Integrated Analysis of Circular RNA Associated Cerna Network Reveals Potential CircRNA Biomarkers in Human Breast Cancer

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Primary research

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Abstract

Background

There is increasing evidence that circular RNA (circRNA) is closely related to tumorigenesis and cancer progression. circRNA has been identified as a sponge of microRNA (miRNA) in a competitive endogenous RNA (ceRNA) network and is involved in the regulation of mRNA expression. However, the roles of cancer specific circRNAs in circRNA-related ceRNA network of breast cancer (BRCA) are still unclear. This study aims to construct a ceRNA network associated with circRNA and to explore new therapeutic and prognostic targets and biomarkers for breast cancer.

Methods

We downloaded the circRNA expression profile of BRCA from Gene Expression Omnibus (GEO) microarray datasets and downloaded the miRNA and mRNA expression profiles of BRCA from The Cancer Genome Atlas (TCGA) database, these data were included in the study for comprehensive analysis. Differentially expressed mRNAs (DEmRNAs), differentially expressed miRNAs (DEmiRNAs) and differentially expressed circRNAs (DEcircRNAs) were identified and a competitive endogenous RNA (ceRNA) regulatory network was constructed based on circRNA–miRNA pairs and miRNA–mRNA pairs. Gene ontology and pathway enrichment analysis were performed on mRNAs regulated by circRNAs in ceRNA networks. Survival analysis and correlation analysis of all mRNAs and miRNAs in the ceRNA network were performed. The STRING search tool was used to predict the interaction between proteins, and the hub genes were screened by the MCODE plugin in Cytoscape.

Results

A total of 72 DEcircRNAs, 158 DEmiRNAs and 2762 DE mRNAs were identified. The constructed ceRNA network contains 60 circRNA-miRNA pairs and 140 miRNA-mRNA pairs, including 40 circRNAs, 30 miRNAs and 100 mRNAs. Functional enrichment indicated that DEmRNAs regulated by DEcircRNAs in ceRNA networks were significantly enriched in PI3K-Akt signaling pathway, MicroRNAs in cancer and Proteoglycans in cancer. Survival analysis and correlation analysis of all mRNAs and miRNAs in the ceRNA network showed that a total of 13 mRNAs and 6 miRNAs were significantly associated with overall survival, and 48 miRNA-mRNA interaction pairs had a significant negative correlation. A PPI network was established and 21 hub genes were determined from the network. After comprehensive analysis, four potential ceRNA regulatory axes were constructed based on three circRNAs, two miRNAs, and three mRNAs.

Conclusions

This study provides an effective bioinformatics basis for further understanding the molecular mechanisms and predictions of breast cancer. A better understanding of the circRNA-related ceRNA network in BRCA will help identify potential biomarkers for diagnosis and prognosis.
Background

Breast cancer is one of the most common cancer among women worldwide [1], with strong invasiveness and metastasis, and the incidence and mortality of breast cancer continue to increase [2]. Currently, treatments for breast cancer include surgery, radiation therapy, endocrine therapy, chemotherapy, and biotargeted therapy. However, the recurrence rate and drug resistance of some patients are still high, and the therapeutic effect and prognosis of breast cancer have not been satisfactory. Therefore, the molecular pathogenesis of breast cancer needs to be further understood, and the identification of new candidate therapeutic targets and biomarkers is urgently needed for breast cancer treatment. An in-depth study of the molecular mechanism of tumors based on bioinformatics analysis has exploited an important method to tumor research. It can not only explore the molecular pathogenesis of tumors in depth, but also identify new biomarkers for tumor pathogenesis and prognosis [3].

In the past few decades, 70%-90% of the transcribed human genome has been identified. Related data indicate that protein-coding genes account for only about 2% of the human genome, and non-coding RNAs make up the majority of the human transcriptome [4]. Non-coding RNAs are a large class of RNA molecules that do not encode proteins, but which serve regulatory roles, mainly includes: circular RNAs (circRNAs), microRNAs (miRNAs), long noncoding RNAs (lncRNAs) and small nuclear RNAs. The competitive endogenous RNA (ceRNA) hypothesis reveals a new mechanism for interaction between RNAs. The main idea of the ceRNA hypothesis is that multiple types of RNA transcripts communicate with each other by competing for binding to shared miRNA-binding sites (miRNA response elements or MREs) [5]. It has been reported that circRNAs contain multiple miRNA-binding sites that bind to miRNAs, which are seen as miRNA sponges that result in inhibition of miRNAs activity and regulation of expression of their downstream target genes [6, 7].

CircRNA is a class of covalently closed single-stranded circular RNA molecules without free 5 or 3 end which makes them well expressed and more stable than their linear counterparts. CircRNA is abundant in eukaryotic cells, highly conserved, structurally stable, and has certain tissue, time and disease specificity. Due to these characteristics, circRNA has become a new hotspot of research [8]. A vast number of circRNAs have been discovered in a variety of cancers and they are activated in inhibiting tumor progression or promoting tumorigenesis. For example, circ-MTO1 can inhibit the progression of liver cancer cells [9]. circ-LARP4 can inhibit cell proliferation and invasion of gastric cancer cells by sponging miR-424-5p and regulating the expression of LATS1 [10]. Circ-FBXW7 suppresses the development of gliomas, and its expression is positively correlated with the overall survival of patients with glioblastoma [11]. The hsa_circ_001783 regulates the progression of breast cancer (in vitro) by sponging miR-200c-3p to regulate ZEB1/2 and ETS1 and is associated with poor clinical outcomes in breast cancer patients [12].

In the current study, we collected the expression profiles of circRNA, miRNA and mRNA from BRCA tissues and adjacent normal mammary gland tissues from the Gene Expression Omnibus (GEO) database and the The Cancer Genome Atlas (TCGA) database. We performed a comprehensive analysis of these
expression profiles to identify differentially expressed mRNAs (DEmRNAs), differentially expressed miRNAs (DEmiRNAs), and differentially expressed circRNAs (DEcircRNAs). After predicting sponging of miRNAs by circRNA and miRNA target genes, we constructed a circRNA-miRNA-mRNA network. To investigate the main functional pathways involved in the development of breast cancer in this ceRNA network, DEmRNAs of the ceRNA network were assessed by gene ontology (GO) annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses, and we have established a protein-protein interaction network, this study will try to better understand the pathogenesis of BRCA. Finally, we performed an overall survival analysis of miRNAs and mRNAs in ceRNA networks to identify prognostic biomarkers associated with breast cancer. Through this study, we can not only further understand the molecular mechanism of breast cancer development, but also provide potential circRNA, miRNA and mRNA biomarkers for the early diagnosis, treatment and prognosis of breast cancer.

