RESEARCH COMMUNICATION

The Role of Immunohistochemistry in Predicting Behavior of Astrocytic Tumors

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Abstract

The purpose of this study was to analyze the significance of p53, bcl-2 and EGFR expression in the grading and biological behavior of astrocytic tumors, especially in the Indian population. A total of 117 cases of astrocytomas graded using the WHO grading system published in 2007 were immunolabeled using p53, EGFR and bcl-2 monoclonal antibodies and analyzed with respect to grade and other relevant parameters. The 117 cases included 16 cases of pilocytic astrocytomas and 25, 15 and 61 cases of diffuse fibrillary astrocytomas WHO grade II, anaplastic astrocytomas WHO grade III and glioblastomas (GBM), respectively. Our results showed that p53 alterations is an early event in astrocytic gliomagenesis, but is not significant in the evolution of pilocytic astrocytomas. Bcl-2 expression did not correlate with grade and no statistical correlation was seen with p53 expression. EGFR protein expression correlated with the severity of tumor grade. Of the GBM cases, 47.5% were p53 positive only, 18% were EGFR positive only, 16.5% were negative for both and 18% were positive for both. The mean age in the dual positive category was significantly higher when compared to the others. EGFR and p53 alterations are not mutually exclusive and mightn act synergistically to promote progression. We also noted a significantly higher p53 expression in females in GBMs. Though most of our findings correlated with those of previous studies, some differences were noted, especially in the pattern of immunoexpression in GBMs, perhaps because of ethnicity.

Keywords: EGFR - p53 - bcl-2 - astrocytomas

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Introduction

Glial tumors are the most common primary tumors of the CNS. They can recur and display malignant progression depending upon the histopathological type, grade of malignancy, location, patient's age and extent of surgical resection. Though the histological grade continues to be one of the most important prognostic indicators, the roles of cytogenetic alterations, various growth factors like epidermal growth factor (EGF), platelet derived growth factor (PDGF), vascular endothelialgrowth factor (VEGF), growth factor receptors like the ep idermal growth factor receptor (EGFR), platelet derived growth factor receptor (PDGFR) and mutations of various oncogenes are also being investigated. The association of genetic alterations with astrocytic glioma behavior has stimulated numerous investigations into the prognostic relevance of genetic markers. The role of the p53 tumor suppressor gene, EGFR, bcl-2 and other oncogenes in gliomagenesis is being extensively studied.

The p53 tumor suppressor gene located on the short arm of chromosome 17 is activated by DNA damage, abnormal growth signals and other intrinsic and extrinsic stresses. In normal cells, the expression of p53 protein is generally below the detection level of immunohistochemical methods. Mutations of the p53 gene are among the most common molecular changes identified in human cancers. These mutations can result in accumulation and overexpresssion of mutant p53 protein. The alterations of p53 gene play a significant role in the initiation and progression of astrocytomas (Pardo et al., 2004; Yue et al., 2009).

The EGFR or erbB1 gene, located on the short arm of chromosome 7 is associated with a wide range of cell functions, such as cell proliferation, migration, maturation and differentiation. EGFR may be deregulated following two distinct mechanisms: point mutations occurring in exons 18-21, corresponding to the tyrosine kinase (TK) domain, and protein overexpression, leading to tumour growth, metastasis, angiogenesis and inhibition of apoptosis. EGFR is deregulated in a variety of solid malignant tumours; in particular its expression has been correlated with disease progression and poor survival. The precise evaluation of EGFR status is very essential due to the prognostic and predictive role of EGFR and to the availability of specific targeted therapies. In contrast to other solid tumours, protein overexpression in gliomas is almost always associated with gene amplification (Martin et al., 2009; Coulibaly et al., 2010).

Bcl-2 gene family is composed of genes, which can

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promote or prevent apoptosis. Bcl-2 gene is antiapoptotic whereas BAX gene is proapoptotic. Bcl-2 proto-oncogene located on chromosome 18 can promote neoplastic transformation. Bcl-2 expression was observed in both benign and malignant tumors of the CNS (Tyagi et al., 2002).

