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ORIGINAL ARTICLE

Hsa_circ_0065149 is an Indicator for Early Gastric Cancer Screening and Prognosis Prediction

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Abstract

Circular RNAs (circRNAs) are an endogenous RNAs with a covalently closed cyclic structure. They have emerged recently as key regulators in the development and progression of human cancers. However, the clinical values of most circRNAs in gastric cancer (GC) are unknown. Hsa_circ_0065149, one of the dysregulated circRNAs in gastric carcinogenesis detected by circRNA microarray, was chose as a targeted circRNA in this study. We firstly enlarged sample size and identified the level changes of hsa_circ_0065149 among four stages of gastric tumorigenesis from healthy gastric mucosa, gastritis, intestinal metaplasia to GC. Then, the potential relationship between hsa_circ_0065149 expression levels and GC patients' clinicopathological factors was investigated. Moreover, the clinical significance of hsa_circ_0065149 in plasma exosomes and gastric juice were explored. Receiver operating characteristic (ROC) curve and Kaplan-Meier survival curve were constructed to evaluate diagnostic and prognostic values. Finally, bioinformatics analysis was performed to excavate the potential functions of hsa_circ_0065149. Hsa_circ_0065149 expression was only significantly down-regulated in gastric cancer, not changed among healthy gastric mucosa and gastritis intestinal metaplasia. Low hsa_circ_0065149 expression levels in GC tissues were significantly associated with tumor diameter ($P = 0.034$) and perineural invasion ($P = 0.037$). GC patients with low hsa_circ_0065149 levels had a much longer overall survival than those in high group ($P = 0.020$). More important, hsa_circ_0065149 levels were significantly decreased in plasma exosomes of early GC patients. As a screening biomarker for early GC, hsa_circ_0065149 in plasma exosomes has higher sensitivity and specificity than traditional clinical biomarkers. Bioinformatics analysis suggest that the abnormal expression of hsa_circ_0065149 may play an important role during gastric carcinogenesis. Those results indicate that hsa_circ_0065149 in exosmoes is an indicator for early GC screening and prognosis prediction.

Keywords CircRNA · Hsa_circ_0065149 · Exosomes · Gastric juice · Biomarker

Introduction

Gastric cancer (GC) is one of the most common malignancy, and ranks the third leading cause of cancer death in the world [1]. Despite the improvement of screening techniques and early intervention, the estimated mortality rate of GC is still high due to cancer metastasis and recurrence, particularly in advanced patients [2, 3]. Possible explanations for such phenomenon may include indistinct molecular mechanisms of GC and lack of tumor-specific molecular targets. Thus, it will be of great significances to identify novel molecular targets for GC.

Circular RNA (circRNA) is an endogenous RNA with a covalently closed cyclic structure [4]. Recently years, more

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functional circRNAs have been identified by new RNA-sequencing technology [5]. According to their origination, circRNAs can be divide into exonic circRNAs, intronic circRNAs and exon-intron circRNAs [6]. Importantly, due to their covalently closed loop structures, circRNAs are more resistant to RNA exonuclease than linear RNA, which endows them with many potential functions and applications [6–8]. Furthermore, emerging evidence has been indicated that dysregulated circRNAs were involved in regulating tumorigenesis, providing novel perspective in therapeutic targets [9].

Hsa_circ_0065149 is one of the dysregulated circRNAs in gastric carcinogenesis detected by our circRNA microarray (data accessible at NCBI GEO database, accession GSE89143, Guo, 2016; https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE89143) [10]. Its gene is named SETD2 (set domain containing 2) and located at chr3:47098310–47,103,836, with 854 nucleotides in final spliced sequence. However, the potential role and clinical significance of hsa_circ_0065149 in gastric carcinogenesis is still unknown. In this study, we firstly enlarged sample size and identified hsa_circ_0065149 expression level changes among four stages of gastric tumorigenesis from healthy gastric mucosa, gastritis, intestinal metaplasia to GC. Then, the potential relationship between hsa_circ_0065149 expression levels and GC patients' clinicopathological factors was investigated; and the clinical significance of plasma exosome and gastric juice hsa_circ_0065149 from different stages of gastric tumorigenesis were explored. Moreover, receiver operating characteristic (ROC) curve and Kaplan-Meier survival curve were constructed to evaluate its diagnostic and prognostic values. Bioinformatics analysis was performed to excavate the potential functions of hsa_circ_0065149. Our results showed that downregulated expression of hsa_circ_0065149 is an indicator for prognosis prediction and early gastric cancer (EGC) screening.

