

Identification of circular RNAs hsa-circ-000696 as a novel biomarker for breast cancer

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Abstract

Background : To investigate the characteristic expression of circular RNAs between breast cancer and para-carcinoma tissue, identified hsa-circ-000696 as a novel biomarker for breast cancer. Methods: 136 patients were recruited in General Hospital of Ningxia Medical University. Of which 3 patients tissue for microarray analysis, 20 and 113 patients for circRNA expression in tissue and peripheral blood, respectively. The candidate circRNAs was verified by qPCR in BC patients' tissue and peripheral blood. GeneSpring 13.0(Agilent) software were used for analysis the circRNA array data. CircRNA structure were performed by circPrimer1.2 software. MiRanda v3.3, RNAhybrid 2.1 and Cytoscape 3.6.0 were utilized as tools to predict the circRNA-miRNA networks. T-test, Curve regression analysis and ROC analysis were performed to determine the diagnostic values of candidate circRNAs. Statistical analysis was performed using SPSS23.0 software. Results: 2021 differentially expressed circRNAs were identified, of which 546 were upregulated and 1475 were downregulated. Four circRNAs were selected with filter criteria for further verify in BC tissues. Hsa-circ-0006969 was significantly downregulated both in breast cancer tissues and peripheral blood. Hsa-circ-0006969 proved a highest diagnostic value in breast cancer tissues(AUC=0.889), peripheral blood(AUC=0.823) for Grade 1(AUC=0.672), Grade 3(AUC=0.675), ER positive(AUC=0.648), TNM I(AUC=0.687) and TNM II(AUC=0.769), TNM III(AUC=0.757), TNM early stage(I, II) (AUC=0.751). Hsa-circ-0006969 show more effective diagnostic values of for tumor metastasis (AUC=0.742) compared with CA153 (AUC=0.712). Conclusion: Hsa-circ-0006969 could be a novel predictive biomarker for diagnostic and treatment of breast cancer.

Background

Breast cancer is one of the most frequently occurring women cancer and the leading cause of cancer-related death among worldwide[1]. Current studies have shown that age, obesity, abnormal estrogen, late childbirth, genetic and epigenetic are associated with an increased risk of breast cancer[2, 3]. Despite the development of diagnosis and treatment of BC, the mortality remains unsatisfactory for BC patients[4]. This discrepancy is mainly because the current diagnostic methods cannot simultaneously achieve high sensitivity and convenience of breast cancer. We need an efficient and sensitivity diagnostic marker and therapeutic target.

Circular RNA(circRNA) is a type of non-coding RNA which have a continuous closed loop structure[5]. circRNA have a large number of miRNA binding sites, which provide its function of regulating miRNA by "sponge" with them. CircRNA are tissue-specific and play important roles in different cancer[5, 6]. Recent reports have showed that circRNA involved in the cancer cells metastasis by directly bounding(sponge) to its target miRNA. For example, Human ciRS-7 is a typical example as miRNA sponge functions with 74 miR-7 binding sites[7]. In Esophageal Squamous Cell Carcinoma, circ-ITCH Inhibit cell proliferation through binding miR-7, miR-17, and miR-214, and active Wnt/ β -catenin signaling pathway[8]. However, the potential functions of a large proportion of circRNAs in cancer still not clear.

In this study, we first screening the circRNAs profile in breast cancer and para-carcinoma tissue by microarray analysis. Then confirmed the differentially expression circRNAs in larger independent cohorts including breast cancer tissue and patients' peripheral blood specimens. Based on the correlation analysis of the target circRNA and patient's clinic characteristic, we identified hsa-circ-0006969(CIRC6969) as a candidate gene for breast cancer diagnostic. Further analysis being used to evaluate the diagnostic value of CIRC6969 in breast cancer.

Methods

Participants and specimens

Both tumor tissue and plasma samples were collected from the General Hospital of Ningxia Medical University, Surgery department. This study were approved by the General Hospital of Ningxia Medical University Ethics Committee(Yinchuan, China). All patients involved in the study signed the informed consent for donating their samples. 23 BC tissues and 113 BC peripheral blood were collected from January 2017 to August 2018. The patients were including (1) women; (2) without previous cancer

history; (3) without HIV/AIDS; (4) >18 years old; (5) after mastectomy. The age of patients ranging between 25-75 years old (average 49.94±9.97 years). All the patients are not received radiotherapy or chemotherapy treatment before specimen collection.

peripheral blood samples were collected from the median cubital vein with an EDTA anticoagulated vacutainer(2mL) after overnight fasting. BC tissues and para-carcinoma tissue were collected from patients who underwent surgical breast resection. The para-carcinoma tissue were located >5 cm from the tumors. Clinical and cancer pathological characteristics were collected including age, Healthy person(as control) were recruited from physical examination department in General Hospital of Ningxia Medical University. Collect the clinical, pathological, and molecular characterization data of all participants.

Cell culture

Human breast cancer cell line MDA-MB-231 were purchased from The Typical Culture Preservation Committee Cell Bank of Chinese Academy of Sciences. The cells were cultured in DMEM (Gibco, USA) with 10% fetal bovine serum (FBS, Gibco, USA) at 37 °C in 5% CO₂.

