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JSM Genetics & Genomics

Research Article

Promoter Hypermethylation of Phosphatase and Tensin Homolog in Glioblastoma without Gene Deletion

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Submitted: 28 March 2016

Accepted: 14 May 2016

Published: 16 May 2016

Keywords
- Methylation
- Glioblastoma
- Epigenetics
- *PTEN*

Abstract

Research efforts have focused on discovering key biochemical pathways of tumorigenesis and identifying genes that are central to the development of brain cancer. *PTEN*, a tumor suppressor in the PI3K/AKT pathway is frequently deleted in glioblastoma. This study aimed to examine epigenetic events, such as hypermethylation of the promoter region of *PTEN* as a contributing mechanism of the malignant phenotype. Analysis was performed on 77 glioma samples. *PTEN* methylation status was measured by pyrosequencing and compared to *PTEN* deletion status previously analyzed by amplification and capillary electrophoresis of linked polymorphic loci. *PTEN* protein expression was assessed by immunohistochemistry.G-CIMP status was determined using the MethyLight procedure. *PTEN* promoter methylation or deletion occurred in 38% and 36% of cases, respectively. 8% of cases had both. G-CIMP was associated with *PTEN* methylation ($p=0.002$) and negatively correlated with *PTEN* deletion ($p<0.001$). Approximation of *PTEN* protein expression by immunohistochemistry revealed slightly decreased expression in cases with either promoter methylation or deletion staining frequency X intensity, (48.4 and 50.8, respectively), compared to cases with neither (54.4). *PTEN* methylation and *PTEN* deletion were found to be mutually exclusive events in brain tumors. These results suggest that tumor suppressive functionality of *PTEN* could be epigenetically hindered, potentially promoting tumorigenesis in glioma in the absence of gene deletion.

ABBREVIATIONS

GBM: Glioblastoma Multiforme; LOH: Loss of Heterozygosity; *PTEN*: Phosphatase and Tensin Homolog

INTRODUCTION

Gliomas are solid tumors that originate from the glial cells of the brain. Gliomas can grow and develop in people of all ages. The most common malignancy in adults are World Health Organization (WHO) Grade IV astrocytomas, also known as glioblastoma multiforme (GBM), which account for 45% of malignant gliomas in the United States [1]. Although treatment with GBM with resection, radiation therapy, and temozolomide (TMZ) chemotherapy shows efficacy, GBM recurrence is high in most patients, with overall survival (OS) of about12-15 months post diagnosis; less than 5% of patients in the mean age group of 65 years survive GBM 5 years after diagnosis [2]. This study focuses on WHO Grade III and Grade IV "high grade" adult gliomas sampled for diagnosis before treatment.

The phosphotidylinositol-3-kinase/protein kinase B (PI3K/AKT) pathway is dysfunctional in a variety of cancers including brain. Phosphatase and tensin homolog (*PTEN*) is a member of the PI3K/AKT pathway and acts as a tumor suppressor. Loss of function of this gene through deletion, point mutations, or methylation occurs in a variety of tumors, including gliomas, and is associated with tumor growth and poor prognosis [3,4].

Studies of both GBM samples and melanoma brain metastases have found monoallelic inactivation of *PTEN* through either deletion or promoter hypermethylation; additionally, the two events were found to be mutually exclusive in melanoma.[5] In light of these findings, assessment of the exclusivity between methylation and deletion might better define whether a breakdown in *PTEN* functionality is due to biallelic inactivation or if methylation and/or deletion is sufficient to render *PTEN* ineffective in GBM. If methylation and deletion of *PTEN* are found to be mutually exclusive events, then methylation analysis of *PTEN* may be of importance in diagnosis and prognosis of GBM.

MATERIALS AND METHODS

GBM Patient Samples

A total of 77 paraffin embedded glioma sections (Grade I through IV) were used in this study (Table 1). The average age at diagnosis in this group differed significantly between those categorized as WHO Grade I, II, and III (low grade) vs. WHO Grade IV (high grade) tumors (t(75)= -3.233, p=0.002, n=77), consistent with previous literature reports [6-8].

