

A Novel Gene “*Niban*” Upregulated in Renal Carcinogenesis: Cloning by the cDNA-amplified Fragment Length Polymorphism Approach

Shuichi Majima,^{1,2} Kazunori Kajino,¹ Tomokazu Fukuda,¹ Fujio Otsuka² and Okio Hino^{1,3}

¹Department of Experimental Pathology, Cancer Institute, 1-37-1 Kami-ikebukuro, Toshima-ku, Tokyo 170-8455 and ²Department of Dermatology, Tsukuba University, 1-1-1 Tennoudai, Tsukuba, Ibaraki 305-0005

A modified AFLP (amplified fragment length polymorphism) method was employed to isolate genes differentially expressed in renal carcinogenesis of *Tsc2* gene mutant (Eker) rats. One gene, selected for further investigation, was named “*Niban*” (“second” in Japanese), because it is the second new gene to be found after *Erc* (expressed in renal carcinoma) in our laboratory. Importantly, “*Niban*” is well expressed even in small primary rat Eker renal tumors, more than in progressed cell lines, and is also expressed in human renal carcinoma cells, but not in normal human or rat kidneys. Chromosome assignment was to RNO 13 in the rat, and HSA 1. This “*Niban*” gene is a candidate as a marker for renal tumor, especially early-stage renal carcinogenesis.

Key words: *Tsc2* gene mutant (Eker) rats — Multistep renal carcinogenesis — cDNA-AFLP — Tumor marker

Various tumor suppressor genes or anti-oncogenes have been identified by the study of hereditary human cancers.¹⁾ Although these genes are recessive, they render heterozygous carriers highly susceptible to particular cancers and so appear in pedigrees as dominantly inherited disorders. Such a dominantly inherited predisposition was described in rats by Eker.²⁾ The hereditary renal carcinoma in the rat, originally reported in 1954, is an example of a Mendelian dominantly inherited predisposition to a specific cancer in an experimental animal. At the histological level, renal carcinomas develop through multiple stages from early preneoplastic lesions (e.g., phenotypically altered tubules, which begin to appear around 2 months of age), to adenomas in virtually all heterozygotes around the age of 1 year.^{3,4)} The homozygous mutant condition is lethal to the fetus.³⁾ The fact that ionizing radiation induced additional tumors with a linear dose-response suggests that in heterozygotes two events (the first inherited and the second somatic) are necessary to produce tumors.³⁾ The predisposing gene in the Eker rat was mapped to the proximal part of rat chromosome (RNO) 10.^{5,6)} We have established a new conserved linkage group on rat (RNO) 10q and human (HSA) 16p13.3, whereby the Eker mutation was found to be tightly linked to the tuberous sclerosis (*Tsc2*) gene,⁷⁾ and finally identified a germline mutation in the *Tsc2* gene.^{8,9)}

Carcinogenesis consists of multiple steps and carcinoma development is associated with multi-gene alterations. To identify the genes associated with multi-step renal carcino-

genesis, we performed subtractive cDNA cloning for two renal carcinoma cell lines using the cDNA-AFLP (amplified fragment length polymorphism) approach. These cell lines, named LK9d(L) and LK9d(R) were established from the same Eker rat,^{10,11)} but differ in many aspects. First, LK9d(L), but not LK9d(R), can only be cultured on collagen-coated culture plates. Second, LK9d(R) is flat and round, whereas LK9d(L) is spindle-shaped. Third, growth of LK9d(R) is much faster. Fourth, loss of the p16/15 region (RNO5), reported for a number of carcinomas,^{12,13)} was only found in LK9d(R).

The modified AFLP method employed here was originally developed to isolate genomic markers in plant genetics.^{14,15)}

Cell and tissue materials: Total RNAs were extracted from cell lines and tissues by the acid guanidine phenol chloroform method using ISOGEN (Nippon Gene, Tokyo). Poly-A tailed RNA was isolated with Oligotex dT Super 30 (TaKaRa, Kyoto), and used as the material for cDNA synthesis. More detailed information is available in our previous reports.^{12,16)}

The strategy of the cDNA-AFLP method: The AFLP method adapted for cDNA was as described in our previous report.¹⁵⁾ PCR products which were preferentially amplified in either of the cell lines were recovered. To prevent biased subcloning, we picked up five independent clones and sequenced them. Northern blotting was performed to confirm differential expression.

For expression analysis, we used cell line RNAs obtained from LK9d(L), LK9d(R), ERC (Eker rat Renal Carcinoma) 33, S-LK9d(L)-SLMs, Hep G2, and human renal carcinoma cells (hRCCs). Tissue RNAs were also

³To whom all correspondence should be addressed.
E-mail: ohino@ims.u-tokyo.ac.jp

obtained from Eker rat small renal tumors and normal Wistar rat organs such as the kidney. LK9d(L) is a slow-growing cell line, but LK9d(R) and ERC33 are fast-growing cell lines.^{17, 18} S-LK9d(L)-SLM (selected lung metastasis) cell lines were established from LK9d(L) *in vivo* using nude mice and are slightly faster-growing than LK9d(L).^{10, 15} Human renal carcinoma cells (hRCCs) were established from human renal carcinomas obtained at surgery (manuscript in preparation). Hep G2 is a commonly employed cell line established from a hepatoblastoma. To check the expression of “*Niban*” in human normal organs, we used Clontech human multiple tissue northern blots membrane.

