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Changes of glucocorticoid receptor isoforms expression in acute lymphoblastic leukemia correlate with glucocorticoid resistance

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Alternative splicing of the glucocorticoid receptor (GR) gene results in several GR isoforms, we examined their expression (GRα, GRβ, GRγ and GR-P) by real-time RT-PCR in glucocorticoid (GC) sensitive (CEM-C7), GC resistant (CEM-C1) cells and adult acute lymphoblastic leukemia (ALL) patients, to determine the association of GR isoform expression profiles and GC resistance in adult ALL patients. With GC treatment, GR levels in C1 cells showed no obvious changes. In C7 cells, the mRNA levels of GRα, GRβ and GRγ first increased and then decreased, whereas GR-P mRNA had a continued rising trend. C7 cells had a higher GRα/GRγ, lower GRα/GR-P and GRγ/GR-P ratios than C1 cells ($P < 0.01$). In adult ALL patients, GRγ mRNA varied in different ALL stages (complete remission CR 15.82 vs. relapsed 8.21 vs. initial 1.93 $P < 0.05$). It also did in the ratios between GR isoforms that GRα/GRγ and GRα/GR-P in initial patients were higher than relapsed and CR ($P < 0.05$), while GRγ/GR-P in CR was higher than initial and relapsed patients ($P < 0.05$). GR-P mRNA in T-ALL patients was much higher than that in B-ALL patients ($P < 0.05$). Peripheral blood hemoglobin (HB) was positively correlated with GRα mRNA and GR-P mRNA ($P < 0.05$), while white blood cells (WBC) negatively correlated with GRγ mRNA ($P < 0.05$). The present study demonstrates that GR autoinduction is more important to GC sensitivity than its basal level expression. GC sensitivity is also significantly correlated with GRα mRNA and mildly associated with GRβ mRNA expression. Both GRγ mRNA and the ratios between GR isoforms (GRα/GRγ, GRα/GR-P and GRγ/GR-P) are correlated with ALL stages. The changes of mRNA expression levels of GRα, GR-P and GRγ may provide valuable information for GC resistance. Peripheral blood HB and WBC affect GR isoform expression.

1. Introduction

Glucocorticoid (GC) can induce lymphoid cell apoptosis and cell cycle arrest, and plays an important role in the treatment of many malignant hematological diseases such as acute lymphoblastic leukemia (ALL) (Greenstein et al. 2002). However, some patients are resistant to GC treatment, which has an adverse effect on ALL therapy. The molecular mechanism of GC resistance in ALL is not completely understood. The cell lytic effect of GC is mediated by the glucocorticoid receptor (GR). Single GR gene generates different GR isoforms, including GRα, GRβ, GRγ, GR-P, GRA and GRB through alterative splicing events (Yudt and Cidlowski 2002).

GRα is thought to be the primary mediator of GC action, while GRβ is an inhibitor of GRα activity because of its inability to bind steroids and to activate GC-responsive genes (Bamberger et al. 1995; Hecht et al. 1997; Kofler et al. 2003; Oakley et al. 1996; Reichardt and Schütz 1998). Studies on GRγ and GR-P showed that their expression may be related to GC sensitivity in hematological malignancies (Beger et al. 2003; Koga et al. 2005; Sánchez-Vega et al. 2006). Therefore, the expression level of GR isoforms is important to GC sensitivity in ALL.

Up to now, the criteria of GC resistance in ALL patients, especially in adult ALL patients, had not reached an agreement. Little research has been conducted in adult ALL patients on GR isoform expression and their changes with GC treatment. Most studies on GR isoform expression in ALL were reported in ALL cell lines and infant ALL patients. Rarely reports are found in adult ALL patients.

In this study, we examined the mRNA abundance of GR isoforms (GRα, GRβ, GRγ and GR-P) in GC sensitive, resistant T-ALL cells and adult ALL patients, and tried to determine whether alternative splicing of the GR contributes to the differential GC resistance profiles observed in ALL.

