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Original Research

Epigenetic promoter methylation of hmlh1 gene in human gut malignancies: A comparative study

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Abstract

Gastrointestinal malignancies (GIT) are a major health hazard globally. The incidence of upper GIT malignancies varies widely based on geographic location, race, and feeding habits etc. GI cancers are most prevalent in Jammu and Kashmir region of India and have multi-factorial etiology involving dietary habits, genetic factors, and gene environmental interactions. Cancer development and progression is dictated by series of alterations in genes such as tumour suppressor genes, DNA repair genes, oncogenes and others. Inactivation of the hmlh1 gene expression by aberrant promoter hypermethylation plays an important role in the progression of various cancers. The present study was a hospital based comparative case-control study and the aim was to analyse the promoter hypermethylation of CpG islands of hmlh1 gene in G cancer patients of ethnic Kashmiri origin. Methylation-specific polymerase chain reaction (MS-PCR) was used for the analysis of the promoter methylation status of hmlh1 gene. DNA was extracted from all the samples and was modified using bisulphite modification kit. The epigenetic analysis revealed that frequency of Promoter region hypermethylation of hmlh1 gene among gastrointestinal malignancies was found to be higher in colorectal cancer (60%) followed by oesophageal (50%) and then gastric cancer (46%) and statically the association of promoter region hypermethylation with GIT malignancies was found to be significant ($p<0.05$). The promoter hypermethylation of the hmlh1 gene was found to be more prevalent in respective male cases of all the three GIT malignancies in a statistically significant manner ($p<0.05$).

INTRODUCTION

GIT malignancies account for about 20% of all cancers worldwide with gastric and oesophageal cancer topping the list [1]. Kashmir has higher incidence of GI cancers owing to peculiar feeding habits and life style. Colorectal cancer in Kashmir valley is the third most common GIT cancer after oesophageal and gastric cancer [2]. The annual incidence of oesophageal cancer in Kashmir is reported as 42 and 27 for men and women, respectively per 100,000 individuals [3]. The development of GIT malignancy is a result of multiple independent processes that add up to give a malignant phenotype and include mutations in various different proto-oncogenes, tumour suppressor genes, and epigenetic changes in DNA [4, 5]. In neoplasms, including GIT cancers, epigenetic changes play a key role in the process of tumorogenesis [6, 7] . Epigenetic changes mean the changes in the DNA that modify gene expression without changing its sequence. More specifically, epigenetics is the study of collective interaction of multiple mechanisms in establishing states of chromatin structure, histone modification, transcriptional activity, and DNA methylation. Evidence accumulated in recent years clearly

demonstrates that epigenetic abnormalities are important factors in the etiology of virtually all human cancer types [8-10]. DNA methylation is the most widely studied epigenetic abnormality in tumorogenesis. It refers to methylation of cytosine at CpG dinucleotides. CpG dinucleotides are not randomly distributed throughout the human genome. CpG-rich regions, known as CpG islands, are usually unmethylated in normal cells (with the exception of imprinted genes and several genes located on chromosome X) and are typically found associated with 5' promoter end and first exon of numerous genes [11]. It is estimated that there are about 29000 CpG islands within the human genome. Approximately 50–60% of all genes contain CpG islands. Alterations of cytosine methylation are prevalent in human sporadic cancers [12, 13]. Methylation defects include genome hypomethylation (resulting in epigenetic activation of oncogenes and retro elements) and localized aberrant hypermethylation of CpG islands, resulting in transcriptional repression of many important genes [14]. Promoter hypermethylation patterns in human cancer show strong specificity with respect to the tissue of origin, and can be found early in tumorogenesis. Tumour suppressor genes that undergo aberrant methylation in multiple tumour types may virtually belong to all cellular pathways, thus aberrant promoter hypermethylation has relevant consequences for carcinogenesis. Moreover, the methylation status of an individual gene promoter may be used for assessing the prediction, general prognosis, and response to therapy, underlying the importance of studies on specific patterns of promoter hypermethylation in tumours [15]. In GI cancers, epigenetic analysis has revealed a number of genes and pathways inactivated by methylation-associated silencing [16, 17]. Human MutL homologue or *hmlh1* is a member of the mismatch repair system whose function is to replicate the genome faithfully [18]. Methylation changes in mismatch repair (MMR) genes affect their function and result in accumulation of damage leading to genomic instability [19, 20]. The MMR system maintains genomic integrity by correcting replicative errors. Generally, it is accepted that defects in MMR genes are responsible for the microsatellite instability (MSI) observed in different diseases including cancer. Point mutations within the MMR genes seem to be infrequent; however, promoter hypermethylation has been suggested as the main cause of MMR gene silencing [21, 22]. Deficiencies in mismatch repair (MMR) system result in mutation rates about 100 fold greater than observed in normal cells [23, 24]. Methylation of the hmlh1 gene has been correlated with the loss of protein expression in many cancers of humans [25].

