Cyclophosphamide–Induced Apoptosis in A431 Cells Is Inhibited by Fucosyltransferase IV

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ABSTRACT

Fucosyltransferase IV (FUT4) is an essential enzyme that catalyzes the synthesis of difucosylated oligosaccharide LeY which is overexpressed in the cancers derived from the epithelial tissues. Our previous studies have shown that FUT4 overexpression promotes A431 cell proliferation through the MAPK and PI3K/Akt signaling pathways, but the relationship between FUT4 and apoptosis remained unclear. Here, we investigated the effect of FUT4 overexpression on cyclophosphamide (CPA)-induced apoptosis in A431 cells. Western blot analysis showed that FUT4 overexpression decreased expression of Bax, Caspase 3, and PARP proteins, and increased anti-apoptotic Bcl-2 protein in A431 cells. The anti-apoptosis effect of FUT4 was confirmed both by Annexin-V/PI and JC-1 assays. The results showed that FUT4 overexpression up-regulated phosphorylation of ERK1/2 and Akt which was inhibited by CPA in dose-dependent manner. By blocking the ERK/MAPK and PI3K/Akt pathways with specific inhibitors, we demonstrated that these two pathways were required in mediating the anti-apoptosis effect of FUT4. We concluded that FUT4 inhibited cell apoptosis induced by CPA through decreasing the expression of apoptotic proteins Bax, Caspase 3, and PARP and increasing the expression of anti-apoptotic protein Bcl-2 via the ERK/MAPK and PI3K/Akt signaling pathways in A431 cells. J. Cell. Biochem. 112: 1376–1383, 2011. © 2011 Wiley-Liss, Inc.

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The members of fucosyltransferase (FUT) gene family participate in the transferring of L-fucose from GDP-fucose to their acceptors in $\alpha 1,2$-, $\alpha 1,3/4$-, and $\alpha 1,6$-linkages. Six human $\alpha 1,3$-fucosyltransferases genes have been identified, including FUT3, 4, 5, 6, 7, and 9 genes [Ma et al., 2006]. LeY is a difucosylated oligosaccharide with the chemical structure $[Fuc\alpha 1 \rightarrow 2Gal\beta 1 \rightarrow 4(Fuc\alpha 1 \rightarrow 3)GlcNAc\beta 1 \rightarrow R]$ [Cao et al., 2001; Escrevente et al., 2006]. The $\alpha 1,3$-fucosylation of LeY is catalyzed by fucosyltransferase IV (FUT4). FUT4 is a critical enzyme that controls LeY oligosaccharide synthesis [Taniguchi et al., 2000; Wang et al., 2001]. FUT4 is mainly expressed in the leukocytes and some epithelial cells [Allahverdian et al., 2006]. Increases in the expression of FUT4 level are seen in different cancers, for example, gastric carcinoma [Petretti et al., 1999], colorectal cancer [Ito et al., 1997; Kudo et al., 1998], pancreatic cancer [Ito et al., 1997], and lung adenocarcinoma [Martin-Satue et al., 1998].

The glycosylation of cell surface proteins is important for cancer biology processes such as cellular proliferation and metastasis [Helenius and Aebi, 2001]. Several reports indicated that fucosyltransferases had a role in cell proliferation and metastasis. The increased fucosylation of EGFR significantly promoted EGF-mediated cellular growth [Matsumoto et al., 2008]. Higher expression of fucosyltransferases (FUT1, FUT4) might be an important step in the formation of surface structures that facilitate metastasis of melanoma [Ciolczyk-Wierzbicka et al., 2007].

Apoptosis is now recognized as an important process for normal development of multicellular organisms and plays a major role in homeostasis [Rokhlin et al., 1997; Li et al., 2005]. The pathways of cellular proliferation and apoptosis appear to be linked to minimize the occurrence of neoplasia [Harrington et al., 1994]. In multicellular organisms, the total number of cells is a balance between the cell-generating effects of mitosis and cell death that is induced through apoptosis. A disruption of this delicate balance can lead to the development of cancer [Cotter, 2009]. Deregulation of apoptotic signaling pathways may suggest a mechanism to promote tumorigenesis by preventing the elimination of these cells through

normal checkpoint control [Skaug et al., 2009; Wagner and Nebreda, 2009].

Cyclophosphamide (CPA) is a chemotherapy drug that is given as a treatment for many types of cancer such as breast cancer [Low et al., 2009; Margolin et al., 2010], ovarian cancer [Kolasa et al., 2009], and multiple myeloma [Auner et al., 2010; Schey et al., 2010], etc. One of the important anti-tumor effects of CPA is to induce tumor cell apoptosis, for example, CPA could induce the apoptosis of human breast cancer cell (MCF-7) mediated by caspase [Singh et al., 2009]; CPA inhibited growth of hepatocarcinoma 22 cells [Pang et al., 2008]. But there is no report about the apoptotic role of CPA in human epidermoid carcinoma A431 cells.

