Inhibition of fatty acid synthase supresses osteosarcoma cell invasion and migration

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Abstract

Background: Fatty acid synthase (FASN) is overexpressed in a variety of human cancers, and may be involved in cancer metastasis. Hence, the strategies targeted on FASN may have therapeutic potential for treating cancer metastasis.

Objectives: The aim of this study is to investigate the correlation of FASN expression with metastasis in human osteosarcoma.

Materials and Methods: Human osteosarcoma cell lines U2-OS and osteosarcoma biopsy specimens were employed in this study. The expression of FASN protein in osteosarcoma specimens was detected by IHC (immunohistochemistry) and the relationship with metastasis was analyzed. We performed the cerulenin, an inhibitor of FASN, to inhibit FASN expression in U2-OS cells. Western blot and RT-PCR were performed to investigate the expression of FASN in U2-OS cells. Cells mobility was detected by wound healing and Transwell assays.

Results: Results showed that the FASN expression level in the cases with pulmonary metastases was significantly higher than in those without metastasis. In vitro, the invasion and migration of U2-OS cells were suppressed by inhibiting FASN. Our findings suggested that FASN may be involved in osteosarcoma metastasis.

Keywords: Fatty acid synthase, immunohistochemistry, metastasis, osteosarcoma

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Introduction

Osteosarcoma (OS) is one of the most common primary malignant bone tumor in childhood and adolescents. It was not until the early 1970s that the introduction of doxorubicin and methotrexate with leucovorin rescue showed promise to improve the survival. After the effective chemotherapy advent, the 5-year survival rate of patients treated with intensive multirdrug chemotherapy and aggressive local control have been reported at 55%-80%. [1][2][3] Despite the encouraging trend to longer survival many patients still face a dismal outcome. Numerous articles have reported that the five years survival rate of patients with metastatic diseases less than 20%. [4][5][6] Clearly, the impact of identifying the factors that govern metastasis is significant on the management of osteosarcoma.

Fatty acid metabolic pathways play an important role in linking to carcinogenesis. [7] Fatty acid synthase (FASN) is an enzyme crucial for endogenous lipogenesis in mammals, responsible for catalyzes the synthesis of long-chain fatty acids. FASN is critical to sustain the biological features of cancer cells. [8] FASN is expressed at high levels in a variety of human tumors, [9][10][11][12][13][14][15][16][17] but low levels in normal tissues. Many studies have reported that inhibition expression of FASN could suppress cancer cells proliferation in vitro and vivo. [18][19][20][21][22][23] Thus, FASN is considered a novel promising target for anticancer therapy. Recent studies revealed that FASN may be contributed to cancer cells metastasis. [24][25] However, whether FASN is involved in OS metastasis has not been reported.

In this study, we investigated the correlation of FASN expression levels with pulmonary metastases among patients with osteosarcoma of extremities. The expression of FASN in OS with pulmonary metastasis was evaluated using IHC, as same as OS without metastasis. Furthermore, the effect of inhibition to FASN on cells invasion and migration in vitro was investigated. We performed an inhibitor of FASN, cerulenin, to inhibit FASN expression in U2-OS cells. And the cells migration and invasion were investigated by using wound healing and Transwell assay. We found that the cells invasion and migration were inhibited by inhibiting FASN. We confirmed that FASN may be involved in osteosarcoma metastasis.

Materials and Methods

Patients and Specimens

A total of 136 cases of extremities OS samples were obtained from patients who underwent surgery in our hospital (The First Hospital Affiliated to Nanchang University, China) from 2005 to 2009. The pulmonary metastasis survey was performed with plain films and chest CT scans at first diagnosis. All the patients do not have a history of prior therapies with anticancer drugs or radiotherapy. According to the Ennecking classification, there were 24 cases in stage Ia (17.6%), 42 in stage Ib tumors
(30.9%), 32 in stage IIa (23.51%), 32 in stage IIb (23.51%), and 6 in stage III (4.4%). There were 78 tumors diameter larger than 8 cm, 58 tumors diameter small than 8 cm: patho-subtype: osteoblastic osteosarcoma 57.4%, fibroblastic osteosarcoma 25%, chondroblastic osteosarcoma 17.6%. The patients’ characteristics included: age ≥16 years, 40.7%, <16 years 59.3%; male 61.8%, femal 38.2%; with pulmonary metastasis 32.4%, without metastasis 67.6%. The samples were fixed with 10% formalin and embedded in paraffin, and then, were cut into 2 μm sections. Informed consent was taken from all subjects, and the Institute Ethics Committee approved the study protocol.

