

Allelic Imbalance in *BRCA1* and *BRCA2* Gene Expression and Familial Ovarian Cancer

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Abstract

Background: Family history is the strongest risk factor for ovarian cancer. Recent evidence suggests that unidentified *BRCA1/2* variations or other genetic events may contribute to familial ovarian cancers. Allelic imbalance (AI) of *BRCA1/2* expression, a result of a significant decrease in the ratios between the expression from one allele of *BRCA1/2* and the other allele, has been observed in breast cancer. The AI of *BRCA1/2* expression could decrease the level of transcripts and thus contribute to an increased susceptibility of developing familial ovarian cancer.

Methods: To test this hypothesis, we applied a quantitative, allelic-specific, real-time PCR method to survey the levels of AI in *BRCA1/2* in lymphoblastoid cell lines (LCL) from 126 familial ovarian cancer patients who are noncarriers of any known *BRCA1/2* and *MLH1/MSH* mutations and 118 cancer-free relative controls.

Results: The AI ratios of *BRCA1*, but not *BRCA2*, in the LCLs from familial ovarian cancer patients were found to be significantly increased as compared with family controls (*BRCA1*: 0.463 ± 0.054 vs. 0.405 ± 0.111 , $P = 0.0007$; *BRCA2*: 0.325 ± 0.124 vs. 0.302 ± 0.118 , $P = 0.328$). Using the cutoff point of 0.458 identified from the receiver operating characteristic (ROC) analysis, higher levels of AI were associated with a 4.22-fold increased risk of familial ovarian cancer (95% CI: 1.60–11.16). In further analysis, we observed that levels of AI were negatively significantly correlated with the age of familial ovarian cancer diagnosis ($\rho = -0.469$, $P < 0.001$).

Conclusion: Taken together, our data suggest that AI affecting *BRCA1* may contribute to familial ovarian cancer. *Cancer Epidemiol Biomarkers Prev*; 20(1); 50–6. ©2011 AACR.

Introduction

Epithelial carcinoma of the ovary is one of the most common gynecologic malignancies in women (1). Family history is the strongest risk factor for ovarian cancer. Compared with a 1.6% lifetime risk of developing ovarian cancer in the general population, women with 1 first-degree relative with ovarian cancer have a 5% risk and women with 2 first-degree relatives have a 7% risk. Familial clustering with an autosomal dominant pattern of inheritance (hereditary ovarian cancer) results from germline mutations in putative tumor suppressor genes (TSG), such as the *BRCA1/2* and *MLH1/MSH2* genes (2–5). However, known mutations in *BRCA1/2* and *MMR* genes can only explain a small part of the familial aggregation of ovarian cancer (5%–13%). This suggests that other genetic events may contribute to familial ovarian cancers.

Allelic imbalance (AI) is defined as the decrease of the ratios between the expressions from the variant allele and the corresponding wild-type one. AI of gene expression is common in humans and is of interest because of its potential contribution to variation in heritable traits. However, the roles of AI in the genesis of human diseases have rarely been studied. A previous study reported that 6 of 13 human genes, including *BRCA1* and *p53*, were expressed with significant difference between the 2 alleles, and that this difference was transmitted by Mendelian inheritance (6). Furthermore, Yan et al. observed that decreased expression of one of the adenomatous polyposis coli (*APC*) tumor suppressor gene alleles was associated with the development of familial adenomatous polyposis (7). Their studies also found that even more modest decreases in the expression of an *APC* allele could contribute to attenuated forms of polyposis.

AI of *BRCA1/2* has been previously reported in patients with mutant *BRCA1/2* mRNAs containing premature stop codons. The mutant *BRCA1/2* were eliminated or destabilized by nonsense-mediated mRNA decay (NMD) and lead to a state of haploinsufficiency (8). Whether AI of *BRCA1/2* exists in individuals without *BRCA1/2* mutations is rarely studied. In a study of breast cancer, AI in *BRCA1/2* was observed in breast cancer patients without known *BRCA1/2* mutations and also healthy controls (9). Considering the

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significant role of *BRCA1/2* in the etiology of ovarian cancer, AI of *BRCA1* or *BRCA2* is extremely relevant to ovarian cancer. In the present study, we hypothesize that a subset of non-*BRCA1/2* or *MLH1/MSH* mutation carriers with a strong family history of ovarian cancer is at increased risk of developing this disease as a result of AI in *BRCA1* and *BRCA2* gene expression. To test the hypothesis, we compared *BRCA1/2* AI in a cohort of *BRCA1/2* or *MLH1/MSH* mutation-negative familial ovarian cancer patients and cancer-free relative controls. Because genetic susceptibility of ovarian cancer is far from being fully understood, our study may help to further identify genetic factors that contribute to ovarian cancer susceptibility.

