Review

Genetic Pathways to Primary and Secondary Glioblastoma

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Glioblastoma is the most frequent and most malignant human brain tumor. The prognosis remains very poor, with most patients dying within 1 year after diagnosis. Primary and secondary glioblastoma constitute distinct disease subtypes, affecting patients of different age and developing through different genetic pathways. The majority of cases (>90%) are primary glioblastomas that develop rapidly de novo, without clinical or histological evidence of a less malignant precursor lesion. They affect mainly the elderly and are genetically characterized by loss of heterozygosity 10q (70% of cases), \textit{EGFR} amplification (36%), \textit{p16} \textit{INK4a} deletion (31%), and \textit{PTEN} mutations (25%). Secondary glioblastomas develop through progression from low-grade diffuse astrocytoma or anaplastic astrocytoma and manifest in younger patients. In the pathway to secondary glioblastoma, \textit{TP53} mutations are the most frequent and earliest detectable genetic alteration, already present in 60% of precursor low-grade astrocytomas. The mutation pattern is characterized by frequent \textit{G:C}→\textit{A:T} mutations at CpG sites. During progression to glioblastoma, additional mutations accumulate, including loss of heterozygosity 10q25-qter (~70%), which is the most frequent genetic alteration in both primary and secondary glioblastomas. Primary and secondary glioblastomas also differ significantly in their pattern of promoter methylation and in expression profiles at RNA and protein levels. This has significant implications, particularly for the development of novel, targeted therapies, as discussed in this review. (Am J Pathol 2007, 170:1445–1453; DOI: 10.2353/ajpath.2007.070011)

Epidemiology and Clinical Features

Glioblastoma (WHO grade IV) is the most frequent histological type of brain tumor, accounting for 69% of all incident cases of astrocytic and oligodendrogial tumors, with 3.55 new cases in Switzerland per 100,000 population per year, adjusted to the European Standard Population.\textsuperscript{5,6} The incidence rate of glioblastomas in the United States, adjusted to the United States Standard Population, is 2.96 new cases per 100,000 population per year (Central Brain Tumor Registry of the United States: Central Brain Tumor Registry of the United States (CBTRUS); \textit{http://www.cbtrus.org}). These incident cases do not include secondary glioblastomas that progressed from low-grade or anaplastic gliomas because only the first diagnosis is considered as an incident case. Primary glioblastomas present at diagnosis as full-blown tumors, without clinical, radiological, or histopatho-
logical evidence of a less-malignant precursor lesion. They are also termed de novo glioblastoma, but this does not suggest a single-step transformation; like other human neoplasms, they result from the acquisition of multiple genetic alterations. Secondary glioblastomas develop slowly through progression from low-grade diffuse astrocytoma (WHO grade II) or anaplastic astrocytoma (WHO grade III). The diagnosis of secondary glioblastoma requires clinical (neuroimaging) or histological (biopic) evidence of an evolution from a less malignant astrocytoma.

At a population level, we found that only 5% of all cases were secondary glioblastomas with histopathological evidence of a precursor low-grade or anaplastic astrocytoma.5,6 This is consistent with observations at the University of Alabama, in which 19 of 392 (5%) cases of glioblastomas had histologically proven prior low-grade gliomas.10 The incidence rate of low-grade and anaplastic gliomas (WHO grade III) was 6.3 months (Table 1).5,6 The time to progression from anaplastic glioma to glioblastoma was ~2 years, and that from low-grade glioma to glioblastoma was ~5 years (Table 1).6

There is a striking difference in the age distribution of patients with primary and secondary glioblastomas (Figure 1). The mean age of primary glioblastoma patients was 62 years, whereas secondary glioblastomas developed in younger patients (45 years).5,6 At a population level, primary glioblastomas developed more frequently in men (male to female ratio, 1.33), whereas secondary glioblastomas were more frequent in women (male to female ratio, 0.65).5 A review of several studies also showed a tendency toward a higher male to female ratio in primary than secondary glioblastomas.7,13-16 This corroborates a previous observation that glioblastomas with TP53 mutations (a genetic hallmark of secondary glioblastoma) are more common in women.17 This is surprising because in hospital-based18,19 and population-based studies11 (CBTRUS; http://www.cbtrus.org), the incidence of precursor low-grade or anaplastic gliomas was reported to be similar to or higher in males than in females. The possibility exists that in female patients gliomas progress more frequently or more rapidly to glioblastoma.

