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Association between genetic variants of EGF-containing fibulin-like extracellular matrix protein1 gene and sporadic breast cancer in a Chinese Han population

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Summary

Purpose of investigation: Genetic susceptibility of breast cancer has been shown to be modulated by inheritance of polymorphic genes. EGF-containing fibulin-like extracellular matrix protein1 (*EFEMP1)* gene played an important role in many tumors, including lung cancer, hepatic carcinoma, and prostate cancer. In addition, it was importantly downexpressed in breast cancer. The present research aimed to assess the association between genetic variations of *EFEMP1* and breast cancer risk. *Materials and Methods:* The authors genotyped 11 common tagging SNPs with an array platform including 960 cases and 972 cancer-free controls of Chinese women, according to the HapMap database based on the pairwise linkage disequilibrium (LD) r^2 threshold of 0.8, minor allele frequency of 0.05. *Results:* Three SNPs were significant associated with breast cancer (rs3791679, p=0.016, OR=1.21, 95%CI=1.04-1.41; rs1346786, p=0.005, OR=1.31, 95%CI=1.08 -1.59; rs727878, p=0.002, OR=1.29, 95%CI = 1.10-1.51). Multivariate logistic regression analysis revealed that, compared with wild-type carriers in a dominant model, a significantly increased breast cancer risk was associated with the three identified risk SNPs. Among the selected tagging SNPs, three haplotype blocks were identified, and the results of haplotype analysis were consistent with the single-locus analysis. The haplotype 'GG' in block 1 and haplotype 'AG' in block 2 were significantly associated with breast cancer, and had a 54% and 28% increased breast cancer risk respectively, compared with their corresponding noncarriers. *Conclusions:* The present results suggested that the polymorphisms of *EFEMP1* gene were associated with breast cancer and might contribute to the susceptibility of the progression of breast cancer in Chinese Han women. Individuals with the risk alleles might increase the risk of breast cancer.

Key worlds: EFEMP1 gene; Single nucleotide polymorphism; Genetic susceptibility; Molecular biology; Breast cancer.

Introduction

Currently, breast cancer remains to be a major contributor to overall morbidity and mortality among women worldwide, accounting for 23% of all cancers in women in 2008 [1]. The etiology of breast cancer is considered a multifactorial disease with a combined effect of genetic and environmental. In familial linkage and twin studies, several high-penetrance low-frequency mutations in genes confer increased susceptibility to breast cancer, including BRCA1, BRCA2, etc.; However, these causative mutations explain only approximately 25% of the familial risk and almost 5% of breast cancer incidence [2, 3]. Therefore, low-penetrance high- frequency genes or loci might have significant associations with breast cancer risk and might contribute to the remaining 75% of the risk. Then, understanding the genetic etiology of breast cancer may help to reveal the mechanism of breast cancer and provide new insight for the diagnosis and treatment. The genes that are relative with the angiogenesis and the formation and development of cancers should be considered.

Fibulins are a seven-member family of secreted glycoproteins, which are characterized by repeated epidermal growth-factor-like domains and a unique C-terminal structure [4]. Studies show different members of the fibulin family have different functions, either tumor-suppressive or oncogenic activity [5]. *EFEMP1* gene is located in chromosome 2 and encodes a member of the fibulin family of extracellular matrix glycoproteins. The gene may play a role in the nature of many malignant tumors and interacts with its partners and modulates their functions [4, 5]. To unravel the role of *EFEMP1*, many researches have been done and most of the results supported a tumor-suppressive role. Albig *et al.* found that *EFEMP1* had an anti-angiogenic function via suppression of endothelial cells' sprouting [6] and down-expression of *EFEMP1* gene or *EFEMP1* promoter methylation occurred in many cancers, including glioma, lung cancer, hepatic carcinoma, prostate cancer, and nasopharyngeal carcinomas [7-13]. However, on the contrary, the gene was overexpressed in the pancreatic adenocarcinoma [14, 15]. Furthermore, *EFEMP1* expression was proved to be correlated with the prognosis, downexpression was correlated with a worse prognosis in hepatocellular and nasopharyngeal carcinoma, and with a better prognosis in cervical cancer [9, 11, 12]. Importantly, Sadr-

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Nabavi *et al.* found *EFEMP1* gene was downexpressed in breast cancer [13]. Overall, all the studies of *EFEMP1* function may help elucidate the molecular mechanisms and find a potential target for cancer therapy, especially for breast cancer. Nevertheless, fewer studies have examined the susceptibility of *EFEMP1* gene with diseases, and no association studies between *EFEMP1* and breast cancer have been published to date. Therefore, to assess the association between genetic variations of *EFEMP1* and breast cancer risk, the present authors performed a hospital-based case-control study in a Han population of Chinese women.

