

Original Article

Interaction between fatty acid synthase and human epidermal growth receptor 2 (HER2) in osteosarcoma cells

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Abstract: Elevated expression of fatty acid synthase (FASN) and human epidermal growth factor receptor-2 (HER2) are observed in human osteosarcoma (OS). The aim in this study is to investigate a possible connection between FASN expression and the activity of HER2. The immunohistochemistry staining was conducted on 24 OS specimens from patients, which revealed a significant positive correlation between FASN and HER2 as well as p-HER2 protein expression. Furthermore, the human OS cell lines MG-63 and U2-OS were treated with FASN-specific RNAi Plasmid or Lapatinib (an inhibitor of HER2). The mRNA of HER2 and FASN was measured using RT-PCR. Western blot was performed to detect the protein expression of HER2, p-HER2 and FASN. The results demonstrated that HER2 modulates FASN expression, inhibition of FASN resulted in down-regulation of HER2 and p-HER2 protein in OS cells. Our findings suggested that there was positive feedback regulation between FASN and HER2 expression and phosphorylation in OS cells.

Keywords: Osteosarcoma, FASN, HER2, positive feedback regulation

Introduction

Fatty acid synthase (FASN) is an enzyme crucial for endogenous lipogenesis in mammals, responsible for catalyzes the synthesis of long-chain fatty acids. As a large protein with a complex structure and multiple catalytic domains, FASN is considered as an important metabolic enzyme and a potential target in human cancers. FASN is elevated expression in a variety of tumors and involved in cell proliferation, apoptosis, migration and invasion [1-4]. Our previous studies demonstrated that FASN may be contributed to osteosarcoma cells proliferation, apoptosis and metastasis [5-7]. However, its potential molecular mechanism of increased expression of FASN in OS cell is still unclear. Substantial evidences revealed that the transcriptional regulation of FASN expression has been considered to be the main cause for the elevated FASN expression in malignant tumor cells [8]. Furthermore, study showed that ligand stimulation induces dimerization of the HER2

receptor (homodimer or heterodimer), which leads to self-phosphorylation on tyrosine residues localized to the C-terminal domain of HER2 receptors [9]. The phosphorylated HER2 receptors activated a variety of downstream signaling pathways, such as the phosphatidylinositol3-kinase (PI3K)/Akt [10], which plays an essential role in regulation FASN expression [11].

HER2 is a 185-kDa transmembrane receptor tyrosine kinase (RTK), belonging to the epidermal growth factor receptor (EGFR) family. Increased expression of HER2 is found in various types of tumor, including breast cancer [12], ovarian cancer [13] and OS [14]. High levels of HER2 expression are associated with recurrence and death in malignant tumor patients [15]. Various studies have revealed that targeting HER2 is an important therapeutic strategy for treating OS [16, 17]. Recently, Nan Li et al reported that inhibition of FASN effectively inhibits the activity of "HER2-PI3K/Akt

Table 1. Primer information

Primer	Sequences 5' to 3'
FASN-262bp-F	GTCGGAGAACTTGCAGGAGT
FASN-262bp-R	TCCTCGGAGTGAATCTGGGT
HER2-108bp-F	CCCTGACCTGCTGGAAAAG
HER2-108bp-R	GGCCGACATTCAGAGTCAAT
GAPDH-199bp-F	CAGGGCTGCTTTAACTCTGGT
GAPDH-199bp-R	GATTTGGAGGGATCTCGCT

axis" and alters the malignant phenotype in colorectal cancer cells [18]. But, how FASN interacted with HER2 in OS was remained unknown.

In this study, we analyzed the potential link between FASN and HER2 expression in OS cells.

Materials and methods

Patient specimens

A total of 24 samples of OS tissues were obtained from patients with pulmonary metastatic disease who underwent surgery in our hospital (First Affiliated Hospital of Nanchang University, China). The pulmonary metastasis survey was performed with plain films and chest CT scans at first diagnosis. All the patients have no history of prior therapies with anticancer drugs or radiotherapy. The samples were fixed with 10% formalin and embedded in paraffin, and then, were cut into 4 μ m sections. In all cases, informed consent was obtained from the relative departments and persons, and the study had the approval of the Ethics Committee of Nanchang University.

