Research article

Novel mutations in the *BRCA1* and *BRCA2* genes in Iranian women with early-onset breast cancer

Vahid R Yassaee[1,2], Sirous Zeinali[3], Iraj Harirchi[4], Soghra Jarvandi[4], Mohammad A Mohagheghi[5], David P Hornby[1] and Ann Dalton[2]

[1]Department of Molecular Biology and Biotechnology, The University of Sheffield, Sheffield, UK
[2]North Trent Molecular Genetics Laboratory, Sheffield Children's Hospital, Sheffield, UK
[3]Department of Molecular Biotechnology, Pasteur Institute, Tehran, Iran
[4]Iranian Centre for Breast Cancer (ICBC), Tehran University of Medical Sciences, Tehran, Iran
[5]Cancer Institute, Tehran University of Medical Sciences, Tehran, Iran

Correspondence: Ann Dalton, PhD, MRCPath, Head of Molecular Genetics Laboratory, North Trent Molecular Genetics Service, Sheffield Children's Hospital, Western Bank, Sheffield S10 2TH, UK. Tel: +44 114 2717004; fax: +44 114 2737467; e-mail: ann.dalton@sch.nhs.uk

Abstract

Background: Breast cancer is the most common female malignancy and a major cause of death in middle-aged women. So far, germline mutations in the *BRCA1* and *BRCA2* genes in patients with early-onset breast and/or ovarian cancer have not been identified within the Iranian population.

Methods: With the collaboration of two main centres for cancer in Iran, we obtained clinical information, family history and peripheral blood from 83 women under the age of 45 with early-onset breast cancer for scanning of germline mutations in the *BRCA1* and *BRCA2* genes. We analysed *BRCA1* exons 11 and *BRCA2* exons 10 and 11 by the protein truncation test, and *BRCA1* exons 2, 3, 5, 13 and 20 and *BRCA2* exons 9, 17, 18 and 23 with the single-strand conformation polymorphism assay on genomic DNA amplified by polymerase chain reaction.

Results: Ten sequence variants were identified: five frameshifts (putative mutations – four novel); three missense changes of unknown significance and two polymorphisms, one seen commonly in both Iranian and British populations.

Conclusions: Identification of these novel mutations suggests that any given population should develop a mutation database for its programme of breast cancer screening. The pattern of mutations seen in the *BRCA* genes seems not to differ from other populations studied. Early-onset breast cancer (less than 45 years) and a limited family history is sufficient to justify mutation screening with a detection rate of over 25% in this group, whereas sporadic early-onset breast cancer (detection rate less than 5%) is unlikely to be cost-effective.

Keywords: *BRCA1*, *BRCA2*, breast cancer, Iranian population, mutation detection methods

Introduction

Worldwide, breast cancer is the most common cancer in women, excluding skin cancers [1], with a lifetime risk of 10% in the general population [2]. In spite of earlier detection and better treatment, largely due to recent technological advances, it is still the second leading cause of cancer death in women, exceeded only by lung cancer [3]. The three commonest cancers, excluding skin cancer, in the Iranian population (females, all ages), are cancers of the oesophagus, breast and cervix, in diminishing order of incidence [4]. A preliminary study showed no significant differences in age-specific

PTT = protein truncation test; SSCP = single-strand conformation polymorphism.

incidences among Iranian women with breast cancer, in comparison with other studies [5,6].

Hereditary breast cancer is suspected in an individual who has a family history of breast cancer, or breast and ovarian cancer, that is consistent with autosomal dominant inheritance [7]. Many efforts are now under way to reduce the high incidence and mortality associated with breast and ovarian cancer by the early detection of women at high risk. These women, once identified, can be targeted for more aggressive prevention programs. Breast cancer is not a systemic disease at its inception but is progressive; screening can arrest its development and the treatment of advanced breast cancer is often futile and disfiguring [8].

In 1990, the first breast cancer susceptibility gene, *BRCA1*, was localised by linkage analysis to chromosome 17q21 [9]. Miki *et al.* [10] isolated the *BRCA1* gene in 1994. Subsequently, Wooster *et al.* [11] identified a new gene, *BRCA2*. Both *BRCA1* and *BRCA2* are large genes, consisting of 24 and 27 exons, respectively. Mutations in *BRCA* genes are distributed throughout the coding region. Women who carry *BRCA1* and *BRCA2* mutations have a significantly increased chance of developing breast cancer before the age of 50 years [12–14]. Some studies indicate that 13% of women who are diagnosed with breast cancer under the age of 30 years, and 7% of women who are diagnosed with breast cancer under the age of 35 years, have germline *BRCA1* alterations [12,15]. No molecular genetics study of *BRCA1* and *BRCA2* germline mutations has been reported in the Iranian population and there are few individuals with strong family histories that have as yet been identified. We therefore selected women who were most likely to harbour germline mutations in these genes.

