

RESEARCH ARTICLE

MSP58 Knockdown Inhibits the Proliferation of Esophageal Squamous Cell Carcinoma in Vitro and in Vivo

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Abstract

Esophageal carcinoma (EC) is one of the most aggressive cancers with a poor prognosis. Understanding the molecular mechanisms underlying esophageal cancer progression is a high priority for improved EC diagnosis and prognosis. Recently, MSP58 was shown to behave as an oncogene in colorectal carcinomas and gliomas. However, little is known about its function in esophageal carcinomas. We therefore examined the effects of MSP58 knockdown on the growth of esophageal squamous cell carcinoma (ESCC) cells in vitro and in vivo in order to gain a better understanding of its potential as a tumor therapeutic target. We employed lentiviral-mediated small hairpin RNA (shRNA) to knock down the expression of MSP58 in the ESCC cell lines Eca-109 and EC9706 and demonstrated inhibition of ESCC cell proliferation and colony formation in vitro. Furthermore, flow cytometry and western blot analyses revealed that MSP58 depletion induced cell cycle arrest by regulating the expression of P21, CDK4 and cyclin D1. Notably, the downregulation of MSP58 significantly inhibited the growth of ESCC xenografts in nude mice. Our results suggest that MSP58 may play an important role in ESCC progression.

Keywords: MSP58 - esophageal squamous cell carcinoma - small hairpin RNA (shRNA)

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Introduction

Esophageal carcinoma (EC) is one of the most fatal cancers in the gastrointestinal tract of the digestive system. Epidemiological evidence reveals that, worldwide, it is the fifth most common cause of death from cancer in men and the eighth most common cause of death from cancer in women (Jemal et al., 2011). The highest incidence rate is found in developing countries, particularly in China (Pickens et al., 2003; Yu et al., 2010; Jemal et al., 2011). Esophageal squamous cell carcinoma (ESCC) is one of the most common pathologic types in EC (about 90%) (Stoner et al., 2001; Enzinger et al., 2003). Although great improvements have been made in the diagnosis, surgical treatment, chemotherapy and radiotherapy of esophageal cancer, the overall survival rate of esophageal cancer remains poor, with a 5-year survival of 15–34% (Allum et al., 2009; Sjoquist et al., 2011; Siegel et al., 2012; Xu et al., 2012). Therefore, it is extremely necessary to investigate the molecular mechanisms of the development of EC. Further studies, which aim to identify novel tumor-related genes and clarify of their roles in EC, will help to elucidate the mechanism of the initiation and progression of esophageal cancer.

MSP58 was first identified as a nuclear protein that interacts with the proliferation-related nucleus protein p120 (Ren et al., 1998). Additional studies showed that, in the nucleus, MSP58 could function to regulate transcription through its interactions with the transcription factors Daxx, STRA13, and the RNA-binding protein FMR (Lin et al., 2002; Ivanova et al., 2005; Davidovic et al., 2006). A study showed that TOJ3, the quail homologue of MSP58, displayed transformation activity in jun-transformed fibroblasts (Bader et al., 2001), whereas the tumor suppressor gene PTEN could suppress its transforming activity (Okumura et al., 2005). In addition, our previous studies demonstrated that MSP58 interacted with N-myc downstream regulated gene 2 (Ndr2) in the nucleus, which exerted important functions on cell differentiation and tumor proliferation (Zhang et al., 2007). Furthermore, we found that the expression of MSP58 was significantly up-regulated in high-grade glioblastoma and colorectal carcinoma tissues and that the over-expression of MSP58 was involved in tumor growth, metastasis, cell cycle control and invasion (Lin et al., 2009; Shi et al., 2009). However, neither MSP58 expression nor its function has been previously examined in ESCC.

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In this study, we established stable ESCC cell lines with MSP58 knockdown and evaluated their biological function. Our results demonstrated that specific down-regulation of MSP58 was sufficient to inhibit the growth of human ESCC cells in vitro and in vivo, indicating its important role in the development of ESCC.

