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EXPERIMENTAL STUDY

Cisplatin plus norcantharidin alter the expression of TGF-β1/ Smads signaling pathway in hepatocellular carcinoma

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ABSTRACT

PURPOSE: To investigate the effects of cisplatin plus norcantharidin on transforming growth factor (TGF)-β1/ Smads signaling pathway in hepatocellular carcinoma cells.

METHODS: Hepatocellular carcinoma cells (Hep3B) were divided into four groups: control group, cisplatin 2.0 μg/ml group, norcantharidin 10 μg/ml group, and cisplatin 2.0 μg/ml plus norcantharidin 10 μg/ml group. All cells were incubated for 24 hours. Cells proliferation was assessed using cell counting kit-8. Relative mRNA expression of TGF-β1, Smad4 and Smad7 were assessed by quantitative RT-PCR. Protein expression of TGF-β1 and Smad4 were investigated by western blotting.

RESULTS: Cisplatin, norcantharidin and cisplatin plus norcantharidin significantly inhibited the proliferation of cells, significantly attenuated both the mRNA and protein expression of TGF-β1 and Smad7, and significantly up-regulated the mRNA and protein expression of Smad4 in Hep3B (all $p < 0.05$), and cisplatin plus norcantharidin exhibited powerful effects than cisplatin and norcantharidin.

CONCLUSIONS: Cisplatin, norcantharidin and cisplatin plus norcantharidin can significantly alter the expression of TGF-β1/Smads signaling pathway and inhibit the proliferation of Hep3B cells. Cisplatin plus norcantharidin exhibited powerful effects than cisplatin and norcantharidin *(Fig. 4, Ref. 23)*. Text in PDF *www.elis.sk*.

KEY WORDS: hepatocellular carcinoma cells, TGF-β1/Smads, cisplatin, norcantharidin.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant neoplasms worldwide (1, 2). Surgical therapy, chemotherapy and radiation have been used for the treatment of HCC. However, HCC remains one of the more difficult cancers to treat. Studies indicate that transforming growth factor-β1 (TGF-β1) / Smads signaling pathway plays an important role in the pathogenesis of HCC (3, 4). Cisplatin (DDP) has been widely used in the treatment of different kinds of cancers, especially in the treatment of HCC (5–7). Norcantharidin (NCTD) has been used in the treatment of HCC (8, 9). Because DDP and NCTD at higher concentrations induces many side effects (such as gastrointestinal reactions etc), low-dose combination therapy is required. In particular, the function of DDP and NCTD in HCC TGF-β1/ Smads signaling pathway have not been elucidated to date. This study investigated whether DDP and NCTD can alter the expression of the TGF-β1/Smads signaling pathway in Hep3B.

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Materials and methods

Key reagents

Hep3B (China Center for Type Culture Collection);The primer sequences for PCR (polymerase chain reaction) amplification (TGF-β1forward primer: 5′- ACAATTCCTGGCGATACCTC-3′, reverse primer:5′- TAAGGCGAAAGCCCTCAAT -3′,product 132 bp; Smad4 forward primer: 5′- ACGAACGAGTTGTATCACCTGG -3′, reverse primer: 5′- TGCACGATTACTTGGTGGATG -3′, product 173 bp ; Smad7 forward primer:5′- GGACGCTGTTG-GTACACAAG -3′, reverse primer: 5′- GCTGCATAAACTC-GTGGTCATTG -3′, product 105 bp ; GAPDH forward primer:5′- TGACTTCAACAGCGACACCCA -3′, reverse primer: 5′- CACCCTGTTGCTGTAGCCAAA -3′, product 121 bp), primers were purchased from life technologies(USA) , DDP(Qilu pharmaceutical, China), NCTD (Sigma, USA).

Grouping and Treatment

The cells from passages 6–8 were used and the cells were randomly divided into 4 groups: control group, DDP 2.0 μg/ml group, NCTD 10 μg/ml group, and DDP 2.0 μg/ml plus NCTD 10 μg/ml group.

The cells were transferred into 6-well plates (cells proliferation assay used 96-well) at a density of 2×10^5/wells (cells proliferation assay used 2×10^4/wells) cells per well and cultured in Dulbecco's modified Eagle's medium(DMEM) supplemented with 8 % fetal bovine serum(FBS). After starving in serum free medium overnight, the cells were cultured in the medium for 24 h.

Proliferation assay

The cells were seeded into 96-well plates and cultured in 100μl DMEM supplemented with 8 % FBS. After treated for 24 h, the cell counting kit-8 (Hyclone, USA) solution (10 μl) was then added to each well (100 μl), and the mixture was incubated at 37 °C for 2.5 h. The optical densities were read by a microplate reader (Bio-Rad, USA) in the plates at 450 nm. All cell counting kit-8 assays were repeated five times.

TGF-β1, Smad4 and Smad7 mRNA expression

The treated cells were used. Total RNA was extracted from the cells by using RNAiso Reagent (TaKaRa, Japan) and then was reversely transcribed to cDNA by using the SYBR® Premix Ex Taq TM reverse transcription–PCR kit.