Mutational spectrum

At present, over 878 distinct mutations, polymorphisms and variants throughout the *BRCA1* gene have been discovered [16]. Over 900 distinct alterations have been identified in *BRCA2* [16] and, as in *BRCA1*, they are not limited to a particular region of the gene. Most mutations in both genes are private [16]. *BRCA1* is considered to be responsible for about one-half of all cases of early-onset breast cancer and for the majority of familial breast and ovarian cancers [9,17]. *BRCA1* somatic mutations have never been reported in sporadic breast cancer [18] although they have occasionally been found in sporadic ovarian cancers [19,20].

Materials and methods
Design

We performed the present study to obtain initial experience in identifying germline mutations in the *BRCA1* and *BRCA2* genes in Iranian women diagnosed with early-onset breast cancer.

With the cooperation of two main centres for cancer research and treatment in Tehran, Iran, namely the Iranian Centre for Breast Cancer and the Cancer Institute at Tehran University of Medical Sciences, 83 samples derived from 82 unrelated Iranian families were selected for screening of germline mutations in *BRCA1* and *BRCA2*.

During 3 years since 1997, the medical records of 152 women diagnosed with breast cancer at the Iranian Centre for Breast Cancer were reviewed and 39 patients were selected for screening. In the 12 months after December 1999, a consecutive series of 44 patients from the Cancer Institute who were diagnosed with breast cancer were chosen for screening. All selected patients from these two centres were under the age of 45 years.

All selected women were informed that their DNA samples would be analysed for known mutations in genes associated with susceptibility to breast cancer; they were offered the opportunity to receive the results and were asked to sign a second consent form if they chose to learn the results.

DNA isolation and mutation analysis

Using a Promega DNA purification kit (catalogue no. LA1620) and in accordance with the manufacturer's protocols, genomic DNA was extracted from peripheral blood lymphocytes at the Pasteur Institute in Tehran, Iran.

We analysed *BRCA1* exon 11 and *BRCA2* exons 10 and 11 by the protein truncation test (PTT) [21–23], and *BRCA1* exons 2, 3, 5, 13 and 20 and *BRCA2* exons 9, 17, 18 and 23 by the single-strand conformation polymorphism (SSCP) assay [24] and heteroduplex analysis [25] by amplification from genomic DNA with the polymerase chain reaction (PCR).

Although mutations are scattered throughout both *BRCA1* and *BRCA2*, some coding regions were particularly chosen for several reasons: first, it has been shown that they have a significant role in protein function (exons 2–5, Ring finger domain [26]; exon 11 and exon 20, BRCT domain) [27]; second, exons 10 and 11 cover a large segment of the gene; third, many putative mutations have been reported in these regions; and fourth, the coding regions listed above have been shown in other significant studies [28–32] to be most likely to harbour germline *BRCA1* and *BRCA2* mutations. A comparison of data from other studies suggests that, with this strategy, about 14% and 22% of mutations in *BRCA1* and *BRCA2*, respectively, would be missed. However, there are no data to indicate the presence of population-specific mutations in Iran; these data must therefore be considered preliminary [28–32].

The PTT method

Most mutations reported in *BRCA1* and *BRCA2* cause premature termination of translation, which is readily

detectable by PTT; PTT is a very sensitive and efficient tool for mutation detection [28].

Modified primers containing a T7 promoter and a perfect Kozak consensus sequence were used to generate PCR products of the whole of exon 11 of *BRCA1* and exons 10 and 11 of *BRCA2* that were suitable for PTT. Each primer pair amplified 3446 base pairs (bp) and 4959 bp for *BRCA1* exon 11 and *BRCA2* exon 11, respectively. Regions (the 5′ and 3′ ends) of exon 11 in both genes were screened separately to identify any potential decrease in the sensivity of PTT resulting from the use of such large fragments.

