

Analysis of molecular aberrations of Wnt pathway gladiators in colorectal cancer in the Kashmiri population

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Abstract

The development and progression of colorectal cancer (CRC) is a multi-step process, and the Wnt pathways with its two molecular gladiators adenomatous polyposis coli (*APC*) and β -catenin 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 the *APC* and β -catenin genes in the pathogenesis of CRC in the Kashmir valley, and to correlate it with various clinicopathological variables. We examined the paired tumour and normal-tissue specimens of 86 CRC patients for the occurrence of aberrations in the mutation cluster region (MCR) of the *APC* gene and exon 3 of the β -catenin gene by polymerase chain reaction–single-strand conformation polymorphism (PCR-SSCP) and/or PCR-direct sequencing. Analysis of promoter hypermethylation of the *APC* gene was also carried out using methylation-specific PCR (MS-PCR). The overall mutation rate of the MCR of the *APC* gene among 86 CRC cases was 12.8 per cent (11 of 86). Promoter hypermethylation of *APC* was observed in 54.65 per cent (47 of 86) of cases. Furthermore, we found a significant association between tumour location, tumour grade and node status and the methylation status of the *APC* gene ($p \leq 0.05$). Although the number of mutations in the *APC* and β -catenin genes in our CRC cases was very low, the study confirms the role of epigenetic gene silencing of the pivotal molecular gladiator, *APC*, of the Wnt pathway in the development of CRC in the Kashmiri population.

Keywords: Wnt pathway, *APC*, β -catenin, colorectal cancer, Kashmir, hypermethylation, mutations, Dukes' stage

Introduction

Colorectal cancer (CRC) is a major cause of mortality and morbidity, and the third most common malignancy in the world.¹ The incidence of this malignancy shows considerable variation among racially or ethnically defined populations in multiracial/ethnic countries. It is the fourth most common cancer in men and the third most common in women worldwide.¹ Kashmir has been reported as being a high-incidence area for gastrointestinal (GIT) cancers.^{2,3} In the Kashmir valley,

CRC represents the third most common GIT cancer after oesophageal and gastric cancer.^{4,5}

It has been suggested that CRC is a multi-step process which arises from cumulative aberrations of a number of different genes (including tumour suppressor genes, proto-oncogenes, DNA repair genes, the genes encoding growth factors and their receptors, cell cycle checkpoint genes and apoptosis-related genes) or from epigenetic changes in DNA at different stages of development and progression.^{6,7} It is

believed that mutations in the gene encoding adenomatous polyposis coli (*APC*) or that encoding β -catenin set the stage for the initiation and transformation of normal colonic epithelial cells. Further accumulation of mutations in other genes then contributes to the progression of cancer through adenoma – carcinoma – metastasis stages. The generally accepted model of CRC tumorigenesis for the majority of tumours has been a stepwise progression, in which mutations in *APC* are followed by several other mutations, including alterations in the genes encoding Kirsten ras (*K-ras*) and tumour protein 53 (*TP53*).^{6–8} During the accumulation of genetic changes, a complex signalling network is established among inactivated and activated cellular pathways.⁹

The Wnt pathway regulates cell adhesion, morphology, proliferation, migration and structural remodelling^{9,10} and plays an important role in a variety of cellular processes, including proliferation, differentiation, survival, apoptosis and cell motility.¹¹ 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.^{12–15} Two of the most important gliadator molecules of the Wnt pathway are *APC* and β -catenin.

APC is a classical tumour suppressor gene, located on 5q21, containing 21 exons. The *APC* transcript is 9.0 kilobases (kb) in length and the most common isoform of the *APC* protein contains 2,843 amino acids, with a molecular weight of 310 kD. Exon 15 of *APC* is most important, as it comprises >75 per cent of the coding sequence of *APC* and hence is the common target for both germline and somatic mutations, which usually span codons 1286–1513 of this exon.^{16,17} This region represents the mutation cluster region (MCR), and 68–77 per cent of somatic mutations in *APC* occur in this region.¹⁸ Mutations in *APC* are considered to be the earliest genetic aberrations in the initiation and progression of CRC,^{7,9,19} and have also been found in ~60–80 per cent of sporadic carcinomas and adenomas.^{20,21} Using mutant mouse models, various genetic studies have demonstrated that mutations in *APC* are responsible for intestinal tumorigenesis.^{22–24} Homozygous *APC*

mutations in mice lead to embryonic lethality,^{23,25,26} and conditional deletion of the gene in the adult mouse disrupts homeostasis, not only in the intestines but also in other tissues.^{27–29}

In addition to the mutational inactivation, hypermethylation of the gene promoter is another important mechanism associated with gene silencing.³⁰ In many tumours the hypermethylation of CpG islands in gene promoters has been found to be a frequent epigenetic change in cancers, and is usually associated with the loss of transcription of *APC*.^{31–38} Hypermethylation of the *APC* gene promoters has been reported in about 20–48 per cent of human CRCs.^{32,37,39,40}

