

RESEARCH ARTICLE

Lack of Influence of MGMT Codon Leu84Phe and Codon Ileu143Val Polymorphisms on Esophageal Cancer Risk in the Kashmir Valley

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Abstract

The enzyme encoded by the *MGMT* gene is involved in the repair of alkylated lesions formed in DNA by carcinogenic nitrosamines. Since dietary items consumed by the Kashmiri population contain high concentrations of these agents, it is biologically plausible that *MGMT* polymorphic variants may be associated with their risk of esophageal cancer. The present study was performed to assess whether non-synonymous SNPs at codon Leu84Phe and codon Ileu143Val of the *MGMT* gene, close to the active site of the protein, might be linked to predisposition of Kashmiris to esophageal cancer. Genotyping was carried out by polymerase chain reaction-restriction fragment length polymorphism on 92 cases and 77 healthy controls. Codon 84 and codon 143 SNPs of the *MGMT* gene were not associated with any increase in risk. While the frequency of the Phe allele at codon 84 in cases was (0.16), slightly higher than controls (0.12), the difference was not statistically significant. Similarly, the frequency of Valine allele in cases at codon 143 (0.08) and controls (0.09) was nearly equal. Moreover, no significant association of *MGMT* genotypes with the clinicopathologic variables of esophageal cancer patients was observed. In conclusion, *MGMT* variants at codon 84 and codon143 may not be involved in the susceptibility of the Kashmiri population to esophageal cancer.

Keywords: Esophageal cancer - Kashmiri population - *MGMT* - PCR-RFLP

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Introduction

Kashmir valley has been reported as a high risk area for esophageal cancer, similar to and in continuity with the "Asian esophageal cancer belt" (Khuroo et al., 1992). While some of the well known risk factors for esophageal cancer such as alcohol consumption, use of tobacco and betel nut, low fruit intake and lack of animal protein are absent in Kashmir, distinct stable dietary habits are notable. Use of sun dried and pickled vegetables, dried and smoked fish, high consumption of red chillies and spices in food and hot salted tea are some of the local features that are suspected to have a strong bearing on the occurrence of esophageal cancer (Siddiqi & Preussmann, 1989).

Since exposure to N-nitroso compounds both exogenous and endogenous, is suspected to be involved in the etiology of esophageal cancer (Bartsch & Montesano, 1984), several studies investigated the presence of preformed volatile and non volatile nitrosamines in raw foods commonly consumed in Kashmir. Data from these studies showed that the levels of nitrosamines present in Kashmiri food are relatively higher than those reported for western foods. It is assumed that exposure to relatively high concentrations of nitrosamines is responsible for the high incidence of this malignancy in Kashmir.

A considerable volume of data now exists which indicates that the formation of O⁶-alkylguanine and O-alkylpyrimidines is biologically more important than N-7-alkylation of guanine in the initiation of the carcinogenic process by nitrosamines in a specific tissue or cell (Swann & Magee, 1968; Loveless, 1969; Goth & Rajewsky, 1974). O⁶-methylguanine is repaired by *MGMT* protein in human cells. If not repaired, O⁶-methylguanine can give rise to cell death, chromosomal aberrations, mutations and cancer. The role of *MGMT* gene in protecting the cells from mutagenic and carcinogenic effect of O⁶ methyl lesions produced by alkylating agents like nitrosamines has drawn the attention of researchers around the world to investigate the molecular status of this gene in normal as well as cancer tissues. Several studies conducted so far have reported that it is rarely mutated in cancers but is silenced by promoter hypermethylation in various tumours (Esteller & Herman, 2004). Moreover, polymorphisms in the *MGMT* gene have also been described but their impact on an individual's sensitivity and cancer susceptibility is unclear (Pegg et al., 2007). In this study, we carried out a case-control study to investigate the polymorphic variants at codon Leu84Phe and codon Ileu143Val of *MGMT* gene for the predisposition of Kashmiri population to esophageal cancer.

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Materials and Methods

Study population

A total of 92 histopathologically confirmed esophageal cancer patients and 72 healthy individuals without overt cancer were recruited in this study. 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 of the cases and controls using the phenol/chloroform extraction after proteinase K digestion (Blin.N, Stafford, D.W. 1976).

