

Effects of RNA silencing of matrix metalloproteinase-2 on the growth of esophageal carcinoma cells *in vivo*

YU-GUANG SHEN^{1*}, WEN FENG^{2*}, YI-JUN XU^{3*}, NA-NA JIAO⁴, DA-QIANG SUN³,
WEN-DONG QU⁴, QUAN TANG⁴, WEI XIONG⁴, YANG TANG⁴, YU XIA⁴, QING-YONG CAI⁴,
DA-XING LIU⁴, XUN ZHANG³, GANG XU⁴ and GUI-YOU LIANG⁴

¹Department of Thoracic and Cardiovascular Surgery, The First People's Hospital of Zunyi, Zunyi, Guizhou 563003;

²Department of Pathology, Henan Tumor Hospital of Zhengzhou University, Zhengzhou, Henan 450000;

³Thoracic Department, Tianjin Chest Hospital, Tianjin 300051;

⁴Department of Thoracic and Cardiovascular Surgery, Affiliated Hospital of Zunyi Medical College, Guizhou 563003, P.R. China

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Abstract. Esophageal carcinoma is one of the most common malignancies in China. Previous studies reported that matrix metalloproteinases (MMPs) have important roles in the progression and invasion of numerous types of solid tumors. Among the MMPs, MMP-2 has been closely associated with tumor growth and invasion. In the present study, a short hairpin RNA (shRNA) lentiviral expression vector targeting the MMP-2 gene was constructed in order to observe the inhibitory effect of MMP-2 gene silencing on the growth of the KYSE150 esophageal carcinoma cell line *in vivo*. Three small hairpin RNA sequences targeting MMP-2 were designed and cloned into lentiviral vectors. Following transfection of the lentiviral vectors into KTSE150 cells, MMP-2 mRNA and protein expression levels were examined by reverse transcription-quantitative polymerase chain reaction and western blotting, and the growth rate of cells was analyzed by MTT assays. Subsequently, tumor growth was assessed in nude mice. Lentivirus-mediated RNA interference effectively inhibited the expression of MMP-2 mRNA and protein in KYSE150 esophageal carcinoma cells, and suppressed the growth of esophageal carcinoma cells *in vivo*. The results of the present study suggested that lentivirus-mediated gene therapy targeting MMP-2 may be an attractive strategy for the treatment of esophageal

carcinoma and justifies the performance of further studies on the application of lentivirus vectors to cancer gene therapy.

Introduction

Esophageal carcinoma is one of the most common malignant cancers in China. As a result of extensive local cancer invasion, lymph node involvement and distant metastasis at the time of diagnosis, patients with esophageal carcinoma typically show rapid cancer progression and a poor prognosis (1). It has previously been demonstrated that the development of esophageal carcinoma is a complex, multi-step process involving a multitude of enzymes (2).

Human matrix metalloproteinases (MMPs) are a group of endopeptidases that degrade various components of the extracellular matrix (ECM) (3). MMPs have been shown to have important roles in tumor metastasis, invasion and angiogenesis (4). MMP-2, which is also known as type IV collagenase and gelatinase A, is a member of the MMP family that is located on the long arm of chromosome 16 (16q), is comprised of 13 exons and 12 introns, and has a molecular weight of 72 kDa (5). MMP-2 degrades type IV collagen within basement membranes, which are the primary barrier to cancer invasion (6). Previous studies have reported a role for MMP-2 in the invasion of pancreatic, ovarian and lung cancer (7-9). However, the function of MMP-2 in esophageal carcinoma remains uncertain.

