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Analysis of frequency of alterations in EGFR exon 18-21 in Glioblastoma multiforme and its prognostic significance – A single centre experience from India.

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ABSTRACT

Background*: Glioblastoma multiforme (GBM) is a disease with poorest outcome among all central nervous system malignancies. Alteration in epidermal growth factor receptors (EGFR) is reported in GBM and may be a prognostic and predictive marker of overall disease outcome.*

Objective: *We aimed to analyze the frequency of alteration of EGFR exon 18-21 in patients with GBM and their outcomes after standard treatment.*

Methods: *Since there are no study from southern part of India this was conceived as a pilot, retrospective study in a single tertiary cancer center in South India.*

Results: *Forty patients with GBM who had their entire treatment done at this centre were identified and their primary tumor tissue blocks were retrieved. Genomic DNA was extracted and polymerase chain reaction (PCR) for high resolution melting (HRM) analysis were performed and analyzed. The results of mutational analysis were correlated with treatment outcome of the patient. Our study found a significant difference in the overall survival (OS) and progression free survival (PFS) between patients with presence or absence of EGFR exon-19 overexpression.*

Conclusion: *This study found EGFR exon-19 overexpression to be an independent negative predictor of OS and PFS in GBM patients treated with present standard of care.*

Key Words: *Glioblastoma Multiforme, Epidermal growth factor receptor, Alteration, Mutation*

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I. Introduction

Glioblastoma multiforme (GBM) and its variants (giant cell glioblastoma, gliosarcoma, epithelioid glioblastoma), classified as grade IV tumors of central nervous system (CNS) are the most malignant forms of primary brain tumors.[1] According to CBTRUS statistical report of CNS tumors in United states for 2011-2015, GBM accounts for 14.7% of all intracranial tumors and at 47.7% is the most common of all malignant brain tumors. [2] India has a limited population based cancer registry and therefore we depend on hospital cancer registry data which generally provides a skewed understanding of incidence and mortality. A decade old study from Tata Memorial Hospital, India involving 656 adult patients with CNS tumors reported 38.7% gliomas, and among these 59.5% were high-grade gliomas.[3] GBM can affect patients at any age but has a peak incidence between ages 45 and 75 years. A multi-institutional study in pediatric brain tumors in India, reported GBM to account for 4.46% of all astrocytomas among children. [4] Our own institutional unpublished data shows the median age to be 51.5 years (16-75 years).

GBM has the poorest overall survival (OS), with only 0.05% to 4.7% of patients surviving five years past their diagnosis. [5] The attempts at aggressive treatment for GBM have yielded modest results. The ineffectiveness of conventional cytotoxic drugs like alkylating agents, topoisomerase inhibitors could be primarily be due to their non-specific, non-targeted nature and its inability to cross the blood-brain and blood-tumor barrier.[6] Since the turn of the century molecular, cytogenetic and array-based assays of comparative genomic hybridization and RNA expression have opened doors to understanding of genetic alterations which are likely to be causative of gliomas. [7] Amplification of EGFR gene leads to over-expression of the transmembrane tyrosine kinase receptor and is a common genetic alteration in GBM. [8]

EGFR tyrosine kinase receptors have been effectively targeted in other tumor types. In a limited therapeutic scenario in GBM treatment, successful targeting of newer sites may provide respite to these patients

who have an otherwise poor prognosis. Patient populations across the continents have different genetic profile; hence it is important to have local data in one's own community, to understand the disease and its varied behavior which would assist in therapeutic decision making.

We analyzed the frequency of alterations of EGFR Exon 18 - 21 amongst our patients of GBM to assess the presence of molecular alterations and its impact on the disease.

II. Methods

This was a single center, non randomized, retrospective pilot study in patients diagnosed to have GBM. Only those patients who received their treatment at this single tertiary care hospital and had documented follow up records until the time of study initiation were included. Patients who were lost to follow up or those with clinical outcomes not available were excluded from the study. A total of forty patient tumor samples were identified and included in the study. The protocol was reviewed and approved by the institutional review board.

