

**Assessment of Survivin and Lactate Dehydrogenase Isoenzyme in
Leukemic Egyptian Patients**

Abstract:

Accurate diagnosis and proper monitoring of cancer patients remain important obstacles for successful cancer treatment. The search for cancer biomarkers is carried out in order to quickly identify tumour cells and predict treatment response, ultimately leading to a favourable therapeutic outcome.

The goal of this study is to highlight the importance of survivin level in cases of Acute lymphoblastic leukemia (ALL) and chronic lymphocytic leukemia (CLL) to find its possible role in pathophysiological development of the disease. Also to assess the oxidative stress in leukemic patients and to elucidate the relationship between erythrocytes Lactate Dehydrogenase (LDH) isoenzymes, antioxidant status, and survivin protein. This was achieved by detection the concentration of survivin, Erythrocyte glutathione peroxidase (E-GP-x), both of vitamin E and C, erythrocyte and serum LDH isoenzymes levels, and malondialdehyde (MDA). This study included 40 patients divided into two groups: 20 patients with CLL and 20 patients with ALL, together with 20 healthy control subjects (two groups). The study showed significant elevation in serum survivin protein, total LDH and MDA levels in patients with acute and chronic leukemia in comparison to healthy subjects. In contrast vit.C levels were decreased significantly in both studied groups. Strong correlation between survivin concentration and MDA was evident in ALL and CLL patients in the other hand a significant negative correlation between survivin and vit. C in ALL patients was observed. A highly significant increase in serum levels of LDH isoenzymes commonly recorded in ALL patients with significant increase in LDH isoenzyme form 1, 3, 4, and 5 in CLL group. While in erythrocytes, LDH isoenzymes 4 and 5 were highly significant increase and highly significant decrease in LDH-2 in both groups. Survivin may play a key role in tumor angiogenesis so, it has an important role in the development of acute and chronic leukemia.

Key Words: Leukemia, survivin, oxidative stress and LDH.

Introduction:

Acute lymphoblastic leukemia (ALL) and chronic lymphocytic leukemia (CLL) are the most common types of leukemia in children and in adults, respectively. ALL is a rare disease with an incidence of 1.2–1.4 per 100,000 populations per year in Europe. It is an aggressive malignancy, characterised by a sudden onset and rapid progression, and diagnosis usually requires urgent medical attention (Deborah *et al.*, 2015). Some ALL patients suffer from refractory or recurrent disease and cannot be cured with conventional chemotherapy. Treatment of acute lymphoblastic leukemia in adults presents a formidable challenge. While overall results have improved over the past 3 decades the long-term 10 year survival for patients aged less than 60 years is only in the range of 30–40% (Manal *et al.*, 2012).

According to blood cancer society (2014), chronic lymphocytic leukemia (CLL) is the result of an overproduction of abnormal lymphocytes, a type of white blood cell. Leukaemia

cells, which are produced in the bone marrow, then spread to other parts of the body through the blood stream. CLL usually develops in older adults, not children. There are two types of

CLL: slow-growing and fast-growing. Slow-growing CLL may not require treatment for years. Fast-growing CLL is much more serious and demands aggressive treatment.

Historically, CLL has been considered as an accumulative disease of lymphocytes defective in apoptosis, and this particular mechanism, but not increased proliferation, was thought to contribute toward leukemogenesis (Agata *et al.*, 2015).

Programmed cell death is a feature of living cells, and damaged cells are eliminated in this way. Inhibitors of programmed cell death aberrantly prolong cell viability, so contributing to the occurrence and growth of tumours. It plays an important role in both carcinogenesis and cancer treatment. Regulation of apoptosis is balanced by signalling pathways between apoptosis-promoting factors, and anti-apoptotic factors (Susan, 2007; Rebecca, 2011 and Marat *et al.*, 2011).

The inhibitors of apoptosis proteins (IAPs) are family of proteins that suppress both mitochondrial and death receptor mediated apoptosis primarily by inhibiting caspases. Currently, eight IAP members have been identified in humans. Among them, survivin is the most potent cellular caspase inhibitor (Ismael *et al.*, 2010).

Survivin (16 kD protein) is a cancer-related molecule usually detected in the cytoplasm of tumour cells. It plays an important role in regulating both apoptosis and cell division. It is undetectable in normal tissues but highly expressed in most forms of human cancer and functions as both an inhibitor of cell death and a mitotic regulator. Overexpression of survivin reduces the sensitivity of neoplastic cells to death stimuli induced by gamma radiation or chemotherapeutic agents. Also it is associated with increased risk of recurrence and poor outcome in a variety of cancers including hematologic malignancies (Stefania *et al.*, 2013 and Mohane *et al.*, 2013).

