Original Article

Reduction of miR-132-3p contributes to gastric cancer proliferation mainly by targeting MUC13

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Received January 16, 2016; Accepted April 6, 2016; Epub May 15, 2016; Published May 30, 2016

Abstract: Abnormal expression of EGFR signaling and miRNAs has been widely seen in gastric cancer. This study mainly focuses on the miRNAs that regulate HER activation through MUC13. The expression of MUC13 was explored using western blot assay or immunohistochemistry. Bioinformatic predictions were conducted using TargetScan. Luciferase reporter assay and western blot were performed to determine whether miRs target MUC13. MiR mimics or inhibitors were used to enhance or inhibit miRNA expression. The effect of miRs or MUC13 on cell proliferation, colony formation and migration were analyzed. The protein level of MUC13 was significantly increased in gastric cancer tissues compared with normal tissues. TargetScan predictions indicated that MiR-212-3p and miR-132-3p may bind to the 3'-untranslated region of MUC13. Further study revealed that miR-132-3p was significantly decreased in gastric cancer tissues, while miR-212-3p showed no obvious changes. Luciferase assay and western blot validated that MUC13 was a target gene of miR-132-3p. Inhibition of miR-132-3p enhanced gastric cancer cell proliferation, colony formation and migration through activation of HER2, ERK and Akt, which was similar with the effect of MUC13 overexpression. Inhibition of MUC13 could reverse miR-132-3p inhibition-induced effects. In summary, reduction of miR-132-3p contributes to gastric cancer proliferation mainly by targeting MUC13.

Keywords: MiR-132-3p, gastric cancer, cell migration, MUC13, HER2

Introduction

Gastric cancer is one of the most malignant tumors among men and women in the world. In 2008, it was reported that about 738,000 people died from gastric cancer [1]. The major factors that contribute to gastric cancer include Helicobacter pylori, diet, alcoholic consumption and smoking [2, 3].

Receptor tyrosine kinase (RTK) pathways play key roles in the progression of various tumors [4-6]. Among the RTKs, the epidermal growth factor receptor (EGFR) is aberrantly changed [7]. In over 60% tumors, it is found that the EGF family is significantly overexpressed [8, 9]. The family members of EGFR mainly include EGFR (HER1), HER2 (ErbB2), HER3 (ErbB3) and HER4 (ErbB4). In gastric cancer, EGFR and HER2 are found to be significantly upregulated and mainly act as well-known oncogenes [10]. Mucin 13 (MUC13) was found to be aberrantly upregulated in various tumors [11-13]. Exogenous expression of MUC13 contributes to abnormal cell proliferation, motility and tumor growth [13]. It was found that overexpression of MUC13 results in the activation of HER2, ERK, Akt, as well as the reduction of p53 [12]. However, little study has been conducted about the expression of MUC13 in gastric cancer.

MicroRNAs (miRNAs, miRs) are small non-coding RNAs that widely control gene expression at the post-transcriptional level [14-16]. Abnormal expression of miRNAs leads to the initiation, formation and progression of tumors due to their oncogenic or tumor suppressive roles. In gastric cancer, many miRNAs are reported to be differentially expressed, such as miR-199a-3p, miR-429 and miR-34a [14-16]. In this study, we mainly focused on potential miRNAs that regulate the expression of MUC13 in gastric cancer. And we found miR-132-3p was significantly decreased in gastric cancer tissues. Through targeting MUC13, reduction of miR-132-3p significantly enhanced the activation of HER2, ERK and Akt signaling.

Materials and methods

Patient selection and biopsies collection

In the present study, 40 patients receiving adenocarcinoma surgery of the stomach or esoph-
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Gastric cancer tissue samples were collected from July 2012 to November 2014. The consent forms were signed by all individuals. The collection of biopsies was approved by the Ethics Committee of the First Hospital of Jilin University in accord with the Helsinki Declaration. Biopsies were taken from both tumor tissue and the adjacent normal tissue. All biopsy samples were reviewed by an experience pathologist to validate the diagnosis.

**Immunohistochemistry**

Sections were incubated in 3% H$_2$O$_2$ for 30 min at room temperature (RT) after washing with PBS for three times (5 min for each). After incubation with goat serum for 30 min, sections were incubated with primary antibody MUC13 (1:80) for 24 hr at 4°C. After washing with PBS, sections were incubated with secondary antibody (biotin-labelled goat anti-mouse IgG, 1:200) for 1 hr at 4°C, washed with PBS and incubated with horseradish peroxidase conjugated streptavidin (1:200) for 1 hr at RT, and then with diaminobenzidine (DAB)/H$_2$O$_2$ for 15 min at RT. After dehydration in gradient alcohol, and transparentizing in xylene, sections were mounted with glycerol and observed under a microscope. In control sections, the primary antibody was replaced with 1% calf serum.

