



## Molecular Testing of Brain Tumor

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The World Health Organization (WHO) classification of central nervous system (CNS) tumors was revised in 2016 with a basis on the integrated diagnosis of molecular genetics. We herein provide the guidelines for using molecular genetic tests in routine pathological practice for an accurate diagnosis and appropriate management. While astrocytomas and IDH-mutant (secondary) glioblastomas are characterized by the mutational status of *IDH*, *TP53*, and *ATRX*, oligodendrogliomas have a 1p/19q codeletion and mutations in *IDH*, *CIC*, *FUBP1*, and the promoter region of telomerase reverse transcriptase (*TERTp*). IDH-wildtype (primary) glioblastomas typically lack mutations in *IDH*, but are characterized by copy number variations of *EGFR*, *PTEN*, *CDKN2A/B*, *PDGFRA*, and *NF1* as well as mutations of *TERTp*. High-grade pediatric gliomas differ from those of adult gliomas, consisting of mutations in *H3F3A*, *ATRX*, and *DAXX*, but not in *IDH* genes. In contrast, well-circumscribed low-grade neuroepithelial tumors in children, such as pilocytic astrocytoma, pleomorphic xanthoastrocytoma, and ganglioglioma, often have mutations or activating rearrangements in the *BRAF*, *FGFR1*, and *MYB* genes. Other CNS tumors, such as ependymomas, neuronal and glioneuronal tumors, embryonal tumors, meningothelial, and other mesenchymal tumors have important genetic alterations, many of which are diagnostic, prognostic, and predictive markers and therapeutic targets. Therefore, the neuropathological evaluation of brain tumors is increasingly dependent on molecular genetic tests for proper classification, prediction of biological behavior and patient management. Identifying these gene abnormalities requires cost-effective and high-throughput testing, such as next-generation sequencing. Overall, this paper reviews the global guidelines and diagnostic algorithms for molecular genetic testing of brain tumors.

**Key Words:** Brain neoplasms; Molecular biology; Next generation sequencing; Pathological diagnosis

The central nervous system (CNS) tumors are those of the brain and spinal cord including the meninges, pituitary gland, pineal gland, and nerves according to the 3rd edition of the International Classification of Diseases for Oncology (ICD-O-3) (<http://codes.iarc.fr/>). The incidence rate of all primary malignant and nonmalignant CNS tumors in the United States was 22.36 cases per 100,000 (5.67/100,000 for 0–14 years, 5.71/100,000 for 0–19 years) according to the U.S. Central Brain Tumor Registry (CBTRUS; <http://www.cbtrus.org/factsheet/factsheet.html>). The proportion of women with CNS tumors is higher than that of men (1.2:1). In the United States, the number of newly diagnosed primary malignant and nonmalignant CNS tumors is expected to reach 79,270 (26,070 cases of primary malignant and 53,200 cases of nonmalignant tumors) by 2017. The average annual mortality rate in the United States between 2009 and 2013 is 4.32 per 100,000 and with 73,450 deaths attributed to primary malignant brain and other CNS tumors. If we extrapolate the U.S. data with its incidence rate to the

Korean population, per year we would expect about 12,500 new cases of primary CNS tumors and 2,160 deaths. According to the nationwide cancer registration (hospital based Korean Central Cancer Registry [KCCR], [http://www.iacr.com.fr/index.php?option=com\\_comprofiler&task=userprofile&user=973&Itemid=498](http://www.iacr.com.fr/index.php?option=com_comprofiler&task=userprofile&user=973&Itemid=498), <http://ncc.re.kr/cancerStatsView.ncc?bbsnum=358&searchKey=total&searchValue=&pageNum=1>), 10,004 patients were diagnosed with primary CNS tumors in Korea in 2010, which means that the incidence of brain tumors in Korea is lower than that of United States.<sup>1,2</sup> Similar to the United States, CNS tumors occurred more frequently in female (female:male, 1.59:1). The four most common tumors were meningioma (35.5%), pituitary tumors (18.7%), gliomas (15.1%), and nerve sheath tumors (10.3%). Glioblastoma (GBM) is the most common and the most malignant glioma, which comprises 34.6% of all gliomas. In children (0–19 years), sellar region tumors (pituitary adenoma and craniopharyngiomas), pilocytic astrocytomas, germ cell tumor, and embryonal tumors/medulloblastoma were

the most common brain tumors.<sup>2</sup>

The 5-year relative survival rate in the United States after the diagnosis of brain or other CNS tumors that are either primary malignant or nonmalignant is 34.7% and 90.4%, respectively. The survival rate of patients with malignant CNS tumors depends on onset age, histologic and molecular diagnosis, and the tumor grade. The overall survival rate decreases with age (0–19 years, 73.8%; 20–44 years, 61.5%; 45–54 years, 33.5%; 55–64 years, 18.5%; 65–74 years, 11.2%; 75 or older: 6.3%). Therefore, children are more likely to survive than adults after malignant CNS tumors develop. Thus, advances must be made to improve the survival rates of young patients with CNS tumors.

The completion of the human genome project led to two powerful tools: the human genome sequence and advanced molecular technologies. Over the past few decades, investigators have been constantly searching for causes of cancer. Through high-throughput studies such as the Cancer Genome Atlas (TCGA) project (<https://cancergenome.nih.gov/>) or the cBioPortal project (<http://www.cbioportal.org/>), a large number of brain tumors have been sequenced and major genetic alterations in brain tumors have been identified,<sup>3</sup> thus allowing for the classification of gliomas. The signaling pathways that are mainly involved in GBM are RTK/RAS/phosphoinositide 3-kinase (PI3K) signaling, p53 signaling, and Rb signaling, which were identified through analyses of common mutations and copy number variations (CNV) in gliomas.<sup>4</sup> In addition, according to the molecular classification by global mRNA expression and DNA methylation, GBMs can be categorized into neural, proneural, classical, and mesenchymal transcriptomic subtypes.<sup>5</sup>

One of the most prominent molecular discoveries was made in 2008 in the genomics of diffuse gliomas. Mutations in *IDH1* and *IDH2* were discovered by whole exome sequencing (WES) of GBMs, thus altering the classification of gliomas.<sup>6</sup> *IDH1* or *IDH2* mutations are found in both astrocytic and oligodendroglial tumors since they act as a starting point for gliomagenesis. Therefore, the 2016 World Health Organization (WHO) classification guidelines combined astrocytic tumors and oligodendroglial tumors into one category.<sup>7–10</sup> Furthermore, each tumor has a typical genetic signature. For example, oligodendrogliomas are characterized by a codeletion in 1p/19q, mutations in *IDH*, and the promoter region of the gene encoding telomerase reverse transcriptase (*TERT*). Furthermore, grade 2 and grade 3 astrocytic tumors are characterized by *ATRX* and *TP53* mutations, while *IDH*-wildtype (primary) GBMs are characterized by the CNV of *EGFR*, *PTEN*, *CDKN2A/B*, *PDGFRA*, and *MET* genes, in addition to a lack of mutations

in *IDH* and a codeletion in 1p/19q.<sup>11–13</sup>

Even in tumors that are morphologically similar, those with mutations in *IDH* and a codeletion in 1p/19q differ in the treatment response and prognosis compared with tumors without these two molecular alterations.<sup>11,14–19</sup> Since only gliomas with mutations in *IDH* and a codeletion in 1p/19q are considered oligodendrogliomas, the so-called pediatric-type oligodendroglioma lacking these two alterations is not considered an oligodendroglioma. The molecular differences of primary *IDH*-wildtype and *IDH*-mutant (secondary) GBMs are well summarized in the paper by Cachia et al.<sup>20</sup> The genetic abnormalities of the *IDH*-mutant GBMs are similar to grade II/III astrocytomas. In other words, they more likely have mutations in *IDH*, *ATRX*, and *TP53*.

Since the prognosis of each tumor depends on the mutational status of *IDH1* and *IDH2*, astrocytic tumors are also classified according to this metric (Fig. 1).<sup>6,8,21</sup> Pediatric GBMs and high-grade gliomas differ from those of adults. No genetic abnormalities in *IDH1* and *IDH2* are found in pediatric GBMs, but mutations are generally found in *H3F3A*, *ATRX* and *DAXX* (Fig. 2). The K27M in *H3F3A* is a major mutation found in diffuse midline gliomas.<sup>22–25</sup> Additionally, a *C11orf95-RELA* fusion was found in supratentorial ependymomas.<sup>26–34</sup> A V600E mutation in *BRAF* was found in circumscribed gliomas, such as pleomorphic xanthoastrocytoma (PXA) (66%),<sup>35–38</sup> gangliogliomas (25%),<sup>35,38</sup> and pilocytic astrocytomas (15%).<sup>35,38</sup> Furthermore, a *BRAF-TIAA1549* fusion was discovered in pilocytic astrocytoma (more than 70%) (Fig. 2).<sup>39–43</sup> *SMARCB1* (*INI1*) or *SMARCA4* (*BRG1*) gene mutations or deletions were observed in atypical teratoid/rhabdoid tumors (AT/RT),<sup>44–46</sup> and a *NAB2-STAT6* fusion was present in solitary fibrous tumor/hemangiopericytomas.<sup>47–49</sup> Meningiomas also have typical gene mutations according to the tumor type and grade. Sixty percent of the sporadic meningiomas have mutations in *TRAF7*, *KLF4*, *AKT1*, *SMO*, and *PIK3CA*.<sup>50–52</sup> Among them, mutations in *TRAF7* and *KLF4* are found in secretory-type meningiomas and mutations in *TRAF7/AKT1/PIK3CA* are found in meningotheial and transitional-type meningiomas.<sup>52,53</sup> Mutations in *TRAF7* are the most common genomic aberrations and are found in 12%–15% of sporadic meningiomas, preferentially fibrous and transitional subtype, which are found concurrently with mutations in *KLF4*, *AKT1*, or *PIK3CA*, but are mutually exclusive with mutations in *SMO* and neurofibromatosis type 2 (*NF2*).<sup>53</sup> *NF2*-associated meningiomas have mutations in *NF2*. Atypical and anaplastic meningiomas usually have mutations in *TERT* or marked copy number aberrations and loss of *CDKN2A/2B*.<sup>53</sup>

These findings demonstrate that tumors are genetic disorders and that certain mutations can represent different biological behaviors and result in different prognoses.

