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Smad2 and Smad4 gene mutations in hepatocellular carcinoma

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TGF-β is a negative regulator of liver growth. Smad family of genes, as mediators of TGF-β pathway, are candidate tumor suppressor genes in hepatocellular carcinoma (HCC). We studied 35 HCC and non-tumour liver tissues for possible mutations in Smad2 and Smad4 genes. Three tumours displayed somatic mutations; two in Smad4 (Asp332Gly and Cys401Arg) and one in Smad2 (Gln407Arg) genes. All three mutations were A:T → G:C transitions suspected to result from oxidative stress as observed in mitochondrial DNA. These observation demonstrate that TGF-β pathway is altered in hepatocellular carcinoma.

Keywords: hepatocellular carcinoma; Smad2; Smad4; TGFβ; tumor suppressor genes; somatic mutation

Introduction

Hepatocellular carcinoma (HCC), one of the ten most frequent cancers world-wide, is associated with well defined viral and non-viral etiological factors. Chronic infection with hepatitis B (HBV) and hepatitis C (HCV) viruses, and oral intake of aflatoxins are the major causes of HCC, but the molecular mechanisms underlying the malignant transformation of hepatocytes are largely unknown (Ozturk, 1995). Long latent period (20–30 years) between viral infections and the development of HCC suggests the multi-step nature of hepatocarcinogenesis (Ozturk, 1995). Genetic studies provide sufficient evidence for this hypothesis. Allelotype studies indicate that many chromosomal regions (i.e. 1p, 1q, 4q, 5q, 6q, 8p, 8q, 9p, 10q, 11p, 13q, 14q, 16p, 16q, 17p) undergo structural changes in HCC (Nagai *et al*., 1997; Piao *et al*., 1998; for review see Grisham, 1996). Some of these chromosomal changes are associated with different tumour stages, suggesting that they are associated with the tumour progression (Tsuda *et al*., 1990). The critical genes located at these chromosomal regions are mostly unknown. To date only a few genes including p53, p16, mannose-6-phosphate/insulin-like growth factor II receptor (M6P/IGFIIR), β-catenin (Miyoshi *et al*., 1998; De la Coste *et al*., 1998) and cyclin D have been shown to be significantly altered in HCC (for review see Grisham, 1996). Both the suspected genetic heterogeneity of HCC and the discordance between allelotype and mutation studies strongly suggest that many other genes undergo somatic mutations in these tumours.

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Genes encoding for proteins involved in the control of hepatocyte growth (i.e. inhibitors of hepatocyte proliferation and the activators of apoptotic cell death) are potential tumour suppressor genes for HCC. Transforming growth factor-β (TGF-β) and activin are potent inhibitors of hepatocyte growth (Fausto *et al*., 1995). TGF-β is also known to induce apoptosis in both hepatocytes and HCC cell lines (Oberhammer *et al*., 1992; Gressner *et al*., 1997). Certain transformed cells, however, show defective response to TGF-β-induced growth inhibition including some liver-derived cell lines (Fynan and Reiss, 1993). Based on these observations, genes involved in TGFβ signalling pathway are candidate tumour suppressor genes in HCC. For instance, M6P/IGFIIR gene was shown to be mutated in about 25% of HCCs displaying LOH at the M6P/IGFIIR locus (De Souza *et al*., 1995). Although the protein product of this gene has a broad range of cellular functions, it is suspected to be an activator of latent TGF-β in the liver. In addition, at least two other genes involved in HCC, namely cyclin D and c-*myc* genes appear to be among the ultimate targets of TGFβ signalling pathway, as their expression level is modified following treatment of different cell lines with TGFβ (Alexandrow and Moses, 1995, Grisham, 1996).

Recently, the Smad family of proteins have been discovered as mediators of TGFβ signalling pathway. Eight distinct members of Smad family have been identified in vertebrates and at least two of them, namely Smad2 and Smad4 act as tumour suppressor genes in humans (Heldin *et al*., 1997; Baker and Harland, 1997). Smad4 and Smad2 are both involved in cytoplasmic signal transduction upon activation of TGF-β and activin receptors by their specific ligands (for review see Heldin *et al*., 1997). Missense mutations within carboxyl terminal effector domains of Smad2 and Smad4 have been identified in different cancers including the cancers of pancreas (Hahn *et al*., 1996; Moskaluk *et al*., 1997), biliary tract (Hahn *et al*., 1998), colon (Eppert *et al*., 1996; Riggins *et al*., 1996; Takagi *et al*., 1996), lung (Uchida *et al*., 1996), as well as head and neck carcinomas (Kim *et al*., 1996). Smad2 and Smad4 appear to be the most critical targets of mutational inactivation because a study based on 167 tumour samples suggested that mutational inactivation of the other Smad genes does not account for the widespread resistance of cancer cells to TGF-β (Riggins *et al*., 1997). Based on these indications, we selected Smad2 and Smad4 (also called JV18-1 and DPC4 respectively) as candidate tumour suppressor genes in HCC and studied 35 primary tumours as well as six hepatoma cell lines for possible mutations. We demonstrate that both genes display somatic mutations in HCC, but the mutation frequency is low. We will discuss possible implications of these observations in

relation with the role of TGFβ signalling pathway in hepatocellular carcinogenesis.

