miR-30a-3p Targets MAD2L1 and Regulates Proliferation of Gastric Cancer Cells

Purpose: This study was done to investigate the inhibition effects of miR-30a-3p on mitotic arrest deficient 2 like 1 (MAD2L1) expression and the proliferation of gastric cancer cells.

Patients and methods: Cluster analysis and the TCGA database were used to screen the key genes highly expressed in gastric cancer. Based on the LinkedOmics website, the correlation between the miR-30a-3p and the cell cycle-related target gene MAD2L1 in gastric cancer was analyzed. The mRNA and protein expression levels were detected with the quantitative real-time PCR and Western blot analysis. The cell proliferation and cell cycle were also detected and analyzed.

Results: Bioinformatics analysis showed that MAD2L1 was highly expressed in tumor tissues compared with normal tissues. Compared with normal tissues, the miR-30a-3p was significantly decreased in the gastric cancer tissues. Moreover, MAD2L1 was significantly negatively correlated with the miR-30a-3p expression. Furthermore, over-expression of miR-30a-3p decreased the expression of MAD2L1 at the protein level, which inhibited the proliferation of AGS and BGC-823 gastric cancer cells. In addition, the cell cycles of AGS and BGC-823 cells were arrested at the G0/G1 phase.

Conclusion: MAD2L1 is a pro-oncogene which is up-regulated in gastric cancer. The miR-30a-3p can down-regulate the MAD2L1 expression, inhibiting the proliferation of gastric cancer cells and affect the cell cycle.

Keywords: gastric cancer, bioinformatics analysis, MAD2L1, miR-30a-3p, cell proliferation

Introduction

Gastric cancer is one of the common tumors in the digestive system. According to the statistics of cancer data worldwide in 2018, the death cases due to gastric cancer is as high as 782,000 per year. Gastric cancer is a malignant tumor originating from the gastric mucosa epithelium. The disease has a very high incidence of malignant tumors in China and poses a serious threat to the health of patients. Although the medical service has been continuously improved in recent years, the five-year survival rate of gastric cancer is still less than 30%. Therefore, it is of great clinical importance and significance to investigate the inactivation mutation of proto-oncogenes and tumor suppressor genes involved in the development of gastric cancer.

Mitotic arrest defective protein 2 (MAD2L1) is an important component of the mitotic checkpoint complex protein, and the MAD2L1 gene is located on chromosome 4 in human beings (chromosome 6 in mice). The miR-30a-5p is a member of the miR-30 family, with the length of 22 bp, which has been reported to regulate the tumor cells proliferation, apoptosis, invasion and migration in various cancers, including the hepatocellular carcinoma, glioma, breast cancer and colon cancer. Based on the
big data screening of the key gene expression in gastric cancer cells, the cycle-related target gene MAD2L1 was selected. Accordingly, we speculate that the high expression of MAD2L1 might be caused by its targeted miRNA. Moreover, bioinformatics analysis showed that miR-30a-3p could bind to the 3'-UTR of MAD2L1, and its decreased expression might be responsible for the increase expression of MAD2L1. Therefore, we hypothesize that MAD2L1 may be a target gene of miR-30a-3p, and miR-30a-3p may affect the function of gastric cancer cells by regulating the expression of MAD2L1.

In this study, with the bioinformatics analysis, a variety of cell biology research techniques were performed to identify the key genes in the development of gastric cancer. Based on our findings herein, the effects of miR-30a-3p on MAD2L1 and gastric cancer cell proliferation were investigated.

Materials and Methods
Bioinformatics Analysis
The data used in this study were derived from the Cancer Genome Atlas Database (TCGA), the Gene Expression Database (GEO), the Kyoto Gene and Genomic Encyclopedia Database (KEGG), and the Gene Ontology Database (GO). Differentially expressed genes were constructed using Cytoscape software to further observe the correlation between genes. The plugin Molecular Complex Detection (MCODE) was used to screen the modules of PPI network in Cytoscape. The standard settings were as follows: MCODE score > 2, and number of nodes > 2. P < 0.05 was considered statistically significant.

