



Effects of shRNA targeting maspin on invasion of gastric carcinoma SGC7901 cell line

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Abstract. Maspin is a member of the serpin (serine protease inhibitor) family of protease inhibitors known to have tumor suppressor activity in diverse human cancers. However, maspin gene function and the molecular aspects in gastric carcinoma remain largely unclear. To investigate the effects of maspin on invasion of gastric carcinoma SGC7901 cell line and the underlying molecular mechanism involved in this process, we cloned short hairpin oligoes (shRNA) targeting maspin into plasmid pGenesil-1.1 eukaryotic expression vector and then transfected the recombinant plasmid pGenesil-maspin into gastric carcinoma SGC7901 cells using Lipofectamine 2000. After the maspin expression was successfully knocked down, the number of cells invading through Matrigel was obviously increased ($P<0.05$) in the Transwell chamber assay. By detection of reverse transcription polymerase chain reaction (RT-PCR) and Western blot analysis, respectively, we found that mRNA and protein of uPA, VEGF-C were increased significantly, and the protein level of MMP7 was also increased ($P<0.05$). These results suggested that maspin gene could inhibit invasion of gastric carcinoma SGC7901 cells and this inhibition maybe result from the interaction between maspin and uPA, MMP7, or VEGF-C.

Introduction

Maspin is a 42 kD tumor suppressor member of the serpin (serine protease inhibitor) family, originally discovered in normal mammary epithelium (1). The protein is encoded by a gene on chromosome 18q21.3 and has been demonstrated in multiple tissues including epithelium of the breast, prostate, epidermis, lung, and in stromal cells of the cornea (2). Depending on cell type, Maspin is found localized to the cytoplasm, nucleus, the membrane of epithelial cells and the extracellular matrix (ECM). Multiple clinical studies have

confirmed that loss of maspin expression was a negative prognostic factor in diverse human cancers (3-8). Reintroduction of maspin into tumor cells inhibits growth, cell migration and invasion, angiogenesis, and increases cell adhesion and the sensitivity to apoptotic stimulation (9-12), all of which are hallmarks of a tumor suppressor.

Despite enhanced methods of detection and treatment, gastric carcinoma remains one of the most aggressive malignancies, with an extremely poor prognosis, and is the second leading cause of cancer death worldwide (13). Most newly diagnosed patients will present with incurable disease, and have a median survival of less than 1 year (14), so it is important to explore carcinogenesis and molecular mechanisms of gastric carcinoma to improve prevention and treatment. However, there are two opposing views on the role of maspin in human gastric carcinoma. One is that maspin is a tumor suppressor because of the loss and reduced expression in gastric carcinoma (15,16); the other is that maspin may be a gastric cancer-causing gene because of the higher expression in gastric carcinoma than that in normal gastric mucosa (17), and there was a significant correlation between the incidence of maspin-positive tumour staining and lymph node metastasis (18). We recently demonstrated that maspin expression was significantly related to the depth of invasion and lymph node metastasis of gastric carcinoma. Furthermore, by immunohistochemical detection, we found that maspin had a negative correlation with MMP7 and uPA in gastric carcinoma.

In the present study, the maspin-specific shRNA expression vector was constructed and transfected into cultured gastric carcinoma cells SGC7901. The efficacy of the vector in interference with maspin was confirmed by RT-PCR and Western blot analysis. By observation of the invasion ability, detection of uPA, VEGF-C and MMP7 mRNA and protein expression, we further studied and discuss the gene function of maspin in gastric carcinoma and the underlying molecular mechanisms.

Materials and methods

Cell culture. Gastric carcinoma SGC7901 cells were cultured in RPMI-1640 medium (Hyclon, USA) supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/ml) and streptomycin (100 μ g/ml) at 37°C in a humidified, 5% CO₂ atmosphere.

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Table I. Primer sequence of target genes for RT-PCR.

