Expression of The Antiapoptotic Gene Survivin in Acute Leukemias

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Abstract: Objectives:To assess the level of expression of the antiapoptotic signal "Survivin" in Egyptian patients with acute leukemias and to delineate any significant correlation between the level of Survivin with the clinical and haematological findings in those patients. Patients and Methods: Survivin expression was quantitatively determined by a real-time PCR technique in 30 acute leukemia patients; ALL and AML in two age groups; pediatric group (<18 years) and adult group (≥18 years) and in age and sex matched control healthy subjects. Results: Statistically significant higher expression was noted in both ALL and AML groups when compared to the control group (p-value = 0.0001). A statistically significant negative correlation was detected between Survivin expression and RBCs count, HB level and Platelet count with p-values = 0.01, 0.01 and 0.0001 respectively. Positive correlations were found with T.L.C, peripheral blood blasts, bone marrow malignant cells, LDH , ALP and uric acid levels with p-values = 0.0001, 0.0001, 0.03, 0.0001, 0.006 and 0.001 respectively. During the post-induction phase, Survivin expression showed a statistically insignificant difference between patients achieving complete remission and those showing unfavorable response with a p-value = 0.7. After follow up, the expression change between patients achieving complete remission and those showing unfavorable response was statistically insignificant with a p-value = 0.6. In summary, the previous data emphasized important correlations between Survivin expression and established risk factors in acute leukemia patients. Thus Survivin could be used as a marker for assessment of bone marrow infiltration that in future could be used to refine treatment stratification.
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1. Introduction

The balance between cell death and cell viability is important in tissue homeostasis. Abnormalities in the control of programmed cell death (apoptosis) play an important role in tumorigenesis (Tazzari et al. 2008). The evolutionarily conserved multi-step apoptosis cascade is regulated by proteins that promote or inhibit apoptotic cell death (Jakubowska et al. 2007). The inhibitor of apoptosis Survivin is a member of the inhibitor of apoptosis protein (IAP) family that suppresses apoptosis or programmed cell death and regulates cell division and thereby integrates apoptosis and cell division (Badran et al. 2004). Six human IAPs have been identified: XIAP, cIAP1, cIAP2, NAIP, livin, and survivin. Because of their important role in regulating apoptosis, IAPs are being investigated as a potential prognostic factor as well as a treatment target in cancer patients (Wrzesień-Kuś et al. 2004). The survivin protein shuttles between the nucleus and the cytoplasm, where it effectively inhibits apoptosis, most likely by binding to second mitochondrial activator of caspase (Smac) (Song et al. 2003).

One of the clinically significant features of survivin is its differential distribution in many cancers compared with its limited expression in normal terminally differentiated tissues (Johnson and Howerth 2004). Very high levels of survivin have been described in a number of different tumors (Ito et al. 2000, Kato et al. 2001, Kawasaki et al. 1998, Lu et al. 1998, Monzo et. 1999, Saitoh et al. 1999, Satoh et al. 2001, Swana et al. 1999, Tanaka et al. 2000 & Yoshida et al. 2001).

Overexpression of several IAPs has been detected in various hematological malignancies, including acute leukemias, myelodysplastic syndrome (MDS), chronic myeloid leukemia (CML), and many types of lymphoid malignancies, such as chronic lymphocytic leukemia (CLL) and diffuse large B-cell lymphoma (DLBCL). Many publications revealed significant correlation between a high level of IAPs, especially of XIAP and survivin, and tumor progression contributing to leukemogenesis due to deregulated apoptosis (Wrzesień-Kuś et al. 2004 &

Troeger et al. 2007). The expression of survivin may be a general feature of cancer and survivin alone or with other antiapoptosis genes such as Bcl-2 may extend the viability of transformed cells and regulate their susceptibility/resistance to apoptosis-based therapy. For this reason survivin may provide an ideal therapeutic target for its selective expression in neoplasia (Ambrosini et al. 1997). Various strategies have been developed to target IAPs for therapeutic purposes in leukemia and lymphoma cells, including small-molecule inhibitors and antisense oligonucleotides (Fulda 2009).

The aim of this study is to assess the level of expression of the antiapoptotic signal "Survivin" in Egyptian patients with acute leukemias and to delineate any significant correlation between the level of Survivin with the clinical and haematological findings in those patients. Hence we can throw some lights upon the prognostic impact of surviving.

