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MicroRNA-1258: An invasion and metastasis regulator that targets heparanase in gastric cancer

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Abstract. Numerous microRNAs (miRNAs/miRs) are involved in suppressing or promoting the formation of cancer. However, to the best of our knowledge, the role of miRNA-1258 in gastric cancer (GC) has not previously been investigated. Our previous study demonstrated an increased expression of heparanase (HPSE) in GC tissues and HPSE-facilitated invasion and metastasis of GC cells. Consequently, in the present study, the function of miR-1258 in the invasion and metastasis of GC cells was investigated to determine whether miR-1258 is associated with GC through HPSE. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was used to determine miR-1258 expression in GC cell lines and tissues. A Transwell cell invasion assay and an MTT proliferation assay were used to investigate the effects of miR-1258 on the invasion and proliferation of one of the cell lines, SGC-7901 cells, *in vitro*. The effect of miR-1258 on SGC-7901 cell metastasis was investigated using an *in vivo* tumor metastasis formation assay. Western blot analysis and RT-qPCR were used to investigate whether HPSE was a target of miR-1258 in GC. The expression of miR-1258 was significantly downregulated in 3 GC cell lines and 116 GC tissues compared with controls (all P<0.001). An association was identified between decreased miR-1258 expression level and increased age (P=0.042), advanced pathological tumor stage (P=0.027) and positive lymphatic vessel invasion (P=0.044) in patients with GC. Furthermore, the upregulation of miR-1258 expression suppressed SGC-7901 cell invasion *in vitro* (P<0.001) and inhibited SGC-7901 cell metastasis *in vivo* (P=0.016). The western blot analysis and RT-qPCR results indicated that miR-1258 downregulated the expression of HPSE at the translational level. The results of the present study indicate that miR-1258 acts as a tumor suppressor to inhibit invasion and metastasis by targeting HPSE. Therefore, miR-1258 may serve as a novel biomarker and therapeutic target in the treatment of GC.

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Introduction

Gastric cancer (GC) is the third most common cancer in China, with the third highest mortality rate (1). Patients are frequently diagnosed at an advanced stage, resulting in a poor prognosis. It is estimated that ~50% of patients with GC exhibit metastasis at diagnosis (2). Tumor metastasis is a common cause of mortality in GC, therefore it is important to identify novel biomarkers that may contribute to the early diagnosis of tumor invasion and metastasis.

MicroRNAs (miRNAs/miRs) were initially described in *Caenorhabditis elegans* in 1993 (3). They are endogenous non-coding small molecule RNAs, between 19 and 24 ribonucleotides in length, which post-transcriptionally regulate gene expression in plants and animals (2,4). miRNAs have important roles in regulating a number of cell processes, including cell differentiation, apoptosis and cell cycle progression (5-9). miRNAs serve as biomarkers in various types of cancer, including GC and prostate cancer (10,11). Although a number of miRNAs have been identified to be dysregulated in GC tissues, the role of miR-1258 in GC has not, to the best of our knowledge, previously been investigated.

Heparanase (HPSE) is a 65-kDa inactive precursor that cleaves heparan sulfate and participates in the degradation and remodeling of the extracellular matrix (ECM) (12). It is widely accepted that tumor metastasis begins with the degradation of the ECM and breakdown of the basement membrane (13). Our previous studies demonstrated an increased expression of HPSE in GC tissues and identified that HPSE facilitates invasion and metastasis of GC cells (13,14). However, the underlying molecular mechanism of the upregulation of HPSE in GC tissues remains unclear. Zhang *et al* (15) identified that miR-1258 suppressed breast cancer brain metastasis by inhibiting the expression and activity of HPSE, by directly targeting the HPSE 3'-untranslated region. Furthermore,

Liu *et al* (16) identified that miR-1258 influenced the morbidity and metastasis of non-small cell lung cancer by regulating the expression of HPSE. However, the potential association between miR-1258 and HPSE in GC remains unclear. The aim of the present study was to investigate the role of miR-1258 in GC and to determine the regulation of HPSE by miR-1258 in GC.

