

Genetic analysis of the BRCA1 region in a large breast/ovarian family: refinement of the minimal region containing BRCA1

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We have analyzed a single multi-affected breast/ovarian cancer pedigree (BOV3) and have shown consistent inheritance of markers on chromosome 17q with the disease confirming that this family is due to the BRCA1 gene. Analysis of 17q haplotypes shows a recombination event in a bilateral breast cancer case which suggests that the BRCA1 gene lies distal to D17S857; D17S857 is thus the new proximal boundary for the region containing BRCA1. Combining this information with previously published mapping information suggests that BRCA1 is contained in a region estimated at 1–1.5 Mb in length. All seven breast tumour/blood pairs examined from this family show loss of heterozygosity in the tumours. The allele retained in each tumour was from the disease-bearing chromosome implicating BRCA1 as a tumour suppressor gene. We have sequenced the 17 β -oestradiol dehydrogenase genes (EDH17B1 and EDH17B2) which have been suggested as candidate genes for BRCA1 in four members of this family. No germline mutations were detected.

INTRODUCTION

The literature contains reports of many families which show autosomal dominant inheritance for breast cancer susceptibility (1, 2, 3, 4). In some of these families, female gene carriers are also at risk of ovarian cancer (5). In 1990, a locus for breast cancer predisposition was mapped provisionally to the long arm of chromosome 17 (6) and subsequently confirmed (7); this locus is now referred to as 'BRCA1' (8). A collaborative study utilizing markers mapping in this region in 214 breast cancer families including 57 breast/ovarian families found that all families containing at least one ovarian cancer case as well as breast cancer cases were consistent with linkage while only 45% of 'breast cancer only' families were attributable to BRCA1 (9). Examination of recombinants in clearly linked extended pedigrees has suggested that the gene lies between THRA1 and D17S183 in a region which is genetically less than 4 cM in length (10).

In this study we have focused on a single extended family with thirteen cases of breast cancer (with ages of onset ranging from 28 to 63 years) and three cases of ovarian cancer (diagnosed at 43, 59 and less than 58 years). We have previously confirmed linkage of this family to BRCA1 (11). Subsequent to our previous analysis (11), we have typed further individuals (in some cases from archival material) and also screened with further microsatellite markers mapping within the THRA1 and D17S579 region in an attempt to refine the localization of BRCA1.

The comparison of tumour DNA content with the blood lymphocytic DNA of an individual using polymorphic DNA markers has identified genomic regions commonly deleted in constitutively heterozygous individuals ('loss of heterozygosity'

or LOH) (12, 13). Studies have shown that high rates of LOH can be detected in breast and ovarian tumours in many regions including chromosome 17 (14, 15, 16). Loss is detected frequently on 17p with the presumptive target being TP53 (17, 18, 19). High rates of loss have also been detected on 17q with the likely target being BRCA1 since in 9 tumours exhibiting LOH from implied BRCA1 carriers the chromosome from the non-transmitting parent for the BRCA1 mutation was lost (20).

With the narrowing of the minimal region containing BRCA1 and the refinement of the genetic map, a number of candidate genes for BRCA1 have been eliminated. These include ERBB2 which is amplified in a proportion of breast tumours, the putative oncogene THRA1, and NME (21). The gene for prohibitin has also been shown to be somatically mutated in a number of sporadic breast tumours (22). Sequencing of the coding region of this gene in BRCA1-linked families revealed no disease-causing germline mutations (Black and Kelsell, unpublished data); this gene has subsequently been excluded genetically (10).

