

Alpha Fetoprotein Plays Antagonistic Role in Benzyl-Isothiocyanate Arresting Cell Cycle in Liver Cancer Cells

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Abstract

Background: To investigate the effect of alpha fetoprotein (AFP) on benzyl-isothiocyanate (BITC) arresting cell cycle in human liver cancer cells line *in vitro*, and explore the possible role mechanism of BITC inhibited proliferation of hepatocellular carcinoma (HCC) cells.

Methods: In this study, we selected HCC cells lines, Bel 7402 and HLE for test. Fluorescent microscopy and Western blotting were applied to observe the transfected effect and expression of proteins; MTT assay the proliferation of HCC cells; Flow cytometry method was used to detect the cellular cycle; RNA interference was used to test and expressed vector constructed technology were performed to silence and induce expression of AFP, respectively.

Results: Analysis showed BITC had a significant inhibitory effect on the proliferation of HCC cells in dose-dependent manner, inhibitory effect was enhanced while Bel 7402 cells were transfected with AFP-siRNA vectors but attenuated in HLE cells while transfected with pcDNA3.1-afp vectors. The growth ratio of AFP-siRNA transfected Bel 7402 cells and pcDNA3.1-afp transfected HLE cells followed treated with 80 $\mu\text{mol/L}$ BITC were $(42.43 \pm 4.92)\%$ ($P < 0.05$ vs Bel 7402 cells group) and $(40.13 \pm 4.99)\%$ ($P < 0.05$ vs HLE cells group). BITC could obviously induce cell cycle G2/M phase arrest in these HCC cells; The induction effect was enhanced in AFP-siRNA transfected Bel 7402 cells but attenuated in pcDNA3.1-afp transfected HLE cells. BITC inhibited the expression of cell cycle related proteins, cyclin B1, CDK1, Cdc25c but stimulated expression of Weel in Bel 7402 cells and HLE cells, and such effect was enhanced in AFP-siRNA transfected Bel 7402 cells but attenuated in pcDNA3.1-afp transfected HLE cells.

Conclusions: BITC could inhibit the growth of HCC cells and induce cell cycle G2/M phase arrest through down regulating the expression of cyclin B1, CDK1, Cdc25c and up regulating the expression of Weel; AFP played an antagonistic role in BITC arresting cell cycle in HCC cells.

Keywords: Alpha-fetoprotein (AFP); Benzyl isothiocyanate (BITC); Hepatocellular carcinoma cells; Cell cycle arrest

Abbreviations:

AFP: Alpha-fetoprotein; BITC: Benzyl-isothiocyanate; ITCs: Isothiocyanates; HCC: Hepatocellular Carcinoma; FCS: Fetal Calf Serum; MTT: Methylthiazolyldiphenyl-tetrazolium Bromide; CDK1: Cyclin Dependent Kinases-1; PI3K: Phosphatidylinositol 3-kinase; AKT: Protein kinase B

Introduction

Benzyl isothiocyanate (BITC) is one of hydrolysis compound of biologically active isothiocyanates (ITCs) documents indicated that ITCs played pivotal roles in detoxification of carcinogens [1-4]. Growth of tumor cells were dependent on boosting of cells cycle, many studies have reported that BITC prevents development of cancers in

laboratory animals and might also be chemoprotective in humans via restraining progress of cells cycle [5-9], BITC displayed anti-tumor activity in many types of tumor, include hepatoma cells [10,11]. These data implicated that BITC was applied to prevent and therapy of cancer.

