Abstract. Chondrosarcoma is a type of malignant bone tumor secreting cartilage-like matrix. In clinical treatment, there is no frequently used drug treatment option except for surgical resection. Hedgehog (HH) pathway is a classical signaling pathway that regulates normal cartilage cell development. In order to detect the role that HH pathway plays in chondrosarcoma, we used immunohistochemistry and found this tumor clearly expressed HH pathway-related proteins. Treatment with HH pathway inhibitor-4 (HPI-4) could significantly decrease human chondrosarcoma cell proliferation, invasion and migration ability. Furthermore, HPI-4 could distinctly disturb HH pathway-mediated ciliogenesis and suppress primary cilia-related protein intraflagellar transport protein IFT88 expression. HH downstream effect molecular GLI2 was restrained to block parathyroid hormone-related protein (PTHrP) and affect MAPK/ERK-regulated matrix metalloproteinases (MMP2 and MMP9). These results indicated that activated HH pathway existed in chondrosarcoma and HPI-4 could be a new therapeutic option specific to chondrosarcoma expressing elevated levels of HH pathway.

Introduction

Chondrosarcoma is the second most common malignant tumor originating from bone tissue. It appears mostly in 30-60-year-old patients. With complex clinical characteristics, pathological manifestations and various differentiation degrees, chondrosarcoma is not sensitive to either radiotherapy or chemotherapy and surgical resection remains the most common treatment in the clinic (1).

Hedgehog (HH) pathway is a crucial signaling pathway in regulating cell growth and differentiation, particularly in bone and cartilage tissues (2,3). Indian hedgehog (Ihh) ligands first bind to patched protein 1 (PTCH1) receptor and then release the repression of Smoothened (Smo) receptor which initiates the activation of downstream transcription factor GLI family (2,4). This process leads to downstream gene expression and regulation of multifarious physiological functions. Our previous studies showed that an HH/parathyroid hormone-related protein (PTHrP) negative feedback loop exists to regulate the development of different levels of cartilage cells. Ihh facilitates PTHrP expression to promote the growth plate cell proliferation, inhibition of cell differentiation and maturation. Otherwise, PTHrP reduces Ihh production conversely (2,3). More specifically, HH pathway controls transcription factor GLI2 to control secretion of PTHrP protein which can promote osteolytic destruction, especially in primary or metastatic bone tumor (5,6). Recently, that abnormal activation of HH pathway induces carcinogenesis was found in various tumor types, such as rhabdomyosarcoma, breast cancer, gastrointestinal malignant tumor and human renal cell carcinomas (7-11). These studies confirmed that overexpression of GLI transcription factors caused the tumorigenesis by affecting cell growth and differentiation progression. Moreover, the malignant properties of these neoplasms were determined by the differentiation and maturation degree of tumors (7-11). In this study, we found that human chondrosarcoma tissue expressed the seemingly abnormal level of HH signaling-related proteins, such as Ihh, GLI1, GLI2 and PTHrP. However, to date, there is no evidence to illustrate how HH signaling pathways affect the growth process of chondrosarcoma and whether targeting the HH pathway could be an optional therapy to treat this tumor (2). Based on our previous findings, we speculated that the HH signaling pathway may be a potential antitumor target for drug therapy. To test this hypothesis, we treated human chondrosarcoma SW1353 cells with the HH pathway inhibitor,
HH pathway inhibitor-4 (HPI-4). HPI-4 is a special type of inhibitor which may affect the downstream of Smo but does not directly act on transcription factor GLIs (13,14).

Primary cilia, as a crucial part of the HH signaling pathway, are special external cellular organelles. Since they contain various receptor proteins, they are always regarded as important extracellular chemical and physical sensors in regulating the cell growth and development process. Cilia can influence the process of differentiation through regulating cell division cycle. Primary cilia’s abnormal occurrence, morphology and function are closely related to tumorigenesis (15-19). We focused on the cilia to study the effects of HPI-4 on chondrosarcoma cells by investigating the effects on tumor malignant characteristics, such as proliferation, invasion and migration ability, so as to determine whether this drug has a negative regulation on human chondrosarcoma. In addition, the potential mechanism of HPI-4 through working on primary cilia to regulate Ihh-PTHrP signaling in vitro was investigated. In conclusion, these results indicated that HPI-4 blocking HH signaling may be a feasible chemotherapeutic option for human chondrosarcoma treatment.

