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Original Paper

Downregulation of microRNA-376a in Gastric Cancer and Association with Poor Prognosis

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Key Words
miR-376a • MicroRNA • Gastric cancer • Prognosis • Biomarker

Abstract
Background/Aims: MicroRNAs have a significant role in the tumorigenesis and progression of cancers, including gastric cancer (GC). Our study aimed to identify a novel biomarker to predict the prognosis of patients with GC. ***Methods:*** The GC microarray dataset, GSE28700, was downloaded from the Gene Expression Omnibus (GEO) database and screened for differentially expressed miRNAs (DEMs). The downregulation of miR-376a expression was verified in GC cell lines and 82 paired GC tissues by performing RT-qPCR and the correlation between its expression and clinicopathological characteristics was also explored. The target genes of miR-376a were predicted using TargetScan7.1, miRDB, and DIANA website tools. A functional enrichment analysis was performed to explore the biological role of the common target genes. ***Results:*** Bioinformatics analysis found that miR-376a was downregulated in GC tissues. Compared with the control group, RT-qPCR results showed that the expression of miR-376a in GC cell lines and tissues were also significantly decreased. The expression of miR-376a was statistically associated with T and N stage. Survival analysis with Kaplan–Meier showed that GC patients in the low expression group had a poorer prognosis than those in the high expression group (median survival of 26.4 and 46.9 months, respectively). Univariate and multivariate analysis demonstrated that low miR-376a expression was an independent prognostic marker for poor survival. Functional enrichment analysis indicated that the common targets genes were involved in cell–cell communication, VEGF and mTOR1-mediated signaling, and epithelial-to-mesenchymal transition (EMT). ***Conclusion:*** The results suggest that miR-376a could play an important role in the tumorigenesis and progression of GC and act as a novel therapeutic target and prognostic indicator in patients with GC.

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Introduction

Gastric cancer (GC) is one of the most common malignant neoplasms worldwide and has a high incidence and mortality. In 2012, the cases of newly diagnosed GC and mortalities were 951, 600 and 723, 100, respectively [1]. Most patients were diagnosed at an advanced stage when the rate of 5-year survival is less than 20% [2]. Therefore, it is urgent to identify an effective therapeutic target and prognostic biomarker for improving the poor survival of GC patients.

MicroRNAs (miRNAs) are endogenous small noncoding RNAs of about 22 nt in length that participate in post-transcriptional regulation [3]. Several studies have reported that miR-21 played an important role in GC cell proliferation, invasion and apoptosis by targeting PTEN [4], PDCD4 [5], and 15-PGDH [6]. Furthermore, over-expressed miR-21 could be used as a diagnostic biomarker of GC [7]. Our previous study also found that miR-497 was downregulated by DNA methylation and acted as a tumor suppressor in GC [8]. Thus, increasing evidence support that aberrant expression of miRNAs is involved in the initiation and development of GC.

The Gene Expression Omnibus (GEO) is a public database that stores raw and processed data of high throughput gene expression and genomics [9]. The Cancer Genome Atlas (TCGA) project contains the genomic sequence, expression, methylation and copy number variation data of more than 11, 000 patients with diverse types of tumor [10]. By processing the GSE28700 dataset from the GEO database, we obtained 42 differentially expressed miRNAs (DEMs) between GC and normal gastric samples. Among these DEMs, miR-376a was downregulated and has not yet been reported. The biological function of miR-376a in GC remains indistinct, therefore, we selected it for further exploration.

In this study, we first confirmed that miR-376a was downregulated in GC cell lines and tissues. The downstream target genes of miR-376a were predicted by three bioinformatics tools, and the consensus genes were used for gene functional enrichment analysis. The results indicated that the target genes of miR-376a involved in several crucial pathways of GC, such as VEGF signaling, mTOR1-mediated signaling, and epithelial-to-mesenchymal transition (EMT). Kaplan–Meier survival analysis showed that GC patients with low expression of miR-376a tend to have a poorer prognosis than patients with high expression. These results suggested that miR-376a could act as a therapeutic target and prognostic indicator in GC.

