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ORIGINAL ARTICLE

Expression pattern of *BIM*, *BCL-6*, and *c-MYC* in adult B-cell acute lymphoblastic leukemia*

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Abstract

Objective We aimed to evaluate the expression pattern of the genes *BIM*, *BCL-6*, and *c-MYC* in adult patients at initial diagnosis of B-cell acute lymphoblastic leukemia (B-ALL).

Methods Relative mRNA levels of *BIM*, *BCL-6*, and *c-MYC* in peripheral blood mononuclear cells (PBMCs) from B-ALL patients were determined by quantitative reverse-transcription polymerase chain reaction (qRT-PCR) using SYBR Green dye. PBMCs from healthy volunteers served as a control. *GAPDH* was used as a reference gene.

Results Relative expression of *BIM*, *BCL-6*, and *c-MYC* mRNA in B-ALL patients was significantly lower than in healthy controls ($P < 0.05$). Furthermore, this result was observed for both newly diagnosed B-ALL patients and those incomplete remission (CR) ($P < 0.05$). There were no statistically significant differences in the expression levels of *BIM*, *BCL-6*, and *c-MYC* between these B-ALL patient groups ($P > 0.05$). Spearman's rank correlation analyses revealed the expression level of *BIM* to be positively correlated with that of *BCL-6* in B-ALL patients.

Conclusion Expression of the genes *BIM*, *BCL-6*, and *c-MYC* is decreased in adult B-ALL patients. Moreover, the expression pattern of these genes may be similar in such patients at initial diagnosis and following CR. The expression characteristics of *BIM*, *BCL-6*, and *c-MYC* may constitute useful markers for the diagnosis of adult B-ALL.

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Human B-cell acute lymphoblastic leukemia (B-ALL), accounting for 83% of all ALLs and 30% to 40% of all childhood cancers, is characterized by clonal expansion of developmentally arrested malignant B-cell precursors [1–2]. Despite current knowledge of the cytogenetics of this disease, obstacles to relapse-free survival remain for up to 15% of children and the majority of adults with this disease [3–4].

Our previous analysis of the global gene expression profile of SUDHL6 diffuse large B cell lymphoma cells following *BCL11A* down regulation revealed altered expression of a broad range of genes, including *BIM*, *BCL-6*, and *c-MYC* [5].

BIM exhibits proapoptotic functions. For instance, Korfi *et al* indicated that *BIM* mediates the synergistic killing of B-ALL cells by MEK inhibition and BCL-2/BCL-XL family inhibitors [6]. *BCL-6* is a proto-oncogene encoding a protein that acts as a sequence-specific transcriptional repressor in B cells [7]. BCL-6 is also thought to suppress genes involved in lymphocyte activation, differentiation, cell cycle arrest, and apoptosis, and is mainly involved in the pathogenesis of germinal-center B-cell-like diffuse large B cell lymphoma [8–9]. Induced activation of

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BCL-6 downstream of the pre-B cell receptor (pre-BCR) results in transcriptional repression of MYC and CCND2. Hence, pre-BCR-mediated activation of BCL-6 limits pre-B cell proliferation and induces cellular quiescence at the small pre-BII stage [10]. *c-MYC* is one of the most prevalent oncogenes, being deregulated in approximately 50% of tumors, although such deregulation is more frequently associated with lymphoma than lymphoblastic leukemia. c-MYC expression appears to be significantly lower in B-ALL than in mature B-cell neoplasms [11].

In the present study, we examined de novo B-ALL patients and B-ALL patients incomplete remission (CR), with the aim of determining the roles of *BIM*, *BCL-6*, and *c-MYC* in this disease. Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) was used to analyze the expression levels of these genes.

Materials and methods

Samples

Seventeen patients with B-ALL (8 men and 9 women) with a median age of 29.5 years (range: 19–69 years) were enrolled in the study. Ten patients were examined at initial diagnosis, whereas seven of the tested patients had achieved and remained in CR. Ten healthy volunteers (7 men and 3 women) with a median age of 31 years (range: 22–62 years) served as controls. All procedures were conducted in accordance with the guidelines of the Medical Ethics Committees of the Health Bureau of Guangdong Province, China. Peripheral blood was collected with heparin anticoagulation, and peripheral blood mononuclear cells were separated using the Ficoll-Hypaque gradient centrifugation method.