**Methods**

**Expression profiling in The Cancer Genome Atlas and Gene Expression Omnibus**

The mRNA and miRNA sequence data of breast cancer were extracted from the TCGA database [https://portal.gdc.cancer.gov/](https://portal.gdc.cancer.gov/). All file data were downloaded using the GDC Data Transfer Tool (Provided by GDC Apps) [https://tcga-data.nci.nih.gov/](https://tcga-data.nci.nih.gov/). The mRNA profiles contained 1097 BRCA tissues and 114 adjacent normal tissues, and the miRNA profiles contained 1092 BRCA tissues and 105 adjacent normal tissues. The exclusion criteria were set as follows: samples without clinical data and samples without complete information of stage and overall survival period.

The circRNA expression profiles of BRCA were downloaded from GEO database [http://www.ncbi.nlm.nih.gov/geo](http://www.ncbi.nlm.nih.gov/geo) by searching keywords ("breast neoplasms" [MeSH Terms] OR breast cancer [All Fields]) AND circRNA [All Fields]) AND ("Homo sapiens" [Organism] AND ("Non-coding RNA profiling by array" [Filter] OR "Non-coding RNA profiling by high throughput sequencing" [Filter])). We selected data according to the following criteria: selected datasets should be circRNA transcriptome data of the whole genome, these data were derived from tumor tissues and adjacent normal tissues of patients with BRCA, and datasets were standardized or raw datasets. The GSE101123 dataset met the screening requirements and was used in this study. The dataset included 3 normal mammary gland tissues and 8 BRCA tissues. These expression profiles do not require ethical approval or informed consent due to we used the publicly available data from TCGA and GEO.

**Identification of differentially expressed mRNAs, miRNA, circRNA in breast cancer compared to adjacent tissues**

Firstly, the difficultly detected mRNAs/miRNAs, which with read count value=0 in more than 50% samples, were filtered and deleted. To obtain the differentially expressed mRNAs (DEmRNAs) and miRNAs (DEmiRNAs) between normal tissues and BRCA, the count data were processed with the Bioconductor package edge R [13] in software. All RNA expression levels were standardized to the sample mean. The $P$ value was corrected with a false discovery rate (FDR). The threshold for the expression of DEmRNAs and
DEmiRNAs was FDR<0.01 and |log₂ fold change|>1. Additionally, the differently expressed circRNAs (DEcircRNAs) were screened using Limma package, the threshold for the expression of DEcircRNAs was P value<0.01 and |log₂ fold change|>1.

**Construction of the ceRNA regulatory network**

The Circular RNA Interactome (CircInteractome) ([https://circinteractome.nia.nih.gov/](https://circinteractome.nia.nih.gov/)) and Cancer-Specific CircRNA (CSCD) ([http://gb.whu.edu.cn/CSCD/](http://gb.whu.edu.cn/CSCD/)) were used to predict miRNA binding sites (MREs). These miRNAs were considered as potential target miRNAs of the DEcircRNAs. These target miRNAs were further screened by DEmiRNA based on the TCGA.

Interactions between miRNA and mRNA were predicted based on the Targetscan [14], miRTarBase [15], and miRDB [16] databases. Only mRNAs recognized by all three database were considered as candidate mRNAs, and were intersected with DEmRNAs to screen the DEmRNAs targeted by DEmiRNAs. The circRNA-miRNA-mRNA regulatory network was constructed using a combination of circRNA-miRNA pairs and miRNA-mRNA pairs. Finally, the network was visualized and mapped using Cytoscape v3.7.0 [17]. Figure 1 shows a flow chart for the development of the ceRNA network.

**Gene ontology and pathway enrichment analysis of DEGs in the ceRNA network**

To assess the function of differentially expressed genes (DEGs) in the ceRNA network in tumorigenesis, we performed Gene Ontology (GO) annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses using the clusterProfiler package [18] of R software. P value<0.01 was set as the cut-off criterion.

**Survival analysis and correlation analysis of DEmiRNAs and DEmRNAs in ceRNA networks**

Each sample in the TCGA was independent of each other and it contained all sample information such as gene expression, prognosis and survival time. We obtained clinical information from breast cancer patients from the TCGA database and combined the expression data of DEmiRNAs and DE mRNAs with clinical data from patients. We used Survival package of R to perform survival analysis of DEmiRNAs and DE mRNAs in the ceRNA network with \( P < 0.05 \) as the threshold. In addition, DEmiRNAs and DEmRNAs with significant overall survival were identified as prognostic biomarkers.

In the TCGA-BRCA dataset, the vast majority of samples were present in both miRNA and mRNA expression profiles, and samples that were only present in one expression profile were deleted. Correlation analysis between the interacting miRNA and mRNA in the ceRNA network was performed using R software, with \( r < 0.3, P < 0.001 \) as the threshold. The miRNA-mRNA pair that satisfies the condition is considered to have a strong negative correlation.

**Construction PPI network and module analysis**
To assess the interactions between the DEGs in the ceRNA network, we constructed a protein-protein interaction (PPI) network using the Search Tool for the Retrieval of Interacting Genes (STRING, http://string.embl.de/) online tool. We used the MCODE plugin to screen modules of hub genes from the PPI network. The interaction network was visualized using Cytoscape software.

