Alterations in the Smad4 gene in hamster pancreatic duct adenocarcinomas and established cell lines

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Abstract. Alterations of the Smad4 gene, identified as a mediator of the transforming growth factor-ß pathway, were investigated in hamster pancreatic duct adenocarcinomas (PDAs) and established cell lines. Female Syrian golden hamsters received 70 mg/kg of *N*-nitrosobis(2-oxopropyl)amine (BOP) followed by repeated exposure to an augmentation pressure regimen consisting of a choline-deficient diet combined with DL-ethionine then L-methionine and a further administration of 20 mg/kg BOP. A total of 12 PDAs obtained 10 weeks after beginning the experiment and three cell lines established from subcutaneously transplantable PDAs in syngeneic hamsters were examined for mutations using reverse transcription-polymerase chain reaction-single strand conformation polymorphism (RT-PCR-SSCP) analysis. A mutation was detected in only one PDA (1/12, 8.3%) in the form of an ACC to ATC (Thr to IIe) transition at codon 73; none were detected in the three cell lines. No reduced or increased expression of the Smad4 gene was detected in any case using real-time quantitative RT-PCR. These results suggest that the Smad4 gene might play a role in limited fraction of BOP-induced pancreatic duct carcinogenesis in hamsters.

Introduction

Smad proteins are central mediators of the transforming growth factor ß (TGFß) signaling pathway (1-3). After activation by ligand binding, TGFß receptors stimulate the phosphorylation of Smad proteins, which form heterometric complexes with Smad4. These complexes accumulate in the nucleus, where they control gene expression in a cell-type specific and ligand dose-dependent manner with transcriptional factors, coactivators and corepressors (4-6). Smad4 has been mapped to chromosome 18q (7), and a high frequency of Smad4 genetic alterations have been observed in human pancreatic carcinomas (7), suggesting that the Smad4 gene is the target tumor suppressor gene of 18q loss of heterozygosity (LOH) (7).

Pancreatic duct adenocarcinomas (PDAs) have one of the lowest cure rates among human malignancies (8,9); therefore, it is of great importance that we understand the molecular mechanisms underlying pancreatic ductal carcinogenesis. However, at present, information on rate-limiting molecular events is exceedingly limited. Experimental models suitable for the investigation of human PDA development have been established in hamsters using the carcinogen, *N*-nitrosobis(2-oxopropyl)amine (BOP), and related compounds (10-12), and we previously developed a rapid production approach to facilitate studies on the underlying mechanisms (13-15). We have reported several genetic changes in this model. For example, a high frequency of Ki-ras mutations were found at early stages, along with p53 mutations, during pancreatic ductal carcinogenesis (16-18). In addition, we provided evidence that overexpression of the midkine and aberrant transcript of Fhit might also be involved in the development of PDAs (19,20). In the present study, to assess involvement of the Smad4 gene in hamster pancreatic duct carcinogenesis, we investigated alterations of the Smad4 gene in hamster PDAs induced by BOP and three established cell lines.

Materials and methods

Animals and treatment. A total of 18 female Syrian golden hamsters, weighing approximately 100 g each, were used (Japan SLC Inc., Shizuoka, Japan). PDAs were induced in 12 animals according to the rapid production model (13-15). Briefly, BOP (30 mg/kg body weight) (Nakalai Tesque, Inc., Kyoto, Japan) was given subcutaneously at initiation, followed by two cycles of augmentation pressure, which consisted of choline-deficient diet administration and ethionine-methionine-BOP injection. To obtain normal control tissue, including the pancreas, the remaining 6 animals were untreated and maintained free from carcinogen exposure throughout the

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experimental period. All hamsters were sacrificed under light ether anesthesia 10 weeks after beginning the experiment, and the pancreas was immediately excised. Macroscopically apparent tumors were dissected from the surrounding tissue and frozen in liquid nitrogen. Portions of the tumors were also fixed in 10% neutrally buffered formalin at 4˚C, routinely processed for paraffin embedding, sectioned and stained with hematoxylin and eosin for histological examination.

