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Original Article

Single nucleotide polymorphisms of let-7-related genes increase susceptibility to breast cancer

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Abstract: Background: Let-7 is a microRNA (miRNA) that targets the β2 adrenergic receptor (*ADRB2*), hypoxia inducible factor 1 subunit alpha inhibitor (*HIF1AN*), and claudin 12 (*CLDN12*) genes. Single nucleotide polymorphisms (SNPs) in the structural or regulatory regions of these miRNA let-7-related genes may be associated with breast cancer carcinogenesis and prognosis. Low let-7 expression may increase breast cancer risk. We investigated the effects of let-7-related gene SNP (mirSNPs) on breast cancer risk and clinical outcomes. Methods: The distribution frequencies of the three SNPs were genotyped in patients with breast cancer and controls. Multivariate logistic regression analysis was used to evaluate the association between the SNPs and susceptibility to breast cancer. We investigated the effects of these mirSNPs prospectively on disease-free survival (DFS) using the Kaplan-Meier method and the extended multivariate Cox model. Results: We found that rs1042713 in the *ADRB2* gene and rs11292 in the 3'-UTR of the *HIF1AN* gene were associated with breast cancer susceptibility ($P<0.05$). The *CLDN12* rs1017105 genotype was associated with estrogen receptor ($P=0.031$) and progesterone receptor status ($P=0.007$). The number of risk alleles was associated with estrogen receptor (P=0.034) status in breast cancer patients. In the survival analysis, the extended Cox model demonstrated that rs1042713 (P=0.000) and rs1017105 (P=0.004) were independent predictors of DFS. The number of risk alleles of the *ADRB2*, *HIF1AN*, and *CLDN12* genes was an independent predictor of DFS (P<0.001). Conclusion: Let-7-related mirSNPs might be associated with carcinogenesis and clinical outcome in breast cancer, suggesting that variants of miRNA let-7-related gene networks coregulate breast cancer characteristics.

Keywords: Breast cancer, miRNA let-7, mirSNP, susceptibility, clinical outcome

Introduction

MicroRNAs (miRNAs) are small, single-stranded molecules approximately 21 nucleotides in length [1]. Inherited variations in miRNAs are associated with increased cancer risk. MiRNAs regulate gene expression by base pairing with sequences within the 3'-untranslated region (UTR), 5'-UTR, and coding sequence regions of target mRNAs [2]. Polymorphisms disrupting miRNA coding sequences [3] or 3'-UTR miRNA binding sites are strong predictors of cancer risk, including breast cancer risk [4, 5].

MiRNA dysregulation has been detected in several malignancies, including breast cancer. Let-7 was one of the first miRNAs discovered and encodes an evolutionarily conserved family of 13 homologous miRNAs frequently deleted in a variety of human cancers. During cell development, let-7 expression gradually increases, playing important roles in many biological processes, including cancer and immune responses [6]. Dysregulation of let-7 has been associated with the development and prognosis of multiple cancer types [7]. Reduced let-7 expression promotes the proliferation and self-renewal of breast-tumor-initiating cells, which may increase the risk of breast cancer [8]. The let-7 family of miRNAs suppresses several oncogenes [9-11]. Let-7f has a dual role in the regulation of β_2-adrenergic receptor (β_2-ADR, encoded by the *ADRB2* gene), and the *ADRB2* gene is a target of let-7f [12]. Under static conditions, let-7f actively represses translation and establishes basal β_2-ADR expression. During agonist

activation, let-7f levels decrease, resulting in depressed *ADRB2* gene silencing and thus an attenuation of agonist-promoted down-regulation. Moreover, let-7f regulates the *HIF1AN* [13, 14] and *CLDN12* genes [15].

ADRs control the cardiovascular and respiratory systems, and β_2-ADR appears to be the most effective regarding the mobilization of lipids [16]. Epinephrine and norepinephrine, typically released during stress, bind to different ADRs. ADRs are composed of two main groups, α- and β-receptors. Hypoxia-inducible factor 1-alpha inhibitor is a protein that is encoded by the *HIF1AN* gene. HIF1AN expression and differential cellular localization are associated with shorter survival in breast cancer [17]. Aakula et al. demonstrated that miR-135b regulated estrogen receptor (ER) alpha, androgen receptor and HIF1AN protein levels through interaction with their 3'UTR regions, aa well as proliferation in ER-alpha-positive breast cancer [18]. Claudins are also linked with cancer progression and metastasis. The *CLDN12* gene encodes a member of the claudin family, claudin-12. Claudins are membrane proteins and components of tight junction strands. Tight junction strands are physical barriers that prevent solutes and water from passing freely through the paracellular space between epithelial or endothelial cell sheets, and maintain cell polarity and signal transduction. Several claudins are shown to be upregulated in various cancer types [19]. The *CIDN10* single nucleotide polymorphism rs1325774 alters the risk of breast cancer in South Chinese women [20].

These findings led us to speculate that gene polymorphisms regulated by the miRNA let-7 consist of a network system. In this system, we hypothesized that these polymorphisms might be associated with carcinogenesis, biological characteristics, or clinical outcomes of breast cancer. Therefore, we carried out a case-control study to examine the polymorphisms of miRNA let-7-related genes, including *ADRB2*, *HIF1AN*, and *CLDN12*, as well as their effect on breast cancer risk and prognosis.

