Analysis of circular RNA-associated competing endogenous RNA network in breast cancer

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Abstract. As the most common type of cancer in female patients, the morbidity and mortality rates of breast cancer (BC) are high, and its incidence is gradually increasing worldwide. However, the underlying molecular and genetic mechanisms involved in the etiopathogenesis of BC remain unclear. Circular RNAs (circRNAs) are a novel type of non-coding RNAs that have been verified to serve a crucial role in tumorigenesis. However, the majority of functions and mechanisms of circRNAs remain unknown. The present study identified 47 differentially expressed circRNAs in a dataset from Gene Expression Omnibus. Using the cancer-specific circRNA database, the potential microRNA (miRNA) response elements, RNA-binding proteins and open reading frames of the candidate circRNAs were predicted. Combing the predictions of miRNAs and target mRNAs, a competing endogenous RNA network was constructed, which may serve as the theoretical basis for further research. Furthermore, the analyses conducted using Gene Ontology terms and Kyoto Encyclopedia of Genes and Genomes pathways indicated that candidate circRNAs may serve a role in transcriptional regulation. Moreover, 20 BC tissue specimens and their paired adjacent normal tissue specimens were used to evaluate the expression levels of the screened circRNAs. Thus, the analyses of the raw microarray data conducted in the present study offer perspectives on the exploration of mechanisms associated with BC tumorigenesis with regard to the circRNA-miRNA-mRNA network.

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Introduction

Breast cancer (BC) is the most frequent type of cancer in female patients, with high morbidity and mortality rates worldwide. It has been estimated that there will be 27,1270 new cases of BC and 42,260 deaths associated with BC in the USA in 2019 (1). Previous epidemiological studies have reported that obesity (2), utilization of estrogen and progestin (3), advanced maternal age at first birth (4) and alcohol abuse (5) increase the risk of developing BC. In addition, genetic mutations and epigenetic modifications are regarded as potential causes of tumorigenesis in BC (6,7). In the past decades, there has been considerable progress in preventing, diagnosing and treating BC. However, the prognosis for BC remains poor. In consequence, understanding the molecular and genetic mechanisms associated with the pathogenesis of BC is essential.

Circular RNAs (circRNAs), a new type of 3'-5' head-to-tail covalently closed non-coding RNA, were identified in 1976 (8,9). However, in subsequent decades, circRNAs were considered to be the product of incorrect splicing (10). Recently, it was recognized that circRNAs are normal co-products of numerous eukaryotic protein-coding genes. Furthermore, circRNAs have extensive functions, including: i) Sponging microRNAs (miRNAs/miRs), thus inhibiting the expression of target genes (11); ii) binding to proteins in order to form a complex and consequently serving a role in transcription (12); iii) post-transcriptional regulation via a variety of mechanisms (13); and iv) translational functions involving the modification of the internal ribosome entry site or N6-methyladenosine (14,15). However, the majority of functions and mechanisms of circRNAs remain unknown, which

suggests that circRNAs may be a promising avenue to explore in medical research (16).

miRNAs are a type of non-coding RNAs with a length of ~22 nucleotides (17). miRNAs perform a repressive function on the expression of their target genes through miRNA response elements (MREs), which exist in their target RNA transcripts (18). Previous studies have demonstrated that circRNAs sponge miRNAs via MREs in numerous diseases. This mechanism has also been reported in cancer initiation and progression (19). Accumulating evidence has demonstrated that circRNAs regulate the growth, development and metastasis of BC by acting as miRNA sponges. For instance, ciRS-7 sponges miR-7 and inhibits the expression of its target gene in numerous tumors (20). In addition, circHIPK3 may function as a miR-124 sponge and inhibit its antineoplastic function, thus inducing the proliferation of BC cells (21). However, further studies are required to explore the potential mechanisms of tumorigenesis, which may aid in the diagnosis and treatment of BC.

The application of microarray technology has enabled the extensive study of gene expression and has facilitated the research of disease susceptibility in favor of treating diseases at the molecular level. Several studies have indicated that microarray technology and *in silico* analysis have broad application in identifying pathogenetic mechanisms and novel diagnostic and therapeutic targets (22,23).

The present study identified novel circRNAs and revealed their underlying mechanisms in BC via the combined application of *in silico* analysis and microarray technology. A diagram representing the overall study design is presented in Fig. 1. First, BC-associated microarray datasets from the Gene Expression Omnibus (GEO) database were screened, the bioinformatic data were analyzed and differentially expressed circRNAs (DECs) were obtained. Next, the potential miRNAs sponged by DECs were identified through the cancer-specific circRNA database (CSCD) database. Moreover, a bioinformatics prediction of target mRNAs was performed, and a circRNA-miRNA-mRNA competing endoegenous network was constructed. Gene enrichment analyses of the candidate mRNAs were performed with the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases, which resulted in the prediction of the signaling pathways involved in BC. The identification of DECs and potential mechanisms reported in the present study may facilitate future research in BC treatment and diagnosis.

Materials and methods

Screening of DECs in BC from the GEO database. The microarray datasets analyzed in the present study, including the circRNA expression profile in BC, were downloaded from the GEO database [National Center for Biotechnology Information (NCBI); http://www.ncbi.nlm.nih.gov/geo/] (24). The GSE101124 dataset was derived from the GEO database, and contained gene expression data from 15 samples, including 4 breast cancer cell lines, 8 cancerous breast tissues and 3 healthy mammary gland tissue samples (25). The GPL19978 platform (Agilent-069978 Arraystar Human circRNA microarray V1; Agilent Technologies, Inc.) was used to identify the GSE101124 dataset. The raw data were pretreated and homogenized, followed by detection, screening and annotation of circRNAs. The Limma package of R/Bioconductor software (version 3.9) (26) was used for the analysis of raw microarray data and the identification of DECs in the dataset. The following filtering criteria were applied: $|\log FC|>2$ and adjusted $P<0.05$.

Prediction of miRNAs and competing endogenous (ceRNA) network construction. The CSCD database (http://gb.whu.edu.cn/CSCD) can calculate predictions of MREs, RNA-binding proteins (RBPs) and open reading frames (ORFs) (27). miRNAs sponged to circRNAs were identified from the GSE101124 dataset. Furthermore, potential ORFs in DECs and RBPs combined with DECs were examined. A ceRNA network was constructed to predict the association between miRNAs and the identified DECs or mRNAs. Using the sequences and annotations of miRNAs obtained from miRBase (http://www.mirbase.org/), the mRNA-miRNA interactions were predicted by miRTarbase (28), TargetScan (29) and miRDB (30). Target genes were selected from the overlapping genes for the construction of the ceRNA network.

The ceRNA network was constructed as a graphical representation by Cytoscape software (version 3.6.1) (31) with the miRNA target genes and DECs.

Functional term and signaling pathway enrichment analyses. GO (http://www.geneontology.org) annotates genes to biological process (BP), molecular function (MF) and cellular component (CC) (32). The identification and annotation of homologous genes and protein sequences in various organisms helps to elucidate the specific roles of certain genes.

The KEGG (http://www.genome.jp/kegg/) database consists of 16 main databases and is comprised of disease information organized in computable forms (33). Among these databases, the KEGG pathway database provides molecular networks for molecular systems by annotating genes to pathways (33). The R (version 3.9) package (http://www.bioconductor.org/) clusterProfiler (34) allows gene classification and calculation of enrichment for GO terms and KEGG pathways. In the present study, GO annotation and KEGG pathway analyses were conducted with clusterProfiler to explore the potential biological roles of target genes. The analysis results were visualized with the ggplot2 package of the R software. Adjusted $P<0.05$ and $q<0.05$ were considered to indicate statistically significant enriched function annotations.