Immunohistochemistry

Immunoperoxidase procedure (S-P procedure) and hematoxylin and eosin (H&E) staining were performed on paraffin-embedded sections. Antigen retrieval was performed with heating the sections in 10 mmol/L citrate buffer (pH 6.0) for 20 min. Rabbit anti-FASN (1:500, Abcam), mouse anti-HER2 (1:200, Abcam) and p-HER2 (Y1248) monoclonal antibodies were used as the primary antibody. 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), 10-49% (score 2), 50-79% (score 3), and 80-100% (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 ($2 \times 2 = 4$). 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 (+++). For HER2 and p-HER2, only specimens with more than 25% highly (+++) or moderately (++) positive cells were classified as positive.

Construction of the recombinant plasmid targeting FASN gene

The human cDNA sequence encoding FASN protein (NM_004104.4) was obtained from GenBank. miRNA and control single strain DNA oligos were designed and synthesized using the following primer sequences: forward 5'-TGCTGAACCTCCTGCAAGTTCTCCGACGTTTTGGCCACTGACTGACGTCGGAGATTGCAGGAGTT-3' and reverse 5'-CCTGAACCTCCTGCAATCT CCGACGTCAGTCAGTGGCCAAAACGTCGGAGA ACTT GCAGGAGTTC-3'. Products were cloned into the express vector pcDNA6.2-GW/EmGFP-miR using the BLOCK-iT™ Pol II miR RNAi Expression Vector kit with EmGFP (K4936-00; Invitrogen Life Technologies, Carlsbad, CA, USA). The DNA sequence of the plasmid was confirmed using the PureLink HiPure Plasmid DNA kit (K2100-03; Invitrogen Life Technologies).

Cell culture

The human OS cell lines MG-63 and U2-OS cells well purchased from American Type Culture Collection (Manassas, VA), and routinely cultured in RPMI-1640 (HyClone) supplemented with 10% fetal bovine serum (Sigma) in a humidified 37°C incubator containing 5% CO₂.

