Based on the Effect of Huazhengsanji Prescription and Cisplatin on Hypoxia-Induced HepG2 Hepatoma Cells HIF-1α and VEGF

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Research

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Abstract

Background

Hepatoma is one of the most common malignant tumors in my country, and its occurrence and development play an important role in the molecular mechanism of hypoxia microenvironment and angiogenesis. Huazhengsanji prescription (HZSJ) is an empirical prescription for the treatment of liver cancer, but its specific anti-tumor molecular mechanism is still unclear, and whether it has a synergistic effect with cisplatin (DDP), a chemotherapy drug for liver cancer, has not been reported yet. This article discusses the inhibition of the proliferation and migration of HepG2 hepatocarcinoma cells and the difference in the expression of HIF-1α and VEGF when the HZSJ, DDP and the two are used in combination, and compares and analyzes the mechanism of the HZSJ in enhancing the anticancer sensitivity of chemotherapeutics.

Methods

HepG2 Hepatoma cells were divided into blank control group, hypoxia model group, DDP group, HZSJ group, HZSJ+DDP group, and the hypoxia environment was induced by the CoCl2 method. MTT method detects cell proliferation ability, scratch test detects cell migration ability, immunofluorescence method and western blot detect HIF-1α and VEGF protein expression.

Results

HZSJ has the effect of inhibiting the proliferation and migration of HepG2 cells, and has obvious concentration-dependent; The combined application of HZSJ and DDP has significantly stronger inhibitory effect on cell proliferation than the HZSJ group (P<0.01) and the DDP group (P<0.01, P<0.001). High-dose HZSJ can inhibit the migration ability of HepG2 cells (P<0.01); the combination of HZSJ and DDP can significantly reduce the migration rate of HepG2 cells after induction (P<0.01, P<0.01, P<0.01). The results of immunofluorescence and western blot showed that: compared with the blank control group, the expression levels of HIF-1α and VEGF protein in the model group were significantly increased (P<0.05, P<0.01, P<0.001); compared with the model group and DDP The expression of HIF-1α protein in the high-dose HZSJ group (200μg/mL) and the combination group decreased (P<0.05, P<0.01, P<0.001), but there was no difference between the groups. Compared with the model group, the high-dose HZSJ group (200μg/mL) can reduce the expression of VEGF (P<0.05); compared with the model group and the DDP group, the combination group can reduce the expression of VEGF (P<0.01, P<0.001), and has obvious concentration dependence.

Conclusions

HZSJ can inhibit the proliferation and migration of HepG2 hepatoma cells under hypoxia, which may be related to the reduction of HIF-1α and VEGF expression, and its increase in the anticancer sensitivity of the chemotherapy drug DDP may be related to both. The synergistic inhibition of VEGF expression is related.
Background

Hepatoma is one of the most common malignant tumors in my country. The hypoxic microenvironment is conducive to the development of tumor growth and metastasis \[1\]. Hypoxia inducible factor-1 (HIF-1) is the main transcription mediator of hypoxia response and the main regulator of O\(_2\) stability \[2\]. HIF-1 is a heterodimer composed of two subunits, \(\alpha\)-subunit and \(\beta\)-subunit, among which one of the main mediators of hypoxia signal transduction is the transcription factor HIF-1\(\alpha\)\[3\]. Vascular endothelial growth factor (VEGF) is considered to be the main effector of tumor angiogenesis. Hypoxia activates the expression of HIF-1\(\alpha\) in tumor cells, and the combination of HIF-1\(\alpha\) and the upstream enhancer sequence of the VEGF gene promotes the expression of VEGF \[4\]. In addition, VEGF can directly participate in the process of tumor cell formation, proliferation and migration \[5\]. HZSJ is an empirical prescription for the treatment of Hepatoma. Pre-clinical studies have shown that Huazhen Sanji prescription can protect liver function, reduce AFP, and prolong the average survival time \[6\]. Experimental studies have shown that Huazhensanji prescription can inhibit hepatoma cell. The growth of \[7, 8\], the protection of liver cells \[9\] and so on.

