

Microarray Profile of Circular RNAs Identifies hsa_circ_0001583 as A New Circular RNA Biomarker for Breast Cancer: A Retrospective Study

Min Zheng, M.M.¹, Wei-Hua Cai, B.M.², Mou-Feng Wang, B.M.¹, Yu-Jie Deng, M.M.¹, Ling-Ling Huang, M.M.¹, Yong-Jin Cao, M.M.^{2*}

1. Department of Oncology, Fujian Key Laboratory of Precision Medicine for Cancer, The First Affiliated Hospital of Fujian Medical University, Fujian, Fuzhou, P. R. China

2. Department of Thyroid and Breast Surgery, The First Affiliated Hospital of Fujian Hospital of Fujian Medical University, Fujian, Fuzhou, P. R. China

*Corresponding Address: Department of Thyroid and Breast Surgery, The First Affiliated Hospital of Fujian Medical University, Fujian, Fuzhou, P. R. China

Email: cyj9803209@fjmu.edu.cn

Received: 22/August/2021, Accepted: 27/April/2022

Abstract

Objective: Breast cancer (BC) is the most common cancer, which is currently the leading cause of cancer death. Circular RNAs (circRNAs) play important roles in cancer, however, circRNAs serving as vital index in BC for guiding treatment have not yet been identified. The aim of our study is to explore a novel kind of potential biomarker for BC.

Materials and Methods: In this retrospective study, the samples used for assays were two groups of breast tumor tissue obtained from four BC patients, including four pairs of tumor tissues and adjacent nontumor samples. The circRNA expression profiles were detected via microarray and validated by real-time quantitative polymerase chain reaction (PCR).

Results: The differentially expressed circRNAs in tested samples were screened and analyzed by using human circRNA microarray. After analysis, considering a fold gene expression change of ≥ 2.0 and $P < 0.05$, results suggested that 256 circRNAs were significantly up-regulated and 277 circRNAs were significantly down-regulated. Besides, the results of the real-time quantitative PCR assay showed that the expression of hsa_circ_0001583 was significantly up-regulated in BC groups ($P < 0.05$) by real-time quantitative PCR. Therefore, we thought hsa_circ_0001583 might serve as a novel kind of biomarker for BC.

Conclusion: Hsa_circ_0001583 showed significant up-regulation in BC patients with paired adjacent tissues. Many cancer immune pathways were related to has_circ_0001583, including autoimmune thyroid disease, chemokine and T-cell receptor signaling pathways.

Keywords: Breast Cancer, circRNA, hsa_circ_0001583

Cell Journal (Yakhteh), Vol 24, No 9, September 2022, Pages: 500-505

Citation: Zheng M, Cai WH, Wang MF, Deng YJ, Huang LL, Cao YJ. Microarray profile of circular RNAs identifies hsa_circ_0001583 as a new circular RNA biomarker for breast cancer: a retrospective study. Cell J. 2022; 24(9): 500-505. doi: 10.22074/cellj.2022.8200.

This open-access article has been published under the terms of the Creative Commons Attribution Non-Commercial 3.0 (CC BY-NC 3.0).

Introduction

As one of the most common cancers in women, breast cancer (BC) is the primary cause of death from cancer at present. In 2020, the number of newly diagnosed cases of BC was more than 279 thousand and the number of new deaths was about 42 thousand, with 276,480 and 42,170 of them being in women, respectively (1). BC patients in early stages can be cured by local and systemic treatment (2). At present, surgery, adjuvant chemotherapy, endocrine therapy and target-directed therapy were used in the treatment of BC, however, the prognosis of BC was poorly understood because of regional advanced recurrence or distant metastasis. Therefore, to better understand the progression of breast cancer, finding novel molecules as potential biomarkers for BC is essential.

Circular RNAs (circRNAs) were initially found as a kind of covalently closed looped RNAs in viruses (3). Particularly, unlike typical splicing of linear RNA,

circRNAs are formed by backward splicing at the 3' and 5' ends of covalent connections (4, 5). Considering the high stability, high specificity and high abundance of circRNAs, previous reports suggested that circRNAs played vital roles in cancers, such as BC, hepatocellular, gastric and colorectal cancer (6-9). CircRNAs usually act as "sponges" of miRNAs which target the 3' UTR region of protein coding RNAs and inhibit target gene expression (4, 10). For example, circBMPR2 can regulate the expression of USP2 via binding to miR-553 as an miRNA sponge (11).