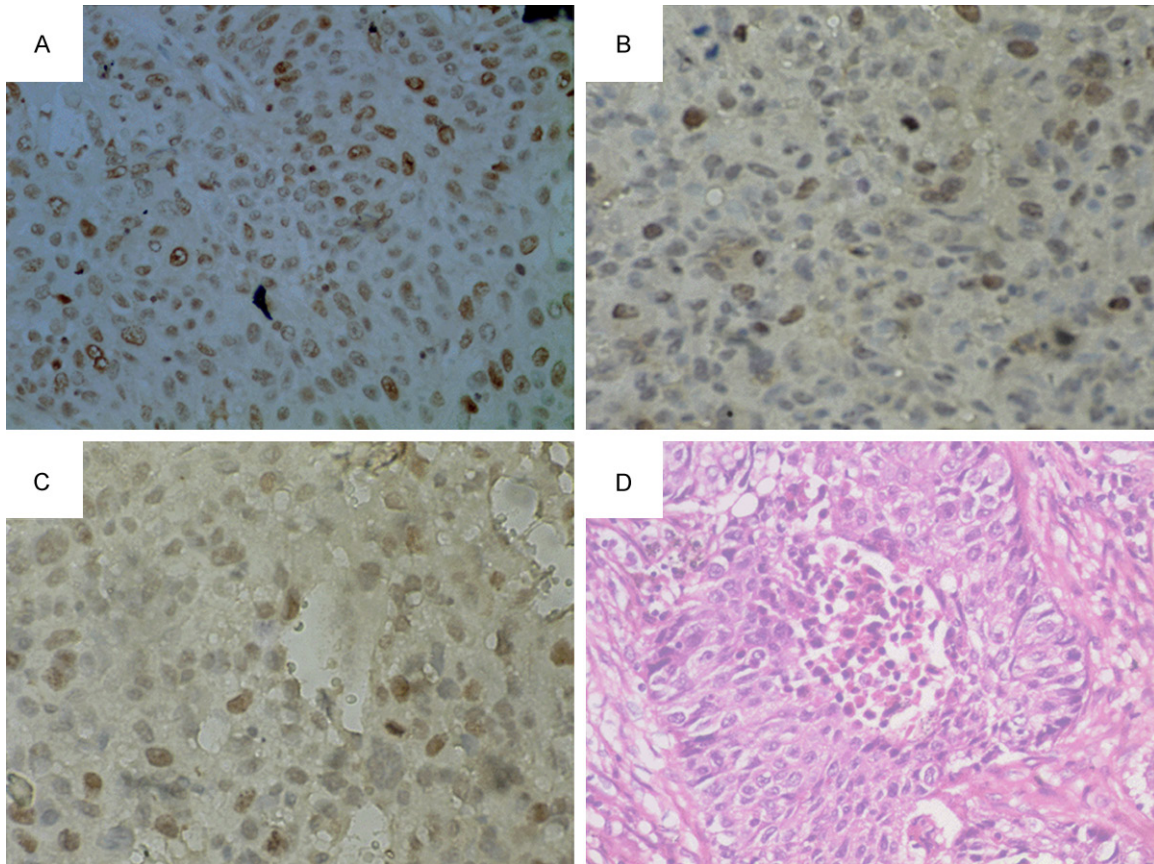


Figure 1. H&E staining (D, $\times 400$) and immunohistochemical staining (A-C, $\times 400$) assays. FASN (A) protein was expressed in the cytoplasm. HER2 (B) and p-HER2 (C) protein were both expressed in cytoplasm and nucleus.

Cell treated with FASN-specific RNAi plasmid or Lapatinib

MG-63 and U2-OS cells were seeded in 6-well plates at 40% confluence on the day prior to deal with FASN specific RNAi plasmid (RNAi-FASN) and the negative control plasmid (Neg) using Lipofectamine 2000 according to the Invitrogen technical bulletin. In additional, We previous study showed that The IC₅₀ values for lapatinib in U2-OS and MG-63 cells at 24 h were 22.150 and 11.646 $\mu\text{mol/l}$, respectively [19]. Therefore, in this study, the U2-OS and MG-63 cells were treated with 20 $\mu\text{mol/l}$ and 10 $\mu\text{mol/l}$ Lapatinib, respectively.

Real-time PCR

Semi-quantitative PCR was used to detect FASN and HER2 mRNA levels. Total RNA was extracted from cells using TRIzol reagent (Invitrogen Life Technologies). Total RNA concentration was determined by spectrophotometry at 260

nm and the purity was determined by calculating the 260/280 ratio with a BioPhotometer (Eppendorf, Hamburg, Germany). The Two-Step kit (Promega Corporation, Madison, WI, USA) was used to obtain cDNA according to the manufacturer's instructions, which was then used as the template for amplification. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal standard. The primer sequences were listed in **Table 1**. All experiments were repeated by six times over multiple days.

Western blot analysis

Total protein from the cells was extracted using RIPA lysis buffer containing 60 $\mu\text{g/ml}$ PMSF. Cell lysates were then subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by western blot analysis according to manufacturer's instructions. The determination of gray scale value was processed by Image J software. All experiments were repeated by six times over multiple days.

