Predictive Factors for BRCA1 and BRCA2 Genetic Testing in an Asian Clinic-Based Population

Edward S. Y. Wong1, Sandhya Shekar1, Claire H. T. Chan1, Lewis Z. Hong2a, Suk-Yean Poon2, Toomas Silla3, Clarabelle Lin4, Vikrant Kumar4, Sonia Davila4, Mathijs Voorhoeve5, Aye Aye Thike5, Gay Hui Ho6, Yoon Sim Yap7, Puay Hoon Tan5, Min-Han Tan7, Peter Ang7,8, Ann S. G. Lee1,9,10*

1 Division of Medical Sciences, Humphrey Oei Institute of Cancer Research, National Cancer Centre, Singapore, Singapore, 2 Institute of Molecular and Cell Biology, Singapore, Singapore, 3 Cancer and Stem Cell Biology Program, Duke-NUS Graduate Medical School, Singapore, Singapore, 4 Human Genetics, Genome Institute of Singapore, Singapore, Singapore, 5 Department of Pathology, Singapore General Hospital, Singapore, Singapore, 6 Department of Surgical Oncology, National Cancer Centre, Singapore, Singapore, 7 Division of Medical Oncology, National Cancer Centre, Singapore, Singapore, 8 OncoCare Cancer Centre, Mount Elizabeth Novena Specialist Centre, Singapore, Singapore, 9 Department of Physiology, Yong Yoo Lin School of Medicine, National University of Singapore, Singapore, Singapore, 10 Office of Clinical and Academic Faculty Affairs, Duke-NUS Graduate Medical School, Singapore, Singapore

* Current address: Molecular Biomarkers & Diagnostics, Translational Medicine Research Centre, Merck Sharp & Dohme, Singapore, Singapore
* dmslsg@nccs.com.sg

Abstract

Purpose

The National Comprehensive Cancer Network (NCCN) has proposed guidelines for the genetic testing of the BRCA1 and BRCA2 genes, based on studies in western populations. This current study assessed potential predictive factors for BRCA mutation probability, in an Asian population.

Methods

A total of 359 breast cancer patients, who presented with either a family history (FH) of breast and/or ovarian cancer or early onset breast cancer, were accrued at the National Cancer Center Singapore (NCCS). The relationships between clinico-pathological features and mutational status were calculated using the Chi-squared test and binary logistic regression analysis.

Results

Of 359 patients, 45 (12.5%) had deleterious or damaging missense mutations in BRCA1 and/or BRCA2. BRCA1 mutations were more likely to be found in ER-negative than ER-positive breast cancer patients (P=0.01). Moreover, ER-negative patients with BRCA mutations were diagnosed at an earlier age (40 vs. 48 years, P=0.008). Similarly, triple-negative breast cancer (TNBC) patients were more likely to have BRCA1 mutations (P=0.001) and
that these patients were diagnosed at a relatively younger age than non-TNBC patients (38 vs. 46 years, \( P=0.028 \)). Our analysis has confirmed that ER-negative status, TNBC status and a FH of hereditary breast and ovarian cancer (HBOC) are strong factors predicting the likelihood of having \( BRCA \) mutations.

**Conclusions**

Our study provides evidence that TNBC or ER-negative patients may benefit from \( BRCA \) genetic testing, particularly younger patients (<40 years) or those with a strong FH of HBOC, in Asian patients.

**Introduction**

The National Comprehensive Cancer Network (NCCN) has recommended various guidelines for the genetic testing of \( BRCA1 \) and \( BRCA2 \), which include specific criteria on the age at diagnosis of the patients and family members; the occurrence of breast, ovarian, pancreatic or prostate cancer in close relatives; and the diagnosis of triple-negative breast cancer (TNBC) [1]. Notably, TNBC patients have higher incidence rates of \( BRCA1 \) and \( BRCA2 \) mutations of up to 30% and 17% respectively [2–4], with younger TNBC patients (aged below 40 years) having an even higher incidence of 36% compared to those diagnosed below 50 years of 27% [5]. Most of these studies were based on Caucasian populations. It is unclear if these guidelines may also be adopted in Asian populations.

Next-generation sequencing (NGS) techniques enable the mutation screening of a larger set of samples in parallel, in a cost effective and accurate manner [6,7]. Recently, the emergence of NGS techniques has played an important role in the simultaneous screening of multiple cancer susceptibility genes including the \( BRCA1 \) and \( BRCA2 \) genes [8,9]. NGS technology has also been widely used in identifying novel genes with mutations related to HBOC [10,11].

Here, we studied 359 breast cancer patients to determine the prevalence of \( BRCA \) mutations in an Asian clinic-based population, using next-generation sequencing and Sanger sequencing. In addition, we evaluated the predictive value of ER-, PR- and HER2- receptor status, age at diagnosis, FH, and histological type for determining the likelihood of mutations in the \( BRCA1 \) and \( BRCA2 \) genes.

**Methods**

**Patients**

Peripheral blood samples were obtained from 359 breast cancer patients attending a risk assessment clinic at the National Cancer Centre Singapore (NCCS). Subjects were eligible if they had a FH of breast and/or ovarian cancer in first- and/or second-degree relatives (\( n = 176 \)), or if they had early-onset breast cancer in the absence of FH (\( \leq 40 \) years of age) (\( n = 183 \)). Patients were accrued from 2002 till 2013. Samples from two earlier studies (accluar from 1992 to 1996 and 2002 to 2006) were also included in this current study [12,13]. Of the 359 breast cancer patients, 321 (89.4%) were Chinese, 16 (4.5%) were Malays, 6 (1.7%) were Indians and 16 (4.5%) were of other Asian ethnicities. ER, PR and HER2 statuses were obtained from clinical databases, and were scored as positive or negative according to previously published criteria [14–16]; ER and PR were considered positive when nuclear staining was present in \( \geq 1\% \) of tumour cells. Her2 was considered as positive when >10% of tumour cells had strong (3+) cell
membrane staining. The information for ER and TNBC status were available for 281 and 206 patients respectively. Written informed consent was obtained from all patients and the study was approved by the SingHealth Centralised Institutional Review Board.

