Review: Diagnostic, prognostic and predictive relevance of molecular markers in gliomas

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Abstract:

The advances of genome-wide "discovery platforms" and the increasing affordability of the analysis of significant sample sizes have led to the identification of novel mutations in brain tumours that became diagnostically and prognostically relevant. The development of mutation-specific antibodies has facilitated the introduction of these convenient biomarkers into most neuropathology laboratories and has changed our approach to brain tumour diagnostics. However, tissue diagnosis will remain an essential first step for the correct stratification for subsequent molecular tests and the combined interpretation of the molecular and tissue diagnosis ideally remains with the neuropathologist. This overview will help understanding the pathobiology of common intrinsic brain tumours in adults and help guiding which molecular tests can supplement and refine the tissue diagnosis of the most common adult intrinsic brain tumours. This article will discuss the relevance of 1p/19q codeletions, *IDH1/2* mutations, BRAF V600E and *BRAF* fusion mutations, more recently discovered mutations in *ATRX, H3F3A, TERT, CIC* and *FUBP1*, for diagnosis, prognostication and predictive testing. In a tumour-specific topic, the role of MAPK pathway mutations in the pathogenesis of pilocytic astrocytomas will be covered.

Key words

Astrocytoma, pilocytic astrocytoma, oligodendroglioma, oligoastrocytoma, glioblastoma, BRAF V600E mutation, *BRAF* fusion mutation, 1p/19q codeletion, *IDH1* mutation, *IDH2* mutation, *ATRX, CIC, H3F3A, TERT, FUBP1* mutations, MGMT promoter methylation, biomarker**.**

Introduction

The central purpose of tissue-based diagnosis is to advise clinicians and patients about the most adequate treatment, by informing them about the nature of the lesion and the prognosis of the disease. The fundament of the WHO classification of brain tumours is the distinction of grades, which since its introduction in 1979 has used a scheme representing a

"malignancy scale" ranging across a wide range of CNS neoplasms [1]. As a rule of thumb, patients with grade II tumours survive more than 5 years, whilst patients with grade III tumours survive 2-3 years and the survival of patients with WHO grade IV tumours depends on the availability of effective treatment. Untreated grade IV tumours are rapidly fatal [1]. In gliomas, the grade is largely based on the presence of certain morphological features including cellular and nuclear atypia, mitotic activity, microvascular proliferation and necrosis [2]. The nomenclature ("typing") of brain tumours according to the WHO classification is still largely based on the resemblance of tumour cell shape, morphology and localization to those of differentiated cells of the brain or expression of glial or neuronal proteins. This has continuously been supplemented by biomarkers that more reliably determine proliferation and more precisely define the differentiation of tumour cells. A significant advance in molecular glioma diagnostics was the discovery of the relatively consistent deletion of chromosomal arms 1p and 19q in oligodendroglial tumours [3-5] (Fig. 1) but it took more than a decade until the predictive and prognostic value was corroborated [6] and verified in randomised clinical trials [7, 8]. Although generally recognised as an essential diagnostic tool, 1p/19q testing is still not as widely available as desirable, mainly because it is a comparatively laborious and thus expensive test method. It will be important that the Neuropathology community encourages also smaller centres to make a case to provide this test locally. More recently, next generation sequencing and methylome arrays became potentially useful diagnostic tests that can complement glioma diagnostics.

A major breakthrough in the diagnosis of gliomas was the discovery of the mutation in the *IDH1* and *2* genes in astrocytomas and oligodendrogliomas. Antibodies, generated against the most common *IDH* mutation (IDH1^{R132H}), has within less than a year after discovery become an affordable test for a biomarker with diagnostic and predictive value [9, 10]. The retrospective analysis of samples of patients with astrocytomas and oligodendrogliomas by large scale genomic methods subsequently identified further genes that were consistently altered, e.g. *CIC* and *FUBP1* [11, 12] in 1p/19q deleted oligodendrogliomas or *ATRX* mutations in *IDH* mutant, 1p/19q-preserved astrocytomas.

The discovery of new biomarkers and their mapping against clinical outcome highlights the importance of the full integration of the tissue diagnosis with biomarkers, and underpins the continued importance of the neuropathologist in giving a full, integrated opinion [13]. These "Haarlem Consensus Guidelines for Nervous System Tumor Classification" suggest that some entities will require molecular information to provide an "integrated" diagnosis, which is based on several layers comprising (i) the integrated diagnosis as top layer, followed by (ii) histological classification, (iii) WHO grade, and (iv) molecular information.

The identification of driver mutations in glial or glioneuronal tumours, such as *IDH1*, *IDH2*, *H3F3A* or *BRAF* point or fusion mutations also widens our understanding of the pathobiology and histogenesis of these tumours and we will have to accept that many of these tumours may represent part of a spectrum of histological phenotypes arising from a defined mutation in the stem/progenitor compartment in the brain [14-18] rather than being predominantly morphologically defined entities that may lack a biological justification. This is likely to have an impact on our approach towards diagnosing brain tumours, and one can be hopeful that ambiguous diagnoses, such as oligoastrocytomas or the glioblastoma (GBM) with oligodendroglial component will disappear in favour of molecularly endorsed entities [13,

19]. Recent consensus meetings have underpinned this notion and highlight the growing importance of molecular markers to support a molecularly enhanced neuropathological diagnosis but there is a lively debate to what extent to integrate (or rely on) molecular data [13, 20].

This review will present data to justify molecular testing of gliomas and will highlight the diagnostic value for tumour classification, and predictive/prognostic values of the molecular tests:

1) What is the benefit of 1p/19q and *IDH* molecular testing for the patient?
2) Is there a benefit to use the markers *ATRX*, *FUBP1*, *H3F3A*, *CIC* and *TERT* in glioma diagnostics?
3) What is the biological and diagnostic role of BRAF V600E and *BRAF* fusion gene mutations in the context of low grade CNS tumours?
4) What is the predictive and prognostic value of the *MGMT* promoter methylation in high grade gliomas?

Incidence and survival rates of common glial tumours

Astrocytic and oligodendroglial tumours (WHO Grades II and III) have an annual incidence of 10.1 new tumours per million population in Western countries, and GBMs have an incidence of 35.5 per million and that of all intrinsic brain tumours is 52.7 per million [21, 22]. Extrapolated for the UK (population 63.7 million), this equates to ~640 new oligodendrogliomas and astrocytomas in adults, and ~2,260 GBM annually. Pilocytic astrocytomas are less common with 3-4 per million, or approximately 220 cases annually in the UK and both the pleomorphic xanthoastrocytoma and the subependymal giant cell astrocytomas are very rare with 2 and 1 cases per million population annually (14 and 7 cases annually in the UK).

Survival rates for pilocytic astrocytomas are generally excellent (96% at 10 years), whilst the diffusely infiltrating gliomas have a less favourable prognosis with median survival times of 5.6 years for WHO Grade II astrocytomas, 1.6 years for anaplastic astrocytomas (WHO Grade III) and 0.4 years for GBM (WHO Grade IV) [22-24]. WHO Grade II oligodendrogliomas have a median survival time of 11.6 years and anaplastic oligodendrogliomas 3.5 years. Of note these epidemiological data were not validated by molecular parameters.