Genes	Primer sequence	Size (bp)
GAPDH	5'-ACGGATTTGGTCGTATTGGG-3' 5'-TGATTTTGGAGGGATCTCGC-3'	230
maspin	5'-CACTGGGCAATGTCCTCTTC-3' 5'-TGGTCTGGTCGTTCACTG-3'	382
uPA	5'-GCT GTG AGA TCA CTG GCT TTG-3' 5'-CCT TGG AGG GAA CAG ACG AG-3'	198
VEGF-C	5'-CTT CTT CTC TGT GGC GTG TTG T-3' 5'-GTC TCT TCT GCT CTT GAG TTG AGG-3'	303
MMP7	5'-AGA TGT GGA GTG CCA GAT GT-3' 5'-TAG ACT GCT ACC ATC CGT CC-3'	358

Construction and transfection of maspin shRNA expression vector. shRNA expression vector pGenesil-1.1 (Genesil Biotechnology, Wuhan, China) which can express enhanced green fluorescent protein (EGFP) was prepared. Oligonucleotides specific for maspin were designed as follows: maspin sense (5'-CAGGGTCCTCTTCTCTCCAATCTTTC AAGACGAGATTGGAGAGAAGAGGACTTTTTTG-3') and maspin antisense (5'-AGCTCAAAAAAGTCCTCTTCT CTCCAATCTCGTCTTGAAAGATTGGAGAGAAGAGG AC-3'). The sequences 5'-GATCCGACTTCATAAGGCGC ATCGTTCAAGACGGCATGCGCCTTATGAAGTCTTTT TTGTCGACA-3' (sense), and 5'-AGCTTGTCGACAAA AAAGACTTCATAAGGCGCATGCCGTCTTGAAGCATG CGCCTTATGAAGTCG-3' (antisense) were used as a scrambled RNAi control. The annealed double stranded oligonucleotides were ligated into the SacI sites of pGenesil-1.1 vector and the constructs were verified by DNA sequencing. SGC7901 cells were transfected with the pGenesil-maspin, pGenesil-scramble and pGenesil-1.1 vector using Lipofectamin 2000 (Invitrogen, USA) according to the manufacturer's instructions. After transfection (12, 24 and 48 h, respectively), EGFP expressions of 5 visual fields (10x20 times) were randomly observed under a fluorescence microscope, and the transfection efficiency was calculated as: green fluorescent cells/total cells x 100%. Stable clones were generated for further experiments by selection in complete culture medium containing 400 μ g/ml G418 (Gibco, USA).

Reverse transcription-polymerase chain reaction (RT-PCR). RT-PCR was performed to access transcripts of maspin, uPA, VEGF-C and MMP7 expressions after transfection. Total RNAs of SGC7901 cells were extracted by TRIzol (Invitrogen) reagent, and according to the manufacturer's protocol of RT reagent kit (Takara, Dalian, China), we synthesized successfully first-strand cDNAs from 1 μ l of total RNA using 0.5 μ l oligo (dt) primer, 1 μ l dNTP mixture, 0.5 μ l AMV Reverse Transcriptase (Promega, Mannheim, Germany), 0.25 μ l RNase inhibitor, 3.75 μ l RNase Free H₂O and 1 μ l 10X RT buffer. Each first-strand cDNA of target genes was amplified under its appropriate parameters.

