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Low Incidence of Point Mutations in H-, K- and N-*ras* Oncogenes and *p53* Tumor Suppressor Gene in Renal Cell Carcinoma and Peritoneal Mesothelioma of Wistar Rats Induced by Ferric Nitrilotriacetate

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An iron chelate, ferric nitrilotriacetate (Fe-NTA), induces renal proximal tubular damage, a consequence of iron-catalyzed free radical reactions, that finally leads to a high incidence of renal cell carcinoma (RCC) in rodents. Previous studies have identified, within 24 h after administration of Fe-NTA, lipid peroxidation products, aldehyde-modified proteins and a variety of modified DNA bases such as 8-hydroxyguanine that may be mutagenic *in vivo*. In the present study, pathological features of the RCCs were studied, and, in an effort to correlate them with carcinogen-specific molecular events in Fe-NTA-induced carcinogenesis, the H-, K- and N-*ras* oncogenes and the *p53* tumor suppressor gene were investigated for the presence of mutations. Fe-NTA-induced RCCs showed similarity to human RCCs in that they are often invasive, metastatic and fatal. None (0 of 12) of the tumors had mutation in codons 12, 13 and 61 of the H-, K- and N-*ras* genes by direct sequencing. Only one (1 of 12) tumor with high grade histology revealed a CGC-to-CTC (Arg to Leu) transversion in codon 246 of the *p53* gene by the use of single strand conformation polymorphism (SSCP) analysis and direct sequencing. High expression of mutant p53 protein was confirmed by Western blotting and immunohistochemistry. Study of three peritoneal mesotheliomas induced by Fe-NTA revealed no mutation in *ras* and *p53* genes. These results suggest that the *ras* and *p53* genes are not the major targets of mutation in Fe-NTA-induced carcinogenesis of kidney and mesothelium. Instead, *p53* mutation may work for potentiation of malignant character in Fe-NTA-induced renal carcinogenesis.

Key words: Iron — Renal cell carcinoma — *ras* — *p53* — Oxidative DNA damage

Nitrilotriacetic acid (NTA) is a synthetic aminotricarboxylic acid that efficiently forms water-soluble chelate complexes with several metal cations at neutral pH and has been used as a substitute for polyphosphates in detergents for household and hospital use in the U.S., Canada and Europe.[1] An experimental model of iron overload was established by the use of ferric nitrilotriacetate (Fe-NTA).[2] Later, repeated i.p. administration of Fe-NTA was reported to induce acute and subacute renal proximal tubular damage and a subsequent high incidence (60–92%) of renal cell carcinoma (RCC) in male rats and mice.[3–5] We have so far demonstrated in this model lipid peroxidation products (thiobarbituric acid-reactive substances, cold Schiff's method, free 4-hydroxy-2-nonenal), aldehyde-modified proteins (carbonyls, 4-hydroxy-2-nonenal-modified proteins)[5–7] and a variety of modified DNA bases such as 8-hydroxyguanine.[8] These results strongly suggest that Fe-NTA-induced renal carcinogenesis is mediated by oxy-radicals since 4-hydroxy-2-nonenal[9] and 8-hydroxyguanine[10] are mutagenic *in vitro*. Thus, it is intriguing to study the mutation spectrum of Fe-NTA-induced tumors to correlate iron-specific molecular events with genetic change. In the present study, we selected the H-, K- and N-*ras* oncogenes and *p53* tumor suppressor gene in search of genetic alteration in iron-induced rat tumors. In addition, we studied the pathological features of Fe-NTA-induced RCCs.

MATERIALS AND METHODS

Animals Male SPF slc:Wistar rats (Shizuoka Laboratory Animal Center, Shizuoka), weighing 80 to 120 g (4 weeks of age) were used. They were kept in a stainless steel cage and given commercial rat chow (Funabashi F-2, Chiba) as well as deionized water (Millipore Japan, Osaka) *ad libitum*. A total of 61 animals were divided into three groups, Fe-NTA (N=31), NTA (N=15) and untreated control (N=15).

