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Cloning of the Rat Homologue of the von Hippel-Lindau Tumor Suppressor Gene and Its Non-somatic Mutation in Rat Renal Cell Carcinomas

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Recently, von Hippel-Lindau (*VHL*) gene mutations were detected in non-inherited, sporadic human renal cell carcinomas (RCs) at a high frequency. In order to determine whether or not the *VHL* gene is also a critical gene in rat RCs, we cloned and sequenced the rat homologue of human *VHL* gene and searched for mutations of the *VHL* gene in rat RCs. Mutations in the *VHL* gene were not detected in spontaneous RCs of the Eker rat model or in ferric nitrilotriacetate-induced rat RCs using the polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) method. These data indicate that mutation of the *VHL* tumor suppressor gene is not an event in rat renal carcinogenesis, at least in our present systems.

Key words: *VHL* gene — Tumor suppressor gene — Eker rat model — PCR-SSCP — Renal carcinogenesis

von Hippel-Lindau (VHL) disease, a rare autosomal dominant disorder, is associated with multiple lesions, including central nervous system hemangioblastomas, pheochromocytomas and renal cell carcinomas (RCs). Recently, the predisposing *VHL* gene, located on human chromosome 3p25-26, was isolated by positional cloning.[1] Mutation analyses suggested that this gene is involved in development of not only the VHL-related RCs, but also sporadic non-papillary RCs.[2] Thus, the *VHL* gene appears to play a key role in human renal carcinogenesis.

On the other hand, rat models have been utilized to analyze spontaneous and chemically induced RCs in greater detail and have proved to be excellent tools for studying the sequence of cellular changes during renal carcinogenesis.[3–5] Therefore, in this study, we cloned the rat homologue *VHL* gene and determined the organization of the entire rat VHL cDNA and genomic DNA in order to analyze intragenic mutations in spontaneous and chemically-induced rat RCs using the polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) method. In particular, using the Eker rat model of dominantly inherited RC carrying a germline mutation of *Tsc2* gene,[6] we examined whether or not the *VHL* gene is an additional genetic target during RC development and/or progression, because rat chromosome 4, where the rat *VHL* gene is located [our unpublished data obtained by using hybrid panels and cytogenetic data[7]], is frequently changed in Eker rat RC cells.[8]

A rat kidney cDNA (λZAP II vector) derived from an adult male (Long Evans strain, Kiwa Breeding Laboratory)[9] was screened with a human VHL cDNA clone g7 (gift of Dr. B. Zbar, National Cancer Institute).[1] Hybridization and washing were carried out as described earlier.[6] A rat genomic cosmid library (provided by Dr. T. Oda, Hamamatsu University School of Medicine)[10] was also screened with g7.

Ten RCs having Eker mutation from F1 hybrids (Eker rat × Long Evans strain, Kiwa) were used for DNA extraction; LK8A [male, 89 weeks (w)], LK8B (male, 104 w), LK20b (female, 91 w), LK17B (male, 103 w), LK18D (male, 101 w), LK19D (male, 97 w), LK12g (female, 97 w), LK19b (female, 99 w), LK15B (male, 95 w), and LK15e (female, 95 w). To obtain chemically induced rat RCs, male Wistar rats (6–7 weeks old) (Shizuoka Laboratory Animal Center) were given 10 mg Fe/kg body weight of ferric nitrilotriacetate (Fe-NTA) twice a week for 3 months and killed 9 months after the completion of the last injection.[11] A portion of the renal tumors was dispersed with collagenase, and cells were maintained in Iscovez's modified Dulbecco's medium with 20% fetal bovine serum. The cells that proliferated were polygonal epithelioid cells. The DNAs were extracted from a frozen primary tumor (T1), and two cultured RCs (T2 and T3) at second passage. Histologically, Eker rat spontaneous and Fe-NTA induced RCs were both of non-papillary, granular type and were basically the same, in spite of their different etiology.[11,12]