Materials and Methods

Cell lines and culture conditions

The human ESCC cell lines Eca-109 and EC9706, which express high levels of MSP58 protein, were obtained from the Shanghai Institute of Cell Biology, the Chinese Academy of Sciences and cultured in RPMI 1640 (Invitrogen, USA) and supplemented with 10% fetal bovine serum (FBS, Sigma, USA). Human embryonic kidney HEK-293T cells were cultured in DMEM containing 10% FBS. Cells were cultured at 37°C in a humidified atmosphere containing 5% CO₂.

Lentiviral packaging and infection

Short hairpin RNA (shRNA) vectors targeting the human MSP58 gene (shMSP58) and scramble shRNA vectors were obtained from Sigma. The shRNA is complementary to a 21-base sequence (shMSP58-1: CCAAGCGTGTGAAGAAGAGTA, shMSP58-2: CCAGTGAGAAGAAGAAGGTAT). The lentivirus system included three plasmids (pLKO.1, psPAX2, and pMD2.G) and was obtained from Addgene (Cambridge, MA, USA). At being 60% confluence, HEK-293T cells were transfected with plko.1 shRNA (2 µg), psPAX2 (1.5 µg) and pMD2.G (0.5 µg) using Lipofectamine2000 (Invitrogen, USA), according to the manufacturer's instructions. After 12 hours of transfection, the media was changed to remove the transfection reagent. The cells were incubated at 37 °C in 5% CO₂ for 48 hours and the media was harvested from the cells and filtered through a 0.45 µm filter. The lentiviral particle solution was added to target cells that were approximately 70% confluent.

Then, the target cells were cultured for 48 h and then grown in media, which contained puromycin (1 mg/mL) (Invitrogen). The media was changed to fresh puromycin-containing media every other day as needed. After 3 weeks of culture, single colonies were picked up, expanded and maintained.

Real-time PCR

Total RNA was extracted using TRIzol reagent (Invitrogen) and cDNA was synthesized from 2 µg of RNA using reverse transcriptase (Promega, WI, USA). Real-time PCR was performed using the ABI 7500 system and the SYBR® Premix Ex Taq™ II Kit (Takara, Japan) according to the manufacturer's instructions.

The relative MSP58 mRNA levels were calculated using the 2^{-ΔΔCt} method and GAPDH was used as a reference gene. The primers of MSP58 were as follows: 5'-CCTCCTGCTCATAAATGCTGTG-3' and 5'-CTGGTTCGATCCCACTTTGCT-3'. The primers for GAPDH were as follows: 5'-GCACCGTCAAGGCTGAGAAC-3' and 5'-TGTTGAAGACGCCAGTGA-3'.

Western blot

Cells were washed twice with ice-cold PBS, harvested by scraping and lysed in RIPA buffer (50 mM Tris-HCl pH 7.4, 1% [v/v] Triton X-100, 1 mM EDTA, 1 mM leupeptin, 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF, 1 mM Na₃VO₄). Protein concentrations were determined using the BCA protein assay (Pierce, Rockford, IL, USA). Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. After blocking in 5% fat-free milk made in 0.1% Tween-20 in Tris-buffered saline (TBS), the membranes were incubated with primary antibodies (MSP58, Mouse, 1:500, Abnova, Taiwan; β-actin, Rabbit, 1:2000, Boster, Wu Han, China; P21, Cyclin D1, CDK2, cyclin E or CDK4, Mouse, 1:1000, Cell Signaling Technology, Danvers, MA) overnight at 4 °C. Next, the membranes were incubated with the corresponding secondary antibodies for 1 h at room temperature and enhanced chemiluminescence (ECL) detection solution was used.

