Infrequent mutation of the tumour-suppressor gene Smad4 in early-stage colorectal cancer

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Smad4 is a candidate tumour-suppressor gene identified recently on chromosome 18q21.1. Both alleles are inactivated in nearly one-half of pancreatic carcinomas, but its role in the tumorigenesis of other tumours is still unknown. The aim of this study was to investigate the potential involvement of the Smad4 locus in early-stage colorectal cancers (stages I–III) in tumour samples from a randomised multicentre trial. Of a large collection of DNA samples, 73 with a loss of one allele of the Smad4 gene were analysed for the presence of point mutations in the remaining gene. Patients, from whom biopsies were isolated, were part of a previous randomised multicentre study of the Swiss Group for Clinical Cancer Research on the benefit of adjuvant chemotherapy (SAKK study 40/81). Mutation analysis was restricted to the highly conserved C-terminal domain (exons 8, 9, 10 and 11) of Smad4, using PCR and single-strand conformational variant analysis. Two of the 73 patients (3%) with loss of one allele of Smad4 had a point mutation in the remaining allele. These results indicate that whereas Smad4 point mutations are prevalent in pancreatic carcinoma, they are infrequent in early stages (I–III) of colorectal cancer.

Keywords: DPC4; loss of heterozygosity (LOH); Smad4; TGFβ; tumorigenesis

Deletion of a chromosomal region is a frequent cytogenetic alteration observed in carcinogenesis. The loss of tumour-suppressor genes has been reported in numerous types of human tumours, in particular those of the gastrointestinal tract (Vogelstein et al., 1988). APC and p53 have been widely recognised as important tumour-suppressor genes inactivated during colorectal carcinogenesis. Several other tumour-suppressor genes have been located on chromosomes 1p, 8p, 18q and 22q. In particular, loss of heterozygosity (LOH) at 18q21 is correlated with carcinomas of the colon, and other tumours such as pancreatic carcinoma, renal cell carcinoma, melanoma and breast carcinoma (Schutte et al., 1996; Barbera et al., 2000). Much of the interest in this region arose because reports indicated that 18q losses are associated with high metastatic potential and reduced patient survival (Iino et al., 1994).

In fact, several potentially cancer-related genes map to the 18q21 region, including bcl-2, gastrin-releasing peptide gene and cellular homologue of yes-1. However, none of these have been observed to be mutated in colorectal cancer (CRC). Fearon et al. (1990) identified another tumour-suppressor gene localised on 18q21, designated DCC for deleted in colorectal cancer. However, there have been several cases in which loss of expression did not correlate with LOH (Kikuchi-Yanoshita et al., 1992), and mutation in the coding region of the DCC gene has been infrequently detected (Cho et al., 1994; Sato et al., 2001). Owing to the controversial evidence as to the role of DCC in cancer, additional genetic analysis of the 18q21 region led to the identification of other potential tumour-suppressor genes, including three candidate tumour-suppressor genes: Smad2, Smad4 and Smad7. These genes are involved in signal transduction of the TGFβ signalling pathway. Members of the transforming growth factor (TGF)β family transmit their signals from the plasma membrane to the nucleus through combinations of serine/threonine kinase receptors and their downstream effectors, known as Smads. After the Smad4 (MADH4) gene was isolated from the same region as a tumour-suppressor gene for pancreatic cancer (Hahn et al., 1996), mutation analysis of this gene has been carried out in various cancers. In recent studies, Smad4 was identified as a genetic target in pancreatic carcinomas, inactivated through homozygous deletion (n = 5), intragenic mutation (n = 3) and lack of protein (n = 2) in 10 out of 16 pancreatic cell lines (Barbera et al., 2000). Furthermore, it could be shown that when genetically inactivated this tumour-suppressor in the TGFβ signalling pathway represents a prognostic factor in invasive pancreatic cancer influenced by Smad4 status (Tascilar et al., 2001). In addition to observations in pancreatic carcinomas, Smad4 is also known as a gene involved in juvenile polyposis tumour predisposition syndrome (Howe et al., 1998; Huang et al., 2000). Mutations of the Smad4 gene have been detected in some colorectal cancers, but its role in this specific cancer remains unclear. The frequencies of mutations (5–45%) have been found to be low (Takagi et al., 1996; MacGrogan et al., 1997; Ohtaki et al., 2001), but data originated from relatively small studies, and the tumour populations examined were inhomogeneous explaining the broad range of incidences found.
The aim of this study was to further expand this data using Smad4 mutation analysis of a large set of early-stage (I–III) colorectal cancer patients treated in a randomised multicentre trial of 5-fluorouracil (5-FU)/Mitomycin C adjuvant chemotherapy of the Swiss Group for Clinical Cancer Research (SAKK study 40/81). Owing to the significance of LOH in colorectal cancer and the role of the remaining gene, this study was focused on patients with an allelic loss of one Smad4.

