

Significant Effect of Anti-tyrosine Kinase Inhibitor (Gefitinib) on Overall Survival of the Glioblastoma Multiforme Patients in the Backdrop of Mutational Status of Epidermal Growth Factor Receptor and *PTEN* Genes

Abstract

Introduction: We aimed to assess the effect of anti-tyrosine kinase inhibitors (TKIs) (gefitinib) in overall survival (OS) of the glioblastoma multiforme (GBM) patients in the backdrop of mutational status of epidermal growth factor receptor (*EGFR*) and *PTEN* genes. **Materials and Methods:** All the patients subjected to resection or biopsies were put on gefitinib, and radiotherapy was delivered as per the hospital protocol. *EGFR* and *PTEN* mutational spectrum was performed by single-strand conformation polymorphism followed by DNA sequencing. **Results:** In total, 50% GBM tumors had mutation either in *EGFR* or *PTEN*. Median progression-free survival (PFS) and OS observed in patients with *EGFR* +ve/*PTEN* -ve were significantly favorable ($P < 0.05$) which aggregated to 9(7, 11) months and 20 (16, 24) months, respectively, than 6 (4, 8) months and 13 (7, 19) months in patients with *PTEN* +ve/*EGFR* -ve. Patients positive for both *EGFR/PTEN* had lower disease-free survival and OS of 6 and 9 months as compared to 6 (5, 7) and 14 (12, 24) months for those negative for both *EGFR/PTEN*. **Conclusions:** We conclude that *EGFR* gene alterations with wild-type *PTEN* are associated with significantly better PFS and OS in patients treated with anti-TKIs (gefitinib). Combined *EGFR* and *PTEN* gene mutation is associated with significantly poor response to gefitinib in terms of median OS.

Keywords: Anti-tyrosine kinases, DNA sequencing, epidermal growth factor receptor, gefitinib, glioblastoma multiforme, mutations, *PTEN*

Introduction

Glioblastoma multiforme (GBM) presents as frequent and aggressive type of primary brain tumor.^[1] Among brain tumors in adults, GBM is the foremost common and found to be implicated more common in western population and among men.^[2,3] The incidence of GBM in India is nearly 3/10⁵ population whereas another estimate accounts for 2–5 new cases/10⁵/year.^[4] In Kashmir (North India), among the brain tumors, glioma (60%) is most common in which GBM is the most common followed by diffuse and anaplastic astrocytoma.^[5] GBM is the foremost deadly sorts of cancers with a median survival of 10–12 months.^[6] In contrast to most different kinds of cancer, GBM seldom metastasizes; rather, they induce death through putting resistance to current therapies and invasion into traditional brain tissues.^[7]

Commonly known two genetic pathways have been established in GBM development: de novo from glial cells and

is mostly common in older patients and secondary GBM develops over months to years from preexisting low-grade astrocytomas and mostly affects younger patients.^[8,9] GBM involves a multistep process that goes through a series of potential genetic alterations. Among these, primary GBM tumors exhibit overexpression (>60% of cases) or amplification (>40% of cases) of the epidermal growth factor (EGF) gene^[10,11] and prominently include deletion or mutation of the *PTEN* gene.^[9] Owing to failure of treatment for GBM by conventional cytotoxic chemotherapy and radiotherapy, a considerable amount of knowledge for aberrant signaling pathways involved in GBM has elucidated new potential therapeutic targets. These targeted drug therapies may augment better treatment modalities for patients with GBM, which particularly involves EGF receptor (*EGFR*) inhibitors currently being tested in clinical trials.^[11]

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Preliminary reports of targeted molecular therapies in GBM have concentrated on the inhibition of tyrosine kinases and growth factor pathways associated with it. In this scenario, gefitinib, a selective small-molecule inhibitor of the *EGFR*,^[12] is generally well tolerated, and patients with GBM in initial clinical trials on this drug produced partial responses after previous radiotherapy.^[13] Varied responses have been demonstrated in the backdrop of loss of the *PTEN* gene and *EGFR* wherein loss of the former was highly correlated with treatment failure. Evidences show that co-expression of *EGFRvIII* and *PTEN* strikingly predicted treatment responses.^[14] Now, it seems plausible that *PTEN* loss could promote resistance to EGFR kinase inhibitors by dissociating *EGFR/EGFRvIII* inhibition from downstream inhibition of the PI3K signaling pathway.^[15]

