miRNA-1207-5p is associated with cancer progression by targeting stomatin-like protein 2 in esophageal carcinoma

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Received December 19, 2014; Accepted February 2, 2015

DOI: 10.3892/ijo.2015.2900

Abstract. Newly discovered intrinsic regulators, the miRNAs regulate gene expression by binding to the 3'-untranslated regions of the genome. Accumulating studies have indicated that miRNAs are aberrantly expressed in various human cancers. We found that miRNA-1207-5p (miR-1207-5p) was markedly downregulated in esophageal carcinoma (EC) tissues, and was correlated with EC differentiation, pathological stage and lymph node metastasis. Rates of apoptosis were increased and cell invasion ability was decreased in EC9706 and EC-1 cells transfected with a miR-1207-5p mimic. Stomatin-like protein 2 (STOML-2) was predicted to be a potential target of miR-1207-5p by bioinformatics analysis and this was confirmed by luciferase assay and western blotting. Our study showed that STOML-2 was negatively regulated by miR-1207-5p. Furthermore, overexpression of STOML-2 abolished the miR-1207-5p anti-invasion function. Based on these results, we proposed that miR-1207-5p might act as a potential therapeutic target in the treatment of EC.

Introduction

MicroRNAs (miRNAs) are a large family of non-coding RNAs, typically 18-25 nucleotides in length, that are highly conserved and endogenously expressed in many species. Newly discovered intrinsic regulators, the miRNAs regulate gene expression by binding to the 3'-untranslated regions (3'-UTR) (1-4). Rapidly accumulating studies have indicated that miRNAs are differentially expressed in various human cancers, including non-small cell lung cancer, esophageal cancer, colorectal cancer, bladder cancer and lymphocytic leukemia, and they function as tumor suppressors and oncogenes (4-8). It has been reported that miRNAs are involved in various cellular processes such as differentiation, migration and apoptosis.

Aberrant expression of miRNAs has been reported in esophageal carcinoma (EC) (9,10), and may play a role in the development of EC. We screened a series of miRNAs in EC tissues and in comparison with their normal counterparts using miRNA microarrays. Notably, miR-1207-5p was expressed at a lower level in EC tissues. Previous reports have shown that miR-1207-5p has differential expression in breast cancer and in human corneal epithelial cells (11,12). In addition, miR-1207-5p acted as a suppressor in gastric cancer, inhibited the growth of tumors by targeting human telomerase reverse transcriptase, and resulted in reduced tumor volume (13); miR-1207-5p was also shown to regulate heparin binding epidermal growth factor expression, to be involved in extracellular matrix accumulation and to be associated with disease severity in nephropathy (14,15). However, the functions of miR-1207-5p in EC have not been reported. We used TargetScan 6.2 (http://www.targetscan.org) and miRBase (http://www.mirbase.org) to predict that the target of miR-1207-5p is STOML-2, which is a member of the stomatin family, but differs from other members of the family by the lack of a hydrophobic membrane anchor at its N-terminus (16-18). Reports have indicated that STOML-2 was upregulated in several kinds of tumor tissue and was involved in invasion and metastasis of cancers including esophageal cancer, gastric cancer, breast cancer and glioma (19-23). Inhibition of STOML-2 was able to decrease cell growth and proliferation, and reduced migratory speed and invasive ability (24,25).

Herein, we report that miR-1207-5p was markedly downregulated in 49 EC specimens, and STOML-2 was correspondingly upregulated. Rates of apoptosis were found to be increased in EC9706 and EC-1 cells transfected with a miR-1207-5p mimic. We further measured the level of phospho-IκBα (p-IκBα), an important molecule in the NF-κB signal pathway, which was downregulated in miR-1207-5p-transfected cells. Taken together, our results suggested that miR-1207-5p affected cell invasion and apoptosis in EC cells by targeting STOML-2.

Materials and methods

Patient information and specimens. Tumor center and marginal tissues (n=49) were collected at the First Affiliated Hospital of Zhengzhou University and Tumor Hospital of Linzhou City.
Cell lines. Human esophageal cancer EC9706 and EC-1 cells were purchased from the Cell Bank of the Chinese Academy of Medical Sciences (Beijing, China). The two cell lines were maintained in RPMI-1640 medium with 100 U/ml penicillin and 100 µg/ml streptomycin (Solarbio, Beijing, China) supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA, USA) and incubated in a humidified atmosphere of 5% CO₂ at 37°C.

