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Original Article

Association between *WT1* polymorphisms and susceptibility to breast cancer: results from a case-control study in a southwestern Chinese population

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Abstract: Wilms' tumor gene 1 (*WT1*) single nucleotide polymorphism (SNP), rs16754, has been considered as an independent prognostic factor in patients with acute myeloid leukemia and renal cell carcinoma. However, its biological role in breast cancer has not been reported. To test whether *WT1* SNPs can be used as a molecular marker in order to improve the risk stratification of breast cancer, we performed a case-control study including 709 female sporadic breast cancer patients and 749 female healthy control subjects in the Southeast China. Five *WT1* SNPs (rs16754, rs3930513, rs5030141, rs5030317, rs5030320) were selected and determined by polymerase chain reaction-ligase detection reaction to assess their associations with breast cancer risk. Results showed the distributions of the alleles of these *WT1* SNPs were consistent with data from Chinese population as suggested by the International HapMap Project. Individuals with the minor alleles of rs16754, rs5030317 and rs5030320 showed a significant decrease of breast cancer risk in codominant model (OR = 0.6370, 95% CI: 0.4260-0.9520 for rs16754; OR = 0.5940, 95% CI: 0.3890-0.9070 for rs5030317; OR = 0.5870, 95% CI: 0.3850-0.8960 for 5030320, respectively) and recessive model. Stratified analyses showed the protective effects were more evident in the subjects with age $\leq$ 50 years or in pre-menopausal status. To explore the potential mechanism, we conducted bioinformatics genotype-phenotype correlation analysis, and found that the mRNA expression level for homozygous rare allele of *WT1* gene was lower than that in wild-type and heterozygous group (P = 0.0021) in Chinese population. In summary, our findings indicated that minor alleles of rs16754, rs5030317 and rs5030320 are associated with reduced risk of breast cancer, suggesting that *WT1* SNPs may be a potential biomarker of individualized prediction of susceptibility to breast cancer. However, large prospective and molecular epidemiology studies are needed to verify this correlation and clarify its underlying mechanisms.

Keywords: WT1, polymorphism, breast cancer, susceptibility, risk

Introduction

Breast cancer is the most common cancer among women worldwide, with an estimated 1.67 million newly diagnosed cases in 2012 accounting for 25% of all cancers, and it is the most frequent cause of cancer death in women in less developed regions (324,000 deaths, 14.3% of total) [1]. It is reported that breast cancer has become the major burden of the health for Chinese women [2], and approximately 12.2% of all newly diagnosed breast cancers and 9.6% of all deaths from breast cancer worldwide occurred in China [3]. It is well-known that breast cancer has a complex pathogenesis affected by many epidemiological, genetic, and epigenetic factors [4-8]. For genetic factors, single nucleotide polymorphisms (SNPs) of candidate genes have been believed to be responsible for a large percentage of breast cancers [8, 9].

The Wilms' tumor gene 1 (*WT1*), located at chromosome 11p13, was firstly cloned in 1990 as a suppressor in Wilms' tumor [10-12], and plays an important role in cell proliferation, differen-

tiation, apoptosis, organ development and the maintenance of several adult tissues [13-16]. Despite its role in Wilms' tumor, a growing body of evidences indicated that *WT1* might play an oncologic role in hematologic malignancies and a variety of solid tumors, and over-expression of WT1 indicated worse outcomes for patients with these cancers [17-27].

Sequencing analysis demonstrated that *WT1* mutations were found in sporadic Wilms' tumor [28] as well as urogenital abnormalities, such as Denys-Drash syndrome and Frasier syndrome [29, 30]. In addition, *WT1* mutations occurred in approximately 15% of acute myeloid leukemia (AML) [31], and correlated with poor outcomes in these patients [32-34]. Most recently, rs16754, a *WT1* SNP in exon 7, was shown to predict significantly improved outcomes in patients with favorable-risk pediatric AML [35], cytogenetically normal AML [36] and clear cell renal cell carcinoma [37], which suggested that it might be biologically involved in the expression process of WT1 [35]. However, to the best of our knowledge, the potential roles of *WT1* SNPs in breast cancer have not been clarified.

To explore whether *WT1* SNP genotypes are associated with the risk of breast cancer in females, we carried out a case-control study involving 709 breast cancer patients and 749 healthy controls in the Southeast China. A total of five *WT1* SNPs (rs16754, rs3930513, rs5030141, rs5030317, rs5030320) were selected as targets and characterized to assess their associations with breast cancer risk. We found minor alleles of rs16754, rs5030317 and rs5030320 could predict low susceptibility to breast cancer, especially in the subjects with age equal or less than 50 years old or in premenopausal status.

Materials and methods

Subjects

A total of 709 sporadic breast cancer patients and 749 healthy controls were enrolled from January 2012 to August 2013 from the Southwest Hospital, Third Military Medical University, Chongqing, China. All subjects were genetically unrelated Chinese females in Chongqing City and its surrounding regions. The inclusion criteria included histopathologically confirmed newly diagnosed breast cancer patients, who did not receive any kind of therapy before blood sampling, regardless of their age and pathological types. The exclusion criteria included pregnancy, being unwilling to undergo biopsy/surgical procedures, congestive heart failure, ischemic heart disease, severe hepatic or renal dysfunction and altered mental status other malignancies. The inclusion criteria for controls were healthy individuals and frequency matched to the cases for age (± 5 years), who were randomly selected from medical examination persons at the same hospital and during the same time. The study was approved by the Clinic Ethics Review Committee of Southwest Hospital, Third Military Medical University. Written informed consent was obtained from all participants involved in the study.

SNPs selection

One *WT1* SNP, rs16754 in exon region was selected based on previously reports [35-37]. Bioinformatics analysis with Haploview software 4.2 (Mark Daly's lab of Broad Institute, Cambridge, MA, Britain) was performed to analyze the haplotype block based on the CHB (Chinese Han Beijing) population data of HapMap (http://hapmap.ncbi.nlm.nih.gov/). Four tag SNPs were found in the WT1 gene: rs3930513 and rs5030141 in intron region, rs5030317 and rs5030320 in the 3'UTR, with a minimum allele frequency (MAF) of 0.05 in CHB population.

