[CANCER RESEARCH 63, 4583–4587, August 1, 2003]

Inactivation of *BHD* in Sporadic Renal Tumors[1]

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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 tumorigenesis of the skin and of different cell types of kidney. To additionally evaluate the role of *BHD* in kidney tumorigenesis, 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 (*i.e., 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 tumorigenesis.

INTRODUCTION

RCC[3] 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 oncocytoma, 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 composing 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 contributions 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 tumorigenesis. 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,

Received 1/27/03; accepted 5/6/03.

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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 Caki-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% CO_2 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 flanking 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 $MgCl_2$, 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, 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 μl 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 near the *p53* region as well. D17S1678 is 40-kb telomeric to *p53*. The PCR and genotyping conditions were similar to the previous markers.

Genomic Characterization of *BHD* Promoter Region. The presence of CpG islands was analyzed using the CpG promoter program,[4] which is an expansion of the CpG plot program from EMBOSS (24). The CpG promoter program is based on the results of discriminant analysis between the promoter-associated and nonassociated 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 sulfonated in 3 M 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′-ATGTGGATAGGAAGTTTTAG-GTTGGTTATATTT-3′) as the forward primer and BHD-BISF-OR (5′-ACAAAATCACAC CCAAAACCCCC-3′) as the reverse primer. Two μl of the bisulfite-treated product was added to 25 μl of total reaction volume containing 2 mM $MgCl_2$, 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′-GAAATGGTTTTTTT-TAGTATTTTTAGTTGGTG-3′) and BHD-BISF-IR (5′-CCCAAAACCCC-CAAACCCA-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 *Rsa*I (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 *Rsa*I 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 χ^2 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.

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%) chromophobe 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 χ^2 contingency test showed that there was a statistically significant difference in the percentage of LOH among different cell types of renal tumors ($\chi^2 = 9.45$; $0.05 < P < 0.10$). 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. 1*A*. *BHD*

[4] Internet address: ftp://cshl.org/pub/science/mzhanglab/ioshikhes.

Table 1 *Inactivation mechanisms of BHD in four cell types of sporadic renal tumors*

Cell type	Sample ID	Mutation	LOH	Methylation
Renal Oncocytoma	ORC-1[a]	–[b]	NA[c]	–
	ORC-2[d]	–	–	–
	ORC-3	–	–	–
	ORC-4	–	–	+
	ORC-5	–	–	–
	ORC-6	–	NA	–
	ORC-7	–	NA	+
Chromophobe RCC	**CRC-1**	–	–	–
	CRC-2	–	NA	–
	CRC-3	–	NA	–
	CRC-4	–	NA	–
	CRC-5	–	+	–
	CRC-6	–	–	–
	CRC-7	–	–	–
	CRC-8	–	–	–
	CRC-9	–	+	+
Papillary RCC	PRC-1	–	NA	–
	PRC-2	–	NA	–
	PRC-3	c1939_1966delinsT	NA	–
	PRC-4	–	+	–
	PRC-5	–	+	–
	PRC-6	–	NA	–
	PRC-7	–	+	+
	PRC-8	–	+	+
	PRC-9	–	NA	+
	PRC-10	–	+	+
	PRC-11	–	–	–
Clear cell RCC	**CCRC-1**	–	–	–
	CCRC-2	–	–	–
	CCRC-5	–	–	–
	CCRC-7	–	–	–
	CCRC-9	–	–	–
	CCRC-13	–	–	+
	CCRC-15	–	–	+
	CCRC-16	–	–	+
	CCRC-19	–	+	–
	CCRC-22	–	+	+
	CCRC-23	–	–	–
	CCRC-24	–	+	–

[a] Nonbold lettering, tumor without matched normal tissue.
[b] –, absent; +, present.
[c] NA, not applicable.
[d] Bold lettering, tumor with matched normal tissue.