Finally, although many putative candidate genes have been ruled out genetically, the 17 β -oestradiol dehydrogenase genes do lie in the current minimal region for BRCA1. In fact, two 17 β -oestradiol dehydrogenase genes (EDH17B1 and EDH17B2) are localised within a 13 kb genomic DNA fragment on 17q (23). 17 β -Oestradiol dehydrogenase catalyses the interconversion between the weak oestrogen, oestrone, and the more potent oestradiol, the levels of which have been shown to be much higher in breast tumours than in normal breast cells (24, 25). Two mRNA species have been identified, one of 1.3 kb and another

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of 2.2 kb (26), in varying abundance in most tissues, including the breast and ovary (23). Therefore, regulation of 17 β -EDH expression could plausibly be important in the initiation and/or progression of breast (and ovarian) tumours making these genes candidates for BRCA1 (21). Nucleotide sequence comparison of the two genes over their exons and introns shows 89% homology. EDH17B1 has a stop codon at position 218 instead of the amino acid Gln as in EDH17B2 and is likely to be a pseudogene. However, it could still transcribe and encode a protein of 214 amino acids (26). No sequence analysis of these candidate genes has been reported to date, so we have performed this comparison for three gene carriers and one non-carrier from BOV3.

RESULTS

Haplotype analysis

Twenty nine DNA markers mapping to 17q have been screened across this family. For the purpose of this study, the results of thirteen are reported as well as one 17p marker (TP53). The map of some of the closest 17q markers to the BRCA1 region is shown in Figure 1.

The individual 3552 was at 50% prior risk of being a gene carrier since her mother carried the BRCA1 mutation (Figure 2). As reported previously (11), haplotype analysis using the results from the typing of relatives and archival material from this individual 3552 confirms this individual to be a sporadic ovarian case (diagnosed at 59 years) since she inherited the maternal chromosome containing the wildtype BRCA1 allele (Figure 2). The fine-scale typing reported in this analysis removes any possibility that an unusual double recombination event had taken place.

Haplotype analysis also shows that the affected individual 3684 has inherited the (maternal) disease-bearing haplotype distal to the marker D17S857 and the maternal non-disease chromosome proximal to D17S846 (Figure 2). Individual 3684 had bilateral breast cancer diagnosed at 36 and 41 years respectively, while her mother had breast cancer and her grandmother, ovarian cancer. This suggests that this individual is unlikely to be a sporadic case and the BRCA1 gene lies distal to D17S857. The cross-over event between D17S857 and D17S846 was unrecognized previously (11) because of homozygosity of the only two proximal markers, D17S250 and THRA1, typed in the mother of individual 3684 (3677). This homozygosity masked the recombination event proximal to D17S579.

The affected daughters of the uncle–niece marriage of 5352 and 5355 were at risk of being homozygous for the BRCA1 mutation since both parents were putative carriers. However, analysis of DNA from a whole section containing both tumour and apparently normal tissue from individual 5367 revealed that this individual was heterozygous (Figure 3, panel b, track T2). The typing of offspring from both affected daughters also infers they were in fact heterozygous at the BRCA1 locus. Thus, the disease transmission is likely to be coming from the unaffected mother 5355 who had a sister with breast cancer (5361), diagnosed at age 50. Unfortunately archival material is unavailable to confirm this inferred transmission.

We examined the carrier status of the stomach cancer case, 3542, by examining various markers using DNA from the tumour and were able to show that this individual has not inherited the BRCA1 mutation (Figure 2).

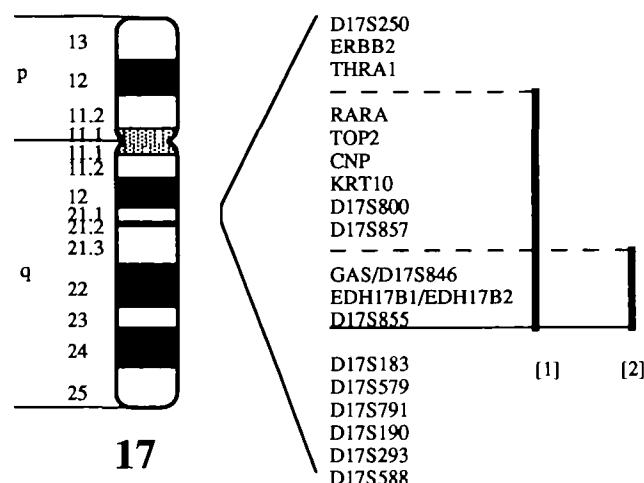


Figure 1. The 17q map of loci relevant to this analysis. All anonymous polymorphisms listed here plus polymorphisms in THRA1, EDH17B1 and EDH17B2 have been typed; loci referring to other known genes (ERBB2, RARA, TOP2, CNP, KRT10 and GAS) which are located in this region are included for completeness but were not typed. The minimal published region containing BRCA1 (10) (labelled as [1]) is shown as well as the minimal region incorporating the recombination event observed in this family (labelled as [2]).

