Jpn. J. Cancer Res. **89**, 814–820, August 1998

Development of High-grade Renal Cell Carcinomas in Rats Independently of Somatic Mutations in the *Tsc2* and *VHL* Tumor Suppressor Genes

Shinya Toyokuni,[1,3] Kunihiko Okada,[1,4] Shohei Kondo,[1] Hiroaki Nishioka,[5] Tomoyuki Tanaka,[1] Yasuyuki Nishiyama,[1] Okio Hino[2] and Hiroshi Hiai[1]

[1]Department of Pathology and Biology of Diseases, Graduate School of Medicine, Kyoto University, Yoshida-Konoe-cho, Sakyo-ku, Kyoto 606-8501 and [2]Department of Experimental Pathology, Cancer Institute, 1-37-1 Kami-ikebukuro, Toshima-ku, Tokyo 170-0012

Ferric nitrilotriacetate (Fe-NTA) induces renal proximal tubular damage that ultimately leads to a high incidence of renal cell carcinoma (RCC) in rats. The RCCs are characterized by 1) high incidence of pulmonary metastasis and peritoneal invasion, 2) high incidence of tumor-associated mortality and 3) possible involvement of reactive oxygen species in carcinogenesis. The present study investigated the possible role of *Tsc2* and *VHL* tumor suppressor genes in this model. Thirty-four Fe-NTA-induced primary RCCs and 20 other primary or metastatic tumors of rats were searched for genetic alteration in all the coding exons of both genes by polymerase chain reaction-single-strand-conformation polymorphism analysis and sequencing in conjunction with morphological evaluation. In the Fe-NTA-induced RCCs, frequency of metastasis or invasion was proportionally associated with the nuclear grade of the tumor (grades 1–3). Only one Fe-NTA-induced RCC of grade 1 revealed missense mutations with loss of heterozygosity in exon 10 of the *Tsc2* gene (codons 334, GTG (Val) to GCG (Ala), and 336, TAT (Tyr) to CAT (His)). No mutation was found in the *VHL* gene. The results suggest that 1) high-grade RCCs can develop in the absence of mutations in the *Tsc2* and *VHL* genes in rats, and that 2) *Tsc2* gene somatic mutation can nonetheless be one of the causes of non-Eker rat RCCs.

Key words: Renal cell carcinoma — Rat — Reactive oxygen species — *Tsc2* — *VHL*

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 US, Canada and Europe.[1)] Intraperitoneal injection of ferric nitrilotriacetate (Fe-NTA) induces renal proximal tubular damage that ultimately leads to a high incidence of renal cell carcinoma (RCC) in rodents.[2–5)] The Fe-NTA-induced rat renal carcinogenesis model is a unique animal model characterized by 1) high incidence of pulmonary metastasis and peritoneal invasion, 2) high incidence of tumor-associated mortality either by respiratory failure due to massive pulmonary metastasis, or intraperitoneal hemorrhage due to tumor rupture, and 3) possible involvement of reactive oxygen species in the carcinogenic process. In the kidney after Fe-NTA treatment, we have previously reported an increase in oxidative DNA base modifications such as 8-oxoguanine,[6)] thymine-tyrosine cross-links,[7)] thiobarbituric acid-reactive substances,[8)] saturated and unsaturated mutagenic aldehydes such as 4-hydroxy-2-nonenal (HNE) and malondialdehyde (MDA),[9, 10)] and HNE- or MDA-modified proteins.[9, 11)] Fe-NTA-induced RCCs were shown to have no mutations in H-, K- and N-*ras* oncogenes and a low incidence of mutation in the *p53* tumor suppressor gene.[12)] Therefore, it would be of interest to find the target gene(s) in this model.

In the present work, we have focused on the two tumor suppressor genes associated with human and rat hereditary RCC diseases, *VHL* and *Tsc2* tumor suppressor genes. The genetic defect responsible for hereditary RCCs in von Hippel-Lindau (VHL) disease has been identified as residing in the *VHL* tumor suppressor gene.[13)] Among human non-hereditary non-papillary clear-cell subtype RCCs, 33%,[14)] 57%[15)] and 56%[16)] have been reported to contain alterations in the *VHL* gene. Hereditary RCC in the rat, originally reported by Eker in 1954, is an example of a dominantly inherited Mendelian predisposition to a specific cancer in an experimental model. A germline retrotransposon insertion in the *Tsc2* gene is responsible for the Eker rat model of hereditary RCC.[17, 18)]

To determine whether the *Tsc2* and *VHL* genes are involved in the development of Fe-NTA-induced RCCs,

