RESEARCH ARTICLE

Characterization and Resistance Mechanisms of A 5-fluorouracil-resistant Hepatocellular Carcinoma Cell Line

Wei Gu, Fan-Fu Fang, Bai Li, Bin-Bin Cheng, Chang-Quan Ling*

Abstract

    Purpose: The chemoresistance of human hepatocellular carcinoma (HCC) to cytotoxic drugs, especially intrinsic or acquired multidrug resistance (MDR), still remains a major challenge in the management of HCC. In the present study, possible mechanisms involved in MDR of HCC were identified using a 5-fluorouracil (5-FU) -resistant human HCC cell line. **Methods:** BEL-7402/5-FU cells were established through continuous culturing parental BEL-7402 cells, imitating the pattern of chemotherapy clinically. Growth curves and chemosensitivity to cytotoxic drugs were determined by MTT assay. Doubling times, colony formation and adherence rates were calculated after cell counting. Morphological alteration, karyotype morphology, and untrastructure were assessed under optical and electron microscopes. The distribution in the cell cycle and drug efflux pump activity were measured by flow cytometry. Furthermore, expression of potential genes involved in MDR of BEL-7402/5-FU cells were detected by immunocytochemistry. **Results:** Compared to its parental cells, BEL-7402/5-FU cells had a prolonged doubling time, a lower mitotic index, colony efficiency and adhesive ability, and a decreased drug efflux pump activity. The resistant cells tended to grow in clusters and apparent changes of ultrastructures occurred. BEL-7402/5-FU cells presented with an increased proportion in S and G2/M phases with a concomitant decrease in G0/G1 phase. The MDR phenotype of BEL-7402/5-FU might be partly attributed to increased drug efflux pump activity via multidrug resistance protein 1 (MRP1), overexpression of thymidylate synthase (TS), resistance to apoptosis by augmentation of the Bcl-xl/Bax ratio, and intracellular adhesion medicated by E-cadherin (E-cad). P-glycoprotein (P-gp) might play a limited role in the MDR of BEL-7402/5-FU. **Conclusion:** Increased activity or expression of MRP1, Bcl-xl, TS, and E-cad appear to be involved in the MDR mechanism of BEL-7402/5-FU.

Keywords: Hepatocellular carcinoma - multidrug resistance - 5-fluorouracil - ATP-binding cassette transporters

Introduction

    Hepatocellular carcinoma (HCC) is currently the sixth most common type of cancer and the third leading cause of cancer-related death in the world. More than 747000 new cases of HCC and 695000 deaths were estimated to have occurred in 2008. In China, more than 401000 new patients (53.7% of the world) are diagnosed with HCC and more than 371000 patients (53.4% of the world) are killed by the terrible disease annually (Ferlay et al., 2010). Even worse are approximately 80% of patients diagnosed initially with an advanced stage where the lesions were more often unresectable. Furthermore, nearly half of patients who underwent hepatectomy would have a recurrent or metastatic disease within two years. Therefore, effective therapeutic approaches are urgently desired for patients with HCC (El-Serag, 2011; Livraghi et al., 2011).

    Chemotherapy with cytotoxic drugs (fluoropyrimidines, anthracyclines, platinum complexes, etc.) plays an essential role in the management of advanced HCC

(Wrzesinski et al., 2011). Particularly, 5-fluorouracil (5-FU) -based regimens have shown encouraging advantages in tumor response and survival for advanced HCC patients recently (Frustaci et al., 2003; Qin et al., 2010). However, the overall response of HCC to chemotherapeutics still remains rather low. The chemoresistance of HCC cells, especially multidrug resistance (MDR), either intrinsic or acquired, still remains a major challenge and difficult problem in the management of HCC (Marin et al., 2010; Ryu and Chung, 2010). In the present study, to elucidate the mechanism of MDR in HCC, a 5-FU-resistant human HCC cell line with MDR phenotype was established as closely as possible to the MDR actually happened in clinical setting. The biological characteristics and possible mechanisms involved in MDR were identified.

Materials and Methods

Drugs and reagents
    Five-FU, adriamycin (ADM), methotrexate (MTX),

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vincristine (VCR), and oxaliplatin (L-OHP) were obtained from Shanghai Xudong Haipu Pharmaceutical Co., Shenzhen Wanle Pharmaceutical Co., Shanghai Hualian Pharmaceutical Co., Zhejiang Haizheng Pharmaceutical Co., China and Sanofi-Aventis, France, respectively. The ready-to-use antibodies against thymidylate synthase (TS), multidrug resistanec protein 1 (MRP1), P-glycoprotein (P-gp) were purchased from Fuzhou Maxim Biotech Ltd. (Fuzhou, Fujian, China). Mouse monoclonal antibodies against human E-cadherin (E-cad), B-cell lymphoma-extra large (Bcl-xl), and Bax were purchased from Sigma-Aldrich (St Louis, MO, USA).