It functions in the cell cycle as an essential mitotic regulator being a member of the chromosomal passenger complex. Also cytoplasmic survivin is predominantly involved in preventing apoptosis (Jong *et al.*, 2016).

The role of survivin in apoptosis inhibition has been the subject of controversy. Initially, survivin and other IAPs were postulated to selectively bind to and promote the degradation of active caspases (Marat *et al.*, 2011).

Increased generation of reactive oxygen species (ROS) and an altered redox status have long been observed in cancer cells, and different studies suggest that this biochemical property of cancer cells can be exploited for therapeutic benefits. Cancer cells in advanced stage tumours frequently exhibit multiple genetic alterations and high oxidative stress (Dunyaporn *et al.*, 2009).

Excessive amounts of ROS can cause irreversible oxidative damage to lipids, proteins, and DNA, which lead to cell death. It still remains unclear whether the production of ROS in apoptosis is an essential component of the apoptotic pathway primarily by inducing mitochondrial damage, or an epiphenomenon of mitochondrial dysfunction that is unrelated to the key signalling events leading to apoptotic cell death (Fruehauf and Meyskens 2007; and Jamie *et al.*, 2012).

This study was conducted to evaluate the survivin as anti-apoptotic marker, antioxidant status and LDH isoenzymes in acute lymphocytic leukemia patients as dependable tool in pathophysiology and prognosis of the disease.

Patients and methods

This study included 40 subjects were selected from those attending at National cancer institute (NCI) Egypt, Cairo, and 20 apparently healthy normal volunteers.

Patient groups divided into two subgroups. Group I, Included 20 patients with *de novo* chronic lymphocytic leukemia with mean 51.3 ± 14.1 years (8 females and 12 males). Group II, Included 20 children patients with *de novo* acute lymphocytic leukemia with mean 6.2 ± 4.1 years (5 females and 15 males). Leukemia was diagnosed and classified according to laboratory tests used to diagnose; all patients had not been treated with therapeutic drugs before the study. The healthy subjects were divided into two groups: (C1) control group, 10 subjects with mean 47.9 ± 15.3 years (4 females and 6 males). (C2) a second control group with mean 7.1 ± 6.9 years (3 females and 7 males). C1 and C2 are related to group I and group II respectively. The study protocol was approved by Ain Shams University and National Cancer Institute (NCI) Committee.

Blood specimens:

Venous blood samples were collected from lymphocytic leukemia patients and healthy control in the morning after an overnight fasting. Each blood sample was divided into the following portions:

- 1) 0.5 ml Lithium-heparinized blood was kept at $2 - 8^{\circ}\text{C}$ and assayed in the same day of the collection for erythrocyte glutathione peroxidase (E-GP-x).
- 2) 4.5 ml Lithium-heparinized blood centrifuged immediately at $1000 \times g$ for 5 minutes and the plasma samples were separated rapidly. 0.25 ml of plasma was mixed with 1.0 ml of 6 g/dl meta-phosphoric acid (MPA), for stabilizing ascorbic acid. The plasma-MPA mixture was stored at -20°C until assayed. The remaining of the plasma was divided into several aliquots, for the determination of malondialdehyde (MDA), and stored at -70°C to be thawed only once for analysis.
- 3) 2 ml EDTA blood was kept at $2-8^{\circ}\text{C}$ and assayed within two hours of the collection for survivin, complete blood count (CBC) and LDH isoenzymes.
- 4) 2.0 ml clotted blood (tubes without additives) for the determination of biochemical investigations. Samples were left to clot at 37°C for 20 minutes, and then centrifuged at $2000 \times g$ for 10 minutes. The serum was then separated and stored at -20°C to be thawed only once for analysis of vitamin E, total lactate dehydrogenase enzyme (LDH) .

Biochemical Assays: whole blood, plasma or sera were subjected to the following investigation:

Detection of survivin in human blood lymphocytes by flow cytometry:

Samples were assayed for the presence of Survivin using the Human Survivin Fluorescein-conjugated Antibody (IC6472F) flow cytometric kit (R&D Systems, Inc., Minneapolis, MN), according to the manufacturer's instructions. Human

peripheral blood lymphocytes were stained with Mouse Anti-Human Survivin Fluorescein-conjugated Monoclonal Antibody or isotype control antibody for a negative control (This isotype control has been derivatized with a quantity of fluorochrome that matched the F/P (fluorochrome/protein) ratio of R&D Systems monoclonal reagents). To facilitate intracellular staining, cells were fixed with Flow Cytometry Fixation Buffer (1% paraformaldehyde) and permeabilized with Flow Cytometry Permeabilization/Wash Buffer. Flowcytometric analysis was generated a signal which could be detected in the FITC signal detector.

