Worse Outcome in Primary Glioblastoma Multiforme With Concurrent Epidermal Growth Factor Receptor and p53 Alteration

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Abstract

Primary glioblastoma multiforme (GBM), in contrast with secondary GBM, has been associated with the presence of EGFR amplification and absence of p53 mutation. In this study, we analyzed relevant molecular and clinical variables in 194 primary GBMs and tested them for survival analysis. Although most of the tumors showed a mutually exclusive pattern, concurrent alterations of EGFR and p53 were detected. Survival analysis of CDK4 amplification revealed a highly significant association with a worse clinical outcome (P = .01), whereas MDM2, CDK6, PTEN, and p21 were not associated with patient survival. Multivariate analysis including the significant clinical and molecular variables revealed CDK4 amplification, age, and radiotherapy to be markers with independent prognostic value. In addition, the primary GBM tumors showing simultaneous EGFR and p53 alterations were significantly associated with worse survival (P < .01). These results highlight the prognostic value of CDK4 amplification and of simultaneous EGFR-p53 alterations in the clinical outcome of patients with primary GBM.

Glioblastoma multiforme (GBM) is the most frequent primary brain tumor in adults. GBMs are defined by the histopathologic features of cellular atypia, mitotic figures, necrotic foci with peripheral cellular pseudopalisading, and microvascular hyperplasia, which distinguish them from lower-grade astrocytic tumors.1 Scheré7 first classified GBMs into primary and secondary tumors in 1940. Primary GBMs occur de novo without a recognizable precursor lesion. The clinical history of the disease is usually short, with a median survival of approximately 1 year following diagnosis. In contrast, secondary GBMs develop slowly and arise from preexisting, lower-grade astrocytomas.2-4 Although these GBM subtypes are histologically indistinguishable, molecular genetic analyses suggest that there are at least 2 distinct pathways contributing to the tumorigenesis of GBM. Primary GBMs frequently show amplification and/or overexpression of the epidermal growth factor receptor (EGFR), PTEN mutations, and partial or complete loss of chromosome 10. Otherwise, secondary GBMs generally feature mutations of the p53 gene, overexpression of platelet-derived growth factor receptors, and loss of heterozygosity at 17p, 19q, and 10q. In addition, they rarely overexpress EGFR.5-8 Therefore, EGFR amplification and/or overexpression and p53 mutation are 2 genetic events that seem to molecularly differentiate these clinical subtypes of GBM. Nevertheless, while the association of these 2 hallmarks has been considered to be almost mutually exclusive, fluorescence in situ hybridization (FISH) and reverse transcription–polymerase chain reaction expression studies have shown coexistence of p53 mutation and EGFR amplification in subsets of primary GBM tumors.9-11
knowledge, the prognostic significance of these concurrent alterations has not been studied.

On the other hand, frequent alterations of genes coding for proteins involved in G1-S cell-cycle transition control have been reported in primary and secondary GBMs. This is the case for the human homologue of the mouse double minute 2 (MDM2), cyclin-dependent kinases 4 and 6 (CDK4 and CDK6), and the inhibitor of cyclin-dependent kinases p21. While coamplification of MDM2 and CDK4 has been observed in gliomas, MDM2 amplification and/or overexpression is more frequent in primary than in secondary GBMs. Conversely, CDK4 amplification is considered a late event during astrocytoma progression and is frequently found in secondary GBMs.17,18

This study was undertaken to explore the prognostic relevance of EGFR and p53 and other growth-control molecules such as MDM2, CDK4, CDK6, PTEN, and p21 in a large series of primary GBMs.

Materials and Methods

Cases
The study involved 194 GBM samples obtained between 1999 and 2008 from 3 hospitals in Spain: Virgen de la Salud (Toledo), Clinic (Barcelona), and Xeral-Cies (Vigo). All cases were reviewed by 3 of us (T.R., C.F., and M.M.) to verify tumor viability and to confirm the diagnosis according to the 2007 World Health Organization diagnostic criteria.17

The selected patients did not have a history of brain tumor, so the clinical and genetic profiles of the cases are characteristic of a primary (de novo) GBM subtype. The patients received conventional therapy consisting of surgical resection of as much of the tumor as clinically and technically possible, followed by radiotherapy and/or chemotherapy. The median age of the patients at the time of diagnosis was 59.5 years (range, 16-81 years), the median overall survival was 10 months (range, 1-72 months), and the median postsurgical Karnofsky performance score (P-KPS) was 80 (range, 50-100). Clinical information is summarized in Table 1. The investigation was performed following the approval of the institutional review board.