[3] To whom correspondence should be addressed.
[4] Present address: Laboratory of Food and Biodynamics, Nagoya University Graduate School of Bioagricultural Sciences, Furoucho, Chikusa-ku, Nagoya 464-8601.
[5] Present address: Department of Geriatric Medicine, Graduate School of Medicine, Kyoto University, 54 Shogoin-Kawaharacho, Sakyo-ku, Kyoto 606-8507.

we used polymerase chain reaction-single-strand-conformation polymorphism (PCR-SSCP) and sequencing analyses to detect *VHL* and *Tsc2* gene alterations in a panel of Fe-NTA-induced primary and metastatic RCCs and other tumors. PCR-SSCP analyses revealed no alteration in the *VHL* gene (none of 34 primary RCCs) and a low incidence of alteration in the *Tsc2* gene (one of 34 primary RCCs). These data suggest that high-grade RCCs can develop via a pathway that does not involve mutations in the *VHL* and *Tsc2* tumor suppressor genes.

MATERIALS AND METHODS

Animals Male specific-pathogen-free Wistar rats (Shizuoka Laboratory Animal Center, Shizuoka), or F1 hybrids of Wistar rats and Long-Evans rats from a randomly bred closed colony originally outbred from the Ben May Laboratory for Cancer Research (University of Chicago) in 1973[19] were used. They were kept in stainless steel cages in an air-conditioned room (22–24°C) with a light/dark cycle of 12 h each and given commercial rat chow (Funabashi F-2, Chiba) as well as deionized water (Millipore Japan, Osaka) *ad libitum*. A total of 102 animals (31 male Wistar rats, 36 male F1 hybrids and 35 female F1 hybrids) were registered for the study.

Materials Ferric nitrate enneahydrate and sodium carbonate were from Wako (Osaka); nitrilotriacetic acid disodium salt was from Nacalai Tesque Inc. (Kyoto). All the chemicals used were of analytical quality; deionized water was used throughout.

Preparation of Fe-NTA and tumor induction protocol Fe-NTA solution was prepared as previously described.[9] Fe-NTA was injected i.p. into the 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. Injections were withheld when animals showed marked weight loss (≥5% of the body weight of the previous day). Animals were thereafter kept under close observation until each animal appeared seriously ill. Animals were killed by decapitation when they were found dying. Parts of the induced tumors were fixed with 10% phosphate-buffered neutral formalin for histological examination. The remaining parts were kept frozen at –80°C until use.

Analyzed rat tumor samples A total of 56 samples appropriate for genetic analyses were selected: primary RCCs (≥10 mm, *n*=34), metastatic or invasive lesions (lung, *n*=3; peritoneum, *n*=2), Leydig cell tumor of testis (*n*=4), peritoneal mesothelioma (*n*=3), leukemia (*n*=2, spleen), leiomyosarcoma (*n*=1), basal cell carcinoma (*n*=1), pituitary adenoma (*n*=1), and normal kidney and brain. Three primary RCCs induced by cupric nitrilotriacetate[20] were also used for analyses. All the specimens were diagnosed under the microscope (hematoxylin and eosin staining) by two independent pathologists. Grading of nuclear atypia in RCC was according to the "General Rule for Clinical and Pathological Studies on Renal Cell Carcinoma."[21] Grading is dependent solely on nuclear morphology; briefly, grade 1, nuclei are similar to those of normal proximal tubular epithelia, and sometimes show pycnosis; grade 2, nuclei are larger than those of grade 1, sometimes show irregularity or slight pleomorphism, often have prominent nucleoli, but are neither evidently atypical nor bizzare; grade 3, nuclei reveal prominent irregularity and pleomorphism, and many bizarre or giant nuclei are observed.

Oligonucleotide primers for the *VHL* and *Tsc2* genes Oligonucleotide primers for the *VHL* (4 pairs covering 3 coding exons) and *Tsc2* (45 pairs covering 41 coding exons and one non-coding exon) genes were synthesized according to the published data.[22, 23]

PCR-SSCP analysis DNA was extracted from each frozen sample and amplification was carried out in a 12.5 μl reaction mixture including 50 ng of genomic DNA of sample tissue, 5 pmol of each pair of primers, 0.125 U of AmpliTaq DNA polymerase (Perkin Elmer, Branchburg, NJ), 0.25 m*M* dNTP, 10 m*M* Tris-HCl at pH 8.3, 50 m*M* KCl, 1.5 m*M* $MgCl_2$ and 0.001% (w/v) gelatin. Mixtures were denatured at 95°C for 10 min followed by 30 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min. In the last cycle, the 72°C step was extended to 10 min. After amplification, 4 μl of the products was electrophoresed on 4% NuSieve GTG agarose gel (FMC BioProducts, Rockland, ME) to confirm specific amplification of the targeted fragment. The annealing temperature was modified when specific amplification was not accomplished. The second PCR amplification was then carried out in a 10 μl reaction mixture with modification of the first procedure by using 0.1 μl of the amplified fragment as a template, 0.025 m*M* dCTP in the presence of 2.5 μCi of [α-^{32}P]dCTP, and 6 cycles for amplification. Samples were then boiled for 5 min after addition of 180 μl of loading buffer (95% formamide, 20 m*M* EDTA, 0.05% xylene cyanol, 0.05% bromophenol blue), and cooled on ice. Two microliter aliquots of each sample were loaded onto 6% polyacrylamide gels of two different compositions (with or without 10% glycerol). The gel electrophoresis was performed at 15 W in 0.5× Tris-borate-EDTA buffer at 20°C. After drying, the gels were exposed to X-ray film.

