ONLINE MUTATION REPORT

Loss of five amino acids in *BRCA2* is associated with ovarian cancer

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Hereditary breast and ovarian cancer (HBOC) syndrome is characterised by an early age of onset and an autosomal dominant pattern of inheritance. Mutations in *BRCA1* or *BRCA2* account for the majority of families with HBOC, with carriers bearing a lifetime risk of approximately 50–80% for breast cancer and 15–45% for ovarian cancer.[1 2] Most deleterious mutations are small insertions/deletions, nonsense mutations, or splice site mutations that result in a frame shift and/or premature protein truncation. However, minor alterations such as missense mutations or small in frame insertions/deletions in the coding regions of *BRCA1* and *BRCA2* are often the only change detected. These present a clinical dilemma in cancer risk counselling because of their classification as variants of unknown significance (VUS), which are essentially uninformative findings.[3]

A number of analytical strategies exist to elucidate the significance of a VUS. If the variant has been reported previously, the commercial vendor of the gene test provides ancillary data about the VUS, such as the number of observations, predominant racial or ethnic origin, whether seen concurrently with a known deleterious mutation, and whether the VUS tracks with disease in families where such testing is possible. In addition, given the presumed tumour suppressor function of the BRCA proteins, it has been suggested that somatic allelic deletion of the wild type allele in a tumour, as detected by loss of heterozygosity (LOH) analysis, can provide evidence for the deleterious nature of the respective germline mutations.[4] Finally, the degree of evolutionary conservation of the amino acid sequence and the presence of functional domains in a given region of the protein can be considered circumstantial evidence for the importance of the amino acids encompassing that domain.[5]

BRCA gene sequencing (Myriad Genetic Laboratories Inc, Salt Lake City, UT, USA) of a 50 year old ovarian cancer patient from an HBOC family revealed a previously unreported 15 bp in frame deletion in exon 18 of *BRCA2* (8457del15), classified as a VUS. This mutation is predicted to result in the deletion of five amino acids (2744–2748; RLTVG) in the carboxyterminal region of the protein. We report here studies to elucidate the clinical significance of this mutation.

Key points

- Mutations in *BRCA1* and *BRCA2* are responsible for the majority of families with hereditary breast and ovarian cancer (HBOC) syndrome, and most deleterious mutations are small insertions/deletions, nonsense mutations, or splice site mutations that result in a truncated protein.
- The significance of minor alterations in coding regions such as missense mutations and small in frame insertions/deletions frequently cannot be inferred, so they are classified as variants of unknown significance, which creates a clinical dilemma in genetic counselling.
- We confirmed the probable clinical significance of a 15 bp in frame deletion in exon 18 of *BRCA2* (8457del15) that was discovered through *BRCA* gene sequencing of a 50 year old ovarian cancer patient from an HBOC family.
- The variant was found to track exclusively in affected family members. Tumour samples from two carriers showed selective loss of the wild type allele at the *BRCA2* locus, and the extent of chromosomal loss determined by LOH was distinct for each tumour. The region of BRCA2 harbouring the mutation revealed high evolutionary conservation across species and two overlapping functional domains that may be disrupted in carriers of this variant.

METHODS

Personal and family history, blood samples, and archival tumours were obtained from the patient and first degree relatives after written informed consent for participation in an institutional review board approved prospective hereditary cancer registry (IRB#96144). To investigate whether the mutation tracked with disease in the family, we isolated leukocyte DNA from the proband, two unaffected sisters, and a daughter, using a salting out method (AGTC; Denver, CO, USA). Paraffin embedded ovarian tumour tissue blocks from the proband and her deceased mother (history of breast cancer at 57 years of age and ovarian cancer at 62 years) were sectioned and microdissected to yield material with >90% tumour or normal cells, and DNA was extracted using a standard phenol/chloroform procedure following digestion with proteinase K. Exon 18 of *BRCA2* was amplified using published primer sequences and a modified touchdown PCR program. Nested primers encompassing the 8457del15 mutation were designed to amplify a 161 bp fragment from the initial PCR product (forward: 5′-CAGATGGGTGGTATGCTGTTAAGG and reverse: 5′-CATAAGAGATTCTGGGGCTTCAAG). This 161 bp amplicon was then used for LOH experiments and for sequencing on an ABI 377 automated sequencer (Applied Biosystems; Foster, CA, USA). A single PCR amplification (using only the inner primers) was sufficient for DNA extracted from whole blood.

