Allelic imbalance in \textit{BRCA1} and \textit{BRCA2} gene expression is associated with an increased breast cancer risk

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The contribution of \textit{BRCA1} and \textit{BRCA2} to familial and non-familial forms of breast cancer has been difficult to accurately estimate because of the myriad of potential genetic and epigenetic mechanisms that can ultimately influence their expression and involvement in cellular activities. As one of these potential mechanisms, we investigated whether allelic imbalance (AI) of \textit{BRCA1} or \textit{BRCA2} expression was associated with an increased risk of developing breast cancer. By developing a quantitative approach utilizing allele-specific real-time PCR, we first evaluated AI caused by nonsense-mediated mRNA decay in patients with frameshift mutations in \textit{BRCA1} and \textit{BRCA2}. We next measured AI for \textit{BRCA1} and \textit{BRCA2} in lymphocytes from three groups: familial breast cancer patients, non-familial breast cancer patients and age-matched cancer-free females. The AI ratios of \textit{BRCA1}, but not \textit{BRCA2}, in the lymphocytes from familial breast cancer patients were found to be significantly increased as compared to cancer-free women (\textit{BRCA1}: 0.424 versus 0.211, \textit{P} = 0.00001; \textit{BRCA2}: 0.206 versus 0.172, \textit{P} = 0.38). Similarly, the AI ratios were greater for \textit{BRCA1} and \textit{BRCA2} in the lymphocytes of non-familial breast cancer cases versus controls (\textit{BRCA1}: 0.353, \textit{P} = 0.002; \textit{BRCA2}: 0.267, \textit{P} = 0.03). Furthermore, the distribution of under-expressed alleles between cancer-free controls and familial cases was significantly different for both \textit{BRCA1} and \textit{BRCA2} gene expression (\textit{P} < 0.02 and \textit{P} < 0.02, respectively). In conclusion, we have found that AI affecting \textit{BRCA1} and to a lesser extent \textit{BRCA2} may contribute to both familial and non-familial forms of breast cancer.

\textbf{INTRODUCTION}

Breast cancer is the most common cancer affecting women, with a lifetime risk among females ~10\% by the age of 80 years. In the USA, it has been reported that there will be approximately 180 510 new cases of breast cancer, and more than 40 910 breast cancer-related deaths in 2007 (1). Current estimates suggest that family history is associated with 10–20\% of breast cancer (2,3). \textit{BRCA1} (OMIM: 113705) and \textit{BRCA2} (OMIM: 600185) are two of the most prominent breast cancer susceptibility genes and deleterious mutations in these two genes are estimated to account for about 15–30\% of familial breast cancer (4–6).

Germline mutations affecting the coding region of \textit{BRCA1} and \textit{BRCA2} are thought to lead to expression of mutant proteins, which are either inactive or function as dominant negatives. However, these scenarios have not been supported by functional studies (7–9). In fact, \textit{Brca1} and \textit{Brca2} knockout mouse models have demonstrated that elimination of Brca1 or Brca2 proteins is sufficient for the development of mammary cancer (10,11). Previously, we have reported that mutant \textit{BRCA1} mRNAs containing premature stop codons were eliminated or destabilized by nonsense-mediated mRNA decay (NMD) (12) and lead to a state of haploinsufficiency. As a result, the ratios between the expressions from the mutant alleles and the corresponding wild-type alleles were...
significantly decreased, resulting in what was referred to as allelic imbalance (AI). AI of BRCA1 or BRCA2 expression could decrease the level of both transcripts and proteins and thus contribute to increased susceptibility of developing breast cancer.

There is growing evidence to support this concept. Epigenetic studies have shown that loss of BRCA1 expression due to promoter hypermethylation is associated with ~10% of sporadic cases of breast and ovarian cancer (13–18). However, screens to evaluate AI have not been applied in depth to study its potential role in the genesis of familial forms of these diseases. A previous study reported that 6 out of 13 human genes, including BRCA1 and p53, were expressed with significant difference between the two alleles, and this difference was transmitted by Mendelian inheritance (19). Furthermore, Yan et al. (20) observed that decreased expression of one of the adenomatous polyposis coli tumor suppressor gene (APC) alleles was associated with the development of familial adenomatous polyposis. Their studies also found that even more modest decreases in the expression of one APC allele could contribute to attenuated forms of polyposis (20). Based on these findings, we hypothesize that a subset of non-BRCA1/2 mutation carriers with a strong family history of breast cancer are at increased risk of developing this disease as a result of AI in BRCA1 and BRCA2 gene expression.

