

RESEARCH ARTICLE

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Significant Pattern of Promoter Hypermethylation of UNC5C Gene in Colorectal Cancer and Its Implication in Late Stage Disease

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Abstract

Background: The development of Colorectal Cancer (CRC) is a complex multistep process involving an accumulation of multiple genetic and epigenetic alterations. Epigenetic modifications, particularly DNA methylation in selected gene are recognized as common molecular alterations in human tumors. Netrin-1 receptors are aberrantly methylated in primary colorectal cancer. Epigenetic alterations in the netrin-1 receptors have been found to be related with the malignant potential of CRC. **Purpose:** In the present study, we evaluated the role of promoter hypermethylation of UNC5C gene (one of the netrin-1 receptors) in colorectal cancer patients of Kashmiri population (North India). Hypermethylation in tumour tissue was detected by Methylation-Specific Polymerase Chain Reaction (MS-PCR). **Results:** UNC5C promoter hypermethylation was significantly found to be associated with colorectal cancer cases where frequency was 62% (31 of 50) and 38% (19 of 50) patients were unmethylated ($p < 0.0001$). UNC5C methylation was significantly higher in CRCs with a frequency of 62% than 10% in corresponding normal mucosa of ($p < 0.0001$). Further, UNC5C hypermethylation was found to be significantly associated with stage-III/IV as compared to stage I/II with a frequency of 75.8% and 42.8% respectively ($p > 0.05$). **Conclusion:** We conclude that UNC5C hypermethylation is implicated in CRC which plays a role in its tumorigenesis and may predict the late stage disease.

Keywords: Colorectal cancer- hypermethylation- UNC5C, Netrin-1- DCC

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Introduction

CRC is the second leading cause of cancer causing death worldwide after breast cancer (Hanahan et al., 2000). Colorectal cancer is the third leading cause of cancer-related deaths in the United States when men and women are considered separately, and the second leading cause when both sexes are combined. It was expected to cause about 49,190 deaths during 2016 (Cancer Facts and Figures, 2016; Ferlay et al., 2004). In India, for colon and rectal cancer, the annual incidence rates (AARs) in men are 4.4 and 4.1/105 while as in women, the it is 3.9/105 (Sharma et al., 2011). According to recent study, the 5 year trend of different cancer studies shows that CRC is the fourth most common cancer in Kashmir valley having a frequency of 11% among different cancers (Arshad et al., 2012).

Epigenetic alterations of specific genes are emerging as important candidate biomarkers for the early detection of cancer (Croce et al., 2008; Knudson et al., 2001). Aberrant methylation of CpG (cytosines preceding guanines) islands in promoter regions of genes is associated with epigenetic transcriptional silencing of tumor suppressor genes,

which is important in early stages of CRC development (Kondo et al., 2004). Gene promoter methylation has a clinical potential for molecular diagnostics of CRC. DNA hypomethylation has been linked to CRC and plays an important role in carcinogenesis (Baylin et al., 2000).

Netrins main role is in axonal guidance, neuronal migration and morphogenesis of different branching structures (Serafini et al., 1994). Netrin-1, a diffusible laminin-related protein, has been shown to play a major role in the control of neuronal navigation during the development of the nervous system, by interacting with its main receptors, DCC and UNC5H (Kennedy et al., 1994). However, netrin-1 has rapidly emerged as a multifunctional protein implicated in multiple functions beyond the brain (Rajasekharan et al., 2009). Studies have shown that a large part of its activity is associated with the fact that netrin-1 regulates endothelial and epithelial cell survival by inhibiting the pro-apoptotic activity of the dependence receptors DCC and UNC5H, i.e., UNC5H1, UNC5H2, UNC5H3, UNC5H4 also called UNC5A, B, C, D (Mehlen et al., 2011; Wang et al., 2008; Castets et al., 2009; William et al., 2013).

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Results implicate that epigenetic alteration in the netrin-1 receptors do not occur randomly in CRC and may be related to the malignant potential of CRC (Vogelstein et al., 1988). Given the apparent importance of the Netrin pathway in the maintenance of gut epithelium, a better understanding of the molecular mechanisms that mediate loss of expression of these genes, and the consequence of loss of these receptors in malignant colonic tissues, is warranted (Bos et al., 1987; Hibi et al., 1996; Hibi et al., 1997).

This study thus investigated the hypermethylation status of Netrin-1 receptor gene UNC5C to analyse its pattern and role in a series of 50 colorectal cancer patients of Kashmir Valley.