The β -catenin gene is located at 3p22–p21.3 and encompasses 23.2kb of DNA. It contains 16 exons, with a mRNA transcript of about 2343 base pairs (bp), encoding a 781-amino-acid-residue protein with a molecular weight of 92 kD.⁴¹ This gene is mutated in up to 10 per cent of all sporadic CRC by point mutations or in frame deletions of the serine and threonine residues that are phosphorylated by glycogen synthase kinase 3-beta (*GSK3 β*).⁴² These mutations result in the stabilisation of β -catenin and the activation of Wnt signalling. Mutations in the β -catenin gene occur in exclusivity to *APC* aberrations, as both molecules are components of the same pathway.¹⁹

Based on the hypothesis that CRC carcinogenesis is a multi-step and multi-gene event, we designed this study to elucidate the role of *APC* and β -catenin in the development and progression of CRC in the Kashmiri population, and to correlate the gene aberrations and hypermethylation with the clinicopathological parameters of CRC cases.

Materials and methods

Patients and specimens

Out of 104 patients who were diagnosed with CRC by clinicians using either sigmoidoscopy or colonoscopy and confirmed by MRI, a total of 86 CRC tissue specimens, comprising tumour tissues and corresponding adjacent normal tissues as controls, were collected for analysis. All samples were surgically resected and collected fresh at the Department

of Surgery of the Sher-I-Kashmir Institute of Medical Sciences, Srinagar, Kashmir. Tissue samples were divided into two parts; one part was sent for histopathological diagnosis and the other was snap-frozen at -70°C 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 pre-designed questionnaire (available on request). The study was carried out in accordance with the principles of the Helsinki Declaration. The study protocol was approved by the Research Ethics Committee of the Sher-I-Kashmir Institute of Medical Sciences, Kashmir.

DNA isolation

Genomic DNA was extracted from blood and tissue samples (previously stored at -70°C) from CRC patients using DNA Extraction Kit II (Zymo Research, Orange, CA). The tissue for DNA extraction from the tumour sample was chosen by an experienced pathologist and was ascertained to comprise more than 90 per cent of the tumour cells.

Polymerase chain reaction (PCR)

APC and *β -catenin* gene analysis was carried out on all of the extracted DNA samples. The MCR region of *APC*, comprising codons 1260 to 1596, and exon 3 of *β -catenin*, which encompasses the region for GSK-3 β phosphorylation, were amplified using specific oligonucleotide primers (Table 1). PCR was performed in a 50 μl total volume reaction mixture containing 50 ng of genomic DNA, 100 ng of each primer, 100 μM of each deoxynucleotide triphosphate (dNTP), 1.5 mM MgCl_2 , 10 \times Taq buffer and 2 U of Taq DNA polymerase (Fermentas Inc, Glen Burnie, MD). The conditions of PCR were as follows: initial denaturation at 95°C for 5 minutes, 35 cycles of denaturation at 95°C , annealing at 52 – 58°C (see Table 1) and extension at 72°C , for 30 seconds each, and final extension at 72°C for

7 minutes in a Biorad *icycler*. The PCR products were run on 2–3 per cent agarose gel and analysed under an ultraviolet illuminator.

Mutation analysis of the *APC* gene

Mutation analysis of the *APC* gene was performed on all cases. Four sets of oligonucleotide primers that have previously been reported were used for fragment-wise amplification (APCA, B, C and D) of codons 1260 to 1596.⁴³ All amplicons were 300 bp in length (Figure 1A–D, Table 1), and were then subjected to single-strand conformation polymorphism (SSCP) analysis. For the samples showing an aberrant band in the SSCP analysis, *APC* BF and *APC* DR primers were used to amplify the 890 bp target region (Figure 1E) and then were subjected to direct sequencing (Macrogen Inc, Seoul, Korea), including the original 300 bp amplicons; however, studying the aberrations of only the MCR was a limitation of this study.

Mutation analysis of the *β -catenin* gene

Genomic DNA from each sample was amplified by PCR using the previously reported primer pair, which amplified a 200-bp amplicon of exon 3 of the *β -catenin* gene (Figure 2A, Table 1).⁴³

Base substitutions in codons 32 and 33 were further confirmed by the *Hinf*I restriction endonuclease assay (Fermentas). The 200-bp PCR product for *β -catenin* contains two *Hinf*I restriction endonuclease sites, yielding 7-bp, 55-bp and 138-bp DNA fragments after digestion of the wild-type allele. *β -catenin* gene mutations in codons 32 and 33 yield only 62-bp and 138-bp fragments after digestion because of ablation of the first *Hinf*I site. The digested products were run on 10 per cent polyacrylamide gel electrophoresis (PAGE) (Figure 2B) to assess the digested fragments.