Sequencing analysis Shifted bands were dissected from the gel and DNA was extracted by boiling with 100 μl of distilled water. DNA fragments were then reamplified by PCR with the same pair of primers as used for SSCP, and were subcloned into pBluescript SK(+) (Stratagene, La Jolla, CA). Multiple subclones were sequenced with an ABI Prizm™ 377 DNA sequencer (Perkin Elmer) in each case.

RESULTS

Fe-NTA-induced RCCs Tumor incidence and incubation period in male Wistar rats after Fe-NTA administration have been separately reported.[20] Thirty-four Fe-NTA-induced primary RCCs of appropriate size (≥10 mm) were selected, and classified into grades 1 to 3 for nuclear atypia. Typical histological appearances of grades 1 to 3 RCCs are shown in Fig. 1. RCCs with histology of human clear cell subtype are rare in the Fe-NTA-induced rat renal carcinogenesis model. There was no RCC of pure clear cell subtype, but 6 RCCs were of mixed granular and clear cell subtype. RCCs of each grade were analyzed microscopically as well as macroscopically for the presence of metastasis or invasion. These two factors were closely associated (correlation coefficient $r=0.999$, $P<0.0001$; Table I). All the cupric NTA-induced RCCs available for the present study were of grade 3, as previously reported.[20]

Analysis of *Tsc2* gene Only one Fe-NTA-induced RCC showed a band shift in the PCR-SSCP analyses. Two wild-type bands were replaced by a single shifted band in exon 10 (Fig. 2A). Multiple sequence analyses of the shifted band revealed only one clone with two missense transition mutations: codon 334, GTG (Val) to GCG (Ala), and codon 336, TAT (Tyr) to CAT (His) (Fig. 2, B and C). This result was therefore interpreted as reflecting point mutations with loss of heterozygosity (LOH). This RCC (case 10-13-1) was 60 mm in diameter and presented a histology of papillotubular structure pattern, finely reticular cytoplasm with distinct cell boundary,

Table I. Invasion/metastasis and Grade of RCCs Induced by Fe-NTA

Carcinogen	Nuclear grade	Numbelr of cases	Cases of invasion/metastasis
Fe-NTA	Grade 1	8	0
	Grade 2	16	6 (37.5 %)
	Grade 3	10	8 (80.0 %)

Refer to Fig. 1 for the nuclear grade.

