BRCA1 and *BRCA2* Mutations in an Asian Clinic-based Population Detected Using a Comprehensive Strategy

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Abstract

Background and objective: **Genetic testing for germ line mutations in the *BRCA1* and *BRCA2* genes for some families at high risk for breast and/or ovarian cancer may yield negative results due to unidentified mutations or mutations with unknown clinical significance. We aimed to accurately determine the prevalence of mutations in these genes in an Asian clinic-based population by using a comprehensive testing strategy.**

Materials and Methods: **Ninety-four subjects from 90 families were accrued from risk assessment clinics. In addition to conventional mutational screening of *BRCA1* and *BRCA2*, multiplex ligation-dependent probe amplification for the detection of large genomic rearrangements, evaluation of splice site alterations using transcript analysis and SpliceSiteFinder prediction, and analysis of missense mutations of unknown significance by multiple sequence alignment, PolyPhen analysis, and comparison of Protein Data Bank structures were incorporated into our testing strategy.**

Results: **The prevalence rates for clearly deleterious *BRCA1* and *BRCA2* mutations were 6.7% (6 of 90) and 8.9% (8 of 90), respectively, or 7.8% (7 of 90) and 11.1% (10 of 90), respectively, by including missense mutations predicted to be deleterious by computational analysis. In contrast to observations from European and American populations, deleterious mutations in *BRCA2* (10 families) were more common than for *BRCA1* (7 families). Overall, the frequency of mutations was 12.2% (n = 11) by conventional screening. However, by including deleterious mutations detected using multiplex ligation-dependent probe amplification (n = 1), transcript analysis (n = 2), and computational evaluation of missense mutations (n = 3), the frequency increased substantially to 18.9%. This suggests that the comprehensive strategy used is effective for identifying deleterious mutations in Asian individuals at high risk for breast and/or ovarian cancer.** (Cancer Epidemiol Biomarkers Prev 2007;16(11):2276–84)

Introduction

Breast cancer is the most frequent cancer among females in many Caucasian populations and in Singapore. Increased susceptibility for breast cancer is usually associated with deleterious mutations of the breast cancer susceptibility genes *BRCA1* and *BRCA2*, particularly in families with both breast and ovarian cancer. Such germ line mutations in the *BRCA1* and *BRCA2* genes have been documented in most populations (1-3). Clinical genetic testing for *BRCA1* and *BRCA2* mutations after genetic counseling is the standard of care in North America and Europe but is not available in many countries in Asia.

Genetic testing of the *BRCA1* and *BRCA2* genes typically involves the DNA sequencing of all exons and intron-exon junctions. However, some high-risk pedigrees will have ''negative'' results, possibly due to unidentified mutations or mutations with unknown clinical significance, thus presenting a dilemma in risk assessment and genetic counseling (4).

Large genomic rearrangements, such as exon duplications or deletions found in high frequencies in European populations, are not detected by conventional genetic testing strategies (5). Recently, the multiplex ligation-dependent probe amplification (MLPA) assay has been used by many studies to detect large genomic rearrangements in the *BRCA1* and *BRCA2* genes (6, 7). In a large study of 300 families with four or more cases of breast or ovarian cancer but who were commercially tested negative for *BRCA1* and *BRCA2* mutations, MLPA detected genomic rearrangements in 12% of the probands (8), thus highlighting the importance of screening for such aberrations in addition to conventional PCR-sequencing protocols.

Intronic alterations that are located within or near to intron-exon junctions may affect mRNA splicing fidelity (9). These alterations are categorized as unclassified variants unless they have been evaluated by transcript analysis to determine their effect on mRNA splicing

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(10, 11). Other unclassified variants include missense mutations, which have unclear pathogenicity and which form between a third to half of all genetic variants documented in the Breast Cancer Information Core database. Pathogenicity of these variants may be established from family studies of cosegregation, absence in unaffected controls, or by using biochemical criteria, such as conservation of amino acid across species, severity of amino acid change, and involvement of an amino acid within a functional domain (12-14).

In this study, we aimed to accurately determine the prevalence of mutations in the *BRCA1* and *BRCA2* genes in a cohort of Singaporean women accrued at risk assessment clinics by using a comprehensive testing strategy. In addition to conventional screening of the coding exons of the *BRCA1* and *BRCA2* genes and their intron-exon boundaries, MLPA analysis for large genomic rearrangements, RNA analysis, *in silico* prediction of intronic alterations, and evaluation of missense mutations of unknown clinical significance by computational analyses were done. Although these techniques have been used for studies to evaluate the prevalence of *BRCA1/BRCA2* mutations, to our knowledge, there have not been any reports incorporating all of these techniques for a comprehensive testing strategy for *BRCA1* and *BRCA2* mutation detection.

Materials and Methods

A flow chart summarizing the methodology of the study, from patient accrual to validation of mutations, is shown in Fig. 1.

