Downregulation of miR-153 contributes to epithelial-mesenchymal transition and tumor metastasis in human epithelial cancer

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The epithelial-mesenchymal transition (EMT) is a crucial step in epithelial cancer invasion and metastasis. The aims of this study were to investigate and validate unidentified micro RNAs (miRNAs) that regulate EMT and to reveal their clinical relevance in epithelial cancer patients. By applying miRNA array screening in a natural epithelial-mesenchymal phenotype cell line pair and in a transforming growth factor-β (TGF-β)-induced EMT cell model, we found miR-153 was markedly downregulated in the cells that underwent an EMT. A close association was confirmed between inhibition of miR-153 and the EMT phenotype, as well as the invasive ability of epithelial cancer cells. Ectopic expression of miR-153 in mesenchymal-like cells resulted in an epithelial morphology change with decreased cellular invasive ability. On the contrary, transfection of a miR-153 inhibitor in epithelial-like cells led to a mesenchymal phenotype change. In vivo ectopic expression of miR-153 significantly inhibited tumor cell metastasis formation. Data from the dual-luciferase reporter gene assay showed, for the first time, that SNAI1 and ZEB2 were direct targets of miR-153. Inverse correlations were also observed between miR-153 and SNAI1 and ZEB2 levels in oral cancer patients’ samples. Furthermore, low expression level of miR-153 was found to be significantly related to metastasis and poor prognosis in oral cancer patients. These data demonstrate that miR-153 is a novel regulator of EMT by targeting SNAI1 and ZEB2 and indicate its potential therapeutic value for reducing cancer metastasis.

Introduction

Metastases are the main cause of 90% of human cancer deaths (1). A characteristic of metastasizing cells is their transition from an epithelial to a mesenchymal state, a process known as epithelial-mesenchymal transition (EMT) (2). EMT is involved in embryonic development and cancer progression. In EMT, the loss of epithelial functions, such as cell–cell contacts, undergoing cytoskeletal remodeling and polarity changes is complemented by the acquisition of mesenchymal functions, which result in increased cell motility and invasion. Loss of expression of epithelial-specific proteins, such as E-cadherin, and increased expression of mesenchymal-specific proteins, such as vimentin, can be used as markers that an epithelial cell has undergone an EMT (3). During tumor progression, cancer cells can acquire motility and invasive ability by initiating an EMT process, which can potentially lead to distant metastases (4). Recent evidence indicates that EMT is a critical step in cancer cell invasion and metastasis and that it positively correlates with poor prognosis in cancer patients (5,6). Previous reports noted that carcinoma cells with properties of early cancers have an epithelial-like phenotype, whereas cells resembling more advanced cancer have a more mesenchymal-like phenotype (7).

The EMT-associated transcription factors ZEB1, ZEB2, SNAI1, SNAI2 and TWIST repress E-cadherin expression and promote cancer cell migration and invasion (8). Transforming growth factor-β (TGF-β) controls cell proliferation and differentiation and is a major inducer of EMT in epithelial cells during embryonic development and cancer progression (9). A further level of control in the regulation of this transcription network is exerted by micro RNAs (miRNAs) (10). miRNAs are an evolutionarily conserved group of small non-coding RNAs (~22 nucleotides) that downregulate gene expression at the post-transcriptional level (11). miRNAs are emerging as master regulators of tumorigenesis and have also been linked to the control of EMT in the development of cancer (12,13). For example, recent work suggests that members of the miR-200 family play key roles in mediating the effects of TGF-β and other EMT regulators on EMT in a number of different malignancies (14,15).

The miR-200 family was shown to target the transcriptional regulators of E-cadherin, ZEB1 and ZEB2, thus leading to an increase in E-cadherin expression (16).

Given that EMT is a process regulated by numerous factors, it is reasonable to assume that a variety of different miRNAs are involved in its regulation. Although the effects of the miR-200 family on EMT have been well elucidated, studies on the roles of miRNAs other than the miR-200 family in EMT are limited.

The overall goal of this study was to find and validate unidentified miRNAs that regulate EMT. By comparing and analyzing the miRNA expression profiles of epithelial phenotype cancer cells, mesenchymal phenotype cancer cells and TGF-β-induced EMT cell models, miR-153 has been identified as a key EMT suppressor that targets SNAI1 and ZEB2, thereby inhibiting cancer cell invasion and metastasis. Furthermore, we demonstrate that the low expression of miR-153 in human oral epithelial cancer is associated with clinical metastasis and poor survival.

