Inactivation of BHD in Sporadic Renal Tumors

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ABSTRACT

Studies of families with Birt-Hogg-Dubé syndrome (BHD) have recently revealed protein-truncating mutations in the BHD gene, leading to tumor-igenesis of the skin and of different cell types of kidney. To additionally evaluate the role of BHD in kidney tumorgenesis, we studied 39 sporadic renal tumors of different cell types: 7 renal oncocytomas, 9 chromophobe renal cell carcinomas (RCCs), 11 papillary RCCs, and 12 clear cell RCCs. We screened for BHD mutations and identified a novel somatic mutation in exon 13: c.1939_1966delinsT in a papillary RCC. We performed loss of heterozygosity (LOH) analysis on 28 matched normal/tumor sets, of which 10 of 28 (36%) demonstrated LOH: 2 of 6 (33%) chromophobe RCCs, 5 of 6 (83%) papillary RCCs, 3 of 12 (25%) clear cell RCCs, but 0 of 4 renal oncocytomas. BHD promoter methylation status was examined by a methylation-specific PCR assay of all of the tumors. Methylation was detected in 11 of 39 (28%) sporadic renal tumors: 2 of 7 (29%) renal oncocytomas, 1 of 9 (11%) chromophobe RCCs, 4 of 11 (36%) papillary RCCs, and 4 of 12 (33%) clear cell RCCs. Five tumors with methylation also exhibited LOH. Mutation and methylation were absent in 9 kidney cancer cell lines. Our results showed that somatic BHD mutations are rare in sporadic renal tumors. The alternatives, LOH and BHD promoter methylation, are the two possible inactivating mechanisms involved. In conclusion, unlike other hereditary kidney cancer-related genes (e.g., VHL and MET), which are cell type-specific, BHD is involved in the entire spectrum of histological types of renal tumors, suggesting its major role in kidney cancer tumorgenesis.

INTRODUCTION

RCC is the sixth leading cause of cancer deaths in the United States and accounts for 3% of adult malignancies (1). Approximately 32,000 Americans were diagnosed with RCC in 2001; nearly 40% of those will die because of metastasis (2). The most common RCC is the clear cell subtype (~75%), followed by the papillary (~15%), and the chromophobe (~5%) subtypes. Renal oncocytomas, on the other hand, is a benign neoplasm accounting for ~5% of renal tubular neoplasms.

Genetic studies of hereditary kidney cancer syndromes such as VHL, hereditary papillary renal carcinoma, and hereditary leiomyomatosis and renal cell cancer have led to the identification of a number of kidney cancer-related genes (3–5). These genes are cell type-specific and, interestingly, they are also involved in the sporadic counterparts of the same tumor types observed in hereditary cases. In clear cell RCC, 57% demonstrate somatic mutations in the von Hippel-Lindau gene (VHL; Refs. 3, 6, 7), and 19% show inactivation of the VHL gene by methylation (8). In addition, LOH of the VHL locus at chromosome 3p25 has been detected in >90% of the clear cell subtype (6, 9). The MET and FH genes were found to cause hereditary papillary renal carcinoma and hereditary leiomyomatosis and renal cell cancer, respectively (4, 5), and their somatic mutations have been identified in the sporadic counterparts of their hereditary papillary subtypes (10, 11).

BHD is an inherited, autosomal dominant neoplasia syndrome characterized by a triad of cutaneous lesions comprising multiple fibrofolliculomas, trichodiscomas, and acrochordons (12). A wide spectrum of phenotypic features has been found related to BHD (13). Besides spontaneous pneumothorax (14–17) and colorectal neoplasia (14, 16, 18), diverse classes of renal tumors such as renal oncocytoma, chromophobe, papillary, and clear cell RCCs have been reported in BHD patients (15–17, 19, 20). Most recently, the Birt-Hogg-Dubé gene (BHD) has been identified (20), and germ-line mutations of BHD were shown to cause hereditary predisposition to a wide histological spectrum of renal tumors (19, 20). Because BHD is a newly identified novel kidney cancer-related gene, little is known about its contribution to sporadic kidney tumorigenesis. Somatic inactivations of BHD in sporadic renal tumors have yet to be investigated.

