotide length differences in DNA strands dozens or hundreds nucleotides in length, (2) sequence-specific complementary primers, with one primer used in the forward reaction and the other used in reverse reaction for each DNA template strand, and (3) the addition of small proportions of dideoxynucleoside triphosphates (ddNTPs) in addition to the conventional deoxyribonucleoside triphosphates (dNTPs) used in polymerase chain reaction. Dideoxynucleotides differ from deoxynucleotides by having a hydrogen atom attached to the 3' carbon rather than an OH group, which is present on the deoxynucleotide. Because the ddNTPs lack a 3'-hydroxyl group, elongation of the newly polymerized chain cannot occur once a ddNTP has been incorporated. The end result is a set of fragments of different lengths complementary to the parent DNA strand. The sequencing reaction products are most frequently detected by capillary electrophoresis on a DNA sequencing instrument.

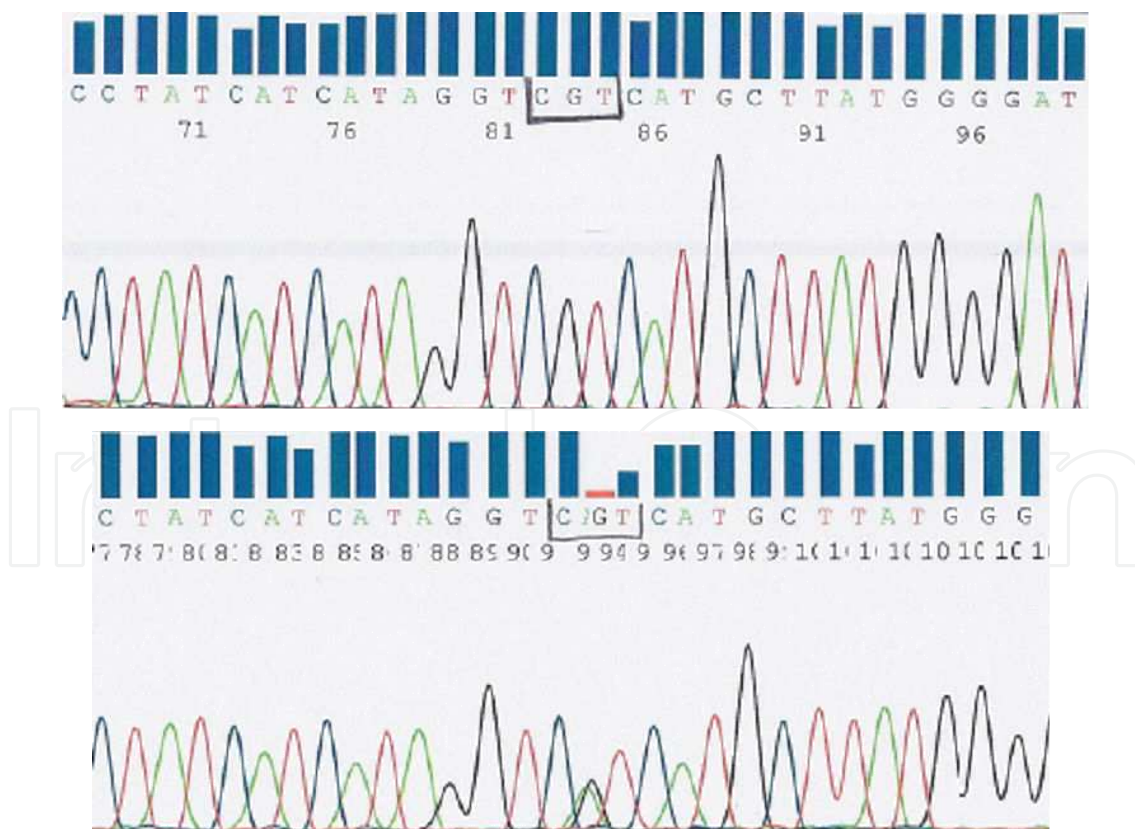


Figure 4. Sanger sequencing for *IDH1* mutation detection: *IDH1* wild type sequence (top) and CGT>CAT, p.R132H mutant (bottom)

Immunohistochemistry: Approximately 90% of IDH1 mutated proteins can be detected by using immunohistochemistry with a monoclonal antibody that detects p.R132H, the most common IDH1 mutation [47].

1.5. EGFRvIII mutation detection in glioblastomamultiforme

The epidermal growth factor receptor (EGFR) is an attractive molecular target in glioblastoma because it is amplified, overexpressed, and/or mutated in up to 40% to 50% of patients. EGFR variant III (EGFRvIII) is an oncogenic, constitutively active mutant form of EGFR that is commonly expressed in glioblastoma. *EGFRvIII* is generated by in-frame genomic deletion of 801bp from exons 2 to 7 of the coding region of *EGFR* which produces a truncated receptor lacking a portion of extracellular ligand binding domain. *EGFRvIII* mutations in gliomas typically lead to unique signal transduction properties to the receptor, particularly enhanced downstream activation of a phosphatidylinositol 3'-kinase (PI3K) signaling pathway with concurrent loss of PTEN. Cell culture and in vivo models of glioblastoma have demonstrated *EGFRvIII* as defining prognostically distinct subgroups of glioblastomas. Additionally, the presence of *EGFRvIII* has been shown to sensitize tumors to EGFR tyrosine kinase inhibitors when the tumor suppressor protein PTEN is intact [48, 49].

