Splice site and Germline variations of the MGMT gene in Esophageal cancer from Kashmir Valley: India

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Abstract

Objectives: The aim of our investigation was to detect mutation or genetic polymorphisms in *MGMT* gene of esophageal cancer patients from Kashmir Valley (India)

Methodology: The genetic polymorphisms or mutations in the coding exons 2, 3, 4 and 5 of *MGMT* gene were searched for in DNA samples from the frozen tumor tissues of 30 esophageal cancer patients from Kashmir. The PCR products were sequenced with fluorescently labelled terminators and separated on automatic sequencer. We developed a new PCR based RFLP approach for genotyping c.459A>G (p.Gly153Gly) variation in 71 esophageal cancer patients and 60 healthy controls.

Results: Two somatic variations c.274 +4G>A and c.274 + 22G>A were identified in Exon3-intron 4 boundary. A novel germline variation c.459A>G (p.Gly153Gly) was found in the exon 5 of an esophageal cancer patient. This germline variation was not found in any of the studied esophageal cancer patients and healthy controls except the patient where it has been found by direct sequencing.

Conclusion: We identified novel sequence variants of the *MGMT* gene in esophageal cancer patients from Kashmir valley-India.

Key words: Esophageal cancer; Kashmiri population; *MGMT*; Splice site mutations; Germline variation

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Introduction

Esophageal cancer is one of the most common cancers occurring globally [1] and is a major cause of cancer related deaths in India. The high incidence areas in India includes North-East India [2] and Kashmir valley [3] where environment and dietary habits play an overwhelming role in the development of Esophageal cancer over the genetic factors. These include intake of sun-dried vegetables of Brassica family (Hakh), pickled vegetables (Anchar) and hot salted tea, which contains potentially high content of carcinogenic compounds like nitrosamines. [4-8]

Nitroso compounds are the alkylating agents which induce the formation of O^6 alkylguanine adducts. The ability of these adducts to pair with thymine instead of cytosine during DNA replication and is responsible for the increase in the frequency of transition mutations following exposure to alkylating agents, and determines their mutational spectrum. The toxic and recombinogenic effect of O6- alkylguanine lesions, in particular the methyl version, is determined by the action of the post-replication mismatch repair system on O^6-methylguanine (O^6-meG): T mispairs, although the precise mechanisms of these effects remain to be established.

The DNA repair protein O^6-alkylguanine-DNA alkyltransferase [ATase; also known as AGT, AGAT and MGMT (O^6-methylguanine-DNA methyltransferase; E.C.2.1.1.63)] reverses O^6-alkylation damage in DNA simply by removing the offending alkyl group by covalent transfer to the ATase protein, inactivating it and targeting it for ubiquitination and proteasome-mediated degradation. Therefore, constitutive levels of MGMT determine the initial repair capacity of a cell. Several reviews have discussed the role of MGMT in protecting the cells against the pleiotropic biological effects of O^6Methylguanine DNA adducts. These include defence against point mutations, cancer initiation and progression, sister chromatid exchanges, chromosomal aberrations and apoptosis. [9-14]

Genetic analysis of *MGMT* gene has shown that it is not frequently mutated in human cancers. There has been only two reports describing *MGMT* mutations and deletions and this was in a substantial proportion of tumours of the oesophagus and colon cancer. [15, 16] Moreover, several studies have reported the existence of five genetic variants of the coding regions of the *MGMT* gene, four of which lead to alterations in the amino acid sequence of the protein. The currently known variants affecting the primary structure of MGMT are: W65C; L84F; I143V/K178R and G160R. [17-29] The SNPs leading to the I143V and K178R changes are in almost perfect disequilibrium [19,20,26,28] and it can be assumed that both changes occur in the protein derived from this gene but in some cases only one of the alterations was tested for. There is also a silent mutation with a C to T variation at codon 53 encoding Leu.

The genetic status of *MGMT* gene has been analysed in various types of cancers and in different populations. In this study, we made an attempt to investigate the molecular status of *MGMT* gene in esophageal cancer patients from Kashmiri population which is highly exposed to dietary as well as tobacco specific nitrosamines.

