Revisiting the Role of p53 in Primary and Secondary Glioblastomas

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Abstract. Glioblastoma multiforme (GBM) develops from astrocytes and is the most aggressive primary cancer in humans. Invading cells grow rapidly and form their own blood vessels making them difficult to surgically remove or treat. GBM may develop de novo (primary) or through progression from a low-grade or anaplastic astrocytoma (secondary). Mutational inactivation of the p53 gene and presence of aberrant p53 expression are reported in GBM, suggesting that p53 has a role in tumor progression. This study of seven de novo GBM and four secondary GBM patients, indicated that nine out of eleven (82%) had overexpression of p53. Our histopathological analysis showed that the expression of p53 in three out of four (75%) secondary GBM was confined to the nucleus and the p53 positive cells were randomly distributed throughout the tumor. The expression of p53 in four out of seven (57%) de novo GBM was cytoplasmic, diffusive, and confined to the perivascular region of the tumor. In two (29%) de novo samples both nuclear as well as cytoplasmic staining that was not confined to the perivascular area was observed. The results suggest that cytoplasmic p53 may contribute to the formation and maintenance of de novo GBM by virtue of its control of the vasculature of tumors. Furthermore, cytoplasmic p53 may reflect an association of p53 with Cullin 7, PARC, or with the sequestering partner of p53, mortalin. These results underscore the significance of p53 in the tumorigenesis of de novo GBM.

Glioblastoma multiforme (GBM) remains one of the most lethal forms of cancer, with a median survival of 10 to 12 months (1). It is a primary brain tumor comprising of 12-15% of all intracranial neoplasms and 50-60% of astrocytic tumors. Reported incidence in North America are 2-3 cases/100,000 people each year. Unlike most other types of cancer, GBM rarely metastasize, instead, it induces death through invasion (2) into normal brain tissues and by striking resistance to current therapies. Histological gliomas are graded based on the presence of specific histological markers including necrosis, nuclear pleomorphism, mitotic activity, and vascular proliferation (3). Several clinical markers, such as patient age and Karnofsky performance status, often serve as prognostic indicators of the disease. Two genetic pathways have been described in GBM development: primary and secondary (4, 5). Primary (de novo) GBMs grow rapidly without evidence of a preexisting low grade tumor and represent the most frequent presentation. Patients tend to be older (mean age 62 years) and have short clinical history (6). On the other hand, secondary GBMs develop more slowly by progression from a low grade (WHO grade II), or anaplastic astrocytoma (7), along with a longer clinical course in younger patients (mean age 45 years) (6). Although both types of GBM have a lethal course, primary GBM is marked by a more rapid progression to death. Common treatment options include gross total resection and radiation therapy, which have been shown to improve survival, whereas chemotherapy has limited benefits. Novel therapies, which act on specific molecular targets are currently under development for many cancers, including GBM (8). Temozolomide, a promising new drug now used in the treatment of GBM, has shown to improve survival benefit in patients, when used in conjunction with radiotherapy (9).

Distinct genetic alterations associated with primary and secondary GBMs have been described in previous studies (4-7). These alterations include EGFR amplification, mutations of EGFR, p53, and PTEN genes, p16INK4A, and loss of heterozygosity (LOH) on chromosome 9p, 10p, 10q and 17p. Of these, LOH of chromosome 10 is the most commonly encountered genetic aberration, demonstrated in GBM (69%) (7). Attempts have been made to identify specific cell signaling pathways associated with GBM (10-13). These studies have yet to identify the genes that

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uniquely distinguish primary from secondary GBM. A proteomic analysis of primary and secondary GBM demonstrated that 11 proteins are uniquely expressed in either primary or secondary GBM, suggesting distinct origins (14). In spite of the potential genetic variations, no major difference in patient survival has been noted between de novo and secondary GBMs, when controlled for age (6). In fact, there have been no widely validated prognostic genetic markers for GBM patients as of yet.