Materials and Methods

Study population

The research consisted of a total of 972 Chinese women with breast cancer and 960 healthy controls. The patients diagnosed with breast cancer were from The Fifth People's Hospital of Shanghai between June 2009 and October 2013. Breast cancer was diagnosed from surgical and pathological symptoms. The control subjects were from annual check-up visitors at the same hospital during the similar time period and frequency matched to cases by age (± five years) and residence (urban or rural areas). The controls with a self-reported history of cancer or autoimmune disorders were excluded. Samples were obtained from subjects after they had provided written informed consent.

The authors collected five ml peripheral venous blood sample from each subject for DNA extraction and genotyping. The estrogen receptor (ER) and progesterone receptor (PR) status of breast cancers was determined by immunohistochemistry examinations available in the medical records of the hospitals.

Selection of tagging SNPs and genotyping assays

Eleven tagging SNPs in this region were selected based on the date of Chinese Han population (CHB) in HapMap Phase 3 data with the criteria of minor allele frequency³0.05 and r²³0.80. A total of 11 tagging SNPs in this region were selected for this study which met the above criteria.

Genomic DNA was extracted from five ml frozen whole blood using a DNA extraction kit, according to the manufacturer's protocol. Fragments containing polymorphisms were amplified by the PCR and genotyped with an array platform using allele-specific matrix-assisted laser desorption ionization–time-of-flight mass spectrometry assay [16]. Primers for amplification and extension reactions were designed using an assay design version 3.1 software. The authors examined genotyping quality by means of a detailed quality control procedure that ensured over 95% successful call rate with duplicate calling of genotypes, and subsequent Hardy-Weinberg equilibrium testing.

Statistical analysis

Statistical analysis system (SAS) software was used to perform the statistical analysis. The differences of demographic characteristics and alleles frequencies between cases and controls were calculated by c^2 (for categorical variables) or the Student *t*-test (for continuous variables). The c^2 test also was used to assess *p*-value of HWE in each group. Unconditional logistic regression analysis was performed to calculate ORs and 95% CIs as estimates of the relative risk. SNPs were excluded if they were out of HWE ($p < 0.01$) and had a minor allele frequency of less than 5%. All *p*-values presented were two-sided test, and the level of $p < 0.05$ was considered significant. The authors used Haploview 3.2 software to reconstruct haplotypes and estimate haplotype frequencies in the unrelated cases and controls. In order to obtain a measure of significance corrected for multiple testing bias, the authors ran 10,000 permutations to compute *p*-values using the Haploview program.

Table 1. — *Demographic and clinical date of the studied subjects.*

Variables	No. of cases N=960	%	No. of controls N=972	%	*p* value
Age, years (mean ± SD)	53.24±13.2		51.02±10.24		0.54
Age					
> 45	524	54.6	510	52.5	0.36
≤ 45	436	45.4	462	47.5	
Age at menarche, years (mean ± SD)	15.25±2.12		15.96±1.94		**0.0031**
Menopausal status					
Premenopausal	450	46.9	471	48.5	0.50
Postmenopausal	510	53.1	501	51.5	
Estrogen receptor (ER) ‡					
Positive	473	49.3			
Negative	388	40.4			
Progesterone receptor (PR) ‡					
Positive	460	48.0			
Negative	396	38.4			
Axillary lymph node metastasis§					
Positive	442	48.9			
Negative	461	48,0			

* Two-sided χ^2 test. † Independent-samples *t*-test.
‡Information on ER and PR was available in 862 and 856 cases, respectively.
§Information on axillary lymph node metastasis status was available in 903 breast cancer cases.

Results

Demographic and clinical data of the studied subjects

The distribution of demographic characteristics of the 960 cases and 972 controls are presented in Table 1. Because participants were frequency-matched for sex and residential area under the present study design, the distribution of age (age at diagnosis for cases and age at recruitment for controls) was matched between cases and controls. The mean ± SD of age was 53.24 ± 13.2 for cases and 51.02 ± 10.24 for controls. Early age at menarche was a risk factor for breast cancer ($p = 0.003$), 15.25 ± 2.12 for cases and 15.96 ± 1.94 for controls. Of the 960 cases, the authors obtained information about ER and PR status for 862 cases and axillary lymph node metastasis status for 903 cases from their medical records. The cutoff level used to define positive ER/PR status was more than 10% positive staining of the immunohistochemical assay.