The formalin fixed paraffin embedded (FFPE) tumor blocks were retrieved from the department of pathology archives and were tested for the EGFR sequences (Exon 18-21). Tissue sections of 5 µm thickness were obtained from FFPE blocks and stained with methyl green. The tumor rich areas were micro-dissected using a 21G needle and the samples were subjected to proteinase K digestion in a rotating incubator at 56°C for 3 days. Genomic DNA was extracted using the DNeasy Tissue kit (Qiagen, Hilden, Germany) and was kept at 4°C before use.

High resolution melting (HRM) analysis technique was used as it detects almost all alterations at DNA level. In HRM positive controls are not necessary since this technique is not specific for any particular mutation. PCR for HRM analysis was performed in 0.1 ml tubes on the Rotor-Gene 6000TM (Corbett Research, Sydney, Australia) in the presence of the fluorescent DNA intercalating dye, SYTO 9 (Invitrogen, Carlsbad, CA). The reaction mixture in a 20 µl final volume contained; 1× PCR buffer, 2.5 mM MgCl2, 200–400 nM forward primer, 200–400 nM reverse primer, 5 ng of genomic DNA, 200 µM of dNTPs, 5 µM of SYTO 9, 0.5 U of HotStarTaq (Qiagen) polymerase and PCR grade water. The cycling and melting conditions for *EGFR* exons 18 and 19 were as follows; one cycle of 95°C for 15 min; 45–50 cycles of 95°C for 10 s, 65°C for 10 s with an initial 10 cycles of touchdown (1°C/cycle), 72°C for 30 s; one cycle of 97°C for 1 min and a melt from 70°C to 95°C rising 0.2°C per second. The genomic DNA was diluted to 2.5 ng/µl (5 ng tested) to provide a consistent testing condition. All samples were tested in duplicate.

HRM analysis

High resolution melting analysis was performed on the Rotor-Gene 6000 Software (v1.7). The normalized graph and the difference graph were used to analyze the data. The normalized graph was generated by the monitoring of dissociation of the fluorescent dye from double-stranded DNA as the temperature increased. The dye (SYTO 9) used in the current study can only fluoresce when it is intercalated into double-strand DNA. The normalized graph shows the degree of reduction in fluorescence over a temperature range (typically 70°C to 95°C).

All samples including the wild-type were plotted according to their melting profiles. In the difference graph, the melting profiles of each sample were compared to that of the wild-type which was converted to a horizontal line. Significant deviations from the horizontal line (relative to the spread of the wild type controls) were indicative of sequence changes within the amplicon analyzed. Samples with aberrant melting curves were recorded as HRM mutation positive. Wild type DNA controls were used in analyzing the HRM data and the fragments showing a pattern change in melting curve from wild type fragment were only reported as mutation positive.

The HRM analysis was done on isolated tumor DNA. So the changes detected in our study are essentially at the DNA level. The results of mutational analysis were correlated with patient demographics and treatment outcome of the patient.

III. Statistical Analysis

Descriptive statistics were used to describe patient characteristics as frequencies. The actual values relating to the patient characteristics are mentioned in mean or median values. Statistical analysis was carried out using the IBM SPSS version 20 software. Survival outcome analysis was done using the Kaplan Meier method. Association between the groups and various parameters (age, extent of surgical excision, size of the tumor etc) was looked at using the Log Rank test.

IV. Results

This retrospective study included forty patients. All patients received conventional treatment with maximal safe resection followed by chemoradiation and adjuvant Temozolomide. The radiation dose was 6000

cGy in 30 fractions. The concurrent Temozolomide was dosed at 75 mg/m^2 and the adjuvant was administered at 150 - 200 mg/m^2 days 1-5 every 4 weeks for six cycles.

EGFR exon 18 and 19 alterations were detected in the 12.5% (n=5) and 42.5% (n=17) of tumor samples respectively. No alterations were detected in EGFR exons 20 and 21. The clinical characteristic features of subjects with the alterations in exon 19 are tabulated in Table 1.

