Identification of seven circRNAs that are repeatedly down-regulated in gastric cancer as a biomarker and their potential miRNA targets

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Research

Keywords: Differentially-expressed circRNAs, differentially-expressed miRNAs, Gastric cancer

Posted Date: September 16th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-880098/v1

Identification of seven circRNAs that are repeatedly down-regulated in gastric cancer as a biomarker and their potential miRNA targets

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Abstract: Objective: This study aimed to screen and identify the differentially-expressed circRNAs (DE-circRNAs) in multiple microarray data sets of gastric cancer (GC), predict their corresponding miRNAs, and explore their regulatory relationship, thereby identifying potential molecular markers for early diagnosis of GC. **Results:** Seven DE-circRNAs were screened and identified: hsa_circ_0007991, hsa_circ_0013048, hsa_circ_0048607, hsa_circ_0050745, hsa_circ_0054971, hsa_circ_0059802 and hsa_circ_0067934. The host genes of these DE-circRNAs are enriched in cancer-related signaling pathways, including ERb signaling, mTOR signaling, chemokine signaling, and B cell receptor signaling. Moreover, these seven DE-circRNAs were all down-regulated in GC tissues. The 7 miRNAs predicted by DE-circRNAs were expressed in GC tissues, namely hsa-miR-1182, hsa-miR-1225-3p, hsa-miR-1275, hsa-miR-193a-5p, hsa-miR-543, hsa-miR-622 and hsa-miR-630. Among them, miR-193a targets were abundant and highly enriched in cancer-related pathways (ERb signaling and mTOR signaling), which was highly consistent with the enrichment of the host gene where the DEcircRNA was located. These results suggested that circRNA may regulate the occurrence and development of GC through the regulation of the expression of the host genes and miRNA. In clinical samples, DE-circRNAs were less expressed in GC tissues when compared to normal tissues. **Conclusion:** Seven down-regulated DE-circRNAs and 7 targeted miRNAs were identified in GC, which contributed to the understand of the underlying molecular mechanism of GC and provided a basis for the early diagnosis of GC and the development of t

argeted drugs.**Methods:** Four sets of circRNA microarray data and one set of miRNA microarray data in GC and adjacent tissues were downloaded from the Gene Expression Omnibus (GEO) and analyzed using GEO2R tool to identify DE-circRNAs and differentially-expressed miRNAs (DE-miRNAs). Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were used to analyze the enrichment pathway of host mRNAs of DE-circRNAs. Miranda and TargetScan were used to predict miRNA-binding DE-circRNAs. The predicted DE-circRNA interacting miRNAs were intersected with DE-miRNAs, and KEGG and Reactome analyzed the targeted mRNAs corresponding to the obtained miRNAs. DE-circRNAs in GC tissues and adjacent tissues was verified in clinical samples.

Keywords: Differentially-expressed circRNAs, differentially-expressed miRNAs, Gastric cancer

1. Introduction

GC is one of the most common gastrointestinal malignancies globally, with a morbidity and mortality that ranks 5th and 3rd among all malignancies[1]. Early GC has no specific clinical manifestations, leading to the lack of[2]. Clinical tumor markers, such as carcinoembryonic antigen (CEA), CA199(Serum Antigen 199) , and CA125 (Serum Antigen 125) have low specificity and sensitivity, and most need an approach that involves combined detection[3]. Invasive "endoscopic biopsy" is the gold standard for GC diagnosis[4], however, the invasive nature of an operation limits patients with contraindications and complications.

With the development and application of high-throughput RNA sequencing and bioinformatics analysis, circRNA has been shown to be expressed in various tumors[5], and has become a hot spot for diagnosis and prognostic markers of GC. Reports on the underlying mechanisms of circRNA in GC are limited[6].

The number of GC-related circRNAs with validated biological functions and their underlying mechanisms of action is growing. The main means include miRNA sponges[7], regulation of alternative splicing, and regulation of parental gene transcription. The protein production of miRNA sponges has been extensively researched[8], and miRNAs are able to directly bind to target mRNA by base-pairing to trigger cleavage of mRNAs or repress mRNA translation[9]. CircRNAs located in the cytoplasm also contain complementary miRNA binding sites and thereby serve as competitive miRNA inhibitors[10]. Moreover, oncogenic circRNAs and antioncogenic circRNAs have been discovered to regulate the proliferation, migration, invasion, and apoptosis of GC cells[11]. Most circRNAs could regulate GC-related signaling pathways through miRNA sponges[12]. CiRS-7 acts as oncogenic circRNA by antagonizing the miR-7-mediated PTEN/PI3K/AKT pathway in GC[13]. In addition, circLARP4 acts as an antioncogenic circRNA by antagonizing miR-424-5p in modulating the Hippo-Yap pathway[14]. Due to their abundance, high stability, tissue- and developmental stage-specific expression patterns, and wide distribution in various body fluids and exosomes[15, 16], circRNAs exhibit a great potential to serve as biomarkers for GC.