Expression of EGFRvIII is associated with favorable clinical response to the EGFR kinase inhibitors gefitinib and erlotinib when the tumor suppressor protein PTEN is intact

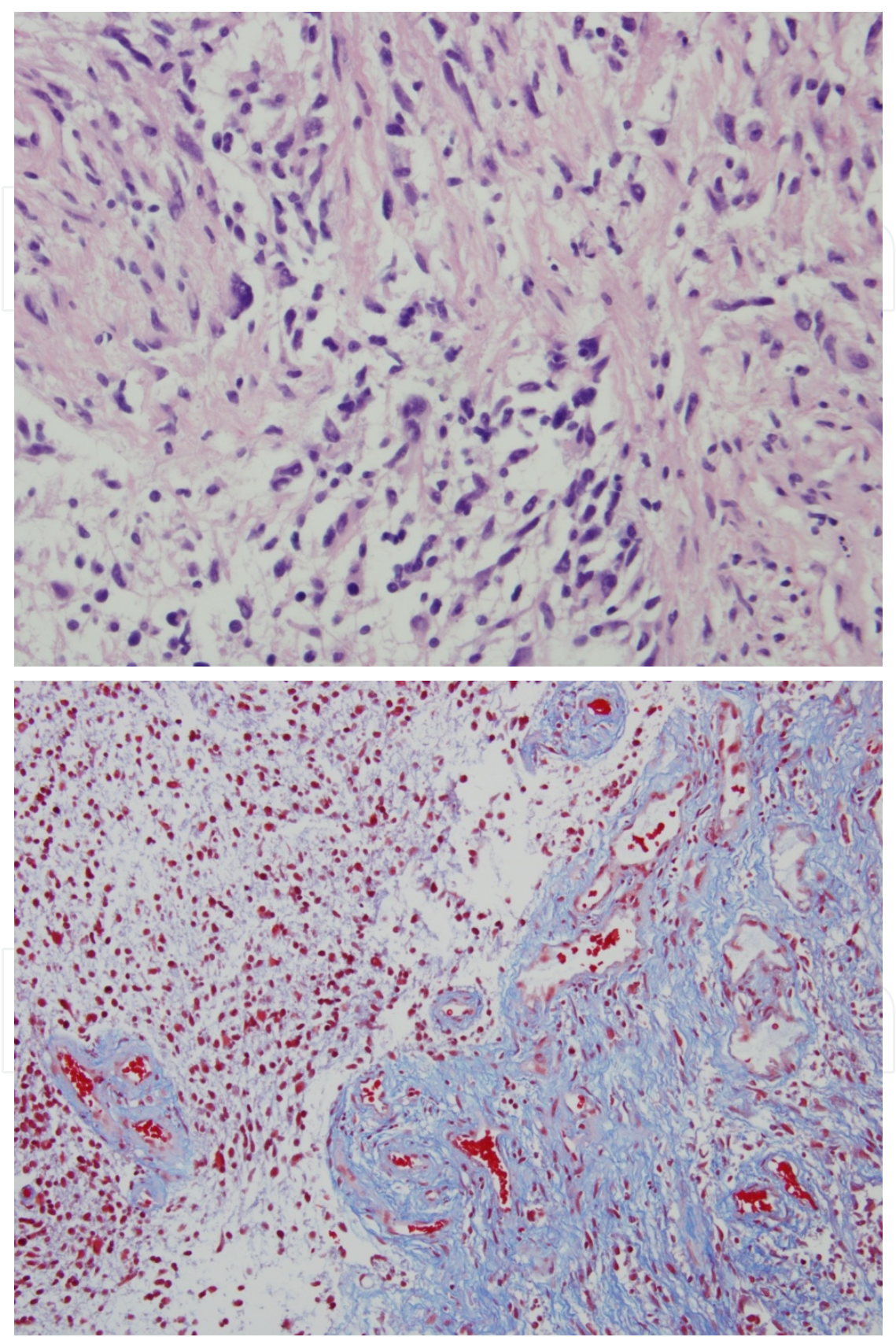
EGFRvIII also presents a unique antigenetic target on tumor cells that is currently being therapeutically used with anti- EGFRvIII vaccines as a molecularly targeted treatment approach. In addition to its recently shown relevance for defining prognostically distinct subgroups, *EGFRvIII* detection is likely to be increasingly important for determining treatment decisions for patients with glioblastoma and potentially for those with other types of cancer.

RT-PCR-Based Detection - Reverse transcription-polymerase chain reaction (RT-PCR) is an RNA-based PCR assay [50]. Reverse transcriptase catalyzes DNA synthesis using RNA as the template, producing a DNA strand complementary to the RNA template, called complementary DNA (cDNA). Because cDNA is not subject to RNase degradation, it is much more stable in laboratory environment than corresponding RNA. For EGFRvIII detection, RNA is extracted from FFPE tissue and reverse transcribed into cDNA. PCR is performed and PCR products are detected by gel electrophoresis. PCR primers are designed to detect EGFRvIII sequences but not unmutated sequences. A portion of a control gene is amplified in parallel to test for RNA/cDNA yield and integrity.

2. Testing and quality control issues

2.1. Specimen types

Formalin-fixed paraffin-embedded tissue - most frequently used in clinical testing, both for DNA-based and RNA-based analysis. Fresh and frozen tissue can be used to extract DNA or RNA, however it is less frequently used in clinical molecular laboratories.