2. Patients and Methods

Thirty patients with untreated de' novo acute leukemia were included in the analysis for survivin expression. Patients were selected from the out patient clinic of medical and pediatric oncology departments of the National Cancer Institute (NCI), Cairo University and followed up for one year. According to the French-American-British (FAB) standard there were 15 patients with acute myeloblastic leukemia (AML) and 15 patients with acute lymphoblastic leukemia (ALL) consisting of 25 males and 5 females . The leukemic patients were divided into two age groups; pediatric group (<18 years): 16 patients and adult group (≥18 years): 14 patients. The control group included 20 age and sex matched normal healthy volunteers, they were 14 males and 6 females. All patients of this study were treated according to the institute ongoing induction and consolidation regimens. Patients were followed up for one year and outcome was designated either favorable or unfavorable. Favorable outcome was considered when complete remission (CR) was achieved; CR status was determined 4 weeks after induction chemotherapy, it is defined by neutrophil count at least (1.5 $x10^3/\mu L$), platelet count (> 100 $x10^3/\mu L$), BM aspiration and biopsy that demonstrate at least 20% cellularity, < 5% blasts and no auer rods as well as absence of extramedullary infiltration.

Unfavorable outcome was considered with failure of remission, occurrence of relapse or death.

An informed consent was obtained after complete description of the study from each adult participant and from parents or legal guardians of the children participants at the time of enrolment, according to guide line of the local ethical committee of the National Research Center.

Diagnosis of de novo' acute leukemias was made based on morphological and cytochemical assessment [Myeloperoxidase (MPO), Nonspecificesterase (NSE) and NSE with sodium fluoride inhibition] of peripheral blood and bone marrow smears. Complete Immunophenotyping was routinely performed using Flowcytometer Patec III DAKO cytomation for evaluation of acute leukemia. In addition, all patients were subjected to CSF examination, chest x-ray and pelvi-abdominal ultrasound for extramedullary involvement detection. Sera were analyzed for lactate dehydrogenase (LDH), alkaline phosphatase (ALP) and uric acid levels using Synchron CX9PRO.

Survivin Gene expression was quantitatively determined by real-time PCR technique. Extra peripheral blood samples were taken into tubes with K_3EDTA from the patients (study group) and from healthy subjects (control group).

RNA Extraction and complementary(c) DNA synthesis:

Total RNA was extracted from whole blood using QIAamp RNA Blood Mini kit supplied by QIAGEN worldwide companies (Cat No. 52304) according to manufacture's protocol. The concentration and purity of RNA was determined by measuring its absorbance at 260 nm (A_{260}) and 280 nm (A_{280}) in a UV visible spectrophotometer. Total RNA (2 µg) was reverse-transcribed to complementary DNA (cDNA) according to manufacturing instructions using Thermal Cycler (Biometra, Germany) with kit provided from Applied Biosystems. cDNAs were stored at -20°C until real-time quantitative PCR was performed.

Gene expression relative quantitation: Polymerase chain reaction (PCR) amplification reaction mixtures (50 µL) contained cDNA, survivin forward primer (SF) 5`-ACCAGGTGAGAAGTGAGGGA-3` and Survivin Reverse Primer (SR)5`-AACAGTAGAGGAGCCAGGGA-3`. The Survivin RNA Genbank accession number is NM001168. The TaqMan Probe sequence was GCACCCCGGAGCGGAT. GAPDH primers were: GAPDH Forward Primer (GF) 5`-ACATCG CTCAGACACCATGG-3` and GAPDH Reverse Primer (GR) 5`-GTA GTTGAGGTCAATGAAGGG-3`.GAPDH RNA Genbank accession number is NM33197. The TaqMan Probe sequence CAAGCTTCCCGTTCTCAGCC. All primers and probes were synthesized by Applied Biosystems-Perkinelmer .

Thermal cycle conditions included holding the reactions at 50°C for 2 minutes and at 95°C for 10 minutes and cycling for 40 cycles between 95°C for 15 seconds and 60°C for 1 minute. Results were collected and analyzed by Applied Biosystems Perkin Elmer 7300 sequence detection system.