RNA Isolation

Tissue RNA were isolated from BC tissue and adjacent normal tissue with Trizol reagent (Invitrogen, Inc., USA). Peripheral blood RNA were isolated with Total RNA Rapid Extraction Kit (Biotek, Inc., China). The quality of RNA were measured using Nanodrop 2000 (Thermo Scientific, USA). RNA integrity was determined by 1.3% agarose gel electrophoresis (120V, 15 min, 1×FA buffer).

circRNA Microarray

Human CircRNA Array v2 microarray(Beijing Capital Bio Biotechnology Corporation, China) has been used for circRNA microarray expression profiling. GeneSpring 13.0 software(Agilent) were used for circRNA array data analysis. Three pairs of BC tissues and para-carcinoma tissues were collected for circRNA microarray analysis. The labelled RNAs were hybridized on the microarray containing 162351 human circRNA probes. In order to improve the screening efficiency, differentially expressed of circRNAs were screened by the filter criteria $FC \geq 4$, $P\text{-value} < 0.05$, Original Fluorescence value ≥ 100 .

qPCR

The cDNA were synthesized by the RevertAid First strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc., USA). qPCR were performed using LightCycler480II quantitative system(Roche, USA). The primers were synthesized Sangon biotech(Shanghai) Co.,Ltd, all circRNA primer was list in Table S1. The reaction in a total volume of 20 μ L system including 1 μ L total RNA (500 ng), 1 μ L random primer, 10 μ L ddH₂O, 2 μ L dNTP, 4 μ L Reaction buffer, 1 μ L Ribolock RNase Inhibitor, 1 μ L RevertAid M-MuLV RT. The cycling program is 25°C 5min, 42°C 60min, 70°C 5min. GAPDH Expression were used as an internal control. Each reaction was repeated three times and the mean relative expression of genes were calculated using $\Delta\Delta$ CT method[9]. The PCR production was stored at -80°C before use.

Microarray data analysis

The circRNA microarray data were extracted using Feature Extraction software(CapitalBio). Normalization, Fold change and P value were performed using GeneSpring software V13.0 (Agilent). Heat map were analysed using Cluster 3.0 software. ScatterPlot and VolcanoPlot were analysed using ggPlot2 software(R)[10]. CircRNA structure were performed by circPrimer1.2 software. Miranda v3.3a (<http://miranda.org.uk/>) and RNAhybrid 2.1 (<https://directory.fsf.org/wiki/RNAhybrid>) were utilized to predict the

target miRNAs of CIRC6969. The graphs of circRNA-miRNA interaction networks were drawn by cytoscape 3.6.0 (<https://cytoscape.org/>)[11]. GO analysis and genecards database(<https://www.genecards.org/>) were used to annotate the genesymbol of all differentially expressed circRNA, to explain their biological processes, cellular components and molecular functions. □□□□□□□□

Statistical analysis

Data statistical analysis were performed by SPSS23.0 software (IBM,USA), GraphPad Prism version 8.0 (GraphPad Software, USA), data were presented as mean \pm SD. The categorical variables were tested by the Chi-square test, and the continuous variables were tested by the *t*-test. ROC were performed to verify the clinical diagnostic value of circRNA. The correlation between circRNA expression and the breast cancer pathological characteristics were evaluated by Pearson`s correlation test and Curve regression analysis. Differences were considered significant if $p < 0.05$ (* $p < 0.05$; ** $p < 0.01$).

Results

circRNAs Expression profiles in the BC patients

To identify the specific circRNA expressed in breast cancer, 3 paired BC tissues samples (CA1-CA3) and para-carcinoma tissues samples (AP1-AP3) were collected for circRNA microarray assay. Based on the circRNA microarray results, 2021 differential expressed circRNAs being identified. With the filter criteria as $FC \geq 2$ and P -value < 0.05 , 546 were upregulated and 1475 were downregulated (Figure S1). The cluster analysis divided the differential expressed circRNA in two groups (Figure S1A). According to the different fluorescence signal values to screening differential expressed circRNAs in two group (Figure S1B).

We use the filter criteria as $FC \geq 4$, P -value < 0.05 , Original fluorescence value ≥ 100 to verify the candidate circRNA. Symbol gene functional annotation was associated with tumor occurrence and development. The four candidate circRNAs selected included two upregulated circRNAs, Hsa-circ_0044513 and Hsa-circ_0101692, two downregulated circRNAs, Hsa-circ-0006969 and Hsa-circ_0054020 as candidate circRNAs in a subsequent validation utilizing a larger cohort(Table S1).

Verify the candidate circRNAs by qPCR

To verify the four selected circRNA, 20 pairs of breast carcinoma and para-carcinoma tissues samples were used in an independent cohort. qPCR proved that hsa-circ-0006969, hsa-circ-0054020 were significantly decreased while another hsa-circ-0044513 and hsa-circ-0101692 were increased in breast carcinoma. The expression FC of hsa-circ-0006969 was 5.4, hsa-circ-0054020 was 3.1, hsa-circ-0044513 was 5.7 and hsa-circ-0101692 was 9.6, respectively. ROC curve analysis performed that hsa-circ-0006969(CIRC6969) have higher AUC and lower P values than other three circRNAs, indicated that expression of CIRC6969 was significantly correlated with BC (Figure 1).