DNA preparation and genotyping analysis

According to the manufacturer's instructions, DNA was extracted from peripheral blood leukocytes using Wizard® Genomic DNA Purification Kit (Promega, Madison, Wisconsin, USA). All DNA samples were stored in aliquots at -80°C for further use.

The selected 5 SNPs were genotyped with the method of polymerase chain reaction (PCR)-ligase detection reaction (LDR) on an ABI Prism 377 Sequence Detection System (Applied Biosystems, Foster City, CA, USA), as previously reported [38, 39] with technical supports from the Shanghai Genesky Biotechnology Company (Shanghai, China). Two multiplex PCR reactions were designed. The first PCR reaction in 20 μl contained 1x PCR buffer, 3.0 mM Mg^{2+}, 0.3 mM dNTP, 1 U of Hot-Start Taq DNA polymerase (Takara, Dalian, Liaoning, China), 1 μl of primer

Table 1. Products size and primers of *WT1* SNPs

SNPs	Product size (bp)	PCR primer sequence[a]	Ligase reaction primer sequence
rs16754	244	rs16754F: TGTGCATCTGTAAGTGGGACAGC rs16754R: CCTGCCACCCCTTCTTTGGATA	rs16754RC: TTCCGCGTTCGGACTGATATCTGCCTGCAGGATGTGAGG rs16754RP: CGTGTGCCYGGAGTAGCCCTTTTTTTTT rs16754RT: TACGGTTATTCGGGCTCCTGTCTGCCTGCAGGATGTGAGA
rs3930513	162	rs3930513F: AAAGCTCGTGCCTCCTTCCACT rs3930513R: TGGATAGCTCCCGCGTATGGTA	rs3930513FA: TACGGTTATTCGGGCTCCTGTCTGGTCCCGCATAGCTTGCAA rs3930513FC: TTCCGCGTTCGGACTGATATCTGGTCCCGCATAGCTTGTAC rs3930513FP: TCGGATAAGTCAAGTTSTCTTCCATCTTTTTT
rs5030141	277	rs5030141F: TGGAGGTGCTCCTGGACATTTT rs5030141R: GAGCCTGACTGTTCGCAAGAGC	rs5030141FG: TCTCTCGGGTCAATTCGTCCTTCCAGAGTCCAGACGTCTGAAAATCG rs5030141FP: CTACGCTTGGTGACAATTTGGCTTTTTT rs5030141FT: TGTTCGTGGGCCGGATTAGTCCAGAGTCCAGACGTCTGAAAACCT
rs5030317	153	rs5030317F: TCAGGGGGACATGATCAGCTATG rs5030317R: TGCCTGGAAGAGTTGGTCTCTG	rs5030317RG: TACGGTTATTCGGGCTCCTGTAAAAGCCCATTGCCATTTGTTC rs5030317RC: TTCCGCGTTCGGACTGATATAAAAGCCCATTGCCATTTGTTG rs5030317RP: TGGATTTTCTACTGTAAGAAGAGCCATAGCTTTT
rs5030320	278	rs5030320F: CCCCTCCATTTGTGCAAGGA rs5030320R: GCCAGGCTGCTAACCTGGAAA	rs5030320RC: TCTCTCGGGTCAATTCGTCCTTCATGCATTTCAAGCAGCTGAAGACAG rs5030320RP: AATCAGAACTAACCAGTACCTCTGTATAGAAATCT rs5030320RT: TGTTCGTGGGCCGGATTAGTCATGCATTTCAAGCAGCTGAAGACAA

[a]F indicates forward primer and R indicates reverse primer.

Table 2. Demographics of the subjects included in the case-control study

Characteristics	Controls (N = 749, %)	Cases (N = 709, %)	*P*
Age			
Mean ± SD	44.8144 ± 10.0065	48.3695 ± 10.3534	< .0001[a]
≤ 50	556 (74.23)	460 (64.88)	0.0001[b]
> 50	193 (25.77)	249 (35.12)	
Menopausal status			
Pre-menopausal	519 (69.29)	447 (63.05)	0.0117[b]
Post-menopausal	230 (30.71)	262 (36.95)	

[a]t test; [b]chi-square test.

mixture 1 and about 20 ng of genomic DNA. The second PCR reaction in 20 μl volume contained 1x GC Buffer I, 3.0 mM Mg^{2+}, 0.3 mM dNTP, 1 U of Hot-Start Taq DNA polymerase (Takara, Dalian, Liaoning, China), 1 μl of primer mixture 2 and about 20 ng of genomic DNA. The PCR program for both reactions was as follows: 95°C 2 min; 11 cycles x (94°C 20 s, 65°C -0.5°C/cycle 40 s, 72°C 1 min 30 s); 24 cycles x (94°C 20 s, 59°C 30 s, 72°C 1 min 30 s); 72°C 2 min; hold at 4°C. The two PCR products were equally mixed and purified by 1 U of shrimp alkaline phosphatase's digestion at 37°C for 1 hr and at 75°C for 15 min. The ligation reaction in 20 μl volume contains 1x ligation buffer, 80 U of Taq DNA Ligase, 1 μl of labeling oligo mixture, 2 μl of probe mixture and 5 μl of purified PCR product mixture. The ligation cycling program was 95°C 2 min; 38 cycles x (94°C 1 min, 56°C 4 min); hold at 4°C. And 0.5 μl of ligation product was loaded in ABI 3730xl and then the raw data were analyzed by GeneMapper 4.1 (Applied Biosystems, Foster City, CA, USA). The primer sequences for PCR reaction and ligase reaction were described in **Table 1**.

The genotyping was carried out blinded to group status. For quality control, a random sample accounting for 5% of the total participants were selected (n = 29) and genotyped twice by different researchers, yielding a reproducibility of 100%.

