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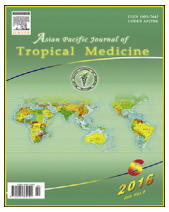

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# The expression of MYH9 in osteosarcoma and its effect on the migration and invasion abilities of tumor cell

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ABSTRACT

**Objective:** To determine the expression of non-muscle myosin heavy chain 9 (MYH9) in osteosarcoma and its effect on the migration and invasion abilities of tumor cell.

**Methods:** A total of 65 cases of osteosarcoma and 20 cases with benign osteochondroma who underwent resection operation in the Orthopaedics Department of our hospital from January 1st 2009 to January 1st 2015 were selected. Their mRNA levels of MYH9 were tested by qrt-PCR. Immunohistochemical method was used to examine the expression of MYH9 in osteosarcoma and the correlation between the positive expression of MYH9 and the clinicopathological features of patients was illustrated by statistical analysis. MYH9 was compounded artificially. The expression of MYH9 in SAOS2 osteosarcoma cells was decreased by siRNA. Scratch test was used to determine the change of SAOS2 cell migration ability after MYH9 silence. Transwell assay was employed to detect the change of cell invasion ability after MYH9 silence.

**Results:** The expression levels of mRNA of MYH9 and protein in osteosarcoma tissues were significantly higher than those in benign osteochondroma tissues. The high expression of MYH9 in osteosarcoma tissues was apparently related to the high Enneking classification (III classification) and lung metastasis. SiRNA of MYH9 could evidently decrease the expression level of MYH9 in SAOS2. The down-regulated expression of MYH9 could inhibit the migration and invasion abilities of SAOS2 cells.

**Conclusions:** MYH9 shows a trend of high expression in osteosarcoma tissues, and its high expression is associated with features such as tumor invasion and metastasis. The down-regulated MYH9 can realize an anti-tumor effect by inhibiting the migration and invasion of osteosarcoma cells.

## 1. Introduction

Osteosarcoma is a common malignant tumor in bone and cartilaginous tissues [1]. It is the second factor causing cancerous deaths among children and teenagers [2]. Although the application of new chemotherapy and radiation therapy has improved the five-year survival rate for patients, the fatality and disability rates caused by osteosarcoma migration still maintain at high levels [3]. Therefore, searching for a molecular therapeutic target for preventing osteosarcoma cells from migration becomes one of the important methods to break this "bottle neck" of the treatment of osteosarcoma.

Non-muscle myosin heavy chain 9 (MYH9) is located on human chromosome 22q22.1 [4]. Protein products expressed by it were called NMIA [Non-muscle myosin (IIA)] [5]. Previous researches have manifested that MYH9 has played an important regulatory role in diseases such as hereditary diseases [6], hematologic diseases [7–10] and inflammations [11]. Researchers have found in recent years that, to some degree, MYH9 can promote the migration and invasion of tumor cells. For example, Katono *et al* [12] found in non-

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small cell lung cancer that lung cancer tissue of positive expressed MYH9 possessed a higher ratio of intra-tumor vascular invasion and tumor node metastasis, and patients with positive expressed MYH9 showed poorer prognosis, which indicated that it was definitely worth exploring the value of MYH9 in the aspects of tumor prognosis and migrant. However, the expression of MYH9 in osteosarcoma tissues and its influences on the tumor migrant and invasion remain unknown yet.

In this study, the correlation between the expression state of MYH9 and clinical features and prognosis of tumor patients was illustrated by analyzing the expression condition of MYH9 in osteosarcoma tissues. SiRNA technology was employed for the silence of the expression level of MYH9 in SAOS2 cells *in vitro* and detecting the influence of down-regulated MYH9 expression on the invasion ability of osteosarcoma cells, so as to provide certain experiment basis to make MYH9 a prognosis evaluation indicator and a biological therapeutic target for patients with hepatocellular carcinoma.

