MicroRNA-21 stimulates gastric cancer growth and invasion by inhibiting the tumor suppressor effects of programmed cell death protein 4 and phosphatase and tensin homolog

Ling Li1, Liya Zhou1, Yuwen Li1, Sanren Lin1, Ciprian Tomuleasa2

1Department of Gastroenterology, Third Hospital of Peking University, Health Science Center, Beijing, P.R. China; 2Research Center for Functional Genomics and Translational Medicine of the Iuliu Hatieganu University of Medicine and Pharmacy, Cluj Napoca, and Department of Hematology, Ion Chiricuta Cancer Center, Cluj Napoca, Romania

Summary

Purpose: MicroRNA-21 (miR-21) is abnormally expressed in many solid cancers, such as gastric adenocarcinoma, and regulates some targets involved in cancer initiation and progression. In this study, we investigated the function of miR-21 in two gastric cancer cell lines, as well as its potential targeting of the tumor suppressor genes phosphatase and tensin homolog (PTEN) and programmed cell death protein 4 (PDCD4).

Methods: The first step was to use quantitative (q) RT-PCR in order to verify the overexpression of miR-21 in two different gastric cancer cell lines (SGC-7901 and MKN-45) transfected with miR-21 mimic. Western blotting confirmed the qRT-PCR data in a set of rescue experiments in which miR-21 mimic, inhibitor, and non specific mimic (NSM) were used to transfect the two gastric cancer cell lines. The protein levels of miR-21 targets PTEN and PDCD4 were estimated. Then, we evaluated its effect on tumor growth and invasion potential on the two different gastric adenocarcinoma cell lines.

Results: qRT-PCR results proved that miR-21 was overexpressed in gastric cancer cells transfected with miR-21 mimic. Western blot results further suggested that PTEN and PDCD4 were regulated by miR-21, as miR-21 inhibitor increased the expression of PTEN and PDCD4 proteins and significantly reduced cell proliferation, migration, and invasion. In the control experiment miR-21 mimic significantly inhibited the expression of PTEN and PDCD4 proteins in the two gastric cell lines, leading to an increase in cell invasion and migration. Furthermore, miR-21 mimic inhibited the apoptosis of the two gastric cancer cell lines.

Conclusions: miR-21 is overexpressed in gastric cancer and its aberrant expression may have important role in gastric cancer growth and dissemination by modulating the expression of the tumor suppressors PTEN and PDCD4, as well as by modulating the pathways involved in mediating cell growth, migration, invasion and apoptosis. Targeting miR-21 may help develop novel therapeutics for gastric cancer, once its pathophysiology is completely investigated.

Key words: gastric cancer, miR-21, PDCD4, PTEN, tumor suppressor genes

Introduction

Gastric cancer is one of the most common and deadly malignancies worldwide, with approximately 870,000 new cases [1] accounting for approximately 650,000 deaths per year [2]. Its prognosis is poor [3], partly due to late diagnosis [4]. A better understanding of the molecular mechanisms underlying gastric carcinogenesis, leading to the development of better early diagnostic tools, is thus imperative. Earlier gastric cancer diagnosis will likely be a consequence of an improved understanding of the specific carcinogenetic pathways leading to the appearance of this disease.

Recent studies support the crucial role of miRs in the genesis and homeostasis of a variety of solid tumors, including gastric cancer [5-8]. As early diagnosis and improved treatment are key elements in future anticancer research, the role
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The aim of this paper was to investigate the role of miR-21 in human gastric cancer in modulating the expression of the tumor suppressor genes PTEN and PDCD4. This may lead to better understanding of the genetic and epigenetic mechanisms of gastric cancer initiation and progression, which in turn may provide new targets for diagnosis and treatment.

Methods

Cell lines and cell culture

Human gastric cancer cell lines SGC-7901 and MKN-45 were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen, Carlsbad, CA, USA). Cells were cultured in a humidified atmosphere in an incubator with 5% CO2 at 37°C.

Cell transfection

Hsa-miR-21 mimic, Hsa-miR-21 inhibitor and NSM (Table 1) were purchased from GenePharma (Shanghai, China). The pyrimidine nucleotides in the miR-21 mimics, miR-21 inhibitors and NSM were substituted by their 2-O-methyl analogs to improve RNA stability. SGC-7901 and MKN-45 cells were plated one day before transfection. A final concentration of 50 nM of RNA mimic or 200 nM of inhibitor and their respective NSM were transfected using LipofectamineRNAi-max (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After 48 or 72 h, cells were harvested and cultured for further experiments.