Molecular testing, such as molecular profiling of brain tumors, improves diagnostic accuracy, allows for predictive prognosis, and enables target identification. At present, target therapy has become one of the most promising treatment options for many tumors. Here, we summarize and provide guidelines for the molecular testing of brain tumors. High-throughput genomic studies, such as next-generation sequencing (NGS), can be useful financially and practically by rapidly replacing time-consuming and labor-intensive conventional methods since multiple molecular genetic abnormalities must be studied in order to diagnose various brain tumors.

## IMPORTANT MOLECULAR GENETICS FOUND IN BRAIN TUMORS

The indications and methods of each genetic study common to brain tumors are summarized in Tables 1–6. Integration of morphological criteria and genetic alterations is the guideline for an accurate diagnosis, risk classification, and development of therapeutic strategies.

### Mutations in *IDH1/2*

The discovery of mutations affecting the enzymatic function of IDH in gliomas provided a fundamentally new insight into the biology of gliomagenesis and triggered molecular classification of gliomas by a somatic mutation. IDH catalyzes the oxidative decarboxylation of isocitrate to produce  $\alpha$ -ketoglutarate

**Table 1.** Mutation rate in various genes commonly mutated in brain tumors<sup>3,41,55-61</sup>

	WHO grade	Altered genes	Genomic alteration (%)
Astrocytoma	II, III	<i>IDH</i> mutation	65
		<i>P53</i> mutation	96
		<i>ATRX</i> mutation	96
Glioblastoma	IV	<i>EGFR</i> amplification	57
		<i>PDGFRA</i> amplification	13
		<i>EGFRvIII</i> mutation	20
		<i>PTEN</i> homozygous deletion	25–35
		<i>CDKN2A</i> homozygous deletion	61
		<i>BRAF</i> V600E mutation	1–2 (epithelioid GBM)
Oligodendroglioma	II, III	<i>IDH</i> mutation	100
		1p/19q codeletion	100
		<i>CIC/FUBP1</i> mutation	56/29
		<i>TERTp</i> mutation	80–96
Subependymal giant cell astrocytoma	I	<i>TSC1</i> and <i>TSC2</i> mutation	100
Pilocytic astrocytoma	I	<i>BRAF-KIAA1549</i> fusion	75 (cerebellar tumor)
		<i>BRAF</i> V600E mutation	13–15
Pleomorphic xanthoastrocytoma	II, III	<i>BRAF</i> V600E mutation	66
		<i>CDKN2A</i> homozygous deletion	50
Angiocentric glioma	I	<i>MYB-QK1</i> fusion	100
Ganglioglioma	I, III	<i>BRAF</i> V600E mutation	25
		<i>TSC1</i> and <i>TSC2</i> mutation	Unknown
Craniopharyngioma, papillary type	I	<i>BRAF</i> V600E mutation	100
Craniopharyngioma, adamantinomatous type	I	<i>CTNNB1</i> mutation	100
AT/RT	IV	<i>SMARCB1</i> deletion/mutation	>95
	IV	<i>SMARCA4</i> mutation	<5
Cribriform tumor		<i>SMARCB1</i> deletion/mutation	100
Meningioma, fibrous type		<i>NF2</i> inactivation mutation (no hot spot)	45–55 <sup>50</sup>
		<i>TRAF7/KLF4</i> mutation	100 (secretory meningioma)
		<i>AKT1</i> (p.Glu17Lys) mutation	2.5
		<i>SMO</i> (p.Trp535Leu) mutation	5
		<i>TERTp</i> mutation	10
Langerhans cell histiocytosis	I, III	<i>BRAF</i> V600E mutation	50–57 <sup>54</sup>

WHO, World Health Organization; GBM, glioblastoma; TSC, tuberous sclerosis complex; AT/RT, atypical teratoid/rhabdoid tumor.

**Table 2.** Common pediatric molecular subgroups with molecular features<sup>7,23,30,43,56,62,66</sup>

Tumor	Molecular subgroup	Molecular feature	Prognosis
LGG	<i>BRAF</i> V600E	70% of PXA, GG, DA	Good
	<i>KIAA-BRAF</i> fusion; <i>BRAF</i> duplication	90% of PA	Good
	<i>MYB-QKI</i> rearrangement	High proportion of AG	Good
	<i>FGFR1</i> duplication	PA, DA, DNT	Good
HGG	K27M-mutant	H3.1 and H3.3 K27 mutation, <i>PDGFRA</i> focal amplification, <i>TP53</i> mutation, <i>ACVR1</i> mutation	Poor
	G34R/V-mutant	H3.3 G3 and <i>TP53</i> mutation	Poor
	<i>RTK1</i> amplified	<i>PDGFRA</i> and <i>EGFR</i> focal amplification, <i>CDKN2A/2B</i> homozygous deletion	Poor
	Mesenchymal	<i>NFI</i> mutation, <i>PDGFRA</i> and <i>EGFR</i> focal amplification, <i>CDKN2A/2B</i> homozygous deletion	-
Ependymoma	C11orf95- <i>RELA</i> + (70% of ST E)	<i>RELA</i> fusion transcripts	Poor
	<i>RELA</i> fusion – (30% of ST E)	Possibly <i>YAP1</i> fusion transcripts	Good
	Posterior fossa E	Group A: <i>LAMA2</i> overexpression, Group B: <i>NELL2</i> overexpression	Poor Good
	Spinal cord E (10% of child E)	<i>NF2</i> mutation, myxopapillary histology	Good
Medulloblastoma	WNT (10%)	Nuclear $\beta$ -catenin positive, <i>CTNNB1</i> mutations in exon 3, monosomy 6	Excellent
	SHH (30%)	Heterogeneous molecular features depending on age of presentation; <i>PTCH1</i> , <i>SMO</i> , and <i>SUFU</i> mutations, <i>GLI2</i> and <i>MYCN</i> amplification; germline <i>TP53</i> mutations	Intermediate (except in infants who have a good prognosis)
	Group 3 (25%)	By some unknown mechanism; <i>MYC</i> amplification and over expression	Poor
	Group 4 (35%)	i(17)q; <i>MYCN</i> amplification	Intermediate
AT/RT		<i>SMARCB1/SHARCA4</i> mutation	Poor
ETMR		<i>C19MC</i> amplification	Poor

LGG, low grade glioma; PXA, pleomorphic xanthoastrocytoma; GG, ganglioglioma; DA, diffuse astrocytoma; PA, pilocytic astrocytoma; AG, angiocentric glioma; DNT, dysembryoplastic neuroepithelial tumor; HGG, high grade glioma; ST, supratentorial; PF, posterior fossa; E, ependymoma; WNT, wingless signaling pathway; i, isochromosome; AT/RT, atypical teratoid/rhabdoid tumor; ETMR, embryonal tumor with multilayer rosettes.

( $\alpha$ -KG) and CO<sub>2</sub>, but mutant *IDH1/2* has a preferential affinity for  $\alpha$ -KG instead of isocitrate outside of the citric acid cycle, thus leading to the production and accumulation of the oncometabolite 2-hydroxyglutarate (2HG).<sup>67-70</sup> Dang et al.<sup>68</sup> hypothesized that 2HG induces redox stress due to damage to the respiratory chain, which subsequently promotes the mutagenesis and development of gliomas. The  $\alpha$ -KG-dependent prolyl hydroxylases modulate HIF1 $\alpha$  levels and changes in the HIF1 $\alpha$  downstream pathway, leading to an increase in reactive oxygen species levels and potentially contributing to the risk of cancer.<sup>71</sup>

The glioma-CpG island methylator phenotype (G-CIMP) subset is distinctively and invariably found in the gliomas with mutant *IDH*, which is the proneural transcriptional group.<sup>72</sup> 2HG, the oncometabolite produced by mutant *IDH1/2*, inhibits  $\alpha$ -KG-dependent dioxygenases including histone demethylases and the Ten-Eleven Translocation (*TET*) family of histone 5-methylcytosine hydroxylase, which directly induce the hypermethylated state.<sup>73</sup> The G-CIMP subgroup of GBMs is common in younger patients and has a longer lifespan.<sup>13,17,30,67,74-76</sup>

All *IDH1/2* mutations are common in 70%–80% of type II and III infiltrating gliomas and are found in 100% of oligodendroglomas and IDH-mutant GBMs.<sup>67</sup>

droglomas and IDH-mutant GBMs.<sup>67</sup>

#### *TP53* mutation

*TP53* is a typical tumor suppressor gene located in 17p13.1, which encodes the nuclear protein p53. The p53 protein responds to diverse cellular stresses to regulate the expression of its target genes, thereby inducing cell cycle arrest, apoptosis, senescence, DNA repair, or metabolic changes. Mutated *TP53* genes and overexpressed abnormal p53 protein, which has a longer half-life than wild type p53, are associated with a variety of human cancers, including Li-Fraumeni syndrome and many hereditary gliomas. As mentioned in the introduction, the p53 signaling pathway is one of the major abrogated pathways of astrocytic tumors including GBMs. WHO class II and III astrocytic tumors show high levels of *TP53* mutations (94%) and/or p53 overexpression (Tables 3, 4).<sup>3</sup> However, these alterations are rare in oligodendroglomas, well-circumscribed astrocytic tumors including pilocytic astrocytomas, pleomorphic xanthoastrocytomas, and subependymal giant cell astrocytomas, ependymomas, and embryonal tumors such as medulloblastoma, except for the SHH-type, p53-activated subgroup.