In our previous studies, we reported that overexpression of FUT4 promoted A431 cell growth [Yang et al., 2007, 2010]. However, few studies have been focused on the role of anti-apoptosis of FUT4 and the mechanism. In the present study, we aimed to provide more data on the effect of FUT4 in the course of the apoptosis induced by CPA in A431 cells. These studies showed that the anti-apoptosis effect of FUT4 against CPA-induced A431 cell apoptosis was mediated by the activation of the ERK/MAPK and PI3/Akt pathways. This conclusion gave us a suggestion that epidermoid carcinoma patients develop resistance to cyclophosphamide and if this resistance is due to FUT4 increase then FUT4 can be a viable target for therapy in these patients.

MATERIALS AND METHODS

MATERIALS

A431 cell line was obtained from American Type Culture Collection (Manassas, VA). DMEM/F12 (1:1), fetal bovine serum (FBS), Lipofectamine™ Reagent and Plus™ Reagent were purchased from Invitrogen. Enhanced chemiluminescence (ECL) assay kit was purchased from Amersham. G418, LY294002, and PD98059 were obtained from Sigma Chemical Company. The antibodies against ERK1/2, phosphorylated ERK1/2 (pERK1/2), Akt, phosphorylated Akts (pAkt308 and pAkt473), and PARP were purchased from Santa Cruz. The antibodies against Bcl-2, Bax, and PARP were purchased from BiYunTian Company. Caspase 3 (sc-56053) was purchased from Santa Cruz. Horseradish peroxidase (HRP)-conjugated anti-mouse secondary antibody and anti-rabbit secondary antibody were purchased from Santa Cruz. All the other reagents were of the highest purity commercially available. FUT4 interfered plasmid (FUT4-RNAi) was constructed in our own lab [Zhang et al., 2008]. Briefly, the RNAi sequences of FUT4 were: 5′-GATCCGCCTGGC-AAGTAACCTCTTCTCAAGAGAAAGAGGTTACTTGCCAGGCTTA-3′, 5′-AGCTTAAAGCCTGGCAAGTAACCTCTTTCTCTTGAGAAGAGGT-TACTTGCCAGGCG-3′. These oligonucleotides were annealed before cloning into the linearized pSilencer 4.1-CMV neo-vector using T4 DNA ligase per manufacturer's instructions. The plasmid containing RNAi sequences for FUT4 was referred as FUT4 RNAi.

CELL CULTURE

A431 cells were cultured in DMEM/F12 (1:1) supplemented with 10% FBS, 100 U/ml penicillin and 50 µg/µl streptomycin at 37°C under 5% CO_2 in humidified air. A431 cells were transfected with pEGFP-N1-FUT4 and screened by G418 for FUT4 stably transfected cells, which has been described elsewhere [Yang et al., 2007].

DETECTION OF APOPTOSIS BY HOECHST 33342 STAINING

Morphological evaluation of cell apoptosis was performed using Hoechst 33342 staining which detected the nuclei of both apoptotic and living cells. Cells grown on the glass coverslips were fixed with 4% paraformaldehyde/PBS for 30 min, washed for 15 min in 0.1% Triton X-100/PBS, and incubated in dark with Hoechst 33342 (10 µg/ml) for 15 min. The stained cells were studied using a fluorescence microscope. The rate of apoptotic cells was recorded in 10 random nonoverlapping fields by two blinded observers.

FLOW CYTOMETRY BY ANNEXIN V–PI STAINING

Adherent and floating cells were harvested, washed twice with ice-cold PBS, and then double-labeled with 5 µl Annexin V-fluorescein and 5 µl PI in 100 µl of binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, and 2.5 mM $CaCl_2$). After 15 min incubation at room temperature, 400 µl of binding buffer was added in each sample and cells were then analyzed by a FACScan flow cytometer of 20,000 cells in each group. Data analysis was performed with the standard Cell Quest Software. All experiments were performed in duplicate and reproducibility was checked in three independent experiments.

JC–1 ASSAY FOR MITOCHONDRIAL MEMBRANE POTENTIAL

The mitochondrial membrane potential was determined using the dual-emission mitochondrial dye JC-1, (5,5′,6,6′-tetrachloro-1,1′, 3,3′-tetraethylbenzimidazolocarbocyanine iodide). 1×10^6 cells/ml were incubated with JC-1 dye for 20 min at 37°C, then washed for 15 min in JC-1 dye buffer. After incubation, samples were immediately assessed for red and green fluorescence using a flow cytometer. Photomultiplier settings were adjusted to detect green fluorescence ($\lambda_{em} = 525$ nm) of JC-1 monomer on the filter 1 (FL-1 detector) and the red fluorescence ($\lambda_{em} = 590$ nm) of JC-1 aggregates on the filter 2 (FL2 detector). In each experiment, at least 10,000 events were analyzed. The relative aggregate:monomer (red:green) fluorescence intensity values were used for data presentation.

