Abstract. The present study was designed to investigate the synergistic inhibitory effects on hepatocellular carcinoma with recombinant human adenovirus Aspp2 (Aspp2-ad) and oxaliplatin via p53-independent pathway in vitro and in vivo. After being treated with Aspp2-ad and/or oxaliplatin for 24-48 h, HepG2 P53-/- and Hep3B cells showed a significant growth inhibition compared with vehicle control. Combination group showed a synergetic effect, the inhibitory rates were all above 80% at 48 h point in HepG2 P53-/- and Hep3B cells. The apoptotic cell numbers of Aspp2-ad and/or oxaliplatin treatment groups were increased remarkably, especially for the combined therapy group in the liver cancer cells. The Hep3B xenograft experiment also showed similar inhibition of Aspp2-ad and/or oxaliplatin to the in vitro experiment. H&E results showed that combination group had the least mitotic indexes and the most necrosis. The immunohistochemistry results showed that PCNA, CD31 expression decreased greatly in treatment groups. These results suggested that Aspp2-ad might inhibit proliferation and vascular growth of hepatocarcinoma. Aspp2 induced apoptosis protein expression in Aspp2-ad and combination groups, the Aspp2, Bax and activation of caspase-3 expression increased greatly both in vitro and in vivo. But interestingly, the autophagy proteins showed different responses not only in HepG2 P53-/- and Hep3B cells but also in vitro and in vivo. We found that Aspp2-ad down-regulated the p-ERK, p-STAT3 expression, the synergistic effects were observed in combination group, while there was not response of mTOR to Aspp2-ad. In conclusion, Aspp2-ad, in P53-independent manner, regulated ERK and STAT3 signal moleculars to inhibit hepatocarcinoma in coordination with oxaliplatin by influencing the protein expression of prolife ration, apoptosis, autophagy and vascular growth. Aspp2-ad has the potential to be developed in gene therapy for HCC, especially for P53 deletion or mutation in HCC.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors in the world and causes significant mortality, rapidly rising in incidence in China (1,2). Surgical resection and transplantation are the cornerstone of therapy in early-stage hepatocellular carcinoma, while locoregional therapy and chemotherapy (such as sorafenib and oxaliplatin) are beneficial in those with more advanced disease or those who are not surgical candidates (3,4). Oxaliplatin is a new platinum anticancer drug, commonly used in metastatic colorectal cancer treatment, or adjuvant treatment in resection of primary colon cancer (5). The mechanism of oxaliplatin, is not yet entirely clear, but some research suggest that oxaliplatin produced hydration derivatives, acting on DNA, to form inter and intra chain cross-linking, which inhibits DNA synthesis, induced cytotoxicity and antitumor activity (6). Oxaliplatin-based regimens were permitted by CFDA (China Food and Drug Administration) as systemic therapies for advanced HCC since 2013 in China, which bring more benefit for patients with advanced HCC. Clinical research has shown that oxaliplatin-based regimens are safe and efficacious in patients with HCC (7-9).

It has long been recognized that tumor does not generally respond to conventional chemotherapy, especially to P53 mutation or deletion tumors. With the development of cancer genetics, gene therapy has stood out as a promising multidisciplinary treatment approach against tumors (10). P53, TK and other gene products have been used in clinical treatment of liver cancer, and achieved certain therapeutic effect alone or in coordination with other treatments (11,12). However, the treatment to tumors of P53 mutation is not ideal, so it is necessary to develop new gene therapy.
Aspp2 (apoptosis stimulating protein of p53-2) is one of p53 binding proteins. Effects of inhibiting tumor cell growth and inducing apoptosis are already confirmed. Aspp2 is lowly expressed in a variety of tumors and is related to the occurrence and development of tumors. These studies suggest that Aspp2 is a very important tumor suppressor and may be a candidate gene for gene therapy of HCC (13-17).