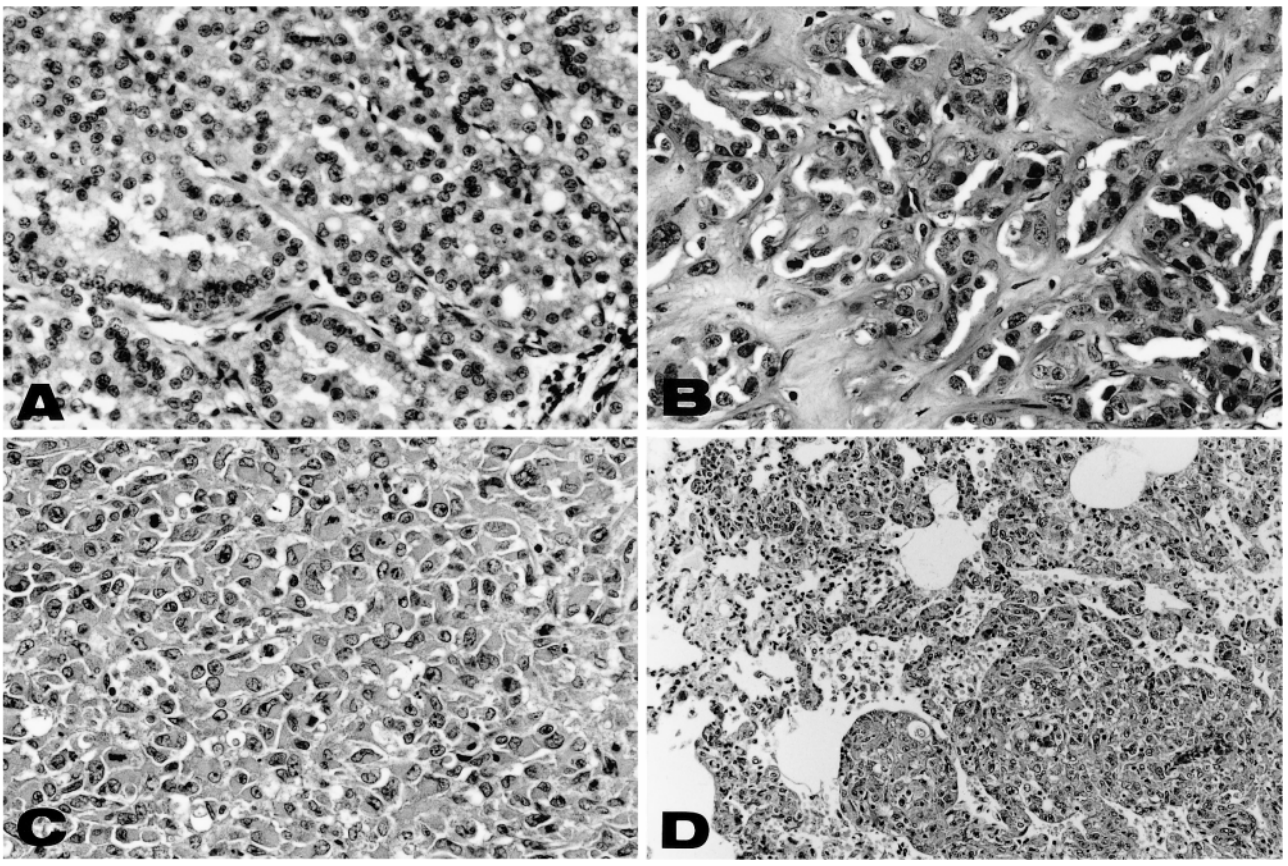

Fig. 1. Histology of nuclear grades 1–3 renal cell carcinoma induced by Fe-NTA. A, grade 1; B, grade 2; C, grade 3; D, pulmonary metastasis of grade 3 renal cell carcinoma. HE, ×257 (A–C), ×129 (D).

grade 1 nuclear atypia and low infiltrating activity (INF α) (Fig. 3, A and B). The same kind of histology was not observed in any other RCC analyzed in the present experiment.

Analysis of *VHL* gene All the sample DNAs showed a normal wild-type pattern in PCR-SSCP analyses (data not shown). To confirm the absence of mutations in the *VHL* gene in the tumors, 10 randomly selected Fe-NTA-induced primary RCCs were sequenced for the 3 coding exons, but no alterations were found. The results indicate that there is little possibility of mutation in the *VHL* gene in Fe-NTA-induced primary and metastatic RCCs, or in the other tumors studied.

DISCUSSION

RCCs comprise approximately 2% of all the malignant neoplasms in human adults. Human RCCs have high metastatic potential so that metastasis may be the presenting manifestation of an unsuspected renal primary in approximately 10–25% of RCC patients.[24] First, we investigated the incidence of invasion or metastasis in Fe-NTA-induced rat RCCs. The results showed that 41% of the induced RCCs (≥10 mm in diameter) exhibited either invasion or metastasis, and further that the incidence of invasion or metastasis was proportionally associated with the histological nuclear grade. As far as we know, this is the only rodent model of chemical renal carcinogenesis that shows a high incidence of invasion and metastasis. In this sense, Fe-NTA can induce high-grade RCCs in rats.

In the present study, SSCP analyses were used for the detection of genomic alterations since hot spots have not yet been observed in the *Tsc2* gene, which consists of 42 exons.[23] We have tried to raise the sensitivity of SSCP by changing the gel composition. There was only one Fe-NTA-induced RCC that contained two point mutations in exon 10 with LOH. This is so far the second report of *Tsc2* somatic mutation in chemical renal carcinogenesis of non-Eker rat, after a recent report on somatic mutation of *Tsc2* gene in a rat RCC cell line.[25] We believe that our

