RESEARCH ARTICLE

Insulin Resistance Reduces Sensitivity to Cis-Platinum and Promotes Adhesion, Migration and Invasion in HepG2 Cells

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Abstract

The liver is normally the major site of glucose metabolism in intact organisms and the most important target organ for the action of insulin. It has been widely accepted that insulin resistance (IR) is closely associated with postoperative recurrence of hepatocellular carcinoma (HCC). However, the relationship between IR and drug resistance in liver cancer cells is unclear. In the present study, IR was induced in HepG2 cells via incubation with a high concentration of insulin. Once the insulin-resistant cell line was established, the stability of HepG2/IR cells was further tested via incubation in insulin-free medium for another 72h. Afterwards, the biological effects of insulin resistance on adhesion, migration, invasion and sensitivity to cis-platinum (DDP) of cells were determined. The results indicated that glucose consumption was reduced in insulin-resistant cells. In addition, the expression of the insulin receptor and glucose transportor-2 was downregulated. Furthermore, HepG2/IR cells displayed markedly enhanced adhesion, migration, and invasion. Most importantly, these cells exhibited a lower sensitivity to DDP. By contrast, HepG2/IR cells exhibited decreased adhesion and invasion after treatment with the insulin sensitizer pioglitazone hydrochloride. The results suggest that IR is closely related to drug resistance as well as adhesion, migration, and invasion in HepG2 cells. These findings may help explain the clinical observation of limited efficacy for chemotherapy on a background of IR, which promotes the invasion and migration of cancer cells.

Keywords: Hepatocellular carcinoma - insulin resistance - proliferation - pioglitazone hydrochloride

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Introduction

Physical activity appears to impact all stage of carcinogenesis. Insulin promotes the utilization of glucose and synthesis of glycogen by inhibiting gluconeogenesis. Additionally, insulin plays a role in catalyzing the synthesis of fats and proteins and inhibits their degradation. Normally, insulin binds to its receptor to initiate signal transduction. Insulin resistance (IR) can be induced by any of the following events: reduction in the number and affinity of insulin receptors, mutations of encoding genes, downregulation of the glucose transporter, and defects in insulin signal transduction (Yoshikawa et al., 2001; Wilcox, 2005; Draznin, 2006; Leclercq et al., 2007). IR refers to a decrease in the sensitivity of the whole organism, organs, tissues, or cells to insulin stimulation. IR is involved in the chronic pathological process of glucose metabolism disorders, and it ultimately leads to type 2 diabetes. Moreover, insulin plays a role in metabolic syndrome and the drug resistance of tumors (Wilcox, 2005). IR is an established risk factor for patients with hepatocellular carcinoma (HCC) due to the hypoxic and ischemic conditions encountered during tumor progression. Several studies revealed that IR was a prognostic factor for the postoperative recurrence of HCC (Yoshikawa et al., 2001; Keku et al., 2005; Soliman et al., 2006; Turati et al., 2013). However, the specific mechanism of IR and its relationship with multidrug resistance are unclear.

Adhesion, invasion, and migration are the most distinctive and prominent features of cancer cells, and these features are involved in cancer progression and subsequent death. A hypothetical 'three-step' mechanism of tumor cell invasion was proposed, and it has been confirmed that tumor cells must first destroy the original tissues, infiltrate adjacent tissues, and then migrate to distant sites (Tsujimoto et al., 1984; Liotta and Stetler-Stevenson, 1991). Given this mechanism, therapies targeting the regulation of invasion can arrest tumor progression and improve prognosis. Thus far, there is no evidence about the roles of IR acquisition in the regulation of the biological characteristics of liver cancer cells. In the present study, our results demonstrated that HepG2/IR cells were resistant to cis-platinum (DDP), a commonly

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used chemotherapeutic drug. Most importantly, HepG2/IR cells exhibited enhanced adhesion, migration, and invasion. By contrast, pioglitazone hydrochloride (PH) treatment decreased IR and inhibited the invasiveness of HepG2 cells. These findings illustrated the connection between IR and drug resistance in liver cancer cells.