Materials and Methods

Study population

A total of 30 surgically resected esophageal tumor tissues and their adjacent normal tissue were used for mutational analysis. To screen the novel polymorphism, identified after mutational analysis, the blood samples of 71 esophageal cancer patients and 60 healthy individuals without overt cancer were recruited in this study. The histological type of the tumors was diagnosed on the basis of resected specimens in the Department of Pathology of the same hospital. The healthy participants, who have no history or diagnosis of cancer or genetic disease, were recruited from individuals who visited the same hospital for physical examination during the same period. The cancer patients and control participants were all of unrelated Kashmiri nationality from Kashmir Valley and its surrounding regions.

All subjects were interviewed using a structured questionnaire to obtain information on patients' age, area of residence, lifetime history of tobacco, occupational history, family history of malignancy, dietary habits etc. The study was approved by the Ethics Committee

of Sher-I-Kashmir Institute of Medical Sciences and informed consent was obtained from all recruited participants.

DNA extraction

DNA was isolated from the peripheral blood or normal and cancerous esophageal tissues of subjects using the phenol/chloroform extraction after proteinase K digestion. [30]

PCR amplification and DNA sequencing

The *MGMT* coding regions (exons 2-5), were amplified by PCR. The primers used for exons 2-4 were designed using primer3software and included splice junctions also. The primer for exon5 has been described as previously. [31] The primer sequences and the size of amplicons are listed in the Table1. Each PCR was performed under standard conditions in a 25 µL reaction mixture containing 100 ng of template DNA, 2.5 µL of 10 × PCR buffer, 1.5 mM $MgCl_2$, 1 unit of *Taq*-DNA polymerase, 200 µM of deoxynucleotide triphosphate (dNTP) and 5 pmol of each primer. Each PCR was performed for 5 min at 95°C followed by 35 cycles at 95°C for 35 s, 54°C (for exon2 and exon 4) or 65°C (for exon3) or 62 °C (for exon5) for 35 s, and 72°C for 1 min (for exons 2, 3 and 4) or 30 s (for exon5), followed by a final extension step at 72°C for 7 min in a Biorad thermal cycler. Nucleotide sequencing of 30 tumor and their adjacent normal tissues was performed in both directions using the Big Dye method and an automatic sequencer ABI 377 (Applied Biosystems). Sequencing primers were the same as those used for PCR.

Table 1. Primer sequences and the size of the amplicon

Exon	Sense primer (5'-----3')	Antisense primer (5'-----3')	Amplicon size (bp)
Exon2	GCTGCTCTAGGTAATGG	TCGTGGTTGCGGTTGTG	463
Exon3	TCCTTGCCTCACCAGTCC	TGTTGGAGTGGGTGGAG	491
Exon4	GTGTAGATGCGTTTCCTG	CATCTGGCATAATGGTCC	390
Exon5	CTTGACCCCAAAGACCTCGT	TGTCGCTCAAACATCCATCC	306

Genotyping

We developed a new PCR-RFLP approach for genotyping *MGMT* c.459A>G (p.Gly153Gly) variation. Briefly, two primers, 5'-TTGACCCCAAAGACCTCGT-3' (sense) and 5'- AGTCCTCCGGAGTAGTTGC -3' (antisense), were used to produce a 134-bp fragment. The fragment was amplified in a 25 µl PCR reaction mixture containing approximately 50 ng genomic DNA, 200 µM dNTPs, 1X PCR buffer solution, 1.5mM $MgCl_2$, 5 pmol of each primer, and 1U of Taq DNA polymerase (Sigma, USA). The PCR conditions were as follows: an initial melting step of 95^0C for 6 min was followed by 35 cycles of melting at 95^0C for 30 s, annealing at 57^0C for 35 s and extension at 72^0C for 45 s. A final extension step at 72^0C for 7 min terminated the process. A 10-µl amplicon was digested at 37^0C overnight with 10U of *Bsu*RI *(Hae*III*)* in a total volume of 30 µl. The digested product was run on a 2.5% agarose gel at 85 V for 1 h. The genotypes were identified according to the banding pattern observed. The variant allele c.459A>G is recognized by single bands at 108 bp and 26 bp. The reference allele is identified by the absence of the *Bsu*RI *(Hae*III*)* cutting site indicative of the reference allele sequence.

