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

Context: MGMT, is a DNA repair protein involved in removing the mutagenic and cytotoxic adducts from O6-guanine in DNA, which otherwise can lead to the mutation, primarily due to the tendency of O6-methylguanine to pair with thymine during replication, resulting in the conversion of GC to AT pairs, if left unrepaird 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

INTRODUCTION

MGMT, also known as AGT, is a DNA repair protein that removes mutagenic and cytotoxic adducts from O6-guanine in DNA.[1-2] MGMT is a large gene spanning approximately 300 kb, including five exons at chromosomal region 10q26 (accession number: NT_008815,2499397-2799297); mRNA is 866 bases long, encoding 207 amino acids (accession number: NM_002412.2 and NP_002403.1). Alkylation of DNA at the O6 position of guanine is an important step in the appearance of mutations in cancer, primarily due to the tendency of the O6-methylguanine to pair with thymine during replication, resulting in the conversion of GC to AT pairs in DNA.[3] Furthermore, the O6-alkylguanine–DNA adduct (especially the O6-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 O6-guanine in DNA to an active cysteine within its own sequence in a reaction that inactivates one MGMT molecule for each lesion repaired.[5]

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[8] 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,[8] 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 O6-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 R-ARF, 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 KitTM.

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 MgCl2, 100 mM of each kind of dNTP and 0.2 mM of mixture containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 2 mM for the reaction (PCR) method for promoter methylation analysis. 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 MgCl2 concentration was reduced to 1 mM and each sample was amplified in two reactions, with one reaction containing primers specific for methylated cytosine (forward, TTTGACGTTGTAAGTTCG; reverse, GCACCTTTCGAAAACGAAACG) and the other reaction containing primers specific for unmethylated cytosine (forward, TTTGTTGGTTTGTGTTGTTG; reverse, AAACCTCAATTCTTCGAAAACGAAAACA). 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 BstUI for the MGMT gene — using the reagents and conditions provided by the manufacturer (New England Biolabs, Beverly, MA, USA).[18] 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>
<thead>
<tr>
<th>Characteristic</th>
<th>N (%)</th>
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<tbody>
<tr>
<td>Gender</td>
<td></td>
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<tr>
<td>Male</td>
<td>35 (70)</td>
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<tr>
<td>Female</td>
<td>15 (30)</td>
</tr>
<tr>
<td>Age</td>
<td></td>
</tr>
<tr>
<td>&lt;50 years</td>
<td>21 (42)</td>
</tr>
<tr>
<td>≥50 years</td>
<td>29 (58)</td>
</tr>
<tr>
<td>Smoking status</td>
<td></td>
</tr>
<tr>
<td>Nonsmokers</td>
<td>13 (26)</td>
</tr>
<tr>
<td>Former smokers</td>
<td>16 (32)</td>
</tr>
<tr>
<td>Current smokers</td>
<td>21 (42)</td>
</tr>
<tr>
<td>Histological cell type</td>
<td></td>
</tr>
<tr>
<td>SCC</td>
<td>33 (66)</td>
</tr>
<tr>
<td>AD</td>
<td>8 (16)</td>
</tr>
<tr>
<td>Large cell carcinoma</td>
<td>5 (10)</td>
</tr>
<tr>
<td>Bronchioalveolar carcinoma</td>
<td>4 (8)</td>
</tr>
<tr>
<td>Clinical stages</td>
<td></td>
</tr>
<tr>
<td>Stage I</td>
<td>10 (20)</td>
</tr>
<tr>
<td>Stage II</td>
<td>14 (28)</td>
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<tr>
<td>Stages III and IV</td>
<td>26 (52)</td>
</tr>
<tr>
<td>Pesticide exposure</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>32 (64)</td>
</tr>
<tr>
<td>No</td>
<td>18 (36)</td>
</tr>
</tbody>
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SCC=Squamous cell carcinoma, AD=Adenocarcinoma
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 O6-guanine in DNA\(^{[3,4]}\). Alkylation of DNA at the O6 position of guanine is an important step in the formation of mutations in cancer, primarily because of the tendency of O6-methylguanine to pair with thymine during replication, resulting in a conversion of guanine–cytosine to adenine–thymine pairs in DNA.\(^{[4,9]}\) 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\(^{[3,4]}\) and with loss of protein expression in primary human neoplasia.\(^{[3]}\) 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 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 (69% 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\(^{[23]}\) 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, Graffstrom\(^{[29]}\) 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\textsuperscript{6} position of guanine and pyridoxalpyruvate adducts, both repaired by MGMT.[10] 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.[12,33]

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.[16,20] 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.

REFERENCES


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