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Alterations of the Transforming Growth Factor-β Signaling Pathway in Hepatocellular Carcinomas Induced Endogenously and Exogenously in Rats

Yasutaka Sasaki, Toshifumi Tsujiuchi, Nao Murata, Masahiro Tsutsumi and Yoichi Konishi[1]

Department of Oncological Pathology, Cancer Center, Nara Medical University, 840 Shijo-cho, Kashihara, Nara 634-8521

To elucidate involvement of the transforming growth factor-β (TGF-β) signaling pathway in endogenous and exogenous liver carcinogenesis, we investigated mutations of TGF-β receptor type II (TGF-βRII), *Smad2* and *Smad4* genes, and expression of TGF-βRII in hepatocellular carcinomas (HCCs) induced by a choline-deficient L-amino acid-defined (CDAA) diet and by N-nitrosodiethylamine (DEN). Male Fischer 344 rats received a CDAA diet continuously and HCCs were sampled after 75 weeks. Administration of DEN was followed by partial hepatectomy (PH), with colchicine to induce cell cycle disturbance and a selection pressure regimen, HCCs being obtained after 42 weeks. Total RNAs were extracted from individual HCCs and mutations in TGF-βRII, Smad2 and Smad4 were investigated by reverse transcription (RT)-polymerase chain reaction (PCR)-restriction-single-strand conformation polymorphism (SSCP) analysis followed by sequencing analysis. Mutations of Smad2 were detected in 2 out of 12 HCCs (16.7%) induced by the CDAA diet, a GGT-to-GGC transition (Gly to Gly) at codon 30 and a TCT-to-GCT (Ser to Ala) transversion at codon 118, without any TGF-βRII or Smad4 alterations. No mutations of TGF-βRII, Smad2 and Smad4 were encountered in eleven HCCs induced by the exogenous carcinogen. Semi-quantitative RT-PCR revealed reduced expression of TGF-βRII in 2 HCCs (16.7%) without Smad2 mutations out of 12 HCCs induced by the CDAA diet and none of 11 induced by DEN. These results suggest that the TGF-β signaling pathway may be disturbed in endogenous liver carcinogenesis in rats.

Key words: TGF-β receptor type II — Smad2 — Smad4 — HCC — Rat

Liver carcinogenesis can be divided into two categories, i.e., those due to endogenous changes occurring without any established carcinogen exposure, and those caused by an exogenous carcinogen. We have demonstrated high yields of hepatocellular carcinomas (HCCs) associated with cirrhosis on chronic administration of a choline-deficient L-amino acid-defined (CDAA) diet without any known carcinogen.[1] This diet is almost completely devoid of choline and has a greater capacity than a semisynthetic choline-deficient, methionine-low diet to cause hepatocarcinogenesis, as well as oxidative stress.[1,2] As an exogenous agent, N-nitrosodiethylamine (DEN) is one of the best-known liver carcinogens in rats. We have reported that a cell cycle disturbance induced in DEN-initiated hepatocytes by colchicine gives a growth advantage to formation of putative preneoplastic lesions under conditions of partial hepatectomy (PH) and selection pressure, so that a high incidence of HCCs can be obtained within a short period.[3,4] Since our studies revealed differential effects of chemopreventive agents in our two liver models,[5,6] the possibility arises of different mechanisms underlying endogenous and exogenous hepatocarcinogenesis in rats.

Transforming growth factor-β (TGF-β) is a multifunctional polypeptide which regulates a wide variety of cell characteristics, including cell proliferation, differentiation, apoptosis, migration and adhesion.[7–10] TGF-β action is primarily mediated by binding to specific cell surface proteins, the TGF-β receptors (TGF-βRs), TGF-βRI and TGF-βRII, both of which belong to an emerging family of transmembrane serine/threonine kinases.[11,12] TGF-β binds directly to TGF-βRII, whereas TGF-βRI appears to recognize only this complex, being then recruited into a ternary signaling complex. In this complex, TGF-βRII phosphorylates TGF-βRI, resulting in the propagation of further downstream signals.[12–14] The most important postreceptor event in TGF-β signaling is mediated by members of the Smad family.[15–17] After activation by ligand binding, TGF-β family receptors are considered to phosphorylate a Smad, resulting in its translocation into the nucleus, where expression of growth-regulatory genes is induced.[18,19] Eight distinct members of the Smad family have been identified in mammals.[20,21] Recently, mutations of TGF-βRII, Smad2 and Smad4, and reduced expression of TGF-βRII have been reported in several human cancers,[22–36] these alterations resulting in resistance of cancer cells to the effects of TGF-β inhibition. Therefore, it is conceivable that *TGF-βRII*, *Smad2* and *Smad4* can act as tumor suppressor genes.