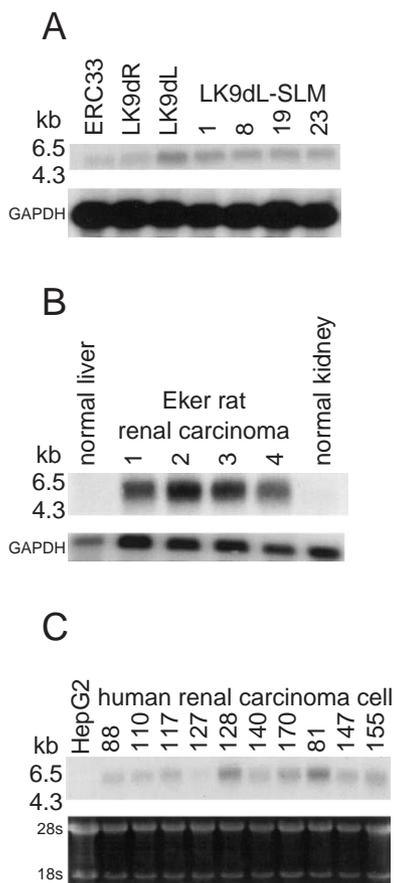


Fig. 1. Northern blot analysis of “*Niban*.” A human glyceraldehyde-3 phosphate dehydrogenase (GAPDH) probe was utilized as the loading control for northern blot analysis. (A) Note the strong expression in LK9d(L), the intermediate level expression in S-LK9d(L)-SLM, but very faint bands for LK9d(R) and ERC33. (B) In tissues, there is strong expression in Eker rat small renal tumors, but none in Wistar rat normal kidney and Eker rat normal liver. (C) In human renal carcinoma cells, there is strong-moderate expression, while Hep G2 cells are negative.

The cloned cDNAs were ³²P-labeled by a random hexamer method, and used as probes for northern and Southern blotting. Two hundred nanograms of poly-A tailed RNA or 5 μg of total RNA derived from LK9d(L) and LK9d(R) was run on formalin denaturing gels and transferred to Biodyne B nylon membranes (Pole, East Hills, NY).

BLAST and FASTA homology searches were performed with the nucleotide sequence information. For the unknown clones, longer cDNAs were obtained using the Marathon cDNA amplification system (Clontech, Palo Alto, CA) based on rapid amplification of cDNA ends (RACE) with long distance PCR or the ZAP-cDNA synthesis system (Stratagene, La Jolla, CA).¹⁵

Genomic DNA isolation and Southern blot analysis: DNAs were isolated from LK9d(L) and LK9d(R) by the sodium dodecyl sulfate (SDS)/proteinase K method with phenol extraction. After restriction enzyme digestion, the DNAs

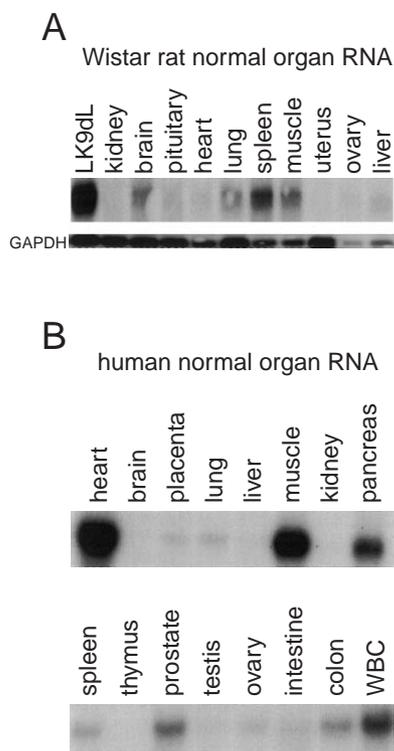


Fig. 2. Northern blot analysis with poly-A rich RNAs in normal tissues. (A) In the normal Wistar rat, there is expression in brain, lung, spleen, and skeletal muscle, but not in kidney, pituitary gland, heart, uterus, ovary, and liver. LK9d(L) is the positive control. (B) In normal human tissues, there is expression in heart, skeletal muscle, pancreas, white blood cell (WBC) and prostate, moderate expression in colon and spleen and none in thymus, testis, ovary, small intestine, brain, placenta, lung, liver, or kidney.