The median survival of secondary glioblastoma patients was 7.8 months, significantly longer than that of primary glioblastoma patients (4.7 months; \( P = 0.003 \)). However, this difference is considered largely due to the younger age of secondary glioblastoma patients, because younger age is a consistent and significant predictive factor of longer survival of glioblastoma patients.5,6 After age adjustment, multivariate analyses showed no significant difference in survival of patients with primary and secondary glioblastomas.5,6

Genetic Pathways to Primary and Secondary Glioblastoma

Genetic pathways to primary and secondary glioblastomas at a population level are summarized in Figure 2. LOH 10q is most frequent in both primary and secondary glioblastomas. Epidermal growth factor receptor (EGFR) amplification and PTEN mutations are genetic alterations typical of primary glioblastomas, whereas TP53 mutations are early and frequent genetic alterations in the pathway leading to secondary glioblastomas.

EGFR/PTEN/Akt/mTOR Pathway

The EGFR/PTEN/Akt/mTOR pathway is a key signaling pathway in the development of primary glioblastomas.20 Amplification of the EGFR occurs in ~40% of primary glioblastomas5,21 but rarely in secondary glioblastomas.5,15 EGFR overexpression is also more common in primary glioblastomas (>60%) than in secondary glioblastomas (<10%).15 All primary glioblastomas with EGFR amplification show EGFR overexpression, and 70 to 90% of those with EGFR overexpression have EGFR amplification.22,23 The age distribution of cases with EGFR amplification closely follows that of primary glioblastoma patients. In a large, population-based study, EGFR amplification was not detected in any glioblastoma from patients younger than 35 years of age (Figure 1).5 This confirms studies on pediatric glioblastomas in which EGFR amplification is either absent or very rare.24 EGFR amplicons are often mutated; variant 3 (EGFRvIII), with deletion of exons 2 to 7 is the most frequent type. It is associated with constitutive activation of the receptor and failure to attenuate signaling by receptor down-regulation, causes mitogenic effects, and has more powerful transforming activity.25 The constitutively active EGFRvIII can enhance cell proliferation in part by down-regulation of p27 through activation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway.26 This variant occurs only in glioblastomas with concurrent wild-type EGFR amplification,21 ie, primary glioblastomas (Table 1).

EGFR becomes activated through the binding of growth factors (epidermal growth factor, transforming growth factor-\(\alpha\)) to its extracellular domain, resulting in recruitment of PI3K to the cell membrane (Figure 3). PI3K phosphorylates phosphatidylinositol-4,5-bisphosphate to the respective 3-phosphate (PIP3), which activates downstream effector molecules such as AKT (protein kinase B) and mTOR, the mammalian target of rapamy-
cin; this results in cell proliferation and increased cell survival by blocking apoptosis. PTEN inhibits the PIP3 signal, thereby inhibiting cell proliferation (Figure 3). Response to EGFR kinase inhibitors requires coexpression of EGFRvIII and PTEN.

The PTEN (phosphatase and tensin homology) gene, located at 10q23.3, encodes a central domain with homology to the catalytic region of protein tyrosine phosphatases, which is important in the function of protein phosphatase and 3'-phosphoinositid phosphatase activities. The amino terminal domain of PTEN, with homology to tensin and auxilin, is important in regulating cell migration and invasion by directly dephosphorylating focal adhesion kinase.

