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Molecular Pathology Techniques

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The field of neuro-oncology has undergone a number of significant changes over the past decades. One of the most striking, however, has been the rapid pace of discovery in the field of molecular genetics, especially over the past few years. As a result, the genomic landscape of the most common entities has been defined, including a discovery of recurrent genetic alterations, leading to the establishment of diagnostic, prognostic, and predictive biomarkers. Some of these genetic markers, such as 1p/19q loss, O^6-methylguanine-DNA-methyltransferase (MGMT) promoter methylation, and isocitrate dehydrogenase 1 (*IDH1*) mutations, have already entered routine clinical diagnostics and are considered a standard of care. While the clinical utility of other molecular genetic biomarkers, such as epidermal growth factor receptor (*EGFR*) amplification, proto-oncogene B-Raf (*BRAF*) mutation/duplication, or molecular subclassification based on gene expression profile is not firmly established yet, some can be utilized for diagnostic purposes. Furthermore, given the development of targeted therapy, the molecular signature can be also utilized to identify the appropriate target population, substratify patients for clinical trials, and validate candidate predictive biomarkers. As in other tumors and diseases, advanced molecular diagnostics will not replace traditional histopathology, but provide valuable additional information to increase diagnostic accuracy and precision.

Given the limitations of standard cytotoxic therapies, such as chemotherapy and radiation therapy, in the treatment of brain tumors, it has become clear that major progress will require novel approaches. As a result, significant efforts are being made to develop more targeted or selective approaches, based on the specific molecular signature of the tumor. A variety of technical assays have been designed to analyze gene expression, as well as large chromosomal losses and gains, gene rearrangements, focal copy-number changes, point mutations, and epigenetic changes. Genome, transcriptome, and epigenome analyses will likely become a focus for diagnostics to identify new therapeutic targets.

Gliomas are the most common tumors of the central nervous system (CNS) and often require additional molecular work-up, either for diagnosis or biomarkers. In clinical practice, focused assays are usually performed. The most commonly used assays include analyses of 1p/19q loss, *MGMT* promoter methylation, and *IDH1* mutation status. Most commonly performed technical assays are fluorescence in situ hybridization (FISH), polymerase chain reaction (PCR) and its variants, variety of methylation specific assays and sequencing or immunohistochemistry (IHC). These assays are particularly useful in clinical management and diagnostics of adult diffuse gliomas. Although genetic changes and expression profiles have been well studied in other brain tumors as well, the routine clinical use of molecular testing in meningiomas, ependymomas, or medulloblastomas is not yet established. Whole genome expression profiling and DNA analysis of medulloblastomas have pioneered molecular and biological subclassification of a morphologically relatively uniform disease. Similar results were shown in gliomas and specific molecular classes have also been defined in meningioma and ependymoma using next-generation sequencing (NGS) and/or expression profiling. With the costs of whole genome approaches decreasing, we can expect a decline in number of single target assays in molecular laboratories in favor of broad genome-wide analyses in the future.

Molecular Techniques in Clinical Practice

Copy-Number Analysis

Fluorescence In Situ Hybridization

Fluorescence in situ hybridization (FISH) is one of the oldest and most commonly used techniques in molecular pathology [1]. FISH uses fluorescently labeled DNA probes which attach/hybridize to specific targets in the DNA, providing the

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information on copy-number changes on the level of single cells while preserving the morphology of the underlying tissue. Using different fluorescent dyes enables the investigation of multiple DNA targets simultaneously. FISH can be used on formalin-fixed paraffin-embedded tissue (FFPE) and allows identification of genomic changes in situ. A disadvantage is that FISH probes/signals have to be relatively large to be detected by fluorescent-light microscopy and therefore are not useful for identifying small genomic changes, such as small insertions/deletions. Also, due to the relatively broad optical spectrum of fluorescent dyes, the number of dyes (and therefore, probes) is limited to four at most on a single slide.

FISH offers numerous applications for the routine detection of cytogenetic biomarkers. It can assess ploidy, large chromosomal gains and losses, focal amplifications/deletions, and large structural gene rearrangements. Because the assay is performed directly on the tissue, it allows for the detection of genetic changes even in a small biopsy, or when only a limited number of tumor cells are present among normal tissue. In contrast with whole genome assays, FISH also provides information of whether different genetic changes are present in the same tumor cell or in a different tumor subclone, i.e., genetic mosaicism.

Because of the diagnostic utility of FISH in clinical practice, its application for a variety of tumors is now considered standard of care, and standard protocols are well established. Therefore, any molecular pathology or neuropathology laboratory should be able to implement it. Probes are available commercially and automated systems are used in large laboratories. However, several issues and limitations have to be noted. The assay is labor intensive, with the maximum number of slides managed by a single technician ranging between 10 and 20 per single run, depending on the technician's experience. Larger sample volumes can be managed more efficiently with deparaffinization, protease digestion, and pre- and post-hybridization washes performed by an automated system. Automated systems also allow for standardized and uniform treatment of all specimens. Protease digestion is particularly important, since the brain tissue has a strong autofluorescence and insufficient digestion will result in strong background and weak hybridization signals. On the other hand, excessive digestion will damage the tissue and may lead to a technical failure. Also, the tissue fixation and processing can have a deleterious effect on the ability to perform FISH. Particularly heavy acid decalcification, which is fortunately rare in CNS specimens, almost always leads to FISH failure. The time required for scoring can vary greatly. While the 1p/19q assay, for example, is quite time-consuming and requires scoring ~100 nuclei and two slides, one for chromosome 1 and one for chromosome 19, EGFR and other amplification assays can be detected relatively quickly. However, in the light of recent observations about minor amplified subpopulations and the potential impact of different levels of EGFR amplification on survival, careful scoring of the entire tumor specimen is warranted [2].

