Original Article

Cisplatin suppresses the growth and proliferation of breast and cervical cancer cell lines by inhibiting integrin β5-mediated glycolysis

Shaojia Wang¹,³*, Jie Xie²,³*, Jiajia Li¹,³, Fei Liu¹,³, Xiaohua Wu¹, Ziliang Wang¹,²,³

¹Cancer institute and Department of Gynecological Oncology, Fudan University Shanghai Cancer Center, Shanghai 200032, China; ²Department of Medical Oncology, Fudan University Shanghai Cancer Center, Shanghai 200032, China; ³Department of Oncology, Shanghai Medical College, Fudan University, 270 Dong’an Road, Shanghai 200032, China. *Equal contributors.

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Abstract: Cancer cells harbor lower energy consumption after rounds of anticancer drugs, but the underlying mechanism remains unclear. In this study, we investigated metabolic alterations in cancer cells exposed to cisplatin. The present study exhibited cisplatin, known as a chemotherapeutic agent interacting with DNA, also acted as an anti-metabolic agent. We found that glycolysis levels of breast and cervical cancer cells were reduced after cisplatin treatment, resulting in cells growth and proliferation inhibition. We demonstrated that cisplatin suppressed glycolysis-related proteins expression, including glucose transporter 1 (GLUT1), glucose transporter 4 (GLUT4) and lactate dehydrogenase B (LDHB), through down-regulating integrin β5 (ITGB5)/focal adhesion kinase (FAK) signaling pathway. ITGB5 overexpression rescued cisplatin-induced inhibition of cancer cell glycolysis, growth and proliferation. Conclusively, we reveal a novel insight into cisplatin-induced anticancer mechanism, suggesting alternative strategies to the current therapeutic approaches of targeting ITGB5, as well as of a combination of cisplatin with glucose up-regulation chemotherapeutic agents to enhance anticancer effect.

Keywords: Cisplatin, ITGB5, integrin, glucose metabolism

Introduction

Breast and cervical cancer are two main malignancies that threaten women's health worldwide [1-3]. In addition to surgical treatment, chemotherapy has been one of the primary treatments. Cisplatin (cis-diamminedichloroplatinum II), a platinum-derived agent exerting cytotoxicity via apoptosis, is highly effective in rapidly proliferating cancer cells [4]. At present, cisplatin has been found to accumulate rapidly in mitochondria thus deteriorate the mitochondrial structure and metabolic function [5], leading to significant changes in the levels of metabolites involved in the tricarboxylic acid cycle (TCA cycle) and glycolysis pathway [6, 7]. Although the anticancer effects of cisplatin have been widely investigated [6-8], the underlying mechanism of cisplatin-induced metabolic toxicity still remains elusive.

Integrins are heterodimeric transmembrane matrix receptors modulating cell adhesion to extracellular matrix (ECM) and ECM-induced intracellular signaling. Some studies show that β-integrins, such as β1, β3 and β5, play an important role in cell growth, proliferation, invasion and migration [9, 10]. It has been demonstrated that integrin levels are frequently elevated in aggressive tumors [11-13], implying these proteins might be promising targets for cancer treatments [14, 15]. However, the function of specific integrin is not fully illustrated. Growing studies exhibited that integrin β5 (ITGB5) contributed to chemoresistance in malignant disease [16]. ITGB5 promoted intracellular signaling by recruiting and activating integrin-associated kinases, including focal adhesion kinase (FAK). FAK, interacting with Src at Tyr861, played a vital role in the ITGB5-mediated signaling in response to vascular endothelial growth factor (VEGF) and Ras transformation in fibroblasts. [10, 17-19]. Thus, ITGB5 and its signaling components might be potential therapeutic targets in cancer treatment.
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In this study, we show that cisplatin suppresses breast and cervical cancer cell growth and proliferation by inhibiting cell glucose metabolism. Our study also provides evidence that ITGB5 facilitates glycolysis in cancer cell through the induction of FAK/p-FAK signaling. Meanwhile, the up-regulation of ITGB5 expression can remarkably weaken the anticancer effect of cisplatin. Taken together, our results show that ITGB5 may be an attractive therapeutic target.