Total RNA isolation and quantitative reverse transcription PCR analysis. Total RNA was extracted from tissues and cells using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA); cDNA was synthesized using the RevertAid First Strand cDNA (K1621; Thermo Fisher Scientific, Waltham, MA, USA) in 20 µl containing 2 µl of RNA, 1 µl of random hexamer primer, 9 µl of Nuclease-free water, 4 µl of 5X reaction buffer, 1 µl of RNase inhibitor (20 U/µl), 2 µl of 10 mM dNTPs mix and 1 µl of reverse transcriptase (200 U/µl). The mixture was incubated for 5 min at 25°C, followed by 60 min at 42°C, and then at 70°C for 5 min. qRT-PCR was performed using SYBR Green I (DRO041A; Takara, Dalian, China) following the kit manual. A specific two step Stemaim-it miR qRT-PCR quantitation kit (LM-0101A; Novland Co. Ltd., Shanghai, China) was used to detect miR-1207-5p. The primers for miR-1207-5p were 5'-GTCGTATCCAGTGAGGTCCAGGATTCG (stem-loop RT primer), 5'-TCCGAGGCGGGAGGAC-3' (forward), and 5'-GTCCAGGGTGCCAGGT-3' (reverse). The primers for U6 were 5'-GTCGATCCAGTGAGGTCCAGGATTCG (stem-loop RT primer), 5'-TCCGATCCAGTGAGGTCCAGGATTCG (reverse). Reaction solution contained 15 µl of master mix (2X), 1 µl of enzyme mix, 1 µl of miR primer mix (0.1 µM), 2 µl of cDNA and 11 µl of RNase free water in a final volume of 30 µl, which was pretreated for 3 min at 94°C, then incubated for 20 sec at 94°C, followed by 40 sec at 62°C for 40 cycles. qRT-PCR was carried out on 7500 Fast Real-time PCR system (Applied Biosystems), the relative expression levels of the miRNA were calculated using the comparative Ct (2-ΔΔCt) and were normalized to U6 small nuclear RNA.

Western blot analysis. Total protein from cultured cells were obtained using NP-40 lysis buffer (P0013F; Beyotime, Haimen, China) with 1 mM phenylmethanesulfonyl fluoride (ST506; Beyotime). The protein concentrations were determined using a BCA protein assay kit (P0012; Beyotime). Protein lysates were subjected to SDS-PAGE, and were transferred into polyvinylidene fluoride (PVDF) membranes. Membranes were blocked with 5% non-fat milk in TBST for 2 h at room temperature and incubated overnight at 4°C with anti-STOML-2 (1:2,000, Proteintech™, Wuhan, China), anti-beta-actin (1:2,000, Proteintech), or anti-pIκBα (pSer53, 1:2,000, Epitomics, Burlingame, CA, USA). Horseradish peroxidase-conjugated goat anti-rabbit IgG (1:3,000, Proteintech) was used for detection of immunoreactive proteins. Signals were detected using ECL kit (P0018; Beyotime). An antibody against alpha-tubulin (1:2,000, Proteintech) served as endogenous control.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>No. of patients (n=49)</th>
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<tbody>
<tr>
<td>Gender (male/female)</td>
<td>33/16</td>
</tr>
<tr>
<td>Age (years) (≥60/&lt;60)</td>
<td>31/18</td>
</tr>
<tr>
<td>Tumor location (middle/lower)</td>
<td>38/11</td>
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<tr>
<td>Lymph node metastasis (negative/positive)</td>
<td>33/16</td>
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<tr>
<td>Differentiation (well/moderate/poor)</td>
<td>15/25/9</td>
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<tr>
<td>TNM stage (I/II/III)</td>
<td>13/24/12</td>
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Apoptosis assay. Cells were collected at 24 h post-transfection and stained with Annexin V-FITC apoptosis detection kit (KGA107; KeyGen, Nanjing, China) according to the instruction manual. Apoptosis was analyzed using a FACScan® flow cytometer equipped with CellQuest software (BD Biosciences).