The nucleotide sequences of recombinant clones were determined by the dideoxy chain termination method

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12A	5'-AACGAGCGTCCGGTTCCAAT-3'	204bp
11B	5'-GCTGAGGCTCACCATCAAAG-3'	
14A	5'-GCGTCGTGCTGCCTTTGTGG-3'	178bp
14B	5'-TGAGTAGGGACCTGGTGCTC-3'	
21A	5'-ACTGCTGTTGCCTTGCTCAG-3'	302bp
21B	5'-TCCTCAGCCCCAAGGTCTTA-3'	
31A	5'-TGACTGGAGCCTGCCTCAGA-3'	284bp
31B	5'-TCAATTTCAGACCATCAAGG-3	

Fig. 1. The primers that were used in PCR-SSCP analysis. (A) and (B) indicate forward and reverse primers, respectively; the primer sets were 12A and 11B, 14A and 14B, 21A and 21B, and 31A and 31B. The right column shows the size of PCR products.

using a SequiTherm Sequencing kit (Epicentre Technologies).

Primers were designed using genomic DNA sequence data (Fig. 1) and made with a DNA synthesizer (Milligen/Biosearch, Division of Millipore). All PCR reactions were carried out with Tth DNA polymerase (Pharmacia-Biotech) in a volume of 25 μl containing 100 ng of the template DNA, 25 pmol of each primer, 200 μM dNTPs, 1.5 mM $MgCl_2$, 0.185 MBq of [α-^{32}P]dCTP (>3000 Ci/mmol, Amersham), and the supplied buffer. Reactions were carried out using a QTII or QTPI thermal cycler (Nippon Genetics) as follows; at 92°C for 3 min, followed by 35 cycles of 92°C for 1 min, 58°C (exon 1) or 55°C (exon 2 and 3) for 1 min, and 72°C for 2 min, terminated with a final extension at 72°C for 5 min. Following PCR, the samples were diluted fifty-fold using dilution buffer containing 95% formamide, 20 mM EDTA, 0.1% BPB and 0.1% XC. Diluted samples were denatured for 5 min by boiling, then 1 μl was taken and run on 6% polyacrylamide gels containing 10% glycerol and 1×TBE buffer in an AE-6160 Genoquencer SSCP

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     CGTAAACCCGCACGTCGCGCCGCCCACGTCCAGCTCGCGAACGAGCGTCCGGTTCCAATA

     ATGCCCCGGAAGGCAGCTAGTCCAGAGGAGGCAGAAAGGATGCCGGGCTCTGAAGAGATA
  1  MetProArgLysAlaAlaSerProGluGluAlaGluArgMetProGlySerGluGluIle   20

     GAGGCTGGGCGGCCGCGGCCGGTTTTACGCTCTGTGAACTCGCGCGAACCCTCTCAGGTC
 21  GluAlaGlyArgProArgProValLeuArgSerValAsnSerArgGluProSerGlnVal   40

     ATCTTCTGCAACCGCAGCCCGCGCGTCGTGCTGCCTTTGTGGCTCAACTTTGATGGTGAG
 41  IlePheCysAsnArgSerProArgValValLeuProLeuTrpLeuAsnPheAspGlyGlu   60

     CCTCAGCCCTACCCGACCTTACCACCGGGCACCGGCCGCCGCATCCACAGCTACCGAGGT
 61  ProGlnProTyrProThrLeuProProGlyThrGlyArgArgIleHisSerTyrArgGly   80

     CACCTTTGGCTCTTCAGGGATGCGGGGACCCATGATGGACTTCTGGTTAACCAAACGGAA
 81  HisLeuTrpLeuPheArgAspAlaGlyThrHisAspGlyLeuLeuValAsnGlnThrGlu  100

     CTGTTTGTGCCATCCCTCAATGTTGATGGACAGCCTATTTTTGCCAACATCACATTGCCA
101  LeuPheValProSerLeuAsnValAspGlyGlnProIlePheAlaAsnIleThrLeuPro  120