2. Investigations and results

2.1. Cell growth inhibition and apoptosis with DEX

After treatment with different concentrations of dexamethasone (DEX) for 48 h, CEM-C1 and CEM-C7 cells displayed variable growth inhibition. As shown in Fig. 1, C1 cells had lower growth inhibition rates than C7 cells at all DEX concentrations. With rising concentration of DEX, the growth inhibition rates of both cell types increased gradually. The growth inhibition rates of C7 being higher than that of C1 indicates that C7 was more sensitive to GC, most significantly at the concentration of 10^{-7} M DEX (C1 $21.68 \pm 5.19\%$ *vs* C7 $47.28 \pm 7.69\%$, $P < 0.05$). Therefore it was chosen as the final testing concentration.

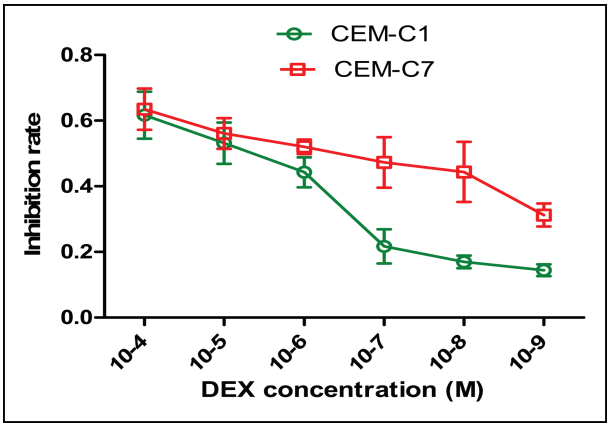

Fig. 1: Growth inhibition of different concentrations of DEX on C1 and C7 cells.

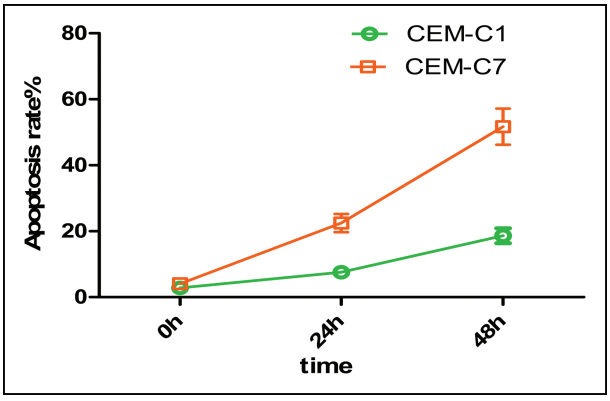

Fig. 2: Cell apoptosis induced by DEX.

Both type of cells were cultured with 10^{-7}M DEX for 48 h and then examined for apoptosis by flow cytometry at indicated time points. As shown in Fig. 2, DEX induced dramatic apoptosis in C7 cells (48 h 51.70 ± 5.47%), whereas only minor effects were observed in C1 cells (48 h 18.61 ± 2.26%). The difference is statistically significant ($P < 0.05$).

2.2. GR mRNA isoforms expression in CEM-C1 and CEM-C7 cells

Quantitative RT-PCR showed that the relative expression levels of GR isoforms changed with DEX treatment in C1 and C7 cells, and statistical significance was found between the two cell types ($P < 0.01$) (Fig. 3). The mRNA levels of GRα (0 h 3.19 ± 0.39, 48 h max level 72.49 ± 3.65, 72 h 34.37 ± 1.76), GRβ (0 h 0.01 ± 0.00, 24 h max level 0.02 ± 0.00, 72 h 0.00 ± 0.00) and GRγ (0 h 0.46 ± 0.07, 24 h max level 5.24 ± 0.54, 72 h 2.81 ± 0.41) showed a trend of first increasing and then decreasing in C7 cells, but had no obvious changes in C1 cells (GRα 0 h 8.35 ± 1.04, 48 h max level 10.52 ± 0.72; GRβ 0 h 0.01 ± 0.00, 24 h 0.00 ± 0.00 and GRγ 0 h max level 2.24 ± 0.24, 24 h min level 0.65 ± 0.07). GR-P mRNA levels showed continued rise in C7 cells (0 h 0.59 ± 0.03, 72 h max level 12.61 ± 0.63), but minor changes in C1 cells (0 h max level 2.02 ± 0.15, 24 h min level 0.53 ± 0.05) ($P < 0.01$).