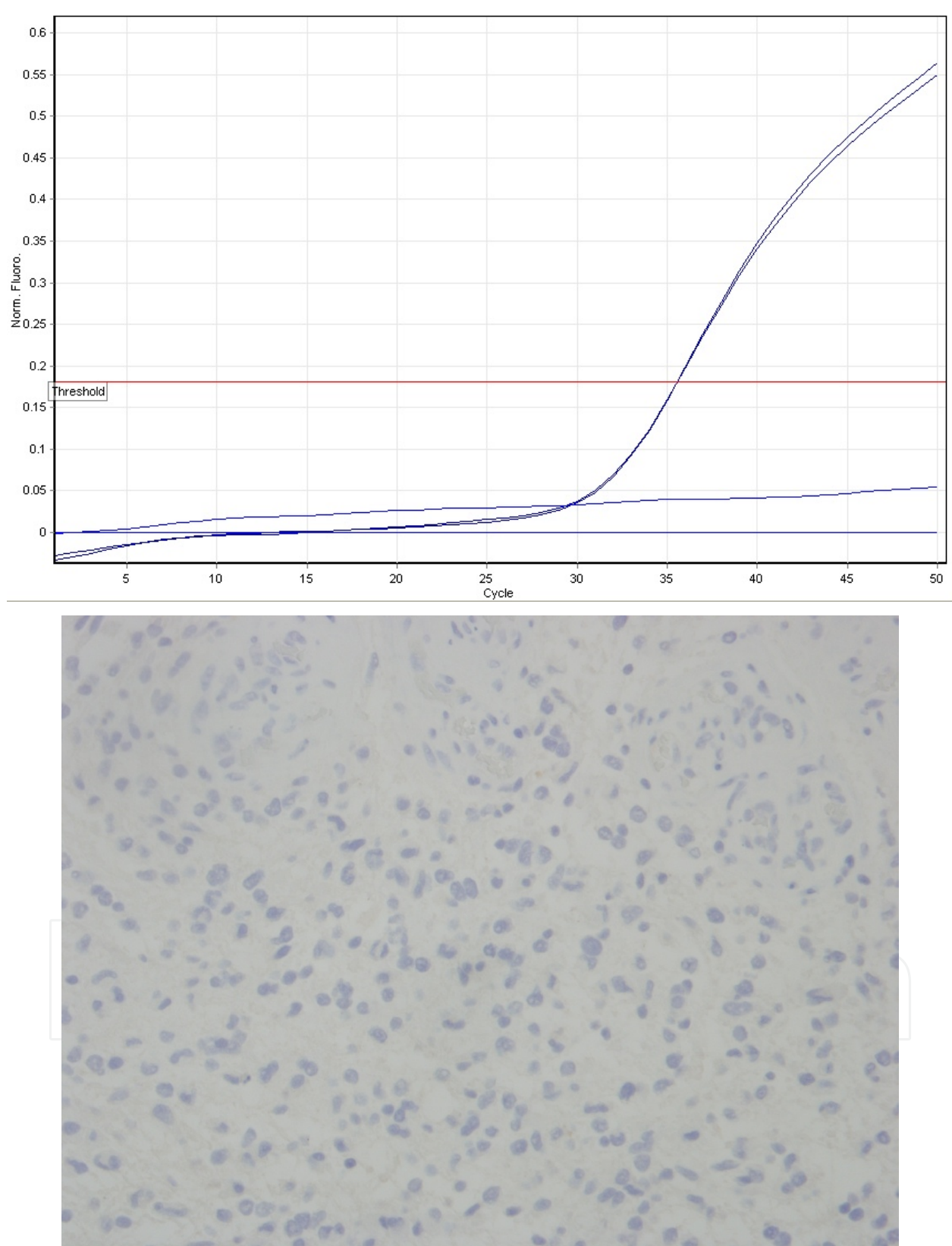


Figure 5. Gliosarcoma, WHO grade IV. (A and B) Sections show a moderately pleomorphic, biphasic glial tumor. Tumor tissue tested positive for MGMT promoter methylation (C) and negative for IDH1 mutation (D).

Microdissection may need to be performed to enrich for tumor content. If a specimen contains too few tumor cells or too many background reactive cell types, the sensitivity of the test may be diminished. In the past 15 years, various techniques of microdissection have been employed to isolate cells of interest in order to increase test sensitivity. Most frequently, a pathologist will examine an H&E stained tissue section and mark areas of tumor involvement. Tissues from tumor rich areas are then manually scrapped off the slides and used for nucleic acid extraction. Less frequently, laser capture microdissection might be performed, however this method is not practical for use in routine clinical molecular laboratories.

2.2. Nucleic acid extraction

Nucleic acid purification begins with lysis of the cells in the sample. Cell lysis liberates cellular macromolecules including proteins, lipids, and nucleic acids. Cell lysis can be accomplished using a detergent solution to break cell membranes and remove lipids. Proteins are enzymatically degraded with protease, usually proteinase K, or selectively precipitated. Protein digestion is performed at about 56°C and will permanently denature many proteins but does not affect nucleic acids. This process is followed by selective extraction that takes advantage of the physical and chemical differences between nucleic acids and other cellular molecules, forming the basis for their isolation. The nucleic acid is then purified from the soluble contaminants produced in the extraction process by precipitation in an ethanol-salt solution. The isolated nucleic acid is then resuspended in a dilute salt buffer.

2.3. DNA

PCR-based genetic analyses most frequently require isolated genomic DNA. Molecular analyses of nucleic acids have traditionally required DNA derived from blood, bone marrow aspirate, and fresh or frozen tissues. New developments in DNA extraction methods now make it possible to also use DNA extracted from formalin-fixed, paraffin-embedded (FFPE) tissues. The purpose of DNA isolation/extraction procedures is to obtain useful samples of DNA that are free of contaminating molecules which could hinder downstream DNA analysis. DNA is a hardy molecule present at stable cellular levels. It is relatively easy to isolate and store because deoxyribonucleases (DNases) that could potentially degrade isolated DNA are easily denatured by heating or inhibited by sequestration of divalent cations.

2.4. RNA

Gene expression protocols (RT-PCR based genetic analyses) require isolated human RNA. RNA analysis depends on successful RNA isolation and preservation. Total RNA is purified with the use of RNA purification kits by first adding the white blood cells or tissue samples to a detergent/salt solution to lyse and homogenize the cells and eliminate endogenous RNase activity. Homogenization disrupts the cell membranes releasing RNA into the lysing solution, and shears the genomic DNA to reduce its ability to bind to the purification column with the RNA. The lysates are then passed through a purification column to bind the RNA and wash away proteins, DNA and other contaminants. Residual DNA is removed by an on-column DNase treatment. Finally, the purified RNA is eluted with DEPC-treated water.

2.5. Limitations of nucleic acid-based procedures

The following can affect the quality of lab results and should ideally be addressed during assay validation:

1. The accumulation of normal cells in the tumor, including infiltrating lymphocytes, may complicate accurate assessment of *MGMT* promoter methylation. Review of tissue morphology by a pathologist prior to testing will ensure that best suited tissue block are examined.
2. Excessive necrosis of tumor tissue will complicate analysis; a different tissue block needs to be selected, when available.
3. Bisulfite treatment of DNA is technically the most challenging part of this protocol. Since DNA loss routinely occurs during bisulfite treatment; it is important to select cases with minimal necrosis to ensure adequate yield of DNA. In order to control for bisulfite effect, methylated and unmethylated controls must be treated in parallel to patient samples to ensure that complete conversion occurred.

Appropriate specimen handling is critical to ensure specimen integrity and the accuracy of quantitative and qualitative nucleic acid detection. Inappropriate specimen handling can result in nucleic acid degradation, which can lead to erroneous quantitation of target from the patient. For example, RNA is rapidly degraded by a variety of ribonuclease (RNase) enzymes that are abundantly present within cells, on the skin surfaces, and possibly laboratory bench tops and equipment. RNases are very stable, active in virtually any aqueous environment, and can regain their activity after denaturation, and steps need to be developed to prevent RNA degradation by exposure to contaminating RNases.