Thus, in the backdrop of mutational status of *EGFR* and *PTEN* genes, the aim of this study conducted first time from Indian subcontinent was to assess the effect of anti-tyrosine kinase inhibitor (TKI) (gefitinib) in combination with surgery on the recurrence and overall survival (OS) of the GBM patients

Materials and Methods

Patients

The present study was carried jointly by the Departments of Neurosurgery, Medical Oncology and Immunology, and Molecular Medicine, Sher-i-Kashmir Institute of Medical Sciences, Srinagar, Jammu and Kashmir, between 2009 and 2012. All consecutive patients with the GBM seen at our institution were considered for the study, and the sample size was calculated as per the hospital records which showed a power of the study >75.

Patients were included in the study after written informed consent. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards, and ethical approval was obtained from Institutional Ethical Committee.

The surgically resected tissue samples taken through stereotactic/open biopsy of GBM tumors, were collected directly into sterile vials containing chilled phosphate-buffered saline (pH = 7.2), and frozen at 70°C for molecular investigations. The normal brain tissue was a 2 mm × 2 mm × 1 mm block procured while performing corticectomy for the same lesion.

After entry into the study, patients were evaluated for detailed history, physical and systemic examination. All the patients were subjected to radiological examinations such as X-ray chest, contrast-enhanced computed tomography (CECT) brain, and contrast-enhanced magnetic resonance imaging (MRI) brain.

All the patients were subjected to gross-total resection, subtotal resection, or biopsy depending on the patient's status and tumor location. Once the pathology was confirmed, all the patients were put on gefitinib at an initial oral dose of 250 mg/day^[16] and radiotherapy was delivered as per the hospital protocol. Patients were treated with concurrent chemoradiotherapy that included temozolomide. Radiotherapy was delivered as 60 Grays in 30 fractions at 2 Gray per fraction, 5 days a week for a period of 6 weeks. The gross tumor volume (GTV) was determined by pre- and post-operative MRI imaging using enhanced T1 and fluid-attenuated inversion recovery/T2. The GTV was expanded by 2–3 cm to generate clinical target volume, to account for subdiagnostic tumor infiltration. Radiation fields were reduced after 46 Grays to prescribe boost radiation to gross disease. Patients received oral temozolomide 75 mg/m²/day for the duration of radiotherapy. Four weeks after the completion of concurrent chemoradiotherapy, patients received 3-weekly six cycles (175 mg/m² orally daily 5 days) of temozolomide. All patients received oral premedication during treatment. Patients who received dexamethasone and/or enzyme-inducing antiepileptic drugs without toxicities after 2 weeks of receiving gefitinib had the gefitinib dose escalated to 500 mg/day. Therapy was continued until disease progression, significant clinical decline, unacceptable toxicity, or patient decision. Toxicity was graded using the National Cancer Institute Common Toxicity Criteria, version 2.0.^[17]

For Grade 2 skin rashes and diarrhea that were unacceptable to the patient for symptomatic reasons, gefitinib was temporarily withheld until resolution and subsequently restarted at the same dose. If symptomatic Grade 2 diarrhea and skin rash recurred after reinstating gefitinib at the same dose, treatment was held until resolution to Grade 1 or less, and gefitinib was reinstated at a lower dose. If a patient dose was lowered, no increase was undertaken.

All the patients were monitored initially biweekly, thereafter monthly for complete blood count, liver function tests, kidney function test, and X-ray chest. All the patients were subjected to follow-up MRI/CECT head at the 1st week and every 3 months thereafter. Survival time of the patients was deduced which was taken as the length of time from either the date of diagnosis or the start of treatment for GBM that patients diagnosed with the disease are still alive. 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. Follow-up ranged from 6 months to a maximum of 25 months. Progression-free survival (PFS) was defined as the time from first surgery to first evidence of tumor progression on CT or MRI or to death whereas 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.^[18]

Histopathologically confirmed GBM tissues and corresponding normal tissues were used for mutational analysis of *PTEN* and *EGFR* gene.

Detection of mutations in epidermal growth factor receptor and *PTEN* genes

DNA was extracted from the tissues by phenol-chloroform method and by Qiagen DNA extraction kit (Zymo Research Corporation, USA).

EGFR and *PTEN* genes were amplified 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 MgCl₂, 100 mM 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 (Genscript; Piscataway, NJ, USA).