Subjects. All subjects were recruited from clinics at the National Cancer Center and KK Women's and Children's Hospital in Singapore under the Risk Evaluation and Prevention program. Detailed pedigree risk assessment was done with a combination of counselor assessment and the BRCAPRO model (15). Family histories of all cancers for three generations were recorded, including ages of diagnoses of all cancers. Subjects were eligible if there is a personal or family history of breast and ovarian cancer, if there are two primaries, if there is a personal and family history of breast (at least one premenopausal) or ovarian cancer(s) in a close relative from the same side of the family, or if the subjects have breast or ovarian cancer and were ages 39 years and below. One unaffected subject was tested as her sister had recent breast cancer at age 35 and was being evaluated for suspected ovarian cancer. Informed consent was obtained, and the research protocol was approved by the ethics review committee at both hospitals.

Between March 2002 and April 2006, 95 eligible subjects were accrued from 90 families. All subjects were of Asian descent. Subjects were predominantly Chinese (75.8%), followed by Malays (11.6%), Indians (4.2%), and others (8.4%), reflecting the ethnic composition of Singapore. The median age of participants at entry was 36 years, with a range from 19 to 72 years. Half of the subjects were below 40 years old.

Mutational Analysis of *BRCA1* and *BRCA2*. Genomic DNA was isolated using standard techniques as previously reported (16). Purified DNA was amplified as

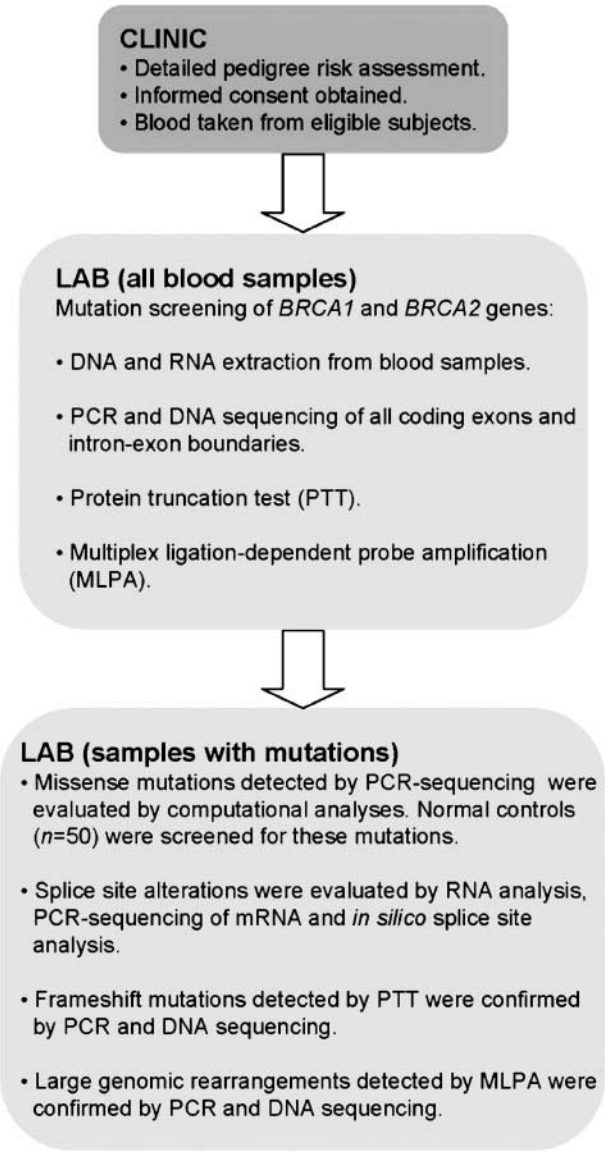

Figure 1. Flow chart of the methodology used for the screening of mutations in the *BRCA1* and *BRCA2* genes.

described using published primers (17, 18) and primers described at the Breast Cancer Information Core Web site[6] (16). Direct sequencing of the amplified products was done using the CEQ Dye Termination Cycle Sequencing quick start kit (Beckman Coulter), and the products were analyzed using the CEQ 8000 System (Beckman Coulter) according to the manufacturer's recommendations. The PCR products of exon 11 in *BRCA1* and exons 10 and 11 in *BRCA2* were *in vitro* transcribed/translated using the protein truncation test (TNT®T7 Quick for PCR DNA, Promega). The translation products were electrophoresed

[6] http://research.nhgri.nih.gov/bic/

on 10% SDS-polyacrylamide gels. Any mutations detected were confirmed by separate PCR amplifications done at least once.

MLPA. MLPA was done with the MLPA P002-BRCA1 and P0045-BRCA2 test kits and the MLPA P087 confirmation kit (MRC Holland) according to the manufacturer's recommendations, using alternative protocol 2 for multiplex PCR. Fragment analysis was done on the Beckman CEQ 8000 System (Beckman Coulter). Peak profiles for each sample were compared with a normal control and with other samples within the same experimental batch.