Materials and methods

Study populations

We investigated the relationship between the single nucleotide polymorphisms (SNPs) of *ADRB2*, *HIF1AN*, and *CLDN12* genes and the risk of breast cancer through a case-control study. All the participants were genetically unrelated Han Chinese living in China. The hospital-based case-control study included 741 patients with pathologically confirmed primary breast cancer, who were consecutively recruited between September 2009 and March 2012. In total, 315 women with pathologically-confirmed nonmalignant breast diseases (including fibroadenoma, hyperplasia of the usual type and intraductal papilloma) were matched to the cases according to age and menopausal status. These women were from the same ward of the hospital during the same period. Informed consent, data on family history of cancer and reproductive history were obtained, and 3-5 mL of peripheral venous blood were collected from all participants. All participants (study population) provided written informed consent. The DNA was extracted from the peripheral blood samples of all subjects using the Qiagen DNA blood kit (Qiagen NV, Venlo, the Netherlands) according to the manufacturer's protocols. This study was approved by the Ethics Committee of Fudan University Shanghai Cancer Center (FUSCC). Detailed characteristics of the subjects enrolled in this study are shown in **Table 1**.

Selection of SNPs in the exon of the ADRB2 gene and 3'UTR of the HIF1AN and CLDN12 genes

The SNPs in the *ADRB2* gene were surveyed using the NCBI-dbSNP (www.ncbi.nlm.nih.gov/SNP/) and Hapmap (www.hapmap.org) databases. The NCBI-dbSNP and Hapmap databases have genotyped a large number of SNPs in different populations and have provided a set of tag SNPs (tSNPs) representing evolutionarily linked genetic variants [21]. Using the SNPbrowser™ v4.0 software developed by ABI, we selected one tSNP (rs1042713) in an ADRB2 exon.

We searched the miRBase (www.mirbase.org Release 22), TargetScan (http://www.targetscan.org/Release 7.2) and UCSC Genome Browser (http://genome.cse.ucsc.edu) databases to identify the potential target genes of miRNA let-7. We selected the potential target genes of miRNA let-7 that related to malignant characteristics of breast cancer, such as prolif-

Table 1. Main characteristics of the enrolled participants

Variables	Cases n=741 (%)	Controls n=315 (%)	*P* value
Age, years (range)	23-77	31-85	0.662
Menstrual status[a]			
Premenopausal	363 (49.0)	165 (52.4)	0.149
Postmenopausal	347 (46.8)	129 (41.0)	
Unknown	31 (4.2)	21 (6.6)	
Estrogen receptor (ER)			
Positive	480 (64.8)		
Negative	162 (21.9)		
Unknown	99 (13.3)		
Progesterone receptor (PR)			
Positive	487 (65.7)		
Negative	165 (22.3)		
Unknown	89 (12.0)		
Axillary lymph node metastasis			
Positive	290 (39.1)		
Negative	424 (57.2)		
Unknown	27 (3.7)		
Tumor size			
≤2 cm	409 (55.2)		
>2 cm	332 (44.8)		
HER2			
0-2+	651 (87.9)		
3+	90 (12.1)		

a: Menopause is generally the permanent cessation of menses. Reasonable criteria for determining menopause include prior bilateral oophorectomy, age ≥60 years old and age <60 years old and amenorrheic for 12 or more months.

eration, angiogenesis, invasion, metastasis or inhibition of apoptosis. We screened the SNP loci that cover the extension of 2 kb at both sides of the let-7-binding sites in the target genes. We then screened for SNPs and selected three mirSNPs, including rs11292 located in the 3'-UTR of the *HIF1AN* gene (**Figure 1A**), rs1017105 located in the 3'-UTR of the *CLDN12* gene (**Figure 1B**), and rs1042713 (c.46A>G, p.Arg16Gly) located in the exon of the *ADRB2* gene.

SNP genotyping

SNP genotyping was performed at Shanghai Bene-gene Biotechnology Co., Ltd. (Shanghai, People's Republic of China), using the MassARRAY system (Sequenom, San Diego CA, USA) by the matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry method. Detailed primer information is in **Table 2**.

Prediction of the protein structure

The SWISS-MODEL (https://swiss-model.expasy.org/) was used to predict the protein structure.

Statistical analysis

Genotype distributions of all cases and controls were tested for Hardy-Weinberg equilibrium (HWE), with a *P*-value >0.01. Associations between SNPs and tumor-relevant biomarkers were evaluated using a Student's t-test for continuous variables and the chi-square or Fisher's exact tests for unordered categorical variables. The modified odds ratios (ORs) with their 95% confidence intervals (CIs) were calculated using a multivariate logistic regression analysis to estimate the relative risk of breast cancer associated with each genetic model.

Disease-free survival (DFS) was defined as the time from random assignment to recurrence (either local, regional, or distant), new primary breast tumors (ductal carcinoma in situ or invasive), second primary non-breast cancer or death due to any cause. Follow-up information was prospectively collected. DFS curves were estimated using the Kaplan-Meier method. Statistical significance of a relationship between genetic polymorphism and clinical outcome was assessed using the log-rank test. The proportional-hazards (PH) assumption was used for estimating whether the SNPs examined in the study were time-dependent factors. The extended Cox model was used for time-dependent factors to identify significant prognostic clinical factors. For graphical display of DFS, hazard rates were estimated using a Kernel smoothing method. All statistical analyses were calculated by Stata 12.0 (StataCorp LP, College Station, TX, USA).