Cell proliferation assay

Cell proliferation was measured by the MTT assay. Cells were plated at a density of 1×10⁴ cells/well in 96-well plates. After a 4-hour incubation with MTT (Sigma), at a final concentration of 0.5 mg/ml, the cells were lysed in 150 µl of DMSO (Sigma) for 10 min. The absorbance was determined at 490 nm using a multiscanner reader (TECAN-spectra mini Grodig, Austria).

Plate colony formation assay

For colony formation assays, 1×10³ cells were plated in 60-mm dishes containing 5ml of RPMI 1640 supplemented with 10% FBS. The dishes were incubated at 37 °C in a 5% CO₂ atmosphere. Medium were changed every 2-3 days. After 14 days, the resulting colonies were fixed with methanol at -20 °C for 5 min and stained with Giemsa (Sigma-Aldrich Co.) for 20 min. Only clearly visible colonies (diameter > 50 µm) were counted.

Cell cycle analysis

Flow cytometric analysis was carried out as described previously (Shi, Chen et al. 2009). Cells were seeded into 25-ml flasks in complete medium overnight. To synchronize the cells, cells were cultured in serum-free medium for 24 h and then cultured in complete medium for another 24 h. After washing with ice-cold PBS twice, cells were fixed in 70% alcohol overnight at 4 °C. The suspension was filtered through a 50µm nylon mesh and the DNA content of the stained nuclei was analyzed using a flow cytometer (EPICS XL; Coulter, Miami, FL, USA). The cell cycle was analyzed using Multicycle-DNA Cell Cycle Analyzed Software.

Tumorigenicity in nude mice

The tumorigenicity of nude mice was measured as described previously (Lin et al., 2009). For tumorigenicity assays, the nude mice (Shanghai Experimental Animal Center, Chinese Academy of Sciences, Shanghai, China) were separated randomly into two groups of five mice per group. The cells (5×10⁶) were inoculated subcutaneously into the right flank of the nude mice to establish xenografts.

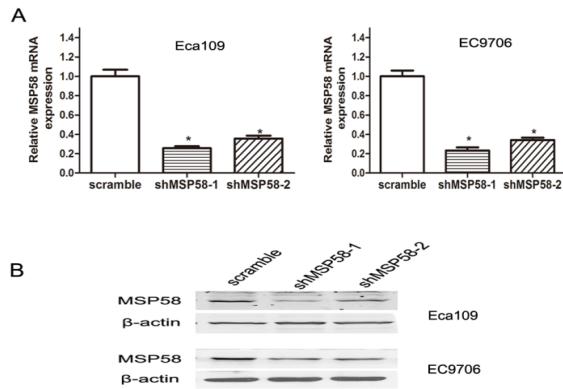


Figure 1. Lentiviral-Mediated shRNA Targeting MSP58 Effectively Knocked Down the Expression of MSP58 in the ESCC Cells at Both the mRNA and the Protein Level. (A) MSP58 mRNA levels were assessed by realtime-PCR in ESCC cells with MSP58 depletion. (B) MSP58 protein levels were assessed by western blot analysis. β -actin was used as a loading control. Values are expressed as mean \pm SD of three independent experiments. * $P < 0.05$, as compared with both control groups

Tumor volumes were measured every 3 days with a slide caliper and calculated using the formula: length \times width² / 2. Animals were sacrificed 28 days after the first inoculation. All animal studies were performed in accordance with the international guidelines for the care and treatment of laboratory animals.

Immunohistochemistry

Immunohistochemical staining was performed as described previously (Lin et al., 2009). Tumor tissues from nude mice were fixed with 10% formalin and embedded in paraffin. For immunohistochemistry, 5- μ m thick tissue sections were cut, dewaxed in xylene and rehydrated. For Ki67 staining, an anti-Ki67 monoclonal antibody (Neomarkers, Fremont, CA, USA) was used at a 1:100 dilution and incubated at 4 °C overnight. Next, the sections were incubated with an anti-mouse HRP conjugate IgG (1:500 dilution) for 1 hr at room temperature. After washing, sections were incubated with streptavidin-peroxidase, lightly counterstained with hematoxylin, and observed under a photomicroscope.

