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Knockdown of ERK/Slug signals sensitizes HL-60 Leukemia cells to Cytarabine via upregulation of PUMA

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Abstract. – OBJECTIVE: To study the mechanism of early chemosensitivity and later chemoresistance of Cytarabine (Cyt) to HL-60 cells to regulate ERK/Slug signal pathway.

MATERIALS AND METHODS: HL-60 cells were stably transfected with NF-κBp65 siRNA P53 up-regulated modulator of apoptosis (PUMA) siRNA (PUMA siRNA) and Slug siRNA respectively or exposed to 25 nM U0126 for 24h, then the cells were exposed to Cyt (250 nM) respectively. In different time point after Cyt treatment, the activity of NF-κB and the expression of Erk1/2, NF-κBp65, Slug and PUMA protein were detected. The cells survival rate and apoptotic index were detected by MTT and TUNEL methods.

RESULTS: After the HL-60 cells were exposed to Cyt (250 nM) for 24 h, the activity of NF-κB reached maximum value, and reached minimum value for 48 h. The expressions of NF-κBp65 and PUMA reached maximum value after exporser to Cyt (250 nM) for 24 h, and reached minimum value after 48 h. Erk1/2 phosphorylation and Slug expression began to increase after exporser to Cyt (250 nM) for 24 h, and reached maximum value for 48 h. The cells survival rate was decreased, and arrived to minimum value (29.5 ± 4.6, F = 12.74, $p < 0.01$). The cells apotosis rate was increased, and arrived to maximum value (14.2 ± 2.8, F = 15.02, $p < 0.01$). By inhibiting NF-κBp65 or PUMA by siRNA transfection, the cells survival rate was enhanced and the apotosis rate was significantly declined after exporsed to Cyt (250 nM). When NF-κBp65 was inhibited, the expression of PUMA was decreased. By inhibiting Erk1/2 phosphorylation and Slug expression, the cells survival rate began to decrease for 24 h, and reached the fewest at 48 h after exporsed to Cyt (250 nM) (19.8 ± 2.7, F = 11.4, $p < 0.01$ and 17.4 ± 0.6, F = 15.3, $p < 0.01$). The cells apotosis rate began to increase from 24 h, and reach a head at 48 h after exporser to Cyt (250 nM) (28.6 ± 4.7, F = 9.84, $p < 0.01$ and 27.6 ± 6.4, F = 10.31, $p < 0.01$. After Erk1/2 phosphorylation inhibited, Slug expression was lowered and PUMA expression was significantly increased. The changes are most distinct after the cells were exporser to Cyt (250 nM) for 48 h. By Slug inhibition had no effect on Erk1/2 phosphorylation, but increase the expression of PUMA.

CONCLUSIONS: The chemosensitiveness of HL-60 cells to Cyt were relation to the up-regulation of PUMA induced by NF-κB. The chemoresistance to Cyt was related to the up-regulation of Slug induced by Erk1/2, which inhibited the expression of PUMA.

Key Words:
Acute myeloid leukaemia; P53 up-regulated modulator of apoptosis (PUMA); Chemotherapy; NF-κB; ERK1/2

Introduction

Acute myeloid leukemia (AML) accounts for one-fourth of acute leukemia in children, but is responsible for more than half of the leukemia deaths in this patient population[1]. Standard treatment options for AML are chemotherapy and hematopoietic stem cell transplantation[2]. The majority of patients with AML does not achieve complete remission (CR) or are expected to relapse even with intensive chemotherapy. This is mainly due to the development of drug resistance in tumor cells[3]. Resistance to cytarabine (Cyt)-based chemotherapy is a major cause of treatment failure in this disease[4,5]. Therefore, new therapies for children with AML are urgently needed.

