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EGFR and *PTEN* Gene Mutation Status in Glioblastoma Patients and their Prognostic Impact on Patient's Survival

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Abstract

Glioblastoma multiforme (GBM) is the most aggressive form of glioma. Genetic analysis of GBM tumorigenesis has identified several alterations in particular *EGFR* and *PTEN* genes. The purpose of the present study was to analyze the frequency and distribution of *EGFR/PTEN* mutations in GBM and to determine their relationship with different clinicopathological characteristics.

The paired tumor and adjacent normal tissue specimens of 40 consecutive patients with GBM were examined and DNA preparations were evaluated for the occurrence of *EGFR*/*PTEN* gene mutations by PCR-SCCP and DNA sequencing.

In total, 20 of 40 (50%) GBM tumours had mutation of either an *EGFR* or *PTEN*. *EGFR* gene mutation was present in 13 (32.5%) and *PTEN* gene mutations in 07 of 40 (17.5%) patients. Both *EGFR/PTEN* mutations were found in 03 of 40 samples (7.5%). The samples which showed *EGFR* mutations but were negative for *PTEN* were detected in 10 of 40 (25%) patients (*EGF*R+ve/*PTEN*-ve). The samples with *PTEN* +ve/*EGFR* –ve were present in 04 of 40 (10%) patients. Median PFS and Median OS was better in patients with *EGFR* +ve/*PTEN* -ve (p>0.05).

EGFR and *PTEN* gene mutations exist in our population with GBM and play a significant role in its development with better survival for patients for *EGFR*+ve/PTE –ve mutation status.

Keywords: Glioblastoma multiforme; Mutations; Overall survival; DNA sequencing

Introduction

Glioblastoma multiforme (GBM) is the most common primary brain tumor in adults [1]. Although GBM occurs in patients of all ages, the incidence is highest in the elderly, and GBM is slightly more common in whites and men [1,2]. Astrocytic tumors are the most common glial neoplasms, with an annual incidence of 3-4/100,000 inhabitants, and approximately 80% are glioblastomas [3]. In India the incidence is about 3/100,000 population [4]. In Kashmir (North India), among the brain tumors glioma (60%) is the commonest in which GBM is the most common followed by diffuse and anaplastic astrocytoma [5]. Glioblastomas remain one of the most lethal forms of cancers with a median survival of 10 to 12 months [6]. Unlike most other types of cancer, glioblastomas rarely metastasize; rather, they induce death through striking resistance to current therapies and invasion into normal brain tissues [7]. Recent therapies in GBM have focused on the inhibition of tyrosine kinases and associated growth factor pathways. Over activity of the epidermal growth factor receptor (*EGFR*) pathway is associated with resistance to treatment with RT and chemotherapy [8,9]. Therefore, combining targeted *EGFR* therapy with RT or chemotherapy may increase the effectiveness of treatment.

It has been suggested that genetic alterations of certain genes are critical events behind the pathogenesis of gliomas. The *EGFR* is a receptor tyrosine kinase that regulates fundamental processes of cell growth and differentiation. Mechanisms for oncogenic conversion of *EGFR* in cancer include *EGFR* gene amplification, structural rearrangements of the receptor, overexpression of epidermal growth factor (*EGF*)–family ligands by tumor cells and/or surrounding stroma, and activating mutations in the EGFR kinase domain [10]. *EGFR* gene amplification (>40% of cases) and over-expression (>60% of cases) are a striking feature of GBM. The most common *EGFR* mutant is named *EGFR*vIII (*EGFR* type III, *EGFR*vIII, del2-7, Δ*EGFR*) [11]. Phosphatase and tensin homolog (*PTEN*), a tumor suppressor gene is a dual-specificity phosphatase. In general, *PTEN* negatively regulates the anti-apoptotic action of akt phosphorylation. In addition, other types of cancer and the inherited predisposition to cancer, Cowden disease, are associated with *PTEN* mutations [12]. It has been reported that mutations of *PTEN* have been implicated in the malignant progression of astrocytic gliomas, as these alterations are most frequently observed in GBM and very rare in the lower grade astrocytoma [13].

