Recent advances in the molecular understanding of glioblastoma

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Abstract
Glioblastoma is the most common and most aggressive primary brain tumor. Glioblastomas are characterized by rapid invasion, profound angiogenesis and heterogeneity in histological appearance. Despite maximal treatment, patients only have a median survival time of 15 months due to the tumor’s resistance to therapeutic approaches. Thus far, methylation of the O6-methylguanine-DNA methyltransferase (MGMT) promoter has been the only confirmed molecular predictive factor in glioblastoma. However, novel ‘genome-wide’ techniques have identified additional important molecular alterations. This review summarizes genetic, epigenetic, transcriptional and proteomic studies of glioblastoma. It provides the clinician with an up-to-date overview of recent advances that should ultimately lead to new therapeutic targets and more individualized treatment approaches.

Introduction
Glioblastoma, or astrocytomas WHO grade IV, is the most fatal primary brain cancer found in humans. It is preferentially located in the cerebral hemispheres.1 The incidence of glioblastoma is about 3.5 per 100,000 people per year, with a slight male predominance. Glioblastoma may manifest at any age, but preferentially affects adults, with a peak of incidences between 45 and 75 years. Most glioblastomas are sporadic although they do occasionally occur in hereditary syndromes, such as neurofibromatosis types 1 and 2, Li-Fraumeni and Turcot’s syndromes.1 The only proven risk factor for gliomas is therapeutic irradiation of the head such as during treatment of head and neck tumors.3

Most glioblastoma manifest rapidly de novo, without recognizable precursor lesions. These primary glioblastomas present in elderly patients with a brief clinical history and are characterized by rapid progression and short survival time. A small group of young patients has a history of epilepsy caused by low-grade gliomas, which, within years, progress to secondary glioblastoma. A secondary glioblastoma occurs in ~5% of glioblastoma patients, and can only be diagnosed with clinical (neuroimaging) or histological evidence of its evolution from a less malignant glioma.4

In more than 50% of the cases, the clinical history of a patient presenting with a glioblastoma is less than three months. Symptoms are either general, such as headache, nausea/vomiting and papilledema as a result of raised intracranial pressure, or focal, such as hemiparesis, aphasia and visual deficits, depending on the tumor’s location. Up to one third of these patients experience epileptic seizures. The clinical diagnosis of brain tumors is preferentially made by magnetic resonance imaging (MRI) with gadolinium contrast. To obtain a histological diagnosis and to reduce the space-occupying effect, resection of most of the tumor (gross total removal) is the preferred initial intervention.

Complete removal is impossible due to the cancer cells’ widespread invasion into the surrounding brain. If the tumor is located in the proximity of an eloquent area, or if the patient’s condition does not allow extensive surgical intervention, a biopsy is taken to obtain a histological diagnosis. There is mounting evidence that there is a correlation between the
extent of resection and the prognosis.\textsuperscript{5} Surgical treatment is followed by radiotherapy, which is administered in 30 daily fractions of 2 Gy.\textsuperscript{6} Since 2005, Temozolomide (TMZ), an alkylation cytostatic drug, is given concomitantly with and adjuvant to radiotherapy. This combination, referred to as chemoradiation, is widely accepted as the standard treatment for newly diagnosed glioblastoma patients.\textsuperscript{7} Differences between patients and their performance status lead to variation in survival rates, which can be calculated for individual patients by means of nomograms.\textsuperscript{8} A better prognosis is associated with a younger age, better performance status, more extensive surgical resection and chemoradiation following resection.\textsuperscript{8} Although a rare group of long-term survivors (2-5%), generally characterized by young age and good condition, shows a survival time exceeding 3 years without recurrence,\textsuperscript{9,10} the median overall survival is only 15 months.\textsuperscript{7}

There is no standardized treatment for recurrent tumors and most patients selected for treatment at recurrence enter clinical trials. Trials for both primary and recurrent tumors frequently include small molecules or compounds that inhibit the oncogenic pathways activated in glioblastoma. Contrary to many other malignancies, however, there have only been small improvements in the glioblastoma patient’s prognosis over the last decades. Cancer stem cells (CSCs) have been identified in glioblastoma by the expression of stem cell markers such as CD133, as was done in other tumor types.\textsuperscript{11} The question, however, whether CSCs arise from developmentally stalled neural progenitor cells, or from dedifferentiated oligodendrocytes or astrocytes, is still unanswered.\textsuperscript{12}

Nevertheless, the understanding of molecular alterations in signaling pathways and the consequent pathology in glioblastoma has greatly increased and begins to parallel that of other types of cancer. Many of the alterations that have thus far been identified in glioblastoma are clustered in three pathways, the P53, RB and PI3K-AKT, downstream of the receptor tyrosine kinases (Figure 1).\textsuperscript{13,14} The present review provides an overview of the molecular alterations in glioblastoma. They are grouped according to various mechanisms that underlie the transformation to the neoplastic phenotype and are discussed in order of development in relation to gliomagenesis and glioma progression and in relation to clinical subclasses. Finally, the application of these new insights is discussed in the light of future prospects for experimental and clinical practice in neuro-oncology.