Materials and Methods

Data processing and DEM identification

The miRNA expression profiles GSE28700 dataset contains 22 pairs of GC samples and gastric normal samples. The data were downloaded from the GEO database, then the data were processed for screening DEMs with the limma package in R. The cut-off criterion are fold change (FC) > 2.0 and $P < 0.05$.

Gastric cancer cell lines and tissue samples

A normal gastric mucosa cell line (GES-1) and four GC cell lines (MKN-45, SGC-7901, MGC-803, and BGC-823) were purchased from the Chinese Academy of Sciences (Shanghai, China). All cells were cultivated with RPMI 1640 medium (Gibco, Germany) containing 10% fetal bovine serum and maintained in an incubator with 5% CO2 at 37°C. The 82 paired samples of GC tissue and adjacent normal tissue were collected from patients who had received surgical resection at the Fourth Affiliated Hospital of China Medical University. All patients signed the informed consent and the study was authorized by the Ethics Committee of China Medical University. Tissues were stored at −80°C immediately after resection and were confirmed by histopathology.

Quantitative real-time PCR

Total RNA of tissues and GC cell lines was isolated by TRIzol reagent (Invitrogen, CA, USA). Reverse transcription and qRT-PCR were conducted with SYBR-Green (GenePharma). The level of expression was measured by the $2^{-\Delta\Delta Ct}$ method (U6 snRNA was selected as internal reference). The U6 primers were forward: 5'-CTCGCTTCGGCAGCACA-3' and reverse: 5'AACGCTTCACGAATTTGCGT-3', the Stem-loop RT primer of miR-376a was 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACGTGG-3' and forward: 5'-GTGCAGGGTCCGAGGT-3' and reverse: 5'-ATCATAGAGGAAAATCCACG-3'. All the steps were conducted according to the manufacturer's instructions.

Association analysis between miR-376a expression and GC patients' survival

The website Firebrowse (http://firebrowse.org/) operated by the Broad Institute of Massachusetts Institute of Technology (MIT) and Harvard using a Representational State Transfer (REST) Application Programmable Interface (API) Firebrowse service is making large-scale multi-platform omics data analysis results publicly available [11]. For instance, it provides a more accessible way of downloading and integrating the analysis of TCGA data. Thus, the mature miRNA sequencing data and merged clinical information of stomach adenocarcinoma were downloaded from Firebrowse (update 2016.01). The exclusion criteria were: (1) the expression of miR-376a is not available; (2) lack of necessary information, such as age at diagnosis and gender; (3) a survival time of more than 3000 days. Finally, a total of 296 GC patients were involved in this study. The median expression of miR-376a was selected as the cut-off value, and then 296 GC patients were classified into groups of high or low expression. The survival analysis was performed with the Kaplan–Meier method and log-rank test.

Consensus target gene prediction of miR-376a and gene function analysis

Three online tools of TargetScan7.1 [12] (http://www.targetscan.org/vert_71/), miRDB [13], (http://www.mirdb.org/) and DIANA [14] (http://www.microrna.gr/microT-CDS), which are widely used in predicting target genes of microRNAs, were selected for predicting the feasible target genes of miR-376a. All the steps were conducted according to instructions in the relevant literature. A Venn plot was then applied for obtaining the consensus target genes. FunRich is an open-access tool for analyzing the functional enrichment and networks of genes [15]. The functional analysis of consensus target genes was carried out in the domains of Cellular Component, Biological Process, Molecular Function, and Biological Pathways.

Statistical analysis

We used IBM SPSS version 19.0 to conduct the statistical analysis. An unpaired t-test was used to analyze the difference between the two groups. The differences between miR-376a expression and clinicopathological characteristics were assessed by the chi-square test. The Kaplan–Meier curve was analyzed by the log-rank test. $P<0.05$ was considered to have a significant difference.