RNA Analysis of Splice-Site Alterations. Total RNA was isolated from peripheral blood lymphocytes using Trizol reagent (Invitrogen). cDNA was then reverse transcribed from 150 ng of total RNA using the AMV reverse transcription system (Promega). cDNA (25 ng) was amplified using the following primers by standard procedures with HotStarTaq (Qiagen Gmbh). For amplification of exons 5 to 9 of *BRCA1*, the primers 5′-CTGAAACTTCTCAACCAGAAGAAA-3′ (E6-8F) and 5′-GTAACAATTCTTGATCTCCCACAC-3′ (E6-8R) were used. For amplification of exons 14 to 16 of *BRCA2*, the primers 5′-TCATGTTTCTTTAGAGCCGATTAC-3′ (2E14-16F) and 5′-ATTTTAGTTGAAGAAGCACCCTTT-3′ (2E14-16R) were used.

Amplification consisted of 35 cycles, each of 1 min, at 94°C, 56°C, and 72°C. PCR products were electrophoresed on a 3% NuSieve GTG agarose gel (Cambrex; for exons 5-9 of *BRCA1*) or 2% agarose gel (for exons 14-16 of *BRCA2*), and PCR fragments were purified by gel extraction with the PureLink Quick Gel extraction kit (Invitrogen). Direct sequencing was done as described above.

***In silico* Splice Site Analysis.** SpliceSiteFinder[7] was used to determine the presence and relative efficiencies of donor, acceptor, and branch point sites.

Computational Analyses of Missense Mutations. BRCA1 and BRCA2 sequences from various organisms that contain the region that encompasses the site of missense mutation were obtained from Swiss-Prot. Multiple sequence alignment was then conducted using ClustalX 1.83. The Swiss PDB-Viewer was used for rendering and viewing of protein database structures of mutation sites that were available for analysis. When a mutation occurred at the vicinity of putative phosphorylation sites as reported in the literature, NetPhos 2.0 was used to predict possible sites of phosphorylation (19). Comparison of relevant protein structures deposited at the Protein Data Bank[8] was done. These Protein Data Bank structures are protein structures which are derived by X-ray crystallography or by nuclear magnetic resonance. Deductions from these analyses were compared with the predictions of the effects of the mutation made by PolyPhen (20). PolyPhen (polymorphism phenotyping) combines information on sequence features, structural variables, and contacts to characterize nucleotide substitutions (20).

[7] http://www.genet.sickkids.on.ca/~ali/splicesitefinder.html
[8] http://www.rcsb.org/pdb

Results

The clinicopathologic features of the breast and ovarian tumors are summarized in Table 1. As expected, *BRCA1* or *BRCA2* mutations were more likely to be present in patients from hereditary breast and ovarian cancer (HBOC) families than in those with no family history. All of the patients with *BRCA1* or *BRCA2* mutations were *Her2* negative. All but one *BRCA1* mutation carrier had estrogen, progesterone, and *Her2*-negative breast cancer.

Prevalence and Mutational Spectrum of *BRCA1* and *BRCA2* Mutations. Deleterious mutations are indicated in bold in Table 2. All frameshift and nonsense mutations generating premature termination codons were classified as deleterious. Missense mutations that were absent in at least 50 normal individuals and which involved a highly conserved amino acid were classified as deleterious. An exon 13 duplication in *BRCA1* (21) and the IVS7-15del10 in *BRCA1* were deemed deleterious, as there was linkage with breast and/or ovarian cancer within the respective families.

Fifteen novel genetic alterations were detected in the *BRCA1* and *BRCA2* genes, with 11 classified as

Table 1. Clinicopathologic features of cases with and without *BRCA1* or *BRCA2* mutation

	With *BRCA1* or *BRCA2* mutation*	Without mutation
Pedigree diagnosis (n = 90)		
Breast and ovarian cancer family	9	17
Breast cancer only family	3	31
Ovarian cancer only family	0	1
Early onset breast/ovarian cancer with no family history	2	27
Breast cancer (n = 84) †		
Histology		
Infiltrating ductal	12	58
Infiltrating lobular	0	2
Mixed infiltrating ductal/lobular	0	1
Papillary	0	1
Tubular	0	1
Mucinous	0	3
Ductal carcinoma *in situ*	0	1
Not specified	2	3
Estrogen receptor status (n = 78) ‡,§		
Positive	7	43
Negative	5	23
Progesterone receptor status (n = 77) ‡,§		
Positive	7	36
Negative	5	29
HER2 status (n = 69) ‡,§		
Positive	0	22
Negative	10	37
Ovarian cancer (n = 13) †		
Histology		
Mixed mucinous and clear cell	0	1
Not specified	5	7

*Nonsense and frameshift mutations, exon duplication, and splice site mutations.
†Ten patients had both breast and ovarian cancer.
‡ER, progesterone receptor, and HER2 status were determined for available tissue samples only. HER2 positivity was defined as 3+ staining by immunohistochemistry or amplification detected by fluorescence *in situ* hybridization.
§One patient with bilateral breast cancer had two tumors evaluated for ER, progesterone receptor, and HER2 status.

deleterious mutations and four as polymorphisms (Tables 2 and 3).

