RESEARCH ARTICLE

Metformin Down-regulates Endometrial Carcinoma Cell Secretion of IGF-1 and Expression of IGF-1R

Yu Zhang*, Meng-Xiong Li, Huan Wang, Zheng Zeng, Xiao-Mao Li*

Abstract

As metformin can inhibit endometrial carcinoma (EC) cell growth and the insulin growth factor (IGF) system is active in EC, the question of whether it can regulate endometrial carcinoma cell secretion of IGF-1 or expression of IGF-1 receptor (IGF-1R) is of interest. In this study, serum IGF-1 levels in EC patients were found to be comparable with that in the non EC patients ($p>0.05$). However, the IGF-1 level in the medium of cultured cells after treatment with metformin was decreased ($p<0.05$). IGF-1R was highly expressed in human endometrial carcinoma paraffin sections, but IGF-1R and phosphor-protein kinase B/protein kinase B (p-Akt/Akt) expression was down-regulated after metformin treatment ($p<0.05$). In summary, metformin can reduce the secretion of IGF-1 by Ishikawa and JEC EC cell lines and their expression of IGF-1R to deactivate downstream signaling involving the PI-3K/Akt pathway to inhibit endometrial carcinoma cell growth.

Keywords: Metformin - endometrial carcinoma - IGF-1, IGF-1R

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Introduction

Endometrial cancer is a most common gynecologic malignant tumor and the fourth malignant tumor for women in developed country (Barakat et al., 2007). Risk factors for the development of this cancer include unopposed estrogen stimulation, obesity, and diabetes among others (Morrow et al., 1993; Bershtein, 2014). Growth factors are polypeptides that act by autocrine, paracrine, and endocrine pathways to regulate cell proliferation and death (Goustin et al., 1986; Liang K et al., 2014).

The major players in extracellular signal are growth factor receptors. The type-1 insulin-like growth factor receptor (IGF-1R) is one member of tyrosine protein kinase receptor family, which is important for the establishment of a malignant cell phenotype, cell metastasis, protection from apoptosis and enhancement of cell proliferation (Baserga, 1995; Morrison et al., 2002; Samani et al., 2004). In human endometrial carcinoma, levels of IGF-1R have been correlated with tumor progression through phosphatidylinositol-3-kinase/protein kinase B/the mammalian target of Rapamycin, PI-3K/Akt/mTOR and rat sarcoma/ rapidly accelerated fibrosarcoma/extracellular regulated protein kinases (Ras/Raf/Erk) signaling pathways (Casamassima et al., 1998; Sachdev et al., 2004). High IGF-1R expression in endometrial carcinoma has also been found to be an important prognostic factor (Hirano et al., 2004), and our previous study testified the inhibitory effect of siRNA targeting IGF-1R on endometrial carcinoma (Shu et al., 2011). In addition, endometrial carcinoma cells can synthesize and secrete IGF-I and IGF-II, which can integrate with their IGF-1R membrane receptor system and thereby cause continuous proliferation of tumor cells (Pavelic et al., 2007).

Metformin is a biguanide, commonly used for treating type 2 diabetes mellitus, increases insulin sensitivity and improves glycemic control (Nathan., 2009; Nevadunsky et al., 2014). Research shows that metformin can reduce the risks of some kinds of cancers including endometrial carcinoma (Goodwin et al., 2008; Pollak., 2010) and inhibit endometrial carcinoma cells growth by the mechanism that metformin acts as adenosine monophosphate-activated protein kinase (AMPK) agonist to inhibit mTOR phosphorylation (Xie et al., 2011; Arian et al., 2013). Moreover, researchers think that metformin prevented lung cancer caused by smoking is based on metformin reducing IGF-1 and blood levels of insulin to suppress mTOR effect, while mTOR is a protein that promotes the growth of lung cancer cells (Leone et al., 2014).