Results and Discussion

To investigate the potential involvement of the Smad2 and Smad4 genes in HCCs, we screened 35 HCC samples as well as six hepatoma cell lines for possible genetic alterations of these genes. Initially, six hepatoma cell lines were tested by RT–PCR for the expression of Smad2 and Smad4 genes. All cell lines expressed both Smad2 and Smad4 genes and RT–PCR products did not show size alteration in the respective coding region (data not shown). Homozygous and large deletions of Smad2 and Smad4 genes have been reported for other cancers (Hahn *et al*., 1996, Riggins *et al*., 1996). But the hepatoma cell lines tested here did not show such alterations.

Next, we studied genomic DNA from 35 primary tumours. The majority of the previously identified Smad2 and Smad4 gene mutations are located within the region coding for the highly conserved carboxyl terminal domains, corresponding to exon 8–11 of both genes (Hahn *et al*., 1996; Riggins *et al*., 1996; Schutte *et al*., 1996, Hahn *et al*., 1998). In addition, two mutations, one located in exon 4 of Smad2 and another located in exon 2 of Smad4 affecting the N-terminal regions of Smad2 and Smad4 (Arg133Cys and Arg100Thr, respectively) proteins have been described in colon and pancreas cancers (Hata *et al*., 1997; Shi *et al*., 1997). To test whether HCCs display similar mutations, we studied exon 4 of Smad2, exon 2 of Smad4, in addition to exons 8–11 of both genes by SSCP analysis. A total of seven HCC samples showed altered migration pattern (see Figure 1 for exon 8 alterations as an example). Four of these alterations (three in exon 8 and one in exon 10 of Smad4 gene) were also present in the non-tumour liver samples of the respective patients. DNA sequence analysis of these four tumours did not reveal any alteration in exonic sequences and immediately flanking intronic regions (data not shown) suggesting that constitutional polymorphisms affecting intronic sequences were present in these patients. The other three SSCP alterations were seen only in tumour DNA, but not in non tumorous liver DNA. Sequence analysis of tumour and non-tumour liver DNA samples from

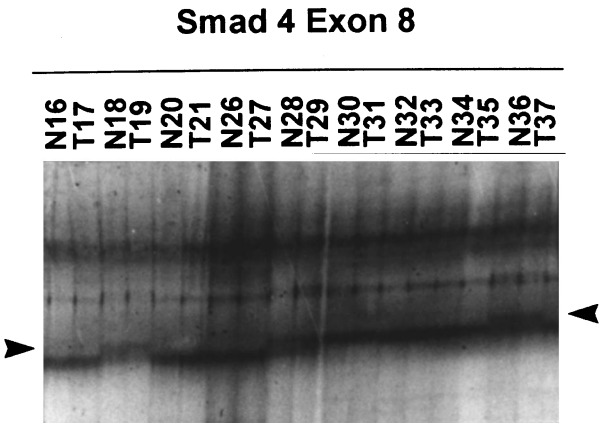

Figure 1 SSCP analysis of Smad4 gene (exon 8) in hepatocellular carcinomas. T and N denotes tumour and matching non-tumour liver DNA, respectively. T19, T29 and T37, as well as their matching non-tumour liver DNAs (N18, N28 and N36, respectively) display abnormally migrating bands