Materials and methods

Cells and reagents. The human chondrosarcoma cell line SW1353 was purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Dulbecco’s modified Eagle’s medium/nutrient mixture F-12 (DMEM/F12) with 10% fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 U/ml) at 37°C with 5% CO₂. HPI-4, also known as Ciliobrevin A, whose molecular formula is C₁₇H₉Cl₂N₃O₂, was purchased from Sigma-Aldrich (St. Louis, MO, USA). The study was approved by the Ethics Committee of Tongji Medical College, Huazhong University of Science and Technology (Wuhan, Hubei, China).

Cell proliferation-toxicity test. To evaluate the change of cell proliferation ability, we used the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2H-tetrazolium (MTS-8) assay to measure cell proliferation and the toxicity of this drug. The procedure was as follows: 2,000 cells were plated in 96-well plates per well. HPI-4 was added to cells at concentrations of 0, 5 and 10 µM in 100 µl DMEM/F12 with 10% FBS and incubated for 0, 1, 3, 6 and 9 days. Then, 10 µl MST-8 (Beyotime, China) was added to the media in each well and incubated in an environment without light for 90 min. The absorbance value was measured using an enzyme microplate reader at 450 nm wavelength. The relative viability of cells was expressed by OD value.

Tablet colony formation assay. SW1353 cells were harvested by trypsin enzyme digesting and 200 cells were seeded in a 6-well plate. After cells attached, HPI-4 was added to cells at different concentrations of 0, 5 and 10 µM in each well. Cells were cultured in DMEM/F12 medium with 10% FBS and incubated at 37°C with 5% CO₂ and fresh medium was changed every 3 days. Four weeks later, cells were fixed with 4% paraformaldehyde and stained with 0.2% crystal violet. Finally, images were captured to record the size and number of colonies.

Transwell invasion assay. The invasion assay was conducted using Transwell plates. The filter membrane (pore size, 8 µm) of the Transwell plates was coated with 20 µg Matrigel (BD Biosciences, USA) at 37°C for 30 min and the lower chamber was filled with culture medium DMEM/F12 containing 10% FBS. SW1353 cells were starved with serum-free DMEM/F12 for 6 h and then 1x10⁵ cells were transferred onto the upper surface of the chamber. HPI-4 was added at different concentrations of 0, 5 and 10 µM. Twenty-four hours later, the culture medium was gently removed and the upper surface Matrigel was wiped by cotton swab. We used 4% paraformaldehyde to fix the cells that had invaded through and adhered to the lower surface for 15 min, then stained with 0.2% crystal violet. Ten random fields in each plate were selected and counted using an Olympus microscope.

Wound healing assay. Tumor migratory behavior was imitated with a scratch assay by measuring migration distance. First, SW1353 cells were seeded in 6-well plates at a density of 1x10⁵/well. When they reached nearly confluent monolayer, a single scratch was made vertically through each well by use of a sterile pipette tip. After washing with PBS three times, the cells were incubated with 2% bovine serum DMEM/F12 and cells were stimulated with different concentrations of HPI-4, and observed under a microscope (10x objective lens) after 0, 24 and 48 h. Images were recorded at the same position in order to assess the repair process accurately. Finally, the percentage of cell migration distance was calculated.