At present, most studies on circRNA biomarkers of GC use independent ci rcRNA microarray detection, and circRNA detected by different microarrays als o have significant differences. Therefore, the circRNAs identified in studies are expected to become a more stable and reliable molecular biomarker for GC. I n this study, 4 circRNA microarray data sets that were differentially expressed in GC tissues, and adjacent tissues and 1 miRNA microarray data set were do wnloaded from the GEO database. The network interactions of circRNA-miRN A-mRNA constructed and verified by qRT-PCR in GC tissues, and 7 down-reg ulated DE-circRNAs in GC were identified. The targeted regulation of miRNAs was predicted, which may be molecular markers for early diagnosis of GC.

2 RESULTS:

2.1 Analysis of circRNAs detected in four published sets of GC data

A total of 84979 circRNAs obtained by analyzing 4 sets of chips met the general requirements of bioinformatics screening of differentially-expressed circ RNAs(Figure 1-A).Dimensional analysis of 4 sets of chip data showed that sa mples of different batches of data had good similarity (PCA diagram, Figure 1 -B). There were significant differences in the expression of circRNAs between GC tissues and the adjacent tissues in the heat map analysis (Figure 1-C).

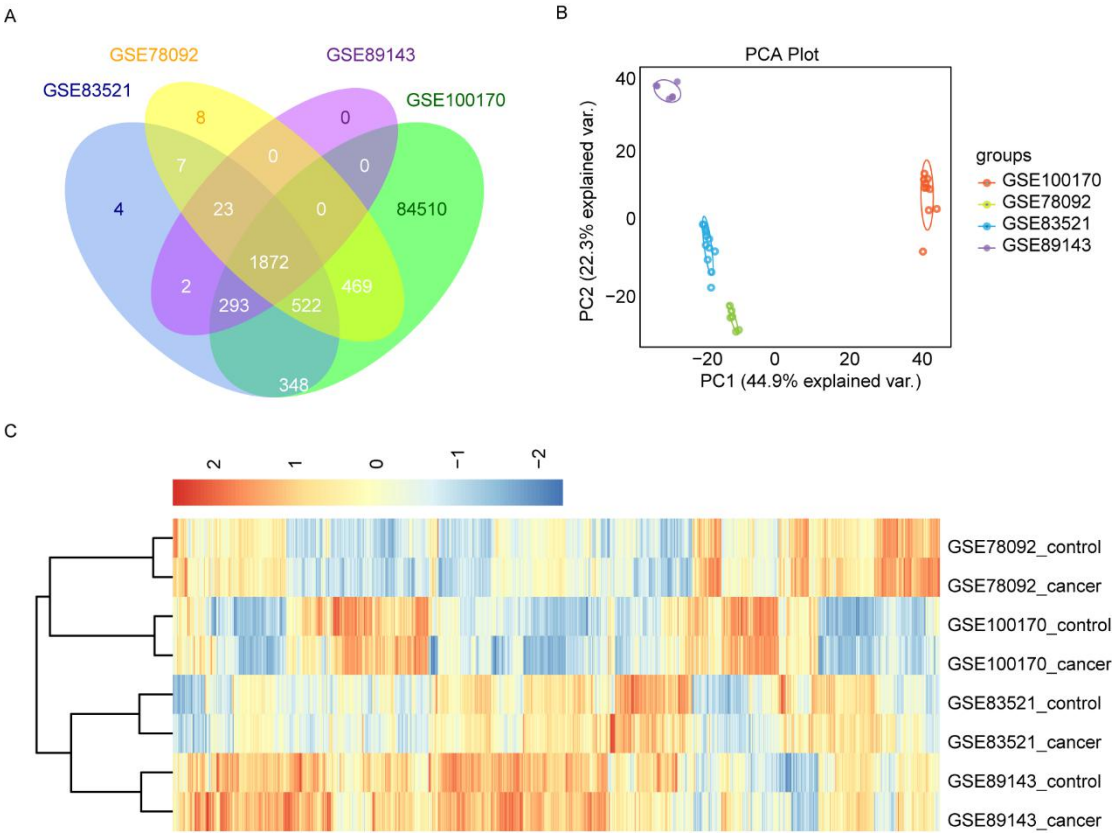

Figure 1 Analysis of circRNAs detected in four published sets of gastric cancer data. (A).Venn diagram showing the overlap of circRNAs detected from GSE78092, GSE100170, GSE83521, GSE89143; (B). Principal component analysis (PCA) plot of the expression of circRNAs in each data set. Ellipses and shapes show the clustering of the samples. Samples of the same set of data come together. (C). Clustered Heatmap of the expression of circRNAs in gastric cancer (GC) tissues and para-carcinoma in each set of data. Red indicated high clustering; blue indicated low clustering. The average expression level of cancer or para-carcinoma samples in each data set was calculated as input.