Alpha fetoprotein (AFP) is an early biomarker for diagnosis of hepatocellular carcinoma (HCC) occurrence. High expression of AFP was emerged in 70-80% HCC patients, and AFP was thought as an important factor for the malignant behaviors of HCC cells, included resistance of chemodrugs cytotoxicity [12,13]. Previously, we had found that AFP was able to activate transduction of phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) signal pathway through inhibiting activity of PTEN, led to HCC cells resistance the apoptosis induced by all trans retinoic acid [14], and researchers had proved that PTEN has a trait to restrain the progression of cell cycle [15-17]. Although investigations had found that BITC was capability to inhibit proliferation of tumor cells,

whether BITC suppressed growth of HCC cells via arresting the cell cycle is still unclear. We have found that AFP inhibited the function of PTEN [14], in the present study, further, we investigated the role of AFP in BITC regulating the cell cycle of hepatoma cells, and we found that AFP antagonized the role of benzyl-isothiocyanate in arresting cell cycle in liver cancer cells.

Material and methods

Cell culture

Human HCC cell lines, Bel 7402 (AFP-producer) and HLE (non-AFP-producer) were friendly gift from Department of Cell Biology, Peking University Health Science Center. In this study, we selected these cells for test. These cells were cultured with RPMI-1640 medium and supplemented 10% heat-inactivated fetal calf serum (FCS), the cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂.

RNA interference assay, AFP-expressed vectors construction and transient transfection

The AFP-siRNA vectors and AFP-expressed vectors (pcDNA3.1-afp) were constructed as described in previous study [18,19]. Bel 7402 cells and HLE cells were transfected with AFP-siRNA vectors and transfected with AFP-expressed vectors for 48 h, respectively, the transfected efficiency was observed by fluorescent microscope, and the expression of AFP was detected by Western blotting.

MTT methods analyzed the growth of the cells

The Methylthiazolyldiphenyl-tetrazolium Bromide (MTT) methods were referred to previously [18]. Briefly, total 1.5×10^4 cells per well of Bel 7402 cells or HLE cells were plated into 96-well plates and cultured in RPMI-1640 medium supplemented with 10% FCS at 37°C in a humidified atmosphere of 5% CO₂ for 48hrs. The cultured cells were replaced with medium without FCS for another 24hrs. Bel 7402 cells were transfected with AFP-siRNA vectors and HLE cells were transfected with AFP-expressed vectors for 24 hrs, respectively, followed treatment with differential concentration of BITC (10-80 µmol/L) for 24hrs. The effects of AFP on BITC (Sigma, UAS) regulating the growth of the cells were measured by MTT assay as described [18], which were performed following a regular procedure. The growth ratio of the cells = (control group A490-treated group A490) / control group A490 × 100%.

Flow cytometry analysis

2.5×10^4 cells of Bel 7402 cells and HLE cells were cultured in RPMI-1640 medium supplemented with 10% FCS at 37°C in a humidified atmosphere with 5% CO₂. The cells were treated with BITC (40 µmol/L) or BITC (80 µmol/L) for 48hrs, and Bel 7402 cells were transfected with AFP-siRNA vectors, HLE cells were transfected with AFP-expressed vectors for 24hrs followed treatment with BITC (40 µmol/L) or BITC (80 µmol/L) for 48hrs. The cell cycle of Bel 7402 cells or HLE cells were analyzed by flow cytometry. The detailed procedure as described in previous study [18].

Western blotting analysis

In order to estimate the influence of BITC and AFP on the expression of cell cycle related proteins, Bel 7402 cells and HLE cells

were treated with BITC (40 µmol/L) or BITC (80 µmol/L) for 48hrs; Bel 7402 cells were transfected with AFP-siRNA vectors or HLE cells were transfected with AFP-expressed vectors for 24hrs followed treatment with BITC (40 µmol/L) or BITC (80 µmol/L) for 48hrs, the expression of cell cycle related proteins, such as Cyclin B1, CDK1, Cdc25c and Weel in Bel 7402 cells or in HLE cells were analyzed by Western blotting. The detailed procedure as described in previous study [18,19].

