Original Article

Downregulation of hsa_circ_0006220 and its correlation with clinicopathological factors in human breast cancer

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Background: Circular ribonucleic acids (circRNAs) are highly stable and conserved forms of RNAs present in all eukaryotes. They can modulate the expression of genes by sponging specific micro RNAs (miRNAs), thereby affecting various disease processes. However, their expression pattern in human breast cancer has not been elucidated.

Methods: In this study, differentially expressed circRNAs in breast cancer tissues and paired noncancerous tissues were analyzed using an Arraystar Human circRNA Microarray, and hsa_circ_0006220 was selected for its 27-fold downregulation in breast cancer tissues. Its expression was also verified in 50 breast cancer and paired noncancerous tissues using real-time polymerase chain reaction (RT-PCR). An analysis of the expression of hsa_circ_0006220 and the clinicopathological factors in breast cancer was conducted. A receiver operating characteristic (ROC) curve of hsa_circ_0006220 was constructed. The interaction between hsa_circ_0006220 and five possible target miRNAs was predicted, and their expression were verified when overexpressing hsa_circ_0006220 by RT-PCR.

Results: Hsa_circ_0006220 was found to be significantly downregulated in breast cancer tissues compared to the paired noncancerous tissues by microarray and RT-PCR. The expression of hsa_circ_0006220 was significantly inversely correlated with histological type (P=0.0028) and lymph node metastasis (P=0.0341). The area under the ROC curve (AUC) was 0.706. Five miRNAs that might be sponged by hsa_circ_0006220 were predicted. MiR-197-5p was significantly downregulated after overexpression of hsa_circ_0006220.

Conclusions: Our results indicated that hsa_circ_0006220 may play a role in human breast cancer and might be a potential tumor marker for breast cancer screening.

Keywords: Breast cancer; circular ribonucleic acid (circRNA); hsa_circ_0006220; clinicopathological factors

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Introduction

Breast cancer is one of the most common malignant tumors. In China, the incidence of breast cancer-related death has been rapidly increasing (1,2). Local recurrence and distant metastasis of breast malignancies occur frequently, resulting in a poor prognosis for some patients with breast cancer. At present, no specific biomarker is available for the early diagnosis of breast cancer. Therefore, exploring new tumor markers for breast cancer screening is a top priority.

Circular ribonucleic acids (circRNAs), a novel class of endogenous RNA molecules (3), are more conservative and stable than linear RNAs (4). Accumulating evidence has illustrated that circRNAs can combine with and inhibit micro RNAs (miRNAs) to exert biological effects by acting as a miRNA "sponge" or can serve as transcription regulators (3,5-7). According to previous studies, a handful of human diseases, particularly multiple malignancies, are related to circRNAs (8-16). The circRNAs hsa_circ_001569 and hsa_circ_001988 are correlated with colorectal cancer (10,11). Hsa_circ_0001895 decreases in human gastric cancer and is correlated with clinical significance (17). Lü *et al*. (12) reported that several circRNAs screened using microarray were associated with breast cancer. Although, several circRNAs were reported having correlation with clinicopathological factors in human breast cancer, the understanding of how circRNAs influencing the progression of breast cancer remains limited (18,19). Hence, the expression pattern of circRNAs in breast cancer requires further exploration.

Herein, we found the 27-fold downregulation of hsa_circ_0006220 in breast cancer tissues using a circRNA microarray. The results were validated using real-time polymerase chain reaction (RT-PCR). Subsequently, the possible relationship between hsa_circ_0006220 and clinical outcomes was analyzed. This study provides new evidence for understanding the role of hsa_circ_0006220 in breast cancer. Hsa_circ_0006220 may serve as a potential tumor marker for breast cancer screening. We present the following article in accordance with the MDAR checklist (available at http://dx.doi.org/10.21037/gs-21-42).

