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The BH3-only protein, PUMA, is involved in oxaliplatin-induced apoptosis in colon cancer cells

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ABSTRACT

Oxaliplatin, the first line chemotherapeutic of colon cancer, induces damage to tumors via induction of apoptosis. PUMA (p53 up-regulate modulator of apoptosis) is an important pro-apoptotic member of Bcl-2 family and regulated mainly by p53. Here we investigated the role of PUMA in oxalipaltin-induced apoptosis and the potential mechanism. We showed that oxaliplatin-induced PUMA expression in a time- and dose-dependent manner and suppression of PUMA expression by stable transfecting anti-sense PUMA plasmid decreased oxaliplatin-induced apoptosis in colon cancer cells. By abrogating the function of p53, we further demonstrated that the induction was p53-independent. We also found that oxaliplatin could inactivate ERK and suppression of ERK activity by its specific inhibitor (PD98059), and dominant negative plasmid (DN-MEK1) enhanced the oxaliplatin-induced PUMA expression and apoptosis in a p53-independent manner. Taken together, our data suggest that PUMA plays an important role in oxaliplatin-induced apoptosis and the induction could be both p53-dependent and p53-independent. Moreover, PUMA expression and apoptosis in oxaliplatin-treated colon cancer cells could be regulated partly by ERK inactivation. Identification of the molecular components involved in regulating the cellular sensitivity to oxaliplatin may provide potential targets for development of novel compounds that may be useful in enhancement of oxaliplatin cytotoxicity in p53 deficient colon cancer.

1. Introduction

Oxaliplatin (1R, 2R-diaminocyclohexane) is the first of several analogs from the diaminocyclohexane platinum family to be successfully developed in the clinic and has a non-hydrolyzable diaminocyclohexane (DACH) carrier ligand which is maintained in the final cytotoxic metabolites of the drug [1,2]. It produces the same type of inter- and 1, 2-GG intrastrand cross-links like cisplatin but has a spectrum of activity and mechanisms of action and resistance different from those of cisplatin and carboplatin [3]. Oxaliplatin has shown a wide spectrum of antitumor effects both in vitro and in vivo and has a better safety profile than cisplatin. A lack of cross-resistance with

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cisplatin and carboplatin makes it a promising chemotherapy agent [4,5].

Inducing cancer cells to apoptosis is an important action of oxaliplatin [6], but the precise molecular mechanism is not very clear. As Bcl-2 family members play a pivotal role in the intrinsic apoptotic cascade [7–9], therefore, we investigated the roles of PUMA (p53 up-regulate modulator of apoptosis) in oxiliplatin-induced apoptosis. PUMA was identified by Yu and Nakano et al. in 2001 [10]. It belongs to BH3-only protein family and is found to locate at mitochondrial membrane and interacts with Bcl-2 and Bcl-X_L through a BH3 domain [11,12]. When apoptotic stimuli induces PUMA expression, it will make Bax translocates to the mitochondria membrane and multimerizes [13], cytochrome c releases and apoptosis appears. Previous studies have shown that PUMA is a direct mediator of p53 through the cytochrome c/Apaf-1-dependent pathway [14] and plays an important role in stress-induced apoptosis [15,16]. The balance between $p21^{WAF1/CIP1}$ and PUMA is pivotal in determining the cells to cycle arrest or apoptosis in human cancer.

Mitogen-activated protein kinases (MAPKs) are serine/threonine kinases that play an important role in signal transduction from the cell surface to the nucleus. The mammalian MAPKs can be divided into extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs), and p38 MAPKs. In response to a wide range of extracellular stimuli, the MAPK cascades determine cell fate, including cell growth, differentiation, and apoptosis [17]. Generally, the ERK cascade is mainly involved in the regulation of cell proliferation in a variety of cells [18], while the JNK and p38 MAPK pathways are responsible for induction of differentiation and apoptosis [19]. In our present study, we determined the role of PUMA in oxaliplatin-induced colon cancer cell apoptosis and attempted to find the potential role of MAPK signaling pathway in this process. Our data showed that oxaliplatin induced PUMA expression in a p53-independent manner. Suppression of PUMA expression decreased oxaliplatin-induced apoptosis in colon cancer cells. Blockade of ERK activity increased the PUMA expression and apoptosis in oxaliplatin-treated colon cancer cells in a p53-independent manner. These results suggest that oxaliplatin-induced ERK inactivation is involved in the regulation of oxaliplatin-induced PUMA expression and apoptosis.

2. Materials and methods

2.1. Materials

RPMI 1640 medium, FBS (fetal bovine serum) were purchased from GIBCO. Oxaliplatin (1*R*, 2*R*-diaminocyclohexane) and pifithrin-alpha (p53 inhibitor) were obtained from Sigma (Poole, Dorset, UK). SP600125 (2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one) and PD98059 (2V-amino-3V-methoxyflavone) were purchased from Calbiochem (La Jolla, CA). p53 (DO-1) antibody, p73 antibody, p21/Waf-1 antibody, p38 MAPK (Tyr183/Tyr185) antibody, phospho-specific JNK (Tyr183/Tyr185) antibody, phospho-specific ERK1/2 antibody, total (unphosphorylated) ERK1/2, JNK1/2 antibodies, actin antibody and secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). PUMA antibody and Hoechst dye 33258 were obtained from Sigma. All other chemicals were of analytical grade. The plasmid pEF HA (hs) Puma hygro containing PUMA full sequence was a gift from Andreas Strasser (The Walter and Eliza Hall Institute of Medical Research Melbourne, Australia). pSUPER-p53 plasmid was a gift from Thijn R. Brummelkamp (Division of Molecular Carcinogenesis, The Netherlands Cancer Institute, Netherlands). DN-MEK1 plasmid was constructed as previously described [20].

2.2. Cell culture

Human colon cancer cell lines lovo and SW1116 were obtained from American Type Culture Collection (Manassas, VA). Cells were cultivated in 75 cm^2 flasks in RPMI 1640 medium supplemented with 10% (v/v) FBS and penicillin-streptomycin (100 U/ml). Cells were maintained at 37 °C in a humidified 5% CO_2 atmosphere until confluency and subcultured using trypsin (0.05%, w/v)/EDTA (0.02%, w/v). The inhibitors SP600125 and PD98059 were dissolved in dimethyl sulphoxide (DMSO). Control was treated with 0.1% DMSO alone. Cells for Western blotting were grown in six-well cluster dishes, whereas cell viability assays were performed using 96-well plates. All treatments were carried out on cells at 85–90% confluence, p53 inhibitor and kinases inhibitors were added an hour before cytotoxic drug subsequently added.