Interaction between FASN and HER2 in OS cells

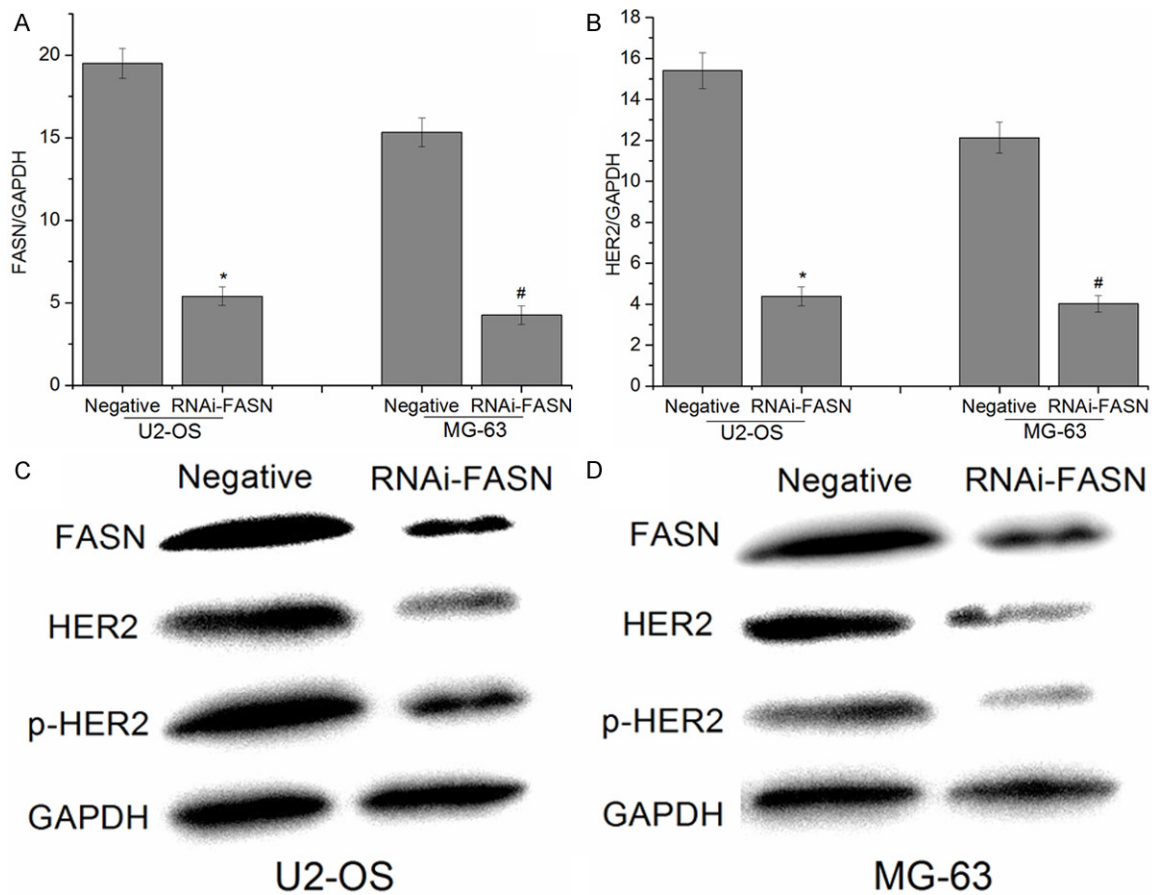


Figure 2. The FASN specific RNAi plasmid was performed to inhibit the FASN expression in U2-OS and MG-63 cells. The mRNAs of FASN (A) and HER2 (B) were significantly down-regulated (Columns, mean (n = 6); bars, S.D; *P < 0.05, #P < 0.05 VS negative). FASN, HER2 and P-HER2 proteins expression in U2-OS cells (C) and MG-63 cells (D) were also inhibited. Results suggested that inhibiting FASN could down-regulate the expression and phosphorylation of HER2 in OS cells.

Statistical analyses

Statistical comparisons were performed using SPSS Version 13.0 (SPSS Inc, Chicago, IL, USA). The correlation of FASN with p-HER2 and HER2 protein in OS tissues was evaluated using the Wilcoxon rank Sum Test. All Measurement data were presented as $\bar{x} \pm SD$. The Independent-samples T-test was performed for statistical analysis. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Positive correlation between FASN and p-HER2 (Y1248), HER2 protein in tissues of OS with pulmonary metastasis

To explore a possible relationship of FASN with HER2 and p-HER2 in OS with pulmonary meta-

static disease, the FASN, HER2 and p-HER2 protein in 24 samples from patients with pulmonary metastatic disease was detected by immunohistochemistry. FASN protein was expressed in the cytoplasm (**Figure 1A**), which was consistent with previous reports [5]. HER2 and p-HER2 were both in cytoplasm and nucleus (**Figure 1B and 1C**). The relationship was significant between FASN and HER2 as well as p-HER2 protein expression level (Spearman's rho, $r_s = 0.502$, $r_s = 0.444$). It suggested that a possible connection between FASN expression and HER2 expression and phosphorylation may exist in OS cells.

Inhibition of FASN down-regulate phosphorylation and expression of HER2

To investigate the effect of silencing FASN on the phosphorylation and expression of HER2 in

