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Review

Colorectal cancer, TGF-β signaling and SMADs

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Colorectal cancer (CRC) being the commonest cancer, is the major cause of mortality and morbidity worldwide. *TGF-β* pathway is one of the important pathways that play a prominent role in cell proliferation, differentiation, migration and apoptosis. Smad dependent *TGF- β* signaling cascade is responsible for the regulation and expression of almost 500 odd genes, which in turn play important role in the proper development of intestinal mucosa. Here in this review we have discussed the overall machinery of the *TGF- β* pathway and the advances in the mutational research on *SMAD4* gene in cancers with special look on our own research in CRC cases of Kashmiri population.

Key words: Colorectal cancer, Kashmir, SMADs, mutations, PCR-SSCP.

INTRODUCTION

CRC (CRC), also called colon cancer or large bowel cancer includes cancerous growths in the colon, rectum and appendix. CRC is a commonly diagnosed cancer in both men and women and is the third most common form of cancer and the second leading cause of cancer-related death in the western world. CRC causes 655,000 deaths worldwide per year, including about 16,000 in the UK, and about 50,000 in United States, where it is the second most common site (after lung) to cause cancer death (World Health Organization, 2006; American Cancer Society, 2008).

Two kinds of observations indicate a genetic contribution to CRC risk: a) increased incidence of CRC among persons with a family history of CRC; and b) families in which multiple family members are affected with CRC, the pattern indicates an autosomal dominant inheritance of cancer susceptibility (Burt et al., 1996; Lynch et al., 1996; Utsunomiya et al., 1990; Herrera, 1990; Schoen, 2000).

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About 75% of patients with CRC have sporadic dis-ease, with no apparent evidence of having inherited the disorder. The remaining 25% of patients have a family history of CRC that suggests a genetic contribution, common exposures among family members, or a combination of both. Genetic mutations have been identified as the cause of inherited cancer risk in some colon cancer–prone families; these mutations are estimated to account for only 5 - 6% of CRC cases overall. It is likely that other undiscovered major genes and background genetic factors contribute to the development of CRC, in conjunction with non-genetic risk factors (NCI, 2008).

High incidence rates are found in western world populations, that is Western Europe, North America, and Australia. The lowest rates of CRC are found in the sub-Saharan Africa, South America and Asia, but are increasing in countries adopting western life-style and dietary habits (Vainio et al., 2003).

Colorectal tumors present with a broad spectrum of neoplasms, ranging from benign growths to invasive cancer, and are predominantly epithelial-derived tumors (that is, adenomas or adenocarcinomas). Pathologists have classified the lesions into three groups: nonneoplastic polyps, neoplastic polyps (adenomatous polyps,

adenomas), and cancers (O'brien et al., 2004; Zauber et al., 2002).

More than 95% of CRCs are carcinomas and among them 95% are adenocarcinomas. While there is no direct proof that most CRCs arise from adenomas, adenocarcinomas are generally considered to arise from adenomas (Howe *et al.,* 1998) based upon these two important observations: a) benign and malignant tissue occur within colorectal tumors; and b) when patients with adenomas were followed for 20 years, the risk of cancer at the site of the adenoma was 25%, a rate much higher than that expected in the normal population (O'Brien et al., 1990; Winawer et al., 2000).

The etiology of CRC is multifactorial, and is likely to involve the actions of genes at multiple levels along the multistage carcinogenesis process. Examples of genes involved in pathogenesis of CRC include *p53*, *p16*, *p14*, *APC*, *β-catenin*, *E-cadherin*, *transforming growth factor* (*TGF)-β*, *SMADs*, *MLH1*, *MSH2*, *MSH6*, *PMS2*, *AXIN*, S*TK11*, *PTEN*, *DCC* and *K-RAS* (Sayar et al., 2007).

REVIEW

CRC is a common disease in both men and women. Because 5% of persons are predisposed to development of CRC, this disease is an important public health issue. CRC is the third most common cause of cancer-related death in the western world (Paula et al., 2002; Zoe et al., 2004).

Worldwide, CRC represents 9.4% of all incident cancers in men and 10.1% in women. CRC, however, is not equally common throughout the world. If the westernized countries (North America; those in northern, southern, and western Europe; Australasia; and New Zealand) are combined, CRC represents 12.6% of all incident cancers in westernized countries in men and 14.1% in women. Elsewhere CRC represents 7.7% and 7.9% of all incident cases in men and women respectively (Boyle et al., 2001).

The lifetime risk of developing colon cancer in the United States is about 7%. Certain factors increase a person's risk of developing the disease. The most important of these are the age, diet, obesity, diabetes and smoking, personal cancer history, alcohol consumption, large intestinal polyps, family history of colon cancer, race and ethnic background, genetic or family predisposition. 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 CRC 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 pre- disposition 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 (FAP) and the hamartomatous polyposis syndromes. The nonpolyposis predominant syndromes include hereditary nonpolyposis CRC (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 CRC. 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 (Paula et al., 2002).

