MicroRNA-335 acts as a metastasis suppressor in gastric cancer by targeting Bcl-w and specificity protein 1

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Aberrant expression of miR-335 has been frequently reported in cancer studies, suggesting that there is a close correlation between miR-335 and cancer during its development, progression, metastasis and prognosis. The expression of miR-335 in gastric cancer and its effects are not known. Relative expression of miR-335 in 4 gastric cancer cell lines and in 70 gastric cancer tissues was confirmed by real-time quantitative reverse transcriptase-PCR compared with controls. Transwell cell migration and Matrigel invasion assay in vitro and metastasis formation assay in vivo were used to examine the effects of miR-335 expression on gastric cancer cell invasion and metastasis. The effect of miR-335 expression on gastric cancer cell proliferation was estimated by the 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay. Luciferase reporter assay and western blot were used to examine the potential target genes and related pathways. Gene silencing with small-interfering RNA was used to examine the effects of target genes on gastric cancer cell invasion. miR-335 was dramatically downregulated in gastric cancer cell lines than in the normal gastric cell line GES-1. Low expression of miR-335 was significantly associated with lymph-node metastasis, poor pT stage, poor pN stage and invasion of lymphatic vessels. Overexpression of miR-335 suppressed gastric cancer cell invasion and metastasis in vitro and in vivo, but has no significant effects on cell proliferation. Furthermore, miR-335 might suppress gastric cancer invasion and metastasis by targeting Bcl-w and specificity protein 1 (SP1). Taken together, our results provide evidence that miR-335 might function as a metastasis suppressor in gastric cancer by targeting SP1 directly and indirectly through the Bcl-w-induced phosphoinositide 3-kinase-Akt-SP1 pathway. miR-335 showing altered expression at different stages of gastric cancer could be a target for gastric cancer therapies and could be further developed as a potential prognostic factor.

Keywords: MicroRNA; gastric cancer; miR-335; SP1; Bcl-w

Introduction

Until the mid-1990s, gastric cancer was the most common cause of cancer death worldwide (Bertuccio et al., 2009). Although the incidence of gastric cancer has been declining in the past several decades, it remains the second most common cause of cancer death (Parkin et al., 2005). The molecular mechanisms of gastric cancer remain to be further elucidated.

Recently, the classical categories of oncogenes and tumor-suppressor genes have been expanded to include a new family of RNAs known as microRNAs (miRNAs), which may regulate a vast number of protein-coding genes, including tumor-related genes (Liu et al., 2008; Wu et al., 2010). miRNAs are a large family of highly conserved small non-coding RNAs that can have important regulatory roles in animals and plants. They trigger translational repression and/or mRNA degradation mostly through complementary binding to the 3′-untranslated (3′-UTR) regions of target mRNAs (Calin et al., 2002; Bartel, 2004; Wu et al., 2006). More than half of human miRNA genes are in cancer-associated genomic regions or in fragile sites (Calin et al., 2004). Evidence has shown that miRNAs repress the expression of important cancer-related genes and that downregulation or upregulation of miRNAs is correlated with various human cancers, indicating that miRNAs can function as tumor suppressors or oncogenes (Costinean et al., 2006; Volinia et al., 2006; Bommer et al., 2007; Gironella et al., 2007; Chen et al., 2010). Recently, the aberrant expression of miRNA has been frequently reported in gastric cancer, suggesting that there is a close correlation between miRNAs and the development, progression, metastasis and prognosis of gastric cancer (Petrocca et al., 2008; Du et al., 2009; Kim et al., 2009; Chiang et al., 2010; Wada et al., 2010; Wu et al., 2010; Song et al., 2011).

The low expression of miR-335 has a close correlation with cancer development (Marcucci et al., 2008; Sorrentino et al., 2008; Soon et al., 2009; Wang et al., 2010). In addition, miR-335 has been identified as a
metastasis-suppressor miRNA in breast cancer. The expression of miR-335 is lost in the majority of primary breast tumors from patients who relapse, and the loss of miR-335 expression is associated with poor distal metastasis-free survival. It regulates a set of genes, which are SOX4, PTPRN2, MERTK and possibly TNC, the collective expression of which in a large cohort of human tumors is associated with the risk of distal metastasis (Tavazoie et al., 2008).

In our study, a significant low expression of miR-335 was found in gastric cancer cell lines. The relative expression of miR-335 in gastric cancer tissues showed that low expression of miR-335 is associated with lymph-node metastasis, poor pT stage, poor pN stage and invasion into lymphatic vessels. Transwell cell migration and Matrigel invasion assay in vitro and metastasis formation assay in vivo demonstrated that overexpression of miR-335 has the effect of suppressing gastric cancer cell invasion and metastasis. Furthermore, we used a luciferase reporter assay and western blot to confirm that miR-335 may function as a metastatic suppressor in gastric cancer by targeting specificity protein 1 (SP1) directly and indirectly through a Bcl-w-induced signaling pathway that sequentially involves phosphoinositide 3-kinase (PI3K), Akt and Sp1.