Correlation analysis for WT1 SNPs and mRNA expression levels

To elucidate possible underlying mechanisms, we analyzed the correlation between *WT1* mRNA expression levels and variant genotypes as previous report [40] following the instructions of SNPexp (http://app3.titan.uio.no/biotools/tool.php?appsnpexp) [41]. The genotyping data were from the International HapMap Project (http://hapmap.ncbi.nlm.nih.gov/) [42] consisting of 3.96 million SNP genotypes from 270 individuals in four ethnic groups, and the data on transcript expression levels were from genome-wide expression arrays (47294 transcripts) for EBV-transformed lymphoblastoid cell lines of the same 270 HapMap individuals [41].

Statistical analysis

Chi-square and *t* tests were used to assess differences in the distributions of age and menopausal status between breast cancer cases and controls. *Chi*-square and fisher exact tests were used to explore the relationship between characteristics of breast cancer and the alleles and genotypes of *WT1* gene. The Hardy-Weinberg equilibrium (HWE) was tested by a goodness-of-fit chi-square test to compare the expected genotype frequencies with observed genotype frequencies in healthy controls.

The associations between case-control status and each specific *WT1* SNP were measured by the odds ratio (OR) and its corresponding 95% confidence interval (CI) using an unconditional logistic regression model with adjustments of age (continuous variable) and menopausal status (pre- or post-menopause). The ORs were performed for codominant model, dominant model, recessive model and allele contrast, respectively. Stratified analyses were done by age (≥ 50 or < 50 years old) and menopausal status (pre- or post-menopause) to evaluate the stratum variable-related ORs among the *WT1* genotypes and alleles. When analysis was stratified by menopausal status, the ORs were calculated only with adjustment of age (continuous variable).

GraphPad Prism (version 6.0; GraphPad Software Inc., La Jolla, CA, USA) was used to explore the differences between the distributions of *WT1* genotypes and mRNA expression level using one-way ANOVA test and make the

Table 3. Characteristics of WT1 SNPs selected in the case-control study

Gene	SNP	Chr.	SNP Property	Allele	*P* for HWE[a]	MAF			
						Allele	Controls (%)	Cases (%)	CHB[b] (%)
WT1	rs16754	11	exon7	G/A	0.1442	A	29.17	26.59	25.20
WT1	rs3930513	11	intron1	T/G	0.3464	G	26.84	24.33	22.10
WT1	rs5030141	11	intron1	A/C	0.3369	C	31.71	28.84	27.70
WT1	rs5030317	11	3'-UTR	C/G	0.1203	G	27.70	25.04	23.70
WT1	rs5030320	11	3'-UTR	G/A	0.0826	A	27.70	24.96	23.20

HWE: Hardy Weinberg Equilibrium; MAF: minimal allele frequency; [a]goodness-of-fit chi-square test for HWE for genotype distribution in controls. [b]Chinese Han in Beijing, data from http://hapmap.ncbi.nlm.nih.gov/.

graphic. All other kinds of tests were done using the SAS software (version 9.3; SAS Institute, Cary, NC, USA). All tests were all two-sided, and $P < 0.05$ was considered statistically significant.

Results

Population characteristics

A total of 709 female breast cancer cases and 749 controls were enrolled in this study. As shown in **Table 2**, the mean age was 48.3695 ± 10.3534 years for cases and 44.8144 ± 10.0065 years for controls ($P < 0.0001$). There were more women older than 50 years (35.12% vs. 25.77%, $P = 0.0001$) and more post-menopausal women (36.95% vs. 30.71%, $P = 0.0117$) in breast cancer group than in control group.

Frequencies and features of WT1 SNPs in breast cancer patients and controls

The detailed information of these five SNPs was described in **Table 3**. The MAF of these SNPs in controls were 29.17% for rs16754, 26.84% for rs3930513, 31.71% for rs5030141, 27.70% for rs5030317, 27.70% for rs5030320, respectively. Similar frequencies of these SNPs were presented in breast cases. The distributions of the minor alleles were consistent with the data from CHB. The observed genotype frequencies of the five polymorphisms in controls conformed to the HWE ($P = 0.1442$ for rs16754, 0.3464 for rs3930513, 0.3369 for rs5030141, 0.1203 for rs5030317, 0.0826 for rs5030320, respectively).

Correlation between WT1 SNPs alleles and genotypes and risk of breast cancer

As shown in **Table 4**, individuals with the minor alleles of rs16754, rs5030317 and rs5030320 showed a significant decrease of breast cancer risk in codominant model (OR = 0.6370, 95% CI: 0.4260-0.9520 for rs16754; OR = 0.5940, 95% CI: 0.3890-0.9070 for rs5030317; OR = 0.5870, 95% CI: 0.3850-0.8960 for 5030320, respectively) and recessive model (OR = 0.6420, 95% CI: 0.4340-0.9490 for rs16754; OR = 0.5990, 95% CI: 0.3960-0.9060 for rs5030317; OR = 0.5920, 95% CI: 0.3920-0.8950 for 5030320, respectively), compared with the corresponding controls.

The same significant results were also found in the population with age equal or less than 50 years old under codominant model (OR = 0.5960, 95% CI: 0.3690-0.9620 for rs16754; OR = 0.5510, 95% CI: 0.3330-0.9120 for rs5030317; OR = 0.5440, 95% CI: 0.3290-0.8980 for 5030320, respectively) and recessive model (OR = 0.6110, 95% CI: 0.3840-0.9730 for rs16754; OR = 0.5660, 95% CI: 0.3460-0.9250 for rs5030317; OR = 0.5570, 95% CI: 0.3420-0.9090 for 5030320, respectively), compared with the corresponding controls (**Table 5**). However, no significant differences were demonstrated in the population older than 50 years.

