ONLINE MUTATION REPORT

BRCA1 c.2845insA is a recurring mutation with a founder effect in Singapore Malay women with early onset breast/ovarian cancer

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Breast cancer is the third most common form of cancer worldwide, after lung and stomach cancer, and is the most common cause of cancer death in women.[1] The age-adjusted incidence rates of breast cancer are 176% higher in developed than developing countries.[2] In Singapore, breast cancer is the most common cancer in women and the incidence rate in 1993–1997 was 2.3 times higher than in 1968–1972, with an annual increase significantly higher in pre-menopausal than post-menopausal women (5.7% *v* 3.9%).[3–4] Of note, the Malay ethnic group in Singapore has shown the greatest increase in the incidence of breast cancer (4.4%) over the past three decades compared with the Chinese and Indian subpopulations. In addition, breast cancer in Malay women has shown a bimodal distribution in age specific incidence, at 45 and 65 years, and a strong birth cohort effect has been noticed in this group.

The majority of breast cancer is sporadic, but about 5% to 10% of all breast and ovarian cancer cases are believed to be attributable to the inheritance of germline mutations in high penetrance, autosomal dominant susceptibility genes.[5–7] The identification and cloning of one such gene *BRCA1* (OMIM # 113705)[8–9] has rapidly led to the characterisation of mutations in this gene among high risk families, as well as early onset breast and/or ovarian cancer patients or tumour series in populations worldwide.[10–14] Several founder and recurrent mutations have now been identified in specific ethnic groups, such as African-Americans, Ashkenazi Jews, Dutch, Finns, French-Canadians, Icelanders, Japanese, Norwegians, Swedes, and Russians, among others.[15–17] However, almost all molecular studies of *BRCA1* performed have defined breast cancer risks and related genetic factors in Caucasian women of European descent, and studies in Asian populations, although on the increase, have been few.[18–24] Among these Asian populations, recurrent and founder mutations have now been reported for Japanese, Filipinos, Thais, and Chinese.[18 22 24–25]

Given the increase in the incidence of breast cancer, together with the rise in the number of patients presenting with early onset breast cancer among Singapore Malays, we decided to characterise germline *BRCA1* mutations and polymorphic variants in patients presenting with early onset breast or ovarian cancer, with or without a family history, in order to evaluate the proportion of cancers attributable to mutations in this gene.

MATERIALS AND METHODS
Study patients and determination of family history
From 2001 to 2002, 62 consecutive unrelated Malay patients presenting to the National University Hospital were eligible for *BRCA1* testing if they had a diagnosis of breast or ovarian cancer before the age of 45 and 50 years respectively. This study had institutional ethics committee approval, and signed written informed consent was obtained from each participant before blood collection. The family histories on number and age of relatives, numbers of affected relatives,

Key points

- Breast cancer is the third most common form of cancer worldwide, after lung and stomach cancer, and is the most common cause of cancer death in women.

- Most studies have been carried out in women of European descent, and as yet, few Asian studies have been published.

- We characterised germline *BRCA1* mutations and polymorphic variants in 62 unrelated Singapore Malay patients presenting with early onset breast or ovarian cancer, with or without a family history, in order to evaluate the proportion of cancers attributable to mutations in this gene.

- Ten distinct *BRCA1* sequence alterations were identified in 12 patients. One, the frameshift mutation c.2845insA, which produces a truncated protein signal at codon 914, was found to occur in six apparently unrelated patients, but was not found in 114 unaffected females (matched on basis of ethnicity and age) with no family history of cancer.

- Haplotype analysis of all six patients with the recurrent c.2845insA mutation showed a shared haplotype for all three intragenic polymorphic markers, strongly suggesting the presence of a founder effect at this locus.

- If this gene is indeed a founder effect, it may open the possibility of widescale gene screening for Singapore Malays.

and age of onset of cancers were determined by direct interview. To determine the frequency in the general Malay population of the recurring genetic variant detected in the study group, blood samples were collected from 114 unrelated normal subjects randomly selected from the general population. This control set was matched to the cases on basis of race, gender, and 5 year age intervals.

