Low-dose apatinib and CPT synergistically optimize the tumor microenvironment and enhance the antitumor effects of PD-1 inhibitors in hepatocellular carcinoma

Hankang Wang
Shandong First Medical University, Shandong Academy of Medical Sciences

Congcong Gao
Center for Disease Control and Prevention

Feng Chen (fengxiao6556@126.com)
Shandong First Medical University, Shandong Academy of Medical Sciences

Guijie Li
The First Affiliated Hospital of Shandong First Medical University

Xiaodong Li
Shandong First Medical University, Shandong Academy of Medical Sciences

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Abstract

Background

Apatinib is a selective vascular endothelial growth factor receptor 2-tyrosine kinase inhibitor (TKI) that has been shown to apply to a variety of solid tumors, including advanced hepatocellular carcinoma (HCC). Clinical outcome prove that the combination of apatinib and anti programmed death 1 (PD-1) inhibitors has a cooperate with anti-tumor effect. In this study, we investigated the synergistic enhancement of the antitumor effect of PD-1 inhibitor in HCC by camptothecin (CPT) and low-dose apatinib.

Methods

The effect of low-dose apatinib in combination with CPT on the antitumor effects of PD-1 inhibitor was evaluated in making use of the H22 mouse model (n = 32), which was divided into four treatment groups. Immunohistochemical staining and western blotting were used to detect nuclear factor erythroid 2-related factor 2 (Nrf2) as well as sequestosome 1 (p62), vascular endothelial growth factor A (VEGFA), vascular endothelial growth factor receptor 2 (VEGFR2), PD-1, and programmed cell death ligand 1 (PD-L1).

Results

The results showed that the average size of the tumor of the combination group was signicantly less than that of the apatinib + PD-1 inhibitor group. The expression levels of Nrf2, p62, VEGFA, VEGFR2, PD-1, and PD-L1 in the apatinib + PD-1 inhibitor group were lower than those in the control group (P < 0.05). The expression levels of these genes were significantly lower in the combination group (P < 0.05).

Conclusion

There was no obvious difference in body weight and liver and kidney functions between the four groups of mice. In conclusion, CPT synergistically enhanced the antitumor effect of PD-1 inhibitor in HCC with low-dose apatinib.

Introduction

In 2020, primary liver cancer was the sixth most common cancer (4.7%) and third among cancer-related deaths (8.3%), with HCC accounting for 75–85% of cases (1). HCC patients often experience recurrence of tumors, which account for approximately 90% of HCC-related deaths (2, 3). Apatinib is an orally administered small-molecule TKI that very selectively blocks VEGFR2 and inhibits VEGF-mediated endothelial cell proliferation and migration (4). VEGFR2 is thought to be the primary receptor for pro-angiogenic signaling downstream of VEGFA. Apatinib also promotes reactive oxygen species (ROS)
production and inhibits the expression of Nrf2 and p62, leading to autophagy and apoptosis. Apatinib induces autophagy and apoptosis in tumor cells by regulating the VEGFR2/PD-L1 and ROS/Nrf2/p62 signaling pathways (5). Sun et al. demonstrated that apatinib promotes ROS-dependent ovarian cancer cells by negatively regulating Nrf2 and p62 apoptosis and autophagy (6). Other studies have also reported that apatinib inhibits cell growth and metastasis and promotes apoptosis by regulating autophagy in a variety of human cancers (7, 8). Currently, a large number of studies have shown favorable therapeutic effects of apatinib in a variety of solid tumors (9–13). Compared with other VEGFR TKIs, apatinib is easy to administer and less economically burdensome, making it an interesting emerging antiangiogenic therapy.

Recently, immunotherapy has brought revolutionary changes to cancer treatment, effectively controlling previously incurable highly invasive cancers. Antibodies against PD-1 and its ligand, PD-L1, are widely used to treat malignant tumors, including HCC (14, 15). Immune checkpoint inhibitor therapies represent a great method in the treatment of a variety of solid tumors by inhibiting the interaction between PD1, mainly expressed on activated CD8 + T cells, and PD-L1 ((16, 17). PD-1 inhibitor reactivates damaged T cells and restores its ability to kill tumor cells (18, 19). However, the result of single-agent PD-1 inhibitor is 20–40% in a variety of solid tumors (19, 20). Roger et al. (21) found that the vasoproliferative tumor microenvironment (TME) is strongly associated with resistance to PD-1 inhibitor. Hypoxia, pro-angiogenesis and epithelial-mesenchymal transition are correlates of PD-1 resistance, the most important of which is the high expression of VEGFA (21).