Single-SNP analysis

Eleven common tagging SNPs of *EFEMP1* gene were genotyped, which could cover all of the gene. Table 2 shows the information of the 11 tagging SNPs. The geno-

Table 2. — *Information of eleven genotyped SNPs in* EFEMP1 *gene.*

Gene name, OMIM no.*, and locus	SNP ID	Chromosome position	SNP location†	Base change	Minor allele frequency‡ Database	Control	Case	*p*-value for §	*p*-value for χ^2	OR (95%CI)
	rs3791679	55950396	Intron10	G>A	0.207	0.199	0.231	0.72	**0.016**	1.21 (1.04-1.41)
	rs17047290	55959438	Intron5	A>G	0.122	0.120	0.127	0.54	0.508	1.07 (0.88-1.30)
	rs1346786	55961837	Intron5	A>G	0.122	0.112	0.142	0.76	**0.005**	1.31 (1.08-1.59)
EFEMP1,	rs3791675	55964813	Intron4	A>G	0.220	0.214	0.232	0.63	0.179	1.00 (0.95-1.29)
OMIM:601548,	rs10865291	55971550	Intron4	A>G	0.195	0.201	0.186	0.12	0.238	0.91 (0.77-1.07)
and located in	rs727878	55973161	Intron4	A>G	0.183	0.174	0.213	0.39	**0.002**	1.29 (1.10-1.51)
chromosome 2	rs1344733	55981531	Intron4	A>G	0.329	0.335	0.321	0.57	0.354	0.94 (0.82-1.07)
	rs3791661	55983374	Intron4	A>C	0.411	0.374	0.380	0.31	0.699	1.03 (0.90-1.17)
	rs3791660	55983384	Intron4	C>A	0.061	0.059	0.067	0.49	0.308	1.15 (0.88-1.49)
	rs1430195	55984144	Intron4	A>G	0.341	0.357	0.342	0.96	0.328	0.94 (0.82-1.07)
	rs7559906	55986544	Intron4	A>G	0.098	0.092	0.103	0.43	0.247	1.13 (0.92-1.40)

Abbreviations: *EFEMP1*=EGF-containing fibulin-like extracellular matrix protein1: SNP=single nucleotide polymorphism; HWE=Hardy-Weinberg equilibrium. The significant differences are indicated in **bold**.
* OMIM=Online Mendelian Inheritance in Man (http://www.ncbi.nlm.nih.gov/omim/?term=EFEMP1).
† SNP position in National Center for Biotechnology Information Genome build 37.3 (http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?).
‡ Minor allele frequency from dbSNP databases. § HWE P-value in control group.

Table 3. — *Genotype frequencies of three SNPs between cases and controls and their associations with breast cancer risk.*

SNP ID	Genotype	No. of cases	%	No. of controls	%	*p*-value for χ^2 test	Logistic regression OR (95%CI)	*p*-value
rs3791679	GG	656	83.96	702	87.65	**0.010**	1.00 (reference)	
	AG	252	15.83	216	11.90		**1.39 (1.07-1.80)**	**0.014**
	AA	50	0.21	54	0.41		0.53 (0.10-2.90)	0.462
Dominant	AA/AC+CC	154	16.04	120	12.31	**0.022**	**1.36 (1.05-1.76)**	**0.020**
rs1346786	AA	813	84.69	866	89.09	**3.36E-04**	1.00 (reference)	
	AC	146	15.21	102	10.49		**1.53 (1.16-2.00)**	**0.002**
	CC	1	0.10	4	0.41		0.266 (0.03-2.39)	0.237
Dominant	AA/AC+CC	147	15.31	106	10.91	**0.005**	**1.48 (1.13-1.93)**	**0.004**
rs727878	AA	827	86.15	844	88.83	0.779	1.00 (reference)	
	AC	131	13.65	124	12.76		1.08 (0.83-1.40)	0.576
	CC	2	0.21	4	0.41		0.51 (0.09-2.79)	0.438
Dominant	AA/AC+CC	133	13.86	128	13.17	0.690	1.06 (0.82-1.38)	0.659

Abbreviations: SNP=single nucleotide polymorphism; OR=odds ratio; CI=confidence interval. The significant differences are indicated in **bold**.
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Table 4. — *The frequency of haplotypes in each block of* EFEMP1 *gene, and association with breast cancer risk.*

