Knockdown of myosin VI by lentivirus-mediated short hairpin RNA suppresses proliferation of melanoma

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Abstract. Myosin VI has been reported to be associated with the progression of ovarian and prostate cancer. The aim of the present study was to reveal the role of myosin VI in the proliferation of melanoma. Briefly, lentivirus-mediated short hairpin RNA (shRNA) was designed specifically to silence myosin VI in A375 and A431 cell lines. Expression levels of myosin VI were then analyzed in the two cell lines by quantitative polymerase chain reaction and western blot analyses. Cell viability was assessed using MTT and colony formation assays. In addition, the cell cycle distribution was determined by flow cytometry. The results demonstrated that knockdown of myosin VI significantly suppressed melanoma cell viability and proliferation, and induced cell cycle arrest in G0/G1 phase. To the best of our knowledge, the present study was the first to assess the role of myosin VI in the growth of melanoma. Knowledge of the underlying mechanism of the role myosin VI in skin cancer cells may aid in the development of novel methods of melanoma diagnosis and therapy in the future.

Introduction

Cutaneous melanoma is the most common malignant neoplasm of human skin, with the number of cases increasing (1). An estimated 76,690 novel cases of melanoma occurred in the USA in 2013 (2). Conventional treatment is commonly performed through tumorectomy along with resection of lymph nodes in the neighboring area. Although the five-year survival rate was improved, it has remained as low as 70%

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in African Americans (3). Other treatments, including chemotherapy and radiotherapy, have been proved to be applicable and are widely adopted, but serious side-effects cannot be avoided (4,5). Therefore, a novel therapy against skin cancer with better prognostics is required.

Shiu *et al* (6) reported that the survival rate of pregnant patients and parous women was significantly low compared with that of nulliparous patients with stage II melanoma. This observation indicated that the effect of cell endocrine in melanoma is closely associated with exocytosis (7), which in turn is regulated by myosin VI. Myosin VI is an unconventional motor protein, which moves towards the minus end of actin filaments. It manipulates a wide range of cellular processes, including exocytosis, cell migration, cell division, endocytosis and cytokinesis (8,9). Myosin VI interacts with specific adaptor proteins and is regulated by multiple regulatory signals and modifications, including actin filament geometry, which determines the myosin VI binding sites (10), and disturbance or block of these cellular signaling pathways leads to the development of diseases, including deafness and cancer (8).

Several studies have found abnormal expression of myosin VI in ovary and prostate cancer; however, the underlying mechanism and the association and of myosin VI and human cancer remained poorly understood until Yoshida *et al* (11) discovered the overexpression of myosin IV in ovary carcinoma, but not in normal ovaries. Through inhibition of myosin VI expression, the spreading and migration of high-grade ovary carcinoma cells was impeded *in vitro*. Wei *et al* (12) found that the expression levels of myosin IV were elevated in the Golgi apparatus in prostate cancer, but not in normal cells. Furthermore, Dunn *et al* (13) impaired the migration and colony formation *in vitro* by silencing myosin VI in prostate cancer cells. All of these findings suggested that myosin VI is involved in tumor invasion and may be a diagnosis biomarker and therapeutic target of ovary and prostate cancer.

In spite of the studies available on prostate and ovary cancer, the specific effect of myosin VI on skin cancer cell viability has remained to be clearly demonstrated. In the present study, the role of myosin VI in melanoma was firstly studied. Specific knockdown of myosin VI was performed by RNA interference (RNAi), and its effect on the proliferation, colony formation and cell cycle of the melanoma cell lines

A375 and A431 was investigated. Knockdown of myosin VI may be a novel therapeutic method for melanoma in future, which may be the key to fundamentally cure cancer with little side-effects by regulating the tagged gene.

Materials and methods

Cell culture. The melanocytes (A375 and A431) and human embryonic kidney cells (293T) were obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). All cell lines were cultured with the Dulbecco's Modified Eagle's Medium (Hyclone, Logan, UT, USA) with 10% FBS (Gibco Life Technologies, Carlsbad, CA, USA) at 37°C in an incubator with humidified air and 5% CO_2.

