Chapter 14

Possible Role of Proto-Oncogenes in Colorectal Cancer — A Population Based Study

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Additional information is available at the end of the chapter

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1. Introduction

Cancer is not just one disease, but a generic term used to encompass a group of more than two hundred diseases sharing common characteristics. From a clinical point of view, cancer is a large group of diseases, that vary in their age of onset, rate of growth, state of cellular differentiation, diagnostic detectability, invasiveness, metastatic potential, response to treatment, and prognosis. From a molecular and cell biological point of view, however, cancer may be a relatively small number of diseases caused by similar molecular defects in cell function resulting from common types of alterations to a cell's genes. Ultimately, cancer is a disease of abnormal gene expression. There are a number of mechanisms by which this altered gene expression occurs. These mechanisms may occur via a direct insult to DNA, such as a gene mutation, translocation, amplification, deletion, loss of heterozygosity, or via a mechanism resulting from abnormal gene transcription or translation. The overall result is an imbalance of cell replication and cell death in a tumor cell population that leads to an expansion of tumor tissue. Cancers (carcinomas) are characterized by their unregulated growth and spread of cells to other parts of the body [1,2]. Treatment of an individual diagnosed with cancer is not only dependent upon which type of malignancy (cancer) they have, but also on the extent of its spread, together with its sensitivity to treatment [3].The total care of the patient will involve assessment of their physical, psychological and social needs, so that a complete care package can be developed to support them and their carer(s) throughout the whole of their patient.

1.1. Colorectal cancer

Colorectal cancer (CRC), less formally known as bowel cancer, is a cancer characterized by neoplasia in the colon, rectum, or vermiform appendix. CRC is a leading cause of cancer mortality in the Western World. In the United States, CRC is the third most commonly diagnosed cancer in men and women and the second leading cause of cancer-related mortality [4]. Because 5% of persons (1 in 20 persons) will develop colorectal cancer, this disease is an important public health issue.

1.2. Incidence of colorectal cancer

Globally, cancer of the colon and rectum is the third mostcommon cancer in males and in females with mortality paralleling incidence [5]. An estimated 141,210 cases (71,850 male and 69360 female) of CRC were expected to occur in 2011.An estimated 49, 380 deaths (25,250 male and 24,130 female)of CRC were expected to occur in 2011, accounting for about 9% of all cancer deaths (Table 1). The5-year survival is 90% when CRC is diagnosed at an early stage however, less than 40% cases are diagnosed when the cancer is still localized [6]. The frequency of CRC varies remarkably among different populations. The incidence of colorectal cancer is increasing in certain countries where risk was historically low (Japan, Puerto Rico). In high-risk countries, trends are gradually increasing (England), stabilizing (New Zealand), or declining (United States) with time. The greatest increases in the incidence of colorectal cancer are in Asia (Japan, Hong Kong, Singapore), Eastern Europe (Hungary, Poland), Israel, and Puerto Rico. In contrast to the recent decrease in rates seen in some western and northern European countries, relatively large increases have been observed in Spain. The decrease in incidence in the United States partially reflects the increase in detection and removal of precancerous lesions; the increase in several Asian and Eastern European countries may reflect changes in the prevalence of obesity and dietary patterns. Age standardized incidence of colorectal cancer around the world is depicted in graph 1.

In India, CRC does not figure amongst the 10 most common malignancies. The age-standardized rates of CRC in India have been estimated to be 4.2 and 3.2/100,000 for males and females, respectively.

Inter-regional differences in the incidence of CRC, including difference among population groups living in geographic proximity but with different life styles, suggest that environment plays a role in the development of the disease [7]. Change in the location of these tumours is seen with increasing age. The proportion of tumours beyond the reach of sigmoidoscopy increases with age [8]. Sub site distribution also may differ according to ethnicity [9]

1.3. Risk factors

Epidemiologic studies have revealed a number of risk factors for colorectal cancer including age, family history of colon cancer or inflammatory bowel disease, smoking, alcohol consumption, obesity, and diet.

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Estimated New cases		Estimated Deaths	
Male	Female	Male	Female
Prostate	Breast	Lung & bronchus	Lung & bronchus
240,890 (29%)	230,480 (30%)	85,600 (28%)	71,340 (26%)
Lung & bronchus	Lung & bronchus	Prostate	Breast
115,060 (14%)	106,070 (14%)	33,720 (11%)	39,520 (15%)
Colon & rectum	Colon & rectum	Colon & rectum	Colon & rectum
71,850 (9%)	69,360 (9%)	25,250 (8%)	24,130 (9%)
Urinary bladder	Uterine corpus	Pancreas	Pancreas
52,020 (6%)	46,470 (6%)	19,360 (6%)	18,300 (7%)
Melanoma of the skin	Thyroid	Liver & intrahepatic bile duct	Ovary
40,010 (5%)	36,550 (5%)	13,260 (4%)	15,460 (6%)
Kidney & renal pelvis	Non-Hodgkin lymphoma	Leukemia	Non-Hodgkin lymphoma
37,120 (5%)	30,300 (4%)	12,740 (4%)	9,570 (4%)
Non-Hodgkin lymphoma	Melanoma of the skin	Esophagus	Leukemia
36,060 (4%)	30,220 (4%)	11,910 (4%)	9,040 (3%)
Oral cavity & pharynx	Kidney & renal pelvis	Urinary bladder	Uterine corpus
27,710 (3%)	23,800 (3%)	10,670 (4%)	8,120 (3%)
Leukemia	Ovary	Non-Hodgkin lymphoma	Liver & intrahepatic bile duct
25,320 (3%)	21,990 (3%)	9,750 (3%)	6,330 (2%)
Pancreas	Pancreas	Kidney & renal pelvis	Brain & other nervous system
22,050 (3%)	21,980 (3%)	8,270 (3%)	5,670 (2%)
All sites	All sites	All sites	All sites
822,300 (100%)	774,370 (100%)	300,430 (100%)	271,520 (100%)

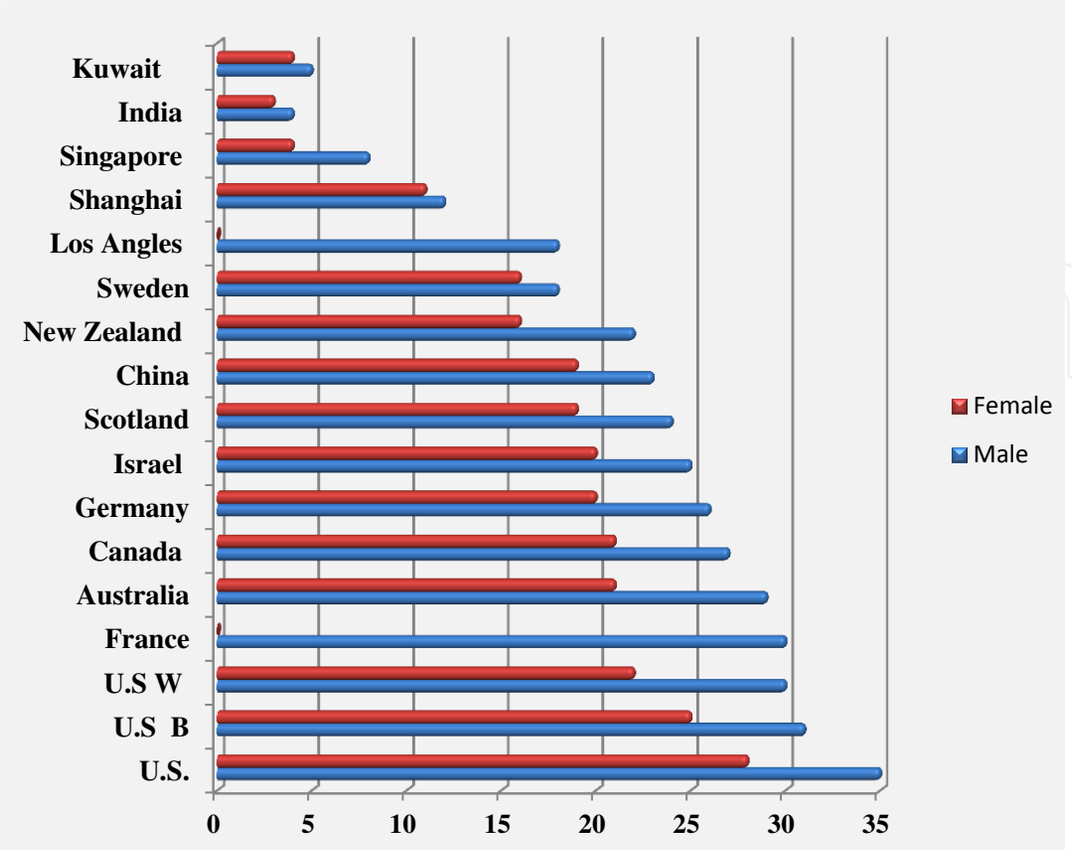
Table 1. Leading Sites of New Cancer Cases and Deaths (2011, American Cancer Society, Inc., Surveillance) 2011.

Age

Colorectal cancer is most commonly found in those aged 50 years and over.

Sex

Men are more likely than women to develop colorectal cancer. The incidence rate of colorectal cancer between 2000 and 2004 was 69.2 per 100,000 population among men and 45.8per 100,000 populations among women [10]



Graph 1 Family History

Graph 1: Age standardized incidence of colorectal cancer/100,000populations around the world (Arshad et al., 2011)

According to the CDC (Centre for Disease Control and Prevention), those who have a family history of colorectal cancer are at higher risk for developing colorectal cancer themselves. In addition to particular genetic pathways that are activated in the development of colon cancer, there are also known genetic mutations that can be inherited and make up approximately10% of all colorectal cancer cases [11]

Smoking

Tobacco use does not only put persons at risk for higher rates of lung, mouth, and esophageal cancers, it has also been associated with higher risk for developing colon cancer [11,12]

Diet

There have been a number of different dietary factors that have been linked to a higher risk of colorectal cancer including higher levels of red meat consumption, low levels of fruit and vegetable consumption, and diets that are low in fiber.