Materials Ferric nitrate enneahydrate and sodium carbonate were from Wako (Osaka); nitrilotriacetic acid disodium salt was from Nacalai Tesque Inc. (Kyoto). Enhanced chemiluminescence (ECL) immunoblotting

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reagents were obtained from Amersham (Buckinghamshire, England). Protein concentration was measured using the BCA protein assay reagent obtained from Pierce (Rockford, IL). All the chemicals used were of analytical quality; deionized water was used throughout.

Preparation of Fe-NTA solution and tumor induction protocol Fe-NTA solution was prepared as previously described.[6] Fe-NTA or an equivalent dose of NTA was injected i.p. into animals as follows; 5 mg iron/kg body weight for 3 days, 10 mg iron/kg body weight for the next 2 days and then 5 days a week for 11 weeks. Thereafter, the animals were observed every day till each animal appeared seriously ill or died. Injections were withheld when animals markedly lost weight (more than 5 % of the body weight) because of acute or subacute renal damage by Fe-NTA. Animals were killed by decapitation when they were found dying. Some of the induced tumors were fixed with phosphate-buffered neutral formalin (10%) for histological examination. The rest of the tumor was kept frozen at −80°C. Some of the frozen tumors were re-buried in Tissue-Tek optimum cutting temperature compound (Miles Inc., Elkhart, IN) for immunohistochemistry.

Oligonucleotide primers and probes for polymerase chain reaction (PCR) As primers, the pairs of appropriate oligonucleotides shown in Table I were used for amplification of H-, K- and N-*ras* gene fragments from genomic DNA and the *p53* gene fragments from cDNA.

PCR reaction of *ras* oncogenes DNA was extracted from each frozen tissue as previously described.[11] For amplification of *ras* genes, PCR was done according to the manufacturer's protocol using a GeneAmp reagent kit (Perkin-Elmer, Branchburg, NJ). PCR products of *ras* oncogenes were directly sequenced.

RNA preparation and reverse transcription-polymerase chain reaction (RT-PCR) of the *p53* gene Total RNA was isolated from each frozen tumor and from normal kidneys by means of the modified acid guanidinium phenol chloroform method (Isogen, Nippon Gene, Tokyo). Messenger RNA of the *p53* gene including the conserved coding regions was specifically reverse-transcribed to cDNA by the use of a first-strand cDNA synthesis kit (Pharmacia-LKB, Uppsala, Sweden). Total RNA (0.5 μg) was used in a 15-μl reaction mixture containing a downstream primer (1 μM; reverse primer of seg. 6) complementary to the 3′ end of the p53 mRNA, as described.[12] PCR was thereafter performed using 5 μl of the first strand cDNA product containing an upstream primer (0.1 μM; forward primer of seg. 1) with reaction buffer (GeneAmp, Perkin-Elmer) in a total volume of 50 μl. The PCR conditions were: 95°C for 5 min, then 40 cycles of 95°C for 1 min, 55°C for 1min and

Table I. PCR Fragments and Oligonucleotide Primers of *ras* and *p53*

Gene		Primer	Product size (bp)	Reference
K-*ras*	exon 1	F AGGCCTGCTGAAAATGACTG R GCAGCGTTACCTCTATCGTA	134	34)
	exon 2	F CCTACAGGAAACAAGTAGTA R TAAACCCACCTATAATGGTG	184	34)
H-*ras*	exon 1	F TGATTCTCATTGGCAGGTTGG R GAGCTCACCTCTATAGTGGGA	176	34)
	exon 2	F AGGACTCCTACCGGAAACAG R ACCTGTACTGATGGATGTCT	174	34)
N-*ras*	exon 1	F ATGACTGAGTACAAACTGGT R AAGGACCAGGCAGTGGATTG	140	34) 35)
	exon 2	F GATTCTTACCGAAAGCAAGT R AGAGTGCACATACAAAGG	192	34) 35)
p53	exons 4 and 5 (seg. 1)	F AACTTACCAAGGCAACTATG R CTTGTAGATGGCCATGGCAC	190	12) 33)
	exons 5 and 6 (seg. 2)	F TCACCTCCACACCTCCACCT R CTCAGGTGGCTCATACGGTA	233	33) 12)
	exons 5–7 (seg. 3)	F AGCGTTGCTCTGATGGTGAC R TGGATAGTGGTATAGTCGGA	160	12) 33)
	exons 6–8 (seg. 4)	F TGGTACCGTATGAGCCACCT R CGGGCAATGCTCTTCTTTTT	242	33) 12)
	exons 8 and 9 (seg. 5)	F TTCGTGTTTGTGCCTGTCCT R CTTAAGGGTGAAATATTC	179	12) 33)
	exons 9 and 10 (seg. 6)	F CACTGCCCACCAGCACAAGC R CTTCTTGGTCTTCGGGTAGC	200	33) 12)