Cell lines. Details of the establishment of the three cell lines, HPD-1NR, HPD-2NR and HPD-3NR, were reported previously (21). Frozen cell lines were cultured in Dulbecco's modified Eagle's medium (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) containing 10% fetal bovine serum (Flow Laboratories, McLean, VA), 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin sulfate.

RNA extraction and detection of hamster Smad4 gene cDNA. Total RNA was prepared from frozen normal liver tissue using an Isogen kit (Nippon Gene, Inc. Toyama, Japan), and first-strand cDNA was synthesized from 1 μg aliquots using Ready-to-Go Your-Prime first-strand beads (Amersham Pharmacia Biotech Co. Ltd., Tokyo, Japan). To determine the sequence of the open reading frame (ORF) and 5' upstream and 3' downstream regions, PCR amplifications were performed with primer sets based on the rat Smad4 cDNA sequence (GenBank accession no. AF170064) as described previously (22). The amplified products were separated on 2% NuSieve agarose gels (BMA, Rockland, ME) containing 0.05 μg/ml ethidium bromide and then extracted and directly sequenced using a BigDye Terminator v3.0 cycle sequencing ready reaction kit (Applied Biosystems Japan Ltd., Tokyo, Japan) and an ABI PRISM 310 genetic analyzer (Applied Biosystems Japan Ltd.).

Reverse transcription-polymerase chain reaction single-strand conformation polymorphism (RT-PCR-SSCP) analysis. Total RNA was prepared from the 12 frozen PDA samples, 3 cell lines and 3 normal pancreas specimens using an Isogen kit (Nippon Gene, Inc.), then first-strand cDNA was synthesized from 0.2 μg aliquots with Ready-to-Go Your-Prime First-Strand beads (Amersham Pharmacia Biotech Co. Ltd.). To eliminate possible false positives caused by residual genomic DNA, all samples were treated with DNase.

RT-PCR-SSCP analysis was performed using the primers listed in Table I. All were designated from the hamster Smad4 cDNA sequence obtained in the above analysis. The MH-1 domain was amplified with 1F-1R and 2F-2R primer sets, and the MH-2 domain with 3F-3R, 4F-4R, and 5F-5R primer sets. Briefly, PCR for SSCP was performed in 10 μl of reaction mixture containing 1 μM of each primer, 200 μM of each dNTP, 1X PCR buffer (Perking-Elmer, Applied Biosystems Division, Foster City, CA), 2.5 units of Ampli Taq (Perking-Elmer) and 0.5 μl of synthesized cDNA mixture under the following reaction conditions; primary denaturation for 2 min at 95˚C, 30 cycles of 30-sec denaturation at 95˚C, 30-sec annealing at 56-64˚C, 1-min extension at 72˚C, and a final extension for 10 min at 72˚C. PCR products were diluted with 10 μl of loading solution containing 90% formide, 20 mM EDTA and 0.05% xylene cyanol and bromophenol blue.

Table I. Primers used for the RT-PCR-SSCP and real-time RT-PCR analyses.

	Primers	Annealing temp (°C)
1F	5'-GCCTGTCTGAGCATTGTACAT-3'	62
1R	5'-CCTCCATAGACGGGCATAG-3'	
2F	5'-TCAGGTGGCTGGTCGGAAAG-3'	58
2R	5'-TACTTGGTGGAGCATTACTCT-3'	
3F	5'-TCCTGCTCCTGAGTATTGG-3'	56
3R	5'-AGTAACTCTGCACAAAGACTG-3'	
4F	5'-GTTTGGGTCAGGTGCCTTAG-3'	63
4R	5'-CAGCAGCAGACAGACTGATTG-3'	
5F	5'-CTGGGTCCGTAGGTGGAATAG-3'	64
5R	5'-GCATGGTGTGAAGCACTTCAT-3'	
FR	5'-CTGGCGACGCTGTTCATAAGA-3'	64
RR	5'-GCTGCATCTGCCGGTGAC-3'	

Aliquots containing 6 μl of diluted products were electrophoresed on polyacrylamide gel using a GeneGel Excel 12.5/24 kit (Amersham Pharmacia Biotech Co. Ltd.) at 5˚C, 10˚C, 15˚C and 20˚C for 90 min at 15W with a GenePhor electrophoresis unit (Amersham Pharmacia Biotech Co. Ltd.). After electrophoresis, the gels were stained using a DNA silver-staining kit (Amersham Pharmacia Biotech Co. Ltd.).

