RESEARCH ARTICLE

CD133 and *BMI1* expressions and its prognostic role in primary glioblastoma

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Abstract

Glioblastoma is the most common malignant brain tumour, generated by bulk of malignant cancer stem cells, which express various stem cell factors like CD133, BMI1 and nestin. There are several studies which show the importance of CD133 in cancer, but the function and interaction with other major oncogenes and tumour suppressor genes is still not understood. This study aimed to analyse the expression of *CD133* mRNA and its correlations with BMI1 protein expression and *TP53* mutations in newly diagnosed glioblastoma patients and its role in prognosis. Overexpression of *CD133* mRNA and BMI1 protein was found in 47.6 and 76.2% patients respectively and *TP53* mutations was seen in 57.1% of patients in our study. There was no correlation among *TP53* mutations and expressions of *CD133* and BMI1. We found that high level of BMI1 expression was favourable for the patient survival ($P = 0.0075$) and high *CD133* mRNA expression was unfavourable for the patient survival ($P = 0.0226$). *CD133* mRNA and BMI1 protein expression could independently predict the glioblastoma patient survival in multivariate analysis. In conclusion, the overexpression of these stem cell markers is a common event in glioblastoma progression and could be used as potential prognostic markers.

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Introduction

Glioblastomas are high grade tumours (WHO Grade IV) which accounts for 50–60% astrocytic tumours and is the most common malignant brain neoplasm (Vougiouklakis *et al.* 2006). Even with advanced neurosurgical procedures and adjuvant therapeutic treatments, which specifically kill cancer cells, glioblastoma has the worse prognosis among cancers with a median survival of 14 months (Louis *et al.* 2007; Nicholas 2007). Over the last few years, the focus is to look for mechanisms in treatment-resistant properties, which are originated from the group of cells known as the cancer stem cells (Galli *et al.* 2004; Singh *et al.* 2004).

CD133 was first identified as prominin protein family member and its role is essential for selfrenewal and proliferation of glioblastoma stem cells (Brescia *et al.* 2013). Currently, CD133 in combination with other markers is used for the isolation of stem cells from numerous tissues. Monoclonal antibodies against CD133 are already in use to identify and to isolate cancer stem cell population from brain and use this as a potential therapeutic target (Singh *et al.* 2003, 2004). Recent studies show that CD133 can change its subcellular location between the cytoplasm and plasma membrane of neurosphere cells and is present in both CD133+ and CD133− cells. Silencing of CD133 in those cells could reduce the tumourigenicity and selfrenewal properties (Brescia *et al.* 2013).

There are a group of important transcription regulators called polycomb group proteins (PcG), which are classified into two categories, polycomb repressive complex 1 (PRC1) and polycomb repressive complex 2 (PRC2) (Orlando 2003). BMI1, is an important oncogene which is a component of PRC2, contains the Ezh2 and Eed. During embryonic development, both PcG complexes are found to be functionally redundant in the control of HOX gene expression, but helps in the proliferation of hematopoietic stem cells (Lessard *et al*. 1999). The experiments performed in double transgenic mice showed that BMI1 is involved in lymphoma genesis (Haupt *et al.* 1991; Van Lohuizen *et al.* 1991). BMI1

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convert mouse embryo fibroblasts into immortal neoplastic cells by preventing senescence (Jacobs *et al.* 1999). The BMI1 gene amplification and overexpression were found in certain mantle cell lymphomas and varied expression pattern was found in nonsmall cell lung cancer, colorectal carcinomas, multiple myelomas, medulloblastoma and even in glioblastomas (Beá *et al*. 2001; Vonlanthen *et al*. 2001; Molofsky *et al*. 2003; Kim *et al*. 2004; Jiang *et al*. 2013). Recently it is shown that selfrenewal of neuronal stem cells and cancer stem cells require the sustained expression of BMI1 (Molofsky *et al.* 2003; Smith *et al.* 2003; Glinsky *et al.* 2005; Jiang *et al.* 2013). The BMI1 expression pattern in various tissues is different from the expression of other PcG genes and it is a distinguishing factor in various cancers (Lessard *et al.* 1998).