72°C for 1 min. The 817-bp fragments were electrophoresed on 1.5% agarose gel, cut out and purified using Qiaex (Qiagen, Inc., Chatsworth, CA).

Single strand conformation polymorphism (SSCP) analysis of the *p53* gene Utilizing the gel-purified 817-bp cDNA fragment as a template, six internally primed fragments were amplified for analysis by SSCP (Fig. 1). PCR amplification was performed using a primer pair of seg. 1 to yield a 190-bp product (exons 4 and 5); a primer pair of seg. 2 to yield a 233-bp product (exons 5 and 6); a primer pair of seg. 3 to yield a 160-bp product (exons 5–7); a primer pair of seg. 4 to yield a 242-bp product (exons 6–8); a primer pair of seg. 5 to yield a 179-bp product (exons 8 and 9); a primer pair of seg. 6 to yield a 200-bp product (exons 9 and 10). The PCR mixture contained 0.025 pmol of each primer, 6.25 nmol of dNTP, Taq polymerase (0.05 U), cDNA template (<1 μg) and [α-^{32}P]dCTP (1 μCi) with reaction buffer (GeneAmp, Perkin-Elmer) to a final volume of 5 μl. The PCR conditions were: 94°C for 5 min, then 30 cycles of 94°C for 1 min, 55°C for 1 min (except for the primer set of seg. 5; 50°C) and 72°C for 1 min in a thermal cycler (Perkin-Elmer Cetus). The ^{32}P-labeled PCR fragments (2 μl) were diluted with 18 μl of stop solution (95% formamide, 20 m*M* EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol). Samples were then heated to 98°C for 5 min and placed on ice, followed by electrophoresis (2 μl/well) on 5% polyacrylamide gel with or without 5% glycerol at a constant 300 or 500 V for 13 h at room temperature in 1× Tris-borate buffer (TBE). The gels were dried on filter paper and exposed to X-ray film in the presence of an intensifying screen. The DNA fragment was extracted from the mobility-shifted band in the polyacrylamide gel and reamplified.

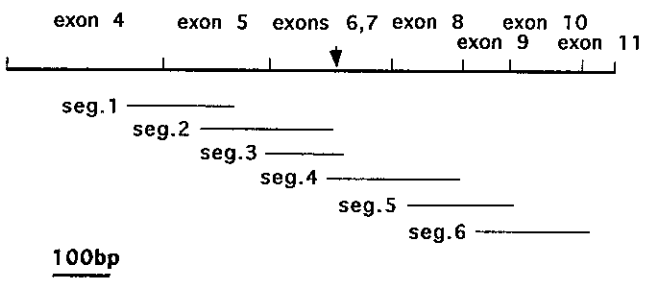

Fig. 1. Schematic representation of exons 4–10 of the rat p53 cDNA with location of PCR fragments. The rat gene lacks intron 6.[12,33] The arrow indicates the position of the human- and mouse-specific intron 6 splice site.