Obesity

Obesity is an important risk factor to consider based on the recent trends in the U.S. A number of studies have shown that being overweight is associated with increased risk of colorectal

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cancer. A case-control study conducted by Caan *et al* [13] found that men who had a BMI in the highest quintile were almost 2 times as likely to develop colon cancer as men with a BMI in the lowest quintile.

1.4. Classification and grade of CRC

Staging describes the extent or spread of the disease at the time of diagnosis. It is essential in determining the choice of therapy and in assessing prognosis. Stage is based on the primary tumour's size and location and whether it has spread to other areas of the body. A number of different staging systems are used to classify tumours. For CRC patients' pathologic stage represents one of the most important prognostic factors. The Dukes' system was the classic staging method for CRC, however the tumour, node, metastasis (TNM) staging system is more detailed and is most commonly used today. On occasion, Roman numerals I through IV are used in CRC staging (Table 2). These numerals correspond with Dukes' classes.TNM staging system is useful for descriptive and statistical analysis of tumour registry data. If cancer cells are present only in the layer of cells where they originated and have not penetrated the basement membrane of the tissue, the stage is in situ; otherwise it is invasive. Stage is categorized as local if cancer cells are confined to the organ of origin, regional if the cells have spread beyond their original (primary) site to nearby lymph nodes or tissues, and distant if they have spread from the primary site to distant organs or distant lymph nodes.

AJCC stage	TNM stage	TNM stage criteria for colorectal cancer
Stage 0	Tis N0 M0	Tis: Tumour confined to mucosa; cancer-in-situ
Stage I	T1 N0 M0	T1: Tumour invades submucosa
Stage I	T2 N0 M0	T2: Tumour invades muscularispropria
Stage II-A	T3 N0 M0	T3: Tumour invades subserosa or beyond (without other organs involved)
Stage II-B	T4 N0 M0	T4: Tumour invades adjacent organs or perforates the visceral peritoneum
Stage III-A	T1-2 N1 M0	N1: Metastasis to 1 to 3 regional lymph nodes. T1 or T2.
Stage III-B	T3-4 N1 M0	N1: Metastasis to 1 to 3 regional lymph nodes. T3 or T4.
Stage III-C	any T, N2 M0	N2: Metastasis to 4 or more regional lymph nodes. Any T.
Stage IV	any T, any N, M1	M1: Distant metastases present. Any T, any N.

Table 2. TNM staging for colorectal cancer

1.5. Genetics of CRC

Fifteen years ago, Fearon and Vogelstein [14] proposed a genetic model to explain the stepwise formation of CRC from normal colonic tissues. This model states that 1) CRC is the result of changes (mutations) of genes with important functions in regulating cell proliferation or repair

of DNA damages, 2) mutations in more than one gene are required, and 3) the sequence of mutations is important in determining the eventual formation of CRC. The model is illustrated in (Figure 1), which also incorporated information from more recent studies.

The genes involved in the genetic paradigm leading to CRC can be broadly divided into two classes: tumour suppressor genes (TSGs) and oncogenes. TSGs encode proteins that either inhibit cell proliferation or promote apoptosis.

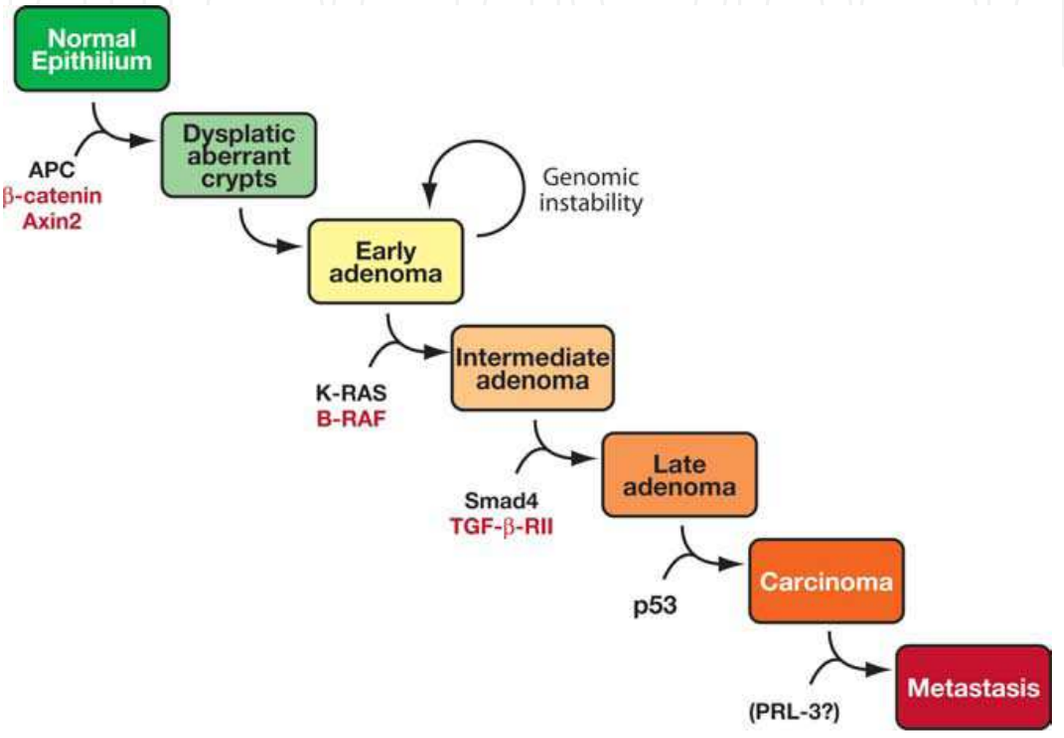

Figure 1. Correlation between CRC Progression and the accumulation of genetic alterations according to Fearon & Vogelstein (1990). The genetic alterations frequently found in CIN tumours are depicted in black; genetic alterations more common in MIN tumours are depicted in red.

TSGs are often inactivated in CRC. In contrast, oncogenes are activated versions of proto-oncogenes, which are often involved promoting cell proliferation or development. Once activated, oncogenes can lead to accelerated cell growth and contribute to tumour formation [15]. It is widely accepted that the molecular genetics of human cancers can be used to categorize colorectal carcinomas into two major types of genomic instabilities, chromosomal instability (CIN) and microsatellite instability (MSI) [16].The majority of colorectal carcinomas are categorized into the CIN pathway, which is characterized by a high frequency of allelic losses, deletions, and/or mutations of tumour suppressor genes such as APC and p53, and abnormal tumour DNA (Figure 2) [16]. Aneuploidy in CIN phenotype tumours had been demonstrated in colorectal cancer cell lines and tumour tissues. Although CIN is a common finding in colorectal carcinomas, the mechanism of CIN has not been clearly elucidated. Defects in DNA replication check point genes and many other genes increase the rate of genome rearrangement and it is suggested to be associated with CIN [17].

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The other pathway, namely the MSI pathway, begins with the inactivation of one of a group of genes responsible for DNA nucleotide mismatch repair, which leads to extensive mutations in both repetitive and non-repetitive DNA sequences with low frequencies of allelic losses and rare alterations of tumour DNA content [18]. The mechanism of tumorigenesis in high-microsatellite instability (MSI-H) tumours is thought to involve frame shift mutations of microsatellite repeats within coding regions of the affected target genes, and the inactivation of these target genes is believed to directly contribute to tumour development and progression. Although these two distinct major genetic pathways of genetic instabilities are widely accepted, some tumours reveal different genetic pathways i.e., some tumours show both types of genomic instabilities and some tumours do not show any of these two instabilities. Further evidence for alternative pathways come from studies which show that mutations in *APC, KRAS* as well as *p53* do not occur in all tumours and some tumours may only contain a mutation in one of these genes. Another novel pathway has been described termed the CpG island methylator phenotype (CIMP) [17]. Two groups of tumours were identified. CIMP-positive tumours show a high degree of CpG island methylation in genes such as *p16* and *hMLH1* and are accompanied by mutations in *KRAS* and *TGF RII.* CIMP-negative tumours, which by definition do not contain a high degree of methylation, are characterized by *p53* mutations. CIMP-positive tumours may show a degree of correlation with the MSI pathway. Finally, colorectal cancers, arising from ulcerative colitis, do not develop from adenomas suggesting that they follow yet another different pathway 9 (Figure 2) [19]

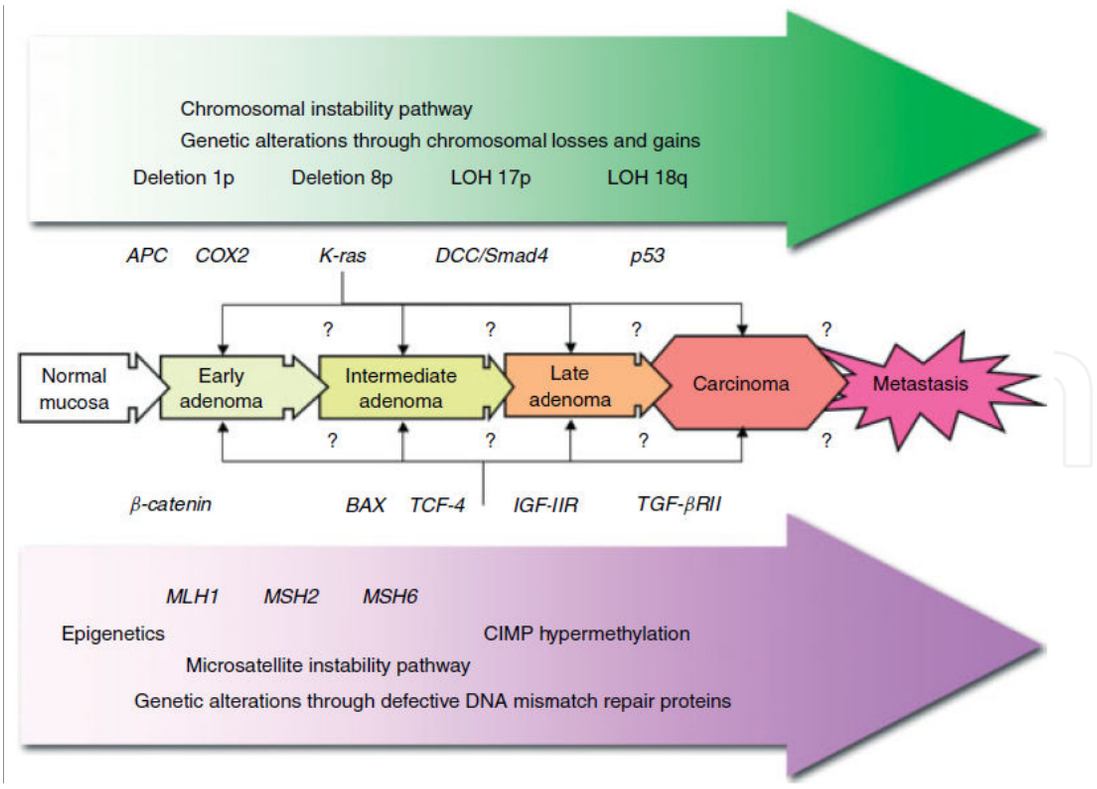

Figure 2. Characteristics of the two major pathways in CRC.