Materials and methods

Cell culture materials

HN-4 cancer cells have an epithelial phenotype and low invasive capacity; these cells were derived from a primary squamous cell carcinoma of the tongue. HN-12 cancer cells exhibit a mesenchymal phenotype and have high invasive capacity; they were derived from a nodal metastasis in the patient from whom the HN-4 cells originated (17). Both these cell lines were obtained from the American Type Culture Collection. All the cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum and 0.4 µg/ml hydrocortisone, and the cells were maintained at 37°C in 5% CO2.

Transfection of miRNA mimics and inhibitors

Cells were seeded at 2 × 105 cells per well in a six well plate and transfected with synthetic miRNA double-stranded mimics or inhibitors at a final concentration of 100 nM using Lipofectamine 2000 (Invitrogen). Total RNA and protein were collected for assay 3 days post-transfection.

The sequences of miR-153 were as follows: sense: 5′-UUGCAUAGUC ACAAAGUGAUUC-3′; antisense: 5′-GAUCACUUUUGGACAUUGCCACAA3′. The sequences of miR-200c were as follows: sense: 5′-UAUUAUCUGCC GGGAUAUGAUGA-3′ and antisense: 5′-UCACACAUUACCCGGACGU AUUA-3′.
The sequence of the miR-153 inhibitor was as follows: 5′-AUCACUU UUGUGACUUAGCU-3′, and the sequence of the miR-200c inhibitor was as follows: 5′-CCAUUUAUCCCCGGCAUUU-3′.

Transwell invasive assay

Cell invasion behavior was evaluated using 24 well transwell units with 8 μm porosity polycarbonate filters. All the filters were coated with 50 μg reconstituted basement membrane matrix (Matrigel; BD Biosciences). The coated filters were air dried at 4°C prior to the addition of the cells. The basement membrane (coated filters) was hydrated with 50 μl serum-free DMEM medium 30 min before use (18–20). Cells were digested with trypsin, and cell density was adjusted to 1 × 10^6/ml using serum-free DMEM medium. A total of 200 μl of cell suspension was added into each upper transwell chamber, and 600 μl of DMEM medium containing 5% fetal bovine serum was added into the lower chamber. There were three duplicates for each cell group. The cells were incubated for 24 h in a humidified atmosphere of 5% CO₂ at 37°C. Cells were fixed with methanol and stained with Giemsa stain. Cells on the upper surface of the filter were removed by wipping with a cotton swab, and invasion was determined by counting the cells that migrated to the lower side of the filter using optical microscopy at ×200. A total of five visual fields at the center and in the surrounding areas were counted, and the average was calculated. The experiment was repeated three times.

RNA extraction and real-time PCR

Total RNA from cultured cells was extracted using Trizol (Invitrogen), and total RNA from formalin-fixed, paraffin-embedded samples of the oral squamous cell carcinoma (OSCC) was extracted using a High Pure miRNA Isolation Kit (Roche) according to the manufacturer’s instructions. For analysis of SNAI1 and ZEB2 messenger RNA (mRNA) expression, complementary DNA (cDNA) synthesis was performed using random primers under standard conditions. The specific primers for SNAI1, ZEB1, and GAPDH were designed as follows: SNAI1: 5′-GCTGGAGCAGCTCTAATCCGCA-3′/5′-ATCCGGGAGTGTTGATG-3′, ZEB2: 5′-AGGAGCTGTCTCGCTGTGT-3′/5′-GGAAAACAGTCCTGGGTGT-3′ (GAPDH: 5′-GCATGAGAAGTATGACCAAC-3′/5′-CTCTGTGGTGTTCAGGCTTA-3′). The mRNA expression was quantified using the ΔΔCt method. GAPDH served as the internal control. For miRNA analysis, real-time PCR was performed using PrimeScript™ miRNA RT-PCR Kit (Takara) according to the manufacturer’s instructions. Briefly, all the miRNAs in the total RNA samples were polyadenylated and reverse transcribed. The resulting cDNA was then subjected to real-time PCR. The entire sequences of miR-153 (TGTCAGATCTGAAAAGGTAGCT), miR-200c (TAATCTGGGGGTAATGATGGA) or U6 (CGCAAGGATGACACGTGAAG) were used as specific forward primers in combination with the reverse primers against E-cadherin (Invitrogen), vimentin (Sigma), ZEB1 (Gene Tex), SNAI1 (Santa Cruz), ZEB1 (Sigma) and SNAI2 (Cell signaling). The membranes were stripped and probed with monoclonal antibodies for β-actin as loading controls according to standard protocols.