As shown in Fig. 4, C1 and C7 cells exhibited different mRNA ratios of GRα/GRγ and GRα/GR-P at different time points after GC treatment. The ratios in C1 cells showed a trend of first increasing and then decreasing, while those in C7 cells first decreasing then increasing and finally decreasing. C7 cells had a higher mRNA ratio of GRα/GRγ than C1 cells at time point 48 h ($P < 0.01$) and lower ratio of GRα/GR-P at time point 24 h ($P < 0.01$). In contrast, both C7 and C1 cells had similar variation pattern of GRγ/GR-P ratio (Fig. 4): both had the trend of first increasing and then decreasing. The ratio of GRγ/GR-P mRNA in C1 cells (min level 0.61 ± 0.01, max level 1.22 ± 0.05) was higher than that in C7 cells (min level 0.22 ± 0.04, max level 0.97 ± 0.19) ($P < 0.01$).

2.3. GR mRNA isoforms expression in patients

Studies of GR mRNA isoforms expression in the control and adult ALL patients showed no statistical differences between the two groups in the relative mRNA expression of GRα, GRβ, GRγ and GR-P ($P > 0.05$) and the two groups had the same order of GR isoform mRNA expression (GRα > GR-P > GRγ > GRβ) (Table 1).

In ALL patients, no statistical differences was found between T-ALL and B-ALL in the relative mRNA expression level of GRα, GRβ and GRγ ($P > 0.05$). However, GR-P mRNA in T-ALL was much higher than that in B-ALL ($P < 0.05$) (Table 1). No significant difference was found in the ratios of GR isoforms (GRα/GRβ, GRα/GRγ, GRα/GR-P and GRγ/GR-P) between T-ALL and B-ALL patients. No significant difference was found in the expression levels of GR isoforms between poor prognosis group (PPG) and good prognosis group (GPG) ($P > 0.05$) (Table 1).

Further analysis showed no statistical difference in the mRNA expression of GRα, GRβ and GR-P among different stages (initial, relapsed and complete remission CR) of ALL, except that GRγ mRNA expression level was significantly different among the three stages ($P < 0.01$), with the order of CR > relapsed > initial ($P < 0.016$) (P value for correction) (Table 2).

The ratios between GR isoforms were analyzed, as shown in Table 2. No significant difference was found in GRα/GRβ ratio among ALL stages ($P = 0.50$). The ratio of GRα/GRγ was significantly different among the three stages (initial > relapsed > CR) with all P values less than 0.016 (P value for correction). The ratio of GRα/GR-P was also significantly different among the three stages ($P = 0.000$): the initial group was significantly higher than the relapsed group ($P = 0.001$) and the CR group ($P = 0.000$), but no significant difference was found between relapsed and CR groups ($P = 0.924$). GRγ/GR-P was different in ALL patients among the three stages ($P = 0.000$), and the CR group was significantly higher than the initial group ($P = 0.000$) and the relapsed group ($P = 0.000$), and no significant difference was found between initial and relapsed group ($P = 0.057$).

As shown in Table 3, positive correlations were found among GRα, GR-P and GRγ mRNA levels in ALL patients. Hemoglobin (HB) in peripheral blood was also found to be positively correlated with GRα and GR-P expression, white blood cell (WBC) count in peripheral blood was negatively correlated with GRγ expression, no correlation was found between GR isoforms expression and the serum level of cholesterol (CHOL), triglyceride (TG), high-density lipoprotein (HDL), low-density lipoprotein (LDL); lactic dehydrogenase (LDH) and β2 microglobulin (β2 MG); blast count in bone marrow (BM), age of patients and platelet count in peripheral blood.

3. Discussion

GR gene mutation leading to GC resistance was mostly documented in ALL cell lines but rarely in ALL patients, which suggests that gene mutation is not the primary cause of GC resistance (Beesley et al. 2009a; Irving et al. 2005). Different GR isoforms generated by alternative splicing and translation initiation have different functions. The changes of GR isoforms during the process of GC application may be associated with GC resistance (Koga et al. 2005; Sánchez-Vega et al. 2006).