Relative quantitative real time PCR was performed using the ABI StepOne system (Life technologies, USA). Threshold cycles at which emission rises above baseline were automatically calculated by the real-time PCR system. The expression of GAPDH was used as internal control. Relative quantization was expressed as fold-induction compared with control. Melting curves were generated after each run to confirm amplification of specific transcripts.

TGF-β1 and Smad4 protein expression

The treated cells were used. The protein concentrations were detected using the Micro BCA assay (Pearce Perbio Science, Germany). Thirteen μg of cell lysate were separated on a 10 % gradient bis-tris polyacrylamide (Life technologies, USA) under reducing conditions and transferred onto polyvinylidene fluoride membrane (Sigma, USA). To detect TGF-β1, Smad4 and β-actin, a monoclonal anti-human antibody (ab92486, ab40759, ab8227) from Abcam(UK) were used followed by goat anti-rabbit IgG-HRP antibodies (ab6721,abcam,UK), chemiluminescence was used by an ECL kit (Amersham, Germany). The emission amount of chemiluminescence was quantified. β-actin was used as internal control.

Statistics

All cellular experiments were performed three to five times. Data were expressed as mean ($\bar{\chi}$) ± standard deviation. Statistical analyses were performed by SPSS 16.0. Data were compared using the one-way Analysis of Variance (Bonferroni post hoc test). $p < 0.05$ was considered to be statistically significant.

Results

Effects of DDP and NCTD on the proliferation of fibroblasts

Hep3B in the treatment group of DDP, NCTD and DDP plus NCTD showed a significantly lower optical density than in the control (0.384 ± 0.009 vs 0.519 ± 0.007; 0.406 ± 0.014 vs 0.519 ± 0.007; 0.269 ± 0.011 vs 0.519 ± 0.007; all $p < 0.01$), DDP plus NCTD had more powerful effects than DDP and NCTD (Fig. 1).

Relative mRNA expression of TGF-β1, Smad4 and Smad7

The relative mRNA expression of TGF-β1(0.817 ± 0.012 vs 1.000 ± 0.087; 0.805 ± 0.023 vs 1.000 ± 0.087; 0.567 ± 0.027 vs 1.000 ± 0.087; all $p < 0.01$) and Smad7 (0.751 ± 0.029 vs 1.000 ± 0.048; 0.803 ± 0.015 vs 1.000 ± 0.048; 0.580 ± 0.037 vs 1.000 ± 0.048; all $p < 0.01$) in Hep3B following DDP, NCTD and DDP plus NCTD treatment were significantly down-regulated compared with control group, but Smad4 (0.815 ± 0.017 vs 0.527 ± 0.009; 0.767 ± 0.034 vs 0.527 ± 0.009; 1.000 ± 0.025 vs 0.527 ± 0.009; all $p < 0.01$) in Hep3B following DDP, NCTD and DDP plus NCTD treatment were significantly up-regulated compared with control group, DDP plus NCTD had more powerful effects than DDP and NCTD (Fig. 2).

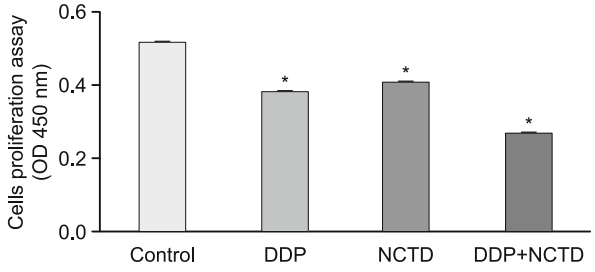

Fig. 1. The fibroblasts proliferation assay in different groups. Data were presented as mean ± SD. * VS control $p < 0.01$.

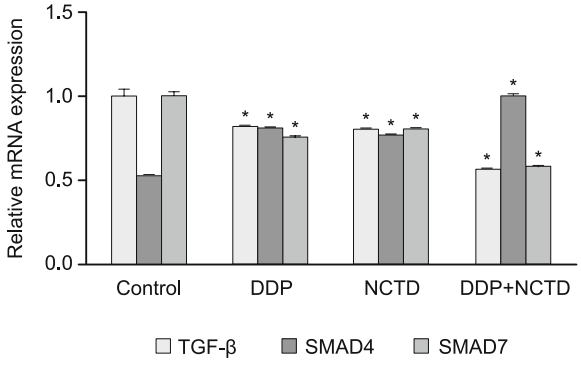

Fig. 2. The relative mRNA expression of TGF-β1, Smad4 and Smad7 in different groups. Data were presented mean ± SD, * VS control $p < 0.01$.

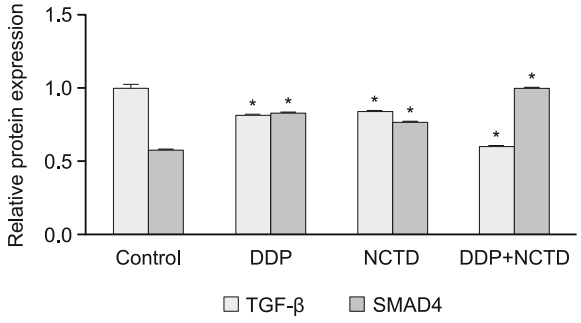

Fig. 3. The relative protein expression of TGF-β1 and Smad4 in different groups. Data were presented mean ± SD,* VS control $p < 0.01$.

