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Original Research

Hsa_circRNA_0059655 plays a role in salivary adenoid cystic carcinoma by functioning as a sponge of miR-338-3p

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Abstract: Circular RNAs(circRNA) are recently demonstrated to have a close relationship with tumors.To investigate the role of circular RNA in the pathogenesis of salivary adenoid cystic carcinoma(SACC), ten SACC tissues and paired normal submandibular gland(SMG) tissues were collected as the tumor group and the control group. Total RNA was extracted and then measured using ceRNA microarray (including mRNA, lncRNA, and circRNA) and miRNA microarray. Gene Ontology(GO) analysis and Kyoto Encyclopedia of Gene and Genomes (KEGG) pathway analysis were performed in order to investigate the function of the differential expressing genes. The ceRNA regulatory network was constructed to find the core circRNAs. Then the role of circRNA on proliferation was examined in the SACC cell line SACC-83 using CCK-8,qRT-PCR and western blotting, and its roles on migration and invasion were examined using wound healing assay and transwell assay. The results of the microarrays showed that 3792 mRNAs, 7649 lncRNAs, 11553 circRNAs, and 132 miRNAs expressed differentially. The ceRNA regulatory network analysis showed that hsa_circ_0059655 and other 14circRNAs derived from PYGB target on several similar genes by miR-338-3p.Among the 15 circRNAs derived from PYGB, hsa_circ_0059655has the most relationships in the ceRNA network. Furthermore, after hsa_circ_0059655 was knocked down in SACC-83 cells, the expression of hsa-miR-338-3p was up-regulated while CCND1was down-regulated. The proliferation, migration, and invasion of SACC-83 cells also decreased after hsa_circ_0059655 knock-downed.Taken together, the circRNAs derived from PYGB may regulate the tumorigenesis and development of SACC through competing with miR-338-3p.

Key words: Salivary adenoid cystic carcinoma; hsa_circRNA_0059655; ceRNA.

Introduction

Salivary adenoid cystic carcinoma (SACC) is one of the most common malignant tumors in salivary glands, which mainly occurs in small salivary glands and parotid glands, followed by submandibular glands(1). It is characterized by neurotropic, local invasion, and distal metastasis.The lung is the main targetfor the metastasis of SACC(2). Despite it grows slowly, SACC has a poor prognosis because of the high rate of invasion and distant metastasis.Therefore, further in-depth and systematic research is needed to improve effective therapies for patients with SACC.

The theory “competitive endogenous RNA (ceRNA)” was proposed by professor Pandolfi in 2011(3). This hypothesis suggests that some endogenous RNAs, including mRNAs, pseudogenes, lncRNAs can inhibit the function of target genes through competitively binding with miRNA. It provides a new perspective for the mechanism of tumorigenesis and development in the tumor.

CircRNA is a type of endogenous non-coding RNA with a covalently closed loop. Sanger first discovered single-stranded circular viruses in 1976(4). Then circRNAs were found in bacteria(5), yeast mitochondria(6), mouse testis, and human cells(7). With the development of sequencing technology, more and more circRNAs have been found in different species, tissues, cells, and even exosomes(8, 9). In 2013, Memczakand Hansen studied that some circRN Asabound with “miRNA response elements (MRE)”can act as “molecular sponges” to combine with miRNA. Which is to say, these circRNAs regulate gene expression and function through competitive combined with miRNA(7, 10, 11). More and more researches have shown that circRNA has an important impact on tumor development (12-18).

The stable structure of circRNA can make it easier to be measured. However, the role of circRNA in SACC has not been reported. In this study, we measured the expression of circRNAs in SACC, and explore the role of circRNA in the development of the tumor.

Materials and Methods

Human tissue samples and cell lines

We collected 10 pairs of SACC and paired SMG tissues from patients who received surgical treatment without preoperative chemotherapy or radiotherapy in Peking University Hospital of Stomatology. Samples were stored into liquid nitrogen immediately after surgical re-

section and pathologically confirmed. The research was conducted after we got informed consent of all involved patients. This research was approved by the Institution Review Board of Peking University Hospital of Stomatology (permit number: PKUSSIRB-201522040).