With regard to neoplasia in rodents, there have been few reports of *Smad* gene alterations. In mouse lung tumors, reduced expression of TGF-βRII,[37] but no mutations of

[1] To whom requests for reprints should be addressed.
E-mail: ykonishi@nmu-gw.cc.naramed-u.ac.jp

Smad2 and Smad4 were found.[38] Recently, however, we detected mutations of TGF-βRII and reduced expression (unpublished results), along with mutations of Smad2 and Smad4, in lung adenocarcinomas induced by N-nitrosobis(2-hydroxypropyl)amine (BHP) in rats.[39] In the present study, to clarify involvement of the TGF-β signaling pathway in rat liver carcinogenesis due to endogenous and exogenous agents, we investigated mutations of TGF-βRII, Smad2 and Smad4, and the expression of TGF-βRII, in HCCs induced by the CDAA diet and by DEN.

MATERIALS AND METHODS

Animals Male Fischer 344 rats, 5 weeks old, were purchased from Japan SLC Inc. (Shizuoka) and housed in stainless-steel, wire-bottomed cages in an air-conditioned room, with a constant temperature of 25°C and a 12-h light-dark cycle. Food and water were given *ad libitum* throughout the study. After a 1-week acclimation period on a basal diet in pellet form (Oriental MF Diet; Oriental Yeast Co., Ltd., Tokyo), the animals were allocated to experimental groups.

Diets and chemicals CDAA diet, with the composition described previously,[1,2] was purchased from Dyets Inc. (Bethlehem, PA; product number 518753), and stored at 4°C immediately on arrival. DEN was purchased from Wako Pure Chemical Co., Ltd. (Kyoto) and diluted with a 0.9% NaCl solution to a concentration of 0.1%. Colchicine was purchased from Sigma Chemical Co. (St. Louis, MO) and dissolved in a 0.9% NaCl solution to a concentration of 0.05%. 2-Acetylaminofluorene (AAF) and carbon tetrachloride (CCl_4) were purchased from Nacalai Tesque, Inc. (Kyoto), and the latter was diluted 1:1 with corn oil. Diet containing 0.02% AAF was prepared by admixing the chemical with Oriental MF powdered basal diet.

Animal treatments For endogenous carcinogenesis, animals were continuously given the CDAA diet and killed under ether anesthesia 75 weeks after the beginning of the experiment. With the exogenous agent, the method for the production of HCCs was as previously described.[3,4] Animals received DEN intraperitoneally at a dose of 10 mg/kg body weight followed after 4 h by PH performed by the method of Higgins and Anderson.[40] Colchicine at a dose of 0.5 mg/kg body weight was injected intraperitoneally 1 and 3 days after DEN treatment. After an 11-day recovery period, rats were placed on the selection regimen, comprising feeding of 0.02% AAF diet for 2 weeks and a single intragastric administration of CCl_4 at 1 ml/kg body

Table I. Sequences of Primers for *TGF-βRII*, *Smad2* and *Smad4* Used in This Study

