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Wild type p53 increased chemosensitivity of drug-resistant human hepatocellular carcinoma Bel7402/5-FU cells

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KEY WORDS hepatocellular carcinoma; 5-Fluorouracil; drug resistance; apoptosis; p53 genes; transfection

ABSTRACT

AIM: To study the effect of wild type (wt) p53 gene transfection on drug resistant human hepatocellular carcinoma (HCC) cells induced by 5-Fluorouracil (5-FU). **METHODS:** The cytotoxicity of anticancer drugs on Bel7402 and Bel7402/5-FU cells was assessed using SRB assay. p53 expression was detected at its mRNA level by RT-PCR assay and at its protein level Western blot or immunocytochemistry assay in Bel7402/5-FU cells transfected with either control vector or wt p53. AnnexinV-FITC/PI double labeled assay was performed to detect apoptosis. The chemosensitivity of Bel7402/5-FU cells transfected with wt p53 was assessed using SRB assay. **RESULTS**: Bel7402/5-FU cells exhibited cross-resistance to vincristine, doxorubicin, paclitaxel, and so on. wt p53 gene transfection upregulated the expression of p53 in Bel7402/5-FU cells. wt p53 was able to greatly inhibit cell proliferation and significantly induce apoptosis in Bel7402/5-FU cells. Moreover, wt p53 gene transfection increased the chemosensitivity of Bel7402/5-FU cells to some anticancer drugs. **CONCLUSION:** These results indicated that the wt p53 gene transfection not only induced suppression of cell growth, but also increased the sensitivity of Bel7402/5-FU cells to 5-FU, vincristine, and doxorubicin.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common human cancers in the world and there is a pronounced geographic variation in its incidence[1]. It is highly prevalent in Asia (China and Singapore) and Africa (Mozambique, Zimbabwe, and Ethiopia), and during recent years, its incidence has been on the rise in the western world [1]. HCC is the third and fourth most common cancer in Chinese men and women, respectively[3]. An estimated 260 000 new HCC cases will occur every year in the world, of which Chinese HCC cases accounts for 42.5 %[2,3].

The survival rate of hepatoma patients was very low. Surgery is considered the best option but unfortunately a majority of patients with HCC are not amenable to surgery at diagnosis. Cytotoxic chemotherapy is an integral part of the therapeutic approach for treatment of many of the solid tumors. 5-Fluorouracil (5-FU) still represents the most widely employed chemotherapeutic drug in the management of hepatoma[4-8]. However, because HCC is less sensitive or becomes resistant to anticancer drugs after consecutive treatments, most trials failed to find a therapy that produced responses higher than 25 % among hepatoma patients[7,8]. It is therefore clear that there is an urgent need to develop new approaches to treat drug-resistant

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HCC[9]. Gene therapy may offer a new therapeutic option for HCC[9]. The p53 gene is central in governing a tumor cell's decision to arrest and wait for alleviation of the deleterious condition, re-enter the cell cycle, or commit to apoptosis[10,11]. Tumor suppressor gene p53 may be a good candidate.

Bel7402/5-FU subline, which was the first cell line about the establishment of acquired resistance of 5-FU in HCC, was selected from HCC Bel7402 cells after long term exposure to 5-FU[12]. No study on the effect of wt p53 gene transfection in drug resistant HCC cells has been reported so far. In this article, we investigated whether drug-resistant HCC cells induced by 5-FU might be effectively treated with wt p53 gene transfection.

MATERIALS AND METHODS

Drugs 5-FU (Sigma), doxorubicin (Sigma), vincristine (Sigma), Paclitaxel (Beijing Four-Rings Pharmaceutical Science and Technology Co, Ltd), daunorubicin (Pharmacia & Upjohn Company), cytarabine (Shanghai Hualian Pharmaceutical Co, Ltd), methotrexate (Shanghai Hualian Pharmaceutical Co, Ltd), cisplatin (Qilu Pharmaceutical Factory) were purchased from the sources indicated. Above drugs were dissolved in 0.9 % sodium chloride.

Cell lines and cell cultures The human HCC cell line Bel7402 cells and the 5-FU selected drug-resistant Bel7402/5-FU cells (generously provided by Prof LIU Geng-Tao) were cultured in RPMI1640 (Gibco BRL, Life Technologies, Inc) supplemented with 10 % heat-inactivated fetal bovine serum in a humidified incubator at 37 ºC with 5 % CO_2. The cells grew as monolayers in tissue culture flasks and were subcultured approximately once every four days by 0.25 % trypsin plus 0.02 % edetic acid.

