

# Diminished expression of *MGMT* & *RASSF1A* genes in gastric cancer in ethnic population of Kashmir

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**Background:** Cancer initiation and progression are accompanied by profound changes in DNA. DNA methylation that was the first epigenetic alterations identified in cancer. DNA hypermethylation at promoter sites is closely associated with down regulation of protein and as major participant in the development and progression of series of human tumors. Therefore we hypothesized that promoter hypermethylation of *RASSF1A* & *MGMT* gene could influence susceptibility to gastric cancer (GC) as well, and we conducted this study to test the hypothesis in Kashmiri population.

**Methods:** A hospital based case-control study; including 200 GC cases and 200 matched controls from patients who went surgical resection. Promoter hypermethylation was determined by Methylation Specific Polymerase chain reaction. The expression of *MGMT* & *RASSF1A* protein was examined by Western blotting technique.

**Results:** Frequency of promoter region hypermethylation of *MGMT* gene were 46.5% in cases and 5.5% in controls ( $P < 0.05$ ) while as in case of *RASSF1A* frequency was 44% in cases and 4.5% in controls ( $P < 0.05$ ). Further, frequency of hypermethylation of both genes was found predominant in males, aged and advanced pathological stage subjects. Loss of *MGMT* expression was found in 46.5% cases ( $P < 0.05$ ) while as loss of *RASSF1A* expression was found in 40.5% cases ( $P < 0.05$ ). In both genes a positive correlation was observed between promoter CpG island hypermethylation and down regulation of respective proteins.

**Conclusions:** These findings indicate that promoter hypermethylation at CpG island may be responsible for reduction of expression at protein level which may be an initial event in carcinogenesis and the progression of GC.

**Keywords:** Methylation specific PCR; epigenetics, hypermethylation; CpG island; gastric cancer (GC)

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## Introduction

Gastric cancer (GC) is the fifth most common cancer in the world and is the second and third-most common cancer among males and females in Asia and worldwide (1-3), where as in Kashmir valley this cancer is 3rd in males and sixth in females (4). Carcinogenesis is a multistep process in which genetic and epigenetic alterations accumulate, a major epigenetic modification that is strongly involved in the control of gene expression and is an early landmark event in carcinogenesis is promoter region CpG island hypermethylation (5). Understanding mechanism of epigenetics holds great promise for cancer prevention, detection and therapy (6). CpG island hypermethylation at promoter site has been studied in various malignancies for several genes, and the spectrum of genes involved suggests that specific tumors may have their own distinct pattern of methylation (7). O6-methylguanine-DNA methyltransferase (*MGMT*) is a ubiquitous DNA repair protein that removes mutagenic and cytotoxic adducts from the O6-guanine in DNA, the preferred point of attack of many carcinogens and alkylating chemotherapeutic agents (8,9). Alkylating of DNA at the O6 position of guanine is an important step in the formation of mutations in cancer, primarily due to the tendency of the O6-methylguanine to pair with thymine during replication, resulting in a conversion of guanine-cytosine to adenine-thymine pairs in DNA (10). Furthermore, the O6 alkyl guanine-DNA adduct may cross-link with the opposite cytosine residues, blocking DNA replication (11-13) while as *RASSF1A* is a putative tumor suppressor gene located on 3p21 and functions in cell cycle control, microtubule stabilization, cellular adhesion, motility and apoptosis (14). Promoter region CpG island methylation, which is an epigenetic change, is the predominant mechanism of *RASSF1A* gene inactivation and has been recognized in many human solid tumors (15-21), thus, *RASSF1A* promoter methylation may be a prognostic indicator in such tumors. Several studies have shown the presence of promoter region CpG island hypermethylation and loss of protein expression (22-25), but to our best knowledge, no data concerning the expression of *MGMT* and *RASSF1A* gene and their association with promoter region CpG island hypermethylation status in GC in Kashmiri population are available. In the present study, we have studied promoter region CpG island hypermethylation of *MGMT* & *RASSF1A* and their association with expression at protein level in Kashmiri population with GC.

