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Pathology Section

Immunohistochemical Analysis of TP53 and PTEN Expression in Glioblastoma Multiforme Patients of Western Rajasthan, India: An Observational Study

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ABSTRACT

Introduction: Glioblastoma Multiforme (GBM) is the most aggressive brain tumour with a dismal prognosis. Very few studies on its diagnosis, prognosis and therapeutics have been attempted in the past on Indian population. Standard treatment of this highly aggressive tumour yields little survival benefit; hence, greater attention is now being paid to personalised treatment and, correspondingly, to the expression of specific molecular markers with the goal of assessing their possible therapeutic as well as prognostic significance.

Aim: To estimate the proportion of Tumour Protein 53 (TP53) and PTEN (Phosphatase and Tensin Homolog) expression in GBM patients of Western Rajasthan, India.

Materials and Methods: This study was a ambispective, single institutional observational study done on 35 brain tissue biopsies of histopathologically diagnosed and confirmed cases of GBM based on the World Health Organisation (WHO) classification, 2007 received in Department of Pathology, Dr. Sampurnanand Medical College (Dr. SNMC), Jodhpur, Rajasthan, India, from January 2015 to December 2020 (January 2015 to September 2018:Retrospective and October 2018 to December 2020:Prospective) after applying inclusion and exclusion criteria. Immunohistochemical (IHC) analysis was done for TP53 and PTEN markers in all cases of GBM. The results were then applied for statistical analysis using Statistical Package for the Social Sciences (SPSS) version 22.0 software package (SPSS Inc., Chicago, IL, USA). The association between age and gender with TP53 and PTEN positive cases, respectively, was analysed by using Fisher's-exact test and the results of the same were compiled.

Results: The TP53 expression was seen in 32 cases (91.4%). The mean age of presentation of TP53 positivity was 50.3 years. Most of the TP53 cases occurred in the 41 to 50 and 61 to 70 years of age groups. In TP53 mutated cases, males were found more commonly affected as compared to females. Overall, the majority of cases (eight cases) occurred in the temporal region of the brain (25%). The relationship between age and gender with TP53 expression was found to be insignificant. PTEN expression was found absent (mutation positive) in 11 cases (32.4%). Mean age of these patients was 52.5 years. The male to female ratio was found to be 1.8:1. Regarding tumour location in the brain, most of the cases occurred unilaterally, with 45.4% (n=5) of cases occurring in the temporal region. The association between PTEN mutation with age and gender was not found to be significant.

Conclusion: The higher prevalence of TP53 expression in the population suggests distinct genetic pathways that need to be studied in detail to have a better understanding of this highly aggressive tumour. The PTEN mutation was discovered to be prevalent in the population of Western Rajasthan and needs to be studied further on a large scale. The simultaneous presence of molecular markers in GBM cases would need to be considered before initiating any gene therapy for its improved effectiveness.

Keywords: Genetic pathways, Immunohistochemical grading, Molecular markers, Nuclear staining, Targeted therapy, World Health Organisation classification 2007

INTRODUCTION

The incidence of brain tumours in India ranges from 5 to 10 per 100,000 people [1,2]. High-grade gliomas, mostly grade IV gliomas, such as GBM, are common types of primary brain tumours [3]. Histopathologically, glioblastoma shows high mitotic activity, cellular and nuclear pleomorphism, intravascular microthrombi, microvascular proliferation and necrosis with or without cellular pseudopallisading [4]. Molecular markers play an important role in diagnosis, prognosis, and predicting therapeutic effects of treatment. These may be proteins, antigens, or might have genetic, epigenetic, or cytogenetic origins. Currently, the importance of biomarkers is being prioritised utilising various molecular approaches that may be relevant for both prognostic and diagnostic purposes. In this study, two IHC markers, namely TP53 and PTEN, were included for analysis in GBM.