Genotyping by PCR-RFLP

Nucleotide sequence of the Primers and the Restriction enzymes for the codon84 and codon143 SNPs were obtained from the literature. 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 MgCl₂, 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, 60 °C (for codon84) or 62 °C (for codon143) for 35 s, and 72 °C for 1 min, followed by a final extension step at 72 °C for 7 min in a Biorad thermal cyclor.

10 µl of the PCR products were subjected to restriction digestion with EarI and BstF5I for codon84, and codon143SNPs respectively. The reaction conditions were set up according to the supplier of restriction enzymes. The restriction digestion with EarI and BstF5I produced 68bp and 34bp in the presence of wild type allele (CTT) at codon 84 and 157bp and 22bp for wild type allele (ATC) at codon143 respectively. The digested PCR products were visualized on 8% Native PAGE for better resolution (Figure 1).

Statistical Analysis

Odds ratios and 95% confidence intervals were calculated to measure the degree of association and Chi-square test was used as the significance test. P value < 0.05 was considered as statistically significant. All analyses were conducted using SAS (SAS institute, Inc., Cary, NC).

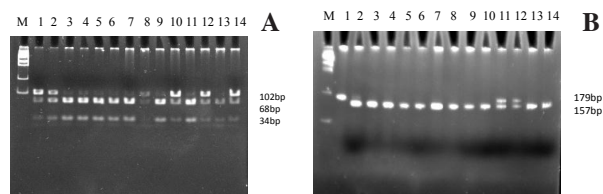


Figure 1. Showing MGMT Codon84 and Codon143 Polymorphisms. A: RFLP picture of codon84 after restriction digestion with EarI enzyme (8%PAGE). Lane M: 100bp DNA marker, Lanes 3-7, 9, 11 & 13: wild (CC) genotype, Lanes 1, 2, 8, 10, 12, 14: heterozygous (CT) genotype

Results

Subject characteristics

The clinicopathologic characteristics of study subjects are summarized in Table 1. The mean age of the patients and controls were 58±11.1 and 51.8±13.5 years respectively. The subjects were considered former smokers if they had quit the smoking since last 1 year before sample collection and current if they are smoking presently or had quit smoking since last 6 months or less before sample collection. There were no significant differences among cases and controls in terms of mean age, gender distribution and smoking, although, more current smokers were present in cases than in controls (n=52; 56.5% Vs n=38; 49.3%). The cases which consumed snuff were less (n=27, 29.3%) as compared to snuff user (n=65, 70.6%). On the basis of the daily consumption of salted tea, the cases were divided into <6 cups/day and ≥ 6 cups/day consumers. 71.7 % (n=66) belonged to the latter group while as 28.2% (n=26) belonged to the former group. Since the consumption of hot beverages, is considered as a possible risk factor for esophageal cancer, we designated the cases which drank the salted tea immediately after preparation as hot salted tea drinkers (n=49, 53.2%) and the cases which drank the salted tea after approximately 5 min. as moderate salted tea drinkers (n=43, 46.7%). There was no significant difference among the cases which consumed high spicy food (n= 42, 45.6%) as compared to the cases which consumed less spicy food (n=50, 54.3%). 95.6% (n=88) of the cases were having Grade

Table 1. Clinicopathologic Characteristics of Esophageal Cancer Patients and Healthy Controls used for Polymorphic Analysis of Codon Leu84Phe and Codon Ile143Val

Characteristic: Subgroup	Cases, n=92(%)	Controls, n=77(%)	P value
Gender: Male	64(69.5)	52(67.5)	0.77
Female	28(30.4)	25(32.4)	
Age: <60 years	45(48.9)	40(51.9)	0.28
≥60 years	47(51.1)	37(48.0)	
Smoking Status: Never	31(33.7)	27(35.0)	
Former	09(9.78)	12(15.5)	0.45
Current	52(56.5)	38(49.3)	0.6
Snuff: Yes	27(29.3)	-	-
No	65(70.6)		
Salted tea: <6 cups/day	26(28.2)	-	-
≥6 cups/day	66(71.7)		
Salted tea: Moderate	43(46.7)	-	-
Hot	49(53.2)		
Food: Moderate spicy	50(54.3)	-	-
High Spicy	42(45.6)		
Dysphagia: Grade 0 & I	88(95.6)	-	-
Grade II & III	04(4.35)		
Histologic type: SCC	58(63.0)	-	-
AD	34(36.9)		
Histologic grade: Well Diff.	28(30.4)	-	-
Mod. & Poorly Diff.	64(69.5)		
Clinical Stage: I & II	63(68.4)	-	-
III & IV	29(31.5)		

