Abstract. – OBJECTIVE: Gastric cancer (GC) is the third leading cause of cancer-related deaths while the mechanisms underlying its metastasis are not fully understood. In this study, we aimed to explore the relationship between miR-217 and GC metastasis.

PATIENTS AND METHODS: We examined miR-217 level in gastric tumor tissues of 48 patients with GC and in cell lines including gastric mucosa epithelial cell line (GES-1), gastric cancer cell line (BGC-823), and gastric cancer cell line (SGC-7901). The effects of miR-217 on EMT conditions were detected using cell migration and invasion assays. The potential regulatory target of miR-217 was determined by prediction tool, target protein expression and Luciferase reporter assay.

RESULTS: We found a lower expression of miR-217 in the tumor tissues of GC patients with metastasis. Increased expression of miR-217 markedly suppressed GC cell metastasis and invasion in vitro. We observed a strongly negative correlation between expressions of miR-217 and PTPN14 mRNA in GC tissues, and miR-217 repressed PTPN14 expression by directly targeting its 3’UTR. Furthermore, the loss of PTPN14 induced by miR-217 or si-PTPN14 reduced the metastasis and invasion of GC cells, whereas restoration of PTPN14 led to the enhanced metastases and invasion of GC cells. MiR-217-induced the loss of PTPN14 modulated the epithelial-to-mesenchymal transition (EMT) in GC cells, as indicated by the modulated expression of E-cadherin.

CONCLUSIONS: We concluded that miR-217 suppressed the EMT through directly binding to the PTPN14-3’UTR in GC progression, and might be a novel biomarker for the detection of GC metastasis.

Key Words: Gastric cancer, microRNA-217, PTPN14, Metastasis, Epithelial-to-mesenchymal transition.

Introduction

Gastric cancer (GC) is one of the most common malignant tumors worldwide and the third leading cause of cancer-related deaths. Tumor progression and metastasis are the main causes of cancer-associated deaths and more than 90% of mortality in GC patients is caused by distal metastasis. In spite of the developments in the treatment of GC, the prognostic of advanced gastric cancer patients, especially those with metastasis, remains rather poor. Consequently, it is urgent to investigate the molecular mechanisms underlying metastasis, drug resistance, and histological heterogeneity to develop novel markers for the diagnosis and treatment for GC. MicroRNAs (miRNAs), small non-coding RNAs, are endogenous approximately 22 nt RNAs that can play important regulatory roles as negative regulators of protein coding genes in animals and plants by targeting mRNAs for cleavage or translational repression. Increasing evidence has indicated that miRNAs play an important role in cell proliferation, differentiation, apoptosis, migration and invasion in tumor development. Under the molecular mechanisms, cancer progression has similarities with the process of epithelial-to-mesenchymal transition (EMT) found during embryonic development, during which cells down-regulate...
E-cadherin and up-regulate Vimentin expression, EMT contributes to promoting invasion and migration in various cancers\textsuperscript{9,11}. EMT is a complex process, including a loss of E-cadherin expression and gains in expression of the mesenchymal markers fibronectin, such as ZEB1, resulting in cell motility and a change in cell morphology\textsuperscript{12,13}. Previous research\textsuperscript{11,14} indicated that miR-200 family regulates EMT process during cancer progression and metastasis through targeting ZEB1 and ZEB2, which repressed E-cadherin\textsuperscript{11,14}. To date, in-depth studies on the molecular mechanisms of gastric cancer involving miRNAs are still not well illustrated. Previous studies\textsuperscript{15} reported that downregulation of microRNA-217 (miR-217) was strongly associated with hepatocellular carcinoma migration and invasion, miR-217 could inhibit tumor progression and metastasis by downregulating EZH2 and predicts favorable prognosis in gastric cancer\textsuperscript{3}, and miR-217 might play as a potential tumor suppressor in pancreatic ductal adenocarcinoma by targeting KRAS\textsuperscript{16}. However, the molecular mechanism of miR-217-induced EMT in GC metastasis still needs to be further understood. Therefore, we examined the level of miR-217 in tumor tissues of GC patients, and investigated its effects on the migration and invasion of GC cells \textit{in vitro}.