RNA interference (RNAi) using small interfering (si)RNAs to inhibit the expression of specific genes is a powerful and promising technology for basic research and therapeutic intervention (10-13). Our previous study demonstrated that MMP-2 knockdown using synthesized oligonucleotides inhibited the invasion and migration of the KYSE150 esophageal carcinoma cell line *in vitro* (14). In the present study, lentiviral vectors targeting the MMP-2 gene were constructed and transfected into KYSE150 cells, in order to observe the inhibitory effect of MMP-2 silencing on the growth of esophageal carcinoma cells in nude mice. The present study aimed to further clarify the

Correspondence to: Professor Xun Zhang, Thoracic Department, Tianjin Chest Hospital, Xi'an Road, Tianjin 300051, P.R. China
E-mail: zhangxuntj@126.com

*Contributed equally

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Table I. Three hairpin DNA oligonucleotide sequences targeting matrix metalloproteinase-2 mRNA.

shRNAs	Sequences
shRNA-1	Sense: 5'-TGC GACAAGAAGTATGGCTTCTTTCAAG AGAAGAAGCCATACTTCTTGTGCGCTTTTTTC-3' Antisense: 5'-TCGAGAAAAAAGCGACAAGAAGTATGGCTT CTTCTCTTGAAAGAAGCCATACTTCTTGTGCGCA-3'
shRNA-2	Sense: 5'-TGGAGATACAATGAGGTGAAGATTC AAGAGATCTTCACCTCATTGTATCTCTTTTTTTC-3' Antisense: 5'-TCGAGAAAAAAGGAGATACAATGAGGT GAAGATCTCTTGAATCTTCACCTCATTGTATCTCCA-3'
shRNA-3	Sense: 5'-TGCAAACAGGACATTGTATTTGTTCAAGA GACAAATACAATGTCTGTTTGTCTTTTTTTC-3' Antisense: 5'-TCGAGAAAAAAGCAAACAGGACATTGTATT TGTCTCTTGAACAAATACAATGTCTGTTTGTCA-3'
Non-targeting control	Sense: 5'-TGTAGCGACTAAACACATCAATTCAAG AGATTGATGTGTTTAGTCGCATTCTTTTTTTC-3' Antisense: 5'-TCGAGAAAAAATAGCGACTAAACACATCAA TCTCTTGAATTGATGTGTTTAGTCGCATGCA-3'

shRNA, small hairpin RNA.

role of MMP-2 in esophageal carcinoma *in vivo* and to provide experimental evidence for pre-clinical gene therapy for esophageal carcinoma.

Materials and methods

Cell culture. The human embryonic kidney 293T packaging cell line and KYSE150 esophageal carcinoma cell line were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C in a humidified incubator containing 5% CO₂.

Lentivirus vector construction and transfection into KYSE150 cells. The lentiviral vector system is composed of four plasmids: The expression plasmid and three packaging vectors, including pMD2.g (VSVG), pRSV-REV and pMDLg/pRRE (Shanghai Telebio Biomedical, Co., Ltd., Shanghai, China). The human MMP-2 gene (Gen Bank ID: 4313, NM_001127891.1, NM_004530.4) interference sequence was obtained using small hairpin (sh)RNA analysis software (<http://www.invitrogen.com/rnai>), and a Basic Local Alignment Search Tool analysis of the NCBI database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) confirmed that it had no homology with other genes. Three self-complementary hairpin DNA oligonucleotides and a negative control targeting MMP-2 mRNA were synthesized. The sequences are shown in Table I. Subsequently, the DNA oligonucleotides were cloned into the lentiviral vectors and they were confirmed by DNA sequencing.

Lentiviral vectors and packaging vectors were transfected into 293T cells. Following transfection, the cells were cultured for 8 h, after which the culture medium was exchanged with

Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc.). After 48 h, the supernatant containing the retroviral particles was collected and then concentrated by centrifugation at 4,000 x g and 4°C. A total of 2x10⁵ KYSE150 cells/well were transduced with viral supernatants, and the transfection efficiency was detected directly by assessing the expression ratio of green fluorescent protein (GFP) by fluorescence microscopy. Stable cell lines were obtained after selection by culture in medium containing 100 µg/ml ampicillin for 18 days.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from cultured cells using the RNA purification kit (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany). DNase I was used to remove any contaminating DNA. Subsequently, RNA was reverse transcribed into cDNA. The primers for the PCR were designed using Primer Premier 3 software (Premier Biosoft International, Palo Alto, CA, USA) and were synthesized by Beijing SBS Genetech, Co., Ltd. (Beijing, China). PCR was performed using Quantitative RT-PCR ReadyMix (Sigma-Aldrich; Merck Millipore). The primers for MMP-2 were as follows: Forward, 5'-TCCAGAGGCAATGCAGTGGGG-3' and reverse, 5'-CAGCTCTCCTTGGGGCAGCCA-3'. Primers for β-actin were as follows: Forward, 5'-ACCACAGTCCATGCCATCAC-3' and reverse, 5'-TCCACCACCTGTTGCTGTGA-3'. The PCR conditions involved denaturing the DNA at 94°C for 3 min, followed by 30 cycles of amplification: 94°C for 30 sec, 55°C for 1 min, 72°C for 1 min and a final extension step at 72°C for 10 min. The data were analyzed using the 2^{-ΔΔC_q} method (15).

Western blotting. The cells were washed with cold PBS and lysed in pre-cooled radioimmunoprecipitation assay buffer (Pierce; Thermo Fisher Scientific, Inc.) containing proteinase