P53 is a well-established tumour suppressor protein, which influence cell cycle pathway and network which encompasses tumour suppressor genes that monitors the fate of a cell to either proliferate or remain quiescent (Vogelstein *et al.* 2000, 2010). Role of *TP53* gene in the prevention of cancer development has been well established, as it is found to play a major role in acute stress response with other proteins (Vousden and Lane 2007). *TP53* mutations are more prevalent in cancer and it is the single most common event in human cancers including glioblastoma (Vogelstein *et al.* 2010).

Even though, cancer stem cells are found to play a major role in glioblastoma prognosis, its molecular characterization is not executed properly. In this study, we analysed the role of major stem cell factor *CD133* and its correlations with BMI1 protein expression, and *TP53* mutations. We have looked into the role of all these major genes independently in the prognosis of primary glioblastoma.

Materials and methods

Patients and tissue samples

From the year 2010 to 2012, 21 newly diagnosed primary glioblastoma patients who underwent tumour resection and treatment at the National Institute of Mental Health and Neurosciences (NIMHANS) Hospital, Bengaluru, India, were enrolled in this study. Histopathological diagnosis was performed according to the guidelines of World Health Organization (Louis *et al.* 2007). Informed consent was obtained from all the patients. This study was approved by the Institute Ethics Committee of NIMHANS. Tissue samples were collected and stored in RNA later at −80°C. Detailed clinical and pathology data were collected from medical record section for each patient, which includes sex, age, tumour location, amount of resection, grading and treatment. The follow up of the patient was done once in three months until death or up to 30 months.

RNA isolation and qRT-PCR for CD133

Total RNA was extracted from the tissue samples using the Qiagen DNA/RNA isolation kit and 1 μg of this RNA was reverse transcribed into cDNA using the cDNA conversion kit according to the manufacturer's instructions. Power SYBR green universal master mix and 7500 fast real-time PCR system (Applied Biosystems, Foster City, USA) were used for real-time PCR work. Each sample was assayed in triplicate. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as a reference gene. The primer sequences were 5′-GCATTGGCATCTTCTATGGTT-3′, 5′-CGCCTTGTCCTTGGTAGTGT-3′ for *CD133* and 5′-GAAGGTGAAGGTCGGAGTCAAC-3′, 5′-CAGAGTTAAAAGCAGCCCTGGT-3′ for GAPDH. Cycling conditions were standardized to 10 min at 95°C followed by 40 cycles of amplification for 15 s at 95°C and 1 min at 60°C. Comparative analysis by $2^{-\Delta\Delta Ct}$ method was used to compare the expressions of both genes.

Western blotting for BMI1: Whole cell protein extracts were obtained from 21 tissues. Cell lysates were prepared in cold lysis buffer (50 mM Tris, 150 mM NaCl, 1 mol/L EDTA, 0.1% sodium dodecyl sulphate, 1% Triton X-100, 1 mol/L phenylmethylsulphonyl fluoride (pH 8)). The protein content in the lysates was measured using Bradford assay. For Western blot analysis, 50 μg protein was resolved in 12% SDS-PAGE gels, transferred onto PVDF membranes (Biorad, Hercules, USA) and subsequently incubated in blocking buffer (5% BSA, 1% Tween 20; in 20 mol/L TBS, pH 7.6) for 2 h. The blots were incubated with monoclonal antibody BMI1 (1 in 1000) overnight and anti-mouse secondary antibody conjugated to horseradish peroxidase (1 in 5000, Santacruz Biotech, Dallas, USA) for 1 h and detected by enhanced chemiluminescence kit (Biorad) according to the manufacturer's recommendations. Equal loading of protein was confirmed by stripping the blots and reprobing with β-tubulin (1 in 2000, Santacruz Biotech). Bands were analysed using Image J software.