Molecular studies
DNA isolation
Peripheral blood lymphocytes were isolated from blood samples using Ficoll-Paque according to the manufacturer's instructions (Pharmacia Biotech, Uppsala, Sweden). Total genomic DNA was extracted from the lymphocytes using standard procedures.[26]

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Abbreviations: BIC, Breast Cancer Information Core; PCR, polymerase chain reaction; PTT, protein transcription–translation analysis; SSCP, single strand conformational polymorphism

Mutation analysis of *BRCA1*

The entire coding sequence, splice junctions and neighbouring intronic regions of *BRCA1* were screened using a combination of methods, primarily single strand conformational polymorphism (SSCP) analysis and the protein truncation test. SSCP analysis, using primer pairs spanning the *BRCA1* coding region[14] including the intron–exon boundaries, was performed for all coding exons except for exon 11. Polymerase chain reaction (PCR) amplification of genomic DNA was carried out in 10 µl volumes containing 50 ng of genomic DNA, 1.5 mmol/l $MgCl_2$, 50 mmol/l KCl, 10 mmol/l Tris-HCl (pH 8.3), 200 µmol/l dNTPs (Promega, Madison, WI, USA), 0.8 µmol/l of each primer and 0.75 U of *Taq* polymerase (Promega). An initial denaturation step at 94℃ for 3 min was followed by 35 cycles of amplification (30 s at 94℃, 1 min at the respective annealing temperature, and 1 min at 72℃), and a final extension at 72℃ for 5 min in a thermal cycler (GeneAmp® PCR System 9700; PE Applied Biosystems, Foster City, CA, USA). A 1.2 µl aliquot of the PCR product was diluted into 4.9 µl of denaturing loading buffer (95% formamide, 10 mmol/l NaOH, 0.05% xylene cyanol FF and 0.05% bromophenol blue), heated at 94℃ for 5 min, and loaded for electrophoresis. SSCP gels consisting of 0.5 × mutation detection enhancement solution (FMC Bioproducts, Rockland, ME, USA) in 0.6 × TBE buffer at 4 W for 18–22 h at 4℃. After electrophoresis, the SSCP bands were visualised by silver staining and the DNA bands of variant mobility were excised from the gels and eluted into 50 µl TE (pH 8.0). The eluted DNA (10 µl) was used as a template for reamplification and the DNA product was sequenced.

Protein transcription–translation (PTT) analysis was used to detect truncating mutations in exon 11.[27 28] Exon 11 was amplified in three overlapping fragments, ranging in size from 1275 to 1600 bp. PCR products were purified, and the mRNA was translated into radiolabelled peptides using the TnT™ T7 Quick System (Promega). [^{35}S]-methionine/cysteine (NEN Research Products, Boston, MA, USA) was used for radioactive labelling of the translation products. The products were size separated on 12% SDS-PAGE gels. After electrophoreses, the gels were dried and autoradiographed on Hyperfilm™ (Amersham Biosciences, Amersham, Bucks, UK) overnight at −70℃.

DNA sequencing

All PCR products showing an electrophoretic variant pattern by SSCP or PTT were reamplified from the original genomic DNA, and both strands were directly sequenced by the fluorometric method with automated sequencing procedures according to the manufacturers' instructions. All the deleterious mutations and genetic variants were named according to the practice of the BIC database, with the nucleotide numbering starting at the first transcribed base of *BRCA1* according to GenBank entry U14680.[29] All insertions or deletions were designated according to HUGO recommendations.[30]

Analysis of recurrent mutation

We screened 114 unaffected females (matched on basis of ethnicity and age) with no family history of cancer for the presence of the recurring mutation found in the patients. The region of exon 11, which contains the recurring mutation, was amplified from the genomic DNA and the subsequent PCR products were analysed by SSCP.