Materials and Methods

Study population

Both ovarian cancer cases and cancer-free relative controls were obtained from the Gilda Radner Familial Ovarian Cancer Registry (GRFOCR). One hundred twenty-six nonrelated women with familial ovarian cancer were included in this study as the cases. They were identified from families with inherited ovarian cancer in which at least 2 first- or second-degree relatives had epithelial ovarian cancer diagnosed at any age. All of the samples were noncarriers of *BRCA1/2* or *MLH1/MSH2* mutations. Different methods have been used to determine the mutation status of *BRCA1/2* in GRFOCR in the past and current. For samples collected before 2002, mutation status was determined by screening all exons and intron/exon splice junctions of *BRCA 1/2* by a combination of SSCP and HD analysis. In addition, exon 11 of *BRCA 1* was assayed by the protein truncation test for stop generating mutations. If alterations were found, the altered fragment was sequenced. Since 2002, sequencing of exons and splice junctions was used. All samples (old and new) not showing a mutation were assayed for *BRCA 1* large rearrangements in the last 5 years. One hundred eighteen cancer-free women were included in this study as the controls. They were family relatives to the cases, including mothers, sisters, and nieces. All study subjects donated blood samples when they were enrolled in the GRFOCR. LCLs were established by Epstein-Barr virus (EBV) transformation using the isolated lymphocytes from the blood samples. The cell lines have not been authenticated. The study was approved by the institutional review board.

Lymphoblastoid cell line culture and RNA extraction

Lymphoblastoid cell lines (LCL) were maintained in RPMI 1640 (Gibco BRL) media supplemented with 15% fetal calf serum and antibiotics at 37°C, 5% CO₂ atmospheric condition, and 95% humidity. Total cellular RNAs were isolated from LCLs using TRIzol reagent according to the protocols provided by the manufacturer (Invitrogen Corp.). Purified RNAs were further processed to remove any contaminating DNA (DNA-free kit; Ambion, Inc.). Two micrograms of total

RNA from each sample was used as a template to be reverse-transcribed (RT) in a 20 µL reaction (containing 5 µmol/L random hexamers, 500 µmol/L of deoxynucleoside triphosphate mix, 1× reverse transcriptase buffer, 5 mmol/L of MgCl₂, 1.5 units of RNase inhibitor, and 7.5 units of MuLV reverse transcriptase). The RT reaction conditions were 10 minutes at 25°C, 1 hour at 42°C, and 5 minutes at 94°C.

Genotyping analysis to identify informative individuals

The AI analysis has to be carried out in the informative individuals who are heterozygous for at least 1 genetic variant of *BRCA1/2* genes. For the *BRCA1* gene, a common polymorphism, *BRCA1*-c.4308T/C (rs1060915) was genotyped to identify the informative (heterozygous) study subjects. For the *BRCA2* gene, we conducted genotyping analyses on the *BRCA2*-c.3396A/G (rs1801406) polymorphism to identify informative individuals who are heterozygous for the polymorphism. Genotyping analysis was carried out using StepOnePlus Real Time PCR System and Assays-on-Demand SNP Genotyping products for fluorogenic polymerase chain reaction allelic discrimination (Applied Biosystems). Each PCR reaction plate included negative controls, positive controls, and unknown samples.