SSCP analysis

SSCP analysis of PCR products was carried out on 6 per cent non-denaturing polyacrylamide gel (PAG) utilising either non-radioactive silver staining or radioactive procedures, as explained

Table 1. Primer sequences used for the mutational analysis of β -catenin and APC genes in the Wnt pathway

Gene	Amplicon	Primer sequence	Amplicon size (bp)	Annealing temperature ($^{\circ}$ C)
β -catenin	Exon 3	BCat F: 5'-ATG GAA CCA GAC AGA AAA GC-3' BCat R: 5'-GCT ACT TGT TCT TGA GTG AAG-3'	200	58
APC	Exon 15	APC A F: 5'-CAGACTTATTGTGTAGAAGA- 3'	295 for codons	52
		APC A R: 5'-ATCCTGAAGAAAATTCAACA-3'	1260 to 1359	
		APC B F: 5'-AGGGTTCTAGTTTATCTTCA-3'	293 for codons	55
		APC B R: 5'-TCTGCTTGGTGGCATGGTTT-3'	1339 to 1436	
		APC C F: 5'-GGCATTATAAGCCCCAGTGA-3'	290 for codons	55
		APC C R: 5'-AAATGGCTCATCGAGGCTCA-3'	1417 to 1516	
		APC D F: 5'-ACTCCAGATGGATTTTCTTG-3'	300 for codons	55
APC D R: 5'-GGCTGGCTTTTTTGCTTTAC-3'	1497 to 1596			

previously.^{4,44} In non-radioactive SSCP analysis, PCR products were mixed together in denaturing buffer (95 per cent formamide, 10 mM NaOH, 0.05 per cent xylene-cyanol FF and 0.05 per cent bromophenol blue) in a 1:1 ratio, heat denatured at 95 $^{\circ}$ C for 5 minutes and immediately cooled on ice for 20 minutes. Of the resulting product, 6 μ l was loaded on 6 per cent PAG and electrophoresed in 0.5 \times Tris-borate EDTA buffer at \pm 17 $^{\circ}$ C at 4 W constant power for 18–22 hours. Gels were then silver stained. In radioactive SSCP analysis,

radiolabelled PCR products (using α 32-pCTP) were mixed in a denaturing loading buffer (95 per cent formamide, 20 mM EDTA, 0.05 per cent xylene-cyanol FF and 0.05 per cent bromophenol blue) in a 1:10 ratio and heat denatured at 95 $^{\circ}$ C for 5 minutes. Of the resulting product, 3 μ l was loaded on 6 per cent PAG and electrophoresed at 4 W constant power in 0.5 \times Tris-borate ethylene diamine tetra-acetic acid (EDTA) buffer at \pm 17 $^{\circ}$ C for 18–22 hours. The gel was then transferred onto 3 mm Whatman paper, covered with Saran wrap

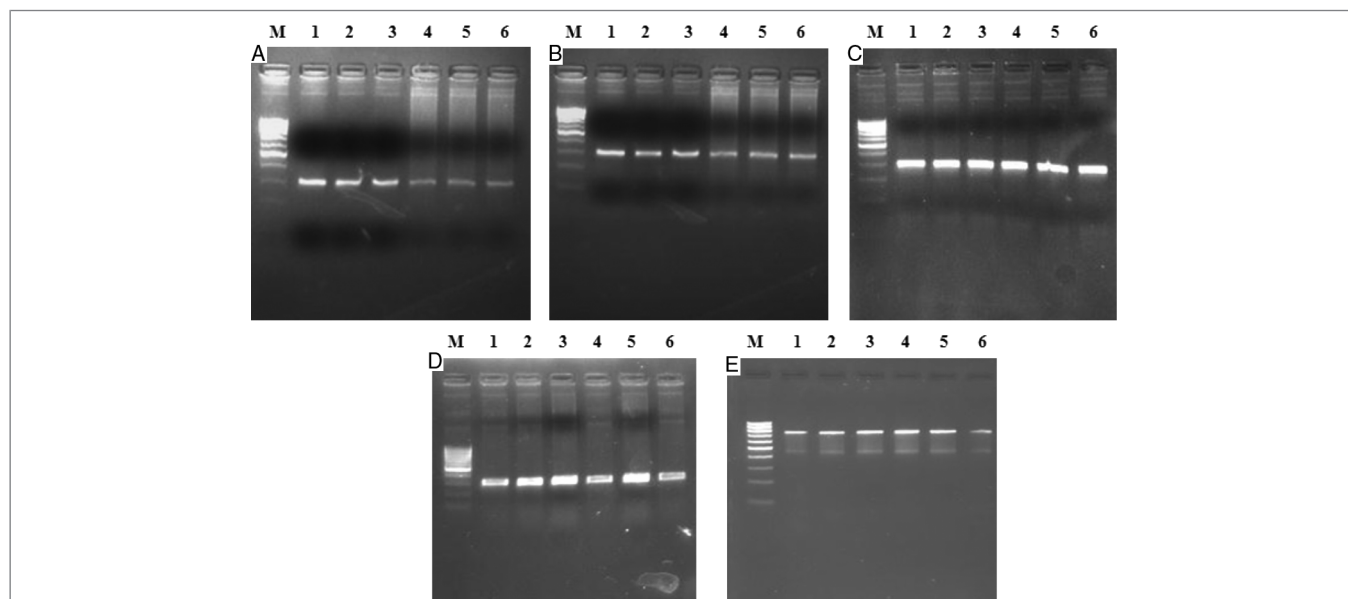


Figure IA–E. Representative gel picture of mutation cluster regions of APC gene comprising of Exon 15 APCA (295bp); APCB (293bp); APCC (290bp); APCD (295bp); and APC Full (890bp) fragments. Lane M: Molecular size marker 100bp (Middle Prominent Band =500bp) Lane 1–6: Amplified product from cancer samples.