Immunohistochemical studies. We collected 10 human chondrosarcoma tissues from surgical resection and these tissues were firstly fixed in 4% paraformaldehyde. They were then embedded in paraffin and sectioned for immunohistochemical assays. All experimental processes were conducted using standard techniques (12). Ihh (1:100; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), GLI1 (1:400; Epitomics Inc., Burlingame, CA, USA), GLI2 (1:100; Boster, Wuhan, China) and PTHrP (1:100; Santa Cruz Biotechnology, Inc.) were used as markers for activation of HH signaling pathway. Ki67 staining was used to detect proliferation cells in tissue sections at a dilution of 1:200 (Cell Signaling Technology, USA). All sections were observed under a microscope at x200 magnification.

Immunofluorescence assay. SW1353 cells were cultured on the cover glass and treated with different concentrations of HPI-4. Twenty-four hours later, they were fixed with 4% paraformaldehyde for 15 min, blocked with 5% BSA at room temperature for 60 min and then incubated with acetylated α-tubulin antibody (1:400; Abcam, Cambridge, UK) overnight at 4°C. Subsequently, the SW1353 cells were incubated with Cy3-conjugated goat anti-Mouse IgG secondary antibody (Boster) at room temperature and nuclei were stained with 1 µg/µl DAPI. We used PBS to wash cells three times during each step during this process. In the end, images were visualized with fluorescent microscope at x200 magnification.

Quantitative real-time PCR (qRT-PCR). HH signaling pathway-related gene (GLI1, PTCH1, Smo, IFT88, PTHrP) expression was measured by RT-PCR. Total RNA was
extracted from cells with TRIzol reagent after 48 h incubation with HPI-4 in 6-well plates and then 2.5 µg of total RNA was used to synthesize cDNA with the SuperScript II cDNA synthesis kit (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. The total PCR system contained cDNA, SYBR-Green, no RNA enzyme water and primers; the primer sequences are listed in Table I.

**Western blot analysis.** These procedures were based on previously described ones (6). Total cell proteins (45 µg/lane) were loaded and separated by sodium dodecyl sulfate (SDS) polyacrylamide gels, transferred onto PVDF membranes. We used the following primary antibodies: MMP2 (1:1,000), MMP9 (1:1,000) (both from Cell Signaling Technology), IFT88 (1:1,000; Abgent, USA), CyclinD1 (1:100; Boster) and PTHrP (1:100; Santa Cruz Biotechnology, Inc.); β-actin (1:400; Boster), along with secondary antibody horseradish peroxidase-labeled goat anti-rabbit and goat anti-mouse IgG (1:5,000; Boster). We used ECL western blotting detection kit (Thermo Fisher Scientific, USA) to detect all the protein bands and were visualized using an enhanced chemiluminescence system (Bio-Rad, Hercules, CA, USA). All values were expressed relative to β-actin.

**Statistical analysis.** Each experiment was performed at least three times independently. These data are represented as mean ± SD. We used the Student's t-test or one-way analysis of variance (ANOVA) to analyze the differences between means, and P<0.05 was considered to indicate a statistically significant difference. All statistical analyses were performed using SPSS 20.0.

**Results**

**Human chondrosarcoma prominently expresses Ihh-PTHrP signaling-related proteins.** Previous studies demonstrated that a variety of tumors exist with abnormal activation of the HH signaling pathway (7-11). Using immunohistochemistry assay, we detected the expression of the Ihh-PTHrP-related proteins in human chondrosarcoma tissues. These tumor tissues exhibited significantly high level of staining intensity for Ihh, GLI1, GLI2 and PTHrP (Fig. 1B-E) compared with the negative control (Fig. 1A). At the same time, tumor tissues had a considerable percentage of positive Ki67 staining in approximately half of total tumor cells (44.30±4.52%). Obvious expression of Ki67 confirmed that chondrosarcoma had considerable proliferation ability (Fig. 1F). On the other hand, apparent expression of Ihh and PTHrP simultaneously indicated that there appeared no natural negative feedback regulation between Ihh and PTHrP proteins. These results demonstrated that the distinct activation of HH signaling pathway may have a positively correlated relationship with elevated proliferation ability in chondrosarcoma.