Results

Relationship of the SNPs in the ADRB2, HIF1AN and CLDN12 genes with the risk of breast cancer

The genotype distribution of cases and controls showed no deviation from the HWE for the three

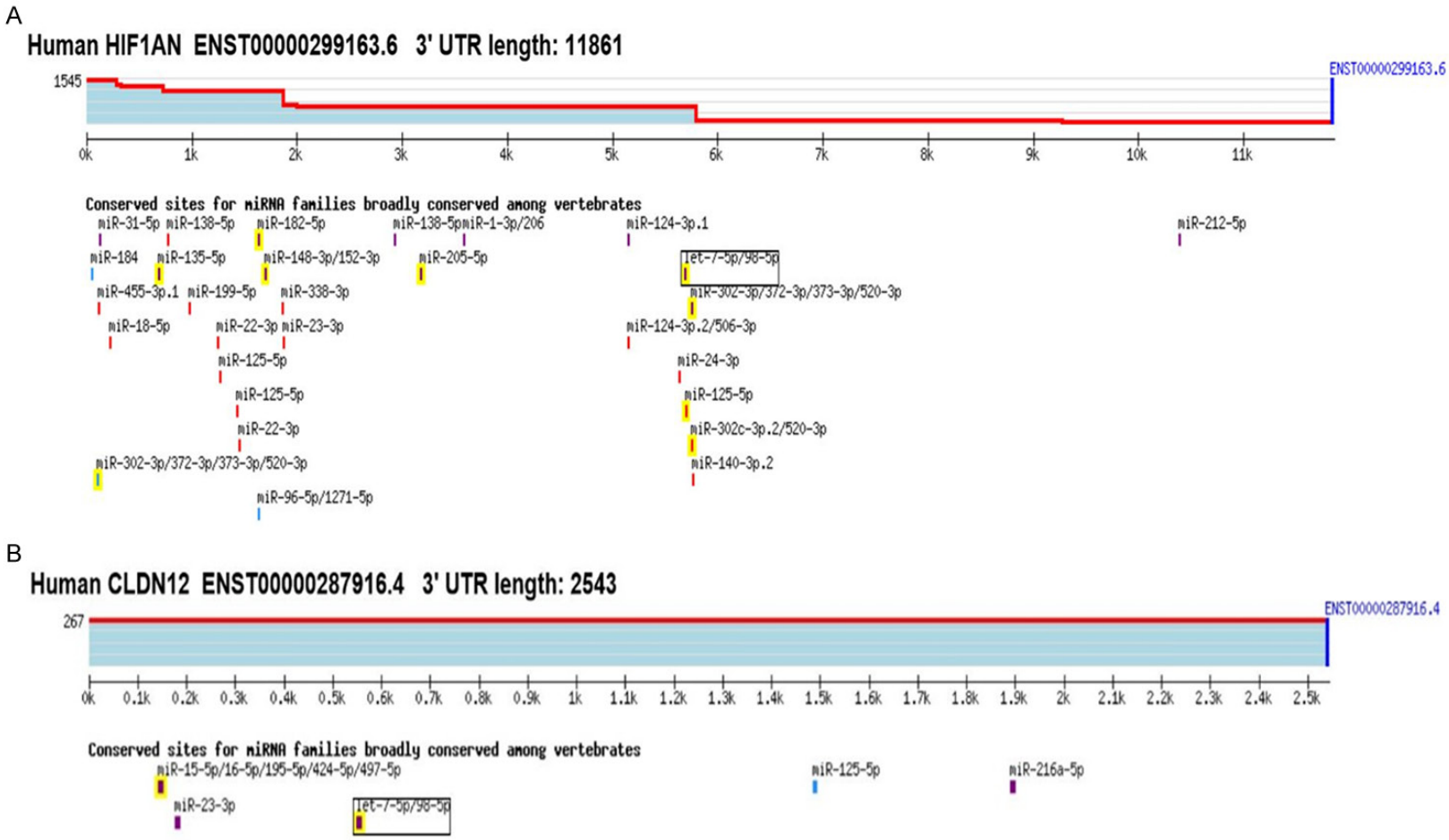

Figure 1. The binding sites of miRNA let-7 with the 3'-UTR of HIF1AN and CLDN12. A. The binding sites of miRNA let-7 with the 3'-UTR of HIF1AN. B. The binding sites of miRNA let-7 with the 3'-UTR of CLDN12.

Table 2. Sequences of primers

SNP	Primer 1	Primer 2	Extension of primer	Single-base primer
Rs1042713	ACGTTGGATGACCCACACCTCGTCCCTTT	ACGTTGGATGAGCGCCTTCTTGCTGGCA	R	ACGTTGGATGAGCGCCTTCTTGCTGGCA
Rs11292	ACGTTGGATGGGAGGGAGGCATGTTGAAAA	ACGTTGGATGGCACTGTGCTAAAGCTTTAC	R	ACGTTGGATGGCACTGTGCTAAAGCTTTAC
Rs1017105	ACGTTGGATGTCTTCTACCTCATGCCACTG	ACGTTGGATGGGTGATATTTAGAATGGTAG	F	ACGTTGGATGGGTGATATTTAGAATGGTAG

