

Original Research Article

MiR-148a-3p suppresses the progression of gastric cancer cells through targeting ATP6AP2

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Abstract

Purpose: Gastric cancer (GC) is one of the most frequent tumors with high mortality rate, worldwide. A proper understanding of the mechanism underlying its progression is required for its diagnosis and development of novel treatment option. MicroRNAs are associated with the development and advancement of different types of cancer, including GC. The current research was aimed at investigating the molecular and biological function of miR-148a-3p in GC development.

Methods: A human normal gastric epithelial cell line, GES-1 (control) as well as four GC cell lines (NUGC-4, SNU-520, STKM-2 and MKN-74) were employed for the study. MiR-148a-3p and ATP6AP2 expression levels in GC cell lines were examined by RT-qPCR technique. Transfection procedure was used to upregulate miR-148a-3p expression in the MKN-45 cell line. MTT assay was utilized to evaluate cell viability in GC cell lines. The molecular interaction between miR-148a-3p and ATP6AP2 was predicted using bioinformatics system and the prediction was then validated by luciferase reporter assay.

Results: Expression levels of miR-148-3p was low, whilst that of ATP6AP2 was high in GC cell lines. MiR-148a-3p overexpression resulted in the reduction of cell viability in GC cell lines. More so, it was confirmed that miR-148-3p, as a post-transcriptional regulator inhibited ATP6AP2 expression by having a negative association with it in GC cells. More so, ATP6AP2 was found to be a direct target of miR-148a-3p.

Conclusion: Our results revealed that miR-148a-3p plays a crucial function in GC development through targeting ATP6AP2. This finding could be explored in the discovery of new therapeutic approaches for GC treatment.

Keywords: ATP6AP2, Cell viability, Gastric cancer, miR-148a-3p, Progression

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INTRODUCTION

Gastric cancer (GC) is recorded to be the number five most frequent tumor and the number three chief source of mortalities, caused by

cancers worldwide [1]. For most widespread malignancies, reappearance as well as metastasis are the principal hindrances to gastric cancer treatment[2]. Environmental features, for instance smoking, *Helicobacter pylori* infection,

as well as genetic variations are believed to be the main risk factors for GC development[3]. The occurrence of GC as well as the resultant deaths may be reduced as a result of developments in treatment, diagnosis as well as healthier environments and conditions of living [4]. Generally, GC is identified at the progressive stage because of the occurrence of primary indicative markers[5]. Nonetheless, the fundamental molecular systems are not totally elucidated in this procedure. Consequently, more researches are required to investigate the molecular mechanism underlying the initiation as well as the progression of GC, and to discover new indicative makers and targets for therapy.

MicroRNA (miRNA) is a kind of small noncoding RNA, which is normally made of 18–25 nucleotides. Mechanically, miRNA controls target gene expression via binding to the 3'-UTR of the mRNAs, thereby inhibiting translation as well as degrading target mRNAs[6]. Recently, more researches proposed that the functions played by miRNA in the carcinoma progression include modulating growth of tumor, metastasis, as well as epithelial–mesenchymal transition (EMT). According to earlier studies, miRNA expression is linked to the development and advancement of GC tumor[7]. For example, miR 143-3p targets QKI-5 and could apply its tumor repressing impact and hinders esophageal cancer cell growth, invasion and migration. Nonetheless, the role of miR-148a-3p in the development of GC cells is still not discovered. The study was aimed at investigating the function of miR-148a-3p expression in the progression of gastric cancer.

In the current study, we explored the expression level of miR-148a-3p in GC cell lines using the RT-qPCR analysis. Subsequently, we investigated the effects of miR-148a-3p overexpression on GC cell viability. Also, MTT assay was carried out following transfection procedure. Furthermore, we examined the expression levels of ATP6AP2 in GC cell lines using RT-qPCR assay. Then bioinformatics analysis was carried out to find out if there were matching binding sequences between miR-148a-3p and ATP6AP2, which was followed by the luciferase reporter assay to determine luciferase activity.