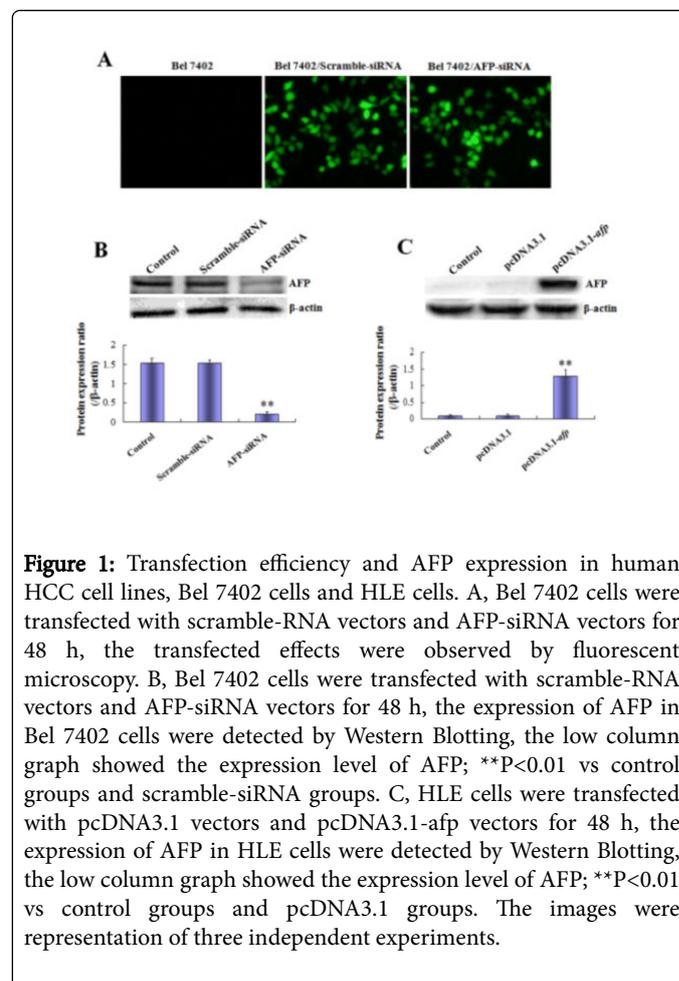
Statistical analysis

Data are presented as mean ± S.D. Statistical analysis was performed using the Student t test (for two experimental groups). Significance was set at P<0.05. Statistical significance was determined using Student's t-test and One-way ANOVA (SPSS 11.5 software for Windows, SPSS Inc., Chicago, IL, US).

Results

Successful suppressed expression of AFP in Bel 7402 cells and overexpression of AFP in HLE cells

In the present study, in order to observe the effect of AFP on the growth of HCC cells, we constructed AFP-siRNA vectors and AFP-expressed vectors (pcDNA3.1-afp) and transfected into Bel 7402 and HLE cells respectively.



Because siRNA vectors carried a green fluorescent protein gene, fluorescent microscope was applied to observe the transfected efficiency of siRNA vectors, the results displayed that high transfected efficiency of siRNA vectors in Bel 7402 cells (Figure 1A), Western blotting analysis displayed that AFP-siRNA vectors was able to significantly suppress the expression of AFP in Bel 7402 cells compared to scramble-siRNA vectors and non-treated groups (Figure 1B). Western blotting detection also displayed that pcDNA3.1-afp vectors was capable of inducing overexpression of AFP in HLE cells (Figure 1C). These results demonstrated that successfully inhibited expression of AFP in Bel 7402 cells and overexpression of AFP in HLE cells.

AFP harbors a trait for antagonizing BITC restrained growth of HCC cells

BITC was used to treat Bel 7402 cells or HLE cells, MTT analysis indicated that BITC had a significant inhibitory effect on the growth of HCC cells in dose-dependent manner. Bel 7402 cells were treated with concentration of BITC (10-80 $\mu\text{mol/L}$) for 24 h, the growth ratio of Bel 7402 cells were $(95.43 \pm 8.62)\%$ - $(53.06 \pm 7.97)\%$.