**Genomic and genetic variants**

Genomic instability is a hallmark of cancer, and is broadly differentiated into chromosome instability (CIN) and microsatellite instability (MIN or MSI). Cytogenetic studies of glioblastoma have shown that most tumors are near-diploid, and that numerical and structural chromosomal abnormalities are common.\textsuperscript{15} Non-inherited newly diagnosed glioblastomas rarely exhibit MSI due to inactivation of mismatch repair (MMR) genes.\textsuperscript{16,17} However, in recurrent glioblastomas following TMZ treatment, inactivating mutations have been observed in $\text{MSH6}$, one of the MMR genes. $\text{MSH6}$ mutations do not result in detectable MSI as manifested by changes in the length of microsatellite sequences, but in a hypermutator phenotype.\textsuperscript{13,14,18,19}
Different techniques have identified copy number alterations (CNAs), varying from small intragenic deletions to complete chromosomal changes in glioblastoma. Karyograms have revealed gross chromosomal changes, such as a gain of chromosome 7 and a loss of chromosome 10. These abnormalities have been confirmed by fluorescent in situ hybridization (FISH) analyses, using specific DNA probes to label chromosome centromeres and suspected DNA loci. Various technologies based on comparative genomic hybridization (CGH) have also been applied. CGH is based on the principle that test and reference DNA are labelled with distinct fluorescent probes and hybridized to reference DNA; subsequent differences in hybridization pinpoint CNAs. Hybridization to metaphase spreads showed a low resolution. For a higher sensitivity, genomic libraries have been hybridized to membranes or arrays (aCGH), thus identifying more discrete chromosomal changes. Last generation array-based CGHs have identified even amplified and deleted regions of a
few thousands base pairs. Nowadays, whole genome single nucleotide polymorphism (SNP)-based arrays are used. In this technique, DNA is hybridized to chips containing short oligonucleotide probes that contain SNPs. In this way, depending on the density of the oligonucleotides, even smaller CNAs can be detected. Furthermore, these arrays have the ability to detect signals from individual alleles and can reveal copy-number-neutral (CNN) loss of heterozygosity (LOH). For example, 17p, which contains TP53, is a significant region of CNN LOH.

**Amplifications**

Amplification of the epidermal growth factor receptor (EGFR) gene is a characteristic finding in primary glioblastomas (Table 1). Focal (restricted to a few Mb) and broader (from several Mbs to whole chromosomes) CNAs that include the EGFR gene may have different molecular consequences. Focal amplification of EGFR correlates with EGFR overexpression or mutations and deletions in the EGFR gene, and the subsequent activation of the PI3K-AKT pathway. In contrast, the amplification of chromosome 7, containing EGFR, MET and its ligand HGF, has been found to correlate with activation of the MET axis in vitro and is less frequently associated with EGFR mutations or deletions. The genes encoding the receptor tyrosine kinases KIT, KDR and PDGFRα, adjacentlly located on chromosome 4q12, are frequently found to be (co)amplified. Amplification of 12q13-15, where the oncogenes CDK4 and MDM2 are located, results in the disruption of both the RB and P53 pathways. Other amplified regions containing oncogenes, such as AKT3 and CCND2, are listed in Table 1.