Lentiviral vector design and construction. Two lentiviral vectors were designed to knock down the myosin VI gene (NM_004999) and to avoid the non-specific knockdown effect, respectively. The sequence of the shRNA designed to silence myosin VI was 5'-GTGAATCCAGAGATAAGTTTACTCGAG TAAACTTATCTCTGGATTCACTTTTT-3', and that of the control siRNA was 5'-TTCTCCGAACGTGTCACGT-3'. The stem-loop-stem oligos (shRNAs) were synthesized, annealed and ligated into the Nhe I/Pac I-linearized pFH-L vector (Shanghai Hollybio, Shanghai, China). After confirmation of the vectors by nucleotide sequencing, the successfully constructed vectors, packaged by pVSVG-I and pCMVΔR8.92 plasmids (Shanghai Hollybio), were transfected into 293T cells to avail larger quantities of usable lentivirus, which were subsequently harvested 72 h after transfection and then purified.

Lentivirus infection and gene knockdown. A375 and A431 cells ($5x10^4$ cells/well) were seeded in six-well plates and infected with MYO6 shRNA lentivirus (Lv-shMYO6) or control shRNA lentivirus (Lv-shCon) at a multiplicity of infection of 20 or 25, respectively, supplemented with poly-brene (Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Purification was conducted as previously described (14). A BX50 fluorescent microscope (Olympus Corporation, Tokyo, Japan) was then used to observe infection efficiency 96 h post-infection.

Quantitative polymerase chain reaction (qPCR). qPCR was performed on a Bio-Rad MyCycler Connect Real-Time PCR platform (Bio-Rad Laboratories, Hercules, CA, USA). Total RNA was extracted from the A375 and A431 cells with TRIzol® (Invitrogen Life Technologies, Carlsbad, CA, USA). The RNA (2 µg) was then reverse transcribed using an M-MLV Reverse Transcriptase kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. Moloy murine leukemia virus reverse transcriptase (cat no. M1705; Promega) was used as the reverse transcriptase in this method. The qPCR reaction system consisted of 2X SYBR premix ex taq (10 µl), forward and reverse primers (2.5 µM; 0.8 µl), cDNA (5 µl) and ddH$_2$O (4.2 µl). For actin, the forward primer was 5'-GTGGACATCCGCAAAGAC-3' and the reverse primer was 5'-AAAGGGTGTAACGCAACTA-3'. For myosin VI, the forward primer was 5'-AATCACTGGCTCACATGCAG-3' and the reverse primer was 5'-AATGCGAGGTTTGTGTCTCC-3'. All primers were obtained from 3D-HTS Company

(Shanghai, China). The qPCR program was as follows: Initial denaturation at 95°C for 1 min, 40 cycles of denaturation at 95°C for 5 sec and annealing extension at 60°C for 20 sec. The cycle threshold (Ct) normalized to that of actin was used to determine the relative expression of myosin VI using the $2^{-\Delta\Delta Ct}$ method.

Western blot analysis. Lentivirus-transduced cells were lysed in 2X SDS sample buffer (100 mM Tris-HCl (pH 6.8), 10 mM EDTA, 4% SDS and 10% glycine; Sigma-Aldrich, St. Louis, MO, USA). Proteins were separated by 10% SDS-PAGE (Sigma-Aldrich). In each lane of the gels, 30 µg of protein was added and electrophoresis was performed under 50 V for 3 h. The transmembrane procedure was performed under 300 mA for 1.5 h. Next, the membrane was incubated with rabbit monoclonal anti-MYO6 (1:500; cat. no. M0691; Sigma-Aldrich) and mouse polyclonal anti-GADPH (1:3,000; cat. no. 10494-1-AP; Proteintech Group, Inc., Chicago, IL, USA) primary antibodies at 4°C overnight, and horseradish peroxidase-conjugated goat anti-mouse/rabbit IgG secondary antibodies (cat. no. sc-2054; Santa Cruz Biotechnology, Inc.) at room temperature for 2 h. GAPDH protein was used as a loading control.

