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Systematical Analysis of Circular RNAs in Triple Negative Breast Cancer

Li XJ[1], Yao YF[1], Ren Z[2], Bao J[3] and Yu Q[1]*

[1]Department of General Surgery, Jiangsu Cancer Hospital, China

[2]Department of Pathology, Jiangsu Cancer Hospital, China

[3]Department of Medical Oncology, Jiangsu Cancer Hospital, China

Abstract

Background/Aims: This study aims to investigate circRNA expression profiles and their potential biological functions in Triple Negative Breast Cancer (TNBC).

Methods: High-throughput RNA sequencing was used to assess circRNA expression profiles in TNBC, and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) was used to validate dysregulated circRNA s. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were employed to determine the biological functions of differentially expressed circRNA s. Bioinformatic analyses were applied to predict interactions between circRNA s and microRNAs (miRNAs), and a circRNA -miRNA-mRNA network was constructed using Cytoscape software.

Results: We identified a number of differentially expressed circRNA s in TNBC tissues compared with paired normal tissues, with chr11:32589546:32596047:+, chr18:36195256:36203189:+, chr7:22266963:22318037:-, chr9:22046750:22064018:+, and chr10:836402:845055:- being up regulated, and chr12: 55700898:55701154:-, chr15:54012647:54015886:+, chr5:55467868:55491114:-, chr4:61934839:62044549:+ and chr7:152145152:152149152:- being down regulated. These findings were confirmed by qRT-PCR. GO and KEGG pathway analyses showed that some of these circRNA s are related to cancers. Additionally, bioinformatic analyses revealed a potential competing-endogenous-RNA-regulating network among circRNA s, miRNAs, and mRNAs.

Conclusion: Our study results depict the landscape of circRNA expression profiles in TNBC and also provide potential biomarkers for TNBC. Further functional and mechanistic studies of this circRNA s may improve our understanding of TNBC tumorigenesis.

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Correspondence:

Bao J, Department of Medical Oncology, Jiangsu Cancer Hospital, Baiziting, Nanjing, Pin: 42, 210009, China,

E-mail: 343485967@qq.com

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Introduction

Breast cancer is a progressive disease that is a leading cause of cancer-related mortality in women. As we all know, the basal-like subtype has the poorest prognosis in breast cancer [1], therefore, we focused on this subtype in this study. The basal-like subtype is generally negative for ER, PR and HER2 and therefore also called as triple-negative breast cancer (TNBC) [2]. Moreover, current targeted therapy strategy is available for breast cancers that are ER, PR or HER2 positive, but there are no specific targeted therapy options available for TNBC. Therefore, it is urgent and necessary that new treatments are found based on the specific mechanisms of TNBC to improve treatment efficiency and avoid the adverse effects of conventional methods. Accumulating evidence demonstrates that non coding RNAs, such as micro RNAs (miRNAs) and long non coding RNAs (lncRNAs), play an important regulatory role in many physiological and pathophysiological processes. More recently, a novel class of non coding RNA termed circular RNA (circRNA) has become a hot topic in RNA research. CircRNAs are commonly found in mammalian cells and can regulate gene expression at the transcriptional or post-transcriptional level by acting as miRNA sponges or by interacting with other molecules [3]. Recent studies have shown and emphasized the importance of circRNA s in regulating cancer-related signaling pathways [4,5]. Additionally, circRNA s may be associated with tumor types and serve as risk factors for some types of cancer [6,7].

To date, little is known about the associations between circRNA s and TNBC. Therefore, in the current study, we screened the circRNA profiles of TNBC patients using high-throughput RNA sequencing (RNA-Seq) and validated dysregulated circRNA s in TNBC tissues by quantitative real-time polymerase chain reaction (qRT-PCR). Bioinformatic analysis was then performed to predict

