Basic research

Expression of beclin-1 and apoptosis-related genes in childhood acute lymphoblastic leukemia

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Submitted: 9 November 2017
Accepted: 19 November 2017

Arch Med Sci Civil Dis 2017; 2: e168–e173
DOI: https://doi.org/10.5114/amscd.2017.72535

Abstract

Introduction: Autophagy was found to play a major role in the pathogenesis of acute lymphoblastic leukemia (ALL). In this study we investigated the expression of beclin-1, Bad, Bax, Bcl-2, and Bcl-xL in patients with ALL.
Material and methods: This was a comparative study conducted on 100 ALL patients (age 8–15) divided into 2 groups. The first group, the ALL group, comprised ALL cases at their initial diagnosis (46 patients), while the second group, the Remission group, comprised in-remission cases (50 patients). mRNA expression levels in patients' blood samples were determined using real-time polymerase chain reaction (PCR).
Results: Beclin-1 levels were significantly lower in the ALL group than in the Remission group (0.22 ±0.03 vs. 196.8 ±32.47; $p = 0.001$). Bad levels were significantly lower in the ALL group (1.0 ±0.18 vs. 163.6 ±36.2; $p = 0.001$), while Bax levels were significantly higher in the ALL group than in the Remission group (131.52 ±31.4 vs. 4.29 ±0.64; $p = 0.001$). Bcl-2 levels were significantly higher in the ALL group (2678.91 ±575.5 vs. 7.56 ±2.9; $p = 0.001$), and Bcl-xL levels were also significantly higher in the ALL group (142.99 ±24.43 vs. 0.99 ±0.2; $p = 0.001$). There was negative correlation between immunophenotyping with beclin-1 ($r = –0.725$; $p < 0.001$), while there was a positive correlation with Bcl-2 ($r = 0.533$; $p < 0.001$).
Conclusions: Our findings reveal potential prognostic value for these markers in pediatric ALL, with regard to the delicate mutual balance among them.

Key words: autophagy, acute lymphoblastic anemia, acute lymphoblastic leukemia, beclin-1, Bcl-2, Bcl-xL, Bad, Bax.

Introduction

Acute lymphoblastic leukemia (ALL) is considered one of the most common pediatric malignancies, representing 30% of newly diagnosed

cases annually. Acute lymphoblastic leukemia constitutes 70% of pediatric leukemias. It is more commonly observed in the 2–10 years age group. Acute lymphoblastic leukemia is divided into different subtypes according to the presence of chromosomal rearrangements, DNA deletions, gains and sequence mutations that target common cellular pathways. It is known to be a polyclonal disease, with multiple genetic alterations which affect response to treatment, treatment failure and disease relapses. Current available therapy regimens result in 5-year disease-free survival in more than 85% of children (aged 1–21); nevertheless, disease relapse is associated with poor outcome [1, 2]. Acute lymphoblastic leukemia is still the leading cause of cancer-related death in children and young adults between the ages of 21 to 39 [3]. Although ALL is less common in adults, the outcomes of treatment are significantly inferior to those in children [4].

Autophagy represents a catabolic pathway, involving lysosomal degradation and recycling of cell proteins and organelles [5]. It is considered an important survival mechanism for both normal cells and cancer cells in response to metabolic stress or chemotherapy. In hematologic malignancies, autophagy can have contradictory roles in cancer pathology. It either acts as a chemo-resistance mechanism or has tumor suppressive functions [6]. One of the current treatment strategies for leukemia is modulating the autophagy pathway. It is reported that autophagy is downregulated in BCR-ABL leukemic cells. Some studies showed that the activation of autophagy in these leukemias and chronic lymphoblastic leukemia led to serious complications, relapse, as well as resistance to chemotherapy [7].

The Bcl-2 oncoprotein acts a suppressor of apoptosis [7], in multiple cell types and under a variety of conditions, such as treatment with γ irradiation, chemotherapeutic drugs, glucocorticoids, cytotoxic lymphokines, and heat shock [8], suggesting that it acts at a step common to many cell death routes.