2.3. Reverse transcriptase-polymerase chain reaction

0.5 μg total RNA was isolated from the cells using TRIZOL (Gibco). PUMA mRNA expression was determined by RT-PCR analysis using a RevertAid First Strand cDNA Synthesis Kit (MBI) and PCR kit (SBS Genetech Co. Ltd., Beijing, China). The two gene-specific primers used for amplification were as follows: (upper) gacgacctcaacgcacagta and (lower) ccagggtgtcaggaggtg. PCR products were electrophoresed in 1.5% agarose gel. Beta-actin mRNA was also amplified as an internal control. The experiment was repeated twice.

2.4. Western blotting analysis of PUMA, p73 and ERK1/2, JNK, p38 MAPK activation

Cells were washed with ice-cold PBS twice and lysed with ice-cold lysis buffer (20 mM Tris–HCl, 1 mM EDTA, 1 mM EGTA, 1 mM sodium vanadate, 0.2 mM phenylmethylsulphony1 fluoride, 0.5% NP-40, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml Pepstatin) and PMSF (1:100). The lysates were transferred to 1.5 ml eppendorf microcentrifuge tubes on ice and vortex 5–6 times in 20 min. Insoluble material was removed by centrifugation. Soluble protein was removed to a new tube and stored at −20 °C until required. Protein samples (15 μg) were separated by sodium dodecyl sulphate/polyacrylamide gel electrophoresis (SDS/PAGE; 12% acrylamide gel) using a Bio-Rad Mini-Protean III system (45 mA for about 2 h). Proteins were transferred to PVDF membranes using a Hoefer® semiphor™ system, 92 mA for 3.5 h in transfer buffer (48 mM Tris base, 39 mM glycine,

0.037% SDS and 20% methanol). Following transferring, the membranes were blocked for 1 h at room temperature with 5% (w/v) skimmed milk powder in Tris buffered saline–Tween 20 (0.1% by volume, TBS-T). Blots were then incubated at room temperature with primary antibodies in 1% (w/v) skimmed milk powder dissolved in TBS-T (1:500 dilution). Primary antibodies were removed and the blots were extensively washed with TBS-T for three times. Blots were then incubated for 1 h at room temperature with the secondary antibodies (donkey anti-goat or anti-rabbit antibody coupled to horseradish peroxidase, 1:2000 dilution) in 1% (w/v) skimmed milk powder dissolved in TBS-T. Following removal of the secondary antibody, blots were extensively washed as above for an hour and developed using the Enhanced Chemiluminescence detection system (Amersham Pharmacia Biotech, Piscataway, NJ) and quantified using the GeneTools systems.

2.5. Construction of PUMA inserted into pcDNA3.1- vector

To make the construct for stable transfections in lovo cells, PUMA anti-sense cDNA was inserted into the mammalian expression vector pcDNA3.1- (Invitrogen) (pcDNA3.1-/PUMAAS). Briefly, a 592-bp PUMA cDNA fragment that included the start codon region was obtained by digesting the plasmid pEF HA (hs) Puma hygro with BamH1 and XbaI sites of pcDNA3.1-. This vector contains a neomycin gene for selection of genetincin resistant colonies. The orientation of the insert was confirmed by sequencing.

2.6. Transient and stable transfections

Transient transfection was performed 24 h before oxaliplatin was added using Lipofectamine reagent (Invitrogen) according to the manufacturer's instructions. Briefly, cells were seeded at 80% density in six-well plates and a DNA/liposome mix containing 1 μg plasmid (if necessary) and 6 μl lipofectmine 2000 was used. Transfections were allowed to proceed for 3 h in the absence of serum and the cells recovered in medium supplemented with reduced serum level (2.5%) for 21 h. For the stable transfections, the transfection mix (serum-free) was removed from 10 cm dishes and replaced with RPMI 1640 medium with 10% FBS after 3 h. Cells were passaged at 1:5 into fresh medium 24 h after transfection. Stable transfectants were selected in the presence of 600 μg/ml G418 48 h later. After selection, a resistant colony to G418 that we chose as total population was cloned by limiting dilution to a single-cell/well until that anti-sense expression was stable along the passages.

2.7. Flow cytometric analysis of apoptosis

A TACS^{TM} Annexin V-FITC Apoptosis Detection Kit (R and D systems) was used to detect apoptosis. Briefly, 1×10^6 cells harvested by trypsinization, were pooled, washed in 500 μl cold phosphate-buffered saline, and re-suspended in 200 μl incubation reagent. After incubate in the dark for 15 min at room temperature, stained cells were added another 800 μl 1× binding buffer and immediately analyzed by flow cytometry.

2.8. Cell morphology and immunofluorescence staining for apoptosis

For analysis of apoptosis by nuclear staining with Hoechst dye 33258 (Sigma Chemical Co.), cells were stimulated according to experimental protocols, washed once with PBS and then fixed with ice-cold methanol (500 μl/well) for 10 min. After fixing, cells were washed twice with PBS, stained with 1 μM Hoechst dye 33258 for 10 min, and then extensively washed twice with PBS and distilled water. Apoptosis was indicated by the presence of condensed or fragmented nuclei which bound the Hoechst dye with high affinity. For analysis of cell structure by photo-microscopy, coverslips were washed once with PBS and then inverted and mounted on to glass slides. Cells were visualized using an Olympus microscope. Two hundred cells in three randomly chosen fields were counted and scored for the incidence of apoptotic chromatin changes under fluorescence microscopy.

2.9. MTT assay

Cultured cells were harvested from 90% confluent monolayer cultures by a brief trypsinization with 0.1% trypsin and 0.1% EDTA. The cells were seeded at a density of 2000 cells per well of 96-well tissue culture plates and cultured for 12 h in the regular medium. The cells were washed with PBS twice and treated under conditions mentioned in Section 3. Cell growth was monitored after 24 h by 3-(4, 5-dimethylthiazol-2yl)-2, 5 diphenyltetrazolium bromide (MTT) assay [21].