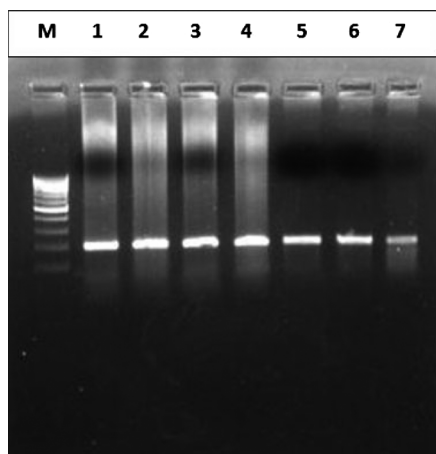


Figure 2A. Amplified DNA fragment (200 bp amplicon) of exon 3 of β -catenin gene. Lane M: 100 bp molecular ladder, Lanes 1–7: Amplicons from different tumour tissues.

and dried in a vacuum drier at 90°C for 1 hour. The Saran wrap was then replaced by X-ray film and kept at –70°C for 48 hours.

The mobility shift in DNA bands was visualised by developing the X-ray film. Purified PCR products of the samples showing mobility shift on SSCP analysis and randomly chosen samples were used for direct DNA sequencing.

Methylation-specific PCR (MS-PCR) of APC promoters

Both normal and tumour DNAs were subjected to sodium bisulphite modification using the EZ DNA Methylation Kit (Zymo Research, Irvine, CA).

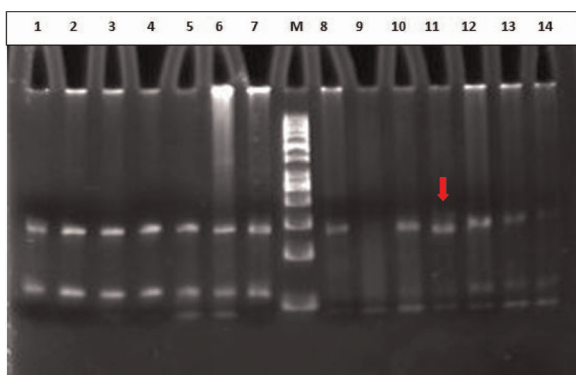


Figure 2B. PAGE analysis of Hinf I digest of 200 bp amplicon of exon 3 of the β -catenin gene. Lane M: 100 bp molecular ladder, Lanes 1–14: Hinf I digested amplicons of the β -catenin gene. Lane 11 shows the codon 32 mutant.

About 10 μ l of DNA from each sample was modified as described in the protocol. Previously reported primer sets were used for amplification of the two promoters – 1A and 1B of the APC gene (Table 2).^{32,37}

PCR was performed in a 50 μ l total volume reaction mixture containing 10 ng of modified genomic DNA, 100 ng of each primer, 100 μ M of each dNTP, 1.5 mM MgCl₂, 5 per cent dimethyl sulphoxide (DMSO), 10 \times Taq buffer and 2 U of Taq DNA polymerase (Fermentas). The reactions were hot started at 97°C for 10 minutes before the addition of 0.75 units of Taq polymerase (Fermentas). The PCR conditions were as follows: 40 cycles of denaturation at 95°C for 40 seconds, annealing at the temperatures specified in Table 1 for 45 seconds, extension at 72°C for 45 seconds and a final extension at 72°C for 10 minutes to complete each PCR. The PCR amplicons were electrophoresed on 2.5 per cent agarose gels and were visualised after staining with ethidium bromide (Figure 3A and 3B).

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.

Direct sequencing

PCR amplicons of the tumour samples and from randomly chosen normal samples were first purified using the DNA Recovery Kit (Zymo Research) and then used for direct DNA sequencing. DNA sequencing was carried out at Macrogen Inc. To minimise the sequencing artefacts by PCR, amplicons from at least two different PCRs were sequenced using forward and reverse primers.

Statistical analysis

All statistical analysis was performed using PASW software, version 18 (IBM, New York, NY). Pearson's chi-square two-proportion test was used to evaluate the hypothesis of equal distribution of molecular alterations with different

Table 2. Primer sequences used for hypermethylation analysis of the promoter region of the *APC* gene

Gene	Amplicon	Primer sequence	Amplicon size (bp)	Annealing temperature (°C)
APC	IA Promoter	APC IA UF: 5'-TGTTTTATTGTGGAGTGTGGGTT-3'	108	60
		APC IA UR: 5'-CCAATCAACAACTCCCAACAA-3'		
		APC IA MF: 5'-TATTGCGGAGTGCGGGTC-3'	98	55
		APC IA MR: 5'-TCGACGAACTCCCGACGA-3'		
APC	IB Promoter	APC IB UF: 5'-GATAGAATAGTGAATGAGTGTTT-3'	195	55
		APC IB UR: 5'-CTTCCAACAACCACACCCCA-3'		
		APC IB MF: 5'-TAGAATAGCGAACGAGTGTTTC-3'	190	55
		APC IB MR: 5'-TCCGACGACCACACCCCG-3'		

clinicopathological variables. A Fisher's two-tailed test (p values) of 0.05 or less was considered to be statistically significant.