Relative quantitation using comparative threshold (C_t) was used to determine the change in expression of a nucleic acid sequence (target) in the test and control samples. The C_t value for any sample was normalized to the endogenous housekeeping gene GAPDH. A negative control was included in each experiment. Survivin Gene expression levels were expressed as copy number per cell (c/cell).

Statistical Methods :

All data were collected from the patient charts and entered into a computerized spreadsheet. The fit of the data to the normal distribution were tested with Kolmogorov-Smirnov Test. Data was statistically described in terms of range, mean, standard deviation (SD), frequencies (number of cases) and relative frequencies (percentages). Comparisons were made using Student's t-test, One-way ANOVA test and Pearson's correlation coefficients were calculated between the assessed variables. Statistical Package for Social Science SPSS Inc., Chicago,IL,U.S.A.version 9.0 was used for analysis of data. The null hypothesis was rejected with a two sided P value of < 0.05.

3. Results

Frequency distribution of demographic and clinical data of ALL and AML patients are shown in Table 1. Median percentage of leukemic blast cells in peripheral blood was 80% in ALL patients and 53% in AML patients. Frequency distribution of FAB subtypes and therapy response data of AML and ALL patients included in the study are shown in Table 2.

Comparisons between haematological and biochemical data in the pediatric ALL patients, AML patients and control subjects are demonstrated in Table 3. Comparisons between haematological and biochemical data in the adult ALL patients, AML patients and control subjects are demonstrated in Table 4.

Survivin gene was expressed in 60.0% of ALL patients and in in 73.3% of AML patients. Statistically significant higher expression was noted in both ALL and AML groups when compared to the control group (p-value = 0.0001) as shown in Figure 1and Table 5. Survivin gene expression was significantly higher in pediatric groups of both ALL and AML patients than pediatric group of control subjects with a p-value = 0.004 (Figure 2). Also Survivin gene expression was significantly higher in adult groups of leukemia patients than adult group of control subjects with a p-value =0.0001 (Figure 3).

In the correlation studies, no statistically significant correlation was found between Survivin gene expression and demographic and clinical data of patients.

A statistically significant negative correlation was detected between Survivin gene expression and RBCs count, HB level and Platelet count with p-values = 0.01, 0.01 and 0.0001 respectively. Positive correlations were found with T.L.C, peripheral blood blasts, bone marrow malignant cells, LDH , ALP and uric acid levels with p-values = 0.0001, 0.0001, 0.03, 0.0001, 0.006 and 0.001 respectively (Table 6) .

No statistically significant difference could be determined between L_1 and L_2 FAB subtypes (p-value= 1.0). Regarding immunophenotyping, Survivin expression level in C-ALLA patients showed a mean value of 1.3 ± 0.7c/cell, whereas Pro B and Pre B patients showed a mean Survivin expression level of 0.6 ± 0.8 c/cell and 0.8 ± 0.7 c/cell respectively with a p-value = 1.0 which is statistically insignificant.

In an attempt to correlate between Survivin expression and some prognostic factors of AML: AML patients with normal LDH level showed a mean Survivin level of 0.4 ± 0.3 c/cell, whereas AML patients with elevated LDH level showed a mean Survivin level of 1.6 ± 0.6 c/cell with a p-value = 0.003 which is statistically highly significant.

During the post-induction phase, Survivin gene expression showed a statistically insignificant difference between leukemic patients achieving complete remission and those showing unfavorable response with a p-value = 0.7. After the follow up period, the difference between mean Survivin gene expression in patients achieving complete remission and those showing unfavorable response was statistically insignificant with a p-value = 0.6 (Table 7).

4. Discussion

Survivin has been identified as one of the top 4 transcripts among 3.5 million human transcriptomes uniformly up-regulated in cancer tissues but not in normal tissues (Sugahara et al. 2004). Therefore, we quantitatively determined the expression of survivin by a real-time polymerase chain reaction technique in 30 patients with acute leukemias ; acute lymphoblastic leukemia (ALL), and acute myeloblastic leukemia (AML) in two age groups; pediatric group (<18 years) and adult group (≥18 years) and in age and sex matched control healthy subjects. Age-related acute leukemia groups might be associated with distinct expression patterns

of apoptosis-related molecules leading to the distinct prognosis in adult and childhood acute leukemia.

