RESEARCH COMMUNICATION

Association Between *p16*, *hMLH1* and *E-cadherin* Promoter Hypermethylation and Intake of Local Hot Salted Tea and Sun-dried Foods in Kashmiris with Gastric Tumors

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Abstract

The aim of this study was to evaluate the methylation status of three important cancer related genes viz. *p16, E-cadherin* and *hMLH1* promoters and to associate the findings with specific dietary habits in Kashmiris, a culturally distinct population in India, with gastric cancer. The study subjects were divided into three age groups viz. 0-30yrs (1st), 31-60yrs (2nd) and 61-90yrs (3rd). A highly significant association between the intake of local hot salted tea in 2nd (p=0.001) and 3rd (p=0.009) age groups was observed with the promoter hypermethylation of *E cadherin*. Again a highly significant association between the aberrant methylation of *hMLH1* (p=0.000) and *p16* (p=0.000) promoters and the intake of local hot salted tea was observed in the 2nd age group of gastric cancer patients. The intake of sun-dried food was also significantly associated with the promoter hypermethylation of *E cadherin* (p=0.003) and *p16* (p=0.015) genes in 3rd age group. The results of the present study suggest a close association between the aberrant methylation of *p16, E-cadherin* and *hMLH1* promoters and the intake of local hot salted tea and sun-dried foods in Kashmiri population.

Keywords: Gastric carcinoma - Kashmir - methylation

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Introduction

DNA hypermethylation is the most important epigenetic alteration in the malignant transformation. It includes global hypermethylation and the hypermethylation of CpG islands restricted in the regulatory regions of most human genes (Baylin et al 1999, Santos et al 2007). An aberrant promoter methylation in cancer-related genes is frequently detected in gastric tumors, signifying its involvement in the induction/promotion of gastric cancer (Suzuki et al 1999, Leung et al 1999, Leal et al 2007). Interestingly, some studies in past have indicated DNA methylation induction by N-nitroso compounds (Lawson et al 1981, Leung et al 1985, Milligan et al 1989, Cloutier et al 1999). *p16, E-cadherin* and *hMLH1* being the three widely studied genes for promoter hypermethylation in gastric carcinoma, it was predicted to analyze the association of hypermethylation status of these three genes with the dietary risk factors as suggested by previously concluded studies.

During the process of carcinogenesis, the role of several oncogenes, tumor suppressor genes and DNA repair genes has been strongly implicated. Of several tumor suppressor genes that are associated with the development of variety of human cancers, the *p16* (CDKN2a/INK4a) gene is one of the important tumor-suppressor genes and its protein is considered to be a negative regulator of G1 phase progression (Rocco et al 2001). Promoter hypermethylation, in addition to gene deletion and point mutation of *p16* locus, has been found to be one of the main mechanisms of *p16* inactivation (Cairns et al 1995, Heinzel et al 1996, El-Naggar et al 1997 Sanchez et al 2000).

An equally important gene is *E-cadherin* which is a transmembrane glycoprotein expressed on epithelial cells, and is responsible for calcium dependent homotypic cell adhesion. Cell-cell and cell-matrix interactions are crucially involved in neoplastic transformation and metastasis (Takeichi et al 1991, Pignatelli et al 1994, Hirohashi et al 1998, Wijnhoven et al 2000). The importance of *E-cadherin* in maintaining cell adhesion implies that its dysfunction may play an important part in tumorigenesis (Levenberg et al 1999) .

MutL homolog 1 also known as *MLH1/hMLH1* is a human homolog of the *E. coli* DNA mismatch repair gene *MutL*, located on Chromosome 3 of humans and identified as a locus frequently mutated in hereditary nonpolyposis colon cancer (HNPCC). A study suggests that high-

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frequency microsatellite instability (MSI) in sporadic gastric cancer is mostly due to epigenetic inactivation of *hMLH1* in association with promoter methylation, and the loss of *hMLH1* protein is a significant event in the development of invasive tumor (Leung et al 1999).