Frameshift, nonsense, and intronic mutations and an exon duplication were identified in 16 of 95 subjects (16.8%) or in 14 of 90 families (15.6%). The 90 families composed of 26 HBOC families, 34 breast cancer only families, one ovarian cancer only family, and 29 unrelated subjects with early-onset breast cancer and no family history (Table 1). The prevalence of clearly deleterious *BRCA* mutations in these groups were 35% (9 of 26) in HBOC families, 8.8% (3 of 34) in breast cancer only families, and 6.9% (2 of 29) in subjects with early-onset breast cancer and no family history (Table 2). The prevalence rates for clearly deleterious *BRCA1* and *BRCA2* mutations were 6.7% (6 of 90) and 8.9% (8 of 90), respectively, or 7.8% (7 of 90) and 11.1% (10 of 90), respectively, by including probably deleterious missense mutations. Deleterious mutations in *BRCA1* and *BRCA2* were observed in 7 and 10 families, respectively.

By ethnicity, these mutations were detected in 12 of 67 Chinese families (17.9%), 1 of 11 Malay families (9.1%), and 1 of 4 Indian families (25.0%; Table 2). The mutation in the Indian family was the 185delAG mutation frequently found in Ashkenazi Jews and in studies from India (22-28). The Malay founder mutation previously identified in Singapore was not identified in this cohort (16, 29).

Six different *BRCA1* deleterious mutations were identified in six families (Table 2). Five of these have been previously reported either in the Breast Cancer Information Core or in the literature and comprised three frameshift mutations, one nonsense mutation, and an

Table 2. Mutations in *BRCA1* and *BRCA2*

Case	Age (y) at diagnosis	Proband's cancer type	Family history	Race	Exon	Nucleotide change*	Amino acid change*	No. citations in BIC
BRCA1								
Nonsense and frameshift mutations								
1	37; 39	OC; BC	HBOC	C	16	**A4920T**	**K1601X**	1 †
2	41 and 51	BL BC	3 sisters with BC	C	11	**3977del4**	**Stop 1305**	1
3	26	BC	2 paternal aunts with BC	C	11	**2000del4**	**Stop 630**	3
4 ‡	46	BC and OC	HBOC, mother with OC	I	2	**185delAG**	**Stop 39**	1596
					16	G5075A	M1652I (missense)	38
Splice error								
5	56	BC	HBOC, sister of case 5a	C	Intron 7	**IVS7-15del10**	**Stop 182**	0
5a	55	BC and OC	HBOC, sister of case 5	C	Intron 7	**IVS7-15del10**	**Stop 182**	0
Exon duplication								
6	32 and 41	BL BC	HBOC, sister with OC	C	13	—	**Exon 13 duplication**	0 (Novel)
Missense								
7	55	BC	HBOC, 2 sister with OC	C	5	**C291G**	**P58A**	0 (Novel)
8	33	BC	Early onset BC with no family history	C	17	A5187C	K1690Q	0
9	28	BC	Mother with BC	M	9	G690A	V191I	6
10	35	BC	Early onset BC with no family history	M	9	G690A	V191I	6
BRCA2								
Frameshift								
11	—	No cancer	HBOC, half sister of case 11c	C	11	**4379delT**	**Stop 1387**	0 (Novel)
11c	40	BC	HBOC, half sister of case 11	C	11	**4379delT**	**Stop 1387**	0 (Novel)
12	37	BC	Early onset BC with no family history	C	22	**9118insA**	**Stop 3017**	0 (Novel)
13	51	BC	2 sisters and a maternal aunt with BC	C	11	**5804del4**	**Stop 1861**	20
14	52; 53	OC; BC	HBOC	C	11	**2822del4**	**Stop 873**	0 (Novel)
15	29	BC	Early onset BC with no family history	M	11	**6862del4**	**Stop 2228**	0 (Novel)
16	35	BC	HBOC, mother with BC and maternal aunt with BC and OC	C	22	**9143delT**	**Stop 2975**	0 (Novel)
17	49; 53	BC; OC	HBOC, paternal aunt with BC	C	23	**9325delA**	**Stop 3061**	0 (Novel)
Splice error								
18	51 and 52; 65	BL BC; OC	HBOC	C	Intron 15	**IVS15+1 G>A**	**Deletion of exon 15**	0
Missense								
19	36	BC	Paternal aunt with BC	C	6	**T711G**	**C161W**	0 (Novel)
9	28	BC	Mother with BC	M	16	**G7859A**	**G2544D**	0 (Novel)
20	56	BC	Sister with BC	C	18	G8415T	K2729N	16
21	53 and 54	BL BC	Mother with BC	C	18	G8415T	K2729N	16
22	42	OC	HBOC, sister with BC	C	14	C7280G	A2351G	4

Abbreviations: BIC, Breast Cancer Information Core; BC, breast cancer; BL, bilateral; OC, ovarian cancer; HBOC, hereditary breast and ovarian cancer; C, Chinese; I, Indian; M, Malay.

