Original Article
Non-antiviral effect of entecavir on hepatocellular carcinoma

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Abstract: We discovered that entecavir (ETV), nucleos(t)ide analogue against hepatitis B virus (HBV), possessed liver cancer stem cell suppression function in addition to its antiviral function. However, its molecular mechanisms underlying the antitumor effect on hepatocellular carcinoma (HCC) have not been clarified. First of all, the experiments of ETV effect on tumor cells were performed. Cell counts kit 8 (CCK8) was applied to detect the ETV inhibitory effect with different concentrations on Huh7 cells which is a human hepatocellular carcinoma cell line in vitro. Besides, the tumorigenic potential of the cells in vivo were investigated by assessing growth of xenograft tumors in nude mice. Metastatic features of Huh7 were assessed in Matrigel invasion assays and wound healing analyses. Cell apoptosis and cell cycle profile were detected by flow cytometry (FCM). Western blot was used to analyze the expression of p21, caspase 8, caspase 9, and pAKT in the cells. ETV inhibited the growth of hepatic cancer cells in a concentration-dependent and a time-dependent manner. ETV activated caspase cleavage in a concentration-dependent manner to induce apoptosis. Also, we found that ETV can promote G1/S phase arresting accompanied with the upregulation of p21. The ability of migration, invasion and tumorigenesis of ETV treated huh7 cells were decreased. More importantly, the effect of ETV on cell proliferation was confirmed to be associated with AKT phosphorylation inhibition. In summary, ETV reduces the malignant transformation of HCC by promoting p21 at least partially through the AKT inhibition in addition to its antiviral effect.

Keywords: Hepatocellular carcinoma, nucleos(t)ide analogues, entecavir, p21, AKT, hepatitis B virus

Introduction

Hepatocellular carcinoma (HCC) is one of the most common human cancers and mainly related to chronic hepatitis B (CHB) in Asian [1]. Long-term anti-HBV therapy could not only reduce CHB patients’ risk of developing HCC, but also prevent or delay the recurrence of HCC [2]. Three nucleoside analogs (NAs) [lamivudine, entecavir (ETV), and telbivudine] which can selectively target HBV DNA are FDA-approved agents for the treatment of hepatitis B. However, other nucleoside analogs, such as fludarabine, cladribine, emcitabine and cidofovir, open new perspectives for the use of antitumor [3]. The nucleoside analogs can be divided into sub-classes based on their structural similarity to purine bases (adenine and guanine) or pyrimidine bases (cytosine, uracil, or thymine). They comprise a class of rationally designed agents that play essential role in corrupting key cellular processes and become possible to kill rapidly dividing cancer cells [4]. ETV is a cyclopentyl guanosine analogue that inhibits both the priming and elongation steps of viral DNA replication and commonly used clinically for the treatment of anti-HBV. However, few people pay attention to non-antiviral effect of ETV on HCC. In our previous work, we discovered that ETV can decline the CD133+ cells which are considered to be liver cancer stem cells (CSCs) [5]. CSCs, a subpopulation of tumor cells that possess unique self-renewal activity and mediate tumor initiation and propagation
are not only the resource of HCC but also take part in tumor recurrence, metastasis and progression. The researches have shown the role that AKT signal pathway played in the maintenance of CSCs [6, 7]. The phosphatidylinositol-3-kinase (PI3K)/Akt and the mammalian target of rapamycin (mTOR) signaling pathways are crucial to many cancer physiological and pathological process, including proliferation, angiogenesis, metabolism, differentiation and survival. Studies have exhibited that AKT signaling was involved in CD133 expression in gastrointestinal cancer cells [8]. AKT/mTOR pathway was crucial in the generation and the differentiation of tumorigenic CSCs.