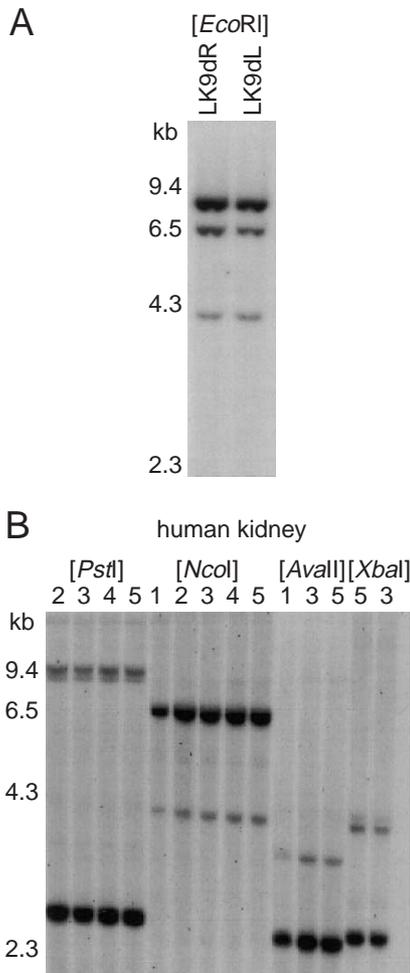


Fig. 3. Southern blot analysis to define that "Niban" is not a gene complex. (A) Three clear bands with *EcoRI* digestion of rat DNA from LK9d(R) and LK9d(L). Note no "smear" or "ladder." (B) Two or three bands are evident with *PstI*, *NcoI*, *AvaI*, and *XbaI* digestion of human kidney DNAs.

were separated on 1% agarose gels and transferred onto nylon membranes under alkaline conditions. Pre-hybridization and hybridization were performed in 0.2 M phosphate buffer (pH 7.2), 1 mM EDTA, 1% bovine serum albumin, and 7% SDS, at 65°C. After addition of ³²P-labeled probes, hybridization was performed in the same solution at 65°C. The filters were then washed twice in 1× standard saline citrate (SSC; 0.15 M NaCl and 15 mM sodium citrate) and 0.1% SDS for 15 min at room temperature and then washed once in 1× standard saline citrate and 0.1% SDS for 30 min at 65°C. The filters were exposed to X-ray film with an intensifying screen at -50°C for 1-3 days. More detailed information is available in our previous reports.¹⁹⁾



Fig. 4. Map of the open reading frame (ORF). The codon "ATG" is indicated by ▽, and the termination codons "TAA, TAG, and TGA" are indicated by |. Top frame is the ORF.

Cloning of differentially expressed genes in LK9d(L): Comparison of cDNA-AFLP patterns revealed different cDNA fragments between LK9d(L) and LK9d(R). These bands were excised from gels and cloned into the plasmid. We picked out and further analyzed one fragment that exhibited especially prominent differences, with greater expression in LK9d(L). Three other bands are obviously not novel genes, resembling mitochondrial DNA, atrial natriuretic peptide and ribosomal RNA in a homology search. So we focused only on the "Niban" gene.

Sequencing and homology evaluation with the BLAST and FASTA programs in DDBJ were performed. The size of "Niban" transcript was about 6.5 kb (Fig. 1A). Northern blot analysis revealed strong expression in LK9d(L), very low expression in LK9d(R) and ERC33, and an intermediate level in S-LK9d(L)-SLM (Fig. 1A). Importantly strong expression was also found in Eker rat small renal tumors, but not in normal Wistar kidney or Eker rat liver (Fig. 1B). Furthermore human renal carcinoma cells (hRCCs) were positive with a rat probe, while the Hep G2 line was negative (Fig. 1C).

Northern blot analysis with poly-A rich RNAs demonstrated expression in normal brain, lung, spleen, and skeletal muscle, but not in kidney, pituitary gland, heart, uterus, ovaries and liver of Wistar rats. LK9d(L) was the positive control in this experiment (Fig. 2A). In normal human tissues, strong expression was found in heart, skeletal muscle, pancreas, white blood cells, and prostate, moderate in colon and spleen and none in thymus, testis, ovary, small intestine, brain, placenta, lung, liver or kidney (Fig. 2B).

To define "Niban" as a single gene, we performed Southern blot analysis. If it were a multi-gene complex, a "smear" or "ladder" might be expected on Southern blot analysis. With LK9d(R) and LK9d(L), as rat sources of DNA, we observed three clear bands on *EcoRI* digestion (Fig. 3A). With human kidney DNAs, we observed two or three bands on *PstI*, *NcoI*, *AvaI*, and *XbaI* digestion (Fig. 3B).