Cell line and cell culture

A well-established Chinese human hepatocellular carcinoma, BEL-7402 (Chen et al., 1980), were obtained from the Chinese Academy of Sciences (Shanghai, China) and cultured in RPMI-1640 (GIBCO), supplemented with 10% fetal bovine serum (FBS, Sijiqing, China) at 37 °C in a humidified atmosphere containing 5% CO_2.

Induction of the 5-FU-resistant HCC cell line BEL-7402/5-FU

The BEL-7402 cells were cultured with 0.1 μmol/L of 5-FU for five days, and after the removal of dead cells (floating cells) and culture medium containing 5-FU, the surviving cells (attached to flask) were cultured continuously in RPMI-1640 medium supplemented with 10% FBS free of 5-FU. When a confluence of 60%-70% has been reached, the cells were exposed to a pulse selection with 10 μmol/L 5-FU for 24 hours. After the surviving cells restored to exponential growth without pressure of 5-FU, the cells were cultured with 1 μmol/L 5-FU until the cells could grow and passage steadily. The above-mentioned induction pattern with low concentrations of 5-FU in a gradually increasing manner combined with intermittent exposure to high concentrations of 5-FU were performed repeatedly. After an induction of 10 months, a 5-FU-resistant HCC cell line BEL-7402/5-FU was established. The resistant cells was then cultured without 5-FU for three passages and frozen in the liquid nitrogen. Freeze-stored cells were recovered three passages later for the identification of biological characteristics and following experiments.

Cell viability assay

The cell viability and cytotoxicity of 5-FU, ADM, MTX, VCR, and L-OHP were determined by using 3-[4,5-Dimethyl-2-thiazol]-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. Cells in the exponential growth phase following trypsinization were seeded in 96-well plates at a density of 1×10^4 per well and incubated for 12 hours. Thereafter, six different concentrations for each drug mentioned above were added and six wells were used for each determination. Medium without drug was added to the control and blank wells. After an incubation of scheduled time, 10 μl MTT (Amresco, MA, USA) solutions with a concentration of 5 mg/ml were added to each well for 4 hours at 37 °C. Then culture medium was removed and the insoluble formazan crystals were dissolved in 150 μl of dimethyl sulfoxide (DMSO). The absorbance (optical density, OD) was measured at 490 nm of the wave length using a microplate reader. The cell growth inhibition rate calculated using the following formula: growth inhibition rate = (OD value of control - OD value of experiment)/(OD value of control - OD value of blank)×100%. The 50% inhibitory concentration (IC_{50}) was estimated by Probit analysis. Resistance indices (RIs) were determined by the ratio of the IC_{50} values of BEL-7402/5-FU to BEL-7402 cells.

Population doubling time

Cell density was adjusted for 1×10^5/ml, and seeded at 10 ml per flask. Cells were cultured in a humidified atmosphere of 5% CO_2 at 37 °C for 100 hours. The cells were then trypsinized and counted by trypan blue exclusion. Cell doubling time (Td) was calculated according to the formula: $Td = t \times [\lg 2/(\lg N_t - \lg N_0)]$, where N0 is the number of cells inoculated, Nt is the number of cells harvested, and t is the culture time in hours (Dai et al., 2007).

Colony formation assay

Cells were trypsinized, counted, and seeded into six-well plates at 400 cells per well. Plates were incubated at 37 °C in 5% CO_2 for nine days. Cells were then fixed for 15 minutes with 95 % ethanol and cell colonies (≥60 cells) were counted under microscopy and the colony formation rate was calculated as (colony counts)/(cells inoculated) ×100%.

Adherence analysis

Cell density was adjusted for 2×10^5/ml, and seeded at 5 ml per flask. Aliquots of cells were cultured in a humidified atmosphere of 5% CO_2 at 37 °C and one of them was taken to trypsinization and counting every hour from the 1st to 6th hour. The adherence rate was calculated as the ratio of adherent cells to inoculated cells.

Morphological observation

Exponentially growing cells were observed under the inverted light microscopy (Olympus, Japan). For transmission electron microscopy, cells were trypsinized, washed, collected, and fixed with 3% glutaric dialdehyde for 2 hours and refixed with 1% osmium tetroxide for 2 hours. The cells were dehydrated with increasing concentrations of ethanol from 50% to 100% and anhydrous acetone. The cells were then embedded in epoxy resin and sections were cut on a LKB Nova Ultramicrotome (LKB, Bromma, Sweden). Ultrathin sections (50 nm) were stained with uranyl acetate and lead citrate and examined in a Tecnai 12 BioTWIN electron microscopy (FEI Co., The Netherlands).