Familial adenomatous polyposis patients inherit a mutated copy of the adenomatous polyposis gene (*APC*), whereas hereditary non-polyposis colon cancer is caused by inheritance of defective DNA mismatch repair genes (*MLH1*, *MSH2*, *PMS2* and *MSH6*). Germ line mutations of the *LKB1/STK11* gene have been shown to cause the Peutz–Jeghers syndrome (PJS), and mutation of *SMAD4* or *ALK3* underly juvenile polyposis (JPS). In contrast, the *MYH*-associated polyposis (MAP) syndrome has autosomal recessive inheritance and results from bi-allelic mutations in the *MYH* gene. Taken together, these syndromes account only for ~2 - 6% of CRC cases. The great majority of CRCs do not have a recognizable inherited cause, but a number of studies have suggested a role for genetic factors in predisposition to a substantial minority of colorectal tumors. The relatives of patients with ‘sporadic’ CRC are themselves at increased risk of the disease and segregation analysis has suggested dominant inheritance of the uncharacterized susceptibility genes. (Cannon-Albright *et al.,* 1988; Houlston et al., 1992; Johns et al., 2001; Hans et al., 1996).

CRC – An overview

The majority of CRCs develop from benign pre-neoplastic lesions: the adenomatous polyps or adenomas. Progression from a benign adenoma to a malignant carcinoma passes through a series of well-defined histological stages, which is referred to as the adenoma-carcinoma sequence (Vogelstein et al., 1988). Two major mechanisms of genomic instability have been identified that give rise to colorectal carcinoma development and progression: chromosomal instability (CIN) and microsatellite instability (MIN). CIN is associated with a series of genetic changes that involve the activation of oncogenes as k-ras and inactivation of tumor suppressor genes as *p53*, *DCC/SMAD4* and *APC* and contributes predominantly to carcinogenesis in the distal segments of the colorectum (conlin et al., 2005; Esteller et al., 2001; Hsieh et al., 2005). Familial Adenomatous Polyposis

Table 1. Association of various aberrant molecules of TGF-β pathway with different cancers.

TGF-β component	Cancers
TGF-β	Aggressiveness because of enhanced invasion and metastasis
Type I receptor	Implicated in all cancers; Colorectal (30%), gastric (15%), prostate, breast, lung, pancreatic, head and neck etc
Type II receptor	Breast (16%), pancreatic, biliary, cervical
Smad 2	Colorectal (11%), lung (7%), liver
Smad4	Implicated in all cancers; Pancreatic (50%), Colorectal (30%), Lung (10%), Breast, Ovarian, Gastric, Prostate, Esophageal, Liver, Head and Neck etc

represents the hereditary syndrome dealing with *APC* mutation (Vogelstein et al., 1988; Fearon et al., 1990). Mutations in DNA mismatch repair (MMR) genes result in a failure to repair errors that occur during DNA replication in repetitive sequences (microsatellites), resulting in an accumulation of frameshift mutations in genes that contain microsatellites. This failure leads to MIN type of tumor and is the hallmark of hereditary non-polyposis CRC (HNPCC) (Boland et al., 1998). MIN is also found in 12 - 15% of sporadic CRCs. In addition to the genetic disparity of CIN and MIN, MIN tumors are more frequently right-sided and poorly differentiated, and more often display unusual histological type (mucinous), and marked peri-tumoral and intra-tumoral lymphocytic infiltration (Dolcetti et al., 1999; Benatti et al., 2005).

Classification and grading

The most common colon cancer cell type is adenocarcinoma which accounts for 95% of cases. Other, rarer types include lymphoma and squamous cell carcinoma. Cancers on the right side (ascending colon and cecum) tend to be exophytic, that is, the tumor grows outwards from one location in the bowel wall. Left-sided tumors tend to be circumferential, and can obstruct the bowel much like a napkin ring. Pathology has an essential role in the staging of CRC. Two classification systems are being used for the staging of the CRC- Dukes classification and TNM (Tumors/Nodes/Metastases) system. Dukes' classification, first proposed by Dukes et al., (1932), identifies the stages as: A - Tumor confined to the intestinal wall; B - Tumor invading through the intestinal wall; C - With lymph node(s) involvement and D - With distant metastasis, which is the commonest in use still. There has been a gradual move from using Dukes's classification to using the TNM classification system as this is thought to lead to a more accurate, independent description of the primary tumors and its spread (Hardy et al., 2001).