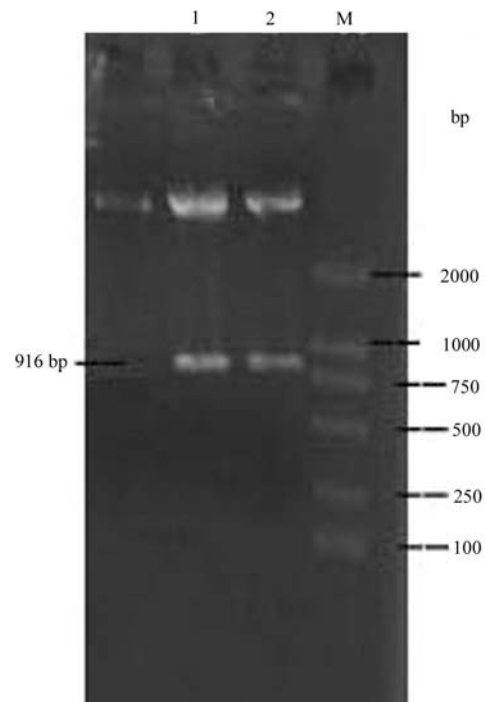


Figure 1. Electrophoresis of the recombinant after restriction endonuclease digestion. M, DL2000 marker; lanes 1 and 2, maspin.

RT-PCR products were analyzed by 2% agarose gel electrophoresis. Images were captured with Bio-Rad and analyzed with Quantity One Software. The entire experiments were repeated at least three times. Amplification of human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene transcripts was performed simultaneously to confirm RNA integrity, efficiency and for quantification of cDNA. All the primers of target genes for RT-PCR are listed in the Table I.

Western blot analysis. Western blot analyses were performed to detect the expression of maspin, uPA, VEGF-C and MMP7 protein after transfection. SGC7901 cells were collected by centrifugation and homogenized in 400 μ l extraction buffer

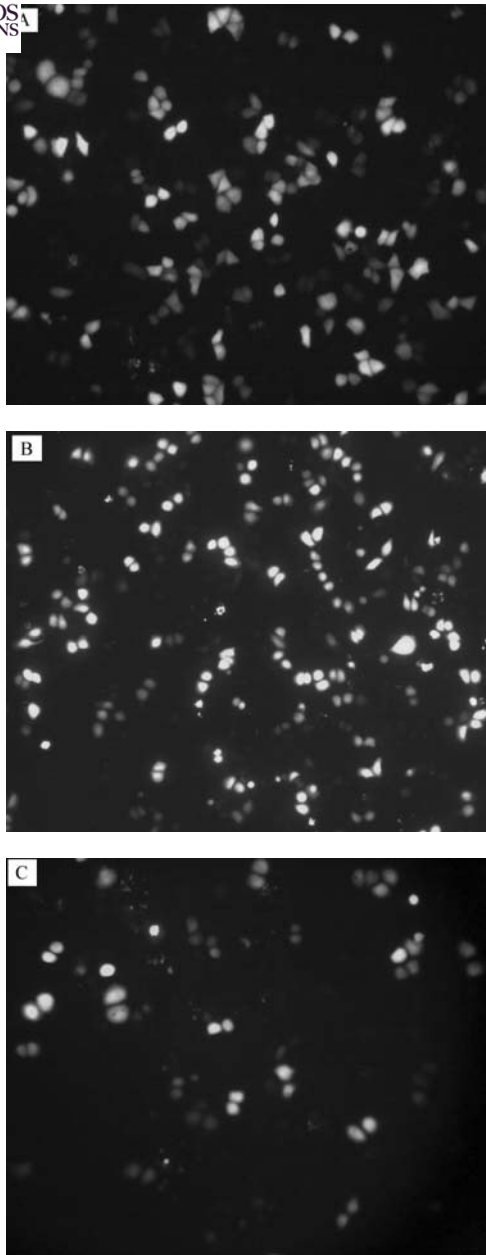


Figure 2. Phase contrast and GFP expression under fluorescence microscope. (A) 12 h; (B) 24 h; (C) 48 h (original magnification, x200).