Cancer is a major disease burden worldwide but there are marked geographical variations in incidence and frequency overall and at specific organ sites. The valley of Kashmir situated at an altitude of about 1800 m to 2400 m above sea level is among one of the provincial territories of India. Kashmir, regarded worldwide as paradise on earth, with over 5 million populations is heavily burdened with cancer. This mountain locked region presents a strikingly different pattern of distribution of cancers not only from India but from most parts of world. So this study aims at finding the mutations, if any, in the hotspot regions of both *EGFR* and *PTEN* genes and their correlation in GBM patients owing to the fact that there is no data on genetic alterations in GBM available either in our population. It is the first initiative to study the recurrence and overall survival of the GBM patients in light of *EGFR* and *PTEN* mutations.

Materials and Methods

Patients

A total of 40 histologically confirmed, previously untreated GBM patients attending Department of Neurosurgery of Sher-I-Kashmir Institute of Medical Sciences (SKIMS), Srinagar were included in this study. The diagnosis of GBM was considered on the histopathological examination. Blood samples were available for 15 cases for ruling out the germ line mutations. A written pre informed consent was obtained from all cases. Demographic and clinicopathological characteristics of each patient were recorded. This study was approved by the Ethical committee of the SKIMS.

Sample collection/storage

05 ml of peripheral blood was obtained from each subject in EDTA containing vials (200 µl of 0.5 M, pH=8.0) and stored at -20°C till use. The surgically resected tissue samples taken through stereotactic/open biopsy of GBM tumors and adjacent normal tissues were collected directly into sterile vials containing chilled PBS (pH=7.2) and frozen at -70°C for molecular investigations. Histopathologically confirmed GBM tissues and corresponding normal tissues were used for mutational analysis of *EGFR* and *PTEN* gene.

Extraction and Quantitation of genomic DNA

DNA was extracted from the tissues by Phenol-Chloroform method and by Qiagen DNA extraction kit (Zymo Research Corporation, USA) while salting out method was used for the extraction of DNA from blood samples. The concentration of the DNA obtained was measured in a spectrophotometer at 260nm wavelength by using the formula: DNA µg/ml=A_{260} × 50 × dilution factor. The purity of DNA was checked by using A_{260}/A_{280} ratio. The quality of the DNA obtained from the tissue specimens and blood samples was analyzed on 1% agarose gel.

PCR amplification of EGFR and PTEN genes

Four hot spot coding exons (18, 19, 20 and 21) of *EGFR* gene and three coding exons of *PTEN* family of genes were amplified (exons 5, 7 and 8) using previously described specific primers (Table 1). PCR amplification was carried out in a 50 µL volume container with 50 ng of genomic DNA, 1XPCR buffer containing 15 mM $MgCl_2$, 100 µM each of dATP, dGTP, dTTP, dCTP, and 1.5 U of *Taq* DNA polymerase (Biotools; Madrid, Spain), and 1 µM of forward and reverse primers (Genescript; Piscataway, NJ, USA). The thermal conditions and product sizes of each exon for both *EGFR* and *PTEN* gene are given in Table 1. The PCR products were run on 2% agarose gel and analyzed under an ultraviolet illuminator. The single-strand conformation polymorphism (SSCP) analysis of the amplicons of exons 18, 19, 20 and 21 of *EGFR* gene and three coding exons 5, 7, and 8 of *PTEN* family of genes was performed on 6% non-denaturing polyacrylamide gel (PAGE) utilizing nonradioactive silver staining. The purified PCR amplicons of the tumor samples showing mobility shift on SSCP analysis and randomly chosen normal samples were used for direct DNA sequencing, using the automated DNA sequencer ABI Prism 310 Genetic Analyzer (Macrogen Korea).

Statistical analysis

Statistical analysis was performed by using SPSS 16.0 software. Fisher's exact test, Chi Square test for homogeneity of proportions and Odds ratio was used wherever applicable. Statistical significance was considered when $P<0.05$. Kaplan Meier curve for Progression free survival was drawn by using SPSS software. Survival time was calculated from the date of first surgery to the date of death or date of last contact if lost to follow up evaluation or Jan 31, 2011. Follow up ranged from 6 months to a maximum of 25 months. Progression-free survival was defined as the time from first surgery to first evidence of tumor progression on CT or MRI or to death [14]. Tumor progression was defined as the appearance of new lesions, an increase in tumor extension by 25% on CT or MRI, a worsening in the clinical/neurological condition, or an increased need for corticosteroids [15].