Codeletion of chromosomal arms 1p and 19q

Summary: The codeletion of 1p/19q, in the past often also referred to as combined loss of chromosomal arms (LOH, loss of heterozygosity) is a significant predictor of outcome for patients with tumours of oligodendroglial and oligoastrocytic histology. 1p/19q codeletion is mainly observed in classical oligodendrogliomas (Fig. 1, 2A). Response to first line procarbazine, CCNU, and vincristine (PCV) or to temozolomide (TMZ) chemotherapy strongly correlated to loss of 1p [25, 26], whilst response to second line alkylating treatment is modest even in tumours with 1p/19q codeletion.

Prognostic and predictive value: The 1p/19q status is prognostically highly relevant. It is associated with longer progression free survival and better chemotherapy response. This was reported and validated in multiple studies [25, 27-31]. Patients with tumours lacking 1p

and 19q (46%) compared with those with preserved 1p and 19q had longer median survival times (7 v 2.8 years, respectively; p <0.001) and longer progression-free survival was most apparent in this group. In current clinical practice patients with gliomas (grade II/III) harbouring a 1p/19q codeletion are often treated with first line chemotherapy (CT) (temozolomide or PCV) with a high chance of good clinical response. It has been reported that many centres tend to defer radiotherapy (RT) particularly in patients with predicted long-term survival [32, 33]. CT alone or in combination with RT shows no significant difference for overall survival in some studies [34], but CT/RT shows survival benefit over RT alone in codeleted oligodendrogliomas [35, 36]. In contrast, patients with non-codeleted 1p/19q will be usually offered first line RT with or without combined CT due to the limited chance of response to chemotherapy [32]. *IDH1/2* mutations (see below) and 1p/19q codeletion are of major prognostic significance for outcome .

Diagnostic value: Histological distinction of oligodendrogliomas from astrocytomas is highly subjective, and there is an established interobserver variability to discriminate astrocytomas, oligoastrocytomas and oligodendrogliomas [37, 38]. This makes the 1p/19q test of indispensable diagnostic value (Fig. 3). Although the test cannot discriminate between oligodendrogliomas and oligoastrocytomas, it will help narrowing down diagnostic options, for example in small, non-representative (but tumour containing) biopsies. It is increasingly recommended, based on molecular evidence to minimise the diagnosis of oligoastrocytomas and to favour a classification into astrocytomas and oligodendrogliomas [19, 39-41]. The diagnostic value of 1p/19q tests is further enhanced by the combined use of the biomarkers *IDH1, IDH2, ATRX, CIC* and *FUBP1* (see below, and Fig. 4).

Molecular Mechanisms: Deletions of segments of the chromosomes 1p and 19q are closely associated with the oligodendroglial histological phenotype (Fig. 3A, B, and Fig. 4). Chromosome losses occur in the majority of oligodendrogliomas and are often associated with a pericentromeric translocation of chromosomes 1 and 19 [42, 43] (Fig. 1). The translocation results in a 1p and 1q t(1;19) fused at the centromere (q10;p10) and a chromosome derivative (Fig. 1). This translocation is unbalanced, leaving the cells with one copy of the short arm of chromosome 1 and one copy of the long arm of chromosome 19. Whilst most cancer translocations involve one of the genes residing near a break- point, and produce a fusion gene product, the der(1;19) (q10;p10) breakpoints are in gene-poor centromeric regions and always associated with codeletion [42, 43].

Mutations in isocitrate dehydrogenase genes *IDH1* and *IDH2*

Summary: Mutations at the codon 132 in the isocitrate dehydrogenase 1 (*IDH1*) genes, primarily of the IDH1^{R132H} type, occur early, with a high frequency, in WHO grade II and III astrocytic and oligodendroglial tumours and in secondary GBMs, which develop from astrocytomas. *IDH* mutations in gliomas are early, if not initiating events in their pathogenesis, and are associated with several clinically relevant parameters including patient age, histopathological diagnosis, combined 1p/19q deletion, TP53 mutation, MGMT promoter hypermethylation and patient survival [44-48].

Prognostic and predictive value: The association between *IDH1/2* mutation and a favourable prognosis is better established in high grade gliomas [49-51], whilst the prognostic value in WHO grade II tumours seems is less clear [50, 52, 53]. The majority of studies reporting mutant *IDH1* as a favourable factor in WHO grade II tumours often include oligodendroglial tumours [50, 52, 54]. Studies comprising low grade astrocytomas only showed no prognostic value of mutant *IDH1/2* [55, 56]. The NOA4 trial identified that MGMT promoter methylation is prognostic for patients with *IDH1/2*-mutant WHO grade III gliomas, but in patients with *IDH*–wild-type tumours, MGMT promoter methylation was predictive for benefit from alkylating chemotherapy [57].

Diagnostic value: Testing of the *IDH* status is highly relevant for the diagnosis of primary brain tumours. An antibody, specific for the IDH[R132H] mutation has been developed in 2009 and is commercially available for diagnostic testing on paraffin sections (Fig. 3) [10, 58, 59]. Using this antibody, 90% of *IDH* mutations can be detected [9]. It is recommended to test the remaining 10% by sequencing (Fig. 3) [46, 60]. Considering the easy and cost-effective testing by immunohistochemistry, *IDH* sequencing is not considered an essential routine test in many neuropathology laboratories. However, in selected low grade gliomas in a patient group where *IDH* mutation status would impact on treatment decisions [46], additional testing of IHC-negative astrocytomas and oligodendrogliomas should be considered [46, 59]. The routine testing for *IDH* mutations can help differentiating low-grade or anaplastic oligodendrogliomas from other intrinsic neoplasms with focal or entire clear cell morphology, including primary GBM, clear cell ependymomas, neurocytomas or pilocytic astrocytomas with oligodendroglial-like differentiation, and high grade astrocytomas or oligodendrogliomas from "primary GBMs with oligodendroglial differentiation" as described in [9] (Fig. 3, 4).