Materials and Methods

Clinical Specimens

All tissue samples, plasma and gastric juice were collected from the cancer centers for gastroenterology, the Affiliated Hospital of Medical School of Ningbo University, between January 2012 and December 2017. A total of 96 paired gastric cancer tissues and non-tumorous tissues were collected from surgical patients. The adjacent normal tissues were 5 cm away from the margins of the tumor. Furthermore, 29 healthy gastric mucosa, 40 gastritis and 15 intestinal metaplasia were collected by biopsy specimens. All specimens were immediately stashed in RNA fixer (Bioteke, Beijing, China) at −80 °C until use. Peripheral blood was obtained from 41 healthy volunteers and 39 early gastric cancer patients who were diagnosed as locally cancerous by pathology. Gastric juice samples were obtained from 22 healthy volunteers, 24 gastric ulcer patients, 12 chronic atrophic gastritis patients, and 16 GC patients. Separation steps of plasma were following previously description [10, 11].

Tumor clinical stages and histological grades were assessed according to Tumor-Node-Metastasis (TNM) staging system (7th ed.) and National Comprehensive Cancer Network clinical practice guideline of oncology (V.1.2012), respectively. Written informed consent was obtained from all subjects. The Human Research Ethics Committee of Ningbo University approved all aspects of this study (IRB No. 20120303).

Plasma Exosome Isolation

Plasma exosomes were isolated by total exosome isolation reagent (Invitrogen, Karlsruhe, Germany). The operating steps and conditions were following the manufacturer's instructions.

RNA Extraction and Reverse Transcription

Tissue RNA and exosome RNA were extracted using TRIzol reagents (Ambion, Carlsbad, CA, USA). Gastric juice total RNA were extracted using TRIzol LS reagents (Ambion). Then, total RNA was reverse transcribed to cDNA by GoScript Reverse Transcription (RT) System (Promega, Madison, WI, USA) following the manufacturer's instruction [10].

qRT-PCR Detection

Quantitative real time RT-polymerase chain reaction (qRT-PCR) analyses were performed with the GoTaq qPCR Master Mix (Promega) on an Mx3005P Real-Time PCR System (Stratagene, La Jolla, CA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used to normalize the level of the circRNA. Primers for hsa_circ_0065149 and GAPDH were synthesized by Sangon Biotech (Shanghai, China). Their sequences were as follows: 5′-TGTCCAACAGTCTATGGTGTGA-3′and 5′-GTGAGAGGGAGCTTCTTCGTT-3′for hsa_circ_0065149; 5′-ACCCACTCCTCCACCTTTGAC-3′ (sense) and 5′-TGTTGCTGTAGCCAAATTCGTT-3′ (antisense) for GAPDH. ΔC_t method was used to normalize circRNA levels. All results were expressed as the Mean ± SD through at least two independent experiments.

Prediction for hsa_circ_0065149 Pathways

Hsa_circ_0065149/microRNA (miRNA) interaction was predicted with Arraystar's home-made miRNA target prediction software based on TargetScan and miRanda [12, 13]. Gene ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis were carried out

