MicroRNA-21 expression is associated with the clinical features of patients with gastric carcinoma and affects the proliferation, invasion and migration of gastric cancer cells by regulating Noxa

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Abstract. The expression levels of microRNA-21 (miR-21) are increased in a number of types of solid tumors. However, the association between miR-21 expression and clinical features of patients with gastric carcinoma, and gastric cancer proliferation, invasion and migration remains to be elucidated. The present study investigated the effect of miR-21 on the clinical features, proliferation, invasion and migration of gastric cancer and the underlying mechanisms associated with Noxa. Reverse transcription quantitative polymerase chain reaction (RT-qPCR) was performed to detect the expression levels of miR-21 and Noxa in samples of gastric cancer tissue and matched, adjacent, non-tumor tissue. The association between miR-21 expression and the clinical features of patients with gastric carcinoma, as well as the correlation between the mRNA and protein expression levels of miR-21 and Noxa were analyzed. SGC-7901 gastric cancer cells were cultured in vitro and transfected with an miR-21 mimic. The effect of miR-21 upregulation on proliferation and the cell cycle was determined using the MTT assay and flow cytometry. In addition, migration and invasion of SGC-7901 cells were observed using the Transwell assay. The target gene of miR-21 was identified using bioinformatics software and a dual luciferase reporting system. The effect of miR-21 upregulation on Noxa expression levels in SGC-7901 cells was also analyzed by RT-qPCR and western blotting. Increased levels of miR-21 expression and decreased levels of Noxa expression were observed in gastric cancer tissue samples when compared with the adjacent non-tumor tissue samples. An increased miR-21 expression level was identified as a risk factor for advanced stage gastric cancer, lymph node metastasis and larger primary tumors. Furthermore, the overexpression of miR-21 inhibited Noxa expression levels in SGC-7901 cells. Therefore, high levels of miR-21 expression may induce gastric cancer migration and invasion via the downregulation of Noxa expression.

Introduction

Gastric cancer is a common type of gastrointestinal cancer worldwide (1). In China, the incidence of gastric cancer is the highest of the digestive system neoplasms and second for all malignant tumors (2). Recent studies have observed that there are ~500,000 diagnoses of gastric cancer in China every year, accounting for ~50% of diagnoses worldwide. Furthermore, stomach cancer results in 10% of cancer-associated mortalities (3). Investigations into the association between microRNA (miR) and stomach cancer have increased. miRs are a class of non-coding RNA 18-21 nt in length. They are highly expressed in eukaryotic cells, and contribute to the regulation of cell growth, development and apoptosis (4,5). Numerous studies have shown that the occurrence and progression of tumors is accompanied by an abnormal change in miR expression levels (6,7). A number of studies have demonstrated increased expression levels of miR-21 in various solid tumors, for example, Volinia et al (8) observed that miR-21 was upregulated in breast and lung cancer, and was associated with a poor prognosis. However, the association between miR-21 expression and gastric cancer proliferation, invasion and migration has not been widely investigated, and the underlying mechanism of the potential target protein miR-21 remains to be elucidated. The present study aimed to validate the association between miR-21 and the clinical features of patients with gastric cancer, as well as the proliferation, invasion and migration of gastric cancer cells by analyzing samples of gastric cancer tissues and the results of in vitro cellular experiments. Bioinformatics and dual luciferase report analysis in the SGC-7901 gastric cancer cell line was performed in the current study and demonstrated that Noxa, a member of the B-cell lymphoma 2 (Bcl-2) family with pro-apoptotic effects (9-11) is the target gene of miR-21.
Materials and methods

Clinical data of patients. Paraffin-embedded tissue samples were collected from 40 patients, who had undergone radical resection for gastric cancer in the Fifth Affiliated Hospital of Zhengzhou University (Zhengzhou, China) between January 2012 and May 2014. Patients included 22 males and 18 females, with a median age of 60 years (range, 43-76 years). All patients were diagnosed by endoscopy and pathology. In eight cases the tumor size was <3 cm, in 10 cases the tumor size was 3-5 cm, and in 22 cases the tumor size was ≥5 cm; 24 cases were intestinal-type and 16 cases were diffuse-type. There were 14 cases with lymph node metastasis, and 26 cases without lymph node metastasis. TNM staging was conducted (12) and 7 cases were stage I, 9 cases were stage II, 18 cases were stage III, and 6 cases were stage IV (Table I). The patients had not received preoperative chemotherapy or radiotherapy. The present study was approved by the ethics committee of the Fifth Affiliated Hospital of Zhengzhou University (Zhengzhou, China).

