RESEARCH ARTICLE

Cisplatin Combined with Metformin Inhibits Migration and Invasion of Human Nasopharyngeal Carcinoma Cells by Regulating E-cadherin and MMP-9

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Abstract

Metformin has been shown to be useful in reducing insulin resistance by restoring sensitivity. Recent evidence suggests that metformin might also possess anti-tumour activity. This study aimed to investigate the effects of cisplatin combined with metformin on the proliferation, invasion and migration of HNE1/DDP human nasopharyngeal carcinoma (NPC) cells, and to provide a new target for treating metastasis. The MTT assay was used to assess viability of HNE1/DDP cells after exposure to different concentrations of 2, 5-diaminopyrimidine-4, 6-diol (DDP; 2, 4, 8, 16, and 32 μmol·L^{-1}), metformin (5, 10, 15, 20, and 25 μmol·L^{-1}), and 4 μmol·L^{-1} of DDP combined with metformin. Wound healing and transwell migration assays were performed to assess cell migration and invasion, and expression of E-cadherin and MMP-9 was detected using Western blotting. MTT assay results showed that DDP could inhibit the proliferation of HNE1/DDP cells in a time- and concentration-dependent manner, with an IC50 of 32.0 μmol·L^{-1} at 24 h ($P < 0.05$), whereas low concentrations of DDP had almost no inhibitory effects on cell invasion and migration. DDP combined with metformin significantly inhibited cell invasion and migration. In addition, genes related to migration and invasion, such as those of E-cadherin and MMP-9, showed differential expression in the NPC cell line HNE1/DDP. In the present study, with an increasing concentration of metformin, the expression of MMP-9 was downregulated whereas that of E-cadherin was significantly upregulated. Taken together, our results show that cisplatin combined with metformin has effects on proliferation, invasion, and migration of human NPC cells.

Keywords: Metformin - cisplatin - NPC - invasion - metastasis

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Introduction

Nasopharyngeal carcinoma (NPC) is a distinct type of head and neck cancer prevalent in Southeast Asia and Southern China, where it constitutes a significant health burden (Yu et al., 2002; Chen et al., 2012). In conventional clinical therapy, radiotherapy is the most commonly used, followed by combined chemotherapy. However, the 5-year survival rate is low, which is mainly attributable to resistance, and it limits the use of chemotherapy as a treatment option (Chen et al., 2012). The morbidity and mortality of malignant tumours in our country has been recently increasing, with the increased mortality being caused by invasion and metastasis, especially in late tumour development. Therefore, it is important to explore the mechanisms of tumour invasion and metastasis and find new therapeutic targets.

Metformin (1, 1-dimethylbiguanidehydrochloride) is considered to be an insulin sensitizer, useful in the reduction of insulin resistance by restoring insulin sensitivity (Fedorcsak et al., 2003). However, metformin also has anti-tumour effects in vivo and in vitro (Decensi et al., 2010; Hsieh et al., 2012). In fact, some studies have found that metformin combined with chemotherapy drugs may enhance the effectiveness of chemotherapy (Jiralerspong et al., 2009). However, the effects and possible mechanisms of action of metformin in the proliferation and metastasis of NPC cells have not been explored. Therefore, we explored the effects of different concentrations of metformin combined with the chemotherapy drug, cisplatin, on the proliferation and metastasis of NPC cells. Our study may provide new ideas and methods for the treatment of NPC.

Materials and Methods

Reagents and antibodies

Metformin were obtained from Calbiochem (La Jolla, CA, USA). MTT-based colorimetric assay kit was purchased from Roche (Indianapolis, IN, USA). RPMI-

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1640 medium, fetal bovine serum (FBS), and phosphate buffered saline (PBS) were purchased from Gibco (Grand Island, NY). Primary antibodies for E-cadherin, MMP-9 (1:500), and β-actin (1:2000) and β-actin antibody were obtained from Santa Cruz Biotechnology (Santa Cruz CA, US).

Matrigel was purchased from BD Biosciences (Bedford, MA, U.S.). The 24-well Transwell insert (8 μm) was obtained from Corning Inc (Corning, NY, USA).

Cell lines

HNE1/DDP, a drug-resistant NPC cell line more tolerant to cisplatin that the parental cell line, HNE1, was obtained from Zhongshan University and cultured in our laboratory. Cells were cultured in RPMI-1640 supplemented with 10% FBS and 100 U of penicillin-streptomycin with 5% CO_2 in a humidified incubator at 37°C.