Apoptosis, or programmed cell death, is a biological process essential for the regular development and maintenance of tissue homeostasis[6]. Disturbance in the regulation of apoptosis machinery contributes to the development of tumor and subsequent multi-drug resistance[7]. As the

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majority of cytotoxic drugs mainly kill malignant cells by the activation of apoptosis, recent anticancer approaches are focusing their efforts on specifically targeting the mediators involved within the respective apoptotic pathways.

Mitogen-activated protein kinase/extracellular signal regulated kinase (ERK) pathway plays an essential role in the development and progression of various tumors. ERK1/2 is a key component of this pathway. The hyperactivation of ERK1/2 also has been shown to promote resistance to chemotherapy drugs in many cancer cells[8-10]. Inhibiting the action of ERK1/2 prevents tumor cell proliferation, promote apoptosis and reverse resistance to therapy[11,12]. Previous reports have demonstrated that the primary effect of ERK downmodulation was a cell cycle arrest followed by the apoptosis of a significant percentage of the leukemic blasts[13]. And blocking the ERK pathway sensitizes AML cells to lovastatin-induced apoptosis[14]. Nishioka et al[15] has reported concomitant administration of cytarabine and the inhibitor of MEK/ERK signaling may be useful for treatment of individuals with AML. However, how ERK signaling functions is not clear.

Recent evidence has suggested that processes of the epithelial to mesenchymal transition (EMT) may play a role in the development of chemoresistance[16]. EMT is a critical process in embryogenesis and has been well studied in that context. It is characterized by up-regulation of extracellular matrix components, a loss of intercellular cohesion, increased rate of cellular migration and invasion, as well as increased resistance to apoptosis, and is modulated by a number of transcription factors, namely Snail and Slug[17-20]. Mancini et al[21] has found that Slug has a central role in a complex network involved in prolonged survival and IM resistance of CML progenitors. Many studies have reported[22-25] that Slug was regulated by ERK signals, and Slug is the downstream of ERK signal. We therefore suggested that positive relation might exit between ERK1/2-Slug signal and chemoresistance. The molecular mechanisms underlying the effect of ERK1/2 silencing on HL-60 cell chemosensitivity might via its downstream signaling targets Slug.

p53 upregulated modulator of apoptosis (PUMA) is a downstream target of p53 and a BH3-only Bcl-2 family member[26-27]. It is induced by p53 following exposure to DNA-damaging agents, such as γ-irradiation and commonly used chemotherapeutic drugs[26-27]. Hydrogen peroxide-induced apoptosis is also associated with the PUMA upregulation[28]. Many studies have proved[29-33] that Slug significantly inhibits PUMA-induced apoptosis. We, therefore, suggested that ERK1/2/Slug signal activation inhibits its downstream target PUMA, only to inhibit cell apoptosis.

NF-κB is a ubiquitous transcription factor that is regulated by a vast array of stimuli, including growth factors, inflammatory mediators, cytotoxic agents like chemotherapeutic drugs, oxidative stress, UV light, and many others. NF-κB is a dimer composed of various combinations of the five mammalian Rel proteins, namely, p65/RelA, c-Rel, RelB, NF-κB1/p50, and NF-κB2/p52[34]. The most common form of NF-κB is a dimer of p65/relA and p50,and this dimer is often referred to simply as NF-κB.

A large body of evidence has demonstrated a protective role of NF-κB in most tissues and cell types[35]. For example, targeted deletion of *p65* in mice leads to increased apoptosis in several tissues. The protection by NF-κB is due to transcriptional activation of a number of antiapoptotic proteins, such as c-FLIP, Bcl-2, Bcl-X_L, cIAP2, and A1/Bfl-2[35]. Recently, NF-κB has been found to promote apoptosis under certain conditions. However, the mechanisms and physiological significance of NF-κB in apoptosis regulation remain controversial and poorly understood. Recently, it has found[28,37-40] that NF-κB promote apoptosis by activating the expression of proapoptotic proteins, such as PUMA.