Methods

Specimens

All specimens were obtained from The First Affiliated Hospital of China Medical University between January 2005 and December 2012. A total of 50 female patients were included in the study (from 27 to 81 years old). The inclusion criteria were as follows: (I) patients with primary breast cancer; (II) patients with no previous history of other malignancies; (III) patients that had not undergone chemotherapy, radiotherapy, endocrine therapy, or targeted therapy prior to surgery; and (IV) patients with no distant metastasis. Fresh breast cancer lesions and their paired noncancerous tissues were collected from patients who underwent surgical breast resection. The corresponding paired noncancerous tissues were over 5 cm from the edge of the tumors. The tissues were frozen and stored in ultra-low temperature freezer (Haier, China) at –80 ℃ until further use.

All pathological results were confirmed by two experienced histopathologists. The clinical information of the patients included age, histological type, tumor size, lymph node metastasis, histological grade, as well as the expression of estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER-2), and Ki67. The number of tumor cells positive for Ki67 expression was determined. The threshold of Ki67 was set at 14% according to the St. Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer (20). All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Ethics Committee of The First Affiliated Hospital of China Medical University (No. AF-0G-03-1.0-02). Written informed consent was retrieved from all participants in the study.

Cell culture

Human breast cancer cell line MDA-MB-231 was purchased from Cell bank of Chinese Academy of Sciences. MDA-MB-231 cellswere cultured in DMEM (Gibco, Carlsbad, CA, USA), containing 10% fetal bovine serum (Hyclone, Logan, USA), and were maintained at 37 ℃ with 5% CO_2 in a humidified incubator.

CircRNA microarray

A total of 2,465 circRNAs in three breast cancer tissues and paired noncancerous tissues were analysed using an Arraystar Human circRNA Microarray (KangChen Bio-tech, Shanghai, China). A NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) was used to quantify total RNA in each sample, and

microarray hybridization was performed according to the Arraystar standard protocol. The inclusion criteria were as follows: (I) fold change >2; (II) P value <0.05; (III) group raw intensity >200; and (IV) conserved expression levels. A 27-fold downregulation of circRNA hsa_circ_0006220 was selected for the next validation.

RT-PCR

Total RNA was extracted from tissues using a simple total RNA kit (BioTeke, Beijing, China), and was then reverse transcribed into complementary deoxyribonucleic acid (cDNA) with Moloney Murine Leukemia Virus (M-MLV) Reverse Transcriptase (BioTeke). The relative expression of circRNA was performed using SYBR®Premix Ex TaqTMII (Takara Bio, Dalian, China) and calculated using the $2^{-\Delta\Delta Ct}$ method via a Light Cycler 480 II sequence detection system (Roche Applied Science, Indianapolis, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control for normalization of circRNA while U6 for normalization of miRNAs. The primers for hsa_circ_0006220 were as follows: 5'-CTACCCTGCTGAACCTGAAACA-3' (forward) and 5'-TTCTCACACTCCTCCTTGGTCTT-3' (reverse). All the experiments were repeated thrice.

Vector construction and cell transfection

The mature sequence of hsa_circ_0006220 was synthesized and then cloned into vector (Genscript, Nanjing, China). The mock vector without its sequence served as control. Vectors were transiently transfected into MDA-MB-231 cells conducted with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocols.

Bioinformatics prediction

The target miRNAs and circRNA/miRNA binding sites were predicted using target prediction software from Arraystar (KangChen Bio-tech), which refers to miRanda (http://www.microrna.org/). The top five putative target miRNAs were selected to combine with hsa_circ_0006220 according to the prediction.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 7.0 software (GraphPad Software, LaJolla, CA, USA). The Wilcoxon signed-rank test was used to identify the differential expression of hsa_circ_0006220 in breast cancer tissues and paired noncancerous tissues. One-way ANOVA was used to verify the level changes of the five target miRNAs of hsa_circ_0006220. The Kruskal-Wallis and Mann-Whitney tests were used to identify the association between hsa_circ_0006220 levels and clinicopathological features. The statistical data were expressed as mean ± standard deviation (SD). The receiver operating characteristic (ROC) curve was constructed using the Statistical Product and Service Solutions (SPSS) 19.0 software (SPSS, Chicago, IL, USA). P<0.05 was regarded as statistically significant.