Nrf2 is encoded by the nuclear factor erythroid-derived 2-like 2 (NFE2L2) gene (22–26). In normal conditions, Kelch-like ECH-associated protein (Keap1) binds to Nrf2, causing it to be degraded by proteasomes in the cytoplasm (22–28). The antioxidant response element-mediated cytoprotective proteins include antioxidant enzymes, stress-responsive proteins, metal-binding proteins, drug-metabolizing enzymes, and drug-transport proteins (26). Carcinogenesis is also a novel function of Nrf2 (22, 23, 26). Activation of Nrf2 protects cells from oncogenic chemicals for a short period of time(29–32).

CPT is a natural alkaloid that is a potent antitumor agent. It binds to DNA topoisomerase I to inhibit DNA replication (30, 33, 34). Previous studies by the authors confirmed that CPT is an Nrf2 inhibitor that is effective at lower concentrations, thereby reducing drug toxicity (30, 34). CPT can be used in combination with anticancer drugs that increase Nrf2 levels to treat HCC (34). Based on these studies, we hypothesized that CPT synergizes with low-dose apatinib to enhance the antitumor effects of PD-1 inhibitors in HCC. In this study, we established a mouse liver tumor H22 model to test this hypothesis by comparing low-dose apatinib + PD-1 inhibitor against CPT with its combination with regard to effects on TME.

Materials and methods

H22 HCC model.
Animal experiments were conducted in accordance with the ARRIVE guidelines (Approval No. S0007) approved by the Animal Protection and Utilization Committee of the First Hospital of Shandong First Medical University (Jinan).

Male BALB/c mice (3–5 weeks old, 18–22 g) were divided into two batches (n = 20/batch), housed under standard animal husbandry conditions with room temperature, sufficient air, 12/12 h light/dark cycles, and permitted to use sterilized water and feed ad libitum. Mice were subcutaneously inoculated with H22 cells (1 × 10^6/200 µl saline).

When the tumors reached the size of soybean grains, the mice received ultrasound to measure the volume of the tumors, with tumor volume (mm^3) = π/6 × length × width (35). Sixteen mice with similar tumor size were randomly divided into 4 groups (group A) (saline), CPT group (group B) (3 mg/kg CPT), apatinib + PD-1 inhibitor group (group C) (60 mg/kg apatinib + 10 mg/kg PD-1 inhibitor), and apatinib + PD-1 inhibitor combined with CPT group (group D) (60 mg/kg apatinib + 10 mg/kg PD-1 inhibitor + 3 mg/kg CPT) (36–38). CPT was injected intraperitoneally every 3 days, and apatinib was administered daily by gavage, while PD-1 inhibitor was injected intraperitoneally every 3 days. A total of 40 mouse models were established, of which 32 were eligible for enrollment; the 8 animals not enrolled were euthanized by sodium pentobarbital injection. A total of 32 mice survived in good condition until the end of the experiment and were euthanized by sodium pentobarbital injection. Mice were euthanized by slow intraperitoneal injection of 2% sodium pentobarbital until death. Finally, tumors were issected after euthanasia and weighed after tumor removal to analyze tumor volume and body weight. Analyses were performed using GraphPad Prism software (version 8; GraphPad Software, Inc.).

The mice were purchased by Beijing Viton Lihua Company. H22 tumors were provided by the laboratory of Qianfoshan Hospital. All animal welfare was taken into account, including minimizing pain and suffering, the use of painkillers or anesthetics, or special housing conditions. The experimental duration of the mouse model was 30 d. After the completion of the experimental objectives, the animals were treated in a scientific and humane manner to minimize their panic and suffering, and euthanasia was performed gently and quickly. By observing respiration, cardiac arrest, pupil, nerve reflexes and other indicators, the death was comprehensively judged, and it was confirmed that the experimental animals had died.