Loss of heterozygosity

Seven breast tumours and one ovarian tumour were analyzed from this family and allele loss was detected in all cases for markers located around the BRCA1 region on 17q. All tumour DNA analyzed was extracted from archival slide preparations; care was taken to minimize the number of non-tumour cells scraped off the slide for PCR analysis. On the basis of the linkage information and tracing inheritance of haplotypes through the family, the seven breast cancer cases had each inherited the disease-bearing haplotype and LOH in each case involved loss of the chromosome inherited from the non-carrier parent (Figures 2, 3 and 4). For instance, analysis of tumour-enriched material from the breast block obtained from 5367 showed significant reduction in intensity of the allele from the non-disease bearing chromosome (Figure 3, panel b, track T1). Combining results from all tumours showed that allele loss was independent of the size of the alleles inherited from each parent and related only to the parental origin. This suggests the results are a true indication of tumour DNA status and not an artifact of PCR. The single sporadic ovarian tumour (from 3552; see 'Haplotype analysis') was also hemizygous for the BRCA1 region, having retained the maternally derived chromosome. Two out of five informative tumours exhibited LOH for TP53 (Figure 4).

EDH17B1 and EDH17B2 sequence analysis

Genomic DNA from lymphoblastic cell lines from three affected gene carriers (3550, 3543 and 3677) and one unaffected non-carrier (3079) were sequenced for the whole coding region of both 17 β -oestradiol dehydrogenase genes, EDH17B1 and EDH17B2, for detection of disease-causing mutations in their constitutive DNA. No variation from the published sequences was detected (23). All individuals were homozygous for the published exon polymorphisms (27). Also, the 5' untranslated region of the two genes from –34 bp to the initiation codon of