Cell Lines and Cell Culture
Human gastric cancer cell lines AGS and BGC-823, and the HEK-293 model cells, were provided by the Biomedical Experiment Center of Xi’an Jiaotong University (China). The use of these cell lines was approved by Ethics Committee of Yan’an University College of Medicine. The AGS and BGC-823 human gastric cancer cells were cultured in the DMEM medium (PAA Laboratories, Pasching, Australia) containing 10% fetal bovine serum (FBS) and the 1640 medium (PAA Laboratories), in a 37°C, 5%CO2 incubator. The culture medium was changed once every 2–3 d. The AGS and BGC-823 cells in the logarithmic growth phase were collected and subjected to the following experiments.

Cell Transfection
The gastric cancer cells in the logarithmic growth was digested and inoculated onto the 6-well culture plate. When 60–80% confluence was reached, the desired transfected fragments (miR-30a-3p-mimics was purchased from GenePharma, Shanghai, China) were mixed and added into the corresponding wells, for further culture for 24–48 h.

Quantitative Real-Time PCR
RNA was extracted from gastric cancer cells using Trizol. The cDNA was obtained with the reverse-transcription using the commercially available kits, according to the manufacturer’s instructions. Quantitative real-time PCR was performed with the PrimeScript™ RT Reagent kit (Takara Bio, Japan) on the iQ5 Optical real-time PCR System machine (Bio-Rad, USA). The following primer sequences were used for amplification: RT miR-30a-3p, 5'-GTCGTATCCAGTGCGTGTCGTGGA GTCGGCAATTGCACTGGATACGACgctgcaa-3'; RT U6 5'-CGCTTTCAGAATTTGCGTGTCGTGGA AATTTGCGTGTCAT-3'; miR-30a-3p, forward 5'-ATCCAGTGGCTGTGTCGTG-3' and reverse 5'-TGCT CTTTCAGTCGGATGTG-3'; U6, forward 5'-GCTTCGGCA GCACATATACAAAT-3' and reverse 5'-CGCTTCAGC AATTTGCGTGTCAT-3'; MAD2L1, forward 5'-GTTCTT CTCAATTCGGCATA-3' and reverse 5'-GAGTGCCGTA TTTCAGTCGGATGTG-3'; and GAPDH, forward 5'-TGAAGG TCGGAGTCAGCAGATT-3' and reverse 5'-CCTGGAAGA TGGTGATGGGATT-3'. The 20-μL PCR system consisted of 10-μL 2×RealStar Green Power Mixture, 1-μL Forward-primer (10 μM), 1-μL Reverse-primer (10 μM), 2-μL cDNA and 6-μL ddH2O. The amplification conditions were as follows: 95°C for 10 min; 95°C for 15 s, 60°C for 1 min, and 72°C for 30 s, for totally 40 cycles. Relative expression levels of the target genes were calculated with the 2^-ΔΔCt method. GAPDH was used as internal reference.

Western Blot Analysis
Cells were harvested and lysed with lysis. Total protein concentration was determined with the BCA method. The protein samples were separated with 7.5–12.5% SDS-PAGE, and electronically transferred onto the PVDF membrane. The
membrane was incubated with anti-β-actin, anti-MAD2L1 primary antibody (Sanying Biological Co., Ltd., Wuhan, Hubei, China), at 4°C overnight. The membrane was then incubated with TBST-conjugated secondary antibody (Sanying Biological Co., Ltd.) at room temperature for 1 h. Color development was performed with the chemiluminescence detection method, and protein bands were imaged and analyzed with the Q550CW software (Leica, Heidelberg, Germany). β-actin was used as internal reference.

**MTT Assay**

Cell proliferation was assessed with the MTT kit (Sigma, St Louis, MO, USA). The cells in the logarithmic growth phase were harvested and seeded onto the 96-well plate. At 24 h, 48 h, and 72 h after seeding, respectively, 10 μL MTT was added into each well to incubate the cells for 4 h. The well was added with 150 μL DMSO, and the absorbance at 490 nm was determined on a microplate reader.

**Cloning Formation Detection**

The transfected cells in the logarithmic growth phase were seeded onto the 6-well plate. After 2 w of culture, the cells were fixed with 4% paraformaldehyde, and stained with crystal violet. The stained cells were then observed, photographed, and counted.