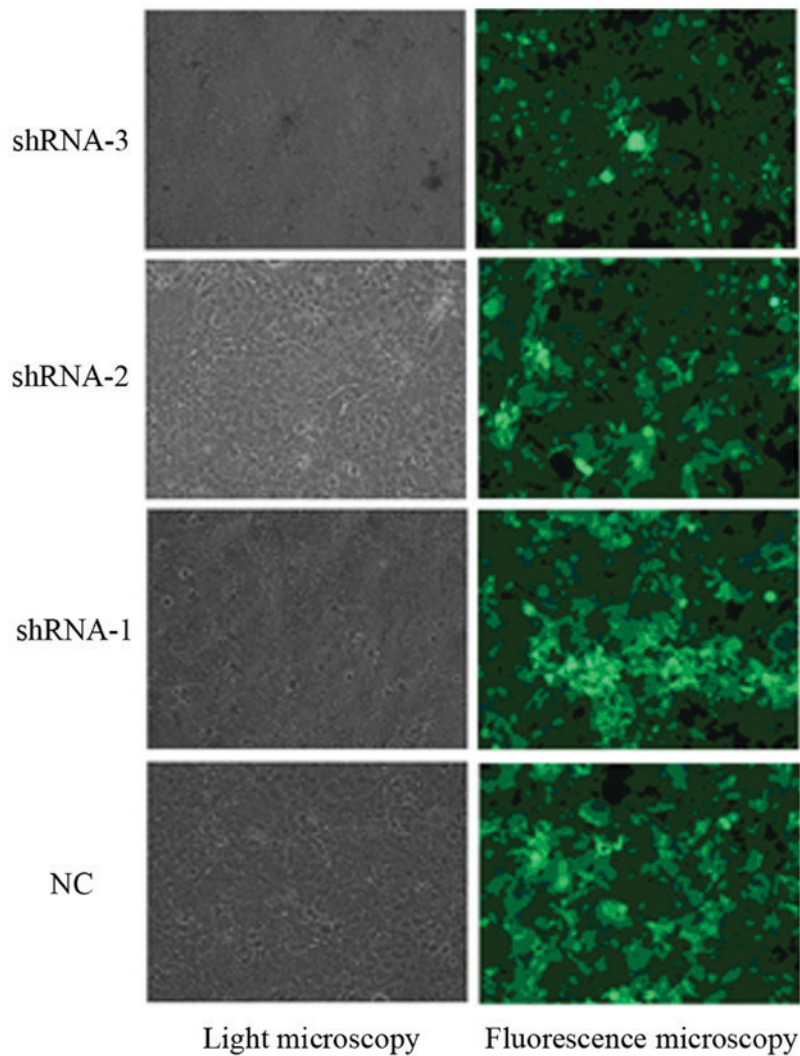


Figure 1. Micrographs of KYSE150 cells infected with recombinant lentiviruses. After being infected with the recombinant lentiviruses carrying the reporter GFP gene, the transfected cells expressed GFP proteins, as observed under a fluorescence microscope. The results indicated that 90% of KYSE150 cells were transfected with the recombinant lentiviruses. GFP, green fluorescent protein; shRNA, short hairpin RNA; NC, normal control.

inhibitors. The mixture was incubated for 30 min on ice, after which cell lysates were cleared of cell debris by centrifugation at $140,009 \times g$ for 5 min at 4°C . Protein concentrations were determined using the BCA protein assay kit (Pierce; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The samples were mixed in 59 loading buffer (Pierce; Thermo Fisher Scientific, Inc.), denatured at 96°C for 10 min and chilled on ice. Subsequently, equal amounts of protein ($60 \mu\text{g}$) were separated by 15% SDS-PAGE and blotted onto nitrocellulose membranes. The membranes were stained with Ponceau Red (Sigma-Aldrich; Merck Millipore) to verify that the proteins had been transferred. Subsequently, membranes were blocked with 5% nonfat milk and incubated with anti-MMP-2 (cat. no. HPA001939-100UL; 1:10,000 dilution; Sigma-Aldrich; Merck Millipore) and anti- β -actin (cat. no. CBL171; 1:10,000 dilution; Sigma-Aldrich; Merck Millipore) primary antibodies overnight at 4°C . After washing with PBS, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:5,000; cat. no., ZDR-5118; Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China) for 60 min at room temperature. Proteins

were detected and quantified using the enhanced chemiluminescence detection system (ChemiDoc XRS System; Bio-Rad Laboratories, Inc., Hercules, CA, USA) and expression levels were normalized to β -actin

MTT assays. The viability of KYSE150 cells transfected with MMP-2-lentivirus and control lentivirus vectors, as well as blank control cells, were measured using MTT assays. Briefly, KYSE150 cells were infected with lentivirus in 6-well plates and re-seeded into 96-well plates at a density of 2,000 cells per well. After 1, 2, 3 or 4 days, the cells were treated with $20 \mu\text{l}$ MTT solution (5 mg/ml) for 4 h, after which the cell supernatants were removed and $150 \mu\text{l}$ dimethyl sulfoxide was added to each well. After 15 min, the optical density (OD) of each well was measured using a microplate reader with an absorbance wavelength of 570 nm. All experiments were performed in triplicate.

In vivo experiments. A total of 24 female BALB/c nude mice (age, 6-8 weeks; weight, 18-22 g) were supplied by Beijing HFK Bioscience Co., Ltd. (Beijing, China). The mice were

