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CORRELATION OF MOLECULAR GENETIC ANALYSIS OF *P53, MDM2, P16, PTEN,* AND *EGFR* AND SURVIVAL OF PATIENTS WITH ANAPLASTIC ASTROCYTOMA AND GLIOBLASTOMA

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1. ABSTRACT

This article reviews studies on the correlation between genetic abnormalities in malignant astrocytic tumors and patient survival. It is almost certain that alterations of *PTEN* on chromosome 10 represent a significant unfavorable prognostic factor in glioblastoma patients. The association of alterations in *p53, MDM2, p16* or *EGFR* with the survival of patients with anaplastic astrocytoma or glioblastoma remains controversial. It is possible that the *p16* alteration and *EGFR* amplification are associated with poor survival in certain groups of patients and that there might be a relationship with age. Malignant transformation of astrocytic cells are driven by the sequential acquisition of genetic alteration. Therefore, it is reasonable to subgroup gliomas by their patterns of genetic alterations. However the studies that correlated the multiple genetic alterations with survival are still limitted.

Further studies on large cohorts are necessary to elucidate the genetic factors that affect the prognosis and response to therapy of patients with malignant gliomas and to develop effective management strategies.

2. INTRODUCTION

Malignant gliomas are the most common primary malignant neoplasms of the central nervous system. Most of these are anaplastic astrocytomas or glioblastomas. As these tumors are highly resistant to current treatment modalities including surgery, radiotherapy and chemotherapy, their prognosis is dismal. Even after multidisciplinary treatment, the median survival of patients with anaplastic astrocytoma is only around 4-5 years; for patients with glioblastoma it is less than 2 years. In each histological group, there is a considerable difference in response to therapy and wide range in survival, and the tumors are thought to be highly heterogeneous. In an attempt to establish individualized treatments and to improve the clinical management of these tumors, efforts have been made to subgroup them genetically (1). One of the approaches to the goal is to identify genetic alteration(s) that are relevant to tumor sensitivity to the treatment. This article reviews studies that investigated the correlation between the survival of patients with anaplastic astrocytoma and glioblastoma and the presence of single or multiple alterations of major genes thought to be importantly involved in tumorigenesis and/or tumor progression.

3. *P53* MUTATION

Analyses of the *p53* tumor suppressor status in brain tumors have demonstrated frequent alterations of the *p53* gene as well as stabilization of mutant and intact *p53* proteins in tumor cells. *P53* mutations are predominantly found in astrocytic tumors; approximately 50-60 % of anaplastic astrocytomas (2,3) and 25-30% of glioblastomas (4,5) manifested these mutations. Tumor cells carrying *p53* mutations are resistant to apoptosis induced by DNA damage; overexpression of wild-type *p53* enhances the radiosensitivity of glioma cells. However, the effect of *p53* mutations on the radio- and chemosensitivity of gliomas, especially glioblastomas, remains controversial. There are reports that *p53* gene mutations were associated with poor outcomes in pediatric brain tumors (6) and adult gemistocytic astrocytomas (7). Other studies using single-strand conformation polymorphism (SSCP) analysis or yeast functional assay found that the presence of *p53* mutations was a positive predictor for a response to radiation therapy (8) and for a good prognosis in patients with glioblastomas (9). On the other hand, in patients with astrocytic tumors, *p53* mutations were reportedly not associated with survival (10,11). We employed yeast functional assay and DNA sequencing to study the *p53* gene status in glioblastoma patients and found no significant difference in the average age of patients with and without the *p53* mutation. Multivariate analysis

adjusted for prognostic factors including the age, gender, and the Karnofsky performance status (KPS) of glioblastoma patients revealed that the mutation did not affect their progression-free or overall survival (12). These conflicting data may be attributable to differences in the patient populations studied, variations in the accuracy of histological diagnoses or tumor sampling, and the reliability of methodologies used for study.