Analysis of cytotoxicity by sulforrodamine B (SRB) assay Growth inhibition of Bel7402 and Bel7402/5-FU cell lines was determined by using the modified SRB assay as described by Skehan *et al*[13]. Briefly, cells were seeded at a density of 5×10^3 cells/well in 96-well microplates. After 24 h, a serial dilution of anticancer drug solution were added in quadruplicate for each concentration. The cells were exposed to drugs for continuous 3 d. The culture medium was removed and trichloroacetic acid (10 %) was added for fixation. Then the plates were air-dried and 0.4 % SRB (sigma) in 1 % acetic acid were added for 30 min. Unbound dye was washed out with 1 % acetic acid. After air-drying, SRB dye within cells were dissolved with 150 µL solution of Tris-base 10 mol/L (pH 10.5). The optical density of the extracted SRB dye was measured with a microplate reader (model 550, Bio-rad) at 490 nm. The 50 % inhibitory concentration (IC_{50}) of the test drugs was calculated using a Probit analysis program. Chemosensitivity of Bel7402/5-FU cells transfected with either control vector or wt p53 was determined by SRB assay described above.

Preparation of pcDNA-wt p53 plasmid wt p53 cDNA fragment covering entire coding region (1.8 kb) was excised with BamHI from The pCMV-wt p53 plasmid (gifted by Bert Vogelstein) and inserted into the mammalian expression vecter pcDNA3.0 (Invitrogen). The correctness of insertion was confirmed by SmaI digestion and DNA sequencing done on an ABI-373A automated DNA sequencer.

Liposome-mediated transient and stable transfection Liposome (lipofectamine™2000, Gibco BRL) mediated transfection was carried out as recommended by the supplier with the following modifications. The transfection time was 8 h. In transient transfection, different concentrations of pcDNA-wt p53 plasmid or pcDNA3.0 control vector complexed with Liposome were used to detect the effect of wt p53 gene transfection on Bel7402/5-FU cells. According to the experiments, the ratio of plasmids-lipofectamine 3:2 was held constantly in the following experiments. The establishment and the selection of Bel7402/5-FU clones transfected stably with the pcDNA-wt p53 plasmid or pcDNA3.0 control vector was carried out as described previously[14]. G418 300 mg/L (Gibco BRL) was added to the cultures for selection. After 3 weeks, single, independent clones of cells were randomly isolated, and each clone was plated separately.

RNA extraction and semi quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis The total RNA were extracted with TRIzol reagent (Gibco BRL) according to the manufacturer's protocol. The concentration of RNA was determined spectrophotometrically (DU640, BECKMAN). RNA 5 µg was reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Invitrogen). Five percent of the cDNA produced was used as template for PCR. GAPDH was used as an internal positive control. The cycle number of PCR amplification was 30, which was chosen to ensure that amplification of all specific cDNA products was exponential. Specific primer sequences

of p53[15], or GAPDH[16] were as follows and the size of production were 604, or 309 bp, respectively:
p53 sense primer: 5'-ATTTGCGTGTGGAGTATTTG -3';
p53 antisense primer: 5'-GGAACAAGAAGTGGA-GAATG-3';
GAPDH sense primer: 5'-GGGAAGCTCACTGGCAT-GGCCTTCC-3';
GAPDH antisense primer: 5'-CATGTGGGCCATGAG-GTCCACCAC-3'.

All of the products were electrophoresed on 1.2 % agarose gel and stained with ethidium bromide. The expression intensities of two coamplified bands were quantified with Gel Doc 2000 system and Quantity One software (BIO-RAD), and expressed as a ratio (p53 *vs* GAPDH).

SDS-polyacrylamide electrophoresis and Western blot The total protein from cells was extracted as described previously[17]. Protein contents were determined with Bradford reagent. Equal amounts of protein (20 µg) were subject to 10 % SDS-PAGE and transferred to nitrocellulose membrane (Amersham UK). The membranes were subsequently preblocked in TBS containing 5 % nonfat milk power and then incubated with mouse monoclonal anti-p53 antibody (Santa Cruz Biotechnology, Inc) at dilution of 1:100 followed by peroxidase-conjugated goat anti-mouse IgG antibody (Santa Cruz Biotechnology, Inc). The antigen-antibody complex was visualized with Western blotting luminol reagent (ECL, Santa Cruz Biotechnology, Inc). The bands were quantified with Gel Doc 2000 system and Quantity One software (BIO-RAD).