**Quantitative real-time PCR validation**

Ten pairs of breast cancer tissues and corresponding adjacent non-tumor tissues from BRCA patients were obtained from Department of Breast Disease, The First Affiliated Hospital of Jiaxing University. The study was approved by the ethics committee and written informed consent was obtained from all patients. In this ceRNA network, we randomly selected six circRNAs, miRNAs and mRNAs respectively, and verified the reliability and validity of the prediction results in BRCA patients using qRT-PCR. Total RNA was isolated using Trizol reagent (Invitrogen, USA) according to the manufacturer’s protocol, and RNA purity was detected by NanoDrop 2000 spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). Based on SuperReal PreMix Plus (Invitrogen, USA) in StepOneplus Real-time PCR Detection System (Applied Biosystems, Foster City, CA, USA), the qRT-PCR reactions were performed. The relative gene expression was calculated by $2^{-\Delta\Delta Ct}$. The human $\beta$-actin and human U6 was used as endogenous controls for mRNA and miRNA expression in analysis, respectively. The human GAPDH was used as endogenous controls for circRNA expression in analysis.

**Results**

**Identification of differentially expressed RNAs in breast cancer**

Compared to adjacent tissues, a total of 2762 DEmRNAs (1118 upregulated and 1644 downregulated miRNAs) and 158 DEmiRNAs (71 upregulated and 87 downregulated miRNAs) were identified in BRCA with FDR<0.01 and $|\log_2\text{fold change}|>1$. A total of 72 DEcircRNAs (51 upregulated and 21 downregulated circRNAs) were obtained in BRCA compared to adjacent tissues with $P$ value<0.01, $|\log_2\text{fold change}|>1$. The RNAs hierarchical clustering analyses are presented in Figure 2, and it was demonstrated that the expression levels of these three type of RNAs were significantly differentiated compared with the normal tissues. Finally, volcano plots were generated, and differences between the normal and tumor groups were identified (Figure 2). Table 1, 2 and 3 show the top 10 up and down regulation of DEmRNAs, DEmiRNAs and DEcircRNAs in BRCA, respectively.

**Table 1** Top 10 upregulation and downregulation DEmRNAs in BRCA.
| mRNA      | logFC     | P value    | FDR         | Regulation |
|-----------|-----------|------------|-------------|------------|
| COL10A1   | 6.870373  | 1.01E-60   | 1.67E-59    | Up         |
| CST1      | 6.616865  | 1.52E-56   | 2.15E-55    | Up         |
| MMP13     | 6.354082  | 8.94E-57   | 1.27E-55    | Up         |
| IBSP      | 5.863152  | 3.64E-104  | 1.91E-102   | Up         |
| MMP11     | 5.781753  | 2.35E-84   | 7.56E-83    | Up         |
| COL11A1   | 5.337783  | 1.91E-37   | 1.39E-36    | Up         |
| MMP1      | 5.235857  | 1.55E-39   | 1.21E-38    | Up         |
| PLPP4     | 5.184921  | 2.61E-88   | 9.12E-87    | Up         |
| SLC24A2   | 4.98746   | 2.18E-73   | 5.43E-72    | Up         |
| COMP      | 4.598021  | 7.01E-40   | 5.58E-39    | Up         |
| ADH1B     | -6.86393  | 2.57E-114  | 1.69E-112   | Down       |
| TUSC5     | -6.81912  | 1.32E-161  | 3.10E-159   | Down       |
| ADIPOQ    | -6.79919  | 3.51E-120  | 2.65E-118   | Down       |
| CIDE1     | -6.63588  | 3.51E-174  | 1.19E-171   | Down       |
| SCARA5    | -6.48854  | 3.00E-239  | 1.53E-235   | Down       |
| FABP4     | -6.14086  | 1.66E-110  | 1.01E-108   | Down       |
| PLIN1     | -6.00856  | 4.59E-146  | 6.21E-144   | Down       |
| GPD1      | -5.99957  | 5.75E-169  | 1.57E-166   | Down       |
| AQP7      | -5.81861  | 1.75E-206  | 1.91E-203   | Down       |
| PLIN4     | -5.79315  | 4.21E-115  | 2.84E-113   | Down       |

**Table 2** Top 10 upregulation and downregulation DEmiRNAs in BRCA.
| miRNA          | logFC  | P-value    | FDR         | Regulation |
|---------------|--------|------------|-------------|------------|
| hsa-miR-592   | 3.424794 | 2.41E-76   | 6.71E-75    | Up         |
| hsa-miR-1307-5p | 3.197336 | 1.96E-88   | 6.73E-87    | Up         |
| hsa-miR-96-5p  | 3.155099  | 3.09E-90   | 1.25E-88    | Up         |
| hsa-miR-141-3p | 3.087332  | 4.21E-97   | 1.88E-95    | Up         |
| hsa-miR-429   | 2.994871  | 1.10E-70   | 2.32E-69    | Up         |
| hsa-miR-190b  | 2.963382  | 6.87E-26   | 2.73E-25    | Up         |
| hsa-miR-183-5p | 2.924208  | 6.80E-108  | 5.05E-106   | Up         |
| hsa-miR-200a-3p | 2.638975  | 4.00E-63   | 6.60E-62    | Up         |
| hsa-miR-184   | 2.553121  | 1.13E-13   | 2.68E-13    | Up         |
| hsa-miR-200a-5p | 2.512744  | 2.55E-65   | 4.53E-64    | Up         |
| hsa-miR-486-5p | -4.09846  | 1.82E-115  | 1.62E-113   | Down       |
| hsa-miR-139-3p | -3.60464  | 2.43E-132  | 5.41E-130   | Down       |
| hsa-miR-204-5p | -3.53102  | 4.25E-102  | 2.70E-100   | Down       |
| hsa-miR-139-5p | -3.19493  | 2.46E-156  | 1.10E-153   | Down       |
| hsa-miR-451a  | -3.07530  | 1.49E-76   | 4.42E-75    | Down       |
| hsa-miR-5683  | -2.96346  | 7.12E-51   | 7.73E-50    | Down       |
| hsa-miR-144-5p | -2.79822  | 2.63E-71   | 5.84E-70    | Down       |
| hsa-miR-1247-3p | -2.73934  | 1.42E-45   | 1.27E-44    | Down       |
| hsa-miR-452-5p | -2.48651  | 2.97E-54   | 3.67E-53    | Down       |
| hsa-miR-145-5p | -2.45593  | 1.41E-123  | 1.57E-121   | Down       |