Karyotype analysis

For cytogenetic analysis, cells in an exponential growth phase were synchronized and blocked with 0.4 μg/ml colchicines (Sigma) for 3 hours, harvested with 0.25% trypsin, treated with hypotonic solution (0.075 mol/L KCl) for 8 minutes at 37 ºC, and fixed with 3:1 methanol/acetic acid. Slides were submitted to standard trypsin-Giemsa banding (Yunis, 1981). The results

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were evaluated according to the International System for Human Cytogenetic Nomenclature (Shaffer and Tommerup, 2005).

Flow cytometry analysis for cell cycle

BEL-7402 and BEL-7402/5-FU cells were seeded into six-well plates at a density of 2×10^5 cells per well and then incubated for 24 hours. Cells were trypsinized, pelleted by centrifugation, washed twice with cold phosphate buffered saline (PBS) and adjusted to 1×10^6 cells/ml. Cells were then fixed by suspending the cells in ethanol at 4 °C overnight. After washing and centrifugation, cells were incubated with 400 μl propidium iodide (50 μg/ml, Sigma) and 10 μl DNase-free RNase (1 mg/ml, Sigma) in PBS in the dark for 30 minutes. The 2×10^4 cells were measured using a FACScan flow cytometer (BD Biosciences, San Jose, CA, USA), and the data were analyzed using Modfit LT for Mac version 3.0 software (Verity Software House, Topsham, ME).

Intracellular ADM accumulation assay

Intracellular accumulation of ADM was determined by flow cytometry as an index of drug efflux pump activity (Durand and Olive, 1981). Cells were plated in six-well plates with a concentration of 1×10^5/ml in a volume of 2 ml per well. After an incubation of 36 hours, ADM was added to a final concentration of 20 μg/ml for 2 hours at 37 °C. Cells were harvested with trypsin after twice washes with cold PBS, resuspended and subsequently subjected to flow cytometry with excitation measured at 488 nm and emission measured at 575 nm. The data of 2×10^4 cells were analyzed using CellQuest software (BD Biosciences, San Jose, CA, USA).

Rhodamine 123 retention assay

Intracellular retention of rhodamine 123 (Rh-123) was determined by flow cytometry as a functional index of P-gp activity (Pétriz and García-López, 1997). Cells were plated in six-well plates in a concentration of 1×10^5/ml in 2 ml per well. After an incubation of 36 hours, Rh-123 (Sigma) was added to a final concentration of 0.25 μg/ml for 30 minutes at 37 °C. Cells were harvested with trypsin after twice washes with cold PBS, resuspended with 400 μl PBS and immediately used for flow cytometric analysis of rhodamine fluorescence at an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

Fluorescein accumulation assay

Intracellular accumulation of fluorescein (FLU) was measured by flow cytometry as a functional index of MRP1 activity (Huai-Yun et al., 1998). Cells were plated in six-well plates in a concentration of 1×10^5/ml in 2 ml per well. After an incubation of 36 hours, FLU (Sigma) was added to a final concentration of 100 μmol/L for 3 hours at 37 °C. Cells were harvested with trypsin after twice washes with cold PBS and resuspended rapidly with 400 μl PBS, and intracellular fluorescence of FLU was immediately measured with flow cytometry at an excitation wavelength of 488 nm and an emission wavelength of 520 nm.

Immunocytochemistry and evaluation

Cells grown on coated glass coverslips in a six-well plate with a concentration of 2×10^5/ml in a volume of 2 ml per well for 24 hours. The cells on coverslips were fixed with 4% paraformaldehyde for 20 minutes, permeabilized by 1% Triton X-100 for 30 minutes, and incubated sequentially for 1 hour at 37 °C with primary antibody against human TS (ready-to-use), MRP1 (ready-to-use), P-gp (ready-to-use), E-cad (1:100), Bcl-xl (1:100), and Bax (1:100) respectively. The immunocytochemistry was visualized according to protocol of Envision System Horseradish Peroxidase kits (DAKO Corp., Carpinteria, CA). The expression of the six proteins was represented as positive indices (PIs) which were analyzed semi-quantitatively by an IMS cell image system (Shanghai Shenteng Information Technology Co. Ltd., China). Six fields were assessed randomly under a low power view (×100) and the PIs were calculated as positive area rate × average optical density.

Statistical analysis

Each experiment was performed at least in triplicate, and the measurements were performed in three independent experiments. Data are expressed as means ± standard deviation (SD). Student's t-test was used to compare the means of two groups. $P<0.05$ was considered statistically significant. All analyses were performed using SPSS 13.0 statistical packages (SPSS Inc., Chicago, IL).

Results

Establishment and MDR phenotype of BEL-7402/5-FU cells

After a continuous induction with low concentration of 5-FU in a gradually increasing manner combined with intermittent exposure to high concentration of 5-FU for 10 months, the BEL-7402/5-FU cells could grow and passage steadily in medium containing 50 μmol/L 5-FU. BEL-7402/5-FU cells represented a typical MDR phenotype compared to its parental cell BEL-7402. It was not only refractory to 5-FU with a resistance index of 18, but also cross-resistant to ADM, VCR, MTX, and L-OHP (Table 1).