2.2 Identification of DE-circRNAs repeatedly detected in multiple sets of data

To identify GC DE-circRNAs, we downloaded 4 sets of GC circRNA gene chips from the GEO database. The results showed that four chips expressed

circRNA1872. The circRNAs of 713,532,379 and 423 differentially-expressed ci rcRNAs were obtained in the GSE100170, GSE78092, GSE83521, and GSE891 43 chip (Figure 2-A). KEGG analysis of up- and down-regulated circRNA host genes (top10)(Figure 2-B, Figure 2-C)After cross-over, no up-regulated circRN As was expressed in three and four chips, 15 up-regulated circRNAs were exp ressed in two chips, no down-regulated circRNAs were expressed in four chips. However, 7 circRNAs (hsa_circ_0007991hsa_circ_0013048, hsa_circ_0048607, hsa_circ_0050745, hsa_circ_0054971, hsa_circ_0059802, and hsa_circ_006, 7934) were down-regulated in the three chips. Note that in the GSE7809, GSE83521, and GSE89143 chips, the circRNA expression threshold was set to |FC|>1.5 P <0.05. Because the GSE100170 data cannot be calculated using the GEO2R, th e threshold was set to |FC|>2, P<P 0.05). The host mRNA of DE-circRNAs w asanalyzed by KEGG analysis in four sets of chips, which showed that they c ould be enriched into cancer signaling pathways, such as ERb signaling, mTO R signaling, and the PI3K-AKT signaling pathway (Figure 2-D UP). The host mRNA of up-regulated and down-regulated DE-circRNAs in both chips was an alyzed by KEGG analysis, and the top 10 signal pathways were enriched in E Rb signaling, mTOR signaling, and chemokine signaling (Figure 2-E and 2-F). The expression of 7 DE-circRNAs was lower in GC tissue compared with adja cent tissue of GSE78092, GSE83521, and GSE89143 (P＜0.050). Furthermore, the expression of hsa_circ_0054971 in GC tissue was lower compared to that i

n adjacent tissue (P<0.05) of the GSE100170 chip, and no significant differenc es were observed in the other 6 DE-circRNAs.