Further validation of the expression of CIRC6969 in peripheral blood samples from 113 patients with BC and 50 healthy person. The results proved the expression of CIRC6969 was down-regulated in BC patients compared with healthy person(Figure 2A, Table S2). Based on the ROC curve analysis, AUC of CIRC6969 was 0.823 with sensitivity of 82.3% and specificity of 68.0% (Figure 2B). The results suggested that CIRC6969 was a blood-specific circRNA in breast cancer.

Bioinformatics analysis of CIRC6969

It was all known that circRNA derived from the reverse splicing of exons of different mRNA. In this study, circPrimer 1.2 software being used to identified the origin mRNA of hsa-circ-0006969. The results demonstrated that hsa-circ-0006969 was made by reverse splicing of exon 10 and 11 of ARHGEF28 gene (Figure 3A). The circ-0006969 RT-qPCR product can not be digested by RNase R, proved its continuous closed loop structure(Figure 3B). We further used miRanda and RNAhybrid softwares to predicted

the binding miRNA of CIRC6969. The results showed that 56 microRNAs were found as a potential binding target of hsa-circ-0006969 (Figure 3C). Based on overlapping of two databases, 8 miRNAs with the significant scores and P-value were identified (Figure 3D). The information of the 8 miRNAs with Hsa-circ-0006969 are shown in Table 1, the biological processes of these miRNAs were all related to cell cycle and cell proliferation. The results suggested that Hsa-circ-0006969 related to cell proliferation and differentiation, and may intervene in tumor occurrence and development through binding these miRNAs as a “sponge”.

The diagnostic values of CIRC6969 as a biomarker in breast cancer

The clinical characteristics of 113 breast cancer patients were obtained to verify the correlation between CIRC6969 expression and breast cancer classification and prognosis. Association analysis demonstrated that CIRC6969 expression were significantly related with tumor grading ($P < 0.05$), ER positive ($P < 0.05$), tumor metastasis ($P < 0.01$) and TNM stage ($P < 0.01$) (Table 2). Pearson's correlation test and Curve regression analysis indicated that CIRC6969 expression was significantly correlated to CA153 level in peripheral blood ($P < 0.05$) and no obvious correlation with CEA level (Figure S2). It is suggested that CIRC6969 was specific expression in blood, and have high correlation with BC tumor stage, grade and metastasis.

To determine the diagnostic values of CIRC6969 for BC, ROC curve analysis were performed. The results showed AUC of Hsa-circ-0006969 for diagnosis of Grade 1 (High differentiated tumor), Grade 2 (moderately differentiated tumor) and Grade 3 (low differentiated tumor) were 0.672 ($P = 0.009$), 0.476 ($P = 0.667$) and 0.675 ($P = 0.004$). The AUC of CIRC6969 for diagnosis of ER positive was 0.648 ($P = 0.031$), and for diagnosis of tumor metastasis was 0.742 ($P = 0.001$) compared with CA153 for tumor metastasis was 0.714 ($P = 0.001$). The AUC of CIRC6969 for diagnosis of TNM I, TNM II, TNM III, TNM IV and TNM early stage (I, II) were 0.687 ($P = 0.017$), 0.649 ($P = 0.007$), 0.757 ($P = 0.001$), 0.599 ($P = 0.208$) and 0.751 ($P = 0.001$). (Figure 4 and Table S3). This suggested that CIRC6969 have diagnostic values in BC.

Discussion

CircRNA was a kind of non-coding RNA with a closed continuous loop and lack of 3' and 5' ends. CircRNA worked as a ceRNA or miRNA “sponge” to regulate the mRNA expression. It involved in many biological process, like gene transcription, protein translation and miRNA expression regulation through multiple mechanism [12]. Present studies indicated that circRNA expression are tissue-specific and stability in most cancer tissue and plasma [13, 14]. These properties providing circRNA have the potential to become biomarkers for diagnosis and prognosis of cancers.

The most important functions of circRNAs is working as miRNA ‘sponge’ through engaging in competitive combination with miRNA. Based on the sponge function, circRNA could regulate cancer cell proliferation, differentiation, migration, angiogenesis, protein cracking and apoptosis [15, 16]. Recent research demonstrated that circRNA was related to the malignant characteristics of the cancer through regulating miRNA. The first characterized circRNA to support this model was ciRS-7, which could inhibited normal miR-7's function through acting as an miRNA sponge [17]. CircRAK3 promoted cell migration, invasion and metastasis in vitro and in vivo by targeting miR-3607 [18].

