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Absence of *ras* Mutations and Low Incidence of *p53* Mutations in Renal Cell Carcinomas Induced by Ferric Nitrilotriacetate

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Renal cell carcinomas induced in male Wistar rats by iron chelate of nitrilotriacetate (Fe-NTA) were examined for mutations in *ras* oncogenes and *p53* tumor suppressor gene. Fourteen primary tumors and two metastatic tumors from 11 animals were evaluated. Exons 1 and 2 of the H-, K-, and N-*ras* genes were amplified by polymerase chain reaction (PCR), and the presence of mutations was examined by direct sequencing. Exon 5 through exon 7 of *p53* gene, including the 3′ half of the conserved region II and the entire conserved region III through V, were surveyed for point mutations by PCR-single stranded conformation polymorphism (SSCP) analysis. Direct sequencing of the *ras* genes showed no mutations in codon 12, 13, or 61 among the tumors evaluated. SSCP analysis of *p53* gene exon 6 indicated conformational changes in two primary tumors. One tumor had a CCG-to-CTG transition at codon 199, and the other had an ATC-to-ATT transition at codon 229 and two nonsense C-to-T transitions. These results suggest that neither *ras* genes nor *p53* gene play a major role in the development of renal cell carcinomas induced by Fe-NTA.

Key words: Chemical carcinogenesis — Nitrilotriacetate — *ras* gene — *p53* gene — Single strand conformation polymorphism

Multistep losses of normal cell regulation are believed to be key events in the development of human cancer. Amplification or activation of oncogenes, and inactivation of tumor suppressor genes result in deregulated growth of the cancer cells. Activation of c-*ras* genes by single point mutation in codon 12, 13 or 61, and inactivation of *p53* tumor suppressor gene by mis-sense mutations or deletions are among the most frequently found genetic alterations in human malignancies.[1–3] Likewise, mutations in the *ras* genes and the *p53* gene have been widely studied in experimental tumor models, and their mutational patterns, namely, presence or absence of mutations, clustered sites of mutations, and type of base substitutions, have been evaluated in terms of the experimental conditions used for the tumor induction. The mutational patterns of an experimental tumor depend on the type of chemical carcinogen,[4, 5] the animal species,[6] the site and the organ of tumor development, and the histological type of the tumor.[7] It was also disclosed that the mutational patterns of rodent tumors did not always coincide with those of human analogues.[8]

Human renal cell carcinoma is exceptional among epithelial malignancies in that neither *ras* oncogenes nor *p53* gene is strongly involved in its development. Alterations of the *ras* genes were rarely found in renal cell carcinomas,[9, 10] and the prevalence of *p53* mutations is in the lowest group of all malignancies.[3, 10] Similar tendencies were also observed in studies of experimental renal cell carcinomas of rodents.[11–13] Renal cell carcinomas induced by N-ethyl-N-hydroxyethyl-nitrosamine or N-nitrosomorpholine showed no mutation in the *ras* genes,[11] while carcinomas induced by N-nitrosodimethylamine exceptionally contained a high frequency of G-to-A transition at codon 12 of the K-*ras* gene.[12] Few studies had evaluated the *p53* gene alteration in experimental renal cell carcinoma, and no mutation of *p53* gene was reported in the tumors induced by nitrosamine.[13] Thus, neither activation of *ras* genes nor inactivation of *p53* tumor suppressor gene has significance in experimental models of renal carcinogenesis, as in human renal cell carcinoma.

Iron chelate of NTA[3] is nephrotoxic and induces renal cell carcinomas in experimental animals.[14, 15] We showed previously that the toxicity of Fe-NTA resulted from radical-induced lipid peroxidation, and we suggested that the carcinogenicity of Fe-NTA was also related to its ability to form oxygen free radicals in renal tubules.[16, 17] Iron and other transition metals play a significant role in free radical chemistry,[18] and several transition metals other than iron have been reported to have mutagenicity or carcinogenicity.[19, 20] While mutagenic activities of chemical carcinogens mostly result from direct DNA-carcinogen complex formation, the mutagenic actions of metals are believed to be indirect, namely, radical-induced oxidative modification of DNA.[21] Since these

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[3] The abbreviations used are: NTA, nitrilotriacetic acid; Fe-NTA, ferric nitrilotriacetate; PCR, polymerase chain reaction; SSCP, single strand conformation polymorphism; 8OHdG, 8-hydroxydeoxyguanosine.