Serum total LDH was assayed colorimetrically according to **Mathieu (1982)**. Serum and erythrocyte Lactate Dehydrogenase Isoenzymes were assayed by gel electrophoresis according to the method of **Opher *et al.*, (1996)**. Both serum vitamin E and vitamin C levels were determined according to **Baker& Frank (1968)** and **Roe & Kuether (1973)** respectively. Erythrocyte glutathione peroxidase (E-GP-x) concentration and lipid peroxidation product (Malondialdehyde) were assayed according to **Paglia and Valentine (1967)** and **Draper and Hadley (1990)** respectively.

Statistical analysis

Data was analyzed using SPSS win statistical package version 22. Numerical data were expressed as mean $\pm$ standard deviation, and range.

For quantitative data, comparison between two groups was done using Mann-Whitney test (non-parametric test corresponding to student t-test for variables. Comparison between groups was done using non-parametric ANOVA test). *P* Values were two-tailed and were considered significant if < 0.05 .

Results

Data presented in table (1) and figure (2) Showed that, blood survivin was highly significantly increased in CLL and ALL groups ($P<0.001$) in comparison with control groups. In addition, there was significant difference between patient groups ($P<0.05$).

serum T.LDH (table 1) was significantly increased in CLL patients ($P<0.05$) and highly significantly increased in ALL patients by 159% and 435% ($P<0.001$) in comparison with control groups, with significant difference between patient groups ($P<0.001$).

WBCs and lymphocyte counts were highly significantly increased in CLL by 322 %, 996% respectively. While, in ALL group lymphoblast count was significantly increases by 455% ($P<0.05$) but WBCs count was not significantly change in comparison with control group. In addition, there was no significant difference between patient groups. The results in table (2) also revealed that, Plasma malondialdehyde was highly significantly increased in group-I and II by 54% and 120% respectively ($P<0.001$) in comparison with control groups. There was a highly significant difference between patient groups ($P<0.001$). While, E.GP-x and serum vitamin E showed non-significant difference either in CLL or ALL groups in comparison with control groups or between the two patient groups.

On the other hand, Serum vitamin C was highly significantly decreased in groups I and II (-40 and -26% respectively) ($P<0.001$) when compared with control groups. But there was no significant difference between the two patient groups.

Table (1): Blood Survivin, serum T. LDH levels, lymphocyte and WBC's count in CLL, ALL and control groups:

Parameters Groups	Survivin* (%)	T.LDH (U/L)	Lymphocytes(CLL) Lymphoblast (ALL)	WBC's
(C1) control				
Range	0.25 – 1.2	310- 355	1.3 – 3.7	3.2- 11
Mean $\pm$ SD	0.3 $\pm$ 0.06	330 $\pm$ 25.1	2.4 $\pm$ 0.07	7.1 $\pm$ 1.9
(C2) control				
Range	0 – 1	306-348	1.9 -4.1	3.8 - 10
Mean $\pm$ SD	0.3 $\pm$ 0.05	327.5 $\pm$ 13.3	2.9 $\pm$ 0.11	6.3 $\pm$ 2.2
CLL Group				
Range	49.1- 90.9	654-979	0.63 - 93.7	5.17 - 100
Mean $\pm$ SD	16.8 $\pm$ 2.2	855.5 $\pm$ 74.9	25.67 $\pm$ 9.45	30.02 $\pm$ 10.2
% Change	$\uparrow$ 16.5	$\uparrow$ 159	$\uparrow$ 996	$\uparrow$ 322
P<	0.001	0.05	0.001	0.01
ALL Group				
Range	30 - 69.7	691-2651	2.76 $\pm$ 91	5 - 101
Mean $\pm$ SD	33.4 $\pm$ 1.8	1757 $\pm$ 181	16.1 $\pm$ 2.33	26.8 $\pm$ 11.6
% Change	$\uparrow$ 33.1	$\uparrow$ 435	$\uparrow$ 455	$\uparrow$ 325
P<	0.001	0.001	0.01	0.05
CLL vs ALL				
P<	0.001	0.001	N.S	NS

NS: non-significant ($P>0.05$); $P<0.05$: significant; $P<0.001$: highly significant.