2.6. Contamination prevention measures in a PCR laboratory

Millions of copies of target DNA are generated when PCR and other in vitro nucleic acid amplification techniques are used. If precautions are not taken, amplicons from previous reactions can be introduced into new amplification reactions and act as substrates for new DNA synthesis. The contaminating amplicons, amplified along with the patient samples will produce false positive results. Clinical molecular laboratories must have strict policies regarding contamination prevention and unidirectional work flow. Amplicon contamination and false positive results are prevented by using physical barriers and chemical and ultraviolet (UV) techniques to destroy amplicons or make them unsuitable for amplification. The physical barriers involve doing separate procedure steps in specially designated areas. For example, DNA isolation and PCR set-up are done in areas, separated by a wall, from areas used in downstream processing (thermal cycling, data analysis). This is also known as unidirectional work flow. Each area must have designated equipment and supplies to avoid cross contamination. Laminar flow hoods and other biological containment boxes equipped with UV light must be available in pre-PCR areas. Small scale physical separation techniques also include the use of barrier pipette tips, frequent glove changes, designated lab coats, and PCR tube openers or careful, slow opening of tubes to prevent aerosolization of contents. Chemical techniques include daily cleansing with bleach and other specialized de-

contaminants of work areas before and after use. Use of UV light will further degrade any residual nucleic acids on work surfaces. A no template PCR reaction must be included with every assay as a quality control check for amplicon contamination.

3. Assay validation

For a test to become generally useful, it must have demonstrated analytic validity and clinical utility. Analytic validation focuses on determining how accurately and reliably the assay measures the molecular event of interest. The assay must be reliable in routine laboratory setting using a variety of specimen types. Even assays that are routinely performed in the laboratory require analytic validation within the clinical setting of each laboratory when test results are used for clinical decisions. To ensure reproducible findings, clinical laboratories need to understand the impact that preanalytic variables and specimen processing have on assay performance. Analytic validation ensures that the same answer will be produced for the same sample within predefined technical variation. In recognition of the critical importance of analytic validation for biomarkers, multiple groups have developed recommendations and frameworks with which to standardize the assessment.

Clinical validation assesses the strength of association between the assay results and the clinical outcome of interest, whether it is diagnostic, prognostic, or predictive. A large number of measures are used to assess these associations. These analyses address whether one can be sure the clinical state is positive if the test is positive (positive predictive power) and that the clinical state is negative if the test is negative (negative predictive power).

Evaluation of new biomarkers can be aided if tumor biomarker studies and the journals reporting them adhere to the Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK). These guidelines were produced by a working group convened as part of the NCI-EORTC joint meeting on Cancer Diagnostics in Nyborg, Denmark, in 2000 [51]. REMARK criteria include specifications of patient populations, biological specimen under study, assay methods, study design, and statistical methods, and detailed guidelines for analysis and presentation of data. This reporting standard is now requested for manuscripts being submitted to many journals. Although the tissue sources are not always available to allow the most rigorous validation of assays, providing a standardized means to communicate the level of clinical validation is critical to biomarker development. Using the above reporting standards should help establish the clinical validity of new biomarkers being used in cancer research and treatment.

An emerging standard for the adoption of new molecular tests is the demonstration of clinical utility. Clinical utility refers to the ability of the assay to improve clinical decision making and patient outcomes. Clinical utility depends on the clinical situation, availability of effective therapies, magnitude of clinical benefit, and relative value the patient, caregiver, and society place on the differences in benefits and risks in these separate groups. For example, if a marker clearly distinguishes differences between positive and negative results but the evidence for differential treatment is not available, there is no reason to test for the

marker. Likewise, if therapy is effective regardless whether the patient tests positive or negative, the marker does not have clinical utility. Additionally, a novel assay might have outstanding analytic validity and proven clinical validity but there is already an established method to test for the same parameters, which makes the new assay unnecessary.

Associations such as Clinical Laboratory Standards Institute (CLSI) have published several guidelines dealing with molecular genomic testing in clinical laboratories. As more molecular tests are being introduced for patient management, CLSI will likely continue to publish new and expanded guidelines addressing current and possibly future considerations and best practices.

3.1. Issues with MGMT assay validation

Molecular markers in general are developed to address a variety of indications [52]. Diagnostic markers are a large category of molecular tests that aid in the diagnosis or subclassification of a particular disease state. Diagnostic subclassification may result in different management of the disease, but the marker is used primarily to establish the particular disease that is present in the patient sample. An example of a diagnostic marker is BCR/ABL t(9;22) translocation in chronic myelogenous leukemia. Prognostic markers have an association with some clinical outcomes, such as overall survival or recurrence-free survival, independent of the treatment rendered. An example of a prognostic marker is the *FLT3* IDT mutation in acute myeloid leukemia, which identifies a subset of patients who will have a more aggressive disease course regardless of current treatment options. Predictive markers predict the activity of a specific class or type of therapy, and are used to help make more specific treatment decisions. They are used as indicators of the likely benefit of a specific treatment to a specific patient. Human epidermal growth factor receptor (EGFR) is an example of a predictive marker. Patients with lung cancers whose tumors exhibit adenocarcinoma histology and carry one of the sensitizing *EGFR* mutations are likely to respond well to treatment with tyrosine kinase inhibitors erlotinib and gefitinib. Some of these markers may also be used as companion markers to identify a subgroup of patients that are likely to respond to a specific therapy. One example of a companion diagnostic is the *BRAF* V600E mutation test which is coapproved with the kinase inhibitor vemurafenib. *BRAF* mutations are found in 30% to 60% of melanomas, and the kinase activating *BRAF* V600E mutation confers sensitivity to vemurafenib, a small molecule inhibitor.