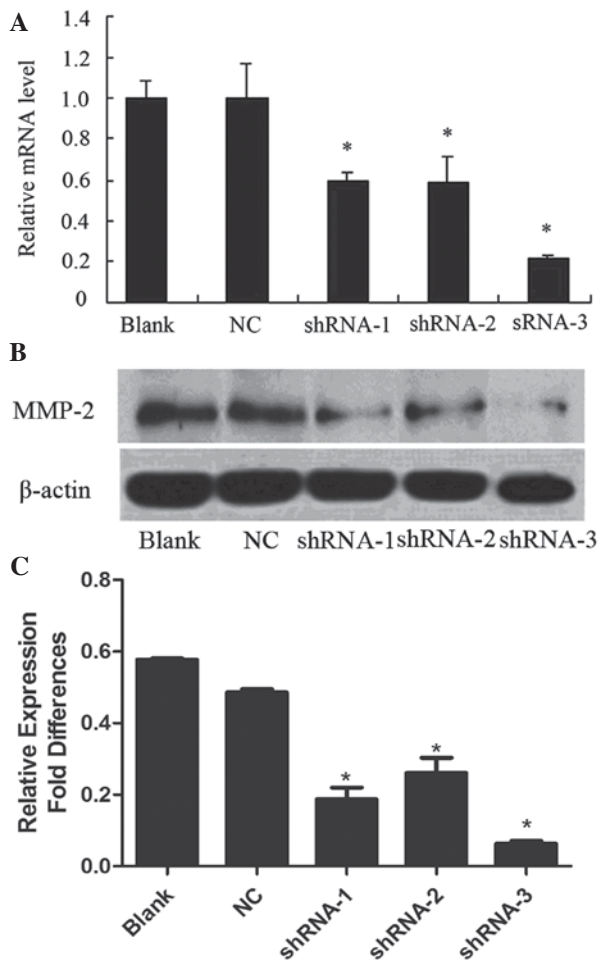


Figure 2. (A) Reverse transcription-quantitative polymerase chain reaction was used to determine the relative expression level of MMP-2 mRNA in five groups of KYSE150 cell lines. The mRNA expression levels of MMP-2 in the three groups transfected with MMP-2-specific shRNA were significantly downregulated ($P < 0.05$), as compared with the blank group, while the NC cells showed no obvious change. (B) Western blotting demonstrated the downregulation of MMP-2 protein expression by shRNA in the KYSE150 esophageal cancer cell line. (C) Quantification of western blots. * $P < 0.05$ vs. blank. MMP-2, matrix metalloproteinase-2; shRNA, short hairpin RNA; NC, normal control.

maintained under sterile conditions at 27°C, exposed to 10 h light/dark cycles and fed a sterilized mouse diet and water *ad libitum*. Non-transfected control KYSE150 cells (blank group), MMP-2-non-targeting shRNA control (NC group) and MMP-2-shRNA-2-transfected KYSE150 cells (2×10^6 cells in 0.1 ml) were injected subcutaneously into the axilla of each BALB/c nude mouse. Tumor size was measured every 2 days in two perpendicular dimensions using vernier calipers and tumor volume was calculated according to the following formula: Tumor volume (mm^3) = $1/2 \times (a \times b^2)$, where a and b refer to the longest and shortest dimensions, respectively.

Statistical analysis. All of the experimental data were analyzed using SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA). Data are expressed as the mean \pm standard deviation. Statistical significance between the different groups was determined using the Student's t-test. $P < 0.05$ was considered statistically significant.

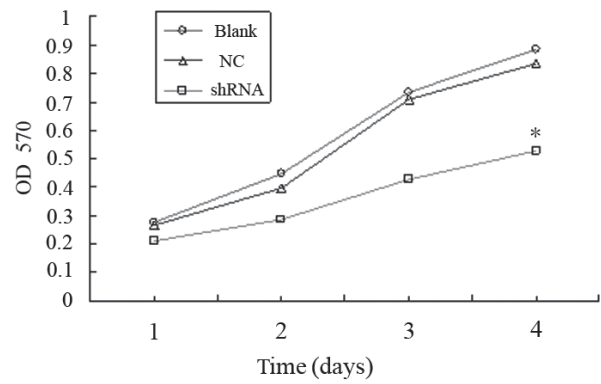


Figure 3. Viability of KYSE150 cell lines following lentivirus-mediated RNA interference of MMP-2, as assessed using MTT assays. KYSE-150 cells infected with lentiviruses carrying MMP-2-specific shRNA showed reduced cell viability, as compared with the cells in the blank and NC groups. * $P < 0.05$ vs. blank or NC group. MMP-2, matrix metalloproteinase-2; shRNA, short hairpin RNA; OD, optical density; NC, normal control.

