Original Article

The decreased expression of hsa_circ_0043278 and its relationship with clinicopathological features of breast cancer

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Background: Breast cancer is one of the most significant causes of death in women around the world. Circular RNAs (circRNAs), which are a novel class of conserved RNA molecules, are involved in the occurrence and development of various diseases, especially malignancies; however, researchers rarely report their roles in human breast cancer.

Methods: In the present study, the differentially expressed levels of circRNAs in human breast cancer tissues and paired noncancerous tissues were screened by circRNA microarray. Hsa_circ_0043278 was downregulated 43-fold in breast cancer and was selected for further analysis. The expression of hsa_circ_0043278 was verified in breast cancer specimens and paired noncancerous tissues by quantitative reverse transcription polymerized chain reaction (qRT-PCR) technique. The relationship between the expression of hsa_circ_0043278 and the clinicopathological features was analyzed.

Results: Among the 520 differentially expressed circRNAs, 292 significantly upregulated circRNAs and 228 downregulated circRNAs in the breast cancer tissues compared with the paired noncancerous tissues. The area under the receiver operating characteristic (ROC) curve of hsa_circ_0043278 was 0.690. The results of the bioinformatics prediction showed five target miRNAs that might be sponged by hsa_circ_0043278. The expression of hsa_circ_0043278 was associated with lymph node metastasis and histological type of the patient. Patients with lymph node metastasis have tumors with significantly downregulated expression of hsa_circ_0043278 (P=0.0201).

Conclusions: Our results suggest that hsa_circ_0043278 is downregulated and may play a key role in human breast cancer.

Keywords: Breast cancer; circRNA; hsa_circ_0043278; clinicopathological features; miRNA

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Introduction

Breast cancer is one of the most common cancers worldwide and is a significant cause of death in women (1,2). Although comprehensive treatment strategies and the diagnosis of breast cancer have improved significantly, local recurrence and distant metastasis still occur frequently. The absence of biomarkers for early detection is one reason for the poor prognosis. Therefore, the exploration of new biomarkers and therapeutic targets for breast cancer treatment is urgently needed.

Compared with linear RNAs, circular RNAs (circRNAs) are stable and conserved RNA molecules (3,4). CircRNAs act as sponges of micro RNAs (miRNAs), thus interfering with the expression of their target mRNAs, and are associated with the development of multiple human diseases (3,5-9). Accumulating evidence shows circRNAs play pivotal roles in cancer biology. Li *et al.* (10) suggested circDDX17 functioned as a tumor suppressor in colorectal cancer. Circ_0030235 might play a crucial role in human pancreatic ductal adenocarcinoma by sponging miR-1253 and miR-1294 (11). In human breast cancer, hsa_circ_0001982 affected breast cancer cell proliferation, invasion, and induced apoptosis by targeting miR-143 (12). Gao *et al.* (13) suggested that circ_0006528 promoted breast cancer growth, invasion, and migration through the circ_0006528/miR-7-5p/Raf1/MEK/ERK network in breast cancer. However, few reports have been published on the role of circRNAs in human breast cancer.

Here, the results of our study showed that hsa_circ_0043278 was downregulated in breast cancer tissues using a microarray. The expression of hsa_circ_0043278 was verified using quantitative real-time polymerase chain reaction (qRT-PCR). The relationship of hsa_circ_0043278 with the clinicopathological characteristics in human breast cancer was confirmed. This study supplies evidence that hsa_circ_0043278 may play a key role and might be a potential marker for human breast cancer. We present the following article in accordance with the MDAR checklist (available at http://dx. doi. org/10. 21037/gs–20–825).