Detection of miR-21 in tissue samples. The miR was extracted and purified from cancer tissues and matched adjacent non-tumor tissues of the 40 patients using a paraffin-embedded tissue microRNA rapid extraction kit (Beijing Bioteke Biotechnology Co., Ltd., Beijing, China) according to the manufacturer's protocols. In brief, tissue samples were treated with dimethylbenzene to remove the paraffin and then guanidine isothiocyanate was added for tissue lysis. Lysates were treated with chloroform and centrifuged at 12,000 x g for 15 min according to the manufacturer's instructions of the protein extract kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA). miRNA products were purified using a silicon substrate membrane adsorption column from the extraction kit. Reverse transcription (RT) of miR into cDNA was performed using a One Step PrimeScript miRNA cDNA Synthesis kit (Takara Biotechnology, Co., Ltd., Dalian, China) according to the manufacturer's protocols. For each sample, 2 µg miR was reverse transcribed in the reaction system, including miR reaction buffer mix (containing oligo-dt and RT primers miRNA) and PrimeScript® RT enzyme mix (containing poly(A) polymerase and reverse transcriptase). For setting the RT control, the same system was used, however, water was used to replace miR. The reaction conditions were as follows: 60 min poly(A) polymerase reaction and reverse transcription at 37˚C and 5 sec at 85˚C for inactivating the enzymes. The cDNA was amplified by RT quantitative polymerase chain reaction (qPCR) with the SYBR® Premix Ex Taq™ II kit (Takara Biotechnology, Co., Ltd.) according to the manufacturer's protocols. The primers were as follows: Sense, 5'-GCTGCTTATCATAGCTGTG-3' for miR-21; and sense, 5'-CTCGCTTGGCGACACA-3' for U6 snRNA, which was used to normalize the data. The antisense primer, Uni-miR qPCR, was obtained from the One Step PrimeScript miRNA cDNA Synthesis kit. The relative expression level of miR-21 was expressed by 2^ΔΔCq (for tumor tissue/non-tumor tissues), where 2^ΔΔCq=Cq (target)-Cq (U6 snRNA) (13).

Detection of Noxa expression levels in tissue samples. The total RNA of tumor and non-tumor tissues was extracted using TRIzol® (Thermo Fisher Scientific, Inc.), and RT of miR into cDNA was performed using the GoScript Reverse Transcription kit [Promega (Beijing) Biotech Co., Ltd., Beijing, China] according to the manufacturer's protocols. Fluorescent RT-qPCR was implemented using a GoTaq® qPCR kit [Promega (Beijing) Biotech Co., Ltd.] according to the manufacturer's protocols. Primers used were as follows: Sense, 5'-TTGTTGTTCAGTCGGTCC-3' and antisense, 5'-CTCGGTTGATGCCTTCTTGCC-3' for Noxa; and sense, 5'-ACACAGTCCATGCCATCAC-3' and antisense, 5'-TCCCACACCTGTTGGTGA-3' for GAPDH, which was used to normalize the data. The relative expression level of Noxa was expressed by 2^ΔΔCq (for tumor tissue / non-tumor tissue).

Total protein of the tissue sample was extracted by radioimmuno precipitation assay (RIPA) lysis buffer (Thermo Fisher Scientific, Inc.) containing 1% protease inhibitor cocktail (500 µl/well; Thermo Fisher Scientific, Inc.). Electrophoresis was performed with 12% polyacrylamide gel (Applygen Technologies Inc., Beijing, China) at 100 V for 1.5 h and for each sample, 45 µg protein was loaded onto each lane. Following electrophoresis, the protein bands were transferred to a polyvinylidene fluoride membrane (Applygen Technologies, Inc.) with a 200-ma constant current for 1.5 h. The membranes were blocked for 1 h with 5% skimmed milk powder at room temperature prior to incubation with mouse monoclonal anti-human Noxa antibody (1:500; Santa Cruz Biotechnology, Inc., Dallas, USA, TX; cat. no. sc-398873) and mouse monoclonal anti-human β-actin antibody (1:1,000; Santa Cruz Biotechnology, Inc.; cat. no. sc-47778) at 4˚C overnight. The membranes were washed three times with phosphate-buffered saline with Tween-20 (Applygen Technologies, Inc.) to remove excess antibodies, and the horseradish peroxidase-labeled goat anti-mouse IgG (1:500; Santa Cruz Biotechnology, Inc; cat. no. sc-2005) secondary antibody was added and incubated for 2 h at room temperature. The membranes were washed to remove excess antibodies and color development of bands was visualized using an enhanced chemiluminescence color development kit (Applygen Technologies, Inc.), and the Noxa protein band intensity was analyzed using the BandScan system (Tanon 5200 Image Analysis System; Tanon Science & Technology Co., Ltd., Shanghai, China).