## 2. Materials and methods

### 2.1. Materials

A total of 65 cases (45 males and 20 females) of osteosarcoma with benign osteochondroma who had been treated with resection operation in the Orthopaedics Department of our hospital from January 1st 2009 to January 1st 2015 were selected. There were 15 males and 20 females aging from 13 to 46 years. The median age was 33 years. At the same time, 20 cases of benign osteochondroma were selected, of which 15 cases were males and 5 were females aging from 17 to 44 years. The median age was 31 years. The surgical specimens drawn its materials from many parts and they were stored in 4% paraformaldehyde solution or liquid nitrogen. RNA extraction kit Fast200 was purchased from Shanghai Fastagen Biotechnology Co., Ltd.; qRT-PCR kit (SYBR Green Quant qRT-PCR kit, FP302) was bought from Beijing Tiangen Biotech Co., Ltd.; and MYH9 and β-actin commercial primers were from Beijing Aoke Biotech Co., Ltd. Human osteosarcoma cells SAOS2 were store by our laboratory research center. DMEM liquid cell cultivation medium was bought form Hyclone (USA). Fetal calf serum and penicillin streptomycin were form Gibco (USA); and lipidosome 2000 was from Invitrogen Corporation (USA). Specific siRNA od MYH9, negative control (NC) siRNA, rabbit-anti-human MYH9 polyclonal antibody (sc-47201) and mouse anti human β-actin monoclonal antibody (sc-47778) were purchased from Santa Cruz (USA). Transwell essay were from Corning Corporation (USA) and matrigel was gotten from BD Corporation (USA).

### 2.2. Methods

#### 2.2.1. qRT-PCR

According to the instruction of Fast200 reagent, tissues and the total RNA of cells were extracted. In accordance with the instruction, 20 μL reverse transcription PCR system were prepared at first and incubated at 37 °C for 60 min to synthesize cDNA. Then, 2 μL cDNA was taken as a template to prepare 20 μL real-time PCR reaction system. Bio-Rad Real-time PCR instrument was used for the reaction. β-actin was served as a reference object and the relative expression quantity of MYH9 mRNA was counted with the $2^{-\triangle\triangle Ct}$ method.

#### 2.2.2. Immuno-histochemical staining

Paraffin-embedded tissue sections were given antibody thermal remediation, inhibited the activity of endogenous peroxidase by hydrogen peroxide and then closed by goat serum after dewaxing and hydration. 1:100 rabbit-anti-human MYH9 compounded on the basis of 1 × PBS solution was employed to cover those sections; they were reacted overnight at 4 °C and then the residual non-combined primary antibody were eliminated. HRP-second antibody was used to combine the specific primary antibody and DAB was applied for coloration. Ten visions of every section chosen randomly under the ×400 amplification were scored in accordance with the standards described previously [13]. It was defined as the positive expression of MYH9 when the score was no less than one.

#### 2.2.3. SiRNA transfection

Before conducting the experiment, SAOS2 cells were cultivated and went down to 2–3 generations on 1 × DMEM medium with 10% FBS. SAOS2 cells were incubated in 6-well culture plates and cultured by a complete medium. After the confluence reached 50%–60%, the culture plates were poured out. PBS solution was used to wash cells adequately. The transfection groups included the MYH9 group that 200 pmol DMEM-made MYH9 siRNA and 5 μL Lipofectamine™ 2000 were added in each well and the NC group that 200 pmol DMEM-made NC siRNA and 5 μL Lipofectamine™ 2000 were added in each well. The final culture volume was 2 mL. After cultivated in a suitable incubator for 6 h, they were cultured continuously with another culture medium.

#### 2.2.4. Western blot

RIPA was used to dissociate cells for 10 min at 4 °C. The dissociation solution was centrifuged for 10 min at 4 °C with a centrifuged speed of 14000 r/min. BCA assay was applied to determine the total protein concentration of the supernate. After that, 50 μg protein samples were added in 10% SDS-PAGE gel of every well and the protein was isolated by vertical electrophoresis. BIO-RAD wet transfer system was used to transfer membrane for 80 min with 250 mA. And then they were closed at room temperature with 5% skim milk for 2 h. The expressions of corresponding proteins were detected by 1:1000 diluted MYH9 and β-actin primary antibodies, respectively; 0.01M TBST was used to wash for 5 min three times. HRP-goat anti-rabbit second antibody diluted with a ratio of 1:5000 was combined with target primary antibody and they were incubated 1 h at 37 °C. Also, 0.01M TBST was used to wash three times with 5 min once. ECL solution was dropped on the membrane in a dark room, and the protein content was examined by BIO-RAD image system.

