MicroRNA-454 inhibits tumor cell proliferation, migration and invasion by downregulating zinc finger E-box-binding homeobox 1 in gastric cancer

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Abstract. Gastric cancer is the fourth most common malignancy and the third leading cause of cancer-associated mortality globally. Accumulating studies have identified the involvement of microRNAs in the initiation and progression of gastric cancer. This study was aimed to investigate the expression, functional roles of microRNA-454 (miR-454) and its direct target gene in gastric cancer. According to the results, the expression level of miR-454 was demonstrated to be reduced in gastric cancer tissues and cell lines compared with corresponding distant non-tumor gastric tissues and human immortalized gastric epithelial, respectively. miR-454 mimic transfection led to inhibition of gastric cancer cells proliferation, migration and invasion in vitro. Bioinformatic analysis predicated that zinc finger E-box-binding homeobox 1 (ZEB1) is a potential target gene of miR-454. Luciferase reporter assays revealed that miR-454 directly targeted the 3′UTR of ZEB1. miR-454 overexpression significantly decreased the ZEB1 mRNA and protein expression levels. ZEB1 knockdown could mimic the tumor suppressive roles induced by miR-454 overexpression on gastric cancer cell proliferation, migration and invasion. In conclusion, the present study suggested that miR-454 under expression may be involved in gastric cancer initiation and progression, by promoting proliferation, migration and invasion by directly targeting ZEB1. miR-454/ZEB1-based targeted therapy may be a potential strategy for the treatment of gastric cancer.

Introduction

Gastric cancer, known as stomach cancer, is the fourth most common malignancy and the third leading cause of cancer-associated mortality globally (1,2), with an estimated 1,000,000 novel cases and 700,000 mortalities annually worldwide (3). Increasing evidence has demonstrated that a number of genetic alterations, *Helicobacter pylori* infection, smoking, alcohol drinking, habitual excessive salt intake and environmental factors, are contributed to gastric cancer carcinogenesis and progression (4). However, the molecular mechanisms underlying the pathogenesis of gastric cancer remain to be completely understood. Presently, the major treatment strategies for patients with gastric cancer are surgery, chemotherapy, radiotherapy and targeted therapy (5). Despite the progress in therapeutic treatments, the prognosis for patients with advanced stage gastric cancer remains disappointing, with a 5-year overall survival rate of 4% (6). Therefore, further research is required into the molecular mechanisms that lead to gastric cancer initiation and progression, and to identify novel therapeutic targets for the treatment of this disease.

Recent studies have identified the involvement of microRNAs (miRNAs/miRs) in the initiation and progression of several types of human cancer, including gastric cancer (7-9). miRNAs are a group of non-coding, endogenous, highly conserved and short RNAs of ~19-25 nucleotides, which act as important regulators of gene expression (10). To date, >2,000 miRNAs have been validated in humans and bioinformatic analysis indicated that miRNAs may regulate >60% protein-coding genes in the human genome (11). miRNAs accelerate the degradation and/or inhibit the translation of their target genes to induce transcriptional and/or post-transcriptional gene repression, and therefore to participate in various physiological and pathological processes, including cellular proliferation, cell cycle, apoptosis, invasion, metastasis, metabolism and differentiation (12-14).

In the context of cancer, miRNAs may act as oncogenes or tumor suppressors in tumorigenesis and tumor development, through negative regulation of tumor suppressors or oncogenes, respectively (15,16). miR-524-5p inhibits proliferation and invasion, and induces apoptosis in gastric cancer cells, by targeting matrix metalloproteinase (MMP)-2 and MMP-9 (17). miR-106a functions as an oncogene in gastric cancer by promoting tumor cell proliferation, migration and invasion, and inhibiting apoptosis in vitro and in vivo, via inhibition of metalloproteinase inhibitor 2 (18). Therefore, the investigation of miRNA expression patterns, roles and molecular mechanism may provide a therapeutic strategy against gastric cancer and other human cancer.
In the present study, miR-454 expression was evaluated in gastric cancer tissues as well as in gastric cancer cell lines. In addition, the effects of miR-454 on gastric cancer cell proliferation, migration and invasion in vitro were investigated. Furthermore, the molecular mechanism underlying the tumor suppressive roles induced by miR-454 in gastric cancer cells was investigated.

**Materials and methods**

**Ethics statement.** The present study was approved by the Research Ethics Committee of Cangzhou Central Hospital (Cangzhou, China), and followed the guidelines of the Declaration of Helsinki. Patients with gastric cancer agreed to participate in the present study and provided written informed consent.