**Immunohistochemical staining.**

The reagents and steps used for tissue fixation, paraffin embedding, sectioning, and hematoxylin and eosin staining were as described elsewhere. Paraffin sections (4 µm) of H22 tumors were deparaffinized with xylene and rehydrated with a descending ethanol series. The sections were blocked with bovine serum albumin for 30 min at 37°C and covered with anti-Nrf2 antibody (1:2,000; cat. no. GB113808), VEGFA (1:500; cat. no. GB14165), p62(1:1,000; cat. no. GB11239-1), PD-1(1:1,000; cat. no. GB12338), cMyc (1:200; cat. no. GB13076), TGF-β(1:500; cat. no. GB11179), CD4(1:500; cat. no. GB15064), and CD8(1:500; cat. no. GB15068), overnight at 4°C. Antibodies against Nrf2, VEGFA, p62, PD-1, cMyc, TGF-β, CD4, and
CD8 were obtained from Wuhan Servicebio Technology Co. Sections were then incubated with HRP-labeled goat anti-mouse IgG solution (cat. no. G1214-100UL; from Wuhan Servicebio Technology Co., Ltd.) diluted at a 1:200 dilution for 30 min at 37°C, and next, DAB substrate was added. Cell nuclei were counterstained with hematoxylin. Cell nuclei were counterstained with hematoxylin. Images were captured under a light microscope. Staining was visualized using Image-ProPlus 6 software (Media Cybernetics, Inc.) and integrated optical density/area values were used to determine protein expression levels in the tumors.

**Western blotting**

H22 tumors were pulverized in RIPA (Wuhan Servicebio Technology Co., Ltd.) buffer with 1 mM PMSF on ice and then centrifuged as previously described (26). Protein concentration was determined using Bicinchoninic acid (BCA). Protein samples (15 µg samples per lane) were loaded into 30% SDS - PAGE gels and transferred to PVDF membranes. The membranes were blocked with 3% bovine serum albumin for 1 h at room temperature and then incubated at 4°C with the following primary antibodies (obtained from Wuhan Servicebio Technology Co., Ltd.) incubated overnight at 4°C with the following primary antibody: Nrf2,P62,VEGFA,VEGFR2,PD-1, and PD-L1. The membrane was then incubated with HRP-labeled goat anti-mouse IgG solution at a dilution of 1:5,000 for 1 h at room temperature. Finally, the membranes were covered with enhanced chemiluminescence (ECL) substrate and scanned. ECL substrate was obtained from Merck Millipore. Quantification of the results normalized to β-actin was conducted using Image J software (version 1.8.0.345; National Institutes of Health).

**Statistical analysis**

Using for GraphPad Prism software to analyze all experiments. Data are presented as the mean ± SD. Comparisons between groups were performed using one-way ANOVA with the post hoc test Tukey’s multiple comparison test. P < 0.05 was considered as indicating a statistically significant difference.

**RESULTS**

**CPT enhances low-dose apatinib sensitivity in HCC by inhibiting the Nrf2/p62 pathway**

When apatinib, PD-1 inhibitor, and CPT were administered, Nrf2 protein in mouse H22 tumor tissues was reduced and tumor growth was also inhibited after inhibiting Nrf2 protein expression. The effects of the combination of apatinib and PD-1 inhibitor and the combination of CPT are shown (Fig. 1.A). Measured under ultrasound in each group, the pre-treatment tumor volumes were 30.958 ± 2.315 mm³, 31.087 ± 2.470 mm³, 31.154 ± 1.251 mm³, 31.416 ± 2.113 mm³ (P ≥ 0.05) in groups A, B, C, and D, respectively (Fig. 1). Tumor volumes after treatment were 161.531 ± 24.616 mm³, 92.285 ± 5.353 mm³, 45.684 ± 2.959 mm³, 10.865 ± 1.552 mm³. There was no statistically significant difference in the comparison of tumor volume between group A and group B (P ≥ 0.05). In contrast, the difference between groups A and C and
groups C and D was statistically significant (P < 0.05) (Fig. 1.B,C). This indicated that apatinib combined with PD-1 inhibitor inhibited tumor growth, and this inhibitory effect was even more pronounced after the combined administration of CPT.

To detect the effects of apatinib, PD-1 inhibitor and CPT on the regulation of Nrf2 and p62 in vivo, western blotting and IHC staining were performed on mouse H22 tumor tissues. Treatment down-regulated Nrf2 expression in group B (P < 0.05) and decreased Nrf2 expression in group C (P < 0.05) compared with group A. However, Nrf2 expression was significantly decreased in group D (P < 0.05) compared with groups A and C. IHC and western blotting yielded comparable results (Fig. 2.A-D). In addition, p62 was also affected in a similar manner (Fig. 2.E-H). Apatinib combined with PD-1 inhibitor had an inhibitory effect on Nrf2 and p62, and the inhibition was more pronounced after the combined administration of CPT, suggesting that CPT could enhance HCC sensitivity to apatinib.