Table 3. Genotype frequencies of the three let-7 mirSNPs and breast cancer risk

SNPs	Model	Genotype	No. Cases (%)	No. Controls (%)	OR (95% CI)[a]	*P* value[a]
rs1042713	Codominant	AA	270 (36.4)	91 (28.9)	1.00	
		AG	371 (50.1)	168 (53.3)	0.75 (0.56-1.02)	0.067
		GG	100 (13.5)	56 (17.8)	0.79 (0.64-0.97)	0.025
	Dominant	AA	270 (36.4)	91 (28.9)	1.00	
		AG+GG	471 (63.6)	224 (71.1)	0.72 (0.54-0.96)	0.028
	Recessive	AA+AG	641 (86.5)	259 (82.2)	1.00	
		GG	100 (13.5)	56 (17.8)	1.39 (0.97-1.98)	0.074
	Overdominant	AA+GG	370 (49.9)	147 (46.7)	1.00	
		AG	371 (50.1)	168 (53.3)	1.01 (0.78-1.33)	0.920
rs11292	Codominant	TT	579 (78.1)	263 (83.5)	1.00	
		TC	153 (20.7)	49 (15.6)	1.44 (1.01-2.06)	0.044
		CC	9 (1.2)	3 (0.9)	1.12 (0.58-2.18)	0.733
	Dominant	TT	579 (78.1)	263 (83.5)	1.00	
		TC+CC	162 (21.9)	52 (16.5)	1.43 (1.01-2.02)	0.044
	Recessive	TT+TC	732 (98.8)	312 (99.1)	1.00	
		CC	9 (1.2)	3 (0.9)	0.64 (0.16-2.53)	0.530
	Overdominant	TT+CC	588 (79.3)	266 (84.4)	1.00	
		TC	153 (20.7)	49 (15.6)	0.96 (0.68-1.35)	0.819
rs1017105	Codominant	TT	322 (43.6)	137 (43.5)	1.00	
		TC	318 (43.0)	138 (43.8)	0.94 (0.71-1.26)	0.697
		CC	99 (13.4)	40 (12.7)	1.03 (0.84-1.27)	0.765
	Dominant	TT	322 (43.6)	137 (43.5)	1.00	
		TC+CC	417 (56.4)	178 (56.5)	0.97 (0.75-1.27)	0.850
	Recessive	TT+TC	640 (86.6)	275 (87.3)	1.00	
		CC	99 (13.4)	40 (12.7)	0.93 (0.62-1.39)	0.721
	Overdominant	TT+CC	421 (57.0)	177 (56.2)	1.00	
		TC	318 (43.0)	138 (43.8)	1.14 (0.86-1.49)	0.363

a: OR and 95% CI was analyzed by logistic regression and adjusted by age and menstrual status. Common genotype was taken as reference.

SNPs either in controls or in cases (Supplementary Table 1). Differences were detected in rs1042713 and rs11292 genotypes between these two groups (**Table 3**, $P<0.05$), suggesting that the genotypes of these two SNPs are associated with breast cancer risk. However, no difference was detected in the rs1017105 genotype distribution.

Women carrying the GG (OR=0.79, 95% CI: 0.64-0.97, $P=0.025$) and AG+GG (OR=0.72, 95% CI: 0.54-0.96, $P=0.028$) genotypes of *ADRB2* rs1042713 had a lower risk of breast cancer than those carrying the AA genotype (**Table 3**). Women carrying the TC (OR=1.44, 95% CI: 1.01-2.06, $P=0.044$) and TC+CC (OR=1.43, 95% CI: 1.01-2.02, $P=0.044$) genotypes of *HIF1AN* rs11292 had a higher risk of breast cancer than those carrying the TT genotype (**Table 3**). Differences in the A/G allele of *ADRB2* rs1042713 ($P=0.018$) indicated that the G allele of *ADRB2* rs1042713 reduced the risk of breast cancer (OR=0.79, 95% CI: 0.66-0.96) (**Table 4**). Marginal significance in the C/T allele of *HIF1AN* rs11292 was detected between the case and control population ($P=0.057$), suggesting that the C allele of *HIF1AN* rs11292 might increase breast cancer risk.

Differences were observed in the *ADRB2* rs1042713 genotype between the patients with different hormone receptor (HR) status and the control group ($P<0.05$), indicating that this SNP contributes to the risk of both HR-positive and HR-negative breast cancer (Supplementary Tables 2, 3). Women carrying the GG (OR=0.79, 95% CI: 0.63-0.98) and AG+GG (OR=0.73, 95% CI: 0.53-0.99) genotypes had a lower risk of HR-positive breast cancer than those carrying the AA genotype (Supplementary Table 2). Women carrying the GG (OR=0.67, 95% CI: 0.50-0.89) and AG+

Table 4. Allele frequencies of the three let-7 mirSNPs and breast cancer risk

SNP	Allele	Frequency		OR (95% CI)[a]	*P* value[a]
		Cases	Controls		
rs1042713	A	0.615	0.556	1.00	
	G	0.385	0.444	0.79 (0.66-0.96)	0.018
rs11292	T	0.885	0.913	1.00	
	C	0.115	0.087	1.17 (1.00-1.37)	0.057
rs1017105	C	0.651	0.654	1.00	
	T	0.349	0.346	1.01 (0.83-1.23)	0.932

a: OR and 95% CI was analyzed by logistic regression and adjusted by age and menses status.

