RESEARCH ARTICLE

EGF Reverses Multi-drug Resistance via the p-ERK Pathway in HepG2/ADM and SMMC7721/ADM Hepatocellular Carcinoma Models

Feng Yan*, Li-Ping Bai, Hua Gao, Chang-Ming Zhu, Li Lin, Xiang-Peng Kang

Abstract

Aim: To investigate signaling pathways for reversal of EGF-mediated multi-drug resistance (MDR) in hepatocellular carcinoma (HCC) models. Materials and Methods: HCC MDR cell strain HepG2/adriamycin (ADM) and SMMC7721/ADM models were established using a method of exposure to medium with ADM between low and high concentration with gradually increasing concentration. Drug sensitivity and reversal of multi-drug resistance by EGF were determined and the cell cycle distribution and apoptosis were analyzed by flow cytometry. Phosphorylation of ERK1, ERK2, ERK5 and expression of Bim were detected by Western blotting. Results: The results showed that HepG2/ADM and SMMC7721/ADM cells were resistant not only to ADM, but also to multiple anticancer drugs. When used alone, EGF had no anti-tumor activity in HepG2/ADM and SMMC7721/ADM cells *in vitro*, while it increased the cytotoxicity of ADM. EGF induced cell apoptosis and G0/G1 phase cell cycle arrest in HepG2/ADM And SMMC7721/ADM cells, while enhancing activity of p-ERKs and up-regulated expression of BimEL. Conclusions: EGF might enhance the chemosensitivity of HepG2/ADM and SMMC7721/ADM cells via up-regulating p-ERKs and BimEL protein.

Keywords: Hepatocellular carcinoma - multi-drug resistance - EGF - ERK - BimEL

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Introduction

Hepatocellular carcinoma (HCC) is one of the most widespread malignant diseases and the fifth most common solid tumor and the fourth leading cause of cancer-related death in the world (Chow et al., 2013) HCC is a hypervascular solid cancer characterized by a high degree of drug resistance (Wakamatsu et al., 2007). Multidrug resistance (MDR) to chemotherapeutic agents plays a major role in the failure of cancer therapy (Perez-Tomas et al., 2006).

There are three major subfamilies of mitogen-activated protein kinases (MAPK): the extracellular-signal-regulated kinases (ERK MAPK); the c-jun N-terminal kinase or stress-activated protein kinases (JNK or SAPK); and MAPK14. The ERK MAPK pathway is one of the most important for cell proliferation. The MAPK pathways are located downstream of many growth-factor receptors, including that for epidermal growth factor (Fang et al., 2005). The activity of ERK family has been implicated in the regulation of cell proliferation, embryonic morphogenesis, tumor transformation and cell apoptosis (Lancet et al., 2005). Our previous research proved that ERK activities were down-regulated in P-gp-mediated MDR HCC cells. ERK1, ERK2 or ERK5 might be a potential drug target for circumventing MDR HCC cells (Feng et al., 2005).

It was our aim to investigate the effects of ERK activator (EGF) on MDR cells and the influence on the expression of related proteins in HepG2/ADM and SMMC7721/ADM cells in vitro.

Materials and Methods

Cell culture

Hepatocarcinoma cell line HepG2 and SMMC7721 were obtained from Cell Bank of Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. HepG2 and SMMC7721 cells were cultured respectively with DMEM and RPMI-1640 (Gibco, Grand Island, NY, USA). Both media were supplemented with 10% calf serum (Gibco, Grand Island, NY, USA) and maintained at 37°C in a humidified atmosphere containing 50 mL/L CO_2 and 950 mL/L air.

Multidrug resistant human HCC cell lines, HepG2/adriamycin (ADM) and SMMC7721/ADM were established. To develop the HepG2/ADM and SMMC7721/ADM cells, ADM (Melone Pharmaceutical Co., Ltd, Dalian, China) was added respectively to HepG2 and SMMC7721 cells at a stepwise increasing concentration

*Department of Gastrointestinal Surgery, Zhongshan Hospital, Xiamen University; Xiamen University Digestive Diseases Institute; Digestive Diseases Center of Xiamen City, Xiamen, China *For correspondence: yanfeng361004@126.com*

from 0.001 to 0.5 mg/L for six month consequently. Resistant cells were selected by removing the non-resistant dead cells. Multidrug resistance was maintained by culturing the cells with low concentration (0.10mg/L) and the culture medium was changed every other day. MDR cells were named HepG2/ADM and SMMC7721/ADM.