Materials and Methods

Establishment and reversal of IR in HepG2 cells

Human hepatocarcinoma HepG2 cells (obtained from the American Type Tissue Culture Collection) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Hyclone, USA) and cultured at 37°C with 5% CO_2. IR was induced in HepG2 cells according to a previously described method (Cousin et al., 1987; Melin et al., 1990). To select induction condition with IR lasting for 72h, the cells were incubated in serum-free DMEM for 6h prior to treatment with 0.5 and 1 μmol/L insulin for 48 and 72h, respectively, and then cultured without insulin for an additional 72h. Afterward, the medium was replaced, and cells were further cultured in DMEM without phenol red for 24h. The glucose content in the culture supernatant was detected by the GOD-POD method (RANDOX Laboratories, UK) using a Hitachi 7600-020 automatic biochemical analyzer. To eliminate the influence of cell viability, we conducted an MTT assay to normalize glucose consumption to the cell number (glucose consumption/OD_{MTT}).

To test the stability of IR, relative glucose consumption (the ratio of glucose consumption in HepG2/IR cells and the corresponding control HepG2 cells) was determined in cells that had been induced, and those that had been induced and then cultured for 72h. The stability of the acquired IR was then determined by assessing whether the relative glucose consumption was stable after 72h.

HepG2/IR cells were treated with PH (10 mmol/L) for 24h to inhibit IR, after which the adhesion, migration, and invasion of the cells (HepG2/IR+PH cells) were analyzed.

Flow cytometry (FCM)

To determine the expression of the insulin receptor (InsR) and glucose transporter-2 (GLUT-2), HepG2 cells were incubated with anti-InsR-FITC (Invitrogen, USA) and anti-GLUT2-FITC (Biological, USA). Cells were treated with 0.5 μmol/L insulin for 72h, cultured without insulin for 72h, harvested, and incubated with the appropriate antibodies for 30 min. Protein expression, including the positive expression rate (PR, %) and mean fluorescent intensity (MFI), was detected using an Epics XL-4 flow cytometer (BD Biosciences, USA) (Macaulay et al., 1995).

An annexin V/propidium iodide (PI) double staining assay (Invitrogen, USA) was used to determine cell apoptosis. HepG2, HepG2/IR, and HepG2/IR+PH cells were treated with DDP (Sigma, USA) for 48h and harvested. The cells were washed with PBS, and FITC-labeled anti-Annexin-V and PI were added at room temperature in the dark for 30 min to stain nuclei. The control, early apoptotic, and late apoptotic cells were analyzed by FCM (Sawai and Domae, 2011).

MTT assay to determine DDP sensitivity (Stockert et al., 2012)

HepG2, HepG2/IR, and HepG2/IR+PH cells were harvested and inoculated in Costar 96-well plates at the density of 1×10^5 cells/mL. DDP (1.0-64.0 mg/L) was added to the culture plate, which was placed in a saturated humidity incubator at 37°C with 5% CO_2 for 44 or 68h. MTT solution (10 μL, 5 g/L) was added to each well, and cells were cultured at 37°C for an additional 4h. The supernatant was removed, and DMSO was added. Cells were incubated on a shaker for 15 min at room temperature. Optical density (OD) was quantified at 490 nm using a Powerwave X plate reader (Bio-Tek, USA). Cell proliferation inhibition rates were calculated using the following formula: cell proliferation inhibition rate=[(ODcontrol-ODexperiment)/ODexperiment] × 100%. The half-maximal inhibitory concentration (IC_{50}) was calculated.