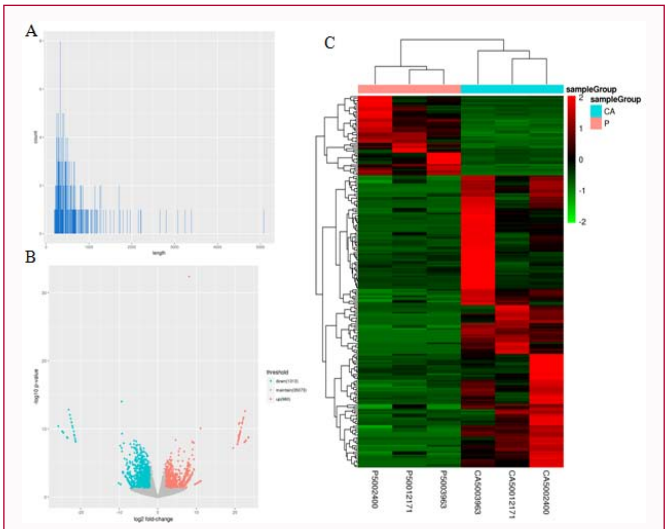

Figure 1: Overview of circRNA profiles (CA: TNBC tissues; P: normal breast tissues), **A:** Density of spliced length of the detected circRNAs, **B:** Volcano plot of differentially expressed circRNAs in TNBC. The vertical green lines correspond to two-fold increased and decreased expression, and the horizontal green line represents P=0.05. The red points represent circRNAs that were differentially expressed with statistical significance, **C:** Hierarchical cluster analysis of differentially expressed circRNAs. The red and green colors indicate high and low expression, respectively.

their potential roles in TNBC and lay a foundation for future studies of circRNA s that are related to TNBC.

Materials and Methods

Patient samples

A total of 20 pairs of TNBC and adjacent normal breast tissues were recruited from the Jiangsu Cancer Hospital. The diagnosis of TNBC was independently confirmed by two pathologists. The patients did not receive any anticancer treatment prior to surgical resection. All tissue samples were snap-frozen and stored in liquid nitrogen until further experiments were performed. Written informed consent was obtained from all patients, and this study was approved by the ethics committee of the Jiangsu Cancer Hospital.

RNA extraction and quality control

Total RNA was isolated from each tissue sample using TRIzol reagent following the manufacturer's instructions. The quality of the RNA samples was determined by OD 260/280 assessment using a Nano Drop ND-2000 instrument (Thermo Fisher Scientific, USA). RNA integrity was determined by denaturing agarose gel electrophoresis.

CircRNA sequencing and bioinformatic analysis

Three pairs of TNBC and adjacent normal breast tissues were selected for RNA-seq analysis. Total RNA from each sample was used to prepare circRNA sequencing libraries. Briefly, circRNA s were detected and annotated with DCC software using two public circRNA databases: circBase and circ2Traits [8-10]. The differentially expressed circRNAs was determined using the R software limma package [11]. CircRNAs that exhibited Fold Changes (FCs) ≥ 2.0 with P values <0.05 were considered to be significantly differentially expressed. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were performed for the host genes of the differentially expressed circRNAs. CircRNA-miRNA interactions and miRNA targets were predicted with popular target prediction software, and a network was constructed with Cytoscape software [12-16].

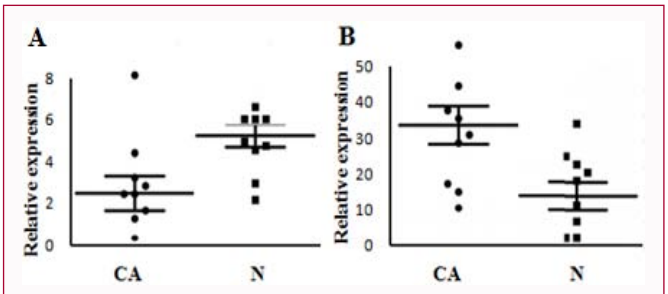

Figure 2: qRT-PCR validation of two differentially expressed circRNAs. The relative expression levels of circRNAs in 9 TNBC tissues and paired normal tissues, A: chr12:55700898:55701154 **B:** chr11:32589546:32596047:+. (N: normal breast tissues; CA: TNBC tissues; P< 0.05).

Figure 3: Gene Ontology (GO) terms for differentially expressed circRNA genes. The bar plot presents the enrichment scores (−log10 [P value]) of the top 10 significantly enriched GO terms.

Real-time quantitative PCR

Qualified total RNA was reverse transcribed to synthesize cDNA using Prime Script RT Master Mix (Perfect Real Time, TaKaRa, Japan) and then analyzed by qRT-PCR with SYBR Premix Ex Taq II (Tli RHaseH Plus, TaKaRa) and a Light Cycler 480 system (Roche, Basel, Switzerland). Divergent primers were designed for each circ RNA. β -actin was used as the internal control. The relative expression level of each circRNA was calculated using the 2–ΔΔCt method.

Data analysis

Statistical analyses were performed using SPSS 19.0. $P<0.05$ was considered statistically significant. Differences between groups were assessed using Student's t test or Mann–Whitney U test, as appropriate.