Since the discovery of Bcl-2, a large number of genes have been identified whose products are similar to Bcl-2 and have been named the Bcl-2 family of proteins. These proteins either suppress or promote apoptosis. Those, like Bcl-2, that suppress apoptosis include Bcl-xL, MCL-1, Bcl-W, and BFL-1, and those that promote apoptosis include BAX, BAD, BAK, NBK/BIK, BID, HRK and BOK [9, 10]. This family of proteins controls apoptosis by a complex process involving multistep protein-protein interactions. They can form homodimers or heterodimers with each other and sometimes with structurally unrelated proteins [11]. There is evidence that the relative expression and competitive dimerization between these proteins may alter the cellular response to apoptotic stimuli.

A body of evidence currently exists indicating that dysregulation of autophagy is strongly implicated in the pathogenesis of malignancies. Also, cytotoxic drugs that are used in the treatment of leukemias work through the triggering of apoptotic pathways [12]. Moreover, the expression of Bcl-2 transcripts is related to clinical outcome in hematological malignancies. However, the relative expression of members of the Bcl-2 family is still unclear. In addition, the relationship between autophagy and apoptosis is not clearly understood and needs to be thoroughly investigated, especially in leukemias. So, the aim of our work is to study the expression of beclin-1, Bcl-2, Bcl-xL, Bad, and Bax in patients having acute lymphoblastic leukemia and study the correlations between them.

Material and methods

Patients

This is a comparative study conducted on 100 ALL patients divided into 2 groups, each group comprising 50 subjects. The first group represents acute lymphoblastic leukemia cases (age 8–15), while the second group represents cases in remission [8–15].

Patients were randomly selected from Kasr al Ainy, University Hospital, Cairo University, between July 2015 and January 2016.

The study protocol was developed in accordance with the ethical guidelines of the Research Ethics Committee at the Faculty of Medicine, Cairo University, 2015, and was approved by the committee before the start of the work. Patients were asked to give written informed voluntary consent and permission to participate in the study and to publish their results.

RNA extraction and cDNA synthesis

From each recruited subject, total RNA was extracted from EDTA anticoagulated blood using a High pure total RNA preparation kit (Roche Applied Science, Germany), followed by cDNA synthesis for each extracted sample by a Transcriptor first strand cDNA synthesis kit (Roche Applied Science, Germany) according to the manufacturer's instructions.

Real-time polymerase chain reaction

Gene expression for each of Bad, Bax, Bcl-2, Bcl-xL, and beclin-1 was measured using SYBR Green based real-time PCR, and consequent relative quantification analysis with the aid of β-actin as a house keeping gene on a LightCycler 2.0 instrument (Roche Applied Science, Germany). The 20-µl PCR reaction mixture for each of the 5 genes

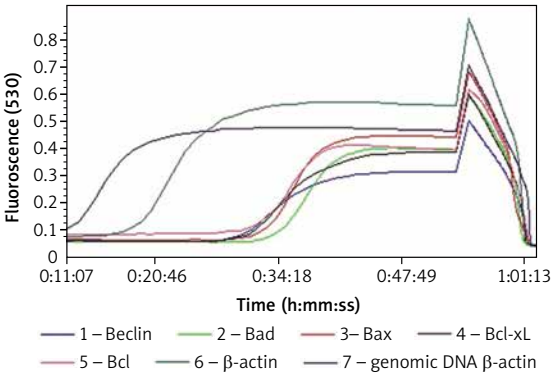

Figure 1. Samples of LightCycler 2.0 Real Time PCR amplification curves and melting curves. Gene expression for each of Bad, Bax, Bcl-2, Bcl-xL, and beclin-1 was measured using SYBR Green based real-time PCR, and consequent relative quantification analysis with the aid of β-actin as a house keeping gene on a LightCycler 2.0 instrument (Roche Applied Science, Germany)

