Abstract

The tumor suppressor gene Smad4 (DPC4) at chromosome 18q21.1 belongs to the Smad family, which mediates the TGFβ signaling pathway suppressing epithelial cell growth. This review summarizes the mutational events of the Smad4 gene in human cancer. The Smad4 gene is genetically responsible for familial juvenile polyposis, an autosomal dominant disease characterized by predisposition to gastrointestinal polyps and cancer. In this syndrome, polyps are formed by inactivation of the Smad4 gene through germline mutation and loss of the unaffected wild-type allele. In pancreatic and colorectal cancer, inactivation of the Smad4 gene through homozygous deletion or intragenic mutation occurs frequently in association with malignant progression. However, mutation of this gene is seen only occasionally in the rest of human cancers. The majority 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 the hetero-oligomer formation between Smad4 and Smad2 proteins, resulting in disruption of TGFβ signaling. Supporting evidence for the above observation was provided by genetically manipulated mice carrying either a heterozygote of the Smad4 gene or a compound heterozygote of the Smad4 and APC genes, which develop either gastrointestinal polyps/cancer mimicking familial juvenile polyposis or progressed colorectal cancer, respectively.

Keywords: Smad4; Mutation; Homozygous deletion; TGFβ signaling; Human cancer; Pancreatic cancer; Colorectal cancer; Familial juvenile polyposis

Isolation of the DPC4 gene and its identification as a member of the Smad family

Smad4 was first identified as a tumor suppressor gene of pancreatic cancer by Harn et al. in 1996 and designated as DPC4 (homozygously deleted in pancreatic carcinoma, locus 4) [1]. Nearly 90% of pancreatic cancer show loss of heterozygosity (LOH) at chromosome 18q [2], in which the candidate tumor suppressor gene DCC (deleted in colorectal carcinoma) is located. Since mutation of the DCC gene has not been found in pancreatic and other cancers, a search for a novel target gene of the 18qLOH was initiated by analysis of pancreatic cancer showing a homozygous deletion. The markers D18S46 and D18S363, centromeric to DCC, were found to be lost, suggesting the presence of a new locus of the target gene at 18q21.1. These two markers were used to screen a YAC library and a cosmid (c917-46) at the smallest consensus of the deleted region was obtained. Using this cosmid, potentially expressed sequences were identified after several additional cloning processes. Hybridization of one of these sequences to a human fetal brain cDNA library revealed a novel 525-bp transcript. This new gene was found to be homozygously deleted in 25 of 84 pancreatic cancers. Moreover, 6 of 27 cancers without homozygous deletion exhibited potentially inactivating mutations.

The human DPC4 gene contains 11 exons with a predicted 552 amino acid coding sequence [1]. This DPC4 protein sequence has similarities to the Drosophila melanogaster Mad (mothers against dpp) protein and to the Caenorhabditis elegans Mad homologs sma-2, sma-3, and sma-4 [3]. DPC4 is the human homolog of sma-4, being called in general Smad4.
Smad proteins are the critical components of the TGFβ signaling pathway, which negatively regulates the growth of epithelial cells (for a review see [4]). As illustrated in Fig. 1, TGFβ signals are transduced by two kinds of receptors, Receptor I and II, which have serine/threonine kinase activity. Upon the binding of TGFβ to TGFβRII, this receptor activates TGFβRI by phosphorylation. TGFβRI in turn phosphorylates the intracellular target Smad2 or Smad3. These proteins have an SSXS motif in their carboxyl-terminal region, which is the phosphorylation target of the activated TGFβRI. Phosphorylated Smad2 or Smad3 forms a hetero-oligomer with Smad4 and the resulting Smad complex translocates to the nucleus. This Smad protein complex interacts with DNA directly or indirectly through other DNA-binding proteins, regulating transcription of the target genes and thus leading to the regulation of cellular proliferation. Smad6 and Smad7 are assumed to inhibit the phosphorylation of Smad2 and Smad3.

Smad2, Smad3, and Smad4 proteins have two regions, termed mad homology 1 and 2 (MH1 and MH2), at the amino-terminal and at the carboxyl-terminal, respectively, which are conserved among diverse species. The MH1 domain is involved in DNA binding, while the MH2 domain participates in homo- and hetero-oligomerization. Analysis of the crystal structure of the Smad4 MH2 domain suggests that this molecule exists as a homo-trimer [5]. The MH2 domain of Smad4 is also involved in transcriptional activation and nuclear location [6].