METHODS

Patients

Patients from whom biopsies were isolated, were part of a previous randomised multicentre study of the SAKK on the benefit of treatment with adjuvant chemotherapy between 1981 and 1987 (Laffer, 1995). Deoxyribo nucleic acid (DNA) samples of these patients were extracted from tumour as well as from healthy tissue derived from the same patient in order to perform genetic analyses. Paraffin-embedded material was available from 329 of the 505 patients. To investigate genetic alterations in the 18q21 region in these tumours, a gene dosage study of the tumour-suppressor genes Smad2, Smad4 and DCC was performed (Boulay et al, 1999). For technical reasons, high-quality DNA for analysis was available from 294 patients only. Individual dosage of the Smad4 gene showed a total deletion frequency (one or both alleles) of 68% when compared to normal tissue. In total, 167 patients (= 57%) were detected with an allelic loss of one Smad4 copy. In this study, we randomly chose 73 of these 167 patients to search for the presence of point mutations in the remaining gene. After analysis of these 73 out of 167 patients, two point mutations of Smad4 had been detected, and for statistical reasons, further mutation analysis in the remaining 94 out of 167 patients did not seem necessary to substantiate our finding.

Gene copy status scoring

Genomic samples from 294 patients were tested for copy dosage of the Smad4 gene using TaqMan quantitative real-time PCR (Perkin-Elmer, Huenenberg, Switzerland). Copy status of the Smad4 gene was determined by comparing tumour DNA to DNA from normal tissue derived from the same patient as described previously (Boulay et al, 1999).

Duplex PCR

Polymerase chain reaction (PCR) amplification on DNA was performed in 15 µl reaction volume, containing 1.5 µl 10 × PCR buffer (Perkin-Elmer, Huenenberg, Switzerland), 10 mM 2’-desoxynucleosoid-5’-triphosphate (dNTPs), 20 µM of each primer, 1 U of AmpliTaq Gold (Perkin-Elmer, Huenenberg, Switzerland), 32P Oligo (2.5 µl 10 × Buffer, 20 µM forward primer, 1 µl PNK and 1 µl γ32P-adenosine triphosphate (ATP) incubated 30 min at 37°C) and 100 ng DNA. Duplex PCR for Smad4 gene was done using primers EX 8/1 and EX 8/2, EX 9/1 and EX 9/2, EX 10/1 and EX 10/2, and finally EX 11/1 and EX 11/2 (Table 1). Polymerase chain reaction conditions were as follows: 40 amplification cycles of denaturation at 94°C for 45 s, annealing at 55°C for 60 s, and extension at 72°C for 60 s, followed by one cycle at 72°C for 10 min. Amplification products were loaded on a 0.4 mm acrylamide gel in a ‘Model S2 Sequencing Gel Electrophoresis Apparatus (Life Technologies, Switzerland)’. Electrophoresis settings are: 1800 V, 35 – 40 mA, 60 VA and 120 min. Polymerase chain reactions without DNA templates were performed as negative controls. Bands were subsequently cut out from the single-strand conformation polymorphism (SSCP)-gel and reamplified in a PCR.

Sequencing analysis

Reamplification of DNA was performed in a 50 µl reaction volume, containing 5 µl 10 × PCR buffer (Perkin-Elmer, Huenenberg, Switzerland), 10 mM dNTPs, 20 µM forward and backward primer, 1 U of AmpliTaq Gold (Perkin-Elmer, Huenenberg, Switzerland) and 38 µl H2O. PCR conditions were as follows: 35 amplification cycles of denaturation at 94°C for 45 s, annealing at 55°C for 60 s and extension at 72°C for 60 s, followed by one cycle at 72°C for 10 min. Sequencing analysis was performed by Microsynth (Basel, Switzerland) on a fluorescence-based DNA sequencer that utilises capillary electrophoresis with 96 capillaries operating in parallel.