*SCC: Squamous cell carcinoma, AD: Adenocarcinoma, Well Diff.: Well differentiated tumours, Mod. & Poorly Diff.: Moderately and Poorly Differentiated tumours

0 & I Dysphagia and only 4.35% (n=4) presented with the clinical symptom of Grade II or III Dysphagia. The main histological types were squamous cell carcinoma (SCC) (n=58; 63.0%) and adenocarcinoma (AD) (n=34; 36.9%). Based on the histopathologic grading, the cases were classified into well differentiated (n=28, 30.4%) and moderately and poorly differentiated (n=64, 69.5%) tumours. 68.4% (n=63) of the cases were at clinical stages I and II compared to 31.5% (n=29) of the cases at stage III & IV.

Analysis of codon Leu84Phe and Ile143Val polymorphism

The genotype distribution of Leu84Phe and Ile143Val for cases and controls is shown in Table 2. The genotype distribution among cases and controls at codon84 (p=0.93

Table 2. Distribution and Frequency of MGMT Codon84 and Codon143 Genotypes among Esophageal Cancer Cases and Healthy Controls

Genotype	Cases (n=92, %)	Controls (n=77, %)	OR (95%CI)	P value
Leu84Leu	64 (69.5)	57(74.0)	Reference	
Leu84Phe	26 (28.2)	20(25.9)	-	
Phe84Phe	2 (2.17)	0	-	
Leu84Phe/Phe84Phe	28 (30.4)	20(25.9)	1.24 (0.63-2.43)	0.60
Phe frequency	0.16	0.12		
Hardy- Weinberg p value	0.93	0.35		
Ileu143ileu	77 (83.7)	63(81.8)	Reference	
Ileu143val	15 (16.3)	14(18.8)	0.87 (0.39-1.92)	0.74
Val143val	0	0	-	
Val.frequency	0.08	0.09		
Hardy -Weinberg p value	0.68	0.67		

and p=0.35) and at codon143 (p= 0.68 and p= 0.67) were consistent with Hardy- Weinberg equilibrium. The frequency of Phe allele in cases at codon84 was 0.16 which was slightly higher than controls (0.12) and this difference was not statistically significant [OR & 95%CI =1.24(0.63-2.43), P=0.60]. The frequency of Valine allele at codon143 in cases (0.08) and controls (0.09) was nearly equal with a non-significant p value [OR & 95% CI = 0.87(0.39-1.92), p=0.74]. Thus, in the present study, codon84 and codon143 SNPs of the MGMT gene were not associated with the increase in the risk of esophageal cancer in Kashmiri population.

We further divided the data into the subgroups based on gender, age, smoking status, snuff use, salted tea drinking status, consumption of spicy food, dysphagia, histopathological type, tumour grade and clinical stage of the disease. We evaluated the esophageal cancer risk for each subgroup by estimating the ORs associated with the combined codon Leu84Phe/Phe84Phe and the codon Ile143Val variant genotypes compared with the codon Leu84Leu and the codon Ile143Ile genotypes respectively (Tables 3 & 4). When we used the codon Leu84Leu as the reference, the risk associated with the combined Leu84Phe/Phe84Phe variant genotype was more evident for subjects younger than 60 years (OR=1.31, 95% CI= 0.52-3.29), females (OR=1.5, 95% CI = 0.45-4.88), never smokers (OR=2.35, 95% CI= 0.78-7.00), snuff users (OR=1.42, 95% CI = 0.56-3.62), <6 cups/day salted tea drinkers (OR = 1.50, 95% CI = 0.59-3.86), moderate salted tea drinkers (OR=1.37, 95% CI = 0.61-3.08), high spicy food consumers (OR = 1.75, 95% CI = 0.79-3.89), Grade 0 & I dysphagia (OR = 1.26, 95% CI =0.64-2.48), SCC (OR = 1.28, 95% CI = 0.60-2.70), well differentiated tumors (OR = 1.58, 95% CI= 0.63-3.94), and subjects with