**Tissue specimens and cell lines.** Primary gastric cancer tissues and corresponding distant non-tumor gastric tissues were obtained from 46 patients who underwent surgical resection in the Department of General Surgery at the Cangzhou Central Hospital. None of these patients had received chemotherapy, radiotherapy or targeted therapy prior to surgery. All fresh tissues were immediately frozen in liquid nitrogen following resection and stored at -80˚C until use.

The human gastric cancer cell lines (SGC-7901, BGC-823, AGS and MKN-45), human immortalized gastric epithelial (GES-1) and 293T cell line were purchased from American Type Culture Collection (Manassas, VA, USA), and were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) in a humidified atmosphere with 5% CO₂ at 37˚C.

**Oligonucleotide transfection.** The miR-454 mimic and miR-454 mimic negative control (NC) were purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China). The miR-454 mimics sequence was 5′UAG UGC AUA UGU CUU CT3′ and the NC sequence was 5′UUC UCCGAA CGGUCAGCTT3′. The small interfering RNA (siRNA) targeting ZEB1 and its NC were obtained from Guangzhou RiboBio Co., Ltd. (Shanghai, China). The ZEB1 siRNA sequence was 5′GUC GCU ACA AAC AGU UGU ATT3′ and the NC sequence was 5′GUC GCU ACA AAC AGU UGU ATT3′. Cells were transfected with miR-454 mimics (100 pmol), NC (100 pmol), ZEB1 siRNA (100 pmol) or NC siRNA (100 pmol) using Lipofectamine 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) in a humidified atmosphere with 5% CO₂ at 37˚C.

**Total RNA extraction.** Total RNA was extracted from tissues or cells with TRIzol agent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. To evaluate miRNA expression, cDNA was synthesized by using the TaqMan MicroRNA Reserve Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. qPCR was carried out using the Taqman MicroRNA assay kit (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermocycling conditions were as follows: 50˚C for 2 min, 95˚C for 10 min, 40 cycles of denaturation at 95˚C for 15 sec and annealing/extension at 60˚C for 60 sec. To evaluate mRNA expression, reverse transcription was performed using the M-MLV RT kit (Promega Corporation, Madison, WI, USA), followed by qPCR with using the SYBR® Green PCR Master mix (Toyobo Life Science, Osaka, Japan). The thermocycling conditions were as follows: 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 1 min. Relative expression of miR-454 and ZEB1 mRNA was normalized to U6 or β-actin, respectively. The primers were designed as follows: miR-454, 5′-ACCCCTATCAATA TTGTCTCTGC-3′ (forward) and 5′-GCCAGCACAGAATTA TACGAC-3′ (reverse); U6, 5′-CTCGTTCTCGCAGCACAT ATACT-3′ (forward) and 5′-ACGCTTCCAGAATTTTGCG TGTC-3′ (reverse); ZEB1, 5′-GCTTCTCAGACTCTGGGT CTTA-3′ (forward) and 5′-GTGTTCTCATCCTCTCCTCTTGG TG-3′ (reverse); GAPDH, 5′-GGGAGGTCGGTGTGAA CG-3′ (forward) and 5′-AGCCCTTCTTCCATGTTGAGTGA AGAC-3′ (reverse). All RT-qPCR reactions were performed using the ABI PRISM 7900 Real-time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The relative expression was calculated by the 2^ΔΔCq method (19).

**Cell Counting Kit-8 (CCK8) assay.** The CCK8 (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) assay was performed to determine cellular proliferation, according to the manufacturer's protocol. Cells were seeded in 96-well plates at a density of 2x10⁴ cells/well. Following incubation overnight, cells were transfected with miRNA or siRNA. Transected cells were incubated at 37˚C in a humidified incubator containing 5% CO₂ for 24, 48, 72 and 96 h. At different time points, 10 µl CCK8 solution was added to each well and cultured for an additional 2 h at 37˚C. The absorbance at 450 nm was recorded with a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