GENETIC BACKGROUND OF CRC

TGF-β signaling

The TGF-β superfamily consists of more than 35 members in vertebrates, including TGF-β, BMPs (bone morphogenetic proteins), GDFs (growth differentiation factors), activins, inhibins, MIS (Mullerian inhibiting substance), nodal, and leftys (Table 1). These proteins were identified mainly through their roles in development; they regulate the establishment of the body plan and tissue differentiation through their effects on cell proliferation, differentiation and migration (Figure 1 and 2). The growth inhibitory effect of TGF-β signaling in epithelial cells explains its role as a tumor suppressor in carcinomas, although TGF-β expression by tumor cells contributes to cancer progression as well (Derynck et al., 2003; Massague et al., 1998). The TGF-β family ligands are translated as prepropeptide precursors with an N-terminal signal peptide followed by the prodomain and the mature domain (Hogan et al., 1996; Padgett et al., 1997; Change et al., 2002).

TGF-β superfamily ligands signal through a family of transmembrane serine/threonine kinases known as the receptors for the TGF-β superfamily, which are divided into two subfamilies: type I and type II receptors. The extracellular regions of these receptors contain about 150 amino acids with 10 or more cysteines that determine the folding of this region (Chang et al., 2002; Wrana et al., 1994; Lastres et al., 1996; Cheifetz et al., 1991; Segarini et al., 1989).

TGF-β signaling pathways are broadly categorized into two types depending upon the main mediators in intracellular environment as Smad dependent and Smad independent. Smad dependent pathway is the most characterized of all the TGF-β signaling pathways. In Smad dependent pathway signaling is initiated when the ligand in dimeric form induces assembly of a heteromeric complex of type II and type I receptors. The type II kinase then phosphorylates the type I receptor in a conserved

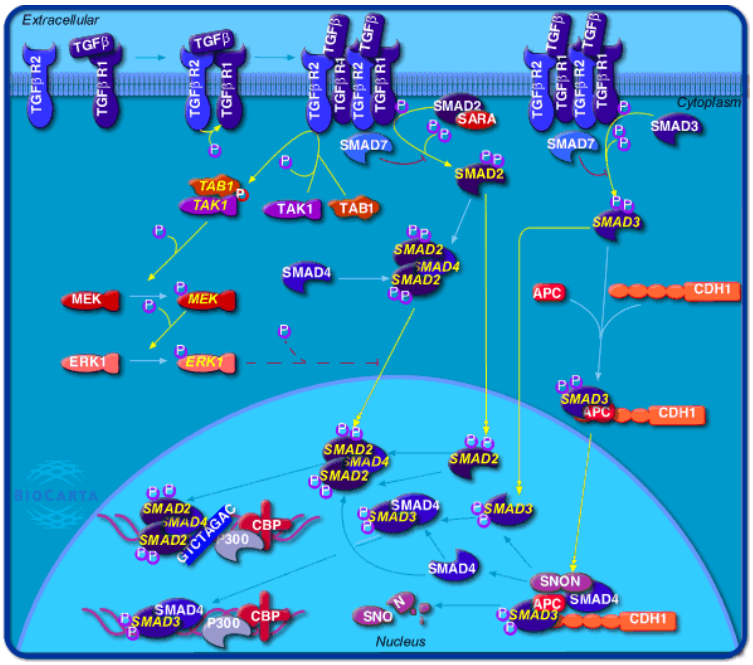

Figure 1. Overview of TGF-β pathway (Courtesy: Biocarta.org).

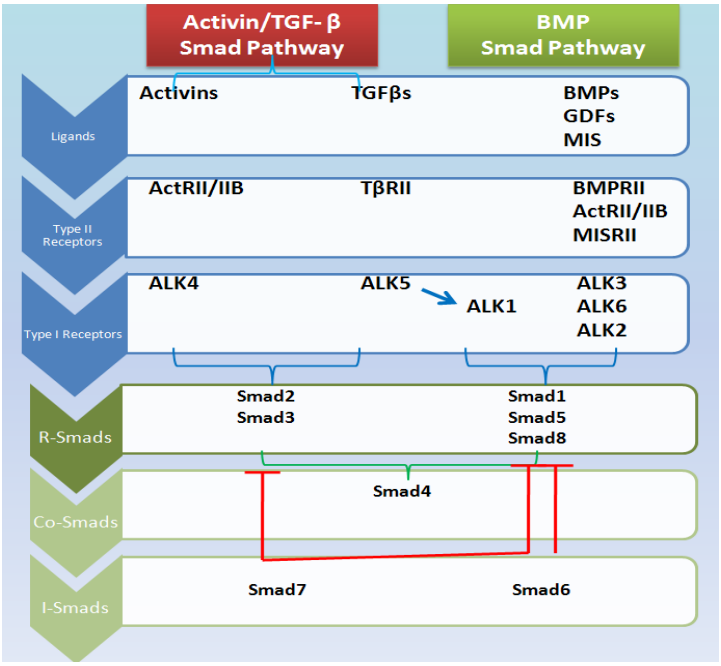

Figure 2. Signaling specificity of TGF-B superfamily (Adapted from Moustakas A. et al. J. Cell Sci. 2001 114: 4359-4369).