Molecular Mechanisms: Pathogenic mutations in the *IDH* genes 1 and 2 were discovered in next-generation sequencing studies of 22 GBM [49]. The analysis led to the discovery of a variety of genes that were not known to be altered in GBMs but importantly these *IDH* mutations were discovered in secondary GBM. Subsequent studies, first on a wide spectrum of brain tumours [61], and subsequently on selected astrocytic and oligodendroglial tumours [62-67] revealed that this mutations is likely to be a tumour initiating or driver mutation [68] in astrocytomas and oligodendrogliomas, even in the presence of a pre-existing mutation of the tumour suppressor gene *p53* [69]. The most common mutation (~90%) in glial brain tumours causes a substitution of the amino acid Arginine to Histidine at codon 132 of the *IDH1* gene (IDH1 R132H) and together with the rarer mutations in codon 132 renders mutant *IDH* as useful diagnostic biomarker [64, 70]. These mutations change the enzymatic activity of the cytoplasmic and peroxysomal *IDH1*. The same holds true for codon 172 mutations in the mitochondrial *IDH2* gene. These homologous enzymes decarboxylate isocitrate to α-ketoglutarate (αKG) and this "neomorphic" mutation renders the IDH enzyme to reduce αKG into 2-hydroxyglutarate (2-HG) in an NADPH-dependent manner [71]. The product of this novel reaction, 2-HG, is a poorly understood metabolite which accumulates to high levels in glioma tissues and it has been suggested that 2-HG enhances proliferation and impairs differentiation [72, 73]. 2-HG can inhibit many 2OG-dependent enzymes, including various histone demethylases and the 5'-methylcytosine hydroxylase TET2. Mutant *IDH1* or *IDH2* are associated with increased histone methylation, suggesting to cause epigenetic alterations in

both, DNA and histones, altering gene expression and promoting oncogenic transformation. The presence of an *IDH* mutation in gliomas leads to a reorganization of the methylome and transcriptome. The hypermethylation of cytosine in the promoter region occurs in CpG islands, where the nucleotide Cytosine is located next to Guanine, separated by one phosphate. Cytosines in CpG dinucleotides can be methylated to form 5-methylcytosine. In this context, hypermethylation represents a *change* in 5-methyl-cytosine distribution across the genome *rather than an overall increase* in the total amount of methylation [74]. Subsequently it has been shown that mutant *IDH* is the molecular basis of the CpG island methylator phenotype (CIMP) in gliomas, leading to global dysregulation of gene expression [75-77]. The analysis of epigenetic changes in the cohort of The Cancer Genome Atlas (TCGA) identified a subgroup of GBM tumours with highly concordant CpG island methylator phenotype which were strongly associated with *IDH* mutations [77, 78]. Methylation arrays have also helped identifying novel subgroups of GBMs, with *IDH* mutant tumours showing a G-CIMP phenotype [79, 80].

Further characterisation of gliomas with the biomarkers ATRX, FUBP1, and CIC

These biomarkers have been recently identified in the context of gliomas and allow for a further diagnostic accuracy and potential stratification for clinical outcome.

ATRX is frequently lost in *IDH* mutant astrocytomas

Summary: ATRX loss is a useful biomarker in the refinement of the diagnosis of *IDH* mutant astrocytomas, and may be used to delineate these tumours from oligoastrocytomas and oligodendrogliomas (Fig. 3, 4) [19, 41]. *ATRX* and *IDH* mutant anaplastic astrocytomas have a favourable prognosis compared to anaplastic astrocytomas with *IDH* mutation only [39].

Prognostic and predictive value: Analysis of *ATRX* mutations in the "biomarker cohort" of the NOA-04 clinical trial [39] shows a survival benefit of *ATRX* mutant astrocytomas. The trial compared efficacy and safety of radiotherapy versus chemotherapy with either PCV or TMZ as initial therapy in patients with newly diagnosed, supratentorial anaplastic gliomas (WHO grade III) and examined the clinical relevance of 1p/19q codeletion, *MGMT* promoter methylation and *IDH1* mutations in anaplastic astrocytomas [26]. Based on the molecular profiles and the clinical outcome, a modified diagnostic algorithm was proposed, suggesting the terms "molecular" astrocytomas, oligodendrogliomas and GBMs, which may not necessarily directly correspond to histological entities [39, 41]. Introducing ATRX immunohistochemical staining into routine glioma testing may be an important step forward as is may help eliminating the ambiguity of the vaguely defined group of oligoastrocytomas (Fig. 3, 4).

Diagnostic value: ATRX loss occurs almost exclusively in *IDH* mutant tumours, and ATRX loss and 1p/19q codeletion are largely mutually exclusive (Fig. 4) [39, 41, 81]. Exome sequencing of *IDH* mutant, 1p/19q intact astrocytomas and oligoastrocytomas showed a high incidence of mutations in the *ATRX* gene, whilst *ATRX* mutations are rarely seen in 1p/19q co-deleted oligodendrogliomas [41]. Assessment of ATRX loss by immunohistochemical staining captures the majority of mutations, 27 % by IHC vs 33% by sequencing in grade II tumours and 41 % by IHC vs 46% by sequencing in grade III astrocytomas in adults [82], indicating that

the use of immunohistochemical testing in routine neuropathology diagnostics gives a reasonable sensitivity. Another study described a higher mutation rate of 67% (10/15) in grade II astrocytomas, and 73 % (32/44) in anaplastic astrocytomas, whilst *ATRX* mutations are rarely present in primary GBM or pure oligodendrogliomas (25% in morphologically diagnosed OII and 7% in OIII) [12], but only two of the 70 oligodendroglial or oligoastrocytic tumours had an *ATRX* mutation combined with 1p/19q codeletion. Recent, larger studies on more than 400 oligodendroglial and astrocytic gliomas further strengthened the notion of *ATRX* being a diagnostically useful and important marker [19, 40, 41]. In contrast to paediatric GBM where *ATRX* mutations occur around a hotspot near the carboxy-terminal helicase domain [15], mutations in adult gliomas are distributed evenly across the gene [12].

Molecular Mechanisms: The ATRX (alpha-thalassemia/mental retardation syndrome X-linked) protein [83] and its binding partner DAXX (death-associated protein 6) are essential members of a multiprotein complex with a role in regulating chromatin remodelling, nucleosome assembly, telomere maintenance (see TERT paragraph below) and incorporation of Histone H3.3 proteins into the telomeric regions of chromosomes [84]. ATRX loss has been described in pancreatic neuroendocrine tumours, [85], and neuroblastoma [86]. *ATRX* loss of function mutations were detected also in a small fraction of adult (presumably *IDH* mutant, "secondary") [87] and paediatric GBM [15].

Markers that facilitate and may in the future complement the diagnosis of oligodendrogliomas: CIC and FUBP mutations segregate with *IDH* mutation and 1p/19q codeletion.

A search for candidate genes on chromosomes 1p and 19q by exome sequencing of oligodendrogliomas revealed inactivating mutations in two tumour suppressor genes: (i) homolog of Drosophila *capicua* (*CIC*) and (ii) *far-upstream binding protein 1* (*FUBP1*) in 53% and 15% of oligodendrogliomas, respectively [11, 12, 88, 89]. *CIC* is located on chromosomal arm 19q (19q13.2) and *FUBP1* is located on chromosomal arm 1p (1p31.1) and thus 1p/19q codeletion is thought to be a mechanism to inactivate *CIC* and *FUBP1* (Fig. 1). Across three studies [12, 89, 90], of 80 oligodendrogliomas (OII) and 89 anaplastic oligodendrogliomas (OIII) 39% of OII and 51% OIII had a *CIC* mutation. In two studies [12, 89] 14% OII (36 analysed) and 25% OIII (49 analysed) had a *FUBP1* mutation. Of these, 9 tumours had a mutation on both genes [12, 89]. To implement mutational analysis into diagnostic practice in neuropathology diagnostics, Baumgarten et al [91] correlated loss of FUBP1 immunoreactivity with the mutation status and found a 100% sensitivity and 90% specificity, i.e. all immunonegative tests had a mutation and IHC would miss 10% of mutations detected by sequencing.