In the present study, we have developed a quantitative approach to measure the allele-specific expression of BRCA1 and BRCA2. We compared BRCA1/2 allelic variation in a cohort of BRCA1/2 mutation-negative familial breast cancer patients, non-familial breast cancer patients and age-matched cancer-free volunteers. Since susceptibility to breast cancer is far from being fully understood, our study may help to further identify genetic factors which contribute to breast cancer susceptibility.

RESULTS

Development of a quantitative allelic imbalance assay

In order to determine if allele-specific real-time PCR is able to quantitatively measure the AI in BRCA1 and BRCA2 gene expression from the blood lymphocytes of two individuals determined by genotype and sequence analysis to be homozygous for either BRCA1-c.4308T/T or BRCA1-c.4308C/C (Fig. 1A). This polymorphism was chosen since it is relatively common, based on NCBI dbSNP data. The samples were then reverse transcribed and the cDNAs were mixed at various ratios (8:1, 4:1, 2:1, 1:1, 1:2, 1:4 and 1:8) as described in the Materials and Methods section. BRCA1-c.4308T/T was detected by the VIC fluorescence signal and BRCA1-c.4308C/C was detected by the FAM fluorescence signal. As shown in Figure 1B, with decreasing cDNA ratios of c.4308T to c.4308C, the VIC curve (detecting the c.4308T allele) shifted to the right with the increasing value of CT-c.4308T (VIC), while the curve of FAM (detecting c.4308C allele) shifted to the left with the decreasing value of CT-c.4308C (FAM). At the same time, the value of ΔCT (CT-c.4308T (VIC) − CT-c.4308C (FAM)) changed from the negative to the positive.

By the regression analysis, a linear relationship between Log2 ratio of cDNAs c.4308T to c.4308C and ΔCT was identified: Log2 (c.4308T/C) = -0.0877 + 1.57897 * ΔCT (P < 0.001) (Fig. 1C). The Pearson correlation coefficient (r) between Log2 (c.4308T/c.4308C) and ΔCT was 0.9798. To establish a similar standard curve for BRCA2 allele expression, cDNAs from two individuals, who were either homozygous for BRCA2-c.3396A/A or BRCA2-c.3396G/G, were mixed at the following ratios: 8:1, 4:1, 2:1, 1:1, 1:2, 1:4 and 1:8 (c.3396A/A allele:c.3396G/G allele). BRCA2-c.3396A was detected by the VIC fluorescence signal and BRCA2-c.3396G was detected by the FAM fluorescence signal. As shown in Figure 1D, with decreasing ratios of c.3396A to c.3396G, the VIC curve (detecting c.3396A allele) shifted to the right while the FAM curve (detecting c.3396G allele) shifted to the left. After regression analysis, a linear relationship between Log2 (c.3396A/c.3396G) and ΔCT was identified: Log2 (c.3396A/G) = 0.11726 + 1.26458 * ΔCT (P < 0.001) (Fig. 1E). The Pearson correlation coefficient (r) between Log2 (c.3396A/G) and ΔCT was 0.9868.

