Correlations between *lncRNA-SOX2OT* polymorphism and susceptibility to breast cancer in a Chinese population

Aim: We evaluated the effect of *lncRNA SOX2OT* expression and its SNP in tumorigenesis and development of breast cancer (BC) in a case-control study. **Methods:** A total of 1106 individuals, 505 newly diagnosed BC patients and 601 age-matched controls (± 2 years) were testified. Real-time PCR was adopted for testing tissues' *SOX2OT* expression. Genotyping of rs9839776 were conducted by using SNaPshot assays. **Results:** *SOX2OT* were overexpressed in BC tissues ($p < 0.001$). *SOX2OT* SNP rs9839776 was strongly associated with the higher expression of *SOX2OT* and an increased risk of BC in Chinese women (odds ratio: 1.42; 95% CI: 1.06–1.90; $p = 0.018$). **Conclusion:** These results confirmed that BC was conspicuously associated with higher *SOX2OT* expression. *SOX2OT* SNP rs9839776 was significantly associated with the onset of BC possibly via influencing the expression of *SOX2OT*.

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Keywords: breast cancer • long noncoding RNA • polymorphism • *SOX2OT*

Breast cancer (BC), also known as breast tumor, is one of the most frequently diagnosed malignant tumors and the major life-threatening diseases for women worldwide. According to the statistics of International Agency for Research on Cancer, there were 1.38 million new cases of BC, which accounted for 22.9% of all female malignant tumor incidences. A total of 460 thousand deaths were caused by BC, accounting for 13.7% of all female malignant tumor mortality worldwide in 2008 [1]. BC is the second most common malignant tumor for Chinese women. In China, there were approximately 169 thousand new female patients with BC and 45 thousand died of BC each year [2]. For the BC survival rate, according to the statistics from 1995 to 2007 in developed countries such as Australia, Canada, Denmark and the UK, the 5-year survival rate of BC patients could be up to 85–90% [3]. In the urban areas of China, the 5-year survival rate of BC patients was 70% and was increasing gradually. Although traditional treatments such as

radiotherapy, chemotherapy and surgery have evolved and improved substantially, the cure rate of BC is still poor [4]. Recent studies have provided evidence that the pathogenesis of BC is complicated and environmental and genetic factors (as well as their potential interactions) contribute to the occurrence and development of BC [5]. Therefore, it is urgently required to promote effective markers of BC for early prognosis and intervention.

Long noncoding RNAs (*lncRNAs*, from 200 bases to 100 kb in length) are transcribed RNA molecules. Lacking an open reading frame, *lncRNAs* have no protein-coding capability [6]. *lncRNAs* are demonstrated to involve in numerous cellular and carcinogenesis processes, including cell growth [7], tumorigenesis [8] and transcriptional regulation [9]. *lncRNAs* are also associated with a number of diseases such as cardiovascular disease [10], neurodegeneration disease [11] and carcinoma [12]. So far, 17 *lncRNAs* have been identified for their crucial role in the tumor bio-

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logy of BC, including BCAR4, PVT1, MALAT1 and GAS5 according to the lncRNA disease database [13].

The effects of SNPs on functional lncRNAs are drawing more attention because of their potential influence on the risks of cancers, including BC: e.g., rs12325489 in the BC-related lncRNA-ENST00000515084 [14]. These evidences support the hypothesis that the SNPs in lncRNAs impact the occurrence and development of BC and other human cancers.

SOX2 overlapping transcript (SOX2OT) is an lncRNA located in the intronic region of *SOX2* gene. Although recent studies have already demonstrated that the alterations of *SOX2OT* expression and successive change in *SOX2* expression are associated with BC [15], the function of *SOX2OT* is not fully understood. The

rs9839776 is an intron variant in the *SOX2OT* gene, and the only SNP reported in *SOX2OT* that has statistical significance for the expression of *SOX2OT* in one genome wide association study [16]. So far no study combining the effect of *SOX2OT* polymorphisms and BC risk has been performed in a Chinese population. In order to better understand its potential association with *SOX2OT* expression, we performed a case–control study to evaluate and quantify the association between rs9839776 C>T and the BC risk in a Chinese population.

