RESEARCH ARTICLE

Promoter Methylation of MGMT Gene in Serum of Patients with Esophageal Squamous Cell Carcinoma in North East India

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Abstract

Background: Promoter hypermethylation is a common event in human cancer. O6-methylguanine-DNA methyltransferase (MGMT) is a gene involved in DNA repair, which is methylated in a variety of cancers. We aimed to explore the methylation status of MGMT gene among the North Eastern population where esophageal cancer incidence and exposure to carcinogens like nitrosamines is high. Materials and Methods: A total of 100 newly diagnosed esophageal cancer cases along with equal number of age, sex and ethnicity matched controls were included in this study. Methylation specific PCR was used to determine the MGMT methylation status in serum samples. Results: Aberrant promoter methylation of the MGMT gene was detected in 70% of esophageal cancer cases. Hypermethylation of MGMT gene was found to be influenced by environmental factors like betel quid and tobacco which contain potent carcinogens like nitrosamines. Tobacco chewing and tobacco smoking habit synergistically with MGMT methylation elevated the risk for esophageal cancer development [adjusted OR=5.02, 95% CI=1.35-18.74; p=0.010 for tobacco chewing and Adjusted OR=3.00, 95% CI=1.22-7.36; p=0.014 for tobacco smoking]. Conclusions: Results suggest that the DNA hypermethylation of MGMT is an important mechanism for MGMT gene silencing resulting in esophageal cancer development and is influenced by the environmental factors. Thus MGMT hypermethylation can be used as a biomarker for esophageal cancer in high incidence region of North East India.

Keywords: Esophageal cancer - North East India - MGMT - methylation - biomarker

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Introduction

Esophageal cancer is the eighth most common form of cancer in the world with 456,000 new cases in 2012 (Ferlay et al., 2012). National cancer registry program report of India during 2009-2011 reported the highest incidence of esophageal cancer in the East Khasi Hills District in Meghalaya (Age Adjusted Rate (AAR) 71.4 among males and 30.2 among females) followed by Aizawl District in Mizoram (AAR 42.0 among males and 7.0 among females) and Kamrup Urban District in Assam (AAR 27.0 among males and 18.3 among females) in the North East India (NCRP 2009-2011). There is a wide geographical disparity in the incidence of esophageal cancer (Sharma et al., 2014a). This variation may be attributed to diverse ethnicity, environmental factors and dietary habits in the Northeast India (Talukdar et al., 2013). Environmental and dietary factors like smoking and smokeless tobacco consumption, betel quid chewing, alcohol intake, poor nutrition, etc., are considered to be associated with Esophageal Squamous Cell Carcinoma (ESCC) in the high risk region of North East India (Phukan et al., 2001a, b, 2005, 2006, 2014; Saikia et al., 2014).

Tobacco and betel quid which is mostly consumed by the North Eastern population contains polycyclic aromatic hydrocarbons like nitrosamines which form DNA adducts like O6 methylguanine (Zhang et al., 2003). These adducts intercalate with DNA and cause mispairing leading to mutations mostly G:C to A:T mutation (Baumann et al., 2006). The mutation caused by these adducts need to be repaired by DNA repair enzymes during replication. Inability to repair these mutations lead to uncontrolled growth of the damaged DNA resulting in tumorigenesis (Fang et al., 2005). MGMT (O6- methyl gyuanine methyl transferase) is an important DNA repair enzyme which specifically repairs the DNA adducts caused by alkylating agents like nitrosamines (Du et al., 2013). It specifically removes the alkyl group from the O6 position of guanine residue by transferring it to specific cytosine residue within the MGMT protein itself (Larijani et al., 2014). Impaired activity of the DNA repair enzyme MGMT may result in impaired DNA repairing capacity thereby resulting in accumulation of damaged DNA causing tumor growth. Mutation, homozygous deletions and hypermethyl¬ation of the promoter are major mechanisms behind the MGMT gene inactivation (Ye et al., 2006; Khatami et al., 2009;

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Chen et al., 2012; Xiong et al., 2013). A number of studies in different populations have shown the association of MGMT hypermethylation with cancer development and progression. Hypermethylation of MGMT gene have been reported in a number of cancers like gastric cancer, esophageal cancer, lung cancer, colorectal cancer hepatocellular carcinoma (Katayama et al., 2007; Khatami et al., 2009; Chen et al., 2012; Gao et al., 2013).