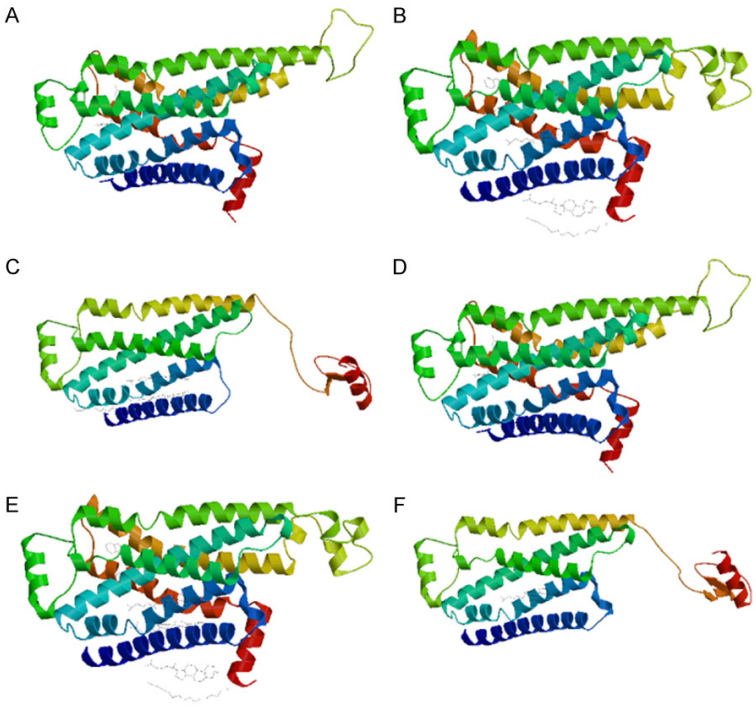

Figure 2. Three possible models of the protein structure encoded by ADRB2 gene. SWISS-MODEL (https://swissmodel.expasy.org/) was used to predict the protein structure encoded by ADRB2 gene. A. Model 01 (QMEAN: -3.99, Cβ: -0.99, All Atom: -0.82, Solvation: 1.50, Torsion: -4.13). B. Model 02 (QMEAN: -3.07, Cβ: -0.65, All Atom: 0.23, Solvation: 2.60, Torsion: -3.51). C. Model 03 (QMEAN: -2.99, Cβ: -1.77, All Atom: -0.08, Solvation: 1.53, Torsion: -3.06). Three possible models of the protein structure encoded by ADRB2 gene with the SNP of rs1042713. Rs1042713 is an A/G single-nucleotide variation at nucleotide position 46 of the ADRB2 gene (AGA to GGA) that substitutes the 16th amino acid of the translated protein chain from arginine to glycine and alters receptor function. The protein product may be altered. SWISS-MODEL (https://swissmodel.expasy.org/) was used to predict the possible protein structure encoded by ADRB2 gene with the SNP of rs1042713. D. Model 01 (QMEAN: -4.01, Cβ: -1.07, All Atom: -0.80, Solvation: 1.49, Torsion: -4.13). E. Model 02 (QMEAN: -3.38, Cβ: -0.53, All Atom: 0.39, Solvation: 2.68, Torsion: -3.88). F. Model 03 (QMEAN: -2.79, Cβ: -1.68, All Atom: 0.54, Solvation: 2.08, Torsion: -3.03).

GG (OR=0.61, 95% CI: 0.41-0.90) genotypes also had a lower risk of HR-negative breast cancer than those who carrying the AA genotype (Supplementary Table 3).

Prediction of the protein structure encoded by the ADRB2 gene

We used the SWISS-MODEL (https://swissmodel.expasy.org/) to predict the protein structure encoded by a normal *ADRB2* gene and by an *ADRB2* gene with the rs1042713 SNP (**Figure 2**). In Model 01, Model 02 and Model 03, we can see that after the substitution of arginine, the predicted protein structure changes.

Associations between genotypes and clinical outcome

To evaluate the effects of the genetic variants in miRNA let-7-related genes on clinical outcomes of breast cancer, we prospectively examined the association between the three SNPs and the DFS of breast cancer patients. Median follow-up was 41 (range 1-68) months. Univariate analysis showed that either *ADRB2* rs1042713 or *CL-DN12* rs1017105 was associated with the DFS. The PH assumption was checked, and the results indicated that the PH assumption had been violated and that *ADRB2* rs1042713 (P=0.009) and *CLDN12* rs10-17105 (P=0.001) were time-dependent variates. In the extended Cox model, *ADRB2* rs1042713 (P=0.000; **Table 5**) and *CLDN12* rs1017105 (P=0.004; **Table 6**) were independent predictors of DFS.

We also investigated a combined effect of *ADRB2* rs10-42713, *HIF1AN* rs11292, and *CLDN12* rs101-7105 genotypes on the DFS by classifying the

Table 5. Extension of the Cox proportional hazards model for rs1042713 in the ADRB2 gene

Variable	Hazard Ratio	95% CI	*P* value
Rs1042713	1.16[a]	1.08-1.24	0.000
Age (<50 vs. ≥50)	0.99	0.96-1.02	0.400
ER	0.54	0.21-1.36	0.192
PR	1.59	0.82-3.07	0.171
Lymph node (negative vs. positive)	2.45	1.40-4.28	0.002
Tumor size (≤2 cm vs. >2 cm)	1.07	0.61-1.90	0.807
HER2	0.90	0.50-1.64	0.741

a: In the extended Cox model, the hazard at time t is modeled as $\lambda(t, X) = \lambda_0(t) \exp\{\beta'(t)X\}$, where β vary according to time (t) or a function of time.

Table 6. Extension of the Cox proportional hazards model for rs1017105 in the CLDN12 gene

Variable	Hazard Ratio	95% CI	*P* value
Rs1017105	1.42[a]	1.27-2.32	0.004
Age (<50 vs. ≥50)	0.98	0.95-1.01	0.131
ER	0.55	0.23-1.29	0.171
PR	0.24	0.77-2.90	0.625
Lymph node (negative vs. positive)	2.26	1.30-3.93	0.004
Tumor size (≤2 cm vs. >2 cm)	1.18	0.67-2.08	0.577
HER2	0.98	0.55-1.76	0.948

a: In the extended Cox model, the hazard at time t is modeled as $\lambda(t, X) = \lambda_0(t) \exp\{\beta'(t)X\}$, where β vary according to time (t) or a function of time.