**Combination of CPT, apatinib, and PD-1 inhibitor inhibits angiogenesis**

In the mouse H22 tumor model, the expression levels of VEGF A and VEGFR2 in group B were lower than those in group A (P < 0.05), and those in group C were significantly lower than those in group A (P < 0.05), while in group D, where CPT was administered in combination, the expression levels of VEGFA and VEGFR2 were significantly lower than those in groups A and C (P < 0.05) (Fig. 3.A-F). Similarly, the downstream target of VEGFR2, c-Myc, showed similar changes (Fig. 3.G and H). This indicated that apatinib combined with PD-1 inhibitor could inhibit tumor angiogenesis by inhibiting the expression of VEGFA and VEGFR2, and then c-Myc, and the inhibitory effect was even more evident with the combined administration of CPT.

**Combination of CPT, apatinib and PD-1 inhibitor improves tumor microenvironment**

In the mouse H22 tumor model, the expression levels of CD4 and CD8 were higher in group B than in group A (P < 0.05), and the expression levels of CD4 and CD8 were significantly higher in group C than in group A (P < 0.05), whereas in group D, with the combined administration of CPT, the expression levels of CD4 and CD8 were significantly higher than those in group A and group C (P < 0.05) (Fig. 4.A-D). This indicated that the combined treatment had a synergistic effect and enhanced the activity of T cells. TGF-β showed similar changes (Fig. 4.E and F). The expression levels of PD-1 and PD-L1 in group B were lower than those in group A (P < 0.05), and those in group C were significantly lower than those in group A (P < 0.05), whereas in group D, in which CPT was administered in combination, the expression levels of PD-1 and PD-L1 were significantly lower than those in group A and group C (P < 0.05) (Fig. 5.A-F). This indicated that the combination therapy could improve the tumor microenvironment and promote immune activation.
Safety and tolerability of the combination of CPT, apatinib, and PD-1 inhibitor

There was no difference in body weight between mice in the four treatment groups after treatment and tumor resection. The body weights of the mice were 20.25 ± 1.79 g (A), 18.75 ± 0.83 g (B), 18.88 ± 1.05 g (C) and 19.25 ± 1.30 g (D) (P ≥ 0.05) (Fig. 6.A). Serum analysis of mice showed that blood urea nitrogen, creatinine, total bilirubin, alanine transferase, and aspartate transferase were at normal levels without any treatment affecting liver and kidney functions (Fig. 6.B-F)

Discussion

Tumor angiogenesis is essential for tumor growth and metastasis. VEGFA is the most important angiogenic factor in the vascular endothelial growth factor family, playing an important role in the occurrence and development of tumors. Studies have confirmed that high levels of VEGFA are related to poor prognosis in tumors such as in gastric cancer, ovarian cancer, HCC, non-small cell lung cancer, and endometrial cancer (39–42). During tumor growth, high metabolism leads to a hypoxic microenvironment within the tumor, which activates growth factors and induces angiogenesis. Previous studies have shown that downregulation of Nrf2 reduces angiogenesis (43, 44), CPT also inhibited the vessel density (30). Apatinib is a highly selective multiple TKI that very selectively blocks VEGFR2. It has been reported that apatinib effectively inhibits tumor proliferation and migration by blocking the VEGF axis (45). Chen et al. (46) reported that the inhibitory effect of apatinib on tumorigenesis may be bound up with the downregulation of VEGF and VEGFR2 expression in HCC. In this study, the expression levels of VEGFA and VEGFR2 in the CPT group were lower than those in the control group, confirming that CPT could inhibit the VEGFA signaling pathway. The expression levels of VEGFA, VEGFR2, and c-Myc were lower in the apatinib + PD-1 inhibitor group than in the control group, while the expression levels of VEGFA, VEGFR2, and c-Myc were significantly lower in the combination group than in the other three groups. This suggests that combination therapy can inhibit HCC angiogenesis by inhibiting the VEGF axis.

