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RESEARCH ARTICLE

CLINICAL SIGNIFICANCE AND PROGNOSTIC VALUE OF SURVIVIN AND P53 IN CHILDREN WITH ACUTE LYMPHOBLASTIC LEUKEMIA

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Abstract

Background : Survivin is a member of the inhibitors of apoptosis (IAP) family, while P53 is a tumor suppressor protein which rapidly increases in response to cell stress, such as DNA damage. Both of Survivin and P53 are overexpressed in cancers including hematologic malignancy.

Objective: To evaluate the level of survivin and P53 in children with acute lymphoblastic leukemia and to correlate it with clinical and hematological findings and response to treatment.

Methods : The level of survivin was measured by ELISA and P53 expression was measured by flowcytometry in 37 children with acute lymphoblastic leukemia and 12 healthy children as a control group. The level of these two parameters was measured before and after treatment of patients and also correlated with clinical and hematological findings and response to treatment.

Results : There was a highly significant difference in both serum survivin level and P53 expression between children with acute lymphoblastic leukemia at diagnosis compared to the control group, and the level has been significantly reduced after complete remission. Moreover, a significant positive correlation was found between serum survivin and LDH, WBCs count and percentage of blast cells in peripheral blood and bone marrow. Also, a significant positive correlation was found between survivin and P53 levels.

Conclusion: The level of both survivin and P53 is significantly high in children with acute lymphoblastic leukemia and is positively correlated with poor prognostic factors, also survivin and P53 are positively correlated with each other.

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Introduction:-

Acute lymphoblastic leukemia (ALL) is characterized by clonal proliferation and accumulation of malignant blast cells in the bone marrow and peripheral blood [1]. It is the most common malignancy in children, representing nearly one third of all pediatric cancers. [2]

Despite the very good overall prognosis of children with acute lymphoblastic leukemia (ALL), whose long-term survival is now 70 - 80%, treatment of relapsed disease remains a challenge. [3]

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Programmed cell death (apoptosis) is a feature of living cells, and damaged cells are eliminated in this way. Inhibitors of apoptosis aberrantly prolong cell viability, so contributing to the occurrence and growth of tumors. [4]

During the last decade, complex networks of pro-apoptotic and anti-apoptotic proteins that strictly govern the regulation of apoptosis pathways have been identified. [5, 6]

Survivin is a small 16 kilodalton (kd) protein that belongs to the inhibitors of apoptosis (IAP) family and also functions as a member of the chromosome passenger complex. Survivin is a unique member of the IAPs in that it is both the smallest member and may not directly interact with caspases. Instead, it may interact with another IAP, Smac/Diablo, to regulate apoptosis within the mitochondria. [7] Survivin was described in some tumors, including hematologic malignancies, where it correlates with poor prognosis. [8]

Rödel et.al [9] reported that survivin is an anti-apoptotic gene which is overexpressed in most human tumors and involved in mitotic checkpoint control. High levels of survivin expression have been associated with cancer progression, drug resistance, poor prognosis, and short patient survival.

Most chemotherapeutic agents induce tumor cell death by apoptosis. [10] Poor response to induction therapy or persistence of minimal residual disease, which is responsible for the subsequent relapse, may be caused by the resistance of leukemic blasts to induction of apoptosis. [11]

P53 is a tumor suppressor protein that in humans is encoded by the TP53 gene located on the short arm of chromosome 17. [12]

P53 has many anticancer mechanisms, and plays a role in apoptosis, genetic stability, and inhibition of angiogenesis. In its anti-cancer role, p53 works through several mechanisms. It can activate DNA repair proteins when DNA has sustained damage, induce growth arrest by holding the cell cycle at the G1/S regulation point on DNA damage recognition and can initiate apoptosis if DNA damage proves to be irreparable. [13]

While p53 levels are kept low in unstressed cells, they rapidly increase in response to stressors, such as DNA damage. P53 will then become activated through posttranslational modifications and tetramerization following genotoxic or cytotoxic stress. [14, 15]

The aim of this study was to evaluate the level of survivin and P53 in children with acute lymphoblastic leukemia (ALL) and to correlate it with clinical and hematological findings and response to treatment.