Gene	cDNA location	Primer	Size of amplified product (bp)	Annealing temperature (°C)	Enzyme digestion	Size of digested product (bp)
TGF-βRII[a]	nt 33–413	1-F: 5′-GCTGCACATCGTCCTGTGGA-3′ 1-R: 5′-CAGGAGCACATGAAGAAGGT-3′	381	62	*Pst*I	182, 199
	nt 333–862	2-F: 5′-CACTCTGGAAGATGCCACTT-3′ 2-R: 5′-TCCACGAGGAGTATTCCTCG-3′	530	62	*Bcn*I	286, 244
	nt 813–1352	3-F: 5′-TGAGACCGTGGCTGTCAAGA-3′ 3-R: 5′-GCCATGGAGTAGACATCCGT-3′	540	64	*Sma*I	254, 286
	nt 1256–1734	4-F: 5′-TGGGAACAGCGAGATACATG-3′ 4-R: 5′-GGAGTCTTGGCCCAGCCTGC-3′	479	64	*Mbo*I	277, 202
Smad2[b]	nt −10–588	21-F: 5′-TTGGTAAGAAAATGTCGTCCAT-3′ 21-R: 5′-ATAGTCATCCAGAGGCGGCAGT-3′	599	62	*Rsa*I, *Hha*I	275, 134, 190
	nt 543–1068	22-F: 5′-TCGGCACACAGAGATTCTAA-3′ 22-R: 5′-AAAGATCGCACTATCACTTA-3′	526	54	*Msp*I, *Hha*I	194, 274, 58
	nt 1005–1512	23-F: 5′-AGTGCGCTTGTATTACATAG-3′ 23-R: 5′-GTTTTCGCTCTGGGTTTTGA-3′	508	55	*Msp*I	274, 234
Smad4[b]	nt −55–504	41-F: 5′-GATCAAAATTACTCCAGAAA-3′ 41-R: 5′-TCCTTCAAAGTCATGAACAT-3′	560	53	*Hin*fI, *Pst*I	219, 85, 256
	nt 455–934	42-F: 5′-CTCCACCAAGTATGTTAGTG-3′ 42-R: 5′-GCTGGAATGCAAGCTCATTG-3′	480	55	*Msp*I	219, 261
	nt 890–1389	43-F: 5′-ATCCTGGACATTACTGGCCGGTTCA-3′ 43-R: 5′-TGCCGCCTGGGCAGCAGCTGCGGCT-3′	500	65	*Bam*HI, *Hae*III	173, 194, 133
	nt 1345–1786	44-F: 5′-CAGCAGGCGGCCACCGCGCA-3′ 44-R: 5′-ACATCTTTCAACCCTTTATG-3′	442	56	*Pst*I	257, 185

a) T. Tsujiuchi *et al*., submitted for publication.
b) Ref. 39).

weight, following the procedure described by Cayama *et al.*,[41] and were killed under ether anesthesia 42 weeks after the beginning of the experiment.

Liver samples At sacrifice, the livers were immediately excised and grossly apparent tumors were dissected from surrounding tissue. Samples were frozen in liquid nitrogen, and stored at −80°C until analysis. Portions of the tumors were also fixed in 10% formalin for routine processing and staining with hematoxylin and eosin (H&E) for histological examination.

Reverse transcription-polymerase chain reaction-restriction-single-strand conformation polymorphism (RT-PCR-restriction-SSCP) analysis of the *TGF-βRII*, *Smad2* and *Smad4* genes Total RNAs were extracted from frozen tissue using ISOGEN (Nippon Gene, Inc., Toyama) and first-strand cDNAs were synthesized from 5 μg samples with Ready-To-Go Your-Prime First-Strand Beads (Pharmacia Co., Ltd., Tokyo). To eliminate possible false positives caused by residual genomic DNA, all samples were treated with DNase.

RT-PCR-restriction-SSCP analysis was carried out using the primers listed as described earlier (unpublished results)[39] (Table I). All primers were designed from rat Smad2 and Smad4 cDNA sequences (GenBank accession numbers for *TGF-βRII*, *Smad2* and *Smad4* are L09653, AB010147 and AB010954, respectively). The PCR amplification was performed in 10 μl of reaction mixture consisting of 1 μ*M* of each primer, 200 μ*M* of each dNTP, 1× PCR buffer (Perkin Elmer, Applied Biosystems Division, Foster City, CA), 68 n*M* [α-^{32}P]dCTP, 2.5 units of Ampli Taq (Perkin Elmer) and 0.5 μl of synthesized cDNA mixture under the following reaction conditions; a denaturation step for 5 min at 95°C, 35 cycles of 1 min at 95°C, 1 min at 53–65°C and 2 min at 72°C, and a final extension for 10 min at 72°C. All PCR reactions were performed at least twice, using individual original DNAs to confirm the results. Amplified fragments were digested to shorter than 300 bp with a restriction enzyme before electrophoresis,[39] as indicated in Table I. PCR products were diluted with 90 μl of loading solution containing 90% formide, 20 m*M* EDTA, and 0.05% xylene cyanol and bromophenol blue, denatured at 90°C for 2 min and applied to 6, 8, or 10% polyacrylamide gels containing 0.5× Tris-borate EDTA buffer with or without 10% glycerol. Electorophoresis was performed at 40 W for about 2.5 h at 30°C. Gels were dried on filter paper and used to expose X-ray films at −80°C.

Cloning and sequence analysis DNA fragments of mobility-shifted bands on SSCP analysis were extracted from the gels and reamplified. The PCR products obtained were cloned using a TOPO TA cloning kit (Invitrogen Corp., CA) and recombinant plasmid DNA clones were sequenced with Sequencing Pro (Toyobo Co., Ltd., Tokyo). In each experiment, 5 to 10 clones from different bacterial colonies were investigated.

