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The Expression of HSPD1, SCUBE3, CXCL14 and Its Relations with the Prognosis in Osteosarcoma

Wenqing Liang · Chengwei Yang · Jiahao Peng ·
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Abstract The aim of the study was to explore the expression of three genes, HSPD1, SCUBE3, and CXCL14, in osteosarcoma cells and tissue, as well as their association with the prognosis of patients with osteosarcoma. The expression of HSPD1, SCUBE3, and CXCL14 in osteosarcoma cells was detected by using Western blotting method. siRNA was used to knockdown the expression of the three genes. CCK8 cell proliferation assay was used to observe the effect of siRNA interference on U2OS cell proliferation. The expression of the three genes in osteosarcoma tissue was detected employing immunohistochemical method. Kaplan–Meier survival analysis was used to compare the relations between the expression of the three genes and prognosis. The Western blotting results showed that the expression of Hsp70, SCUBE3 protein, and CXCL14 chemotactic factor in osteosarcoma cells was significantly higher than that in normal osteocytes ($p < 0.05$). After the three genes were interfered by siRNA, the mRNA and protein expression levels of these genes in osteosarcoma cells were significantly decreased ($p < 0.05$). The growth rate of U2OS cell after the siRNA interference was significantly lower than that before interference and that in the control group transfected with negative control siRNA ($p < 0.05$). The result of immunohistochemistry demonstrated that the expression of Hsp70, SCUBE3 protein, and CXCL14 chemotactic factor in osteosarcoma tissues was significantly higher than that in adjacent muscle tissue ($p < 0.05$). Kaplan–Meier survival analysis indicated that the survival rate of the patients with high expression of those three kinds of genes was obviously lower than that of other patients ($p < 0.05$). There was no significant difference between the survival rates of patients with high or low expression of two genes ($p > 0.05$). The expression of HSPD1, SCUBE3, and CXCL14 was all high in osteosarcoma tissues and cells; moreover, the three kinds of genes had close correlations with the prognosis of the patients. Targeted inhibition of these three genes could inhibit the proliferation of the tumor, which may become a new therapeutic target.

Keywords HSPD1 · SCUBE3 · CXCL14 · Osteosarcoma · Prognosis

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Introduction

In recent years, targeted therapy has become a direction of cancer therapy which progresses rapidly. The appearance of targeted therapy drugs, such as gefitinib, herceptin, and rituximab, significantly improved the prognosis of patients with cancer [1]. At present, there is no specific targeted

therapy drug against osteosarcoma that has higher incidence, accounting for 33 % of osteal malignant cancer. Osteosarcoma usually occurs in teenagers. Pulmonary metastasis occurs after neoadjuvant chemotherapy and limb salvage surgery in 40 % of the patients, which die ultimately [2]. Therefore, it is of great significance for improving the survival rate of patients with osteosarcoma to explore the locus and drug of targeted therapy [3]. HSPD1, SCUBE3, and CXCL14 are the reported genes most related to osteosarcoma. In this study, the expression of HSPD1, SCUBE3, and CXCL14 in osteosarcoma tissues and cells was observed, as well as the effect of RNA interference of these genes on the proliferation of tumor cells and their correlations with the prognosis of the patients.

Materials and Methods

Experimental Materials

Cell culture reagents such as DMEM medium and fetal bovine serum were purchased from Invitrogen. CCK8 was purchased from Beyotime. Immunohistochemical kit was purchased from Zhongshan Golden Bridge Company. RNA extraction kit, RNA reverse transcription kit, and PCR kit were purchased from Takara. Fluorescence quantitative PCR kit was purchased from Toyobo. HSPD1, SCUBE3, and CXCL14 antibodies were purchased from KGI Biotechnology Company. Normal human osteoblasts hFOB 1.19 and the human osteosarcoma cell line U2OS were purchased from American Type Culture Collection (ATCC). Fifty-two cases of osteosarcoma samples in the group were all from surgical resection specimens of the department from February 2011 to February 2013.