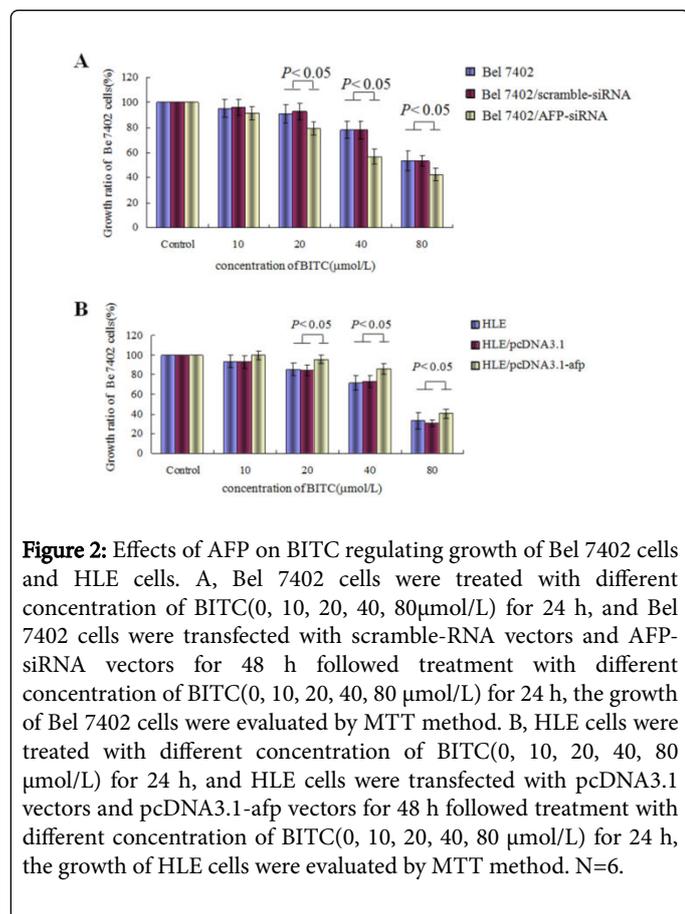


Figure 2: Effects of AFP on BITC regulating growth of Bel 7402 cells and HLE cells. A, Bel 7402 cells were treated with different concentration of BITC(0, 10, 20, 40, 80 $\mu\text{mol/L}$) for 24 h, and Bel 7402 cells were transfected with scramble-RNA vectors and AFP-siRNA vectors for 48 h followed treatment with different concentration of BITC(0, 10, 20, 40, 80 $\mu\text{mol/L}$) for 24 h, the growth of Bel 7402 cells were evaluated by MTT method. B, HLE cells were treated with different concentration of BITC(0, 10, 20, 40, 80 $\mu\text{mol/L}$) for 24 h, and HLE cells were transfected with pcDNA3.1 vectors and pcDNA3.1-afp vectors for 48 h followed treatment with different concentration of BITC(0, 10, 20, 40, 80 $\mu\text{mol/L}$) for 24 h, the growth of HLE cells were evaluated by MTT method. N=6.

While transfected with AFP-siRNA vectors follow treated with 80 $\mu\text{mol/L}$ BITC for 24 h, the growth ratio of the cells was $(42.43 \pm 4.92)\%$ ($P<0.05$ vs Bel 7402 cell group) (Figure 2A). HLE cells were treated with concentration of BITC (10-80 $\mu\text{mol/L}$) for 24 h, the growth ratio of HLE cells were $(94.74 \pm 11.26)\%$ - $(32.91 \pm 8.17)\%$. While HLE cells were transfected with pcDNA3.1-afp vectors followed treatment with 80 $\mu\text{mol/L}$ BITC for 24 h, the growth ratio of the cells was $(42.43 \pm 4.92)\%$ ($P<0.05$ vs HLE cell group) (Figure 2B). These results indicated

that BITC inhibitory effect was enhanced in AFP-siRNA transfected Bel 7402 cells but attenuated in pcDNA3.1-afp transfected HLE cells, implicated that AFP harbors a function to antagonize BITC restrained growth of HCC cells.