WESTERN BLOT

To prepare whole cell extracts, cells at 90% confluence were washed in phosphate-buffered saline (PBS) before incubation with lysis buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA, pH 8.0, 0.2 mM Na_3VO_4, 0.2 mM PMSF, and 0.5% Nonidet P-40) on ice for 10 min. The cell lysates were clarified by centrifugation at 9,000g for 10 min, and the supernatants were collected. Protein concentration was determined with the Coomassie Protein Assay Reagent using bovine serum albumin (BSA) as a standard. Cell lysate (50 µg) was separated by 10% SDS–PAGE mini-gel. Samples were transferred electrophoretically to nitrocellulose membranes, blocked with TTBS (50 mM Tris–HCl, pH 7.5, 0.15 M NaCl, and 0.1% Tween-20) containing 5% fat-free dry milk for 2 h and incubated for 3 h with the appropriate primary antibodies at the dilutions recommended by the supplier. The dilutions for primary antibodies of Bcl-2, Bax, Caspases 3, and β-actin are 1:100. The

dilutions for primary antibodies of ERK, pERK, Akt, pAkt, and PARP are 1:1,000. After incubation with a HRP-conjugated anti-goat secondary antibody, immunoreactive proteins were visualized with ECL detection system.

STATISTICS

Results are expressed as the mean $\pm$ the standard error of the mean (SEM) of at least three independent experiments. Statistical significance of difference between test groups was assessed by one-way ANOVA followed by Scheffe's test (posthoc). Statistical significance was defined at $P < 0.05$.

RESULTS

CPA–INDUCED APOPTOSIS IN A431 CELLS

To investigate morphological changes, A431 cells were treated with 0–200 μg/ml CPA for 24 h, then observed under fluorescence microscope after staining with Hoechst 33342. Observations under the microscope showed apoptotic cells in CPA-treated groups exhibited nuclear condensation, nuclear fragmentation, and chromatin margination in dose-dependent manner (Fig. 1A). Quantitation analysis revealed that the percentage of apoptosis induced by CPA (100 μg/ml) was increased almost 50-fold higher than controls (Fig. 1A). At the same time, we evaluated the effect of CPA on the expression levels of the proteins that played roles in the events of the apoptotic response. A431 cells were treated with CPA at different concentration (0, 50, 100, and 200 μg/ml) for 24 h. Cell lysates were prepared, then analyzed by Western blot with antibodies against Bcl-2, Bax, caspase 3, and PARP. The results showed that the expression of Bcl-2 was decreased and Bax, Caspase 3, and PARP were increased in dose-dependent manner (Fig. 1B). Taken together, though the percentage of apoptosis was the highest at the CPA concentration of 200 μg/ml, the cells were necrotic at that concentration. So we choose the CPA concentration of 100 μg/ml as the apoptosis-induced concentration in this study.

FUT4 HAD ANTI-APOPTOSIS EFFECT ON A431 CELLS

In this study, we investigated the effect of anti-apoptosis of FUT4. Firstly, we analyzed the levels of FUT4 in FUT4-overexpressed and FUT4-silenced A431 cells by Western blot. The result showed that the expression of FUT4 was increased in FUT4-overexpressed cells and decreased in the FUT4-silenced cells (Fig. 2A). Next, we analyzed the effect of FUT4 on cell morphological changes induced by CPA. As showed in Figure 2B, FUT4 overexpression can decrease the degree of nuclear condensation and nuclear fragmentation. But FUT4-silenced can increase the intendancy.

In addition, we evaluated the effect of FUT4 on the expression levels of the proteins which played roles in the immediate events of the apoptotic response. The control A431 cells, FUT4-overexpressed cells, and FUT4-silenced cells were treated with 100 μg/ml CPA. The proteins were collected and subjected to Western blot analysis. The results showed that the expression of apoptotic proteins Bax, Caspase 3, and PARP was decreased in FUT4-overexpressed cells and increased in FUT4-silenced cells, whereas the expression of anti-apoptotic protein Bcl-2 was increased in FUT4-overexpressed cells and decreased in FUT4-silenced cells (Fig. 2C).

Further, cell apoptosis was evaluated after 6 h incubation by flow cytometry analysis using Annexin-V and PI staining. In the early stages of apoptosis, changes occur at the cell surface. One of these plasma membrane alterations is the translocation of phosphatidylserine (PS) from the inner part of the plasma membrane to the outer layer, by which PS becomes exposed at the external surface of the cell. The analysis of PS on the outer leaflet of apoptotic cell-membranes is performed by using Annexin-V-Fluorescein and propidium iodide (PI) for the differentiation from necrotic cells or labeling with a cell surface marker for cell characterization. The results showed that the percentage of apoptosis was $19.85 \pm 3.72\%$ in A431 cells at a CPA concentration of 100 μg/ml, while the percentages of apoptosis were decreased to $10.92 \pm 2.51\%$ in FUT4-overexpressed cells (A431-FUT4) and increased to $46.32 \pm 4.87\%$ in FUT4-silenced cells (FUT4-RNAi), respectively ($P < 0.05$) (Fig. 2D). A431-FUT4-mock and FUT4-silenced-mock were the vector controls for respective A431-FUT4-overexpressed or FUT4-silenced transfectant.