For interpretation, appropriate cutoffs must be determined according to specificity and sensitivity for each test. The most common findings in neuropathology FISH are deletions, low-level copy-number gains/losses, and high-level copy-number gains, i.e., amplifications (Fig. 3.1). Gene rearrangements are less common. For 1p/19q deletions, one of the possible methods of interpretation is the median percentage of nuclei with two reference/control signals (e.g., 1q or 19p) and one test signal (e.g., 1p or 19q) plus three standard deviations as the cutoff values for deletion. Another possibility is to use the ratio of target versus reference signals, with most control specimens being near 1.0 and cutoff around 0.75–0.85, depending on the laboratory standards. In addition, there is increasing evidence in the 1p/19q literature that there are different clinical implications of absolute deletions with one target and two reference signals per nucleus compared to so-called relative deletions in tumors with polyploidy/aneuploidy. These tumors can show variable numbers of target and reference signals such as duplication with two target signals and four reference signals per nucleus, or 3:6, 4:8 ratios. This finding would be misinterpreted as absolute deletions by PCR loss of heterozygosity (LOH) methods. However, recent studies have shown that relative deletion, also known as a superloss, is an important prognostic marker and patients with relative deletion have shorter progression-free survival [3, 4].

Gene amplification testing is most commonly applied for *EGFR*, although the other two most commonly amplified receptor tyrosine kinase (RTK) genes platelet-derived growth factor receptor alpha (*PDGFRA*) and mesenchymal–epithelial transition factor (*MET*) RTK gene are gaining increasing attention, partly due to the increasing clinical availability of kinase inhibitors against these targets [5]. Also, while *EGFR* amplification is generally limited to adult GBMs, *PDGFRA* amplification is common in lower grade and pediatric gliomas [6]. Typically, RTK gene amplifications involve the majority of cells within a given tumor and with high levels of amplification. However, tumors with scattered amplified cells, which represent a minority of the tumor, are also encountered in clinical practice. This phenomenon is most commonly observed in *MET*-amplified GBMs, where cells with amplification can be rare and scattered throughout the tumor. Another recently described phenomenon is mosaic heterogeneity, where tumors are composed of subclones with mutually exclusive amplification of RTK genes [7–9]. Up to three coexisting subclones with amplifications of *EGFR*, *MET*, and *PDGFRA* within a single tumor have been described. More importantly, these studies have shown that during glioma progression, subclones have different propensities to infiltrate normal brain and genomic changes can vary widely among different parts of the same tumor [7]. In addition, studies have also shown that some GBM cells contain simultaneous amplification of different RTK genes. Although the significance of these findings is not clear, they emphasize the complexity of the disease and raise several