Patient samples. For the validation of DECs in clinical samples, RNA was extracted from 20 BC tissue specimens and their paired adjacent normal tissue specimens to perform reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. These samples were collected at The Affiliated Hospital of Qingdao University between April 2018 and December 2018. Inclusion criteria of the patients were as follows: i) Age ≥18 years; ii) BC was confirmed via pathological examination; iii) routine blood count, liver and kidney function and electrocardiogram examination met the treatment requirements before operation; and iv) no serious complications prior to surgery. The exclusion criteria were: i) No pathological diagnosis and unknown TNM stage; ii) received chemotherapy

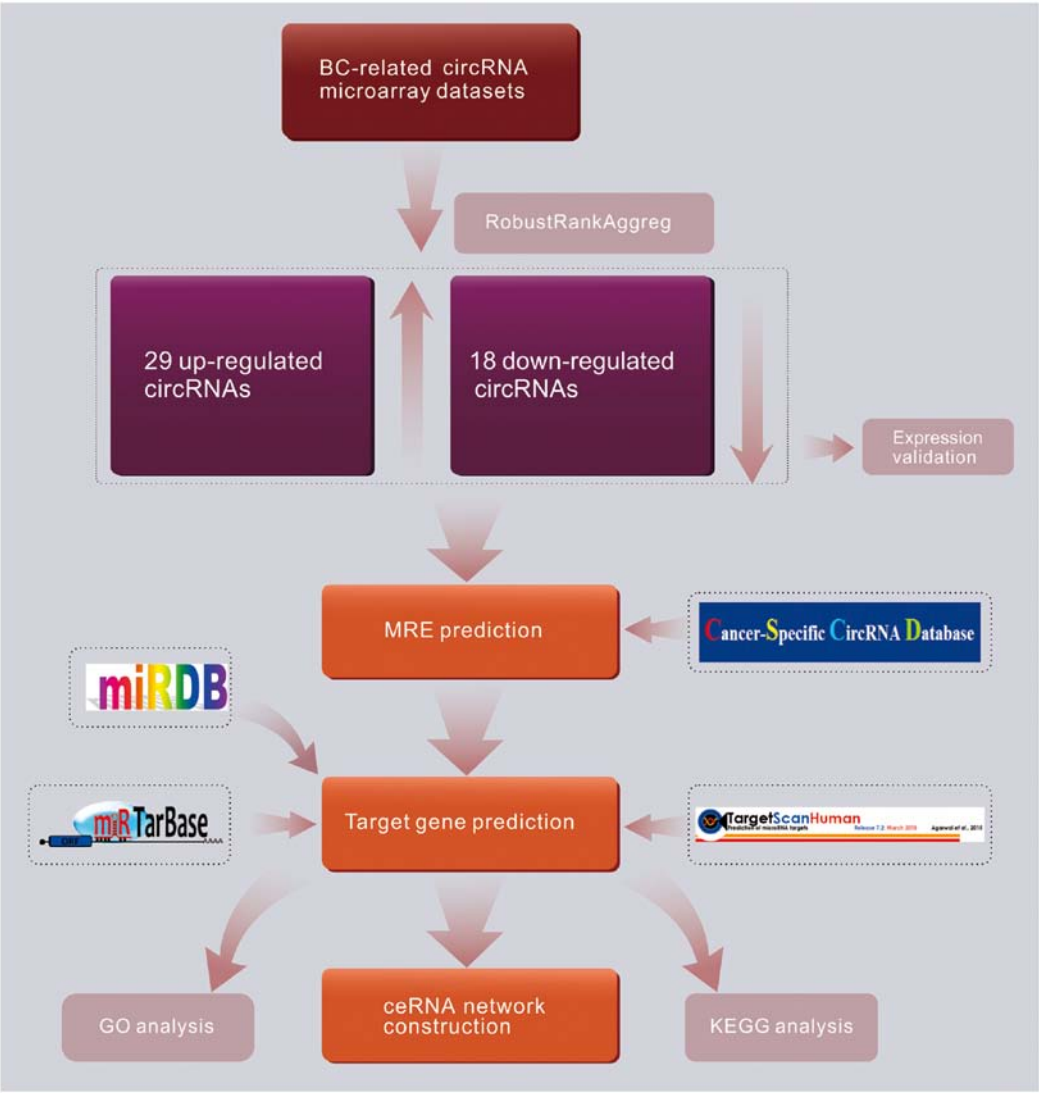

Figure 1. Sequence diagram summarizing the present study. BC, breast cancer; MRE, microRNA response element; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; circRNAs, circular RNAs; ceRNA, competing endogenous RNA.

or radiotherapy prior to surgery; iii) presence of other tumors; and iv) poor compliance of patients. Tissue specimens located ≥5 cm from the tumor margin were obtained via excision surgery. Patients were all female. The median age of patients was 45 years (range, 30-56). Immediately after obtaining the specimen, tissues were frozen in liquid nitrogen and stored at -80˚C for preservation. Ethical approval was obtained from the Ethics Committee of The Affiliated Hospital of Qingdao University. The study participants approved the use of clinical samples by providing written informed consent.

Cell culture. BC cell lines (MCF-7 and MDA-MB-231) and a human normal mammary epithelial cell line (MCF-10A) were obtained from the American Type Culture Collection. MCF-10A cells were used as the control. BC cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS (both Gibco; Thermo Fisher Scientific, Inc.). MCF-10A cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS. All cell lines were used within 6 months and cultured in a 5% CO_2 incubator at 37˚C. RNA was extracted when the cell confluence reached ~80%. All experiments were repeated in triplicate.

RT-qPCR. TRIzol reagent (Takara Bio, Inc.) was used for the extraction of total RNA from tissues or cells following the manufacturer's protocols. After treatment with DNAse I (Takara Bio, Inc.), reverse transcription of 1 μg total RNA was conducted with reverse transcriptase (RT reagent Kit; cat. no. RR037A, Takara Bio, Inc.).

qPCR was subsequently performed using SYBR Premix Ex Taq (Takara Bio, Inc.) on a CFX96 Real-Time PCR Detection system (Bio-Rad Laboratories, Inc.). The conditions of the amplification reaction procedure were set as follows: Pre-denaturation at 95˚C for 30 sec; 95˚C for 10 sec; and 60˚C for 40 sec for a total of 40 cycles. The expression of circRNAs was normalized to that of internal control GAPDH, using the $2^{-\Delta\Delta Cq}$ method (35). The following primer pairs were used for qPCR: GAPDH forward, 5'-CGCTCTCTGCTCCTCCTG TTC-3' and reverse, 5'-ATCCGTTGACTCCGACCTTCAC-3'; *Homo sapiens* (hsa)_circ_0000520 forward, 5'-AGACTA GGGCCAGAGGCG-3' and reverse, 5'-GAGCTTCCCTCC

Table I. Divergent and convergent primers used for PCR.

circRNA ID	Primer type	Sequence (5'-3')
hsa_circ_0000520	Div	F: GGTCTGAGACTAGGGCCAGAGG
		R: GGAGTGGAGTGACAGGACGCAC
	Con	F: ACGAGCTGAGTGCGTCCTGT
		R: AAGCTCAGGGAGAGCCCTGT
hsa_circ_0006220	Div	F: CTACCCTGCTGAACCTGAAACA
		R: TCACACTCCTCCTTGGTCTTGG
	Con	F: GCAGGATGTAGCCAATCAAATGT
		R: TTTTTGCTTCCTCTGCTTGTTTC
hsa_circ_0000977	Div	F: TTTACTTCCTTGGAGCCAGAGC
		R: CAAACATTATTCTCCGCAGCAT
	Con	F: GGAACAACCACAGGGCAGGT
		R: ATGCTCTGGCTCCAAGGAAGTAA
hsa_circ_0043278	Div	F: GAAACAAGCAGAGGAAGCAAAA
		R: CATTTGATTGGCTACATCCTGC
	Con	F: GCAGGATGTAGCCAATCAAATGT
		R: TTTTTGCTTCCTCTGCTTGTTTC

hsa, *Homo sapiens*; circRNA, circular RNA; div, divergent; con, convergent; F, forward; R, reverse.

CCGAAG-3'; hsa_circ_0006220 forward, 5'-ATTCCATTT CACTGCAGGATGTAGC-3' and reverse, 5'-ACATCCTGC AGTGAAATG-3'; hsa_circ_0000977 forward, 5'-ATGTGG AATAAGAACTCC-3' and reverse, 5'-AACCTATAAACT TCAGAATGGAATG-3'; and hsa_circ_0043278 forward, 5'-GCATTTCATCAATAACCCTC-3' and reverse, 5'-TAG TGAAATGGAATGGCTGT-3'. The expression of DECs was normalized to that of GAPDH.

Validation of circRNAs. The expression levels of DECs were verified in clinical samples and cells by RT-qPCR. In addition, to demonstrate the circularized junction of DECs, divergent (circular) primers were designed for the spliced junction of circRNAs and PCR was performed as previously described (36). The cDNA of MCF-7 cells was reverse transcribed (RT reagent Kit, Code No. RR037A, Takara Bio, Inc.), as above. The thermocycling conditions were as follows: Initial denaturation at 95˚C for 5 min, and 38 cycles at 95˚C for 30 sec, 61˚C (annealing temperature) for 30 sec and 72˚C for 30 sec, followed by a 7-min extension at 72˚C (36). Convergent (linear) primers were used as the control. The spliced junction in the PCR product of DECs was then confirmed by Sanger sequencing (37). Combined with agarose gel electrophoresis, these results validated the existence of circRNAs. The primer sequences used in the present study were synthesized by the Beijing Genomics Institute and are listed in Table I.

Statistical analysis. Data are presented as the mean ± standard error of the mean of ≥3 independent experiments. For the comparison of two groups, a two-tailed paired Student's t-test was performed. One-way analysis of variance (ANOVA) was used to analyze more than two groups. Dunnett's test was performed as the post hoc test. $P<0.05$ was considered to indicate a statistically significant difference. All statistical analyses were performed with GraphPad Prism software (version 6; GraphPad Software, Inc.).