PRO-PREP™ (SBS Genetech Co., Ltd., Beijing, China) at -20°C for 20 min. Equal amounts of proteins (according to Bradford assay) and molecular mass marker were denatured and subjected to a 10% SDS-polyacrylamide gel electrophoresis. Proteins were electrotransferred to $0.45\ \mu\text{m}$ nitrocellulose membranes (Millipore, Bedford, MA, USA) in 25 mM Tris-base, 190 mM glycine, and 20% methanol using a semi-dry blotter. Non-specific binding was blocked overnight with 20% non-fat milk and Tris-buffered saline, containing 0.01% Tween-20. The membranes were incubated with primary antibody at 4°C overnight, washed and incubated at room temperature for 1 h with appropriate horseradish peroxidase-conjugated secondary antibody (Biosynthesis Biotechnology Co., Ltd., Beijing, China), then detected by chemiluminescence (ECL; ChemiDoc XRS). Bands corres-

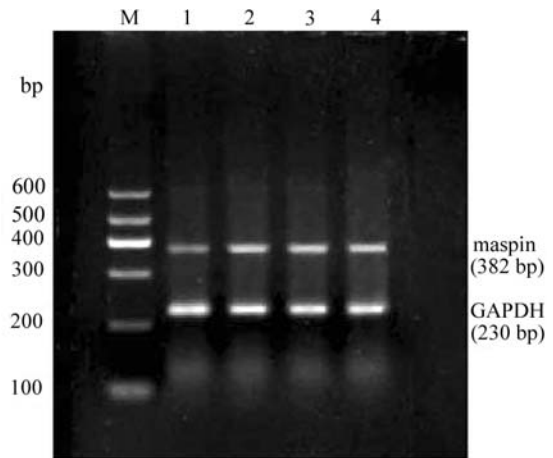


Figure 3. RT-PCR analysis of the maspin mRNA: maspin gene was cloned from the cDNA library with production of 382 bp by PCR. M, DNA marker; lane 1, transfection with pGenesil-maspin; lane 2, lipofectin group; lane 3, transfection with pGenesil-scramble; lane 4, transfection with pGenesil-1.1.

ponding to different proteins were scanned and the respective areas and optic density (OD) were determined by Quantity One 4.5.2. The relative densities were calculated by normalizing the OD of each blot with that of GAPDH. Primary antibodies used: anti-maspin, anti-uPA, anti-VEGF-C, anti-MMP7 and anti-GAPDH (Boster Biological Engineering Co., Ltd., Wuhan, China).

Invasion assays. For Transwell chamber-based invasion assays, equal amounts of cells were loaded into an insert provided with serum-free medium and allowed to pass through an $8\text{-}\mu\text{m}$ pore polycarbonate filter, which had been either pre-coated with $100\ \mu\text{g}$ of Matrigel (Becton-Dickinson, San Jose, CA) for invasion assay or left uncoated for motility assay. Medium supplemented with 10% fetal calf serum was added to the bottom chamber. Cells on the upper surface of filters were wiped out after 24 h, and those on the undersurface were stained with 1% amino toluene blue and counted under a microscope.

Statistical analyses. All data are expressed as mean \pm SEM. Statistical analysis of the data was performed using Student's t-test. $P < 0.05$ was considered significant.

Results

Identification of maspin shRNA expression vector. After restriction endonuclease *Sall* digestion, a small band of DNA (916 bp) was detected by electrophoresis (Fig. 1). DNA sequencing of the constructs (Invitrogen) showed that there were the same sequences as the target fragments.

Transfection efficiency. Forty-eight hours after transfection, 85% of SGC-7901 cells appeared high expression of EGFP, and it demonstrated that transfection by Lipofectamine 2000 was very effective (Fig. 2).

Effect of maspin shRNA on maspin expression. As shown in Figs. 3 and 4, maspin mRNA and protein was detected in the

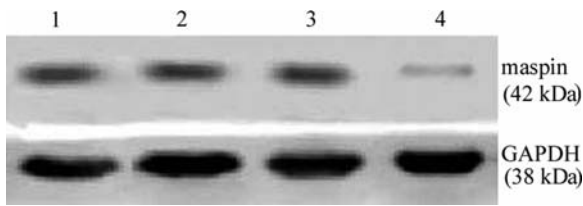


Figure 4. Western blot analysis of maspin protein expression level. Lane 1, Lipofectin group; lane 2, transfection with pGenesil-scramble; lane 3, transfection with pGenesil-1.1; lane 4, transfection with pGenesil-maspin.