In this study, HepG2 hepatocellular carcinoma were treated with CoCl\(_2\) in order to increase the expression of HIF-1\(\alpha\) and VEGF. The effect of Huazhensanji prescription, DDP and the combination of the two in inhibiting the proliferation and migration of HepG2 hepatocellular carcinoma and the differences in the expression of HIF-1\(\alpha\) and VEGF protein were observed. The mechanism of action of HZSJ to enhance the anti-cancer sensitivity of chemotherapy drugs.

Materials And Methods

Material

HepG2 hepatoma cells were purchased from Wuhan Punuosai Life Technology Co., Ltd.; HZSJ was purchased from Beijing Tongrentang Changchun Drugstore Co., Ltd. Hongqi street main store; DDP was purchased from Shanghai Yuanye, the item number is S31072-250 mg; HIF-1\(\alpha\) was purchased from abcam, item number EPR16897; VEGF was purchased from abcam, item number D5B1.

Cell culture

The DMEM medium containing 10% FBS was cultured in an incubator with 5% CO\(_2\), 37 °C and saturated humidity.

MTT method to detect cell viability

After inoculating \(6 \times 10^3\) cells into a 96-well plate for 24 hours, adding different concentrations of drugs for treatment and incubating for 24 hours, MTT detects the absorbance (OD) value of each group of cells at a wavelength of 490 nm. The experiment was repeated three times.
Cell survival rate (%) = \([\text{OD (dosing)} - \text{OD (blank)}]/[\text{OD (control)} - \text{OD (blank)}]\) × 100%.

**Scratch test**

HepG2 cells are seeded in a 12-well plate at a cell density of \(3 \times 10^5\) cells/well. When the cell monolayer grows to cover the bottom of the plate, use a pipette tip to make a “1” mark along the bottom of the culture plate. Incubate for 0 h and 24 h at 37°C and 5% CO\(_2\). Detect the relative distance of cell migration to the injured area under the microscope and take pictures to calculate the cell migration rate.

Cell migration rate (%) = (cell migration area of the negative control group - cell migration area of the experimental group)/cell migration area of the negative control group × 100%.

**Immunofluorescence**

After HepG2 cells were cultured at cell density for 24 hours, they were washed twice with PBS; fixed with paraformaldehyde fixative solution at 4 °C for 60 minutes, added 0.1–0.2% Triton for 10 minutes, washed once with TBST, and blocked with BSA solution for 1 hour; primary antibody, Overnight at 4 °C; wash with TBST 5 times; incubate the secondary antibody for 1 h; wash 5 times with TBST; add DAPI and store in the dark for 5 minutes; wash 3 times with TBST. The high-content system takes pictures and quantitative analysis.

Relative fluorescence intensity (100%) = \(\sum (\text{HIF-1}\alpha, \text{VEGF fluorescence intensity of each cell in the well})/\text{number of cells}\)

**Western Blot**

Cells in each group were cultured for 24 h. The protein lysate was used to extract the total protein of each group of cells, and the protein concentration was determined by BCA method. After quantification, perform SDS-PAGE vertical electrophoresis, wet PVDF transfer membrane, 5% skim milk blocking for 1 hour, 4 °C and incubate with primary antibody dilution (1:1000) overnight, TBST washing 5 times, and incubation with secondary antibody dilution for 1 hour TBST was washed 5 times, ECL glowed, developed, and analyzed with quantityone software after scanning.

**Establishment of fingerprint spectrum and identification of mass spectrometry**

Fingerprints obtained from precision experiments, repeatability experiments, and stability experiments.

**Statistical analysis**

The data was analyzed using IBM SPSS Statistics 22.0 software, and \(p < 0.05\) was considered statistically significant. Measurement data are expressed by (x ± s), One-way-ANOVA is used for comparison between multiple groups, and unpaired t test is used for comparison between two groups.
Results

Fingerprint establishment and mass spectrometry identification

According to the fingerprints obtained from precision experiment, repeatability experiment, and stability experiment, the similarity is all above 0.9, and the sample stability and batch sample repeatability are good.