Results

Identification of DECs in BC. GEO is an international public database containing high-throughput functional genomic data (24). GEO may be used for searching, analyzing and visualizing data. The present study screened the microarray datasets of BC from the GEO database in NCBI. The retrieval terms used in GEO were as follows: (‘breast’ OR ‘mammary’ OR ‘HBL’) and (‘tumor’ OR ‘cancer’ OR ‘carcinoma’ OR ‘neoplasm’ OR ‘malignant'). ‘Homosapiens’ in the ‘Top Organisms’ and ‘Non-coding RNA profiling by array’ were selected in the ‘Study type'.

circRNA microarray datasets were selected as the subject of analysis. Data consisting only microarray samples of tissues or cells were excluded. As a result, the final search identified GSE101124, which included information from 15 microarray samples of BC cells, and tissues and paired adjacent normal tissues, and was consequently selected for further analysis. Subsequently, the CSCD was used to identify the DECs in the GSE101124 microarray dataset. Limma, an R/Bioconductor software package, offers an integrated solution for processing large datasets with advanced computational algorithms (26). Pretreatment and homogenization of the raw data was performed with the Llimma package of R software. After filtering the data based on the inclusion criteria ($|logFC|>2.0$ and adjusted $P<0.05$), 47 circRNAs were revealed to be differentially expressed between BC and paired adjacent normal tissues. As demonstrated in Table SI, 29 upregulated and 18 downregulated circRNAs were screened out in the GSE101124 dataset. The circRNAs that ranked in the top four according to the $|logFC|$ were hsa_circ_0000520, hsa_circ_0006220,

Table II. Essential characteristics of hsa_circ_0000520, hsa_circ_0006220, hsa_circ_0000977 and hsa_circ_0043278.

circRNA ID	circRNA type	Genomic length, bp	Spliced length, bp	Best transcript	Gene symbol	Type of regulation
hsa_circ_0043278	Exonic	2,925	250	NM_001488	TADA2A	Downregulation
hsa_circ_0000977	Exonic	24,404	562	NM_024894	NOL10	Downregulation
hsa_circ_0006220	Exonic	158	158	NM_001488	TADA2A	Downregulation
hsa_circ_0000520	Exonic	123	123	NR_002312	RPPH1	Upregulation

circRNA, circular RNA; TADA2A, transcriptional adaptor 2A; NOL10, nucleolar protein 10; RPPH1, ribonuclease P RNA component H1.

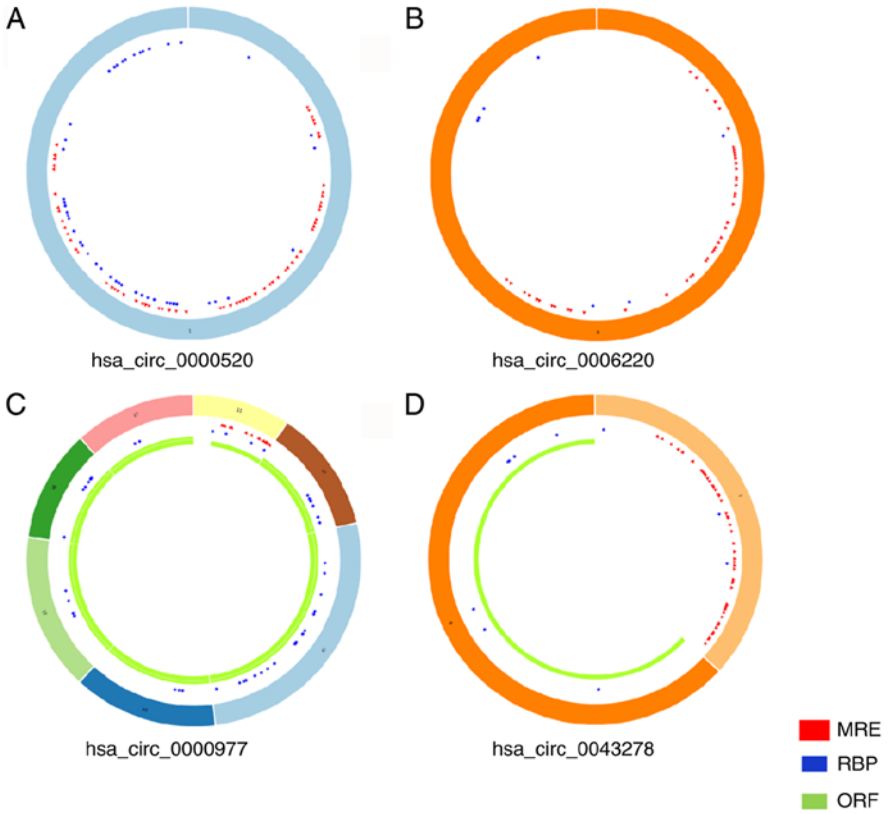

Figure 2. Structural patterns of DECs. The essential characteristics and basic structural patterns of four DECs were analyzed by the cancer-specific circRNA database: (A) hsa_circ_0000520, (B) hsa_circ_0006220, (C) hsa_circ_0000977 and (D) hsa_circ_0043278. DECs, differentially expressed circRNAs; circRNA, circular RNA; hsa, Homo sapiens; MRE, miRNA response element; RBP, RNA binding protein; ORF, open reading frame.

hsa_circ_0000977 and hsa_circ_0043278. The essential characteristics and basic structural patterns of these four circRNAs are presented in Table II and Fig. 2, respectively. Furthermore, a Circos plot (http://circos.ca/) demonstrating DECs in human chromosomes was generated to exhibit the expression of DECs (Fig. 3). The results revealed that the DECs were extensively distributed in all chromosomes, including chromosome X.

Prediction of a circRNA-miRNA-mRNA network. RNA transcripts with the same MREs can competitively sponge miRNAs and subsequently inhibit their downstream functions (38). circRNAs are able to participate in miRNA-mediated post-transcriptional regulation by sponging miRNAs (36).

circRNAs are emerging as essential modulators of ceRNA networks (39). For example, circGFRA1 may sponge miR-34a to serve its regulatory role in triple-negative BC (TNBC) (40). Furthermore, circEPSTI1 may affect the proliferation and apoptosis of TNBC cells by sponging miR-4753 and miR-6809 (41). The next step in the present study was to explore whether the identified DECs acted as miRNA sponges.

CSCD supports the prediction of MREs, RBPs and ORFs for circRNAs (27). The predictions are integrated from TargetScan (29), starBase (http://starbase.sysu.edu.cn/index.php) and ORFfinder (https://indra.mullins.microbiol.washington.edu/sms2/orf_find.html) making CSCD the first comprehensive database specific for cancer-associated circRNAs. This database serves a vital role in the research of the mechanism of action of these molecules. The present study predicted the MREs, RBPs and ORFs of DECs using CSCD (Tables SII-SIV). Moreover, the target genes of miRNAs were predicted and a ceRNA network