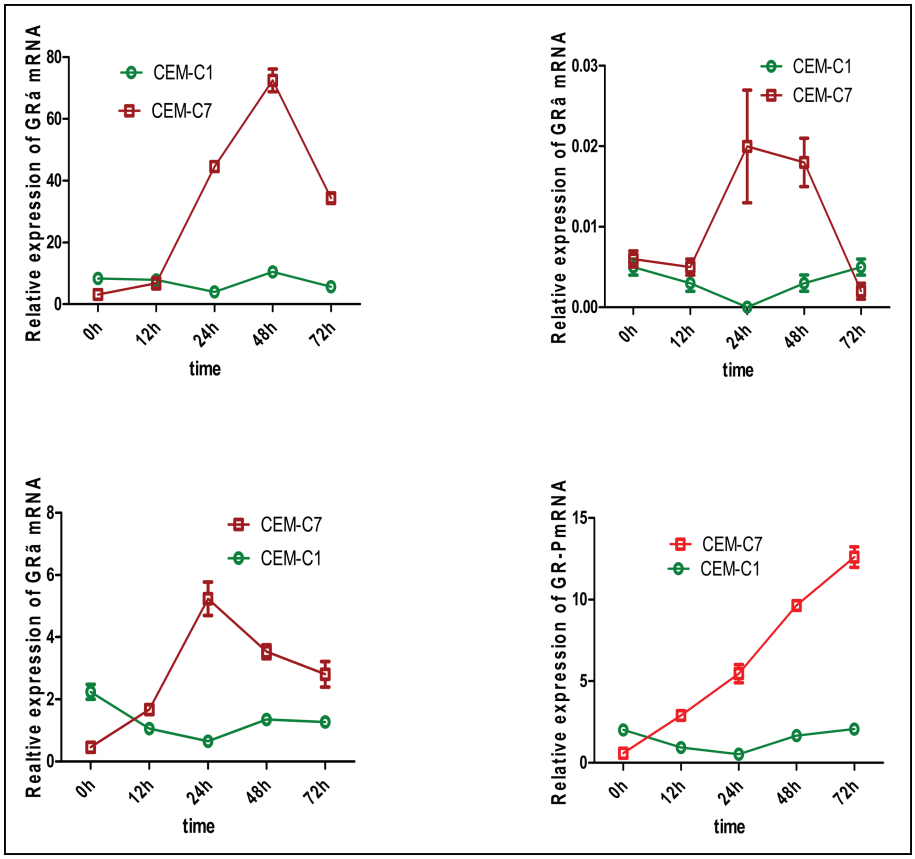

Fig. 3: Relative expression of GR mRNA isoforms in C1 and C7 cells.

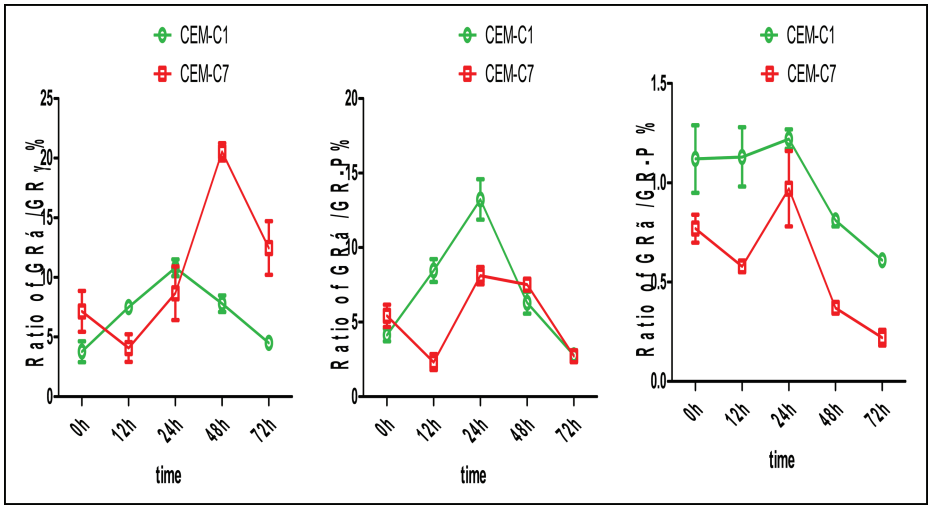

Fig. 4: The ratios between GR mRNA isoforms in C1 and C7 cells.