Inactivating mutations, LOH, and promoter methylation studies are some of the standard approaches to verifying TSGs. LOH, defined as the loss of one allele at a constitutional (germ-line) heterozygous locus, has been accepted as a hallmark of one of the two hits required for the inactivation of TSGs in cancer (21). A high frequency of consistent LOH has been used as a reliable DNA marker for diagnosis and prognosis of cancer. On the other hand, DNA methylation is an epigenetic alteration that disrupts TSG functions and leads to tumorgenesis. CpG islands are CG-rich areas of ~1 kb, usually located in the vicinity of genes and often found near the promoter of widely expressed genes (22–24). Methylation of CpG dinucleotides in the promoter region of TSGs inhibits transcription by interfering with transcription initiation and induces inactivation of the TSGs (25).

In this study, we evaluated the role of BHD in 39 cases of sporadic renal tumors. We screened the BHD mutation from 7 renal oncocytomas, 9 chromophobe RCCs, 11 papillary RCCs, and 12 clear cell RCCs. LOH analysis was performed on 28 matched normal/tumor samples and the BHD promoter methylation profile was determined in all of the tumors. We also examined the mutation and methylation status of 9 kidney cancer cell lines.

MATERIALS AND METHODS

Tissue Samples and DNA Extractions. Thirty-nine tumor samples (28 of which have matched normal tissues) consisting of 7 renal oncocytomas, 9 chromophobe RCCs, 11 papillary RCCs, and 12 clear cell RCCs were collected from the Cooperative Human Tissue Network of the National Cancer Institute, and from Spectrum Health Hospital and Metropolitan Hospital. Each participating patient was examined, and each provided written informed consent. This study was approved by the Institutional Review Board of the Van Andel Research Institute. Genomic DNA was extracted from fresh-frozen tumor tissues using a Wizard Genomic DNA purification kit (Promega, Madison, WI) in accordance with the manufacturer’s instructions.

Kidney Cancer Cell Lines. We obtained nine tumor-derived cell lines from the American Type Culture Collection (Manassas, VA): SW-839, Caki-2, 786-O, and 769-P (derived from clear cell kidney carcinomas); A-704, A-498,

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3 The abbreviations used are: RCC, renal cell carcinoma; VHL, von Hippel-Lindau; LOH, loss of heterozygosity; BHD, Birt-Hogg-Dubé; TSG, tumor suppressor gene.
and ACHN (derived from kidney carcinomas); SW-156 (derived from a kidney hypernephroma); and Cal-1 (derived from a skin metastatic site of clear cell kidney carcinoma). We grew all cells in DMEM containing 10% fetal bovine serum, 100 units/ml penicillin G, and 100 µg/ml streptomycin (all reagents from Life Technologies, Inc., Rockville, MD). All of the cultures were kept in a 5% CO₂ incubator at 37°C.

**Sequencing Analysis.** The entire coding region of the BHD gene (exons 4–14) was screened for mutations. Primer sequences and PCR conditions were according to Nickerson et al. (20). PCR was performed using a DNA Engine Tetrad (MJ Research, Waltham, MA). PCR products were analyzed on standard 1.5% agarose gels stained with ethidium bromide (0.5 µg/ml) before purification with Multiscreen PCR cleanup plates (Millipore, Molsheim, France). Sequencing reactions were performed using Big Dye Terminator (Applied Biosystems, Foster City, CA), purified through Sephadex G-50 (Amersham Biosciences, Uppsala, Sweden), and run on an ABI 3700 genetic analyzer (Applied Biosystems). We aligned and analyzed all of the sequences by Blast 2 Sequences (26) and manually verified them again. All of the sequence changes were verified by reamplification of the corresponding BHD fragment and sequencing of both DNA strands.