2.10. Statistical analysis

Data were presented as mean ± S.D. of three independent experiments. Comparison of the effects of various treatments was performed using one-way analysis of variance and a two-tailed *t*-test. Differences with a p value of <0.05 were considered statistically significant.

3. Results

3.1. Oxaliplatin induced PUMA expression in colon cancer cells in a time- and dose-dependent manner

PUMA plays an important role in stress-induced apoptosis, but its role in oxaliplatin-induced apoptosis is unknown. To assess the effects of oxliplatin on PUMA expression, we exposed lovo cells to a dose range of oxaliplatin at different time points as described in Section 2 and measured its expression by Western blotting. As shown in Fig. 1, oxaliplatin induced PUMA expression in colon cancer cells and the induction was in a time- and dose-dependent manner. The PUMA expression reached a maximum of 2.5-fold increase at a concentration of 4.8 μM (Fig. 1A), and oxaliplatin-induced up-regulation of PUMA expression reached the peak at 24 h (Fig. 1B). To investigate whether oxaliplatin increases PUMA mRNA at the level of transcription, a standard RT-PCR experiment described in methods

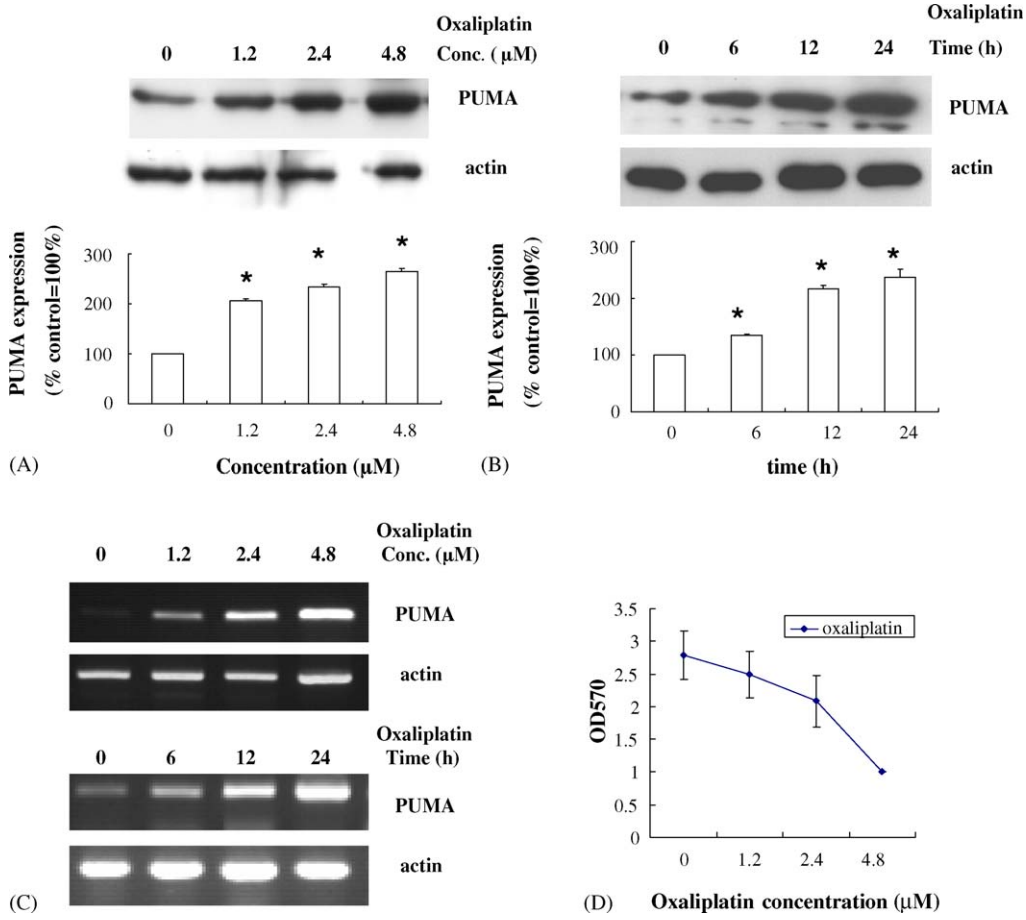


Fig. 1 – Oxaliplatin induced PUMA expression in a time- and dose-dependent manner in colon cancer cells. (A) Lovo cells were treated with indicated concentrations of oxaliplatin for 24 h and PUMA expression was assessed by Western blotting. (B) Lovo cells were treated with 4.8 μM oxaliplatin at the time points shown. The cells were then lysed and PUMA expression was assessed by Western blotting. Data were presented as the percentage of the basal level of PUMA expression (control = 100%) in the absence of oxaliplatin and expressed as mean ± S.D. from three independent experiments. *$p < 0.05$, significant difference between cells treated with oxaliplatin and untreated cells. (C) Lovo cells were treated with indicated concentrations of the drug. mRNA expression was detected by RT-PCR using 0.5 μg total RNA. Beta-actin mRNA was amplified as an internal control. (D) An oxaliplatin dose-response curve on cell viability. Lovo cells were harvested from 90% confluent monolayer cultures by a brief trypsinization and were reseeded into 96-well culture dishes for 12 h. The cells were then treated with oxaliplatin for 24 h and growth was assessed by MTT assay. Each value is the mean of three independent experiments.

was performed. As seen in Fig. 1C, oxaliplatin increased PUMA mRNA in a time- and dose-dependent manner. Therefore, the data suggested that oxaliplatin up-regulated PUMA expression at transcription level. An oxaliplatin dose-response curve on cell viability was also determined by MTT assay (Fig. 1D), which indicated that oxaliplatin induced cell death in a dose-dependent manner.