The high morbidity of breast cancer and its recurrence severely threaten human health. Early diagnosis and type differentiation are helpful to the treatment of breast cancer patients. Gao et al. found hsa-circ-0006528 was upregulated and related to chemotherapeutic resistance in BC [19]. Yin WB, et al. reported that hsa-circ-0001785 can be a diagnostic biomarker for breast cancer [20]. Previous research reported that circ-ABCB10 promotes BC cells proliferation through sponging miR-1271 [21].

Overexpression of hsa-circ-0136666 will promote BC cells progression by sponging miR-1299 and targeting CDK6 [22]. Zhang HD et al. found hsa-circ-0072995 promotes breast cancer cell migration and invasion through sponge for miR-30c-2-3p [23]. These suggest that circRNA plays an important role in the occurrence and development of breast cancer, which is worthy of us to further screening and functional verification.

In this study, circRNA microarray was using to profile the differentially expressed circRNAs in breast cancer. With the cluster analysis and GO analysis, 546 upregulated and 1475 downregulated circRNAs being identified. Based on FDR correction and the

filter criteria, 4 candidate circRNAs being selected. After verified by qPCR in an independent cohort in BC patients' tissue and peripheral samples, CIRC6969 proved to have a good consistency in BC tissue and peripheral blood. CIRC6969 have the highest diagnostic value for breast cancer tissues(AUC=0.889, 95%CI=0.780-0.998, P=0.0001) and peripheral blood (AUC=0.823, 95%CI=0.757-0.887, P=0.0001).

Further research proved that Hsa-circ-0006969 was a novel circRNA encoded by the Rho Guanine Nucleotide Exchange Factor 28 (ARHGEF28) gene, and located in 73128162-73136585 sequence position of sense strand on Human chromosome 5. Previous research indicated that ARHGEF28 gene could work as a RHOA specific guanine nucleotide exchange factor involved in cell adhesion formation, cell motility, aggregation and apoptosis by regulating integrins and growth factor receptors[24]. Present research indicated that CIRC6969 was found in cerebellum, Hela cell, HepG2 cell[25, 26]. But there is no evidence that CIRC6969 could play any functions in metabolism and tumor. To our knowledge, this is the first positive demonstration the relationship of CIRC6969 and BC. With the bioinformatics analysis, 8 possible CIRC6969 targets miRNA being selected. Functional analysis revealed that the biological processes of these 8 miRNAs were all related to cell cycle and cell proliferation, proved that CIRC6969 related to cell proliferation and differentiation, and may intervene in tumor occurrence and development through binding these miRNAs as a "sponge". ROC curve analysis revealed CIRC6969 have higher diagnostic accuracy in BC tissues and patients's peripheral blood samples. The results proved that CIRC6969 have high correlation with BC metastasis and classification, and CIRC6969 can be used as a potential biomarker for BC diagnosis and treatment.

Conclusions

In summary, the study proved that circRNA hsa-circ-0006969 was downregulated both in the BC tissues and peripheral samples. Hsa-circ-0006969 have high diagnostic value and may serve as a promising predictive biomarker for BC diagnosis and treatment. Further research are needed to investigate the mechanism of hsa-circ-0006969 in BC tumorigenesis and metastasis.

Abbreviations

AUC Area Under Curve

AIDS Acquired immune deficiency syndrome

BC Breast cancer

CEA Carcinoembryonic antigen

CA153 Carbohydrate antigen 153

CIRC6969 Hsa-circ-0006969

CeRNA Competing endogenous RNAs

DMEM Dulbecco's Modified Eagle Medium

EDTA Ethylene diamine tetraacetic acid

ER Estrogen receptor

FDR False discovery rate

FC Fold Change

HIV Human immunodeficiency virus

qPCR Quantitative real-time polymerase chain reaction

ROC Receiver operating characteristic

RHOA Ras homolog gene family, member A

95%CI 95% Confidence interval

Declarations

Ethics approval and consent to participate

The study protocol was reviewed and approved by ethics committees of General Hospital of Ningxia Medical University Ethics Committee(Yinchuan, China). (Ethics code number:2018-118). Informed consent was obtained from all individual participants included in the study.

Consent for publication

I would like to declare on behalf of my co-authors that the work described was original research that has not been published previously, and not under consideration for publication elsewhere, in whole or in part. The manuscript is approved by all authors for publication.

Availability of data and material

We guarantee the authenticity and validity of all data and results. Open up some of the raw data upload as the supplementary files.

Competing interests

This work does not have any relationships with business related issues, and no conflict of interest exists in the submission of this manuscript.

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Authors' contributions

W LB: Designed project, revised article, and coordinated all aspects of work.

Z X*: Designed project, participated in all experiment, wrote article.

L XH: Screening circRNA expression in peripheral blood specimens. ROC curve analysis.

W DN: Screening circRNA expression in tissues and peripheral blood specimens. prepared figure.

F HM: Collected tissue and peripheral blood samples. Total RNA and cDNA extraction.

M F: Collected tissue and peripheral blood samples. Total RNA and cDNA extraction.

C J: Collected clinicopathological characteristics of patients with breast cancer.

T JH: CircRNA Microarray analysis. screening candidate circRNAs.

Y JJ: CircRNA Microarray analysis. screening candidate circRNAs. prepared figure.