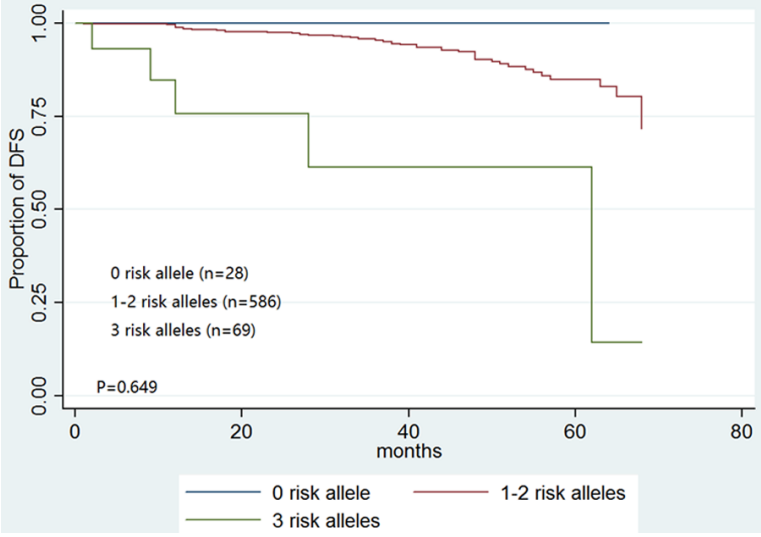

Figure 3. Kaplan-Meier estimates of disease-free survival for the combination effect of ADRB2 rs1042713, HIF1AN rs11292, and CLDN12 rs1017105 genotypes. The patients were classified into three groups (zero, one to two, and three risk allele groups). In univariate survival analysis, there was no association between the number of risk alleles of the three genes and DFS of breast cancer (log-rank P=0.649).

patients into three groups (zero, one to two, and three risk allele groups). Among the patients, 28 were in the zero risk allele group, 586 were in the one to two risk alleles group, and 69 were in the three risk alleles group. In univariate survival analysis, there was no association between the number of risk alleles of the three genes and DFS of breast cancer (log-rank P=0.649; **Figure 3**). The PH assumption and the results showed that the number of risk alleles was a time-dependent variate (P<0.001). In the extended Cox model, the number of risk alleles of the three genes was an independent predictor of DFS (P<0.001; **Table 7**). Using the hazard curves (**Figure 4**), we found that women with zero risk alleles had a decreased likelihood of recurrence compared with women with one to two and three risk alleles. Women with three risk alleles showed an early recurrence peak at the 1.5 year mark after surgery, followed by a decline until year 3.5 and then another increase. The hazard rate for women with one to two risk alleles displayed a gradual increase during the follow-up of approximately 5 years.

Association of the polymorphisms in the ADRB2, HIF1AN and CLDN12 genes with the histopathological characteristics of breast cancer patients

We examined the association between genotype distribution in the three SNPs and histopathological characteristics of breast cancer patients. The genotype of *CLDN12* rs1017105 was associated with ER (*P*=0.031) and progesterone receptor (PR) status (*P*=0.007) (**Table 8**). No association was detected between the genotype of *CLDN12* rs1017105 and age, tumor size, or lymph

Table 7. Multivariate Cox proportional hazards regression analysis for the number of risk alleles of three genes

Variable	Hazard Ratio	95% CI	*P* value
Risk allele	1.02[a]	0.96-2.10	0.000
Age (<50 vs. ≥50)	0.97	0.96-1.01	0.149
ER	0.59	0.23-1.50	0.268
PR	1.19	0.54-2.65	0.663
Lymph node (negative vs. positive)	2.70	1.50-4.86	0.001
Tumor size (≤2 cm vs. >2 cm)	1.02	0.57-1.81	0.948
HER2	0.84	0.44-1.58	0.579

a: In the extended Cox model, the hazard at time t is modeled as $\lambda(t, X) = \lambda_0(t) \exp\{\beta'(t)X\}$, where β vary according to time (t) or a function of time.

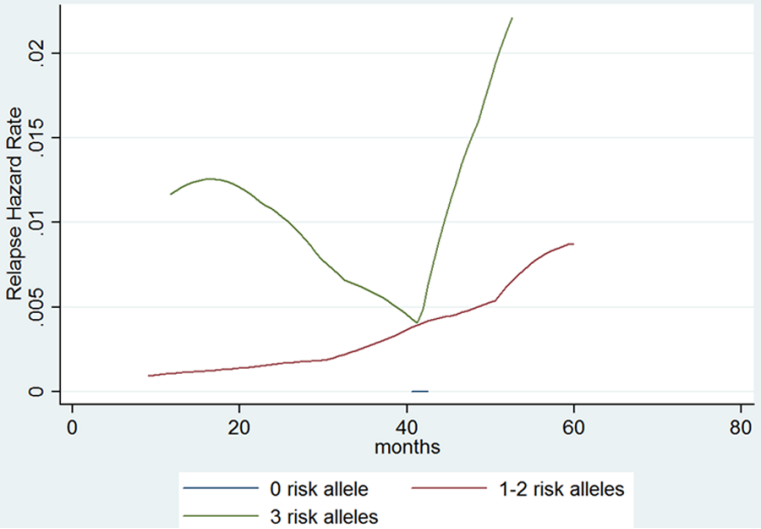

Figure 4. Relapse hazard rate for the combination effect of ADRB2 rs1042713, HIF1AN rs11292, and CLDN12 rs1017105 genotypes. The patients were classified into three groups (zero, one to two, and three risk allele groups). Women with zero risk alleles had a decreased likelihood of recurrence compared with women with one to two and three risk alleles. Women with three risk alleles showed an early recurrence peak at the 1.5 year mark after surgery, followed by a decline until year 3.5 and then another increase. The hazard rate for women with one to two risk alleles displayed a gradual increase during the follow-up of approximately 5 years.

node status (**Table 8**). Next, we investigated the association between the number of risk alleles of the *ADRB2*, *HIF1AN* and *CLDN12* genes and the histopathological characteristics of breast cancer patients. The number of risk alleles was associated with ER status (P=0.034) in breast cancer patients (**Table 9**). No significant association was detected between the number of risk alleles and age, tumor size, or lymph node status (**Table 9**).

Discussion

Genetic variants of miRNA let-7-related genes such as *ADRB2* and *HIF1AN* increase the susceptibility to breast cancer. We initially found that polymorphisms of rs1042713 located in the *ADRB2* gene and rs11292 located in the *HIF1-AN* gene were associated with the risk of breast cancer. The rs1042713 *ADRB2* polymorphism is associated with the risk of both HR-positive and HR-negative breast cancer. We also found that three SNPs of miRNA let-7 related genes were associated with the prognosis of breast cancer patients.