**Immunohistochemical Analysis**

Histological sections cut at 2 μm were stained with hematoxylin and eosin (H and E) staining and detected by immunohistochemical analysis that was performed with S-P procedure. Briefly, antigen retrieval was performed by heating the deparaffinized rehydrated sections in 10 mm citrate buffer (pH 6.0) for 20 min, followed by blocking with 10% goat serum. Then sections were incubated overnight at 4 °C with the primary antibody (rabbit anti-FASN monoclonal antibody, Santa Cruz) at a final dilution of 1:800. For negative controls, sections were incubated with PBS instead of antibodies. After washing with PBS for three times, sections were incubated with biotinylated secondary antibody for 40 min, followed by incubation with HRP-conjugated streptavidin for half an hour. Then the sections were chemiluminescence stained and counterstained using hematoxylin. Stained sections were evaluated and scored by two pathologic doctors in a blind manner without prior knowledge of the clinical pathological features of patients. According to the staining intensity by examining at least 500 cells in five representative areas, the expression level of FASN was judged and the intensity scores were recorded as follows: none, 0; weak, 1; moderate, 2; and intense, 3. According to the percentage of tumor cells with positive expression of FASN, the percentage scores were recorded: 0% (score 0); less than 10% (score 1), 10% to 49% (score 2), 50% to 79% (score 3), and 80% to100% (score 4). The final score was averaged with the scores from the two pathologic doctors; these scores were calculated by multiplying the intensity score to the percentage score. For example, when a specimen contained 50% of the tumor cells with moderate intensity, the final score is 4 (2 × 2 = 4). The section with a final score less than 4 were considered as (?), score 4 were considered as (+), score 6 score as (++), and much 6 were considered as (+++).

**Cell Lines and Cell Culture**

We obtained the human OS cell line U2-OS from American Type Culture Collection (Manassas, VA), and routinely cultured in DMEM medium (HyClone) supplemented with 10% fetal bovine serum (FBS) (Sigma) in a humidified 37 °C incubator containing 5% CO2.

**Cell Growth Assay**

U2-OS cell line was cultured in 96-well tissue culture plates at a cell density of 5000 cells per well in MEM containing 10% fetus bovine serum and 2 mM L-glutamine. After attachment overnight, the medium was replaced and cells were incubated with increasing concentrations (0, 1, 5, 10, 20 μg/mL) of Cerulenin. After treatment for 24 h, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assays were carried out at 490 nm wave length in triplication.
Flow Cytometry

Human OS U-OS cells, 5x10^5 cells/mL, were seeded in T25 culture flask for 24 h. The cells were then treated with 1.0, 5.0, 10.0 μg/mL cerulenin for 24 h. After incubation, the cells were trypsinized, then washed with PBS and fixed overnight in ice-cold 70% ethanol. After fixation, the cells were washed twice with 1% BSA in PBS, resuspended in 1 mL DNA-binding propidium iodide (PI) solution (10 mg/mL in PBS, containing 0.05 mg/mL RNase A), incubated at room temperature in the dark for 15 min and analyzed with EPICS XL flow cytometer (Beckman Coulter, Miami, FL). Apoptotic cells were measured using the control software of flow cytometer.