As shown in Fig. 1, the chromatographic peaks with larger peak areas and better resolution were calibrated, and a total of 32 common peaks were calibrated. Through mass spectrometry combined with reference methods, 6 components in 15 batches of HZSJ samples were identified, among which peak 7 is catalpol, Peak 9 is paeoniflorin, peak 14 is glycyrrhizin, peak 28 is harpagoside, peak 30 is ammonium rhein, and peak 31 is ammonium glycyrrhizinate.

Figure 1 Total 15 batches of HZSJ atlas

HZSJ combined with DDP inhibits HepG2 cell viability

From Fig. 2A, it can be seen that DDP can effectively inhibit the viability of HepG2 cells, with an IC50 of about 17.36 µg/mL. When the concentration of DDP reaches 2.5ug/mL, it starts to have an inhibitory effect. Therefore, it was selected as a fixed dose in the remaining experiments. Drug dosage: HZSJ can inhibit HepG2 cell viability at high concentrations. As shown in Fig. 2B, the two drugs combined with HZSJ can significantly enhance the sensitivity of HepG2 cells to DDP. When the concentration of HZSJ is 50, 100, 200 (µg/mL), the combined effect The IC50 of the latter DDP decreased to 4.08, 2.08, 1.86 (µg/mL) respectively. HZSJ makes DDP have a strong inhibitory activity on HepG2 at low concentrations.

HZSJ and DDP can synergistically inhibit the migration ability of HepG2 cells

As shown in Fig. 3, after CoCl2 hypoxia stimulation, the migration ability of HepG2 cells increases; low-dose DDP (2.5ug/ml) alone has no inhibitory effect; high-dose Huazhengsanjican inhibit the migration ability of HepG2 cells \((P<0.01)\); The combination of HZSJ and DDP can significantly reduce the migration rate of HepG2 cells after induction \((p<0.01, p<0.01, p<0.01)\), indicating that HZSJ can synergize with low doses DDP inhibits the migration ability of HepG2 cells.

Effects of HZSJ and DDP on the expression of HIF-1α and VEGF protein

HIF-1α and VEGF are important factors that affect tumor cell proliferation and migration. Therefore, the effect of Huazhen Sanji prescription and DDP on the expression of HIF-1α and VEGF protein in hypoxia-stimulated HepG2 cells was further tested. As shown in Fig. 4, according to the average fluorescence intensity of cells, the expression level of HIF-1α protein in the model group \((127.56 \pm 0.03)\) was significantly higher than that in the control group \((100 \pm 0.06) \ (P<0.001)\), indicating that it can secrete under hypoxic conditions A large amount of HIF-1α protein; HZSJ \((100 \mu g/mL, 200 \mu g/mL)\) and the combination of the HIF-1α protein expression in each group was lower than that of the hypoxia model
group \( P < 0.05, P < 0.001 \), but between the groups there is no difference. The expression level of VEGF protein in the model group \( 136.54 \pm 0.08 \) was significantly higher than that of the control group \( 100 \pm 0.01 \) \( P < 0.05 \); compared with the model group in the HZSJ group \( 200 \mu g/mL \) and the combination medication groups, it can reduce the expression of VEGF \( P < 0.05, P < 0.001 \); the combination group \( HZSJ 100 \mu g/mL + DDP, 200 \mu g/mL + DDP \) can reduce the expression of VEGF compared with the DDP group \( P < 0.001 \). Figure 5. Western blot results showed that the expression of HIF-1\( \alpha \) and VEGF protein in HepG2 cells was significantly increased under hypoxia \( P < 0.001, P < 0.01 \). Compared with the model group, HZSJ \( 200 \mu g/mL \) showed that the expression level of HIF-1\( \alpha \) and VEGF protein \( P < 0.01, P < 0.05 \), the expression of HIF-1\( \alpha \) and VEGF protein in the combination group was significantly down-regulated compared with the model group and the DDP group \( P < 0.01, P < 0.001 \)), verification. The results of immunofluorescence detection. It shows that HZSJ can positively cooperate with low-dose DDP to reduce the protein expression levels of HIF-1\( \alpha \) and VEGF in HepG2 cells induced by hypoxia.