**LOH Analysis.** LOH was performed on 28 matched normal/tumor tissue pairs. Allelic deletions of the chromosome 17p region containing the BHD gene were assessed using the microsatellite markers D17S740 and D17S2196. The region is ~0.3 Mb. PCR was performed in a 7.5 µl reaction volume containing 0.17 µM each of fluorescence-labeled forward and unlabeled reverse primer, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 4 mM MgCl₂, 0.3 units AmpliTaq Gold polymerase (Applied Biosystems), 0.25 mM deoxynucleoside triphosphates (Invitrogen Life Technologies, Inc., Gaithersburg, MD), and 15 ng of genomic DNA. Amplification was done in a DNA Engine Tetrad (MJ Research) with an initial cycle of 95°C for 10 min; followed by 10 cycles of 94°C for 15 s, 55°C for 15 s, and 72°C for 30 s; and 20 cycles of 89°C for 15 s, 55°C for 15 s, and 72°C for 30 s, with a final extension at 72°C for 10 min.

One µl of each PCR product was added to 5 µl of DNase-free, RNase-free distilled water, 10 µM of Hi-Di formamide, and 0.2 µl of ROX 400HD size standard, and denatured at 95°C for 5 min before loading the samples into an ABI Prism 3700 Genetic Analyzer (Applied Biosystems). Analysis of raw data and assessment of LOH were performed with Genescan v. 3.7 and Genotyper v. 3.7 software (Applied Biosystems). LOH was defined according to the following formula: LOH index = (T2/T1)(N2/N1), where T was the tumor sample, N was the matched normal sample, and 1 and 2 were the intensities of smaller and larger alleles, respectively (27). If the ratio was <0.67 or >1.3, the result was determined to be LOH. Tumors with positive LOH around the BHD region will be analyzed with another marker, D17S1678, to evaluate the LOH result was determined to be LOH. Tumors with positive LOH around the BHD region as well. D17S1678 is 40-kb telomeric to the BHD region, and the region was analyzed by scoring the presence of the smaller and larger alleles, respectively (27). If the ratio was >0.6, the result was determined to be LOH.

**Genomic Characterization of BHD Promoter Region.** The presence of CpG islands was analyzed using the CpG plot program (4), which is an expansion of the CpG plot program from EMBOSS (24). The CpG plot program is based on the results of discriminant analysis between the promoter-associated and non-associated CpG islands (28). This program enables an efficient mapping of human promoters with 2-kb resolution, if there is a CpG island in the interval (~500 to +1500) around a transcription start site. In this study, a CpG-rich region is defined as stretches of DNA with both the average of G+C content >50% and the average of CpG ratio (observed/expected) >0.6.

**Methylation Analysis.** We examined the promoter methylation status of BHD in all 39 of the tumor samples. DNA methylation was determined by a methylation-specific PCR approach (29, 30). DNA was treated with sodium bisulfite, which converted all of the unmethylated cytosines to uracils, whereas methylated cytosines remained unchanged. Briefly, 2 µg of DNA was denatured by incubation with 0.2 M NaOH at 37°C for 10 min. Cytosines were methylated in 3 µM sodium bisulfite (adjusted to pH 5.0; Sigma Chemical Co., St. Louis, MO) and 10 mM hydroquinone (Sigma) in a 50°C water bath for 16 h. The samples were then purified through columns (Microcon YM-100; Millipore, Bedford, MA), desulfonated in 0.3 M NaOH, precipitated with ethanol with glycogen as a carrier, and resuspended in 20 µl of sterile water before storing at −20°C. The specific primers for methylated sequences were designed as follows: BHD-BISF-OF (5'-ATGTGATAGGAAGTTTAGGTGTATATAT-3') as the forward primer and BHD-BISF-OR (5'-ACAAATCACACCAAAACCCC-3') as the reverse primer. Two µl of the bisulfite-treated product was added to 25 µl of total reaction volume containing 2 mM MgCl₂, 0.24 mM of each deoxynucleoside triphosphate (Invitrogen), 0.02 units of TaqDNA polymerase (Invitrogen), and 0.1 µM of each primer. PCR conditions were 95°C for 5 min followed by 35 cycles of 94°C (30 s), 60°C (30 s), and 72°C (45 s), and then 72°C for 7 min.