glycine–serine-rich domain (GS domain). This activates the type I kinase, which subsequently recognizes and phosphorylates members of the intracellular Smad signal transduction pathway. Activation of the Smad pathway occurs when the activated type I kinase associates with the MH2 domain of specific R-Smads. The type I kinase

then phosphorylates the R-Smads on a conserved carboxy-terminal SSXS motif. This causes dissociation of the R-Smad from the receptor, stimulates the assembly of a heteromeric complex between the phosphorylated R-Smad and the Co-Smad, Smad4, and induces the nuclear accumulation of this heteromeric Smad complex. In the nucleus, Smads function to regulate transcriptional responses by directly interacting with a host of resident DNA binding proteins. Here, the R-Smads mediate the interaction of the Smad complex with DNA binding proteins. Once recruited to specific regulatory elements, Smads can then stabilize ternary DNA binding complexes by contacting DNA at adjacent sites and can directly regulate transcription by recruiting coactivators or corepressors to the promoter. Thus, Smads function to transmit signals directly from the cell-surface receptors into the nucleus, where they act as effectors of the transcriptional response to TGFβ-related factors (Grady et al., 2000; Attisano et al., 2000).

SMAD4 – (Mothers against decapentaplegic homolog 4 (drosophila)) and CRC

SMAD4 gene - also known as *MADH4*, *DPC4* & *JIP*, is located on the long arm (q) of chromosome 18 at band 21.1. The gene encompasses 49.5 kb of DNA with 13 exons, out of which first two exons do not code for any amino acid and hence constitute 5'-UTR of the *SMAD4* gene. *SMAD4* mRNA transcript constitutes 3220 nucleotides. The protein of *SMAD4* gene - *Smad4* belongs to the Darfwin family of proteins which harbours two conserved amino- and carboxyl-terminal domains known as MH1 and MH2, respectively. Smad4 in the basal state is found mostly as a homo-oligomer, most likely a trimer. It is ubiquitously expressed within the human body. Smad4 is an intracellular mediator of TGF-β family and activin type 1 receptor. Smad4 mediate TGF-β signaling to regulate cell growth and differentiation. TGF-β stimulation leads to phosphorylation and activation of Smad2 and Smad3, which form complexes with Smad4 that accumulate in the nucleus and regulate transcription of target genes. By interacting with DNA-binding proteins, Smad complexes then positively or negatively regulate the transcription of target genes (Attisano et al., 2000, 2001; Massague et al., 2000; Wrana et al., 2000; Shi, 2001; Saffroy et al., 2004).

The discovery of human homologues of the Drosophila Mad gene, called Smad genes (Hahn *et al.,* 1996), has been a milestone for understanding the genetics of the CRC whether of familial origin or sporadic. It has opened the Pandora's Box for both developmental and cancer biologists. Mutations in two Smad family member genes – Smad4 (also known as *DPC4*) and Smad2 (also known *MADR2*, and hMAD-2) have been identified in human cancers and more importantly with high frequency in pancreatic and CRCs (Riggins et al., 1996). This raises the possibility that one or more of these genes can act as tumor suppressors as well as developmental regulators. Approximately 50% of pancreatic carcinomas, 20% of colon carcinomas, and 10% of lung cancers exhibit mutations in Smad4, and mutations in Smad2 have been found in ~7% of colorectal and lung cancers (Hahn et al., 1996; Riggins et al., 1996, 1997; Uchida et al., 1996).

The Smads are a group of related intracellular proteins which play a critical role in transmitting the signals from the transforming growth factor-β (TGF-β) superfamily located at the cell surface onto the nucleus (Attisano et al., 2000; Massague et al., 2000; Wrana et al., 2000; Dijke et al., 2000; Padgett et al., 1999; Zhang et al., 1999; de Caestecker et al., 2000). Although related to each other, Smads are structurally distinct from other intracellular effector proteins. The prototypic members of the Smad family, *Mad* and *Sma*, were first described in *Drosophila* and *Caenorhabditis elegans*, respectively (Padgett et al., 1999). Related proteins in *Xenopus*, humans, mice and rats were subsequently identified, and all family members are now known as Smads, a contraction of the invertebrate gene names. More recently, related proteins have also been described in zebra-fish and the helminth parasite *Schistosoma mansoni* (Raftery et al., 1999).