3.2. *Oxaliplatin-induced PUMA expression was p53-independent*

PUMA is a direct mediator of p53 in stress-induced apoptosis. To determine the role of p53 in oxaliplatin-induced PUMA expression in colon cancer cells, pifithrin-alpha (PFT-alpha) was used, which was a stable, water soluble inhibitor of p53-dependent apoptosis and was also shown to reduce the activation of p53-regulated genes, including cyclin G, p21/Waf-1 and MDM2 [22]. The effect of PFT-alpha on oxaliplatin-treated colon cancer cells was assessed by MTT assay. PFT-alpha enhanced cell survival after oxaliplatin treatment (Fig. 1A). p21 expression was also decreased in the cells treated with PFT-alpha (20 μM), suggesting that p53 signaling was inhibited. Moreover, oxaliplatin could induce PUMA expression even when the function of p53 was abrogated by PFT-alpha (Fig. 2B). We also used SW1116, a p53 mutated colon cancer cell line, to confirm the result shown in Fig. 2B. We found that oxaliplatin induced PUMA expression in both p53 wide-type cell line (lovo) and p53 mutated cell line (SW1116) (Fig. 2C). These results suggested that oxaliplatin-induced PUMA expression could be both p53-dependent and p53-independent.

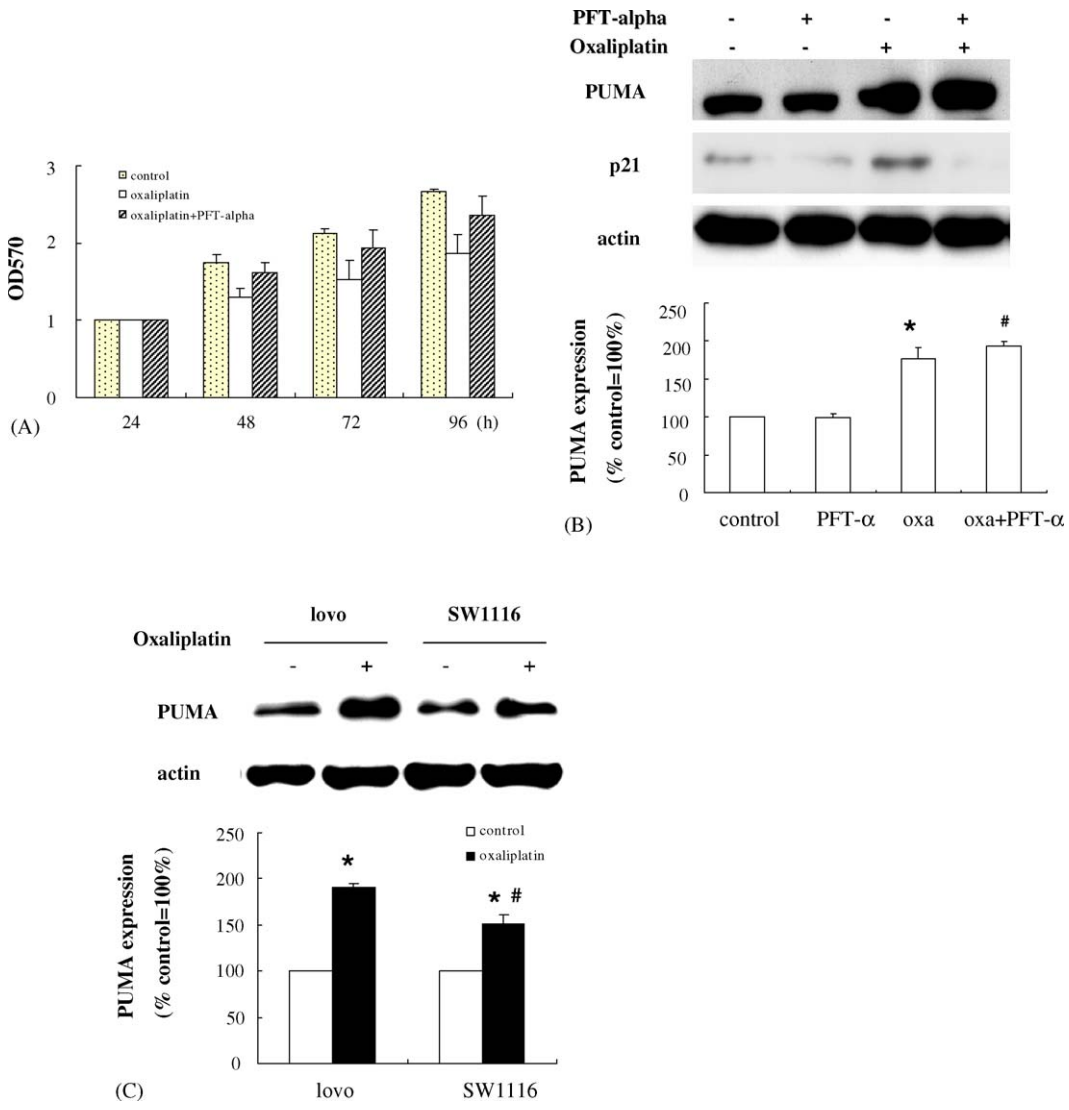


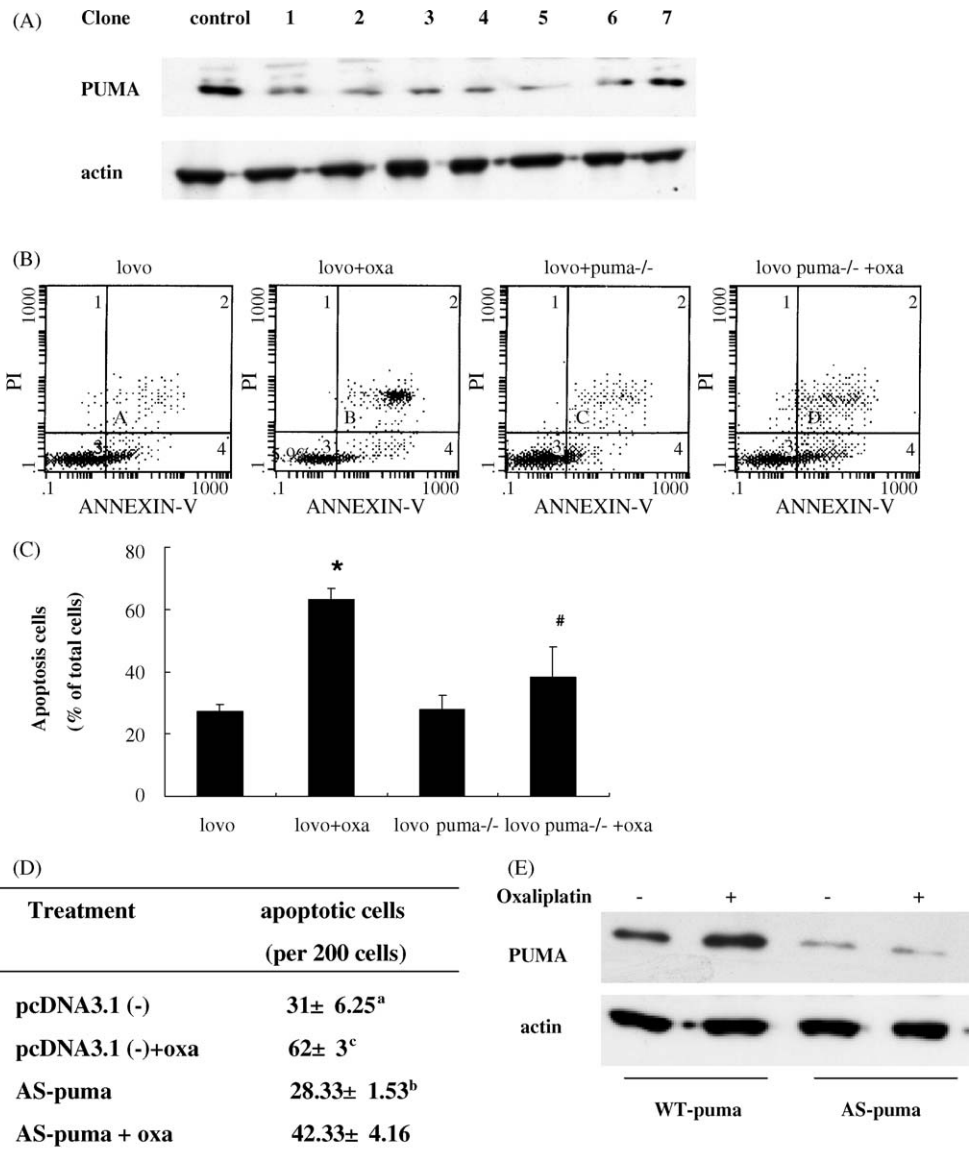
Fig. 2 – Oxaliplatin induced PUMA expression in a p53-independent manner. (A) Lovo cells were incubated with 4.8 μM oxaliplatin at the time point indicated in the presence (+) or absence (−) of 20 μM PFT-alpha and cell survival was assessed by MTT assay. (B) Lovo cells were incubated with 4.8 μM oxaliplatin for 24 h in the presence (+) or absence (−) of 20 μM PFT-alpha and expression of PUMA and p21 was assessed by Western blotting analysis. (C) Oxaliplatin-induced PUMA expression was detected in both lovo (p53 wild-type) and SW1116 (p53 mutated) cell lines by Western blotting analysis. Data were presented as the percentage of the basal level of PUMA expression (control = 100%) in the absence of oxaliplatin and expressed as mean ± S.D. from three independent experiments. $^{*}p < 0.05$, significant difference between cells treated with oxaliplatin and untreated cells. $^{\#}p > 0.05$ no significant difference was observed between cells treated with PFT-alpha and untreated. $^{\$}p > 0.05$, no significant difference between lovo cells and SW1116 cells treated with oxaliplatin.

3.3. *Suppression of PUMA expression decreased oxaliplatin-induced apoptosis*

To determine the role of PUMA in oxaliplatin-induced apoptosis, we suppressed PUMA expression by stable transfection of PUMA anti-sense vector into lovo cells. As shown in Fig. 3A, six clones expressing low level of PUMA protein, named as lovo puma−/− cells, were confirmed by Western blotting. The effects of PUMA suppression in oxaliplatin-induced apoptosis in lovo cells were assessed by flow cytometry and Hoechst 33258 staining. As shown in Fig. 3B–D, comparing with lovo puma wide-type cells, oxaliplatin-induced apoptosis was significantly decreased in lovo puma−/− cells. To verify the function of PUMA in this process, we assessed PUMA expression by Western blotting and found that oxaliplatin-induced PUMA expression was abrogated in lovo puma−/− cells (Fig. 3E).