Results

Microarray analysis for the identification of DEMs in GC

With the cut-off criteria of a fold change > 2.0, a total of 42 DEMs including 29 downregulated and 13 up-regulated microRNAs were identified by analyzing the GSE28700 microarray dataset from the GEO database (Table 1). The up and downregulated miRNAs were distinguished and are presented in a volcano plot (Fig. 1). We found that the expression of miR-376a in GC samples was less than in normal samples with a fold change of 2.2.

Table 1. 42 differentially expressed miRNAs (DEMs) identified by processing the data of microarray GSE28700

DEMs	microRNAs
Down-regulated	miR-376a, miR-133b, miR-204, miR-145, miR-381, miR-29c, miR-99a, miR-30e-5p, miR-148a, miR-143, miR-154, miR-1, miR-133a, miR-497, miR-363, miR-125b, miR-100, miR-195, miR-139, miR-218, miR-368, miR-767-3p, miR-375, miR-129, miR-302c, miR-329, miR-586, miR-328, miR-551b
Up-regulated	miR-18a*, miR-196a, miR-523, miR-604, miR-611, miR-196b, miR-9*, miR-135b, miR-514, miR-369-3p, miR-550, miR-181a*, miR-224

miR-376a is downregulated in GC tissue samples and cell lines by qRT-PCR

For further confirmation, the expression of miR-376a was detected in GC tissues and adjacent normal gastric tissues. The results of RT-qPCR indicated that miR-376a was significantly reduced in GC tissues compared with paired adjacent tissues (Fig. 2A). Furthermore, we measured the expression level of miR-376a in GC cell lines (MKN-45, SGC-7901, MGC-803, and BGC-823) and a normal gastric mucosa cell line (GES-1). Compared with GES-1, the expression levels of miR-376a in four GC cell lines were all significantly lower (Fig. 2B). All the results demonstrated that miR-376a was frequent downregulated in GC and could be associated with GC progression.

Low expression of miR-376a is associated with advanced clinicopathological characteristics of GC

We explored the association between miR-376a and clinicopathological characteristics of 82 paired GC samples. The data showed that the expression of miR-376a is significantly associated with T and N stage. However, no association was observed between miR-376a expression and age, gender, M stage or pathologic differentiation grade (Table 2). Our results indicated that miR-376a might play an important role in the progression of GC.

Low miR-376a expression in GC patients correlates with poor prognosis

To investigate whether miR-376a could act as a biomarker in the prognosis of GC patients, we collected miRNA expression profiles and clinical information of 296 GC patients derived from the TCGA database. The patients were divided into two groups by the median expression of miR-376a and the Kaplan–Meier curve was performed for survival analysis. As a result, we found that the group of GC patients with low miR-376a expression had a less overall survival time than the groups with high expression (Fig. 3). Furthermore, we also investigated the relevant risks in the prognosis of GC. The univariate and multivariate Cox regression analyses revealed that low expression of miR-376a was related with a poorer prognosis of GC patients compared to patients with high expression of miR-376a. In addition, univariate analysis also indicated that pathological stage and T and N stage were strongly associated with the prognosis of GC patients (Table 3).

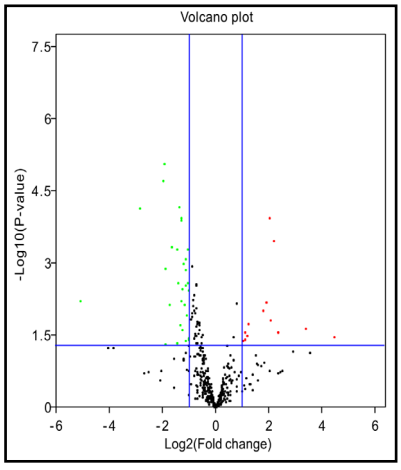

Fig. 1. A total of 42 differentially expressed miRNAs shown in a volcano plot. The green and red spots represent 29 low and 13 high expression miRNAs, respectively (cut-off criteria are P<0.05 and |FC|>2.0). FC: fold change.