Subjects and Methods:-

This study was carried out at Clinical Pathology Department and Pediatric Department (Hematology & Oncology Unit), Zagazig University Hospital, Egypt. It included 49 subjects classified into 2 groups:

Group I (Control group); consisted of 12 age and sex matched apparently healthy children, their age ranged from 6 to 14 years with a mean value of 8.9 ± 2.5 years. It included 7 males and 5 females.

Group II (Patients group); consisted of 37 children with de novo Acute Lymphoblastic Leukemia (ALL), their age ranged from 4 to 13 years with a mean value of 8.6 ± 2.5 years. It included 21 males and 16 females.

All patients had received the appropriate chemotherapy protocol according to the applied guidelines. Serum samples (for survivin assay and biochemical tests) and EDTA samples (for CBC and P53 assay by flowcytometry) were collected from patients twice; one sample before the start of chemotherapy and another sample at complete remission. Similar samples were collected once only from control group.

Patients and controls were subjected to the following:

Full history taking

Clinical examination for signs of anemia, bleeding tendency, hepatosplenomegaly and lymphadenopathy

Laboratory investigations:

A- Routine investigations:

1. Complete Blood Count (CBC) using Sysmex KX 21. N. cell counter and examination of Leishman stained peripheral blood smear
2. Bone marrow aspiration for patients only, with examination of Leishman stained smears at diagnosis and after 28 days of induction of chemotherapy
3. Immunophenotyping on B.M. samples by flowcytometry for patients of acute leukemia at diagnosis using the following monoclonal antibodies:
 - a. Markers of immature cells: HLA-DR and CD 34.
 - b. Myeloid markers: CD 13 and CD 33.
 - c. B-cell markers: CD 10, CD 19, CD 20 and CD 79b.
 - d. T-cell markers: CD 2, CD 5 and CD 7.
4. Biochemical investigations using Cobas Integra 400 chemistry analyzer and including Liver function tests , serum LDH and serum creatinine.

Specific investigations:

Measurement of serum survivin levels by Enzyme Linked Immunosorbent Assay (ELISA) using Quantikine human survivin R & D System kit , it was done once for controls and twice for ALL patients (at diagnosis and at complete remission).

Principle of the assay : Quantitative sandwich enzyme immune assay technique using a microplate pre-coated with monoclonal antibody specific for survivin. Standards and samples are pipetted into the wells and any survivin present is bound by the immobilized antibody. After washing of any unbound substances , an enzyme-linked polyclonal antibody specific for survivin is added to the wells. Any unbound antibody-enzyme reagent is then removed by washing followed by adding a substrate solution. A color will develop in proportion to the amount of survivin bound in the initial step.

Assay procedure:

1. Standards with concentrations of 2000 , 1000 , 500 , 250 , 125 , 62.5 and 31.25 pg/ml were added to the appropriate wells.
2. 100 ul of assay diluent were put into each well
3. 100 ul of controls were pipetted into appropriate wells
4. 100 ul of the samples were pipetted into appropriate wells
5. The plate was tapped gently for mixing , then sealed and incubated at room temperature on a plate shaker for 2 hours.
6. The wells were washed 5 times
7. 200 ul of survivin conjugate were pipetted into each well
8. Plate was sealed and incubated at room temperature on a plate shaker for 2 hours.
9. The wells were washed 5 times
10. 200 ul of substrate solution were pipetted into each well , then incubated at room temp. for 30 min. protected from light.
11. 50 ul of stop solution were pipetted into each well.
12. Optical density was measured within 30 min. by microplate reader at 450 nm

Calculation of results:

Standard curve was created by plotting the mean absorbance of each standard concentration on the y-axis against the survivin standards concentration on the x-axis. A curve was drawn through the points on the graph and the concentration of each sample was calculated from the curve.

Measurement of P 53 levels by flowcytometry:

It was done once for controls and twice for ALL patients (at diagnosis and at complete remission).

Peripheral venous blood samples were collected into vacutainer EDTA tubes , leukemic cells were separated by Ficoll-Hypaque gradient centrifugation (Sigma , St. Louis , MA) , washed 3 times with saline solution (NaCl 0.9 %) , then resuspended in RPMI-1640 medium (Sigma).

