A germ-line insertion in the Birt–Hogg–Dubé (*BHD*) gene gives rise to the Nihon rat model of inherited renal cancer

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A rat model of hereditary renal carcinoma (RC) was found in a rat colony of the Sprague–Dawley strain in Japan and named the ''Nihon'' rat. In heterozygotes, RCs, predominantly the clear cell type, develop from early preneoplastic lesions, which began to appear as early as 3 weeks of age, to adenocarcinomas by the age of 6 months. The Nihon rat is an example of a Mendelian dominantly inherited predisposition for development of RCs like the Eker (*Tsc2* gene mutant) rat. We have previously shown that the Nihon mutation was tightly linked to genes that are located on the distal part of rat chromosome 10. The order of the genes is the Eker (*Tsc2* gene (human 16p13.3)–*Il3* gene–Nihon gene–*Llgl1* locus–*Myhse* gene. We now describe a germ-line mutation in the Birt–Hogg–Dubé gene (*Bhd*) (human 17p11.2) caused by the insertion of a single nucleotide in the Nihon rat, resulting in a frameshift and producing a stop codon 26 aa downstream. We found that the homozygous mutant condition was lethal at an early stage of fetal life in the rat. We detected a high frequency of loss of heterozygosity (LOH) in primary RCs (10/11) at the *Bhd* locus and found a point mutation (nonsense) in one LOH-negative case, fitting Knudson's ''two-hit'' model. The Nihon rat may therefore provide insights into a tumor-suppressor gene that is related to renal carcinogenesis and an animal model of human BHD syndrome.

Hereditary cancer was described in the rat by Eker and Mossige in Oslo 50 years ago (1). The Eker rat model of hereditary renal carcinoma (RC) was the earliest example of a Mendelian dominantly inherited predisposition to a specific cancer in an experimental animal. We and others identified a germ-line mutation in the rat homologous to the human tuberous sclerosis gene (*TSC2*) as the predisposing Eker gene (2, 3). Recently, we found a hereditary RC model in the Sprague–Dawley rat in Japan. We have named this RC model the ''Nihon'' rat (4). In heterozygotes, RCs develop from early preneoplastic lesions, seen as early as 3 weeks of age, into adenomas by 8 weeks of age with complete penetrance for this RC gene by the age of 6 months (5). The Nihon rat is also an example of a Mendelian dominantly inherited predisposition for development of RCs, which are predominantly of the clear cell type, and carries a single gene mutation like the Eker rat (4, 5).

The predisposing inherited gene in the Nihon rat was mapped to rat chromosome 10 between *Il3* (human 5q23–31) and *Llgl1* (human 17p11.2)/*Myhse* (human 17p13.1) loci, 4.4 centimorgans (cM) distal and 0.9 cM/5.3 cM proximal, respectively (6) (Fig. 1a). At that time, we did not know the human chromosome to which it corresponded (e.g., human chromosome 5 or 17). However, it was noted that the predisposing gene of the Birt–Hogg–Dubé (BHD) syndrome associated with renal cancer had been mapped to human chromosome 17p11.2 or 17p12-q11.2 (7, 8). In the present study, we narrowed the Nihon locus to a region of the rat chromosome 10 homologous with human chromosome 17p11.2, and we identified a rat *BHD* homologue, mutations in which predispose to the renal cancer phenotype in the Nihon rat.