TP53 mutation analysis

DNA was extracted from the frozen tissue samples using Qiagen All Prep DNA/RNA kit and exons 5–6, 7 and 8–9 of *TP53* were amplified separately. PCR reaction mix used was 25 μL, which contained 100 ng genomic DNA, 20 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM deoxynucleoside triphosphates, 0.2 μM primers and 1.5 U *Taq* polymerase. After initial 10 min denaturation at 95°C, it was followed by each cycle of 95°C for 1 min, annealing for 1 min, and at 72°C for 1 min. PCR reaction was carried out on Applied Biosystem Veriti Thermal cycler. The amplicons obtained were checked using agarose gel electrophoresis and then sequencing. Primer sequence is given in table 1.

Statistical analysis: Statistical software R.3.0.1 (R foundation for statistical computing, Vienna, Austria) and MedCalc ver. 13.2.2 (Acacialaan, Ostend, Belgium) were used for analysis. Categorical variables were presented as percentage and association between them were analysed using chi-square

Table 1. Primer sequence used for amplification of p53 exons.

Exon		Sequence (5′ → 3′)	Length	T_m	Product length (bp)
5–6	Forward	GTTGGGAGTAGATGGAGCCT	20	58.50	455
	Reverse	GGCATTTTGAGTGTTAGACTG	21	55.15	
7	Forward	CTGCTTGCCACAGGTCTC	18	58.02	283
	Reverse	TGGATGGGTAGTAGTATGGAAG	22	56.14	
8–9	Forward	CGCTAGTGGGTTGCAGGA	18	59.34	550
	Reverse	CACTGACAACCACCCTTAAC	20	56.27	

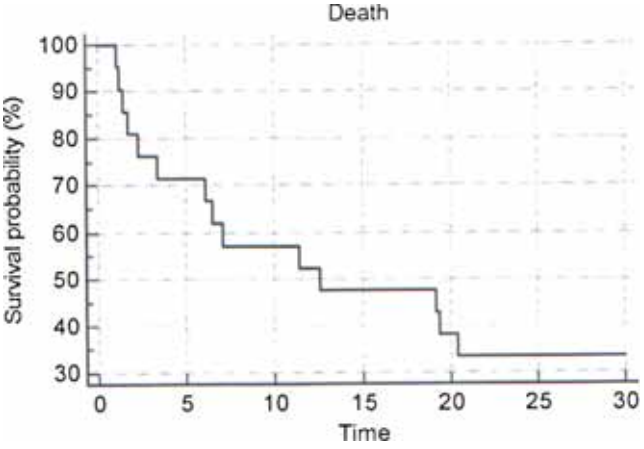

Figure 1. Overall survival of primary glioblastoma patients in the cohort.

Table 2. Details of patient and gene alterations.

Variable	No. of patients (%)
Age	14 (66.7)
≥40	7 (33.3)
<40	
Sex	12 (57.1)
Male	9 (42.9)
Female	
P53 mutated	12 (57.1)
Yes	9 (42.9)
No	
CD133 expression	11 (52.4)
Low	10 (47.6)
High	
Bmi1 expression	5 (23.8)
Low	16 (76.2)
High	

test and Fisher's exact test. Kruskal–Wallis test was used to check the expression levels of BMI1 and *CD133* in different groups. Survival analysis was done using Kaplan Meier survival analysis and survival curves were analysed using log-rank test. Cox proportional hazard model was used to check the prognostic value of *CD133*.

Results

Patient details

Twelve male and nine female patients were enrolled in this study. The median age was 46 years (range 18–68). All tumours were primary glioblastoma and confirmed by MRI and histopathological evaluations. The tumours were located in various regions and gross total resection was achieved in all the patients. The median survival was 12.6 months (range 6.1–20.4 months) (figure 1) and follow up data of all the patients were available. At the last follow up, seven patients were still alive. Five nontumour brain tissues resected during epilepsy surgery, were used as control in this study. Patient's details and gene alterations are given in table 2.

