De novo recurrent germline mutation of the *BRCA2* gene in a patient with early onset breast cancer

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Abstract

Germline mutations in either of the two major breast cancer predisposition genes, *BRCA1* and *BRCA2*, account for a significant proportion of hereditary breast/ovarian cancer. Identification of breast cancer patients carrying mutations of these genes is primarily based on a positive family history of breast/ovarian cancer or early onset of the disease or both. In the course of mutation screening of the *BRCA1* and *BRCA2* genes in a hospital based series of patients with risk factors for hereditary breast/ovarian cancer, we identified a germline mutation in the *BRCA2* gene (3034del4) in a patient with early onset breast cancer and no strong family history of the disease. Subsequent molecular analysis in her parents showed that neither of them carried the mutation. Paternity was confirmed using a set of highly polymorphic markers, showing that the proband carried a de novo germline mutation in the *BRCA2* gene. Interestingly, 3034del4 is a recurrent mutation occurring in a putative mutation prone region of the *BRCA2* gene. Our study presents the first case in which a de novo germline mutation in the *BRCA2* gene has been identified, and supports previous results of haplotype studies, confirming that the 3034del4 mutation has multiple independent origins.

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Keywords: breast cancer; *BRCA2* gene; de novo mutation

Mutations in the two currently known major breast cancer predisposition genes, *BRCA1* and *BRCA2*, account for a significant proportion of the families with hereditary breast/ovarian cancer. Mutation studies of the *BRCA1* and *BRCA2* genes are usually performed in patients with a positive family history of breast/ovarian cancer. In patients without a positive family history, mutation testing of these genes may be considered if possible hereditary features such as an early onset, multifocality, multicentricity, bilaterality, and accompanying lobular carcinoma in situ are present.

Since the identification of the *BRCA1* and *BRCA2* genes in 1994¹ and 1995² respectively, more than 860 distinct genetic variants (including deleterious mutations, polymorphisms, and unclassified variants) have been

reported for each of these genes (The Breast Cancer Information Core (BIC) Database). Although both genes exhibit profound allelic heterogeneity, a large number of recurrent mutations have been reported. Some of the repeatedly identified *BRCA1* and *BRCA2* gene mutations represent founder mutations, which probably arose more than 2000 years ago. The most well known examples of *BRCA* founder mutations include the Ashkenazi Jewish mutations 185delAG and 5382insC in *BRCA1* and 6174delT in the *BRCA2* gene. Specific founder mutations in the *BRCA1* and *BRCA2* genes have been reported in many other populations.³ Some of the frequently observed mutations are likely to have arisen independently owing to the presence of mutational hotspots in the coding sequence of both genes. Haplotype analysis of recurrent mutations in the *BRCA1*⁴ and *BRCA2*⁵ genes provided evidence for multiple origins of several of these mutations. Despite the large number of *BRCA* gene mutations identified and the enormous number of investigations carried out world wide, to our knowledge there is only a single published report of a de novo mutation in the *BRCA1* gene,⁶ while de novo mutation in the *BRCA2* gene has not been reported to date.

During the course of a prospective study on risk factors for hereditary breast cancer in a hospital based series of breast cancer patients,^{7,8} we identified a proband with early onset breast cancer and no strong history of breast or ovarian cancer in her family. Mutation analysis of the *BRCA2* gene resulted in the identification of a deleterious germline mutation. In this report, we provide evidence that the mutation identified in the proband represents a de novo mutational event in the *BRCA2* gene. The mutation occurred at a site within the gene previously found to be involved in the aetiology of breast cancer in geographically diverse breast cancer families.⁵

Materials and methods

SUBJECTS

The patient was referred to our department as a result of her participation in a study on risk factors for hereditary breast cancer in a hospital based series of breast cancer patients. The study was approved by the Medical Ethical Committee of our hospital. All patients in the study had been referred for radiotherapy as part of their curative treatment. For patient selection, a check list with risk factors was used. Risk factors included breast cancer <45

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years, bilateral breast cancer, multicentricity, multifocality, accompanying lobular carcinoma in situ, ovarian cancer, a positive family history of breast cancer, and a positive family history of ovarian cancer. If patients had one or more risk factors, a pedigree was composed. Patients fulfilling the criteria for referral to the family cancer clinic of the University Medical Centre Utrecht were offered genetic counselling and DNA analysis after informed consent was obtained. The proband was diagnosed with multifocal breast cancer with axillary node metastases at the age of 39 years. The patient's father and mother, aged 70 and 71 years respectively, were both healthy. The mother had no family history of breast/ovarian cancer. The father of the proband had a cousin on the paternal side with breast cancer diagnosed at the age of 54.