Gastric cancer is the fourth leading cancer worldwide and is endemic in certain Asian countries such as China, Japan, Iran and India. An epidemiological survey conducted on 966 gastric carcinoma patients (789 men and 177 women) between years 1986 to 1989 in Kashmir revealed incidence rates of 43.6/100,000 per annum and 9.9/100,000 per annum in men and women respectively, which is 3-6 times higher than that was recorded in other Indian cities (Khuroo et al 1992). Kashmir, harboring a unique ethnic population with special and stable dietary habits makes it an interesting field area for studying relevance of diet in human gastric carcinogenesis. Due to the temperate climatic conditions, the people of Kashmir have become habituated to the storage of food by methods like sundrying, pickling, smoking etc. However, previous studies after analysis have concluded that the preserved food in Kashmir contain low to considerable amounts of N-nitroso compounds particularly N-Nitrosodimethylamine (NDMA), *N*-nitrosoproline (NPRO), *N*-nitrosothiazolidine-4-carboxylic acid (NTCA) and *N*-nitrosopipecolic acid (NPIC) which have been simultaneously associated with esophageal carcinogenesis (Siddiqi et al 1988, Siddiqi et al 1988, Siddiqi et al 1989).

Keeping the above mentioned studies in view, the present work was aimed at studying the promoter hypermethylation in three cancer associated genes viz. *p16, E-cadherin* and *hMLH1* and their association with specific dietary variables in the gastric carcinoma patients of Kashmir valley.

Materials and Methods

Patients

A total of 130 patients diagnosed with gastric carcinoma admitted to Sheri-Kashmir Institute of Medical sciences, Srinagar and Government Medical College, Srinagar were considered for the study. Patients undergoing surgery as the primary treatment were recruited representing approximately 90% of this malady. The characteristics of the studied patients are listed in Table 1.

Epidemiological characteristics

Age grouping: Gastric carcinoma patients were divided in three different age groups viz. 0-30yrs (1st), 31-60yrs (2nd) and 61-90yrs (3rd).

Local hot salted tea: Local hot salted tea is a traditional tea of Kashmir and to analyze the association of local hot salted tea with the hypermethylation of the studied genes, the patients were divided into two groups based on the consumption of this tea per day. The individuals who consumed 1-4 cups of tea per day were considered under the 1 group and the ones who consumed >4 cups a day were clubbed under the second group.

Sundried food/Traditional pickles: Sundried food being one of the most common forms of food consumed in Kashmir owing to the temperate climatic conditions, the consumption of the same among the gastric cancer patients was surveyed and calculated as Kilogram per capita per year. The patients consuming 0-60 kg per capita per year were grouped under 'low-intake', the patients who consumed 61-130 kg per capita per year were placed in 'moderate-intake', whereas the one's consuming ≥131 kg per capita per year were placed under 'high-intake' group.

Table 1. Characteristics of Study Subjects (130)

	Characteristic	No.(%)
Gender:	Male	100 (76.9)
	Female	30 (23.1)
Age (years):	0-30	2 (1.53)
	31-60	75 (57.6)
	61-90	53 (40.7)
Local Tea:	1-4 cups	46 (35.4)
	>4 cups	84 (64.6)
Traditional Food habits		
A)Sun dried foods:	1. Low	11 (8.4)
	2. Moderate	13 (10)
	3. High	106 (81.5)
B) Traditional Pickle:	1. Low	62 (47.6)
	2. Moderate	38 (29.2)
	3. High	30 (23)

The consumption of traditional pickles was surveyed among the gastric carcinoma patients and the patients consuming 0-7 kg per capita per year were considered under 'low-intake', the patients consuming 8-14 kg per capita per year were considered under 'moderate-intake' where as the patients consuming ≥15 kg per capita per year were considered under 'high-intake'. The risk, safe and critical values were established after the consumption of all the three food items were surveyed in the healthy Kashmiri population besides the consumption survey of these food items among the patients.

Tissue collection

Tumor samples 5mm^3 were excised from resected specimens within the tumor mass, excluding the margin. Normal samples of similar dimension were taken from the resection margin, approximately 10mm from the macroscopic tumor edge and subsequently confirmed as benign by routine histopathology. A total of 130 tumor and 130 normal tissue samples were collected, and stored at -20^{0}C until analysis.