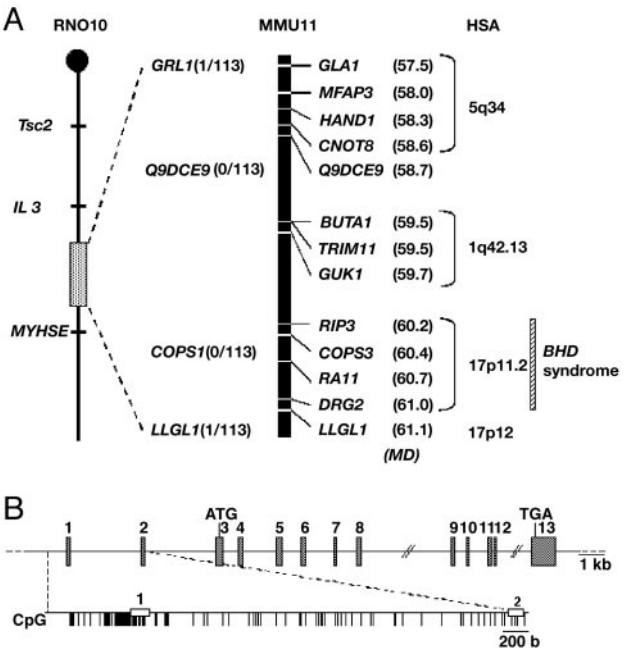

Fig. 1. Genetic mapping and genomic structure of the rat *Bhd* homologue. (*A*) Linkage analysis of rat (*Rattus norvegicus*) chromosome 10 (RNO10). Gene names and genetic distances from the Nihon gene are indicated. *GLR1*, *Q9DCE9*, *COPS3*, and *LLGL1* indicate polymorphic markers (8). MMU11, mouse (*Mus musculus*) chromosome 11; HSA, human (*Homo sapiens*) chromosome loci. (*B*) The genomic structure of rat *Bhd* homologue. Exons are denoted by shaded boxes with numbers. Below the overall exon–intron structure, a CpG island found around exon 1 is shown in an enlarged view. Each vertical line marks a CpG sequence.

Materials and Methods

Tissues Samples, Mapping, and RC-Derived Cell Lines. Rat tissues were dissected and immediately frozen in liquid nitrogen and stored at −80°C until use. The RC gene of Nihon rat (the Nihon gene) is being carried in the Sprague–Dawley (SD) strain of rats. A genealogically unrelated strain, inbred Brown Norway (BN)

Abbreviations: RC, renal carcinoma; BHD, Birt–Hogg–Dubé; LOH, loss of heterozygosity.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AB096213).

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Table 1. Primers for amplification of exons

Exon	5′ primer	3′ primer	Product size, bp
3	GATGCCGTCCTTCCCTACA	AAGGCTAAAACCAGCTCACG	329
4	ATTTTGTCGTCCTTTGTGTGC	ACAGCAATGAACCTATCTGTC	232
5	ACTTCCCCTAAAGTTACTGTG	CCAGCATCCATGCCAAACG	298
6	ACTGAAAGGTTCGGTGGTAG	TGTTCTAATGGCTCACAGATG	264
7	AGCCTCAGGTTCAGTTTTCC	TATGGCAGCCACAAGCCAG	221
8	ATGCTCACTCCATACCTGTC	TCTTCTCAGTTCGGACCACA	259
9	CCTAAAGAACTATGTGCTGTG	AGTCAAGCATTAGCATACAGG	175
10	TTCTGAGCACCTTGTTGTCC	GGCAGTACGTGTCTCACAG	178
11, 12	ACTTTACAAGCTAGCACGTGT	AGCTGTCAAGGCAAGGCTG	381
13	TGTGGGTCTAAGTGTTCATCT	ATGTACACCACCTTCCAGAC	269

(Charles River Breeding Laboratories), which does not carry the mutation, was used in mating with the Nihon male (Nihon/+, SD strain) to produce F_1 hybrids. Nihon rats are diagnosed by detecting microscopic and/or macroscopic kidney tumors after a unilateral nephrectomy. Then, one heterozygote (Nihon/+) F_1 male (designated BX0011-203 in this study) was in turn mated to BN (+/+) females to produce backcross progeny. Among 113 backcross {F_1 [SD (Nihon/+) × BN (+/+)] × BN (+/+)} animals, 63 (56%; 30 males and 33 females) were histologically found to have multiple renal tumors (adenomas) at 13 weeks of age and 50 (44%; 24 males and 26 females) were negative, which is not significantly different from the expected 50%. We selected 13 weeks of age for phenotype assessment in this study because histologically adenomas were observed and differential diagnosis was easy to perform between carrier and noncarrier rats. Moreover, phenotypic expression had reached a plateau by 13 weeks of age, and this critically avoided false negatives in carrier rats. Control animals never developed adenomas/RCs at this age.