The clinical utility of the *MGMT* methylation status as a biomarker for benefit from alkylating agent therapy in gliomas is still being evaluated and there is no consensus as to which procedure is most suitable for routine clinical testing. While most protocols measure the level of methylation accurately, presence of contaminating normal cells in every specimen makes it difficult to set up a consistent cut off point between low positive and negative test results. The extent of methylation required and the sets of CpGs that are crucial for complete silencing are still under investigation. Given the complexity of the biological relationship between promoter methylation and gene silencing and the difficulties to integrate these features into a test that is in addition complicated by presence of nontumoral tissue, there are unavoidable drawbacks for any technology attempting to predict loss of *MGMT* expression

for potential benefit from alkylating agent therapy. Key to introducing tests for diagnostics is their careful prospective validation.

3.2. MGMT methylight assay validation - A one lab experience

Epigenetic silencing of the *MGMT* gene through promoter hypermethylation and resulting transcriptional inactivation is now routinely used as a prognostic and possibly predictive biomarker in evaluating treatment choices in patients diagnosed with glioma. Patients enrolling in clinical trials are also evaluated for the *MGMT* promoter methylation status. For that reason, a sensitive, clinically validated assay for detection of *MGMT* promoter methylation can be of great help in patient management. We previously developed a simplified *MGMT* MSP protocol [53] that utilized formalin fixed paraffin embedded specimens. This MSP protocol proved reliable with majority of glioma patients, however, result interpretation was sometimes challenging when evaluating specimens with few malignant cells and/or extensive necrosis, both of which gave faint bands on agarose gels. To further enhance our ability to accurately assess methylation status of the *MGMT* promoter region in glioma specimens, we investigated the use of real-time PCR technology as a way of enhancing assay sensitivity. Our ultimate goal was to develop a more sensitive detection method that would give reliable, semi-quantitative results, even with suboptimal specimens. Previous studies have shown sodium bisulfite conversion to be a reproducible method, with subsequent quantitative real-time PCR methylation assays having acceptable precision for clinical work [54].

The goal of this study was to evaluate the MethyLight protocol, as compared to the *MGMT*-MSP protocol, and appropriateness of the MethyLight protocol for use in *MGMT* promoter methylation detection in routine clinical testing of glioma cases.

3.3. Materials and methods

3.3.1. Tumor samples

Thirty archival brain resection cases were selected for the study: glioblastoma multiforme (18), oligodendroglioma (3), anaplastic oligodendroglioma (3), and astrocytoma (6). For all tumor samples, histology was reviewed to confirm diagnosis and select blocks with greatest tumor involvement. Microdissection was performed on all samples showing an estimated tumor cell content of less than 50% except in cases where infiltrative pattern of tumor growth made this step unreliable.

3.3.2. DNA extraction and bisulfite treatment

Genomic DNA was isolated from 2 to 3 twenty micrometer thick paraffin sections after confirmation of the histology. DNA from formalin fixed, paraffin embedded tissue was extracted using Puregene kit (Gentra Systems, Minneapolis, MN). *MGMT* promoter methylation status was assessed using a two step approach. The first step involved bisulfite conversion of isolated DNA (200-500 ng) which was done using EZ DNA Methylation Gold kit (Zymo Research, Orange, CA). The second step involved detection of methylated and unmethylated

ed DNA sequences, and was done using the following methods: 1) Methylation specific PCR (MSP) and 2) Real-time PCR amplification (MethyLight).

3.3.3. Methylation-specific PCR

MGMT MSP amplification was performed as previously reported [53] with specific primers designed to distinguish methylated from unmethylated DNA. Methylated and unmethylated DNA sequences were detected on 2.5% agarose gels. Samples giving signals approximately equivalent to the positive methylated control were designated as methylated. Samples giving no signals with positive methylated control, but demonstrating presence of unmethylated DNA, similar to the negative control, were designated as unmethylated.

3.3.4. Quantitative real-time PCR (MethyLight)

Real-time PCR assays were set-up in parallel to measure *MGMT* methylation. Two sets of primers and probes designed specifically for bisulfite-converted DNA were used [55]: a set for *MGMT* gene and a set for collagen 2A1 (*COL2A1*) to normalize for the amount of input DNA. The *MGMT* forward primer is 5'-GCG TTT CGA CGT TCG TAG GT-3', the *MGMT* reverse primer is 5'-CAC TCT TCC GAA AAC GAA ACG-3', and the *MGMT* probe is 6FAM-5'-CGC AAA CGA TAC GCA CCG CGA-3'BHQ1. *COL2A1* forward primer is 5'-TCT AAC AAT TAT AAA CTC CAA CCA CCA A-3', the *COL2A1* reverse primer is 5'-GGG AAG ATG GGA TAG AAG GGA ATA T-3', and the *COL2A1* probe is 6FAM-5'-CCT TCA TTC TAA CCC AAT ACC TAT CCC ACC TCT AAA-3'BHQ1. We used Rotor Gene 3000 real-time PCR instrument (Qiagen, Germantown, MD, USA). The PCR conditions were as previously described [56, 57]. Briefly, PCR amplification was performed in 0.2 ml PCR tubes with a final reaction mixture of 25 µl consisting of 12.5 µl of TaqMan Universal Master mix without uracil DNA glycosylase (Applied Biosystems, Foster City, CA), 3 µl of respective forward and reverse primers (10 µM) for either *MGMT* or *COL2A1*, 1 µl of probe, 5 µl (about 50 ng) of bisulfite-modified DNA, and water. PCR conditions were as follows: 95°C for 10 min, followed by 50 cycles at 95°C for 15 s and 60°C for 1 min.

3.3.5. Assay controls

For assay controls, methylated DNA and unmethylated DNA were purchased from Chemicon International, Temecula, CA, and used as positive and negative controls for methylated sequences. The control DNA was subjected to bisulfite treatment and PCR amplification in parallel with patient samples for every run. Controls without DNA were also performed for each set of reactions. Additionally, for the MethyLight protocol, collagen 2A1 (*COL2A1*) gene was used as the internal reference to assess the quality and quantity of input DNA.