Culture of the SGC-7901 gastric cancer cell line. The SGC-7901 human gastric cancer cell line was purchased from the cell bank of the Shanghai Institute of Biochemistry and Cell Biology (Chinese Academy of Sciences, Shanghai, China). Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Inc.) was used for cell culture and 0.25% trypsin (Thermo Fisher Scientific, Inc.) was used for cell digestion. Cells were cultured at 37˚C in 5% CO₂ for 24-48 h.

Upregulation of miR-21. miR-21 mimics were designed and synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). SGC-7901 cells in the logarithmic growth phase were transfected with the miR-21 mimic and the miR-negative control (NC) using a Lipofectamine™ 2000 transfection kit (Thermo Fisher Scientific, Inc.). The transfection was conducted according to the manufacturer's protocol. Overexpression of miR-21 was verified using RT-qPCR 48 h following transfection.
Effect of miR-21 upregulation on SGC-7901 cell proliferation and the cell cycle. The non-transfected (control), miR-21 mimic-transfected and miR-NC-transfected SGC-7901 cells were maintained in 96-well plates at a density of 1x10^4/well for 6, 12, 24 or 48 h. MTT reagent (10 µl) was added to each well, and after 4 h, DMSO (100 µl) was added to each well. The optical density (OD) values at 490 nm were determined using a microplate reader (UV/Vis Absorbance Spectra; BMG Labtech, Ltd., Cary, NC, USA). For cell cycle analysis, transfected and non-transfected cells were fixed in cold 70% ethanol and stained with 50 µg/ml propidium iodide (Sigma-Aldrich, St. Louis, MO, USA) and incubated for 30 min at room temperature. Analysis was performed using a BD FACS Calibur flow cytometer (Becton, Dickinson and Company, San Jose, CA, USA) within 15 min after incubation. CellQuest software (version 5.1; Becton, Dickinson and Company) was used to analyze the cell cycle and apoptosis rate.

Effect of miR-21 upregulation on SGC-7901 cell migration and invasion. The non-transfected (control), miR-21 mimic-transfected and miR-NC-transfected SGC-7901 cells were cultured in an uncoated Transwell chamber at a density of 1x10^5/well in the upper layer (membrane pore size, 8 µm) to detect the migration of cells. The above-mentioned cells were also cultivated in a Matrigel-coated Transwell chamber (Corning Inc., Corning, NY, USA) in the upper layer (membrane pore size, 8 µm) to detect the invasive ability of the cells. The serum-free DMEM medium was used for the upper chamber and the medium containing 10% FBS was placed in the lower
chamber. Following culture for 6 h, the cells that had remained in the upper chamber were removed, and the number of cells that had migrated or invaded the lower layer was estimated using Giemsa (Applygen Technologies, Inc.) staining.

Prediction and verification of miR-21 targets. The target gene of miR-21 was predicted using the bioinformatics prediction software, PicTar (http://pictar.mdc-berlin.de/) and TargetScan5.0 (Whitehead Institute for Biomedical Research, Cambridge, MA, USA). Following integration of the results from the software, the candidate target genes were further confirmed by the dual luciferase reporting system. Shanghai Sangong Pharmaceutical Co., Ltd. (Shanghai, China) designed and constructed the luciferase reporter plasmid containing wild-type Noxa 3'-untranslated region (UTR) and mutant Noxa 3'-UTR, designated pMIR-Noxa-wild and pMIR-Noxa-mut, respectively. The SGC-7901 cells were divided into four groups as follows: miR-21 mimic + pMIR-Noxa-wild; miR-NC + pMIR-Noxa-wild; miR-21 mimic + pMIR-Noxa-mut; and miR-NC + pMIR-Noxa-wild. Following transfection, cells were cultivated in 96-well plates at a density of 1x10^4/well and cultured for 48 h, prior to the addition of 100 µl lysis buffer (Applygen Technologies, Inc.) to each well. The supernatant was collected following lysis, and 10 µl of supernatant was added to the 40 µl firefly luciferase substrate and mixed evenly for 15 sec prior to determining the fluorescence intensity. The remaining supernatant was added to 40 µl Renilla luciferase substrate, and mixed evenly before determining the fluorescence intensity. The ratio of firefly to Renilla luciferase intensity was calculated to demonstrate the effect of miR-21 on the expression level of Noxa mRNA.