**HPI-4 decreases chondrosarcoma SW1353 cell proliferation ability.** As shown in Fig. 2, in order to examine the effect of HPI-4 treatment on human chondrosarcoma cell growth, SW1353 cells were stimulated respectively with 5 and 10 µM HPI-4 and the changes were explored using MTS-8 assay. With the progress of time, SW1353 cells cultured with HPI-4 revealed an obvious reduction in proliferation rate compared with the empty control group (Fig. 2A). In addition, in order to detect the effect of this drug on a single cell to form a tumor mass, we conducted a tablet colony formation assay (Fig. 2B). After 4 weeks of incubation, the empty control group number was 272±19.8 per well compared with 5 µM (191.00±8.49, P<0.05) and 10 µM (90.50±16.26, P<0.05). The average size showed the same trends at different doses (Fig. 2B). These results confirmed that HPI-4 clearly reduced the ability of a single cancer cell to generate a colony by continuous division, including numbers and sizes of colonies (Fig. 2C). Moreover, western blot assay presented a similar conclusion that inhibiting HH signaling eventually suppressed cell cycle protein CyclinD1 in HPI-4-treated cells along with the higher concentration (Fig. 2D). CyclinD1 is considered a vital symbol of cell cycle progression.

**HPI-4 inhibits the invasion and migration ability of human chondrosarcoma cells.** Migration and invasion are important indicators to evaluate the degree of tumor malignancy, they determine the severity of the tumor and the prognosis of treatment (7,8). In order to illustrate the influence of HPI-4 compound on tumor migration and invasion ability, we performed the Transwell and cell scratch experiment in vitro. Fig. 3A-D shows that the number of cells penetrating through the Matrigel matrix decreased significantly as the drug concentration increased (the white arrows). Matrigel matrix is regarded as the best component to imitate extracellular matrix. Scratch experiment was used to simulate the migration of tumor cells. The results showed that a higher concentration of HPI-4 observably inhibited tumor cell migration rate as time passed. HPI-4 effectively delayed scratch healing time (Fig. 4A and B). At the same time, we conducted western blot experiment to test the expression of matrix metalloproteinases (MMPs) which reflects tumor cell decomposition of the extracellular matrix ability to promote invasion and migration (22,23). The results demonstrated that after stimulating for 3 days, the expression level of MMP2, MMP9 decreased in HPI-4-treated cells. Moreover, as MMPs upstream signaling

<table>
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<th>Gene</th>
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Figure 1. Immunohistochemical staining of HH pathway-related proteins in human chondrosarcoma tissues. (A) Representative staining as negative control without first antibody in human chondrosarcoma tissues. HH signaling pathway target protein (B) Ihh, (C) GLI1 and (D) GLI2 were clearly stained positive. (E) Downstream target protein PTHrP was also stained clear brown and this indicated an activation of PTHrP-regulated proliferation. (F) Nearly half of total tumor cells (44.30±4.52%) presented significantly positive Ki67 expression. Similar to the white arrow, a brown color in chondrosarcoma tissue is defined as a positive staining.

Figure 2. Decrease in proliferation and colony formation abilities of human chondrosarcoma cell line SW1353 following treatment with HPI-4. OD value (absorbance at 450 nm) was used as an index to evaluate cell proliferation ability. (A) The first line chart shows that higher concentration of HPI-4 inhibited tumor cell proliferation with time (1, 3, 6 and 9 days). (B) HPI-4 clearly inhibited proliferation ability so that a single cell could not form a colony with a large range after treating with HPI-4 for 4 weeks and (C) the histogram displays the average number of colonies. The test groups were compared with the control group (P<0.05).
pathway, the MAPK/ERK pathway was also restrained by HPI-4, mainly through inhibiting ERK and phosphorylated-ERK (p-ERK) proteins to downregulate downstream MMPs (Fig. 6A-D).