However, circRNAs in BC that can guide treatment have not yet been identified. In this study, a new circRNA, hsa_circ_0001583 which was screened by circRNA microarray attracted our attention. Hsa_circ_0001583 is also associated with many immune response pathways in KEGG analysis. Their roles in BC are still unknown, so it needs to be further explored. And it might act as a novel kind of biomarker for BC.

Material and Methods

CircRNA microarray assay

In this retrospective study, for circRNAs expression screening, we obtained four pairs of tumorous and paracancerous tissues from four triple negative BC patients and extracted total RNA for circRNA microarray assay. In detail, four pairs of BC tissues and paracancerous tissues were sectioned from clinical patients. TRIzol reagent was used for RNA extraction from tissues, and NanoDrop-1000 and agarose gel electrophoresis were used for RNA concentration and integrity detecting, respectively. According to standard microarray hybridization instructions, the RNA was first purified, and then reverse transcribed to cDNA, cDNA was further transcribed into cy3-fluorescent cRNA, and finally cRNA was hybridized with Human circRNA Arrays (Arraystar). The hybridized microarray was washed with Gene Expression Wash Buffer (Agilent p/n 5188-5325) and the images were scanned by Agilent Scanner G2505C software.

Data analysis by circular microarray

Agilent Feature Extraction software (V11.0.1.1, Agilent santa clara, CA, USA) was used to collect the raw data, then normalized through the quantile method. circRNA expression levels were analyzed by GeneSpring GX V12.1. The circRNAs presenting differential expression between tumors and adjacent tissues with fold gene expression change ≥ 2.0 and $P < 0.05$ were identified via statistical analysis. Hierarchical clustering and volcano plotting were constructed for global overview of the significant differentially expressed circRNAs.

Clinical specimens

There are a total of 4 pairs of triple negative BC tissues and paracancerous normal tissues were sectioned from four clinical patients. This research proposal was approved by The First Affiliated Hospital of Fujian Medical University in 2018.

Total RNA extraction and real-time quantitative polymerase chain reaction

TRIzol reagent (Sigma, USA) was used for total RNA extraction from BC tissues and normal tissues, followed by reverse transcription to cDNA using SuperScriptTM III Reverse Transcriptase Kit (Invitrogen, USA) according to the manufacturer's protocol. Real-time quantitative polymerase chain reaction (PCR) assay was performed with QuantStudioTM 5 System (Thermo Fisher Scientific, USA) using 2X PCR master mix (Arraystar, AS-MR-006-25). The relative circRNAs expression level was normalized to internal control gene *ACTB*. Primer sequences are listed in Table S1 (See Supplementary Online Information at www.celljournal.org).

Prediction for BC-related circRNA-miRNA-target gene axis

Targetscan and miRDB (<http://www.targetscan.org>; <http://www.mirdb.org>) were used for predicting the miRNAs combined with circRNAs together with the target genes of miRNAs. For analyzing the potential function of circRNAs, the GO enrichment analysis (www.geneontology.org/) and KEGG pathway analysis (www.genome.jp/kegg/) were performed by DAVID (<https://david.ncifcrf.gov/tools.jsp>) online software.

Ethical statement

The clinical samples were obtained from The First Affiliated Hospital of Fujian Medical University. This study was approved by the Ethics Committee of The First Affiliated Hospital of Fujian Medical University (Fuzhou, China, project number [2016]073), and written consent was obtained from all patients involved.

Data availability

The circRNA expression profile data are available in the GEO database (accession number GSE165884).

Statistical analysis

All statistical data plotting was performed with Graphpad Prism V8.0 (GraphPad Software Inc., San Diego, CA, USA). Every experiment was conducted in three replicate. Significant differences between data was analyzing by Student's t test ($P < 0.05$) and the significance of gene function enrichment was determined by Fisher's Exact Test.

Results

The circRNAs presenting differential expression were identified in BC

Four pairs of BC patient samples and paracancerous tissues were used to screen the potential involvement of circRNAs in BC via the high-throughput circRNA microarray assays. The results shown in Figure 1A and Figure 1B were the quantile normalization and total detected circRNA types. The scatter plot suggested that 360 circRNAs were up-regulated, while 344 circRNAs were discovered with down-regulation (Fig.1C). Additionally, by comparing the case group with the control group, a volcano plot revealed that a total of 533 circRNAs presented differential expression. In detail, 256 circRNAs were found to be significantly up-regulated and 277 circRNAs were significantly differentially down-regulated based on fold expression change ≥ 2.0 and $P < 0.05$ (Fig.1D). Figure 1E indicates the distinct disparity from BC tissue to their adjacent samples. The results in Figure 1F showed the number and distribution of these circRNAs on the different human chromosomes.

