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Review

Genetic analysis to complement histopathological diagnosis of brain tumors

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Summary. Gliomas, the most frequent tumors originating in the human nervous system, are divided into various subtypes. Currently, microscopic examination alone is insufficient for classification and grading so that genetic profiles are increasingly being emphasized in recognition of the emerging role of molecular diagnostic approaches to glioma classification. Glioblastomas (WHO grade IV) may develop *de novo* (primary glioblastomas) or through progression from lower-grade astrocytomas (secondary glioblastomas), while both glioblastomas show similar histological features. In contrast, they do constitute distinct disease entities that evolve through different genetic pathways, and are likely to differ in prognosis and response to therapy. Oligodendrogliomas (WHO grade II) account for 2.7% of brain tumors and 5-18% of all gliomas. Since this tumor is recognized as a particular subtype of glioma that shows remarkable responses to chemotherapy, a correct diagnosis is of prime importance. The difficulty is that histological differentiation of oligodendrogliomas from diffuse astrocytomas is highly subjective in cases without typical morphological features and there is a lack of reliable immunohistochemical markers. While histological distinction of low-grade gliomas from reactive astrocytes is also often difficult, reactive astrocytes usually lack genetic alterations. More biological and molecular approaches to glioma classification thus appear warranted to provide improved means to achieve correct diagnoses.

Key words: Primary glioblastoma, Secondary glioblastoma, Oligodendroglioma, Diffuse astrocytoma, Reactive astrocyte

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Introduction

Gliomas are divided into astrocytic, oligodendroglial, and ependymal tumors, according to their histologic morphology and similarities of their component cells with differentiated glial cells (Kleihues et al., 2000, 2002). Tumor grading is based on morphologic characteristics such as nuclear atypia, mitotic figures, microvascular proliferation, and focal pseudopalisading necrosis. However, histological diagnosis often depends on subjective judgement and therefore more reliable approaches are needed. Recent advances in molecular biology and molecular genetics may provide the answer in a potential new means to classify gliomas by augmenting the traditional histopathologic classification system (Ichimura et al., 2000, 2004; von Deimling et al., 2000; Louis et al., 2001; Ohgaki et al., 2004; Ohgaki and Kleihues, 2005).

Various genetic alterations that characterize astrocytomas have been extensively studied over the past decade (Collins, 1999; Louis et al., 2001; Ichimura et al., 2004). It has been shown that the apparent heterogeneity of genetic abnormalities initially observed in astrocytic tumors in fact corresponds to distinct, mutually exclusive molecular profiles, suggesting that alternative molecular pathways are involved in progression to anaplasia (von Deimling et al., 1993).

Oligodendrogliomas constitute 2.7% of brain tumors and 5-18% of all central nervous system (CNS) gliomas; another 5% to 10% of gliomas are mixed gliomas (oligoastrocytomas) (Maher et al., 2001; Kleihues et al., 2002; Louis et al., 2002). Their differentiation from diffuse astrocytoma is sometimes difficult without typical morphological features. While histopathological grading is currently the most accurate prognostic indicator, it is generally less helpful in predicting optimum therapy or clinical responses. The idea of genetic subsets may be applicable, however, because extensive studies have shown particular alterations, such as loss of chromosome 1p and 19q, are associated with a good prognosis (Cairncross et al., 1998; Smith et al.,

2000). Underlying molecular mechanisms responsible for this relationship remain to be determined.

Histological distinction of low-grade gliomas from reactive astrocytes is also often difficult, because of the close resemblance at the cell morphology level, and this is important given the widespread and increasing use of stereotactic biopsy techniques placing ever greater diagnostic demands on pathologists. In this context it should be stressed that reactive astrocytes theoretically should lack glioma-associated mutational damage and, therefore, microdissection-based molecular approaches could contribute objective information to complement histopathological analyses.

This article will review and highlight some of the genetic analyses presently used to distinguish gliomas based on their molecular characteristics. Identifying biologically relevant molecular subsets of gliomas can not only convey predictive information, but also guide optimal therapy.

Glioblastomas

Glioblastoma (WHO grade IV) is the most frequent malignant brain tumor. Despite progress in surgery and adjuvant therapy, glioblastoma patients have a very poor prognosis due to incomplete resection and resistance to radio- and chemotherapy (Kleihues et al., 2002). Correlations between the classic histological features of glioblastomas and currently established molecular abnormalities are largely incomplete.