Cell adhesion assay (Yao et al., 2007)

For the adhesion assay, Matrigel (200 μg/mL; BD Biosciences) was added to each well of a 96-well flat-bottomed plate (25 μL/well). The plate was incubated at 37°C for 1h, washed with PBS, and dried at room temperature, and 1% BSA (Sigma) was added to plate (20 μL/well). The plate was then incubated at 37°C for 1h, washed with PBS, and dried at room temperature for 1h. HepG2, HepG2/IR, and HepG2/IR+PH cells were digested with trypsin, and cells (5×10^5/mL) were suspended in 0.1% BSA-DMEM and added to the Matrigel (100 μL/well)-coated wells. After incubation for 0.5 and 1h, non-adherent cells were removed by aspiration and washed three times with PBS. The values of all wells were measured by the MTT assay at 490 nm by using a microplate reader. The adhesiveness of the cells was expressed as follows: adhesion rate (%)=ODexperiment/ODcontrol×100%.

Cell migration and invasion assay (Yao et al., 2007)

The invasiveness of HepG2 cells was assayed using a Transwell migration assay system (Millipore) in which the chambers contained polyvinylpyrrolidone-free polycarbonate filters with a pore diameter of 8.0 μm. To reconstitute the basement membrane, Matrigel (20 μg/mL) was added to the upper surface (100 μL/filter) to form a matrix barrier. Coated filters were incubated at 37°C and dried before using. HepG2, HepG2/IR, and HepG2/IR+PH cells were harvested with DMEM supplemented with 0.1% BSA. Tumor cell suspensions (2×10^6 cells/mL, 100 μL) were added to the upper compartment of the chamber and incubated for 26h, and 600 μL of complete culture medium supplemented with 10% FBS were added to the lower chamber. After incubation, the filters were harvested, fixed with paraformaldehyde, and stained with crystal violet. Tumor cells on the upper surfaces of the filters were removed by wiping with cotton swabs. Cells that had passed through the Matrigel and filter to the lower surface were counted under a microscope in five fields at ×200 magnification. Each assay was performed

in triplicate and repeated at least three times. For the *in vitro* migration experiment, the Matrigel basement membrane matrix was not coated, and the inoculated cell concentration was 8×10^5 cells/mL. Other procedures were performed as described for the invasion experiment.

Statistical analysis

Data are expressed as means±SD. Statistical and graphical analysis was performed using Student's t-test and SPSS 15.0. *P* value less than 0.05 was considered statistically significant.

Results

Construction of HepG2/IR cells by exposure to insulin

HepG2 cells were incubated with 0.5 or 1 μmol/L insulin for 48 or 72h, respectively, to induce IR and then cultured for 72h in insulin-free medium. The glucose consumption was determined, and the results indicated that glucose consumption was decreased in a concentration and time-dependent manner compared with the control ($p<0.05$ and $p<0.01$, respectively). This effect was obvious in cells exposed to insulin for 72h. Based on the glucose consumption, cells were incubated with 0.5 or 1 μmol/L insulin for 72h to establish HepG2/IR cells (Figure 1A). The relative glucose consumption was evaluated to observe the stability of IR. There were no obvious change observed between induced cells and those that were cultured without insulin for an additional 72h after induction (Figure 1B). As a result, the final induction concentration was 0.5 μmol/L insulin for 72h in subsequent experiments according to the cell growth and glucose consumption data.

We applied FCM to determine the expression of InsR and GLUT-2 after culturing the cells in medium without insulin for 72h. Compared to HepG2 cells, the expression levels of InsR and GLUT-2 proteins were significantly decreased in HepG2/IR cells by 82.2 and 27.9% respectively (Figure 1C). This further indicated that HepG2/IR cells had acquired stable IR that could be maintained for at least 72h.

