Role of BIRC5 Gene as a Prognostic Marker in Pediatric Acute Lymphoblastic Leukemia

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Abstract

Background and objectives: Acute lymphoblastic leukaemia (ALL) is the most common malignancy diagnosed in children. Survivin, a small inhibitor-of-apoptosis protein (IAP) encoded by the baculoviral inhibitor of apoptosis repeat-Containing 5 (BIRC5) gene, plays critical roles in malignancy by preventing apoptosis through blocking caspase activity. The aim of this study was to assess the prognostic role of BIRC5 gene in paediatric ALL.

Subjects and methods: The present study was carried out on 42 children with the' novo ALL who were followed up for two years and 10 apparently healthy children of matched age and sex (controls). Each child was subjected to complete history taking, clinical examination, laboratory investigations the form of CBC, Leishman-stained peripheral blood smears, Lactate dehydrogenase (LDH), Bone marrow (BM) aspiration and examination of Leishman-stained smears, Immunophenotyping on BM samples for routine panel of ALL determined by flow cytometer and quantitative determination of survivin gene expression by real time PCR on BM samples.

Results: Patients with ALL had a significantly higher BIRC5 expression than did the control group (P=0.0004). There was a significant increase in survivin gene expression level in T-ALL when compared to common BALL and pre B-ALL (P=0.001). A significant positive correlation was found between survivin expression and LDH, uric acid and white blood cells (WBCs) (r=0.47, P=0.002; r=0.31, P=0.05, r=0.62, P=0.001), respectively. A significant higher survivin expression levels, LDH and WBCs was found in children with unfavorable outcome. 94.5% of ALL patients with high survivin expression had an unfavorable outcome while, in ALL children with low survivin expression, only 25% had an unfavorable outcome (P=0.001).

Conclusion: BIRC5 gene expression was correlated with unfavorable outcome of childhood ALL. So, its measurement at diagnosis may detect a high risk ALL subgroup.

Keywords: Acute lymphoblastic leukaemia (ALL); BIRC5 gene

Introduction

Acute lymphoblastic leukaemia (ALL) is a malignant disorder of lymphoid progenitor cells that proliferates and replaces the normal hematopoietic cells of the bone marrow [1]. ALL is the most common malignancy diagnosed in children, representing nearly one third of all paediatric cancers [2]. In ALL, B cell origin is the most frequently diagnosed (B cell ALL) representing 83%, and T cell ALL comprises 15% [3]. The classification into B- and T-ALL is important for risk stratification and treatment [4]. The improvement in ALL cure rates can be in part attributed to the assessment of prognostic factors and molecular markers associated with a better response to therapy [2]. Survivin, a small inhibitor-of-apoptosis protein (IAP) encoded by the baculoviral inhibitor of apoptosis repeat-containing 5 (BIRC5) gene plays a critical role in apoptosis, cell division, and proliferation [5]. Survivin gene newly named BIRC5 can be regarded as an oncogene as its overexpression in cancer cells contributes to their resistance to apoptosis and therapies, thus contributing to their ongoing survival [6]. It prevents apoptosis by blocking caspases activity [7]. Overexpression of survivin was found in some studies to correlate with poor prognosis in many hematologic malignancies [8] and could serve as therapeutic targets eliminating those cells that drive the disease and trigger relapse [9].This study aimed to assess the level of expression of survivin gene (BIRC5) in childhood ALL and to evaluate its association with the different demographic, clinical and laboratory data, as well as studying its role in therapy outcome (prognostic value).

Subjects and Methods

Patients and controls

Patients were selected from the outpatient clinic of Minia oncology centre. This group consists of 42 newly diagnosed children with ALL, their ages ranged from 3 to 15 years with mean ± SD 8.2 ± 3.5 and included 23 males (54.8%) and 19 females (45.2%). The diagnosis of ALL was confirmed by immunophenotyping and morphological assessment of peripheral blood and bone marrow (BM) smears. This group was sub-classified into five groups according to immunophenotyping, group I_A, I_B, I_C, I_D and group I_E. Patients' group was as well sub-sorted according to survivin gene expression level into IF: Patient with low survivin expression and IG: Patient with high survivin expression.

Patients were followed up for two years and the outcome was designated either favorable or unfavorable. Favorable outcome was

considered when complete remission (CR) was achieved. CR is defined by neutrophil count at least ($1.5 \times 10^3/\mu L$), platelet count (>100 × $10^3/\mu L$), BM aspiration that demonstrates at least 20% cellularity and <5% blasts [10]. Unfavorable outcome was considered to failure of remission, occurrence of relapse or death.