Table 3 Top 10 upregulation and downregulation DEcircRNAs in BRCA.
| circRNA           | Alias           | logFC   | P value   | Regulation |
|------------------|-----------------|---------|-----------|------------|
| hsa_circRNA_001846 | hsa_circ_0000520 | 3.80033 | 0.0004347 | Up         |
| hsa_circRNA_000167 | hsa_circ_0000518 | 3.742085| 0.0016513 | Up         |
| hsa_circRNA_002172 | hsa_circ_0000514 | 3.365067| 0.0042528 | Up         |
| hsa_circRNA_002144 | hsa_circ_0000511 | 2.995608| 0.0044046 | Up         |
| hsa_circRNA_000166 | hsa_circ_0000512 | 2.883005| 0.0044287 | Up         |
| hsa_circRNA_000585 | hsa_circ_0000515 | 2.788805| 0.0012788 | Up         |
| hsa_circRNA_001678 | hsa_circ_0000517 | 2.6775  | 0.0001633 | Up         |
| hsa_circRNA_101967 | hsa_circ_0041732 | 2.335477| 0.002966  | Up         |
| hsa_circRNA_101233 | hsa_circ_0008784 | 1.958382| 0.0035194 | Up         |
| hsa_circRNA_002178 | hsa_circ_0000519 | 1.90691 | 0.00011   | Up         |
| hsa_circRNA_102049 | hsa_circ_0043278 | 3.924372| 0.0099265 | Down       |
| hsa_circRNA_102619 | hsa_circ_000977  | 3.646793| 0.0080463 | Down       |
| hsa_circRNA_102051 | hsa_circ_006220  | 3.398483| 0.0091516 | Down       |
| hsa_circRNA_103345 | hsa_circ_0065173 | 2.507172| 0.0025211 | Down       |
| hsa_circRNA_102651 | hsa_circ_0008911 | 2.179549| 0.001517  | Down       |
| hsa_circRNA_001153 | hsa_circ_0001455 | 2.059051| 0.0016539 | Down       |
| hsa_circRNA_104653 | hsa_circ_0008303 | 1.776765| 0.0009081 | Down       |
| hsa_circRNA_101381 | hsa_circ_004781  | 1.771974| 0.0029019 | Down       |
| hsa_circRNA_100685 | hsa_circ_0020080 | 1.714172| 0.0034233 | Down       |
| hsa_circRNA_000554 | hsa_circ_0000376 | 1.615084| 0.0001321 | Down       |

**Construction of ceRNA regulatory network in BRCA**

To elucidate the regulatory mechanism of BRCA, a circRNA-miRNA-mRNA related ceRNA network of BRCA was developed according the above results. First, we searched for the target miRNAs of the 72 DEcircRNAs in the CircIteractome and CSCD databases, and found 295 interactive circRNAs-miRNAs pairs after intersecting with the DEMiRNAs. The circRNA-miRNA relationship pairs were screened according to a negative regulatory pattern, and positively co-expressed circRNA-miRNA pairs were discarded. The results showed that 162 interactive circRNA-miRNA pairs were screened, of which 72 DEMiRNAs were confirmed to interact with 59 DEcircRNAs. Following this, we predicted that 1626 mRNAs were targeted by these 72 DEMiRNAs in all three target predicting databases (TargetScan, miRTarBase and miRDB). these 1626 target mRNAs intersected with the 2762 DEMRNAs, and target mRNAs not
contained in DEmRNAs was excluded, resulting in a total of 327 interactive miRNA-mRNA pairs. At the same time, we also screened miRNA-mRNA pairs based on negative regulatory patterns and discarded positively co-expressing pairs. The results showed that eventually 30 DEmiRNAs and 100 DEmRNAs formed 140 interactive miRNA-mRNA pairs. The circRNA-miRNA and miRNA –mRNA relationship pairs (Tables 4 and 5) were combined into the ceRNA network following the pattern of negative regulation. Finally, we constructed the ceRNA regulatory network of BRCA comprised of 200 edges among 40 DEcircRNAs, 30 DEmiRNAs and 100 DEmRNAs. The ceRNA network in BRCA was visualized using Cytoscape software (Figure 3).

Table 4 Interaction between circRNA and miRNA in the ceRNA network.
| circRNA            | miRNA                         |
|--------------------|-------------------------------|
| hsa_circ_0000069   | hsa-miR-193a-5p               |
| hsa_circ_0000376   | hsa-miR-142-5p                |
| hsa_circ_0000511   | hsa-miR-296-5p                |
| hsa_circ_0000512   | hsa-miR-296-5p                |
| hsa_circ_0000514   | hsa-miR-296-5p                |
| hsa_circ_0000515   | hsa-miR-204-5p, hsa-miR-296-5p|
| hsa_circ_0000517   | hsa-miR-193a-5p, hsa-miR-296-5p|
| hsa_circ_0000519   | hsa-miR-204-5p, hsa-miR-296-5p|
| hsa_circ_0000520   | hsa-miR-296-5p                |
| hsa_circ_0001455   | hsa-miR-142-5p                |
| hsa_circ_0001806   | hsa-miR-139-5p                |
| hsa_circ_0002702   | hsa-miR-100-5p, hsa-miR-99a-5p, hsa-miR-296-5p |
| hsa_circ_0003528   | hsa-miR-224-5p                |
| hsa_circ_0003645   | hsa-miR-335-5p                |
| hsa_circ_0004313   | hsa-miR-365a-3p, hsa-miR-365b-3p |
| hsa_circ_0004315   | hsa-miR-145-5p, hsa-miR-195-5p, hsa-miR-497-5p, hsa-miR-218-5p |
| hsa_circ_0004538   | hsa-miR-503-5p, hsa-miR-7-5p  |
| hsa_circ_0005273   | hsa-miR-328-3p                |
| hsa_circ_0005397   | hsa-miR-10b-5p, hsa-miR-1-3p  |
| hsa_circ_0005699   | hsa-miR-143-3p                |
| hsa_circ_0006220   | hsa-miR-342-3p                |
| hsa_circ_0006758   | hsa-miR-224-5p, hsa-miR-1-3p  |
| hsa_circ_0008365   | hsa-miR-328-3p                |
| hsa_circ_0008784   | hsa-miR-193a-5p, hsa-miR-139-5p|
| hsa_circ_0014624   | hsa-miR-7-5p                  |
| hsa_circ_0016201   | hsa-miR-33a-5p, hsa-miR-33b-5p|
| hsa_circ_0020080   | hsa-miR-7-5p                  |
| hsa_circ_0022587   | hsa-miR-193a-5p               |
|                |                                                                 |
|----------------|-----------------------------------------------------------------|
| hsa_circ_0025388 | hsa-miR-139-5p, hsa-miR-144-3p, hsa-miR-335-5p                  |
| hsa_circ_0028190 | hsa-miR-451a                                                    |
| hsa_circ_0031724 | hsa-miR-143-3p                                                  |
| hsa_circ_0041732 | hsa-miR-193a-5p                                                 |
| hsa_circ_0041821 | hsa-miR-144-3p                                                  |
| hsa_circ_0049998 | hsa-miR-205-5p, hsa-miR-145-5p                                  |
| hsa_circ_0054021 | hsa-miR-141-3p, hsa-miR-200a-3p                                 |
| hsa_circ_0058753 | hsa-miR-218-5p                                                  |
| hsa_circ_0069104 | hsa-miR-451a, hsa-miR-296-5p                                   |
| hsa_circ_0082564 | hsa-miR-205-5p                                                  |
| hsa_circ_0084429 | hsa-miR-129-5p, hsa-miR-224-5p                                 |
| hsa_circ_0084443 | hsa-miR-129-5p                                                  |