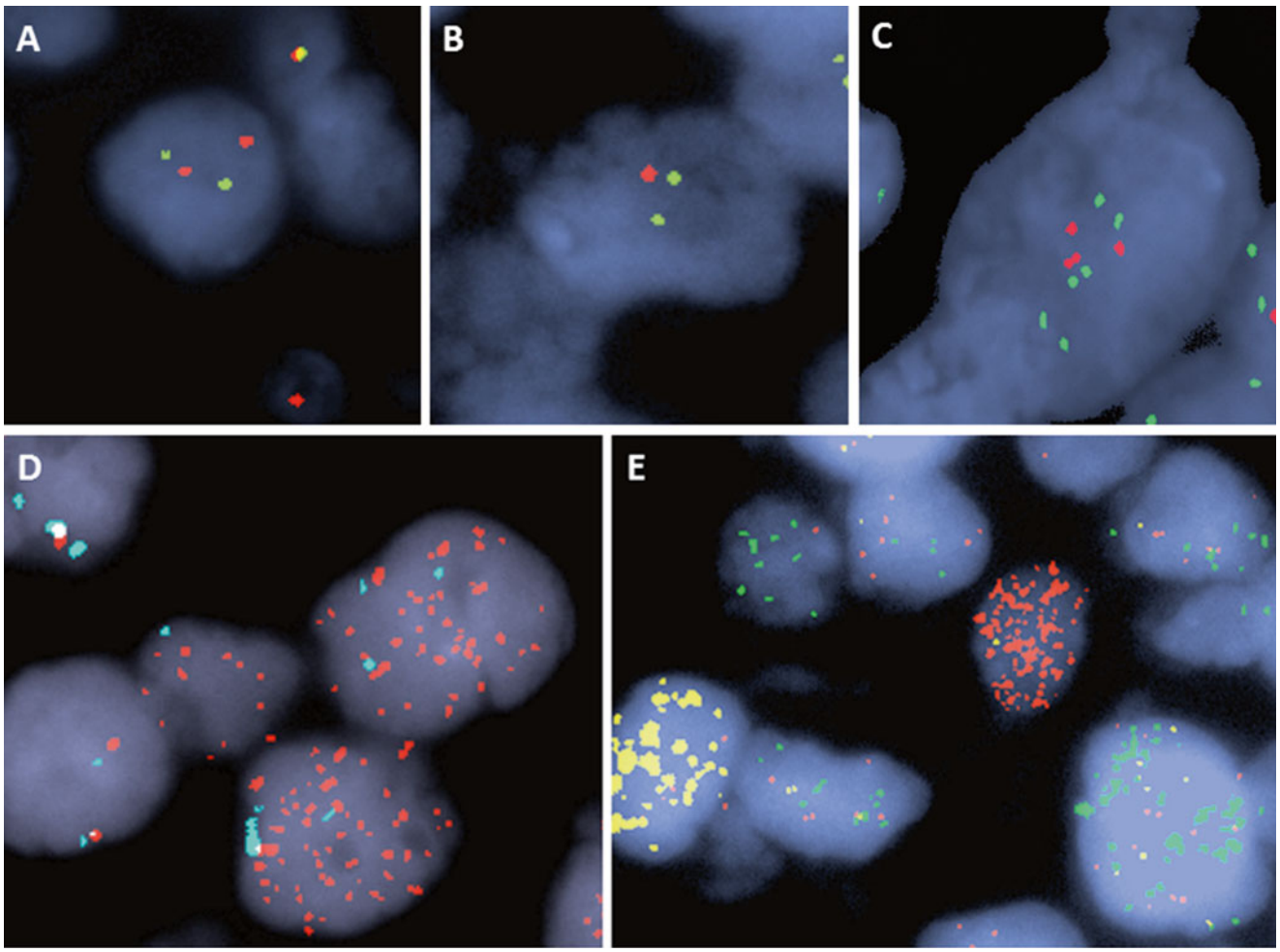

FIG. 3.1. Examples of FISH applications in molecular neuropathology: (**a**–**c**) 1p evaluation, 1p red, 1q green: (**a**) Maintenance of 1p (2:2 signals), (**b**) 1p loss (1:2 signals), (**c**) relative deletions/superloss (4:6). (**d**, **e**) RTK genes evaluation: (**d**) high level amplification of *EGFR* (*red*: *EGFR*, *blue*: CEP7), (**e**) mosaic heterogeneity with mutually exclusive amplifications of *EGFR* (*red*), *PDGFRA* (*green*), and *MET* (*yellow*) in subclones within a GBM. All panels: nuclei are counterstained with DAPI.

challenging implications for molecular pathology and clinical practice. This also raises an important practical question: whether the molecular analysis should be focused on a single target or multiplexed, i.e., analyzing several targets, or even the whole genome. This issue is also discussed in the section on array comparative genomic hybridization (aCGH) below. While specific criteria may differ among laboratories, it is reasonable to suggest that the presence of any subpopulation with gene amplification should be reported. Lastly, FISH can be used to evaluate for translocations. The most typical indication would be for *EWS* gene rearrangement in small, round blue cell tumors when Ewing's sarcoma/peripheral primitive neuroectodermal tumor (PNET) is in the differential.

The 1p/19q analysis can be performed by several techniques, most commonly by FISH, single nucleotide polymorphism (SNP) array, or PCR-based microsatellite LOH. However, FISH is the most commonly used assay and offers additional prognostic information compared to PCR LOH. Fluorescent test probes are commercially available and hybridize to so-called minimally deleted regions [10, 11]. The test probe localizes to 1p36 and a control/reference probe localizes on the opposite arm to 1q25. Target and control/reference probes for chromosome 19 localize to 19q13 and 19p13, respectively. One FFPE section cut at 4–5 μm is used for each chromosome. A few caveats apply for FISH 1p/19q. A normal copy-number LOH resulting from mitotic recombination would not be detected by FISH and could in theory result in false negatives [12]; however, this would be rare in 1p/19q co-deleted oligodendroglioma. More importantly, FISH cannot assess multiple markers to cover the entire arm of the chromosome. Therefore the observed loss might only represent a relatively small "probe-size" deletion on 1p or 19q. However, only the whole arm deletions are truly associated with a favorable prognosis. While the result would be read as positive technically, biologically this would represent a false-positive finding. Many tumors with these

minimal deletions are in fact astrocytomas, rather than oligodendrogliomas, and are actually associated with a worse prognosis. GBMs in particular contain these minimal deletions, and a misdiagnosis of GBM with oligodendroglial component could be made based on a biologically false-positive finding. To avoid this pitfall, some laboratories avoid commercially available probes and choose home-brewed probes on 1p32 and 19q13.4, which are outside the minimal regions of deletion. Although the sensitivity is decreased, this strategy increases specificity of the assay.

The size of FISH probes, ~1 Mb, and staining with either a green or orange/red spectrum fluorescent dye, allows localization against the DAPI counterstained nucleus. As discussed above, four main patterns can be recognized: maintenance of 1p and 19q with two control probes and two target probes, absolute deletion with two control probes and one target probe, polysomy with several copies of target and control regions, and polysomy with deletion of target regions. This pattern known also as relative loss or superloss consists of four control signals and two target signals, for example. However, the ratio of signals can vary and show rations such as 6:3 or 8:4.

Multiple studies have confirmed high reproducibility between SNP/LOH analysis and FISH [13]. While SNP/LOH analysis has an advantage of analyzing multiple markers on chromosomal arms, FISH offers the ability of evaluating the tumor in situ, with small biopsies and without patient's matched normal blood. With growing evidence of implications of polysomy, FISH seems to offer additional prognostic value compared to PCR LOH. There is a strong association between histology and 1p/19q loss. Tumors with classic oligodendroglial features have a higher likelihood of 1p/19q codeletion [14, 15]. It is important to keep in mind that there is no need to select the most oligo-like area when choosing the best section for 1p/19q analysis. It seems that 1p19q codeletion is a very early event in the tumor development, and therefore is present in both oligodendroglial and astrocytic components of an oligoastrocytoma. Another interesting association exists between tumor site and genetics, with frontal oligodendrogliomas having a significantly higher likelihood of 1p/19q loss than temporal lobe tumors [3].