Whether metformin can regulate endometrial carcinoma cells secreting IGF-1 or expressing IGF-1R remains unknown. To answer this question, we tested the serum IGF-1 level in endometrial carcinoma patients before surgery and the medium of cells culture after treated with metformin. We also tested the IGF-1R in the human endometrial carcinoma paraffin sections and the change of IGF-1R expression, the signal pathway in vitro.

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Materials and Methods

Patients and blood samples

12 cases of newly diagnosed as EC by pathology were grouped in the endometrial carcinoma (EC) group, and 12 cases of diagnosed as myoma or benign tumor of ovary without endometrial diseases were grouped in the control group during March 2013 to December 2013 at the Third Affiliated Hospital of Sun Yat-sen University. The average age was (51±13.65) years in the EC group and (47±10.23) years in the control group without significant difference. Diabetes and hormone taken in the past three months were excluded in all the cases. All EC cases were diagnosed as adenocarcinoma with high differentiated (5 cases), middle differentiated (5 cases) and low differentiated (2 cases) at the stagelafter surgery. The morning fasting venous blood 5ml of all the patients collected before surgery were allowed to clot for 2 h at room temperature and centrifuged for 20 min at 1000 rpm. Serum was removed and stored at -20°C until assayed for the detection of IGF-1 value by ELISA . All patients provided written informed consent for participation in this study. The study protocol was approved by the Ethics Committees of the Third Affiliated Hospital of Sun Yat-sen University.

Human endometrium paraffin section

Human normal endometrium tissues and endometrial carcinoma tissues were obtained respectively from above patients. The tissues were paraffin-embedded, formalin-fixed and cut into 3μm sections for immuno-histochemistry staining.

Endometrial carcinoma cell lines, chemicals and antibodies

Human endometrial carcinoma cell lines Ishikawa and JEC were provided by American Type Culture Collection (Manassas, USA). The cells were routinely cultured in Dulbecco's modification of Eagle's medium (Gibco, USA) supplied with 10% fetal calf serum (Hyclone, USA), at 37 °C in the humidified atmosphere of a 5% CO_2 incubation.

Metformin was obtained from Sigma (USA). Mouse/Rat IGF-1 Immunoassay was obtained from Quantikine (USA). IGF-1Rβ Antibody #3027, Akt Antibody #4691, and Phospho-Akt (Ser473)#4060 were obtained from Cell Signaling Technology (USA), GAPDH Antibody was obtained from Santa Cruze (USA).

Immunoassay for IGF-1

Patients serum stored at -20°C were assayed for the detection of IGF-1. Cells were cultured as above and allowed to proliferate until about 70-90% confluence. The serum containing medium was removed, cells were washed with PBS, and serum-free medium and 0mM or 10mM of Metformin were added. After 4 days, cells and medium was separated by centrifuging at 150g for 5 min. Protein degradation in the supernatant was prevented with protease inhibitors (0.2 mM phenylmethylsulfonyl fluoride, 1mM EDTA, and 0.5 mg/liter leupeptin). The conditioned medium was dialyzed overnight at 4°C using membrane tubing with a cutoff of 3500 molecular weight. Aliquots of dialyzed conditioned media were stored at -20°C until assayed.

The IGF-1 Immunoassay was operated to detect IGF-1 as described in the kit protocols.

Immunohistochemistry

Paraffin sections were incubated with IGF-1Rβ Antibody (1:100 dilution) according to the manufacturer's instructions. Slides were examined with a Leica microscope, scanned using an Aperio ScanScope instrument, and analyzed in ImageScope viewing software.

MTT assay

For 3- (4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) viability assay, 2~3×10^3 cells/well were plated in 96-well plates, cultured in the appropriate media, and viability was evaluated using MTT.

Colony assays

Fifty cells per well were plated on 96-well plates 24 h later, medium was replaced, and cells were incubated with chemicals at indicate concentration. Medium was replaced every day, and at day 14, the cells were fixed and stained using 0.5% crystal violet (Sigma).