*Represents percentage of survivin protein population

Table (2): Antioxidants levels in CLL and ALL groups in comparison to the control group:

Parameters Groups	E-(GP-x) (U/g-Hb)	Vitamin C (mg/dl)	Vitamin E (mg/L)	MDA (μ M/L)
(C1) control				
Range	33 – 46	0.7 – 1.9	12.3 – 14.9	1.9- 4.9
Mean $\pm$ SD	14.3 $\pm$ 1.7	1.7 $\pm$ 0.38	13.7 $\pm$ 1.1	3.1 $\pm$ 0.4
(C2) control				
Range	31 – 42	0.74-1.6	11.4-14.7	2.0 – 4.32
Mean $\pm$ SD	16.2 $\pm$ 3.8	1.5 $\pm$ 0.3	13.2 $\pm$ 0.9	2.9 $\pm$ 0.6
CLL Group				
Range	30 - 45.5	0.7-1.7	11 -14.8	2.95 - 7.1
Mean $\pm$ SD	37.8 $\pm$ 4.9	1.02 $\pm$ 0.2	12.9 $\pm$ 1.04	4.8 $\pm$ 1.2
% Change	$\uparrow$ 164.3	$\downarrow$ 40	$\downarrow$ 5.8	$\uparrow$ 54.8
<i>P</i> <	NS	0.001	NS	0.001
ALL Group				
Range	31.1 – 51.5	0.9-1.6	11.3-13.5	4.1 – 12.6
Mean $\pm$ SD	39.1 $\pm$ 5.4	1.1 $\pm$ 0.2	12.4 $\pm$ 0.71	6.4 $\pm$ 2.2
% Change	$\uparrow$ 141	$\downarrow$ 26	$\downarrow$ 6	$\uparrow$ 120
<i>P</i> <	NS	0.001	NS	0.001
CLL vs ALL				
<i>P</i> <	NS	NS	NS	0.01

NS: non-significant ($P > 0.05$); $P < 0.05$: significant; $P < 0.001$: highly significant