DNA Methylation Analysis by Pyrosequencing

Sodium bisulfite conversion was performed using the EZ DNA Methylation KitTM (Zymo Research, Irvine, CA) according the manufacturer's instructions. The *PTEN* promoter region in the converted DNA was amplified in a standard reaction mix containing 0.4 µM forward (GGATGTGGGTGTTTGTGTAATTA) and reverse (AATTCCCACTCCCCAATAATAAC-biotin) primers (IDT, Coralville, IA). PCR was performed using the Veriti® Thermal cycler (Applied Biosystems-Life Technologies) and pyrosequencing of the PCR products on the Pyromark Q24 Pyrosequencer (Qiagen, Valencia, CA). The *PTEN* sequencing primer, TTTGTGTAATTAGTTTTTTA, was directed at five positions of the *PTEN* promoter region: -1333, -1324, -1318, -1310, and -1295 (translation start = +1). MethyLight sequence-specific qPCR was used to detect Glioblastoma CpG Island Methylator Phenotype (G-CIMP). The panel was comprised of four test gene promoters (ANKRD43, FAS1, HFE, and DOCK5) and an internal control (COL2A1).

Immunohistochemical Methods (IHC)

IHC staining was performed on four-micron sections of formalin-fixed tissues. Prior to slide staining of brain tissue samples, the slides were dewaxed and hydrated using a standard xylene and decreasing percent ethanol protocol. The slides were manually stained with rabbit monoclonal anti-*PTEN* antibody, clone EPR9941 (AbCam®, Cambridge, MA), showing specificity to cytoplasmic *PTEN*, using the Ventana® iView DAB detection kit (Roche, Tucson, AZ) according to standard immunohistochemical methods found on the manufacturer's website [9]. Following staining, slides were examined by a pathologist to assess expression within tumor cells. *PTEN* expression was categorized as negative, low positive (10-50% intensity) and high positive (>50% positivity).

Statistical Methods

Analysis of the data was performed with SPSS© Statistics software (IBM©). For categorical calculations, *PTEN* promoter hypermethylation was dichotomized (methylated vs. unmethylated). Chi-Square analysis was used to compare *PTEN* promoter hypermethylation to deletion (yes/no). A Kruskal-Wallis test was performed for K independent variable was used to analyze *PTEN* protein expression to *PTEN* methylation status.

RESULTS

PTEN deletion vs. *PTEN* methylation

Chi- squared analysis of the relationship between *PTEN* deletion and *PTEN* methylation in samples of GBM support the idea that *PTEN* deletion and *PTEN* methylation are mutually exclusive events ((Table 2); $\chi^2_{(1)}$=16.105, p<0.001) with a large effect size (Φ =-0.506). Methylation across all of the *PTEN* promoter CpG sites interrogated was found to be significantly exclusive of *PTEN* deletion. The site that showed the closest correlation to the mean *PTEN* methylation levels within the samples was the last site interrogated at -1295.

PTEN promoter methylation was associated with low grade tumors, while *PTEN* deletion was found in high grade tumors ((Table 2); $\chi^2_{(1)}$ =16.105, p<0.001) with a large effect size (Φ =-0.506).

Five samples had detectable levels *PTEN* methylation despite allelic deletion (Table 2). Methylation and deletion, therefore, do occur concurrently, albeit significantly less often than either alone. It is interesting to note that the *PTEN* promoter region percent methylation in these samples (n=5, methylation=10.8%) was approximately half the level of that found in samples that were undeleted and methylated (n=24, methylation=22%). These cases were all females with high grade disease.

Table 1: Study sample demographic data.

Gender	**Males** **Females**	**45, 58.4%** **32, 39.5%**
Grade of Tumor	Benign Grade I Grade II Grade III Grade IV	2 1 23 8 43
Low (WHO Grade I, II, III) vs. High Grade Tumors (WHO Grade IV)	 Low Grade High Grade	 33 44
Average age of diagnosis		49.4 years
Average age of diagnosis by tumor type (low vs. high)[a]	Low Grade High Grade	43.4 years 53.8 years
Total participants (n)		77

[a]p= 0.002
WHO, World Health Organization

Table 2: *PTEN* deletion (deletion) and *PTEN* promoter methylation in glioblastoma.