**Table 5** Interaction between miRNA and mRNA in the ceRNA network.
| miRNA          | mRNA                                      |
|----------------|-------------------------------------------|
| hsa-miR-100-5p | FGFR3                                     |
| hsa-miR-10b-5p | GATA3, SDC1                               |
| hsa-miR-129-5p | CBX4, COL1A1                              |
| hsa-miR-139-5p | TPD52, ZNF367                             |
| hsa-miR-1-3p   | ADAM12, AP1S1, CERS2, E2F5, FAM102A, FN1, RIMS4, SLC25A22, GPR137C, TRPS1 |
| hsa-miR-141-3p | EPHA2, QKI, STAT5A, USP53, YAP1, ZEB2     |
| hsa-miR-142-5p | CREBRF, FIGN, SLITRK4, TNS1, ZBTB20       |
| hsa-miR-143-3p | COL1A1, ERBB3, LIMK1, SERPINE1, TTYH3     |
| hsa-miR-144-3p | EZH2, KPNA2, NACC1, SIX4                  |
| hsa-miR-145-5p | ABHD17C, ABRACL, RTKN, SERPINE1, SOX11, TPM3 |
| hsa-miR-193a-5p | NUP210                                   |
| hsa-miR-195-5p | CBX2, CBX4, CCNE1, CDC25A, CDC4A, CEP55, CHEK1, CLSPN, E2F7, ENTPD7, HMGA1, KIF23, MYB, RASEF, RET, SLC25A22, ZNF367 |
| hsa-miR-200a-3p | DLC1, EPHA2, HGF, QKI, THRBB, USP53, YAP1, ZEB2 |
| hsa-miR-204-5p | AP1S1, EZR, RUNX2                         |
| hsa-miR-205-5p | CENPF, ERBB3, EZR, LPCAT1, PARD6B, RUNX2  |
| hsa-miR-218-5p | BCL9, CDH2, CNTNAP2, FBN2, FBXO41, LMNB1, RET, NACC1, PRLR, RUNX2, TPD52, TTYH3 |
| hsa-miR-224-5p | DIO1                                      |
| hsa-miR-296-5p | HMGA1                                     |
| hsa-miR-328-3p | H2AFX                                     |
hsa-miR-335-5p  
CDH11

hsa-miR-33a-5p  
ABCA1, ARID5B, DSC3, GAS1, ZC3H12C

hsa-miR-33b-5p  
ABCA1, ARID5B, GAS1

hsa-miR-342-3p  
FIGN, ID4, MBNL3

hsa-miR-365a-3p  
MCOLN2, SIX4

hsa-miR-365b-3p  
MCOLN2, SIX4

hsa-miR-451a  
CDKN2D, MIF

hsa-miR-497-5p  
ANLN, CBX2, CBX4, CCNE1, CDC25A, CDCA4, CEP55, CHEK1, CLSPN, E2F7, KIF23, RASEF, ZNF367

hsa-miR-503-5p  
AKT3, CCND2, CYP26B1, FGF2, PIK3R1, RECK

hsa-miR-7-5p  
EGFR, FNDC4, IRS1, IRS2, KLF4, RBMS3, RRAS2, SNCA, SOCS2

hsa-miR-99a-5p  
FGFR3

**Functional annotation of the DEGs in the ceRNA network**

In order to better understand the potential functional significance of differentially expressed genes in the ceRNA network, we performed GO and KEGG functional enrichment analysis. In the GO analysis we identified a total of 162 enriched GO terms (FDR<0.01). The top 8 significantly enriched GO terms in the biological process (BP), cellular components (CC) and molecular function (MF) are shown in Figure 4. The biological processes of these differentially expressed genes were primarily associated with regulated by protein kinase B signaling, phosphatidylinositol phosphorylation, protein kinase B signaling and lipid phosphorylation. Meanwhile, the genes related to cellular components were mostly involved in nuclear transcription factor complex, focal adhesion, cell-substrate adherens junction and cell-substrate junction. In terms of molecular function, these differential genes were mostly enriched in phosphatidylinositol-4,5-bisphosphate 3-kinase activity, phosphatidylinositol bisphosphate kinase activity, phosphatidylinositol 3-kinase activity and 1-phosphatidylinositol-3-kinase activity.

Additionally, KEGG signal pathway analysis showed that 24 signal pathways were significantly enriched (FDR<0.01). The top 15 significantly enriched pathways are shown in Figure 5. Among these pathways, the ‘PI3K-Akt signaling pathway’, ‘MicroRNAs in cancer’, ‘Proteoglycans in cancer’, ‘Cellular senescence’,
‘FoxO signaling pathway’, ‘Central carbon metabolism in cancer’ and ‘Cell cycle’ are closely correlated with the carcinogenesis and development of BRCA.