and the corresponding housekeeping gene for each sample included 15 µl of master mix with the following components: 9 µl of PCR grade water, 1 µl of forward primer for each parameter and the housekeeping gene (20 pmol/µl), 1 µl of reverse primer for each parameter and the housekeeping gene (20 pmol/µl), 4 µl of ready to use MasterPLUS SYBR Green I (Roche Applied Science, Germany), and 50 ng of cDNA. The thermal profile was as follows: initial denaturation at 95°C for 10 min, followed by 45 cycles of amplification, starting by denaturation at 95°C for 10 s, annealing at 66°C for 20 s and extension at 72°C for 25 s. Following amplification, an extra cycle of melting curve analysis was done for product characterization by heating the reaction mixture from 65°C to 95°C at a rate of 0.2°C/s (Figure 1). LightCycler 2.0 Real-Time PCR System software automatically calculates the gene expression values by relative quantitative analysis.

Statistical analysis

The data were analyzed using the statistical package SPSS version 22.0 (SPSS IBM., Chicago, IL). ALL and Remission groups are expressed as mean ± SE with 95% confidence interval with medians for quantitative variables, and using the frequencies and percentage for qualitative ones; a *p*-value of 0.05 is considered statistically significant. Spearman's rank correlation coefficient and Pearson's correlation coefficient (*r*) were calculated. Diagnostic parameters of subjects were compared using the non-parametric Wilcoxon-Mann-Whitney *U*-test.

Results

In the present study, we found no statistically significant difference between the ALL group and the Remission group in age at onset, sex, HB, HCT, RBCs, PLT and immunophenotyping. On the other hand, we found a highly statistically significant difference between ALL and Remission patients in WBC at onset (p-value < 0.001), and FAB (p-value < 0.001), shown in Table I.

The pro-apoptotic Bad and Bax, anti-apoptotic Bcl-2 and Bcl-xL transcripts and autophagy gene beclin-1 mRNA levels expressed in the ALL and Remission groups are shown in Figure 2.

Beclin-1 levels were significantly lower in ALL than in Remission patients (0.22 ±0.03 vs. 196.8 ±32.47; p = 0.001). Bad levels were significantly lower in ALL than in Remission patients (1.0 ±0.18 vs. 163.6 ±36.2; p = 0.001), while Bax levels were significantly higher in ALL than in Remission patients (131.52 ±31.4 vs. 4.29 ±0.64; p = 0.001). Bcl-2 levels were significantly higher in ALL than in Remission patients (2678.91 ±575.5 vs. 7.56 ±2.9; p = 0.001), while Bcl-xL levels were significantly higher in ALL than in Remission patients (142.99 ±24.43 vs. 0.99 ±0.2; p = 0.001).

Evaluation of the correlation between beclin-1 and each laboratory parameter (Bad, Bax, Bcl-2, Bcl-xL) in each group of the study showed that there is no correlation between beclin-1 with other laboratory parameters (Bad, Bax, Bcl-2, Bcl-xL) in the ALL group, but in the remission group there is a negative correlation between beclin-1 and Bax (r = –0.254; p < 0.04) and Bcl-xL (r = –0.247; p < 0.05) (Table II).

Evaluating clinical correlations between FAB, immunophenotyping with the 5 laboratory parameters (beclin-1, Bad, Bax, Bcl-2, Bcl-xL) in the ALL group, we found no correlation between FAB and laboratory parameters (Bad, Bax, Bcl-2, Bcl-xL) in the ALL group, but there is a negative correlation between FAB and beclin-1 (r = –0.318; p < 0.03), while there is no correlation between immunophenotyping and laboratory parameters (Bad, Bax, Bcl-xL) in the ALL group, but there is a negative correlation between immunophenotyping and beclin-1 (r = –0.725; p < 0.001), while there is a positive correlation with Bcl-2 (r = 0.533; p < 0.001) (Table III).

We evaluated the clinical correlation between WBC count at onset (> 100,000) and the 5 laboratory parameters (beclin-1, Bad, Bax, Bcl-2, Bcl-xL). In the remission group, we found no correlation between WBC count at onset and laboratory parameters (beclin-1, Bad, Bax, Bcl-xL), but there is a negative correlation between WBC count at onset and Bcl-2 (r = –0.258; p < 0.05) (Table IV).