Haplotype*	Overall Case (%)	Control (%)	*p*-value for χ^2 test	Logistic regression OR (95%CI) ‡	*p*-value	Global score test†
Block1			**0.025**			χ^2=9.732, df = 3,
AG	730 (0.761)	781 (0.803)		1.00 (reference)		**p-val = 0.026**
AA	130 (0.135)	118 (0.122)		1.18 (0.90-1.54)	0.231	**sim[b]=0.022**
GG	92 (0.096)	64 (0.067)		**1.54 (1.10-2.15)**	**0.012**	
Block2			**0.032**			χ^2=11.714, df = 3,
AA	531 (0.554)	586 (0.603)		1.00 (reference)		**p-val = 0.011**
AG	321 (0.334)	276 (0.284)		**1.28 (1.05-1.57)**	**0.013**	**sin[b]= 0.009**
GG	87 (0.091)	101 (0.105)		0.95(0.70-1.30)	0.749	
Block3			0.428			
AC	705 (0.734)	696 (0.716)		1.00 (reference)		χ^2=4.435, df = 3,
AA	196 (0.204)	217 (0.223)		0.90 (0.72-1.11)	0.306	p-val = 0.462
CC	58 (0.060)	50 (0.051)		1.15 (0.77-1.70)	0.498	sim[b]= 0.413

Abbreviations: OR=odds ratio; CI=confidence interval.
*Polymorphic bases are in 3'-5' order listed in Table 1. Loci chosen for block 1: SNP1-2; Loci chosen for block 2: SNP4-5; Loci chosen for block 3: SNP10-11.
† Generated by permutation test with 10,000 times simulation. The significant differences are indicated in **bold**.

Table 5. — *The frequency of diplotypes in each block of EFEMP1 gene and association with breast cancer risk.*

Haplotype *	Overall Case (%)	Control (%)	*p*-value for χ^2 test	Logistic regression OR(95%CI) ‡	*p*-value
Block1	0.770				
GG,GA	618 (64.38)	631 (64.92)		1.00 (reference)	
AG,GA	199 (20.73)	216 (22.22)		0.94 (0.75-1.18)	0.590
AA,GA	122 (12.71)	118 (12.14)		1.06 (0.80-1.39)	0.701
Block2			**0.004**		
AA,AA	562 (58.54)	572 (58.85)		1.00 (reference)	
AA,AG	187 (19.48)	135 (13.89)		**1.41 (1.10-1.81)**	**0.007**
AA,GG	190 (19.80)	224 (23.05)		0.86 (0.69-1.08)	0.202
Block3			0.527		
AA,AA	714 (74.38)	745 (76.65)		1.00 (reference)	
GG,AA	206 (21.46)	224 (23.05)		0.96 (0.77-1.19)	0.707
GG,GG	25 (2.610)	19 (1.95)		1.37 (0.75-3.50)	0.305

Abbreviations: OR=odds ratio; CI=confidence interval. The significant differences are indicated in **bold**.
*Polymorphic bases are in 3'-5' order listed in Table 1. Loci chosen for block 1: SNP1-2; Loci chosen for block 2: SNP4-5; Loci chosen for block 3: SNP8-9.