Materials and methods

Cell lines and cell culture

The established human breast cancer cell line MDA-MB-231 and human uterine cervical cancer cell line Siha were both obtained from American Type Culture Collection (ATCC, U.S.A.). All cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM, HyClone, Thermo Scientific, U.S.A.) supplemented with 10% fetal bovine serum (Gibco, Life technologies, U.S.A.), 100 U/ml penicillin (Biowest, Nuaillé, France), and 100 U/ml streptomycin (Biowest, Nuaillé, France) and incubated at 37°C in a humidified atmosphere with 5% CO₂.

Chemical agents

Cisplatin was purchased from Sigma-Aldrich (St Louis, MO) and its store concentration was 5 mM. Cisplatin was used at concentration of 20 μM for MDA-MB-231 and 3 μM for Siha respectively during research if without specific notion. All samples were collected 48 hrs after the treatment of cisplatin. Small interference RNA (siRNA) pools against ITGB5 were from Santa Cruz (Santa Cruz, Biotechnology Santa Cruz, CA). Relative experiments were performed as previously described [17].

Plasmids construction and viral infection

The recombinant plasmid pENTER-ITGB5, containing human full cDNA sequence of ITGB5, was purchased from Vigene Biosciences (Jinan, China), and then the cDNA sequence of ITGB5 was subcloned into lentivirus vector pCDH-CMV-MCS-EF1-PURO, generating the recombinant plasmid pCDH/ITGB5oe. Lentivirus carrying ITGB5 cDNA were generated and harvested as described previously [20]. Briefly, the cells were infected twice for a total of 4 days (2 days for each infection) and the positive clones were selected with puromycin (200 ng/mL) for 7-10 days. Control cell lines were generated by infection with viruses containing the empty vector following the same protocol.

Real-time PCR

Total RNA from 3 × 10⁶ cells for each cell line was isolated by Trizol reagent (Invitrogen, Carlsbad, CA). All RNAs were then reversely transcribed into cDNAs that were suitable for real-time PCR analysis using the ExScript RT-PCR kit (TaKaRa, Japan). Oligonucleotide primers for ITGB5 were 5’-GTGCTTTATGCTCAGAG-3’ (Forward primer) and 5’-TGTCAAGTACGGCTCTCTGG-3’ (Reverse primers). Oligonucleotide primers for GAPDH were 5’-GGCGTTCAAGGATACGTTGACG-3’ (forward primer) and 5’-CCCCTATTGATGAGACCG-3’ (reverse primers). Oligonucleotide primers for LDHB were 5’-GGAAGGAAGTGAATGATGTGG-3’ (forward primer) and 5’-CCCATTATGCTCAGACG-3’ (reverse primers). Oligonucleotide primers for GLUT1 were 5’-CTGATTGCTTCCTGAGGAATG-3’ (forward primer) and 5’-CCACACGTGCTCCAATGTA-3’ (reverse primers). Oligonucleotide primers for GLUT4 were 5’-GGAACGAGAGGCAAGGTAAGAT-3’ (forward primer) and 5’-CCACACGTGCTCCAATCAGG-3’ (reverse primers). All amplifications and detections were carried out in the Applied Biosystems Prism 7900 system (Applied Biosystems, Foster City, CA) using the ExScript Sybr green QPCR kit (TaKaRa) and the following program: 95°C for 10 s, one cycle; 95°C for 5 s, 62°C for 31 s, 40 cycles; followed by a 30-min melting curve collection to verify the primer dimers. Statistical analysis was performed using the 2^ΔΔCT relative quantification method.

Colony formation assay

There were 1 × 10³ cells seeded in six-well plates at a single cell density and the fresh medium was added to allow cells to grow for at least one week. The colonies with more than 50 cells were counted after staining with gentian violet (Solarbio).

Cell proliferation

Cells were detached by using trypsinization and washed twice with phosphate buffered solution (PBS). 3 × 10³ cells per well were seeded in 96-well culture plates (Corning Inc., Corning,
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NY) in 100 μl medium and cultured for 1, 2, 3, 4, 5 days. Cell growth was detected using Cell Counting Kit-8 (CCK-8) (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer’s instructions. Absorbance at 450 nm was measured with a microplate reader. The assay was independently repeated three times.

Glycolysis analysis

Glucose Uptake Colorimetric Assay Kit (Biovision, U.S.A.) and Lactate Colorimetric Assay Kit (Biovision, USA) were purchased to examine the glycolysis process in breast and cervical cancer cells according to the manufacturer’s protocol.