Table II. Statistical analysis of maspin mRNA expression (n=3).

Group	OD ($\bar{x} \pm s$)	P-value		
Lipofectin	0.752 \pm 0.128			
pGenesil-scramble	0.745 \pm 0.123	>0.05 ^a		
pGenesil-1.1	0.738 \pm 0.116	>0.05 ^a	>0.05 ^b	
pGenesil-maspin	0.168 \pm 0.090	<0.05 ^a	<0.05 ^b	<0.05 ^c

^aP vs. Lipofectin; ^bP vs. pGenesil-scramble; ^cP vs. pGenesil-1.1.

Table III. Statistical analysis of maspin protein expression (n=3).

Group	OD ($\bar{x} \pm s$)	P-value		
Lipofectin	0.54 \pm 0.10			
pGenesil-scramble	0.58 \pm 0.15	>0.05 ^a		
pGenesil-1.1	0.57 \pm 0.13	>0.05 ^a	>0.05 ^b	
pGenesil-maspin	0.22 \pm 0.08	<0.05 ^a	<0.05 ^b	<0.05 ^c

^aP vs. Lipofectin; ^bP vs. pGenesil-scramble; ^cP vs. pGenesil-1.1.

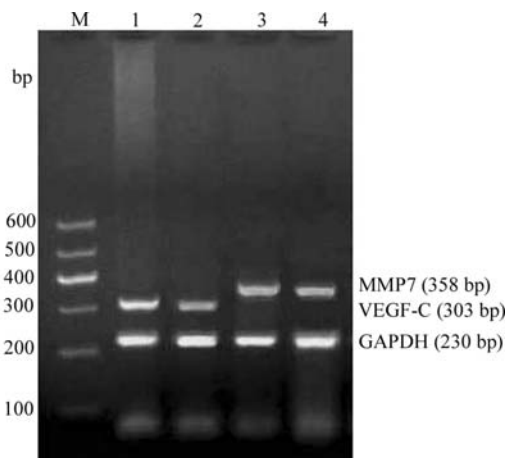


Figure 5. RT-PCR analysis of the VEGF-C, MMP7 mRNA expression. M, DNA marker; lane 1, VEGF-C expression in transfection with pGenesil-maspin; lane 2, VEGF-C expression in transfection with pGenesil-1.1; lane 3, MMP7 expression in transfection with pGenesil-maspin; lane 4, MMP7 expression in transfection with pGenesil-1.1.

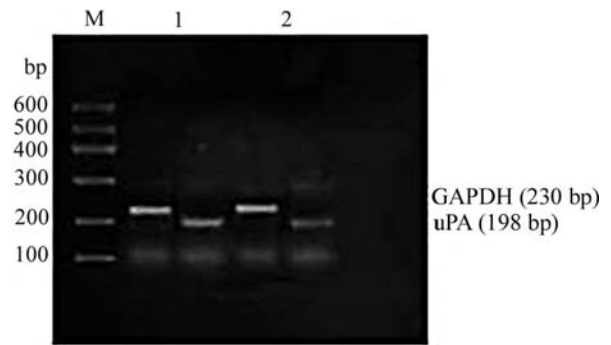


Figure 6. RT-PCR analysis of the uPA mRNA expression. M, DNA marker; lane 1, transfection with pGenesil-maspin; lane 2, uPA expression in transfection with pGenesil-1.1.

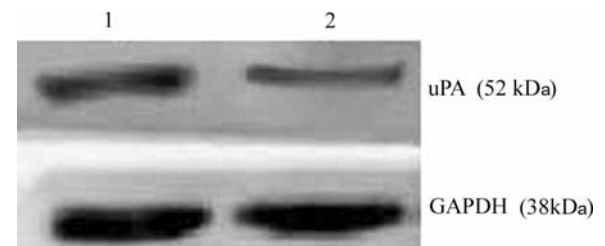


Figure 7. Western blot analysis of uPA protein expression. Lane 1, transfection with pGenesil-maspin; lane 2, transfection with pGenesil-1.1.