Western blot analysis

Cells were harvested and total protein was extracted with RIPA buffer together with a protease inhibitor cocktail (Sigma). Lysates were resolved on 10% SDS-PAGE and immunoblotted with the indicated antibodies. Band intensities were quantified using Image J software.

Statistical analysis

The data were compared using the Student t test or analysis of variance (ANOVA), as appropriate. All tests were two-tailed, and $p<0.05$ was considered significant.

Results

Serum IGF-1 in endometrial carcinoma

Serum IGF-1 concentration was (159.88±102.32)ng/ml in the endometrial carcinoma (EC) group, and was (167.92±112.26)ng/ml in the control group. There was no significant difference between the two groups ($p>0.05$, Figure 1).

IGF-1R highly expressed in endometrial carcinoma tissue

IGF-1R expressed abundantly in normal endometrial glands epithelial cells and endometrial carcinoma tissue, and most highly expressed in endometrial carcinoma tissue than in normal endometrial glands epithelial cells (Figure 2).

Metformin inhibited endometrial carcinoma cells Ishikawa and JEC viability

Effects of different concentration metformin on endometrial carcinoma cells Ishikawa and JEC growth were determined by MTT assay. Ishikawa cells were treated with metformin as indicated concentrations. Metformin attenuated the growth of Ishikawa and JEC cells (*$p<0.05$, **$p<0.01$, Figure 3). And the clone assay

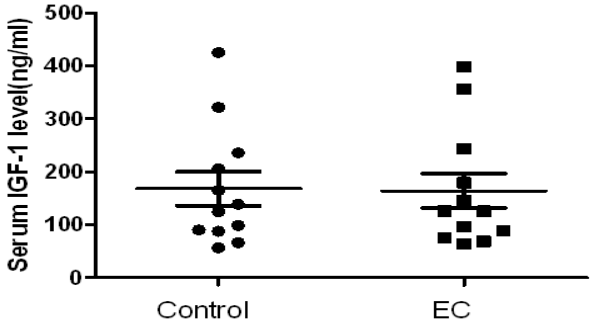

Figure 1. Serum IGF-1 In Endometrial Carcinoma (EC) Group was the Same as that of the Control Group

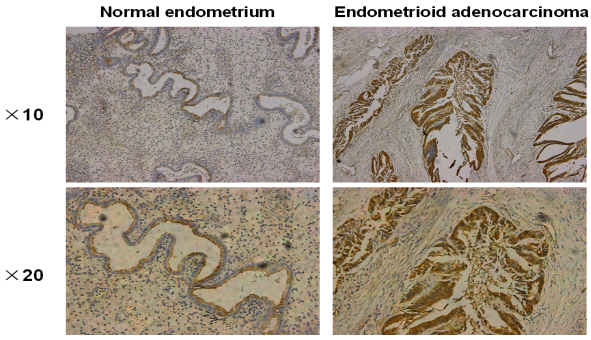

Figure 2. IGF-1R Expressed Highly in Endometrial Carcinoma Tissue and the Brown Yellow Staining in the Gland Cells Indicates IGF-1R

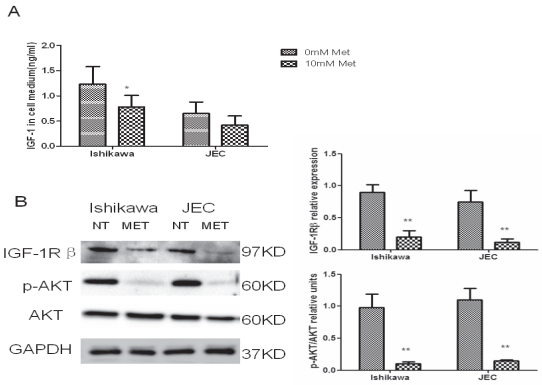

Figure 5. Metformin Reduced IGF-1 concentration in culture medium and down-regulated IGF-1Rβin Ishikawa and JEC cells. A, Ishikawa and JEC cells were treated with Metformin (0mM, 10mM) for 48 h, culture medium were harvested and assayed for IGF-1 RIA as described in the"Materials and Methods" section. Results of three independent experiments are shown in bars(*p<0.05). B, At the same time as A, Ishikawa and JEC cells were harvested and analyzed by Western blotting analysis for the expression of IGF-1Rβand p-AKT/AKT(*p<0.05, **p<0.01)

also showed that clone numbers in the 10mM of metformin treated group was significantly less than that in the control group (*p<0.05, Figure 4).