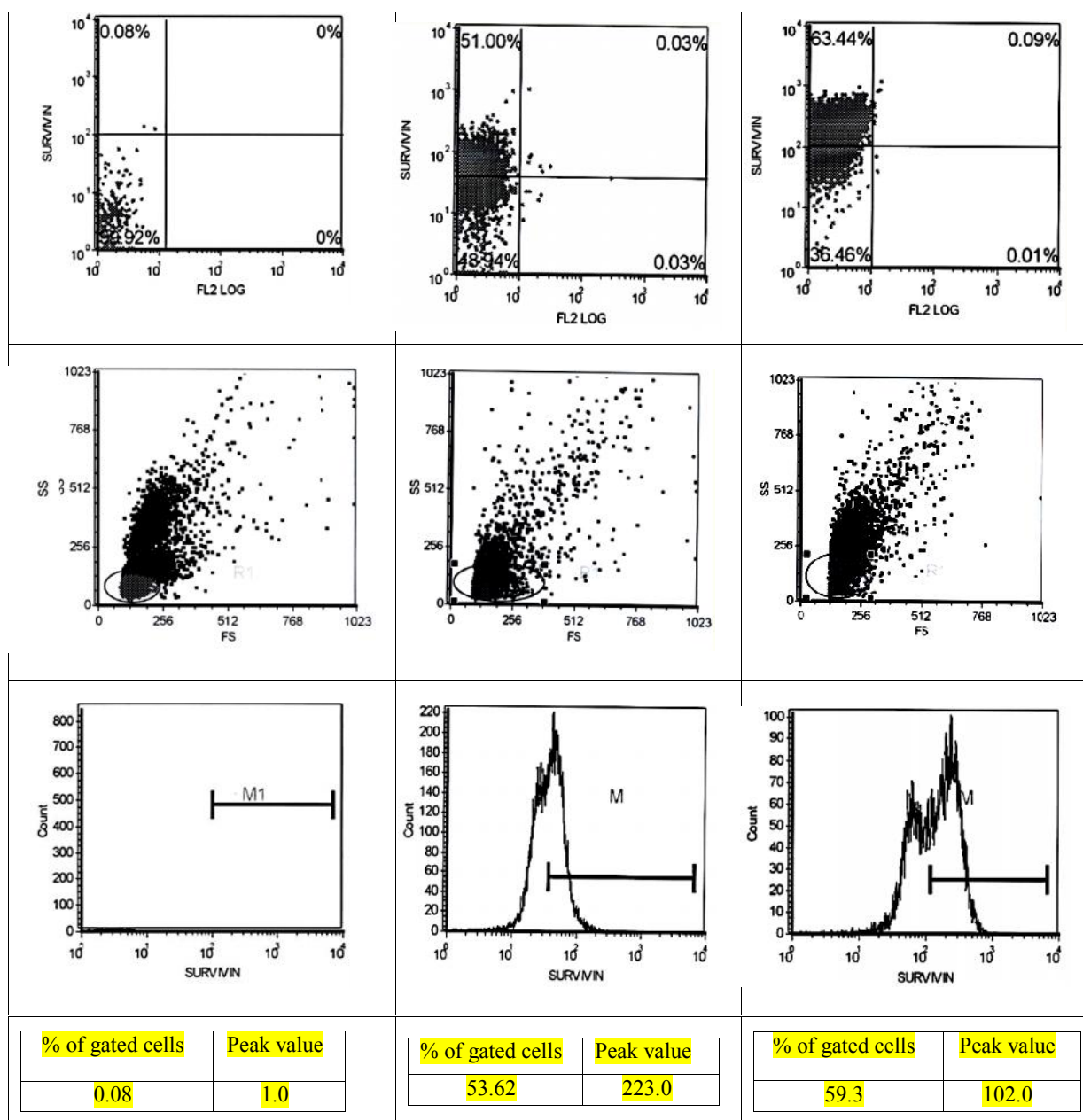
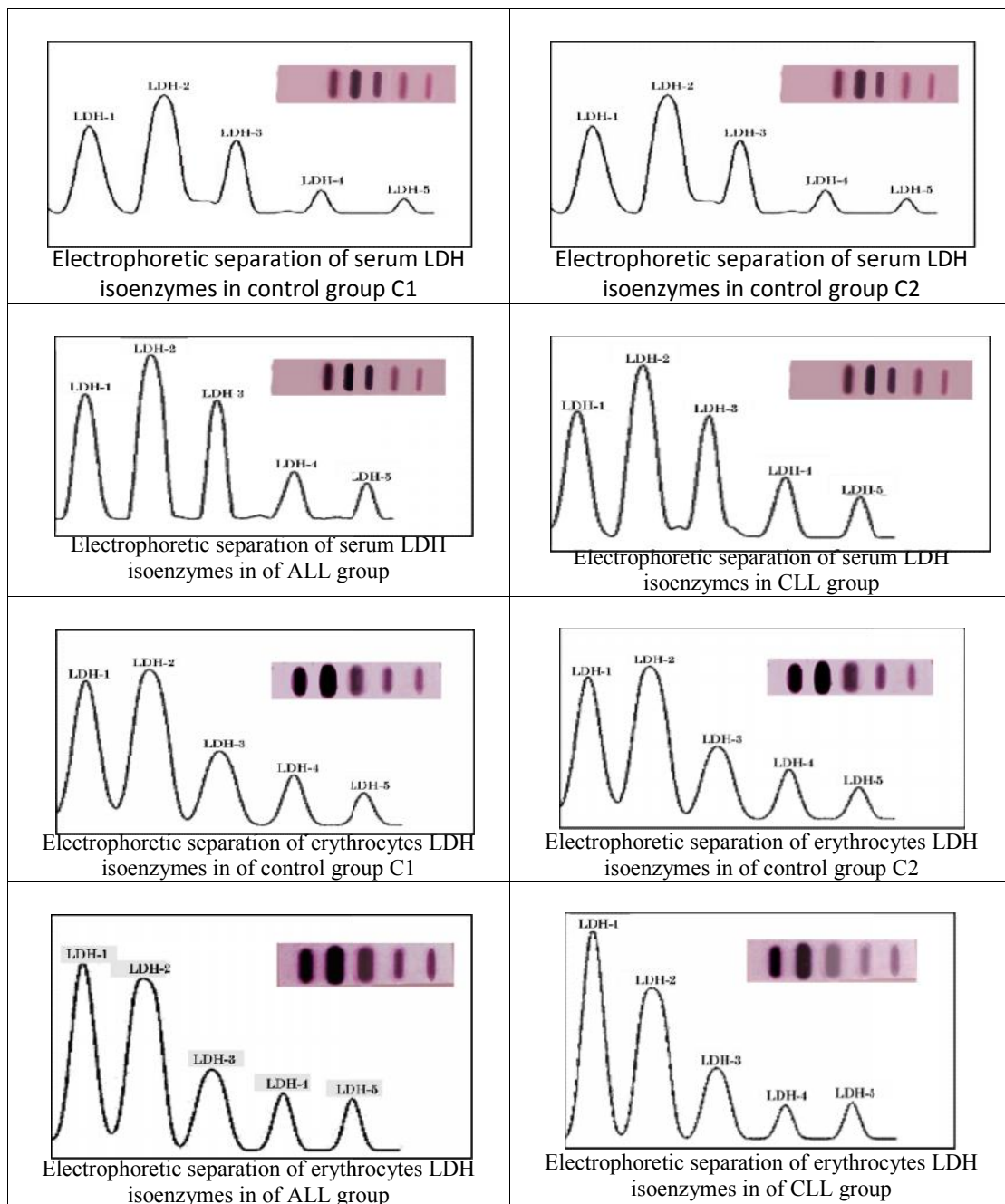


Fig (1): Flow cytometry data analysis of survivin protein in CLL and ALL patients group (b & c) in comparison to control groups (a). FS: forward scatter. SS: side scatter.