Development concepts

From both clinical and biological points of view, the distinction between primary and secondary glioblastomas is important. The majority of lesions develop after a short clinical history, without clinical or histopathological evidence of a less malignant precursor lesion (primary or *de novo* glioblastoma). Another type of glioblastoma, the so-called secondary glioblastoma, progressively develops more slowly from the low-grade diffuse (WHO grade II) or anaplastic (WHO grade III) astrocytoma. Since glioblastoma subtypes share morphologic features, they usually cannot be distinguished histologically, making it difficult to estimate the relative frequencies at which they occur (Kleihues and Ohgaki, 1999). For example, pseudopalisading necrosis is a histologic hallmark of glioblastomas (Lantos et al., 1996) which is observed at similar frequencies in primary and secondary glioblastomas (Tohma et al., 1998). In contrast, large areas of geographic necrosis containing necrotic tumor cells and vessels (Lantos et al., 1996), resulting from an insufficient blood supply, are observed in almost all primary glioblastomas, but in only one-half of the secondary glioblastomas (Tohma et al., 1998). The presence and extent of large ischemic necrosis in glioblastomas in fact correlates with poor clinical outcome, and its absence is associated with younger patients and a more favorable prognosis (Barker et al., 1996).

The concept of different genetic pathways leading to the glioblastoma as a common phenotypic endpoint has gained general acceptance (Watanabe et al., 1996; Kleihues and Ohgaki, 1999; Kleihues et al., 2002; Godard et al., 2003). To elucidate relationships between morphological phenotype and genetic profile, we have also screened several genetic and epigenetic alterations in a series of gliomas. Genetic analyses have shown two glioblastoma subtypes carrying different genetic alterations (Watanabe et al., 1996; Kleihues and Ohgaki, 1999) (Fig. 1). Primary glioblastomas occur in older patients with EGFR amplification/overexpression, *PTEN* mutations, homozygous *p16INK4a* deletion, and loss of heterozygosity (LOH) on entire chromosome 10, while secondary glioblastomas develop in younger patients and with frequent *p53* mutations, LOH preferentially on chromosomes 10q and 19q, and promoter methylation of the *RB1* gene (Fujisawa et al., 2000; Nakamura et al., 2000, 2001a-c).

Recently, we showed that a 957 kb locus, located at 22q12.3, may contain the putative tumor suppressor gene (TSG), *TIMP-3*, which appears to be relevant to progression to secondary glioblastoma (Nakamura et al., 2005). In addition, the possibility of other putative TSGs on 22q12.3–13.2 and 22q13.31 that may also be involved in the development of primary glioblastomas cannot be ruled out.

Glioblastomas typically are very resistant to induction of apoptosis and, consequently, do not respond well to conventional chemotherapy. *HRK* was originally identified as a proapoptotic gene induced by diminished levels of cytokine in hematopoietic cells and cultured neurons and repressed by expression of death-repressor proteins (Inohara et al., 1997). We have demonstrated that hypermethylation of the *HRK* promoter in conjunction with 12q13.1 LOH down-regulates gene expression, which sheds new light on the mechanism by which tumors expressing wild-type p53 escape apoptosis. Inactivation of *HRK* could be one of the important events associated with the development and progression of secondary glioblastoma via abrogation of an apoptotic pathway, but it does not appear to play a major role in the evolution of primary glioblastomas.

Predictive value of histological features and genetic alterations in glioblastomas

Histologically, whether large ischemic necrosis exists or not may predict poor survival of patients (Barker et al., 1996). It is also proposed that undifferentiated small cells constitute a histological feature of primary glioblastomas associated with a poor clinical course. From current knowledge on survival of glioblastoma patients, LOH 10q is also associated with shorter survival (Schmidt et al., 2002; Terada et al., 2002; Ohgaki et al., 2004). In contrast, *PTEN* mutations did not correlate with prognosis in population-based

studies (Ohgaki and Kleihues, 2005). This suggests that an additional, not yet identified, tumor suppressor gene(s) on 10q25qter may be critically involved in evolution of the glioblastoma phenotype. The functional mechanisms and roles of *PTEN* in glioma development have been widely studied and mutations correlate with poor survival in a subset of high-grade gliomas (Rustia et al., 2001; Smith et al., 2001). The protein encoded by the *PTEN* gene regulates several processes that play critical roles in angiogenesis, migration and invasion (Kotelevets et al., 2001; Koul et al., 2001; Ermoian et al., 2002; Park et al., 2002), so that *PTEN* should be investigated as a therapeutic target for glioblastomas.