RC cell lines from Nihon rat were established by limited dilution (one cell per well in 96-well plates) cloning and maintained in DMEM/high glucose/10% fetal calf serum. A tumor cell line, Lk9d(L), from the Eker rat was maintained in medium described previously (9).

RNA Isolation, Northern Blot Analysis, and cDNA Amplification. Total RNAs were isolated by TRIzol reagent (Invitrogen). Northern blotting and RT-PCR were performed as described previously (2). For amplification of mouse *Bhd* cDNA fragments, total RNAs from whole mouse embryos at embryonic day 13.5 and the following primer sets were used: primer set 1, B2-46A, 5′-CAAGAAGTCGGACATGTGTG-3′ (forward), and B2-46B, 5′-CCCACAAGTTGTCATCACTG-3′ (reverse); primer set 2, B2-50A, 5′-TCATGGGGAATCAGGTGATC-3′ (forward) and B2-50B, 5′-CGTCATCCAGAACTTCAGCA-3′ (reverse). For amplification of rat *Bhd* cDNA fragments, the following primer sets were used: primer set 1, BHDRI, 5′-CCCTCTGCCACTTCTGCGA-3′ (forward), and BHDRG, 5′-AAGCCATGTTGCTCATCACC-3′ (reverse); primer set 2, BHDRA, 5′-GCACCCAGGTTATATCAGTC-3′ (forward), and BHDRB, 5′-AGACAGGTTCTGGTTGGTCA-3′ (reverse); primer set 3, BHDRH, 5′-TTGTGGTGACCAGCGGTAG-3′ (forward), and BHDRF, 5′-CTCCGTGACTCTGTAGCTG-3′ (reverse). Amplified cDNA fragments were characterized by direct sequence analysis. 5′- and 3′-RACE were performed as described previously (10). Briefly, for 5′-RACE, first-strand cDNAs were synthesized by using total RNAs from a whole rat embryo with primer BHDG7 (5′-GGCTGACGTACTTGATAGAG-3′). After circularization by RNA ligase, first inverse PCR was performed by using a primer set, BHDG8 (5′-CAAGAAGTCAGACATGTGCG-3′, forward) and BHDG9 (5′-CTTCCTCAGCCTGCTCAAC-3′, reverse). Then, portions of first PCR mixture were subjected to second PCR using a primer set, BHDRA and BHDG10 (5′-GAGCGTGTAGAACCTCCGT-3′, reverse). For 3′-RACE, first-strand cDNA was synthesized by using a primer, cDNA-1 (5′-GAGAGAGAAGAGAGAGAGAACTAGTGCGGCCGCTTTTTTTTTTTTTTTT-3′). For first PCR and second, nested PCR, the following primers were used: BHDRH, 5′-TTGTGGTGACCAGCGGTAG-3′ (forward) and NOT3-1, 5′-AGAACTAGTGCGGCCGCTT-3′ (reverse) for first PCR; BHDRK, 5′-CAAGGAGGACACCCAGAAG-3′ (forward) and NOT3-1 for second PCR. cDNA fragments amplified by RACE were sequenced both directly and after subcloning in plasmid vectors.

DNA Isolation, Southern Blot Analysis, and Loss of Heterozygosity (LOH) Analysis. Tail DNA was extracted from 113 backcross animals at 13 weeks of age (8). DNA was isolated by digestion with proteinase K and extraction with phenol/chloroform as described previously (2). Southern blotting and genomic PCR were performed as described previously (4). Primers used for detection of LOH by genomic PCR were BHDG5, 5′-ATCATCCTTTCTCTCAGCCTGTGG-3′ (forward), and BHDG6, 5′-CGGAATGGACAAGGTTCACATCTC-3′ (reverse). For analysis of the germ-line insertion in "C tract" by genomic PCR, primers BHDRI and BHDRJ (5′-CTCGCACATGTCTGACTTCT-3′, reverse) were used. For search of second-hit mutations, genomic PCR and direct sequence analysis were performed by using DNAs extracted from tumor and matched normal kidney tissue. The entire coding regions of rat *Bhd* (exons 3–13) were screened for mutation. Primers used are listed in Table 1.