*Deleterious mutations are shown in bold.

†The mutation reported in Breast Cancer Information Core originates from this case.

‡Case 4 had two mutations, one being a deleterious frameshift mutation.

Table 3. Polymorphisms in *BRCA1* and *BRCA2*

Frequency in subjects, % (n = 95)	Frequency in normal controls, % (n = 50)	Exon	Nucleotide change	Amino acid change	Mutation type	No. citations in BIC
BRCA1						
1	0	Intron 1	IVS1-22 A>G	None	No effect	0 (Novel)
6.3	—*	3	G233A	L38L	No effect	5
100	—	Intron 6	IVS6-26-7 CA>AC	None	No effect	0 (Novel)
100	100	Intron 7	IVS7-34 T>C	None	No effect	7
69.5	64	Intron 8	IVS8-58delT	None	No effect	4
59	—	13	T4427C	S1426S	No effect	12
60	74	16	A4956G	S1613G	Polymorphism	33
53.7	66	Intron 16	IVS16-68 A>G	None	No effect	4
53.7	66	Intron 17	IVS17+37 G>A	None	No effect	0 (Novel)
57.9	—	Intron 18	IVS18+66 G>A	None	No effect	6
BRCA2						
58.9	—	2	5′UTR 203 G>A	None	No effect	12
1	0	3	A306G	P26P	No effect	0 (Novel)
5.3	—	Intron 8	IVS8+56 C>T	None	No effect	2
58.9	—	14	A7470G	S2414S	No effect	10
9.5	—	Intron 14	IVS14+53 C>T	None	No effect	1
72.6	—	Intron 16	IVS16-14 T>C	None	No effect	14
1	—	Intron 20	IVS20-26 A>G	None	No effect	1
61.1	72	Intron 21	IVS21-66 T>C	None	No effect	2
4.2	6	27	A10462G	I3412V	Polymorphism	110

*"—" indicates that the mutation was not screened in normal controls because transcript analysis showed only normal transcripts or because the alteration was a synonymous substitution.

intronic deletion within intron 7, causing a frameshift with a predicted stop at codon 182 (Fig. 2; ref. 30). A novel genomic rearrangement resulting in the duplication of exon 13 of *BRCA1* was identified by MLPA and confirmed by DNA sequencing of the breakpoint (21).

Of the eight deleterious mutations in *BRCA2*, six were novel (Table 2). There were seven frameshift mutations and a GT to AT 5′ splice site mutation in intron 15, resulting in skipping of exon 15 detected in one subject (Fig. 2; ref. 31).

Eight missense mutations of unknown significance were detected in both *BRCA1* and *BRCA2*, and these were evaluated by computational analyses to determine if they were deleterious (Tables 2 and 4).

None of the mutations listed in Table 2 were detected in 50 normal individuals, matched for ethnicity. Several polymorphisms were detected and are listed in Table 3.

Evaluation of Splice Site Alterations. All cases with *BRCA1* and *BRCA2* intronic splice site alterations were