Statistical analysis

Each experiment was carried out in triplicate and the data were expressed as mean \pm SD. All statistics were performed with the SPSS 13.0 (SPSS company, IN). Comparisons among all groups were performed with a one-way ANOVA test. $P < 0.05$ was considered statistically significant.

Results

Stable knockdown of MSP58 expression by lentivirus-mediated shRNA in ESCC cells

To understand the role of MSP58 in ESCC cells, we constructed Eca-109 and EC9706 cell lines with stable knockdown of MSP58 expression by lentivirus-mediated shRNA (shMSP58-1 and shMSP58-2). MSP58 expression was detected in MSP58 knockdown cells by realtime-PCR

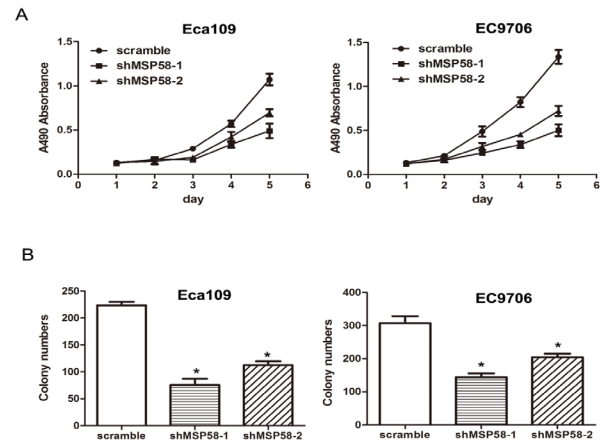


Figure 2. Down-Regulation of MSP58 Inhibited ESCC Cell Proliferation and Colony Formation in Vitro. (A) Cell growth curve. The growth of the ESCC cells was determined by the MTT assay for five consecutive days. * $P < 0.05$ compared with control cells. (B) Approximately 1×10^3 cells were cultured in a 60 mm plate. After 14 days, the cells were fixed and stained with Giemsa. Clone numbers are indicated as mean \pm SD of three independent experiments. * $P < 0.05$ when compared with control groups

and western blotting compared with the control cells (scramble). As shown in Figure 1A and B, the results demonstrated that shMSP58 inhibited MSP58 expression in Eca-109 and EC9706 cell lines effectively at both the mRNA and the protein level.

Down-regulation of MSP58 inhibited cell proliferation and colony formation of ESCC cells

The effect of shMSP58 on the proliferation of ESCC cells was evaluated by the MTT assay. The MTT assay showed that depletion of MSP58 expression caused a significant reduction in viability of both the Eca109 and EC9706 ESCC cell lines (Figure 2A). The inhibitory effect of shMSP58 on ESCC cell growth was also confirmed by the colony formation assay (Figure 2B). Collectively, our results demonstrate that down-regulation of MSP58 suppresses ESCC cell proliferation and colony formation in vitro.

Down-regulation of MSP58 induced cell cycle arrest in the ESCC cells

To further elucidate the mechanism of the inhibition of ESCC cells by the shMSP58, flow cytometry was used to study the effect of MSP58 expression on the cell cycle. Our results showed that 24 hours after release from synchronized culture, the cell cycle distribution of the ESCC cells was altered by MSP58 knockdown. As shown in Figure 3A, the MSP58 knockdown cells showed a significant increase in the percentage of cells in the G1/G0 phase compared with control cells.