**Table 3.** Mutation rate of commonly mutated genes in various brain tumors<sup>7,9,48,55-57,63,77-81</sup>

Genes and molecules	Biomarker type	PA (%)	PXA (%)	DA/AA (%)	IDH-wildtype GBM (%)	IDH-mutant GBM (%)	DMG, H3 K27M (%)	ODG (%)	Epen (%)	MB (%)	AT/RT (%)	Test method
WHO grade		I	II, III	II, III	IV	IV	IV	II/III	II/III	IV	IV	
<i>IDH1</i> mutation	Diagnostic and prognostic	0	-	>80	5–6	>95	0	98	0	0	0	IHC, sequencing
<i>IDH2</i> mutation	Diagnostic and prognostic	0	-	-	-	2–5	0	2	0	0	0	Sequencing
<i>TP53</i> mutation	Diagnostic and prognostic	0	6	94	25–35	62–65	45–50	9–44	0	-	0	Sequencing, IHC
<i>ATRX</i> mutation	Diagnostic and prognostic		-	86	0	90–95	50	-	0	-	0	IHC, sequencing
Histone K27M mutation	Diagnostic and prognostic	0	-	0	Pediatric HGG	0	100	0	0	0	0	IHC, sequencing
Histone G34 mutation	Diagnostic and prognostic	0	-	-	Uncertain	-	-	0	0	-	-	Sequencing
Histone K36 mutation	Diagnostic and prognostic	0	-	-	-	-	-	0	0	-	-	Sequencing
1p/19q codeletion	Diagnostic, prognostic, and predictive	0	0	0	0	0	0	100	0	0	0	FISH, CGH
<i>CIC</i> mutation	Diagnostic, prognostic, and predictive	0	-	-	-	-	-	49	0	-	-	Sequencing, IHC
<i>FUBP1</i> mutation	Diagnostic, prognostic, and predictive	0	-	-	-	-	-	29	0	-	-	Sequencing, IHC
<i>EGFR</i> amp	Prognostic	0	-	-	35–45	4	-	-	0	-	-	FISH, CGH, IHC
<i>PDGFRA</i> amp	Diagnostic		-	-	13	-	50	-	-	-	-	FISH, CGH, IHC
<i>PTEN</i> HoD	Prognostic	0	-	-	25–35	5	-	-	0	-	-	FISH, CGH, IHC
<i>CDKN2A</i> HoD	Predictive	0	60	11	35–50	-	<5	-	0	-	-	FISH, CGH, IHC
<i>BRAF</i> V600E mutation	Diagnostic and prognostic	15	67	0	1–2	0	0	0	0	0	0	Sequencing, IHC
<i>BRAF-KIAA1549</i> fusion	Diagnostic and prognostic	70	-	-	-	-	-	-	-	-	-	RT-PCR, FISH (gain)
<i>TERT</i> promoter mutation	Diagnostic and prognostic	-	-	-	>80	26	-	96	0	21	-	Sequencing
<i>C11orf95-RELA</i> fusion	Diagnostic and prognostic	0	-	-	-	-	-	-	<5	-	-	RT-PCR, IHC, RNA seq
$\beta$ -Catenin mutation	Diagnostic and prognostic	0	-	-	-	-	-	-	0	6–9	-	IHC, sequencing
<i>MYC</i> amp	Prognostic	0	-	-	-	-	-	-	-	10–15	-	FISH, CGH, IHC
<i>MYCN</i> amp	Prognostic	0	-	-	-	-	-	-	-	10–15	-	FISH, CGH
<i>SMARCB1</i> mutation	Diagnostic and prognostic	0	-	-	-	-	-	-	0	0	98	IHC, sequencing
<i>SMARCA4</i> mutation	Diagnostic and prognostic	0	-	-	-	-	-	-	0	0	1–2	IHC, sequencing
<i>MGMT</i> methylation	Predictive	-	-	78.5	48.5	60–80	-	85	0	0	0	MSP-PCR, pyrosequencing, IHC

PA, pilocytic astrocytoma; PXA, pleomorphic xanthoastrocytoma; DA, diffuse astrocytoma; AA, anaplastic astrocytoma; GBM, glioblastoma; DMG, diffuse midline glioma; HGG, high grade glioma; ODG, oligodendroglioma; MB, medulloblastomas; AT/RT, atypical teratoid/ rhabdoid tumor; WHO, World Health Organization; IHC, immunohistochemistry; FISH, fluorescent *in situ* hybridization; CGH, comparative genomic hybridization; RT-PCR, reverse transcription polymerase chain reaction; -, not known; MSP-PCR, methylation-specific polymerase chain reaction.

### *ATRX* is an X-linked gene of $\alpha$ -thalassemia and mental retardation syndrome

*ATRX* is located in Xq21.1, encodes a 280-kDa nuclear protein, and has been shown to be involved in a wide range of cellular functions such as DNA recombination, repair, and transcription regulation.<sup>82</sup> It binds strongly within the promyelocytic leukemia body of the nucleus. When *ATRX* interacts with *DAXX*, this complex functions as a histone chaperone complex for the deposition of histone variant H3.3 into heterochromatic

repeats including pericentric, telomeric, and ribosomal DNA repeat sequences.<sup>83</sup> Functional mutations of this gene have been associated with sister chromatid clumps and defects, abnormal DNA methylation, and the maintenance of telomerase-independent telomeres, resulting in an alternative lengthening of telomeres (ALT). Decreased nuclear expression of *ATRX* and *ATRX* mutations are observed in grade 2 and 3 astrocytic tumors including *IDH* mutant gliomas (86%), *IDH*-mutant GBMs (85%), and pediatric high grade gliomas such as diffuse midline

**Table 4.** Summary of the mutation specific antibodies and their indication in glioma diagnosis<sup>12,36,48,81,84</sup>

Antibody	Positive loci	Mutated gene status	Positive result	Tumors
ATRX	Nucleus	Loss of function mutation	Negative	Diffuse and anaplastic astrocytoma
β-Catenin	Nucleus	Gain of function mutation	Focal positive	WNT type medulloblastoma, adamantinomatous craniopharyngioma
BRAF VE1 (BRAF V600E)	Cytoplasm	Gain of function mutation	Positive	Pleomorphic xanthoastrocytoma, ganglioglioma, pilocytic astrocytoma, epithelioid glioblastoma, papillary craniopharyngioma
BRG1	Nucleus	Gain of function mutation	Negative	Atypical teratoid rhabdoid tumor
CIC	Nucleus	Loss of function mutation	Negative	Oligodendroglioma (47%) <sup>15</sup>
c-MET	Membrane	Overexpression	Positive	Glioblastoma, anaplastic astrocytoma
EGFR	Membrane	Overexpression	Positive	Glioblastoma, anaplastic astrocytoma
EGFRvIII	Membrane	Overexpression	Positive	Glioblastoma, anaplastic astrocytoma
H3 K27M	Nucleus	Gain of function mutation	Positive	Diffuse midline glioma
FUBP1	Nucleus	Loss of function mutation	Negative	Oligodendroglioma (16%) <sup>15</sup>
IDH1 (H09)	Nucleus and cytoplasm	Gain of function mutation	Positive	Astrocytoma and oligodendroglioma
INI1	Nucleus	Loss of function mutation	Negative	Atypical teratoid rhabdoid tumor
P16	Nucleus and cytoplasm	Loss of function mutation	Negative	High grade glioma
P53	Nucleus	Overexpression	Positive	Astrocytic tumors
PDGFRA	Membrane	Overexpression	Positive	Glioblastoma, anaplastic astrocytoma
PTEN	Cytoplasm	Loss of function mutation	Negative	Glioblastoma
RELA (NFκB3)	Cytoplasm	Gain of function mutation	Positive	Cerebral ependymoma
STAT6	Nucleus	Gain of function mutation	Positive	Solitary fibrous tumor/hemangiopericytoma
MLH1	Nucleus	Loss of function mutation	Negative	Gliomas
MSH2	Nucleus	Loss of function mutation	Negative	Gliomas
PMS2	Nucleus	Loss of function mutation	Negative	Gliomas
Ki67	Nucleus	Overexpression	Positive	Brain tumors (for an ancillary test for tumor grading)
pHH3	Nucleus	Overexpression	Positive	Brain tumors (for counting mitoses)
GFAP	Nucleus	Expression	Positive	Astrocytic tumors
Olig2	Nucleus	Expression	Positive	Gliomas including astrocytomas and oligodendroglioma Neither neuronal nor ependymal tumors

gliomas (DMGs) (Figs. 1, 2).<sup>3</sup>

### Histone *H3F3A* (K27M, K36, and G34) mutations

Histone protein H3.1 and H3.2 are expressed only in the S phase and are integrated into the chromatin by chaperone CAF1 during DNA replication only, but H3.3 is expressed at all stages of the cell cycle and can be integrated into the chromatin independently of DNA replication. H3.3 is associated with active chromatin such as H3K4 trimethylation, which promotes open chromatin structure for the binding of co-factors and transcription factors to activate transcription.<sup>85</sup> Recurrent mutations in *H3F3A*, which encodes the replication-independent histone 3 variant H3.3, were first detected in the pediatric diffuse infiltrating pontine glioma (DIPG) by a comprehensive WES analysis. DIPG was later renamed as DMG and is considered a H3 K27M mutation in the new 2016 WHO classification (Fig. 2).<sup>7,23</sup> Eighty percent of pontine gliomas (DIPG) and 70% of other loci DMGs contain a mutation in one allele of the *H3F3A* gene, which is a mutation on the histone tail (K27M, K36, G34R/G34V), an important post-translational modification factor.<sup>25</sup>