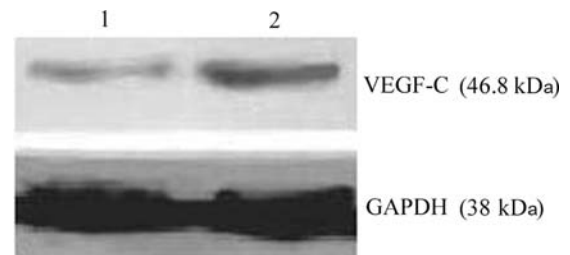


Figure 8. Western blot analysis of VEGF-C protein expression. Lane 1, transfection with pGenesil-1.1; lane 2, transfection with pGenesil-maspin.

SGC7901 cells. There was no difference among stable transfection of pGenesil-scramble, pGenesil-1.1 vector and Lipofectin ($P > 0.05$). However, maspin was significantly decreased in the stable maspin shRNA-transfected cells ($P < 0.01$). All of the data (OD) are shown in Tables II and III. These results indicated that the maspin shRNA expression vector used in this study was efficient in knockdown of the expression of maspin in gastric cancer cells SGC7901.

Effect of maspin shRNA on uPA, VEGF-C and MMP7 expression. Compare with transfection of pGenesil-1 vector, stable transfection of pGenesil-maspin showed higher expression of uPA and VEGF-C mRNA ($P < 0.05$), but there was no difference in MMP7 mRNA expression between them ($P > 0.05$). In addition, uPA, VEGF-C and MMP7 protein expression of pGenesil-maspin transfection were higher than those of pGenesil-1 vector transfection, and the difference

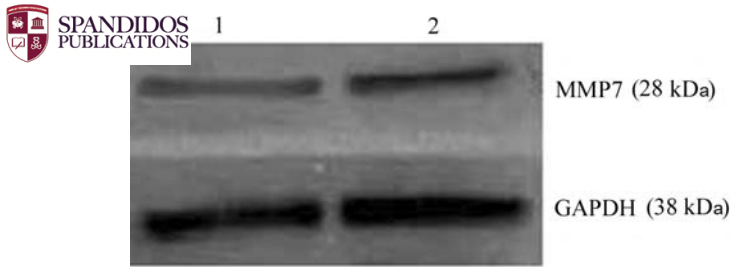


Figure 9. Western blot analysis of MMP7 protein expression. Lane 1, transfection with pGenesil-1.1; lane 2, transfection with pGenesil-maspin.

was significant ($P<0.05$) (Figs. 5-9). The data (OD) are shown in Tables IV and V.

Effect of maspin shRNA on cell invasion. In the invasion assay, the average number of migrated cells per field of pGenesil-maspin transfection was 86.8 ± 2.36 , which was much higher than that of the pGenesil-1 vector transfection

(161.80 ± 17.06). The difference between them was significant ($P<0.05$) (Fig. 10).

Discussion

RNA interfering (RNAi) is the process by which double-stranded RNA induces potent and specific inhibition of eukaryotic gene expression through the degradation of complementary messenger RNA, and is functionally similar to the processes of post-transcriptional gene silencing (19,20). In recent years, RNAi has been widely applied by researchers to the study of gene function and gene therapy in oncology because of its high specificity and apparent non-toxicity (21). Small hairpin RNA (shRNA) expression vector systems have been established to induce RNA interference (RNAi) in mammalian cells (22), and it was proved to provide long-lasting silencing and maximal inhibition of gene expression at low concentration (23). In the present study, a maspin shRNA expression vector was constructed and transfected into SGC7901 cells successfully, and the obvious inhibition of maspin expression was confirmed by RT-PCR and Western blot detection.