### Table 1: Incidence, Age, Survival, and Genetic, Epigenetic Changes, and Expression Profiles in Primary and Secondary Glioblastomas

<table>
<thead>
<tr>
<th></th>
<th>Primary glioblastoma</th>
<th>Secondary glioblastoma</th>
<th>References</th>
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<tbody>
<tr>
<td>Incidence rate*</td>
<td>3.531</td>
<td>0.199</td>
<td></td>
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<tr>
<td>Incidence rate†</td>
<td>2.575</td>
<td>0.167</td>
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<tr>
<td>Mean age</td>
<td>62 years</td>
<td>45 years</td>
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<tr>
<td>M/F ratio</td>
<td>1.33</td>
<td>0.65</td>
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<tr>
<td>Clinical history</td>
<td>&lt;3 months: 68%</td>
<td>From grade II:</td>
<td></td>
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<tr>
<td></td>
<td>3 to 6 months: 16%</td>
<td>5.3 years</td>
<td></td>
</tr>
<tr>
<td></td>
<td>&gt;6 months: 16%</td>
<td>From grade III:</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(mean, 6.3 months)</td>
<td>1.4 years</td>
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<tr>
<td>Survival</td>
<td>Median, 4.7 months</td>
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<td>Genetic alterations</td>
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<tr>
<td>TP53 mutations</td>
<td>28%</td>
<td>65%</td>
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<td>DUOX2</td>
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<td>HRNPA3</td>
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<td>WNT-11 protein precursor</td>
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<td>Cachectin-related tumor suppressor homolog precursor</td>
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<tr>
<td>ADAMTS-19</td>
<td>Low</td>
<td>High</td>
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M/F, male/female; PDGF-AB, platelet-derived growth factor AB.

*Adjusted to the European Standard Population (per 100,000 persons per year).
†Adjusted to the World Standard Population (per 100,000 persons per year).
*Immunohistochemistry, ‡2-DGE, F enzyme-linked immunosorbent assay, ‡DNA array, ‡reverse transcriptase-polymerase chain reaction.
gene is mutated in 15 to 40% of glioblastomas and almost exclusively in primary glioblastomas (Table 1).\(^5\,^{22}\)

**TP53/MDM2/p14\(^{ARF}\) Pathway**

The TP53 pathway plays a crucial role in the development of secondary glioblastomas. TP53 mutations occur in patients of any age group, whereas EGFR amplification occurs in older patients. Note that there is no single case of glioblastoma with EGFR amplification in patients younger than 35 years of age. (Modified from Ohgaki et al.\(^5\)).

Amplification of MDM2 is present in <10% of glioblastomas,\(^{36}\,^{37}\) exclusively in primary glioblastomas that lack a TP53 mutation.\(^{36}\,^{37}\) Loss of p14\(^{ARF}\) expression has frequently been observed in glioblastomas (76%), and this typically correlates with homozygous deletion or promoter methylation of the p14\(^{ARF}\) gene.\(^{38}\) Promoter methylation of p14\(^{ARF}\) was more frequent in secondary than primary glioblastomas, but there was no significant difference in the overall frequency of p14\(^{ARF}\) alterations (homozygous deletion and promoter methylation) between glioblastoma subtypes.\(^{38}\) The analysis of multiple biopsies from the same patients revealed p14\(^{ARF}\) methylation already in one-third of precursor low-grade astrocytomas.\(^{38}\)

The TP53 gene at 17p13.1 encodes a 53-kd protein that plays a role in several cellular processes (Figure 3), including the cell cycle, response of cells to DNA damage, cell death, cell differentiation, and neovascularization.\(^{38}\) After DNA damage, TP53 is activated and induces transcription of genes such as\(^{40},^{41}\) p21\(^{Waf1/Cip1}\).\(^{40},^{41}\) The MDM2 binds to mutant and wild-type TP53 proteins, thereby inhibiting the ability of wild-type TP53 to activate transcription from minimal promoter sequences.\(^{42},^{43}\)

Conversely, transcription of the MDM2 gene is induced by wild-type TP53.\(^{44},^{45}\) In normal cells, this autoregulatory feedback loop regulates both the activity of the TP53 protein and the expression of MDM2.\(^{46}\) The p14\(^{ARF}\) gene product binds to MDM2 and inhibits MDM2-mediated p53 degradation and transactivation silencing.\(^{47},^{51}\) Conversely, p14\(^{ARF}\) expression is negatively regulated by TP53 and inversely correlates with TP53 function in human tumor cell lines.\(^{41}\) Thus, loss of normal TP53 function may result from altered expression of any of the TP53, MDM2, or p14\(^{ARF}\) genes (Figure 3).