1.6. Axin

Axin1 (also simply called Axin), which encodes isoformaand b, and *Axin2* (also called Axil or Conductin) have 45%identity at the nucleotide level and the proteins they encode appear to be functionally similar. However, whereas *Axin1* is expressed ubiquitously during mouse embryogenesis, *Axin2* is expressed in a restricted pattern [20]. *Axin1* is the constitutively expressed component of the degradation complex and is essential for the maintenance of low Wnt signalling activity in the basal state. In contrast, *Axin2* is upregulated in response to increased β-catenin concentrations and thus serves to limit the duration and intensity of the Wnt signal [21]. *Axin* is downregulated in a Wnt dependent manner and is dephosphorylated after Wnt stimulation, which leads to *Axin1* destabilisation over time. Cells that receive Wnt ligand signals have low concentrations of *Axin*. Biochemical studies show that the intracellular concentrations of *Axin* are approximately 1000 times lower than other destruction complex components, suggesting that *Axin* is the limiting factor in this pathway [22]

1.6.1. Role of Axin in signaling pathways

Axin has emerged as a major scaffold protein for regulating a variety of signaling pathways and biological functions (Figure 3). In Wnt signalling, *Axin* binds to many components in the pathway, including the Wnt co-receptor LRP (low-density lipoprotein-related protein receptor) [23] Dishevelled or Dvl [24], tumour suppressor adenomatous polyposis coli (APC), GSK-3β, β-catenin [25], Casein kinases [26], protein phosphatase 2A (PP2A) [27], Diversin [28] *Ccd1* [29], and *Axam* [30].Interestingly, *Axin* itself is regulated with its stability being modulated by Wnt receptors, Dvl [31], and phosphorylation by GSK-3β. Inaddition, *Axin* also interacts with proteins that have no close relevance to Wnt signalling, including MAP kinase kinase (MEKK) [32, 33], I-MFA [34], DCAP [35], SH2/3 adaptor protein Grb4 [36], and Smad3. Interaction of *Axin* with MEKK leads to JNK activation, proceeding through a cascade from Axin, MEKK, and MKK to JNK[37].The most intriguing aspect of JNK activation by Axin is that multiple seemingly concrete structural elements of Axin are required [38]. Axin interacts with Smad3 and affects TGF-β signalling pathway.

1.6.2. Mutation of Axin in colorectal cancers

Alterations in both *Axin1* and *Axin2* have been detected in several different tumours. Mutations are found in most *Axin* domains including the APC (RGS) and β-catenin-binding domains. *Axin* sequence variants have also been found in colon, ovarian, endometrioid, adenocarcinoma, and HCC cell lines. Biochemical and functional studies have shown that these mutations interfere with the binding of GSK3 and that they also alter the interaction between *Axin* and two upstream activators of TCF-dependent transcription, Frat1, and DVL. Many components of the Wnt signalling system are mutated in colorectal cancer. Germ line loss of function mutations in the APC gene are associated with an inherited form of colorectal cancer —familial adenomatous polyposis— with 90–95% penetrance. Somatic APC mutations are also found in most sporadic colorectal cancers [39]. Alterations in other components of Wnt

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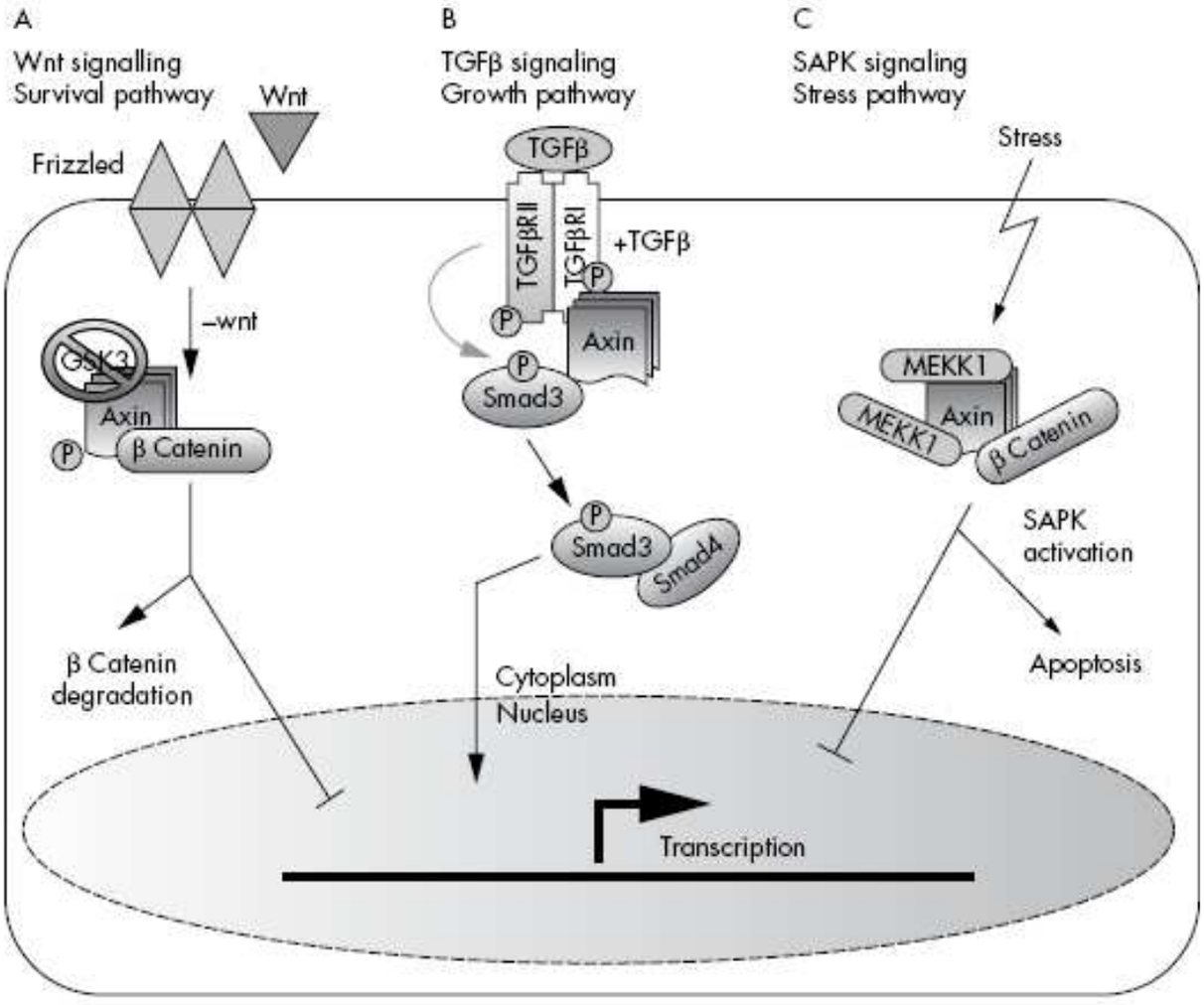

Figure 3. Regulation of three signalling pathways by Axin (1) Axin in the absence of Wnt ligand stimulates β-catenin degradation by proteosome complex and halts its transcriptional activity (2) The presence of transforming growth factor receptor signals Axin and stimulates Smad phosphorylation by TGF-β receptor I & II. The activated Smads then translocate into nucleus and stimulates transcription of downstream target gene. (3) Cells subjected to stress Axin bind to mitogenactivated protein and stimulate stress-activated protein kinase (SAPK/Jun) mediated apoptosis. (courtesy. S Salahshor et al. 2004)

signalling, including β-catenin, TCF, *Axin1*, and *Axin2*, found in colorectal cancer indicate the important role that this pathway plays in the etiology of this disease [40]. Most *Axin1* mutations in colorectal cancer occur between exon 1 and 5, where the APC, GSK3, and β-catenin-binding domains are located. Mutations in *Axin2* have been found in approximately 20% of mismatch repair deficient colorectal tumours [41]. In most cases, one base deletion or insertion occurs in the mononucleotide repeat sequences located in exon 7, leading to a frame shift and premature protein truncation [42]. These mutations lead to elimination of the DIX domain, where DVL binds and negatively regulates *Axin* activity. This domain is also essential for homo-oligomerization of *Axin*. The mutant form of *Axin2* appears to be more stable than the wild-type

protein. Transfection of normal fibroblasts with *Axin2* mutants led to the accumulation of β-catenin in the nuclei. Li-Hua Jin *et al*.2003analysed 54 colorectal tumour tissues for *Axin1* mutation and reported 11% missense mutation suggesting *Axin* mutation may contribute to the onset of colorectal tumourigenesis. Webster *et al* [43] screened *Axin* gene in a range of human tumour cell lines including colon cancer cell lines. They identified two sequence variants carrying a substitution in four colon cancer cell lines. Biochemical and functional studies carried out by them showed that the L 396M change interfered with Axin'sability to bind GSK-3. Interestingly, this mutation and a neighboring L392M change differentially altered Axin'sability to interfere with two upstream activators of TCF dependent transcription factor Frat-1 and Dishevelled. Suraweera *et al* [44] reported heterozygous frame shift mutation and an in frame deletion in exon 7 of *Axin2*. They also reported 8% mutation of *Axin* in colon cancer cell lines. These studies indicates role of *Axin* gene in colorectal carcinogenesis.