DNA extraction and bisulphite treatment

DNA was extracted from 2mm^3 tissue samples using EZ DNA extraction kit (Zymo Research). DNA concentration was measured by spectrophotometry and 2 μg of each sample was modified by Bisulphite treatment using the EZ DNA Methylation Kit (Zymo Research) as per manufacturer's instructions.

Methylation specific PCR (MSP)

Amplification of the promoter regions of the genes *p16, E-cadherin* and *hMLH1*, was carried out in eppendorf gradient minicycler in a 25μl reaction mix containing 1 μl (400ng/μl) bisulphide treated genomic DNA, hotstart Taq DNA polymerase {10X hotstart PCR buffer (200mM Tris HCl, 200mM KCl, 50 mM, $(NH_4)_2 SO_4$) supplied with 25mM $MgCl_2$, Fermentas} and Nuclease free distilled

Table 2. Methylated and Unmethylated Primer Sequences of p16, hMLH1 and E-cadherin Genes

Gene	Mgcl2 concentration (mM)	Annealing Temp (° C)	Amplicon size (bp)
p16 Flank:	2	64	193
Primers: F – 5'-AGAAAGAGGAGGGGTTGGTTGG-3'			
R – 5'-ACRCCCRCACCTCCTCTACC-3'			
p16-M:	2	70	151
Primers: F- 5'-TTATTAGAGGGTGGGGCGGATCGC-3'			
R- 5'-GACCCCGAACCGCGACCGTAA-3'			
p16-U:	1.2	64	150
Primers: F- 5'-TTATTAGAGGGTGGGGTGGATTGT-3'			
R- 5'-CAACCCCAAACCACAACCATAA-3'			
hMLH1 Flank:	2	58	182
Primers: F-5`-GGAGTGAAGGAGGTTAYGGGTAAGT-3`			
R-5`-AAAAACRATAAAACCCTATACCTAATCTATC-3`			
hMLH1-M:	2	60	124
Primers: F-5`-ACGTAGACGTTTTATTAGGGTCGC-3`			
R-5`-CCTCATCGTAACTACCCGCG-3`			
hMLH1-U:	2	60	115
Primers: F-5`-TTTTGATGTAGATGTTTTATTAGGGTTGT-3`			
R-5`-ACCACCTCATCATAACTACCCACA-3`			
E-cadherin Flank:	2	55.7	186
Primers: F-5`-GTGTTTTYGGGGTTTATTTGGTTGT-3`			
R-5`TACRACTCCAAAAACCCATAACTAACC-3`			
E-cadherin-M:	2	57	112
Primers:			
F-5'-TGTAGTTTACGTATTTATTTTTAGTGGCGTC-3`			
R-5'-CGAATACGATCGAATCGAACCG-3`			
E-cadherin-U:	2	57	120
Primers:			
F-5`TGGTTGTAGTTATGTATTTGTTTTTAGTGGTGTT-3`			
R-5'- ACACCAAATACAATCAAATCAAACCAAA-3`			

U, Umethylated DNA specific primers; M, Methylated DNA specific primers; F, Forward primer; R, Reverse primer

water. To facilitate MSP analysis, DNA was first amplified with flanking PCR primers that amplify bisulfate-modified DNA. The resulting template was used as a template for MSP reaction. The primers and annealing temperatures used is given in Table 2.

Statistical analysis

Statistical analyses were performed using the $\chi 2$ test or Fisher's exact test to assess associations between the methylation status and dietary, and characteristics. P values less than 0.05 were considered significant.

Results

Hypermethylation profile of p16, E-cadherin and hMLH1 genes

Methylation-specific PCR was done to examine the methylation status of *p16, E-cadherin* and *hMLH1*. In 83.1% (108/130) of tumors, hypermethylation of the promoter region was present in at least one gene, only twenty two cases (16.9%) showed no evidence of hypermethylation. The electrophoretic pattern of PCR products of methylated and unmethylated promoter regions of *E-cadherin, hMLH1* and *p16* is displayed in Figures 1, 2 and 3 respectively.