**Deletions**

LOH of chromosome 10q is the most common genomic alteration found in both primary and secondary glioblastomas (Table 1) and is associated with poor survival. LOH 10q is considered to be a late event in secondary glioblastomas. Different regions are regularly lost at chromosome 10, including the regions containing PTEN and MGMT, but these loci probably contain other, thus far unidentified, tumor suppressor genes. Another frequent deletion is the loss of chromosome 9p, which contains various tumor suppressor genes, including CDKN2A and CDKN2B. These two genes encode three important cell cycle proteins, P14ARF and p16INK4A, and P15INK4B, respectively, which play a role in the RB and P53 pathways. Deletion of CDKN2A and CDKN2B at chromosome 9 is often accompanied by the deletion of CDKN2C at chromosome 1p32, which encodes another cell cycle protein p18INK4C. Deletion of any part of chromosome 1p is found in primary as well as secondary glioblastomas, generally in a heterozygous manner. Homozygous deletions at 1p36 have been found in 10% of glioblastomas. Thus far, no candidate tumor suppressor gene has been identified at 1p36. LOH 19q is frequently observed in secondary glioblastoma, and seems to correlate with improved survival rates. Occasionally, co-deletion of 1p and 19q is observed in glioblastomas, although this is not associated with prolonged survival or translocations as in oligodendrogliomas. Reports on incidental translocations are rare; consequently, translocations may not play an important role in the development of glioblastoma.
<table>
<thead>
<tr>
<th>Cytoband</th>
<th>Amp/Del</th>
<th>Freq</th>
<th>Gene</th>
<th>Gene name</th>
<th>Function of encoded protein</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>1p32</td>
<td>Del</td>
<td>3-16%</td>
<td>CDKN2C</td>
<td>Cyclin-dependent kinase inhibitor 2C</td>
<td>Regulator of cell cycle</td>
<td>13,27,30</td>
</tr>
<tr>
<td>1p36</td>
<td>Del</td>
<td>14-40%</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Unknown</td>
<td>28,29</td>
</tr>
<tr>
<td>1q32</td>
<td>Amp</td>
<td>4-15%</td>
<td>MDM4</td>
<td>Mdm4 p53 binding protein homolog</td>
<td>Apoptosis</td>
<td>13,22,27</td>
</tr>
<tr>
<td>1q44</td>
<td>Amp</td>
<td>2-11%</td>
<td>AKT3</td>
<td>V-akt murine thymoma viral oncogene homolog 3</td>
<td>Regulator of cell signaling involved in cell proliferation and survival</td>
<td>13,27,28</td>
</tr>
<tr>
<td>3q26</td>
<td>Amp</td>
<td>0-16%</td>
<td>PIK3CA</td>
<td>Phosphoinositide-3-kinase, catalytic, alpha polypeptide</td>
<td>Regulator of cell signaling involved in cell proliferation and survival</td>
<td>13,14,22,24</td>
</tr>
<tr>
<td>4q12</td>
<td>Amp</td>
<td>15%</td>
<td>KIT</td>
<td>V-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog</td>
<td>Regulator of cell signaling involved in cell proliferation and survival</td>
<td>26</td>
</tr>
<tr>
<td>4q12</td>
<td>Amp</td>
<td>15%</td>
<td>KDR</td>
<td>Kinase insert domain receptor</td>
<td>Angiogenesis, vasculogenesis and endothelial cell growth</td>
<td>26</td>
</tr>
<tr>
<td>4q12</td>
<td>Amp</td>
<td>5-18%</td>
<td>PDGFRA</td>
<td>Platelet-derived growth factor receptor, alpha polypeptide</td>
<td>Regulator of cell signaling involved in cell proliferation and survival</td>
<td>13,22,26,27</td>
</tr>
<tr>
<td>7p11</td>
<td>Amp</td>
<td>23-43%</td>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
<td>Regulator of cell signaling involved in cell proliferation and survival</td>
<td>21,13,22,27,65</td>
</tr>
<tr>
<td>7q21</td>
<td>Amp</td>
<td>1%</td>
<td>CDK6</td>
<td>Cyclin-dependent kinase 6</td>
<td>Regulator of cell cycle</td>
<td>13,24</td>
</tr>
<tr>
<td>7q31</td>
<td>Amp</td>
<td>3-19%</td>
<td>MET</td>
<td>Met proto-oncogene</td>
<td>Regulator of cell signaling involved in cell proliferation and survival</td>
<td>13,22,24,27</td>
</tr>
<tr>
<td>9p21</td>
<td>Del</td>
<td>26-50%</td>
<td>CDKN2A</td>
<td>Cyclin-dependent kinase inhibitor 2A</td>
<td>Regulator of cell cycle</td>
<td>2,13,14,27,62</td>
</tr>
<tr>
<td>9p21</td>
<td>Del</td>
<td>31-46%</td>
<td>CDKN2B</td>
<td>Cyclin-dependent kinase inhibitor 2B</td>
<td>Regulator of cell cycle</td>
<td>13,27</td>
</tr>
<tr>
<td>9p23</td>
<td>Del</td>
<td>14-46%</td>
<td>PTPRD</td>
<td>Protein tyrosine phosphatase, receptor type, D</td>
<td>Regulator of cell signaling involved in cell proliferation and survival</td>
<td>27,30</td>
</tr>
<tr>
<td>10q23-24</td>
<td>Del</td>
<td>5-70%</td>
<td>PTEN</td>
<td>Phosphatase and tensin homolog</td>
<td>Regulator of cell signaling involved in cell proliferation and survival</td>
<td>21,13,14,22,27,30</td>
</tr>
<tr>
<td>12p13</td>
<td>Amp</td>
<td>2-14%</td>
<td>CCND2</td>
<td>Cyclin D2</td>
<td>Regulator of cell cycle</td>
<td>13,22,24</td>
</tr>
<tr>
<td>12q14</td>
<td>Amp</td>
<td>7-24%</td>
<td>CDK4</td>
<td>Cyclin-dependent kinase 4</td>
<td>Regulator of cell cycle</td>
<td>13,14,22,27</td>
</tr>
<tr>
<td>12q14-15</td>
<td>Amp</td>
<td>7-22%</td>
<td>MDM2</td>
<td>Mdm2 p53 binding protein homolog</td>
<td>Apoptosis</td>
<td>13,22,27</td>
</tr>
<tr>
<td>13q14</td>
<td>Del</td>
<td>3-47%</td>
<td>RB1</td>
<td>Retinoblastoma 1</td>
<td>Regulator of cell cycle</td>
<td>13,14,22,29</td>
</tr>
<tr>
<td>17p13</td>
<td>Del</td>
<td>1-22%</td>
<td>TP53</td>
<td>Tumor protein p53</td>
<td>Apoptosis</td>
<td>13,14,22,29</td>
</tr>
<tr>
<td>17q11</td>
<td>Del</td>
<td>0-11%</td>
<td>NF1</td>
<td>Neurofibromin 1</td>
<td>Regulator of cell signaling involved in cell proliferation and survival</td>
<td>13,14</td>
</tr>
<tr>
<td>19q</td>
<td>Del</td>
<td>11-35%</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Unknown</td>
<td>22,27,29</td>
</tr>
<tr>
<td>22q12.3</td>
<td>Del</td>
<td>53%</td>
<td>TIMP3</td>
<td>TIMP metalloproteinase inhibitor 3</td>
<td>Extracellular matrix</td>
<td>76</td>
</tr>
</tbody>
</table>