Table 1. Multidrug Resistant Phenotype of BEL-7402/5-FU Cells Compared to Its Parental BEL-7402 Cells

Drugs	IC_{50} at 48 hours (μmol/L)[a]		RI^b	P^c
	BEL-7402	BEL-7402/5-FU		
5-FU	23.7±9.4	427.6±110.5	18	0.003
ADM	1.3±0.3	2.8±0.6	2.1	0.018
VCR	0.9±0.3	8.8±2.5	9.8	0.006
L-OHP	43.2±12.3	7.7±3.6	5.6	0.009
MTX	60.9±29.4	497.2±100.4	8.2	0.002

5-FU, 5-fluorouracil; ADM, adriamycin; MTX, methotrexate; VCR, vincristine; L-OHP, oxaliplatin; IC50, 50% inhibitory concentration; SD, standard variation; RI, resistance index; [a]The IC50 values were presented as mean ± SD; [b]RIs were determined by the ratio of IC50 values of BEL-7402/5-FU to that of BEL-7402 cells; [c]P values were calculated by Student's t-test between the IC50 values of both cell lines
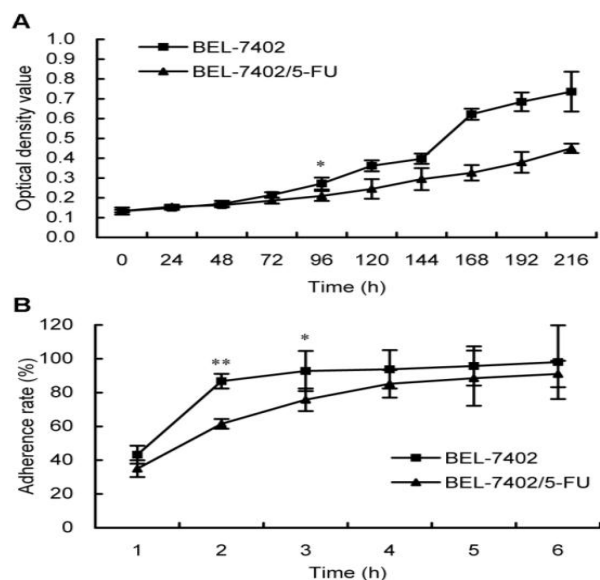
Figure 1. Growth and Adherence Curves of BEL-7402 and BEL-7402/5-FU. (A) The BEL-7402/5-FU cells began to grow slower than BEL-7402 cells from the 48th hour of incubation, and the difference of growth speed between the two cell lines was significant after 96 hours (*P<0.05). (B) Both BEL-7402 and BEL-7402/5-FU cell lines had gradually increasing adherence rates from the 0 to 6th hour after incubation. The adherence rate of BEL-7402/5-FU was always lower than that of BEL-7402, but significant difference was found only at the 2nd (**P<0.001) and the 3rd hour (*P<0.05)

Cell growth and doubling time analysis

The growth curves of BEL-7402/5-FU cells and their parental cells are shown in Figure 1A. From the 48th hour of incubation, the BEL-7402/5-FU cells began to grow slower compared with BEL-7402 and the difference of growth speed became significant after 96 hours (P<0.05). The population doubling time for BEL-7402/5-FU was 42.3±3.3 hours which was prolonged significantly than that of BEL-7402 cells (31.2±1.1 hours, P<0.001).

Adherence rate analysis and colony efficiency

As far as adherence rate was concerned, both BEL-7402/5-FU and BEL-7402 cells had gradually increasing adherence rates from the 0 to 6th hour after inoculation. As shown in Figure 1B, the adherence rate of BEL-7402/5-FU was always lower than that of BEL-7402, but significant difference was found only at the 2nd (P<0.001) and the 3rd hour (P<0.05). The colony formation rate of BEL-7402/5-FU was 53.25%, which was significantly decreased compared to a colony formation rate of 69.08% for BEL-7402 after a culture of nine days (P<0.05).