3.3.6. Results

To determine the limit of detection of the MethyLight protocol, bisulfite treated methylated control DNA was serially diluted into bisulfite treated unmethylated control DNA. DNA mixing study with methylated and unmethylated DNA showed good linearity. The limit of

detection of the MethyLight assay was determined to be 1% of methylated DNA in the background of unmethylated DNA. The MSP protocol was shown to be slightly less sensitive, with the limit of detection of 5% of methylated DNA in unmethylated background [53].

| Tube No. | % of Methylated DNA | % of Unmethylated DNA | Cycle Threshold (Ct) |
|----------|---------------------|-----------------------|----------------------|
| 1 | 100 | 0 | 28.80 |
| 2 | 50 | 50 | 29.77 |
| 3 | 20 | 80 | 30.77 |
| 4 | 10 | 90 | 31.83 |
| 5 | 5 | 95 | 32.74 |
| 6 | 1 | 99 | 35.22 |
| 7 | 0 | 100 | 0 |
| 8 | 0 | 0 | 0 |

Table 3. Cycle threshold values of serial dilution of methylated DNA into unmethylated control DNA

To evaluate run-to-run variations, we tested the reproducibility of the MethyLight assay by performing eight independent runs of the serially diluted methylated control in the negative control background. Acceptable reproducibility was demonstrated between runs.

Of the 30 tumor specimens in the study group, we were able to extract DNA of sufficient quantity and quality to allow us to determine *MGMT* promoter methylation status for all samples. There was a complete concordance in test results between the two methods for 25/30 cases (83%). Overall, methylation specific PCR (MSP) identified 16 (53%) specimens as positive (methylated *MGMT* promoter was present) and 14 (47%) of tumors as negative (having unmethylated *MGMT* promoter). The MethyLight protocol identified 15 specimens (50%) as positive for *MGMT* promoter methylation. Among the discordant cases, two samples tested negative by MSP and were low positive by MethyLight; low DNA recovery was observed for both of these samples, and the small amount of bisulfite treated DNA that was available for analysis might have contributed to the discordance in results. The other three discordant cases tested as weak positive with MSP and were negative by MethyLight; all three of these cases had significant amount of necrotic tissue, which complicated interpretation of the MSP results.

As regards assay controls, in the MSP protocol, *MGMT* promoter hypermethylation was always accompanied by amplification in the unmethylated reaction as well. This is to be ex-

pected since the original tissue sections contained a mixture of tumor and non-malignant tissue. The presence of unmethylated promoter served as an internal amplification control that confirmed that the quality and quantity of bisulfite treated DNA was acceptable for clinical testing. Only tumor samples that contained a clearly visible methylated signal, with or without an additional unmethylated signal, were interpreted as positive for the *MGMT* promoter methylation. All the samples that only amplified with unmethylated primers were interpreted as negative. *COL2A1* internal control was used with the MethyLight protocol to assess the quality and quantity of DNA. All samples demonstrated positive signals with the *COL2A1* PCR amplification.

In comparing the two quality control methods, we observed 100% concordance between the two systems, with both methods indicating that all samples in the study group contained sufficient amount of bisulfite converted DNA for clinical testing. The gel signals observed with unmethylated DNA as an indicator of DNA quantity with the MSP protocol, and the expression of *COL2A1* as an internal control standard in the MethyLight protocol proved to be equivalent and reliable indicators of quality of input DNA.

This study reports an improvement on our previously published MSP protocol for the detection of *MGMT* promoter methylation in glioma specimens. MethyLight protocol, a real-time PCR based approach, was used in a number of application [54, 55-57]. In our laboratory, the MethyLight assay proved to be easy to perform, reproducible, and sensitive in detecting the amount of methylated DNA sequences in formalin fixed, paraffin embedded brain tumor specimens. Furthermore, with the use of the real-time PCR approach, we were able to eliminate post PCR processing that is integral to the MSP protocol.

Recently Ogino and colleagues [54] performed an in-depth investigation of critical parameters that influence the success of quantitative DNA methylation analysis after sodium bisulfite conversion of DNA samples from archived formalin-fixed paraffin-embedded tumor specimens, demonstrating the value of percentage of methylated reference (PMR) estimation. Our system, however, proved impractical for measurement of PMR. To obtain PMR measurement in their study, the investigators microdissected tumor tissue to obtain pure populations of tumor cells. Exclusion of normal tissue allowed for precise calculation of PMR values, and consequently quantitation of the amount of methylation in each tumor sample. This approach proved to be impractical for some of the samples in our study due to the pattern of growth of gliomas, particularly GBM tumors, which grow in an infiltrating pattern, with many benign cells surrounding a few infiltrating tumor cells. Consequently, sections of these tumors contain both malignant and normal cells, with no easy way of dissecting out a pure tumor population, unless one performs laser capture microdissection. Since our aim was to optimize a simple *MGMT* promoter methylation detection assay for routine clinical use, we continued our practice of histology review and preselecting tissue blocks with greatest amount of tumor involvement and smallest amount of necrosis prior to analysis. Using the entire tissue block section proved to be a practical approach for routine testing in our laboratory, even when microdissection was not possible. At 1% sensitivity, we were able to detect *MGMT* promoter methylation even when tumor cells were in minority, as assessed by review of histology.

While this study group consisted of a relatively small number of samples, we were able to develop a testing protocol using MethyLight technique, that proved to be reliable and reproducible in a clinical setting. When applying this method to clinical use, it is important that proper controls be included with every assay run. After bisulfite treatment of isolated DNA, patient samples are tested in duplicates for the evidence of *MGMT* promoter, and for the expression of *COL2A1* internal control sequences to normalize for input DNA. Additionally, three different concentrations (100%, 10% and 1%) of methylated DNA in unmethylated background are run as reference standards. This approach allows clear distinction between positive and negative signals. The assay results are interpreted as positive or negative for *MGMT* promoter methylation. In terms of patient management, temozolomide is given to all patients, regardless of *MGMT* promoter methylation status. It has been established that strongly positive patients will respond to temozolomide, however it is less clear how low positive patients will respond. When very low positive results are reported for a glioma specimen, the amount of tumor involvement in that particular sample, as assessed by review of histology, can serve as an additional guide for the clinical care provider.

In summary, while both MSP and MethyLight detection methods proved acceptable for clinical testing, with 83% concordance between the two methods, the MethyLight method proved superior in several different areas. It allowed easier interpretation of low positive results, because there was a clear distinction between positive and negative signals. This real-time, quantitative approach also allowed for reduced turn around time and high throughput specimen processing, because post PCR gel analysis steps were eliminated. Finally, the MethyLight method appeared to be more sensitive, even though the significance of very low positive results is unknown. The limit of detection of the MethyLight assay was determined to be 1% of methylated DNA in the background of unmethylated DNA, compared to 5% with the MSP assay.