RNA extraction and cDNA synthesis

RNA was extracted using a TRIzol kit (Invitrogen, Carlsbad, CA, USA) and reverse transcribed into first-strand cDNA using random hexamer primers and a SuperScript II reverse transcriptase kit (Invitrogen, USA), according to the manufacturer's instructions. RNA purity and concentration were measured with a spectrophotometer, and RNA integrity was assessed on a 1% agarose gel. qRT-PCR of the *GAPDH* gene was used to determine the quality of the synthesized cDNA.

qRT-PCR with SYBR Green I

Relative changes in gene expression can be calculated from qRT-PCR data using the $2^{-\Delta CT} \times 100\%$ method [12]; however, it should first be verified that target and reference gene amplification efficiency is high. Therefore, a validation experiment was conducted using serial dilutions of cDNA template covering five orders of magnitude. Standard curves were generated by plotting each resulting Ct value against the logarithm of the corresponding cDNA template starting quantity. The slope and correlation coefficient (R^2) were then determined by linear regression analysis. The primer sequences for the genes targeted in this study are listed in Table 1. Each reaction, the volume of which totaled 20 μL, began with enzyme activation at 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s, 58 °C for 20 s, and 72 °C for 30 s. At the end of each run, a melting curve was generated by increasing the temperature from 65 °C to 95 °C in increments of 1 °C every 2 s to verify primer specificity and ensure the absence of primer dimers. At least three independent qRT-PCR experiments were performed.

Table 1 Sequences of primers used for quantitative reverse-transcription polymerase chain reaction

Gene	Forward (5'–3')	Reverse (5'–3')	Size (bp)
BIM	tcaaccactatctcagtgcaatgg	gcgttaaactcgtctccaatacg	112
BCL-6	ttgttgtgagccgtgagcagtt	aaggttgcatttcaactggtctgt	101
c-MYC	tcccctaccctctcaacgacag	gaggagagcagagaatccgagg	94
GAPDH	acccagaagactgtggatgg	ttcagctcagggatgacctt	114

Statistical analysis

The Mann-Whitney *U* test was used to compare relative mRNA expression levels between samples, and Spearman's rank was employed to analyze correlations between *BIM*, *BCL-6*, and *c-MYC* expression using the statistical software package SPSS 13.0 (SPSS Inc., Chicago, IL, USA). Differences were considered statistically significant at $P < 0.05$.

Results

PCR product analysis

The R^2 value of the standard curve for each of the target gene reactions (*BIM*, *BCL-6*, and *c-MYC*) was above 0.990. In all cases, the efficiency with which these genes and the reference gene *GAPDH* were amplified was above 95%. The high amplification efficiency of the target genes was consistent with that of the reference gene.

BIM, *BCL-6*, and *c-MYC* expression in newly diagnosed B-ALL patients and B-ALL patients in CR

A broad range of *BIM* mRNA expression values (range: 0.003–5.49; median: 0.263) (Fig. 1a) was observed. *BIM* mRNA expression was significantly lower in adult B-ALL patients than healthy controls ($P = 0.0003$). Moreover,