The Far Upstream Element [FUSE] Binding Protein 1 (FUBP1) regulates cell cycle regulators MYC and p21 [92, 93]. FUBP1 binds to single-stranded DNA, in particular the far upstream element (*FUSE*) of the oncogene *MYC* [94]. Whilst overexpression of *FUBP1* can stimulate *MYC* expression [94], later studies also suggest that FUBP1 forms part of a complex with the FUBP1 interacting repressor (FIR, a.k.a. PUF60) that negatively regulates *MYC* expression [93]. The recent finding of inactivating mutations of *FUBP1* in the context of oligodendroglioma are in line with the notion that *FUBP1* mutations lead to *MYC* activation

by relieving the suppressing effects of the complex formed by FUBP1, FUBP1 interacting repressor (FIR) and FUSE [93].

The protein encoded by *capicua*, CIC is a downstream component of receptor tyrosine kinase (RTK) pathways including EGFR, Ras, Raf, and MAP kinases [95]. RTK signalling, (e.g. EGFR) blocks the function of CIC thus activating targets that are usually repressed by it. 8 of 11 [89], 7 of 10 [12] and 14 of 15 [90] missense mutations observed in oligodendroglial tumours are located in the highly conserved HMG box functional domain of the CIC protein. Immunohistochemical detection of nuclear CIC is absent in *CIC* mutant tumours [96], although the correlation between DNA mutation and loss of protein expression has not been validated in this study.

In conclusion, *CIC* and *FUBP* mutation analysis, specifically when the immunohistochemical tests are available, in combination with ATRX immunostaining, may prove as useful additional markers that can help classify astrocytic and oligodendroglial tumours ahead of a 1p/19q test, and can be helpful in those settings where the availability of 1p/19q tests is limited or associated with delays. A prognostic value for CIC and FUBP1 has yet to be determined.

TERT mutations

Summary: Telomerase reverse transcriptase (TERT) is essential in maintaining telomere length and its activity is pathologically increased in a number of human cancers, including GBM. *TERT* and *IDH* mutations are mutually exclusive in GBM, but co-occur in oligodendrogliomas (Fig. 4). Two large studies show conflicting results with regard to *TERT* mutation status and survival, but it is possible that *TERT* may in the future become a useful prognostic marker.

Prognostic and predictive value: In a large study comprising more than 400 GBM, astrocytomas and oligodendrogliomas, *TERT* promoter mutations were seen in 74% of GBM, but only in a minority of Grade II-III astrocytomas (18%) [97]. This and other studies [41, 98] also show that *TERT* and *IDH* mutations are largely mutually exclusive in GBM and astrocytomas, but co-occur in most oligodendrogliomas (Fig. 4). In the study by Killela et al [97] Patients with *TERT* promoter mutations alone (i.e. no *IDH* mutation) had the poorest OS (median 11.3 months), patients with tumours without *TERT* or *IDH1/2* mutations had a slightly better survival (median 16.6 months), whilst patients with *IDH*-only mutant GBM had the best survival (median 42.3 months). Although an earlier study with 358 patients found no significant difference in overall survival between *TERT* mutant and *TERT* wild-type (*IDH* wt) GBM [98], the role of *TERT* mutations may in the future provide a tool to identify non-*IDH1/2* mutant GBMs and suggests that combined *IDH1/2* and *TERT* promoter genotyping will be useful for patient management.

Diagnostic value: Knowledge of the *TERT* promoter status in tumours is likely to become of growing interest for molecular classification and as a potential target for therapy. Analysis of *TERT* promoter mutations in 1,515 CNS tumours showed 327 mutations, predominantly in adult patients, with a strong association with older age (p < 0.0001) [99]. Mutations were seen in gliosarcomas (81 %), oligodendrogliomas (78 %), oligoastrocytomas (58 %), and

primary GBMs (54 %). *TERT* promoter mutations are also strongly associated with 1p/19q codeletion (p < 0.0001), and inversely associated with loss of ATRX expression (p < 0.0001) and *IDH1/IDH2* mutations (p < 0.0001) [41, 99] (Fig. 4).

Molecular Mechanisms: The enzyme telomerase reverse transcriptase (TERT), part of the telomerase protein complex plays a critical role in extending the telomeres, repetitive nucleotide sequences at the ends of chromosomes. Telomere length shortens with each cell division, ultimately leading to replicative senescence. Thus, the ability to maintain telomere length is a feature of neoplasia. Mutations in the *TERT* promoter, resulting in overexpression of *TERT* is a feature of most human cancers including gliomas [41, 97, 99, 100]. It has been suggested that tumours derived from cell populations with low self-renewal capacity generally rely on alterations that restore telomerase activity, while epigenetic mechanisms maintain telomerase activity in tumour types derived from self-renewing stem cells [101]. Intriguingly, *ATRX* or *DAXX* mutations have been shown to underlie a telomere maintenance mechanism not involving telomerase ("alternative lengthening of telomeres"; ALT) the mechanisms responsible for telomerase activity in cancers. Killela et al [101] suggest two options to maintain telomere lengths as cells divide: through epigenetic regulation of telomerase activity, in stem cells of tissues that are rapidly renewing, and (in pathological context) through somatic mutations that maintain telomere lengths, such as mutations in the *TERT* promoter or mutations in *DAXX* or *ATRX*.

Histone H3 mutations

Summary: Initially detected in paediatric malignant brain stem glioma, H3 histone family 3A (*H3F3A*) mutations also occur in adult GBM and in locations other than brain stem. The frequency has not yet been established but an estimate by the authors ranges around 5% of all adult GBM harbouring this alteration (Fig. 4). Similar to paediatric GBM, *H3F3A* mutations in adult patients frequently co-occur with *ATRX* mutations (Fig. 3B, Fig. 4). These patients rarely exhibit gain of 7p, and usually carry 10q loss. *EGFR* amplification usually does not occur in this group. The average patient age at manifestation is significantly younger than that of typical GBM patients.

Other frequent alterations in glioblastomas

A recent study of more than 400 gliomas with Illumina 450k CpG island methylation arrays showed that combined 10q loss and 7p gain are a feature of 60% (81/136) primary, *IDH* wild-type GBM (Fig. 4) and nearly all gliosarcomas (93%, 13/14) [41] (Fig. 2 B, C). *EGFR* amplification is a feature of 28 % (38/136) of primary *IDH* wild-type GBM, most of which also have 7p gains and 10q loss (Fig. 4).

Mutations in the *BRAF* gene and the MAP kinase pathway

Summary: *BRAF* gene mutations activate the MAP kinase pathways and stimulate growth. Point mutations of the *BRAF* gene (resulting in the BRAF protein V600E mutation) are present in a range of low grade glial and glioneuronal tumours, such as pleomorphic xanthoastrocytomas (PXA), gangliogliomas, subependymal giant cell astrocytomas (SEGA), desmoplastic infantile gliomas and pilocytic astrocytomas. *BRAF* gene rearrangements and generation of a fusion gene product are signature mutations in pilocytic astrocytomas.