Promoter hypermethylation is an epigenetic modification in which methyl group is covalently added to the C5 position of cytosine ring of DNA (Chen et al., 2012). Addition of methyl group changes the structural conformation of the chromatin network thereby making the promoter site of the gene inaccessible to the transcription factors. The gene is thus made nonfunctional or in other words DNA methylation culminates in gene silencing (Gao et al., 2013; Liu et al., 2013).

Thus this study was conducted to explore the possible association between MGMT hypermethylation with tobacco and betel quid chewing and smoking habit. Further, we aimed to investigate the potency of MGMT hypermethylation to be used as a biomarker among this unique population of North East India where the intake of betel quid and tobacco is rampant and the incidence of esophageal cancer is high.

Materials and Methods

Sample Collection

100 newly diagnosed esophageal cancer cases along with equal number of age (±5 years), sex and ethnicity matched controls were included in this study. 2ml of intravenous blood samples were collected aseptically from histopathologically confirmed esophageal cancer cases who visited Aditya Hospital, Dibrugarh and Assam Medical College and Hospital, Dibrugarh, Assam, India. The collected samples included cases from different North Eastern states of India viz. Assam, Arunachal Pradesh, Meghalaya, Manipur and Mizoram who visited these hospitals for diagnosis and treatment. Blood from age (±5 years), sex and ethnicity matched healthy controls were also collected from the community. Written informed consent was taken from all subjects for participation in the study in a protocol approved by the Institutional Ethical Committee of Regional Medical Research Centre, N.E Region (Indian Council Medical Research). The blood samples were stored at -80^{0}C until further processing.

DNA extraction

Genomic DNA was extracted from the samples using Qiagen Dneasy® Blood and Tissue kit following the protocol provided by the manufacturer.

Bisulphite conversion

Conversion of unmethylated, cytosines to uracils in DNA samples was carried out using the Epitect Bisulphite Kit (Qiagen, Hilden, Germany) according to the manufacturer's specifications.

After treatment, methylated DNA sequence differs from unmethylated DNA, which is used to design methylation specific primers.

Methylation specific PCR (MSP)

The methylation status at the promoter region of MGMT gene was assessed by methylation-specific PCR (MSP) as described by Herman et al. (2001); Wang et al. (2008) with slight modifications in a final volume of 25μL PCR reaction with 100ng of bisulfite-treated DNA, 1.5 μl of each primer (Sigma, United States), 4.5μl of water, and 12.5μL DreamTaq Master Mix from Fermentas. The primers used for MSP are shown in Table 1 alongwith the annealing temperature. Epitect Methylated control DNA from Qiagen was used as a positive control for methylated samples and water was used as negative control.PCR products were loaded onto 2% gels, stained with ethidium bromide, and directly visualized in gel documentation system (Cell Biosciences).

Statistical analysis

Multiple logistic regression analysis was used to analyze the data. The conditional maximum likelihood method was used to estimate the parameters of the regression model because of the matched design and significance was taken at $p \leq 0.05$ (two tailed). Difference in distribution of demographic characteristics and methylation frequencies between cases and controls as well as distribution of clinicopathological characteristics on the basis of methylation status of cancer patients were evaluated using Chi Square (χ^2) test, Fisher's Exact test and independent samples T-test wherever appropriate. The crude measure of association between single putative risk factors and esophageal cancer was expressed by deriving odds ratio (OR) and its corresponding 95% confidence interval (CI) calculated from the standard error of the regression coefficient. To control for confounding variables such as betel-quid chewing, tobacco chewing, tobacco smoking and other covariates, the data was analyzed by conditional multiple logistic regression to evaluate the extent of risk association.

OR with 95% CIs were used to assess the strength of association between the MGMT methylation status and esophageal cancer risk among different subgroups with different chewing and smoking habits. The statistical package used for the analysis was SPSS version 17.