3.4. *Oxaliplatin induced inactivation of ERK1/2 and activation of JNK1/2, but it had no effect on p38 or the expression of p73*

Since MAPKs pathway is associated with cell survival and stress-induced apoptosis, we determined the effects of



Treatment	apoptotic cells (per 200 cells)
pcDNA3.1 (-)	31± 6.25[a]
pcDNA3.1 (-)+oxa	62± 3[c]
AS-puma	28.33± 1.53[b]
AS-puma + oxa	42.33± 4.16

Fig. 3 – Suppression of PUMA expression conferred resistance to oxaliplatin-induced apoptosis. (A) PUMA expression was suppressed by stable transfection of the PUMA anti-sense vector as described in the methods and suppression of PUMA expression was confirmed by Western blotting. Lovo cells stable transfected with pcDNA3.1- vector were as a control. (B) Apoptosis was assessed by flow cytometry. 1×10^6 lovo puma wide-type cells and lovo puma−/− cells were treated with oxaliplatin (4.8 μM) for 24 h and were analyzed using Annexin V-FITC and propidium iodide (PI) staining as described in the methods. Each panel showed a typical flow cytometric histogram of 1000 cells/sample from a representative experiment. (C) Composite data (mean ± S.D.) from three experiments performed as in (B). *$p < 0.05$, significant difference between oxaliplatin treated and untreated lovo cells; #$p < 0.05$, significant difference between lovo and love puma−/− cells after oxaliplatin treatment. (D) Apoptosis was assessed by Hoechst 33258 staining. Two hundred cells in three different views were chosen to count the apoptotic cells. Results were shown in the table and expressed as mean ± S.D. from three independent experiments. (E) Lovo puma wide-type cells and love puma−/− cells were treated with oxaliplatin (4.8 μM) for 24 h and PUMA expression was assessed by Western blotting. (a) $p < 0.05$, significant difference between cells stable transfected with pcDNA3.1- vector and cells treated with pcDNA3.1- vector plus oxaliplatin (4.8 μM). (b) $p < 0.05$, significant difference between lovo puma−/− cells and lovo puma−/− cells plus oxaliplatin (4.8 μM). (c) $p < 0.05$, significant difference between cells stable transfected with pcDNA3.1- vector plus oxaliplatin and lovo puma−/− cells plus oxaliplatin. (oxa: oxaliplation; AS-puma: lovo puma−/− cells; WT-puma: lovo puma wide-type cells).

oxaliplatin on the activities of ERK1/2, JNK1/2 and p38 MAPK in colon cancer cells. Lovo cells were starved in serum-free RPIM 1640 medium for 12 h before incubating with 4.8 μM oxaliplatin. Our data showed that ERK1/2 was inactivated and JNK1/2 was activated by oxaliplatin in a dose-dependent manner (Fig. 4A and B). However, oxaliplatin had no effect on the expression of p73 expression (Fig. 4C) or p38 activation (data not shown).