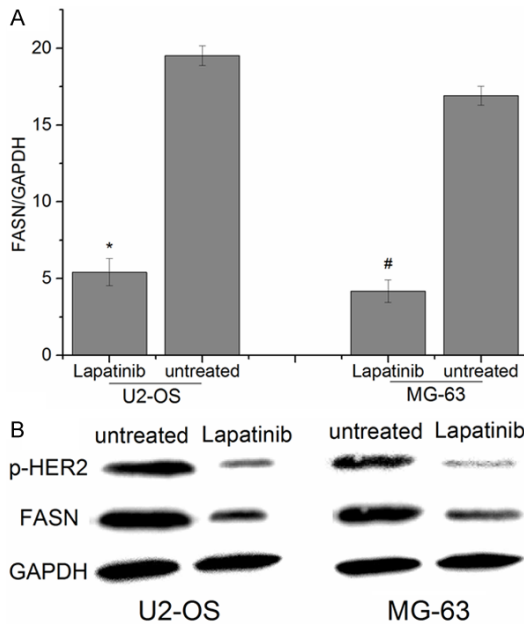


Figure 3. The tyrosine kinase inhibitor Lapatinib was used to inhibit HER2 phosphorylation. The mRNAs of FASN (A) in OS cells treated with Lapatinib was significantly down-regulated (Columns, mean (n = 6); bars, S.D; * $P < 0.05$, # $P < 0.05$ VS untreated OS cells). The expression of FASN protein (B) was also inhibited in OS cells treated with Lapatinib. Results suggested that inhibiting HER2 phosphorylation could down-regulate the expression of FASN in OS cells.

OS cells. The FASN specific RNAi plasmid was performed to inhibit the FASN expression in U2-OS and MG-63 cells. The mRNA of FASN and HER2 was detected by RT-PCR, and western was used to measure the protein expression of FASN, HER2 and P-HER2. As show in **Figure 2**, knockdown of FASN result in down-regulation the expression and phosphorylation of HER2 in OS cells.

Inhibition of HER2 phosphorylation down-regulate FASN expression in OS cells

To investigate whether down-regulation phosphorylation of HER2 could decrease the expression of FASN in OS cells, the U2-OS and MG-63 cells were treated with Lapatinib (tyrosine kinase inhibitor). The mRNA and protein of FASN were measured using RT-PR and western blot. The results showed that both of mRNA and protein of FASN were significant lower in cells treated with Lapatinib than those in untreated cells. It indicated that decreased HER2 phosphorylation could inhibits FASN expression in OS cells (**Figure 3**).

Discussion

Increasing evidences indicated that FASN and HER2 elevated expression play an important role in cancer cell proliferation, survival and metastasis [1-4, 12-15]. Our previous study showed that inhibition of FASN or HER2 could suppress OS cells proliferation, migration and invasion [5-7, 11, 19]. Thomas W et al reported that there was an interaction between fatty acid synthase and ErbB-systems in ovarian cancer cells [20]. In this study, we firstly investigate the interaction between FASN and HER2 in OS cells. To evaluate the potential relationship between FASN expression and HER2 activity, the expression of FASN, HER2 and P-HER2 protein in 24 OS tissues was detected by IHC and the correlation of FASN with HER2 and p-HER2 protein was analyzed. The results revealed that there was positive relationship between FASN and HER2 as well as p-HER2 protein expression. It indicated that a possible connection between FASN expression and HER2 activity may exist in OS cells.

Accumulated evidences revealed that the oncogenic effects of HER2 mainly depend on the preservation of the “lipogenic phenotype”. HER2 activates downstream PI3K/AKT, MAPK and mTOR signaling pathways with subsequent transcriptional activation of FASN expression [21, 22]. To investigated whether inhibition of HER2 phosphorylation could decreases FASN expression in OS Cells, the lapatinib, an inhibitor of HER2 phosphorylation, was used to down-regulate the phosphorylation of HER2 in U2-OS and MG-63 cells. The mRNA and protein of FASN were measured by RT-PCR and Western blot. The results show that both of FASN mRNA and protein was significant inhibited in cells treated with lapatinib, which suggest that phosphorylated HER2 may regulate FASN by affecting transcription and translation in OS cells.

Interesting, various studies show that inhibiting FASN caused a marked decrease in the active forms of HER2 protein [23]. To explore the effect of inhibition of FASN on the phosphorylation and expression of HER2 in OS cells, the FASN specific RNAi plasmid was performed to inhibit the FASN expression in U2-OS and MG-63 cells. We found that the expression of both p-HER2 and HER2 protein were decrease in FASN-silenced OS cells, and the expression

of HER2 mRNA was also suppressed in cells transfected with FASN specific RNAi plasmid when compared with those treated with Negative plasmid. The results suggest FASN may regulate HER2 at the translational and transcriptional level.

In summary, we confirmed that there was a positive feedback loop between HER2 and FASN in OS cells. However, the detailed mechanism on bidirectional connection between FASN expression and HER2 activity in OS cell is currently unknown which requires and deserves further investigation.

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Disclosure of conflict of interest

None.

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