Finally, to assess whether FUT4 affected the function of mitochondria, potential changes in mitochondrial membrane were analyzed by employing a mitochondria fluorescent dye, JC-1. An early indication of apoptosis involves a collapse in the electrochemical gradient across the mitochondrial membrane. Loss of mitochondrial membrane potential can be detected by a unique fluorescent cationic dye known as JC-1. JC-1 is selectively taken up into mitochondria and so is a reliable indicator of change in $\Delta\psi m$. At hyperpolarized $\Delta\psi m$, JC-1 forms J aggregates in a rapidly reversible manner, emitting red fluorescence. During depolarization of mitochondria, JC-1 leaks and consequently reduces dye concentration in the mitochondrial matrix, and emits a green fluorescence. Radiometric measurements of the red to green JC-1 fluorescence indicate $\Delta\psi m$. As shown in Figure 2E, the ratio between red and green fluorescence was increased in the FUT4-overexpressed cells by approximately $97.07 \pm 4.23\%$ and decreased in the FUT4-silenced cells by $55.49 \pm 5.54\%$ compared with the A431 cell control ($84.74 \pm 5.85\%$, $P < 0.05$). This suggested that FUT4 overexpression resulted in significant increase of $\Delta\psi m$ and FUT4-silencing induced $\Delta\psi m$ dissipation. Taken together, we concluded that FUT4 showed anti-apoptosis effect in A431 cells.

ERK/MAPK AND PI3K/AKT ACTIVITIES WERE INHIBITED BY CPA AND RECOVERED BY FUT4

It is well known that phosphorylation of MAPK and Akt is associated with protection of cells from apoptosis [Chen et al., 2009; Watson et al., 2009; Zhang et al., 2009]. We postulated that CPA could inhibit MAPK and Akt activities and consequently lead to apoptosis in A431 cells. To confirm this, A431 cells were treated with CPA at different concentrations (0, 50, 100, and 200 μg/ml) followed by Western blot analysis for phosphorylated and nonphosphorylated ERK1/2 and Akt proteins. In A431 cells, phosphorylation of ERK1/2 and Akt was inhibited with increased CPA (Fig. 3A). In addition, the level of phosphorylation of ERK1/2 and Akt was increased in the FUT4-overexpressed cells and decreased under the treatment with CPA (Fig. 3B), indicating that phosphorylation of ERK1/2 and Akt was dose-dependently inhibited by CPA and this phosphorylation was recovered in the FUT4-overexpressed cells.
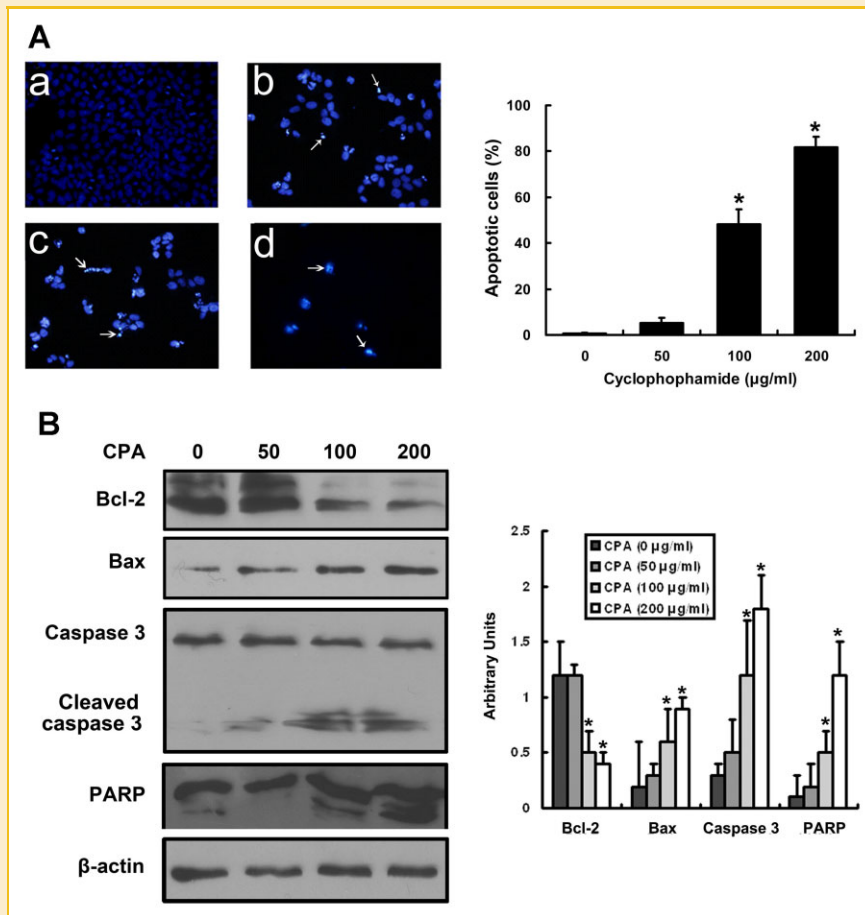
Fig. 1. CPA–induced apoptosis in A431 cells. A431 cells were treated with CPA at different concentration (0, 50, 100, and 200 μg/ml) for 24 h. A: After staining with Hoechst 33342, the cells were observed under a fluorescent microscope. a–d: Cells were treated with CPA at 0, 50, 100, or 200 μg/ml. The arrow indicates the apoptotic bodies of the apoptotic cells. Apoptotic nuclei were counted and data are presented as a percentage of total nuclei. Values are expressed as mean ± SEM of three independent experiments in which more than 1,000 cells were examined ($^*P < 0.01$). B: A431 cells were treated with CPA at different concentration for 24 h. Cell lysates were prepared as described in the "Materials and Methods" Section, and then detected with antibodies against Bcl-2, Bax, Caspase 3, and PARP by Western blot (left panel) and density analysis (right panel). β–actin served as an internal control. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