This study deals with GIT malignancies predominantly in rural and socioeconomically disadvantaged population of all regions of Kashmir and focuses on the analysis of aberrant promoter hypermethylation of CpG islands of *hmlh1* gene in GI cancers, with special emphasis on similarities and differences of promoter hypermethylation of this mismatch repair gene with respect to the tissue of origin. There are various controversial reports of its association with gastrointestinal cancers [26, 27]. The study also emphasized recent advances in promoter hypermethylation profiling in regard to prediction and general prognosis in GI cancers. The Study was a case control comparative study in which a candidate gene approach was used to study a key cancer gene (*hmlh1*) undergoing epigenetic change in gastrointestinal cancers. The Study was undertaken to understand the etiology of gastrointestinal cancers in the population of Kashmiri origin. Also, association of promoter hypermethylation of *hmlh1* gene with gastrointestinal cancers and its relation to clinico-pathological parameters like age and gender was evaluated.

MATERIALS AND METHODS

Collection of tissue samples: The study included 300 surgically/endoscopically obtained gastrointestinal (Gastric, Colorectal and Oesophageal) samples among which 180 were cases (60 from each group) and 120 (40 from each group) were normal gastrointestinal samples. The carcinoma and control samples were obtained in sterilized plastic vials (50 ml volume) containing 10 ml of normal saline from the Department of Surgery and Endoscopic Section of Shri Maharaja Hari Singh (S.M.H.S) hospital, an associated hospital of Government Medical College Srinagar and were stored at -80^{o}C. A part of each sample was sent to histopathology laboratory of S.M.H.S hospital for histopathological confirmation. The information regarding the age and gender for each sample was collected from the histopathological reports and is shown in Table 1.

Extraction and Modification (Bisulfite treatment) of genomic DNA: For the isolation of genomic DNA, kit based method was used. The kit used was Quick- g DNA^{TM} MiniPrep supplied by ZYMO RESEARCH. The DNA eluted was stored at 4^{0}C for a short time and at -20^{o}C for longer duration storage. The integrity of the genomic DNA was examined using 1 % agarose gel. The quantity and quality of the isolated DNA was determined by measuring optical density at 260 nm and 280 nm by double beam spectrophotometer (Evolution 60S from Thermo Scientific). DNA was modified by kit based method and the kit used was EZ DNA Methylation^{TM} Kit supplied by ZYMO RESEARCH. DNA modification (i.e. sodium bisulfite treatment) converted unmethylated cytosines to Uracil and hence

enabled to distinguish between the methylated and non-methylated cytosine residues. The modified DNA was then stored at -20^{0}C for further analysis.

Methyl specific polymerase chain reaction (MSP): The methylation status of the *hmlh1* gene was analysed by using methylation-specific PCR (MSP) approach [28]. The bisulfite-treated DNA was used as a template for PCR. Two sets of primers described previously (39-42) one specific for methylated and the other specific for unmethylated version of the gene were used. All unmethylated cytosines in the unmethylated product were converted to thymines after bisulfite treatment and PCR amplification, suggesting that the *hmlh1* gene is unmethylated. However, the cytosines in the CpG dinucleotides of methylated product remained unchanged, as methylated cytosines are resistant to bisulfite treatment, which indicated that the CpG islands of the gene are methylated. The primers used for amplification along with PCR-annealing temperatures, PCR product sizes are shown in Table 2.