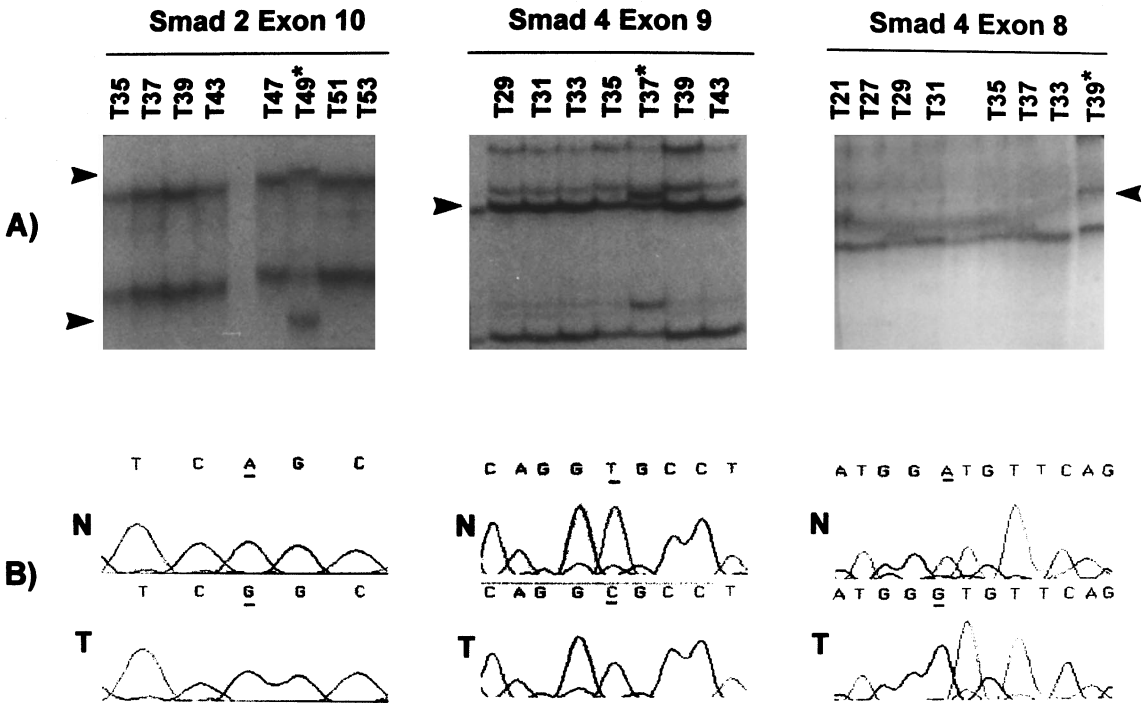

Figure 2 SSCP and DNA sequence analyses of Smad2 (exon 10) and Smad4 (exons 8 and 9) genes indicate the presence of somatic mutations in three hepatocellular carcinomas. (**a**) SSCP analysis of tumours demonstrating mutant allelic shifts in tumours T49, T37 and T39 (the asterisk indicates the samples displaying band-shifts); (**b**) Sequence analysis of hepatocellular carcinoma (T) and non tumour liver tissue (N) DNAs of the samples identified in **a**. Somatic single base-pair mutations (A:T → G:C transitions) were underlined

three patients revealed three different somatic mutations (Figure 2). Two tumour DNAs (T37 and T39) displayed missense mutations in Smad4 gene. An A→G transition at codon 332, leading to a Asp332Gly change was observed in T39. The other Smad4 mutation was a T→C transition at codon 401 leading to a Cys401Arg replacement in T37. One tumour DNA (T49) carried a transition mutation (A→G) at codon 407 of Smad2 gene leading to a Gln407Arg change (Figure 2).

All three of these somatic mutations were novel and have not been described previously. They all appear to be deleterious mutations because of the strong side chain charge changes in the replaced amino acid residues resulting from each mutation. Crystal structure of C-terminal domain of Smad4 reveals that Asp 332 residue is located in the trimeric interface region and forms a hydrogen bond with His 371 (Shi *et al*., 1997). Because this interface region is crucial for trimeric complex formation, it is likely that Asp332Gly change, breaking the hydrogen bond, blocks the TGFβ signal by preventing trimer formation of Smad4 protein. The Cys401 residue of Smad4 is highly conserved between Smad family members. This residue is located within the β6 sheet which makes the hydrophobic core, the β sandwich of Smad4 protein. Amino acid change from cysteine to arginine will most probably affect Smad4 protein function by changing the conformation of the β sandwich. Gln407 of Smad2 is located in a region that is homologous to H4 helix domain of Smad4, also in trimeric interface region (Shi *et al*., 1997).

All three mutations are located in the MH2 domain of Smad proteins. These proteins consist of three structurally and functionally distinct domains. The amino and carboxyl terminal regions of Smad proteins are referred to as MH1 and MH2 domains, respectively. The central region serves as a linker domain between MH1 and MH2 domains. The MH2 domain of Smad 1, 2 and 3 mediates homomeric interactions and is responsible for heteromeric interactions with Smad4. The MH2 domain also called effector domain is able to mimic the activity of the full length proteins (Heldin *et al*., 1997). Thus it is very likely that the three mutations described here are not random mutations and that they either affect protein-protein interactions or destabilise the whole structure of the protein. Taken together, all three mutations described here appear to be deleterious mutations which may affect TGFβ signalling pathway in these tumours.

