SPANDIDOS PUBLICATIONS

ONCOLOGY REPORTS 29: 1579-1587, 2013

Downregulated microRNA-200a promotes EMT and tumor growth through the Wnt/β-catenin pathway by targeting the E-cadherin repressors ZEB1/ZEB2 in gastric adenocarcinoma

NINGNING CONG[1], PING DU[1], ANLING ZHANG[2], FAJUAN SHEN[1], JUAN SU[1], PEIYU PU[2], TAO WANG[1], JIE ZJANG[1], CHUNSHENG KANG[2] and QINGYU ZHANG[1]

[1]Department of Gastroenterology, Tianjin Medical University General Hospital; [2]Department of Neurosurgery, Tianjin Medical University General Hospital and Laboratory of Neuro-Oncology, Tianjin Neurological Institute, Tianjin 300052, P.R. China

Received December 5, 2012; Accepted December 28, 2012

DOI: 10.3892/or.2013.2267

Abstract. In a previous study, we found that microRNA (miRNA)-200a suppresses Wnt/β-catenin signaling by interacting with β-catenin, thereby inhibiting migration, invasion and proliferation. However, the mechanism involved in this suppression remains unclear. In the present study, we investigated the underlying mechanism of miR-200a regulation of epithelial-mesenchymal transition (EMT) in gastric carcinoma cells, and confirmed the tumor suppressor role of miR-200a *in vivo*. The expressions of miRNA-200a, -200b and -200c, identified by fluorescent *in situ* hybridization, were downregulated and inversely correlated with WHO grades of gastric adenocarcinoma (GA). The expression of the potential miR-200a target genes ZEB1 and ZEB2 was detected immunohistochemically. These examinations used the same tissue microarrays to analyze the relationships between miR-200a and potential target genes. The expression of miR-200a and ZEB1/ZEB2 in the same GA tissue microarrays was inversely related. Restored miR-200a expression inhibited tumor growth in nude mice harboring subcutaneous SGC7901 xenografts. The expression of N-cadherin, β-catenin, Twist1 and Snail2 decreased, and E-cadherin levels increased, when miR-200a was elevated, as tested by fluorescence microscopy and immunohistochemistry. Similar results were observed *in vivo*. We found upregulated miR-200a expression to increase E-cadherin and suppress the Wnt/β-catenin pathway by targeting ZEB1 and ZEB2 in GA, thus delaying tumor growth *in vivo*. The effect of miR-200a on Wnt/β-catenin signaling may provide a therapeutic target against EMT.

Correspondence to: Dr Qingyu Zhang, Department of Gastroenterology, Tianjin Medical University General Hospital, 154 An-Shan Road, Heping, Tianjin 300052, P.R. China
E-mail: zhangqy@tijmu.edu.cn

Dr Chunsheng Kang, Department of Neurosurgery, Tianjin Medical University General Hospital and Laboratory of Neuro-Oncology, Tianjin Neurological Institute, 154 An-Shan Road, Heping, Tianjin 300052, P.R. China
E-mail: kang97061@gmail.com

Key words: gastric adenocarcinoma, epithelial-mesenchymal transition, miRNA-200a, Wnt/β-catenin pathway

Introduction

Gastric cancer is the second most fatal malignancy worldwide (1). Despite recent advances in surgical technique, diagnostic methods and chemotherapy regimens, no effective targeting therapy is available for gastric cancer, as the molecular mechanisms underlying gastric cancer development remain unclear (2,3).

microRNAs (miRNAs, miRs) are short, 19-22 nucleotide long, on-coding RNAs that influence cellular processes at the post-transcriptional level by targeting the 3' untranslated region of mRNA, causing reduced translation of proteins or degradation of mRNAs (4-6). miRNAs are dysregulated in various human types of cancer, including gastric adenocarcinoma (GA), and play crucial roles in tumorigenesis, cell growth, differentiation and apoptosis (7,8). Thus, miRNAs may function as tumor suppressor genes or oncogenes (9). Accumulating evidence suggests that the miRNA-200 family serve as tumor suppressors in cancer cells. The miR-200 family consists of two clusters: miR-200b, miR-200a and miR-429 located on chromosome 1; and miR-200c and miR-141 located on chromosome 12 (10,11).

Epithelial-mesenchymal transition (EMT) is a pathological event associated with tumor progression and is considered to influence and promote certain steps in the metastatic cascade. Characterized by loss of cell-cell adhesion and apex-base polarity, EMT enhances cell motility and metastasis (12,13). Its prominent hallmarks are loss of expression of epithelial markers such as E-cadherin and induction of mesenchymal markers such as N-cadherin and vimentin (14). Ectopic expression of ZEB1, ZEB2, Snail and Twist result in loss of E-cadherin-mediated cell-cell adhesion, and induction of cell motility, suggesting that activation of these transcriptional factors results in induction of EMT phenotypes (15).

In this study, we confirmed the expression of miR-200a in GA and normal tissue using fluorescent *in situ* hybridization (FISH); we also showed that ZEB1 and ZEB2 are involved in the EMT process and identified target genes of miR-200a. Transcriptional factors such as Twist1 and Snail2 play key roles in EMT. We further confirmed reduced miR-200a levels in GA through downregulated E-cadherin expression and upregulation of the major activator of the Wnt signaling pathway, β-catenin. We determined that miR-200a acted as a tumor-suppressor factor and inhibited the process of EMT and tumor growth.

Materials and methods

Patients and samples. A GA tissue microarray was obtained from Shaanxi Chaoying Biotechnology Ltd., (Xi'an, China). Pathologic tumor grades on the microarray were defined, according to the 2007 WHO criteria, as follows: 4 cases with grade I, 22 with grade II, 34 with grade III and 4 with grade IV; 10 cases were normal gastric tissue. Each dot was a 1.5-mm diameter tissue sample from an individual specimen that had been pathologically confirmed. All microarrays were stored in the dark at 4˚C.

In situ hybridization. Using antisense locked nucleic acid (LNA) modified oligonucleotide probes, *in situ* hybridization was performed with the In-Situ Hybridization kit (Boster, Wuhan, China). Sequences of the LNA/DNA oligonucleotides contained locked nucleic acids at eight consecutive centrally located bases (indicated by the underline) as shown: hsa-miR-200a 5'-ACA TCG TTA CCA GAC GAC AGT GTT A-3'; hsa-miR-200b 5'-TCA TCA TTA CCA GGC AGT ATT A-3'; and hsa-miR-200c 5'-TCC ATC ATT ACC CGG CAG TAT TA-3'. Sections were deparaffinized and deproteinated, and then prehybridized for 2 h in hybridization liquid in a humidified chamber (50% formamide, 5X SSC). Sections were incubated with 20 µl LNA-miR-200a, LNA-miR-200b and LNA-miR-200c hybridization solution at 42˚C for 16 h, after washing with phosphate-buffered saline (PBS) 3 times. miR-200a, miR-200b and miR-200c were labeled with Cy3-avidin at 0.5 mg/ml and incubated for 2 h at room temperature in the dark. Nuclei were counterstained with a DAPI karyotyping kit (GenMed, Boston, MA, USA). After washing with PBS 3 times, sections were sealed, detected under a fluorescence microscope with an OptiGrid system and analyzed by IPP6.1 (Olympus, Tokyo, Japan).