**Cell culture**

Gastric cancer cell lines, MKN28, MKN45, MKN74, NUGC2, NUGC3, and KATOIII Cells as well as normal gastric mucosa cells GES-1 were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), streptomycin (100 mg/ml) and penicillin (100 IU/ml) at 37°C in a humidified atmosphere containing 5% CO$_2$.

**RNA extraction**

Total RNA were extracted from gastric tissue or cells using Trizol reagent according to the manufacturers’ instructions (Life Technologies, Carlsbad, CA, USA).

**Reverse transcription quantitative polymerase chain**

To quantify the amount of miRNA, total RNA were reverse transcribed using Takara MicroRNA Reverse Transcription Kit (Takara) with specific primers for miR-132-3p and U6. Subsequently, the PCR amplification was performed. 1 ml of cDNA was used for qPCR using SYBR green Master mix (Roche, Basel, Switzerland) on a Roche lightcycler 480 at: 95°C, 10 min; 50 cycles of 95°C for 10 sec, specific annealing temperature for 10 sec, 72°C for 5 sec; 99°C for 1 sec; 59°C for 15 sec; 95°C for 1 sec; cooling to 40°C. Relative miRNA expression of miR-132-3p was normalized against the endogenous control, U6, using the delta-delta CT method.

**Cell viability analysis**

To examine cell viability, MKN28 cells were seeded in 96-well at the density of 1.0×10^4 cells/per well. MiR-132 mimics or inhibitors or negative control (NC) were transfected into cells at 24, 48, 72 hrs after seeding of cells. MTT assay were performed as previously described [17].

**Western blotting analyses**

Tissue or cell protein was extracted using RIPA buffer (SolarBio, Beijing, China). The proteins were resolved by 10% SDS-PAGE gel and transferred onto a PVDF membrane. The protein was detected with primary anti-bodies overnight at 4°C. GAPDH was used as the internal control. Signals were explored with enhanced chemiluminescence according to the manufacturer’s instructions (Millipore, Billerica, MA, USA).

**Bioinformatic predictions**

To determine the potential miRNAs that target MUC13, bioinformatic prediction was performed using TargetScan (http://www.targetscan.org).

**Cell invasion assay**

Invasion of cells were explored using a Transwell system (CHEMICON). The cells were transfected with miR-132 inhibitors or ad-MUC13 were cultured in the lower chamber with fresh medium containing 10% FBS. After incubation for 24 hr at 37°C, the cells on the upper chamber was stained and dissolved in 10% acetic acid for measurement of A560 nm.

**Cell migration assay**

The in vitro wound healing assay was performed as previously described [18]. Briefly,
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MKN28 cells were seeded in 6-well plates to form a confluent monolayer. The monolayer was scratched with a sterile 10 μl pipette tip, and the floating cells were carefully removed by PBS. Then, the cells were cultured in RPMI-1640 medium without FBS at 37°C in a 5% CO₂ atmosphere. The wound scratches were photographed at 0 hour and 12 hours after scraping.

**Adenovirus vector construction**

The adenovirus vector (Ad)-MUC13 and Ad-con were purchased from the Chinese National Human Genome Center (Beijing, China).

**Establishment of MUC13-expressing MKN28 stable cell line**

MKN28 cells were transfected with pcDNA3.1-MUC13 or pcDNA3.1-empty-vector using VigoFect transfection reagent (Vigorus, Beijing, China). Individual G418 resistant clones were selected and applied for further study.

**Figure 1.** The expression of MUC13 was obviously increased in gastric cancer tissues. A. Western blot analysis. B. Immunohistochemistry analysis. n=5 independent tissues, *P<0.05 versus control.

**Statistical analysis**

The data were expressed as the mean ± SEM. The number of independent experiments was represented by "n". Multiple comparisons were performed using one-way ANOVA followed by Tukey’s multiple-comparison test, where P<0.05 was considered significant.

**Results**

**Upregulation of MUC13 in gastric cancer tissues**

We first explored the expression of MUC13 in gastric cancer tissues. Western blot analysis demonstrated that MUC13 was significantly upregulated in gastric cancer tissues in comparison with adjacent normal tissues (Figure 1A). Immunohistochemistry analysis also demonstrated the enhanced expression of MUC13 in gastric cancer tissues (Figure 1B).

**MUC13 is a target gene of miR-132-3p in gastric cancer**

To identify the potential miRNAs that regulate the expression of MUC13, TargetScan online prediction program was applied. As shown in Figure 2A, two putative conserve binding miRNAs, miR-132-3p and miR-212-3p, were identified on the 3’ untranslated region (3’UTR) of MUC13. We found that miR-132-3p was reduced in gastric cancer tissues, and miR-212-3p did not demonstrate significant changes (Figure 2B). Then, the 3’UTR of MUC13 was cloned into the pmirGLO plasmid. Dual luciferase reporter assay showed that miR-132-3p significantly decreased the relative luciferase units (RLU) of pmirGLO-MUC13-3’UTR in comparison with the empty vector of pmirGLO (Figure 2C). Then, miR-132-3p mimics or inhibitors were transfected into MKN28 cells. Overexpression of miR-132-3p significantly decreased the protein level of MUC13 (Figure 2D). In comparison, we found that inhibition of miR-132-3p increased the expression of MUC13 (Figure 2D). These data indicated that reduction of miR-132-3p led to enhanced MUC13 expression in gastric cancer tissues.