A recent study has revealed an increasing trend of CNS cancers in India (Yeole, 2008).

Though many studies have been undertaken in the West to understand the molecular basis of gliomagenesis, there is lesser number of Indian and Asian studies. Studies have proved that there is a relationship between ethnicity and the pattern of expression of these immunomarkers (Wiencke et al., 2005) and hence this study was undertaken. The aim of this study was to analyze the significance of expression of p53, bcl-2 and EGFR proteins in the grading and biological behavior of astrocytic tumors, especially glioblastomas, in the Indian population.

Materials and Methods

A total of 117 cases of astrocytomas were included and graded using the WHO classification published in 2007. Representative blocks of formalin fixed paraffin embedded tissue of these 117 cases were selected and 4μm thick paraffin sections were floated on to slides previously coated with poly-L-lysine. Monoclonal antibodies against the following antigens were used:

P53 (Novocastra, clone DO-7, dilution of 1:100).
EGFR (Novocastra, clone EGFR.25, dilution of 1:100)
Bcl-2 (Novocastra, clone bcl-2/100/D5, dilution of 1:50)

Antigen retrieval was done by boiling in the pressure cooker using Sodium Citrate Buffer (0.01 M, PH=6.0). Immunostain visualization was achieved with the standard streptavidin -biotin peroxidase technique.The slides were stained with diaminobenzidine, counterstained with hematoxylin, and mounted.

During each batch of staining, appropriate positive and negative controls were used. For p53 a known case of colonic adenocarcinoma was used. Tonsil was used for Bcl-2 and a case of squamous cell carcinoma was used as a positive control for EGFR. A negative control slide in which the primary antibody was excluded was used for each batch of slides.

Immunostaining was evaluated in the fields consisting of regions of the tumor having the greatest number of immuno reactive cells as assessed qualitatively at low power examination. Labeling index for p53 was calculated as follows:

$$\text{P53 L I} = \frac{\text{number of nuclei showing positive staining}}{\text{Total number of nuclei counted}} \times 100$$

LIs for glioblastomas were calculated from areas of sections that were free from necrosis or capillary endothelial proliferation. The infiltrative edge of the tumour where neoplastic cells surround normal neurons and glia was also avoided. Where an uneven distribution of immunohistochemical labeling was evident, fields from the area of maximal labeling were chosen for counting. The bcl-2 labeling index was calculated as a percentage of tumour cells with positively stained cytoplasm divided by the total number of tumour cell nuclei counted .The denominator for each labeling index was at least 1000 tumour cells.

For EGFR, although cytoplasmic staining of the tumor cells was observed, only staining of the tumor cell membranes was considered to be specific. The staining pattern of tumor cell membranes was further classified as incomplete staining when tumor cells displayed staining of a part of their membrane and complete staining when tumor cells displayed a circumferential staining of the membrane.

The following scoring approach in the assessment of EGFR immunostaining was used: 0 for nonspecific staining of the tumor cells, 1 for weak (intensity) and incomplete staining (quality) of more than 10% tumor cells, 2 for moderate and complete staining of more than 10% of tumor cells and 3 for strong and complete staining of more than 10% of tumor cells. Cytoplasmic staining of the tumor cells was not regarded and considered as negative. For statistical analysis, a score of 0 and 1 was treated as "negative" and a score of 2 or 3 was considered "positive" (see Figures 1-3).