Materials & methods

Study subjects

In the current study, a total of 1106 female participants, 505 patients with newly diagnosed BC and 601 age-

Table 1. Demographic characteristics and clinical features.

Variables	Cases (n = 505)		Controls (n = 601)		p-value [†]
	n	%	n	%	
Age (years):					
– Mean ± SD	56.9 ± 8.9		57.0 ± 8.4		
– <55	219	43.3	256	42.6	0.852
– ≥55	286	56.7	345	57.4	
Histological types:					
– Invasive ductal carcinoma	309	61.1	–	–	–
– Invasive lobular carcinoma	196	38.9	–	–	–
Differentiation grade:					
– Well (G1)	55	11.0	–	–	–
– Moderate (G2)	352	69.7	–	–	–
– Poor (G3)	98	19.3	–	–	–
Tumor stage:					
– pT1 (<2 cm)	238	47.1	–	–	–
– pT2 (2–5 cm)	207	41.0	–	–	–
– pT3 (≥5 cm)	35	6.9	–	–	–
– pT4 (exophytic)	25	5.0	–	–	–
Nodal status:					
– pN0	312	61.8	–	–	–
– pN1	144	28.5	–	–	–
– pN2	49	9.7	–	–	–
Estrogen receptor:					
– Positive	306	60.6	–	–	–
– Negative	199	39.4	–	–	–
Progesterone receptor:					
– Positive	276	54.7	–	–	–
– Negative	229	45.3	–	–	–

[†]Two-sided χ^2 test for the frequency distributions of selected variables between cases and controls.
SD: Standard deviation.

Figure 1. Expression of long noncoding RNA SOX2 overlapping transcript in both breast cancer tissues and adjacent normal tissues. (A) The expression of SOX2 overlapping transcript relative to β -actin in 41 patients measured by quantitative real-time PCR. (B) The expression of SOX2 overlapping transcript in estrogen receptor (+) and estrogen receptor (-) patients. (C) The expression of SOX2OT in progesterone receptor (+) and progesterone receptor (-) patients. ER: Estrogen receptor; lncRNA: long noncoding RNA; PR: Progesterone receptor; SOX2OT: SOX2 overlapping transcript.

matched (± 2 years) controls, were recruited. All of the blood samples of subjects were collected by Suzhou Center for Disease Prevention and Control and Tianjin Medical University Institute and Hospital between June 2012 and April 2015. None of the patients had received radiotherapy, chemotherapy or endocrine therapy before blood collection. The control participants were free of any type of cancer history and were selected from 10,000 individuals who participated in a community-based chronic disease program of Suzhou

Center for Disease Prevention and Control during the same period as the cases were collected. At recruitment, informed consent was obtained from each participant. The study is in compliance with the Declaration of Helsinki and approved by the Research Ethics Committee of Jiangsu Provincial Center for Disease Prevention and Control (no. 2012025).

Cancerous tissues and noncancerous tissues were collected from 41 patients, involved in the total 503 patients, for further investigation of the SOX2OT

expression in BC tissues and the association of *SOX2OT* SNP with the *SOX2OT* expression (data were shown in Supplementary Table 1).

DNA & RNA extraction

A total of 5 ml peripheral blood was collected in EDTA and taken for DNA isolation and genotyping. Genomic DNA was extracted from the blood samples of subjects by using the QIAcube HT Plasticware and QIAamp 96 DNA QIAcube HT Kit® (Qiagen, Dusseldorf, Germany) following the manufacturer’s protocol and then stored at -80° until use.