**Discussion**

Hepatoma is the second most fatal malignant tumor in the world\(^{10}\). Early hepatoma is generally treated with surgery, including liver resection and liver transplantation. However, patients with hepatoma are often in the middle and late stages when they are diagnosed. Adjuvant treatments include local ablation, radiotherapy, Chemotherapy, gene targeted therapy, hepatic artery interventional therapy, traditional Chinese medicine therapy, etc.\(^{11}\), adjuvant radiotherapy, chemotherapy alone can not significantly improve the survival rate of patients after cancer\(^{12}\). This experiment found that HZSJ can increase the sensitivity of chemotherapy drugs and reduce the pain of patients by reducing the dosage of chemotherapy drugs.

Hypoxia is one of the characteristics of the tumor microenvironment, which can promote tumor angiogenesis\(^{13}\), tumor infiltration and metastasis\(^{14}\). Hypoxia-inducible factor-1\( \alpha \) is an important mediator of hypoxia response. It regulates the expression of various chemokines involved in tumor growth, angiogenesis and metastasis\(^{15}\). Studies have shown that\(^{16}\) hypoxia can promote HIF-1\( \alpha \) mRNA and protein levels in Hepatoma cells. This experiment found that the expression of HIF-1\( \alpha \) protein in the model group was significantly higher than that in the control group. The expression of HIF-1\( \alpha \) protein in the HZSJ group \( 100 \mu g/mL, 200 \mu g/mL \) and the combination medication group was lower than that of the hypoxia model group. VEGF secreted by tumor cells and surrounding stroma stimulates the proliferation and survival of endothelial cells, leading to the formation of new blood vessels\(^{17}\). VEGF (now called VEGF-A) is a member of the protein family, including VEGF-B, VEGF-C, VEGF-D, VEGF-E and placental growth factor (PIGF)\(^{18}\), VEGF-A play an important role in angiogenesis and disease, so it is called VEGF. Studies have found that VEGFmRNA is overexpressed in hepatoma and is associated with invasion, vessel density, metastasis, recurrence and prognosis\(^{19}\). The expression of VEGFR1 in some tumor cell lines mediates the proliferation response of VEGF or PIGF\(^{20}\). VEGF is considered to be one of the most important stimulators of angiogenesis and has been identified as a key angiogenesis signal in HCC\(^{21}\). The expression of VEGF is mainly controlled by two main transcriptional activators: one of them...
is hypoxia-inducible factor-1α (HIF-1α). Studies have found that in the signal transduction pathway of hypoxia regulating VEGF, HIF-1α plays a central role. It not only increases the stability of VEGF mRNA, but also increases the transcriptional activity of VEGF [22]. The role of these two factors in tumor angiogenesis may be related to the mechanism of tumor angiogenesis. This experiment found that hypoxia can promote the secretion of VEGF protein. Compared with the model group, the HZSJ group (200 µg/mL) and the combination group can reduce the expression of VEGF.

The results of this study confirm that HZSJ has the effect of inhibiting the proliferation and migration of HepG2 hepatocellular carcinoma cells under hypoxia, which may be related to the reduction of HIF-1α and VEGF expression. It may increase the anticancer sensitivity of the chemotherapy drug DDP It is related to the synergistic inhibition of VEGF expression.

To sum up, the reduction of HIF-1α and VEGF protein expression may be one of the important mechanisms of high-dose HZSJ and HZSJ combined with DDP to inhibit the proliferation and migration of HepG2 hepatoma cells. Its in-depth study Need to be further demonstrated.

**Conclusions**

HZSJ has the effect of inhibiting the proliferation and migration of HepG2 hepatocellular carcinoma cells under hypoxia, which may be related to the reduction of HIF-1α and VEGF expression. It may increase the anticancer sensitivity of the chemotherapy drug DDP It is related to the synergistic inhibition of VEGF expression.

**Abbreviations**

HZSJ
Huazhengsanji prescription
DDP
Cisplatin
DMEM
Dulbecco's modified eagle medium
HIF-1α
Hypoxia Inducible Factor-1α
VEGF
Vascular Endothelial Growth Factor

**Declarations**

**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.
Authors’ contributions

WSS, LYY, XXH, LTJ, and SZH performed the experiments and analyzed the data; XZ were the major contributor in designing the research and writing the manuscript. All authors read and approved the final manuscript.

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Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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