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ORIGINAL ARTICLE

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.

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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

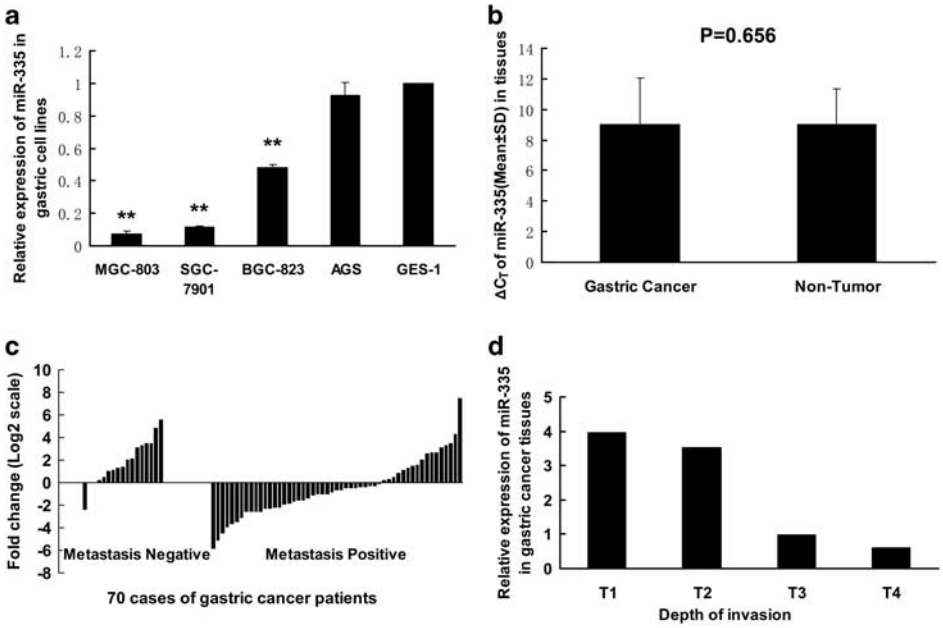

Figure 1 The expression of miR-335 in tissues and cell lines. (**a**) Relative expression of miR-335 in four gastric cancer cell lines (MGC-803, SGC-7901, BGC-823 and AGS). Quantification of miR-335 was measured by qRT–PCR. Data are presented in gastric cancer cell lines relative to GES-1 (which is a 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.

Table 1 Comparison of expression levels of miR-335 with clinicopathological features in patients with gastric cancer

	N	*miR-335*[a]	P-*value*
Sex			
Male	57	0.8 (0.2–3.3)	0.5
Female	13	1.1 (0.5–4.4)	
Age (years)			
⩽55	27	1.1 (0.5–6.2)	0.1
>55	43	0.8 (0.2–2.6)	
Tumor size (cm)			
⩽3.5	14	2.7 (0.3–10.0)	0.1
>3.5	56	0.8 (0.3–2.3)	
Macroscopic type			
Eraly stage	1	4.0	0.6
Borrmann I + II	6	0.8 (0.2–2.5)	
Borrmann III + IV	63	0.8 (0.3–3.9)	
Histological grade			
Well	14	0.8 (0.4–7.2)	0.7
Poor	56	0.9 (0.2–2.9)	
Lauren's grade			
Intestinal	22	0.8 (0.3–4.6)	0.9
Diffuse	48	0.9 (0.3–2.7)	
pT stage			
T1	1	4.0	0.03*
T2	12	3.5 (0.7–10.6)	
T3	21	1.0 (0.4–3.3)	
T4	36	0.6 (0.2–2.0)	
pN stage			
N0 + N1	23	2.1 (1.0–8.4)	0.01*
N2 + N3	47	0.6 (0.2–2.2)	
Metastasis lymph node			
Negative	17	2.6 (1.2–10.2)	0.000*
Positive	53	0.6 (0.2–2.0)	
Invasion into lymphatic vessels			
Negative	54	1.1 (0.3–6.0)	0.02*
Positive	16	0.5 (0.2–0.9)	

*Indicated statistical significance ($P<0.05$).
[a]Median of relative expression, with 25th–75th percentile in parenthesis.

(Figure 1b). The non-parametric test revealed that the low expression of miR-335 was significantly associated with lymph-node metastasis ($P<0.001$), poor pT stage ($P<0.05$), poor pN stage ($P<0.05$) and invasion into lymphatic vessels ($P<0.05$; Table 1). The data showed that the high expression of miR-335 was clearly found in 82.4% of the 17 cases that were found to be metastasis free. On the contrary, of the 53 cases that were found to have metastasis lymph nodes, only 32.1% showed high expression of miR-335 (Figure 1c). The non-parametric test demonstrated that patients with a better pT stage had significantly higher miR-335 expression (4.0-fold with T1 and 3.5-fold with T2) than did those with a poor pT stage (1.0-fold with T3 and 0.6-fold with T4; $P<0.05$; Figure 1d). Similarly, patients with a better pN stage had significantly higher miR-335 expression (2.1-fold with N1 + N2) than did those with a poor pN stage (0.6-fold with N3 + N4; $P<0.05$). Moreover, patients with a low expression of miR-335 tended to have metastasis invasion into lymphatic vessels.