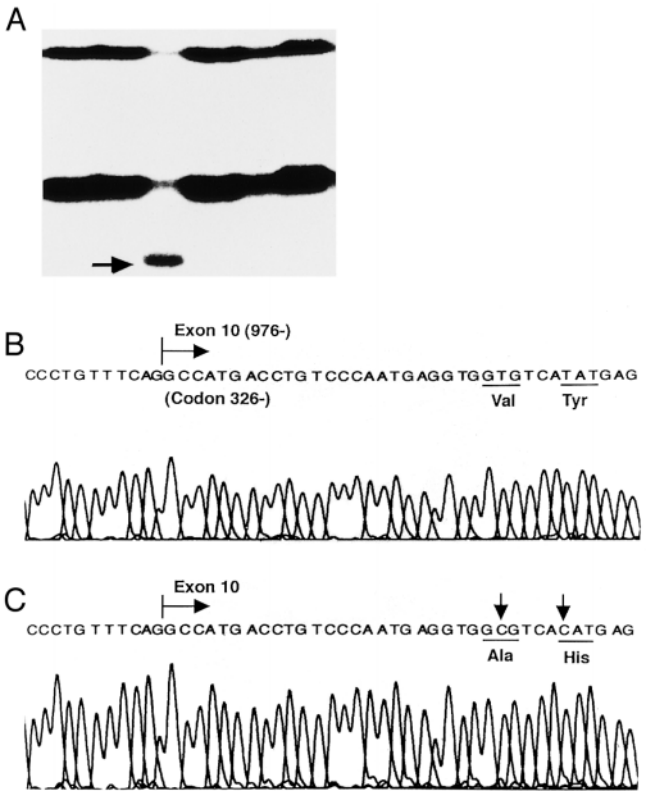

Fig. 2. Mutation analysis of *Tsc2* in Fe-NTA-induced renal cell carcinoma. A. SSCP analysis of exon 10. Arrow shows a shifted extra band with LOH (case 10-13-1). B. Sequence of exon 10 from DNA of normal kidney. C. Sequence of exon 10 from DNA of RCC (case 10-13-1). Two missense mutations are observed.

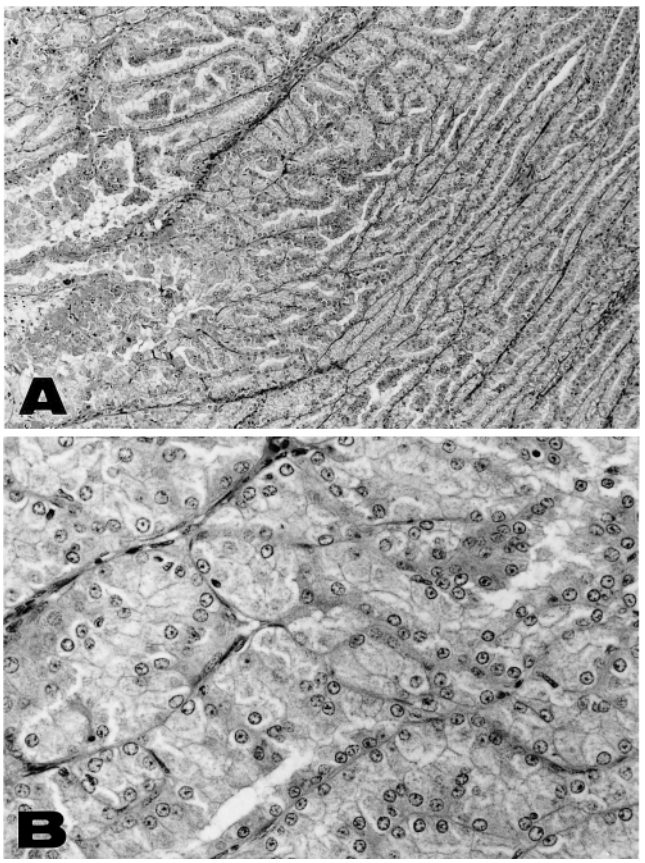

Fig. 3. Histology of case 10-13-1. A. Low magnification. Note papillotubular structure. HE, ×64. B. High magnification. Grade 1 nuclei and finely reticular cytoplasm. ×257.

finding is another example in support of "Knudson's two-hits hypothesis." [26] The function of the *Tsc2* gene product (called tuberin in human) has not been well elucidated, though the protein contains a short region of amino acid sequence homology to *ras* family GTPase-activating proteins (Rap1-GAP) located downstream of the Eker insertion site.[27, 28] Transcriptional activation domains (AD1 and AD2) in the carboxyl terminus of the Tsc2 product were recently identified, and the Eker insertional mutation disrupts their transcriptional activities.[29] At present, it is difficult to predict what effect the two transition mutations might have. However, regarding amino acid sequence homology, codons 334 and 336 are conserved in human and mice.[27, 30, 31] In addition, at least missense mutation of codon 336 is a non-conservative substitution which may induce either blockage of protein folding or loss of a phosphorylation site, or may somehow alter the stability of the protein, leading to susceptibility to proteolysis or posttranslational modification. Further analysis of tuberin in the RCC (case 10-13-1) has been hindered by the absence of appropriate antibodies that recognize each functional domain of this large-molecular-weight protein.