AI assay

A similar AI analysis method used by Chen et al. was used in this study (9). Briefly, 1.25 µL of the cDNA synthesized in the RT reaction was used in a real-time PCR reaction (25-µL total volume), conducted with the ABI StepOnePlus Real Time PCR System following the methods recommended by the manufacturer. Optimal conditions were as follows: step 1, 95°C for 10 minutes; step 2, 92°C for 15 seconds, 60°C for 60 seconds with optics; repeated for 40 cycles. The same primer and probe sets used in the genotyping analysis were also used for the real-time PCR reaction to detect *BRCA1*-c.4308T/C (rs1060915) and *BRCA2*-c.3396A/G (rs1801406) allelic expression. Each 96-well PCR plate included negative controls, positive controls, no-RT controls, and unknown samples in triplicate. We did not observe any amplification on randomly selected no-RT controls, indicating DNA contamination was not an issue in this study. Real-time PCR data was analyzed with ABI SDS 2.2.2 software. A similar method as that described by Chen et al. was used in the AI analysis. In brief, to produce the *BRCA1* allelic expression standard curve, cDNAs from the 2 samples with homozygous genotypes, *BRCA1*-c.4308T/T and *BRCA1*-c.4308C/C, were mixed at the following ratios: 8:1, 4:1, 2:1, 1:1, 1:2, 1:4, and 1:8 (c.4308T/T allele:c.4308C/C allele). For the same purpose, cDNAs from the 2 samples with homozygous genotypes, *BRCA2*-c.3396A/A and *BRCA2*-c.3396G/G, were mixed in 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). The principles of quantitative real-time PCR provide the basis

Table 1. Selected characteristics of the study population

Characteristic	Cases	Controls
Total number	126	118
Age, ^a median (range)	48 (25–78)	52 (23–93)
Ethnicity		
Caucasians	126	118
Others	0	0
<i>BRCA1/2, MLH1/MSH2</i> status		
Negative	126	
Positive	0	
Genotype		
<i>BRCA1</i> -c.4308T/C	60	53
<i>BRCA2</i> -c.3396A/G	58	51

^aFor cases, age refers to age of disease diagnosis; for controls, age refers to age of enrollment in GRFOCR.

of this linear relation between \log_2 ratio and C_T established in our approach to detect AI. Theoretically, allele 1 gene copy number (detected by FAM): $\log_2[\text{Allele} - 1] = -A_1 \times C_{T1} + B_1$. Allele 2 gene copy number (detected by VIC): $\log_2[\text{Allele} - 2] = -A_1 \times C_{T2} + B_2$. If the fluorescence probes have the same efficiency to hybridize with matched target sequences, that is, $A_1 = A_2 = A$, then the ratio of mRNA expression between the 2 alleles: $\log_2[\text{Allele} - 1/2] = -A \times (C_{T2} - C_{T1}) + (B_2 - B_1)$.

BRCA1 mRNA expression

TaqMan-based quantitative real-time PCR was applied to assess the mRNA expression levels in the LCLs. The assay was purchased from Applied Biosystems (ID number: Rn00560871_m1). Briefly, 0.5 μ L of the cDNA synthesized in the RT reaction was used in a real-time PCR reaction (25- μ L total volume), carried out with the ABI StepOnePlus Real Time PCR System following the methods recommended by the manufacturer. Real-time PCR data was analyzed with ABI SDS 2.2.2 software.

Statistical analysis

Student's *t* test was used to compare the mean *BRCA1/2* AI levels for significant differences between familial ovarian cancer patients and family noncancer controls. ROC analysis was carried out to determine the sensitivity

and specificity for the arbitrary cutoff point. The cutoff point that had the highest value of Youden's index (= sensitivity + specificity - 1) was used in the stratified analysis. In stratified analysis, to assess the strength of the association between familial ovarian cancer risk and levels of AI, we calculated the odds ratio (OR) and its 95% confidence interval (CI) by using unconditional logistic regression analysis. Spearman correlation was carried out to assess the relationship between levels of AI in *BRCA1* and *BRCA1* mRNA gene expression. Linear regression analysis was carried out to further investigate the relationship between age at diagnosis of familial ovarian cancer and levels of AI. $P < 0.05$ was considered statistically significant, and all statistical tests were 2-sided. All analyses were conducted using STATA software (Version 9.0, STATA Inc.).