The controls were ten apparently healthy subjects, matched for age and sex with patient group. Their ages ranged from 4 to 15 years with mean ± SD 7.01 ± 3.4 and included 5 males (50%) and 5 females (50%).

Laboratory investigations

Complete blood count (CBC) determined by automated cell counter Cell Dyn 1700 (USA); Lactate dehydrogenase (LDH) and uric acid levels using ACE automated quantitative multistation chemistry Analyzer (Schiapparelli Biosystems. INC; USA); Bone marrow aspiration and examination of Leishman-stained smears; Immunophenotyping for patients only on BM samples determined by Flow cytometer (FACS Calibur BD bioscience, USA).

Determination of survivin gene

RNA was extracted using Gene JET™ whole blood RNA purification Mini kit, supplied by Sermentas Life Sciences G according to the manufacturer's instructions. Briefly, bone marrow was centrifuged for 5 min. 400 × g (2000 rpm) at 4°C, the pellet was resuspended in 600 μL of lysis buffer, mixed well. After the addition of 700 μL of 97% ethanol, the liquid was repeatedly run in a spin-column more than once until it was finished. Finally, RNA was eluted in 50 μL of RNase-free water and, was stored at -80°C until use. Total RNA was reverse-transcribed to complementary DNA (cDNA) using QuantiTect Reverse Transcription Kit supplied by Qiagen (Germany).

DNA from all samples, were amplified by RT-PCR assay, run in an ABI Prism 7000 SDS Real-Time apparatus (Applied Biosystems, USA) using the Superscript III.

Platinum Two-step qRT-PCR kit (Invitrogen, USA). The 25 μL reaction volume contained 12.5 μL 2x QuantiFast Probe PCR Master Mix, 1.25 μL 20x QuantiFast Probe Assay (FAM), 1.25 μL 20x QuantiFast Probe Assay (MAX), 0.5 μL High-ROX Dye Solution, 4.75 μL Template DNA or cDNA and 4.75 μL RNase-free water. The following thermal profile was used: a single cycle for 5 min at 90°C for initial activation step, 30 seconds at 95°C for denaturation, followed by 40 amplification cycles of 30 seconds at 95°C and 30 seconds at 60°C each (annealing-extension step). Each reporter signal was measured against the internal reference dye (ROX) signal to normalize for non-PCR-related fluctuations between samples. The data were collected at the annealing step of each cycle and the threshold cycle (Ct) for each sample was calculated by determining the point at which the fluorescence exceeded the threshold limit.

The quantification of transcripts was carried out by the comparative CT (ΔΔCT) method, the theoretic basis of which was previously described [11,12]. For each experimental sample, the difference between survivin CT value and the CT value of β-actin (housekeeping gene) was used to normalize for differences in the amount of total nucleic acid added to each reaction and the efficiency of the RT step (ΔCT). For relative quantification by the comparative CT method, values are expressed relative to a sample called calibrator. The calibrator is the weakest signal from the normalization (ΔCT) in each cell line. The ΔCT for each experimental sample was subtracted from the ΔCT of the calibrator (ΔΔCT). The amount of survivin (linear value), normalized to an endogenous reference (β-actin) and relative to the calibrator, was determined by evaluating the expression 2-ΔΔCT. CT a numerical value given as parameters by real time PCR software (Corbett Research).

Statistical Methods

Statistical analyses were performed using the SPSS (Statistical Package for Social Science) statistics version 19. Differences in the mean of continuous variables, e.g., age, survivin, LDH, etc., were analysed using parametric tests (independent sample T Test). Differences between categorical variables, e.g., sex, splenomegaly, etc., were analysed using a χ^2 test. The Mann-Whitney U-test was used for statistical comparisons of survivin expression between two subgroups of the cohort of patients, while the Kruskal-Wallis test was applied when comparing more than two subgroups. All tests were considered statistically significant when P value was <0.05.

Results

Survivin expression in controls and ALL patients

Survivin expression was negligible or very low in normal BM with a range (0- 15.0) & mean ± SD (10.4 ± 5.7). In contrast, survivin expression was significantly elevated in patient's BM with a range (10.9-1560), mean ± SD (295.6 ± 257.5) & (P=0. 0004) (Table 1).