Results

Differential expression profiles of circRNAs in breast cancer tissue samples

A total of 2,465 circRNAs in three matched breast tissue samples were detected using a microarray. The distribution of the intensities of circRNAs in all tissue samples was contrasted using box plots, and the distributions were approximately the same in all tested samples (*Figure 1A*). Hierarchical clustering of circRNA expression profiles showed the expression pattern of circRNAs in matched breast tissue samples. According to the initial array data analysis, the differential expression of 520 identified circRNAs was statistically significant (P<0.05), with fold-change values ≥2.0. Among the differentially expressed circRNAs, 292 markedly upregulated circRNAs and 228 notably downregulated circRNAs were identified in the breast cancer tissues compared with paired noncancerous tissues (*Figure 1B*). The top 20 up- and down-regulated circRNAs and the fold changes were listed in Table S1.

Among all of the downregulated circRNAs, hsa_circ_0006220 was selected with a 27-fold downregulation in breast cancer based on the circRNA microarray. It was located at chr17:35800605-35800763, and its associated gene symbol was TADA2A. Its sequence was: TGACACAGCCAT TCCATTTCACTGCAGGATGTAGCCAATCAAATGT.

Verification of hsa_circ_0006220 expression in breast cancer tissues

The level of hsa_circ_0006220 in each cancer and paired noncancerous tissue was determined using RT-PCR. In

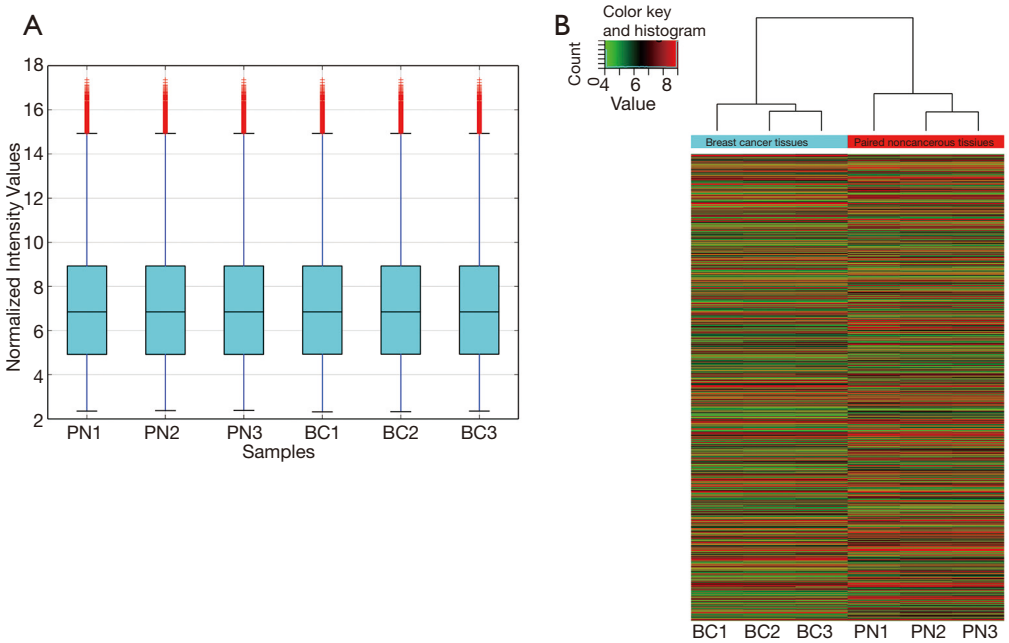

Figure 1 Different expression of circRNAs screened by microarray in human breast tissues. (A) Distribution of the intensities of circRNAs in all tissue samples was contrasted using box plots; (B) Hierarchical clustering analysis of distinguishable circRNA expression profiles of the samples obtained from the microarray data. Each column represents one sample and each row represents one circRNA. circRNAs, circular ribonucleic acids; BC, breast cancer tissues; PN, paired noncancerous tissues.