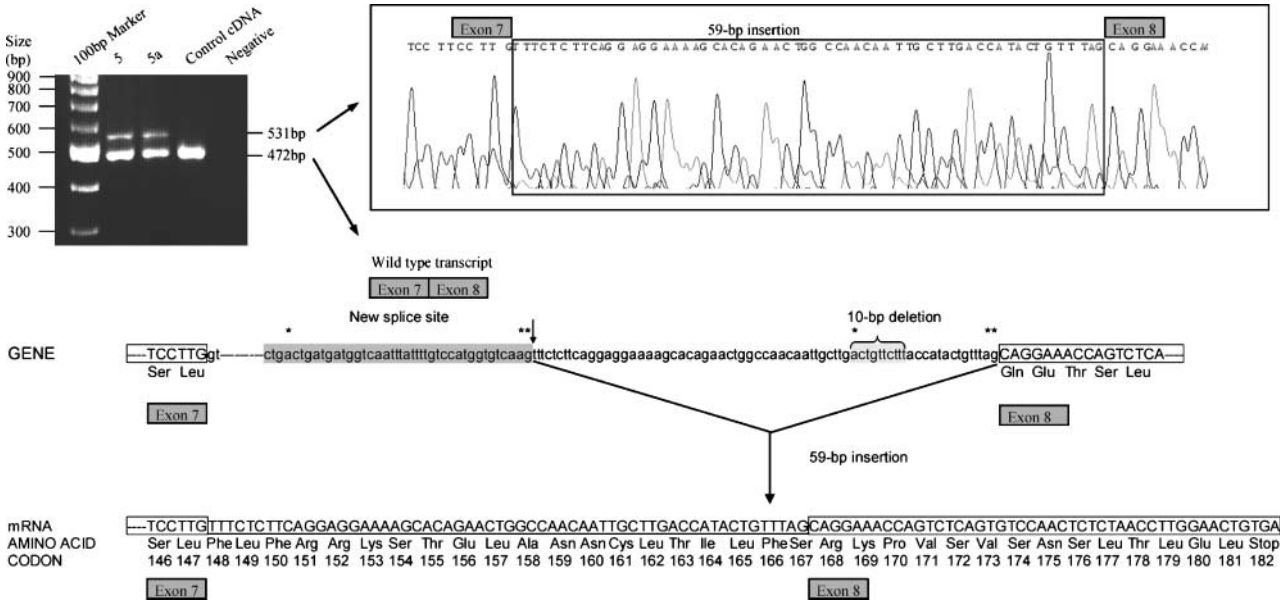

Figure 2. IVS7-15del10 in *BRCA1*. Ethidium bromide–stained gel of reverse transcription–PCR products with the corresponding sequencing chromatogram of the aberrant transcript, and the gene, mRNA, and amino acid sequences for the aberrant transcript. The branch and acceptor sites (a and ag) are indicated by asterisks. Lanes 5 and 5a of the gel correspond to cases 5 and 5a listed in Table 2.

Table 4. Alignment of *BRCA1* and *BRCA2* amino acids across species at sites of missense mutations, percentages of conservation, PolyPhen, and overall predictions and pedigree diagnosis

No.	Species	Species	*BRCA1* accession no*	*BRCA2* accession no*	*BRCA1*				*BRCA2*			
					P58A	V191I	M1652I	K1690Q	C161W	A2351G	G2544D	K2729N
1	Dog	*Canis familiaris*	Q95153	Q8MKI9	P†	V	M	K	C	A	G	K
2	Cat	*Felis cattus*		Q864S8	—	—	—	—	C	A	G	K
3	Human	*Homo sapiens*	P38398	P51587	P	V	M	K	C	A	G	K
4	Mouse	*Mus musculus*	P48754	P97929	P	V	M	K	S	S	G	R
5	Rat	*Rattus norvegicus*	O54952	O35923	P	V	M	K	S	S	G	K
6	Chicken	*Gallus gallus*		Q8UW79	—	—	—	—	Y	L	G	R
7	Cow	*Bos taurus*	Q864U1	—	P	V	M	K	—	—	—	—
8	Chimp	*Pan troglodytes*	Q9GKK8	—	P	V	M	K	—	—	—	—
9	Gorilla	*Gorilla gorilla*	Q6J6I8	—	P	V	M	K	—	—	—	—
10	Orangutan	*Pongo pygmaeus*	Q6J6J0	—	P	V	M	K	—	—	—	—
11	Rhesus monkey	*Macaca mulatta*	Q6J6I9	—	P	V	L	K	—	—	—	—
% Similarity conservation‡					100	100	100	100	100	67	100	100
% Identical conservation§					100	100	89	100	100	50	100	67
PolyPhen prediction					**PD**	B	**PD**	B	**PD**	B	B	B
Overall prediction including comparison of Protein Data Bank structures					**PD**	U	PB	U	**PD**	PB	**PD**	U
Pedigree diagnosis (total no. cases)					HBOC (1)	BC (1), EO (1)	HBOC (1)	EO (1)	BC (1)	HBOC (1)	BC (1)	BC (2)

Abbreviations: PD, probably damaging (bold); B, benign; U, unclear; PB, probably benign; EO, early onset breast cancer without family history.
*Swiss-Prot accession numbers.
†Amino acid residues.
‡Similar residues are defined here as amino acid residues that are classified within the same classic hydrophobic, polar, basic, or acidic groupings. Percentage conservation is calculated by the number of similar or identical residues found at the same position in the alignment over the total number of sequences available for comparison.
§Identical residues are amino acid residues that are the same as that found in human at the same position in the alignment. Percentage conservation is calculated by the number of similar or identical residues found at the same position in the alignment over the total number of sequences available for comparison.

subjected to RNA analysis. Of the 15 intronic alterations detected, two variants, IVS7-15del10 in *BRCA1* and IVS15+1G>A in *BRCA2*, showed aberrant splicing and were classified as deleterious (Table 2; Fig. 2). The remaining 13 variants with normal transcripts were classified as benign polymorphic alterations (Table 3).