For MSP, the total reaction volume was 25 µl containing 50–100 ng of bisulfite modified DNA, 20 pmol of each primer, 25 mM dNTPs, 1 U Taq Polymerase, 2.5 µl 1X PCR buffer (Bangalore Genei, Bangalore). PCR reactions were started by denaturation at 95^{0}C for 5 min, followed by 30 cycles of 94^{0}C for 30s, 60^{0}C (for unmethylated *hmlh1)* and 58^{0}C (for methylated *hmlh1)* for 30s and 72^{0}C for 30s with a final extension at 72^{0}C for 4 min. DNA from normal lymphocytes was used as negative control for methylated alleles of *hmlh1*, and placental DNA treated in vitro with SssI methyltransferase (New England Biolabs, Beverly, MA, USA) was used as positive control for methylated alleles of *hmlh1*. 15 µl of PCR products were analyzed by electrophoresis on a 3% agarose gel and visualized by ethidium bromide staining.

Statistical analysis: The χ^2-test with Odds ratio was used to examine the differences in the distribution of *hmlh1* gene promoter hypermethylation and non hypermethylation between cases and controls of three different GIT malignancies and Fishers exact test was used in case of studying the male and female groups. Odds ratios with 95% CIs were computed using unconditional logistic regression using Graph Pad Prism Software Version 5.0 by Graph Pad Software 2236, Avenida de la Playa, La Jolla, CA 92037, USA.

RESULTS

Analysis of promoter hypermethylation of *hmlh1* gene was carried out in 180 invasive primary gastrointestinal cancer cases and 120 normal samples. Among the cases, more than half were amplified with methylated primer thus were methylated. However, few cases were amplified with both methylated and unmethylated primers which can be probably explained by the presence of infiltrating lymphocytes and/or non-malignant epithelial cells in the primary tumours or by the presence of hemi-methylated DNA. 52.2% of the gastrointestinal cancer cases showed bands in methylated (M) wells which confirmed that promoter region of mismatch repair gene (*hmlh1)* in these cases was hypermethylated. Fig. 1 shows MS-PCR results of few representative GIT cancer cases. However in 47.8% of the gastrointestinal cancer cases, bands were visible in unmethylated wells which confirmed that promoter region of *hmlh1* gene in these cases was not methylated. Among histopathologically confirmed gastrointestinal normal samples, 85.8% of the samples were found to be unmethylated and 14.2% were found to be methylated. Fig. 2 shows MS-PCR results of few representative GIT normal samples. Statistically the association of promoter hypermethylation of mismatch repair gene *hmlh1* with gastrointestinal cancer was evaluated by χ^2-test and was found to be significant (p=0.0001). Frequency of Promoter hypermethylation of *hmlh1* gene in gastrointestinal cancers was found to be higher in colorectal (60%) followed by oesophageal (50%) and then gastric (46%) cancer (Table 3 and Fig. 3). Also, the frequency of promoter hypermethylation of *hmlh1* gene was found to be higher in respective male cases of all GIT malignancies and its association with respective GIT malignancies was found to be significant (Table 4 and Fig. 4). However, among females the frequency of promoter hypermethylation was found to be lower and the association of promoter hypermethylation with respective GIT malignancies was found to be statistically insignificant (Table 5).