Cell proliferation assay

The cytotoxic effects of DDP and metformin on NPC cancer cells were determined using the MTT assay, as previously described. Cells were seeded at a density of 7×10^3 cells/well in 96-well plates with growth medium. After 24 h, the cells were exposed to different concentrations of DDP (2, 4, 8, 16, and 32 μmol·L^{-1}), metformin (5, 10, 15 20, and 25 mmol·L^{-1}), and DDP combined with metformin. After 24 h, 48 h, and 72 h, each well was incubated with MTT (5 mg/mL) in PBS for 4 h at 37°C. After 4 h, the MTT solution was removed and replaced with 150 μL of dimethyl sulfoxide (DMSO). The plates were shaken for 10 min to dissolve the MTT formazan crystals. The optical density (OD) of each well was determined using a scanning multi-well spectrophotometer at a wavelength of 490 nm. Experiments were repeated in triplicate, and 4 parallel samples were measured each time.

Cell migration assay

Boyden chambers were placed into 24-well chambers containing 600 μL of RPMI-1640 medium supplemented with 10% FBS. Cells were seeded at a density of 5×10^4 cells per insert after exposure to DDP, metformin, or DDP combined with metformin, each of which was resuspended in 200-μL serum-free medium and placed in the upper chambers. After 24 h under normoxic conditions, cells that had migrated were stained and photographed under a light microscope at 200× magnification. The number of cells that had migrated was counted from 5 randomly selected fields. The experiments were performed in triplicate.

Cell invasion assay

The invasion assay was performed using a 24-well cell culture plate with 8.0-μm pore membrane inserts. Cells were seeded in the inserts, as described above, placed in the upper chambers at a density of 5×10^4 cells, and normal growth media were placed in underneath chambers. Twenty-four hours after treatment, the cells on the upper surface of the membrane were removed with cotton swabs, and the cells on the lower chamber were incubated with 4% paraformaldehyde in PBS buffer and stained with 0.1% crystal violet. Finally, migratory and invasive cells were counted at 200× magnification in 5 different fields per filter. The experiments were performed in triplicate.

Wound healing assay

Cells were seeded in a 6-well plate and grown overnight to confluence at 5×10^5 cells/well. After 24 h, the cells were exposed to DDP, metformin, or DDP combined with metformin. Cells were washed with PBS, and wounds were created using a sterile 20 μL pipette tip. The cells were then washed 3 times with PBS and incubated in RPMI-1640 medium containing 10% FBS. The medium was then replaced with medium without serum. The rate of wound closure was assessed and photographed 24 h later. Each value is derived from 3 randomly selected fields. The experiments were performed in triplicate.

Real-time PCR

Total RNA was extracted with TRIzol Reagent (Invitrogen). Accumulated PCR products were detected directly by monitoring the increase of the reporter dye (SYBR) in accordance with the manufacturer's instructions. One-fifth of the cDNA was used as a template for PCR performed using the SYBR Green PCR kit (Takara, Kyoto, Japan) in an ABI StepOne™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The mRNA expression levels of E-cadherin and MMP-9 in the treated cells were compared to the expression levels in control cells at each time point using the comparative cycle threshold (Ct) method (Johnsonet al., 2000). The primer sequences for target genes were as follows: E-cadherin (forward, 5'-TCGACACCCGATTCAAAGTGG-3'; reverse, 5'-TTCCAGAAACGGAGGCCTGAT-3'); GAPDH (forward, 5'-ACGGGAAGCTCACTGGCATGG-3'; reverse, 5'-GGTCCACCACCCTGTTGCTGTA-3'), and MMP-9 (forward, 5'-CGGAGTGAGTTGAACCAG-3'; reverse, 5'-GTCCCAGTGGGGATTTAC-3'). The quantity of each transcript was calculated as described in the instrument manual and normalized to the amount of GAPDH, a housekeeping gene. All of the assays were performed in triplicate. The $2^{-\Delta\Delta Ct}$ method was used for relative quantification of gene expression and to determine the levels of E-cadherin or MMP-9 mRNA expression.