p53 gene alterations are one of the distinct features seen in primary, as well as in secondary GBM (6). It has been concluded that p53 mutations are involved in disease progression, especially from low grade astrocytomas or anaplastic astrocytomas (AA) (15). A study of a single GBM tumor, which had heterogenous regions of low grade (well-differentiated) and high grade areas (poorly-differentiated) showed a distinct genetic profile, such as mutation of p53 gene or allelic loss of 17p13.1 (locus of p53 gene), which is a frequent event in GBM (16). Furthermore, germ line mutations of the p53 gene, a hallmark of Li- Fraumeni Syndrome in which patients are predisposed to multiple neoplasms including brain tumors, suggest that loss of wild-type (wt) p53 mutations can initiate malignant transformation (17). The p53 gene encodes a 393-amino acid protein that possesses five conserved regions. The p53 protein is a transcription factor that plays a vital role in regulating cell growth, DNA repair, and apoptosis, in response to stressful conditions (18). p53 is a short-lived protein under complex regulation, including reversible cycles of post-translational modification, such as phosphorylation, acetylation, and ubiquitination. Under physiological conditions, p53 is degraded in an ubiquitin-dependent manner by binding to Mdm2 protein (19), a major ubiquitin ligase for p53.

Abnormalities of p53 have been reported in de novo GBM; 28% of these tumors were found to possess a p53 mutation while 65% of secondary GBM show p53 mutations (6). The incidence of p53 protein accumulation is more frequently seen than p53 mutations are (20-22). p53 mutations are also higher in secondary (>90%) than in primary (<35%) GBM (21). The percentage of glioma cells, in which p53 protein accumulation is found to increase from first biopsy to recurrent tumors (21), may suggest clonal expansion of glioma cells with p53 gene mutations (23). Loss of the p53 gene in GBM is either due to mutations in exons 5-8 or homozygous/heterozygous deletion of chromosome 17 (20). p53 is a phosphoprotein that resides in the nucleus and is involved in DNA repair. In consequence, loss of this protein leads to genomic instability. p53 expression in GBM may accumulate in the cytoplasm, suggesting that cytoplasmic p53 may have a role in de novo GBM. Although there are various opinions of the role of p53 in this location, some authors believe that it is inactive (20), while others speculate about its involvement in progression and maintenance (24) or that the accumulated p53 is actually wt p53 (25).

The main objectives of this study are to evaluate the discrete expression of p53 in primary and secondary GBM and to further investigate its role in gliomagenesis.

Patients and Methods

Patient history. Eleven patients (six males and five females) with diagnosed GBM (WHO grade IV astrocytoma) underwent tumor resection at Westchester Medical Center (Valhalla, NY, USA) from 2003 to 2006. Patient ages ranged from 27 to 70 years with a mean age of 51. Of the eleven tumors, seven were classified as de novo (primary) and four were progressive (secondary). Primary GBM were classified as those, which had a short clinical history in association with histological features of GBM at first biopsy. Patients with secondary GBM were categorized based on the history of prolonged clinical course, along with other histopathological evidence of progression from a lower grade astrocytoma (6). One sample from each patient was taken from pathological discard or tumor specimen, when available for appropriate analysis. All tumor specimens were catalogued following the HIPAA guidelines.

Paraffin-embedded tumor specimens were sectioned at 5 μm thickness. Sections were stained with H&E, reviewed, and graded by a neuropathologist following the standard criteria.

Immunohistochemistry (IHC). The standard IHC technique was utilized to determine the expression of p53 in tumor samples. In brief, slides with tumors were baked at 60°C for 30 min. Specimens were deparaffinized in xylene and rehydrated in graded concentrations of ethanol. Antigen retrieval was done by using citrate buffer and incubating the slides at the highest temperature, in a pressure cooker for 10 min. Tumor sections were then incubated at room temperature for 30 min with the anti-p53 antibody, DO-7(dilution 1:40; Cell Marque, Hot Springs, AZ, USA), which detects both wt and mutant p53 protein.

Detection of p53 was done using a universal detection kit (Ventana, Tucson, AZ, USA). Bound antibody was detected by 3,3-diaminobenzidine tetrahydrochloride (DAB) and counterstained with hematoxylin. Each slide was run with a positive control, which was obtained from tumors that often display mutant p53, such as those of the colon or breast. Negative controls were acquired by incubating tumor sections without the primary antibody.