Interestingly, p21 is located in the downstream of AKT and partially take part in the AKT signal pathway. p21 is another molecule which is associated with cancer cell stemness and is first found having a near relationship with senility. p21 together with p53 constitute a G1 cell cycle checkpoint, so damaged DNA without repair cannot be passed. p21 reduce the replication and accumulation of damage of DNA, playing an important role in cell cycle control and cancer [9]. At the same time, once the cells are damaged by toxins or radiation, p21 will stop cell growing, giving the cells time to rest and self repair. In addition, when the cells are getting “old”, p21 also inhibits the cell division, and start a natural process of cell death, which calls the “aging” process. Many studies demonstrated the role of p21 on inhibition of cell proliferation in several kinds of cancers [10-12], including hepatocellular carcinoma [13].

Whether ETV suppressing HCC is not entirely through its antiviral role? Given all above, we assume that ETV promotes p21 through AKT signaling pathway to suppress HCC. Therefore, we explored biological behavior change of Huh7 in vitro and the molecular expression related to AKT signal pathway of liver cancer cells treated with ETV in order to verify our conjecture.

Materials and methods

Cell culture

Among the cell lines studied, the CD133 antigen was found to be expressed on the surface of Huh7 cells. CD133+ cells from Huh7 performed a higher in vitro proliferative potential and it’s a HBV negative HCC cell line, and often used in the experimental research [14, 15]. Taken together, we choose huh7 as the main study object. Huh7 cells were cultured in high-glucose Dulbecco’s modified Eagle medium (DMEM; Gibco) with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin-streptomycin (PS; Gibco), in an incubator under 37°C, 5% CO₂ and saturated humidity condition. The cells were digested with 0.25% trypsin-EDTA (trypsin; Gibco) for passaging. Cells used in the experiments were in logarithmic growth phase.

CCK8

For the determination of cell growth, 2000 cells stimulated with different concentration of ETV or without in 96-well plates per well were cultured for certain time. The cell count kit8 (CCK8, Dojindo, Japan) was used to evaluate cell viability and the obtained growth curves were used to establish the proliferation of cells. Cells incubated with CCK8 were examined under the indicated conditions after 1 hour. The CCK8-derived formazan developed by cells was measured by the optical density with the microplate reader (EON, BioTek, USA) at wavelength of 450 nm. The calculation method of inhibition rate is [1-(treatment group-blank group)/(negative control group-blank group)]*100%.

Wound-healing assay

Cell migration was assessed by wound-healing assay measuring the movement of cells into a scraped acellular area. We scraped horizontal lines with 100 µl tips (Thermo, QSP, USA) across the entire diameter at the bottom of 6-well plates. Cell media was removed and the cells were gently washed 3 times with PBS to remove unattached cells. The wound area was photographed at 0, 6, 12, 24, 48 h respectively after scraping. To compare cell motility of cancer cells, we measured the gap distance with In-verted Microscope (CKX31, Olympus, Japan) and determined the wound-closing rate.

Invasion assays

For transwell invasion assay, we used 24-well Corning BioCoat Matrigel Invasion Chambers (8 um, Corning Costar, Cambridge, MA, USA) according to the manufacturer’s instructions. 10000 cells in DMEM without FBS were added
to the top chamber, and 10% FBS in DMEM was added to the bottom chamber as an attractant. After 24 hours of incubation, the invaded cells were fixed and stained with 0.1% crystal violet (Sigma-Aldrich, St. Louis, MO, USA) and 10% methanol (Sinopharm Chemical Reagent Co., Shanghai, China), and then observed under a microscope.

**Flow cytometry (FCM)**

Huh7 incubated with and without ETV for 48 h were harvested and then, cell apoptosis and cell cycle profile were detected by flow cytometer (BD LSRFortessa). The labeled cells were analyzed and then the data were analyzed using FlowJo 7.6 software.

**Cell apoptosis assay**

Briefly, 10^6 cells were trypsinized and resuspended in 100 µl binding solution containing 5 µl propidium iodide (BD, Becton, USA) and 5 µl Annexin V (FITC Annexin V Apoptosis Detection Kit, BD, Becton, USA).

**Cell cycle analysis**

Briefly, 10^6 cells were trypsinized, washed phosphate-buffered saline (PBS; Corning, Cambridge, MA, USA), and fixed in 70% ethanol for 24 h, and then resuspended in 100 µl stain buffer (BD) containing 5 µl 7-AAD (BD).