The objective of this study was to investigate the mechanism of early chemosensitivity (activation of NF-κB/PUMA signal pathway) and later chemoresistance (activation of ERK/Slug signal pathway) of Cytarabine (Cyt) to HL-60 cells. To investigate whether the down-regulation of ERK/Slug signal pathway by siRNA could sensitize AML cells to Cyt.

Materials and Methods

Cell Culture Conditions

The HL-60 leukemic cells were maintained in Roswell Park Memorial Institute (RPMI)-1640 culture medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Sigma- Aldrich), 1% antibiotics (100 IU/ml penicillin, 100 μg/ ml streptomycin) (Sigma-Aldrich), 2 mM of glutamine, and 1% sodium pyruvate at 37°C in 95% humidified atmosphere containing 5% CO_2. The cells were sub-cultured with an initial concentra-

tion of 5×10^4 cells/ ml and used in the logarithmic growth phase in whole.

SiRNA Transfection (HL-60/siRNA)

The NF-κBp65 siRNA, PUMA siRNA and Slug siRNA specific and negative control (NC) were purchased from Dharmacon (Lafayette, CO, USA). Just before transfection, the cells were cultivated in RPMI-1640 medium free of serum and antibiotics. siRNA transfection (at a final concentration of 80 nM in all experiments) was performed using Lipofectamine™2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations. Briefly, siRNAs and lipofectamine (4 μl/ml of transfection medium) were diluted in Opti-MEM I Reduced Serum Medium (Invitrogen) separately and incubated for 10 min at room temperature. The diluted solutions were then mixed and incubated for 20 min at room temperature. Subsequently, the mixtures were added to each well containing cells and medium. Moreover, the treated cells with only the transfection reagent were considered as a blank control. The cell culture plates were then incubated for 6 h at 37˚C in a CO2 incubator. Following on, RPMI-1640 medium containing FBS (final FBS concentration of 15%) was added, with cells being incubated under the above mentioned conditions. To evaluate the effects of siRNAs on gene silencing, transfections (5×10^5 cells/well) were performed in 6-well cell culture plates for 24-48h. The suppression of NF-κBp65, PUMA and Slug expression was then assessed by Western blotting.G418 (400 ng/ml) (Life Technologies, Carlsbad, CA, USA) was added to the medium after 48h of transfection, and the cells were cultured for two weeks to permit selection.

Drug Treatments

The HL-60, NF-κBp65 siRNA/HL-60, PUMA siRNA/HL-60 and Slug siRNA/ HL-60 and its control cells were treated with Cyt (250 nM) or for 2, 4, 6, 8, 24, 36, 48 and 72 hs, or treated with 25 nM U0126 for 24 hs followed by Cyt (250 nM) treatment for 2, 4, 6, 8, 24, 36, 48 and 72 hs.

Western Blotting

Cells were serum starved for 12 to 16 hours before the experiment. The whole-cell lysate (60 ug) or fractions thereof (prepared using the Biovision cell fractionation kit (Milpitas, CA, USA) according to the manufacturer's protocol) were fractionated by 4% to 15% gradient SDS-PAGE. After SDS-PAGE, the proteins were electrotransferred onto nitrocellulose membranes, blotted with each antibody (Anti-NF-κBp65, PUMA, Slug, ERK1/2 and pERK1/2, 1:200), and detected with enhanced chemiluminescence reagent (Amersham Biosciences, Piscataway, NJ, USA). Some of the membranes were stripped using Restore stripping buffer (Pierce, Rockford, IL, USA) for reprobing with another antibody. The protein bands obtained were quantified using AlphaEase FC (Fluor Chem 8900) software from Alpha Innotech (San Leandro, CA, USA).

