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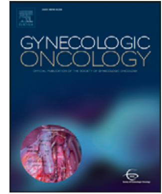

Visfatin stimulates endometrial cancer cell proliferation via activation of PI3K/Akt and MAPK/ERK1/2 signalling pathways

Yingmei Wang [1], Chao Gao [1], Yanfang Zhang, Jinping Gao, Fei Teng, Wenyan Tian, Wen Yang, Ye Yan, Fengxia Xue *

Department of Gynecology and Obstetrics, Tianjin Medical University General Hospital, Tianjin, China

HIGHLIGHTS

- Effects of visfatin on endometrial carcinoma progression were examined.
- Visfatin stimulated proliferation and inhibited apoptosis of both Ishikawa and KLE cells.
- Visfatin promoted Ishikawa xenograft growth in vivo, exhibiting with stronger proliferation index (Ki-67) expression.
- Visfatin effects were abrogated by inhibiting PI3K (LY294002) and MEK (U0126).
- Visfatin promotes endometrial cancer progression via PI3K/Akt and MAPK/ERK signalling.

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ABSTRACT

Objective. Endometrial carcinoma is one of the most common malignancies of the female reproductive system, but the aetiology and pathogenesis are not well understood, although adipokines such as visfatin may be involved. Our study provides insight into the mechanism underlying the tumorigenic effects of visfatin in endometrial carcinoma.

Methods. We investigated the effect of visfatin on endometrial carcinoma cell proliferation, cell cycle, and apoptosis using well-differentiated Ishikawa cells and poorly differentiated KLE cells. We also assessed the effect of visfatin on tumour growth in vivo.

Results. Visfatin stimulated the proliferation of both Ishikawa and KLE cells, and visfatin treatment promoted G1/S phase progression and inhibited endometrial carcinoma cell apoptosis. Visfatin promoted endometrial carcinoma tumour growth in BALB/c-nu mice. Transplanted tumour tissues from an endometrial carcinoma mouse model were analysed using immunohistochemical staining, which revealed much stronger positive signals for Ki-67 with over-abundant visfatin. Western blot analysis revealed that insulin receptor (IR), insulin receptor substrate (IRS)1/2 and key components of the phosphoinositide 3-kinase (PI3K)/AKT and mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK)1/2 signalling pathways were highly expressed in endometrial carcinoma cells exposed to visfatin. Treated cells showed increased C-MYC and cyclin D1 and reduced caspase-3 expression. The effects of visfatin on proliferation and apoptosis were abrogated by treatment with the PI3K inhibitor LY294002 and MEK inhibitor U0126.

Conclusions. Visfatin promotes the malignant progression of endometrial carcinoma via activation of IR and PI3K/Akt and MAPK/ERK signalling. Visfatin may serve as a therapeutic target in the treatment of endometrial carcinoma.

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1. Introduction

Endometrial carcinoma is one of the most common malignancies of the female reproductive system, with an incidence that has been steadily rising each year. In the U.S., the number of new cases is estimated to be 60,050 in 2016, with 10,470 cases leading to death [1]; these numbers are higher than those reported in 2015 [2]. The situation in China is also worsening [3]. Despite these trends, the aetiology and

* Corresponding author at: Department of Obstetrics and Gynecology, Tianjin Medical University General Hospital, 154 Anshan Road, He Ping District, Tianjin 300052, China
E-mail address: fengxiaxue1962@gmail.com (F. Xue).

[1] Contributed equally to this work.

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pathogenesis of endometrial carcinoma remain poorly defined. It is known that an imbalance in oestrogen/progesterone is involved, but epidemiologic studies have found that obesity, hypertension, and diabetes mellitus, which are all metabolic syndromes, are also risk factors. Metabolic syndromes are characterized by insulin resistance [4] and are linked to tumour occurrence [5], including endometrial carcinoma [4].

Previous studies have revealed that high insulin levels and obesity are both independent risk factors for endometrial carcinoma. Insulin stimulates the proliferation and migration of endometrial carcinoma cells and inhibits their apoptosis via phosphoinositide 3-kinase (PI3K)/Akt or mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) signalling pathways [6,7]. Nonetheless, insulin resistance does not underlie all cases of endometrial carcinoma. Moreover, some patients with combined endometrial carcinoma and obesity do not exhibit hyperinsulinemia, implying that other factors are associated with the occurrence and development of this endometrial carcinoma.

Visfatin is a recently discovered adipokine that has been reported to have insulin-mimetic effects and is overexpressed in metabolic syndrome-associated malignancies [8–10] such as colon, breast, gastric, and endometrial cancers. Our previous study reported that endometrial carcinoma patients exhibit higher serum visfatin and insulin levels than normal control subjects; endometrial carcinoma samples also show higher visfatin expression than normal endometrial tissues, and this expression is further increased with endometrial carcinoma progression. Thus, visfatin may be a key molecule linking metabolic syndromes to tumourigenesis, and is a potential serum biomarker for endometrial carcinoma [11].