The biological character of the RCC with *Tsc2* mutation was low-grade (Fig. 3), and metastasis was not observed. This is consistent with the biological behavior and histological appearance of hereditary RCCs of Eker rats, in that RCCs are characterized by abundant eosinophilic cytoplasm and rare metastasis.[32, 33] RCCs in Eker rats are thus rarely fatal, so that additional tumors such as pituitary adenoma, sarcoma of spleen or uterus are observed.[32, 33] In contrast, a large proportion of Fe-NTA-induced RCCs was high-grade (Table I).

There was no mutation in the *VHL* tumor suppressor gene in any of the tumors in the present study. This is consistent with studies by other investigators on hereditary or non-hereditary RCCs of rats.[22, 25, 34] Since mutation of the *VHL* gene is associated with human RCCs of the clear cell-subtype,[14–16] a difference of cell origin in RCCs might be the cause of the failure to detect any somatic mutation in the *VHL* gene.

The present results suggest that neither *Tsc2* nor *VHL* is the main target gene in this renal carcinogenesis model. This result is also consistent with our recent study of the detection of LOH using F1 hybrid rats that revealed no preference for chromosome 4 (*VHL* locus)[22] or chromosome 10 (*Tsc2* locus)[17] as candidates for the responsible tumor suppressor gene(s) in the Fe-NTA-induced renal carcinogenesis model (unpublished data).

The Fe-NTA-induced renal carcinogenesis model is distinct in that involvement of reactive oxygen species in the carcinogenic process is highly likely.[4, 5] The mutations observed were two T-to-C transitions. This might be explained by the increase in thymine glycol content in DNA at the acute phase after Fe-NTA administration,[6] since thymine glycol may induce T-to-C transition mutation at DNA replication.[35] How exposure to reactive oxygen species affects cell proliferation and leads to cancer is another intriguing issue associated with this renal carcinogenesis model.

In humans, there have been few studies on the gene alterations in non-clear cell subtype RCCs. Reportedly, *ras* genes, *p53* gene and *VHL* gene are not responsible for RCCs of this subtype.[15, 36] The Fe-NTA-induced rat renal carcinogenesis model offers a good opportunity to find new gene(s) responsible for high-grade non-clear cell subtype RCCs.

ACKNOWLEDGMENTS

This work was supported in part by a Grant-in-Aid from the Japanese Ministry of Education, Science, Sports and Culture, a grant from the Program for Promotion of Basic Research Activities for Innovative Bioscience (PROBRAIN), and a grant from the Japanese Owners' Association. We thank Ms. Noriko Shibata (Department of Pathology and Biology of Diseases, Graduate School of Medicine, Kyoto University) for technical assistance, and Dr. James E. Strickland (NCI, NIH, Bethesda, MD) for a critical reading of the manuscript.

(Received May 6, 1998/Revised June 10, 1998/Accepted June 12, 1998)

REFERENCES

1) Anderson, R. L., Bishop, W. E. and Campbell, R. L. A review of the environmental and mammalian toxicology of nitrilotriacetic acid. *Crit. Rev. Toxicol.*, **15**, 1–102 (1985).
2) Ebina, Y., Okada, S., Hamazaki, S., Ogino, F., Li, J.-L. and Midorikawa, O. Nephrotoxicity and renal cell carcinoma after use of iron- and aluminum-nitrilotriacetate complexes in rats. *J. Natl. Cancer Inst.*, **76**, 107–113 (1986).
3) Li, J.-L., Okada, S., Hamazaki, S., Ebina, Y. and Midorikawa, O. Subacute nephrotoxicity and induction of renal cell carcinoma in mice treated with ferric nitrilotriacetate. *Cancer Res.*, **47**, 1867–1869 (1987).
4) Toyokuni, S. Iron-induced carcinogenesis: the role of redox regulation. *Free Radic. Biol. Med.*, **20**, 553–566 (1996).
5) Okada, S. Iron-induced tissue damage and cancer: the role of reactive oxygen free radicals. *Pathol. Int.*, **46**, 311–332 (1996).
6) Toyokuni, S., Mori, T. and Dizdaroglu, M. DNA base modifications in renal chromatin of Wistar rats treated with a renal carcinogen, ferric nitrilotriacetate. *Int. J. Cancer*, **57**, 123–128 (1994).
7) Toyokuni, S., Mori, T., Hiai, H. and Dizdaroglu, M. Treat-

ment of Wistar rats with a renal carcinogen, ferric nitrilotriacetate, causes DNA-protein cross-linking between thymine and tyrosine in their renal chromatin. *Int. J. Cancer*, **62**, 309–313 (1995).