Analysis of apoptosis by annexin staining Cells were each seeded at a density of 5×10^4 cells/ml in 25 cm^2 culture flasks and incubated for 24 h in a CO_2 incubator. Cells were incubated for 8 h in the presence or absence of plasmid (3 µg)-liposome (2 µg) complex in 2.5 µL of serum- and antibiotic-free DMEM. The culture medium was replaced after 8 h by complete medium and each plate was incubated for an additional 48 h. Then, Apoptosis was determined by an Annexin-V apoptosis detection kit (Baosai Biotech) according to the manufacturer's protocol. Cells were analyzed by flow cytometry with FACScan (Becton-Dickinson) and analysis software CELLQest (Becton-Dickinson).

Immunocytochemistry assay To identify wt p53 stable transfectant, the streptavidin-biotin complex immunoperoxidase technique was performed with p53 immunohistochemical kit (Zhong Shan Biotechnology Co, Ltd) according to the manufacturer's protocol.

Statistical analysis Data were expressed as mean±SD and comparisons were done by Independent Sample *t* test. All statistical analysis were done in SPSS version 10.0, $P<0.05$ was considered to be statistically significant.

RESULTS

Determination of cross resistance When cytotoxicity assay was performed, it was found that Bel7402/5-FU cells were resistant not only to 5-FU but also to some other anticancer drugs. Here, we have tested 5-FU, vincristine, doxorubicin, paclitaxel, daunorubicin, cytarabine, methotrexate, and cisplatin. The IC_{50} values of some drugs to Bel7402/5-FU increased significantly when compared with non-resistant Bel7402 cells (Tab 1).

Tab 1. Determination of IC_{50} of different anticancer drugs to Bel7402 and Bel7402/5-FU cells. n=4. Mean±SD. Relative resistance (RR)=$IC_{50\ Bel7402/5\text{-}FU}$ /$IC_{50\ Bel7402}$.

Anticancer drugs	IC_{50}/µmol·L^{-1} Bel7402	Bel7402/5-FU	RR
5-Fluorouracil	2.32±0.18	788±173	339.7
Doxorubicin	0.093±0.006	1.25±0.13	13.4
Vincristine	0.025±0.005	0.29±0.08	11.6
Paclitaxel	0.033±0.015	0.83±0.05	25.2
Daunorubicin	1.37±0.24	6.5±1.5	4.7
Cisplatin	17±5	29±20	1.7
Methothexate	0.027±0.003	0.0348±0.0012	1.3
Cytarabine	14±8	80±29	5.7

Recombinant pcDNA-wt p53 gene transfection After construction of pcDNA-wt p53 expression vector, the correctness of insertion was confirmed by enzyme digestion and DNA sequencing (data not shown). Bel7402/5-FU cells were transfected *in vitro* with pcDNA3.0 or pcDNA-wt p53 complexed with lipofectamine. RT-PCR and western blot analysis were performed to determine whether the transfected p53 gene was expressed in Bel7402/5-FU cells. Bel7402/5-FU cells transfected with pcDNA-wt p53 gene expressed a significant amount of amplification product for p53 mRNA compared to transfected with control pcDNA3.0 vector (Fig 1). Bel7402/5-FU cells transfected with pcDNA-wt p53 gene increased significantly in p53 protein expression, compared to transfected with control