DDP sensitivity of HepG2/IR cells

The MTT assay revealed that HepG2/IR cells exhibited significantly decreased sensitivity to DDP. The IC_{50} values HepG2/IR cells after 48 and 72h of exposure to DDP were 165.9 and 158.8% higher than those of HepG2 cells, respectively ($p<0.05$, Figure 2A). This finding suggested that HepG2/IR cells became resistant to DDP after acquiring IR. When HepG2/IR cells were treated with 10 mmol/L PH for 24h, their sensitivity to DDP was restored to a level similar to that of HepG2 cells (Figure 2A), as their IC_{50} after 48 and 72h of exposure to PH were 32.0 and 25.6% lower than those of HepG2/IR cells, respectively. After treatment with 16 mg/L DDP for 48h, FITC-annexin V/PI staining indicated that HepG2/IR cells displayed 50.29% less DDP-induced apoptosis than HepG2 cells. After IR was reversed by PH treatment, the DDP-induced apoptosis rate of these cells was increased. The apoptosis rate of HepG2/IR+PH cells was 72.56% higher than that of HepG2/IR cells before IR reversal ($p<0.01$, Figure 2B) and similar to that of HepG2 cells in the control group. These results indicate that HepG2/IR cells developed resistance to DDP due to the acquisition of IR, which could be reversed by the insulin sensitizer PH. These findings suggested that IR conferred tolerance to chemotherapy in HepG2 cells.

Adhesion was enhanced in HepG2/IR cells

HepG2 and HepG2/IR cells were cultured in Matrigel-coated 96-well plates. MTT assay analysis revealed that the adhesiveness of HepG2/IR cells was significantly enhanced. The adhesion rates were increased by 74.0 (30 min) and 75.3% (60 min) compared with those of HepG2

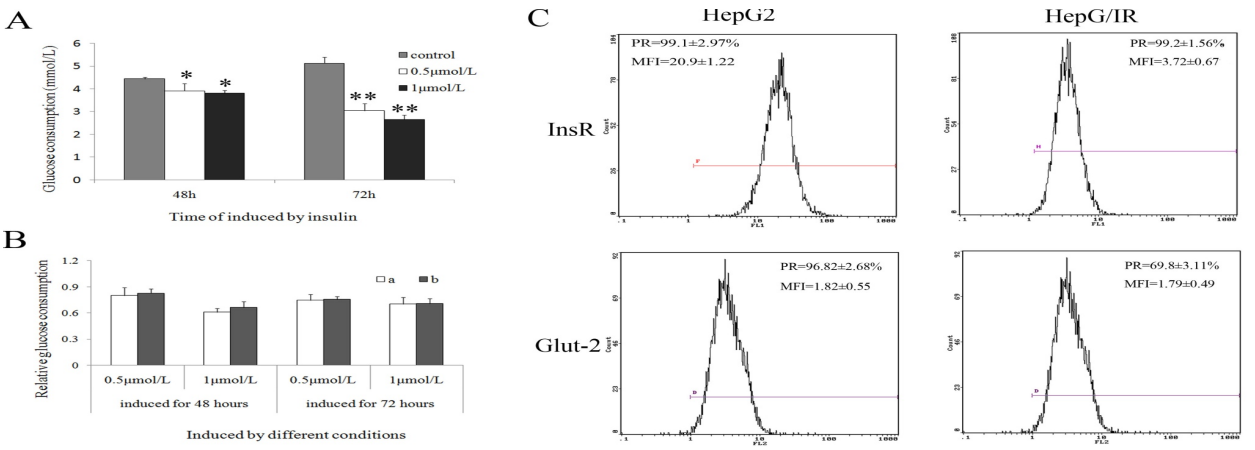

Figure 1. Induced Insulin Resistance (IR) in HepG2 Cells. A) To induce insulin resistance, HepG2 cells were cultured in medium containing 0 (control), 0.5, or 1 μmol/L insulin for 48 or 72h after which they were cultured in insulin-free medium for 72h, and then the GOD-POD assay was used to measure glucose concentrations in HepG2 cells. The glucose consumption of insulin-resistant HepG2 was significantly decreased in a concentration- and time-dependent manner compared with the findings in control cells. **B)** The stability of HepG2/IR cells was >72h. Relative glucose consumption (glucose consumptionexperiment/glucose consumptioncontrol) was compared between cells that had been induced (a), and those that had been induced and then cultured for 72h (b). No significant difference was noted between the two groups. **C)** Flow cytometry was used to assess the expression of the insulin receptor (InsR) and glucose transporter-2 (GLUT-2), and their expression was decreased in HepG2/IR cells after incubation for 72h in completed medium. Experiments were repeated three times with similar results, and results are presented as the mean±SD for triplicate experiments.*$p<0.05$; **$p<0.01$, compared to control