For statistical analysis, Fisher's exact test, Mann-

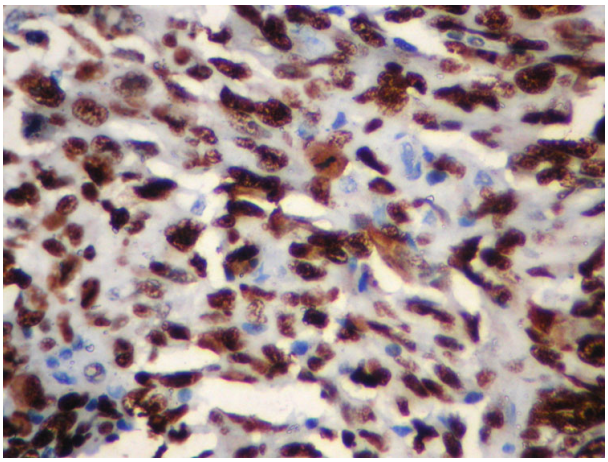

Figure 1. Immunostaining for p53 Showing Strong Nuclear Positivity × 400

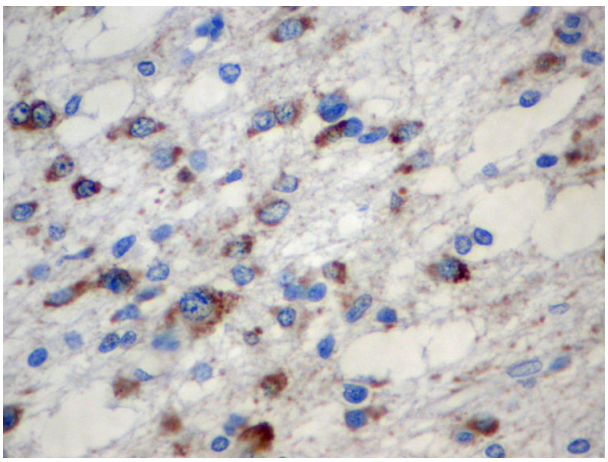

Figure 2. Immunostaining for Bcl-2 Showing Cytoplasmic Positivity × 400

Table 1. Age Distribution of Astrocytic Tumors

Grade	Mean ± SD (in years)	Range (in years)
Pilocytic astrocytoma	10.68±9.54	1.0-32.0
Diffuse astrocytomas	37.16±10.51	19.0-64.0
Anaplastic astrocytomas	42.73±14.89	22.0-73.0
Glioblastomas	50.55±15.32	2.0-78.0

Table 2. P53 Immunoreactivity in Astrocytic Tumors

Grade	No. of cases positive for p53 protein	p53 LI Mean ± SD
Pilocytic astrocytoma	4(25.00%)	3.97±10.77
Diffuse astrocytomas	20(80.0%)	14.24±14.40
Anaplastic astrocytomas	11(73.33%)	22.55±26.55
Glioblastomas	41(67.21%)	17.77±23.89

Table 3. Bcl-2 Immunoreactivity in Astrocytic Tumors

Grade	No. of cases positive for Bcl-2 protein	Bcl 2 LI Mean ± SD
Pilocytic astrocytoma	2 (12.5%)	1.06±2.97
Diffuse astrocytomas	10 (40.0%)	5.08±7.32
Anaplastic astrocytomas	4 (26.7%)	3.93±7.52
Glioblastomas	19 (31.2%)	3.71±7.68

Table 4. Distribution of EGFR Scores in Various Grades of Astrocytomas

Grade	0	1	2	3
Pilocytic astrocytoma	15 (93.8%)	1(6.25%)	0	0
Diffuse astrocytomas	15 (60%)	5(20%)	2 (8%)	3 (12%)
Anaplastic astrocytomas	8(53.33%)	1(6.67%)	3 (20%)	3 (20%)
Glioblastomas	34(55.74%)	5(8.20%)	4(6.56%)	18(29.50%)

		No. of cases	Mean age± SD
P53 -	EGFR -	9 (14.8%)	53.4±13.9
P53 -	EGFR+	11 (18.0%)	46.0±14.0
P53 +	EGFR-	30 (49.2%)	46.6±16.0
P53 +	EGFR+	11 (18.0%)	63.5±7.9

Whitney test, Kruskal Wallis test and Pearson's correlation coefficient were employed.