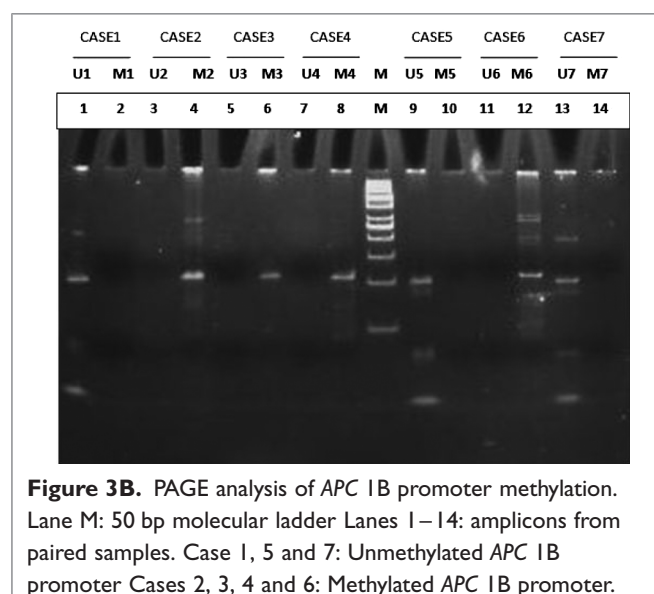
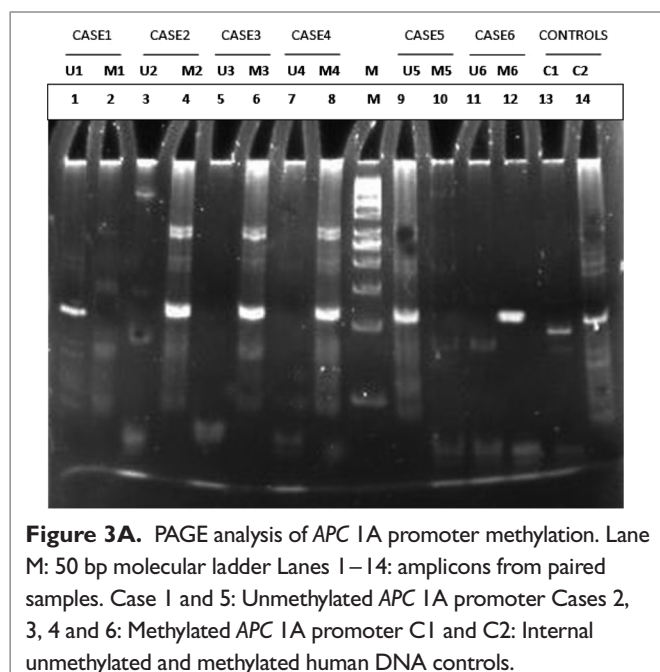
Results

Of 86 confirmed cases of CRC, 38 were of Dukes' A + B stage and 48 were of C + D stage. All patients presented with constipation and bleeding per rectum as their chief complaint. Furthermore, 81 of these cases were sporadic, four had familial adenomatous polyposis and one had Lynch syndrome. All but one case had adenocarcinoma and only one had squamous cell carcinoma (SCC) of

the basal cell type. Thirty-seven patients were female and 49 male; 59 were rural and 27 urban; 36 cases had carcinoma in the colon and 50 in the rectum; and 55 were smokers and 31 non-smokers (Table 3).

Mutation analysis of *APC*

The overall mutation rate of the MCR of *APC* among the 86 patients was 12.8 per cent (11 of 86). This is in contrast to other studies that have reported *APC* as the main gene to undergo aberration in CRC, with a frequency of about 60 per cent. DNA sequencing revealed four missense mutations, three nonsense mutations and four frameshift mutations, including three deletions and



one insertion (Table 4, Figure 4). Among the three nonsense mutations, two were Leu > Stop and one was Lys > Stop.

Table 3. Clinico-epidemiological variables of the 86 CRC patients versus 47 hypermethylated phenotypes of APC (1A and 1B promoter) gene

Variable	Total n = 86	Mutants ^a n = 11 (12.79%)	Methylated ^b n = 47 (54.65%)	p value ^c
Age group				
≤ 60	52 (60.5%)	5	20	<0.05
> 60	34 (39.5%)	6	27	
Gender				
Female	37 (43.0%)	4	19	0.85
Male	49 (67.0%)	7	28	
Dwelling				
Urban	27 (31.4%)	5	17	0.70
Rural	59 (68.6%)	6	30	
Tumour location				
Colon	36 (41.9%)	8	29	<0.05
Rectum	50 (58.1%)	3	18	
Nodal status				
Involved	48 (55.8%)	6	38	<0.05
Not Involved	38 (44.2%)	5	9	
Tumour grade				
A + B	38 (44.2%)	5	9	<0.05
C + D	48 (55.8%)	6	38	
Smoking status				
Never	31 (36.0%)	4	14	0.56
Ever	55 (64.0%)	7	33	
Bleeding PR/Constipation				
No	26 (30.2%)	2	16	0.69
Yes	60 (69.8%)	9	31	
Pesticide exposure				
Never	33 (38.4%)	3	19	0.85
Ever	53 (61.6%)	8	28	

^aOther than G > A transition at codon 1492 of APC.