9 18 27 36 45 54
 5' ACT OCT CTC CCT CCG GTG CAG CAC CGC GGC CAT GGG CGC CTG AGC CTC CAC CCA
 63 72 81 90 99 108
 ACT GGA TGA GGG CAA GTG CGC CTA TAT CCG AGG GAA AAC TGA GGC TTC TAT CAA
 117 126 135 144 153 162
 AAA CTT CAG TCC CTA CTA CAG OCC CCA ATA TTC TOG GCT TTC TGC AAC CAT GTG
 171 180 189 198 207 216
 CGC AGC GAG GTG GAA CAG CAG AGA GAT TTA ACG TCA CAG TTT CTG AAC ACC AAG
 225 234 243 252 261 270
 CCC CCA CTG GAA CCA GGA ACT GTT TGG TAT GAA GCT GAG CTC TCA CAG TTT GCT
 279 288 297 306 315 324
 GAG GAC ATC AGG AAA TGG AAC GAC AGA TAC CTT GTG GTT AAA AAC GAT TTT GCT
 333 342 351 360 369 378
 GTG GAA AGC TAT GAG AAC AAA GAG GCC TAT CAG AGA GGA GCT GTT CCT AAA AGC
 387 396 405 414 423 432
 AGA ATT CTT CCA GCT GGT GGC AAG CTC TTA ACC TCA GAA GAG GAA TAT AGT CTC
 441 450 459 468 477 486
 TTA TCT GAT AAG CAT TTC CCA GAC CCC ACT GCT TCC AGT GAG AAG AAC TCT GAG
 495 504 513 522 531 540
 CCC TTC CTG GTC CTA CCC AAG GCA TTC CCA CTG TAC CTC TGG CAG CCT TAC CTC
 549 558 567 576 585 594
 AGG CAC GGC TAC TTC TGT TTC CAC GAG GCT GGC GAG CAG CAG AAG TTC AGC GAT
 603 612 621 630 639 648
 CTT CTC AAC GAC TGC ATC AGA CAC CTG AAT CAC GAT TAC ATG AAG CAG ACC ACA
 >>>
 Met Lys Gln Thr
 657 666 675 684 693 702
 TTC GAA GCC CAA GCC TTT TTG GAA GCT GTG CAG TTC TTC CCG CAG GAG AAG GGT
 Phe Glu Ala Gln Ala Phe Leu Glu Ala Val Gln Phe Phe Arg Gln Glu Lys Gly
 711 720 729 738 747 756
 CAC TAC GGC GCC TGG GAA GTG ATT ACT GGA GAT GAA GTC CAG ATC CTG AGT AAA
 His Tyr Gly Ala Trp Glu Val Ile Thr Gly Asp Glu Val Gln Ile Leu Ser Lys
 765 774 783 792 801 810
 CTG GTG ATG GAG GAG CTC CTG CCG ACC CTC CAG ACT GAG CAG CTG CCT AAA CTG
 Leu Val Met Glu Glu Leu Leu Pro Thr Leu Gln Thr Asp Leu Leu Pro Lys Leu
 819 828 837 846 855 864
 AAG GGG AAG AAG AAT GAC AGA AAG AGA GCC TGG TTT GGA CTC CTG GAG GAA GCC
 Lys Gly Lys Lys Asn Asp Arg Lys Arg Ala Trp Phe Gly Leu Leu Glu Glu Ala
 873 909 918 927 936 945 954 963 972
 TAC AAT CTG GTT CAG CAC CAA GTT TCA GAA GGA TTA ACT GCT TGG AAG GAG GAG
 Tyr Asn Leu Val Gln His Gln Val Ser Glu Gly Leu Ser Ala Leu Lys Glu Glu
 927 936 945 954 963 972
 TGC AGA GCT CTG ACA AAG GAC CTG GAA GGG ACC ATC CGC TCA GAC ATG GAT CAG
 Cys Arg Ala Leu Thr Lys Asp Leu Glu Gly Thr Ile Arg Ser Asp Met Asp Gln
 981 990 1008 1017 1026
 AAT GTG AAC TCA AAG AAG TTT CTA ACT GGG AAG ATC AGA GCA ATG GTG GCT CAG
 Ile Val Asn Ser Lys Asn Phe Leu Thr Gly Lys Ile Arg Ala Met Val Ala Gln
 1035 1044 1053 1062 1071 1080
 CCG GCC GAG AAT CGC TGT GGG GAA AGT GTG CAG CCC TTC CTG GCA TCC AAT CTG
 Pro Ala Glu Asn Arg Cys Gly Glu Ser Val Gln Pro Phe Leu Ala Ser Ile Leu
 1089 1098 1107 1116 1125 1134
 GAG GAG CTA ATG GCG CCG GTG AGC TCC GGT TTC AGT GAA TTC CGT GCA CTT TTC
 Glu Glu Leu Met Gly Pro Val Ser Ser Gly Phe Ser Glu Val Arg Ala Leu Leu
 1143 1152 1161 1170 1179 1188
 GAA AAA GAA GTA GAT GAC CTC ACT GAG AGC TTC CAG ACC ACC CAG CAC GGT GCC
 Glu Lys Glu Val Asp Glu Leu Ser Gln Ser Phe His Thr Thr Gln Asp Gly Ala
 1197 1206 1215 1224 1233 1242
 CAG CTC AAG GAG TGT CTA GAC CAG CTA ATG AAG CTT CCT CTG GAT TCT GTG AAG
 Gln Leu Lys Glu Cys Leu Asp Gln Leu Met Lys Leu Pro Leu Asp Ser Val Lys
 1251 1260 1269 1278 1287 1296
 ATG GAG CCT TGT TAC ACT AAA GTC ACC CTG CTT CCA GAG CGC CTG CTC GAC CTC
 Met Glu Pro Cys Tyr Thr Lys Val Thr Leu Leu Pro Glu Arg Leu Leu Asp Leu
 1305 1314 1323 1332 1341 1350
 CAG AGT CGC TTC AGA TTC CCT CAC GTT GAT CTC GTG GTC CAG AGG ACT CAG AAC
 Gln Ser Arg Phe Arg Phe Pro His Val Asp Leu Val Val Gln Arg Thr Gln Asn
 1359 1368 1377 1386 1395 1404
 TAC ATG CAG GAG CTC ATG GAG AAT GCT GTG TTC ACA TTT GAG CAG TTC CTC TCC
 Tyr Met Gln Glu Leu Met Glu Asn Ala Val Phe Thr Phe Glu Gln Leu Leu Ser
 1413 1422 1431 1440 1449 1458
 CCA TAT CTT CAA GGA GAG GCT TCC AGA ACA GCA GTG GCC ATC CAG AAG GTT AAG
 Pro Tyr Leu Gln Gly Glu Ala Ser Arg Thr Ala Val Ala Ile Glu Lys Val Lys
 1467 1476 1485 1494 1503 1512
 CTC CGT GTC TTA AAG CAA TAC GAT TAC GAC AGC AGC ACC ATC CGC AAG AAG ATC
 Leu Arg Val Leu Lys Gln Tyr Asp Tyr Asp Ser Ser Thr Ile Arg Lys Lys Ile
 1521 1530 1539 1548 1557 1566
 TCC CAG GAG CCA TTG AAT CAG ATC ACA CTA CCC ACT GTG CAG AAG GCG CTG GCG
 Phe Gln Glu Ala Leu Ile Gln Ile Thr Leu Pro Thr Val Gln Lys Ala Leu Ala
 1575 1584 1593 1602 1611 1620
 TCC ACA TGC AAA CCA GAG CTT CAG AAA TAT GAG CAG TTC ATC TTC CCA GAC CAC
 Ser Thr Cys Lys Pro Glu Leu Gln Lys Tyr Glu Gln Phe Ile Phe Ala Asp His
 1629 1638 1647 1656 1665 1674
 ACC AAT ATG ATC CAT GTT GAG AAC ATC TAT GAA GAG AAT TTG TAT CAG ATC CTC
 Thr Asn Met Ile His Val Glu Asn Ile Tyr Glu Glu Ile Leu Tyr Gln Ile Leu
 1683 1692 1701 1710 1719 1728
 CTC GAT GAG ACC CTC AAA GTG ATA ACG GAA CCA GCT ACT TTG AAG AAA CAC AAC
 Leu Asp Glu Thr Leu Lys Val Ile Thr Glu Ala Ala Ile Leu Lys Lys Lys Ile
 1737 1746 1755 1764 1773 1782
 TTA TTT CAA CAC AAC ATG CCA TTA CCC AGT GAA AGT GTG TCC AGT CTG ACA GAC
 Leu Phe Glu Asp Asn Met Ala Leu Pro Ser Glu Ser Val Ser Ser Leu Thr Asp