H Q: Prepared table and supplementary file.

M R: Statistical analysis.

W J: Prepared reagent and instrument. Designed and screened primers.

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Tables

Table 1. Predicted miRNA response elements regarding the top 8 of has_circ_0006969

	Predicted miRNA response elements(MREs)							
Micro-RNA ID	hsa-miR-140	hsa-miR-15a	hsa-miR-185	hsa-miR-320a	hsa-miR-320b	hsa-miR-490	hsa-miR-1266	hsa-miR-759
Source(Database)	miRanda and RNAhybrid							
Binding P-value	0.005	0.001	0.012	0.003	0.006	0.025	0.019	0.027
Related function	proliferation	proliferation	proliferation	proliferation	proliferation	proliferation	proliferation	proliferation
	cell cycle	apoptosis	apoptosis	Resistance	Resistance	invasiveness	apoptosis	apoptosis
	methylation							

Table 2. The associations between the has_circ_0006969 expression level and clinicopathological characteristics of patients with breast cancer

Parameter	N	Has_circ_0006969 level (Mean ± SD)	T-test	P
			<i>t</i>	
Age status			0.664	0.508
≥50	52	14.48±0.88		
<50	61	14.37±0.83		
CA153 status				
CA153 positive	9	14.62±1.20		
CA153 negative	104	14.40±0.82	0.738	0.462
CEA status			1.608	0.111
CEA positive	17	14.72±0.97		
CEA negative	96	14.37±0.82		
Tumor size status(cm)				0.669
≥2c	61	14.45±0.84	0.428	
< 2c	52	14.38±0.86		
Tumor grading (G,n)				
G1	25	14.98±0.89	G1 VS G2 = -2.312	0.023*
G2	55	14.42±0.74		
G3	33	14.76±0.87	G2 VS G3 = -1.987	0.050*
Triple Negative Breast Cancer(TNBC,n)				0.877
Yes	9	14.98±0.89	-0.156	
No	104	14.98±0.89		
Her-2 Positive				0.156
Yes	50	14.29±0.82	-1.143	
No	63	14.52±0.86		
estrogen receptor(ER,n)				0.041*
Positive	91	14.49±0.87	2.070	
Negative	22	14.08±0.689		
progesterone receptor(PR,n)				0.528
Positive	77	14.45±0.83	0.634	
Negative	36	14.34±0.90		
Androgen Receptor (AR,n)				0.330
Positive	93	14.45±0.86	0.978	
Negative	20	14.24±0.77		
Tumor metastasis status			3.670	0.000**
Yes	83	14.58±0.84		
No	30	13.95±0.70		
TNM stage			I VS II = -0.710	0.480
I	16	14.02±0.68		
II	53	14.19±0.84	II VS III = -4.146	0.000**
III	28	14.93±0.62		
IV	16	14.64±0.94	III VS IV= 1.238	0.222
Edmondson grading			-4.482	0.000**
early stages (I-II)	69	14.15±0.80		
advanced stages (III-IV)	44	14.83±0.75		

Abbreviations: TNM= Tumor Node Metastasis . HER-2= human epidermal growth factor receptor-2. CEA=carcino-embryonic antigen . CA153=carbohydrate antigen 15-3. NA=NOT applicable. The diagnostic cutoff values for metastasis of CA153 was 100U/mL. The diagnostic cutoff values of CEA was 20ng/mL. * $P < 0.05$; ** $P < 0.01$.