Target prediction of miR-376a and gene function analysis

The target prediction websites TargetScan7.1, miRDB, and DIANA were used to predict the targets genes of miR-376a. In order to improve the reliability of the prediction, we obtained the common genes of the three websites tools (Fig. 4). Finally, a total

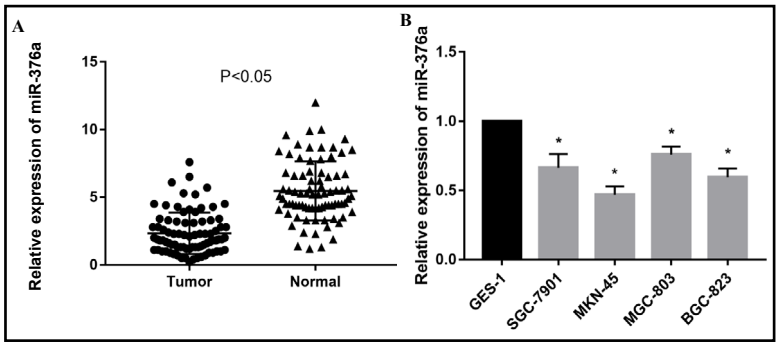

Fig. 2. The expression of miR-376a in gastric cancer (GC) tissues and cell lines. (A) miR-376a was downregulated in GC tissues. (B) miR-376a was downregulated in GC cell lines. * P<0.05.

Table 2. The relationship between miR-376a expression and clinical features of 82 patients with gastric cancer. *P<0.05, statistically significant

Variables	miR-376a expression		Total samples	P value
	Low (n,%)	High (n,%)		
age				
<60	14 (17.1)	11 (13.4)	25	0.757
≥60	23 (28.0)	34 (41.5)	57	
Gender				
Male	27 (32.9)	23 (28.1)	50	0.297
Female	21 (25.6)	11 (13.4)	32	
T stage				
T1+T2	8 (9.8)	13 (15.9)	21	0.027*
T3+T4	40 (48.8)	21 (25.6)	61	
N stage				
N0	11 (13.4)	16 (19.5)	27	0.022*
N1-3	37 (45.1)	18 (22.0)	55	
M stage				
M0	23 (28.0)	13 (15.9)	36	0.384
M1	25 (30.5)	21 (25.6)	46	
Differentiation grade				
Well and moderate	14 (17.1)	15 (18.3)	29	0.163
Poor	34 (41.5)	19 (23.2)	53	

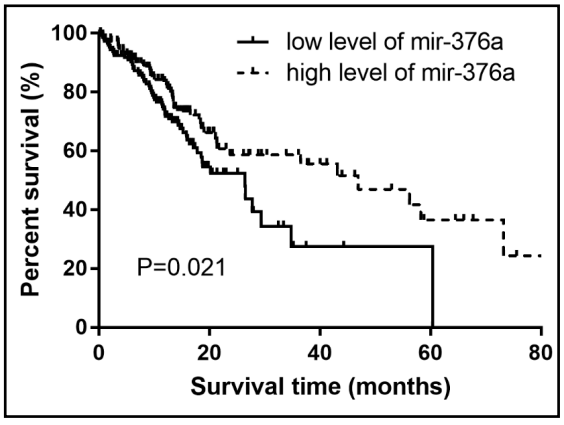

Fig. 3. Kaplan–Meier survival curves according to the median expression of miR-376a in 296 gastric cancer patients.