^bEither 1A or 1B promoter hypermethylation.

^cFisher's two-tailed test for hypermethylation status of APC.

We also found a novel single nucleotide polymorphism (SNP) in our study, a G > A polymorphism in codon 1492 of APC. The polymorphism changes ACG to ACA, without changing the resulting amino-acid residue. We found, that among 86 CRC cases, only 14 (16.3 per cent) had the homozygous wild-type (GG) genotype, while 53 (61.6 per cent) had the homozygous variant (AA) genotype and 19 (22.1 per cent) had the heterozygous (GA) genotype (Table 5, Figure 3). In addition, 72 cases had G > A variants, 67 were variants for G > A at codon 1492 only, while five also had mutations at other sites.

Mutation analysis of the β -catenin gene

The overall mutation rate of the β -catenin gene was 8.1 per cent (seven of 86). Of these seven mutations, three affected codon 32, three affected codon 49 and one affected codon 45 (Figure 5, Table 6); five were missense and two were nonsense mutations. Both nonsense mutations affected codon 49, changing lysine to a stop codon leading to truncation of the protein. In addition, five of the seven patients with β -catenin mutations had higher-grade tumours (C + D). One also had a mutation in APC, but six β -catenin had an intact APC gene.

Hypermethylation of APC promoters

Methylation analysis of APC carried out on two promoters, 1A and 1B, revealed a high methylation status of these two promoters. Forty-seven (54.65 per cent) of the tumours were methylated at either one of the two promoter regions, while 39 (45.35 per cent) of the tumours were not methylated at any of the promoters (Tables 3 and 7). Among the tumours which were methylated, only nine (19.1 per cent) were found to be methylated at the APC 1A promoter exclusively, 15 (31.9 per cent) were found to be methylated at the APC 1B promoter exclusively, while 23 (48.9 per cent) tumours were methylated at both promoters. Furthermore, we also found that only four tumours were mutated as well as hypermethylated for the APC gene

Table 4. Nature of APC mutation cluster region mutations in 11 CRC patients from the Kashmir valley

Patient ID	Mutation ^a	Amino acid change	Affected codon	Effect
A6	<u>TTA</u> > TAA	Leu > Stop	1277	NS
A8	AC <u>CAA</u> > ACA	Del CA	1448/49	FS
A9	<u>TTA</u> > GTA	Leu > Val	1489	MS
A22	<u>AGT</u> > ATT	Ser > Ile	1494	MS
A25	<u>AGA</u> > AGT	Arg > Ser	1336	MS
A27	<u>AGT</u> > ATT	Ser > Ile	1494	MS
A28	<u>TTA</u> > TAA	Leu > Stop	1277	NS
A31	TAAAAG <u>AAAAG</u> > TAAAAGA	Del AAAAG	1307/08/09	FS
A33	TAAAAG > TAAG	Del AA	1307/08	FS
A37	<u>AAG</u> > TAG	Lys > Stop	1449	NS
A77	ATG > AT <u>A</u> G	Ins A	1525	FS
XX ^b	<u>ACG</u> > ACA	Thr > Thr	1492	S

^aMutated, deleted or inserted nucleotide underlined.

^bXX refers to any general tumour sample.

Abbreviations: MS, missense mutation; NS, nonsense mutation; S, silent mutation; FS, Frameshift mutation.

(Table 8). Statistical analysis showed a significant association between *APC* methylation status and the age group, tumour location (colon) and tumour grade (C + D) of the patients (Table 3).

Discussion

The Kashmir valley, located in the northern division of India, has a unique ethnic population,