Evaluation of p53 staining. Tumor sections were examined for p53 immunoreactivity under the microscope at 20x and 40x magnifications. Presence of mutant p53 is evident by its nuclear detection, whereas wt p53 is undetectable. Sections were distinguished based on the type of p53 staining, either cytoplasmic or nuclear. Nuclear staining was further graded using the criteria as described by Elledge et al., (26) with a slight modification. In this study, sections were graded as the sum of the proportion of cells expressing nuclear p53 and the intensity of staining. The grading of intensity ranged from 0-3 and the proportion of positive cells were graded as follows: 0=less than 10%, 1=10-32%, 2=33-66%, and 3=greater than 66%. The numbers for the two criteria were then added, and a total of 3 or more was considered as a significant expression of p53.
Results

Analysis of p53. Analysis of p53 expression revealed that nine (82%; 6 de novo and 3 secondary) tumors displayed p53 expression (Table I). The remaining two (18%) samples (one de novo and one secondary) were negative for p53 staining. Four out of seven (57%) de novo tumors showed p53 expression. However, the expression in these tumors was specifically localized in the cytoplasm and was distributed in the perivascular areas within the tumor region (Figure 1C). The staining in these de novo tumors appears to be diffused and confined to the areas surrounding the vessel wall (Figure 1c). An intense nuclear staining of p53 was displayed along with some cytoplasmic staining in patient numbers 2 and 10 with de novo GBMs (Table I). However, in these tumors, cytoplasmic staining was not confined to the perivascular regions, but appeared randomly distributed. An insignificant number of cells (less than 10 per field) had nuclear p53 staining in de novo patients 3 and 4, therefore, this was considered as an absence of nuclear p53 (according to the criteria of Elledge et al. (26); see “Patients and Methods” section). As shown in Table II, three out of four (75%) secondary GBM which had p53 expression had staining which was confined to the nucleus and distributed randomly throughout the tumor region (Figure 1D and 1d). In these samples, the staining grading was done according to the criteria of Elledge et al., (26) and were between 4 and 6 (Table I).

Discussion

Among tumor suppressor genes, p53 appears to play an important role in the pathogenesis of several common malignancies (27) including brain cancer. p53 has been shown to exert tumor suppressor activity by inducing apoptosis (19), activating the cell cycle (19), stimulating cell differentiation (28), and being involved in DNA repair pathways (29). Mutations in the p53 gene are detected in about 65% of secondary and 28% of de novo GBM (6), thus indicating that p53 abnormalities are common in the progression from a low grade lesion to a high grade lesion (6, 30). An association between p53 mutations and GBM progression remains unclear. An epidemiological study showed that 57% of gene mutations in secondary GBM were in “hot spot” codons such as 248 and 273 and mutations were more equally distributed throughout exons 5-9 and G:C → A:T transitions at CpG sites were more common in secondary GBM than de novo GBM (7). This study suggests that the acquisition of p53 mutations in these two types of GBM occurs through different mechanisms. In GBM p53, mutations occur commonly in residues at codons 175, 245, 248, 249 and 273 (6) and may confer a poor outcome (31). A population-based study of 715 patients

Table I. Analysis of p53 IHC in de novo and secondary GBM.

<table>
<thead>
<tr>
<th>Patient#</th>
<th>Age*</th>
<th>Classification of GBM</th>
<th>Recurrence</th>
<th>p53 expression</th>
<th>Type of staining</th>
<th>Pattern of staining</th>
<th>Staining grade**</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>49</td>
<td>de novo</td>
<td>recurred</td>
<td>positive</td>
<td>Cytoplasmic</td>
<td>Perivascular</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>33</td>
<td>de novo</td>
<td>recurred</td>
<td>positive</td>
<td>Nuclear/Cytoplasmic</td>
<td>Perivascular</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>69</td>
<td>de novo</td>
<td>recurred</td>
<td>positive</td>
<td>Cytoplasmic</td>
<td>Perivascular</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>27</td>
<td>de novo</td>
<td>NKR</td>
<td>positive</td>
<td>Cytoplasmic</td>
<td>Perivascular</td>
<td>NA</td>
</tr>
<tr>
<td>5</td>
<td>70</td>
<td>secondary</td>
<td>NKR</td>
<td>negative</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>6</td>
<td>68</td>
<td>secondary</td>
<td>recurred</td>
<td>positive</td>
<td>Nuclear</td>
<td>Random</td>
<td>6</td>
</tr>
<tr>
<td>7</td>
<td>36</td>
<td>de novo</td>
<td>NKR</td>
<td>negative</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>8</td>
<td>48</td>
<td>de novo</td>
<td>recurred</td>
<td>positive</td>
<td>Cytoplasmic</td>
<td>Perivascular</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>48</td>
<td>secondary</td>
<td>NKR</td>
<td>positive</td>
<td>Nuclear</td>
<td>Random</td>
<td>4</td>
</tr>
<tr>
<td>10</td>
<td>42</td>
<td>de novo</td>
<td>NKR</td>
<td>positive</td>
<td>Nuclear/Cytoplasmic</td>
<td>Random</td>
<td>6</td>
</tr>
<tr>
<td>11</td>
<td>67</td>
<td>secondary</td>
<td>NKR</td>
<td>positive</td>
<td>Nuclear</td>
<td>Random</td>
<td>4</td>
</tr>
</tbody>
</table>