     GTGTATACCCTGAAAGAGCGGTGCCTTCAGGTTGTACGGAGCCTGGTCAAGCCTGAGAAC
121  ValTyrThrLeuLysGluArgCysLeuGlnValValArgSerLeuValLysProGluAsn  140

     TACAGGAGGCTGGACATCGTCAGGTCGCTCTATGAAGACTTGGAAGACCACCCAAATGTG
141  TyrArgArgLeuAspIleValArgSerLeuTyrGluAspLeuGluAspHisProAsnVal  160

     CGGAAAGACATACAGCGGCTGACCCAAGAGCACCTCGAGAATCAGGCCCTGGGAGAGGAG
161  ArgLysAspIleGlnArgLeuThrGlnGluHisLeuGluAsnGlnAlaLeuGlyGluGlu  180

     CCTGAAGGAGTCCACTGAGATTACTGGTCCTGAATTTCGGCCCTTGATGGTCTGAAATTG
181  ProGluGlyValHis  *                                            185
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Fig. 2. The nucleotide and deduced amino acid sequences of *rVHL1* coding region. The nucleotide numbers start from the 1st AUG codon in the VHL mRNA.[17] * indicates stop codon.

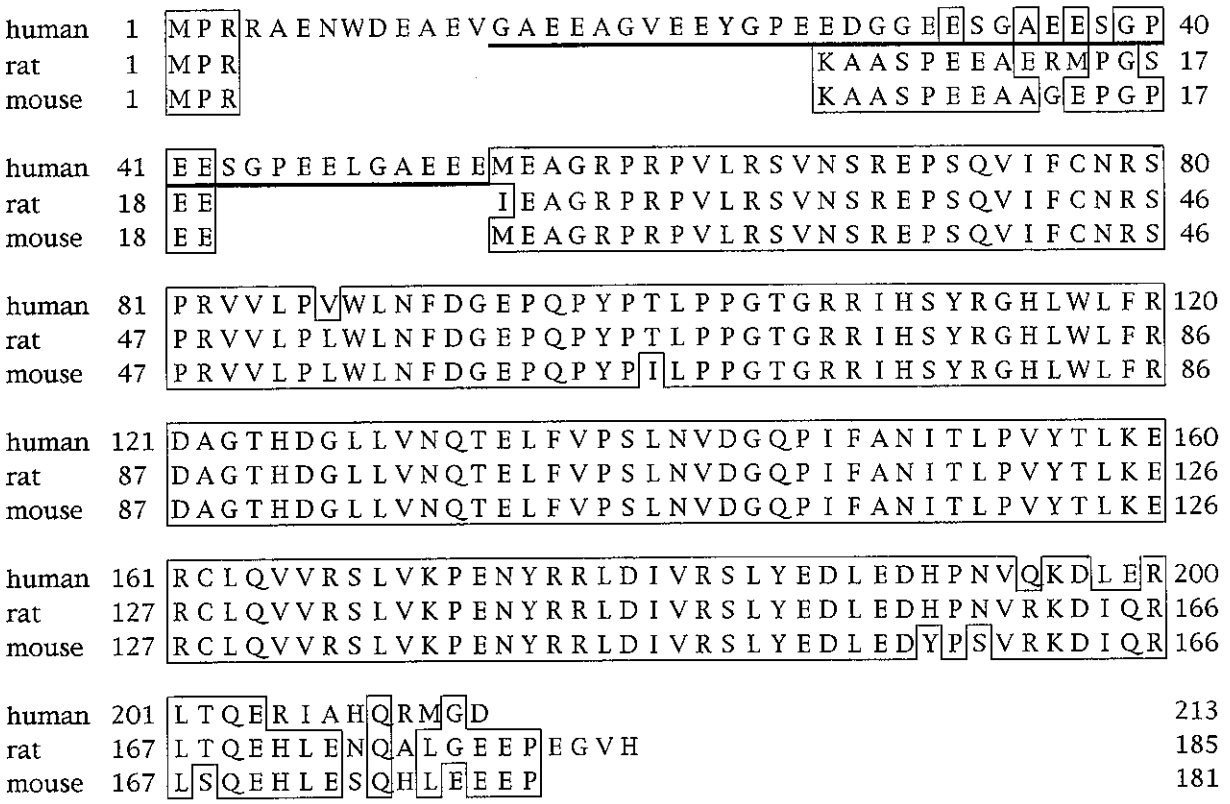


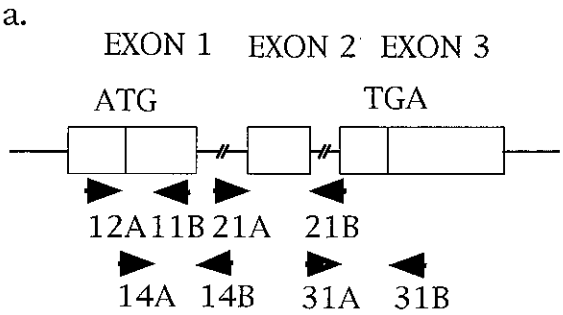
Fig. 3. A comparison of the predicted amino acid sequences of human, rat and mouse *VHL* gene products. The boxed areas show the homologies. Underlining indicates the pentamer repeat sequences in the case of human *VHL*.

(Atto Corp.) at 40 W for 6 h. The gels were dried and exposed to X-ray films overnight.

Two cDNA clones (2.5 kb and 2.9 kb in length) were isolated in plaque hybridization using human VHL g7 clone as a probe. The 5′ end of the 2.5 kb cDNA clone was sequenced using the primer in the plasmid. Then, sequencing-specific primers were designed. The positive genomic DNA clones were digested completely with *Eco*RI and subcloned into pHGS298. Two positive subclones were isolated, with lengths of 4.0 kb and 2.5 kb, respectively. The 2.9 kb cDNA and two genomic DNA subclones were sequenced using the sequencing-specific primers (exon 1 and exon 2/3 were determined from 4.0 kb subclone and 2.5 kb subclone, respectively).