IVS7-15del10 in *BRCA1*, 10-bp deletion in intron 7 of *BRCA1*, was identified in two subjects who were sisters (Table 2; Fig. 2). Reverse transcription–PCR of the region spanning exons 5 to 9 amplified two fragments, a fragment of the expected size of 472 bp and another fragment of 531 bp of reduced intensity possibly due to nonsense-mediated mRNA decay, which occurs if an alternative transcript has a premature termination codon >55 nucleotides upstream of the last exon-exon junction (32). Sequencing of both fragments revealed that the aberrant fragment had a 59-bp insertion between exons 7 and 8 in the mRNA sequence. Based on this, a possible mechanism for the partial inclusion of intron 7 is shown in Fig. 2. The deletion of the original branch site within the 10-bp intronic deletion causes the utilization of a new branch site upstream with the insertion of the 59-bp intronic sequence, causing a frameshift and a predicted stop at codon 182. SpliceSiteFinder predicted that the strength of the new 3′ splice acceptor site (score, 64.4) and new branch point (maximum score, 100) are at least comparable with or higher than the original scores of 67.8 and 83.9, respectively. Both the putative normal and new branch points are based on the consensus sequence YTRAY (33).

The *BRCA2* IVS15+1G>A mutation is located in the consensus sequence of the 5′ donor splice site and resulted in the generation of an altered transcript by skipping of exon 15.

Evaluation of Missense Mutations of Unknown Clinical Significance by Computational Analyses. Table 4 shows the amino acid sequence comparison for sites of missense mutations used to establish the degree of conservation and the results of computational analyses for assessing the significance of the missense mutations. Of the eight missense mutations, three are predicted to be damaging mutations (*BRCA1* P58A, *BRCA2* C161W, and *BRCA2* G2544D), two are predicted to be benign (*BRCA1* M1652I and *BRCA2* A2351G), and the remaining three are of unclear status (*BRCA1* V191I, *BRCA1* K1690Q, and *BRCA2* K2729N; Table 4).

Codon 58 of *BRCA1* lies within the RING domain, which is a highly conserved region among the nine mammalian sequences examined. The domain is implicated in interactions with at least five different proteins (34, 35).

Codon 161 of *BRCA2* has polar residues conserved in all species represented at that site. A mutation to the large hydrophobic Tryptophan residue is likely to be damaging, as it may remove possible covalent or polar bonding.

The G2544D mutation involves a conserved glycine that lies in a highly conserved region, which plays a structural role in forming a loop turn and is likely to affect the binding affinity of BRCA2 with DSS1. The residues involved in the BRCA2-DSS1 interaction are highly conserved, and two DSS1-interacting BRCA2 residues (Ala2564 and Arg2580) were previously found to be mutated in cancer (36). This BRCA2-DSS1 complex directly functions in RAD51-mediated recombination double-stranded break repair (36). However, PolyPhen, which did not examine the Protein Data Bank structure 1mje, classified this mutation as benign.

Codon 1652 of *BRCA1* lies in the hydrophobic core of the protein, and its hydrophobicity is shown to be conserved in the alignment (Table 4). Because the mutation to isoleucine is a conservative mutation, it is most probably a benign mutation. PolyPhen, however, has not evaluated the similarity between the leucine found in rhesus monkey in the alignment and isoleucine, and predicted the mutation to be probably damaging (Table 4).

The A2351G mutation of *BRCA2* is a conservative mutation in a nonconserved region and hence is likely to be a benign mutation.

Codon 191 of *BRCA1* is within a putative site of interaction with oncogene and cell cycle regulators, such as c-myc and estrogen receptors (37). Although V191I is conserved in hydrophobicity and PolyPhen predicted this mutation to be benign, it is still possible that the V191I mutation may affect the phosphorylation state of BRCA1 because NetPhos 2.0 predicted its neighbor T190 to be a likely site of phosphorylation.

The K1690Q mutation in BRCA1 is predicted to be benign by PolyPhen and unclear by our analysis (Table 4). The Protein Data Bank structure 1jnx of BRCA1's BRCT domain showed a salt-bridge interaction between the lysine residue and the glutamic acid residue at codon 1661 (38). A mutation from lysine to glutamine will replace the salt-bridge interaction with a hydrogen bond between the mutated glutamine residue at codon 1690 with the glutamic acid residue at codon 1661. However, the effect of this mutation on the structure and dynamics of the protein is unclear.

The K2729N mutation of *BRCA2* is a relatively conservative mutation in a conserved region. Hence, the effect of the mutation on the structure and function of the protein remains unclear.

Discussion

This is the first comprehensive study of both the *BRCA1* and *BRCA2* genes, involving complete mutation screening, evaluation for large genomic rearrangements and splice site variants, and analysis of missense mutations by computational analysis, in an Asian population. In our cohort of 90 families, the combined frequency of frameshift/nonsense mutations in the *BRCA1* and *BRCA2* genes was 12.2% by conventional mutation screening. However, by including deleterious mutations detected using MLPA analysis (n = 1), RNA analysis (n = 2), and computational evaluation of missense mutations (n = 3), the overall frequency of deleterious mutations increased substantially to 18.9%. The comprehensive mutation testing strategy used was most successful in families with both breast and ovarian cancer.