Semi-quantitative RT-PCR analysis for expression of TGF-βRII mRNA For semi-quantitative RT-PCR analysis, the PCR amplification was carried out in a reaction volume of 20 μl containing 1 μl of first-strand cDNA synthesized in the above experiment. Amplification products comprising a portion of nt 333 to 1352 for TGF-βRII (primer: 2-F and 3-R), and exons 5 through 8 of the glyceraldehyde-3-phosphate dehydrogenase gene (*GAPDH*)[42] were generated from the cDNA template in parallel PCRs. The RT-PCR condition was as follows; a denaturation step for 5 min at 95°C, 26 cycles of 1 min at 95°C, 1 min at 67°C, and 2 min at 72°C, with a final extension step for 10 min at 72°C, using both the TGF-βRII and GAPDH primers. The PCR products were then separated in a 2% agarose gel containing 0.05 μg/ml ethidium bromide. Each RT-PCR assay was repeated at least twice for confirmation.

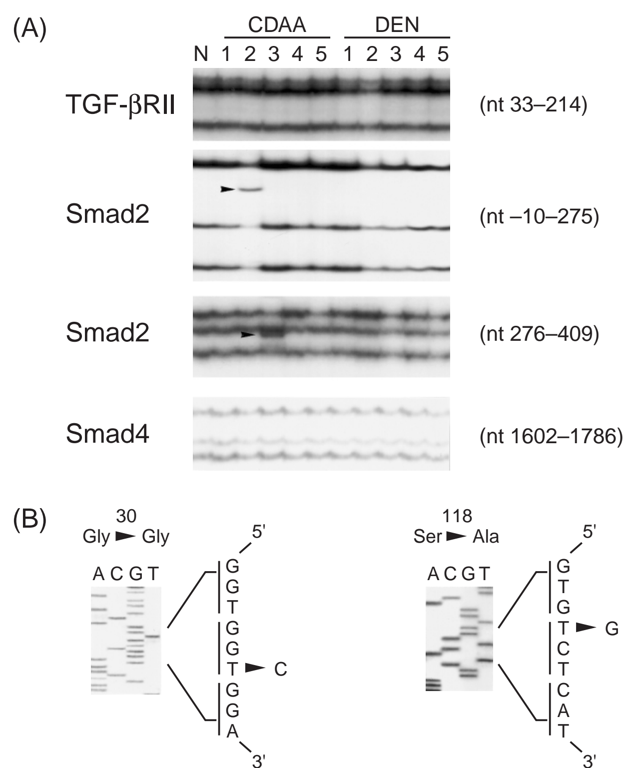

Fig. 1. (A) Representative results of SSCP analysis of TGF-βRII, Smad2 and Smad4 in HCCs induced by the CDAA diet and by DEN. Samples 2 and 3 with the CDAA diet demonstrate band shifts in the regions of nt −10–275 and nt 276–409 of Smad2, respectively (arrowheads). N: normal liver. CDAA: HCCs induced by the CDAA diet. DEN: HCCs induced by DEN. (B) Results of sequencing analysis for Smad2 mutations in HCCs induced by the CDAA diet. Sample CDAA-2 shows a GGT-to-GGC (Gly-to-Gly) transition at codon 30, and sample CDAA-3 a TCT-to-GCT (Ser-to-Ala) transversion at codon 118.

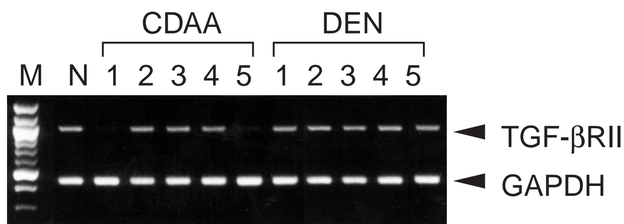

Fig. 2. Representative results of semi-quantitative RT-PCR analysis of TGF-βRII expression in HCCs induced by the CDAA diet and by DEN. Note reduced expression of TGF-βRII in samples 1 and 5 with the CDAA diet. M: 100 bp DNA size marker. N: normal liver. CDAA: HCCs induced by the CDAA diet. DEN: HCCs induced by DEN.