PCR programme
PCRs were performed with genomic DNA containing 50 ng of genomic DNA, 1 μl of each primer at 5 pmol/μl, 2 μl of a mixture of dNTPs (each at 2.5 mM), 2.5 μl of 5 × PCR buffer A [300 mM Tris-SO$_4$ (pH 9.1 at 25°C), 90 mM (NH$_4$)$_2$SO$_4$ and 5 mM MgSO$_4$] and 2.5 μl of buffer B [300 mM Tris-SO$_4$ (pH 9.1 at 25°C), 90 mM (NH$_4$)$_2$SO$_4$ and 10 mM MgSO$_4$], 1 μl of Elongase® Enzyme Mix (Invitrogen, Paisley, UK) in accordance with manufacturer's recommendations, and distilled water was added to a final volume of 25 μl. For amplification, each sample was denatured at 94°C for 1 min and subjected to 31 cycles of PCR (94°C for 25 s, 56°C for 1 min, and extension at 68°C for 4 min on an Applied Biosystems DNA thermal cycler, Applera Europe BV, Cheshire, UK); this was followed by incubation at 68°C for 10 min. The PCR products were checked on a 0.7% agarose gel and a 1 μl sample was used for a coupled transcription–translation reaction (TNT® T7 Quick for PCR DNA kit from Promega, Southampton, UK; catalogue no. L5540), in accordance with the instructions of the manufacturer. Full-length artificial proteins of BRCA1 and BRCA2 exon 11, 126 and 182 kDa, respectively, labelled with ^{35}S, were detected after SDS-PAGE [33] (see Fig. 2) on a 2 × Mini-Protean system (Bio-Rad, Hertfordshire, UK, catalogue no. 165-3301).

The SSCP assay
BRCA1 exons 2, 3, 5, 13 and 20, *BRCA2* exons 9, 17, 18 and 23 and the 5′ and 3′ ends of exon 11 of *BRCA1* and *BRCA2* were analysed with the SSCP method.

PCR was performed on 50 ng of genomic DNA, 1 μl of 5 pmol/μl forward primer, 1 μl of 5 pmol/μl reverse primer, 1.6 μl of a mixture of dNTPs (each at 2.5 mM), 2 μl of 10 × PCR reaction buffer [750 mM Tris-HCl (pH 8.8 at 25°C), 200 mM (NH$_4$)$_2$SO$_4$ and 0.1% (v/v) Tween 20], 0.7 unit of Red Hot *Taq* DNA polymerase, made up to a final volume of 20 μl with distilled water. For amplification, each sample was denatured at 94°C for 2 min and subjected to 28–30 cycles of PCR (at 94°C for 30 s, at 55–60°C for 1 min, and extension at 74°C for 1 min on a

Perkin-Elmer-Cetus DNA thermal cycler); this was followed by incubation at 72°C for 5 min. Annealing temperatures varied according to the melting temperature, T_m, of the primer template.

SSCP analysis for point mutations was performed under the following conditions: a mixture of 5 μl of a 28-cycle PCR product and 5 μl of loading buffer was denatured for 10 min at 95°C, cooled rapidly on ice and separated on a non-denaturing 14% polyacrylamide gel (57:1 acrylamide:bisacrylamide, 3–10% glycerol) in 0.5 × Tris-borate-EDTA buffer at 12–16°C for 16–20 h at 245 V. Bands were revealed by silver staining.

Direct sequencing
All sequence variants were confirmed by using the PCR products of each sequence variant and Big Dye dideoxy-terminator chemistry (Perkin-Elmer) on an ABI 377 DNA sequencer (Applied Biosystems).

Primer design
For SSCP, splice junctions were included in the analysis in all cases. In addition, four sets of primers were used to screen the 5′ and 3′ ends of exon 11 of *BRCA1* and *BRCA2*. For PTT, two sets of primers were designed to screen the whole of exon 11 of *BRCA1* and *BRCA2*.

Results
We have identified ten sequence variants in this cohort: five frameshifts, four of which were novel (Figs 1 and 2), three missense changes of unknown significance and two polymorphisms.

A common polymorphism in *BRCA2* [IVS16-14T>C] was identified in both Iranian and British populations. No sequence variant was detected at the 5′ and 3′ ends of exon 11 in *BRCA1* and *BRCA2* by SSCP analysis, suggesting that there was no loss of sensitivity in analysing such large fragments by PTT.

