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

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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. CRC is one of the most studied cancer types and its underlying aetiology best elucidated. Colorectal tumorigenesis involves a multistep process including genetic and epigenetic alterations of numerous CRC related genes that may act as either oncogenes or tumour suppressor genes. The majority of sporadic CRCs are characterised by deletions of large chromosomal segments, which are thought to represent the loss of wild type tumour suppressor genes. About 15% of sporadic CRCs, on the other hand, show microsatellite instability (MSI), characterised by the insertion and/or deletion of simple repeat sequences and indicative of the involvement of defective mismatch repair.

Birt-Hogg-Dubé syndrome (BHD, OMIM 135150) is an inherited autosomal dominant syndrome characterised by a triad of cutaneous lesions consisting of fibrofolliculomas, trichodiscomas, and acrochordons. A wide spectrum of neoplastic and non-neoplastic features has been described in BHD patients, including diverse types of kidney tumours and spontaneous pneumothorax. BHD has also been reported to be associated with colonic polyposis and colorectal neoplasia, although a large study of 223 patients from 33 BHD families could not establish such a relation. 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. The BHD gene has been mapped to chromosome subband 17p11.2 and recently identified to encode a novel protein named folliculin. Based on the presence of inactivating BHD mutations in BHD patients, and the detection of LOH in a significant proportion of BHD related tumours, the BHD gene was considered to be a tumour suppressor gene. A 44% frequency of frameshift mutations within a mononucleotide (C) tract (nt 1733-1740) has been detected in BHD patients, and this repeat tract represents a BHD mutational hot spot. Other studies have reported the presence of frameshift mutations within intragenic mononucleotide tracts of the TGFBR2 and BAX genes in CRC cell lines and tumours with high level MSI. The poly C tract of the BHD gene may therefore be a potential site of mutation in CRC characterised by MSI.

We have evaluated the role of the BHD gene in 47 unselected colorectal tumours (10 polyps and 37 carcinomas) by screening all coding exons of the BHD gene for mutations and analysing 46 of the tumours for LOH in the chromosome region surrounding the BHD locus. Furthermore, alterations in 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 two novel somatic missense mutations of the BHD gene and LOH in 81% of primary sporadic colorectal tumours with no change in promoter methylation profile. All mutations were detected in MSS tumours.

Key points

- A high incidence of colorectal tumours 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 tumours (10 polyps and 37 carcinomas). One polyp and seven carcinomas showed microsatellite instability (MSI) while all other tumours 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 four of 10 (40%) polyps and 29 of 36 (81%) carcinomas. All four colon polyps showing LOH showed chromosomal loss in the corresponding carcinomas from the same patients. However, LOH was also present in the corresponding carcinomas of six other polyps that did not show LOH, suggesting the involvement of LOH in colorectal tumour 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 tumour progression.

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 tumour 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 magnification.
power light microscopy (20–40×) by scraping of individual cell populations with a 28 gauge needle. DNA was isolated from microdissected tumour 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 leucocytes using the DNA isolation kit for mammalian blood (Roche Molecular Biochemicals).

**Analysis of microsatellite instability (MSI) status**

Paired colorectal carcinoma, polyt, and constitutional DNA samples (n=47) were analysed using a panel of 10 microsatellite markers comprising mononucleotide (BAT25, BAT26), dinucleotide (D2S123, D5S346, D18S34, D3S1611), and tetranucleotide (D15S18, D7S1808, D5S2432, D15S1846) repeats. Amplification was performed in a final volume of 10 μl containing 25 ng DNA, 20 pmol each primer, 16 μmol/l dATP, 0.2 mmol/l remaining dNTPs, 0.4 μl of α-33P [dATP], 50 mmol/l KCl, 10 mmol/l Tris-HCl (pH 8.3), 1.5 mmol/l MgCl2, and 0.5 U Taq polymerase (Amersham-Pharmacia Biotech). PCR was initiated by a five minute denaturation (94°C) followed by 34 cycles of denaturation (94°C, 45 seconds), primer annealing (55–65°C, 45 seconds), and extension (72°C, 45 seconds). PCR cycling was ended with a 10 minute extension (72°C) step. Radioisotope labelled PCR products were electrophoresed on 6% sequencing gels and visualised 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.32

**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.33 PCR was performed using a DNA Engine Tetrad (MJ Research, Waltham, MA). PCR products were analysed 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 analysed on an ABI 3700 genetic analyser (Applied Biosystems). We aligned and analysed all sequences by Blast 2 analysis 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/tumour tissue pairs, as well as 10 matched normal/polyt 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.34 One μl of each PCR product was added to a cocktail containing 5 μl of DNease 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 five minutes 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 tumour sample, N was the matched normal sample, and 1 and 2 were the intensities of smaller and larger alleles, respectively.35 If the ratio was <0.67 or >1.3, the result was determined to be LOH. Initially, the two closest markers (D17S740 and D17S2196) were analysed 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).36 37 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 mol/l NaOH (37°C, 10 minutes). Cytosines were then modified in 3 mol/l sodium bisulfite (adjusted to pH 5.0; Sigma Chemical Co, St Louis, MO) and 10 mmol/l hydroquinone (Sigma) at 50°C for 16 hours. DNA samples were then purified through columns (Microcon YM-100, Millipore, Bedford, MA), treated again in 0.3 mol/l NaOH, precipitated with ethanol using glycogen as a carrier, and resuspended in 20 μl DNease free, RNase free distilled water before storing at −20°C. The specific primers for methylated sequences were designed as follows: BHD-BISF-OF (5′-AGTGAGGATGAGCAATGTTGGTATATTT-3′) as the forward primer, and BHD-BISF-OR (5′-ACAAAATCACACCCAAAAACCCC-3′) as the reverse primer. An aliquot of the bisulphite treated product (2 μl) was amplified in a 25 μl reaction containing 2 mmol/l MgCl2, 0.24 mmol/l each dNTP (Invitrogen), 0.02 U Taq DNA polymerase (Invitrogen), and 0.1 μmol/l of each primer. PCR conditions were 95°C for five minutes followed by 35 cycles of 94°C (30 seconds), 60°C for five minutes followed by 35 cycles of 94°C (30 seconds), and 72°C (45 seconds). PCR was ended with a seven minute extension (72°C). A nested PCR was then performed using 1 μl of the initial amplification reaction. The primers used were BHD-BISF-IF: 5′-GAAATGGTTTTTTTAGTTG-3′, BHD-BISF-IF: 5′-TATTTTAGTTGGTG-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 RoT1 (New England BioLabs Inc, Beverly, MA) for two hours at 37°C. 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 visualised under UV illumination. The sizes of the RoT1 digestion products were 160 and 254 bp.