Materials and methods

Patients and tissue specimens. The present study was approved by the First Hospital of China Medical University (Shenyang, China) and all specimens were obtained from patients who provided informed consent. The present study complied with the principles of The Declaration of Helsinki, and received approval from the Research Ethics Committee of China Medical University. GC was diagnosed according to histopathological evaluation, and the histological grade was staged according to the seventh tumor-node-metastasis (TNM) staging (17). None of the patients in the present study received preoperative therapy, and all received surgery at the time of hospitalization between January 2007 and December 2009. A total of 116 pairs of GC tissue were obtained, and the tissues adjacent to the proximal excision margin were regarded as matched non-tumor adjacent tissues (NATs). The tumor size was represented by the maximum diameter and this measurement was obtained from the pathological reports of the patients. The paired and GC tissues were preserved in liquid nitrogen and stored at -80˚C immediately.

Cell culture. Three GC cell lines (MGC-803, BGC-823 and SGC-7901) were obtained from the Institute of Biochemistry and Cell Biology at the Chinese Academy of Sciences (Shanghai, China). For all assays, the cells were cultured at 37˚C and 5% CO_2 using RPMI-1640 medium (HyClone; GE Healthcare Life Sciences, Logan City, UT, USA) supplemented with 10% fetal bovine serum (FBS).

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA of the paired specimens and cultured cells was isolated using the TRIzol® reagent (Ambion; Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's protocol. A Poly (A) Tailing kit (Ambion; Thermo Fisher Scientific, Inc.) was used to add a poly (A) tail, according to the manufacturer's protocol. RNA was reverse-transcribed into cDNA using a PrimeScript™ RT Reagent kit (Takara Biotechnology, Co., Ltd., Shiga, Japan), according to the manufacturer's protocol. Subsequently, qPCR was employed using 2 μl diluted cDNA products were added to 12.5 μl SYBR Premix Ex Taq II (Takara Biotechnology, Co., Ltd.), 0.5 μl forward and reverse primers (10 μM) and 9.5 μl nuclease-free water in a final volume of 25 μl on a Light Cycler 480 II Real-Time PCR system (Roche Diagnostics, Basel, Switzerland) with a thermocycling protocol of 94˚C for 5 sec, 58˚C for 20 sec and 72˚C for 30 sec, for 45 cycles. U6 small nuclear RNA (U6) expression was used as an internal control and the $2^{-\Delta\Delta Cq}$ method was used to determine expression levels (18). The primers used were: RT-primer1, 5'-GCTGTCAACGATACGCTACGTAACGGCATGACAGTGTTTTTTTTTTTTTTTTTTTTTTA-3'; RT-primer2, 5'-GCTGTCAACGATACGCTACGTAACGGCATGACAGTGTTTTTTTTTTTTTTTTTTTTTTC-3'; RT-primer3, 5'-GCTGTCAACGATACGCTACGTAACGGCATGACAGTGTTTTTTTTTTTTTTTTTTTTTTG-3'; miR-1258-F, 5'-AGTTAGGATTAGGTCGTGGAAAA-3' miR-1258-R, 5'-GCTGTCAACGATACGCTACGT-3'; U6 primer-F, 5'-CGCTTCGGCAGCACATATAC-3'; and U6 primer-R, 5'-TTCACGAATTTGCGTGTCAT-3'.

Transfection of GC cells. miR-1258 mimic was purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China). The cells were seeded in 6-well plates. SGC-7901 cells at a concentration of $2x10^5$ cells/well were transfected with ~50 nM miR-1258 mimic or negative control miRNA (NC) using Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The sequence of miR-1258 mimic was 5'-AGUUAGGAUUAGGUCGUGGAA-3' and the sequence of the NC was 5'-UUCUCCGAACGUGUCACGUdTdT-3'.

Cell proliferation assay. The proliferation ability of untransfected or transfected SGC-7901 cells was detected using an MTT assay. A total of ~$8x10^3$ cells were seeded in 96-well plates, and cultured for 24, 48, 72 or 96 h. Following culture, cells were incubated with 20 μl 5 mg/ml MTT at 37˚C for 4 h prior to dissolution of the formazan crystals generated with 150 μl dimethylsulfoxide for 20 min at room temperature. The optical density was determined at a wavelength of 490 nm using a Model 680 microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

In vitro cell invasion assay. A Transwell invasion assay was used to determine the invasion ability of the GC cells. Untransfected or transfected SGC-7901 cells were cultured for 24 h. A 50 μl volume of Matrigel (BD Biosciences, San Jose, CA, USA) diluted 1:12 was added to the upper chamber of a 24-well tissue culture plate (Corning Incorporated, Corning, NY, USA), and allowed to solidify for 4 h at 37˚C. Subsequently, 200 μl medium containing $5x10^4$ cells was added on top of the solidified Matrigel. Simultaneously, ~600 μl medium with 10% FBS was added to the lower chamber, to act as a chemoattractant. Following incubation at 37˚C and 5% CO_2 for 24 h, the cells remaining on the upper surface were removed using a wet cotton swab. Cells attached to the lower surface were fixed using methanol for 1 min and stained with 0.4% hematoxylin and 0.5% eosin (H&E) separately for 3 min and 30 sec. These steps were performed at room temperature. The cells were examined and counted under a light microscope. All the experiments were performed independently and in triplicate.