EXPERIMENTAL

Cell culture

A total of four gastric cancer cell lines (NUGC-4, SNU-520, STKM-2 and MKN-74) as well as the human normal gastric epithelial cell line GES-1 were acquired from the Beyotime Institute of

Biotechnology, Haimen, China. Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Thermo Fisher Scientific, New Jersey, USA) containing 5 percent fetal bovine serum (fbs; ABCAM, San Francisco, United States), 5 percent CO₂ and 95 percent air in a moistened incubator (Beyotime Institute of Biotechnology, Beijing, China) were used for culturing of the cells.

RT-qPCR

Trizol reagent (Invitrogen, Thermo Fisher Scientific, Philadelphia, USA) was used for total RNA extraction from the MKN-74 cell line. Then, the UV2401 spectrophotometer (Beyotime Institute of Biotechnology, Haimen, China) was used to determine the quality and concentration of the RNA. Particular RNA quantities (1 mg) were reverse-transcribed into cDNA using the Primer Script RT reagent kit (Thermo Fisher Scientific, Philadelphia, USA). PCR intensification for microRNA and mRNA were done by SYBR Premix EX Taq II kit (Beyotime Institute of Biotechnology, Beijing, China).

The house keeping gene GAPDH was used for normalization of the qPCR. Primers (Table 1) were invented by Thermo Fisher Scientific, New Jersey, USA. MiR-NC mimics, miR-148a-3p mimics, as well as miR-148a-3p inhibitor were created by ABCAM, San Francisco, United States. The 2^{-DDCt} system was used to analyse the ultimate comparative quantities of target mRNAs and miRNAs.

Table 1: RT-qPCR primer sequences

Gene name	Direction	Primer sequence
MiR-148a-3p	Forward	5'-GCTCAGTGCCTACAGAAC-3'
	Reverse	5'-GTGCAGGGTCCGAGGT-3'
ATP6AP2	Forward	5'-TATGGTGGGAATGCAGTGGT-3'
	Reverse	5'-CAAGGCCAAGGCGATCA TTA-3'
GAPDH	Forward	5'-ACCCAGAAGACTGTGGA TGG-3'
	Reverse	5'-TCAGCTCAGGGATGACC TTG-3'

Plasmid constructs and cell transfection

RNA overexpression plasmid was intended to precisely target ATP6AP2 by means of oe-RNA inventing apparatuses (ABCAM, San Francisco, United States). Consideration of oe-RNA targeting only the ATP6AP2 was confirmed using BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). RNA-NC and oe-ATP6AP2 were created by Ribo Bio (Beyotime Institute of Biotechnology, Haimen, China). The miR-148a-3p mimics was produced by Ambion (Invitrogen, ABCAM, San Francisco, United States) and transfected into MKN-45 cell to a last solution of 100 nM using Lipofectamine 2000 (Invitrogen; Beyotime Institute of Biotechnology, Haimen, China) for 2 days at 37 °C, following the instructions of the manufacturer. The pre-miR-Con control sequences were bought from ABCAM, San Francisco, United States and used as a negative control.

MTT assay

The MKN-45 cells were planted into the 96-well plate for 1, 2, and 3 days following transfection with oe-RNA-NC or oe-ATP6AP2, premiR-Con or miR-148a-3p mimics. Then, the MTT Kit (Beyotime Institute of Biotechnology, Haimen, China) was used to measure cell viability. The Elx800 Reader (Thermo Fisher Scientific, Philadelphia, USA) was used for recording the absorbance at 450 nm.

Luciferase reporter gene assay

Ribo Bio (Beyotime Institute of Biotechnology, Haimen, China) was used to synthesize the wild-type (WT) and mutant-type (MUT) 3'-UTR of ATP6AP2. Then, they were implanted into the numerous sites of cloning of the pMIR-REPORT vectors (ABCAM, Cambridge, United Kingdom), that expresses the luciferase. These were inserted into the AGS cell with Lipofectamine 2000 (Invitrogen; ABCAM, Cambridge, United Kingdom) for 2 days at 37 °C following the instructions of the manufacturer. To determine the luciferase activity, the Dual Luciferase Reporters Assay System (Thermo Fisher Scientific, New Jersey, USA) was used on a Luminoskan TM Ascent Microplate Luminometer (Thermo Fisher Scientific, New Jersey, USA).