**Mutational screening of BRCA1 and BRCA2**

S1 Fig shows a flow chart of the strategy used to detect mutations in the BRCA1 and BRCA2 genes, to predict damaging mutations and to identify driver/passenger mutations. Frameshift and nonsense mutations were considered to be deleterious.

Sanger sequencing of the BRCA1 and BRCA2 genes was performed as described previously [13], using the CEQ 8000 System (Beckman Coulter, Inc, CA, USA) or the ABI 3130 Genetic Analyzer (AB-Life Technologies; Thermo Fisher Scientific Corporation, MA, USA). The sequenced data were analyzed using the SeqMan Pro v.8.1.2 (Lasergene; DNASTAR, Madison, WI) software.

More recent DNA samples were sequenced by next-generation sequencing, either by Sure-Select capture (Agilent Technologies Inc, CA, USA) followed by sequencing on the Illumina MiSeq platform, or SeqCap EZ capture (Roche Nimblegen, Basel, Switzerland) with sequencing on the Illumina HiSeq platform.

**Bioinformatic Analysis**

For samples sequenced by NGS, reads were aligned to the UCSC human reference genome (hg 19) using the BWA aligner (version 0.5.6). Variant calling was done using the GATK Unified Genotyper [17], and CRISP pipelines [18] (for HiSeq).

All mutations identified from Sanger sequencing or NGS were annotated using the ANNOVAR tool, which provides tools such as SIFT, PolyPhen-II HDIV, PolyPhen—II HVAR, LRT and Mutation Taster to predict the effect of amino acid substitution for each missense mutation. Every missense mutation was scored as damaging or benign with each of the five prediction tools. If the missense mutation was scored as damaging by three or more of the prediction tools, the mutation was classified as a 'Damaging' mutation and if less than three, the mutation was classified as 'Benign'. S1 Table shows the scores for the predictions from the various tools. All missense mutations were also checked against the BIC (http://research.nhgri.nih.gov/bic/), HGMD (http://www.hgmd.org/) and ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/) databases, and were regarded as ‘pathogenic’ if classified as such in two or more databases. All deleterious or pathogenic mutations detected were confirmed by re-sequencing the samples by conventional Sanger sequencing, as described above.

**Multiplex Ligation-dependent Probe Amplification (MLPA)**

All DNA samples were screened for large genomic rearrangements by MLPA using the SALSA MLPA P002-C2 BRCA1 and SALSA MLPA P045-BRCA2 test kits, and validated using the MLPA P087 and P077 confirmation kits (MRC-Holland, Amsterdam, Netherland), respectively. The MLPA analyses were done by DNA fragment analysis on the ABI 3130 Genetic Analyzer and comparative analysis of samples using the Coffalyser freeware v.131123.1303 (MRC-Holland, AM, Netherland).

**Statistical Analysis**

Statistical analysis was done using SPSS version 18.0.2 (SPSS IBM, Armonk, NY). The non-parametric test, i.e., Mann-Whitney U-test was used to compare the median age of the carriers and non-carriers. The Fisher’s exact test was used to determine significant associations between
clinico-pathologic features and the BRCA mutation status. Binary logistic regression analysis was used to estimate the predictive effects of the significantly associated factors for predicting the probability of BRCA mutations. P-values of <0.05 were considered statistically significant.

**Results**

**Mutations in the BRCA1 and BRCA2 genes**

Deleterious mutations detected in BRCA1 and BRCA2 are listed in S1 and S2 Tables. Frame-shift and nonsense mutations, splice-site errors and large genomic rearrangements were classified as deleterious (n = 33). S3 Table shows the list of damaging missense mutations identified. Eleven of 68 missense mutations were predicted to be damaging.

Of 359 patients, 45 (12.5%) had deleterious or damaging missense mutations in the BRCA1 and/or the BRCA2 genes. One patient (case 79) had two deleterious mutations, a BRCA2 nonsense (c.5645C>A; p.S1882X) and a BRCA1 splice-site error (IVS7-15del10) (S1 and S2 Tables). Two patients had the same BRCA1 deleterious mutation (c.67_68delinsAG; p. E23Rfs718).

Three novel BRCA1 mutations, including one frameshift, one nonsense and one large genomic rearrangement (S2 Fig) were detected as well as 11 BRCA1 mutations that have been previously identified (S1 Table) [7,13,19–24]. Eight novel BRCA2 frameshift mutations were identified, together with 10 mutations previously reported (S2 Table) [13,22,23].

**Clinico-pathological characteristics and mutational status**

Table 1 shows the clinico-pathological features of cases with and without BRCA1 and BRCA2 mutations. The median age at diagnosis for BRCA mutation carriers was slightly higher than for non-carriers (41 vs 38) although not statistically significant.

Among 359 patients, 43 (12%) had a FH of HBOC, 132 (37%) had a FH of breast cancer, 1 (0.3%) had a FH of ovarian cancer and 183 (50.9%) were early-onset breast cancer patients without a FH (Table 1). BRCA mutation carriers were more likely to have a FH of HBOC than non-carriers (39.4% vs 9.2%). Conversely, BRCA carriers were less likely to have early-onset breast cancer in the absence of FH as compared to non-carriers (21.2% vs 54%).