Table 3. Univariate and multivariate Cox regression analyses of prognostic parameters in GC patients. *P<0.05, statistically significant

Variables	Univariate analysis		Multivariate analysis	
	HR (95%)	P value	HR (95%)	P value
Gender	0.797 (0.524-1.212)	0.289	0.676 (0.430-1.061)	0.088
Histologic grade	1.267 (0.864-1.859)	0.226	1.117 (0.740-1.687)	0.598
Pathological stage	1.644 (1.289-2.099)	<0.001*	1.475 (0.884-2.463)	0.137
T stage	1.526 (1.187-1.961)	0.001*	1.180 (0.830-1.677)	0.356
N stage	1.385 (1.159-1.654)	<0.001*	1.114 (0.864-1.436)	0.407
M stage	1.623 (0.777-3.388)	0.197	0.734 (0.252-2.139)	0.571
miR-376a expression	0.636 (0.428-0.946)	0.025*	0.626 (0.404-0.968)	0.035*

of 57 consensus target genes were conducted for functional enrichment analysis to clarify the potential role of miR-376a in GC. Cellular component analysis showed that these genes were part of a micro-ribonucleoprotein complex, RNA-induced silencing complex, and nucleus and mRNA cap-binding complex (Fig. 5A). Molecular function and biological process analysis indicated that these genes participated in transcription and translation regulator activity, morphogenesis, cell migration, and regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism (Fig. 5B and 5C). Biological pathway analysis revealed that the genes were involved in cell–cell communication, VEGF, and mTOR1-mediated signaling, and EMT (Fig. 5D).

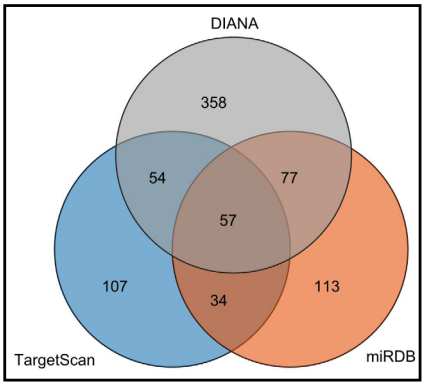

Fig. 4. The Venn plot for 57 common target genes of miR-376a from three website tools (TargetScan7.1, miRDB, and DIANA).