**Protein extraction and western blot**

Cells after treatment with or without ETV were washed with PBS and lysed in RIPA buffer containing 1% NP-40, 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% SDS, protease inhibitor cocktail (Thermo), and PhosSTOP phosphatase inhibitor cocktail (Roche). The total concentration of the cell protein was measured by using bovine serum albumin as standard. The same amount of protein (50 µg) were separated in 10% SDS-PAGE and then transferred to a PVDF membrane; the target proteins were detected with different antibodies (4°C overnight). After washing off the primary antibodies, the membrane was incubated with HRP-conjugated secondary antibody for 1 h at room temperature. Immobilon Western Kit (MILLIPORE, USA) was used to develop the immuno-reactive bands. Then β-actin was used as an internal control to determine the changes in p21 and AKT phosphorylation level in huh7 cells. Relative signal intensity was analyzed by ImageJ launcher software.

The following antibodies were used: rabbit anti-CDKN1A interacting zinc finger protein 1 polyclonal antibody (P21, 1:1000, Abcam, Cambridge, UK), rabbit anti-Phospho-Akt (Ser473) polyclonal antibody (1:1000, CST, USA), rabbit anti-Akt antibody (1:1000, CST, USA), mouse anti-β-actin monoclonal antibody (1:10000, ABGENT, USA), HRP labeled Goat anti-Mouse IgG (H+L) (1:10000, secondary antibodies, Pufei, China) and HRP-conjugated Goat anti-rabbit IgG (H+L) (1:10000, secondary antibodies, Pufei, China).

**Tumor formation in BALB/c nude mice**

To determine the ability of tumorigenic ability in vivo, we inject 1*10^7 huh7 cells treated with ETV (1 mM) subcutaneously into the right side of the hips in male BALB/c nude mice (6 w, 6 per group), respectively. The volume of tumors was measured twice a week. The mice were sacrificed at the 20th day after injection, and the xenografts were removed and photographed. All animal experiments were approved by and performed in accordance with the Committee of the Use of Live Animals in Teaching and Research at the Shanghai public health clinical center.

**Statistical analysis**

Experimental data of normal distributions were expressed as mean ± SD; Independent sample T test method was used to determine the difference between the means of the treatment group and its control. Nonparametric test was used to analyze and compare data of non-normal distributions. One-way ANOVA was carried out for comparison among three groups. \( P < 0.05 \) was determined as with statistically significant differences. GraphPad Prism 5 software was used for all the above analysis.

**Results**

**ETV inhibits the growth of hepatic cancer cells in vitro**

Figure 1B demonstrates that ETV inhibited the growth of Huh7 cells in a concentration-dependent and a time-dependent manner. The inhibition rate was 8.31%, 32.43% in Huh7 with 1 mM ETV, while inhibition rate was 24.98%,
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Figure 1. ETV treatment inhibits the viability of Huh7 cells in vitro. A: Chemical structure of ETV (209216-23-9 (CAS Database Reference)). B: The viability of Huh7 cells treated with ETV over time at the indicated concentrations was determined using a CCK8 assay. C: Cell cycle analysis was accomplished and demonstrated G1/S cell cycle arrest in huh7 treated with ETV. D: Cell apoptosis profiles were measured by flow cytometry following treatment of the cells with or without ETV for 48 h. Cells in the higher left quadrant (Annexin V+/PI-) represent early apoptotic cells, and those in the upper right quadrant (Annexin V+/PI+) represent late apoptotic cells. The induction of apoptosis was established. E: Cleaved caspase-8 and caspase-9 were increased in Huh7 cells treated with ETV. ETV activates caspase cleavage to induce apoptosis in a concentration-dependent manner. *P<0.05, **P<0.01 and ***P<0.001, compared with the control.
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Figure 2. The ability of migration and invasion of Huh7 cell. A, B: Wound-healing assay; C: Matrigel invasion (purple cells mark invaded cells). *P<0.05, **P<0.01 and ***P<0.001 compared with the control.