Table 1. Number of Cases and Controls in Gastrointestinal Cancers

Type of Cancer	Case			Control		
	Male	Female	Total	Male	Female	Total
Gastric	35	25	60	25	15	40
Esophageal	43	17	60	27	13	40
Colorectal	40	20	60	25	15	40

Table 2. Shows primers used along with their annealing temperatures and product sizes

Nature of Primer		Sequence (5□-3□)	*Annealing Temp*(◦C)	Size (bp)
Unmethylated	F	5'-TTTTGATGTAGATGTTTTATTAGGGTTGT-3'	60	115
	R	5'-ACCACCTCATCATAACTACCCACA-3'		
Methylated	F	5'-ACGTAGACGTTTTATTAGGGTCGC-3'	58	110
	R	5'-CCTCATCGTAACTACCCGCG-3'		

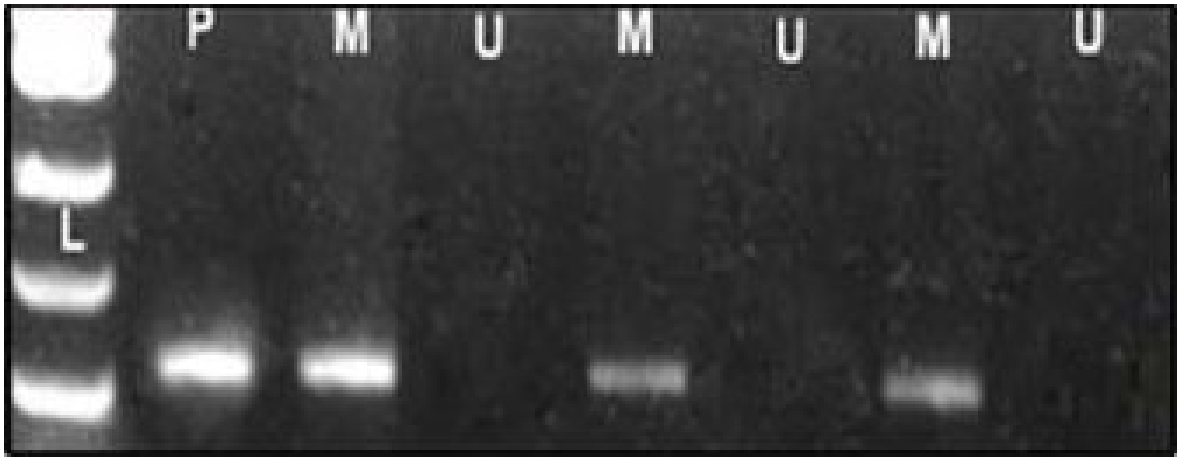

Figure 1. MS-PCR results of few representative gastrointestinal malignant cases
Lane 1 represents 50 Bp ladder
Lane 2 represents positive control amplified with methylated primer
Lane 3 and 4 represents gastric cancer case (GC) amplified with methylated primer only
Lane 5 and 6 represents colorectal cancer case (CC) amplified with methylated primer only
Lane 7 and 8 represents oesophageal cancer case (EC) amplified with methylated primer only

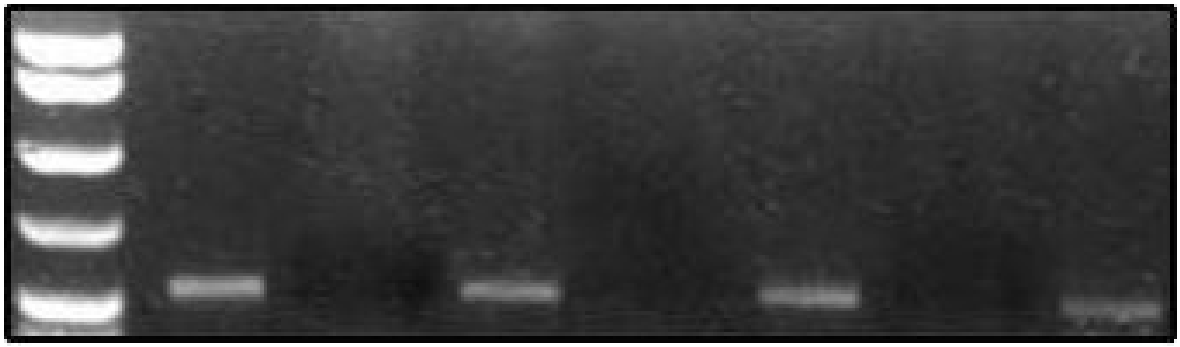

Figure 2. MS-PCR results of few representative gastrointestinal normal samples
Lane 1 represents 50 Bp ladder
Lane 2 represents negative control amplified with unmethylated primer
Lane 3 and 4 represents normal gastric case (GN) amplified with unmethylated primer only
Lane 5 and 6 represents normal colorectal case (CN) amplified with unmethylated primer only
Lane 7 and 8 represents normal oesophageal case (EN) amplified with unmethylated primer only