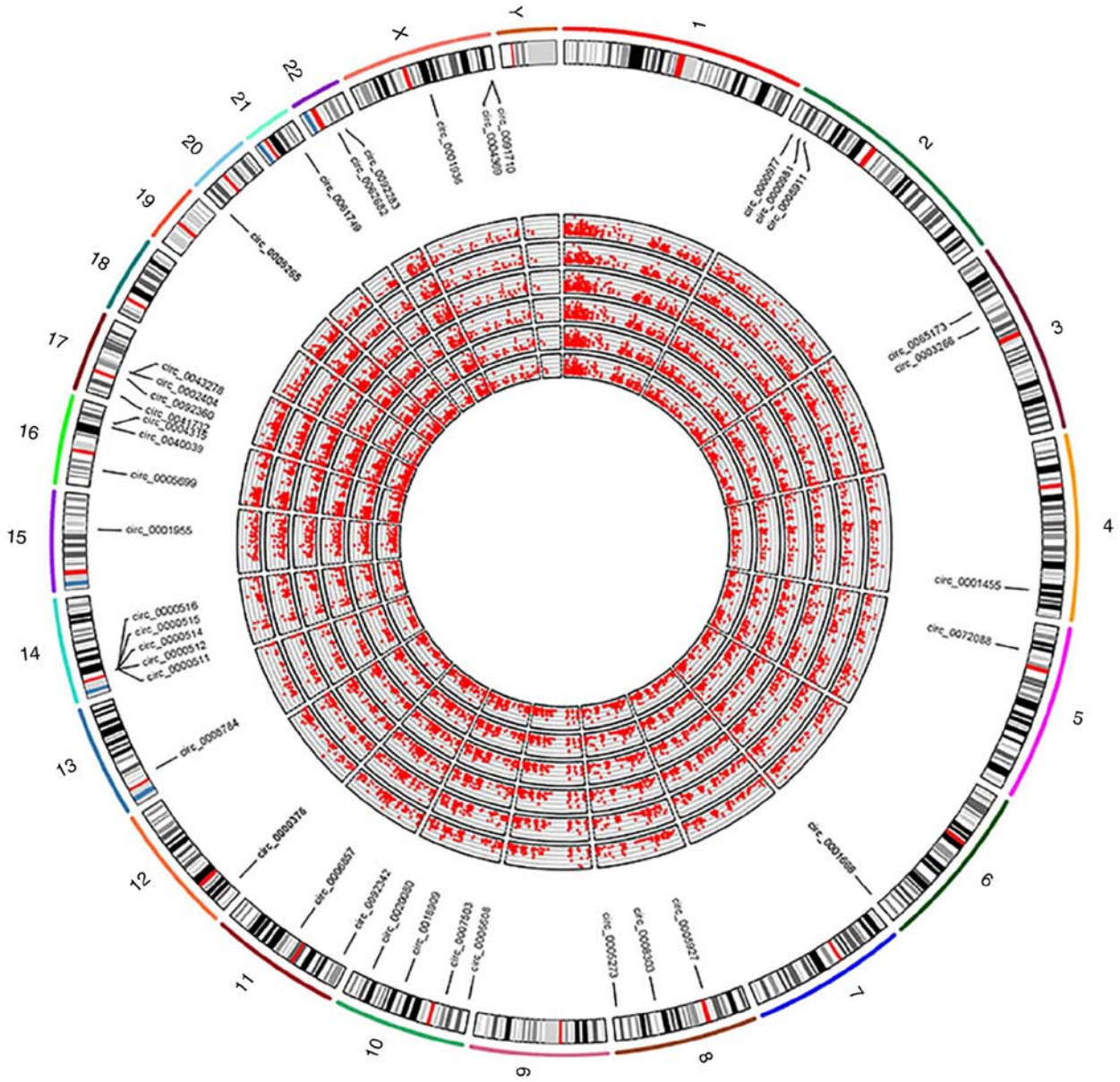

Figure 3. General expression characteristics of DECs. Circos plot displaying DECs in human chromosomes. The outermost layer corresponds to the chromosome map of the human genome; black and white bars represent chromosomal cytobands and red bars represent centromeres. The top 29 upregulated and top 18 downregulated DECs are displayed in the inner layer according to their chromosome locations. All DECs in every chromosome are clustered in the third circle. DECs, differentially expressed circular RNAs; circRNAs, circular RNAs.

was constructed to describe the overall functions of DECs. The predicted target genes are demonstrated in Tables SV-SVIII. The specific mRNAs that bound the target miRNAs were identified using miRTarBase, TargetScan and miRDB. The regulatory network was constructed by Cytoscape and consists of DECs, miRNAs and mRNAs (Fig. 4).

GO annotation and KEGG pathway analysis. In cases where the functional role of the aforementioned DECs has not yet been determined, the roles of mRNAs have been explored to understand the circRNAs. In particular, GO functional term and KEGG pathway enrichment analyses of significantly differentially expressed mRNAs facilitate the understanding of circRNAs (42).

The clusterProfiler software of the R package was used in the present study in order to perform the GO analysis of target genes of the identified DECs. The results for the four aforementioned circRNAs are presented in Fig. 5. For hsa_circ_0000520, the most enriched GO term for target genes in BP was 'transcriptional activator activity, RNA polymerase II transcription regulatory region sequence-specific DNA binding' (Fig. 5A and Table SIX). For hsa_circ_0006220, the most enriched GO term in BP for target genes was 'proximal promoter sequence-specific DNA binding' (Fig. 5B and Table SX). For hsa_circ_0000977 and hsa_circ_0043278, the most enriched GO term for target genes in BP was 'transcription factor activity, RNA polymerase II proximal promoter sequence-specific DNA binding' (Fig. 5C and D; Tables SXI and SXII). In summary, GO enrichment analysis revealed the potential regulatory role of circRNAs in transcription. Recently, Ding *et al* (43) verified that circ-DONSON is capable of activating the transcription of SOX4, a transcription promoter, to

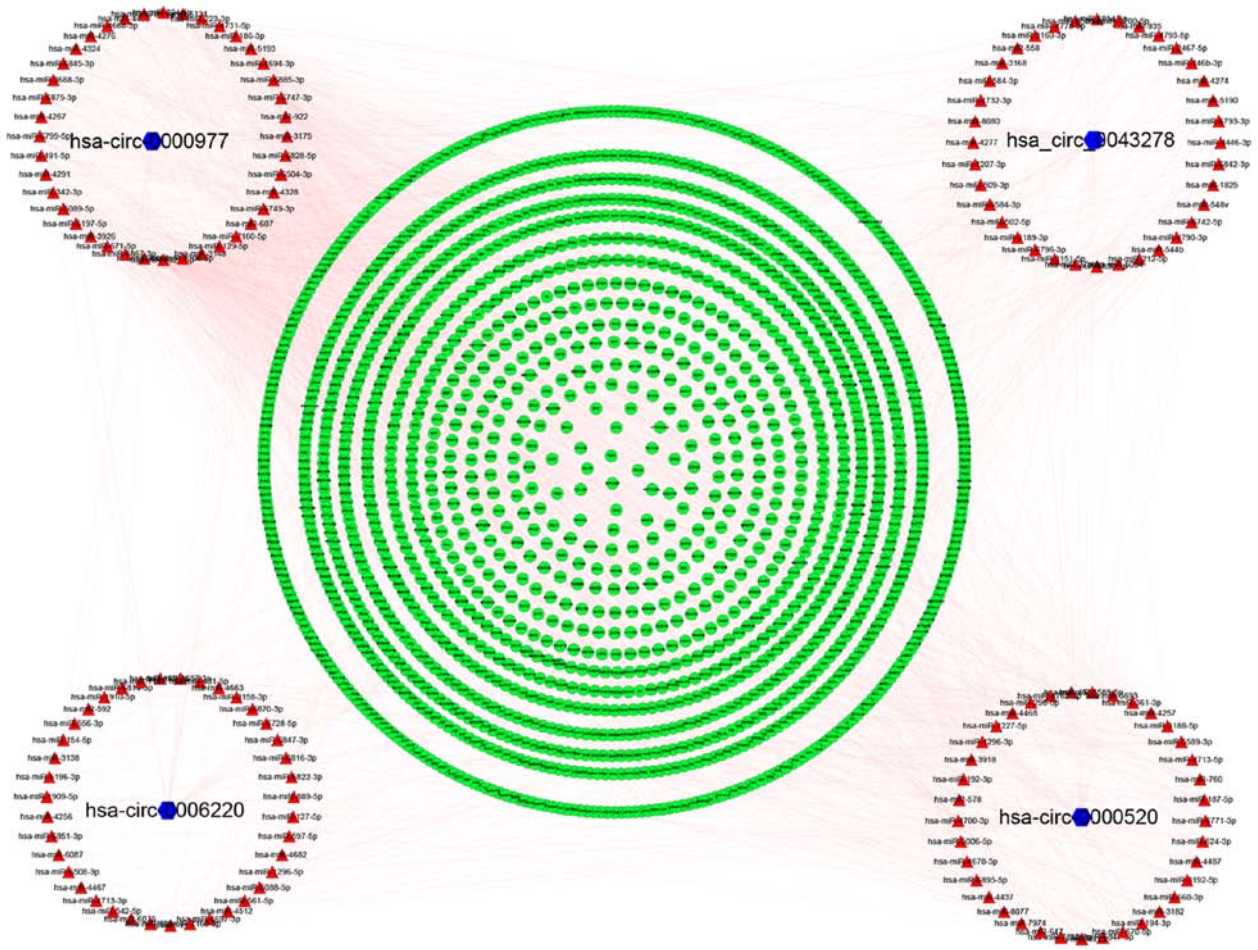

Figure 4. ceRNA network constructed by Cytoscape. The ceRNA network was constructed as a graphical representation with Cytoscape software. The red triangles represent the potential microRNAs sponged to DECs. The green circles represent the target mRNAs. hsa, *Homo sapiens*; circ, circular RNA; ceRNA, competing endogenous RNA.

promote gastric cancer progression. Thus, the results of the present study suggest the potential use of circRNAs as therapeutic biomarkers for patients with BC.

The present study also performed KEGG pathway analysis (Fig. 6). For hsa_circ_0000520, the main enriched pathways for target genes were 'endocytosis' and the 'cell cycle' (Fig. 6A). For hsa_circ_0006220, the most enriched pathway for target genes was the 'PI3K-AKT signaling pathway' (Fig. 6B). For target genes of hsa_circ_0000977, the 'MAPK signaling pathway' was the most enriched pathway (Fig. 6C). For target genes of hsa_circ_0043278, KEGG pathway analysis demonstrated that the main association was with the 'PI3K-AKT signaling pathway' and the 'signaling pathway regulating pluripotency of stem cells' (Fig. 6D). These results suggested that these DECs may be involved in tumor development.

