Heat shock transcription factor 1 promotes the proliferation, migration and invasion of osteosarcoma cells

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Abstract

Objectives: Osteosarcoma is the most commonly diagnosed primary malignancy of bone and its overall survival rate is still very low. The molecular mechanisms underlying the progression of osteosarcoma have not been clearly illuminated. Heat shock transcription factor 1 (HSF1) is a key regulator of the heat shock response and also plays important roles in many cancers, but its function in osteosarcoma remains unexplored.

Materials and methods: In this study, the proliferation of osteosarcoma cells was determined by Cell Counting Kit-8 assays and colony formation assays. Transwell assays were used to demonstrate the migration and invasion abilities of osteosarcoma cells. A tumour formation assay in a nude mouse model was performed to assess the effect of HSF1 on osteosarcoma cell growth in vivo. The protein levels of HSF1 were analysed with immunohistochemical staining in samples from osteosarcoma patients.

Results: We demonstrated that knockdown of HSF1 reduced the proliferation, migration and invasion of osteosarcoma cells, while overexpression of HSF1 promoted the proliferation, migration and invasion of osteosarcoma cells. Furthermore, HSF1 promoted the proliferation of osteosarcoma cells in vivo. In addition, high levels of HSF1 were associated with a poor prognosis in osteosarcoma.

Conclusions: These data highlight an important role of HSF1 in proliferation, migration and invasion of osteosarcoma cells. Moreover, the expression of HSF1 was associated with prognosis in osteosarcoma.

1 | INTRODUCTION

Osteosarcoma is the most commonly diagnosed primary malignancy of bone, particularly in adolescents and young adults.¹ It is the most common radiation-induced sarcoma usually originating in the long bones of the extremities, such as the distal femur and proximal tibia.² A very aggressive malignancy and it frequently metastasizes to the lungs.³ Current treatments include surgery and neoadjuvant chemotherapy.

Abbreviations: CCK-8, Cell Counting Kit-8; DFS, disease-free survival; DMEM, Dulbecco's modified Eagle's medium; HSF1, heat shock transcription factor 1; HSPs, heat shock proteins; OS, overall survival.

Zhenhua Zhou, Yan Li and Qi Jia contributed equally to this work.

Despite some advances in treatment strategies, the overall survival rate of osteosarcoma patients is still very low.^{1,4,5} It is therefore important to clarify the molecular mechanisms underlying the initiation, progression and metastasis of osteosarcoma with a view to improving patient outcomes.

A series of protein-coding genes have recently been reported to be associated with the proliferation and metastasis of osteosarcoma. *MAPK7* and *EZH2* promote osteosarcoma cell proliferation, migration and invasion,^{6,7} whereas *SRCIN1* suppresses osteosarcoma cell proliferation and invasion.⁸ Furthermore, many signalling pathways have also been implicated in the process of osteosarcoma proliferation and metastasis, such as the beta-Catenin/Wnt,⁹ NF-kappaB¹⁰ and PI3K/Akt signalling pathways.¹¹ The

expression of *EZH2* and *FKBP14* is associated with a poor prognosis.^{7,12} Additionally, many microRNAs also play important roles in the proliferation and metastasis of osteosarcoma, including miR-422a, miR-181a, miR-1, miR-320 and miR-10b.^{13–17} However, the molecular mechanisms underlying these processes remain to be comprehensively elucidated.

The gene heat shock transcription factor (HSF1), as a transcription factor, can promote the transcription and expression of heat shock proteins (HSPs).¹⁸ Many studies have revealed that HSF1 is also involved in a series of non-stress process. In this study, we explored the functions of HSF1 in osteosarcoma. We found that HSF1 promoted proliferation, migration and invasion of osteosarcoma cells in vitro and that it also promoted the proliferation of osteosarcoma cells in vivo. Importantly, high expression of HSF1 was identified to be associated with a poor prognosis in osteosarcoma patients.

2 | MATERIALS AND METHODS

2.1 | Patients and tumour samples

A total of 65 tissue samples from osteosarcoma patients were obtained from the Human Tumour Tissue Biobank of Shanghai between January 2008 and January 2014. None of the patients had received preoperative chemotherapy or radiotherapy. Tissue samples were immediately snap-frozen and then stored in liquid nitrogen until analysis. All human tissues were obtained with informed consent, and the project was approved by the Clinical Research Ethics Committee of Changzheng Hospital.

2.2 | Cell culture

Human osteosarcoma cell lines MNNG/HOS Cl #5[R-1059-D] (abbreviated as MNNG in this paper), MG-63, U-2 OS, Saos-2 and HEK-293T were purchased from the American Type Culture Collection (ATCC). MNNG, U-2 OS and HEK-293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C and 5% CO₂. MG-63 cells were cultured in minimum essential medium with 10% FBS.