Figures

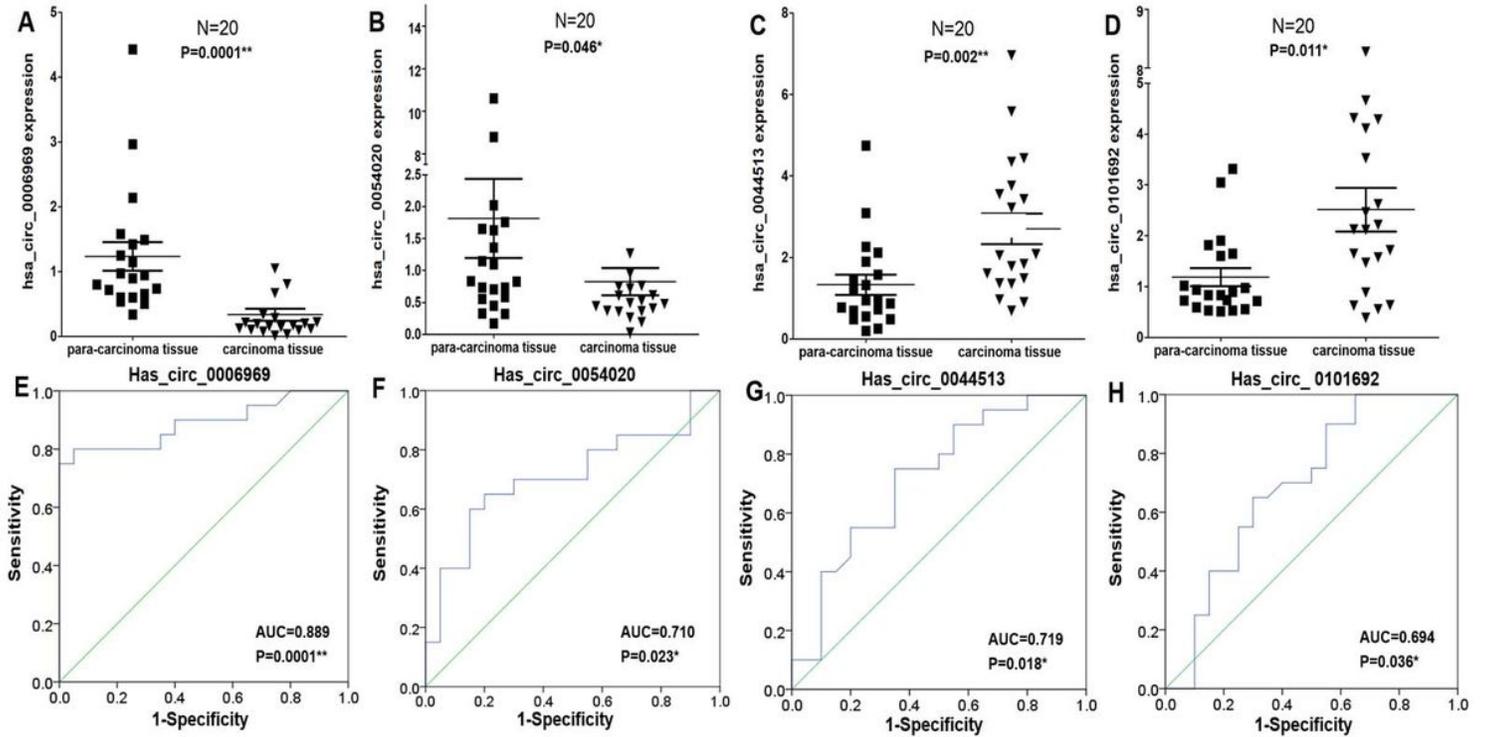


Figure 1

The diagnostic capability of 4 candidate circRNAs. (A-D) 4 candidate circRNAs expression between breast carcinoma tissue and para-carcinoma tissue; (E-H) The ROC analysis results of 4 candidate circRNAs; (A-E) hsa-circ-0006969, (B-F) hsa-circ_0054020 (C-G) hsa-circ_0044513 (D-H) hsa-circ_0101692; *P<0.05, **P<0.01.