BITC induced cell cycle G2/M arrest in HCC cells and AFP antagonized this effect of BITC

In the present study, we applied flow cytometry assay to observe the influence of BITC on the cell cycle of HCC cells. In the experimentation, we selected the 40 $\mu\text{mol/L}$ and 80 $\mu\text{mol/L}$ concentration of BITC to treat Bel 7402 cells and HLE cells for 24h. The results indicated that BITC could significantly induce cell cycle G2/M phase arrest in Bel 7402 cells ($P<0.05$ vs non treated groups) (Figure 3A); silenced expression of AFP by siRNA follow treated with BITC (80 $\mu\text{mol/L}$) was able to significantly enhance cell cycle G2/M phase arrest in Bel 7402 cells contrast to BITC (80 $\mu\text{mol/L}$) treated groups ($P<0.05$ vs BITC alone treated groups) (Figure 3B).

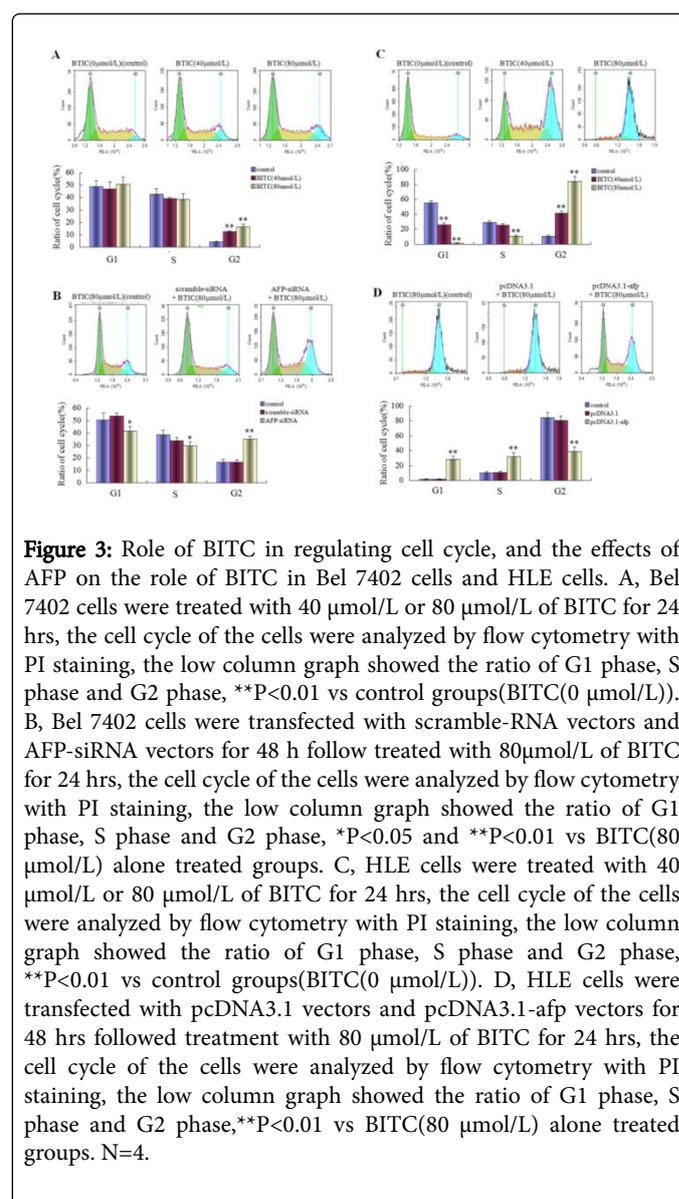


Figure 3: Role of BITC in regulating cell cycle, and the effects of AFP on the role of BITC in Bel 7402 cells and HLE cells. A, Bel 7402 cells were treated with 40 $\mu\text{mol/L}$ or 80 $\mu\text{mol/L}$ of BITC for 24 hrs, the cell cycle of the cells were analyzed by flow cytometry with PI staining, the low column graph showed the ratio of G1 phase, S phase and G2 phase, $**P<0.01$ vs control groups(BITC(0 $\mu\text{mol/L}$)). B, Bel 7402 cells were transfected with scramble-RNA vectors and AFP-siRNA vectors for 48 h follow treated with 80 $\mu\text{mol/L}$ of BITC for 24 hrs, the cell cycle of the cells were analyzed by flow cytometry with PI staining, the low column graph showed the ratio of G1 phase, S phase and G2 phase, $*P<0.05$ and $**P<0.01$ vs BITC(80 $\mu\text{mol/L}$) alone treated groups. C, HLE cells were treated with 40 $\mu\text{mol/L}$ or 80 $\mu\text{mol/L}$ of BITC for 24 hrs, the cell cycle of the cells were analyzed by flow cytometry with PI staining, the low column graph showed the ratio of G1 phase, S phase and G2 phase, $**P<0.01$ vs control groups(BITC(0 $\mu\text{mol/L}$)). D, HLE cells were transfected with pcDNA3.1 vectors and pcDNA3.1-afp vectors for 48 hrs followed treatment with 80 $\mu\text{mol/L}$ of BITC for 24 hrs, the cell cycle of the cells were analyzed by flow cytometry with PI staining, the low column graph showed the ratio of G1 phase, S phase and G2 phase, $**P<0.01$ vs BITC(80 $\mu\text{mol/L}$) alone treated groups. N=4.

40 $\mu\text{mol/L}$ and 80 $\mu\text{mol/L}$ concentration of BITC also significantly induced cell cycle G2/M phase arrest in HLE cells ($P < 0.05$ vs non treated groups) (Figure 3C), but the arrested effect of BITC were attenuated while HLE cells were transfected with pcDNA3.1-afp vectors, ratio of G2 phase was significantly decreased contrast with BITC alone treated groups ($P < 0.05$) (Figure 3D). These results demonstrated that BITC was able to arrest cell cycle of HCC cells in G2/M phase, and AFP antagonized the effect of BITC.