Genotype analysis

In the current study, we conducted genotyping of SNP rs9839776 by using SNaPshot assays. We first purified 2 µl PCR product with 0.3 µl of shrimp alkaline phosphatase® (Thermo Fisher Scientific, MA, USA) and incubated at 37°C for 100 min in turn 75°C for 15 min which will inactivate the enzyme. Then the purification of SNaPshot extension reactions was conducted in a volume of 5µl mixture which contains 2µl SNaPshot ready reaction mix® (Applied Biosystems, CA, USA), 1.5 µl PCR product, and 1 µl of extension primers. The PCR cycling conditions were 96°C for 10 s, 51°C for 5 s and 60°C for 30 s (25 cycles). A total of 0.5 µl of shrimp alkaline phosphatase® (TAKARA, Dalian, China) was incubated with the ultimate product at 37°C for 1 h and at 75°C for 15 min which will inactivate the enzyme. A total of 8.5 µl of Hi-Di Formamide® and 0.5 µl of GeneScan-120 LIZ size standard® (Applied Biosystems) were mixed with 1 µl of the purified minisequencing products. And then, the compound was denatured at 95°C for 5 min. The raw data were collected using 3730 Genetic Analyzer Data Col-

lection Software version 3.0® and then analyzed with GeneMapper Software v4.1® (Applied Biosystems).

Analysis of *SOX2OT* expression in BC tissues

Total RNA were extracted from 41 patients’ cancerous and noncancerous tissues using TRIzol reagent® (Invitrogen, CA, USA) following manufacturer’s protocol. The quality and quantity of RNA were measured by Nanodrop ONE^c spectrophotometer® (Thermo Fisher Scientific.) and stored at -80°. The cDNA was synthesized with Moloney murine leukemia virus (M-MLV) reverse transcriptase® (Invitrogen, Carlsbad, USA).

The expressions of *SOX2OT* in tissues were detected by ABI 7900HT fast Real-Time PCR system® (Applied Biosystems) with SYBR Green Assay (TAKARA, Dalian, China); the levels of β-actin were adopted as internal reference for normalization. All reactions were performed in triplicate.

Statistical analysis

Statistical analysis was performed by SPSS 23.0 software (IL, USA). Categorical variables are represented as percentages and continuous variables are described as the mean ± standard deviation. A χ² test was conducted for the Hardy–Weinberg equilibrium rule of the SNP loci of the rs9839776 in *SOX2OT* gene. Odds ratios (ORs) and 95% CI for genotypes were achieved under conditional logistic regression models. We considered p-values <0.05 as statistically significant.

Results

Participants’ characteristics

Demographic and clinical characteristics of study subjects (505 BC cases and 601 controls) are illustrated in Table 1. There is no significant difference in age between

Table 2. Distribution of genotype of rs9839776 polymorphism and the association with breast cancer risk.							
Genetic models	Genotypes	Cases		Controls		p-value [†]	Adjusted OR (95% CI) [†]
		n = 505	%	n = 601	%		
Codominant	CC	220	43.6	315	52.4	–	1.00 (Ref.)
	CT	243	48.1	247	41.1	0.028	1.40 (1.04–1.90)
	TT	42	8.3	39	6.5	0.144	1.54 (0.86–2.73)
Dominant	CC	220	43.6	315	52.4	–	1.00 (Ref.)
	CT/TT	285	56.4	286	47.6	0.018	1.42 (1.06–1.90)
Recessive	CC/CT	463	91.7	562	93.5	–	1.00 (Ref.)
	TT	42	8.3	39	6.5	0.348	1.30 (0.75–2.27)
Alleles	C	683	67.6	877	73.0	–	1.00 (Ref.)
	T	327	32.4	325	27.0	0.022	1.31 (1.04–1.66)
[†] Adjusted for age in logistic regression model. OR: Odds ratio.							

Table 3. Association between rs9839776 and clinical characteristics of breast cancer in a dominant model.