Cell culture and transfection. Human stomach adenocarcinoma cell lines, SGC7901, were obtained from the Laboratory of Neuro-Oncology, Tianjin Neurological Institute. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco, Carlsbad, CA, USA) supplemented with 12% fetal bovine serum (Invitrogen, Carlsbad, CA, USA), and incubated at 37˚C with 5% CO_2. The miRNA mimics and negative controls were synthesized by GenePharma (Shanghai, China). Transfections with hsa-miR-200a mimics were performed in serum-free medium 24 h after plating. Cell transfection used Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Mimic sequences were: miR-200a mimic sense, 5'-UAA CAC UGU CUG GUA ACG AUG U-3' and anti-sense, 5'-AUC GUU ACC AGA CAG UGU UAU U-3'; negative control sense, 5'-UUC UCC GAA CGU GUC ACG UTT-3' and anti-sense, 5'-ACG UGA CAC GUU CGG AGA ATT-3'. The mixture was then added to cells; after 4 h, the medium was changed to complete medium.

Quantative real-time PCR analysis. Total RNA was harvested using TRIzol (Invitrogen) according to the manufacturer's protocol. Concentrations of RNA were determined using NanoDrop® ND-1000. Total RNA (1 µg) was used to synthesize cDNA by reverse transcription using MMLV reverse transcriptase (Promega Corp., Madison, WI, USA) following the manufacturer's protocol. Real-time PCR analysis was performed to determine the expression of miR-200a in SGC7901 cells 48 h after transfection with miR-200a mimic or scrambled negative control. qRT-PCR primers were purchased from GenePharma. All PCR reactions were performed using standard PCR conditions: stage 1, 95˚C for 3 min (1 cycle); stage 2, 95˚C for 12 sec, followed by 62˚C for 40 sec; stage 3, from 62 up to 95˚C, followed by 0.2˚C for 2 sec (1 cycle). Expression of U6 was used as an internal control.

Immunohistochemical analysis. Immunostaining was performed on paraffin-embedded gastric tissue microarrays and paraffin sections of tumor specimens using the avidin-biotin complex (ABC)-peroxidase method. The sections were incubated with primary antibodies against ZEB1, ZEB2 and Twist (1:100 dilution, Abcam), E-cadherin and β-catenin (1:100 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA), N-cadherin (1:100 dilution, Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China), and Slug (1:100 dilution, Cell Signaling Technology) overnight at 4˚C. They were then treated with biotinylated secondary antibody (1:100) for 1 h at room temperature, followed by incubation with ABC-peroxidase for a further 1 h. After washing with Tris-buffer, sections were stained with 3,3'-diaminobenzidine (DAB) for 5 min, rinsed in water and counterstained with hematoxylin. Negative controls were obtained by substituting primary antibodies with non-immune serum. Sections with no labeling or with <5% labeled cells were scored as 0; as 1 with 5-30% of cells labeled; as 2 with 31-70% of cells labeled; and as 3 with ≥71% of cells labeled. Staining intensity was scored similarly, with 0 for negative staining, 1 for weakly positive, 2 for moderately positive and 3 for strongly positive. Scores for percentage of positive tumor cells and for staining intensity were added to generate an immunoreactive score for each specimen. The products of the quantity and intensity scores were calculated so that a final score of 0-1 indicated negative expression (-), 2-3 indicated weak expression (+), 4-5 indicated moderate expression (++) and 6 indicated strong expression (+++). Each sample was examined separately and scored by two pathologists. Cases with discrepancies in the scores were discussed to reach a consensus.

Western blot analysis. Following transfection, cells were washed with ice-cold PBS three times. The cells were then solubilized in 1% Nonidet P-40 lysis buffer (20 mM Tris, pH 8.0, 137 mM NaCl, 1% Nonidet P-40, 10% glycerol, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride,

1 mM sodium fluoride, 1 mM sodium orthovanadate and a protease inhibitor mixture), then centrifuged at 20,000 x g for 15 min at 4˚C. Protein concentrations were measured by Nanodrop spectrophotometer (Gene, USA). Proteins were harvested and 40 μg from each sample were subjected to SDS-PAGE separation, and then transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bellerica, MA, USA). The membranes were incubated with primary antibody against ZEB1 and ZEB2 (1:1,000 dilution, Santa Cruz Biotechnology), followed by incubation with HRP-conjugated secondary antibody (1:1,000 dilution, Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd.). Specific proteins were detected using the SuperSignal Protein Detection kit (Pierce, Rockford, IL, USA). After washing with stripping buffer, the PVDF membrane was reprobed with anti-GAPDH antibody (1:1,000 dilutions, Santa Cruz Biotechnology).

Immunofluorescence staining. Forty-eight hours after transfection, cells were seeded onto sterile cover slips and washed with cold PBS twice, fixed with 4% paraformaldehyde in PBS, permeabilized with 0.1% Triton X-100 for 10 min, and blocked in 1% BSA for 30 min at room temperature to block non-specific binding. Cells were incubated in appropriate primary antibodies (ZEB1, ZEB2, Snail2, Twist1, E-cadherin, N-cadherin and β-catenin) overnight at 4˚C. Samples were washed, incubated with species-specific secondary rhodamine-labeled antibodies in PBS (1:100 dilution) for 60 min. Nuclei were stained with DAPI at room temperature for 10 min. Immunofluorescence was examined using a confocal microscope (Leica Microsystems, Heidelberg, Germany).