**Patients and Methods**

**Patients and Samples**

All samples including gastric cancer tissues and paired normal tissues were collected from consenting individuals according to the protocols approved by the Ethics Review Board at Yantai Yuhuangding Hospital (Yantai, Shandong Province, China), and written informed consent was obtained from all participants.

**Cell Culture**

Cell lines includingGES-1, BGC-823 (with the highest level of miR-217) and SGC-7901 (with relatively higher miR-217 expression level), and HEK293T cell were purchased from ATCC, and cultured according to the instruction at 37°C in 5% CO\textsubscript{2}.

**RNA Extraction and Real-time PCR**

Total RNA was extracted from tissue samples and cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol. For the measurement of miR-217, the All-in-One\textsuperscript{TM} miRNA qRT-PCR Detection Kit was obtained from GeneCopoeia (Rockville, MD, USA) and it was used according to the Manufacturer’s instructions. For PTPN14 mRNA amplification, the reverse transcription reaction was carried out using PrimeScript\textsuperscript{TM} RT Reagent Kits (TaKaRa, Dalian, Liaoning, China), and the reactions were performed via the following parameters: 37°C for 15 min followed by 85°C for 5 min. SYBR\textsuperscript{®} green Real-time PCR Master Mix (TOYOBO, Shanghai, China) was used to perform the polymerase chain reaction (PCR) reactions, and the following parameters were used: 95°C for 2 min followed by 40 cycles of 95°C for 10 s, 60°C for 10 s and 72°C for 20 s. GAPDH served as an endogenous control. Sequences of the qPCR primer pairs are as follows:

- PTPN14-F: 5’ GTGGACGAACCAAAAGCCAC 3’;
- PTPN14-R: 5’ GCCCAGACAAAAAGGTGCTTG 3’;
- GAPDH-F: 5’ GTTGAAGGTGGAGTCAACGG 3’;
- GAPDH-R: GAGGTCAATGAAGGGGTTCATTG.

**Oligonucleotide Transfection**

MiR-217 control, mimics and inhibitors were purchased from GenePharma Co. Ltd., (Shanghai, China), and si-PTPN14 was obtained from Ribobio Co. Ltd., (Guangzhou, China). Transfections were carried out by the liposome 2000 method (Invitrogen, Carlsbad, CA, USA).

**Luciferase Assay**

We contrasted vectors containing the wild-type 3’UTR or a mutant 3’UTR of PTPN14 mRNA and cloned into the pcDNA3.1 vectors (Invitrogen, Carlsbad, CA, USA) after amplified by polymerase chain reaction (PCR). The Luciferase report vectors were constructed via the synthesized DNAs and a pMIR-REPORT\textsuperscript{TM} Luciferase vectors. The mutant PTPN14-3’UTR served as a control. HEK293T cells were transfected with 0.8 μg of wide-type pMIR-PTPN14-3’UTR or mutant pMIR-PTPN14-3’UTR, and 0.04 μg of Renilla Luciferase control vector pRL-TK (Promega, Madison, WI, USA) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), followed by the cells were transfected with miR-217 control (100 nM) or mimics (100 nM), respectively, for 24 h, and finally the cells were lysed via dual Luciferase reporter assay system, and the fluorescence activity was measured via GloMax 20/20 Lumi-
nometer. The firefly Luciferase activity was normalized to the renilla Luciferase activity.

**Cell Migration and Invasion Assays**

SGC-7901 cells were seeded in 6-well plates and transfected with miR-217 control (100 nM), miR-217 mimics (100 nM), si-PTPN14 (100 nM) or PTPN14 vectors (200 ng) for 24 h. Then cells were collected to perform the migration and invasion assays by using QCMTM Laminin Migration Assay (ECM220) and Cell Invasion Assay Kit (ECM550) according to the manufacturer’s protocols, respectively.

**Western Blot Assay**

The proteins of PTPN14, ZEB1 E-cadherin, and Vimentin were detected by Western blot assay. Samples of 30 μg of protein per lane were fractionated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), electro-transferred onto polyvinylidene fluoride (PVDF) membranes, and then protein levels were detected using dilutions of the primary antibodies. These were next incubated with HRP-conjugated secondary antibodies and the bound antibodies were visualized using an enhanced chemiluminescence reagent (Millipore, Billerica, MA, USA) and quantified by densitometry using ChemiDoc XRS+ image analyzer (Bio-Rad, Hercules, CA, USA). Densitometric analyses of bands were adjusted with β-actin as loading control. Triplicate experiments with triplicate samples were performed.