## Methods

### *Study subjects*

This hospital based case-control study includes 200 GC patients consisted of 117 males and 83 females from 20 to 80 years of age group. Cases and matched control tissue sample from these patients were collected from the operation theatre of Department of Surgery, Government SMHS Hospital Srinagar. Written informed consent was obtained from each participant and was carried out in accordance with the principles of the Helsinki Declaration of the world medical association. There were no age, gender, or stage restrictions, but patients with other malignant disease and not belonging to Kashmir were excluded. Only histopathologically confirmed cases and matched controls were included in study.

### *Molecular analysis*

Genomic DNA from Cases and controls were extracted by kit based method (Quick- g DNA<sup>TM</sup> MiniPrep supplied by ZYMO RESEARCH) in Molecular Biology Lab. of Department of Biochemistry, Government Medical College Srinagar (26).

### **Bisulphite modification and methylation-specific polymerase chain reaction (MS-PCR)**

DNA methylation patterns in the CpG islands of promoter region of genes were determined by chemical treatment with sodium bisulfate and subsequent MSPCR (27). The Bisulphite modification was done by an EZ DNA Methylation-Direct<sup>TM</sup> Kit supplied by ZYMO Research (28). The primers used were listed in the literature (11,29,30). For MS-PCR, the total reaction volume was 25  $\mu$ L containing 2.5  $\mu$ L of 1  $\times$  *Taq* buffer, 1.5  $\mu$ L dNTPs (1.25 mM/L), 2  $\mu$ L of each forward and reverse primer (150 ng/reaction), 1.25  $\mu$ L modified DNA (50 ng/reaction), 1  $\mu$ L *Taq* DNA Polymerase (1 U/ $\mu$ L) and 14.75  $\mu$ L De ionized water. PCR amplification was achieved using a Thermal cycler (*Gradient thermal cycler from Eppendorf Master cycler pro*) and reactions were hot-started at 95  $^{\circ}$ C for 10 min, followed by addition of *Taq* Polymerase, followed by 35 cycles of melting (95  $^{\circ}$ C for 45 s), annealing (59  $^{\circ}$ C for 45 s) and extension (72  $^{\circ}$ C for 45 s) and by final extension step at 72  $^{\circ}$ C for 4 min for *MGMT* gene and for *RASSF1A* gene the PCR conditions consisted of one incubation of 15 min at 95  $^{\circ}$ C, followed

by 40 cycles of a 30 s denaturation at 94 °C, 50 s at an annealing temperature (64 °C for methylated and 59 °C for unmethylated specific primers), and a 30 s extension at 72 °C, and a final extension at 72 °C for 10 min. DNA from normal lymphocytes used as negative Control while as methylated DNA as positive Control (ZYMO RESEARCH). 10 µL of each MS-PCR product was directly loaded on 5% agarose and visualized under UV illumination.

### **Western blotting**

Fifty mg of GC tissue mixed with 500 µL of Trypsin-EDTA (1×), heated for 5–10 min at 37 °C, followed by centrifugation for 1 min and supernatant was discarded. Addition of 250 µL of ice-cold lysis buffer and protease inhibitors to pellet again incubated on ice for 45 min with vortexing after every 10 min. Again Centrifugation and Supernatant containing the protein was collected. Protein concentration was then measured using Nano-drop spectrophotometer and normalization was carried out with appropriate dilution to make the concentration of the proteins equal in all cases (100 µg) and was subject to SDS-polyacrylamide gel electrophoresis (12%) and transferred to polyvinylidene difluoride (PVDF) filter membranes for 30 min by blotting. The PVDF membrane was incubated for 1.5 h in blocking solution at room temperature, followed by overnight incubation at room temperature with primary antibody to *MGMT* and *RASSF1A* 1:3,000 (mouse polyclonal antibody to *MGMT* and *RASSF1A* (NP\_002403.1 and NP\_009113.3 Sigma Aldrich, Inc. Spruce Street, Saint Louis, USA). The excess antibody was removed, followed by 2 h incubation with anti-mouse secondary antibody (LI-COR, USA) with dilution factor 1:30,000. The PVDF membranes were washed again and the immunoreactive bands were detected by enhanced chemiluminescence and visualized by image analysis system (Bio-Rad Laboratories, Inc. Hercules, CA, USA).  $\beta$ -actin (Abcam Biotechnology, Cambridge, UK) was used as loading control.