HPI-4 blocks the HH signaling pathway by disturbing ciliogenesis. We previously demonstrated that an apparent activation of HH signaling pathway exists, which may be associated with tumorigenesis in human chondrosarcoma (Fig. 1). The mechanism of HPI-4 as an inhibitor of HH signaling pathway is not fully clear, although some studies have demonstrated that this drug does not act on conventional Smo receptor (14). However, primary cilia are an indispensable part of the HH signaling pathway. This cell organelle can be counted and described under the field of vision and was regarded as an observed morphology symbol of cell cycle (15,16). In order to detect whether HPI-4 realizes its function through interacting with primary cilia, we used fluorescence microscope to observe the expression percentage of primary cilia. Normal chondrosarcoma SW1353 cells present primary cilia 20.55±4.40%, while adding 5 µM HPI-4 resulted in a decrease to 15.06±5.15% (P<0.05) and 10 µM to 7.38±1.91% (P<0.001). The results confirmed that HPI-4 distinctly downregulates ciliogenesis, particularly at high concentrations (Fig. 5A-D). Moreover, RT-PCR analysis showed that the expression of cilia microtubule transporters IFT88 significantly decreased (P<0.05) and the HH pathway target genes PTCH1 and GLI1 decreased distinctly (P<0.05), but only the higher concentration of HPI-4 suppressed Smo receptor expression. Thus, we speculated that HPI-4 may inhibit the action of the HH pathway by disrupting ciliogenesis rather than by suppressing Smo (Fig. 7E and F).

HPI-4 inhibits the expression of PTHrP regulated by reducing GLI2. HH and PTHrP play an important role in regulating the growth process of bone and cartilage. In normal cartilage tissue, an Ihh/PTHrP negative feedback loop exists. HH signaling pathways activate downstream GLI transcription factors to regulate the generation of PTHrP (2-6). We previously showed that GLI2 and PTHrP have a certain collaborative expression in the human chondrosarcoma tissues with apparent activation of the HH signaling pathway (Fig. 1E). As shown in Fig. 7, after stimulating chondrosarcoma cells with different concentrations of HPI-4, ciliogenesis-related microtubules transporter IFT88 was inhibited, which led to primary cilia dependency. HH pathway transduction was blocked and downregulated transcription factor protein GLI2. Thus, chained along with suppression of PTHrP protein and mRNA expression simultaneously (Fig. 7A-D).
As a type of malignant tumor secreting cartilage-like matrix, human chondrosarcoma has different general properties from other bone tumors. Depending on the tumor differentiation degree, the malignant severity is also different. Chondrosarcoma always occurs with a proclivity for long and flat bones, the main parts of weight bearing structures. This tumor can invade surrounding tissues and the consequences caused by mechanical structure damage cannot be ignored. Due to secreting cartilage-like matrix and lacking vascular distribution, chondrosarcoma is not sensitive to radiotherapy or chemotherapy and has high risks of recurrence and metastasis after surgical resection (1). Therefore, controlling the invasion and metastasis is key in guiding the treatment and improving prognosis of chondrosarcoma.

The HH signaling pathway is an important pathway in the progression of bone and cartilage development. Previous studies have confirmed the existence of HH/PTHrP negative feedback loop in normal growth plates to regulate cell growth and differentiation (1-3), but it is absent in cartilage neoplasia (2,12). Furthermore, abnormal activation of HH signaling was found in different sources of malignant tumors, such as breast cancer, human renal cell carcinomas and gastrointestinal malignant tumor (7-11). Nevertheless, the specific pathogenesis for the abnormal activation of HH pathway that induces oncogenesis remains unclear. In the present study, we detected that apparent expression of protein Ihh, GLI1, GLI2 and PTHrP existed in human chondrosarcoma and this level of HH signaling activation was accompanied by strong proliferation ability. By using HH signaling pathway inhibitor HPI-4 to stimulate chondrosarcoma cell line SW1353

