Expression of p53 & epidermal growth factor receptor in glioblastoma

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Background & objectives: Glioblastoma (GB) is the most frequent brain tumour, manifesting at any age, with a peak incidence between 45 and 75 years. Primary and secondary GBs constitute relatively distinct disease entities in evolution, in expression profiles and in therapeutic response. Histopathologically, primary and secondary GBs are indistinguishable. The aim of this investigation was to study the immunohistochemical (IHC) expression of p53 and epidermal growth factor receptor (EGFR) in GB with the objective of categorizing the morphological variants of GB into primary and secondary based on the presence of low-grade areas and knowing the variable expression of p53 and EGFR in primary and secondary GB.

Methods: A total of 28 patients with GB were studied and categorized into primary and secondary based on the presence of low-grade areas, i.e. discernible astrocytic morphology, gemistocyte and oligodendroglia. Tumours with the presence of combination of the above features or any one of the above features were taken as secondary GB, whereas tumours with highly pleomorphic areas were considered as primary GB. IHC was done on the representative tissue blocks for p53 and EGFR.

Results: Majority of the patients were in the fifth and sixth decades of life with a mean age of 46.96±13 yr with male preponderance (male:female 2.5:1). Mean age of presentation was 48.93±12 yr in primary and 44.69±15 yr in secondary GB. All cases of GB were classified into primary (53.57%) and secondary (46.43%) based on morphology. EGFR was more frequently expressed than p53. Based on IHC, 50 per cent of cases were classified into primary, three per cent into secondary and 47 per cent as unclassified.

Interpretation & conclusions: Histopathological features, i.e. presence of low-grade areas, may play a role in classifying GB into primary and secondary. EGFR has a pivotal role in gliomagenesis. Combination of p53 and EGFR alone may not be sufficient to clarify GB into primary and secondary.

Key words Epidermal growth factor receptor - glioblastoma - immunohistochemistry - p53

Glioblastoma (GB) is the most frequent brain tumour, accounting for approximately 12-15 per cent of all intracranial neoplasms and 65-75 per cent of astrocytic tumours. It manifests at any age, preferentially with peak incidence between 45 and 75 yr of age. The most common genetic alterations in GB include loss of heterozygosity on chromosome 10 (LOH 10), mutations in p53, amplification and rearrangements of epidermal growth factor receptor (EGFR) gene, murine double minute 2 (MDM2) amplification, phosphatase and tensin (PTEN) homology mutations, tumour suppressor genes p16INK4a/p14ARF loss and
prominent microvascular proliferation and/or necrosis with marked nuclear atypia, brisk mitotic activity, differentiated, pleomorphic astrocytic tumour cells are indistinguishable with the presence of poorly histopathologically, primary and secondary GBs. p53 mutations occur in >65 per cent of secondary GBs. Primary GB constitutes around 90 per cent of cases, whereas secondary GB constitutes 10 per cent of cases.

Primary and secondary GBs constitute relatively distinct disease entities that evolve primarily through different genetic pathways and show different expression profiles and are likely to differ in response to therapy but share a high frequency of LOH 10q, which is likely to be associated with the overall GB phenotype. The secondary GBs developed in astrocytomas must be distinguished from primary GBs because these are probably responsible for most of the GBs of long clinical duration. However, histopathologically, primary and secondary GBs are indistinguishable with the presence of poorly differentiated, pleomorphic astrocytic tumour cells with marked nuclear atypia, brisk mitotic activity, prominent microvascular proliferation and/or necrosis. The lack of histopathological separation has made it difficult to estimate the relative frequency at which both subsets of GB occur. Because of these reasons, GBs are classified into primary and secondary based on clinical, radiological and morphologic evidence of less malignant precursor lesion. However, in the 2016 World Health Organization (WHO) classification of CNS tumours, a few histopathological findings which are different in primary and secondary GB are mentioned, i.e. extensive areas of necrosis seen in isocitrate dehydrogenase (IDH)-wild type is primary GB and IDH mutant type with limited areas of necrosis is secondary GB.