TP53 mutations in glioblastoma

Mutation screening was done on exons 5–9 of *TP53* gene. We amplified three fragments spanning from exons 5–8 and analysed using direct sequencing. Twelve of 21 (57.1%) cases showed mutations. Nine samples showed exonic mutations and eight showed intronic mutations. There were a total of 53 mutations of which 38 were exonic and 15 were intronic. Exons 5 and 6 showed 19 mutations each and none in exons 7, 8 and 9. Introns 5 and 8 showed 11 and four mutations each. We have found three novel mutations which were K132F, K139F and S185T. Other exonic mutations are given in table 3.

CD133 gene expression

We assessed mRNA expression of *CD133* using real-time RT-PCR in 21 cases of primary glioblastoma. Overexpression was found in 10 out of 21 cases (47.6%). The expression levels of *CD133* range from 0.08 to 278.04 fold with a median of 48. *CD133* expression did not correlate with sex, age and tumour position.

BMI1 protein expression

Western blotting was performed to check the BMI1 protein expression in primary glioblastoma samples. Overexpression was found in 16 of 21 cases (76.2%). The expression of BMI1 protein ranged from 0.11 to 7.84 fold with a median of 2.64. The protein expression did not correlate with sex, age and tumour position. The Western blot image of few samples are shown in figure 2.

Correlation between CD133 mRNA expression, BMI1 protein and TP53 mutations

We examined the correlation of these major genes with each other in primary glioblastomas. We found that there was no statistically significant correlation between *CD133*

Table 3. P53 exonic mutations in glioma with its consequence.

Nucleotide change	AA change	Type of mutation	
ATC → ATT	I195I	Transition	–
AAG → AAA	K132F	Transition	Missense
AAG → AAA	K139F	Transition	Missense
CAG → CAC	Q144H	Transversion	Missense
GTC → TTC	V157F	Transversion	Missense
CGC → CCC	R158P	Transversion	Missense
GCC → CCC	A159P	Transversion	Missense
AAG → AAC	K164N	Transversion	Missense
CAG → CAT	Q165H	Transversion	Missense
CAG → CAC	Q167H	Transversion	Missense
GAG → GAC	E180D	Transversion	Missense
CGC → CCC	R181P	Transversion	Missense
CAG → CAA	Q183Q	Transition	–
AGC → ACC	S185T	Transversion	Missense
GAT → AAT	D186N	Transition	Missense
CAG → CAT	G192H	Transversion	Missense
CGA → CAA	R196Q	Transition	Missense
GTG → TTG	V197L	Transversion	Missense
GAA → AAA	E198K	Transition	Missense
CGT → CTT	R202L	Transversion	Missense
GAC → AAC	D208N	Transition	Missense
AGA → AAA	R209K	Transition	Missense
CGA → CAA	R213Q	Transition	Missense
AGT → ATT	S215I	Transversion	Missense
CAG → GAC	E221D	Transversion	Missense
CCG → CCC	P222P	Transversion	–
CCG → ACG	P152T	Transversion	Missense
GAG → GAA	E171E	Transition	–
CAG → CAT	Q192H	Transversion	Missense
CGA → CAA	R196Q	Transition	Missense
AGT → ATT	S215I	Transversion	Missense
GAG → GAA	E171E	Transition	–
CGC → CAC	R175H	Transition	Missense
CAG → CAT	Q192H	Transversion	Missense
CGA → TGA	R213*	Transition	Nonsense
CGA → TGA	R196*	Transition	Nonsense