Effect of miR-21 overexpression on Noxa expression levels. The total RNA of non-transfected, miR-21 mimic-transfected and miR-NC-transfected SGC-7901 cells was extracted using TRIzol®. The RT of miR into cDNA was performed using the GoScript Reverse Transcription kit [Promega (Beijing) Biotech Co., Ltd.] according to the manufacturer's protocols. Fluorescent RT-qPCR was implemented using a GoTaq® qPCR kit according to the manufacturer's protocols. The sequence of the primers for Noxa was as described above. Statistical analysis was performed using SPSS 13.0 statistical software (SPSS, Inc., Chicago, IL, USA). The normality data are expressed as means ± standard deviation and the non-normal data are expressed as medians. Data were compared between two or multiple groups using Student's t-test and one-way analysis of variance, respectively. The non-parametric test was performed by Mann-Whitney rank-sum test and χ² test. P<0.05 was considered to indicate a statistically significant difference.

Results

Increased expression levels of miR-21 in gastric cancer tissue are associated with the clinical features of gastric cancer. As presented in Fig. 1, RT-qPCR detection demonstrated that the miR-21 expression level in the cancer tissue was significantly increased (P<0.05). Using the median of the miR-21 expression levels (2^(-ΔΔCq)) in gastric cancer tissue as the limit, all patients with gastric cancer were divided into an miR-21 high expression group [2^(-ΔΔCq) (miR-21)>4.23] and an miR-21 low expression group [2^(-ΔΔCq) (miR-21)<4.23]. Analysis of the association between the clinical features of patients (gender, age, tumor size, histological type, lymph node metastasis and TNM staging), and the level of miR-21 expression (Table I) demonstrated that the expression level of miR-21 was not associated with the patient's age, gender, and histological type (P>0.05); however was associated with the tumor size, TNM stage and presence of lymph node metastasis (P<0.05). Univariate analysis demonstrated that increased expression levels of miR-21 were associated with
larger tumor size [diameter, >5 cm; odds ratio (OR), 5.34; 95% confidence interval (CI), 1.35‑21.15; P<0.05], high TNM stage (stage III and IV; OR, 5.00; 95% CI, 1.27‑19.68; P<0.05), and the emergence of lymph node metastasis (OR, 8.33; 95% CI, 1.90‑6.44; P<0.05).

Expression levels of Noxa in gastric cancer tissue were decreased and negatively correlated with the expression level of miR‑21. As presented in Fig. 2, RT‑qPCR detection
demonstrated that the mRNA level of Noxa in tumor tissue was significantly lower than that of the adjacent non-tumor tissue samples (P<0.05). Consistent with the RT-qPCR results, the western blot analysis indicated that the Noxa protein expression level in the tumor tissue was decreased (Fig. 3). The correlation analysis (Fig. 4) demonstrated that the mRNA expression level of Noxa in the gastric cancer tissue samples was inversely proportional to the level of miR-21 mRNA expression (r=-0.782; P<0.05).

Upregulation of miR-21 promotes growth of SGC-7901 cells. As presented in Fig. 5, MTT analysis indicated that following upregulation of miR-21, the rate of proliferation of SGC-7901 cells was significantly increased. Following culture for 48 h, the OD value of SGC-7901 cells following upregulation of miR-21 was significantly higher than that of the non-transfected (control) and miR-NC-transfected SGC-7901 cells (P<0.05). Cell cycle analysis revealed that following transfection of the miR-21 mimic, the ratio of G2/M of SGC-7901 cells decreased, while the proportion of cells in the G1 phase increased, indicating active DNA synthesis in the cells (Fig. 6).

Increased expression levels of miR-21 promoted the migration and invasion of SGC-7901 cells. As presented in Fig. 7, the Transwell chamber culture analysis demonstrated that, compared with the non-transfected SGC-7901 cells and SGC-7901 cells transfected with miR-NC, the SGC-7901 cells transfected with miR-21 mimic exhibited marked migration and invasion.