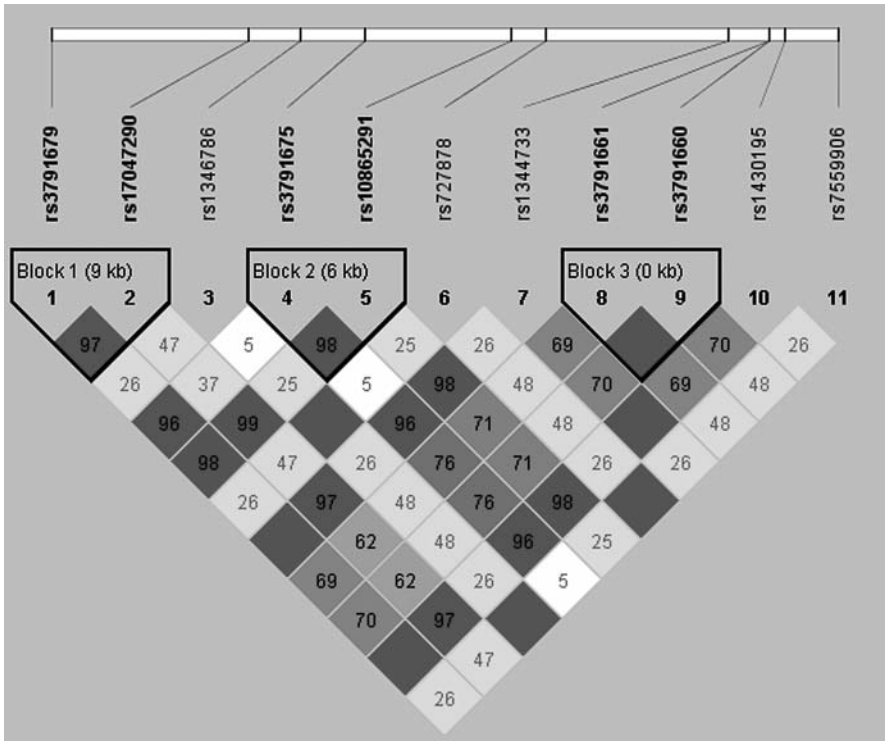

Figure 1. — Graphical representation of the SNP locations and LD structure of *EFEMP1* gene. The SNP distribution and haplotype block in *EFEMP1* gene are shown, respectively. The exact SNP positions are listed in Table 2. Haplotype blocks (colored) were defined according to the criteria laid out by the Haploview software. The rs number (top; from left to right) corresponds to the SNP name and the numbers in squares are the r^2 values.

typing rate of eleven SNPs was more than 95% and all SNPs were in Hardy-Weinberg equilibrium in control subjects. Allele frequencies of three SNPs showed a significant difference between the cases and controls (rs3791679, p = 0.016, OR = 1.21, 95%CI = 1.04-1.41; rs1346786, p = 0.005, OR = 1.31, 95%CI = 1.08-1.59; rs727878, p = 0.002, OR = 1.29, 95%CI = 1.10-1.51). Multivariate logistic regression analysis revealed that, compared with wild-type carriers in a dominant model, two identified risk SNPs (rs3791679, p = 0.008, OR = 1.29, 95%CI = 1.07-1.55; rs1346786, p = 0.006, OR = 1.35, 95%CI = 1.09-1.67) showed a significantly increased breast cancer risk, while rs727878 was not obvious. The information is shown in Table 3.

Haplotype analysis

Haplotype and diplotype with frequencies ³5% are shown in Tables 4 and 5. The LD plot of eleven SNPs in 972 controls is shown in Figure 1, three blocks were defined (rs37916789 was located in block1, rs1346786 was in block2). The logistic regression analysis revealed that the risk of breast cancer was significantly increased among in-

dividuals carrying the haplotype 'GG' (OR = 1.54, p = 0.012, 95%CI = 1.10-2.15) and 'AG' (OR = 1.28, p = 0.013, 95%CI = 1.05-1.57), and had a 54% and 28% increased breast cancer risk respectively, compared with the most common haplotype 'AG' in block 1 and 'AA' in block 2. And the variant genotypes exhibited a statistically significance increased risk of breast cancer individually even after 10,000 time permutation test. Furthermore, the diplotype 'AA, AG' (OR = 1.41, p = 0.007, 95%CI = 1.10-1.81) had a 41% increased breast cancer risk, compared with the most common diplotype 'AA, AG' in block 2. The results of haplotype and diplotype were consistent with single-locus analysis.

Discussion

The determination of genetic polymorphisms is a new means to study the etiology of polygenetic disorders with complex inheritance patterns, such as cancer, diabetes, and hypertension [17]. In this case-control study of breast cancer in Chinese women, the authors investigated the role of multiple common variants of *EFEMP1* and their association with the risk of breast cancer. They found that three SNPs (rs3791679, p = 0.016; rs1346786, p = 0.005; rs727878, p = 0.002) of the 11 selected SNPs in *EFEMP1* showed a significant association with breast cancer. In addition, the results of haplotype and diplotype of the three identified SNPs with breast cancer risk were consistent with the single-SNP analysis, which was the significant finding in this study. Haplotype analysis revealed the haplotype 'GG' (OR = 1.54, p = 0.012) in block 1 carrying risk allele A of rs3791679, and the haplotype 'AG' (adjusted OR = 1.65, p = 0.0004) in block 2 carrying risk allele G of rs1346787 were associated with breast cancer, and had a 54% and 28% increased breast cancer risk, respectively. These results supported the present authors' hypothesis that *EFEMP1* polymorphisms were associated with the risk of breast cancer and provided evidence that common SNPs in *EFEMP1* may be used as candidate biomarkers for breast cancer susceptibility in the Han population of Chinese women.