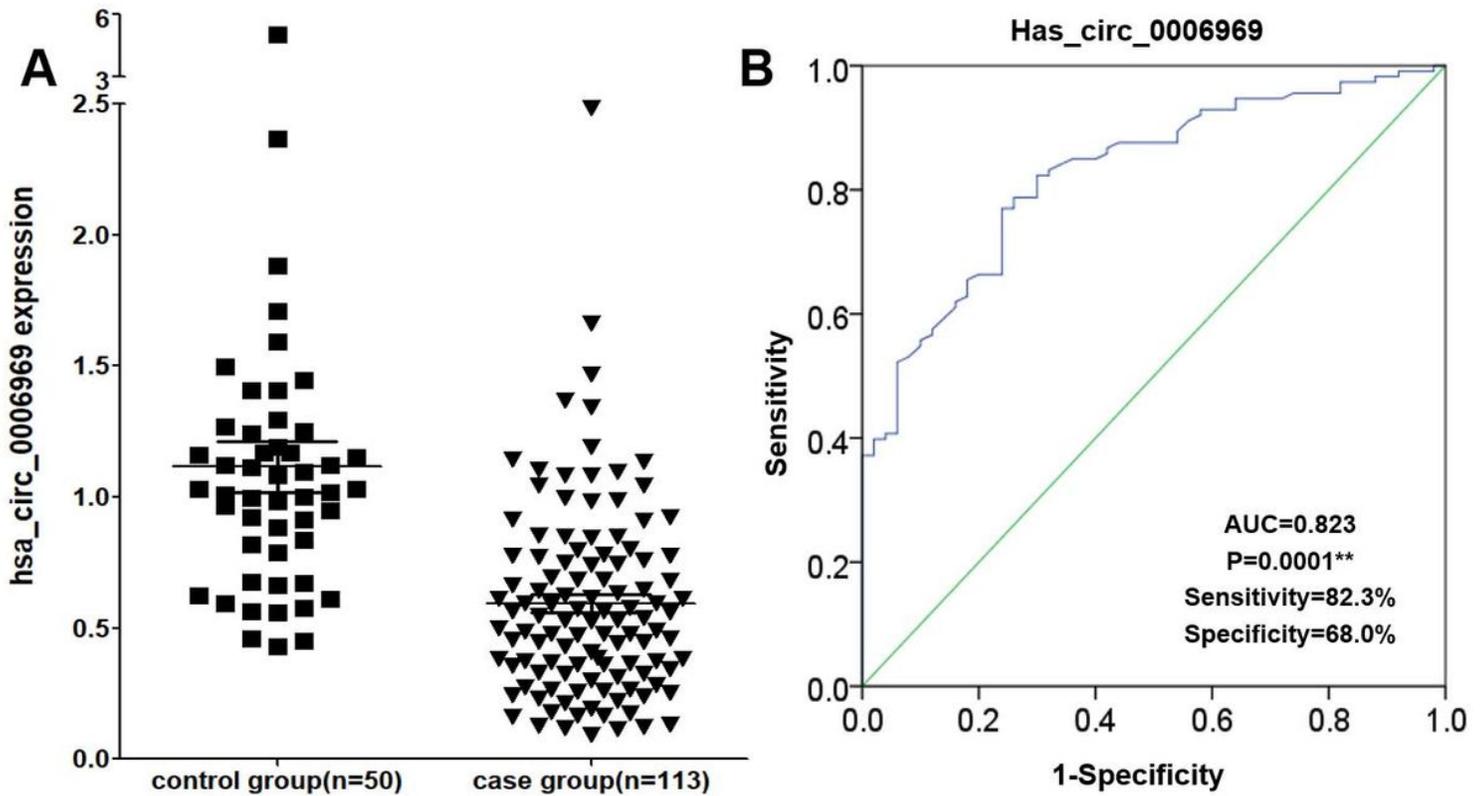


Figure 2

The biological structure of hsa-circ-0006969. (A) Schematic(circPrimer1.2) showed that hsa-circ-0006969 is derived from ARHGEF28 exons 10-11. (B) The expression of hsa-circ-0006969 and ARHGEF28 mRNA in MDA-MB-231 cells treated with RNase R. (C) The potential binding miRNAs of hsa-circ_000696. (D) The candidate binding miRNAs TOP 8(P-value) of hsa-circ-0006969 related with cancer cell cycle, proliferation and apoptosis. *P<0.05