TP53 expression: TP53 is a nuclear phosphoprotein which is involved in classical pathways of cellular growth and differentiation, including induction of apoptosis, instigation of Deoxyribonucleic Acid (DNA) repair, and transient arrest of the cell cycle in the G1 phase. As such, the gene encoding TP53, is classified as a tumour suppressor gene as loss of function leads to tumour inception. In GBM, the majority of TP53 alterations are missense mutations in the DNA binding domain (DBD) limiting transcription factor activity. A mutant TP53 gene product may result in constitutive upregulation of TP53 nuclear expression with potential loss of TP53 function, gain of TP53 function with partial conservation of wild-type protein function, or dominant negative regulation [5].

PTEN expression: PTEN is a tumour suppressor gene that is involved in many signalling pathways, most notably the PI3K/Akt pathway, where it acts as a phosphatase and thus dephosphoryles PIP3, producing a PIP2-molecule and thus maintaining inactivity in the Akt pathway [6-8]. PIP3 is a key regulator of the PI3K/Akt signalling pathway and acts by recruiting Akt to the membrane surface, an event critical for Akt activation. Activated Akt regulates several downstream pathways, controlling cell cycle progression,

protein synthesis, survival, apoptosis, and migration [9-11]. Loss of PTEN expression, through deletion, mutation, or methylation, essentially mimics activation of the Akt pathway as a result of the accumulation of PIP3, while retention of PTEN maintains Akt inactivity [9,11,12]. Although PTEN expression is ubiquitous across all tissues, only in certain tumour types has it been shown to play a role in tumourigenesis-such as tumours of the breast, ovaries, prostate, skin, pancreas, and most notably, the brain [13-22].

As standard medical practice in the treatment of this highly aggressive tumour yields little survival benefit, greater attention is now being paid to personalised treatment and, correspondingly, to the expression of specific molecular markers with the goal of assessing their possible therapeutic as well as prognostic significance [6,23-26]. Very few molecular studies on GBM have been done in the past on Indian population [27,28]. Also, the present study was the first study of such type on GBM patients of Rajasthan, India. Hence this study was an attempt to contribute to the better management of GBM patients in the hope that these patients might achieve improved survival in the future by getting specific target therapies depending on the type of molecular defect present in their tumour. The present research was a single Institutional study which aimed to estimate the proportion of TP53 and PTEN expression in GBM patients.

MATERIALS AND METHODS

The present study was a ambispective (January 2015 to September 2018:Retrospective and October 2018 to December 2020:Prospective), single Institutional observational study which was done on brain tissue biopsies of histopathologically diagnosed and confirmed cases of GBM based on the WHO Classification, 2007 [4] received in the Department of Pathology, Dr. S.N. Medical College, Jodhpur, Rajasthan, India, from January 2015 to December 2020. The proposal for the study was reviewed thoroughly and approved by the Institutional Ethical Committee (IEC) (EC/MC/JU/2018/319) before the commencement of the study.

Inclusion criteria: All brain biopsies from January 2015 to December 2020 with a histopathological diagnosis of GBM were included in the study.

Exclusion criteria: Ultra small biopsies, GBM cases with single tissue block, brain biopsies with histopathological diagnosis other than GBM and GBM cases which were registered outside the above mentioned time frame were all kept under exclusion criteria.

After applying inclusion and exclusion criteria, paraffin embedded tissue blocks of a total of 35 cases of histopathologically confirmed GBM from January 2015 to December 2020 were retrieved from the Department of Pathology. Epidemiological data (age and sex) for the selected cases was procured from the patient records kept in the pathology department.

Immunohistochemistry

For IHC, tissue sections of 5 µm thickness were taken on Bond Max albumin coated microscope slides. These slides were incubated first and processed further for dewaxing with ethanol and xylene. The IHC technique was applied for TP53 and PTEN expression on Leica Bond Max Immunostainer installed in the Department of Pathology using rabbit anti-human p53 Monoclonal Antibody (Clone SP5) and mouse anti-PTEN Monoclonal Antibody (Clone 6H2.1), respectively. IHC slides were mounted and examined microscopically by three histopathologists. Positive controls were taken as carcinoma breast for TP53 and endometrium for PTEN. The cases whose slides reported non uniformity in interpretation were processed twice for IHC. All the cases were reviewed thoroughly by histopathologists as per below mentioned criterias. For TP53 expression, total 35 were analysed while only 34 cases were estimated for PTEN expression as PTEN staining of one case was found to be non contributory (repeated twice). The results were compiled and applied for statistical analysis. Slides showing non contributory staining were not counted for statistical analysis.