#### 2.2.5. Scratch test

After transfected for 24 h, SAOS2 cells were cultivated in 6-well plates overnight till the fusion degree reached over 90%. The original medium was sucked out and cells were washed by PBS solution twice. Cell scratches were made by a 100 uL axygen and then those residual cells were washed away by PBS solution. 1 × DMEM culture medium with 5% serum was used

to cultivate cells for 48 h and invert microscope was implied to observe the healing condition of the cell scratches.

#### 2.2.6. Transwell assay

DMEM nutrient solution was used to dilute matrigel with a ratio of 1:8. After that, it was used to cover on the upper surfaces of Transwell cell membrane with 100 uL of each well. SAOS2 cells transfected for 72 h were collected and suspended by non-serum medium to $5 \times 10^5$/mL. Then, 250 uL cell suspension was added into every upper small well while medium with 10% fetal calf serum was added in the lower wells and they were incubated in a 5% $CO_2$ incubator at 37 °C. Cells without invasion on membranes and Matrix matrigel were cleared with cotton swabs and then washed by PBS solution twice, fixed by 4% meta-formaldehyde and dyed by crystal violet dye. Ten visions were chosen randomly under times light microscopic to count the cell number of the well surface. The mean value was used to represent the invasion ability of SAOS2 cells.

#### 2.2.7. Statistical analysis

All experiment data were analyzed statistically by SPSS 22.0. Continuous variables were presented by Mean ± SD. Differences between groups were tested by student's *t*-test or variance analysis. Enumeration data were examined by Chi-square test. $P < 0.05$ indicated that differences were statistically significant.

## 3. Results

### 3.1. Expression of MYH9 in osteosarcoma tissues

The results of qRT-PCR showed that the relative expression quantity of MYH9 mRNA in osteosarcoma tissues was 6.556 ± 0.291, while the benign osteochondroma's was 3.173 ± 0.106. It was proved by statistical analysis that their relative expression quantities were significantly different ($t = 3.448$, $P < 0.001$). A further immunohistochemical staining was conducted to detect MYH9 protein of osteosarcoma and osteochondroma tissues of the 65 cases. MYH9 proteins were mainly located in cytoplasm and cell membrane. Statistical results revealed that the positive expression rate of MYH9 in osteosarcoma tissues was 75.38% (49/65), while the positive expression rate of MYH9 in osteochondroma tissues was 30.00% (6/20). The results of *Chi*-square analysis demonstrated that the expression of MYH9 in osteosarcoma tissues increased obviously ($\chi^2 = 13.794$, $P < 0.001$).

### 3.2. The positive expression of MYH9 in osteosarcoma tissues and its correlation with clinical features of patients

The expression condition of MYH9 in osteosarcoma tissues and the corresponding clinical data of patients were analyzed by correlation. The statics results showed that the positive expression of MYH9 protein in osteosarcoma tissues was positively correlated with the high Enneking classifications (III classification, $\chi^2 = 7.127$, $P = 0.008$) and lung metastasis ($\chi^2 = 5.639$, $P = 0.018$), while it revealed no distinct correlations with age ($\chi^2 = 3.840$, $P = 0.050$), gender ($\chi^2 = 0.968$, $P = 0.325$), tumor diameter ($\chi^2 = 2.942$, $P = 0.086$) and histological grade ($\chi^2 = 3.265$, $P = 0.071$), which prompted that MYH9 protein might have something to do with tumor migration and invasion.

### 3.3. The expression of MYH9 in siRNA silencing SAOS2 cells

With the application of lipidosome method, MYH9 specific siRNA and NC siRNA were transferred in the SAOS2 cells. It was found in the detection of qRT-PCR that MYH9 specific siRNA could down-regulate the expression of MYH9 mRNA of cells significantly (1.000 *vs.* 0.347 ± 0.023, $P < 0.001$). Western blot also confirmed that MYH9 specific siRNA could obviously down-regulate the expression of protein of cells (0.978 ± 0.031 *vs.* 0.322 ± 0.010, $P < 0.001$).