Results

Identification of differentially expressed circRNAs

A total of 34674 circRNAs were identified by RNA-Seq in three pairs of TNBC and normal breast tissue samples. The density of the length of this circRNAs is presented in Figure 1A. The raw read counts were normalized and differentially expressed circRNAs were filtered using DESeq2. We got 229 circRNAs with a p-value <0.05, including 180 up regulated circRNAs and 49 down regulated circRNAs (Figure 1B and 1C). All the 229 different expressed circRNAs are presented in Supplementary Material 1.

Validation of circRNA expression

Two circRNA s (two were the most differentially expressed) were selected to verify the RNA-Seq in 9 pairs of samples by qRT-PCR. The results revealed that expression of chr11:32589546:32596047:+ was up regulated (Figure 2B), whereas that of chr12:55700898:55701154:- was down regulated (Figure 2A). The qRT-PCR data were strongly consistent with the circRNA sequencing data, indicating the reliability of the RNA-Seq results.

Analysis of involved pathways

GO and KEGG pathway enrichment analysis were conducted with gene symbols of differentially expressed circRNA s (Figure 3 and 4). We observed that these networks were enriched in Pathways in cancer. The enriched pathways of focal adhesion, Extracellular Matrix (ECM)-receptor interaction, PI3K–Akt signaling pathway and p53 signaling pathway have been showed signifcantly associated with migration and invasion of breast cancer [17]. The p53 signaling pathway was validated to be associated with energy metabolism reprogramming and cancer cells survival in anoxic environment [18]. In addition, the p53 signaling pathway was reported to be activated in basal-like subtype in previous studies [19].

Prediction of circRNA/miRNA/mRNA interaction

Because circRNA s contain corresponding miRNA binding sites and can function as miRNA sponges, to evaluate their potential functions in TNBC, we investigated miRNAs potentially associated with the identified differentially expressed circRNA s using miRNA target prediction software. The predicted miRNAs for the top one dysregulated circRNA are listed in Figure 4. For example, circRNA chr11:32589546:32596047:+ is predicted to harbor hsa-miR-33a-3p, hsa-miR-29b-1-5p, hsa-miR-29b-1-5p, hsa-miR-107, and hsa-miR-134-3p (Figure 5). Additionally, a circRNA -miRNA- mRNA network was also constructed (Figure 6), in which each circRNA has certain associated miRNAs and every miRNA has certain predicted mRNA targets. The network indicates the potential competing endogenous

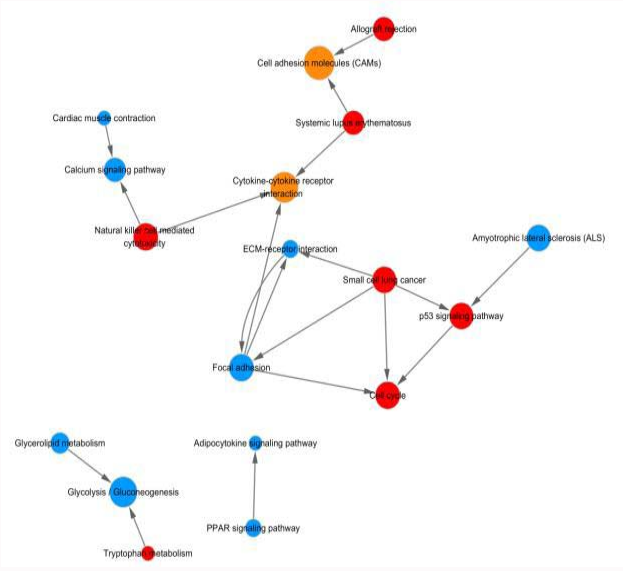

Figure 4: Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways of differentially expressed circRNA genes. The top 10 significantly enriched pathways and their scores (−log10 [P value]) are listed as the y axis and the x axis, respectively.

