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TCF4 MAY ACT AS A TUMOR SUPPRESSOR GENE IN COLORECTAL CARCINOGENESIS

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ABSTRACT

Introduction: Colorectal cancer (CRC) is one of the leading malignancies as being the third most cause of cancer causing death in the world. Carcinogenesis is a complex process caused by various genetic and epigenetic changes in various genes like tumor suppressor genes, DNA repair genes, translation regulatory genes and others. The Wnt/β-catenin pathway plays multiple and diverse roles in development by regulating gene expression via T-cell factor/Lymphoid enhancer-binding factor (Tcf/Lef) DNA binding factors. Misregulation of this pathway is thought to initiate colon adenoma formation. The Wnt pathways with its molecular gladiator Tcf4 plays an important role in transforming a normal tissue into a malignant one. In this study, we aimed to investigate the role of aberrations in Tcf4 gene in the pathogenesis of CRC in the Kashmir valley. **Materials and Methods:** We examined the paired tumor and normal-tissue specimens of 100 CRC patients from Apr 2010- June 2013, from Department of Surgery, Government Medical College, Srinagar and its associated hospital Shri Maharaja Hari Singh Hospital (SMHS) and analysis of promoter hypermethylation for exon 1 and intron 8 of Tcf4 gene was also carried out using Methylation-specific PCR (MS-PCR). **Results and Conclusion**: Promoter hypermethylation of Tcf4 of the exon 1 of Tcf4 gene among 100 CRC cases was found to be 66 per cent (66 of 100) and for intron 8, it was 70%. Besides in both cases of males and females, the hypermethylation status of intron 8 is more than exon 1 of

Tcf4, the study confirms the role of epigenetic gene silencing of the pivotal molecular gladiator, Tcf4, of the Wnt pathway in the development of CRC in the Kashmiri population. So, it was concluded that Tcf4 promoter may be used as a diagnostic tool for colorectal carcinogenesis and therefore can be used a marker for its diagnosis.

KEY WORDS: Wnt pathway, Tcf4 , b-catenin, colorectal cancer, MS-PCR, SMHS.

INTRODUCTION

Colorectal cancer (CRC) is the major cause of mortality and morbidity worldwide as it causes 655,000 deaths worldwide per year (World Health Organization, 2006; American Cancer Society, 2008). It is the fourth most common cancer in men and the third most cancer in women worldwide[1]. Gastrointestinal cancer (GIT) arises through the accumulation of multiple genetic alterations, leading to activation of oncogenes and loss of function of tumor suppressor genes [2].CRC is a multi-step process which arises from cumulative aberrations of a number of different genes or from epigenetic changes in DNA at different stages of development and progression[3,4]. Kashmir valley known as the land of paradise, has been reported as a high-incidence area of GIT cancers[5,6]. CRC represents the third most common GIT cancer [7,8].

Many factors are responsible for carcinogenesis which includes genetics and epigenetics [9,10], apart from diet and other environmental factors. The main epigenetic modification is DNA methylation of cytosine residues within CpG dinucleotides . Methylation of CpG islands within the promoter region may lead to silencing of gene transcription through a complex process involving chromatin condensation and histone deacetylation[11,12]. Epigenetic silencing through DNA methylation can begin very early in tumorogenesis. Abberations in DNA methylation patterns, like global genome hypomethylation[13,14] and hypermethylation of tumor suppressor genes at the CpG islands in their promoter regions, are increasingly found in various different types of cancers .Many genes undergoes silencing due to hypermethylation like cell cycle regulatory genes, apoptotic genes, etc and in turn leads to uninterrupted cell division[15,16]. CRC carcinogenesis is a multi-step and multi-gene event.