Measurement of cellular sensitivity to chemotherapeutics

CCK-8 (Dojindo Molecular Technologies, Minato-ku, Tokyo, JAPAN) assay was used to determine drug sensitivity. Sensitivity of cultured HepG2/ADM and SMMC7721/ADM cells to chemotherapeutics, including ADM, fluorouracil (5-Fu), cisplatin (CDDP) (Melone Pharmaceutical Co., Ltd, Dalian, China), was detected respectively. Resistance index (RI) was calculated according to the formula, that is, IC_{50} for MDR cells divided by IC_{50} for parental cells makes RI.

To determine whether EGF can reverse the drug resistance of MDR cells, ADM was then added with varying concentrations. The experimental group was treated with EGF (Sigma-Aldrich, St. Louis, MO, USA) at a concentration of 10 μg/L. Verapamil (5mg/L) and Ciclosporin A (2.5mg/L) (Sigma-Aldrich, St. Louis, MO, USA) treatment served as positive control. The cells were analyzed using the CCK-8 method. Reverse fold (RF) was evaluated according to the formula: RF=IC_{50} before reversal/IC_{50} after reversal.

Apoptosis assay by statistical FCM

Cell apoptosis was analyzed to confirm if EGF can enhance cell killing sensitivity by ADM. MDR Cells were exposed to EGF (10 ug/L), ADM (0.2 mg/L) and both at 48h. The apoptosis rates were measured using flow cytometric assay. Cell labeling was performed using annexin V conjugated to FITC, which binds to phosphatidylserine exposed on the surface membrane of cells undergoing apoptosis. HepG2/ADM and SMMC7721/ADM cells were collected respectively through trypsinization, washed twice with PBS and centrifuged at 500×g for 5min. The cells were suspended in 500 μL binding buffer, 5 μL annexin V-FITC and 10 μL (20 μg/mL) PI solution (Gibco, Grand Island, NY, USA), and incubated at room temperature for 20 min in the dark. The samples were measured using a flow cytometer with FACS software.

Cell cycle distribution assay by Statistical FCM

Treated and collected the MDR cells as above, then fixed in 75% ethanol for 2 h at 4°C. Samples were rehydrated with PBS and the cells were incubated with 500 μL (200μg/mL) PI solution for 30 min at room temperature. Each sample and the percentage of cells in G0/G1, S and G2/M phases of the cell cycle were calculated using a flow cytometer with FACS software.

Western blot analysis of protein expression

After treatment with the different drugs (10 μg/L EGF, 0.2mg/L ADM and both) at 37°C for 48h, total protein was isolated and subjected to sodium dodecyl sulfate PAGE analysis and transferred to a polyvinylidene difluoride membrane. Membranes were blocked with 5% dry milk in TBS-T (TBS containing 0.05% Tween 20) for 1h at room temperature. The blots were stained with primary antibodies (1:200-500, rabbit anti-human mdr-1 antibody, rabbit anti-human P-gp antibody, rabbit anti-human ERK1/2, goat anti-human p-ERK1/2, rabbit anti-human ERK5, goat anti-human p-ERK5, and rabbit anti-human Bim) (Santa cruz, California, USA) overnight at 4°C. After incubation with the respective primary antibodies, the membranes were exposed to species-specific horseradish peroxidase-labeled secondary antibodies at room temperature, and developed using the ECL plus Western blotting reagent (Santa cruz, California, USA). Parallel membranes were incubated with primary antibodies (1:3000-5000 rabbit anti-human GAPDH and rabbit anti-human β actin) (Santa cruz, California, USA) and HRP-coupled rabbit anti- rabbit secondary antibody.