In the present study statistically significant higher survivin expression was noted in both ALL and AML groups when compared to the control group. We found statistically insignificant change in Survivin expression level between ALL and AML groups.

Thus, the results of this study provide further evidence that survivin plays a role in the malignant process of acute leukemias. Survivin gene was expressed in 60.0% of ALL patients and in 73.3% of AML patients. This could be attributed to dysregulation of Survivin gene which is normally controlled by p53 and transcriptional factors as the Kruppel-like factor 5 (KLF5). Zhu et al. (2006) demonstrated that the transcription factor KLF5 is widely expressed in childhood ALL and is a positive regulator inducing the expression of the anti-apoptotic protein Survivin. Suppression of apoptosis contributes to leukemogenesis by different mechanisms, including prolonging cell life span, thus facilitating the accumulation of gene mutations, permitting growth factor-independent cell survival, promoting resistance to immune-based cytotoxicity, and allowing disobeyance of cell cycle checkpoints which would normally induce apoptosis (Tamm et al. 2004). Carter et al.(2001) demonstrated that Survivin is highly expressed and is cytokine regulated in myeloid leukemias . In their study cytokine stimulation increased survivin expression in leukemic cell lines and in primary AML samples. In cultured primary samples, single-cytokine stimulation substantially increased survivin expression in comparison with control cells, and the combination of G-CSF, GM-CSF, and SCF increased survivin levels even further. Also Badran et al. (2003) reported that the analysis of Ph (+) blastic crisis CML patients (4 patients) revealed positive Survivin expression in all patients (100%) . Data collected by Mori et al (2002) showed that Survivin was detected in 17 out of 31 AML patients (54.8%) and 5 out of 7 patients with CML blastic crisis (71.4%). The variation in the frequency of Survivin expression among different investigators is a consequence of variation in the procedures used and the number of cycles performed in PCR assays which may influence the sensitivity and specificity of the detection method. However Tamm et al. (2004) put few limitations and concluded that cryopreservation may alter protein expression in samples. Freezing and DMSO are proapoptotic stimuli potentially influencing expression levels of the proteins measured. Also the average leukocyte count could influence the expression level.

We did not find significant correlation between Survivin expression level and sex, organomegaly and lymphadenopathy. Survivin expression level showed statistically significant negative correlations with RBCs count, HB level and platelets count. Significant positive correlations with T.L.C, peripheral blood blasts, bone marrow malignant cells, LDH, ALP and uric acid were identified. This could be explained by the antiapoptotic function of Survivin thus offering a survival advantage to malignant cells that dominate the bone marrow at expense of other hematological elements with high cell destruction. These results are in accordance with Oto et al (2007).

With respect to blast immaturity, a significant variation of Survivin expression level could not be identified in ALL patients. Also Troeger et al. (2007) showed that Survivin expression levels were comparable between the different immunophenotypes.

Concerning the outcome of our studied patients, difference in Survivin expression level during the postinduction period between patients who achieved CR and those who didn't was statistically insignificant. After the follow up period of one year, the leukemic patients who achieved CR showed insignificant change of expression when compared to those with unfavourable response. Tamm et al. (2004) could not determine a prognostic role for survivin in adult AML. Survivin is a negative prognostic marker in a variety of solid tumors and diffuse large B-cell lymphomas (Adida et al. 2000a) For example, it has been reported that survivin expression in neuroblastomas correlates with clinically more aggressive, histologically unfavorable disease (Adida et al. 1998) . Adida *et al.* (2000b) found no significant difference in remission rate or survival in adult AML patients expressing high versus low levels of survivin. Also difference in outcome correlations between investigators could be attributed to various treatment strategies and duration of follow up.

In summary:, The previous data emphasized important correlations between Survivin expression level and established risk factors in acute leukemia patients. Thus Survivin could be used as a marker for assessment of bone marrow infiltration that in future could be used to refine treatment stratification. Elevated survivin levels could be cytokine regulated, thus explaining the controversy in its prognostic impact. In future Survivin may even serve as a therapeutic target itself. Patients should be assessed for a considerable period of follow up to compare between Survivin level before , during and after therapy and to correlate between patients' outcome and Survivin level on a larger scale.