Methods

Samples and clinicopathological information

The clinicopathological information of 50 breast cancer patients and 38 benign breast tumor patients was collected from the First Affiliated Hospital of China Medical University (from January 2005 to December 2012). The clinicopathological information, including age, tumor size, lymph node metastasis, histological type, histological grade, estrogen receptor (ER) status, progesterone receptor (PR) status, human epidermal growth factor receptor 2 (HER-2, ErbB-2) status, and Ki67 expression was collected. The expression of ER, PR, HER-2, and Ki67 were following the pathological results. Fresh breast cancer tissues and paired noncancerous tissues were retrieved from 50 patients who underwent surgical treatment. The paired noncancerous tissues were collected over 5 cm from the edge of the cancer tissues. Benign breast tumor tissues were collected from 38 patients, including 28 cases of fibroadenoma, 7 cases of intraductal papilloma, and 3 cases of hyperplastic nodules. The samples were stored at –80 ℃.

All malignant clinical data samples were primary female breast cancer (from 27 to 81 years old), and none of the patients had received any therapeutic interventions, including chemotherapy or radiotherapy. The study was approved by the Ethics Committee of The First Affiliated Hospital of China Medical University (No. AF-0G-03-1.0-02). All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013). Written informed consent was retrieved from all participants in the study.

circRNA microarray

The Arraystar Human circRNA Microarray (KangChen Biotech, Shanghai, China), which covered 2465 human circRNAs, was used to identify the differential expression of circRNAs among three pairs of breast cancer tissues and paired noncancerous tissues. The total number of RNA samples was quantified by NanoDrop ND-1000 (Thermo, USA). The samples were subjected to microarray hybridization according to Arraystar's standard protocols. Differentially expressed circRNAs were found, and the images and data were collected and analyzed by software.

QRT-PCR

We verified hsa_circ_0043278 expression by qRT-PCR in 50 breast cancer tissues and paired noncancerous tissues, and 38 benign breast tumor tissues as control. Total RNA was extracted by TRIZOL reagent (Takara Bio, Japan) following the manufacturer's instructions. Each of the cDNAs was reverse transcribed from 2 μg of total RNA using a PrimeScriptTM RT reagent kit with gDNA eraser (Perfect

Table 1 Primers of hsa_circ_0043278 and GAPDH

RNA	Forward (5'-3')	Reverse (5'-3')
Hsa_circ_0043278	CCCTGCTGAACCTGAAACAAG	AGGGCCATTTCTTCTTGAGC
GAPDH	GTGGAGTCCACTGGCGTCTT	GTGCAGGAGGCATTGCTGAT

GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Real Time) reverse transcriptase (Takara Bio). SYBR performed the reaction method-based qRT-PCR using SYBR®Premix Ex TaqTMII (Takara Bio). A Light Cycler 480 II sequence detection system (Roche Applied Science, Indianapolis, USA). The hsa_circ_0043278 RNA expression levels were normalized to those of GAPDH. The hsa_circ_0043278 and GAPDH primers are shown in *Table 1*. The relative expression of hsa_circ_0043278 is analyzed using $2^{-\Delta CT}$ value. The experiment was repeated thrice.

Bioinformatics prediction

The target miRNAs of hsa_circ_0043278 are predicted by miRNA target prediction software, including TargetScan (http://www.targetscan.org/) and miRanda (http://www.microrna.org/). The top 5 of the target miRNAs were selected from the result of the prediction.

Statistical analysis

Statistical data was analyzed using GraphPad Prism 7.0 software (GraphPad Software, LaJolla, CA, USA). One-way ANOVA was used to verify the circRNA expression in breast cancer tissues, paired noncancerous tissues, and benign breast tumors. Kruskal-Wallis test and Mann-Whitney test were performed for the association between circRNA expression and clinicopathological data. The results for qRT-PCR were presented as mean ± SEM, and the other data were presented as mean ± SD. The receiver operating characteristic (ROC) curve was constructed by Statistical Product and Service Solutions (SPSS) 19.0 software (SPSS, Chicago, IL, USA). A P value <0.05 was considered statistically significant.