Haplotype analysis

The disease associated haplotypes in all the patients with the recurrent mutation and 76 controls were constructed by genotyping three intragenic *BRCA1* microsatellite markers: D17S855, D17S1322, and D17S1323. The primer sequences for the amplification of the markers were obtained from Anderson *et al*[31] and Neuhausen *et al*.[32] The fluorescently labelled PCR products were electrophoresed on an Automatic Laser Fluorescent Express machine (Amersham Biosciences) with a fluorescence labelled DNA fragment size standard.

RESULTS

The study involves the molecular analysis of the *BRCA1* gene in 62 consecutive Malay patients affected with early onset breast and/or ovarian cancer, with/without family history. Table 1 summarises the characteristics of the incident cases from the Surgery and Oncology clinics of the National University Hospital, Singapore. The mean age of diagnosis was 38.6 years (range 25–45 years) and 41.8 years (range 19–50 years) for breast cancer and ovarian cancer cases respectively. Fifty patients were early onset breast or ovarian cases with no family history of these cancers, while the remaining twelve patients had affected relatives with either breast or ovarian cancer. Of these twelve patients, nine presented with breast cancer under the age of 45 years and three with ovarian cancer under the age of 50 years.

Characterisation of alterations in *BRCA1* sequence

In the 62 patients with early onset breast or ovarian cancer under the ages of 45 and 50 years respectively, 10 distinct *BRCA1* sequence alterations were identified (table 2) in 12 patients. These alterations comprised one disease causing mutation, two missense changes, three neutral polymorphisms, and four intronic variants. Of these, one missense mutation and three of the intronic variants were novel mutations that have not yet been reported in the Breast Cancer Information Core (BIC) database.[14]

BRCA1 mutation causing disease

The frameshift mutation *BRCA1* c.2845insA, which produces a truncated protein signal at codon 914, was found to occur in six apparently unrelated patients. Four of the six cases (patients N536, N587, N610, N521; 66.7%) had at least a first or second degree relative with breast or ovarian cancer (table 2). Three patients (N536, N587, N610) were early onset breast cancer patients with a positive family history of breast/ovarian cancer (among first and second degree relatives), while the other three (N521, N576, N590) were ovarian cancer patients, of whom only one (N521) reported a family history of breast carcinoma among first and third degree relatives. The breast cancer patients, N536 and N587, and the ovarian cancer patient N521 showed a predominance of breast cancer in their families. Patient N610 was diagnosed with breast cancer at the age of 35 years, while her mother had ovarian cancer at the age of 49 years. Two ovarian cancer patients, N576 and N590, had no reported family history of breast/ovarian cancer. This particular exon 11 mutation was first reported in a retrospective study of apparently sporadic breast cancer patients (BIC database) and subsequently described in another study where it was found in two of seven unrelated subjects of Malay ethnicity.[33] In addition, our study also showed that none of the 114 unaffected controls were found to carry this mutation.

The genotypes of the six unrelated affected patients with the c.2845insA mutation were examined at three *BRCA1* intragenic markers. There were no available relatives to establish linkage phase, but for each of the loci, one allele was present in all six mutation carriers. For D17S1323 and D17S855, this allele was also common in the control individuals, but for D17S1322, this allele was only found twice in 152 chromosomes (1.3%). These data are consistent with all the c.2845insA mutation being located on a chromosome with a common haplotype, supporting an ancestral link (table 3). In addition, of the 76 controls

Table 1 Clinical and pathologic characteristics of early onset breast and ovarian cancer cases in Singapore Malay patients studied retrospectively