Table 1: GR mRNA isoforms expression between different groups

Group	n	GRα mRNA	GRβ mRNA	GRγ mRNA	GR-P mRNA
ALL	64	24.95(17.18–44.54)	0.01(0.00–0.02)	9.54(1.99–15.30)	10.92(5.10–24.33)
Control	20	28.78(20.03–41.69)	0.01(0.00–0.05)	4.13(2.49–6.34)	16.17(9.91–24.63)
B-ALL	54	19.87(15.05–38.96)	0.01(0.00–0.02)	7.17(1.81–15.56)	10.21(5.01–20.42)*
T-ALL	10	37.96(29.65–65.66)	12.16(4.41–16.67)	22.81(12.69–30.92)*	0.00(0.00–0.06)
PPG	34	25.70(13.73–38.57)	0.01(0.00–0.02)	4.62(1.95–15.23)	10.6(4.76–21.70)
GPG	30	2.85(17.43–47.70)	0.01(0.00–0.04)	11.81(1.97–16.45)	13.06(6.31–26.97)

Median expression values were summerized (range in parentheses). *means $P < 0.05$

Table 2: GR isoforms in different stages of ALL patients

Group	Initial	Relapsed (n = 26)	CR (n = 18)	*P values*
GRα mRNA	19.75 (13.58–33.78)	37.96 (14.19–65.66)	20.19 (18.07–37.51)	0.41
GRβ mRNA	0.00 (0.00–0.02)	0.01 (0.00–0.06)	0.01 (0.00–0.02)	0.62
GRγ mRNA	1.93 (1.61–4.50)	8.21 (2.62–14.99)	15.82 (12.98–20.32)	0.00*
GR-P mRNA	7.63 (4.99–10.38)	22.22 (4.54–28.83)	12.43 (9.21–18.95)	0.06
GRα/GRβ	6657 (1002–10567)	5056 (890–14162)	2505 (818–6756)	0.50
GRα/GRγ	10.80 (6.13–18.30)	4.45 (2.65–6.87)	1.62 (1.35–1.88)	0.00*
GRα/GR-P	3.18 (2.42–4.68)	1.87 (1.59–2.31)	1.88 (1.64–2.01)	0.00*
GRγ/GR-P	0.31 (0.12–0.51)	0.52 (0.28–0.71)	1.17 (0.95–1.25)	0.00*

Median expression values were summerized (range in parentheses). * means $P < 0.05$

Table 3: Correlation of GR mRNA isoforms with clinical factors in ALL patients

	GRα	GRβ	GRγ	GR-P
GRα	–	0.036/0.847	0.727/(0.000)*	0.916/(0.000)*
GRβ	0.036/0.847	–	0.103/0.581	0.017/0.928
GRγ	0.727/(0.000)*	0.103/(0.581)	–	0.738/0.000*
GR-P	0.916/(0.000)*	0.017/0.928	0.738/0.000*	–
CHOL	−0.101/(0.604)	−0.040/0.838	0.015/0.937	0.059/0.761
TG	0.321/(0.089)	−0.229/0.232	0.101/0.601	0.325/0.085
HDL	0.066/(0.735)	−0.040/0.836	0.179/0.354	0.234/0.222
LDL	−0.174/(0.366)	−0.133/0.490	−0.063/0.746	−0.010/0.958
LDH	0.050/(0.805)	0.008/0.967	−0.110/0.584	0.049/0.809
β2MG	0.404/(0.107)	0.001/0.996	0.206/0.428	0.338/0.184
AGE	0.001/0.994	−0.040/0.831	−0.024/0.899	0.088/0.637
WBC	0.036/0.849	−0.047/0.802	−0.39/0.030*	0.027/0.883
HB	0.424/0.018*	0.125/0.504	0.342/0.06	0.437/0.014*
PLT	−0.251/0.173	0.052/0.78	0.077/0.68	−0.284/0.121
BM	0.165/0.375	0.099/0.596	−0.242/0.189	0.030/0.875

Abbreviations: CHOL, cholesterol; TG, triglyceride; HDL, high-density lipoprotein; LDL, low-density lipoprotein; LDH, lactic dehydrogenase; β2MG, β2 microglobulin; BM, blast count in bone marrow. HB: hemoglobin
Results were expressed as coefficient of association r/(*P* value). * means $P < 0.05$