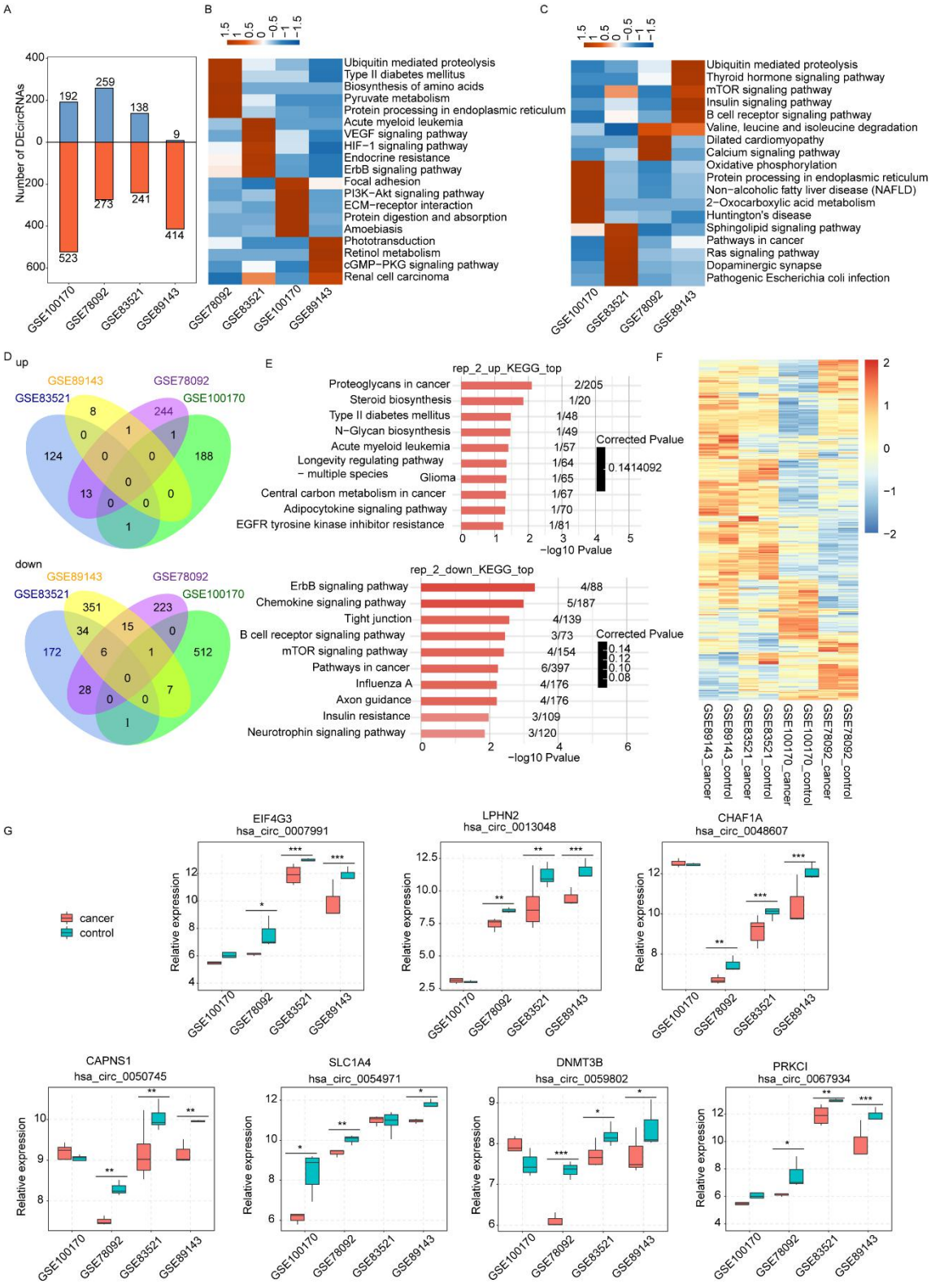

Figure 2. Highly reliable differential expression of circRNA and functional analysis. (A). The number of circRNAs that were differentially expressed between cancer samples and paracancer samples in each data set. Blue indicated up-regulated DE-circRNAs, Red indicated down-regulated DE-circRNAs; (B-C). The top 10 most enriched KEGG pathways illustrated for up-regulated and down-regulated circRNA host genes in cancer. The color scale showed the row-scaled significance (-log10 corrected P value) of the terms. (D). Venn diagram displaying an overlap of up-regulated circRNAs and down-regulated circRNAs in 4 data sets. (E). KEGG analysis of host genes of overlapped up- (left panel) or down (right panel)-regulated circRNAs in at least two data sets in cancer compared with control samples (-log10 is P value). (F). Clustered heatmap of all overlapped up- (left panel) or down (right panel)-regulated circRNAs at least in two data sets. Average expression level of cancer or control samples in each data set was calculated as input. (G). Box plots showing the expression profile of overlapped DE-circRNAs: 7 DE-circRNAs in at least three sets of data showed that the expression level in cancer tissues was lower than that of adjacent tissues, with statistical significance (*P < 0.5, **P < 0.05 and ***P < 0.01, compared with control group).

2.3 Identification of miRNAs targeted by circRNAs differentially expressed in GC tissues

circRNA can be used as a miRNA "adsorption sponge[17]". To predict targeting miRNAs, we downloaded a set of differentially-expressed miRNA chips of GC tissues (GSE26645 chips). In the four sets of data , there are a total of 8 circRNAs in all three sets (one circRNA was raised in two sets and down in a set of data , this circRNA rounding and does no analysis Analysis with the 7 remaining).Analysis of 961 differences miRNAs, 7 DE-circRNAs and pred

icted 83 miRNAs.(Figure 3-A). The predicted 83 miRNAs were verified in th e GSE26645 microarray data, while 7 miRNAs (hsa-miR-1182, hsa-miR-1225-3 p, hsa-miR-1275, hsa-miR-193a-5p, hsa-miR-543, hsa-miR-622 and hsa-miR-630) were expressed in GC (Figure 3-B) . The signal pathway analysis of the 83 miRNAs corresponding targeted mRNA, indicated that genes were significantly enriched in KEGG pathways, including TGF-beta signaling pathway, regulation of actin cytoskeleton, ERBB signaling pathway, renal cell carcinoma and mTO R signaling pathway, which were associated with cancer (Figure 3-C). Reactom e enriched into ERBB2 and ERBB4-related signaling pathways were highly ass ociated with cancer (Figure 3-D).). Using miRanda, we predicted that 5 out o f 7 circRNAs De-circRNAs could adsorb targeted miRNAs, namely hsa_circ_00 48607, hsa_circ_0059802, hsa_circ_0013048, hsa_circ_0007991 and hsa_circ_000 7992. (Figure 3-E). TargetScan analysis showed that in the circRNA-miRNA-mRNA axis, most of the top 10 enriched KEGG pathways were derived from hsa_miR_543 and hsa_miR_193a_2, which were mainly adsorbed to hsa_circ_0 048607 (Figure 3-F).