Criteria for TP53 expression interpretation [29,30]: Presence of nuclear staining in ≥10% of tumour cells in most representative areas were considered positive for TP53 mutation.

Scoring criteria: <10%-negative
10-30%-1+
31-50%-2+
>50%-3+

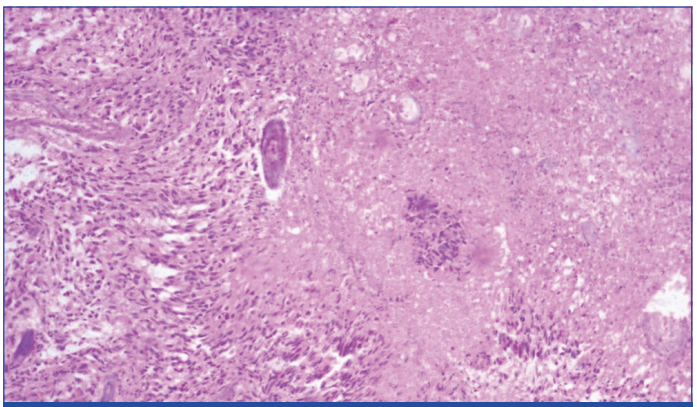
Criteria for PTEN expression interpretation [31]: Absence of nuclear staining in entire or vast majority of tumour was considered positive for PTEN mutation whereas presence of nuclear staining either partially or in entire tumour was considered negative.

STATISTICAL ANALYSIS

The results were then applied for statistical analysis using SPSS 22.0 software package (SPSS Inc., Chicago, IL, USA) using Fisher's-exact test.

RESULTS

Most of the GBM cases [Table/Fig-1] occurred unilaterally, with 45.4% of cases (n=5) occurring in the temporal region.



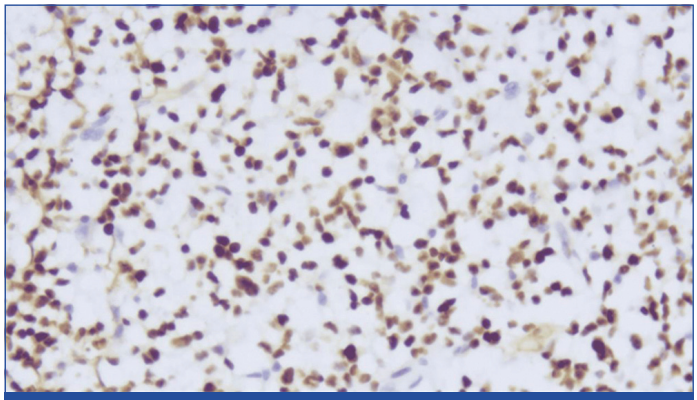
[Table/Fig-1]: Photomicrograph of glioblastoma multiforme showing cellular and nuclear pleomorphism, vascular proliferation, necrosis and pseudopallisading (H&E, 100X).

TP53 expression: Interestingly, TP53 expression was seen in the majority of cases, i.e., 32 out of a total of 35 cases (91.4%). The mean age of presentation of TP53 positivity was 50.3 years. Most of the TP53 cases occurred in the 41 to 50 years and 61 to 70 years age groups (10 cases each). The majority of positive cases were beyond 40 years of age (n=25, 78.1%). Further, among eight cases in the age group less than 40 years, 87.5% (7 cases) showed TP53 expression, whereas it came out to be 92.6% (25 cases) in cases more than 40 years of age (27 cases). However, the association between age and TP53 positivity was not significant (Fisher's-exact test, p-value=0.553). Further p-values of association of distinct age groups with TP53 expression were also found insignificant [Table/Fig-2]. Among TP53 positive cases, 9.4% (3 cases) were showing ≤30% stained tumour cells (Grade 1), 15.6% (5 cases) were showing staining in 31-50% of tumour cells (Grade 2) while in 75% (24 cases), >50% tumour cells were found stained for TP53 (Grade 3) [Table/Fig-3,4a-c].