The Wnt/β-catenin signaling pathway plays an important role during embryonic development and carcinogenesis. Loss of regulation of the Wnt pathway has been implicated in the development of several types of cancers, including colon, lung, breast, thyroid and prostate cancers and leukemia[17-20].The Wnt pathway plays an important role in various cellular

processes, including proliferation, differentiation, survival, apoptosis and cell motility[21] and also regulates cell adhesion, morphology, proliferation, migration and structural remodeling[22,23]. TCF4 is a downstream target of the WNT/b-catenin/TCF pathway and, may when deregulated in human colon cancers[24].TCF4 belongs to bHLH family of proteins ,also known as E-proteins as it makes specific contacts with consensus DNA sequences known as E-boxes(CANNTG) [25]. These proteins are critical regulators in a diverse array of biological processes such as cell growth, differentiation, tissue specific gene expression and programmed cell death [26-28]. It is controversial whether Tcf4 (Tcf7L2) functions as an oncogene or tumor suppressor gene in colon carcinogenesis. It has been also observed that Tcf4 acts as a negative regulator of cell proliferation as the enforced expression of Tcf4 suppresses the colony-forming efficiency of cells in several cell lines[28]. Tcf4 mutation also causes Pitt-Hopkins syndrome, a neurodevelopmental disease [29-31], besides may also contribute to Type 2 Diabetes Mellitus[32]. Tcf4 null mice show developmental defects of the small intestine[33]. Loss of Tcf4 in adult colon results in increased cell proliferation. This suggests that Tcf4 normally modulates proliferation of the colonic epithelium and that disruption of Tcf4 activity increases proliferation, leading to colon tumorigenesis. Tcf4 has different splicing isoforms which are expressed differentially in tissues and during cancer progression [34,35]. The Tcf4 has recently been found to be mutated in clear cell renal cell carcinoma (CCRCC), gastric carcinoma, and breast cancer [36-38]. Tcf mutations have been found in primary CRCs and these enhance cell growth in cell lines[39-41].

In this study, we identify Tcf4 as a hypermethylated gene in CRC cancers using MS-PCR .We demonstrate prominent hypermethylation of CpG dinucleotides in Tcf4, which significantly correlates with gene inactivation in CRC cancers.

MATERIALS AND METHODS

Patients and specimens

Out of 100 patients who were diagnosed with CRC by clinicians using colonoscopy and confirmed by senior pathologist, a total of 100 CRC tissue specimens, comprising tumor tissues and corresponding adjacent normal tissues as controls, were collected for analysis. All samples were surgically resected and collected fresh at SMHS, Srinagar, Jammu & Kashmir. Tissue samples were divided into two parts; one part was sent for histopathological diagnosis and the other was snap frozen at -80^0C immediately until needed for further analysis. Only histopathologically confirmed cases were included for molecular analysis. No follow-up of

the CRC patients was carried out after the curative surgery. Written informed consent was obtained from all the subjects (and/or their guardians) included in the study, recorded on a predesigned questionnaire. The study protocol was approved by the Research Ethics Committee of the SMHS, Kashmir.

DNA isolation

Genomic DNA was extracted from tissue samples (previously stored at -80^{0}C) from CRC patients using DNA Extraction Kit (Zymo Research, Orange, CA). The tissue for DNA extraction from the tumor sample was chosen by an experienced pathologist.

Bisulfite Treatment

The extracted Genomic DNA was modified by EZ DNA Methylation–Direct^{TM} Kit supplied by Zymo Research. Sodium bisulfite treatment converts unmethylated cytosines to uracil. DNA, however, remains unmodified at places where DNA was methylated. This modification can help us to differentiate between methylated and unmethylated DNA using specific primers in MS-PCR. DNA was stored at or below -20°C for later use.

Methyl Specific Polymerase Chain Reaction (MSP)

The status of Tcf4 promoter hypermethylation in CRC cases from Kashmir valley was exploited by Methyl Specific PCR (MSP) using Gradient thermal cycler (Eppendorf) in 100 surgically resected colorectal cancer cases and compared with that of 100 histopathologically confirmed normal colorectal tissues. The primers used were shown in Table I and II [42]. The Ta for both sites was standardized at 59-62^{0}C. Universal Methylated Human DNA (Zymo Research) was used as positive control for methylated alleles whereas DNA from normal lymphocytes was used as a control for unmethylated alleles. Water was used as a negative PCR control in both reactions.