**Cell migration and invasion assay.** Transwell chambers (8-mm pores; BD Biosciences, San Jose, CA, USA) were used to evaluate the cell migratory and invasive ability. For the cell invasion assay only, transwell chambers were coated with Matrigel (BD Biosciences, San Jose, CA, USA) on the upper surface of the polycarbonate membranes. In both assays, 4x10⁴ transfected cells in 200 µl FBS-free culture medium were added to the upper chambers, while culture medium containing 20% FBS was added to the lower chamber as a chemotractant. Cells were incubated for 48 h at 37˚C in a humidified incubator with 5% CO₂, and the non-migratory or non-invasive cells on the upper surface of the chambers were wiped away with a cotton-tipped swab. The migratory and invasive cells were fixed with methanol at room temperature for 15 min, stained with 0.5% crystal violet at room temperature for 15 min and washed with PBS (Gibco, Grand Island, NY). A total of five fields from each chamber were randomly chosen under an inverted microscope (IX71; Olympus Corporation, Tokyo, Japan) and each experiment was repeated at least three times.
Western blotting. Cells were transfected with the miR-454 mimic, NC, ZEB1 siRNA or NC siRNA for 3 days. Transfected cells were lysed in radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) supplemented with proteinase/phosphatase inhibitors (Thermo Fisher Scientific, Inc.). Protein concentration was measured using the Bradford assay (Bio-Rad Laboratories, Inc.). Equal amounts of protein (30 µg/lane) were subjected to SDS-PAGE on 10% gels, transferred to polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA) and then blocked in 5% non-fat dry milk in TBS containing 0.05% Tween-20 (TBST) at room temperature for 2 h. Subsequently, the membranes were probed with mouse anti-human monoclonal ZEB1 (1:1,000; cat. no. sc-81428; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and mouse anti-human monoclonal GADPH (1:1,000; cat. no. sc-365062; Santa Cruz Biotechnology, Inc.) primary antibodies overnight at 4˚C. Following washing with TBST three times, the membranes were incubated with goat anti-mouse horseradish peroxidase-conjugated secondary antibody (sc-2005; 1:5,000; Santa Cruz Biotechnology, Inc.) for 1 h at room temperature. Membranes were washed again in TBST three times and protein bands were visualized using Pierce™ ECL Plus Western Blotting Substrate (Pierce; Thermo Fisher Scientific, Inc.). GADPH was used as an internal control to evaluate ZEB1 protein expression. ImageJ 1.49 (National Institutes of Health, Bethesda, MD) was used to analyze the densitometry.

Bioinformatics analysis and Luciferase reporter assay. The miR-454 target gene was predicted by Targetscan (www.targetscan.org).

Luciferase reporter vectors, PGL3-ZEB1-3'UTR wild type (Wt) and PGL3-ZEB1-3'UTR mutant (Mut), were synthesized and confirmed by Shanghai GenePharma Co., Ltd. (Shanghai, China). 293T cells were cotransfected with PGL3-ZEB1-3'UTR Wt or PGL3-ZEB1-3'UTR Mut, and miR-454 mimics or NC using Lipofectamine 2000 reagent. Transfected 293T cells were harvested at 48 h post transfection, and the luciferase activities were detected using the Dual-Luciferase Reporter assay system (Promega, Manheim, Germany), according to the manufacturer's instructions. Renilla luciferase activities were normalized to firefly luciferase activities.

Statistical analysis. Each experiment was repeated at least three times. Statistical analysis was performed with SPSS 15.0 (SPSS Inc., Chicago, IL, USA) using Student's t tests or one-way analysis of variance. The Student-Newman-Keuls test was used as a post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

miR-454 is downregulated in gastric cancer tissues and cell lines. To evaluate the expression level of miR-454 in gastric cancer, RT-qPCR was performed to determine its expression in gastric cancer tissues and corresponding distant non-tumor gastric tissues. Abnormal downregulation of miR-454 was observed in gastric cancer tissues compared with in corresponding distant non-tumor gastric tissues (P<0.05; Fig. 1A). The expression of miR-454 was also measured in four gastric cancer cell lines (SGC-7901, BGC-823, AGS and MKN-45) and a human immortalized gastric epithelial (GES-1). The results demonstrated that the miR-454 expression levels were reduced in the four gastric cancer cell lines examined compared with GES-1 (all P<0.05; Fig. 1B). Therefore, miR-454 may exhibit a suppressive property in gastric cancer.

miR-454 inhibits gastric cancer cell proliferation. To investigate whether miR-454 has the propensity to inhibit gastric cancer carcinogenesis and progression, an miR-454 mimic or NC was introduced into SGC-7901 and AGS cells, which express relatively decreased levels of miR-454. The upregulation of miR-454 in SGC-7901 and AGS cells 48 h following mimic transfection was confirmed using RT-qPCR (P<0.05; Fig. 2A).