Materials and Methods

Samples

The study comprised of total 50 patients with colorectal carcinoma who were subjected to various types of curative surgical procedures depending upon the site of lesion. Histopathologically confirmed tumor specimens from resected tissues and paired normal tissues were analysed for hypermethylation status in the promoter region of UNC5C gene. CRC tissue specimens and adjacent normal were collected in sterile polypropylene vials and stored at -70 °C. The samples were stored at -80 °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.

DNA extraction

Tissue samples (both tumor and adjacent normal) were immediately snap-frozen and stored at -70 °C until use. DNA was extracted from the tissues using a purelink genomic DNA mini kit (Invitrogen, Van Allen Way Carlsbad, CA) according to the manufacturer's protocol.

Methylation Specific-PCR

Amplification of bisulfite-converted DNA by MS-PCR Methylation in promoter region of UNC5C gene was assessed by subjecting the bisulfite treated DNA to PCR amplification using methylation specific primers. Two primer pairs discriminating between the methylated and non-methylated DNA was used (Hibi et al., 2009). Both the tumor and normal DNAs were subjected to sodium bisulfite modifications following the instructions of the kit (DNeasy, Qiagen, USA). Methylation Specific-PCR (MS-PCR) was performed to examine methylation at promoter sequence of UNC5C gene, using method as described previously (Hibi et al., 2009). Both methylated and unmethylated primers were used for normal as well as cancerous tissue. For each sample two PCR reactions were set with each primer pair. Successful amplification with either of the two pairs indicated the methylation or non-methylation in the promoter region. An aliquot of each PCR product was separated on 2% agarose gel. The gel was stained with ethidium bromide and photographed

under UV illumination. The reproducibility of the results was confirmed by repeating MSP analyses for each DNA sample.

Statistical analysis

Statistical analysis was performed by using SPSS software (V.16). Fisher's exact test, Chi Square test for homogeneity of proportions and Odds ratio was used wherever applicable. Statistical significance was considered when $P < 0.05$.

Results

This study comprised of 50 CRC cases and was jointly conducted by the Department of General and Minimal Access Surgery, SK, Institute of Medical Sciences, J&K, India after obtaining detailed history. Among the recruited patients 60% were males (30/50) and 40% were females (20/50) as depicted in Table 1. Mean age of cases was 53.38 ± 16.6 years. The most common age group comprised of cases of age 61 to 80. There was an increase in the prevalence of colorectal carcinoma with an increase in the age as shown in Table 1.

Hypermethylation in promoter region of UNC5C gene was analysed by MS-PCR in CRC patients. Hypermethylation at the promoter region of UNC5C gene was seen higher in CRC cases with 62% as against 38% unmethylated ($p < 0.0001$) (Table 2). Further study found UNC5C gene promoter hypermethylation in 66.66% of males as compared to 55% in females ($p > 0.05$). The relationship between hypermethylation of UNC5C gene and age does not correlate statistically ($p = 0.76$) (Table 1). In colorectal carcinoma, among patients with smoking history, increased number of cases, 70.83% exhibited hypermethylation in the promoter region of UNC5C gene whereas 29.16% of smokers were non-methylated. Methylation status did not differ statistically when compared with smoking among colorectal carcinoma patients ($p = 0.25$) (Table 2). In CRC cases, hypermethylation correlated with 51.85% of well differentiated adenocarcinoma and 73.91% of moderately differentiated adenocarcinoma/ poorly differentiated adenocarcinoma ($p > 0.05$) (Table 2). Among patients with active lifestyle, there was increased number of hypermethylation in cases, 55.88% in the promoter region of UNC5C gene whereas 75% of patients with sedentary lifestyle also exhibited hypermethylation. There was statically no difference in methylation status among colorectal carcinoma patients ($p = 0.25$) (Table 2). In patients with stage I and II disease, 42.85% exhibited hypermethylation in the promoter region of UNC5C gene as compared to 75.86% of patients with stage III /IV. This pattern among two histological stages was found to be significantly associated ($p = 0.01$) (Table 2).

Discussion

Colorectal cancer is one of the most aggressive malignancies and occurs at a high incidence in most countries (Curado et al., 2007; Parkin et al., 2010).