Whilst diagnostically useful, in particular to discriminate from diffuse astrocytomas, they are not prognostically relevant.

BRAF V600E point mutation:

Summary: Unlike the discovery of *IDH* mutations through deep sequencing [49], the discovery of the BRAF V600E mutation in a sizeable proportion of gangliogliomas, PXA and in a small number of anaplastic PXA/giant cell GBM was made through a conventional and hypothesis-driven approach, following the previous discovery of the *BRAF* point mutation to be present in 7-15% of human cancers, including melanoma, colon and ovarian cancer [102]. Using a PCR multiplexing tumour genotyping assay, tumour specimens tested prospectively guided clinical decision making toward targeted therapies [103]. A similar study, conducted at the same time, looked into the presence of the BRAF V600E mutation in a wider range of intrinsic glial and glioneuronal tumours and found the BRAF V600E mutation in addition to PXA and anaplastic PXA also in gangliogliomas and anaplastic gangliogliomas [104]. More recently the BRAF V600E mutation was detected in papillary craniopharyngiomas [105], in contrast to activating mutations of the *β-catenin* gene (*CTNNB1*) in adamantinomatous craniopharyngiomas. In specific instances the detection of the BRAF V600E mutation can help discriminating craniopharyngiomas from Rathke cleft cysts with unusual presentations [106].

Prognostic and predictive value: A wide spectrum of low grade glial and glioneuronal tumours, many clinically presenting with long-standing epilepsy in children and adults have been identified as BRAF V600E mutant. The mutation is most commonly seen in PXA and the anaplastic form of PXA (65%) [107, 108], followed by gangliogliomas (25%) [104, 109, 110] and DNT (30%) [109], and it is occasionally seen in desmoplastic infantile ganglioglioma/astrocytoma (16%) [111, 112] and pilocytic astrocytomas (10%) [104, 109 , 113]. Small series of cases of GBMs with giant cell components and morphological variants thereof were reported [114-117]. The prognostic value of the BRAF V600E mutation is less well established than that of *IDH* mutations in astrocytomas and oligodendrogliomas. This may be attributed to the overall small number and the less well established survival data of the significantly more diverse group of tumours with the BRAF V600E mutation. Case series of gangliogliomas suggest a slightly worse survival of BRAF V600E mutant tumours [118].

Diagnostic value: Unusual forms of temporal astrocytomas, clinically presenting with frequent, sometimes refractory seizures and no other neurologic deficit have been reported with BRAF V600E mutations (Table 1). Many of these tumours had a spindle cell component and were fairly well demarcated [119]. Unlike the multiple *IDH1* and *IDH2* mutations, which require sequencing [46, 60], the detection of the single BRAF V600E mutation is more straightforward. Although the VE1 antibody is fraught with more technical difficulties [120] than the IDH^{R132H} antibody, it may be superior to the sole use of sequencing, as it can be done as part of the clinical routine and it has been reported to detect the mutation in sequencing-negative cases or in cases where DNA had to be extracted from selected microdissected areas to enrich for mutant cells [110, 111]. It has been suggested that the use of the antibody may reveal a higher proportion of BRAF V600E mutant gangliogliomas than previously thought [118]. The advantages of immunohistochemistry over sequencing are the wide availability and the rapid turnaround time, enabling a molecular diagnosis on

the same or next day. More recently, the BRAF V600E mutation has been identified in 94% of papillary craniopharyngiomas (34/36) [105], while adamantinomatous craniopharyngiomas frequently 96% (51/53) show *CTNNB1* mutations resulting in nuclear localisation of β-catenin [105]. It has been suggested that the mutual exclusivity of mutations in *BRAF* and *CTNNB1* and the easily available immunohistochemistry for BRAF V600E and nuclear β-catenin could be used to distinguish papillary from adamantinomatous craniopharyngiomas in morphologically ambiguous cases [106] and may also be used to direct patients to appropriate clinical trials.

Molecular Mechanisms: Cells respond to extracellular signals by transmitting intracellular instructions to coordinate appropriate responses. Highly conserved mitogen-activated protein kinase (MAPK) or extracellular signal regulated protein kinase (ERK) cascades are often used to transduce these signals [121]. These cascades consist of a three-kinase module that includes a MAPK, which is activated by a MAPK/ERK kinase (MEK), which in turn is activated by a MEK kinase (MEKK). The first and best characterized MAPK cascade consists of Raf isoforms, MEK1/2, and ERK1/2, and is regulated by Ras (Fig. 5). The RAS–RAF–MEK–ERK pathway mediates cell responses to growth signals, and regulates cell proliferation, survival, and senescence in response to extracellular signals [121]. RAS is a small G protein that is attached to the inner surface of the plasma membrane, whereas RAF, MEK, and ERK are cytoplasmic protein kinases that consecutively activate each other, forming a three-tiered signalling cascade. There are three RAF isoforms in mammals, *A-RAF, B-RAF, and C-RAF*. The three *RAF* genes code for cytoplasmic serine/threonine kinases that are regulated by binding RAS [122]. *RAS* is mutated to an oncogenic form in about 15% of human cancer. All *B-RAF* (*BRAF*) mutations occur within in the kinase domain of the BRAF protein. The most common mutation in BRAF is a glutamic acid for valine substitution at position 600 (V600E, previously V599E) [102].The mutation is phospho-mimetic, resulting in an activated protein that signals to MEK–ERK constitutively, stimulating cell proliferation and survival (Fig. 5).

BRAF Fusion gene mutations

Summary: A different type of mutation involving the *BRAF* gene is found predominantly in pilocytic astrocytomas (PA). The mutation is associated with a gain at the locus 7q34. The tandem duplication produces a fusion gene of the genes *KIAA1549* and the *BRAF* gene. The most common fusion is between *KIAA1549* exon 16 and *BRAF* exon 9 (*KEx16BEx9*; 20 cases) followed by *KIAA1549: BRAF 15-9* and *KIAA1549:BRAF 16-11* [123, 124]. Very rare mutations are *KIAA1549–BRAF 18–10* and *KIAA1549:BRAF 19-9* [124].

Prognostic and predictive value: So far, no differences in survival between tumours with and without *BRAF* duplication/fusion have been found [123, 125, 126]. However despite this prognostically uninformative molecular signature, a beneficial result from these findings could be the possibility to develop targeted treatment options. Inhibitors of the MAPK pathway, such as AZD6244 and Sorafenib, have been developed and approved for the treatment of several other cancers and may be useful for the treatment of children with tumours that are difficult to resect or are aggressive [124]. Somatic duplication of 7q34 is specific to pilocytic astrocytomas. The frequency of the mutation varies with the tumour location, cerebellar being most frequent (80%) followed by brainstem and optic pathway

tumours (~60%), whereas it is rare in hemispheric juvenile PA (10-20%) [127]. In contrast only 9% BRAF V600E mutations are seen in adult or paediatric PA [104].