2.3 | Assays of cell proliferation and colony formation

Cell proliferation assays were performed with the Cell Counting Kit-8 (CCK-8) (Dojindo Laboratories, Kumamoto, Japan). Osteosarcoma cells (1×10^3 /well) were plated into 96-well plates. Ten microlitres of CCK-8 solution were added to each well and incubated at 37°C for 2 hours. The absorbance at 450 nm was measured. Each experiment was repeated three times.

For the colony formation assays, cells (1×10^3) were trypsinized and plated onto six-well plates and maintained in media containing 10% FBS for 10 days. Colonies were fixed with methanol and stained with 0.1% crystal violet in 20% methanol. The number of colonies was counted using an inverted microscope.

2.4 | Assays of cell migration and invasion

Assays of cell migration and invasion were performed using a Transwell chamber with an 8- μ m pore size (BD Biosciences, Franklin Lakes, NJ, USA) in a 24-well plate. For the migration assay, 5×10^4 cells were placed in the upper chamber of the insert. For the invasion assay, 1×10^5 cells were seeded into the upper chamber of the inserts coated with Matrigel. DMEM supplemented with 10% FBS (800 μ L) was used as the chemoattractant in lower chamber. The cells that had moved to the basal side of the membrane were stained with crystal violet and then imaged using an IX71 inverted microscope (Olympus, Tokyo, Japan).

2.5 | Assays of tumour formation in a nude mouse model

Athymic BALB/c nude mice were maintained under specific pathogen-free conditions. They were manipulated and housed according to protocols approved by the Shanghai Medical Experimental Animal Care Commission. MNNG cells stably expressing the HSF1 gene or the vector control were trypsinized and washed with PBS and then resuspended in DMEM at a cell concentration of 1×10^7 /mL. About 0.2 mL of the cell suspension was then subcutaneously injected into the flanks of 6-week-old nude mice (six mice in each group). After transplantation, tumour growth was assessed once a week. After 7 weeks, the mice were sacrificed. The tumours were then fixed with phosphate-buffered neutral formalin and prepared for histological examination.

2.6 | Oligonucleotide transfection

The small-interfering RNAs against HSF1 were synthesized by Genescript (Shanghai, China). The sequences used were as follows: sense 5'-GACCCATCATCTCCGACATTT-3' and antisense 5'-ATGTCGGAGATGATGGGTCTT-3'. Cells were transfected using Lipofectamine 2000 according to the manufacturer's (Invitrogen, Carlsbad, CA, USA) instructions. Proliferation, migration and invasion assays were performed 48 hours after transfection.

2.7 | Western blot

Proteins were separated on 10% sodiumdodecyl sulphate-polyacrylamide gel and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). The membranes were blocked with 5% non-fat milk and incubated with rabbit anti-HSF1 antibody (Cell Signaling Technology, Danvers, MA, USA). The antigen-antibody complex was detected with enhanced chemiluminescence reagents (Pierce, Rockford, IL, USA).

2.8 | Statistical analysis

The results were presented as mean $\pm$ SEM. Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA, USA) and SPSS v.20.0 (SPSS Inc., Chicago, IL, USA). The

data were subjected to Student's *t*-test unless otherwise specified. Differences in patient survival were determined by the Kaplan-Meier method. A value of $P < .05$ was considered statistically significant.

3 | RESULTS

3.1 | HSF1 promotes osteosarcoma cell proliferation, migration and invasion in vitro

First, we examined the protein expression levels of HSF1 in four osteosarcoma cell lines (Figure S1). The results showed that HSF1 expression level was relatively high in MNNG and U-2 OS cells. We therefore knocked down the expression of HSF1 by siRNA in these two osteosarcoma cell lines and confirmed the interference efficiency by immunoblotting (Figure 1A). After knockdown of HSF1, changes in cell proliferation were determined by CCK-8 and colony formation assays. The results showed that knockdown of HSF1 significantly reduced the proliferation of osteosarcoma cells (Figure 1B). Colony

formation was also decreased when the HSF1 gene was downregulated in MNNG and U-2 OS cells (Figure 1C). Transwell assays demonstrated that knockdown of HSF1 inhibited the migratory and invasive abilities of osteosarcoma cells (Figure 1D).

Next, HSF1 was overexpressed by lentiviral infection in MNNG and U-2 OS cells, and this was confirmed by western blotting (Figure 2A). We found that HSF1 overexpression led to a significant increase of proliferation ability in MNNG and U-2 OS cells (Figure 2B,C). Transwell assays demonstrated that overexpression of HSF1 promoted cell migration and invasion in both MNNG and U-2 OS (Figure 2D).

