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Expression of DNA methyltransferases 1 and 3B correlates with EZH2 and this 3-marker epigenetic signature predicts outcome in glioblastomas

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ABSTRACT

This study aims to analyze expression of EZH2 and DNA-methyltransferases (DNMT1, 3A and 3B) in astrocytic tumors and investigate their link as well as their correlation with survival, especially in GBMs. Expression of EZH2 and DNMTs (DNMT1, DNMT3A and DNMT3B) in different grades of astrocytomas (n = 93) was assessed by qRT-PCR and immunohistochemistry. GBM-U87MG cell line was used for functional studies. Strong immunopositivity (LI ≥ 25%) for EZH2, DNMT1 and DNMT3B was detected in 52%, 56% and 64% cases of GBMs respectively, which was significantly higher as compared to Grade II/III cases. Similarly, their median fold change of mRNA expression was also significantly higher in GBMs. There was also a significant positive correlation between DNMT1/DNMT3B and EZH2 mRNA and protein expression, which was in concordance with TCGA data set. Inhibition of DNMTs in cell line by Azacytidine resulted in down-regulation of EZH2, while knock-down of EZH2 by siRNA was not associated with any significant alteration of DNMTs, indicating that EZH2 expression in GBMs is possibly regulated by DNMTs, but not the reverse. Strong immunopositivity for EZH2, DNMT1 and DNMT3B were individually associated with significantly shorter survival and showed no correlation with IDH1 mutation status. In addition, the combination of these 3 markers represented an independent prognostic signature with cases having weak/negative expression of all 3 markers being associated with best prognosis. For the first time, the present study describes an epigenetic prognostic signature in GBMs based on immunohistochemical expression of EZH2, DNMT1 and 3B which can be used easily in routine neuropathology practice.

1. Introduction

Epigenetic systems involve heritable changes in DNA and DNA associated proteins which ultimately regulate gene expression. DNA methylation and histone modifications are two principal factors in epigenetic phenomena. DNA methylation is an essential regulatory mechanism of gene expression by which transcriptional activity of DNA decreases and DNA stability increases. DNA methyltransferases (DNMTs) are a group of enzymes which catalyze the transfer of a methyl moiety from S-adenosyl-L-methionine to the 5-position of cytosines in the CpG dinucleotide and are responsible for the establishment and regulation of global patterns of DNA methylation (Robertson, 2001). A number of DNMTs have been identified in mammals, of which DNMT1, DNMT3A and DNMT3B are the major and best known. DNMT1 acts as a maintenance methyltransferase and is responsible for copying methylation patterns after DNA replication while DNMT3A and DNMT3B are responsible for de novo methylation (Chuang et al., 1997; Okano et al., 1999). Altered expression of DNMTs leads to abnormal methylation of CpG islands which can efficiently repress transcription of the tumor suppressor genes. Overexpression of DNMT1, 3A, and 3B have been reported in various malignancies, including gastric (Etoh et al., 2004), hepatocellular (Saito et al., 2003; Sun et al., 1997), colorectal (De Marzo et al., 1999), breast (Girault et al., 2003), lung (Lin et al., 2007), cervical (Sawada et al., 2007) and prostate (Gravina et al., 2013) carcinomas. Furthermore, DNMT overexpression has also been correlated with decreased survival and aggressive biological behavior (Etoh et al., 2004; Saito et al., 2003; De Marzo et al., 1999; Girault et al., 2003; Lin et al., 2007). Only single study by Rajendran et al. (2011) is available on various grades of CNS tumors which reported significant overexpression of DNMT1 and DNMT3B in various tumors including 4 cases of pilocytic astrocytoma, single case of Grade II astrocytoma and 20 cases of GBMs. Strikingly the expression levels correlated with the grade of the tumors (Rajendran et al., 2011). Furthermore, few other studies on GBMs also demonstrated that DNMT-1 and 3B are responsible for promoter region hypermethylation of a large number of genes involved in cell cycle regulation and apoptosis (Foltz et al., 2009; Hervouet et al., 2010). However, to the best of our knowledge,

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there has been no study regarding the immunohistochemical expression of DNMT1, DNMT3A, and DNMT3B in different grades of astrocytomas, and their prognostic significance. As DNA methylation is a reversible biochemical phenomenon, DNMT may be an important target for the treatment of these challenging malignancies.

Although the primary role of DNMTs is to methylate DNA, DNMTs are also reported to be intricately inter-related with histone modification in terms of affecting chromatin structure and gene expression (Hashimshony et al., 2003). Polycomb group of proteins which comprise polycomb repressive complex (PRC) 1 and 2, are key factors that translate DNA methylation patterns into histone modifications. PRC2 group proteins are involved in the initiation of gene silencing, while PRC1 stabilize and maintain gene repression. DNMTs, such as DNMT3B, interface directly with the histone code by interacting with the histone methylases SUV39H1 and EZH2, which impart transcriptionally repressive H3K9 and H3K27 trimethylation marks, respectively (Fuks et al., 2003; Lehnertz et al., 2003; Viré et al., 2006). EZH2 is the main catalytic subunit of PRC2 complex (Chen et al., 2012). Viré et al. (2006) in an in vitro study on U2OS and 293 GP cell lines showed that EZH2 directly interacts with DNMTs and associates with its activity in vivo and that the presence of EZH2 is required for the binding of DNMTs to several EZH2-repressed genes. Taken together, it is evident that the complex interaction between DNMTs, PRCs and histone code is implicated in normal gene expression as well as in carcinogenesis and tumor progression. However, little is known about the connection among these epigenetic regulatory systems in gliomas. Hence, this study was undertaken to assess the correlation of expression of DNMTs with EZH2 in different grades of astrocytomas and to investigate the link between them as well as their correlation with clinical outcome, especially in GBMs.