RNA extraction and RT-PCR

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The RT and PCR primers for miR-21 and U6 were purchased from Applied Biosystems (Foster City, CA, USA) and the expression of U6 and beta-actin (forward) were used as internal control. Primers were purchased from SBS Gentech (Beijing, China). RT-PCR was performed using Taqman kit.

MTT proliferation assay

Cells were seeded at a density of 10,000 cells per well into 96-well culture plates, one day after transfection with the miR-21 mimic or inhibitor. Cells were further cultured for another 6, 24, 48 and 72 h. To assess cell proliferation, cells were incubated with 20 µl of MTT at a final concentration of 0.5mg/ml for 4 h at 37°C. After removing the culture supernatant, 150 µl of dimethyl sulfoxide (DMSO) were added to solubilize the crystals for 20 min at room temperature. Absorbance was measured at a wavelength of 492 nm with a fluorescence plate reader (Molecular Devices, Sunnyvale, CA, USA).

Apoptosis assay

5 x 10^5 SGC-7901 and MKN-45 cells were transfected with 60 nM mimic or inhibitor of miR-21 or NSM, respectively. After 72 h, cells were double-stained with propidium iodide and annexin-V (Vybrant Apoptosis Assay Kit, Invitrogen, USA). Fluorescence intensity was measured using a flow cytometer to assess early apoptotic cells, defined as those staining only with annexin-V.

Cell invasion assay

Matrigel invasion assay was utilized to estimate the in vitro invasion ability of both parental and transfected gastric cancer cells. Serum-free RPMI1640 medium was mixed with Matrigel (1:10; BD Biosciences, Bedford, MA, USA). The bottom of the culture inserts (8-µm pores) in 24-well tissue culture plates (Transwell, Corning, Corning, NY, USA) was coated with 50 µl of the mixture. Afterwards, the Matrigel was allowed to solidify at 37°C for 4 h. After solidification, 5x10^4 cells were harvested by trypsinization, washed with serum-free medium and placed in the upper chamber. The lower chamber contained 10% fetal bovine serum and was used as a chemo-attractant. After incubation at 37°C with 5% CO2 for 48 h, cells in the inner side of the chamber were removed using cotton swabs.

Table 1. PCR primers used in this study

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primer sequence</th>
</tr>
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<tbody>
<tr>
<td>PDCD4 FP:</td>
<td>5’-GTGCCAACCAGTCCAA-3’</td>
</tr>
<tr>
<td>PTEN FP:</td>
<td>5’-CCAAAGTCAGACAGCAATTC-3’</td>
</tr>
<tr>
<td>miR-21 RT:</td>
<td>5’-GTCGTATCTCCAGGGTCGAGGGTCCGAGTTATTCGACCTGATACGACAAAAATATG-3’</td>
</tr>
<tr>
<td>miR21 (mature sequence)</td>
<td>5’-UAGCUUAUACAGCUAGUGUUGUA-3’</td>
</tr>
<tr>
<td>U6 RT:</td>
<td>5’-GTCGTATCTCCAGGGTCGAGGGTCCGAGTTATTCGACCTGATACGACAAAAATATG-3’</td>
</tr>
<tr>
<td>GAPDH FP:</td>
<td>5’-ACACCTCTCCACCTATGAC-3’</td>
</tr>
</tbody>
</table>

RT: reverse transcription primer, PCR: polymerase chain reaction, FP: forward primer; RP: reverse primer.

of miRs is expected to be ever-growing in future gastrointestinal oncology.
number of cells that invaded to the basal side of the membrane was quantified by counting 16 independent symmetrical visual fields under the microscope. Cell morphology was observed after staining with 0.1% Crystal violet.