Results

The grading of the117 cases of astrocytomas included in our study comprised of 16 cases of pilocytic astrocytomas, 25 cases of diffuse astrocytomas WHO grade II, 15 cases of anaplastic astrocytomas WHO grade III and 61 cases of glioblastoma WHO grade IV.

There was significant variation in age for the four categories.

Frequency of p53 immunopositivity and mean p53 LI was significantly lower in pilocytic astrocytomas when compared to the other three categories. No significant variation was observed with the tumor grade when the other three categories were compared with each other.

No significant statistical variation was seen with the mean of bcl-2 LI in all the four grades of astrocytic tumors. There was no significant correlation between bcl-2 and p53 protein labeling indices.

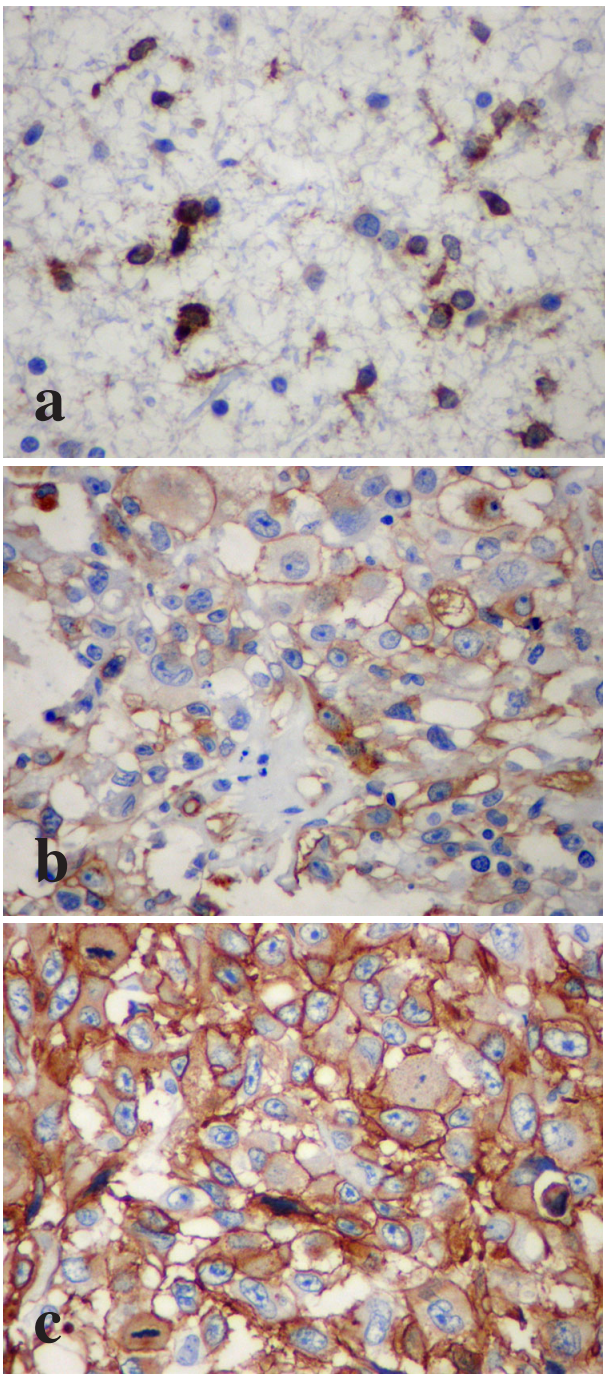

Figure 3. EGFR Immunopositivity. a) 1+ Showing Weak, Incomplete Staining of Tumor Cell Membranes in >10% of cells; b) 2+ Moderate staining >10% of Cells; c) 3+ Strong, staining >10% of cells ×400

EGFR protein over expression was seen in none of the cases of pilocytic astrocytomas, 5 (20.0%) cases of diffuse astrocytomas, 6 (40.0%) cases of anaplastic astrocytomas and 22 (36.1%) cases of glioblastoma. The analysis of intensity of EGFR staining revealed that EGFR protein over expression was significantly higher in the high grade astrocytomas compared to the low grade ones.