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 ($P_{BGC-823}=0.2$, $P_{SGC-7901}=0.2$, $P_{AGS}=0.9$; Figure 2).

miR-335 suppresses gastric cancer metastasis in vivo

We used a metastasis formation assay in nude mice *in vivo* approach. In all, 5×10^5 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

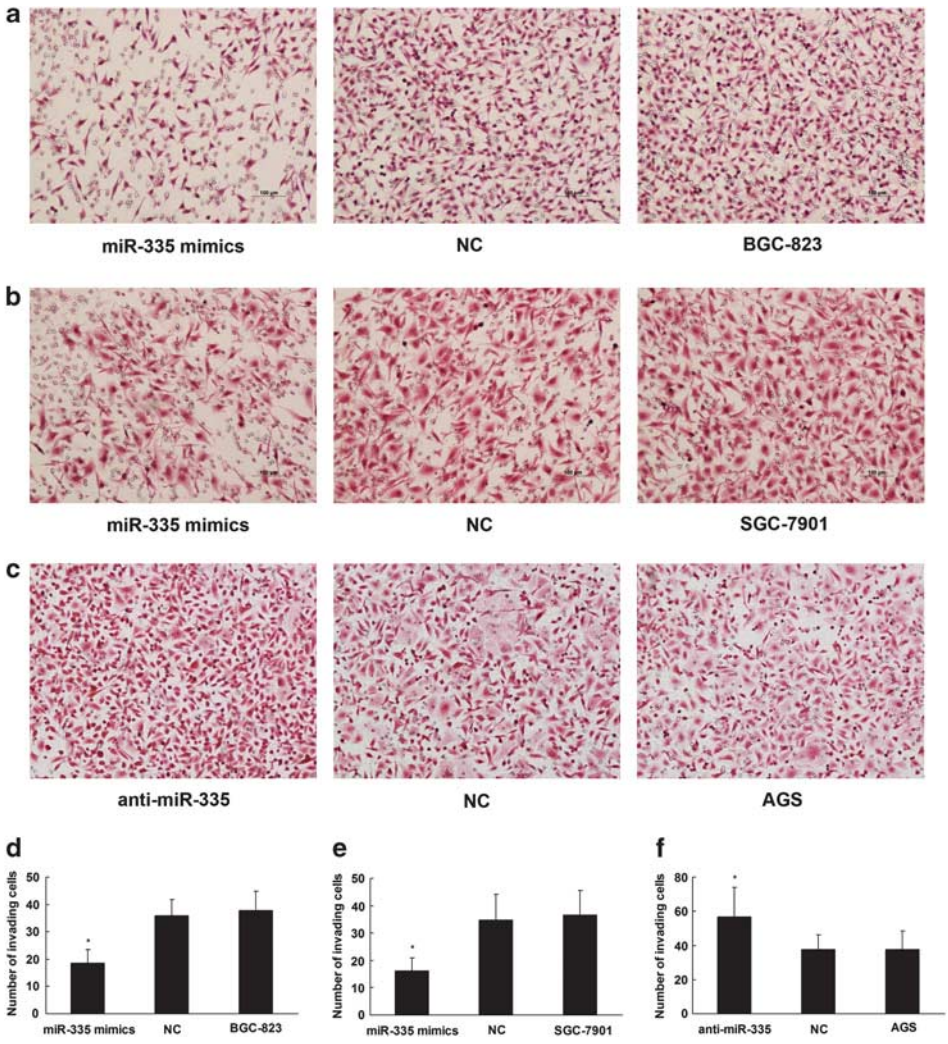

Figure 2 miR-335 inhibits cell invasion *in vitro*. (**a**) Representative photomicrographs of transwell results for BGC-823 cells were taken under $\times 100$ original magnification. (**b**) Representative photomicrographs of transwell results for SGC-7901 cells were taken under $\times 100$ original magnification. (**c**) Representative photomicrographs of transwell results for AGS cells were taken under $\times 100$ original magnification. (**d**) The numbers of miR-335-transfected BGC-823 cells passing through the Matrigel were significantly lower than those of NC-transfected and parental BGC-823 cells. The number of cells was counted in 16 independent symmetrical visual fields under the microscope ($\times 400$ original magnification) from three independent experiments. Results represent the means of the values from three independent experiments. Bars indicate s.d. * Refers to statistical significance between groups ($P<0.05$). (**e**) The numbers of miR-335-transfected SGC-7901 cells passing through the Matrigel were significantly lower than those of NC-transfected and parental SGC-7901 cells. The number of cells was counted in 16 independent symmetrical visual fields under the microscope ($\times 200$ original magnification) from three independent