Results

The present study comprised forty histologically confirmed cases of GBM. Majority of the patients, i.e., 28 (70.0%) were male. The common presenting symptoms included headache (55%), vomiting (45%), and convulsions (37.5%), and other symptoms included altered sensorium, amnesia, slurred speech, and forgetfulness and their details are provided in Table 1.

Tumor location on the left side was seen in 19 (47.5%) as against 17 (42.5%) on the right side whereas midline tumors were observed in only 4 (10%) of patients. Majority of the tumors, i.e., 12 (30%) involved more than one lobe whereas single-lobe involvement was most common (11:27.5%) in the temporal lobe. Other sites involved were frontal, parietal, midline, occipital, and thalamic [Table 1]. Majority of the patients, i.e. 22 (55%) were subjected to gross-total resection whereas 13 (32.5%) were subjected to subtotal resection, and biopsy only was performed in 5 (12.5%) patients. Twenty-seven (67.5%) patients had Karnofsky performance score (KPS) of >70 and 13 (32.5%) had ≤70 with mean KPS as 78.2 ± 9.8 [Table 1].

Overall mutations of *EGFR* and *PTEN* genes identified in this study have been previously published.^[19] The overall frequencies of *EGFR* and *PTEN* mutations in GBM patients are shown in Table 2. In total, 50% (20 of 40) GBM tumors studied had mutation of either in *EGFR* or *PTEN* gene.

EGFR mutation was present in 13 (32.5%) and *PTEN* gene mutations in 7 (17.5%) patients. Both *EGFR* and *PTEN* mutations were found in 3 samples (7.5%). The samples which showed *EGFR* mutations and negative for *PTEN* were detected in 10 (25%) patients (*EGFR* +ve/*PTEN* -ve). The samples which showed *PTEN* mutations but absent in *EGFR* (*PTEN* +ve/*EGFR* -ve) were present in 4 (10%) patients. No mutations were seen in both the genes (*EGFR/PTEN* both -ve) in 23 patients (57.5%) [Table 2].

All the patients were put on gefitinib 250–500 mg/day. Most common side effects noted were rash (26%) and

Table 1: Clinico-pathological characteristics of GBM patients

Parameter	Number (%)
Age (yr)	
<60	21 (52.5)
≥60	19 (47.5)
Gender	
Male	28 (70.0)
Female	12 (30.0)
Dwelling	
Urban	9 (22.5)
Rural	31 (77.5)
Symptoms	
Headache	22 (55%)
Vomiting	18 (45%)
Focal neurodeficit	15 (37.5%)
Convulsions	13 (32.5%)
Giddiness	7 (17.5%)
Altered sensorium	3 (7.5%)
Others	5 (12.5%)
Location of Tumor	
Frontal	6 (15.0)
Temporal	11 (27.5)
Parietal	4 (10.0)
Frontal	6 (15.0)
Multiple Lobe	12 (30.0)
Others	7 (17.5)
Procedure	
Gross total resection	22 (55.0)
Sub total resection	13 (32.5)
Biopsy	5 (12.5)
Karnofsky performance score	
≤70	13 (32.5)
>70	27 (67.5)
Median age	55.0

Table 2: Frequency and distribution of EGFR and PTEN mutations 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

diarrhea (22%). Rash and diarrhea occurred mostly at dose of 500 mg/day. Other toxicities that were encountered included aspartate transaminase and alanine transaminase elevation, conjunctivitis, anorexia, and weight loss. To assess the impact of TKIs, patients put on gefitinib were prospectively followed for their OS and disease-free survival (DFS).

Kaplan–Meier (KM) curves were constructed to assess OS, and differences among groups were analyzed by