Results

Socio-demographic characteristics of the subjects under study

In this case control study, 66% of the subjects were males and 34% were females. There was no statistically significant difference in terms of mean age of cases (59.98±8.24 years, range=40-75) and controls (58.32±8.91 years, range=36-75) (p=0.173) of the study subjects. Most of the cases belonged to rural areas (57%) and were from low financial income group. The esophageal cancer cases included in the study were ESCC. Table 2 summarizes the socio demographic characteristics of the subjects under study.

Logistic regression analysis of risk factors for ESCC

The distribution and the individual effects of the various exogenous factors under study is represented in

Table 1. Primers and Annealing Temperature Used for PCR

GENE	M/U	S/AS	Primer sequences (5'-3')	Annealing temperature (°C)	Product size (bp)
MGMT	M	S	TTTCGACGTTCGTAGGTTTTCGC	66°C	81bp
		AS	GCACTCTTCCGAAAACGAAACG		
	U	S	TTTGTGTTTTGATGTTTGTAGGTTTTTGT	60°C	93bp
		AS	AACTCCACACTCTTCCAAAAACAAAACA		

Table 2. Distribution of Socio-Demographic Characteristic and Risk of Esophageal Cancer

Category	Case (n)	Control (n)	p-value
Sample size	100	100	---
Sex			0.559†
Male	66	66	---
Female	34	34	---
Age (years)			
Range	40-75	36-75	
Means±SD	59.98±8.24	58.32±8.91	0.173‡
Dwelling			
Semi urban	43	60	---
Rural	57	40	---
Income per Month			
>9787	3	5	---
7323-9787	21	27	---
4894-7322	20	24	---
2936-4893	33	30	---
980-2935	23	14	---

†Based on Fisher's Exact test; ‡For independent samples T-test.

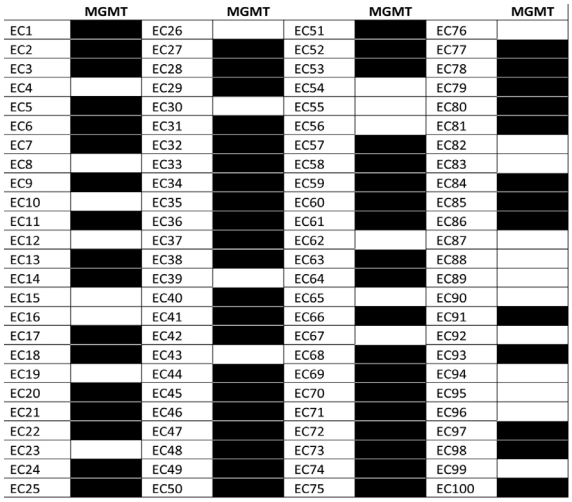

Figure 1. Promoter Methylation Profile of MGMT Gene of 100 Esophageal Cancer patients. Each column and row represents the gene indicated on top and individual patients. The number indicated on the left corresponds to the patient number. Black rectangles are methylated samples and white rectangles are unmethylated samples

Table 3. Betel-quid chewing [Adjusted OR=3.29, 95% CI=1.17-9.21; p=0.023] was found to have a significant risk for esophageal cancer after adjusting for potential confounding factors like tobacco chewing and tobacco smoking. Tobacco chewing [Adjusted OR=3.32, 95% CI=1.21-9.14; p=0.020] too conferred a significant risk for esophageal cancer after adjusting for the potential confounding factors like betel quid chewing and tobacco smoking. Tobacco smoking had individual risk for ESCC but after adjusting for the potential confounders the risk of tobacco smoking for ESCC was not found to be significant.