Metformin reduced IGF-1 concentration in culture medium and down-regulated the expression of IGF-1Rβ

Cells culture medium were collected and processed, and acid-alcohol was extracted to remove IGF-binding proteins as described under Materials and Methods, after which radioimmunoassays for IGF-1 were performed. The concentration of IGF-1 measured was lower in the group treated with 10mM of metformin than that in control group

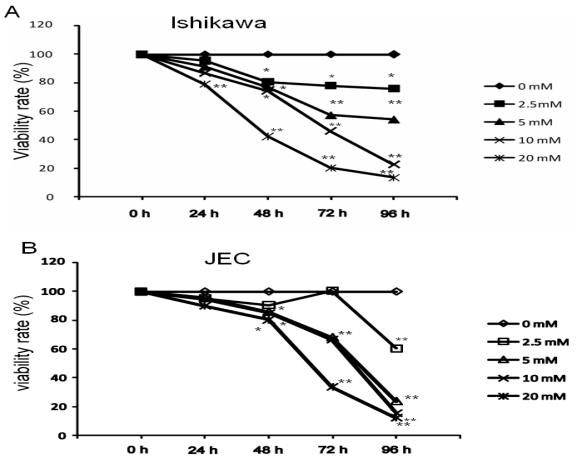

Figure 3. Metformin suppressed endometrial cancer cells Ishikawa and JEC viability. MTT assay was conducted at 24h,48 h,72h and 96h with 0mM,2.5mM,5mM,10mM and 20nM of Metformin. The growth curves were applied to absorbance at 490 nm (A for Ishikawa, B for JEC). Each point represents the means±SD of 3 independent experiments (*p<0.05, **p<0.01)

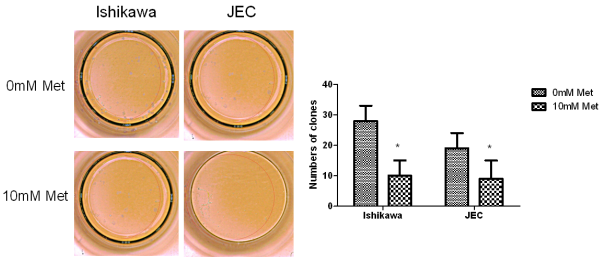

Figure 4. Metformin Inhibited Endometrial Cancer Cells Ishikawa and JEC Clones Formation. Ishikawa and JEC were seeded (50 cells per well, 96-well plate) and treated every day with Metformin (0mM, 10mM) in regular medium. After 14 days, cells were fixed and colonies stained with crystal violet. Quantification of the colonies was performed by Image J(*p<0.05)

(*p<0.05, Figure 5A).

And the intracellular β subunit of IGF-1R was significantly down-regulated when treated with 10mM of metformin than that with 0mM of metformin, resulting in lowering the ratio of p-AKT/AKT (*p<0.05, Figure 5B).

Discussion

IGF-1 is a polypeptide of 70 amino acids, mainly synthesized by the liver, and local organizations such as endometrium also secrete IGF-1 through autocrine and paracrine. And human serum IGF-1 level decrease by age and change by the phase of endometrium (Zhou et al., 1994). Studies demonstrate that IGF-1 is associated with the cancer development, progress and prognosis (Creighton et al., 2008; Torng et al., 2008), but some studies show no change (Lukanov et al., 2008; Lacey et al., 2004; Weiderpass et al., 2003) or decrease (Gunter et al., 2008) of serum IGF-1 level in endometrial carcinoma cases. In our study, serum IGF-1 level in the EC group was also comparable to that in the control group (Figure 1). So, till now serum IGF-1 can not be the serum predictor