MTT assay. Following lentiviral infection, A375 ($2x10^3$ cells/well) and A431 ($3x10^3$ cells/well) were seeded into 96-well plates, respectively. The number of cells was measured at time-points indicated in the figures. 20 µl MTT solution (5 mg/ml) was added into each well. MTT solution was removed by aspiration following incubation, and 100 µl acidic isopropanol (10% SDS, 5% isopropanol and 0.01 mol/l HCl; Sigma-Aldrich) was added. The absorbance of each plate was measured at 595 nm using an ELx800 spectropho-tometer (BioTek Instruments, Inc., Winooski, VT, USA).

Colony formation assay. Following lentiviral infection, A375 cells were seeded into six-well plates at a concentra-tion of 600 cells/well and maintained at 37°C for seven days. The culture media were changed every 2-3 days. When the colonies were formed, the plate was washed and fixed with 4% paraformaldehyde (Sangon Biotech Co., Ltd., Shanghai, China), stained with crystal violet (Beyotime Institute of Biotechnology, Shanghai, China) for 20 min and washed three times with ddH$_2$O. The number of colonies (defined as >50 cells/colony) was counted.

Flow cytometric analysis. Following lentiviral infection, A375 cells were seeded into 6-cm dishes at a concentration of $5x10^4$ cells/dish. Cells were harvested following trypsin-ization, washed with phosphate-buffered saline (PBS) and fixed in 80% ethanol (Sigma-Aldrich) at -20°C for 24 h. Cells were then collected by centrifugation at 1,300 x g for 5 min, resuspended in the staining solution (100 µg/ml RNase A and 50 µg/ml propidium iodide in PBS), and incubated for 1 h at 37°C. The stained cells were subjected to flow cyto-metric analysis using a FACSCalibur II flow cytometer and Cell Quest Pro v. 3.3 (BD Biosciences, San Jose, CA, USA).

Statistical analysis. Values are expressed as the mean ± stan-dard deviation (SD) of at least three independent experiments
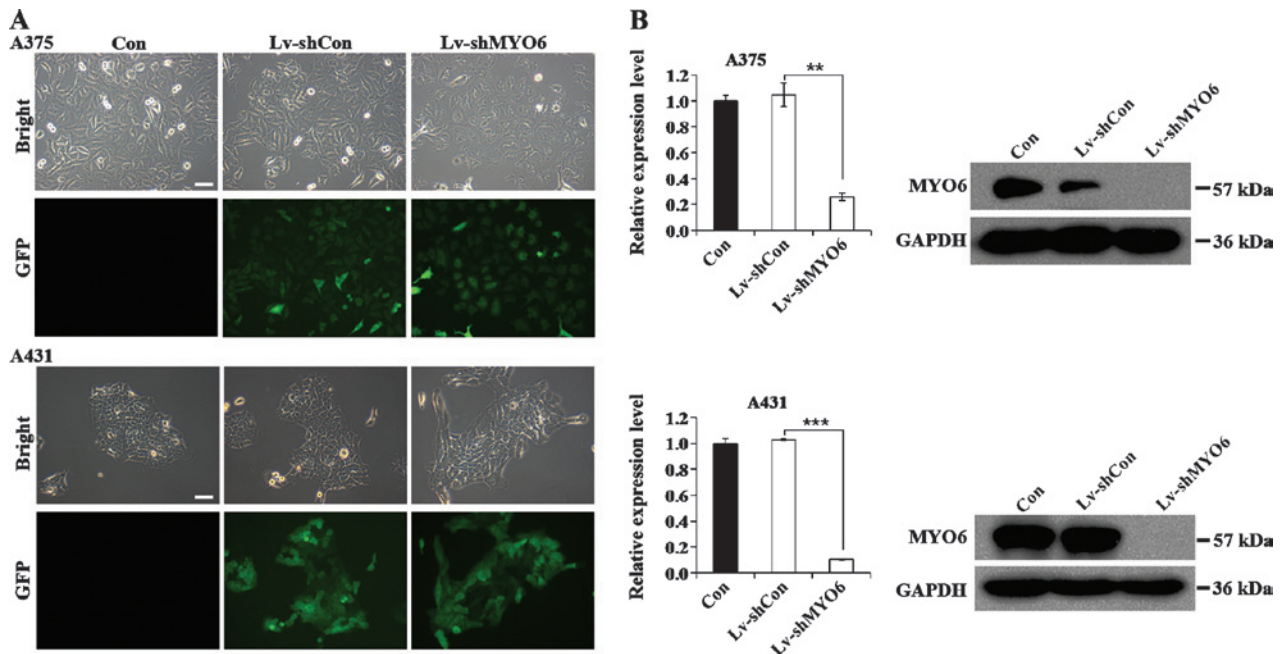
Figure 1. Lentivirus infection efficiency and knockdown efficiency in melanoma cells. Bright field and fluorescence photomicrographs after transduction of (A) A375 and A431 cells in Con, Lv-shCon and Lv-shMYO6 groups, respectively (scale bar, 100 μm). (B) Relative mRNA expression levels of myosin VI in A431 and A431 cells by small interfering RNA assessed using quantitative polymerase chain reaction (bar graphs) and protein levels assessed using western blot analysis (blots). The data are presented as the mean ± standard deviation of three independent experiments. **P<0.01, ***P<0.001, compared with Lv-shCon. Con, control; LV, lentivirus, GFP, green fluorescent protein; sh, small hairpin; myo, myosin.
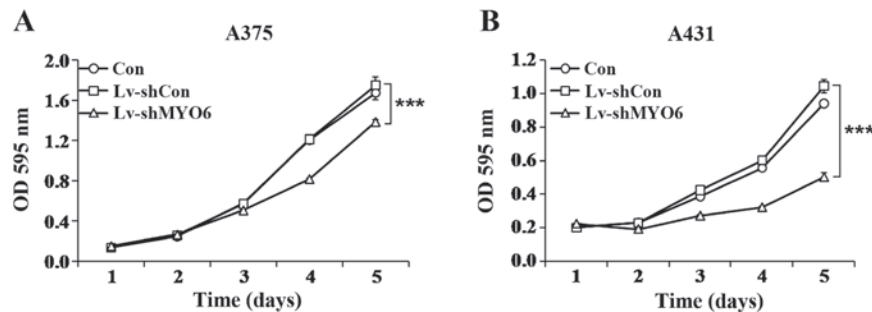