Array Comparative Genomic Hybridization

DNA arrays provide a whole genome analysis of copy number changes. Many arrays offer both copy-number variant and SNP content for LOH analysis in a single array. Genomic DNA can be isolated from FFPE tissue after deparaffinization and protease digestion. A normal male/female DNA standard is usually used for comparison. However, the patient's germline DNA from the peripheral blood can also be utilized. This is particularly useful for SNP analysis. The cancer arrays usually contain a high-resolution backbone with an average spacing approximately one oligo probe every 25–50 kb, which ideally avoid regions containing common copy-number variants (CNV) to minimize detection of benign CNVs. The probe density is usually higher: one every 5 kb in regions defined by International Standards of Cytogenomics Arrays (ISCA). Furthermore, some arrays contain an increased density of probes in known cancer-related genes with up to a single exon resolution, where the density of the probes can be up to one probe every 50 bp. This is particularly useful for genes with known specific deletions, duplications or mutations in cancer. One must keep in mind that although aCGH is a genome-wide technique, the distribution of probes highly depends on the purpose of the array. The design is specific for each clinical indication, and therefore laboratories performing aCGH testing for different clinical questions cannot use the same array for all of them. Although the backbones might be the same or very similar, DNA coverage distribution with highest probe density are significantly different based on whether the array was designed for autism, epilepsy, or cancer, for example.

While a simple PCR LOH does not provide a significant advantage, the aCGH + SNP arrays offer several advantages compared to FISH. The aCGH + SNP provides a whole genome view of the DNA (Fig. 3.2). The same reaction can be performed for all gliomas in the laboratory, regardless whether the diagnosis is GBM or oligodendroglioma, which decreases costs necessary for storing, optimizing, and running several different FISH probes. For example, in a small cell GBM variant where three separate FISH reactions, 1p, 19q, and EGFR, are needed, a single array can provide a definitive answer. In medulloblastoma, aCGH can be utilized for the subgroup classification since different subgroups carry characteristic chromosomal changes. In addition, the array provides information about other genomic changes in brain tumors such as *PTEN*, *CDKN2A/p16*, *PDGFRA*, *NF1*, and *MET*, which are not routinely tested. This information, while not utilized in current clinical care, will increasingly play a role for design of molecularly driven studies, including clinical trials. For example, clinical outcome predictions can be made by evaluating several loci of DNA rearrangements in medulloblastomas, where a number of FISH reactions could be replaced by a single aCGH [16]. If all potential targets are to be tested by FISH, the costs and labor intensity would be significantly higher than a single aCGH + SNP array. An additional advantage is the interpretation software which allows quick review of genomic changes and automated variant call. The software allows manually adjusting levels at which variants can be called and minimizes the possibility of false negatives. While the genome still has to be reviewed manually, the amount of time spent analyzing the array data seems to be equal or shorter in comparison with 1p/19q analysis, which is clearly the most labor-intensive assay in regard to data evaluation. A disadvantage of aCGH technique in comparison with FISH is that it might not be able to detect changes if only scattered infiltrating cells are present in the tissue [7] and might be challenging with small biopsies since approximately 1.5 μg of DNA is needed.

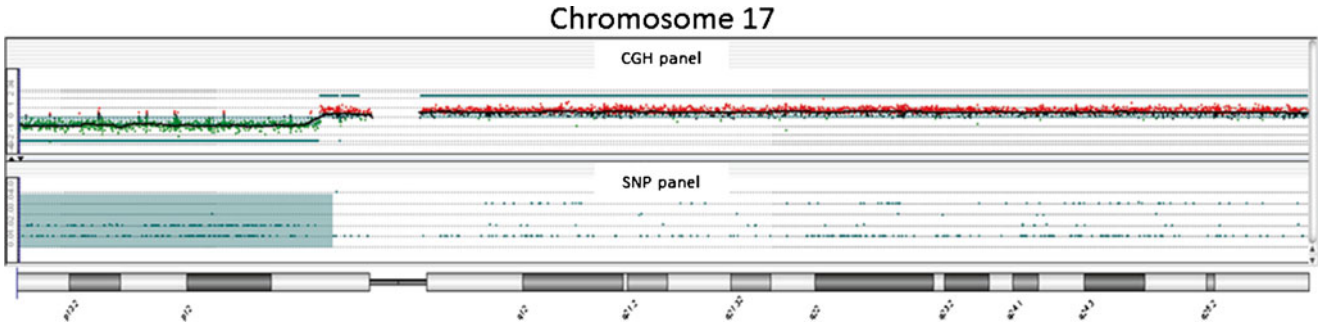

FIG. 3.2. Example of aCGH result in molecular neuropathology: view at chromosome 17 in a medulloblastoma shows a deletion of the short arm of the chromosome 17. This loss occurs in ~25–50 % of medulloblastoma. 17p loss has been associated with a poor survival in some studies suggesting that loss of a tumor suppressor gene located on 17p plays a role in the genesis or progression of medulloblastoma. A novel candidate gene, CTD nuclear envelope phosphatase 1 (*CTDNEP1*), was identified as a recurrent target of mutation in Group 3 and Group 4 medulloblastomas. *CTDNEP1* is located on chromosome 17p13.1 in a hotspot of deletion and LOH.