The most common histological type of breast cancer in our study was infiltrating ductal carcinoma (IDC), at 72.2%, followed by infiltrating lobular carcinoma (ILC) (3.3%) and medullary cancer types (3.3%) (Table 1). Only 1 patients with ILC had BRCA mutations and none of the medullary cases had BRCA mutations. In patients with IDC, the percentage of BRCA mutation carriers was higher at 57.6%, as compared to other histological types of breast cancer.

The percentages of ER-positive and ER-negative patients were 72% (202/281) and 28% (79/281) respectively. BRCA mutation carriers, were likely to be ER-Negative than non-carriers (50% vs 25.9%). All BRCA mutation carriers with known Her2 status had HER2 negative tumors. Of 206 patients with known ER, PR and HER2 status, 13.6% were TNBC patients. Among our 28 TNBC patients, eight (40%) were BRCA mutation carriers.

**Associations between BRCA1 and BRCA2 mutation status with ER or TNBC status**

There was a significant association of ER-negativity with BRCA1 mutation carriers (61.5% vs 26.5%, P = 0.01, (Table 2); however, no difference was observed in BRCA2 mutation carriers compared to the non-carriers. Furthermore, ER-negative patients (8/79) were more likely to have BRCA1 mutations than ER-positive patients (5/202) (10% vs 2.5%, P = 0.01).
### Table 1. Characteristics of 359 breast cancer patients by mutational status.

|                      | Total \n| = 359 | With Mutation \n| = 33 | Without Mutation \n| = 326 |
|----------------------|--------|----------------|--------|----------------|--------|
| **Age at Diagnosis (Years)** |        |                |        |                |        |
| Median (range)        | 38 (19–76) | 41 (20–60) | 38 (19–76) |        |        |
| ≤ 40 years            | 239    |                |        |                |        |
| > 40 years            | 120    |                |        |                |        |
| **Family History**    |        |                |        |                |        |
| Breast and Ovarian Cancer (HBOC) | 43 (12.0%) | 13 (39.4%) | 30 (9.2%) |        |        |
| Breast Cancer (BC)    | 132 (36.8%) | 13 (39.4%) | 119 (36.5%) |        |        |
| Ovarian Cancer (OC)   | 1 (0.3%) | 0 (0.0%) | 1 (0.3%) |        |        |
| Early Onset Breast Cancer | 183 (50.9%) | 7 (21.2%) | 176 (54%) |        |        |
| **Histology**         |        |                |        |                |        |
| Infiltrating Ductal Carcinoma (IDC) | 259 (72.2%) | 19 (57.6%) | 240 (73.6%) |        |        |
| Infiltrating Lobular (ILC) | 12 (3.3%) | 1 (3.0%) | 11 (3.3%) |        |        |
| Medullary (IMC)       | 12 (3.3%) | 0 (0.0%) | 12 (3.7%) |        |        |
| Others                | 40 (11.1%) | 5 (15.2%) | 35 (10.7%) |        |        |
| Unspecified           | 36 (10.1%) | 8 (24.2%) | 28 (8.6%) |        |        |
| **ER Status**         | n = 281 | n = 26 | n = 255 |        |        |
| Positive              | 202 (72.0%) | 13 (50%) | 189 (74.1%) |        |        |
| Negative              | 79 (28.0%) | 13 (50%) | 66 (25.9%) |        |        |
| **PR Status**         | n = 279 | n = 25 | n = 254 |        |        |
| Positive              | 177 (63.4%) | 13 (52%) | 164 (64.6%) |        |        |
| Negative              | 102 (36.6%) | 12 (48%) | 90 (35.4%) |        |        |
| **HER2 Status**       | n = 206 | n = 20 | n = 186 |        |        |
| Positive              | 49 (23.8%) | 0 (0%) | 49 (26.3%) |        |        |
| Negative              | 157 (76.2%) | 20 (100%) | 137 (73.7%) |        |        |
| **Patients with ER, PR & HER2 Status** | n = 206 | n = 20 | n = 186 |        |        |
| TNBC                  | 28 (13.6%) | 8 (40.0%) | 20 (10.7%) |        |        |
| Non-TNBC              | 178 (86.4%) | 12 (60.0%) | 166 (89.2%) |        |        |

doi:10.1371/journal.pone.0134408.t001

### Table 2. Association between ER status, TNBC status, with BRCA mutation status.

|                      | BRCA1 |                  | BRCA2 |                  |
|----------------------|-------|-----------------|-------|-----------------|
|                      | Carriers | Non-BRCA1 carriers | P-value* | Carriers | Non- BRCA2 Carriers | P-value* |
| ER-positive (n = 202) | N = 13 | 38.5 | N = 268 | 73.5 | N = 14 | 64.3 | N = 267 | 72.3 |
| ER-negative (n = 79) | N = 11 | 61.5 | N = 195 | 36.5 | N = 9 | 35.7 | N = 197 | 64.3 |
| Non-TNBC (n = 178) | 5 | 45.5 | N = 173 | 88.7 | 7 | 77.2 | N = 171 | 86.8 |
| TNBC (n = 28) | 6 | 54.5 | 22 | 11.3 | 2 | 22.8 | 26 | 13.2 |

*P-values that were statistically significant are indicated in **bold.**

doi:10.1371/journal.pone.0134408.t002
Similarly there was a strong association between BRCA1 carriers and TNBC status (54.5% vs 11.3%, \( P = 0.001 \), (Table 2). TNBC patients were more likely to have BRCA1 mutations (6/28) than non-TNBC patients (5/178) (21.4% vs 2.8%, \( P = 0.001 \)).