### **Data analysis and statistics**

The  $\chi^2$ -test with Odds ratio was used to examine the differences in the distribution of hypermethylation in promoter regions of both the genes between cases and controls and Fishers exact test was used for studying the male and female groups using Graph Pad Prism Software Version 6.0 by Graph Pad Software 2236, Avenida de la Playa, La Jolla, CA 92037, USA.

## **Results**

### ***Relationship between hypermethylation and GC***

In GC, promoter region CpG island hypermethylation of *MGMT* gene was detected in 46.5% cases and 5.5% controls while as in *RASSF1A* gene 44% in cases and 4.5% in controls. A positive correlation between promoter region CpG island hypermethylation and GC was found ( $P < 0.0001$ ) suggesting that promoter region CpG island hypermethylation may play an important role in GC.

### ***Relationship between hypermethylation and clinicopathological parameters***

The clinicopathological data were tested for correlation with the methylation results in both genes but no significant correlation were observed between the presentation of promoter region CpG island hypermethylation in GC and patient age, gender, smoking, residence but a positive correlation was seen between CpG island hypermethylation and pathological stage in both genes (*Table 1, Figures 1-3*).

### ***Relationship between hypermethylation and protein expression in GC***

*MGMT* protein expression was found in 73% (79 of 107) non-hypermethylated cases and only 30% (28 of 93) in hypermethylated cases while as *RASSF1A* protein expression was 74% (83 of 112) in non-hypermethylated and only 36% (41 of 88) in hypermethylated cases. Statistically association of promoter region CpG island hypermethylation of *MGMT* & *RASSF1A* and down regulation of respective proteins in GC was found significant ( $P < 0.05$ ) (*Figures 4, 5*).

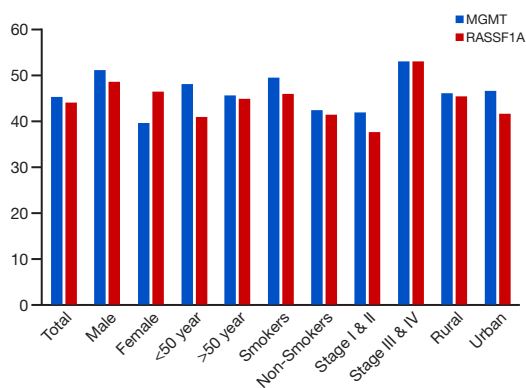
## **Discussion**

In spite of the gradually declining incidence of GC worldwide in recent years, it still remains the fourth most common cancer and the second leading cause of cancer related deaths worldwide (31). This Cancer shows a male preponderance in almost all countries, with rates two to four times higher among males than females (32) which are in concordance with our study in which males are almost double than females. *MGMT* & *RASSF1A* gene, serving as molecular switches in pivotal processes governing cellular growth and differentiation. Several molecular alterations in these genes have been identified such as minisatellites, mutation and SNP, however epigenetic alterations

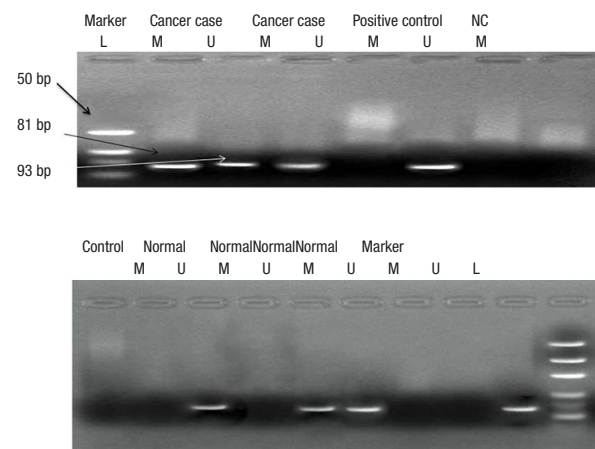
**Table 1** Association of *MGMT* & *RASSF1A* gene hypermethylation with clinicopathological parameters of GC