MUTATION ANALYSIS OF THE *BRCA2* GENE

Peripheral blood samples were obtained from the proband and her parents. Genomic DNA was prepared from 10 ml EDTA blood samples according to standard procedures. For each subject, mutation and polymorphic marker analyses were repeated on a second, independent DNA sample.

Exon 11 of the *BRCA2* gene was screened for mutations using a non-radioactive protein truncation test (PTT). To generate templates for the PTT, four overlapping PCR fragments of the 4.9 kb exon 11 of the *BRCA2* gene were amplified from genomic DNA using the T7 modified oligonucleotide primer pairs designed by Ligtenberg *et al.*⁹ PCR reactions were performed in a GeneAmp 9700 PCR System (PE Biosystems) in a 20 µl volume containing approximately 100 ng genomic DNA as template, 5 pmol of each oligonucleotide primer, 0.2 mmol/l of each dNTP, and 0.5 unit AmpliTaq® DNA polymerase (Perkin Elmer), in 1 × Opti-prime™ buffer No 6 (Stratagene). PCR was performed by an initial denaturation of five minutes at 94°C, followed by 38 cycles of one minute at 94°C, one minute at 55°C, four minutes at 72°C, and a final extension step of seven minutes at 72°C. In vitro transcription and translation using the TnT® T7 Coupled Reticulocyte Lysate System (Promega) and biotinylated tRNA-lysine (transcend, Promega) was essentially performed as described previously.^{9,10}

Further characterisation of the mutation was performed by semi-automated direct sequencing. First, the region of *BRCA2* exon 11 in which a truncating mutation was detected by PTT was amplified from genomic DNA using the forward primer 5'-CGC TAA TAC GAC TCA CTA TAG GAA CAG ACC ACC ATG GTG CAT TCT TCT GTG AAA AGA AGC-3' and the reverse primer 5'-AAG CTG TTC TGA AGC TAC CTC C-3' (nt 3244-3265 in *BRCA2* cDNA sequence U43746 (GDB)). The latter oligonucleotide was used as sequencing primer. The 1159 bp PCR product was purified using the High Pure PCR Product Purification Kit (Roche Diagnostics GmbH). Approximately 90 ng of template was used for sequencing using the dRhodamine Terminator

Figure 1 Detection of a protein truncating mutation in exon 11 of the *BRCA2* gene by the PTT. The normal *in vitro* synthesised protein corresponds to codons 639 to 1144 of the *BRCA2* protein (nt 2144-3661 in the cDNA sequence). In addition to the normal product, the patient (P) shows a shortened protein, indicating a protein truncating mutation in the corresponding region of the *BRCA2* gene. Lane N contains a normal control. Bands marked with an asterisk represent endogenous biotinylated proteins present in the reticulocyte lysate used for *in vitro* transcription and translation. Other background bands correspond to *in vitro* translated products generated by secondary initiation owing to internal start sites.

Cycle Sequencing Ready Reaction - DNA Sequencing Kit (PE Biosystems) according to the manufacturer's instructions. Cycle sequencing PCR was performed by an initial denaturation of one minute at 96°C, followed by 25 cycles of 10 seconds at 96°C, five seconds at 55°C, and four minutes at 60°C. Sequence reactions were purified using a Sephadex™ G-50 superfine column (Amersham Pharmacia Biotech AB) in a Multi-Screen® plate (Millipore) according to the manufacturer's instructions. Sequence reactions were analysed on an ABI Prism™ 3700 DNA Analyzer (PE Applied Biosystems) using the Sequence Analysis 3.1 software (Perkin Elmer).