**p16\(^{INK4a}/RB1 Pathway**

The p16\(^{INK4a}/RB1 pathway seems to be important in pathways to both primary and secondary glioblastomas. Homozygous p16\(^{INK4a}\) deletions were more frequent in primary than in secondary glioblastomas,\(^{38}\,^{52}\) but there was no significant difference in the overall frequency of p16\(^{INK4a}\) alterations (homozygous deletion and promoter methylation).\(^{38}\) Promoter methylation of the RB1 gene was significantly more frequent in secondary (43%) than in primary glioblastomas (14%).\(^{53}\) There was a significant correlation between loss of RB1 expression and promoter methylation of the RB1 gene in glioblastomas.\(^{53}\) RB1 promoter methylation was not detected in low-grade and anaplastic astrocytoma, indicating that it is a late event during astrocytoma progression.\(^{53}\)

RB1 protein controls progression through G\(_1\) to S phase of the cell cycle. The CDK4/cyclin D1 complex phosphorylates the RB1 protein, thereby inducing release of the E2F transcription factor that activates genes involved in the G\(_1\)→S transition.\(^{40}\) p16\(^{INK4a}\) binds to CDK4, inhibits the CDK4/cyclin D1 complex, and thus inhibits the G\(_1\)→S transition.\(^{40}\) Thus, loss of normal RB1 function may result from altered expression of any of the RB1, p16\(^{INK4a}\), or CDK4 genes (Figure 3).
Loss of Heterozygosity (LOH)

LOH 10q is the most frequent genetic alteration occurring in both primary and secondary glioblastomas at similar frequencies (60 to 80%), with a common deletion at 10q25-qter. In contrast, LOH 10p is largely exclusively present in primary glioblastomas, and complete loss of the entire chromosome 10 is typical for primary glioblastomas. Several LOH studies identified at least three commonly deleted loci, i.e., 10p14-p15, 10q23-24 (PTEN), and 10q25-pter, suggesting the presence of several tumor-suppressor genes that may play significant roles in the pathogenesis of glioblastomas. Because LOH 10q25-qter is associated with histologically recognized transition from low-grade or anaplastic astrocytoma to glioblastoma phenotypes and is commonly deleted in primary and secondary glioblastomas, the tumor suppressor gene(s) at these loci seem to be involved in the pathogenesis of both glioblastoma subtypes. This locus contains several putative tumor suppressor genes, including LGI1 at 10q24, BUB3 at 10q24-26, MXI1 at 10q25.1, h-neu at 10q25.1, abLIIM or LIMAB1 at 10q25.1, and DMBTI at 10q26.1 genes, but their role remains unclear because mutations of these genes have been rarely detected in glioblastomas.

LOH 22q is significantly more frequent in secondary glioblastomas (82%) than in primary glioblastomas (41%). Characterization of the 22q deletions in primary glioblastomas identified two minimally deleted regions at 22q12.3-13.2 and 22q13.31. The small (957 kb) deletion was also present in 22 of 23 secondary glioblastomas, a region in which the human tissue inhibitor of metalloproteinases-3 (TIMP-3) is located. TIMP-3 promoter methylation was observed with significantly higher frequency in secondary than in primary glioblastomas and correlated with loss of TIMP-3 expression. LOH 19q is more frequent in secondary glioblastomas (54%) than primary glioblastomas (6%), whereas LOH 1p and 13q occurred at a similar frequency in primary and secondary glioblastomas.