Results

Chemotherapeutics sensitivity

Sensitivity assay obsvered that HepG2/ADM and SMMC7721/ADM were resistant not only to ADM but also to multiple anticancer drugs, including fluorouracil (5-FU) and cisplatin (CDDP). Their lethal dose (IC_{50}) was significantly higher for HepG2/ADM and SMMC7721/ADM cells than for non-resistant parental cells (Table 1.a and b). The ability of EGF at 10 μg/L to enhance the cytotoxicity of adriamycin (ADM) in HepG2/ADM and SMMC7721/ADM was examined. The higher the

Table 1. Comparison of Sensitivities to Different Chemotherapeutic Drugs in HepG2 (a), SMMC7721 (b), HepG2 (c), HepG2 (d), and the Resistant Cells (n=6, Mean±sd)

Drug (mg/L)	IC_{50} HepG2	IC_{50} HepG2/ADM	RI
a. HepG2			
ADM	0.040±0.012	0.516±0.076	12.9
5-FU	0.102±0.031	0.759±0.041	7.4
CDDP	0.158±0.049	0.936±0.103	5.3
Drug (mg/L)	IC_{50} SMMC7721	IC_{50} SMMC7721/ADM	RI
b. SMMC7721			
ADM	0.011±0.004	0.156±0.036	14.2
5-FU	0.071±0.024	0.892±0.124	12.6
CDDP	0.088±0.032	0.836±0.093	9.5
Cell line		IC_{50} ADM (mg/L)	Reverse fold
c. HepG2			
HepG2/ADM		0.516±0.076	
HepG2/ADM+EGF		0.403±0.045	1.28
HepG2/ADM+Verapamil		0.083±0.031	6.23
HepG2/ADM+Ciclosporin A		0.065±0.024	7.98
Cell line		IC_{50} ADM (mg/L)	Reverse fold
d. HepG2			
SMMC7721/ADM		0.156±0.036	
SMMC7721/ADM+EGF		0.087±0.020	1.78
SMMC7721/ADM+Verapamil		0.054±0.015	2.89
SMMC7721/ADM+Ciclosporin A		0.048±0.016	3.24

*IC_{50}: Half inhibition concentration; RI: Resistance index; ADM: adriamycin; 5-FU: 5-fluorouracil; CDDP: cisplatin

concentration of EGF used, the better the inhibitive activity (Table 1.c and d, Figure 1).

Apoptosis statistical FCM assay

After incubation with either EGF (10 μg/L) and ADM (0.2 mg/L) for 48h, the double staining with both Annexin V-FITC and PI was employed to distinguish the apoptotic cells from others. As shown in Figure 2 and 3, compared with untreated group, the proportion of apoptosis increased progressively in HepG2/ADM and SMMC7721/ADM cells. The results indicated that EGF could increase the sensitivity to cell killing induced by ADM in HepG2/ADM and SMMC7721/ADM cells.

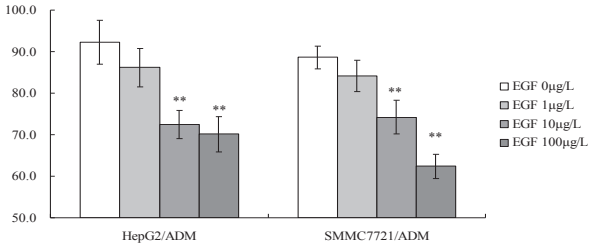

Figure 1. The Concentration-Dependent Reversal Effect of EGF on the HepG2/ADM and SMMC7721/ADM

Cell cycle distribution FCM assay

Cell cycle phase distribution was detected by flow cytometry to determine whether there is any difference in cell cycle kinetics between HepG2/ADM and SMMC7721/ADM cells. Co-treatment of HepG2/ADM and SMMC7721/ADM cells with EGF and ADM resulted in a decreased trend of cells in S phase, along with a