3.4. Regulation of molecular genomic testing

Molecular testing falls under high complexity testing and is highly regulated. In the United States two federal agencies, the Food and Drug Administration (FDA) and the Centers for Medicare and Medicaid Services (CMS) have jurisdiction over this type of testing. The laws and policies concerning the development and implementation of molecular tests continue to evolve. Medical tests are regulated by the Center for devices and Radiologic Health, a branch of FDA. Devices are divided into 3 classes based primarily on the risks associated with their intended use. Class I tests are low risk, usually of a simple design. Class II tests pose moderate risks, are evaluated by the FDA through review of a 510(k) premarket notification, and are cleared for marketing once they are found to be substantially equivalent to a legally marketed device that was previously cleared by the FDA. Class III tests are those associated with the highest clinical risk. Each Class III test is reviewed through application for premarket approval (PMA). A demonstration of safety and effectiveness is needed to gain FDA approval. The FDA approval/clearance process provides reasonable assurance of safety and effectiveness, and that the test will provide clinically significant results.

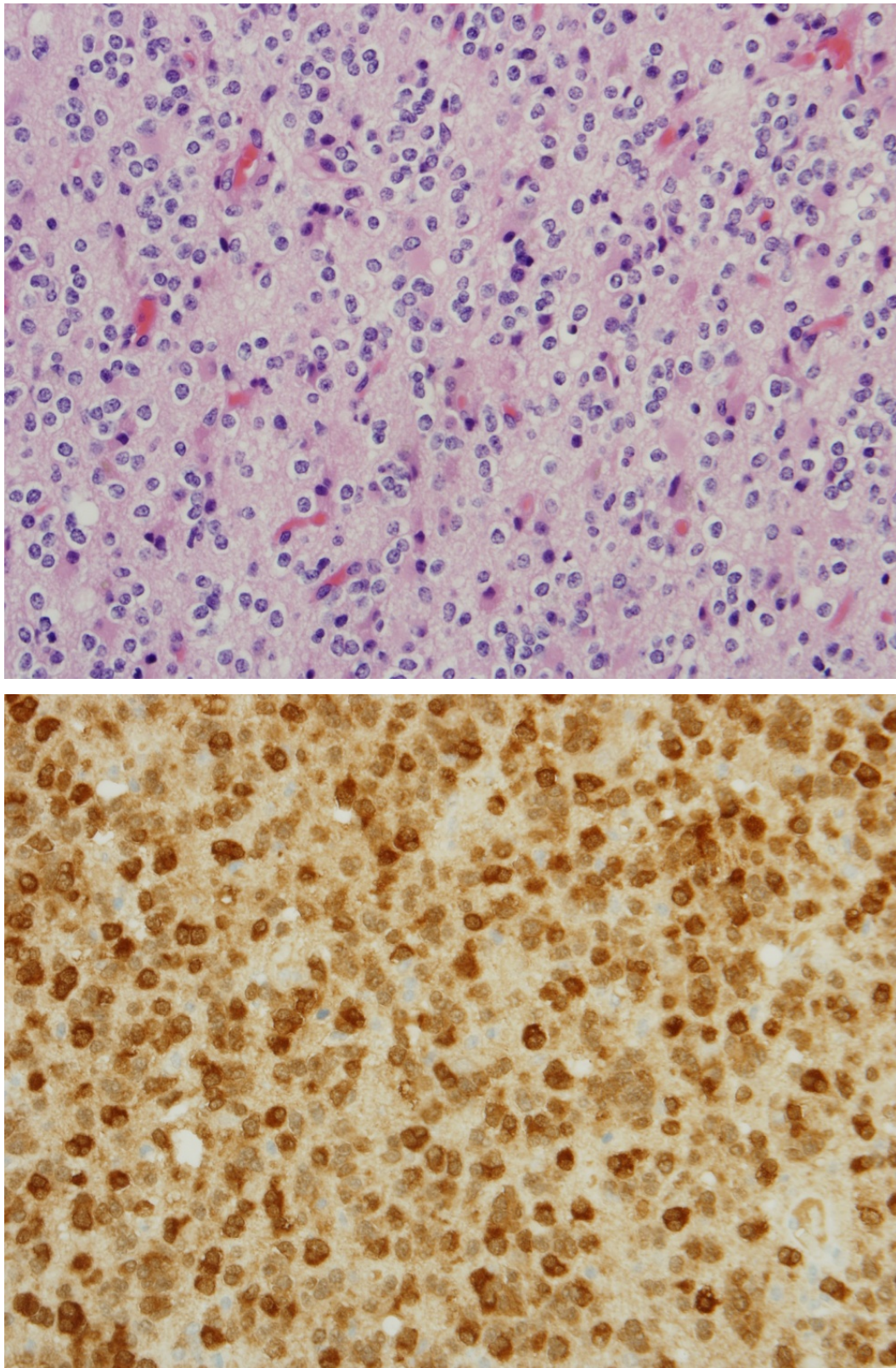


Figure 6. Oligodendroglioma, WHO grade II. (A) Sections show moderately cellular glial neoplastic proliferation. Tumor tissue tested positive for MGMT promoter methylation and 1p19q codeletion. (B) The IDH1 R132H is positive.

The rapid transition of new scientific knowledge to medical practice has led to development and use of diagnostic tests that are often called laboratory developed tests (LTDs). The FDA retains jurisdiction over this category of tests and until recently the oversight was basically directed towards critical reagents used to build these tests. With LTDs being used more widely in clinical decision making it is likely the FDA will develop clinical guidelines on Companion Diagnostics or molecular tests which directly impact the use of a pharmaceutical or biologic drug.