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Fig. (2): LDH isoenzyme patterns of serum and erythrocytes in both control groups and ALL and CLL patients

Figure (2), showed the electrophoretic separation of serum LDH isoenzyme, it revealed that, in CLL patients, serum LDH isoenzymes 1, 3, 4 and 5 showed significantly increase ($P<0.05$) and highly significant increase in LDH-2 ($P<0.001$). Highly significant increase ($P<0.001$) in all LDH isoenzyme forms were recorded in ALL patients.

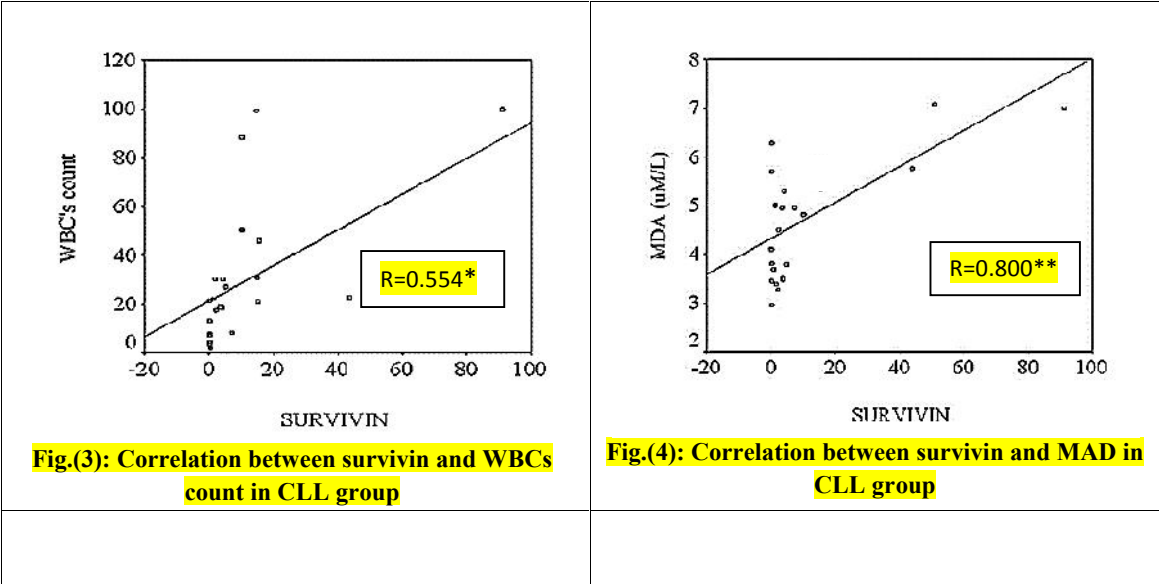
Figure (2) also represented the variations in erythrocyte LDH isoenzymes in the studied groups where, in CLL patients, LDH-1, LDH-4 and LDH-5 were significantly increased. In contrast, there was highly significantly decrease in LDH-2 and a non-significantly changes in LDH-3 when compared to control groups.

In ALL patients, LDH-4 and LDH-5 were significantly increased ($P<0.05$), but LDH-2 showed highly significantly decrease ($P<.001$). In the same time, LDH-1 and LDH-3 were non-significantly changes when compared to control groups.

Figures (3-6) showed the correlation matrix of the different measured parameters and survivin, vitamin C and MDA for the different investigated groups of patients.

The data revealed that:

1. In CLL group: A significant positive correlation was shown between survivin, lymphocyte count and malondialdehyde.
2. In ALL group: A Significant positive correlation was shown between survivin and malondialdehyde. On the other hand, there were significant negative correlations between survivin and vitamin C.