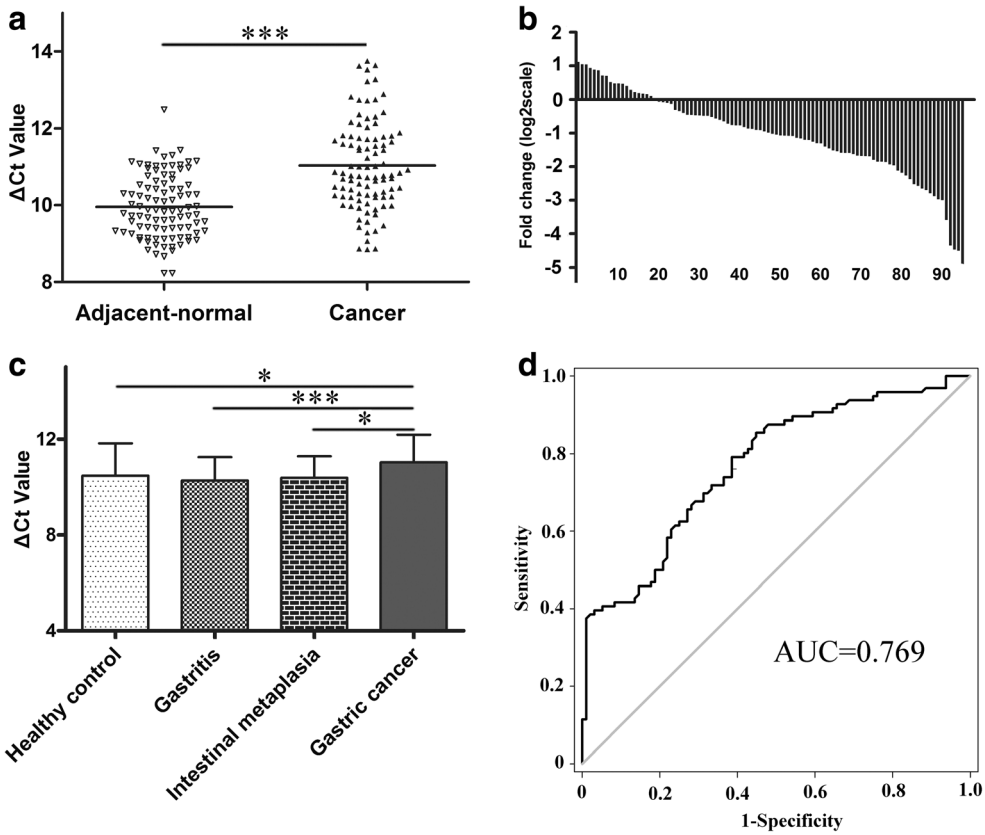

Fig. 1 Hsa_circ_0065149 expression levels in gastric cancer tissues. a The expression levels of hsa_circ_0065149 in cancer tissues ($n = 96$) and adjacent normal tissues ($n = 96$). Higher ΔC_t value indicates lower expression. **b** Hsa_circ_0065149 expression level was significantly down-regulated in 80.21% (77/96) gastric cancer tissues compared with the adjacent normal tissues. **c** Hsa_circ_0065149 expression levels in tissues from various stages of gastric carcinogenesis. Hsa_circ_0065149 was only significantly decreased in gastric cancer tissues ($n = 96$) compared with healthy controls ($n = 29$), gastritis ($n = 40$) and intestinal metaplasia ($n = 15$). (D) ROC curve of hsa_circ_0065149 in differentiating gastric cancer tissues from controls. The area under the curve was up to 0.769. Data are means ± SD of two independent experiments. Asterisks represents significant statistical difference (***$P < 0.001$, *$P < 0.05$)

based on DIANA-miRPath [14]. Network map was drawn by Cytoscape Software. The common downstream targets of miRNAs were displayed by using Venny 2.1 (http://bioinfogp.cnb.csic.es/tools/venny/). $P < 0.05$ was used as the criterion for statistical significance.

Statistical Analysis

Statistical analysis were performed by Statistical Program for Social Sciences (SPSS) 20.0 Software (SPSS, Chicago, IL, USA) and GraphPad Prism 5.0 (GraphPad Software, CA, USA). Student's *t* test, one-way analysis of variance (ANOVA) were flexibly used according to actual conditions. ROC and Kaplan-Meier survival plot were established to evaluate diagnostic and prognostic values. $P < 0.05$ was deemed as statistically significant.

Results

Hsa_circ_0065149 Expression Was Down-Regulated in Gastric Cancer

By using qRT-PCR, we firstly detected expression levels of hsa_circ_0065149 in 96 GC tissues and paired adjacent normal tissues. The results demonstrated that hsa_circ_0065149 was significantly downregulated in GC tissues ($P = 0.001$; Fig. 1a). Among them, the lower expression samples account for more than 80.21% (Fig. 1b). Then, we investigated hsa_circ_0065149 expression levels in tissues from four stages of gastric tumorigenesis. Our results showed that hsa_circ_0065149 expression levels were only reduced in gastric cancer stage (Fig. 1c). Nevertheless, hsa_circ_0065149 levels were not significantly changed among healthy gastric mucosa, gastritis and intestinal metaplasia.