1791 1800 1809 1818 1827 1836
 CTC AAA ACC TCC ATG CCG TCA AAC CAC CCC AGC CCA CCC AGA GGA GCA TCT GCC
 Leu Lys Thr Ser Met Gly Ser Asn Gln Ala Ser Pro Ala Arg Gly Ala Ser Ala
 1845 1854 1863 1872 1881 1890
 ATT CTC CCA GGA GCT CCA GGC GAT GAG GCT CCA GGC AGC GAA GTG TTC CAG GGG
 Ile Leu Pro Gly Ala Pro Gly Asp Glu Ala Pro Gly Ser Glu Val Phe Gln Gly
 1899 1908 1917 1926 1935 1944
 CCT GAG GAA AAG CAG CAG CAG CCG GGT GTC CCT GGC TCG CTG GCC AGA GAA GAA
 Pro Glu Glu Lys Gln Gln Gln Pro Gly Val Pro Gly Ser Leu Ala Arg Glu Glu
 1953 1962 1971 1980 1989 1998
 AGT GCT TCC ATC TCT GGG TCC AGC CCT CCT TCA GGT GAG GAT GGG CAG GTG TCT
 Ser Ala Ser Ile Ser Gly Ser Ser Pro Ser Gly Glu Asp Gly Gln Val Ser
 2007 2015 2025 2034 2043 2052
 GTG TCC GGT GTG GAT AAC TCT GCC GGG AAC CCT CTC ACT CCA GAT AAT TCA GCA
 Val Ser Gly Val Asp Asn Ser Ala Gly Asn Pro Leu Ser Ala Asp Asn Ser Ala
 2061 2070 2079 2088 2097 2106
 GGA CCT CTC AGC TCA CAC TTC TCA GAG CCA GAA GCT GGG GAG CCC CCT AAA ATG
 Gly Pro Leu Ser Ser His Leu Ser Glu Ala Glu Ala Gly Glu Pro Pro Lys Asp
 2115 2124 2133 2142 2151 2160
 GAG GAA ACA GCC CAC AAA AGG CCA GAG TCT AGC GCT GTG CCA GGG TCC TTG AGG
 Glu Glu Thr Ala His Lys Arg Pro Glu Ser Ser Ala Val Pro Gly Ser Leu Arg
 2169 2178 2187 2196 2205 2214
 GAA CTG AAG GAG TTG TTG ACT CTC AGC CTG TTC CTC GAG TCT GCC CCA GAG ATT
 Glu Leu Lys Glu Leu Leu Thr Val Thr Val Phe Val Glu Ser Ala Pro Glu Ile
 2223 2232 2241 2250 2259 2268
 GGA AAT GAT ACA CTC AAC GGG ACA CCT GGT CCC CAA GAA GAT AAA AAA GAA GAA
 Gly Asn Asp Thr Leu Asn Gly Thr Pro Val Pro Gln Glu Asp Lys Lys Glu Glu
 2277 2286 2295 2304 2313 2322
 CAA GAG GAG GAG GAA AGC AAG ATC CAC CCA GAA GCC ACC GCC CCA GCT ACC ATC
 Glu Glu Glu Glu Glu Ser Lys Ile His Pro Glu Ala Ser Gly Pro Ala Ala Ile
 2331 2340 2349 2358 2367 2376
 CAG CAG GAC AGC TGT GAA GAA AGT GAA GTC AGA CAG AGG GAG GCC CAT CCT ATG
 Gln Gln Asp Ser Cys Glu Glu Ser Glu Val Arg Glu Arg Glu Ala His Pro Met
 2385 2394 2403 2412 2421 2430
 CCT TTG GAA GCT GAG GCT CCT GCG GTG AAC TTG GGG ACA CTG CCA GAG GGT AAG
 Pro Leu Glu Ala Glu Ala Pro Gly Val Asn Leu Gly Thr Leu Pro Glu Gly Arg
 2439 2448 2457 2466 2475 2484
 GGG CCT ACC TCT CAG TCT ACC GGT GAG GGA CTC ACT GAG AAC ACC AGC TGT CTA
 Gly Pro Thr Ser Gln Ser Thr Gly Glu Gly Leu Thr Glu Asn Thr Ser Cys Leu
 2493 2502 2511 2520 2529 2538
 GCC CCC ATA GAG GAG CCA TCT GAG GCT CAA GGG CCC ACT CAG GAG CTG CTC CTA
 Gly Pro Ile Glu Glu Pro Ser Glu Ala Gln Gly Pro Thr Glu Glu Val Leu Leu
 2547 2556 2565 2574 2583 2592