Noxa is a potential target of miR-21. The analysis results using PicTar and TargetScan5.0 software indicated that the microRNA gene binding free energy of the Noxa gene binding with miR-21 was lower than other potential miR-21-targeted mRNAs, indicating that miR-21 can easily bind with Noxa, and thus Noxa may be a target of miR-21. The complementary base pairing existed between seven bases of miR-21 and the 3’-UTR. Thus, the Noxa gene may be a potential target gene of miR-21. The dual luciferase report analysis (Fig. 8) demonstrated that the fluorescence intensity of SGC-7901 cells transfected with miR-21 mimic + pMIR-Noxa-wild was significantly lower than that of the cells transfected with miR-NC + pMIR-Noxa-wild (P<0.05). The fluorescence value of SGC-7901 cells transfected with miR-21 mimic + pMIR-Noxa-mut was not decreased significantly compared with the cells transfected with miR-NC + pMIR-Noxa-mut, suggesting that miR-21 may exert an inhibitory effect by functioning at the 3’-UTR of Noxa. RT-qPCR and western blotting (Figs. 9 and 10) confirmed that Noxa may be a target gene of miR-21.

Overexpression of miR-21 reduced the expression levels of Noxa. As presented in Fig. 9, RT-qPCR analysis demonstrated that the mRNA level of Noxa in SGC-7901 cells transfected with the miR-21 mimic was decreased compared with the non-transfected SGC-7901 cells and SGC-7901 cells transfected with miR-NC. It was decreased by 0.63±0.03-fold vs. the non-transfected SGC-7901 cells (P<0.05). The results of the western blot analysis were consistent with those of the RT-qPCR, demonstrating that an increased level of miR-21 augments the expression level of Noxa protein in SGC-7901 cells (Fig. 10).

Discussion

miRs have wide-ranging applications as tumor biomarkers. However, the association between miRs and tumorigenesis, metastasis and infiltration, as well as the underlying mechanisms, requires further investigation. miR-21 is present in a variety of solid tumors. Numerous studies have demonstrated increased expression levels of miR-21 in breast, hepatocellular, lung, pancreatic and ovarian cancer, and glioma (14,15). Furthermore, increased expression levels of miR-21 are associated with poor patient prognosis (16-19). In the present study, the change of miR-21 expression level in patients with gastric cancer was analyzed. The tissue sample analysis demonstrated that the expression level of miR-21 in the cancer tissue samples was significantly increased compared with that of non-tumor tissue samples. Further analysis also indicated that the expression level of miR-21 was associated with the clinical features of patients with gastric cancer; for example, the miR-21 expression level of patients at an advanced TNM stage and patients with lymph node metastasis was increased. Following upregulation of miR-21, SGC-7901 proliferation was observed to accelerate. In addition, the cell cycle analysis indicated an increased proportion of cells in S phase with greater invasion and migration capability.

Noxa is a member of the Bcl-2 family, which is located in the mitochondrial membrane and exerts pro-apoptotic effects (20,21). Its mechanism of pro-apoptosis is associated with the mitochondrial cytochrome c signaling pathway. Noxa increases the permeability of the mitochondrial membrane, promoting the release of cytochrome c (9,10). It is a downstream gene of p53 and when cells are damaged following stimulation, p53 binds with the upstream promoter sequence of Noxa to enhance expression levels. Thus, the pro-apoptotic effects of Noxa are dependent on p53 (22-24). In tumor cells, Noxa expression levels decrease, which may affect tumor cell apoptosis and reduce the effect of therapeutic agents (25).

For example, Kuwahara et al (26) found that decreased Noxa expression levels may result in decreased sensitivity to chemotherapeutic agents in malignant rhabdoid tumor. Ehrhardt et al (27) observed that following p53 and Noxa gene knockout, apoptosis of acute lymphoblastic leukemia, induced by conventional cytotoxic therapeutic agents combined with betulnic acid, decreased. Consistent with these previous findings, decreased expression levels of Noxa were observed in gastric cancer tissue samples in the present study. In addition, Noxa expression levels in gastric cancer patients were observed to be negatively correlated with the miR-21 expression level. The bioinformatics software and dual luciferase reporter enzymes indicated that Noxa is the target gene of miR-21 and upregulation of miR-21 in SGC-7901 cells reduced the Noxa expression levels. Therefore, miR-21 and Noxa may serve as targets for the treatment of gastric cancer. However, the present study did not clarify the effect of variations in the expression level of Noxa on the gastric cancer cells, which requires further investigation.

In conclusion, miR-21 affects the proliferation, invasion and migration of gastric cancer cells via Noxa. Increased expression levels of miR-21 and decreased expression levels of Noxa decreases apoptosis of tumor cells and results in a poor prognosis for gastric cancer patients.