Results

We sequenced exons: 2, 3, 4 and 5 of *MGMT* gene from 30 esophageal resected tumor and their adjacent normal tissues. No

mutations or polymorphisms were detected in exon 2 and 4. Six patients showed L53L and L84F polymorphism in exon3.The two polymorphisms were in linkage disequilibrium. Among the 6 patients, heterozygous variant was found in 4 patients (13.33%) and homozygous variant in 2 patients (6.66%). Moreover, 5 patients showed I143V and L178R polymorphisms in exon 5 which were in linkage disequilibrium. The two polymorphisms in all the 5 patients in exon 5 were heterozygous variants (16.66%).

We also identified two somatic mutations in the intron 4, one in the 5' splice donor site c.274 +4G>A. (Fig.1) and another at c.274 + 22G>A. (Fig.2). Splice site mutation was found in 2 out of 30 cases (6.66%) and c.274 + 22G>A mutation was found in only 1 out of 30 cases(3.33%).

Fig.1 Sequencing results of the *MGMT* gene showing c.274 +4G>A splice site mutation (a) Tumor tisue (b) Paired Normal tissue

Fig.2. Sequencing results of the *MGMT* gene showing c.274 + 22G>A mutation (a) Tumor tissue (b) Paired Normal tissue

A novel germline variation c.459A>G (p.Gly153Gly) was found in only one case (coded as T2) in exon5 (Fig.3).This germline variation was screened in 71 esophageal cancer patients and 60 healthy controls by a novel PCR-RFLP approach. RFLP confirmed the presence of this novel variation in the patient T2 (1.4%) (Fig.4). But none of the 70 patients and 60 healthy controls showed this variation.

Fig 3. Sequencing results of the T2 patient showing c.459A>G variation (a) Tumor tissue (b) Paired Normal tissue

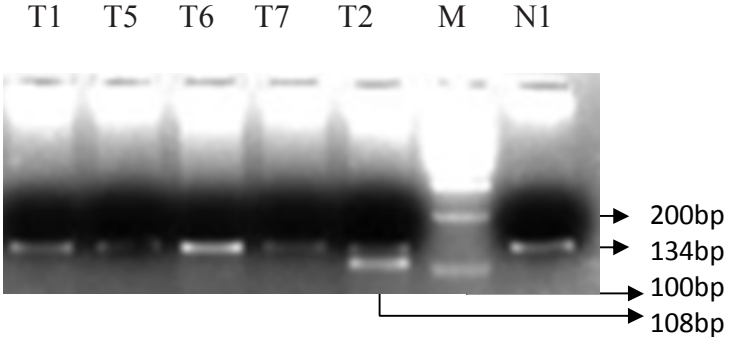

Fig 4: PCR-RFLP genotyping of the *MGMT* c.459A>G variation
T1, T5, T6, T7 & N1 are AA homozygotes
T2 is AG heterozygote
M is 100bp DNA ladder

Discussion

The absence of mutations and any polymorphisms in the exon 2 and 4 of *MGMT* gene as found in our study are not in consistence with the other two studies conducted on esophageal cancer in Chinese population and colorectal cancer in British population. Wang et al have reported point mutations in the codons 121,123 and 132 of Exon 4 of *MGMT* gene in esophageal cancer tissues from Northern China. They have also found the deletion of entire *MGMT* gene in two esophageal cancer patients. [15] In another study conducted by Halford et al., on colorectal cancer tissues and 32 colon cancer cell lines, mutations of the *MGMT* gene have been reported in the codons 23 (exon2), 55, 75(exon3), 104(exon4) and 156(exon5). [16] This inconsistency could be due to the fact that the three studied populations belong to different geographical regions.