Results

Differentially expressed circRNAs screened by microarray

A total of 2,465 human circRNAs in three breast cancer tissues and paired noncancerous tissues were screened by microarray (*Figure 1A*). The expression pattern of all circRNAs in breast cancer tissues and paired noncancerous tissues was shown by hierarchical clustering (*Figure 1A*). Furthermore, 520 differentially expressed circRNAs, including 292 upregulated and 228 downregulated circRNAs in breast cancer tissues, were detected according to the filter criteria (fold-change ≥2 and P<0.05). The distribution of the differential expressed circRNAs between breast cancer tissues and paired noncancerous tissues was evaluated by a scatter plot (*Figure 1B*) and a volcano plot (*Figure 1C*).

Validation of hsa_circ_0043278 in breast cancer tissues by qRT-PCR

According to the microarray results, a novel circRNA hsa_circ_0043278 downregulated 43-fold in breast cancer was chosen for further validation by qRT-PCR. It is located at chromosome 17, and its gene symbol is TADA2A. The result showed that the expression of hsa_circ_0043278 was downregulated in 68% (34/50) of breast cancer tissues compared to paired noncancerous tissues. As shown in *Figure 2*, the expression level of hsa_circ_0043278 is significantly downregulated in breast cancer tissues compared with paired noncancerous tissues (P<0.01). This result was consistent with the microarray and suggested hsa_circ_0043278 might play a role in the occurrence of breast cancer.

The relationship between hsa_circ_0043278 levels and clinicopathological characteristics

Furthermore, the relationship between hsa_circ_0043278 expression levels and the clinicopathological characteristics was examined. As shown in *Table 2*, the results demonstrated that the expression of hsa_circ_0043278 was associated with lymph node metastasis and histological type of the patient. Patients with lymph node metastasis have tumors with significantly downregulated expression of hsa_circ_0043278 (P=0.0201). Also, the expression levels of hsa_circ_0043278 are significantly downregulated in invasive ductal carcinoma