The entire nucleotide and deduced amino acid sequences of the cDNA, named rat VHL (*rVHL1*), are shown in Fig. 2. The cDNA has an ORF corresponding to 185 amino acids. A comparison of the amino acid sequence among the predicted human,[1] mouse[13] and rat gene products is shown in Fig. 3.[13] The *rVHL1* cDNA shares a high homology with the mouse VHL cDNA: 92.1% (500/543) sequence identity at the nucleotide level and 93.4% (169/181) at the amino acid level. Interestingly, rat and mouse *VHL* gene products lacked the eight copies of an acidic tandemly repeated pentamer Gly-X-Glu-Glu-X, which was seen in the human case and which resembles a pentamer (Gly-Pro-Glu-Glu-Pro) in the procyclic surface membrane protein of *Trypanosoma brucei*.[1,2] In the region after codon 20, the *rVHL1* cDNA shares high homology with the human VHL cDNA: 82.3% (395/480) sequence identity at the nucleotide level and 91.9% (147/160) identity at the amino acid level. In VHL-related and human sporadic RCs, nucleotide substitutions, deletions, insertions, and nonsense mutations were detected in this counterpart of human *VHL* gene.[2] Four codons (182, 183, 184 and 185) were not reported in human or mouse *VHL* gene (Fig. 2). A schematic organization of the *rVHL1* gene structure including the sequences at the intron-exon boundaries, and the positions of PCR-SSCP primers are shown in Fig. 4.

All RC DNAs were tested using four overlapping sets of PCR products; RVSS (rVHL primers for SSCP analysis) 12A and 11B, 14A and 14B, 21A and 21B, 31A and 31B. These four products covered the entire coding region of rat *VHL* gene, including splicing donor and



b.