Table 3. Frequency of *hMLH1* gene promoter region hypermethylation in gastrointestinal malignancies:

Type of Cancer	Parameter	Cases			Control		
		M	F	T	M	F	T
Gastric	Number	35	25	60	25	15	40
	Hypermethylation	17	11	28	05	02	07
	Frequency (%)	48	44	46	20	13	17.5
Esophageal	Number	43	17	60	27	13	40
	Hypermethylation	22	08	30	04	02	06
	Frequency	51	47	50	14	15	15
Colorectal	Number	40	20	60	25	15	40
	Hypermethylation	28	08	36	02	02	04
	Frequency	70	40	60	08	13.3	10

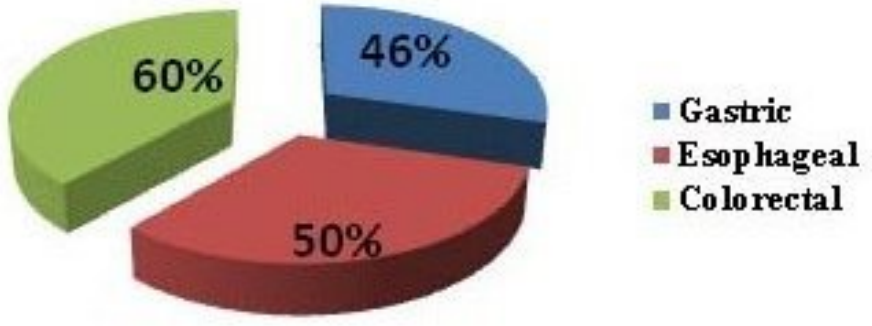

Figure 3. Frequency of *hMLH1* gene hypermethylation in gastrointestinal malignancies

Table 4. Promoter Hypermethylation of Male Cases of GIT malignancies.

Type of Cancer	Gastric		Esophageal		Colorectal	
Gender	Male Cases	Male Controls	Male Cases	Male Controls	Male Cases	Male Controls
Total	35	25	43	27	40	25
Hypermethylation	17	05	22	04	28	02
Non-Hypermethylation	18	20	21	23	12	23
p-value	0.0310		0.0024		0.0001	

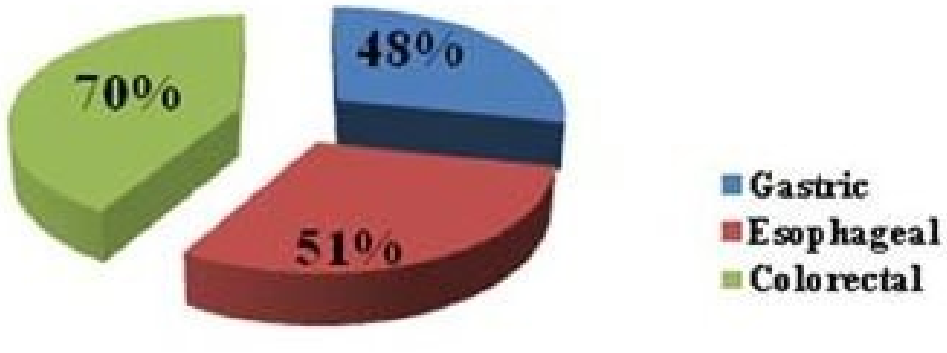

Figure 4. Frequency of hMLH1 gene hypermethylation in male gastrointestinal cancer cases