D17S250
THRA1
D17S800
D17S857
GAS/D17S846
D17S855
D17S183
D17S579
D17S293

○ Allele lost in tumour

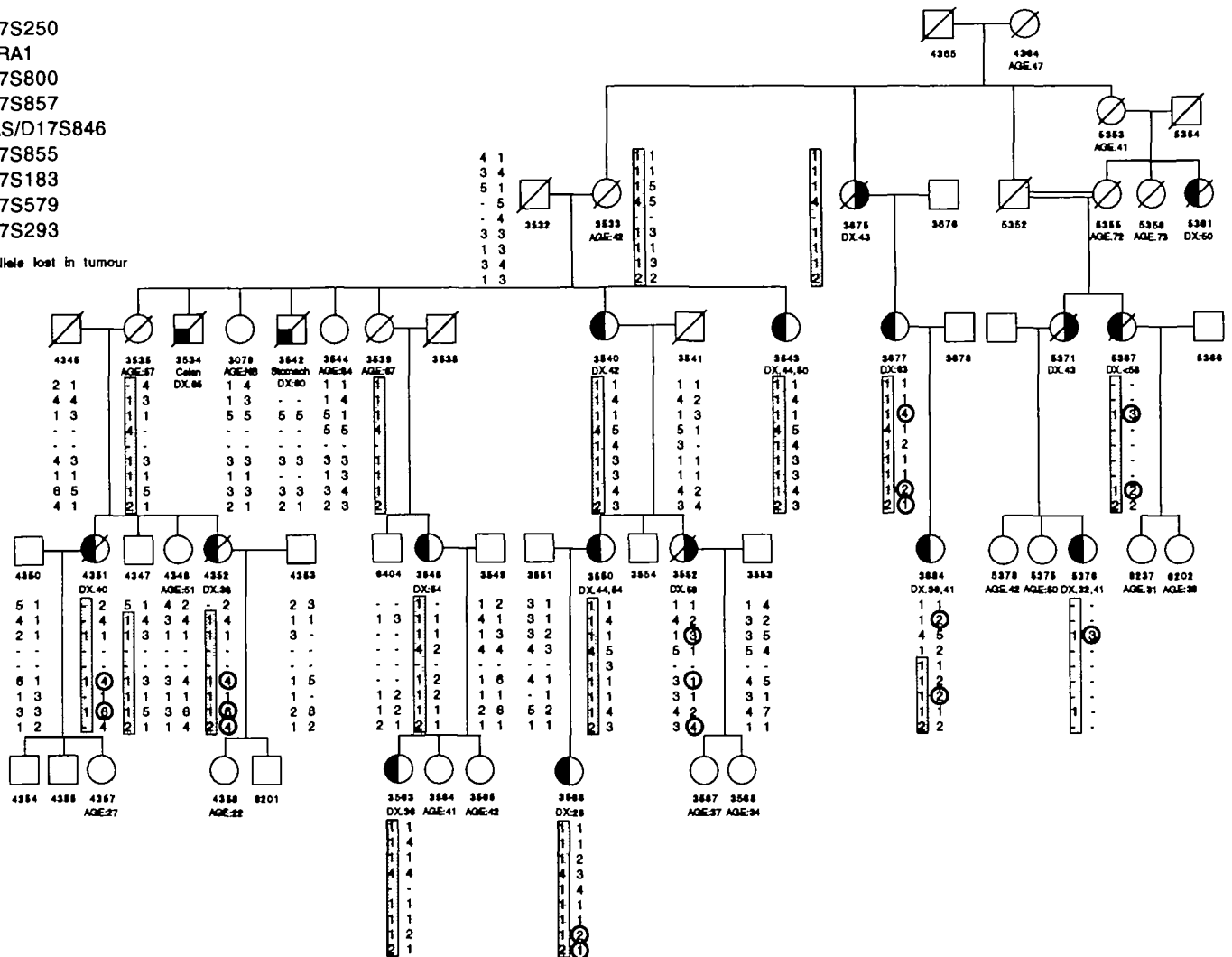


Figure 2. Pedigree of the breast/ovarian family named BOV3. Beneath each pedigree symbol is the personal identifier. Within pedigree symbols, left shading indicates breast cancer cases and right shading indicates ovarian cancer cases. Other cancers are indicated by quarter shading. The age at diagnosis of each case (n years) is shown underneath the personal identifier as DX:n, while current age or age at death is written as AGE:n. The order of typed markers in the orientation centromere to telomere is shown in the left corner of the figure. All offspring marked as alive within the family have been typed but their haplotypes have been omitted to remove clinically relevant information. The status of individuals with marker typings who are marked as deceased have been inferred from information on their relatives. However, for deceased individuals 3552, 4351 and 4352, archival material was available to confirm the genotype inferred from other relatives. For 3542, 5367 and 5376, archival material was typed for markers listed here plus those in Figure 4. The alleles deleted in the LOH analysis (see Figure 4) are indicated by open circles in the haplotypes of each individual's tumour studied.

exon 1 was sequenced and revealed no germline mutations. Finally, extensive Southern blot analysis of a large number of familial and sporadic tumours revealed no DNA rearrangements (Black, unpublished data).

DISCUSSION

In this family (BOV3, Figure 2), we have previously shown co-inheritance of susceptibility to disease with a marker haplotype spanning the region in which the BRCA1 gene is located (9, 11). The current published minimal region (Figure 1) in which BRCA1 maps is between the markers THRA1 and D17S183 (10). As closer flanking markers are identified, informative

recombinants are more difficult to find. There is also the added complexity for gene mapping of a disease with a high frequency of sporadic disease that putative recombinant events may be confused with phenocopies. However, the recombination event involving individual 3684 in this family is particularly convincing because this woman developed breast cancer initially at age 36 years and, subsequently, bilateral disease which is unusual for sporadic disease. The recombination event suggests that, of the markers that we have examined, D17S857 is the new proximal boundary. We conclude that the boundaries are now D17S857 (this work) and D17S183 (10), a distance we estimate to be between 1.0 and 1.5 Mb on the basis of our physical map (28, Karen Jones, personal communication) (Figure 1). This