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

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,

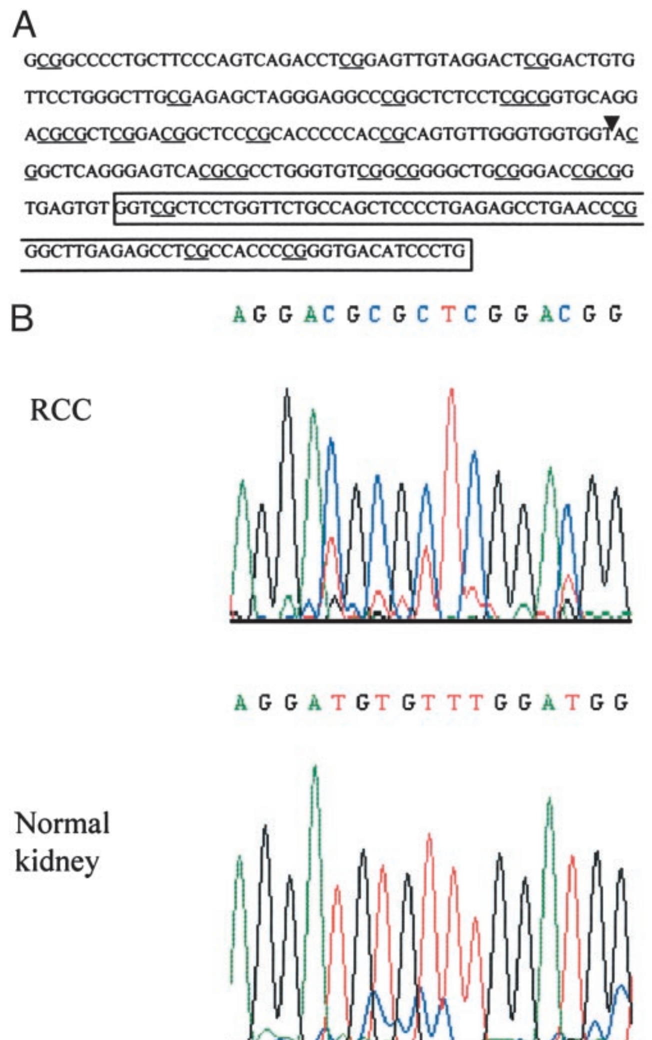

Fig. 1. *A,* sequence of the *BHD* promoter. All 25 of the CpG islands are *underlined*. The *arrow* represents the *Rsa*I recognition site. The beginning of exon 1, which is also part of the promoter region, is *boxed*. *B,* sequences of methylated and unmethylated cytosines in kidney tumor (CCRC-15) and its matched normal tissue, respectively. Methylated cytosines remain unchanged, whereas unmethylated cytosines are converted to thymines in the sequences.

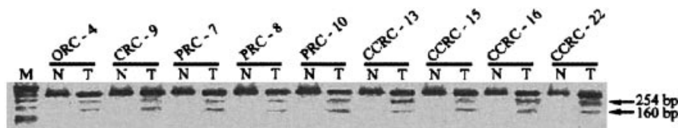

Fig. 2. *BHD* promoter methylation profile of different cell types of sporadic renal tumors. *Rsa*I digestion products are shown as 160 bp and 254 bp. *M* shows the 100 bp DNA ladder. *N,* matched normal tissue; *T,* tumor tissue; *ORC,* renal oncocytoma; *CRC,* chromophobe RCC; *PRC,* papillary RCC; *CCRC,* clear cell RCC.

10). We found 1 novel frame-shift mutation (c.1939_1966delinsT in exon 13 of *BHD*), 5 LOH cases, and 4 *BHD* promoter methylations in 11 papillary RCC samples (6 with matched sets). All three of the methylated samples showed LOH around the *BHD* region, elucidating the two common hits of *BHD* in sporadic papillary RCC. A statistically significant difference between the percentages of LOH among the different cell types demonstrated that it might serve as a molecular biomarker to improve classification of tumor types and subtypes, and is worth additional investigation in a larger sample size.

Genetically, chromophobe RCC is characterized by widespread LOH on chromosomes 1, 2, 6, 10, 13, 17, and 21 (33, 34), as well as hypodiploidy (35). To date, no specific gene mutation in sporadic chromophobe RCC has been described. In this study, we detected LOH around *BHD*, which is located on chromosome 17p, and, thus, our results were consistent with previous reports. In addition, we found one chromophobe RCC with *BHD* promoter methylation, along with LOH. Unlike the novel somatic mutation c.1732delTinsA, which we reported in a BHD-related chromophobe RCC (19), this is the first evidence of epigenetic inactivation revealed in sporadic chromophobe RCC.

In renal oncocytoma, genetic changes include loss of chromosome in 1p, 14q, X, and Y (36–39), or translocations involving 5q35 and 11q13 (40). We did not detect any LOH in our renal oncocytoma samples. However, one *BHD* promoter methylation was identified. Biallelic methylation or haploinsufficiency may address this possibility and is worth additional investigation. Our results suggest that *BHD* plays a major role in kidney tumorigenesis. Unlike other cell type-specific kidney cancer genes (*e.g., VHL* for clear cell RCC and *MET* for papillary RCC), *BHD* alterations occur in four cell types of renal tumors, namely renal oncocytoma and chromophobe, papillary, and clear cell RCCs. This is consistent with the findings in the familial cases where mutations can be found in BHD patients with a wide histological spectrum of renal tumors (19, 20).

p53 is approximately 9 cM telomeric to *BHD*. We found LOH near the *p53* region in all of the tumors with LOH in markers flanking *BHD*. Therefore, in this study, we could not prove conclusively that the inactivation of *BHD* is because of LOH of the gene, as there is a possibility that *p53* and/or other TSGs in that region might be involved. The effects of LOH on the *BHD* gene expression and regulation in renal tumors merit additional investigation.

It has been found that 15 of 88 (17%) hereditary renal tumors demonstrated LOH at five informative 17p microsatellite markers (41). 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 MeCP1) 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 interesting gene should allow better understanding of the tumorigenesis of renal tumors.

ACKNOWLEDGMENTS

We thank the Cooperative Human Tissue Network of National Cancer Institute (CHTN) for providing the renal tumor samples. We also thank Drs. Richard Kahnoski, John Anema, David Kracklau, Philip Wise, Brian Roelof, and Jon Curry, who are the members of the Grand Rapids Urology Study Group, as well as the pathologists at the Spectrum Health and Metropolitan hospitals.

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