FUT4 HAD EFFECT ON APOPTOSIS–RELATED PROTEINS THROUGH THE ERK/MAPK AND AKT SIGNALING PATHWAYS

To test whether the ERK/MAPK and Akt signaling pathway mediates the effect of FUT4 on the apoptosis in A431 cells induced by CPA, FUT4-overexpressed cells were induced by 100 μg/ml CPA with or without the inhibitors of ERK1/2 (PD98059) and/or PI3K (LY294002). The level of apoptosis-related proteins was analyzed by Western blot. The apoptotic proteins Bax, Caspase 3, and PARP were increased and the anti-apoptotic protein Bcl-2 was decreased in FUT4-overexpressed cells induced by CPA. This change was prevented by pretreatment with the ERK1/2 inhibitor PD98059 (10^{-5} μmol/L) and/or the PI3K inhibitor LY294002 (10^{-6} μmol/L) (Fig. 4). The results suggested that the expression of Bax, Caspase 3, and PARP, as well as Bcl-2, was regulated by ERK/MAPK and PI3K signaling pathways with FUT4-expression regulation. It thus concluded that FUT4 may play an anti-apoptosis effect on cell apoptosis via ERK/MAPK and PI3K signaling pathways.

DISCUSSION

In this study, the effects of FUT4 on apoptosis induced by CPA in human epidermoid carcinoma A431 cells were investigated. Our data demonstrated that CPA resulted in the apoptosis in a dose-dependent manner in A431 cells. We found that CPA-induced apoptosis was inhibited by FUT4 through affecting apoptosis-related proteins via the ERK/MAPK and PI3K/Akt signaling pathways.