FISH and Immunohistochemical Analysis on Tissue Microarrays
Tissue microarrays (TMAs) were constructed using formalin-fixed, paraffin-embedded archival tissue blocks as reported elsewhere.15 We included 5 nontumoral control samples (tissue from 4 normal brains and 1 tonsil), H&E-stained full sections from each donor block were used for morphologic selection of the representative areas of each case. FISH and immunohistochemical analyses were performed on 194 GBMs arrayed on TMAs, although the number of tissue cores (samples) valid for assessment varied on each specific analysis.

FISH assays on TMAs were performed using EGFR, MDM2, and CDK4 gene-specific bacterial artificial chromosome clones (RP11-339F13 for EGFR, RP11-611O2 and RP11-450G15 for MDM2, and RP11-571M6 for CDK4). In addition, control clones for each of the genes tested were used: p2750a7a (centromere of chromosome 7) and RP11-467M14/RP11-467M14 (12p13). BAC clones were selected from the EnsEMBL (http://www.ensembl.org) and UCSC (http://www.genome.ucsc.edu) databases. The specificity and location of the probes were confirmed by FISH on normal metaphases before hybridization on the TMA. Gene and control probes were labeled in red and green, respectively, and hybridized as previously described.15

Fluorescence signals were scored by two of us (Y.R. and B.M.) in accordance with the criteria described in previous reports.19,20 For each sample, only well-defined nuclei were analyzed, and the numbers of single-copy gene and control probe signals were scored. Gene amplification was considered positive when 5 or more unbalanced gene copies were detected in more than 5% of tumor cells.

Immunohistochemical assays were performed on deparaffinized TMA sections as reported elsewhere.15 The primary antibodies used were EGFR (Oncogene Research Products, Boston, MA), p53 (Novocastra Laboratories, Newcastle upon Tyne, England), CDK6 (BD Biosciences Pharmingen, San Diego, CA), PTEN (DAKO Denmark A/S, Glostrup, Denmark), and p21 (Oncogene Research Products).

### Table 1

<table>
<thead>
<tr>
<th>Summary of Clinical, Immunohistochemical, and Molecular Data for 194 Patients With Glioblastoma Multiforme*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical data</td>
</tr>
<tr>
<td>Median (range) age (y)</td>
</tr>
<tr>
<td>No. of females/males</td>
</tr>
<tr>
<td>Median (range) overall survival (mo)</td>
</tr>
<tr>
<td>Median (range) postsurgical Karnofsky performance score</td>
</tr>
<tr>
<td>No. alive/dead</td>
</tr>
<tr>
<td>No. of partial/radical surgical resections</td>
</tr>
<tr>
<td>Immunohistochemical and molecular data</td>
</tr>
<tr>
<td>EGFR alteration (FISH and IHC)</td>
</tr>
<tr>
<td>p53 expression (IHC)</td>
</tr>
<tr>
<td>MDM2 amplification (FISH)</td>
</tr>
<tr>
<td>CDK4 amplification (FISH)</td>
</tr>
<tr>
<td>CDK6 expression (IHC)</td>
</tr>
<tr>
<td>p21 nonexpression (IHC)</td>
</tr>
<tr>
<td>PTEN nonexpression (IHC)</td>
</tr>
</tbody>
</table>

* Data are given as number/total evaluable cases (percentage) unless otherwise indicated.

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p53 Mutational Analysis

Genomic DNA was extracted from paraffin-embedded microdissected sections as previously described. Mutations in exons 5 to 8 of the \( p53 \) gene were screened by direct sequencing in an ABI PRISM 310 DNA Analyzer (Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions. Primers and polymerase chain reaction conditions were the same as reported previously.

Statistical Analysis

Univariate and multivariate Cox regression analyses were performed to identify the clinical and molecular variables related to survival. Hazard ratios (HRs) and their associated 95% confidence intervals were computed for selected variables using standard options for the Cox regression analysis. Kaplan-Meier survival curves and Pearson correlation coefficients were also calculated. All analyses were done using SPSS, version 11.0 (SPSS, Chicago, IL).

Results

Molecular Findings

EGFR gene amplification was detected in 75 (51.0%) of 147 GBMs, and strong EGFR overexpression was detected in 120 (65.9%) of 182 GBMs. There was a significant correlation between the FISH and immunophenotypic results for EGFR (\( \rho = 0.526; P < .01 \)). Therefore, taking into account any of these techniques, EGFR was altered in 127 (76.0%) of 167 GBMs. Strong and extensive nuclear immunostaining for p53 protein was found in 21 (11.2%) of 187 GBMs (Table 1). Concurrent alteration of EGFR and p53 was found in 12 of 21 p53-immunopositive samples. A subsequent mutational analysis confirmed \( p53 \) mutation in 8 of the tumors available for analysis (Table 2).