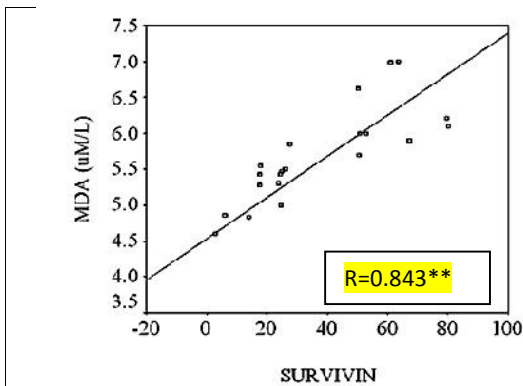


Fig.(5): Correlation between survivin and MDA in ALL group.

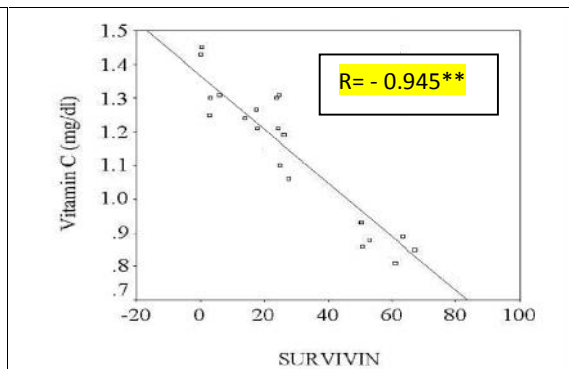


Fig.(6): Correlation between survivin and vitamin C in ALL group.

Discussion

American Cancer Society (2016) has been defined leukemia as the uncontrolled proliferation of hematopoietic cells that have lost the capacity to differentiate normally to mature blood cells. It is usually invade the blood fairly quickly. They can then spread to other parts of the body, including the lymph nodes, liver, spleen, central nervous system and testicles (in males).

Survivin associated with increased risk of recurrence, loco regional lymph node invasion, and metastasis. In addition to its direct role in carcinogenesis, survivin may also play a key role in tumor angiogenesis because it is strongly expressed in endothelial cells during the remodeling and proliferative phase of angiogenesis (**Gine *et al.*, 2010**).

Hence, it act as bifunctional protein through regulation of cell division and suppress apoptosis. Ant apoptotic properties of survivin play an important role in oncogenesis via direct or indirect inhibition of caspases. These properties would clinically provide a significant growth advantage and disclose malignant behavior in tumors (**Marat *et al.*, 2011**).

The results of this study shows a highly significant elevation of protein survivin level in ALL patients compared to control and significantly increased in CLL groups. Also, there was a highly significantly increase of blood survivin in ALL group when compared with CLL group. These results are in agreement with **Raida *et al.*, (2012)**, who reported that; over-expression of survivin was significant in CLL patients compared with the control group. In the meantime, **Duffy *et al.*, (2007)** also conclude that, survivin expression is up-regulated in approximately 90% of ALL cases. It is highly upregulated in most cancers. Particularly in lung cancer, expression levels of survivin appear to relate with disease prognosis (**Fan *et al.*, 2008**).

Our results also were confirmed by **Yao-Kuang *et al.*, (2014)** who reported that the expression of survivin is present in most human neoplasm, but it is absent in normal and differentiated tissues. It is over expressed in majority of lung cancer.

Also, **Sun *et al.*, (2013)** and **Huang *et al.*, (2015)** cleared that overexpression of Survivin are closely related with occurrence and development of acute leukemia, and may be used as an indicator of prognosis evaluation.

Data of the present study showed high significant positive correlations between total leucocyte count and in CLL group and significant positive correlation with lymphoblast in ALL group. These findings indicate that survivin has important role in the pathogenesis and severity of the leukemia disease since the increase of lymphocyte in CLL and lymphoblast count in ALL is parallel with high survivin **protein** level which is responsible for prolonging the life span and the accumulation of these cells by enabling them to escape from their apoptotic fate.

Agata *et al.*, (2015) also declared that an increasing in lymphocyte number is due to decreased apoptosis and slightly increased proliferation of B cells where this was observed in proliferation centers of pseudo follicles located in bone marrow, spleen, and lymph nodes.

Leukocytes count and lymphoblast level were found statistically significant to be increased in ALL patients compared to controls. This finding must be indicating presence the disease (**Escherich *et al.*, 2010**).

LDH plays a major role in hematological malignancies such as leukemia; lymphoma and myeloma that originate in the bone marrow or lymphatic tissues. As cells die, their LDH is released and finds its way into blood. Hence almost all hematological malignancies show elevated levels of serum LDH (**Hala *et al.*, 2014**). Also LDH, considered as a marker of anaerobic metabolism is associated with highly invasive and metastatic cancer (**Katarina *et al.*, 2013**).

The results of the current study revealed that, serum total LDH activity was highly significantly increased in ALL group and significantly increased in CLL group when compared to control groups.