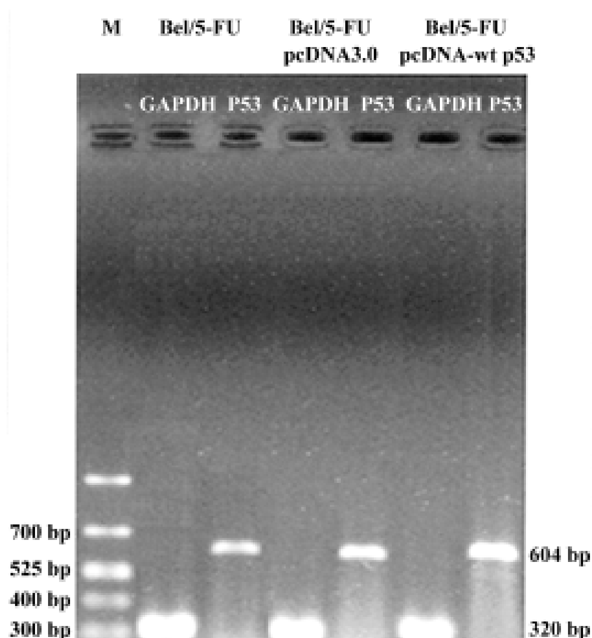

Fig 1. Expression of p53 mRNA in Bel7402/5-FU cells untransfected and transiently transfected with control vector pcDNA3.0 or pcDNA-wt p53.

pcDNA3.0 vector (Fig 2). These data suggest that efficient p53 gene expression was obtained in Bel7402/5-FU cells.

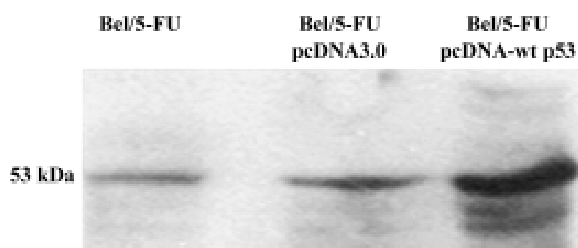

Fig 2. Protein expression of P53 in Bel7402/5-FU cells untransfected and transiently transfected with either control vector pcDNA3.0 or pcDNA-wt p53.

Effect of wt p53 on Bel7402/5-FU cell growth Different concentrations of pcDNA-wt p53 plasmid or pcDNA3.0 control vector complexed with lipofectamine™2000 (2 mg/L) were used to detect the effect of wt p53 gene transfection on Bel7402/5-FU cells. Tab 2 showed that four concentrations of (0.8, 1.6, 3.2, or 6.4 mg/L) could significantly inhibit Bel7402/5-FU cell proliferation when compared with pcDNA3.0 control. pcDNA-wt p53 doses as high as 3.2 mg/L were extremely toxic to Bel7402/5-FU cells with 69 % growth inhibition. These results showed optimal ratio of plasmid DNA to lipofectamine™2000 for transfection was about 3:2 for Bel7402/5-FU cells.

Tab 2. Cytotoxic effect of wt p53 gene transfection on Bel7402/5-FU cell growth for 48 hr. n=3. lipofectamine™ 2000 2 mg/L. Mean±SD. [b]P<0.05 *vs* Bel7402/5-FU pcDNA3.0 cells. [c]P<0.01 *vs* Bel7402/5-FU pcDNA3.0 cells.

Plasmid concentration/ $mg \cdot L^{-1}$	Cell viability ratio/% Bel7402/5-FU pcDNA3.0	Bel7402/5-FU pcDNA-wt p53
0	100±6	100.0±2.2
0.4	88±14	85±6
0.8	84±6	74±3[b]
1.6	89±8	65±5[b]
3.2	90±11	31.0±2.0[c]
6.4	86.9±2.2	57±7[c]

Apoptosis The annexin staining was used to determine the effect on early stage apoptosis 2 d after transfection. Control vector transfection did not induce increase of early stage apoptosis in Bel7402/5-FU cells, and wt p53 gene transfection induced significant early stage apoptosis in Bel7402/5-FU cells (Fig 3).

Identification of stably wt p53-expressing clone To investigate the effect of wt p53 expression on the chemosensitivity of Bel7402/5-FU, Bel7402/5-FU cells were stably transfected with pcDNA-wt p53 plasmid or pcDNA3.0 control vector. Western blot analysis (Fig 4) and immunohistochemistry staining (Fig 5) showed the level of p53 protein was increased significantly in pcDNA-wt p53 stably transfected Bel7402/5-FU cells, compared with control pcDNA3.0 stably transfected cells. These results demonstrated stably p53 expressing clone was successfully generated.

Sensitivity to 5-FU, vincristine, doxorubicin, and paclitaxel The chemosensitivity of Bel7402/5-FU cells transfected stably with wt p53 gene was assessed using SRB assay (Tab 3). IC_{50} of Bel7402/5-FU cells to 5-FU, vincristine, and doxorubicin decreased 49.5 %, 34.7 %, and 41.8 %, respectively $[(IC_{50\ Bel7402/5\text{-}FU\ pcDNA3.0} - IC_{50\ Bel7402/5\text{-}FU\ pcDNA\text{-}wtp53})/IC_{50\ Bel7402/5\text{-}FU\ pcDNA3.0} \times 100\ \%]$ when Bel7402/5-FU cells were transfected with pcDNA-wt p53 gene. This showed that wt p53 gene increased the chemosensitivity of Bel7402/5-FU cells to some anticancer drugs.