In vivo cancer cell metastasis assay. In order to investigate the effect of miR-1258 expression on GC cell metastasis, SGC-7901 cells transfected with miR-1258 mimic or NC, and untransfected SGC-7901 cells were separately injected into the lateral tail vein of 3 groups of four-week-old female BALB/c mice with an average weight of 15 g, which were purchased from HFK Bio-Technology Co., LTD (Beijing, China) and housed in the animal care facility of the China Medical University under specific pathogen-free conditions.

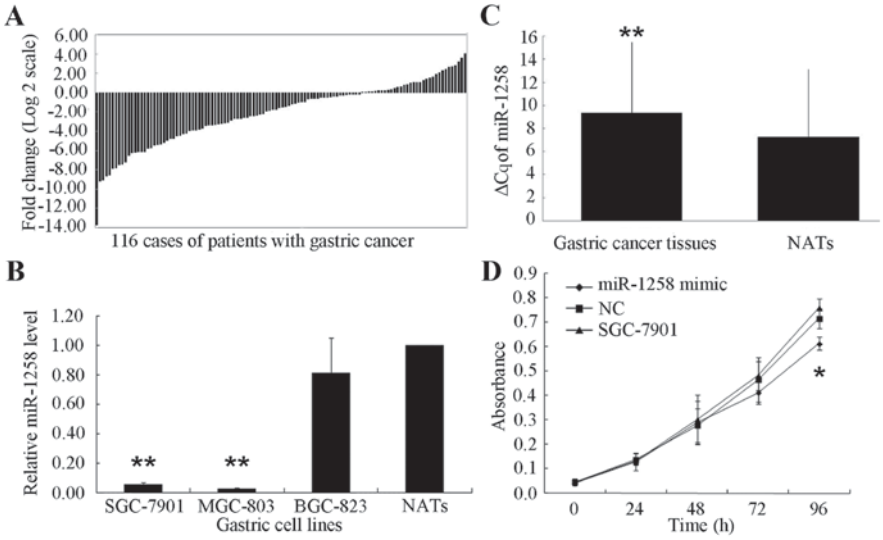

Figure 1. Expression of miR-1258 in tissues and cell lines. (A) miR-1258 was detected in 116 patients with gastric cancer using the reverse transcription-quantitative polymerase chain reaction. Results are presented as $\log_2$ fold change of gastric cancer relative to NATs. (B) Significantly increased ΔCq values for miR-1258 were observed between gastric cancer tissues and NATs. (C) Mean expression of miR-1258 in gastric cancer cell lines (MGC-803, SGC-7901 and BGC-823) relative to NATs. (D) MTT proliferation assay in SGC-7901 cells. *P<0.05; **P<0.01 vs. NAT. miR-1258, microRNA-1258; NAT, non-tumor adjacent tissue; NC, negative control.

Each group included 8 mice that were injected with $1x10^6$ cells resuspended in 0.1 ml PBS. After 5 weeks, the mice were sacrificed. The lungs were isolated and fixed in 4% paraformaldehyde in PBS at room temperature for >24 h. Following embedding in paraffin and sectioning, 4 μm sections were stained with H&E. The H&E staining is performed using separate stains of 0.4% hematoxylin and 0.5% eosin, for 5 min and 2 min, respectively, at room temperature. The visible lung metastases were counted under a light microscope. All procedures involving animals were performed in accordance with the institutional animal welfare guidelines of the Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication no. 80-23, revised 1996).