There were 61 GBM cases in our study, out of which 35 were males and 26 were females. Thirteen cases (21.3%) were seen in the category of ≤40 years age group, 30 cases (49.2%) were in 41-59 years age group and 18 (29.5%) cases were in the ≥60 years age group.

EGFR protein overexpression was seen more frequently in older individuals compared to the younger ones. Only 2 cases (15.4 %) in the ≤40 years age group showed EGFR expression ,whereas 12 cases (40.0%) in 41-59 years age group and 8 cases (44.4%) in the ≥60 years age group expressed EGFR. The mean age of EGFR positive GBMs was 54.7 whereas for EGFR negative GBMs it was 48.2.However this difference of 6 years between the two categories was not quite significant statistically (p value -0.056). There was no clear association between p53 expression and age in our study. The expression of p53 was more in females (80.8% of females) when compared to males (57.1 % of males) in cases of glioblastomas. Even the mean p53 LI was greater in females (27.7) when compared to males (10.4).This difference was statistically significant (p value -0.02).

When the simultaneous expression of p53 and EGFR protein was analyzed in cases of GBM, four subsets were evident. Of the 61 GBM cases, 47.5% were p53 positive only, 18% were EGFR positive only, 16.5% were negative for both and 18% were positive for both. In the dual positive category, the majority of the EGFR positive tumors showed strong positivity for p53 with p53 LI>10. The mean age in the last (dual positive) category was significantly higher when compared to the others. The frequency of GBMs exhibiting dual positivity increased as age increased. No cases of dual positivity were noticed in ≤40 years age group category. There was no significant variation in age for the other three categories.

We did not classify our cases as primary and secondary glioblastomas because the exact duration of the symptoms was not known in some patients.

Discussion

Absence of p53 mutations and p53 immunostaining in pilocytic astrocytomas has been reported in some studies (Tihan et al., 2000; DiSapio et al., 2002; Nayak et al., 2004), but others have identified p53 alterations in pilocytic astrocytomas (Nakamizo et al., 2002; Tibbetts et al.,2009). A recent extensive study on pilocytic astrocytomas has also documented p53 alterations, but could not find a statistically significant relationship between p53 immunoreactivity and event free survival (Tibbetts et al., 2009). Another study also could not correlate p53 expression with outcome (Horbinski et al., 2010). Thus the role of p53 expression and p53 mutation in pilocytic astrocytomas appears controversial and also appears to differ by the methodology used to detect alterations of p53 gene in pilocytic astrocytomas.

Our study detected p53 immunostaining in 25.0% of pilocytic astrocytomas. Frequency of p53 immunopositivity and p53 LI was significantly lower in pilocytic astrocytomas when compared to the other three grades. Thus our study showed that though p53 expression is not totally absent, it does not seem to play a significant role in the evolution of pilocytic astrocytomas as compared to the diffuse and high-grade astrocytomas.

The present study documented p53 immunopositivity in 80.0% of diffuse fibrillary astrocytomas, 73.3% of anaplastic astrocytomas and 67.2 % of GBM cases (refer table 2 for details). Thus p53 immunopositivity was demonstrated in more than two-thirds of tumors of all the three categories. Mean labeling indices of p53 for the three grades were 14.2, 22.5 and 17.7 respectively. The frequency of p53 immunopositivity in previous studies ranges from 56 to 75% for fibrillary astrocytomas, 53 to73 % for anaplastic astrocytomas and 50 to 83% for cases of GBMs (Rodriguez-Pereira et al., 2001; Nayak et al., 2004; Wiencke et al., 2005). Our study showed no correlation between p53 and malignancy grade. A previous study also indicated absence of correlation between p53 LI and the tumor grade (Nayak et al., 2004).