DISCUSSION

A major obstacle in the treatment of malignant tu-

(% *vs* Lipofectin + AngII) (% *vs* Lipofectin + AngII)

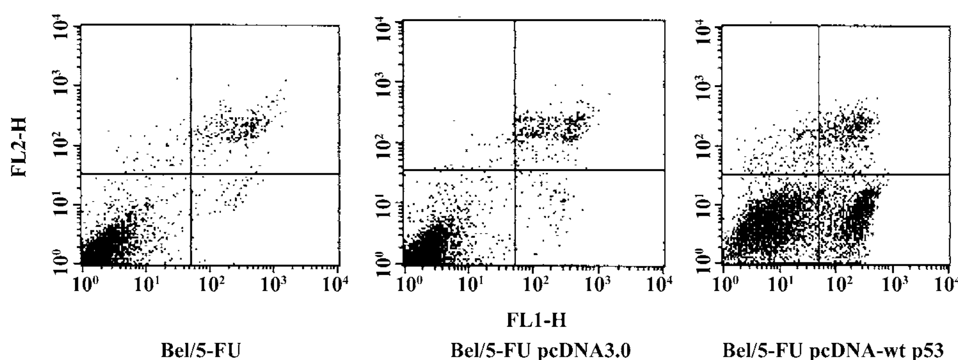

Fig 3. Evaluation of early stage apoptosis by annexin V-FITC/PI staining and analysis by Flow Cytometry in Bel7402/5-FU cells untranfected and transfected with either control vector pcDNA3.0 or pcDNA-wt p53.

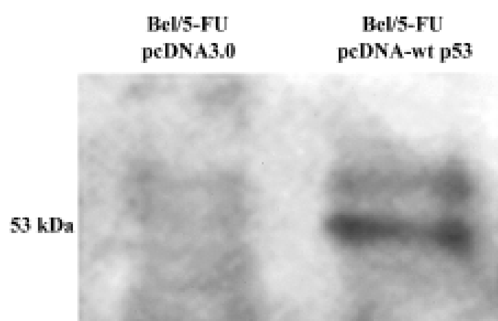

Fig 4. Expression of P53 protein in Bel7402/5-FU cells stably transfected with control vector pcDNA3.0 or pcDNA-wt p53.

Tab 3. Cytotoxicity of some anticancer drugs on Bel7402/5-FU cells stably transfected with either control vector pcDNA3.0 or pcDNA-wt p53. *n*=4. Mean±SD. ^{b}*P*<0.05 *vs* Bel7402/5-FU pcDNA3.0 cells. ^{c}*P*<0.01 *vs* Bel7402/5-FU pcDNA3.0 cells.

Anticancer drugs	IC_{50}/μmol·L^{-1} Bel7402/5-FU pcDNA3.0	Bel7402/5-FU pcDNA-wt p53
5-Fluorouracil	776±102	392±62 ^{c}
Doxorubicin	1.01±0.22	0.66±0.07 ^{b}
Vincristine	0.22±0.04	0.128±0.012 ^{c}
Paclitaxel	0.899±0.019	0.76±0.10

