RESEARCH ARTICLE

Promoter Methylation Status of DNA Repair Gene (hMLH1) in Gastric Carcinoma Patients of the Kashmir Valley

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Abstract

Cancer is a multi-factorial disease and variation in genetic susceptibility, due to inherited differences in the capacity to repair mismatches in the genome, is an important factor in the development of gastric cancer (GC), for example. Epigenetic changes, including aberrant methylation of 5/CpG islands in the promoter regions of mismatch repair (MMR) genes like hMLH1, have been implicated in the development of various types of GC. In the present study we evaluated the role of hMLH1 promoter hypermethylation in Kashmiri GC patients and controls, and assessed correlations with various dietary and lifestyle factors. The study included 70 GC patients (56 males and 14 females; age (mean±S.D) 50±11.4 years). Distinction between methylated and unmethylated was achieved with MS-PCR and DNA band patterns. The Chi-square test was applied to assess the risk due to promoter hypermethylation. We found a strikingly high frequency of promoter hypermethylation in GC cases than in normal samples (72.9% (51/70) in GC cases vs 20% (14/70) in normal samples (p=0.0001).We also observed a statistically significant association between methylated hMLH1 gene promoter and smoking, consumption of sundried vegetables and hot salted tea with the risk of GC. This study revealed that hMLH1 hypermethylation is strongly associated with GC and suggested roles for epigenetic changes in stomach cancer causation in the Kashmir valley.

Keywords: Gastric cancer - promoter hypermethylation - CpG - MMR - Kashmir valley

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Introduction

Although gastric cancer (GC) incidence and mortality rates have shown a consistent decline over several decades in most countries, GC is still one of the common cancers worldwide, accounting for about 8% of new cancers annually (Jemal et al., 2011). There is a wide variation in the geographical distribution of gastric cancer worldwide. Despite great advances in the treatment, the prognosis of the GC is still dismal and the overall five year survival rates remain low (Siewert, 1998; Wagner et al., 2006). Several factors have been associated with the prevalence of GC which includes diet, geographical location and the genetic makeup of the individual (Nagini, 2012). Genetic and epigenetic alterations which have been implicated in the cancer development affect tumor suppressor genes, protooncogenes and DNA repair genes (Tahara, 1993). Epigenetic changes like methylation of regulatory elements are now thought to be just as important as gene mutations in cancer development. DNA methylations caused due to DNA methyltransferases commonly occur in the cytosine residues of the CpG dinucleotides found in the 5/ region of the gene promoter and have been associated with transcriptional repression of the genes (Bird, 1996). The role of promoter hypermethylation in gene silencing of DNA mismatch repair genes (Cunningham et al., 1998; Leung et al., 1999) and cell cycle regulatory genes (Herman et al., 1995; Lee et al., 1997) is now well established and the list of epigenetically altered genes which have important role in determining the fate of the cell is growing. Genes like hMLH1 have been found to be inactivated by promoter hypermutation in various types of gastric cancer (Shim, 2000). Several studies suggest that silencing of the hMLH1 gene by promoter hypermethylation is a major causative event in the development of human gastric cancers with microsatellite instability (Leung et al., 1999; Fleisher et al., 2001; Mir et al., 2012).

Till date no study has been done to understand the role of hMLH1 promoter hypermethylation in high incidence rate of gastric cancer in Kashmir. Therefore the main aim of the present study was to analyze the influence of the hMLH1 promoter hypermethylation on the susceptibility of gastric cancer and also to correlate various dietary and lifestyle factors with the methylation status of hMLH1 gene in Kashmiri population.

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Materials and Methods

Samples

The study was started following approval by the ethical committee of the Sher-I-Kashmir Institute of Medical Sciences (SKIMS). Tumor specimens of 70 resected gastric carcinoma and paired normal tissue were obtained from the Minimal Access Surgery Department of SKIMS. Blood samples were also taken for constitutive DNA. The samples were stored at -80º C until DNA was obtained. A well drafted questionnaire was used to collect information related to the dietary, occupational and lifestyle habits from the patients. All the patients were interviewed by the same person to reduce any possible bias. Only those patients who willingly participated were included in the study.

Methylation Specific-PCR

Both the tumorous and normal DNAs were subjected to sodium bisulfite modifications following the instructions of the kit (DNeasy, Qiagen). Methylation Specific-PCR (MS-PCR) was performed to examine methylation at promoter sequence of hMLH1 gene, using method as described previously (Fleisher et al., 1999). Both methylated and unmethylated primers were used for normal as well as cancerous tissue.