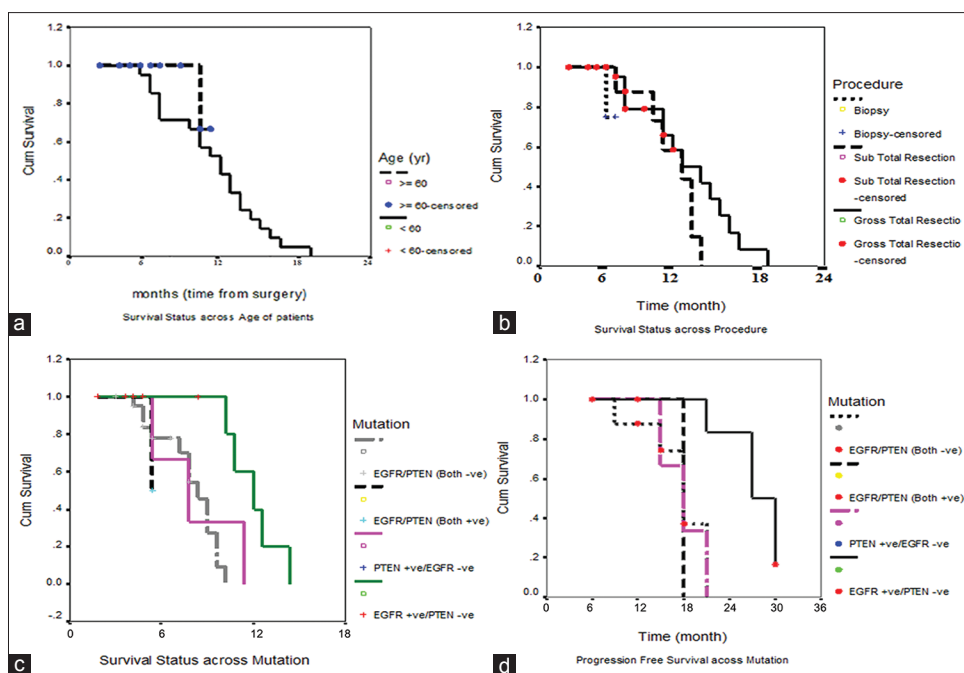


Figure 1: (a) Kaplan–Meier curve of overall survival and progression-free survival as a function age. (b) Kaplan–Meier curve of overall survival and progression-free survival as a function gender. (c) Kaplan–Meier curve to evaluate effect of mutational spectrum of epidermal growth factor receptor/*PTEN* as a component of overall survival. (d) Kaplan–Meier curve to evaluate effect of mutational spectrum of epidermal growth factor receptor/*PTEN* in relation to progression-free survival

Table 3: Pattern of mutations and survival across age and gender

Parameter	EGFR +ve/ <i>PTEN</i> -ve (n%)	<i>PTEN</i> +ve/ <i>EGFR</i> -ve (n%)	<i>EGFR</i> / <i>PTEN</i> (both +ve) (n%)	<i>EGFR</i> / <i>PTEN</i> (both -ve) (n%)	Overall mutations (n%)	Survival (month)
Age (yr)						
<60	3 (42.9)	0 (0.0)	0 (0.0)	4 (57.1)	3/7 (42.9)	16 (15-17)
≥60	7 (21.2)	4 (12.1)	3 (9.1)	19 (57.6)	14/33 (42.4)	14 (12-16)
Gender						
Male	8 (28.6)	2 (7.1)	2 (7.1)	16 (57.1)	12/28 (42.9)	15 (12-18)
Female	2 (16.7)	2 (16.7)	1 (8.3)	7 (58.3)	5/12 (41.7)	15 (12-18)

Table 4: Different characters affecting progression free survival and overall survival

Factors	PFS		OS	
	Month	Log rank <i>P</i>	Month	Log rank <i>P</i>
Age (yr)				
<60	6 (5, 7)	0.0339 (Sig)	15 (12, 18)	0.2125 (NS)
≥60	(-----)		14 (-----)	
Karnofsky performance score				
≤70	6 (3, 9)	0.4584 (NS)	13 (7, 19)	0.6207 (NS)
>70	6 (5, 7)		15 (13, 17)	
<i>EGFR</i> +ve/ <i>PTEN</i> -ve	9 (7, 11)	0.0058 (Sig)	20 (16, 24)	0.0078 (Sig)
<i>PTEN</i> +ve/ <i>EGFR</i> -ve	6 (4, 8)		13 (7, 19)	
<i>EGFR</i> / <i>PTEN</i> (both +ve)	6 (-----)		9 (-----)	
<i>EGFR</i> / <i>PTEN</i> (both -ve)	6 (5, 7)		14 (12, 24)	
Procedure				
Gross total resection	7 (6, 8)	0.0001 (Sig)	17 (12, 22)	0.1112 (NS)
Sub total resection	5 (3, 7)		15 (10, 20)	
Biopsy	3 (----)		8 (----)	

*95% CI at (---) could not be developed due to the sub sample that was too small

the log-rank test. Kaplan–Meier survival analysis was performed to evaluate any possible association between mutational spectrum of *EGFR/PTEN* genes and its impact on treatment with anti-TKIs (gefitinib) and OS of patients. Median PFS was 6 (5, 7) and median OS was 15 (12, 18) months in patients who were <60 years of age compared to 14 months in patients ≥ 60 years of age [Figure 1a]. 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 [Table 3].