Since the ADR gene polymorphism rs1042714 can decrease the risk of breast cancer [17], we investigated an additional ADR SNP and breast cancer. Rs1042713 is an A/G single-nucleotide variation at nucleotide position 46 of the *ADRB2* gene (AGA to GGA) that substitutes the 16th amino acid of the translated protein chain from arginine to glycine. The three-dimensional structure and receptor function may be altered. We observe that following the substitution of arginine, the predicted protein structure changes, thus indicating that the protein function may theoretically change. However, these changes are predictions that require experimental confirmation. Connor et al. investigated the association between *ADRB2* variants (rs1042713, rs1042714) and breast cancer risk in non-Hispanic white and Hispanic women. The results showed that two copies compared to one or zero copies of the *ADRB2* G-G haplotype were associated with non-Hispanic white women, but with reduced risk for Hispanic women [22]. The data suggested that ethnicity modified the association between the *ADRB2* G-G haplotype and breast cancer risk. In our study, we investigated the effects of let-7-related genes SNPs (mirSNPs) on breast cancer risk and clinical outcomes in Chinese women. The results of the study by Connor et al. were con-

Table 8. Association of the polymorphism of CLDN12 rs1017105 with histopathological characteristics of breast cancer patients

Variable	TT n (%)	TC+CC n (%)	χ^2	*P* value
Patient age				
<50	158 (45.3)	191 (54.7)	0.777	0.210
≥50	164 (42.1)	226 (57.9)		
Tumor size				
≤2 cm	112 (40.0)	168 (60.0)	1.335	0.142
>2 cm	129 (44.8)	159 (55.2)		
Lymph node				
Negative	176 (41.6)	247 (58.4)	1.819	0.102
Positive	135 (46.7)	154 (53.3)		
ER				
Negative	60 (37.0)	102 (63.0)	3.792	0.031
Positive	219 (45.8)	259 (54.2)		
PR				
Negative	58 (35.2)	107 (64.8)	6.328	0.007
Positive	225 (46.4)	260 (53.6)		
HER2/neu				
0-2+	214 (43.3)	280 (56.7)	0.012	0.493
3+	64 (43.8)	82 (56.2)		

sistent with our results. We demonstrated that rs1042713 in the *ADRB2* gene was associated with the susceptibility to both HR-positive and HR-negative breast cancer. One explanation is that the protein conformation change induced by rs1042713 in the *ADRB2* gene may change its interaction with some signaling pathways, leading to carcinogenesis in both HR-positive and HR-negative breast cancer.

HIF1AN rs11292, a let-7 miRNA-related SNP (mirSNP), was associated with breast cancer risk. MirSNPs are polymorphisms in pre-microRNAs that flank regions or target sites to affect certain physiological processes. Our results suggest that different genetic variants in miRNA let-7 binding sites up- or downregulate HIF1AN expression, thus altering the risk of breast cancer.

We demonstrated rs1017105 *CLDN12* was associated with the clinical outcome of breast cancer patients. Our results showed rs1017105 *CLDN12* was an independent predictor of DFS in breast cancer patients, including both ER-positive and ER-negative patients. These results align with other studies that found CLDN12 expression can be an independent predictor of poor overall survival in ER-negative breast cancer patients [23].

We also investigated the combined effect of *ADRB2* rs1042713, *HIF1AN* rs11292, and *CLDN12* rs1017105 genotypes on DFS according to the number of risk alleles. In the extended Cox model, the number of risk alleles of the three genes was an independent predictor of DFS. According to the hazard curves, we observed that women with zero risk alleles had a decreased likelihood of recurrence compared to women with one to two and three risk alleles in the *ADRB2*, *HIF1AN*, and *CLDN12* genes. In clinical practice, we must identify the women who are at low risk of recurrence after surgery and do not require adjuvant chemotherapy. Our results suggest that the three SNPs in miRNA let-7-related genes may be used to identify women with a low risk of recurrence. These results suggest that variants of miRNA let-7-related genes (**Figure 5**) influence prognosis of breast cancer.

We showed that rs1017105 located in the *CLDN12* gene was associated with ER and PR status. In addition, we found that the number of risk alleles of *ADRB2*, *HIF1AN*, and *CLDN12* genes was associated with ER status in breast cancer patients. Taken together, our findings suggest that the system regulated by let-7 is associated with HR expression and function. Estrogen is one of the risk factors associated with risk of ER-positive breast cancer. Antiestrogen drugs treat breast cancer effectively, and reduce the risk of contralateral breast cancer in women with early stage breast cancer [24, 25]. Several large phase III cancer prevention trials [25-28] demonstrated that antiestrogen drugs might reduce the risk of ER-positive breast cancer. Therefore, the three SNPs of miRNA let-7-related genes may be useful in the precise prevention of ER-positive breast cancer. These findings require independent replication and assessment in a study specifically designed to test such a hypothesis.