Mutation Analysis

Mutation-Specific Antibodies

Until recently, the only way to analyze point mutations was by Sanger sequencing. A truly revolutionary event was the introduction of mutation-specific IDH1 R132H antibody into clinical practice. That was quickly followed by a novel BRAF V600E mutation-specific antibody [17–20]. The advantage of using a mutation-specific antibody is undisputable. The staining can be performed in a clinical immunohistochemistry laboratory on FFPE on standard 5 µm sections (Fig. 3.3). Provided the antibody is robust and validated as being highly sensitive and specific, detection is fast, inexpensive, reliable, and allows identification of single infiltrating tumor cells. In comparison with rather nonspecific antibodies such as p53, the mutant protein is not expected to be present in any reactive or inflammatory conditions that may lead to overexpression of nonspecific markers. As a consequence, tumor mutation-specific antibodies are of great value in distinguishing not only reactive astrocytes from tumor cells but also oligodendroglioma/oligoastrocytoma from their morphological mimickers [21]. Although there is strong correlation between *IDH1* mutation and 1p/19q loss, the 1p/19q testing cannot be replaced by IDH1 antibody and several caveats must be noted. For IDH1, the antibody detects only one of several known mutations. While R132H is the most common mutation and represents ~90 % of *IDH1* mutations, other mutations at that site will not be detected by the antibody. Furthermore, mutations of *IDH2* at the residue R172 can also be found in gliomas, although rarely [22]. The R172 residue in *IDH2* is the exact analogue of the R132 residue in *IDH1*. The residue is located in the active site of the enzyme and forms hydrogen bonds with the isocitrate substrate [23]. Therefore, *IDH1* and *IDH2* sequencing provides a definitive answer in IDH1 R132H antibody-negative tumors.

BRAF V600E antibody can be used for the same purpose. However, it is most useful in supratentorial tumors. Although BRAF alterations in pilocytic astrocytoma of the cerebellum are common, they are usually due to a tandem repeat producing a fusion BRAF:KIAA1549 gene [24, 25], which would not be detected by the antibody. BRAF V600E is present in supratentorial pilocytic astrocytoma and pilomyxoid astrocytoma, pleomorphic xanthoastrocytoma (PXA), ganglioglioma and dysembryoplastic neuroepithelial tumor [26]. The antibody could be particularly useful in distinguishing between a PXA and a GBM on a small biopsy, since BRAF V600E mutation would be highly unusual in a GBM, but they are common in PXA [27].

Another example of a clinically important antibody detecting a molecular aberration is *INI1*. The loss of protein expression in an embryonal brain tumor is virtually diagnostic of atypical teratoid-rhabdoid tumor (AT/RT), a highly aggressive neoplasm of early childhood. Immunohistochemistry for INI1 should be performed on every medulloblastoma or primitive embryonal tumor in childhood to avoid misdiagnosis of the AT/RT [28].

Sequencing

Until recently, Sanger sequencing represented the most common way to investigate mutations in brain tumors. Considering that many genes commonly mutated in gliomas such as *TP53* and *NF1* are large and can be altered by several different mutations and the predictive value is unknown, sequencing played a minimal role in clinical laboratories for brain tumors. One of the relevant applications is *IDH1* and *IDH2* sequencing for tumors negative for IDH1 R132H by immunohistochemistry, when the suspicion for less common mutations is high based on clinical presentation. NGS methods are still mostly used in research. However, they are being adopted by clinical laboratories, usually as focused cancer gene panels (Fig. 3.4). As the cost of sequencing continues to decline, and the methods themselves including data analysis become easier to manage in the clinical setting, they will

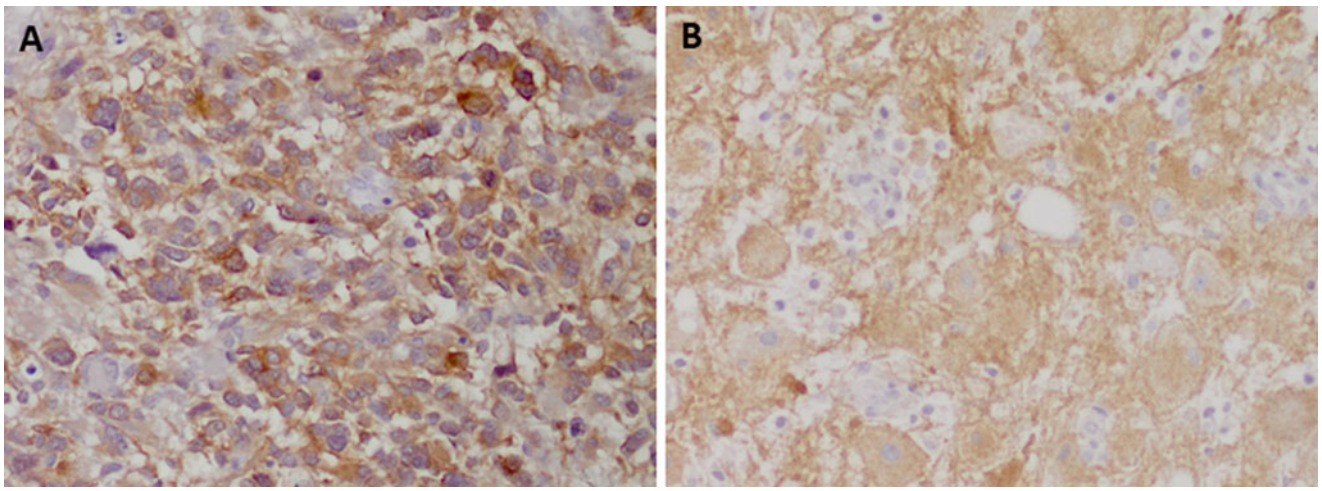

FIG. 3.3 Examples of utility of mutation specific antibodies in neuropathology: the immunohistochemistry with a specific antibody against (**a**) IDH1 R132H in a case of a diffuse astrocytoma and (**b**) BRAF V600E in a cerebellar ganglioglioma shows strong immunoreactivity specific for tumor cells. Reactive cells in the background are negative (**b**)