61.71% in Huh7 with 2 mM ETV in 24, 48 h, respectively. To confirm the occurrence of apoptosis, we performed an Annexin V-FITC/PI double-staining assay. The cells in Annexin V+/PI- represent early apoptotic cells, and those in Annexin V+/PI+ represent late apoptotic cells.
Apoptosis was 9.229% in negative control and increased to 19.05% when treated with 1 mM ETV (Figure 1D). We performed western blot to examine caspase activation since caspase activation as a hallmark of apoptosis. β-actin was used as a loading control. ETV activates caspase 8 and caspase 9 in a concentration-dependent manner to induce apoptosis (Figure 1E). To investigate whether the antiproliferative effect of ETV on Huh7 cells was triggered by cell cycle arrest, we also measured the cell cycle phase ratio by flow cytometry with 7-ADD staining. As shown in Figure 1C, ETV treatment induced significant G1/S-phase accumulation compared with the control. The percentage of G1/S cells increased significantly, from 7.3%/9.05% to 10.6%/53.91%, while the percentage of G2 cells decreased from 42.5% to 29.95%. Therefore, we conclude that ETV can inhibit the growth of hepatoma carcinoma cells through inducing apoptosis and cell cycle arresting.

ETV suppresses migratory and invasive abilities of huh7 cells

Wound-healing assay at different time (12 h, 24 h, 48 h) is showed in Figure 2A, and the ability of migration in Huh7 treated with ETV is obviously weakened in a dose and time dependent manner (P<0.001) (Figure 2B). The gap distances at 12 h were (209.4±2.646), (224.5±6.089), (255.3±5.511) µm in control, ETV 1 mM, ETV 2 mM group, respectively. The gap distances of the control, ETV 1 mM, ETV 2 mM group were (157.5±4.334), (228.9±5.309), (249.3±1.380) µm at 24 h and (25.24±8.072), (190.4±4.489), (229.7±4.756) µm at 48 h, respectively. The migration rate of huh7 treated with 0 mM ETV, 1 mM ETV, 2 mM ETV is 4.74, 1.75, 0.91 µm/h, respectively. Transwell matrigel invasion is showed in Figure 2C, the ability of invasion of huh7 treated with ETV was decreased significantly.

ETV reduces the ability of tumorigenesis in nude mice

Huh7 cells were treated with ETV or without ETV, and then transplanted to the nude mice. We compared the tumorigenesis in nude mice. The tumor formation rate on the third week was much lower in ETV group than that without ETV group (3/6 vs 5/6). The tumor size in without ETV group (153±134.5 mm) was much bigger than that in ETV group (45.7±20.36 mm) in the second week (P=0.02). However, in the third week, the tumor size was (990.0±750.4 mm) in without ETV group while the tumor volume was (27.98±17.88 mm) in ETV group (P=0.0017). Mice bearing huh7 pretreated with ETV were much weaker in tumorigenic ability. The tumor sizes of both groups were showed in Figure 3.

ETV promotes P21 expression via inhibiting p-AKT (Ser473)

Since ETV affected the cell cycle and apoptosis, we detected the expression of cell cycle-related protein (p21). We found that ETV promoted the expression level of p21 in Huh7 (Figure 4A). Western blot analysis confirmed that P21 was up-regulating when using β-actin as control for normalization. To investigate the molecular mechanism of ETV inhibiting tumor cells, we analyzed the expression of pAKT (Ser473). As is shown in Figure 4C, phosphory-
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Discussion

ETV has shown its effects in HBV DNA inhibition and patients whose abnormal liver function own to HBV infection get better after received ETV. To test whether ETV could inhibit HCC in additional to antiviral activity, functional assays were applied to study Huh7 cells treated with ETV. The results demonstrated that a significant reduction in proliferation assay, G1/S cell cycle arresting and declining tumour formation ability of Huh7 cells treated with ETV in nude mice. Interestingly, we also found that ETV can promote p21 in Huh7 cells treated with ETV via inhibiting the AKT signal pathway. This is the first time to explore the molecular mechanisms underlying the direct antitumor effect of ETV on HCC. As an anti-virus agent in the therapy of CHB, ETV opens new perspectives for the application in the therapy of HBV-related HCC.