Table 5. Promoter Hypermethylation of Female Cases of GIT malignancies

Type of cancer	Gastric		Esophageal		Colorectal	
Gender	Female Cases	Female Controls	Female Cases	Female Controls	Female Cases	Female Controls
Total	25	15	17	13	20	15
Hypermethylation	11	02	08	02	08	02
Non-Hypermethylation	14	13	09	11	12	13
p-value	0.0801		0.1194		0.130	

DISCUSSION

GIT malignancy arises as a result of dysregulated cellular signalling which is in turn dictated by alterations in different proto-oncogenes, tumour suppressor genes and also include epigenetic changes in DNA [4, 5]. In neoplasms, including GI cancers, epigenetic changes play a key role in the process of tumorogenesis [6, 7]. DNA methylation in cancer has become the topic of intense investigation. Promoter hypermethylation is an alternative mechanism of gene inactivation in carcinogenesis [29]. Several studies have suggested that aberrant methylation of the promoter causes transcriptional silencing of some important suppressor genes, such as p16 [30], E-cadherin [31], and *von Hippel Lindau* (*vhl*) gene [32], and this has been implicated in the carcinogenic process in many cancers [29]. There are several protective mechanisms that prevent the hypermethylation of the CpG islands. These include active transcription, active demethylation, replication timing, and local chromatin structure preventing access to the DNA methyltransferase [33]. To date, nearly 50% of numerous genes have been found to undergo hypermethylation in cancer. The genes that are susceptible are the genes involved in cell cycle regulation (*P16INK4A*, *p15INK4a*, *Rb*, *p14ARF*), genes associated with DNA repair (*hMLH1*, *BRCA1*, *MGMT*), apoptosis (*DAPK*, *TMS1*), angiogenesis (*THBS1*, *VHL*), invasion (*CDH1*, *TIMP3*), drug resistance, detoxification, differentiation, and metastasis [34] .

The hmlh1 protein, a mismatch repair enzyme, maintains the fidelity of the genome during cellular proliferation. It has no known enzymatic activity and probably acts as a 'molecular matchmaker', recruiting other DNA-repair proteins to the mismatch repair complex [35]. Dysfunction of a mismatch repair system such as *hmlh1* and *hmsh2* could alter microsatellites, short tandem repetitive sequences [36]. The mismatch repair system is composed of a highly diverse group of proteins that interact with numerous DNA structures during DNA repair and replication [37]. Considering the importance of promoter hypermethylation in inactivation of *hmlh1* which is one of the frequently altered genes in many human cancers, a study was designed to analyse promoter methylation status of this mismatch repair gene (MMR) and its frequency in various GIT malignancies in an ethnic of Kashmiri origin. To determine the promoter methylation status of mismatch repair (*hmlh1) gene,* methylation specific polymerase chain reaction was performed on a DNA isolated from 180 surgically resected and endoscopically obtained gastrointestinal cancer cases and was compared with that of 120 histopathologically confirmed normal tissues. In the present study MSP was used for analysis of the methylation status of *hmlh1* gene. This method provided significant advantages over previous ones used for assaying methylation. MSP is much more sensitive than Southern analysis, facilitating the detection of low numbers of methylated alleles and the study of DNA from small samples. MSP allows examination of all CpG sites, not just those within sequences recognized by methylation sensitive restriction enzymes.

The frequency of promoter hypermethylation of *hmlh1* gene in gastrointestinal cancers was found to be higher in colorectal cancer (60%) fallowed by oesophageal cancer (50%) and gastric cancer (46%) where as among normal samples, the frequency was found to be higher in gastric (17.5%) fallowed by oesophageal (15%) and colorectal (10%) samples respectively. However, on reviewing the literature, the methylation frequency ranged from 8 to 50% for *hmlh1* gene in GIT cancers. The lower methylation profile reported in these earlier studies compared with our study may be due to the techniques employed. The association of promoter hypermethylation with GIT malignancies was found to be significant. The genetic analysis revealed that unlike other high-risk regions, Kashmiri population has a different hypermethylation profile of *hmlh1* gene promoter in males and females. Occurrence of *hmlh1* promoter hypermethylation was found to be unequally distributed in males and females with more frequency in males than in females. Frequency of promoter region hypermethylation of *hmlh1* gene in gastrointestinal cancers was found to be higher in colorectal cancer males (70%) followed by oesophageal cancer males (51%) and gastric cancer males (48%). Gastrointestinal carcinogenesis is a stepwise process of the accumulation of genetic and epigenetic abnormalities.