A highly significant increase in serum levels of LDH isoenzymes commonly found in ALL patients with significant increase in LDH isoenzyme form 1, 3,4, and 5 in CLL group. While in erythrocytes, LDH isoenzymes 4 and 5 were highly significant increase in both ALL and CLL patient groups. In contrast, there was a highly significant decrease in LDH-2 in both groups.

The previous variations in serum LDH level encountered in different malignant neoplasms and the markedly elevated levels found in patients with ALL may reflect basic differences in cell proliferation and turnover in these disorders. In a condition such as ALL and CLL with rapid cell turnover, the serum LDH may raise, and the isoenzyme distribution usually reflects the site of the damaged organ and cells.

There is a good relationship between neoplasia and increased cellular LDH activity. It is moderately elevated in many cases of acute leukemia, irrespective to their cell type. Increased

LDH reflects a shift towards anaerobic metabolism and increased glycolysis in the cytoplasm of malignant cells accompanied by a high turnover rate. The variations in the levels of serum LDH encountered in different malignant neoplasms and the markedly elevated levels found in patients with ALL may reflect basic differences in cell proliferation and turnover in these disorders (Golam and Mannan, 2007).

It can be inferred from this results that, serum LDH plays an important role in hematological malignancies.

Reactive Oxygen Species (ROS) exert a key role affecting several hallmarks of cancer. ROS are involved in tumor angiogenesis, through the release of vascular endothelial growth factor and angiopoietin for evading apoptosis (Giannoni *et al.*, 2009 and Go, 2015).

In cancer cells, high levels of ROS can result from increased basal metabolic activity, mitochondrial dysfunction due to hypoxia or mitophagy, peroxisome activity, uncontrolled growth factor of cytokines signaling, and oncogene activity, as well as from enhanced activity of known ROS sources (Tania and Paola, 2012).

In the present study a significant increase in the plasma levels of MDA were observed in patients with ALL and CLL as compared with healthy subjects.

Elevated levels of lipid peroxidation products support the hypothesis that the cancer or malignant cells produce large numbers of ROS and that there exists a relationship between ROS activity and malignancy (Rizwan *et al.*, 2008).

It is a well-known fact that activated oxygen species (AOS) are generated as a result of oxygen metabolism and energy production; they are also implicated in tumor progression (Valko *et al.*, 2007).

In acute leukemia, increased ROS formation may introduce a signal transduction pathway to induce leukemic cells to proliferate but also lead to apoptosis. Serum contains a number of many different enzymatic and non-enzymatic antioxidants such as ascorbic acid, alpha tocopherol, beta carotene, uric acid, cortisol and albumin. Enzymatic antioxidants include Glutathione peroxidase, Glutathione reductase, Catalase and Superoxide dismutase (SOD) (Al-Tonbary *et al.*, 2009 and Sarmento-Ribeiro *et al.*, 2012).

In this study we have examined the activities of GP-x as enzymatic antioxidant and both vitamin E and C as non-enzymatic antioxidant defense system in ALL and CLL patients. Non-significant difference in GP-x activities was observed either in CLL or ALL groups in comparison with control groups or between the two patient groups.

With respect to Vitamin C levels in plasma, it was demonstrated that serum vitamin C was highly significantly decreased in groups I and II compared to healthy subjects

Also our data recorded a non- significant change in vit. E concentration in patient groups in comparison to control health subjects. Where it is an endogenous membrane bound lipid soluble chain breaking antioxidant. It can prevent the propagation of lipid peroxidation chain

reaction by scavenging lipid peroxyl radical. It described as anticarcenogenic factor (Al-Tonbary *et al.*, 2009).

Our results are similar to those obtained by Mahmood *et al.*, (2015) who have shown a decrease in both vit. E and C with significant reduction in the activities of GP-x in ALL and AML patients .

The increase in free radical generation in leukemic patients and the decrease in antioxidant defenses are indicative of oxidative stress involvement in the pathogenesis of human leukemia

Conclusion

Survivin protein is related to anti-apoptotic proteins and has dual role in both apoptosis and cell division, it is considered one of the important factors in the pathophysiology of leukemia. The present work provides evidence for the increased level of survivin protein and special association on the lymphocytes of CLL and lympho-blasts in ALL makes it a reliable marker in the assessment and detection of leukemia. From our results, we concluded that the serum LDH and oxidative damage play an important role in the development of hematological malignancies. For confirmation of this finding, we require further more studies on larger sample size and long-term follow-up in patients more studies are necessary to confirm whether these alterations are the cause or the consequence of carcinogenesis.

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