Moreover, ROC curve was constructed to investigate the potential values of hsa_circ_0065149. The area under the ROC curve (AUC) was up to 0.769 (95% confidence interval [CI], 0.704–0.835; $P < 0.001$; Fig. 1d). The cut-off value, sensitivity and specificity were 10.19, 79.2% and 61.5%, respectively (Fig. 1d).

Potential Prognostic Values of hsa_circ_0065149 in GC

Next, we analyzed its levels with clinicopathological features of GC patients. As shown in Table 1, hsa_circ_0065149 levels in GC tissues were significantly associated with tumor diameter ($P = 0.034$) and perineural invasion ($P = 0.037$).

To further evaluated the prognostic significance of hsa_circ_0065149 in GC, GC patients were classified as two groups, low or high group, according to the hsa_circ_0065149 expression levels in cancer tissues. Interestingly, the results showed that GC patients with low hsa_circ_0065149 levels

Table 1 Relationship of Hsa_circ_0065149 expression levels (ΔC_t) in cancer tissues with clinicopathological factors of gastric cancer patients

Characteristics	No. of case (%)	Mean ± SD	*P* value
Age (y)			
≥60	61 (63.5)	11.095 ± 1.101	0.454
<60	35 (36.5)	10.911 ± 1.243	
Gender			
Male	65 (67.7)	11.114 ± 1.106	0.292
Female	31 (32.3)	10.848 ± 1.241	
Tumor location			
Sinuses ventriculi	49 (51.1)	11.071 ± 1.204	0.425
Cardia	10 (10.4)	11.042 ± 0.964	
Corpora ventriculi	25 (26.0)	10.754 ± 1.058	
Others	12 (12.5)	11.413 ± 1.263	
Diameter (cm)			
≥5	47 (49.0)	11.282 ± 1.235	0.034
<5	49 (51.0)	10.785 ± 1.021	
Differentiation			
Well	12 (12.5)	10.866 ± 1.059	0.345
Moderate	47 (49.0)	11.204 ± 1.064	
Poor	37 (38.5)	10.857 ± 1.277	
Stage			
Early	24 (25.0)	11.155 ± 1.050	0.535
Advanced	72 (75.0)	10.986 ± 1.188	
Borrmann type			
I&II	19 (26.4)	11.117 ± 1.152	0.578
III&IV	53 (73.6)	10.939 ± 1.208	
Pathologic diagnosis			
Signet ring cell cancer	15 (15.6)	10.534 ± 1.173	0.070
Adenocarcinoma	81 (84.4)	11.119 ± 1.132	
Invasion			
T_1&T_2	36 (37.5)	11.137 ± 1.065	0.474
T_3&T_4	60 (62.5)	10.962 ± 1.205	
Lymphatic metastasis			
N_0	38 (39.6)	10.872 ± 1.073	0.286
$N_{1–3}$	58 (60.4)	11.130 ± 1.199	
Distal metastasis			
M_0	82 (85.4)	11.042 ± 1.121	0.774
M_1	14 (14.6)	10.946 ± 1.365	
Venous invasion			
Absent	53 (55.2)	11.006 ± 1.168	0.833
Present	43 (44.8)	11.056 ± 1.145	
Perineural invasion			
Absent	47 (49.0)	11.356 ± 0.989	0.037
Present	49 (51.0)	10.809 ± 1.260	
CEA (Tissue)			
Positive	74 (77.1)	11.124 ± 1.121	0.134
Negative	22 (22.9)	10.704 ± 1.220	
CA19–9 (Tissue)			
Positive	54 (56.3)	11.161 ± 1.112	0.202
Negative	42 (43.7)	10.857 ± 1.193	

CEA carcinoembryonic antigen, *CA19–9* carbohydrate antigens

had a much longer overall survival than those in high group ($P = 0.020$; Fig. 2).