 GGC ACA GTT TCC ACA CAG GAC ACC GAG CCA GGA GGT GAG GCT GTG CAG TCT
 Ala Thr Val Ser Thr Gln Asp Ser Thr Glu Ala Gly Gly Glu Ala Val His Ser
 2601 2610 2619 2628 2637 2646
 GTG ACA GTC ACA CCT CAA GAA GAT GCC ACG TTA AGT TCT AAC CCC ATC TGT CAC
 Val Thr Val Thr Pro Gln Glu Asp Ala Thr Leu Ser Ser Asn Pro Ile Cys Pro
 2655 2664 2673 2682 2691 2700
 CTC GAG AAT AAT GAG GGG CCC CAG GTT TCT GAG GAT CAG GAA GTG CTG CGA GGG
 Val Glu Asn Asn Glu Gly Pro Gln Val Ser Glu Asp Gln Glu Val Leu Gly Gly
 2709 2718 2727 2736 2745 2754
 AAT GAT AGC CCA GCC CTT CCT ATG GAT ACA GAG CAA ATC AAC GAT GCC CAG GTG
 Asn Asp Ser Pro Ala Leu Ala Met Asp Thr Glu Gln Ile Asn Asp Ala His Val
 2763 2772 2781 2790 2799 2808
 TAT GAG TGC CAC TGG GAG GTA GAA GAT GCC CCG AGC GCC GAC ATC CTA GAT GTT
 Tyr Glu Cys His Trp Glu Val Glu Asp Ala Pro Ser Ala Asp Ile Leu Asp Val
 2817 2826 2835 2844 2853 2862
 CAC GAT TGT GAT GTG GGC TCC CCA GGG GAG TGG TAG AAT CAG TTT GCC GGT CTT
 His Asp Cys Asp Val Gly Ser Pro Gly Glu Trp ***
 2871 2880 2889 2898 2907 2916
 TIT TCT TTT AAA ACT CTC AGT CAA GGG GTA TTA GTT ATG GGT ATC CTT AGG GGG
 2925 2934 2943 2952 2961 2970
 TTG CAT GTG ACT CGC TAT AAA AAT TAT GAA ATG TTT CTT TAA TTT GAA AGA TGA
 2979 2988 2997 3006 3015 3024
 AGC CAG AGT GCA CAG ACG GTG GGA GCA GTG AGC AGC CTT TGG GCG GCT GAA GTG
 3033 3042 3051 3060 3069 3078
 CTC CCC ATG AAT GAT CTA TCA CTT TGG CAT TTT AAT AGG AAT GGT AAG AGA ACT
 3087 3096 3105 3114 3123 3132
 GAA CCA TTG GGC CAA AGT CAA AGG GCA TCC AGA AAG GGC AGC ASA AAA TGA CAC
 3141 3150 3159 3168 3177 3186
 ATC AAT AAT CTC TTC ACT GAA ACG TCG TAC ACA TTT TGA ACT GAG TGT TAA GGC
 3195 3204 3213 3222 3231 3240
 CTT TTT AGC AAG CAT GAG AAG GAC TCC AGC CTC AAC ACC TGC TCA GCT GTC ACT
 3249 3258 3267 3276 3285 3294
 CCT CAG CTG GTC GGC TGC ACT CTC TCC TGT CCT TAG ACA GGT CAC AAG AAA GAG
 3303 3312 3321 3330 3339 3348
 GAA GTC ATG AAC CAG GCA CCC CTG TCC TGC AAA GTA CAG AGC CAG TCA CCG CTG
 3357 3366 3375 3384 3393 3402
 GTA GAG CTC AGG GGA TGC AGA GCA GAA ACC CAA GGT GGA AGG TCT GTG GAA CTA
 3411 3420 3429 3438 3447 3456
 TGC ACC CCT GTT CCT AAC CCT GCT AGC AAT CCC TTG CTG GTT AAT TCT GCT GGT
 3465 3474 3483 3492 3501 3510
 TTA CAT TTA ATC ACC CTT TAA GGA AAT TCA GGC AGG GTA AGA GAT TCT ACT
 3519 3528 3537 3546 3555 3564
 GGC ATG AAT CAG CCT TAG GTT GTA CAA AGT GCT GTT CTA AAT TTT CTA GCT CCA
 3573 3582 3591 3600 3609 3618
 ACC CTA CCA GTG ACA CAG CAG TGA CTT GAA CAA CTG CAT GCT CTG TTA GTC
 3627
 GTC ATA ATA AAT AAA 3'