Western blot analysis

The cells were plated in 6-well culture dishes at a density of 5×10^5 cells/well. The harvested cells were then lysed on ice for 30 min in 100mL of lysis buffer (120 mM NaCl, 40 mM Tris (pH 8), 0.1% NP40) and centrifuged at 12000 rpm for 30 min. The protein concentrations were detected using a bicinchoninic acid (BCA) assay. A total of 50 μg of protein was separated using 10% SDS-PAGE and electro-blotted onto PVDF membranes using a semi-dry blotting apparatus. After blocking in 5% non-fat milk, the membranes were incubated overnight at 4°C with primary antibodies. The membranes were then incubated in the secondary anti-bodies for 2 h at room temperature on a shaker. The bands were visualized using Western Lightning ECL Pro with horseradish peroxidase (HRP). β-actin was used as a loading control.

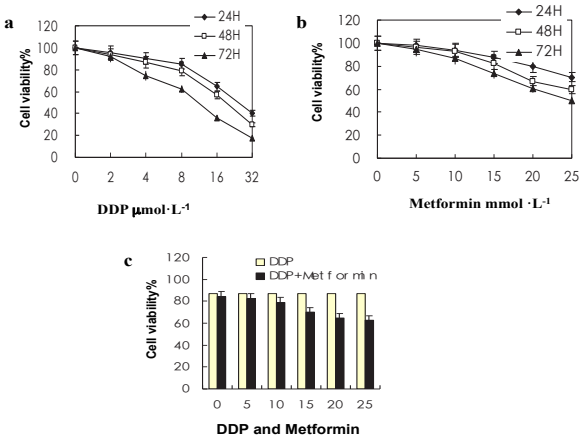

Figure 1. Effects of Cisplatin (DDP) and Metformin on the Viability of HNE1/DDP Cells. a) HNE1/DDP cells were treated with DDP at different concentrations, ranging from 2 to 32 μmol·L^{-1}, and measured after 3 days of treatment. b) HNE1/DDP cells were treated with metformin at different concentrations (0, 5, 10, 15, 20, and 25 mmol·L^{-1}) and measured after 3 days of treatment. c) HNE1/DDP cells were co-treated with DDP (4 μmol·L^{-1}) and metformin (0, 5, 10, 15, 20, and 25 mmol·L-1) for 24 h and then measured for viability using the MTT assay

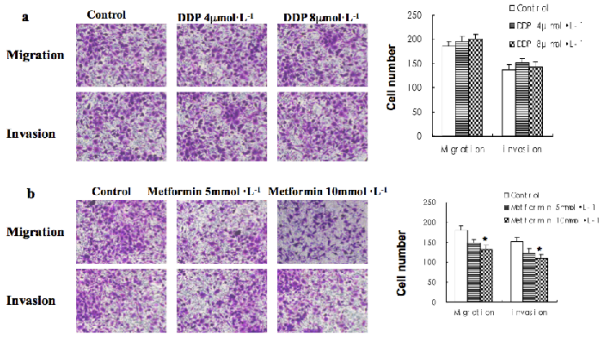

Figure 2. Effects of DDP and Metformin on the Invasion and Migration of HNE1/DDP Cells. After 48 h, invasion and migration of HNE1/DDP cells were detected by performing the transwell assay. Five visual fields were chosen randomly for each insert and photographed under a light microscope (magnification, 400×)

Statistical analysis

All experiments were repeated at least three times. Data are presented as the mean ± SEM. The differences between means were analyzed using the two-tailed Student's t-test. All statistical analyses were performed using SPSS 13.0 software (Chicago IL, US). Differences with $P < 0.05$ were considered statistically significant.

Results

Effects of DDP and DDP combined with metformin on cell proliferation

In this study, we detected the effects of different concentrations of DDP (2, 4, 8, 16, and 32 μmol·L^{-1}), different concentrations of metformin (5, 10, 15, 20, and 25 mmol·L^{-1}), and DDP (4 μmol·L^{-1}) combined with metformin (5, 10, 15, 20, and 25 mmol·L^{-1}) on HNE1/DDP cells by performing the MTT assay. We found that DDP inhibited cell proliferation in a concentration- and time-dependent manner (Figure 1a, $P < 0.05$), whereas