Antibody Production and Western Blot Analysis. Antibodies against 15 amino acid residues (Leu-Met-Ser-Thr-Val-Arg-Ser-Pro-Thr-Ala-Ser-Glu-Ser-Arg-Asn) of human folliculin (human exon 14) were generated by immunizing rabbits. This sequence differs by one residue from the rat sequence (rat exon 13). Antibodies were affinity-purified from serum. Cell extracts were obtained by lysis with sample buffer for SDS/PAGE, and protein concentration was determined by DC (detergent compatible) protein assay (Bio-Rad). After addition of 2-mercaptoethanol, equal amounts of proteins were separated by SDS/PAGE (10% gel) and transferred onto nylon membranes. Reaction of primary and secondary antibodies and chemiluminescent detection were performed as described previously (11).

Results and Discussion

Fine Mapping Identifies the Rat *Bhd* Gene Locus with Zero Recombination. In this study, we extended the previous Nihon rat linkage analysis through fine mapping with markers close to the Nihon gene locus and obtained zero recombination (0/113) at two loci (*Q9DCE9*, human chromosome unknown, and *COPS3*, human chromosome 17p11.2, respectively) (6). During our analysis, the

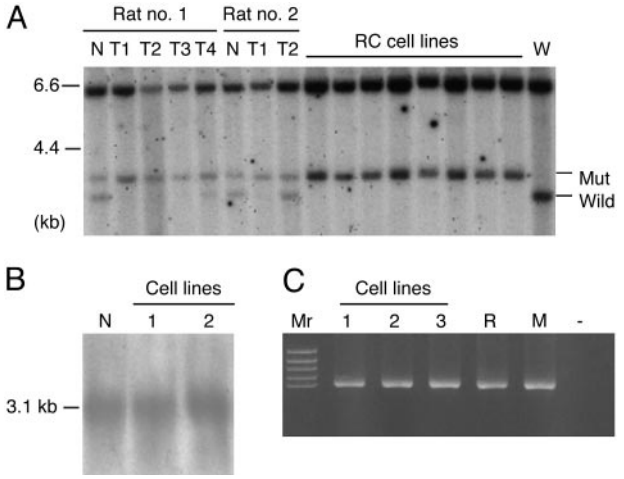

Fig. 2. LOH and expression of the *Bhd* gene in Nihon rat RCs. (*A*) Representative Southern blot analysis of RCs with a *Bhd* probe. DNAs from normal tissues (N) and primary RCs (T) from two Nihon rats and Nihon rat-derived RC cell lines were analyzed by *Hin*cII digestion. Positions of Nihon rat-associated band (Mut) and wild-type band (Wild) are shown on the right. (*B*) Northern blot analysis of Nihon rat-derived RC cell lines (NR). Total RNAs from two NR with LOH at the *Bhd* locus (lanes 1 and 2) and normal kidney (lane N) were analyzed by *Bhd* probe. (*C*) RT-PCR analysis of Nihon rat-derived RC cell lines. Total RNAs from three cell lines with LOH of *Bhd* (lanes 1–3) and rat and mouse embryos (lanes R and M) were subjected to amplification of *Bhd* cDNA. Lane Mr, molecular weight markers; lane −, negative control without first-strand cDNA template.