	Number of samples[a]	Mean % *PTEN* promoter methylation[b]	G-CIMP+/Total	Grade IV
PTEN undeleted and not hypermethylated	11	4	1/11 (p=0.079)	5/11 (p=0.515)
PTEN hypermethylatedonly[c]	24	22	17/24 (p=0.001)	8/24 (p=0.003)
PTEN deleted only	23	4	3/23 (p<0.001)	20/23 (p<0.001)
PTEN deleted and hypermethylated	5	10.8	0/5 (p=0.150)	5/5 (p=0.148)
TOTAL	63		21/63	38/63

[a]Out of the 77samples that were investigated for methylation, 63 had *PTEN*deletion, methylation, G-CIMP and staging data.
[b]Average percent methylation across five CpG sites in the *PTEN* gene promoter (p<0.001 vs. *PTEN* deletion)
[c]Hypermethylation was defined as >20% methylation by pyrosequencing

Although a weak relationship between MGMT and *PTEN* methylation status was observed in the current patient group, there was a positive association between G-CIMP+ status and *PTEN* methylation. G-CIMP+ status and *PTEN* deletion were negatively associated (Table 2).

Immunohistochemical (IHC) Analysis

Four micron sections of tumor tissue were stained for *PTEN* protein expression. Seventy slides were stained for *PTEN* expression, of which 54 produced staining of adequate quality. Thirty five percent of the cases showed positive cytoplasmic staining for *PTEN* protein; 16% were strongly positive (Figure 1). In cases without *PTEN* promoter hypermethylation, 27% was strong positive, compared to 11% if cases with *PTEN* promoter methylation. There was no strong association found between *PTEN* deletion and *PTEN* expression (47% with deletion vs. 54% without).

Figure 2 shows the results of frequency time's intensity of staining in cases with and without *PTEN* promoter methylation and *PTEN* deletion. Staining was slightly decreased by *PTEN* promoter methylation or *PTEN* gene deletion alone. None of the five samples with both deletion and methylation yielded adequate staining results. Further analysis will be required to confirm expression data. Proclivities of brain tissue include increases in small fatty areas that may negatively affect staining, as *PTEN* antibodies may not react as well with these areas. *PTEN* antibody binding may cross-react or even be inhibited with other cytoplasmic proteins, resulting in false positives as well as false negatives due to lack of specificity; future studies utilizing a more specific and sensitive method or a different monoclonal antibody may be considered when pursing this avenue of study.

DISCUSSION

Studies on sporadic mutations have suggested a "two-hit" mechanism of biallelic activation of *PTEN* either by genetic or epigenetic means can lead to haploinsufficiency and tumorigenesis in GBM. Newer studies on GBM as well as studies on other cancers such as CRC and MBM's, however, have contradicted this observation, and have proposed that biallelic inactivation of *PTEN* is unnecessary for tumorigenesis in GBM and that either methylation or deletion events are sufficient [10-12].

The results of this study support the latter observation, which was that hypermethylation of the *PTEN* promoter region may be mutually exclusive from loss of heterozygosity due to deletion in high-grade gliomas [13]. Also of interest was the observation that, in those samples in which *PTEN* was both methylated and deleted, the level of methylation was about half the level of those samples that were methylated and showed no deletion of *PTEN*. This supports the notion of biallelic methylation in cases where the second allele is not deleted. Differences in protein expression as measured by IHC were not strongly supportive of the effects of neither deletion nor methylation, however, these assessments were difficult to quantify. Further studies will confirm as well as to find a possible explanation for this observation.

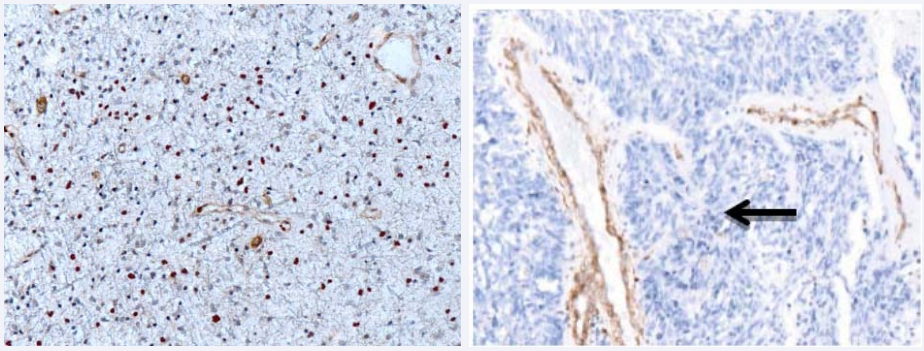

Figure 1 Cytoplasmic expression of *PTEN* in brain tumors, positive (left) and negative (right) the arrow shows expected positive staining of endothelial cells surrounding blood vessels.