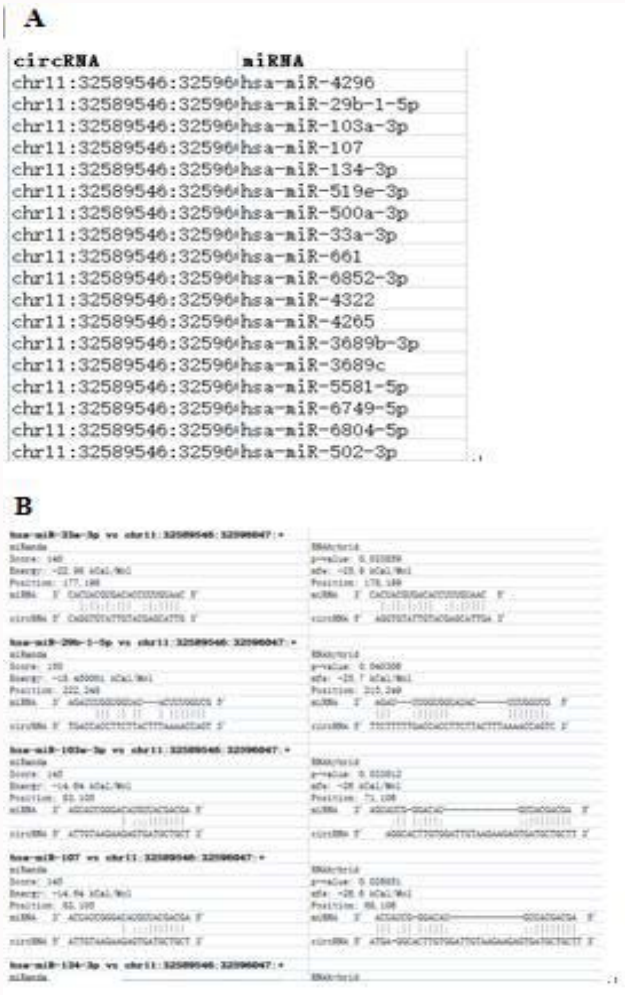

Figure 5: A: Predicted miRNAs for the chr11:32589546:32596047:+ and **B:** Detailed annotation of circRNA-miRNA interactions between chr11:32589546:32596047:+ and its target miRNAs.

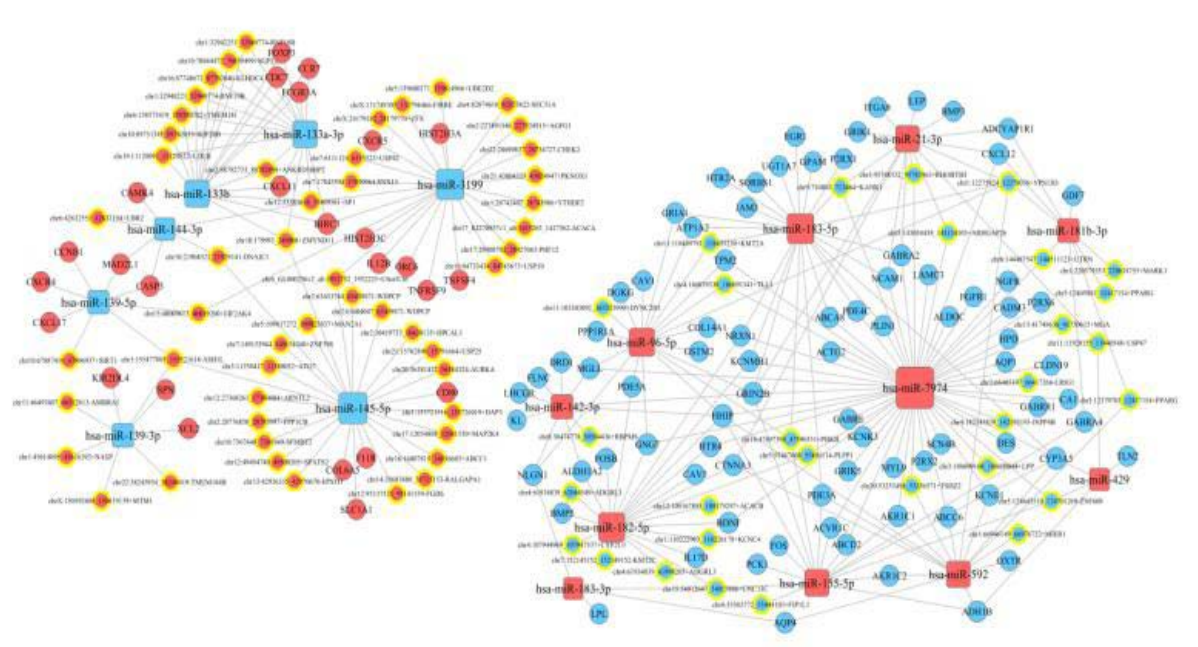

Figure 6: Predicted circRNA-miRNA-mRNA interaction network for the dysregulated circRNAs in TNBC.

RNA (ceRNA) relationships among circRNA s, miRNAs, and mRNAs in TNBC.