Six microsatellite markers spanning chromosome 13 (D13S742, D13S218, D13S171, D13S267, D13S263, and D13S248; listed in physical map order) were amplified from matched normal and tumour sample pairs using published primer sequences. The markers were also amplified in

Abbreviations: HBOC, hereditary breast and ovarian cancer; LOH, loss of heterozygosity; OB, oligonucleotide/oligosaccharide binding; VUS, variant of unknown significance

unaffected relatives to gain haplotype information to determine the phase of the wild type allele. PCR products were analysed by PAGE on 8% (30 cm×40 cm, 0.8 mm spacers) gels in 1×TBE and visualised by ethidium bromide staining. LOH was determined semi-quantitatively (>50% allelic imbalance), and loci were scored as uninformative (homozygous in normal tissue), informative (heterozygous), or LOH.

Analysis of evolutionary conservation for BRCA2 was performed by a multisequence alignment using the SeqWeb comparison program Pile-Up (Accelrys; San Diego, CA, USA). Protein sequences were obtained by linking out to "Protein Neighbors" from human *BRCA2* on the NCBI website (www.ncbi.nlm.nih.gov). We conducted a PubMed (National Library of Medicine) search and reviewed published manuscripts reporting on functional and structural studies of BRCA2.

RESULTS

This was the first report of the *BRCA2* (8457del15) VUS by the vendor, therefore no ancillary data were available. Sequencing of the PCR product from the affected mother's tumour block confirmed the presence of the 15 bp deletion (fig 1). The smaller, mutated allele was clearly detected in both the proband and her mother by electrophoresis of the 161 bp fragment on a native 8% acrylamide gel. Conversely, the unaffected siblings and daughter of the proband were all negative for the mutation (fig 2). The probability of the variant tracking solely with disease in this family (that is, carrier status of the five individuals, and their respective affected/unaffected status) *v* chance (the 50:50 odds of inheriting a specific autosomal allele) is 1/32 (0.03). Excluding the 24 year old daughter, the probability is 1/16 (0.06), although the current ages of the other unaffected members may not be adequate to exclude potential ovarian cancer expression (the age at onset is not reliably earlier in *BRCA* carriers). In addition, tumours from both the proband and her mother showed loss of the wild type allele compared with normal tissue (fig 2C).

Analysis of six microsatellite markers along chromosome 13 revealed selective loss of the wild type allele in both the proband and her mother (data not shown). In the proband's tumour, all informative markers revealed LOH including distal markers on either end of the chromosome, indicating that complete somatic loss of chromosome 13 may have occurred. The mother's tumour was uninformative for the 13p terminal marker (D13S742) and retained heterozygosity at D13S218. All other markers analysed displayed LOH. Thus, the somatic loss in each tumour was unique.

An alignment of BRCA2 amino acid sequences from five species (human, mouse, rat, dog, and chicken) indicated significant evolutionary conservation in the region, with 39/50 amino acid identity (78%) between mammals (human, mouse, rat, and dog), including four of the five amino acids involved in the deletion (fig 3). A conservative change, from arginine to lysine, was seen at amino acid position 2744 in mouse. All other residues are identical across the five species including chicken.

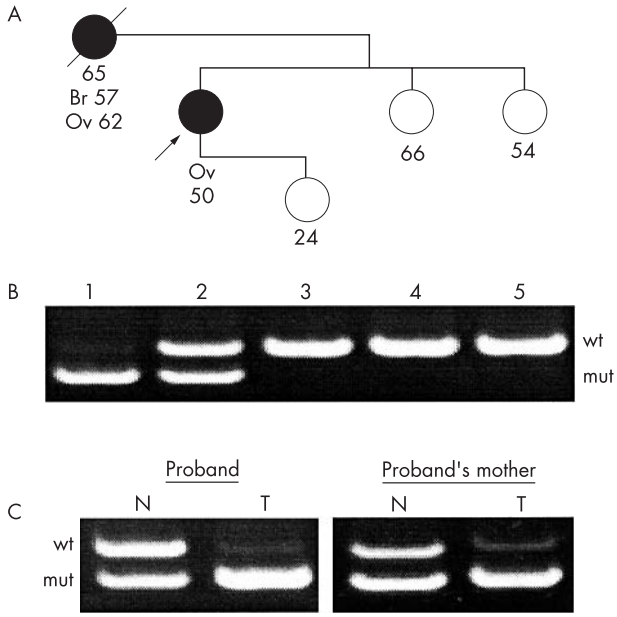

Figure 2 Segregation of the 8457del15 mutation in the family. (A) Abbreviated pedigree of the family with ages and affected status (filled symbols) indicated. (B) The PCR product from the section of exon 18 encompassing the mutation was analysed by PAGE for each individual. Lane 1 is from a tumour sample from the proband's deceased mother. Only the mutant allele is represented owing to the selective loss of the wild type allele. Lane 2, the proband, depicts the presence of the wild type and mutated allele in genomic DNA. Lane 3 is the proband's daughter, and lanes 4 and 5 are the unaffected siblings of the proband, all of whom are apparently homozygous for the wild type allele. (C) Comparison of normal *v* tumour DNA for both the proband and her mother at the 8457del15 locus reveal loss of heterozygosity, with selective loss of the wild type allele in each case.