Table I*:* Primers for exon 1 and length of fragments obtained in Methylation Specific PCR of TCF4 gene .

Nature of Sequence	Primer sequence		Size of amplicon
UNMETHYLATED PRIMER	Forward primer	5’- TGA ATT TGT STTT GTG TGT TTTT G - 3’	259bp
	Reverse primer	5’- AAA AAA AAC TCT CCA TAC ACCACC - 3’	
METHYLATED PRIMER	Forward primer	5’- GAA TTT GTA ATT TCG TGC GTT TC - 3’	258bp
	Reverse primer	5’- AAA AAA AAC TCT CCG TAC ACC G - 3’	

Table II: Primers for intron 8 and length of fragments obtained in Methylation Specific PCR of TCF4 gene.

Nature of Sequence	Primer sequence		Size of amplicon
UNMETHYLATED PRIMER	Forward primer	5'- TTTTAGAGTGGAGAATGTGTGT-3'	199bp
	Reverse primer	5'- AAACAAAA TAACAATACAACCCACC - 3'	
METHYLATED PRIMER	Forward primer	5'- GAA TTT GTA ATT TCG TGC GTT TC - 3'	198bp
	Reverse Primer	5'- AAATAACAATACGACCCGCC - 3'	

Statistical analysis

The Fischer's exact test with Odds ratio was used to examine the differences in the distribution of Tcf4 gene promoter hypermethylation and non hypermethylation between cases and controls. Odds ratios with 95% CIs were computed using unconditional logistic regression using Graph Pad Prism Software Version 6.0 (Graph Pad Software 2236, La Jolla, CA, USA).

RESULTS

MS-PCR was done to examine the hypermethylation status of the promoter region of *Tcf4* gene. The hypermethylated amplicons of 258 bp of exon 1 of Tcf4 gene shown in fig I and for intron 8,the unhypermethylated amplicon of 199bp shown in fig II.

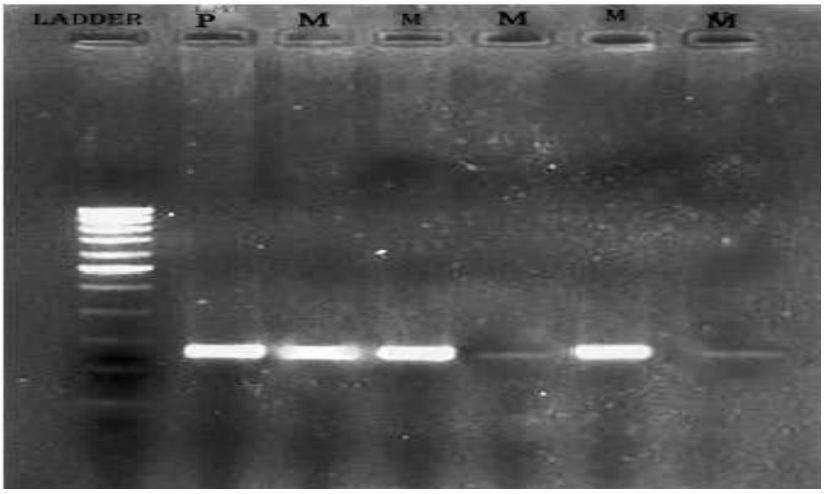

Fig I: Representative gel picture shows methylated amplicons (Amplified by methylated primers only) of exon 1 of Tcf4 gene and the size is of 258 bp.Ladder is of 100 bp