The effect of miR-454 on gastric cancer cell proliferation was evaluated using CCK8 assays. Results from the CCK8 assays demonstrated that the growth rate of SGC-7901 and AGS cells was significantly suppressed by miR-454 overexpression at 96 h following transfection (P<0.05; Fig. 2B). These results suggested that miR-454 may act as a tumor suppressor in gastric cancer growth.

miR-454 inhibits gastric cancer cell migration and invasion. The roles of miR-454 on gastric cancer cell metastasis

Figure 1. Downregulation of miR-454 in gastric cancer tissues and cell lines. (A) miR-454 was downregulated in gastric cancer tissues compared with the corresponding distant non-tumor gastric tissues. (B) Expression levels of miR-454 were reduced in SGC-7901, BGC-823, AGS and MKN-45 cell lines, in comparison with the human immortalized gastric epithelial cell line GES-1. *P<0.05 vs. the respective control. miR, microRNA.
were assessed using cellular migration and invasion assays. The results revealed that restoration of miR-454 expression significantly inhibited cell migratory and invasive abilities in SGC-7901 (P<0.05; Fig. 3A) and AGS (P<0.05; Fig. 3B) cells. These results indicated that miR-454 may serve tumor suppressive roles in gastric cancer.

ZEB1 is negatively regulated by miR‑454 in gastric cancer by targeting putative binding sites in the 3'UTR. To elucidate the molecular mechanism underlying the biological functions of miR-454 in gastric cancer, Targetscan was used for predicating the potential target genes of miR-454. The results demonstrated that the 3'UTR of ZEB1 contained a putative binding site for miR-454 (Fig. 4A).

To investigate whether miR-454 binds directly to the 3'UTR of ZEB1, luciferase reporter assays were performed. 293T cells were transfected with PGL3-ZEB1-3'UTR Wt or PGL3-ZEB1-3'UTR Mut, together with an miR-454 mimic or NC. The results revealed that the luciferase activity of PGL3-ZEB1-3'UTR Wt was significantly inhibited by miR-454 overexpression (P<0.05; Fig. 4B), while upregulation of miR-454 exhibited no effect on the luciferase activity of PGL3-ZEB1-3'UTR Mut.

Subsequently, the regulatory effect of miR-454 on endogenous ZEB1 expression was investigated. SGC-7901 and AGS cells were transfected with an miR-454 mimic or NC, and RT-qPCR and western blotting were performed. The results demonstrated that, compared with NC group, ZEB1 mRNA (both P<0.05; Fig. 4C) and protein (both P<0.05; Fig. 4D) levels were reduced in SGC-7901 and AGS cells transfected with the miR-454 mimic. These results demonstrated that miR-454 was capable of decreasing ZEB1 expression by directly targeting its 3'UTR.

Knockdown of ZEB1 inhibits gastric cancer cell proliferation, migration and invasion. To investigate the roles of ZEB1 in gastric cancer, SGC-7901 and AGS cells were transfected
with ZEB1 siRNA or NC siRNA. Following transfection, the results of the RT-qPCR and western blot analysis demonstrated that ZEB1 siRNA decreased ZEB1 mRNA (both P<0.05; Fig. 5A) and protein (both P<0.05; Fig. 5B) expression in SGC-7901 and AGS cells. Subsequently, CCK8 assays, and cell migration and invasion assays were performed to investigate the effects of ZEB1 on gastric cancer cell growth and metastasis. The results revealed that proliferation 96 h following transfection (both P<0.05; Fig. 5C), and migration and invasion (all P<0.05; Fig. 5D) were significantly inhibited in ZEB1 siRNA-transfected SGC-7901 and AGS cells. These results may suggest that miR-454 inhibited proliferation, migration and invasion in gastric cancer cells by negatively regulating ZEB1.

Discussion

Increasing research into the role of miRNA on gastric cancer has indicated that abnormal expression of miRNAs appears in gastric cancer, and among these miRNAs, certain function as oncogenes or tumor suppressors (8,20,21). In the present study, the expression of miR-454 in gastric cancer tissues and cell lines was evaluated, and miR-454 was identified to be significantly downregulated in gastric cancer tissues and cell lines, suggesting that miR-454 may serve important roles in gastric cancer. Functional studies revealed that ectopic expression of miR-454 inhibited cell proliferation, migration and invasion of gastric cancer. Further studies demonstrated through bioinformatic analysis, luciferase reporter assays, RT-qPCR analysis and western blotting that ZEB1 was a direct target of miR-454. Furthermore, the effects of ZEB1 under expression were similar to those induced by miR-454 overexpression in gastric cancer cells. These results suggested that miR-454 functions as a tumor suppressor in gastric cancer through negative regulation of ZEB1.