### Associations between clinical characteristics with ER or TNBC status

The median age at diagnosis for ER-positive and ER-negative patients was 40 years and 39 years respectively (Table 3). In addition, the age at diagnosis for ER-negative patients with BRCA mutations was significantly younger than for ER-positive patients (40 vs 48, \( P = 0.008 \)). When stratified by BRCA1 and BRCA2 mutational status independently, age at diagnosis for ER-negative patients with BRCA1 and BRCA2 mutations was significantly younger than for ER-positive patients (39.5 vs 50, \( P = 0.053 \) and 40 vs 48, \( P = 0.031 \), respectively) (Table 3).

The median age at diagnosis for TNBC patients was younger than for non-TNBC patients although not statistically significant (38 vs 40) (Table 3). The median age at diagnosis for TNBC patients with BRCA mutations was significantly younger than for non-TNBC patients with BRCA mutations (38 vs 47, \( P = 0.03 \)). When stratified by BRCA1 or BRCA2 mutational status independently, age at diagnosis for TNBC patients with BRCA1 and BRCA2 mutations was significantly younger than for non-TNBC patients (38 vs 46 and 38.5 vs 48, respectively, \( P = 0.028 \)).

---

**Table 3. Association between clinical characteristics of breast cancer patients with ER or TNBC status.**

|                       | ER Status | TNBC Status |
|-----------------------|-----------|-------------|
|                       | Total     | Positive    | Negative   | \( P \)-value* | Total     | TNBC       | Non-TNBC   | \( P \)-value* |
| **Age at Diagnosis (Years)** | n = 281  | n = 202 (72%) | n = 79 (28%) |             | n = 206  | n = 28 (13.6%) | n = 178 (86.4%) |             |
| BRCA                  |           |             |             |             |           |             |             |             |
| Non-carriers          |           | 40 (22–76) | 39 (19–65) | 0.284       |           | 38 (22–65) | 40 (19–74) | 0.236       |
| Carriers              |           | 48 (29–60) | 40 (22–52) | 0.008       |           | 38 (22–52) | 47 (29–60) | 0.03        |
| **Among carriers**    |           |             |             |             |           |             |             |             |
| BRCA 1                |           | 50 (35–57) | 39.5 (22–52) | 0.053     |           | 38 (22–52) | 46 (43–57) | 0.028       |
| BRCA 2                |           | 48 (29–60) | 40 (35–40) | 0.031       |           | 38.5 (37–40) | 48 (29–60) | 0.359       |
| **Family History**    |           |             |             |             |           |             |             |             |
| Breast and Ovarian Cancer (HBOC) | 32 (11.4%) | 21 (10.4%) | 11 (13.9%) | 0.408       | 29 (14.1%) | 8 (28.6%) | 21 (11.8%) | 0.035       |
| Breast Cancer (BC)    | 115 (40.9%) | 87 (43.1%) | 28 (35.4%) | 0.281       | 96 (46.6%) | 12 (42.9%) | 84 (47.2%) | 0.690       |
| Ovarian Cancer (OC)   | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) | 0.000       | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) | 0.000       |
| Early Onset Breast Cancer | 134 (47.7%) | 94 (46.5%) | 40 (50.6%) | 0.596       | 81 (39.3%) | 8 (28.6%) | 73 (41.0%) | 0.298       |
| **Histology**         |           |             |             |             |           |             |             |             |
| Infiltrating Ductal Carcinoma (IDC) | 224 (79.7%) | 156 (77.2%) | 68 (86.1%) | 0.102       | 159 (77.2%) | 22 (78.6%) | 137 (77.0%) | 1           |
| Infiltrating Lobular (ILC) | 10 (3.6%) | 8 (4.0%) | 2 (2.5%) | 0.731       | 8 (3.9%) | 1 (3.6%) | 7 (3.9%) | 1           |
| Medullary (IMC)       | 12 (4.3%) | 10 (5.0%) | 2 (2.5%) | 0.519       | 11 (5.3%) | 0 (0.0%) | 11 (6.2%) | 0.367       |
| Others                | 29 (10.3%) | 23 (11.4%) | 6 (8.0%) | 0.393       | 24 (11.7%) | 4 (14.3%) | 20 (11.2%) | 0.750       |
| Unspecified           | 6 (2.1%) | 5 (2.5%) | 1 (1.3%) | 1           | 4 (1.9%) | 1 (3.6%) | 3 (1.7%) | 0.445       |

*\( P \)-values that were statistically significant are indicated in **bold.**

doi:10.1371/journal.pone.0134408.t003
Furthermore, the percentage of TNBC patients with a FH of HBOC was higher than for non-TNBC (28.6% vs 11.8%, P = 0.035). However, there was no statistical difference between the TNBC and non-TNBC patients for patients with other FH.

Predictive factors for BRCA1 and BRCA2 mutations in ER and TNBC patients

Table 4 shows the potential predictive factors for BRCA1 and BRCA2 mutation carriers determined by binary logistic regression analysis. The analyses showed that of all the clinico-pathological characteristics (ER status, age at diagnosis, pedigree diagnosis and histological data), ER-negative status and a FH of HBOC were the strongest predictors for BRCA1 or BRCA2 mutations. The likelihood of patients with HBOC having BRCA1/2 mutations was higher than for other patients (Odds ratio [OR] 3.898; 95% confidence interval [CI] 1.518–10.011; P = 0.005). The odds of having a BRCA1 or BRCA2 mutation in ER-positive patients was 0.390 times (95% CI 0.172–0.887; P = 0.025) less than ER-negative patients (OR 2.562; 95% CI 1.127–5.826; P = 0.025). Neither of the beta coefficients of both factors exceeded the absolute constant value (1.890), indicating that a single factor was insufficient to predict the mutation status. Both ER negative status and a FH of HBOC are required to predict the likelihood of having a BRCA1/2 mutation.