Discussion

TNBC is a subtype of breast malignant tumor with poorer prognosis than other molecular subtypes. Even the development of chemotherapy has made progress and the patients can achieve pathological complete response after neoadjuvant chemotherapy, TNBC patients still have a higher metastasis rate and poorer prognosis than other breast cancer subtypes. Therefore, to find the proven effective target therapies for TNBC, we performed a comprehensive analysis of the global changes in the expression pattern of circRNAs in TNBC. Systemic bioinformatics analysis was conducted to identify circRNAs essential for the biological processes of TNBC, which may provide potential targets for the development of novel therapeutic strategies against TNBC.

A total of 34674 human circRNAs were detected. There were 229 differentially expressed circRNA s, consisting of 180 up-regulated and 49 down-regulated circRNAs. Among them, some differentially expressed circRNAs were selected for validation. The great consistency between the real-time qPCR results and microarray data confirmed the high reliability of the RNA-Seq, which supports the further functional analysis of the differentially expressed circRNAs.

Following circRNA detection within the tissue samples, we found that chr11:32589546:32596047:+ was up-regulated in TNBC tissue samples, while chr12:55700898:55701154:- was down-regulated. This interesting result drove us to focus on these two circRNAs. The result indicates that chr11:32589546:32596047:+ and chr12:55700898:55701154:- may play an important role in the biological processes of TNBC, such as cell proliferation, differentiation, and invasion, among other similar processes. Therefore, both prediction of the circRNA/microRNA/mRNA interaction network and bioinformatics analysis were conducted for a more comprehensive understanding of these two circRNAs.

The function of circRNAs remains unclear. An intriguing possibility is that circRNAs act as microRNA sponges. Oncogenic miRNAs like hsa-miR-339-5p, hsa-miR-143-5p, hsa-miR-409-3p, hsa-miR-153-3p and hsa-miR-145-5p have been reported to be down regulated in breast cancer [20,21]. These miRNAs were matched with up regulated circRNA s in our study. Other miRNAs, like hsa-miR-298, hsa-miR-485-3p, and hsa-miR-100, which were matched with down regulated circRNA s in our study, have also been reported to be related to breast cancer [22].

Intracellular circRNA s with Competing Endogenous RNAs (ceRNAs) activity may compete with linear RNAs by binding miRNAs, which strongly reduce the ability of miRNAs to bind their target mRNAs and result in increased expression levels of these genes [19,20]. In this study, we have found that chr11:32589546:32596047:+ expression was significantly correlated with the expression of Cyclin-Dependent Kinase 6 (CDK6), Cyclin D1 (CCND1) and serine/threonine kinase PIM-1. This result suggests that chr11:32589546:32596047:+ may increase the expression of Cyclin-Dependent Kinase 6 (CDK6), cyclin D1 (CCND1) and serine/threonine kinase PIM-1 through competitive binding with hsa-miR-33a-3p, whose target gene are Cyclin-Dependent Kinase 6 (CDK6), Cyclin D1 (CCND1) and serine/threonine kinase PIM-1. And chr11:32589546:32596047:+ may act as an oncogene and promote the incidence and development of TNBC by chr11:32589546:32596047:+/hsa-miR-33a-3p/CDK6/CCND1/PIM-1 pathway, which highly reflects the effect of ceRNA regulatory network.

At the same time, GO and KEGG pathway analyses revealed that there were certain pathways associated with TNBC, such as PI3K-Akt signaling pathway and ErbB signaling pathway [17]. Previous studies have suggested that the crosstalk between the PI3K/Akt and ErbB pathways can overly activate to cause cell proliferation, which impacts the prognosis of TNBC patients. Therefore, related circRNA s may participate in the tumorigenesis of TNBC by regulating their parent genes.

We also predicted the potential miRNA targets of circRNA s and constructed a circRNA -miRNA-mRNA network to explore the function of dysregulated circRNA s in TNBC. The network illustrates the potential ceRNA relationships among circRNAs, miRNAs, and mRNAs. For example, chr11:32589546:32596047:+ as well as chr12:55700898:55701154:-, are the top one up- and down regulated circRNA s and possibly regulate expression of certain mRNAs by binding to their target miRNAs. Dysregulated circRNA s may participate in the pathogenesis of TNBC by regulating miRNAs and their target mRNAs. Future studies should focus on these circRNAs and their related miRNAs and mRNAs.

In summary, the present study identified the comprehensive expression profile of circRNAs in TNBC. The ceRNAs network prediction and bioinformatics analysis could provide a comprehensive understanding of differentially expressed circRNA s, which may be involved in the initiation and progression of osteosarcoma. Further functional and mechanistic studies of this circRNAs will improve our understanding of tumorigenesis in TNBC.

Acknowledgement

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