Detection of allelic imbalance caused by nonsense-mediated mRNA decay

To examine whether the allele-specific real-time PCR assay is able to detect AI of BRCA1 and BRCA2 gene expression in cell lines, we evaluated RNAs isolated from lymphoblastoid cell lines (LCLs) which were derived from deleterious mutation carriers heterozygous for BRCA1-c.3671ins4 or BRCA2-c.796delT. These frame-shift mutations create the premature stop codons, which are predicted to activate the NMD pathway and thus lead to decreased levels of mRNAs from the mutant alleles (12). As shown in Figure 2A and B, the ratios of BRCA1-c.4308T to -c.4308C between wild type and BRCA1-c.3671ins4 heterozygous samples were 0.93 ± 0.04 and 2.07 ± 0.06, respectively (P < 0.01). By subcloning and sequencing the individual transcripts, we found that the under-expressed allele contained both the BRCA1-c.3671ins4 mutation and the BRCA1-c.4308C polymorphism (detected by the FAM signal) (data not shown). To further examine if the loss of BRCA1-c.3671ins4 was associated with NMD, we treated the BRCA1-c.3671ins4 LCLs with puromycin, a translational inhibitor, 14 h prior to RNA isolation. The ratio of BRCA1-c.4308T to -c.4308C in BRCA1-c.3671ins4 heterozygous cells decreased ~30%, in comparison to the non-treatment group (1.50 ± 0.05 versus 2.07 ± 0.06, P < 0.01) (Fig. 2B). Our data indicated that treatment with puromycin was able to partially recover the AI caused by NMD. The ratio of BRCA2-c.3396G to -c.3396A between wild-type and BRCA2-c.796delT heterozygous samples were 0.98 ± 0.06 and 6.59 ± 1.31, respectively (P < 0.01). After treating the BRCA2-c.796delT LCLs with puromycin, the ratio of BRCA2-c.3396G to -c.3396A in BRCA2-c.796delT heterozygous cells decreased ~31%, in comparison to the non-treatment group (4.90 ± 0.87 versus 6.25 ± 1.17) (Fig. 2C and D). Our results suggested that the loss of expression of BRCA1 or BRCA2 mutant alleles via NMD significantly contributed to the observed AI.
Figure 1. Standard curves for BRCA1 and BRCA2 allelic imbalance.
(A) Allele-specific real-time PCR amplification plot analyses of BRCA1-c.4308T (VIC) and -c.4308C (FAM) was performed in cDNAs generated by RT-PCR using RNAs from blood lymphocytes of two individuals homozygous for either the BRCA1-c.4308T/T or BRCA1-c.4308C/C. DNA sequencing chromatograms confirming the genotype are shown in the right panel. (B) Allele-specific real-time PCR amplification plot was analyzed in mixed cDNAs of BRCA1-c.4308T/T (detected by VIC) and BRCA1-c.4308C/C (detected by FAM) at the following ratios: 8:1, 4:1, 2:1, 1:1, 1:2, 1:4 and 1:8, respectively. (C) The standard curve for BRCA1 allelic imbalance: Log2 (c.4308T/C) = $0.0877 + 1.57897 \times \Delta C_T$. The Pearson correlation coefficient ($r$) between Log2 (c.4308T/c.4308C) and $\Delta C_T$ was 0.9798 (Data expressed as Mean ± SD, n=3; the mean value of $\Delta C_T$ for c.4308T/C=1 has been adjusted to zero). (D) Allele-specific real-time PCR amplification plot was analyzed in mixed cDNAs of BRCA2-c.3396A/A (detected by VIC) and BRCA2-c.3396G/G (detected by FAM) at the following ratios: 8:1, 4:1, 2:1, 1:2, 1:4 and 1:8, respectively. (E) The standard curve for BRCA2 allelic imbalance: Log2 (c.3396A/G) = $0.11726 + 1.26458 \times \Delta C_T$. The Pearson correlation coefficient ($r$) between Log2 (c.3396A/G) and $\Delta C_T$ was 0.9868 (Data expressed as Mean ± SD, n=3; the mean value of $\Delta C_T$ for c.3396A/G = 1 has been adjusted to zero).
**BRCA1 and BRCA2 allelic imbalance is associated with breast cancer risk**

To evaluate AI of *BRCA1* and *BRCA2* gene expression, genotype analysis of the two common polymorphisms, *BRCA1*-c.4308T/C and *BRCA2*-c.3396A/G, was performed on DNA samples isolated from fresh-frozen peripheral blood lymphocytes from 85 unrelated *BRCA1/2* mutation-negative familial breast cancer carriers (median age at sample collection: 47), 112 non-familial breast cancer carriers (median age at sample collection: 52) and 102 age-matched cancer-free females (median age at sample collection: 51) (Table 1). From these analyses, 37 (43.5%), 48 (42.9%) and 41 (40.2%) of the samples evaluated were determined to be heterozygote for the *BRCA1*-c.4308T/C polymorphism for familial breast cancer patients, non-familial breast cancer carriers and cancer-free controls, respectively (Table 1). Furthermore, 39 (45.9%), 44 (39.3%) and 36 (35.3%) of the samples above were found to be heterozygous for the *BRCA2*-c.3396A/G polymorphism (Table 1).