Morphological changes of BEL-7402/5-FU compared with BEL-7402

Microscopic observation found BEL-7402 cells were relatively uniform in size and shape in monolayer culture (Figure 2A). However, BEL-7402/5-FU cells lost contact inhibition, tended to grow in clusters and arranged irregularly (Figure 2B). The cells often represented as fusiform or oval forms and were more sensitive to trypsin digestion but hard to be monodispersed compared to BEL-7402. Electron microscopy revealed that the parental BEL-7402 cells, represented as polygonal form, were
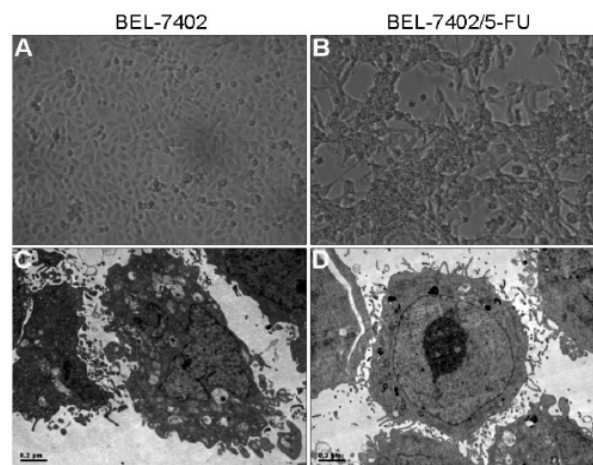
Figure 2. Morphological Observations of BEL-7402 and BEL-7402/5-FU. (A) BEL-7402 cells were relatively uniform in size and shape in monolayer culture. (B) BEL-7402/5-FU cells tended to grow in clusters and arranged irregularly and often represented as fusiform or oval forms. (C) Representative electron microscopy image of BEL-7402 cells, which are usually polygonal with a few microvilli and protuberance on cell surface, big and irregular nuclei composing big and more nucleolus, loose chromatin structure, simple cellular organs and small mitochondria. (D) Representative electron microscopy image of BEL-7402/5-FU cells, which usually are fusiform or oval with numerous microvilli and protuberance on cell surface

characterized with a few microvilli and protuberance on cell surface, big and irregular nuclei composing big and more nucleolus. Loose chromatin structure, simple cellular organs, and small mitochondria were also noticed in BEL-7402 cells (Figure 2C). However, the resistant cells represented as fusiform or oval forms, and characterized with more microvilli and protuberance on cell surface, and numerous microvilli (Figure 2D). No other apparent differences were found in cell ultrastructures.

Karyotype analysis

Possible chromosomal changes during the establishment of BEL-7402/5-FU were determined by karyotype analysis. The results indicated BEL-7402/5-FU cells showed a lower mitotic index than its parental cells. However, both cells showed aneuploid chromosome pattern and no obvious differences were found in the numbers and morphology of chromosomes between them.

Cell cycle distribution

Flow cytometry showed the proportion of cells in G0/G1, S and G2/M phases were 61.4%, 25.5%, and 13.1% for BEL-7402; and 48.3%, 31.7% and 20.0% for BEL-7402/5-FU, respectively. The BEL-7402/5-FU cells exhibited a longer S and G2/M phases with a concomitant shorter G0/G1 phase in cycle distribution than BEL-7402 cells (Figure 3).

Efflux pump activity analysis

The efflux pump activity of both cell lines was compared by quantification of intracellular ADM fluorescence. As shown in Figure 4, the intracellular ADM intensity of BEL-7402/5-FU decreased significantly compared to BEL-7402 (P<0.001), which indicated an
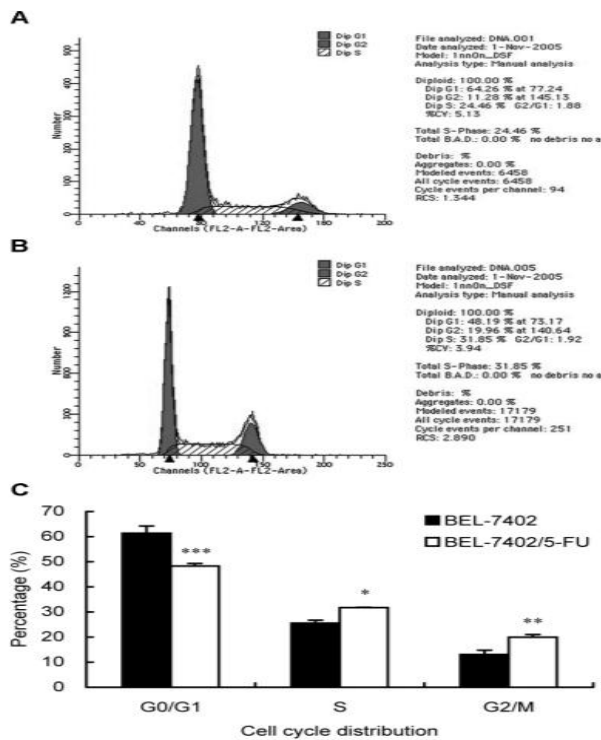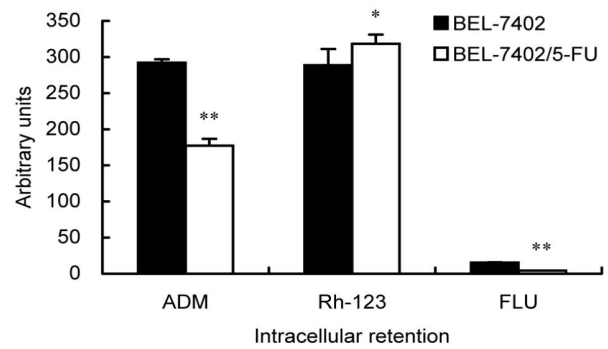
Figure 3. Cell Cycle Distribution of BEL-7402 and BEL-7402/5-FU Revealed by Flow Cytometry. The proportion of BEL-7402/5-FU cells in the S and G2/M phases were increased significantly compared with BEL-7402 cells (*P=0.001, **P=0.004), with a concomitant reduction in G0/G1 phase significantly (***P=0.002)