Diagnostic value: With one exception [128] *BRAF* gene fusions were to date identified only in PA and not in any other types of brain tumours, such as diffuse astrocytoma and oligodendroglial tumours, GBM, ependymoma, medulloblastoma, atypical teratoid/rhabdoid tumour (AT/RT), choroid plexus papilloma and -carcinoma, dysembryoplastic neuroepithelial tumour, ganglioglioma, or papillary glioneuronal tumour [129]. PCR-based methods such as fusion detection in genomic DNA [130] or in cDNA [123] and tissue based methods such as FISH (fluorescence in situ hybridization) [64] can be used to detect the fusion product. The combination of *BRAF* fusion tests and *IDH* mutation analysis can be diagnostically useful in low grade astrocytomas lacking characterising morphological hallmarks.

Molecular mechanisms: In each fusion protein the N-terminus of RAF is replaced by that of KIAA1549. The N-terminal autoinhibitory domain of BRAF is lost whilst the C-terminal kinase domain is retained, resulting in a constitutive kinase activity of the *BRAF* fusion gene product [124]. It is thought that the gene fusions arise from tandem duplication events [125, 126, 131]. Two mechanisms are implicated in the creation of tandem duplications in cancers, non-allelic homologous recombination and non-homologous end joining [132], of which the former is the more likely mechanism in the *BRAF* fusion generation in pilocytic astrocytomas [125].

The role of MAP kinase activation in the pathogenesis of pilocytic astrocytomas

Pilocytic astrocytomas occur in infra and supratentorial compartments in children and adults. The role of *BRAF* gene fusions [123-127]; and to a lesser extent, BRAF V600E point mutations [104] and *NF2* mutations [133] is well established. A recent study [134] has added significant new information about genes other than *BRAF*, that activate the MAPK pathway. Whole-genome sequencing with matched RNA sequencing discovered 4 new *BRAF* fusions, all resulting in loss of the N-terminal regulatory region of BRAF (*RNF130-BRAF* fusion, *RUFY1* (encoding RUN and FYVE domain–containing 1) and *TMEM178B* (encoding transmembrane protein 178B).

Other mutations occurred in *KRAS*. 19% of the non-cerebellar tumours showed two new gene fusions involving the region encoding the kinase domain of TrkB. The downstream effects of TrkB activation are mediated, at least in part, via MAPK pathway activation. A new mutation was found in the kinase domain of FGFR1 and in in the phosphatase gene *PTPN11* (*SHP-2*) encoding a RAS-MAPK–related adaptor protein.

The study concludes that all pilocytic astrocytomas in their cohort harboured a MAPK pathway alteration; in addition to *BRAF* also *FGFR1*, *KRAS* and *NF1*. The mutations were almost always single pathway mutations, suggesting that BRAF kinase activation alone is a driver mutation in pilocytic astrocytomas. All *FGFR1*-mutant tumours were extracerebellar, mostly in midline locations, suggesting a link between cell of origin and/or microenvironment with *FGFR1*-driven tumourigenesis [134].

The role of the methylation of the MGMT promoter

Summary: *MGMT* promoter methylation in GBM is a prognostic and predictive biomarker indicating response to chemoradiation. It has no diagnostic value.

Prognostic and predictive value: *MGMT* promoter methylation is a prognostic and predictive biomarker [135]. This was demonstrated in the EORTC NCIC registration trial for TMZ in newly diagnosed GBM where patients with *MGMT* promoter methylated tumours derived most benefit when treated with TMZ [136]. The addition of the integrin antagonist cilengitide to radiation and TMZ chemotherapy in *MGMT* methylated GBM was initially promising [137], but was not confirmed in a larger clinical trial which did not show a benefit for progression-free or overall survival [138, 139]. The trial of the effect of TMZ on newly diagnosed GBM [136, 140] showed that *MGMT* promoter methylation was an independent favourable prognostic factor. Patients with tumours with methylated *MGMT* promoter had a survival benefit when treated with TMZ and radiotherapy, compared to those who received radiotherapy only, whilst absence of *MGMT* promoter methylation resulted in a smaller and statistically insignificant difference in survival between the treatment groups. Further studies showed that patients with *MGMT* promoter-unmethylated tumours had no survival benefit from chemotherapy, regardless of whether given at diagnosis together with RT or as a salvage treatment [141, 142]. Prospective randomized trials, (NOA-08) [26], the Nordic trial [143] and RTOG 0525 [144] concluded that *MGMT* promoter methylation is a useful predictive biomarker to stratify elderly GBM patients for RT versus alkylating agent chemotherapy. Accordingly, these consistent trial results suggest that elderly GBM patients eligible for either RT or TMZ should undergo *MGMT* promoter methylation testing prior to clinical decision making.

Diagnostic value: Testing *MGMT* promoter methylation has currently no diagnostic value. The "MGMT status" can be tested by assessing promoter methylation, mRNA or protein expression or enzymatic activity. Methylation-specific PCR (MSP)-based detection of *MGMT* promoter methylation has provided robust results as judged by clinical correlations [78, 135, 145, 146] and is currently the only prospectively validated test for *MGMT* promoter methylation assessment in GBM [147]. However an internationally accepted consensus about the most appropriate diagnostic method is still missing. Most methodologies lack published validation according to guidelines for molecular diagnostics. In practice, the choice of method thus preferentially depends upon the individual experience, equipment of each laboratory (eg. availability of a pyrosequencer, real-time PCR machine or a molecular facility to process methylome chips), and the level of validation [80]. Previous attempts to simplify the tests by detecting MGMT protein by imunohistochemistry had failed, in that it showed a poor inter-observer agreement and thus no correlation to molecular test results [146]. Quantitative or semiquanitative methods include methylation-specific pyrosequencing, methylation-specific multiplex ligation-dependent probe amplification (MS-MPLA) [148], methylation-sensitive high-resolution melting (MS-HRM) [149] bead array- mass spectroscopy, and denaturing high-performance liquid chromatography (HPLC) as reviewed in [135]. Whole-genome Illumina beadchips (HM450) can identify the "MGMT status" as part of the cancer methylome [78, 80].

Molecular Mechanisms: The O(6)-Methylguanine-DNA Methyl Transferase (MGMT) is a DNA repair enzyme that reverts the naturally occurring mutagenic O6-methylguanine back to guanine. This prevents errors during DNA replication. In the context of chemotherapy with

alkylating agents (e.g. TMZ) it removes a cytotoxic lesion, thus counteracting the chemotherapeutic effects of the drug. Aberrant, cancer-related methylation of the *MGMT* promoter region leads to its silencing, a reduction of the MGMT enzyme expression and subsequently to less repair activity of DNA damage, including that induced by TMZ (Fig. 6).

Summary, conclusions and outlook:

The advances in identifying biomarkers have significantly shaped our perception of the histopathogenesis of brain tumours. The presence of a common mutation in a spectrum of brain tumours, for example *IDH* mutations in astrocytomas and oligodendrogliomas (Fig. 3,4), or of the BRAF V600E mutation in an even wider range of low grade intrinsic tumours (Table 1) has highlighted the need for a redefinition of the classification of brain tumours. However, it reassuringly underlines the importance of the correct histological diagnosis (Fig. 3), essential to guide for appropriate molecular tests and subsequent provision of an integrated report and combined tissue and molecular based opinion to guide the neuro-oncological management of these tumours. Genome wide assays, such as the 450k Illumina beadchips that assays 450.000 CpG islands, or next generation sequencing are now being tested in a few large centres. This may define new (molecularly and histogenetically characterised) brain tumour entities, and at the same time remove morphologically similar but biologically diverse entities. Certainly there will be immense opportunities but also significant challenges to the way we will diagnose brain tumours in the future.