**Flow Cytometry**

The transfected cells in the logarithmic growth phase were inoculated onto the 6-well plate, and cultured for 1 d. Cells were fixed in 70% ethanol for 24 h, which were then treated with PI and RNase within the kit. Cell cycle distribution was detected by flow cytometry.

**Dual Luciferase Reporter Assay**

The HEK-293 cells were divided into the miR-30a-3p and pmirGLO empty vector, miR-30a-3p and pmirGLO-MAD2L1-WT (GenePharma), miR-30a-3p and pmirGLO-MAD2L1-MuT (GenePharma) co-transfection groups, respectively. Cells without treatment were used as control. The MAD2L1 wild-type and mutant fragments were synthesized by Genechem, Shanghai, China, as follows: wild-type MAD2L1, up 5′-cTATAGACATGCATGCTGAAAAATGT TTTATTAGTATAATGc-3′ and down 5′-tcgagCATTATACT AATAAAAAACATTTCACGATCATGTCATAgagct-3′; and mutant MAD2L1, up 5′-cTATAGACATGCATGCTGAAAAATGTT TTATTATTAGTATAATGc-3′ and down 5′-tcgagCATTATACT AATAAAAAACATTTCACGATCATGTCATAgagct-3′. These cells were inoculated onto the 96-well plates, and cultured for 24 h. The luciferase activity was detected by the microplate reader. Renilla was used as internal reference.

**Statistical Analysis**

The SPSS22.0 software was used for statistical analysis. Bioinformatics analysis was performed by the R language ggstatsplot package. Experimental data were processed by the GraphPad Prism7.0 software. Comparison was conducted with the Independent t-test. P<0.05 was considered as statistically significant.

**Results**

**Bioinformatics Analysis of Key Genes in Gastric Cancer**

First, the GEO database, including 22 gastric cancer samples and 8 normal gastritis epithelial samples, was used to analyze the differentially expressed genes in gastric cancer. The results showed that there were 876 differentially expressed genes in the normal sample and the tumor sample, including 423 up-regulated genes and 453 down-regulated genes (Supplementary Figure 1A). Then, the TCGA database, including 32 gastric cancer samples and 32 normal gastritis epithelial samples, was used. As shown in Supplementary Figure 1B, there were 904 differentially expressed genes in normal samples and tumor samples, including 438 up-regulated genes and 466 down-regulated genes. The Venn diagram was drawn with Venny2.1, and the differentially expressed genes obtained from the GEO database and the TCGA database were crossed to obtain 96 differentially expressed genes, including 62 up-regulated genes (Supplementary Figure 2A) and 34 down-regulated genes (Supplementary Figure 2B). These 96 differentially expressed genes were further analyzed to construct the PPI network (Supplementary Figure 3). By cluster analysis of the PPI network map using MCODE, 6 groups of genes were obtained (Supplementary Table 1). The first group of genes with the highest scores, including KIF11, NCAPD2, TOP2A, MCM3, MCM2, KIF23, and MAD2L1, was selected as the key genes of gastric cancer to be further investigated in this study.

**Bioinformatics Prediction and Validation of Interaction Between MAD2L1 and miR-30a-3p**

Based on the cluster analysis, MAD2L1, a cell cycle-associated gene, ranked the first and was selected for further analysis (Figure 1A and B). TCGA data showed that, compared with
the normal control group, the expression level of MAD2L1 was significantly increased in the tumors \( (P<0.001) \) (Figure 1C), which might be caused by decreased expression of its target miRNAs. Based on the linkedomics website, the miRNAs negatively correlated with MAD2L1 expression in gastric cancer were analyzed and studied (Figure 2A and B). In combination with the targetscan analysis, miR-30a-3p was selected and considered as the regulatory miRNA targeting on MAD2L1 (Figure 2C). Therefore, bioinformatics analysis was performed to further predict and investigate the relationship between miR-30a-3p and MAD2L1. The results from the TCGA data analysis showed that, compared with the normal control group, the expression level of miR-30a-3p in the gastric cancer tissue was significantly declined (Figure 3A), and the expression level of MAD2L1 was significantly negatively correlated with miR-30a-3p (Figure 3B).