Similar analyses were performed to investigate the potential contribution of PR status, as a predictive factor, to predict the likelihood of having BRCA1/2 mutations. However, no statistical significance was found (data not shown).

A similar analysis was done to evaluate the potential predictive factors for BRCA1 and BRCA2 mutations carriers in TNBC patients (Table 4). The analyses showed that of all the clinico-pathological characteristics (including age of diagnosis, family history and histology), TNBC status and a FH of HBOC were the strongest predictors for BRCA1 or BRCA2 mutations. The likelihood of patients with HBOC being diagnosed with BRCA1/2 mutations was 3.164 times higher than for other patients (OR 3.164; 95% CI 1.080–9.268; P = 0.036). The odds of TNBC patients being diagnosed with BRCA1/2 mutations was 4.651 higher compared to non-TNBC patients (OR 4.651; 95% CI 1.643–13.163; P = 0.004). Similarly, neither of the beta coefficients of both factors exceeded the absolute constant value (2.835), indicating that a single factor was insufficient to predict the mutation status. Thus, both TNBC status and a FH of HBOC are required to predict the probability of having BRCA1/2 mutations.

| Table 4. Potential predictive factors for BRCA1 and BRCA2 mutations in patients stratified by ER status and TNBC status. |
| Factor | Beta | Standard Error | Odds ratio | 95% C.I. for Odds ratio | P-value* |
|--------|------|----------------|------------|------------------------|---------|
|        |      |                |            | Lower                  | Upper   |
| ER status (n = 281) |      |                |            |                        |         |
| Estrogen Receptor Status (Positive) | -0.941 | 0.419 | 0.39 | 0.172 | 0.887 | 0.025 |
| Hereditary Breast and Ovarian Cancer (HBOC) | 1.36 | 0.481 | 3.898 | 1.518 | 10.011 | 0.005 |
| Constant | -1.89 | 0.334 | 0.151 | 0.001 | |
| TNBC status (n = 206) |      |                |            |                        |         |
| Triple Negative Breast Cancer (TNBC) | 1.537 | 0.531 | 4.651 | 1.643 | 13.163 | 0.004 |
| Hereditary Breast and Ovarian Cancer (HBOC) | 1.152 | 0.548 | 3.164 | 1.08 | 9.268 | 0.036 |
| Constant | -2.835 | 0.332 | 0.059 | <0.001 | |

*P-values that were statistically significant are indicated in bold.

doi:10.1371/journal.pone.0134408.t004
Predictive factors for \textit{BRCA1} and \textit{BRCA2} mutations, with the inclusion of damaging missense mutations

Binary logistic regression analysis was performed as before but with deleterious \textit{BRCA} mutations as well as damaging missense mutations (S4–S7 Tables). However, unlike the previous analyses, the median ages did not show any significant difference. Moreover, the analysis showed that only the FH of HBOC is necessary in patients with known ER status, to predict the likelihood of \textit{BRCA} mutations (S7 Table). For patients with known TNBC status, having a TNBC status and a FH of HBOC are required as predictive factors for \textit{BRCA} mutation testing (S7 Table).

\section*{Discussion}

There are few Asian studies that have evaluated the association of \textit{BRCA} mutation status and clinical characteristics. This current Singapore study, based on 359 Asian breast cancer patients prospectively accrued from a risk-assessment clinic, has identified ER-negativity, TNBC status and a FH of HBOC as predictive factors to increase the likelihood of detecting \textit{BRCA1} and \textit{BRCA2} mutations.

Approximately 70–80\% of \textit{BRCA1}-associated breast cancer cases are ER-negative [25–29]. We found that \textit{BRCA1} carriers are more likely to be ER-negative as has been reported previously in western populations [30]. ER-negative status has been suggested to be intrinsic to \textit{BRCA1}-related cancer as it has been found that the proportion of ER-negative patients with \textit{BRCA1} mutations was significantly higher than for ER-positive patients [31].

Patients with \textit{BRCA} mutations were diagnosed at an earlier age in this study. \textit{BRCA1}-associated breast cancers have been shown to be more likely ER-negative for each age group (<45, 45–54, and 55–64 years), with an increase in ER-positive breast cancers with increasing age [31]. Our data concurs with these findings. Furthermore, we provide evidence that ER-negative patients with either \textit{BRCA1} or \textit{BRCA2} mutations were significantly younger than ER-positive patients.

In our cohort, 14\% (28/206) of our patients were TNBC, of which approximately 21.4\% (6/28) and 7.1\% (2/28) had \textit{BRCA1} and \textit{BRCA2} mutations, respectively. A higher frequency of \textit{BRCA1} mutations (20.9\%) as compared to \textit{BRCA2} mutations (3.6\%) was also observed in another study on TNBC patients from Malaysia [32]. A literature review by Pershkin \textit{et al} has reported that among TNBC patients, the proportion of \textit{BRCA1} and \textit{BRCA2} carriers ranged from 9 to 100\% and 2 to 12\%, respectively [33].

Among our \textit{BRCA1} mutation carriers, 37.5\% (6/16) were TNBC patients; whilst among our \textit{BRCA2} carriers, 10.5\% (2/19) were TNBC patients. This frequency of \textit{BRCA} mutations in our TNBC patients is slightly lower than that reported by Peshkin \textit{et al} (2010) of between 42\% to 100\% and 14\% to 35\%, for \textit{BRCA1} and \textit{BRCA2} mutations respectively [33].