The frequency of methylation of promoter regions of *E-cadherin, hMLH1* and *p16* ranged between 0.4 - 0.8

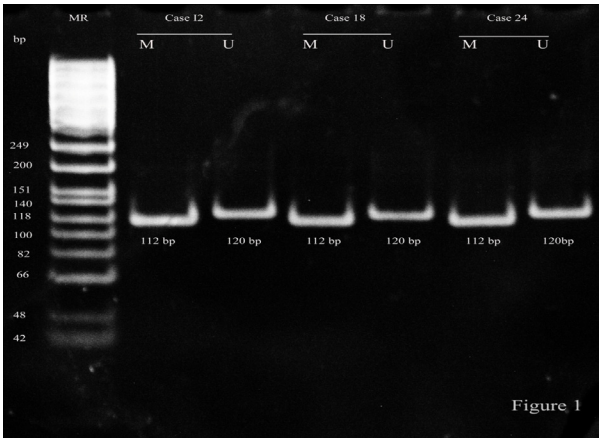

Figure 1. Methylation of *E-cadherin* in Gastric Carcinoma. MSP analysis of methylation of promoter regions of *E-cadherin* gene. After bisulfite modification each sample is amplified using primer specific for methylated (M) – 112bp and umethylated sequences (U) – 120 bp. The samples were electrophoresed on 8% Polyacrylamide Gel Electrophoresis and then stained with ethidium bromide

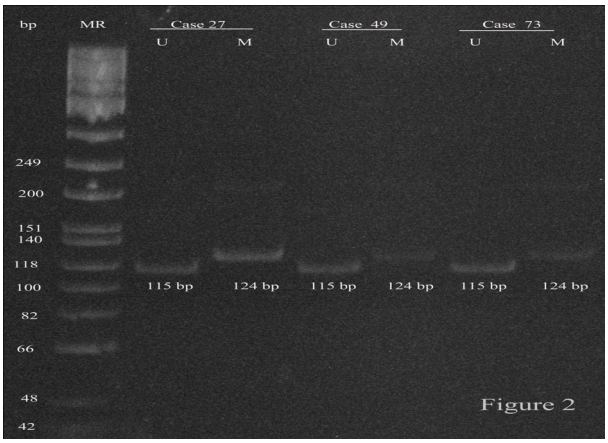

Figure 2. Methylation of *hMLH1* in Gastric Carcinoma. MSP analysis of methylation of promoter regions of *hMLH1* gene. After bisulfite modification each sample is amplified using primer specific for methylated (M) – 124 bp and umethylated sequences (U) – 115 bp. The samples were electrophoresed on 8% Polyacrylamide Gel Electrophoresis and then stained with ethidium bromide

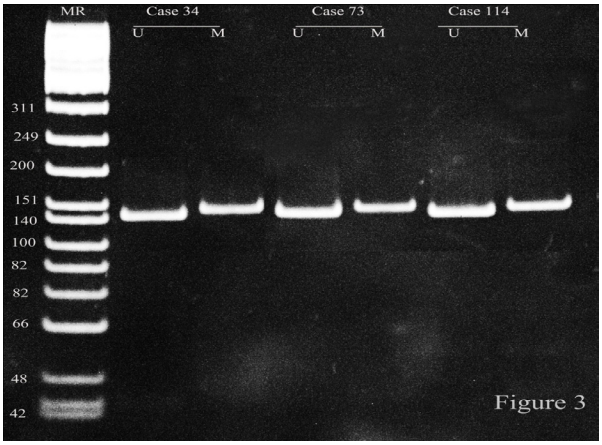

Figure 3. Methylation of *p16* in Gastric Carcinoma. MSP analysis of methylation of promoter region of 6 gene. After bisulfite modification each sample is amplified using primer specific for methylated (M) – 151 bp and umethylated sequences (U) – 150 bp. The samples were electrophoresed on 12% Polyacrylamide Gel Electrophoresis and then stained with ethidium bromide

Table 3. Frequency of Hypermethylation of *E-cadherin*, *hMLH1* and *p16* in Neoplastic and Adjacent Non-neoplastic Gastric Tissue

Genes	Neoplastic (Frequency)	Non-neoplastic (Frequency)	P- value
E-cadherin	0.58	0.38	0.001
hMLH1	0.8	0.68	0.004
P16	0.4	0.24	0.008

in neoplastic tissue and 0.24 – 0.68 in adjacent non-neoplastic tissue. In neoplastic tissues hMLH1 was the most frequently affected gene (80%; 104/130) followed by E-cadherin (58.4%; 76/130) and p16 (40%; 52/130). Methylation percentage of these genes in non-neoplastic gastric tissues was significantly lower than that observed in their adjacent tumor counterparts; *hMLH1* (68%; 82/130), *E-cadherin* (37%; 48/130) and *p16* (24%; 31/130). The methylation of all three genes was significantly associated with gastric carcinoma as displayed in Table 3. The aberrant methylation of these three genes with different age groups did not reveal any significance as shown in Table 4.