Since our initial validation studies were performed using immortalized LCLs, we first compared AI in RNA isolated from 20 fresh-frozen lymphocytes versus 20 established Epstein-Barr Virus (EBV)-lines. No significant differences were detected between these two sample sets (*BRCA1*: $0.424 \pm 0.129$ versus $0.409 \pm 0.127$ ($n=11$); *BRCA2*: $0.212 \pm 0.180$ versus $0.225 \pm 0.209$ ($n=10$)). However, to limit any AI variation potentially introduced by EBV transformation, all subsequent AI assays were performed using RNAs isolated from peripheral blood lymphocytes. Next, RNA isolated from *BRCA1*-c.4308T/C ($n=126$) and *BRCA2*-c.3396A/G ($n=119$) heterozygotes, including single heterozygotes and double heterozygotes, were evaluated for integrity and quantity. Those samples demonstrating high quality and the necessary quantities were used in the AI assay, as described in the Materials and Methods section.

To evaluate the AI, we used the absolute values of Log$_2$ (*BRCA1*-c.4308T/C) or Log$_2$ (*BRCA2*-c.3396A/c.3396G).
Figure 2. BRCA1 and BRCA2 allelic imbalance caused by NMD. (A) Allele-specific real-time PCR amplification plots of BRCA1-c.4308T (VIC) and -c.4308C (FAM) for non-template control, BRCA1 wild-type lymphoblastoid cells (WT), BRCA1 mutant (heterozygous BRCA1-c.3671ins4) lymphoblastoid cells without [PC (−)] or with [PC (+)] puromycin treatment. (B) Allelic expression ratios of BRCA1-c.4308T to BRCA1-c.4308C (a: versus WT; b: versus PC (+); t-test, P < 0.05). (C) Allele-specific real-time PCR amplification plots of BRCA2-c.3396A (VIC) and -c.3396G (FAM) for non-template control, BRCA2 wild-type lymphoblastoid cells (WT), BRCA2 mutant (heterozygous BRCA2-c.796delT) lymphoblastoid cells without [PC (−)] or with [PC (+)] puromycin treatment. (D) Allelic expression ratios of BRCA2-c.3396G to BRCA2-c.3396A (a: versus WT; b: versus PC (+); t-test, P < 0.05).
The mean value of \( \log_2 (c.4308T/C) \) of \( BRCA1 \) in the lymphocytes from familial breast cancer carriers was found to be \( \sim 104\% \) higher than that in the lymphocytes from cancer-free controls [0.424 \( \pm \) 0.157 \( (n = 32) \) versus 0.211 \( \pm \) 0.169 \( (n = 40) \), \( P = 0.00001; t \)-test] (Table 2 and Fig. 3A and B). \( \log_2 \) of \( BRCA1 \)-c.4308T/C in the lymphocytes from non-familial breast cancer carriers was 73\% higher than that in cancer-free controls [0.353 \( \pm \) 0.209 \( (n = 32) \), \( P = 0.002 \) versus control] (Table 2 and Fig. 3A and C). In comparison, the mean value of \( \log_2 \) of \( BRCA2 \)-c.3396A/G in the lymphocytes from familial breast cancer carriers was moderately higher (10\%) than that in cancer-free controls [0.206 \( \pm \) 0.180 \( (n = 37) \) versus 0.172 \( \pm \) 0.123 \( (n = 31) \), \( P = 0.38; t \)-test] (Table 2 and Fig. 4A and B). A similar result (38\% higher) was observed for \( \log_2 \) (c.3396A/G) of \( BRCA2 \) in the lymphocytes of non-familial breast cancer carriers [0.267 \( \pm \) 0.171 \( (n = 26) \), \( P = 0.03 \) versus control] (Table 2 and Fig. 4A and C).

Interestingly, the distribution of under-expressed alleles of \( BRCA1 \) and \( BRCA2 \) was found to be significantly different between cancer-free control and familial breast carriers, but not between cancer-free control and non-familial breast carriers. As shown in Table 3 and Figure 3, under-expressed...
addition, under-expressed BRCA2 of familial breast cancer carriers, respectively (37 of 31) and (17 of 31) of cancer-free controls as compared to 0.375 + 0.06, respectively) (Table 3). Furthermore, we have demonstrated that the AI patterns for BRCA1 expression, albeit in a small number of families, can be transmitted by Mendelian inheritance (Table 4). Although these findings are consistent with a previous study (19), future evaluations will benefit from evaluating AI in large families for evidence of disease segregation.