The human SACC cell line SACC-83 was established in our lab (19). Cells were cultured in RPMI 1640 (Gibco, California, US) with 10% fetal bovine serum (Gibco, California, US) and incubated in a humidified atmosphere of 5% CO^2 air at 37°C.

RNA extraction

Total RNA was extracted from the human sample tissues and SACC-83 cells using TRIzol (Invitrogen, California, US) following the manufacturer's instructions. Then the concentration and quality were measured by NanoDrop8000 (Thermo, California, USA). Besides, RNA was checked by Agilent Bioanalyzer 2100 (Agilent Technologies, California, US) with a RIN number inspecting RNA integration for microarray.

Microarray assay

The Shbio Human ceRNA microarray v1.0 (for mRNA/circRNA/lncRNA detection) (Shanghai Biotechnology, China) and the SurePrint Human miRNA microarray v21.0 (for miRNA detection) (Shanghai Biotechnology, China) were used to determine differentially expressed mRNA, lncRNA, circRNA and miRNA between SACC and SMG tissues. The fold change ≥ 2.0, $P< 0.05$ and FDR < 0.05 were recommended.

qRT-PCR

We performed qRT-PCR using FastStart Universal SYBR GreenMaster (ROX) reagent (Roche, Basel, Switzerland) on an ABI 7500 Sequence Detection System. The expression of mRNA and circRNA were normalized to GAPDH, the expression of miRNA was normalized to U6. All the results were represented as fold change using the 2-ΔΔCt method. Paired t-tests were applied and $P< 0.05$ was considered to be significant. The primers are shown in table 1.

ceRNA regulatory network analysis

Based on the expression values of genes, we established a network among mRNA, circRNA, and miRNA to identify their correlation through regression model analysis. Furthermore, the miRNAs must be correlated with mRNA and circRNA in the network through seed sequence matching analysis. The regulatory network of ceRNA was based on the theory of ceRNA that the circRNA competitively binds to the same miRNA with mRNA (3, 20, 21).

GO analysis, KEGG pathway analysis, and binding sites analysis

The host genes of circRNAs were obtained from circBase (http://www.circbase.org/). The potential miRNAs binding circRNAs were predicted based on the TargetScan, miRanda, PicTar, MirTarget2, and PITA. For function analysis, we conducted Gene Ontology (GO) analysis (http://www.geneontology.org/) and pathway analysis (http://www.genome.jp/kegg/) to annotate differential expressing circRNAs. The binding sites of circRNA and miRNA were conducted through Targetsacan.

RNA interference and cell transfection

The siRNAs (si-1, si-2) targeting the back-splice junction of hsa_circRNA_0059655 were designed and synthesized by RiboBio (Guangzhou, China). The sequence of si-1 was 5' TGTGGTTGGCTGAGTTCAA 3', the sequence of si-2 was 5'TTGGCTGAGTTCAAGGTGT 3'. SACC-83 cells were transfected using Lipofectamine 2000 (Invitrogen, CA, US) according to the manufacturer's protocol. Cells were harvested after 24h.

Cell proliferation, migration, and invasion assays

Cell proliferation was detected using CCK-8 reagents (Dojindo Laboratories, Kyushu Island, Japan) according to the manufacturer's instructions. Transfected cells and control cells were seeded in 96-well plates at 5000 cells/well with 100 μl media. After incubated with the CCK-8 reagent for 2h, the plates were measured at 450

Table 1. Primers in this article.