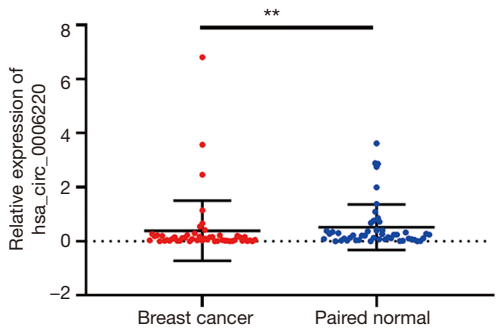

Figure 2 Verification of the differential expression levels of hsa_circ_0006220 between breast cancer and paired noncancerous tissues using RT-PCR (n=50). The expression of hsa_circ_0006220 was significantly downregulated in breast cancer tissues. $2^{-\Delta CT}$ values were used to calculate the expression; values were means ± SD; **, P<0.01.

50 paired tissues, hsa_circ_0006220 levels were downregulated in 72% (36/50) of breast cancer tissues relative to the paired noncancerous tissues. The levels of hsa_circ_0006220 in breast cancer were significantly lower than those in the paired noncancerous tissues (P<0.01, *Figure 2*). These results suggested a correlation between hsa_circ_0006220 and breast cancer.

Association between hsa_circ_0006220 levels and clinicopathological features

Consequently, the possible association between hsa_circ_0006220 levels and the clinicopathological features of breast cancer was investigated. As suggested in *Table 1*, the expression of hsa_circ_0006220 was related to histological type (P=0.0028) and lymph node metastasis (P=0.0341), indicating that hsa_circ_0006220 might represent a predictor for breast cancer prognosis. No association was found between the expression levels of hsa_circ_0006220 and other important clinicopathological features, including age (P=0.9375), tumor size (P=0.6018), ER status (P=0.3695), PR status (P=0.9065), HER-2 status (P=0.1866), Ki67 status (P=0.2331), and histological grade (P=0.1041).

ROC curve of hsa_circ_0006220

A ROC curve was established to estimate the diagnostic value of hsa_circ_0006220 in breast cancer. The area under

Table 1 Relationship between hsa_circ_0006220 expression and the clinicopathological features in patients with breast cancer

Characteristics	No. of cases [%]	hsa_circ_0006220 expression (mean ± SD)	P value
Age (year)			0.9375
≥60	12 [24]	0.408±1.002	
<60	38 [76]	0.399±1.152	
Tumor size (cm)			0.6018
<2	5 [10]	0.224±0.251	
2–5	38 [76]	0.399±1.211	
≥5	7 [14]	0.537±0.945	
Lymphatic metastasis			*0.0341*
N0	30 [60]	0.591±1.402	
N1–3	20 [40]	0.116±0.152	
Pathological type			*0.0028*
Ductal carcinoma *in situ*	6 [12]	1.856±2.578	
Invasive ductal carcinoma	40 [80]	0.211±0.564	
Others	4 [8]	0.123±0.0782	
Histological grade (invasive ductal carcinoma) (n=40)			0.1041
I	3 [7.5]	0.071±0.029	
II	33 [82.5]	0.223±0.620	
III	4 [10]	0.017±0.024	
Estrogen receptor			0.3695
Absent	9 [18]	0.276±0.366	
Present	41 [82]	0.428±1.213	
Progesterone receptor			0.9065
Absent	14 [28]	0.199±0.308	
Present	36 [72]	0.480±1.288	
HER-2			0.1866
Absent	8 [16]	0.594±1.207	
1+	17 [34]	0.251±0.579	
2+	17 [34]	0.609±1.626	
3+	8 [16]	0.084±0.085	
Ki-67			0.2331
<14%	6 [12]	0.207±0.174	
≥14%	44 [88]	0.427±1.178	

Italic P values indicate P<0.05. HER-2, human epidermal growth receptor 2.