Cell Culture

Normal human osteoblasts and human osteosarcoma cells were cultivated at the condition of 37 °C and 5 % of CO_2 in DMEM high glucose medium that contained 10 % fetal bovine serum, 100-U/mL penicillin, 100-U/mL streptomycin, and 2-mmol/L L-glutamine. When the cells reached around 80 % confluence, they were digested by trypsin and subcultured. The cells in logarithmic growth phase were selected for further experiment [4].

Western Blot Analysis

RIPA lysis buffer was used for lysing normal human osteoblasts and human osteosarcoma cells. After the concentration of protein lysate was detected, 60 μg of total protein was resolved on 10 % SDS-PAGE gel by electrophoresis at 120 V for 2 h, and then electrophoretically transferred to membranes. The membranes were blocked and incubated with specific primary antibodies at 4 °C overnight. After being washed, the membranes were further incubated with appropriate secondary antibodies coupled to horseradish peroxidase and developed using enhanced chemiluminescence detection reagents. The expression levels of chemotactic factors Hsp70, SCUBE3, and CXCL14 in normal human osteoblasts and human osteosarcoma cells were detected [3].

RNA Interference Experiments

HSPD1, SCUBE3, and CXCL14 siRNAs as well as a random negative siRNA (used as a negative control) were synthesized by Shanghai GenePharma Technology Co. U2OS cells in logarithmic growth phase were inoculated in 6-well plate at 2×10^5 cells per well. The medium was replaced with the new medium without double antibiotics when the cells reached 30–40 % confluence. 0.25 mL of OPTI-MEM respectively containing 100-pmol siRNA and 5-μL Lipofectamine 2000 was incubated for 5 min at room temperature and then mixed. Consequently, the mixture was incubated for another 20 min and added into 6-well plate. The medium was replaced after 6 h of regular cultivation. Protein expression level was detected 48 h after transfection [5].

Detection of HSPD1, SCUBE3, and CXCL14 Expression Using RT-q PCR

The RNA from cells, which was transfected with HSPD1, SCUBE3, CXCL14 siRNAs or random negative siRNAs, was extracted. Synthesized cDNA was used to prepare 10-μL reaction solution. Then ABI gradient PCR amplification instrument was used at the following conditions: 37 °C for 15 min, 85 °C for 5 s. 2-μL cDNA was added with Taq, upstream and downstream primer, dH_2O sequentially. Then ABI gradient PCR amplification instrument was used to amplify the cDNA at the following conditions: pre-denaturation at 94 °C for 3 min, followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 57 °C for 15 s, extension at 72 °C for 15 s. The final step was extension at 72 °C for 10 min. Then agarose gel electrophoresis and UV analyzer were used for detection [6].

CCK8 Cell Proliferation Assay

U2OS cells in logarithmic growth phase were inoculated in 96-well plate at 2×10^3 cells per well in 200 μL of culture medium. The cultivating condition in incubator was 37 °C and 5 % CO_2. 5 days before cultivation, 20-μL CCK8

solution was added to cultivate for 4 h every day, and then optical density was detected at 490 nm [7].

Immunohistochemistry of Osteosarcoma Tissue

The obtained 52 cases of osteosarcoma tissue samples and peritumoral muscle tissue samples were sliced, and then baked, dewaxed, and rehydrated followed by antigen repair by using pepsin. Then hydrogen peroxide, HSPD1 antibody, SCUBE3 antibody, and CXCL14 antibody were added for incubation overnight with the antibody titer of 1:200 and incubation temperature of 4 °C. After incubation with horseradish peroxidase at room temperature for 30 min, the slices were stained with DAB, counter stained with hematoxylin, dehydrated, and mounted. The tissue sections were read double-blind by at least two professional pathologists. The sections were graded according to the proportion of positive staining osteosarcoma cells: (−) no positive cells, (+) positive cells <10 %, (++) 10–50 % of positive cells, (+++) positive cells >50 % [8].