Cell surface molecules undergo specific changes during cell maturation, proliferation, and apoptosis, including the expression level of some proteins and alterations in sugar chains [Dreyfuss et al., 2009]. Among these, the fucosylation of the glycoproteins plays an important role in the cell apoptosis. For example, Gregory T.C. Moore reported that hFUT1 increased TCR signaling and apoptosis in hFUT1 transgenic mice [Moore et al., 2008]. Hao Wang reported that α1,3 fucosyltransferase-VII modified the susceptibility of apoptosis induced by ultraviolet and retinoic acid in human hepatocarcinoma cells [Wang et al., 2007]. Fucosyltransferase VII
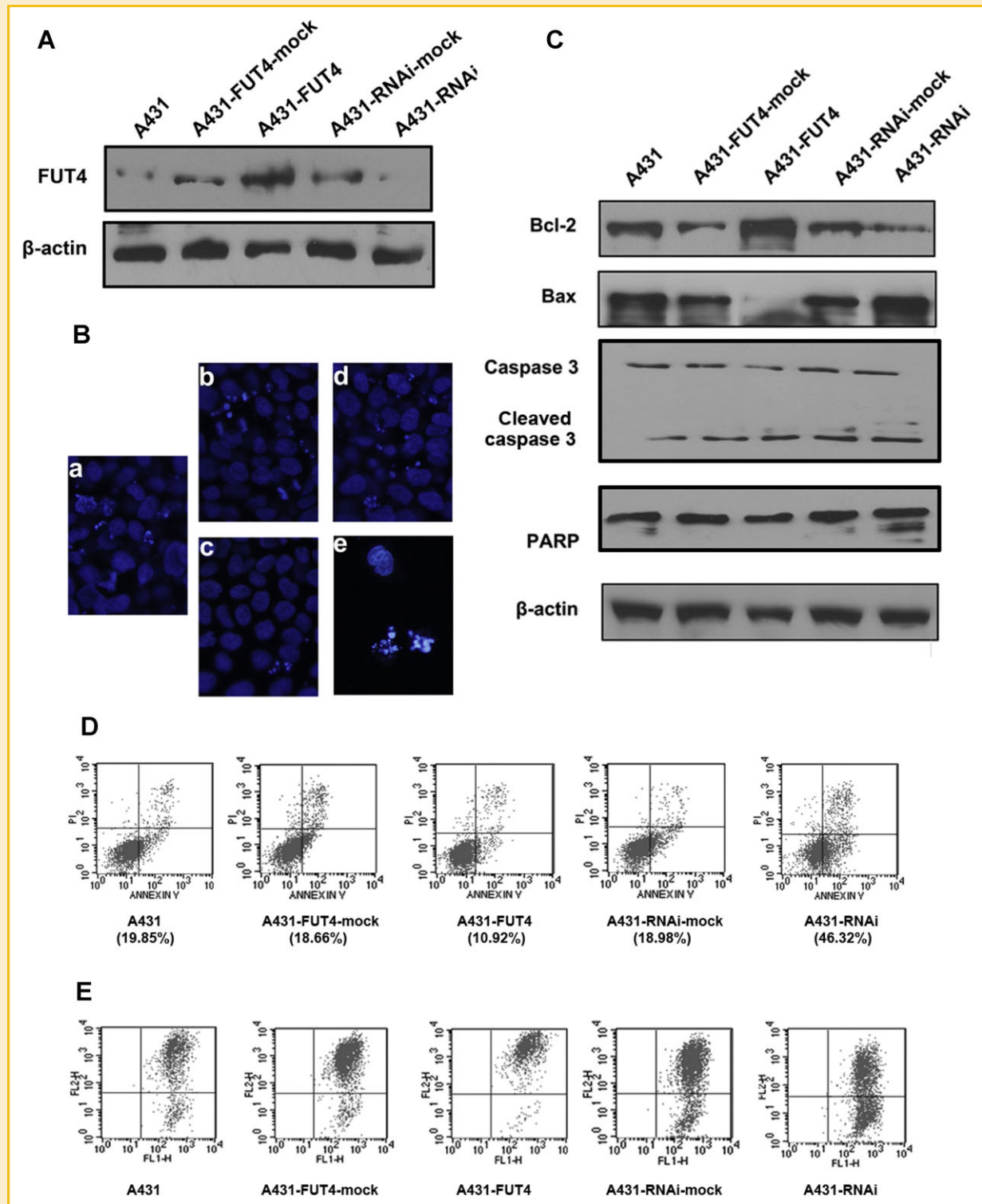
Fig. 2. FUT4 expression inhibited the apoptosis of A431 cells. A: Western blot analysis showing the levels of FUT4 in untreated A431 cells or transfected with FUT4 cDNA and FUT4 interfered plasmid as well as relative vector control. B: Hoechst 33342 staining treated as indicated above. (a) A431: untreated cell control; (b) A431–FUT4 mock: cells transfected with vector for A431–FUT4 transfectant; (c) A431–FUT4: FUT4 overexpression cells; (d) A431–RNAi mock: cells transfected with vector for FUT4 RNAi transfectant; (e) A431–RNAi: cells transfected with FUT4 interfered plasmid cells. C: Effect of FUT4 on expression of Bcl–2, Bax, Caspase 3 and PARP proteins in A431 cells. D: Cell apoptosis were induced with CPA (100 μg/ml). After 24 h cells were stained with Annexin V–fluorescein and PI. Percentage of Annexin–V/PI stained cells was determined by flow cytometry. E: Analysis of mitochondrial membrane potential (Δψm). Cells were treated with CPA (100 μg/ml). Change in mitochondrial membrane potential was determined by flow cytometric analysis with JC–1 dye. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
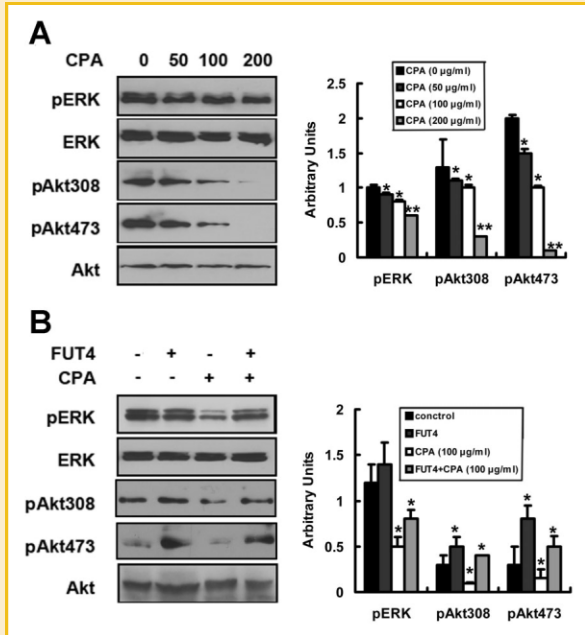
Fig. 3. Effect of FUT4 on the activities of ERK/MAPK and Akt in CPA treated A431 cells. A: CPA inhibited ERK1/2 and Akt phosphorylation. Cells were treated with different concentrations of CPA (0, 50, 100, and 200 μg/ml) for 24 h. Cell lysates were prepared as described in the "Materials and Methods" Section, and then analyzed by Western blot with antibodies against pERK1/2, pAkt308, and pAkt473. B: The effect of FUT4 on the expression of pERK, pAkt308, and pAkt473 induced by CPA. FUT4 overexpression cells were treated with CPA of 100 μg/ml. Western blot analysis was used to show the pERK, pAkt308, and pAkt473 expression levels. *P < 0.05; **P < 0.01.
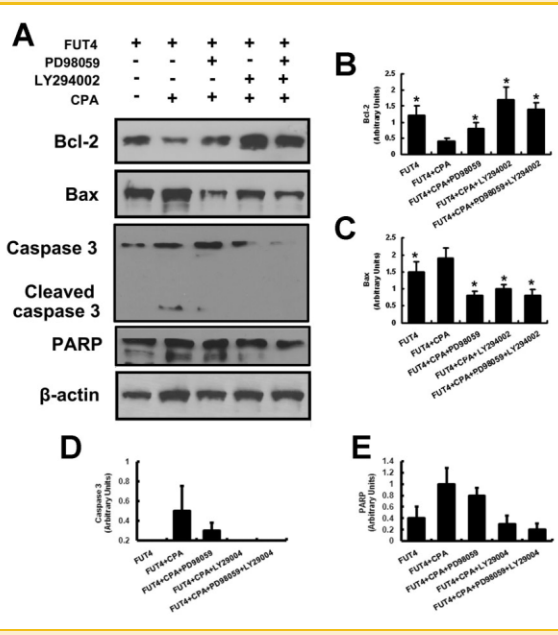