Discussion

We aimed in the current study to evaluate the expression of beclin-1, one of the main autophagocytic genes, which bridges autophagy and

Table I. Clinical and pathological features of ALL and Remission groups

Parameter		ALL group Mean ± SE or *n* (%) *N* = 46	Remission group Mean ± SE or *n* (%) *N* = 50	*P*-value
Age at onset		6.7 ±0.45	6.4 ±0.41	0.6
Gender	Female	10 (21.7%)	18 (36.0%)	0.1
	Male	36 (78.3%)	32 (64.0%)	
WBC at onset		129.2 ±23.05	88.6 ±14.4	0.001**
Hb		8.08 ±0.3	7.8 ±0.3	0.4
HCT		25.3 ±0.9	23.9 ±0.8	0.2
RBCs		3.04 ±0.12	2.9 ±0.1	0.7
Platelets		40304.4 ±4087.08	48140.0 ±4303.8	0.1
FAB	L1	9 (19.6%)	4 (8.0%)	0.001**
	L2	28 (60.9%)	42 (84.0%)	
	L1/L2	9 (19.6%)	4 (8.0%)	
Immuno-phenotyping	B	29 (63.0%)	34 (68.0%)	0.6
	T	17 (37.0%)	16 (32.0%)	
mRNA expression	Bad	1.0 ±0.18	163.6 ±36.2	0.001**
	Bax	131.52 ±31.4	4.29 ±0.64	0.001**
	Bcl-2	2678.91 ±575.5	7.56 ±2.9	0.001**
	Bcl-xL	142.99 ±24.43	0.99 ±0.2	0.001**
	Beclin 1	0.22 ±0.03	196.8 ±32.47	0.001**

***Correlation is significant at the 0.01 level (2-tailed).*

apoptosis and both pro- (Bad, Bax) and anti-apoptotic (Bcl-2, Bcl-xL) factors in childhood ALL. We studied whether the levels of these biomarkers could be of prognostic relevance.

Beclin-1, a Bcl-2 homology 3 (BH3) domain only protein, is an essential initiator of autophagy. Beclin-1 recruits key autophagic proteins to a pre-autophagosomal structure [13]. We found in the present study that the beclin-1 mRNA level is significantly lower in the ALL group than in the Remission group (p = 0.001). Yuan *et al.* found that activation of autophagy by rapamycin inhibits *in vitro* pre-B ALL cells in part through down-regulating DNA and RNA polymerases [14]. Therefore, activation of autophagy could be of a great value in fighting ALL.

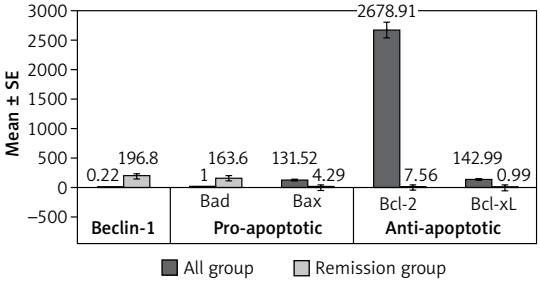

Figure 2. Significant difference between groups of the study (ALL, Remission) in laboratory parameters. The pro-apoptotic Bad and Bax, anti-apoptotic Bcl-2 and Bcl-xL transcripts and autophagy gene Beclin-1 mRNA levels expressed in the ALL and Remission groups were measured using real-time PCR

The anti-apoptotic proteins Bcl-2 and Bcl-xL bind and inhibit beclin-1, which leads to inhibition of autophagy [9]. Our findings showed that both Bcl-2 and Bcl-xL mRNA levels were significantly higher in the ALL group than in the Remission group (p = 0.001). The Bcl-2 results were consistent with what was found by Barakat *et al.* [15], and Hogarth *et al.* [16]. The increase in Bcl-2 con-

Table II. Correlation of beclin-1 with Bad, Bax, Bcl-2, and Bcl-xL in both ALL group and Remission group

Parameters		Acute ALL group		Remission group	
		r	*P*-value	*r*	*P*-value
Beclin-1	Bad	–0.017	0.908	0.21	0.143
	Bax	–0.102	0.501	–0.254	0.04*
	Bcl-2	–0.124	0.411	–0.092	0.527
	Bcl-xL	0.096	0.525	–0.247	0.05*