AGCTACCGAGgtacgagacgcgcgctaggc--
--intron 1--
--tcctctgtttccattgacagGTCACCTTTG

ACATTGCCAGgttgatagtcaatcttacat--
--intron 2--
--ttggtttcctacctttgcagTGTATACCCT

Fig. 4. The structure of the *rVHL1* gene (a) and nucleotide sequences at exon-intron junctions (b). Capital letters; exon sequences, lower-case letters; intron sequences.

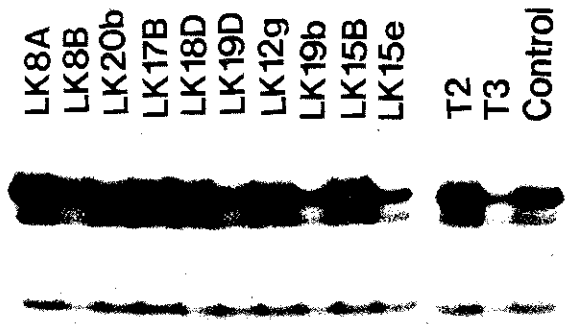


Fig. 5. Representative PCR-SSCP analysis. The primer set was 14A and 14B. There were no band shifts from the normal control.

acceptor sites. All RC DNAs showed the normal wild-type pattern (Fig. 5). The results indicate that there were non-somatic mutations of the *VHL* gene in 10 spontaneous and 3 chemically induced RCs derived from 10 Eker rats and 3 Fe-NTA-treated Wistar rats, respectively.

Although the function of human *VHL* gene is not yet understood, the homology between human and mouse/rat *VHL* genes suggest that the *VHL* gene might have a significant role in rodents, too. In this study, however, mutations in *rVHL1* gene were not detected by the PCR-SSCP method or by conventional Southern blot analysis (data not shown). Thus, *rVHL1* gene mutation does not appear to be a high frequency event in the development of rat RCs. At the present time, we do not know the functional role of the pentamer repeats present in the human *VHL* gene, but not in rat or mouse *VHL* gene.

The human RCs have been classified into papillary, tubular or solid growth of clear or granular cells, or a mixture of these. Recently, a new classification of human RCs based on the genetic alterations has been proposed.[14] The common type is the non-papillary, clear cell RCs which are characterized by the loss of human chromosome 3p, where human *VHL* gene is located. The various types of RCs apparently originate from different segments of this system (e.g., from the proximal tubule and the collecting duct system). It is evident from several studies in rats that the clear cell RCs are similar to the predominant cell types of human RCs that arise from the collecting duct system,[3] while human clear cell type RCs originate from the proximal tubule.[15] This is an intriguing discrepancy between human and rat clear cell type RCs. Atypical cysts lined by clear cells and associated with RCs showing a similar clear cell phenotype have been described in some VHL disease or in tuberous sclerosis patients.[16] As mentioned above, the Eker rat is an animal model of tuberous sclerosis (*Tsc2* gene) and its RCs are granular rather than clear cell type.[12] It has been proposed that diversity in the cellular phenotypes of RCs does not indicate an origin from a special segment of the tubular system, but might reflect an abnormal gene expression associated with neoplastic changes of cell clones, or result from options for differentiation of a common precursor stem cell.[3]

Our present findings imply a role of different genetic events including *Tsc2* gene mutations in human and rat RCs, which are not related to the *VHL* gene.