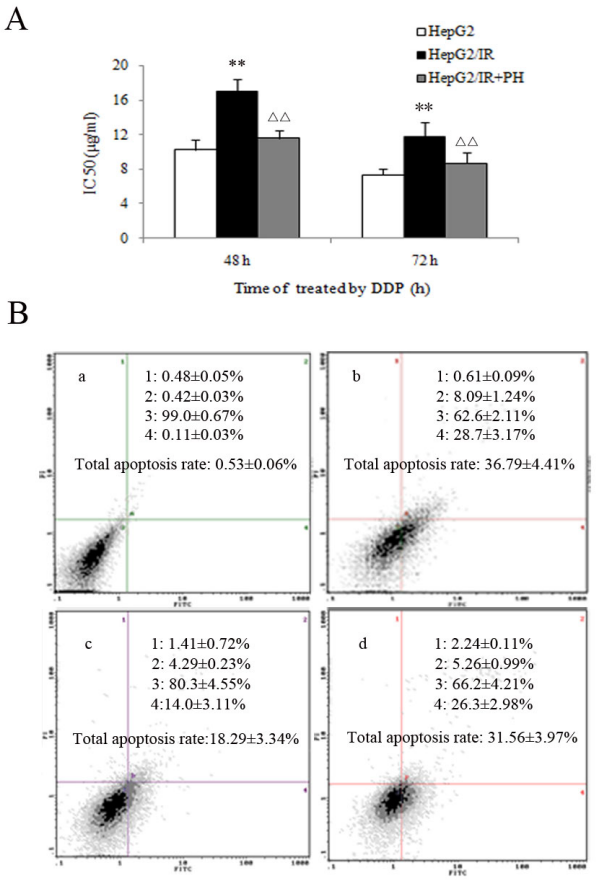

Figure 2. Insulin Resistance (IR) Resulted in Tolerance to DDP in HepG2/IR Cells. (A) HepG2/IR cells were resistant to cis-platinum (DDP; 1.00-64.00 mg/L), which could be reversed by treatment with the insulin sensitizer pioglitazone (PH). An MTT assay was used to assess the viability of HepG2/IR (exposed to 0.5 μmol/L insulin for 72h) and HepG2/IR+PH cells (exposed to 0.5 μmol/L insulin for 72h and then incubated with 10 mol/L PH for 24h) in response to DDP treatment. a, HepG2 cells; b, DDP-treated HepG2 cells; c, DDP-treated HepG2/IR cells; d, DDP-treated HepG2/IR+PH cells. (B) Flow cytometry spot diagram shows HepG2, HepG2/IR and HepG2/IR+PH cells after incubation for 48h with or without 16.00 mg/L DDP. The early apoptotic index was calculated as the percentage of annexin V-positive and propidium iodide (PI)-negative cells. The late apoptotic index was assessed as the percentage of annexin V-positive and PI-positive cells. The cells were gated into four regions according to annexin V and PI staining. The experiments were independently repeated three times with similar results, and the results are presented as the mean±SD.*p<0.05 and **p<0.01; $^{\triangle}p$<0.05; and $^{\triangle\triangle}p$<0.01

cells. On the contrary, when PH induced a reversal of IR in HepG2/IR cells, adhesion was significantly inhibited. The adhesion rates were decreased by 26.7 (30 min) and 29.2% (60 min) compared to those of HepG2/IR cells (p<0.01, Figure 3). It was thus implied that IR increases adhesion in HepG2/IR cells.