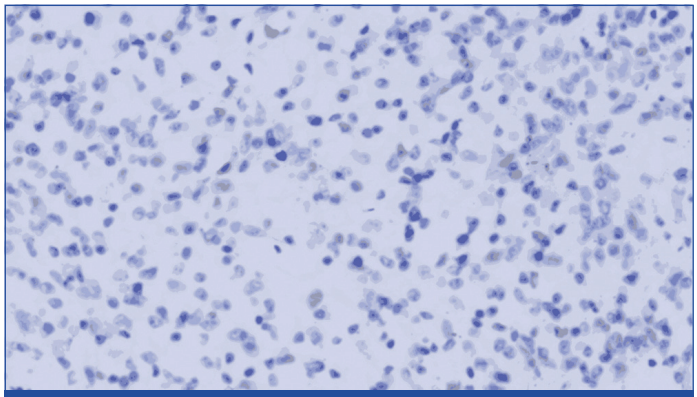
The mean age of presentation in Grade 1, Grade 2 and Grade 3 were 52 years, 50.4 years, and 51.1 years respectively. Most of the cases occurred beyond 40 years of age and showed a peak age of presentation between 41-50 years of age in Grade 1 (n=2, 66.67%), while it was 41-60 years (n=4, 80%) in Grade 2. In Grade 3, most of the cases were in the 61-70 year age group (n=9, 37.5%).

Age (yrs)	TP53 grading						Total		p-value
	Grade I		Grade II		Grade III				
	N	%	N	%	N	%	N	%	
17-30	0	0	0	0	3	100	3	9.4	0.576
31-40	0	0	1	25	3	75	4	12.5	0.709
41-50	2	20	2	20	6	60	10	31.2	0.306
51-60	0	0	2	40	3	60	5	15.7	0.224
61-70	1	10	0	0	9	90	10	31.2	0.257
Total	3	9.38	5	15.63	24	75.00	32	100	

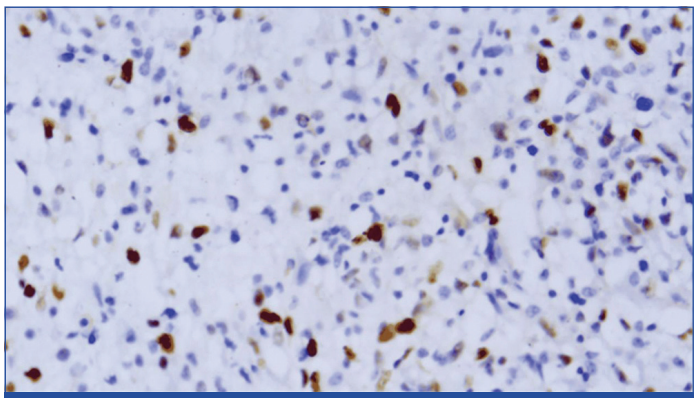
[Table/Fig-2]: Age distribution in grades of TP53 positive cases.



[Table/Fig-3]: Photomicrograph of immunostained tissue section of carcinoma breast taken as TP53 positive control showing strong diffuse nuclear staining (X400).



