Original Article

Promoter hypermethylation of methyl guanine methyl transferase in lung cancer patients of Kashmir valley

Abstract

Context: *MGMT*, is a DNA repair protein involved in removing the mutagenic and cytotoxic adducts from O^6-guanine in DNA, which otherwise can lead to the mutation, primarily due to the tendency of O^6-methylguanine to pair with thymine during replication, resulting in the conversion of GC to AT pairs, if left unrepaired due to epigenetic silencing of its promoter. **Aims:** To study the status of *MGMT* in Kashmiri lung cancer patients **Materials and Methods:** In this study we analyzed the promoter status of this gene in 50 lung cancer patients using methylation specific-PCR and confirmed by restriction digestion. **Results:** The promoter was found to be methylated in 52% of the cases, more in male patients (54.2%) than in female patients (46.6%). When stratified according to smoking status, current smokers showed a strong association with methylation (OR = 6.0, *P* = 0.005) than former and never smokers. We also compared the promoter methylation of the *MGMT* gene with pesticide exposure, and found that patients with pesticide exposure had statistically significant (OR = 7.7 and *P* = 0.003) association with promoter methylation. **Conclusions:** Our results indicate that *MGMT* promoter methylation is associated with smoking exposure and there appears to be an association of *MGMT* promoter methylation with pesticide exposure in Kashmiri lung cancer patients.

Key words: Kashmiri population, lung cancer, *MGMT*, NSCLC, pesticide exposure

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INTRODUCTION

MGMT, also known as AGT, is a DNA repair protein that removes mutagenic and cytotoxic adducts from O^6-guanine in DNA.[1-2] *MGMT* is a large gene spanning approximately 300 kb, including five exons at chromosomal region 10q26 (accession number: NT_0088.15,2499397-2799297); mRNA is 866 bases long, encoding 207 amino acids (accession number: NM_002412.2 and NP_002403.1). Alkylation of DNA at the O^6 position of guanine is an important step in the appearance of mutations in cancer, primarily due to the tendency of the O^6-methylguanine to pair with thymine during replication, resulting in the conversion of GC to AT pairs in DNA.[3] Furthermore, the O^6-alkylguanine–DNA adduct (especially the O^6-chloroethylguanine) may cross-link with the complementary cytosine residues, blocking DNA replication.[4] *MGMT* protects cells against these lesions, transferring the alkyl group from the O^6-guanine in DNA to an active cysteine within its own sequence in a reaction that inactivates one *MGMT* molecule for each lesion repaired.[1]

The epigenetic silencing of *MGMT* by promoter hypermethylation in cancer cell lines and primary human tumors has been reported by several groups,[5-7] and has been correlated with the loss of MGMT protein, lack of mRNA expression[5] and loss of enzymatic activity.[8] Furthermore, the CpG island hypermethylation-associated silencing of *MGMT* occurs very early in human tumorigenesis, such as in small colon adenomas,[5] strongly supporting its relevant role in carcinogenesis. The transcriptional silencing of *MGMT* by promoter hypermethylation causes an important mutator pathway in human cancer because the O^6-methylguanine adducts, resulting from alkylating agents, are not removed and this consequently causes G: C to A: T transitions. The first gene described to have G: C to A: T transitions as a consequence of *MGMT* inactivation in human tumors was *K-ras*.[9] The mutation distribution in *K-ras* strongly resembles the pattern of *MGMT* promoter hypermethylation. The association between *MGMT* promoter hypermethylation and *K-ras* mutations has been reported not

only in colon cancer[10] but also in gastric and gallbladder cancers.[11,12] Another gene that was reported to have G: C to A: T transitions caused by the epigenetic silencing of *MGMT* in human cancer was the tumor suppressor gene *TP53*.[13]

Because DNA methylation changes occur at the early stages of cancer development and are detected in virtually any kind of tumor tissue, hypermethylation markers may be used for the detection of both primary and metastatic or recurrent cancer cases.[14] Hypermethylation of *MGMT, p16, RASSF1A, DAPK* and *RAR*β, detected in the serum of patients with lung cancer, appeared to be a moderately good marker of cancer with a sensitivity of 50.9% in comparison with 11.3% exhibited by serum protein tumor markers.[15] In another study, hypermethylation of *p16* and *MGMT* in patients with squamous cell lung carcinoma was detected in sputum samples 3 years prior to disease diagnosis.[16] Keeping these in view, we studied the promoter hypermethylation status of *MGMT* gene in lung cancer patients of our population.

MATERIALS AND METHODS

Fifty histologically confirmed, previously untreated lung cancer patients attending the Departments of Cardio-Vascular and Thoracic Surgery, Medical Oncology and Internal Medicine of Sher-I-Kashmir Institute of Medical Sciences (SKIMS), Srinagar, were included in this study. A written pre-informed consent was obtained from all the patients.

DNA extraction from fresh–frozen tissues was performed by the Proteinase K, phenol/chloroform method[17] and the bisulphite modification of DNA, including positive and negative controls, was carried out by EZ DNA Methylation Gold Kit™.