Results

The present study comprised of 40 histologically confirmed cases of GBM. There were 28 (70%) males and 12 (30%) females with a male female ratio of 2.3:1. The mean age of the patients was 52.9 ± 12.4 years. The patients ranged in age from 26-70 years (Table 2). Majority of the patients 27 (67.5%) had Karnofsky Performance Score of >70 and 13 (32.5%) had ≤ 70. Mean KPS was 78.2 ± 9.8. Majority of the tumors 19 (47.5%) were located on the Left side and 17 (42.5%) were present on the Right side. Midline tumors were observed in only 04 (10%) of patients. Majority of the tumors 12 (30%) involved more than one lobe. Among the single lobe involvement, temporal lobe 11 (27.5%) was the commonest. Other sites involved were frontal, parietal, midline; occipital and thalamic. Most of the patients 22 (55%) were subjected to gross total resection while as 13 (32.5%) were subjected to sub total resection and only biopsy was performed in 05 (12.5%) patients. All the patients were put on Gefitinib 250-500 mg/day.

Overall mutations in exon 18, 19, 20 and 21 of *EGFR* identified in this study aggregated to 32.5% (13 of 40). In all there were 13 missence mutations, seven were C>A transversions and six were A>T transversions. We detected two different single-nucleotide substitutions in 13 of the 40 GBM patients (Table 3). Codon 691 contained 21.42% (3/14), codon 737 comprised of 46.15% (6/13) mutations while as codon 742 contained 30.76% (4/13) mutations. In *PTEN* gene the present study looked for mutations in exon 5, 7 and 8 which are reported to be the hot spot exons. In *PTEN* gene we found mutations in 07 of 40 (17.5%) GBM patients. In all we detected 07 missence mutations in exon 5 and 7 but could not detect any mutations in exon 8. The mutations comprised of only transitions (3 A>G and 4 G>A) involving codon 449, 384 and 628. Codon 449 and 384 contained 28.57% (2 of 7) mutations each while as codon 628 comprised of 42.85% (3 of 7) mutations (Table 3).

Gene	Exon	Primer sequence	Tm (C)	Product size (bp)
EGFR	18	F-5´CCAAATGAGCTGGCAAGTG 3' R-5´TCCCAAACACTCAGTGAAACAAA 3'	58	397
	19	F-5´CCCAGTGTCCCTCACCTTC 3' R-5´GCAGGGTCTAGAGCAGAGCA 3'	62	306
	20	F-5´CATTCATGCGTCTTCACCTG 3' R- 5´CATATCCCCATGGCAAACTC 3'	58	377
	21	F-5´GCTCAGAGCCTGGCATGAA 3' R-5´CATCCTCCCCTGCATGTGT 3'	62	348
PTEN	5	F-5'GCAACATTTCTAAAGTTACCTACTTG 3' R-5'CCAATAAATTCTCAGATCCAGG 3'	50	378
	7	F-5'TGGTATGTATTTAACCATGC 3' R-5' CCTTATTTTGATATTTCTCCC 3'	57	231
	8/1	F-5'TGCAAATGTTTAACATAGGTGA 3' R-5'CCTTGTCATTATCTGCACGC 3'	55	246
	8/2	F-5 GGAAGTCTATGTGATCAAGA 3' R-5' CGTAAACACTGCTTCGAAATA 3'	53	286

Table 1: Primer sequences and annealing temperatures for sequencing of *EGFR* and *PTEN* genes.

Age (years)	Male		Female		Total		P value
	N	%	n	%	n	%	
30	4	14.3	0	0.0	4	10.0	0.135
31 to 40	2	7.1	1	8.3	3	7.5	
41 to 50	7	25.0	3	25.0	10	25.0	
51 to 60	8	28.6	2	16.7	10	25.0	
>60	7	25.0	6	50.0	13	32.5	
Total	28	70.0	12	30.0	40	100.0	
mean ± SD	50.9 ± 12.8		57.4 ± 10.7		52.9 ± 12.4		

Table 2: Age and gender distribution of the patients.