The tumours described here have been previously examined for the presence of p53 gene mutations and HBV DNA (Unsal *et al*., 1994). As shown in Table 1, HBV DNA was present in all three tumours with Smad mutations. p53 was mutant (Arg249Ser) in T37 but not in two other tumours. Thus, it appears that Smad mutations described here are associated with HBV infection and that there appears to be no correlation with p53 mutations. On the other hand, it is noteworthy that all three mutations described here affected A:T pairs with a consistent change to G:C pairs. This pattern was recently shown to be a common type of mitochondrial DNA mutation in colorectal tumours and explained by high level of reactive oxygen species (ROS) in mitochondria (Polyak *et al*., 1998). Similarly, Smad gene mutations observed here may be caused by high levels of ROS in the precursor cells of these tumours.

Previous studies showed that Smad4 is altered in a significant portion of pancreatic (Hahn *et al*., 1996) and common bile duct cancers (Hanh *et al*., 1998) and a minority of colorectal cancers (Eppert *et al*., 1996; Takagi *et al*., 1996) but rarely in other tumours (Schutte *et al*., 1996). Smad2 gene alterations are also limited to a small fraction of colorectal and lung cancers (Riggins *et al*., 1996, 1997; Uchida *et al*., 1996. Our data suggest that, Smad2 and Smad4 gene mutations may contribute to a fraction of HCCs. Low prevalence of these alterations may be explained with genetic heterogeneity of HCC (Unsal *et al*., 1994). Indeed, Kawate *et al*. (1999) reported very recently that mutations of TGFβ-receptor II, Smad2 and Smad4 mutations are rare in HCCs from Japan. In addition, Smad mutations in HCC could occur at late stages during tumor progression. In an animal model of compound heterozygosity for APC and Smad4, inactivation of Smad4 gene resulted in the malignant progression of the intestinal and colonic polyps initiated by LOH in the APC gene. This observation suggested the inactivation of Smad4 as a late event in carcinogenesis (Takaku *et al*., 1998).

In conclusion, we identified Smad gene mutations in HCC at a frequency of $\sim$10%. The occurrence of these mutations provides evidence that TGFβ pathway is altered in HCC. In conformation to our hypothesis, TGFβ itself was identified as a new form of tumour suppressor for liver and lung cancers in mice heterozygous for deletion of the TGFβ gene (Tang *et al*., 1998). However, the low frequency of Smad gene mutations in human HCC also suggest that other genes of TGFβ pathway may also be alterated in these tumours.

Materials and methods

Tumour samples and cell lines

We analysed a total of 35 pair of DNA samples isolated from HCC and non-tumour liver tissues from patients living in different geographical regions including Mozambique ($n=7$), South Africa ($n=11$), China ($n=8$), Japan ($n=6$) and Germany ($n=3$). Characteristics of these tumours and methods for DNA isolation have been described previously (Unsal *et al*., 1994). We also analysed six established

Table 1 Smad gene mutations in hepatocellular carcinoma

Tumour	*Age*	*Country*	*Gene*	*Exon*	*Mutation*	*Predicted effect*	*p53 Status*	*HBV DNA*	*Stage*
T49	14	South Africa	Smad2	10	CAG to CGG	Gln-407-Arg	WT	+	Late
T37	36	Mozambique	Smad4	9	TGC to CGC	Cys-401-Arg	Arg-249-Ser	+	Late
T39	34	Swaziland	Smad4	8	GAT to GGT	Asp-332-Gly	WT	+	Late

hepatoma cell lines (HepG2, Huh7, Mahlavu, PLC/PRF/5, Hep3B and Focus). Genomic DNA from cell lines was isolated as described (Unsal *et al*., 1994). Total RNA from cell lines were isolated using Ultraspect RNA extraction kit (Biotecx) according to the manufacturer's directions.

RT–PCR

cDNAs were synthesised by MMLV reverse transcriptase (SuperScript™, Gibco) using oligo $(dT)_{12-18}$ primers. The entire coding region of Smad2 and Smad4 genes were amplified using primers indicated in Table 2. PCR was performed in 50 μl reaction mixture containing 1 μl of the complementary DNA mix, 1 × Taq buffer (MBI), 1.5 mM $MgCl_2$, 1 unit of Taq DNA polymerase, 20 pmol of each primer and 10 mM each of dNTPs. PCR conditions consisted of 35 cycles of denaturation at 94°C for 60 s, annealing at temperatures varying between 55–56°C (see Table 2 for primer sequences and annealing temperatures) for 30 s, followed by an extension at 72°C for 90 s. The final cycle included an additional 3 min extension at 72°C.