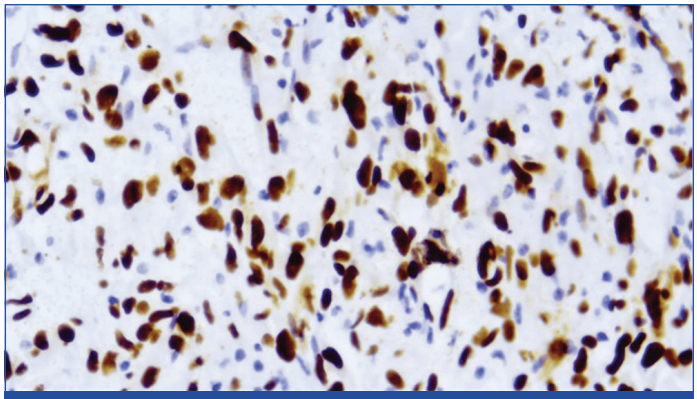
[Table/Fig-4a]: Photomicrograph of immunostained tissue section of GBM showing TP53 nuclear staining with grade 1 positivity (X400).



[Table/Fig-4b]: Photomicrograph of immunostained tissue section of GBM showing TP53 nuclear staining with grade 2 positivity (X400).

In TP53 positive cases, males were found more commonly affected as compared to females in total, with a male to female ratio of 1.5:1. In Grade 1, the gender distribution (male: female) is 1:2, 4:1 in Grade 2, and 1.4:1 in Grade 3 [Table/Fig-5]. Freeman-Halton extension of Fisher's-exact test was used to see if there was a link between TP53 expression and gender for which p-value was found to be non significant (p-value=0.497).

Overall, the majority of cases (eight cases) occurred in the temporal region of the brain (25%), out of which two cases were seen in the



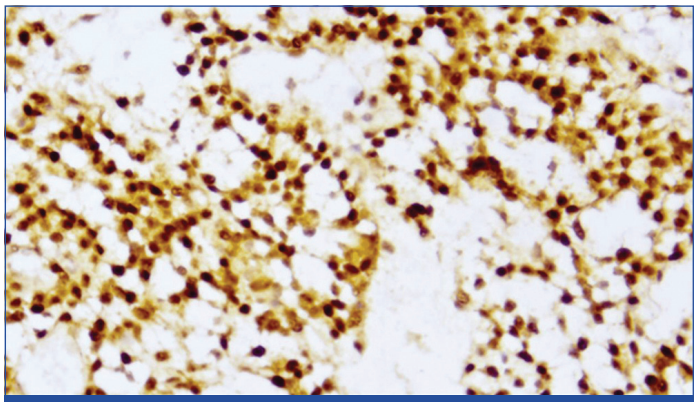
[Table/Fig-4c]: Photomicrograph of immunostained tissue section of GBM showing TP53 nuclear staining with grade 3 positivity (X400).

Gender	Grade 1	Grade 2	Grade 3	Total	p-value
Male	1	4	14	19	0.497
Female	2	1	10	13	

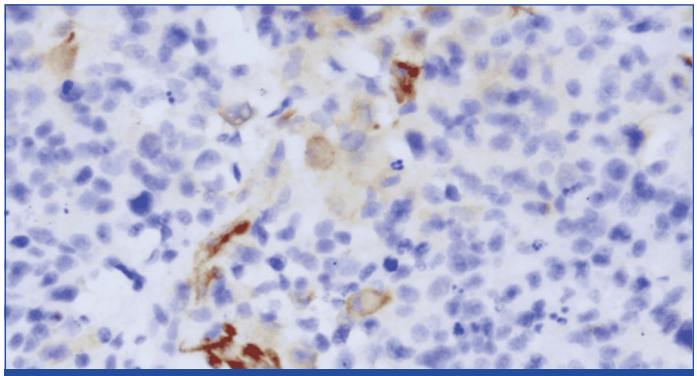
[Table/Fig-5]: Gender distribution in Grades of TP53 positive cases.

right temporal region and six cases in the left temporal region. There was no particular preference for any one side of the brain by this tumour for TP53 expression.

PTEN expression: For PTEN expression, one case was found to be non contributory as its staining (repeated twice) was not clearly defined. Out of the rest of 34 cases, 11 (32.4%) of them were found to be negative for staining, which mean that they had a PTEN mutation [Table/Fig-6,7].