We tested and proved the different apoptosis rates between GC sensitive cells (CEM-C7) and GC resistant cells (CEM-C1) under the concentration of 10^{-7} M DEX. We found that these two types of cells had different temporal patterns of GR isoform expression after GC treatment. The sum GR in C7 cells was lower than that of C1 cells at time point 0 h, but gradually increased with time and became much higher than that of C1 cells during the 24-48 h GC treatment. These results indicate that the up-regulation of GR isoform expression after GC application (GR autoinduction) is more significant than basal GR expression in predicting GC resistance, which is consistent with the results reported by Beesly (2009a) and Schmidt (2006). The expression level of GRα mRNA was the highest and GRβ mRNA was the lowest among GR isoforms in C1, C7 cells and ALL patients. GRα mRNA in C7 cells increased more dramatically than that in C1 cells after GC treatment. This remarkable ratio of GRα/GRβ led us to conclude that GRα had a leading role in GC therapeutic effect, as compared to GRβ which had a minimal value to GC resistance. The same conclusion was also found in adult immune thrombocytopenia (Ma et al. 2013). In C1 cells, GR-P mRNA expression was slightly lower than GRγ mRNA within the first 24 h of DEX treatment, it then upregulated and finally became higher than GRγ mRNA. While in C7 cells, GR-P mRNA expression was higher than GRγ mRNA throughout the DEX treatment. The ratios of GR isoforms to sum GR in C1 cells were GRα > GRγ > GR-P > GRβ during 0-24 h of GC treatment, and GRα > GR-P > GRγ > GRβ during 48-72 h; while in C7 cells the order of GRα > GR-P > GRγ > GRβ was exhibited throughout the time. Thus, we concluded that the changes of GR-P and GRγ expression may be related to GC resistance in T-ALL cells.

Further analysis found that C7 cells displayed a much higher GRα/GRγ mRNA ratio, and lower GRα/GR-P, GRγ/GR-P ratios than those in C1 cells. Therefore, we deduce that the up-regulation of GRα, GRγ mRNA or the downregulation of GR-P mRNA may lead to GC resistance in C1 and C7 cells, and the interaction among GR isoforms may be relevant to GC resistance.

A study in infant ALL patients showed that changes of GR isoforms expression with GC treatment may be correlated with GC therapeutic effects (Lauten et al. 2009). Patients were divided into two groups: prednisone good responders (PGR) and prednisone poor responders (PPR) by their peripheral blood blasts counts after 8 days of GC treatment. The expression levels of GRα and GRγ were higher in PGR group than those in PPR group, and no significantly difference was found in GR-P expression between the two groups. Although these results were different from ours, they also proved that GR isoform expression and their changes with GC treatment correlated with GC resistance.

Another study on infant ALL patients GR isoform expression (Haarman et al. 2004) found that it was the GRγ mRNA level, but not GRα or GRβ, that was related to GC resistance. GC resistant group expressed higher level of GRγ mRNA than other groups (patients were grouped by testing the IC50 values after culturing patients' blasts with GC *in vitro*).

In adult ALL patients, we found that GRγ mRNA was related to ALL stages, and no significant difference was found in

other GR isoforms expression. The orders of GR isoforms mRNA expression levels in both the initial and relapsed groups were the same: GRα > GR-P > GRγ > GRβ, but different in the CR group: GRα > GRγ > GR-P > GRβ. Further analysis of the ratios of GRα/GRβ, GRα/GRγ, GRα/GR-P and GRγ/GR-P in different groups found that GRα/GRγ had the order of initial > relapsed > CR group ($P < 0.01$). The order of GRα/GR-P was initial > relapsed and CR groups, and GRγ/GR-P was CR > relapsed and initial groups; while GRα/GRβ was not significantly different among the three groups.

These results indicate that the mRNA expression of GRα, GR-P and GRγ varies in different groups and no clear conclusions can be drawn. We speculated that GC resistance was significantly correlated with GRα mRNA expression and mildly associated with GRβ mRNA because of their marked differences in expression levels. The mRNA expression of GR-P and GRγ varied greatly in different groups, especially the ratios between GR isoforms, suggesting that these GR isoforms (GRα, GR-P and GRγ) may contribute to GC resistance, and their interactions may affect their expression, influence GC therapeutic effects and predict ALL stages. Because of the lack of clear criteria of GC sensitivity and resistance in adult ALL patients, we only analyzed the correlation between ALL stages and GR isoforms expression.