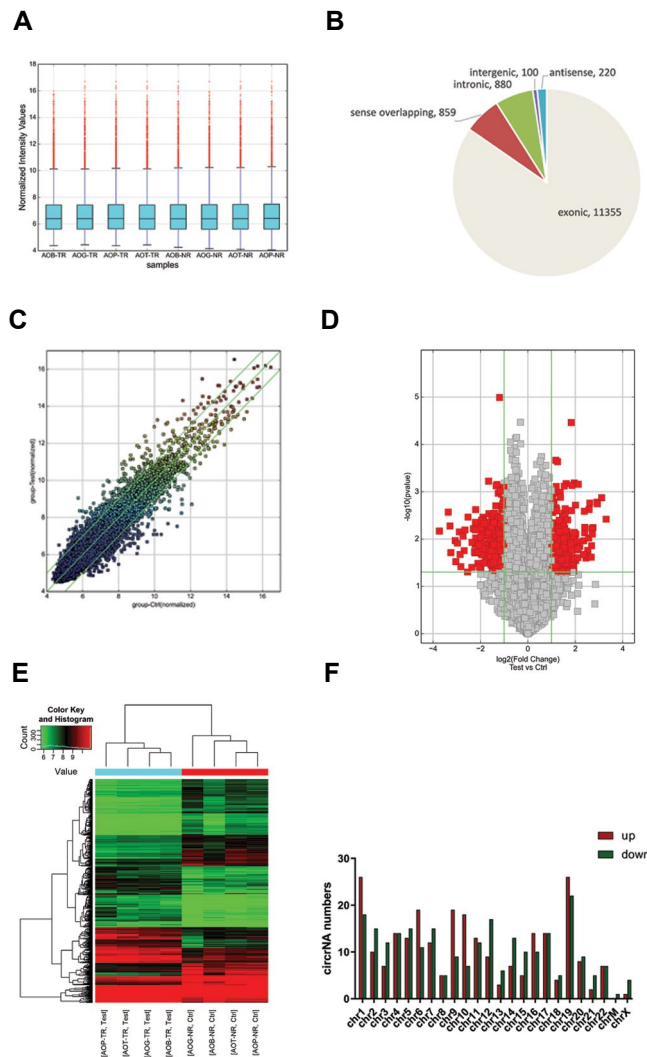


Fig.1: circRNA expression profile and differentially regulated circRNAs in BC. **A.** The quantile normalization results of Arraystar circRNA microarray for 8 samples. **B.** Different types in all circRNA expression profile. **C.** Scatter plot for assessing the variation in circRNA expression. **D.** Volcano plot indicated the differentially expressed circRNAs in BC samples and adjacent tissues based on fold expression change ≥ 2 and $P < 0.05$. **E.** Hierarchical clustering heatmap for differentially expressed circRNAs. **F.** The distribution of differentially expressed circRNAs on human chromosomes. circRNA; Circular RNAs (circRNAs) and BC; Breast cancer.

Potential functional analysis of differentially expressed circRNAs

To evaluate the functions of differentiated expressed circRNAs, we performed GO and pathway enrichment analysis using the circRNA target genes. As the results show in Figure 2A, by biological process analysis, these circRNAs might act in negative transcriptional regulation associated with the RNA polymerase II promoter, protein ubiquitination modification in ubiquitin-dependent protein catabolism, cell shape, regulation of cellular signal transduction mediated by small GTPase, GTPase activity regulation, protein nuclear export, protein folding in the endoplasmic reticulum, actin cytoskeleton organization, protein polyubiquitination, and cell-cell adhesion. Cellular component enrichment results showed that the host genes can be part of the cytosol, focal adhesion, cytoplasm, membrane, stress fibers, extracellular exosomes, the actin cytoskeleton, the mitotic spindle, the extracellular

matrix, and cell-cell adhesion junctions (Fig.2B). Molecular function results of the differentially expressed circRNAs are shown in Figure 2C.

In addition, KEGG pathway analysis showed that differentially expressed circRNAs could be involved in focal adhesion, actin cytoskeleton regulation, bacterial invasion of epithelial cells, ubiquitin mediated proteolysis, proteoglycans in cancer, ribosome, and amoebiasis (Fig.2D).