Direct sequencing analysis of the *ras* and *p53* genes Both pairs of PCR primers were labeled with [γ-^{32}P] ATP by polynucleotide kinase, and sequencing was carried out using a Circumvent DNA sequencing kit (New England Biolab, Ontario, Canada) in a thermal cycler according to the manufacturer's protocol. Samples were denatured at 90°C for 3 min and applied to 6% or 8% polyacrylamide gel containing 8.3 *M* urea. Electrophoresis was

Table II. Rat Renal Cell Carcinomas Produced by Fe-NTA

No. of case	Histological type[a]				Metastasis and invasion	*p53* mutation
	Structure	Cell type	Grading	Size (mm)[b]		
1[c]	Papillary	common, granular	G1>G2	10	(−)	(−)
2[d]	Solid and tubular	common, granular	G2>G3	30	lung, kidney, liver, peritoneum	(−)
3[d]	Solid and papillary	granular and pleomorphic	G2	10	(−)	(−)
4	Cystic and solid	common, granular	G2>G3	40	lung, peritoneum	(−)
5	Tubular	common, granular	G1>G2	15	(−)	(−)
6	Papillary, tubular and cystic (mixed)	common, granular	G2	20	lung	(−)
7	Solid	pleomorphic	G3	50	lung, peritoneum	CGC→CTC (Arg→Leu, codon 246)
8	Solid	pleomorphic	G3	20	lung	(−)
9[d]	Cystic and tubular	common, mixed	G1	25	(−)	(−)
10[d]	Tubular and cystic	common, mixed	G1	5	(−)	(−)
11[c]	Solid and tubular	common, granular	G1>G2	35	kidney	(−)
12	Solid	granular and pleomorphic	G2>G3>G1	20	(−)	(−)

a) Histological classification was according to the modified WHO classification.[13] Refer also to Fig. 2.
b) Size indicates maximal diameter.
c) Mesothelioma was also induced.
d) Cases 2 and 3 and cases 9 and 10 are multiple RCCs of a single rat, respectively.

done at a constant power of 20 W for 2–6 h at room temperature. The gel was dried and an autoradiogram was prepared.

Western blot Tumor or normal kidney homogenate with 1.15% KCl was used without further purification. Western blotting was done with a standard procedure[7] after