2. Materials and methods

2.1. Patients and tissue samples

A retrospective study was conducted. A total of 95 cases of astrocytic tumors of different grades diagnosed over a period of 6 years (2009–2014) in the Neuropathology Laboratory of the Department of Pathology, All India Institute of Medical Sciences, New Delhi, India wherein adequate tumor tissue was available in formalin fixed paraffin embedded (FFPE) blocks along with snap frozen tumor tissue stored at −80 °C were taken up for the study. The hematoxylin and eosin (H&E) stained slides of these cases were reviewed and a concordant agreement was established for the confirmation of the diagnosis between three trained pathologists, based on the WHO classification (2007).

Patient records were reviewed to obtain demographic data, including age, sex and tumor location. Follow-up data was available only for GBMs. All the patients included for survival analysis (n = 54) underwent total to near total surgical resection and received radiotherapy along with concurrent chemotherapy and had a KPS score more than 70. Post-operative radiation therapy was started within four to six weeks of surgery. A dose of 50 Gy in 25 fractions was prescribed for CTV1 (enhancing tumor + edema + 2.5 cm margin all around as seen in the pre-operative T2 weighted MRI scan) followed by a boost of 10 Gy in 5 fractions over one week. Concurrent Temozolomide was given at a dose of 75 mg/m^2 daily in empty stomach following antiemetic and ranitidine 1 h before radiation. The maintenance Temozolomide was started after a gap of one month in patients without any recurrence. The first cycle was given at 150 mg/m^2 and depending on the tolerance increased to 200 mg/m^2 in the next cycle for a minimum of six cycles every 4 weeks. The incidence date was defined as the date of surgery. The recurrences were defined as the cases, which showed evidence of progression by magnetic resonance imaging that required a second resection or adjuvant treatment. Patient outcome was characterized in terms of progression-free survival (PFS) and overall survival (OS).

2.2. Immunohistochemical analysis

Immunohistochemical staining was performed for DNMT1, DNMT3A, DNMT3B, and EZH2. Immunohistochemical analysis was performed on serial 5 μm sections mounted on poly-L-lysine coated slides. The sections were deparaffinized in xylene and rehydrated through a decreasing concentration of alcohol. Antigen retrieval was performed by transferring the sections into 0.01 mol/l citrate buffer (pH 6.0) inside a 600-watt microwave oven on full power for 30 min. Peroxidase activity was blocked with 3% H_2O_2 in methanol for 30 min at room temperature. The sections were then incubated with adequate amount and dilution of primary antibodies at 4 °C in a humidity chamber for 2 h (Table 1). Sections were washed in Tris, treated with the biotin-labeled secondary antibody for 60 min at RT, and then washed in Tris buffer. Peroxidase conjugated streptavidin was applied to cover the sections and incubated at room temperature for 30 min. Then the slides were rinsed with three changes of Tris HCl buffer for 5 min each. Sections were then stained with diaminobenzidine for 10 min, washed with distilled water, counterstained in hematoxylin for 1 min, and then mounted.

2.3. Quantification of immunohistochemistry

Two experienced pathologists blinded to the clinico-pathologic information performed the scoring. Immunostaining was first grouped into negative and positive. In the immunopositive cases, the LI was calculated as a percentage of positively stained nuclei. For this, one thousand tumor cells were counted under ×400 magnification in the areas with highest density of positive nuclei (at least 10 representative microscopic fields). Care was taken to exclude the vascular endothelial cells and inflammatory cells in the counts. Immunopositive cases were further divided into those with weak expression (LI < 25%) and strong expression (LI ≥ 25%) (Smits et al., 2010; Purkait et al., 2015).

2.4. Real time quantitative PCR for mRNA expression of DNMTs and EZH2

mRNA expression status was assessed for DNMT1, DNMT3B and EZH2 by quantitative real time PCR.

Snap frozen tumor tissue collected at the time of surgery and stored at −80 °C was used for RNA extraction and real time PCR studies. Total RNA was isolated using miRVANA total RNA isolation kit (M/S Ambion®, Life Technologies, Cat. No. AM1560) as per manufacturer's protocol. DNA contamination was removed by DNase I treatment (M/S Ambion®, Life Technologies, Cat. No. AM1560). A total of 2 μg RNA was used to perform reverse transcription reaction using a high capacity SuperScript VILO cDNA synthesis kit (SuperScript® VILO™ cDNA

Table 1
Details of primary antibodies and primers used for the study.

Primary antibodies				
Antibody	Company	Antigen retrieval	Dilution	Localization
EZH2	Cell Signalling	Citrate	1:100	Nucleus
DNMT1	Sigma Aldrich	Citrate	1:100	Nucleus
DNMT3A	Sigma Aldrich	Citrate	1:100	Nucleus
DNMT3B	Sigma Aldrich	Citrate	1:300	Nucleus
Primers details				
EZH2	F: 5′-GCCAGACTGGGAAGAAATCTG-3′ R: 5′-TGTGTTGGAAAATCCAAG TCA-3′			
DNMT1	F: 5′ AAGACAAAGACCAGGATGAGAAG 3′ R: 5′ GGGTGTTGGTTCTTTGGTTTG3′			
DNMT3B	F: 5′ CCATTCGAGTCCTGTCATTG 3′ R: 5′ GCAATGGACTCCTCACACAC 3′			
HPRT1	F: 5′-TGAGGATTTGGAAAGGGTGT-3′ R: 5′-GAGCACACAGAGGGCTACAA-3′			
TBP	F: 5′-GAGCTGTGATGTGAAGTTTCC-3′ R: 5′-TCTGGGTTTGATCATTCTGTAG-3′			