PATERNITY CONFIRMATION

To confirm paternity, a set of highly polymorphic short tandem repeat (STR) markers was analysed using the AmpFISTR® Profiler Plus™ PCR Amplification Kit (PE Applied Biosystems). The STR markers analysed were D3S1358, vWA, FGA, D8S1179, D21S11, D18S51, D5S818, D13S317, and D7S820. PCR amplification and fragment analysis of STR markers were performed according to the manufacturer's instructions. Markers were analysed on an ABI Prism 377™ DNA Sequencer (PE Applied Biosystems) using the GeneScan® Analysis 3.1 and GenoTyper 2.1 software (Perkin Elmer). According to the AmpFISTR® Profiler Plus™ PCR Amplification Kit (PE Applied Biosystems) user's manual, the combined probability that a random alleged father will be excluded from paternity after DNA typing of these nine STR loci is 0.999982.

Figure 2 DNA sequence analysis of the protein truncating mutation in exon 11 of the *BRCA2* gene detected in the proband. Note that the sequence is shown in the reverse orientation. Analysis of the patient's DNA (P) shows a frameshift (arrow) caused by a 4 bp deletion within the sequence TTGTTT (AAACAA in the forward sequence, corresponding to nt 3034-3039). In contrast, both the father (F) and mother (M) of the patient show a normal sequence for this region of the *BRCA2* gene.

Results

Mutation screening of the *BRCA2* gene by PTT in the proband indicated the presence of a premature termination of translation in fragment C (nt 2144-3639) of exon 11 (fig 1). To identify the molecular nature of the protein truncation, direct sequencing of the corresponding region of exon 11 was performed. The patient was found to carry a 4 bp deletion within a 6 bp sequence (AAACAA) located between positions 3034 and 3039 (fig 2). The deletion results in a frameshift and a premature termination at codon 958.

Table 1 Results of genotype analysis using nine highly polymorphic markers*

STR marker†	Father‡	Proband	Mother
D3S1358	14-16	14-17	15-17
FGA	19-22	19-22	22-25
D5S818	11-12	11-11	11-12
D7S820	8-9	8-9	8-11
D8S1179	11-15	11-15	15-17
VWA	15-15	15-19	17-19
D13S317	8-11	8-12	12-14
D18S51	14-16	13-16	10-13
D21S11	28-33	33-33	29-33

*Both alleles of the corresponding STR marker are shown for each person. Marker alleles are designated according to the user's manual of the AmpFISTR® Profiler Plus™ PCR Amplification Kit (PE Applied Biosystems).
†Alleles transmitted to the proband are shown in bold.
‡The combined probability of paternity exclusion for the multi-locus genotype shown in bold is 0.999999979.

Subsequently, the father and mother of the patient were investigated for carriership of the mutation by direct sequence analysis. Surprisingly, neither of the parents was found to carry the mutation, suggesting that this germline mutation had occurred de novo. Analysis of nine highly polymorphic STR markers supported the claimed paternity with high probability (table 1). This confirms that the deletion identified in the proband represents a de novo germline mutation in *BRCA2*.

To determine whether the mutation had occurred on the maternal or paternal allele, we investigated a polymorphism (3199A→T, Asn991Asp) located near the site of mutation in the *BRCA2* gene. However, we could not take advantage of this single nucleotide polymorphism because both parents were homozygous (data not shown).

Discussion

Since the cloning of the *BRCA1* and *BRCA2* genes, a continuously increasing number of disease causing mutations, common polymorphisms, and variants of uncertain clinical significance (usually missense mutations) has been reported. Using haplotype analysis, some of the recurrent mutations in these genes were found to have multiple independent origins. It is assumed that mutational hotspots, such as tandemly repeated sequences, give rise to some of the recurrent mutations. Yet, there is only one published report of a proven de novo mutation in the *BRCA1* gene,⁶ while de novo mutations in the *BRCA2* gene have not been described.