We applied the two-stage methylation-specific polymerase chain reaction (PCR) method for promoter methylation analysis. The first stage PCR amplification was carried out in a 25-μL reaction mixture containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 100 mM of each kind of dNTP and 0.2 mM of forward primer GGATATGTTGGGATAGTT and reverse primer CCAAAAACCCCAAACCC. The reaction was heated at 95°C for 10 min, then amplified for 40 cycles [95°C/30 s, 52°C (annealing temperature)/30 s, and 72°C/30 s], followed by a final 10-min extension at 72°C. An aliquot of each round 1 PCR product was diluted (by Tris EDTA buffer) 10-fold and 1 μL was used for round 2 PCR, using the same reagents and conditions as for round 1 PCR, except that the $MgCl_2$ concentration was reduced to 1 mM and each sample was amplified in two reactions, with one reaction containing primers specific for methylated cytosine (forward, TTTCGACGTTCGTAGGTTTTCGC; reverse, GCACTCTTCCGAAAACGAAACG) and the other reaction containing primers specific for unmethylated cytosine (forward, TTTGTGTTTTGATGTTTGTAGGTTTTTGT; reverse, AACTCCACACTCTTCCAAAAACAAAACA). Each reaction was heated at 95°C for 10 min, then amplified for 40 cycles, each consisting (for the reaction containing methylated primers) of 95°C/30 s, 64°C/30 s and 72°C/30 s and (for the reaction containing unmethylated primers) of 95°C/30 s, 64°C/30 s and 72°C/30 s. An aliquot of each round 2 PCR products was separated on an 8% polyacrylamide gel. The gel was stained with ethidium bromide and photographed under UV illumination. The reproducibility of the results was confirmed by repeating methylation specific polymerase chain reaction analysis for each DNA sample.

Round 2 PCR products were also analyzed by restriction fragment length polymorphism to confirm their "methylated" status. For this purpose, a 2-μL aliquot from each round 2 PCR product was treated in a final 10-μL reaction — with the restriction enzyme *TaqI* and *BstU1* for the *MGMT* gene — using the reagents and conditions provided by the manufacturer (New England Biolabs, Beverly, MA, USA).[1] The digestion products were separated on an 8% polyacrylamide gel. The gel was stained with ethidium bromide and photographed under UV visualization.

RESULTS

For this study, fifty lung cancer tissues and their corresponding normal tissues were analyzed. The clinicopathological characteristics of the studied subjects are given in Table 1. In case the promoter region was highly methylated (both alleles), only the methylated band was detected (22%). When the promoter was partially methylated, both methylated and unmethylated bands were detected (30%).

The promoter region of *MGMT* gene was found to be methylated in 52% (n = 26) of the lung cancer patients studied [Table 2]. The promoter methylation was found to be higher in male

Table 1: Clinicopathological and other characteristic of lung cancer patients (50)

Characteristic	*N* (%)
Gender	
Male	35 (70)
Female	15 (30)
Age	
<50 years	21 (42)
≥50 years	29 (58)
Smoking status	
Nonsmokers	13 (26)
Former smokers	16 (32)
Current smokers	21 (42)
Histological cell type	
SCC	33 (66)
AD	8 (16)
Large cell carcinoma	5 (10)
Bronchioalveolar carcinoma	4 (8)
Clinical stages	
Stage I	10 (20)
Stage II	14 (28)
Stages III and IV	26 (52)
Pesticide exposure	
Yes	32 (64)
No	18 (36)

SCC=Squamous cell carcinoma, AD=Adenocarcinoma

Table 2: Association of MGMT promoter methylation with different variables of lung cancer patients (*N*=50)

Variable	MGMT methylation		OR (CI 95%)	*P* value
	Positive	Negative		
Gender				
Male	19	16	Ref.	0.760
Female	7	8	1.3 (0.415-4.440)	
Age				
<50	10	11	Ref.	0.775
≥50	16	13	1.3 (0.446-4.112)	
Smoking status				
Non	4	9	Ref.	0.005
Former	6	10	2.3 (0.720-7.837)	
Current	16	5	6.0 (1.761-20.832)	
Histopathological type				
SCC	17	16	Ref.	0.250
AD	6	2	3.3 (0.666-15.848)	
Others	3	6	2.5 (0.603-10.643)	
Clinical stage				
Stage I and II	7	17	Ref.	0.004
Stage III and IV	19	7	6.5 (1.953-22.246)	
Pesticide exposure				
Yes	22	10	Ref.	0.003
No	4	14	7.7 (2.084-28.069)	

SCC=Squamous cell carcinoma, AD=Adenocarcinoma, Others=Large cell carcinoma, brochioalveolar carcinoma, MGMT= Methy guanine methyl , OR=Odds ratio

patients (n = 19; 54.2%) than in female patients (n = 7; 46.6%). When methylation was compared with age of patients, more patients with greater than or equal to 50 years of age had methylated promoter region than those with age less than 50 years (n = 16; 55.1% vs. N =10; 47.6%), but this was statistically insignificant. When patients were grouped according to smoking status, current smokers showed strong association with methylation (OR = 6.0, P = 0.005) than former and non-smokers. Among different histological types, methylation was found to be higher in adenocarcinoma patients (n = 6; 75%) than in squamous cell carcinoma (SCC) (n = 17; 51.1%) and other histopathological types, although this was statistically insignificant. In clinical stages of lung cancer, promoter methylation was strongly associated with advanced stages (stage III and IV) (OR = 6.5 and P = 0.004) than in early clinical stages (stage I and II). Besides, when we compared the promoter methylation of *MGMT* gene with pesticide exposure, it was found that patients with pesticide exposure had a statistically significant (OR = 7.7 and P = 0.003) association with promoter methylation.