When stratified by menopausal status (**Table 6**), the significant association with decreased breast cancer risk was additionally identified in rs3930513, except for rs16754, rs5030317 and rs5030320, in pre-menopausal persons under codominant model (OR = 0.5400, 95% CI: 0.3290-0.8860 for rs16754; OR = 0.5350, 95% CI: 0.3120-0.9180 for rs3930513; OR = 0.5170, 95% CI: 0.3090-0.8660 for rs5030317; OR = 0.5080, 95% CI: 0.3040-0.8500 for rs5030320, respectively) and recessive model (OR = 0.5420, 95% CI: 0.3350-0.8780 for rs16754; OR = 0.5540, 95% CI: 0.3270-0.9400 for rs3930513; OR = 0.5080, 95% CI: 0.3040-0.8500 for rs5030317; OR = 0.5130, 95% CI:

Table 4. Overall analyses of the associations between WT1 genotypes and breast cancer risk

SNPs	Genetic model	Genotype	Controls (n = 749)	Cases (n = 709)	Adjusted OR[a]	*P*
rs16754	Codominant	GG	384	378	reference	
		GA	293	285	0.9830 (0.7880-1.2260)	0.8777
		AA	72	46	0.6370 (0.4260-0.9520)	0.0280
	Dominant	GG	384	378	reference	
		GA + AA	365	331	0.9140 (0.7410-1.1270)	0.3992
	Recessive	GG + GA	677	663	reference	
		AA	72	46	0.6420 (0.4340-0.9490)	0.0261
	Allele contrast	G	1061	1041	reference	
		A	437	377	0.8720 (0.7390-1.0280)	0.1034
rs3930513	Codominant	TT	406	405	reference	
		TG	284	263	0.9270 (0.7430-1.1570)	0.5046
		GG	59	41	0.6810 (0.4440-1.0450)	0.0790
	Dominant	TT	406	405	reference	
		TG + GG	343	304	0.8840 (0.7160-1.0920)	0.2538
	Recessive	TT + TG	690	668	reference	
		GG	59	41	0.7020 (0.4620-1.0680)	0.0981
	Allele contrast	T	1096	1073	reference	
		G	402	345	0.8710 (0.7340-1.0320)	0.1098
rs5030141	Codominant	AA	355	364	reference	
		AC	313	281	0.8770 (0.7020-1.0950)	0.2467
		CC	81	64	0.7800 (0.5410-1.1250)	0.1841
	Dominant	AA	355	364	reference	
		AC + CC	394	345	0.8570 (0.6950-1.0570)	0.1501
	Recessive	AA + AC	668	645	reference	
		CC	81	64	0.8280 (0.5820-1.1770)	0.2933
	Allele contrast	A	1023	1009	reference	
		C	475	409	0.8770 (0.7460-1.0310)	0.1116
rs5030317	Codominant	CC	400	394	reference	
		CG	283	275	0.9770 (0.7840-1.2190)	0.8398
		GG	66	40	0.5940 (0.3890-0.9070)	0.0158
	Dominant	CC	400	394	reference	
		CG + GG	349	315	0.9040 (0.7320-1.1150)	0.3453
	Recessive	CC + CG	683	669	reference	
		GG	66	40	0.5990 (0.3960-0.9060)	0.0152
	Allele contrast	C	1083	1063	reference	
		G	415	355	0.8590 (0.7260-1.0160)	0.0758
rs5030320	Codominant	GG	401	395	reference	
		GA	281	274	0.9780 (0.7840-1.2210)	0.8457
		AA	67	40	0.5870 (0.3850-0.8960)	0.0135
	Dominant	GG	401	395	reference	
		GA + AA	348	314	0.9020 (0.7310-1.1130)	0.3367
	Recessive	GG + GA	682	669	reference	
		AA	67	40	0.5920 (0.3920-0.8950)	0.0129
	Allele contrast	G	1083	1064	reference	
		A	415	354	0.8550 (0.7230-1.0120)	0.0688

[a]adjusted by age (continuous variable) and menopausal status.

Table 5. Stratified analyses of the associations between *WT1* genotypes and breast cancer risk by age of 50 years

SNPs	Genetic model	Genotype	≤ 50 years				> 50 years			
			Controls (n = 556)	Cases (n = 460)	adjusted OR[a]	*P*	Controls (n = 193)	Cases (n = 249)	adjusted OR[a]	*P*
rs16754	Codominant	GG	283	249	reference		101	129	reference	
		GA	218	180	0.9430 (0.7240-1.2290)	0.6652	75	105	1.1240 (0.7490-1.6890)	0.5718
		AA	55	31	0.5960 (0.3690-0.9620)	0.0340	17	15	0.7960 (0.3710-1.7090)	0.5584
	Dominant	GG	283	249	reference		101	129	reference	
		GA + AA	273	211	0.8710 (0.6780-1.1200)	0.2813	92	120	1.0670 (0.7230-1.5730)	0.7450
	Recessive	GG + GA	501	429	reference		176	234	reference	
		AA	55	31	0.6110 (0.3840-0.9730)	0.0378	17	15	0.7560 (0.3600-1.5880)	0.4597
	Allele contrast	G	784	678	reference		277	363	reference	
		A	328	242	0.8370 (0.6860-1.0200)	0.0778	109	135	0.9920 (0.7300-1.3480)	0.9607
rs3930513	Codominant	TT	301	266	reference		105	139	reference	
		TG	211	168	0.9060 (0.6950-1.1810)	0.4660	73	95	1.0290 (0.6830-1.5490)	0.8921
		GG	44	26	0.6240 (0.3710-1.0500)	0.0759	15	15	0.8810 (0.4030-1.9270)	0.7512
	Dominant	TT	301	266	reference		105	105	reference	
		TG + GG	255	194	0.8560 (0.6650-1.1020)	0.2283	88	110	1.0050 (0.6800-1.4840)	0.9811
	Recessive	TT + TG	512	434	reference		178	234	reference	
		GG	44	26	0.6500 (0.3900 -1.0810)	0.0967	15	15	0.8710 (0.4060-1.8690)	0.7225
	Allele contrast	T	813	700	reference		283	373	reference	
		G	299	220	0.8420 (0.6860-1.0320)	0.0979	103	125	0.9800 (0.7170-1.3400)	0.8994
rs5030141	Codominant	AA	263	239	reference		92	125	reference	
		AC	232	178	0.8540 (0.6540-1.1140)	0.2437	81	103	0.9870 (0.6550-1.4850)	0.9483
		CC	61	43	0.7690 (0.4990-1.1870)	0.2359	20	21	0.8440 (0.4230-1.6840)	0.6297
	Dominant	AA	263	239	reference		92	125	reference	
		AC + CC	293	221	0.8360 (0.6510-1.0740)	0.1615	101	124	0.9590 (0.6500-1.4140)	0.8318
	Recessive	AA + AC	495	417	reference		173	228	reference	
		CC	61	43	0.8260 (0.5440-1.2530)	0.3684	20	21	0.8490 (0.4370-1.6500)	0.6291
	Allele contrast	A	758	656	reference		265	353	reference	
		C	354	264	0.8630 (0.7110-1.0470)	0.1349	121	145	0.9440 (0.7010-1.2720)	0.7044
rs5030317	Codominant	CC	296	260	reference		104	134	reference	
		CG	209	173	0.9390 (0.7200-1.2240)	0.6410	74	102	1.1150 (0.7430-1.6730)	0.6000
		GG	51	27	0.5510 (0.3330-0.9120)	0.0204	15	13	0.7520 (0.3340-1.6900)	0.4899
	Dominant	CC	296	260	reference		104	134	reference	
		CG + GG	260	200	0.8600 (0.6690-1.1070)	0.2428	89	115	1.0560 (0.7150-1.5580)	0.7854