Figure 4. Efflux Pump Activity Analysis. The drug efflux pump activity, P-gp activity, and MRP1 activity of BEL-7402 and BEL-7402/5-FU cells were compared by quantification of intracellular ADM fluorescence, retention of Rh-123, and accumulation of FLU, respectively. The intracellular ADM intensity of BEL-7402/5-FU decreased significantly compared to BEL-7402 (**P<0.001). The Rh-123 intensity in BEL-7402/5-FU cells was slightly higher than that in BEL-7402, but no significant difference was found between them (*P=0.118). The intracellular accumulation of FLU was greatly decreased in BEL-7402/5-FU cells compared to its parental cells (**P<0.001)
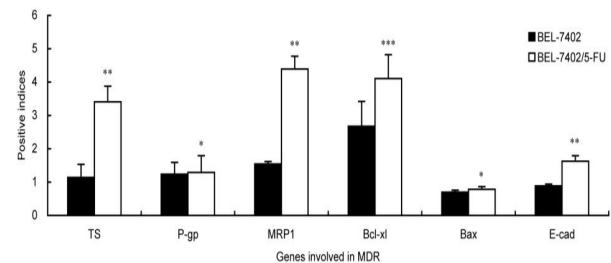
Figure 5. Immunocytochemistry of TS, P-gp, MRP1, Bcl-xl, Bax, and E-cad in BEL-7402 and BEL-7402/5-FU Cells. The positive indices of the six genes quantified by IMS system showed that expressions of TS, MRP1, Bcl-xl, and E-cad in BEL-7402/5-FU cells were significantly upregulated compared to its parental cells (**P<0.001, ***P=0.006), but the expression of P-gp and Bax in the resistant cell line were equal to that in BEL-7402 cells (*P>0.05)

enhanced efflux pump activity was acquired by BEL-7402/5-FU cells. Although the Rh-123 intensity in BEL-7402/5-FU cells was slightly higher than that in BEL-7402 cells, no significant difference was found between them (P>0.05). The results suggested that the activity of P-gp might play little role in the acquired MDR of BEL-7402/5-FU. Reversely, the intracellular accumulation of FLU, a functional index of MRP1 activity, was greatly decreased in BEL-7402/5-FU cells compared to its parental cells (P<0.001) which indicated that activity of MRP1 were markedly enhanced during acquisition of MDR phenotype. TS, MRP1, Bcl-xl, and E-cad involved in MDR of BEL-7402/5-FU

Immunocytochemistry were performed to determine the expression of six genes potentially involved in MDR of BEL-7402/5-FU. Staining results found that TS, P-gp, MRP1, Bcl-xl, Bax, and E-cad were all expressed with different intensity on the cell surface or in the cytoplasm of BEL-7402 and BEL-7402/5-FU cells. The PIs of the six genes quantified by IMS system are reported in Figure 5. There were significantly upregulated expressions of TS, MRP1, Bcl-xl, and E-cad in BEL-7402/5-FU cells compared to its parental cells (P<0.01), but the expression of P-gp and Bax in the resistant cells were equal to that in BEL-7402 cells (P>0.05). In addition, the Bcl-xl/Bax ratio towards apoptosis was significantly augmented accordingly (data no shown).

Discussion

MDR in cancer is a condition in which cancer cells are resistant to distinct anticancer drugs of a wide variety of structure and function targeted at cancer cells. The MDR phenotype of cancer cells might be innate or acquired after chemotherapy treatment using cytotoxic agents (Baguley, 2010). MDR has been a major obstacle for the success of chemotherapy, especially for HCC patients. Although chemotherapy with cytotoxic agents are not yet regarded as a standard treatment for advanced HCC (Bruix and Sherman, 2005), efforts to promote the efficacy of chemotherapy by circumventing MDR phenotype of HCC have been making for several decades (Ryu and Chung, 2010). It has been well acknowledged that a comprehensive understanding of possible molecular mechanisms and signal pathways involved MDR of HCC could be useful to overcome MDR and improve the chemotherapeutic efficacy for HCC patients. Based on these considerations, a MDR human HCC cell line established as closely as possible to the MDR actually happened in clinical setting would be greatly useful to elucidate the mechanism of the MDR of HCC and screen potential agents or approaches to reversing chemoresistance of HCC.