**Prognostic characteristics of RNAs in the ceRNA regulatory network**

Survival analysis based on Survival package of R found that 13 mRNAs – CCNE1, TPD52, SDC1, ANLN, ZNF367, SOX11, IRS2, EZR, DSC3, CCND2, KPNA2, CBX2 and CEP55 – among the 100 DEmRNAs in the ceRNA network were closely associated with the overall survival of breast cancer patients. The low expression of CCNE1, TPD52, SDC1, ANLN, ZNF367, SOX11, EZR, KPNA2, CBX2 and CEP55 was associated with high survival, whereas for IRS2, DSC3 and CCND2, high expression was associated with high survival. Six miRNAs (hsa-miR-204-5p, hsa-miR-335-5p, hsa-miR-100-5p, hsa-miR-195-5p, hsa-miR-328-3p and hsa-miR-342-3p) of 30 DEmiRNAs were associated with prognosis. High expression of hsa-miR-204-5p, hsa-miR-335-5p, hsa-miR-100-5p, hsa-miR-195-5p and hsa-miR-342-3p indicated long survival time, while high expression of hsa-miR-328-3p indicated a relatively short survival time. Survival analysis results are shown in Table 6 and Figure 6. Notably, based on the ceRNA network, we found that the hsa_circ_0004315-hsa-miR195-5p axis was associated with four mRNAs associated with breast cancer prognosis.

**Table 6** Prognostic value of the differentially expressed mRNAs and miRNAs.
| Name          | HR (95% CI)         | P value |
|--------------|---------------------|---------|
| CCNE1        | 1.606 (1.169-2.208) | 0.0038  |
| TPD52        | 1.578 (1.148-2.169) | 0.0053  |
| SDC1         | 1.516 (1.102-2.086) | 0.0102  |
| ANLN         | 1.489 (1.083-2.046) | 0.0154  |
| ZNF367       | 1.475 (1.073-2.025) | 0.0176  |
| SOX11        | 1.443 (1.105-1.983) | 0.0244  |
| IRS2         | 0.705 (0.512-0.971) | 0.0299  |
| EZR          | 1.418 (1.030-1.950) | 0.0311  |
| DSC3         | 0.718 (0.521-0.988) | 0.0398  |
| CCND2        | 0.718 (0.522-0.987) | 0.0400  |
| KPNA2        | 1.395 (1.015-1.917) | 0.0410  |
| CBX2         | 1.391 (1.101-1.912) | 0.0433  |
| CEP55        | 1.384 (1.107-1.902) | 0.0474  |
| hsa-miR-195-5p | 0.629 (0.455-0.870) | 0.0046  |
| hsa-miR-204-5p | 0.648 (0.469-0.894) | 0.0086  |
| hsa-miR-335-5p | 0.664 (0.481-0.916) | 0.0134  |
| hsa-miR-342-3p | 0.696 (0.504-0.961) | 0.0280  |
| hsa-miR-100-5p | 0.704 (0.509-0.972) | 0.0323  |
| hsa-miR-328-3p | 1.410 (1.021-1.947) | 0.0356  |

Interaction between miRNA and mRNA from the ceRNA network

According to ceRNA theory, circRNA could indirectly affect mRNA through miRNA. At the expression level, miRNA was negatively correlated with circRNA and mRNA. In order to verify that the network we built was consistent with ceRNA theory, we needed to perform correlation analysis on different kinds of RNA. The expression information of circRNA in this study was from the GSE101123 dataset, while the expression information of miRNA and mRNA were from the TCGA dataset. Since the expression information of RNAs in the correlation analysis must be from the same sample, this study could only analyze the correlation between the expression levels of miRNA and mRNA. We performed a correlation analysis of miRNA-mRNA pairs in the ceRNA network based on R software, and the results showed that there were 48 miRNA-mRNA pairs with strong negative correlation ($r$<0.3, $P$<0.001) (Table 7). For instance, hsa-miR-141-3p negatively correlated with ZEB2 ($r$=-0.599, $P$<0.001) and QKI ($r$=-0.535, $P$<0.001), hsa-miR-195-5p
negatively correlated with CEP55 ($r=-0.547$, $P<0.001$) and CLSPN ($r=-0.525$, $P<0.001$), hsa-miR-200a-3p negatively correlated with ZEB2 ($r=-0.520$, $P<0.001$) as well as QKI ($r=-0.513$, $P=0.001$) (Figure 7).

**Table 7** Correlation analysis of the relationship between miRNA and mRNA.
| miRNA          | mRNA  | R      | P_value |
|---------------|-------|--------|---------|
| hsa-miR-141-3p| ZEB2  | -0.59875 | 0       |
| hsa-miR-195-5p| CEP55 | -0.54744 | 0       |
| hsa-miR-141-3p| QKI   | -0.53509 | 0       |
| hsa-miR-195-5p| CLSPN | -0.52524 | 0       |
| hsa-miR-200a-3p| ZEB2 | -0.52049 | 0       |
| hsa-miR-200a-3p| QKI  | -0.51254 | 0       |
| hsa-miR-195-5p| HMGA1 | -0.49915 | 0       |
| hsa-miR-497-5p| CEP55 | -0.49525 | 0       |
| hsa-miR-195-5p| CHEK1 | -0.49341 | 0       |
| hsa-miR-195-5p| CDC25A | -0.49147 | 0       |
| hsa-miR-195-5p| CCNE1 | -0.48761 | 0       |
| hsa-miR-497-5p| ANLN  | -0.45814 | 0       |
| hsa-miR-139-5p| TPD52 | -0.44566 | 0       |
| hsa-miR-195-5p| E2F7  | -0.44235 | 0       |
| hsa-miR-139-5p| ZNF367 | -0.43672 | 0       |
| hsa-miR-497-5p| CLSPN | -0.43102 | 0       |
| hsa-miR-145-5p| TPM3  | -0.42875 | 0       |
| hsa-miR-195-5p| CBX2  | -0.42701 | 0       |
| hsa-miR-145-5p| RTKN  | -0.42632 | 0       |
| hsa-miR-195-5p| ZNF367 | -0.42269 | 0       |
| hsa-miR-200a-3p| DLC1 | -0.4224  | 0       |
| hsa-miR-497-5p| CCNE1 | -0.42016 | 0       |
| hsa-miR-7-5p   | RBMS3 | -0.41862 | 0       |
| hsa-miR-497-5p| CDC25A | -0.4139  | 0       |
| hsa-miR-342-3p | ID4   | -0.4063  | 0       |
| hsa-miR-497-5p| CHEK1 | -0.40444 | 0       |
| hsa-miR-141-3p| STAT5A | -0.39451 | 0       |
| hsa-miR-218-5p | LMNB1 | -0.3945  | 0       |
The STRING database was used to unveil the interrelationships between the DEmRNAs in the ceRNA network by constructing PPI network. This PPI network involves a total of 75 nodes and 283 edges. Visualization was performed with Cytoscape (Figure 8A). In order to identify hub genes in the process of BRCA carcinogenesis, the MCODE plugin in Cytoscape was used to identify the core subnetwork in the PPI network. Two core sub-networks were obtained, including 21 genes and 49 edges (Figure 8B). We used these 21 genes as potential hub genes.