Table 3. Association of Codon84 Polymorphic Alleles with Clinicopathologic Characteristics of Study Subjects

Variable	Subgroup	Cases (Wild/mutant)	Controls (Wild/mutant)	OR (95%CI)	P value
Overall	-	64/28	57/20	1.24 (0.63-2.43)	0.6
Gender	Male	45/19	38/14	1.14 (0.51-2.56)	0.74
	Female	19/09	19/06	1.50 (0.45-4.88)	0.51
Age	<60 years	30/15	29/11	1.31 (0.52-3.29)	0.56
	≥60 years	34/13	28/09	1.19 (0.45-3.12)	0.73
Smoking Status	Never	17/14	20/07	2.35 (0.78-7.00)	0.12
	Former	1-Aug	3-Sep	0.37 (0.04-3.38)	0.42
	Current	39/13	28/10	0.93 (0.36-2.38)	0.88
Snuff	Yes	18/09	57/20	1.42 (0.56-3.62)	0.46
	No	46/19	57/20	1.17 (0.56-2.44)	0.66
Salted tea	<6 cups/day	17/09	57/20	1.50 (0.59-3.86)	0.45
	≥6 cups/day	47/19	57/20	1.15 (0.55-2.39)	0.7
Salted tea	Moderate	29/14	57/20	1.37 (0.61-3.08)	0.44
	Hot	35/14	57/20	1.14 (0.51-2.52)	0.74
Food	Moderate spicy	38/12	57/20	0.90 (0.39-2.03)	0.8
	High Spicy	26/16	57/20	1.75 (0.79-3.89)	0.16
Dysphagia	Grade 0 & I	61/27	57/20	1.26 (0.64-2.48)	0.5
	Grade II & III	1-Mar	57/20	0.95 (0.13-7.16)	0.96
Histologic type	SCC	40/18	57/20	1.28 (0.60-2.70)	0.51
	AD	24/10	57/20	1.18 (0.49-2.87)	0.70
Histologic grade	Well Diff.	18/10	57/20	1.58 (0.63-3.94)	0.33
	Mod. & Poorly Diff	46/18	57/20	1.11 (0.53-2.33)	0.85
Clinical Stage	I & II	47/16	57/20	0.97 (0.45-2.06)	0.93
	III & IV	17/12	57/20	2.01 (0.83-4.88)	0.12

*Wild, Leu84Leu genotype; Mutant, Leu84Phe or Phe84Phe genotype; Well Diff, Well differentiated tumours; Mod. & Poorly Diff., Moderately and Poorly Differentiated tumours; SCC, Squamous cell carcinoma; AD, Adenocarcinoma

Table 4. Association of Codon143 Polymorphic Alleles with Clinicopathologic Characteristics of Study Subjects

Variable	Subgroup	Cases (Wild/mutant)	Controls (Wild/mutant)	OR (95%CI),	P value
Overall		77/15	63/14	0.87 (0.39-1.92)	0.74
Gender	Male	56/08	42/10	0.60 (0.22-1.61)	0.44
	Female	21/07	21/04	1.75 (0.46-6.46)	0.42
Age	<60 years	34/11	34/06	1.83 (0.62-5.33)	0.22
	≥60 years	43/04	29/08	0.38 (0.09-1.16)	0.08
Smoking Status	Never	23/08	22/05	1.53 (0.45-5.16)	0.5
	Former	08/01	1/11	1.37 (0.12-15.2)	0.83
	Current	46/06	30/08	0.49 (0.16-1.49)	0.21
Snuff	Yes	23/04	63/14	0.78 (0.24-2.51)	0.69
	No	54/11	63/14	0.91 (0.39-2.15)	0.84
Salted tea	<6 cups/day	18/08	63/14	2.00 (0.74-5.42)	0.18
	≥6 cups/day	59/07	63/14	0.53 (0.20-1.38)	0.24
Salted tea	Moderate	33/10	63/14	1.36 (0.55-3.35)	0.5
	Hot	44/05	63/14	0.51 (0.17-1.47)	0.22
Food	Moderate spicy	46/04	63/14	0.39 (0.12-1.21)	0.12
	High Spicy	31/11	63/14	1.59 (0.66-3.87)	0.3
Dysphagia	Grade 0 & I	73/15	63/14	0.92 (0.41-2.03)	0.84
	Grade II & III	04/00	63/14	-	-
Histologic type	SCC	46/12	63/14	1.17 (0.50-2.73)	0.71
	AD	31/03	63/14	0.43 (0.12-1.53)	0.2
Histologic grade	Well Diff.	23/05	63/14	0.97 (0.33-2.92)	1
	Mod. & Poorly Diff	54/10	63/14	0.83 (0.34-1.99)	0.82
Clinical Stage	I & II	53/10	63/14	0.84 (0.35-2.03)	0.71
	III & IV	24/05	63/14	0.93 (0.31-2.79)	0.91