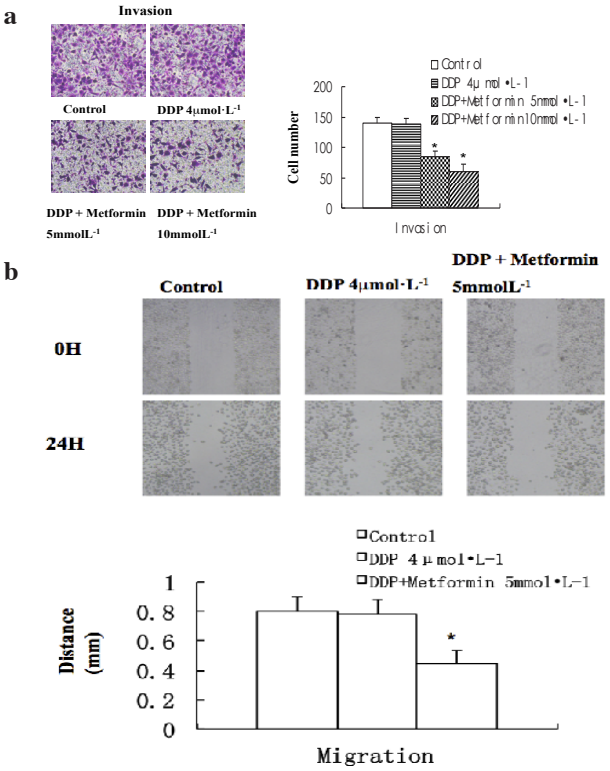

Figure 3. Effects of DDP (4 μmol·L^{-1}) and Metformin on Invasion and Migration of HNE1/DDP Cells. a) After 48 h, invasion of HNE1/DDP cells was detected by performing a transwell assay. Five visual fields were chosen randomly for each insert and photographed under a light microscope (magnification, 400×). b) After 24 h, the invasion and migration were detected by performing a wound healing assay

metformin slightly inhibited cell proliferation on HNE1/DDP (Figure 1b, $P < 0.05$). However, cell viability decreased when the cells were treated with DDP combined with metformin, unlike that in the cells treated with DDP only (Figure 1c, $P < 0.05$).

Effects of DDP and metformin on cell migration and invasion

The transwell migration and invasion assay revealed that exposure to DDP (4 and 8 μmol·L^{-1}) had little effect on cell migration and invasion (Figure 2a). Metformin (5 and 10 mmol·L^{-1}) slightly inhibited cell migration and invasion (Figure 2b).

Effects of DDP combined with metformin on cell migration and invasion

In order to analyse the effects of DDP (4 μmol·L^{-1}) or DDP (4 μmol·L^{-1}) combined with metformin (5 and 10 mmol·L^{-1}) on cell migration and invasion, we determined the cell numbers in the transwell assay. The migration and invasion assay showed that DDP could enhance the effect of metformin in a dose-dependent manner. As shown in Figure 3a, metformin, when combined with DDP, attenuated the number of invading cells. As shown in Figure 3b, a wound healing assay used to detect the mobility of NPC cell lines showed that metformin inhibited wound gap closure in a time-dependent manner. Relative expression of migration and invasion genes in human NPC cells

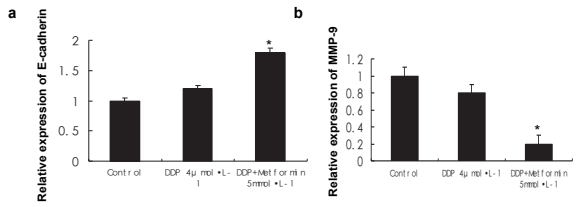

Figure 4. Relative Expression of Migration and Invasion Genes in Human NPC Cells. a) HNE1/DDP cells were exposed to DDP (4μmol·L^{-1}) and DDP (4μmol·L^{-1}) combined with Metformin (5, 10 mmol·L^{-1}). The relative expression of E-cadherin were detected by qRT-PCR. b) The relative expression of MMP-9 were detected by qRT-PCR

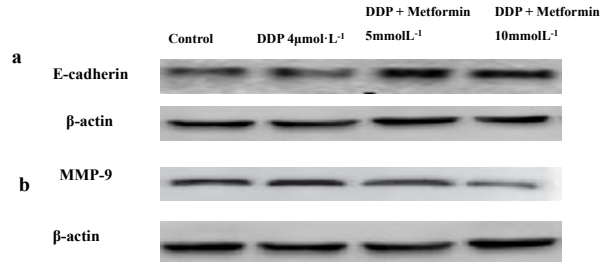

Figure 5. DDP (4 μmol·L^{-1}) and Metformin and Proteins Related to Migration and Invasion. a) HNE1/DDP cells were exposed to DDP (4 μmol·L^{-1}) and DDP (4 μmol·L^{-1}) combined with metformin (5 and 10 mmol·L^{-1}). The levels of E-cadherin protein were analysed by western blotting at 48 h post-treatment. b) HNE1/DDP cells were exposed to DDP (4 μmol·L^{-1}) and DDP (4 μmol·L^{-1}) combined with metformin (5 and 10 mmol·L^{-1}). The levels of MMP-9 protein were analysed by western blotting at 48 h post-treatment