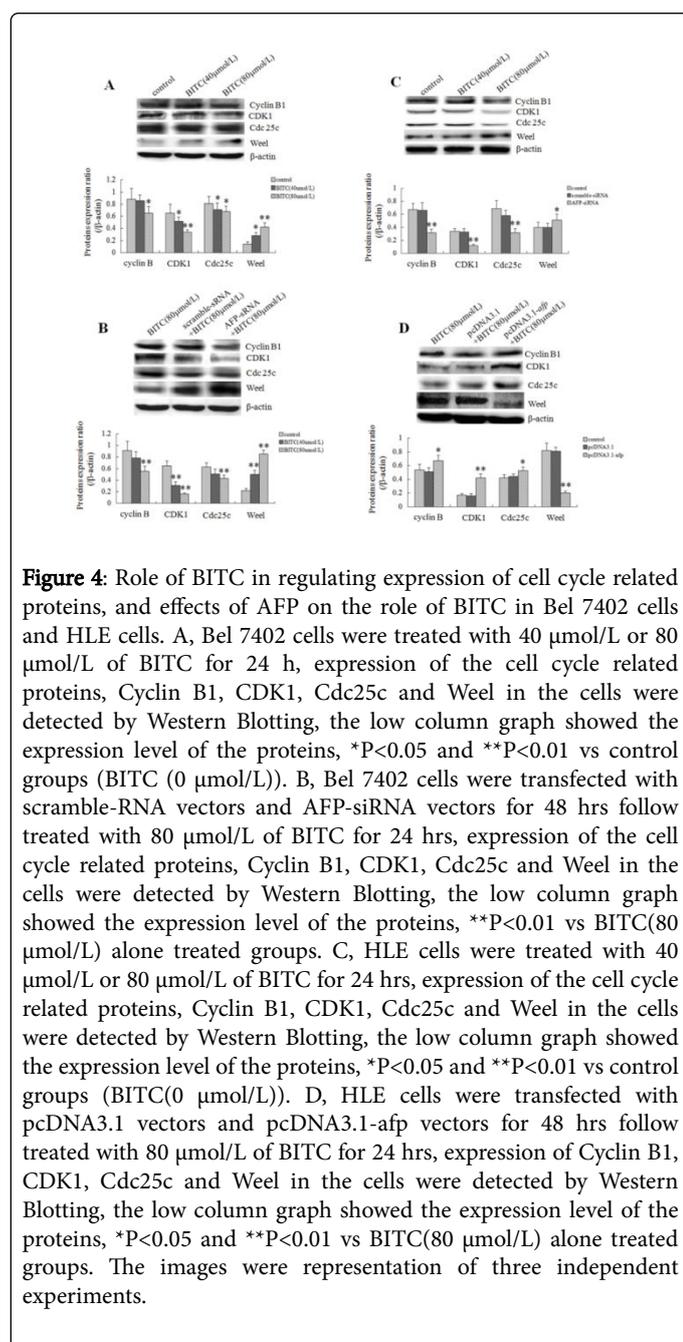


Figure 4: Role of BITC in regulating expression of cell cycle related proteins, and effects of AFP on the role of BITC in Bel 7402 cells and HLE cells. A, Bel 7402 cells were treated with 40 $\mu\text{mol/L}$ or 80 $\mu\text{mol/L}$ of BITC for 24 h, expression of the cell cycle related proteins, Cyclin B1, CDK1, Cdc25c and Weel in the cells were detected by Western Blotting, the low column graph showed the expression level of the proteins, * $P < 0.05$ and ** $P < 0.01$ vs control groups (BITC (0 $\mu\text{mol/L}$)). B, Bel 7402 cells were transfected with scramble-RNA vectors and AFP-siRNA vectors for 48 hrs follow treated with 80 $\mu\text{mol/L}$ of BITC for 24 hrs, expression of the cell cycle related proteins, Cyclin B1, CDK1, Cdc25c and Weel in the cells were detected by Western Blotting, the low column graph showed the expression level of the proteins, ** $P < 0.01$ vs BITC(80 $\mu\text{mol/L}$) alone treated groups. C, HLE cells were treated with 40 $\mu\text{mol/L}$ or 80 $\mu\text{mol/L}$ of BITC for 24 hrs, expression of the cell cycle related proteins, Cyclin B1, CDK1, Cdc25c and Weel in the cells were detected by Western Blotting, the low column graph showed the expression level of the proteins, * $P < 0.05$ and ** $P < 0.01$ vs control groups (BITC(0 $\mu\text{mol/L}$)). D, HLE cells were transfected with pcDNA3.1 vectors and pcDNA3.1-afp vectors for 48 hrs follow treated with 80 $\mu\text{mol/L}$ of BITC for 24 hrs, expression of Cyclin B1, CDK1, Cdc25c and Weel in the cells were detected by Western Blotting, the low column graph showed the expression level of the proteins, * $P < 0.05$ and ** $P < 0.01$ vs BITC(80 $\mu\text{mol/L}$) alone treated groups. The images were representation of three independent experiments.

BITC regulated the expression of cell cycle related proteins and AFP inhibited the effects of BITC

In order to explore the mechanism of BITC influenced on the progress of cell cycle, in the present investigation, we applied Western

blotting to detect the expression of cell cycle related proteins, such as cyclin B1, Cyclin dependent kinase 1(CDK1), Cdc25c and Weel. The results indicated that BITC was capable of inhibiting expression of cyclin B1, CDK1, Cdc25c, and stimulating expression of Weel in Bel 7402 cells(Figure 4A), the effect was enhanced while silenced expression of AFP by siRNA in Bel 7402 cells (Figure 4B). BITC also could inhibit expression of cyclin B1, CDK1, Cdc25c, and stimulating expression of Weel in HLE cells (Figure 4C). However, while AFP-expressed vectors were transfected into HLE cells, the effect of BITC on regulating the expression of the cell cycle related proteins was significantly weaker than BITC alone treated groups (Figure 4D). These results demonstrated that BITC could regulated the expression of cell cycle related proteins in HCC cells, and AFP played a role in inhibiting the effects of BITC.