Table 1: Characteristics of EGFR Exon 19 alteration positive and negative patients

		Exon 19 POSITIVE (%)	**Exon 19 NEGATIVE (%)**	**pValue**
Total		17 (42.5)	23 (57.5)	
Sex	Males	12(70.6)	12(52.2)	
	Females	5(29.4)	11(47.8)	0.240
Age		46.41 years	51.26 years	0.320
Size	< 5 cms	8(47.1)	15(65.2)	
	> 5 Cms	9(52.9)	8(34.8)	0.250
Resection	Total	9(52.9)	16(69.6)	
	Near total	2(11.8)	5(21.7)	0.100
	Suboptimal	6(35.3)	2(8.7)	
Outcome	Alive	4 (23.5)	12 (52.2)	
	Dead	13 (76.5)	11 (47.8)	<0.001

The median progression free survival (PFS) time among the whole cohort was 10.53 months. The median PFS time in the EGFR exon 19 altered group was 7.36 months and the negative group was 13.0 months (p <0.001) [Figure 1]. The median OS time in the EGFR exon 19 mutation positive patients was 7.3 months compared to 15.4 months in negative group. This survival difference was statistically significant (p <0.001) [Figure 2]. There was no statistically significant difference in the exon 18 altered and normal patients.

Fig 1: Kaplan Meier Graph for Progression Free Survival in EGFR Exon 19

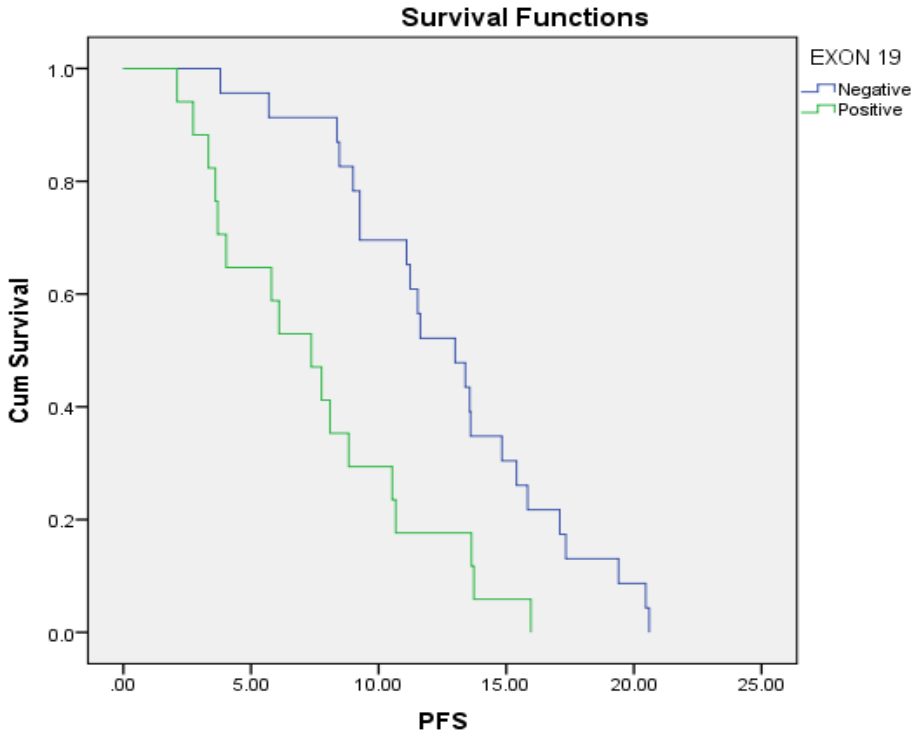
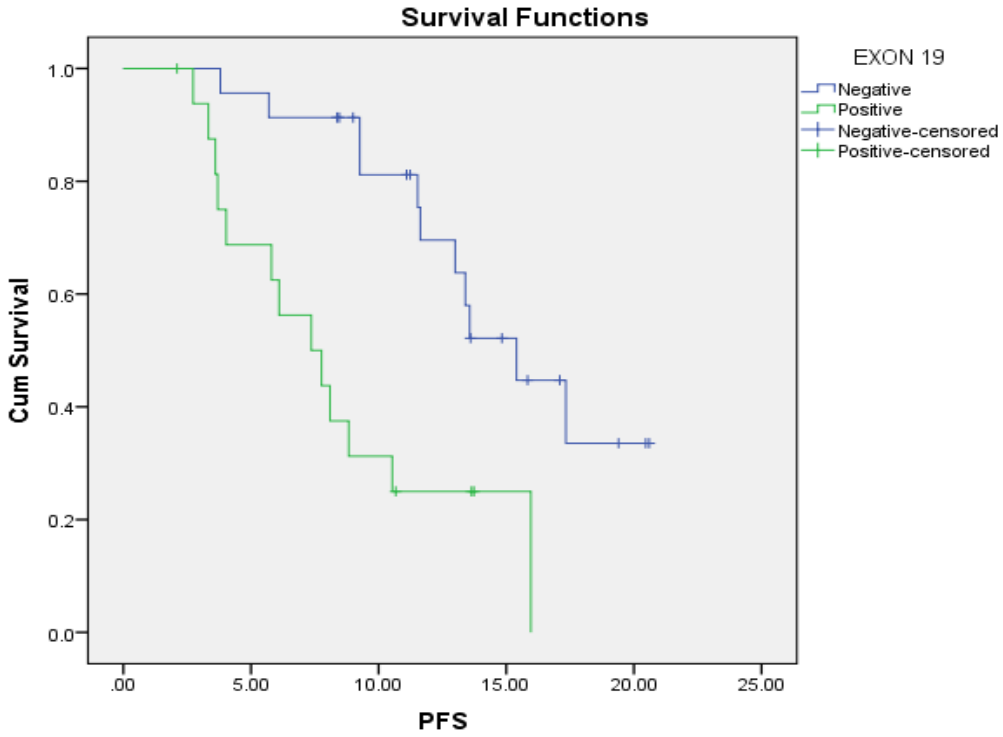