Results

A total of 126 familial ovarian cancer cases and 118 cancer-free relatives were included in the analysis (Table 1). In the case group, the median age at cancer diagnosis was 48 (ranging from 25 to 78). All of them were noncarriers of *BRCA1/2* or *MLH1/MSH2* mutations. In the control group, the median age at enrollment in GRFOCR was 52 (ranging from 23 to 93). All of the cases and controls were White women. To evaluate the AI of *BRCA1* and *BRCA2* gene expression, genotype analysis of the 2 common polymorphisms *BRCA1*-c.4308T/C and *BRCA2*-c.3396A/G was carried out on DNA samples isolated from cases and controls. From these analyses, 60 (47.6%) and 53 (44.9%) of the familial ovarian cancer patient and cancer-free relative samples, respectively, evaluated were determined to be heterozygous for the *BRCA1*-c.4308T/C polymorphism. Fifty-eight (46.0%) and 51 (43.2%) of these samples were found to be heterozygous for the *BRCA2*-c.3396A/G polymorphism. Next, RNA was isolated from individuals who were determined to be heterozygotes for the *BRCA1*-c.4308T/C ($n = 113$) and *BRCA2*-c.3396A/G ($n = 109$) gene.

To evaluate the AI, we used the absolute values of $\log_2(\text{BRCA1-c.4308T/C})$ or $\log_2(\text{BRCA2-c.3396A/c.3396G})$. The mean value of c.4308T/C allelic ratio of *BRCA1* in the LCLs from familial ovarian cancer carriers was found to be significantly higher than that in the LCLs from cancer-free relative controls [1.600 ± 0.189 ($n = 60$) vs. 1.501 ± 0.081 ($n = 53$), $P = 0.0007$; Table 2]. Using log transformation, the mean value of $\log_2(\text{c.4308T/$

Table 2. Allelic imbalance in *BRCA1* and *BRCA2* expression in cases and controls

Genes	Status	Sample size	AI (mean \pm SD)	<i>P</i> value	$\log_2(\text{AI})$ (mean \pm SD)	<i>P</i> value
<i>BRCA1</i>	Cases	60	1.600 \pm 0.189	0.0007	0.463 \pm 0.054	0.0007
	Controls	53	1.501 \pm 0.081		0.405 \pm 0.111	
<i>BRCA2</i>	Cases	58	1.395 \pm 0.432	0.783	0.325 \pm 0.124	0.328
	Controls	51	1.369 \pm 0.548		0.302 \pm 0.118	

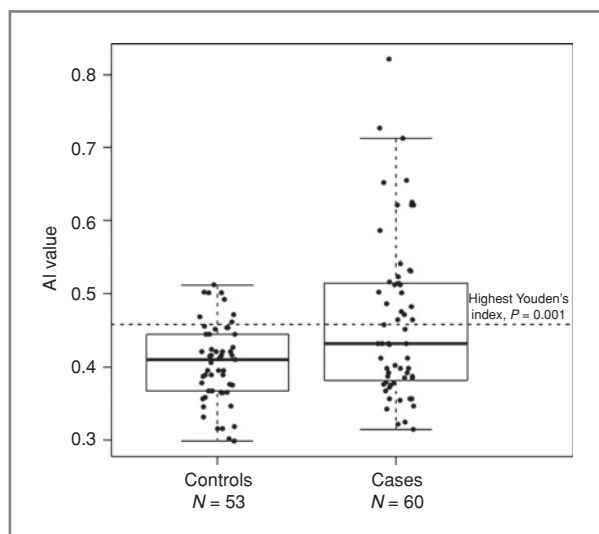


Figure 1. *BRCA1* AI distribution in 60 familial ovarian cancer cases and 53 controls. The AI cutoff value of 0.458, chosen to categorize the cases, is indicated together with its associated *P* value obtained from comparing the proportions of cases (27/60) and controls (8/53) above the indicated value.

C) of *BRCA1* in the LCLs from familial ovarian cancer carriers was found to be significantly higher than that in the LCLs from cancer-free relative controls [0.465 ± 0.054 ($n = 60$) vs. 0.405 ± 0.111 ($n = 53$), $P = 0.0007$; Table 2]. In comparison, the mean value of $\log_2(BRCA2\text{-}c.3396A/G)$ was not significantly different between the LCLs from familial ovarian cancer patients and ones from cancer-free relative controls [0.325 ± 0.124 ($n = 58$) vs. 0.302 ± 0.118 ($n = 51$), $P = 0.328$; Table 2].