Combination of gene therapy with other therapeutic approaches such as chemotherapy might provide more treatment benefit (18,19). Our previous research showed that Aspp2 enhanced oxaliplatin-induced colorectal cancer cell apoptosis in a p53-independent manner by inhibiting cell autophagy (20). Moreover, we successfully prepared the human Aspp2 recombinant adenovirus (Aspp2-ad) (21). In the present study, human Aspp2 recombinant adenovirus (Aspp2-ad) preparation was used to investigate whether Aspp2 could enhance the inhibitory effect of oxaliplatin on hepatocellular carcinoma. To confirm whether Aspp2 exerted the inhibitory effect in a p53-independent way, we chose the cell lines of Hep3B (P53 natural deficiency) and HepG2P53-/- (constructed by our laboratory) as the objects of the study.

**Materials and methods**

**Reagents and antibodies.** Human Aspp2 recombinant adenovirus (Aspp2-ad) was constructed by the Beijing Institute of Hepatology (Beijing, China); oxaliplatin was obtained from Qilu Pharmaceutical Co., Ltd. (Jinan, China); trypsin-ethylenediaminetetraacetic acid and Dulbecco's modified Eagle's medium (DMEM) were purchased from Gibco (Grand Island, NY, USA); fetal bovine serum (FBS) was from China Hangzhou Sijiqing Biological Technology Co., Ltd. (Hangzhou, China); 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were provided by Sigma-Aldrich (St. Louis, MO, USA); p53 primary monoclonal antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA); activation of caspase-3, Bax, Bcl-2, LC3B, Beclin1, ERK, p-ERK, mTOR, p-mTOR, STAT3, p-STAT3 and GAPDH primary monoclonal antibody were from Cell Signaling Technology (Boston, MA, USA); CD31 and PCNA immuno-histochemistry kits were obtained from Beijing Zhongshan Gold Bridge Biotechnology Co., Ltd. (Beijing, China); Annexin V apoptosis Kit-PE stain. Cells in the logarithmic growth phase were plated in 96-well plates in a seeding density of 5,000 cells/well and incubated in a 37°C incubator with 5% CO₂ overnight. After cells were pretreated with Aspp2-ad for 24 h, the cells were then incubated with oxaliplatin for 24-48 h. The culture medium in each well was abandoned, incubating with 0.5 g/l MTT 100 µl for 4 h. Then, each well was added with 150 µl DMSO and vibrated for 10 min, then absorbance of each well was detected with microplate reader (ELX800 type; BioTek Instruments Inc., Winooski, VT, USA) at the 490 nm wavelength. The inhibition rate (IR) was calculated as follows: IR (%) = (1-ODtreatment /ODcontrol) x 100%.

**Annexin-PE stain.** Cell culture was as described above. After treated with Aspp2-ad and oxaliplatin, the cells were stained with 10 µl Annexin-PE at 37°C for 15 min. The Annexin-PE stained cells of 5 same visual fields were counted under inverted fluorescence microscope (Cytation 3; BioTek Instruments).

**Xenograft experiment.** Hep3B cells (5x10⁶) were inoculated in nude mice subcutaneously to establish xenograft tumors to be transplanted into nude mice. Three days after the transplantation, the mice were divided into groups according to the tumor sizes. Each group contained 6 mice. When the tumor sizes were ~100 mm³, the test mice were injected with 25 mg/kg oxaliplatin intraperitoneally and/or Aspp2-ad intratumorally (1x10⁵-1x10⁶ pfu/mouse according to the tumor size) 2 times every week. The control mice were given the vehicle. The sizes of subcutaneous tumors were measured with calipers and internal constructions of tumors were observed with ultrasonic machine (S-sharp Prospect). At the endpoint of the experiment, nude mice were dissected and the tumor samples were collected according to the follow-up experiments. The tumor growth inhibition rates and relative tumor volumes (RTV) is calculated: RTV = Vt/V0 (where Vt is the volume of the tumor after the initial administration and V0, the volume of the tumor before the initial administration).