Oligodendroglial tumors

Oligodendroglial versus astrocytic tumors

Histologic hallmarks of oligodendrogliomas include a perinuclear halo, which may present as a so-called "honeycomb" or "fried egg" appearance. Dense networks of branching capillaries resembling chicken wire are also common. Based on these classical histologic criteria, oligodendrogliomas have been estimated to account for 5–18% of all gliomas (Kleihues et al., 2000). However, the morphology of tumor cells is not often typical and often varies even within a single case, with more astrocytic features in some areas and oligodendroglial features in others. For cases showing various degrees of mixed morphology, histological separation of low-grade diffuse astrocytomas from oligodendrogliomas shows high inter-observer variability (Fig. 2). This is particularly true for diffuse astrocytomas that contain small areas of oligodendroglial differentiation. Such tumors are variably diagnosed as low-grade diffuse astrocytomas, oligoastrocytomas, or even oligodendrogliomas.

Unfortunately there is a lack of reliable markers available for specific and sensitive recognition of oligodendrocytes and their neoplasms. Expression of Olig1/Olig2, helix-loop-helix transcription factors specifically expressed in cells of oligodendroglial lineage, has been examined as a potential molecular marker (Lu et al., 2001; Marie et al., 2001), but despite promising earlier reports recent studies have demonstrated expression in a wide range of gliomas, including astrocytomas (Bouvier et al., 2003; Ligon et al., 2004; Riemenschneider et al., 2004). Popko et al. found that both individually and in combination, 3 of 4 myelin transcripts (CGT, MBP, CNP and PLP) can be useful in establishing the differential diagnosis of astrocytoma and oligodendroglioma (Popko et al., 2002). Glioblastomas may show strikingly more YKL-40, a member of the 18 glycosyl hydrolase family, than anaplastic oligodendrogliomas on immuno-histochemistry (Nutt et al., 2005). Thus, combinations of molecular markers may be useful in distinguishing the two subtypes of gliomas.