In gliomas, not only mutant but also wild-type *p53* proteins (13) are often stabilized by mechanisms that are currently not entirely understood. The turnover is sustained by proteolytic degradation which takes place in the cytoplasm and involves targeting of *p53* at proteosomal degradation by the murine double minute 2 (*MDM2*). The increased stability of wild-type proteins is partly due to an alternatively spliced *MDM2* which cannot degrade the target *p53* protein (14,15). On the other hand, *MDM2* overexpression with or without gene amplification(s) is observed mainly in glioblastomas without *p53* gene mutations (9,16). This is suggestive of *p53* dysfunctional mechanisms other than mutation. *P14ARF* is an inhibitor of *p53* proteolysis by *MDM2* encoded on the *INK4a* locus that is frequently deleted in human tumors. *P53* mutations and *p14ARF* deletions have been shown as mutually exclusive in human glioblastoma (17). Ichimura *et al.* (2) reported that 76% of glioblastomas, 72% of anaplastic astrocytomas, and 67% of low-grade diffuse astrocytomas manifested deregulation of the *p53* pathway either by *p53* gene mutation, *MDM2* amplification, or homozygous deletion/mutation of p14ARF. This leads to the speculation that the *p53* tumor suppression pathway is inactivated not only by *p53* mutation in the early stages of astrocytic tumorigenesis, and that most glioblastomas have acquired impairment of tumor suppression functions. Our preliminary data showed that anaplastic astrocytomas and glioblastomas differed in the distribution of the *p53* mutation locus, suggesting the existence of different mechanisms for the *p53* mutation in these two types of high-grade astrocytic tumors (9).

4. *MDM2* AMPLIFICATION / OVEREXPRESSION

MDM2 oncoprotein promotes cell survival and cell-cycle progression by inhibiting the *p53* tumor suppressor protein (18). The *MDM2* gene which locates on 12q13-14 was found to be amplified in about 10-15% of high-grade gliomas, thus it represents the second most frequently amplified gene after the *EGFR* gene in these tumor types (19,20). Previous attempts to correlate *MDM2* gene amplification with survival produced contradictory results. There is a report that immunohistochemical positivity for *MDM2* protein was associated with longer survival in patients with malignant astrocytic tumors(21); in other studies, immunohistochemical positivity for *MDM2* protein or *MDM2* gene amplification was associated with shorter survival time (9,22). Others (23-25) found *MDM2* gene amplification to have no significant relevance to overall patient survival.