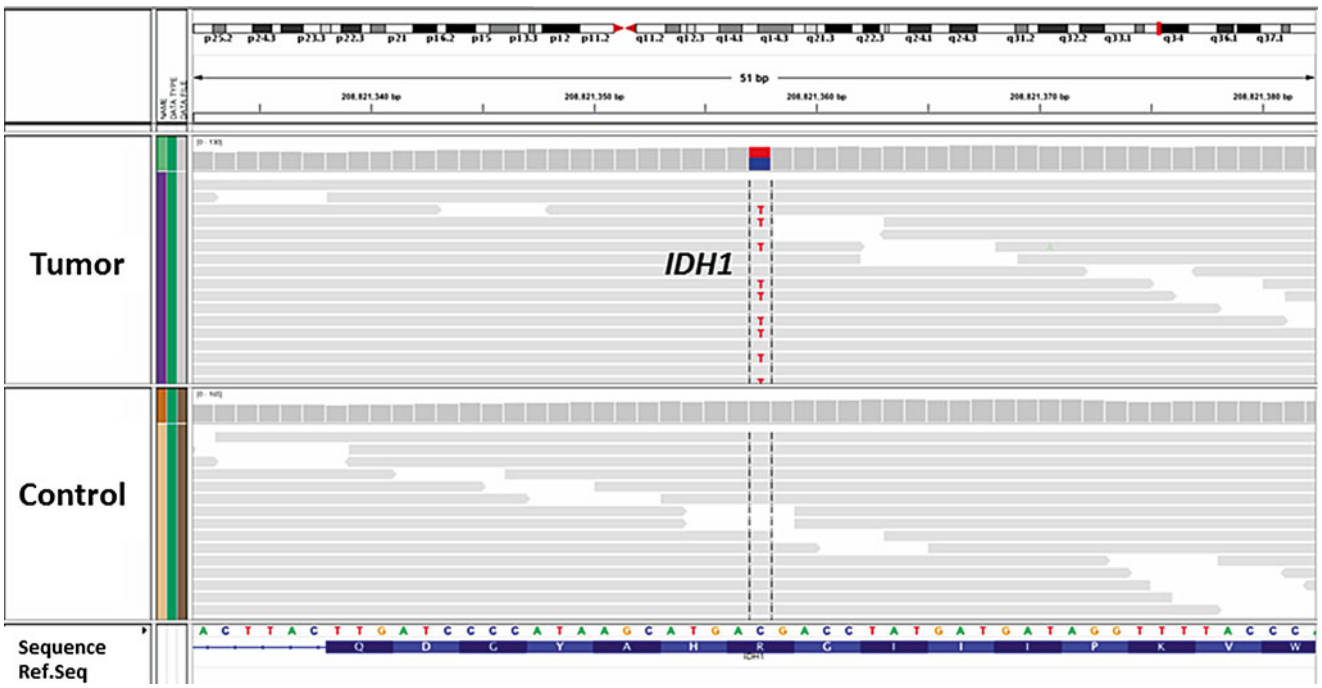

FIG. 3.4 Example of the next-generation sequencing in molecular neuropathology: in oligodendroglioma, whole genome sequencing (Illumina platform) identifies mutation in the IDH1 (c. 395 C>T, p.R132H) gene. The majority of *IDH1* mutations in gliomas are p.R132H. The example shown is in the form of the IGV browser view (Courtesy of Dr. Stephen Yip, BC Cancer Agency)

likely become increasingly available for routine use. In the future, NGS will most likely cover tens to hundreds of cancer-specific genes. However it is only the matter of time before the whole exome or whole genome sequencing cost will not be that much different from a focused panel. Additionally, whole exome/genome sequencing will allow identification of gene rearrangements, which were previously unappreciated phenomena in gliomas [29].

MGMT Testing

MGMT promoter methylation has been confirmed by several clinical studies as a biomarker in patients with gliomas. The MGMT gene is located on chromosome l0q26.34 and contains five exons, the first of which is noncoding. Transcription of the MGMT gene is initiated at a single site within a GC-rich, non-TATA box-containing promoter. Expression of the MGMT gene is epigenetically regulated by methylation-dependent silencing.

Temozolomide (TMZ) methylates DNA at position 6 of guanine nucleotides. The resultant O^6-methylguanine adducts pair with thymidine, and when DNA mismatch repair (MMR) enzymes attempt to excise O^6-methylguanine, they generate single- and double-strand breaks which lead to apoptosis. MGMT can rescue the cell by restoring the normal guanine, which leads to resistance to alkylating chemotherapy. Gliomas with MGMT promoter methylation are less capable to repair DNA and are more sensitive to TMZ.

Several methods have been established for detection of MGMT in glioma (reviewed in [30]). In general, they can be divided into methods requiring or not requiring bisulfite treatment. The three most common methods are methylation-specific PCR (MSP), real-time PCR or MethyLight PCR, and methylation-specific sequencing or pyrosequencing, and they all require bisulfite treatment of the tissue. Detection can be performed on FFPE tissue, as well as on the frozen tissue. For practical purposes, FFPE-based methods are preferable. Other methods that do not require bisulfite conversion and can be used in the clinical setting are methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) and IHC for MGMT protein expression.

There is a significant heterogeneity of MGMT expression and promoter methylation within a glioma. In contrast to 1p/19q testing, MGMT testing requires careful sample selection with a neuropathologist evaluating the case, providing an estimate of the percentage of neoplastic cells and selecting the section with the least amount of necrosis and contaminating non-tumor cells. If normal brain is present on the same section, microdissection of the tumor from the unstained slide is warranted. Many laboratories also require a minimum 50 % of a viable tumor in a given sample to perform testing.