Patients and Follow-Up

There were 64 cases of patients with osteosarcoma treated in our hospital during April 2010–April 2011, including 34 cases of males and 30 cases of females at the age of 8–38, with the average age of 15.4 ± 8.6. Tumor sites: There were 28 cases of distal femur, 18 cases of proximal tibia, ten cases of proximal humerus, eight cases of proximal femur, and ten cases of the other. All the patients were followed up by telephone or outpatient. The follow-up stopped after 3 years or when the patient died. The survival of the patients was recorded in the process of follow-up. 1-year survival rate, 3-year survival rate, and medium survival time of the two groups were calculated.

Statistical Analysis

SPSS 14.0 was implemented for statistical analysis. χ^2 test was used for comparison of enumeration data. Measurement data were compared using t test or analysis of variance. Kaplan–Meier method and log-rank test were used for survival analysis. Wilcoxon rank sum test was used for comparison between ranked data. Measurement data are represented as $\bar{x} \pm s$. Significance level was set at $\alpha = 0.05$. $p < 0.05$ indicated statistical difference.

Results

Gene Expression Detection Using Western Blot

As shown in Fig. 1, the protein expression of Hsp70, SCUBE3 protein, and CXCL14 chemotactic factor in

osteosarcoma cells was significantly higher than that in normal osteoblasts ($p < 0.05$).

Knockdown of HSPD1, SCUBE3, and CXCL14 Expression by siRNA

The mRNA and protein expression of HSPD1, SCUBE3, and CXCL14 in U2OS cells were markedly down-regulated after siRNA interference ($p < 0.05$) (Table 1).

CCK8 Cell Proliferation Assay Before and After siRNA Interference

After U2OS cells were transfected with siRNA, cell proliferation rate was significantly inhibited ($p < 0.05$). There was no significant difference of the cell proliferation rate between un-transfected cells and cells transfected with negative control siRNA ($p > 0.05$). No significant difference of cell proliferation rate was found among the cells transfected with specific siRNA against HSPD1, SCUBE3, or CXCL14 ($p > 0.05$) (Fig. 2).

Comparison of Immunohistochemistry Between Osteosarcoma Tissue and Peritumoral Muscle Tissue

As shown in Table 2, the positive expression rates of HSPD1, SCUBE3, and CXCL14 in osteosarcoma tissue were significantly higher than that in peritumoral muscle tissue ($p < 0.05$).

Correlations Between Expression of HSPD1, SCUBE3, CXCL14, and Prognosis

There were six cases of loss to follow up in 64 patients. The survival rate and medium survival time in the patients with high expression of all the three genes were significantly lower than those in other patients ($p < 0.05$). The
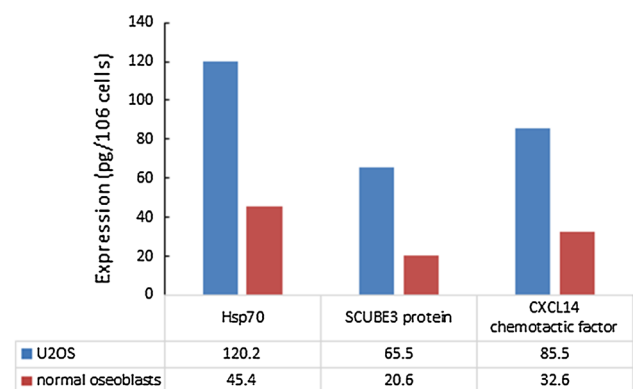

	Hsp70	SCUBE3 protein	CXCL14 chemotactic factor
U2OS	120.2	65.5	85.5
normal oseoblasts	45.4	20.6	32.6

Fig. 1 Comparison of protein expression of Hsp70, SCUBE3, and CXCL14 chemotactic factor in osteosarcoma cells and in normal osteoblasts

Table 1 Comparison of mRNA and protein expression of HSPD1, SCUBE3, and CXCL14 in human osteosarcoma cells before and after siRNA interference