RESULTS

Twelve HCCs induced by the CDAA diet in 12 rats and 11 HCCs induced by DEN in 11 rats used for the analysis were all histologically well-differentiated carcinomas. Representative results of RT-PCR-restriction-SSCP analysis and sequencing analysis for *TGF-βRII*, *Smad2* and *Smad4* gene mutations are shown in Fig. 1, (A) and (B). Two out of 12 HCCs induced by the CDAA diet showed band shifts in the regions of nt −10–275 and nt 276–409 in Smad2, indicative of mutations (16.7%) (Fig. 1 (A)). These were established to be a GGT-to-GGC (Gly to Gly) transition at codon 30 and a TCT-to-GCT (Ser to Ala) transversion at codon 118, respectively (Fig. 1 (B)). However, no mutations in TGF-βRII or Smad4 were found in any of the HCCs induced by the CDAA diet. The eleven HCCs induced by DEN showed no abnormal band shifts for TGF-βRII, Smad2 and Smad4 (Fig. 1 (A)).

Representative results of semi-quantitative RT-PCR for the expression of TGF-βRII are shown in Fig. 2. Two HCCs (16.7%) without Smad2 mutations out of 12 HCCs induced by the CDAA diet showed reduced expression. However, no change was evident in the 11 HCCs induced by DEN.

DISCUSSION

Recently, mutations of the *TGF-βRII* gene have been reported in several human cancers, with high frequencies detected in colorectal cancers and cell lines, and gastric cancer cell lines.[28–32] In human HCCs, however, no mutations of TGF-βRII were found.[43] Reduction of TGF-βRII expression has also been reported. In a number of neoplasms, including gastric cancer cell lines,[31] thyroid tumors,[33] and lung cancers,[34–36] decrease at the RNA or protein level was observed without apparent structural mutation. In HCCs, a similar reduction has been described,[44] although in another report, there were no changes of TGF-βRII expression in HCCs compared with normal liver tissue, while expression of TGF-β ligands was elevated.[45] In the present study, although no mutations of TGF-βRII were found in HCCs induced by the two regimens, reduced expression of TGF-βRII was apparent in the case of the CDAA diet. Therefore, reduced expression of TGF-βRII rather than its mutation may be involved in endogenous liver carcinogenesis.

Smad2 and Smad4 have been considered as the most critical targets of mutational inactivation, since recent studies suggested that mutational inactivation of the other *Smad* genes does not account for the widespread resistance of cancer cells to TGF-β.[46] Therefore, in this study, we examined mutations of Smad2 and Smad4 among the eight members of the *Smad* gene family. Mutations of Smad2 and Smad4 in human HCCs are either lacking[43] or infrequent.[47] Where found, they were located in the MH2 domain, which is responsible for homo- and hetero-oligomerization.[48, 49] In the present study, we found Smad2 but not Smad4 mutations in HCCs induced by the CDAA diet, and these were located in the MH1 domain. Since this domain exhibits sequence-specific DNA binding activity and negatively regulates the MH2 domain function,[20, 21] missense mutations in this region may influence the function of Smad protein. However, one of the two mutations in the *Smad2* gene was not associated with amino acid replacement, so no disturbance of the TGF-β signaling pathway should have occurred.

Previously, we have reported oxidative damage to liver DNA and extra-DNA subhepatocellular components due to reactive oxygen species in animals fed the CDAA diet.[2, 50] Oxidative DNA damage as evidenced by 8-hydroxydeoxyguanine (8-OHdG) formation is detectable after only one day and progressively accumulates at least up to day 84,[2, 50] inducing specific G/C-to-T/A and A/T-to-C/G transversions.[51] Moreover, T/A-to-C/G transitions were shown to be a common type of mitochondrial DNA mutation in colorectal tumors; this may be related to the high level of reactive oxygen species in these organellas.[52] In the present study, the Smad2 mutations were a T/A-to-C/G transition and a T/A-to-G/C transversion. Therefore, they may have been due to oxygen species generated during endogenous liver carcinogenesis.

Recently, we have reported different frequencies and patterns of β-catenin mutations in rat HCCs induced by DEN and the CDAA diet.[53] The former showed a high frequency of β-catenin mutations with amino acid alterations, whereas the latter showed a low frequency of silent mutations. Therefore, it has been suggested that different genetic pathways underlie exogenous and endogenous liver carcinogenesis in rats.[53] In this study, we have demonstrated disturbance of the TGF-β signaling pathway in endogenous liver carcinogenesis, but the differences in the pathway in the two cases should be further clarified, since

they might have relevance to chemoprevention and novel therapeutic approaches.

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