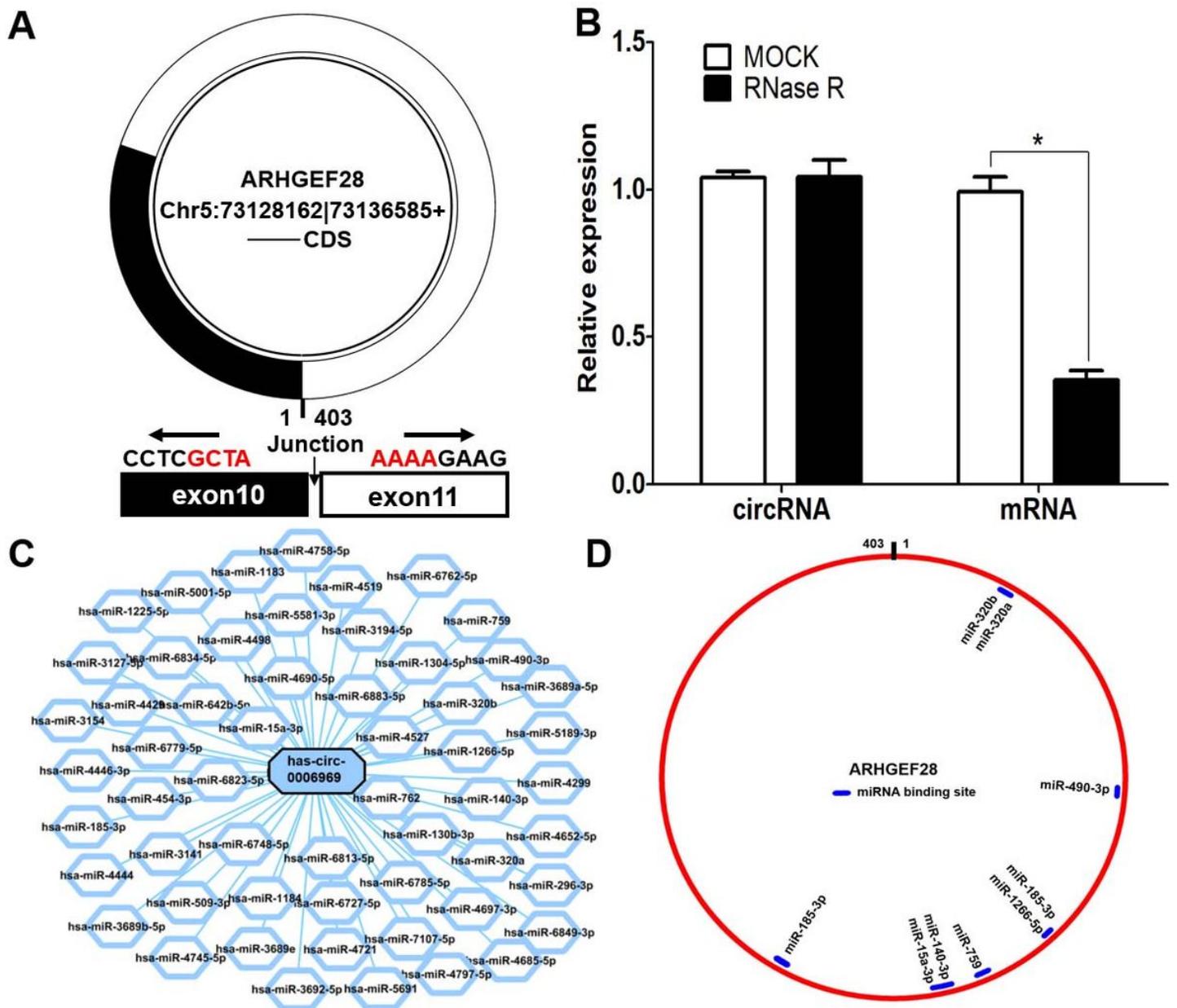


Figure 3

The diagnostic capability of hsa-circ-0006969 in peripheral blood. (breast cancer peripheral blood, case group, n=113; healthy person peripheral blood, control group, n=50; A Expression levels of hsa-circ-0006969 quantified by qPCR; B the ROC analyses of hsa-circ-0006969, The AUC values are given on the graphs; *P<0.05, **P<0.01.

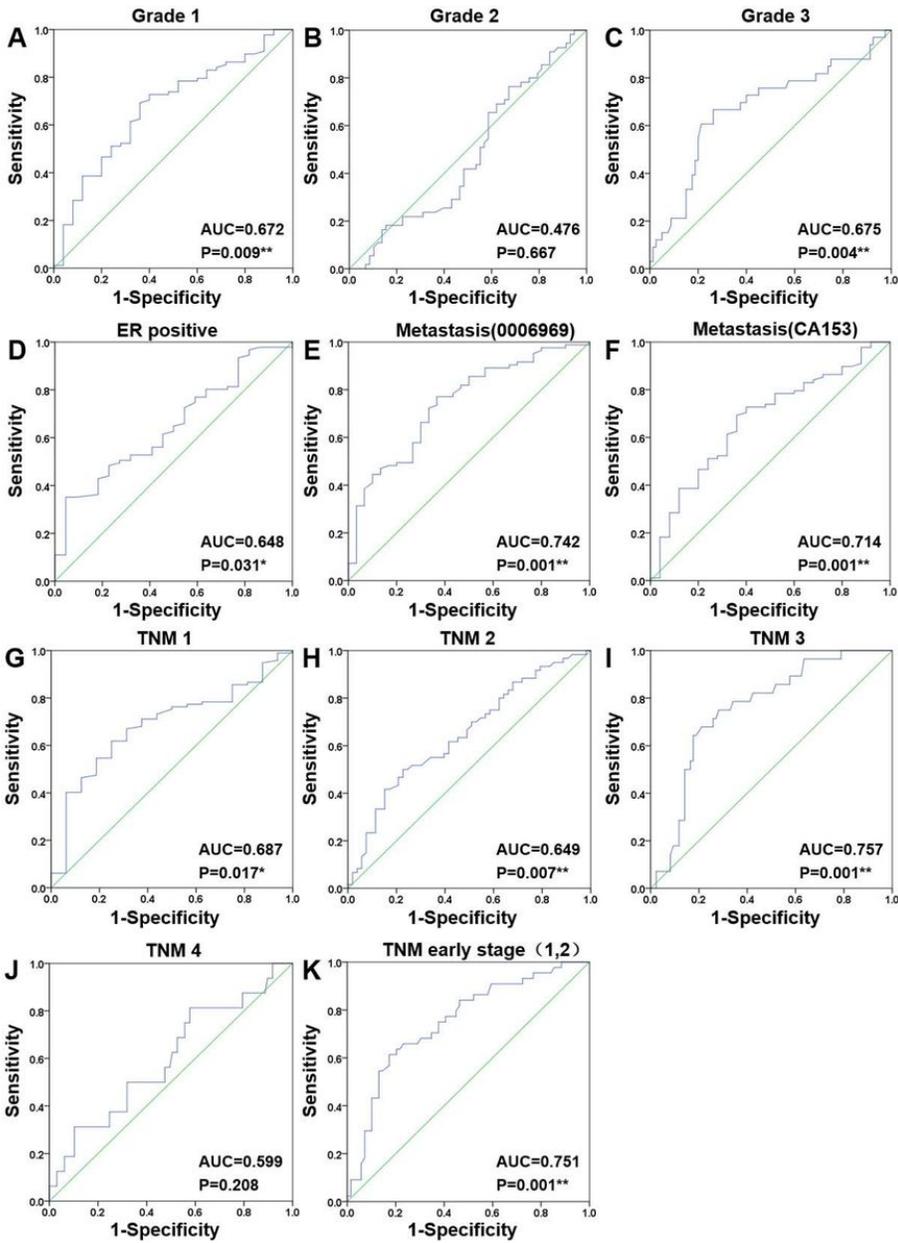


Figure 4

ROC curve analyses of hsa-circ-0006969 in peripheral blood with breast cancer. The AUC values are given on the graphs. sensitivity and specificity were showed in table S4; *P<0.05, **P<0.01.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [microarraydata.xlsx](#)
- [FigureS2.tif](#)
- [TableS3.docx](#)
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