DISCUSSION

MGMT is a DNA repair gene coding for a protein that removes mutagenic and cytotoxic adducts from the O^6-guanine in DNA.[1] Alkylation of DNA at the O^6 position of guanine is an important step in the formation of mutations in cancer, primarily because of the tendency of O^6-methylguanine to pair with thymine during replication, resulting in a conversion of guanine–cytosine to adenine–thymine pairs in DNA.[3,4] Loss of *MGMT* expression is not commonly because of deletion or rearrangement of the gene[18-20] but rather methylation of CpG islands in the *MGMT* promoter region. Methylation of the CpG islands of *MGMT* promoter has been associated with silencing of the *MGMT* gene in cell lines,[5,6] and with loss of protein expression in primary human neoplasia.[7] It has also been reported that the methylation status of the *MGMT* promoter is associated with clinical outcome in human cancer, where they showed that inactivation of the *MGMT* gene by promoter methylation is a predictor of overall survival and response to alkylating agents in patients with gliomas, additionally supporting the promise of methylated markers as prognostic tools in human cancers.[9]

In the present study, we studied the *MGMT* promoter methylation in 50 primary lung tumors. The frequency of *MGMT* promoter methylation in this tumor set is relatively higher than some studies.[21,22] However, the prevalence of *MGMT* promoter methylation is same as reported by Pulling *et al.* (52% vs. 51% and[23] 52% vs 50%). Besides, our results show that the prevalence of *MGMT* promoter methylation is not statistically different between two distinct histological types of adenocarcinoma and SCC patients (75% vs. 51.1%). This is consistent with other reports,[22] which showed that the frequency of promoter methylation of *MGMT* does not differ between adenocarcinoma and SCC (31% vs 27%), but inconsistent with others,[23] which showed that the frequency of promoter methylation is significantly higher in SCC than in adenocarcinoma (60% vs. 40%, P = 0.003). The reason of higher frequency could be because, in our case, there were lesser adenocarcinoma patients than SCC patients and a population of different ethnic background.

When the patients were stratified according to smoking status, we observed a significantly higher prevalence of promoter methylation in current smokers than in former and nonsmokers. This is consistent with other reports.[22,24] However, our results are inconsistent with that of others, like[25] Pulling's, who has reported that *MGMT* promoter is significantly methylated among nonsmokers compared with the smokers. The reason for disagreement could be because some studies consider a patient as nonsmoker if he has smoked less than 100 cigarettes,[24-26] but, in our case, it refers to a patient who has never smoked during his lifetime. Besides, environmental/geographical and ethnic differences could be a factor of disagreement. Also, tobacco smoke contains many carcinogens, some of which have been shown to affect gene promoter methylation.[27,28] Although there has been no direct evidence linking tobacco smoke carcinogens with *MGMT* promoter methylation, Grafstrom[28] *et al.*, however, showed that human epithelial cells treated with acetaldehyde (carcinogen present in tobacco smoke) had a significantly decreased activity of *MGMT* gene. Further, tobacco smoke is a mixture of various carcinogens, some of which have been found to form DNA adducts at CpG site guanines,[30] especially tobacco-specific nitrosamine NNK,

which is a potent lung carcinogen forming methyl adducts at the O^6 position of guanine and pyridyloxobutyl adducts, both repaired by *MGMT*.[31] Also, cigarette smoking is known to increase *MGMT* expression in both normal and neoplastic lung tissue, suggesting that *MGMT* may protect the lung from carcinogen-induced guanine alkylation.[32,33]

Our results also indicate that promoter methylation is significantly higher in patients with an advanced stage of lung cancer. This is consistent with other studies.[34] We also observed that promoter methylation of *MGMT* is significantly associated with pesticide exposure. Although there has been no study showing an association of promoter methylation with pesticide exposure, it could be implicated that pesticides might be interacting with the *MGMT* gene, more like tobacco smoke carcinogens thus inducing promoter methylation.

In conclusion, our results showed a higher frequency of *MGMT* promoter methylation in lung cancer from smokers compared with non-smokers, indicating an association between tobacco smoking and increased frequency of promoter methylation. To the best of our knowledge, the present study is reporting, for the first time, an association of pesticide exposure with promoter methylation of *MGMT* gene in lung cancer patients. These results need to be confirmed in a larger group of sample size that may be useful for understanding the etiology and genetics of lung cancer in other ethnic populations as well as our population of Kashmir.

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