	Recessive	CC + CG	505	433	reference		178	236	reference	
		GG	51	27	0.5660 (0.3460-0.9250)	0.0231	15	13	0.7170 (0.3250-1.5830)	0.4106
	Allele contrast	C	801	693	reference		282	370	reference	
		G	311	227	0.8210 (0.6710-1.0050)	0.0560	104	128	0.9830 (0.7200-1.3420)	0.9148
rs5030320	Codominant	GG	297	261	reference		104	134	reference	
		GA	207	172	0.9420 (0.7220-1.2280)	0.6571	74	102	1.1150 (0.7430-1.6730)	0.6000
		AA	52	27	0.5440 (0.3290-0.8980)	0.0173	15	13	0.7520 (0.3340-1.6900)	0.4899
	Dominant	GG	297	261	reference		104	134	reference	
		GA + AA	259	199	0.8600 (0.6680-1.1060)	0.2397	89	115	1.0560 (0.7150-1.5580)	0.7854
	Recessive	GG + GA	504	433	reference		178	236	reference	
		AA	52	27	0.5570 (0.3420-0.9090)	0.0193	15	13	0.7170 (0.3250-1.5830)	0.4106
	Allele contrast	G	801	694	reference		282	370	reference	
		A	311	226	0.8180 (0.6680-1.0010)	0.0508	104	128	0.9830 (0.7200-1.3420)	0.9148

[a]adjusted by age (continuous variable) and menopausal status.

Table 6. Stratified analyses of the associations between WT1 genotypes and breast cancer risk by menopausal status

SNPs	Genetic model	Geno-type	Pre-menopause				Post-menopause			
			Controls (n = 519)	Cases (n = 447)	adjusted OR[a]	*P*	Controls (n =2 30)	Cases (n = 262)	adjusted OR[a]	*P*
rs16754	Codominant	GG	262	236	reference		122	142	reference	
		GA	203	182	0.9890 (0.7520-1.3010)	0.9387	90	103	0.9750 (0.6710-1.4180)	0.8955
		AA	54	29	0.5400 (0.3290-0.8860)	0.0148	18	17	0.8410 (0.4140-1.7100)	0.6329
	Dominant	GG	262	236	reference		122	142	reference	
		GA + AA	257	211	0.8900 (0.6860-1.1550)	0.3817	108	120	0.9530 (0.6670-1.3630)	0.7927
	Recessive	GG + GA	465	418	reference		212	245	reference	
		AA	54	29	0.5420 (0.3350-0.8780)	0.0127	18	17	0.8500 (0.4260-1.6980)	0.6456
	Allele contrast	G	727	654	reference		334	387	reference	
		A	311	240	0.8290 (0.6760-1.0170)	0.0727	126	137	0.9440 (0.7100-1.2540)	0.6903
rs3930513	Codominant	TT	277	255	reference		129	150	reference	
		TG	198	168	0.9180 (0.6980-1.2080)	0.5418	86	95	0.9470 (0.6500-1.3810)	0.7782
		GG	44	24	0.5350 (0.3120-0.9180)	0.0232	15	17	1.0100 (0.4840-2.1120)	0.9779
	Dominant	TT	277	255	reference		129	150	reference	
		TG + GG	242	192	0.8450 (0.6510-1.0980)	0.2082	101	112	0.9560 (0.6680-1.3700)	0.8084
	Recessive	TT + TG	475	423	reference		215	245	reference	
		GG	44	24	0.5540 (0.3270-0.9400)	0.0284	15	17	1.0320 (0.5010-2.1250)	0.9313