**Results**

miR-335 is downregulated in human gastric cancer cell lines

We detected the expression of miR-335 in five human gastric cell lines (namely SGC-7901, MGC-803, BGC-823, AGS and GES-1) by real-time quantitative reverse transcriptase-PCR (RT–PCR) and found that miR-335 was downregulated in gastric cancer cell lines SGC-7901, MGC-803, BGC-823 and AGS compared with a normal gastric epithelial cell line GES-1 (MGC-803, 0.07 ± 0.02-fold ($P < 0.01$); SGC-7901, 0.1 ± 0.006-fold ($P < 0.01$); BGC-823, 0.5 ± 0.02-fold ($P < 0.01$); AGS, 0.9 ± 0.08-fold ($P = 0.3$); Figure 1a).

Relative expression of miR-335 in gastric cancer tissues and its correlation with clinicopathological features

To determine whether miR-335 expression is associated with gastric cancer, we examined miR-335 expression in a large cohort consisting of 70 primary gastric cancer tissues relative to their pair-matched adjacent non-tumor tissues using real-time quantitative RT–PCR (qRT–PCR). No statistical significance was found with miR-335 expression between gastric cancer tissues and their pair-matched adjacent non-tumor tissues.

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**Figure 1** The expression of miR-335 in tissues and cell lines. (a) Relative expression of miR-335 in four gastric cancer cell lines (MGC-803, SGC-7901, BGC-823 and AGS). Quantification of miR-335 was measured by qRT–PCR. Data are presented in gastric cancer cell lines relative to GES-1 (which is a normal gastric epithelial cell line that was chosen as a control). Results represent the means of the values. Bars indicate s.d. ** Refers to statistical significance between groups ($P < 0.01$). (b) There is no statistical significance with miR-335 expression between gastric cancer tissues and their pair-matched adjacent non-tumor tissues ($P = 0.7$). miR-335 was normalized by U6RNA, $\Delta C_T = C_T$ miR-335 $- C_T$ U6RNA. Results represent the means of the values. Bars indicate s.d. (c) Relative expression of miR-335 in 70 patients with gastric cancer. Quantification of miRNAs was measured by qRT–PCR. Data that were transformed to log2 values in gastric cancer tissues relative to their matched non-tumor adjacent tissues were divided into two groups of metastasis free and metastasis positive. The non-parametric test revealed that the low expression of miR-335 was significantly associated with lymph-node metastasis ($P < 0.001$). (d) The non-parametric test demonstrated that patients with better pT stage had significantly higher miR-335 expression than did those with poor pT stage ($P < 0.05$). Quantification of miRNAs was measured by qRT–PCR. Results represent the median of the values, which are the relative expressions of miR-335 associated with the pT stage.
miR-335 suppresses gastric cancer invasion in vitro

To explore the role of miR-335 in gastric cancer, BGC-823 and SGC-7901 cell lines were transfected with hsa-miR-335 mimics or stable negative control (NC), and AGS cells were transfected with anti-miR-335 or anti-NC. Transfection efficiency was perfect (Supplementary Figures S1a–c). We estimated the effects of miR-335 on the invasion of cells derived from gastric cancer using a transwell cell migration and Matrigel invasion assay. The data demonstrated that overexpression of miR-335 obviously inhibited the invasion and metastasis of BGC-823 and SGC-7901 cell lines, and that invasion of AGS cells transfected with anti-miR-335 was increased compared with that of cells transfected with anti-NC and blank controls. The numbers of miR-335-transfected BGC-823 cells (19 ± 5; P < 0.01) passing through the Matrigel were significantly lower than those of NC-transfected BGC-823 cells (36 ± 6) and parental BGC-823 cells (38 ± 7) (Figures 2a and d). A similar result was found with SGC-7901 cells; the numbers of miR-335-transfected SGC-7901 cells (16 ± 5; P < 0.01) passing through the Matrigel were significantly lower than those of NC-transfected SGC-7901 cells (35 ± 9) and parental SGC-7901 cells (37 ± 9) (Figures 2b and e). On the contrary, the numbers of anti-miR-335-transfected AGS cells (57 ± 17; P < 0.01) passing through the Matrigel were significantly higher than those of anti-NC-transfected AGS cells (38 ± 9) and parental AGS cells (37 ± 11) (Figures 2c and f). There were no significant differences between all NC-transfected and parental cells (P_BGC-823 = 0.2, P_SGC-7901 = 0.2, P_AG121S = 0.9; Figure 2).

miR-335 suppresses gastric cancer metastasis in vivo

We used a metastasis formation assay in nude mice in vivo approach. In all, 5 × 10^6 viable parental SGC-7901 cells or SGC-7901 cells transfected with hsa-miR-335 mimics or stable NC were resuspended in 0.1 ml phosphate-buffered saline and injected into the lateral tail vein. Five weeks after injection, we killed the mice and dissected the lungs for microscopic histology. The numbers of lung metastases in mice that were injected with miR-335-transfected SGC-7901 cells were significantly lower than those in mice injected with NC-transfected SGC-7901 cells (P < 0.01; Figure 3), demonstrating that miR-335 overexpression obviously inhibited the metastasis effect of the SGC-7901 cell line in vivo.

miR-335 has no significant effects on cell proliferation of gastric cancer

An MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay was used to estimate the proliferation ability of miR-335-transfected, NC-transfected and parental SGC-7901 cells. The result...
showed that overexpression of miR-335 in SGC-7901 cells has no significant effects on cell proliferation (Supplementary Figure S2).