Terminal Deoxynucleotidyl Transferase-Mediated Nick end Labeling (TUNEL) Assay

Analysis of apoptosis by the terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) assay was performed as described previously[41]. Briefly, the cells were exposed for the indicated times, harvested, and fixed by incubation in 4% formaldehyde for 15 minutes on ice. Subsequently, the cells were centrifuged, washed, resuspended in 70% ethanol (EtOH), and stored at −20°C for up to 1 week. For analysis, 10^6 cells were incubated with 0.02 mmol/L biotin-dUTP and 12.5 units of terminal deoxynucleotidyltransferase enzyme in a reaction buffer [200 mmol/L potassium cacodylate, 25 mmol/L Tris-HCl, and 25 μg/mL bovine serum albumin: BSA (pH 6.6)], 2.5 mmol/L CoCl2, and 0.01 mmol/L dTTP (Roche Applied Science, Laval, Quebec, Canada) for 45 minutes at 37°C. The cells were then washed twice with PBS, labeled with avidin-FITC for 60 minutes at room temperature, washed again, and analyzed using a FACScalibur cytometer (Becton Dickinson, Hercules, CA, USA).

MTT Assays

In different group at different time point, the number of viable cells in each well was quantified using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described[41]. The absorbance was read at 570 nm and experiments conducted at least thrice. The results were expressed as a percentage of the control where the absorbance value of the untreated cells was normalized to 100%.

Statistical Analysis

Each assay was performed in triplicate. Data are expressed as the mean ± standard deviation (SD). Statistical significance of all data was evaluated using the Student's *t*-test, $p < 0.05$.

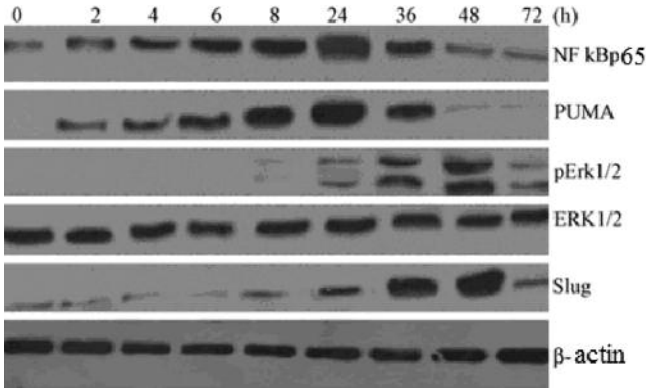

Figure 1. The effect of Cyt treatment on NF-κBp65, ERK1/2, PUMA and Slug expression in HL-60 cells.

Results

The Effect of Cyt Treatment on NF-κBp65, ERK1/2, PUMA and Slug Expression in HL-60 Cells

The HL-60 cells were treated with Cyt (250 nM) for 2, 4, 6, 8, 24, 36, 48 and 72 hs, NF-κBp65, ERK1/2, PUMA and slug expression was detected by western blot assay. The results showed that NF-κBp65 and PUMA expression was increased in a time-dependent way, and reached the highest value at 24 hs, then decreased gradually, and reached the lowest value at 48 hs (Figure 1). However,p-ERK1/2 and Slug began to increase at 24 hs after Cyt treatment, and reached the highest value at 48 hs, then decreased gradually (Figure 1).

The Effect of Cyt Treatment on Apoptosis and Proliferation in HL-60 cells

The HL-60 cells were treated with Cyt (250 nM) for 2, 4, 6, 8, 24, 36, 48 and 72 hs, the survival rate began to decline at 2 hs. At 24 hs, the survival rate was (29.5 ± 4.6) % ($F = 15.02$, $p < 0.01$). After 24 hs, the survival rate was gradually increased (Table I). TUNEL assay showed that cell apoptotic rate was increased in a time-dependant way, and reached the highest value at 24 hs [14.2% 2.8%, $F = 12.74$, $p < 0.01$)], then decreased gradually (Table I).