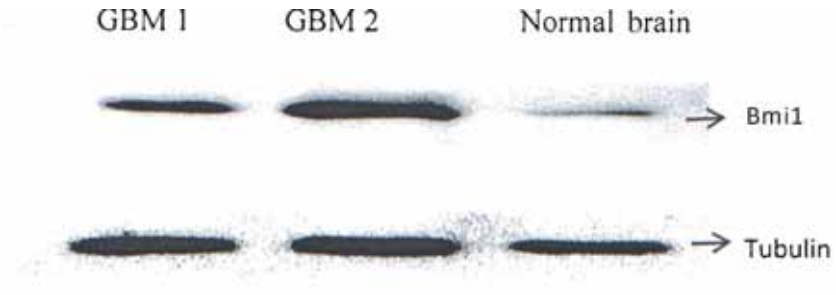

Figure 2. Western blot showing Bmi1 overexpression in primary glioblastoma.

mRNA expression and BMI1 protein expression in our cohort (Spearman correlation test, rho = −0.307, *P* value = 0.1755). There was no difference between *CD133* gene expressions in *TP53* mutated and nonmutated cases (Kruskal–Wallis test, *P* value = 0.393) and BMI1 expression in *TP53* mutated and nonmutated cases (Kruskal–Wallis test, *P* value = 0.145).

Age, sex, TP53 mutation, CD133 and BMI1 expression in the prognosis of glioblastoma

Patient survival from first diagnosis to the end was estimated with Kaplan Meier method. Age, sex and *TP53* mutations could not predict the patient survival when analysed using log-rank test in Kaplan Meier survival analysis. Male patients aged below 40 years and with no *TP53* mutations

Table 4. Kaplan Meier survival analysis to check the role of variables as prognostic markers.

Variable	Median	95% CI	Long rank (*P* value)
Age			
>40	19.200	6.500–19.200	0.5040
≤40	11.400	2.300–20.400	
Sex			
M	20.400	12.600–20.400	0.1198
F	6.100	1.700–11.400	
P53 mutation			
Yes	11.400	3.400–12.600	0.6542
No	19.400	6.500–20.400	
CD133			
Low	–	–	0.0226
High	6.300	1.700–12.600	
BMI1			
Low	1.700	1.100–19.400	0.0075
High	19.800	7.100–20.400	

were tend to show good prognosis, but it was not statistically significant. *CD133* expression found to be a good prognostic marker in glioblastoma patients. Low *CD133* mRNA expression had a better prognostic value with higher survival rate, whereas high *CD133* expression had shorter survival. *BMI1* gene also proved to be a potential prognostic marker in primary glioblastoma. Patients with high BMI1 protein expression were favourable for longer patient survival in our cohort (table 4; figure 3).

Cox proportional hazard regression analysis

To identify independent prognostic roles of BMI1 and *CD133*, we investigated the expression data of both the genes in Cox proportional hazard regression model. We found that *CD133* and BMI1 expression could independently predict the glioblastoma patient survival (table 5).

Discussion

Glioblastoma is an aggressive tumour with high expression levels of various cancer stem cell proteins (Hemmati *et al.* 2003; Yuan *et al.* 2004; Tso *et al.* 2006; Chen *et al.* 2010; Liu *et al.* 2011). *CD133* is the most important in this category and its mRNA expression was not studied in detail in relation with the expression of other stem cell proteins. We have analysed mRNA expression of *CD133*, protein expression of BMI1 and mutations of tumour suppressor gene *TP53* in 21 primary glioblastoma samples. The alterations in these genes were correlated with the survival status of the patients.

We selected 21 adult patients with primary glioblastoma, as it is the most common and severe form of glioma. We excluded patients below 18 years, as the paediatric gliomas have a different gene signature and survival. All the patients were treated uniformly during radiotherapy and chemotherapy after gross total resection of tumour.