Our logistic regression analyses indicated that the odds ratio of TNBC patients with HBOC having \textit{BRCA1}/2 mutations was 3.164 (95\% CI 1.080–9.268; \textit{P} = 0.036), highlighting the importance of FH when estimating \textit{BRCA} mutations prevalence. This is consistent with another study from the US that reported that TNBC patients with a FH of breast cancer or ovarian cancer had a higher probability of having \textit{BRCA} mutations as compared to those without any FH of breast cancer or ovarian cancer (57\% vs 29\%; \textit{P}<0.001 and 77\% vs 41\%; \textit{P}<0.001, respectively) [34].

The NCCN guidelines have proposed the inclusion of TNBC patients aged 60 years or younger for \textit{BRCA} mutation testing. Recently, a Korean study demonstrated that TNBC patients are more likely to be diagnosed at a younger age than non-TNBC patients in the cohort (42 vs 44.1) although the association was not statistically significant [35]. Nevertheless, in the mutation carriers, the mean age at diagnosis of TNBC patients was older than for the non-TNBC
patients (39.2 vs 34.6 for BRCA1 and 51.5 vs 44.0 for BRCA2). Our data, however, are in contrast to these findings. We showed that the median age at diagnosis for TNBC patients with either BRCA1 or BRCA2 is younger than for non-TNBC patients (38 vs 46 for BRCA1 and 38.5 vs 48 for BRCA2), suggesting that BRCA1- and BRCA2-associated breast cancer is most likely early-onset. A study from Malaysia showed that TNBC patients aged below 35 years had a higher prevalence of BRCA1 and BRCA2 mutations compared to non-TNBC patients (28% vs 9.9%) [32]. However, additional studies in larger populations from Asia are warranted to verify these findings from Malaysia and Singapore.

Collectively, and confirming previous findings in western populations, our results showed that the likelihood of TNBC patients being diagnosed with BRCA mutations was higher compared to non-TNBC patients, and the inclusion of additional criteria like a FH of HBOC may increase the probability of identifying BRCA1/2 mutations. A study from Malaysia showed an improvement in the sensitivity and specificity of the Manchester scoring system with the combination of negative ER status, FH and TNBC status [32].

In conclusion, our data showed that almost half of our BRCA mutation carriers in our cohort, are ER-negative. We also found that 29% (8/28) of TNBC patients are BRCA mutation carriers, with the majority being BRCA1 mutation carriers. In addition, we have shown that our TNBC patients with either BRCA1 or BRCA2 mutations were diagnosed at an earlier age. The discovery of the predictive factors, ER-negative status, TNBC status and HBOC, in our study, warrants confirmation in additional Asian populations.

Supporting Information

**S1 Fig. Flow chart of the strategy used for the detection and analysis of mutations in the BRCA1 and BRCA2 genes.** * Computational algorithms used were SIFT, Polyphen-II HDIV, Polyphen-II HVAR, LRT and Mutational Taster; † Filtration criteria is explained in the methods section.

**(TIF)**

**S2 Fig. Deletion of Exons 16 to 19 in BRCA1.** A) Gel photo of PCR products obtained from the amplification of a 563bp-target region from the sample FH42 and control cDNA template; and a sequencing chromatogram of the 272bp-band observed from FH42. (B and C) Changes in mRNA sequence brought about by the deletion of 291bp in FH42 and its corresponding amino acid sequence.

**(TIF)**

**S1 Table. Deleterious mutations in BRCA1.** C, Chinese; I, Indian; B, Burmese; M, Malay; BC, Breast Cancer; OC, Ovarian Cancer; PC, Pancreatic Cancer; IDC, Invasive Ductal Carcinoma; D&L, Mixed Ductal and lobular; ILC, Invasive Lobular Carcinoma; HR, Hormone Receptor; ER, Estrogen Receptor; PR, Progesterone Receptor; TNBC, Triple Negative Breast Cancer; Unk, Unknown; Fs, Frameshift; Del, Deletion of exon; Dup, Duplication of exon; N, Nonsense; SE, Splice-site Error; Ref, References.

**(XLSX)**

**S2 Table. Deleterious mutations in BRCA2.** C, Chinese; I, Indian; B, Burmese; M, Malay; BC, Breast Cancer; OC, Ovarian Cancer; PC, Pancreatic Cancer; IDC, Invasive Ductal Carcinoma; D&L, Mixed Ductal and lobular; ILC, Invasive Lobular Carcinoma; HR, Hormone Receptor; ER, Estrogen Receptor; PR, Progesterone Receptor; TNBC, Triple Negative Breast Cancer; Unk, Unknown; Fs, Frameshift; Del, Deletion of exon; Dup, Duplication of exon; N, Nonsense; SE, Splice-site Error; Ref, References.

**(XLSX)**
S3 Table. Damaging missense mutations in the BRCA1 and BRCA2 genes. Ca, Cancer; C, Chinese; M, Malay; BC, Breast Cancer; OC, Ovarian Cancer; IDC, Invasive Ductal Carcinoma; ILC, Invasive Lobular Carcinoma; DCIS, Ductal Carcinoma in situ; HR, Hormone Receptor; ER, Estrogen Receptor; PR, Progesterone Receptor; TNBC, Triple Negative Breast Cancer; Unk, Unknown; Ref, References; OS, Overall Scores for the prediction of damaging mutations; D, Damaging; P, Potential damaging; N, Neutral.

S4 Table. Characteristics of 359 breast cancer patients by mutational status. * P-values that were statistically significant are indicated in bold; NS: Not Significant.

S5 Table. Association between ER status, TNBC status, with BRCA mutation status. * P-values that were statistically significant are indicated in bold; NS: Not Significant.

S6 Table. Association between clinical characteristics of breast cancer patients with ER or TNBC status. * P-values that were statistically significant are indicated in bold; NS: Not Significant.