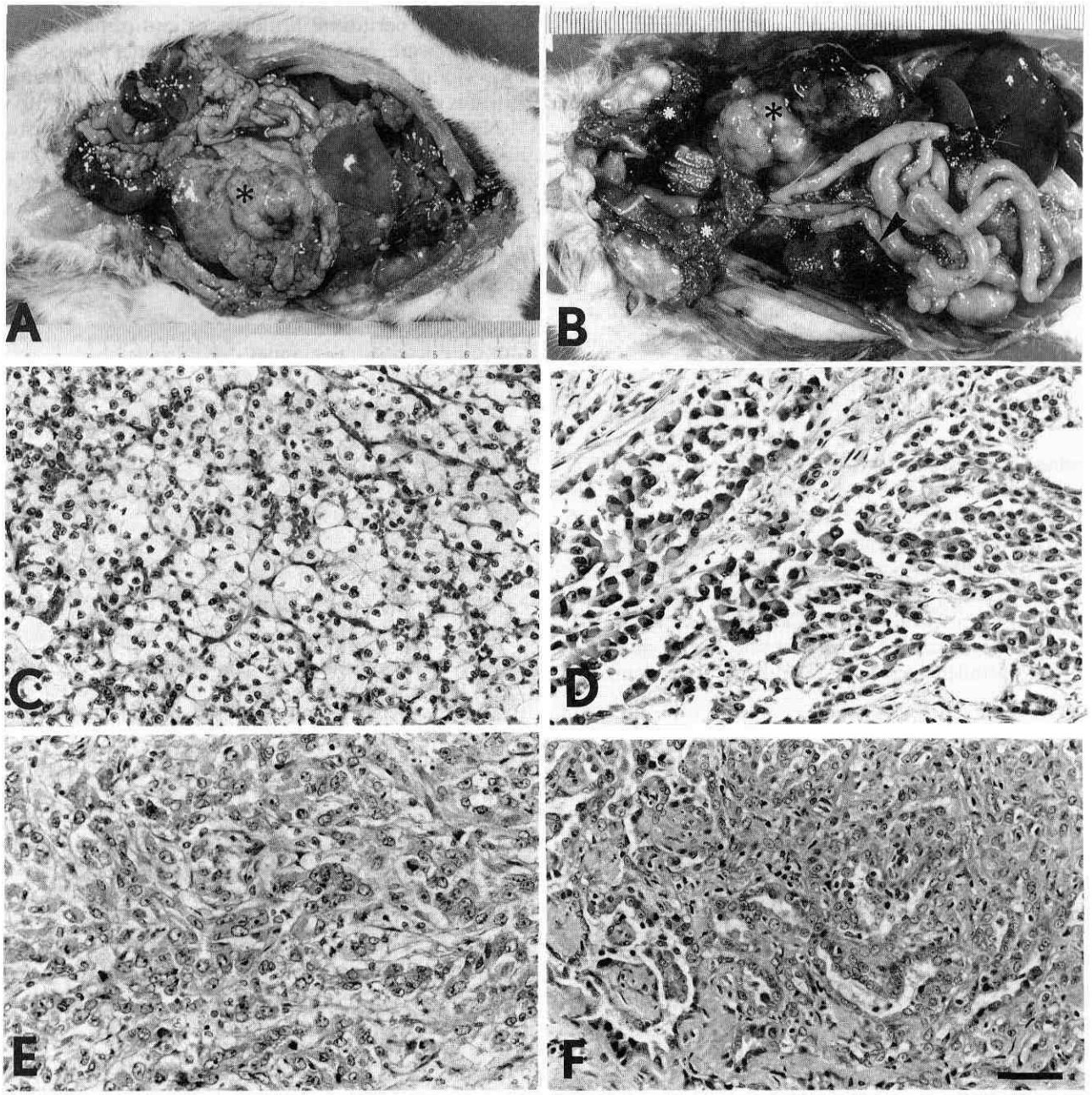

Fig. 2. Pathological features of Fe-NTA-induced renal cell carcinoma and mesothelioma. A, macroscopic appearance of case 7 of Table II. The left kidney is replaced by a huge tumor (asterisk). Peritoneal dissemination and lung metastasis are also noted; B, macroscopic appearance of case 11 of Table II. Renal cell carcinoma (black asterisk) is found in the right kidney. A large cyst (arrow) is found in the left kidney. Peritoneal mesothelioma (white asterisks) arising in the tunica vaginalis of the bilateral testes are also seen; C, renal cell carcinoma, common type, mixed subtype, grade 1 (location of the clear cell subtype is shown in the photograph); D, renal cell carcinoma, common type, granular cell subtype, grade 2; E, renal cell carcinoma, pleomorphic cell type, grade 3 (case 7); F, mesothelioma, biphasic type (hematoxylin and eosin stain). Bar, 50 μm.

12.5 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions (25 μg protein for each lane) using 10 μg/ml primary antibody (PAb421, Oncogene Science, Cambridge, MA), horseradish peroxidase-labeled anti-mouse IgG serum (Dako Japan Co., Ltd., Kyoto ; diluted to 1:5000) and the ECL detection system as described by the manufacturer.

Immunohistochemistry The avidin-biotin complex method was used as previously described with a slight modification.[6] Normal rabbit serum (Dako, diluted to 1:75) for the inhibition of non-specific binding of secondary antibody, anti-p53 monoclonal antibody, biotin-labeled rabbit anti-mouse IgG serum (Dako; diluted to 1:300) and avidin-biotin-alkaline phosphatase complex (Vector Laboratories, Burlingame, CA; diluted to 1:100) were sequentially used. The substrate for alkaline phosphatase (black) was obtained from Vector. Frozen sections cut at 10 μm were postfixed with acetone for 5 min before the immunohistochemistry procedures.

RESULTS

Carcinogenesis study All the animals of the Fe-NTA group died by day 683 after birth (day 571 after the final administration of Fe-NTA). RCC was found in 16 rats (51.6%) in the present experiment. Among animals that survived 300 days after birth, 72.7% of the population had RCC. A total of 26 RCCs was obtained, since Fe-NTA induces multiple RCCs. Of these RCCs, 12 primary tumors (size $\geq$ 5 mm) were selected for mutation analyses. Detailed pathological and mutation data (see below) of 12 primary RCCs are summarized in Table II. In the Fe-NTA group, three mesotheliomas (biphasic type) were induced. Representative macroscopic and microscopic findings of RCCs and mesotheliomas are shown in Fig. 2. Some of the RCCs showed prominent invasion, peritoneal dissemination and lung metastasis, resulting in peritoneal hemorrhage and respiratory failure that were responsible for the animal's death. The histology of RCCs was quite similar to that in humans. We thus applied the modified WHO classification[13] to rat RCCs induced by Fe-NTA. The carcinogenesis study was terminated at day 957 after birth. Neither RCCs nor mesotheliomas were found in the NTA or control group. Leukemia, Leydig cell tumor of the testis and soft tissue sarcomas, such as leiomyosarcoma, that were not specific to the Fe-NTA group were also included for mutation analyses.

