Abstract. Prostate cancer is the second most frequently diagnosed cancer among males around the world. Myosin VI (MYO6), as a motor protein, has been reported to be implicated in cancer-related cell migration and cellular functions. To investigate the role of MYO6 in prostate cancer, immunohistochemical analysis was firstly applied to prostate cancer tissues and revealed that MYO6 was closely related with the Gleason score in prostate cancer. Then we used specific short hairpin RNA (shRNA) to downregulate MYO6 expression in DU145 and PC-3 cells and found that decreased MYO6 expression significantly suppressed cell proliferation, as determined by MTT and colony formation assays. Flow cytometry confirmed that the suppression of MYO6 promoted cell cycle arrest at the G2/M and sub-G1 phase in the DU145 cells. Furthermore, PathScan intracellular signaling array analysis demonstrated that the phosphorylation of ERK1/2 and PRAS40 was downregulated in the DU145 cells following MYO6 knockdown. Knockdown of MYO6 downregulated the expression of AKT3 and upregulated the expression of PARP, as confirmed by western blot analysis. These results suggest that MYO6 plays an essential role in the progression of prostate cancer and silencing of MYO6 may be a promising therapeutic approach for prostate cancer.

Introduction

Prostate cancer is the second leading cause of cancer-related mortality in males, and the incidence has been rising rapidly worldwide, including the incidence in low-risk populations (1,2). The etiological factors for prostate cancer include genetic changes, sex hormones, diet and environment (3,4). Based on prostate cancer pathogenesis and characteristics, various advanced strategies have been applied in clinical practice. However, deleterious side effects frequently occur and make current strategies ineffective against stage T3 prostate cancer (5). Therefore, exploring effective management strategies and identifying therapeutic targets are urgently needed for the treatment of prostate cancer.

Myosins are motor proteins that move along cytoskeletal filaments by using energy derived from ATP (6,7). Myosins constitute a superfamily of more than 18 known members (8). Myosin VI (MYO6) is a member of the unconventional myosin protein, which moves towards the minus ends of polarized actin filaments in the opposite direction to all other myosins. Previous studies indicate that it can promote cancer-related cell migration and cellular functions (9-11). For instance, the in vitro migration and colony formation were impaired in LNCap human prostate cancer cells after MYO6 knockdown (12). The cell spreading and migration of high-grade ovarian carcinoma cells were impeded by knockdown of MYO6 (13). The overexpression of cancer-specific MYO6 has been shown primarily restricted in human prostate and breast cancers (12). In addition, MYO6 was shown to regulate protein secretion in prostate cancer cells (14).

To explore the relationship between MYO6 and prostate cancer, the association among MYO6 expression profiles with clinical and pathological features of prostate cancer was analyzed. Then, lentivirus-based shRNA was used to efficiently knock down the expression of MYO6 in prostate cancer DU145 and PU-3 cells. We aimed to investigate its possible function to impact growth in prostate cancer cells in vitro.

Materials and methods

Immunohistochemistry. A total of 148 cases of prostate cancer tissues used in this research were biopsy samples obtained from Fuzhou General Hospital of the Nanjing Military Command (Fuzhou, China). Immunohistochemistry was carried out as previously described (15) using Histostain-Plus 3rd Gen IHC...
Detection contained 2X SYBR Premix Ex Taq 10 µl, forward and reverse, AATGCGAGGTTTGTGTCTCC) and MYO6 Primers for Quantitative real-time PCR (qRT-PCR) analysis. Total RNA was centrifuged for 20 min at 4,000 x g at 4˚C. 45-µm filter and the viral concentrate was collected by filtration for 10 min at 4˚C). Then, the particles were filtered through a vector particles were harvested by centrifugation (4,000 x g, 15 h). Two days later, the supernatant was collected and the lentiviral groups shMYO6 (S1), shMYO6 (S2) and shCon. Forty-eight hours later, the supernatant was collected and the lentiviral shRNA encoding MY06 shRNA s1 and 5'-CCAGATTTAACCATTCCATACT CGAATTATGGATGTTAATCTGTTTTT-3' for MYO6 shRNA s1 and 5'-GTGAATCCAGAGATAAGTTTACT CGAATTCATGCAGAATATCTCGAGATATTCTTTCAA ACCTCCTCGTTTTTTT-3'. Three nucleotide sequences were cloned into the pFH-L lentiviral vector (Shanghai Hollybio, China), respectively. For lentivirus packaging, the HEK293T cells were transfected with pFH-L-MYO6 shRNA s1, pFH-L-MYO6 shRNA s2, or control shRNA with virion-packaging elements (pSVSVG-I and pCMVΔR8.92; Shanghai Hollybio) using Lipofectamine 2000 (Invitrogen) to generate three groups shMYO6 (S1), shMYO6 (S2) and shCon. Forty-eight hours later, the supernatant was collected and the lentiviral vector particles were harvested by centrifugation (4,000 x g, 10 min at 4˚C). Then, the particles were filtered through a 45-µm filter and the viral concentrate was collected by filter centrifuged for 20 min at 4,000 x g at 4˚C.