Survivin expression levels were compared in different groups of patients. Common B ALL showed survivin gene expression ranging from 12.0 to 385 with a mean of 121.7 ± 120.5. Patients with pre B-ALL had survivin expression values ranging from 10.9 to 832 with a mean of 231.6 ± 228.9. Patients with T-ALL had values ranging from 15 to 1560 with a mean of 882.6 ± 529.6. Mean survivin expression levels showed highly significant increases in T-ALL when compared with common B ALL and pre B ALL (P=0.001) (Table 2).

Correlation of survivin with disease characteristics

There were significant associations between survivin expression level and WBCs (P=0.001), LDH (P=0.002) and uric acid (P=0.05). There was no correlation with other parameters (Table 3).

Survivin levels and patient outcome

There was a significant correlation between elevated WBCs count, LDH, survivin expression levels and unfavorable outcome (P=0.04, P=0.04, P=0.0002) respectively. Common B ALL had statistically

		Patients	Controls	P-value
		Group I N=42	Group П N=10	
Survivin	Range	10.9-1560	0-15.0	0.0004**
	Mean ± SD	295.6 ± 257.5	10.4 ± 5.7	
P value is highly significant (when p-value < 0.001).				

Table 1: Comparison between patients and control subjects regarding survivin gene expression (normalized mRNA).

		Group IB (Common B) N=17	Group IC(Pre B) N=17	Group IE (T-ALL) N=6	P-value		
Survivin	Range mean ± SD	12.0- 385	10.9 -832	15-1560	**IB vs. IC**	**IB vs. IE**	**IC vs. IE**
		121.7 ± 120.5	231.6 ± 228.9	882.6 ± 529.6	0.7	0.001**	0.001**
P value is highly significant (when P < 0.001).							

Table 2: Survivin Gene expression in different immunophenotyping subgroups of ALL.

Variables	Survivin	
	r	P
Sex	0.061	0.7
L.N	0.05	0.7
Splenomegaly	0.12	0.4
Hepatomegaly	0.12	0.4
HB	0.14	0.4
Platelets	-0.19	0.2
WBCs	0.62	0.001**
B.M blast	0.14	0.4
LDH	0.47	0.002*
Uric acid	0.31	0.05*

Hb; Haemoglobin: WBCs; White blood cells: L.N; Lymph node: B.M.; Bone marrow: LDH; Lactate dehydrogenase
*P value is significant (when P < 0.05).
**P value is highly significant (when P < 0.001).

Table 3: Correlation of survivin expression to clinical, biochemical and hematological data of patients.

	Favorable N=19	Un favorable N=23	P
Age	6.1 ± 2.9	7.9 ± 3.7	0.9
Sex			
Male	10(52.6%)	12(57.1%)	0.7
Female	9(47.4%)	9(42.9%)	
L.N.	9(47.4%)	12(57.1%)	0.5
Splenomegaly	11(57.9%)	15(71.4%)	0.3
Hepatomegaly	11(57.9%)	8(38.1%)	0.2
HB (g/dL)	7.4 ± 1.9	7 ± 1.9	0.4
Plt × 10³/mm³	46.5 ± 32.9	49.3 ± 29.8	0.7
WBCs × 10³/mm³	21.9 ± 17.8	40.9 ± 27.5	0.04*
LDH (U/L)	921.7 ± 409.3	1634.2 ± 1494.9	0.04*
Uric acid(mg/dL)	5.3 ± 3.8	5.2 ± 3.5	0.9
B.M. blast	77.2 ± 19.8	79.3 ± 15.2	0.6
Survivin	87.0 ± 77.7	475.9 ± 427.2	0.0002**
Immunophenotyping subgroup			
Early precursor B	0 (0%)	1(100%)	
Common B	12(70.6%)	5(29.4%)	0.04*
Pre B	6(35.3%)	11(64.7%)	0.1
Mature B	0 (0%)	1(100%)	
T-ALL	1(16.7%)	5(83.3%)	0.03*

Hb; Haemoglobin: WBCs; White blood cells: L.N; Lymph node: B.M; Bone marrow: LDH; Lactate dehydrogenase
*P value is significant (when P < 0.05)
**P value is highly significant (when P < 0.001)

Table 4: Relation of clinical, biochemical and hematological data to outcome of patients.

significant favorable outcome (P=0.04) while T-ALL had an unfavorable outcome (P=0.03) (Table 4).

There isn't any significant association between survivin expression level at diagnosis and response to induction therapy (P=0.6). Patients suffering unfavorable outcome in the form of failure of remission, occurrence of relapse or death, had highly significant higher survivin expression levels with a mean ± SD of (608 ± 478.7), (254.1 ± 144.5) and (1004 ± 243.2) respectively, compared to those remaining in complete remission (favorable outcome) mean ± SD: (87 ± 77.2) (p=0. 001) (Table 5).