Characteristics	Cases (%)	No. with BRCA1 sequence alterations† (%)	No. with the BRCA1 2841/5insA mutation (%)
Total number (breast and ovarian)‡	62	12 (19.4)	6 (9.7)
Breast cancer cases§	50	9	3
Age of onset (years)			
<35	11 (22.0)	3 (33.3)	—
35–44	39 (78.0)	6 (66.7)	3 (100)
Family history (first, second, or third degree relatives)			
Breast cancer (diagnosis at any age)	8 (16.0)	2 (22.2)	2 (66.7)
Breast cancer (diagnosis<51 years)	5 (10.0)	1 (11.1)	1 (33.3)
Breast cancer (>1)	1 (2.0)	1 (11.1)	1 (33.3)
Ovarian cancer	1 (2.0)	1 (11.1)	1 (33.3)
Other types of cancer	10 (20.0)	4 (44.4)	2 (66.7)
Tumour histology			
Infiltrating ductal carcinoma	30 (60.0)	7 (77.8)	3(100.0)
Infiltrating lobular carcinoma	2 (4.0)	1 (11.1)	—
Mucinous	3 (6.0)	—	—
Other	11 (22.0)	1 (11.1)	—
Unknown	4 (8.0)	—	—
Nuclear grade			
1	2 (4.0)	—	—
2	4 (8.0)	—	—
3	33 (66.0)	5 (55.6)	1 (33.3)
Unknown	11 (22.0)	4 (44.4)	2 (66.7)
Presence of lymph node involvement¶			
Yes	20 (40.0)	3 (33.3)	—
No	17 (34.0)	3 (33.3)	1 (33.3)
Unknown	13 (26.0)	3 (33.3)	2 (66.7)
Oestrogen receptor¶			
Positive	17 (34.0)	2 (22.2)	—
Negative	19 (38.0)	5 (55.6)	2 (66.7)
Unknown††	14 (28.0)	2 (22.2)	1 (33.3)
Progesterone receptor¶			
Positive	23 (46.0)	3 (33.3)	—
Negative	12 (24.0)	4 (44.4)	2 (66.7)
Unknown††	15 (30.0)	2 (22.2)	1 (33.3)
Ovarian cancer cases (age of onset <50 years)‡‡	12	3	3
Family history (first, second, or third degree relatives)			
Breast cancer (diagnosis <51 years)	3 (25.0)	1 (33.3)	1 (33.3)
Breast cancer (>1)	2 (16.7)	1 (33.3)	1 (33.3)
Other types of cancer	3 (25.0)	1 (33.3)	1 (33.3)

†Sequence alterations include frameshift and missense mutations; polymorphisms and intronic variants.
‡Based on total number of early onset breast cancer and ovarian cancer cases with/without family history.
§Based on total number of incident breast cancer cases.
¶Percentages were calculated from cases in which the lymph node involvement status and receptor status were examined.
††Inclusive of ductal carcinoma in situ cases which are not routinely assessed for oestrogen/progesterone receptor status.
‡‡Based on total number of incident ovarian cancer cases.

haplotyped, none shared the same haplotype as the six positive samples, which leads us to infer that there may be a specific subpopulation within the Malay population in which this mutation occurs.

Missense mutations of unknown significance

Two missense mutations of unknown significance were detected. The first, c.335C→A (S72R) is not a common mutation, having only been described once before in the BIC database. This amino acid change is located within the BRCA1 RING finger structural domain, in close proximity of a RING finger motif. The other mutation, c.778C→A (S220Y) is novel, and was found in patient N536, who also harbours the recurring frameshift mutation c.2845insT.

Common polymorphisms and intronic variants

Three neutral polymorphisms were detected in this study. Two patients harboured the single base substitution, c.233G→A, and two other breast cancer patients, N599 and N616, had the same two intronic polymorphisms, IVS16-68A→G and IVS16-92A→G. These three polymorphisms have been reported previously in the BIC database, and they are among the top 20 mutation frequencies that have been described by designation. The remaining four intronic variants were IVS6+26CA→AC, IVS5-44G→A, IVS7-34T→C, and IVS18+72G→C, three of which are single-base substitutions, are novel and have never been reported. The intronic variant IVS18+72G→C was detected in a breast cancer patient, N587, who not only had the recurring mutation but also a family history of breast cancer (mother and maternal aunt).