KP analysis showed that median OS was better, 15 months in patients with KPS >70 compared to 13 (7, 19) months in patients with KPS ≤ 70 but was not statistically significant. In our study, median PFS was better 7 (6, 8) months in patients with gross-total resection compared to patients who were subjected to subtotal resection 5 (3, 7) months or biopsy only 3(--) months ($P > 0.05$). Median OS was also better 17 (12, 22) months in patients who were subjected to gross-total resection compared to the patients subjected to subtotal resection 15 (10, 20) months or biopsy only 8 (--) months, respectively [Figure 1b].

Survival score (OS and DFS) was deduced from the GBM patients put on gefitinib by KM curves to evaluate effect of mutational spectrum of *EGFR/PTEN* [Figure 1c and d]. A better median PFS and median OS were observed in patients who were EGFR +ve/PTEN –ve which aggregated to 9 (7, 11) months and 20 (16, 24) months, respectively, as compared to patients with *EGFR –ve/PTEN +ve* where survival was 6 (4, 8) months and 13 (7, 19) months, respectively. Patients positive for both *EGFR/PTEN* (+) had lower DFS and OS of 6 (--) and 9 (--) months whereas patients negative for both *EGFR/PTEN* (–) had 6 (5, 7) months and 14 (12, 24) months, respectively. The finding was observed to be significantly associated with the response to gefitinib in patients with *EGFR +ve/PTEN –ve* in terms of survival [Table 4].

Discussion

The association of genetic alterations with brain tumor behavior has aroused multiple investigations into the prognostic value of various genetic markers.^[20] The main focus of investigations has led more deep into GBM pathogenesis, and in this relation, elucidation of the *PTEN* and *EGFR* oncogenes has been the primary target of analysis. Recent evidence shows that about 10%–20% of unselected GBM patients showed significant tumor regression in response to EGFR kinase inhibitors.^[14,21] A definite connection has been established between a response and resistance to EGFR kinase inhibitors in GBM patients that is mediated by loss of the *PTEN* gene derangement.^[15] To understand the molecular basis for drug response in the backdrop of their mutation spectrum in *EGFR* and *PTEN* gene, we conducted a prospective analysis of tumor tissues from patients who responded and/or did not respond to EGFR kinase inhibitor

therapy in combination with surgery and radiotherapy to analyze PFS and OS of the GBM patients.

GBM incidence occurs mostly between the sixth and seventh decade, but these tumors appear to show its increasing trend in young adults.^[22,23] Incidence in men is approximately 40% more than in women, and accordingly, we observed that in our study, 28 (70%) were male and 12 (30%) were female with a male:female ratio of 2.3:1. All the patients were put on gefitinib 250–500 mg/day. Among the most common side effects, noted rash (26%) and diarrhea (22%) occurred mostly at dose of 500 mg/day, which are in agreement with the previous studies.^[24-26]

Fukuoka *et al.* (2003) and Ranson *et al.* (2002),^[26,27] in two large randomized phase II trials, analyzed the effectiveness and toxicity of gefitinib (250 mg or 500 mg/day) wherein the former conducted trial of gefitinib for nonsmall cell lung cancer patients. The study observed no difference between rate of response and survivals following the two completely different dose schedules whereas the adverse event rates were higher in 500 mg/day. In phase I studies, gefitinib at doses lower than 250 mg/day showed better responses and disease stabilizations, and gefitinib at 500 mg/day in intermittent schedules has been shown to be effective.^[28,29]

In our series of GBM patients, both *PTEN* and *EGFR* gene mutations aggregated to 50% (20 of 40). Although a major proportion of the samples were exclusive for mutations in *PTEN* and *EGFR*, 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 of GBM cases harbor both mutations implicating *EGFR* and *PTEN* as mutually inclusive genetic events. Our study thus is in agreement with Smith *et al.* who also observed the same frequency of genetic alterations of *EGFR* and *PTEN* in GBM patients.^[30]