Fig. 4. Anti-apoptosis effect of FUT4 through ERK/MAPK and PI3K/Akt signaling pathways in CPA treated cells. Cell apoptosis were induced by CPA (100 μg/ml) followed by with or without the specific inhibitor of ERK1/2 (PD98059) and inhibitor of PI3K (LY294002) for 24 h. Cell lysates were immunobloted and detected with the antibodies for apoptosis analysis. A: Representative blots showing Bcl-2, Bax, Caspase 3, and PARP expression. B–E: The values were enumerated by densitometry of the bands of Bcl-2 (B), Bax (C), Caspase 3 (D), and PARP (E). The data showed the mean ± SEM of three independent experiments. *P < 0.05; **P < 0.01.

alone participated in the E-selectin-mediated growth inhibition and apoptosis observed in hematopoietic progenitor cells [Winkler et al., 2004]. But the role of FUT4 in the apoptosis of A431 cells was not reported.

Apoptosis is a controlled physiological process which is inhibited in cancer cells [Lowe and Lin, 2000; Schulze-Bergkamen and Krammer, 2004]. Many molecules and factors have been reported to be linked with the regulation of apoptosis, in which Bcl-2 and Bax genes are suggested to play a major role in determining cell survival or death after apoptotic stimuli [Hector and Prehn, 2009; Kang and Reynolds, 2009]. Many anti-apoptosis molecules, such as Bcl-2 family, have been shown to inhibit the apoptosis [Chang et al., 2009; Zhao et al., 2009]. Apoptosis in A431 cells induced by different stimuli showed the expression change of Bcl-2 and Bax [Ishimaru et al., 2009; Prasad et al., 2009]. In this study, we found that FUT4 overexpression increased anti-apoptotic protein (Bcl-2) expression and reduced apoptotic protein (Bax) expression in A431 cells. Caspase, a family of cysteine-dependent proteases, is the executioner of apoptosis, which is activated by a cascade mechanism. Among the members of the caspase family, Caspase 3 is most popular and acts the final protease in the caspase cascade [Cho and Choi, 2002]. Functionally, active Caspase 3 forms a proteolytic cascade capable of cleaving and activating specific substrates, including PARP [Cho and Choi, 2002; Jin and El-Deiry, 2005; Vermeulen et al., 2005]. In our study, we investigated the change of

Caspase 3 and PARP expression induced by CPA in A431 cells, indicating that the change of caspase cascade was involved in apoptosis of A431 cells. The apoptosis inducing effect of CPA in FUT4 expression cells was weakened, indicating the expression level of FUT4 may influence the sensitivity of CPA. Taken together, we concluded that FUT4 showed the anti-apoptosis effect on A431 cells.

