RESEARCH ARTICLE

Methylation of O^6-Methyl Guanine Methyltransferase Gene Promoter in Meningiomas - Comparison between Tumor Grades I, II, and III

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Abstract

Background: Meningiomas are the second most common primary intracranial tumors after gliomas. Epigenetic biomarkers such as DNA methylation, which is found in many tumors and is thus important in tumorigenesis can help diagnose meningiomas and predict response to adjuvant chemotherapy. We investigated aberrant O6-methyl guanine methyltransferase (MGMT) methylation in meningiomas. Materials and Methods: Sixty-one patients were classified according to the WHO grading, and MGMT promoter methylation status was examined via the methylation-Specific PCR(MSP) method. Results: MGMT promoter methylation was found in 22.2% of grade I, 35% of grade I with atypical features, 36% of grade II, and 42.9% of grade III tumors. Conclusions: There was an increase, albeit not statistically significant, in MGMT methylation with a rise in the tumor grade. Higher methylation levels were also observed in the male gender.

Keywords: Meningioma - methylation - MGMT - cancer - MS- PCR

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Introduction

Meningiomas are neoplasms arising from meningothelial cells of the meninges and account for about 16-30% of intracranial neoplasms. The WHO 2007 grading system categorizes meningiomas into gradeI (90%), grade II (7%), and grade III (3%).

Afshin Moradi et al. (2008) reviewed intracranial lesions that underwent biopsy in Shohada Hospital in a 10-year period between 1997 and 2006 and reported that meningiomas accounted for 378 out of 4885 (7.74%) studied specimens. Of these, 329 cases were grade I, 41 cases grade II, and 8 cases grade III, according to the WHO 2000 classification.

The WHO grade I tumors are generally well-defined, slow in progress, and curable by surgery, whereas grade II lesions have ill-defined borders with a slow growth rate and greater probability of recurrence and grade III lesions have malignant histological features and require aggressive adjuvant therapy.

We defined a group of tumors that show some degrees of anaplasia, which is insufficient for the diagnosis of meningioma grade II. In the WHO grading system, three out of the five criteria of increased cellularity, small cells with a high nuclear-to-cytoplasmic ratio, prominent nucleoli, patternless growth, and necrosis must be present for the definition of meningioma grade II (Louis et al., 2007). We hypothesized that if a tumor fulfills fewer than three criteria and does not meet the criteria of grade two, it may be in a less differentiated grade than grade I tumors and named it"meningioma with atypical features (I /A)". These tumors may be a source of the reported unpredictable behavior of meningiomas.

There are numerous reports that show low-grade tumors (WHO grade I) behave like malignant ones and even occasionally metastasize to distant organs. Asiolis et al. (2007) reported a case of benign meningioma metastasizing to the lung twelve years after the resection of a primary intracranial tumor. Be Figueroa et al. (1999) described a metastatic transitional meningioma (WHO grade I) after tumor recurrence: the primary tumor, recurrence, and metastatic lesions had the same

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morphology. In 2002, Ramakishnamurthy et al. (2002) reported an intraventricular meningioma with benign histology that spread through the cerebrospinal fluid pathways and the recurrence of the tumor also had the same benign morphology. Nakano et al. (2012) described a case of 34-year-old man with a bilateral parasagittal meningioma that developed pulmonary metastasis with the tumor histology of transitional (WHOgradeI) meningioma.

Several genetic changes such as inactivation mutations in neurofibromatosis 2 gene (merlin) on chromosome 22q have been well-known in meningioma for many years (Perry et al., 2004), but there is currently a dearth of data on epigenetic changes. Although the importance of epigenetic alterations has opened a new era for a better recognition of tumorigenesis and there have been studies on such intracranial neoplasms as glioblastomas, there are only scant researches on small groups of meningiomas. Furthermore, most of these investigations have been performed on low-grade tumours.

Epigenetic alterations include reversible heritable changes in the gene function without alteration in primary DNA sequences (Russo et al., 1996). There are four major epigenetic mechanisms affecting gene transcriptions in the human genome: chromatin modification (Li 2002);histone code (Jenuwein, Allis 2001); micro RNAs (Sato et al., 2011) and DNA methylation (Bird 2002). DNA methylation is defined as the addition of the methylgroup to cytosine before guanine, which is carried out by DNA methyltransferase enzyme.