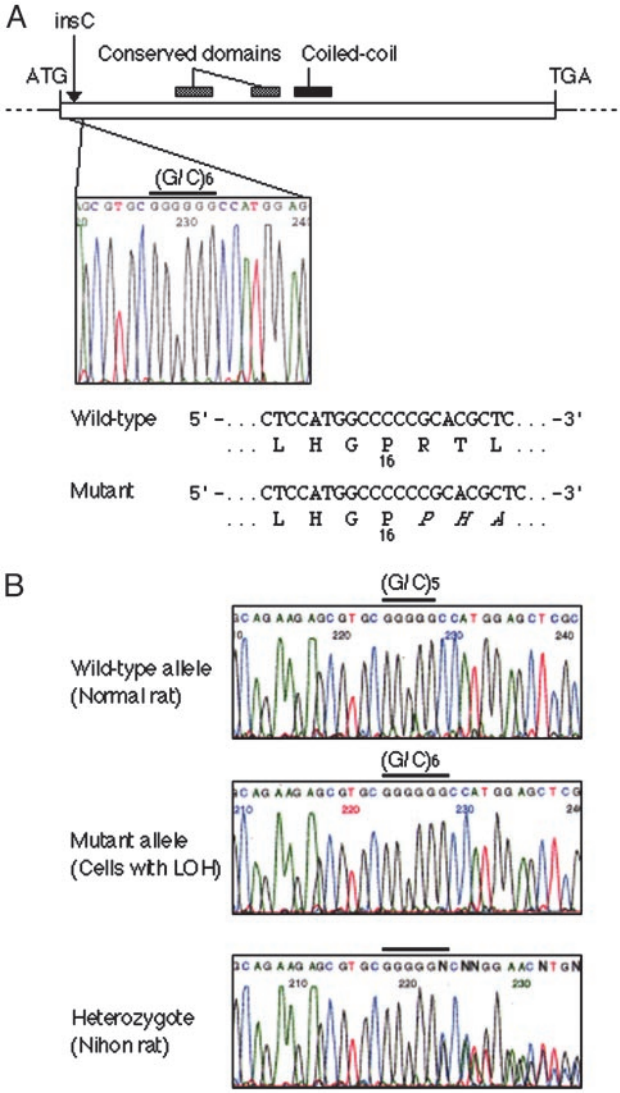

Fig. 3. A germ-line mutation of *Bhd* homologue found in the Nihon rat. (*A*) A cytosine (C) insertion in the *Bhd* cDNA found in RC cells in exon 3. The *Bhd* cDNA from the Nihon rat-associated allele in RC cells is schematically represented. The coding region of *Bhd* is shown by an open box. Regions encoding evolutionarily conserved domain and coiled-coil motif are denoted by shaded and filled boxes, respectively. The position of the cytosine insertion is marked by an arrow (insC). A representative chromatograph (reverse sequence) of direct sequence analysis of the amplified *Bhd* cDNA product is shown. A C/G_6 tract replaces wild-type C/G_5 tract in the sequence (bar). Below the chromatograph, reading frames of wild-type and mutant cDNAs are compared. As a result of the C insertion, a frameshift occurs after codon 17. Italics denote incorrect amino acid sequence generated by insertion. (*B*) Genomic sequence analysis of *Bhd* gene in the Nihon rat. Genomic DNAs of wild-type rat, RC cell with LOH of *Bhd* and Nihon rat heterozygote are analyzed for exon 3 sequence. Representative chromatographs (reverse sequence) are shown. In the Nihon rat, peaks are overlapped after the insertion site.

human *BHD* gene and its mouse homologue were identified (7, 8, 12). Mouse *Bhd* cDNA fragments were amplified and used as probes for linkage analysis to localize homologous sequence in the rat genome. Complete concordance of segregation between putative rat *BHD* homologue (*Bhd*) and renal phenotype of the Nihon rat was found. Thus, rat *Bhd* was localized on RNO10 and tightly linked to the causative gene of the Nihon rat (0/113 = zero recombination, Fig. 1*A*). Interestingly, the Eker (*Tsc2*) gene is located on the proximal part of the same chromosome 10 (2). Thus, RC-related genes are located on rat chromosome 3 (*Tsc1* and *Wt1*), rat chromosome 4 (*Vhl* and *c-Met*), and rat chromosome 10 (*Tsc2* and Nihon).