Although in our study, we did not find a major difference in PFS and OS between two age groups, OS was better in patients <60 years of age compared to those with age ≥ 60 years ($P > 0.05$). Median PFS could not be calculated in patients with age ≥ 60 due to the subsample that was too small. Most of the previous studies concluded that patient's age had the greatest effect on survival. In a study conducted by Donato *et al.*,^[31] it was observed that patients under 61 years of age had a significantly prolonged survival. Filippini *et al.* (2008)^[32] observed a strong evidence for the effectiveness of surgery even for elderly patients, provided that they had an adequate performance status.^[33]

We observed that median OS was better 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 preoperative KPS.^[31]

Whether the extent of resection is a factor significantly associated with the survival advantage is much debated,

but some reports found that more extensive resection was associated with longer survival^[33,34] whereas others showed no relation.^[35,36] Our results also support a significant increase in PFS and a nonsignificant increase in OS associated with extensive surgical resection compared with partial resection/biopsy although precise evaluation of PFS after surgery by postoperative imaging was not available in all patients. In our study, median PFS was better 7 (6, 8) months in patients with gross-total resection compared to patients who were subjected to subtotal resection 5 (3, 7) or biopsy only 3 (--) months, ($P < 0.05$). Median OS was also better 17 (12, 22) months in patients who were subjected to gross-total resection compared to the patients subjected to subtotal resection 15 (10, 20) months or biopsy only 8 (--) months, respectively.

Although gefitinib is generally well tolerated, patients with GBM in initial clinical trials with gefitinib had minimal tumor response and no improvement in OS.^[37] In one phase II study, response to gefitinib showed that 13% patients remained progression free for a minimum of 6 months.^[37] In the following phase I/II study conducted by the North American Brain Tumor Consortium, a partial response was shown after previous RT in around 13% patients with GBM.^[25] In our study, we observed that median PFS and median OS was better 9 (7, 11) and 20 (16, 24) months, respectively, in patients who were *EGFR* +ve/*PTEN* -ve as compared to patients with *PTEN* +ve/*EGFR* -ve 6 (4, 8) and 13 (7, 19) months, *EGFR/PTEN* (both +ve) 6 (--) and 9 (--) months, and *EGFR/PTEN* (both -ve) 6 (5, 7) and 14 (12, 24) months, and this difference was found statistically significant ($P < 0.05$). Hence, *EGFR* kinase inhibitor (gefitinib) showed significant response in terms of PFS and OS in our patients who had alterations in *EGFR* gene and intact *PTEN* gene. Data till date analyzed from various clinical trials and preclinical models^[14,38] report that treatment with *EGFR* kinase inhibitors is irresponsive in *PTEN* deficient tumors even if they derive activating *EGFR* mutations. This potentially causes upfront resistance to *EGFR* kinase inhibitors in highly *PTEN*-deficient tumors or when resistance is acquired in molecularly heterogeneous tumors wherein *PTEN* deficient cells develop a selective growth advantage during chemotherapy.

Median PFS and median OS in patients who were *PTEN* +ve/*EGFR* -ve were 6 (4, 8) months and 13 (7, 19) months, respectively, and in patients who were *PTEN/EGFR* (both +ve) were 6 (--) and 9 (--) months, respectively. Hence, patients with mutation in *PTEN* gene showed poor response to gefitinib in terms of median PFS and median OS than patients without alterations in *PTEN*. The fact that *EGFR* gene derangements with intact wild-type *PTEN* are associated with significantly better PFS and OS in patients treated with *EGFR* inhibitors (gefitinib). These observations are in agreement with the previous studies.^[39-41] The *PTEN* gene is lost or mutated in ~40% of GBM,^[1] and retention of *PTEN* protein expression has been

linked with responses to *EGFR* TKIs in GBM patients^[21] suggesting that the detection of functional *PTEN* may inform the successful deployment of targeted therapeutics in this currently intractable disease.

Conclusions

We conclude that *EGFR* gene alterations with wild-type *PTEN* are associated with significantly better PFS and OS in patients treated with *EGFR* inhibitors (gefitinib). Combined *EGFR* and *PTEN* gene mutation is associated with significantly poor response to gefitinib in terms of median OS. Molecularly targeted therapies can potentially provide novel cancer therapies by selectively inhibiting these aberrant pathways, but this needs to be evaluated in further studies in GBM patients of the ethnic Kashmiri population.

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Conflicts of interest

There are no conflicts of interest.

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