Figure 2. Viability of melanoma cells following myosin VI silencing. OD at 595 nm of (A) A375 and (B) A431 cells in Con, Lv-shCon and Lv-shMYO6 groups respectively. The data are presented as the mean ± standard deviation of three independent experiments. ***P<0.001, compared to Lv-shCon. OD, optical density; Con, control; LV, lentivirus, sh, small hairpin; myo, myosin.

performed in triplicate. Statistical significance was assessed with Student's t-test. All statistical analyses were performed using Prism 5 for Windows (GraphPad Software, Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference between values.

Results

Myosin VI knockdown by lentivirus-mediated RNAi in melanoma cells. A specific lentivirus-shRNA system (Lv-shMYO6) was designed to knockdown myosin VI, and was then used to investigate whether myosin VI expression was required for the growth and viability of human melanoma cells. In addition, a control shRNA (Lv-shCon) was constructed to avoid the non-specific gene-silencing effect of the lentivirus alone (15,16). Either of the two lentivirus systems was transfected into A375 and A431 cells. The efficiency of Lv-shMYO6 transfection was observed under

a fluorescent microscope, and >80% of cells expressed GFP, whereas the non-infected control group showed no green fluorescence at all (Fig. 1A), which suggested that the efficiency of lentiviral infection was stable and substantial.