The overall frequencies of *EGFR* and *PTEN* mutations in GBM patients are shown in Table 4. In total, 20 of 40 (50%) GBM tumours studied had mutation of either an *EGFR* or *PTEN*. *EGFR* mutation was present in 13 of 40 (32.5%) patients and *PTEN* gene mutations in 07 of 40(17.5%) patients. Both *EGFR* and *PTEN* mutations were found in 03 samples (7.5%). The samples which showed EGFR mutations but were negative for *PTEN* were detected in 10 of 40 (25%) patients (*EGFR* +ve/*PTEN*-ve). The samples which showed *PTEN* mutations but were absent in *EGFR* (*PTEN* +ve /*EGFR*-ve) were present in 04 (10%) patients. No mutations were seen in both the genes (*EGFR*/*PTEN* both -ve) in 23 patients (57.5%) (Table 4).

GENE	Case No.	Sex	Age (years)	Exon	Nucleotide Change	Amino Acid Change
EGFR	1	M	62	19	742C>A	Pro>His
	2	M	26	19	742C>A	Pro>His
	3	M	50	18	691C>A	Pro>Thr
	4	M	45	19	737A>T	Pro>Thr
	8	M	65	20	737A>T	Lys>Ile
	14	F	62	21	742C>A	Pro>His
	17	M	35	21	742C>A	Pro>His
	18	F	60	20	737A>T	Lys>Ile
	22	M	26	21	691C>A	Pro>Thr
	24	M	45	20	737A>T	Lys>Ile
	28	M	60	20	737A>T	Lys>Ile
	34	F	70	21	691C>A	Pro>Thr
	37	M	65	20	737A>T	Lys>Ile
PTEN	1	M	62	5	449G>A	D152N
	3	M	50	5	449G>A	D152N
	6	F	65	5	384G>A	G128R
	10	F	50	7	628A>G	T211A
	18	F	60	7	628A>G	T211A
	26	M	58	7	628A>G	T211A
	30	M	57	5	384G>A	G128R

Table 3: Genetic alterations in *EGFR* and *PTEN* genes in GBM patients.

Mutations	No	Percentage
EGFR Mutation	13	32.5
PTEN Mutation	7	17.5
EGFR/*PTEN* (Both +ve)	3	7.5
EGFR +ve/*PTEN* -ve	10	25
PTEN+ve/*EGFR*-ve	4	10
EGFR-ve/*PTEN*-ve	23	57.5

Table 4: Distribution of patients as per the type of mutation.

Median Progression free survival (PFS) was 6 (5,7) and Median Overall Survival (OS) was 15 (12,18) months in patients who were <60 years of age compared to 14months in patients ≥ 60 years of age. Median Overall survival was better i.e., 15 months in patients with KPS >70 compared to 13 months in patients with KPS ≤ 70 ($p > 0.05$). Median PFS was better i.e. 7 months in patients with gross total resection compared to patients who were subjected to subtotal

resection (5 months) or Biopsy (3 months) and it was statistically significant. Median PFS and median OS was better (9 and 20 months) in patients who were *EGFR*+ve/*PTEN* -ve as compared to patients with *PTEN* +ve/*EGFR* -ve (6 and 13 months), *EGFR*+ve/*PTEN* +ve (6 and 13 months respectively) and *EGFR*-ve/*PTEN*-ve (6 and 14 months). The findings were statistically significant (Table 5).

Factors		PFS		OS	
		Months	Log rank p	Months	Log rank p
Age (yr)	<60	6	0.0339	15	0.2125
		14		14	
Karnofsky	≤ 70	6	0.4584	13	0.6207
Performance Score	>70	6		15	
EGFR +ve/PTEN -ve		9	0.0058	20	0.0078
PTEN +ve/EGFR -ve		6		13	
EGFR/PTEN (Both +ve)		6		9	
EGFR/PTEN (Both -ve)		6		14	
Procedure	Gross Total Resection	7	0.0001	17	0.1112
	Sub Total Resection	5		15	
	Biopsy	3		8	

Table 5: Factors affecting progression free survival and overall survival. *95% CI at (---) could not be developed due to the sub sample that was too small.

Median overall survival (OS) was better i.e., 16 months in patients who were <40 years of age as compared to 14 months in patients ≥ 40 years of age. There was no difference in the median OS between the two genders in our study. As far as the overall mutations are concerned, they were almost equally present in both the genders and also in patients who were <40 or ≥ 40 years of age (Table 6). Kaplan Meier curves of OS as a function of age and KPS are shown in Figure 1. Kaplan Meier curve of OS and PFS as a function of mutation (D and E) are shown in Figure 2.