**Histological observation.** The tumor tissues were fixed in 4% paraformaldehyde solution for conventional hematoxylin and eosin (H&E) staining and immunohistochemistry. CD31,
PCNA expression were measured according to the kit instruction. Nuclear mitotic index (MI) was calculated in 3 slices of each animal. MI (%) = number of mitotic cells/1,000 cells.

Western blot analysis. Proteins of cells and tissues were conventionally extracted. The protein concentration of lysates was determined using the bicinchoninic acid method. Cell lysates (40 µg per lane) were separated using 10% SDS-PAGE and transferred electrophoretically to polyvinylidene difluoride membrane. Membranes were blocked with Tris-buffered saline/0.1% Tween-20 containing 5% bovine serum albumin (BSA) and then incubated overnight at 4˚C with primary antibodies (1:1,000). Membranes were washed three times with TBS/T and incubated for 1 h at room temperature with the appropriate secondary antibody conjugated to goat anti-rabbit horseradish peroxidase (1:2,000). Membranes were then washed and immunoreactive band were developed with ECL and visualized by autoradiography. Protein loading was normalized using β-actin antibody.

Luminex assay. The protein concentrations of sample tissues were diluted below 2 µg/ml. Assay buffer (200 µl) was added into each well of 96-well microtiter plate to block for 10 min with shaking. Another 25 µl of assay buffer, 25 µl sample and 25 µl mixed antibody microspheres were added into each plate after the assay buffer was removed. After incubation overnight at 4˚C, the residual liquid was discarded. A total of 200 µl of wash buffer was used to wash the plate; 25 µl secondary antibody was applied and incubated for 2 h at room temperature, then 25 µl streptavidin-phycocerythrin was added and incubated for 30 min. All the liquid was discarded, each plate was washed with 200 µl of wash buffer twice; 150 µl of sheath liquid was added with shaking for 5 min. The mean fluorescence intensity was measured with the FLEXMAP 3D™ system (Luminex Corp., Austin, TX, USA).

Statistical analysis. The data were expressed as the mean ± SD. The results were subjected to one-way ANOVA test using SPSS software (17.0 version; SPSS, Inc., Chicago, IL, USA).

Results

P53 expression in Hep3B and HepG2P53-/- cells. We constructed a HepG2 cell line with P53 deletion, as shown in Fig. 1. P53 expression in wild-type HepG2 cells was normal, but was absent in the Hep3B and HepG2P53-/- cells (Fig. 1). This suggested we successfully constructed the P53 deficient cell line.

Aspp2-ad enhances the inhibition of oxaliplatin on hepatocellular carcinoma cells. After pretreated with 1.25x10⁸, 6.125x10⁷ pfu/ml Aspp2-ad for 24 h, these hepatocellular carcinoma cells were then added with oxaliplatin for 24 and 48 h.

For 24 h in HepG2P53-/- cells, the inhibitory rate (IR) of 20 µg/ml oxaliplatin and 1.25x10⁸, 6.125x10⁷ pfu/ml Aspp2-ad were 20.10, 37.87 and 20.62%, respectively. Synergistic inhibitory rates of 1.25x10⁸, 6.125x10⁷ pfu/ml Aspp2-ad with oxaliplatin were 60.37 and 50.54%, respectively. For 48 h in HepG2P53-/- cells, the IR of 20 µg/ml oxaliplatin and 1.25x10⁸, 6.125x10⁷ pfu/ml Aspp2-ad with oxaliplatin were 60.37 and 50.54%, respectively. For 24 h in Hep3B cells, the IR of 10 µg/ml oxaliplatin and 1.25x10⁸, 6.125x10⁷ pfu/ml Aspp2-ad were 32.93, 60.71 and 42.46%, respectively. Synergistic inhibitory rates of 1.25x10⁸, 6.125x10⁷ pfu/ml Aspp2-ad with oxaliplatin were 77.81 and 74.01%, respectively. For 48 h in HepG2P53-/- cells, the IR of 20 µg/ml oxaliplatin and 1.25x10⁸, 6.125x10⁷ pfu/ml Aspp2-ad with oxaliplatin were 69.52, 76.91 and 66.15%, respectively. Synergistic inhibitory rates of 1.25x10⁸, 6.125x10⁷ pfu/ml Aspp2-ad with oxaliplatin were 82.82 and 82.31%, respectively (Fig. 2A).