Results

Transfection efficiency of lentiviral vectors in KYSE150 cells. The recombinant lentivirus vector was successfully constructed and confirmed by DNA sequencing. After being infected with the recombinant lentiviruses carrying the reporter GFP gene, the transfected KYSE150 cells were shown to express GFP, and the proportion of transfected cells was analyzed under a fluorescence microscope. The results indicated that 90% of KYSE150 cells were transfected with the recombinant lentiviruses (Fig. 1).

Lentivirus-mediated RNAi inhibits MMP-2 gene expression in KYSE150 cells. To evaluate the inhibition of MMP-2 mRNA expression, RT-qPCR was performed 72 h following transfection. The MMP-2 mRNA expression levels in shRNA-1-, shRNA-2- and shRNA-3-transfected KESY150 cells were reduced by 40.5, 41.4 and 78.9%, respectively, as compared with the non-transfected control (blank group) ($P < 0.05$; Fig. 2A). In addition, no significant difference was observed between the blank group and the MMP-2-non-targeting shRNA control (NC group) (Fig. 2A). The results indicated that the shRNA-3 oligonucleotide was the most effective for silencing of MMP-2 mRNA expression (Fig. 2A).

Lentivirus-mediated RNAi inhibits MMP-2 protein expression in KYSE150 cells. Western blotting demonstrated a significant reduction in MMP-2 protein expression in the KESY150 cells infected with shRNA-1 (0.187 ± 0.072 of β -actin), shRNA-2 (0.261 ± 0.095 of β -actin) and shRNA-3 (0.063 ± 0.016 of β -actin), as compared with a non-targeting control (0.486 ± 0.021 of β -actin) and blank control (0.577 ± 0.009 of β -actin) ($P < 0.05$; Fig. 2B and C). These results indicated that shRNA-1, shRNA-2 and shRNA-3 all blocked MMP-2 expression, although shRNA-3 showed the most effective inhibition of MMP-2 expression. No obvious inhibition of MMP-2 protein was observed in the non-transfected control and blank control cells.

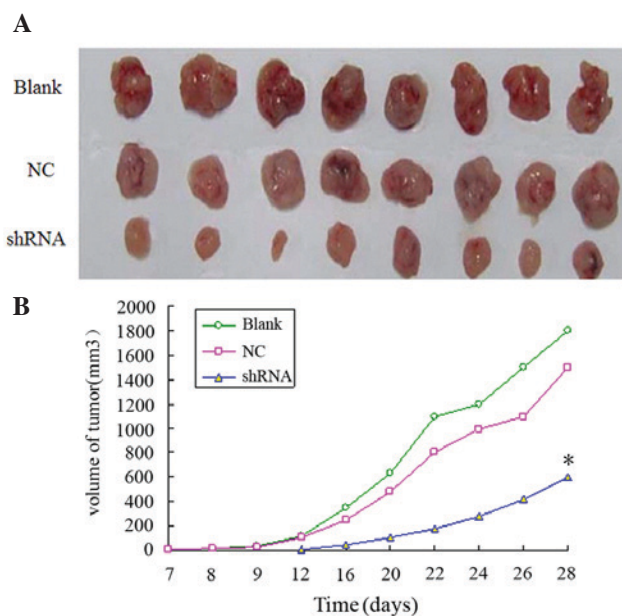


Figure 4. Lentivirus-mediated RNA interference targeting MMP-2 inhibits tumor growth *in vivo*. (A) Images of xenotransplant tumors. (B) The growth rates of MMP-2-knockdown nude mice tumors were significantly slower compared with those of blank control and NC groups (* $P < 0.05$). MMP-2, matrix metalloproteinase-2; NC, normal control.

Lentivirus-mediated RNAi against MMP-2 reduces KYSE150 cell viability in vitro. As the shRNA-3 oligonucleotide showed the most effective inhibition of MMP-2 expression, shRNA-3 was selected for MTT assays. As shown in Fig. 3, the viability of KYSE150 cells infected with lentivirus carrying shRNA-3 was significantly reduced, as compared with the NC and blank groups ($P < 0.05$). These results suggest that knockdown of MMP-2 reduces the viability of KYSE150 cells *in vitro*.