There are eight Smad family members in mammals, and a search of human genome database suggests that this represents the full complement. The eight human Smad genes have been mapped to four chromosomes. Three of the Smad genes - Smad2, Smad4 and Smad7 - are closely clustered at 18q21.1, a region that is frequently deleted in human cancers. Three are found on chromosome 15, with Smad3 and Smad6 mapping to 15q21 - 22 and Smad5 to 15q31. The remaining Smad genes, Smad1 and Smad8, are located on chromosomes 4 and 13, respectively (Attisano et al., 2001). Smads are ubiquitously expressed throughout development and in all adult tissues, and many of them (Smad2, Smad4, Smad5, Smad6 and Smad8) are produced from alternatively spliced mRNAs (Luukko et al., 2001).

Functional studies have demonstrated that Smads, which range from about 400 to 500 amino acids in length, can be grouped into three subfamilies: a) the receptor-regulated Smads (*R-Smads*: Smad1, Smad2, Smad3, Smad5, Smad8), which become phosphorylated by the type I receptors; b) the common Smads (*co-Smads*: Smad4), which oligomerise with activated R-Smads; and c) the inhibitory Smads (*I-Smads*: Smad6 and Smad7), which are induced by TGF-b family members. Each of these Smads plays a distinct role in the pathway (Figure 3) (Attisano et al., 2001; Moustakas et al., 2001).

Smads have two conserved domains, the N-terminal Mad homology 1 (*MH1*) and C-terminal Mad homology 2 (*MH2*) domains. The MH1 domain is highly conserved among R-Smads and Co-Smads; however, the N-terminal

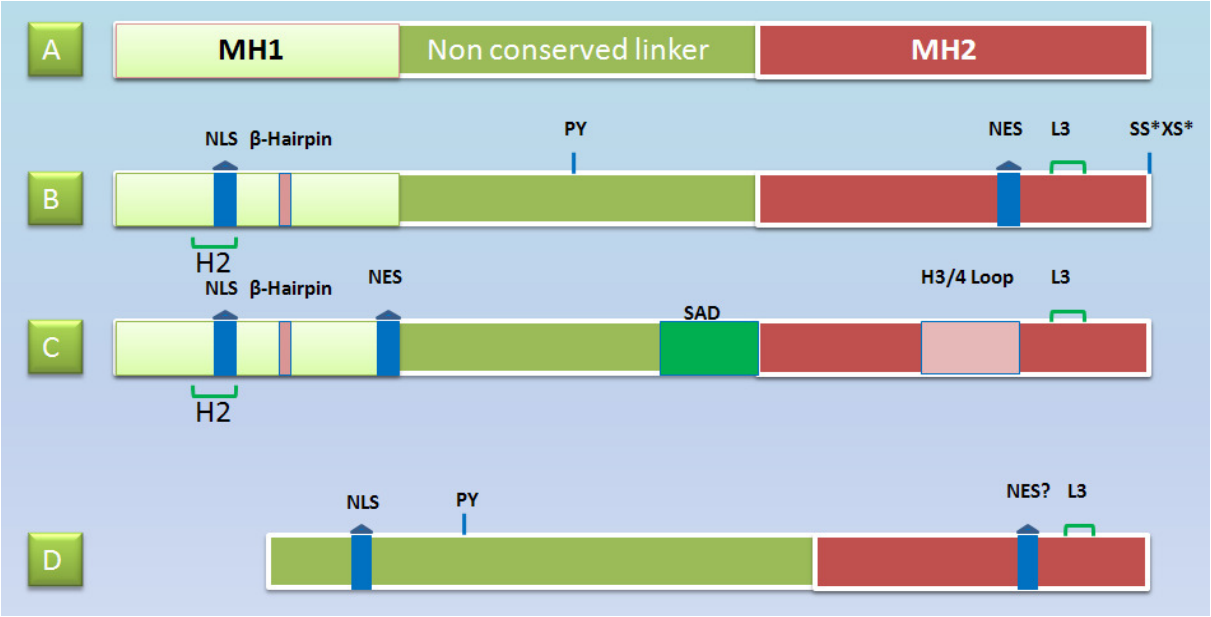

Figure 3. The Smad family. Diagramatic representation of structure of three subfamilies of Smads.
A. General structure of Smads
B. R-Smads (Smad1, Smad2, Smad3, Smad5 and Smad8)
C. Co-Smads (Smad4)
D. I-Smads (Smad6 and Smad7)
(Adapted from Moustakas A. et al., J. Cell Sci. 2001 114: 4359-4369).

parts of I-Smads have only weak sequence similarity to MH1 domains. Sequence and structural analyses indicate that the MH1 domain is homologous to the diverse His-Me (histidine-metal-ion) finger family of endonucleases, and it may have evolved from an ancient enzymatic domain that had lost its catalytic activity but retained its DNA-binding properties (Grishin *et al.,* 2001). The MH1 domain regulates nuclear import and transcription by binding to DNA and interacting with nuclear proteins. The MH2 domain is highly conserved among all Smads. Its structure contains several α-helices and loops, which surround a β-sandwich, and it resembles the fork head associated (FHA) domain, a phosphopeptide-binding domain common in transcription and signaling factors. The MH2 domain regulates Smad oligomerisation, recognition by type I receptors and interacts with cytoplasmic adaptors and several transcription factors (Shi Y, 2001; Moustakas et al., 2001; Li et al., 2000).