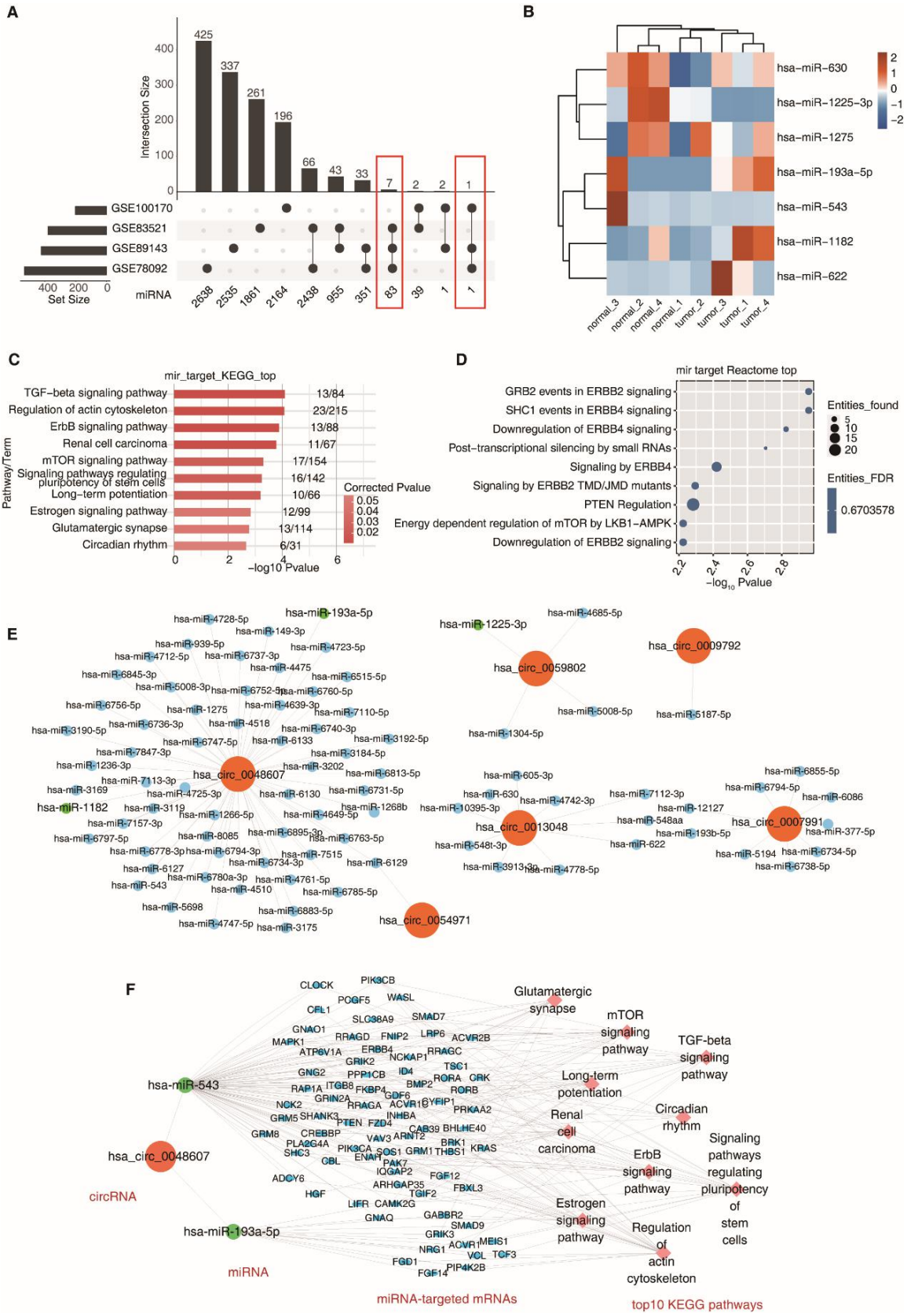
A
Intersection Size
425
337
261
196
66
43
33
7
2
2
1
GSE100170
GSE83521
GSE89143
GSE78092
Set Size
miRNA
2638
2535
1861
2164
2438
955
351
83
39
1
1
B
hsa-miR-630
hsa-miR-1225-3p
hsa-miR-1275
hsa-miR-193a-5p
hsa-miR-543
hsa-miR-1182
hsa-miR-622
normal_3
normal_2
normal_4
normal_1
tumor_2
tumor_3
tumor_1
tumor_4
C
mir_target_KEGG_top
TGF-beta signaling pathway
Regulation of actin cytoskeleton
ErbB signaling pathway
Renal cell carcinoma
mTOR signaling pathway
Signaling pathways regulating pluripotency of stem cells
Long-term potentiation
Estrogen signaling pathway
Glutamatergic synapse
Circadian rhythm
13/84
23/215
13/88
11/67
17/154
16/142
10/66
12/99
13/114
6/31
Pathway/Term
Corrected Pvalue
−log10 Pvalue
D
mir target Reactome top
GRB2 events in ERBB2 signaling
SHC1 events in ERBB4 signaling
Downregulation of ERBB4 signaling
Post-transcriptional silencing by small RNAs
Signaling by ERBB4
Signaling by ERBB2 TMD/JMD mutants
PTEN Regulation
Energy dependent regulation of mTOR by LKB1-AMPK
Downregulation of ERBB2 signaling
Entities_found
Entities_FDR
0.6703578
−log10 Pvalue
E
hsa-miR-193a-5p
hsa_circ_0048607
hsa-miR-1182
hsa-miR-1225-3p
hsa_circ_0059802
hsa_circ_0009792
hsa_circ_0013048
hsa_circ_0007991
hsa_circ_0054971
F
hsa-miR-543
hsa_circ_0048607
hsa-miR-193a-5p
circRNA
miRNA
miRNA-targeted mRNAs
top10 KEGG pathways
Glutamatergic synapse
mTOR signaling pathway
TGF-beta signaling pathway
Long-term potentiation
Renal cell carcinoma
Circadian rhythm
ErbB signaling pathway
Signaling pathways regulating pluripotency of stem cells
Estrogen signaling pathway
Regulation of actin cytoskeleton