Immunofluorescence

Cells were grown on coverslips, washed twice with phosphate-buffered saline, fixed in 4% paraformaldehyde for 20 min, permeabilized using 1% sodium dodecyl sulfate (SDS) in a blocking buffer containing 0.25% Triton X-100 in phosphate-buffered saline (pH 7.4) and probed with an E-cadherin antibody (1:1000; Invitrogen) or vimentin antibody (1:1000; Sigma). The primary antibody was detected using goat-anti-mouse-Alexa 594-conjugated antibodies (1:500; Invitrogen). To detect nuclei, cells were costained with 4′,6-diamidino-2-phenylindole (Invitrogen). Cells were observed on a Leica microscope, and images were analyzed with Leica software.

miRNA microarray

After completing an overnight incubation, samples from Trizol-extracted RNA (20 μg) were harvested for miRNAs, using a kit specifically designed to capture these low molecular weight species (Exiqon). The resulting samples were frozen and sent for miRNA microarray analysis (Exiqon, mercury LNA). At the time of analysis, Exiqon was capable of probing for 1355 separate miRNA species. The miRNA data were analyzed as the ratio of EMT/non-EMT cell lines. The time of analysis, Exiqon was capable of probing for 1355 separate miRNA species. The miRNA data were analyzed as the ratio of EMT/non-EMT cell lines. The average was calculated. The experiment was repeated three times.

Western blot analysis

Cells were lysed in RIPA buffer (50mM Tris pH 7.5, 150mM NaCl 1% Triton X-100, 5mM ethylenediaminetetraacetic acid). Protein concentration was determined using the BCA Kit (Pierce). Twenty-five micrograms of protein was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to nitrocellulose. The blots were then probed with antibodies against E-cadherin (Invitrogen), vimentin (Sigma), ZEB1 (Gene Tex), SNAI1 (Santa Cruz), ZEB1 (Sigma) and SNAI2 (Cell signaling). Following this, the blots were incubated with 1:5000 fluorescent secondary antibody (IRDye 800 anti-mouse antibody; Rockland). Images were acquired with the Odyssey infrared imaging system and analyzed by the software program as specified by Odyssey. The membranes were stripped and probed with monoclonal antibodies for β-actin as loading controls according to standard protocols.

Rescue experiment

To further validate that SNAI1 and ZEB2 targeting is involved in miR-153-mediated EMT regulation, functional rescue experiments were performed by cotransfection with miR-153 and plasmid constructs expressing SNAI1 and ZEB2. EMT-related markers and phenotypes were then measured. The expression plasmid pcDNA3-ZEB2, encoding human ZEB2, was constructed as follows. A cDNA clone of human ZEB2 was purchased from Proteintech Group as the template for the next PCR. The full-length ZEB2 gene was amplified by high-fidelity PCR (PrimeSTAR; Takara) with a set of primers (5′-CGGGTACCATGCGGCTCTTCTTCTTCTTTCTCTC-3′/5′-CGGATCCCATGCGGCTCTTCTTCTTCTTTCTCTTCTTCTC-3′). The amplified product was then digested with KpnI and cloned into the eukaryotic expression vector pcDNA3, pcDNA3-SNAI1, encoding human SNAI1, was constructed as follows. Full-length SNAI1 cDNA was amplified by PCR using the reverse transcription product of RNA from MDA-MB-231 cells as the template with a set of primers (5′-CGGATCCATGCGGCTCTTCTTCTTCTTCTTCTTCTTCTC-3′/5′-GAAGATCTCAGCGGCTCTTCTTCTTCTTCTTCTTCTTCTC-3′). The amplified product was then digested with BamHI and cloned into the BamHI cloning site of pcDNA3. The presence of complete ZEB2 and SNAI1 coding regions was confirmed by DNA sequencing.

Experimental metastasis assay

All animal procedures were performed according to guidelines approved by the Shanghai Jiao Tong University School of Medicine. Female athymic mice (Shanghai Laboratory Animal Center) were used for the studies. Cells evaluated included MDA-MB-231 cells stably transfected with miR-153 or miR-200c, alone or in combination, as well as MDA-MB-231 cells transfected with empty plasmid. Cells (2 × 10^6 cells in 0.2 ml phosphate-buffered saline) were injected intravenously into the lateral tail vein of 4-week-old athymic mice, and lung colonization was evaluated. Each treatment group consisted of seven mice. At termination, the lungs were removed and fixed in Bouin’s fixative diluted 1:5 with neutral-buffered formalin (21). Surface metastatic nodules per lung were then determined.