Interference genes	Time	Before interference	After interference	t	p
HSPD1	mRNA	3.3 ± 0.5	1.1 ± 0.8	7.3744	0.0000
	protein	120.2 ± 18.9	56.3 ± 10.2	9.4088	0.0000
SCUBE3	mRNA	1.2 ± 0.2	0.4 ± 0.1	11.3137	0.0000
	protein	65.5 ± 8.4	23.2 ± 5.6	13.2498	0.0000
CXCL14	mRNA	2.2 ± 0.3	0.8 ± 0.2	12.2788	0.0000
	protein	85.5 ± 10.2	35.1 ± 6.7	13.0599	0.0000
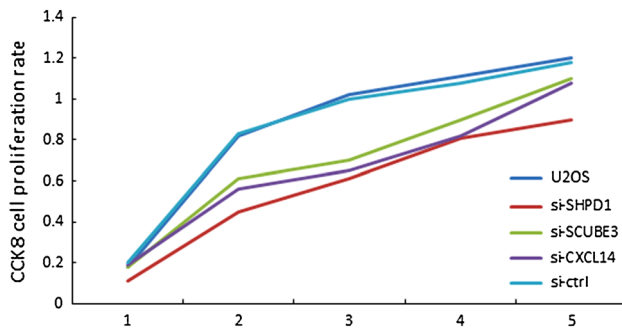
Fig. 2 CCK8 cell proliferation assay before and after siRNA interference

survival rate and medium survival time in the patients with low expression of all the three genes were significantly higher than those in other patients ($p < 0.05$). There was no significant difference of survival rate and medium survival time among the patients with high expression or low expression of two of the three genes ($p < 0.05$, Table 3).

Discussion

With the continuous progression in the field of genetic research, the genetic relatedness of tumor is undoubted. New oncogenes, tumor suppressor genes are constantly being discovered. More importantly, the above discoveries need to be translated into cancer therapy to improve the prognosis of the patients with cancer. Osteosarcoma is the most common malignant bone tumor belonging to stromal cell tumors and is one of the most common malignancies in

teenagers accounting for 5 % of all malignant tumors of the minors [9]. The prognosis of osteosarcoma is not good. According to the reports, the 5-year survival rate of osteosarcoma is 32–48 %. Moreover, it may cause permanent disability to teenagers [8]. In recent years, targeted therapy has become an important progress in cancer therapy. Targeted therapy drugs have significantly improved the survival of patients with various malignant tumors, such as lung cancer, breast cancer and lymphoma. The discovery of new targeted locus is the most important condition of targeted therapy [10]. In this study, we explored the expression of HSPD1, SCUBE3, and CXCL14 in osteosarcoma tissues and cells, the effect of RNA interference on tumor cell proliferation, and its relatedness to the prognosis of patients.

HSDP1 gene mainly encodes heat shock proteins (Hsp) such as Hsp60 and Hsp70. Heat shock protein belongs to stress protein, which widely exists in human body. It has vital physiological functions such as regulating the activity and function of various proteins, regulating cell proliferation and differentiation, regulating embryonic development, etc. [3, 11–13]. Numerous studies have indicated that Hsp has close relations with the genesis and development of various tumors, including non-small-cell lung carcinoma [14], small cell lung carcinoma [4], and breast ductal carcinoma [6]. In this study, we proved that HSPD1 gene expressed high in osteosarcoma at mRNA level, protein level, cell level, and tissue level. Moreover, the proliferation rate of U2OS cells was inhibited by the transfection of HSPD1 siRNA, indicating the relatedness between HSPD1 and osteosarcoma cell proliferation. SCUBE3 is found first in human umbilical vein endothelial cells, which belongs to

Table 2 Comparison of immunohistochemistry analysis of HSPD1, SCUBE3, and CXCL14 expression between osteosarcoma tissue and peritumoral muscle tissue