Figure 4. Tumor cell migration ability is downregulated by HPI-4. Wound closure assay was used to illustrate tumor cell migration ability. Confluent monolayer of SW1353 cells was wounded by scratching with a pipette tip. (A) Under different concentrations of HPI-4 treatment (0, 5 and 10 µM), cell migration speed recorded visible differences at 24 and 48 h. (B) Line chart shows average migration rate of cells. HPI-4 clearly inhibited tumor cell migration ability and delayed wound healing time.
**Figure 5.** HPI-4 perturbs chondrosarcoma cell ciliogenesis. Human chondrosarcoma cells SW1353 were stained with acetylate-α tubulin (red) antibody to detect primary cilia. Comparison of the (A) control group and the (B and C) HPI-4-treated groups showed a lower manifestation of primary cilia (the white arrows) and (D) the histogram shows the percentage of cilia present. Cells were counted in 10 randomly selected microscopic fields and a P-value <0.05 was considered statistically significant compared with the control group (*P<0.05).
in vitro, we illustrated that suppression of HH signaling could decrease tumor cell proliferation activation as time passed. Furthermore, MAPK/ERK signaling mediated tumor invasion-related proteins matrix metalloproteinases MMP2 and MMP9 (22,23) expression declined visibly by use of HH signaling inhibitor HPI-4. This phenomenon indicates that there may exist a downstream crosstalk between HH signaling and MAPK/ERK signaling pathways. HH signaling inhibitor HPI-4 could have an obvious inhibitory effect on tumor invasion ability by regulating the expression of MMPs eventually. Therefore, we concluded that HH signaling pathway activation is closely linked with tumor cell proliferation activity and enhancing the decomposition of the extracellular matrix, thereby affecting tumor invasion and metastasis.

In the HH pathway inhibitor family, HPI-4 has a different interaction mechanism from other HH signaling pathway inhibitors. For example, the classical inhibitor cyclopa mine is able to block Smo receptor and GANT51/GANT61 directly inhibits the expression of GLI family to interrupt HH pathway (13,14), while HPI-4 may affect ciliogenesis to block this signaling pathway. Primary cilia are believed to regulate cell cycle progression and are considered a sign of ending cell division (15-18). In normal cells, primary cilia only present on non-proliferating cells and play an important role in initiating cilia-dependent HH pathway (4,15-19). Cilia related IFT88 transfer in primary cilia and reflect the activity of material transportation in cilia (16). Theoretically, cilia microtubules disassembling during cell division progression and proliferation cells should lack primary cilia. However, after stimulating with HPI-4, the number of extracellular cilia decreased and intra-cilia transport protein IFT88 expressed in significant decline on gene and protein level accompanied by multiplication ability suppression. This illustrated that the drug might disrupt the normal function of cilia and inhibit cell proliferation simultaneously, accompanied by restraining the expression of HH downstream target genes GLI1 and PTCH1. Hence, we can infer that HPI-4 can downregulate HH activation by disrupting ciligenesis and affecting their function. Primary cilia may be a crucial cell organelle involved in tumor initiation and progression.
Regarded as a vital factor in the regulation of osteogenesis and osteolysis, PTHrP is closely related to HH signaling pathway (5,6,20,21). Previous studies revealed that PTHrP expression could be increased by TGF-β stimulation, which could upregulate HH downstream transcription molecule GLI2 expression. GLI2 could be activated and transferred into nucleus to launch a variety of cytokines transcription, especially PTHrP (6). By inhibiting the activity of the HH signaling, we found its downstream GLI2 expression decreased and endogenous PTHrP was restrained. As a result, we infer that the HH pathway inhibitor could affect to block osteolysis caused by PTHrP overexpression in tumor.

In summary, this study revealed that abnormal HH signaling pathway existed in human chondrosarcoma tissues. HPI-4, a new special antitumor compound, can perturb tumor cell ciliogenesis to block HH signaling pathway, consequently suppressing chondrosarcoma cell proliferation, invasion and migration abilities. These conclusions indicate that HPI-4 may be an effective therapy option to treat chondrosarcoma with abnormal activation of the HH signaling pathway.

Acknowledgements

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