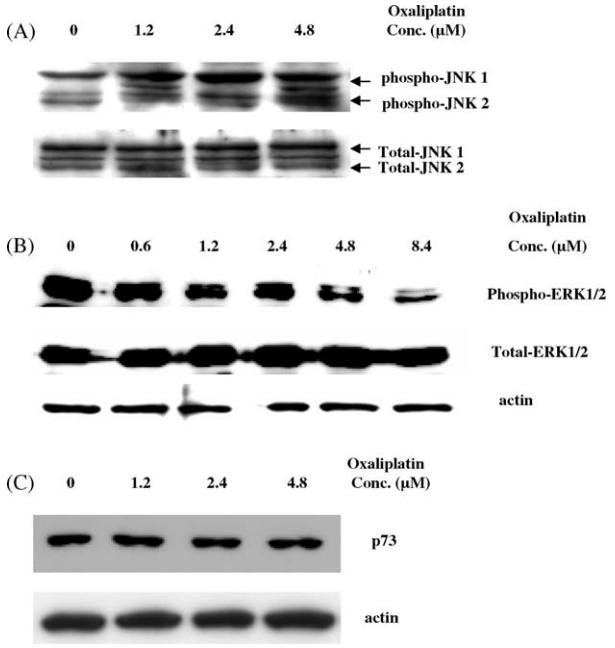


Fig. 4 – Oxaliplatin induced activation of JNK1/2 (A) and inactivation of ERK1/2 (B), but not p38 (data not shown) or expression of p73 (C) in colon cancer cells. 2×10^5 per well lovo cells were seeded in six-well-plate one day before the experiment and incubated with serum-free RPIM1640 medium for 12 h. The starved cells were treated with oxaliplatin for 24 h under the indicated concentrations, thereafter cells were lysed, MAPKs activity and p53 expression were assessed by Western blotting analysis.

3.5. *Suppressing the activation of ERK enhanced oxaliplatin-induced PUMA expression and apoptosis in a p53-independent manner*

Starved cells were treated with JNK inhibitor SP600125 (20 μM) and ERK inhibitor PD98059 (20 μM) separately an hour before oxaliplatin (4.8 μM) was added. PUMA expression was determined by Western blotting analysis. Fig. 5A indicated that PD98059 enhanced oxaliplatin-induced PUMA expression, which is consistent with the result that oxaliplatin inactivated ERK. And SP600125 had no effect on PUMA expression. To confirm this result, we suppressed the activation of ERK by transient transfection of DN-MEK1 plasmid into the cells and our data suggested that suppressing the activation of ERK further enhanced oxaliplatin-induced PUMA expression (Fig. 5B). Both lovo (p53 wide-type) and SW1116 (p53 mutated) cells were treated with PD98059. As shown in Fig. 5B, PD98059 up-regulated oxaliplatin-induced PUMA expression in both p53 wide-type cells (lovo) and p53 mutated cells (SW1116), suggesting that the PUMA induction by oxaliplatin is p53-independent. Subsequently, we inhibited p53-dependent gene transcription by PFT-alpha (20 μM). PD98059 up-regulated PUMA expression in lovo cells even the function of p53 was abrogated (Fig. 5C), further suggesting that the induction is p53-independent and that ERK signaling pathway is involved in oxaliplatin-induced PUMA expression.

To investigate the role of PD98059 and the relationship between ERK and p53 status in oxaliplatin-induced apoptosis, lovo p53 wide-type cells and lovo p53 deficient cells (lovo p53−/− cells) were treated with PD98059 an hour before 4.8 μM oxaliplatin was added. As shown in Fig. 5D, oxaliplatin and PD98059 could induce apoptosis synergistically and there was no significant difference between p53 wide-type and p53 deficient cells.