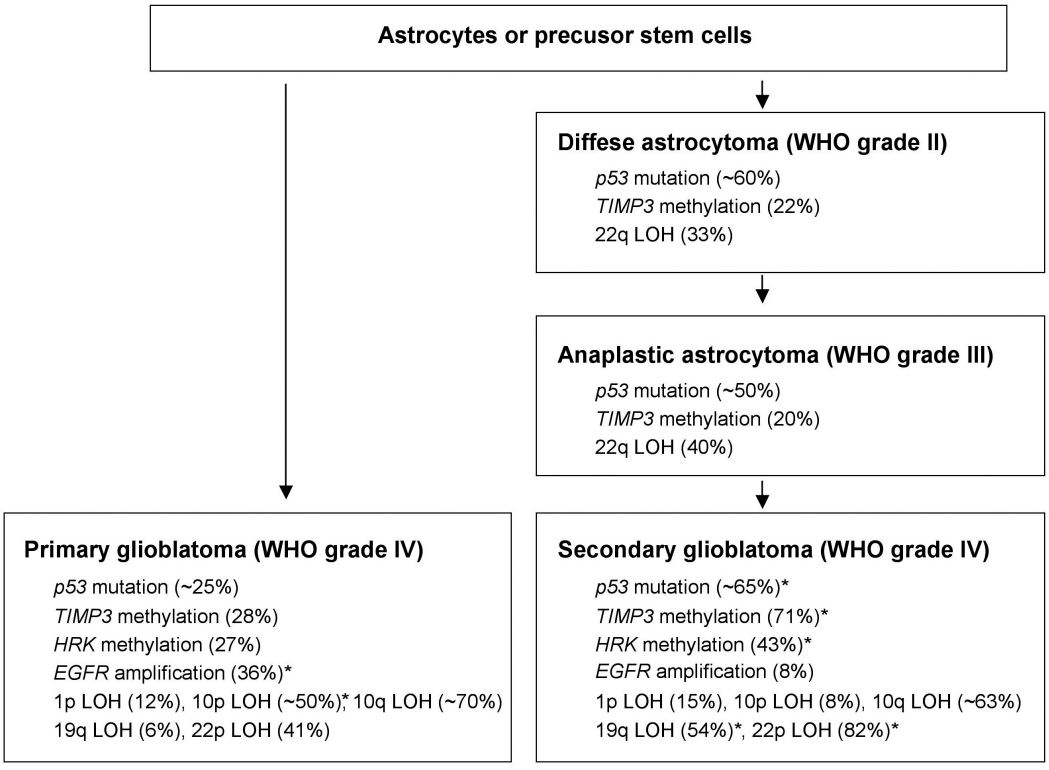

Fig. 1. Genetic pathways to primary and secondary glioblastomas. LOH 10q and 22q is frequent in both primary and secondary glioblastomas, but common deleted regions vary between the two subtypes. *p53* mutations, *TIMP3* methylation and LOH 22q are early and frequent genetic alterations in the pathway leading to secondary glioblastomas. *, Genetic alterations that are significantly different in frequency between primary and secondary glioblastomas. Modified from Ohgaki et al. (Ohgaki et al., 2004; Ohgaki and Kleihues, 2005).

Oligodendrogliomas have attracted much attention in recent years, because they are recognized as a particular subtype of gliomas that shows remarkable responses to procarbazine, CCNU, and vincristine (PCV) treatment, resulting in longer survival of patients (Mason et al., 1996; Streffer et al., 2000) and radiotherapy. In contrast, astrocytic tumours are highly resistant to chemotherapy (Forsyth and Roa, 1999). There has been a recent tendency to expand the histological criteria of oligodendrogliomas to include features such as nuclear regularity and roundness, often accompanied by a thin, eccentric rim of eosinophilic cytoplasm and apparent lack of cell processes (Coons et al., 1997; Daumas-Duport et al., 1997; Fortin et al., 1999). Coons et al. has argued that a significant fraction of tumours previously diagnosed as low-grade diffuse astrocytomas are oligodendrogliomas, representing 25–33% of all glial tumours (Coons et al., 1997; Donahue et al., 1997; Fortin et al., 1999). Such expansion of diagnostic criteria may be misleading with regard to chemosensitivity and