Variables	CC (case/control)		CT/TT (case/control)		p-value [†]	Adjusted OR (95% CI) [†]
	n	%	n	%		
Age (years):						
– ≤55	93/140	42.5/54.7	126/116	57.5/45.3	0.035	1.62 (1.04–2.52)
– >55	127/175	44.4/50.7	159/170	55.6/49.3	0.193	1.29 (0.88–1.90)
Histological types:						
– Invasive ductal carcinoma	139/315	45.0/52.4	170/286	55.0/47.6	0.094	1.33 (0.95–1.87)
– Invasive lobular carcinoma	81/315	41.3/52.4	115/286	58.7/47.6	0.026	1.58 (1.06–2.35)
Differentiation grade:						
– Well (G1)	31/315	56.4/52.4	24/286	43.6/47.6	0.598	0.83 (0.42–1.64)
– Moderate (G2)	155/315	44.0/52.4	197/286	56.0/47.6	0.037	1.41 (1.02–1.95)
– Poor (G3)	34/315	34.7/52.4	64/286	65.3/47.6	0.013	2.00 (1.16–3.45)
Tumor stage:						
– pT1 (<2 cm)	108/315	45.4/52.4	130/286	54.6/47.6	0.127	1.33 (0.92–1.93)
– pT2 (2–5 cm)	81/315	39.1/52.4	126/286	60.9/47.6	0.008	1.71 (1.15–2.53)
– pT3 (≥5 cm)	15/315	42.8/52.4	20/286	57.1/47.6	0.399	1.44 (0.62–3.36)
– pT4 (exophytic)	16/315	64.0/52.4	9/286	36.0/47.6	0.337	0.61 (0.22–1.68)
Nodal status:						
– pN0	149/315	47.8/52.4	163/286	52.2/47.6	0.264	1.21 (0.87–1.69)
– pN1	58/315	40.3/52.4	86/286	59.7/47.6	0.033	1.64 (1.04–2.58)
– pN2	13/315	26.5/52.4	36/286	73.5/47.6	0.008	2.93 (1.33–6.47)
Estrogen receptor:						
– Positive	132/135	43.1/52.4	174/286	56.9/47.6	0.032	1.45 (1.03–2.04)
– Negative	88/315	44.2/52.4	111/286	55.8/47.6	0.102	1.39 (0.94–2.06)
Progesterone receptor:						
– Positive	115/315	41.7/52.4	161/286	58.3/47.6	0.018	1.53 (1.07–2.17)
– Negative	105/315	45.9/52.4	124/286	54.1/47.6	0.161	1.31 (0.90–1.90)

[†]Two-sided χ^2 test for the frequency distributions of selected variables between cases and control.
OR: Odds ratio.

the two groups. In case group, 61.1% of BC patients are in invasive ductal carcinoma and 38.9% are in invasive lobular carcinoma. For differentiation, 11.0% of BC cases are in well grade (G1), 69.7% in moderate grade (G2) and 19.3% in poor grade (G3). Besides, 238 (47.1%), 207 (41.0%), 35 (6.9%) and 25 (5.0%) patients are with a pT1, pT2, pT3 and pT4 tumor stage, respectively. For nodal status, 312 patients are in pN0 (61.8%), 144 patients in N1 (28.5%) and 49 patients (9.7%) in N2. With respects to hormone receptors, 60.6% patients show positive ER and 54.7% patients show positive PR.

SOX2OT expression in BC

To confirm the role of SOX2OT in BC, we tested the SOX2OT RNA in 41 patients' both carcinoma and adjacent noncarcinoma tissues. In **Figure 1A**, the lncRNA SOX2OT displayed a significantly higher level in carci-

noma tissues than in adjacent normal tissues ($p < 0.001$, **Figure 1A**). Considering the effect of hormones in BC, we evaluated the associations between SOX2OT expression and both estrogen receptor (ER) and progesterone receptor (PR). As shown in **Figure 1B** and **1C**, upregulations of SOX2OT existed in ER positive (ER⁺) group, PR positive group and PR negative groups ($p < 0.05$), but not in ER negative (ER⁻) group ($p = 0.065$); the average expression of SOX2OT in BC tissues showed an unexpressed trend in the majority of ER⁻ patients' tissues (data were shown in **Supplementary Data**).