Fig. 5. The cDNA sequence containing the open reading frame (ORF) and the amino acid sequence.

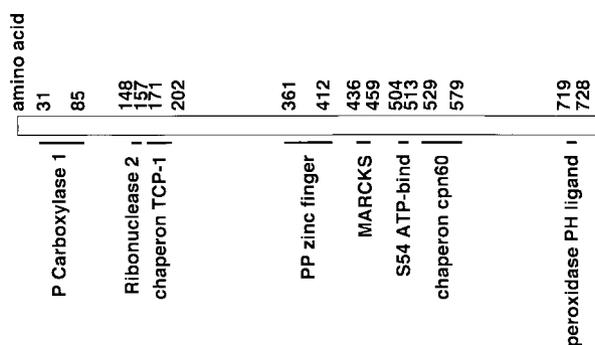


Fig. 6. Similarity of amino acid sequence encoded by "Niban" to known proteins. P Carboxylase is phosphoenolpyruvate carboxylase protein 1 (BL00781C, similarity score 1054). Ribonuclease 2 is ribonuclease 2 family proteins (BL01175C, similarity score 1011). Chaperon TCP-1 is chaperonins TCP-1 protein (BL00750C, similarity score 1010). PP zinc finger is poly(ADP-ribose)polymerase zinc finger domain protein (BL00347B, similarity score 1020). MARCKS is myristoylated alanine-rich C-kinase substrate family proteins (BL00826A, similarity score 1015). S54 ATP-binder is Sigma-54 interaction domain ATP-binding region A (BL00675D, similarity score 1006). Chaperon cpn60 is chaperonins cpn60 protein (BL00296E, similarity score 1014). Peroxidases PH ligand is peroxidases proximal heme-ligand protein (BL00435A, similarity score 1062).

The chromosomal assignment of the "Niban" gene was determined by Southern blot analysis of a human/rat somatic cell hybrid panel.²⁰ The membrane filters for hybrid cell panel analysis were kindly provided by Dr. G. Levan (University of Goteborg, Goteborg, Sweden).^{21,22} The chromosome assignment was to RNO13 in the rat and HSA1 in the human case (data not shown).

Cloning of longer cDNA fragments and identification of the "Niban" gene: We obtained longer cDNA fragments from conventional library screening and 5' or 3'-RACE reactions based on long-distance PCR. Two overlapping cDNA clones (2.3 kb and 3.0 kb) contained a complete open reading frame (ORF) of 2748 bp (Fig. 4 and Fig. 5). We have not determined the initiation codon in this ORF. If the first ATG at nt 634 is the initiation codon, this gene codes for 737 amino acid. The mRNA size was estimated to be 6.5 kb by northern blotting. Homology search revealed several sequences with some homology to the ORF of "Niban." These sequences are listed in the Genbank database as EST (expressed sequence tags) or HTG (high throughput genome). Among them, those coded by

Genbank accession number AA191493 (EST; zp88e01.s1 Stratagene HeLa cell s3 <540 nt>, 86.296% identity in 540 nt overlap), AW503842 (EST: UI-HF-BN0-alb-c-07-0-UI.r1 NIH-MGC <436 nt>, 84.988% identity in 433 nt overlap) and AL136086 (HTG: human DNA sequence sequencing <97 777 nt>, 70.244% identity in 615 nt overlap) showed higher homology to "Niban," but their functional significance has not yet been reported. Possible functional domains in "Niban" was searched by use of the MOTIF program (<http://www.genome.ad.jp/>). It revealed no known functional domains in "Niban," but showed the existence of some similarity to known proteins by ungapped multiple sequence alignments (BLOCK program).^{23,24} Details are shown in Fig. 6.

The "Niban" gene showed unique band pattern in Southern blots. It was found to be well conserved between human and rat. We conclude that it is a new single gene.

The present study showed "Niban" to be expressed highly in the LK9d(L) line and at an intermediate level in S-LK9d(L)-SLM. The LK9d(R) and ERC33 cells, on the other hand, had low expression. The absence of expression in normal rat/human kidneys and in a large tumor (data not shown) and the results in cultured RCC cells suggest an inverse relation between expression of "Niban" and progression of renal carcinogenesis. We think "Niban" expression is most dramatically increased in the early stage of renal carcinogenesis and might decline during malignant progression. It would be worthwhile to do a function analysis of "Niban."