Cell apoptosis is tightly controlled by the complex regulatory networks [Jin and El-Deiry, 2005]. However, the signaling pathways via which FUT4 exerts anti-apoptosis effect are not fully understood. Two signaling pathways ERK/MAPK and PI3K/Akt are involved in apoptosis. ERK/MAPK is found to regulate cellular activities, such as cell growth, differentiation, and apoptosis in mammalian cells [Abe et al., 2000; Baines and Molkentin, 2005]. PI3K/Akt pathway plays a critical role in cell survival by regulating caspase-mediated apoptosis, and the inhibition of this signaling leads to apoptosis in various cell types [Lusis, 2000]. Several studies demonstrated that activation of PI3K and its downstream effector Akt had been shown to suppress the apoptosis and promote cell survival [Brunet et al., 1999; Mullonkal and Toledo-Pereyra, 2007]. In this study, we demonstrated that FUT4 overexpression up-regulated phosphorylation of ERK and Akt which was inhibited by CPA in dose-dependent manner. When blocking the ERK/MAPK and PI3K/Akt pathways with the specific inhibitors (PD98059 and LY294002), we found that the expression of Bcl-2 increased and BAX, Caspase 3, and PARP decreased (Fig. 4). During the apoptosis in this study, there are many

factors to affect the apoptosis such as FUT4, CPA, ERK, and Akt. In Figure 2B, we could learn that CPA decreased the phosphorylation of ERK and Akt significantly. While in the FUT4-overexpressed cells, the degree of decreasing the phosphorylation of ERK and Akt by CPA was less than that of the A431 cells. So we concluded that the effect of these factors had the quantitative relationship. Our observations revealed that FUT4 overexpression did not completely abolish CPA-induced apoptosis, suggesting that the other regulators might contribute to CPA-induced apoptotic cell death. But the real result demonstrated that FUT4 played an important role during the course. We thought FUT4 had an antagonistic function to CPA. So FUT4 overexpression decreased the function of PD98059 and LY294002 when treated with CPA. Then the changes of apoptosis relative proteins were shown in Figure 4. That further confirmed that the ERK/MAPK and PI3K/Akt pathways were required in mediating the anti-apoptosis effect of FUT4 on the apoptosis of A431 cells induced by CPA. The results supported the notion that both the ERK/MAPK and PI3K/Akt pathways contributed independently to CPA-induced apoptosis in A431 cells were affected by FUT4 expression. The hypothetical schematic demonstrated the relationship of all these apoptosis-related molecules and the effect of CPA and FUT4 on cell apoptosis (Fig. 5).

According to the results above, we concluded that FUT4-induced resistance to the chemotherapeutic agent CPA. Chemoresistance is a major obstacle of chemotherapy of cancer. It is known that chemotherapy induces drug resistance in cancer cells, resulting in treatment failure [Gatti and Zunino, 2005].

FUT4 is an essential enzyme that catalyzes the synthesis of LeY oligosaccharide which is a difucosylated oligosaccharide. Our previous reports indicated that up-regulation and down-regulation of FUT4 could change the level of LeY, and LeY of EGFR changes the activation of EGFR/MAPK signaling pathway [Zhang et al., 2008]. We also demonstrated that LeY synthesis was increased in FUT4-overexpressed cells and decreased in FUT4-RNAi cells (data were not shown). In this study, we mainly discussed the effect of FUT4 on cell apoptosis. We concluded that FUT4 exerted anti-apoptosis effect.

We deduced that the anti-apoptosis effect of FUT4 probably is through regulating the synthesis of LeY. LeY oligosaccharide may produce alteration in CPA–tumor cell interaction, or change in cellular response in apoptotic pathways, or increased expression of defense factors involved in reducing intracellular drug concentration. Finally, FUT4 showed the chemoresistance effect. Further study is needed to demonstrate this mechanism.

In summary, the data presented here suggest that FUT4 inhibits cell apoptosis induced by CPA through decreasing the expression of apoptotic proteins Bax, Caspase 3, and PARP and increasing the expression of anti-apoptotic protein Bcl-2 via the ERK/MAPK and PI3K/Akt signaling pathways in A431 cells. Study of the effect of FUT4 on apoptosis will be helpful to understand the molecular mechanism of cancer and supply the research basis and ways for the specific treatment of cancer with abnormally elevated FUT4 level.

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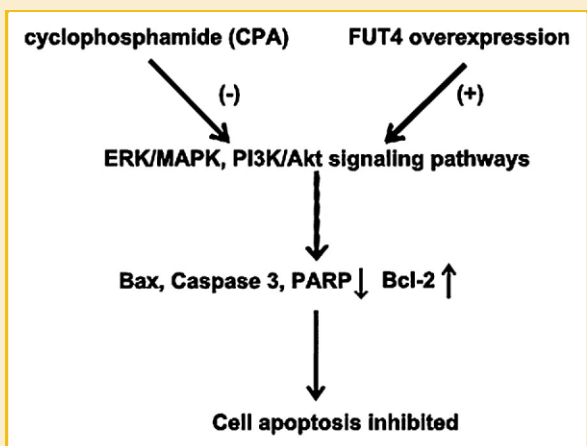
Fig. 5. The schematic illustration summarized the effect of CPA and FUT4 on cell apoptosis of A431 cells through ERK/MAPK and PI3K/Akt signaling pathways.

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