Discussion

Glioblastomas remain one of the most lethal forms of cancers with a median survival of 10 to 12 months [6]. Despite clinical and technological advances in the understanding and treatment of brain tumours over the last three decades, the survival of patients with GBM has not notably improved. Therefore, research focused on the development of new targeted agents and approaches is needed.

A prospective study was undertaken to see the mutational profile of *EGFR* and *PTEN* in Glioblastoma multiforme patients in Kashmir and the impact of *EGFR* inhibitors in combination with surgery and radiotherapy on progression free survival and overall survival of the Glioblastoma multiforme patients in light of *EGFR* and *PTEN* mutations.

In the present research study, mutational spectrum of *EGFR* gene (exon 18, 19, 20 and 21) were studied in 40 confirmed GBM cases. The frequency of mutations in this series aggregated to 32.5% (13 of 40). *EGFR* mutations in GBM have been extensively studied. In May 2004, two independent groups of investigators reported the discovery of somatic mutations in the TK domain (exons 18–23) of *EGFR* [16,17]. Practically all mutations that have been reported are on exons 18 through 21. Among all mutations, four predominantly result in TKI drug sensitivity by in vitro and in vivo studies. These include point mutations in exons 18 (G719A/C) and 21 (L858R and L861Q) and in-frame deletions in exon 19, which eliminate four amino acids (LREA) downstream of the lysine residue at position 745; other mutations appear to be associated with variable or less sensitivity [16-18].

In the present study, we looked for *EGFR* mutations in Kashmiri GBM patients. EGFR mutations in GBM were more frequent in men than in women. All the hot spot exons 18-21 were observed to contain the mutations which were of missense in nature. A meta-analysis of nine published studies showed that EGFR mutations are limited to the first four exons (exons 18-21) of the TK domain, which encode the N-lobe and the 5' portion of the alpha C-lobe of *EGFR* [19]. In our study the mutations were all transversions of two types only which comprised of 07 C>A (60%) and 06 A>T (40%) involving a range of change in the native amino acids into different amino acids thereby markedly changing the overall structure of the kinase domains of *EGFR*.

A better understanding of genetics and biology of glioblastoma is critical for development of molecular targeted drugs and rationalization of their delivery to glioblastoma patients whose prognosis remains poor. Indeed, several molecular targeted drugs, particularly antigrowth factor receptors, are already under preclinical or clinical evaluations. Up to now, few patients respond to these drugs [20]. This suggests that other critical gliomagenesis actors remain to be targeted and predictors of response might be precisely identified and validated [21,22]. In that way, the present study contributes to a better understanding of glioblastoma genetics, confirming the relatively high frequency of *EGFR* extracellular domain mutations. Indeed, novel missense mutations of the *EGFR* extracellular domain have been recently reported in glioblastomas [23-25] and few of our mutations that were detected in GBM patients are consistent to these studies. The 13.5% rate of missense mutations of the EGFR extracellular domain in our series is in agreement with the findings of Lee et al. [22] but the frequency observed was very less as compared with Lori et al. [24].

Screening of *PTEN* genes was conducted in a series of 40 GBM samples. The mutations found were only seen in the two hot spot exons (5 and 7) of *PTEN* and all the mutations that were identified in this study were of missense nature. The overall mutations in both exons in this study aggregated to 17.5% (07 of 40). *PTEN* mutations have been found in 15 to 40% of glioblastoma [26,27]. In several previous studies *PTEN* mutations were not associated with prognosis of glioblastoma [28,29]. In our series we report 7 missense mutations mostly in the region of homologous tensin, auxlin and dual specificity phosphatases thereby possibly resulting in the gliomogenesis in the samples harboring these mutations. The frequency of mutations detected in our study, are in tune with the previous studies in GBM [26,27].

		EGFR +ve/ *PTEN* -ve		*PTEN*+ve/ *EGFR* -ve		*EGFR*+ve/ *PTEN*+ve		*EGFR*-ve/ *PTEN*-ve		Overall Mutations		Survival (months)
		n	%	n	%	n	%	n	%	n	%	
Age (years)	< 40	3	42.9	0	0.0	0	0.0	4	57.1	3/7	42.9	16
	≥ 40	7	21.2	4	12.1	3	9.1	19	57.6	14/33	42.4	14
Gender	Male	8	28.6	2	7.1	2	7.1	16	57.1	12/28	42.9	15
	Female	2	16.7	2	16.7	1	8.3	7	58.3	5/12	41.7	15

Table 6: Mutations and survival across age and gender.