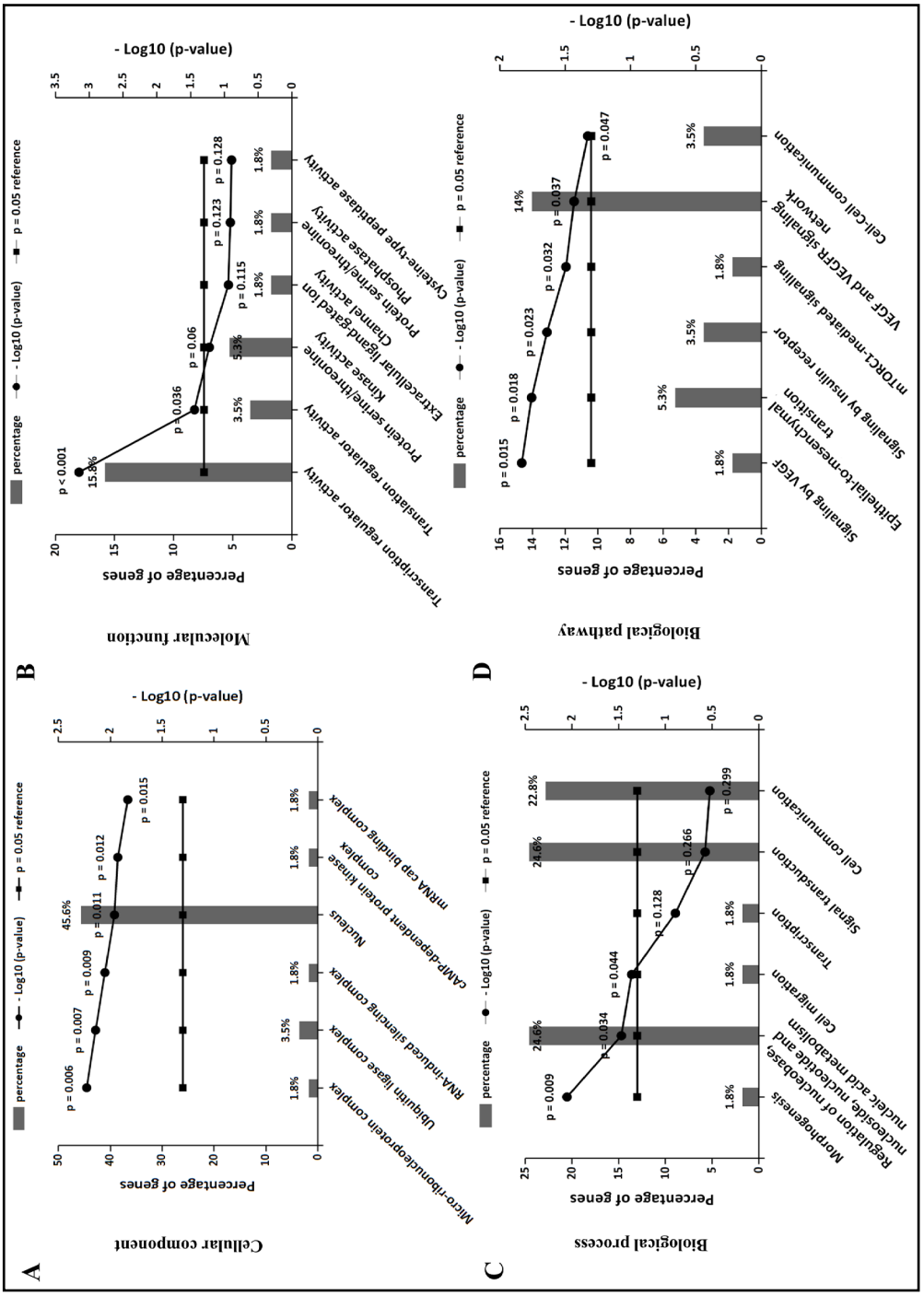

Fig. 5. Genetic functional enrichment analysis. (A) Cellular component analysis indicated that the genes are part of a micro-ribonucleoprotein complex, RNA-induced silencing complex, nucleus and mRNA cap binding complex; (B) Molecular Function analysis showed that target genes participate in transcription and translation regulator activity; (C) Biological Process analysis indicated that the genes participate in morphogenesis, cell migration and regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism; (D) Biological Pathway analysis revealed that genes are involved in cell-cell communication, VEGF and mTOR1-mediated signaling, and epithelial-to-mesenchymal transition (EMT).

Discussion

Increasing studies have revealed that the dysregulation of miRNAs is strongly associated with the tumorigenesis and progression of cancers, including GC [16-18]. Additionally, miRNAs have been reported to be used as biomarkers of diagnosis [19] and prognosis [20, 21] in GC. In this study, we first analyzed a microarray miRNA chip, GSE28700, from the GPL9081 platform and the bioinformatics results indicated that miR-376a was downregulated in GC. Then, we confirmed by a qRT-PCR assay that miR-376a was significantly downregulated in GC cell lines and 82 paired GC tissue and adjacent normal tissue collected from patients, and found that low miR-376a expression was correlated with advanced clinicopathological characteristics of GC. The result showing that miR-376a was downregulated in GC was supported by bioinformatics evaluation and qRT-PCR assay, which was a high-level of evidence.

Moreover, we obtained the clinical information of the GC patients from TCGA and explored the potential association between aberrant expression of miR-376a and prognosis. The results from TCGA database demonstrated that the patients with low expression of miR-376a had a poorer prognosis (median survival of 26.4 months) than those with high expression (median survival of 46.9 months). The univariate and multivariate Cox regression analyses revealed that low expression of miR-376a was an independent risk factor of poor prognosis. A total of 296 GC patients were included in the statistic analysis, which made our study more convincing and authentic.