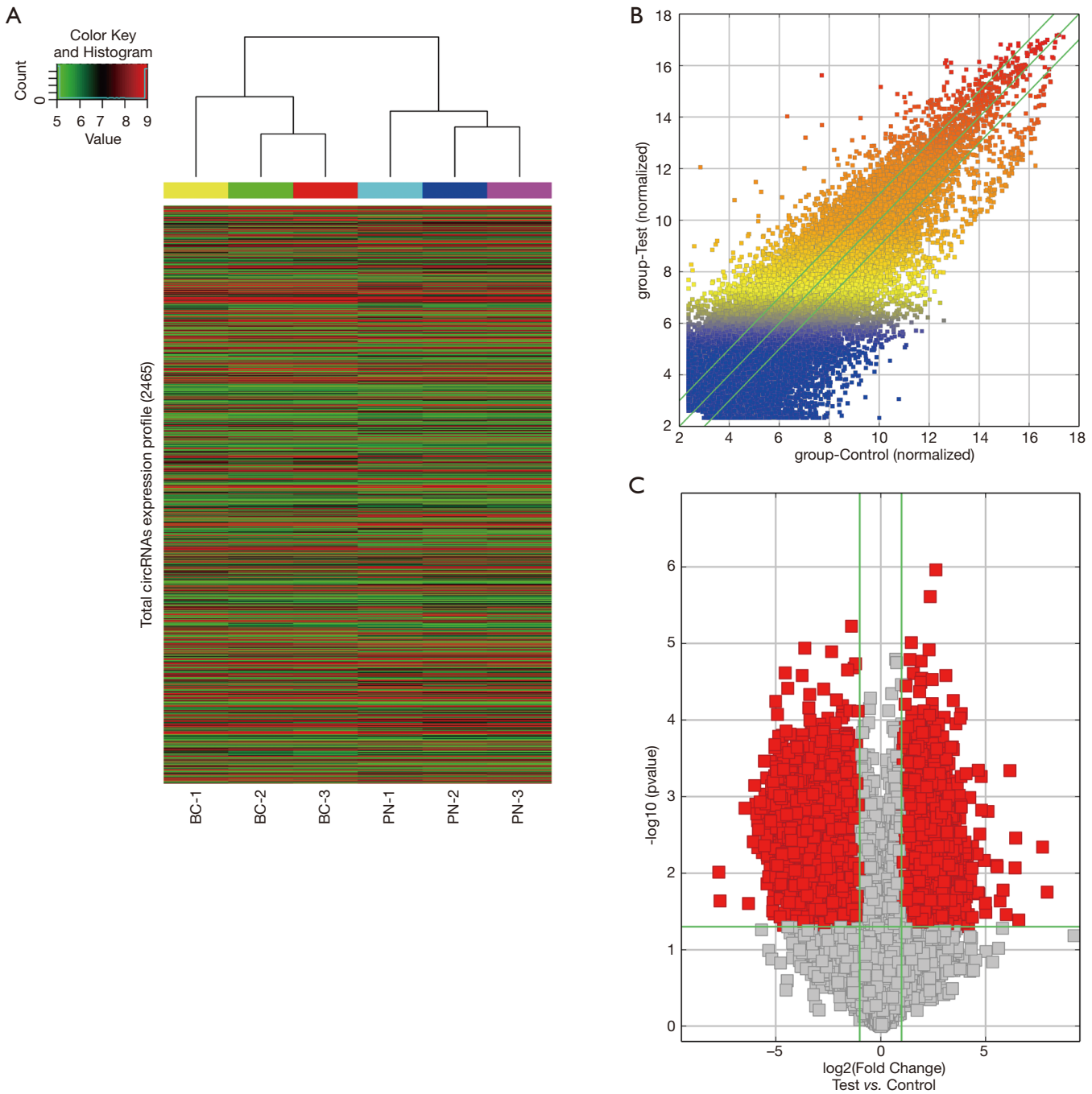

Figure 1 Total circRNAs expression profile screened by microarray. (A) The cluster heat map of all circRNAs expression between breast cancer and paired noncancerous tissues from microarray data. BC, breast cancer tissues; PN, paired noncancerous tissues. Each column is one sample, and each row is one circRNA. (B) Scatterplot of circRNA signal values between breast cancer and paired noncancerous tissues. The group-Test shows breast cancer tissues; the group-Control shows paired noncancerous tissues. The values spotted on the X and Y axes are normalized. The green lines are fold-changes. The circRNAs above the upper green line and under the lower green line represent changes greater than 2.0-fold. (C) The volcano plot shows the differential expression of circRNAs. The vertical green lines show changes greater than 2.0-fold. The left side of the green line are downregulated circRNAs, and the right side of the green line represents upregulated circRNAs (breast cancer *vs.* paired noncancerous tissues). The horizontal green line shows a P value of 0.05. The red boxes show significantly different expression of circRNAs.

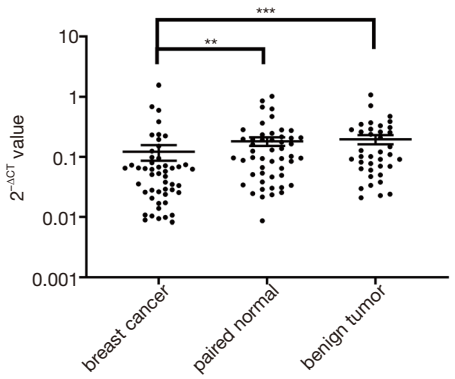

Figure 2 Verification of the differential expression of hsa_circ_0043278 between breast cancer, paired noncancerous tissues, and benign breast tumors by qRT-PCR. $2^{-\Delta CT}$ values were used to calculate the expression of hsa_circ_0043278. Values were means ± SEM. **, P<0.01; ***, P<0.001.

compared with ductal carcinoma *in situ* (P=0.0021). However, the expression levels of hsa_circ_0043278 have no relationship with other clinicopathological characteristics.