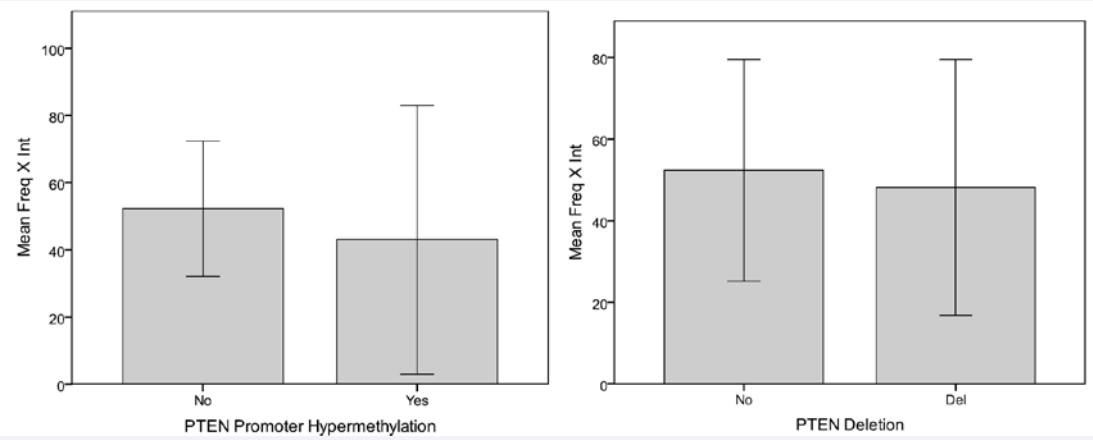

Figure 2 Expression of *PTEN* in brain tumors, measured as frequency (0->50 % of cells) times intensity (weak, 1, strong, 2). *PTEN* promoter methylation (left), and *PTEN* deletion (right).

Hypermethylation of the promoter region of tumor suppressor genes like *PTEN* lowers their expression in cancer cells. Conversely, hypomethylation of the intergenic regions causes transposable elements and oncogenes to be inappropriately expressed leading to chromosomal instability and over proliferation of cells [14]. The effects of epigenetic changes, such as methylation on tumorigenesis have been found to be a valid alternative explanation to mutation or deletion of tumor suppressor genes in various cancers, including GBM.

As GBM has been increasingly characterized by genetic and molecular changes, a global hypermethylator or Glioma CpG Island Methylator phenotype (G-CIMP) has been described [15]. The G-CIMP phenotype was likely to be associated with *IDH1* mutations predominantly found in secondary glioblastoma and recurrent GBM after treatment [15,16]. A global hypermethylation state, as seen in G-CIMP, involving multiple genes within various biochemical pathways may contribute to oncogenesis and progression of aberrant cellular events in many cancers but may also be associated with positive treatment outcomes and longer survival.[11] Methyl guanine methyl transferase (MGMT) hypermethylation is currently utilized in the clinical molecular diagnosis of GBM as a prognostic marker of responsiveness to Temozolomide (TMZ) chemotherapy and longer survival [17]. Are MGMT and *PTEN* methylation reflecting the hypermethylator phenotype? Although a weak relationship between MGMT and *PTEN* methylation status was observed in the current patient group, there was a positive association between G-CIMP+ status and *PTEN* methylation. G-CIMP+ status and *PTEN* deletion were negatively associated (Table 2). G-CIMP+ may exert an effect that includes hypermethylation of *PTEN*, although this was not a primary finding in studies investigating the most highly hypermethylated loci in G-CIMP+ samples. Further, a hypermethylator phenotype would compromise the effects of specific drug interactions; such efficacy of TMZ in MGMT methylated brain cancers [18].

CONCLUSION

An interdependence between *PTEN* deletion and *PTEN* promoter methylation to transcriptionally silence *PTEN* (biallelic inactivation) would be in accordance with the "two-hit" hypothesis. This study has found, however, that either hypermethylation or deletion alone may contribute to the malignant cell phenotype. Anti methylation agents might reverse this effect. Multigene epigenetic investigations will address these complex interactions.

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Cite this article

Choi E, Roye R, Alnajar H, Buckingham L (2016) Promoter Hypermethylation of Phosphatase and Tensin Homolog in Glioblastoma without Gene Deletion. JSM Genet Genomics 3(1): 1011.