In the present study, a well-established Chinese human

HCC cell line BEL-7402 was used as the parent cell line to be induced by 5-FU in vitro (Chen et al., 1980). As a cell-cycle-specific drug, 5-FU selectively inhibits cancer cells in S phase and it is recommended to be administered by continuous intravenous infusion instead of bolus injection clinically (Guglielmi et al., 1996; Meta-analysis Group In Cancer, 1998). Thereafter, a persistent culturing the parental cell line in medium with relatively low concentration of 5-FU in a gradually increasing manner were firstly applied to imitating the continuous administration of 5-FU for HCC patients during the treatment period. Simultaneously, intermittent exposure to high concentration of 5-FU was also integrated to simulate the treatment cycles patients received clinically. The development of MDR induced by the continuous combined with intermittent pattern was hypothesized to have more similarity with MDR actually happened in clinical setting than that produced by continuous exposure only.

As a result, after an induction of 10 months a 5-FU-resistant HCC cell line BEL-7402/5-FU with a moderate RI of 18 was established with a typical MDR phenotype. It is easy to notice that the RIs of BEL-7402/5-FU to cytotoxic drugs in our study (Table 1) are much smaller than that reported by other studies where their RIs of BEL-7402/5-FU cells ranged 160-339.7 folds for 5-FU and 13.4-26.90 folds for ADM (Jin et al., 2002; Li et al., 2004; Tong et al, 2012). The wide variation between RIs of BEL-7402/5-FU established by different research teams might be mainly due to the difference in induction methods. Actually, HCC patients characterized with such high resistance to 5-FU is rare to see in practice and the clinical relevance of these resistant HCC cell lines with super high RIs is difficult to be evaluated. The RI of our established HCC cell line is a moderate value of 18 which might be more similar to actual RIs for HCC clinically. Our results were consistent with another two 5-FU-resistant HCC cell lines with 4- and 10-fold RIs (Uchibori et al., 2012). Besides the RI values, the time to development of MDR (10 months), and the maximum concentration of 5-FU to maintain the MDR phenotype (50 μmol/L) have more clinical similarities to clinical practice, which indicate that the BEL-7402/5-FU cells established by us could reflect the MDR development accurately for HCC patients received chemotherapy with 5-FU. It might be served as an ideal model for studying the possible mechanisms concerning MDR and screening potential agents to overcome MDR for HCC.

Compared to its parental cells, the most prominent feature of BEL-7402/5-FU lies in the slower proliferation (Figure 1A) which has been verified by other MDR HCC cell lines. In our study, BEL-7402/5-FU had a doubling time of 42.3 hours, which was prolonged by 35% than the doubling time of 31.2 hours for its parental cells. The difference in adherence ability, colony efficiency and mitotic index between BEL-7402/5-FU and its parental cells further confirmed a significantly reduced viability of BEL-7402/5-FU. However, analysis of cell cycle kinetics showed that the resistant cells presented a significant increase in the proportion of cells in S and G2/M phases accompanied with a clear decreasing proportion of G0/

G1 phase than its parental cells (Figure 3). The same alterations in cell cycle distribution were also found in two distinct MDR human HCC cell lines, SMMC-7721/ADM and SK-Hep-1/CDDP (Yan et al, 2010; Zhou et al, 2010). The former was developed by culturing SMMC-7721 cells in the presence of gradually increasing concentrations of ADM while the latter was selected from SK-Hep-1 cells by intermittent pulse exposure with 5 µg/ml CDDP for six times. The changes in cell cycle meant that more time was needed for resistant HCC cells to synthesize DNA prior to mitosis. The redistribution of cell cycles might be partly attributed to adaptive response of HCC cells to cytotoxicity effects to survive. A delay in the cell cycle might lead to cellular escape from the cytotoxicity of cell cycle-specific agents (e.g. VCR and 5-FU) and the development of MDR (Yan et al., 2010).