ROC curve of hsa_circ_0043278

A ROC curve was constructed to evaluate the diagnostic value of hsa_circ_0043278 in breast cancer. The area under the ROC curve was 0.690 [95% confidence interval (CI) =0.585–0.795; P=0.001; *Figure 3*]. It suggested that hsa_circ_0043278 might have a potential impact on the occurrence of breast cancer.

Prediction of hsa_circ_0043278 target genes

To find the target miRNAs, we used bioinformatics tools to

Table 2 Relationship between hsa_circ_0043278 expression and clinicopathologic features in breast cancer patients

Characteristics	No. of cases [%]	Mean ± SD	P value
Age (years)			
≥60	12 [24]	0.09256±0.108	0.6296
<60	38 [76]	0.1315±0.2794	
Diameter (cm)			
<2	5 [10]	0.1158±0.08635	0.5535
2–5	38 [76]	0.1185±0.2712	
≥5	7 [14]	0.1465±0.2143	
Lymphatic metastasis			
N0	30 [60]	0.173±0.3111	0.0201
N1–3	20 [40]	0.04591±0.04019	
Pathological type			
Ductal carcinoma *in situ*	6 [12]	0.3096±0.2705	0.0021
Invasive ductal carcinoma	40 [80]	0.09771±0.2477	
Others	4 [8]	0.08544±0.102	
Histological grade (invasive ductal carcinoma, n=40)			
I	3 [7.5]	0.1013±0.04939	0.0777
II	33 [82.5]	0.1062±0.2718	
III	4 [10]	0.0248±0.01611	
Estrogen receptor			
Absent	9 [18]	0.2298±0.5058	0.9209
Present	41 [82]	0.09853±0.1464	

Table 2 (*continued*)

Table 2 (*continued*)

Characteristics	No. of cases [%]	Mean ± SD	P value
Progesterone receptor			
Absent	14 [28]	0.16±0.4087	0.3296
Present	36 [72]	0.1074±0.1541	
HER-2			
Negative	8 [16]	0.1288±0.1254	0.3673
1+	17 [34]	0.09135±0.1397	
2+	17 [34]	0.1892±0.3916	
3+	8 [16]	0.03849±0.02715	
Ki 67			
<14%	6 [12]	0.3224±0.6106	0.2705
>14%	44 [88]	0.09486±0.1441	

HER-2, human epidermal growth receptor 2.

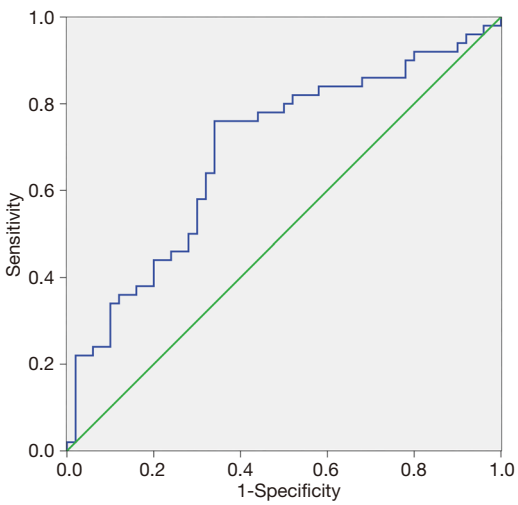

Figure 3 Assessment of the diagnostic value of hsa_circ_0043278 by ROC curve analysis between breast cancer and paired noncancerous tissues.

predict the target miRNAs of hsa_circ_0043278. As shown in *Figure 4*, we have selected five miRNAs likely to match hsa_circ_0043278: miR-103a-2-5p, miR-302b-3p, miR-302c-3p, miR-455-3p, and miR-520d-3p. The possible target sites were then predicted, and the binding sites were represented by vertical solid lines (*Figure 4*). The result suggests hsa_circ_0043278 possibly functions through these target miRNAs.

