Alterations of the Birt-Hogg-Dubé gene (BHD) in sporadic colorectal tumors

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Short title: BHD and sporadic colorectal tumors
Key points

- A high incidence of colorectal tumors was recently reported in patients with Birt-Hogg-Dubé syndrome (BHD), implicating a potential role for the BHD gene in colorectal tumorigenesis.

- We have screened the BHD gene for genetic (mutations and loss of heterozygosity (LOH)) and epigenetic (altered promoter methylation status) alterations in 47 unselected primary sporadic colorectal tumors (10 polyps and 37 carcinomas). One polyp and 7 carcinomas demonstrated microsatellite instability (MSI) while all other tumors were microsatellite stable (MSS).

- We identified two novel missense mutations, S79W and A445T, in two MSS carcinomas. Methylation status, examined by methylation specific PCR (MSP) analysis of 23 matched normal/carcinoma tissues showed an absence of any BHD promoter methylation differences.

- Genotyping of microsatellite markers encompassing the BHD gene showed LOH in 4 of 10 (40%) polyps and 29 of 36 (81%) carcinomas. All 4 colon polyps showing LOH demonstrated chromosomal loss in the corresponding carcinomas from the same patients. However, LOH was also present in the corresponding carcinomas of 6 other polyps that did not show LOH, suggesting the involvement of LOH in colorectal tumor progression.

- Our results suggest that the BHD gene is involved in the tumorigenesis of a subset of MSS sporadic colorectal carcinomas, and that allelic loss in the region close to the BHD gene may play a role in colorectal tumor progression.

Key words: BHD, colorectal tumors, mutation, MSI, LOH
Colorectal cancer (CRC) is the third most common cancer diagnosed in both men and women, and the second most common cause of cancer deaths in the United States. There were approximately 150,000 new cases resulting in 57,000 deaths in 2002 [1]. CRC is one of the most studied cancer types and its underlying etiology best elucidated. Colorectal tumorigenesis involves a multi-step process including genetic and epigenetic alterations of numerous CRC-related genes that may act as either oncogenes or tumor suppressor genes [2-5]. The majority of sporadic CRCs are characterized by deletions of large chromosomal segments, which is thought to represent the loss of wild-type tumor suppressor genes [6, 7]. About 15% of sporadic CRCs, on the other hand, are show microsatellite instability (MSI), characterized by the insertion and/or deletion of simple-repeat sequences and indicative of the involvement of defective mismatch repair [8-9].

Birt-Hogg-Dubé syndrome (BHD, OMIM 135150) is an inherited autosomal dominant syndrome characterized by a triad of cutaneous lesions consisting of fibrofolliculomas, trichodiscomas, and acrochordons [10]. A wide spectrum of neoplastic and non-neoplastic features has been described in BHD patients [11], including diverse types of kidney tumors [12-17] and spontaneous pneumothorax [12-16, 18]. BHD has also been reported to be associated with colonic polyposis and colorectal neoplasia [13, 19-22], although a large study of 223 patients from 33 BHD families could not establish such a relation [23]. We recently reported a high incidence of colorectal polyps and carcinomas in patients with confirmed BHD germline mutations, indicating that the BHD gene may be involved in colorectal tumorigenesis [13]. The BHD gene has been mapped to chromosome sub-band 17p11.2 [12, 14], and recently identified to encode a novel protein named folliculin [15]. Based on the presence of inactivating BHD
mutations in BHD patients, and the detection of LOH in a significant proportion of BHD-related tumors, the \textit{BHD} gene was considered to be a tumor suppressor gene. A 44\% frequency of frameshift mutations within a mononucleotide (C)\textsubscript{8} tract (nt 1733–1740) has been detected in BHD patients [15], and this repeat tract represents a \textit{BHD} mutational hot spot [13, 15]. Other studies have reported the presence of frameshift mutations within intragenic mononucleotide tracts of the \textit{TGFBR2} and \textit{BAX} genes in CRC cell lines and tumors with high level MSI [24, 25]. The poly C tract of the \textit{BHD} gene may therefore be a potential site of mutation in CRC characterized by MSI.

We have evaluated the role of the \textit{BHD} gene in 47 unselected colorectal tumors (10 polyps and 37 carcinomas) by screening all coding exons of the \textit{BHD} gene for mutations and analyzing 46 of the tumors for LOH at the chromosome region surrounding the \textit{BHD} locus. Furthermore, alterations in \textit{BHD} promoter methylation profiles were determined in 23 cases of matched normal/carcinoma tissues where a sufficient quantity of DNA was available. We report the detection of 2 novel somatic missense mutations of the \textit{BHD} gene and LOH in 81\% of primary sporadic colorectal tumors with no change in promoter methylation profile. All mutations were detected in MSS tumors.
MATERIALS AND METHODS

Tissue samples and DNA extraction

Forty-seven matched samples (from 37 patients), of which 10 were colonic polyps with their matched carcinomas from the same patients, and 37 colorectal carcinomas, were obtained from the South Western Sydney Colorectal Tumour Bank (Liverpool Hospital, Australia). All tissue samples were collected prospectively with the informed consent of patients who underwent surgery in the South Western Sydney Area Health Service during the period 2000-2002. The lack of a family history of colorectal cancer or other familial cancer syndrome was ascertained by detailed questionnaire. This study was approved by the Institutional Review Board of the Van Andel Research Institute.