Gene	Forward primer	Reverse primer
GAPDH	CGACAGTCAGCCGCATCTT	CCAATACGACCAAATCCGTTG
ADIPOQ	CCCATTCGCTTTACCAAGAT	GGCTGACCTTCACATCCTTC
ACOX2	GTGACCCAGAGGCAAAGGT	AATGGCAGTGTAGGAGTGCTG
AQP7	TTGCCACCTACCTTCCTGAT	AGTGCTGGGTTGTTCTCCTG
BAMBI	AGACATCTGCCAAGCCAAAC	TGGGAGGAGAGAGAACATCG
BCL2	ATGTGTGTGGAGAGCGTCAA	GAGACAGCCAGGAGAAATCAA
CCL19	CCAATGATGCTGAAGACTGCT	TGGATGATGCGTTCTACCC
CCL21	GCCTCAAGTACAGCCAAAGG	GGGCAAGAACAGGATAGCTG
CCL5	GAAAGAACCGCCAAGTGTGT	GCAAGCAGAAACAGGCAAAT
CCND1	CCTGTCCTACTACCGCCTCA	TCCTCCTCTTCCTCCTCCTC
CCR7	TGTGGTCGTGGTCTTCATAGTC	TCGTAGGCGATGTTGAGTTG
U6	CTCGCTTCGGCAGCACA	CTCGCTTCGGCAGCACA
hsa_circ_0059655	TACAGCCTTTTATTGAGTGGGG	TGTTCCTGATGACCTTCTTGG
miR-338-3p	CGGTACGTCCAGCATCAGTGATT	ATCCAGTGCAGGGTCCGAGG
miR-338-3p RT primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACT GGATACGACCAACAA	

nm using an ELx808 Absorbance Microplate Reader (BioTek, Vermont, US).

For wound healing assay, a linear wound was made using a tip after the transfected cells and the control cells had been cultured in 6-well plates for 24h. The detached cells were moved by washing twice with D-Hank's media. Images were captured using an Eclipse TE2000-inverted microscope (Nikon, Tokyo, Japan).

For invasion assay, cells were seeded at 5000 cells/well with 200 µl FBS free media in the upper chamber of the 8.0 µm pore size Transwell inserts (Millipore, MA, US), which had been coated with Matrigel (BD Bioscience, NJ, US) for 4h. After incubation for 20h, cells on the upper surface were wiped off with a cotton swab. Cells on the bottom surface were fixed with 95% ethanol and stained with 1% crystal violet (solarbio, Beijing, China), and counted with a BX51 microscope (Olympus, Tokyo, Japan).

Western blot

Total protein was extracted using RIPA Lysis Buffer (Beyotime, China) according to the standard procedure, then separated on 12% SDS-PAGE gel and transferred to a PVDF (polyvinylidene fluoride) membrane. After being blocked for 2 h with 0.5% skim milk powder, the membrane was hybridization with monoclonal antibodies at 4°C overnight. The following primary antibodies were used: CCND1 (1:10000; cat. no. ab134175; 34 kDa), (Abcam), p53 (1:1000; cat. no. ab32389; 53 kDa), (Abcam). Equal protein sample loading was determined using anti-β-actin (ZSGB-Bio, China) for total protein lysates. The membrane was incubated with a secondary antibody anti-RabitIgG H&L (1:4,000; cat. no. ab205718) (Abcam) at RT (room temperature) for 1h and ECL detection followed after being washed three times. The bands were quantified using the Gel-Pro analyzer.

Statistical analyses

All statistical analyses were carried out using SPSS Version 19.0 software (IBM, New York, US). All numerical data were expressed as the mean ± standard deviation (SD) and compared by students' t-test unless otherwise noted. $P < 0.05$ was considered statistically significant.

Supplementary Materials

Supplementary materials can be found in this section.

Results

mRNA, circRNA, lncRNA, and miRNA expression profiles in SACC

Firstly, we performed the ceRNA microarray (including circRNA, mRNA, and lncRNA) and miRNA microarray to describe the circRNA, mRNA, lncRNA, and miRNA expression profiles in SACC.Scatter plots (Figure 1) showed the expression levels of circRNA,mRNA, lncRNA, and miRNA between SACC (group 1) and SMG (group 2) tissues. In total, 11553 circRNAs, 3792 mRNAs, 7649 lncRNAs and 132 miRNAs were detected to be differentially expressed with a fold change ≥ 2.0, $P< 0.05$ and FDR < 0.05. Among them, 6809 circRNAs, 2337mRNAs, 2915lncRNAs, and 73 miRNAs were up-regulated and 4744 circRNAs, 1455 mRNAs, 4734 lncRNAs, and 59 miRNAs were-down-regulated.Hierarchical clustering of circRNA, mRNA, lncRNA, and miRNA revealed the consistent results with scatter plots.