Discussion

circRNAs are a particular type of conserved RNA and are a popular topic in the field of RNA research. However, at present, our understanding of circRNA is still limited (14,15). Few circRNAs have been reported in breast cancer. In our paper, downregulated circRNA hsa_circ_0043278 was found using a microarray and verified using qRT-PCR in breast cancer tissues. Further research revealed that the expression of hsa_circ_0043278 was correlated with histological type and lymph node metastasis of breast cancer. The results suggested that hsa_circ_0043278 played a role in breast cancer and had a potential research value.

Hsa_circ_0043278 was downregulated 43-fold in breast cancer tissues, and its biological function has not yet been reported. The results of microarray and qRT-PCR suggested hsa_circ_0043278 might be related to the progression of breast cancer. However, due to the low expression level of circRNAs in the human body, it is difficult to avoid the potential for false negatives in evaluating circRNA expression. Further experiments are needed to verify these findings.

Consequently, we found that the expression of hsa_circ_0043278 was much lower in patients with greater lymph node involvement and poorly differentiated breast cancer. Despite the limitation in the sample size in this study, our results showed that hsa_circ_0043278 expression might be correlated with lymph node invasion

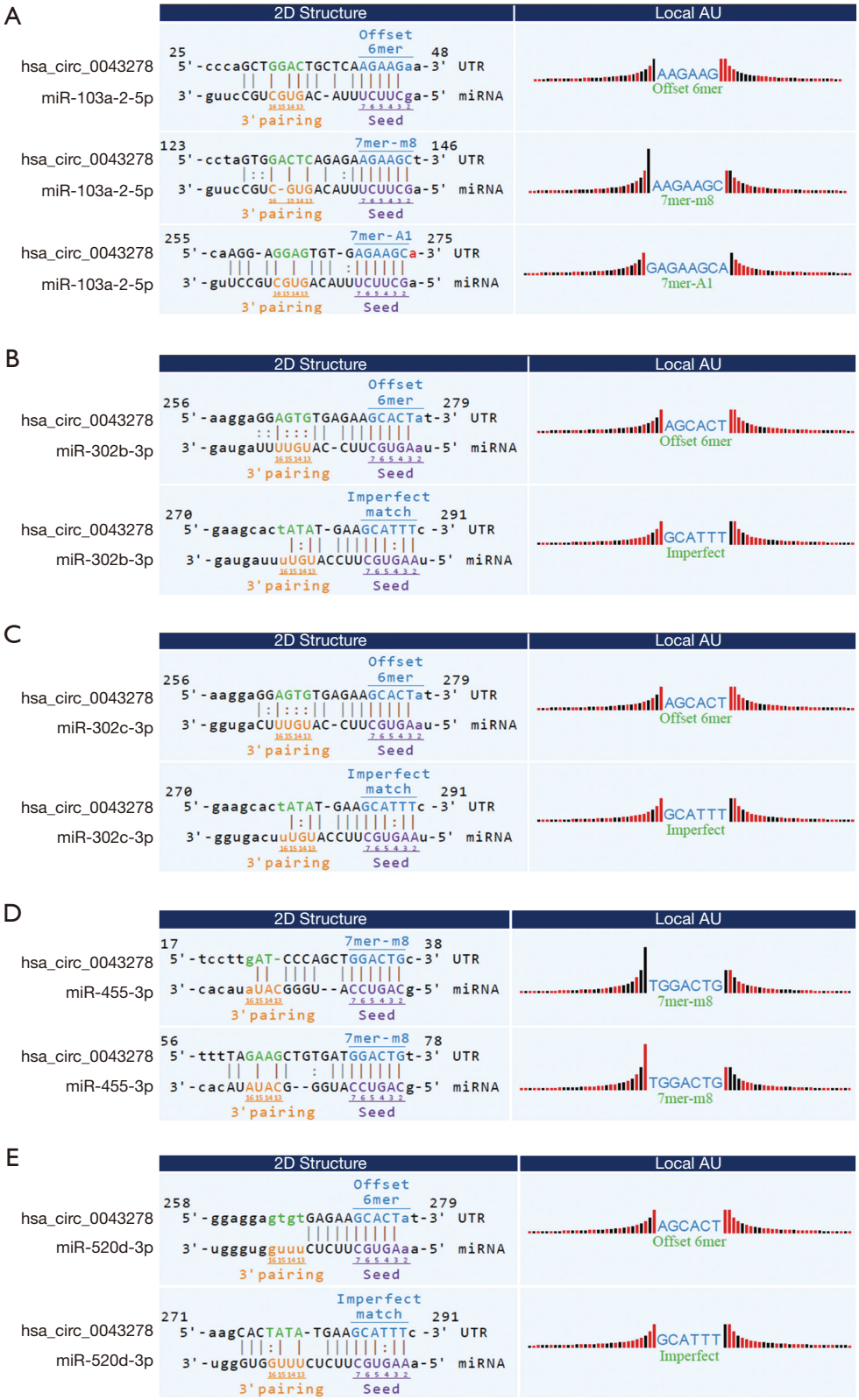

Figure 4 Prediction of the target microRNAs of hsa_circ_0043278 using bioinformatics tools. miR-103a-2-5p (A), miR-302b-3p (B), miR-302c-3p (C), miR-455-3p (D) and miR-520d-3p (E) are matched to hsa_circ_0043278 by solid lines.

and the degree of malignancy of breast cancer. Our results showed that low expression of hsa_circ_0043278 might be correlated with a poorer prognosis in breast cancer. These findings will need further verification.

Tumor biomarkers and their combined detection are beneficial for the screening of tumors, but few reports of tumor biomarkers in breast cancer have been published. In our study, the ROC curve was constructed, and the result showed that the AUC was 0.690, which indicates that hsa_circ_0043278 might have potential diagnostic value for breast cancer. However, we need to expand the sample size for further verification.