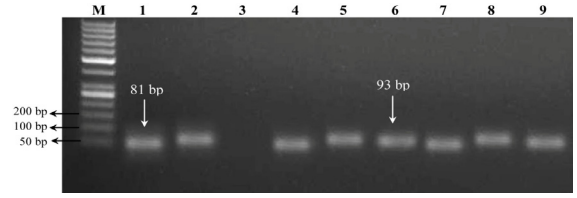

Figure 2. 2% Agarose Gel Photograph Showing Representative Results of Methylation-Specific PCR Analysis for MGMT Gene Using Both Methylated and Unmethylated Specific Primers. Lane M is 50 bp ladder. Lane 1 and 7 are methylated bands in cases. Lane 2 and 8 are unmethylated bands in cases. Lane 3 is a negative control. Lane 4 is Epitect methylated DNA positive control from Qiagen, Lane 5 is Epitect unmethylated DNA positive control from Qiagen. Lane 6 and 9 are unmethylated bands in controls

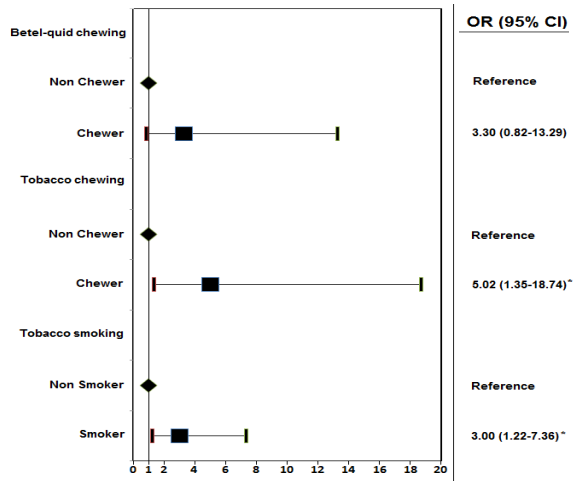

Figure 3. Associations of MGMT Gene with Esophageal Cancer Among Different Subgroups with Different Chewing and Smoking Habits

Table 4. MGMT Promoter Methylation Profile and Clinicopathological Characteristics of Esophageal Cancer Patients

Category	Cases without MGMT promoter methylation (n %)	Cases with MGMT promoter methylation (n %)
Sample size	30 (100)	70 (100)
Pathological grade		
G1: Well differentiated	10 (33.3)	18 (25.7)
G2: Moderately differentiated	19 (63.3)	50 (71.4)
G3: Poorly differentiated	1 (3.3)	2 (2.9)
Tumor topography		
C15.3: Upper third of esophagus	4 (13.3)	6 (8.6)
C15.4: Middle third of esophagus	19 (63.3)	43 (61.4)
C15.5: Lower third of esophagus	7 (23.3)	21 (30.0)

MGMT promoter methylation profile of ESCC patients

Aberrant promoter methylation of the MGMT gene was detected in 70% esophageal cancer cases (Figure 1). Agarose gel picture depicting methylation status of cases

Table 3. Association of Esophageal Cancer with Exogenous Factors

Category	Case (n)	Control (n)	Crude OR (95% CI)	p-value	Adjusted† OR (95% CI)	p-value
Betel-quid chewing						
Nonchewer	9	40	1.0 (Reference)		1.0 (Reference)	
Chewer	91	60	6.74 (3.05- 14.90)	<0.001	3.29 (1.17-9.21)	0.023*
Tobacco chewing						
Nonchewer	11	43	1.0 (Reference)		1.0 (Reference)	
Chewer	89	57	6.10 (2.91-12.81)	<0.001	3.32 (1.21-9.14)	0.020*
Tobacco smoking						
Nonsmoker	48	59	1.0 (Reference)		1.0 (Reference)	
Smoker	52	41	1.56 (0.89-2.73)	0.119	0.74 (0.38-1.44)	0.375

*Significant; †Adjusted for betel-quid chewing, tobacco chewing and tobacco smoking in conditional multiple logistic regression model

in representative samples is shown in Figure 2.

Clinicopathological characteristics of ESCC patients

71.4% of the ESCC were moderately differentiated followed by 25.7% well differentiated and 2.9% poorly differentiated. Most of the tumor topography comprised of the C15.4: middle third of the esophagus (61.4%) followed by C15.5: lower third of esophagus (30%) and C15.3 upper third of esophagus (8.6%) (Table 4).