**Quantitative real-time PCR (qRT-PCR) analysis.** Total RNA was extracted from cells using TRIzol reagent ( Gibco-BRL). Primers for MYO6 (forward, AATCACTGGCTCAGATCGACG and reverse, AATGGCATGTGTTCTCCTC) and actin (forward, GTGGACATCCGCAAAAGAC and reverse, AAAGGTGTGTAACGCAACTA) were designed to evaluate mRNA expression of MYO6 on BioRad Connet Real-Time PCR platform (Bio-Rad, Hercules, CA, USA). Each 20 µl reaction contained 2X SYBR Premix Ex Taq 10 µl, forward and reverse primers (2.5 µM) 0.8 µl, cDNA5 µl, and dH2O 4.2 µl. The qPCR procedures were as followed: initial denaturation for 1 min at 95°C, denaturation for 5 sec at 95°C, and annealing for 40 cycles at 60°C. Relative expression of MYO6 mRNA was calculated by using the 2^-ΔΔCt method.

Western blot assay. Lentivirus-transduced cells were washed twice with ice-cold PBS and lysed in 2X SDS sample buffer (10 mM EDTA, 4% SDS, 10% glycerine in 100 mM Tris-HCl buffer, pH 6.8) at 4˚C. Cell proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). The membranes were blocked by 4% nonfat dry milk and incubated with mouse anti-MYO6 (1:1,000 dilution, cat #M0691; Sigma), rabbit anti-Akt3 (1:500 dilution, 21641-1-AP; Proteintech), rabbit anti-PARP (1:1,000 dilution, #9542; Cell Signaling Technology), rabbit anti-GAPDH (1:100,000 dilution, 10494-1-AP) and mouse anti-β-actin (1:2,000 dilution, 60008-1-lg) (both from Proteintech). After washing in PBS with 0.05% Tween-20, the membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse (1:5,000 dilution, SC-2005) and HRP-conjugated goat anti-rabbit (1:5,000 dilution, SC-2054) (both from Santa Cruz Biotechnology), respectively. MYO6 protein was visualized by enhanced chemiluminescence (ECL; Amersham) according to the manufacturer's protocol.

**MTT assay.** Tetrazolium (MTT) colorimetric assay was used to measure cell viability and activity. DU145 and PC-3 cells were plated at a concentration of 3,000 cells/dish and 2,000 cells/dish in 96-well plates. Afterwards, DU145 and PC-3 cells were infected by the lentivirus encoding MYO6 shRNA or the control shRNA for 96 h. Then, 20 µl MTT solution (stock solution 5 mg/ml PBS) was added to each well and incubated for 5 h at 37°C. The MTT-containing medium was then removed, and 150 µl DMSO was added to each tube. The optical density at 595 nm was detected using a microplate reader (Bio-Rad).

**Colony-forming capability analysis.** DU145 and PC-3 cells transfected with shMYO6 (S1) and shCon were grown in 6-well plates at a density of 500 cells/well and incubated for 8 and 9 days, respectively. The medium was replaced every 2-3 days until 8-9 days in culture of the DU145 and PC-3 cells, and then the cells were washed in PBS before being fixed in 4% paraformaldehyde for 10 min. After treatment, the cells were stained with crystal violet (0.5% crystal violet in 20% methanol) for 20 min. A fluorescence microscope (Zeiss) was used to detect cell colonies. The result showed that more than 50 cells were counted per colony.

**Analysis of the cell cycle distribution of prostate DU145 cells.** To investigate cell cycle distribution, flow cytometry of propidium iodide (PI) staining was carried out. After infection with shMYO6 (S1) or shCon for 5 days, the DU145 cells were then adjusted to a concentration of 2x10^5 cells/dish in 6-cm dishes and cultured for 40 h at 37°C. After being washed with ice-cold PBS, the cells were fixed with 1 ml of 70% cold alcohol and kept at 4°C for 20 min. The supernatant was discarded by centrifugation, resuspended in a mixture of 1 ml PI (10 µg/ml) and DNase-free RNase (20 µg/ml) and incubated for 20 min. The cell cycle progression was analyzed by flow cytometry (FACSCalibur; Becton Dickinson) after the sample was filtered through a 50-µm nylon mesh.