Subcutaneous tumor assay. BALB/c-A 6-week-old nude mice, bred at the laboratory animal facility, were purchased from the animal center of the Cancer Institute of Chinese Academy of Medical Science. All experimental procedures were carried out according to the regulations and Internal Biosafety and Bioethics Guidelines of the Tianjin Medical University and the Tianjin Municipal Science and Technology Commission. The SGC7901 subcutaneous tumor xenografts were established as previously described (16). When the subcutaneous tumors reached 50 mm^3 in size, 18 mice were randomly selected for the control, scrambled miR-treated (scramble), and miR-200a-treated groups (n=6 for each group). A mixture of 10 μl oligonucleotides containing scrambled miR-200a mimics and 10 μl Lipofectamine 2000 was injected into the xenograft tumors in a multi-site injection manner. The mice in the control group received 10 μl of PBS only. Treatment was conducted at 2-day intervals, twice. Tumor volume was measured with a caliper every 2 days, using the formula: volume = 1/2 (length x $width^2$). At the end of a 21-day observation period, the mice were sacrificed and tumor tissues were removed for formalin fixation and preparation of paraffin-embedded sections for immunohistochemical analysis.

Statistical analysis. Data were analyzed with the SPSS 10.0. The one-way analysis of variance (ANOVA), the χ^2 test and the Pearson's correlation were used to analyze significance between groups. P<0.05 was considered to indicate a statistically significant difference.

Results

miRNA-200s expression and their association with pathologic grade in gastric carcinoma. Analysis using FISH showed miR-200a, miR-200b and miR-200c were expressed in gastric carcinoma; their positive rates were 70.27% (52/74), 66.21% (49/74) and 68.91% (51/74), respectively. Levels of miR-200a, miR-200b and miR-200c decreased significantly in high grade GA (WHO grades III-IV) compared to low grade GA (WHO grades I-II). Indeed, detectable levels of miR-200a, miR-200b and miR-200c were found in 84.62% (22/26), 80.77% (21/26) and 80.77% (21/26) low-grade gastric carcinomas, respectively, but were detectable in 55.26% (21/38), 50.00% (19/38), 52.63% (20/38) high-grade GAs, respectively (P<0.05; Fig. 1A). miR-200a, miR-200b and miR-200c expression negatively correlated with WHO GA grades (Fig. 1A).

miR-200a inhibits xenograft tumor growth in vivo. Our previous study revealed that overexpression of miR-200a significantly inhibited SGC7901 cell growth, invasion and induced G_0/G_1 phase arrest *in vitro* (17). To further investigate the effect of miR-200a on tumor growth *in vivo*, SGC7901 cells were injected into three groups of nude mice to construct SGC7901 gastric carcinoma xenografts. Main tumor volume prior to injection with miR-200 mimic-transfected SGC7901 cells was 120±33.11 mm^3. During the first 3 days of the observation period, tumor sizes did not differ significantly among the three groups (P<0.05). Following treatment, tumors in the miR-200a-treated group grew slower than those in the control and scramble group. On Day 7, tumors of the miR-200a-treated group started to become significantly smaller than in the control groups (P<0.05). At the end of the study, differences in tumor mass between the miR-200a-treated group and the control group were marked (P<0.01); however, tumor volume between control and scramble mice did not differ significantly (Fig. 1C). Three weeks after injection, the group with miR-200a mimics formed substantially smaller tumors (1499.9±361.0 mm^3) than did the control (3128.5±309.0 mm^3) and the scramble groups (3325.8±278.1 mm^3) (Fig. 1B).

Expression of potential miR-200a target genes. Forty-eight hours after transfection with miR-200a mimics in SGC7901 cells, RT real-time PCR showed relative expression of miR-200a in the miR-200 mimic-transfected group to be upregulated ~10-fold compared to control groups (P<0.05) (Fig. 2A). To further determine the mechanism by which miR-200a regulates EMT and inhibits tumor growth, we performed a miRNA target search using TargetScan. Some predicted and validated target genes, such as ZEB1 and ZEB2, which probably participate in EMT in GA, have been suggested as direct targets for miR-200a in kidney tubular cells, ovarian cancer and breast cancer (18-20). We tested the effects of miR-200a elevation on ZEB1 and ZEB2 protein expression in SGC7901, using western blot analysis. Overexpression of miR-200a reduced ZEB1 and ZEB2 protein levels (Fig. 2B). Immunofluorescence staining of ZEB1 and ZEB2 showed them to be expressed in the nucleus, but decreased when miR-200a expression increased (Fig. 2C).

Since finding ZEB1 and ZEB2 to be targets of miR-200a, we immunohistochemically investigated the relationship