1.7. Deleted in colorectal cancers (DCC) gene

The development of human cancer has been proposed to be a multistep process [45]. Vogelstein et al., 1988 showed that colonic tumorigenesis provides the systematic course to the multistep hypothesis at the molecular level. Several genes have been identified that alter during tumour progression. Frequent and consistent loss of heterozygosity (LOH) of specific chromosomes in human cancers has been associated with the presence of tumour suppressor genes [46]. In particular, the long arm of chromosome 18 has been shown to be lost in about 75% of colonic cancers [47].The tumour-suppressor gene *DCC* (deleted in colorectal carcinoma),located on the long arm of chromosome 18 (Figure 2) encodes a cell surface protein containing homology with N-CAM [14].*DCC* a putative tumour suppressor gene has been mapped on the long arm of 18th chromosome (18q). In normal conditions, *DCC* induced apoptosis limits cellular lifespan in the intestinal crypt and thereby inhibits the initiation of malignant transformation. Transfection of *DCC* cDNA into a human cell line lacking *DCC* expression suppresses tumour growth and results in apoptosis and cell cycle arrest [48].

1.7.1. Loss of heterozygosity of DCC gene

Human cancers arise by a combination of discrete mutations and chromosomal alterations. Loss of heterozygosity (LOH) of chromosomal regions bearing mutated tumour suppressor genes is a key event in the evolution of epithelial and mesenchymal tumours. The term Loss of heterozygosityv (LOH), refers to a technique widely used in cancer research.LOH relies upon an individual processing two non-identical alleles for specific genetic marker, which can be distinguished from each other. These individuals are referred to as heterozygote with respect to this allele. Distinguishing between alleles can be done by the presence of a restriction site on one allele or through polymorphic microsatellite repeats (also referred to as microsatellite markers).In the latter the alleles differ from one another based on their size. Using LOH, a comparison is made between the DNA extracted from normal and tumour tissue. If an allele is present in the normal DNA but missing in the tumour than we can suggest that this region of DNA has been lost or deleted through mutation. Therefore the tumour cells have lost an

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allele as only one is detected, hence loss of heterozygosity. Most commonly the deletion of DNA will not be isolated to just this marker but will more than likely also involve the loss of gene surrounding that region. This is important if the surrounding region contains one or more tumour suppressor genes. In fact LOH studies are often used to examine neoplasms to locate frequent chromosomal regions that are lost and hence may harbor putative tumour suppressor genes pivotal in the development of cancer. The greater the degree of LOH, the more genetically unstable the tumour type and more aggressive it is likely to be.

Global patterns of LOH can be understood through allele typing of tumours with polymorphic genetic markers. Simple sequence length polymorphisms (SSLPs or microsatellites) are reliable genetic markers for studying LOH. Microsatellites are short repetitive sequences of DNA that are scattered throughout the genome and are stably inherited, unique to each individual and have low inherent mutation rate [49]. Several studies have shown that alterations due to mutations in the simple repeat sequences or microsatellites are a feature in a number of cancers [50].Researchers working on colon cancers found the length of microsatellite DNA in tumour tissue vary from matching normal tissue. This variation in length of microsatellite represents a mutational process of insertion or deletion within tumour DNA [51].Loss of heterozygosity (LOH) i.e., loss of one allele at a constitutional heterozygous locus indicates the probability of loss of a tumour suppressor gene, which might promote neoplastic progression [52].

2. Aim and objectives

India is heavily burdened with CRC. Most of the genes implicated in CRC (like *APC, KRAS, SMAD* etc) have been studied in CRC patients of this population. Results therein have depicted either some semblance or little discrepancies in CRC in comparison to other studies conducted in other ethnic groups. The important genes like *Axin 1*, *Axin 2* and *DCC* have been reported to be involved in etio-pathology of CRC, but their role is yet to be elucidated in CRC patients of North India. Keeping in view of this, we carried out this study with following objectives

- To analyse the mutations, if any, in the coding exons (1a,1b,1c,2,4,6 and 10) of *Axin*1 gene
- To analyse the mutations, if any, in exon 7 of *Axin2* gene.
- To establish the correlation of *Axin1* and *Axin2* gene mutation with clinicopathological variables of CRC patients
- To analyse expression of *Axin* in CRC patients using western blotting technique andto correlate the altered expression of *Axin* with clinico -pathological characteristics of CRC patients.
- To analyse Loss of Heterozygosity of *DCC* gene at VNTR and D18S8-M2 markers in CRC patients and to correlate LOH of *DCC* gene with clinicopathological variables.
- Polymorphic studies of SNPs at codon 399 of *XRCC1* genes.

The main goals of this work are based on the hypothesis to understand

- What is the role of *Axin* 1 and *Axin* 2 gene aberrations in CRC?
- To understand the pattern of Axin expression in CRC tumours with respect to normal samples?
- What is the role of *DCC* gene aberrations in CRC?
- What is the role of Arg399Gln SNP of *XRCC1* gene in CRC?

3. Methodology and results

3.1. Mutational analysis of *Axin 1* and *Axin 2* gene

Characteristics of the study subjects

A total of fifty (n=50) tissue samples of colorectal carcinoma and their adjacent normal samples were used for mutational analysis of *Axin1* and *Axin2* gene. Same samples were used for analysis of Axin protein expression. Tumour and adjacent normal tissue samples were collected in the General Surgery Department (SKIMS) after surgical resection. All the resected tissue specimens were histologically confirmed to be colorectal carcinomas by a panel of 2 expert pathologists. Median age at the time of diagnosis was 52 years (range 30-75); and male: female ratio was 1:1. Clinico-pathological characteristics of patients are given in table 3.On the basis of age, the patients were grouped into two categories, less than 50 years (<50) and greater than or equal to 50 years of age (≥50). The number of cases in the age group of ≥50 were 62 % (31/50) and less than <50 years were 38 % (19/50).In this study 29(58%) patients had cancer in the colon while as cancer of rectum accounted for 21(42%) of CRC cases. 33(61%) cases of CRC were well differentiated and 17(34%) were poorly/moderately differentiated. 31(62%) of CRC patients belonged to rural area and 19 (38%) to urban area. Based on the smoking status, 21(42%) patients were non-smokers and 29 (58%) were smokers. Almost all the patients with left colon carcinoma had attended the hospital with a clinical presentation of bleeding per rectum.

3.2. Molecular analysis of *Axin* 1 and *Axin 2* gene

High molecular weight genomic DNA isolated from the samples (tumour tissues and corresponding normal tissues) (Figure 4) were subjected to PCR to amplify the exon 1a, 1b, 1c, 2, 4, 6 and 10 of *Axin1* and exon 7 of *Axin2*.The representative gel pictures of each amplified exon of *Axin1* and *Axin2* genes are given in figure 5. PCR products were purified manually and then purified samples were subjected to DNA sequence analysis. To identify the sequence variations, the electrophoregram obtained after sequencing of the PCR products were compared manually with the reference sequence of the *Axin1* and *Axin2* gene deposited in the NCBI Gene Bank database (Accession No.NC 000016 & NC 000017).

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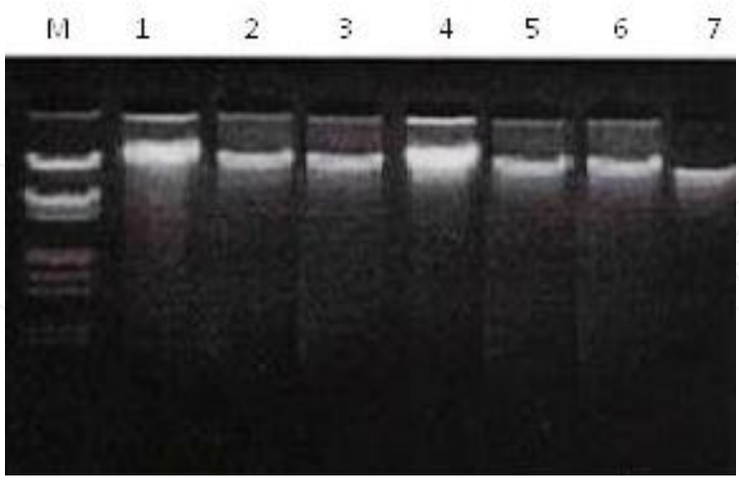
Clinico-epidemiological Parameters	Subgroup	Cases (n=50)
Grade/Differentiation	WD	33 (66%)
	MD/PD	17 (34%)
Stage	I/II	28(56%)
	III/IV	22(44%)
Location	Colon	29 (58%)
	Rectum	21 (42%)
Dwelling	Rural	31(62%)
	Urban	19 (38%)
Age	<50	19 (38%)
	≥50	31 (62%)
Sex	Male	24 (48%)
	Female	26 (52%)
Smoking status	Never	21(42%)
	Ever	29(58%)

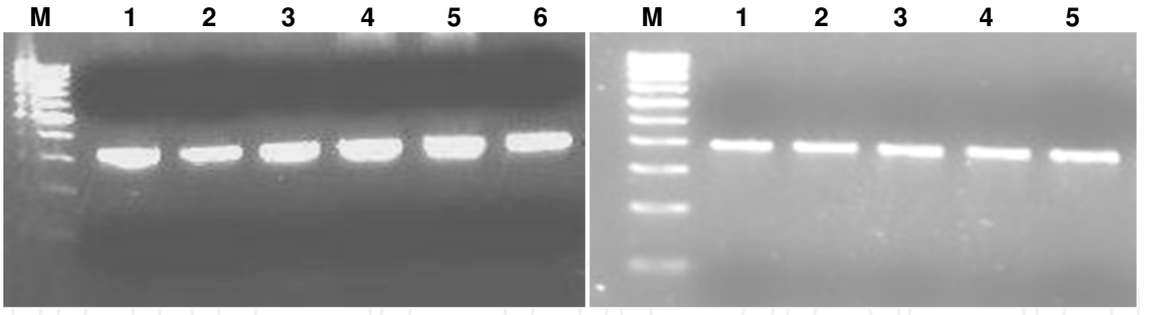
WD: well differentiated; MD: moderately differentiated, PD: poorly differentiated