SMAD4 and aberrations

The role of Smad4 gene as an important tumor suppressor gene came out by the novel study of the allelotype loss in pancreatic adenocarcinoma (Shi Y, 2001). This study showed that about 90% of these tumors show allelic loss of chromosome 18q. In the same year another study identified the genetic target of these allelic losses as the *DPC4* gene (*DPC-Deleted in Pancreatic Carcinoma, locus 4*). The study analyzed 338 tumors, originating from 12 distinct anatomic sites, for alterations in the *DPC4* gene. An alteration of the *DPC4* gene sequence was identified in one of eight breast carcinomas and one of eight ovarian carcinomas. *DPC4* was found to be homozygously deleted in about 30% of pancreatic carcinomas and inactivated by intragenic mutation in another 20% of the tumors. The tissue restriction of alterations in *DPC4*, as in many other tumor-suppressor genes, emphasizes the complexity of rate-limiting checkpoints in human tumorigenesis (Schutte et al., 1996).

Smad4 was proposed to be a tumor suppressor gene that may function to disrupt TGF-β signaling. Mutant Smad4 proteins, identified in human carcinomas, were found to be impaired in their ability to regulate gene transcription. Most of Smad4 gene mutations in human cancer are missense, nonsense, and frameshift mutations at the mad homology 2 region (MH2) which interfere with the homo-oligomer formation of Smad4 protein and hetero-oligomer formation between Smad4 and Smad2 proteins, resulting in disruption of TGF-β signaling (Table 2) (Shi, 2001; Woodford-Richens et al., 2001; Roth et al., 2003).

Moskaluk et al. (1997) later on described the optimized primers and conditions used in polymerase chain reaction

Table 2. Nature of *SMAD4* MCR region mutations in colorectal carcinoma patients (Reported from across the globe).

SMAD4 Exon	Mutation	Amino acid change	Affected codon	Effect
1	GGA>GTA	Gly>Val	*64*	*MS*
1	GCT >GTT	Ala >Val	*86*	*MS*
2	TAT>AAT	Tyr> Asn	*94*	*MS*
2	TGG>CGG	Trp>Arg	*99*	*MS*
2	AAA>AAAA	Insertion (1bp)	*106*	*FS*
2	TGT>CGT	Cys>Arg	*115*	*MS*
2	GCG>GAG	Ala>Glu	*118*	*MS*
2	GCG>GTG	Ala>Val	*118*	*MS*
2	TTA >TA	Deletion (1bp)	*121*	*FS*
2	TTA >TTAA	Insertion (1bp)	*121*	*FS*
2	GTC> GCC	Val to Ala	*127*	*MS*
2	AAT>AAG	Asn>Lys	*129*	*MS*
2	CGA>TGA	Arg>Stop	*135*	*NS*
4	GGA>TGA	Gly>Stop	*168*	*NS*
4	TAC>TAA	Tyr>Stop	*195*	*NS*
4	CAG>TAG	Gln>Stop	*245*	*NS*
4	ACT to A	Deletion (2bp)	*259*	*FS*
6	ACT>ACTT	Truncation at codon 271/72	*269-270*	*FS*
7	TGG>CGG	Trp>Arg	*302*	*MS*
8	AGT>AAT	Aberrant splicing	*Intron-Exon region*	*Splice site change*
8	TAC> TAA	Tyr >Stop	*328*	*NS*
8	GAA>GCA	Glu>Ala	*330*	*MS*
8	GAA>AAA	Glu>Lys	*330*	*MS*
8	GAT>GGT	Asp>Gly	*332*	*MS*
8	AAG>GAG	Lys>Glu	*340*	*MS*
8	AT….AA>AA	Deletion (15bp)	*339-343*	*FS*
8	GAGAGA>GAGA	Truncation at codon 339-40	*336-338*	*FS*
8	GTT>GAT	Val>Asp	*350*	*MS*
8	GAT>CAT	Asp>His	*351*	*MS*
8	TAC>TGC	Tyr>Cys	*353*	*MS*
8	GAC>GAA	Asp>Glu	*355*	*MS*
8	CGC>AGC	Arg>Ser	*361*	*MS*
8	CGC>CAC	Arg>His	*361*	*MS*
8	TGT>AGT	Cys>Ser	*363*	*MS*
8	GTT>GAT	Val>Asp	*370*	*MS*
9	TGC>CGC	Cys>Arg	*401*	*MS*
9	TTT>TCT	Phe>Ser	*408*	*MS*
9	CAG>CACAG	Insertion (2bp)	*410*	*FS*
9	AGACAGAG>AGAG	Deletion	*415-16*	*FS*
9	GCA>GTA	Ala>Val	*433*	*MS*
10	CAG>TAG	Gln>Stop	*442*	*NS*
10	CGA>TGA	Arg>Stop	*445*	*NS*
10	GC…..AGC>GC	Deletion (25bp)	*447-455*	*FS*
10	CA…CT>CT	Deletion (28bp)	*450-459*	*FS*
11	GGT>GTT	Gly>Val	*491*	*MS*
11	GTT>TTT	Val>Phe	*492*	*MS*
11	GAT>GCT	Asp>Val	*493*	*MS*
11	CGC>CAC	Arg>His	*497*	*MS*
11	TGC>TAC	Cys>Tyr	*499*	*MS*