The expression profile of miR-454 has been studied in multiple types of human cancer. For example, Niu et al (22) reported that expression levels of miR-454 were reduced in osteosarcoma tissues and cell lines compared with adjacent non-tumor tissues and a human osteoblast cell line, respectively. miR-454 was also identified to be downregulated in glioblastoma tissues (23). However, in colorectal cancer and uveal melanoma, miR-454 was significantly upregulated in tumor tissues and cell lines (24,25). Yu et al (26) revealed that miR-454 expression was increased in hepatocellular carcinoma tissues and cell lines. Zhou et al (27) demonstrated that high levels of miR-454 in hepatocellular carcinoma tissues were correlated with a poor 5-year overall survival. In addition, patients with hepatocellular carcinoma with high miR-454 expression exhibited decreased disease-free survival rates. Furthermore, multivariate analysis demonstrated that upregulation of miR-454 was an independent prognostic factor for 5-year overall survival and 5-year...
SONG et al: miR-454 IN GASTRIC CANCER

These studies suggested that miR-454 expression pattern differences have tissue specificity. The detailed functions of miR-454 in human cancer have been sufficiently investigated. Liang et al (24) demonstrated that miR-454 overexpression enhanced the proliferation and anchorage-independent growth of colorectal cancer cells, whereas miR-454 under expression decreased this effect. In osteosarcoma, ectopic expression of miR-454 suppressed cell proliferation and invasion (22). A study by Yu et al (26) revealed that miR-454 knockdown inhibited cell growth, invasion and epithelial-mesenchymal transition in hepatocellular carcinoma. Sun et al (25) demonstrated that restoration of miR-454 expression promoted cell proliferation, colony formation, invasion and induction of cell cycle in uveal melanoma. Fang et al (23) reported that upregulation of miR-454 inhibited glioblastoma cell proliferation and arrested cells at the G0/G1 phase. However, to the best of the authors’ knowledge, no previous studies have investigated the roles of miR-454 in gastric cancer. The present study indicated that miR-454 acted as a tumor suppressor in gastric cancer by inhibiting tumor cell growth and metastasis. These results suggested that miR-454 performs an essential function in these types of cancer, and may have the potential to be developed as an anticancer therapeutic agent.

The identification of miR-454 target genes is essential for understanding its molecular mechanism in carcinogenesis and tumor development, and for the development of novel targeted
disease-free survival (28). These studies suggested that miR-454 expression pattern differences have tissue specificity.

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The identification of miR-454 target genes is essential for understanding its molecular mechanism in carcinogenesis and tumor development, and for the development of novel targeted
therapies. Multiple target genes have been validated, including CYLD lysine 63 deubiquitinase (24) in colorectal cancer, MET proto-oncogene receptor tyrosine kinase (22) in osteosarcoma, chromodomain helicase DNA binding protein 5 (26) in hepatocellular carcinoma, phosphatase and tensin homolog in uveal melanoma (25) and pyruvate dehydrogenase kinase 1 (23) in glioblastoma. In the present study, ZEB1 was successfully identified to be a novel direct target gene of miR-454. ZEB1, a member of the zinc finger family, is located on the short arm of human chromosome 10 (28). In gastric cancer, ZEB1 was highly expressed and high ZEB1 expression was correlated with peritoneal dissemination, and was an independent prognostic factor. Logistic regression analysis demonstrated that overexpression of ZEB1 was an independent risk factor for peritoneal dissemination. Furthermore, immunohistochemical analysis indicated that ZEB1 was highly expressed in primary cancer and peritoneal dissemination samples (29). Another study also demonstrated that ZEB1 expression was associated with an increased tumor node metastasis stage and increased invasion in gastric cancer. Downregulation of ZEB1 markedly decreased the invasive and metastatic ability of gastric cancer cells (30). In the present study, ectopic miR-454 expression in gastric cancer cells demonstrated that miR-454 inhibits cell proliferation, migration and invasion via downregulation of ZEB1. These results suggested that miR-454/ZEB1 may present a therapeutic approach for patients with gastric cancer.

In conclusion, the results of the present study demonstrated that miR-454 acted as a tumor suppressor that is involved in the carcinogenesis and progression of gastric cancer. Restoration of miR-454 expression inhibited cell proliferation, migration and invasion in gastric cancer. Therefore, miR-454 may be a novel therapeutic target in gastric cancer.

References