Table I. Summary of the Tumor Location, Pathological Typing, Grade and Mutations

Animal	Tumor	Location	Pathological typing[a]	Grade[a]	Mutations	
					ras	*p53*
A1	1	Kidney	Alveolar/Granular	G3	—	—
A1	2	Kidney	Alveolar/Granular	G2	—	—
A1	3	Kidney	Alveolar/Granular	G3	—	—
A1	4	Lymph node	Alveolar/Granular	G3	—	—
A2	5	Kidney	Alveolar/Granular	G2	—	Exon 6
A3	6	Kidney	Mixed/Granular	G2	—	—
A4	7	Kidney	Mixed/Granular	G2	—	Exon 6
B1	8	Kidney	Alveolar/Granular	G3	—	—
B1	9	Kidney	Alveolar/Granular	G2	—	—
B1	10	Lung	Alveolar/Granular	G2	—	—
B2	11	Kidney	Papillary/Granular	G2	—	—
C1	12	Kidney	Papillary/Granular	G2	—	—
C2	13	Kidney	Papillary/Granular	G2	—	—
D1	14	Kidney	Alveolar/Granular	G2	—	—
D2	15	Kidney	Alveolar/Granular	G2	—	—
D3	16	Kidney	Alveolar/Granular	G2	—	—

a) Tumors were assessed according to the General Rules for Clinical and Pathological Studies on Renal Cell Carcinoma, Japan.

Table II. Primers for Amplification of Rat *ras* Exons 1 and 2

Gene	Exon	Strand	DNA sequence 5′ → 3′
H-*ras*	1	sense	TGATT CTCAT TGGCA GGTGG
H-*ras*	1	antisense	GAGCT CACTC TATAG TGGGA
H-*ras*	2	sense	AGGAC TCCTA CCGGA AACAG
H-*ras*	2	antisense	ACCTG TACTG ATGGA TGTCT
K-*ras*	1	sense	AGGCC TGCTG AAAAT GACTG
K-*ras*	1	antisense	GCAGC GTTAC CTCTA TCGTA
K-*ras*	2	sense	CCTAC AGGAA ACAAG TAGTA
K-*ras*	2	antisense	TAAAC CCACC TATAA TGGTG
N-*ras*	1	sense	ATGAC TGAGT ACAAA CTGGT
N-*ras*	1	antisense	GGCAG TGGAT TGGGC CTCAC
N-*ras*	2	sense	GATTC TTACC GAAAG CAAGT
N-*ras*	2	antisense	TCAGA AAACA TTCCC AGTAC

differences in mutagenic mechanisms might be reflected in the mutational patterns, it is of special interest to evaluate the mutations in Fe-NTA induced tumors, despite the low incidence of the *ras* and *p53* mutations expected in renal cell carcinoma. In the present study, we examined the mutations of the *ras* oncogenes and *p53* tumor suppressor gene in rat renal cell carcinomas induced by Fe-NTA.

MATERIALS AND METHODS

Tumors and DNA extraction Renal cell carcinomas were induced by intraperitoneal injections of Fe-NTA in male Wistar rats (Shizuoka Laboratory Animal Center, Shizuoka). Ferric chelate of NTA was prepared by mixing ferric nitrate solution with nitrilotriacetic acid disodium salt solution.[14, 15] Rats received daily injections of Fe-NTA (5–15 mg Fe/kg body weight) for three months, and were killed over a nine-month period. Details of the tumor induction were described in our previous reports.[14, 15, 17] Fourteen primary tumors and 2 metastatic tumors from 11 animals were analyzed in the present study. Their pathological typings and grades are summarized in Table I.