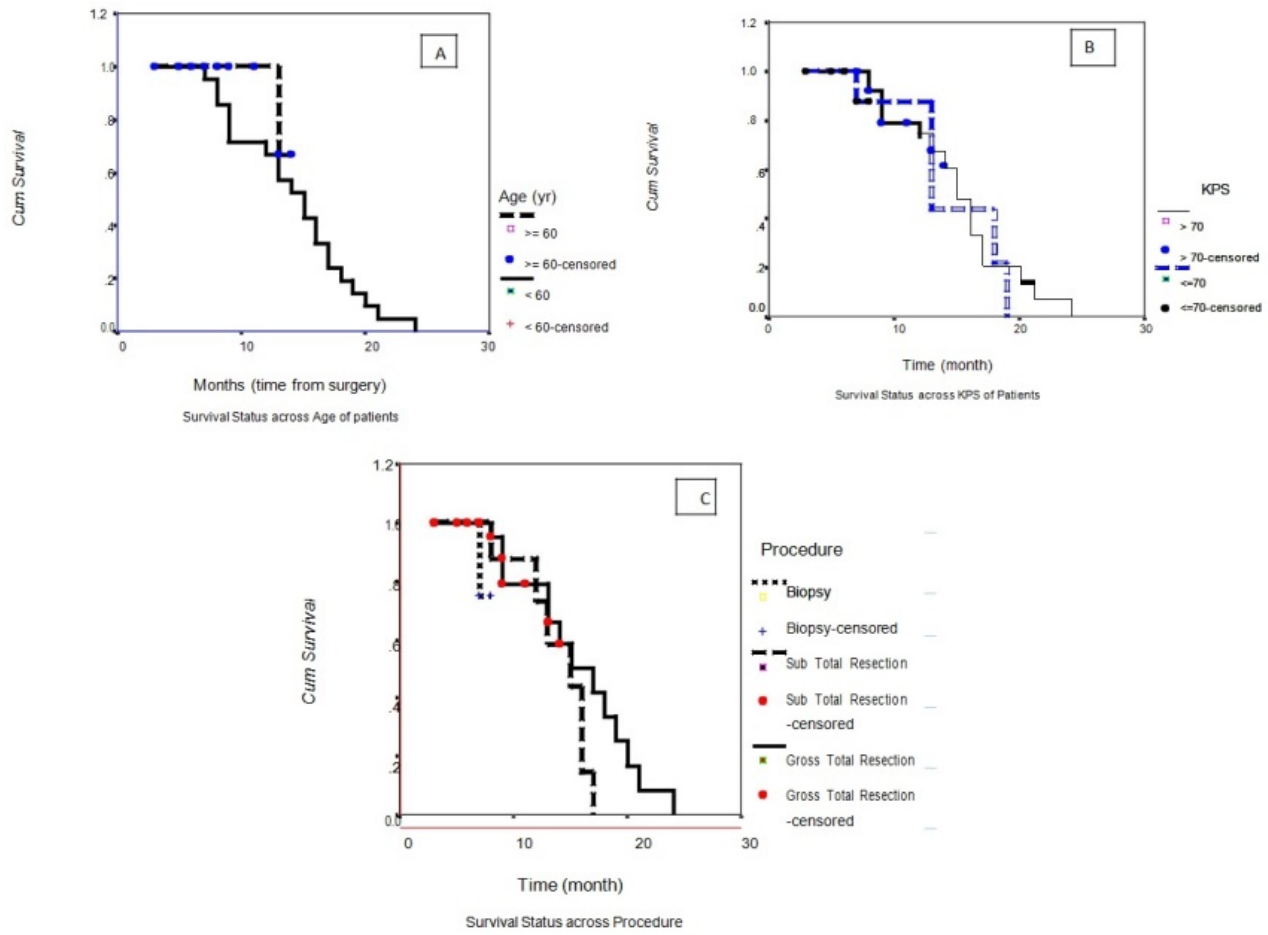

Figure 1: Kaplan Meier curves of OS as a function of age (A) and KPS (B) and procedure (C).