Tables 1 and 2 describe the distribution of *BRCA1* and *BRCA2* mutations respectively by the type of mutations.

Discussion
This is the first report to describe mutations in the *BRCA* genes in the Iranian population. The cohort studied all had early-onset (less than 45 years) breast cancer; a subgroup had a family history that was quite limited (Tables 1 and 2) in comparison with most other studies [34–37]. These preliminary data suggest that the spectrum of mutations identified differs little from that seen in other studies, with no recurrent mutation and the mutations spread throughout the genes. Five putative (frameshift) mutations were detected in the group as a whole (6.02% detection rate) in which four mutations were detected among 14 individuals with a family history (28.6% detection rate with 95%

Figure 1

(a)

(b)

(c)
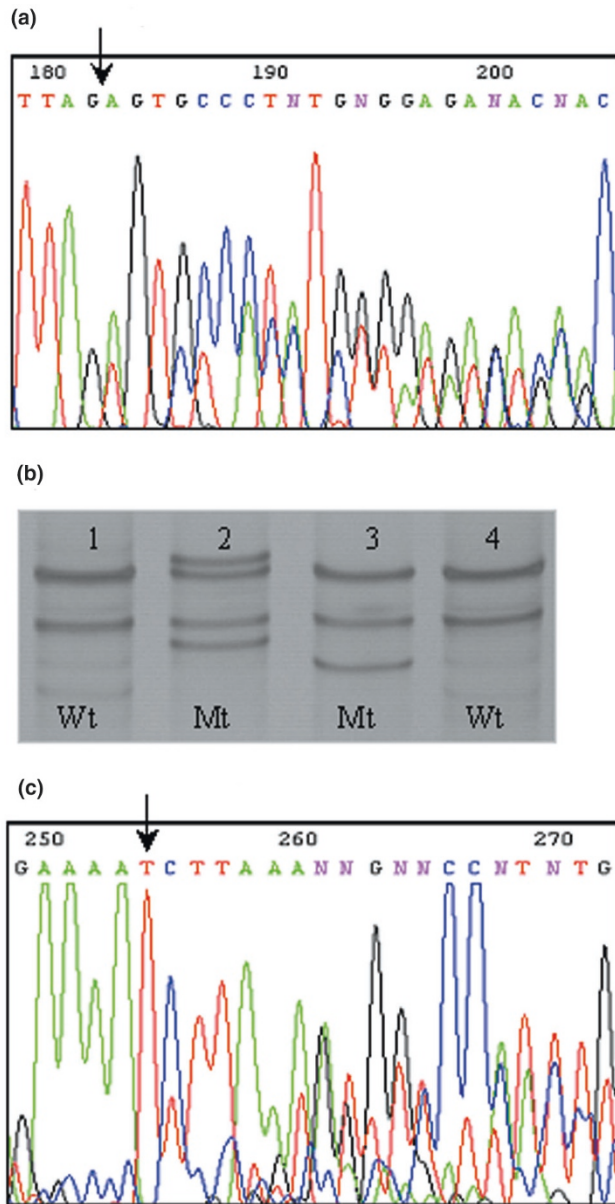
Single-strand conformation polymorphism (SSCP) assay for germline mutation in *BRCA1* exon 2. Lanes 1 and 4 show normal patterns, and lane 2 and 3 depict the abnormal patterns of single-strand DNA mobility seen on a polyacrylamide gel **(b)**. Frameshift mutation in sample (lane 2) confirmed by direct sequencing **(a)** that shows a 2 bp (AG) deletion in *BRCA1* exon 2 at nucleotides 185–186 that leads to the formation of TGA at codon 39. **(c)** A frameshift mutation (lane 3) identified by direct sequencing, which revealed a 1 bp (T) insertion in *BRCA1* exon 2 between nucleotides 181 and 182, leading to the formation of TGA at codon 40. These frameshift mutations are likely to disrupt the function of the *BRCA1* proteins.

binomial confidence interval 0.09–0.58), whereas one mutation was seen in a patient among 69 individuals with early onset breast cancer but without a family history