Table 3. Clinico-epidemiological characteristics of the CRC patients

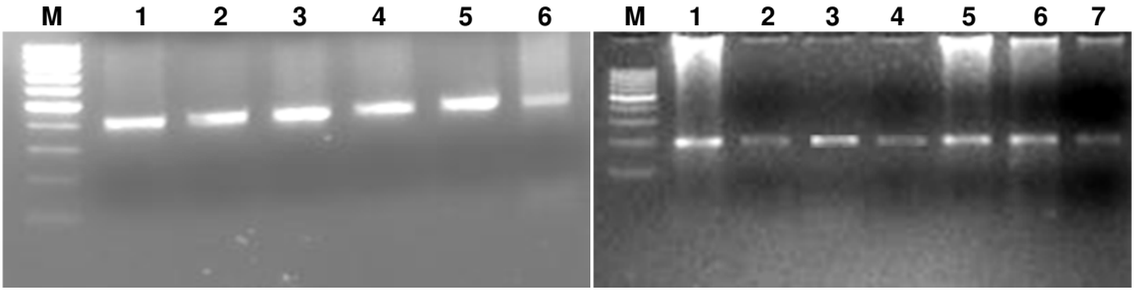


Lane M: 100bp DNA ladder; Lane 1: DNA derived from blood of CRC patients; Lane 2: DNA derived from blood of a normal healthy control; Lane 3 and 4: DNA derived from Tumour Tissue; Lane 5, 6 and 7: DNA derived from adjacent Normal Tissue

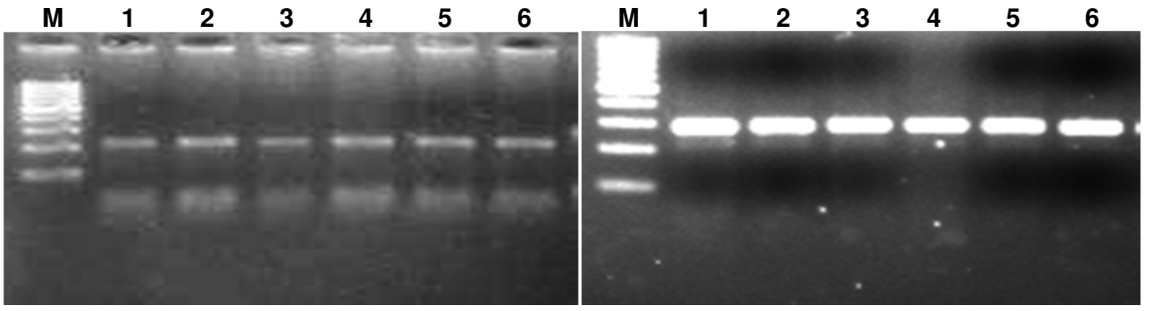
Figure 4. Agarose gel electrophoresis of DNA isolated from blood, tumour tissue, and adjacent normal tissue of CRC patients.



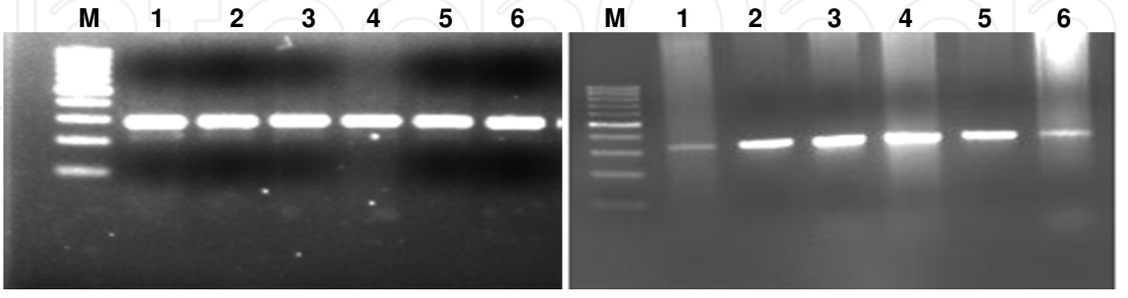
A: *Axin 1*; Exon 1a (311bp) **B:** *Axin 1*; Exon 1b (387 bp)



C: *Axin* 1; Exon 1c (405bp) **D:** *Axin* 1; Exon 2 (224bp)



E: *Axin1*; Exon 4 (253 bp) **F:** *Axin1*; Exon 6 (256 bp)



G: *Axin 1*;Exon 10 (260 bp) **H:** *Axin 2*; Exon 7 (320bp)

Lane M Molecular size marker 100bp; Lane 1-5, 6 and 7 Amplified product of DNA.

Figure 5. PCR amplification of different exons of Axin1 and Axin2 genes

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3.3. Mutational spectrum of *Axin* 1 and *Axin* 2 gene

In this study DNA sequencing was used to analyze the exon 1a, 1b, 1c, 2, 4, 6 and 10 of *Axin1* and exon 7 of *Axin2* in a series of 50 CRC patients. No previously reported mutations were detected in any of the analysed exons of *Axin1* and *Axin2* genes in CRC patients except two SNPs mentioned below. However, an interesting finding of this study was that we detected a novel mutation of G>T (GCT>TCT) transversion in exon 7 of *Axin2* gene at codon G695T (p.alanine>serine) which has not been reported before this study [53] This G695T novel mutation was further confirmed by reverse sequence of the same samples. This novel mutation was found at a frequency of 6% (3/50). Among these three patients two were chronic smokers with mean age of fifty seven years. All the three patients had well differentiated adenocarcinoma. Clinico-pathological characteristics of patients having novel mutation are given in *Table 4*. In the same exon of *Axin2* gene a single nucleotide polymorphism (SNP) (rs 35415678) of C>T transition was detected in codon L688L (CCT>CTT) at a frequency of 18/50(36%). In exon1c of Axin1 we detected a SNP of T>C transition at codon D726D (GAT>GAC) at a frequency of 31/50 (62.5%) (Table 5.). This SNP is synonymous and does not lead to any change of amino acid (Figures 6, 7 & 8). Table 4.3 shows the changes in nucleotides of *Axin1* and *Axin2* genes observed in our study. No significant association of these SNPs was found in this report with any clinico-epidemiological characteristics (Table 6 & 7).

3.4. Analysis of protein expression of Axin

In the present study, 50 colorectal cancer tissues and their adjacent normal samples previously studied for mutation spectrum were analysed for the protein expression of the *Axin*. The clinicopathological characteristics of the studied subjects are given in Table 3. The representative picture of the proteins extracted that were run on SDS page is shown in the figure 9. Out of 50 cases of CRC, 26% (13/50) showed reduced expression of *Axin* (Figure 10) and the rest 74% (37/50) of the cases showed normal protein (*Axin*) expression. Among 13 cases of CRC with reduced expression,27% (9/33) were of well differentiated grade and 24% (4/17) of moderately/poorly differentiated grade. Reduced expression of Axin was found to be 25% (7/28) and 27% (6/22) of cases of stage I/II and III/IV respectively. Reduced expression of Axin was found to be in 4/21 (19%) of never smokers and 9/29 (31%) of ever smokers. Reduced expression of Axin in males was observed as 5/24 (21%) and in females as 8/26 (31%). 24% (7/29) of the CRC cases with colon carcinoma and 29% (6/21) cases of rectal carcinoma showed reduced expression of *Axin*. Association of reduced expression of Axin with clinicopathological characteristics is shown in Graph 2. No significant association of reduced expression of Axin with any of the clinicopathological characteristic was found ($p>0.05$) (Table 8).

3.5. Loss of heterozygosity (LOH) of *DCC* gene

Loss of heterozygosity of *DCC* gene was determined by PCR-LOH assay in eighty samples of colorectal carcinoma and corresponding adjacent normal tissue. Mean age at the time of diagnosis was 52 years (range 30-80) with male: female ratio of 1:1.All the tumour samples included in this study were histopathologically confirmed cases of CRC. Histopathological findings of the CRC cases revealed 51 of 80 (64%) as well differentiated grade and 29 of 80

(36%) as moderately/poorly differentiated grade. In order to analyse LOH of *DCC* gene at two markers both the markers were amplified the amplified PCR products for D18S8-M2 (396bp) was digested by *Msp*I restriction enzyme and analyzed on 8% polyacrylamide gel whereas amplified product of VNTR region was directly run on 8% PAGE (Figure 12) and photographed under ultraviolet light.

S. code	Age	Gender	S. Status	Dwelling	Location	HPG	C.change	A.Achange	N. change
CRC 29	50	Male	C.smoker	Urban	A. Colon	WD	GCT→TCT	Alanine →serine	2397 G→T
CRC 32	65	Male	C.smoker	Urban	Rectum	WD	GCT→TCT	Alanine →serine	2397 G→T
CRC 38	57	Female	N.smoker	Rural	Colon	WD	GCT→TCT	Alanine →serine	2397 G→T

Abbrevations: S.code=sample code;S.Status=Smaoking status; C.smoker=chronic smoker;

N.smoker=non-smoker HP G = Histopathological grade;WD=well differentiated; A.A change=Amino acid change;

N.N change =nucleotide change C.Change=codon change; A. Colon= ascending colon.

Table 4. Clinico-epidemiological characteristics of the patients with novel mutation in Axin 2 gene

Gene/Exon	Nucleotide change	Codon change	Amino Acid change	Frequency
Axin1 Exon 1c	1134 T→C	GAT→GAC	Asp→ Asp	31/50(62.5%)
Axin2 Exon 7	2376 C→T	CCT→CTT	Leu→ Leu	18/50(36%)

(Transcript ID of Axin1 gene ENSG00000103126, NCBI Reference Sequence NC_000016.9) (Transcript ID of Axin2 gene ENSG00000168646, NCBI Reference Sequence: NC_000017.10)

Table 5. Single nucleotide changes in Axin1 & Axin2 genes in CRC patients.