**RESULTS**

**Tumour MSI status**

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

**BHD mutations**

Screening of the **BHD** coding region identified two 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 non-conservative amino acid changes. In both cases the carcinomas were MSS and tumours 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 tumours.

**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% (four of 10) of colon polyps (table 1). The four colon polyps with LOH were from the same people who showed LOH in their colorectal carcinomas (CRC-34, 34P; CRC-42, 42P; CRC-56, 56P; and CRC-59, 59P). CRC-28 showed 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 owing to insufficient DNA being available.

**DISCUSSION**

Early studies have reported several cases of colorectal neoplasia in patients with **BHD**. However, one recent study 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 six cases of colonic polyps and two cases of possible colon cancer in a BHD family with confirmed BHD germline mutations, indicating that the BHD gene is involved in the tumorigenesis of these BHD related colorectal tumours. In this study, we show that the BHD gene is also involved in a subset of sporadic colorectal cancers. Two cases of MSS colorectal cancer were found to harbour two novel somatic missense mutations, S79W and C105R, in the hypermutable poly-A tract, particularly in the MSI carcinomas, as this region is a potential site for insertion or deletion in cancers with defective DNA 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 tumours is small in this series and further investigation is warranted.

The missense mutations detected were non-conservative amino acid substitutions (S79W and C105R) in the BHD gene product which could cause conformational changes in the structure of the protein, leading to dysfunction. 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 Thr may lead 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 tissues, we suggest that LOH negative polyps are more likely to occur in the vicinity of the BHD gene region. We propose that LOH surrounding the BHD locus may be involved in colorectal cancer progression, although other tumour 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.

Therefore, the effects of LOH on BHD gene expression and regulation in colorectal tumours merits further investigation.

Sample CRC-28 was found to harbour a mutation in the BHD gene which was not tested in this study. Sample CRC-54 was found to harbour a mutation in the BHD gene in accordance with Knudson’s classical two hit theory. Apparent biallelic alteration of the BHD gene which were not tested in this study.

In summary, we have shown 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 tumours.

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A gene locus for branchio-otic syndrome maps to chromosome 14q21.3-q24.3


Branchio-oto-renal syndrome (BOR, OMIM 113650) is an autosomal dominant disorder characterised by the association of hearing loss (HL), structural ear anomalies, branchial arch defects, and renal anomalies. The prevalence approximates 1:40 000 in the general population, and has been reported in about 2% of deaf children. Age of onset for deafness varies from childhood to early adulthood. The clinical expression of BOR exhibits wide intra- and inter-familial variability. In addition, reduced penetrance for BOR has been assumed. The major feature of BOR, which occurs in 93% of patients, is HL, which can be conductive, sensorineural, or mixed. Besides the classical ear, kidney, and branchial arch anomalies, different developmental manifestations of BOR in other organ systems have been described. Among these, dysfunction of the lacrimal duct system is a common association. Thus, BOR represents a clinically and genetically heterogeneous disease complex that manifests predominantly during organogenesis. A gene locus for autosomal dominant BOR had been localised on chromosome 8q13. Subsequently, mutations in the human homologue of the Drosophila eyes absent gene (EYA1) have been shown to cause BOR and BOS. A locus (BOS2) for autosomal dominant BOS has been localised to chromosome 1q31.

We performed a genome wide search for linkage in a large pedigree with BOS with more than 40 affected subjects and mapped a new gene locus (BOS3) to chromosome 14q21.3-q24.3. The highest multipoint lod score was Zmax=4.81 (θ=0) for marker D14S980.

Identification of the gene causing branchio-otic syndrome type 3 will offer new insights into the development and molecular mechanisms of hearing.

Key points

- Branchio-oto-renal syndrome (BOR) is an autosomal dominant developmental disorder characterised by the association of hearing loss, branchial arch defects, and renal anomalies. Branchio-otic syndrome (BOS) represents a related disorder presenting with the same clinical features without renal anomalies.

- Recessive mutations in the human homologue of the Drosophila eyes absent gene (EYA1) have been shown to cause BOR and BOS. A locus (BOS2) for autosomal dominant BOS has been localised to chromosome 1q31.

- We performed a genome wide search for linkage in a large pedigree with BOS with more than 40 affected subjects and mapped a new gene locus (BOS3) to chromosome 14q21.3-q24.3. The highest multipoint lod score was Zmax=4.81 (θ=0) for marker D14S980.

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