In our study, we have found the already well known 2SNPs L53L and L84F in the exon 3 of the *MGMT* gene. The frequency of these SNPS as mentioned in the results cannot be taken as absolute, because only 30 esophageal cancer tissues were directly sequenced. The frequency of these SNPs is well documented in the literature. [17-22] However, preliminary observations reveal that the 2SNPs L53L and L84F are in linkage disequilibrium in our study which is in contrast to other studies. Again this could be due to small number of samples involved in our study.

The sequencing analysis of the exon5 of the *MGMT* gene in our study led to the identification of two well documented SNPs I143V and Ll78R which are in linkage disequilibrium.[19,20,26,28] The association of these two non-synonymous SNPs with the susceptibility of various types of cancers have been studied by various workers. [17-29] Our preliminary results of these SNPs seem to be no different from others.

To the best of our knowledge, we are for the first time reporting (a) the presence of Splice site mutation in the donor site (b) the germline variation at codon 153 (exon5) of the *MGMT* gene in esophageal cancer patients from Kashmir Valley-a high Incidence area.

The identification of the somatic splice mutation in the donor site of the *MGMT* gene in our study is in agreement with the fact that somatic splice-site mutations are known for a number of familial cancer syndromes and the corresponding tumor suppressor genes such as *BRCA1*, *APC*, p53, *FHIT*, and *LKB1* and, the prostate cancer susceptibility gene *KLF6.* [32-36]

Most disease causing single nucleotide substitutions in donor or acceptor splice sites involve the +1/+2 or -1/-2 position, respectively. [37] But in our study the single nucleotide substitution is at +4 position. However, the +4 mutation has been found in a number of genes like *BTK*, *IVD*, *NF1*, *SPTB* and *WT1*. In our study we have also identified a splice site mutation at +22 position of intron 4. Mutation at this position is usually rare such as in *IDS* gene. [38]

The functional studies of the splice site mutations at donor or acceptor sites and at the positions which are close or fairly distant from these sites have been carried out by a number of workers. It has been established that mutations in splice sites decrease recognition of the adjacent exon and consequently inhibit splicing of the adjacent intron. [39, 40] Splice site mutations may result in exon skipping, activation of cryptic splice sites, creation of a pseudo-exon within an intron, or intron retention. [41] The functional reason why alterations in positions close to the splice sites may cause aberrant splicing is usually due to the disruption of interactions with U1 snRNP, U6 snRNP, and U2AF 65 or 35 in the splicing process. [42]

There is possibility that the splice site mutations found in our study may lead to the formation of splice variants in the *MGMT* gene. However, effect of these splice mutations on splicing of *MGMT* gene can be validated by functional studies.

The presence of germline variation c.459A>G (p.Gly153Gly) in only one among the 71 esophageal cancer patients and not in 60 healthy controls, suggests that it could be a rare polymorphism or a germline mutation. It could not be a PCR artefact because it has been confirmed by new PCR based RFLP approach and non-radioactive SSCP (data not shown). The significance of this synonymous germline variation with respect to the etiology of esophageal cancer can be understood on a

large case control study. Recently, Kimchi-Sarfaty et al. observed that synonymous single-nucleotide polymorphisms (SNPs) that retain the amino acid sequence can nevertheless result in a protein with an altered structure and function. [43] This finding underscores the fact that synonymous mutations are as important as non-synonymous.

There are 2835 germline variants of *MGMT* gene deposited in the human SNP database of NCBI. Besides this, Mihi Yang et al has found an apparent 10 bp-deletion in the intron before exon 5 of the *MGMT* gene by DNA sequencing. Because this ‘‘deletion’’ was observed in all sequenced samples (*N* = 20), they have suggested that the previously reported human (Caucasian) *MGMT* gene sequence should be revised to exclude this 10 bp segment. [44] From this, it can be concluded that c.459A>G (p.Gly153Gly) germline variation may be another germline variant of the *MGMT* gene from Kashmiri population-India.

In conclusion, our study has found the new sequence variants of the *MGMT* gene in esophageal cancer patients from Kashmir valley whose functional significance needs to be validated by other assays.

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Disclosure statement

The authors declare that they have no conflict of interest.

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