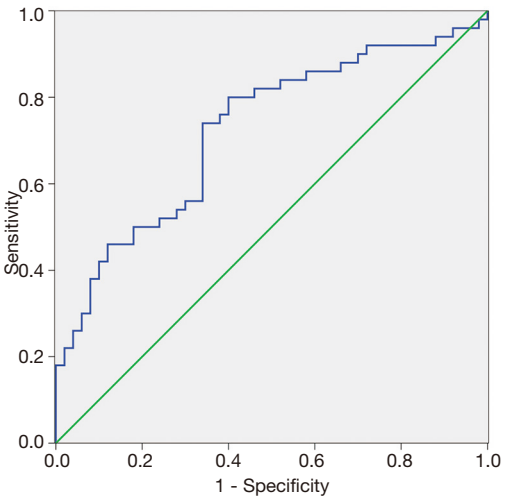

Figure 3 Assessment of the diagnostic value of hsa_circ_0006220 using a ROC curve comparing breast cancer and paired noncancerous tissues. The AUC was 0.706 (95% CI =0.603–0.808; P<0.001). ROC, receiver operating characteristic.

the ROC curve (AUC) was 0.706 [95% confidence interval (CI) =0.603–0.808; P<0.001; *Figure 3*].

Prediction of target genes of hsa_circ_0006220

Next, the target miRNAs of hsa_circ_0006220 were predicted using bioinformatics tools. Five miRNAs mostly affected by hsa_circ_0006220 were selected. The potential targeted miRNAs were miR-197-5p, miR-302b-3p, miR-302c-3p, miR-302d-3p, and miR-520d-3p. The binding sites were predicted and represented by vertical solid lines (*Figure 4*).

Verification of the five target miRNAs of hsa_circ_0006220

The expression of miR-197-5p, miR-302b-3p, miR-302c-3p, miR-302d-3p, and miR-520d-3p were measured when overexpressing hsa_circ_0006220 by RT-PCR in MDA-MB-231. The level of miR-197-5p was significantly downregulated after overexpression of hsa_circ_0006220 compared to the control group (P<0.0001), while the other 4 predicted miRNAs showed no obviously changes (*Figure 5*). Thus, the result shows miR-197-5p might be a target of hsa_circ_0006220 in breast cancer.

Discussion

CircRNAs are a special type of stable, conserved, endogenous RNA, and are ubiquitous in molecular cell biology (3,21,22). However, studies on breast cancer are few, and the understanding of circRNAs remains limited (18,19). In this study, a novel circRNA, hsa_circ_0006220, was selected using a microarray. The expression of hsa_circ_0006220 was found to be correlated with histological type and lymph node metastasis. These results indicated a potential value of hsa_circ_0006220 in the diagnosis of breast cancer.

The expression of hsa_circ_0006220 was significantly downregulated in breast cancer samples, suggesting its correlation with breast cancer. Lymph node metastasis is a known factor affecting tumor invasion and metastasis ability in breast cancer patients (23-26), and lymph node metastasis indicates a poor prognosis in breast cancer (23,24). Our results illustrated that hsa_circ_0006220 may be correlated with lymph node metastasis. Intraductal carcinoma is a precancerous lesion of invasive ductal carcinoma and can develop into invasive ductal carcinoma, which exhibits higher malignancy (27). Our results showed that hsa_circ_0006220 might indicate the degree of malignancy in patients with breast cancer.

Traditional biological diagnostic factors, including cancer antigen 15-3 (CA15-3), cancer antigen 12-5 (CA12-5), and carcinoembryonic antigen (CEA), as well as their combined detection, are used for screening and diagnosis of patients in clinical practice. However, these factors cannot conclusively determine the clinical diagnosis of breast cancer (28-34). Thus, new biomarkers are needed to provide references for breast cancer diagnosis. In the present study, ROC curve analysis showed that the AUC was 0.706, which indicated that hsa_circ_0006220 might be a potential biomarker of breast cancer. However, the sample size needs to be expanded for further verification.