The primer sequences of hMLH1 for the methylated reaction were 5' TTAATAGGAAGAGTGGATAGTG-3' (sense) and 5'-TCTATAAATTACTAAATCTCTTCA-3' (antisense), whereas for the unmethylated reaction the primer sequences were 5'-TTAATAGGAAGAGCGGATAGC-3' (sense) and R5'-CTATAAATTACTAAATCTCTTCG-3' (antisense).

Bisulfite modified DNA (50ng) was amplified in a total volume of 25 μl containing 1.0 mM MgCl2, 20 mM of each primer, 0.2 mM dNTPs, and 1 unit of Taq polymerase (Fermentas). The reaction was hot started at 95ºC for 3 minutes. The amplifications were carried out in a thermal cycler (Veriti- Applied Biosystems) for 35 cycles of 30 seconds at 95ºC (denaturation), 30 seconds at 56ºC (for methylated primers) and 30 seconds at 58ºC (for unmethylated primers), 30 seconds at 72ºC (extension) and a final extension of 4 minutes at 72ºC. The PCR products were eletrophoresed on a 4% agarose gel stained with ethidium bromide and visualized under UV illumination.

Statistical analysis

Statistical comparisons were performed using Fisher's exact test. A probability value (P-value) of <0.05 was taken statistically significant. All the statistical calculations were done using GraphPad Prism 5.

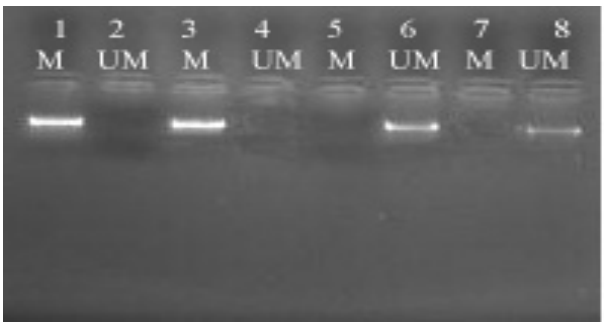

Figure 1. 4% Agarose Gel Demonstrating The Hypermethylation Of Hmlh1 Gene Lane No. 1&2: Positive Control (M & UM), Lane No. 3&4: Tumor Tissue (M & UM) Lane No. 5&6: Normal Tissue (M & UM), Lane No. 7&8: Constitutive DNA Sample (Blood) (M & UM).

Results

The study was conducted over a period of two and half years starting from August 2009 to January 2012. During this period total of confirmed 70 patients with gastric

Table 1. Methylation Status of the Hmlh1 Gene in Gastric Cancer Patients and their Paired Normal Samples

Samples	Methylation Status		P value
	Methylated n(%age)	Unmethylated n(%age)	
Normal tissue	14(20)	56(80)	
Cancer tissue	51(72.9)	19(27.1)	0.0001

*n: No of samples, P value <0.05 was considered statistically significant

Table 2. Demographic and Histopathological Charecteristics of Gastric Cancer Patients and their Promoter Hypermethylation Status of hMLH1 Gene

Variables	Methylation status of tumor		P value
	Methylated	Unmethylated	
Number of GC cases (%age)	51 (72.9)	19 (27.1)	
Gender			
Male	46 (82.1)	10 (17.9)	
Female	5 (35.7)	9 (64.3)	0.063
Dwelling			
Urban	16 (69.56)	7 (30.43)	
Rural	35 (74.46)	12 (25.53)	0.41
Family History			
Present	5 (7.14)	6 (8.57)	
Absent	46 (65.71)	13 (18.57)	0.17
Smoking status			
Smoker	43 (81.13)	10 (18.86)	
Non-smoker	8 (47.05)	9 (52.94)	0.005
Blood group			
A^+	22 (78.57)	6 (21.42)	0.188
B^+	11 (78.57)	3 (21.42)	
AB^+	(87.50)	1 (12.50)	
O^+	11 (55.00)	9 (45.00)	
Histopathology (Adenocarcinoma)			
Normal tissue	14 (20)	56 (80)	-
Well differentiated	17 (77.27)	5 (22.72)	0.0001
Moderately differentiated	11 (78.57)	3 (21.42)	0.0001
Poorly differentiated	8 (50.00)	8 (50.00)	0.0235
Signet ring cell carcinoma	3 (75)	1 (25)	0.0358
Mucinous	5 (100)	0 (0.00)	0.0007
Intestinal type	6 (85.71)	1 (14.28)	0.001

*Student's t test was used for age comparison and all other comparisons were done by Fisher's exact test.