Figure 3. Identification of miRNAs targeted by circRNAs that are differentially expressed in GC tissues. (A);Upset plot shows in the four data sets, three data sets (GSE83521, GSE89143 and GSE78092), there are 83 mirnas in the intersection, among which 7 mirnas are expressed in gastric cancer tissues in the other three data sets (GSE100170, GSE89143 and GSE78092), there is 1 miRNA in the intersection, and 1 miRNA in gastric cancer tissues. (B). Clustered heatmap shows expression of detected 7 miRNAs in GSE26645 dataset.(C).The top 10 most enriched KEGG pathways were illustrated for miRNA-targeted mRNAs. The colour scale shows the row-scaled significance (-log10 corrected P value) of the terms.(D). The top 10 most enriched Reactom pathways were illustrated for miRNA-targeted mRNAs.The colour scale shows the row-scaled significance (-log10 corrected P value) of the terms.(E). The network of DEcircRNAs (Red dot) and DEmiRNAs (lightgreen dot).(F). The interaction network of circRNA-miRNAs-mRNAs, Hsa_circ_0048607 adsorbs hsa_miR_543 and Hsa_mir_193a-5p,These two miRNA-targeted mRNAs involved in the top10 enriched KEGG terms .

2.4 predicted DE-circRNAs were validated in gastric cancer and normal tissues

qRT-PCR detection of 7 DE-circRNAs in 60 tissue samples (30 GC tissues and 30 adjacent tissues). The expression of 7 DE-circRNAs (hsa_circ_0007991, hsa_circ_0013048, hsa_circ_0048602, hsa_circ_0050745, hsa_circ_0054971, hsa_circ_0059802, and hsa_circ_0067934) in GC tissues was significantly lower than that in control tissues (Figure 4-A).The expression of circ48607 was lower than that in normal tissue. (Figure 4-B).

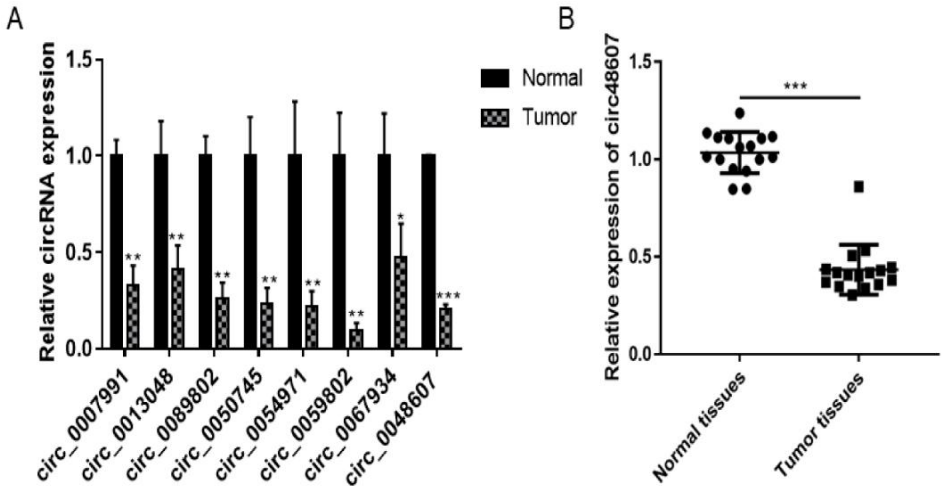

Figure 4. The confirmation of the sustained downregulation of DE-circRNAs in cancer tissues. A. The expression of DE-circRNA in GC and normal tissue B. The expression of circ48607 by qRT-PCR analysis. *P<0.05, ** P<0.01, ***P<0.001compared with normal group.

3. Discussion

GC is one of the malignant type of tumors that affects human health, accompanied by easy metastasis and recurrence. Because of its insidious onset and, patients often miss diagnosis in the early stage due to the absence of obvious symptoms [18]. Clinical tumor markers such as CA199, CEA and CA125 are not specific enough to be tested in combination. circRNA has been shown to play an indispensable regulatory role in tumor gene expression[19]. circRNAs→miRNAs "networks become more diverse" circRNAs→miRNAs→mRNA scomplex regulatory networks[20]. CircRNAs competitively combine with miRNA to reduce the inhibitory effect of miRNA on target gene mRNA, thus participating in a series of biological processes, including tumorigenesis and development[20, 21]. CircRNAs are differentially expressed in GC because of their diverse functions, strong conservatism, and higher stability compared to linear RNA. CircRNAs

are potential molecular markers for GC[22, 23]. Therefore, the circRNAs of scree ning co-expressed circRNAs from multiple chips and their targeted miRNA, ide ntification is expected to become a more stable and reliable molecular marker of GC.