Validation of candidate circRNAs. The circRNAs (hsa_circ_0000520, hsa_circ_0006220, hsa_circ_0000977 and hsa_circ_0043278) that ranked in the top four according to |logFC| (>2.0) were selected for validation experiments. First, the expression levels of these four DECs were evaluated in 20 BC tissue specimens and their paired adjacent normal tissue specimens. Subsequently, the expression levels of these four DECs were also examined in BC cells (MCF-7 and MDA-MB-231) and a human normal mammary epithelial cell line (MCF-10A). The relative expression of hsa_circ_0000520 was significantly higher in BC than that in the adjacent normal tissues and MCF-10A cells (Fig. 7A and E). The relative expression levels of hsa_circ_0006220, hsa_circ_0000977 and hsa_circ_0043278 were significantly lower in BC than in the adjacent normal tissues and MCF-10A cells (Fig. 7B-E). To validate whether these circRNAs were actually circular transcripts, PCR was performed using divergent and convergent primers specific for spliced junctions of circRNAs. The cDNA and genomic DNA (gDNA) extracted from MCF-7 cells were used as templates. The results revealed that these circRNAs were amplified with the divergent primers on cDNA instead of gDNA. Furthermore, the sequences of the spliced junctions were confirmed by Sanger sequencing (Fig. 8A-D).

Discussion

As the most common type of cancer among females, BC has high morbidity and mortality rates, and its incidence is gradually increasing worldwide. A previous study in the USA estimated 27,1270 new cases and 42,260 deaths caused by BC in 2019 (1). The main reasons for morbidity and mortality in patients with BC include cancer cell invasion, migration and metastasis. The mechanisms of tumorigenesis remain unclear

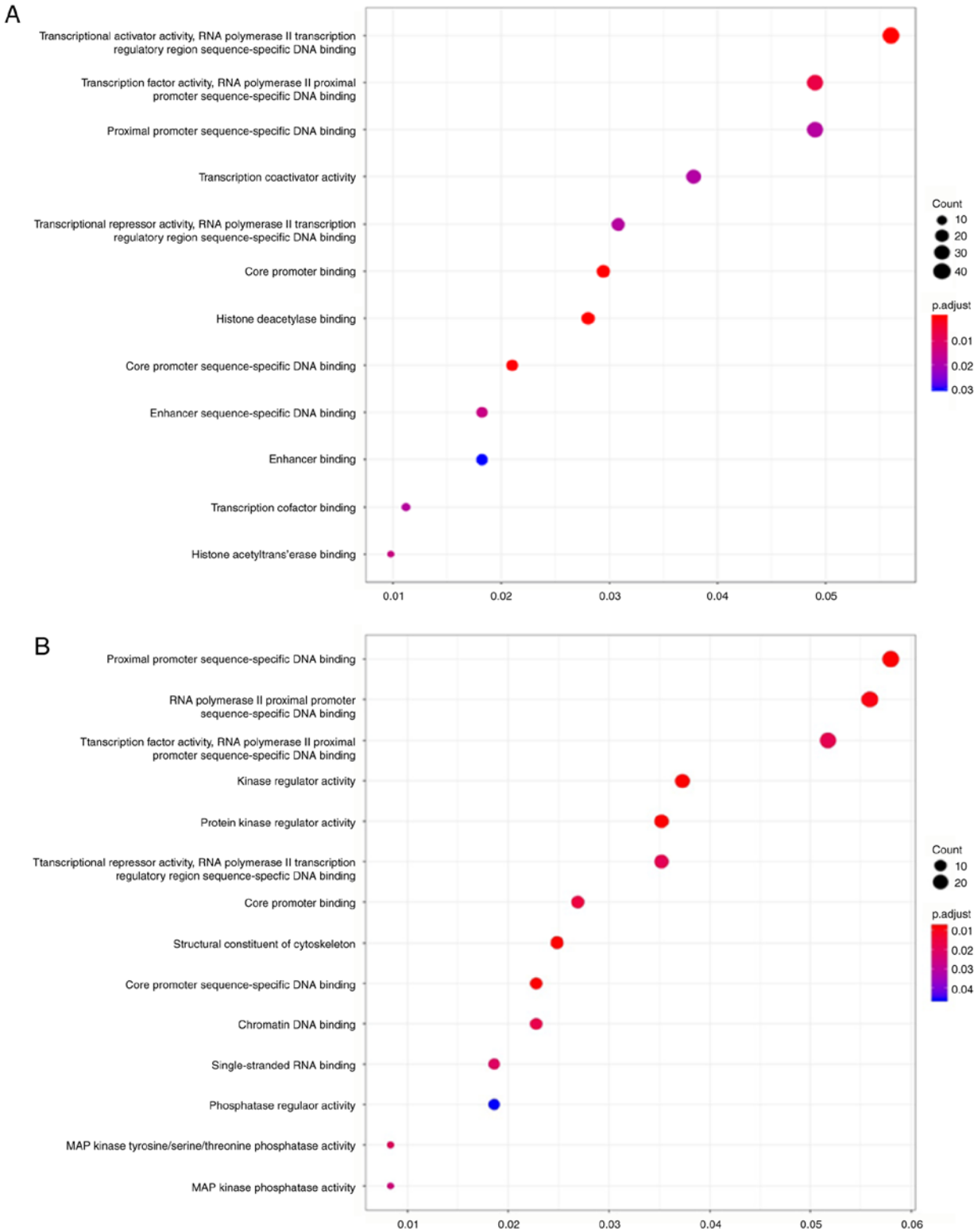

Figure 5. GO enrichment annotations for the target mRNAs of the competing endogenous RNA network. GO analysis of (A) hsa_circ_0000520, (B) hsa_circ_0006220 on the basis of the regulatory network. GO analysis was performed with the clusterProfiler package of the R software and visualized with the ggplot2 package of the R software (version 3.9). Adjusted P<0.05 was considered to indicate significantly enriched GO annotations. p.adjust, adjusted P-value; MAP, mitogen-activated protein; UTR, untranslated region; GO, Gene Ontology; circ, circular; hsa, *Homo sapiens*.

due to the complex genetic mutations and epigenetic alterations involved in this process.

The development of effective therapies for patients with BC requires the identification of therapeutic and prognostic targets. Recent studies have focused on the identification of differentially expressed non-coding RNAs, including long non-coding RNAs, miRNAs and circRNAs, by gene chip and high-throughput sequencing (44-46). It is essential to identify early clinical diagnostic markers in order to understand the molecular mechanism underlying, and facilitate the diagnosis of, BC.