To validate the interaction between miR-30a-3p and MAD2L1, dual luciferase reporter assay was performed. Our results showed that, compared with the normal control group, the luciferase was significantly down-regulated in the wild-type MAD2L1 group \( (P<0.05) \), while no significant changes were observed in the mutant group (Figure 4). Taken together, these results suggest that, MAD2L1 is a direct target of miR-30a-3p.

**Effects of miR-30a-3p Over-Expression on MAD2L1 in AGS and BGC-823 Cells**

To investigate the effects of miR-30a-3p on the expression of MAD2L1, the AGS and BGC-823 cells were transfected with miR-30a-3p, and the expression levels of MAD2L1 were detected. Our results from the quantitative real-time PCR indicated the significant over-expression of miR-30a-3p in the AGS and BGC-823 cells, compared with the normal control group \( (P<0.05) \) (Figure 5A and B). Moreover, our results from the Western blot analysis showed that, compared with the normal control group, the MAD2L1 protein expression levels were significantly reduced in both the AGS and BGC-823 cells over-expressed with miR-30a-3p \( (P<0.05) \) (Figure 5C and D).

MAD2L1 was significantly decreased at the protein level compared with the control group after over-expression of miR-30a-3p in AGS and BGC-823 cells (Figure 5C and D, \( P<0.05 \)). These results suggest that miR-30a-3p over-expression could
down-regulate the MAD2L1 expression in gastric cancer cell lines.

**Effects of miR-30a-3p Over-Expression on Proliferation and Cell Cycle of Gastric Cancer Cells**

To investigate the effects of miR-30a-3p on the proliferation ability and cell cycle on AGS and BGC-823 cells, the MTT assay, clone formation assay, and flow cytometry were performed, respectively. Our results from the MTT and clone formation assays showed that, after the transfection of miR-30a-3p, the proliferation of AGS and BGC-823 cells was significantly declined ($P<0.05$) (Figure 6A–F). On the other hand, our results from the flow cytometry showed that, compared with the normal control group, the cell proportions in the G0/G1 phase in the NC+ovMAD2L1 groups were significantly declined for both the AGS and BGC-823 cell lines, which were significantly elevated in the miR-30a-3p-mimics+ctrl groups (Figure 7A and B). However, no significant differences were observed in the G0/G1 cell proportions for the miR-30a-3p-mimics+ovMAD2L1 groups, for either of these two cell lines. Taken together, these results suggest that, MAD2L1 over-expression could encounter the effects of miR-30a-3p over-expression on the gastric cancer cells.

**Effects of miR-30a-3p and MAD2L1 Simultaneous Over-Expression on Gastric Cancer Cells**

Based on the above findings, the effects of miR-30a-3p and MAD2L1 simultaneous over-expression on the proliferation ability and cell cycle of gastric cancer cells were investigated. MTT showed that, compared with the normal control group, the proliferation abilities of both the AGS and BGC-823 cells in the miR-30a-3p-mimics+ctrl groups were significantly depressed, which were, however, enhanced in these two cell lines from the miR-30a-3p-mimics+ovMAD2L1 over-expression groups ($P<0.05$) (Figure 8A and B). On the other hand, our results from the cytometry showed that, compared with the normal control group, the cell proportions in the G0/G1 phase in the NC+ovMAD2L1 groups were significantly declined for both the AGS and BGC-823 cell lines, which were significantly elevated in the miR-30a-3p-mimics+ctrl groups (Figure 9A and B). However, no significant differences were observed in the G0/G1 cell proportions for the miR-30a-3p-mimics+ovMAD2L1 groups, for either of these two cell lines. Taken together, these results suggest that, MAD2L1 over-expression could encounter the effects of miR-30a-3p over-expression on the gastric cancer cells.

**Discussion**

Gastric cancer is one of the most common malignant tumors throughout the world, the mortality rate of which ranked the second place. Factors associated with gastric cancer development might include lacking nutrition, high salt and nitrate intake, low vitamin A and C diets, heavily smoking, food lacking refrigeration and drinking water with poor quality. Although the diagnosis and treatment of gastric cancer have made great progress, the disease prognosis is still poor, mainly...
due to the tumor cell metastasis, invasion and recurrence. Recently, studies have shown that a variety of genes and cellular pathways may be involved in the occurrence and development of gastric cancer. However, the limited knowledge of the exact molecular mechanisms underlying gastric cancer progression limits the treatment development of advanced gastric cancer. Therefore, deeper understanding the disease pathogenesis is essential for developing more effective diagnostic and therapeutic strategies of gastric cancers.