The histone *H3F3A* mutant gliomas have a poor prognosis

regardless of histopathological grade. K27M-H3.3 mutations occur mainly in young patients (median age, 11 years) and G34R/V-H3.3 mutations occur in older children and young adults (median age, 20 years).<sup>22</sup> All of the cases with G34-H3.3 mutations are in pediatric GBMs (13/13), and especially associated with mutations in *ATRX* and *DAXX*.<sup>22</sup> Loss of *ATRX* is associated with the *ALT* phenotype; thus, *ALT* often coexists with mutations in *ATRX/H3F3A/TP53*.<sup>22</sup>

### Codeletion of chromosome 1p/19q and mutations in *CIC* and *FUBP1*

Simultaneous deletion of chromosome 1p/19q is a characteristic and early genetic event in oligodendroglial tumors, which is associated with better prognosis and is also a good indicator of the patient response to specific combination chemotherapy of procarbazine, lomustine, and vincristine (PCV). Recurrent mutations in *CIC* and *FUBP1* were found in 46%–53% and 15%–24% of oligodendroglomas, respectively, in addition to a codeletion in 1p/19q and mutations in *IDH1* or *IDH2* (Fig. 1).<sup>86-88</sup>

*FUBP1* is located in 1p31.1, and mutations in *FUBP1* are predicted to disrupt *FUBP1* protein function (Table 1). *FUBP1*

acts as an RNA binding protein and alterations of its normal function can lead to tumorigenesis, which has been suggested to act either as a protooncogene or a tumor suppressor gene depending on the tumor type.<sup>36,87</sup> Through the c-Myc pathway, *FUBP1* promotes cell migration and protects cells from apoptosis.

The *CIC* gene on chromosome 19q13.2 represses genes induced by RTK pathway activation.<sup>89</sup> Although the function of

human *CIC* protein is not known, a recent study in cultured cells demonstrated that *CIC* acts together with *IDH1* to regulate citrate levels in the cytoplasm. *CIC* mutations promote the accumulation of 2HG and reduce clonogenicity in the setting of *IDH1* mutations. Loss of 19q in oligodendroglial tumors unmasks mutations in the *CIC* gene.<sup>87,90</sup> In this regard, the co-existence of *IDH1* with *CIC* or *FUBP1* mutations may partially

**Table 5.** The current sequencing conditions commonly used for brain tumor diagnosis<sup>35,45,59,68,84,91,92</sup>

Gene	Full name	Gene location	Forward primer	Reverse primer	Product (bp)	Indication	Function
<i>IDH1</i>	Isocitrate dehydrogenase 1	2q33.3	5'-M13-GTA AAA CGA CGG CCA GTC GGT CTT CAG AGA AGC CA-3'	5'-GCG GAT AAC AAT TTC ACA CAG GGC AAA ATC ACA TTA TTG C-3'	180–190	Astrocytic and ODG	Affect citrate metabolism, leading to 2-HG metabolite
<i>IDH2</i>	Isocitrate dehydrogenase 2	15q26.1	5'-GCT GCA GTG GGA CCA CTA TT-3'	5'-TGT GGC GTT GTA CTG CAG AG-3'	295–305	Astrocytic and ODG	Same with <i>IDH1</i>
<i>H3F3A</i> (K27M/K36/G34)	H3 Histone family 3A	1q42.12	5'-CTG GTA AAG CAC CCA GGA AGC-3'	5'-CAT GGA TAG CAC ACA GGT TGG T-3'	330–340	DMG	Chromatin structure, gene transcription
<i>BRAF</i> V600E	V-RAF murine sarcoma viral oncogene homolog B1	7q34	1st: 5'-GCT TGC TCT GAT AGG AAA ATG AG-3' 2nd: 5'-TCA TAA TGC TTG CTC TGA TAG GA-3'	1st: 5'-GTA ACT CAG CAG CAT CTC AGG-3' 2nd: 5'-GGC CAA AAA TTT AAT CAG TGG A-3'	245–255 230–235	Gliomas including PXA, GG, PA, epithelioid GBM	MAPK signaling
<i>CTNNB1</i>	Catenin, beta-1	3p22.1	5'-GAT TTG ATG GAG TTG GAC ATG G-3'	5'-TGT TCT TGA GTG AAG GAC TGA G-3'	230–235	Medulloblastoma, WNT subtype, craniopharyngioma, adamantinomatous type	The transmission of the 'contact inhibition' signal
<i>TERTp</i>	Telomerase reverse transcriptase	5p15.33	5'-M13-GGC CGA TTC GAC CTC TCT-3' (M13: TGT AAA ACG ACG GCC AGT)	5'-AGC ACC TCG CGG TAG TGG-3' (M13: GCG GAT AAC AAT TTC ACA CA)	300–310	Gliomas, especially ODG and GBM	Telomerase maintenance
<i>MGMT</i>	O6 methylguanine-DNA methyltransferase	10q26.3	5'-TTT CGA CGT TCG TAG GTT TTC GC-3'	5'-GCA CTC TTC CGA AAA CGA AAC G-3'	80–90	Gliomas	Promoter methylation

ODG, oligodendroglioma; 2-HG, 2-hydroxyglutamate; DMG, diffuse midline glioma; PXA, pleomorphic xanthoastrocytoma; GG, ganglioglioma; PA, pilocytic astrocytoma; GBM, glioblastoma; MAPK, mitogen-activated protein kinase.

**Table 6.** Probes and reading criteria of FISH studies used for brain tumor diagnosis

	Target probe	Control	Cut off	Indication	Biomarker
1p deletion	Chr1p36	1q25	1p < 0.8 and Deleted nuclei > 50%	ODG	Diagnostic, prognostic, and predictive
19q deletion	Chr19q13	19p13	19q < 0.8 and Deleted nuclei > 50%	ODG, HGG	Diagnostic, prognostic, and predictive
<i>BRAF</i> gain	Chr7q34	CEP7	Gold signal > 3	PA	Diagnostic, prognostic, and predictive
<i>CDKN2A</i> (9p21.3) homozygous/hemizygous deletion	Chr9p21.3	CEP9	HoD ≥ 10% HeD ≥ 50%	HGG	Diagnostic and prognostic
<i>EGFR</i> amplification	Chr711.2	CEP7	Ratio ≥ 2.0	HGG	Diagnostic and prognostic
<i>PTEN</i> HoD /HeD	Chr10q23.31	CEP10	HoD ≥ 10% HeD ≥ 50%	HGG	Diagnostic and prognostic
<i>RELA-C11orf95</i> fusion	Chr11q13.1	CEP11	Break apart	S-ependymoma	Diagnostic and prognostic
<i>C19MC</i> amplification	Chr19	CEP19	Ratio ≥ 2.0	ODG, GBM	Diagnostic and prognostic
<i>SMARCB1</i>	Chr22q11.23	CEP22	NF2 < 0.8	AT/RT	Diagnostic and prognostic

FISH, fluorescent *in situ* hybridization; Chr, chromosome; ODG, oligodendroglioma; HGG, high grade glioma; PA, pilocytic astrocytoma; HoD, homozygous deletion; HeD, hemizygous deletion; S-ependymoma, supratentorial ependymoma; GBM, glioblastoma; AT/RT, atypical teratoid/rhabdoid tumor.

explain the slower tumor growth and longer survival of oligodendrogliomas with mutations in *IDH1* and a codeletion of 1p/19q when compared to other diffuse gliomas.<sup>90</sup> The overall survival rate of patients with oligodendrogliomas with *CIC* mutations was lower than that of patients without *CIC* mutations, and the *FUBP1* mutation was significantly associated with unfavorable progression-free survival.<sup>15</sup> However, oligodendrogliomas cannot be diagnosed with *CIC* or *FUBP1* mutations only.<sup>15</sup>