Cell invasion assays. Cell invasion assays were performed using a transwell assay (8.0 µm, Corning, NY, USA); the upper chambers were coated with Matrigel basement membrane matrix (356234; BD Biosciences, San Jose, CA, USA) in serum-free RPMI-1640 for 3 h at 37°C. EC9706 and EC-1 cells were suspended in serum-free medium at a density of 2x10⁴ cells/ml at 48 h post-transfection and placed into the upper chamber (200 µl/well). Medium containing 10% FBS (500 µl) was added to the lower chamber, followed by incubation at 37°C in 5% CO₂ for 30 h. Following this, the medium and cells in the upper chamber were removed, and the cells that had migrated to the other side of the membrane were fixed in 4% paraformaldehyde, stained with 10% crystal purple for >30 min, and counted in three random fields under an inverted microscope (Leica Microsystems, Wetzkar, Germany).
3′-UTR luciferase reporter assay. To construct the STOML-2 3′-UTR luciferase reporter vector, the 3′-UTR of human STOML-2 (NM_013442, bases 1173-1431) fragment containing the seed sequence of mature miR-1207-5p was amplified by PCR from human genomic DNA. The primers for STOML-2 3′-UTR were as follows: 5′-AAGCTCGAGTGGAGCTGGGCTTGGCCAGGGAGTCTG-3′ (forward), 5′-GGGTCTAGATGGTTTGCCACTGGTGAGTTTATTACA-3′ (reverse). The fragment was cloned into the pmirGLO vector (E1330; Promega, Madison, WI, USA) downstream of the luciferase reporter gene to construct the recombinant vector, which was named STOML-2-3′-UTR-WT. The mutant STOML-2 3′-UTR fragment was generated by overlap extension PCR. The primers were as follows: 5′-AAGCTCGAGTGGAGCTGGGCTTGGCCAGGGAGTCTG-3′ (primer 1 forward), 5′-CTAGCTGGTTTGCCACTGGTGAGTTTATTACA-3′ (primer 1 reverse), 5′-AATCTACCGTCCCAAGCTAGAGCAGAATCAGG-3′ (primer 2 forward), 5′-GGGTCTAGATGGTTTGCCACTGGTGAGTTTATTACA-3′ (primer 2 reverse). In detail, with the STOML-2 3′-UTR fragment serving as a template, primer 1 and primer 2 were used to amplify fragment 1 and fragment 2; the two fragments and primer 3 were used to generate the full-length mutant fragment. Subsequently, the mutant fragment was inserted into the pmirGLO vector (E1330; Promega) and named STOML-2-3′-UTR-MT. For the luciferase reporter assay, human HEK293T cells were transiently co-transfected with 50 nM of miRNA (miR-1207-5p mimic or miR-1207-5p NC) and 50 nM of recombinant vectors (STOML-2-3′-UTR-WT or STOML-2-3′-UTR-MT) using Lipofectamine 2000. Luciferase activities were analyzed with a Dual-Luciferase Reporter assay system (E1910; Promega) and a CentroXS3 LB960 luminometer (Berthold, Germany) at 24 h post-transfection.

Statistical analysis. Statistical analyses were carried out using SPSS 17.0 software. Numerical results were presented as mean ± standard deviation. One-way analysis of variance was used to evaluate the data. Results were considered significant when P<0.05.

Results

The levels of miR-1207-5p expression are downregulated and STOML-2 are upregulated in EC tissues. To determine whether miR-1207-5p was involved in the tumorigenesis of EC, we investigated miR-1207-5p expression in 49 matched EC specimens and adjacent non-tumor tissues by qRT-PCR. As shown in Fig. 1A, the levels of miR-1207-5p were lower in tumor tissues than in adjacent non-tumor tissues (P<0.01). We also examined the possible correlation of miR-1207-5p expression with the occurrence of poor tumor differentiation and lymph node metastasis (P<0.05; Fig. 1B and Table II) in all patients with EC. However, there were no significant associ-
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Correlations between the levels of miR-1207-5p expression and gender, age or tumor location (P>0.05; Table II). Compared to the counterpart tissues, the levels of STOML-2 in EC tissues were greatly increased (P<0.05; Fig. 1C and E and Table II). STOML-2 expression levels in EC tissues were related to lymph node metastases and the differentiation status (P<0.05; Fig. 1D and Table II), but not to gender, age or tumor location (P>0.05; Table II). The expression of miR-1207-5p and STOML-2 exhibited significant differences at different TNM stages (P<0.05; Fig. 1F and G and Table II). The results also showed that miR-1207-5p was negatively correlated with STOML-2. Thus, these results indicated that downregulation of miR-1207-5p might play an important role in the progression and development of EC.