Fig 2: Kaplan Meier Graph for Overall Survival in EGFR Exon 19



V. Discussion

GBM being a disease with a very short median survival, elucidating both prognostic and predictive biomarkers have a very important role. Amplification of the EGFR gene is a common genetic event in high-grade astrocytomas and occurs in about half of GBM. It leads to overexpression at both the mRNA and protein level, however, over expression without this genetic event occurs as well. [9]

The mean age in our cohort was 50-years and the mean age among patients who expressed the EGFR alteration was 47 years. This was a similar observation as in other larger studies. [10] The influence of EGFR alterations, EGFR gene amplification in patient prognosis has been highly controversial for gliomas. [11] We observed that the frequency of GBM with EGFR alteration in exon 18 (12.5%) and exon 19 (42.5%) were higher in comparison with the published literature. We have represented for comparison some of the available studies in literature in table 2. [12-23]

In a study on non-small cell lung cancer where EGFR has a well-established role, Eberhard et al and Chen et al recorded most of the EGFR mutations to be localized in the TK domain on exons 18 through 21. This holds value since these mutations have been found to be sensitive to treatment with newer tyrosine kinase inhibitors. [24, 25] Based on this information we studied the frequency of alteration of EGFR with respect to exon 18-21. Our finding of EGFR alteration is contrary to those reported in literature. Marie Y et al reports not finding any mutations in exons 19 and 21 of the EGFR tyrosine kinase domain in 95 gliomas including glioblastomas, anaplastic oligodendrogliomas, and low-grade gliomas. [26] An even larger study of tumors from patients treated on North American Brain Tumor Consortium trials 01-03 and 00-01 by Lassman et al did not find any lung signature mutations of EGFR exons 18 to 21. [27] However two recently reported study by Umesh S et al (2009)[22] and Arif SH et al (2015)[23] from India recorded EGFR overexpression in significant proportion of patients and it translated into poorer outcomes. This suggests that there is a definite geographic variation in the genetic behavior of tumors. This was clearly demonstrated in lung malignancy where Asian patients were found to have a higher frequency of EGFR mutation. [28]