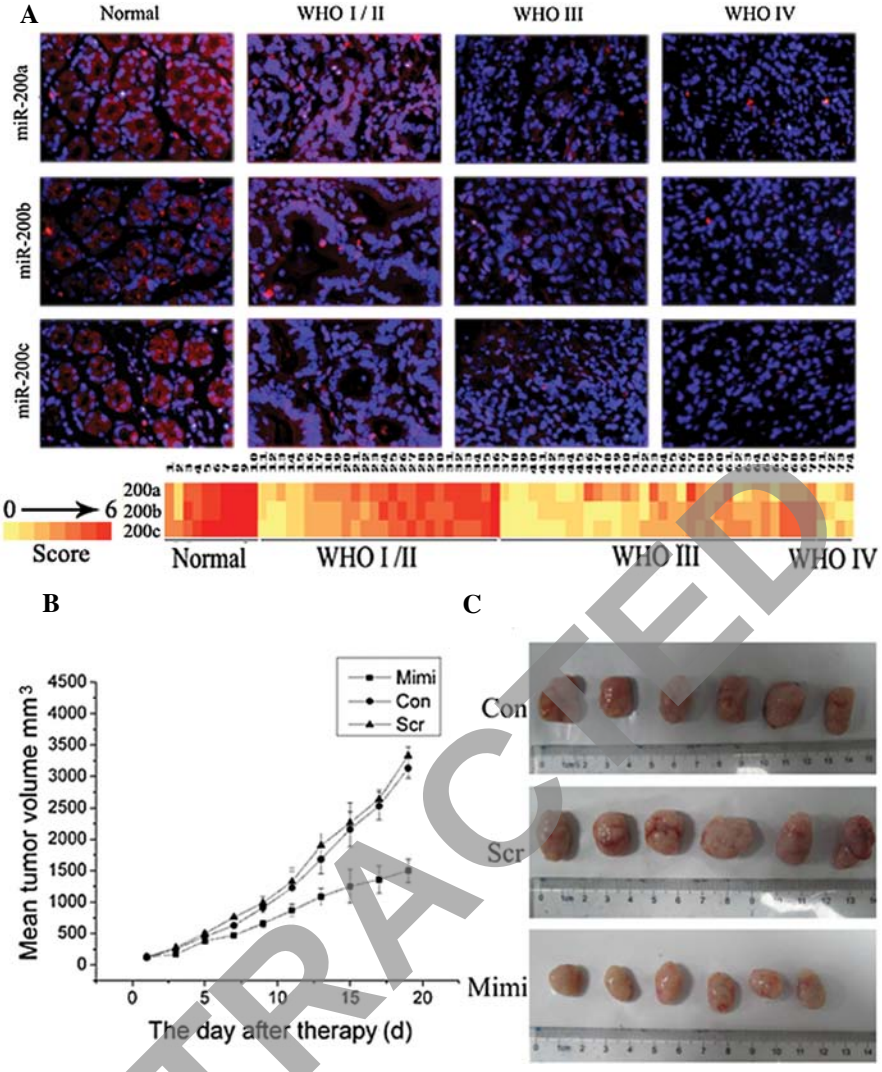

Figure 1. Expression of miR-200a, miR-200b, miR-200c in normal gastric mucosa and in GA and upregulation of miR-200a inhibits tumor growth *in vivo*. (A) Relationships between expression of miR-200a, miR-200b and miR-200c were detected by *in situ* hybridization. Normal gastric tissues and GA of different WHO grades were compared; levels of miR-200a, miR-200b and miR-200c decreased markedly in high grade GAs (WHO grades III-V) compared to low-grade GAs (WHO grades I-II) and normal gastric tissue. (B) When subcutaneous tumors were established, mean tumor volume was compared after treatment with miR-200a mimics. (C) Tumor sample from the 3 groups at the end of the study.

between miR-200a and ZEB1/ZEB2 expression in the same GA tissue microarray. We have shown that miR-200a negatively correlates with WHO grades. Expression of ZEB1 and ZEB2 increased significantly in high-grade GA (WHO grades III-IV) compared to low-grade GA (WHO grades I-II) (Fig. 2D). Given the *in vivo* data of SGC7901 cells following treatment with miR-200a mimics, immunohistochemical staining analysis of xenograft tumors taken 21 days after treatment revealed that ZEB1 and ZEB2 levels were downregulated in the miR-200a-treated group compared to tumors from the scramble and the control groups (Fig. 2E).

According to these data, we found an inverse correlation between expression of miR-200a and ZEB1/ZEB2 protein in cells, tissue samples and xenograft tumors. We postulate that ZEB1/ZEB2 are the targets of miR-200a in GA.

miRNA-200a effects on E-cadherin and the Wnt/β-catenin pathway inhibits EMT. miR-200a reportedly regulates both the EMT of the cells and the activity of the Wnt/β-catenin signaling pathway (17). To further determine the mechanism by which miR-200a regulates EMT and the activity of the Wnt/β-catenin signaling pathway, we transfected miR-200a mimics into SGC7901 cells, and then used immunofluorescence staining to investigate expression levels and locations of E-cadherin, N-cadherin and β-catenin proteins. Re-expression of miR-200a inhibits EMT by upregulating E-cadherin and downregulating N-cadherin in the cytomembrane; the location of β-catenin shifts from nucleus to cytoplasm (Fig. 3A). When E-cadherin is reduced or deleted, β-catenin is released from catenin/cadherin complexes and is translocated to the nucleus, activating the Wnt/β-catenin signaling pathway. miRNA-200a acts as a tumor suppressor through its effects on E-cadherin, thereby suppressing the Wnt/β-catenin pathway. Re-expression of miR-200a could result in reversal of EMT to MET.

These regulators play a crucial role in EMT. The E-box-binding factor Snail functions as a repressor transcription factor by directly binding to an E-box in the E-cadherin promoter, thus directly repressing its transcription. Twist, a basic helix-loop-helix transcriptional factor, suppresses E-cadherin