Quantitative real-time PCR validation

Finally, we randomly selected four DEcircRNAs, DEmiRNAs and DEmRNAs respectively in the ceRNA network to verify the reliability and validity of the above analysis results. These results showed that
CCNE1, CEP55, ANLN, hsa-miR-592, hsa-miR-141-3p, hsa_circ_0000069, hsa_circ_0000518 and has_circ_0000520 were up-regulated in BRCA tumor tissues compared to adjacent non-tumor tissues, while ADIPOQ, hsa-miR-195-5p, hsa-miR-204-5p and has_circ_0000977 were down-regulated in BRCA tumor tissues (Figure 9). The results of qRT-PCR validation from new breast cancer patients were consistent with the above bioinformatics results, indicating that our bioinformatics analysis was credible.

**Discussion**

Abnormal expression of circRNA has been widely observed in various diseases. Studies have shown that dysregulated circRNA plays a key role in the important biological properties of cancer [19]. However, only a few studies have described the profile of circRNA in BRCA by microarray analysis. The constructed BRCA-related circRNA-associated ceRNA network provides important hints for detecting the key RNAs of ceRNA-mediated gene regulatory network in the initiation and development of BRCA.

We obtained BRCA mRNA, miRNA expression profile and circRNA expression profile from TCGA database and GEO database respectively. After statistical analysis, 2762 DEmRNAs, 158 DEmiRNAs and 72 DEcircRNAs were identified. Next, we screened the circRNAs-miRNAs interaction pairs through CircIteractome and CSCD databases, screened the miRNA-mRNA interaction pairs by TargetScan, miRTarBase and miRDB databases, and then took the intersection, and finally constructed a specific circRNA-miRNA-mRNA ceRNA regulatory network. We have found that specific circRNAs in this ceRNA network, such as hsa_circ_0000376, hsa_circ_0000069, hsa_circ_0000520 and hsa_circ_0008365, have also been reported as potential diagnostic markers in certain cancers. Hsa_circ_0000376 is highly expressed in gastric cancer tissues [20], and hsa_circ_0000069 is up-regulated in colorectal cancer tissues, which can promote the proliferation, migration and invasion of tumor cells [21]. Hsa_circ_0000520 was up-regulated in breast cancer and cell lines (T47D, MCF-7, MDA-MB-231, BT549 and SKBR3), and hsa_circ_0000520 high expression was associated with poor overall survival[22]. Hsa_circ_0008365 (Circ-SERPINE2) is a novel proliferative promoter that can regulate YWHAZ through sponge miR-375 to promote the development of gastric cancer [23]. To understand the potential functional significance of differentially expressed mRNA in ceRNA networks, we performed GO analysis and KEGG analysis. It was worth noting that KEGG analysis found that some enriched signaling pathways were closely related to the development of cancer, such as 'PI3K-Akt signaling pathway' [24], 'MicroRNAs in cancer', 'Proteoglycans in cancer', 'Cellular senescence', 'FoxO signaling pathway' [25], 'Central carbon metabolism in cancer' and 'Cell cycle' [26]. Functionally annotated results also indicate that circRNAs that regulate these key mRNAs may play an important role in the initiation and development of BRCA and pathways associated with cancer genes.

In order to further identify the key genes involved in the regulatory network, we established a PPI network and screened two core sub-networks through the MCODE plug-in, which contained 21 genes, which will be used as potential hub genes. At the same time, we analyzed the relationship between DEmRNAs and DEmiRNAs in ceRNA networks and overall survival of breast cancer patients, and found that 13 mRNAs (CCNE1, TPDS2, SDC1, ANLN, ZNF367, SOX11, IRS2, EZR, DSC3, CCND2, KPNA2, CBX2 and CEP55) and 6
miRNAs (hsa-miR-204-5p, hsa-miR-335-5p, hsa-miR-100-5p, hsa-miR-195-5p, hsa-miR-328-3p and hsa-miR-342-3p) are significantly associated with the prognosis of breast cancer patients. Most of these mRNA molecules related to patient survival are thought to be related to the molecular pathogenesis of various tumors, and were closely related to the occurrence, development, proliferation, metastasis and prognosis of cancer [27-31]. For example, the DNA copy number of TPD52 is amplified in prostate cancer cells, and the level of TPD52 protein may be regulated by androgen. Studies have shown that genomic amplification and dysregulation of TPD52 caused by androgen induction may play a role in the progression of prostate cancer [28]. Gui X found that SDC1 is overexpressed in breast cancer and may be a potential prognostic indicator for breast cancer [29]. It has been reported that the upregulation of ANLN is a common feature in the carcinogenesis of lung tissue; ANLN can play a key role in the development of human lung cancer by activating RHOA and participating in the phosphoinositide 3-kinase/AKT pathway; the expression of ANLN is also associated with low survival in patients with NSCLC [30]. CEP55 is a determinant of mitosis in breast cancer cells [31]. By immunohistochemical analysis, it was found that EZR is up-regulated in breast cancer and can be used as a potential marker for overall survival of breast cancer [32].