The majority of CpG islands (CpG I) in an active gene are normally unmethylated.

The methylation of selected CpG sites within a CpG I in the promoter of a gene is associated with decreased gene expression (Feinberg, Vogelstein 1983). CpG I is at least 200 bps stretch of DNA that contains a high frequency of CpG dinucleotide C+G content (above 50%) and an observed/expected CpG ratio of greater than 60% (Bird 1986). O6-methylguanine-DNA methyltransferase (MGMT) protein removes the alkyl group from the O6 of guanine residue by transferring it to specific cytosine residue within the protein (Ludlum, 1990; Pegg et al., 1995).

Silencing of the DNA can damage repair genes by hypermethylation and the promoters of CpG I can contribute to tumorigenesis (Baylin, Herman 2000). Previous reports have shown that MGMT hypermethylation occurs in many tumor types, including gliomas, large B-cell lymphomas, retinoblastomas, and cancers of the breast, lung, prostate, stomach, and colon (Herfarth et al., 1999; Wu et al., 2008; Hibi et al., 2009; Sharma et al., 2009). Many studies have observed a high frequency of MGMT methylation in tumors, but there are only a few studies on the status of MGMT methylation in meningiomas.

The present study aims to evaluate the hyper methylation of MGMT promoter as an accessory tool, in addition to the tumor grade and proliferative indices, to predict the tumor behavior and response to alkylating chemotherapeutic agents with a view to a better patient management; comparison of the hypermethylation rates between the different grades and evaluation of its role in the tumor genesis and comparison of the degree of hypermethylation in meningiomas with atypical features with three well-established histological grades.

Materials and Methods

Tissue samples

Paraffin-embedded blocks of meningiomas were collected from Department of Pathology , Shohada Hospital affiliated to Shahid Beheshti University of Medical Sciences (SBMU) between 1996 and 2010. All of the samples have been fixed in 10% buffered formalin,and embedded according to routine standards of pathology. All the samples have been prepared under the same protocol for fixation and embedding.Meningioma slides from each case were re-reviewed by three pathologists to confirm the histological diagnosis and revision of the grading in accordance with the WHO 2007 classification of the central nervous system tumors. The representative sample was selected by a pathologist. The meningioma samples comprised 9 benign (WHO grade I), 20 grade I/A (WHO grade I), 25 atypical (WHO grade II), and7 anaplastic (WHO grade III) tumors.

DNA extraction

DNA methylation in the CpG I of MGMT gene was examined using the Methylation-Specific PCR (MSP) method. Each paraffin block was cut at 10μM (3-5 sections) and collected in an autoclaved plastic tube. To avoid the cross-contamination of the samples, the Microtome Blade was carefully cleaned with xylene and ethanol. Genomic DNA was isolated from the tumor sections using QIAamp DNA FFPE Tissue Kit (QIAGEN, Germany) following the manufacturer's instructions.

Bisulfite treatment

The DNA extracted from the tumor samples was subjected to bisulfite treatment and DNA purification using the EpiTect Bisulfite (QIAGEN, Germany) in accordance with the manufacturer's instructions. Two hundred ng bisulfite-modified DNAs from the same treatment were used as the template for PCR. The modified DNA was amplified using primers specific for either methylated or unmethylated MGMT promoter sequences. The primers were used in earlier reports (Bello et al., 2004) and are listed in Table 1.

Methylation-specific PCR

Amplifications were performed in a 25-µl reaction volume and contained 1.5 mM of $MgCl_2$, 1.5 units of HotStarTaq Plus DNA Polymerase (QIAGEN, Germany),

Table 1. PCR Primer Sequences (Bello et al., 2004)

Primer set	Primer sequence	Product size
Unmethyl		93bps
MGMT Sense	5′-TTTGTGTTTTGATGTTTGTAGGTTTTTGT-3′	
MGMT Antisense	5′-AACTCCACACTCTTCCAAAAACAAAACA-3′	
Methyl		81bps
MGMT Sense	5′-TTTCGACGTTCGTAGGTTTTCGC-3′	
MGMT Antisense	5′-GCACTCTTCCGAAAACGAAACG-3′	

200 μM of dNTPs, 10 pmol of each primer, and 200 ng of bisulfite-treated DNA.