AKT signal pathway has become a hot anti-tumor target during recent years. Phosphorylated AKT could promote the growth and proliferation, invasion and metastasis of cancer cells, trigger the blood vessel formation and reduce the apoptosis of cancer cells after radiotherapy and chemotherapy. Researchers also discover that AKT pathway had taken an important role in the occurrence and progress in liver cancer [16, 17]. Our experiments show the similar results, which phosphorylated AKT was declined in the ETV treated Huh7 cells, and which may result in the invasive ability reduction of Huh7 cells stimulated with ETV.

Located downstream of the AKT signaling pathway, p21 bound to cyclin-dependent protein kinase (CDKs) and inhibited CDKs' activity, blocking the growth of the cell cycle at specific stages [18, 19] and the initiating DNA replication by inhibiting the CDK complex. Acting as a well-known tumor suppressor, p21 could inhibit the growth of the cancer cell and was observed in 37% of HCC tissues [13].

Antiviral therapy is a key component in the management of CHB. In clinical works, doctors usually use ETV just for anti-HBV to achieve the effect of anti-virus. Recent advances in the function of cidofovir, one kind of NAs, had showed the evaluation in not only the treatment of HPV-associated tumors, but also can be used as an anti-tumor agent in the therapy of tumors which are not associated with onco-

Figure 4. ETV promoted p21 expression via inhibiting p-AKT (Ser473). A: Western blotting was performed to detect p21. β-actin was used as a loading control. B: ImageJ Launcher software was used to detect the relative signal intensity of p21. C: Western blotting was performed to detect p-AKT. β-actin was used as a loading control. D: Image J Launcher software was used to detect the relative signal intensity of p-AKT (Ser473). *P<0.05, **P<0.01 and ***P<0.001 compared with the control.
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Research also shows overall survival, decompensation-free survival, and recurrence-free survival were better in the ETV-treated patients than in the lamivudine treated-patients [20], indicating that the potent antiviral drug should be the preferred choice in HBV-related HCC patients.

We have to point out that the floating dead cells can be obviously seen under a microscope when the concentration of ETV being added up. We deduced that the floating dead cells were not entirely caused by apoptosis and ETV with high concentration may be poisonous to Huh7 cells. p21 has different effects depends on its subcellular localization. Nuclear p21 is anti-proliferative, while cytoplasmic p21 may exert oncogenic role [21]. This study only investigated the total cellular protein p21. To determine the tumorigenic ability in vivo of huh7 treated with ETV, we evaluated the volume of tumors in the nude mouse xenograft model. The results showed that the ability of tumorigenicity and tumor growth in vivo were suppressed in nude mouse xenograft model injected huh7 treated with ETV. In addition, we also found out the sizes of xenograft tumor get smaller over time. This is an ambivalent phenomenon and we think this may be associated with immune state of the nude mouse. The immunological feature of the nude mouse is characterized by thymus aplasia, but not totally immunodeficiency. Adult nude mice of more than 6-8 w have higher activity of Natural killer (NK) cells. NK cells serve an important role in the suppression of carcinogenesis. With increasing activity of NK cells, the non-specific immunity to tumor increase [22].

In conclusion, ETV exerts a growth-inhibitory effect on HCC cells. Our research reveals that ETV induces tumor cell apoptosis through the caspase-dependent pathway and G1/S phase arrest. Its mechanisms of action may be related to upregulation of p21, and inhibition of p-AKT (Ser473). As its effects on inhibiting AKT signal pathway, which is a prospect anti-tumor target, and increasing the expression of tumor suppressor molecule p21, ETV may also play a potential anti-tumor role in HCC patients with HBV infection. Attempts to seek for the clear mechanism are urgently needed.

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Disclosure of conflict of interest

None.

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