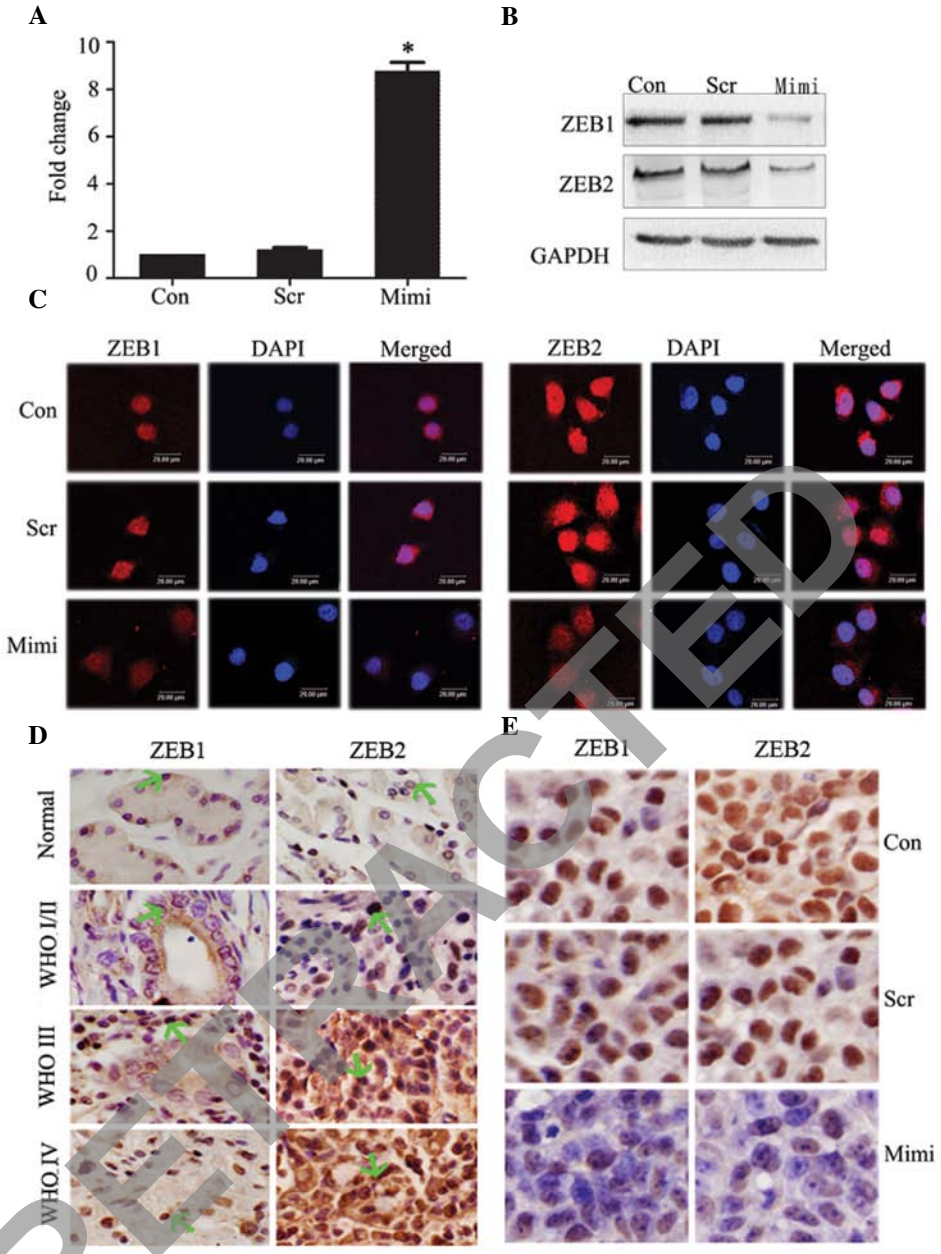

Figure 2. Expression of target genes of miR-200a. (A) Real-time PCR analysis of miR-200a expression in SGC7901 cells. SGC7901 cells transfected with miR-200a mimics and scrambled negative controls. The RT-PCR results indicate that miR-200a expression is higher in cells treated with miR-200a mimics. (B) Western blot analysis of ZEB1 and ZEB2 in cells treated with miR-200a mimics. Upregulation of miR-200a decreased expression of ZEB1 and ZEB2. (C) Immunofluorescence assay for ZEB1 and ZEB2 indicated that nuclear ZEB1/ZEB2 and expression of ZEB1/ZEB2 decrease when miR-200a expression is increased. (D) Expression of ZEB1/2 was detected in GA and in normal gastric samples using immunohistochemistry. ZEB1/2 was upregulated in GA compared to normal gastric mucosa, and was positively correlated with the WHO GA grades. (E) Immunohistochemistry of xenograft tumors after treatment with miR-200 mimics. Expression of ZEB1 and ZEB2 was suppressed in the miR-200a mimic-treated group.

transcription, to promote EMT. An analysis of correlations between miRNA and mRNA expression in publicly available data for NCI60 cell lines showed Twist1 to be in the top 25 genes negatively correlated with miR-200 expression (21). To confirm whether transcription factors change expression when treated with miR-200a mimics, and thus affect EMT regulation, immunofluorescence staining of Twist and Snail2 showed that they decreased in the nucleus, when miR-200a expression is elevated (Fig. 3A). However, the underlying mechanism requires further investigation.

We tested expression of E-cadherin, N-cadherin, β-catenin, Twist and Snail2 immunohistochemically in the gastric carcinoma tissue microarray, and found that levels of N-cadherin, β-catenin, Twist1 and Snail2 protein were positively correlated with the WHO GA grades. However, the expression of E-cadherin was negatively correlated with the WHO GA grades (Fig. 3B). To verify gene expression levels induced by miR-200a mimics *in vivo*, we determined protein expression of these genes immunohistochemically. N-cadherin, β-catenin, Twist and Snail2 were prominently downregulated, and E-cadherin was upregulated in tumor specimens of the miR-200a mimic-treated group (Fig. 3C).

We confirmed that miR-200a regulates EMT through the Wnt/β-catenin signaling pathway.