Figure legends:

Figure 1: Chromosomal translocations leading to 1p/19q codeletions (based on data from [42]. A, a normal cell contains two copies of chromosomes 1 and 19, each of which has a short ("petit"; p) and long (q) arm. The location of *CIC* (on 19q13.2) and *FUBP1* (on 1p31.10) are indicated, as well as the most commonly used probes on 1p36, 19q13 and controls on 1q24 and 19p13 [43, 150]. B, unbalanced translocation leads to a transposition at centromere level of 19q to 1p (left, shaded) and its subsequent loss, and of the formation of a derivative chromosome consisting of 1q and 1p and, on centromere level, form the "derivative ("der") chromosome der(1;19)(p10;q10), where "p10" and "q10" indicate centromeric location on the chromosome. C, the loss of chromosomal arms 1p and 19q results in one copy of 1p and of 19q, and two copies of 1q and 19p.

Figure 2: Typical copy number profiles of oligodendroglioma and glioblastoma: A, classical profile of an oligodendroglioma with 1p/19q codeletion. B, classical profile of a GBM with 7p gain with *EGFR* amplification and 10q deletion. C, GBM with segmental loss of 1p, and again

the characteristic 7p gain and 10q deletion. In addition there are also deletions of 10p, and 15q. Small segmental deletions on Chr 1p can mislead when using a single FISH or qPCR probe in the 1p.36 region [151].

Figure 3: **Algorithm for the integrated diagnosis of astrocytomas, oligodendrogliomas and glioblastomas**. A, diagnostic pathway for tumours that correspond histologically to oligodendrogliomas or astrocytomas. Following initial assessment on H&E stained sections, immunostaining for IDH1 R132H and ATRX can positively identify the majority of oligodendrogliomas and astrocytomas (Col 2, 4). It is recommended that IDH1 R132H immunonegative tumours are followed up by sequencing to identify rare *IDH1* and all *IDH 2* mutations (Col 1, 3, 5, 6) [46, 60]. Testing for 1p/19q codeletions is the next essential step (red outline) to identify oligodendrogliomas (Col. 1, 2a) and discriminate them from a small number of *IDH* mutant, ATRX retained astrocytomas (Col 2b, red outline, hatched filling). The group of *IDH* mutant, ATRX-negative (=mutant) and 1p/19 non-codeleted tumours corresponds to astrocytomas (Col 3, 4). The remaining groups of *IDH* wild-type tumours may represent infiltrating GBM, or other tumours (Col 5). A small number of *IDH* wild-type and ATRX negative (mutant) GBM show mutations in the *Histone H3* gene (Col 6). Clinically, patients with H3 mutant (anaplastic) astrocytomas have a prognosis similar to those with GBM.

B, Diagnostic pathways for tumours with histological appearance of a GBM. All tumours undergo testing with antibodies for mutant IDH1 R132H and *ATRX* to identify a small number of anaplastic oligodendrogliomas or GBM with *IDH* mutations (Col 2, 4). Depending on the resources, subsequent sequencing may be added to identify a small proportion of these tumours that carry a rare *IDH* mutation not detected by immunostaining (Col. 1, 3, 5, 6) [46, 60]). The majority of "*IDH* mutant GBM" have an ATRX loss (Col 3, 4) whilst a small number of GBM are *IDH* mutant with ATRX retained (Col 2b). Col 5 represents the most common form of morphological GBM: *IDH* wild-type, ATRX retained and 1p/19q non-codeleted (retained). A small number of GBM are *IDH* wild-type, with ATRX loss and Histone mutation (Col 6) and have a similar prognosis as the GBMs as shown in Col 5. This scheme also suggests that the diagnosis of oligoastrocytomas can be phased out, in particular with support of molecular data (See also Fig. 4).

Figure 4: Integrated diagnosis based on molecular data of more than 400 gliomas. The data were obtained from the supplementary material of [41] and were further analysed and colour coded in MS Excel. Primary sorting criterion was the "integrated diagnosis", followed by *IDH*, 1p/19q, ATRX, TERT and H3 status. All samples were analysed with Illumina 450k methylation arrays as described in [41]. The top row shows the diagnosis according to the WHO 2007 classification of brain tumours, and below the reclassification ("integrated diagnosis") based on histology and combinations of molecular markers, which has now resulted in the elimination of oligoastrocytomas and GBM-O and their reclassification into astrocytomas, oligodendrogliomas, "GBM-IDH" and "primary GBM". Below, age of the patients with a mean age and the mutation profiles of the tumour classes and grades. *IDH* mutations define astrocytomas, oligodendrogliomas and a small proportion of GBM ("GBM-IDH"; 9.3%, 14/150). Additional 1p/19q codeletions, *TERT* mutations and retained ATRX

expression define oligodendrogliomas. ATRX loss, retained 1p and *TERT* wild-type are a feature of astrocytomas (of which approximately 25% have a 19q deletion). *IDH* mutant GBM show 10q loss and no *TERT* mutations. The majority of *IDH* wild-type ("primary") GBM show 7p gain, and 10q loss. *EGFR* amplification is seen in primary GBM only. ATRX mutant GBM are *IDH* wild type and often have a Histone H3F3 (K27M or G34R) mutation and occur in younger patients within the GBM group. This schematic reflects the changes that are stipulated by the International Society of Neuropathology-Haarlem Consensus Guidelines for Nervous System Tumor Classification [13], in that (i) disease entities should be defined as precisely and objectively as possible in order to establish highly biologically and clinically uniform groups, (ii) some entities will require molecular information to provide an "integrated" diagnosis, (iii) resulting in a "layered" diagnosis. These layers will comprising (a) the integrated diagnosis (top layer), followed by (b) histological classification, (c) WHO grade, and (d) molecular information.

Figure 5: **Activating mutation in the MAP kinase (MAPK) pathway and its role in pilocytic astrocytomas pathogenesis** [123, 131, 134]: MAPKs are involved in directing cellular responses to regulate proliferation, gene expression, differentiation, mitosis, cell survival, and apoptosis. Under physiological conditions, the pathway is activated by ligand binding to receptor tyrosine kinases. Here, activating point mutations of FGFR1 (red star) and activating fusion mutations fusions of the TRKB receptor have been identified. Physiological ligand binding and both mutations result in phosphorylation of specific intracellular tyrosine residues and creates binding sites for the adapter proteins (GRB2, GAB1, SOS, and SHP2). Mutations in SHP2 (also known as PTPN11, blue star) have been found in conjunction with FGFR1 mutations but are not thought to be sufficient to induce oncogenic signals themselves [134]. Activation of the adapter proteins result in an activation of Ras which subsequently activates Raf and BRAF. Under pathological conditions, Raf can be activated by mutant KRas or by relief of the inhibitory action of NF1 (inactivating NF1 mutation indicated by yellow star). The BRAF V600E mutation and the *BRAF* fusions both activate BRAF signalling and act on downstream targets, similar to the physiological action of Raf and BRAF. Both mechanisms activate the downstream cascade of MEK (Mitogen-activated protein kinase kinase) and ERK (extracellular signal-regulated kinases) which trigger intracellular growth-stimulating transcription factors. The percentages relate to the frequencies of mutations in juvenile pilocytic astrocytomas [134].