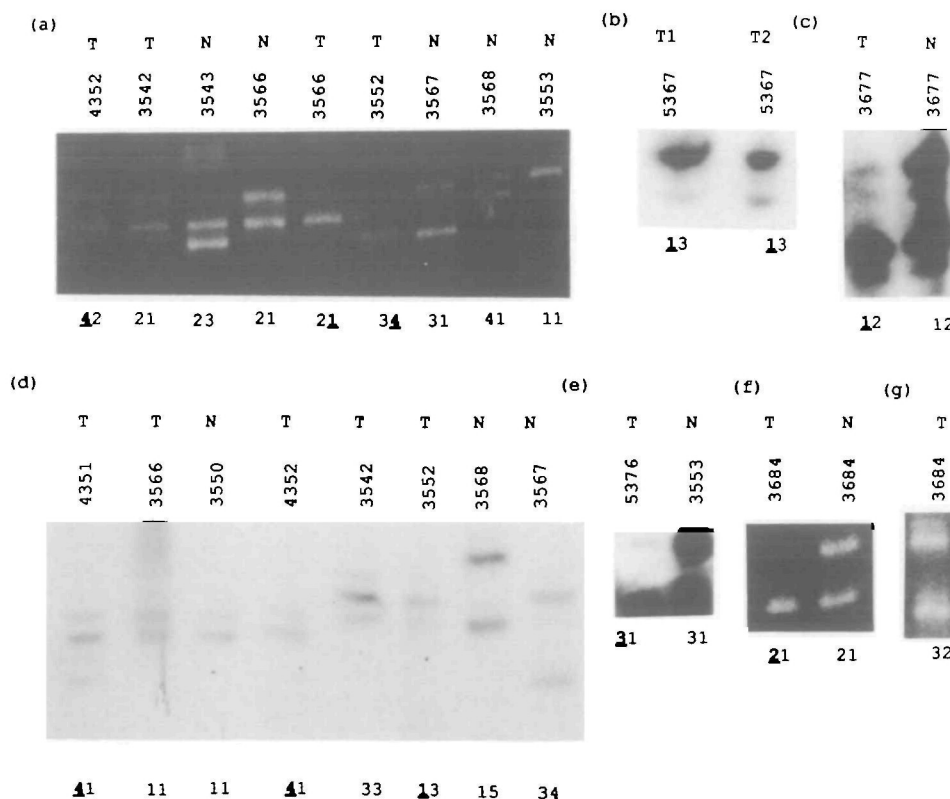


Figure 3. Examples of the results from the PCR microsatellite analysis of nine tumours from the BOV3 pedigree. The personal identifier is indicated above each lane. T = tumour DNA, T1 = approximately 90% tumour cell content, T2 = approximately 70% tumour cell content, N = normal lymphocyte DNA. Allele typing is shown beneath each lane; allele in bold and underlined indicates the allele deleted in each tumour; (a) D17S293 (allele sizes approximately 120 bp): Allele 2 is from the disease haplotype. (b) D17S800 (allele sizes 168–178 bp): Allele 1 is from the disease haplotype, (c) D17S293 (allele sizes approximately 120 bp): Allele 2 is from the disease haplotype, (d) D17S855 (allele sizes approximately 150 bp): Allele 1 is from the disease haplotype, (e) D17S190 (allele sizes approximately 190 bp): Allele 1 is from the disease haplotype. No normal control was available, but 3553 is shown to represent a typical 3–1 typing, (f) D17S183 (allele sizes approximately 100 bp): Allele 1 is from the disease haplotype, (g) representation of allele retention with the TP53 microsatellite (allele sizes approximately 110 bp).

recombination event excludes RARA1, TOP2, CNP and KRT10 as candidate genes for BRCA1 (28, 29). Analysis of a polymorphism defined by the marker D17S846 which maps within 40 kb of the gastrin gene (GAS) shows that the recombination event lies between this marker and D17S857 (Figure 2) so that gastrin lies within the minimal region.