Statistical analysis

Mean \pm SEM was used for reporting the data. IBM SPSS Statistics Version 19.0 (SPSS Inc., USA) as well as GraphPad Prism Version 7.0 (GraphPad Software, Inc., USA) were both used for executing the statistical analysis. The

Student's t-test was used to examine the differences between two groups; the one-way analysis of variance was used to analyze the inter-group variances, then a post hoc Tukey test for numerous contrasts.

RESULTS

miR-148a-3p is downregulated in gastric cancer cells

Firstly, RT-qPCR assay was performed in order to determine expression levels of miR-148a-3p in GC cell lines. The results (Figure 1) revealed that miR-148a-3p expression was lower in GC cell lines (MKN-45, SNU-520, NUGC-4 and STKM-2) than in the typical gastric epithelial cell line (GES-1). MKN-45 cells had the lowest expression of miR-148a-3p, hence they were selected for all the subsequent experiments.

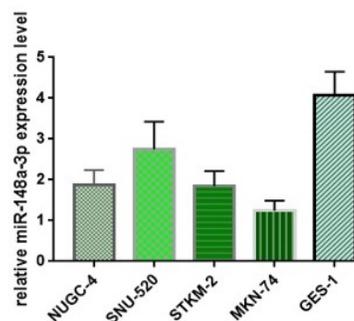


Figure 1: miR-148a-3p is downregulated in human gastric cancer cell lines. Expression levels of miR-148a-3p were examined in gastric cancer cell lines (NUGC-4, SNU-520, STKM-2, MKN-74) and normal cell line (GES-1) by RT-qPCR. All the experiments were repeated three times. **: $P < 0.01$

Effects of miR-148a-3p on gastric cancer cell viability

Moreover, to investigate the biological function of miR-148a-3p in GC cell viability, the MKN-45 cell line was transfected with miR-148a-3p mimics. Afterward, RT-qPCR method was used to verify the overexpression of miR-148a-3p in the transfected MKN-45 cell line. The outcomes (Figure 2A) showed that miR-148a-3p expression was significantly higher in the miR-148a-3p overexpressed MKN-45 cells in comparison to the relative negative control group. Furthermore, the results obtained for the MTT assay revealed that the MKN-45 cells transfected with miR-148a-3p overexpression reflected a low cell viability, compared to the negative control group (Figure 2B). These outcomes suggest that upregulation of miR-148a-3p could inhibit viability of GC cells.

ATP6AP2 is upregulated and is a direct target of miR-148a-3p in gastric cancer cells

RT-qPCR assay was carried out in order to determine expression level of ATP6AP2 in GC cells. The outcomes (Figure 3A) revealed that ATP6AP2 expression was higher in GC cell lines (MKN-45, SNU-520, NUGC-4 and STKM-2) compared to the control typical gastric epithelial cell line (GES-1). Then, bioinformatic system (<http://www.targetscan.org>) was used to discover whether miR-148a-3p could directly bind to ATP6AP2. MiR-148a-3p and its possible putative binding sequence in the 3'-UTR of ATP6AP2 was revealed as shown in Figure 3B. Overexpression of miR-148a-3p diminished the activity of firefly luciferase that had the wild-type, but not the mutant 3'-UTR of ATP6AP2 (Figure 3C).