PUMA is Required for Cyt -induced Apoptosis in HL-60 cells

Although Cyt (250 nM) treatment significantly induced apoptosis and inhibited proliferation in HL-60 cells (Table I), the cell apoptitic rate was significantly decreased and the cell proliferation rate was significantly increased after the HL-60 cells was transfected into NF-κBp65 siRNA or PUMA siRNA to block NF-κBp65 or PUMA expression (Table II-III). There was no significant increase or decrease of NF-κBp65 siRNA or PUMA siRNA transfection on apoptosis and proliferation in HL-60 cells (data not shown). Western blot assay showed that PUMA expression was significantly increased after NF-κBp65 was knockdown. No significant activation or in activation of pErk1/2 and Slug following NF-κBp65 silencing (Figure 2).

Cyt Upregulates pERK1/2-Slug Signal and Inhibits PUMA Expression in HL-60 Cells

After the HL-60 cells were treated with Cyt (250nM) for 24 hs, p-ERK1/2 and Slug began to increase ,and reached the highest value at 48 hs, then reached the lowest value at 72 hs (Figure 3a). On the contrary, PUMA expression was significantly increased at 24 hs, and undetectable at 48 hs (Figure 3a). When the HL-60 cells were exposed to 25 nM U0126 for 24h to inhibit the p-ERK1/2 activity, Slug was significantly inhibited and PUMA was significantly upregulated (Figure 3b).When Slug was blocked via siRNA transfection, PUMA was also upregulated (Figure 3b), however, no significant activation or in activation of pErk1/2 following Slug silencing (data not shown). The results showed PUMA was negatively regulated by pERK1/2-Slug signal.

Knockdown of pErk1/2-Slug Signal Promotes Cyt-Induced Apoptosis

Treatment with 25nM U0126 or transfection with slug siRNA increased Cyt-induced apoptosis, and inhibited proliferation in HL-60 cells (Table IV, $p < 0.01$, respectively). After PUMA was inhibited by siRNA transfection, Cyt-induced apoptosis in 25nM U0126 or Slug siRNA transfected HL-60 cells was inhibited (Table IV).

Table I. The effect of Cyt treatment on apoptosis and proliferation in HL-60 cells (%, $x \pm s$).

Group	0 h	2h	4h	6h	8h	24h	36h	48h	72h
Survival rate (%)	100	95.2 ± 2.8	80 ± 5.3	71.4 ± 5.8	47 ± 3.6	29.5 ± 4.6[a]	41.5 ± 5.7	59.4 ± 7.5	63.4 ± 4.8
Apoptosis rate (%)	0.7 ± 0.1	2.1 ± 0.16	4.0 ± 0.8	7.6 ±1.2	10.3 ± 2.6	14.2 ± 2.8[b]	10.4 ± 2.3	7.3 ± 1.6	3.5 ± 0.48

Ps: Vs 0 h, F = 12.74, [a]$p < 0.01$; Vs 0 h, F = 15.02, [b]$p < 0.01$.

Table II. TUNEL assay Cyt-induced apoptosis in HL-60 cells (%, $x \pm s$).

Group	0 h	2h	4h	6h	8h	24h
Cyt	0.76 ± 0.12	2.1 ± 0.16	4.0 ± 0.8	7.6 ± 1.2	10.3 ± 2.6	14.2 ± 2.8
[a]NF-kBp65 siRNA/Cyt	0.74 ± 0.11	0.78 ± 0.12	0.80 ± 0.12	0.92 ± 0.16	1.80 ± 0.36	3.28 ± 0.78
[b]PUMA siRNA/Cyt	0.78 ± 0.12	0.80 ± 0.11	0.87 ± 0.13	1.03 ± 0.18	1.89 ± 0.47	3.46 ± 0.84

Ps: Vs Cyt, F = 10.83, [a]$p < 0.01$, Vs Cyt, F = 9.92, [b]$p < 0.01$.

Table III. MTT assay Cyt-induced proliferation in HL-60 cells (%, $x \pm s$).