Co-Presence of Genetic Alterations

LOH 10q typically co-presents with any of the other genetic alterations in glioblastomas. In contrast, TP53 mutations, EGFR amplification, and PTEN mutations show inverse associations with each other, whereas there is a positive correlation between p16 deletion and EGFR amplification. These observations suggest that LOH 10q plus at least one or two other genetic alterations...
may contribute to the development of both primary and secondary glioblastomas. In the pathway to secondary glioblastoma, TP53 mutations occur early, and LOH 10q is a late event (Figure 2). However, the sequence of genetic alterations during the development of primary glioblastomas is unknown.

Correlation between Genetic Alterations and Histopathological Features

Glioblastoma is heterogeneous, with remarkable variability of histological features. Some of these tend to be associated with glioblastoma subtypes. The small cell phenotype is often associated with EGFR amplification and is thus a typical component of primary glioblastomas. Tumor necrosis, in particular large ischemic necroses, are significantly more frequent in primary glioblastomas, whereas secondary glioblastomas more frequently contain an oligodendroglioma component.

Promoter Methylation

Promoter methylation of p16, p14, RB1, TIMP-3, and MGMT genes has been comparatively assessed in primary and secondary glioblastomas. Overall, secondary glioblastomas showed a higher frequency of promoter methylation than primary glioblastomas (Table 1). O6-Methylguanine-DNA methyltransferase (MGMT) is a repair protein that specifically removes promutagenic alkyl groups from the O6 position of guanine in DNA. MGMT therefore protects cells against carcinogenesis induced by alkylating agents, and an inverse correlation has been reported between MGMT activity and tissue-specific tumorigenesis induced by alkylating agents in rats. Repair of O6-alkylguanine adducts by tumor cells has been implicated in drug resistance because it reduces the cytotoxicity of alkylating chemotherapeutic agents. Loss of MGMT expression caused by methylation of promoter CpG islands was detected in 75% of secondary glioblastomas, significantly more frequently than in primary glioblastomas (36%). The difference in frequency of MGMT methylation between primary and secondary glioblastomas is clinically relevant because patients with glioblastoma containing a methylated MGMT promoter were shown to have a substantially greater benefit from adjuvant temozolomide treatment.

A correlation between the presence of TP53 mutations and MGMT promoter methylation has been reported in lung cancer and between the presences of G→A mutations in the K-ras gene in colon carcinomas. Similarly, the majority of low-grade astrocytomas with MGMT methylation (92%) contained a TP53 mutation, whereas only 39% of cases without MGMT methylation carried a TP53 mutation. Furthermore, G:C→A:T transition mutations at CpG sites were significantly more frequent in low-grade astrocytomas with MGMT methylation (58%) than in those without (11%). These findings suggest the possibility that TP53 mutations at CpG sites in low-grade gliomas may result, at least in part, from exogenous or endogenous factors that produce DNA adducts at the O6 position of guanine. Various adducts at this position are substrates for repair by MGMT. Such adducts typically result from exposure to N-nitrosamides and related alkylating agents that cause brain tumors in rats, but there is currently no evidence indicating that alkylating carcinogens are involved in the etiology of human brain tumors.

Gene Expression and Proteomic Profiles

Different genetic alterations in primary and secondary glioblastomas reflect different expression patterns at the RNA and protein levels (Table 1). In a cDNA expression array analysis with 1176 cancer-related genes, low-grade astrocytomas showed rather specific and similar expression profiles, whereas primary glioblastomas showed larger variation, and secondary glioblastomas displayed features of both groups. Prominent genes expressed at a significantly higher level in primary than in secondary glioblastomas include vascular endothelial growth factor (VEGF) fms-related tyrosine kinase 1 (involved in angiogenesis) and IGFBP2. Because VEGF is induced by hypoxia-inducible factor (HIF-1), this difference explains the higher frequency and greater extent of necrosis in primary glioblastomas. Using an array containing 14,500 genes, Tso and colleagues showed that secondary glioblastomas primarily include mitotic cell cycle components, suggesting the loss of function in prominent cell cycle regulators, whereas primary glioblastomas preferentially express genes typical of a stromal response.
suggesting the importance of extracellular signaling. Immunohistochemical staining of glioblastoma tissue arrays confirmed expression differences.9