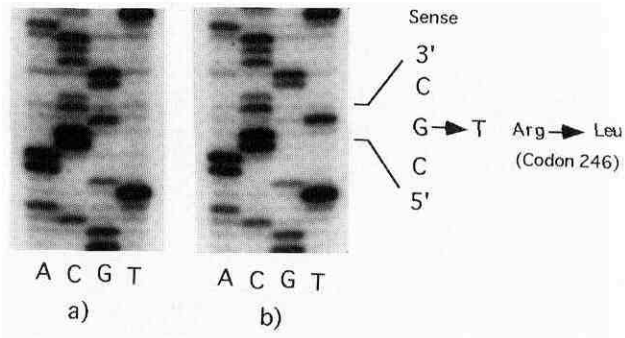

Fig. 4. Sequence pattern of mutated *p53* genes in case 7 of Table II. a) wild type; b) case 7. A missense CGC→CTC transversion (Arg→Leu) in codon 246 of exon 7 is shown.

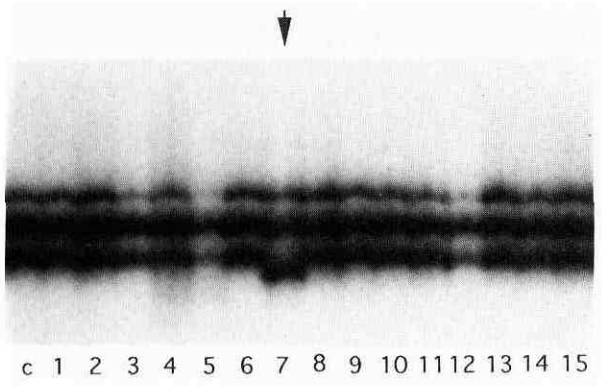

Fig. 3. PCR-SSCP pattern of cDNA studied for mutation in the exons 6 and 7 (seg. 4) region of the *p53* gene in Fe-NTA-induced rat renal cell carcinomas and mesotheliomas. The arrow indicates the presence of a possible mutation in case 7. c, control rat kidney; lanes 1–12, renal cell carcinomas (the numbers correspond to those of Table II); lanes 13–15, mesotheliomas.

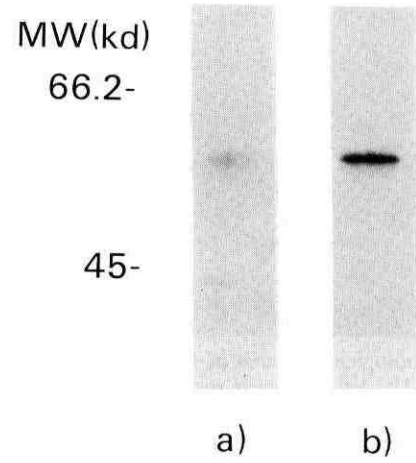

Fig. 5. Western blot analysis of p53 protein. a) control kidney; b) case 7. Refer to Table II for the case number.