Table (1): Frequency distribution of demographic and clinical data of ALL and AML patients included in the study

Variables	ALL patients N (%)	AML patients N (%)
Sex:		
Males	13 (86.7)	12 (80)
Females	2 (13.3)	3 (20)
Organomegaly: Hepatomegaly		
Negative	3 (20)	9 (60)
Positive	12 (80)	6 (40)
Splenomegaly		
Negative	2 (13.3)	8 (53.3)
Positive	13 (86.7)	7 (46.7)
Lymphadenopathy		
Negative	5 (33.3)	11 (73.3)
Positive	10 (66.7)	4 (26.7)
Extramedullary involvement: CNS manifestations		
Negative	15 (100)	15
Positive	0 (0.0)	0
Testicular enlargement		
Negative	14 (93.3)	-
Positive	1 (6.7)	-

CNS : Central Nervous System
N : Number
% : Percentage

Table (2): Frequency distribution of FAB subtypes and therapy response data of AML and ALL patients included in the study

Variables	ALL Patients N (%)	AML Patients N (%)
FAB Subtypes:		
M1		2 (13.3)
M2		10 (66.7)
M3		2 (13.3)
M5a		1 (6.7)
L1	2 (13.3)	
L2	13 (86.7)	
I.P.T:		
C-ALLA	8 (53.4)	
ProB	2 (13.3)	
PreB	5 (33.3)	
Postinduction response:		
CR	10 (66.7)	9 (60)
PR	4 (26.7)	5 (33.3)
Death	1 (6.6)	1 (6.7)
Follow up after one year:		
CR	8 (53.3)	4 (28.6)
Relapse	3 (20)	7 (50)
Death	4 (26.7)	3 (21.4)

N.B : one AML patient was lost, so 14AML patients only were assessed after 1 year of follow up.

FAB	:	French American British
I.P.T	:	Immunophenotyping
C-ALLA	:	Common Acute Lymphoblastic Leukemia Antigen
CR	:	Complete Remission
PR	:	Partial Remission
N	:	Number
%	:	Percentage

Table (3): Comparisons between haematological and biochemical data in pediatric groups (< 18 years) of ALL patients, AML patients and control subjects included in the study

Variables	AML pediatric patients Mean ± SD N = 5	ALL pediatric patients Mean ± SD N = 11	Control pediatric subjects Mean ± SD N = 9	P-value
Age (years)	11.4 ± 4.9	6.4 ± 5.6	8.8 ± 4.7	0.4
Sex (M/F)	5/-	9/2	7/2	
RBCs ($x10^6$/uL)	1.9±0.4a	2.5±0.8a	5.0±0.4b	0.0001*
HB (gm/dL)	5.4±1.4a	6.5±2.0a	13.8±1.5b	0.0001*
T.L.C. ($x10^3$/uL)	110.3±128.4a	39.8±54.6a	9.0±2.0b	0.04*
Platelets ($x10^3$/uL)	32.0±30.5a	46.0±28.0a	281.1±85.1b	0.0001*
P.B blasts (%)	48.4 ±27.4	52.8 ±38.6	-	0.9
Bone Marrow malignant cells (%)	56.4 ±26.5	86.6 ±17.4	-	0.06
LDH (U/L)	1636.0±1313.9a	798.0 ±566.7b	297.2 ±37.6b	0.007*
ALP (U/L)	161.2±61.1	153.0 ±106.0	89.0 ±19.7	0.1
Uric acid (mg/dL)	6.8±1.3a	6.9±1.7a	4.6±0.7b	0.001*

RBCs Red Blood Corpuscles
HB Haemoglobin
T.L.C Total Leucocytic Count
P. B blasts Peripheral Blood blasts
LDH Lactate Dehydrogenase
ALP Alkaline Phosphatase
N Number
SD Standard Deviation
P-value is significant if <0.05*
Different symbol indicates significance

Table (4): Comparisons between haematological and biochemical data in adult groups (> 18 years) of ALL patients, AML patients and control subjects included in the study

Variables	AML adult patients Mean ± SD N = 10	ALL adult patients Mean ± SD N = 4	Control adult subjects Mean ± SD N = 11	P-value
Age (years)	35.0 ± 12.4	51.3 ± 8.5	35.5 ± 12.3	0.4
Sex (M/F)	7/3	4/-	7/4	-
RBCs ($x10^6$/uL)	2.3 ± 0.7a	2.1 ± 0.4a	4.7 ± 0.5b	0.0001*
HB (gm/dL)	6.5 ±1.5a	5.2 ± 0.9a	13.9 ±1.2b	0.0001*
T.L.C. ($x10^3$/uL)	44.8 ± 41.4a	126.0 ± 115.5b	6.7±1.8a	0.002*