EGF-containing fibulin-like extracellular matrix protein1, officially namely *EFEMP1,* is a member of the fibulin families. Fibulins are a seven-member family of secreted glycoproteins and are characterized by repeated epidermal growth-factor-like domains and a unique C-terminal structure, glycoproteins constitute a complex network structure, support and connect the organizational structure, regulate tissue and cell physiological activity, and then may play a role in the formation and development of cancers [4]. Previous studies found different members of the fibulin family showed different functions, either tumor-suppressive or oncogenic activity [4, 5]. Up to now, to explore the role of *EFEMP1* in cancer biology, many studies have been done; *EFEMP1* was demonstrated performing as either a tumor-suppressive or oncogenic activity factor. In support of a possible tumor-suppression role, Albig *et al.* found that *EFEMP1* had an anti-angiogenic function via suppression of endothelial cells' sprouting [6]. The overexpression of EFEMP1 could inhibit tumorigenicity of fibrosarcoma cells. In addition, in glioma, lung cancer, hepatic carcinoma, prostate cancer, and nasopharyngeal carcinomas, *EFEMP1* was low-expressed or *EFEMP1* promoter was methylated [7-12]. On the contrary, a potential cancer-promoting function of EFEMP1 was implied in the Seeliger *et al.* study. *EFEMP1* overexpression was found to promote xenograft formation in pancreatic adenocarcinoma [14, 15]. In addition, down- expression of *EFEMP1* in hepatocellular and nasopharyngeal carcinoma was correlated with a worse prognosis [11, 12]. De Vega *et al.* demonstrated that the overexpression of *EFEMP1* was correlated to the poor prognosis in cervical cancer [4]; these findings suggested that the gene expression may be further diminished or enhanced in different cancers.

In breast cancer, Sadr-Nabavi *et al.* found *EFEMP1* gene was downexpressed and promoter methylation was demonstrated as the major cause of this downregulation by bisulphite genomic sequencing in breast cancer cell lines and primary breast cancer tissues [13]. Overall, through reviewing previous studies and the present research about *EFEMP1* in cancers, considering that *EFEMP1*'s function either promoted or inhibited cancer growth, which may be dependent on a molecular context that differs by cancer cell type and malignancy stage, the authors can conclude that *EFEMP1* gene plays an important role in breast cancer.

Introns in eukaryotes fulfill a broad spectrum of functions, such as functioning as transposable elements, and are involved in virtually every step of mRNA processing [18, 19]. In the present study, three positive SNPs were located in the intron region and might perform certain function, ultimately affecting the expression level of *EFEMP1*. Rs3791679 was located in the Intron10, which was originally discovered in individuals of European ancestry and associated with adult height [20-23]. Rs1346786 and rs727878 were located in the Intron5 and Intron4, respectively. No functional relevance of the two SNPs was reported until now; this might possibly increase the affinity of transcription activators or decrease that of transcription suppressors to the intron enhancer, thus upregulating the expression levels of *EFEMP1*. Actually, to date, the majority of the positive SNPs that were found in Genome Wide Association Studies or other association studies were located in intron regions, which meant intron played an important role in all genome and further mechanism researches of introns should be considered.

Finally, several limitations must be noted in the present research. First, to some extent, due to hospital-based controls, selection bias cannot be ruled out, although the authors recruited the samples by matching the controls to the cases based on age, sex, and residential area (urban or

rural). Second, the information on other factors such as occupational exposure and certain dietary components was not available in this research, which might interact with *EFEMP1* genotypes or act as potential confounding factors. At the same time, the present study also had several strengths. The sample size (960 breast cancer patients and 972 controls) is relatively large among breast cancer association studies published to date, and all samples were from homogeneous populations of the same ethnicity. In addition, 11 SNPs in *EFEMP1*, which captured the entire gene, were examined in this study and could represent all the genetic variability of the *EFEMP1* gene.

In conclusion, the present research provided evidence that genetic variations of *EFEMP1* were associated with the risk of breast cancer in a Han population of Chinese women. Considering that the limited sample size may produce relative risk estimates with bias, extended analyses with larger sample size should be carried out from different ethnic origins to further verify this association. In addition, both variants and genes should also be considered in further studies to elucidate the molecular basis of the relationship between *EFEMP1* and breast cancer susceptibility.

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