Validation of microarray by qRT-PCR

To verify the microarray results,qRT-PCR was conducted using the same samples as in microarrays. Ten mRNAs (BAMBI, BCL2, CCND1 ACOX2, ADIPOQ, AQP7, CCL19, CCL21, CCL5, and CCR7)were chosen randomly. As shown in Figure 2, the results of qRT-PCR were consistent with the microarray results,as three mRNAs were up-regulated while seven were down-regulated, demonstrating the high reliability of the profiles.

Gene ontology (GO) analysisand KEGG pathway analysis

To find the significant functions and pathways of the

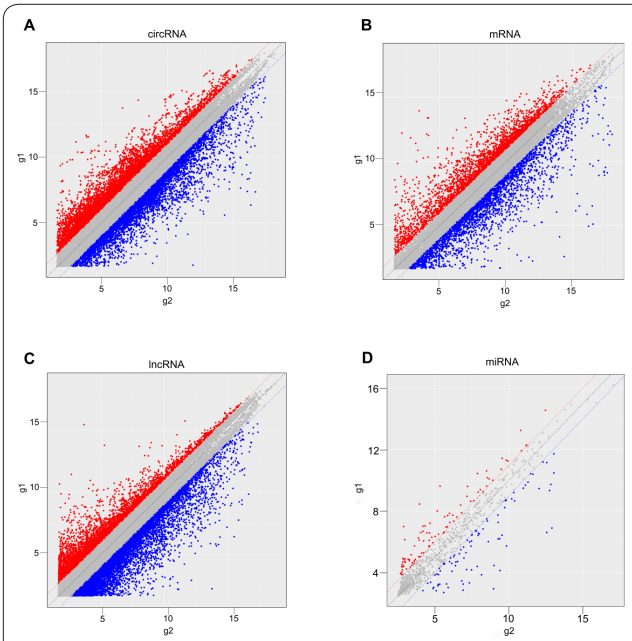

Figure 1. Differentially expression profiles of RNAs in SACC. The scatter plots were used for assessing the variation in circRNA(A), mRNA(B), lncRNA(C), and miRNA(D) between SACC and SMG tissues. The values of x and y axes were the normalized signal of the samples (log2 scaled). The dotted lines are fold-changed lines. The RNAs above the top dotted line and below the bottom dotted line indicated more than 2.0-fold change of RNAs between the two compared samples. g1= SACC group; g2 = SMG group.

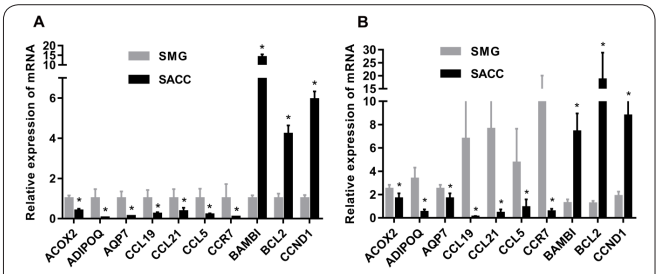

Figure 2. Validation of microarray profiles by qRT-PCR. (A) Relative expression of ACOX2, ADIPOQ, AQP7, CCL19, CCL21, CCL5, CCR7, BAMBI, BCL2, and CCND1 from microarray assay. (B) Relative expression of ACOX2, ADIPOQ, AQP7, CCL19, CCL21, CCL5, CCR7, BAMBI, BCL2, and CCND1 in SACC and SMG tissues with qRT-PCR.

differential expressing circRNAs, we performed GO analysis and KEGG pathway analysis.Figure 3A showed the top 30 items of GO enrichment. It revealed that the differential expressing circRNAs were enriched inDNA duplex unwinding, cell-substrate junction assembly, extracellular matrix component and other functions. Figure 3B showed the top 30 items of KEGG pathways, among which cell cycle, focal adhesion, and ECM-receptor interaction were significant. Based on the results, these functions and pathways were related with the differential expressing circRNAs and could contribute to the development of SACC.