In this study, some target genes highly expressed in gastric cancer were screened based on the bioinformatics analysis, including the KIF11, NCAPD2, TOP2A, MCM3, MCM2, KIF23 and MAD2L1. KIF11 has been considered to be one of the pivotal genes with the highest connectivity. KIF11 is a conserved molecular regulator of cell growth and movement, which promotes the growth of refractory glioblastoma tumor-initiating cells and non-tumor initiating cells. Moreover, KIF11 could also accelerate the tumorigenesis and self-renewal of tumor-initiating cell populations. The localization of KIF11 in photoreceptor cells suggests the retinal ciliary disease. Progressive retinal degeneration of KIF11-associated retinopathy suggests that KIF11 play important roles not only in the eye development, but also in maintaining the retinal morphology and function. Moreover, for NCAPD2, it has been shown to be significantly associated with the risk of Parkinson’s disease (PD) in Chinese Han population and may serve as a potential genetic marker for the sporadic cases. The central granule proximal centrosome agglomerator affects the primary cilia formation, which is part of the DNA damage responses. For the gene of TOP2A, it has been reported to be involved in several molecular pathways in nasopharyngeal carcinoma, which can activate the carcinoma pathogenesis.
TOP2A induces the malignant features of pancreatic cancer by activating the β-catenin signaling pathway. MCM3 is one of the microsomal maintenance proteins that initiate DNA replication in eukaryotes, as a part of pre-replication complex. It has been reported that MCM3 is mainly involved in cell proliferation and tumorigenesis. In addition, MCM3 may play a vital role in neuronal apoptosis. Studies have also shown that MCM3 mutations can impair the neuronal proliferation and memory function in Drosophila. Another gene, MCM2, is a cancer-proliferating biomarker that is a permissive factor initiating the DNA replication. MCM2 promotes the conformational changes and drives the DNA unfolding at the origin of DNA replication. MCM2 over-expression is associated with low survival rate of lung cancer patients, and MCM2 dysregulation can affect lung cancer cell proliferation, cell cycle and migration. Furthermore, KIF23 is a member of microtubule-dependent molecules transporting the organelles and chromosomes within cells during cell division. Moreover, KIF23 is highly expressed in a variety of human malignancies, such as the primary lung cancer and liver cancer. However, the biological function of KIF23 in gastric cancer remains unclear. In addition, MAD2L1 is an important component of the spindle assembly checkpoint protein, which significantly activates the protein structural regulation and leads to the susceptibility of chromophobe cells. In addition, MAD2L1 mutations prevent the proliferation of aneuploid cells by activating the p53 pathway, and trigger the early maturation of human primary fibroblasts. It has been reported that, in the early stage of mitosis, MAD1L1 can directly recruit MAD2L1 and mediate chromosome centromere separation, which is very important for the interaction between the MAD2L1 and the anaphase promoting complex regulator.
CDC20.29 The direct interaction of CDC20 with the MAD2L1 and MAD1L1 complexes could inhibit the complex activity in the late stage of mitosis, eventually leading to loss of adhesion of sister chromatids during the mid-to-late conversion.30,31 Current studies have shown that disrupted MAD2L1 function in the mammalian cells can affect the spindle checkpoint function, which in turn leads to cell aneuploidy or tumorigenesis. MAD2L1± cells are accompanied by chromosome instability (CIN), which ultimately leads to the formation of lung cancer in mice.32 Moreover, over-expression of MAD2L1 could also lead to chromosome instability, cellular aneuploidy and tumorigenesis,33 and the chromosomal instability is one of the major driving factors of tumorigenesis.34 At the same time, previous studies have shown that up-regulation or down-regulation of MAD2L1 expression levels might increase the probability of mitotic errors and affects the recurrence-free survival,35,36 which is consistent with our bioinformatics predictions.