A variety of mechanisms are thought to be involved in the MDR of cancer cells. It has been demonstrated that an increased efflux of cytotoxic drugs out of cancer cells is responsible for MDR mainly (Baguley, 2010). The remarkably enhanced drug efflux pump activity acquired by the resistant cells was also verified by our results (Figure 4). The enhanced efflux pump activity is attributed to an overexpression of membrane transport proteins that belong to the ATP-binding cassette (ABC) transporters which includes P-gp (encoded by human multidrug resistance gene 1, MDR1, also ABCB1), MRP1 (encoded by ABCC1), lung resistance-related protein (LRP), breast cancer resistance protein (BCRP, encoded by ABCG2), and so on. Among them, P-gp and MRP1 have been widely investigated in MDR of HCC. It has been suggested that overexpression of P-gp but not MRP1 mainly contributes to the MDR of SMMC-7721/ADM cells (Yan et al., 2010). The upregulation of P-gp and BCRP but not MRP1 and LRP are involved in the MDR of HepG2/ADM cells (Li et al., 2007; Sun et al., 2010). Overexpression of P-gp is also a predominant mechanism to explain the MDR in a taxol-resistant cell line, QGY-TR50 (Zhou et al., 2001). SK-Hep-1/CDDP was closely related to the overexpression of MDR1 and MRP1 (Zhou et al., 2010). Controversial results also could be found in other studies. An over-expression of MRP1 in addition to P-gp was also observed in MDR of SMMC-7721/ADM cells (Li et al., 2006). For HepG2/ADM cells, the levels of P-gP, MRP and LRP were significantly enhanced (Zhai et al., 2006). In a cisplatin-resistant HCC cell line (QGY/CDDP), MDR is not associated with P-gp expression (Yang et al., 2009). The varied mechanism of efflux pump proteins might be attributed to some extent to complexity of MDR of HCC. To explore which ABC transporters involved in BEL-7402/5-FU we established, Rh-123 and FLU accumulation are employed to check the function of P-gp and MRP1 respectively. A significant decrease of intracellular FLU accumulation was observed in BEL-7402/5-FU cells compared to its parental cells, which indicated that activity of MRP1 were markedly enhanced in BEL-7402/5-FU cells. While no significant difference as to intracellular retention of Rh-123 was found between the two cell lines, which suggested activity of P-gp might play little role in the acquired MDR of BEL-7402/5-FU. The unique mechanism of 5-FU resistance via MRP1 was further

confirmed by our findings from immunocytochemistry (Figure 5). Although MRP1 is also a member of the ABC superfamily of membrane transporters, the MDR pattern conferred by MRP1 is similar but not identical to that of P-gp (Jin et al., 2002). The difference might be due to the fact that 5-FU is different from those drugs known to be P-gp substrates such as VCR, ADM, and taxol. The detailed mechanism how MRP1 regulates the MDR of 5-FU are warranted to be explored.

TS is the central target of 5-FU. Its inhibition causes a blockade in DNA synthesis, DNA strand breaks, and cell death. In MDR of cancer cells induced by 5-FU, the expression and activity of TS should be clarified to determine its possible role in MDR development. The immunocytochemistry (Figure 5) in this study expectedly suggested a significant increase of TS expression in BEL-7402/5-FU compared to its parental cells. Although the upregulated expression of TS might be associated with the resistance to 5-FU, the BEL-7402/5-FU cells still need more time to synthesize DNA which contributed to a prolonged S phase in cell cycle (Figure 3) than its parental cells. These results indicate that the overexpression of TS is only one of the causes for the MDR of BEL-7402/5-FU cells.

Resistance to apoptosis has been known to be associated with MDR of cancer cells (Wilson et al., 2009). It is well-known the Bcl-2 family such as Bcl-2, Bcl-xL, Bax and Bad plays a crucial role in the control of apoptosis. The ratio of Bcl-2/Bax is a critical determinant for cell apoptosis. In our previous study, a loss of Bcl-2 expression was found in BEL-7402 cells (Han et al., 2007). Therefore, the expression of Bcl-xl instead of Bcl-2 was examined combined with Bax, and the ratio of Bcl-xl/Bax were analyzed in this study. An overexpression of Bcl-xl was determined in BEL-7402/5-FU cells but the expression of Bax in the resistant cells was equal to that in parental cells (Figure 5). Consequently, the Bcl-xl/Bax ratio towards apoptosis was augmented accordingly which lead to MDR of BEL-7402/5-FU cells.

Additionally, an evident morphological change of BEL-7402/5-FU cells was the cells tended to grow in clusters without contact inhibition. Previous research has demonstrated that tumor cells cultured in aggregates are resistant to cytotoxic drugs (Jianmin et al., 2002). Because this form of resistance is dependent on intercellular adhesion (Green et al., 2004), we have investigated the expression of E-cad which is an important adhesive molecule responsible of intercellular adhesion. Compared to its parental cells, BEL-7402/5-FU showed a significantly upregulation of E-cad (Figure 5). The results suggest that intercellular adhesion mediated by E-cad is also involved in MDR mechanisms of BEL-7402/5-FU cells.

In conclusion, a MDR human HCC cell line BEL-7402/5-FU was successfully established as closely as possible to the MDR actually happened in clinical setting. The MDR phenotype of BEL-7402/5-FU might be partly attributed to increased drug efflux pump activity via MRP1, overexpression of TS, resistance to apoptosis by augment of Bcl-xl/Bax ratio, and intracellular adhesion medicated by E-cad. BEL-7402/5-FU would be greatly useful to elucidate the mechanism of the MDR in HCC and screen potential agents or approaches to reversing chemoresistance of HCC.

Acknowledgements

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