Quantitative real-time RT-PCR was used to detect the levels of mRNA expression of genes r-elated to migration and invasion. Compared to Ctrl and DDP (4 μmol·L^{-1}), exposed to DDP combined with metformin (5 mmol·L^{-1}) obviously increased the expression of E-cadherin (Figure 4a). On the contrary, DDP combined with metformin (5mmol·L^{-1}) decreased the expression of MMP-9 (Figure 4b).

Effects of DDP combined with metformin on the expression of E-cadherin and MMP-9 proteins

Metformin has been shown to inhibit proliferation in some cancer cell lines through the activation of the AMPK pathway (Zakikhani, Dowling et al. 2006); however, the precise mechanisms underlying NPC metastasis are not well understood. The expression of genes related to migration and invasion may contribute to specific stages of the metastatic cascade. In order to elucidate the molecular mechanisms of the effect of metformin on the metastasis of NPC, E-cadherin and MMP-9 expression levels were assessed using the western blot analysis. Exposure to metformin increased the rate of E-cadherin expression and reduced the levels of MMP-9 expression (Figure 5a and b).

Discussion

DDP is a non-specific cell-cycle anticancer drug that inhibits tumour growth through the inhibition of DNA and RNA synthesis and mitosis and is a commonly used drug for treating NPC. Although DDP can significantly inhibit the proliferation of NPC, toxicity effects increase with increased drug doses, and it has no effect on cancer cell invasion and migration, which limits its use in cancer therapy.

Rencently, the main reason of the cancer death is the increased mortality being caused by invasion and metastasis of the cancer cells. A large number of studies have demonstrated that epithelial-mesenchymal transition (EMT) is a crucialevent in many cancers, such as hepatocellular carcinoma (HCC).The present study revealed that nuclear translocation of PKM2 regulated HCC cells migrationin vitro (Fan et al., 2014). These discoveries are very important to the later research about cancer cell migration and invasion.

Metformin has been widely used as an oral biguanide agent for the treatment of type 2 diabetes (Witters 2001). This drug has been shown to inhibit the energy-sensitive AMPK/mTOR signalling pathway that leads to reduced protein synthesis and cell proliferation (Zakikhani et al., 2006). Numerous studies have shown that metformin has significant effects on tumourigenesis and cancer cell growth and can inhibit the in vitro and in vivo proliferation of various cancer cells, such as breast cancer (Anisimov et al., 2005; Ben et al., 2010), ovarian cancer (Rattan et al., 2011; Rattan et al., 2011), prostate cancer (Ben Sahra et al., 2008), gastric cancer (Kato et al., 2012), and human glioblastoma tumour-initiating cells (Wurth et al., 2013). However, the mode of action and the biological consequences of the anti-diabetic drug, metformin, in cancer cell migration and invasion are poorly understood. Indeed, only a few studies performed in fibrosarcoma, carcinoma, and ovarian carcinoma cells have addressed the role of metformin in this process (Hwang et al., 2010; Tan et al., 2011; Wu et al., 2012), and the effects of metformin on invasion by NPC remain unknown. In our study, we found that metformin could enhance the effects of cisplatin on proliferation and invasion of human NPC cells.

The study was designed to explore the anti-invasive potential of metformin combined with DDP in HNE1/DDP cells and to analyse the molecular mechanisms involved in this process. The MTT assay showed that DDP inhibited cell proliferation in a concentration- and time-dependent manner, whereas metformin slightly inhibited HNE1/DDP cell proliferation. However, metformin enhanced the effects of DDP on the inhibition of cell proliferation. In the present study, E-cadherin and MMP-9 expression levels were assessed using the western blot analysis. Exposure to metformin increased the rate of E-cadherin expression and reduced the rate of MMP-9 expression.

In summary, we showed, for the first time, that metformin inhibits NPC invasion and metastasis development by regulating the expression of E-cadherin and MMP-9. Metformin, therefore, has the potential to be a potent anti-cancer drug in therapeutic strategies for NPC metastasis.

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