Frozen sections (15 μm) were prepared from stored tumor specimens. The first, middle and last slides (5 μm) were stained as reference slides. Manual microdissection was carried out on the unstained slides under low power light microscopy (20-40X) by scraping of individual cell populations with a 28-gauge needle. DNA was isolated from microdissected tumor cells and specimens of normal colonic mucosa using the Qiagen DNeasy Mini system (Qiagen, Valencia, CA), according to the manufacturer’s instructions. DNA was extracted from peripheral blood leukocytes using the DNA isolation kit for mammalian blood (Roche Molecular Biochemicals).

Analysis of microsatellite instability (MSI) status

Paired colorectal carcinoma, polyp and constitutional DNA samples (n=47) were analyzed using a panel of 10 microsatellite markers comprising mononucleotide (BAT25, BAT26), dinucleotide (D2S123, D5S346, D18S34, D3S1611), and tetranucleotide (D1S518, D7S1808, D3S2432, D10S1426) repeats. Amplification was
performed in a final volume of 10 µl containing 25 ng DNA, 20 pmol each primer, 16 µM dATP, 0.2 mM remaining dNTPs, 0.4 µCi of α-33P [dATP], 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl2 and 0.5 U Taq polymerase (Amersham-Pharmacia Biotech). PCR was initiated by a 5 min denaturation (94 ºC) followed by 34 cycles of denaturation (94 ºC, 45 s), primer annealing (55-65 ºC, 45 s) and extension (72 ºC, 45 s). PCR cycling was ended with a 10 min extension (72 ºC) step. Radio isotope-labeled PCR products were electrophoresed on 6% sequencing gels and visualized by autoradiography. Samples were classified as MSI-L (low level microsatellite instability) if instability was observed at 20–40% of loci assayed or MSI-H (high level microsatellite instability) if instability was observed at over 40% of loci assayed [26].

**Mutation analysis**

Mutation screening was performed on all 47 matched samples. The entire coding region of the *BHD* gene (exons 4–14) was screened. Primer sequences and PCR conditions were according to Nickerson *et al.* [14]. 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 the Big Dye Terminator system (Applied Biosystems, Foster City, CA), purified through Sephadex G-50 (Amersham Biosciences, Uppsala, Sweden) and analyzed on an ABI 3700 genetic analyzer (Applied Biosystems). We aligned and analyzed all sequences by Blast 2 analysis [27] and manually verified all sequences again. All sequence changes were verified by reamplification of the corresponding *BHD* fragment and sequencing of both DNA strands.
Analysis of loss of heterozygosity (LOH) status

LOH was performed on 36 matched normal/tumor tissue pairs, as well as 10 matched normal/polyp pairs. Allelic deletions of the chromosome 17p region flanking the BHD gene were assessed using microsatellite markers D17S1857, D17S740, D17S2196, and D17S620. The relative distances between each marker and their relationship to the BHD gene were calculated using the UCSC Genomic Bioinformatics site (fig. 1). PCR conditions were according to Khoo et al. [13]. One microliter of each PCR product was added to a cocktail containing 5 µl of DNAse-free, RNAse-free distilled water, 10 µl of Hi-Di formamide and 0.2 µl of ROX 400HD size standard. The mixture was denatured at 95°C for 5 min before loading into an ABI Prism 3700 Genetic Analyzer (Applied Biosystems). Analysis of raw data and assessment of LOH were carried out using 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, 1 and 2 were the intensities of smaller and larger alleles, respectively [28]. If the ratio was < 0.67 or > 1.3, the result was determined to be LOH. Initially, the 2 closest markers (D17S740 and D17S2196) were analyzed for LOH. A designation of LOH was given when at least one of the markers had a ratio that was < 0.67 or > 1.3. If the LOH value was close to these thresholds (0.67 + 0.1; 1.3 – 0.1), a further two markers, D17S1857 and D17S620, were examined to confirm the LOH status.