Protein extraction and western blot analysis. To extract the total protein from cells, a Total Protein Extraction kit (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China) was used according to the manufacturer's protocol. Protein (50 μg/lane) was separated by SDS-PAGE (12% gels) prepared using an SDS-PAGE Gel Preparation kit (Nanjing KeyGen Biotech Co., Ltd.), according to the manufacturer's protocol, then transferred onto 0.2 μm pore size polyvinyl difluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked using the 5% skimmed milk powder at room temperature for 2 h. Then the membranes were incubated overnight at 4˚C with rabbit anti-HPSE polyclonal antibody (1:200; sc-25825; Santa Cruz Biotechnology, Dallas, TX, USA) or rabbit anti-β-actin monoclonal antibody (1:5,000; A5441; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) as a control. Following washing three times for 20 min in Tris-buffered saline containing Tween 20 (TBST), membranes were incubated with the horseradish peroxidase-conjugated AffiniPure goat anti-mouse or anti-rabbit IgG secondary antibody (1:5,000; ZB-2305 and ZB-2301, respectively; OriGene Technologies, Inc., Beijing, China) for 2 h at room temperature, then washed again in TBST three times for 20 min. SuperSignal Chemiluminescent substrates made by the mixture of the Stable Peroxide Solution and Luminol/Enhancer Solution (Thermo Fisher Scientific, Inc.) were used, according to the manufacturer's protocol, to detect protein bands, which were observed using GelCapture software (version 2.0.0.0; DNR Bio-Imaging Systems, Ltd., Jerusalem, Israel), and the relative protein expression was analyzed using FluorChem software (version 2.01; ProteinSimple, San Jose, CA, USA).

Statistical analysis. All results are presented as the mean ± standard deviation and were analyzed using SPSS software (version 18.0; SPSS, Chicago, IL, USA). Student's t test and non-parametric tests (Mann-Whitney U and Kruskal-Wallis tests) were used for statistical analysis. All the experiments were performed at least in triplicate. P<0.05 was considered to indicate a statistically significant difference.

Results

miR-1258 expression is downregulated in human GC cells and patient tissues. Among the 116 patients with GC, a decreased expression of miR-1258 was exhibited in 83 cases (71.55%) compared with their NATs (Fig. 1A). The ΔCq for miR-1258 was significantly increased in GC tissues compared with NATs (9.3957±6.1351 and 7.2786±5.8543, respectively; P<0.001; Fig. 1B). Furthermore, compared with the NATs from the patients with GC, a significantly decreased expression of miR-1258 was identified in SGC-7901 cells (0.05±0.04-fold; P<0.001) and MGC-803 cells (0.02±0.01-fold, P<0.001) (Fig. 1C).

As shown in Table I, according to results of the Mann-Whitney U test and the Kruskal-Wallis test concerning the relative miR-1258 expression levels and the clinicopathological characteristics, older patients exhibited a decreased miR-1258 expression (P=0.042), and patients with the decreased miR-1258 expression tended to be classified

Table I. Associations between the expression of miR-1258 with clinicopathological characteristics in 116 patients with gastric cancer.

Characteristic	n	miR-1258 T/N[a]	P-value
Gender			0.410
Male	90	1.048 (0.068-1.068)	
Female	26	1.577 (0.044-2.034)	
Age, years			0.042[c]
<62	57	1.568 (0.083-1.330)	
≥62	59	0.778 (0.032-0.899)	
Tumor size, cm			0.579
<5	53	1.342 (0.081-1.310)	
≥5	63	1.018 (0.051-1.045)	
Macroscopic type[b]			0.560
Borrmann I+II	12	1.061 (0.035-1.073)	
Borrmann III+IV	100	1.181 (0.057-1.081)	
Histological grade			0.920
Well and moderately differentiated	28	1.582 (0.028-1.961)	
Poorly differentiated	88	1.034 (0.057-1.058)	
pT stage			0.027[c]
T1+T2	23	2.312 (0.138-2.104)	
T3+T4	93	0.883 (0.045-0.963)	
pN stage			0.343
Negative	30	1.821 (0.086-1.851)	
Positive	86	0.938 (0.050-0.891)	
pN stage			0.350
N0	28	1.914 (0.092-2.020)	
N1	17	1.401 (0.086-2.087)	
N2	28	0.486 (0.046-0.761)	
N3	43	1.030 (0.027-0.889)	
pTNM stage			0.199
I	13	2.077 (0.055-1.936)	
II	27	1.586 (0.103-2.099)	
III	76	0.862 (0.038-0.873)	
Invasion into lymphatic vessels			0.044[c]
Negative	75	1.395 (0.103-1.176)	
Positive	41	0.748 (0.030-0.752)	

miRNA-1258, microRNA-1258; pT, pathological tumor; pN, pathological node; pTNM, pathological tumor-node-metastasis. [a]Median relative expression of tumor tissues/non-tumor (adjacent) tissues, with the 25th-75th percentile in parentheses. [b]A total of 4 patient cases classified as early stage tumor in Borrmann's gross type was excluded. [c]P<0.05.