Analysis of human oral epithelial cancer samples

Seventy-five formalin-fixed, paraffin-embedded tissue specimens were obtained from patients who were diagnosed with OSCC and underwent surgical resection between 2006 and 2007 in the Department of Oral Pathology, Ninth People’s Hospital, Shanghai Jiao Tong University School of Medicine. Follow-up ended on December 11th at or death. Overall survival was defined as the time from...
miR-153 is markedly downregulated in the TGF-β-induced mesenchymal phenotype of epithelial cancer cells

We employed tumor cell lines with different metastatic potentials to screen out the metastasis-associated miRNAs from epithelial cells. HN-4 cells were derived from a primary squamous cell carcinoma of the tongue, and the HN-12 cells were derived from a nodal metastasis in the patient from whom the HN-4 cells originated. Both MCF-7 and MDA-MB-231 cells were derived from the pleural effusion of patients with breast cancer. Morphologically, the HN-4 and MCF-7 cells are typical multilateral paving stone-like epithelial cells. In contrast, HN-12 and MDA-MB-231 cells have a spindle-shaped fibroblast-like morphology (Figure 1A). Immunofluorescence and western blotting results showed loss of the epithelial protein marker, E-cadherin, and increased expression of the mesenchymal-specific protein, vimentin, in HN-12 and MDA-MB-231 cells compared with HN-4 and MCF-7 cells (Figure 1A and B). Transwell assays demonstrated that the HN-12 and MDA-MB-231 cells were 4.9-fold and 5.4-fold more invasive than HN-4 and MCF-7 cells, respectively (Figure 1C). As noted above, evidence indicates that the HN-4 and MCF-7 cells are typical epithelial phenotype carcinoma cells, whereas HN-12 and MDA-MB-231 cells have undergone an EMT and display a more mesenchymal phenotype. Upon treatment with TGF-β, HN-4 and MCF-7 cells acquire a spindle-type cell morphology with inhibition of cell–cell contacts (Figure 1A). Decreased protein levels of E-cadherin and increased levels of vimentin were also observed and accompanied by an enhanced invasive ability (Figure 1A–C). These results suggest that TGF-β can effectively induce an EMT in HN-4 and MCF-7 cells. To examine the potential participation of miRNAs in EMT of epithelial cancer cells, the miRNA profiles in a natural epithelial-mesenchymal phenotype cell line pair (HN-4/HN-12) and in the TGF-β-induced EMT models (HN-4/HN-4 treated by TGF-β) were determined by miRNA microarray. Downregulated miRNAs (>2-fold change downward) were revealed in both the TGF-β-induced EMT model and the natural mesenchymal phenotype epithelial carcinoma cells (Figure 1D). The results also showed that 51 miRNAs were downregulated in HN-12 cells compared with HN-4 cells and 40 miRNAs were downregulated in TGF-β-induced HN-4 cells compared with the untreated HN-4 cells. A Venn diagram analysis indicated 18 overlapping genes including miR-200 family members (miR-200c and miR-200a), a group well known to be powerful negative regulators of EMT (Figure 1E). The miRNA array data were further validated by real-time PCR. High consistency was found in these two assays (Supplementary Tables I and II, available at Carcinogenesis Online). Therefore, the miRNA array results can provide some valuable information. In addition to downregulation of members of the miR-200 family, miR-153, a miRNA with, as yet, no reported role in EMT, was found to have one of the highest levels of downregulation of miRNAs in the cells that underwent EMT. These results were also verified by quantitative RT-PCR (Figure 1F). Thus, it can be postulated that this miRNA plays a specific functional role in EMT. Accordingly, miR-153 was selected for further analysis.

miR-153 is an important EMT regulator in epithelial cancer cells

To determine whether the miR-153 downregulation is essential for TGF-β-induced EMT, HN-4 and MCF-7 cells were treated both with miR-153 mimics and TGF-β, whereas miR-200c served as a positive control. Coincubation of miR-153 and miR-200c mimics with TGF-β prevented the morphological (Figure 2A) and protein marker (Figure 2B and C) changes associated with EMT; this suggests that treatment with miR-153 could block TGF-β-induced EMT in epithelial carcinoma cells. We, subsequently, investigated whether miR-153 was required for epithelial cancer cells to maintain an epithelial phenotype and if overexpression of miR-153 had an effect on mesenchymal-to-epithelial transition (MET), a reversal of the EMT process. For this purpose, two functional cell lines were generated. First, stable HN-4 and MCF-7 cell lines inhibiting the expression of miR-153 and miR-200c, and second, stable HN-12 and MDA-MB-231 cell lines overexpressing miR-153 and miR-200c. As shown in Figure 3A and C, overexpression of miR-153 and miR-200c in HN-12 and MDA-MB-231 cells resulted in an epithelial type morphology change from an elongated, spindle-shaped, mesenchymal phenotype to a more rounded, epithelial-like phenotype, with cells aggregating in groups. This morphological change was associated with reduced vimentin expression and increased E-cadherin expression. Ectopic expression of miR-153 and miR-200c also caused significantly decreased invasive ability of the cells. The strongest changes occurred in the miR-153 and miR-200c combined treatment group. After transfection with the miR-153 and miR-200c expression inhibitors, HN-4 and MCF-7 cells began to adopt a mesenchymal-like morphology, accompanied by a substantial increase in vimentin and a decrease in E-cadherin. These cells also showed a higher invasive ability in vitro, compared with parental cells. Similarly, combined inhibition of miR-153 and miR-200c led to the strongest EMT-associated changes (Figure 3B and D). These data indicate that downregulation of miR-153 is important in the acquisition or maintenance of the mesenchymal morphology and ectopic expression of the miR-153 in cells with a mesenchymal-like phenotype that would promote a MET. In addition, an additive effect was observed when miR-153 and miR-200c were combined.