The frequencies of p53 immunopositivity and p53 labeling indices in diffuse and high-grade astrocytomas in our study were similar. In fact mean p53 LI in grade III astrocytomas was slightly higher when compared to GBM. Thus our study confirms that inactivation of p53 tumor suppressor gene is an important early genetic event in formation of astrocytomas as proved by earlier studies (Nayak et al., 2004; Pardo et al., 2004).

Though p53 protein overexpression correlates well with p53 gene status, it can occur for reasons other than mutation in the p53 gene (Chen et al., 2001; Pardo et al., 2004; Wiencke et al., 2005). Alterations in MDM2 gene and $p14^{ARF}$ gene (other components of the p53 pathway) can also lead to abnormal accumulation of p53 protein (Ichimura et al., 2000; Chen et al., 2001; Wiencke et al., 2005; Hollstein and Hainaut, 2010).

Bcl-2 expresssion has been studied in many tumours, especially hematological malignancies. The expression of bcl-2 has been examined immunohistochemically in benign and malignant CNS tumors. Bcl-2 oncoprotein expression did not correlate with glial tumor type or grade and significant bcl-2 expression has been observed in low-grade glial tumors also (Tyagi et al., 2002). In our study bcl-2 protein expression was observed in all grades of astrocytomas. However bcl-2 protein expression did not significantly vary in low and high-grade astrocytomas and there was no correlation between bcl-2 and p53 labeling indices also. a

Apoptosis is an important determinant of tumor growth, which can be regulated by the bcl-2 and p53 genes. But no relationship between bcl-2 and p53 expression could be established in astrocytomas (Rodriguez-Pereira et al., 2001; Ardeleanu et al., 2005).n. Although overexpression of bcl-2 can protect neoplastic cells from damage by radiotherapy and chemotherapy, no significant relationship has been found between bcl-2 expression and length of survival of patients in gliomas (Rodriguez-Pereira et al., 2001; Ardeleanu et al., 2005). In our study, p53 expression was seen in a higher percentage of tumors compared to bcl-2. Antiapoptotic proteins and proteins inhibiting apoptosis may be preferentially expressed in gliomas compared to proapoptotic bcl-2 family proteins leading to deregulation of pathways regulating apoptosis (Steinbach and Weller, 2004).

In our study EGFR overexpression was significantly greater in high-grade astrocytomas compared to the low grade ones. None of the pilocytic astrocytomas in our study expressed EGFR. Thus EGFR overexpression increased with grade of malignancy. Our results were

similar to other studies (Wiencke et al., 2005; Maiti et al., 2008).

Several studies have analyzed the complex relationship between age and p53 and EGFR expression in malignant gliomas, especially GBMs, the most common and most malignant subtype of astrocytomas. Some studies have shown that ethnicity has a role to play in patterns of expression of oncoproteins in GBMs, especially in relation to age, thus delineating different genetic pathways (Chen et al., 2001; Das et al., 2002; Wiencke et al., 2005, Xie et al., 2005). Hence we analyzed the relationship between age and p53 and EGFR expression in cases of GBMs in our study.

There were 61 GBM cases in our study. EGFR protein overexpression was seen more frequently in older individuals compared to the younger ones. The mean age of EGFR positive GBMs was 54.72 whereas for EGFR negative GBMs it was 48.20.However this difference of 6 years between the two categories was not quite significant statistically (p value -0.056). There was no clear association between p53 expression and age in our study.

In some studies, EGFR overexpression and amplification in GBMs were seen more commonly in older individuals whereas TP53 mutations and p53 protein accumulation in GBMs were observed more commonly in younger ones (Barker et al., 2001; Stark et al., 2003). However, Wiencke et al found little difference in age of patient by EGFR amplification or expression among GBM cases. Similarly the ages of TP53 immunohistochemically positive and negative cases were not markedly different from one another for GBM. On the other hand, GBM cases with TP53 mutation were found to be younger compared with cases that did not have mutations (Wiencke et al., 2005).