**EGFR amplification and EGFRvIII truncation mutations**

*EGFR*, also called *Erb1* or *HER1*, is located on chromosome 7q12 and acts as an *ErbB* family receptor tyrosine kinase. *EGFR* amplification is closely related to *EGFR* overexpression. *EGFRvIII*-positive tumor cells exhibit particularly high levels of *mTOR* signal and exhibit the most proliferative and aggressive phenotype similar to GBMs (Fig. 1). The *EGFRvIII* mutation is a 801 bp frame deletion from exons 2 to 7 of the *EGFR* gene, which is associated with *EGFR* amplification and the response to antibody therapy as well as poor prognosis.<sup>93</sup> In addition to *EGFR* amplification, *EGFRvIII*-positive GBM cell lines are sensitively suppressed by first-generation *EGFR* inhibitors (erlotinib, gefitinib, vandetanib) and second-generation *EGFR* inhibitors (NT113) as compared to GBM cell lines in which *PTEN*

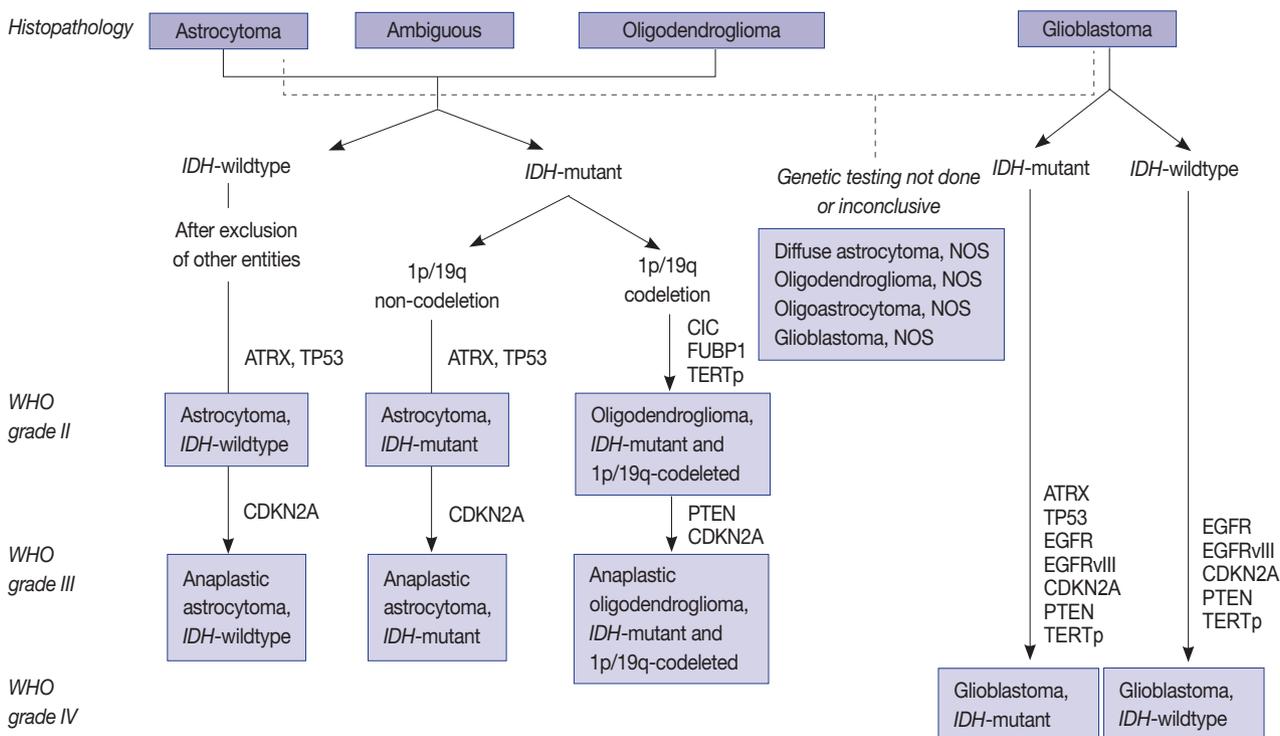
tumor suppressor genes are lost *in vitro*.<sup>94</sup>

*EGFR* immunohistochemistry is usually uniform in tumors expressing high levels of the protein, however, *EGFRvIII* staining is heterogeneous and usually present in only a subset of tumor cells. Therefore, representative sampling of tumor tissue is important to avoid false-negative results.<sup>56,57</sup>

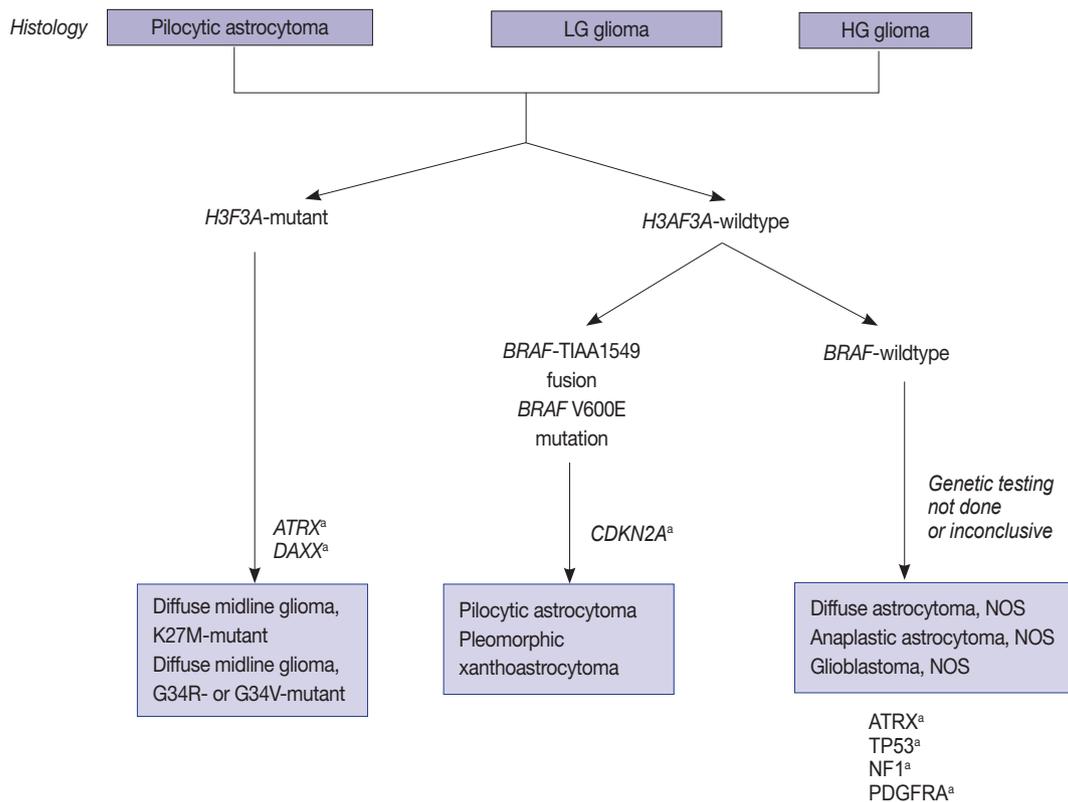
Unlike *EGFR* point mutations, *EGFR* fusions with *SEPT14*, *PSPH*, or *SEC61G* has also been shown to play a role in GBMs, offering a unique opportunity to investigate fused oncogene dependencies.<sup>94</sup>

**Deletions in CDKN2A/2B**

*CDKN2A/2B* is a tumor suppressor gene located on chromosome 9p21.3, because it encodes a cyclin-dependent kinase inhibitor (p16) and is a cell cycle regulator of the *Rb* pathway. In 60% of GBMs and 11% of low-grade gliomas including oligodendroglioma, pleomorphic xanthoastrocytoma, and pilocytic astrocytoma, the loss or inactivation of p16 protein as a result of a homozygous deletion or promoter methylation of *CDKN2A/2B* is observed (Fig. 1).<sup>55</sup> It is also one of the more commonly altered genes in PXA and high grade gliomas in both adults and children (Fig. 2).<sup>55,95</sup> After adjusting for the *IDH* mutational status, sex, and age, *CDKN2A* deletions were strongly associated with poorer overall survival in astrocytomas but not in oligodendrogliomas.<sup>18</sup>



**Fig. 1.** Schematic view of the classification of adult diffuse glioma according to the status of key genes.



**Fig. 2.** Schematic view of the classification of pediatric diffuse gliomas according to the status of key genes. LG, low grade; HG, high grade; NOS, not otherwise specified. <sup>a</sup>Characteristic but not required for diagnosis.

### **BRAF mutations and BRAF-KIAA1549 fusions**

*BRAF* V600E mutations and fusions of *BRAF* with *KIAA1549* or *FAM131B* are characteristics of pilocytic astrocytomas. In 2008, a tandem duplication was confirmed in 7q34, and a new fusion gene was found to be generated by a fusion between the *KIAA1549* gene and *BRAF* genes, which was previously uncharacterized in pilocytic astrocytomas.<sup>58</sup> Fusions between *KIAA1549* exon 15 and *BRAF* exon 9 and those between *FAM131B* exon 2 and *BRAF* exon 9 are derived from a 2.0 Mb tandem duplication in chromosome band 7q34, which can be detected by real-time polymerase chain reaction (PCR) or fluorescent *in situ* hybridization (FISH) (Fig. 2).<sup>62</sup>

The *BRAF* V600E mutation was found in two-thirds of all pleomorphic xanthoastrocytomas, one-fourth of gangliogliomas, and one-seventh of pilocytic astrocytomas.<sup>35</sup>

In children, *CDKN2A* deletions and *BRAF* mutations are early events in low-grade gliomas (pediatric low-grade glioma [PLGG]) undergoing malignant transformation (Fig. 2).<sup>96</sup> The *BRAF* V600E mutant PLGG has longer transformation latency periods than the *BRAF* wild type PLGG (median latency period, 6.65 years vs 1.59 years, respectively).<sup>96</sup> As a result, all of the

patients with secondary high-grade glioma (sHGGs) containing mutant *BRAF* were diagnosed at age 9 or older.<sup>96</sup> The sHGG in children showed recurrent changes of *BRAF* V600E mutations and *CDKN2A* deletions in 39% and 57%, respectively.