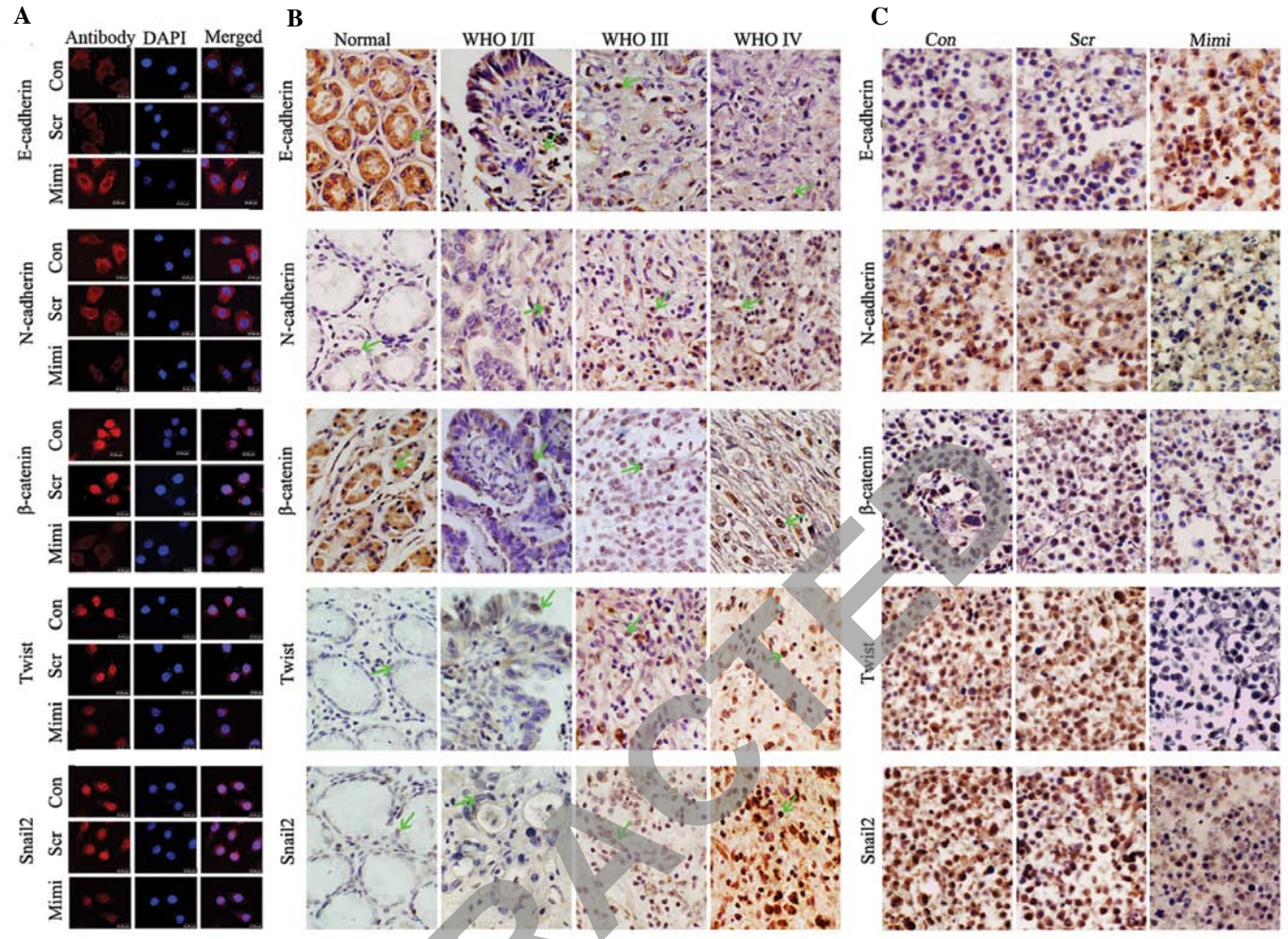

Figure 3. Impact of elevated miR-200a on the expression of the EMT-associated proteins. (A) Location of β-catenin shifted from nucleus to cytoplasm in SGC7901 cells when miR-200a was upregulated, while levels of Snail2 and Twist1 decreased in the nucleus and N-cadherin decreased in cell membranes, compared to control vector. E-cadherin increased in cell membranes compared to control vector. (B) Expression of E-cadherin, N-cadherin, β-catenin, Twist1 and Snail2 was detected in GA and in normal gastric samples using immunohistochemistry. E-cadherin was downregulated in GA compared to normal gastric mucosa, and was negatively correlated with the WHO GA grades. However, levels of N-cadherin, β-catenin, Twist1 and Snail2 protein were positively correlated with the WHO GA grades. (C) Immunohistochemical analyses of xenograft tumors after treatment with miR-200a mimics. Expression of N-cadherin, β-catenin, Twist1 and Snail2 was suppressed in xenograft tumors of the miR-200a-mimic treatment group; however, the level of E-cadherin was increased compared with control groups.

Discussion

microRNAs are dysregulated in cancer and may play essential roles in tumorigenesis. In this study, we focused on the miR-200 family, which is reportedly downregulated in prostate cancer, breast cancer and lung adenocarcinoma (22-24), but is elevated in ovarian adenocarcinoma and endometrial carcinoma (25,26). To study the expression of the miR-200 family during GA progression, we performed *in situ* hybridization in a GA tissue microarray. We found that miR-200a, miR-200b and miR-200c were downregulated in GA compared to normal gastric mucosa, and were negatively associated with gastric carcinoma grade.

Our previous study showed that elevated miR-200a in SGC7901 cells inhibited cell growth and invasion and induced G_0/G_1 phase arrest (17). In this study, we found that upregulated miR-200a suppressed tumor growth *in vivo*, confirming our *in vitro* results. However, the mechanism involved in this suppression was unclear. Little is known regarding the effect of miR-200a on ZEB1 and ZEB2 expression in GA. From western blotting and immunofluorescence staining in SGC7901 cells, and immunohistochemistry *in vivo*, we inferred that ZEB1 and ZEB2 are targets of miR-200a. To verify the significance of the Wnt/β-catenin signaling pathway and EMT in GA, we performed immunofluorescence staining in SGC7901, immunohistochemistry in gastric carcinoma tissue microarray and a tumor xenograft mouse model. The Pearson's correlation showed significant inverse correlations existed between miR-200a expression and those of ZEB1/ZEB2 (rs=-0.9343, P<0.0001 and rs=-0.8153, P<0.0001, respectively), N-cadherin (rs=-0.8411, P<0.0001), β-catenin (rs=-0.7957, P<0.0001), Snail2 (rs=-0.7957, P<0.0001) and Twist (rs=-0.8130, P<0.0001), and a significant positive correlation existed between miR-200a expression and E-cadherin expression (rs=0.7014, P<0.0001; Fig. 4A) in the same gastric carcinoma tissue microarray. This evidence, both *in vitro* and *in vivo*, indicated that miR-200a inhibition caused downregulation of E-cadherin by targeting ZEB1/ZEB2, meanwhile elevating expression of β-catenin and

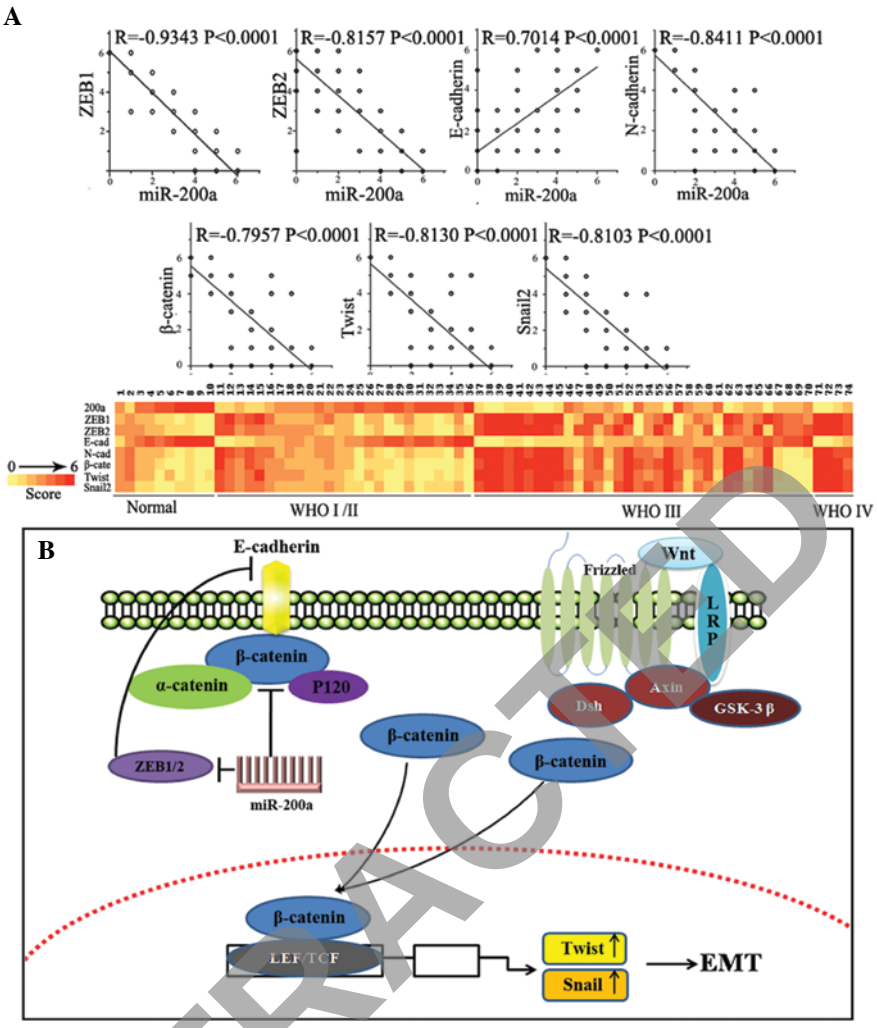

Figure 4. miR-200a regulates the Wnt/β-catenin signaling pathway. (A) Correlation of miR-200a expression with ZEB1/2, E-cadherin, N-cadherin, β-catenin, Twist1 and Snail2 expression in GA. A trend line is provided in each plot, which represents a 'best fit', as determined by simple linear regression (P<0.0001 for all; Pearson's correlation analysis). (B) Mechanism of how miR-200a downregulation could activate the Wnt/β-catenin signaling pathway and promote EMT.