Association between SNP rs9839776 & BC risk

In this case–control study, SNP rs9839776 in the *SOX2OT* gene were selected and quantified. The allele and genotype distribution for rs9839776 among participants were shown in **Table 2**. The genotype dis-

Figure 2. Relevance of lncRNA *SOX2OT* SNP rs9830776 and expression of lncRNA *SOX2OT* expression levels in breast cancer tissues. CC genotype (n = 20) versus CT+TT genotypes (n = 21), $p = 0.011$ in a codominant model.

tribution of rs9839776 in controls conform to the Hardy–Weinberg equilibrium ($p = 0.396$). Patients with T allele of rs9839776 in the *SOX2OT* gene had higher risk of BC compared with individuals carrying C allele in Chinese women, with OR of 1.31 (95% CI: 1.04–1.66). After adjusted for age, the CT and TT genotypes showed significantly higher risk for BC compared with the CC genotype in the dominant model (adjusted OR: 1.42; 95% CI: 1.06–1.90; $p = 0.018$). In the codominant model, the CT genotype significantly increased BC risk compared with the CC genotype (adjusted OR: 1.40; 95% CI: 1.04–1.90; $p = 0.028$). However, in the recessive model, rs9839776 had no significant association with BC ($p = 0.348$).

Stratified analysis of SNP genotypes & BC risk characteristics

We further examined the impacts of rs9839776 genotypes in BC on various disease characteristics in the patient cohort, including age, histological types, differentiation grade, tumor stage, nodal status, ER and PR in a dominant model. The results are shown in Table 3. For the patients younger than 55, in the dominant model, combined genotypes (CT/TT) of rs9839776 had a significant 1.62-fold increase of BC risk compared with the genotype CC individuals (adjusted OR: 1.62; 95% CI: 1.04–2.52; $p = 0.035$). Besides, carriers with CT/TT genotypes and invasive lobular carcinoma histological type had a significantly higher BC risk (adjusted OR: 1.58; 95% CI: 1.06–2.35; $p = 0.026$). Furthermore, individuals with CT/TT were more prone to BC com-

pared with CC (adjusted OR: 1.41; 95% CI: 1.02–1.95; $p = 0.037$ for G2 and adjusted OR: 2.00; 95% CI: 1.16–3.45; $p = 0.013$ for G3, respectively). Based on these discoveries, we conclude that CC genotype has lower BC risk than CT/TT genotypes.

The relationship of rs9839776 with lncRNA *SOX2OT* expression

We revealed the impact of lncRNA-related SNP on *SOX2OT* expression. The influences of rs9839776 different genotypes on *SOX2OT* expression is presented in Figure 2. Individuals with CT+TT genotype have significantly higher *SOX2OT* expression levels in BC tissues ($p = 0.011$) than CC genotype.

Discussion

In this study, we have identified the impact of *SOX2OT* in BC. There is a higher expression level of *SOX2OT* mRNA in BC tissues, especially in ER⁺ patients. The SNP rs9839776 polymorphism in *SOX2OT* gene is genotyped, and evaluated for its association with BC risk in a Chinese women population. We have revealed that SNP rs9839776 in the *SOX2OT* gene is significantly associated with increased BC risk in Chinese women population. Patients carrying T allele of rs9839776 have higher risk of BC compared with C allele. For women at age ≤ 50 years, significantly higher risk of BC was found in individuals with CT/TT genotypes of rs9839776 than CC genotype (OR: 1.62; 95% CI: 1.04–2.52), which is consistent with the conception that BC is a complex disease and its etiology is related to both genetic and reproductive factors.

SOX2OT gene locates in the 3q26.3–q27 locus of human chromosome and harbors *SOX2* gene, which is one of the major regulators of pluripotency [17]. Unfortunately, the mechanism of *SOX2* regulation has not been fully understood. So far, *SOX2* has been discovered to participate in the generation process from adult human somatic cells to induced pluripotent stem cells, along with OCT4, c-Myc and Klf4 [18]. Recent research on squamous cell carcinoma of the skin tumors suggests that *SOX2* could take part in the promotion of tumor initiation and control of cancer stem cell properties [19]. While the exact role of *SOX2OT* is not yet clear, emerging evidence has demonstrated its positive role in transcription regulation of *SOX2* gene. Several studies have demonstrated that the alterations of *SOX2OT* expression and corresponding expression change of *SOX2* have been associated with BC [15]. A previous study analyzed the expression of lncRNA *SOX2OT* in BC tumor tissues and their corresponding adjacent noncancerous tissues and found that lncRNA *SOX2OT* showed significant overexpression in BC tumor tissues