It is clear that promoter hypermethylation of mismatch repair genes is as important for this multistep process as genetic changes in the progression of these cancers. Our study has supplemented the steadily growing list of genes inactivated by promoter hypermethylation in gastrointestinal cancers. These provide not only new insights into the molecular basis of the diseases but also list of interesting candidate genes for the development of molecular markers which might contribute to the improvement of diagnosis and also prognosis. In addition, the fact that methylation can be reversed *in vitro* and the effect of the demethylating agent 5-aza-2□-deoxycytidine *in vitro* raise hope for new treatment strategies for gastrointestinal cancer patients.

CONFLICT OF INTEREST

The authors declare that there are no competing interests.

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REFERENCES:

1. Chan AO, Rashid A. CpG island methylation in precursors of gastrointestinal malignancies. Curr Mol Med 2006; 6(4):401-408.
2. Sameer AS, Chowdhri NA, Siddiqi MA. Adenocarcinoma of the colon and rectum in the Kashmiri population. Indian J Hum Genet 2009; 15(3):143-144.
3. Khuroo MS, Zargar SA, Mahajan R, Banday MA. High incidence of oesophageal and gastric cancer in Kashmir in a population with special personal and dietary habits. Gut 1992; 33(1):11-15.
4. Vogelstein B, Fearon ER, Hamilton SR, Kern SE, Preisinger AC, Leppert M, Nakamura Y, White R, Smits AM, Bos JL. Genetic alterations during colorectal-tumor development. N Engl J Med 1988; 319(9):525-532.
5. Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. Cell 1990; 61(5):759-767.
6. Baylin S, Bestor TH. Altered methylation patterns in cancer cell genomes: cause or consequence? Cancer Cell 2002; 1(4):299-305.
7. Baylin SB, Ohm JE. Epigenetic gene silencing in cancer - a mechanism for early oncogenic pathway addiction? Nat Rev Cancer 2006; 6(2):107-116.
8. Laird PW. Cancer epigenetics. Hum Mol Genet 2005; 14 Spec No 1R65-76.
9. Laird PW, Jaenisch R. The role of DNA methylation in cancer genetic and epigenetics. Annu Rev Genet 1996; 30441-464.
10. Goldberg AD, Allis CD, Bernstein E. Epigenetics: a landscape takes shape. Cell 2007; 128(4):635-638.
11. Plass C. Cancer epigenomics. Hum Mol Genet 2002; 11(20):2479-2488.
12. Costello JF, Plass C. Methylation matters. J Med Genet 2001; 38(5):285-303.
13. Costello JF, Vertino PM. Methylation matters: a new spin on maspin. Nat Genet 2002; 31(2):123-124.
14. Ting AH, McGarvey KM, Baylin SB. The cancer epigenome--components and functional correlates. Genes Dev 2006; 20(23):3215-3231.
15. Esteller M, Corn PG, Baylin SB, Herman JG. A gene hypermethylation profile of human cancer. Cancer Res 2001; 61(8):3225-3229.
16. Esteller M. Profiling aberrant DNA methylation in hematologic neoplasms: a view from the tip of the iceberg. Clin Immunol 2003; 109(1):80-88.
17. Kang GH, Lee S, Kim JS, Jung HY. Profile of aberrant CpG island methylation along the multistep pathway of gastric carcinogenesis. Lab Invest 2003; 83(5):635-641.
18. Ahuja N, Li Q, Mohan AL, Baylin SB, Issa JP. Aging and DNA methylation in colorectal mucosa and cancer. Cancer Res 1998; 58(23):5489-5494.
19. Razin A, Riggs AD. DNA methylation and gene function. Science 1980; 210(4470):604-610.
20. Kang YH, Bae SI, Kim WH. Comprehensive analysis of promoter methylation and altered expression of hMLH1 in gastric cancer cell lines with microsatellite instability. J Cancer Res Clin Oncol 2002; 128(3):119-124.
21. Deng G, Chen A, Hong J, Chae HS, Kim YS. Methylation of CpG in a small region of the hMLH1 promoter invariably correlates with the absence of gene expression. Cancer Res 1999; 59(9):2029-2033.
22. Cunningham JM, Christensen ER, Tester DJ, Kim CY, Roche PC, Burgart LJ, Thibodeau SN. Hypermethylation of the hMLH1 promoter in colon cancer with microsatellite instability. Cancer Res 1998; 58(15):3455-3460.
23. Thomas DC, Umar A, Kunkel TA. Microsatellite instability and mismatch repair defects in cancer. Mutat Res 1996; 350(1):201-205.
24. Jiricny J. The multifaceted mismatch-repair system. Nat Rev Mol Cell Biol 2006; 7(5):335-346.
25. Leung SY, Yuen ST, Chung LP, Chu KM, Chan AS, Ho JC. hMLH1 promoter methylation and lack of hMLH1 expression in sporadic gastric carcinomas with high-frequency microsatellite instability. Cancer Res 1999; 59(1):159-164.
26. Hayashi M, Tamura G, Jin Z, Kato I, Sato M, Shibuya Y, Yang S, Motoyama T. Microsatellite instability in esophageal squamous cell carcinoma is not associated