**miR-335 suppresses gastric cancer invasion and metastasis by targeting Bcl-w and SP1**

For miRNA target gene prediction, we used TargetScan Release 5.1 online software (http://www.targetscan.org, Whitehead Institute for Biomedical Research, Cambridge, MA, USA). Among a total of 146 genes that were potentially targeted by miR-335, we found that SP1 and Bcl-w may contribute to the metastasis of gastric cancer.

To confirm that SP1 and Bcl-w are direct targets of miR-335, we constructed luciferase reporter pGL3-SP1-3’-UTR and pGL3-Bcl-w-3’-UTR. Scrambled target sites (pGL3-SP1-MUT and pGL3-Bcl-w-MUT) were also constructed as controls for sequence specificity. All the reporters were transfected in SGC-7901 cells. The luciferase activity of pGL3-SP1-3’-UTR and pGL3-Bcl-w-3’-UTR reporters was significantly suppressed in miR-335-transfected SGC-7901 cells than in NC-transfected cells.
In vitro gastric cancer invasion and metastasis
The above results showed that miR-335 suppresses in vitro cancer invasion than in NC-transfected and parental SGC-7901 cells. Bcl-w proteins in miR-335-transfected SGC-7901 cells showed a clear reduction in the level of the endogenous SP1 and was shown in Supplementary Figure S1d. We observed time qRT–PCR (transfection efficiency of anti-miR-335 and parental SGC-7901 cells by western blot and real-fected, anti-miR-335-transfected, their respective NC

Downregulation of miR-335 in gastric cancer

In support of these results, we examined the SP1 and Bcl-w proteins and mRNA levels in miR-335-transfected, anti-miR-335-transfected, their respective NC and parental SGC-7901 cells by western blot and real-time qRT–PCR (transfection efficiency of anti-miR-335 was shown in Supplementary Figure S1d). We observed a clear reduction in the level of the endogenous SP1 and Bcl-w proteins in miR-335-transfected SGC-7901 cells than in NC-transfected and parental SGC-7901 cells normalized to an endogenous reference β-actin protein (Figure 4e). Overexpression of SP1 and Bcl-w proteins was also found in anti-miR-335-transfected SGC-7901 cells compared with anti-NC-transfected and parental SGC-7901 cells (Figure 4f). These results demonstrated that miR-335 may target SP1 and Bcl-w in gastric cancer. Despite the effect of miR-335 on SP1 and Bcl-w protein levels, no effect on SP1 and Bcl-w mRNA levels was detected (Figures 4c and d).

Knockdown of the SP1 and Bcl-w genes suppresses gastric cancer invasion in vitro
The above results showed that miR-335 suppresses gastric cancer invasion and metastasis in vitro and in vivo. Moreover, SP1 and Bcl-w are potential targets of miR-335. Do SP1 and Bcl-w genes have any effects on gastric cancer cell invasion? To this end, the expressions of the SP1 and Bcl-w genes of SGC-7901 cells were knocked down by transfection with small-interfering RNA (siRNA) (Supplementary Figures S3a–c). A transwell cell migration and Matrigel invasion assay showed that knockdown of SP1 and Bcl-w gene expressions significantly inhibited the invasion and metastasis of SGC-7901 cells in vitro (Supplementary Figures S3d and e).

Discussion
The low expression of miR-335, which has a close correlation with the development, progression, metastasis and prognosis of cancer, has been frequently reported. With a RT–PCR assay, miR-335 has been found to be downregulated in breast cancer tissues and sera (Wang et al., 2010). Similarly, the low expression of miR-335 was significantly associated with adrenocortical carcinomas and acute myeloid leukemia (Marcucci et al., 2008; Soon et al., 2009). Sorrentino et al. (2008) revealed that there is a direct correlation between development of ovarian cancer cell chemoresistance and low expression of miR-335. In this study, we found that miR-335 was dramatically downregulated in gastric cancer cell lines, suggesting that the low expression of miR-335 is significantly associated with gastric cancer. However, overexpression of miR-335 may also have an important role in the development of several cancers, including colonic cancer, pediatric acute leukemia and multiple myeloma (Ronchetti et al., 2008; Zhang et al., 2009; Wang et al., 2010). This distinction may be caused by tissue specificity or ethnic diversity.