Germline mutation of the Smad4 gene in familial juvenile polyposis

Familial juvenile polyposis is an autosomal dominant disease characterized by predisposition to hamartomatous polyps and gastrointestinal cancer. This disease usually occurs during childhood. In 1998, Howe et al. [7] reported germline mutations in the Smad4 gene among 5 of 9 familial juvenile polyposis families, suggesting that Smad4 is the causative gene of this disease. To date, germline mutations have been detected in nearly 20 families and account for 25–60% of the cases analyzed. The majority of germline mutations are located in the MH2 domain: six families were reported to have the same AGAC 4 bp deletion at the codon 414–416, which is most likely a mutational hot spot. Woodford-Richens et al. [8] reported that Smad4 was not detected immunohistochemically in 63 of 64 polyps from Smad4 mutation carriers, including those with frameshift and missense germline mutations. In families without the Smad4 mutation, however, 37 of 38 polyps expressed Smad4. These data suggest that germline mutation of the Smad4 gene is accompanied with the second somatic mutation of Smad4 in the unaffected allele, implying that Smad4 acts as a tumor suppressor gene in the development of familial juvenile polyposis.

Supporting evidence for the above observation was provided by Taketo and his colleagues [9], who constructed knock-out mice with the Smad4 gene. Although Smad4-null mice (−/−) were embryonically lethal, the heterozygotes of Smad4 (+/−) were fertile and appeared normal up to the age of 1 year. At the age of 50 weeks, however, gastric polyps developed in 3 of 15 heterozygous mice, and at the age of 100 weeks, in all heterozygous mice. In addition, duodenal polyps were found in mice older than 50 weeks. Those polyps losing the wild-type allele were not stained for the Smad4 protein. Morphologically, these polyps resembled those of human juvenile polyposis. These results suggest that inactivation of Smad4 is one of the early events in polyp formation in the Smad4 (+/−) mice, which is analogous to human familial juvenile polyposis.

Somatic alterations of the Smad4 gene in pancreatic cancer

Prior to isolation of the DPC4 gene, the same group demonstrated in 1995 frequent LOH at chromosomes 1p (70%), 9p (80%), 17p (90%), and 18q (90%) by an allelotype analysis of 18 xenografts of human pancreatic cancer [2]. This suggested that possible tumor suppres-
sor genes are present in these regions. As mentioned above, the tumor suppressor gene, Smad4, was identified at the homozygously deleted region 18q21.1. Additional genetic alterations in pancreatic cancer include mutations of the K-ras gene (over 80%), mutations of the p53 gene at 17p (70%), and mutations or homozygous deletions of the p16 gene at 9p (nearly 80%).

Mutational events of the Smad4 gene in pancreatic cancer were demonstrated during the period from 1996 to 2000. Homozygous deletions of the Smad4 region were detected in 30% (25/84) [10] and 37% (14/38) [11]. In two studies [11,12], intragenic inactivating mutations of the Smad4 gene were accompanied by a loss of the other allele. These mutations include nonsense, missense, and frameshift mutations, more than 90% of which are located in the MH2 region. In total, approximately 55% of pancreatic cancers show inactivation of the Smad4 gene.

Wilentz et al. [13] demonstrated that inactivation of Smad4 gene is well correlated with a loss of the expression of its protein. Immunohistochemical staining revealed that 91% (21/23) of primary carcinomas with inactivated Smad4 genes did not express the Smad4 protein, whereas 94% (17/18) of the tumors with wildtype alleles expressed the Smad4 protein. A further study from the same group [14] revealed that the expression of Smad4 protein is associated with histopathological grades of pancreatic cancer. Of 188 pancreatic intraepithelial neoplasias examined, Smad4 protein was not expressed immunohistochemically in 31% (9/29) of the high-grade lesions (Pan IN-3), while all 159 histopathologically low-grade neoplasias (Pan IN-1 and -2) expressed it. Based on these observations, the following stages of genetic alterations were proposed for the progression of pancreatic cancer: the K-ras mutation is the earliest change, followed by alteration of p16, and at a later stage p53 and Smad4 alterations occurring.

Somatic alterations of the Smad4 gene in colorectal cancer

In 1990, Fearon and Vogelstein [15] proposed multistage genetic alterations in colorectal carcinogenesis, typically in an adenoma–carcinoma sequence. In this sequence, the APC gene (5q) is inactivated at an early stage, followed by activation of the K-ras gene during the development of moderate to severe adenoma, and inactivation of the p53 gene (17p) at the stage of conversion from adenoma to carcinoma. Additional frequent LOH at chromosomes 1p, 8p, 18q, and 22q suggests that inactivation of possible tumor suppressor genes at these regions may also be involved in colorectal carcinogenesis [16–18]. Miyaki et al. [17,18] demonstrated that the LOH on chromosome 18 was observed in 46% of invasive carcinomas, but not in adenomas or intramucosal carcinomas, suggesting that 18qLOH occurs at the stage of progression to invasive carcinoma.