Western blot analysis

Western blot analysis was performed to determine the expression levels of various proteins in cells. Cells were harvested, washed with cold 1 × PBS, and lysed with RIPA lysis buffer (Beyotime) for 30 min on ice, then centrifuged at 12,000 g for 15 min at 4°C. The total protein concentration was determined by BCA protein assay kit (Beyotime). Equal amounts (30 μg per load) of protein samples were subjected to SDS-PAGE electrophoresis and transferred on to polyvinylidene fluoride (PVDF) membranes (Millipore). The blots were blocked in 10% non-fat milk, and incubated with primary antibodies, followed by incubation with secondary antibodies conjugated with horseradish peroxidase (HRP). The protein bands were developed with the chemiluminescent reagents (Millipore). Antibodies to ITGB5 were from Abgent, GLUT1, GLUT4 and LDHB were from Proteintech. The antibody for β-Actin was purchased from Sigma-Aldrich (St Louis, MO). FAK and phospho-FAK Y925 were purchased from Santa Cruz (Santa Cruz, Biotechnology, Santa Cruz, CA). Phospho-FAK Y861 was purchased from Invitrogen (BioSource International Invitrogen).

Animal assays

The MDA-MB-231 or siha cells were stably transfected with ITGB5 cDNA and their corresponding controls by retroviral infection for animal assay. To generate tumor growth in vivo, 5 × 10⁶ cells of each cell line were subcutaneously injected into 4- to 6-week-old BALB/c athymic nude mice (Department of Laboratory Animal, Fudan University). The animal experiments were approved by the Institutional Animal Care and Use Committee of Fudan University and performed following Institutional Guidelines and Protocols. Each cell line was injected into ten mice. Mice were treated with cisplatin at 25 mg/kg by intraperitoneal injection every 3 days for 5 times 2 weeks after cancer cell injection. PBS was injected into peritoneal cavity as control group. One week after the last cisplatin injection, mice were sacrificed and the tumors were weighed. The longest diameter “a” and the shortest diameter “b” of tumors were measured and the tumor volume was calculated with the use of the following formula: tumor volume (in mm³) = a × b² × 0.52, where 0.52 is a constant to calculate the volume of an ellipsoid. Three tumors per cell line were excised, fixed in 10% formalin overnight, and subjected to routine histological examination by investigators who were blinded to the tumor status. Animal assays were repeated twice.

Immunohistochemical staining

Samples from xenograft mouse tumors were used for immunohistochemical staining of ITGB5, LDHB, GLUT1 and GLUT4 expression. Antibodies to ITGB5 were from Abgent, GLUT1, GLUT4 and LDHB were from Proteintech. The paraffin-embedded sections were pre-treated and stained with antibodies by using the previously reported method [21]. The secondary antibodies against mouse or rabbit IgG were supplied in an IHC kit (#CW2069) from Beijing Co Win Bioscience Co. Ltd (Beijing, China).

Statistical analysis

Results were reported as the mean ± SD or the mean ± SEM as indicated in the figure legends. All statistical analyses were performed using SPSS 22.0 software (SPSS, U.S.A.) and PRISM 6.0 (GraphPad Software Inc, U.S.A.). Variance between the groups was analyzed with Student’s t-test to quantitative data or χ² test to qualitative variables. P < 0.05 was considered to be significant.

Results

Cisplatin inhibits cell glycolysis in breast and cervical cancer cells

To evaluate the anticancer effect of cisplatin, both MDA-MB-231 and siha cells were treated
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with varying concentrations of cisplatin from $1 \times 10^{-4}$ to $1 \times 10^{-4} \mu M$. A dose-dependent cytotoxicity was observed in both cancer cell lines (Figure 1A, 1B). The half-maximal inhibitory concentration ($IC_{50}$) value for MDA-MB-231 cells and siha cells were 25.28 μM and 4.49 μM, respectively, demonstrating that siha cells tended to be more sensitive to cisplatin treatment compared with MDA-MB-231 cells. Based on results above, multiple low-toxic concentrations calculated from dose-response curves were used in subsequent experiments.