These results suggested that Aspp2-ad could significantly enhance the inhibitory effect of oxaliplatin on hepatocellular carcinoma cells.

Aspp2-ad enhances the apoptosis of oxaliplatin on hepatocellular carcinoma cells. After the cells were stained with Annexin V-PE, we found that the apoptotic cells in Aspp2-ad, oxaliplatin, combination (Aspp2-ad+oxaliplatin) groups increased greatly. In HepG2P53-/- cells, the apoptosis rates of control, Aspp2-ad, oxaliplatin and combination groups were 0.01±0.30, 15.87±6.79, 12.09±3.02 and 38.09±7.33%, respectively (Fig. 3A). In Hep3B cells, the apoptosis rates of control, Aspp2-ad, oxaliplatin and Aspp2-ad with combination groups were 0.04±0.21, 17.33±4.57, 20.4±5.75 and 40.95±10.10%, respectively (Fig. 3B).

These results suggested that Aspp2-ad could significantly enhance the inhibitory effect of oxaliplatin on hepatocellular carcinoma cells.

Aspp2-ad enhances the growth inhibition of oxaliplatin in hepatocellular carcinoma xenograft models. We successfully established the Hep3B subcutaneous xenograft tumor models (Fig. 4A). All the mice in different groups were
 LIU et al: ASPP2-ad EXERTS INHIBITION OF OXALIPLATIN ON HEPATOCELLULAR CARCINOMA

tolerant to the treatment. The body weights showed no significant difference in the groups (Fig. 4C). The relative tumor volume (RTV) was calculated to assess the efficacy of Aspp2-ad and/or oxaliplatin. The results showed that RTVs of oxaliplatin, Aspp2 and combination groups all tended to reduce, but only significantly differed in combination groups compared with control group (Fig. 4D). The ultrasonic images showed that small vessel quantity in interior tumors were also decreased in oxaliplatin, Aspp2 and combination groups (Fig. 4B).
Figure 4. Aspp2-ad enhances the growth inhibition of oxaliplatin in hepatocellular carcinoma xenograft models. (A) Gross observations of tumors at the end point of experiments. (B) Ultrasonic observations of interior tumors at the end point of experiments. (C) Dynamical changes of body weight of nude mice of different groups. (D) The relative tumor volume (RTV) was calculated to assess the efficacy of Aspp2-ad and/or oxaliplatin. Compared with control group, *P<0.05.

Figure 5. Synergistic influence of Aspp2-ad and oxaliplatin on morphology and CD31, PCNA protein expression in hepatocellular carcinoma xenograft models. (A) H&E staining. Red arrow, mitotic nucleus; black arrow, necrosis. (B) MI of different groups. (C) PCNA expression of tumors in different groups. (D) CD31 expression of tumors in different groups. The magnification of A, C and D was x200. Compared with control group, *P<0.05.
Synergistic influence of Aspp2-ad and oxaliplatin on pathology and CD31, PCNA protein expression in hepatocellular carcinoma xenograft models. H&E results showed that MI decreased greatly in Aspp2-ad, oxaliplatin and combination groups compared with control group. The mitotic indexes of tumors in control, Aspp2-ad, oxaliplatin and combination groups were 0.051, 0.030, 0.028 and 0.018, respectively. Necrosis areas of tumors in combination group were increased compared to those in other groups (Fig. 5A and B).

Compared with those in control group, immunohistochemistry results showed that CD31 and PCNA expression of tumors in treatment groups decreased greatly, which were consistent with H&E and ultrasonic results (Fig. 5C and D).