**Statistical Analysis**

Statistical software SPSS18.0 (SPSS Inc. Chicago, IL, USA) was used for the assessment. Mann-Whitney test was used to compare the expressions of miR-217 and PTPN14 in GC tissues. The differences between two groups in GC cells were determined via Two-tailed Student’s t-test. Pearson’s correlation was used to analyze the relationship between the expressions of miR-217 and PTPN14 mRNA in GC tissues. p<0.05 was considered as statistically significant.

**Results**

**Patients’ Information**

48 patients with GC, including 33 patients with GC metastasis and 15 patients without GC metastasis, were registered in our study between 2012 and 2014. The information of the patients was shown in Table I.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Gastric cancer, N = 48 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymph node status</td>
<td></td>
</tr>
<tr>
<td>– Metastasis</td>
<td>33 (69)</td>
</tr>
<tr>
<td>– No metastasis</td>
<td>15 (31)</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
</tr>
<tr>
<td>– Male</td>
<td>30 (63)</td>
</tr>
<tr>
<td>– Female</td>
<td>18 (37)</td>
</tr>
<tr>
<td>Age</td>
<td></td>
</tr>
<tr>
<td>– Median (range)</td>
<td>54 (35-72)</td>
</tr>
<tr>
<td>– ≥55</td>
<td>27 (56)</td>
</tr>
<tr>
<td>– &lt; 55</td>
<td>21 (44)</td>
</tr>
<tr>
<td>Tumor location</td>
<td></td>
</tr>
<tr>
<td>– Body</td>
<td>25 (52)</td>
</tr>
<tr>
<td>– Antrum</td>
<td>13 (27)</td>
</tr>
<tr>
<td>– Cardia*</td>
<td>10 (21)</td>
</tr>
<tr>
<td>Histology</td>
<td></td>
</tr>
<tr>
<td>– Adenocarcinoma</td>
<td>39 (81)</td>
</tr>
<tr>
<td>– Mucinous adenocarcinoma</td>
<td>9 (19)</td>
</tr>
<tr>
<td>TNM stage</td>
<td></td>
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<tr>
<td>I</td>
<td>5 (10)</td>
</tr>
<tr>
<td>II</td>
<td>14 (29)</td>
</tr>
<tr>
<td>III</td>
<td>18 (38)</td>
</tr>
<tr>
<td>IV</td>
<td>11 (23)</td>
</tr>
</tbody>
</table>

Note: The cardia is part of the alimentary canal, the interface part of the esophagus and stomach. It’s the upper entrance to the stomach.

**miR-217 Level in Tumor Tissues of GC Patients**

To determined miR-217 expression in gastric tumor tissues of GC patients with or without metastasis, we analyzed the miR-217 expression in the gastric tumor tissues with or without metastasis, and the matched normal tissues. Our data showed that the miR-217 expression was much lower in the metastatic and non-metastatic GC tissues when compared with the matched normal tissues, and the miR-217 expression in the metastatic GC tissues was much lower than that in the non-metastatic GC tissues (Figure 1A, normalized with normal tissues). We also compared miR-217 expression in the tumor tissues of patients with different TNM stage, and we observed lower miR-217 expression in II, III and IV stage. The difference between stage III/IV and stage I was statistically significant (p=0.024, p=0.015), and the lowest expression of miR-217 was observed in the tumor tissues of patients with TNM stage IV (Figure 1B, normalized with TNM stage I). Therefore, we associated with tumor metastasis in patients with GC.
MiR-217 Promotes the Migration and Invasion of GC Cells in Vitro

To assess whether miR-217 contributes to the metastasis of GC cells, we selected the GC cell lines of BGC-823 and SGC-7901 to perform the migration and invasion assays. First we analyzed miR-217 expression in GES-1, BGC-823 and SGC-7901 cells. As shown in Figure 1C, the lowest expression of miR-217 was found in SGC-7901 cells, and the miR-217 expression in BGC-823 cells was significantly lower than that in the GES-1 cells (Figure 2A). Then BGC-823 and SGC-7901 cells were transferred with miR-217 controls or mimics for 24 h, followed by the detection of cell migration and invasion ability. As shown in Figure 2B and Figure 2C, miR-217 mimics significantly reduced the metastasis and invasion of BGC-823 and SGC-7901 cells (p=0.022, p=0.018). These data suggested that miR-217 suppressed GC cell metastasis and invasion.