To further analyze the effects of AI of *BRCA1* on familial ovarian cancer, we applied ROC analysis, which estimates the sensitivity and specificity of the cutoff point, to define a cutoff point. As shown in the Figure 1, the value of 0.458 maximizes both characteristics, providing the highest Youden's index. Although there is no overall need to define a firm cutoff point at this stage because it is not possible to determine whether the degree of predisposition to familial ovarian cancer is proportional to the degree of AI of *BRCA1*, we still used the value of 0.458 to categorize familial ovarian cancer cases and controls

Table 3. Allelic imbalance in *BRCA1* and risk of familial ovarian cancer

<i>BRCA1</i> ^a	Number of cases	Number of controls	OR (95% CI)
<0.458	33	45	Reference
≥0.458	27	8	4.22 (1.60–11.16)

^a0.458 was used as the cutoff point.

into AI and non-AI as an exploratory analysis. Using our definition, AI occurred in 27 of 60 (45%) of informative ovarian cancer cases and 8 of 53 (15%) of informative cancer-free relative controls. Thus, AI of *BRCA1* was associated with 4.22-fold increased risk of familial ovarian cancer (95% CI: 1.60–11.16; Table 3).

In further analysis, we found that the distribution of underexpressed alleles of *BRCA1* was significantly different between cancer-free relative control and familial ovarian patients (Table 4). As shown in Table 4, underexpressed *BRCA1*-c.4308T [i.e., $\log_2(4308T/C) < 0$] and *BRCA1*-c.4308C [i.e., $\log_2(4308T/C) > 0$] alleles were found in 47% (25/53) and 53% (28/53) of cancer-free controls as compared with 25% (15/60) and 75% (45/60) of familial ovarian cancer carriers, respectively ($P = 0.014$). Intriguingly, all the individuals who were defined as having AI had underexpressed *BRCA1*-c.4308C allele. In contrast, underexpressed *BRCA2*-c.3396A [i.e., $\log_2(3396A/G) < 0$] and *BRCA2*-c.3396G [i.e., $\log_2(3396A/G) > 0$] alleles were found in 49% (25/51) and 51% (26/51) of cancer-free relative controls as compared with 45% (26/58) and 55% (32/58) of familial ovarian cancer carriers ($P = 0.66$; Table 4).

To further explore the biological significance of AI in *BRCA1*, we assessed the correlation between AI of *BRCA1* and *BRCA1* mRNA expression in LCLs from both informative cases and controls. We observed a significant reverse correlation between levels of AI in *BRCA1* and *BRCA1* overall mRNA expression in both cases and controls (correlation coefficient $\rho = -0.432$, $P = 0.026$ for cases and $\rho = -0.472$, $P = 0.022$ for controls). High levels of AI in *BRCA1* were linked to the low overall expression of *BRCA1* mRNA.

Table 4. Distribution of underexpressed alleles of *BRCA1* and *BRCA2*

Gene	Group	Underexpressed alleles	<i>P</i>
<i>BRCA1</i>	Cases	c.4308T, $\log_2(4308T/C) < 0$	0.014
	Controls	c.4308C, $\log_2(4308T/C) > 0$	
<i>BRCA2</i>	Cases	c.3396A, $\log_2(3396A/G) < 0$	0.66
	Controls	c.3396G, $\log_2(3396A/G) > 0$	

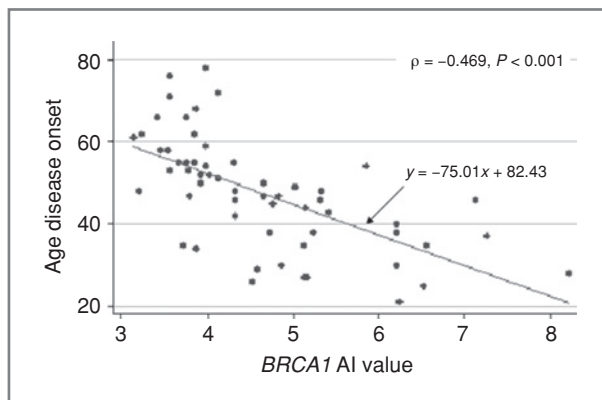


Figure 2. Correlation between *BRCA1* AI and age of ovarian cancer onset. Linear regression between *BRCA1* AI and age of disease onset: y (age of disease onset) = $-75.01 \times x$ (*BRCA1* AI value) + 82.43.