In conclusion, the present study demonstrated that the cDNA-AFLP technique is a useful tool to search for additional genes specifically involved in *Tsc2* gene mutant (Eker) renal carcinogenesis. Analysis of the molecular function of the "Niban" gene and of the relationship with *Tsc2* gene mutation should provide new insights into multi-step carcinogenesis in the kidney. Thus, the *Tsc2* mutant (Eker) rat provides a promising model for analyzing the key events of carcinogenesis at different stages.

We thank Drs. Haruo Sugano, Tomoyuki Kitagawa and Alfred G. Knudson for encouragement throughout this work, which was supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Education, Science, Sports and Culture of Japan and a grant from the Organization for Pharmaceutical Safety and Research (OPSR).

(Received May 30, 2000/Revised July 31, 2000/Accepted August 4, 2000)

REFERENCES

- 1) Knudson, A. G., Jr. Hereditary cancer, oncogenes, and antioncogenes. *Cancer. Res.*, **45**, 1437-1443 (1985).
- 2) Eker, R. and Mossige, J. A dominant gene for renal adenomas in the rat. *Nature*, **189**, 858-859 (1961).
- 3) Hino, O., Klein-Szanto, A. J., 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).
- 4) 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).
 - 5) Hino, O., Mitani, H., Nishizawa, M., Katsuyama, H., Kobayashi, E. and Hirayama, Y. A novel renal cell carcinoma susceptibility gene maps on chromosome 10 in the Eker rat. *Jpn. J. Cancer Res.*, **84**, 1106–1109 (1993).
 - 6) Yeung, R. S., Buetow, K. H., Testa, J. R. and Knudson, A. G., Jr. 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).
 - 7) Hino, O., Kobayashi, T., Tsuchiya, H., Kikuchi, Y., Kobayashi, E., Mitani, H. and Hirayama, Y. The predisposing gene of the Eker rat inherited cancer syndrome is tightly linked to the tuberous sclerosis (TSC2) gene. *Biochem. Biophys. Res. Commun.*, **203**, 1302–1308 (1994).
 - 8) 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 [published erratum appears in *Nat. Genet.*, 1995 Feb; **9** (2): 218]. *Nat. Genet.*, **9**, 70–74 (1995).
 - 9) 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).
 - 10) Fukuda, T., Hirayama, Y., Mitani, H., Maeda, H., Tsutsumi, M., Konishi, Y. and Hino, O. Generation of metastatic variants of Eker renal carcinoma cell lines for experimental investigation of renal cancer metastasis. *Jpn. J. Cancer Res.*, **89**, 1104–1108 (1998).
 - 11) Orimoto, K., Tsuchiya, H., Kobayashi, T., Matsuda, T. and Hino, O. Suppression of the neoplastic phenotype by replacement of the Tsc2 gene in Eker rat renal carcinoma cells. *Biochem. Biophys. Res. Commun.*, **219**, 70–75 (1996).
 - 12) 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.*, **14**, 23–27 (1995).
 - 13) Kitagawa, T., Miyasaka, K., Kanda, H., Yasui, H. and Hino, O. Hepatocarcinogenesis in rodents and humans. *J. Cancer Res. Clin. Oncol.*, **121**, 511–515 (1995).
 - 14) Vos, P., Hogers, R., Bleeker, M., Reijans, M., van de Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M. and Zabeau, M. AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Res.*, **23**, 4407–4414 (1995).
 - 15) Fukuda, T., Kido, A., Kajino, K., Tsutsumi, M., Miyauchi, Y., Tsujiuchi, T., Konishi, Y. and Hino, O. Cloning of differentially expressed genes in highly and low metastatic rat osteosarcomas by a modified cDNA-AFLP method. *Biochem. Biophys. Res. Commun.*, **261**, 35–40 (1999).
 - 16) 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).
 - 17) 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.*, **121**, 602–605 (1995).
 - 18) 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).
 - 19) 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).
 - 20) Satake, N., Kobayashi, T., Kobayashi, E., Izumi, K. and Hino, O. Isolation and characterization of a rat homologue of the human tuberous sclerosis 1 gene (Tsc1) and analysis of its mutations in rat renal carcinomas. *Cancer Res.*, **59**, 849–855 (1999).
 - 21) Szpirer, J., Levan, G., Thorn, M. and Szpirer, C. Gene mapping in the rat by mouse-rat somatic cell hybridization: synteny of the albumin and alpha-fetoprotein genes and assignment to chromosome 14. *Cytogenet. Cell Genet.*, **38**, 142–149 (1984).
 - 22) Kobayashi, T., Kawaguchi, T., Kishino, T., Matsumoto, N., Niikawa, N., Mori, M., Levan, G., Klinga-Levan, K. and Hino, O. Isolation of microdissection clones from rat chromosome 10. *Mamm. Genome*, **6**, 216–218 (1995).
 - 23) Henikoff, S., Henikoff, J. G. and Pietrokovski, S. Blocks+: a non-redundant database of protein alignment blocks derived from multiple compilations. *Bioinformatics*, **15**, 471–479 (1999).
 - 24) Henikoff, J. G., Henikoff, S. and Pietrokovski, S. New features of the Blocks Database servers. *Nucleic Acids Res.*, **27**, 226–228 (1999).