Platelets (x10^3/uL)	39.3 ± 24.6a	28.8 ± 15.4a	227.2 ± 49.2b	0.0001*
P.B blasts (%)	50.7 ± 27.7	77.0 ± 17.2	-	0.3
Bone Marrow malignant cells (%)	71.5 ± 21.4	89.8 ± 4.0	-	0.3
LDH (U/L)	973.5 ± 848.3a	2218.8 ±1259.7b	311.6 ± 45.2C	0.001*
ALP (U/L)	146.0 ± 57.8a	291. 8 ± 146.0b	80.4 ± 20.0C	0.0001*
Uric acid (mg/dL)	7.2 ± 1.7a	8.6 ± 1.1a	4.9 ± 1.3b	0.0001*

RBCs Red Blood Corpuscles
HB Haemoglobin
T.L.C Total Leucocytic Count
P.B blasts Peripheral Blood blasts
LDH Lactate Dehydrogenase
ALP Alkaline Phosphatase
N Number
SD Standard Deviation
P-value is significant if < 0.05*
Different symbol indicates significance

Table (5): Comparison Between mean Survivin Gene expression levels (c/cell) in ALL patients, AML patients and Control subjects included in the study

Variables	Minimum	Maximum	Mean ± SD	P-value
ALL patients	0.02	2.3	1.0±0.8a	0.0001*
AML patients	0.03	2.4	1.2 ±0.8a	
Control subjects	undetectable	0.6	0.2 ± 0.2b	

c/cell copy number per cell
SD standard deviation
P-value is significant if < 0.05*
Different symbol indicates significance

Table (6): Correlation of Survivin Gene expression level to biochemical and haematological data of AML patients included in the study

Variables	r	P-value
LDH (U/L)	0.8	0.0001*
ALP (U/L)	0.3	0.2
Uric acid (mg/dL)	0.7	0.002*
RBCs (x10^6/µL)	- 0.7	0.004*
HB (gm/dL)	- 0.7	0.002*
T.L.C. (x10^3/µL)	0.7	0.004*
Platelets (x10^3/µL)	- 0.4	0.1
P.B blasts (%)	0.8	0.0001*
Bone Marrow malignant cells (%)	0.7	0.008*

LDH : Lactate Dehydrogenase
ALP : Alkaline Phosphatase
RBCs : Red Blood Corpuscles
HB : Haemoglobin
T.L.C : Total Leucocytic Count

P.B blasts : Peripheral Blood blasts
% : Percentage
P-value is significant if < 0.05*

Table (7): Relation of Survivin Gene expression level (c/cell) to therapy response data of acute leukemia patients (n=30)

Variables		Mean ± SD	P-value
Postinduction response :			
Favourable (CR)	n = 19	1.2 ± 0.8	0.7
Unfavourable (PR, Relapse, Death)	n = 11	1.1 ± 0.8	
Follow up after one year:			
Favourable (CR)	n = 12	1.1 ± 0.8	0.6
Unfavourable (PR, Relapse, Death)	n = 17	1.2 ± 0.7	

CR : Complete Remission
PR : Partial Remission
n : number
SD : Standard Deviation
c/cell : copy number per cell
P-value is significant if < 0.05*

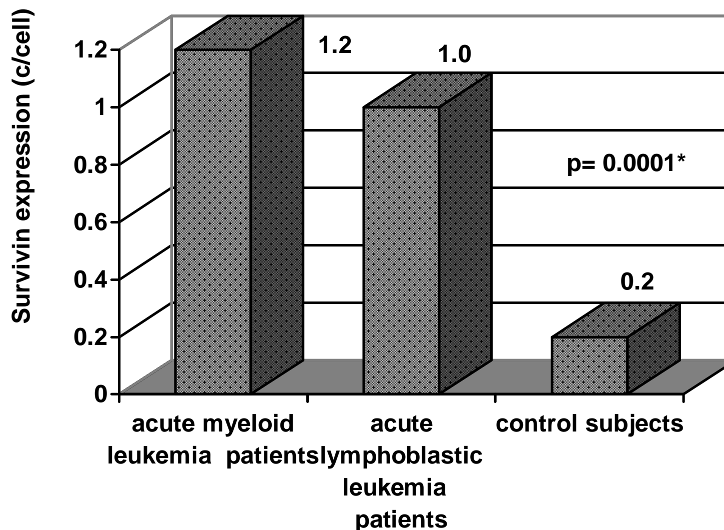

Figure 1. Comparison between mean Survivin gene expression levels (c/cell) of AML patients, ALL patients and Control subjects