Western blotting

All antibodies were purchased from Abcam (Abcam, Cambridge, UK). Whole cell lysates were prepared in RIPA buffer (Byotime, Haimen, China) and afterwards protease inhibitor was added. The protein concentrations of the lysates were measured using a Bradford protein assay kit (Bio-Rad, Hercules, CA, USA). In the next step, 50 µg of each protein were denatured in 2X loading buffer at 100 °C for 10 min, mixed with 6× sodium dodecyl sulfate–polyacrylamide gel electrophoresis sample buffer, electrophoresed in a 4–20% linear gradient Tris-HCl-ready gel (Bio-Rad), and then transferred to nitrocellulose membranes. The membranes were blocked with 5% non fat dry milk in Tris-buffered saline, pH 7.4, containing 0.5% Tween 20 and were incubated with primary antibodies and IRDye700- and IRDye800-labeled secondary antibodies (Rockland, Gilbertsville, PA, USA) according to the manufacturer’s instructions. The protein of interest was visualized and quantitated using the LI-COR Odyssey Infrared Imaging System (LI-COR Bioscience, Lincoln, NE, USA).

Statistics

Data were expressed as mean±standard error of the mean (SEM). Statistical significance for the mean of each group was assessed by one-way analysis of variance (ANOVA) with a p-value < 0.05 being considered significant.

Results

Cell proliferation assay

To investigate the potential cancer-related biological effects of miR-21, cell proliferation assay was performed using the MTT assay. Transfection of miR-21 mimic induced significantly increased growth rates in both SGC-7901 and MKN-45 gastric cancer cells. Cells transfected with miR-21 inhibitor decreased the growth potential of cancer cells (Figure 1A -1B).

Cell apoptosis assay

After transfection with both miR-21 mimic and inhibitor, it was clearly shown that both types of gastric cancer cells displayed a decreased apoptotic rate in comparison with the controls (NSM) (Figure 2).

Cancer cell invasion assay

Cells transfected with miR-21 mimic had a far more aggressive phenotype, as shown in Figure 3, where the number of cancer cells is noticeably higher in comparison with the cancer cells trans-
Figure 2. Apoptosis assay shows miR-21 mimic decreased the apoptosis of gastric cancer cell lines. SGC-7901 and MKN-45 were transfected with miR-21 mimic, miR-21 inhibitor and NSM. Apoptosis was measured 72 h post transfection by Annex V and PI labeling via flow cytometry. X-axis is Annex V (FL1-H) staining cells, Y-axis is PI staining cells. The apoptotic cells are in the right lower site (LR). 2A-2D: the apoptotic rate of SGC-7901-miR-21 mimic is 3.84%, while in SGC-7901 cells transfected with NSM the apoptotic rate is 6.45%; in addition, in SGC-7901-miR-21 inhibitor cells apoptosis reached 11.46%. 2E-2H: the apoptotic rate of MKN-45-miR-21 mimic is 0.89%, in MKN-45 NSM of apoptotic cells is 4.11%, while in MKN-45-miR-21 inhibitor cells apoptosis increased to 12.02%. A representative experiment performed in triplicate is shown and this experiment was repeated three times (Student’s t-test).

Figure 3. Invasion assay demonstrated that miR-21 enhances the invasion ability of SGC-7901 and MKN-45 gastric cancer cells. The number of SGC-7901 and MKN-45 cells passing through Matrigel were counted at 100x magnification. 3A-3D: shows miR-21 mimic can enhance SGC-7901 invasion ability, whereas miR-21 inhibitor decreases the invasion capacity of SGC-7901 (SGC-7901 miR-21 mimic vs SGC-7901 NSM, p=0.0012; SGC-7901 miR-21 inhibitor vs SGC-7901 NSM, p=0.0058). 3E-3H: MKN-45 miR-21 mimic shows higher invasion ability (MKN45 miR-21 mimic vs MKN45 NSM, p=0.0001), while the invasion ability of MKN-45 miR-21 inhibitor was lower than NSM (MKN-45 miR-21 inhibitor vs MKN-45 NSM, p=0.0006). Data are shown as mean±SEM from 3 independent experiments (Student’s t-test).
Figure 4. RT-PCR shows miR-21 expression in gastric cancer cell lines transfected with miR-21 mimic or NSM. MiR-21 expression in SGC-7901-miR-21 mimic and MKN-45-miR-21 mimic were significantly higher than in NSM transfected cells. A: SGC-7901-miR-21 mimic vs SGC-7901 NSM, p <0.01. B: MKN-45 miR-21 mimic vs MKN-45 NSM, p<0.01. Experiments were performed in triplicate (Student’s t-test).