DNA nucleotide sequencing. Following RT-PCR-SSCP analysis, DNA fragment from the abnormal shift band in the gel was extracted and reamplified. The obtained PCR product was directly sequenced using a BigDye Terminator v3.0 Cycle Sequencing Ready Reaction kit (Applied Biosystems Japan Ltd.) and ABI PRISM 310 genetic analyzer (Applied Biosystems Japan Ltd.). To confirm the results, PCR amplification was repeated using the same samples and each PCR product was sequenced with the forward and reverse primers at least twice.

Preparation of a cDNA probe and Southern blot analysis. A 1.775-kb cDNA fragment of the hamster Smad4 gene was obtained by RT-PCR using primer 5'-AGAAACTGGAGA GTTTGATT-3' and 5'-CTTCAGATTATAAACAGGGT-3'. The amplified product was subcloned and confirmed by sequencing and used as the hybridization probe for Smad4.

Genomic DNAs were extracted from frozen tissue of 6 PDAs, 3 cell lines and 3 normal pancreas samples using a DNeasy tissue kit (Qiagen, Hilden, Germany). After digestion with the restriction enzyme *Hind*III (Takara, Kyoto, Japan), which does not cut the ORF of hamster Smad4 cDNA, 7-μg DNA samples were fractioned by size in 1% agarose gel, blotted onto Hybond-XL membranes (Amersham Pharmacia Biotech, Buckinghamshire, UK), and hybridized with a [α-^{32}P]dCTP radiolabeled Smad4 probe using a Rediprime II random prime labeling system (Amersham Pharmacia Biotech). Blots were washed and then placed in contact with Hyperfilm MP (Amersham Pharmacia Biotech) at -80˚C.

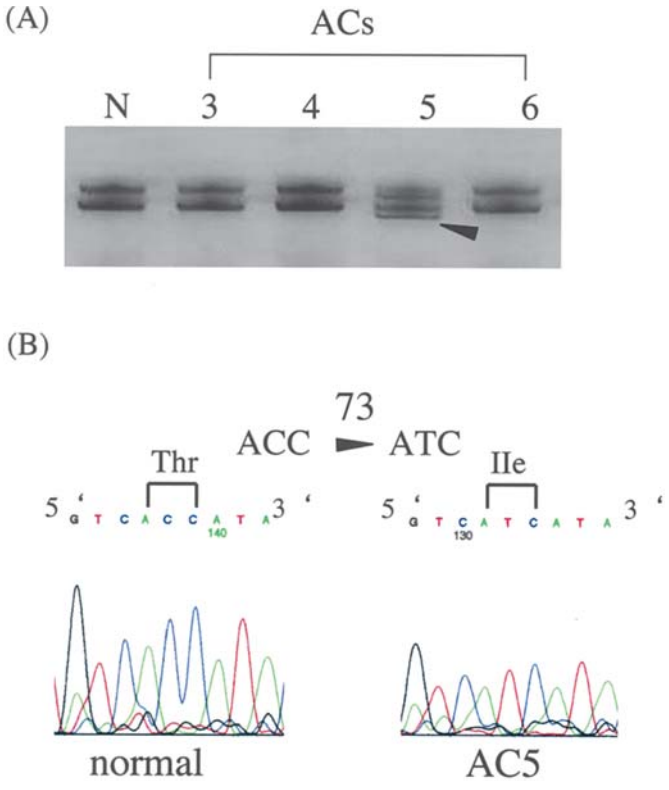

Figure 1. Point mutation of the Smad4 gene. (A) Representative results of RT-PCR-SSCP analysis. The arrowhead indicates an abnormal band shift. N, normal pancreas; ACs, adenocarcinomas. (B) The mutation patterns of the Smad4 gene detected by sequencing analysis. Normal, normal pancreas; AC, adenocarcinoma.