	Allele contrast	T	752	678	reference		344	395	reference	
		G	286	216	0.8130 (0.6590-1.0040)	0.0544	116	129	0.9760 (0.7300-1.3060)	0.8717
rs5030141	Codominant	AA	243	231	reference		112	133	reference	
		AC	216	176	0.8730 (0.6630-1.1500)	0.3344	97	105	0.9010 (0.6190-1.3130)	0.5886
		CC	60	40	0.6920 (0.4410-1.0860)	0.1096	21	24	0.9800 (0.5160-1.8610)	0.9497
	Dominant	AA	243	231	reference		112	133	reference	
		AC + CC	276	216	0.8330 (0.6420-1.0810)	0.1689	118	129	0.9150 (0.6410-1.3070)	0.6263
	Recessive	AA + AC	459	407	reference		209	238	reference	
		CC	60	40	0.7360 (0.4770-1.1350)	0.1653	21	24	1.0270 (0.5530-1.906)	0.9337
	Allele contrast	A	702	638	reference		321	371	reference	
		C	336	256	0.8400 (0.6880-1.0260)	0.0881	139	153	0.9530 (0.7240-1.2560)	0.7342
rs5030317	Codominant	CC	275	247	reference		125	147	reference	
		CG	194	174	0.9830 (0.7470-1.2950)	0.9056	89	101	0.9630 (0.6630-1.4000)	0.8435
		GG	50	26	0.5170 (0.3090-0.8660)	0.0123	16	14	0.7570 (0.3540-1.6170)	0.4717
	Dominant	CC	275	247	reference		125	147	reference	
		CG + GG	244	200	0.8830 (0.6800-1.1470)	0.3517	105	115	0.9320 (0.6510-1.3330)	0.6991
	Recessive	CC + CG	469	421	reference		214	248	reference	
		GG	50	26	0.5080 (0.3040-0.8500)	0.0110	16	14	0.7680 (0.3650-1.6160)	0.4876
	Allele contrast	C	744	668	reference		339	395	reference	
		G	294	226	0.8220 (0.6670-1.0120)	0.0645	121	129	0.9180 (0.6870-1.2250)	0.5600
rs5030320	Codominant	GG	275	248	reference		126	147	reference	
		GA	193	173	0.9760 (0.7410-1.2860)	0.8644	88	101	0.9800 (0.6740-1.4240)	0.9139
		AA	51	26	0.5080 (0.3040-0.8500)	0.0099	16	14	0.7620 (0.3570-1.6280)	0.4829
	Dominant	GG	275	248	reference		126	147	reference	
		GA + AA	244	199	0.8740 (0.6730-1.1350)	0.3135	104	115	0.9460 (0.6610-1.3540)	0.7628
	Recessive	GG + GA	468	421	reference		214	248	reference	
		AA	51	26	0.5130 (0.3110-0.8480)	0.0092	16	14	0.7680 (0.3650-1.6160)	0.4876
	Allele contrast	G	743	669	reference		340	395	reference	
		A	295	225	0.8130 (0.6600-1.0020)	0.0520	120	129	0.9270 (0.6940-1.2380)	0.6066

[a]adjusted by age (continuous variable) and menopausal status.

Table 7. Associations between *WT1* polymorphisms and the clinicopathological characteristics of breast cancer patients

Parameter	rs16754			rs3930513			rs5030141			rs5030317			rs5030320		
	GG + CA (N=, %)	AA (N=, %)	P^a	TT + TG (N=, %)	GG (N=, %)	P^a	AA + AC (N=, %)	CC (N=, %)	P^a	CC + CG (N=, %)	GG (N=, %)	P^a	GG + GA (N=, %)	AA (N=, %)	P^a
Age			0.7121			0.8395			0.6852			0.7208			0.7208
≤ 50	429 (64.71)	31 (67.39)		434 (64.97)	26 (63.41)		417 (64.65)	43 (67.19)		433 (64.72)	27 (67.50)		433 (64.72)	27 (67.50)	
> 50	234 (35.29)	15 (32.61)		234 (35.03)	15 (36.59)		228 (35.35)	21 (32.81)		236 (35.28)	13 (32.50)		236 (35.28)	13 (32.50)	
Menopausal status			0.9996			0.5377			0.9243			0.7922			0.7922
pre-menopause	418 (63.05)	29 (63.04)		423 (63.32)	24 (58.54)		407 (63.10)	40 (62.50)		421 (62.93)	26 (65.00)		421 (62.93)	26 (65.00)	
post-menopause	245 (36.95)	17 (36.96)		245 (36.68)	17 (41.46)		238 (36.90)	24 (37.50)		248 (37.07)	14 (35.00)		248 (37.07)	14 (35.00)	
T stage			0.0530[b]			0.3201[b]			0.6784[b]			0.1075[b]			0.1075[b]
T0	57 (8.6)	0 (0.00)		55 (8.32)	24.88)		53 (8.37)	3 (4.69)		57 (8.52)	0 (0.00)		57 (8.52)	0 (0.00)	
T1 + T2	576 (86.88)	45 (97.83)		582 (87.13)	39 (95.12)		562 (87.13)	59 (92.19)		582 (87.00)	39 (97.50)		582 (87.00)	39 (97.50)	
T3 + T4	30 (4.52)	1 (2.17)		31 (4.64)	0 (0.00)		29 (4.50)	2 (3.13)		30 (4.478)	1 (2.50)		30 (4.478)	1 (2.50)	
N stage			0.5247			0.4938			0.2945			0.5745			0.3709
negative	401 (60.48)	30 (65.22)		404 (60.48)	27 (65.85)		396 (61.40)	35 (54.69)		405 (60.54)	26 (65.00)		404 (60.39)	30 (67.50)	
positive	262 (39.52)	16 (34.78)		264 (39.52)	14 (34.15)		249 (38.60)	29 (45.31)		264 (39.46)	14 (35.00)		262 (39.61)	16 (32.50)	
M stage			0.7607[b]			0.5111[b]			0.6031[b]			0.5080[b]			0.5080[b]
M0	618 (93.21)	44 (95.65)		622 (93.11)	40 (97.56)		603 (93.49)	59 (92.19)		623 (93.12)	39 (97.50)		623 (93.12)	39 (97.50)	
M1	45 (6.79)	2 (4.35)		46 (6.89)	1 (2.44)		42 (6.51)	5 (7.81)		46 (6.88)	1 (2.50)		46 (6.88)	1 (2.50)	
TNM stage			0.3589			0.252			0.4124			0.2925			0.2925
I + II	462 (69.68)	35 (76.09)		465 (69.61)	32 (78.05)		455 (70.54)	42 (65.63)		466 (69.66)	31 (77.50)		466 (69.66)	31 (77.50)	
III + IV	201 (30.32)	11 (23.91)		203 (30.39)	9 (21.95)		190 (29.46)	22 (34.38)		203 (30.34)	9 (22.50)		203 (30.34)	9 (22.50)	
ER			0.1064			0.0039			0.0049			0.3305			0.3305
-	222 (36.51)	21 (48.84)		220 (35.95)	23 (58.97)		211 (35.64)	32 (54.24)		226 (36.87)	17 (44.74)		226 (36.87)	17 (44.74)	
+	386 (63.49)	22 (51.16)		392 (64.05)	16 (41.03)		381 (64.36)	27 (45.76)		387 (63.13)	21 (55.26)		387 (63.13)	21 (55.26)	
Unknown	58 (8.18)			58 (8.18)			58 (8.18)			58 (8.18)			58 (8.18)		
PR			0.0176			0.0064			0.0085			0.1200			0.1200
-	254 (41.91)	26 (60.47)		255 (41.80)	25 (64.10)		245 (41.53)	35 (59.32)		259 (42.39)	21 (55.26)		259 (42.39)	21 (55.26)	
+	352 (58.09)	17 (39.53)		355 (58.20)	14 (35.90)		345 (58.47)	24 (40.68)		352 (57.61)	17 (44.74)		352 (57.61)	17 (44.74)	
Unknown	60 (8.46)			60 (8.46)			60 (8.46)			60 (8.46)			60 (8.46)		
HER-2			0.7497			0.9134			0.5479						
-	420 (69.08)	30 (71.43)		422 (69.18)	28 (70.00)		405 (68.88)	45 (72.58)		423 (69.00)	27 (72.97)	0.6115	422 (68.84)	28 (75.68)	0.3818
+	188 (30.92)	12 (28.57)		188 (30.82)	12 (30.00)		183 (31.12)	17 (27.42)		190 (31.00)	10 (27.03)		191 (31.16)	9 (24.32)	
Unknown	59 (8.32)			59 (8.32)			59 (8.32)			59 (8.32)			59 (8.32)		
Ki-67			0.6965			0.5839			0.2225						
-	314 (51.90)	22 (48.89)		317 (51.97)	19 (47.50)		309 (52.46)	27 (44.26)		320 (52.37)	16 (41.03)	0.1692	321 (52.45)	15 (39.47)	0.1203
+	291 (48.10)	23 (51.11)		293 (48.03)	21 (52.50)		280 (47.54)	34 (55.74)		291 (47.63)	23 (58.97)		291 (47.55)	23 (60.53)	
Unknown	59 (8.32)			59 (8.32)			59 (8.32)			59 (8.32)			59 (8.32)		