Relative protein expression of TGF-β1 and Smad4

The relative protein expressions of TGF-β1 in Hep3B treated with DDP, NCTD and DDP plus NCTD were significantly down-

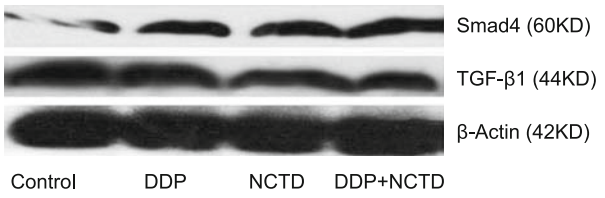

Fig. 4. The relative protein expression of TGF-β1 and Smad4 in different groups.

regulated compared with control group (0.814 ± 0.030 vs 1.000 ± 0.072; 0.840 ± 0.028 vs 1.000 ± 0.072; 0.604 ± 0.026 vs 1.000 ± 0.072; all $p < 0.01$), but the relative protein expressions of Smad4 in Hep3B treated with DDP, NCTD and DDP plus NCTD were significantly up-regulated compared with control group (0.830 ± 0.039 vs 0.580 ± 0.037; 0.766 ± 0.027 vs 0.580 ± 0.037; 1.000 ± 0.027 vs 0.580 ± 0.037; all $p < 0.01$), DDP plus NCTD had more powerful effects than DDP and NCTD (Figs 3 and 4).

Discussion

TGF- β regulates the proliferation and differentiation of cells, wound healing, angiogenesis etc (10). There are three isoforms of TGF-β: TGF-β1, TGF-β2, and TGF-β3. TGF-β1 mRNA is expressed in connective-tissue cells, hematopoietic, and endothelial; TGF-β2 mRNA in neuronal cells and epithelial; and TGF-β3 mRNA primarily in mesenchymal cells (11).Smads are important mediators of TGF-β signaling transduction pathway. The Smad family in humans includes eight members, five of which (Smads 1, 2, 3, 5, and 8) are called receptor-regulated Smads (R-Smads),Smad4 is common-mediator Smad(co-Smad), Smad6 and Smad7 are inhibitory Smads (I-Smads) (12). TGF-β/Smads signaling components include TGF-β, TβR I, TβR II, Smad4, Smad3/Smad2, Smad6/Smad7 and endoglin, and TGF-β/Smads signal transduction pathway can regulate itself by positive and negative feedback regulation loops (4). The up-regulation of TGF-β1, Smad7 and down-regulation of Smad4 play an important role in the pathogenesis of HCC (3, 13). Smad4 plays a central role in TGF-β signaling, Smad4 can mediate an antitumor function by activating ERK and JNK MAP kinases and Ras/MEK/MAP kinase (14–16). Modulating the TGF-β/Smad pathway has been shown to be an important way to suppress HCC (17–19).

DDP has been widely used in the treatment of different kinds of cancers, especially in the treatment of HCC (5–7). DDP can enhance NK cells immunotherapeutic efficacy to suppress HCC progression via altering the androgen receptor (AR)-ULBP2 signals (20). DDP also inhibits transcription activity of NF-κB and increases apoptosis in human hepatocellular carcinoma cells (21). This study demonstrated that DDP (2.0 μg/ml) can significantly down-regulate the relative mRNA/protein expression of TGF-β1 and Smad7, significantly up-regulated the relative mRNA and protein expression of Smad4 compared with the control group ($p < 0.01$) and significantly inhibit the proliferation of Hep3B cells ($p < 0.01$).

NCTD, the demethylated form of cantharidin, has been used in the treatment of HCC (8, 9). NCTD can induce mitotic arrest and apoptosis in human hepatoma cells (22), which also can induce apoptosis in human Hep3B hepatoma cells through a p53 independent pathway via TRAIL/DR5 signal transduction (9). The anticancer activity and mechanisms of NCTD on hepatoma may be connected with modulating Bax, p21, Bcl-2 protein expression (8) and inhibiting N-acetyltransferase activity (23). This study demonstrated that NCTD (10 μg/ml) can significantly down-regulate the relative mRNA/protein expression of TGF-β1 and Smad7, significantly up-regulated the relative mRNA and protein expression of Smad4 compared with the control group ($p < 0.01$)and significantly inhibit the proliferation of Hep3B cells ($p < 0.01$). DDP plus NCTD exhibited more powerful effect than DDP and NCTD.

Conclusion

This study demonstrated that DDP, NCTD and DDP plus NCTD significantly inhibited the proliferation of cells, significantly attenuated both the mRNA and protein expression of TGF-β1 and Smad7, and significantly up-regulated the mRNA and protein expression of Smad4 in Hep3B cells, DDP plus NCTD exhibited more powerful effects than DDP and NCTD.

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