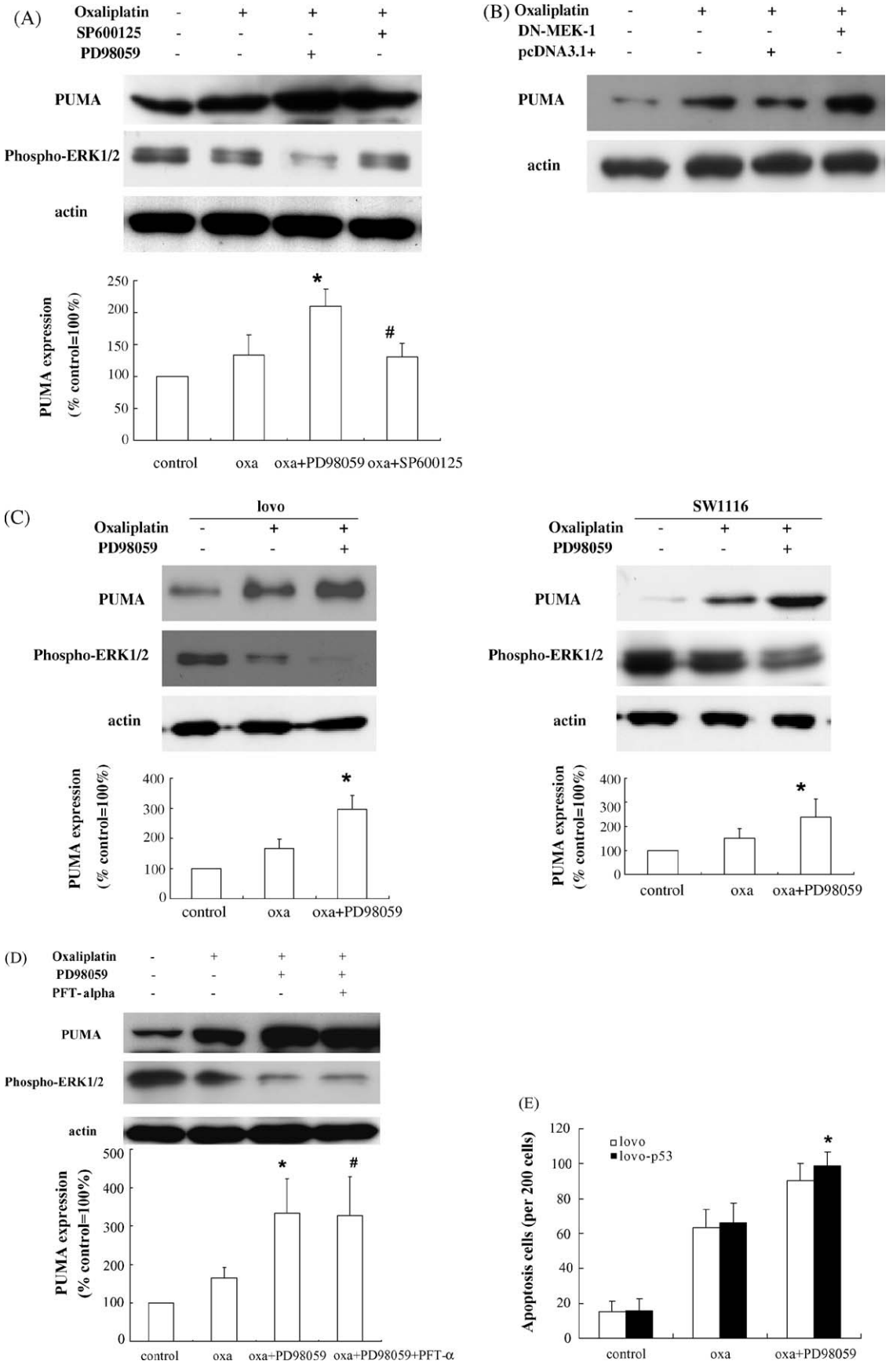
3.6. *PUMA played a role in ERK inhibitor enhanced-apoptosis in colon cancer cells*

Having shown that PD98059 can enhance oxaliplatin-induced PUMA expression and apoptosis in colon cancer cells, we subsequently determined the role of PUMA in PD98059 enhanced apoptosis. Apoptosis was evaluated by Hoechst dye 33258. When PUMA expression was suppressed by transfecting PUMA anti-sense vector, apoptosis induced by oxalipaltin and PD98059 was significantly reduced in puma−/− cells comparing with that in the control puma wide-type cells (Fig. 6).

4. Discussion

In the present study, we determined the role of PUMA in oxaliplatin-induced colon cancer cell apoptosis and the potential mechanism in this process. Our data showed for

Fig. 5 – Inhibition of ERK up-regulated the oxaliplatin-induced PUMA expression in a p53-independent manner and ERK inhibitor enhanced oxalipaltin-induced apoptosis in colon cancer cells. (A) ERK inhibitor up-regulated oxaliplatin-induced PUMA expression while JNK inhibitor had no effect. Cells were incubated with serum-free PRIM1640 medium for 12 h. Starved cells were treated with SP600125 (20 μM) and PD98059 (20 μM) an hour before oxaliplatin (4.8 μM) was added. PUMA expression was determined by Western blotting analysis 24 h later. (B) To confirm the result in (A), DN-MEK1 plasmid was transiently transfected into lovo cells 24 h before oxaliplatin (4.8 μM) treatment to suppress the activation of ERK. PUMA expression was determined by Western blotting analysis. (C) Lovo and SW1116 cells were treated with 4.8 μM oxaliplatin and ERK inhibitor up-regulated PUMA expression in both lovo and SW1116 cell lines. (D) Lovo cells were treated with pifithrin-alpha two hours before oxaliplatin was added and with PD98059 an hour before the drug was added. PUMA expression was assessed by Western blotting analysis 24 h later. (E) p53 wide type and p53 deficient cells were exposed to 4.8 μM oxaliplatin for 24 h in the absence (control) or presence of PD98059 (20 μM). Inhibitor was applied an hour prior to the treatment of oxaliplatin. Each column is the mean ± S.D. from three independent experiments. $^{*}p < 0.05$, significant difference between PD98059 treated cells and untreated cells. $^{\#}p > 0.05$, no significant difference between SP600125 treated and untreated cells. $^{\$}p > 0.05$, no significant difference between PFT-alpha treated and untreated cells.


(A)
Oxaliplatin - + + +
SP600125 - - - +
PD98059 - - + -
PUMA
Phospho-ERK1/2
actin
PUMA expression (% control=100%)
250
200
150
100
50
0
*
#
control
oxa
oxa+PD98059
oxa+SP600125
(B)
Oxaliplatin - + + +
DN-MEK-1 - - - +
pcDNA3.1+ - - + -
PUMA
actin
(C)
lovo
Oxaliplatin - + +
PD98059 - - +
PUMA
Phospho-ERK1/2
actin
PUMA expression (% control=100%)
400
300
200
100
0
*
control
oxa
oxa+PD98059
SW1116
Oxaliplatin - + +
PD98059 - - +
PUMA
Phospho-ERK1/2
actin
PUMA expression (% control=100%)
400
300
200
100
0
*
control
oxa
oxa+PD98059
(D)
Oxaliplatin - + + +
PD98059 - - + +
PFT-alpha - - - +
PUMA
Phospho-ERK1/2
actin
PUMA expression (% control=100%)
500
400
300
200
100
0
*
#
control
oxa
oxa+PD98059
oxa+PD98059+PFT-α
(E)
Apoptosis cells (per 200 cells)
120
100
80
60
40
20
0
lovo
lovo-p53
*
control
oxa
oxa+PD98059