Two candidate genes for BRCA1 that are not ruled out genetically by this or other recombinants are the 17 β -oestradiol dehydrogenase genes (EDH17B1 and EDH17B2). In this study we have performed sequence analysis of them both. No mutations were detected in the coding region of either gene in the germline DNA from three affected individuals from the family. There remains the possibility of an inherited mutation in an upstream promoter or control region or other non-coding region of this gene leading to aberrant expression of 17 β -EDH protein. To eliminate completely this locus as a candidate for BRCA1 requires sequencing the remainder of the normally untranslated region or identifying a recombination event between this locus and BRCA1. A number of polymorphisms at this locus have been identified so that genetic analysis is possible (27).

This study has also given more support to the suggestion that BRCA1 acts as a tumour suppressor gene requiring inactivation by 'knock-out' of the wildtype allele (20). The seven breast tumours analyzed from BOV3 gene carriers were consistent with

the tumour suppressor hypothesis. All showed LOH in this region with retention of the chromosome bearing the BRCA1 mutation. Further LOH analysis of these tumours (in particular of 3566, 4251 and 4352) is in progress to determine the limits of each deletion which may provide further mapping information as to the location of BRCA1. LOH at the TP53 locus on 17p was only detected in two out of the five informative BRCA1 familial tumours suggesting that there is a focus of LOH on 17q.

This family shows a similar phenomenon as the family reported elsewhere (20) in that all affected individuals whose tumours have been examined and loss detected have inherited disease susceptibility from their mothers. Thus it is not inconceivable that LOH represents loss of the paternal allele rather than loss of the wildtype allele per se. We think that this is an unlikely explanation especially as no evidence of a parental origin effect in age of onset has been observed (Bishop, unpublished data concerning the families reported in (9)).

An interesting speculation is the risk of cancer in male carriers of the BRCA1 mutation. In one study, male carriers appeared to be at increased risk of prostate cancer (30). While there are few obligatory male carriers within this family, there are two males at 50% prior risk who were diagnosed with cancer. No material was available for the colon cancer case, 3534, but haplotype analysis of the stomach cancer case, 3542, shows the

MARKER	BREAST TUMOURS FROM GENE CARRIERS							SPORADIC OVARIAN TUMOUR	GENETIC LOCATION
	4352	4351	3677	3684	3566	3567	5376		
TP53	●	●		⊗	⊗	⊗	●	●	17p
THRA1			●	●	●				17q proximal to BRCA1 region
D17S800	●		●			●	●	●	
D17S855	●	●	●		●		●	●	17q BRCA1 region
D17S183	●	●	●	●	●				17q distal to BRCA1 region
D17S579	●	●	●	●	●	●	●		
D17S791	●	●	●	●		●	●		
D17S190			●	●			●	●	
D17S293	●		●	●	●			●	
D17S688	●	●	●		●	●	●		

● uninformative or loss not determinable
 ● loss of heterozygosity
 ⊗ retention of heterozygosity

Figure 4. The summary of loss of heterozygosity results for tumours taken from seven women who on the basis of the haplotyping were considered BRCA1 carriers and a sporadic ovarian tumour from a family member. The lack of a symbol for a marker indicates that LOH was not determined in this tumour.

individual had not inherited the disease-bearing chromosome. Larger scale studies are presently in progress to address the overall issue of cancer risk to male carriers.

MATERIALS AND METHODS

Family ascertainment

The identification of this family has been described previously (11). Fifty ml blood samples were collected from family members. These were used for the preparation of lymphoblastoid cell lines from which genomic DNA was prepared. Medical records were obtained to verify the majority of cancers diagnosed in family members (11).

Microsatellite marker analysis

A total of thirty markers (29 on 17q and 1 on 17p) were typed in this study although only fourteen (13 on 17q and 1 on 17p) are reported here. Those omitted are either reported in our previous study (11) or do not add significantly to those reported here. The details of the majority of the markers are summarised elsewhere (see appendix of (9)). The remaining markers (D17S855 and D17S857) are available through GDB (Johns Hopkins University). D17S791 and D17S800 are published elsewhere (31). Figure 1 shows the genetic map of markers reported in this analysis as well as various genes known to lie in this region. The map order was determined from published literature (9, 11, 28, 29) and from Karen Jones (unpublished information) although there is some dispute about the order of D17S800 and D17S857 with some evidence pointing to the opposite orientation to that presented in Figure 1 (Dr Simon Smith, personal communication). D17S846 is known to lie within 40 kb of the gastrin gene, GAS (33).