We also analyzed the relative expression of miR-335 in gastric cancer tissues and demonstrated that the low expression of miR-335 was significantly associated with lymph-node metastasis, poor pT stage, poor pN stage and invasion into lymphatic vessels. Lymph-node metastasis, pT stage and pN stage are independent prognosis factors associated with the overall survival rates of gastric cancer patients, and lymphatic vessel invasion is identified as an independent factor predicting lymph-node metastasis. Gastric cancer patients with lymph-node metastasis, poor pT stage, poor pN stage and invasion into lymphatic vessels tend to have a low survival rate (Xu et al., 2007; Sun et al., 2009, 2010; Jiang et al., 2010). This suggests that the expression of miR-335 may act as an independent prognosis factor that is significantly associated with overall survival rates of gastric cancer patients. Further studies demonstrated that overexpression of miR-335 has the effect of suppressing gastric cancer cell invasion and metastasis. These results are consistent with a report that showed that the expression of miR-335 is lost in the majority of primary breast tumors from patients who relapse, and that the loss of the expression of miR-335 is associated with poor distal metastasis-free survival (Tavazoie et al.,
Our findings suggest that miR-335 may function as a metastasis suppressor in gastric cancer. What is the molecular mechanism by which miR-335 acts as a metastasis suppressor in gastric cancer? We used luciferase reporter assay and western blot to confirm that SP1 is a target of miR-335 in gastric cancer cells. SP1 is a sequence-specific DNA-binding protein that is an extremely versatile protein involved in the expression of many different genes (Suske, 1999). It is overexpressed in a number of human cancers, and its overexpression contributes to malignant transformation by regulating the expression of a number of genes participating in multiple aspects of tumorigenesis, such as angiogenesis, cell growth and resistance to apoptosis (Lou et al., 2005; Wang et al., 2005; Kanai et al., 2006). Recent studies have shown that SP1 has important roles in gastric cancer development and progression. Its high expression level is correlated with advanced disease stage, lymph-node metastasis and poor survival rates (Wang et al., 2003; Yao et al., 2004). Knockdown of SP1 gene expression significantly inhibited the invasion and metastasis of SGC-7901 cells in vitro. These results demonstrate that miR-335 may act as a metastasis suppressor in gastric cancer by targeting SP1.

Bcl-w is an anti-apoptotic member of the Bcl-2 protein family. The mechanism by which Bcl-w suppresses apoptosis is that Bcl-w interacts directly with pro-apoptotic members to block their apoptotic activities (Gross et al., 1999; Antonsson, 2001; Uren et al., 2007; Bae et al., 2009). Recent evidence suggests that the expression of Bcl-w is significantly associated with infiltrative morphotypes (Lee et al., 2003). It can activate the PI3K/Akt pathway, which is involved in tumor cell invasion, by elevating PI3K activity and Akt phosphorylation. Inhibiting either PI3K or Akt both abolish Bcl-w-induced Sp1 activation. These suggest that overexpression of Bcl-w in gastric cancer cells results in an increase in their migratory and invasive potentials by activating the PI3K-Akt-Sp1 pathway (Lee et al., 2003; Bae et al., 2006, 2009). We have confirmed
that Bcl-w is a target of miR-335 in gastric cancer cells; knockdown of Bcl-w gene expression significantly inhibited the invasion and metastasis of SGC-7901 cells in vitro. These observations demonstrate that miR-335 not only targets SP1 directly but also targets SP1 indirectly through a Bcl-w-induced signaling pathway that sequentially involves PI3K, Akt and Sp1. Bcl-w inducing matrix metalloproteinase-2 expression through the Bcl-w-induced PI3K-Akt-Sp1 pathway has an important role in tumor invasion and angiogenesis (Bae et al., 2006). However, SP1 can regulate not only matrix metalloproteinase-2 but also a number of other genes that are important to gastric cancer development and progression (Qin et al., 1999; Yu et al., 2002; Lou et al., 2005; Wang et al., 2005; Kanai et al., 2006; Kuo et al., 2006). Maybe Bcl-w could regulate the expression of a number of genes by inducing SP1 expression through the PI3K-Akt-Sp1 pathway. Our findings also suggest that miRNA target genes may increase in the roles that they have by interacting with each other.

Although SP1 and Bcl-w are negatively regulated directly by miR-335 in gastric cancer cells, miR-335 has no effect on the SP1 and Bcl-w mRNA levels detected by qRT–PCR. These results highlight that miR-335 negatively regulates SP1 and Bcl-w expression at the translational level in gastric cancer.

In conclusion, our study showed that miR-335 is dramatically downregulated in gastric cancer cell lines and that the low expression of miR-335 is significantly associated with lymph-node metastasis, poor pT stage, poor pN stage and invasion into lymphatic vessels. Moreover, overexpression of miR-335 has the effect of suppressing gastric cancer cell invasion and metastasis. miR-335 may act as an important metastasis suppressor in gastric cancer.

Materials and methods

Tissue samples

Samples of human gastric cancer tissues and paired-adjacent non-tumor gastric tissues that were farther than 5 cm from the tumors were obtained from 70 patients who underwent radical resection at the First Hospital of China Medical University. The median age of patients (57 men and 13 women) was 60 years (range, 26–80 years). Fresh samples were snap frozen in liquid nitrogen immediately after resection and stored at −80 °C. All samples were obtained with patients’ informed consent and were histologically confirmed by staining with hematoxylin–eosin. The histological grade of cancers was assessed according to criteria set by the World Health Organization. The tumor–node–metastasis stage was performed using standard criteria of the seventh tumor–node–metastasis staging system.

Cell lines and cell culture

Human gastric cancer cell lines SGC-7901, MGC-803, BGC-823, AGS and one normal gastric epithelial cell line GES-1 (as control) were purchased from the Institute of Biochemistry and Cell Biology at the Chinese Academy of Sciences (Shanghai, China). SGC-7901, MGC-803 and BGC-823 were propagated in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA). AGS was propagated in F-12K medium (Invitropen) and GES-1 was propagated in Dulbecco’s modified Eagle’s medium (Invitrogen). All the media were supplemented with 10% fetal bovine serum. Cell lines were cultured at 37 °C in a humidified incubator of 5% CO₂.