Analysis of BHD promoter methylation profile

We examined the promoter methylation status of the BHD gene in 23 matched normal/carcinoma sample sets. DNA methylation status was determined by a methylation-specific PCR approach (MSP) [29, 30]. DNA was treated with sodium
bisulfite, which converts all unmethylated cytosines to uracils, leaving methylated cytosines unchanged. Briefly, 2 µg of DNA was denatured by incubation in 0.2 M NaOH (37 ºC, 10 min). Cytosines were then modified in 3 M sodium bisulfite (adjusted to pH 5.0; Sigma Chemical Co., St. Louis, MO) and 10 mM hydroquinone (Sigma) at 50 ºC for 16 h. DNA samples were then purified through columns (Microcon YM-100; Millipore, Bedford, MA), treated again in 0.3 M NaOH, precipitated with ethanol using glycogen as a carrier, and resuspended in 20 µl DNAse-free, RNAse-free distilled water before storing at -20ºC. The specific primers for methylated sequences were designed as follows: BHD-BISF-OF (5’-ATGTGGATAGGAAGTTTTAGGTTGGTTATATTTT-3’) as the forward primer, and BHD-BISF-OR (5’-ACAAAAATCACACCCAAAACCCCCC-3’) as the reverse primer. An aliquot of the bisulfite treated product (2 µl) was amplified in a 25 µl reaction containing 2 mM MgCl₂, 0.24 mM each dNTP (Invitrogen), 0.02 U Taq DNA polymerase (Invitrogen) and 0.1 µM of each primer. PCR conditions were 95 ºC for 5 min followed by 35 cycles of 94 ºC (30 s), 60 ºC (30 s), and 72 ºC (45 s). PCR was ended with a 7 min extension (72 ºC). A nested PCR was then performed using 1 µl of the initial amplification reaction. The primers used were BHD-BISF-IF: 5’-GAAATGGTTTTTTTAGTATTTTATAGTTGGTG-3’ and BHD-BISF-IR: 5’-CCCAAAACCCCACCCCA-3’, with conditions similar to those described for the preceding PCR amplification, with the exception that 40 amplification cycles were carried out. The PCR products were purified using Microcon YM-100 columns (Millipore). After amplification, 20 µl of the 414 bp PCR product was incubated with 0.3 U of RsaI (New England BioLabs, Inc., Beverly, MA) for 2 h at 37 ºC. λDNA (0.3 µg) and distilled water were used respectively as positive and negative controls. Products of restriction digestion (20 µl) were electrophoresed on 2% agarose gels
containing ethidium bromide, and visualized under UV illumination. The sizes of the
RsaI digestion products were 160 and 254 bp.
RESULTS

Tumor MSI status

Analysis of MSI status showed that 8 of 47 tumors tested demonstrated MSI (table 1). This represents approximately 17% of the sporadic colorectal tumor cases evaluated in this study. Five carcinomas (CRC-7, CRC-17, CRC-18, CRC-46, and CRC-52) showed high frequency of MSI (MSI-H), while 2 carcinomas (CRC-23 and CRC-42) exhibited low frequency of MSI (MSI-L). CRC-42 also demonstrated low level of MSI in its corresponding polyp (42P). All other tumors (39 of 47) were microsatellite stable (MSS).

BHD Mutations

Screening of the BHD coding region identified 2 novel somatic mutations in exon 4 (c.691C>G) and exon 12 (c.1788G>A) of CRC-28 and CRC-54, respectively (table 1, fig. 2). Both are missense mutations (S79W and A445T), leading to nonconservative amino acid changes. In both cases the carcinomas were MSS and tumors with BHD mutations represented approximately 7% of the MSS colorectal carcinomas tested (n=30). No mutations were detected in the (C)8 repeat tract (nt 1733–1740), known to be a mutational hot spot within the BHD gene, in either the MSI or MSS tumors. BHD mutations were absent in all colon polyps.

LOH status

LOH at the chromosomal region surrounding the BHD locus was identified in 81% (29 of 36) of the sporadic colorectal carcinomas, and 40% (4 of 10) of colon polyps (table
1). The 4 colon polyps with LOH were from the same individuals who showed LOH in their colorectal carcinomas (CRC-34, 34P; CRC-42, 42P; CRC-56, 56P; and CRC-59, 59P). CRC-28 demonstrated LOH, along with somatic mutation S79W.