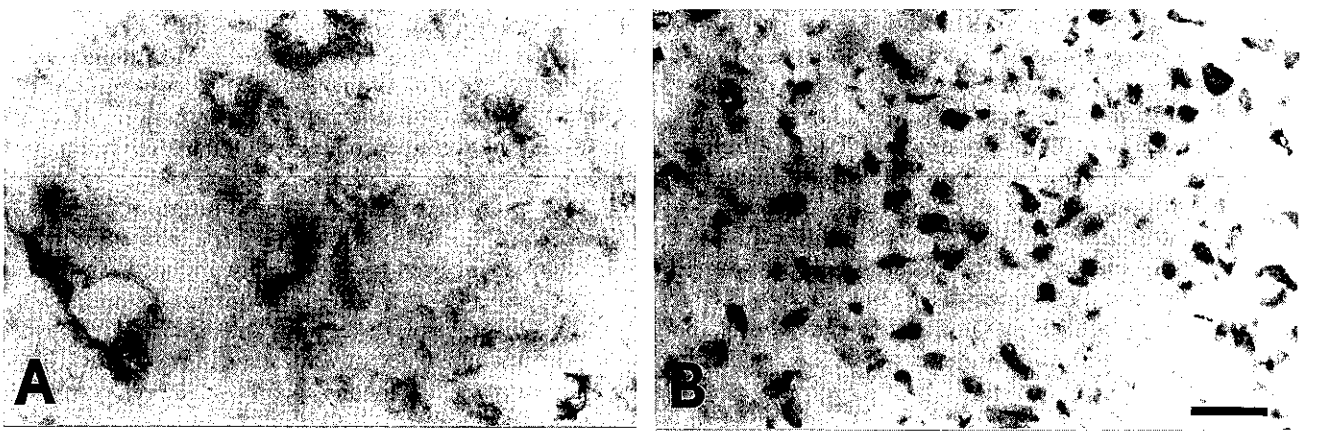

Fig. 6. Immunohistochemical detection of p53 protein. A, case 11; B, case 7. Refer to Table II for the case number. Bar, 25 μm, frozen section, no nuclear counterstaining.

Mutations of the K-, H- and N-*ras* genes Twelve primary RCCs, three mesotheliomas and eight other tumors (three Leydig cell tumors, two leukemias, one leiomyosarcoma, one fibrosarcoma and one pituitary adenoma) were examined. No mutations were detected at codon 12, 13, or 61 of the H-, K-, and N-*ras* genes in the cases of RCCs and mesotheliomas. In one case (Leydig cell tumor in Fe-NTA group), a point mutation of K-*ras* gene was found (AGG [Arg] at codon 68 to ACG [Thr] at codon 68).

Mutations of the *p53* gene Six overlapping fragments (smaller than 250 base pairs) were amplified. By SSCP analysis, mobility shifts were noted in two cases. One case was RCC (Fig. 3). A point mutation was found in case 7 at codon 246 with a change in amino acid from wild-type CGC (Arg) to CTC (Leu) (Fig. 4). The other case was leukemia in the Fe-NTA group. The sequence GCC (Ala) at codon 157 was changed to GTC (Val) (data not shown).

Western blot and immunohistochemistry of the p53 protein We used a monoclonal antibody (PAb421) that recognizes the carboxyl terminal region (codon 371–380) of p53 protein. In case 7 of RCC that exhibited point mutation, overexpression of p53 protein was observed in the Western blot (Fig. 5) and strong nuclear staining was seen in the immunohistochemistry (Fig. 6).

DISCUSSION

The Fe-NTA-induced renal carcinogenesis model in rodents, established by Okada and colleagues,[3,4] is unique in two respects, as confirmed in the present study: 1) induced renal tumors are similar to those of humans in that they are of epithelial origin and possess invasive and metastatic potential. In contrast, nickel-induced renal tumors are sarcomas.[14] Multiple RCCs are induced spontaneously or chemically in Eker rats that have a hereditary insertional mutation in one allele of *Tsc2* gene.[15] However, the RCCs almost never metastasize and are rarely fatal, being followed by the appearance of second and third malignant tumors of extra-renal origin.[16] 2) Biochemical studies on the early stages of the Fe-NTA-induced carcinogenesis model strongly suggest that free radicals or oxidative stress are involved in this renal carcinogenesis. It is well established that a "catalytic" or "free" form of iron, such as Fe-NTA, catalyzes the production of free radicals via the Fenton reaction. Our previous studies support the view that these reactions are indeed induced *in vivo* by Fe-NTA.[5,6,8] Furthermore, several epidemiological studies have found an association of increased body iron stores and increased risk of cancer.[17]