DNA can be extracted from the FFPE tissue using available protocols and kits. The most common methods for MGMT promoter methylation require sodium bisulfite treatment of DNA, which converts unmethylated cytosine into uracil. Methylated cytosine in a CpG island remains unchanged. This bisulfite-modified DNA is used as a template for PCR and sequence differences between methylated and unmethylated DNA after bisulfite treatment allowing for the design of PCR primers that are specific for each template. Bisulfite treatment of DNA is the most difficult part of the assay since it causes further DNA fragmentation. Furthermore, partial conversion could lead to false-positive results. Therefore, appropriate methylated and unmethylated controls are necessary and must be treated in parallel to patient samples to ensure that complete conversion occurred.

MSP is the most commonly used method and allows for the evaluation of methylation status at 6–9 CpG sites. Two primer sets are usually used. One pair is used for amplification of sequences with converted cytosine after bisulfite treatment detects an unmethylated MGMT. A second pair of primers is used for sequences with unconverted cytosine (mC) and detection of a methylated MGMT. PCR product can be visualized by capillary gel electrophoresis, after fluorescent labeling, or by agarose gel electrophoresis (Fig. 3.5). With numerous established protocols available, this method can be easily established in a molecular laboratory and does not require specialized laboratory equipment. An advantage is an easy-to-read result; however a disadvantage is lack of the quantitative assessment of methylation. Another disadvantage is that this method does not include a control for bisulfite conversion, and incomplete conversion of unmethylated cytosines may be interpreted as methylation, leading to false-positive results.

The qMethylation-Specific RT-PCR-MethyLight assay is a simple, quantitative real-time PCR method to determine the methylation status of MGMT CpG islands. It utilizes the TaqMan PCR with forward and reverse primers. It also contains a fluorescent oligo probe, which emits only after it is degraded by the 5′–3′ exonuclease activity of Taq polymerase. It requires a second set of primers and a probe, for amplification of a housekeeping gene, which are used as amplification controls for quality and quantity of the DNA. Control gene primers and probes are designed for the regions with no CpG islands and complementary to the

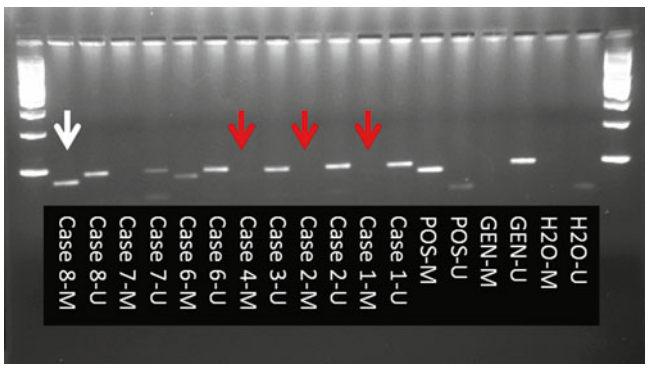

FIG. 3.5. Example of methylation specific PCR evaluation of MGMT in GBM: Agarose gel electrophoresis show examples of MGMT evaluation. *H_2O* water, *GEN* genomic control (negative for promoter methylation), *POS* positive control (positive for MGMT promoter methylation), *U* unmethylated primers, *M* methylated primers. Cases 1, 2, and 3 show no PCR product in the methylated lane and are examples of tumors without MGMT promoter methylation (*red arrows*). Case 6 shows a product in the methylated lane and is an example of a tumor with MGMT promoter methylation (*white arrow*).

bisulfite-converted sequence. This allows control for assessing the efficiency of bisulfite conversion and quantifying MGMT methylation.

The qRT-PCR assay is more specific, and rarely produces false-positive results. The assay is relatively easy to set up, but requires a real-time PCR instrument, which is available in most laboratories. The results are easy to interpret, and inclusion of the standard curve gives numerical values for copy numbers of methylated MGMT sequences, as well as housekeeping gene sequences. However, the percentage of contaminating stromal cells cannot be accurately assessed, and therefore quantitation of MGMT promoter methylation by qRT-PCR is not recommended.

Primers can be designed to cover both the upstream and downstream regions of CpGs, as well as methylated and unmethylated sequences. Sanger bidirectional DNA sequencing can be used to provide a semiquantitative measure of MGMT promoter. However, standard Sanger sequencing has not been established in MGMT analyses compared to methylation specific PCR. On the other hand, pyrosequencing, or sequencing by synthesis, has been used in some laboratories. Pyrosequencing also requires bisulfite treatment of genomic DNA and PCR amplification with primers surrounding CpGI, followed by pyrosequencing. The main advantage of pyrosequencing is the ability to quantify methylation at each CpG site and identify cases with low levels of methylation reliably. However, costs of equipment are high and it is therefore more appropriate for high volume laboratories. NGS methods are still mostly research applications; however, they will likely become available in clinical settings, and can be used for methylation analysis.

Methylation-specific MLPA utilizes unique approach with the ligation of oligonucleotide probes, followed by a digestion of the genomic DNA-probe hybrid complex with methylation-sensitive endonucleases. When the CpG locus is not methylated, methylation-sensitive restriction endonuclease cleaves its restriction site, resulting in lack of PCR amplification. When the CpG locus is methylated, the restriction site is protected from endonuclease digestion and PCR product is generated. The methylation-specific probes are designed so that the sequences detected contain a methylation-sensitive restriction site GCGC. The advantage of this semiquantitative method, in which the level of methylation at each site can be determined, is that it does not require bisulfite treatment. MLPA can detect changes in both CpG methylation and copy-number of up to 40 chromosomal sequences in a single reaction. Capillary electrophoresis is necessary to identify and quantify PCR products of the individual probes. The sample DNA is split and one part is subjected to a single ligation step, whereas for the other part ligation is combined with the methylation-sensitive digestion. Subsequent PCR reaction amplifies either total DNA or the methylated fraction. Comparison of the peaks of the ligated fraction and the fraction that is digested with endonuclease provides the methylation ratios. The disadvantage of this method is the need for special equipment and expensive reagents. However, for laboratories performing MLPA assays for other indications, the methylation-specific MLPA is easy to establish.

