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

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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 carcinogenesis, 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

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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.

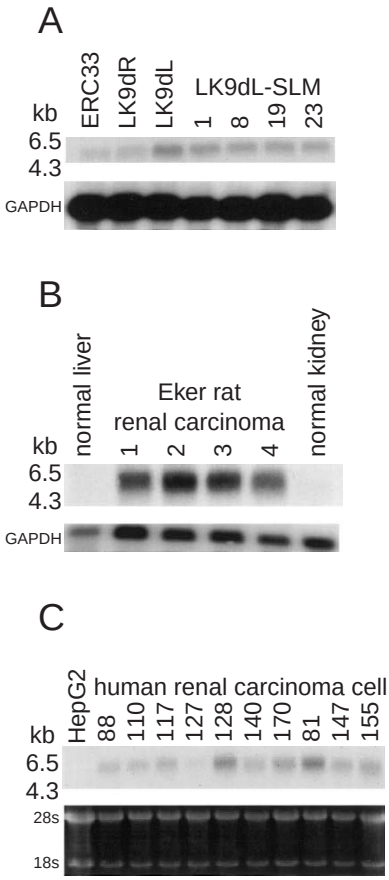

Fig. 1. Northern blot analysis of "*Niban*." A human glyceraldehyde-3 phosphate dehydrogenase (GAPDH) probe was utilized as the loading control for northern blot analysis. (A) Note the strong expression in LK9d(L), the intermediate level expression in S-LK9d(L)-SLM, but very faint bands for LK9d(R) and 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 ^{32}P-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

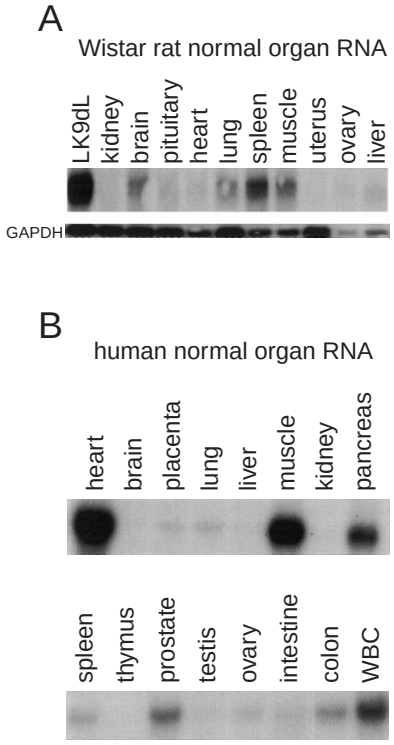

Fig. 2. Northern blot analysis with poly-A rich RNAs in normal tissues. (A) In the normal Wistar rat, there is expression in brain, lung, spleen, and skeletal muscle, but not in kidney, pituitary gland, heart, uterus, ovary, and liver. LK9d(L) is the positive control. (B) In normal human tissues, there 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.

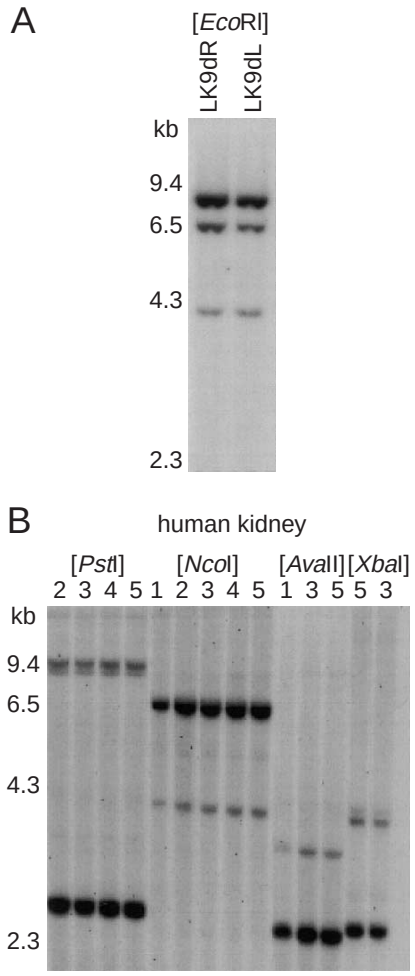

Fig. 3. Southern blot analysis to define that "*Niban*" is not a gene complex. (A) Three clear bands with *Eco*RI digestion of rat DNA from LK9d(R) and LK9d(L). Note no "smear" or "ladder." (B) Two or three bands are evident with *Pst*I, *Nco*I, *Ava*I, and *Xba*I digestion of human kidney DNAs.

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 m*M* EDTA, 1% bovine serum albumin, and 7% SDS, at 65°C. After addition of ^{32}P-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 m*M* 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 *Eco*RI digestion (Fig. 3A). With human kidney DNAs, we observed two or three bands on *Pst*I, *Nco*I, *Ava*I, and *Xba*I digestion (Fig. 3B).