in an advanced pathological tumor (pT) category (P=0.027). Furthermore, miR-1258 expression was significantly decreased in cases in which lymphatic vessel invasion was positive (P=0.044). However, no significant association between the expression level and gender, tumor size, macroscopic type, histological grade, pathological node (pN) category or TNM stage was identified (Table I). Although no significant association between miR-1258 expression and pN classification was identified, a decreased miR-1258 expression was observed in 76.74% of the 86 cases with metastatic lymph nodes. By contrast, of the 30 cases with no metastatic lymph nodes, only 56.67% exhibited a decreased expression of miR-1258.

Association between miR-1258 and cell proliferation in GC. An MTT assay of the effect of miR-1258 on SGC-7901 cell proliferation following transfection with miR-1258 mimic or NC revealed that the miR-1258 mimic exhibited a limited effect on the cell growth. An MTT assay was performed at 24, 48, 72 and 96 h. No significant differences were identified in the first 72 h. Only at 96 h was a significant decrease in the number of SGC-7901 cells identified in the miR-1258-transfected cells

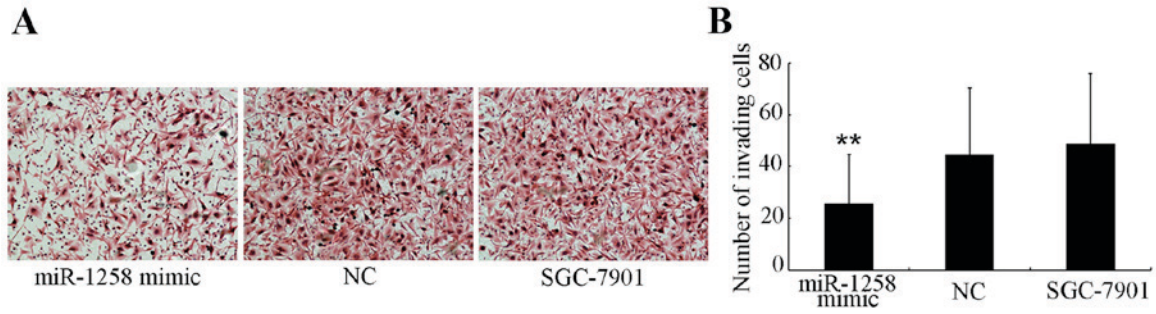

Figure 2. Overexpression of miR-1258 suppresses SGC-7901 cell invasion *in vitro*. A Transwell invasion assay was performed to estimate the effect of miR-1258 on the invasive potential of gastric cancer cells. (A) Representative images and (B) quantification of the cells that had migrated to the basal side of the membrane. Magnification, x200. **P<0.01, miR-1258 transfected vs. untransfected SGC-7901 cells. miR-1258, microRNA-1258; NC, negative control.

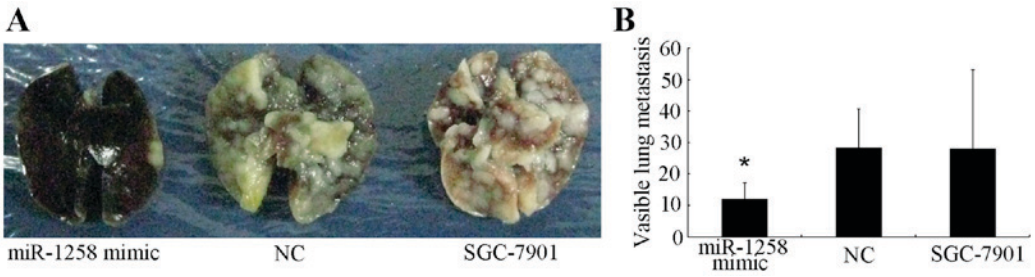

Figure 3. Overexpression of miR-1258 inhibits SGC-7901 cell metastasis *in vivo*. A total of 5 weeks after injection, mice were sacrificed and the lungs were dissected for microscopic histology. (A) Representative images of the lungs of mice from each of the 3 groups. (B) Quantification of lung metastases in the 3 groups. Magnification, x2. *P<0.05, miR-1258 transfected vs. untransfected SGC-7901 cells. miR-1258, microRNA-1258; NC, negative control.

compared with the NC-transfected and untransfected cells (P<0.05; Fig. 1D).