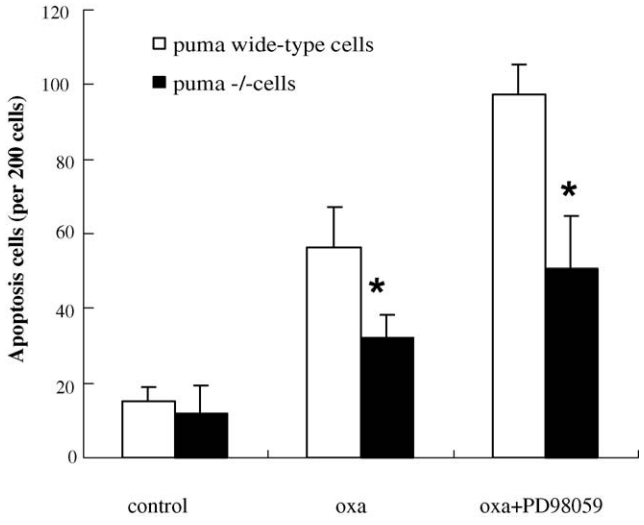


Fig. 6 – Suppression of PUMA expression abrogated the apoptosis induced by PD98059. lovo puma wide-type cells and puma−/− cells were treated with PD98059 (20 μM) an hour before oxaliplatin (4.8 μM) was added. Each column is the mean ± S.D. from three independent experiments. *$p < 0.05$, significant difference between lovo puma wide-type cells and puma−/− cells.

the first time that oxaliplatin could induce PUMA expression and the induction is p53 independent. ERK signaling pathway also appears to be essential in the regulation of oxaliplatin-induced PUMA expression and apoptosis.

Induction of apoptosis in cancer cells is a critical feature of chemotherapeutics [23]. The major mechanism of oxaliplatin-induced damage to tumors is via induction of apoptosis, but the cellular and molecular mechanism of oxaliplatin action has not yet been fully elucidated. Recent evidence indicated that PUMA, an important member of Bcl-2 family protein, was an important mediator of apoptosis induced by a number of chemotherapeutics and radiation [24,25]. These findings suggested that PUMA may be a potential common target for stress induced apoptosis and led us to hypothesize that PUMA may also play a role in platinum-induced apoptosis in colon cancer cells.

Our data showed that oxaliplatin but not cisplatin (data not shown) treatment of colon cancer cells resulted in induction of PUMA expression in a time- and dose-dependent manner and the up-regulation was at transcriptional level, suggesting that the mechanisms of cytotoxicity of these two drugs are different and need to be further studied. Furthermore to determine the effects of PUMA expression on oxaliplatin-induced apoptosis, we suppressed PUMA expression by stably transfecting the anti-sense PUMA vector into lovo cells. In consistent with our hypothesis, oxaliplatin-induced apoptosis was reduced by the suppression of PUMA expression. These data indicated that oxaliplatin-induced apoptosis was mediated by PUMA in colon cancer cells.

Previous study has shown that p53 plays an important role in the apoptotic cascade initiated by oxaliplatin and inactivation of p53 can lead to significant increased resistance to oxaliplatin [26]. Recently, PUMA protein was shown to be mainly responsible for the apoptotic responses of colorectal cancer cells to p53 induction [27]. However, it has also been shown that PUMA is an essential mediator of both p53-dependent and -independent apoptotic pathways [28,29] and other factors such as E2F1 are involved in the physiological regulation of PUMA expression [30]. Therefore, we determined the role of p53 in oxaliplatin-induced PUMA expression. To abrogate the function of p53 in lovo cells, PFT-alpha, a reversible inhibitor of p53 was used. Recent research showed that neither PFT-alpha nor beta can be regarded as a ubiquitous inhibitor of p53 function [31]. We determined the effect of PFT-alpha on p53 function in lovo cells by analyzing the expression of p21 and determined the cell survival after PFT-alpha treatment by MTT assay. Our result showed that PFT-alpha can abrogate p53 function effectively in lovo cells and found that oxaliplatin could induce PUMA expression even p53 function was abrogated. Similar data were obtained in SW1116 cells, a p53 mutated colon cancer cell line, suggesting that the induction is not linked with a particular p53 status and that there were other factors to be involved in oxaliplatin-induced PUMA expression. p73, like its homologue p53, is able to induce apoptosis in several cell types by directly transactivating PUMA [32]. We therefore determined the expression of p73 after oxaliplatin treatment and found that oxaliplatin has no effect on its expression. Previous studies have shown that MAP kinase signaling cascades are associated with cell proliferation, differentiation, apoptosis [33,34] and response to exposure to the DNA damage-inducing chemotherapeutic agent in both p53-dependent and p53-independent manner [35], we analyzed the activities of MAPKs with oxaliplatin treatment in colon cancer cells. We found that oxaliplatin induced inactivation of ERK and activation of JNK, but had no effect on the activity of p38. These results were inconsistent with the previous study [36], suggesting that the effect of p73 on PUMA and the role of p53 in the cellular response to oxaliplatin was cell type- and context-dependent. To further explore the roles of JNK and ERK in oxaliplatin-induced PUMA expression and apoptosis, we inhibited the activation of JNK and ERK separately by selective MAPKs inhibitors SP600125 and PD98059. Our results showed that ERK inhibitor could enhance PUMA expression and apoptosis induced by oxaliplatin, even when the p53 function was inhibited by stable transfection of the pSUPER-p53 vector, which can reduce endogenous and over-expressed p53 protein [37]. This data was confirmed by transient transfection of DN-MEK1 plasmid into lovo cells. Furthermore, oxaliplatin and ERK inhibitor-induced apoptosis was abrogated by suppression of PUMA expression. These data therefore suggest that the inactivation of ERK in colon cancer cells may partly contribute to the oxaliplatin-induced PUMA expression and apoptosis in a p53-independent manner. But the precise mechanisms need to be further investigated.

p53 plays a pivotal role in controlling cell cycle checkpoint regulation, DNA repair, transcription, and induced apoptosis. Lack of p53 function impairs these cellular processes, and increases resistance to chemotherapeutic regimens [38–40]. Our results showed that PUMA plays an important role in oxaliplatin-induced apoptosis in colon cancer cells and ERK signaling is also involved in oxaliplatin-induced PUMA expression and apoptosis in a p53-independent manner.

Identification of the molecular components involved in regulating the cellular sensitivity to oxaliplatin may provide potential targets for development of novel compounds that may be useful in enhancement of oxaliplatin cytotoxicity in p53 deficient colon cancer. Further investigation will be required to elucidate the downstream factors by which the ERK pathway modulates the oxaliplatin-induced PUMA expression.

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