PCR conditions were as follows: one step at 95°C for 5 minutes; 40 cycles at 94°C for 45 seconds; 59°C for 45 seconds; 72°C for 45 seconds; and final extension at 72°C for 10 minutes.

Unmethylated and methylated DNA (QIAGEN, Germany) served as negative and positive controls, respectively. A negative control without DNA was also included and each PCR was repeated twice. The hypermethylation status of MGMT promoter CpG I was determined through an analysis of the PCR products in 12% polyacrylamide gel after silver nitrate staining.

This study was approved by Cancer Research Center Ethics Committees of Shahid Beheshti University of Medical Sciences (8730-111-1-90).

Table 2. Patient Characteristics

Characteristics	Patients (n=61)
Age, years	
All patients (n=61)	
Mean ±SD	48.41±15.20
Median (range)	48.00 (2-80)
Female (n=31)	
Mean ±SD	44.84±13.19
Median (range)	46.00 (17-75)
Male (n=30)	
Mean ±SD	52.10±16.45
Median (range)	49.50 (2-80)
Gender, no. (%)	
Female	31 (50.80%)
Location of tumor, no. (%)	
Lateral convexity &sagittal sinus	51 (83.61%)
Sphenoid ridge & base of skull	7 (11.47%)
Spinal	3 (4.92%)
MGMT status, no. (%)	
Methylated	21 (34.40%)
Unmethylated	40 (65.60%)
Grade of meningioma, no. (%)	
Grade I	9 (14.75%)
Methylated	2
Unmethylated	7
Grade I/A	20 (32.79%)
Methylated	7
Unmethylated	13
Grade II	25 (40.98%)
Methylated	9
Unmethylated	16
Grade III	7 (11.48%)
Methylated	3
Unmethylated	4

Statistical analysis

In this study, two logistic regression models were conducted to assess the effect of the tumor grade on methylation status. In model I, the tumor grade was considered a categorical variable with four categories (I, I/A, II, and III), by using indicator variables for the various categories (grade 1 was regarded as the reference group). In model II, the grade of the tumor was treated as an ordered variable by coding the categories as follows: grade=1 if grade I; grade =1.5 if grade I/A; grade=2 if grade II; and grade =3 if grade III. Thereafter, the two models were compared to test whether the ordered scale provided an adequate fit by comparison with the use of the indicator variables. Furthermore, unadjusted binary logistic regression models were conducted to assess the association between the patients' characteristics and morphological features and MGMT gene methylation status.

Results

Study population

Sixty-one patients were recruited into this study. The patients' characteristics are summarized in Table 2. The result of the MSP analysis for MGMT gene are shown in Table 4. A total of 21 of the 61 samples (34.40%) showed hypermethylation.The median age of the patient was 48.00 years with a range of 2-80 years.MGMT promoter hypermethylation was detcted in all grades of meningiomas.there were 31 (50.80%) female and 30 (49.20%) male patients.

Results of methylation on meningiomas

The frequency of MGMT methylation in our study was 22.22% (2/9) in grade I, 35% (7/20) in grade I/A, 36% (9/25) in grade II, and 42.85% (3/7) in grade III, which indicated that MGMT was more frequently methylated in grade I/A than gradeI and showed that MGMT methylation frequency in grade I/A was more similar to grade II than grade I.

Association between methylation status and tumour grade

Table 3 depicts the results of the logistic regression analyses to assess the effect of the tumor grade on methylation status. Using the likelihood ratio test, the

Table 3. The Results of Logistic Regression Analyses

Model (1)	B	S.E.	Wald	df	Sig.	OR	95% CI for OR	
							Lower	Upper
Constant	-1.25	0.8	2.44	1	0.12	0.29		
Grade			0.82	3	0.84			
Grade I/A (*vs*. grade I)	0.63	0.93	0.47	1	0.49	1.88	0.3	11.64
Grade II (*vs*. grade I)	0.68	0.9	0.56	1	0.45	1.97	0.33	11.57
Grade III (*vs*. grade I)	0.96	1.11	0.76	1	0.38	2.62	0.3	22.1
Model (2)								
Constant	-1.34	0.93	2.08	1	0.15	0.26		
Grade	0.38	0.48	0.62	1	0.43	1.47	0.57	3.79

Model I: The grade of disease was considered a categorical variable with four categories (I, I/A, II, and III), by using indicator variables for the various categories (grade 1 was considered a reference group), Model II: The grade of disease was treated as an ordered variable by coding the categories, as follows: grade =1 if grade I; grade=1.5 if grade I/A; grade=2 if grade II; and grade=3 if grade III.