RNA extraction and real-time qRT–PCR

Total RNA, inclusive of the small RNA fraction, was extracted using the mir-Vana miRNA Isolation Kit (Ambion, Austin, TX, USA) according to the manufacturer’s instructions. The concentration and purity of RNA were controlled by ultraviolet spectrophotometry (A260/A280 > 1.9) using a Nano-Photometer ultraviolet/Vis spectrophotometer (Implen, Schatzbogen, Germany). The 3′-termini of RNA were polyadenylated by Escherichia coli poly(A) polymerase (E-PAP) at 37 °C for 30 min using the Poly(A) Tailing Kit (Ambion) following the manufacturer’s instructions. Subsequently, RNA was extracted with phenol–chloroform and precipitated with ethanol.

Real-time qRT–PCR was used to confirm the expression level of mRNAs. Reverse transcription was performed using a superscript III first-strand synthesis system for a RT–PCR kit (Invitrogen), and real-time qRT–PCR was performed on a Rotor-gene 2000 Real-time Cycler detection system (Corbett Research, Sydney, Australia) supplied with analytical software, using an Express SYBR Green primer Universal Kit (Invitrogen) according to the manufacturer’s instructions. The PCR reaction for amplification of miR-335 was conducted at 95 °C for 20 s, followed by 45 cycles of 95 °C for 5 s and 53 °C for 20 s. U6 mRNA levels, as an endogenous reference, were used for normalization. The PCR reaction for amplification of SP1 and Bcl-w was conducted at 95 °C or 20 s, followed by 45 cycles of 95 °C for 5 s and 60 °C for 20 s. Glyceraldehyde 3-phosphate dehydrogenase mRNA levels were used for normalization as an endogenous reference. After the final cycle, a melting curve analysis was conducted within the range of 55-95 °C. The expression level of miR-335 in cancer relative to its non-tumorous control was calculated using the equation 2^{−ΔΔCt} in which ΔΔCt = Ct 335 − Ct U6 (Livak and Schmittgen, 2001). The value of the relative expression ratio < 1.0 was considered as low expression in cancer relative to the non-tumorous control, whereas others were considered as high expression. Primers for RT–PCR are given in Table 2 and Supplementary Table S1.

RNA oligoribonucleotides and cell transfection

All RNA oligoribonucleotides and their respective NCs (Supplementary Table S2) were purchased from GenePharma (Shanghai, China). The pyrimidine nucleotides in the miR-335 mimics, anti-miR-335 and their respective NCs were substituted by their 2-O-methyl analogs to improve RNA stability. The siRNAs were used to lower SP1 and Bcl-w expressions, respectively. The target SP1 sequence of the siRNA is 5′-AATGAGAACAGCAACAACCTC-3′ (Bond et al., 2004) and the target Bcl-w sequence of the siRNA is 5′-AAGGGTGTGCTGTGAGCT-3′ (Yao et al., 2005).

SGC-7901, BGC-823 and AGS cells were plated 1 day before transfection. A final concentration of 50 nm of RNA mimics or 200 nm of inhibitor or 100 nm of siRNA and their respective NCs were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. After 24 or 48 h, cells were harvested for further experiments.

Cell invasion assay

We used a transwell cell migration and Matrigel invasion assay to estimate the invasion ability of parental and transfected
were harvested by trypsinization, washed with serum-free RT-primer-3 GCTGTCAACGATACGCTACGTAACGGCATGACAGTGTTTTTTTTTTTTTTTTTTTTTTTTC
RT-primer-2 GCTGTCAACGATACGCTACGTAACGGCATGACAGTGTTTTTTTTTTTTTTTTTTTTTTTTG

In all, 8 103 viable cells were resuspended in 0.1 ml phosphate-buffered saline. Paraffin embedding, sectioning and tail vein. Five weeks after injection, mice were killed and the visible lung metastases were measured and counted using a microscope. All experimental procedures involving animals were in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication no. 80-23, revised 1996) and were performed according to the institutional ethical guidelines for animal experiment.

BGC-823, SGC-7901 and AGS cell lines, respectively, in vitro. Serum-free RPMI1640 or F-12K medium was mixed with Matrigel (1:10; BD Biosciences, Bedford, MA, USA). The bottom of the culture inserts (8-μm pores) in 24-well tissue culture plates (Transwell, Corning, Corning, NY, USA) was coated with 50 μl of the mixture, and the Matrigel was allowed to solidify at 37 °C for 4 h. After solidification, 5 × 104 cells were harvested by trypsinization, washed with serum-free medium to 25 × 106/ml and placed in the upper chamber. The lower chamber contained 10% fetal bovine serum for use as a chemoattractant. After 24 h of incubation at 37 °C with 5% CO2, the number of cells that had migrated to the basal side of the membrane was quantified by counting 16 independent symmetrical visual fields under the microscope, and cell morphology was observed by staining with hematoxylin–eosin.