Cells were incubated for 10 minutes at room temperature with 2 ml of mixture of 4 % paraformaldehyde and Becton Dickinson's FACS lysing solution. This procedure leads to lysis of RBCs and fixation and permeabilization of other

cells. Cells were centrifuged for 5 min. , the supernatant was discarded , and cells washed with 2 ml of 0.5 % Tween 20 in protein buffered saline (PBS) and centrifuged for 5 min. [16]

P53 protein immunostaining : Fixed and permeabilized cells were incubated with 10 ml of anti-p53 Monoclonal Ab labeled with FITC (DO7 / DAKO, Carpintaria , CA) for 30 min. , followed by 2 washes with Tween 20/PBS , resuspended in 500 ul of 1 % formaldehyde / PBS and analyzed by FC.

Analysis was done with Fluorescence-activated cell analyzer (FACScan , San Jose , CA) with cell Quest software (Cell Quest™ software , Becton Dickinson Immunocytometry systems , San Jose , CA)

Stained samples were analyzed through forward scatter (FSC) and side scatter (SSC) gated set around the blast cells population.

Expression was evaluated as cell percent (The number of stained cells minus the number of cells stained by irrelevant negative control).

Results:-

Table 1:- Age distribution of studied groups.

	Control (N=12)	Patients (N=37)	t	p
Mean ± SD	8.9 ± 2.5	8.6 ± 2.5	0.08	0.93
Range	(6 – 14)	(4 – 13)		

This table shows that there is no significant difference in age distribution.

Table 2:- Comparison between hematological data in patients at diagnosis and the control group..

	Control (N=12)	Patients at diagnosis (N=37)	t	p
R.B.Cs. (10^6 /ul)	4.2 ± 0.25 (3.82 ± 4.6)	2.4 ± 0.23 (1.9 – 3.0)	24.1	0.00*
Hb. (g/dl)	12.2 ± 0.4 (11.6 – 13)	6.4 ± 0.51 (5.4 – 7.4)	35.1	0.00*
Platelets (10^3 /ul)	289 ± 54.3 (200 – 390)	(44 ± 7) (29 – 61)	27.5	0.00*
W.B.Cs. (10^3 /ul)	8.1 ± 1.6 (58 – 10.5)	36.5 ± 6.3 (28 – 56.2)	15.3	0.00*
Blast cells % in peripheral blood	---	35.3 ± 9.7 (18 – 56)		

The table shows that there is a highly significant difference regarding red blood cells (RBCs) , Hemoglobin , platelets and white blood cells (WBCs)

Table (3):- Comparison between hematological data in patients at diagnosis and at remission .

	Patients at diagnosis (N=37)	Patients at remission (N=37)	t	P
R.B.Cs. (10^6 /ul)	2.4 ± 0.23 (1.9 – 3.0)	3.5 ± 0.29 (2.99 – 4.1)	18.5	0.00*
Hb. (g/dl)	6.4 ± 0.51 (5.4 – 7.4)	9.8 ± 1.1 (7.5 – 12.4)	17.4	0.00*
Platelets (10^3 /ul)	(44 ± 7) (29 – 61)	206.1 ± 20.8 (168 – 249)	44.9	0.00*
W.B.Cs. (10^3 /ul)	36.5 ± 6.3 (28 – 56.2)	7.9 ± 0.89 (6.6 – 10.2)	27.2	0.00*
Blast cells % in peripheral blood	35.3 ± 9.7 (18 – 56)	--		
Blast cells % in bone marrow	81.9 ± 5.9	2.1 ± 0.67	82.4	0.00*

	(69 – 93)	(1 – 3.5)		
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This table shows that there is a highly significant difference regarding RBCs , Hemoglobin , platelets , WBCs and bone marrow blast cells.

Table (4):- Comparison between LDH level (U/L) in the control group and patients at diagnosis.

	Control group	Patients at diagnosis	t	p
Mean ± SD	247.6 ± 11.7	1022.7 ± 172.5	15.4	< 0.001***
Range	(230 – 265)	(730 – 1320)		(0.00)

This table shows that there is a highly significant difference in LDH level (U/L) between the 2 groups.