In this study only informative cases were included (cases in which normal samples were heterozygous at M2-D18S8 marker), whereas uninformative cases (cases in which normal sample showed no heterozygosity) (Figure 11) were excluded from the study. Digested product of D18S8-M2 region yielded products of size 396, 257 and 139bp. LOH was considered positive for samples with absence of 396 bp bands and presence of 257 and139 bp (Figure 11). PCR product of VNTR when run directly on 8% PAGE generated a spectrum of alleles ranging

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Variables	Cases (n=50)	Wild allele 19(38%)	Variant allele 31(62%)	OR(95%CI)	*P-Value*
Grade					
WD	33(66%)	13(39%)	20(61%)	Reference	0.77
MD/PD	17(34%)	06(35%)	11(65%)	1.2(0.3-4.7)	
Age Group					
<50	19(38%)	08(42%)	11(58%)	Reference	0.63
≥50	31(62%)	11(35%)	20(65%)	1.3(0.4-4.1)	
Gender					
Male	24(48%)	10(42%)	14(58%)	Reference	0.63
Female	26(52%)	09(35%)	17(65%)	1.3(0.4-4.1)	
Smoking					
Never	21(42%)	09(43%)	12(57%)	Reference	0.62
Ever	29(58%)	10(34%)	19(66%)	1.4(0.4-4.0)	
Residence					
Rural	31(62%)	12(39%)	19(61%)	Reference	0.33
Urban	19 (38%)	10(53%)	09(47%)	0.5(0.09-2.9)	
Tumor site					
Colon	29(58%)	10(34%)	19(66%)	Reference	0.54
Rectum	21 (42%)	09(43%)	12(57%)	0.7(0.2-2.2)	

WD=Well Differentiated; MD= Moderately differentiated

Table 6. Single nucleotide changes in Axin1 & Axin2 genes in CRC patients.

in size from 150 to 210bp (Figure 12) depending on insertion or deletion. LOH at both D18S8-M2 and VNTR markers was observed as 39% (20/51) in samples with well differentiated grade and 86% (25/29) in moderately/poorly differentiated samples. 47 of 80 (59%) cases of CRC were of stage I-II and 33 of 80 (41%) of stage III-IV. LOH was found 47% (22/47) and 70% (23/33) at both the markers in stage I-II and III-IV respectively. The overall combined frequency of LOH at two markers (D18S8-M2 and VNTR) in CRC cases was reported to be 56.25 % (45/80) (Table 10; see Graph 3 also). LOH of DCC was found to be highly frequent in patients with higher stage/grade of CRC and this association was found to be significant ($p<0.05$). However no association of LOH was observed with any of the etiological parameter as depicted in Table 9.

Variables	Cases (n=50)	Wild allele 32(64%)	Variant allele 18(36%)	OR(95%CI)	*P*-value
Grade					
WD	33(66%)	19(58%)	14(42%)	Reference	0.19
MD/PD	17(34%)	13(76%)	04(24%)	0.4(0.2-1.5)	
AgeGroup					
<50	19(38%)	13(68%)	06(32%)	Reference	0.10
≥50	31 (62%)	19(61%)	12(39%)	1.3(0.4-4.8)	
Gender					
Male	24(48%)	16(67%)	08(33%)	Reference	0.7
Female	26 (52%)	16(62%)	10(38%)	1.3(0.3-4.2)	
Smoking					
Never	21(42%)	13(62%)	08(38%)	Reference	0.79
Ever	29(58%)	19(66%)	10(34%)	0.8(0.24-2.6)	
Residence					
Rural	31(62%)	20(65%)	11(35%)	Reference	0.9
Urban	19 (38%)	12(63%)	07(37%)	1(0.3-3.2)	
Tumorsite					
Colon	29(58%)	18(62%)	11(38%)	Reference	0.7
Rectum	21 (42%)	14(67%)	07(33%)	0.8(0.24-3.0)	

Table 7. Clinico-epidemiological Characteristics of the CRC Patients with single nucleotide polymorphism at codon 688 CCT>CTT Axin2 gene.

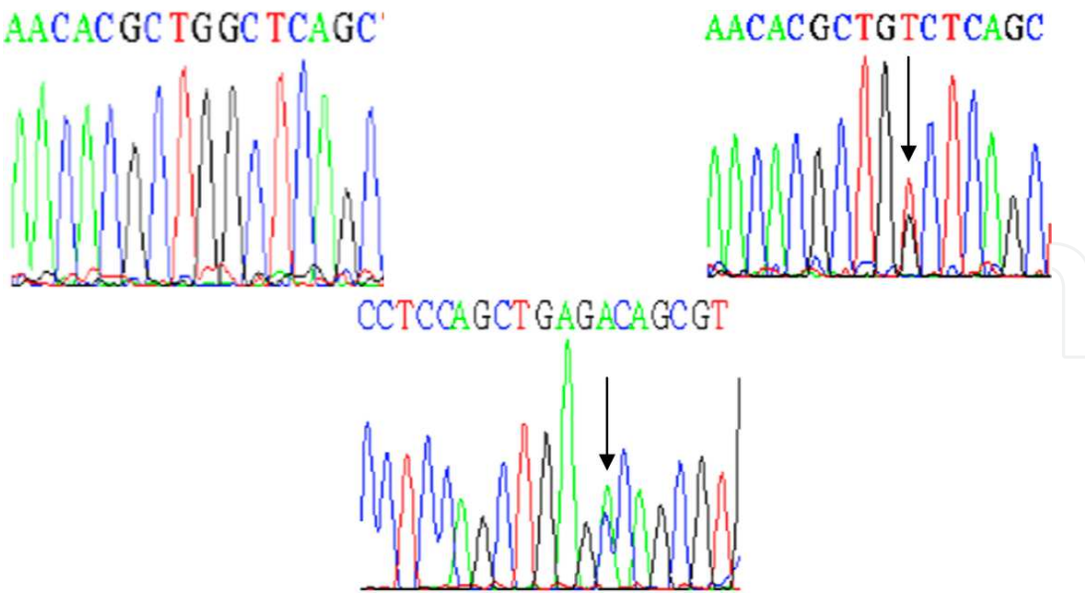

Figure 6. Partial nucleotide sequences in Exon 7 of normal (left) and of the mutants in (right) of the Axin 2 gene codon (GCT>TCT) Partial reverse sequence of the same mutation (below). Arrow points toward base change in mutants with respect to normal sequence.

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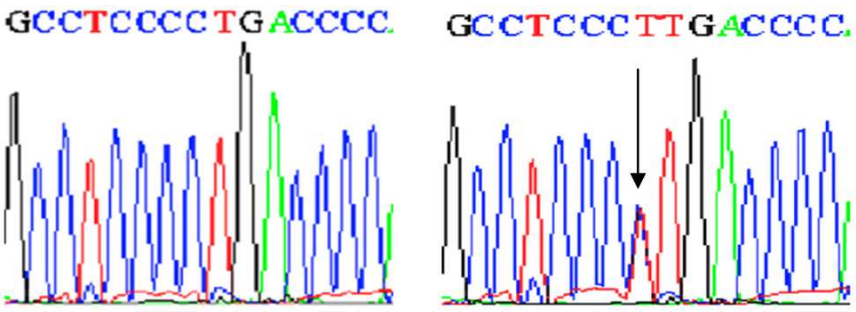


Figure 7. Partial nucleotide sequences in Exon 7 of the normal (left) and mutants in exon 7 of the Axin2 gene codon (CCT→CTT). Red arrow points toward base change in mutants with respect to normal sequence.

		Normal expression N (%)	Reduced expression N (%)	OR(95%CI)	P-Value
Clinico pathological variables	Overall results Case (n=50)	37(74%)	13(26%)	-	-
Age				Reference	0.3
<50	19(38%)	15(79%)	4(21%)	1.9(0.57-7)	
>50	31(62%)	22(71%)	9(29%)		
Sex				Reference	0.4
Male	24(48%)	19(79%)	5(21%)	1.68(1.0-2.3)	
Female	26(52%)	18(69%)	8(31%)		
Dwelling				Reference	0.2
Rural	31(62%)	21(68%)	10(32%)	0.39(0.09-1.6)	
Urban	19(38%)	16(84%)	03(16%)		
Smoking				Reference	0.3
Never	21(42%)	17(81%)	4(19%)	1.9(0.57-7.2)	
Ever	29(58%)	20(69%)	9(31%)		
Grade				Reference	0.77
WD	33(66%)	24(73%)	9(27%)	0.8(0.2-3.0)	
MD/PD	17(34%)	13(76%)	4(24%)		
Stage				Reference	0.8
I/II	28(56%)	21(75%)	7(25%)	1.1(0.3-3.8)	
III/IV	22(44%)	16(73%)	6(27%)		
Location				Reference	0.7
Colon	29(58%)	22(76%)	7(24%)	1.25(0.35-4.3)	
Rectum	21(42%)	15(71%)	6(29%)		

χ^2 was used to calculate the p-value of the variables. *P-Value <0.05 was considered statistically significant

Table 8. Association of Clinic pathological characteristics with reduced expression of Axin

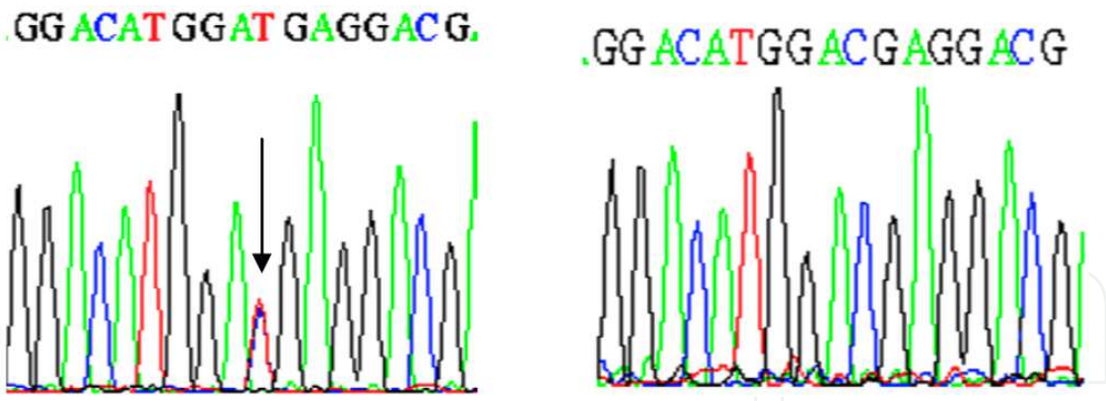


Figure 8. Partial nucleotide sequences in Exon1c of the normal (left) and mutants in exon 1c of the Axin1 gene codon (GAT →GAC).Arrow points toward base change in mutants with respect to normal sequence.