PCR amplification reactions were in 100 µl volume containing 200 ng of genomic DNA, 200 µM of each dATP, dCTP, dGTP and dTTP (Boehringer), 600 ng of each primer, 1×PCR buffer (Promega) containing 1.5 mM MgCl₂ and 1 unit Taq DNA polymerase (Promega). Thirty five amplification cycles consisting of

30 seconds at 90°C, 30 seconds at 55°C and 1 minute at 70°C were carried out on a Perkin-Elmer 9600 thermocycler. All the amplification products were analysed by one of two methods, either 20 µl of the PCR reaction product was run on a 10% nondenaturing acrylamide gel followed by ethidium bromide staining and visualised by UV transillumination (32) or, alternatively, 2 µl of PCR amplification product was run on a 6% denaturing sequencing gel. The DNA was transferred by blotting in 10×TBE onto Hybond N+ membrane (Amersham International). After 3–12 hours the DNA on the membranes was denatured in 0.4 M NaOH for 15 minutes and neutralised in 5×SSC for 10 minutes. This was followed by 12 hours of hybridisation at 42°C with one of the oligonucleotide primers used in the initial PCR amplification. The hybridisation buffer contained 7% SDS, 10% PEG 6000, 250 mM NaCl and 12 mM phosphate buffer at pH 7.0. Five hundred ng of primer was labelled with [α -³²P] dCTP (Amersham International) using 15 units of terminal transferase (Gibco-BRL) at 37°C for 30 minutes. After hybridisation the membranes were washed once in 2×SSC and 0.1% SDS for 10 minutes at room temperature and the reaction products were detected by 3–12 hours of autoradiography.

DNA extraction from archival samples

Ten µm histological sections mounted on glass slides were stained with haematoxylin and eosin to confirm the pathology of the tumours. Tumour cells were scraped from slides of sections taken either side of the stained section into a 1.5 ml sterile tube. DNA was extracted by either of the following two methods:

1. Overnight incubation at 55°C in 500 µl of lysis buffer purchased from Applied Biosystems Limited and 100 µg/ml proteinase K was followed by standard extraction with phenol/chloroform, precipitation with absolute ethanol and resuspension in 50 µl H₂O (34). 1–5 µl (approximately 10–50 ng) was added to PCR amplification reactions, as described previously.
2. Overnight incubation at 55°C in 250 µl of 10 mM Tris pH 8.0, 0.5 mM KCl, 1.5 mM MgCl₂, 100 µg/ml BSA, 0.45% Tween 20 and 100 µg/ml proteinase K was followed by boiling the samples for 5 minutes and 5 µl added to PCR amplification reactions. This method was adapted from (20).

Loss of heterozygosity (LOH) determination

LOH was determined as complete or almost complete reduction to hemizygosity in constitutively heterozygous individuals. When a normal DNA control was unavailable for an individual, genotypes were confirmed from the analysis of close relatives. A microsatellite marker mapping within the TP53 gene was used to determine TP53 DNA status in the tumours (35).

EDH17B1 and EDH17B2 DNA sequence analysis

Genomic DNA isolated from lymphoblastoid cell lines was sequenced directly following PCR amplification. Oligonucleotide primers 5' and 3' to the coding region and specific for either EDH17B1 or EDH17B2 were designed from the published sequences (23). These are shown in Figure 5. Primers (a) and (c) were used for EDH17B1 amplification and primers (b) and (d) for EDH17B2. The two gene specific templates produced using these primer sets were added to a series of nested PCR reactions to generate PCR products suitable for DNA sequencing using a combination of the primers (e)–(m) (Figure 5). The initial gene specific targets were generated by PCR amplification cycles consisting of 30 seconds at 90°C, 30 seconds at 55°C and 3 minutes at 70°C repeated 35 times. Due to the high nucleotide homology of the two genes (74% over introns and exons), the identical sets of internal primer pairs could be used to amplify the exons for both genes. The specific primers used are shown in Figure 5 using the same amplification conditions as that used to generate the starting templates with the exception that the elongation time at 70°C was shortened to 30 seconds. DNA sequencing was carried out using Sequenase version 2 (USB) following the method described by (36).