[a]Chi-square test; bfisher exact tests.

Table 8. Distribution of *WT1* variant genotypes and mRNA expression levels in Chinese Han Beijing population

SNPs	Wild-type			Heterozygous			Homozygous		
	N	Mean	SD	N	Mean	SD	N	Mean	SD
rs16754	23	6.1700	0.1751	19	6.1370	0.0100	3	6.0670	0.1340
rs3930513	24	6.1590	0.1296	17	6.1560	0.1705	3	6.0670	0.1340
rs5030141	22	6.1450	0.1253	18	6.1750	0.1702	5	6.0720	0.1219
rs5030317	24	6.1740	0.1720	17	6.1290	0.1018	3	6.0670	0.1340
rs5030320	24	6.1740	0.1720	17	6.1290	0.1018	3	6.0670	0.1340

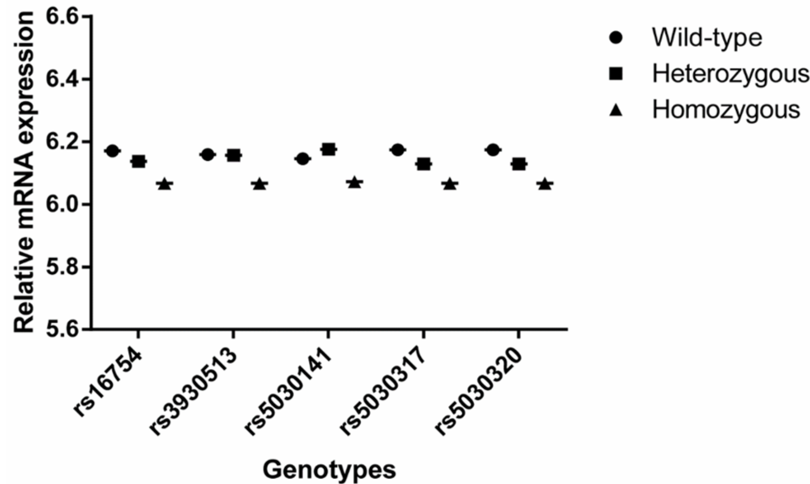

Figure 1. Effect of WT1 SNPs on mRNA expression in EBV-transformed lymphoblastoid cell lines derived from Chinese Han Beijing population.

0.3110-0.8480 for 5030320, respectively), compared with the corresponding controls. However, no significant differences were found in post-menopause group.

Association between WT1 SNPs and genotypes and clinicopathological characteristics in breast cancer patients

WT1 SNPs genotypes under recessive model and their relations to clinical and pathological characteristics of breast cancer patients were shown in **Table 7**. Patients with homozygous minor allele of rs3930513 and rs5030141 were more likely to be ER negative ($P = 0.0039$ and 0.0049, respectively), while, carriers with that of rs16754 and rs5030141 tended to be PR negative ($P = 0.0176$ and 0.0085, respectively). No significant differences were found between patients with wild-type/heterozygous minor allele and the patients with homozygous minor allele of the five *WT1* SNPs, with regard to age, menopausal status, T stage, N stage, M stage, TNM stage, HER-2 status and Ki-67 status ($P > 0.05$).

Correlation of WT1 mRNA expression levels with variant genotypes in CHB population

To explore possible underlying molecular mechanism, we performed genotype-phenotype correlation analysis using the available data on the *WT1* genotypes and mRNA expression levels of lymphoblastoid cell lines derived from CHB population. According to the distributions of *WT1* genotypes and mRNA expression level (**Table 8**), we found a significantly low level of *WT1* mRNA expression for homozygous rare allele of *WT1* gene, compared with wild-type and heterozygous group ($P = 0.0021$) as shown in **Figure 1**, which supports the finding of an association between *WT1* SNPs and reduced breast cancer risk.