Real-time quantitative RT-PCR analysis. To assess expression of the Smad4 gene, real-time quantitative RT-PCR analysis using a Smart Cycler II System (Takara Bio, Inc., Shiga, Japan) and SYBR Premix Ex Taq (Takara) was performed according to the manufacturer's protocol. Six normal pancreas tissues were used as controls in this analysis. One μl of the cDNA synthesized from each sample was used in the following assay. The primers for Smad4 were newly designed as FR and RR (Table I). The rat glyceraldehyde-3-phosphate dehydrogenase (Gapdh) gene was used as an internal control (23). To obtain the standard curve for each gene, cDNA synthesized from hamster normal liver tissue was used. The amplification plots of the PCR reaction were used to determine the threshold cycle (Ct), which represents the first cycle in which there was a significant increase in fluorescence above the background fluorescence. The initial copy number of the target mRNA was calculated using a plot of the Ct against the input target quantity. Target gene data were normalized to that of Gapdh. Each assay was repeated at least twice for confirmation. Data were statistically analyzed using the Student's t-test.

Results

PDAs developed in all 12 hamsters treated with BOP, and one from every hamster was obtained for RT-PCR-SSCP and RT-PCR analyses. All PDAs used in this study were histologically well-differentiated. Of these, 6 were >7 mm in diameter and could be used in additional Southern blot analysis. Since PDAs include normal fibroblasts, three cell lines were also used for RT-PCR-SSCP, RT-PCR and Southern blot analyses. We detected the ORF of the hamster Smad4 gene cDNA sequence (GenBank accession no. AB209970) and were thus able to designate primers for the RT-PCR-SSCP analysis.

Using primers to amplify the MH1 and MH2 domains (Table I), the PCR products showed a clear single band in 1% agarose gels (data not shown). Representative results of the RT-PCR-SSCP and sequencing analysis are shown in Fig. 1A and B, respectively. One of 12 PDAs (8.3% incidence) indicated an abnormal band shift in the MH1 domain using a primer set of 1F and 1R. Sequence analysis revealed the mutation to be an ACC to ATC (Thr to IIe) transition at codon 73. This sample was one of the smaller tumors (<7 mm). No mutations were found in the MH2 domain, and no homozygous deletions were apparent in the MH1 and MH2 domains. No mutations or homozygous deletions were detected in the three cell lines. Normal sized PCR products amplified from the MH1 and MH2 domains indicated no mutations (data not shown). Southern blot analysis using a probe including the MH1 and MH2 domains showed no abnormal restriction patterns such as gross genomic rearrangement or homozygous deletion in the 6 PDAs and 3 cell lines (data not shown).

The results of semi-quantitative RT-PCR analysis are shown in Fig. 2. No reduced or increased expression of the Smad4 gene was detected in the 12 PDAs or 3 cell lines compared with 6 normal pancreas samples. There were no statistically significant differences.

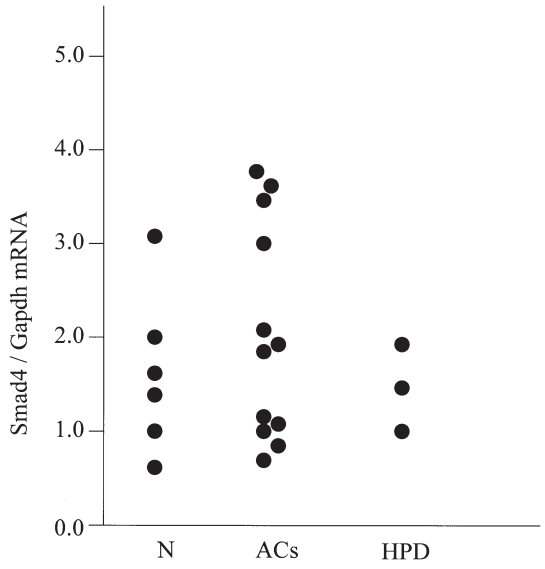

Figure 2. Expression levels of Smad4 mRNA relative to Gapdh mRNA. N, normal pancreas; ACs, adenocarcinomas; HPD, hamster PDA cell line.