*Wild, Ile143Val genotype; Mutant, Val143Val genotype; Well Diff, Well differentiated tumours; Mod. & Poorly Diff, Moderately and Poorly Differentiated tumours; SCC, Squamous cell carcinoma; AD, Adenocarcinoma

clinical stage III & IV (OR = 2.01, 95% CI = 0.83-4.88). When we used the codon Ile143Ile as the reference, the risk associated with the Ile143Val variant genotype was more evident for subjects younger than 60 years (OR=1.83, 95% CI=0.62-5.33), females (OR=1.75, 95% CI=0.46-6.46), never (OR=1.53, 95% CI=0.45-5.16) and former smokers (OR = 1.37, 95% CI = 0.12- 15.23), <6 cups/day salted tea drinkers (OR = 2.00, 95% CI = 0.74-5.42), moderate salted tea drinkers (OR=1.36, 95% CI = 0.55-3.35), high spicy food consumers (OR = 1.59, 95% CI = 0.66-3.87) and subjects with SCC (OR = 1.17, 95% CI = 0.50-2.73). We did not find statistical significant association (p value >0.05) between the combined codon Leu84Phe/Phe84Phe, codon Ile143Val variant genotypes and esophageal cancer risk among any subgroup.

Discussion

There is an increasing realization that polymorphisms in genes involved in either the metabolism of carcinogens/toxins or the responses to damage caused by these agents including DNA repair may have a profound effect on sensitivity to these agents and thus on human health. Identification of polymorphic variants in such genes and understanding the mechanistic implications of such alterations is now a major research area. In the present study, we analysed the two non-synonymous SNPs Leu84Phe and Ileu143Val which lie close to the active site Cys145 residue of the MGMT protein. In the present study, the frequency of Phe allele in cases was 0.16 and 0.12 in controls. The frequency of valine allele in cases was 0.08 and 0.09 in controls. The difference in allele frequencies was not statistically significant and hence is not associated with increase in the risk of esophageal

cancer. The frequency of the Phe and Val as reported in other studies are 21% (12–27%) and 24% (11–28%) respectively. However, these variants are much rarer in Asian and African populations which support our findings (Bugni et al., 2007).

Few studies have analysed the association of codon84 and codon143 genotypes of MGMT gene with the risk of esophageal cancer. Since ESCC is the most frequent type of esophageal cancer in Asia, two studies, one from China (Xing et al., 2003) and another from Iran (Akbari et al., 2009), analyzed the effect of MGMT polymorphisms on the risk of ESCC. No main effects were observed for MGMT codon84 and codon143 polymorphisms with the risk of esophageal cancer in Chinese or Iranian population. However, the Iranian study reported the association of the A allele of the rs7087131 variant, which is an intronic SNP, of MGMT gene with a decreased risk of ESCC under a dominant model (odds ratio, 0.79; 95% confidence interval, 0.64-0.96; P = 0.02). Recently, association of intronic SNPs with the ESCC risk has also been reported from china (Wen-Jing et al., 2010). By contrast, a multicenter case-control study conducted in six centres from five Central and Eastern European countries: Bucharest (Romania), Lodz (Poland), Moscow (Russia), Banska Bystrika (Slovakia), and Olomouc and Prague (Czech Republic), reported the association of codon53 and codon84 variant genotypes of MGMT gene with the risk of ESCC (Hall et al., 2007).