*Age at time of tumor resection. **For nuclear p53, cytoplasmic staining not graded. NKR = no known recurrences; NA = not applicable.

Table II. Expression of p53 in GBM.

<table>
<thead>
<tr>
<th></th>
<th>de novo</th>
<th>Secondary</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of tumors*</td>
<td>7 (64%)</td>
<td>4 (36%)</td>
</tr>
<tr>
<td>p53 expression</td>
<td>6 (86%)</td>
<td>3 (75%)</td>
</tr>
<tr>
<td>Cytoplasmic**</td>
<td>4 (57%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Nuclear***</td>
<td>0 (0%)</td>
<td>3 (75%)</td>
</tr>
<tr>
<td>Both****</td>
<td>2 (29%)</td>
<td>0 (0%)</td>
</tr>
</tbody>
</table>

*Represents percentage analyses for total number of samples including those which did not express p53. **p53 expressed in the cytoplasm. ***p53 expressed in the nucleus. ****p53 expressed in cytoplasm and nucleus.
Figure 1. Expression of p53 in de novo and secondary GBM. A. H&E stain of de novo GBM. B. H&E stain of secondary GBM. C. p53 staining in de novo GBM depicting intense cytoplasmic staining surrounding the perivascular region; boxed area from C (20x) is magnified in c (40x); arrow indicates the expression of p53. D. p53 staining in the nucleus distributed randomly throughout secondary GBM tumors; boxed area from D (20x) is magnified in d (40x); arrow indicates nuclear staining of p53.
revealed that the presence of mutant p53 predicted a longer survival in GBM patients compared to those with the presence of wt p53 (7). A univariate analysis indicated the presence of p53 mutations predicted a longer survival in a population-based study but, a multivariate analysis, when adjusted for age, failed to sustain such an association (7). Alternatively, status of p53 had little effect on the survival of GBM patients (15). In another study, 41 patients with long-term survival (> 3 years) versus 48 patients with short-term survival (< 1.5 years) showed that p53 expression was more common in the long-term survival irrespective of the specific types of p53 mutation (32). There was also no association of p53 status and time to tumor progression (33). Several initial studies have indicated that p53 was significantly altered in patients with malignant transformation rather than those with no apparent progression (34, 35). For example, Chozick et al., (34) have shown that among the six patients with malignant degeneration, the average percentage of p53 immunoreactivity was about 27%, as compared to only about 4% of patients with no apparent progression. The rationale of the association and better prognosis is apparently due to the ability of wt p53 to repair DNA damage defects, a downstream target of p53 and p21 may be associated with radiosensitivity (33).