More differentiated neoplastic astrocytes are usually discernible, at least focally in GB resulting from the progression of diffuse astrocytoma WHO grade II, i.e. in secondary GBs. GBs with oligodendroglioma component are frequently secondary neoplasms with higher frequency of IDH-1 mutations and a lower frequency of PTEN mutations. However, diagnostic difficulties may arise due to the heterogeneity of the tumour, morphological overlap with other gliomas or partial sampling of the lesion. As a result, several studies have used molecular techniques aiming to find biomarkers with diagnostic and/or prognostic relevance. The subtypes of GB are classified partially based on p53 and EGFR status. p53 negative and EGFR positive is the characteristic immunohistochemical (IHC) profile of primary GB, and p53 positive and EGFR negative is the characteristic IHC profile of secondary GB.

p53 mutations are also noted in precursor low-grade lesions (59%) and anaplastic astrocytomas (53%)2. It indicates that p53 mutations are early and frequent genetic alterations in pathways, leading to secondary GB. Only the abnormal or mutated variant of p53 becomes visible by IHC. Cells with impaired function of p53 may develop genetic aberrations leading to the development of malignancies. In secondary GBs in which mutations in the TP53 gene are more common than in primary lesions, p53 may be mutated in more than 65 per cent of the cases.

EGFR is a transmembrane receptor with several major truncated variants. The most common is variant III. EGFR mutations are tumour-specific and provide promising target for therapy. EGFR expression is a characteristic of primary GB. The molecular-targeted therapy has enabled significant progress, especially with EGFR-targeted therapy. EGFR-targeted therapy includes monoclonal antibodies (mAbs) and tyrosine kinase inhibitors (TKIs). Several phase I and phase II clinical trials of EGFR TKIs and mAbs have been conducted in malignant gliomas.

High protein levels of EGFR occur in about 90 per cent of astrocytic tumours, suggesting that alterations in transcription and translation of these genes may also participate in tumorigenesis. Alterations in EGFR gene are more commonly found in primary GBs. Specifically, EGFR is amplified in 40 per cent, overexpressed in 60 per cent and mutated in 20-30 per cent of the patients. Amplifications and rearrangements of EGFR are highly indicative of high-grade gliomas, with a worse prognosis than estimated from just histopathological grading. This fact has prompted the investigation of EGFR inhibitors aiming to promote apoptosis of cancer cells and increasing tumour sensitivity to possible adjuvant therapies. EGFR gene amplification is the hallmark of primary GB.

In this study an attempt was made to classify GBs into primary and secondary based on low-grade areas
in histopathological examination and to study the expression of p53 and EGFR in histopathologically classified primary and secondary GB.

**Material & Methods**

A prospective study was conducted from May 2012 to September 2014 in the department of Pathology, Sri Venkateswara Institute of Medical Sciences (SVIMS), Tirupati, India, in all the patients of GB diagnosed on histopathological examination of surgically excised specimens and classified according to 2007 WHO guidelines. All eligible patients diagnosed to have GB including small cell variant, gliosarcoma and giant cell variant based on light microscopy were included in the study. Patients not willing to participate were excluded from the study. The study was conducted after obtaining prior approval from the Institutional Ethics Committee (I.E.C No: 309/13-05-2013) and written informed consent from the patients.

A total of 28 patients were studied. All patients were categorized into primary and secondary based on the presence of low-grade areas (i.e. discernible astrocytic morphology, gemistocyte and oligodendroglia). Tumours with the presence of combination of the above features or any one of the above features were taken as secondary GB whereas tumours with highly pleomorphic areas were considered as primary GBs. Secondary GB cases were differentiated from oligoastrocytomas, gemistocytic astrocytomas based on the presence of areas of necrosis and microvascular proliferation. For IHC, representative paraffin blocks were selected based on hematoxylin and eosin (H & E)-stained slides and IHC was done for the markers p53 and EGFR according to the procedure provided in the kit (Biogenex, India). Peroxidase, antiperoxidase technique was adopted for IHC analysis

For p53 clone, D07 mouse monoclonal antibody, and for EGFR, polyclonal rabbit antibody were procured from Biogenex, India. Scoring of p53 and EGFR was done by taking proportional and intensity score 0-3. p53 scoring was done using four-point scale. Scores 0 and 1 were considered negative and scores 2 and 3 were considered positive. EGFR scoring was done by three-point scale, 0-2. Score 0 was considered negative, score 1 and 2 were considered positive.

**Statistical analysis:** The difference between expression of p53 and EGFR in primary and secondary GB was evaluated using Chi-square test and Fisher’s exact test. Agreement between the results in histopathology and IHC methods was evaluated by performing Spearman rank correlation. Statistical analysis was carried out using SPSS version 20 (IBM Corp., Armonk, NY, USA).