Tissues	n	HSPD1				SCUBE3				CXCL14			
		−	+	++	+++	−	+	++	+++	−	+	++	+++
Osteosarcoma	52	1	18	20	13	3	8	12	29	2	10	12	28
Peritumoral muscle	52	32	10	8	2	28	10	10	4	32	12	6	2
u		6.1678				6.2121				7.1054			
p		0.0000				0.0000				0.0000			

Table 3 Correlations between HSPD1, SCUBE3, CXCL14, and prognosis (cases, %)

HSPD1	SCUBE3	CXCL14	n	1-year survival rate (%)	2-year survival rate (%)	3-year survival rate (%)	Medium survival time (months)
+	+	+	9	3 (33.3)	2 (22.2)	1 (11.1)	8.9 ± 4.3
−	+	+	6	5 (83.3)	3 (50.0)	2 (33.3)	26.8 ± 5.7
+	−	+	6	4 (66.7)	4 (66.7)	2 (33.3)	28.9 ± 6.1
+	+	+	8	6 (75.0)	4 (50.0)	2 (25.0)	24.5 ± 4.8
+	−	−	9	6 (66.7)	5 (55.6)	3 (33.3)	26.8 ± 5.7
−	+	−	8	5 (62.5)	4 (50.0)	2 (25.0)	24.0 ± 8.0
−	−	+	8	4 (50.0)	3 (37.5)	3 (37.5)	12.8 ± 5.6
−	−	−	4	4 (100)	3 (75.0)	3 (75.0)	36.5 ± 10.2

secretory cell surface glycoprotein [15]. In normal human bodies, SCUBE3 expresses in gonads, central nervous system, heart, skeleton, vascular endothelial cells and plays roles in cell recognition, cell remodeling, cell differentiation, etc. Studies have indicated that SCUBE3 significantly overexpressed in tumor tissues. Chou et al. showed that SCUBE3 overexpressed in invasive lung carcinoma tissues and had close correlations with the angiogenesis of lung tumor tissues [16]. Wu et al. proved that SCUBE3 promoted the angiogenesis of tumor tissues and the process of epithelial-mesenchymal transition mainly via TGF-α pathway [17]. Zhao et al. demonstrated the association between high expression of SCUBE3 and the poor prognosis of patients with lung cancer. In this study, we observed the expression of SCUBE3 in osteosarcoma cells and tissues. Our results demonstrated that both mRNA and protein expression of SCUBE3 were significantly upregulated. After knockdown by siRNA, the proliferation of osteosarcoma was inhibited, which was consistent with the above conclusions obtained in lung cancer. Except for the above-related genes, in this study, we also discovered the effect of CXCL14 expression and knockdown on osteosarcoma cells and tissues. CXCL14 gene belongs to chemokine family. Chemokine family plays very important roles in inflammation, immunomodulation, angiogenesis, cell proliferation, invasion, and metastasis of tumor [5]. The study of Gu et al. found the low expression of CXCL14 in breast tumor tissue. After overexpression of CXCL14 gene, the cell proliferation of breast tumor cell was inhibited [18]. However, other scholars have obtained the opposite conclusion in other tumors. Zeng et al. found that in resection specimens of colorectal carcinoma, the CXCL14 expression was significantly higher than that in the surrounding normal tissues. The CXCL14 levels were significantly different in different TNM stages. The prognosis of patients with high CXCL14 expression was significantly poor [19]. The conclusions of Park et al. are consistent with the conclusions of this article, considering that CXCL14 promoted proliferation and metastasis of various tumors such as lung cancer

[20]. The correlations between the prognosis and the three genes were explored in the study. The prognosis was the poorest in patients with high expression of all the three types of genes. And the prognosis was the best in patients with low expression of all the three types of genes. There was no significant difference among the other patients, demonstrating the independence among the three genes and their capability of predicting the prognosis of patients.

In summary, HSPD1, SCUBE3, and CXCL14 all expressed high in osteosarcoma tissues and cells. Moreover, the three types of genes had close correlations with the prognosis of the patients. Targeted inhibition of the three genes could inhibit the proliferation of the tumor, which may become new therapeutic targets.

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