Table 2. Contd.

SMAD4 Exon	Mutation	Amino acid change	Affected codon	Effect
11	AAA>CAA	Lys>Gln	*507*	*MS*
11	AGA>GGA	Arg>Gly	*515*	*MS*
11	CTC>GTC	Leu>Val	*533*	*MS*
11	GAT>TAT	Asp>Tyr	*537*	*MS*
11	CTA>CGA	Leu>Arg	*540*	*MS*
11	CT…CC>CC	Deletion (7bp)	*540-542*	*FS*

MS: Missense mutation; NS: Nonsense mutation and FS: Frameshift mutation.

and cycle sequencing of the entire *DPC4/SMAD4* coding sequence. In another study, a subset of juvenile polyposis syndrome (JPS) families was identified to carry germ line mutations in *SMAD4* gene. The mutant *SMAD4* proteins were predicted to be truncated at the carboxyl-terminus and lack sequences required for normal function. These results confirmed an important role for *SMAD4* in the development of gastrointestinal tumors (Howe et al., 1998). However another study carried out in England (Houlston et al., 1998), having the same design as the previous one reported somatic missense mutations affecting codon 361 (CGC/arg→TGC/Cys) in *DPC4/SMAD4* gene in juvenile polyposis tumors.

In the same year, a study on the mutational spectrum of the *SMAD2* gene was carried out in National Cancer Center Institute, Japan on human colon cancers (Takenoshita et al., 1998). The study revealed that though there was no mutation within all exons of the *SMAD2* gene, two of 60 sporadic CRCs displayed deletions in the polypyrimidine tract preceding exon4. Deletions of this region were also detected in colon cancer cell lines, and were clustered within cells exhibiting microsatellite instability.

Koyama et al. (1999) investigated the potential role of *DPC4/SMAD4* gene in CRCs. LOH was identified in 50 - 78% of the tumors that were informative for polymorphic markers in the region. Somatic mutations were identified in seven of those tumors: two frameshift mutations, a 1-bp deletion (326 del T) in exon8 and a 1-bp insertion (50 - 51 ins A) in exon1; two nonsense mutations, Arg445Ter in exon10 and Glu538Ter in exon11; and three missense mutations, Asn129Lys in exon 2, Tyr95Asn in exon 2, and Asp355Glu in exon8. Three of the seven mutations were observed in the MH1 domain encoded by exons1 and 2. The results demonstrated that inactivation of both alleles of the *DPC4/SMAD4* gene occurs in a substantial proportion of advanced CRCs, and that the *DPC4/SMAD4* gene probably exerts a tumor-suppressor effect for colorectal carcinogenesis that fulfills the criterion of the two-hit concept proposed by Knudson A.G. (1985).

With time, a large number of researchers' detected mutations in *SMAD2* and *SMAD4* genes in some colorectal carcinomas (Riggins et al., 1997; Houlston et al., 1992; Thiagalingam et al., 1996; Takagi et al., 1996; Mac Grogan et al., 1997), however the frequencies of these mutations have been found to be low, and the role of these genes in colorectal carcinogenesis is still unclear.

In order to clarify the contribution of *SMAD* genes in colorectal carcinogenesis, Miyaki et al. (1999) analyzed mutations of Smad2, 3, 4, 6 and 7 in different stages of tumor. Their study revealed twenty-one Smad4 mutations and one Smad2 mutation, whereas mutation of Smad 3, 6 and 7 genes was not detected. Smad4 mutations included seven frameshift, one inframe deletion, four nonsense and nine missense mutations, 95% of which resulted in alteration of Smad4 protein regions included in homo-oligomer and hetero-oligomer formation. Frequencies of tumors with Smad4 mutation were 0/40 (0%) in adenoma, 4/39 (10%) in intramucosal carcinoma, 3/44 (7%) in primary invasive carcinoma without distant metastasis, 6/17 (35%) in primary invasive carcinoma with distant metastasis, and 11/36 (31%) in distant metastasis. In a similar study Yakicier et al. (1999) analyzed mutations in *SMAD2* and *SMAD4* in hepatocellular carcinoma (HCC). The study was carried out on 35 HCC and non-tumor liver tissues. The results revealed that three tumors displayed somatic missense mutations, all of which were transistions of A: T →G: C type; two were in *SMAD4* (Asp332Gly and Cys401Arg) and one was in *SMAD2* (Gln407Arg).