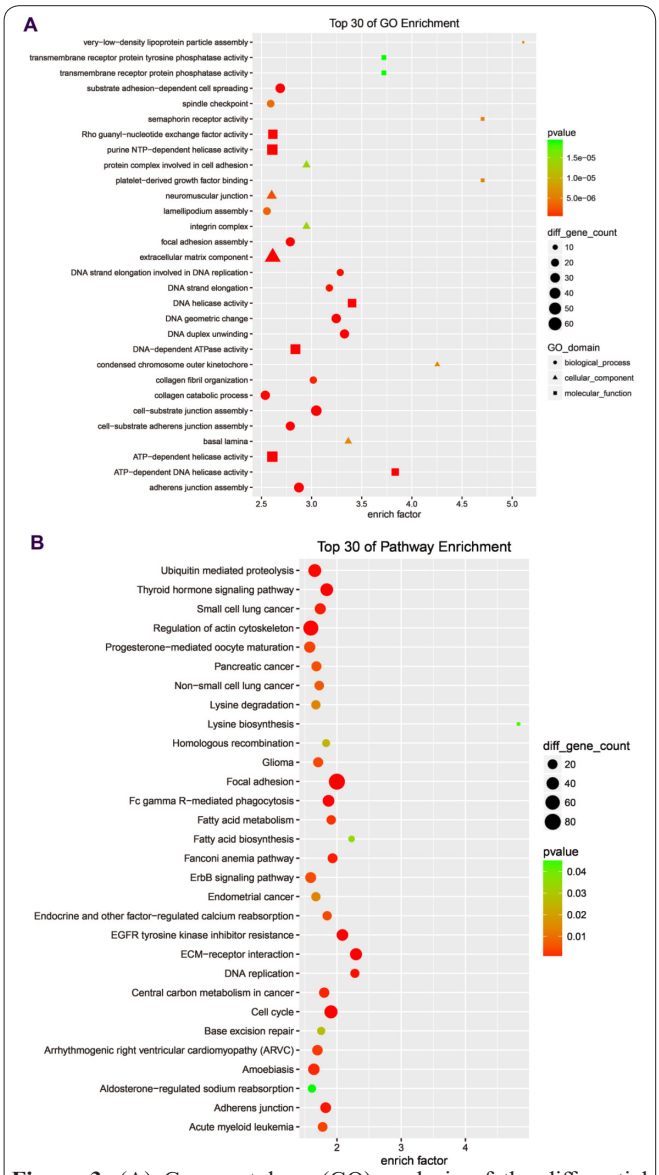

Figure 3. (A) Gene ontology (GO) analysis of the differential expressing circRNAs by microarray. GO annotation of biological process, cellular component, and molecular function with the top 30 enrichment factors. (B) KEGG pathway analysis of the differential expressing circRNAs by microarray.

Construction of ceRNA regulatory network and binding sites between hsa_circ_0059655 and miR-338-3p

We established a computational model based on the theory of ceRNA: supposing circRNAs competitively combined with miRNAs targeting mRNAs.Then the ceRNA regulatory network was constructed including 59 mRNA, 122 circRNA, and 10 miRNA according to the microarray results.

In the network, we found 15 circRNAs targeting some genes through the same miRNA, which was