Finally, a comprehensive PubMed search was conducted to collect information on mutations occurring within the region of interest, as well as any pertinent structural data. Of note, examination of recent crystallographic data from the BRCA2 carboxyterminus[6] revealed that in addition to being located within one of three oligonucleotide/oligosaccharide binding (OB) folds, the 8457del15 variant obliterates three DSS1 contacting residues.

DISCUSSION

VUS in the *BRCA* genes continue to be problematic in cancer risk counselling. If the VUS tracks exclusively with disease in

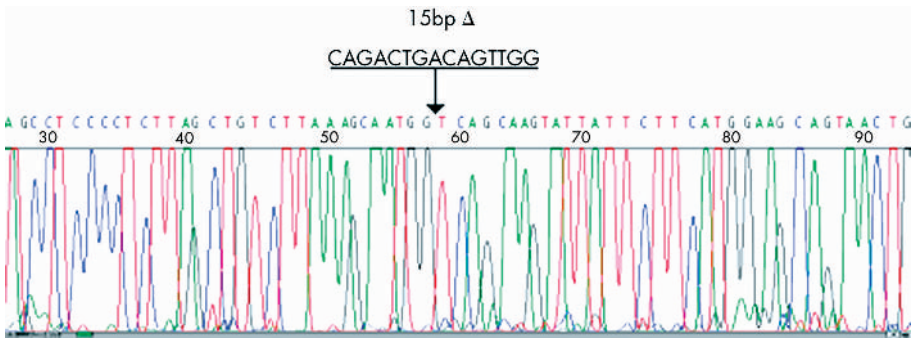

Figure 1 Chromatogram of the 161 bp BRCA2 exon 18 PCR product. Sequence data for tumour sample from proband's mother showing almost exclusively the mutant sequence (8457del15).

Figure 3 Multisequence alignment of BRCA2 orthologs. The five amino acids deleted in 8457del15 (2744–2748), depicted by the solid black bar, are highly conserved with only a conservative change from arginine to lysine in the first amino acid position in mouse, in addition to high conservation across the region, with 39/50 amino acid identity.

the family, as it does here, the likelihood of pathogenicity is increased. However, studies of tracking within a single family are seldom definitive because of limited statistical power, phenocopies, and incomplete age dependent penetrance. The strength of the association can be augmented by informative LOH studies demonstrating loss of the wild type allele in tumours. Furthermore, microsatellite markers can be useful for determining the extent of the somatic chromosomal loss. The use of sequence databases to determine the degree of evolutionary conservation in the amino acid sequence region in which the variant occurs is a complementary approach. High conservation indicates probable biological significance of the region in relation to protein function.[7]

The 8457del15 variant reported here has not previously been observed in published literature or in the Breast Cancer Information Core database. Our analysis indicated that the mutation tracked exclusively with the affected members in this family, and that there was somatic allelic deletion of the wild type allele in each case. This mutation is predicted to result in the deletion of five of the 3418 amino acids that constitute the BRCA2 protein, and lies within the highly conserved carboxyterminal domain. Conservation of the five specific amino acids seen here indicates that the region harbouring the deletion may have functional significance.

With respect to known functional domains, 8457del15 lies well downstream of the ovarian cancer cluster region[8] and the BRC repeats (Rad 51 binding sites). Recent crystallographic data[6] indicates that this variant results in the loss of three DSS1 contacting residues. Although DSS1 has not been linked to carcinogenesis to date, it may function by stabilizing BRCA2 within the cell or have some other as yet undefined role in DNA repair. Crystallography has also revealed that the 8457del15 variant lies within one of three OB folds, which are present in most single stranded DNA binding proteins. Consequently, disruption of this OB fold by the variant could compromise BRCA2 binding to ssDNA during homologous recombination.

Based on the facts that the *BRCA2* 8457del15 variant tracks exclusively with affected family members, with concurrent loss of the wild type allele in tumours from both affected carriers, coupled with the data on evolutionary conservation and possible disruption of functional domains, we conclude that the 15 bp deletion is highly likely to be deleterious. This information has clinical relevance for at risk members of this family and others who may have alterations affecting this region of BRCA2.

ELECTRONIC DATA ACCESS

Breast Cancer Information Core (accession number 4993): http://www.nhgri.nih.gov/Intramural_research/Lab_transfer/Bic/
PubMed: http://www.ncbi.nlm.nih.gov./PubMed/
Online Mendelian Inheritance in Man (OMIM). *BRCA1* (OMIM 113705) and *BRCA2* (OMIM600185). http://www.ncbi.nlm.nih.gov/Omim/

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