Figure 5. mRNA qRT-PCR for PDCD4 and PTEN in gastric cancer cells. Experiments were performed in triplicate in SGC-7901 as well as in MKN-45 cell lines. SGC-7901 and MKN-45 cells were transfected with miR-21 mimic,miR-21 inhibitor and NSM. PTEN and PDCD4 level were assayed by qRT-PCR. Data were normalized to GAPDH. The experiments were done in triplicate (Student’s t-test). A-B show miR-21 mimic can significantly downregulate the expression of PDCD4 in both cell lines (SGC-7901-miR-21 mimic vs SGC-7901-NSM, p=0.0018; MKN-45-miR-21 mimic vs MKN45 NSM, p=0.0066), while miR-21 inhibitor can upregulate PDCD4 expression in both cell lines (SGC-7901-miR-21 inhibitor vs SGC-7901 NSM, p=0.0018; MKN-45-miR-21 inhibitor vs MKN-45 NSM, p=0.001). C-D demonstrate that miR-21 mimic decreases the expression of PTEN in both cell lines. SGC-7901-miR-21 mimic vs SGC-7901 NSM, p=0.007; MKN-45-miR-21 mimic vs MKN-45 NSM, p=0.002), while miR-21 inhibitor increases PTEN expression in both cell lines (SGC-7901-miR-21 inhibitor vs SGC-7901 NSM, p=0.007; MKN-45-miR-21 inhibitor vs MKN-45 NSM, p=0.036).
Figure 6. Western blotting for PDCD4. SGC-7901 and MKN-45 were transfected with miR-21 mimic, miR-21 inhibitor or NSM for 72 h. PDCD4 protein was detected by Western blotting. GAPDH was detected on the same blot as loading control. A-B: PDCD4 protein expression was reduced in both cell lines by miR-21 mimic (SGC-7901-miR-21 mimic vs SGC-7901 NSM, p=0.003; MKN-45-miR-21 mimic vs MKN-45 NSM, p=0.0001). C-D show miR-21 inhibitor can increase PDCD4 protein in both cell lines. SGC-7901-miR-21 inhibitor vs SGC-7901 NSM, p=0.031; MKN-45-miR-21 inhibitor vs MKN-45-NSM, p=0.0003. Data are mean±SEM from 3 independent experiments (Student’s t-test).

Figure 7. Western blotting for PTEN in SGC-7901 and MKN-45 cells. The cells were transfected with miR-21 mimic, miR-21 inhibitor or NSM for 72 h. Experiments were performed in triplicate. A-B: PTEN protein expression was downregulated in both cell lines by miR-21 mimic (SGC-7901-miR-21 mimic vs SGC-7901 NSM, p=0.047; MKN-45-miR-21 mimic vs MKN-45 NSM, p=0.002. C-D: miR-21 inhibitor increased PTEN protein in both cell lines (SGC-7901-miR-21 inhibitor vs SGC-7901-NSM, p=0.002; MKN-45 miR-21 inhibitor vs MKN-45 NSM, p=0.019). Data are mean±SEM from 3 independent experiments (Student’s t-test).
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fected with the NSM or with the miR-21 inhibitor.

Investigation of the molecular mechanisms of miR-21 on target genes PTEN and PDCD4

The quality control RT-PCR of SGC-7901 and MKN-45 cells after transfection with miR-21 mimic proved that the transfection was indeed successful (Figure 4A-4B), as the miR-21 levels were increased in the cells transfected with the active mimic when compared with the gastric cancer cells that were transfected with the NSM. Figure 5A-5B shows the results of the qRT-PCR for PDCD4 messenger RNA expression. For both gastric cancer cell lines, cell transfected with miR-21 mimic downregulated the expression of this gene when compared with the ones transfected with the NSM and also with the miR-21 inhibitor. The results were also confirmed for PTEN, where miR-21 inhibited its expression when compared with the NSM (Figure 5C-5D).

After confirmation of our initial hypothesis that overexpression of miR-21 can downregulate tumor suppressors PDCD4 and PTEN and their protein synthesis in gastric cancer at the molecular level, we confirmed once again the previous results at the protein synthesis compartment. Cells transfected with miR-21 mimic which exerted an increased invasive potential, had a statistically significant downregulating level of both protein synthesis when compared with the control groups (Figure 6 and 7). Similar experiments, but with cancer cells transfected with the miR-21 inhibitor, showed that PTEN and PDCD4 protein synthesis was increased when compared with the NSM.