Because inherited cancers are more likely to be diagnosed at a younger age, we examined the relationships between AI of *BRCA1* and age of familial ovarian cancer diagnosis (Fig. 2). Significant trends of decreasing age at cancer diagnosis with increasing levels of AI in *BRCA1* were observed in familial ovarian cancer patients. ($\rho = -0.469$, $P < 0.001$). In comparison to the age of disease onset between AI and non-AI groups, the median age of disease onset in the AI group was significantly earlier than that in the non-AI group (39 vs. 55, $P < 0.001$), indicating AI of *BRCA1* is associated with early age of familial ovarian cancer onset. Then, we looked at the relationship between the levels of AI in *BRCA1* and age of disease onset in the AI group and the non-AI group. A significant trend of decreasing age at cancer diagnosis with increasing levels of AI in *BRCA1* was observed in the AI group ($\rho = -0.421$, $P = 0.015$). A similar trend was also observed in the non-AI group, but the correlation did not reach statistical significance ($\rho = -0.322$, $P = 0.101$).

Discussion

In this study, we applied a TaqMan-based quantitative AI assay to compare the levels of AI in *BRCA1* and *BRCA2* genes between patients with familial ovarian cancer and cancer-free relative controls. The AI ratios of *BRCA1* in familial ovarian cancer cases were significantly higher than those from cancer-free relative controls ($P = 0.0007$). In contrast, the AI ratios of *BRCA2* were not significantly different between familial ovarian cancer cases and cancer-free controls ($P = 0.328$). The distribution of underexpressed alleles between cancer-free controls and familial cases was significantly different for *BRCA1* gene expression but not for *BRCA2* gene expression ($P < 0.014$ for *BRCA1* and $P = 0.66$ for *BRCA2*). Using a cutoff point of *BRCA1* AI (0.458) obtained from ROC analysis, higher levels of AI in *BRCA1* were associated with a 4.22-fold increased risk of familial ovarian cancer. Furthermore, we have

observed a significant trend of decreasing age at cancer diagnosis with increasing levels of AI in *BRCA1* ($P < 0.001$).

Our study is consistent with a previous report in breast cancer by Chen et al. (9). In their study, they found that the AI ratios of *BRCA1* in the lymphocytes from familial breast cancer patients were significantly increased as compared with cancer-free women (0.424 vs. 0.211, $P = 0.00001$). Similarly, the AI ratios were greater for *BRCA1* and *BRCA2* in the lymphocytes of nonfamilial breast cancer cases versus controls (*BRCA1*: 0.353 vs. 0.211, $P = 0.002$; *BRCA2*: 0.267 versus 0.211, $P = 0.03$). Thus, AI affecting *BRCA1/2* may contribute to both familial and nonfamilial forms of breast cancer. Interestingly, our results showed the similar difference of AI ratio in *BRCA1* between familial cases and controls but at a less degree (0.463 vs. 0.405). This might be due to the difference between control populations, unrelated cancer-free women in their study versus cancer-free family relatives in our study. Because cancer-free family relatives are genetically close to familial cancer cases, they theoretically will share more genetic risk factors than the unrelated cancer-free women. One of the genetic risk factors might be AI of *BRCA1*. This is probably the reason why the levels of *BRCA1* AI is much higher in our controls than in their controls (0.405 vs. 0.211) and the levels of *BRCA1* AI are very similar in our cases and their cases (0.463 vs. 0.424). Recently, the AI of another tumor suppressor gene, *APC* gene, was reported in adenomatous polyposis families (10). Castellsagué et al. reported that the germline AI of *APC* was observed in the *APC* mutation-positive and -negative families. Thus, AI of tumor suppressor genes might represent a novel genetic mechanism that contributes to human cancer development.