Parameter	N	<i>MGMT</i> hypermethylation	P value (association)	<i>RASSF1A</i> hypermethylation	P value (association)
Subjects			<0.0001 (significant)		<0.0001 (significant)
Case	200	93		88	
Control	200	11		9	
Gender			0.115 (insignificant)		0.115 (insignificant)
Male	117	60		57	
Female	83	33		31	
Age			0.878 (insignificant)		0.636 (insignificant)
<50 years	56	27		23	
>50 years	144	66		65	
Habit			0.392 (Insignificant)		0.397 (insignificant)
Smokers	111	55		51	
Nonsmokers	89	38		37	
Residence			1.000 (insignificant)		0.663 (insignificant)
Rural	121	56		55	
Urban	79	37		33	
Stage			0.149 (insignificant)		0.042 (Significant)
I & II	119	50		45	
III & IV	81	43		43	

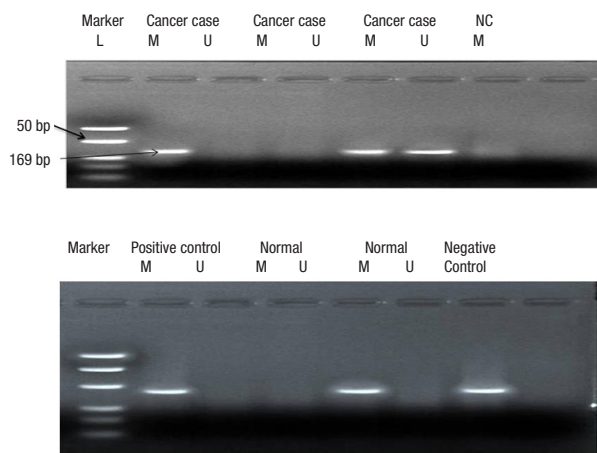
GC, gastric cancer.

**Figure 1** Methylation of *MGMT* & *RASSF1A* gene with clinicopathological parameters in gastric cancer.

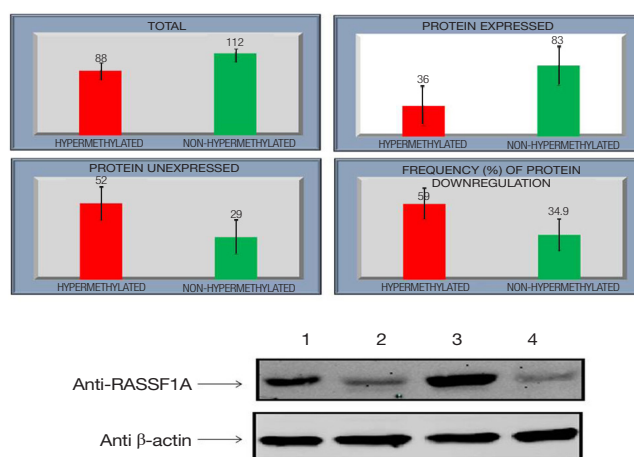
have been acknowledged as an important mechanism contributing to early carcinogenesis. These genes are probably inactivated by mechanisms of methylation. Since the research on epigenetic alteration (hypermethylation) in the above mentioned genes is rare, in the present study we provided evidence that *MGMT* & *RASSF1A* expression in GC was reduced at protein level and the methylation

**Figure 2** Representative gel picture of amplified products of *MGMT* gene in Gastric cancer cases & controls. (A) MSPCR of gastric cancer cases; (B) MSPCR of gastric controls. L, 50 bp ladder, M, methylated 81 bp; U, unmethylated 93 bp.