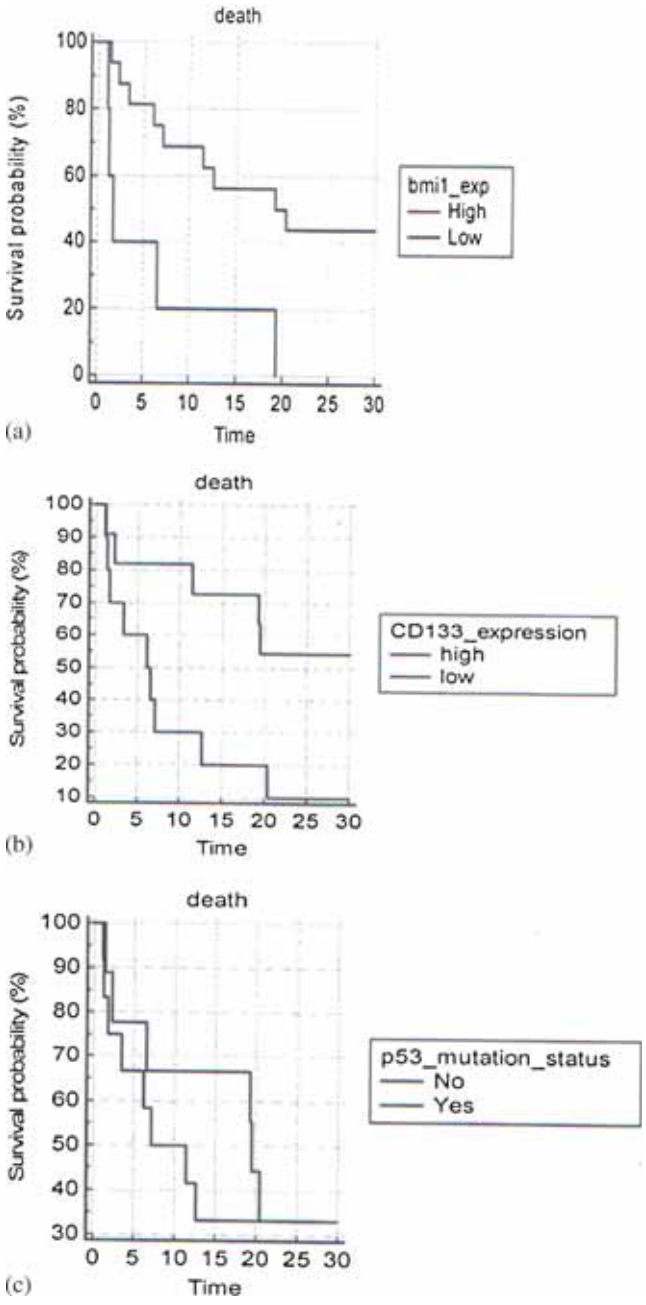

Figure 3. Kaplan Meier survival curve showing (a) BMIl expression in primary glioblastoma, (b) CD133 expression in primary glioblastoma and (c) p53 mutations in primary glioblastoma.

Table 5. Multivariate analysis for BMI1 and CD133 as prognostic markers.

Variable	HR	CI	*P* value
CD133			
Low	1	1.1266–11.3535	0.0315
High	3.2366		
BMI1			
Low	3.9048	0.0710–0.7472	0.0150
High	1		

CD133 is the first identified member of the Prominin family which is a penta-transmembrane glycoprotein (Miraglia *et al.* 1997). The function and specific ligands of the Prominin protein is still not well understood. CD133 is an important biomarker of cancer stem cells which promote various malignancies (Ong *et al.* 2010; Grosse-Gehling *et al.* 2013). It is implicated in radio- and chemo-resistance of various tumours (Liu *et al.* 2006). The influence of CD133 in cancer stem cells isolated from glioblastoma patients is clinically useful for prognostic studies. Recently, a metaanalysis was carried out by Han *et al.* (2015) with 21 published studies to reach a conclusion about the role of CD133 in gliomas. They found that high expression of CD133 in glioma patients was associated with high grades and there was an association between CD133 high expression and poor overall survival (OS) and progression free survival (PFS) (Han *et al.* 2015). One of the earlier studies was by Metellus *et al.* (2011), who used real-time PCR to quantify the mRNA expression of *CD133* from glioblastoma and its role in prognosis. In their study, they found 31.6% of samples were overexpressing *CD133* and the high expressing patients had unfavourable outcome. Similar observations are found in our study exclusively on adult primary glioblastoma; where, expression of *CD133* directly correlates with bad prognosis.