Discussion

The incidence of gastric cancer is second only to lung cancer worldwide, with about 650,000 deaths per year, and a 5-year overall survival of resectable cases in specialized centers in Europe and the US of approximately 35%. Research has brought relatively little improvement in the survival rates of this malignancy due to the fact that primary tumors must be detected and treated at an early stage. Unfortunately, most gastric cancers are diagnosed at a late stage [9], when surgery is no longer an option. Current diagnostic tests have relatively low sensitivity, indicating a dire need for developing new disease markers [10]. In addition, the pathogenesis and homeostasis of gastric cancer are incompletely understood, further complicating the treatment [11]. In the current paper, we investigated the role of miR-21 in gastric cancer progression and dissemination, in the hope of understanding its biology, as well as providing new diagnostic strategies.

miRs are small non-coding RNAs, 19-24 nucleotides in length [12]. miRs are synthesized from primary miRs in two stages by the action of Drosha in the nucleus and Dicer in the cytoplasm [13,14]. Recent research strongly supports the role for miRs in the regulation of crucial processes such as cell proliferation, apoptosis, development, differentiation and metabolism [15]. By extension, the role of miRs has been investigated in a variety of solid tumors [16]. These studies strongly suggest that miRs are critical regulators of cancer homeostasis, including cell cycle regulation [17], proliferation [18], invasion and metastasis [19]. Lastly, miRs recently emerged as attractive therapeutic targets [20].

miRs bind to mRNA targets by nucleotide complementarity [21]. The effects of miRs on their targets are exerted through either degradation of the target mRNA or inhibition of its translation into proteins [22]. While every miR has hundreds to thousands of in silico predicted mRNA targets, it is likely that only a fraction of these are functionally important [23]. Nonetheless, miRs emerged as subtle regulators of mRNA translation and it is likely that their effect is exerted through a highly coordinated, multi-target downregulation [24].

In the current study, we investigated the relationship between miR-21 and its targets PDCD4 and PTEN. According to our data, PDCD4 and PTEN have been found to be negatively regulated by miR-21. PDCD4 has previously been shown to be upregulated in apoptosis [25-27], whereas miR-21 is known to act as an anti-apoptotic factor [28,29] in human ontogenesis. In cancer biology, this was confirmed by Menget al. [30] who proved that gemcitabine-induced apoptosis is specifically inhibited by miR-21 via PTEN.

Our data show that miR-21 is a negative regulator of PDCD4, and revealed also its antiapoptotic role via negative regulation of PDCD4. The transfection experiments confirmed this very fact, where PDCD4 protein synthesis was inhibited when compared with cancer cells transfected with NSM. Still, the complete mechanism by which miR-21 induces PDCD4 regulation is far from being elucidated and needs further investigation. PDCD4 might stimulate the invasive capacity of gastric cancer cells regulated by miR-21, which may act as an activator of tumor cell proliferation. This feature was observed in our in vitro studies, but the mechanisms and potential target of miR-21 besides PDCD4 are still largely unknown.

As miR-21 is one of the most prominent miRs implicated in the genesis and progression of hu-
man cancer [31-34], our study further investigated the basic pathophysiology of this small non-coding RNA for gastric cancer. We suggest that tumor suppressors PDCD4 and PTEN are negatively regulated by miR-21. Furthermore, due to the finding that miR-21 mimic induces an increased invasion potential for gastric cancer cells, we hypothesize that a potential inhibitor targeted against miR-21 may provide good therapeutic applications in gastric cancer in the future, via a positive loop with the tumor suppressor genes PDCD4 and PTEN.

**Conclusion**

Gastric cancer is one of the most common and lethal carcinomas in the world. miRs, a type of non-coding RNAs, are important specific regulators of various tumor suppressor genes and are thus involved in numerous bioprocesses of an organism. miR-21, one of the most widely studied non-coding RNAs in translational oncology and gastroenterology, was identified as a very suitable potential choice for further investigation because it is overexpressed in nearly all solid tumors.

The present paper confirms data obtained on other types of cancers and emphasizes the need for further research on this topic, in order to develop new, more suitable approaches in the clinic for the early detection, as well as for a better therapy of this disease.

**References**

23. Rosenfeld N, Aharonov R, Meiri E et al. MicroRNAs ac-

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26. Ferris WF, Marriott CE, Ali T, Landy C, Campbell SC, Macfarlane WM. Tumor suppressor Pdcd4 is a major transcript that is upregulated during in vivo pancreatic islet neogenesis and is expressed in both beta-cell and ductal cell lines. Pancreas 2011;40:61-66.