There are some limitations in the present study. This was a hospital-based case-control study,

Table 9. Association of the number of risk alleles of three genes with histopathological characteristics of patients

Variable	0 risk allele (%)	1 risk allele (%)	2 risk alleles (%)	3 risk alleles (%)	χ^2	*P* value
Tumor size						
≤2 cm	12 (42.9)	92 (36.4)	125 (37.9)	24 (37.5)	1.564	0.953
>2 cm	16 (57.1)	161 (63.6)	205 (62.1)	40 (62.5)		
Lymph node						
Negative	18 (64.3)	139 (54.9)	208 (63.2)	40 (62.5)	5.217	0.454
Positive	10 (55.7)	114 (45.1)	121 (36.8)	24 (37.5)		
ER						
Negative	5 (17.9)	62 (24.7)	96 (30.1)	20 (31.3)	18.379	0.034
Positive	23 (82.1)	189 (75.3)	223 (69.9)	44 (68.7)		
PR						
Negative	5 (17.9)	82 (32.7)	122 (37.5)	18 (28.1)	10.963	0.096
Positive	23 (82.1)	169 (67.3)	203 (62.5)	46 (71.9)		
HER2/neu						
0-2+	20 (71.4)	172 (69.6)	236 (74.1)	45 (71.4)	2.642	0.746
3+	8 (28.6)	75 (30.4)	81 (25.9)	18 (28.6)		

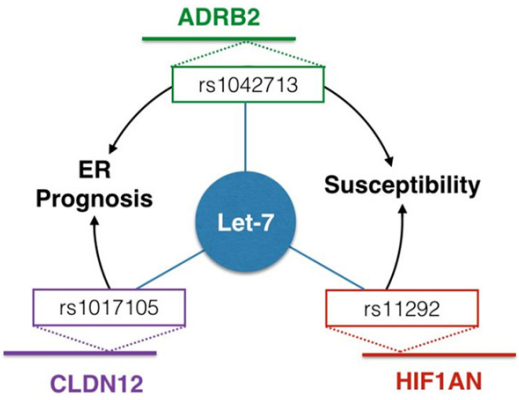

Figure 5. Possible regulatory association of miRNA let-7-related gene networks. Genetic variants of miRNA let-7-related genes such as ADRB2 and HIF1AN affect the susceptibility to breast cancer. For breast cancer patients, rs1042713 ADRB2 and rs1017105 CLDN12 are the independent predictors of DFS in a time-dependent model. The genotype of CLDN12 rs1017105 is associated with ER- and PR-status in breast cancer patients. Strong β2 AR expression might indicate an active ER signaling pathway.

in which the controls were all patients with nonmalignant breast diseases. As nonmalignant breast diseases may include precancerous lesions [29], our results may not reflect the breast cancer susceptibility of the whole population. On the other hand, if a difference had been detected in genotype distributions between breast cancer cases and nonmalignant controls, it may be suggestive of a more obvious difference in the whole population.

ADRB2 and *HIF1AN* genetic variants regulated by miRNA let-7 can promote breast cancer risk. Rs1042713 in the *ADRB2* gene and rs1017105 in the *CLDN12* gene might be important predictors for the prognosis of patients with breast cancer. The combined effect of the *ADRB2* rs1042713, *HIF1AN* rs11292, and *CLDN12* rs1017105 genotypes showed that the number of risk alleles was associated with the ER status and prognosis of breast cancer patients.

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Disclosure of conflict of interest

None.

Abbreviations

ADRB2, beta2 adrenergic receptor; miRNA, microRNA; ER, estrogen receptor; SNP, single nucleotide polymorphism; DFS, disease-free survival; HR, hormone receptor; UTR, untranslated region; FUSCC, Fudan University Shanghai Cancer Center; OR, odds ratio; HWE, Hardy-Weinberg equilibrium; CI, confidence interval; PH, proportional-hazards.

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Supplementary Table 1. Hardy-Weinberg equilibrium analysis for the cases and controls

SNP	Allele	Case	Control
Rs1042713	G>A	0.121	0.156
Rs11292	T>C	0.756	0.672
Rs1017105	C>T	0.148	0.570

Supplementary Table 2. Genotype frequencies of rs1042713 and HR-positive breast cancer risk

SNPs	Model	Genotype	No. Cases (%)	No. Controls (%)	OR (95% CI)[a]	*P* value[a]
rs1042713	Codominant	AA	173 (35.9)	91 (28.9)	1.00	
		AG	245 (50.8)	168 (53.3)	0.77 (0.55-1.06)	0.105
		GG	64 (13.3)	56 (17.8)	0.79 (0.63-0.98)	0.035
	Dominant	AA	173 (35.9)	91 (28.9)	1.00	
		AG+GG	309 (64.1)	224 (71.1)	0.73 (0.53-0.99)	0.042
	Recessive	AA+AG	418 (86.7)	259 (82.2)	1.00	
		GG	64 (13.3)	56 (17.8)	0.98 (0.66-1.45)	0.904
	Overdominant	AA+GG	237 (49.2)	147 (46.7)	1.00	
		AG	245 (50.8)	168 (53.3)	1.28 (0.96-1.70)	0.920

a: OR and 95% CI was analyzed by logistic regression and adjusted by age and menstrual status. Common genotype was taken as reference.

Supplementary Table 3. Genotype frequencies of rs1042713 and HR-negative breast cancer risk

SNPs	Model	Genotype	No. Cases (%)	No. Controls (%)	OR (95% CI)[a]	*P* value[a]
rs1042713	Codominant	AA	97 (37.5)	91 (28.9)	1.00	
		AG	126 (48.6)	168 (53.3)	0.74 (0.57-1.04)	0.062
		GG	36 (13.9)	56 (17.8)	0.78 (0.62-0.98)	0.023
	Dominant	AA	97 (37.5)	91 (28.9)	1.00	
		AG+GG	162 (62.5)	224 (71.1)	0.72 (0.55-0.98)	0.026
	Recessive	AA+AG	223 (86.1)	259 (82.2)	1.00	
		GG	36 (13.9)	56 (17.8)	1.10 (0.96-1.97)	0.071
	Overdominant	AA+GG	133 (51.4)	147 (46.7)	1.00	
		AG	126 (48.6)	168 (53.3)	1.03 (0.81-1.42)	0.890

a: OR and 95% CI was analyzed by logistic regression and adjusted by age and menstrual status. Common genotype was taken as reference.