### **TERTp mutations**

Human telomerase is a ribonucleoprotein that regulates the length of telomeric DNA at the ends of chromosomes; therefore, it plays an important role in cellular immortalization and oncogenesis.<sup>13</sup> Mutations in *TERTp* are molecular hallmarks of glioma, occurring in more than 80%–96% of IDH-wildtype GBMs and oligodendrogliomas, but less frequently present in grade II and III astrocytomas (38.5%) (Table 1, Fig. 1).<sup>97</sup> Chan *et al.* (2015)<sup>98</sup> found that mutations in *TERTp* are present in 41 of 142 (28.9%) grade II gliomas and 20 of 72 (27.8%) grade III gliomas. The mutations were always found in two hotspots (chr5: 1 295 228 C > T and 1 295 250 C > T), resulting in a somatic gain-of-function mutation and an enhancement of *TERTp* activity.<sup>99</sup> A relative telomere length is strongly correlated with *TERTp* mutations. These two hot spot mutations are mutually exclusive in gliomas and *TP53* mutations, but coincident with

a 1p/19q codeletion.<sup>100</sup>

The prognostic effect of the *TERT* mutation is controversial. Some studies have suggested that *TERT* mutations in low-grade gliomas are associated with shorter progression-free survival.<sup>101</sup> Other researchers have found that *TERT* mutations are a predictor of a poorer response to temozolomide.<sup>102</sup> *TERT* mutations are associated with good outcome in gliomas with mutant *IDH*, but it is also related to poor outcome in gliomas with wild type *IDH*.<sup>97</sup> In gliomas with wildtype *IDH* or GBMs with hypomethylated *MGMT*, *TERT* mutations were found to predict poor prognosis.<sup>103</sup> Therefore, when using the *TERT* mutational status as a prognostic factor, other factors such as mutations in *IDH* and tumor grade should be considered.<sup>101</sup> Primer sequences for *TERT* mutation is summarized in Table 5.

### *C11orf95-RELA* fusion

More than two-thirds of supratentorial ependymomas contain oncogenic fusions between *RELA* (the principle effector of canonical nuclear factor  $\kappa$ B [NF- $\kappa$ B] signaling) and *C11orf95* (an uncharacterized gene) (Table 2).<sup>32,34</sup> Ependymomas carrying the *C11orf95-RELA* fusion are characterized by a nuclear accumulation of p65RelA (NF $\kappa$ B) indicating a pathological activation of the NF $\kappa$ B signaling pathway.<sup>34</sup> *C11orf95-RELA* fusions result from chromothripsis involving chromosome 11q13.1. Furthermore, the *C11orf95-RELA* fusion protein was found to spontaneously translocate to the nucleus to activate NF- $\kappa$ B target genes, and to rapidly transform neural stem cells, the cell of origin for ependymomas, to form these tumors in mice, which is a poor prognostic marker (Table 2).<sup>34</sup>

### *CTNNB1* mutations

Mutations in *CTNNB1* on chromosome 3p21, which encodes  $\beta$ -catenin, promote the stabilization and nuclear translocation of itself, therefore activating the WNT signaling pathway (Table 2). The nuclear translocation of  $\beta$ -catenin is present in medulloblastomas, solitary fibrous tumors/hemangiopericytomas, and adamantinomatous-type craniopharyngiomas. Nuclear expression of  $\beta$ -catenin is always focal. Overall, these mutations can act as future therapeutic targets in nuclear  $\beta$ -catenin-positive tumors.

### *MYC* and *MYCN* amplification

*MYC* is a regulator gene that encodes for transcription factors for c-myc, Mycn, and Mycl.<sup>77</sup> Nuclear myc protein has multiple functions such as cell cycle progression, apoptosis, and cellular transformation. *MYC/MYCN* amplification or overexpression is found not only in type-3 medulloblastomas, but also in other

aggressive subtypes of medulloblastomas, which account for approximately 10% of medulloblastomas.<sup>78,104</sup>

### *SMARCB1* and *SMARCA4* mutations

AT/RT are highly malignant CNS embryonal tumors with rhabdoid morphology, where biallelic inactivation of *SMARCB1* results in a loss of INI1 (BAF47) nuclear expression (Tables 2–4). The loss of BRG1 nuclear expression in AT/RT with mutations in *SMARCA4* is rarely reported.<sup>105</sup> The tumor suppressors *SMARCB1* and *SMARCA4* are located on 22q11.23 and 19p13.2, respectively.

The biallelic loss of *SMARCB1* and *SMARCA4* can be recognized by nuclear negativity of the INI1 and BRG1 immunostaining. However, FISH and DNA sequencing can also be used to detect these genetic alterations.

WES and RNA sequencing of AT/RT revealed few somatic mutations and several deregulated signaling pathways related to the *SMARCB1* deficiency.<sup>106</sup>

### *C19MC* amplification

Chromosomal 19 micro-ribosomal clusters (*C19MC*), localized at 19q13.42, are produced by chromothripsis, a phenomenon of chromosomal breakage and inaccurate recombination, resulting in the rearrangement of thousands of clustered chromosomes in a single event to a localized and restricted genomic region on one or more chromosomes.<sup>107</sup> *C19MC* amplification is a genetic feature of embryonal tumors with multilayer rosettes (ETMR), supratentorial ependymomas, and medulloepitheliomas (Table 2).<sup>108</sup> This abnormality can be detected by FISH.

### *MGMT* promoter hypermethylation

One of the most clinically important DNA methylation markers in GBMs is the promoter of *MGMT*, a DNA repair enzyme that can abrogate the effects of alkylating chemotherapy such as temozolomide. More than 50% of oligodendrogliomas and 30%–40% of adult high-grade gliomas (approximately 40% of IDH-wildtype GBMs) reveal *MGMT* methylation. *MGMT* promoter hypermethylation induces gene silencing and susceptibility to combined temozolomide and radiation therapy. In cases of oligodendroglioma with promoter methylation of the *MGMT* gene, whether or not PCV chemotherapy is advantageous remains controversial.<sup>59</sup> However, it is still considered to be the most accurate predictive factor for survival during PCV chemotherapy.<sup>60</sup>

## METHODS OF MOLECULAR TESTING

The molecular testing methods commonly used in pathology laboratories include immunohistochemical staining, direct sequencing, FISH, chromosomal genomic hybridization, and NGS.

### Immunohistochemical studies

Some of the genetic changes can be detected by immunohistochemical studies. *IDH* R132H, H3 K27M, *BRAF* V600E, and *EGFRvIII* can be diagnosed using mutation-specific monoclonal antibodies (mAbs). IDH1 (H09), K27M, and BRAF VE1 antibodies have 100% sensitivity and specificity to detect gene mutations.<sup>36</sup> The INI-1 (*SMARCB1*), BRG1 (*SMARCA4*), p16 (*CDKN2A*), *CIC*, and *FUBP1* mutations and *MGMT* methylation can be detected by the loss of expression. *TP53* mutations are detected by the complete loss of expression or overexpression via the stabilization of the mutant protein. In addition, *STAT6* (*NAB2-STAT6* fusion), *LICAM* (protein marker for *RELA-c9ORF95* fusion), and the amplifications of *EGFR*, *MET*, and *PDGFR* can be analyzed by overexpression assays (Table 4).<sup>21,24,36,55</sup> Mutations in several genes give rise to the overexpression or loss of proteins, depending on whether the mutation is a gain of function or a loss of function mutation, respectively. For example, p53 nuclear overexpression reflects a *TP53* mutation, and p16 nuclear and cytoplasmic losses are associated with a *CDKN2A* homozygous deletion. Furthermore, the nuclear loss of *ATRX* is associated with an *ATRX* mutation, and *MLH1*, *MSH2*, and *PMS* nuclear losses are associated with methylation of these genes. Among these, all but *TP53* have high correlations between immunohistochemical results and molecular genetic studies. The correlation rate of p53 between immunohistochemistry and mutation studies is low. This is because p53 immunoreactivity may have resulted from the prolongation of half-life either due to p53 mutations or the accumulation of wild type protein brought about by mechanisms other than those caused by mutations, such as a complex formation with *MDM2* overexpression products,<sup>109</sup> whereas p53 negativity can be observed by the methylation of the *TP53* promoter. Therefore, overexpression of p53 protein is not always associated with mutations in conserved exons of the p53 gene.