Discussion

To the best of our knowledge, the associations between breast cancer susceptibility and *WT1* polymorphisms have not been detected in any population using case-control studies. Our results obtained from 709 breast cancer patients and 749 controls showed that minor alleles of rs16754, rs5030317 and rs5030320 were associated with reduced risk of breast cancer, especially in participants with age equal or less than 50 years old or in pre-menopausal status, which indicated that *WT1* SNPs could serve as a potential molecular marker for stratifying risk of breast cancer.

The *WT1* gene, which encodes the protein consisting of four zinc finger domains at the C terminus and a glutamine and proline-rich domain at the N terminus [43], was recently found to

play an oncogenic role in leukemogenesis and tumorigenesis, despite originally identified as a tumor suppressor gene [15, 29, 30, 44]. As for breast cancer, accumulating evidences have demonstrated that wild-type *WT1* gene plays an important role in the development and progression of breast cancer [45]. It was reported that *WT1* could be detected in 87% of primary breast carcinomas, but not in normal breast epithelium [21]. In addition, prognostic studies showed that high *WT1* mRNA level significantly correlated with worse outcomes of breast cancer [18, 25], and *WT1* could promote the proliferation and restrain the apoptosis of breast cancer cells [46-49], all indicating that *WT1* might serve as an oncogene in breast cancer.

SNPs are the most frequent sequence variations in the human genome, accounting for much of the phenotypic diversity among individuals. SNPs may lead to a change of the encoded amino acids (nonsynonymous) or be silent (synonymous) [50]. They may influence promoter activity (gene expression), mRNA stability, and subcellular localization of mRNAs and/or proteins [51, 52]. Evidences from population studies have shown that some SNPs can affect breast cancer risk [53-56]. A synonymous SNP of *WT1* gene in exon 7, rs16754, consisting of a substitution of the nucleotide adenine with guanine at nucleotide position 1293 [57], has recently been considered as a prognostic factor in patients with acute myeloid leukemia [35, 36, 58] and renal cell carcinoma [37] in different populations. These findings may be due to the fact that rs16754 genotype variations could affect translation kinetics [35] and/or drug sensitivity [36], which indicated that *WT1* SNPs might play certain roles in the biology of cancer; however, the potential impacts of *WT1* SNPs in the development of breast cancer have not been uncovered.

To explore the correlation between *WT1* SNPs and risk of breast cancer, we carried out the case-control study, and found that minor alleles of rs16754, rs5030317 and rs5030320 were significantly associated with reduced risk of breast cancer. The protective effects of these SNPs were more evident in participants with age equal or less than 50 years old or in pre-menopausal status; the deceased susceptibility to breast cancer was also demonstrated for rs3930513 in pre-menopausal subjects. In addition, our results also showed that breast cancer patients with homozygous minor allele of rs16754, rs3930513 and rs5030141 were more likely to be ER and/or PR negative. All of these findings suggest that *WT1* SNPs could be used as potentially promising biomarkers of individual genetic background to predict the risk of breast cancer, and support our hypothesis that *WT1* SNPs may be involved in the initiation, development and even progression of breast cancer, although molecular mechanisms underlying this observed association still need to be clarified.

According data from the International HapMap Project [42], the G(C) allele frequency of rs16754 detected in our study was in concordance with that reported in the CHB population. Together with the results from another Chinese population [58] and Thai [59] and Korean [60] populations, it can be concluded that G(C) is the major allele in Asian population, while, A(T) is the major allele in Western populations, which could be attributed to the differences in ethnicities [37]. In rs16754, it is reported that the rare codon CGA (6.2 per thousand) is replaced by the more frequently used codon CGG (11.4 per thousand) [37], leading to increased translation kinetics that could potentially affect protein folding and thereby protein [35]. In fact, it has been proposed that synonymous SNPs may change protein amount, structure or function through alterations in RNA stability, folding, or splicing; differences in tRNA selection; or binding of noncoding RNAs [36]. Therefore, it is biologically plausible that rs16754 could take part in the regulation of WT1 expression, and thereby, affect the development of cancer, including breast cancer. The distributions of minor allele of other four SNPs are also in accordance with the data from HapMap in CHB population. As for the their putative functions as predicted by SNP Function prediction (http://snpinfo.niehs.nih.gov/snpinfo/snpfunc.htm) [61] and F-SNP database (http://compbio.cs.queensu.ca/F-SNP/) [62], rs3930513 and rs5030141 might be transcription factor binding sites, while, rs5030317 and rs5030320 might be miRNA (hsa-miR-600, hsa-miR-936) binding sites, indicating the potential involvements of these SNPs in transcriptional regulation of target genes.

To identify the potential mechanism, we conducted bioinformatics genotype- phenotype correlation analysis in CHB population, and

found a relatively low level of *WT1* mRNA expression for homozygous rare allele of *WT1* gene, which could partially explain the association between *WT1* SNPs and decreased risk of breast cancer since *WT1* mRNA was shown to be over-expressed in breast cancer tissues and correlate with poor prognosis of breast cancer patients [18, 19, 21, 25]. Nevertheless, additional molecular researches are needed to finally establish the links between *WT1* genotypes and mRNA expression, because our preliminary results were only based on cell lines and bioinformatics analyses. In fact, even for rs16754, the most extendedly studied SNP of *WT1*, its impacts on *WT1* mRNA expression [35-37] as well as targeted gene- and microRNA-expression patterns [63] have been largely unknown and inconsistent. Of course, the possibility that these SNPs may be in linkage disequilibrium with other untyped functional polymorphisms or susceptibility gene could not be excluded, which are ongoing to be identified by researchers.

In summary, our findings provide strong evidence linking minor alleles of rs16754, rs5030317 and rs5030320 to reduced risk of breast cancer, and the protective effects are more evident in the subjects with age equal or less than 50 years old or in pre-menopausal status. Therefore, *WT1* SNPs could be used as a potential biomarker to predict the susceptibility to breast cancer of individuals. Large prospective and molecular epidemiological studies are needed to verify the observed relationship and clarify its underlying mechanism.

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Disclosure of conflict of interest

Authors have no relevant, potential conflicts of interest to declare.

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