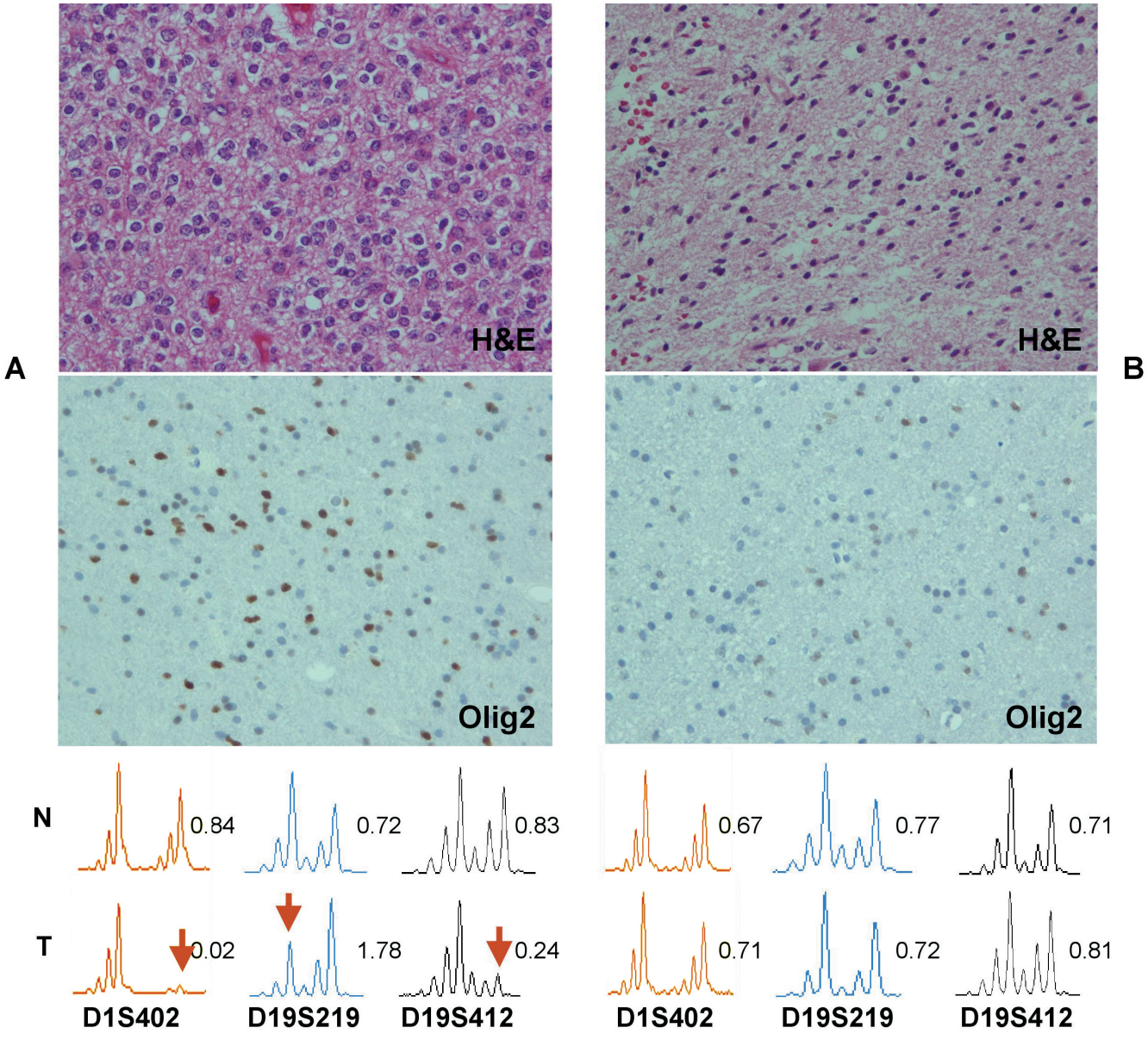

Fig. 2. H&E and Olig2 staining with GeneScan electropherogram of 1p and 19q markers for normal (N) and tumor (T) genotypes in oligodendrogliomas. **A.** Oligodendrogliomas with typical honeycomb appearance with Olig2-expression feature loss of 1p/19q. **B.** Equivocal oligodendrogliomas harbor p53 mutations with weak Olig2-expression, but not 1p/19q loss. Red arrows indicate LOH.

prognosis.

Tumor histology and molecular genetics

Many molecular and genetic events associated with oligodendroglial tumors have been identified over the past years (Reifenberger and Louis, 2003). Among the documented alterations, oligodendrogliomas are genetically characterized by LOH on chromosomes 1p and 19q in 40–90% of cases (Kraus et al., 1995; Maintz et al., 1997; Bigner et al., 1999; Smith et al., 1999, 2000; von Deimling et al., 2000; Reifenberger and Louis, 2003), whereas p53 mutations occur in 5–15% of this subtype (Watanabe et al., 2002; Reifenberger and Louis, 2003; Ohgaki and Kleihues, 2005). Low-grade diffuse astrocytomas are genetically characterized by *p53* mutations that occur in approximately two-thirds of cases (Ohgaki et al., 2004; Ohgaki and Kleihues, 2005).

We have analyzed the molecular genetic status of 14 oligodendrogliomas (Watanabe et al., 2002), the majority (13 of 14, 93%) with chicken-wire vascular pattern and typical perinuclear halo in >50% of tumour cells showing LOH on 1p and/or 19q. *p53* mutations were less frequent (21%) and typically found in lesions lacking chicken-wire vascular pattern and having occasional but not extensive perinuclear halo formation (Fig. 2). This suggests that oligodendrogliomas with classical histologic features are likely to be chemosensitive. Sasaki et al. detected 1p loss in 19 of 22 (86%) classical oligodendrogliomas, whereas 16/22 (73%) oligodendrogliomas with astrocytic components retained heterozygosity of 1p alleles (Sasaki et al., 2002). Ueki et al. analyzed 91 nonselected gliomas, and compared the results with histological diagnoses by four independent neuropathologists (Ueki, 2005). They also provided evidence that analyses of 1p/19q/10q/p53 have significant diagnostic value, especially for distinguishing oligodendroglial tumors. In addition, 1p LOH and *p53* mutations in gliomas may be markers of oligodendroglial and astrocytic pathways, respectively.