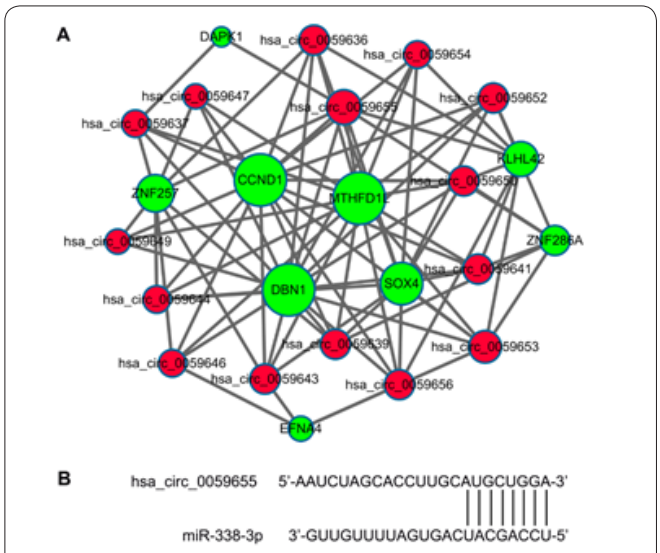

Figure 4. (A) CeRNA regulatory network of circRNAs from PYGB. red nodes = circRNAs, green nodes = mRNAs, gray lines = miR-338-3p. (B) Binding site of has_circ_0059655 and miR-338-3p by Targetscan.

Table 2.CircRNAs derived from PYGB target multiple genes through miR-338-3p.

circRNA	miRNA	mRNA	host gene
hsa_circ_0059636	hsa-miR-338-3p	DBN1 CCND1 MTHFD1L SOX4 KLHL42 ZNF257	PYGB (glycogen phosphorylase B)
hsa_circ_0059637		DAPK1 DBN1 CCND1 MTHFD1L ZNF257	
hsa_circ_0059639		DBN1 CCND1 MTHFD1L SOX4 ZNF257 ZNF286A	
hsa_circ_0059641		DBN1 CCND1 MTHFD1L SOX4 KLHL42 ZNF286A	
hsa_circ_0059643		DBN1 CCND1 MTHFD1L SOX4 EFNA4 ZNF257	
hsa_circ_0059644		DBN1 CCND1 MTHFD1L SOX4 ZNF257	
hsa_circ_0059646		DBN1 CCND1 MTHFD1L ZNF257 EFNA4	
hsa_circ_0059647		DBN1 CCND1 MTHFD1L ZNF257	
hsa_circ_0059649		DBN1 CCND1 MTHFD1L ZNF257	
hsa_circ_0059650		DBN1 CCND1 MTHFD1L SOX4 KLHL42 ZNF286A	
hsa_circ_0059652		DBN1 CCND1 MTHFD1L SOX4 KLHL42 ZNF286A	
hsa_circ_0059653		DBN1 CCND1 MTHFD1L SOX4 KLHL42 EFNA4 ZNF286A	
hsa_circ_0059654		DBN1 CCND1 MTHFD1L SOX4 KLHL42	
hsa_circ_0059655		CCND1 SOX4 MTHFD1L ZNF257 KLHL42 ZNF286A DAPK1 DBN1	
hsa_circ_0059656		DBN1 CCND1 MTHFD1L SOX4 KLHL42 EFNA4	

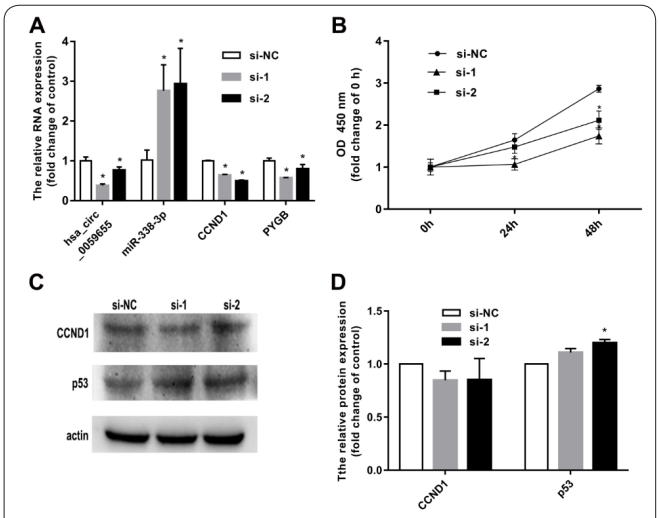

Figure 5. Knocking down of hsa_circ_0059655 inhibits the proliferation of SACC-83 cells. (A) qRT-PCR: the relative expression of hsa_circ_0059655, miR-338-3p and target genes in SACC-83 cells after knocking down hsa_circ_0059655 with siRNAs. (B) CCK-8 assay. (C,D) WB: the relative expression of CCND1 and p53.