[Table/Fig-6]: Photomicrograph of immunostained tissue section of endometrium taken as PTEN positive control showing strong diffuse nuclear positivity (X400).



[Table/Fig-7]: Photomicrograph of immunostained tissue section of GBM showing loss of PTEN nuclear staining taken as positive for PTEN mutation (X400).

The mean age of patients showing PTEN mutation was 52.5 years. Among these, the majority of cases (76.4%) were above 40 years of age. Three (37.5%) out of eight patients under the age of 40 were found to have PTEN mutations, while this mutation was found positive in eight (30.8%) of 26 patients over the age of 40 years [Table/Fig-8]. The association between PTEN mutation and age was found to be non significant (Fisher's-exact test, p-value=1.000). The

majority of the cases that tested negative for PTEN mutations were also discovered after the age of 40 age group (18 cases).

Age (yrs)	PTEN mutation				Total	
	Positive		Negative			
	N	%	N	%	N	%
≤40	3	37.50	5	62.50	8	23.53
>40	8	30.77	18	69.23	26	76.47
Total	11	32.35	23	67.65	34*	100

[Table/Fig-8]: PTEN mutation with age.
*One case was not included due to ill defined, non uniform staining (repeated twice)

The male to female ratio in total PTEN mutation positive cases was found to be 1.8:1 [Table/Fig-9]. In PTEN mutation negative cases, the male female ratio was reported as 1.3:1. The association between PTEN mutation and gender was found to be non significant (Fisher's-exact test, p-value=0.729).

Gender	PTEN mutation				Total	
	Positive		Negative			
	N	%	N	%	N	%
Male	7	35	13	65	20	58.82
Female	4	28.57	10	71.43	14	41.18
Total	11	32.35	23	67.65	34	100

[Table/Fig-9]: Gender distribution of PTEN mutation.

DISCUSSION

TP53 expression: The results of TP53 positivity were interestingly found to be very similar to the study by Das A et al., who reported 96% of TP53 mutation positivity in their GBM cases [32]. This study by Das A et al., was also on the Asian population and was mainly on Chinese, Malaysians, and Indians. Similar to present study, they also performed IHC as a diagnostic technique for TP53 mutation detection. The results of these studies on the Asian population are much higher than most of the previous studies in the literature on non Asian patients [32]. In support of the conclusion of the study by Das A et al., it is suggested that there might be distinct genetic pathways responsible for tumourigenesis in GBM tumours of the Asian population and hence they need to be further investigated. Another reason for such variation in results might be the use of different diagnostic criteria for TP53 mutation reporting. While on the one hand, few studies reported positivity for any staining on IHC, others took >80% positivity as diagnostic criteria [32-35]. Such a huge variation results in heterogeneity in results. Gülten G et al., took ≥80% staining as criteria for positivity for the TP53 gene mutation and reported 12.05% positivity [33]. In present study, 7/35 (20%) cases were shown ≥80% of TP53 staining. Similarly, Montgomery RM et al., examined 36 GBM cases and reported 86% positivity by taking >50% staining as criteria for TP53 staining positivity [34]. These results appear to be in line with present study, which shows 68.6% of cases as positive when applying the same criteria. Thus, it is suggested that there should be a uniform standard criteria for reporting of TP53 mutations worldwide to minimise such "pseudo-variations" in TP53 reporting to avoid misinterpretation of results. Previous studies on TP53 and PTEN mutations are enlisted in [Table/Fig-10,11], respectively [27,28,30-44].

The M:F ratio was around 1:1 in Das A et al., study [32], while it was 1.5:1 in present study. In both studies, no significant association was noted between age, gender, and TP53 overexpression. There was no significant association found between age and TP53 expression in Montgomery RM et al., study, which was in line with present study [34].