Statistically significant higher expression was noted in both ALL and AML groups when compared to the control group (p-value = 0.0001)

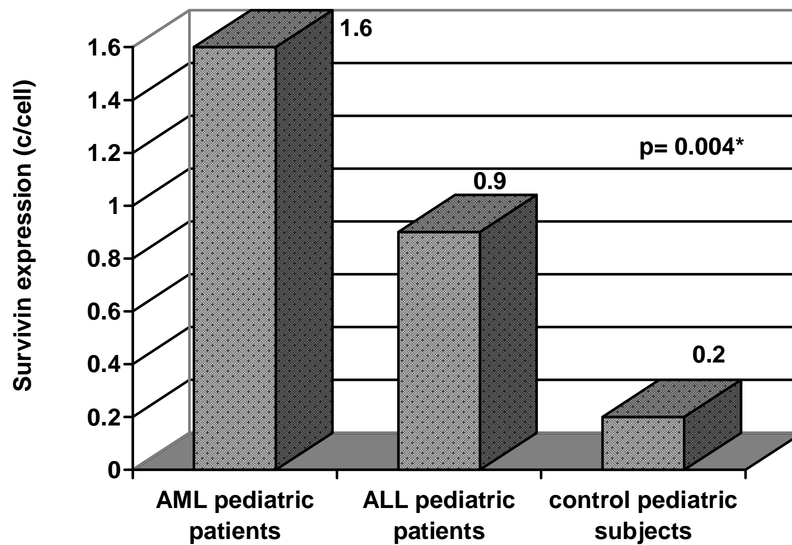

Figure 2. Comparison between mean Survivin gene expression levels (c/cell) in pediatric groups (<18 years) of AML patients, ALL patients and control subjects

Survivin gene expression was significantly higher in pediatric groups of both ALL and AML patients than pediatric group of control subjects with a p-value = 0.004

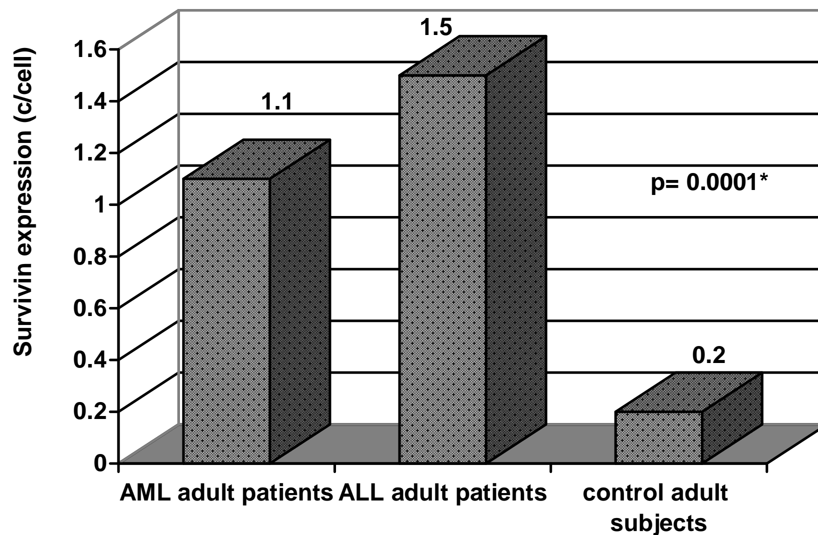

Figure 3. Comparison between mean Survivin gene expression levels (c/cell) in adult groups (>18 years) of AML patients, ALL patients and control subjects

Survivin gene expression was significantly higher in adult groups of leukemia patients than adult group of control subjects with a p-value =0.0001

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