qPCR and western blot analyses were performed to evaluate the knockdown efficiency five days after infection. There was no significant difference between the expression of myosin VI in the Lv-shCon group and that in the control (Con) group in the two cell lines. However, the expression of myosin VI was reduced by 75.0% in A375 cells (P<0.01; Fig. 1B, top) and 89.9% in A431 cells (P<0.001; Fig. 1B, bottom), respectively, compared to that in the Lv-shCon group. These results showed that the expression of myosin VI was effectively and specifically switched off by the lentivirus system in the two cell lines. Therefore, Lv-shMYO6 was able to be safely used as a downregulator to examine the role of the myosin VI gene in human skin cancer cells.
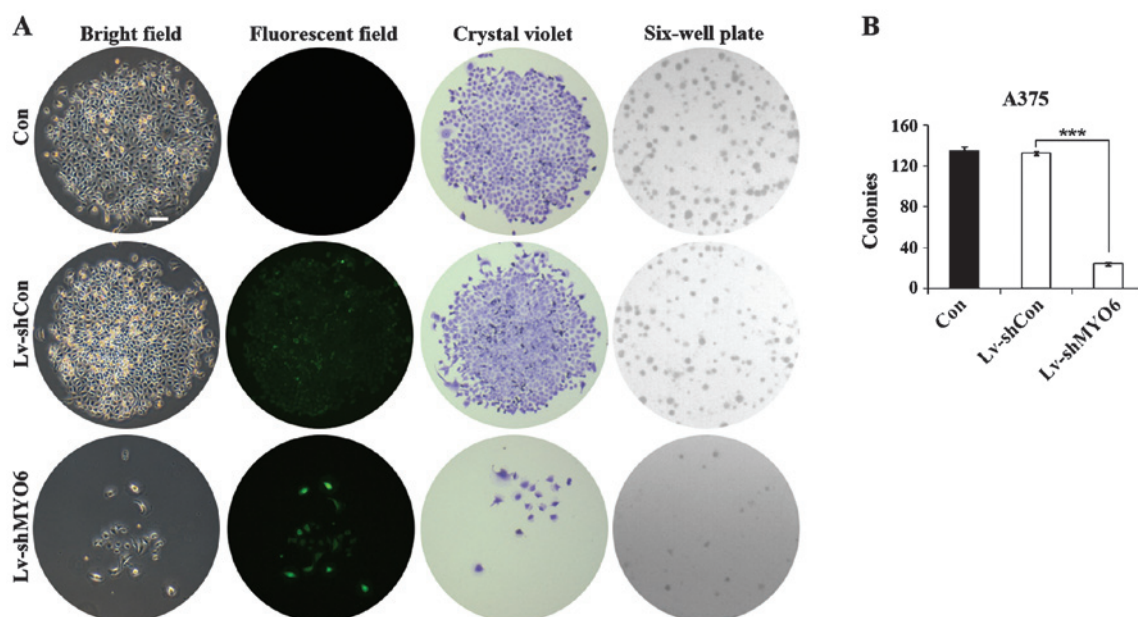
Figure 3. Colony formation ability of A375 cells following myosin VI silencing. (A) Colony formation of A375 cells in Con, Lv-shCon and Lv-shMYO6 groups. Cells were visualized by bright field or fluorescent field microscopy, following crystal violet staining or in a six-well plate, respectively (scale bar, 250 µm; magnification, x40). (B) Statistical evaluation of colony numbers in Con, Lv-shCon and Lv-shMYO6 groups. The data are presented as the mean ± standard deviation of three independent experiments. ***$P<0.001$, compared to Lv-shCon. Con, control; LV, lentivirus; sh, small hairpin; myo, myosin.
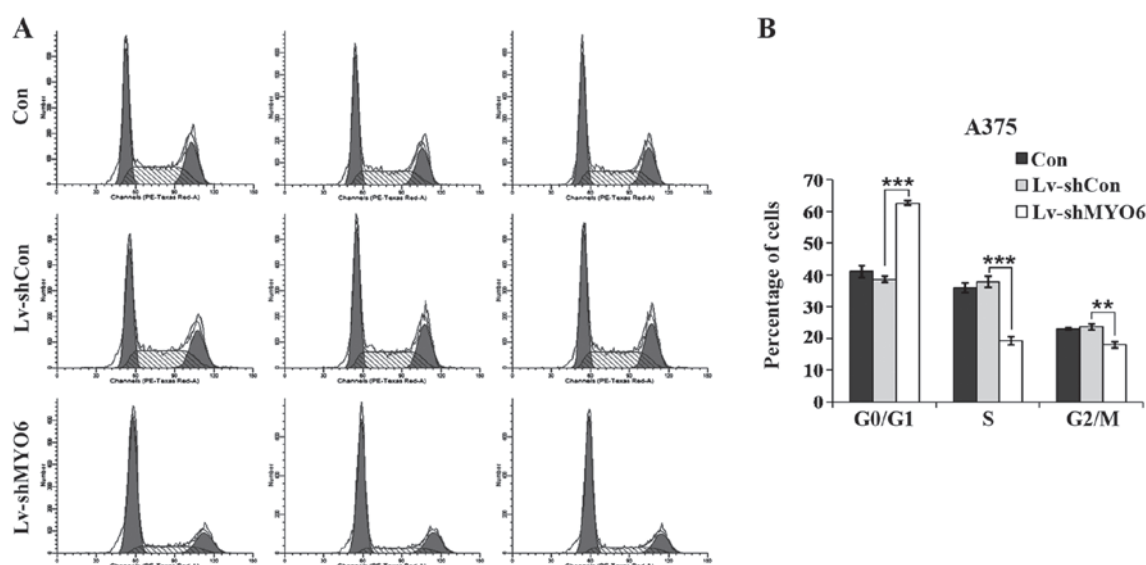