The use of IHC for the detection of MGMT has been investigated in a number of studies, with lack of concordance between MGMT expression by immunohistochemistry and MGMT promoter methylation. Furthermore, lack of MGMT expression by IHC was not as robust of a biomarker as MGMT promoter methylation. The conclusion from these studies is that MGMT promoter methylation and MGMT protein expression detected by immunohistochemistry cannot be used interchangeably to predict survival for patients with malignant gliomas [31]. Therefore, immunohistochemistry is not the method of choice for the detection of MGMT activity and its use should be discouraged.

Expression Profiling

Gene signatures have been shown to be capable of distinguishing molecular subtypes of tumors that appear indistinguishable histologically, but reflect different disease biologies as evidenced by differences in clinical presentation and/or outcomes. A number of groups have attempted to identify individual genes as well as signaling pathways from microarray data that are prognostic in malignant glioma and medulloblastoma [32–34]. Expression profiling was able to identify specific subgroups within each disease that were associated with improved or decreased survival. Despite some prognostic value [35], the application in clinical practice has been limited due to a variety of reasons such as costs, equipment requirement, and the need of frozen material for good-quality whole genome expression profile studies. Overall, as with all molecular tests in clinical practice, the use of FFPE-based assays is critical to the wide-scale acceptance of a biomarker due to the limited availability of fresh/frozen tissues. In GBM, a multigene profile compatible for FFPE samples is currently used as a stratification factor in a large Phase III clinical trial (RTOG-0825) [36]. The 9-gene set was validated with an independent sample set and was shown to be an independent predictor of clinical outcome after adjusting for clinical factors and MGMT status. Another approach is to use a selected set of genes that have been firmly associated with the subtype using FFPE samples on a platform such as NanoString® to molecularly classify brain tumors such as medulloblastoma or GBM (Fig. 3.6). RNA-based methods such as NanoString® that can utilize FFPE tissues are easier to implement in clinical laboratories than assays that require high-quality RNA from the frozen tissue, which will likely not be implemented in standard clinical practice.

Summary

Several well-established techniques are currently used in molecular neuropathology. Analyses include copy-number changes, mutations, and epigenetic modification assessed

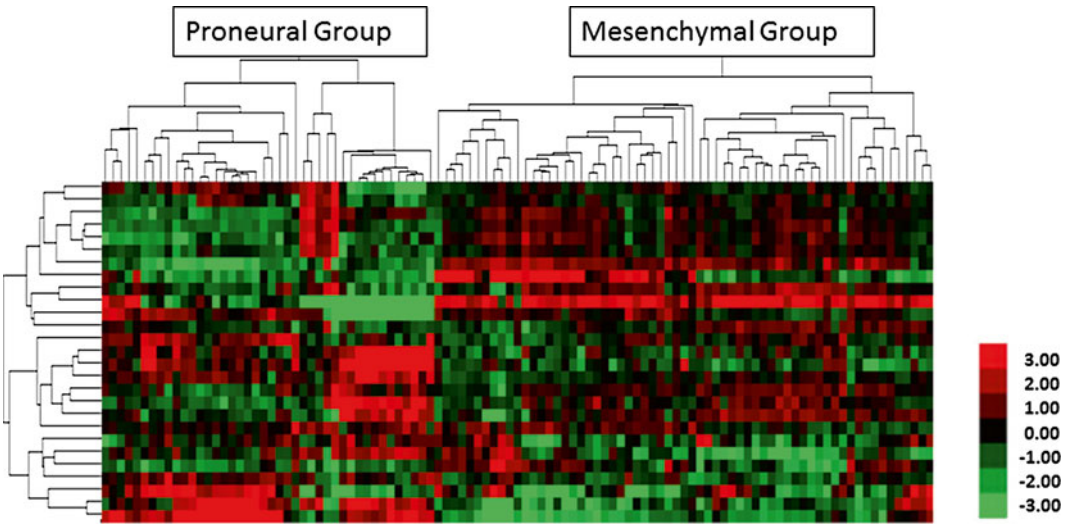

FIG. 3.6 Example of expression profile evaluation of GBM: RNA isolated from the FFPE tissues and can be used for the next-generation of expression profile techniques. Using an expression of a core of validated genes for GBM and unsupervised hierarchical clustering, tumors are segregating into different molecular subgroups such as Proneural and Mesenchymal. *Red–green* scale shows the change in the expression level (Figure created in collaboration with Dr. Stephen Yip, BC Cancer Agency)

most commonly by FISH, PCR, sequencing of mutation-specific antibodies. From the technical perspective, once validated, these techniques are robust and require minimal troubleshooting. The most commonly performed tests with the largest clinical impact include MGMT promoter methylation, 1p/19q status and *IDH1* mutation. These assays should be incorporated both within routine clinical care and within clinical trial designs. When used in the right clinical context after neuropathology review and with appropriate interpretation guidelines, they can provide diagnostic, prognostic, and predictive information that can help guide clinical management.

At the present time, the number of assays that can be performed on brain tumors and the information that can be obtained is significantly greater than what can be used for practical diagnostic and clinical purposes. Careful retrospective and prospective validation of molecular genetic alterations and profiles for prognostic and predictive value will be required in clinical studies before implementation into routine diagnostics and clinical care.

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