A Germ-Line Mutation in the Rat *Bhd* Homologue in the Nihon Rat and LOH in the Tumors. By searching the rat genomic database at the National Center for Biotechnology Information (released on July 21, 2002), two bacterial artificial chromosome (BAC) clones, CH230-13G20 and CH230-61A17, were found to show homology with the human/mouse *Bhd* cDNA sequence. Collectively, sequences corresponding to exons 5–13 of the human *BHD* gene were found (data not shown). Considering these putative exon sequences in rat BACs and homology between 5′- and 3′-terminal parts of human and mouse *Bhd* coding regions, primer sets were designed to amplify rat *Bhd* cDNA fragments by RT-PCR and 5′- and 3′-RACE. These primers successfully amplified rat *Bhd* cDNAs, and sequence analysis revealed a gene of 2,846 nucleotides predicted. The rat *Bhd* gene is predicted to encode a gene product (folliculin) of 579 amino acid residues showing 93% and 97% sequence identity with human (GenBank accession no. NP 659434) and mouse (GenBank accession no. AAH25820) folliculins, respectively (data not shown; rat *Bhd* gene product, GenBank accession no. AB096213). Two 5′ noncoding exons were found in the rat *Bhd* gene by database search, whereas three noncoding exons were found in human *BHD* (Fig. 1*B*) (12).

By Southern blot analysis using these cDNAs as probes and by genomic PCR using a sequence-length polymorphism (SLP) found in intron 8 (corresponding to intron 9 in the human genomic sequence), primary RCs and RC cell lines (NRs) from the Nihon rat (male, 10 months old) were examined for LOH at the *Bhd* locus (Fig. 2*A* and data not shown). Eleven primary tumors (from four Nihon rats) and NRs (seven lines from one tumor) were examined. Ten of 11 tumors and all NRs showed LOH at the *Bhd* locus. Importantly, in all the LOH-positive cases, the Nihon rat mutant allele was retained (Fig. 2*A*).

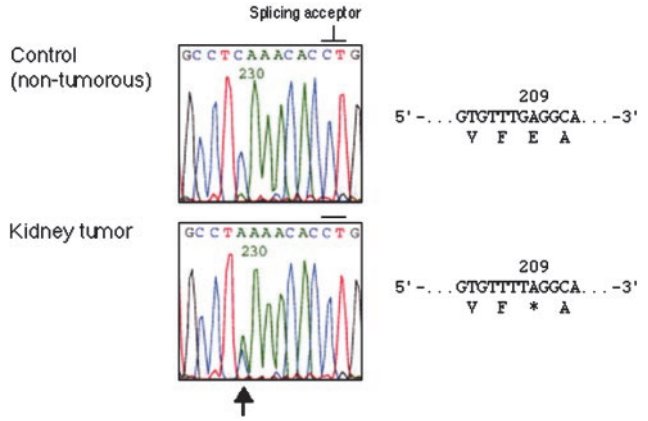

Fig. 4. An intragenic *Bhd* mutation found in an LOH-negative primary RC (T2 in Fig. 2*A*) in the Nihon rat. (*Left*) Chromatographs of direct sequence analysis for exon 6. Reverse sequences of exon 6 in a LOH-negative primary RC and control tissue are shown. Near the splicing acceptor site, C/A overlapped peak was detected in the RC sample (arrow), indicating that a mutation was introduced at this site. (*Right*) A nonsense mutation found in exon 6. First G of codon 209 (GAG for Glu) in the wild-type sequence is replaced by T in the RC. This G → T transversion creates a premature stop codon (asterisk).

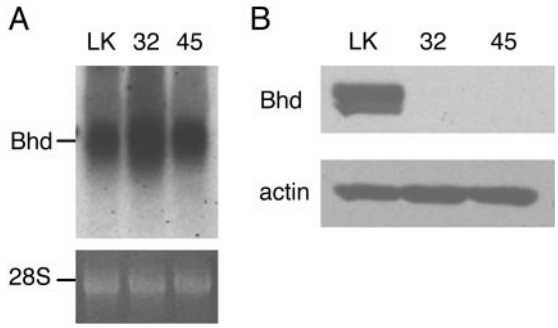

Fig. 5. Loss of expression of the folliculin proteins in Nihon rat RC cell lines. (*A*) Northern blot analysis of *Bhd* expression in RC cells. Total RNAs from the Eker rat RC cell line (LK) and two Nihon rat RC cell lines (NR 32 and 45) were analyzed by a *Bhd* probe. 28S RNA bands stained with ethidium bromide are shown below. (*B*) Western blot analysis of folliculin expression in RC cell lines. Total extracts from cells shown in *A* were analyzed by Western blotting with anti-folliculin antibody. Bands of ≈66-kDa folliculin are absent in Nihon rat RC cells (NR 32 and 45). Results of Western blot analysis for β-actin are shown below as a control.