**Tumor metastasis formation assay in vivo**

We used the nude mice xenograft model to estimate the metastatic ability of the parental and transfected SGC-7901 in vivo. Female BALB/c mice (4–5 weeks) were purchased from the Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China) and housed in the animal care facilities of the China Medical University under specific pathogen-free conditions. After 1 week, 5 × 105 viable cells were resuspended in 0.1 ml phosphate-buffered saline and injected into the lateral tail vein. Five weeks after injection, mice were killed and the lungs were extracted and fixed in 4% paraformaldehyde in phosphate-buffered saline. Paraffin embedding, sectioning and staining with hematoxylin–eosin of the lungs were performed at the First Affiliated Hospital of the China Medical University. Visible lung metastases were measured and counted using a microscope. All experimental procedures involving animals were in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication no. 80-23, revised 1996) and were performed according to the institutional ethical guidelines for animal experiment.

**MTT proliferation assay**

An MTT assay was used to estimate the proliferation ability of the parental and transfected SGC-7901 cell line. In all, 8 × 104 cells were seeded into 96-well culture plates for 24, 48, 72 and 96 h. Cells were then incubated with 20 μl of MTT (5 mg/ml) for 4 h at 37 °C. After removing the culture supernatant, 150 μl of dimethyl sulfoxide was added to solubilize the crystals for 20 min at room temperature. Absorbance was measured at a wavelength of 495 nm using a spectrophotometer (Multiskan MK3; Thermo, Shimadzu, Japan).

**Bioinformatics analyses**

For miRNA target gene prediction, we used TargetScan Release 5.1 online software (http://www.targetscan.org/). Among all the genes that were compiled, we determined which genes may contribute to gastric cancer metastasis.

**Vector construction and luciferase reporter assay**

For luciferase reporter experiments, 3’-UTR segments containing the miR-335-binding sites of SP1 and Bcl-w were amplified by PCR from human genomic DNA and inserted into the pGL3-control vector (Promega, Madison, WI, USA) using the XbaI site immediately downstream of the stop codon of luciferase. DNA segments with scrambled target sites (SP1-MUT and Bcl-w-MUT) designed to interfere with seed sequence recognition were also cloned to serve as control for specificity. Primers and DNA segments used for vector construction are indicated in Supplementary Table S3.

**Protein extraction and western blot**

Total protein was extracted from parental and transfected SGC-7901 cells using a Total Protein Extraction Kit (KeyGen, Nanjing, China) according to the manufacturer’s instructions. Measurement of protein concentration was done using a BCA Protein Assay Kit (KeyGen), and 60 μg of each sample was separated by 12% SDS-PAGE and electrophoretically transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). Nonspecific binding sites were blocked by incubating with 5% non-fat dry milk in Tris-buffered saline–0.5% Tween-20 at room temperature for 1 h and incubated at 4 °C overnight with antibodies directed against SP1, Bcl-w (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and β-actin (1:5000; Sigma, St Louis, MO, USA). The antigen–antibody complexes were visualized using horseradish peroxidase-conjugated secondary antibody and an ECL Kit (Pierce, Rockford, IL, USA). Quantification of protein was carried out using FluorChem 2.01 (Alpha Innotech, San Leandro, CA, USA). SP1 and Bcl-w protein levels in transfected SGC-7901 cells were presented as fold change normalized to an endogenous reference (β-actin protein) and relative to parental SGC-7901 cells. Therefore, a fold change of <1.0 was considered as low expression.

**Statistical analysis**

Correlations between miRNA expression and clinicopathological features were analyzed by non-parametric tests: Mann–Whitney U-test between two groups and Kruskal–Wallis test.
for three or more groups. All the other results are presented as mean ± s.d. from at least three separate experiments and were submitted to analysis using Student’s t-test. All statistical analyses were performed using SPSS 16.0 computer software (SPSS Inc., Chicago, IL, USA). P-values <0.05 were considered as statistically significant.

**Conflict of interest**

The authors declare no conflict of interest.

**References**

Antonsson B. (2001). Bax and other pro-apoptotic Bcl-2 family “killer-proteins” and their victim the mitochondrion. *Cell Tissue Res* **306**: 347–361.

Bae JH, Park MJ, Yoon SH, Kang SW, Lee SS, Choi KM et al. (2006). Bcl-w promotes gastric cancer cell invasion by inducing matrix metalloproteinase-2 expression via phosphoinoside 3-kinase, Akt, and Sp1. *Cancer Res* **66**: 4991–4995.

Bae JH, Yoon SH, Lee SB, Park JK, Ho JN, Um HD. (2009). Signaling components involved in Bcl-w-induced migration of gastric cancer cells. *Cancer Lett* **277**: 22–28.

Bartel DP. (2004). MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* **116**: 281–297.

Bertuccio P, Chatenoud L, Levi F, Praud D, Ferlay J, Negri E. (2009). Recent patterns in gastric cancer: a global overview. *Int J Cancer* **125**: 666–673.

Bommer GT, Gerin I, Feng Y, Kaczorowski AJ, Kuick R, Love RE et al. (2007). p53-mediated activation of miRNA34 candidate tumor-suppressor genes. *Curr Biol* **17**: 1298–1307.