Several methods have been developed to evaluate allele-specific expression. The first method combines primer extension and capillary electrophoresis (19,21). The second approach utilizes microarray technology to measure allele-specific mRNA expression (22). Compared to the AI assay presented here, the method of primer extension plus capillary electrophoresis is also accurate but relatively time-consuming and expensive. The microarray approach provides a high-throughput and a powerful platform for the simultaneous analysis of large numbers of genes to analyze allele-specific gene expression, but it has less power to define the AI. Like the majority of allelic expression methods (23), our AI assay also requires a transcribed heterozygous variant in the individuals to be evaluated. In the present study, we targeted two common polymorphisms, BRCA1-c.4308T>C and BRCA2-c.3396A>G in the general population. Therefore, a substantial number of subjects homozygous for the polymorphisms had to be excluded. To overcome this limitation of population selection based on genotypes, other primers and probes will need to be developed to target other common polymorphisms in BRCA1 and/or BRCA2. In addition, our approach could easily be applied for studying AI in other cancer susceptibility genes, such as p53, APC, PTEN, etc.

In this study, we have demonstrated AI for both BRCA1 and BRCA2 in breast cancer populations. Interestingly, the increase of AI ratios in familial and non-familial breast cancer patients was more significant for BRCA1 than BRCA2. Loss of BRCA1 expression in breast cancer has been reported to be related to the pathogenesis of breast cancer (13–17). Loss of BRCA2 expression in cancers, in

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| BRCA1-c.4308T/C          | 37           | 48           | 41          |
| BRCA2-c.3396A/G          | 39           | 44           | 36          |

*aNumber of first and/or second-degree relatives affected with either breast and/or ovarian cancer.

In this study, we have demonstrated AI for both BRCA1 and BRCA2 (Fig. 1). By performing this AI assay with specific primers and probes that target common single nucleotide polymorphisms in BRCA1 and in BRCA2, we were able to detect allelic imbalance associated with NMD in patients carrying frameshift mutations in BRCA1 and BRCA2 (Fig. 2). We next compared AI of BRCA1 and BRCA2 expression among three groups, familial breast cancer patients, non-familial breast cancer patients, and age-matched cancer-free females. AI ratios of BRCA1 in familial breast cancer cases were significantly higher than those from cancer-free controls (P = 0.00001) (Table 2 and Fig. 3). Similar results were observed for AI ratios of BRCA1 in the lymphocytes from non-familial breast cancer patients (P = 0.002). AI ratios of BRCA2 in familial or non-familial breast cancer cases were also higher than those from cancer-free controls (P = 0.38 or P = 0.03, respectively). However, the difference was not statistically significant in the ratios of mRNA expressed from the BRCA2 alleles found in familial breast cancer cases when compared to cancer-free controls (Table 2 and Fig. 4). In addition, the distribution of under-expressed alleles between cancer-free controls and familial cases was significantly different for both BRCA1 and BRCA2 gene expression (P < 0.02 and P < 0.02, respectively) (Table 3). Furthermore, we have demonstrated that the AI patterns for BRCA1 expression, albeit in a small number of families, can be transmitted by Mendelian inheritance (Table 4). Although these findings are consistent with a previous study (19), future evaluations will benefit from evaluating AI in large families for evidence of disease segregation.

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| BRCA1-c.4308T/C | 37           | 48           | 41          |
| BRCA2-c.3396A/G | 39           | 44           | 36          |

*aNumber of first and/or second-degree relatives affected with either breast and/or ovarian cancer.