Table 1. UNC5C Gene Promoter Hypermethylation in Colorectal Carcinomapatients and Their Demographics

Variables	Methylation state of the tumor		P- value	OR (95% CI)
	Methylated n= 31 (62%)	Unmethylated n=19 (38%)		
Age group				
<55 yrs	14 (66.7)	7 (33.4)	0.76	Ref 1.4 (0.37-5.3)
> 55 yrs	17 (58.63)	12 (41.38)		
Gender				
Male	11 (55%)	9 (45%)	0.55	
Female	31 (62%)	19 (38%)		
Smoking status				
Smoker	17 (70.83%)	7 (29.16%)	0.25	Ref 2.0 (0.5-5.8)
Non –smoker	14 (53.84%)	12 (46.15%)		
Histopathological differentiation				
Well differentiated	14 (51.86%)	13 (48.14%)	0.15	Ref 2.6 (0.7-0.8)
Moderately/ Poorly differentiated	17(73.91%)	6 (26.08%)		
Lifestyle				
Active	19 (55.88%)	15 (44.12%)	0.25	1.7 (0.43-4.6)
Sedentary	12 (75%)	4 (25%)		
Clinical Staging				
I/II	9 (42.85%)	12 (57.14%)	0.01	4.2 (1.1-11.2)
III/IV	12 (75.86%)	7 (24.13%)		

Table 2. UNC5C Gene Promoter Hypermethylation in Colorectal Carcinoma Tissue and Adjacent Normal

Tissue	Methylated		Non Methylated		OR (95% CI)	p value
Normal	5	10%	45	90%		
Tumor	31	62%	19	38%	14.6 (4.5-32.1)	<0.0001
Total	50		50			

For this purpose, it is important to identify the occurrence of genetic alterations as new parameters to estimate the malignancy (Shin et al., 2007). CRC occurs as a consequence of the successive accumulation of genetic and epigenetic alterations in multiple genes that control epithelial cell growth and other cellular behaviours (Llambi et al., 2001).

UNC5C belongs to the functional dependence receptor family, members of which share the ability to induce apoptosis in the absence of their ligands (Hong et al., 1999; Thiebault et al., 2003; Bernet et al., 2007). Such a trait has been hypothesized to confer a tumor-suppressor activity. Indeed, the loss of UNC5C expression is particularly prominent in colorectal cancer (Shin et al., 2007). However, the molecular mechanisms responsible for the loss of UNC5C expression are poorly understood. Recently, two reports indicated that UNC5C methylation was closely associated with loss of gene expression in colorectal cancer (Jones et al., 1999; Grady et al., 2007; Hibi et al., 2009). These results prompted us to examine the methylation status of the UNC5C gene in CRC patients of our region.

In the present study conducted first time from here, the methylation status of the UNC5C gene was examined in primary carcinomas and corresponding normal tissues derived from 50 patients with CRC and the correlation between the methylation status and the clinico-pathological findings was evaluated. Hypermethylation of the gene

promoter has been shown to be a frequent epigenetic event in various human cancers. Aberrant UNC5C promoter methylation has also been observed in several cancers (Baylin et al., 2000). In the present study, we have extended the findings to CRC patients of the Kashmir valley for UNC5C promoter methylation. The frequency of promoter hypermethylation in these cases was found to be 62.0%. UNC5C methylation was significantly higher in CRCs (62.0%) than in corresponding normal mucosa (10%; $P < 0.0001$). This frequency is however; lower than reported in other study (Hibi et al., 2009). These differences may be attributed to differences in ethnic populations and/or etiologic factors involved. Additionally, we tested putative correlations between UNC5C hypermethylation and various clinico-pathological characteristics of the colorectal cancer patients. An interesting finding in this study was that hypermethylation of the UNC5C promoter was found to be significantly associated with stage-III/IV ($p < 0.05$). This finding is in agreement with the study of Hibi et al., (2009). They showed that a significantly greater proportion of cases with Dukes' stage C exhibited methylated UNC5C ($p > 0.05$) than earlier stages. Likewise, in another study conducted by Bernet et al., (2007) mice showed that inactivation of UNC5C was found to be associated with increased intestinal tumor progression. Further, very recently, netrins have also been shown to have pivotal role cell adhesion, motility, proliferation, differentiation and, ultimately, cell survival in a number

of non-neuronal tissues (Cirulli et al., 2007). The study of Shin et al., (2007) reported that UNC5C inactivation occurs during early stages of multistep colorectal carcinogenesis. Further, the study conducted by Shin et al showed that UNC5C hypermethylation was associated with age and tumour differentiation (Umut et al., 2017). In contrast our study showed no connection with the age of patients, however methylation was found to be more frequent in cases with moderately/poorly differentiated tumour than those with well differentiated status, but this difference couldn't reach the statistical significance. This discrepancy may be due to difference in ethnicity, sample size or geographic location.

In summary we found that hypermethylation in UNC5C gene can be a key event in CRC which plays an important role in its tumorigenesis and may predict the late stage disease. Owing to the limitation of our small sample size, these results need to be further validated in large number of cohort CRC samples

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