miR-153 targets the SNAI1 and ZEB2

To identify targets of miR-153, a list of predicted target mRNAs obtained through the algorithm TargetScan (www.targetscan.org) were analyzed. Significantly, SNAI1 and ZEB2, which are known to be relevant to EMT and tumor metastasis, were predicted as the targets of miR-153 (Figure 4A). This prediction was validated using reporter gene assays in 293T cells using the dual-luciferase reporter system. A 3′UTR fragments containing the predicted miR-153 targeting sites of SNAI1 (pGL3-SNAI1 3′UTR) and the targeting site of ZEB2 (pGL3-ZEB2 3′UTR) were fused downstream of the firefly luciferase gene. Dual-luciferase reporter vectors with a miR-153 mutated binding site (pGL3-SNAI1 3′UTR-mut, pGL3-ZEB2 3′UTR-mut) were also constructed. Cotransfection of pGL3-SNAI1 3′UTR or pGL3-ZEB2 3′UTR with miR-153 inhibitor caused an elevated luciferase signal in HN-12 cells (Figure 4C). SNAI1 and ZEB2 protein expression increased when HN-4 and MCF-7 cells were treated with miR-153 inhibitor and decreased when HN-12 and MDA-MB-231 cells were treated with miR-153 mimic (Figure 4D). In contrast, miR-153 had no effect on the expression level of SNAI2 and ZEB1, another two important EMT regulators (Figure 4D). Furthermore, overexpression of both SNAI1 and ZEB2 rescued the miR-153 effect on the MET in mesenchymal-like HN-12 and MDA-MB-231 cells (Figure 4E). These data indicate that miR-153 regulates SNAI1 and ZEB2 expression through targeting their 3′UTRs that are complementary to the seed region of miR-153.

Ectopic expression of miR-153 inhibits tumor cell metastasis in vivo

The suppressive effect of miR-153 and miR-200c on tumor metastasis was further investigated in an experimental metastasis animal model.
Fig. 1. Analysis of differentially expressed miRNAs based on the TGF-β-induced EMT phenotype. (A) Morphology and E-cadherin and vimentin staining of HN-4 cells, TGF-β-treated HN-4 cells, MCF-7 cells, TGF-β-treated MCF-7 cells and mesenchymal-like HN-12 cells, MDA-MB-231 cells. (B) Western blot analysis of E-cadherin and vimentin protein levels in cell lines. (C) Invasive ability of cells evaluated by Matrigel invasion assay. (D) Heatmap of differentially
This was performed by injecting MDA-MB-231 cells stably expressing miR-153 and miR-200c alone or in combination into nude mice through the lateral tail vein. The metastatic nodules were counted on the surface of lung after 7 weeks and confirmed by histological examination (Figure 5A and B). Ectopic expression of miR-153 or miR-200c resulted in approximately a 4.2-fold and 3.3-fold reduction, respectively, of the number of metastatic nodules compared with the control group. The greatest reduction (12.5-fold) appeared in the combination treatment groups (Figure 5C). These results indicate that both miR-153 and miR-200c could effectively suppress tumor metastasis in vivo, and that combined treatment with miR-153 and miR-200c had an additive effect.

**Inverse correlation between the expression of miR-153 and SNAI1 and ZEB2 in oral cancer tumors**

To determine in vivo miR-153 regulation of SNAI1 and ZEB2, a correlation analysis between the expression of miR-153 and its targets was performed in oral cancer patients’ samples. A significant inverse correlation between miR-153 and SNAI1 (Pearson’s correlation $R = -0.327$, $P = 0.004$) and ZEB2 (Pearson’s correlation $R = -0.412$, $P < 0.001$) mRNA expression was observed (Figure 6A).