Discussion

Unbalance of cell cycle led to the abnormal proliferation of cancer cells. The normal cell cycle was strictly checked by cycle-related molecules in check-points, included G1/S phase point, S phase point, G2/M phase point and mid-later point. Suppressed the function of checkpoint molecules which driven cell cycle progress maybe prevent development of cancer, these effect implicated that the progression of cells cycle closely associated with tumorigenesis. Researchers have found that ITC could induce cell cycle arrest in tumor cells, such as in G0/G1 point, G2/M point, and BITC have been found arresting the cell cycle in G1 phase and G2/M phase in cancer cells [20]. In the present study, the results indicated that BITC inhibited growth of hepatoma cell lines, Bel 7402 cells and HLE cells, implicated that BITC has a efficacy for preventing the proliferation of HCC cells. In order to explore the role mechanism of BITC in restraining the growth of HCC cells, flow cytometry assay was applied to observe the influence of BITC on the progression of cell cycle in HCC cells, the results indicated that high concentration of BITC(80 $\mu\text{mol/L}$) significantly increased the ratio of G2 phase in Bel 7402 cells and HLE cells, proved that BITC maybe plays a role in arresting G2/M phase in these cells. Cyclin B1 is a member of the cyclin family, evidences showed that cyclin B played important role in promoting progression of tumor cell cycle [21,22]; Cyclin dependent kinases-1(CDK1) and cyclin B1 formed complex to regulate the progression of cell cycle, during the progression of cycle, the G2/M phase initiate and convert is depended the location and activity of CDK1/cyclin B1 complex [23,24]. Protein kinase Weel phosphorylated CDK1 site Tyr15 and Thr 14 to inactivate CDK1/cyclin B1 complex [24], when the mitosis start, Cdc25c dephosphorylation of the CDK1 site Tyr15 and Thr 14 to activate CDK1/cyclin B1 complex drive the progression of cell cycle [23,24]. In this study, the results showed that BITC could arrest G2/M phase in Bel7402 and HLE cells, and downregulate expression of cyclin B1, CDK1and Cdc25c, but upregulate expression of Weel, implicated that BITC arrested G2/M phase through regulating the expression of cell cycle related proteins.

Previously, we have found that AFP selectively inhibited caspases signal pathway caspase-3 molecule led to HCC cells resist apoptosis induced by tumor necrosis-related apoptosis ligand and all trans retinoic acid [18,10]. Silenced expression of AFP could induce expression of caspase-3 and promote apoptosis of HCC cells [25]. Recently, we found that AFP inhibited activity of PTEN to activate PI3K/AKT signal to stimulate proliferation and the malignant behaviours of HCC cells [12,26-29], documents evidence proved that PTEN was able to suppress the progression of tumor cell cycle [15-17].

These results indicated that expression of AFP played an important role in HCC cells resisting apoptosis induced by drugs, but whether AFP plays a role in BITC regulating the growth of HCC cells is still unclear. In this study, the results showed that BITC suppressed growth ratio of Bel 7402 cells lower than that in HLE cells, and silenced expression of AFP was able to increase sensitivity to BITC of Bel 7402 cells, however, the effect was opposite merged in HLE cells while transfected with AFP-expressed vectors. The results also indicated that AFP antagonized BITC regulated expression of cell cycle related proteins. These results implicated that AFP played a antagonistic role in BITC inhibiting growth and arresting HCC cell cycle.

Altogether, to our knowledge, this is the first time to report that BITC inhibited growth of HCC cells through arresting cells cycle in G2/M phase; AFP played a confront role in BITC restraining the proliferation and arresting cell cycle of HCC cells, AFP may be used as a novel biotarget for the therapy of HCC patients.

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