Table (5):- Comparison between LDH levels (U/L) in the control group and patients at remission.

	Control group	Patients at remission	t	p
Mean ± SD	247.6 ± 11.7	284.1 ± 31.5	1.36	> 0.05
Range	(230 – 265)	(275 – 389)		(0.08)

This table shows that there is no significant difference in LDH level (U/L) between the 2 groups (p > 0.05)

Table (6):- Comparison between LDH level (U/L) in patients at diagnosis and at remission..

	Patients at diagnosis	Patients at remission	t	p
Mean ± SD	1022.7 ± 172.5	284.1 ± 31.5	23.9	< 0.001***
Range	(730 – 1320)	(275 – 389)		

This table shows that there is a highly significant difference in LDH level (U/L) in patients at diagnosis and at remission (p < 0.001)

Table (7):- Comparison between the mean values of survivin level (pg/ml) in patients at diagnosis and the control group.

	Control group	Patients at diagnosis	t	P
Mean ± SD	29.8 ± 1.7	106.9 ± 16.9	15.7	< 0.01**
Range	(26.5 – 32.5)	(78 – 138)		

This table shows that there is a highly significant difference in survivin level between the 2 groups (p < 0.01)

Table (8):- Comparison between the mean values of survivin level (pg/ml) in patients at remission and the control group.

	Control group	Patients at remission	t	P
Mean ± SD	29.8 ± 1.7	31.5 ± 3.2	1.11	> 0.05
Range	(26.5 – 32.5)	(28.8 – 44.1)		(0.16)

This table shows that there is no significant difference in survivin level between the 2 groups (p > 0.05)

Table (9):- Comparison between the mean values of survivin level in patients at diagnosis and at remission.

	Patients at diagnosis	Patients at remission	t	P
Mean ± SD	106.9 ± 16.9	31.5 ± 3.2	25.4	< 0.001*
Range	(78 – 138)	(28.8 – 44.1)		

This table shows that there is a highly significant difference in survivin level (p < 0.001)

Table (10):- Correlations between survivin level and the hematological data and LDH in patients at diagnosis.

	r	t
RBCs (10 ⁶ /ul)	-0.81	0.00*
Hb. (g/dl)	-0.70	0.00*
Platelets (10 ³ /ul)	-0.64	0.00*
W.B.Cs. (10 ³ /ul)	0.57	0.00*
Blast cells % in peripheral blood	0.34	0.00*
Blast cells % in bone marrow	0.44	0.00*
LDH (U/L)	0.79	0.00*

This table shows that there is a significant negative correlation with RBCs , Hb and platelets while there is a significant positive correlation with WBCs , Blast cells and LDH

Table (11):- Comparison between the mean values of p53 expression (%) in patients at diagnosis and the control group.

	Control group	Patients at diagnosis	t	P
Mean \pm SD	4.28 \pm 0.36	14.9 \pm 1.5	4.31	< 0.001**
Range	(3.8 – 4.8)	(8.5 – 18.8)		

This table shows that there is a highly significant difference between the 2 groups ($p < 0.001$)

Table (12):- Comparison between the mean values of p53 expression (%) in patients at remission and the control group.

	Control group	Patients at remission	t	p
Mean \pm SD	4.28 \pm 0.36	4.4 \pm 0.53	1.09	> 0.05
Range	(3.8 – 4.8)	(3.6 – 5.6)		(0.28)

This table shows that there is no significant difference in p53 expression between the 2 groups ($p > 0.05$)

Table (13):- Comparison between the mean values of p53 expression (%) in patients at diagnosis and at remission.

	Patients at diagnosis	Patients at remission	t	P
Mean \pm SD	14.9 \pm 1.5	4.4 \pm 0.53	4.33	< 0.001**
Range	(8.5 – 18.8)	(3.6 – 5.6)		

This table shows that there is a highly significant difference between the 2 groups ($p < 0.001$)

Table (14):- Correlations between p53 expression and the hematological data and LDH in patients at diagnosis.

	r	p
RBCs (10^6 /ul)	- 0.47	0.04
Hb. (g/dl)	- 0.54	0.03
Platelets (10^3 /ul)	- 0.74	0.00
WBCs (10^3 /ul)	0.48	0.03
Blast cells % in peripheral blood	0.75	0.00
Blast cells % in bone marrow	0.63	0.00
LDH (U/L)	0.76	0.00

There is a significant negative correlation with RBCs , Hb and platelets while there is a significant positive correlation with WBCs , Blast cells count and LDH.