experiments. Results represent the means of the values from three independent experiments. Bars indicate s.d. * Refers to statistical significance between groups ($P<0.05$). (**f**) The numbers of anti-miR-335-transfected AGS cells passing through the Matrigel were significantly higher than those of NC-transfected and parental AGS cells. The number of cells was counted in 16 independent symmetrical visual fields under the microscope ($\times 200$ original magnification) from three independent experiments. Results represent the means of the values from three independent experiments. Bars indicate s.d. * Refers to statistical significance between groups ($P<0.05$).

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-trans-

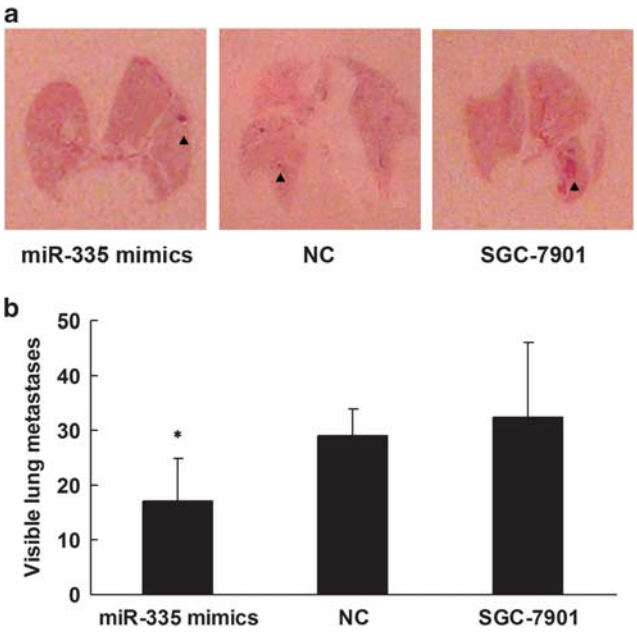

Figure 3 miR-335 suppresses gastric cancer metastasis *in vivo*. (**a**) Representative photographs for lung tissues. Cell aggregates, which were observed by staining with dark nucleus, represent lung metastases. (**b**) The data are shown graphically with the number of lung metastases from each mouse. Results represent the means of the values. Bars indicate s.d. * Refers to statistical significance between groups ($P<0.05$).

fected SGC-7901 cells normalized to a control vector containing Renilla luciferase, pRL-TK. On the contrary, there were no significantly differences of the relative luciferase activity of pGL3-SP1-MUT and pGL3-Bcl-w-MUT reporters in miR-335-transfected SGC-7901 cells compared with NC-transfected SGC-7901 cells (Figures 4a and b). These results showed that SP1 and Bcl-w are negatively regulated directly by miR-335 in gastric cancer cells.