CircRNAs act as transcription regulators or potent miRNA sponges (5), and can also serve as competing endogenous RNAs for miRNAs (35,36). A circRNA may include a handful of miRNA-binding sites and have adsorption effects on several miRNAs. The interaction between circRNAs and disease-associated miRNAs indicates that circRNAs are important for the development of diseases (37). In this study, five possible target miRNAs of hsa_circ_0006220 were predicted using bioinformatics tools.

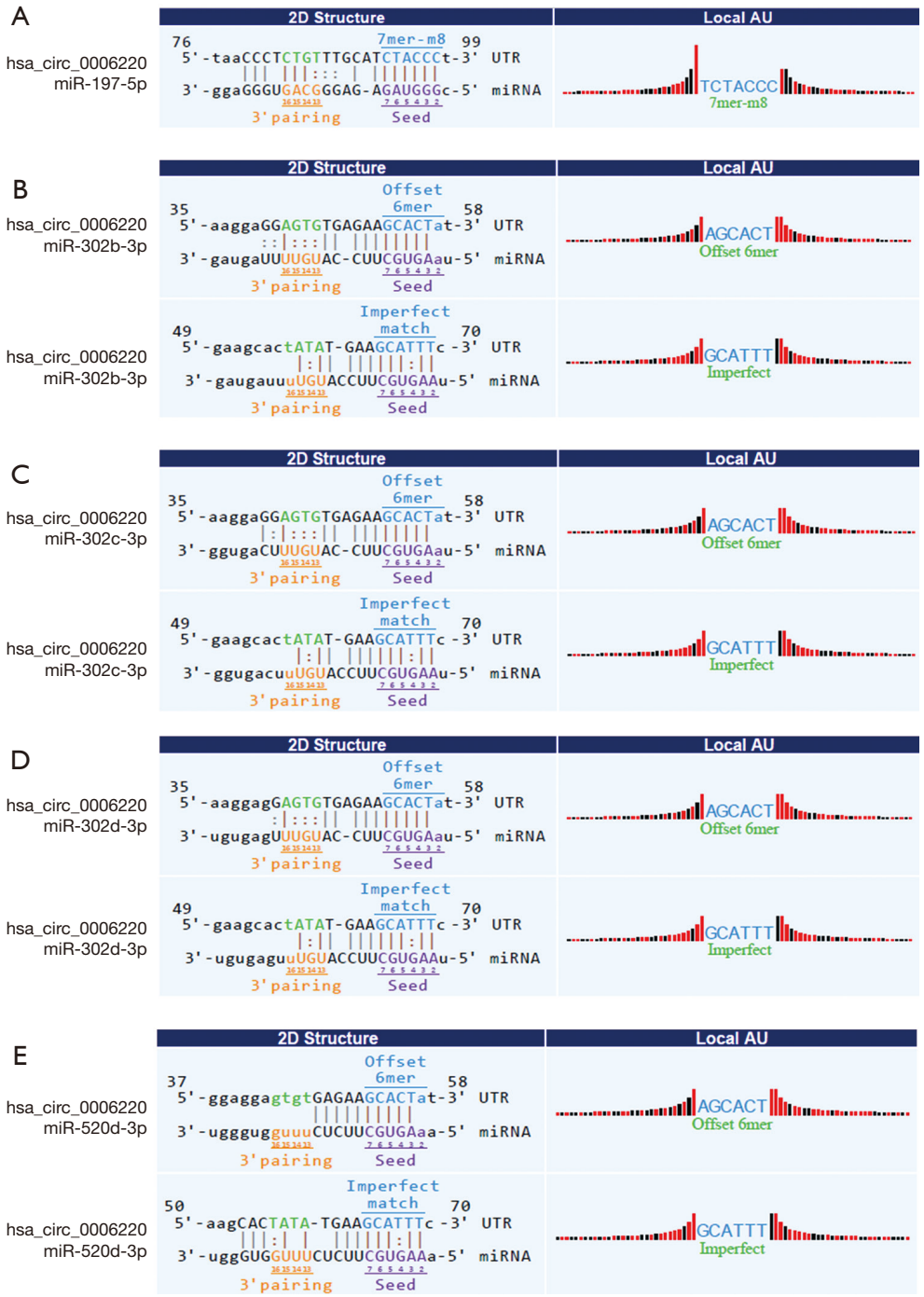

Figure 4 Prediction of target microRNAs of hsa_circ_0006220 using bioinformatics tools. MiR-197-5p (A), miR-302b-3p (B), miR-302c-3p (C), miR-302d-3p (D), and miR-520d-3p (E) were predicted as matches for hsa_circ_0006220. The vertical solid lines between nucleotide sequences represent the predicted binding sites.