Table (15):- Correlation between serum survivin level and P53 expression in patients at diagnosis

r	P
0.84	0.000

The table shows that there is a significant positive correlation between serum survivin level and P53 expression.

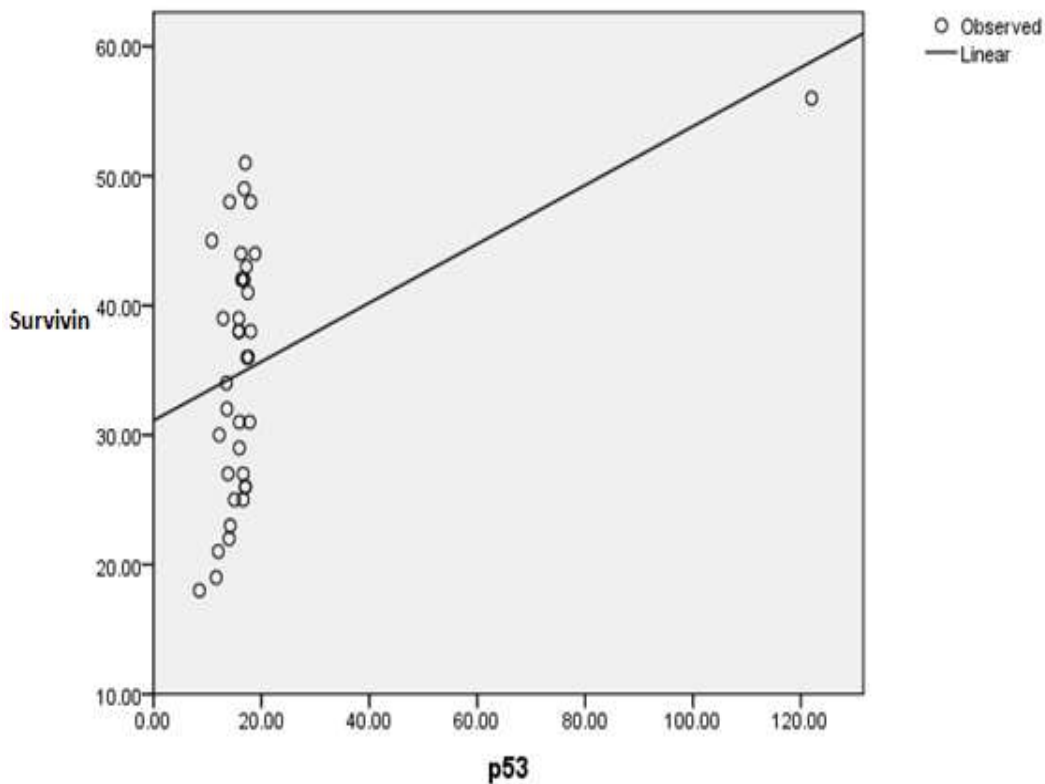


Figure 1:- Correlation between serum survivin level and P53 expression in patients at diagnosis.

The figure shows that there is a significant positive correlation between serum survivin level and P53 expression.

Discussion:-

Survivin is a 16.5 KD protein that belongs to the inhibitors of apoptosis protein (IAP) gene family. It is suggested that survivin plays a major role in linking cell death and proliferation. [17] Overexpression of survivin and other IAPs has been detected in various hematological malignancies including acute leukemia, chronic myeloid leukemia, myelodysplastic syndrome (MDS) and chronic lymphocytic leukemia (CLL). [18]

In our work, we studied the serum survivin level in children with acute lymphoblastic leukemia (ALL) at the time of diagnosis and also at complete remission in addition to a group of healthy children as a control group. Some hematological investigations related to acute leukemia were also done for our subjects.

There was a significant difference between control group and patients group at diagnosis regarding RBCs count, Hb level, platelets count, WBCs count, blast cells in peripheral blood and LDH level.