**Low expression level of miR-153 is related to metastasis and poor prognosis in patients with oral cancer**

Furthermore, the clinical implications of miR-153 and miR-200c were also assessed. The levels of miR-153 and miR-200c in oral cancers with or without metastasis were measured. The primary tumors of patients with metastasis expressed approximately 1.47-fold lower levels of miR-153 ($P = 0.022$, $F = 5.49$) and 1.78-fold lower levels of miR-200c ($P = 0.037$, $F = 4.52$) than those of patients without metastasis (Figure 6B). This result indicates that the loss of either miR-153 or miR-200c may contribute to the metastatic progression of oral cancers. Subsequently, the correlation of these two miRNAs expression levels with survival was conducted. The Kaplan–Meier survival curves show that patients with low expression levels of miR-153 ($P = 0.025$) and miR-200c ($P = 0.023$) had poorer survival than patients with high miR-153 and miR-200c expression. Notably, the dual low expression of miR-153 and miR-200c was significantly associated with a poor overall survival of patients with oral cancer, with a lower $P$ value ($P = 0.007$) compared with miR-153 and miR-200c alone (Figure 6C).

**Discussion**

Consistent with previous reports, our data showed a close correlation between EMT and the invasion potential of tumor cells. Mesenchymal-like cancer cells and TGF-$\beta$-induced EMT cells displayed more invasive ability than epithelial-like cancer cells, confirming that EMT is a key step that facilitates tumor migration and invasion. Recently, miRNAs were demonstrated to be powerful regulators of EMT (22), which encouraged us to study the role of novel miRNAs in the regulation of EMT. To date, more than 1400 miRNAs have been validated in the human genome (23). With respect to cancer, although specific miRNAs are overexpressed in cancer cells, miRNAs are often downregulated in tumors (24,25). Reports have indicated that both human and mouse cancers show a global reduction in mature miRNA expressed miRNAs patterns between HN-4 cells (epithelial-like cell line), HN-12 cells (mesenchymal-like cell line) and TGF-$\beta$-treated HN-4 cells. Red indicates upregulation, and green indicates downregulation. (E) A Venn diagram analysis indicated 18 overlapping genes downregulated in both HN-12 cells and TGF-$\beta$-treated HN-4 cells compared with untreated parental HN-4 cells. (F) Downregulation of miR-153 and miR-200c in both mesenchymal-like cell lines (HN-12 and MDA-MB-231 cells) and TGF-$\beta$-induced cell lines (TGF-$\beta$-treated HN-4 cells and TGF-$\beta$-treated MCF-7 cells) as measured by real-time PCR.
Fig. 3. miR-153 was required for maintaining an epithelial phenotype and promoted MET. (A) Phase contrast microscopy, western blot analysis of E-cadherin and vimentin staining, invasive ability of HN-12 cells transfected with miR-153, miR-200c, alone or in combination. (B) Phase contrast microscopy, western blot analysis of E-cadherin and vimentin staining, invasive ability of HN-4 cells transfected with miR-153 inhibitor, miR-200c inhibitor alone or in combination.
levels compared with normal tissues (24). In addition, this type of reduced mature miRNA expression is associated with the transformed phenotype of cancer cells and can ultimately promote tumorigenesis (26). Therefore, we placed our focus on the downregulated miRNAs associated with EMT.

In this study, which used a comprehensive screening approach that combined analysis of the dysregulation of miRNAs in a natural epithelial-mesenchymal phenotype pair of cell lines and TGFB-induced EMT models, downregulated miRNAs, in the intersections, were screened out for further analysis. This intersection may reflect the aberrantly expressed miRNAs that play an important role in maintaining or obtaining EMT status. Some miR-200 family members (miR-200a and miR-200c) were found in the altered gene set. The miR-200 family has previously been demonstrated to be a powerful negative regulator of EMT. Lower expression of the miR-200 family has been reported in mesenchymal-like cell lines compared with epithelial-like cell lines. By suppressing the mesenchymal-specific repressors of E-cadherin transcription, ZEB1 and ZEB2, the miR-200 family members increase the levels of E-cadherin and are capable of enforcing the epithelial phenotype in mesenchymal-like cells (27,28). The consistency of our observations of the downregulation of the miR-200 family during EMT with previously reported results provide an indication of the reliability of our screening method. In addition to the miR-200 family, miR-153, a miRNA with no reported function in EMT thus far, was also found to be downregulated in the cells that underwent EMT. As previously reported, downregulation of miR-153 is a frequent event in a certain types of tumors, including glioblastoma and advanced ovarian tumor (29–31). By targeting BCL-2 and MCL-1, miR-153 induces apoptosis in these tumors, suggesting that miR-153 functions as a tumor suppressor (30,31). Studies have revealed a tendency toward downregulation of miR-153 in relation to lymph node metastasis in ovarian epithelial tumors (29) and downregulation of miR-153 in high-risk medulloblastomas, which are either metastatic or belong to children >3 years of age (32). Nonetheless, the roles of miR-153 in EMT and metastasis have not been investigated. Our results provide new evidence that reveals roles of miR-153 in cancer cell metastasis.