SSCP analysis

PCR was performed using 50 ng genomic DNA, 1 × Taq buffer (MBI), 1.5 mM $MgCl_2$, 1 unit of Taq DNA polymerase, 20 pmol of each primer, 40 μM each of dNTPs, 2 μCi [α-^{32}P]dATP (1000 Ci/mmol, Amersham) in a final volume of 25 μl. PCR conditions consisted of 30 cycles of denaturation at 94°C for 45 s, annealing at temperatures varying between 50–60°C (see Table 2 for primer sequences and annealing temperatures) for 30 s, followed by an extension at 72°C for 1 min. The final cycle included an additional 3 min extension at 72°C. SSCP analysis of PCR products was done at both 4°C and room temperature, on 10% of acrylamide gel with or without glycerol as well as on MDE gels (FMC Bioproducts). Gels were mounted on a 3M Whatman paper, dried and visualised by autoradiography.

Sequence analysis

For the analysis of tumour PCR products, aberrantly migrating radioactive bands were recovered from SSCP gels in order to enrich for mutant DNA fragments before sequencing. Selected bands were cut with a razor blade, eluted into TE and reamplified as described, except that [α-^{32}P]dATP was omitted. Non-tumour PCR products were analysed directly following non-radioactive PCR amplification. PCR products were purified using Quick PCR purification kit (Qiagen). For sequencing reactions, dye terminator cycle sequencing ready reaction kit (ABI Prism) was used. Reaction products were then analysed using the ABI Prism 377 fluorescent DNA sequencer (Perkin Elmer). Identified mutations were confirmed by DNA sequencing of

Table 2 List of primers and annealing temperatures

Primer name	*Primer sequence*	*Product size* (bp)	T_A (°C)	*Ref.*
Smad4 Ex2 F	TTCTAGGTGGCTGGTCGGAA	175	56	This study
Smad4 Ex2 R	CAGGTGATACAACTCGTTCG			
Smad4 Ex8 F	TTTCTCATGGGAGGATGTTC	264	56	Hahn *et al*., 1998
Smad4 Ex8 R	CAATTTTTTAAAGTAACTATCTGAC			
Smad4 Ex9a F	TATTAAGCATGCTATACAATCTG	194	57	Hahn *et al*., 1998
Smad4 Ex9a R	CTGTCTAAGTAGTAACTCTTG			
Smad4 Ex9b F	CAAAGGTGTGCAGTTGGAATG	234	57	Hahn *et al*., 1998
Smad4 Ex9b R	CTTCCACCCAGATTTCAATTC			
Smad4 Ex10 F	GAATTTTCTTTATGAACTCATAG	213	57	Hahn *et al*., 1998
Smad4 Ex10 R	TTTAAAAAAGAATGAAAAGCATAC			
Smad4 Ex11 F	CTGATGTCTTCCAAACTCTTTTCTG	299	57	Hahn *et al*., 1998
Smad4 Ex11 R	TGTATTTTGTAGTCCACCATC			
Smad2 Ex4 F	TCTGATAGTGGTAAGGGTTT	189	56	This study
Smad2 Ex4 R	GTCTCTTGATGGTCGTCTC			
Smad2 Ex8 F	CAGTTACTTACTCAGAACCT	269	53	This study
Smad2 Ex8 R	GCCTACATTCTGAGTATACAG			
Smad2 Ex9 F	CCAAAGTCACACTGAAATAG	242	57	This study
Smad2 Ex9 R	AGCAAGTTGACATGATAGG			
Smad2 Ex10 F	GCATGCTCATATTCTAAAAC	275	50	This study
Smad2 Ex10 R	ACTGTGGAAATTTAAGAACC			
Smad2 Ex11 F	GCCTGTGGACTTGAATTTCAT	192	56	This study
Smad2 Ex11 R	GGACTTGATTGGTGAAGCTTT			
Smad2 ECR F	GGATCCTAATACGACTCACTATAGGGAGACCAC-CATGGGTAAGAACATGTCGTCCAT	1477	56	Riggins *et al*., 1996
Smad2 ECR R	TTTCCATGGGACTTGATTGG			
Smad4 ECR F	AAGCGGATCCGCTTCAGAAATTGGAGA	1769	55	This study
Smad4 ECR R	AAGCAAGCTTCCATCCTGATAAGGTTAAGGG			

Ex and ECR identify primers used for PCR amplifications of exons and the entire coding regions, respectively

independent PCR products after cloning into pGEM-T plasmid (Promega).

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