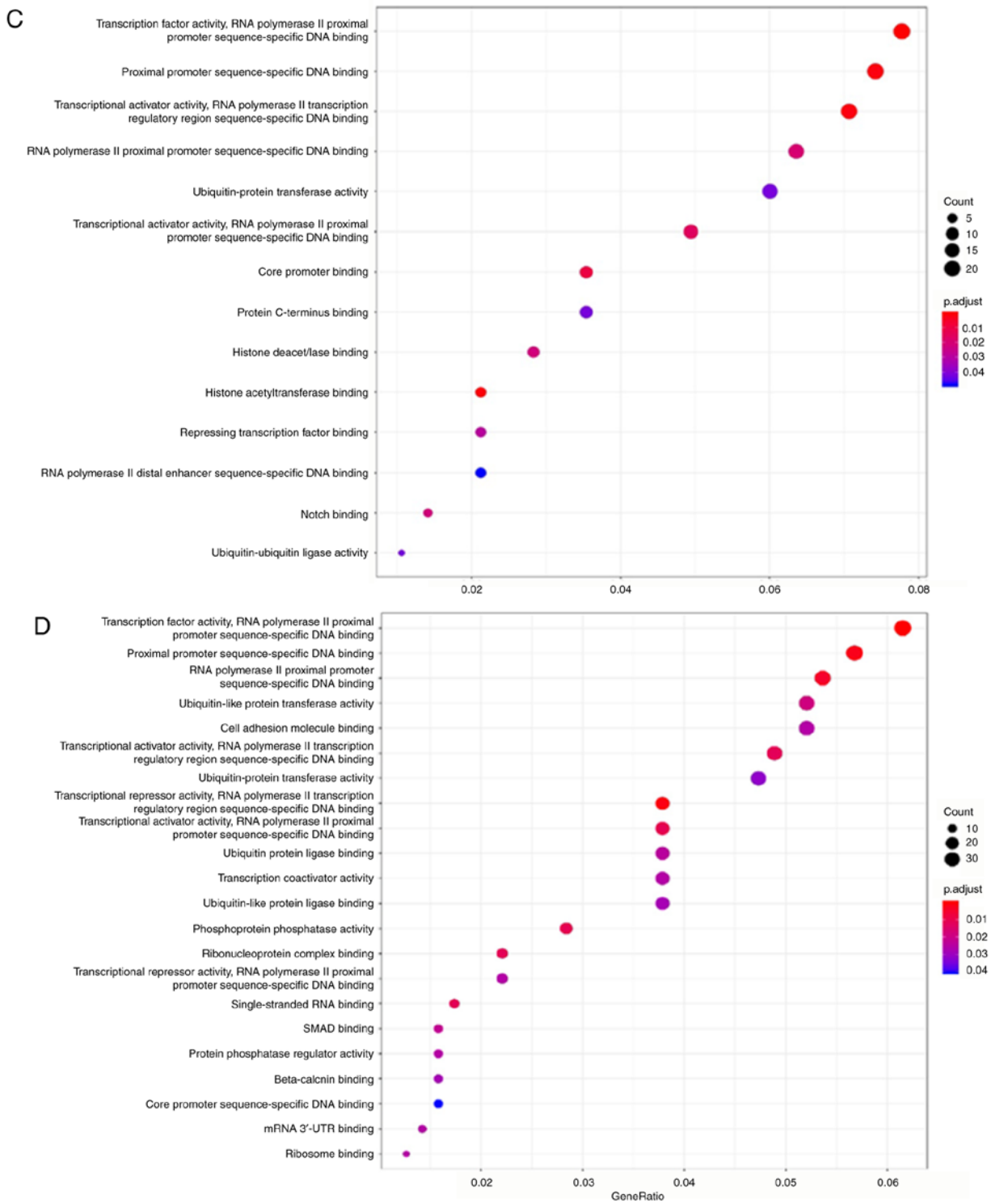

Figure 5. Continued. GO enrichment annotations for the target mRNAs of the competing endogenous RNA network. GO analysis of (C) hsa_circ_0000977 and (D) hsa_circ_0043278 on the basis of the regulatory network. GO analysis was performed with the clusterProfiler package of the R software and visualized with the ggplot2 package of the R software (version 3.9). Adjusted P<0.05 was considered to indicate significantly enriched GO annotations. p.adjust, adjusted P-value; MAP, mitogen-activated protein; UTR, untranslated region; GO, Gene Ontology; circ, circular; hsa, *Homo sapiens*.

As naturally occurring RNAs, circRNAs are a subclass of non-coding RNAs widely expressed in mammalian cells (47). It was recently demonstrated that circRNAs may serve a vital role in the progression of various human cancer types (48-51). For example, circCEP128 promotes bladder cancer progression by sponging miR-145-5p, which inhibits the function of SRY-box transcription factor 11 (48). However, their roles in breast tumorigenesis are not yet well understood. Further research of the molecular mechanisms of circRNAs may provide insight into the diagnosis and treatment of BC.

There are two primary methods of circRNA detection: RNA sequencing (RNA-seq) and high-throughput circRNA microarray. RNA-seq is a common method for genome-wide analysis of circRNAs, though limited by the low reads that

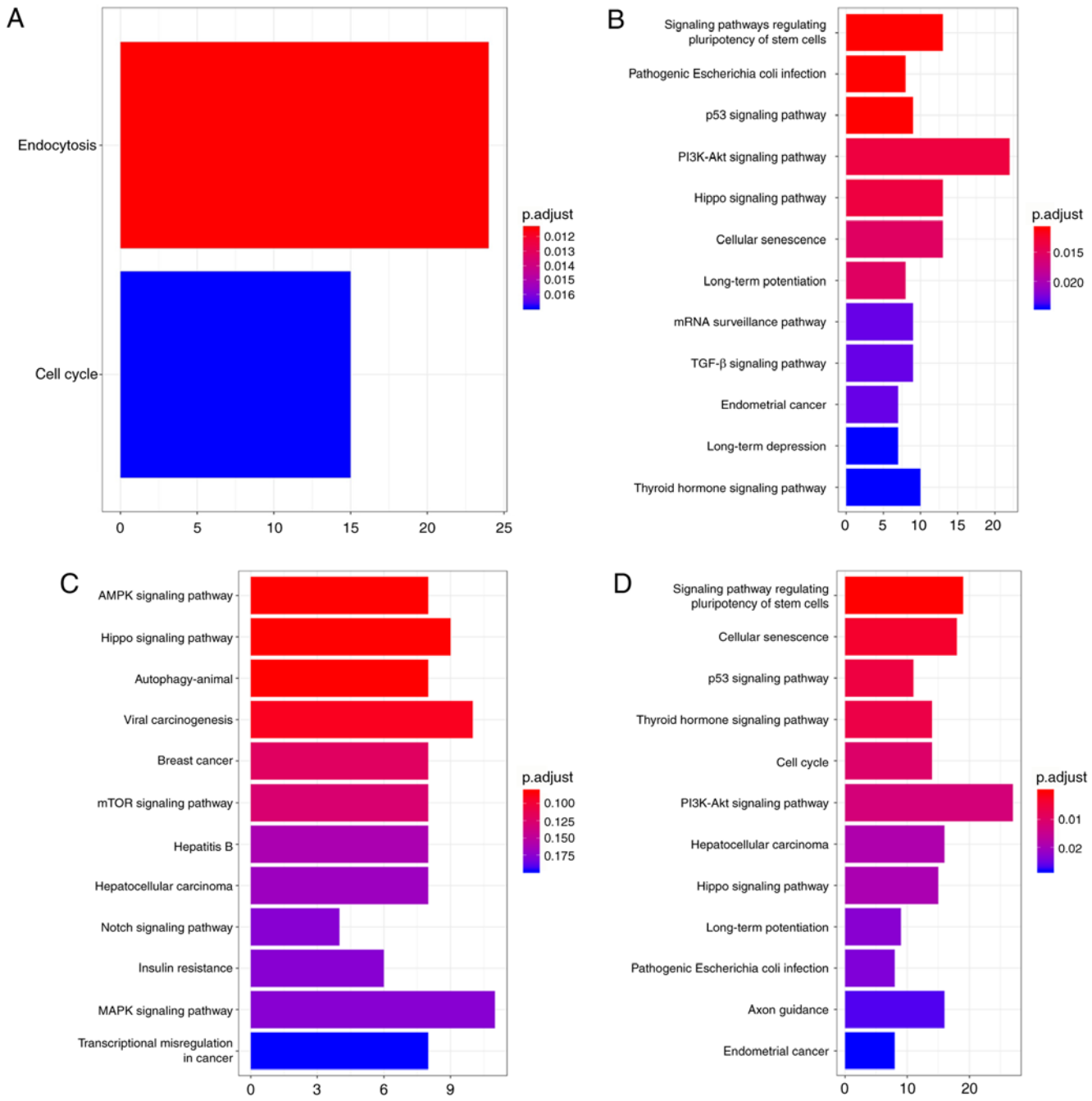

Figure 6. KEGG pathway analysis of the target mRNAs of the competing endogenous RNA network. KEGG analysis of (A) hsa_circ_0000520, (B) hsa_circ_0006220, (C) hsa_circ_0000977 and (D) hsa_circ_0043278 on the basis of the regulatory network. KEGG analysis was performed with the clusterProfiler package of the R software and visualized with the ggplot2 package of the R software (version 3.9). Adjusted P<0.05 was considered to indicate significantly enriched KEGG pathways. p.adjust, adjusted P-value; AMPK, AMP-activated protein kinase; MAPK, mitogen-activated protein kinase; KEGG, Kyoto Encyclopedia of Genes and Genomes; circ, circular RNA; TGF-β, transforming growth factor-β; hsa, *Homo sapiens*.

cover the specific splicing junction (52). In previous studies, Lan *et al* (53) investigated circRNA expression profiles via high-throughput RNA-seq in papillary thyroid carcinoma, while Coscujuela *et al* (45) used RNA-seq to profile and characterize circRNAs in luminal-like BC. Nair *et al* (44) created a comprehensive workflow termed Circ-Seq based on existing bioinformatics approaches to screen expressed circRNAs in BC subtypes.