A minimally lost 18q21 region contains at least two candidate tumor suppressor genes, i.e., DCC and Smad4. Like in pancreatic cancer, the DCC gene seems not to be involved in colorectal carcinogenesis [19]. As for the Smad4 gene, homozygous deletions and/or mutations were reported in xenografts and cell lines derived from colorectal cancer, as well as in primary cancers, at varying frequencies from 10% to 35% [20–23].

To clarify a possible role of the Smad4 gene in the progression of colorectal carcinogenesis, it is important to analyze tumors with varying stages. In 1999, we demonstrated that frequency of mutational events of Smad4 gene increases with the progression of carcinogenesis by analyzing 176 tumors at varying stages [24]: being 0% (0/40) in adenomas, 10% (4/39) in intramucosal carcinomas, 7% (3/44) in invasive carcinomas without distant metastasis, 35% (6/17) in primary invasive carcinomas with distant metastasis, and 31% (11/36) in carcinomas metastasized to the liver and distant lymph nodes, or disseminated (Fig. 2). These Smad4 gene mutations include frameshift, nonsense, and missense mutations, more than 80% of which occur at the MH2 region, as seen in Fig. 3. There is a hot spot of missense mutations at codon 361, which disrupt both homo- and hetero-oligomerization. Loss of the wild-type allele was also detected in 95% of invasive and metastatic carcinomas. Our observation provides convincing evidence that the loss of Smad4 function occurs at later stages of malignancy, playing a role in the acquisition of advanced phenotypes.

![Fig. 2. Frequencies of somatic mutation of the Smad4 gene in colorectal tumors at varying stages.](image-url)
Maitra et al. [25] confirmed our observation by the immunohistochemical staining of Smad4 protein in varying stages of colorectal cancers. Although all adenomas or stage I adenocarcinomas expressed Smad4 protein, 8% (1/13) of stage II, 6% (1/17) of stage III, and 22% (5/23) of stage IV cancers were not stained. These data again suggest that inactivation of Smad4 is a late event in colorectal carcinogenesis.

Using genetically manipulated mice, Taketo and his colleagues demonstrated in 1998 that the Smad4 mutation is a late event in mouse intestinal carcinogenesis [26]. They cleverly constructed compound heterozygotes carrying both APC and Smad4 mutations on the same chromosome by meiotic recombination. The intestinal polyps in the cis-compound heterozygotes, i.e., APC (+/-) Smad4 (+/-) mice, were much larger than those in the simple APC heterozygotes, i.e., APC (+/+) mice, although the polyp numbers were not significantly different between these two genotypes of mice. Moreover, the submucosal invasion and development into adenocarcinomas, which formed tumors in nude mice, were observed in the cis-compound heterozygotes, whereas polyps in the simple APC heterozygotes did not progress. These malignant tumors were found to lose the wild-type allele of both the APC and Smad4 genes. These results in the mouse models demonstrated that inactivation of the Smad4 gene results in the malignant progression of intestinal polyps initiated by inactivation of the APC gene.

### Somatic alterations of the Smad4 gene in various types of human cancer

Table 1 summarizes the Smad4 gene alterations in various human cancers. Somatic alteration of the Smad4 gene is, as mentioned above, most prevalent in pancreatic and colorectal cancer. Intragenic mutations were less frequently observed in acute myeloid leukemia, biliary tract carcinoma, ovarian cancer, and small

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nd, not determined.
intestinal carcinoma, and occasionally in gastric carcinoma, head/neck squamous carcinoma, hepatocellular carcinoma, and lung carcinoma.

As shown in Fig. 3, the majority of Smad4 gene mutations in these human cancer occur at the MH2 domain, interfering with the homo-oligomer formation of Smad4 protein and the hetero-oligomer formation between Smad4 and Smad2 (or Smad3) proteins. Almost all cancers with the Smad4 mutation show a loss of the wild-type allele, indicating that biallelic alterations result in the inactivation of Smad4 protein.

Mutations of other members of the Smad family

Mutations of the Smad2 gene, located at 18q21, have been detected in the MH2 domain in colorectal and other human cancers, but their frequency being far less than those of the Smad4 gene [37]. Mutations of Smad3 (15q21), Smad6 (15q21), and Smad7 (18q21) genes have not been detected so far in human cancers.

References