Our findings showed that ectopic expression of miR-153 in carcinoma cells could prevent TGFB-induced EMT and revert mesenchymal-like cells to an epithelial-like phenotype; miR-153 inhibitors were sufficient to initiate EMT changes in epithelial-like cancer cells. In addition, downregulation of miR-153 contributed to EMT-associated carcinoma cell invasion. These results indicate that miR-153 has important roles in regulating EMT.

Identification of putative targets is important for a complete understanding of the specific functions of miR-153. In this study, by using the miRNA target prediction program and a dual-luciferase report assay, we demonstrated that miR-153 directly downregulated SNAI1 and ZEB2 by binding their 3′UTR sites. Accordingly, the ectopic expression or targeted knock down of miR-153 resulted in downregulation or increased expression of SNAI1 and ZEB2 protein level. These results indicate that SNAI1 and ZEB2 are direct targets for miR-153. Both SNAI1 and ZEB2 are well-characterized transcriptional repressors of E-cadherin by binding to E-box elements present in the E-cadherin promoter. E-cadherin is a central component of the adherens junction complex responsible for cell–cell adhesion, and loss of E-cadherin expression is a key event in EMT. Consistent with this, a decreased expression of E-cadherin was detected in the cells that underwent EMT triggered by the miR-153 inhibitor. In addition, an additive EMT-inducing effect and increased repression of E-cadherin expression was obtained by cotransfecting cells with miR-153 and miR-200c inhibitors in combination. Conversely, ectopic expression of miR-153 led to MET, the reverse of EMT, accompanied by increased levels of E-cadherin. Simultaneously transfecting cells with miR-153 and miR-200c resulted in the largest MET effect, as well as a maximal increase of E-cadherin. This enhanced the EMT regulation effect of the combination of miR-153 and miR-200c, probably due to an increased effect on targets of these two miRNAs (miR-153: SNAI1 and ZEB2; miR-200c: ZEB1 and ZEB2). Furthermore, in the rescue experiment, overexpression of both SNAI1 and ZEB2 restore the phenotype in miR-153-treated mesenchymal-like cells. This study provides a new link between miR-153 and SNAI1 and ZEB2 in the regulation of epithelial carcinoma cell EMT programs.

EMT is a crucial step of tumor metastasis, therefore, inhibition of EMT represents a very promising therapeutic strategy to prevent tumor metastasis (33). In this study, we found forced expression of miR-153 in invasive cell lines could decrease invasive capacity. This finding is of great interest as targeting EMT in invasive cancer cells by miR-153 might provide a valuable tool in development of new therapeutic avenues against metastasis. We also extended this finding in an experimental metastasis model in which ectopic miR-153 expressing MDA-MB-231 cells and parental cells were used to examine the generation of pulmonary metastases. (HN-12 cells were also employed for animal study however, they failed to develop experimental metastasis model data not shown.) Strikingly, miR-153 significantly decreased macroscopic tumor formation in the lung. In both in vivo and in vitro experiments, an additive inhibitory effect on cancer cell invasion or metastasis was observed when miR-153 and miR-200c were treated in combination; these results are consistent with their repressive effects on EMT. In fact, studies indicate that targeting of ZEB1 and ZEB2 by miR-200c and the resulting upregulation of E-cadherin are shown to contribute to inhibition of invasion and metastasis of different cancer cells (34,35). In this study, miR-153 is proved a novel regulator of EMT by targeting SNAI1 and ZEB2. Thus, it can be speculated that the additive effect of these two miRNAs on inhibition of invasion and metastasis is possibly in part due to the combination treatment targets of multiple EMT regulation pathways. These results indicate that miR-153 and miR-200c can suppress the invasion and metastasis of cancer, possibly through EMT inhibition, and could therefore serve as targets for tumor therapy.

To further clarify the clinical prognostic significance of miR-153 and miR-200c, we examined expression of these miRNAs in 75 oral cancer samples to identify the links between these miRNAs and cancer metastasis, as well as survival prognosis. Our data showed the expression of both miR-153 and miR-200c was lower in patients with metastasis than patients without metastasis. This result revealed a clear correlation between miR-153 expression and oral cancer metastasis, rather than the indirect evidence reported previously. Although the role of the miR-200 family in regulating EMT is well established, their expression in metastases is controversial. Some studies have shown that the miR-200 family is downregulated between matched primary cancers versus metastatic breast, colon, lung, bladder and liver cancers (36–38), and lower miR-200 expression levels are reported associated with a higher propensity to lymph node metastases in lung cancer and gastric carcinoma (39,40). Another group reported that the miR-200 family is overexpressed in breast cancer metastases (41). In this study, low levels of expression of miR-200c were detected in oral cancer patients with metastasis. This, together with the in vitro findings that miR-153 and miR-200c are lost in cell lines with high metastatic ability and the results that ectopic expression of miR-153 and miR-200c limited metastasis in vivo, further confirmed the roles of miR-153 and miR-200c in cancer cell metastasis. Moreover, the Kaplan–Meier survival analysis showed that the expression of miR-153 and miR-200c were correlated with overall survival. The patients