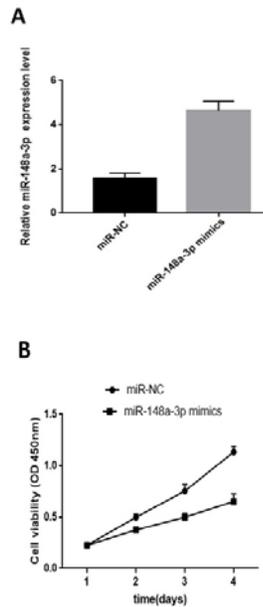


Figure 2: MiR-148a-3p overexpression inhibits gastric cancer cell viability *in vitro*. (A) Expression of miR-148a-3p was determined by RT-qPCR in the MKN-74 cells transfected with the miR-148a-3p mimic. (B) Cell viability was examined by MTT assay in the MKN-74 cells transfected with the miR-148a-3p mimic

Effects of ATP6AP2 on gastric cancer cell viability

Furthermore, after co-transfection with the ATP6AP2 overexpression plasmid and miR-148a-3p mimic, overexpression of ATP6AP2 restored the effects of miR-148a-3p overexpression in GC cell viability. Initially, the results from the RT-qPCR revealed that ATP6AP2 expression was significantly upregulated in miR-148a-3p mimics+oeATP-6AP2 group, compared to the control group

(figure 4A). The results from the MTT assay revealed that cell viability increased in the miR-148a-3p mimics+oeATP6AP2 group, compared to the control group (Fig. 4B). Collectively, these results suggest that transfection of overexpressed ATP6AP2 into the overexpressed miR-148a-3p cells reversed the effects of miR-148a-3p overexpression on GC cell viability.

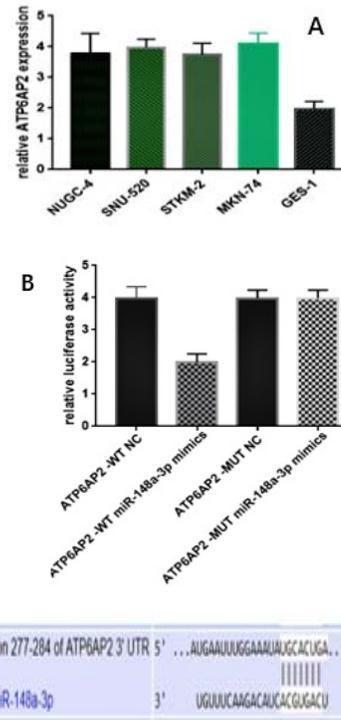


Figure 3: ATP6AP2 is a direct target of miR-148a-3p. (A) Expression levels of ATP6AP2 were examined in GC cell lines (NUGC-4, SNU-520, STKM-2, MKN-74) and normal cell line (GES-1) by RT-qPCR. (B) miR-148a-3p binding site on the wild-type and mutant ATP6AP2 3'UTR was examined by bioinformatics. (C) Relative luciferase activity analysis in the MKN-74 cells co-transfected with WT/Mut-ATP6AP2 reporter plasmid and miR-148a-3p mimics or miR-NC

DISCUSSION

Exploring the molecular system underlying the development of gastric cancer (GC) is crucial for discovering successful means of treatment. Abnormal expressions of miRNAs are usually discovered in human malignancies. They have been stated to have substantial purposes in the development and advancement of tumors [8,9]. Consequently, it is important to find out the decontrolled miRNAs in malignancies. Previous researches revealed that miR-148a-3p possibly contained a tumor-suppressing effect in human cancers. For instance, miR-148a-3p represses the advancement of epithelial ovarian carcinoma

through sponging c-Met [10]. Also, Wang *et al* revealed that miR-148a-3p suppressed the esophageal carcinoma invasion as well as growth through sponging DNMT1[11]. MiR-148a-3p influences suppression in DNA divergence Repair-Deficient Colorectal carcinoma via sponging PD-L1[12]. Furthermore, miR-148a-3p repressed the growth and epithelial-mesenchymal transition development of non-small-cell lung carcinoma via regulating Ras/MAPK/Erk signalling [13]. However, till date, the miR-148a-3p functions in GC development, progression and the molecular system through which miR-148-3p applies its roles are still unknown.

Nevertheless, in this research, it was revealed that the expression of miR-148a-3p was substantially lower in GC cell lines in comparison to adjacent normal cell line. Upregulation of miR-148-3p greatly reduced GC cell viability. These outcomes proposed that miR-148-3p might be a new tumor-repressing miRNA in GC. Our results from this study implicated the tumor-suppressing function of miR-148-3p in GC *in vitro*. Furthermore, as for the downregulation of miRNAs in cancers, numerous researches display that methylation might be one of the sources responsible for it. Thus, additional underlying systems still need to be examined.