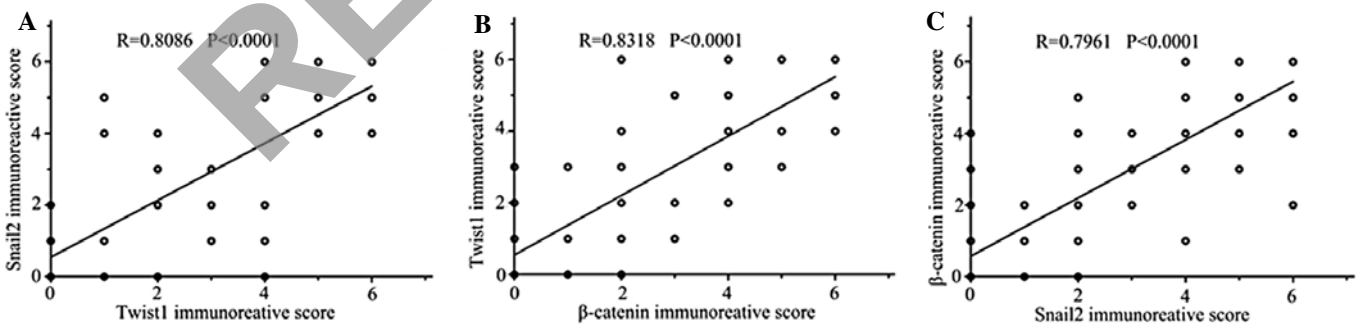

Figure 5. Relationships among β-catenin, Twist1 and Snail2 expression were analyzed in GA. A trend line is provided in each plot, which represents a 'best fit', as determined by simple linear regression. Immunoreactive scores: (A) Twist1 vs. Snail2; (B) β-catenin vs. Twist1; (C) Snail2 vs. β-catenin. (P<0.001 for all; Pearson's correlation analysis).

relocating mostly membrane-associated β-catenin to nucleus, thus activating the Wnt/β-catenin signaling pathway. Aberrant activation of the Wnt/β-catenin signaling pathway promotes cell proliferation, metastasis and tumorigenesis (30). In conclusion, overexpression of miR-200a inhibited EMT and delayed tumor growth by increasing E-cadherin level and reducing expression of N-cadherin and β-catenin.

Our previous study showed that miR-200a can directly target β-catenin, and, thus, inhibit the Wnt/β-catenin signaling pathway. miR-200a inhibits EMT, the initial tumorigenesis step, by targeting ZEB1/ZEB2, which inhibit E-cadherin by binding to an E-box element in the E-cadherin gene promoter (27,28). The cadherin-associated protein β-catenin is critical to the Wnt/β-catenin signaling pathway (29). In

summary, miR-200a regulates the activity of β-catenin through two types of mechanisms (Fig. 4B).

Previous investigations found that Twist1 required Snail2 to suppress E-cadherin, which induces EMT and tumor metastasis in HMLE cells. It is suggested that Twist1 specifically and directly binded the E-box domain 306 bp from the human Snail2 transcription start site to activate its transcription (31). During *Drosophila* mesoderm formation, Twist1 induces Snail1 expression to promote EMT (32,33), and also induces expression of mesenchymal markers, such as N-cadherin and fibronectin. These events appear to be independent of E-cadherin expression during *Drosophila* gastrulation, and may function as a potent transcriptional activator to induce expression of mesenchymal markers (33,34). The repressor Twist1 is reportedly induced by its gene promoter being directly activated by canonical Wnt signaling and the TCF/LEF transcription factors (35). Previous studies suggest that the strong β-catenin/TCF signaling in sparse SW480 cell cultures induce the Slug gene, resulting in repression of E-cadherin transcription (36).

We confirmed for the first time, using SGC7901 cells, that upregulated miR-200 reduced levels of Snail2 and Twist. To further confirm these results, we performed immunohistochemical analyses of a tumor xenograft mouse model and GA tissue microarray. We also found that miR-200 expression was negatively correlated with Snail2 and Twist1 and analyzed relationships among expression of Snail2, Twist1 and β-catenin in the same tissue assay (Fig. 5).

In this study, we found fluctuations in miR-200a expression that were similar to changes in expression of miR-200b and miR-200c in gastric carcinoma and normal gastric mucosa. A recent report suggested that miR-200b regulates EMT and promotes cell proliferation, invasion, and migration by directly targeting ZEB2 in gastric carcinoma (37). ZEB1 and ZEB2 are established direct targets of miR-200c (20). miR-200c could regulate migration and invasion activity through both ZEB1/E-cadherin-dependent and -independent pathways (38). However, further investigations are required to clarify whether miR-200b and miR-200c affect the biological activity of tumor cells or mediate EMT processes.

Acknowledgements

This study was supported by the Chinese National Natural Scientific Fund 81172356 and 81172406, and by the Natural Science Foundation of Tianjin (10JCZDJC18500). The authors thank Dr Daiming Fan for kindly providing SGC7901 gastric cancer cells and the members of the Tianjin Laboratory of Neuro-Oncology, Tianjin Neurological Institute for their technical assistance.