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REFERENCES

1) Latif, F. L., 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.-S. R., Dean, M., Glavac, D., Richards, F. M., Crossey, P. A., Ferguson-Smith, M. A., Paslier, D. L., Chumakov, I., Cohen, D., Chinault, A. C., Maher, E. R., Linehan, W. M., Zbar, B. and Lerman, M. I. Identification of the von Hippel-Lindau disease tumor suppressor gene. *Science,* **260,** 1317–1320 (1993).
2) Linehan, W. M., Lerman, M. I. and Zbar, B. Identification of the von Hippel-Lindau (VHL) gene. *J. Am. Med. Assoc.,* **273,** 564–570 (1995).
3) Bannasch, B. and Zerban, H. Animal models and renal carcinogenesis. *In* "Tumors and Tumor-like Conditions of the Kidneys and Ureters," ed. J. N. Ebel, pp. 1–34 (1990). Churchill Livingstone Inc., New York.
4) Hino, O., Kobayashi, E., Hirayama, Y., Kobayashi, T., Kubo, Y., Tsuchiya, H., Kikuchi, Y. and Mitani, H. Molecular genetic basis of renal carcinogenesis in the Eker rat model of tuberous sclerosis (TSC2). *Mol. Carcinog.,* in press.
5) Hino, O., Kobayashi, E., Nishizawa, M., Kubo, Y., Kobayashi, T., Hirayama, Y., Takai, S., Kikuchi, Y., Tsuchiya, H., Orimoto, K., Kajino, K., Takahara, T. and Mitani, H. Renal carcinogenesis in the Eker rat. *J. Cancer Res. Clin. Oncol.,* in press.
6) 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. *Nature Genet.,* **9,** 70–74 (1995).
7) Yeung, R. S., Buetow, K., Testa, J. R. and Knudson, A. G. Susceptibility to renal carcinoma in the Eker rat involves a tumor suppressor gene on chromosome 10. *Proc. Natl. Acad. Sci. USA,* **90,** 8038–8042 (1993).
8) Funaki, K., Everitt, J., Oshimura, M., Freed, J. J., Knudson, A. G. and Walker, C. Hereditary renal cell carcinoma in the rat associated with nonrandom loss of chromosome 5 and 6. *Cancer Res.,* **51,** 4415–4422 (1991).
9) 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).
10) Oda, T., Funai, T. and Ichiyama, A. Generation from a single gene of two mRNAs that encode the mitochondrial and peroxisomal serine: pyruvate aminotransferase of rat liver. *J. Biol. Chem.,* **265,** 7513–7519 (1990).
11) Deguchi, J., Kawabata, T., Kondo, A. and Okada, S. Transforming growth factor-α expression of renal proximal tubules in Wistar rats treated with ferric and aluminum nitrilotriacetate. *Jpn. J. Cancer Res.,* **84,** 649–655 (1993).
12) Hino, O., Klein-Szanto, A. J. P., Freed, J. J., Testa, J. R., Brown, D. Q., Vilensky, M., Yeung, R. S., Tartof, K. D. and Knudson, A. G. Spontaneous and radiation-induced renal tumors in the Eker rat model of dominantly inherited cancer. *Proc. Natl. Acad. Sci. USA,* **90,** 327–331 (1993).
13) Gao, J., Naglich, J. G., Laidlaw, J., Whaley, J. M., Seizinger, B. R. and Kley, N. Cloning and characterization of a mouse gene with homology to the human von Hippel-Lindau disease tumor suppressor gene: implications for the potential organization of the human von Hippel-Lindau disease gene. *Cancer Res.,* **55,** 743–747 (1995).
14) Kovacs, G. Molecular cytogenetics of renal cell tumors. *Adv. Cancer Res.,* **62,** 89–124 (1993).
15) Obering, C., Riviere, M. and Haguenau, F. Ultrastructure of the clear cells in renal carcinomas and its importance for the demonstration of their renal origin. *Nature,* **186,** 402–403 (1960).
16) Ibrahim, R. E., Weinberg, D. S. and Weidner, N. Atypical cysts and carcinomas of the kidneys in the phacomatoses. *Cancer,* **63,** 148–157 (1989).
17) Kuzmin, I., Duh, F.-M., Latif, F., Geil, L., Zbar, B. and Lerman, M. L. Identification of the promoter of the human von Hippel-Lindau disease. *Oncogene,* **10,** 2185–2194 (1995).