To characterize the possible abnormalities of cell cycle–related molecules involved in the EGFR and p53 pathways, we performed molecular analyses of MDM2, CDK4, CDK6,
Ruano et al / EGFR and p53 Alteration in Primary GBM

Table 2
p53 Mutations in Glioblastoma Multiforme Samples With Concurrent EGFR-p53 Alteration

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Exon</th>
<th>Mutation</th>
<th>Codon</th>
<th>Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>39</td>
<td>8</td>
<td>GAG to AAG</td>
<td>271</td>
<td>Glu to Lys</td>
</tr>
<tr>
<td>110</td>
<td>5</td>
<td>GTG to GAG</td>
<td>143</td>
<td>Val to Glu</td>
</tr>
<tr>
<td>91</td>
<td>5</td>
<td>CGC to CAG</td>
<td>175</td>
<td>Arg to His</td>
</tr>
<tr>
<td>111</td>
<td>8</td>
<td>CCT to CTT</td>
<td>278</td>
<td>Pro to Leu</td>
</tr>
<tr>
<td>114</td>
<td>7</td>
<td>CGG to CAG</td>
<td>248</td>
<td>Arg to Gln</td>
</tr>
<tr>
<td>115</td>
<td>7</td>
<td>TAC to AAC</td>
<td>236</td>
<td>Tyr to Asn</td>
</tr>
<tr>
<td>11</td>
<td>8</td>
<td>CGT to TGT</td>
<td>273</td>
<td>Arg to Cys</td>
</tr>
<tr>
<td>117</td>
<td>7</td>
<td>TGT to TAT</td>
<td>238</td>
<td>Cys to Tyr</td>
</tr>
</tbody>
</table>

EGFR, epidermal growth factor receptor.

Table 3
Univariate Cox Regression Analyses in 194 Primary Glioblastoma Multiforme Cases

<table>
<thead>
<tr>
<th>Variable</th>
<th>Hazard Ratio</th>
<th>95% Confidence Interval</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical data</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>2.4</td>
<td>1.48-3.86</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>Postsurgical Karnofsky</td>
<td>0.25</td>
<td>0.12-0.53</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>performance score</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extent of resection</td>
<td>0.53</td>
<td>0.36-0.78</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>Radiotherapy</td>
<td>6.91</td>
<td>3.74-12.77</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>2.39</td>
<td>1.41-4.03</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>Molecular data</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CDK4 amplification</td>
<td>2.67</td>
<td>1.17-6.08</td>
<td>&lt;.05</td>
</tr>
</tbody>
</table>

p21, and PTEN in our series of primary GBM. We found overexpression and/or amplification of MDM2 and CDK4 in 20 (11.4%) of 175 tumors and 23 (19.8%) of 116 tumors, respectively. CDK6-immunopositive expression was found in 56 (51.4%) of 109 primary GBMs, and there was absence of expression of p21 and PTEN in 34 (37.0%) of 92 tumors and 68 (38.6%) of 176 tumors, respectively (Table 1).

Survival Studies

Univariate Cox regression analyses of clinical variables in these series showed that age, P-KPS, extent of surgical resection, radiotherapy, and chemotherapy were significantly associated with the clinical outcome of the GBM patients (Table 3). Subsequent univariate Cox regression analyses of the molecular variables revealed CDK4 amplification to be a significant predictor of clinical outcome, whereas none of the other molecular variables tested were independently associated with patient survival (Table 3). The Kaplan-Meier survival curves indicated that CDK4 amplification was significantly associated with the worse overall survival of patients with GBM (P =.01; log-rank test) (Figure 1).

In addition, multivariate analysis involving the significant clinical and molecular variables associated with survival probability revealed that age (P <.05; HR, 2.89; 95% confidence interval [CI], 1.01-8.23), radiotherapy (P <.05; HR, 6.93; 95% CI, 1.59-30.20), and CDK4 amplification (P <.05; HR, 3.15; 95% CI, 1.04-9.56) continued to be independent prognostic factors.