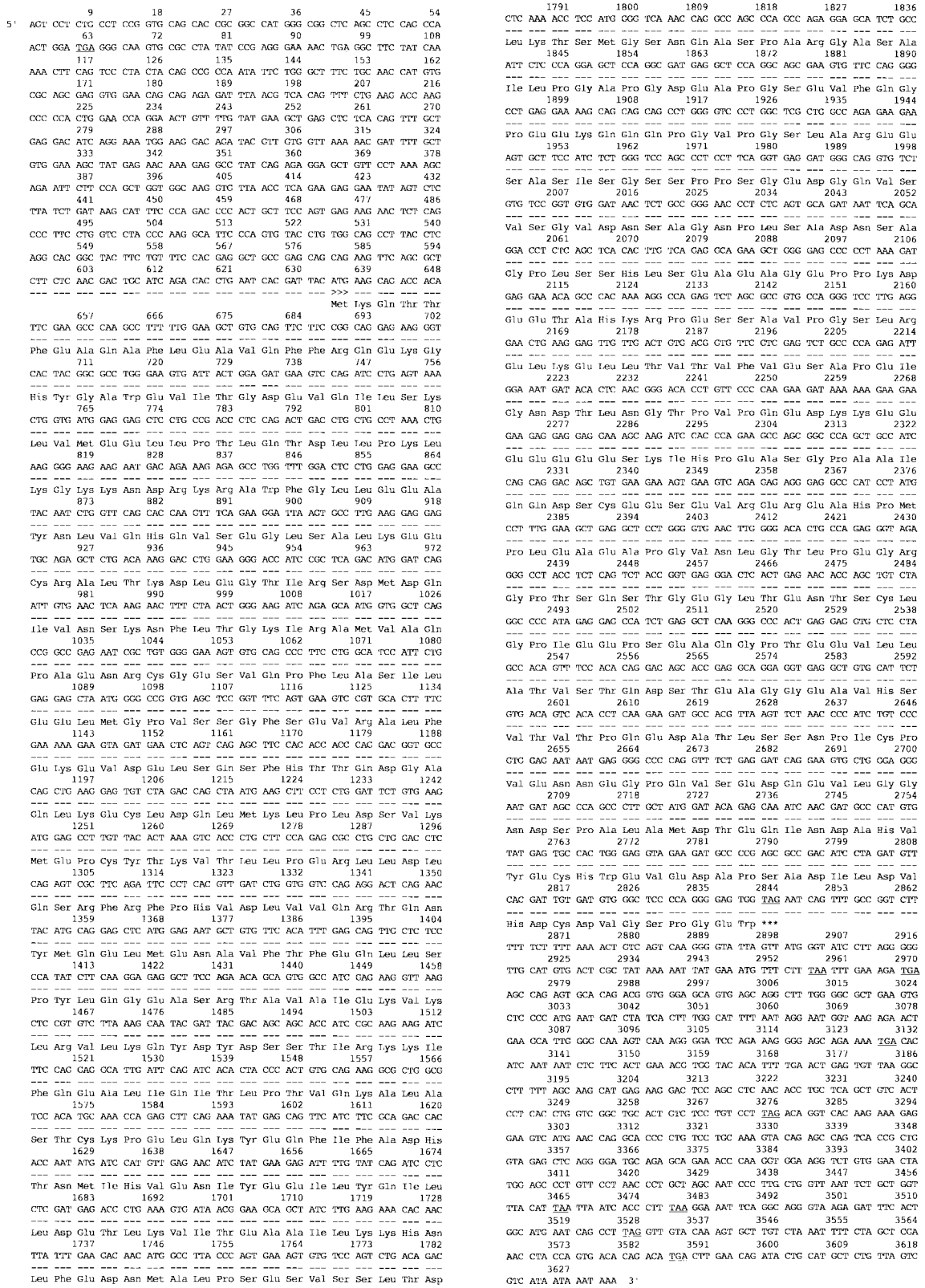

Fig. 5. The cDNA sequence containing the open reading frame (ORF) and the amino acid sequence.

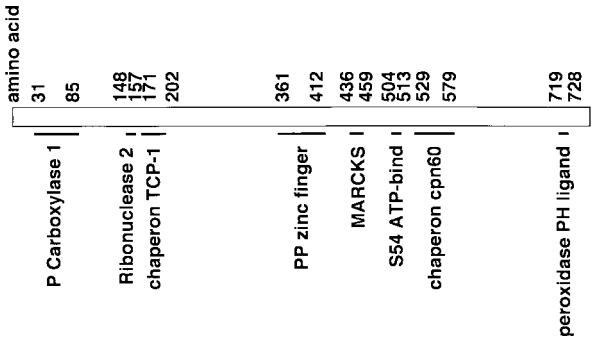

Fig. 6. Similarity of amino acid sequence encoded by "*Niban*" to known proteins. P Carboxylase is phosphoenolpyruvate carboxylase protein 1 (BL00781C, similarity score 1054). Ribonuclease 2 is ribonuclease 2 family proteins (BL01175C, similarity score 1011). Chaperon TCP-1 is chaperonins TCP-1 protein (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.

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