8) Toyokuni, S., Okada, S., Hamazaki, S., Minamiyama, Y., Yamada, Y., Liang, P., Fukunaga, Y. and Midorikawa, O. Combined histochemical and biochemical analysis of sex hormone dependence of ferric nitrilotriacetate-induced renal lipid peroxidation in ddY mice. *Cancer Res.*, **50**, 5574–5580 (1990).
9) Toyokuni, S., Uchida, K., Okamoto, K., Hattori-Nakakuki, Y., Hiai, H. and Stadtman, E. R. Formation of 4-hydroxy-2-nonenal-modified proteins in the renal proximal tubules of rats treated with a renal carcinogen, ferric nitrilotriacetate. *Proc. Natl. Acad. Sci. USA*, **91**, 2616–2620 (1994).
10) Toyokuni, S., Luo, X.-P., Tanaka, T., Uchida, K., Hiai, H. and Lehotay, D. C. Induction of a wide range of C_{2-12} aldehydes and C_{7-12} acyloins in the kidney of Wistar rats after treatment with a renal carcinogen, ferric nitrilotriacetate. *Free Radic. Biol. Med.*, **22**, 1019–1027 (1997).
11) Uchida, K., Fukuda, A., Kawakishi, S., Hiai, H. and Toyokuni, S. A renal carcinogen ferric nitrilotriacetate mediates a temporary accumulation of aldehyde-modified proteins within cytosolic compartment of rat kidney. *Arch. Biochem. Biophys.*, **317**, 405–411 (1995).
12) Nishiyama, Y., Suwa, H., Okamoto, K., Fukumoto, M., Hiai, H. and Toyokuni, S. 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. *Jpn. J. Cancer Res.*, **86**, 1150–1158 (1995).
13) Latif, F., Tory, K., Gnarra, J., Yao, M., Duh, F. M., Orcutt, M. L., Stackhouse, T., Kuzmin, I., Modi, W., Geil, L., Schmidt, L., Zhou, F., Li, H., Wei, M. H., Chen, F., Glenn, G., Choyke, P., Walther, M. M., Weng, Y., Duan, D. R., Dean, M., Glavac, D., Richards, F. M., Crossey, P. A., Ferguson-Smith, M. A., Linehan, W. M., Zbar, B. and Lehman, M. I. Identification of the von Hippel-Lindau disease tumor suppressor gene. *Science*, **260**, 1317–1320 (1993).
14) Gnarra, J. R., Tory, K., Weng, Y., Schmidt, L., Wei, M. H., Li, H., Latif, F., Liu, S., Chen, F., Duh, F. M., Lubensky, I., Duan, D. R., Florence, C., Pozzatia, R., Walther, M. M., Bander, M. H., Grossman, H. B., Bauch, H., Brooks, J. D., Isaacs, W. B., Lerman, M. I., Zbar, B. and Linehan, W. M. Mutations of the VHL tumour suppressor gene in renal carcinoma. *Nat. Genet.*, **7**, 85–90 (1994).
15) Shuin, T., Kondo, K., Torigoe, S., Kishida, T., Kubota, Y., Hosaka, M., Nagashima, Y., Kitamura, H., Latif, F., Zbar, B., Lerman, M. I. and Yao, M. Frequent somatic mutations and loss of heterozygosity of the von Hippel-Lindau tumor suppressor gene in primary human renal cell carcinomas. *Cancer Res.*, **54**, 2852–2855 (1994).
16) Whaley, J. M., Naglich, J., Gelbert, L., Hsia, Y. E., Lamiell, J. M., Green, J. S., Collins, D., Neumann, H. P., Laidlaw, J., Li, F. P., Klein-Szanto, A. J. P., Seizinger, B. R. and Kley, N. Germ-line mutations in the von Hippel-Lindau tumor-suppressor gene are similar to somatic von Hippel-Lindau aberrations in sporadic renal cell carcinoma. *Am. J. Hum. Genet.*, **55**, 1092–1102 (1994).
17) Kobayashi, T., Hirayama, Y., Kobayashi, E., Kubo, Y. and Hino, O. A germline insertion in the tuberous sclerosis (Tsc2) gene gives rise to the Eker rat model of dominantly inherited cancer. *Nat. Genet.*, **9**, 70–74 (1995).
18) Yeung, R. S., Xiao, G. H., Jin, F., Lee, W. C., Testa, J. R. and Knudson, A. G. Predisposition to renal carcinoma in the Eker rat is determined by germ-line mutation of the tuberous sclerosis 2 (TSC2) gene. *Proc. Natl. Acad. Sci. USA*, **91**, 11413–11416 (1994).
19) Osaka, M., Matsuo, S., Koh, T. and Sugiyama, T. Specific N-ras mutation in bone marrow within 48 h of 7,12-dimethylbenz[a]anthracene treatment in Huggins-Sugiyama rat leukemogenesis. *Mol. Carcinog.*, **16**, 126–131 (1996).
20) Toyokuni, S., Tanaka, T., Nishiyama, Y., Okamoto, K., Nakashima, Y., Hamazaki, S., Okada, S. and Hiai, H. Induction of renal cell carcinoma in male Wistar rats treated with cupric nitrilotriacetate. *Lab. Invest.*, **75**, 239–248 (1996).
21) Japanese Urological Association, the Japanese Society of Pathology, and Japanese Radiological Society. "General Rule for Clinical and Pathological Studies on Renal Cell Carcinoma" (1996). Kanehara Publication Co., Ltd., Tokyo.
22) Kikuchi, Y., Kobayashi, E., Nishizawa, M., Hamazaki, S., Okada, S. and Hino, O. Cloning of the rat homologue of the von Hippel-Lindau tumor suppressor gene and its non-somatic mutation in rat renal cell carcinomas. *Jpn. J. Cancer Res.*, **86**, 905–909 (1995).
23) Kobayashi, T., Urakami, S., Hirayama, Y., Yamamoto, T., Nishizawa, M., Takahara, T., Kubo, Y. and Hino, O. Intragenic *Tsc2* somatic mutations at Knudson's second hit in spontaneous and chemically induced renal carcinomas in the Eker rat model. *Jpn. J. Cancer Res.*, **88**, 254–261 (1997).
24) Nasdy, T., Bane, B. L. and Silva, F. G. Adult renal diseases. *In* " Diagnostic Surgical Pathology," ed. S. S. Sternberg, pp. 1711–1739 (1994). Raven Press, New York.
25) Urakami, S., Tokuzen, R., Tsuda, H., Igawa, M. and Hino, O. Somatic mutation of the tuberous sclerosis (Tsc2) tumor suppressor gene in chemically induced rat renal carcinoma cell. *J. Urol.*, **158**, 275–278 (1997).
26) Knudson, A. G., Jr., Hethcote, H. W. and Brown, B. W. Mutation and childhood cancer: a probabilistic model for the incidence of retinoblastoma. *Proc. Natl. Acad. Sci. USA*, **72**, 5116–5120 (1975).
27) Kobayashi, T., Nishizawa, M., Hirayama, Y., Kobayashi, E. and Hino, O. cDNA structure, alternative splicing and exon-intron organization of the predisposing tuberous sclerosis (Tsc2) gene of the Eker rat model. *Nucleic Acids Res.*, **23**, 2608–2613 (1995).
28) Wienecke, R., Konig, A. and DeClue, J. E. Identification