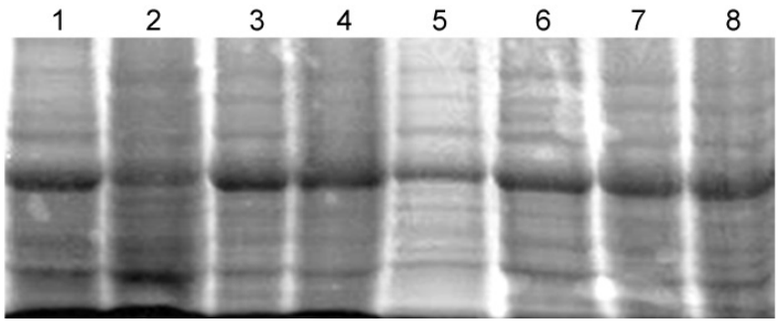
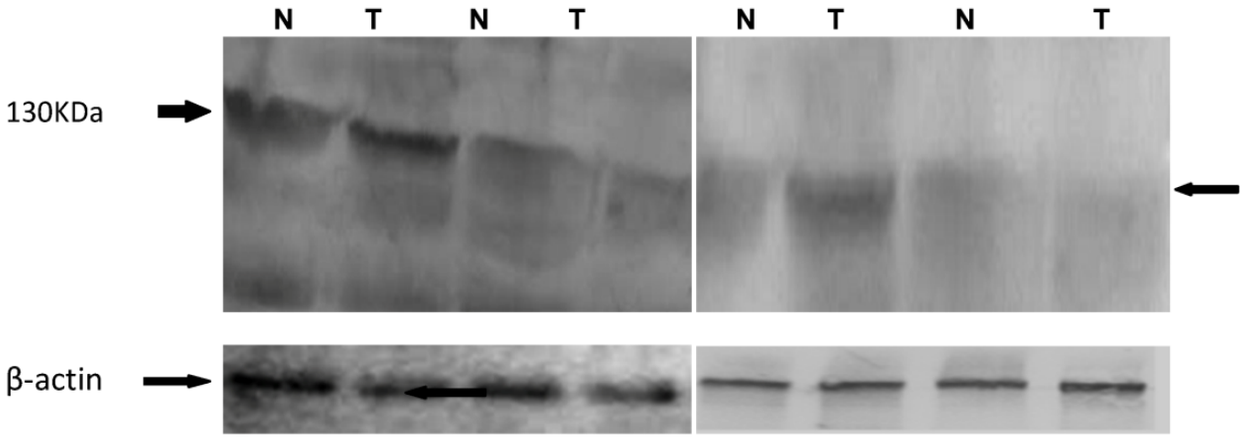


Figure 9. Representative gel picture of 10% SDS-PAGE. In each case 25 µl of the crude protein extract from tumor as well as normal tissue was loaded.



Lanes N: Protein extracted from Normal tissues; Lanes T: Protein extracted from Tumour tissue; Membrane was probed with a polyclonal antibody specific for Axin

Figure 10. Western blot analysis of Axin protein in colorectal tumour and adjacent normal tissues. Figure A-D Representative immunoblot showing the expression of Axin in Colorectal carcinoma as compared to their adjacent normals. Extract from samples was separately run for β-actin protein expression as loading control.

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Variables	Cases n=80	LOH–ve [%]	LOH+ve [%]	P-value
Grade(differentiation)				
Well differentiated	51(64%)	31(61%)	20 (39%)	0.00019*
Mod/Poorly differentiated	29(36%)	4(14%)	25 (86%)	
Clinical staging				
Stages I-II	47(59%)	25(53%)	22(47%)	0.044*
Stages III-IV	33(41%)	10(30%)	23(70%)	
Location				
Colon	42(52.5%)	15 (36%)	27 (64%)	0.12
Rectum	38(47.5%)	20 (53%)	18 (47%)	
Dwelling				
Rural	46(57.5%)	22 (48%)	24 (52%)	0.393
Urban	34(42.5%)	13 (38%)	21 (62%)	
Age				
<50	33(41.25%)	14 (42%)	19 (58%)	0.8412
≥50	47(58.75%)	21 (45%)	26 (55%)	
Sex				
Male	43(53.75%)	21(49%)	22(51%)	0.323
Female	37(46.25%)	14(38%)	23(62%)	

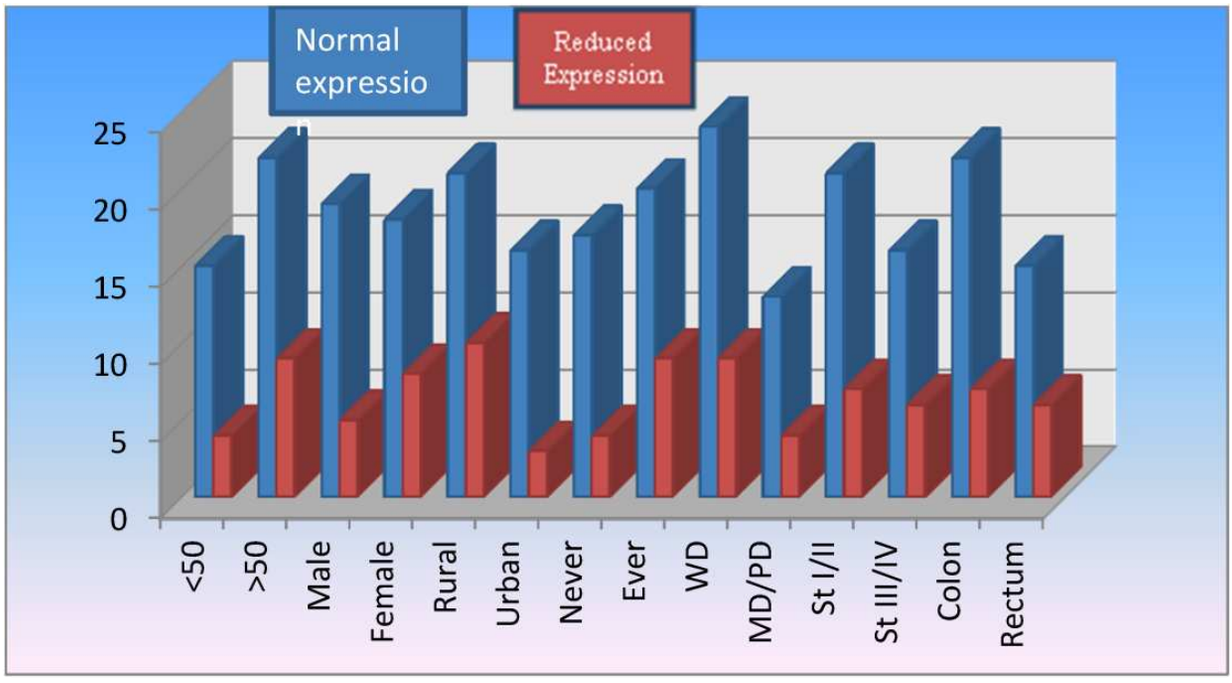
χ^2 was used to calculate the p-value of the variables. *p-Value<0.05 was considered statistically significant

Table 9. Relation of clinico-pathological variables with LOH of DCC gene

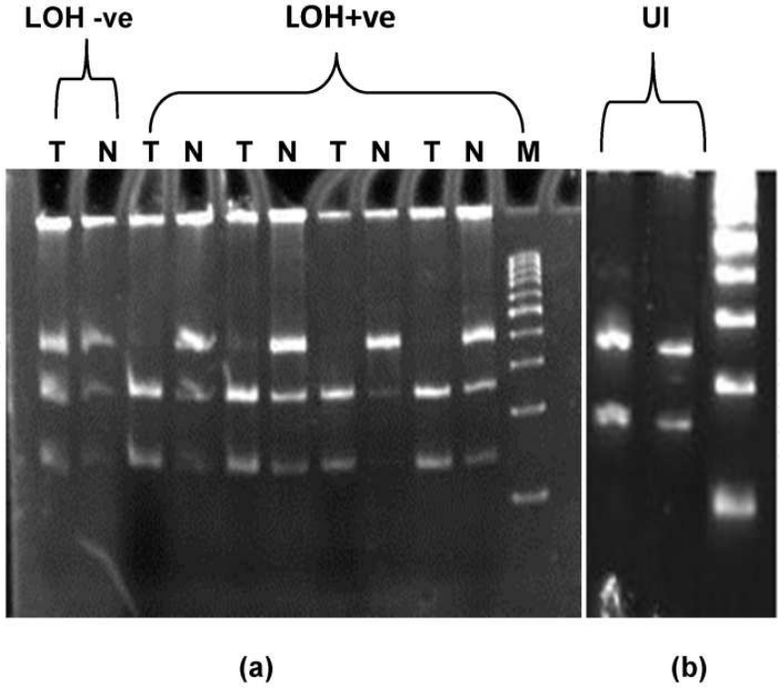
Markers(n=80)	LOH-ve	LOH+ve	p-value
D18S8M2	61(76%)	19(24%)	0.21
VNTR	54(67.50%)	26(32.50%)	

χ^2 was used to calculate the P-value of the variables

Table 10. Percentage of Cases with and without Loss of Heterozygosity at two different markers



Graph 2. Association of reduced expression of Axin with clinic-pathological characteristics



Lane M: 100bp DNA ladder,N=Normal; T=tumour. Normal samples showed three bands (band size 396,257 &139). LOH^{+ve} Informative cases. LOH -ve samples showed no loss of heterozygosity.

Figure 11. (a): LOH of DCC gene at D18S8-M2 region, (b): Uninformative cases (UI) were excluded from the study.