Upregulation of the miR-1258 expression level suppresses SGC-7901 cell invasion in vitro. According to Mann-Whitney U test analysis, a significant association between the expression of miR-1258 and advanced pT category was identified, therefore the influence of miR-1258 on the invasive ability of GC cells was investigated. Cells that were able to invade to the lower side of the membrane in the Transwell assays after a 24-h incubation were harvested (Fig. 2A) and quantified (Fig. 2B). The number of miR-1258-transfected SGC-7901 cells (25.46±19.12) that invaded through the Matrigel was significantly decreased (P<0.001) compared with the number of NC-transfected (44.44±25.77) and untransfected (48.75±27.09) SGC-7901 cells.

Upregulation of miR-1258 expression level inhibits SGC-7901 cell metastasis in vivo. In order to investigate the function of miR-1258 in GC metastasis, a metastasis formation assay was established using nude mice. After 5 weeks, microscopic histological analyses were performed by dissecting the lungs of the sacrificed mice. The number of lung metastases in first group (miR-1258 mimic) was 12.00±5.2, whereas the number of metastases in the second (NC) and third (untransfected SGC-7901) were 28.25±12.40 and 28±25.19, respectively (Fig. 3A and B). These results demonstrate that upregulation of miR-1258 expression significantly inhibits cell metastasis *in vivo*.

miR-1258 may target HPSE in SGC-7901 cells. RT-qPCR and western blot analysis were used to investigate the effect of HPSE expression on mRNA and protein levels. No effect of miR-1258 on HPSE mRNA levels was exhibited 48 h after transfection (Fig. 4A). However, a marked decrease in translational level in cells overexpressing miR-1258 was observed, which was normalized to an endogenous reference β-actin protein (Fig. 4B). The transfection efficiency of miR-1258 mimic was marked (Fig. 5). The results indicate that miR-1258 interacts with HPSE and inversely regulates the expression of HPSE at the translational level.

Discussion

miRNAs have been studied extensively in numerous types of cancer. In the last 20 years, >2,000 miRNAs have been discovered in humans, and are reported to regulate ~1/3 of the genes in the human genome (19). There are numerous miRNAs that exhibit similar functions in different types of cancer. For example, miR-203 is able to inhibit the proliferation, migration and metastasis of triple-negative breast and lung cancer cells (20,21). The expression of miR-203 is associated with tumor size, advanced Borrmann type and pT category of the GC (22). In addition, miR-148b is able to suppress cell growth in GC and colorectal cancer by targeting cholecystokinin B receptor (8,23), indicating that a number of miRNAs have the same function in different types of cancer.

It has been reported that the low expression of miR-1258 is associated with the development, progression, metastasis, and prognosis of cancer: For example, Zhang *et al* (15) identified that miR-1258, a candidate miRNA, directly targeted HPSE and suppressed brain metastatic breast cancer. Furthermore, it has been demonstrated that HPSE is upregulated in GC

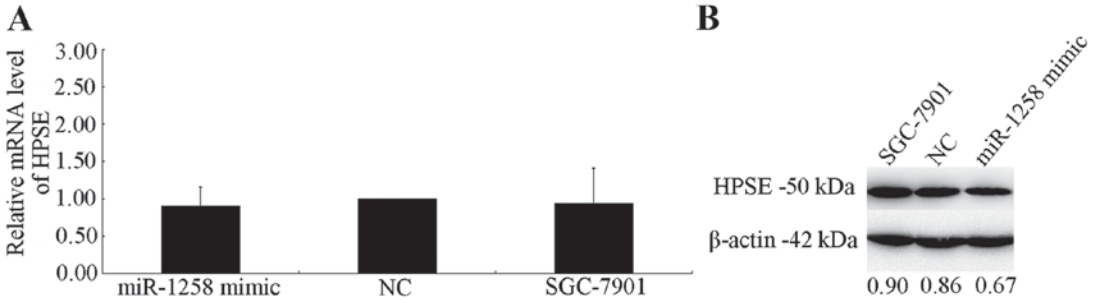

Figure 4. HPSE is a potential target of miR-1258 in SGC-7901 cells. (A) miR-1258 had no effect on HPSE mRNA levels as determined using the reverse transcription-quantitative polymerase reaction. (B) Western blot analysis identified that miR-1258 interacted with HPSE and negatively regulated its expression at the translational level. Densitometric values for the HPSE bands are indicated. β-actin was used as a loading control. HPSE, heparanase; miR-1258, microRNA-1258; NC, negative control.