The uniqueness of this model prompted us to study the mutation spectrum of the induced Fe-NTA-specific tumors. We selected *ras* oncogenes and *p53* tumor suppressor gene for the present study. We obtained only one case with *p53* mutation. In this case, a missense mutation was induced in the DNA binding domain of p53 protein and overexpression of the mutated protein was confirmed by Western blotting and immunohistochemistry (Figs. 5 and 6). In human RCC, previous studies have documented a low incidence of *ras* mutation (0–2%)[18,19] and a relatively low incidence of *p53* mutation (2–33%).[18–20] Therefore, the genetic pathway in human renal carcinogenesis appears to differ considerably from that of other human neoplasms, such as colon, lung and esophageal carcinoma. This may also be the case for Fe-NTA-induced rat renal carcinogenesis. Recently, mutations of the von Hippel Lindau (VHL) tumor suppressor gene were identified at high frequency in the clear cell type of

familial and sporadic human RCCs.[21] Studies need to be done to examine mutation in the rat homologue of VHL tumor suppressor gene.

In a recent study, Oda *et al.*[22] showed a high frequency of *p53* mutations (78%) in sarcomatoid lesion of human RCCs. Thus, loss of *p53* function would be expected to correlate with aneuploidy and increased proliferative rate. Case 7 with *p53* mutation showed highly malignant characteristics, such as high nuclear grade, peritoneal dissemination and lung metastasis (Fig. 2, A and E). Thus, loss of *p53* function may be associated with the biological character of the tumor, as was demonstrated in human RCCs.

The mutation spectrum of *ras* and *p53* genes in chemically induced rat tumors varies with the chemical agents used. Frequent G-to-T transversion of K-*ras* codon 12 was reported for nickel subsulfide/iron-induced tumor (60%).[14] Frequent *p53* mutation (75–100%) and K-*ras* mutation (75%) were reported for alkylating N-nitroso compound-induced tumors.[23,24] On the other hand, a low incidence of *p53* mutation was reported in nickel subsulfide- and iron-induced tumor (0%) and methylnitrosamine-induced tumor (10%).[12] A low incidence of *ras* mutation was reported in N-ethyl-N-hydroxyethylnitrosamine- or N-nitrosomorpholine-induced renal cell tumors.[25]

We and other investigators have previously reported that Fe-NTA in the presence of a reducing agent, such as H_2O_2, ascorbate or cysteine, induces single and double strand breaks in plasmid DNA[26] and a variety of modified DNA bases and thymine-tyrosine cross-links in calf thymus DNA *in vitro*.[27,28] We have also reported that modified DNA bases and thymine-tyrosine cross-links are induced *in vivo* in the target organ of Fe-NTA-induced carcinogenesis after a single i.p. administration.[8,29] In an *in vitro* study using single-stranded DNA templates, the most frequent mutations induced by oxygen free radicals mediated by iron were C-to-T transitions (23%) and G-to-T (18%) and G-to-C (39%) transversions.[30] The G-to-T transversion could be explained by 8-hydroxyguanine and adenine mispairs.[10] Our finding of G-to-T transversion in codon 246 of *p53* gene may be due to production of 8-hydroxyguanine in DNA.

It is presumed that induced mutation patterns in tumors are likely to depend on the character of the chemical agent used and the tumor induced. Since reactive oxygen species mediated by iron induce mutations in plasmid DNA *in vitro* as mentioned above, we suspect that the low incidence of mutations in *ras* and *p53* genes in our study may be a consequence of the renal epithelial nature of the tumor.

Mesothelioma is a tumor specific for iron, as we have reported.[31] In humans its association with asbestosis is well established and genetic alterations of the *p53* gene have been reported.[32] The *ras* and *p53* genes were not targets for mutation in Fe-NTA-induced mesotheliomas in the present study.

In conclusion, pathological features of Fe-NTA-induced RCCs are similar to those of human RCCs except for the low incidence of alveolar structure and the purely clear cell subtype in histology. In this model, *ras* oncogenes and *p53* tumor suppressor gene were not the major targets of mutation. However, the one case of *p53* mutation could mean that *p53* mutation favors progression of the disease, and the mutation spectrum was consistent with the production of 8-hydroxyguanine. Major target genes must be sought in this renal carcinogenesis model.

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