Table 1. Frequently identified copy number alterations in glioblastoma

A deletion can indicate either a CNN-LOH, an LOH or a homozygous deletion; Unknown indicates that the gene of interest has not been identified yet. Abbreviations: Amp, amplification; CNN, copy number neutral; Del, deletion; Freq, frequency; LOH, loss of heterozygosity; Ref, reference.
Somatic mutations
Mutation analysis has identified mutations activating oncogenes as well as those inactivating tumor suppressor genes in glioblastoma. Thus far, the golden standard is direct or Sanger sequencing after amplification of the suspected locus by means of polymerase chain reaction (PCR). Next generation sequencing technologies are being developed and may be rapidly applied to facilitate genome-wide mutation analysis.

The most striking example of a mutation in glioblastoma is the **EGFRvIII** mutant. EGFRvIII lacks 267 amino acids in the extracellular part, causing a constitutively activated receptor that no longer requires its ligand EGF to signal downstream. Recently, additional point mutations have been identified in the EGFR extracellular domain. Two recent extensive mutational studies have provided an overview of the most common mutations affecting glioblastoma (Table 2). ERRB2 is a family member of EGFR and its gene contains mutations in the extracellular domain like EGFR. Mutations in **PIK3CA**, coding for the PI3K catalytic subunit p110α, have already been described. However, recently, mutations have also been found in **PIK3R1** – coding for P85α – the PI3K regulatory subunit. Mutations in **BRAF** and **RAS** genes have rarely been observed in gliomas, probably because inactivating mutations and deletions have frequently been found in the inhibitory tumor suppressor gene **NF1**. Mutations in **IDH1**, which encodes isocitrate dehydrogenase 1, have recently been found in 12% of glioblastomas. Mutations frequently affect young patients with a secondary glioblastoma and a better prognosis. The role of IDH1 in gliomagenesis may be related to its NADPH production, which is necessary to tackle oxidative stress that induces DNA damage.

### Table 2. Genes frequently found to contain point mutations in glioblastoma
<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene name</th>
<th>Function of encoded protein</th>
<th>Freq</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
<td>Regulator of cell signaling involved in cell proliferation and survival</td>
<td>14-15%</td>
<td>11,14,39</td>
</tr>
<tr>
<td>ERBB2</td>
<td>V-erb-b2 erythroblastic leukemia viral oncogene homolog 2</td>
<td>Regulator of cell signaling involved in cell proliferation and survival</td>
<td>0-7%</td>
<td>11,14,18</td>
</tr>
<tr>
<td>IDH1</td>
<td>Isocitrate dehydrogenase 1 (NADP+)</td>
<td>NADPH production</td>
<td>12-20%</td>
<td>14,41,44</td>
</tr>
<tr>
<td>NF1</td>
<td>Neurofibromin 1</td>
<td>Regulator of cell signaling involved in cell proliferation and survival</td>
<td>15-17%</td>
<td>12,14</td>
</tr>
<tr>
<td>PIK3CA</td>
<td>Phosphoinositide-3-kinase catalytic, alpha polypeptide</td>
<td>Regulator of cell signaling involved in cell proliferation and survival</td>
<td>7-10%</td>
<td>13,14</td>
</tr>
<tr>
<td>PIK3R1</td>
<td>Phosphoinositide-3-kinase regulatory subunit 1 (alpha)</td>
<td>Regulator of cell signaling involved in cell proliferation and survival</td>
<td>7-8%</td>
<td>13,14</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homolog</td>
<td>Regulator of cell signaling involved in cell proliferation and survival</td>
<td>24-37%</td>
<td>2,13,14</td>
</tr>
<tr>
<td>PTPRD</td>
<td>Protein tyrosine phosphatase receptor type, D</td>
<td>Regulator of cell signaling involved in cell proliferation and survival</td>
<td>0-6%</td>
<td>14,30</td>
</tr>
<tr>
<td>RB1</td>
<td>Retinoblastoma 1</td>
<td>Regulator of cell cycle</td>
<td>8-13%</td>
<td>13,14</td>
</tr>
<tr>
<td>TP53</td>
<td>Tumor protein p53</td>
<td>Apoptosis</td>
<td>31-38%</td>
<td>2,12,14</td>
</tr>
</tbody>
</table>

Abbreviations: Freq, frequency; Ref, reference.
Figure 2. Molecular pathways from cell of origin to primary and secondary glioblastoma
Mutations causing cancer are thought to occur in a sequential order, implicating different stages of gliomagenesis (Figure 2). For example, PTEN mutations are thought to be important in glioma progression, but not in initiation. In contrast, IDH1 inactivating mutations seem to be an early event in gliomagenesis. The mutation rate in glioblastoma is lower than in other solid tumors, with the exception of the hypermutation phenotype, which, as described above, is found in recurrent glioblastomas after treatment with alkylating agents. This is probably caused by mutational inactivation of DNA repair enzymes, such as MSH6.