High-molecular-weight DNAs were extracted by the phenol/chloroform method after proteinase K digestion from frozen specimens of untreated rat kidney and 3 carcinomas (tumor 14–16) induced by Fe-NTA. Extraction of DNAs from paraffin-embedded sections (tumors 1–13) was done as described by Koshiba *et al.*[22]

Amplification and sequencing of rat *ras* genes All oligonucleotide primers were synthesized using an Applied Biosystems model 392 oligonucleotide synthesizer (Foster City, CA). The primer sequences[23, 24] used for the amplification of the *ras* genes are listed in Table II. Reaction mixtures contained 200 ng of DNA, 12.5 pmol of each primer, 0.2 m*M* dNTPs, 1.25 U of *Taq* DNA polymerase (Boehringer-Mannheim, Mannheim, Germany), and appropriate buffers in a total volume of 50 μl. Amplification from frozen tumors was performed for 40 cycles of 40 s at 94°C, 40 s at 55°C, and 40 s at 72°C using a thermal cycler (PCT-100, MJ Research, Watertown, MS). Amplification from paraffin-embedded tumors was performed for 45 cycles with the same thermal pattern.

Dideoxynucleotide chain-termination sequencing of the purified PCR products was done using a Taq Cycle

Sequencing Kit (Takara, Kyoto) and [γ-^{32}P]ATP (Amersham, Buckinghamshire, UK). Either the sense or antisense PCR primer listed in Table II was used as sequencing primer. Electrophoresis was done on 8 % denaturing polyacrylamide gels, and the dried gels were exposed to X-ray films (Fuji Photo Film, Tokyo).

PCR-SSCP analysis of rat *p53* gene Since rat *p53* gene lacks intron 6, we renumbered the downstream exons accordingly, as reported by Hulla and Schneider[25] (Fig. 1). The primers used for the amplification of the rat *p53* exons 5 through 7 were designed to cover the 3′ region of the conserved region II, and the entire conserved regions III to V (Table III). Most of the transformation-associated substitutional mutations of human *p53* gene are clustered in these regions.[2, 3] To avoid the amplification of the processed *p53* pseudogenes,[25] at least one of the paired primers was on the intron sequences (Fig. 1). The SSCP analysis was done as previously described,[26, 27] with minor modifications. Five μl of reaction mixture contained 20 ng of template DNA, 1.25 pmol of each primer, 100 nmol of dATP, dGTP, and dTTP, 10 nmol of dCTP, 1 μCi of [α-^{32}P]dCTP (Amersham), 0.25 unit of *Taq* polymerase, and appropriate buffer. Thirty-five cycles of amplification (40 s at 94°C, 40 s at 55°C, and 40 s at 72°C) were done and 1 μl aliquots of the amplified products were diluted with 19 μl of denaturing buffer. One μl of diluted products was loaded on 8 % non-denaturing polyacrylamide gel with or without 10 % glycerol, and electrophoresed at 200 V for 15 h at room temperature. Then, the gels were dried and autoradiographed. Possibilities of false negatives in the SSCP analysis were reduced by running samples on two different types of gels. A positive control for SSCP analysis was generated by a primer introduced with a base substitution (primer 5AM, Table III). An area of gel displaying a mobility shift was cut out, and DNAs were extracted by heating the gel in 20 μl of water at 80°C for 15 min.

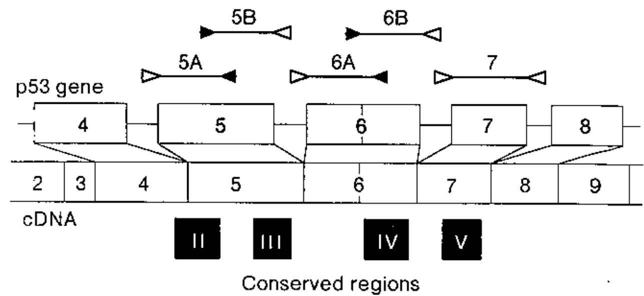

Fig. 1. Design of primers for PCR-SSCP analysis of *p53* gene. Rat exon 6 corresponds to human exons 6 and 7. Exons are shown as shaded boxes; evolutionarily conserved regions are specified by painted boxes; PCR primers are illustrated by arrowheads. Closed arrowheads are exon-based primers; open arrowheads are intron-based primers. In exons 5 and 6, two overlapping fragments from each exon were amplified and designated as regions 5A, 5B, 6A and 6B respectively.

DNAs recovered from SSCP gels were PCR-amplified again, and sequenced as described above. Both sense and antisense PCR primers were used as sequencing primers.

RESULTS

No mutation of the H-, K-, and N-*ras* genes (codon 12, 13, or 61) was detected by direct sequence analysis in the 16 tumors induced by Fe-NTA (data not shown).