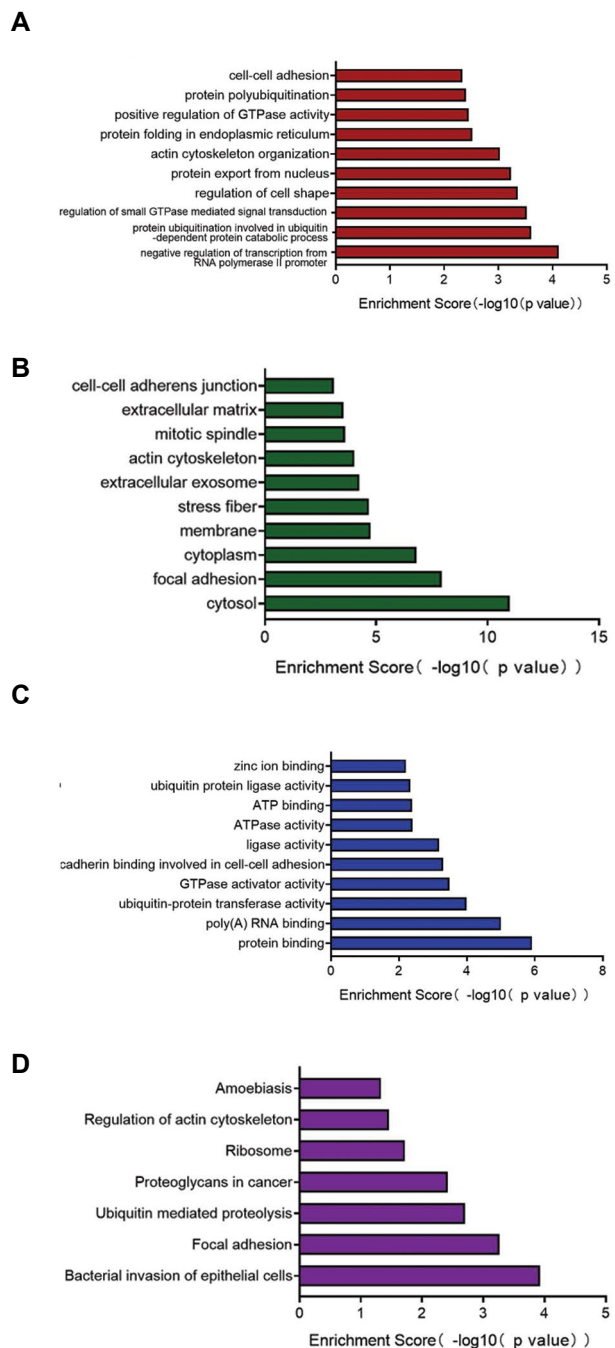


Fig.2: Predicting the functions of differentially expressed circRNA host genes. **A.** Top ten biological processes associated with the differentially expressed circRNAs host genes arranged by enrichment score. **B.** Top ten cellular components of differentially expressed circRNAs host genes. **C.** Top ten molecular functions of differentially expressed circRNAs host genes. **D.** Significant KEGG pathway enrichment results of differentially expressed circRNAs host genes.

Diagnostic values of hsa_circ_0001583 in BC

To further study the importance of circRNAs in BC, we detected the expression change of differentially expressed circRNAs by real-time quantitative PCR, including hsa_circ_0000700, hsa_circ_0089153, hsa_circ_0067301, hsa_circ_0001583, hsa_circ_0001589 and hsa_circ_0039908 (Fig.3A). Interestingly, consistent with the microarray results, hsa_circ_0001583 presented the most significant up-regulation in 4 BC tissues (Fig.3B). In fact, hsa_circ_0001583 is generated from *H1-2* gene, which is a histone H1 protein binding to linker DNA (Fig.3C). Therefore, we thought hsa_circ_0001583 might serve as a novel kind of biomarker for BC.