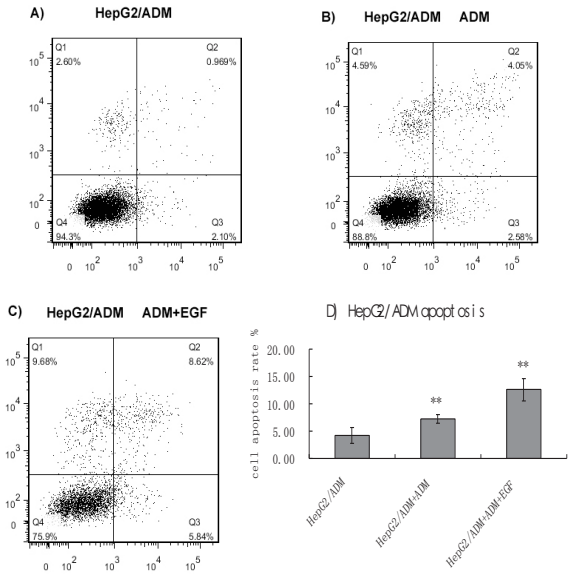

Figure 2. Cell Apoptosis in HepG2/ADM Cells Treated with ADM and EGF for 48h

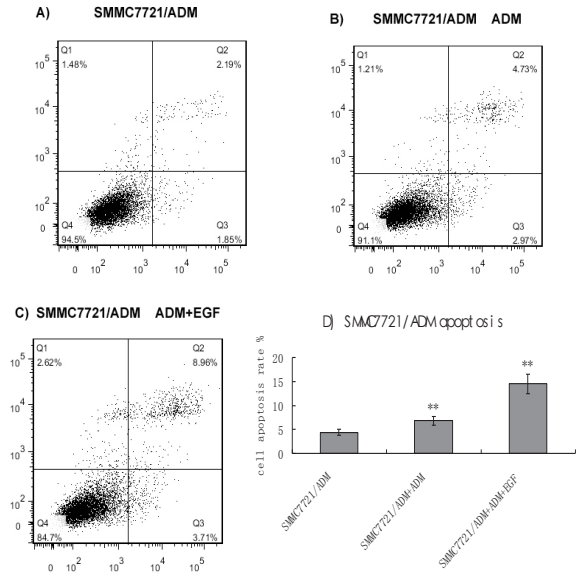

Figure 3. Cell Apoptosis in SMMC7721/ADM Cells Treated with ADM and EGF for 48h

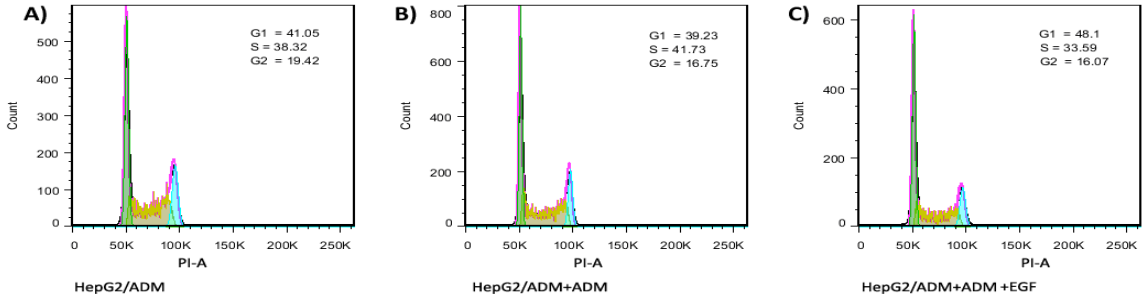

Figure 4. Cell Cycle Distributions in HepG2/ADM Cells Treated with ADM and EGF for 48h

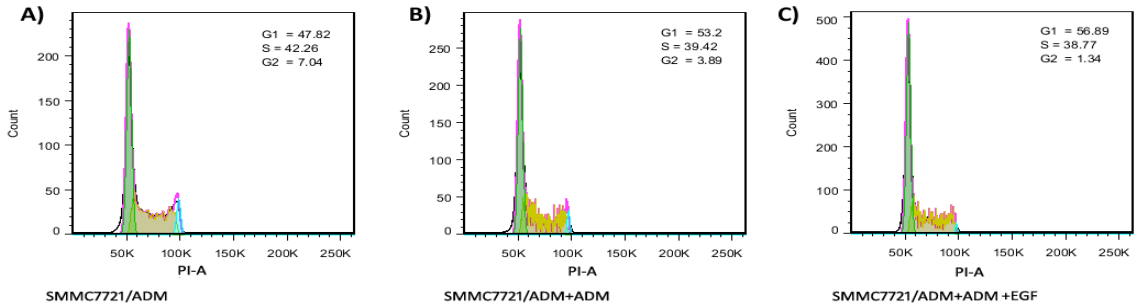

Figure 5. Cell Cycle Distributions in SMMC7721/ADM Cells Treated with ADM and EGF for 48h