Two of the 16 tumors showed conformational band shifts in *p53* exon 6 (Fig. 2). Both tumors were primary renal tumors. No abnormal band shift was observed in exon 5, 7, or 8 of the *p53* gene. Direct sequencing analysis of the fragments showing mobility shifts revealed that one tumor had a C-to-T transition at the second position of codon 199 resulting in replacement of a proline residue by leucine (Fig. 3). The other tumor had a C-to-T

Table III. Primers for Amplification of Rat *p53* Gene

Primer	Region	Strand	DNA sequence 5′ → 3′	
5AF[a]	5A[b]	sense	GATTC TTTCT CCTCT CCTAC	intron
5AR	5A	antisense	CTTGT AGATG GCCAT GGCAC	exon
5BF	5B[b]	sense	TCACC TCCAC ACCTC CACCT	exon
5BR	5B	antisense	ACAGC AGTGC CCAGT GCTCA	intron
6AF	6A[b]	sense	GCCTC TGACT TATTC TTGCT	intron
6AR	6A	antisense	TGGAT AGTGG TATAG TCGGA	exon
6BF	6B[b]	sense	TGGTA CCGTA TGAGC CACCT	exon
6BR	6B	antisense	CCCGG CCTGG CACAC AGCTT	intron
7F	Exon 7	sense	CTCCT CTTGT CCCGG GTAGT	intron
7R	Exon 7	antisense	CTTCT TTGTC CTGCC TGCTC	intron
5AM[a]	5A	antisense	CTTGT AGATG GCCAT GGCACTGAC	exon

a) Primers 5AM and 5AF were used to generate positive control DNA fragment for SSCP analysis.
b) Regions 5A, 5B, 6A and 6B are indicated in Fig. 1.

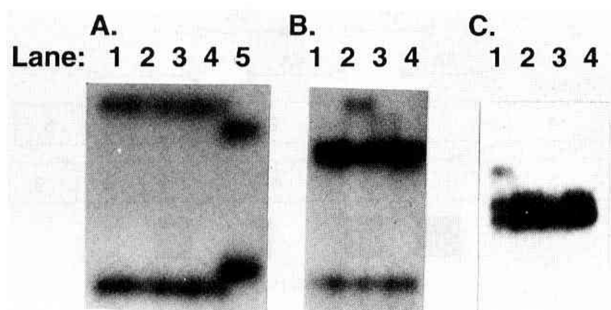

Fig. 2. PCR-SSCP analysis of *p53* gene. DNAs from Fe-NTA induced tumors were amplified with the primers for region 5A of exon 5 (A), region 6A of exon 6 (B), or region 6B of exon 6 (C). (A) Lanes 1–4, Fe-NTA induced tumors; lane 5, a positive control for region 5A. (B) Lanes 1–4, Fe-NTA induced tumors. Lane 2 (tumor 5) showed conformational polymorphism in region 6A of exon 6. (C) Lanes 1–4, Fe-NTA induced tumors. Lane 1 (tumor 7) showed conformational polymorphism in region 6B of exon 6.

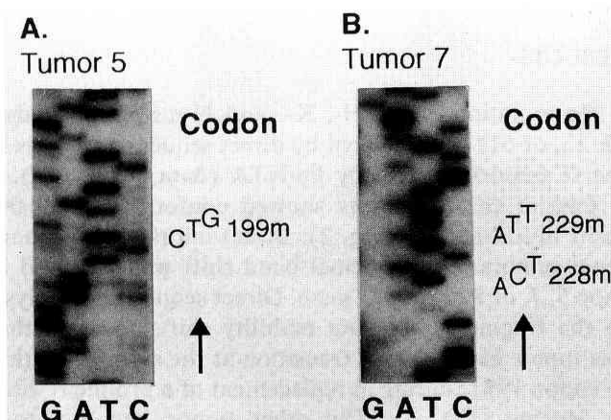

Fig. 3. Direct sequencing of *p53* gene exon 6. (A) Sequence of tumor 5 with CCG-to-CTG transition at codon 199. (B) Sequence of tumor 7 with ACT-to-ATT transition at codon 229, and ACC-to-ACT transition at codon 228.

transition at the second position of codon 229 resulting in replacement of a threonine residue by isoleucine, and two nonsense C-to-T transitions at codons 228 and 249 (Table IV). Non-tandem multiple mutations in the *p53* gene have sometimes been reported in experimental tumors.[8, 28] There was no obvious correlation between genetic alteration and tumor histology or grade (Table I).