Table 3. Dietary Habits of Gastric Cancer Patients and their hMLH1 Gene Promoter Hypermethylation Status

Dietary habits	Methylation status of tumor		P value
	Methylated	Unmethylated	
Usage of Sun dried vegetables			
Frequently[1]	36 (81.82)	8 (18.18)	0.02
Moderately[2]	13 (65)	7 (35)	
Occasionally[3]	2 (33.33)	4 (66.67)	
Consumption of fresh fruits			
<4/week	11 (100)	0 (0.00)	0.02
4-8/week	25 (75.75)	8 (24.24)	
>8/week	15 (57.69)	11 (42.30)	
Salt-tea usage			
<3 cups[#]/day	1 (16.66)	5 (83.33)	0.001
3-6 cups/day	18 (66.66)	9 (33.33)	
>6 cups/day	32 (86.48)	5 (13.51)	

[1]Once in a week during six months of winter, [2]Once in a month in winter, [3]Once or twice in a winter, [#]One cup contains 250ml of tea

Table 4. Clinocopathological Features of Gastric Cancer Patients

		No. of gastric cases	%age
Symptoms	Anemia	19	27.1
	Epigastric pain.	18	25.7
	GI Bleed	17	24.3
	Vomiting	16	22.9
	Weight loss	14	20.0
	Anorexia	0	1.0
Site of Lesion	GE Junction	5	7.1
	Body	7	10.0
	Antrum	29	41.4
	Pylorus	9	12.85
	Lesser curvature	18	25.7
	Greater curvature and Cardia	2	2.9
Type of Lesion	Polypoid	24	34.3
	Ulcerative	26	37.1
	Ulcero infiltrative	4	5.7
	Infiltrative	4	5.7
	Fingating	5	7.1
	Diffuse thickening	7	10.0

carcinoma were included in the study. The mean age of these patients was 50 years (range 32–84 years). The distinction between methylated and unmethylated status at promoter region of hMLH1 gene was done by MS-PCR technique and by DNA band pattern (Figure 1). In this study we observed that 72.9% (51/70) of gastric cancer cases had hypermethylated CpG islands in the promoter region of hMLH1 gene whereas only 20% (14/70) of the normal samples were found to have hypermethylated hMLH1 gene promoter CpGs. hMLH1 methylation was associated with gastric cancer samples compared to nonneoplastic samples (p=0.0001) (Table 1).

The demographic and histopathological characteristics of gastric cancer patients and their promoter hypermethylation status of hMLH1 gene are summarized in Table 2. Assessment of the association between the dietary habits of gastric cancer patients and the promoter hypermethylation of hMLH1 gene is given in the Table 3. Table 4 shows clinical and pathological features and hMLH1 promoter gene hypermethylation status in the studied gastric cancer patients.

Discussion

Gastric cancer which is responsible for 4th largest cancer related deaths in the world (Ferlay et al., 2010) is a highly complex disease and is yet to be understood completely. Several studies suggest that genesis of GC is the result of multistep process which involves both genetic and epigenetic changes and is favored by the individual genetic susceptibility and various environmental factors (David & Meltzer, 2010; Nagini, 2012; Pereira et al., 2012). The role of genetic instability in the development of GC is now well recognized. Although the exact cause of genetic instability is yet to be understood completely but it is known as an essential part of the initiation process leading to the development of the GC. The human MMR genes correct the errors that may have occurred during replication process or caused due to physiochemical changes. Defunct MMR genes can lead to multiple frameshift mutations in various genes (Nobili et al., 2011). Epigenetic changes involving promoter hypermethylation of the MMR genes like hMLH1 have been implicated in the development of various types of gastric cancer (Bacani et al., 2005; Nobili et al., 2011). Several other studies have demonstrated the hMLH1 promoter hypermethylation in gastric cancer (Moura et al., 2008). Studying the methylation status of the MMR genes can help in understanding the role of these epigenetic changes in the cancer development. In the present we sought to determine the methylation status of hMLH1 gene in gastric cancer patients and also any potential association of it with the dietary, clinical and pathological characteristics.

In the present study 51 patients (72.9%) showed the hypermethylation of hMLH1 gene as revealed by the MS-PCR technique. This is in accordance with the study done by Fleisher et al. (1999): (2001), Kang et al. (1999), Leung et al. (1999) and Suzuki et al. (1999) who have found hMLH1 promoter hypermethylation in 62.5-100% of sporadic gastric cancers with microsatellite instability (Fleisher et al., 1999; Kang, 1999; Leung et al., 1999; Suzuki et al., 1999). On analyzing the methylation status we found that the hMLH1 methylation was associated with the gastric cancer as compared to the paired non carcinogenic samples (p=0.0001) (Table 1). Similar results were also reported by Eleonidas et al., 2008, where a significant difference between the methylation status of gastric cancer samples and normal samples was observed (Moura et al., 2008).