Also, we found a significant difference between patients at diagnosis and the same patients at complete remission regarding RBCs count, Hb level, platelets count, WBCs count, blast cells in peripheral blood, blast cells in bone marrow and LDH level.

Haviz and Mannan [19] also had found that level of LDH was significantly higher in patients with ALL at diagnosis than control group, while this level significantly decreased at remission, which agrees with our study.

Regarding serum survivin level, we found a highly significant difference between children with ALL at diagnosis compared to normal control group ($p < 0.001$). Also, survivin level has been significantly reduced after complete remission, so there was no significant difference between children with ALL at diagnosis and at complete remission. This agrees with **Sadek et al.,[20]** and **Raida et al.,[21]**

These findings also correlate with the study of **Muhammad Al Makhbar and Almoutassem Billah Zetoune [22]** who found that serum survivin levels were significantly greater in patients before therapy than after therapy.

Oto et al., [23] had studied serum survivin in patients with acute leukemia and concluded that it was high in those patients and had a bad prognostic effect while normal survivin level was associated with a good clinical outcome. Another study was done by **Zhang et al., [24]** and they stated that survivin level was significantly higher in children with acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML) than in healthy children, and concluded that high survivin level has a role in modulation of apoptosis in patients with acute leukemia.

We also studied the correlations between serum survivin and hematological parameters and LDH level in our patients. A significant positive correlation was found with LDH, WBCs count and percentage of blast cells in peripheral blood and bone marrow (B.M.), while a significant negative correlation was found with Hb, RBCs and platelets. This can be attributed to the anti-apoptotic effect of survivin that allows malignant (leukemic) cells to proliferate and invade B.M.

These correlations agree with the study of **Paydas et al., [25]** who found a positive correlation with LDH and with **Nakayama and Kamihara [26]** who found a positive correlation with WBCs count. Also, our results agree with those of **Kamal Elden et al., [27]** who found a positive correlation of survivin expression with WBCs count and blast cells count in both peripheral blood and bone marrow.

So, according to our study, there is positive correlation of serum survivin with many unfavorable prognostic factors such as high percentage of blast cells, high LDH level, and increased WBCs count which can give important data about disease severity and progression. On the other hand, survivin level was decreased after complete remission so that it can be used for monitoring the response to therapy.

P53 is a very unstable protein due to its degradation by the proteasome after binding to its major negative regulator protein which is murine double minute 2 (MDM 2) [28]. When cells are exposed to stress such as DNA damage, P53 protein conformation will change and can escape the MDM 2 effect, accumulate and become an active transcription factor [29] and [30].

In this study, we found a highly significant increase in expression level of p53 in children with ALL at diagnosis compared to control group, while at complete remission the level decreased nearly to that of control group. This is in accordance with results of **Park et al., [31]** which showed that p53 was overexpressed in both acute lymphoblastic leukemia and acute myeloid leukemia patients by immunohistochemical technique.

Mohamed Abdel-Aziz [32] studied the serum level of p53 in patients with ALL and AML using ELISA technique and he found a significant increase of serum p53 in those patients compared to healthy control.

Another study that employed our technique, which is flowcytometry, for measurement of p53 was done by **Konikova et al., [33]** but it was done for AML cases only, and also it showed a significant high expression of p53 in AML patients compared to healthy people. Another study that agrees with our work and done also by flowcytometry was conducted by **Raida et al., [21]** and it included children with ALL. It revealed a highly significant elevation of p53 expression at diagnosis compared to healthy control while a significant decrease was detected at complete remission.

We also studied the correlation between the level of survivin and p53, there was a significant positive correlation which is in agreement with the results of **Raida et al., [21]** and **Hui et al., [34]**. This correlation suggests that there is an interaction between p53 and survivin that can play a role in pathogenesis of ALL.

Conclusion:-

The level of both survivin and P53 is high in children with acute lymphoblastic leukemia and it drops nearly to normal after complete remission. Also, survivin and P53 are positively correlated with poor prognostic factors in those patients and are also positively correlated with each other. So, both of them can be used for monitoring the response to therapy in those patients.

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