In the present study, we investigated the effects of visfatin on endometrial carcinoma cell proliferation and apoptosis both in vitro and in vivo, as well as the underlying mechanisms. We found that visfatin induces endometrial carcinoma cell proliferation via PI3K/AKT and MAPK/ERK1/2 signalling pathways, thereby promoting the malignant progression of endometrial carcinoma. These results suggest that visfatin could serve as a target for drugs that are developed to treat endometrial carcinoma.

2. Methods

2.1. Drugs, cell lines, and animals

Human visfatin and its inhibitor FK866 were respectively purchased from BioVision (Milpitas, CA, USA) and Sigma-Aldrich (St. Louis, MO, USA). The PI3K inhibitor LY294002 and the MAPK/ERK kinase (MEK) inhibitor U0126 were also from Sigma-Aldrich. The well-differentiated Ishikawa endometrial carcinoma cell line was provided by Dr. Kim K. Leslie (University of New Mexico Health Sciences Center, USA), and the poorly differentiated KLE cell line was provided by Dr. Russell R. Broaddus (University of Texas MD Anderson Cancer Center, USA). Specific pathogen-free BALB/c-nu mice (5 weeks old, 16–17 g) were used for in vivo experiments [production permit no. SCXK (JING) 2014-0004, Beijing HFK Bioscience Co., Beijing, China]. Animal experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institute of Hematology and Hospital of Blood Diseases, Chinese Academy of Medical Sciences, Tianjin, China [license no. SYXK (JIN) 2009-0002].

2.2. Cell culture

Cells were cultured following standard procedures [12] in Dulbecco's Modified Eagle Medium/F-12 containing foetal calf serum (1:1, Gibco/Life Technologies, Carlsbad, CA, USA). Cells were passaged when they reached 80%–90% confluence by using 0.25% trypsin-EDTA (Gibco).

2.3. Cell proliferation assay

Cell Counting Kit (CCK)-8 (Beyotime Institute of Biotechnology, Jiangsu, China) was used to assess cell proliferation [13]. After cell attachment, the cell culture medium was replaced with serum-free medium for 12 h to eliminate interference from serum growth factors. Cells were treated with various concentrations of visfatin (0 [control], 25, 50, 100, 200, 400, and 800 ng/ml) or left untreated; 20 μl of CCK-8 solution was added 24, 48, or 72 h later. After incubation for 2 h, the absorbance was measured using an automatic microplate reader (Gene Company, Hong Kong) at an optical density of 450 nm (OD450) at room temperature.

2.4. Cell cycle analysis

Cells were fixed with ethanol and centrifuged at 1000 rpm for 5 min. The ethanol supernatant was discarded and cells were resuspended in 1 ml of pre-cooled phosphate-buffered saline (PBS) for 1 min, and then centrifuged at 1000 rpm for 5 min. The cells were resuspended in 870 μl of PBS to which 10 μl of 10 mg/ml RNase (final concentration 100 μg/ml), 100 μ1 of 1% (v/v) Triton X-100 (final concentration 0.1%) and 20 μ1 of 1 mg/ml propidium iodide (PI) were added. After incubation in the dark at 37 °C for 30 min [14], cells were sorted on a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). In total, 5000 events were analysed for each sample using ModFit LT software (Verity Software House, Torpsham, ME, USA).

2.5. Cell apoptosis assay

An annexin V-fluorescein isothiocyanate (FITC) apoptosis kit (BD Biosciences) was used to detect apoptosis of Ishikawa and KLE cells. Cells were treated with 25% trypsin without EDTA at 37 °C and washed with PBS, then resuspended in 500 μ1 binding buffer containing 5 μl of annexin V-FITC and 5 μ1 of PI [15,16]. After incubation for 15 min in the dark at room temperature, apoptotic cells were detected within 1 h by flow cytometry at excitation and emission wavelengths of 488 and 530 nm, respectively.

2.6. Tumour growth assay

Female BALB/C mice ($n = 5$) were subcutaneously injected in the right groin area with 200 μl of Ishikawa cell suspension (cell density = 5×10^7/ml). After 6 weeks, when the tumours had reached a diameter of about 10 mm, the mice were sacrificed and the tumours were dissected. After the removal of necrotic tissue, the fresh tissue was cut into pieces 5 mm in diameter, which were then inoculated into the right groin area of nude mice in the experimental group using a trocar. The control and visfatin groups each had eight mice that did not differ in terms of initial body weight. Starting from the date of tumour inoculation, visfatin (2 ng/g weight) and FK866 (20 mg/kg weight) were injected daily into the abdomen of mice in the visfatin group ($n = 8$), whereas mice in the control group ($n = 8$) were injected with saline. Tumour size was measured every 3 days, and tumour volume was calculated according to the equation $V = (a \times b^2)/2$, where a and b are the maximum and minimum diameters [17]. The values were used to generate a growth curve. After 4 weeks of observation, the mice were sacrificed and the tumours were dissected and weighed [18,19].

2.7. H&E staining, immunohistochemistry and enzyme-linked immunosorbent assay (ELISA)

Tumour tissues were fixed in 10% formaldehyde and then embedded in paraffin. After embedding, the tissues were sectioned (4 μm thickness), and the sections were stained with hematoxylin and eosin (H&E) according to standard procedures [20].

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