5. *P16* ALTERATION

The *p16* gene, which maps to chromosome 9p21, is a tumor suppressor gene that has been investigated in many human cancers including gliomas (26,27). In high-grade astrocytic tumors, its inactivation is the most common alteration in the *p16*-cdk4-cyclinD1-Rb pathway and occurs mainly through homozygous deletion (28-30). Homozygous deletion of the *p16* gene has been reported in approximately 10-50 % of anaplastic astrocytomas and 30-70 % of glioblastomas. These divergent results may at least partly be attributable to the different methods employed for the detection of homozygous deletions in clinical tumor samples (2,29,31-38). As the *p16* protein regulates cell-cycle control at the G1-S transition, its inactivation leads to loss of cell-cycle control and consequently, increased proliferation. High-grade astrocytic tumors with *p16* homozygous deletion had higher Ki-67 indices than those without the deletion (34). At the same chromosome 9p21 (*INK4a* locus), the *p14ARF* gene is known to be located and shares exon 2 and 3 with *p16* gene, but encodes a distinct protein which participated in p14-*MDM2*-*p53* pathway (39,40). Recently, it has been elucidated that in astrocytic tumors, homozygous deletion of the *p16* gene is associated with co-deletion of the *p14ARF* gene (2,17,36). Although no studies have addressed the correlation between *p14ARF* alteration alone and survival in patients with high-grade astrocytic tumors, *p16* alteration has been investigated in relation to survival. While the correlation between *p16* inactivation and survival in patients with high-grade astrocytic tumors has been investigated, the prognostic value of *p16* abrogation remains controversial. Studies using multiplex polymerase chain reaction (PCR) assay (37,41), Western analysis (42), or immunohistochemical methods (43) detected no significant correlation between homozygous deletions of the *p16* gene or loss of *p16* protein expression, and survival in patients with high-grade astrocytic tumors (37,41-43). On the other hand, Newcomb *et al.* (11) found that in glioblastoma patients older than 61 years, *p16* immunonegativity tended to signal a poor prognosis. In high-grade astrocytic tumors, *p16* immunonegativity was an independent indicator of a poor prognosis according to univariate and multivariate analyses adjusted for age, tumor histology, extent of surgery, and the Ki-67 labeling index (LI) (44). Using quantitative real-time PCR assay of 105 primary gliomas, Labuhn *et al.* (45) recently demonstrated that deletion of the *INK4a* locus, where *p16*- and *p14ARF* gene are located at the 9p21 locus, affected both genes. They separated glioblastoma patients into those with homozygous deletion at the *INK4a* locus and those with retention of both copies of the locus and compared their survival. The only significant difference in survival was recorded for patients older than 50 years. We studied patients with supratentorial glioblastoma who had been treated with surgery and postoperative radio- and chemotherapy and examined homozygous deletion of the *p16* gene (exon 2) using multiplex PCR (38). We chose this assay because in glioma tissue samples, the results of multiplex PCR were highly concordant with data obtained by fluorescence *in situ* hybridization (FISH) and comparative genomic hybridization (CGH) (46). When we subjected primary tumor tissue to multiplex PCR, we performed titration experiments to confirm the validity of the results (38). We detected *p16* homozygous deletion in 30.4% of all patients; this was true in 30.8% male and

30.0% female patients. Cox proportional hazard regression analysis, adjusted for age at surgery, the KPS score, the extent of surgical resection, and the MIB-1 LI revealed that homozygous deletion of the *p16* gene was associated with neither overall- nor progression-free survival in our glioblastoma patients. However, in male patients, this deletion was significantly correlated with poor prognosis. The results of both Labuhn *et al.* (45) and our study suggest that homozygous deletion of the *INK4a* locus is an unfavorable prognostic factor in certain subgroups of glioblastoma patients (38,45). The gender difference revealed by our study concerning the effect of homozygous deletion of the *p16* gene on survival requires further investigation.

The mechanisms of *p16* inactivation other than homozygous deletion include mutation of the gene and transcriptional repression due to hypermethylation of the 5'CpG island in the promoter region (47-50). However, these abrogations are rare in astrocytic tumors (51-53) and to our knowledge, no studies have addressed the correlation between these abrogations and the survival of patients with high-grade astrocytic tumors. Most glioblastomas manifest molecular genetic alteration of at least one component in each of the Rb and *p53* pathways (2,17,29,30,33). In addition, alterations of individual components appear inversely correlated in each of these pathways (17,29,30,33,35). To understand their possible effects on the survival of patients with high-grade astrocytic tumors, systematic investigation of these pathways is necessary (54). Presumably, homozygous deletion of the *INK4a* locus in high-grade astrocytic tumors is the most effective means of abrogating both the Rb and *p53* pathways.

6. *PTEN* ALTERATION

The most common chromosomal abnormality in high-grade gliomas is the loss of all or part of chromosome 10. This is true in approximately 30-70% of anaplastic astrocytomas and 60-95% of glioblastomas (55-58). Candidate tumor suppressor genes on chromosome 10 that may be involved in tumorigenesis and/or tumor progression have been identified. They include *PTEN* at 10q23.3 (59), FGFR2 at 10q25.3-26 (60), DMBT1 at 10q25.3-26.1 (61), LGI1 at 10q24 (62), and "h-neu" at 10q25.1 (63). The most important tumor suppressor among them is the *PTEN* (also known as *MMAC*1 (64) / TEP1 (65)) gene. *PTEN* negatively regulates the phosphatidylinositol 3-kinase (PI3K)-Akt pathway, and thereby affects control of the cell-cycle and cell survival (66,67). Additionally, *PTEN* inhibits focal adhesion, spreading, and migration by dephosphorylating focal adhesion kinase, and also regulates tumor-induced angiogenesis (68,69).