Western Blotting Analysis

U2-OS cells in the exponential growth phase were treated with different concentrations of cerulenin (0, 1.0, 5.0, 10.0 μg/mL) for 24 h. Cells were washed with cold PBS. Total protein from the cells was extracted using RIPA lysis buffer containing 60 μg/mL PMSF. Cell lysates were then subjected to SDS-PAGE followed by western blot analysis as previously described.[26]

Real-time PCR

RT-PCR was used to detect the level of FASN mRNA. Briefly, U2-OS cells were treated with different concentration of Cerulenin (0, 1.0, 5.0, 10.0 μg/mL) for 24 h. The cellular total RNA was extracted with TRIZOL reagent (Invitrogen, Carlsbad, CA, USA). The concentration of total RNA was determined by spectrophotometry at 260 nm and the purity was determined by the 260/280 ratio with a BioPhotometer (Eppendorf, Hamburg, Germany). RT-PCR was routinely performed with the Two Steps kit (Promega, USA) according to the manual instruction to obtain cDNA, which was then used as the template for amplification. FASN forward primer 5’- CCCACCTACGTACTGGCCTA-3’, and reverse primer 5’- CTTGGCCCTTGGGTGTTACT-3’, 294bp; β-actin forward primer 5’- TGGCATTTGCG ACAGGATGCAGAA-3’, and reverse primer 5’- CTCGTCATACTCCTGCTTG CTG AT -3’, 172bp (Sangon, Shanghai, China) were used for amplification. After amplification, DNA electrophoresis was performed on standard 1% agarose gels. And then DNA was labeled with ethidium bromide and images were acquired using Canon DIGITAL IXUS 900Ti.

Transwell Assay

Invasion of U2-OS cells was measured using the BD BioCoatTM BD Matrigel TM Invasion Chamber (BD Bioscience, NJ, U S A) according to the manufacturer’s protocol. Briefly, the cells were transfected with plasmids and selected by neomycin. The medium in the lower chamber contained 5% fetal calf serum as a source of chemo attractants. Cells were suspended in serum-free medium containing various concentrations of cerulenin (0, 10.0 μg/mL) and added to the upper chambers at the same time. Cells that passed through the Matrigel-coated membrane were stained with Diff-Quik (Sysmex, Kobe, Japan) and photographed. Cell migration was quantified by direct microscopic visualization and counting. The values for invasion were obtained by counting three fields per membrane and represented as the average of six independent
experiments done over multiple days.

**Wound Healing Assay**

We assessed cell migration by determining the ability of the cells to move into a cellular space in a two-dimensional *in vitro* "wound healing assay." In brief, cells were grown to confluence in 6-well tissue culture plastic dishes to a density of approximately 5×10⁶ cells/well. After being treated with different concentration of cerulenin (0, 10.0 μg/mL) for 24 h, the cells were denuded by dragging a rubber policeman (Fisher Scientific, Hampton, NH, USA) through the center of the plate. Cultures were rinsed with PBS and replaced with fresh quiescent medium alone or containing 10% FBS, following which the cells were incubated at 37 °C for 24 h. Photographs were taken at 0 and 24 h, and the migrated distance was measured. The cells migration rate was obtained by counting three fields per area and represented as the average of six independent experiments done over multiple days.

**Statistical analysis**

Data were expressed as the means ±SD. The difference of cells invasion and migration between inhibition FASN and control group were evaluated by A one-way ANOVATwo-Independent-Samples and K Independent Samples Test were used for analysis the correlation of FASN expression levels with clinical pathologic parameters, and a value of *P* <0.05 was considered statistically significant. All analyses were performed using SPSS Version 13.0 (Statistical Software for Social sciences, Chicago, IL).