Polymorphisms
Family members of glioma patients are more susceptible to glioma and other cancer types, suggesting a genetic link. The most common type of genetic variation is formed by single nucleotide polymorphisms (SNPs). A SNP is a single base pair alteration at a specific locus. By hybridizing complementary DNA probes to the SNP site, several applications have been developed to cross-examine SNPs; currently, SNP arrays containing hundreds of thousands of probes on a small chip are commonly used. As described above, these arrays can be used to detect CNAs as well. SNPs have been linked to susceptibility to glioblastomas. In particular, allergy’s and asthma’s inverse associations with glioblastoma have been observed in different studies and have been linked with polymorphisms in HLA and interleukins. This may suggest that immune factors play a role in gliomagenesis. SNP 309 in MDM2 has been associated with an increased risk of various types of cancer, but has not been associated as a risk or prognostic factor in respect of glioblastoma in large studies. Various SNPs have been correlated with survival in glioblastoma, however, these studies’ findings have not been confirmed.

Gene expression profiling
Overexpression or underexpression of genes in glioblastoma compared to that in a normal brain or to low-grade gliomas may be an indication of genes that play a role in gliomagenesis (Table 3). Most of the 20,000-25,000 genes encoded by the human genome are known, and these have been spotted on chips used for micro-arrays. Differences in expression of ‘unknown’ genes can be studied by means of the serial analysis of gene expression (SAGE), using small expression tags. Large-scale expression studies are generally validated by reverse transcription (RT)-PCR for individual genes.

Low-grade astrocytomas have rather specific and consistent expression profiles, whereas primary glioblastomas exhibit much larger variation between tumors. Furthermore, secondary glioblastomas show distinct expression profiles and display features of the other two types. Expression profiling of different types and grades of gliomas has been found to outperform histopathologic grading for prognosis. Distinct molecular subclasses in high-grade glioma (HGG) have been identified, delineating a pattern of disease progression that resembles stages in neurogenesis. The molecular subclasses have been given descriptive names such as proneural (PN), proliferative (Pro) and mesenchymal (Mes). A meta-analysis
confirmed the classification of glioblastoma samples into these subgroups and added a fourth category, ProMes, which includes the overexpression of genes characteristic of the Pro and Mes subgroups. Recurrent tumors are more frequently of the Mes subclass. Patients with a PN expression pattern are mostly younger than 40 years of age and have the best prognosis. This may explain why younger glioblastoma patients have a better survival rate. Expression of mesenchymal markers, such as CHI3L1 and co-expression of double cortex, semaphorin 3B and SPARC have been associated with a poor prognosis (Table 3). The response to concomitant chemoradiation has been associated with a stem-cell-related “self-renewal” signature dominated by HOX genes, including CD133.

Several studies have combined DNA copy number analysis with gene expression changes. This approach has shown a strong association: 76% of the genes within recurrent CNAs show expression patterns that correlate with the copy number. Glioblastomas in the Mes subclass are associated with a gain of chromosome 7 and a loss of chromosome 10.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene name</th>
<th>Function of encoded protein</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD44</td>
<td>CD44 molecule</td>
<td>Cell-cell interactions, cell adhesion and migration</td>
<td>56,57,63</td>
</tr>
<tr>
<td>DLL3</td>
<td>Delta-like 3</td>
<td>Notch signaling</td>
<td>57,63</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
<td>Regulator of cell signaling involved in cell proliferation and survival</td>
<td>54,63</td>
</tr>
<tr>
<td>FABP7</td>
<td>Fatty acid binding protein 7</td>
<td>Fatty acid uptake, transport, and metabolism</td>
<td>63</td>
</tr>
<tr>
<td>IGFBP2</td>
<td>Insulin-like growth factor binding protein 2</td>
<td>Regulation of cell growth</td>
<td>53,56,50,61,63</td>
</tr>
<tr>
<td>MMP9</td>
<td>Matrix metallopeptidase 9</td>
<td>Extracellular matrix</td>
<td>54,63</td>
</tr>
<tr>
<td>SPARC</td>
<td>Secreted protein, acidic cysteine-rich (osteonectin)</td>
<td>Extracellular matrix</td>
<td>61,63</td>
</tr>
<tr>
<td>TNC</td>
<td>Tenascin C</td>
<td>Cell adhesion</td>
<td>56,60,63</td>
</tr>
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<td>VEGFA</td>
<td>Vascular endothelial growth factor A</td>
<td>Angiogenesis, vasculogenesis and endothelial cell growth</td>
<td>53,57,63</td>
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<td>CHI3L1</td>
<td>Chitinase 3-like 1 (YKL-40)</td>
<td>Extracellular matrix</td>
<td>54,56,57,60,63</td>
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<td>VIM</td>
<td>Vimentin</td>
<td>Cytoskeletal element</td>
<td>56,57</td>
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<td>MiR-21</td>
<td>MicroRNA 21</td>
<td>Invasion</td>
<td>80,81</td>
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<td>MiR-221</td>
<td>MicroRNA 221</td>
<td>Regulator of cell cycle</td>
<td>80</td>
</tr>
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</table>

Table 3. Genes frequently found to be overexpressed in glioblastoma
Genes frequently found to be overexpressed in glioblastoma compared to either normal brain tissue or low-grade gliomas. Abbreviation: Ref, reference.
Epigenetics
The epigenetic silencing of tumor suppressor genes is a common phenomenon of genomic instability in cancer. Epigenetics are inherited characteristics of gene expression, not related to nucleotide sequences. Examples are: promoter hypermethylation, histone deacetylation, histone methylation, and other histone modifications which can alter chromatin structure (in)directly and, furthermore, RNA silencing mechanisms such as RNA interference and microRNA regulation of gene expression. In contrast to the global DNA hypomethylation found in glioblastoma and other tumors, tumor suppressor genes are commonly found to be hypermethylated and, hence, silenced. DNA methylation, histone deacetylation and microRNA are best studied in glioblastoma and are discussed next.