By the start of the 21st century the mutational analysis of *smad* genes got a boost and large number of researchers reported the mutations in *SMAD2* and or *SMAD4* genes in CRCs of sporadic or of familial type and of different grades (Woodford-Richens et al., 2001; Roth et al., 2003; Ohtaki et al., 2001; Howe et al., 2001, 2002, 2004; Sayed et al., 2002).

Christine et al. (2004) recently reported the mutational hot spot in *SMAD4* gene and its functional consequences in human tumors. Their study concluded that the homozygous deletion, followed by inactivating nonsense or frameshift mutations, is the predominant form of *SMAD4* inactivation in pancreatic cancers. Among the naturally occurring *SMAD4* missense mutations, the MH2 domain is the most frequent target (77%) of missense mutations in human tumors. A mutational hot spot resides

Table 3. Nature of *SMAD4* MCR region mutations in colorectal carcinoma patients from Kashmir valley.

SMAD4 Exon	Mutation	Amino acid change	Affected codon	Effect
2	TGT>*C*GT	Cys>Arg	115	MS
8	CGC>*A*GC	Arg>Ser	361	MS
8	CGC>C*A*C	Arg>His	361	MS
8	TTT>TT*G*	Phe>Leu	362	MS
8	TGT>*A*GT	Cys>Ser	363	MS
8	GGTT>G*A*GTT	Insertion	341	FS
8	CGC>C*A*C	Arg>His	361	MS
9	TGG>*G*GG	Trp>Gly	419	MS
9	*AGACA*GAG>AGAG	Deletion	415/16	FS
9	AGA>A*A*A	Arg>Lys	415	MS
10	CAG>*T*AG	Gln>Stop	442	NS
10	GCT>GC*C*	Ala >Ala	456	S
10	CGA>*T*GA	Arg>Stop	445	NS
11	AAGGC>AA*TT*GC	Lys> Asn; Gly>Cys	507/8	MS
11	GGC>*A*GC	Gly>Ser	508	MS
11	AAA>*C*AA	Lys>Gln	507	MS

within the MH2 domain corresponding to codons 330 - 370, termed the mutation cluster region (MCR). These findings have important implications for *in vitro* functional studies, suggesting that the majority of missense mutations inactivate Madh4 by protein degradation in contrast to those that occur within the MCR (Christine et al., 2004).

More recently a number of articles have been published on the *SMAD4* mutations and their increased association with the CRC. One of the important observations has been the identification of *SMAD4* gene as the prognostic marker of the subtype of CRC. A recent article published in cancer letters by Qiu et al. (2007) identified the novel nonsense mutations in *SMAD4* gene located in exon 5 codon 245 CAG (glut) →TAG (stop) and in *SMAD2* in exon 8 at codon 276 TCG (ser) → TTG (leu).

In our own study carried out on 86 primary colorectal carcinomas from Kashmiri population, we have found 16 (18.6%) tumors harboring *SMAD4* mutations (Table 3). There were eleven missense mutations, one silent mutation, two nonsense mutations, one silent mutation and two frameshift mutations including eight transitions and four transversions. Among the two frame shift mutations, one was observed in codon 341 (exon 8) due to insertion of A and the other one in codons 415/416 (exon 9) due to deletion of AGACA pentamer respectively (Table 2, Figure 1). Among the transistions, G:A>A:G substitutions were most prevalent followed by C:T>T:C. The two nonsense mutations included, CAG>TAG transition leading to Gln>Stop at codon 442 and other CGA>TGA transition leading to Arg>Stop at codon 445, both occured in exon 10 of *SMAD4* gene. (Manuscript Submitted in BMC Cancer).

In conclusion, considering the important role of *SMAD4* in the colorectal carcinogenesis one can say that *SMAD4* plays a role of important molecular gladiator in the development of normal mucosa. If however, this gladiator losses to the mutations then it creates havoc in the tissue morphology leading to the uncontrolled development of mucosa which in turn leads to progression to tumor. As proved by most studies *SMAD4* may serve as the important prognostic molecule in CRC, laboratories across the world may use it for better treatment of the secondary CRC especially in identification and treatment of Dukes C patients and thus help in increasing the overall survival of the patient.

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