In this study, we have demonstrated AI for several tumor suppressor genes could be transmitted by Mendelian inheritance (19). To test if the AI observed in our study may be inherited, we identified three affected women (i.e. probands) reporting a significant family history of breast and/or ovarian cancer. We therefore, each sister had to be heterozygous for the BRCA1-c.4308T/C polymorphism. As shown in family A (Table 4), sister Sis-02 displayed a similar AI pattern as compared to the proband, while sister Sis-01 displayed no AI. In the two other families, both the affected probands and their corresponding sisters showed AI (Table 4). We further performed a haplotype analysis to determine whether the alleles showing AI were shared between siblings. As shown in Table 4, sisters with the same AI phenotype shared the same haplotype with their affected sister. Importantly, sister Sis-01 in family A did not share the same haplotype. Her blood sample displayed no AI (0.007 ± 0.147) for BRCA1 gene expressions whereas the AI was detected in her unaffected and affected sisters (Sis-02 and Proband, 0.382 ± 0.176 and 0.375 ± 0.06, respectively) (Table 4). The allele frequencies of the microsatellite markers used for haplotype construction are listed in Supplementary Material, Table S1.
contrast, is still controversial (24,25). These findings indicate that AI in BRCA1 appears to be a more common event in breast cancer development than AI involving BRCA2. However, the mechanism(s) leading to the observed AI is for the most part unknown.

We have demonstrated that both BRCA1 and BRCA2 deleterious mutations can activate the NMD pathway and result in AI [Figure 2, and (12)]. However, all the familial breast cancer patients evaluated in the current study were determined to lack a mutation in BRCA1 and BRCA2 that would trigger NMD. Furthermore, we evaluated the BRCA1 and BRCA2 genes in the sporadic breast cancer patients and cancer-free controls demonstrating AI [i.e. allele expression ratio > 0.25 or < −0.25 (Figs 3 and 4)]. Again, no deleterious germline mutations were detected (data not shown). This is not entirely surprising given that germline mutations in BRCA1 and BRCA2 are rare in women affected with breast cancer without a strong family history of the disease (26–29).

Based on these observations, we conclude that NMD is not likely to be responsible for the observed AI in our case-control comparisons. Therefore, other mechanisms are likely to exist to account for the observed increased AI of BRCA1 and BRCA2 gene expression in female breast cancer patients. For example, the 5′ and 3′ non-coding regions of BRCA1 and BRCA2 are rarely evaluated through genetic testing, even though genetic alterations in these non-coding regions could be important in regulating BRCA1 and BRCA2 expression. For instance, genetic alterations within 5′ DNA or the putative promoter regions are able to disrupt the binding of transcription factors to DNA regulatory elements and hence lead to the loss of allelic gene expression. Several studies have shown that large genomic deletions involving the BRCA1 promoter were associated with hereditary breast cancer (30–32). This concept is further supported by studies of Cowden syndrome (CS) showing that ~10% of CS-related PTEN mutations occur in the PTEN promoter and lead to a 50% reduction in PTEN expression (33,34). Also, allele-specific hypermethylation of the BRCA1 promoter region and decreased BRCA1 expression is associated with ~10% of sporadic breast cancer cases (18,30,35). Recent advances have identified a new pathway for gene regulation, i.e. via microRNAs (miRNAs) (36,37). These 21–22 nt RNA molecules are complementary to the 3′-UTR sequence of transcripts and mediate negative post-transcriptional regulation through RNA duplex formation (36,38). By performing in silico analyses in four BRCA1 SNPs and two BRCA2 SNPs (39), we have identified three rare BRCA1 alleles (c.5628G, c.6273T, c.6924A) that could potentially create target sites for selected microRNAs (Supplementary Material, Table S2). Therefore, it is possible that altered mRNA targeting could contribute to AI of BRCA1 gene expression in the absence of frameshift mutations. It will be important in future studies to determine the mechanisms that either disrupt transcription factors binding or alter miRNA binding, leading to constitutively decreased levels of BRCA1 and BRCA2 and an increased risk of developing breast cancer.

In summary, we have developed a quantitative approach to evaluate expression of BRCA1 and BRCA2 from individual alleles, and we have found that AI in BRCA1 and to a lesser extent BRCA2 is associated with increased breast cancer risk. Furthermore, we have demonstrated that the AI patterns for BRCA1 expression could be transmitted by Mendelian inheritance. Since susceptibility to breast cancer is far from being fully understood, our study suggests that alternate mechanisms, other than deleterious coding mutations, may contribute to breast cancer.

### MATERIALS AND METHODS

#### Databases

RefSeqs (GenBank Accession No: NM_007295.2 and NM_000059.1) were used for BRCA1 and BRCA2 mRNA numbering, respectively. The A of ATG translation initiation codon is defined as position +1.