To confirm whether cisplatin regulated glucose metabolism in breast cancer and cervical cancer cells, we treated MDA-MB-231 and siha cells with cisplatin at low toxic dosages and performed glucose uptake and lactate production assays. As expected, both glucose uptake and lactate production dramatically decreased in MDA-MB-231 and siha when compared with their controls ($P < 0.001$) (Figure 1C, 1D). Further, we detected the glycolysis-related protein expression in MDA-MB-231 and siha cells with or without cisplatin treatment by real-time PCR and Western blot. The results of real-time PCR and Western blot exhibited that mRNA level and protein level of GLUT1, GLUT4 and LDHB were down-regulated ($P < 0.05$) when compared with their controls (Figure 1E-G), indicating cisplatin was involved in cancer metabolism. Collectively, these results showed that cisplatin blocked the glucose metabolism in MDA-MB-231 and siha cancer cells by reducing the expression of GLUT1, GLUT4 and LDHB, which further impacted cancer cell growth and proliferation.

**Figure 1.** Cisplatin inhibits glycolysis and lactate metabolism of MDA-MB-231 and siha cells. A, B. The values of $IC_{50}$ of MDA-MB-231 and siha cells treated with cisplatin were determined by CCK8 kit assay. C. Detection of glucose uptake capacity of MDA-MB-231 and siha cells treated with cisplatin by glucose uptake kit ($P < 0.001$). D. Detection of lactate production capacity of MDA-MB-231 and siha cells treated with cisplatin by lactate production kit ($P < 0.001$). E, F. Quantitative real-time PCR analysis of the expression levels of GLUT1, GLUT4, and LDHB in MDA-MB-231 or siha cells with or without the treatment of cisplatin ($P < 0.05$). G. Immunoblotting analysis of GLUT1, GLUT 4 and LDHB proteins in MDA-MB-231 or siha cells with or without the treatment of cisplatin.

ITGB5 rescues the effect of cisplatin on cell growth and proliferation by recovering cell glycolysis

As integrins are found to be involved in chemotherapy agent induced toxicity, we examine whether the expression of integrins was affect-
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ed by cisplatin treatment. Among some members of integrin family were detected, ITGB5 exhibited a markedly changes compared to ITGB2 and ITGB4 (Supplementary Figure 1A) after cisplatin treatment in both MDA-MA-231 and siha cells and significantly increased in cis-

Figure 2. ITGB5 rescues the cisplatin-induced inhibition of cell glycolysis. A, B. Quantitative real-time PCR and immunoblotting analysis of ITGB5 expression in MDA-MB-231 and siha cell with the treatment of cisplatin (P < 0.05). Error bars = 95% confidence intervals (CIs). C, D. Detection of the glucose uptake and lactate production of MDA-MB-231 or siha cell expressing ITGB5 cDNA and their controls with or without the treatment of cisplatin (P < 0.05). E, F. The proliferous capacity of MDA-MB-231 or siha cell expressing ITGB5 cDNA and that of their controls with or without the treatment of cisplatin by CCK8 Kit (P < 0.05). Error bars = 95% CIs. G, H. Detection of colonies formation ability of MDA-MB-231 or siha cell expressing ITGB5 cDNA and their controls with or without the treatment of cisplatin and quantitative analysis of colonies formation (P < 0.05). Error bars = 95% CIs.
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platin resistant sublines (Supplementary Figure 1B). As shown in Figure 2A, 2B, we found that ITGB5 expression was negatively correlated with cisplatin treatment in a dose-dependent manner, verifying ITGB5 might be a downstream effector of cisplatin in MDA-MB-231 and siha cells (P < 0.05).

To investigate the role of ITGB5 in cisplatin-induced glycolysis inhibition, we established MDA-MB-231/ITGB5 and siha/ITGB5 cells stably expressing ITGB5 cDNA, and treated these cells and their corresponding control cells with cisplatin at the same concentration. We found that the introduction of ITGB5 cDNA into MDA-MB-231 and siha cells rescued the effect of cisplatin on cells in glucose uptake and lactate production assays when compared with their controls treated with cisplatin (P < 0.05) (Figure 2C, 2D). The results of CCK8 and Colony formation assay showed that cisplatin-induced suppression on growth and proliferation of MDA-MB-231 and siha cells was obviously reduced by the up-regulation of ITGB5 when compared with their controls treated after cisplatin (P < 0.05) (Figure 2E-G). Further, we found that ITGB5 overexpression enhanced the expression of GLUT1, GLUT4 and LDHB in MDA-MB-231 and siha cells treated with cisplatin (P < 0.05) (Figure 3A-C), compared with their corresponding control cells. These results suggested that ITGB5 promoted the glycolysis-induced growth and proliferation of breast and cervical cancer cells by inducing the expression of GLUT1, GLUT4 and LDHB, thus reversed cisplatin-induced glycolysis inhibition. Additionally, we explored the effect of ITGB5 silencing on cancer cell growth and glucose metabolism. Further study showed that silencing of ITGB5 resulted in about 20-30% colonies reduction (P < 0.05), and exhibited a decrease in glucose uptake and lactate production in both MDA-MB-231 and siha cell lines when compared with their controls (P < 0.05) (Supplementary Figure 2A-E), indicating knockdown of ITGB5 in MDA-MB-231 and siha cells might to be similar to cisplatin treatment.