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*.,

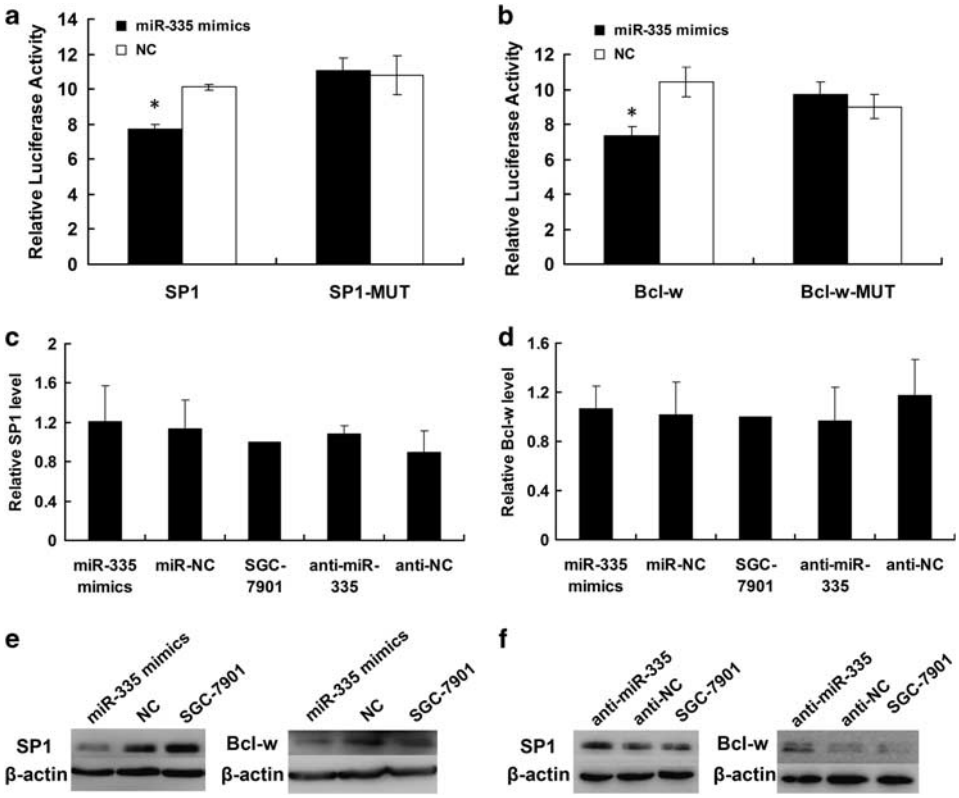

Figure 4 SP1 and Bcl-w are potential targets of miR-335 in gastric cancer cells. (**a**) Analysis of luciferase activity of SP1 and SP1-MUT with miR-335 mimics or NCs in SGC-7901 cells. * Refers to statistical significance between groups ($P<0.05$). (**b**) Analysis of luciferase activity of Bcl-w and Bcl-w-MUT with miR-335 mimics or NCs in SGC-7901 cells. * Refers to statistical significance between groups ($P<0.05$). (**c**) mRNA expression analysis for SP1 in parental and transfected SGC-7901 cells by real-time qRT–PCR. No significant difference in the level of the endogenous SP1 mRNA was found in transfected and parental SGC-7901 cells normalized to an endogenous reference GAPDH mRNA. (**d**) mRNA expression analysis for Bcl-w in parental and transfected SGC-7901 cells by real-time qRT–PCR. No significant difference in the level of endogenous Bcl-w mRNA was found in transfected and parental SGC-7901 cells normalized to an endogenous reference GAPDH mRNA. (**e**) Protein expression analysis for SP1 and Bcl-w in miR-335-transfected, NC-transfected and parental SGC-7901 cells by western blot. A clear reduction in the level of the endogenous SP1 and Bcl-w proteins was demonstrated in miR-335-transfected SGC-7901 cells than in NC-transfected SGC-7901 cells normalized to an endogenous reference β-actin protein. (**f**) Protein expression analysis for SP1 and Bcl-w in anti-miR-335-transfected, anti-NC-transfected and parental SGC-7901 cells by western blot. The level of the endogenous SP1 and Bcl-w proteins was increased in anti-miR-335-transfected SGC-7901 cells than in anti-NC-transfected SGC-7901 cells normalized to an endogenous reference β-actin protein.

2008). 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 (Invitrogen) 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% CO_2.

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 greener qPCR supermix 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 $2^{-\Delta\Delta CT}$ in which $\Delta C_T = C_T$ 335$-C_T$ 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′-AATGAGAACAGCAACAACTCC-3′ (Bond *et al*., 2004) and the target Bcl-w sequence of the siRNA is 5′-AAGGGTT ATGTCTGTGGAGCT-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

Table 2 Real-time qRT–PCR primers for amplification of expression of miR-335

	Primer sequence (5′-3′)
RT-primer-1	GCTGTCAACGATACGCTACGTAACGGCATGACAGTGTTTTTTTTTTTTTTTTTTTTTTA
RT-primer-2	GCTGTCAACGATACGCTACGTAACGGCATGACAGTGTTTTTTTTTTTTTTTTTTTTTTG
RT-primer-3	GCTGTCAACGATACGCTACGTAACGGCATGACAGTGTTTTTTTTTTTTTTTTTTTTTTC
miR-335-F[a]	TCAAGAGCAATAACGAAAAATGT
miR-335-R[b]	GCTGTCAACGATACGCTACGT
U6 RNA-F[a]	CGCTTCGGCAGCACATATAC
U6 RNA-R[b]	TTCACGAATTTGCGTGTCAT

Abbreviation: qRT–PCR, quantitative reverse-transcriptase PCR.
[a]Forward primer.
[b]Reverse primer.

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, 5×10^4 cells were harvested by trypsinization, washed with serum-free medium to 25×10^4/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% CO_2, 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, 5×10^5 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, 8×10^3 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 from 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.

SGC-7901 cells were transfected in 24-well plates using Lipofectamine 2000 according to the manufacturer's instructions with 0.8 μg of the firefly luciferase report vector and 0.08 μg of the control vector containing Renilla luciferase, pRL-TK (Promega). For each well, 20 pm hsa-mir-335 or a NC was used. Firefly and Renilla luciferase activities were measured consecutively using a dual-luciferase reporter assay (Promega) 24 h after transfection on a Centro LB 960 (Berthold, Bad Wildbad, Germany).

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 preformed 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.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)