**Results**

**Correlation of FASN Expression Levels in OS Tissues with Clinical Pathologic Parameters**

FASN was expressed in the tumor cytoplasm [Figure 1], which was consistent with previous reports. [27] The correlation found among FASN expression in OS, the clinical and pathological parameters was summarized in [Table 1]. Of all these 136 cases, 86 cases (63.2%) were positive for FASN expression. More interestingly, the positive expression rate of FASN in the cases with pulmonary metastasis was 86.4% (38/44), but only 52.2% (48/92) in those without pulmonary metastasis, the difference was significant. It indicated that FASN may be involved in OS metastasis. we also investigated the correlation of FASN expression levels with other clinical and pathologic parameters. We found that FASN expression was correlated with the Tumor size (*P* = 0.013). The FASN expression levels were much higher in tumor diameter larger than 8 cm than in smaller than 8 cm. No significant correlation was observed between FASN expression levels and other clinical and pathologic variables, such as age at diagnosis (*P* = 0.0708), gender (*P* = 0.229), clinical stage (*P* = 0.996) and pathologic subtype (*P* = 0.965). Our findings revealed that FASN may be involved in the development and metastasis. It suggested that FASN may be a favorable target for treating OS metastases.
Effect of inhibition FASN on U2-OS cell proliferation and apoptosis

In order to investigate effect of inhibition FASN on U2-OS cells proliferation and apoptosis, a specific inhibitor of FASN, cerulenin was used to suppress FASN expression. The cells were treated with various concentrations (1, 5, 10 μg/mL) cerulenin. Western Blot and RT-PCR were performed to measuring the FASN protein and mRNA expression levels. The results showed that the expression of FASN was suppressed by cerulenin at various concentrations compared with untreated cells [Figure 2]. MTT assay was performed to measure the inhibition effect of cerulenin on U2-OS cells proliferation. The results revealed that cerulenin inhibited U2-OS cell proliferation in dose-dependent and IC50 value is 22.63 μg/mL for 24 h [Figure 3]. FACS analysis was used to examine the mechanism of inhibiting cells growth via inhibition FASN. Different concentrations (1, 5, 10 μg/mL) of cerulenin were added to U2-OS cell cultures in the exponential growth phase. The treated and untreated cell samples were taken and fixed for FACS analysis 24 h later. FACS analysis showed that the effect of inhibition FASN on inducing cells apoptosis was increased along with the decrease of FASN expression [Figure 4]. The data showed that inhibition FASN expression could suppress U2-OS cell growth and induce apoptosis in vitro. It suggested that FASN may be a promising target for treating OS.
Inhibition of FASN suppress U2-OS cells invasion and migration *in vitro*

According to IC50 value, the appropriate cerulenin concentration for wound healing migration and Transwell invasion cell assay was determined. To examine the effect of inhibition FASN on mobility of U2-OS cells, the migration and invasion were measured by wound-healing and Transwell assays. The cells were treated with 10.0 μg/mL cerulenin for 24 h in wound healing and Transwell assay, the results showed that the migration and invasion of cells treated with cerulenin were significantly inhibited when compared with untreated cells. The results suggested that inhibit FASN expression could suppress U2-OS cells migration and invasion [Figure 5]. Our data demonstrated that inhibition FASN expression could suppress U2-OS cell migration and invasion in vitro. It suggested that inhibition FASN is likely to be an effective Strategy for treating OS metastases.

**Figure 5: Inhibition FASN inhibits cell migration and suppresses cells invasion**

Discussion

Osteosarcoma is the most common primary malignant tumor in youth and lung metastasis is the most important factor which affecting the prognosis of patients with osteosarcoma. To study the molecular mechanisms of lung metastasis of osteosarcoma is very important to improve survival in patients with metastatic disease. Currently a large number of studies suggested that the WNT and TGF beta pathway in osteosarcoma transfusion plays an important role. Recent studies have found that the metabolic pathway also plays an important role in tumor metastasis.