**Correlation is significant at the 0.05 level (2-tailed).*

Table III. Correlation of beclin-1, Bad, Bax, Bcl-2, and Bcl-xL with both FAB and immunophenotyping in ALL group

Parameters	FAB		Immunophenotyping	
	r	*P*-value	*r*	*P*-value
Beclin-1	–0.318*	0.03	–0.725**	0.001
Bad	–0.097	0.522	–0.158	0.29
Bax	0.014	0.924	–0.005	0.97
Bcl-2	0.14	0.353	0.533**	0.001
Bcl-xL	0.158	0.293	–0.107	0.48

**Correlation is significant at the 0.05 level (2-tailed), **correlation is significant at the 0.01 level (2-tailed).*

Table IV. Correlation of beclin-1, Bad, Bax, Bcl-2, and Bcl-xL with WBC count at onset in Remission group

Parameters		*r*	*P*-value
WBC count at onset (> 100,000)	Beclin-1	–0.08	0.58
	Bad	0.122	0.399
	Bax	0.171	0.234
	Bcl-2	–0.258	0.05*
	Bcl-xL	0.043	0.766

**Correlation is significant at the 0.05 level (2-tailed).*

centration in newly diagnosed ALL cases was suggested to be due to DNA hypomethylation at the 5 end of the Bcl-2 gene. The hypomethylation is associated with transcriptional upregulation of gene expression leading to inhibition of apoptosis [15]. We think that both Bcl-2 and Bcl-xL need further study, and could be of prognostic significance.

The pro-apoptotic proteins Bad and Bax were found to have the ability to induce autophagy through binding to Bcl-2, causing the cancellation of its inhibitory effect [17]. Bad mRNA levels were found to be lower in the ALL group than in the Remission group ($p = 0.001$). On the other hand, Bax mRNA levels were found to be higher in the ALL group than in the Remission group ($p = 0.001$). These Bax mRNA results are consistent with the results of Barakat *et al.* [15] and Maia *et al.* [18]. We found no similar studies on Bad mRNA. Bax mRNA increase was found to have a significant correlation with increased probability of relapse in ALL patients [16]. The unexpected relationship between high BAX expression and poor prognosis may be explained by the observation that Bcl-2 and BAX have a role in the control of proliferation as well as apoptosis. Mature T cells overexpressing BAX have been shown to have lower levels of p27 Kip1 and enter the S phase more rapidly in response to interleukin-2 stimulation than control T cells. The converse is true for Bcl-2-transfected T cells. Transfection of several mammalian cell lines with Bcl-2 has been associated with reduced cell proliferation and prolongation of the G1 phase of the cell cycle, both of which could be abrogated by the coexpression of BAX [16, 18].

A significant inverse correlation emerged between beclin-1 and Bax mRNA levels ($r = –0.254$; $p = 0.04$) and between beclin-1 and Bcl-xL mRNA ($r = –0.247$; $p = 0.05$) in the Remission group. This is, of course, due to the similar roles of both beclin-1 and Bax in the induction of autophagy [9]. In the ALL group, we found an inverse correlation between beclin-1 and FAB ($r = 0.318$; $p = 0.03$). Moreover, an inverse correlation was observed between beclin-1 and immunophenotyping ($r = –0.725$; $p = 0.001$), and a positive one emerged between Bcl-2 mRNA and immunophenotyping ($r = 0.533$, $p = 0.001$). In the Remission group, an inverse correlation was observed between Bcl-2 mRNA levels and WBC ($r = –0.258$; $p = 0.05$).

In conclusion, this is a study investigating the expression levels of this combined panel of autophagic beclin-1, 2 pro-apoptotic (Bad & Bax), and 2 anti-apoptotic (Bcl-2 & Bcl-xL) parameters, demonstrating the correlation between their levels in ALL. Our findings reveal potential prognostic value for these markers in pediatric ALL with regard to the delicate mutual balance among them.

Conflict of interest

The authors declare no conflict of interest.

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