For the detection of known circRNAs, high-throughput circRNA microarray assays are able to efficiently target the reported back-splice sites in the samples of interest. Microarray data have been largely examined to identify pathogenetic pathways and therapeutic targets in several diseases, including cancer and autoimmune, fibrotic, neurodegenerative and infectious diseases (22,23,54-59). Presti *et al* (22) utilized RNA-seq data of 281 and 283 DNA-sequenced glioblastoma multiforme and low-grade glioma patients, respectively, to evaluate the expression levels of migration inhibitory factor (MIF) and related genes in glioma. After screening for differentially expressed genes, a correlation analysis was performed. Moreover, microarray transcriptomic data were used to assess the mean difference in MIF levels between

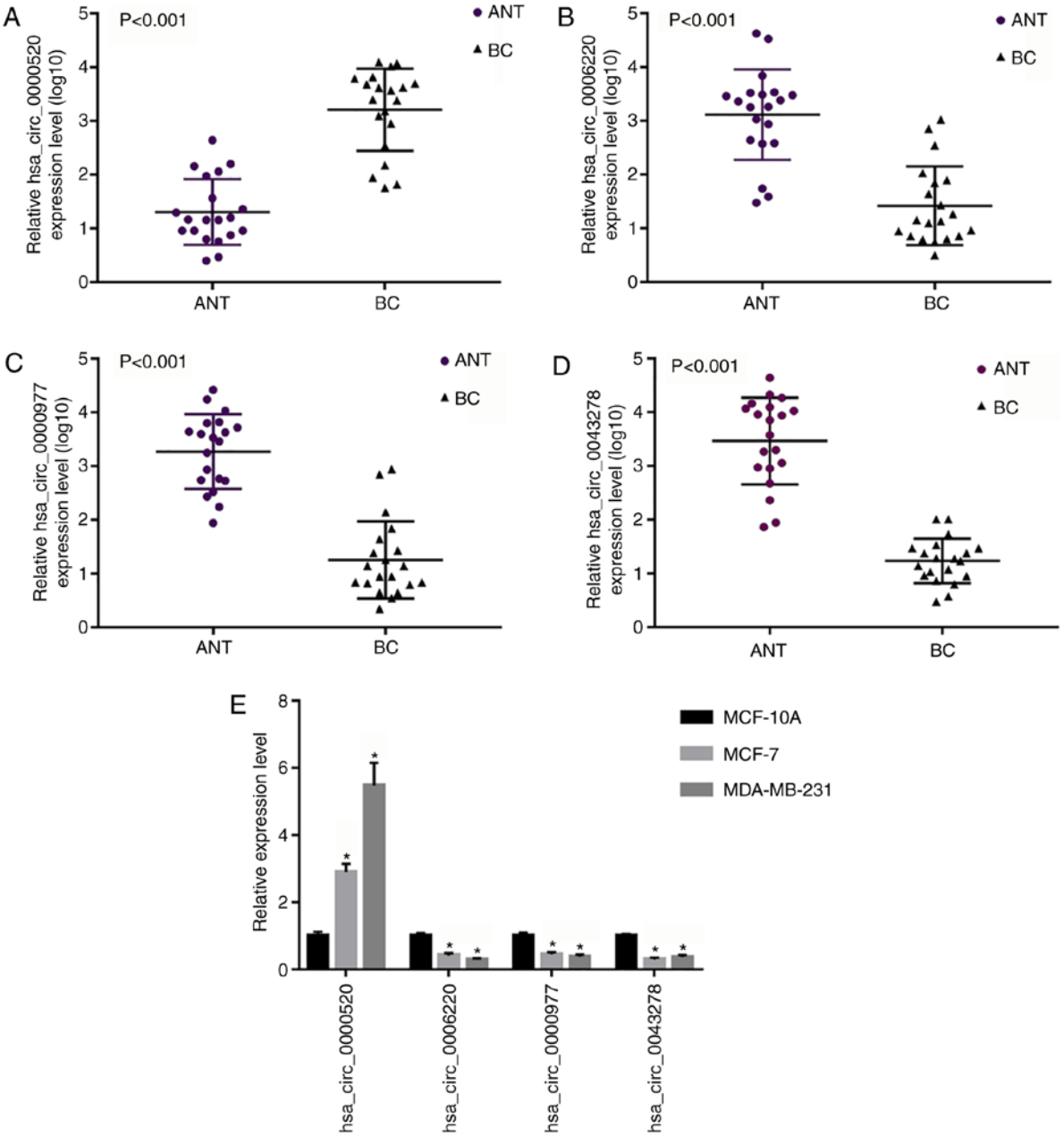

Figure 7. Scatter plots of the expression of DECs. The expression of four DECs in 20 BC tissue specimens and their paired adjacent normal tissue specimens. (A) hsa_circ_0000520, (B) hsa_circ_0006220, (C) hsa_circ_0000977 and (D) hsa_circ_0043278. P<0.001 (n=20). (E) Expression levels of the top four DECs in BC cells (MCF-7 and MDA-MB-231) and in human normal mammary epithelial cells (MCF-10A) were analyzed by RT-qPCR. MCF-10A cells were used as the control. *P<0.05 vs. control. BC, breast cancer; DECs, differentially expressed circRNAs; circRNA, circular RNA; hsa, Homo sapiens; RT-qPCR, reverse transcription-quantitative PCR; ANT, adjacent normal tissue.

neoadjuvant and non-neoadjuvant therapy. In uveal melanoma (UM), Petralia *et al* (54) characterized the pathophysiological role of CD47. CD47 expression levels were found to affect the tumor microenvironment, immune capacity and stroma in UM. Microarray datasets were used to identify 64 differentially expressed genes in metastatic UM. These genes, which are associated with the metastatic properties of UM, may be useful for the verification of potential chemotherapeutic methods.

In colorectal cancer (CRC), KCNMA1 has been reported to be downregulated, and assessment of the GSE35834 microarray dataset has suggested that mir-17-5p may be a target for KCNMA1. Therefore, KCNMA1 has been indicated as a therapeutic target in the early stages of CRC (60). For the study of multiple sclerosis (MS), Fagone *et al* (23) utilized microarray dataset analysis to obtain 45 genes that were differentially expressed between low and high responder $CD4^+$ T cells. The identification of a specific gene signature for natalizumab responsiveness may provide a theoretical basis for suitable treatments for patients with MS, indicating the potential of microarray dataset analysis in improving the understanding of multiple disease types. Fagone *et al* (57) analyzed 254 upregulated genes in activated hepatic stellate cells by screening microarray datasets in order to investigate liver fibrosis. Regarding BC, a recent study systematically

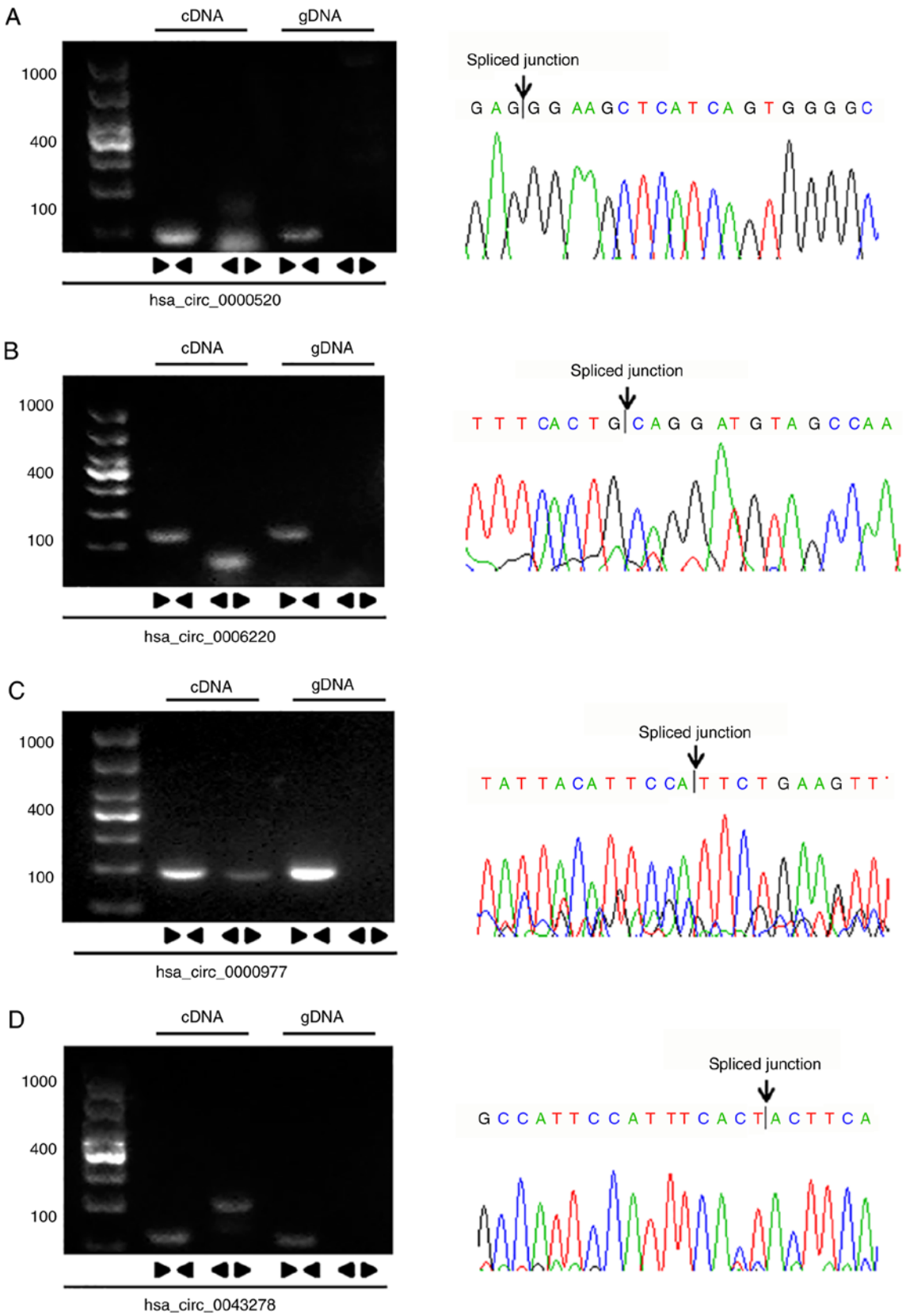

Figure 8. Verification of DECs. PCR products were amplified from the cDNA of MCF-7 cells by divergent primers. The genomic DNA did not produce amplification products by divergent primers. Sanger sequencing was used to validate the head-to-tail splicing of DECs. (A) hsa_circ_0000520. (B) hsa_circ_0006220. (C) hsa_circ_0000977. (D) hsa_circ_0043278. DECs, differentially expressed circRNAs; circ, circular; cDNA, complementary DNA; gDNA, genomic DNA; hsa, *Homo sapiens*.

identified DECs by circRNA microarray, and identified the hsa_circ0087378/miR-1260b/secreted frizzled-related protein 1 axis as a potential regulatory mechanism in estrogen receptor-positive BC (61).