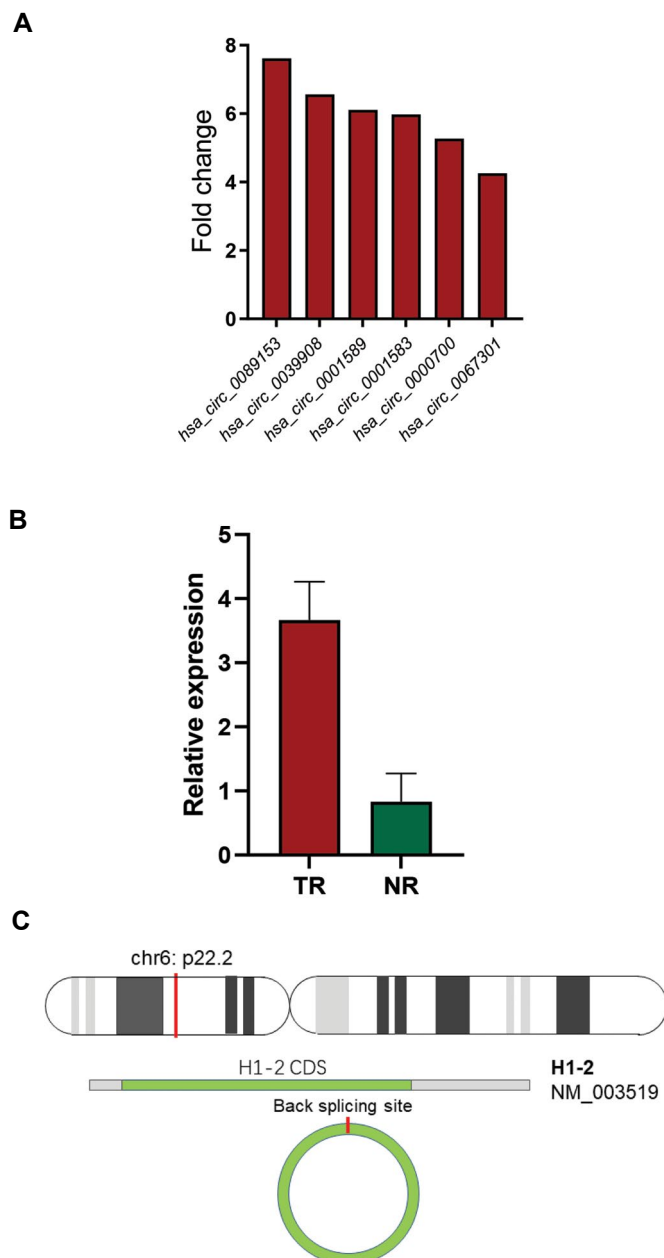


Fig.3: Validation of differentially expressed circRNAs by realtime quantitative polymerase chain reaction (RT-qPCR). **A.** 6 selected up-regulated circRNAs for RT-qPCR validation. **B.** *hsa_circ_0001583* was significantly up-regulated in breast cancer tissues ($P=0.0014$). **C.** The genome locus and host gene of *hsa_circ_0001583*.

Prediction of miRNAs acting on hsa_circ_0001583 and downstream genes

To study the function of *hsa_circ_0001583*, the *hsa_circ_0001583*-miRNA-mRNA network was predicted (Fig.4A). In detail, the top 5 related miRNAs were exhibited in Figure 4B, containing hsa-miR-6815-3p, hsa-miR-324-3p, hsa-miR-103a-2-5p, hsa-miR-4778-3p and hsa-miR-647, while Figure 4B also shows the possible binding sites between *hsa_circ_0001583* and its related miRNAs.