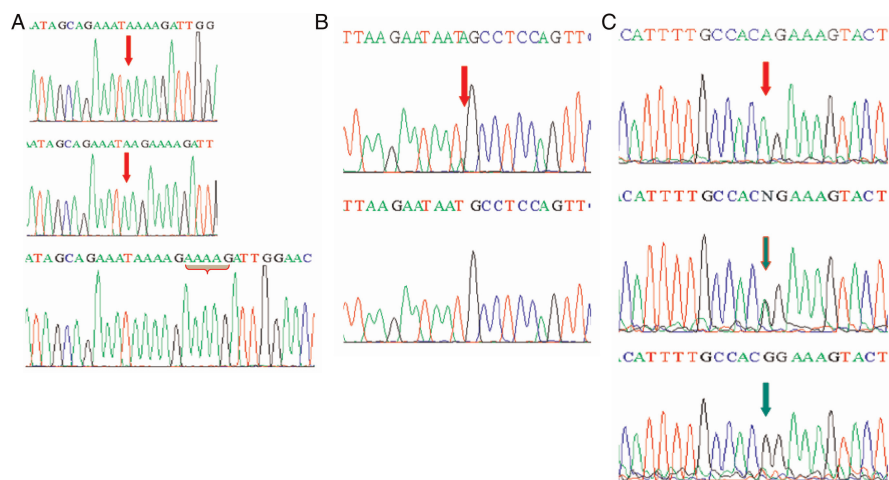


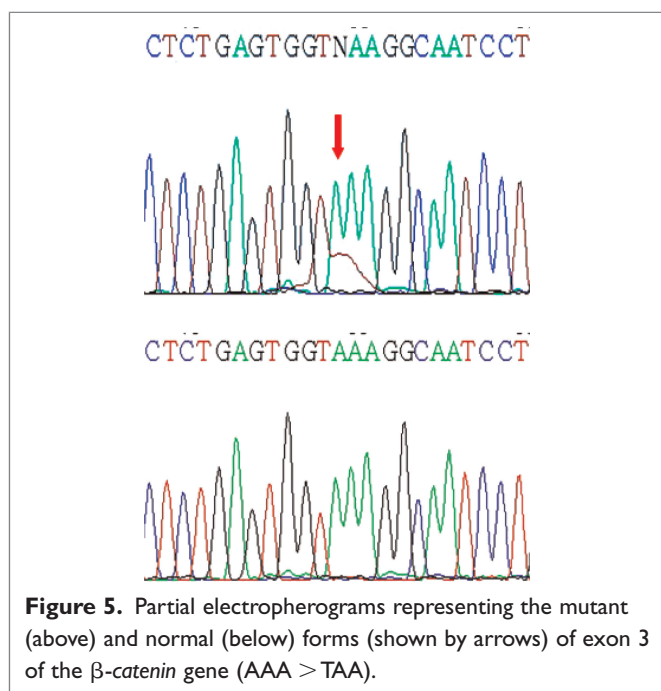
Figure 4. Partial electropherograms representing the mutant (above) and normal (below) forms (shown by arrows) of the mutation cluster region (MCR) of *APC*. (A) Deletion of the AAAAG pentamer at codons 1307/08/09 in the MCR region of *APC* and deletion of the AA dimer at codons 1307/08 in the MCR region of *APC*. (B) Insertion of A at codon 1525 in the MCR region of *APC*. (C) Polymorphism of codon 1492 in the MCR region of *APC*.

Table 5. Codon 1492 status of APC gene in 86 colorectal carcinoma cases in Kashmiri population

APC codon 1492 status	Cases (n = 86)
Variant	53 (61.6%)
Heterozygous	19 (22.1%)
Wild-type	14 (16.3%)

living in temperate environmental conditions and with distinctive food habits, which, along with genetic factors, play a large role in the development of GIT cancers^{3-5,45}. As previously reported, the aetiology and incidence of various GIT cancers in this population has been attributed to a probable exposure to nitroso compounds, amines and nitrates reported to be present in local foodstuffs such as *hoakhe suen* (sun-dried vegetables), *pharei* and *hoggade* (sundried and/or smoked fish and meat), *hakh* (a leafy vegetable of the *Brassica* family), hot *noon chai* (salted tea), dried and pickled vegetables and red chilli, and also through smoking *hukka* (a water pipe).^{2-5, 46}

According to the multi-step model of colorectal tumorigenesis,⁷ the most common and principal causes of APC inactivation are gene aberrations. A

**Figure 5.** Partial electropherograms representing the mutant (above) and normal (below) forms (shown by arrows) of exon 3 of the β -catenin gene (AAA > TAA).**Table 6.** Nature of β -catenin gene in seven colorectal cancer patients from the Kashmir valley

Patient ID	Mutation ^a	Amino acid change	Affected codon	Effect
A1	GAC > GGC	Asp > Gly	32	MS
A5	AAA > TAA	Lys > Stop	49	NS
A9	AAA > TTA	Lys > Leu	49	MS
A10	GAC > GGC	Asp > Gly	32	MS
A62	AAA > TAA	Lys > Stop	49	NS
A74	GAC > GGC	Asp > Gly	32	MS
A82	TCT > TTT	Ser > Phe	45	MS

^aMutated, deleted or inserted nucleotide underlined.