There was a significant increase in WBCs count in patient with high survivin expression than those with low survivin expression (P=0.05).

94.5% of patients with high survivin expression had unfavorable outcome, however, only 25% of patients with low survivin expression had an unfavorable outcome (P=0.001) (Table 6).

Discussion

ALL is most common in childhood, with a peak incidence at 2–5 years of age and another peak in old age [13]. Improvements in diagnosis and treatment, overall cure rates for children with ALL exceed 80%, but despite the improvement about 20% of patients still relapse [14]. The majority of patients in this study were B-ALL 85.8%, while 14.2% T-ALL and this agreed with other studies [15]. In the

	Outcome	N	Mean ± SD	P
Survivin	Post induction response:			
	CR	26	145 ± 138.8	0.6
	RD	16	608 ± 478.7	
Survivin	Follow up for two years:			
	CR (favorable)	19	87.0 ± 77.3	0.001**
	RD (unfavorable)	16	608 ± 478.7	
	R (unfavorable)	5	254.1 ± 144.5	
	Death (unfavorable)	2	1004 ± 243.2	

**P value is highly significant (when p- value < 0.001). CR; complete remission: RD; Residual disease: R; relapse

Table 5: Relation of Survivin expression level to initial post induction response and outcome data of patients after follow up.

		Low survivin expression N=24 (57%)	High survivin expression N=18 (43%)	P-value
Age	Range mean ± SD	3-15	3-15	0.8
		6.6 ± 2.9	6.9 ± 3.9	
Sex	Male	13(54.1%)	10(52.6%)	0.6
	Female	11(45.9%)	8(47.4%)	
L.N	+v	13(54.1%)	9(50%)	0.5
	-ve	11(45.9%)	9(50%)	
Splenomegaly	+v	15(62.5%)	12(66.6%)	0.6
	-ve	9(37.5%)	6(33.4%)	
Hepatomegaly	+v	12(50%)	8(44.4%)	0.3
	-ve	12(50%)	10(55.6%)	
HB (g/dL)	Range mean ± SD	4.2-10.9	4.4-10.8	0.2
		7.3 ± 2.0	8.1 ± 1.8	
Plt × 10³/mm³	Range mean ± SD	5-110	Jul-99	0.3
		52.5 ± 31.9	41.6 ± 29.2	
WBCs × 10³/ mm³	Range mean ± SD	2.5 -100	3.1-285	0.05*
		22.6 ± 20.1	69.9 ± 60.3	
LDH (U/L)	Range mean ± SD	302 - 2250	392 - 6279	0.1
		1001.8 ± 476	1736.8 ± 1680.8	
Uric acid (mg/dL)	Range mean ± SD	2.1-17.79	2.1-16.54	0.6
		5.0 ± 3.4	5.6 ± 3.9	
BM blasts	Range mean ± SD	40-99	60-98	0.1
		75.2 ± 19.6	83.0 ± 12.3	
Outcome	Favorable %	18(75%)	1(5.5%)	0.001**
	Unfavorable %	6(25%)	17(94.5%)	

Hb, Haemoglobin: WBCs, white blood cells;; L.N, lymph node; B.M., bone marrow; LDH, lactate dehydrogenase
* P value is significant (when P<0.05)
** P value is highly significant (when P<0.001)

Table 6: Comparison between low and high survivin expression in ALL patients.

present study, statistically significant higher survivin expression was found in ALL patients when compared to the control group. This may provide an evidence that survivin plays a role in the malignant process of acute leukaemias, this agreed with previous studies [8,16]. We could not identify a significant variation of the survivin expression level in childhood B-ALL subtypes that agreed with Yahya et al., [16]. However, on comparing survivin expression levels in T-ALL and B-ALL, there was a highly significant increase in survivin level in T-ALL (p=0.001), this was consistent with other studies [17]. In the present study, survivin gene expression showed no association with clinical data of ALL patients as; fever, lymph node enlargement, hepatomegaly and splenomegaly (P>0.05), this was agreed with Esh et al., [18] who found no association between survivin expression and clinical data of ALL patients.