for endometrial carcinoma. But IGF-1 can be produced by the uterus endometrial stromal cells, regulating cell proliferation and differentiation in autocrine and paracrine manner (Haining et al., 1991). Moreover, endometrial carcinoma cells can synthesize and secrete IGF-I and IGF-II, which can integrate with their IGF-1R membrane receptor and cause continuous proliferation of the tumor cells (Pavelie et al., 2007; Sarkissyan et al., 2014). And we also verified that high expression of GLP-1R in the EC tissue than that in the normal endometrial tissue (Figure 2). So local high IGF-1 level around the endometrial carcinoma cells matching with high expression of IGF-1R will promotes EC growth.

The fact of that metformin can inhibit cancer cells growth in vitro is accepted wildly, and the mechanism is associated with that metformin acts as AMPK agonist to inhibit mTOR phosphorylation (Zhou et al., 2001; Shaw et al., 2005; Xu et al., 2014), and that metformin can reduce insulin/insulin like growth factor signaling and suppress the PI-3K/Akt/mTOR axis (Luo et al., 2010; Matin et al., 2010). Metformin inhibits EC cells growth just as the results of MTT assay and clones formation in our study. Next, we observed the IGF-1 level in the medium of cells culture, the change of IGF-1R expression and its download signal after treated with metformin to testify whether metformin regulates IGF-1 level around the EC cells and IGF-1R expression to inhibit EC cells growth.

Growth factors are polypeptides that act by autocrine, paracrine, and endocrine pathways to regulate cell proliferation and death (Goustin et al., 1986). When growth factors like epidermal growth factor (EGF), transforming growth factor-a (TGF-a), and IGF-1 binds to its specific receptor, an intracellular signal cascade is initiated, resulting in activation of gene transcription, protein synthesis and mitosis (Derynck et al., 1987). And studies show that these growth factors including IGF-1 are potentially important in the development of endometrial carcinoma (Lelle et al., 1993; Pearl et al., 1993). And IGF-1 is demonstrated in endometrial cancer cell lines by IGF-1 RIA (Eugenio et al., 1999). Our data show IGF-1 secreted by endometrial cancer cell lines of Ishikawa and JEC can be detected in the culture, and the concentration of IGF-1 was lower in the group treated with 10mM of Metformin than that in the control group (Figure 5A). As we know Metformin acts as AMPK activator to decrease protein synthesis (Salani et al., 2014), and the production of IGF-1 may be included. The IGF-1R is composed of extracellular α subunit and intracellular β subunit, which covalent bonds forming tyrosine kinase receptor. Its activation induces the phospharylation of downstream tyrosine residues and itself or transmits growth signal from extracellular to nucleus through PI-3K/Akt/mTOR and Ras/Raf/Erk pathways (Casamassima et al., 1998). In our study, we detected intracellular β subunit of IGF-1R by western blot, and the intracellularβsubunit of IGF-1R was significantly down-regulated when treated with 10mM of metformin than that with 0mM of metformin, resulting in lowering the ratio of p–AKT/AKT ($P < 0.05$, Figure.5B). Other studies has also shown that metformin decrease IGF-1R complex of trastuzumab- resistant breast cancer cells (Liu et al., 2011) and suggested that metformin disrupts crosstalk between insulin/ IGF-1R and G protein-coupled receptors in pancreatic cancer cells (Kisfalvi et al., 2009; Rozengurt et al., 2010; Markowska et al., 2014).

In summary, metformin can reduce endometrial carcinoma cell lines of Ishikawa and JEC secreting IGF-1 and expressing IGF-1R to deactivate downstream signaling involving the PI-3K/Akt pathway to inhibit endometrial carcinoma cell growth. Further detailed analyses and clinical trials will be necessary to identify the specific molecular mechanisms, and to determine whether metformin would have similar effects in vivo. We will explore this mechanism in patients or models of endometrial carcinoma as well in next study.

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