BMI1 gene is a stem cell renewal promoter which plays a major role in chemo-resistance and angiogenesis of glioblastoma (Abdouh *et al.* 2009; Jiang *et al.* 2013). We have observed that BMI1 protein expression was high in glioblastoma samples when compared to the control brain tissue. Age and sex of the patients did not have any significant change in the expression levels of BMI1. Previous studies have shown that BMI1 overexpression is associated with shorter survival (Hayry *et al.* 2008; Farivar *et al.* 2013; Wu *et al.* 2013). But, our observation was interesting, as the overexpression of BMI1 protein showed favourable prognosis in our cohort. Our results were also strengthened by the multivariate analysis, as BMI1 expression could independently predict the overall survival of the patients. The probable explanation for our finding is that the median survival time in primary glioblastoma is comparatively less than secondary glioblastoma. Similar observations were made by Cenci *et al.* (2012) who suggested that other possible proapoptotic role of BMI1 protein in primary glioblastoma. They analysed the C-MYC expression in combination with BMI1 expression and its role in the prognosis of glioblastoma. *C-MYC* is an important gene in cancer, as it regulates epigenetic modification of cancer cells (Amente *et al.* 2011). They found expressions of both genes are correlated with each other and they concluded that c-myc might be able to upregulate the BMI1 (Cenci *et al.* 2012). de Vries *et al.* (2015) demonstrated the importance of EZH2, a member of polycomb repressor pathway. The short-term depletion of EZH2 improved survival without glioblastoma progression. But the prolonged EZH2 depletion triggers switch in cell fate, cell proliferation, DNA damage repair and activation of a part of the pluripotency network. This shows EZH2 is also a strong therapeutic target in GBM (de Vries *et al.* 2015). Interestingly, recent studies have demonstrated that EZH2 may be one of the PcG proteins essential for Bmi1 recruitment to the PcG bodies, suggesting the closed relationship between BMI1 and EZH2 (Hyland *et al.* 2011).

The tumour suppressor *TP53* gene mutation is often seen in astrocytic tumours, especially in malignant gliomas (Fromentel and Soussi 1992; Newcomb *et al.* 1993; Ishii *et al.* 1999). Although, *TP53* gene has been studied extensively in all types of cancers, implications of individual mutation is less understood. We chose exons 5–9 of *TP53* gene for mutation analysis as maximum number of mutations are concentrated in this region (Fromentel and Soussi 1992). We have found that 57.14% of our samples had *TP53* mutations as compared to 13% samples reported earlier (Phatak *et al.* 2002) from the Indian glioma patients. In our study, the *TP53* mutations are concentrated particularly in 5–7 exons and no mutations in 8 and 9 exons. We are reporting new missense *TP53* mutations, K132F, K139F and S185T for the first time in primary glioblastoma patients. There are conflicting reports on the status of *TP53* mutations and prognosis of gliomas. Some reports suggests that there is no correlation between mutations and survival status (Newcomb *et al.* 1998; Kraus *et al.* 2000; Shiraishi *et al.* 2002), but few reports indicate a strong clinical correlations and prognosis in glioblastoma patients (Tada *et al.* 1998; Schiebe *et al.* 2000). Our study on the *TP53* mutations indicates that there is no direct influence on the survival or the prognosis of primary glioblastoma patients.

We conclude that *CD133* expression plays an important role in the prognosis of primary glioblastoma, in terms of overexpression showing dismal prognosis. High BMI1 protein expression is indicative for better prognosis. The *TP53* mutation status does not influence the outcome of survival in our patients. No correlation has been observed between the expression of CD133 in TP53 mutated or nonmutated cases, similar trend was observed in BMI1 expression cases. But, the overexpression of *CD133* mRNA and BMI1 protein expression influence the survival status independently in primary glioblastoma patients.

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