In the present study, circRNA microarray was utilized to construct a general expression profile of BC. Specific circRNAs were analyzed, and a ceRNA network was constructed with the aim of exploring the potential mechanisms of these circRNAs as diagnostic biomarkers.

First, the GSE101124 dataset from the GEO database was screened and 47 DECs were detected using Limma. Among them, hsa_circ_0000520 was found to be upregulated, whereas hsa_circ_0006220, hsa_circ_0000977 and hsa_circ_0043278 were found to be downregulated. hsa_circ_0000520 was spliced from ribonuclease PRNA component H1 gene, the RNA component of the RNase P ribonucleoprotein, which is an endoribonuclease that assists in the formation of the mature 5' termini of tRNA sequences by cleaving tRNA precursor molecules (62). hsa_circ_0006220 and hsa_circ_0043278 were found to be encoded by transcriptional adaptor 2A (TADA2A), which encodes proteins as a transcriptional activator adaptor and as part of the PCAF (human Gcn5 homologue) histone acetylase complex (63). circTADA2A, also known as hsa_circ_0043278, has been demonstrated to serve a role in promoting osteosarcoma progression and metastasis by sponging miR-203a-3p (64). hsa_circ_0000977 was derived from the nucleolar protein 10 (NOL10) gene. Bammert *et al* (65) hypothesized that NOL10 formed a salt-stable protein complex in conjunction with neuroguidin (NGDN) and apoptosis-antagonizing transcription factor (AATF/Che-1/TRB) and demonstrated that the AATF-NGDN-NOL10 complex is involved in ribosome biogenesis. In the present study, the presence and expression level of the four circRNAs were validated in both 20 BC tissues and their paired adjacent normal tissues, using qPCR and RT-qPCR, respectively. hsa_circ_000052 was upregulated in BC, whereas hsa_circ_0006220, hsa_circ_0000977 and hsa_circ_0043278 were downregulated. Back-splicing in the RT-qPCR products of hsa_circ_000052, hsa_circ_0006220, hsa_circ_0000977 and hsa_circ_0043278 was confirmed by Sanger sequencing.

circRNAs have been demonstrated to serve a vital role in cancer cell proliferation (66), migration and invasion (67), anchorage-independent cell growth (68) and cell cycle progression (69). It has been reported that exonic circRNAs are involved in miRNA or protein sponging and are able to inhibit their functional activities in the cytoplasm (70). hsa_circ_0000520, hsa_circ_0006220, hsa_circ_0000977 and hsa_circ_0043278 were both cyclized from exons. To identify whether the identified circRNAs act as miRNA sponges, the bioinformatics database CSCD was employed to analyze the presence of potential miRNA binding sites. In addition, CSCD can also be used for the prediction of RBPs and ORFs for circRNAs. hsa_circ_0000977 and hsa_circ_0043278 were revealed to have ORFs, which implies that these circRNAs may be translated. The results demonstrated that hsa_circ_0000520 has 87 potential MREs, hsa_circ_0006220 has 56 potential MREs, hsa_circ_0000977 has 37 potential MREs and hsa_circ_0043278 has 68 potential MREs. To elucidate the molecular mechanism behind the functions of miRNAs, their hypothetical target genes were analyzed using TargetScan, miRDB and miRTarBase. The results of the analysis were used to construct the ceRNA network.

Currently, there is no direct method of predicting the function of circRNAs. However, the functions of mRNAs in the ceRNA network may be analyzed to identify the potential mechanisms of circRNAs. GO, as a bioinformatics tool, consists of three separate ontologies: BP, MF and CC. The clusterProfiler software of the R package was used to perform GO analysis, and the results demonstrated that the four circRNAs may function as transcriptional regulatory factors. For example, the most enriched GO term for hsa_circ_0000520 in BP was 'transcriptional activator activity, RNA polymerase II transcription regulatory region sequence-specific DNA binding'. Ding *et al* (43) reported that circDONSON recruited the nucleosome remodeling factor complex to the SOX4 promoter and initiated its transcription in gastric cancer. Huang *et al* (71) confirmed that circNfix, as a super enhancer-associated circRNA, may be the key regulator of cardiac regeneration. The results of the GO analysis in the present study provided information on the mechanism of circRNA regulation in BC tumorigenesis.

KEGG analysis revealed that the aforementioned DECs were related to tumor-associated pathways. For hsa_circ_0000520, the main enriched pathways were 'endocytosis' and the 'cell cycle'. Cell cycle arrest results in decreased cell proliferation and increased cell apoptosis. For example, upregulation of circRNA-BARD1 has been identified to inhibit BC cell proliferation, lead to cell cycle arrest and stimulate apoptosis (72). For hsa_circ_0006220, the most enriched pathway was the 'PI3K-AKT signaling pathway'. The PI3K-AKT pathway is a ubiquitous signaling pathway that triggers a series of responses, including cell survival, growth and proliferation (73). Imbalance in the components of this pathway may cause cells to proliferate abnormally, which may facilitate tumor formation and progression (74). Recently, Zhen *et al* (75) revealed that circHMGCS1 serves an important role in hepatoblastoma cell proliferation by regulating IGF2 and IGF1R expression and upregulating the downstream effectors of the PI3K-AKT signaling pathway. Regarding hsa_circ_0000977, the 'MAPK signaling pathway' was observed as the most enriched pathway. Once activated, mitogen-activated protein kinases (MAPKs) phosphorylate other protein kinases and numerous transcription factors in various cellular processes, including cell survival, differentiation, proliferation and migration (76). A previous study has reported that the circSETD3/miR-421/MAPK14 signaling axis prevents the proliferation of hepatocellular carcinoma (77). KEGG pathway analysis demonstrated that hsa_circ_0043278 was mainly associated with the 'PI3K-AKT signaling pathway' and 'signaling pathways regulating pluripotency of stem cells'. One study demonstrated that the combined inhibition of PI3K/Akt/mTOR and sonic hedgehog pathways modulates the activity of pluripotency promoting factors and inhibits the survival, self-renewal and tumorigenic potential of glioblastoma-initiating cells (78).

However, there are various limitations in the present study due to the small sample size of the dataset employed. In the future, integrated analysis of additional datasets may reduce the likelihood of false-positives. In addition, the DECs identified in the present study should be verified in a larger number of clinical samples and other experiments should be conducted, including *in vivo* studies and western blotting. The

present study provides a theoretical basis for the future study of circRNAs-miRNAs-mRNAs in BC.

In conclusion, the present study revealed the expression profiles of specific DECs in BC. Specifically, 47 DECs were identified by circRNA microarray analysis of the GSE101124 dataset. Four specific DECs (hsa_circ_0000520, hsa_circ_0006220, hsa_circ_0000977 and hsa_circ_0043278) were selected for further validation. Following the construction of ceRNA networks and function enrichment analysis, it was concluded that these four circRNAs may serve a role in BC tumorigenesis as transcriptional regulators. In summary, the present study provides further knowledge on the mechanism by which circRNAs may serve as biomarkers in BC.

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Availability of data and materials

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

Authors' contributions

XW contributed to the conception of the present study and completed the draft of the manuscript. All authors participated in the design of the study and conducted the research. YD, ML and WL processed and analyzed the data from the dataset. QW, TL and YW were responsible for the clinical data collection. CL and WX contributed to the experimental design and were responsible for revising the manuscript and approving the version to be published. All authors reviewed all the data and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was conducted in accordance with the Declaration of Helsinki. The Ethics Committee of the Affiliated Hospital of Qingdao University approved the study. The participants approved the use of clinical samples by providing written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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