It has been confirmed that miRNAs play tumor-suppressive or oncogenic function via binding to the 3'-UTR of target genes, thus discovering the targets of miR-148-3p in GC is important, in order to understand its regulatory mechanism. In the current research, the target gene of miR-148a-3p was predicted by using bioinformatics. Finally, ATP6AP2 (ATPase, H⁺ transporting, lysosomal accessory protein 2) appeared to be the target. It was also discovered that miR-148-3p decreased the ATP6AP2 expression level and vice versa, suggesting that miR-140-3p inversely regulated ATP6AP2. Moreover, luciferase reporter gene analysis revealed that ATP6AP2 was directly targeted by miR148-3p in GC cells.

ATP6AP2 gene encodes a protein that is related to adenosine triphosphatases (ATPases). Proton-translocating ATPases have important functions in preservation of energy and transport [14], intracellular sections acidification, as well as cellular pH homeostasis[15]. There are three groups of ATPases-F, P, and V. The vacuolar (V-type) ATPases contains a transmembrane proton-conducting segment as well as an additional layer catalytic segment[16]. ATP6AP2 was discovered to be related to the transmembrane sector of the V-type ATPases. A previous research discovered that the prorenin receptor (PRR) is an element of the Wnt receptor compound and PRR purposes in a renin-sovereign way as a connector amid Wnt receptors and the vacuolar H⁺-adenosine triphosphatase (V-ATPase) (ATP6AP2) compound [17,18].

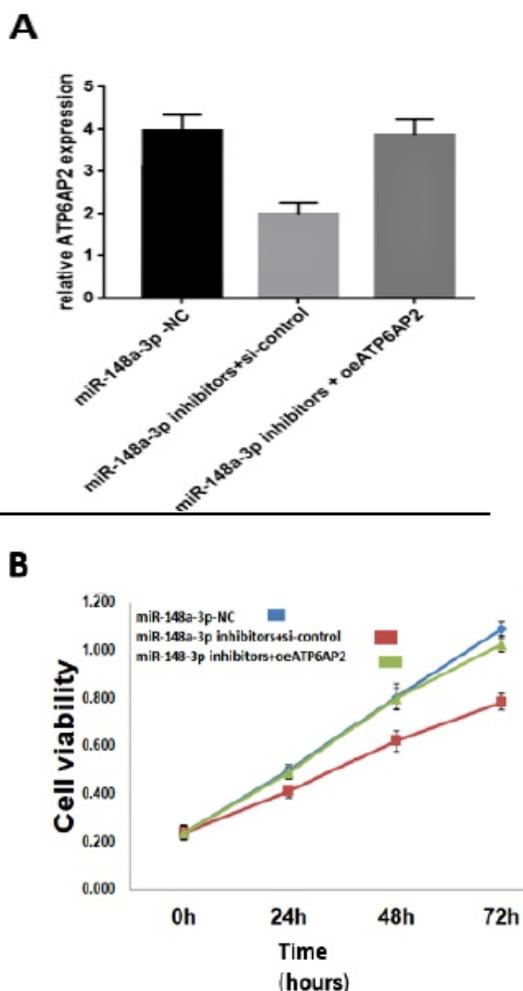


Figure 4: Effects of ATP6AP2 on gastric cancer cell viability. (A) ATP6AP2 expression was examined in MKN-74 cells transfected with miR-148a-3p inhibitors + oeATP6AP2 and the controls by RT-qPCR. (B) Cell viability was examined in MKN-74 cells transfected with miR-148a-3p inhibitors+oeATP6AP2 and the controls by the MTT assay

CONCLUSION

The results obtained from this study revealed that miR-148-3p is remarkably low in GC cell lines and it induced cell viability through direct targeting of ATP6AP2. Thus, miR-148-3p may perhaps be a new therapeutic target for GC treatment.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

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