#### Subjects and genotype analysis

Three populations were used in this study, (i) BRCA1/2 mutation-negative women reporting a personal and family history of breast cancer, i.e. familial; (ii) female breast cancer patients without a significant family history of disease, i.e. non-familial; and (iii) age-matched cancer-free female controls (Table 1). All participants were Caucasian women with European-American ancestry and were from the Delaware Valley, including the greater Philadelphia Metropolitan area in Pennsylvania. For family studies, eligible subjects were women with a personal and family history of cancer (at least two first and/or second-degree relatives affected with either breast and/or ovarian cancer) and were ascertained from the Family Risk Assessment Program (FRAP) at the Fox Chase Cancer Center (FCCC). All relevant institutional

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*To calculate the mean value of AI, all negative value of Log2 (BRCA1-c.4308T/C) and Log2 (BRCA2-c.3396A/c.3396G) in Figures 3 and 4 were changed to positive values.
review boards approved the study protocol and written informed consent was obtained from all participants. Genotype analyses of the two common polymorphisms, BRCA1-c.4308T/C and BRCA2-c.3396A/G were carried out using ABI PRISM 7900HT Sequence Detection System and Assays-on-Demand SNP Genotyping products for fluorogenic polymerase chain reaction allelic discrimination (Applied Biosystems, Foster City, CA, USA).
**Figure 4.** *BRCA2* allelic expression ratios in cancer-free controls, familial and non-familial breast cancer patients. The AI assays were performed using specific primer and probe sets targeting *BRCA2*-c.3396A/G. Log2 ratios of *BRCA2*-c.3396A allele to -c.3396G allele expression were presented in cancer-free controls (A), familial (B) and non-familial cancer patients (C). (Data expressed as Mean ± SD, n = 3; the mean value of allelic expression ratios of total normal samples has been adjusted to zero).
Allelic imbalance assay

A 1.25 μl of the cDNA synthesized in the RT reaction was used in a real-time PCR reaction (25 μl total volume), performed with ABI PRISM 7900HT Sequence Detection System following methods recommended by the manufacturer. Optimal conditions were as follows: Step 1, 95°C for 10 min; Step 2, 92°C for 15 s, 60°C for 60 s with Optics; repeated for 40 cycles. The primer and probe sets used in real-time PCR reaction to detected BRCA1-c.4308T/C (rs1060915) and BRCA2-c.3396A/G (rs1801406) allelic expression were obtained from Applied Biosystem TaqMan® SNP Assay program (Assay ID: C.3178676 and C.7605673.1 for BRCA1 and BRCA2, respectively). Sequence information for primers and probes is available upon request. Each 96-well PCR plate included negative controls, positive controls and unknown samples. Real-time PCR data were analyzed with ABI SDS 2.2.2 software. In order to produce the BRCA1 allelic expression standard curve, cDNAs from the two samples with homozygous genotypes, BRCA1-c.4308T/T and BRCA2-c.4308C/C, were mixed as the following ratios: 8:1, 4:1, 2:1, 1:1, 1:2, 1:4 and 1:8 (c.4308T/C allele). For the same purpose, cDNAs from the two samples with homozygous genotypes, BRCA2-c.3396A/A and BRCA2-c.3396G/G, were mixed as the following ratios: 8:1, 4:1, 2:1, 1:1, 1:2, 1:4 and 1:8 (c.3396A/G allele) (42). In contrast, mismatches between a probe and target are expected to reduce the efficiency of probe hybridization, and AmpliTaq DNA polymerase is more likely to displace a mismatched probe without cleaving it, which does not produce a fluorescent signal.

The theoretically, Allele 1 gene copy number (detected by FAM):

\[ \log_2[\text{Allele} - 1] = -A_1 \times C_{T1} + B_1 \]  

and Allele 2 gene copy number (detected by VIC):

\[ \log_2[\text{Allele} - 2] = -A_2 \times C_{T2} + B_2 \]

If the fluorescence probes have the same efficiency to hybridize with matched target sequence, that is, \( A_1 = A_2 = A \), therefore,

\[ \log_2[\text{Allele} - 1/2] = A \times (C_{T2} - C_{T1}) + (B_1 - B_2) \]