**Overexpression of MUC13 prompted MKN28 cell invasion and migration through activation of HER signaling**

To explore the role of MUC13 on gastric cancer progression, cell invasion and migration were...
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Analyzed. As shown in Figure 3A, the transfection efficiency of ad-MUC13 or ad-con was nearly 100% (Figure 3A). Transwell assay showed that overexpression of MUC13 significantly enhanced cell invasion capacity (Figure 3B). Furthermore, scratch test showed that MKN28 cell migration was significantly enhanced when MUC13 was overexpressed (Figure 3C). We further explored the downstream signaling of MUC13. As shown in Figure 3D, overexpression of MUC13 obviously enhanced the activation of HER2, ERK and Akt (Figure 3D).

Knockdown of MUC13 partially reverse miR-132-3p inhibition-induced MKN28 cell invasion and migration

To explore whether miR-132-3p exerts its role through MUC13, we transfected miR-132-3p inhibitors into MKN28 cells. As shown in Figure 4A, inhibition of miR-132-3p significantly enhanced the protein level of MUC13, thereby enhancing the activation of HER2, ERK and Akt. More importantly, a siRNA targeting MUC13 was selected to suppress the expression of MUC13. Obviously, knockdown of MUC13 could reverse miR-132-3p inhibition-induced HER2, ERK and Akt activation (Figure 4B). Then, we further explored cell invasion and migration under the same circumstances. MUC13 knockdown significantly decreased cell invasion and migration even in cells transfected with miR-132-3p inhibitors (Figure 4C and 4D).

Discussion

Mucins are considered as potential tumor markers and possible therapeutic targets in various malignancies [19-21]. As a high-molec-
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ular-weight transmembrane glycoprotein, MUC13 is reported to be frequently overexpressed in many epithelial carcinomas, such as gastric, colorectal, and ovarian cancers [22]. MUC13 includes three EGF-like domains as well as a cytoplasmic domain with possible phosphorylation sites, which triggers the activation of HER2 signaling [22]. In this study, we explored its expression in gastric cancer tissues and found it was significantly upregulated.

Previous study has found that overexpression of MUC13 significantly enhanced the activation of HER2, ERK and Akt [13]. To validate the role of MUC13 in gastric cancer progression, we exogenously expressed MUC13 in MKN28 cells. We found that overexpression of MUC13 obviously enhanced gastric cancer cell invasion and migration. Due to the three EGF domains, MUC13 was proposed to stabilize the protein level of EGF receptors, especially HER2, thereby enhancing the activation of ERK and Akt. And activation of PI3K/Akt and MAPK signaling through HER2 enhanced the tumorigenesis in various cancers [23, 24].

MiRNAs are increasingly found to be differentially expressed in various tumors either as oncogenes or suppressors [25-27]. In previous study, miR-145 was found to target MUC13 thereby inhibiting the tumor growth and invasion in pancreatic tissues. In the present study, we try to elucidate new miRNAs that regulate the expression of MUC13 in gastric cancer [12]. Bioinformatic predictions suggested that miR-132-3p and miR-212-3p could possibly bind to the 3'UTR of MUC13. Luciferase reporter assay and western blot analysis demonstrated that

Figure 3. Overexpression of MUC13 prompted MKN28 cell invasion and migration through activation of HER signaling. A. The transfection efficiency of ad-MUC13 or ad-control. B. Transwell assay showed that overexpression of MUC13 significantly enhanced cell invasion capacity. C. Scratch test showed that MKN28 cell migration was significantly enhanced when MUC13 was overexpressed. D. Overexpression of MUC13 obviously enhanced the activation of HER2, ERK and Akt. n=3 independent experiments, *P<0.05, **P<0.01 versus control.
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MUC13 is the target gene of miR-132-3p. Further study showed that miR-132-3p was obviously decreased in gastric cancer tissues compared with normal adjacent tissues. In line with MUC13 overexpression, reduced miR-132-3p contributed to gastric cancer cell invasion and migration. More importantly, overexpression of miR-132-3p could reduce the activation of ERK and Akt even in cells transfected the specific siRNA targeting MUC13, suggesting the tumor suppressor role of miR-132-3p in gastric cancer through MUC13.

To conclude, miR-132-3p mainly functions as a tumor suppressor in gastric cancer tissues through targeting MUC13 thereby prompting the activation of HER2 signaling.

Disclosure of conflict of interest

None.

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