(C) Phase contrast microscopy, western blot analysis of E-cadherin and vimentin staining, invasive ability of MDA-MB-231 cells transfected with miR-153, miR-200c alone or in combination. (D) Phase contrast microscopy, western blot analysis of E-cadherin and vimentin staining, invasive ability of MCF-7 cells transfected with miR-153 inhibitor, miR-200c inhibitor, alone or in combination. *P < 0.05 compared with control group, **combination of miR-153 and miR-200c treatment showed significant difference compared with single miRNA treatment.
Fig. 4. miR-153 targeted SNAI1 and ZEB2. (A) SNAI1 and ZEB2 are predicted as the targets of miR-153. (B) A dual-luciferase reporter system analysis was performed to validate miR-153 target genes. A 3′ UTR fragment containing the predicted miR-153 targeting sites of SNAI1 and the targeting site of ZEB2 was fused downstream of the firefly luciferase gene. A miR-153 mutated binding site (mut) was also constructed. Cotransfection of SNAI1 3′UTR or ZEB2 3′UTR...
Role of miR-153 in EMT

with lower levels of miR-153 and miR-200c had shorter survival times, and the poorest prognosis occurred in patients with low expression of these two miRNAs. Described as a tumor suppressor, lower levels of miR-153 have previously been reported to be a poor prognostic marker in multiple types of cancer (29,42); this is consistent with our results. The clinical relevance of miR-200c in cancer patients is not very clear. Data have suggested low miR-200 family expression is linked to poor prognosis in ovarian cancer (43–45) and in lung cancer, and lower miR-200c expression levels were found to be associated with a poor grade of differentiation, an aggressive, invasive and chemoresistant phenotype (39). Although miR-200 was found to be associated with poor prognosis in breast cancer (41), it is possible that tumor type specificity may lead to this difference. Our results indicate the miR-200 level could be a predictive marker for clinical outcome.

Fig. 5. Ectopic expression of miR-153 and miR-200c inhibited tumor cell metastasis in vivo. An experimental metastasis animal model analysis was performed by injecting MDA-MB-231 cells stably expressing miR-153 and miR-200c alone or in combination into nude mice through the lateral tail vein. (A) Images of lungs from all mice in each experimental group (n = 7). (B) Hematoxylin and eosin staining of a representative lung section. (C) The number of tumor nodules on the lung surface. *P < 0.05 compared with control group, **combination of miR-153 and miR-200c treatment showed significant difference compared with single miRNA treatment.
in oral cancer. Prospective clinical studies are necessary to confirm whether these miRNAs are reliable clinical predictors of outcome for cancer patients. Another major finding in clinical samples is that we observed significant inverse correlations between miR-153 and SNAI1 and ZEB2 mRNA expression levels in patient tumors, which supports the target regulation of miR-153 found in vitro. Collectively, this study revealed a critical inhibitory effect of miR-153 on metastasis via regulation of EMT by way of targeting ZEB2 and SNAI1. An additive effect can be acquired by combination with miR-200c. Our results provide insights into the therapeutic value of miR-153 and miR-200c in reducing cancer metastasis. Furthermore, our findings suggest possible roles for miR-153 and miR-200c both as markers of metastasis and prognostic factors in OSCC. Nevertheless, these data should be further validated in independent cohorts and prospective trials.

Supplementary material

Supplementary Tables I and II can be found at http://carcin.oxfordjournals.org/

Fig. 6. Inverse correlation between the expression of miR-153 and its predicted targets, and the clinical implications of miR-153 in patients with OSCC. (A) Significant inverse correlations between miR-153 and SNAI1 (Pearson’s correlation $R = -0.327, P = 0.004$) and ZEB2 (Pearson’s correlation $R = -0.412, P < 0.001$) mRNA expression in oral cancer patient tumors. (B) The levels of miR-153 and miR-200c in the primary tumors of oral epithelial cancers with $(n = 35)$ metastasis were significantly lower than those without metastasis $(n = 40)$ $(P = 0.022, P = 0.037)$. (C) The Kaplan–Meier survival curves indicated that patients with low expression levels of miR-153 $(P = 0.025)$ and miR-200c $(P = 0.023)$ had poorer survival than patients with high miR-153 and miR-200c expression. The dual low expression of miR-153 and miR-200c was significantly associated with the poorest overall survival of patients with OSCC $(P = 0.007)$.

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References


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