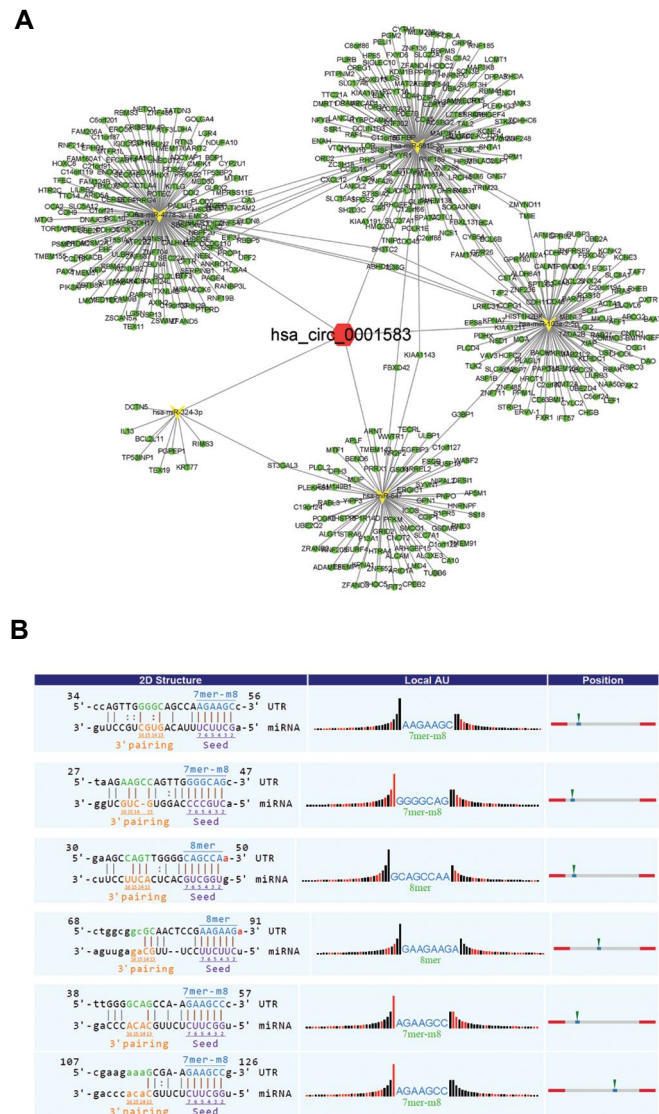


Fig.4: ceRNA network of *hsa_circ_0001583*. **A.** The predicted *hsa_circ_0001583* sponged miRNA and miRNA target genes. **B.** The potential binding sites of *hsa_circ_0001583* and sponged miRNAs.

Analyzing the functions of hsa_circ_0001583 ceRNA genes

GO enrichment analysis revealed that the target genes of those candidate miRNAs are related to many biological processes and pathways, including negative transcriptional regulation of RNA polymerase II promoter, signal transduction mediated by Rho protein, covalent chromatin modification, protein K48-linked ubiquitination, adaptive

immune response, kinase activity regulation, cell motility, ventricular septum development, and cell proliferation (Fig.5A). The cellular component and molecular function enrichment analysis results are shown in Figure 5B and Figure 5C. KEGG analysis revealed that several immune response pathways are associated with candidate miRNAs, including long-term potentiation, T/B-cell receptor signaling pathway, bile secretion, oxytocin and calcium signaling pathway, infection of vibrio cholerae, proteolysis mediated by ubiquitin, axon guidance (Fig.5D). These results suggested that hsa_circ_0001583 was strongly associated with BC development, which will pave the way for further research in the future.

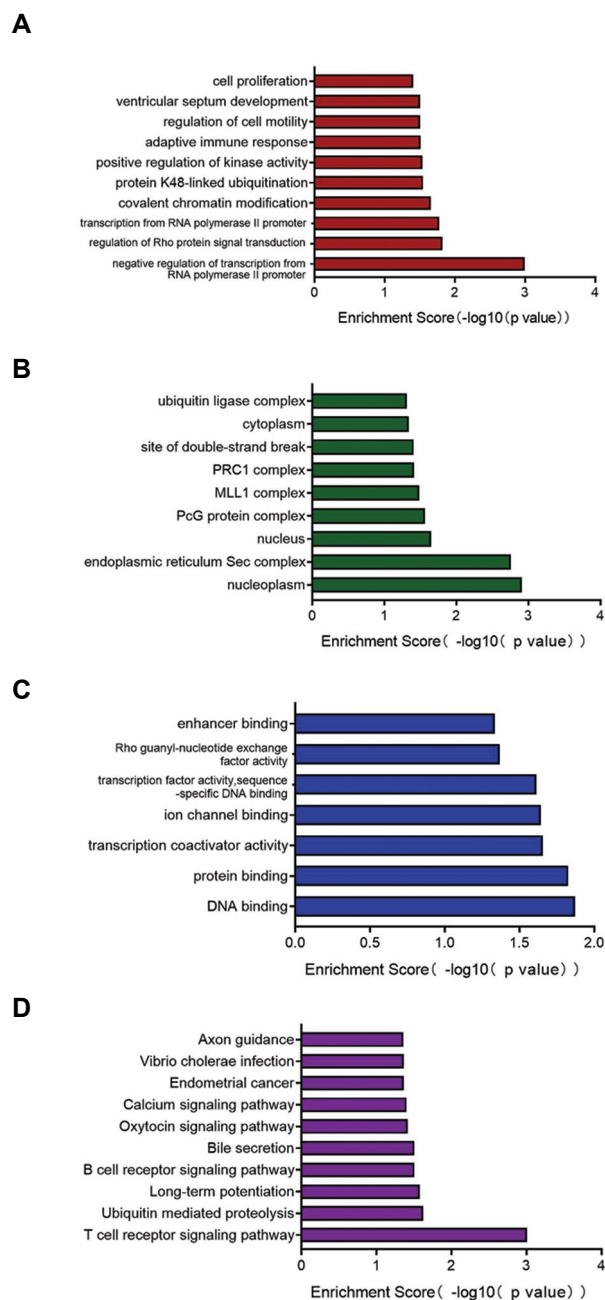


Fig.5: The GO/pathway enrichment analysis of hsa_circ_0001583 ceRNAs. **A.** Top ten GO_BP enrichment terms of candidate circRNA ceRNAs. **B.** The significant GO_CC enrichment results of candidate circRNA ceRNAs. **C.** The significant GO_MF enrichment results of candidate circRNA ceRNAs. **D.** KEGG pathway analysis revealed that several immune related pathways associated with hsa_circ_0001583.