Oligoastrocytomas are defined as neoplasms with a conspicuous mixture of two distinct neoplastic cells resembling neoplastic oligodendrocytes and astrocytes (Kleihues et al., 2000). Approximately 30-50% of cases show LOH on 1p/19q, about 30% feature *p53* mutations and/or LOH on chromosome 17p (Maintz et al., 1997; von Deimling et al., 2000). Oligoastrocytomas with *p53* mutations and/or LOH on chromosome 17p do not show LOH on 1p/19q, and vice versa (Maintz et al., 1997). Oligoastrocytomas with LOH on 1p/19q typically have predominant features of oligodendroglioma, whereas those with *p53* mutations are more often astrocytoma-predominant (Maintz et al., 1997). A subset of tumours diagnosed as low grade diffuse astrocytomas contain small areas with oligodendroglial features, but this was not found to be predictive regarding the presence of either *p53* mutations or LOH on chromosomes 1p/19q (Watanabe et al., 2002). Thus, the presence of small oligodendroglial foci in low-grade diffuse astrocytomas does not necessarily reflect an oligodendroglial genotype. In four astrocytomas with limited oligodendroglial components, samples of DNA extracted separately from astrocytoma and oligodendroglioma areas both showed LOH on chromosomes 1p and 19q. The results suggest that frequent LOH on 1p/19q and infrequent *p53* mutations are genetic alterations in the evolution of oligodendrogliomas.

Mechanisms underlying chemosensitivity

LOH on chromosomes 1p and 19q is correlated with sensitivity to PCV chemotherapy with increased survival in anaplastic oligodendroglioma cases (WHO grade III) (Cairncross et al., 1998). Similarly, combined LOH on 1p and 19q has been identified as a predictor of favorable overall survival in oligodendrogliomas (Smith et al., 2000). However, it is still unclear whether these deletions simply represent surrogate markers of favorable biologic behavior of the neoplasm, or are predictive of improved survival after specific treatment interventions. Chromosome 19q is of particular interest since it contains several important genes involved in DNA repair, apoptosis, and cell survival pathways, although no specific alterations of these genes have been shown convincingly to be associated with gliomas.

Chemoresistance may result from saturation (Kleihues and Margison, 1976) or lack of expression of the DNA repair protein O6-methylguanine-DNA methyltransferase (MGMT), which removes alkyl groups from the O^6-position of guanine, a critical site of alkylation by monofunctional (procarbazine) and bifunctional cross-linking (BCNU, CCNU) nitrosoureas (Pegg, 2000). Loss of MGMT expression may be caused by the methylation of promoter CpG islands (Qian and Brent, 1997; Watts et al., 1997) observed in a variety of human cancers, including gliomas (Esteller et al., 1999; Nakamura et al., 2001a; Watanabe et al., 2002). *MGMT* is down-regulated in the majority of oligodendroglial tumors through promoter methylation, preferentially in tumors with 1p or 19q deletions (Watanabe et al., 2002; Mollemann et al., 2005). The data suggest that *MGMT* hypermethylation and low expression likely contribute to the sensitivity of oligodendroglial tumors to alkylating chemotherapy. Further investigations of larger series of oligodendrogliomas with data on treatment and clinical follow-up are clearly warranted.

Cell origin

Although the origin of oligodendrogliomas is still controversial, it is speculated that a high expression level of Olig2 might play an important role in the maintenance of neoplastic oligodendrocytes, which arise from either putative progenitor cells in the adult CNS or mature oligodendrocytes. Recent studies have demonstrated that common precursor cells exist which can differentiate into both neurons and oligodendroglia (Sanai et al., 2005), in line with histological observations of some

Table 1. Genetic alterations in reactive astrocyte and diffuse astrocytoma.

	p53 mutation	*p14^{ARF}* methylation	*O^6-MGMT* methylation	*TIMP-3* methylation	*HRK* methylation	19q LOH	*22q* LOH
Reactive astrocyte	0/7	0/7	0/7	0/7	0/7	0/7	0/7
Diffuse astrocytoma	54/90 (60%)	17/51 (33%)	26/54 (48%)	8/36 (22%)	7/36 (19%)	3/23 (13%)	12/36 (33%)

oligodendrogliomas with neurocytic differentiation (Perry et al., 2002).