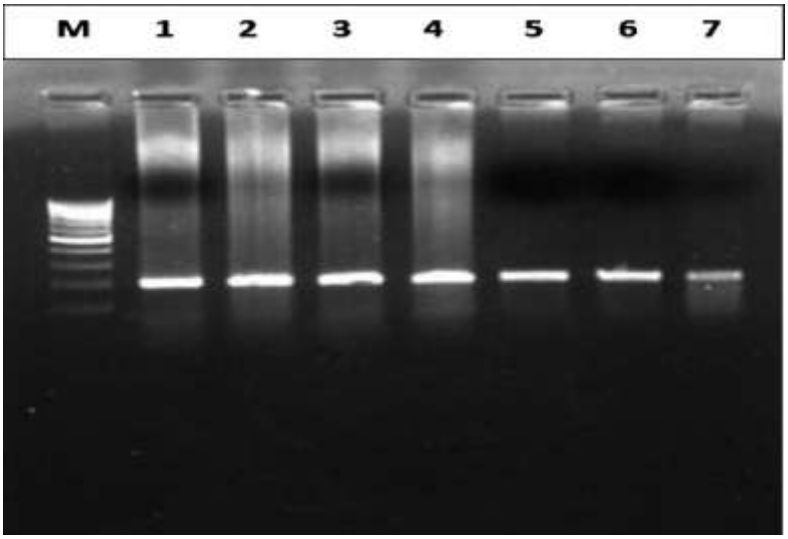

Fig II: Representative gel picture shows the unmethylated amplicons of 199bp of intron 8 of Tcf4 gene . Lane M shows 100bp ladder.

66% (66/100) of the colorectal cancer tissues showed hypermethylated *Tcf4* promoter and 34% (34/100) of the cases however showed unmethylated *TCF4* promoter , while as remaining 100 normal tissues 80% (80/100) showed hypermethylation only (Table III) and the p value was found to be <0.0001, so highly significant association .Table IV showed 70% (70/100) showed hypermethylation of intron 8 of *TCF4* gene, similarly 77% (77/100) of controls showed promoter hypermethylation . The association of promoter hypermethylation with colorectal is evaluated by Fischer's exact test with odds ratio and was found to be significant (p=<0.0001).

Relationship of promoter hypermethylation of Tcf4 gene with colorectal cancer in males and females

Among 58 males, 39 cases were hypermethylated and 19 cases were non-hypermethylated and among 58 male controls, 8 cases were hypermethylated and 50 cases were non-hypermethylated (Table V). The association of promoter hypermethylation with colorectal cancer was evaluated using Fisher's exact test and was found to be significant in males (P =<0.0001) for exon 1. For intron 8, among 58 male cases, 45 cases were hypermethylated and 13 cases were non-hypermethylated and among 58 male controls, 14 cases were hypermethylated and 44 cases were non-hypermethylated (Table VI). The association of promoter hypermethylation with colorectal cancer was evaluated using Fisher's exact test and was found to be significant in males (P =<0.0001). In comparison, among 42 females, 26 cases were hypermethylated and 16 cases were non-hypermethylated and among 42 female

controls 10 cases was hypermethylated and 32 cases were non-hypermethylated (Table VII). The association of promoter hypermethylation with colorectal cancer was again evaluated using Fisher's exact test and was found to be significant in females too (P =0.0008) for exon 1 of the gene. For intron 8,among 42 females,28 were found to be hypermethylated and remaining 14 non hypermethylated and among 42 female controls,25 were found to be non hypermethylated and p value was found to be highly significant (p= <0.0001) as shown in Table VIII.

Table III: Hypermethylation frequency for exon 1 of Tcf4 gene among colorectal cases and controls.

	Cases	**Controls**
Hypermethylation	66% (66/100)	20 %(20/100)
Non hypermethylation	34% (34/100)	80% (80/100)

Table V: Hypermethylation frequency for exon1 of Tcf4 gene among colorectal male cases and controls.

	Cases	**Controls**
Hypermethylation	70% (70/100)	23%(23/100)
Non hypermethylation	30% (30/100)	77% (77/100)
Males	Cases	Controls
Hypermethylation	67.24% (39/58)	13.79%(8/58)
Non hypermethylation	32.75% (19/58)	86.20% (50/58)

Table VI: Hypermethylation frequency for exon1 of Tcf4 gene among colorectal female cases and controls.