References

1. Zhang X, Nie Y, Du Y, Cao J, Shen B and Li Y: MicroRNA-181a promotes gastric cancer by negatively regulating tumor suppressor KLF6. Tumour Biol 33: 1589-1597, 2012.
2. Wang M, Li C, Nie H, *et al*: Down-regulated miR-625 suppresses invasion and metastasis of gastric cancer by targeting ILK. FEBS Lett 586: 2382-2388, 2012.
3. Li Z, Cao Y, Jie Z, *et al*: miR-495 and miR-551a inhibit the migration and invasion of human gastric cancer cells by directly interacting with PRL-3. Cancer Lett 323: 41-47, 2012.
4. Hua Y, Duan S, Murmann AE, *et al*: miRConnect: identifying effector genes of miRNAs and miRNA families in cancer cells. PLoS One 6: e26521, 2011.
5. Bartel DP: MicroRNAs: target recognition and regulatory functions. Cell 136: 215-233, 2009.
6. Kim VN, Han J and Siomi MC: Biogenesis of small RNAs in animals. Nat Rev Mol Cell Biol 10: 126-139, 2009.
7. Schickel R, Boyerinas B, Park SM and Peter ME: MicroRNAs: key players in the immune system, differentiation, tumorigenesis and cell death. Oncogene 27: 5959-5974, 2008.
8. Bartel DP: MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116: 281-297, 2004.
9. Negrini M, Nicoloso MS and Calin GA: MicroRNAs and cancer - new paradigms in molecular oncology. Curr Opin Cell Biol 21: 470-479, 2009.
10. Park SM, Gaur AB, Lengyel E and Peter ME: The miR-200 family determines the epithelial phenotype of cancer cells by targeting the E-cadherin repressors ZEB1 and ZEB2. Genes Dev 22: 894-907, 2008.
11. Howe EN, Cochrane DR and Richer JK: Targets of miR-200c mediate suppression of cell motility and anoikis resistance. Breast Cancer Res 13: R45, 2011.
12. Schliekelman MJ, Gibbons DL, Faca VM, *et al*: Targets of the tumor suppressor miR-200 in regulation of the epithelial-mesenchymal transition in cancer. Cancer Res 71: 7670-7682, 2011.
13. Slabakova E, Pernicova Z, Slavickova E, Starsichova A, Kozubik A and Soucek K: TGF-beta1-induced EMT of non-transformed prostate hyperplasia cells is characterized by early induction of SNAI2/Slug. Prostate 71: 1332-1343, 2011.
14. Xu J, Lamouille S and Derynck R: TGF-beta-induced epithelial to mesenchymal transition. Cell Res 19: 156-172, 2009.
15. Davalos V, Moutinho C and Villanueva A, *et al*: Dynamic epigenetic regulation of the microRNA-200 family mediates epithelial and mesenchymal transitions in human tumorigenesis. Oncogene 31: 2062-2074, 2011.
16. Zhao F, Zhang Q, Kang C, *et al*: Suppression of matrix metalloproteinase-9 expression by RNA interference inhibits SGC7901 gastric adenocarcinoma cell growth and invasion in vitro and in vivo. Med Oncol 27: 774-784, 2010.
17. Su J, Zhang A, Shi Z, *et al*: MicroRNA-200a suppresses the Wnt/β-catenin signaling pathway by interacting with β-catenin. Int J Oncol 40: 1162-1170, 2012.
18. Gregory PA, Bert AG, Paterson EL, *et al*: The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1. Nat Cell Biol 10: 593-601, 2008.
19. Korpal M, Lee ES, Hu G and Kang Y: The miR-200 family inhibits epithelial-mesenchymal transition and cancer cell migration by direct targeting of E-cadherin transcriptional repressors ZEB1 and ZEB2. J Biol Chem 283: 14910-14914, 2008.
20. Burk U, Schubert J, Wellner U, *et al*: A reciprocal repression between ZEB1 and members of the miR-200 family promotes EMT and invasion in cancer cells. EMBO Rep 9: 582-589, 2008.
21. Wiklund ED, Bramsen JB, Hulf T, *et al*: Coordinated epigenetic repression of the miR-200 family and miR-205 in invasive bladder cancer. Int J Cancer 128: 1327-1334, 2011.
22. Barron N, Keenan J, Gammell P, *et al*: Biochemical relapses following radical prostatectomy and miR-200a levels in prostate cancer. Prostate 7: 1193-1199, 2011.
23. Guttilla IK, Adams BD and White BA: ERalpha, microRNAs, and the epithelial-mesenchymal transition in breast cancer. Trends Endocrinol Metab 23: 73-82, 2012.
24. Roybal JD, Zang Y, Ahn YH, *et al*: miR-200 inhibits lung adenocarcinoma cell invasion and metastasis by targeting Flt1/VEGFR1. Mol Cancer Res 9: 25-35, 2011.
25. Snowdon J, Zhang X, Childs T, Tron VA and Feilotter H: The microRNA-200 family is upregulated in endometrial carcinoma. PLoS One 6: e22828, 2011.
26. Mateescu B, Batista L, Cardon M, *et al*: miR-141 and miR-200a act on ovarian tumorigenesis by controlling oxidative stress response. Nat Med 17: 1627-1635, 2011.
27. Saydam O, Shen Y, Wurdinger T, *et al*: Downregulated microRNA-200a in meningiomas promotes tumor growth by reducing E-cadherin and activating the Wnt/beta-catenin signaling pathway. Mol Cell Biol 29: 5923-5940, 2009.
28. Xia H, Ng SS, Jiang S, *et al*: miR-200a-mediated downregulation of ZEB2 and CTNNB1 differentially inhibits nasopharyngeal carcinoma cell growth, migration and invasion. Biochem Biophys Res Commun 391: 535-541, 2010.

29. Han L, Yang Y, Yue X, *et al*: Inactivation of PI3K/AKT signaling inhibits glioma cell growth through modulation of beta-catenin-mediated transcription. Brain Res 1366: 9-17, 2010.
30. Logan CY and Nusse R: The Wnt signaling pathway in development and disease. Annu Rev Cell Dev Biol 20: 781-810, 2004.
31. Casas E, Kim J, Bendesky A, Ohno-Machado L, Wolfe CJ and Yang J: Snail2 is an essential mediator of Twist1-induced epithelial mesenchymal transition and metastasis. Cancer Res 71: 245-254, 2011.
32. Ip YT, Park RE, Kosman D, Yazdanbakhsh K and Levine M: Dorsal-twist interactions establish snail expression in the presumptive mesoderm of the *Drosophila* embryo. Genes Dev 6: 1518-1530, 1992.
33. Leptin M: Twist and snail as positive and negative regulators during *Drosophila* mesoderm development. Genes Dev 5: 1568-1576, 1991.
34. Yang J, Mani SA, Donaher JL, *et al*: Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. Cell 117: 927-939, 2004.
35. Reinhold MI, Kapadia RM, Liao Z and Naski MC: The Wnt-inducible transcription factor Twist1 inhibits chondrogenesis. J Biol Chem 281: 1381-1388, 2006.
36. Conacci-Sorrell M, Simcha I, Ben-Yedidia T, Blechman J, Savagner P and Ben-Ze'Ev A: Autoregulation of E-cadherin expression by cadherin-cadherin interactions: the roles of beta-catenin signaling, Slug, and MAPK. J Cell Biol 163: 847-857, 2003.
37. Kurashige J, Kamohara H, Watanabe M, *et al*: MicroRNA-200b regulates cell proliferation, invasion, and migration by directly targeting ZEB2 in gastric carcinoma. Ann Surg Oncol 19: 656-664, 2012.
38. Radisky DC: miR-200c at the nexus of epithelial-mesenchymal transition, resistance to apoptosis, and the breast cancer stem cell phenotype. Breast Cancer Res 13: 110, 2011.