of tuberin, the tuberous sclerosis-2 product. Tuberin possesses specific Rap1GAP activity. *J. Biol. Chem.*, **270**, 16409–16414 (1995).
29) Tsuchiya, H., Orimoto, K., Kobayashi, K. and Hino, O. Presence of potent transcriptional activation domains in the predisposing tuberous sclerosis (Tsc2) gene product of the Eker rat model. *Cancer Res.*, **56**, 429–433 (1996).
30) The European Chromosome 16 Tuberous Sclerosis Consortium. Identification and characterization of the tuberous sclerosis gene on chromosome 16. *Cell*, **75**, 1305–1315 (1993).
31) Kim, K. K., Pajak, L., Wang, H. and Field, L. J. Cloning, developmental expression, and evidence for alternative splicing of the murine tuberous sclerosis (TSC2) gene product. *Cell. Mol. Biol. Res.*, **41**, 515–526 (1995).
32) Everitt, J. I., Goldsworthy, T. L., Wolf, D. C. and Walker, C. L. Hereditary renal cell carcinoma in the Eker rat: a rodent familial cancer syndrome. *J. Urol.*, **148**, 1932–1936 (1992).
33) Hino, O., Mitani, H., Katsuyama, H. and Kubo, Y. A novel cancer predisposition syndrome in the Eker rat model. *Cancer Lett.*, **83**, 117–121 (1994).
34) Walker, C., Ahn, Y. T., Everitt, J. and Yuan, X. Renal cell carcinoma development in the rat independent of alterations at the VHL gene locus. *Mol. Carcinog.*, **15**, 154–161 (1996).
35) Basu, A. K., Loechler, E. L., Leadon, S. A. and Essigmann, J. M. Genetic effets of thymine glycol: site-specific mutagenesis and molecular modeling studies. *Proc. Natl. Acad. Sci. USA*, **86**, 7677–7681 (1989).
36) Uchida, T., Wada, C., Wang, C., Egawa, S., Ohtani, H. and Koshiba, K. Genomic instability of microsatellite repeats and mutations of H-, K-, and N-ras, and p53 genes in renal cell carcinoma. *Cancer Res.*, **54**, 3682–3685 (1994).