3.2 | HSF1 promotes osteosarcoma cell proliferation in vivo

To assess the effect of HSF1 on osteosarcoma cell growth in vivo, we performed a tumour formation assay in a nude mouse model. MNNG cells stably expressing HSF1 or the vector control were subcutaneously injected into nude mice; the growth of tumours was

FIGURE 1 Silencing of heat shock transcription factor 1 (HSF1) reduced osteosarcoma cell proliferation, migration and invasion in vitro. (A) HSF1 protein levels were detected using Western blot assays in MNNG and U-2 OS cells transfected with HSF1 siRNA or negative control (NC). (B, C) CCK-8 and colony formation assays were performed to observe the effects of HSF1 knockdown on cell proliferation in MNNG and U-2 OS. (D) Transwell migration and invasion assays were performed in MNNG and U-2 OS cells transfected with HSF1 siRNA or NC. Statistical analysis was performed with Student's *t*-test. * $P < .05$; ** $P < .01$; *** $P < .001$. Data represent mean $\pm$ SEM

FIGURE 2 Overexpression of heat shock transcription factor 1 (HSF1) promotes osteosarcoma cell proliferation, migration and invasion in vitro. (A) HSF1 protein levels were determined by Western blotting following lentivirus-mediated overexpression of HSF1 in MNNG and U-2 OS cells. (B, C) CCK-8 and colony formation assays were performed to observe the effects of HSF1 overexpression on cell proliferation in MNNG and U-2 OS cells. (D) Transwell migration and invasion assays were performed in MNNG and U-2 OS cells after lentiviral transduction of HSF1 or empty vector. Statistical analysis was performed with Student's *t*-test. **P*<.05; ***P*<.01; ****P*<.001. Data represent mean ± SEM

then monitored. The mice were sacrificed after 7 weeks. Compared with the empty vector control, overexpression of HSF1 promoted tumorigenesis in nude mice to a remarkable degree (Figure 3A-C,E). Immunohistochemistry staining verified the overexpression of HSF1 in Lenti-HSF1 xenografted tumours compared with Lenti-vector xenografted tumours (Figure 3D).

3.3 | HSF1 expression is associated with poor survival in osteosarcoma

We investigated the relationship between HSF1 expression and osteosarcoma survival. The protein levels of HSF1 were analysed with immunohistochemical staining in 65 samples from osteosarcoma patients; brown nuclear staining was observed in these tissues. The levels of HSF1 expression were grouped according to nuclear staining score. HSF1 expression was high (scored at 2 or 3) in 57% (37/65) and low (scored at 0 or 1) in 43% (28/65) of the tissues (Figure 4A-D). Notably, the levels of HSF1 protein expression were closely correlated with the overall survival and disease-free survival rates of the

osteosarcoma patients (both *P*<.05, Figure 4E,F). Furthermore, we analysed the correlation between the level of expression of HSF1 and the clinicopathological features of osteosarcoma (Table 1) and found that HSF1 expression was positively correlated with tumour size (*P*=.004), recurrence (*P*=.018) and metastasis (*P*=.01) (Table 1).

4 | DISCUSSION

In this study, we demonstrated that the silencing of HSF1 reduced the proliferation, migration and invasion of osteosarcoma cells, while HSF1 overexpression promoted the proliferation, migration and invasion of osteosarcoma cells in vitro. Overexpression of HSF1 also promoted the proliferation of osteosarcoma cells in vivo. Furthermore, high expression of HSF1 was found to be correlated with a poor prognosis in patients with osteosarcoma.

Biological organisms (bacteria, animals and plants) can respond to elevated temperatures and a variety of chemical and physiological stresses; when this happens, HSPs increase rapidly.^{18,19} In eukaryotes,

HSPs are controlled by the HSF.¹⁸ The HSF1 gene has a highly conserved molecular structure in eukaryotic species.²⁰ HSF1 trimers bind to heat shock elements, which comprise inverted repeats of the 5 bp sequence NGAAN to regulate the HSPs.¹⁸

Recently, it is revealed that HSF1 is also involved in a series of non-stress processes, such as metabolism, ageing and cancer. Dai and colleagues²¹ have reported that lack of HSF1 protects mice from tumours induced by ras oncogenes or p53 mutations. Mendillo