Survivin gene expression showed statistically significant positive correlations with WBCs count, that agreed with Xue et al., [19] who found high levels of survivin in tumour cells, but disagreed with Troeger et al., [20] who found that there was no statistical difference of survivin expression level between patients presenting with low initial leukocyte counts and high initial leukocyte counts. While there was no correlation of survivin with Hb level and platelet count, this was consistent with a previous study [19] which reported no association between high surviving expression and age, HB level, and platelet count at diagnosis, but disagreed with Sadek et al., [21] who stated that survivin expression level showed statistically significant negative correlations with RBCs count, HB level and platelet count. Correlation of survivin gene expression level to biochemical data showed statistically significant positive correlations with LDH and uric acid that agreed with Xue et al., [19] who identified survivin expression as an independent predictive parameter on survival in addition to LDH.

There was non-significant relation between age, sex, generalized lymphadenopathy, hepatomegaly, splenomegaly, HB, Plt and uric acid and outcome. That was against National Cancer Institute study [22] which found that certain factors affect prognosis (chance of recovery) and treatment options. This result also against the American Cancer Society study [23], which found that age at diagnosis and initial white blood cell count are thought to be the most important prognostic factors of childhood ALL. This may be due to the limited age range (3-15 years) and a small number of subjects of our study.

There was significant correlation between high WBCs count and unfavourable outcome, this was in agreement with the Mayo Clinic staff study [24] who found that the most important bad prognostic feature is a high WBC count; above 50 × 109/L at time of diagnosis. This result also agreed with American Cancer Society [23] who found that children with ALL with high WBC counts are classified as high risk ALL. There was non-significant correlation between BM blasts at time of diagnosis and outcome of patients that agreed with the Mayo Clinic staff study [24] but was disagreed with Sadek et al., [22] who found that BM blasts have prognostic value in ALL. There was a significant relation between elevated LDH level and unfavorable outcome (P=0.04), so elevated LDH at time of diagnosis can be used as bad prognostic factors. That was in agreement with Kyle et al., [25] and Xue et al., [19] who stated that LDH is one of the important prognostic indices for ALL.

In this study, common B ALL had better prognosis (P=0.04) while T-ALL has a bad prognosis (P=0.03), this was agreed with Locatelli et al., [26] who stated that TALL, are associated with a worse prognosis than B-ALL. This result also agreed with the American Cancer Society study [23] who found that children with pre-B, common, or early pre-

B-cell ALL generally do better than those with mature B-cell (Burkitt) leukemia and T-ALL.

In the present study there was no association between survivin expression level at diagnosis and response to induction therapy (P=0.6). Our result is the same as by Esh et al., [18] who stated that high risk patients are treated more aggressively than low risk patients so the negative effect of survivin over expression in the high-risk group is masked. But this result disagreed with Andrew et al., [27] who stated that survivin overexpression in primary ALL plays a critical role in drug resistance and associated with refractory disease.

Patients suffering unfavorable outcome had significantly higher survivin expression levels compared to those remaining in complete remission (favorable outcome). Significantly higher survivin levels in patients who suffered a relapse suggest that high survivin levels confer a survival advantage to blasts by inhibiting programmed cell death. Our result goes in agreement with Mahsa et al., [8] who revealed a correlation between survivin gene expression and a clinically unfavorable course of paediatric precursor B-cell ALL (BCP-ALL), suggesting that survivin expression is a poor prognostic factor. That also agreed with Zhang Y et al., [6] who found that survivin is overexpressed in relapsed pediatric ALL patients.

It has been shown that transcription factor Sp1 is essential for the suppression of survivin gene in cancers via recruitment of repression complex, including p53, DNMT1, histone methyltransferase G9a, and HDAC1[28]. As recent publication indicates that histone methyltransferase G9a is able to interact with DNMT3a/b [29], de novo DNA methyltransferases DNMT3a and 3b might also be the components of this repression complex. Interestingly, as G9a is involved in the maintenance of DNA methylation at imprinted loci, the established DNA methylation at the promoter of survivin gene might not only be maintained by DNMTs [30], but also histone methyltransferase G9a. Therefore, it is crucial to investigate the way to enhance the Sp1 associated silencing of survivin gene, especially in the children suffered ALL.

Conclusion

Survivin gene showed high expression level in childhood ALL with significantly increased levels in T-ALL than B-ALL; however, there was non-significant variation between common B and Pre B subgroups. Patients suffering unfavorable outcome in the form of failure of remission, occurrence of relapse or death, had significantly higher survivin expression levels. Overexpression of the survivin gene can be considered as an important participant to predict poor prognosis in childhood ALL i.e., measurement of survivin expression level at diagnosis could detect a high risk ALL subgroups.

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