compared with adjacent noncancerous tissues [20]. Another experiment demonstrated that the expression of *SOX2* is concordant with *SOX2OT* expression in BC samples, and ER⁺ and ER⁻ BC samples had distinct *SOX2* and *SOX2OT* expression. They also demonstrated that increased *SOX2* expression level was due to the ectopic expression of *SOX2OT*, which caused a decreased proliferation and increased BC cell anchorage-independent growth [21]. In our study, *SOX2OT* showed a lower expression in ER⁻ BC tissues (Supplementary Data & Figure 1), which partly supported the previous study. Besides, the rs9839776 polymorphism showed no significance in ER⁻ BC tissues in our case-control study. This is a convincing evidence that in ER⁻ BC, the mechanism of *SOX2OT* and *SOX2* could be different from other types of BC.

According to the data of BC gene expression from Cancer Genome Atlas and RNA sequencing data in BC tissues samples, the expression of *SOX2OT* is consistent with *SOX2* in BC through elaborate analysis of the genome-wide RNA transcript profiles [15]. It has been widely accepted that the expression of adjacent antisense and overlapped genes could be regulated by lncRNAs through various mechanisms [22]. The intronic region of *SOX2OT* harbors *SOX2* gene, a hypothesis that *SOX2* expression could be regulated by *SOX2OT* gene was proposed. Several experimental results obtained from gene expression alteration during carcinogenesis have supported this hypothesis [23]. Thus, dysregulation of *SOX2OT* may play an important role in tumorigenesis and development of BC through the regulation of *SOX2* expression. In our study, the rs9839776 CT+TT genotypes are significantly associated with a higher expression of *SOX2OT*, and T allele of rs9839776 in *SOX2OT* gene increased a 1.31-fold risk for BC in Chinese women. These results are mostly consistent with the previous studies and, therefore, we herein conclude that SNP rs9839776 results in ectopic expression of *SOX2OT* which causes higher *SOX2* expression level, with an increased BC cell growth.

As limitations in our study, the inherent selecting bias and information bias were the major unavoidable problems, because all participants were recruited from hospitals. The second limitation could arise from the relatively small sample size, which impact the statistical power of our study. Third, the lack of HER2

information in patients enrolled in 2012 also limited further investigation for triple-negative BC. Last, the explicit mechanism of rs9839776 on regulating *SOX2OT* expression was still bewildered; increasing direct function experiments should be required.

Conclusion

In conclusion, we have confirmed the overexpression of lncRNA *SOX2OT* in BC tissues in Chinese female patients. Furthermore, we have identified significant association between SNP rs9839776 and BC risk. Also, the influence of SNP rs9839776 on *SOX2OT* gene expression was pointed out. Our study provides a new insight on the relationship between *SOX2OT* gene and breast tumorigenesis, and contributes to the potential early detection of BC.

Supplementary data

To view the supplementary data that accompany this paper, please visit the journal website at: www.futuremedicine.com/doi/full/10.2217/bmm-2016-0238

Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The study is in compliance with the Declaration of Helsinki and approved by the Research Ethics Committee of Jiangsu Provincial Center for Disease Prevention and Control (No. 2012025).

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Executive Summary

- Dysregulation of *SOX2* overlapping transcript (*SOX2OT*) was significantly higher in breast cancer (BC) tissues than in normal tissues.
- C allele of *SOX2OT* rs9839776 polymorphism could cause the increased risk of BC.
- CC/CT genotypes of rs9839776 were strongly related to the higher expression of *SOX2OT*.
- SNPs rs9839776 could be a novel potential biomarker of BC.

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