Table 4. Results of Unadjusted Binary Logistic Regression Analyses for Associations Between Subject Characteristics and Methylation Status[1]

Characteristics[2]	Odds ratio (95%CI)	P value
Age, years (*vs*. <50)		
≥ 50 years	1.11 (0.38-3.20)	0.84
Male (*vs*. Female)	3.00 (0.99-9.07)	0.046*
Mitoses (*vs*. <4)		0.86
4-20	0.70 (0.19-2.60)	
>20	0.87 (0.07-10.43)	
N/C ratio (*vs*. Normal)		
High	1.50 (0.30-7.43)	0.62
Nucleolus (*vs*. Negative)		
Positive	0.93 (0.30-2.83)	0.9
Cellularity (*vs*. Low)		
High	0.32 (0.06-1.60)	0.16
Necrosis (*vs*. Negative)		
Positive	2.00 (0.61-6.59)	0.25
Tissue size	1.17 (0.98-1.40)	0.07

[1] Methylation status: Methylated and not methylated, [2] The unadjusted odds ratios were calculated for each variable; the reference group for each variable is given in parentheses. For example, the odds of methylation in the men is 3 times the odds for the females (p value =0.046).

model that related the tumor grade on an ordered scale to the incidence of methylation provided an adequate fit as compared to the use of the indicator variables (p value =0.88, Table 3). According to model II, the tumor grade was not significantly associated with methylation status, although higher risks of methylation were observed in the expected directions (p value =0.43). Based on model II, the odds ratios for grades I/A, II, and III (compared to grade I) were 1.21, 1.46, and 2.14, respectively (Table 3). In the unadjusted binary logistic regression models, only gender showed statistically significant differences in promoter methylation frequency (M=3, 95%CI=0.99-9.07; p value=0.046) (Table 4).There was no independent statistical correlation between methylation status and patients' age, tumor location, mitotic count, nucleo-cytoplasmic ratio, prominent nucleolus, hypercellularity, necrosis, and tumor size. The logistic regression analysis showed that there were no differences in promoter MGMT methylation frequencies between the different meningioma grades.

Discussion

Many studies have shown that DNA methylation profiles vary between normal tissues and derived tumors and between different tumor types. Aberrant DNA hypermethylation at the promoter region DNA repair gene contributes to the progression of some cancers (Herfarth et al., 1999; Wu et al., 2008; Hibi et al., 2009; Sharma et al., 2009). We used MSP (Methylation-Specific PCR) to evaluate the status of the promoter methylation of MGMT gene in the different grades of meningioma tumors. There are several case reports of benign meningiomas (WHO grade I) disseminating through the cerebrospinal fluid or even metastasizing to distant organs, especially the lung. As mentioned previously, we believe that even minor degrees of anaplasia may have some effects on the tumor behavior and we defined such cases as meningioma grade I with atypical features (grade I/A). The status of MGMT methylation in meningioma grade I/A in comparison with the WHO grades I, II, and III of meningiomas was the major subject of this study. The methylation status of MGMT promoter might be a useful predictor marker alongside morphological features for a better characterization of the meningioma grades and, thus, predict responsiveness to alkylating agents such as carmustine, lumastin, and temozolomide.

The frequency of MGMT methylation in our study was 22.22% (2/9) in grade I, 35% (7/20) in grade I/A, 36% (9/25) in grade II, and 42.85% (3/7) in grade III, which indicated that MGMT was more frequently methylated in grade I/A than gradeI and showed that MGMT methylation frequency in grade I/A was more similar to grade II than grade I. No significant differences were, however, observed in the methylation status between grade I and grade I/A and nor was there any significant association between MGMT methylation frequency and different tumor grades.