S7 Table. Potential predictive factors for BRCA1 and BRCA2 mutations in patients stratified by ER status and TNBC status.

Acknowledgments

We are grateful to all the doctors at the National Cancer Centre Singapore who have referred cases for this study, and to the patients who have volunteered. We thank Dr. William F. Burkholder for helpful discussions and Maurice Chan, Delia Chua, Cheng Ying Ying and Faith Ng for technical support.

Author Contributions

Conceived and designed the experiments: AL PA. Performed the experiments: EW CC LH SP CL. Analyzed the data: EW CC SS TS VK. Contributed reagents/materials/analysis tools: MV SD AAT GHH YSY PHT MT PA. Wrote the paper: AL EW SS.

References

1. National Comprehensive Cancer Network I (2014) The NCCN Guidelines Genetic/Familial High-Risk Assessment: Breast and Ovarian. (Version 2.2014). Retrieved 09/23/14.
2. Evans DG, Howell A, Ward D, Laloo F, Jones JL, Eccles DM (2011) Prevalence of BRCA1 and BRCA2 mutations in triple negative breast cancer. J Med Genet 48: 520–522. doi:10.1136/jmedgenet-2011-100006 PMID: 21653198
3. Comen E, Davids M, Kirchhoff T, Hudis C, Offit K, Robson M (2011) Relative contributions of BRCA1 and BRCA2 mutations to “triple-negative” breast cancer in Ashkenazi Women. Breast Cancer Res Treat 129: 185–190. doi:10.1007/s10549-011-1433-2 PMID: 21394499
4. Meyer P, Landgraf K, Hogel B, Eiermann W, Ataseven B (2012) BRCA2 mutations and triple-negative breast cancer. PLoS One 7: e38361. doi: 10.1371/journal.pone.0038361 PMID: 2266503
5. Fostira F, Tsitlaidou M, Papadimitriou C, Pertesi M, Timotheadou E, Stavropoulou AV, et al. (2012) Prevalence of BRCA1 mutations among 403 women with triple-negative breast cancer: implications for genetic screening selection criteria: a Hellenic Cooperative Oncology Group Study. Breast Cancer Res Treat 134: 353–362. doi: 10.1007/s10549-012-2021-9 PMID: 22434525
6. Feiliubadalo L, Lopez-Doriga A, Castellsague E, del Valle J, Menendez M, Tomero E, et al. (2013) Next-generation sequencing meets genetic diagnostics: development of a comprehensive workflow for the analysis of BRCA1 and BRCA2 genes. Eur J Hum Genet 21: 864–870. doi: 10.1038/ejhg.2012.270 PMID: 23249957

7. Chan M, Ji SM, Yeo ZX, Gan L, Yap E, Yap YS, et al. (2012) Development of a next-generation sequencing method for BRCA mutation screening: a comparison between a high-throughput and a benchtop platform. J Mol Diagn 14: 602–612. doi: 10.1016/j.jmoldi.2012.06.003 PMID: 22921312

8. Snape K, Ruark E, Tarpey P, Renwick A, Tumbull C, Seal S, et al. (2012) Predisposition gene identification in common cancers by exome sequencing: insights from familial breast cancer. Breast Cancer Res Treat 134: 429–433. doi: 10.1007/s10549-012-2057-x PMID: 22527104

9. Kurian AW, Hare EE, Mills MA, Kingham KE, McPherson L, Whittimore AS, et al. (2014) Clinical evaluation of a multiple-gene sequencing panel for hereditary cancer risk assessment. J Clin Oncol 32: 2001–2009. doi: 10.1200/JCO.2013.53.6607 PMID: 24733792

10. Castera L, Krieger S, Rousselin A, Legros A, Baumann J-J, Bruet O, et al. (2014) Next-generation sequencing for the diagnosis of hereditary breast and ovarian cancer using genomic capture targeting multiple candidate genes.

11. Walsh T, Lee MK, Casadei S, Thornton AM, Stray SM, Pennil C, et al. (2010) Detection of inherited Castera L, Krieger S, Rousselin A, Legros A, Baumann J-J, Bruet O, et al. (2014) Next-generation sequencing for the diagnosis of hereditary breast and ovarian cancer using genomic capture targeting multiple candidate genes.

12. Leevy-Lahad E, Catane R, Eisenberg S, Kaufman B, Hornreich G, Lishinsky E, et al. (1997) Founder BRCA1/2 mutations in the Europe: implications for hereditary breast-o

13. HIROKI, Phang BH, Ng IS, Law HY, Soo KC, Ng EH (2000) Novel germline BRCA1 mutations detected in women in singapore who developed breast carcinoma before the age of 36 years. Cancer 89: 811–816. PMID: 10951344

14. Kamodo K, Machackova E, De Vos M, Mortier G, De Paepe A, Messiaen L (1999) Mutation analysis of the BRCA1 and BRCA2 genes results in the identification of novel and recurrent mutations in 6/16 Flemish families with breast and/or ovarian cancer but not in 12 sporadic patients with early-onset disease. Genet Med 1: 397–407. doi: 10.1093/g殁.1.397 PMID: 105153

15. Ahmed SS, Iqbal J, Cheok PY, Tse GM, Wong NS, et al. (2011) Mutations in the epidermal growth factor receptor (EGFR) gene in triple negative breast cancer: possible implications for targeted therapy. Breast Cancer Res 13: R35. doi: 10.1186/bcr2857 PMID: 21457545

16. Iqbal J, Cheok PY, Tse GM, Tan PH (2012) Insulin growth factor receptor-1 expression and loss of PTEN protein predict early recurrence in triple-negative breast cancer. Histopathology 61: 652–659. doi: 10.1111/j.1365-2559.2012.04255.x PMID: 22759273