Group	0 h	2h	4h	6h	8h	24h
Cyt	100	95.2 ± 2.8	80.2 ± 5.3	71.4 ± 5.8	47 ± 3.6	29.5 ± 4.6
aNF-kBp65siRNA/Cyt	100	93.6 ± 13.3	92.3 ± 12.8	91.6 ± 13.4	90.3 ± 11.2	85.7 ± 9.6
bPUMAsiRNA/Cyt	100	94.6 ± 13.2	93.6 ± 13.8	92.9 ± 12.4	92.3 ± 12	87.5 ± 11.5

Ps: Vs Cyt, F = 12.3, [a]$p < 0.01$; PS: Vs Cyt, F = 13.7, [b]$p < 0.01$.

Discussion

Leukemia is the most common form of childhood cancer , and cancer is the leading cause of death from disease of Chinese children. Hence, improving leukemia therapy is of utmost importance in pediatric health. This is particularly relevant to AML in which progress has lagged significantly in comparison to childhood acute lymphoblastic leukemia. Owing to the resistance to cytarabine-based chemotherapy in leukemia cells, the majority of patients does not achieve CR or show relapse after first CR, following the standard therapy[3,42-44]. Therefore, new therapies for children with AML must be developed.

In this study, we identified PUMA as a target of NF-κB and a critical mediator of Cyt-induced apoptosis *in vitro* in early time of Cyt treatment. PUMA protein were consistently activated in cells treated with Cyt at 24 hs. The induction of PUMA by Cyt required the p65 subunit of NF-κB. These results established the first case of direct regulation of PUMA by NF-κB in the Cyt response. Our study revealed a new PUMA in-

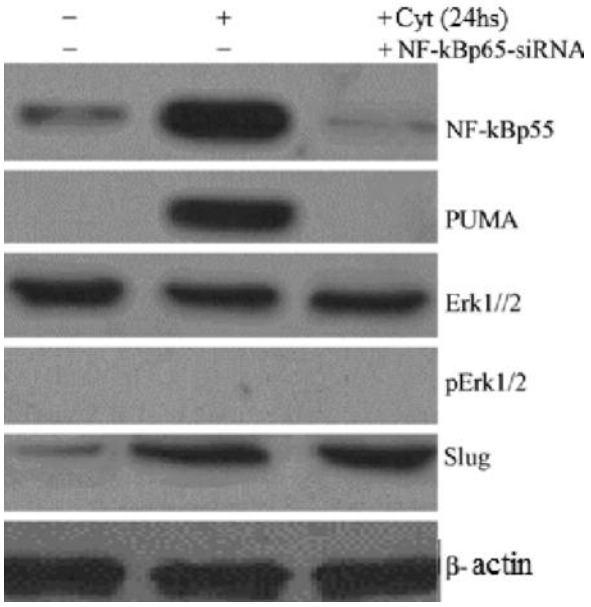

Figure 2. Western blot assay of different protein after NF-κBp65 was knockdown by siRNA transfection in HL-60 cells. PUMA expression was significantly increased after NF-κBp65 was knockdown. No significant activation or in activation of pErk1/2 and Slug following NF-κBp65 silencing.