A nested PCR was then performed using 1 of 25 of the initially amplified products. The primers used were BHD-BISF-IF (5'-GAAATGGTTTTTTTGTACTTCTTTTG-3') and BHD-BISF-IR (5'-CCCAAAACCCC-CAAAACCAAC-3') with conditions similar to those described for the preceding PCR amplification but for 40 cycles. The PCR products were purified using Microcon YM-100 (Millipore). Twenty µl of the 414-bp PCR product were incubated with 0.3 U of RsaI (New England BioLabs, Inc., Beverly, MA) for 2 h at 37°C. λ DNA (0.3 µg) and distilled water were used, respectively, as positive and negative controls. The sizes of the RsaI digestion products were 160 and 254 bp. The restriction enzyme digestion products were then visualized in 2% agarose gels containing ethidium bromide, and the presence of methylation was verified by direct sequencing.

**Statistical Analysis.** The χ² contingency test and Fisher’s exact test were used to compare the occurrence of LOH as well as methylation in each type of renal tumors. Generally, a P < 0.05 is taken as significant.

**RESULTS**

**BHD Mutation Is Not Common in Sporadic Renal Tumors.** Along with another research group, we have identified a number of BHD mutations in BHD patients and in a BHD-related renal tumor (19, 20). To evaluate the occurrence of mutation in sporadic renal tumors, the entire coding region (exons 4–14) of BHD was screened in 39 renal tumors. We found mutation in only one tumor of the papillary type (PRC-3), with an insertion/deletion (indel) c.1939_1966delinsT in exon 13. Unfortunately, there was no matched normal tissue from this 70-year-old female patient. However, the patient did not have any family history or clinical evidence of BHD. The identified mutation represented 2.6% of the total samples, suggesting that somatic mutation of BHD is rare in sporadic renal tumors. We could not detect any mutation in the nine kidney cancer cell lines. Generally, a P < 0.05 is taken as significant.

**LOH Is Observed in Chromophobe, Papillary, and Clear Cell RCCs, but Not in Renal Oncocytoma.** To determine whether BHD is a TSG, we performed LOH on 28 pairs of matched tissue samples. LOH was present in 2 of 6 (33%) chromosome 17q RCCs, 5 of 6 (83%) papillary RCCs, and 3 of 12 (25%) clear cell RCCs. Renal oncocytoma did not exhibit any LOH around the BHD region (0 of 4; 0%). These results are shown in Table 1. In all of the cases of LOH, only one allele of the flanking markers was lost. A χ² contingency test showed that there was a statistically significant difference in the percentage of LOH among different cell types of renal tumors (χ² = 9.45; 0.05 < P < 0.10). Fisher’s exact test confirmed the significant difference between papillary RCC and renal oncocytoma (P = 0.048). All of the tumors with LOH in D17S740 and D17S2196 also demonstrated LOH in D17S1678, the marker close to p53. Nevertheless, the modest frequency (10 of 28; 36%) of LOH observed around the BHD region (0.3 Mb) may still suggest that BHD functions as a TSG in sporadic renal tumors.