IR promoted the migration and invasion of HepG2/IR cells

Invasion and migration were assessed using the Transwell migration assay. In comparison with HepG2 cells, invasion and migration were significantly enhanced in HepG2/IR cells. The numbers of migrating and invading HepG2/IR cells were 1.73-and 1.71-fold higher than those of HepG2 cells, respectively (p<0.05, Figure 4). When PH

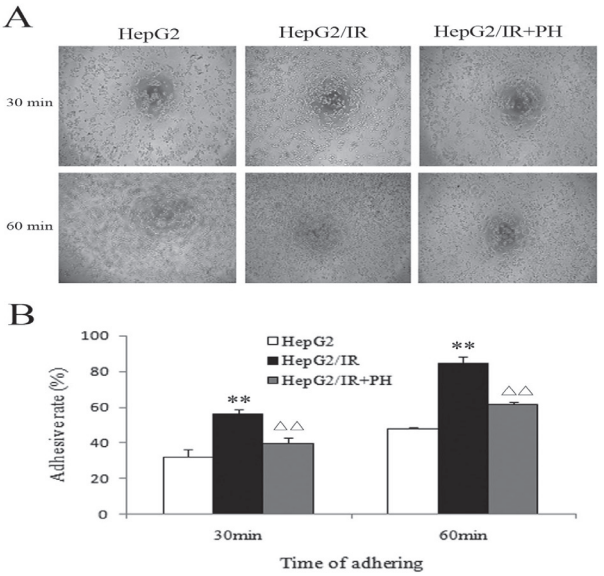

Figure 3. The Effect of Insulin Resistance (IR) on Adhesion in HepG2 Cells. A) Representative phase contrast microscopy images of HepG2, insulin-resistant HepG2 (HepG2/IR), and pioglitazone-treated HepG2/IR (HepG2/IR+PH) cells on a thin layer of Matrigel. The pictures were recorded at 30 and 60 min (magnification, ×100. **B)** An MTT assay was conducted to assess the viability of HepG2, HepG2/IR, and HepG2/IR+PH cells at 30 and 60 min. IR enhanced the adhesiveness of HepG2 cells, whereas HepG2/IR+PH cells displayed reduced adhesiveness. The results are expressed as the inhibition of cell adhesion in six experiments. * **p<0.01, compared to HepG2 cells; $^{\triangle\triangle}p$<0.01 compared to HepG2/IR cells

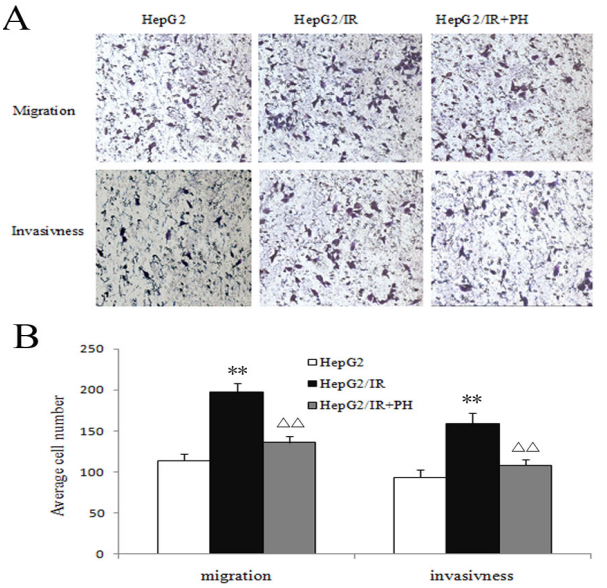

Figure 4. IR Enhanced Migration and Invasion in HepG2/IR Cells. A) HepG2, insulin-resistant HepG2 (HepG2/IR), and pioglitazone-treated HepG2/IR (HepG2/IR+PH) cells were incubated in the upper compartment of a Transwell culture chamber for 28h. Migrating and invading cells were stained by crystal violet (A; magnification, ×200). The image shown is a representative field in an experiment independently repeated three times. **B)** The numbers of migrating and invading cells in five fields were counted under a microscope. Each assay was performed in triplicate and repeated at least three times. The results are presented as the mean±SD for triplicate experiments. **p<0.01, compared to HepG2 cells; $^{\triangle\triangle}p$<0.01, compared to HepG2/IR cells