**Detection of intracellular signaling.** To simultaneously detect 18 vital and well-characterized signaling molecules,
the cell lysates were analyzed by PathScan® Intracellular Signaling Array kit according to the manufacturer's instructions. After infection with shMYO6 (S1) or shCon for 5 days, the DU145 cells were rinsed twice with ice-cold 1X PBS and immediately dissolved in 1X cell lysis buffer. Array Blocking Buffer was then added to each sample and blocked for 20 min. An equal volume of lysate was placed in each samples and incubated for 2 h at room temperature. Before incubation with HRP-linked streptavidin, each reaction was incubated with detective antibody mixture for 1 h at room temperature. The slides were exposed to film for 25 sec after being developed with LumiGLO/Peroxide reagent (Cell Signaling Technology).

Statistical analysis. Statistical analysis was carried out using SPSS 13.0 software. Each experiment was performed at least three times, and the results are presented as mean ± SD. Student's t-test was used to detect the significance of the differences (P<0.05) between the experimental and control groups.

Results

Expression of MYO6 in prostate cancer and normal prostate tissues. Immunohistochemistry was used to clarify the expression of MYO6 in prostate cancer. Representative images of four degrees of MYO6 expression intensity are shown in Fig. 1. The association between MYO6 expression and the clinicopathologic parameters are shown in Table I. Higher expression of MYO6 was found to be significantly related with Gleason score (P<0.01). However, there was no significantly difference between MYO6 expression and patient age.

Expression of MYO6 is significantly suppressed in prostate cancer cells after infection with shMYO6 (S1). To study the potential relationship between MYO6 levels and prostate cancer risk, the expression of MYO6 was knocked down in DU145 and PC-3 cells using lentiviral-mediated RNA interference. As shown in Fig. 2A, most of the cells presented GFP-positive signals suggesting satisfactory infection efficacy for shMYO6 (S1). Then the knockdown efficacy was further determined in the DU145 and PC-3 cells using qRT-PCR and western blot analysis. Levels of MYO6 mRNA were found to be much lower in the DU145 and PC-3 cells after infection with shMYO6 (S1) than that in the shCon-transfected cells (Fig. 2B and C, P<0.001). A further examination of MYO6 protein expression was performed in the DU145 and PC-3 cells following shMYO6 (S1) and shCon infection. Western blot analysis also showed that MYO6 protein was reduced following MYO6 knockdown (Fig. 2D and E). These results suggest that shMYO6 (S1) significantly downregulated MYO6 expression in the DU145 and PC-3 cells.

Knockdown of MYO6 by shMYO6 (S1) inhibits the proliferation of prostate cancer cells. An MTT assay was used to determine the effect of MYO6 knockdown on cell proliferation. As shown in Fig. 3A, the growth curve of the shMYO6 (S1)-treated cells started to decrease from day 2, compared with the shCon-
treated cells in the DU145 and PC-3 cells. The decline reached 83.3% (P<0.001) and 19.8% (P<0.001) on day 5 in the DU145 and PC-3 cells, respectively, compared with the shCon-treated cells. These data indicate that shMYO6 (S1)-mediated MYO6 knockdown obviously suppressed the proliferation of the DU145 and PC-3 cells.

Then, the long-term effect of MYO6 silencing on cell proliferation was determined by colony formation assay. As shown in Fig. 3B, there were fewer and smaller colonies in the shMYO6 (S1)-treated cells than those in the shCon-treated cells. Moreover, statistical analysis further confirmed that the number of colonies that formed in the cells was significantly decreased in the shMYO6 (S1)-treated cells (Fig. 3C, P<0.001). The results showed that MYO6 knockdown mediated by shMYO6 (S1) markedly inhibited the cell proliferation of the DU145 and PC-3 cells.

Knockdown of MYO6 by shMYO6 (S2) suppresses the proliferation of prostate cancer cells. The knockdown efficiency of MYO6 by the other recombinant lentivirus shMYO6 (S2) was determined in DU145 cells. After four days of infection, more than 90% of the DU145 cells strongly expressed GFP fluorescence (Fig. 4A). In addition, the mRNA and protein levels of MYO6 were significantly downregulated in the shMYO6 (S2)-treated DU145 cells (Fig. 4B). MTT assay showed that cell viability was significantly decreased in the shMYO6 (S2)-treated DU145 cells (P<0.001), compared with the cell viability of the shCon-treated cells (Fig. 4C).