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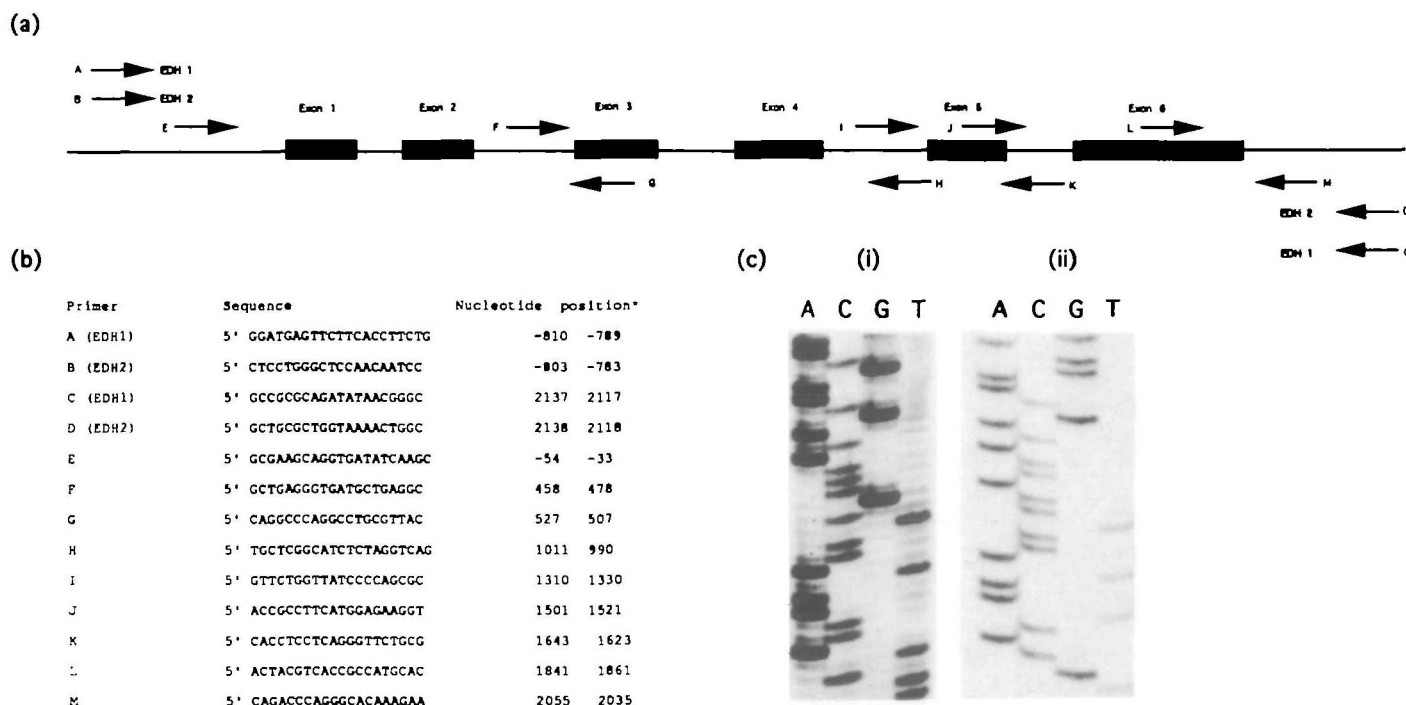


Figure 5. Sequencing of EDH17B1 and EDH17B2. (a) Schematic representation of the EDH17B1 and EDH17B2 genes showing the positions of the primers used in the gene specific amplification and sequencing. (b) Primer sequences. Gene specific primer sequences are indicated which were used to amplify gene specific template *Position relative to the first nucleotide of the translation initiation codon labelled 1. (c) Examples of autoradiograms from sequence analysis of the same region of exon 5 for each gene: (i) EDH17B2 and (ii) EDH17B1.

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