RESULTS

Among the 294 tumours for which gene dosage data (Smad2, Smad4 and DCC) were available, 167 tumours (57%) showed heterozygous loss of Smad4 (Boulay et al, 1999), and 73 out of 167 samples were randomly chosen for further analysis. Of these, only two (3%) carried point mutations in Smad4 in tumour but not the corresponding healthy tissue, as demonstrated by PCR–SSCP (Figure 1). The two mutations were located in the highly conserved C-terminal Smad4 homology region. One confirmed point mutation was found in exon 9 and another point mutation in exon 11. Both mutations were confirmed by direct sequencing analysis showing one mutation resulting in an amino-acid change from arginine to serine; the second mutation led to an exchange of alanine to valine (Table 2).

However, a caveat in the interpretation of our data needs to be mentioned: informative and reproducible data were available from a total of 174 complete exons (8, 9, 10 and 11) derived from the 73 defined patients. This shortcoming of our data was because of technical problems in the analysis, caused by the sometimes poor quality of the DNA, as is often observed with nucleic acids isolated from paraffin-embedded tissue. Nevertheless, since the exons for which interpretable results were available were equally distributed

Table 1 Primer–sequences designed for duplex-PCR

| Primer   | Forward         | Reverse         |
|----------|-----------------|-----------------|
| EX 8/1   | 5’-GAAAGCCTTATATC TTCTCTC-3’ | 5’-CAGTATCCGATCAACACGTA-3’ |
| EX 8/2   | 5’-TCTTTAGGCTGTGCGATG-3’ | 5’-CAATTTTAAAGAATCTATCGA-3’ |
| EX 9/1   | 5’-TATTAGCCATCTAACAATCG-3’ | 5’-GTGGTCACACTAAGGCACCTGA-3’ |
| EX 9/2   | 5’-TAAAGGTGAAAGGTAGTATGTT-3’ | 5’-CAAATAGGCTTTAGCTCTA-3’ |
| EX 10/1  | 5’-GTCAGAGCCTTGTGTTTAAATG-3’ | 5’-ATCTGCGGGAAGGAGGTGTT-3’ |
| EX 10/2  | 5’-AACATCCCTGCCCGCAGATT-3’ | 5’-CAAAATATGTGATCCACACC-3’ |
| EX 11/1  | 5’-AAGAGATCACCCTGTCCTCCTCT-3’ | 5’-CCACCGAGGTTCTCTGTCTTG-3’ |
| EX 11/2  | 5’-GGATTACCAAAGCAGAGCA-3’ | 5’-GTATTTTGTGAGCATTCTAC-3’ |
DISCUSSION

Smad proteins are a novel family of proteins that function downstream of serine/threonine kinase receptors to transduce signals for members of the TGFβ superfamily (Massague, 1996). The three Smads (Smad2, Smad4 and Smad7) encoded in the 18q21 chromosomal region participate in the signalling mechanisms subsequent to TGFβ-receptor complex formation. Smad4, a so-called "co-Smad" of Smad2, is known as a tumour-suppressor gene in different cancer types. Tumour-suppressor genes are often inactivated when one allele acquires a mutation and the second allele is lost, typically through deletion (Cavenee et al, 1983). The importance of genes that encode tumour-suppressor protein (DCC), these findings could not be confirmed (Sato et al, 1997). Our screen of 73 patients with early-stage colorectal cancer (I–III) carrying a loss of one Smad4 allele–initially used to select a population with a presumably high mutation frequency–is one possible theoretical explanation for our findings. However, in pancreatic and biliary tract carcinomas, patients with LOH represent a group with an especially high point mutation frequency in the remaining gene, making this explanation highly unlikely (Hahn et al, 1998; Barbera et al, 2000).

Other possible explanations for the absence of Smad4 point mutations in colorectal cancer at this stage include methylation changes at the promoter and alternative splicing or changes in mRNA stability (Roth et al, 2000). The importance of genes that undergo alterations at low prevalence, however, may as yet be underestimated. Such events may contribute significantly to the genetic variety within a tumour type and, thus, to the complexity of human tumorigenesis. Since it is likely that many alterations of low prevalence exist in human cancers, an individual tumour might still acquire several of these different alterations with a high probability, making low prevalence alterations a powerful driving force of the carcinogenic process.

In conclusion, our findings indicate that Smad4 point mutations are infrequent in early stages of colorectal cancer. However, it cannot be completely ruled out that inactivation of Smad4 could be a common genetic event at later stages of colorectal cancer. Future research comparing early and advanced stages is required to investigate the tumour-suppressor pathway in colorectal cancer and to redefine the role Smad4 signalling plays in tumorigenesis.

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