Several explanations are possible for the apparently low frequency of de novo mutations in the *BRCA1* and *BRCA2* genes. Breast cancer resulting from mutation of these genes does not usually affect the reproductive period to a great extent, given that the mean age at diagnosis is 42.7 years in *BRCA1* gene carriers and 48.7 years in *BRCA2* gene carriers.¹¹ For ovarian cancer, mean age at diagnosis is 52.6 years (*BRCA1*) and 62.8 years (*BRCA2*). Therefore, mutations will remain present in the population for many generations, which results in the presence of detectable founder haplotypes around ancient mutations in various populations.³ Under these circumstances the proportion of de novo mutations among all gene

carriers will be low.¹² The low reported incidence of de novo mutation may also be the result of a selection bias, since mutation studies of the *BRCA1* and *BRCA2* genes are usually performed in the context of a strong family history for breast/ovarian cancer. De novo *BRCA* gene mutations are particularly expected in early onset breast cancer patients without a family history of the disease. Although there are many published reports of *BRCA1* and *BRCA2* germline mutations detected in early onset breast cancer patients without a family history of the disease, these studies do not include results of mutation screening in their parents. In order to obtain further insight into the incidence of de novo mutation of the *BRCA1* and *BRCA2* genes, both parents of each mutation carrier should, wherever possible, be investigated for carrier-ship of the mutation, regardless of the family history of the disease.

The 3034del4 mutation identified in the proband represents a proven de novo alteration that has occurred at a mutation prone sequence in the *BRCA2* gene. Apart from this recurrent 4 bp deletion, at least one other deletion (3038delAA) is frequently found within the same region. It is important to note that a third frequently reported deletion, known as 3036del4, probably represents the same mutation as 3034del4. The 3034del4 and 3036del4 mutations cannot be distinguished, because it is impossible to determine which four bases (AAAC, AACA, or ACAA) are deleted. Any deletion of four adjacent bases between positions 3034 and 3039 in the *BRCA2* gene results in a frameshift and premature stop at codon 958. Moreover, it is unclear whether these mutations are different ones. According to the recommendations for the nomenclature of human gene mutations,¹³ a 4 bp deletion occurring in this sequence of the *BRCA2* gene should be designated 3036del4.

Currently, the number of reports of the 3034del4 and the 3036del4 mutation in the BIC Database is 12 and 24, respectively. In a recent survey of *BRCA2* gene mutations identified in Dutch and Belgian breast and ovarian cancer families, the 3034del4 mutation was reported in five out of 139 mutation positive families, while the 3036del4 mutation was observed twice (Dutch and Belgian Collaborative Group on BRCA Diagnostics (LOB), unpublished data).

Our findings are in accordance with previous haplotype studies, suggesting that the 3034del4 *BRCA2* germline mutation may have arisen on many separate occasions in people of various ethnic origins. In an international study on recurrent *BRCA2* gene mutations, 11 families from seven different western European and North American countries (Belgium, Canada, France, Italy, Spain, Switzerland, and the United States) carrying the 3034del4 mutation were investigated.⁵ Analysis of 10 polymorphic STR markers flanking the *BRCA2* locus showed a considerable amount of haplotype diversity among the families examined. Although no statistically significant evidence was found for multiple independent origins of the

3034del4 mutations, the authors could not rule out the possibility of independent mutations for as many as half of the families. The observation of a de novo mutation reported in the present study provides further evidence that the sequence between nucleotides 3034 and 3039 in the *BRCA2* gene represents a hot spot for small deletions. A variety of different mechanisms have been proposed for the generation of gene deletions mediated by short direct repeats, palindromes, and quasi-palindromes.¹⁴ Owing to the presence of a short repeat motif within the target sequence (nt 3034-3039, AAACAA), misalignment of this short direct repeat during DNA replication may have caused the 4 bp deletion detected in the proband.

Mutations in the *BRCA2* gene are usually detected in patients with a family history of breast/ovarian cancer or in isolated patients with risk factors for hereditary breast/ovarian cancer. Our study underlines the importance of mutation screening of the *BRCA1* and *BRCA2* genes in patients with early onset breast cancer without a strong family history of the disease.

Website addresses: The Breast Cancer Information Core (BIC) Database: http://www.nhgri.nih.gov/Intramural_research/Lab_transfer/Bic/Member/index.html. The Genome Database: <http://www.gdb.org>.

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