Clinical laboratories are governed through the Clinical Laboratory Improvement Amendment (CLIA) of 1988 administered by CMS. CLIA certification is a descriptor which is frequently associated with analytically valid assays performed in a clinical laboratory or a laboratory that has received a CLIA certification. CLIA certification requires that a laboratory adopt specific practices and perform prescribed measures of analytic validation while performing specific assays. Assays must be performed in CLIA certified laboratories if the results of the assays are going to be used to guide patient management. Key components of the regulation and oversight of CLIA accredited laboratories are inspections and proficiency tests. In most hospitals these are provided by the College of American Pathologists (CAP). CAP inspection guidelines essentially set the standards for overall biomarker laboratory operation, including assay validation, quality control, and quality assurance activities. The guidelines mandate regular proficiency testing for every clinical assay, and CAP runs an extensive program providing proficiency testing samples for all commonly used clinical assays.

4. Prognostic and predictive relevance of glioma biomarkers

Cancer-specific DNA methylation changes are hallmarks of human cancers, in which global hypomethylation is often seen concomitantly with hypermethylation of CpG islands. Promoter CpG island hypermethylation generally results in transcriptional silencing of the associated gene [58]. There have been several reports of promoter-associated CpG island hypermethylator phenotype in human GBM and other glioma subtypes [59-61]. Several studies have reported differences between primary and secondary GBMs with respect to epigenetic changes. Overall, secondary GBMs have a higher frequency of promoter methylation than primary GBMs [62]. Analysis of epigenetic changes from The Cancer Genome Atlas study [63] identified the existence of a proportion of GBM tumors with highly concordant DNA methylation of a subset of loci, indicative of a CpG island methylator phenotype (G-CIMP). G-CIMP positive samples were associated with secondary or recurrent (treated) tumors and tightly associated with *IDH1* mutation. G-CIMP tumors also showed a relative lack of copy number variation commonly observed in GBM. Integration of the DNA methylation data with gene expression data showed that G-CIMP tumors represent a subset of proneural tumors. G-CIMP -positive tumors showed a favorable prognosis with GBMs as a whole and also with the proneural subset. These data suggest that G-CIMP-positive status may confer favorable outcome. When lower grade tumors were examined, nearly 10-fold more G-CIMP-positive gliomas were detected among grade II tumors as compared to grade IV GBMs. The study suggests that the improved survival of G-CIMP gliomas at all tumor

grades might be due to certain molecular features within the G-CIMP gliomas that encourage a less aggressive tumor phenotype.

Low grade diffuse gliomas WHO grade II (diffuse astrocytoma, oligoastrocytoma, oligodendroglioma) are characterized by frequent *IDH1/2* mutations (>80%) that occur at a very early stage. In addition, the majority of diffuse astrocytomas (about 60%) carry TP53 mutations, which constitute a prognostic marker for shorter survival. Limited data exists correlating *IDH1/2* mutations with loss of heterozygosity (LOH) on chromosome arms 1p and 19q, methylation of the promoter region of DNA repair enzyme O-6-Methylguanine DNA methyl transferase (M-MGMT) rendering sensitivity to DNA alkylating agents, and epidermal growth factor receptor variant III (*EGFRvIII*), a constitutively active mutant form of *EGFR* frequently present in glioblastoma.

| Diagnosis | GR | Micro dissected | IDH1 (+/total) | IDH2 (+/ total) | MGMT (+/total) | EGFRvIII (+/total) | LOH (+/total) |
|-----------|--------|--------------------|-------------------|--------------------|-------------------|-----------------------|------------------|
| GBM | IV | 5/20 | 0/20 | 0/20 | 10/20 | 3/20 | nd |
| O | II | 5/14 | 12/14 | 1/14 | 10/13 | 0/3 | 10/13 |
| AO | III | 0/2 | 2/2 | 0/2 | 1/2 | 0/1 | 1/1 |
| A | II | 2/6 | 2/6 | 0/6 | 3/6 | 0/4 | 0/2 |
| AA | III | 0/6 | 0/6 | 0/6 | 3/6 | 0/4 | 0/2 |
| OA | II/III | 3/8 | 8/8 | 0/8 | 6/7 | 0/4 | 2/8 |
| AOA | III | 1/6 | 1/6 | 0/6 | 3/5 | 0/3 | 3/3 |

Table 4. Biomarker distribution among different glioma types

High throughput profiling techniques in conjunction with sophisticated bioinformatics integrative tools are emerging to revolutionize our knowledge about the complexity of the disease. Development of novel array-based profiling techniques and next generation sequencing techniques has facilitated development of sophisticated tumor-specific genomic and transcriptional signatures. Integrative analysis of DNA copy number, gene expression and DNA methylation profiling indicate that molecular alterations may impact future treatment strategies. For example, as *PIK3R1* encodes the regulatory protein p85a subunit, response to PI3K inhibitors may depend on whether the tumors bear mutations in this specific gene or not. Also, in predicting sensitivity and the development of resistance to temozolomide the Cancer Genome Atlas network added further support for a role of the DNA mismatch repair system. *MGMT* methylation in conjunction with temozolomide treatment may lead to a loss of mismatch repair function by introduction of mutations in mismatch repair genes [64, 65]. Thus patients who initially respond to front line therapy may evolve treatment resistance by developing a hypermutator phenotype. As a consequence, selective strategies targeting mismatch repair deficient cells might have to be used in combination with alkylating agent therapy to prevent or minimize resistance to temozolomide.

5. Conclusion

The list of clinically useful biomarkers in gliomas is expected to expand as novel markers are validated in large scale clinical trials [52]. *MGMT* promoter methylation is now routinely performed in many institutions. Alkylating agents cause cell death by forming cross-links between adjacent strands of DNA due to alkylation of the O⁶ position of guanine. The cellular DNA repair protein O⁶-methylguanine-DNA methyltransferase (*MGMT*) functions as a DNA repair enzyme that removes the mutagenic alkyl-adducts from the O⁶-position of guanine and thereby causes resistance to alkylating drugs [66-68]. 1p/19q LOH has shown clinical utility, and is a part of guidelines for all oligodendroglioma and mixed tumor management. Some of the newer markers, such as *IDH1/2* mutations and *EGFRvIII* mutations still need further evaluation, and are being used with increasing frequency to provide further definition of tumor stage and possibly subsequent behavior. Molecular laboratories are likely to evolve also, as clinical care providers continue to rely on genomic-type assays for guiding patient treatment.

Acknowledgements

The author gratefully acknowledges Lynda Szymanski, DO for her help with figures and Lisa Whiteley for help with data analysis.

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