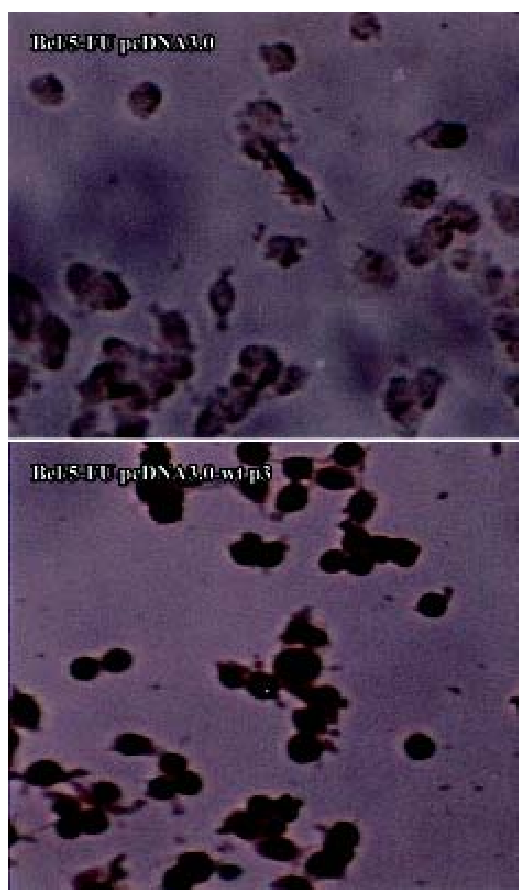

Fig 5. Immunocytochemical staining for the expression of P53 in Bel7402/5-FU cells stably transfected with control vector pcDNA3.0 or pcDNA-wt p53.

mor with conventional anti-cancer agents is the deveopment of drugs resistance and tumor cells resistant to single agents usually become cross-resistant to several other anti-cancer drugs. To date there is no effective single agent or polychemotherapeutic regimen for the treatment of resistant hepatocellular carcinoma which is the leading cancer in Chinese[7,8]. The hypothesis that failure to undergo apoptosis contributes to the development of resistance to anticancer agents has been the subject of extensive research[18,19]. The susceptibility of cells to apoptosis induction appears to be regulated by tumor suppressor gene p53[20,21], Bcl-2 family, as well as other molecules involved in apoptotic pathway. p53 acts as a checkpoint protein to suppress cell growth

by inhibiting G_1 progression to the S phase while the cell attempts to repair the damage, or to promote apoptosis in cells that fail to repair[10,11].

So far, there is no paper studying the effect of p53 on drug- resistant HCC induced by 5-FU. In the present experiments, we investigated the effect of exogenous p53 on 5-FU resistant cell line derived from Chinese hepatocellular carcinoma cells. Bel7402/5-FU cells exhibited not only strong resistant to 5-FU but also cross-resistant to doxorubicin, vincristine, paclitaxel, and so on. The transfection process was mediated by liposome which would facilitate the binding of DNA vector to the cell membrane and hence their uptake. We checked the expression of pcDNA-wt p53 plasmid in Bel7402/5-FU cells. The results demonstrated p53 was overexpressed, indicating that this plasmid was functional in expression in Bel7402/5-FU cells. The results of SRB showed that wt p53 could significantly inhibit Bel7402/5-FU cell proliferation in dose-dependent way. The annexin staining assay data indicated that wt p53 significantly induced apoptosis in HCC Bel7402/5-FU cells. Consistent with our results, Seth and his colleagues had shown that wt p53 induced higher apoptosis in drug-resistant human breast cancer cells[22]. These findings suggested that wt p53 gene therapy might be more effective for some drug- resistant tumors.

It had been reported that wt p53 sensitized breast carcinoma, soft tissue sarcoma, and colorectal carcinoma cells to chemotherapeutic agents, such as doxorubicin, 5-FU, and topotecan[22-24]. However, other studies indicated that the inactivation of p53 enhanced sensitivity to multiple anticancer drugs[25,26]. When we attempted to detect the sensitivity of stably wt p53-expressing clones to some anticancer drugs, we found p53 expression reduced Bel7402/5-FU cells resistance to 5-FU, vincristine, and doxorubicin. According to these conflicting results, it is conceivable that the role of p53 in tumor cell chemosensitivity is decided by tissue-specific differences and/or treatment regimen. The combination of p53 gene therapy and chemotherapy might result in synergy and increase the therapeutic efficacy in the treatment of patients with drug resistance HCC induced by 5-FU. These findings have important implications with respect to cancer chemotherapy. When the resistance of HCC Bel7402/5-FU cells to anticancer drugs reduced, smaller doses of drugs can be applied with greater effects. Therefore, this treatment can reduce the side effects of anticancer drugs.

In conclusion, this study demonstrated that wt p53 gene transfection resulted in the potentiation of cytotoxicity and apoptosis in Bel7402/5-FU cells. Moreover, p53 gene therapy combined with some anticancer agents might result in better inhibition of drug resistant HCC cells growth. Although *in vivo* studies are needed, these findings suggest that manipulation of tumor suppressor gene p53 is feasible and synergistic to some anticancer drugs, providing a way to treat, at least partly, the drug resistant HCC induced by 5-FU.

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