Human FASN is a 270-kDa cytosolic dimeric enzyme that responsible for fatty acid synthesis. Endogenous fatty acid synthesis from the small carbon precursors acetyl-CoA and malonyl-CoA is dependent on the activity of FASN. In the most of the cells, FASN is down-regulated by the dietary fatty acids, with exception of lipogenic tissues as liver, lactating breast, fetal lung and adipose tissue. Recent studies provided compelling evidence that neoplastic lipogenesis is essential for cancer cell survival. Various studies reported that FASN was over expression in variety of human tumors. [28],[29],[30],[31],[32],[33]

In this study, we demonstrated that FASN was positive expression in 86 samples of all 136 OS cases. And total of moderate (++) and intensively (+++) positive expression was 68 cases (50%). We investigated the correlation of FASN expression levels with clinical pathologic parameters. No relationship of FASN expression levels with clinical pathologic parameters (P>0.05), such as pathologic subtype, clinical stage (Ennecking stage), gender and age at diagnosis, was observed. Unfortunately, the OS tissues used in our study were paraffin specimens and the patient’s contact information was incomplete,
the patient's information of postoperation was unable to obtain completely. Result in the correlation between FASN expression levels in OS tissues and prognosis, survival time have not been analyzed. But, Kaste, [35] Ozger [36] reported that with metastasis and tumor size at diagnosis are the important prognosis factors for patients with extremities OS. In present study, the expression levels of FASN was significant higher in tissues of the tumor diameter larger than 8 cm than in small than 8 cm (P=0.013). Our results indicated that FASN over expression should be significantly correlated to the tumor size, but not correlated to other clinical parameters such as gender, age at diagnosis and histological subtypes and grading, which was consistent with previous reported. [27]-[34]

From our results and previous studies, we could infer that FASN may be an important treating target and predictor of prognosis for OS.

Recent studies showed that the FASN expression level was associated with tumor cells metastasis in vivo. [24],[25] In our research, there was a similar result that the relationship of FASN and tumor cells metastasis. FASN was over expressed in 38 samples of all 44 patients with pulmonary metastasis, but only 48 cases positive expression in all 92 patients without metastasis. The difference of expression levels was significant (P=0.002). It suggested that FASN could be involved in OS metastasis.

A large number of studies showed that inhibition of FASN can effectively inhibit tumor cells proliferation in vitro. [21],[27] [38],[39],[40] In our study, to clarify whether the inhibition FASN expression could inhibits OS cells proliferation, we used a specific inhibitor, cerulenin, to silence FASN expression in U2-OS cells. The western blot and RT-PCR results showed that FASN expression was inhibited by cerulenin in dose dependent. MTT cell proliferation assay was performed to investigate the effect of inhibition FASN expression on U2-OS cells proliferation. Our data showed that the cells growth was suppressed by inhibition FASN, and the inhibition effect of inhibiting FASN on U2-OS cells proliferation was increased as the result of the decrease of FASN expression. FACS analysis was used to examine the mechanism of inhibiting cell proliferation via inhibition FASN. The results showed that the rate of apoptotic cells was increased with FASN expression level decreased. The data indicated that inhibition FASN could suppress U2-OS cells proliferation via induce apoptosis. It suggested that inhibition FASN may be a Treatment strategy for treating OS.

To confirm whether FASN was involved in OS metastasis, we performed the wound healing invasion assay and Transwell migration assay in vitro to investigate the effect of inhibiting FASN expression on U2-OS cells mobility. The cerulenin, a specific FASN inhibitor, was performed to inhibit FASN expression in U2-OS cells. According to IC50 value, the appropriate concentration, 10.0 μg/mL cerulenin, for wound healing migration and Transwell invasion cell assay was determined. Western and RT-PCR revealed that FASN protein and mRNA expression in cells treated with 10.0 μg/mL cerulenin for 24 h were significant inhibited. The wound healing assay and Transwell assay showed that the migrated rate and invasion cells of U2-OS cells untreated with cerulenin was significant higher than cells treated with cerulenin. The results, similar to Selvendiran et al. [41] reported, showed that the cells invasion and migration were suppressed by inhibiting FASN. It suggested that inhibition FASN could suppress invasion and migration of OS cells in vitro.

Conclusion
In sum, our results demonstrated that FASN may involve in OS metastasis. However, previous studies showed that tumor microenvironment might influence tumor progression, invasion, and cell migration. So, furthermore experiments in vivo are necessary to be performed to clear that the FASN may be a promising target and predictor of prognosis for treating OS metastases.

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