with hMLH1 promoter hypermethylation. Pathol Int 2003; 53(5):270-276.

27. Geddert H, Kiel S, Iskender E, Florl AR, Krieg T, Vossen S, Gabbert HE, Sarbia M. Correlation of hMLH1 and HPP1 hypermethylation in gastric, but not in esophageal and cardiac adenocarcinoma. Int J Cancer 2004; 110(2):208-211.
28. Herman JG. Hypermethylation of tumor suppressor genes in cancer. Semin Cancer Biol 1999; 9(5):359-367.
29. Baylin SB, Herman JG, Graff JR, Vertino PM, Issa JP. Alterations in DNA methylation: a fundamental aspect of neoplasia. Adv Cancer Res 1998; 72141-196.
30. Merlo A, Herman JG, Mao L, Lee DJ, Gabrielson E, Burger PC, Baylin SB, Sidransky D. 5' CpG island methylation is associated with transcriptional silencing of the tumour suppressor p16/CDKN2/MTS1 in human cancers. Nat Med 1995; 1(7):686-692.
31. Graff JR, Herman JG, Lapidus RG, Chopra H, Xu R, Jarrard DF, Isaacs WB, Pitha PM, Davidson NE, Baylin SB. E-cadherin expression is silenced by DNA hypermethylation in human breast and prostate carcinomas. Cancer Res 1995; 55(22):5195-5199.
32. Herman JG, Latif F, Weng Y, Lerman MI, Zbar B, Liu S, Samid D, Duan DS, Gnarra JR, Linehan WM *et al*. Silencing of the VHL tumor-suppressor gene by DNA methylation in renal carcinoma. Proc Natl Acad Sci U S A 1994; 91(21):9700-9704.
33. Clark SJ, Melki J. DNA methylation and gene silencing in cancer: which is the guilty party? Oncogene 2002; 21(35):5380-5387.
34. Jones PA, Baylin SB. The fundamental role of epigenetic events in cancer. Nat Rev Genet 2002; 3(6):415-428.
35. Modrich P. Mechanisms and biological effects of mismatch repair. Annu Rev Genet 1991; 25229-253.
36. Thibodeau SN, Bren G, Schaid D. Microsatellite instability in cancer of the proximal colon. Science 1993; 260(5109):816-819.
37. Hoffmann ER, Borts RH. Meiotic recombination intermediates and mismatch repair proteins. Cytogenet Genome Res 2004; 107(3-4):232-248.