It has been found in a study of infant ALL patients that GRα mRNA was higher in the relapsed group than in the initial group, while GRγ mRNA expression was not significantly different between the initial and relapsed groups (Haarman et al. 2004). It has also been reported that leukemia blasts with low GRβ/GRα ratio were more easily to undergo apoptosis induced by GC in children (Koga et al. 2005). To our knowledge, no studies in adult ALL patients has been reported.

In our study, we found that adult T-ALL patients had a higher level GR median than B-ALL patients, and both groups of patients had the same order of GR mRNA isoforms (GRα > GR-P > GRγ > GRβ). Especially, the expression of GR-P mRNA in T-ALL was much higher than that in B-ALL ($P < 0.05$). While in infant initial ALL patients, T-ALL was found to have lower GR isoforms than non-T-ALL (Lauten et al. 2009). This discrepancy may be explained by the different patient populations (adult ALL patients including initial, relapsed and CR stages in our study vs infant initial ALL patients), as well as the limited case numbers in our study.

We found that there was no statistical difference in the expression levels of GR isoforms between group PPG and GPG. Both groups had the same order of GR mRNA expression: GRα > GR-P > GRγ > GRβ. Therefore, GR isoform expression seems to have little value to the prognosis of adult ALL patients.

It has been speculated that GC cytotoxicity in lymphoblasts was correlated with suppression of metabolic pathways such as lipid metabolism (Beesley et al. 2009b; Richards and Kilberg 2006; Samuels et al. 2014; Tonko et al. 2001; Tung et al. 2013). We analyzed the association of GR isoform expression with lipid profiles. No correlation was found between GR isoforms and the serum levels of blood-fat, LDH and β2 MG. Nevertheless we found a positive correlation among the mRNA expression levels of GRα, GR-P and GRγ. Positive correlation was also found between peripheral blood HB and GRα mRNA, GR-P mRNA. In contrast, a negative correlation was found between WBC and GRγ mRNA. This is consistent with a previous report that a high peripheral WBC count was negatively correlated with the expression of GRα, GRγ and GR-P (Lauten et al. 2009).

Multiple factors might contribute to the diverse results reported. The sensitivity to GC varies among individuals, tissues, cell types, and even during the cell cycle (Hsu and DeFranco 1995; Lim-Tio et al. 1997); the interaction of GC and GR is closely correlated with the hypothalamic-pituitary-adrenal axis and cell line studies *in vitro* fail to completely reflect the mechanism of GC resistance *in vivo*; specific antibodies to GRγ and GR-P are unavailable commercially. Since our study is limited by the number of cases, larger scale clinical studies are needed for further clarification.

In summary, we conclude that the basal level of GR in ALL cells has little value to GC sensitivity, while GR autoinduction is important and the changes of GR isoform levels after GC treatment may be useful in predicting the efficacy of GC treatment. GC resistance is mainly related to GRα mRNA expression and mildly associated with GRβ expression, it is also related to the mRNA expression of GRγ and GR-P. The interaction between GRα, GRγ and GR-P, peripheral blood WBC and HB may also influence GC effects.

4. Experimental

4.1. Preparation of cell samples

GC sensitive ALL cells (CCRF-CEM-C7) were bought from LONZA, Swiss, and GC resistant cells (CCRF-CEM-C1) were bought from Shanghai bank, Chinese Academy of Sciences. Cells were grown in RPMI 1640 supplemented with 15% fetal bovine serum in 5% CO_2 at 37 °C. Dexamethasone (DEX) sodium phosphate injection was obtained from Tianjin Jingang pharmaceutical factory, China.

Bone marrow samples were obtained from our 64 hospitalized ALL patients between February 2009 and February 2012, including 54 B-ALL/10 T-ALL, 31 males/33 females, 20 initial ALL/26 relapsed ALL /18 complete remission (CR) ALL, with a median age of 41 (16–69). Control group consisted of 20 iron deficiency anemia (IDA) patients (median age of 40 (18–72) years, 9 males/11 females). According to the 2012 National Comprehensive Cancer Network (NCCN) ALL guidelines, ALL patients were divided into two groups: good prognosis group (GPG) and poor prognosis group (PPG). PPG were defined with at least one of these prognostic factors: 1. Philadelphia chromosome positive; 2. failed to CR after two courses treatment or relapsed in six months after CR; 3. chromosome abnormality of t (4;11)/MLL-AF4; 4. hyperleukocytosis (B-ALL: WBC $\geq 30 \times 10^9$/L, T-ALL: WBC $\geq 100 \times 10^9$/L). Patients without the above factors belonged to GPG group. The bone marrow mononuclear cells (MNC) were isolated using Ficoll gradient centrifugation (density, 1.077 g/ml) and stored at -80 °C until RNA was extracted.