RNA samples from RC cells showing LOH and normal control kidney tissues were analyzed for expression of the rat *Bhd* homologue. By Northern blot analysis, one major 3-kb band was detected in normal kidney tissue samples by the *Bhd* probe (Fig. 2*B*). No aberrant *Bhd* mRNA species were detected and *Bhd* expression was at levels comparable with normal tissue. By RT-PCR, cDNA fragments with comparable size were amplified from both normal tissues and RC cells (Fig. 2*C*). Sequence analysis of these fragments revealed that there was an insertion of a cytosine (C) in a C tract in the cDNA from RC cell lines within exon 3 (Fig. 3*A*). Instead of a C_5 tract as seen in the wild-type allele, a C_6 tract was found in the mutant allele. As a result of this insertion, a frameshift occurred and the ORF was disrupted at codon 17, creating a premature termination codon 26 aa downstream. The resulting mutant protein is lacking the vast majority of the normal rat Bhd (folliculin) sequence (Fig. 3*A*). Independent Nihon rats that developed RCs carried this insertion in their genomic DNA sequences as a germ-line mutation (Fig. 3*B*). In one LOH-negative tumor (Fig. 2*A*) without loss of the wild-type allele, a G → T transversion (G926T), which caused a premature termination of the protein at codon 209 (Glu to stop), was found in exon 6 by direct sequence analysis (Fig. 4). Thus inactivation of both alleles of the rat *Bhd* gene supports a tumor-suppressor mechanism.

Interestingly, the type of mutation found in the rat, a C insertion in a homonucleotide tract, is the type of mutation most commonly found in the human BHD syndrome (12). Slippage of the DNA polymerase during replication has been suggested as a possible mechanism of mutation in homonucleotide tracts and may cause such regions of genomic DNA to be hypermutable (13). The hypermutable C tract in exon 11 of the human *BHD* gene is interrupted in the rat *Bhd* homologue.

Anti-Folliculin Does Not Detect a Truncated Mutant BHD Protein. Next, we generated rabbit anti-folliculin antibodies that recognize the carboxyl-terminal peptide of folliculin (exon 13 in rat, exon 14 in human), and we found that the antibody reacted with an ≈66-kDa protein in the extract of the Lk9d(L) RC cell line from the Eker rat by Western blot analysis (Fig. 5*B* and data not shown). This molecular mass is similar to the 64-kDa predicted size of rat folliculin. In contrast to Lk9d(L), an ≈66-kDa protein was not detected in the NR cells (Fig. 5*B*). As NR cells expressed *Bhd* mRNA from the mutant allele at the same level with Lk9d(L) (Fig. 5*A*), this result indicated that the germ-line mutant allele does not produce normal folliculin. In addition, there was no sign of aberrant products initiated from internal initiation codons. Taken together, these results indicate that the loss of folliculin function by a two-hit mechanism is a critical step for the renal carcinogenesis in the Nihon rat.

We could not find homozygous mutants at the 14th day of gestation after matings of two carriers, indicating that homozygous mutant status could be lethal at an early stage of fetal life (6).

The BHD syndrome, originally described by Birt, Hogg, and Dubé in 1977 (14), is characterized by hair follicle hamartomas, kidney tumors, and spontaneous pneumothorax. The recent discovery of the human *BHD* gene (14) is an important first step toward understanding the mechanism of tumorigenesis in BHD patients; however, the function of the *BHD* gene product (folliculin) remained to be elucidated. During the preparation of this manuscript, a missense mutation that changed a highly conserved histidine to arginine at codon 255 was identified in the dog *BHD* homologue associated with canine renal cystoadenocarcinoma and nodular dermatofibrosis (RCND). However, no second-hit mutations or LOH in the dog renal tumors was reported (15). Constructing transgenic Nihon rats with the wild-type *Bhd* gene should allow us to ascertain whether suppression of the Nihon phenotype is possible. Thus, the Nihon rat continues to be a valuable experimental model for studying *BHD* gene function and its role in tumorigenesis.

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