Bond GL, Hu W, Bond EE, Robins H, Lutzker SG, Arva NC et al. (2004). A single nucleotide polymorphism in the MDM2 promoter attenuates the p53 tumor suppressor pathway and accelerates tumor formation in humans. *Cell* **119**: 591–602.

Calin GA, Dumitru CD, Shimizu M, Bichi R, Zupko S, Noch E et al. (2002). Frequent deletions and down-regulation of microRNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. *Proc Natl Acad Sci USA* **99**: 15524–15529.

Calin GA, Sevignani C, Dumitru CD, Hyslop T, Noch E, Yendamuri S et al. (2004). Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers. *Proc Natl Acad Sci USA* **101**: 2999–3004.

Chen Y, Song Y, Wang Z, Yue Z, Xu H, Xing C et al. (2010). Altered expression of MiR-148a and MiR-152 in gastrointestinal cancers and its clinical significance. *J Gastrointest Surg* **14**: 1170–1179.

Chiang Y, Song Y, Wang Z, Chen Y, Yue Z, Xu H et al. (2010). Aberrant expression of miR-203 and its clinical significance in gastric and colorectal cancers. *J Gastrointest Surg* **15**: 63–70.

Costinean S, Zanesi N, Pekarsky Y, Tili E, Volinia S, Heerema N et al. (2006). Pre-B cell proliferation and lymphoblastic leukemia/high-grade lymphoma in E(mu)-miR155 transgenic mice. *Proc Natl Acad Sci USA* **103**: 7024–7029.

Du Y, Xu Y, Ding L, Yao H, Yu H, Zhou T et al. (2009). Down-regulation of miR-141 in gastric cancer and its involvement in cell growth. *J Gastroenterol* **44**: 556–561.

Gironella M, Seux M, Xie MJ, Cano C, Tomasini R, Gommeaux J et al. (2007). Tumor protein 53-induced nuclear protein 1 expression is repressed by miR-155, and its restoration inhibits pancreatic tumor development. *Proc Natl Acad Sci USA* **104**: 16170–16175.

Gross A, McDonnell JM, Korsmeyer SJ. (1999). BCL-2 family members and the mitochondria in apoptosis. *Genes Dev* **13**: 1899–1911.

Jiang CG, Wang ZN, Sun Z, Liu FN, Yu M, Xu HM. (2010). Clinicopathologic characteristics and prognosis of gastric cancer invading the subserosa. *J Surg Oncol* **102**: 737–741.

Kanai M, Wei D, Li Q, Jia Z, Ajani J, Le X et al. (2006). Loss of Krüppel-like factor 4 expression contributes to Sp1 overexpression and human gastric cancer development and progression. *Clin Cancer Res* **12**: 6395–6402.

Kim YK, Yu J, Han TS, Park SY, Namkoong B, Kim DH et al. (2009). Functional links between clustered microRNAs: suppression of cell-cycle inhibitors by microRNA clusters in gastric cancer. *Nucleic Acids Res* **37**: 1672–1681.

Kuo L, Chang HC, Leu TH, Maa MC, Hung WC. (2006). Src oncogene activates MMP-2 expression via the ERK/Sp1 pathway. *J Cell Physiol* **207**: 729–734.

Lee HW, Lee SS, Lee SJ, Um HD. (2003). Bcl-w is expressed in a majority of infiltrative gastric adenocarcinomas and suppresses the cancer cell death by blocking stress-activated protein kinase-c-Jun NH2-terminal kinase activation. *Cancer Res* **63**: 1093–1100.

Liu T, Tang H, Lang Y, Liu M, Li X. (2008). MicroRNA-27a functions as an oncogene in gastric adenocarcinoma by targeting prohibitin. *Cancer Lett* **273**: 233–242.

Livak KJ, Schmittgen TD. (2001). Analysis of relative gene expression using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* **25**: 402–408.

Lou Z, O’Reilly S, Liang H, Maher VM, Sleight SD, McCormick JJ. (2005). Down-regulation of overexpressed spl protein in human fibrosarcoma cell lines inhibits tumor formation. *Cancer Res* **65**: 1007–1017.

Marcucci G, Maharry K, Radmacher MD, Mrózek K, Vukosavljevic T, Paschka P et al. (2008). Prognostic significance of, and gene and microRNA expression signatures associated with, CEBPA mutations in cytogenetically normal acute myeloid leukemia with high-risk molecular features: a Cancer and Leukemia Group B Study. *J Clin Oncol* **26**: 5078–5087.

Parkin DM, Bray F, Ferlay J, Pisani P. (2005). Global Cancer Statistics. 2002. *CA Cancer J Clin* **55**: 74–108.

Petrocca F, Visone R, Onelli MR, Shah MH, Nicoloso MS, de Martino I et al. (2008). E2F1-regulated microRNAs impair TGFbeta-dependent cell-cycle arrest and apoptosis in gastric cancer. *Cancer Cell* **13**: 272–286.

Qin H, Sun Y, Benveniste EN. (1999). The transcription factors p53 and Sp1. *Mol Cell Biol* **19**: 402–408.

Ronchetti D, Lionetti M, Mosca L, Agnelli L, Andronache A, Fabris S et al. (2008). An integrative genomic approach reveals coordinated expression of intronic miR-335, miR-342, and miR-561 with deregulated host genes in multiple myeloma. *BMC Med Genomic* **1**: 37.