So far, the underlying genetic mechanism of AI of *BRCA1/2* is not clear. Because all of the ovarian cancer cases in this study are noncarriers of known *BRCA1/2* and *MLH/MSH* mutations, NMD is not likely to be responsible for the observed AI in our case-control comparisons. In addition, the AI of *BRCA1/2* also occurs in noncancer relative controls. Therefore, other mechanisms are likely to exist to account for the observed AI of *BRCA1/2* in both familial ovarian cancer cases and noncancer relative controls and the increased AI of *BRCA1* gene expression in female ovarian cancer cases. Both *BRCA1*-c.4308T/C (rs1060915) and *BRCA2*-c.3396A/G (rs1801406) are synonymous SNPs, which are unlikely to affect *BRCA1/2* expression. In a recent retrospective study, Dombernowsky et al. (11) show that common SNPs in the *BRCA1/2* coding regions are not associated with risks of breast and ovarian cancers, suggesting that the functional SNPs might be in other regions such as the promoter regulatory regions, 5'UTR, and 3'UTR. Recent studies have shown that regulatory SNPs might be able to explain more than 50% of the population variation in allelic specific gene expression such as AI (12). In the case of *BRCA1/2*, the 5' and 3' noncoding regions of *BRCA1*

and *BRCA2* are rarely evaluated through genetic testing, even though genetic alterations in these noncoding regions could be important in regulating *BRCA1/2* expression. For instance, several studies have shown that large genomic deletions involving the *BRCA1* promoter were associated with hereditary breast/ovarian cancers (13–15). The deletions lead to the disruption of the binding of transcription factors to DNA regulatory elements and hence lead to the loss of allelic gene expression. In addition, microRNAs (miRNA) might regulate *BRCA1/2* expression by binding the 5'UTR, 3'UTR, and coding regions (9). Several rare SNPs have been found at miRNA binding sites at the 3'UTR region of *BRCA1/2*, although the functional significance of these SNPs is unknown. Interestingly, Nicoloso et al. found that a codon SNP (rs799917) in *BRCA1* gene, which is predicted to be a binding site for miR-638, was associated with increased risk of breast cancer (16). Also, allele-specific hypermethylation of the *BRCA1* promoter region and decreased *BRCA1* expression is associated with 5% of the sporadic breast and ovarian cancer cases (17, 18). The assessment of the status of the *BRCA1* promoter hypermethylation in the familial ovarian cancer patients used in this study is ongoing right now. It will be important in future studies to determine the underlying mechanisms of AI of *BRCA1/2* and subsequently understand the roles of AI of *BRCA1* in the development of familial ovarian cancer.

There might be a concern in using LCLs, instead of primary peripheral blood lymphocytes, to study the AI of *BRCA1/2* because EBV immortalization of lymphocytes might change the overall gene expression profile. Unlike LCLs, the gene expression in the lymphocytes might be varied by the environment and dietary factors and can mask the real genetic effects. In our study in breast cancer, we compared the levels of AI in 6 genes (including *BRCA1/2*) between 30 pairs of LCLs and matched lymphocytes. The results were highly concordant between LCLs and the lymphocytes ($\rho = 0.784$ and 0.821 for *BRCA1* and *BRCA2*, respectively; data not shown). In

addition, several reports have shown that the gene expression patterns are similar in LCLs and lymphocytes (19–21).

One limitation of this study is that the true frequency of AI in cases and controls cannot be precisely assessed at present because not all individuals are informative (heterozygous for a transcribed SNP). The accuracy of our PCR-based method to quantify AI is also limited. Quantitative reverse transcription PCR is invariably affected by noise from the nonspecific binding of RNA to the probes. Therefore, in the near future, high-throughput technologies, such as RNA sequencing, are urgently needed to capture more individuals and determine gene expression more accurately. In addition, we will expand our analysis to other known high penetrance genes, such as *MLH1/MSH*. Nevertheless, this is the first report showing the relationship between AI of *BRCA1*, risk of familial ovarian cancer, and age of onset of familial ovarian cancer. The data should be interpreted with caution because of the small sample size and undetermined genetic mechanisms. Future large studies and advanced technologies are warranted to confirm our findings. In brief, our findings underscore the significance of AI of *BRCA1* in familial ovarian cancer and warrant further investigations on the association of AI with human diseases.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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