PTPN14 is a Novel Target of miR-217

We next contrasted vectors containing the wild-type 3’UTR or mutant 3’UTR of PTPN14 mRNA, which was individually fused directly downstream of the firefly Luciferase gene (Figure 3A). For the Luciferase assays, the wild-type or mutant vector and miR-217 control or mimics were co-transfected into HEK293T cells, respectively. The results showed that miR-217 mimics markedly reduced the relative Luciferase activity in HEK293T cells transfected with wild-type 3’UTR of PTPN14 (p<0.05, p=0.024), while did not change the Luciferase activity in HEK293T cells transfected with mutant 3’UTR (Figure 3B). We also found that miR-217 decreased the protein expression of PTPN14 (p<0.05) (Figure 3D). We then detected mRNA expression of PTPN14 in the 48 pairs of GC tissues, and analyzed the correlation between the expressions of miR-217 and PTPN14 mRNA. As shown in Figure 3C, we found a strongly negative correlation between the miR-217 expression and PTPN14 expression (R²=0.2843, p=0.010).

Inhibition of PTPN14 by si-PTPN14 Suppressed Migration and Invasion Ability of GC Cells

We used si-PTPN14 and si-RNA control to determine the role of PTPN14 in modulating the migration and invasion of SGC-7901 cells. Our data showed that si-PTPN14 significantly repressed the PTPN14 expression (Figure 4A). Then the migration and invasion assays showed that si-PTPN14 could notably reduce the metastasis and invasion of SGC-7901 cells, consistent with miR-217 mimics-induced the suppressed metastasis and invasion of the cells (Figure 4B). To further determine miR-217-induced the suppressed metastasis and invasion of GC cells through target-
MicroRNA-217 suppressed EMT in gastric cancer metastasis

Figure 2. MiR-217 in different cell lines and effects of miR-217 on the migration and invasion ability of gastric cancer cells in vitro. Analysis of miR-217 in GES-1, BGC-823, and SGC-7901 showed that in gastric cancer cells the miR-217 level was much lower than normal GEC-1 cells (A). Then BGC-823 and SGC-7901 cells transfected with miR-217 control (100 nM) or miR-217 mimics (100 nM) were used to perform the migration (B) and invasion (C) assays. *p<0.05.

Figure 3. PTPN14 is a novel target of miR-217. A, The 3'-UTR of PTPN14 mRNA contains the binding site mutant of miR-217. B, Co-transfection of the wild type Luciferase report vectors and miR-217 mimics significantly reduced the Luciferase activity in HEK293T cells, while co-transfection of mutant Luciferase report vectors and miR-217 mimics did not affect these levels. C, Linear regression revealed a strongly negative correlation between the mRNA expressions of PTPN14 and miR-217 in the gastric tumor tissues of the 40 patients. D, MiR-217 mimics markedly decreased the protein expression of PTPN14 analyzed by Western blot. *p<0.05.

PTPN14 vectors was restored compared with that in SGC-7901 cells transfected with miR-217 mimics. MiR-217 mimics significantly suppressed the cell metastasis and invasion, while the metastasis and invasion of the cells were restored when the cells were co-transfected with miR-217 mimics and PTPN14 vectors (Figure 4D).
**MiR-217 Modulated EMT and Apoptosis Signal in GC Cells**

We detected the protein expression of E-cadherin in GC cells after transfection with miR-217 control and mimics. As shown in Figure 5, miR-217 mimics markedly increased the E-cadherin expression and decreased the Vimentin expression. Then we examined the protein expressions of E-cadherin and Vimentin in SGC-7901 cells co-transfected with miR-217 mimics and PTPN14 vectors, and we found that the effects of miR-217 on expression of E-cadherin and Vimentin were neutralized. These suggested that miR-217 modulated the EMT in GC cells through targeting PTPN14. Further analysis of the proteins also found that up-regulation...
MicroRNA-217 suppressed EMT in gastric cancer metastasis