**BHD promoter methylation**

Methylation specific PCR analysis of the *BHD* promoter did not detect any promoter methylation profile differences in the 23 matched sets tested (table 1). Unfortunately, methylation profiles for the rest of the samples could not be determined due to insufficient DNA being available.
DISCUSSION

Early studies have reported several cases of colorectal neoplasia in patients with BHD [19-22]. However, one recent study [23] showed a lack of statistical significance when comparing the incidence of colon cancer in 111 BHD-affected and 112 BHD-unaffected subjects, as well as the occurrence of colon polyps in 45 BHD-affected and 38 BHD-unaffected subjects, thus excluding any association between colonic neoplasia and BHD. Nevertheless, we recently reported 6 cases of colonic polyps and 2 cases of possible colon cancer in a BHD family with confirmed BHD germline mutations [13], indicating that the BHD gene is involved in the tumorigenesis of these BHD-related colorectal tumors. In this study, we demonstrate that the BHD gene is also involved in a subset of sporadic colorectal cancers. Two cases of MSS colorectal cancer were found to harbor two novel somatic missense mutations, S79W and A445T, in exons 4 and 12, respectively. Interestingly, no frameshift mutation was identified in the hypermutable poly C tract, particularly in the MSI carcinomas, as this region is a potential site for insertion or deletion in cancers with defective mismatch repair. These results suggest that the BHD gene may be involved in a pathway of colorectal tumorigenesis that is distinct from the pathway of mismatch repair deficiency. However, the sample size of the MSI tumors is small in this series and further investigation is warranted.

The detected missense mutations where nonconservative amino acid substitutions (S79W and A445T) in the BHD gene product which could cause conformational changes in the structure of the protein, leading to dysfunction [31-35]. Protein phosphorylation, a modulator of protein function and stability, can occur at Ser, Thr or Tyr residues and is mediated by specific protein kinases. In CRC-28, the change from
Ser to Trp leads to the loss of a potential site of phosphorylation whereas the Ala to Thr change in CRC-54 leads to the gain of a potential phosphorylation site. These amino acid changes could lead to altered protein phosphorylation status with consequent functional changes.

LOH, which indicates the loss of one functional copy of a gene, has been used as a marker for diagnosis and prognosis of cancer. In this study, we identified LOH at microsatellite loci flanking the BHD gene in 40% of colon polyps and 81% of colorectal carcinomas. Together with the finding of LOH in matched normal/carcinoma samples of LOH negative polyps, we propose that LOH surrounding the BHD locus may be involved in colorectal cancer progression, although other tumor suppressor genes located on chromosome 17p, such as p53, should not be excluded. The p53 gene is located approximately 9 cM telomeric to the BHD gene. Studies have shown that LOH at 17p may be essential for the malignant transformation of benign lesions in colorectal neoplasms [36, 37]. Therefore, the effects of LOH on BHD gene expression and regulation in colorectal tumors merits further investigation.

Sample CRC-28 was found to harbor a mutation in the BHD gene as well as LOH around the BHD region, which could represent two hits of the BHD gene in accordance with Knudson’s classical 2-hit theory. Apparent biallelic alteration of the BHD gene appears to be uncommon and the high frequency of LOH in the rest of tumors without mutations suggests several possibilities. First, loss of a single allele may be the preferred mode of inactivation of the BHD gene and that haploinsufficiency contributes to tumorigenesis. Second, there may be mutations present in the regulatory region of
the *BHD* gene that was not tested in this study. Finally, there may be loss of other tumor suppressor genes in the vicinity of the *BHD* gene.

DNA methylation is an epigenetic alteration that interferes with transcriptional initiation. In general, methylation of CpG dinucleotides in the promoter regions of tumor suppressor genes leads to loss of tumor suppressor gene expression (silencing) and consequent function. Hypermethylation of tumor suppressor genes has been frequently reported in many tumor types. We recently identified the involvement of the *BHD* gene in sporadic renal tumors by showing frequent methylation of the *BHD* promoter in a wide spectrum of sporadic renal tumors [38]. In the present study, we did not detect any *BHD* promoter methylation profile differences in the 23 colorectal carcinoma cases where a sufficient amount of DNA was available for the MSP assay. We conclude that epigenetic alteration of the *BHD* gene is not a common event in colorectal cancer.

In summary, we have demonstrated that the *BHD* gene is mutated in a subset of MSS sporadic colorectal carcinomas, and allelic loss around the region of the gene may play a role in the progression of colorectal tumors.
Acknowledgements

This study was supported by the Van Andel Foundation
REFERENCES


Legends to figures

Figure 1

Schematic map of microsatellite markers encompassing the *BHD* gene. The relative distances (in cM) between each marker and their relationship to the *BHD* locus are indicated.

Figure 2

Detection of mutations within the *BHD* gene in sporadic colorectal cancer. Two novel somatic mutations of the *BHD* gene were detected in two MSS colorectal carcinomas. Each of the mutations is not present in the matched normal tissues. (A) c.691C>G (S79W) in CRC-28 and (B) c.1788G>A (A445T) in CRC-54.
<table>
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P = polyp; – = absent; + = present; H = high level MSI; L = low level MSI; ND = not determined.
A Wild type

690 700 710
CCCAAAAATCCTGCA CATGTGCAGA

720 730 740
CCCAGAAGTCATGGACA CATGTGCAGA

B Wild type

80 90
CCACGCGATCCACCGT

80 90
CCACGCGATCCACCGT

1788G>A (A445T)