### 3.4. The inhibitory effect of the expression of silencing MYH9 on the migration and invasion of SAOS2 cells

After silencing of MYH9 expression in SAOS2 cells, the results of stretch test showed that the migrant ability of SAOS2 cells of the MYH9 group were decreased significantly at 48 h as compared to that of the NC group (surplus distance of migration was 22.591 ± 2.127 *vs.* 72.685 ± 3.032, $t = 9.731$ $P = 0.006$). Furthermore, it was shown by Transwell assay that the number of invasion cells of the MYH9 group within 24 h were reduced as compared to that of the NC group (35.398 ± 2.181 *vs.* 67.184 ± 3.257, $t = 9.625$, $P = 0.007$).

## 4. Discussion

Osteosarcoma is one of the commonest bone tumors possessing the highest case fatality rate [14]. Due to its complicated incidence and development biological mechanism, it is difficult to find a molecular therapeutic target, which makes difficulty in benefiting from targeted therapy [15,16]. MYH9, as a II type myosin, can be assembled as a two-way polar myosin filament after it is activated and then its head and actin combine together [17,18]. Besides, the Mg+-ATP enzyme in its globular head can hydrolyze ATP productivity to move myofilament [19] and generate contractility, which therefore mediates the processes of cell migration and division [20]. Tumor cell migration is a main molecular basis of tumor metastasis. II type myosin plays the key role in the movement and chemotaxis processes of tumor cells [21]. Hence, MYH9 owns great research value in the process of tumor migration and invasion.

This study proved not only the significantly increased expression of MYH9 mRNA in osteosarcoma tissues by the detection of qRT-PCR, but also the positive expression of MYH9 existed in more than 75.38% osteosarcoma tissues according to the results of immuno-histochemical staining. It was discovered after a more specific analysis of the clinical pathological files of patients that the positive expression of MYH9 was closely related to the high Enneking classification and lung metastasis. It could be predicted from these clinical data that the high-expressed MYH9 may play an important in facilitating the invasion and migration of osteosarcoma. Katono *et al* [12] has also found in the researches of lung cancer that the high-expressed MYH9 protein was closely related to tumor migration indexes such as distant metastasis of tumor and late TNM classification.

To inhibit the migration and invasion abilities of cells is one of the main micro-mechanisms of anti-tumor migration [22–24]. Mature MYH9 specific disturbed RNA bought from Santa Cruz Company (USA) was applied *in vitro* for silencing the expression of MYH9 in osteosarcoma SAOS2 cells. The

stretch-healing assay has proved that the migration speed of osteosarcoma cells could be weakened by decreasing the expression of MYH9. Meanwhile, the results of Transwell assay also claimed that the decreased expressed MYH9 could inhibit the invasion ability of SAOS2 cells were inhibited distinctly, which hinted that the process of the invasion and metastasis of osteosarcoma cells could be suppressed effectively by the decreased MYH9. The occurrence of epithelial–mesenchymal transition (EMT) is one of the important molecular mechanisms of the distant metastasis of tumor [25,26]. It was revealed in the research of breast cancer that MYH9 could inhibit the formations of epithelial markers E-cadherin and catenin complexus in MCF-7 cells in breast cancer to mediate the incidence of epithelial–mesenchymal transition of MCF-7 cells, as a result, to accelerate the occurrence of tumor migration [27]. MicroRNA is one of the most worth-studied molecular targeted therapy nucleotides following after siRNA [28,29]. Studies have shown that miRNA could silence its expression by combining with the specificity of the 3′-UTR area of MYH9 mRNA to suppress the invasion and migration of colon cancer cells both *in vivo* and *in vitro* [30].

In conclusion, MYH9 expressed abnormally high in osteosarcoma tissues and its high expression level is significantly associated with the malignant clinical pathological characteristics of tumor. The migration and invasion of osteosarcoma cells can be inhibited effectively by decreasing the expression of MYH9.

## Conflict of interest statement

We declare that we have no conflict of interest.

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