Synergistic influence of Aspp2-ad and oxaliplatin on apoptosis and autophagy protein expression of hepatocellular cells and hepatocarcinoma. In HepG2P53-/- cells, cells treated with Aspp2-ad increased the Aspp2 protein expression, and oxaliplatin enhanced the Aspp2 expression. Aspp2-ad increased the Bax protein expression, while decreased the Beclin1 protein expression. Aspp2-ad and oxaliplatin both increased the LC3B expression. PCNA showed no significance in the treated cells. In Hep3B cells, cells treated with Aspp2-ad increased the Aspp2 protein expression, and oxaliplatin enhanced the Aspp2 expression. Oxaliplatin increased the Bax protein expression while decreased LC3B expression. Aspp2-ad and oxaliplatin both increased Beclin1 expression.
PCNA showed no significance in the different treatments of cells (Fig. 6A-C).

In Hep3B xenograft tumor models, Aspp2-ad and oxaliplatin both increased Bax, caspase-3 cleaved expression. Aspp2-ad exerted oxaliplatin to regulate the LC3B and Beclin1 expression (Fig. 6D and E).

**ASPP2-ad enhances the inhibitory effect of oxaliplatin on hepatocellular carcinoma via regulating the cell signal pathways.** In Hep3B xenograft mice, the western blot results showed that the expression of p-STAT3, p-ERK of Aspp2-ad and combination group decreased greatly. The radio of p-ERK/T-ERK and p-STAT3/T-STAT3 were also decreased in these two groups with multiplexed bead-based immunoassay. Synergetic inhibition of Aspp2-ad and oxaliplatin on these two signal molecules was observed. No changes of p-mTOR/T-mTOR were shown in the different groups (Fig. 7).

**Discussion**

Gene therapy is a biological treatment method which can transfer the foreign gene into the target cells by gene transfer technology, correcting or compensating for the disease caused by the gene defect and abnormality (22). The first gene therapy approved by FDA in the United States occurred in 1990 for a patient with severe combined immunodeficiency disorder (23). In 2004, Gendicine (Recombinant Human Ad-p53) as the first world’s tumor gene therapy drug was successfully developed in China (18,24). Since then, various gene therapy research and several commercially approved medications for cancer have been reported (25-28). Although the research and development of antitumor gene drugs have developed rapidly, the most serious problem of gene therapy is the selection of effective genes. Overexpression of Aspp2 was found to have antitumor effect independently and dependent on P53 pathways (29). Aspp2 was considered an appropriate gene for antitumor treatment in our previous study (20).

In HCC, the frequency of p53 gene mutation/deletion is as high as 50.0% (average, 30.0%). Therefore, analysis of its deregulation is associated with human diseases, including cancer and diabetes. The mTOR pathway integrates signals from nutrients, energy status and growth factors to regulate many processes, including autophagy, ribosome biogenesis and metabolism (42). mTOR activation can promote angiogenesis, proliferation, with peak expression occurring during late G1 and S phases (32). A wide range of functions of PCNA involved in genome maintenance, duplication, transmission and cell-cycle regulation were found later (33). Indeed, PCNA was increased in many tumor tissues involved in the prognosis of cancer patients (34). Platelet endothelial cell adhesion molecule (PECAM-1) also known as cluster of differentiation 31 (CD31). CD31 is mainly used to demonstrate the presence of endothelial cells for assessing tumor angiogenesis, which may imply a rapid increase in the extent of the tumor (35). The immunohistochemistry results showed that PCNA, and CD31 expression decreased greatly in treatment groups. These results suggested that Aspp2-ad might inhibit proliferation and vascular growth of hepatocarcinoma.