Quan et al found no difference in frequency of EGFR amplification in patients younger than age 50 compared with patients who were 50 years of age or older (Quan et al., 2005). Asian studies have also shown different results.

Shinojima et al observed that though the mean age of patients with EGFR overexpression and EGFR amplification was higher than that of patients without EGFR overexpression, although no statistically significant difference was found (Shinojima et al., 2003).

A study from China revealed that overexpression of EGFR may be closely related with the patient's age but not with the tumors'pathological pathway (Xie et al., 2005).

An analysis of GBMs occurring in Singaporean population showed no significant association between age and expression of either EGFR or EGFR truncated type III. Neither was there any significant association between p53 over-expression and age at diagnosis. Three mutations previously undocumented in glioblastomas were also detected (Das et al., 2002). These facts prove that development of glioblastomas in Asian patients may not be identical to previously accepted models. Another interesting fact to be remembered in this context is the different pattern of EGFR expression in lung carcinomas reported to be occurring in Asian patients (Jiang et al., 2009).

Thus some studies showed that EGFR and p53 expression varied significantly with age, whereas other studies failed to reveal significant correlation of p53 and EGFR with age. Ethnicity also appears to influence the pattern of EGFR and p53 alterations observed in relation to age.

In our study, four subsets of GBMs were evident when the simultaneous expression of p53 and EGFR protein was analyzed in our study. Of the 61 GBM cases, 47.5% were p53 positive only, 18% were EGFR positive only, 16.5% were negative for both and 18% were positive for both. Thus maximum number of cases were seen in the p53 + / EGFR-category. GBM cases that showed dual positivity and dual negativity were also evident. A striking feature in our study was a higher mean age in the dual positive category compared to the other three categories. The association between age, p53 and EGFR alterations and survival were analyzed in other studies also. Although some studies showed that p53 and EGFR alterations showed inverse correlation and were mutually exclusive (Barker et al., 2001; Houillier et al., 2006), others have shown that co-expression can be seen. In a recent study of 194 primary GBMs, a worse outcome was observed in cases with concurrent EGFR and p53 alterations (Ruano et al., 2009).

In a study using FISH analysis, it was observed that EGFR gene amplification occurs commonly in glioblastoma cells with TP53 mutation, probably reflecting propensity of mutant p53 to induce genomic stability and gene amplification. EGFR gene amplification was seen preferentially in the infiltrative edges (Okada et al., 2003). Our conventional methods may not demonstrate these subtle effects making it more difficult to categorize gliomas based on p53 and EGFR alterations. In conclusion p53 and EGFR alterations can be seen concurrently and can show overlap especially with respect to age at diagnosis. However, the exact prognostic significance of co-expression of p53 and EGFR in glioblastomas has to be investigated extensively to explore more therapeutic options.

Another interesting finding in our study was that p53 expression was significantly greater in females; whereas this was not observed with EGFR in cases of GBMs. Few studies have investigated this aspect. Previous studies had also documented that tumors from women were somewhat more likely to have p53 mutations than men (Chen et al., 2001; Ohgaki et al., 2004). Is there any relation between hormonal expression and p53 expression? An extensive search of literature reveals that progesterone can act as a neurosteroid and can induce cell growth in astrocytomas by binding to its nuclear receptor (Inoue et al., 2002; González-Aguero et al., 2007; Cabrera-Munoz et al., 2009). Another recent study has also stressed that hormonal changes in pregnancy increases the growth rate of gliomas (Pallud et al., 2010). It is beyond the scope of our study to explain the increased p53 expression in females. However this aspect has to be investigated to explore any possible therapeutic options.

The facts presented in our study emphasize that more extensive research has to be carried in India and other South -East Asian countries to understand the molecular signatures of gliomas, especially glioblastomas.

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