To analyze the influence of concomitant p53 and EGFR alterations studied by FISH and/or immunohistochemical analysis on outcome, we used Kaplan-Meier survival curves. The analysis of the immunophenotypic pattern of p53 in the EGFR– and EGFR+ GBM subgroups revealed that p53 expression was significantly associated with poorer survival in the EGFR+ subgroup (amplified and/or overexpressed; P <.01; log-rank test). Likewise, the analysis of EGFR in the p53-immunopositive and p53-immunonegative subgroups revealed that EGFR alteration (amplified and/or overexpressed) was significantly associated with a worse clinical outcome in the p53-immunopositive subgroup (P <.01; log-rank test) (Figure 1).

Discussion

GBM can be classified as a primary or secondary tumor on the basis of clinical features. This distinction is supported by the finding of mutually exclusive EGFR and p53 alterations. However, coexistence of p53 mutation and EGFR amplification in subsets of primary GBM tumors has been described.\(^9\)\(^{11}\) Moreover, Thomas and coworkers\(^23\) reported that the glioma cell line SKMG-3 had an unusual genotype.
in which a p53 gene mutation coexisted with an amplified EGFR gene. In the present study, we detected EGFR alteration in most of the tumors of this series (76.0%), and p53 nuclear immunostaining was found in 11.2% of the tumors. In addition, concurrent EGFR and p53 alterations were found in approximately half of the p53-immunopositive tumors. Therefore, our results are in agreement with the mutually exclusive pattern of EGFR and p53 in primary GBMs, but also support the existence of a minority subgroup with concurrence of EGFR and p53 aberrations.10,11

The series of 194 primary GBMs analyzed in this study was tested and validated by performing univariate Cox regression analysis using clinical data, such as age, KPS, or extent of surgical resection, that were previously associated with better outcome.24-27 Survival analysis of molecular data revealed CDK4 amplification to be associated with worse survival in GBM. It is important to note that multivariate analysis including significant variables pointed out CDK4 amplification as an independent marker with prognostic value. An aberrant function of CDK4 would be involved in the abnormalities of the p16/RB/CDK4 pathway, which have been previously correlated with astrocytic differentiation and the poorer survival of patients with glioma.28,29

The prognostic significance of EGFR and p53 alteration in astrocytomas has always been a cause of controversy. Several reports have concluded that EGFR amplification and/or overexpression predicts shorter survival,30-33 whereas others have found no statistically significant relationship.34,35 As with EGFR, no consensus has been reached about the prognostic value of p53. Some groups claim no association with survival,32,34,35 whereas others propose that p53 alteration is a negative or positive prognostic factor.30,36-38 In our study, EGFR and p53 were not factors independently associated with survival. However, we detected for the first time that the GBM subgroup showing simultaneous alteration of

![Figure 1](image1.png) Kaplan-Meier survival curve comparing positive and negative gene amplification for CDK4 with survival in patients with glioblastoma multiforme. The median overall survival times were 5.5 and 10 months for cases with positive and negative CDK4-amplified samples, respectively. P = .01; log-rank.

![Figure 2](image2.png) Survival analysis in glioblastoma multiforme. A, Kaplan-Meier analysis comparing positive and negative protein expression of p53 with survival in epidermal growth factor receptor (EGFR)+ samples (amplified and/or overexpressed). P < .01; log-rank. B, Kaplan-Meier survival curve comparing EGFR status, determined by using fluorescence in situ hybridization and/or immunohistochemical analysis, with survival in p53-immunopositive samples. P < .01; log-rank.
EGFR and p53 pathways was characterized by a worse clinical outcome. Thus, our findings may support the hypothesis that mutant p53 induces genomic instability in this fraction of tumors and, consequently, EGFR gene amplification. The simultaneous deregulation of the EGFR and p53 pathways may indicate a relevant cell cycle deregulation that leads to more aggressive GBM formation.

In contrast with our results, Simmons and coworkers reported that younger patients with GBM (<55 years, median series age), with EGFR overexpression and p53 immunonegativity had a statistically significant worse survival. This contradictory result may be due to the higher percentage of p53 immunopositivity in the younger patients (57% vs 16%) or in the overall series (49% vs 11.2%), when both data sets are compared. In this sense, our series showed high EGFR alteration (76.0%) and low p53 expression (11.2%), which are suggestive of a primary GBM pattern.

CDK4 amplification is associated with a significantly worse survival in patients with GBM, and it is a molecular variable showing independent prognostic value. Likewise, although EGFR and p53 are mutually exclusive, there is a primary GBM subgroup exhibiting concurrent EGFR and p53 alterations that is associated with a poorer clinical outcome. These findings may help in the search for useful prognostic and therapeutic markers. However, additional studies will almost certainly highlight further the genetic complexity and importance of the EGFR-p53 positive subgroup in the biology of primary GBMs.

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