Figure 6: **Role of MGMT promoter methylation and response to alkylating agents:**
Expression of the DNA repair protein O6- methylguanine-methyltransferase (MGMT) results in resistance of tumours to alkylating agents. A, MGMT is a cellular DNA repair protein that re verses alkylation (including methylation) at the O^6 position of guanine. B, Methylation of the *MGMT* promoter is an epigenetic event leading to silencing of the promoter and thus reduced expression of the active enzyme, MGMT. C; High levels of MGMT activity in tumour tissue confer with resistance to alkylating agents (left), whilst methylation of the *MGMT* promoter result in reduced MGMT repair activity, and this are associated with increased sensitivity to alkylating agents (right).

Table 1: Meta-Analysis of BRAF V600E mutant case series in intrinsic brain tumours in the literature (excluding case reports of less than 5 cases)

		PXA		aPXA		GG		aGG		DNT		DIG/DIA		PA		SEGA	
	Method	M	WT	M	WT	M	WT	M	WT	M	WT	M	WT	M	WT	M	WT
[113]	Seq	8	4			12	39							7	38		
[109]	Seq, AB	3	2			13	18			6	14			2	38		
[110]	Seq, AB											3	15				
[104]	Seq	42	22	15	8	14	63	3	3					9	88	1	2
[152]	Seq	10	11	2	1					26	25					6	8
[134]	Seq													4	96		
Total		63	39	17	9	39	120	3	3	32	29	3	15	22	260	7	10
Proportion of mutant cases		0.62		0.61		0.25		0.5		0.52		0.167		0.08		0.41	

The upper row indicates the abbreviated histological diagnosis: PXA and aPXA, (anaplastic) pleomorphic xanthoastrocytoma; GG and aGG, (anaplastic) gangliogliomas; DNT, dysembryoplastic neuroepithelial tumour; DIG/DIA, desmoplastic infantile ganglioglioma/ astrocytoma; PA, pilocytic astrocytoma. Second row and second column: Method of the detection of the mutation: seq, sequencing; AB, antibody; Second row: M, mutant; WT, wild-type or non-mutant. The penultimate row indicates the cumulative number of mutant or wild-type cases in the studies, and the last row indicates the proportion of

mutant cases per diagnostic group. The inset graph illustrates the proportion of mutant and non-mutant (wt) tumours for each entity, based on the numbers in the table above.

Table 2: Overview of diagnostically and prognostically relevant molecular tests. Abbreviations: DA diffuse astrocytoma; AA anaplastic astrocytoma; DNT dysembryoplastic neuroepithelial tumour; DIPG (paediatric) diffuse intrinsic pontine glioma, non-BS DIPG non brain stem DIPG; OD oligodendroglioma; OA, oligoastrocytoma; DIA, desmoplastic infantile astrocytoma; HG, high grade; LG low grade; PXA, pleomorphic xanthoastrocytoma.

Reference for application in Neuropathology	Molecular marker or target	Type of mutation	Relevant to confirm which brain tumours	Positive test excludes DD	Frequency	Correlation with other markers	Diagnostically relevant	Prognostically relevant	Test method
[37, 38]	Chr 1p/19q	Codeletion	Oligodendroglioma	Diffuse astrocytomas, neurocytomas, DNT	>80% in OD	Co-segregates with *IDH* mutations	Yes	Yes	qPCR FISH
[9, 10, 58]	*IDH1, IDH2*	Point mutation	Astrocytomas, Oligoastrocytomas, Oligodendrogliomas	Pilocytic astrocytomas	70-85%	*IDH1* and *IDH2* are mutually exclusive	Yes	Yes	Mutation specific IHC Sequencing for IHC neg
[39, 41]	ATRX	Mutation, deletion	Diffuse and anaplastic astrocytoma	Oligoastrocytomas	50% -90 of DA and AA	Largely mutually exclusive with 1p/19q Mainly in *IDH* mut gliomas	Yes	Yes	IHC to detect loss (80-90% sensitivity)
[96]	CIC	Mutations	Oligodendrogliomas (Located on 19q)	Astrocytomas	50% of OD with 1p/19q codeletion	Seen in a subset of 1p/19q codeletion	Yes	Limited (marker for 19q loss). Best in combination with 1p test	IHC (loss of staining) (sensitivity not validated)
[91]	FUBP1	Mutations	Oligodendrogliomas (Located on 1p)	Astrocytomas	14% LG OD, 25% HG OD	Seen in a subset of 1p/19q codeletion	Yes	Yes (marker for 1p loss)	IHC (loss of staining) (sensitivity 90%)

Reference for application in Neuropathology	Molecular marker or target	Type of mutation	Relevant to confirm which brain tumours	Positive test excludes DD	Frequency	Correlation with other markers	Diagnostically relevant	Prognostically relevant	Test method
[104, 109, 110] [120]	BRAF V600E	Point mutation	PXA Ganglioglioma DIA Pilocytic astrocytoma	Diffuse astrocytomas	PXA:60-70% GG: 25% DIA/DIG: 20% PA 5%	Mutually exclusive with *BRAF* Fusion	Yes	No	Mutation specific IHC, Seq
[105]			Papillary craniopharyngioma,	Adamantinomatous craniopharyngioma	PC: 94%	Mutually exclusive with CTNNB mutation		To be confirmed	
[102]			Melanoma	n/a	66%		No	May influence treatment	
[153]	*BRAF* Fusion	Fusion transcript	Pilocytic astrocytoma	PXA, other LGG	Depends on age and localisation.	Mutually exclusive with V600E mutation	Yes	No	RT-PCR Seq FISH
[15, 41, 154]	*Histone H3*	Point mutation	non BS-DIPG, DIPG	Not established	36% non BS-DIPG, 78% DIPG 3-5% adult GBM		DIPG	Yes (DIPG)	Seq, IHC
[41, 97, 98]	*TERT*	Promoter mutation		ATRX mutant astrocytomas	50-74% of GBM 68% OD	Mutually exclusive with ATRX loss	Yes	Contradictory reports	Sequencing
[155]	MGMT	Promoter methylation	Non-diagnostic		n/a	Preference in *IDH* mutant tumours	no	Yes, in GBM	msPCR

Supplementary Material:

Supplementary table: Data format of Figure 4: Data were obtained from the supplementary material of [41] and further analysed and colour coded in MS Excel. Primary sorting criterion was the "integrated diagnosis", followed by *IDH*, 1p/19q, ATRX, TERT and H3 status. All colour coding and sample sorting as described in figure 4.

Supplementary figure: Data as shown in Figure 4, formatted as A3, high resolution printout.

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