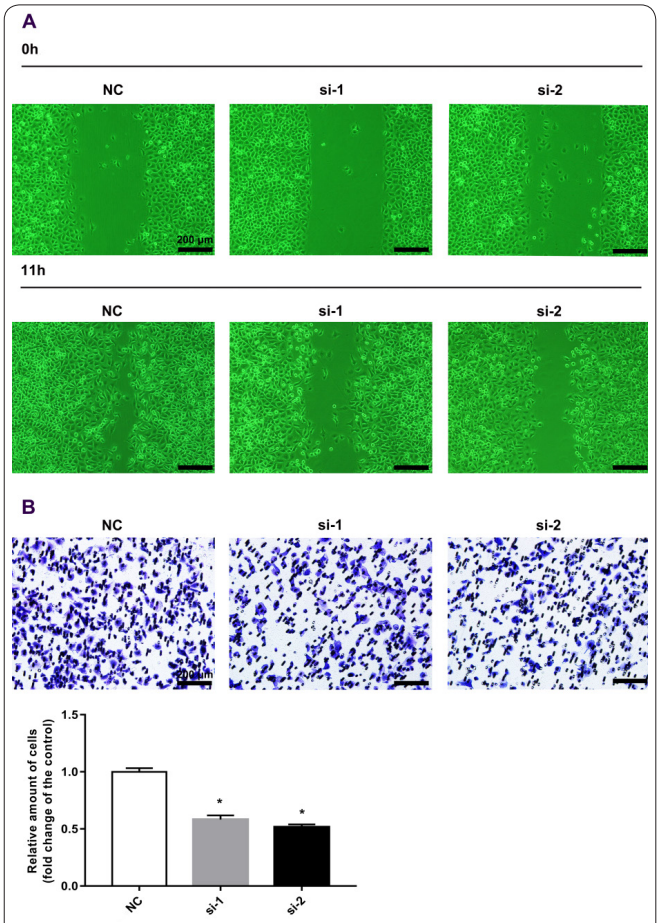

Figure 6. Knocking down of hsa_circ_0059655 inhibits the migration and invasion of SACC-83 cells. (A) Wound healing assay for SACC-83 cells after knocking down hsa_circ_0059655. (B) Invasion assay for SACC-83 cells after knocking down hsa_circ_0059655.

miR-338-3p (Table 2). What's interesting was that all the 15 circRNAs were derived from the same gene PYGB after searching the circBase.From the enlarged network(Figure4), we got that the target genes were CCND1, SOX4, MTHFD1L, ZNF257, KLHL42, ZNF286A, DAPK1, DBN1, and EFNA4. The relationships were listed in Table 2. Among the 15 circRNAs, the target genes of hsa_circ_0059655 were the most, indicating that hsa_circ_0059655 had an important effect on SACC. Then we validated the binding sites between hsa_circ_0059655 and miR-338-3p by Targetscan. Thus we would detect the function of hsa_circ_0059655 in the development of SACC.

Knocking down hsa_circ_0059655 inhibits the proliferation of SACC-83 cells

We knocked down the expression of hsa_circ_0059655 in SACC-83 cells with two siRNAs(Figure 5A), and the result of qRT-PCR showed the expression of miR-338-3p was down-regulated and the expression of CCND1 was up-regulated after hsa_circ_0059655 was knock-downed.CCK-8 was used for detecting the proliferation of SACC-83 cells after transfection for 24h. Figure 5B showed that the proliferation of SACC-83 cells was inhibited after hsa_circ_0059655 was knock-downed.The results of western blotting showed CCND1, p53 were up-regulated (Figure 5C, 5D).

Knocking down hsa_circ_0059655 inhibits the migration and invasion of SACC-83 cells

Wound healingassay results demonstrated that knockdown of hsa_circ_0059655 by siRNAs inhibited the migrationof SACC-83 cells (Figure 6A). Moreover, knockdown of hsa_circ_0059655 also inhibited cell invasion (Figure 6B).