DISCUSSION

The discovery of the BRCA genes has led to major advancements in our understanding of breast cancer carcinogenesis, both in hereditary and sporadic breast cancers. However, the primary clinical application of this new knowledge to date has been the identification of high risk individuals. Data from large, multicancer families have shown that 55–80% of carriers of a germline mutation at the BRCA gene develop breast cancer by the age of 70 years, with a similar level of risk of ovarian cancer.[34–36] Weaker associations have also been found between BRCA mutations and pancreatic, prostatic, and colonic cancers.[37–40]

Widespread BRCA gene testing has unfortunately not been practical, partially owing to the low estimated prevalence in the general population, accounting for less than 5% of all breast cancer in outbred populations.[41] In addition, mutation screening to date has been difficult and costly because of the size and complexity of the BRCA genes. With the exception of some genetically isolated populations, deleterious mutations at the BRCA loci have been shown to occur across the entire length of the genes, requiring full-length analysis for a comprehensive screen.

Earlier studies carried out in the Singapore population by our group and others have shown a similar prevalence of BRCA1 mutations to those in reports from western series.[20 33] Interestingly, one common finding was that the same BRCA1 deleterious mutation 2845insA was found in 2/7 Malay patients in one study, suggesting the presence of a founder mutation.[33] Follow on studies confirming such a possibility, combined with the low incidence of mutations elsewhere in the gene, would have more significant clinical applications, as a simplified mutation test limited to specific regions of the gene would allow for more widespread gene testing and a more accurate estimate of mutation penetrance outside of multiple cancer families.

To test the above hypothesis, we studied the spectrum of BRCA1 mutations in 62 Singapore Malay patients with early onset breast/ovarian cancer. All cases had their treatment at the same hospital, allowing for histological confirmation. The histolological phenotypes of mutation carriers were in keeping with previously published series, with a propensity for BRCA1 related tumours to be of high grade, of no special type, and oestrogen receptor negative. The prevalence of BRCA1 mutation in Southeast Asian breast and ovarian cancer patients, in particular of Malay ethnicity, has not been adequately reported and our study constitutes the largest cohort of such Malay patients to date. In this study, the prevalence of BRCA1 mutation carriers in Malays was found to be similar to that of outbred populations, and despite a full analysis of the entire coding region and adjacent intronic segments, all the deleterious mutations were found to be c.2845insA. In addition, none of the controls was found to

Table 2 *BRCA1* sequence alterations in Singapore Malay patients with early onset breast/ovarian cancer

Case	Exon	Sequence alteration	Amino acid change	Age (years)	Family history Breast /ovarian cancer	Other cancers
BRCA1 sequence variants						
Frameshift mutation						
N536 (Br)	11	c.2845insA	N909fsX5	35	Br33 (S)	Co58 (F)
N587 (Br)	11	c.2845insA	N909fsX5	44	Br60 (M), Br50 (MA)	Nil
N610 (Br)	11	c.2845insA	N909fsX5	35	Ov49 (M)	CSU (MA)
N521(Ov)	11	c.2845insA	N909fsX5	49	Br37 (S), Br30 (MA), Br40 (MC)	Nil
N576 (Ov)	11	c.2845insA	N909fsX5	50	Nil	Nil
N590 (Ov)	11	c.2845insA	N909fsX5	40	Nil	St (MA)
Missense mutations						
N591 (Br)	6	c.335C→A	S72R	41	Nil	Nil
N536 (Br)	10	c.778C→A	S220Y	35	Br33 (S)	Co59 (F)
Neutral polymorphisms						
N33 (Br)	3	233G→A	K38K	38	Nil	Nil
N522 (Br)	3	233G→A	K38K	32	Nil	Nil
N599 (Br)	17	c.5106-68A→G	IVS16-68A→G	29	Nil	Co70 (MGF)
N616 (Br)	17	c.5106-68A→G	IVS16-68A→G	35	Nil	—
N599 (Br)	17	c.5106-92A→G	IVS16-92A→G	29	Nil	Co70 (MGF)
N616 (Br)	17	c.5106-92A→G	IVS16-92A→G	35	Nil	Nil
Intronic variants						
N591 (Br)	6	c.420+26CA→AC	IVS6+26CA→AC	41	Nil	Nil
N591 (Br)	6	c.432-44G→A	IVS5-44G→A	41	Nil	Nil
N511 (Br)	8	c.561-34T→C	IVS7-34T→C	34	Nil	Brn53 (F)
N587 (Br)	18	c.5272+72G→C	IVS18+72G→C	44	Br60 (M), Br50 (MA)	Nil