The prevalence rates for *BRCA1* and *BRCA2* mutations that were observed in this study were 6.7% (6 of 90) and 8.9% (8 of 90), respectively, or 7.8% (7 of 90) and 11.1% (10 of 90), respectively, by including missense mutations predicted to be deleterious by computational analysis. These frequencies are comparable with data from other studies on Chinese women, the majority of which have been small studies with fewer than 50 subjects. In women with early-onset breast cancer, prevalence rates ranging from 8.0% to 9.5% in *BRCA1* (39-42) and 2.4% in *BRCA2* (40) have been observed, although one study reported no mutations in *BRCA1* among 35 Singaporean-Chinese women (43). It should be noted, however, that the age limits set for each of these studies differ and range from <35 to <45 years, which may account for differences in prevalence rates between studies. In addition, a cancer genetics clinic with risk assessment allows risk stratification for genetic testing of a higher risk cohort.

In Chinese cases with a family history of breast or ovarian cancer, mutations in *BRCA1* were detected between 8.1% and 12.5% (30, 40, 44, 45) and between 2.7% and 16.7% for *BRCA2* (30, 40, 44, 45). In one study on 25 HBOC families, 40% of the women had mutations in *BRCA1* (46).

The majority of the *BRCA2* frameshift mutations detected were novel mutations. This may be because there is a paucity of information on *BRCA2* mutations among the Chinese, Malay, and Indian ethnic groups. Furthermore, we found that in our cohort of Singaporean women, deleterious mutations in *BRCA2* (10 families) were more common than for *BRCA1* (seven families). This contrasts with observations from European and American populations, wherein the frequency of *BRCA1* mutations is higher than that of *BRCA2* (1, 3). In a large U.S. study of 1948 families, *BRCA1* and *BRCA2* mutations were detected in 14.6% and 7.4% of the families, respectively (3). However, in Chinese women from Shanghai, a 1:1 ratio for *BRCA1/BRCA2* mutations was observed (44). Thus, our findings underscore the importance of screening for *BRCA2* mutations in individuals at high risk of developing breast and/or ovarian cancer.

Although there have been hospital-based genetic testing studies for *BRCA1/BRCA2*, there have been no Asian risk assessment clinic-based reports with predominantly Asian individuals seeking counseling and genetic testing (22, 47, 48). The advantages of clinic-based ascertainment is, firstly, the use of risk stratification to allow for genetic testing of a higher risk cohort and, secondly, the exclusion of breast cancer families related to other predisposition genes. We excluded one family with Cowden's syndrome and another with Li-Fraumeni syndrome (data not shown). Additionally, we screened for the *CHEK2*1100delC* mutation, which also predisposes to early-onset breast cancer, and all our subjects were negative (unpublished data).

The technology to sequence *BRCA1* and *BRCA2* is increasingly available in Asian countries, and although genetic testing is often done as part of research studies, the set up of formal assessment clinics could increase the scope of hereditary cancers detected.

To ascertain the pathologic effect of the intronic alterations on mRNA splicing fidelity and expression, RNA analysis using reverse transcription–PCR and sequencing and *in silico* splice prediction methods were used (9, 10). This current study identified two deleterious intronic aberrations, IVS7-15del10 in *BRCA1* and IVS15+1G>A in *BRCA2*. The occurrence of these splicing aberrations emphasizes the importance of studying mutations both at the genomic DNA and RNA levels to determine the pathogenic effect of the mutations (49) which would effect on genetic counseling. However, if RNA is not available, then another option would be to use theoretical splicing prediction approaches, such as SpliceSiteFinder, to determine if the *BRCA1* and *BRCA2*

splice site variants identified from genomic sequence would cause aberrant splicing (10).

Missense mutations of unknown clinical significance or unclassified variants pose a challenge in genetic counseling. By using bioinformatic tools to assess such mutations, deleterious mutations may be identified in high-risk individuals, providing information for risk reduction decision making. Such computational tools provide a feasible alternative to the evaluation of missense mutations by segregation analysis, which is particularly difficult in small families or in families for which pedigree information is unknown or limited.

This study has shown that intronic alterations, large genomic rearrangements, and missense mutations in combination contribute significantly to deleterious mutations in our study population. Improved mutation detection by a comprehensive screening approach for the *BRCA1* and *BRCA2* genes, as shown here, may be effective for the identification of deleterious mutations, with implications for genetic counseling and risk assessment of individuals at high-risk for breast and/or ovarian cancer.

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BRCA1 and *BRCA2* Mutations in an Asian Clinic-based Population Detected Using a Comprehensive Strategy

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