The function (3) was confirmed by two standard curves, \( \log_2 (c.4308T/C) = -0.0877 + 1.57897 \times \Delta C_T \) and \( \log_2 (c.3396A/G) = 0.11726 + 1.26458 \times \Delta C_T \), set up by our experimental data (Fig. 1). Besides using function (3) to calculate the ratio of mRNA expression between the two alleles, function (1) and function (2) are able to be applied for examining the absolute value of each allele mRNA expression. However, the direct analysis of single allele expression is often complicated by the potential variations between individuals with different environmental or physiological background rather than genetic factors. Comparing the relative expression levels of two alleles of the same gene within the same biologic sample will help to minimize these variations.
Peripheral blood lymphocytes and LCLs

Lymphocytes were isolated from peripheral blood and stored at $-150^\circ C$ until needed. None of the blood samples from breast cancer patients were collected at the time of chemotherapy or radiation therapy. In addition, a subset of cryopreserved lymphocytes from BRCA1 or BRCA2 mutation carriers (e.g. BRCA1-c.3671ins4 and BRCA2-c.796delT) or disease-free individuals were infected with EBV to establish immortal LCLs. LCLs were maintained in RPMI (GIBCO BRL) media supplemented with 20% fetal calf serum and antibiotics at 37°C, 5% CO2 atmospheric condition and 95% humidity. The immortalized LCLs from cancer-free individuals that had been tested negative for mutations in BRCA1 and BRCA2 served as wild-type controls. To prevent potential degradation of unstable transcripts by NMD a translation inhibitor, puromycin (Sigma, St Louis, MO, USA) was added to the LCL cells as described in a previous study (12).

Subcloning the PCR product and sequence analysis

PCR fragments containing a common polymorphism and deleterious mutation were subcloned directly into pCR®-4-TOPO vector (Invitrogen, Carlsbad, CA, USA). PCR was then performed to identify bacterial colonies containing appropriate inserts. Plasmid DNA was purified using QIAfilter™ Plasmid Maxi Kit (Qiagen Inc., Valencia, CA, USA) and the insert was sequenced using either the universal M13-primers or the primers for PCR reactions.

RNA isolation and reverse transcription (RT)

Total cellular RNAs were isolated from blood lymphocyte pellets using TRIzol reagent according to the protocols provided by the manufacturer (Invitrogen Corp., Carlsbad, CA, USA). Purified RNAs were further processed to remove any contaminating DNA (DNA-free kit, Ambion, Inc., Houston, TX, USA). After quantification with Bioanalyzer-2100 system using RNA 6000 Nano LabChip kits (Agilent Technologies, Palo Alto, CA, USA), 2 μg of total RNA from each sample was used as a template to be reverse-transcribed (RT) in a 20 μl reaction [containing 5 μM random hexamers, 500 μM deoxynucleoside triphosphate mix, 1 × RT (reverse transcriptase) buffer, 5 mM MgCl2, 1.5 units of RNase inhibitor and 7.5 units of MuLV reverse transcriptase]. All reagents were purchased from Applied Biosystems (Branchburg, NJ, USA). The RT reaction conditions were 10 min at 25°C, 1 h at 42°C and 5 min at 94°C.

Haplotype analysis

Haplotypes were constructed for BRCA1 using three polymorphic microsatellite repeat markers located within (D17S855 and D17S1322) or adjacent (D17S1325) to the BRCA1 locus. The sequences of the primer pairs were obtained from the Genome Database (http://www.gdb.org) and PCR reaction was carried out as previously reported (43,44). PCR products with fluorescent dye (HEX) labeled primer were mixed with Hi-Di Formamide and a fluorescent labeled internal size marker. The mixture was subjected to electrophoresis on an ABI 3100 Automated DNA Sequencer (Applied Biosystems, Foster City, CA, USA) and the data were analyzed by the GeneScan (Version 3.7) and GeneMapper (Version 4.0) software provided by the manufacturer.

Statistical analysis

Allele specific real-time PCR data were analyzed with ABI SDS software v2.2.2 (Applied Biosystems, Foster City, CA, USA). Statistical analysis was conducted using the SAS System (version 9) developed by the SAS Institute, Inc. (Cary, NC, USA). Student’s t-test was employed for continuous data and results were presented as the mean ± SD. We compared the distribution of under-expressed alleles in BRCA1 or BRCA2 between cases and controls using χ² 95% confidence intervals (CI) and the difference in distribution of under-expressed alleles was estimated as odds ratios (OR). A value of $P < 0.05$ is considered significant.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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