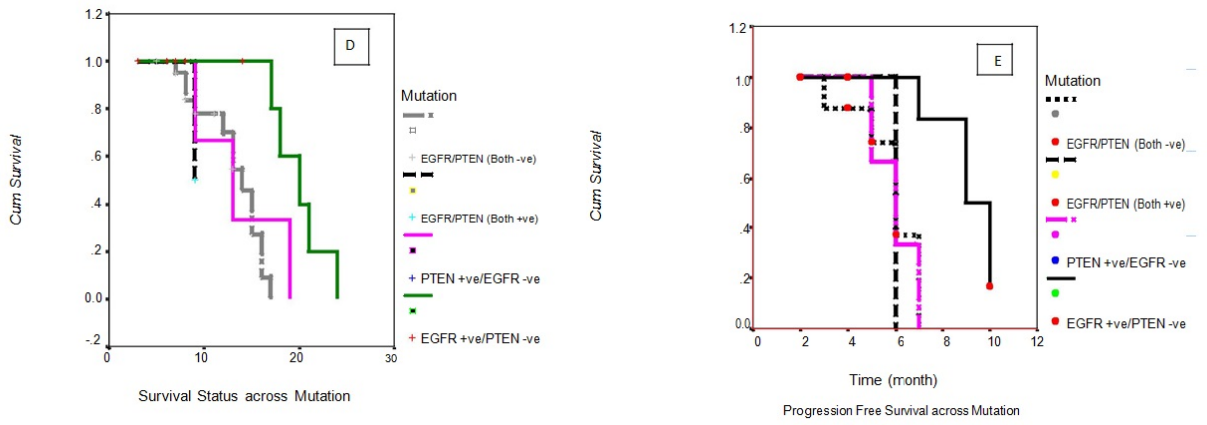

Figure 2: Kaplan Meier curve of OS and PFS as a function of mutation (D and E).

Both *PTEN* and *EGFR* mutations aggregated to 20 of 40 (50%) in our series of GBM patients. Though a major proportion of the samples were exclusive for mutations in *PTEN* and *EGFR*, but three mutations were commonly found in same samples in both genes and were thus observed to be overlapping in 15% of the GBM cases. This shows that a good proportion GBM cases harbour both mutations implicating *EGFR* and *PTEN* as mutually inclusive genetic events. Our study thus is in agreement with Justin et al., [27] who also observed the same frequency of genetic alterations of *EGFR* and *PTEN* in GBM patients.

In our study the overall mutations were almost equally present in both the genders and also in patients who were <40 or ≥ 40 years of age. This is in accordance to Justin S. et al [27] who also observed that there was no association between the incidence of *EGFR* gene alterations and patient's age but contrary to *PTEN* alteration that was observed statistically significantly more common among younger patients with GBM [28].

In our study Median Progression free survival (PFS) was 6 months and Median Overall Survival (OS) was 15 months in patients who were <60 years of age compared to 14 months in patients ≥ 60 years of age. Though OS was better in patients <60 years of age compared to patients with age ≥ 60 years ($p>0.05$). Median PFS could not be calculated in patients with age ≥ 60 due to the small sample size. Most of the previous studies concluded that patient's age had the greatest effect on survival. Vittorio Donato in 2007 observed that patients under 61 years of age had a significantly prolonged survival [29]. We observed that Median Overall survival was better i.e, 15 months in patients with KPS >70 compared to 13 (7,19) months in patients with KPS ≤ 70 ($p>0.05$) . Most of the previous studies have also concluded that Median OS is better in patients with good pre-operative KPS [29,30].

Further, we observed median PFS and median OS was better i.e. 9 and 20 months respectively in patients who were *EGFR* +ve/*PTEN* -ve as compared to patients with *PTEN* +ve/*EGFR* -ve (6 and 13 months), *EGFR*+ve/*PTEN*+ve (6 and 9 months) and *EGFR*-ve/*PTEN*-ve (6 and 14 months) and this association among the mutation pattern was statistically significant ($p<0.05$). These observations are in agreement with the previous studies [31-35]. However contrary to our results some of the studies detected no correlation between the outcome and *PTEN* mutations [28, 36].

Conclusion

We conclude that *EGFR* and *PTEN* gene mutations exist in our patients and play a significant role in the development of GBM in Kashmiri population. Moreover, the wild type samples reflect the involvement of different genetic factors, but this needs to be evaluated in further studies in GBM patients in our region.

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