Our logistic regression models revealed increased odds for the frequency of MGMT methylation from low- to high-grade meningiomas. The increase in the methylation rate in the higher-grade tumors may be one of several genetic and epigenetic changes responsible for neoplasm upgrading. There is a paucity of data in the existing literature on the status of MGMT methylation of meningiomas. Bello et al. (Bello et al., 2004) detected aberrant MGMT methylation in 13%,26%, and 0% of grade I,II and III tumors. In another study Yanli liu et al. (2005) reported the status of aberrant MGMT methylation in 6% (1/16) of grade I,5% (1/19) of grade II, and 8%(1/13) of grade III tumors. In the Robles et al. (2008), none of the meningioma samples showed MGMT promoter methylation. In the present study, the frequency of MGMT methylation was higher than that previously reported. It is worthy of note that the status of MGMT methylation can vary for a particular tumor. For example in glioblastoma patients, previous studies have reported rates of 24%,33%,34%,35%, 40%,45%,47.5%,53%,68%, and 70% for MGMT promoter methylation (Esteller et al., 2000; Hegi et al., 2004; Hegi et al., 2005; Brandes et al., 2008; Dunn et al., 2009; Costa et al., 2010; Rivera et al., 2010; Sciuscio et al., 2011; Havik et al., 2012; Tang et al., 2012). It is possible that variability in the rates of promoter-region gene hypermethylation in meningioma tumors may be influenced by race, high fat food diet, polymorphism, smoking, dietary variables, and other environmental exposures. Enokida et al. (2005) observed that methylation of GSTP1 was significantly high in Caucasians and Asians in prostate cancer. In prostate cancer, Kwabi-Addo et al. (2011) observed significant differences in methylation levels in five genes in African Americans in comparison with Caucasian samples. Wallace et al. (2010) reported that African Americans had lower levels of ERα and SFRP1methylation than did Caucasians and Hispanics, and higher RBC folate levels were associated with higher levels of the methylation of the genes. Accordingly, the utilization of MGMT as ethnic-sensitive biomarkers may be considered for meningiomas.

Brait et al.(2009)demonstrated a statically significant association between RARβ2 promoter methylation and

a high fatty food intake. The Leng et al. (2011) showed that haplotype containing A allele of MGMT promoter-enhancer SNP could serve as apredictorfor the methylation rate along with the process of lung carcinogenesis. Liu et al. (2010) demonstrated that male smokers had more MGMT methylation than did non-smokers. Elsewhere, a positive correlation was shown between environmental tobacco smoke and MGMTmethylation by Brait et al. (Brait et al., 2009; Leng et al., 2011).

We found that our male patients had significantly higher levels of MGMT methylation than did the females. The impact of gender on DNA methylation has been previously studied. For instance, THBS1,TIMP3,E-cadherin,DAP-kinase, RASST1A, MTHFR, and some other genes have been reported to be more frequently methylated in male patients than in females (Kang et al., 2003; Sarter et al., 2005). Wu et al. (2010) observed that tumors with p53 mutation in males contained higher levels of MGMT methylation than those in females. Lai et al. (2009) reported lower rates of ER and MGMT methylation and lower risks for lung cancer in females and contributed it to β-estradiol hormone replacement therapy.

First and foremost among the limitations of the current study was its relatively small sample size. A larger sample size should provide accurate statistical analyses of the possible relationship between methylation status and grades of meningiomas.We suggest experiments on larger samples in each tumor grade. Larger studies are needed to validate the gender differences in MGMT methylation frequency between the different tumor grades. Insufficient information on the patients' lifestyle such as smoking habits and fatty food intake and information on recurrence, treatment, and survival, which rendered the definition of a cancer grade-specific biomarker based on our observation difficult, was another major drawback. The strength of this study, however, lies in the fact that it is the first study of its kind to investigate MGMT methylation WHO grade I with atypical features in comparison with the WHO grades I,II, and III.

In summary, we analyzed and compared MGMT methylation between the WHO grade I/A and grades I,II, and III. We detected a linear increase, albeit not statistically significant, in MGMT methylation grade I, grade I/A, gradeII, and grade III. We also demonstrated a tendency for increase in the MGMT methylation rate in the process of anaplastic transformation and found a statistically significant association between MGMT methylation and the male gender in meningiomas.

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