**Results**

There were 20 (72%) male and 8 (28%) female patients, with a male:female ratio of 2.5:1. The mean age was 46.96±13 yr with a range of 18-72 years. Based on histopathology, 15 (54%) tumours were classified into primary and 13 (46%) as secondary GB. Mean age of patients with primary GB (n=15) was 48.93±12 yr, and that of secondary GB (n=13) was 44.69±15 years.

GB with the presence of low-grade areas were classified as secondary (Fig. 1) and those without low-grade areas and with the presence of highly pleomorphic areas were classified as primary GB (Figs 2-5).

Low-grade areas with discernible astrocytic morphology (Fig. 6) were seen in eight, gemistocytes in seven and oligodendroglial component in seven patients. Combination of low-grade areas with discernible astrocytic morphology and gemistocytes (Fig. 7) was seen in three patients, low-grade areas with discernible astrocytic morphology and oligodendrogial component (Fig. 8) was seen in one and gemistocytes and oligodendroglial component was seen in three patients. All these features were observed in one patient. Based on IHC (Figs 9-12), 14 (50%) patients were classified into primary and one (3%) into secondary GB. Thirteen cases were undetermined or showed overlapping.

Seven tumours could be classified as primary GB and only one as secondary GB based on histopathology.
and IHC. Seven (25%) cases which were classified as secondary GB based on histopathology showed characteristic IHC profile of primary GB. Overall, no significant correlation was observed in classifications made based on two techniques. These results suggested that IHC did not play a major role in classifying cases into secondary GB. However, there was an agreement of histopathology and IHC methods in classifying 25 per cent of the cases as primary GB.

Fig. 2. Primary glioblastoma with highly pleomorphic areas (H & E, 20×10) (arrow).

Fig. 3. Primary glioblastoma with extensive microvascular proliferation (H & E, 10×10) (arrow).

Fig. 4. Glioblastoma with typical pseudopalisading necrosis (H & E, 4×10) (arrow).

Fig. 5. Glioblastoma with extensive areas of coagulative tumour necrosis (H & E, 4×10) (arrow).

Overall, EGFR expression (Figs 11 and 12) was observed in 100 per cent (15/15) of primary GB and 84.62 per cent (11/13) of secondary GB, whereas expression of p53 (Figs 9 and 11) was 53.33 per cent (8/15) in histopathologically classified primary GB and 38.46 per cent (5/13) in histopathologically classified secondary GBs. Expression of both p53 and EGFR was seen in 53.33 per cent (8/15) of histopathologically classified primary GB and 30.77 per cent (4/13) of histopathologically classified secondary GB. One patient with secondary GB did not show p53 and EGFR expression (Table I). Different morphologic variants were observed in both types of GB, i.e. classical, giant cell variant, small cell variant and gliosarcoma. Most frequent variant among both types was classical GB. The results of the present study were compared and analyzed with other studies from world literature (Tables II and III).

Discussion

In the present study, an attempt was made to classify GB into primary and secondary based on histopathological findings. Distribution of low-grade areas with discernible astrocytic morphology was seen in eight cases, with gemistocytes in seven and
oligodendrogial component in seven cases of secondary GB. This emphasizes the fact that histopathological features are important in classifying GB.

Overall EGFR expression was more compared to p53 in our study. Similar results were noted by others\textsuperscript{11,23}. Regarding the immunostaining positivity for EGFR, the pattern of immunostaining obtained is the result of technical pre-analytical issues such as fixation or processing of the material and the labelling may vary according to the technique used, the exposure time to antibodies and lesion sampling\textsuperscript{14}. Overexpression of EGFR contributes to the differentiation, proliferation, survival, migration and invasiveness of cancer cells and increases tumour angiogenesis. All these features reduce the responsiveness to chemotherapy and radiotherapy\textsuperscript{14}.