Alternatively, status of p53 had little effect on the survival of GBM (24). A study (20). Studies have shown the over-expression of p53 in either to the presence of wt p53 gene (37) or to a homozygous deletion of the p53 gene at chromosome 17 (37). In our study, two samples (patient numbers 5 and 7) were devoid of p53. This may be due either to the presence of wt p53 gene (37) or to a homozygous deletion of the p53 gene at chromosome 17 (20). Studies have shown the over-expression of p53 in GBM without an obvious mutation in the gene (24). A study of 26 tumors showed that about 69% had p53-positive staining, of which, 46% had nuclear staining and 23% had cytoplasmic staining, suggesting that the pattern of p53 expression was not confined to a particular region, such as the infiltrating edges or vascularization areas (24). Mutational analyses of exons 5-8 indicated that no mutations were found in those samples which had cytoplasmic expression of p53, thus suggesting that cytoplasmic p53 may be wt (24). This finding is consistent in part with our observation that cytoplasmic p53 expression was exclusively seen in the perivascular areas in 57% of de novo cases and was not seen in secondary GBM (Table II; Figure 1). These authors (24) have also demonstrated a strong correlation between vimentin and cytoplasmic p53. Association of such a marker for p53 may reflect the involvement of p53 in the differentiation of CNS progenitor cells. In fact, wt p53 in the cytoplasm may not be a tumor specific event, since such expression is seen in the embryonic stem cells of mice (38). Furthermore, p53 mRNA and protein accumulation to high levels in proliferating cells without any indication of apoptosis is observed in the embryonic brain tissue of rats (39). Moreover, cytoplasmic wt p53 is involved in differentiation and may reflect a normal as opposed to abnormal event in it (40). Also, the glial cell lineage precursor cells express vimentin (41) and well-differentiated human astrocytomas express vimentin less frequently than AA and GBM do (42). In addition, GBM also exhibit the stem cell markers, such as nestin, whereas grade II astrocytomas do not (43). Thus, the presence of wt p53 in GBM may represent the cells arrested in an early stage of astrocyte differentiation (24). Due to the lack of association between the presence of cytoplasmic p53 and specific clinical features of the disease as well as with the proliferative index, it is assumed that this form of p53 is inactive. However, the p53-dependent pathway appears to be intact in neuroblastoma cell lines, where only p53 tumor cells express cytoplastic p53 (44). Inactivation of wt p53 without any LOH or mutation can occur by mechanisms such as binding to SV40 (45) or by its association with Cullin 7 (46). Recent studies have also indicated that there may be another mechanism involving a hsp70 family protein, mortalin (MOT-2), which binds to the C terminus (containing nuclear localization sequence in the p53 gene) of the p53 protein and sequesters it to the cytoplasm (47).

The expression of cytoplasmic p53 around the perivascular area seen in our study may represent its role in angiogenesis. Wt type p53 down-regulates Vascular Endothelial Growth Factor (VEGF) mRNA levels and suppresses VEGF promoter activity in a dose-dependent fashion, but this effect is not evident in the presence of mutant p53 (48). Interestingly, 86% of 29 GBM and 79% of 14 AA showed VEGF immunoreactivity in tumor cells, which was associated with vascularity and positively correlated with p53 expression, therefore suggesting an association between
mutant p53 and VEGF (49). Furthermore, low grade gliomas are moderately vascularized, whereas high grade gliomas show prominent vascular proliferations in the areas of high vascular density that may be related to high expression of VEGF and its receptors (50). The relationship between p53 expression and relapse-free survival has been found to depend on the level of angiogenic activity. It is noteworthy that the acquisition of p53 mutations in GBM subtypes occurs via distinct mechanisms, which may reflect discrete patterns of p53 expression in these two subtypes. As with our study, it has been widely established that nuclear over-expression of p53 in secondary GBM reflect the presence of mutant p53. However, it remains to be seen whether the cytoplasmic expression of p53 surrounding the perivascular region in de novo GBM, in our study is wt or mutant p53.

The exact mechanisms of p53 sequestration into the cytoplasm and how it remains stable is yet to be determined. There are several proteins such as MOT-2 and PARC, which bind to p53 and sequester it to the cytoplasm (46, 47). Numerous investigators have studied the involvement of p53 in GBM and established that p53 has a role in tumor progression. However, only a handful of studies have indicated the presence of cytoplasmic p53 in GBM samples (24, 37). Sembritzki et al., (24) have demonstrated that cytoplasmic p53 is wt p53, since 38% of total GBM patients expressed cytoplasmic p53 without any obvious mutations in exons 5-8 of the p53 gene. Our study of only eleven patients has shown this unique expression of p53 in the cytoplasm localized to the perivascular area. One explanation could be that mutant p53 expression is generally noticed only in the nucleus, and cytoplasmic expression of p53 was not taken into account. It would be interesting to see if such a pattern holds true in a larger patient population.

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