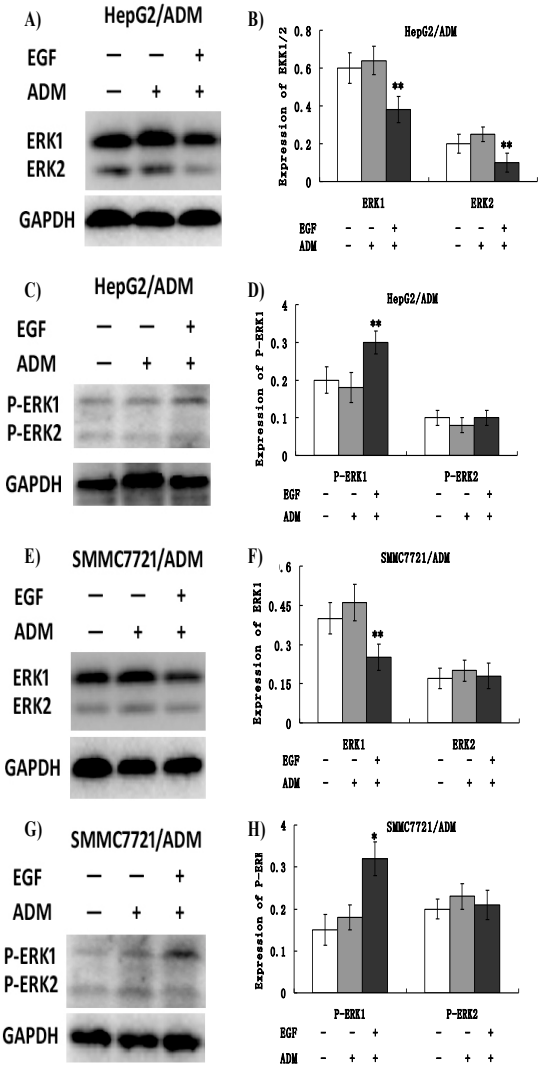

Figure 6. Expression and Phosphorylation of ERK1 and ERK2 in HepG2/ADM and SMMC7721/ADM Cells by EGF for 48h

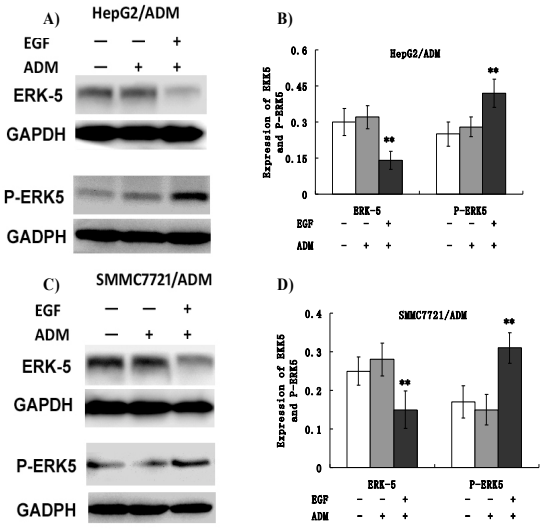

Figure 7. Expression and Phosphorylation of ERK5 in HepG2/ADM and SMMC7721/ADM Cells by EGF for 48h

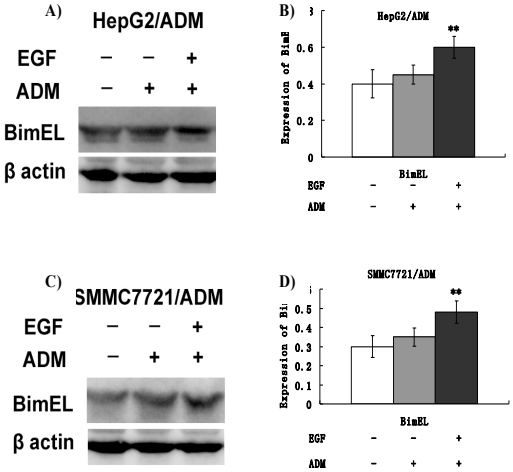

Figure 8. Expression of BimEL in HepG2/ADM and SMMC7721/ADM Cells Treated with ADM and EGF for 48h

pronounced arrest in G0/G1 phase compared with ADM alone. No statistical differences in the percentage of G2 phase cells were noted between the combined drugs group and single ADM (Figure 4 and 5).