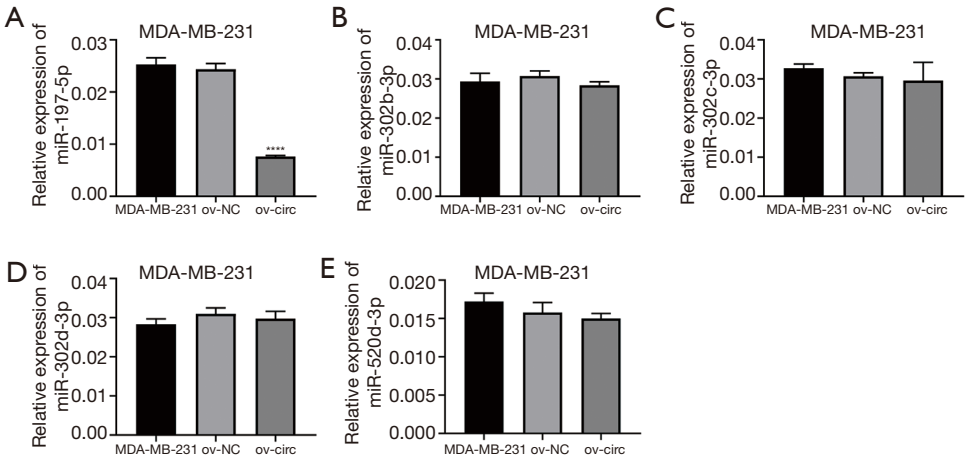

Figure 5 Verification of the five target microRNAs of hsa_circ_0006220 by RT-PCR in MDA-MB-231. The expression of miR-197-5p (A), miR-302b-3p (B), miR-302c-3p (C), miR-302d-3p (D), and miR-520d-3p (E) were measured when overexpressing hsa_circ_0006220. $2^{-\Delta CT}$ values were used to calculate the expression; values were means ± SD; ****, P<0.0001.

The results of RT-PCR showed that miR-197-5p might be regulated by hsa_circ_0006220. Several studies indicated that miR-197 was a biomarker for different cancers (38). The expression of miR-197 was significantly increased in the serum of patients with breast cancer (39) and was upregulated in human male breast cancer tissues (40). Hence, it may be a useful biomarker for breast cancer screening. MiR-302B, miR-302C, and miR-302D were reported to be closely associated with the occurrence and development of breast cancer (41). The overexpression of miR-302 has been shown to sensitize breast cancer cells to adriamycin (42). Furthermore, miR-520d and miR-302c have been shown to be associated with HER2/neu (43). Therefore, we speculated that hsa_circ_0006220 might play a role in breast cancer by regulating its putative target miRNAs. Further research is needed to verify the interaction between hsa_circ_0006220 and its target miRNAs.

In summary, this study shows that hsa_circ_0006220 plays a role in breast cancer and is associated with lymph node metastasis and pathological type. Also, it is likely a potential biomarker and therapeutic target of breast cancer. Nonetheless, the application of hsa_circ_0006220 for breast cancer screening and treatment needs further improvement. The association between hsa_circ_0006220 and miR-197-5p should also be verified by further experiments. The molecular mechanisms and biological functions of circRNAs in breast cancer warrant further investigation.

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Footnote

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/gs-21-42). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Ethics Committee of The First Affiliated Hospital of China Medical University (No. AF-0G-03-1.0-02). Written informed consent was retrieved from all participants in the study.

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