All the patients had no history of GC and immunosuppressive agent use or infection two weeks prior to the study. The study was approved by the Medical Ethical Committee of the First Affiliated Hospital of Dalian Medical University, and informed consent had been signed by patients.

4.2. Detection of DEX-induced cell growth inhibition by MTT test

Both CEM-C1 and CEM-C7 cells in logarithmic growth phase were collected and resuspended in 1×10^5/ml. Cell suspensions (200 μl) were seeded in a 96-well plate with different final concentrations of DEX (10^{-4} M, 10^{-5} M, 10^{-6} M, 10^{-7} M, 10^{-8} M, and 10^{-9} M) and vehicle control. After incubation for 48 h, the cells were treated with 20 μL MTT (4,5-dimethylth iazol-2-yl-2,5-diphenyltetrazolium bromide) solution (5 mg/ml) and incubated for another 4 h. Finally 150 μL DMSO (Sigma, USA) was added to the cell suspension. The absorbance at 570 nm was measured by an ELISA reader. Each test was performed in triplicates. The inhibition ratio of ALL cells was calculated according to the formula: Inhibition rate (%) = (1-absorbance of the experimental group/absorbance of the control group) x 100%.

4.3. Apoptosis measurement by flow cytometry

According to MTT tests, both CEM-C1 and CEM-C7 cells in logarithmic growth phase were incubated with 10^{-7} M DEX, and then collected and washed at different time points (0 h, 24 h, 48 h). After adding and incubating with Annexin V-FITC and propidium iodide (both from Nanjing Keygen biotech company, china) successively, apoptosis was examined by flow cytometry.

4.4. Taq-man real-time quantitative RT-PCR

Total RNA of 1×10^6 MNCs was isolated by Trizol reagent (Takara Biotechnology, Dalian, China) method. Reverse transcription of RNA and RT-PCR was performed as we previously described (Ma et al. 2013)

The primers of GRα, GRβ, GRγ, GR-P and GAPDH were designed according to Koga's protocol (Koga et al. 2005). The sequences are as follows: GRα: forward: 5'-CTATGCATGA-AGTGGTTGA AAA-3', reverse: 5'-TTTCAGCTAACATCT-CGGG-3'; GRβ: forward: 5'-GAAGGAAACTCCAG CCAG-AA -3', reverse: 5'-CCACATAACATTTTCATGCATAGA-3'; GRγ: forward: 5'-TTCAA AAGAGCAGTGGAAGGTA-3', reverse:5'-GGTAGGGGTGAGTTGTGGTAACG-3'; GR-P: forward: 5'-GCTGTGTTTTGCTCCTGATCTGA-3', reverse: 5'-TGACATAAGGTGAAAAGGT GTTCTACC-3'; GAPDH internal control-forward: 5'-GGACCTGACCTGCCGTCTAG-3', reverse: 5'-TAG CCCAGGATGCCCTTGAG-3'. The lengths of the products were 96, 81, 264, 194 and 99 bp, respectively. All reactions were performed in triplicates. The relative expression levels of PCR products were calculated using the comparative Ct method. The target PCR Ct values are standardized against the Ct value of the reference gene (GAPDH) by the following equation: relative expression $= 2^{-\{[Ct(\text{target gene}) - Ct(\text{GAPDH of target gene})] - [Ct(\text{reference gene}) - Ct(\text{GAPDH of reference gene})]\}}$

4.5. Statistical analysis

All statistical analysis was performed using the SPSS 17.0 version and graphs were performed by Graphpad Prism5. The cell line data were reported as the mean ± SD and analyzed by ANOVA. The patient data were expressed as median and interquartile range (P25-P75) and analyzed by Kruskal-Wallis test and Mann-Whitney U test. Spearman's rank correlation test was used to analyze data correlation. All *P* values were two-sided and values < 0.05 were considered statistically significant.

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