MiRNAs can bind and inhibit 3'UTR activity of target mRNAs from repressing these coding genes (16-20). It is generally recognized that circRNAs act as potent miRNA sponges (5) and serves as competing endogenous RNAs (21,22). Therefore, we predicted five target miRNAs of hsa_circ_0043278 using bioinformatics tools. Some miRNAs were found to be associated with breast cancer. Wang (23) revealed miR-455 was downregulated in breast cancer tissues and cells, and that it inhibited cell proliferation by targeting CDK14. This result showed that miR-455 could be a target for breast cancer therapy. MiR-455-3p was reported to play an essential role in melanoma and could thus be a potential target for treatment (24). Preliminary research has indicated that miR-302b and miR-302c were associated with the occurrence and development of breast cancer (25). MiR-302b could enhance the sensitivity of breast cancer cells to cisplatin (26), while miR-302a/b/c/d could also sensitize breast cancer cells to Adriamycin (27). Another analysis predicted that miR-520d and miR-302c corresponded with HER2/*neu* (28). Therefore, we hypothesize that hsa_circ_0043278 might have a role in breast cancer and functions via the regulation of these putative target miRNAs. Nonetheless, further research is needed to verify the interaction between hsa_circ_0043278 and these miRNAs. The expression of these five miRNAs in breast cancer also requires further confirmation.

In summary, circRNAs were found to be highly prevalent spliced transcripts from hundreds of genes (21), and a subset of them are related to multiple malignancies. We detected differentially expressed circRNAs in breast tissue samples by microarray and determined hsa_circ_0043278 was downregulated in breast cancer. The results suggest that hsa_circ_0043278 may play a role in breast cancer and could be a promising marker for breast cancer diagnosis. Nonetheless, how hsa_circ_0043278 affects the biological behavior of breast cancer needs to be further investigated. It will also be essential to determine how hsa_circ_0043278 can be used for future breast cancer screening and treatment.

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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-20-825). 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. The study was approved by the Ethics Committee of The First Affiliated Hospital of China Medical University (No. AF-0G-03-1.0-02). All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013). Written informed consent was retrieved from all participants in the study.

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