was added to the medium to reverse IR, the migratory and invasive abilities of HepG2/IR cells were significantly decreased by 30.95 and 32.00%, respectively ($p<0.05$, Figure 4). The induction of IR could strengthen invasion and migration in liver cancer cells.

Discussion

Physical activity appears to impact all stage of carcinogenesis (Kruk and Czerniak, 2013). Glucose metabolism dysregulation is a feature of malignant cancer that results in decreased insulin sensitivity and IR. IR is a high risk factor for multiple cancers (Hu et al., 2013). The prevention of IR represents one of the most important strategies to reduce the risk of postoperative recurrence. Moreover, several reports indicated that IR helps to promote the progression of cancer. More importantly, IR hinders the efficacy of chemotherapeutic drugs. It had been proven that IR combined with hyperinsulinemia facilitates cancer cell proliferation and inhibits apoptosis. Consequently, IR prevents the improvement of anticancer drug efficacy (Ledoux et al., 2003). However, we do not fully understand the biological effects of IR on cancer cells. As the most important target organ of insulin, reduced insulin sensitivity in liver cells is one of the pathological changes of carcinogenesis (Leclercq et al., 2007). The HepG2 cell line is an HCC cell line that possesses some features of normal liver cells including high InsR expression. In this study, we successfully established stable IR for over 72h by exposing HepG2 cells to a high insulin concentration. The HepG2/IR cells displayed reduced glucose consumption and low InsR and GLUT-2 expression, all of which are typical features of IR.

It is difficult to eradicate HCC cells with chemotherapeutics due to their inherently low sensitivity to anticancer drugs or complex mechanisms of drug resistance. In this study, we found that IR decreased DDP sensitivity and inhibited apoptosis in HepG2 cells. PH, a widely used insulin sensitizer, effectively reverses IR in fat, skeletal muscle, and liver tissues. The reversal of IR significantly elevates glucose consumptions in tumor cells (Cho and Momose, 2008). In the present study, we proved that DDP resistance caused by IR could be reversed by PH in HepG2/IR cells.

The recurrence and metastasis of solid tumors are strongly influenced by tumor cell adhesion, migration, and invasion, which are associated with the poor prognosis of patients with HCC (Su et al., 2006; Loberg et al., 2007). IR encourage fibrosis succession, progress of hepatic steatosis, hyperleptinemia, increased tumor necrosis factor (TNF) production and bring with poor prognosis for HCC patients (Gupta et al., 2013). To our knowledge, no study reported the effects of IR on the migration of HCC cells. Our results suggested that IR enhanced adhesion, migration, and invasion in HepG2/IR cells, and significantly decreased their sensitivity to DDP. Nonetheless, once IR was reversed by the insulin sensitizer PH, adhesion, migration, and invasion were inhibited in HepG2/IR cells. Therefore, these findings indicate that IR might be associated with resistance to chemotherapeutic drugs in patients with HCC who have a poor prognosis. Given that, the monitor level of insulin should be apply to assess the states of HCC patients, so as to provide them with proper treatment.

In conclusion, we established a stable HepG2 cell line with IR, and the induction of IR resulted in resistance to chemotherapeutic drugs. Our results demonstrated that IR enhanced adhesion, migration, and invasion in HepG2 cells. According to these findings, IR might be one of the major causes of chemotherapeutic failure in patients with HCC. For this reason, decreasing insulin resistance will improve the sensitivity of tumor cells to chemotherapy and inhibit their invasiveness, ultimately enhancing the therapeutic efficacy for HCC.

Acknowledgements

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