Clinical Diagnostic Values of hsa_circ_0065149 in Plasma Exosomes

To confirm whether hsa_circ_0065149 exists in human plasma exosomes, we sequenced the qRT-PCR products of plasma exosomes. As expected, we found their sequencing results were completely consistent with the sequence in circBase (http://circrna.org/) (Supplementary Fig. 1). DNA sequence confirmed the existence of hsa_circ_0065149 in plasma exosomes. Then, exosomes from 41 healthy volunteers and 39 EGC patients were isolated and quantified by qRT-PCR. As shown in Fig. 3a, hsa_circ_0065149 levels in plasma exosomes from patents with EGC were significantly decreased than those from healthy control ($P < 0.001$).

ROC curve was constructed to investigate the potential values of plasma hsa_circ_0065149 as a biomarker for EGC screening. As showed in Fig. 3b, the AUC was up to 0.640 (95% CI, 0.509–0.771; $P = 0.031$). The cut-off value, sensitivity and specificity were 6.43, 48.7% and 90.2%, respectively.

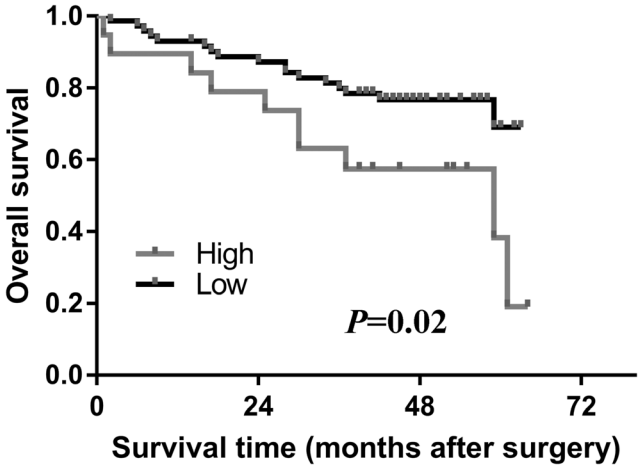

Fig. 2 Kaplan-Meier survival plot. Gastric cancer patients with low hsa_circ_0065149 levels had a much longer overall survival than those in high group ($P = 0.020$)

Clinical Diagnostic Values of hsa_circ_0065149 in Gastric Juice

After confirming the existence of hsa_circ_0065149 in gastric juice by DNA sequence (Supplementary Fig. 2), we detected and compared gastric juice hsa_circ_0065149 levels in healthy volunteers, gastric ulcer patients, chronic atrophic gastritis patients, and GC patients. Unexpectedly, maybe due to small number of samples tested, hsa_circ_0065149 levels have no significant difference among four groups ($P = 0.448$; Supplementary Fig. 3).

Annotation for hsa_circ_0065149 Function

To excavate the potential functions of hsa_circ_0065149, its interaction with miRNAs was predicted. Hsa_circ_0065149 was showed to harbor hsa-miR-197-5p, hsa-miR-222-3p, hsa-miR-330-5p and hsa-miR-486-3p seed sequences