To investigate the underlying molecular mechanism of the cell cycle arrest induced by MSP58 knockdown, the expression of cell cycle-related molecules was detected in the MSP58 depletion cells and the control cells. The expression of cyclin D1, cyclin E, CDK2, CDK4 and p21 were assessed by western blot analysis. Our data demonstrated that MSP58 knockdown inhibited the

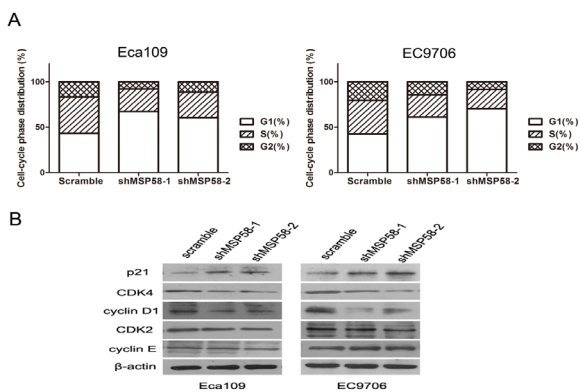


Figure 3. MSP58 Depletion Induced Cell Cycle Arrest and Regulated the Expression of Cell Cycle-Related Proteins. (A) Cell cycle distribution of the indicated cell types was analyzed by flow cytometry. (B) The expression of the p21, CDK4, cyclin D1, CDK2 and cyclin E in the Eca-109 and EC9706 cells with MSP58 depletion was examined by western blot analysis using the corresponding antibodies. β -actin was used as an internal control

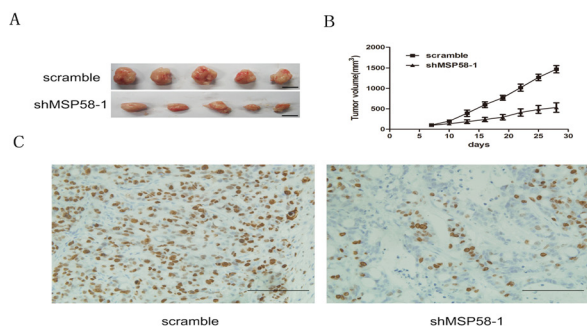


Figure 4. Down-Regulation of MSP58 Expression Significantly Inhibited the Growth of ESCC Cell Xenografts in Nude Mice. (A) Tumor specimens dissected from the nude mice xenografted with different ESCC cells. Scale bar, 1 cm. (B) Tumor growth curve. The data were generated from five mice in three different groups. * $P < 0.05$, as compared with control groups. (C) Immunohistochemical analysis of Ki67 antigen expression in the tumors of nude mice. Scale bar, 100 μ m

expression of cyclin D1 and CDK4 and up-regulated the expression of p21. However, the expression of CDK2 and cyclin E was not affected (Figure 3B). Therefore, the G1/S phase arrest induced by the knockdown of MSP58 in Eca-109 and EC9706 cells may be mediated by the down-regulation of cyclin D1 and CDK4 and the up-regulation of p21.

Tumorigenesis inhibition of MSP58 knockdown in ESCC cells

To test whether the knockdown of MSP58 affected the tumorigenesis of ESCC cells in vivo, we extended our study to evaluate the effect of MSP58-shRNA on xenograft growth. As shown in Figure 4A and B, the shMSP58-1 group led to a significant ($P < 0.05$) shrinkage of tumor development compared with the control groups. We also analyzed the expression of Ki67 antigen, a well-established proliferation marker, in different tumor tissues. As shown in Figure 4C, the Ki67 expression was significantly decreased in the shMSP58-1 group, which clearly demonstrated the proliferation inhibition of MSP58 knockdown. These results demonstrate that knockdown

of MSP58 in Eca-109 cells could inhibit tumor formation in vivo.

Discussion

In the present study, using lentiviral-mediated MSP58 shRNA, we found that the knockdown of MSP58 inhibited the proliferation and colony-forming ability of ESCC cells. In addition, the down-regulation of MSP58 inhibited the tumor formation of ESCC cells in vivo. Moreover, we demonstrated that MSP58 depletion induced cell cycle arrest by regulating the expression of P21, CDK4 and cyclin D1. Taken together, our results demonstrated that MSP58 is involved in esophageal carcinogenesis.