Figure 4. Myosin VI silencing caused cell cycle arrest. (A) Cell cycle analysis of A375 cells by flow cytometry in Con, Lv-shCon and Lv-shMYO6 groups respectively. (B) Quantification of percentage of cells at various stages of the cell cycle. The data are presented as the mean ± standard deviation of three independent experiments. **$P<0.01$, ***$P<0.001$, compared to Lv-shCon. Con, control; LV, lentivirus; sh, small hairpin; myo, myosin.

Knockdown of myosin VI decreases cell viability and growth. The populations of A375 and A431 cells in all groups were routinely observed *in vitro* through MTT assays at indicated time-points following infection (Fig. 2). The viability in the Lv-shMYO6 group was significantly decreased compared with that in the Con and Lv-shCon groups, whereas the Con and Lv-shCon exhibited similar viability. In more detail, on day four, the optical density (OD) value at 595 nm of A375 cells in the Lv-shMYO6 group (0.815±0.014) was lower than that in the Lv-shCon group (1.211±0.030; $P<0.001$) and the Con group (1.203±0.027; $P<0.001$). On day five, the OD value at 595 nm of A375 cells in the Lv-shMYO6 group (1.382±0.025) was markedly lower than that in the Lv-shCon group (1.747±0.084; $P<0.001$) and the Con group (1.672±0.065; $P<0.001$). The population gap between the Lv-shMYO6 and Con groups remained distinctive. The effect on the viability of A431 cells was more marked than that on the viability of A375 cells. The population of A431 cells in the Lv-shMYO6 group was 48.1% of that in the Con group on day five ($P<0.001$). Therefore, it is reasonable to conclude that lentivirus-mediated myosin VI silencing had a suppressive effect on the viability and proliferation of melanoma cells.

In addition, the proliferation of A375 cells was determined by colony formation assay. As shown in the bright field, fluorescent field and crystal violet-stained microscopy images in Fig. 3A, the size of a single colony in the Lv-shMYO6 group was significantly smaller compared with that in the Con and Lv-shCon groups. In addition, the number of colonies in the Lv-shMYO6 group was lower compared with that in the Con and Lv-shCon groups, as shown in the image of the six-well plate experiment. As shown in Fig. 3B, compared with that in the Lv-shCon group, the number of colonies was decreased by 82.0% in the Lv-shMYO6 group (P<0.001), while the colony numbers in the Con group had were similar to those in the Lv-shCon group. The significant decrease in the proliferation of the two cell lines indicated that knockdown of myosin VI impeded the growth of melanoma, which further suggested that myosin VI may be a potential therapeutic target of melanoma.

MYO6 siliencing causes G0/G1 phase arrest. The cell cycle distribution of A375 cells following myosin VI silencing was analyzed by flow cytometric analysis (17). As illustrated in Fig. 4A, myosin VI silencing significantly altered the cell cycle distribution, with the G2/M-phase population significantly decreased in the Lv-shMYO6 group in compared with that in the Con and Lv-shCon groups. Quantification of the cell cycle distributions (Fig. 4B) revealed that the S-phase population in the Lv-shMYO6 group (19.30±1.40%) was markedly decreased compared with that in the Lv-shCon group (37.74±1.76%; P<0.001). Furthermore, the G2/M-phase population in the Lv-shMYO6 group (18.11±0.95%) was decreased compared with that in the Lv-shCon group (23.63±0.96%; P<0.01). However, the G0/G1-phase population in A375 cells infected with Lv-shMYO6 (62.59±0.68%) was markedly increased compared with that in the Con (40.99±0.10%; P<0.001) and Lv-shCon (38.62±0.87%; P<0.001) groups. These results suggested that the impaired cell proliferation and colony formation may have been caused by cell cycle arrest in G0/G1 phase. Cells may have been hindered from entering S and G2/M phases, so that they were no longer able to divide, which led to a decrease in their growth.