Discussion

SACC is one of the most common malignant tumors in the oral and maxillofacial region, and it is characterized by neural invasion, distant metastasis, and recurrence. CircRNA is a class of RNAs with the closed ring structure, and it is commonly found in eukaryotic cells. CircRNA is well conserved among species, but it is specific in tissues and cells(22). CircRNA is not easily degraded by RNA enzyme because it is lack of poly-A tail(23). CircRNA can also be detected in blood, saliva, and exosomes, suggesting that circRNA may play an important role in cell information transfer and tumor metastasis.

In order to explore the role of circRNA in the pathogenesis and development of SACC, 10 pairs of SACC and SMG tissues were collected. CeRNA and miRNA microarrays were used to detect the expression of RNAs. Bioinformatics was used to analyze the microarray data. It was found that circRNAs derived from PYGB gene acted on different target genes through miR-338-3p. SiRNA was then used to knock down hsa_circ_0059655 in SACC-83 cells. It was found that the ability of cell proliferation, invasion and migration was significantly decreased, which suggested that circRNA played an important role in the proliferation, invasion, and migration of SACC.

Hsa_circ_0059655 from PYGB is located on human chromosome 20: 25276239-25278648 and its splice length is 1710 BP(22). According to our data, hsa_circ_0059655 has a binding site of miR-338-3p (Figure 4B).MiR-338-3p is located on chromosome 17 and plays an important role in tumors. In non-small cell lung cancer and other tumors, miR-338-3p affects the proliferation, invasion, and migration of tumors by acting

on different target genes (24-29). Xiaoyu Fu et al detected the 3'end of CCND1 by TargetScan, finding that it contains two binding sites of miR-338-3p.Using double luciferase assay in hepatocellular carcinoma cells,they revealed that 2397-2403 nt of CCND1 was the binding site of miR-338-3p. The inhibition of miR-338-3p on the expression of CCND1 resulted in a decrease in cell proliferation (28, 30).

In this study, we got the ceRNA regulatory network, in which 15 circRNAs from PYGB targeted on CCND1 and other genes through the same miRNA which is miR-338-3p. Previous studies have suggested that both the parent gene PYGB and the target gene CCND1 play an important role in cancer (31-33). Simultaneously, miR-338-3p plays an important role in tumors (34, 35), and can be bound to hsa_circ_0059655 according to our results. From our research, we found that the expression of miR-338-3p was up-regulated after hsa_circ_0059655 was knocked down, but the target gene CCND1 was down-regulated, which was consistent with the expression pattern of ceRNA. These suggest that hsa_circ_0059655 / miR-338-3p / CCND1 could affect the occurrence and development of SACC. The conclusion provides a new idea for the study of SACC.

Studies showed that circRNA may act on its parent gene and affect the disease process(36). The parent gene PYGB is one of the three subtypes of glycogen phosphorylase. With the increase of tumor malignancy, PYGB can promote cell proliferation, inhibit apoptosis, and enhance the migration and tumorigenesis of tumor cells. It is highly expressed in human colorectal cancer cells (37).Detection of PYGB during gastric biopsy may predict the occurrence and development of new lesions after local treatment of early gastric cancer (38, 39). These suggest that PYGB has the function of malignant transformation of cells (40). The relationship of hsa_circ_0059655 and PYGB is deserved to be explored in the future.

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Conflicts of Interest

The authors declare no conflict of interest.

Author Contributions

Conceptualization, XiyuanGe and Shenglin Li; Data curation, Fei Zhao, Chuwen Chen, Wenwen Yang, LihuaXu and Zhihao Du; Funding acquisition, XiyuanGe and Shenglin Li; Methodology, Fei Zhao, Chuwen Chen, Wenwen Yang, LihuaXu and Zhihao Du; Project administration, XiyuanGe and Shenglin Li; Supervision, XiyuanGe and Shenglin Li; Validation, Fei Zhao; Writing – original draft, Fei Zhao; Writing – review & editing, XiyuanGe and Shenglin Li.

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