Females	**Cases**	**Controls**
Hypermethylation	61.90% (26/42)	23.80%(10/42)
Non hypermethylation	38.09% (16/42)	76.19% (32/42)

Table VII: Hypermethylation frequency for intron 8 of Tcf4 gene among male colorectal cases and controls.

Males	Cases	Controls
Hypermethylation	77.58% (45/58)	24.13%(14/58)
Non hypermethylation	22.41% (13/58)	75.86% (44/58)

Table VIII: Hypermethylation frequency for intron 8 of Tcf4 gene among colorectal female cases and controls.

Females	**Cases**	**Controls**
Hypermethylation	66.66% (28/42)	40.47%(17/42)
Non hypermethylation	33.33% (14/42)	59.52% (25/42)

DISCUSSION

Colorectal cancer is one of the most deadly cancer [43]. CRC results from accumulation of both genetic and epigenetic alterations of the cellular genome that transforms normal glandular epithelium into adenocarcinoma [44]. Transcriptional silencing by CpG island hypermethylation affects genes involved in all different cell functions and acts as a critical trigger for neoplastic development and progression [45,46]. Promoter hypermethylation of *Tcf4* gene in colorectal cancer patients has been studied and in several other types of cancers as well[47,48] . Human *Tcf4* gene possesses a CpG island in the promoter region and has been reported that the methylation of exon 1 and intron 8 of CpG island is associated with the silencing of the gene[42]. The exon 1 coding sequences of the *Tcf4* gene resides within 5' CpG islands. This area is not methylated in normal cells but is found to be methylated in many cancers like gastric cancer, colorectal cancers, breast cancer, etc[47].

However, recently developed techniques for detecting changes in DNA methylation have dramatically increased the amount of information available regarding the patterns of methylation that occur as cancers progresses. Aberrant methylation is also known to occur with aging and inflammation as aging affects a large number of CpG islands, age-related methylation might explain the increased incidence of cancer seen among older individuals. Other patterns of methylation are cancer specific and detected only in a subset of tumors exhibiting the CpG island methylator phenotype (CIMP)[49]. Tcf4 mutant mice show a loss of proliferative cells, so data suggests that Tcf4 is important for stem cell renewal in the small intestine50. One study also revealed that silencing of TCF4 caused a significant sensitization of CRC cells to clinically relevant doses of X- rays 51.

In the present study, the level of *Tcf4* promoter hypermethylation was investigated in colorectal carcinoma tissues of patients from Kashmir valley where frequency of colorectal cancer is found to be higher. In the present study MSP technique was used for analysis of the methylation status of *Tcf4* gene. This method is highly sensitive and accurate. In the study, the association of promoter hypermethylation of *Tcf4* gene for both exon 1 and intron 8 was found to be significant as $p < 0.0001$,so *Tcf4* hypermethylation may be involved in colorectal cancer development. We observed *Tcf4* promoter hypermethylation among 39 cases out of 58. Though there was no selection bias in sampling, occurrence of *Tcf4* methylation was found to be unequally distributed in males and females with more frequency in males for both sites of the gene than in case of females. And also it was observed that promoter

hypermethylation in intron 8 is more than in exon 1 of same gene,so intron 8 can be more exploited for diagnostic purposes for CRC.

This study also revealed that *Tcf4* promoter hypermethylation is a frequent epigenetic event in colorectal cancer of the Kashmir region so these results suggest that *Tcf4* aberrant methylation may play an important role in colorectal cancer development. Therapeutic strategies targeting promoter hypermethylation may be highly beneficial in the Kashmiri population and other regions where colorectal cancer is on rise as is associated with high frequency of *Tcf4* promoter methylation. The data gives a clue that *Tcf4* gene expression can be readily and fully restored and growth rate of cancer cells decreased

by treating with demethylating agents. Also the study concludes that *Tcf4* gene can be designated as epigenetic biomarker for the diagnosis and prognosis of this disease. Tcf4 may act as a screening and diagnostic marker for colorectal cancer.

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