It is well known that miRNAs regulate about 60% of human genes and mediate a variety of biological pathways, including pathways critical for tumorigenesis. Here, we found that microRNAs associated with BRCA overall survival in ceRNA networks have been reported to play an important role in tumorigenesis, development, prognosis, and drug resistance. Extracellular vesicles containing miR-335-5p can downregulate the growth and invasion of liver cancer in vitro and in vivo; the exosome miR-335-5p can be used as a novel therapeutic strategy for hepatocellular carcinoma [33]. NABAVI N identified miR-100-5p as one of the key molecular components in the initiation and evolution of androgen ablation therapy resistance in prostate cancer [34]. A research team reported that miR-328-3p is up-regulated in ovarian cancer stem cell (CSC), and high expression of miR-328-3p can directly target DNA damage-binding protein 2 to maintain CSC properties; inhibition of miR-328-3p is a new strategy to effectively eliminate CSC [35]. Enhanced expression of miR-342-3p synergizes with miR-205-5p to inhibit E2F1, thereby reducing tumor chemoresistance [36]. Published studies have shown that hsa-miR-204-5p can be used to predict the prognosis of patients with clear renal cell carcinoma, lung adenocarcinoma and other cancers [37, 38]. Hsa-miR-204-5p directly targets FOXA1 to regulate tumor cell infiltration and metastasis [39], and can affect tumor angiogenesis by interfering with the expression of ANGPT1/TGFBR2 [40]. hsa-miR-195-5p can affect the development of colorectal cancer by inhibiting the Hippo-YAP pathway [41], meanwhile, hsa-miR-195-5p can be a potential diagnostic and prognostic target in breast cancer [42].

We performed a correlation analysis between the expression levels of miRNAs and mRNAs from the same sample in the TCGA database. The results indicate that there are 43 pairs of interconnected miRNAs and mRNAs with a significant negative correlation in the constructed miRNA-mRNA interaction pairs. These links have also been found in some reports. For example, Luo Q found that overexpression of hsa-miR-195-5p can reduce the expression level of CCNE1 and targeting this miRNA may provide a new strategy for the diagnosis and treatment of breast cancer [42]. In addition, reports on circRNA found that circAGFG1 can act as a sponge of hsa-miR-195-5p, which promotes the progression of triple-negative
breast cancer by regulating the expression of CCNE1 [43]. These results also indirectly reflect the feasibility of using bioinformatics to construct regulatory networks.

Here, we identified four ceRNA regulatory axes, indicating competitive regulatory relationships of three circRNAs with the three genes in BRCA. However, given that these results are based solely on bioinformatics models, further in-depth studies are critical to verifying the possible role of these four axes in BRCA.

Conclusions

In this study, we identified aberrant expressed key RNAs by analyzing the RNAs expression profiles of BRCA in public databases. These specific circRNA, miRNA and mRNA molecules may be helpful in the discovery of sensitive biomarkers in BRCA. Importantly, we have constructed a circRNA-miRNA-mRNA ceRNA network that will be used to elucidate the unknown ceRNA regulatory axes in BRCA. Our findings provide novel insights into an in-depth understanding of circRNA-related ceRNA networks in breast cancer as well as potential diagnostic and prognostic biomarkers.

Declarations

Authors’ contributions

Hui Shen and Huan Pan carried out data analysis. Ming Yao participated in study design and data collection. All authors drafted the final manuscript. All authors read and approved the final manuscript.

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Not applicable.

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Consent for publication

Not applicable.

Ethics approval and consent to participate

This study was approved by the ethics committee of The First Affiliated Hospital of Jiaxing University.
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**Figures**
Figure 1

Flow chart of comprehensive bioinformatics analysis in the construction of competing endogenous RNA (ceRNA) regulatory network.
Figure 2

Heatmap and volcano diagrams of breast cancer-related differentially expressed mRNAs, miRNAs and circRNAs. A, mRNA; B, miRNA; C, circRNA. The color from blue to red shows a trend from low expression to high expression. The red dot represents upregulated mRNA, miRNA and circRNA, the green dot represents downregulated mRNA, miRNA and circRNA.
Figure 3

Competing endogenous RNA (ceRNA) (DEcircRNA-DEMsiRNA-DEmRNA) regulatory network. The v nodes, round rectangle nodes and elliptical nodes indicate DEcircRNAs, DEMsiRNAs and DEmRNAs, respectively. Red and blue represent upregulation and downregulation, respectively. Green borders surrounding the nodes indicate prognostic significance. Purple edges indicate good negative correlation between RNAs.
Figure 4

Significantly enriched Gene Ontology (GO) terms of differentially expressed mRNAs in ceRNA regulatory network. BP, biological process; CC, cellular component; MF, molecular function. The x-axis shows counts of host genes enrich in GO terms and the y-axis shows GO terms. The color scale represented p.adjust.
Figure 5

Significantly enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways of differentially expressed mRNAs in ceRNA regulatory network. The x-axis shows counts of host genes enrich in KEGG pathways and the y-axis shows KEGG pathways. The color scale represented p.adjust.
Figure 6

Kaplan-Meier survival curves of differentially expressed miRNAs (DEmiRNAs) and differentially expressed mRNAs (DEmRNAs) in the competing endogenous RNA (ceRNA) network that are significantly associated with overall survival in breast cancer.
Figure 7

Pearson's correlation analysis between the expression level of the interacting miRNA and mRNA in the ceRNA network.
Figure 8

Identification of hub genes from the PPI network with the MCODE algorithm. A. PPI network construction. B. Two core subnets with 21 hub genes. Red nodes represent the upregulated genes, blue nodes represent downregulated genes; PPI, protein-protein interaction.
Figure 9

qRT-PCR validation of the DEmRNAs, DEMiRNAs and DEcircRNAs in breast cancer. The x-axis represents the DEmRNAs/DEmiRNAs/DEcircRNAs and the y-axis represents log2(fold change).