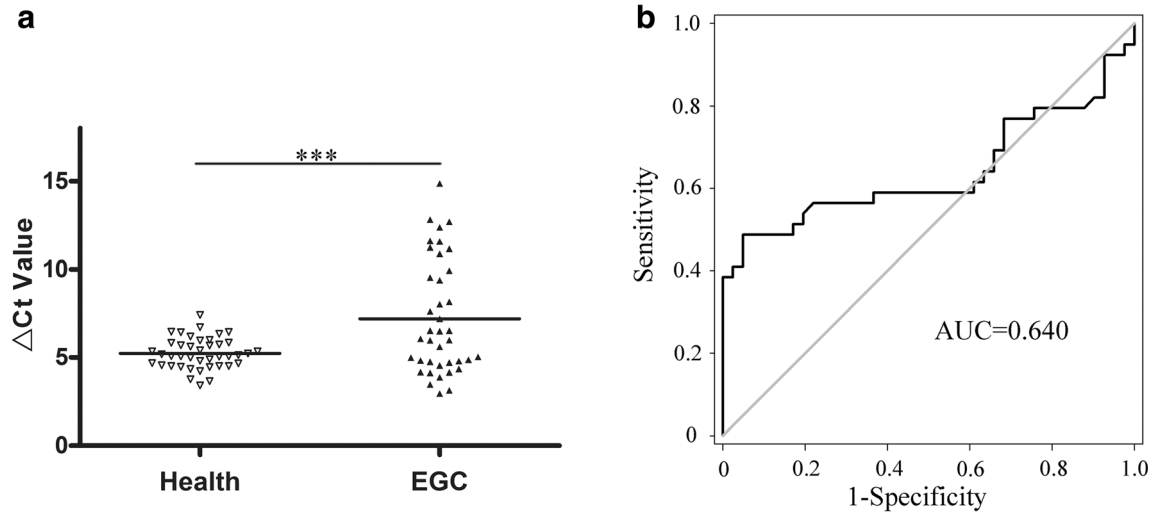

Fig. 3 Clinical diagnostic values of hsa_circ_0065149 in plasma exosomes. a Hsa_circ_0065149 levels in plasma exosomes were significantly decreased in patients with EGC ($n = 39$) than those in healthy control ($n = 41$) (***$P < 0.001$). **b** ROC curve was constructed to investigate the potential values of plasma hsa_circ_0065149 as a biomarker for EGC screening. The AUC was up to 0.640 (95% CI, 0.509–0.771; $P = 0.031$). The cut-off value, sensitivity and specificity were 6.43, 48.7% and 90.2%, respectively

(Fig. 4a). A network map comprising hsa_circ_0065149, four miRNAs and their downstream targets was presented in Fig. 4b generated by using Cytoscape. Venn diagram revealed the number of common downstream targets of four miRNAs (Fig. 4c).GO and KEGG pathway analyses of those target genes showed that they were involved in a variety of biological functions and signaling pathways (Fig. 4d-e).

Discussion

Recently, researchers devote more attention to interpret the molecular mechanisms of GC. Utilization of high-throughput sequencing can be efficient strategies to identify aberrantly expressed circRNAs [7, 15, 16]. As a new study hotspot, circRNAs play a vital role in GC development and may be regarded as potential biomarkers. For example, by performing circRNA expression profile, our previous study have discovered several dysregulated circRNAs and further verified by qRT-PCR [10]. Importantly, we demonstrated that some circRNAs such as hsa_circ_0014717 could be stably exist in gastric juice, which met the need of clinical detection [10]. Lately, Li et al. showed the cell-free circRNAs profile in plasma of GC patients [17]. Two circRNAs, hsa_circ_0001017 and hsa_circ_0061276, were found associated with distal metastasis; and AUC in combinative use of them could reach to 0.966 (specificity and sensitivity were 95.7% and 95.5%) [17]. All these results suggested that dysregulated circRNAs are involved in gastric tumorigenesis and could be valuable blood-based biomarkers for cancer screening.

Following our previously bioinformatics analysis, the dysregulated hsa_circ_0065149 was one of circRNAs associated with GC. In this study, we firstly investigated its expression in the tissues from different stages of gastric tumorigenesis. We found that hsa_circ_0065149 in GC were remarkably reduced than that in other three types of tissues (Fig. 1c), whereas there was no difference of its levels among healthy gastric mucosa, gastritis, and intestinal metaplasia. As an indicator for tissue identification, the sensitivity and specificity of hsa_circ_0065149 can be achieved to 79.2% and 61.5%, respectively. Our data emphasized that hsa_circ_0065149 are closely associated with gastric cancerogenesis and can be a specific indicator for GC.

Several clinicopathologic features such as tumor size and perineural invasion are independent prognostic factors in GC [18, 19]. Tumor size affects the survival rate of GC patients; and the poorer prognosis often combined with larger tumor size [20, 21]. By presented a systemic review and meta-analysis, Deng et al. suggested that perineural invasion could be an independent prognostic factor affecting overall survival (OS) of GC patients who had received the curative resection [19]. Moreover, Zhou et al. indicated that perineural invasion predicted a poor outcome in GC [22]. In our study, we found hsa_circ_0065149 levels were not only associated with tumor size ($P = 0.034$) but also associated with perineural invasion ($P = 0.037$) (Table 1). Moreover, GC patients with low hsa_circ_0065149 levels had a much longer overall survival than high group in Kaplan-Meier survival plot analysis (Fig. 2). Theses implied that downregulated hsa_circ_0065149 is an indicator for prognosis of patients with GC.