Risk assessment of ESCC with MGMT promoter methylation

The association of MGMT hypermethylation with environmental factors and the risk of ESCC are shown in Figure 3. Among the cases, tobacco chewing with MGMT methylation synergistically conferred a significantly increased risk for ESCC [OR=5.02, 95% CI=1.35-18.74; p=0.010 for tobacco chewing]. Tobacco smoking in association with MGMT methylation was also found to confer a significantly increased risk for ESCC [OR=3.00, 95% CI=1.22-7.36; p=0.014] whereas tobacco smoking without MGMT methylation conferred a risk but it was not significant for development for ESCC. Betel-quid chewing with MGMT methylation conferred a risk but it was not found to be significant (Figure 3).

Thus it was seen that MGMT methylation in conjunction with environmental factors elevated the risk of esophageal cancer development.

Discussion

North East India has the highest incidence of esophageal cancer and the number of esophageal cancer cases in this region is increasing at an alarming rate (Chattopadhyay et al., 2007; Sharma et al., 2013; Sharma et al., 2014b). Comparison of esophageal cancer incidence rate in the year 2006-2008 and 2009-2011 revealed a noticeable increase in the incidence rate of esophageal cancer among the North Eastern states of India (NCRP 2006-2008, 2009-2011).

Although esophageal cancer is rampant in North East India, not much work has been done to decipher the molecular pathogenesis of esophageal cancer. Moreover, this is the first report on methylation status of MGMT gene in the North Eastern population.

Epigenetic events like promoter methylation of tumor suppressor genes is recognized to play a key role in tumor development and progression by repression of the tumor suppressor activity of the corresponding genes (Limpaiboon 2012). MGMT is a well-known DNA repair protein whose loss of function leads to impaired DNA repairing capacity resulting in accumulation of mutation causing cancer development (Du et al., 2013). Our study was therefore focused on exploring the possible feasibility of using a methylation marker for detection of cancer. Since cancer can be prevented only if detected early; the need for early detection molecular markers is of utmost importance.

Accordingly, we have studied the promoter methylation status of this important DNA repair gene. We have found a significantly high frequency of MGMT methylation in cases with esophageal cancer. This is in line with earlier studies on MGMT methylation status (Corvalan and Maturana 2010). We have got a slightly higher frequency of MGMT methylation in our study population which might be due to ethnic differences and influence of unique dietary habits like betel nut and tobacco chewing and tobacco smoking habit of the people of North East India.

We therefore explored the possible association between MGMT promoter hypermethylation and environmental risk factors. In our previous study we have earlier reported that betel quid and tobacco chewing is one of the prominent risk factors for esophageal cancer in North East India (Phukan et al., 2001a). In this case control study too, betel quid chewing and tobacco chewing was found to be a significant risk factor for ESCC. Tobacco smoking and alcohol was also found to confer a risk for esophageal cancer development. Further classifying the cases based on MGMT methylation status we have found that methylation was more in chewers (both tobaaco-93.8% and betel quid-95.1%) than non chewers.

The difference in the methylation frequency of MGMT gene among tobacco users and non users can be explained by the invitro study on mouse model and lung cancer tissues which revealed that NNK found in tobacco cause DNA methyl transferase I degradation and induces its nuclear accumulation resulting in hypermethylation of genes (Lin et al., 2010). Moreover, the nitrosamines present in tobacco is also responsible for causing the alkyl DNA damages and because MGMT plays an important role in the repair of O6 methyl guanine adduct formed due to nitrosamine carcinogen, its involvement in esophageal carcinogenesis is biologically plausible.

Hypermethylation of MGMT gene was found to be influenced by environmental risk factors. Tobacco chewing and tobacco smoking with MGMT methylation

synergistically conferred a significantly increased risk for ESCC.

In conclusion, the result in our present study indicated that the MGMT aberrant methylation is a frequent event in the occurrence of ESCC and it also indicated that MGMT methylation may be a putative biomarker for early detection of ESCC in the high incidence region of North East India. Tobacco and betel quid chewing confer a significant risk on ESCC development and it synergistically has a strong effect on promoter methylation leading to esophageal cancer. Therefore there is a strong influence of environmental risk factors on MGMT hypermethylation. Further study on large sample size would help to identify further probable factors behind MGMT hypermethylation and esophageal cancer risk in North east India.

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