In other types of cancers, the dysregulation of miR-376a also played a vital role in tumorigenesis and progression. Zheng et al. reported that miR-376a was downregulated in hepatocellular carcinoma tissues and cell lines and could suppress proliferation and induce apoptosis in hepatocellular carcinoma [22]. Fellenberg et al [23]. and Herr et al [24]. found that miR-376a acted as a tumor suppressor by targeting PDIA6 and the restoration of miR-376a counteracted the neoplastic phenotype of giant cell tumor of bone. In colorectal cancer, miR-376a was reported to be downregulated in tumor tissues and may be used as a prognostic biomarker and therapeutic target [25]. However, Yang et al [26]. demonstrated that miR-376a were significantly increased in ovarian cancer tissues and overexpression of miR-376a promoted cell proliferation, migration, and invasion of ovarian cancer. All the studies suggested that the expression of miR-376a was tissue-specific and had a complicated effect in cancers. The different findings regarding the roles of miR-376a in different cancers raised our concern, moreover, the effect of miR-376a in GC was still vague. All these matters encouraged us to explore further. In this study, we first confirmed that the expression of miR-376a was low in GC and might act as a tumor suppressor in the tumorigenesis and progression of GC.

In general, miRNAs participate in tumorigenesis and progression through binding to the 3′ untranslated regions (UTRs) of target genes and negatively regulate mRNAs. To investigate the potential mechanism of miR-376a in GC, we predicted reliable target genes using bioinformatic methods and conducted gene functional enrichment analysis. The results indicated that genes were frequently involved in cell–cell communication, VEGF and mTOR1-mediated signaling, and EMT. Li et al. revealed that miR-148a-3p could suppress the cytoprotective autophagy through inhibiting the activation of mTOR1 and enhance cisplatin cytotoxicity in GC [27]. A novel oncogene, KIF26B, was confirmed to promote GC cell proliferation and metastasis through the VEGF pathway [28]. EMT had been recognized as a vital process in promoting invasion and metastasis of GC. MiR-30c-5p [29] and miR-646 [30] were observed to suppress EMT in GC by targeting MTA1 and FOXK1, respectively. There are various target gene prediction tools for miRNAs and each tool has its specific algorithm in prediction. In order to enhance the reliability of target genes prediction, we obtained the consensus genes from the results of three tools (TargetScan7.1, miRDB, and DIANA). This operation also contributed to acquiring more accurate results of gene functional enrichment analysis, which is one of the highlights in this research.

Nevertheless, there were several limitations to our study. The data for GC miRSeq and clinical information from Firebrowse were only updated to 2016.01, so we could not obtain the latest data. However, the data used in the present study are still robust and convincing. We will follow up this research once the data in Firebrowse are updated. In addition, the mechanism by which miR-376a is downregulated in GC is unclear. It may be regulated by a number of genetic and epigenetic factors, such as transcription factors, aberrant DNA methylation, and histone modification. All these factors may play a vital role in the regulation of miR-376a solely or together and further exploration would help to determine the malignant behavior of GC cells.

Conclusion

In conclusion, we identified that the expression of miR-376a was reduced in GC tissues and cell lines. In addition, miR-376a might participate in the tumorigenesis and progression of GC. The low expression of miR-376a was significantly associated with an advanced stage of GC and the poor prognosis of patients, which indicate that miR-376a could be a novel therapeutic target and prognostic indicator in GC.

Acknowledgements

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All patients signed the informed consent and the study protocol has been approved by the research institute's committee on human research.

Dai DQ and Zhang C designed the study; Zhang C performed the experiment work; Zhang C, Liang Y, Ma MH and Wu KZ conducted the data analysis; Zhang C, Zhang CD and Dai DQ contributed to the manuscript preparation.

Disclosure Statement

The authors have no conflicts of interest to declare.

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