The rates of EAC in western countries have increased rapidly in recent decades. The primary risk factors for EAC such as gastro-esophageal acid reflux and smoking are potentially genotoxic through the generation of N-nitroso compounds. The MGMT gene is the major cellular defense against alkylating DNA damage. In order to assess the role

of MGMT polymorphisms in EAC, a case control study conducted on Australian population observed significantly higher frequencies of the minor allele for 3 of the MGMT polymorphisms examined (rs12269324, rs12268840 and I143V), among EAC cases than controls. At the genotype level, carriers of homozygous variant genotypes were found to have 60–100% higher risks of EAC than those with consensus genotypes (Doecke et al., 2008).

Besides above mentioned studies, there are conflicting results in studies on the association of the Leu84Phe and Ile143Val polymorphisms with cancer. In order to clarify this paradox, recently, a meta-analysis was conducted with a large collected sample (13,069 cancer patients and 20,290 controls). A significant association was found between the T allele (84Phe) and cancer risk, under the recessive genetic model [P = 0.023, odds ratio (OR) = 1.251, 95% confidence interval (CI) 1.031-1.517, P(heterogeneity) = 0.270], TT versus CC comparison (P = 0.035, OR = 1.239, 95% CI 1.015-1.511, P(heterogeneity) = 0.225) and TT versus CT comparison (P = 0.007, OR = 1.292, 95% CI 1.071-1.559, P(heterogeneity) = 0.374), using the random-effect model. In the ethnicity subgroup analysis, a significant association with cancer among Caucasians was found under the recessive genetic model, homozygote comparison and TT versus TC comparison. In the tumour sites subgroup analysis, only the protective effects of Leu84Phe polymorphism were found in colorectal cancer, under CT versus CC comparison. No significant association between the G allele of Ile143Val and cancer risk was found. The G allele showed an increased lung cancer risk under the dominant genetic model and AG versus AA comparison in all Hardy-Weinberg equilibrium subjects, only when the fixed-effect model was used. However, it was insignificant in the random-effect model (Zhong et al., 2010).

In the present study, we examined the association of Leu84Phe and Ile143Val MGMT polymorphisms with risk of ESCC as well as EAC from Kashmiri population which lies in the “Asian esophageal cancer belt”. To the best of our knowledge, this is the first study from Asia in which association of MGMT polymorphisms with EAC was analysed. Although sample size in our study is small, but our results are in agreement with Chinese and Iranian studies (Xing et al., 2003; Akbari et al., 2009). However, the lack of association of MGMT polymorphisms with the risk of EAC in our study is inconsistent with the European study (Hall et al., 2007). This may be attributed to the difference in the ethnicity of the two populations and /or contributing etiologic factors. Furthermore, our results show concordance with the meta-analysis studies (Zhong et al., 2010).

Based on the fact that tobacco smoking can result in mutagenic O6 alkylguanine DNA lesions that are repaired mainly by MGMT, it is biologically plausible to hypothesise that the potential association between tobacco smoking and esophageal cancer risk may be influenced by heterogeneity in MGMT genotype status. In the present study, we assessed the the association of codon84 and codon143 genotypes with esophageal cancer risk and the interaction with tobacco smoking. Our study revealed no significant association between tobacco smoking and these

polymorphisms in esophageal cancer which is consistent with a number of published studies (Cohet et al., 2004; Shen et al., 2005; Tranah et al., 2006).

Besides, age, gender, smoking and the clinico-pathological features of the cases, we also examined the association of local personal and dietary habits of the cases such as use of snuff, consumption of salted tea, spicy food with MGMT polymorphisms. The rationale for the inclusion of these characteristics was the occurrence of carcinogenic nitrosamines in them. Since thermal irritation of esophagus is a possible risk factor for esophageal cancer, salted tea drinkers were dichotomised into hot salted tea drinkers and moderate salted tea drinkers for analysing the association with MGMT polymorphisms. We found no significant association of these characteristics with either Leu84Phe or Ile143Val variants and the risk of esophageal cancer.

In conclusion: MGMT variants at codon 84 and codon143 may not be involved in the susceptibility of Kashmiri population to esophageal cancer.

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