### Direct sequencing

The rapidly growing number of prognostic and predictive genetic markers in neuro-oncology has led to an increasing need for a more thorough molecular analysis of brain tumor specimens in a modern pathology setting. Although several diagnos-

tically important mutations can be detected by immunohistochemistry, this is not the case for the full spectrum of alterations now known to be of relevance. Therefore, the mutations of *IDH* 1/2, *BRAF*, *CTNNB1*, *H3F3A* (K27M, G34), *TP53*, *ATRX*, and *TERTp* are usually detected by direct sequencing. The sequencing conditions, primer sequences, and PCR product sizes that are currently being used for brain tumor diagnosis are summarized in Table 5.

In order to detect mutations in *CIC* (missense mutations or small in-frame deletions) and *FUBP1* (indel, splicing alteration, and nonsense mutations) in the functional regions such as the HMG box and CI motif, NGS is required because the mutation sites of these two genes are widely distributed along the coding regions.<sup>88</sup>

Sanger sequencing is a method of DNA sequencing based on the selective incorporation of chain-terminating dideoxynucleotides by DNA polymerase during DNA replication *in vitro*, which was developed by Frederick Sanger and colleagues in 1977.<sup>91</sup>

Recently, one-step Sanger sequencing (combining the amplification and sequencing steps) methods such as Ampliseq and SeqSharp have been developed that allows for rapid sequencing of target genes without cloning or prior amplification.

However, covering all potentially clinically relevant genes in a routine diagnostic setting by these methods has become virtually impossible. Reliable high-throughput methods allowing for parallel analysis of multiple targets emerged as an attractive alternative for comprehensive diagnostic neuropathology. NGS provides opportunities to evaluate many genomic targets with high accuracy and sensitivity due to high sequencing coverage. However, the Sanger method is widely used for small-scale projects, and validation of NGS results, especially long serial DNA sequence reads that are greater than 500 nucleotides.

### Pyrosequencing

Pyrosequencing is a method of DNA sequencing based on the “sequencing by synthesis” principle.<sup>110</sup> It differs from Sanger sequencing in that it relies on the detection of pyrophosphates that are released upon nucleotide incorporation rather than chain termination with dideoxynucleotides. Various gene alterations can be detected by pyrosequencing, including *IDH1/IDH2* mutations, *TERTp* mutation and *MGMT* methylation.<sup>92,111-113</sup>

### NGS

More recently, high volume Sanger sequencing has been sup-

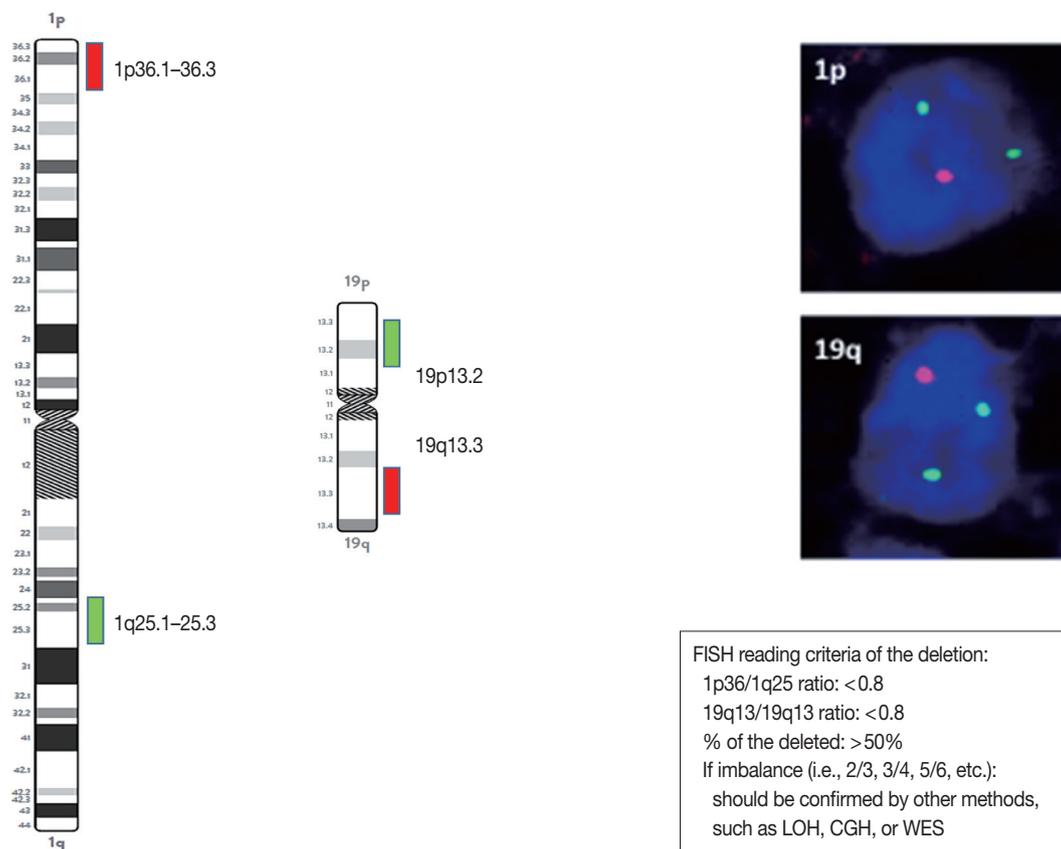
planted by NGS methods, especially for large-scale, automated genome analyses. NGS is a high-capacity parallel sequencing process that handles hundreds to millions of DNA fragments simultaneously.<sup>114,115</sup> Currently, there are second- and third-generation sequencing technologies that continue to reduce the cost of DNA sequencing and improve accuracy. The ability to multiplex several samples also leads to increased throughput and reduced cost. Soon, NGS will enter clinical practices but it is still uncertain whether it can replace the established techniques. Depending on the type of NGS panel, it can only detect single nucleotide variations and indels or embrace CNV and translocations. The performance of the NGS panel requires rigorous validations and strict quality control for its sensitivity, specificity, and accuracy. There are several NGS panels for brain tumor companion diagnosis.<sup>78,79,95,116</sup> Current practice and guidelines for the clinical use of a NGS-based oncology test are described in Strom's 2016 paper.<sup>117</sup> A comparison of different NGS-based diagnostic tools is summarized in Lapin *et al.* paper (2016).<sup>118</sup>

## FISH

FISH studies can be easily used to identify chromosomal aberrations, such as a 1p/19q codeletion, *EGFR* amplification or high polysomy, *PTEN* and *CDKN2A* homozygous or hemizygous deletions, *C-MYC* and *N-MYC* amplifications, *BRAF* fusions or copy gains, *C11orf95-RELA* fusions, *C11orf95-YAP1* fusions, *YAP1-MAMLD1* fusions, and *MYB-QKI* fusions. The probes and reading criteria of currently used FISH for the diagnosis and prognosis of brain tumors are summarized in Table 6.

The presence or absence of the 1p/19q codeletion can be determined by FISH, comparative genomic hybridization (CGH), or microsatellite analyses for a loss of heterozygosity (LOH) on chromosomes 1p and 19q.<sup>119,120</sup>

When the amount of DNA is insufficient to carry out the single nucleotide polymorphism or CGH array, microsatellite analysis is performed to evaluate via PCR to determine whether there is a LOH of chromosome 1p and 19q.<sup>121</sup> In cases of 1p/19q codeletion, the evaluation and interpretation of FISH results are based on International Society of Pediatric Oncology



**Fig. 3.** Schematic view, photographs, and reading criteria of the FISH probes for detecting 1p and 19q deletion. The FISH probes are long enough to find out the whole arm deletion. 1p/19q deleted cases show one orange signal and two green signals. The diagnostic cut-off of 1p and 19q deletion is written in the box. FISH, fluorescent *in situ* hybridization; LOH, loss of heterozygosity; CGH, comparative genomic hybridization; WES, whole exome sequencing.

(SIOP) guidelines for neuroblastoma study.<sup>80</sup> For each hybridization, the number of green and orange signals is assessed for a minimum of 100 nonoverlapping nuclei. An interpretation of a deletion was made when > 50% of nuclei harbored less than a single orange signal (0/2, 1/2, 0/3, 1/3, etc). Such deletions most likely correspond to a LOH (Fig. 3). However, with increasing grades of malignancy, genomic polyploidies may be encountered. These chromosomal polysomies may be balanced (e.g., 3/3, 4/4, 5/5, etc.) or imbalanced (e.g., 3/2, 4/2, 3/5, 4/5, etc.), indicating relative gains or losses. Whether an imbalance situation with relative loss of the target 1p or 19q corresponds to a hemizygous deletion in the presence of reduplication cannot be solved by FISH. In such cases, secondary assays such as PCR-based LOH or CGH studies should be used for better specificity.<sup>80</sup>

#### Methylation specific PCR (O6 methyl guanine methyl transferase)

Methylation can be detected by methylation specific PCR (MSP), pyrosequencing, multiplex ligation-dependent probe assay, or BeadChip microarray.<sup>57,113,122</sup> In the Seoul National University Hospital, MSP is conducted using an EZ DNA methylation Kit (catalog No. D5005, Zymo Research, Irvine, CA, USA) to determine the methylation status of the *MGMT* promoter. The primer set is described in Table 5. The primer sequences for *MGMT* were as follows: methylated forward, 5'-TTT CGA CGT TCG TAG GTT TTC GC-3'; methylated reverse, 5'-GCA CTC TTC CGA AAA CGA AACG 3'; unmethylated forward, 5'-TTT GTG TTT TGA TGT TTG TAG GTT TTT GT-3'; and unmethylated reverse, 5'-AAC TCC ACA CTC TTC CAA AAA CAA AAC A-3'.