Discussion

It has been found in previous studies that circRNAs exhibited pivotal regulatory functions in cancer progression and carcinogenesis (12). The circRNAs are stable due to the lack of 5' or 3' polarities or polyadenylated tails (13). Moreover, circRNAs are also abundant, showing the high conservation, together with structural stability, timing, organization, and disease-specific activity in eukaryotic cells (14). Therefore, circRNAs are extremely suitable to serve as biomarkers. It has been reported that circEPSTI1 is a prognostic marker in triple-negative BC (9). Hsa_circ_0072995, hsa_circ_0072309 and hsa_circ_0001982 have also been reported in BC (15-17). In the cytoplasm, circRNA can function as miRNA sponges that bind miRNAs, thus impeding their binding and suppressing their target genes. In human astrocyte cells, circHIPK2 can bind miR124-HG, which modulate astrocyte cell autophagy and endoplasmic reticulum stress response (18). During differentiation of human epidermal stem cells, circZNF1 functions as a sponge for miR-23b-3p (19), and circBIRC6 is a sponge for miR-34a and miR-145 which then regulate differentiation of human stem cells (20). Although there have been so many studies, there is no known biomarker that is more clinically appropriate. Therefore, this study aimed to identify a potential biomarker in breast cancer.

In our work, the potential participation of circRNAs in BC was investigated using four pairs of BC patient samples and paracancerous tissues. The results suggested that 256 circRNAs were differentially up-regulated and 277 circRNAs were differentially down-regulated. In addition, we verified the expression of six differentially up-regulated circRNAs in the filtered library, finding that hsa_circ_0001583 expression was indeed up-regulated. Hence, we thought that hsa_circ_0001583 might be a novel biomarker in BC. Furthermore, the top 5 related miRNAs are shown by predicting the hsa_circ_0001583-miRNA-mRNA network. Importantly, KEGG analysis revealed that several immune response pathways are associated with candidate miRNA, including long-term potentiation, T/B-cell receptor signaling pathway, bile secretion, oxytocin and calcium signaling pathway, infection of vibrio cholerae, proteolysis mediated by ubiquitin, and axon guidance. These results suggested that hsa_circ_0001583 was strongly associated, which will lay the foundation for further research in the future.

Objectively, this study also has some limitations. At first, this study is a retrospective study with a small sample which would be better with initial or external validation. Second, *in vivo* or *in vitro* functional studies of hsa_circ_0001583 are still lacking. Third, the relationship between hsa_circ_0001583 and long-term outcomes were not investigated in BC patients.

Taken together, we thought hsa_circ_0001583 might act as a biomarker for BC related to the immune response of the body. As is well known, BC immunology has progressed tremendously over the last decade, which is an

important treatment in BC. Therefore, our future research will focus on hsa_circ_0001583 in the immunology and mechanism of BC.

Conclusion

We utilized 4 pairs of BC neoplasm tissues and their paracancerous tissues to investigate the potential of circRNAs biomarkers in BC by applying circRNA microarray and real-time quantitative PCR methods. Importantly, hsa_circ_0001583 was discovered to be significantly up-regulated in breast tumors, and it might be an excellent biomarker for BC diagnosis and may modulate tumor immunology through ceRNA mechanism.

Acknowledgements

We are grateful to Dr. Zong-Cai Wang in the First Affiliated Hospital of Fujian Medical University for clinical sample collection and Dr. Xia Chen for technical support. The present study was financially supported by the Natural Science Foundation of Fujian Province, China (2017J01285). The authors report no conflicts of interest in this work.

Authors' Contribution

Y.-J.C., W.-H.C.; Designed and perform the research, analyzed the data and wrote the manuscript. M.Z., M.-F.W.; Performed the research and analyzed the data. Y.-J.D., L.-L.H.; Collected and analyzed the clinical data. All authors read and approved the final manuscript.