Methylation and histone deacetylation
The predictive factor of the response to TMZ treatment of glioblastomas is the methylation status of the MGMT promoter. TMZ alkylates the O6-position in guanines, which then prohibits cell replication. However, the DNA repair enzyme MGMT is able to remove alkyl groups, thus introducing resistance to TMZ treatment. When the promoter of MGMT is methylated, MGMT is not transcribed and therefore cannot repair DNA damage caused by TMZ, making TMZ more efficient. The best assessment of the MGMT status has been debated; the gold standard is methylation-specific PCR (MS-PCR). MGMT methylation has been observed in 40-57% of glioblastomas; however, specific subgroups have a higher frequency. MGMT methylation has been found to be significantly more frequent in secondary glioblastomas, in females and in long-term survivors (LTS). Conflicting results have been reported regarding the methylation status of MGMT as a prognostic marker.

A large-scale analysis of epigenetically regulated candidate genes has been hampered by the lack of a high throughput approach for analyzing epigenetic patterns. Epigenetically suppressed genes can become re-activated, using histone deacetylase (HDAC) or DNA methyltransferase inhibitors, such as trichostatin A and 5-aza-2'-deoxycytidine. Differences in expression can subsequently be identified by a comparative, genome-wide expression analysis. These approaches have been used to identify epigenetically silenced candidate tumor suppressor genes in different malignant glioma cell lines. Differences in various genes’ promoter methylation have been found between primary and secondary glioblastomas (Table 4), long- and short-term glioblastoma survivors, and primary and recurrent tumors. Previously undetected caspase 8 hypermethylation has been detected in glioblastoma relapses and is associated with a prolonged time to tumor progression.

MicroRNAs
MicroRNAs (miRNAs or miRs) are short non-coding RNAs, consisting of approximately 22 nucleotides, which regulate gene expression. MiRs usually inhibit target genes’ expression, either by inhibiting translation or by triggering the cleavage of the target mRNA. Over 500 miRs have been described in humans. Using the same methods as previously described in respect of gene expression, differences in miR expression between tumors and normal tissue have also been examined. Of various differentially expressed miRs, miR-221 and
the miR-21 have been found to be overexpressed in glioblastomas when compared to normal brain. MiR-21 has been described as promoting glioma invasion. MiR-221 is thought to target cell cyclin-dependent kinase inhibitors p27 and p57. Both MiR-21 and MiR-221 are considered as oncomiRs. MiR-7 and miR-128 are frequently down-regulated miRNAs in glioblastomas. MiR-7 independently inhibits both the EGFR and the AKT pathways, and is a potential tumor suppressor miR in glioblastomas. The delivery of miR-7 may be an appealing approach for therapy, whereas oncogenic miRNAs may be targeted by antagonirs. MiR-128 targets the oncogene BMI1, which has been shown to promote stem cell renewal.

Proteomics

Thus far, only a limited number of proteomic studies have been performed. In general, samples are run on 2D gels, which show protein patterns based on size and charge. Proteins identified in tumor samples but not in normal tissue samples are subsequently analyzed by mass spectrometry and matrix-assisted laser desorption/ionization (MALDI). Thus far, glioma subtypes have been distinguished on the basis of different protein patterns as primary and secondary glioblastomas. Furthermore, based on proteome analysis, survival has been predicted in respect of glioma subtypes. Additionally, proteins’ phosphorylation status is a tool to identify activated proteins. Consequently activated receptor tyrosine kinases and the downstream signaling pathways of EGFRVIII have been identified in glioblastomas.

Clinical consequences of molecular alterations: subgroups of glioblastomas, prognostic and predictive factors

Molecular alterations of long-term survivors

Since various genetic alterations have been linked to prognosis, efforts have been made to identify differences between long-term survivors (LTS) and short-term survivors (STS). On average, tumors from LTS exhibit fewer genetic alterations than STS tumors. In general, 9p loss, 10q loss, a gain of chromosome 7 or amplification of EGFR were less frequently observed in LTS. In contrast, loss of 19q may be a marker of longer survival. Also, LTS have a higher MGMT and TMS1/ASC methylation frequency than STS. Molecular differences between primary and secondary glioblastomas

Primary and secondary glioblastoma subtypes are histopathologically indistinguishable, but differences can be demonstrated by molecular markers at the epigenetic, genetic, expression and proteomic level (Figure 2 and Table 4). Primary glioblastomas have a higher rate of EGFR alterations, MDM2 duplications, PTEN mutations and homozygous deletions of CDKN2A, encoding P14ARF and P16INK4A. MET amplification, overexpression of PDGFRA and mutations in IDH1 and TP53 are more prevalent in secondary glioblastomas. Differences in expression profiles between primary and secondary glioblastomas may be reflected by the presence of CD133+ cells in primary glioblastomas, which have not thus far been detected in secondary glioblastomas. Several genes associated with embryonic and neural stem cells have been found to be overexpressed in primary glioblastomas.
### Table 4. Molecular differences between primary and secondary glioblastoma

Unknown indicates that the gene of interest has not been identified yet. Abbreviations: Amp, amplification; Del, deletion; Meth, methylation; Mut, mutation; Prim, primary; Ref, reference; Sec, secondary.