The commonly used blood-based biomarkers, such as carcinoembryonic antigen (CEA) and carbohydrate antigens 19–9 (CA19–9), do not have satisfactory sensitivity for GC detection especially in early stage [17, 23]. For EGC screening, the positive rate of CEA, CA19–9, and CA125 level were 4.3%, 4.8%, and 1.9%, respectively; and the highest positive rate was only 10.4% for combination of all markers

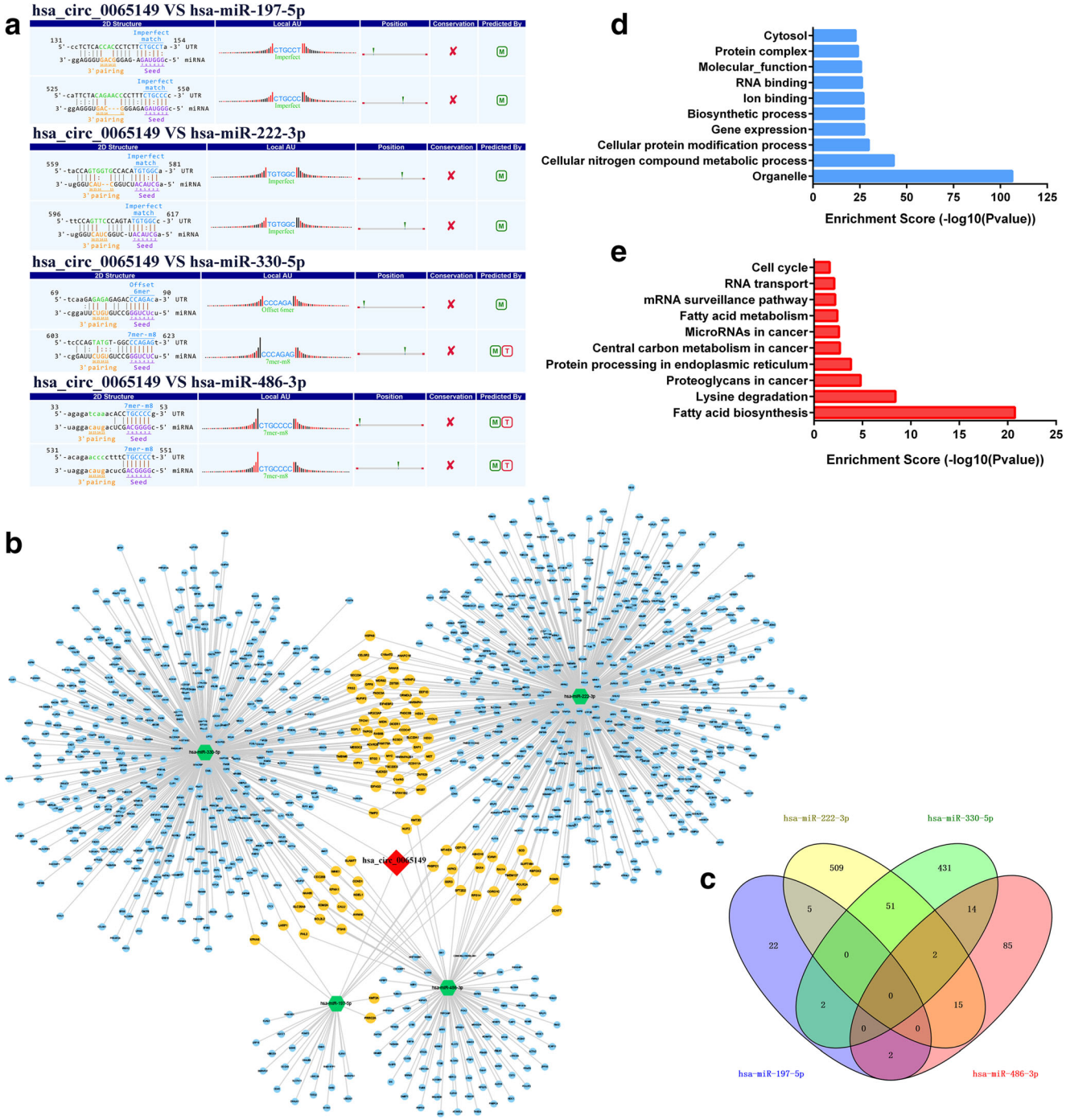

Fig. 4 Prediction for hsa_circ_0065149 pathways. a Prediction for hsa_circ_0065149/miRNAs interactions. **b** A network map comprising hsa_circ_0065149, four miRNAs and their downstream targets was presented. **c** Venn diagram revealed the number of common downstream targets of four miRNAs. **d** The hsa_circ_0065149/miRNAs related GO analysis. **e** The hsa_circ_0065149/miRNAs related KEGG pathway analysis

[23]. The traditional clinical markers cannot satisfy the diagnosis of EGC.