Brn, brain; Br, breast; Co, colon; Ov, ovary; St, stomach; CSU, cancer site unknown; del:deletion; ins:insertion. Affected relatives in parentheses: F, father; M, mother; MA, maternal aunt; MC, maternal cousin; MGF, maternal grandfather; S, sister.

carry this mutation. To the best of our knowledge, all reported *BRCA1* mutations in Malays have been shown to occur at this position, with the exception of the c.5448insC mutation, which was reported in a Malay woman diagnosed with breast cancer at the age of 32 years with no significant family history of the disease.[42] Haplotype analysis of all six patients with the recurrent c.2845insA mutation showed a shared haplotype for all three intragenic polymorphic markers, strongly suggesting the presence of a founder effect at this locus. Interestingly, this haplotype was not seen in 76 controls, indicating that this may be a specific haplotype existing within a specific subpopulation of the Malays, and that this haplotype is associated with increased susceptibility to breast/ovarian cancer. However, more controls will have to be investigated in order to assess the prevalence of this haplotype. Case tracing of family members in the families that harbour the common mutation is presently underway to assess the segregation of this haplotype to the affected members.

Although *BRCA1* mutations have largely been reported to occur throughout the gene, recurrent mutations have now been identified in specific subpopulations. This phenomenon is most likely to occur in populations that have historically been geographically or politically isolated from surrounding populations, and in which reproduction occurs almost solely within the group, such as the presentday Singapore Malays. This ethnic group is part of a race of people inhabiting what is today peninsular Malaysia and portions of adjacent islands in southeast Asia, including the east coast of Sumatra, the coast of Borneo, and the smaller islands that lie between these areas, including Singapore. The Singapore Malays are typically characterised by their ethnically homogenous structure and low propensity towards immigration. In addition, the absence of the carrier haplotype among the 76 controls in our study also raises the possibility of a distinct sub-group at increased risk among the Malays. A similar situation has also been seen in other genetically isolated populations such as in Finland and Iceland, where mutation at the c.999del5 site of the *BRCA2* gene accounts for almost all mutations in both *BRCA* genes.[43–45]

The discovery of founder mutations has significant clinical applications, as a directed search for such mutations is likely to account for the majority of carriers in the population. Further studies will be necessary to estimate more accurately the population frequency of this mutation. The simplification of the mutation screening process will allow for more widespread gene testing outside of hereditary breast and/or ovarian cancer syndromes, which are rare in the general population. In addition, the identification of carriers outside of these families will also allow for a more accurate assessment of the penetrance of *BRCA* mutations, a vital requirement for genetic counselling and clinical decision making regarding intervention, with either closer surveillance or prophylactic surgery.

Nevertheless, the risk estimates among mutation carriers is still likely to fall within wide confidence intervals. In carriers of the Ashkenazi Jewish founder mutations, a wide heterogeneity of risk for both breast and ovarian cancers has been reported, possibly as a result of interaction with genetic or environmental modifiers, as indeed is also the case with the *BRCA2* 999del5 Icelandic founder mutation, which has been reported to have increased penetrance across successive generations. Whether such modifiers are found in Malay carriers will be an area of future research.

Table 3 Genotypes for the recurrent *BRCA1* c.2845insA mutation identified in the Malay subjects

Case	Intron 12 D17S1323		Intron 19 D17S1322		Intron 20 D17S855	
N536 (Br)	155	**159**	**119**	**127**	**142**	148
N587 (Br)	**157**	**159**	115	**127**	**142**	142
N610 (Br)	**153**	**159**	**119**	**127**	**142**	146
N521(Ov)	**153**	**159**	**119**	**127**	**142**	146
N576 (Ov)	**157**	**159**	121	**127**	**142**	144
N590 (Ov)	**153**	**159**	125	**127**	**142**	148

Common haplotypes are shown in **bold**.

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