Physiological processes, such as autophagy and apoptosis are affected in tumors. Most agents might regulate progress of autophagy and apoptosis to influence tumor growth. The regulation of autophagy on cell death is 2-fold: mild autophagy protects cells from harmful conditions and promotes cell survival; severe or rapid autophagy can induce programmed cell death, known as autophagic cell death (autophagy-mediated cell death, ACD). However, the regulation of apoptosis on cell death is unidirectional, and the defect of apoptosis will lead to the occurrence of tumor cell death (36-39). Aspp2 induced apoptosis protein expression in Aspp2-ad and combination groups, Bax and activation of caspase-3 expression increased greatly both in vitro and in vivo. It is worth noting that the intrinsic Aspp2 had no change after treatment with oxaliplatin, but the Aspp2 expression increased greatly in combination group, possibly oxaliplatin exerted the extrinsic Aspp2 to promote intrinsic Aspp2 expression. Notably, the autophagy proteins showed different responses not only in HepG2 but also in Hep3B cells but also in vitro and in vivo, which might relate to the autophagy level of samples.

From the data above, we concluded that Aspp2-ad exerted oxaliplatin to regulate the proliferation, apoptosis, vascular growth and autophagy to inhibit hepatocarcinoma.

To clarify which signal pathway Aspp2 affected, we detected the ERK1/2, m-TOR and STAT3 pathways with western blot analysis and multiplexed bead-based immunoassay. ERK signal transduction pathway is the classical MAPK signaling pathway, ERK could phosphorylate cytoplasmic protein, but also phosphorylated in the nucleus of some transcription factors, which were involved in cell proliferation, differentiation, apoptosis and regulation of aging, migration (40). In the past 30 years, it has become evident that the Ras/Raf/MEK/extracellular signal-regulated kinase (ERK) signaling pathway plays a significant role in the occurrence and development of HCC. ERK phosphorylation activates a variety of target molecules to promote the development of liver cancer (41). The mammalian TOR (mTOR) pathway is a key regulator of cell growth and proliferation and increasing evidence suggests that its deregulation is associated with human diseases, including cancer and diabetes. The mTOR pathway integrates signals from nutrients, energy status and growth factors to regulate many processes, including autophagy, ribosome biogenesis and metabolism (42). mTOR activation can promote angiogenesis,
tumor invasion, metastasis and the cell cycle. It plays a very important role in the occurrence, development and treatment of liver cancer (43-46). Signal transducers and activators of transcription3 (STAT3), activation leads to increased expression of downstream target genes, leading to increased cell proliferation, cell survival, angiogenesis and immune system evasion. In normal cells, STAT3 activation is tightly controlled to prevent dysregulated gene transcription, whereas constitutively activated STAT3 plays an important role in tumorigenesis through the upregulation of genes involved in anti-apoptosis, proliferation and angiogenesis (47-49). We found that Aspp2-ad downregulated the p-ERK1/2, p-STAT3 expression, the synergistic effects were observed in combination group, while there was no response of mTOR to Aspp2-ad. These results were in further confirmed that Aspp2-ad exerted oxaliplatin inhibiting hepatocarcinoma via P53-independent pathway by regulating ERK and STAT3 pathway.

In conclusion, Aspp2-ad, P53-independently, regulated ERK and STAT3 signal molecules to inhibit hepatocarcinoma in combination with oxaliplatin by influencing the protein expression of proliferation, apoptosis, autophagy and vascular growth. Aspp2-ad has the potential to be developed as a gene therapy for HCC, especially for P53 deletion or mutation in HCC.

Acknowledgements

The present study was supported by the Training Plan for High Level of Health Technical Personnel of Beijing Health System (2015-3-101), the Foundation of Beijing Institute of Hepatology (no. BJHH-01712), the National Natural Science Foundation of China (no. 81272266), the National Natural Science Foundation of China (no. 8136120401), the Beijing Municipal Institute of Public Medical Research Development and Reform Pilot Project (no. 2016-2) and the Beijing Precision Medicine and Transformation Engineering Technology Research Center of Hepatitis and Liver Cancer.

References