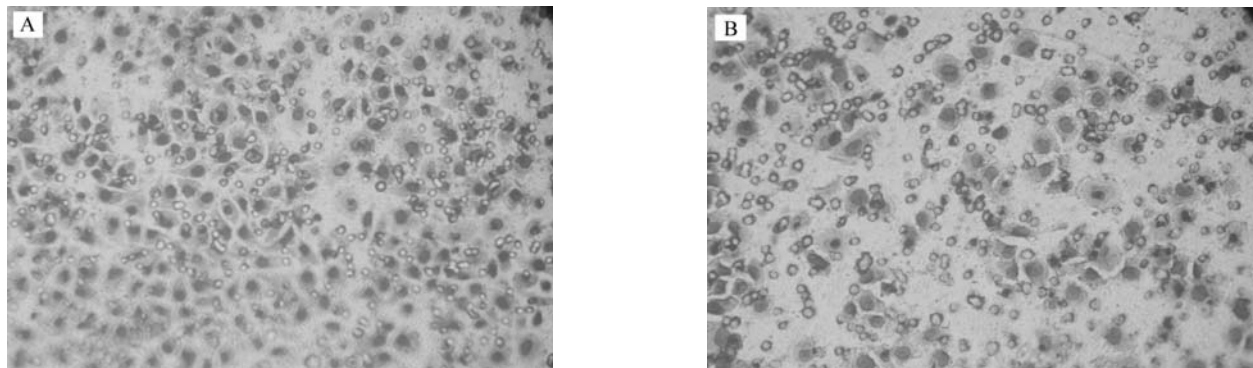


Figure 10. Invasion assays of gastric cancer SGC7901 (original magnification, $\times 200$). (A) Transfection with pGenesil-maspin; (B) transfection with pGenesil-1.1.

Table IV. Statistical analysis of uPA, VEGF-C, MMP7 mRNA expression (n=3).

Group	uPA	VEGF-C	MMP7
pGenesil-1.1	0.322 ± 0.134	0.427 ± 0.164	0.458 ± 0.137
pGenesil-maspin	0.576 ± 0.067^a	0.726 ± 0.126^b	0.437 ± 0.163^c

^a $P<0.05$ vs. pGenesil-1.1; ^b $P<0.05$ vs. pGenesil-1.1; ^c $P<0.05$ vs. pGenesil-1.1.

Table V. Statistical analysis of uPA, VEGF-C, MMP7 protein expression (n=3).

Group	uPA	VEGF-C	MMP7
pGenesil-1.1	0.364 ± 0.107	0.162 ± 0.079	0.211 ± 0.071
pGenesil-maspin	0.872 ± 0.156^a	0.475 ± 0.171^b	0.557 ± 0.124^c

^a $P<0.05$ vs. pGenesil-1.1; ^b $P<0.05$ vs. pGenesil-1.1; ^c $P<0.05$ vs. pGenesil-1.1.

Serpins comprise a large protein family with diverse biological functions. As a member of the serpin family, maspin has been grouped with the ov-serpin subfamily (Clade B), as maspin exhibits significant sequence similarity to chicken ovalbumin (31%) (1). There are currently 13 known human ov-serpins, all of them are known functional protease inhibitor (24). Protease inhibition occurs via the serpin reactive site loop (RSL), which is the primary functional domain of the serpin family and has proven necessary for some of maspin's tumor suppressive functions, including adhesion to the cellular matrix and maspin interactions at the cell surface. In an early study of maspin gene in breast carcinoma, Ngamkitidechakul *et al* (25) found that the RSL peptide alone (amino acids 330-345) is sufficient to induce adhesion of breast cancer MDA-MB-231 cells to fibronectin and that substitution of the ovalbumin RSL with the maspin RSL converted ovalbumin into an active protein able to stimulate adhesion of breast cancer cells to fibronectin.