The patients who were EGFR exon 19 positive had a significantly reduced OS and PFS as compared to the exon 19 negative patients suggesting that mutation in this domain translated into a poorer prognosis. This is in concurrence with a recent metaanalysis done by Li J et al which reported that overexpression of EGFR was an indicator of poor prognosis in glioblastoma multiforme patients (HR =1.57).[29]

Table 2: Comparable studies for EGFR alterations

Study Author / year	Number of cases	EGFR study	Result
S. H. Bigner, 1988 [12]	54	Alterations	No prognostic significance on OS
T. J. Pigott, 1993 [13]	88	Alterations	No prognostic significance on OS.
J. Schlegel,1994, [14]	72	Alterations	No prognostic significance on OS. No significant correlation between alterations and histological malignancy grade.
U. Diedrich, 1995 [15]	75	Alterations	No prognostic significance on PFS Significant correlation between amplification and histological malignancy grade.
A. Zhu, 1996 [16]	71	Alterations	Significant negative prognostic factor on OS and PFS with EGFR.
P . Korkolopoulou, 1997 [17]	51	Alterations	Significant negative prognosis on PFS.
C. Bouvier-Labit, 1998 [18]	63	Alterations	No prognostic significance on PFS and OS.
A. Chakravarti, M. 2001 [19]	81	Alterations	Statistical significant reduced OS.
N. Shinojima, 2003 [20]	87	EGFR V III alterations	Significant unfavorable predictor on OS for amplification. EGFRvIII showed a trend towards shorter OS.
A. B. Heimberger 2005 [21]	196	EGFR V III alterations	No prognostic significance on OS with EGFRvIII
S. Umesh, 2009 [22]	54	Alterations	Significant negative prognostic factor on OS.
S H Arif, 2015 [23]	40	EGFR/PTEN mutations in GBM	Better survival for patients for EGFR positive/PTEN negative mutation status.
Present study	40	Alterations of EGFR Exon sequences 18-21.	Significant OS and PFS difference in EGFR exon 19 subgroup

EGFR targeted treatment has been attempted. Gefitinib did not show objective responses, but provided evidence of disease control. Erlotinib which inhibits wild-type HER1/EGFR and EGFRvIII, on the other hand has shown more promising results. Monoclonal antibodies, radio-immuno conjugates, ligand-toxin conjugates, antisense oligonucleotides and ribozymes are the other agents being studied for its potential treatment utility in glioma. [30] The absence of mutation in exon 19 and 21 where Gefitinib acts was suggested as a likely difference in biology of EGFR in gliomas vis-à-vis lung cancer leading to resistance of glioblastomas to gefitinib. In the light of a higher percentage of patients with EGFR exon 19 mutations in our patient cohort, drugs like Gefitinib and Erlotinib may still hold value in treatment of recurrent glioma in patients in this subcontinent.

EGFR mutations have also been found to promote tumorigenesis through a SOX9 and FOXG1-dependent transcriptional regulatory network in vitro and in vivo models suggesting a role of transcriptional / epigenetic remodeling in EGFR-dependent pathogenesis which could be translated into a basis for epigenetic therapy. [31]

The limitations of our study have been the small sample size. Due to cost constraints we have not been able to verify the HRM positive samples using other real time PCR based assays or next generation sequencing to confirm the alterations identified in our study. We did not do Sanger sequencing due to its lower sensitivity compared to HRM. A number of studies have compared the sensitivity of Sanger and found very low mutation pick up rate with Sanger compared to HRM and Taqman probe based assays. So even if we would have done Sanger sequencing, we could have confirmed only a partial number of mutations from the sample pool.
However in a cost constrained setting we believe this study has a significant role in raising a research question regarding inherent geographical variation in the Indian population which needs to be answered in a larger genomic study.

VI. Conclusions

Based on our study in the Indian context, in patients with GBM, EGFR overexpression is not uncommon and carries a poor prognosis.

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Conflict of Interests Disclosure: None.

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