Demographic and histopathological charecteristics of gastric cancer patients and their promoter hypermethylation status of hMLH1 gene are given in Table 2. We observed a higher representation of GC cases in the age group between 40 and 60. Also an age dependent trend in the methylation status of the hMLH1 gene promoter was observed in gastric cancer cases as the patient group above 60 years of age had highest number of subjects (76%) with methylated hMLH1 gene promoter as compared to the 66.66% in the age group of 20-40 years.

Gastric cancer was found to be more common in males (80%) as compared to females (20%) with Male: Female ratio of 4:1 as against the previously reported ratio of 3.3:1 (Qurieshi, Masoodi, Kadla, Ahmad, & Gangadharan,

2011). Methylation was found to be more prevent in males (82.1%) than in females (35.75%). However, the difference did not reach the statistical significance.

A high proportion (67.15%) of our subjects belonged to rural area of the Kashmir valley and a good number (34.28%) of them practiced farming. Strong et al. (1967) and Haenszel et al. (1976) also reported high incidence of stomach cancer in farmers in Costa Rica and Japan (Strong, 1967; Haenszel, 1976). Increased exposure in the fields to various pesticides in these farmers could to a possible explanation for high rate of GC in them.

Eleven (15.7%) of our GC patients had first degree family history of same or other type of cancer. The methylation status of hMLH1 promoter did not show any association with the family history. Tobacco smoking is a well known risk factor of GC ("Tobacco smoke and involuntary smoking," 2004) and the smokers have been found to be at 1.5-3.0 fold increased risk to develop GC (Fuchs & Mayer, 1995). In our study a sizeable portion 75.7% (53/70) of the GC patients were tobacco smokers, consuming it in one or the other form. We found a strong positive association between the smoking habit and the methylation status of the hMLH1 promoter. It posed a 2.5 fold risk to develop GC in patients who were involved in tobacco smoking. The increased risk could be due to the exposure of these patients to certain carcinogens like polycyclic aromatic hydrocarbons which are present in the tobacco smoke.

Among 70 GC cases, 43.1% were of A^+, 21.5% of B^+, 13.7% of AB^+ and 21.5% of O^+ blood group. The high prevalence of gastric cancer in patients with A^+ blood group suggests the role of genetic factors in the gastric cancer etiology. Similar association of blood group A and gastric cancer was reported by other studies (Cassell & Robinson, 1976). The methylation status among various blood groups did not vary statistically. Histopathological examination revealed that the well differentiated adencarcinoma was the predominant type. We observed an association between the gastric cancers irrespective of type with the higher hMLH1 methylation frequency (Table 2).

Dietary habits of gastric cancer patients and their hMLH1 gene promoter hypermethylation status are shown in the Table 3. Special food items consumed by the local population include sun dried vegetables, red chilies, dried fish etc. Almost all the patients consumed sun dried food either in lesser or greater quantity. We found an association between the consumption of sun dried vegetables and the higher hMLH1 frequency ($p<0.020$). Consumption of fresh fruits was found to be low in GC patients with only 26 cases (37.1%) taking fruits >8/week. We also observed an association between the consumption of high volume (>6 cups/day) of salt-tea and methylated promoter hMLH1 gene with the risk of GC.

Table 3 gives the clinicopathological features of the GC patients. Weight loss which is the most common sign of GC was observed in (20%) of the patients. The various symptoms present in the patients at the time of presentation include epigastric pain with post prandial fullness (25.7%), GI bleed in the form of melena and haematemisis in 24.3%, vomiting in 22.9% and dysphagia in 8% of patients.

The most common site involved was the antral part of the stomach (41.4%), followed by lesser curve (25.7%), pyloris (12.8%), body (10%), GE junction (7.1%) while 2.9% had involvement of cardia and greater curve. The most common type of the tumour was the ulcerative type which occurred in 26% of the patients, followed by the polypoid (24%) and diffuse thickening (7%).

This is the first report highlighting the genetic susceptibility due to promoter hypermethylation of hMLH1 gene and the role of various other suspected factors to which the local population is exposed and the risk to GC. The strength of this study is that it contributes significantly to the understanding of the role of epigenetic changes in the DNA repair genes in modulating the individual susceptibility to GC in the Kashmir valley. The information obtained through this study is valuable as it can help identify the high risk individuals and can also help in early diagnosis of the disease. The disease state can be improved to a certain extent by modifications in lifestyle and dietary habits, and by reducing occupational exposure towards substances known to be risk factors for the gastric carcinoma.

In conclusion, on the basis of above stated facts, we conclude that hypermethylation of hMLH1 gene play a significant role in the causation and progression of Gastric cancer. However a study on larger samples is required for further increasing the power of study that would help in devising molecular diagnostic and treatment modalities in gastric cancer patients.

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