Tumor-derived exosomes, present in all body fluids, are emerging as a new type of cancer biomarker [24]. Exosomes harbor diverse types of nucleic acids such as DNA fragment and RNA [25, 26]. They are currently considered as potentially promising candidates for diagnosis, prognosis and therapeutic responses in cancer [24]. In this study, whether hsa_circ_0065149 exists in plasma exosomes and plasma exosomes hsa_circ_0065149 have unique advantage for EGC screening are not clear. Thus, we isolated total exosomes from plasma and sequenced the qRT-PCR products of

hsa_circ_0065149 in plasma exosomes. Based on sequencing results, we confirmed the existence of hsa_circ_0065149 in exosomes (Supplementary Fig. 1). Then, exosomes hsa_circ_0065149 from 41 healthy volunteers and 39 EGC patients were quantified by qRT-PCR. Our data showed that hsa_circ_0065149 levels in plasma exosomes were decreased significantly in EGC than those in healthy control ($P < 0.001$; Fig. 3a). When as a screening biomarker for EGC, exosomes hsa_circ_0065149 has higher sensitivity and specificity than traditional clinical biomarker such as CEA, CA19–9, and CA125, in which the sensitivity and specificity were 48.7% and 90.2%, respectively.

Gastric juice shows its unique advantage, due to high specificity for the stomach [27]. We detected and compared gastric juice hsa_circ_0065149 level in healthy volunteers, gastric ulcer patients, chronic atrophic gastritis patients, and GC patients. Unexpectedly, hsa_circ_0065149 levels have no significant difference among four groups ($P = 0.448$; Supplementary Fig. 3). Moreover, it is worth noting that hsa_circ_0065149 levels in gastric juice are not consistent with its levels in GC tissues and plasma exosomes. This opposite trend may be related to the function of exosomes; and the mechanism of hsa_circ_0065149 selectively change in different body fluid needs further explore.

Recent studies demonstrated that circRNAs can regulate miRNA functions by sharing miRNA response elements (MREs) [13, 28]. CircRNAs associate with related miRNAs and the circRNA-miRNA axes are involved in a serious of human diseases by regulating pathogenicity-related gene expression [29]. To date, evidences are arising that circRNA-miRNA-mRNA axis participates in many disease pathways such as apoptosis, vascularization, invasion and metastasis during carcinogenesis [28]. In this study, the interaction between hsa_circ_0065149 and miRNAs was predicted to excavate its potential functions. We found that hsa_circ_0065149 has MREs of hsa-miR-197-5p, hsa-miR-222-3p, hsa-miR-330-5p and hsa-miR-486-3p (Fig. 4a). Then, through DIANA mirPath software, we identified that the downstream four miRNAs of hsa_circ_0065149 have many targets, which are closely related with a lot of cancer-related pathways, such as cell cycle, mRNA surveillance pathway, proteoglycans in cancer, etc. (Fig. 4e), and also are involved in a lot of biological function processes such as gene expression, RNA binding, protein complex formation, cellular protein modification process, etc. (Fig. 4d). The network map and Venn diagram revealed that different hsa_circ_0065149/miRNAs axis also have many common downstream targets (Fig. 4b-c). This is likely to enhance the downstream biological effects of hsa_circ_0065149 in cells. Our results of bioinformatics analysis suggested that abnormal expression of hsa_circ_0065149 may play an important role during gastric carcinogenesis.

In conclusion, our results suggested that downregulated expression of hsa_circ_0065149 is an indicator for prognosis prediction and biomarker for early gastric cancer screening.

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Compliance with Ethical Standards

Competing Interests The authors disclose no conflicts.

Ethical Approval This study was approved by the Human Research Ethics Committee of Ningbo University School of Medicine.

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