**ITGB5 activates p-FAK (Tyr861)-induced signaling to regulate cell glycolysis**

Previous studies demonstrated that integrins can recruit and activate FAK and its downstream signaling to regulate cell growth and...
Figure 4. Xenograft tumor burden in mice with overexpression of ITGB5 with or without the treatment of cisplatin. A-D. In vivo tumorigenesis examined by animal assay and subcutaneous tumor growth from mice injected with cells expressing ITGB5 cDNA and the corresponding controls with or without the treatment of cisplatin (n = 10 for each group, P > 0.05 in ITGB5 overexpressing group). Error bars = 95% CIs. Figures show tumor and tumor weights of mice at the end of observation. E. Immunohistochemistry staining of xenograft tumor tissues. Tissues was stained with rabbit anti-ITGB5, GLUT1, GLUT4 and LDHB antibody and visualized with goat anti-rabbit secondary antibody (Magnification × 400).
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proliferation [22-25]. In present study, we also found that cisplatin suppressed the expression of focal adhesion kinase (FAK) and phosphorylated FAK (p-FAK) (Figure 3C), and ITGB5 overexpression weakened cisplatin-induced suppression on FAK and p-FAK (Figure 3C). Furthermore, we found that phosphorylation of FAK at Tyr861 but not at Tyr925 was correlated with ITGB5, indicating p-FAK containing different phosphorylation sites might interact with specific integrin to affect diverse cell functions.

ITGB5 promotes proliferation by restoring cisplatin-induced glycolysis in vivo

To clarify the interaction between cisplatin and ITGB5 in vivo, we inoculated MDA-MB-231/ITGB5 or siha/ITGB5 cells and their corresponding control cells either into the mammary fatpad of nude mice or subcutaneously into nude mice with or without the treatment of cisplatin at the concentration of 5 mg/kg. As shown in Figure 4A-D, the volume and weight of xenograft tumors formed by MDA-MB-231/ITGB5 or siha/ITGB5 cells were obviously increased (n = 10 for each group P < 0.05), compared with their corresponding control cells. After cisplatin treatment, the volume and weight of xenograft tumors formed by MDA-MB-231/ITGB5 (the volume was 913.2 mm³ and the weight was 2.63 g) or siha/ITGB5 cells (the volume was 992.4 mm³ and the weight was 2.96 g) were scarcely diminished (P > 0.05) after the treatment of cisplatin when compared with their ITGB5 overexpressing controls MDA-MB-231/ITGB5 (the volume is 933.2 mm³ and the weight is 2.81 g) or siha/ITGB5 cells (the volume is 1036.3 mm³ and the weight is 3.13 g) without cisplatin treatment (Figure 4A-D). However, the volume and weight of xenograft tumors formed by MDA-MB-231 (the volume is 420.3 mm³ and the weight is 1.24 g) or siha cells (the volume is 402.1 mm³ and the weight is 1.42 g) treated after cisplatin were significantly reduced (P < 0.05), compared with those formed by MDA-MB-231 (the volume is 641.3 mm³ and the weight is 2.32 g) or siha cells (the volume is 821.2 mm³ and the weight is 2.46 g) without cisplatin treatment (Figure 4A-D).

To determine whether cisplatin and ITGB5 affected the expression of GLUT1, GLUT4 and LDHB in vivo, we performed IHC analysis on tumor sections from all the experimental groups. As shown in Figure 4E, compared with corresponding control groups in MDA-MB-231 and siha, ITGB5 overexpression enhanced the expression of GLUT1, GLUT4 and LDHB in MDA-MB-231/ITGB5 or siha/ITGB5 cells with or without cisplatin treatment, respectively. These results in vivo demonstrated that ITGB5 exhibited anti-cisplatin activities through induction of GLUT1, GLUT4 and LDHB in breast and cervical cancer.