Lentivirus-mediated RNAi against MMP-2 inhibits KYSE150 cell tumorigenicity in vivo. To determine whether lentivirus-mediated RNAi against MMP-2 was able to inhibit tumor development *in vivo*, nude mice were injected with MMP-2-shRNA3-infected KYSE150 cells, and the volume of the tumor was measured at 2-day intervals. As compared with the blank and NC groups, the MMP-2-shRNA3-infected KYSE150 cells resulted in significantly smaller tumors in the nude mice ($P < 0.05$; Fig. 4), indicating that MMP-2 knockdown inhibited the tumorigenesis of esophageal cancer cells.

Discussion

Esophageal cancer was the fourth leading cause of cancer-associated mortality and the fifth most common diagnosed cancer in China in 2009, which was increased compared with the data from 2003-2007 (16). Although the results of surgery have improved significantly in recent years, the overall 5-year survival rates remain between 15 and 25% (17). Therefore, novel therapeutic strategies are urgently required for the treatment of this disease. Cancer metastasis is a multi-step process, and destruction or penetration of the basement membrane is thought to be critical for the successful metastasis of tumor

cells (18). Human MMPs are thought to have important roles in tumor metastasis, invasion and angiogenesis (18).

Previous studies have reported a role for MMP-2 in the progression and invasion of numerous types of cancers (19,20). In our previous study, it was demonstrated that MMP-2-knockdown using RNAi with synthesized oligonucleotides inhibited the invasion and migration of the KYSE150 esophageal carcinoma cell line *in vitro* (14). Furthermore, MMP-2 has exhibited a tumor-promoting function in many tumors *in vivo*, including ovarian (21), larynx (22) and brain (23) cancers. However, to the best of our knowledge, few studies have investigated the *in vivo* function of MMP-2 in esophageal cancer.

RNAi is a powerful means for post-transcriptional gene silencing and has been applied to a wide variety of eukaryotic organisms (24). Currently, chemically synthetic siRNAs are being evaluated for their use as highly-specific gene-silencing therapeutics, as well as their traditional role as an extremely powerful instrument for functional genomic analyses (25). However, there are several disadvantages associated with the use of synthesized siRNAs: i) The transduction of siRNA into cells usually leads to only transient silencing effects; ii) the transfection efficiency of siRNA may influence the silencing effects in target cells; and iii) transfected siRNA is expensive, as they must be chemically or enzymatically synthesized (26). To overcome these shortcomings, a stable RNAi DNA vector-based method has been developed (27-29). Approximately 70% of the vectors used in gene therapy clinical trials are represented by viral-based delivery systems (30). However, a number of failures with regard to gene therapy have been observed and thus, further optimization is required for the safe use of these vectors for clinical purposes in the future (31).

In the present study, a shRNA-lentiviral expression vector was used to obtain efficient knockdown of the MMP-2 gene in KYSE150 cells. The results showed that all shRNAs targeting MMP-2, including shRNA-1, shRNA-2 and shRNA-3, effectively inhibited the expression of MMP-2 mRNA and protein in KYSE150 cells, while the non-transfected and blank control groups showed no difference in MMP-2 expression. Notably, shRNA-3 was the most effective at suppressing MMP-2 expression and, therefore, it was selected for further investigation. Subsequently, it was demonstrated that MMP-2-shRNA-3 reduced the viability of KYSE150 esophageal carcinoma cells *in vitro* and tumorigenesis *in vivo*, as compared with the NC and blank control groups.

In conclusion, the results of the present study suggested that MMP-2 is a feasible RNAi target gene for esophageal carcinoma and that stable lentivirus-mediated shRNA targeting MMP-2 may be a promising and novel approach to the treatment of MMP-2-positive esophageal carcinoma. However, to further promote the application of this technique as a therapeutic approach, an effective and safe protocol should be developed.

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