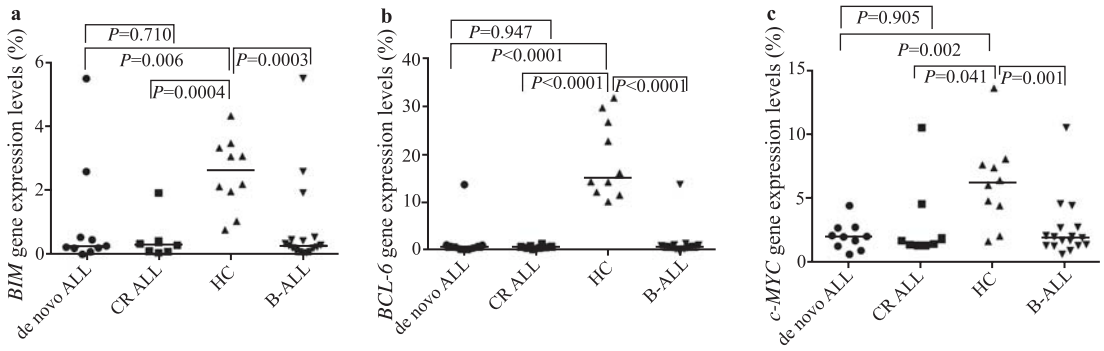

Fig. 1 *BIM*, *BCL-6*, and *c-MYC* gene expression in adult B-ALL patients. (a) *BIM* expression level; (b) *BCL-6* expression level; (c) *c-MYC* expression level. mRNA levels of *BIM*, *BCL-6*, and *c-MYC* among adult B-ALL patients, de novo B-ALL patients, B-ALL patients incomplete remission (CR), and healthy controls (HC) were normalized to those of *GAPDH*.

median *BIM* expression was 0.244 at initial diagnosis and 0.285 in the context of CR. This gene was expressed at lower levels in untreated, newly diagnosed B-ALL patients than healthy controls (P = 0.0068) (Fig. 1a). Similarly, relative median *BIM* expression in B-ALL patients in CR was significantly lower than that inhealthy individuals (P = 0.0004). This suggests that *BIM* downregulation might play an important role in B-ALL among adult patients.

Expression of *BCL-6* mRNA in the adult B-ALL patient group (median: 0.711) was significantly lower than that in the healthy control group (P < 0.0001) (Fig. 1b). Consistent with this, relative *BCL-6* expression among newly diagnosed B-ALL patients was lower than among healthy controls (P < 0.0001). Median *BCL-6* expression was 0.674 at initial diagnosis (Fig. 1b). Furthermore, expression levels of *BCL-6* in B-ALL patients in CR were significantly reduced compared to those in healthy controls (P = 0.0001).

In addition, we noted decreased expression of *c-MYC* mRNA among adult B-ALL patients (median: 1.840) in comparison with healthy control individuals (P = 0.0017) (Fig. 1c). Our data also revealed lower *c-MYC* expression levels in B-ALL patients at initial diagnosis and those in CR following induction therapy (P = 0.002 and P = 0.04, respectively) compared to those in normal controls. The relative median expression level of *c-MYC* in untreated, newly diagnosed B-ALL patients was higher than in B-ALL patients in CR, but this difference was not significant (P > 0.05).

Correlation between relative *BIM*, *BCL-6*, and *c-MYC* expression in B-ALL

Spearman's rank correlation analyses revealed relative expression of *BIM* to be positively correlated with that of *BCL-6* in B-ALL patients (r_s = 0.866, P < 0.0001) (Fig. 2). However, no correlation was established between *BCL-6* and *c-MYC*, or between *BIM* and *c-MYC* in this respect. Therefore, among the data in the present study, a positive correlation exists between *BIM* and *BCL-6* mRNA levels.

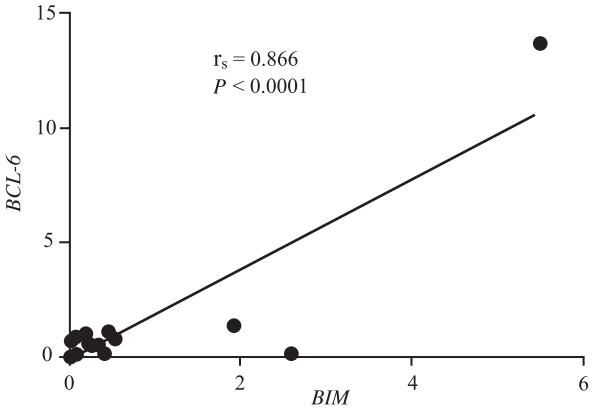

Fig. 2 Correlation analysis of relative *BIM* and *BCL-6* expression levels among B-ALL patients.

Discussion

In this study, *BIM*, *BCL-6*, and *c-MYC* mRNA expression in B-ALL was determined by qRT-PCR. We showed that the median mRNA expression levels of each of these genes were significantly lower in blood samples from B-ALL patients than in those from healthy controls.

In the present work, we observed that *BIM* expression was significantly decreased in untreated, newly diagnosed B-ALL patients and in B-ALL patients in CR compared to that in healthy controls. This implies that loss of *BIM* expression might play an important role in this disease, whether at initial diagnosis prior to treatment or following achievement of CR. Egle *et al* showed that loss of *BIM* accelerates *MYC*-induced lymphomagenesis and identified *BIM* is a tumor suppressor [13].