Overexpression of miR-1207-5p inhibits proliferation in EC9706 and EC-1 cells. To investigate whether miR-1207-5p functions as a tumor suppressor, the effects of upregulation of miR-1207-5p on the proliferation of EC cells were determined in vitro. The levels of miR-1207-5p expression in EC cells were detected by qRT-PCR. The levels of miR-1207-5p in EC9706 and EC-1 cells transfected with the miR-1207-5p mimic were higher than in the control groups, including the non-transfected blank group (blank) and the negative control group (NC) (P<0.01, Fig. 2A). In contrast, the levels of STOML-2 were found to be lower in the miR-1207-5p group when compared with the blank and NC groups (Fig. 2B). As shown in Fig. 2C and D, miR-1207-5p overexpression significantly decreased the growth rate of EC cells. The absorbance at 450 nm for the miR-1207-5p group at 48, 72 and 96 h was significantly decreased (P<0.05) in both EC9706 and EC-1 cells. However, there were no significant differences between the blank and NC groups (P>0.05).

Overexpression of miR-1207-5p induces apoptosis and restricts cell invasion of EC cells. To determine whether miR-1207-5p was contributing to apoptosis, we performed flow cytometric analysis of EC cells after transfection with miR-1207-5p mimic or NC. The results revealed that the level of apoptosis of cells transfected with the miR-1207-5p mimic was significantly increased when compared with the blank and NC groups (P<0.05; Fig. 3A). These results suggested that upregulation of miR-1207-5p mimics was able to induce apoptosis in EC9706 and EC-1 cells.

Transwell assays were used to test the effect of miR-1207-5p on cells invasion. Cells transfected with miR-1207-5p showed a remarkable decrease in invasive capacity compared with cells in the blank and NC groups (P<0.01, Fig. 3B). However, there were no significant differences between the blank and NC groups. In summary, these results indicated that overex-
Figure 2. Effects of miR-1207-5p overexpression in vitro on proliferation of EC cells. Levels of expression of miR-1207-5p were measured by qRT-PCR in three treatment groups: miR-1207-5p, NC, blank. The levels of miR-1207-5p were higher in transfected with miR-1207-5p mimic group than in the other two groups (P<0.01). (B) Levels of STOML-2 expression were determined with western blot assays. The results showed that the level of STOML-2 expression in the miR-1207-5p group was lower than in the control groups. (C and D) The proliferation of cells was detected using a CCK8 assay at 0, 24, 48, 72 and 96 h after transfection (*P<0.05). miR-1207-5p, cells transfected with miR-1207-5p mimic; NC, cells transfected with scrambled miR-1207-5p negative control; blank, untransfected cells.

Figure 3. Effects of upregulation of miR-1207-5p on apoptosis and invasion of EC9706 and EC-1. (A) Cell apoptosis was detected by Annexin V-FITC/PI assay. (B) Transwell assays were used to assess the invasive ability. *P<0.05; miR-1207-5p, cells transfected with miR-1207-5p mimic; NC, cells transfected with scrambled miR-1207-5p negative control; blank, untransfected cells.
Expression of miR-1207-5p decreased the invasive ability of both EC9706 and EC-1 cells.

**miR-1207-5p directly targets the STOML-2 gene by binding with the 3′-UTR.** The TargetScan and miRBase database predicted that the 3′-UTR of STOML-2 mRNA contained the seed region for miR-1207-5p. To verify this prediction, we used a dual-luciferase reporter system. The wild STOML-2 3′-UTR sequence and the mutant STOML-2 3′-UTR sequence were inserted into the pmirGLO vector to construct the recombinant vectors STOML-2-3′-UTR-WT and STOML-2-3′-UTR-MT. The recombinant vectors were co-transfected with miR-1207-5p mimic or NC into human HEK293T cells. The luciferase activity of the reporter was decreased in the group co-transfected with miR-1207-5p mimic and the STOML-2-3′-UTR-WT (P<0.05, Fig. 4A and B). However, the luciferase activity of the reporter was unaffected by co-transfection with miR-1207-5p and the STOML-2-3′-UTR-MT. Western blot analysis indeed showed that STOML-2 protein expression was significantly inhibited in EC9706 and EC-1 cells transfected with the miR-1207-5p mimic, compared with control cells (P<0.05; Fig. 4C and D). These results indicated that the overexpression of STOML-2 abrogated the anti-invasion function of miR-1207-5p.