TABLE 1 Correlation of HSF1 expression with clinicopathological features

Features	Number of cases	HSF1 expression		χ^2	P
		High (n,%)	Low (n,%)		
Gender					
Male	39	23 (59.0)	16 (41.0)	0.167	.683
Female	26	14 (53.8)	12 (46.2)		
Age					
≤20	26	16 (61.5)	10 (38.5)	0.54	.614
20	39	21 (53.8)	18 (46.2)		
Enneking stages					
I	8	3 (37.5)	5 (62.5)	5.357	.147
IIA	16	8 (50.0)	8 (50.0)		
IIB	36	21 (58.3)	15 (46.2)		
III	5	5 (100)	0		
Histologic type					
Osteoblastic	27	19 (70.4)	8 (29.6)	4.064	.255
Chondroblastic	17	9 (52.9)	8 (47.1)		
Fibroblastic	8	4 (50.0)	4 (50.0)		
Mixed	13	5 (62.5)	8 (37.5)		
Location					
Femur	28	17 (60.7)	11 (39.3)	7.004	.072
Tibia	17	13 (76.5)	4 (23.5)		
Humerus	13	4 (30.8)	9 (69.2)		
Others	7	3 (42.9)	4 (57.1)		
Tumour size					
Large (>5 cm)	32	24 (75.0)	8 (25.0)	8.4	0.004*
Small (≤5 cm)	33	13 (39.4)	20 (60.6)		
Tumour recurrence					
Presence	22	17 (77.3)	5 (22.7)	5.616	.018*
Absence	43	20 (46.5)	23 (53.5)		
Tumour metastasis					
Presence	23	18 (78.3)	5 (21.7)	6.609	.01*
Absence	42	19 (45.2)	23 (54.8)		

*P<.05.

and colleagues²² compared human breast cancer cells with high and low malignant potential with non-transformed counterparts using ChIP-Seq; they identified an HSF1-regulated transcriptional programme specific to highly malignant cells and distinct from heat shock. In hepatocellular carcinoma (HCC), it was reported that HSF1 directly activated miR-135b expression and then promoted HCC cell migration and invasion.²³ These studies demonstrate that HSF1 plays a significant role in the development and progression of cancer.

Osteosarcoma is the most common primary malignant tumour of bone and its prognosis remains poor, especially when metastases are present at diagnosis. The poor prognoses of osteosarcoma patients are due to the lack of early diagnostic markers and therapeutic targets. Hence, it is important to identify new biological

markers to improve the outcome in osteosarcoma patients with poor prognosis. It has been reported that HSF1 is associated with the prognosis of breast cancer patients. High expression of HSF1 in the nuclei of breast cancer cells in situ and at invasive stages were correlated with poor prognoses.²⁴ In ER-positive breast cancer patients, high expression levels of HSF1 were associated with shorter overall and relapse-free survival.²⁵ In our study, we analysed the protein expression levels of HSF1 in 65 osteosarcoma patient samples. We found that the expression of HSF1 was high in 57% of osteosarcoma tissues (Figure 4A-D) and high expression level of HSF1 was correlated with the poor overall survival and disease-free survival rates of the osteosarcoma patients (Figure 4E,F). Moreover, HSF1 expression was positively correlated with osteosarcoma recurrence and metastasis (Table 1). These results indicate that HSF1

may provide a new possibility for the early diagnosis and treatment of osteosarcoma.

In addition, it is reported that overexpression of HSF1 significantly increased 3D tumour sphere formation in breast cancer cell lines.²⁶ However, we observed that HSF1 had no obvious effect on the formation of tumour sphere in osteosarcoma (Figure S2). This result suggests that the correlation between high HSF1 expression and poor prognosis may not be due to the influence of HSF1 on cancer stem cells in osteosarcoma.

Triptolide, which was isolated from a Chinese medicinal herb, *Tripterygium wilfordii*, has been used to treat inflammatory and autoimmune diseases.^{27,28} It is reported that triptolide mediates cancer cell death by inhibiting HSP 70 in pancreatic cancer.²⁹ A proteomic analysis of osteosarcoma and primary osteoblastic cells revealed that HSF1 and HSP70 were elevated in osteosarcoma cells.³⁰ Triptolide has very poor solubility in aqueous medium, so a water-soluble pro-drug of triptolide named minnelide was developed. A study revealed that minnelide treatment significantly reduced tumour burden and lung metastasis in the osteosarcoma orthotopic and lung colonization models and HSF1 was decreased in minnelide treatment cells.³¹ However, the precise mechanism of how minnelide kills cancer cells is unknown. Our study revealed the function of HSF1 in osteosarcoma, which may provide some basis to this point.

In summary, we found that HSF1 promoted the proliferation, migration and invasion of osteosarcoma cells and was correlated with prognosis of osteosarcoma. Thus, our results reveal the important role of HSF1 in osteosarcoma and suggest that HSF1 might be a potential target for the treatment of osteosarcoma.

CONFLICT OF INTEREST

The authors declare no competing financial interests.

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SUPPORTING INFORMATION

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