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 adenomas by 8 weeks of age, and 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–Lgl1 locus–Myhse gene. We now describe a germ-line mutation in the Birt–Hogg–Dubé (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 Lgl1 (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 homologues, mutations in which predispose to the renal cancer phenotype in the Nihon rat.

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)
Table 1. Primers for amplification of exons

<table>
<thead>
<tr>
<th>Exon</th>
<th>5' primer</th>
<th>3' primer</th>
<th>Product size, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>GATGCGCCTCCCTCTCCTACA</td>
<td>AAGGCTAAACACGCTCAG</td>
<td>329</td>
</tr>
<tr>
<td>4</td>
<td>ATTTTGTGCTCCTTTTGTTGTCG</td>
<td>AACGACGAAGCTCTAGTCTGC</td>
<td>232</td>
</tr>
<tr>
<td>5</td>
<td>ACTCCCCCTAAAGTACTGTG</td>
<td>CACGACTCATTGCAAAACG</td>
<td>298</td>
</tr>
<tr>
<td>6</td>
<td>ACGTAAAGGTCGGCTGGTAG</td>
<td>TGGTACTAATGGCTCAGAGTG</td>
<td>264</td>
</tr>
<tr>
<td>7</td>
<td>AGCTCAGGTTGCTAAGTTTCCT</td>
<td>TAGTGGACACCAAGCCACG</td>
<td>221</td>
</tr>
<tr>
<td>8</td>
<td>ATGCTCAGTCTACATCTGTGCT</td>
<td>TCTCTCAGTGGGAGCACA</td>
<td>259</td>
</tr>
<tr>
<td>9</td>
<td>CCTAAGAAGACTATGGTGTG</td>
<td>AGTCAGATTAGCATTACAGG</td>
<td>175</td>
</tr>
<tr>
<td>10</td>
<td>TCTGAGACCTCCTGGTTC</td>
<td>GGGAGTACGGTCTCAGAG</td>
<td>178</td>
</tr>
<tr>
<td>11/12</td>
<td>ATCTTACAGAAGCAGTGGTC</td>
<td>ATGGGAGTACGACCTTACGAC</td>
<td>381</td>
</tr>
<tr>
<td>13</td>
<td>TGTGGGTCATAGGGTCACCTCT</td>
<td>ATGTACACACATTTCCAGAC</td>
<td>269</td>
</tr>
</tbody>
</table>

(Charles River Breeding Laboratories), which does not carry the mutation, was used in mating with the Nihon male (Nihon+/+, SD strain) to produce F1 hybrids. Nihon rats are diagnosed by detecting microscopic and/or macroscopic kidney tumors after a unilateral nephrectomy. Then, one heterozygote (Nihon+/+) F1 male (designated BX0011-203 in this study) was in turn mated to BN (+/+ +) females to produce backcross progeny. Among 113 backcross {F1 [SD (Nihon+/+) × BN (+/+ +)] × BN (+/+ +)} animals, 63 (56%; 30 males and 33 females) were histologically detected microscopic and/or macroscopic kidney 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 adenosomas/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'-GACAGGTCTTGTTGTTGAG-3' (forward), and B2-46B, 5'-CCACAAAGTGTGCTACTG-3' (reverse); primer set 2, B2-50A, 5'-CTAGGGAATCAGGTTGAC-3' (forward) and B2-50B, 5'-CGTCACTCCAGAACTTCCAG-3' (reverse). Amplification of rat Bhd cDNA fragments, the following primer sets were used: primer set 1, BHDR1, 5'-CCCTCTGACCT-AGT GC-3' (forward), and BHDRG, 5'-AGGCCATGTT-GCTCATCACC-3' (reverse); primer set 2, BHDRA, 5'-GCACCCAGGTTATATCAGTC-3' (forward); primer set 3, BHDHH, 5'-TTGTGGGTCTAAGTGTTCATCT ATGTACACCACCTTCCAGAC 269

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'-ATCATCCTTCTCTCAGCTGGGTTG3' (forward), and BHDG6, 5'-CGGAATTGGCAGGTGGCTCAGAC 3' (forward) and NOT3-1, 5'-AGAACTAGTGCGGCCGCTT-3' (reverse) for first PCR; BHDKR, 5'-CAAGGGACACCC-AGAAG-3' (forward) and NOT3-1 for second PCR. cDNA fragments amplified by RACE were sequenced both directly and after subcloning in plasmid vectors.

Antibody Production and Western Blot Analysis. Antibodies against 15 amino acid residues (Leu-Met-Ser-Thr-Val-Ar g Ser-Pro-Thr Ala-Ser-Glu-Ser-Arg) 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 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
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. 1A). 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. AAH39820) 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. 1B) (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. 2A 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. 2A).

![Fig. 2. LOH and expression of the Bhd gene in Nihon rat RCs.](image-url)

- **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 HindIII 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.](image-url)

- **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 tract replaces wild-type C/G 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.
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. 2B). 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. 2C). 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. 3D). Instead of a C tract as seen in the wild-type sequence, a C 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 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).

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. 5B 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. 5B). As NR cells expressed Bhd mRNA from the mutant allele at the same level with Lk9d(L) (Fig. 5A), 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 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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4. Okimoto, K., Kouchi, M., Kakawa, E., Toyosawa, K., Kouijitani, T., Tanaka,