The work of Korfi *et al* indicated that MEK inhibition acts synergistically with BCL-2/BCL-XL family inhibitors

to suppress proliferation and induce apoptosis of B-ALL cells. This synergism is mediated by the pro-apoptotic factor BIM, which is dephosphorylated as a result of MEK inhibition, allowing it to bind to and neutralize MCL-1, thereby enhancing BCL-2/BCL-XL inhibitor-induced cell death. These findings imply that BIM protein expression may serve as a convenient biomarker to identify B-ALL patients who might benefit from this combination therapy [6]. It has also been shown that basal levels of BIM expression significantly correlate with treatment response among pediatric ALL patients stratified according to rapid or slow early response to a standard four-drug regimen, including glucocorticoid [14–15]. However, in our study, although *BIM* mRNA expression was lower in untreated, newly diagnosed B-ALL patients than in B-ALL patients in CR, this difference was not statistically significant. Therefore, further studies using larger samples of B-ALL specimens are needed to clarify this issue.

We also found *BCL-6* expression to be significantly lower in B-ALL patients than healthy controls, in agreement with earlier reports [16–17]. In the B-cell lineage, BCL-6 is selectively expressed by germinal-center B cells, but not by immature B-cell precursors or differentiated plasma cells [16]. *BCL-6* mutations are very rarely observed in ALL, whereas they are frequently documented in germinal center and post-germinal center neoplasms [17]. A very recent study [18] demonstrated that BCL-6 may represent a biomarker of pre-BCR$^+$ ALL. In approximately 13.5% of the 830 ALL cases examined, ALL cells expressed a functional pre-BCR (pre-BCR$^+$); however, in the majority of cases, such cells lacked pre-BCR signaling (pre-BCR$^-$) [18]. Therefore, it is possible that the patient sample in our study included more pre-BCR$^+$ than pre-BCR$^-$ B-ALL cases. In a previous investigation, BCL-6 protein expression was observed in approximately 12% of the B-lymphoblastic leukemia specimens examined, which derived from both pediatric and adult patients, and no significant difference in the percentage of BCL-6-positive cases was noted between newly diagnosed and relapsed disease. Furthermore, in the same work, public microarray expression database mining was used to show that *BCL-6* mRNA expression levels in B-lymphoblastic leukemia correlate with BCL-6 protein expression [19]. Our results demonstrated that *BCL-6* expression at initial diagnosis is similar to that following achievement of CR, in agreement with previous studies [19].

Expression of *c-MYC* mRNA was also found to be decreased in B-ALL patients compared to that in healthy controls. In contrast, Cardone *et al* reported coordinated elevated expression of MYC and TEL2 in B-ALL [20], and Ge *et al* observed that *c-MYC* expression correlates with clinical features in adult ALL patients, indicating that raised *c-MYC* expression is associated with high-risk ALL in adults [21]. Moreover, a recent report established that *c-MYC* is an important downstream mediator of pre-BCR signaling in B-ALL [22]. These discordant results may be due to the small numbers and genetic heterogeneity of the B-ALL patients involved. *c-MYC* expression at initial diagnosis did not differ from that following CR, in line with a previous report, in which no statistically significant association between c-MYC expression and risk of relapse or death was evident among B-ALL patients [11].

Among the B-ALL samples in the current work, correlation analyses revealed that the expression level of *BIM* positively correlated with that of *BCL-6*. Despite this positive correlation between *BIM* and *BCL-6* mRNA levels, the existence of an interaction between these genes in the leukemogenesis of B-ALL remains to be demonstrated in further investigations.

In conclusion, our results indicate that the expression patterns of the genes *BIM*, *BCL-6*, and *c-MYC* may be used to distinguish ALL patients at initial diagnosis or having achieved CR from healthy individuals. Furthermore, *BIM* and *BCL-6* expression are positively correlated. While the present work provides valuable data that warrant further in vestigation, larger numbers of samples are needed in future studies of the expression patterns of *BIM*, *BCL-6*, and *c-MYC* in B-ALL.

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Conflicts of interest

The authors indicated no potential conflicts of interest.

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