Knockdown of MYO6 arrests DU145 cells at the G2/M phase and sub-G1 phase. In order to investigate the mechanisms underlying the growth suppressive effect of MYO6 knockdown, the cell cycle distribution of DU145 cells was analyzed using flow cytometric analysis (Fig. 5A). As shown in Fig. 5B, the percentage of cells in G0/G1 was significantly decreased whereas the percentage of cells in the G2/M phase was markedly increased in the shMYO6 (S1)-treated cells compared with those in the shCon-treated cells (P<0.001). Notably, more cells were accumulated in sub-G1 phase, representing early apoptosis in the shMYO6 (S1)-treated cells compared with the number of cells in the shCon-treated cells (Fig. 5C, P<0.001). These data suggest that MYO6 knockdown suppresses prostate cancer cell growth via blockade of cell cycle progression.

MYO6 knockdown inhibits ERK1/2, AKT3, PRAS40 and PARP activation. To further explore the molecular mechanisms underlying MYO6-mediated prostate cancer cell growth, PathScan® Intracellular Signaling Array kit was used to detect the modifications of signaling molecules in the shMYO6 (S1)-treated DU145. As shown in Fig. 6A, the phosphorylated levels of ERK1/2 (Thr202/Tyr204) and PRAS (Thr246) were downregulated in the shMYO6 (S1)-treated cells compared with levels in the shCon-treated cells. Moreover, the expression of AKT3, as a downstream effector molecule of ERK-1/2 and PRAS40, was slightly downregulated in the shMYO6 (S1)-treated cells. Apoptosis marker PARP presented higher expression in the shMYO6
These results indicate that MYO6 knockdown inhibited the growth of prostate cancer cells via blockade of ERK1/2, AKT3, PRAS40 and activation of PARP.

Discussion

Prostate cancer is one of the most heterogeneous cancers histologically and clinically (16). MYO6 is related to actin...
motor and participates in intracellular vesicle trafficking and transport (17,18). The present study aimed to explore a potential link between MYO6 and prostate cancer. Our results showed that higher expression of MYO6 was found to be significantly related with Gleason score, which indicates that MYO6 is associated with the development of prostate cancer. To further explore the biological function of MYO6 in prostate cancer, the expression of MYO6 was specifically knocked down in two prostate cancer cell lines DU145 and PC-3. Decreased MYO6 expression by two shRNAs both impaired cell proliferation and colony formation. Moreover, the DU145 cells were arrested at the G2/M and sub-G1 phases in response to MYO6.
knockdown. Wang et al previously also observed inhibited cell proliferation and impaired colony formation, as well as G2/M and sub-G1 phase arrest in breast cancer cells after MYO6 silencing (19).

To reveal the molecular mechanisms underlying MYO6-mediated prostate cancer cell proliferation, various signaling molecules involved in cell growth and survival in DU145 cells after MYO6 knockdown were investigated. ERK1/2 is a member of the mitogen-activated protein kinase superfamily, and its phosphorylation through the Ras-Raf-MEK-ERK (or ERK pathway) signaling network, can regulate cell motility, invasiveness, and apoptosis (20,21). The Ras-Raf-MEK-ERK pathway is frequently active in cancer through upstream signaling molecule activation to promote human tumor development (22,23). In the present study, the ERK1/2 phosphorylation was decreased in prostate cancer cells by MYO6 knockdown. This indicates that the ERK1/2 is a downstream target of MYO6 and the Ras-Raf-MEK-ERK pathway may be suppressed by knockdown of MYO6.

PRAS40 has been shown to be overexpressed in breast and lung cancer cells, indicating that it plays an important role in cancer growth (24). PRAS40 is also a critical downstream protein of the Akt3 signaling cascade and the elevation of PRAS40 phosphorylation facilitates melanoma tumor cell growth (25,26). A previous report also demonstrated that knockdown of PRAS40 and Akt3 protein levels corresponded to increased levels of cleaved caspase-3, which is a marker of apoptosis (25). PARP is one of the most used diagnostic tools for the detection of apoptosis in cells (27). As a specificity substrate, when PARP is cut by the cleavage of caspases, apoptosis will be induced. The present research found that more cells accumulated in the sub-G1 phase. Furthermore, western blot data revealed that PARP was activated and AKT3 was suppressed by knockdown of MYO6. This result suggested that Akt3 activity was decreased due to deregulated phosphorylation of PRAS40, which led to prostate cancer cell arrest at the G2/M phase.

In conclusion, we firstly identified that MYO6 plays an important role in prostate cancer cell growth. It would be important to confirm the oncogenic function of MYO6 in prostate cancer in vivo. Collectively, MYO6 could be considered as a potential therapeutic target for the treatment of prostate cancer.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (nos. 81272247 and 81372751).

References