DISCUSSION

The present study evaluated the presence of *ras* and *p53* mutations in rat renal cell carcinoma induced by Fe-NTA. None of the sixteen tumors examined had mutations in the *ras* genes, and only two tumors had missense mutations in exon 6 of *p53* gene. One tumor had a CCG-CTG transition in codon 199, resulting in substitution of a leucine for a proline, and the other tumor had a ACT-ATT transition in codon 229 resulting in substitution of isoleucine for threonine. Amino acid sequences in these two regions are not evolutionarily conserved, and missense mutations are rarely found in human malignancies.[2, 3] Thus, the biological relevance of the observed *p53* mutations is unclear, and it can be concluded that neither activation of *ras* genes nor inactivation of *p53* gene is obligatory in renal cell carcinoma induced by Fe-NTA.

There are two possible explanations for the low frequency of *p53* mutations. Posttranslational mechanisms might indirectly inactivate the wild-type p53 protein, or rather, the combined effects of other inactivated tumor suppressor genes would suffice in the development of the renal carcinoma. Studies on the loss of heterozygosity in human renal cell carcinomas led to the prediction that candidate tumor suppressor genes exist on chromosomes 3p, 5q, 6q, and 10q,[29, 30] and that there would be at least three separate tumor suppressor genes on chromosome 3p.[29, 31] Recently, von Hippel Lindau (VHL) disease gene, located at the 3p25-26 region, was identified as a tumor suppressor gene of human renal cell carcinoma.[32] Mutations of *VHL* gene were found in about 50 % of sporadic clear cell renal carcinomas.[32] As for rodent

Table IV. Summary of the Mutations of *ras* and *p53* Genes

Tumor	*ras* mutation	*p53* mutation			
		Exon	Codon	Nucleotide change	Amino acid change
5	—	6	199	CCG-CTG	Pro-Leu
7	—	6	228[a]	ACC-ACT	—
			229[a]	ACT-ATT	Thr-Ile
			249[a]	ATC-ATT	—

a) Three mutations were on the same allele.

models, Hino *et al.* recently reported a germline mutation of tuberous sclerosis gene in Eker rat,[33, 34] which is predisposed to develop renal cell carcinoma. Tuberous sclerosis gene was supposed to be a new tumor suppressor gene of rodent renal cell carcinomas. Further studies should reveal the prevalence of these genes in Fe-NTA induced renal cell carcinomas.

We had shown that nephrotoxicity of Fe-NTA resulted from radical-induced lipid peroxidation, and we suggested that the carcinogenicity of Fe-NTA is related to its ability to form oxygen free radicals.[16, 17] It is well known that oxygen free radicals induce various DNA lesions which are implicated in mutagenesis and carcinogenesis.[21] Among these oxidative DNA lesions, formation of 8OHdG has been highlighted.[35-39] Guanine residues in DNA can be hydroxylated to form 8OHdG by several reducing agents or transition metals, and hydroxyl radical is implicated in this process.[35] Elevated levels of 8OHdG in tissues have been found after treatment with reactive oxygen-producing carcinogens such as $KBrO_3$[36] and Fe-NTA.[37] The 8OHdG in DNA had been reported to induce G-to-T transversions *in vitro*,[38, 39] and G-to-T transversions in experimental tumors were proposed to be a signature mutation of oxidative damage in DNA. Consistent results were obtained in experimental tumors by Higinbotham *et al.*[19] Renal mesenchymal tumors induced with nickel sulfide and iron frequently showed G-to-T transversions of K-*ras* gene codon 12.[19] In the present study, observed mutations of *p53* were solely C:G-to-T:A transitions, and the low incidence of the mutations makes it difficult to interpret the significance of this pattern. Since DNA lesions induced by oxygen radicals are not confined to 8OHdG, and replication of DNA damaged by free radicals results in diverse patterns of mutations under different experimental conditions,[40-42] the assignment of specific mutations to the radical-related tumors needs further elucidation.

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