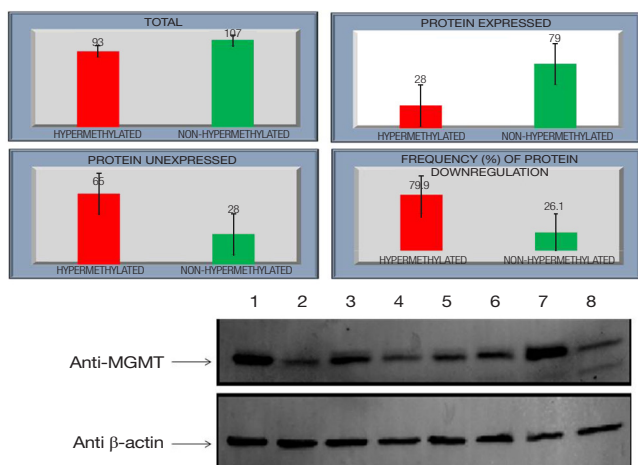
in promoter region of the above genes was 3–5-fold more frequent in primary GC than in corresponding normal tissues. The frequency of hypermethylation of both genes showed male predominance in GC which is in concordance



**Figure 3** Representative gel picture of amplified products of RASSF1A gene in gastric cancer cases & controls. (A) gel picture of gastric cancer cases; (B) gel picture of gastric normal. L, 50 bp ladder; M, methylated; U, unmethylated 69 bp.



**Figure 5** Histogram and representative Western blot of RASSF1A protein in hypermethylated & non-hypermethylated GC cases. L 1 & 3 represents protein expression; L 2 & 4 represents down regulation of protein; lower gel panel represents loading control β-actin. GC, gastric cancer. L, 50 bp ladder.



**Figure 4** Histogram and Representative western blot of MGMT protein in Hypermethylated & non hypermethylated GC Cases. L 1, 3, 5, 6 & 7, from left represents protein expression; L 2, 4 & 8, represents down regulation of protein. Lower gel panel represents loading control β-actin. L, 50 bp ladder.

with other Study (33). It has been documented that the incidence of GC is known to increase with age (34) which is in concordance with our study where we found frequency of hypermethylation of both genes was high in older aged patients but Statistically association of hypermethylation with age in GC was insignificant in both genes ( $P > 0.05$ ). The study conducted by different authors (35,36) have shown association between the cigarette smoking and GC

risk while as in our study frequency of hypermethylation was found high in smokers with GC of both genes ( $P > 0.05$ ). On the basis of pathological stage of GC, frequency of promoter region hypermethylation of both genes was found high in advanced stage (III & IV) compared to early stage. These results are also in concordance with the Study conducted by other authors (37,38). *MGMT* & *RASSF1A* protein was 70% and 60% down regulated in hypermethylated GC cases while as 26% each in non-hypermethylated GC cases ( $P < 0.05$ ). Our study showed that in the corresponding normal tissues, the methylation was seen which may be due to the difference of the samples which we studied were chronic gastritis tissues, especially intestinal metaplasia tissues in corresponding adjacent normal tissues. The study conducted by To *et al.* (25) reported that several genes were frequently methylated in chronic gastritis, especially in intestinal metaplasia tissues. In conclusion, Assessment of both these genes methylation status is clinically useful for identifying GC patients with a poor prognosis. In future, molecular therapies that mediate demethylation of these genes may improve patient prognosis. Less than 25% of GC cases are diagnosed at an early stage, and the 5-year survival rate is approximately 20–25% worldwide. However, the survival rate improves to over 60% if the disease is detected at an early stage, emphasizing the importance of making an early diagnosis of GC.

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## Footnote

*Conflicts of Interest:* The authors have no conflicts of interest to declare.

*Ethical Statement:* The study was in accordance with the principles of the Helsinki Declaration of the world medical association and written informed consent was obtained from all patients.

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