Firstly, we downloaded 4 chips from the GEO database. A total of 7 D E-circRNAs (hsa_circ_0007991, hsa_circ_0013048, hsa_circ_0048607, hsa_circ_0 050745, hsa_circ_0054971, hsa_circ_0059802, and hsa_circ_0067934）were scre ened and showed that the expression of GC tissue was lower compared to that of adjacent tissue. We next validated if the 7 DE-circRNAs were expressed in clinical tissue samples by qRT-PCR. We found that the differential expression abundance of hsa_circ_0048607 was the highest. Secondly, KEGG/Reactome an alysis of the host gene of down-regulated DE-circRNA was performed in at lea st two chips and was enriched to include ERb signaling, mTOR signaling, and chemokine signaling cancer-related signaling pathways. Thirdly, a set of miRN As (GSE 26645) chip data was downloaded using miRanda to predict DE-circ RNAs targeting miRNAs. Seven DE-circRNAs can target 83 miRNAs. Seven miRNAs were expressed in GC tissues. Among them, miR-193a, and miR-1182 were tumor suppressor genes that were up-regulated in cancer tissues, and neg atively correlated with the expression of DE-circRNAs. MiR-193a targets are a bundant and highly enriched in ERb signaling and mTOR signaling of cancer-r elated pathways, which was highly consistent with the enrichment of the host gene where DE-circRNAs were located. In circRNA-miRNA-mRNA axis, most

significant KEGG-enriched signaling pathways originated from hsa_miR_543 an d hsa_miR_193a, which were mainly adsorb to hsa_circ_0048607, suggesting th at circ48607 may play an important role in the functional regulation of miR-19 3a.

In addition to hsa_circ_0048607, totally 7 DE-circRNAs were identified, w hich have not been reported yet. Liu et al.[24] showed that circRNAs expression profiles were obtained from GEO, and GEO2 tools were used for DE-circRN As. The application of circRNAs interaction network tools can predict the miR NA binding sites of De-circRNAs, while miRNA target genes were predicted u sing target scanning and miRNet database. A total of 3 circRNA, 6 miRNA an d 8 hub-genes were identified, and a circRNA-circRNA-hub-gene network was created. Among them, hsa_circ_0048607 was consistent with the results of our study. The research method was similar, which confirmed the identification of t his paper from another angle. At the same time, it also confirmed that the ana lyst has high accuracy in identifying the core nodes of the ceRNA network.

MiRNA participates in the proliferation, apoptosis, and invasion of GC cell s by regulating the expression of target genes[25]. We downloaded a set of miR NA chips (GSE26645), targeting miRNAs by using miRanda to predict DE-circ RNAs and found that miR-193a and miR-1182 were tumor suppressor genes th at were up-regulated in cancer tissues, and negatively correlated with DE-circR NA expression. Mature miRNAs are produced by precursor miRNAs (pre miR NAs)[26]. The total length is about 70 nucleotides, including a 5 p arm, a 3 p

arm, and a terminal cycle[27]. Ectopic expression of miR193a-3p and miR193a-5p inhibited the growth of GC cells. Chou et al[28] identified miR-193a-3p by bioinformatics to inhibit the growth and invasion of GC cells. The proliferation and invasion of GC AGS cells were inhibited by directly targeting the expression of miR-193a-3p and ETS1 in *in vitro*. Wang et al. [18] have clarified that hsa_miR_193a_5p the targeted mRNA regulated, it was found that it can regulate IGF2, and plays a vital role in the development of GC. Zuo identified Hsa_miR_193a_5p regulatory targeting mRNA and found that it can regulate IGF2, and plays a vital role in the occurrence and development of GC[29]. Wei et al. found FOXM1 involvement in GC progression through SIRT7/mTOR/IGF2 pathways[30]. The above findings suggested that the mRNA of miR-193a targeting in this study may be CCND1, ETS1 and IGF2, which provided a new idea for the study of circRNA-circRNA-mRNA [31], and its underlying mechanism of action needs to be further studied.

Analysis of enriched signaling pathways showed that miR-193a targets were abundant and highly enriched in cancer-related pathways, such as ERb signaling and mTOR signaling, which was highly consistent with the fact that DE-circRNAs host genes were enriched to estrogen receptor beta (ERb) signaling. ERb signaling is a nuclear receptor and its ligand is estrogen. When the ligand binds to the receptor, a homodimer is formed. Regulation of transcription with other cofactors. Output signals regulate endocrine signals and participate in endocrine and tumor-related diseases. mTOR signaling is an important part of the

PI3K/Akt/mTOR signaling[32]. After activation of the tyrosine kinase receptor, p hosphatidylinositol 3-kinase (PI3K) catalyzes phosphatidylinositol 3,4,5-trisphosp hate, leading to activation of 3-phos-phoinositide dependent protein kinase 1 (P DK1) and protein kinase B (PKB, also known as Akt). Then, Akt acts on seve ral downstream effector factors, such as mTOR and downstream molecules, the reby influencing growth, proliferation and apoptosis of tumor cells[33].

To sum up, this study predictes circRNA-targeted miRNA and enriched sig naling pathways through bioinformatics screening of DE-circRNA, and further s tudies the regulatory relationship of the miRNA-circRNA-mRNA. This can prov ide the basis for the early diagnosis of GC and the development of targeted dr ugs.

Conclusion: Seven down-regulated DE-circRNAs and 7 targeted miRNAs were identified in GC, which contributed to the understand of the underlying molec ular mechanism of GC and provided a basis for the early diagnosis of GC and the development of targeted drugs

4. Materials and Methods

4.1 The circRNA and miRNAs microarray data

Download GSE141977, GSE121445, GSE83521, GSE100170, GSE78092 and GSE89143 chip data was downloaded from GEO. GSE121445 and GSE14197 7 were unavailable due to sequencing data and unclear annotation information, respectively. CircRNA microarray data of 4 sets of GC were obtained: GSE100 170[34], GSE83521[35], GSE78092[36], and GSE89143[37] (Table1). One set of mi

RNA chip GSE26645 was downloaded (4 GC and adjacent tissues), and a total of 961 miRNAs were expressed.