Discussion

The present investigation demonstrated infrequent mutations, no genomic rearrangement or homozygous deletion and no reduced expression in the Smad4 gene in BOP-induced hamster PDAs and three established cell lines. These results

suggest that alterations in the Smad4 gene might play a role in a limited fraction of nitrosamine-induced hamster pancreatic duct carcinogenesis.

In human PDAs, a high frequency of Smad4 gene mutations has been reported (20%) (7). Mutations of the Smad4 gene have been also found in human colorectal carcinomas (<10%), biliary tract carcinomas (16%) and lung cancer (10%) (7,24-28), albeit at a rather low frequency. Thus, it has been suggested that inactivation of the Smad4 gene might play a role in pancreatic cancer and possibly other human cancers. In the case of rodents, we previously reported a low frequency of Smad4 mutations in rat lung adenocarcinomas induced by *N*-nitrosobis(2-hydroxypropyl)amine (8.3%) (22) and no mutations in rat hepatocellular carcinomas induced by *N*-nitrosodiethylamine (29). The present study showed only one mutation in 12 hamster PDAs and three established cell lines. It is unclear why the frequency of Smad4 mutation is so different in the PDAs of humans and hamsters. It seems that other factors, such as chronic oxidative stress, may be more involved than nitrosocompounds in the induction of Smad4 mutations. In fact, it is well-established that 8-hydroxyguanine, a representative feature of oxidative DNA damage, induces G to T or A to C transversions in *E. coli* (30).

Smad proteins consist of two conserved domains, the *N*-terminal MH1 domain and C-terminal MH2 domain. The MH2 domain is responsible for inactivation and homo- and hetero-oligomerization, whereas the MH1 domain exhibits sequence-specific DNA binding activity and negatively regulates MH2 domain function (6,31,32). The central region simply serves as a linker. Therefore, in the present study, we focused on mutations in the MH1 and MH2 domains of Smad4. In fact, the majority of Smad4 mutations in human cancers are located in the MH2 domain (6,7,24-27). In human PDAs, mutations of the Smad4 gene were shown to be mainly located in the MH2 domain (7). However, one mutation found in this study was located in the MH1 domain and not the MH2 domain.

It is well known that nitrosamines are widely distributed in the environment (33,34). G/C to A/T transition is considered a common mutation induced by methylating *N*-nitroso-compounds (35). Our previous report showed a G/C to A/T transition of the Ki-ras gene in hamster PDAs induced by BOP (57%), suggesting that Ki-ras mutations might be caused by the nitrosocompound (16,17,36). In the present study, the detected smad4 mutation indicated a C/G to T/A transition and might also be the result of nitrosocompound. However, this mutation was found in only one PDA, while Ki-ras mutations were detected in PDAs and preneoplastic lesions, such as hyperplasias and adenomas (16,17). Therefore, it seems that the Smad4 gene might not be the main target of nitrosocompounds in hamster pancreatic duct carcinogenesis. Although homozygous deletions were previously found in 30% of human PDAs (7), we found no DNA rearrangement or homozygous deletions in hamster PDAs (data not shown). It has also been reported that human lung cancers frequently show tumor-specific aberrant hypermethylation at 18q21, demonstrating that smad4 itself might be inactivated by transcriptional repression due to aberrant hypermethylation (37). The present study indicated no reduced expression of the Smad4 gene in hamster PDAs and three established cell lines, suggesting no involvement of aberrant hypermethylation. The involvement of LOH remains to be clarified.

In conclusion, taken together with our previous findings (22,28), the results of this study show that alterations in the Smad4 gene might play a limited role during nitrosamine-related carcinogenesis in rodents. We previously reported alterations in TGFß signaling pathway-associated genes, such as TGFß receptor II, Smad2 and mannose 6-phospate/insuline-like growth factor II receptor genes, in rat lung adeno-carcinomas induced by *N*-nitrosobis(2-hydroxypropyl)amine (22,38,39), with tumor cells expressing higher levels of TGFß than normal lung tissue (40). To understand the involvement of the TGFß signaling pathway in BOP-induced hamster pancreatic ductal carcinogenesis, further studies of alterations in these genes in hamster PDAs are required.

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