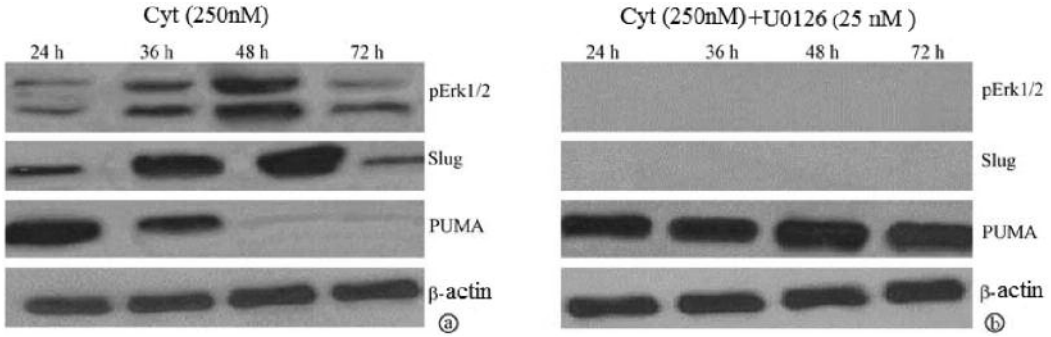

Figure 3. The effect of Cyt treatment on ERK1/2, Slug and PUMA expression in HL-60 cells. ***A,*** HL-60 cells were treated with Cyt (250 nM) for 24-72 hs, ERK1/2, Slug and PUMA were detected by western blot assay. ***B,*** HL-60 cells were treated with 25 nM U0126 for 24 hs followed by Cyt (250 nM) treatment for 24- 72 hs, ERK1/2, Slug and PUMA were detected by western blot assay.

Table IV. The effect of Cyt treatment on apoptosis and proliferation in HL-60 cells (%, $x \pm s$).

Group	0 h		24 h		48 h		72 h	
	Apoptotic rate (%)	Survival rate (%)	Apoptotic rate (%)	Survival rate (%)	Apoptotic rate (%)	Survival rate (%)	Apoptotic rate (%)	Survival rate (%)
Cyt	0.76 ± 0.12	100	14.2 ± 2.8	42.5 ± 6.6	15.3 ± 2.76	53.3 ± 10.4	13.2 ± 1.9	60.2 ± 11.3
U0126	0.82 ± 0.13	100	0.88 ± 0.16	98.7 ± 3.8	0.93 ± 0.12	97.3 ± 4.2	0.98 ± 0.14	96.6 ± 5.2
Cyt + U0126	0.76 ± 0.12	100	24.6 ± 3.8	42.5 ± 7.3	28.6 ± 4.7[a]	19.8 ± 2.7[b]	25.4 ± 5.3	23.7 ± 1.8[b]
Slug siRNA	0.76 ± 0.12	100	0.81 ± 0.12	97.6 ± 3.5	0.93 ± 0.15	96.4 ± 4.2	1.02 ± 0.17	94.4 ± 3.9
Cyt + Slug siRNA	0.76 ± 0.12	100	25.4 ± 3.4	43.5 ± 3.3	27.6 ± 6.4[c]	17.4 ± 4.6[d]	24.3 ± 5.6	19.7 ± 2.1

Ps: Vs Cyt; F = 9.84; [a]$p < 0.01$; F = 11.4; [b]$p < 0.01$; F = p 10.31; [c]$p < 0.01$; F = 15.3, [d]$p < 0.01$.

duction mechanism, which may be important in apoptosis triggered by Cyt. PUMA is required for Cyt-induced apoptosis in HL-60 cells in early time. PUMA is necessary for Cyt-induced apoptosis as it functions as a novel link between the extrinsic and intrinsic apoptotic pathways.

Studies of Cyt-induced apoptosis often rely on using transcription or translation inhibitors, which nonspeci-fically inhibit gene expression and often complicate data interpretation. We found that Cyt simultaneously induced PUMA and pERK1/2-dependent Slug upregulation in HL-60 cells. The apoptotic effect of NF-κB-mediated PUMA induction could be unmasked by inhibition of pERK1/2-Slug signal. Cyt-induced pERK1/2-Slug overexpression can neutralize the pro-apoptotic activity of PUMA, which may allow preneoplastic cells to evade the killing effects of Cyt. This is why HL-60 cells is resistant to Cyt in the latter time.

Conclusions

We demonstrated that PUMA is a direct target of NF-κB and a critical mediator of Cyt-induced apoptosis *in vitro* in early time. The chemosensitiveness of HL-60 cells to Cyt were relation to the up-regulation of PUMA induced by NF-κB. The chemoresistance to Cyt was related to the up-regulation of Slug induced by Erk1/2, which inhibited the expression of PUMA.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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