**miR-1207-5p might be involved in the NF-κB signal pathway.** In the above studies, we found that upregulation of miR-1207-5p decreased the expression of STOML-2. Other researchers have reported that silencing STOML-2 in cells significantly inhibited the NF-κB activity and decreased the levels of expression of NF-κB target genes, including Bcl-xL. Therefore, we examined whether miR-1207-5p has effects on the NF-κB pathway by analyzing the phosphorylation of the main signaling molecule, IκBα, and Bcl-2, one of the NF-κB target genes. As shown in Fig. 6A, p-IκBα was significantly reduced in miR-1207-5p-transfected EC cells compared with the control cells. It was found that Bcl-2 expression was down-regulated in miR-1207-5p-transfected cells (Fig. 6B). These results suggested that downregulation of STOML-2 protein by miR-1207-5p might be involved in the NF-κB activation pathway in EC cells.
Discussion

Ectopic miRNAs have been reported in various cancers, and are involved in various cellular processes such as differentiation, invasion and apoptosis (26-28). Previous studies have indicated that miR-1207-5p was downregulated in several cancers (11,13). Our study showed that the expressions of mature miR-1207-5p were significantly downregulated in central EC.
tissues compared with tissues from the tumor margin, and that it might be a useful biomarker in EC. In addition, the levels of miR-1207-5p in patients with EC were associated with lymph node metastases, the levels of tumor differentiation and pathological stages. To examine the effects of miR-1207-5p, we transfected the miR-1207-5p mimic into EC9706 and EC-1 cells. Upregulation of miR-1207-5p was observed to suppress cell proliferation and invasion and to promote cell apoptosis. These results implied that miR-1207-5p might be an inhibitor of EC, and contributes to the development, progression and metastasis of EC.

Our study showed that STOML-2 was negatively regulated by miR-1207-5p at the posttranscriptional level by binding to the 3’-UTR of STOML-2 mRNA in EC cells. Studies have revealed that this interaction could decrease the expression of STOML-2 in EC cells. Previous experiments showed that STOML-2 was overexpressed in various tumors, such as esophageal carcinoma, gastric adenocarcinoma, breast cancer and glioma. Levels of STOML-2 were much higher in central tumor tissues than in paired tissues, and STOML-2-silenced cells showed decreased cell proliferation, invasive capability and adhesive ability in vitro (25). Accordingly, the level of STOML-2 was associated with tumor metastasis in breast cancer and pulmonary squamous cell carcinoma (29). Our study showed that the effect of STOML-2 on EC invasion needs to be further investigated. Our findings showed that miR-1207-5p might act as a suppressor of metastasis by targeting STOML-2.

Abnormal activation of NF-κB has been found in many types of cancer, including esophageal carcinoma (30-32); however, the activation mechanisms have not been elucidated. Accumulating publications have shown that miRNAs such as miR-146, miR-155, miR-21, and miR-301a have pathological relevance to NF-κB signaling (33-35). The phosphorylation of IκBα is a key step in NF-κB pathway activation, and Bcl-2 is one of the classical NF-κB target genes. Bcl-2 is an anti-apoptotic protein and promotes cell survival (36,37). Previous research has shown that it was related to the early development of EC (38,39). We found that upregulation of miR-1207-5p significantly inhibited STOML-2 expression and IκBα phosphorylation. The expression of Bcl-2 was downregulated in miR-1207-5p-transfected cells. Studies have demonstrated that depletion of STOML-2 in glioma cells reduced NF-κB transcriptional activity (25); this finding was consistent with our results, and indicated that STOML-2 might be involved in the regulation of the NF-κB signaling pathway. The precise molecular mechanism underlying miR-1207-5p/STOML-2 and the NF-κB signaling pathway needs to be investigated further.

In conclusion, this study showed that miR-1207-5p was downregulated in EC, and that upregulation of miR-1207-5p suppressed cell proliferation, invasion and promotes apoptosis. Based on these results, we propose that miR-1207-5p might act as a potential therapeutic target in the treatment of esophageal carcinoma.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (no. 81301726).

References


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