Figure 2

SDS-PAGE (6% polyacrylamide)
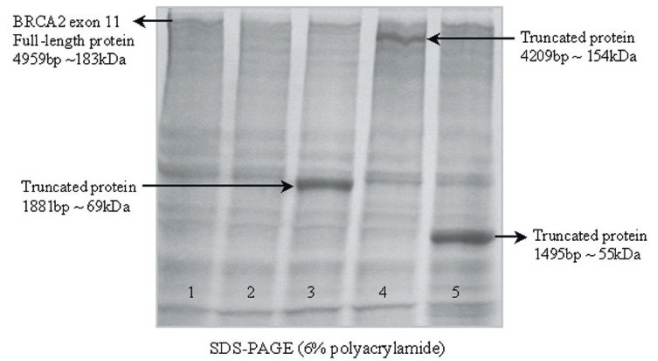
SDS-PAGE analysis of the whole of exon 11 of *BRCA2*, revealing the capacity of the PTT technique to detect mutations within 4959 bp coding sequences in a single reaction with the use of a coupled transcription–translation system, TNT® T7 Quick for PCR DNA kit from Promega. Lanes 1 and 2 show the normal pattern of the full-length (181 kDa) protein from exon 11 of *BRCA2*; lanes 3–5 show three different sizes of truncated protein, which were identified on 6% SDS-PAGE. Arrows show the sizes and positions of the normal and truncated proteins. Lane 4 shows a mutation occurring close to the 3′ end of exon 11 and producing a large protein for which the band migrated close to the top of the gel.

(1.5% detection rate with 95% binomial confidence interval 0–0.09) [38].

In many of the study cohort, we failed to identify a causative mutation, regardless of the presence or absence of a family history and these may simply be sporadic cases. However, it is clear that the known genes cannot explain most of the variation in breast cancer risk in the population. The difficulties of identifying further genes by linkage indicate that the remaining genes might be numerous, with relatively common alleles conferring moderate risks. Only by identifying these genes will the true pattern of risk and the mechanism behind them become clear [39].

Conclusions
The detection rate in those with a family history was high, especially when compared with other studies in which the selection criteria for screening required a much stronger family history. This suggests that the screening, albeit incomplete, was well targeted. The low detection rate in those with apparently sporadic early-onset cancer suggests that early-onset cancer alone is insufficient to justify screening in the Iranian population. If these results were confirmed on a large cohort, molecular methods would form a vital part of any screening programme in Iran.

GenBank accession numbers
These sequence variants have already been submitted to GenBank: accession numbers AF274503, AF284812,

Table 1

Germline mutations in the *BRCA1* gene

Exon	Mutation and nucleotide change	Stop codon at amino acid	Coding effect	Screening method	Family history	Age at diagnosis (years)
2	185-186delAG	39 (TGA)	Frameshift	SSCP/HA	2 BC, 1 PS	37
2	181-182insT	40 (TGA)	Frameshift	SSCP/HA	1 OV	41
11	2335-2336delAA	741 (TAA)	Frameshift	PTT	2 BC <40	42
20*	12bp dup GTATTCCACTCC IVS20+48	–	Polymorphism	SSCP/HA	2 BC <42	27

*This patient also has a frameshift mutation in exon 11 of *BRCA2*. BC, breast cancer (early-onset ages are also shown); HA, heteroduplex analysis; OV, ovarian cancer; PS, prostate cancer; PTT, protein truncation test; SSCP, single-strand conformation polymorphism assay.

Table 2

Germline mutations in the *BRCA2* gene

Exon	Mutation and nucleotide change	Stop codon at amino acid	Coding effect	Screening method	Family history	Age at diagnosis (years)
11*	6261-6262 insGT	2040 (TAA)	Frameshift	PTT	2 BC <40	27
11	3979-3980 insA	1264 (TAA)	Frameshift	PTT	Negative	40
11	5972 C>T T1915M	–	Missense	DS	Negative	41
17†	IVS16-14T>C IVS16-6T>G	–	Close to splice site	SSCP/HA	n.a.	n.a.
18	8345A>G N2706S	–	Missense	SSCP/HA	Negative	38
23	9266C>T T3013I	–	Missense	SSCP/HA	Negative	31

*This patient has also *BRCA1*-IVS20+48 dup GTATTCCACTCC. †This common polymorphism was detected in both Iranian and British populations. BC, breast cancer (early-onset ages are also shown); DS, direct sequencing; HA, heteroduplex analysis; n.a., not applicable; PTT, protein truncation test; SSCP, single-strand conformation polymorphism assay.

AF288936, AF288937, AF288938, AF309413, AF317283, AF348515, AY008850 and AY008851.

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