EGFR and MDM2 overexpression detected by immunohistochemistry are frequent and typical in primary glioblastomas.15,37 Immunohistochemistry revealed active matrix metalloproteinase-9 (MMP-9) in 69% of primary glioblastomas but only 14% of secondary glioblastomas.14 Active MMP-9 expression was strongly correlated with EGFR/VEGF expression,14 which is also typical for primary glioblastomas.21 Survivin (located on 17q25), which was initially identified as a gene with structural homology to a family of genes known as inhibitors of apoptosis,144 is expressed significantly more often in primary (83%) than in secondary glioblastomas (46%).13

Somasundaram and colleagues85 reported that ASCL1 is overexpressed in 86% of grade II diffuse astrocytomas and 88% of secondary glioblastomas, whereas the majority (67%) of primary glioblastomas expressed similar to or less than normal brain levels. ASCL1 up-regulation was accompanied by inhibition of Notch signaling as seen by uninduced levels of HES1, a transcriptional target of Notch1, and increased levels of HES6, a dominant-negative inhibitor of HES1-mediated repression of ASCL1.85 Increased levels of the Notch ligand Delta1 inhibits Notch signaling via formation of intracellular Notch ligand autonomous complexes, suggesting that inhibition of Notch signaling may be an important early event in the pathway to secondary glioblastomas.85

A recent proteomics approach identified additional proteins that are differentially expressed between glioblastoma subtypes (Table 1). Using two-dimensional protein gel electrophoresis (2-DGE) and protein sequencing, Furuta and colleagues8 identified distinct protein patterns in primary and secondary glioblastomas. Proteins unique for primary glioblastomas were tenascin-X precursor (6q21.3), enolase 1 (1pter-p36.13), centrosome-associated protein 350 (1p36.13-q41), and EGFR (7p12.3-p12.1), whereas those unique for secondary glioblastomas were ERCC6 (10q11), DUOX2 (15q15.3), HNRPA3 (10q11.1), WNT-11 protein precursor (11q13.5), cadherin-related tumor suppressor homolog precursor (chromosomal location unknown), and ADAMTS-19 (5q31).8 Quantification of the cytokines in the supernatant of 30 tissue-correspondent glioma cultures revealed a predominant expression of VEGF-A in primary glioblastomas and a significantly higher expression level of platelet-derived growth factor AB in secondary glioblastomas.25 This suggests that optimal anti-angiogenic therapy may require targeting of multiple angiogenic pathways that differ significantly between primary and secondary glioblastomas.16

Conclusions

Primary and secondary glioblastomas are distinct disease entities that affect different age groups of patients and develop through distinct genetic pathways with different mRNA and protein expression profiles. These differences are important, especially because they may affect sensitivity to radio- and chemotherapy and should thus be considered in the identification of targets for novel therapeutic approaches.

Future Directions

There are several chromosomal regions with LOH that probably contain tumor suppressor genes that need to be identified. The most common and extensive deletions involve chromosome 10q, distal of PTEN. It has been suggested that 10q25-pter harbors a putative tumor suppressor that may play a key role in the development of both primary and secondary glioblastomas. Glioblastoma is the first cancer type in The Cancer Genome Atlas project initiated by the United States National Cancer Institute and the National Human Genome Research Institute, which aims at establishing a database of high-resolution expression profiles, LOH, chromosome copy numbers, and sequence alterations in a total of 500 cases. If both primary and secondary glioblastomas were included, this would greatly increase our understanding of their molecular basis and facilitate the development of drugs that specifically target tumor subtypes with divergent genetic profiles.

Acknowledgments

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References


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