17. McKenna A, Hanna M, Banks E, Sivachenko A, Cibulskis K, Kernytsky A, et al. (2010) The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. Genome Res 20: 1297–1303. doi: 10.1101/gr.107524.110 PMID: 20644199

18. Bansal V (2010) A statistical method for the detection of variants from next-generation resequencing of DNA pools. Bioinformatics 26: i318–324. doi: 10.1093/bioinformatics/btq214 PMID: 20529923

19. Cia K, Machackova E, De Vos M, Mortier G, De Paepe A, Messiaen L (1999) Mutation analysis of the BRCA1 and BRCA2 genes results in the identification of novel and recurrent mutations in 6/16 Flemish families with breast and/or ovarian cancer but not in 12 sporadic patients with early-onset disease. Mutations in brief no. 224. Online. Hum Mutat 13: 256.

20. Levy-Lahad E, Catane R, Eisenberg S, Kaufman B, Horreich G, Lishinsky S, et al. (1997) Founder BRCA1 and BRCA2 mutations in Ashkenazi Jews in Israel: frequency and differential penetrance in ovarian cancer and in breast-ovarian cancer families. Am J Hum Genet 60: 1059–1067. PMID: 9150153

21. Janavicius R (2010) Founder BRCA1/2 mutations in the Europe: implications for hereditary breast-o

22. Yeo ZX, Wong JC, Rozen SG, Lee AS (2014) Evaluation and optimisation of indel detection workflows for ion torrent sequencing of the BRCA1 and BRCA2 genes. BMC Genomics 15: 516. doi: 10.1186/1471-2164-15-516 PMID: 24962530

23. Kang PC, Phuah SY, Sivanandan K, Kang IN, Thirthagiri E, Liu JJ, et al. (2014) Recurrent mutation testing of BRCA1 and BRCA2 in Asian breast cancer patients identify carriers in those with presumed low risk by family history. Breast Cancer Res Treat 144: 635–642. doi: 10.1007/s10549-014-2894-x PMID: 24578176

24. Cerami E, Gao J, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA, et al. (2012) The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. Cancer Discov 2: 401–404. doi: 10.1158/2159-8290.CD-12-0095 PMID: 22588977
25. Karp SE, Tonin PN, Begin LR, Martinez JJ, Zhang JC, Pollak MN, et al. (1997) Influence of BRCA1 mutations on nuclear grade and estrogen receptor status of breast carcinoma in Ashkenazi Jewish women. Cancer 80: 435–441. PMID: 9241077

26. Loman N, Johannsson O, Bendahl PO, Borg A, Ferno M, Olsson H (1998) Steroid receptors in hereditary breast carcinomas associated with BRCA1 or BRCA2 mutations or unknown susceptibility genes. Cancer 83: 310–319. PMID: 9669814

27. Eisinger F, Stoppa-Lyonnet D, Longy M, Kerangueven F, Noguchi T, Bailly C, et al. (1996) Germ line mutation at BRCA1 affects the histoprognostic grade in hereditary breast cancer. Cancer Res 56: 471–474. PMID: 8564955

28. Robson M, Gilewski T, Haas B, Levin D, Borgen P, Rajan P, et al. (1998) BRCA-associated breast cancer in young women. J Clin Oncol 16: 1642–1649. PMID: 9586873

29. Verhoog LC, Brekelmans CT, Seynaeve C, van den Bosch LM, Dahmen G, van Geel AN, et al. (1998) Survival and tumour characteristics of breast-cancer patients with germline mutations of BRCA1. Lancet 351: 316–321. PMID: 9652611

30. Atchley DP, Albarracin CT, Lopez A, Valero V, Amos CI, Gonzalez-Angulo AM, et al. (2008) Clinical and pathologic characteristics of patients with BRCA-positive and BRCA-negative breast cancer. J Clin Oncol 26: 4282–4288. doi: 10.1200/JCO.2008.16.6231 PMID: 18779615

31. Foulkes WD, Metcalfe K, Sun P, Hanna WM, Lynch HT, Ghadirian P, et al. (2004) Estrogen receptor status in BRCA1- and BRCA2-related breast cancer: the influence of age, grade, and histological type. Clin Cancer Res 10: 2029–2034. PMID: 15041722

32. Phuah SY, Looi LM, Hassan N, Rhodes A, Dean S, Taib NA, et al. (2012) Triple-negative breast cancer and PTEN (phosphatase and tensin homologue) loss are predictors of BRCA1 germline mutations in women with early-onset and familial breast cancer, but not in women with isolated late-onset breast cancer. Breast Cancer Res 14: R142. doi: 10.1186/bcr3347 PMID: 23116406

33. Peshkin BN, Alabek ML, Isaacs C (2010) BRCA1/2 mutations and triple negative breast cancers. Breast Dis 32: 25–33. doi: 10.3233/BD-2010-0306 PMID: 21778580

34. Bayraktar S, Gutierrez-Barrera AM, Liu D, Tasbas T, Akar U, Litton JK, et al. (2011) Outcome of triple-negative breast cancer in patients with or without deleterious BRCA mutations. Breast Cancer Res Treat 130: 145–153. doi: 10.1007/s10549-011-1711-z PMID: 21830012

35. Seong MW, Kim KH, Chung IY, Kang E, Lee JW, Park SK, et al. (2014) A multi-institutional study on the association between BRCA1/BRCA2 mutational status and triple-negative breast cancer in familial breast cancer patients. Breast Cancer Res Treat 146: 63–69. doi: 10.1007/s10549-014-3006-7 PMID: 24894343