Song X, Yue ZY, Wang ZN, Xu YY, Luo Y, Xu HM et al. (2011). MicroRNA-148b is frequently down-regulated in gastric cancer and acts as a tumor suppressor by inhibiting cell proliferation. *Mol Cancer* **10**: 1.

Soon PS, Talon LJ, Gill AJ, Bambach CP, Sywak MS, Campbell PR et al. (2009). miR-195 and miR-483-5p identified as predictors of poor prognosis in adenocortical cancer. *Clin Cancer Res* **15**: 7684–7692.

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Sorrentino A, Liu CG, Addario A, Peschle C, Scambia G, Ferlini C. (2008). Role of microRNAs in drug-resistant ovarian cancer cells. *Gynecol Oncol* **111**: 478–486.

Sun Z, Li DM, Wang ZN, Huang BJ, Xu Y, Li K *et al.* (2009). Prognostic significance of microscopic positive margins for gastric cancer patients with potentially curative resection. *Ann Surg Oncol* **16**: 3028–3037.

Sun Z, Wang ZN, Zhu GL, Huang BJ, Li K, Xu Y *et al.* (2010). Advanced gastric cancer with early cancer macroscopic appearance: is it worthy of D2 lymphadenectomy? *Ann Surg Oncol* **17**: 1278–1290.

Suske G. (1999). The Sp-family of transcription factors. *Gene* **238**: 291–300.

Tavazoie SF, Alarcon C, Oskarsson T, Padua D, Wang Q, Bos PD *et al.* (2008). Endogenous human microRNAs that suppress breast cancer metastasis. *Nature* **451**: 147–152.

Uren RT, Dewson G, Chen L, Coyne SC, Huang DC, Adams JM *et al.* (2007). Mitochondrial permeabilization relies on BH3 ligands engaging multiple prosurvival Bcl-2 relatives, not Bak. *J Cell Biol* **177**: 277–287.

Volinia S, Culin GA, Liu CG, Amb s S, Cimmino A, Petrocca F *et al.* (2006). A microRNA expression signature of human solid tumors defines cancer gene targets. *Proc Natl Acad Sci USA* **103**: 2257–2261.

Wada R, Akiyama Y, Hashimoto Y, Fukamachi H, Yuasa Y. (2010). miR-212 is downregulated and suppresses methyl-CpG-binding protein MeCP2 in human gastric cancer. *Int J Cancer* **127**: 1106–1114.

Wang F, Zheng Z, Guo J, , Ding X. (2010). Correlation and quantitation of microRNA aberrant expression in tissues and sera from patients with breast tumor. *Gynecol Oncol* **119**: 586–593.

Wang L, Guan X, Gong W, Yao J, Peng Z, Wei D *et al.* (2005). Altered expression of transcription factor Sp1 critically impairs the angiogenic phenotype of human gastric cancer. *Clin Exp Metastasis* **22**: 205–213.

Wang L, Wei D, Huang S, Peng Z, Le X, Wu TT *et al.* (2003). Transcription factor Sp1 expression is a significant predictor of survival in human gastric cancer. *Clin Cancer Res* **9**: 6371–6380.

Wang YX, Zhang XY, Zhang BF, Yang CQ, Chen XM, Gao HJ. (2010). Initial study of microRNA expression profiles of colonic cancer without lymph node metastasis. *J Dig Dis* **11**: 50–54.

Wu L, Fan J, Belasco JG. (2006). MicroRNAs direct rapid deadenylation of mRNA. *Proc Natl Acad Sci USA* **103**: 4034–4039.

Wu Q, Jin H, Yang Z, Luo G, Lu Y, Li K *et al.* (2010). MiR-150 promotes gastric cancer proliferation by negatively regulating the pro-apoptotic gene EGR2. *Biochem Biophys Res Commun* **392**: 340–345.

Xu YY, Huang BJ, Sun Z, Lu C, Liu YP. (2007). Risk factors for lymph node metastasis and evaluation of reasonable surgery for early gastric cancer. *World J Gastroenterol* **13**: 5133–5138.

Yao JC, Wang L, Wei D, Gong W, Hassan M, Wu TT *et al.* (2004). Association between expression of transcription factor Sp1 and increased vascular endothelial growth factor expression, advanced stage, and poor survival in patients with resected gastric cancer. *Clin Cancer Res* **10**: 4109–4117.

Yao M, Nguyen TV, Pike CJ. (2005). Beta-amyloid-induced neuronal apoptosis involves c-Jun N-terminal kinase-dependent downregulation of Bel-2. *J Neurosci* **25**: 1149–1158.

Yu C, Pan K, Xing D, Liang G, Tan W, Zhang L *et al.* (2002). Correlation between a single nucleotide polymorphism in the matrix metalloproteinase-2 promoter and risk of lung cancer. *Cancer Res* **62**: 6430–6433.

Zhang H, Luo XQ, Zhang P, Huang LB, Zheng YS, Wu J *et al.* (2009). MicroRNA patterns associated with clinical prognostic parameters and CNS relapse prediction in pediatric acute leukemia. *PLoS One* **4**: e7826.

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