**BHD Promoter Sequence Contains 25 CpG Islands, and the G+C Content is 70%.** The full-length BHD sequence of 3674 nucleotides (AF517523) has been characterized recently (20), but its promoter region has not been identified. Thus, we were interested in characterizing the BHD promoter sequence for methylation analysis. We searched an interval of 2 kb upstream of exon 1, including exon 1, and found only one region that is best defined as a promoter region. The region is −207 to +79 of the first base of exon 1, and the full sequence of the predicted BHD promoter is shown in Fig. 1A. BHD

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promoter consists of 25 CpG islands, and the percentage of G+C nucleotides is 70. Methylated and unmethylated cytosines are shown in Fig. 1B.

**BHD Promoter Methylation Is Observed in a Wide Spectrum of Renal Tumors.** A lack of somatic mutations in TSG, as shown in our results, suggested that other mechanisms of inactivation may be involved. The importance of CpG island aberrant methylation as an alternative mechanism for the inactivation of TSGs has been recognized and may be the most common mechanism for gene regulation in cancer (25). Aberrant promoter methylation has been associated with loss of expression of a growing number of tumor-related genes in a variety of cancers (30). To investigate the promoter methylation profile of BHD in sporadic renal tumors, we analyzed all 39 of the cases of renal tumor samples. We found methylation in 11 of 39 (28%) samples, with 2 of 7 (29%) in renal oncocytomas, 1 of 9 (11%) in chromophobe RCCs, 4 of 11 (36%) in papillary RCCs, and 4 of 12 (33%) in clear cell RCCs (Table 1; Fig. 2). There was no statistical significant differences among the four cell types of renal tumors (P > 0.05). All of the tested kidney cancer cell lines did not show methylation.

**DISCUSSION**

We describe a comprehensive study of BHD in sporadic renal tumors and demonstrate that somatic BHD mutation is rare. Alternatively, BHD promoter methylation is observed (28%) in many cell types of sporadic renal tumors. Using two microsatellite markers, D17S740 and D17S2196, which are telomeric and centromeric to BHD, respectively, we found LOH in 36% of informative cases. Five of the samples with LOH were methylated, which is consistent with Knudson’s two hits theory.

To date, only four germ-line mutations have been identified in familial BHD: 1087delAGinsC in exon 7, 1378→1405dup in exon 9, C1844G in exon 12, and 1733insC or 1733delC in exon 11 (polyC tract) as a mutational hot spot (19, 20). We had also reported a somatic mutation of BHD in exon 11 (c.1732delTCinsA) in a BHD-related chromophobe RCC (19). Here, we describe a potential somatic mutation of BHD in sporadic renal tumors. Of 39 cases, we found only one frame-shift mutation in exon 13, c.1939_1966delinsT, which was in a papillary RCC.

Somatic mutations and hypermethylation of VHL are found in 70% of clear cell RCCs and cell lines (3, 6–9, 31, 32). In this study, we showed 3 of 12 (25%) LOH cases and 4 of 12 (33%) BHD promoter methylation, with one sample exhibiting both LOH and methylation, indicating the involvement of BHD in sporadic clear cell RCC tumorigenesis.

Somatic mutations in the tyrosine kinase domain of the MET proto-oncogene have been described in a subset of papillary RCC (4,
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REFERENCES


The occurrence of LOH is relatively low in hereditary BHD renal tumors. Our results in this study show that instead of LOH, BHD promoter methylation may be the explanation for the inactivation. Methylation may inhibit binding of certain transcription factors to their CpG-containing recognition sites (42, 43), or proteins or protein complexes (MeCP2 or McCP1) may bind specifically to methylated CpGs and indirectly inhibit the binding of transcription factors by limiting access to a regulatory element (44–47), causing gene silencing.

In conclusion, the modest frequencies of LOH and methylation of the BHD gene in sporadic renal tumors suggest its role as a TSG, and epigenetic inactivation acts as an alternative to mutations to disrupt its functions. Unlike other cell type-specific kidney cancer genes, inactivation of BHD can be detected in several cell types of sporadic renal tumors, indicating that BHD is intimately involved in kidney tumorigenesis. Additional functional analysis of this interest gene should allow better understanding of the tumorigenesis of renal tumors.

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