MicroRNA (miRNA) is a group of small non-coding RNAs consisting of 17–26 nt that mediates the degradation of target mRNA by binding to the 3'-untranslated sequence at the level of reverse transcription, thus inhibiting the expression of target genes.4,5 At present, it has been generally believed that miRNAs play important roles in regulating various biological processes, such as cell differentiation and migration.37–39 In recent years, a large number of studies have shown that the abnormal expression of miRNA is closely related to human cancer, which might be involved in and contribute to the tumor development, including the cell proliferation and migration, as the proto-oncogenes and tumor suppressor genes.40–42 Therefore, circulating miRNAs could be used as alternative biomarkers.43 Since one of the important functions of miRNA is to ensure the ideal target gene expression levels through subtle regulation of the gene expression. Therefore, miRNA may also be involved in the regulation of MAD2L1 expression level.44 Accordingly, the miRNAs negatively regulated to the expression of MAD2L1 in gastric cancer were analyzed by the bioinformatics analysis, with the targetscan analysis. The screening leads to the identification of miR-30a-3p. Moreover, it has been confirmed by the experiments that miR-30a-3p is the key miRNA regulating the expression of MAD2L1, in line with the results from the bioinformatics analysis. Based on these findings, miR-30a-3p inhibits the proliferation of gastric cancer cells by targeting on MAD2L1. After over-expression of miR-30a-3p, the MAD2L1 expression was down-regulated at the protein levels compared with the control group, while the proliferation abilities of AGS and BGC-823 cells were decreased, and cells were arrested in the G0/G1 phase. These results suggest that miR-30a-3p might have
an inhibitory effect on the development of gastric cancer. Moreover, it has been shown that miR-30a-3p can exert anti-cancer effects by targeting on both COX-2 and BCL9, which significantly affects the development of gastric cancer induced by Helicobacter pylori, providing new insights to better understand the mechanism underlying the disease development.\textsuperscript{45} Similarly, miR-944,\textsuperscript{46} miR-139,\textsuperscript{47} miR-511b,\textsuperscript{48} and miR-143-3p\textsuperscript{49,50} have also been shown to be the inhibiting factors of the malignant behavior of gastric cancer.

It has been also shown that MAD2L1 exerts function as a cancer-promoting factor in the gastric cancer. Moreover, miR-30a-3p acts as a tumor suppressor by regulating the MAD2L1 level in the AGS and BGC-823 cells, inhibiting the growth of tumor cells and affecting abnormal mitosis.

Figure 7 Effects of miR-30a-3p over-expression on cell cycle of gastric cancer cells. (A and B) The miR-30a-3p was over-expressed in AGS (A) and BGC-823 (B) cells, and the cell cycle was assessed. Compared with the control group, *P<0.05, **P<0.01.

Figure 8 Effects of miR-30a-3p and MAD2L1 over-expression on proliferation of gastric cancer cells. (A and B) The miR-30a-3p and MAD2L1 were over-expressed in AGS (A) and BGC-823 (B) cells, and the cell proliferation was assessed with the MTT assay. Compared with the control group, *P<0.05, **P<0.01.
(causing cell cycle arrest in the G0/G1 phase). These findings were in line with the above bioinformatics analysis. The results of rescue experiments showed that, after over-expression of miR-30a-3p together with MAD2L1, the AGS and BGC-823 cells exhibited increased proliferation abilities, with no alterations concerning the cell cycle, suggesting that the miR-30a-3p directly targets on and inhibits the expression of MAD2L1, further inhibiting the proliferation and growth of the AGS and BGC-823 cells. However, this study still has certain limitations. For example, miR-30a-3p may not be the only factor regulating the expression levels of MAD2L1. In fact, it has been reported that both miR-433-3p and miR-28-5p target on the MAD2L1 mRNAs.

Conclusion
In conclusion, our results from the bioinformatics analysis and experiments showed that MAD2L1 was a pro-oncogene up-regulated in the pathogenesis of gastric cancer. Moreover, miR-30a-3p down-regulated the expression level of MAD2L1, inhibited the proliferation of gastric cancer cells, and affected the cell cycle. These findings have certain diagnostic value for the clinical guidance of the treatment for gastric cancer.

Abbreviation
MAD2L1, mitotic arrest deficient 2 like 1.

Data Sharing Statement
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Author Contributions
All authors contributed to data analysis, drafting or revising the article, gave final approval of the version, and are accountable for all aspects of the work.

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Disclosure
The authors report no conflicts of interest in this work.

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