Abbreviations: MS, missense mutation; NS, nonsense mutation.

somatic mutation in APC leads to a truncated protein in most sporadic CRCs.^{15,36} Hypermethylation of APC at the promoter region constitutes an alternative mechanism for APC inactivation in breast, lung and GIT cancers, especially CRCs.^{30,33,34,38,40,47} Combined with these two mechanisms of APC inactivation and the aberrations in the β -catenin gene, the Wnt pathway molecules play an important role in CRC development and progression.^{48,49}

The present study involved the mutational analysis of exon 15 (MCR) of APC and exon 3 of the β -catenin gene and also the hypermethylation analysis of two promoters of APC. Although being the important genetic molecule of the Wnt pathway, and implicated in almost 60 per cent of sporadic CRCs, we found APC gene to be aberrant in only 12.79 per cent of CRCs, which was considerably lower than the previously reported frequencies.^{9,19,35,50-53} This low frequency suggests that

Table 7. APC gene methylation status in 86 CRC cases in the Kashmiri population

APC promoter status	Cases (n = 86)
Either methylated (IA and/or IB)	47 (54.65%)
Only IA methylated	9/47 (19.1%)
Only IB methylated	15/47 (31.9%)
Both methylated	23/47 (48.9%)
Neither methylated	39 (45.35%)

Table 8. Correlation of *APC* mutation status versus *APC* methylation status

	APC status		OR; 95% CI; p value
	Wild-type W = 75	Mutant ^a M = 11	
APC promoter methylation^b			
Unmethylated; n = 39	32 (42.7%)	7 (63.6%)	2.35; 0.63–8.72; 0.22
Methylated; n = 47	43 (57.3%)	4 (36.4%)	

^aOther than G > A transition at codon 1492.

^bEither 1A or 1B promoter hypermethylation.

Abbreviations: OR, odds ratio; CI, confidence interval.

APC may not be the foremost gene to be implicated in the development of CRCs in this population.

Furthermore, we found an SNP (G > A) at codon 1492 in 72 (83.7 per cent) CRC cases. Out of 72 cases, 53 were homozygous variants. This was a novel finding, as it has not been reported previously.

We also found a low frequency (8.1 per cent; seven of 86) of β -catenin mutations in CRC. These results were in line with those in the published literature.^{54–57} Exon 3 of *β -catenin* contains a regulatory domain which is the hotspot for genetic aberrations. Mutations in this exon have been reported in various tumours, resulting in its nuclear accumulation and leading to progression of the tumour.^{9,43,58} The mutations in the hotspot codons – 32, 33, 41, 45 and 49 – in exon 3 of *β -catenin* result in an amino acid change at the GSK-3 β phosphorylation sites, which in turn affect the phosphorylation mechanism and result in the decreased sequestration of *β -catenin* by *APC*.⁵⁸ Furthermore, the mutation affecting codon 45 (TCT > TTT; Ser > Phe) was present in a Lynch syndrome patient, as has been reported previously.^{55,56} Also, six of seven tumour samples which harboured *β -catenin* gene mutations were wild-type for *APC* (MCR only), which further corroborated findings in the literature that mutations of the genes encoding these two Wnt pathway molecules are mutually exclusive.^{9,54,57,59,60} Only one case (A9) had mutations in both genes (Tables 4 and 6). Overall, mutations in Wnt pathway molecule genes were found to be present in 20.9 per cent (18 of 86) of CRC cases.

Thus, our observation identifies this pathway as being important in determining the development and progression of CRC but is less important than in other populations, where the mutational frequency of these *Wnt* gliadators is higher.

CpG island hypermethylation is one of the important mechanisms of gene inactivation. Cancer cell lines have in general demonstrated an increased frequency of hypermethylation by comparison with primary tumours.⁶¹ Inactivation of tumour suppressor genes by promoter hypermethylation has been recognised to be as common as gene disruption by mutation in tumorigenesis.^{36,37,62,63} A number of studies on CRC around the globe have demonstrated the role of promoter hypermethylation of a number of different genes in the development and progression of CRC.^{32,64,65} Promoter hypermethylation of *APC*, similarly to that of other genes, plays a pivotal role in the inactivation of *APC*, which in turn enhances tumour development.^{30,33}

In the present study, we found hypermethylation in 54.65 per cent (47/86) of CRC cases, which is consistent with the results found in some other major studies, although markedly higher than reported in others.^{30,32,33} However, only 26.7 per cent (23/86) of the tumours were hypermethylated at both the 1A and 1B promoters. This may be due to the fact that there is less mutational inactivation of *APC* in this population and also because this population is exposed to a special set of environmental challenges, such as extreme temperature, high altitude, special food habits and exposure to agricultural by-products such as pesticides and nitrosamines.^{5,46} As has been revealed in previous studies, promoter hypermethylation constitutes an

alternative hit in the inactivation of *APC* in cancers,^{39,66} and we have identified the same phenomenon as the major cause of *APC* inactivation in our population. Various studies have shown transcriptional repression of *APC* by hypermethylation in tumours as well as cell lines.^{34,67}

Arnold *et al.* demonstrated the loss of protein expression due to the promoter hypermethylation of *APC*.³⁹ In addition, 42.8 per cent (three of seven) of patients with a mutation in β -catenin were also found to have hypermethylation of *APC*. We found the methylation status of the *APC* promoter to be associated with age (>60), tumour location (colon) and nodal status/tumour grade (C + D) in CRC.

Conclusion

We conclude that, in the Kashmir valley population, although mutational aberration of the genes encoding two pivotal molecules of the Wnt pathway – *APC* and β -catenin – occurs at a low frequency in CRC cases, the high level of epigenetic silencing of *APC* plays a pivotal role in the initial tumorigenesis and also enhances the chances of tumour development and progression to advanced stages.

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