Discussion

In this study, we showed cisplatin exerted its anticancer effect on breast and cervical cancer cells by inhibiting cell glycolysis. The overexpression of ITGB5 relieved cisplatin-induced glycolysis inhibition, implying that ITGB5 might be a novel target in cisplatin treatment. Further study found that ITGB5 activated the FAK signaling pathway in cancer cell glycolysis alteration, and resisted the anticancer effect of cisplatin, which disclosed a new mechanism of cisplatin resistance.

Cancer cells harbor lower energy consumption through defective mitochondria after rounds of anticancer agents, thus inefficient ATP synthesis becomes an obstacle for cell growth and proliferation [26]. In present work, we evaluated the cytotoxicity of cisplatin on glycolysis and found that cisplatin, known as a chemotherapeutic agent interacting with DNA, also acted as an anti-metabolic agent. Previous studies showed that ITGB5 contributes to the transforming growth factor β (TGF-β)-induced epithelial-mesenchymal transition (EMT) [10], tumor angiogenesis [27] and resistance to radio- and chemotherapy [28]. Herein, we demonstrated that ITGB5 not only enhanced the cell glycolysis to promote cancer cell growth and proliferation, but also counteracted cisplatin cytotoxicity to breast and cervical cancer cells, indicating ITGB5 to be an attractive therapeutic target combined with cisplatin treatment.

Due to the lack of enzymatic activity, integrins promote signaling transduction by recruiting and activating integrin-associated kinases, including FAK and integrin-linked kinase (ILK) [24]. Some studies revealed that the interaction of Src with FAK at Tyr861 is crucial for ITGB5-mediated signaling in response to vascular endothelial growth factor (VEGF) and Ras transformation in fibroblasts [25]. In other sys-
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tems, FAK-activated mitogen-activated protein kinases (MAPK) promotes cancer cell growth and proliferation [29, 30]. Our results demonstrated that Src-induced phosphorylation of FAK at Tyr861 but not Tyr925 was involved in ITGB5-mediated glycolysis, which further expanded the understanding of ITGB5/FAK signaling.

Additionally, not all chemotherapeutic agents inhibit glycolysis in cancer cells. Previous study showed a significant up-regulation of glucose metabolism under 5-Fluorouracil (5-Fu) treatment in non-small cell lung cancer (NSCLC) cells was observed [31], revealing a novel mechanism of a combined treatment of 5-Fu and cisplatin.

Collectively, our study provides a novel insight into the mechanism of cisplatin-induced toxicity by glycolysis inhibition irrespective of mitochondrial damage or mitochondrial dysfunction, and reveals that ITGB5 modulates glycolysis in both breast and cervical cancer via FAK/p-FAK pathway. These findings suggest alternative strategies to the current therapeutic approaches of targeting ITGB5, as well as of a combination of cisplatin with glucose up-regulation chemotherapeutic agents to enhance anticancer effect.

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Disclosure of conflict of interest

None.

Address correspondence to: Ziliang Wang and Xiaohua Wu, Cancer Institute and Department of Gynecological Oncology, Fudan University Shanghai Cancer Center, Shanghai 200032, China. Tel: 86-21-34777310; E-mail: huf_zlwang@126.com (ZLW); Tel: 86-21-64175590-81006; E-mail: docwuxh@hotmail.com (XHW)

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Supplementary Figure 1. ITGB5 was more sensitive than ITGB2 and ITGB4 to the treatment of cisplatin. A. Immunoblotting analysis of ITGB5, ITGB4 and ITGB2 expression in MDA-MB-231 and siha cell with cisplatin treatment. B. Immunoblotting analysis of ITGB5 in A2780 cisplatin resistant subline and corresponding control cell.
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Supplementary Figure 2. Silencing of ITGB5 inhibited the breast and cervical cell proliferation and glucose metabolism. A. Immunoblotting analysis of ITGB5 expression in MDA-MB-231 and siha cell. B, C. Detection of colonies formation ability of MDA-MB-231 or siha cell silencing ITGB5 and their controls and quantitative analysis of colonies formation (\( P < 0.05 \)). Error bars = 95% CIs. D. Detection of glucose uptake capacity of MDA-MB-231 and siha cells silencing ITGB5 and their controls by glucose uptake kit (\( P < 0.001 \)). Error bars = 95% CIs. E. Detection of lactate production capacity of MDA-MB-231 and siha cells silencing ITGB5 and their controls by lactate production kit (\( P < 0.001 \)). Error bars = 95% CIs.