Table 1. Expression of circRNAs.

GEO	platform	organization	time	journal	Number of samples (N/T)	circRNA detection number
GSE100170	GPL23259	Gastra	Aug,2017	Scientific Reports	5/5	88014
GSE83521	GPL19978	Gastra	2017	Aging-US	6/6	3071
GSE78092	GPL21485	Gastra	Sept, 2017	Molecular Medicine Reports	3/3	2901
GSE89143	GPL19978	Gastra	June, 2017	Cancer Medicine	3/3	2190

4.2 Identification of DE-circRNA, DE-miRNA, and Venn analysis

We used GEO2R (http://www.ncbi.nlm.nih.gov/geo/geo2r) and analyzed DE-circRNA and DE-miRNA. The DEG parameters of 4 sets of circRNA and 1 set microRNA were as follows: $p<0.05$, $FC>1.5$,). CircRNAs and down-regulated circRNA of co-expressed circRNA were analyzed by Venn analysis.

4.3 CircRNA combined miRNA forecasting analysis

We use miRanda (http://cbio.mskcc.org/microrna_data/manual.html) to predict miRNA circRNA targets. Moreover TargetScan (www.targetscan.org) was used to predict interactions between the selected miRNAs and messenger RNAs (mRNAs).

4.4 GO and KEGG pathway analyses

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genome (KEGG) pathway analysis were performed using DAVID (database for annotation, visualization, and integrated discovery) bioinformatics resources. The host mRNA of DE-circRNAs was used for enrichment pathway analysis, while KEGG and Reactome of targeted mRNA corresponded to prediction.

4.5 qRT-PCR verifies DE-circRNA expression in clinical samples

According to the manufacturer's instructions, TRIzol reagent was used to extract total RNA samples. Based on the seven circRNAs published in the circbase database (http：//www.circbase.org), primers were designed (see Table 2), and quantitative real-time fluorescence quantitative PCR (qPT PCR) was performed three times. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression

served as the internal control. Primers used for qRT-PCR analysis are shown in Table 2.

Table 2. QRT-PCR Primers.

circRNA name	Primer Information
circ_0007991	F: CTTCACTCCCGCTGTCTC
	R: TGGTTGGACCTGCATTCT
circ_0013048	F: CAGCAGAGCAGCTTTACC
	R: CCGTCCGACCATAGTTAG
circ_0059802	F: TGATGCTGACCCATTTCA
	R: CCAGTGCCACCAGTTTGT
circ_0050745	F: CAACATTGAGGCCAACGA
	R: CTGGGCAAAGAGTCTCCG
circ_0054971	F: CCATAGGCACTGAGATAGAA
	R: TGAAGAAACGGATGAGGT
circ_0067934	F: AAAACAAATTCGCATACCAC
	R: CAATCAACATTTCGGAAGAA
circ_0048607	F:GGGAAGGTGCCTATGGTG
	R: GGGACGAATGGCTGAGTA

4.6 Statistical analysis Data are expressed as the mean ± standard deviatio n and were processed using the statistical software SPSS 20.0 (SPSS Inc., Chi cago, IL, USA) and GraphPad Prism 6.0. Statistical comparisons were performe

d using one-way analysis of variance. Principal component analysis (PCA) anal ysis was performed by R package factoextra (https:// cloud.r-project.org/ pack age=factoextra) to show the clustering of samples with the first two component s.

Abbreviations

GC : Gastric cancer

DE-circRNAs:differentially expressed circRNAs

DE-miRNAs: differentially-expressed miRNAs

CEA : carcinoembryonic antigen

CA199 :Serum Antigen 199

CA125 :Serum Antigen 125

GEO Gene Expression Omnibus

GO: Gene Ontology

KEGG: Kyoto Encyclopedia of Genes and Genomes

Acknowledgements

Not applicable.

Funding

This study was supported by the National Natural Science Foundation of China () and Qinghai Provincial Scientific Research Project Foundation (2018-ZJ-735) .Qinghai university youth research fund project(2020-QYY-2)

Availability of data and materials

All dNo. 81760730ata generated or used during this study are included in this published article, still, further details are available from the corresponding auth or on reasonable request.

Authors' contributions

Qijing Guo and Yushuang Luo designed this study. Linglin Zhao, Dengfeng Re n, Yan Li, Xiaofeng Zhou , Siai Chen Shenyan Dang, and Xianliang Shen per formed the experiments and were involved in data analysis. Cuiping Guo, Jie Yang ,Xiao qian Chen and Xing zhang collected clinical samples and preforme d experiments. Qijing Guo drafted the manuscript. Yushuang Luo reviewed and edited the final version of the manuscript. All authors have read and approve d the final manuscript.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Qinghai University Affili ated Hospital. All patients signed informed consent to participate in the study .

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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