The cancer stem cell theory proposes that cancer cells showing heteogeneous features originate through asymmetric division of cancer stem cells like stem cells in normal development. Recently, self-renewing, multipotent cells expressing the CD133 cell-surface marker were isolated from human gliomas and transplanted into adult mouse brains, where they recapitulated the parent tumor's histology (Galli et al., 2004; Singh et al., 2004; Yuan et al., 2004; Sanai et al., 2005). Genetic and histological heterogeneity within individual tumors may be due to secondary changes within subclones, but it is possible that the origin is a multipotent tumor cell. Mixed gliomagenesis is not likely to be due to independent transformation of two differentiated cells, but rather to transformation of a single, bipotential progenitor cell (Chekenya and Pilkington, 2002).

Oligodendrogliomas with 1p/19q loss, responding to PCV chemotherapy, appear to be mostly located in the frontal, parietal and occipital lobes, whereas tumors without 1p/19q loss are more likely to be found in the temporal lobe, insula and diencephalon (Zlatescu et al., 2001). This raises interesting questions about tumor initiation. Different types of oligodendrogliomas might originate from different precursor cells that are relatively region-specific in the brain or occur at different stages in development. The existence of at least two oligodendrocyte precursor cells has been reported, one of which is defined by the PDGFRα expression, whereas the other is characterized by the p1p/dm-20 expression and is independent from PDGF-AA for its survival and proliferation (Spassky et al., 2000). The event responsible for initiation (e.g. loss of 1p and 19q) may be tumorigenic in only certain cells or regions of the brain and may be dependent on the expression pattern of the cells (Jeuken et al., 2004).

Reactive astrocytes and diffuse astrocytoma

Benign reactive astrocytic lesions and low-grade diffuse astrocytomas are difficult to distinguish because of overlapping morphological features. Especially, examination of stereotactic biopsy specimens from patients with low-grade diffuse astrocytomas may result in inconclusive diagnoses because of small sample size and histopathologic heterogeneity (Gaudin et al., 1997). Attempts to differentiate between nonneoplastic and neoplastic proliferation of astrocytes have relied on a range of methods, such as the intra-operative cytologic wet smear techniques and additional immuno-histochemical parameters such as p53 and Ki-67 indices (Bigio et al., 1999). No expression of p53 is immunohistochemically detected in normal brain tissue, but some non-neoplastic astrocytic lesions are positive (Wessels et al., 2001). p53 immunopositivity in questionable lesions could raise suspicion of malignancy if a decision cannot be made based on physical, radiologic, and histologic examinations. However, because p53 immunoreactivity is only found in half to two thirds of astrocytomas (Wessels et al., 2001), lack of staining in questionable lesions is less helpful for exclusion of the possibility of malignancy. Ki-67 labeling indices are generally low in diffuse astrocytomas but there may be considrerable heterogeneity within tumors, so that the marker has low sensitivity for the prediction of a malignant clinical course.

Conventional cytogenetic procedures can also be of value in differential diagnosis. Wessels et al. showed that in some inconclusive cases in situ hybridization (ISH) technique allows differentiation between reactive gliosis and diffuse astrocytoma (Wessels et al., 2001).

We have analyzed genetic alterations as markers to discriminate reactive astrocytes from diffuse astrocytomas. None of the samples with reactive gliosis showed genetic and epigenetic aberrations, whereas overall 90% of the samples of evident low-grade astrocytomas were genetically aberrant for *p53*, *p14^{ARF}*, *O^6-MGMT*, 19q LOH and/or 22q LOH (Table 1). The absence of numerical genetic and epigenetic aberrations in the nonneoplastic reactive gliosis corroborates findings of other previous studies of gliotic brain tissue (Wessels et al., 2001). The findings indicate that the detectability of genotypic alterations extends well beyond the histomorphologic border of astrocytomas.

Conclusions

Histological diagnosis has retained benefit over the last several decades, and it is unlikely that molecular diagnosis can provide a complete replacement, particularly in cases with classical histological features. Among indistinct cases, however, objectivity is the obvious strength of molecular diagnosis. In gliomas, chemosensitivity and prognosis seem to be well predicted by molecular diagnosis, therefore, examination of 1p/19q and *p53* status should be performed to provide optimal treatment in cases with oligodendroglial

components. We propose that pathological diagnosis should be based on identification of criteria for combined clinical, histological, and genetic profiling.

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