Effect of dietary habits on methylation profile

Keeping in view the specific traditional dietary trends in the people of Kashmir valley, the association between gene hypermethylation and specific dietary habits was analyzed. A very strong correlation between the consumption of local hot salted tea and the aberrant methylation was observed in case of *E-cadherin* and *p16* genes. This tea consumption displayed a highly significant association with promoter hypermethylation of E-cadherin gene in the 2nd (p=0.001) and 3rd (p=0.009) age groups while as a very strong correlation was observed between the consumption of this tea and aberrant methylation of p16 (p=0.000) and *hMLH1* (p=0.000) promoters in 2nd age group of gastric carcinoma patients (Table 5).

Consumption of sundried food, commonly consumed as traditional food, displayed a significant association with promoter hypermethylation of *E cadherin* (p=0.003) and *p16* (p=0.015) in 3rd age group of patients. The intake of traditional pickles of Kashmir in the gastric carcinoma patients was also evaluated for their association with the aberrant methylation of *E-cadherin, hMLHI* and *p16* genes, however, no significant association between them was observed in any of the age groups.

Table 4. Methylation of the Genes in Different Age Groups of Gastric Carcinoma Patients

	E-cadherin			*hMLH1*			*p16*		
Age group	M	U	P Value	M	U	P Value	M	U	P Value
0-30 (2)	1	1	N.S	1	1	N.S	0	2	N.S
31-60 (75)	42	33		57	18		33	42	
61-90 (53)	33	20		46	7		19	34	

* Since quiet less patients fall in the Ist age group, so it has not been included in the statistical analysis.

Discussion

A better comprehension of the causality of gastric carcinoma can be established by combining epidemiological data with the molecular endpoints. Here, we have examined whether dietary intake of specific traditional Kashmiri food is associated with the hypermethylation of the cancer related genes in surgically excised gastric tumor and adjacent non-tumor samples. Nested MSP analysis was optimized and hypermethylation in the promoter regions of *p16, E-cadherin* and *hMLH1* was analyzed.

Studies on promoter hypermethylation of *p16* gene in gastric carcinoma have reported 26% (Huang et al., 2004), 26.5% (Ksiaa et al., 2009) and 42.2% (Kang et al., 2001) promoter hypermethylation which is nearly in agreement with the hypermethylation percentage of 40 found in the present study. Previously promoter methylation of *E-cadherin* gene in gastric mucosa was associated with *Helicobacter pylori* infection and gastric cancer where 31% of dyspeptic patients having gastric adenocarcinoma possessed promoter hypermethylation in *E-cadherin* gene (Chan et al 2002). In an another study, hypermethylation of the *E-cadherin* promoter was evident in 51% of primary gastric carcinomas examined by MSP–SSCP (Tamura et al 2000) which is comparable with the present work wherein promoter hypermethylation percentage of *E-cadherin* was 58.4. Several studies conducted independently on promoter hypermethylation of *hMLH1* gene in gastric carcinoma have revealed percentages of 71.4 in MSI positive tumors and 29.8 MSI negative tumors (Nan et

Table 5. Association of Gene Methylation with Dietary Variables of Gastric Carcinoma Patients