Because of the pronounced pro-angiogenic effects of VEGFA, high VEGFA expression exacerbates tumor vascular abnormalities, poor perfusion, and inadequate oxygen supply. Hypoxic eventually regulates TME into an immunosuppressive environment (47, 48). Therefore, the hypoxia, angiogenesis, and immunosuppressive tumor microenvironment induced by VEGFA overexpression is definitely detrimental to PD-1 inhibitor (21). Apatinib at low doses significantly alleviated tumor hypoxia, increased CD4 + and CD8 + cell infiltration, and decreased TGF-β levels at certain time points, suggesting that angiogenesis inhibitors have a true immunomodulatory effect (37). Studies by Schmittnaegel et al. (49) and Elizabeth et al. (50) provide evidence that antiangiogenic drugs specifically improve anti-PD-1/PD-L1 therapy when promoting an immunostimulatory tumor microenvironment and tumor vascular normalization in various tumor models. In this study, the CD4 + and CD8 + cell expression levels were higher in the apatinib + PD-1 inhibitor group than in the control group, whereas they were significantly higher in the combination group than in the other three groups. The expression levels of TGF-β, PD-1, and PD-L1 were lower in the apatinib
+ PD-1 inhibitor group than in the control group, while the expression levels of TGF-β, PD-1 and PD-L1 were significantly lower in the combination group than in the other three groups. This suggests that low-dose apatinib and CPT synergistically optimize the TME and enhance the antitumor effect of PD-1 inhibitor in HCC.

CPT was proved as a potent Nrf2 inhibitor among multitudinous agents (34). CPT has a proven safety profile and is used clinically for chemotherapy drug (51, 52). There was no significant change in body weight of mice in all four treatment groups in each model, and there was no significant injury to the liver or kidneys, indicating that the drug toxicity was accepted. In our previous studies, CPT was shown to be effective in inhibiting ROS levels and Nrf2 expression (30, 34). Increasing evidence suggests that Nrf2 plays a important role in autophagy regulation by forming positive feedback with p62. It has been proved that insufficient autophagy leads to p62 accumulation, which further segregates Keap1, a negative regulator of Nrf2, leading to Nrf2 stabilization (53). Apatinib induces cellular autophagy and apoptosis by promoting ROS generation and inhibiting Nrf2 and p62 expression (5). In this study, Nrf2 and p62 expression levels were lower in the CPT group than in the control group, confirming that CPT inhibits the Nrf2 signaling pathway. The Nrf2 and p62 expression levels in the apatinib + PD-1 inhibitor group were lower than those in the control group, whereas those in the combination group were significantly lower than those in the other three groups. This suggests that the combination treatment can downregulate Nrf2 by inhibiting the Nrf2 axis, which is beneficial for inhibiting the growth of HCC as well as inducing autophagy and apoptosis in tumor cells.

In conclusion, this study confirmed that low-dose apatinib synergistically reduces tumor activity with CPT, optimizes the TME, and enhances the antitumor effects of PD-1 inhibitor in HCC.

**Declarations**

**Acknowledgements**

Not applicable.

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**Competing interests**

The authors declare that they have no competing interests.
Authors' contributions

HW, CG designed the study. HW, CG and FC completed the experimental process. HW, HO and GL organized experimental data and analyze it. HW processed data and wrote the manuscript. FC and GL provided final approval. All authors contributed to the article and read and approved the final version of the manuscript. HW confirm the authenticity of all the raw data.

Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

All animal experiments were performed according to the ARRIVE guidelines approved (approval no. S0007) by the Animal Care and Use Committee of the First Hospital of Shandong First Medical University (Jinan, China).

Patient consent for publication

Not applicable.

References


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**Figures**
Figure 1

The effects of the combination of apatinib and PD-1 inhibitor and the combination of CPT in H22 models (A). The tumor volumes before (B) and after (C) treatment in A, B, C and D groups (n=8). * P<0.05 and # P<0.05.

Figure 2
The Nrf2 expressed in (B) IHC staining and (D) WB. The P62 expressed in (F) IHC staining and (H) WB. Magnification, x400. β-actin was the internal control of WB. Panels A, C, E and G demonstrate the statistical analysis for IHC staining and WB. * P<0.05 and # P<0.05.

Figure 3

The VEGFA expressed in (B) IHC staining and (D) WB. The VEGFR2 expressed in (F) WB in H22 models. The c-Myc expressed in (H) IHC staining. Magnification, x400. β-actin was the internal control of WB. Panels A, C, E and G demonstrate the statistical analysis for IHC staining and WB. * P<0.05 and # P<0.05.
Figure 4

The CD4, CD8 and TGF-β expressed in (B, D and F) IHC staining. Magnification, x400. Panels A, C and E demonstrate the statistical analysis for IHC staining. * P<0.05 and # P<0.05.

Figure 5
Figure 6

Panels A, B, C, D, E and F demonstrate the statistical analysis for IHC staining and WB. ns, no statistical significance.