Effects of EGF on the activities of ERK family members

To study the mechanisms of EGF on the reversal of Multi-Drug Resistance, we examined respectively the expression and phoshorylation of ERK1, ERK2 and ERK5 in HepG2/ADM and SMMC7721/ADM cells by Western blot. The remarkable phosphorylation of ERK-1 and ERK5 mmmwas detected in HepG2/ADM and SMMC7721/ADM cells which treated with EGF for 48 h. However, there was significant increase of the phosphorylation of ERK2 protein in HepG2/ADM cells and no obvious change in SMMC7721/ADM cells (Figure 6 and 7).

Expression of BimEL

To further examine whether important proapoptotic regulatory proteins could be related to the reversal of Multi-Drug Resistance by EGF, the expression of Bim was determined. As shown in Fig. 8, the significant increase of BimEL was observed in HepG2/ADM and SMMC7721/ADM cells which treated with EGF for 48h by Western blot.

Discussion

Nearly 50% of human cancers are either completely resistant to chemotherapy or respond to chemotherapy only transiently, after which they are no longer affected by common anticancer drugs (Chen et al., 2012). This phenomenon is referred to as MDR and is inherently expressed by some tumor types, while others acquire MDR after exposure to chemotherapy treatment (Nishimoto et al., 2006; Chen et al., 2013; Ozdemir et al., 2013; Ren et al., 2012; Zhu et al., 2013; Roy et al., 2014). To elucidate if p-ERKs involved in reversal of Multi-Drug resistance by EGF in HepG2/ADM and SMMC7721/ADM cells, cell apoptosis and cell cycle distribution were analyzed by FCM. The results showed that proportion of apoptosis and G0/G1 phase cell cycle arrest increased remarkably

in HepG2/ADM and SMMC7721/ADM cells treated with EGF for 48 h, which probably contributes to the lower ability of cells to proliferate.

The ERK signaling pathway plays a central role in the regulation of various physiological processes such as proliferation, survival or cell motility. Recent studies demonstrated that modulation of ERK activation may be a new method to reverse MDR, however, whether ERK activation is positively or negatively correlated with MDR still remains controversial (Chen et al., 2008; Li et al., 2008 ; Yan et al., 2008). To investigate the mechanism of EGF in HepG2/ADM and SMMC7721/ADM cells, expression of ERK1, ERK2, ERK5 were detected by western blot. The visible phosphorylation of ERK1 and ERK5 was observed in HepG2/ADM and SMMC7721/ADM cells. There was a significant increase of phosphorylation of ERK2 protein in HepG2/ADM cells and obsolete change in SMMC7721/ADM cells.

Pro-apototic Bcl-2 family members promote cell death by neutralizing their anti-apoptotic relatives, which otherwise maintain cell viability by regulating caspase activity (Bustamante et al., 2007). Bim belongs to the BH3-only subgroup of Bcl-2 related proteins, and exists in three distinct isoforms, such as, BimS (short), BimL (long) and BimEL (extra long). It was reported that expression of BimEL was related to ERK pathway. At least three sites for ERK1/2 phosphorylation exist on BimEL, whereas ERK1/2 does not affect BimS and BimL, implying a unique role for BimEL in cell survival signaling (Claessens et al., 2002; Korhonen et al., 2003). In present study, the result showed that EGF can up-regulate expression of BimEL.

In conclusion, the mechanism has demonstrated that EGF can reverse the MDR of HepG2/ADM and SMMC7721/ADM cells in vitro by activating expression of ERK1, ERK2 and ERK5, up-regulating BimEL expression, and inducing cell apoptosis which is concomitant with the G0/G1 arrest, which indicated that EGF might be a promising compound, especially for the treatment of MDR human carcinoma.

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