The correlation between loss of heterozygosity (LOH) for chromosome 10 and the survival of glioma patients has been examined. Using microsatellite analysis, Lin *et al.* (70) studied two loci including *MMAC*/*PTEN* and DMBT1 for LOH in patients with various histological types of glioma. They found that LOH around *MMAC*/*PTEN* was a significant unfavorable prognostic factor in patients with anaplastic astrocytoma and glioblastoma; their survival was not significantly affected by the presence or absence of LOH in the DMBT1 region. In their reverse transcription PCR (RT-PCR) study, Sano *et al.* (71) examined the expression of *MMAC*/*PTEN* in high-grade glioma patients and found that those whose tumors expressed high levels of *MMAC*/*PTEN* had a significantly better prognosis. We used microsatellite analysis to investigate the correlation between survival and LOH for several loci on chromosome 10 where candidate tumor suppressor genes for the oncogenesis of high-grade astrocytomas are thought to reside (72). We found that in patients with glioblastoma, the only statistically significant predictor of overall survival, identified by both univariate and multivariate analyses, was LOH for *PTEN*/*MMAC*1. The LOH frequency at each locus manifested a different pattern in anaplastic astrocytomas and glioblastomas. In anaplastic astrocytomas, telomeric regions deleted more frequently than *PTEN*/*MMAC*1. This finding coincides with the speculation (70,73) that LOH at these loci may represent an early genetic event in the progression of astrocytic tumors. In our study, the frequency of LOH was significantly higher in older than younger patients with anaplastic astrocytomas. This result too was consistent with that of Lin *et al.* (70) who demonstrated that the frequency of LOH for *MMAC*/*PTEN* significantly increased with age in patients with anaplastic astrocytoma as well as those with glioblastoma.

PTEN is altered by mechanisms other than LOH, including mutations, homozygous deletion, and methylation defects. In all informative cases, *PTEN* mutations occured in tumors with LOH on chromosome 10q, suggesting the inactivation of this gene by a 2-hit mechanism. The mutations were reported in approximately 20-40% of glioblastomas (55,56,74). Among studies investigating whether the *PTEN* mutation is associated with survival in patients with high-grade astrocytic tumors, a small cohort of pediatric patients suggested the correlation of *PTEN* mutation and survival (75). Using denaturing gradient gel electrophoresis (DGGE) followed by DNA sequencing, Zhou *et al.* (76) found no significant correlation between the *PTEN* mutation and survival in glioblastoma patients. Other studies showed the same results (41,76).

7. *EGFR* AMPLIFICATION / OVEREXPRESSION

The most frequent oncogenic alteration in glioblastomas is *EGFR* gene amplification resulting in the overexpression of *EGFR*, a transmembrane tyrosine kinase receptor (78-81). *EGFR* amplification is thought to be present in 30-50% of all glioblastomas and to occur more frequently in primary (de novo) glioblastomas (24,78,81-89). It confers to cells advantages of growth and invasiveness, and radio- and chemo-resistance (90-93). Furthermore, glioblastomas with *EGFR* amplification frequently exhibit a variety of *EGFR* alterations and mutations, especially *EGFRv III* (also known as del2-7*EGFR* and ? *EGFR*), whose extracellular ligand-binding domain is truncated; *EGFRv III* is constitutively activated and enhances tumorigenicity *in vivo* (90,93-98). While there is evidence that *EGFR* amplification may signal a less

favorable prognosis in glioblastoma patients, the results of clinical studies were not conclusive.