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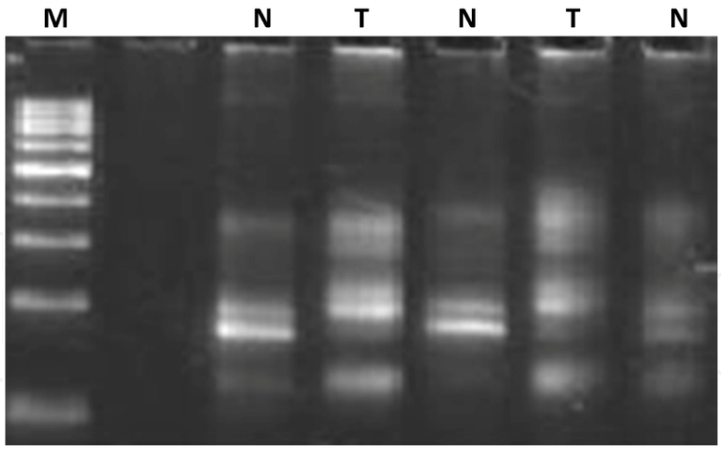
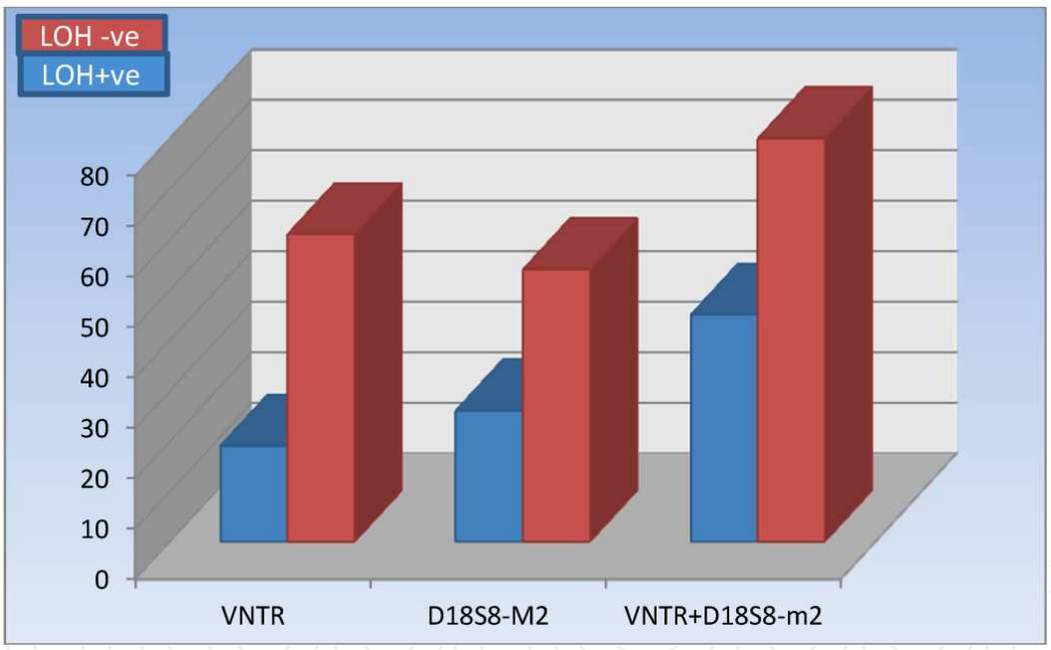


Figure 12. LOH of DCC gene at VNTR region. Lane M: 100bp DNA ladder, N: normal DNA, T: tumor DNA. Strong allelic imbalance is seen in tumor showing range of bands (150-200bp) not in adjacent normal tissues, with dominance of the larger 200-base pair allele.



Graph 3. Frequency of distribution of LOH at two markers D18S8-M2 & VNTR of DCC gene

4. Summary and conclusion

Colorectal cancer (CRC) is a leading cause of death in the western world. [54]. The frequency of CRC varies remarkably among different populations. In India, CRC does not figure amongst the 10 most common malignancies [55]. In Kashmir incidence of cancer is showing an increasing trend and sites among the top ten common cancers [56]

Multiple factors contribute to the development of CRC, dietary and life style factors on one hand and genetic factors on the other [57]. Colon cancer is a common disease in both men and women. Because 5% of persons (1 in 20 persons) will develop colorectal cancer, this disease is an important public health issue. Colon cancer is usually observed in one of three specific patterns: sporadic, inherited or familial. Sporadic disease, with no familial or inherited predisposition, accounts for approximately 70% of colorectal cancer in the population. Sporadic colon cancer is common in persons older than 50 years of age, probably as a result of dietary and environmental factors as well as normal aging. Fewer than 10% of patients have an inherited predisposition to colon cancer. The inherited syndromes include those in which colonic polyps are a major manifestation of disease and those in which they are not. The polyposis syndromes are subdivided into familial adenomatous polyposis and the hamartomatous polyposis syndromes. The non-polyposis predominant syndromes include hereditary non-polyposis colorectal cancer (HNPCC) (Lynch syndrome I) and the cancer family syndrome (Lynch syndrome II). Although uncommon, these syndromes provide insight into the biology of all types of colorectal cancer. The third and least understood pattern of colon cancer development is known as familial colon cancer. In affected families, colon cancer develops too frequently to be considered sporadic colon cancer but not in a pattern consistent with an inherited syndrome. Up to 25% of all cases of colon cancer may fall into this category. CRC is more common in North America, parts of Europe, Australia, New Zeeland and Japan than in eastern Asia and Africa [58] This together with the fact that populations migrating from a low-incidence to a high-incidence geographical area show a similar incidence as those living in the high-incidence area, points towards life style and dietary habits being causative [59] The exact causes are still controversial but epidemiological studies indicate that diets that include low fruit, vegetable or fiber intake, high red meat or saturated fat consumption increase the risk of developing CRC. Exposure to caffeine, cigarette smoke and alcohol has also been suggested to increase risk. Diets high in calcium, folate and regular physical activity are associated with a reduced risk of developing CRC [60]

According to the model developed by Vogelstein and coworkers, colorectal neoplasia evolves through a series of genetic alterations that includes the activation of oncogenes by mutation and the inactivation of tumor suppressor genes by mutation, loss of gene, or methylation [61]. As per their multistage model of colorectal carcinogenesis alteration of genes like Axin, APC, β-Catenin, Smads, TGF-β, B-Raf are early events whereas alteration of p53,DCC are late events in the development of CRC. In our population genes like APC, β-catenin and Smads have been previously studied in relation to the development of colorectal cancer. As per one of the study carried out on Kashmiri population by Sameer et al., 2010 the mutational aberrations of APC and β-catenin were reported to be low in CRC cases in Kashmiri populations however, frequency of the epigenetic silencing of the APC gene was reported to be high. SMAD4 gene aberrations were reported to be the common event in CRC development [62].

We studied genetic alterations of *Axin* 1, *Axin* 2 and *DCC* genes in CRC patients of Kashmiri population. Following are the major findings of our study

- In the present study we studied fifty CRC and adjacent normal samples, we found a novel mutation in exon 7 of *Axin2* gene at codon 695.This G>T transversion leads to the change of codon GCT>TCT.

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- The frequency of this novel mutation was found to be 6 % (3/50).
- This novel mutation leads to the change of amino acid alanine to serine.
- A SNP (rs 35415678) of C>T was found in exon 7 of *Axin2* gene at a frequency of 32% (18/50).This SNP was found at codon L688L resulting in the change of codon CCT>CTT. However this SNP was synonymous and hence does not lead to the change of amino acid.
- A SNP (rs 1805105) of T>C was found in exon 1c of *Axin1* gene at a frequency of 62.5% (31/50).This SNP was found at codon D726D leading to the change of codon GAT>GAC. This SNP was also found to be synonymous and hence does not lead to the change of amino acid
- No other sequence variation in any other analysed exons of *Axin* gene was found
- We did not find any significant association of any of the clinical epidemiological characteristic with the development of CRC.
- Protein expression of *Axin* gene in fifty tumour specimens with respect to their adjacent normal samples was studied. The samples which were studied for protein expression were same studied for mutational analysis.
- 26% (13/50) CRC patients showed reduced expression of Axin.
- No association was found with any of the clinico-pathological characteristic of CRC with the reduced expression of Axin.
- LOH of *DCC* gene at D18S8-M2 and VNTR marker was studied in eighty CRC tumour samples with respect to adjacent normal samples.
- LOH of *DCC* gene at D18S8M2 marker was found to be 23.75 % (19/80).
- LOH of *DCC* gene at VNTR marker was found to be 32.50 % (26/80).
- Aggregate percentage of loss of heterozygosity of *DCC* gene was found to be 55.25%.
- We found a significant association of LOH of *DCC* gene with higher stage and grade ($P<0.05$)
- No significant association of any other clinical pathological parameter was found with the development of CRC.
- Arg/Arg (GG) and Arg/Gln (GA) were found to be significantly associated with higher risk of CRC.
- The frequency of the *XRCC1* allele Gln/Gln was found to be 6(5%) for cases & 34(23.3%) for controls with $P<0.05$
- The frequency of the *XRCC1* allele Arg/Gln was found to be 80(66.7%) for cases & 62(42.5%) for controls.
- No significant association of Arg399Gln SNP with any clinico-pathological parameters was found.

- We found a protective role of Gln/Gln allele against the risk of development of CRC in Kashmiri population.

In conclusion, our study demonstrates significant role of *Axin* in the development of colorectal cancer. Eventhough we did not find any of the reported mutation in *Axin1* gene but we found reduced expression of *Axin1* in majority of CRC cases which clearly suggests its possible role in the development of CRC. Thus, our study points to the fact those other possible genetic alterations other than mutation could be responsible for malfunctioning of *Axin1* gene which may be responsible for the development of CRC. In *Axin*2 gene the novel mutation was found at low frequency of 6% leading to the change of amino acid from alanine to serine. The codon at which this novel mutation was found lies in the region capable of binding to various proteins and thus may somehow render *Axin* incapable of binding various other proteins involved in different pathways. This may lead to the derangement of Wnt, TGF-β, and Jun/SAPK pathways. Aberration in specific binding of these signaling molecules to *Axin* due to the mutation G695T found in our study perhaps may aid in the deregulation of pathways and hence may lead to colorectal carcinogenesis.

Our study also supports the multistep model of colorectal carcinogenesis in which alteration of DCC gene has been reported to be the late event in the development of CRC as observed in our report. In this study we found that LOH has a frequency of 56% in patients with CRC and is highly frequent in patients with higher stage/grade in CRC suggesting that LOH of DCC gene may be one of the genetic events involved in the development of colorectal cancer in Kashmiri population.

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