Year	Study by	Region	Criteria	Positivity
2022	Present study	India	<10%-negative 10-30%-1+ 31-50%-2+ >50%-3+	91.4%
2020	Gülten G et al., [33]	Turkey	<80%-neg ≥80%-pos	Pos-12.05% Neg-87.95%
2017	Karnam S et al., [35]	India	0-1 -neg 2-3-pos	46.4%
2016	Chaurasia A et al., [30]	Korea	0-<10%-no staining 1-10-30% 2-30.1-50% 3->50% 1-Negative 2-&3-pos	48.4%
2015	Montgomery RM et al., [34]	Brazil	1-0-25% 2-26-50% 3-51-75% 4-76-100% 1,2-neg 3,4-pos	86%
2013	Lee KS et al., [36]	Korea	≥10%	49.3%
2009	Ruano Y et al., [37]	Spain	Strong and extensive nuclear staining	11.2%
2008	Cancer Genome Atlas [38]			>90%
2002	Das A et al., [32]	Singapore	No staining-neg Any staining-pos	96%
2001	Simmons ML et al., [39]	Calfornia	0-No staining 1-<5% 2-5-30% 3->30%	56%
1996	Watanabe K et al., [40]	Boston	Any staining-pos	36.8% 97%
1995	Kyritsis AP et al., [41]	U.S	0-No labelling 1-<5% 2-5-30% 3->30%	60%
1994	Ng HK et al., [42]	China	Any staining-pos	36%

[Table/Fig-10]: List of previous studies on TP53 expression in GBM patients [30,32-42].

Year	Study by	Region	Positivity
2022	Present study	India	32.4%
2018	Arif SH et al., [27]	India	17.5%
2013	Gan HK et al., [43]	Australia	40%
2012	Carico C et al., [44]	California	53.5%
2011	Jha P et al., [28]	India	49.3%
2010	Kim B et al., [31]	Korea	21.4%
2009	Ruano Y et al,. [37]	Spain	38.6%

[Table/Fig-11]: List of previous studies on PTEN mutation in GBM patients [27,28, 31,37,43,44].

PTEN expression: In this study, loss or deletion in the PTEN gene was considered a positive staining outcome on IHC, which was seen in 11 out of 34 cases (32.4%), which was in concordance with literature [Table/Fig-11].

In this study, the majority of PTEN mutated cases occurred above 40 years of age, which was in line with the study by Jha P et al., [28]. They also reported that the mean age of GBM cases showing a PTEN mutation was older than the non mutated cases.

In a study by Srividya MR et al., on 73 GBM cases, the median age for PTEN mutation was reported to be 50.97±10.2 years, while non mutated cases of the PTEN IHC marker was 43.52±13.5 years [45]. As a result, the median age of patients with PTEN homozygous deletion was significantly higher than that of patients with the retained status (p=0.019). In present study, a similar age was reported in PTEN mutated cases (52.5 years), but there was no statistically significant correlation found between age and PTEN

mutation status. Furthermore, in previous studies, tyrosine kinase inhibitors were found to be effective only in cases of EGFR vIII mutations with a retained PTEN gene [34].

Limitations(s)

The principal limitation of this study was that this was a non randomised single Institutional study. Another major limitation was small sample size. Hence, the findings could be readily generalised or considered conclusive when confirmed by subsequent studies on Indian population. Moreover, limited clinical data restricted subdivision of tumours in primary and secondary glioblastoma which could have added information regarding association of TP53 and PTEN mutations to subtypes.

CONCLUSION(S)

The higher prevalence of TP53 expression in present study population suggests distinct genetic pathways that need to be studied in detail to have a better understanding of this highly aggressive tumour. The PTEN mutation positivity found in present study was in concordance with the literature. Before starting gene therapy for GBM, it would be important to think about the presence of molecular markers in the same place. To develop targeted therapies against these molecular markers, it is important to know the prevalence of these markers as well as other clinical data in racially diverse GBM patients. This work is a step towards personalised treatment of GBM patients, particularly on population of Western Rajasthan, India. More such studies are needed in the future in India on a large scale, to have a better understanding of this lethal tumour for improved patient survival.

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