References

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2020. *CA Cancer J Clin.* 2020; 70(1): 7-30.
2. Noh JM, Choi DH, Huh SJ, Park W, Yang JH, Nam SJ, et al. Patterns of recurrence after breast-conserving treatment for early stage breast cancer by molecular subtype. *J Breast Cancer.* 2011; 14(1): 46-51.
3. Kolakofsky D. Isolation and characterization of Sendai virus DI-RNAs. *Cell.* 1976; 8(4): 547-55.
4. Memczak S, Jens M, Elefsinioti A, Torti F, Krueger J, Rybak A, et al. Circular RNAs are a large class of animal RNAs with regulatory potency. *Nature.* 2013; 495(7441): 333-338.
5. Wang F, Nazarali AJ, Ji S. Circular RNAs as potential biomarkers for cancer diagnosis and therapy. *Am J Cancer Res.* 2016; 6(6): 1167-1176.
6. Qin M, Liu G, Huo X, Tao X, Sun X, Ge Z, et al. Hsa_circ_0001649: A circular RNA and potential novel biomarker for hepatocellular carcinoma. *Cancer Biomark.* 2016; 16(1): 161-169.
7. Lu J, Zhang PY, Li P, Xie JW, Wang JB, Lin JX, et al. Circular RNA hsa_circ_0001368 suppresses the progression of gastric cancer by regulating miR-6506-5p/FOXO3 axis. *Biochem Biophys Res Commun.* 2019; 512(1): 29-33.
8. Ji W, Qiu C, Wang M, Mao N, Wu S, Dai Y. Hsa_circ_0001649: A circular RNA and potential novel biomarker for colorectal cancer. *Biochem Biophys Res Commun.* 2018; 497(1): 122-126.
9. Chen B, Wei W, Huang X, Xie X, Kong Y, Dai D, et al. circEPST11 as a prognostic marker and mediator of triple-negative breast cancer progression. *Theranostics.* 2018; 8(14): 4003-4015.
10. Hansen TB, Kjems J, Damgaard CK. Circular RNA and miR-7 in cancer. *Cancer Res.* 2013; 73(18): 5609-5612.
11. Liang Y, Song X, Li Y, Ma T, Su P, Guo R, et al. Targeting the circBMP2/miR-553/USP4 axis as a potent therapeutic approach for breast cancer. *Mol Ther Nucleic Acids.* 2019; 17: 347-361.
12. Jeck WR, Sorrentino JA, Wang K, Slevin MK, Burd CE, Liu J, et al. Circular RNAs are abundant, conserved, and associated with ALU repeats. *RNA.* 2013; 19(2): 141-157.
13. Li J, Yang J, Zhou P, Le Y, Zhou C, Wang S, et al. Circular RNAs in cancer: novel insights into origins, properties, functions and implications. *Am J Cancer Res.* 2015; 5(2): 472-480.
14. Rybak-Wolf A, Stottmeister C, Glažar P, Jens M, Pino N, Giusti S, et al. Circular RNAs in the mammalian brain are highly abundant, conserved, and dynamically expressed. *Mol Cell.* 2015; 58(5): 870-885.
15. Zhang HD, Jiang LH, Hou JC, Zhou SY, Zhong SL, Zhu LP, et al. Circular RNA hsa_circ_0072995 promotes breast cancer cell migration and invasion through sponge for miR-30c-2-3p. *Epigenomics.* 2018; 10(9): 1229-1242.
16. Yan L, Zheng M, Wang H. Circular RNA hsa_circ_0072309 inhibits proliferation and invasion of breast cancer cells via targeting miR-492. *Cancer Manag Res.* 2019; 11: 1033-1041.
17. Tang YY, Zhao P, Zou TN, Duan JJ, Zhi R, Yang SY, et al. Circular RNA hsa_circ_0001982 promotes breast cancer cell carcinogenesis through decreasing miR-143. *DNA Cell Biol.* 2017; 36(11): 901-908.
18. Huang R, Zhang Y, Han B, Bai Y, Zhou R, Gan G, et al. Circular RNA HIPK2 regulates astrocyte activation via cooperation of autophagy and ER stress by targeting MIR124-2HG. *Autophagy.* 2017; 13(10): 1722-1741.
19. Kristensen LS, Okholm TLH, Venø MT, Kjems J. Circular RNAs are abundantly expressed and upregulated during human epidermal stem cell differentiation. *RNA Biol.* 2018; 15(2): 280-291.
20. Yu CY, Li TC, Wu YY, Yeh CH, Chiang W, Chuang CY, et al. The circular RNA circBIRC6 participates in the molecular circuitry controlling human pluripotency. *Nat Commun.* 2017; 8(1): 1149.