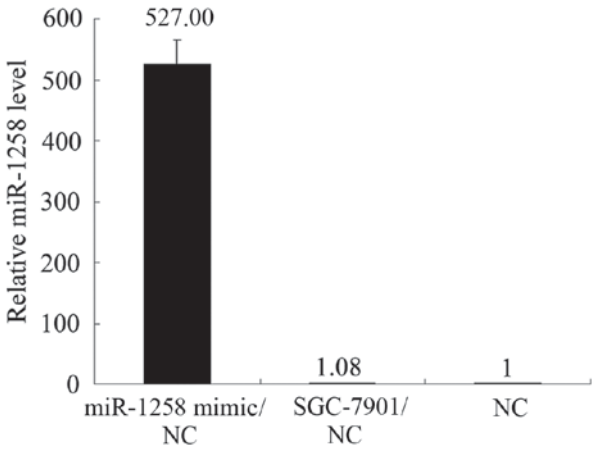

Figure 5. Transfection efficiency of miR-1258 mimic and NC. A total of 48 h after transfection, the efficiency of transfection was determined using the reverse transcription-quantitative polymerase chain reaction. The expression of miR-1258 in SGC-7901 cells transfected with miR-1258 mimic was increased 527±39-fold relative to NC. miR-1258, microRNA-1258; NC, negative control.

and facilitates invasion and metastasis of GC cells (24-27). A number of molecular mechanisms underlying single nucleotide polymorphisms of HPSE have been reported (28,29). Furthermore, Liu *et al* (16) identified that miR-1258 regulates the expression level of HPSE to influence the morbidity and metastasis of non-small cell lung cancer. Therefore, the aim of the present study was to determine whether these effects were associated only with breast cancer and lung cancer, or whether they also existed in GC.

In the present study, the expression level of miR-1258 was significantly decreased in GC tissues and cells. The patients with GC that exhibited decreased expression of miR-1258 may exhibit a more advanced pT category and positive lymphatic vessel invasion. The decreased expression of miR-1258 was inversely associated with increased age of the patients. It is well-known that miR-1258 facilitates invasion and metastasis of breast cancer or non-small cell lung cancer cells (15,16). Migration and invasion assays, and metastasis formation assay identified that the upregulation of miR-1258 suppressed SGC-7901 cell invasion *in vitro* and inhibited SGC-7901 cell metastasis *in vivo*.

RT-qPCR results indicated that miR-1258 exhibited a limited effect on the HPSE mRNA level; however, a marked inverse association was observed between miR-1258 and HPSE protein expression. These results indicate that HPSE is a target of miR-1258, and that miR-1258 negatively regulates HPSE expression at the translational level. Furthermore, it was demonstrated previously that, following knockdown of HPSE by siRNA, SGC-7901 cell invasion was significantly decreased (13). Therefore, the results of the present study suggest that miR-1258 was downregulated in GC, and influenced the invasion and metastasis of GC cells by regulating the expression level of HPSE.

Although an inverse association between miR-1258 and HPSE protein expression has been demonstrated in GC in the present study, a number of previous studies have identified that the same miRNA potentially regulates distinct targets in various cell types or in the same cell type, or is dependent on distinct binding regions (30-34). Similarly, a single target gene may be regulated by a number of miRNAs (35,36). Therefore, further investigation of the target genes of miR-1258 and the other miRNAs that regulate HPSE expression is warranted.

The results of the present study have demonstrated downregulation of miR-1258 in GC tissues and cell lines compared with NATs. Furthermore, decreased miR-1258 expression was identified to be associated with pT stage depth of invasion and positive lymphatic vessel invasion in patients with GC. In addition, it was identified that overexpression of miR-1258 was able to suppress SGC-7901 cell invasion *in vitro* and inhibit SGC-7901 cell metastasis *in vivo*. As miR-1258 downregulates the expression of HPSE protein in GC cells and inhibits cell invasion, miR-1258 may serve as a novel biomarker and therapeutic target for the treatment of GC.

Acknowledgements

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