<table>
<thead>
<tr>
<th>Event</th>
<th>Gene</th>
<th>Gene name</th>
<th>Function of encoded protein</th>
<th>Frequency</th>
<th>Overall</th>
<th>Prim</th>
<th>Sec</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amp</td>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
<td>Regulator of cell signaling involved in cell proliferation and survival</td>
<td>34%</td>
<td>36%</td>
<td>8%</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Del</td>
<td>CDKN2A- P14ARF</td>
<td>Cyclin-dependent kinase inhibitor 2A</td>
<td>Regulator of cell cycle</td>
<td>44%</td>
<td>44%</td>
<td>44%</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CDKN2A- P16INK4A</td>
<td>Cyclin-dependent kinase inhibitor 2A</td>
<td>Regulator of cell cycle</td>
<td>26-31%</td>
<td>31-32%</td>
<td>13-19%</td>
<td>2, 31</td>
<td></td>
</tr>
<tr>
<td>LOH</td>
<td>10q incl PTEN)</td>
<td>Phosphatase and tensin homolog</td>
<td>Regulator of cell signaling involved in cell proliferation and survival</td>
<td>69%</td>
<td>70%</td>
<td>63%</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td></td>
<td>13q incl RB1</td>
<td>Retinoblastoma 1</td>
<td>Regulator of cell cycle</td>
<td>23%</td>
<td>12%</td>
<td>38%</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td></td>
<td>22q incl TIMP3</td>
<td>TIMP metalloproteinase inhibitor 3</td>
<td>Involved in degradation of the extracellular matrix</td>
<td>53%</td>
<td>41%</td>
<td>82%</td>
<td>76</td>
<td></td>
</tr>
<tr>
<td></td>
<td>19q</td>
<td>Unknown</td>
<td>Unknown</td>
<td>27%</td>
<td>54%</td>
<td>6%</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>Meth</td>
<td>MGMT</td>
<td>O6-methylguanine-DNA methyltransferase</td>
<td>DNA repair</td>
<td>44%</td>
<td>43%</td>
<td>73%</td>
<td>67</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CDKN2A- P14ARF</td>
<td>Cyclin-dependent kinase inhibitor 2A</td>
<td>Regulator of cell cycle</td>
<td>14%</td>
<td>6%</td>
<td>31%</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CDKN2A- P16INK4A</td>
<td>Cyclin-dependent kinase inhibitor 2A</td>
<td>Regulator of cell cycle</td>
<td>8%</td>
<td>3%</td>
<td>19%</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NDRG2</td>
<td>N-myc downstream-regulated gene 2</td>
<td>May have a role in neurite outgrowth</td>
<td>46%</td>
<td>62%</td>
<td>0%</td>
<td>67</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PTEN</td>
<td>Phosphatase and tensin homolog</td>
<td>Regulator of cell signaling involved in cell proliferation and survival</td>
<td>32%</td>
<td>9%</td>
<td>82%</td>
<td>75</td>
<td></td>
</tr>
<tr>
<td></td>
<td>RB1</td>
<td>Retinoblastoma 1</td>
<td>Regulator of cell cycle</td>
<td>25%</td>
<td>14%</td>
<td>43%</td>
<td>74</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TIMP3</td>
<td>TIMP metalloproteinase inhibitor 3</td>
<td>Involved in degradation of the extracellular matrix</td>
<td>41%</td>
<td>28%</td>
<td>71%</td>
<td>76</td>
<td></td>
</tr>
<tr>
<td>Mut</td>
<td>IDH1</td>
<td>Isocitrate dehydrogenase 1 (NADP+)</td>
<td>NADPH production</td>
<td>12-20%</td>
<td>7-12%</td>
<td>73-88%</td>
<td>14, 41, 44</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PTEN</td>
<td>Phosphatase and tensin homolog</td>
<td>Regulator of cell signaling involved in cell proliferation and survival</td>
<td>24-37%</td>
<td>25-40%</td>
<td>4%</td>
<td>2, 35</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TP53</td>
<td>Tumor protein p53</td>
<td>Apoptosis</td>
<td>31-38%</td>
<td>28-29%</td>
<td>65%</td>
<td>2, 35</td>
<td></td>
</tr>
</tbody>
</table>
Molecular prognostic and predictive factors
A molecular alteration that has been consistently associated with poor prognosis is LOH 10q, including PTEN. In contrast, the recently discovered IDH1 mutations have been found to correlate with a prolonged survival. The frequently found EGFR alterations and mutations in TP53 mutations and PTEN are not prognostic factors. Other alterations have been found as prognostic markers in single studies, but could not be repeated, including the methylation status of MGMT and deletion of CDKN2A. The expression of the mesenchymal marker CHI3L1 expression signatures showing upregulated AKT and NOTCH signaling, and increased levels of phosphorylated (and, hence, activated) MAPK and PI3K-AKT proteins have been associated with a poor prognosis. In glioblastomas, increased CHI3L1 expression is also associated with increased resistance to radiotherapy and shorter time to progression. Furthermore, higher expression of CHI3L1 and of the protease MMP-9 in the serum of glioblastoma patients has been shown to correlate with poor survival. Although the exact function of the secreted protein CHI3L1 is unclear, it seems to be involved in ECM remodelling and may facilitate invasion, migration and/or angiogenesis and may be a therapeutic target.