Variable	*E-cadherin*									*hMLH1*									*p16*								
	0-30 (2)			31-60 (75)			61-90 (53)			0-30 (2)			31-60 (75)			61-90 (53)			0-30 (2)			31-60 (75)			61-90 (53)		
	M	U	P	M	U	P	M	U	P	M	U	P	M	U	P	M	U	P	M	U	P	M	U	P	M	U	P
Local tea																											
>4 cups(84)	0	0	N.S	35	16	0.001	25	8	0.009	0	0	N.S	48	3	0	29	4	N.S	0	0	N.S	30	21	0	15	18	N.S
1-4 cups(46)	1	1		7	17		8	12		1	1		9	15		17	3		0	2		3	21		4	16	
Sun dried foods																											
High (106)	0	1	N.S	36	27	N.S	31	11	0.003	0	1	N.S	49	14	N.S	41	1	N.S	0	1	N.S	29	34	N.S	11	31	0.015
Moderate (13)	1	0		3	4		1	4		1	0		5	2		3	2		0	1		2	5		4	1	
Low (11)	0	0		3	2		1	5		0	0		3	2		2	4		0	0		2	3		4	2	
Traditional pickle																											
High (30)	0	0	N.S	12	7	N.S	8	3	N.S	0	0	N.S	12	7	N.S	10	1	N.S	0	0	N.S	9	10	N.S	3	8	N.S
Moderate (38)	1	1		11	7		7	11		1	1		13	5		16	2		0	2		8	10		8	10	
Low (62)	0	0		19	19		18	6		0	0		32	6		20	4		0	0		16	22		8	16	

al., 2000) , 100 in high frequency MSI tumors (MSI-H) (Leung et al., 1999 , Sakata et al., 2002, 37 in MSI-H (Carvalho et al., 2003) and 20.3 in low frequency MSI tumors (MSI-L) (Ksiaa et al., 2009), 77.8 of MSI-H and 75 of MSI-L (Fleisher et al., 1999) which is in agreement to the *hMLH1* hypermethylation percentage seen in our study.

The consumption of hot salted tea has always been suspected to be a risk factor for gastro intestinal tumors in Kashmir. The use of sodium bicarbonate at the time of boiling the tea leaves and the addition of common salt to the prepared tea could be a major cause of thermal injury to oesophageal epithelium and common salt alone is a well known irritant of gastric epithelium and has been considered a risk factor for highly prevalent gastric cancer in Kashmir (Khuroo et al., 1992). Interestingly, a study on esophageal carcinoma patients from Kashmir demonstrated that consumption of hot salted tea (with sodium bicarbonate) is significantly associated ($p = 0.002$) with the hypermethylation of *p16* gene (Salam et al., 2009) which is in agreement with the present study where in the aberrant methylation of *p16* promoter in gastric carcinoma patients showed a highly significant association with the intake of hot salted tea in 2nd (p=0.000) age group. Interestingly, highly significant association was observed between E-cadherin in 2nd (p=0.001) and 3rd (p=0.009) age groups and *hMLH1* (p=0.000) in 2nd age group with salted tea consumption. Considering the display of significant association between hot salted tea and cancer related gene hypermethylation in 2nd and 3rd age groups, it becomes very logical to interpret that consumption of this tea may be playing a role in induction of gene methylation and thereby leading to silencing of some important tumor related genes which might be one of the important mechanisms leading to gastric cancer.

The consumption of sundried food was found to display a significant association with aberrant methylation of *E cadherin* (p=0.003) and *p16* (P=0.015) gene in 3rd age group which suggests that prolonged intake of sundried food either might induce the aberrant methylation in *E cadherin* and *p16* gene promoters. It indicates that the sole intake of these foods alone may play a role in development of gastric carcinoma in Kashmiri people. Earlier epidemiological studies on esophageal and gastric carcinoma in Kashmir suggest that the special dietary habits of Kashmiri population could be the most likely etiological factors for the development of stomach cancer (Khuroo et al., 1992; Malik et al., 2000). A study reported that high consumption of pickled foods was associated with a significant increased risk of stomach cancer (Ngoan et al., 2002). However, in the present study, traditional pickle consumption did not show any significant association with any of the gene methylations in any age group of patients.

The differential gene methylation in tumoral tissues aids us to conclude that aberrant gene methylation is associated with gastric carcinogenesis. Aberrant methylation of *p16, E-cadherin* and *hMLH1* promoters could be induced by special dietary variables consumed in Kashmir. Thus promoter hypermethylation of these genes may be the possible epigenetic modification induced by these special dietary features leading to the highly prevalent gastric carcinoma in the valley.

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