Figure 5. MiR-217 modulated the EMT signal and apoptotic proteins in gastric cancer cells. A. MiR-217 mimics markedly increased the E-cadherin expression and decrease Vimentin in SGC-7901 cells after transfection with miR-217 mimics, and when SGC-7901 cells were co-transfected with miR-217 mimics and vector PTPN14, the effects of miR-217 on expressions of E-cadherin and Vimentin were neutralized. B. miR-217 mimics significantly increase apoptotic protein caspase-3, caspase-9, and cleaved-PARP, and when co-transfected with PTPN14 vectors, the increase of the above apoptotic proteins was reversed accordingly.

Discussion

MiRNAs play an important role in GC metastasis. In this study, we confirmed that miR-217 was frequently decreased in tumor tissues of GC patients with metastasis when compared with non-metastatic GC tissues and matched normal tissues, and overexpression of miR-217 could reduce the migration and invasion of GC cells through directly binding to PTPN14-3’UTR, and miR-217-induced the loss of PTPN14 suppressed the EMT in GC cells. Previous researches reported that the downregulated expression of miR-217 was frequently observed in different types of tumors, and was often associated with tumor metastasis; for example, the miR-217 expression was strongly decreased in metastatic hepatocellular carcinoma (HCC) tissues and highly invasive MHCC-79H HCC cells15. The decreased expression of miR-217 could regulate KRAS and function as a tumor suppressor in pancreatic ductal adenocarcinoma16, and the lower expression of miR-217 was associated with higher tumor grade and stage in clear cell renal carcinoma17. In this study, we also confirmed that miR-217 was frequently decreased in tumor tissues of GC patients with metastasis when compared with non-metastatic GC tissues and matched
normal tissues. These results were consistent with previous reports. Then, we examined the effects of miR-217 on the migration and invasion of GC cells in vitro. The level of miR-217 was detected in different cell lines including GES-1, BGC-823 and SGC-7901. Our data revealed the least expression of miR-217 in SGC-7901, then in BGC-823, when compared with the normal GES-1 cells. The invasion and migration assay also proved that decreased miR-217 promoted invasion and migration. When miR-217 mimics were transfected into SGC-7901 and BGC-823 cells, the invasion and migration ability of these cells were significantly decreased as we expected. Identification of miR-217 target, which is associated with GC cell metastasis, is required for determination of the function of miR-217 in GC metastasis. Therefore, we searched for its potential target genes that promoted the metastasis in tumor progression using prediction algorithms such as microRNA, ORG and miRDB. We found that, PTPN14, which is strongly associated with modulating EMT in tumor cell metastasis, is a potential target of miR-217. Early in 2004, six PTPs mutations, including protein tyrosine phosphatase non-receptor type 14 (PTPN14), were found in human cancers, affecting 26% of colorectal cancers and a smaller fraction of lung, breast, and gastric cancers\(^{16}\). It has been reported PTPN14 resides at different subcellular loci subject to a variety of influential factors, such as cell type, cell-matrix adhesion, serine phosphorylation and cell confluence\(^{19-21}\). PTPN14 is crucial in tumorigenesis, EMT and malignant transformation, and it has also been reported that PTPN14 interacted with Yes-associated protein (YAP), which is a transcriptional co-activator amplified in mouse tumor cells to disseminate and spread throughout the body. microRNAs also contribute significantly to gastric cancer EMT modulation\(^{25,26}\). Compelling evidence indicated that PTPN14 played an important role in EMT, so we determined whether miR-217 regulated the EMT signals in GC cells. In this study, we analyzed the level of E-cadherin in GC cells, and the results confirmed that miR-217 inhibited the metastatic ability of GC cells.

### Conclusions

We found that the miR-217 expression was decreased in metastatic GC tissues. MiR-217 suppressed the EMT in GC cells through directly targeting PTPN14, and further repressed the metastatic ability of GC cells.

### Conflict of interest

The authors declare no conflict of interest.

### References

MicroRNA-217 suppressed EMT in gastric cancer metastasis