At this moment, the only confirmed predictive factor of response to chemoradiation for glioblastoma is the methylation status of the MGMT promoter. MGMT methylation is also associated with pseudo-progression after concomitant radiochemotherapy for newly-diagnosed glioblastoma patients. Furthermore, the pattern of recurrence, including time to recurrence and localization of the recurrent tumor, seems to be independently correlated with the MGMT methylation status of the primary tumor. EGFR alterations may be predictive of response to therapy. The clinical response to EGFR inhibitors was found to be associated with co-expression of EGFRvIII and PTEN in recurrent glioblastomas, but not in combination with chemoradiation for newly diagnosed glioblastomas or in glioblastomas treated with erlotinib and TMZ.

Potential therapeutic targets and future perspectives
Looking at all the molecular alterations found in glioblastoma, it is clear that the picture of the changes in the glioma cancer cell becomes more complex as the techniques that enable us to investigate molecular mechanisms develop. The good news is, however, that many of the alterations identified in glioblastoma cluster in three pathways, the P53 (64-87%), RB (68-78%) and the PI3K-AKT (50%), downstream of the receptor tyrosine kinases (altered 88% in total; Figure 1). Most alterations occur in a mutual exclusive fashion: alterations within one tumor affect only a single gene in a pathway, suggesting that different genes in a pathway are functionally equivalent. Functional validation of the identified molecular changes is necessary before they can be assessed as targets for therapy. Taking this into account, it becomes clear that good models are needed to test rationally designed combinations of targeted drugs in a high-throughput fashion. At this point, genetic and epigenetic alterations’ findings are of the utmost importance. For example, several experiments have shown that established glioblastoma cell lines resemble glioblastomas in patients very poorly when compared at the level of DNA alterations or gene expression
However, solutions may be found with the application of tumor neurospheres cultured in stem cell medium, organotypic spheroid cultures, or low-passage monolayer cultures. These resemble original tumors better\textsuperscript{104,105} and do contain the alterations that we find in patients.

The development of new treatment modalities is the most important application of the insights presented in this paper. Rational drug design and rationally designed clinical trials to test these drugs are needed as infinite numbers of compounds, such as small molecules and blocking antibodies, are currently available, which can be tested in limitless numbers of combinations. Rather than single agent therapy, which has shown good responses in other types of cancer but that will probably not suffice in the treatment of glioblastoma, combination treatment is necessary. PTEN deficient glioblastoma patients could, for example, be treated with a cocktail of drugs consisting of an EGFR inhibitor and rapamycin.\textsuperscript{25,107} The chemoradiation response of patients who do not show $MGMT$ methylation may be improved by the addition of $MGMT$ depleting agents, which are currently under investigation.\textsuperscript{108} A putative disadvantage of combination treatment is the potential increase in side effects.\textsuperscript{109} This may in part be solved by application of new drug delivery techniques. In this field, advances have been made with the application of biodegradable wafers, convection-enhanced delivery and strategically-designed liposomes to circumvent the blood brain barrier.\textsuperscript{110-112}

At this moment, we have increased the understanding of the molecular mechanisms underlying subgroups of glioblastoma patients, such as primary and secondary glioblastomas. Moreover, many of the alterations in the aforementioned pathways have been elucidated and the classification into PN, Pro, Mes or ProMes expression profiles predicts prognosis. However, in contrast to many other forms of cancer, the treatment consequences are lagging behind. Nevertheless, the assessment of the molecular profiles of responding versus non-responding patients can, for example, be used to determine predictive factors and may lead to the identification of new therapeutic targets. Subsequently the validation of such new therapeutic approaches will be followed by stratified clinical trials based on such molecular subgroups. Finally, the present insights will lead to more individualized therapies for glioblastoma patients. The combination of the present knowledge of molecular alterations in glioblastoma and the availability of many targeted drugs makes investigations into new treatments more promising than ever before.

**Search strategy and selection criteria**

Original English language articles focussing on adult humans were considered for inclusion. Other articles were identified from the reference lists of relevant papers, from our own literature archives and recent reviews. In different studies, both genomic changes and gene expression profiles have revealed that established glioma cell lines are poor representatives of primary human gliomas; consequently, we have chosen to only include studies on original glioblastoma samples in our analyses, unless otherwise specified. To enhance the reproducibility of our results, we furthermore only included studies on gene copy number alterations and gene expression profiling if at least 20 glioblastoma samples had been assessed, unless otherwise indicated.

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References

Chapter 11

Chapter 11