## DISCUSSION

Among primary adult and pediatric CNS tumors, diffuse gliomas are the largest and most diverse group. They usually arise in the cerebral hemispheres and are defined by their widely infiltrative properties and tendency for biological progression. According to the 2016 WHO criteria, gliomas can be classified using combined histological and molecular markers as diffuse astrocytic and oligodendroglial tumors, other astrocytic tumors, ependymal tumors, or other gliomas. The most aggressive astrocytic tumors with a dismal prognosis are the GBMs with wild type IDH and DMG, histone-mutant (H3 K27, G34, and K36). Less infiltrative gliomas in children and young adults include WHO grade I pilocytic astrocytomas, WHO grade II/III

pleomorphic xanthoastrocytomas, and WHO grade I subependymal giant cell astrocytomas. Other major classes of CNS tumors include neuronal and mixed glioneuronal tumors as well as embryonal tumors such as cerebellar medulloblastomas, and AT/RT, and extra-axial meningiomas.

The diagnosis of CNS tumors is historically primarily based on histopathological features. However, studies have shown that patients with morphologically identical tumors have different clinical outcomes and treatment responses due to the different molecular genetic characteristics of the tumor. Therefore, many molecular markers became deeply integrated into the diagnosis of CNS tumors and are now used to guide prognosis and treatment of patients. If molecular genetic testing is not possible, a diagnosis is made with the nonspecific term “glioma, not otherwise specified (NOS).” In this case, because the grade and the exact diagnosis of the tumor as well as the biological markers are covered, the precision diagnosis and the appropriate treatments are no longer possible. These “NOS diagnostics” must be reclassified using molecular genetic diagnostic tools.

#### Diffuse astrocytic and oligodendroglial tumors

Molecular genetic testing can classify diffuse astrocytic and oligodendroglial tumors (WHO grades II–III) and differentiate IDH-mutant GBMs from IDH-wildtype GBMs based on molecular signature of astrocytic and oligodendroglial tumors. Diffuse astrocytic tumors and IDH-mutant GBMs are characterized by mutations in *IDH1/2*, *TP53*, and *ATRX*, whereas oligodendrogliomas are characterized by mutations in *IDH1/2*, *CIC*, *FUBP1*, and *TERTp*, and a codeletion in 1p/19q (whole chromosomal arm deletion) (Fig. 1). IDH-wildtype GBMs can be identified by demonstrating a dysregulation of several critical signaling pathways and a lack of the above-mentioned genetic alterations except *TERTp* mutations, which are common in IDH-wildtype GBM. The main signaling pathways involved in IDH-wildtype GBMs are RTK/RAS/PI3K pathways (via amplification and mutations in *EGFR* [*EGFRvIII*], *PIK3CA*, *RAS*, *NF1*, and *MET*) and *TP53* and *RB1* suppressor pathways (via mutations in or loss of *TP53*, *CDKN2A*, and *RB1* genes) (Fig. 1).<sup>94</sup>

Mutations in *IDH1/2* should be studied in diffuse gliomas for the WHO classification of CNS tumors. They are favorable prognostic markers and are genomic abnormalities initially present in both astrocytic and oligodendroglial tumors. Therefore, from the revised version of 2016 fourth edition of the CNS Tumor Classification, these two tumor categories have been combined as diffuse astrocytic and oligodendroglial tumors.<sup>7</sup> *P53* and *ATRX* mutations are now the hallmark of WHO

grade II and grade III astrocytic tumors. *TERT* mutations, such as C228T and C250T, are found in more than 90% of oligodendroglial tumors and in more than 80% of GBMs as well as about 30%–40% of lower grade gliomas. Mutations in *TERT* were associated with poor survival and resistance to radiotherapy in GBMs and in lower grade gliomas (grade II and III) without *IDH* mutation; however, *TERT* mutations were associated with a favorable prognosis in lower grade gliomas with *IDH* mutations.<sup>63,123</sup>

### High- and low-grade pediatric gliomas

Pediatric high-grade gliomas are unique, featuring mutations in *H3F3A*, *ATRX*, and *DAXX*, whereas PLGGs contain mutations or rearrangements in *BRAF*, *FGFR1*, or *MYB*. Furthermore, mutations in *IDH* are rare unless the patient is an adolescent (Fig. 2).<sup>22,64,65</sup> The circumscribed low-grade gliomas such as pilocytic astrocytoma, pilomyxoid astrocytoma, ganglioglioma, and PXA often harbor the *BRAF* V600E or *FGFR1* mutation, or gene fusions involving *BRAF* with *KIAA1549* or *FAM131B*.<sup>35,62,65</sup> Histone H3 K27M, K36, and G34 mutations should be studied in pediatric glioma arising in the midline of CNS, i.e., the thalamus, pons, and spinal cord.<sup>124</sup> However, the histone G34 mutation can be present in cerebral high-grade diffuse gliomas of the adult (about 5%) and the histone K36 mutation is rarely found in pediatric midline gliomas.<sup>84</sup>

Finally, the *BRAF* V600E mutation can be studied in low-grade glioneuronal tumors, such as PXA (about 60%), ganglioglioma (about 25%), pilocytic astrocytoma (about 15%), epithelioid GBM (about 50%), and papillary type of craniopharyngioma (about 100%).<sup>35,66</sup>

### Medulloblastomas and other embryonic tumors

Medulloblastomas have been recently divided into several subtypes based on specific driver mutations including *WNT*, *SHH*, group 3, and group 4.<sup>125–127</sup> Mutations in *CTNNB1*, *DDX3X* or monosomy 6 in the Wnt pathway of medulloblastomas tend to lead to a much better prognosis. In contrast, *MYC* or *MYCN/CDK6* amplification is characteristic of group 3 and 4 medulloblastomas, which are far more likely to metastasize and have a poor prognosis even with intensive therapy. Tumors with *PTCH* and *SMO* belong to the *SHH* class, and have a relatively intermediate prognosis between *Wnt* and group 3/4 tumors. *CTNNB1* mutations can be present in classical-type medulloblastomas and adamantinomatous-type craniopharyngiomas.<sup>61,125,128</sup>

*SMARCB1 (INI1)* or *SMARCA4 (BRG1)* gene mutations or deletions are essential for the diagnosis of AT/RT, which show

dismal prognosis.<sup>44–46</sup> The molecular genetic hallmark of ETMR is chromosome 19 miRNA cluster (*C19MC*) amplification.<sup>129</sup> If certain tumors are morphologically similar to these tumors, i.e., rhabdoid feature, but molecular studies do not reveal these genetic abnormalities or molecular studies cannot be done, CNS embryonal tumor with rhabdoid feature or ETMR, NOS can be made as a pathological diagnosis.<sup>7,129</sup> Therefore, the molecular characterization of medulloblastomas and embryonal tumors have lasting clinical value.

### Meningioma and meningeal solitary fibrous tumor/hemangiopericytoma

Meningiomas often have mutations in the *NF2*, *AKT1*, *SMO*, and *KLF4* genes. Furthermore, meningiomas with mutant *NF2* are far more likely to exhibit atypical grade II features than the other subtypes.<sup>50–52</sup> Recurrent mutations in *KLF4*, *AKT1*, and *SMO* genes are often present in *NF2*-negative sporadic meningiomas.<sup>50</sup> Clinical trials involving *SMO/AKT/NF2* inhibitors are open for patients with progressive meningiomas with *SMO/AKT/NF2* mutations.<sup>50,81</sup>

Meningeal hemangiopericytomas and solitary fibrous tumors have the same genetic modification as *NAB2-STAT6* gene fusion and are considered as the same tumor.<sup>48</sup> This gene fusion study is essential for the differential diagnosis of histologically similar tumors.

### Genetic alterations as prognostic and predictive markers and therapeutic targets

Overall, the analysis of multiple molecular markers not only aids in establishing a correct morphological diagnosis, but also highlights the biological differences between morphologically similar tumors. It can also help with the clinical management of patients. For example, patients with *IDH1/TERTp/CIC/FUBP1* positive lower grade gliomas (WHO grades II–III) have a significantly longer median overall survival than those with *IDH1/TP53/ATRX* mutations.<sup>86,130</sup>

In addition, several molecular biomarkers showed promise in predicting responses to targeted therapies.<sup>94,131</sup> For example, clinical trials are now open for GBMs with *EGFRvIII* mutations, vemurafenib is being evaluated in *BRAF* V600E mutant gliomas, and clinical response has already been observed in *FGFR3-TACC3*-positive patients treated with an *FGFR* inhibitor.<sup>78</sup> NGS-based high-throughput molecular testing may provide potential new targets for precision medicine.

## CONCLUSION

We are now in the era of precision medicine, and have slowly caught up with the rapid development of precision medicine. High-throughput assays that can test multiple genes at once are essential for pathological diagnosis in daily practice, and NGS technology has made this possible. Current practices and guidelines for the clinical use of NGS-based oncology tests are well documented in Strom's paper.<sup>117</sup> However, the medical insurance system has not kept up with the speed of technical development, the advances in diagnostic modalities, and newly developed treatment options. These are obstacles that prevents the doctors from implementing the precision medicine. In order to introduce the advanced knowledge and technologies into the daily practice of pathological diagnosis and clinical fields and reduce the costs, the medical insurance system should be formulated accordingly.

### Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

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