RNA interference (RNAi) is a widespread and powerful tool used for the functional regulation of gene expression, and it has been developed as a potential therapeutic strategy in various diseases, including cancers (Pai et al., 2006; Boudreau et al., 2012). However, the knockdown effect of regular synthesized siRNA only lasts for a short time and does not allow the stable inhibition of target gene function. At the present time, for efficient delivery and stable knockdown of target genes, lentiviral vectors are the most appealing tool. The lentiviral vectors for shRNA expression were used in our system. This approach allows for the stable suppression of target gene expression both in cell culture conditions and in animals (Sumimoto et al., 2007; Bos et al., 2009; Chumakov et al., 2010; Matrai et al., 2010).

Our results showed that MSP58 shRNA could effectively downregulate MSP58 expression by up to 80% in ESCC cells with great specificity, indicating that the lentiviral-based RNAi, because of its potency, could be utilized as an effective strategy for cancer therapy. Additionally, the blockage of proliferation in ESCC cells and the inhibition of tumorigenesis in nude mice support the effectiveness of this strategy.

Most anti-proliferative factors control cell growth by altering cell cycle progression (Hartwell et al., 1994; Hanahan et al., 2011). In our study, cell cycle analysis revealed that MSP58 down-regulation inhibited the G0/G1 to S phase transition and caused a G1/S arrest, which might partially explain the mechanism of how MSP58 knockdown suppresses ESCC cell proliferation. Most important, these results were consistent with our previous work in glioma cells (Lin et al., 2009; Shi et al., 2009).

Cell cycle progression is mainly modulated by two classes of proteins: the cyclins and their kinase partners, the cyclin-dependent kinases (CDKs) (Swanton, 2004; Howell et al., 2012). The G1 to S phase progression is regulated by cyclin-dependent kinase inhibitors (CDKI), such as p21, p27 and p57 (Xiong et al., 1993; Polyak et al., 1994; Matsuoka et al., 1995), and the cyclin D family (Ekholm et al., 2000). These CDKI proteins inhibit a broad range of CDKs by binding to several cyclin/CDK complexes, including cyclin D/CDK4 (or CDK6), cyclin E/CDK2 and cyclin A/CDK2. Of note, p21, a strong negative regulator of cell proliferation, inhibits CDK/cyclin activities and the overexpression of p21 causes G1 phase arrest (Xiong et al., 1993; Ferrandiz et al., 2012). Cyclin D1 was known to play an important role

in the G1/S checkpoint of the cell cycle. Overexpression of cyclin D1 in esophageal carcinoma has been reported and cyclin D1 is closely associated with cancer initiation and progression (Anayama et al., 2001). Our results showed that the knockdown of MSP58 significantly down-regulated cyclin D1 and CDK4, but up-regulated p21 in ESCC cells. Therefore, we conclude that MSP58 affected cell cycle progression, at least partly, by regulating the expression of cyclinD1, CDK4 and p21. The detailed mechanism of how MSP58 alter the expression of these proteins requires further study.

MSP58 is identified as an oncogene and is expressed at high levels in high-grade glioma samples and glioma cell lines. Knockdown of MSP58 inhibits proliferation and suppresses multiple tumorigenic characters of glioma cells (Lin et al., 2009). In colorectal carcinoma, MSP58 is also found to express more frequently than adjacent non-cancerous tissue. However, the role of MSP58 needs investigation in other types of cancer and normal cells (Shi et al., 2009). Next, we will evaluate the expression of MSP58 in ESCC samples and analyze the relation with clinicopathological features to further understand the function of MSP58 in ESCC.

In conclusion, our study demonstrated, for the first time, the important role of MSP58 in the proliferation of the ESCC cells. We present solid evidence that MSP58 knockdown inhibits esophageal squamous cancer cell growth by causing a cell cycle G1/S phase arrest. The modulation of cell cycle related proteins, cyclin D1, CDK4 and p21 is most likely one of the underlying mechanisms. Taken together, our results indicate that MSP58 plays an important role in tumorigenic of ESCC.

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