The present study results were similar to Lee et al\textsuperscript{11} in p53 expression, but contrary to Das et al\textsuperscript{22}, who showed 96 per cent.

p53 mutations are genetic hallmarks of secondary GB found in more than 65 per cent of the previous studies\textsuperscript{10,12} and are significantly less frequent (approximately 25 per cent in primary GB)\textsuperscript{3,10}. EGFR amplification occurs in approximately 40 per cent of primary GB\textsuperscript{4} but rarely in secondary GB.

p53+/EGFR− is characteristic IHC profile of secondary GB whereas p53−/EGFR+ is the profile of primary GB\textsuperscript{11}. Fourteen (50%) cases were classified into primary with p53−/EGFR+ IHC profile, one (3%) into secondary GB with p53+/EGFR− IHC profile and 13 (47%) were categorized into unclassified type with p53+/EGFR+ (42.86%) and p53−/EGFR− (3.52%) IHC.
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profile in view of overlapping features. Unexpected IHC expression of p53+/EGFR+ and p53−/EGFR− was also noted by Lee et al11 in 20.7 and 9.3 per cent of cases, respectively, which showed certain limitations to classify GB based on IHC.

The present study showed p53+ and EGFR+ IHC profile in 53.33 per cent (8/15) cases of primary GB and 30.77 per cent (4/13) cases of secondary GB. Frequent overlapping was noted in primary GB. However, coexistence of p53 mutation and EGFR was observed in subsets of primary GB24. Thomas et al25 reported that the glioma cell line SKMG-3 had an unusual genotype in which p53 gene mutation coexisted with an amplified EGFR gene. Ruano et al23 also reported a subgroup of GB showing simultaneous alteration of p53 and EGFR pathways which were associated with worse clinical outcome. In the present study the patients were not followed up on clinical outcome which is a limiting factor. The hypothesis in literature for such results is that mutant p53 induces genomic instability 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 GB formation23. Similar type of overlapping was observed by Das et al22 in the Asian population.

According to Aldape et al26, high-throughput analysis has identified molecular subtypes and has led to progress in more accurate classification of GB. In the WHO 2016 classification of CNS tumours7, GB is classified based on IDH status. GB with wild-type IDH is classified as primary GB, while GB with mutant IDH is classified as secondary GB and GB not otherwise classified as primary or secondary is classified as anaplastic GB.

**Table 1. Immunohistochemical expression in primary and secondary glioblastoma**

|                | Primary glioblastoma | Secondary glioblastoma |
|----------------|----------------------|------------------------|
|                | (n=15)               | (n=13)                 |
| p53            | 8 (53.33)            | 5 (38.46)              |
| EGFR           | 15 (100)             | 11 (84.62)             |
| p53 negative/EGFR positive | 7 (46.67) | 7 (53.85) |
| p53 positive/EGFR negative | 0 | 1 (7.69) |
| p53 positive/EGFR positive | 8 (53.33) | 4 (30.77) |
| p53 negative/EGFR negative | 0 | 1 (7.69) |

EGFR, epidermal growth factor receptor; IHC, immunohistochemistry

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Fig. 10. Epidermal growth factor receptor (control) - squamous cell carcinoma of buccal mucosa with membrane positivity (IHC- score 2; 40×10) (arrow).

Fig. 11. p53 - More than 30 per cent of cells showing nuclear positivity (test) (IHC- score 3; 40×10) (arrow).

Fig. 12. Epidermal growth factor receptor - intense diffuse, membranous and cytoplasmic positivity (test) (IHC- score 2; 40×10) (arrow).
Comparison of immunohistochemical profile

| IHC marker | Present study (n=28) n (%) | Das et al2 (n=50) n (%) | Lee et al1 (n=150) n (%) | Ruano et al3 (n=150) n (%) |
|------------|----------------------------|-----------------------|------------------------|-------------------------|
| p53        | 13/28 (46.43)              | PAb 240* (96)         | 74/150 (49.3)            | 21/187 (11.2)           |
| EGFR       | 26/28 (92.86)              | 26/50 (52)            | 92/150 (61.2)            | 127/167 (76)            |

*PAb 240 - type of antibody. IHC, immunohistochemistry

In conclusion, results of the current study indicate that histopathological features, i.e. presence of low-grade areas, play a role in classifying GB into primary and secondary. Frequent expression of EGFR in GB indicates that this particular receptor has got pivotal role in gliomagenesis. Combination of EGFR and p53 alone may not be sufficient to classify GB on IHC into primary and secondary. Though the IHC expression of p53 and EGFR could not classify GB in majority of the cases, variable expression of these markers suggests further studies to include IDH mutation and may be able to provide preliminary information regarding prognosis as expression of p53 and EGFR carries worse prognosis even without evaluating IDH mutation.

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Conflicts of Interest: None.

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