The relationship between maspin and gastric carcinoma is controversial. Our study showed that maspin gene may be involved in the invasion of gastric carcinoma SGC7901. In invasive assays, the average number of SGC7901 cells which could pass through a filter pre-coated with Matrigel was increased after maspin silencing by RNAi, and as ECM alternatives, Matrigel contains IV collagen, laminin and other components of basement membrane. Crystal structure analysis of maspin has uncovered that the maspin G-helix was shown to adopt both a 'closed' and 'open' configuration. This conformational modification is unique to maspin and results in a reorganization of charged residues to expose a large negatively charged domain centered on the G-helix. The authors hypothesize that this domain might function in ligand binding other domains include a negatively charged region near the D-helix that could represent a possible collagen binding-domain (26).

We found that uPA mRNA and protein in SGC7901 cells were significantly increased after interference of maspin expression. Although maspin does not directly inhibit the activity of urokinase plasminogen activator (uPA), maspin was shown to reduce cell surface-associated urokinase plasminogen activator/urokinase plasminogen activator receptor (uPA/uPAR) by inducing its internalization (27,28). uPA/uPAR complex contributes to transformation from plasminogen to plasmin which can initiate a protease cascade that results in localized degradation of the ECM (such as fibronectin, fibrin and laminin) for the purpose of cell migration (29). Amir *et al* (30) reported that transfection of maspin gene in the highly invasive MDA-MB-231 breast cancer cells can inhibit hypoxia-induced invasion of metastatic cancer cells by blocking the expression of uPA/uPAR complex. In a more recent research (28), it was demonstrated that pro-uPA zymogen can not be activated just because of its combination with maspin.

High amounts of cytokines secreted by tumor cells can induce the new blood vessels and lymphatic vessels formation which are involved in invasion and metastasis of tumor. In an earlier study of the relationship between maspin and vascular endothelial growth factor (VEGF), which act as important chemoattractants during angiogenesis, it was demonstrated that maspin can act directly on the cultured

endothelial cells and prevent mitosis (31,32). However, some researchers found that maspin and VEGF showed no correlation in gastric carcinoma (33). VEGF-C, a member of VEGF family, is the first identified lymph-angiogenic growth factor, and by combination with VEGF-3, it can induce the proliferation and expansion of lymphatic vessels and the following lymphatic invasion and lymph node metastasis (34-36). Our study showed that VEGF-C mRNA and protein expression were increased with the decrease of maspin expression, at the same time the invasive ability of gastric carcinoma cells was enhanced. It suggested that mediated by VEGF-C, maspin can inhibit the invasion and metastasis of gastric carcinoma.

Matrix metalloproteinases (MMPs), a specialized group of enzymes capable of proteolytically degrading extracellular matrix proteins, have been postulated to play an important role in metastasis. MMP7, a member of MMPs, is expressed by tumor cells of epithelial and mesenchymal origin in esophagus, stomach, colorectum, liver, pancreas, lung, skin, breast, and endometrium, it may be used as a biological marker of an aggressive phenotype (37-42). Few studies demonstrated the relationship of maspin and MMP7. In this study, we found that MMP7 protein expression in recombinant plasmid transfection group was significantly increased, whereas there was no significant change in its mRNA expression. MMPs act both upstream and downstream of VEGF (43). An ovarian carcinoma study implied that VEGF could increase MMP7 secretion significantly and enhanced the activity of MMP7, while had no effect on the expression of its mRNA (44). In gastric carcinoma, maspin may also down-regulate MMP7 by inhibiting the expression of VEGF. Moreover, as pro-MMP was activated by uPA/uPAR complex (45), it can be speculated that maspin could down-regulate MMPs by inhibition of uPA.

Overall, maspin gene expression in human gastric cancer SGC7901 cells was inhibited by RNAi technology and the invasive ability of tumor cells was enhanced. It is suggested that maspin gene could inhibit invasion of gastric carcinoma SGC7901 cells and this inhibition maybe result from the interaction between maspin and uPA, MMP7, or VEGF-C.

Acknowledgements

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