Discussion

Melanoma is one of the most fatal types of cancer (1). In spite of the development of therapeutic protocols, the survival rate of colon cancer patients has remained low due to its high potential to form metastasis. As a large number of tumor-inducing mutations and differentially expressed genes have been identified in melanoma, it is important to identify novel molecular targets for the development of novel treatments and biomarkers (4).

Myosin VI is a major gene which demonstrates cancer-specific overexpression, and is closely associated with membrane trafficking, clathrin-mediated endocytosis, exocytosis at the Golgi complex and cell migration (18-20). Numerous studies have been performed to reveal the structure, function and regulatory mechanisms of myosin VI, yet the association between myosin VI and cancer was only appropriately emphasized at the beginning of this

century (21,22). It was found that myosin VI is overexpressed, particularly at late stages of ovary and prostate cancer, but not in normal tissue; therefore, myosin VI is a biomarker for ovary cancer and prostate cancer (11,12). A subsequent study showed that silencing myosin VI had an inhibiting effect on cell colony formation and migration of malignant cancer cells (13). These results inspired us to investigate whether knockdown of myosin VI exhibited any anti-melanoma effect. To the best our knowledge, the present study was the first to investigate the role of myosin VI in melanoma. Lentivirus-based shRNA expression systems were introduced to silence myosin VI in two different melanoma cell lines (A375 and A431) in order to investigate whether myosin VI is a potential therapeutic target of melanoma. The results indicated that the constructed lentivirus-mediated knockdown system was effective in silencing myosin VI at the gene transcription as well as the protein level. Following the specific knockdown of myosin VI, the viability of melanoma cell lines was significantly suppressed. These results suggested that myosin VI has an essential role in melanoma growth and migration. Further study of the cell cycle using flow cytometry was performed in order to investigate the underlying mechanism of the decrease of the cell proliferation rate following myosin VI knockdown. It was found that following myosin VI knockdown, the cell population in G0/G1 was increased. Simultaneously, the cell populations in S phase and G2/M phase were concomitantly reduced compared with those in the control groups. This implied that the cell cycle was arrested at the G0/G1 following myosin VI silencing. With a lower amount of cells entering S and G2/M phase, DNA replication and cell mitosis were impeded, which explains for the decreased cell proliferation and viability observed following myosin VI silencing.

In spite of the lack of extensive knowledge of the underlying mechanism and role of myosin VI in carcinogenesis as well as the anti-cancer effect of its knockdown, previous studies have provided three clues: Firstly, it has been reported that myosin VI interacted with GIPC1 to form a protein complex. GIPC1 regulates trophoblast glycoprotein, which is a prognostic factor in colorectal, ovarian and gastric cancer (23), tyrosinase-related protein 1, which affects melanocyte proliferation and melanocyte cell death (24), and T6BP, which is a binding partner of myosin VI involved in cell adhesion (25). Secondly, myosin VI binds to tumor suppressor gene DAB2, leading to the downregulation of DAB2 in ovarian carcinoma cell lines (26). Finally, myosin VI is a mediator of the p53-dependent cell survival pathway (27), while p53 is recognized as a cancer suppressor gene. These findings are a foundation for further study on the role of myosin VI in cancer.

In conclusion, the present study was the first to reveal that myosin VI is significantly correlated with melanoma. A specific myosin VI-targeting lentivirus system was then constructed to successfully knock down this gene. It was found that silencing myosin VI markedly inhibit colony formation and cell viability along with G0/G1 phase arrest. The results of the present study, supported by previous studies of myosin VI in ovary and prostate cancer (11-13), indicated that myosin VI is a prospective biomarker of various types of cancer and a promising therapeutic target for melanoma.

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