In a study using differential PCR assay, multivariate analysis assigned no statistical value to *EGFR* amplification with respect to the survival of patients with astrocytic gliomas (85). Simmons *et al.* (99), who assessed *EGFR* overexpression in glioblastoma patients by immunohistochemical- and *p53* mutation by immunopositivity studies as well as by SSCP found no association of these alterations with survival. However, when they separated patients according to age (younger vs. older than median age), univariate analysis revealed that *EGFR* overexpression was associated with better prognosis in the older group; there was no such association in the younger patient group. Their multivariate analysis demonstrated that *EGFR* overexpression was an independent unfavorable prognostic factor in younger patients whose *p53* statuses was normal. Barker *et al.* (100) found positive *EGFR* immunoreactivity to be an independent significant predictor of a poor radiation response in glioblastoma patients; their multivariate analysis detected no significant correlation between the immunoreactivity or mutation status of *p53* and tumor radiation sensitivity. Feldkamp *et al.* (101), who assessed *EGFR* and *EGFRv III* expression by RT-PCR, Western blotting, and immunohistochemistry in a small cohort of glioma patients, reported that those with *EGFR*v III-positive tumors survived for shorter periods than did patients with *EGFRv III* -negative tumors, although the difference was not statistically significant by the Log-rank test.

8. COMBINED GENETIC ALTERATIONS

There is general consensus that malignant transformation of astrocytic cells is a multistep process driven by the sequential acquisition of genetic alterations. Therefore, it is reasonable to sub-categorize gliomas by their patterns of genetic alterations. The observed different combinations of *p53* mutations, LOH on chromosomes 17p or 10, and *EGFR* amplification, have led to the hypothesis that there exist subsets of glioblastomas with distinct genetic alterations; i.e. primary (de novo) and secondary glioblastomas (87,89). The former are seen more frequently in elderly patients and are characterized by *EGFR* amplification and LOH on chromosome 10 without *p53* mutation; the latter occur more frequently in younger patients and are characterized by *p53* mutation and LOH of chromosome 17p. While there was general agreement regarding the proposed sub-classification of gliomas, there are only a few reports that studied survival significance related to the two subsets.

An extensive study on 80 glioblastoma patients showed no significant association of altered expression of *p16*, *p53*, *EGFR*, *MDM2* or Bcl-2 with survival (11). Leenstra *et al.* (102) studied *EGFR* alteration by dual-probe FISH and differential PCR, *PTEN* abnormality by FISH and PCR-based analysis, and *p53* by PCR-based analysis. They classified their 75 patients with high-grade astrocytoma into 4 groups according to genetic changes in the tumors: *p53* gene alterations without complete LOH of chromosome 10 (Group 1); complete LOH of chromosome 10 only (Group 2); *p53* gene alterations plus complete LOH of chromosome 10 (Group 3); complete LOH for chromosome 10 plus *EGFR* gene amplification (Group 4). Multivariate analysis adjusted for age and gender revealed that complete LOH for chromosome 10 plus *EGFR* gene amplification (Group 4) was a significantly more unfavorable prognostic factor than *p53* gene alteration alone. Simmons *et al.* (99), who investigated *EGFR* overexpression in glioblastoma patients by immunohistochemical methods, *p53* immunopositivity, and *p53* mutation by SSCP analysis, found a statistically significant association of *EGFR* overexpression with worse survival in younger patients with wild-type *p53* but not those with *p53* alterations.

Investigations to correlate gene alterations in malignant gliomas with survival have produced contradictory results. Although the reasons for these divergent findings remain unclear, they reflect different methodologies and differences among the patient populations studied. Systematic large-scale studies are needed to obtain important information regarding the various genetic factors that affect the prognosis and response to therapy of patients with malignant gliomas. Such studies will also facilitate the development of better management strategies.

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