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Evaluation of *Survivin* and *Vascular Endothelial Growth Factor* (*VEGF*) Expression in Egyptian Children with Acute Lymphoblastic Leukemia

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ABSTRACT

Background: Acute lymphoblastic leukemia (ALL) is the most common form of childhood cancer. Over-expression of *survivin* is associated with increased risk of recurrence in a variety of cancers including hematologic malignancies. *Vascular endothelial growth factor* (*VEGF*) has been demonstrated to be a significant promoter of tumor neo-vascularity.

Objectives: The aim of the current study was to evaluate the prognostic significance of expression of *survivin* and *VEGF* in pediatric patients with ALL before induction chemotherapy as well as their correlation to minimal residual disease (MRD) following chemotherapy.

Patients and Methods: This study was conducted on 100 patients with de novo ALL and 50 age and gender matched patients were selected as a control group. *Survivin* and *VEGF* expression were analyzed before and after induction chemotherapy. Patients were examined for MRD by flowcytometry to determine those prone to relapse.

Results: Using real time polymerase chain reaction, there was an over-expression of both *survivin* and *VEGF* in patients before induction chemotherapy than controls. After induction, *survivin* as well as *VEGF* expression levels declined significantly. Patients with MRD had significantly higher expression of both genes than those who were MRD negative. A significant positive correlation was detected between both genes before and after induction chemotherapy.

Conclusion: These data support the association between a high co-expression of survivin and *VEGF* and disease activation as well as their correlation with a higher tendency to relapse in pediatric ALL.

Key Words: *Survivin – VEGF – Pediatric ALL – MRD.*

INTRODUCTION

One of the most common forms of childhood cancer is acute lymphoblastic leukemia (ALL). Relapse of drug-resistant ALL remains a significant problem despite the fact that overall survival for patients has improved to approximately 80% for children **[1]**. At relapse, risk stratification depends on the time from initial diagnosis to relapse, the anatomic site of relapse, and immunophenotype **[2]**. In addition, the most recent relapse protocols have integrated therapy response with minimal residual disease (MRD) for further treatment adjustments **[3]**.

One of the regulators of both cell-cycle progression and apoptosis is *survivin*, which is over-expressed in practically all human cancers, but is low in most normal terminally differentiated tissues except for the liver, ovary, testes and hematopoietic progenitor cells **[4]**. Furthermore, survivin over-expression has been associated with resistant and refractory disease in many different malignancies including ALL **[5]**. Expression of *survivin* has been reported to be up to ten-fold higher in ALL blasts than in normal peripheral blood and bone marrow **[6]**.

Poor prognosis has been associated with elevated *survivin* expression in cancer. In particular, gene-expression analysis of matched diagnosis-relapse pairs of ALL samples revealed higher expression levels of *survivin* at relapse than at diagnosis **[7]**. Several clinical trials on survivin; employing different approaches including antisense oligonucleotides, small molecule inhibitors and immunotherapy; are offered as a treatment option for terminally ill relapsed B-ALL patients in the context of clinical trial **[8]**.

Vascular endothelial growth factor (VEGF) is a cytokine involved in angiogenesis **[9]**. According to a number of studies, acute leukemia cells secrete significant amounts of VEGF in the serum and malignant hematopoietic cells were found to express *VEGF* and its receptors **[10]**. Since *VEGF* and its receptor levels are lower in lymphoma I-II stages than in III-IV stages, thus *VEGF* expression has a certain correlation with the degree and the stage of tumor malignancy **[11]**. It also has been shown, that high VEGF secretion is necessary for the growth of leukemia cells, while VEGF inhibition led to apoptosis **[12]**. In addition, *VEGF* expression levels were found higher during recurrences compared to that at the time of diagnosis, but without any effects on prognosis **[13]**. High *Survivin* and *VEGF* co-expression levels have been associated with a wide range of clinical disorders, including cancer and cardiovascular diseases **[14]**. The aim of the current study was to evaluate the prognostic significance of expression of the anti-apoptotic factor, survivin and the angiogenic factor, *VEGF* in pediatric patients with ALL before and after induction and their correlation with minimal residual disease (MRD) following chemotherapy.

MATERIAL AND METHODS

This study was conducted on 100 patients with de novo ALL. Patients were selected from the outpatient clinic of Alexandria University Children Hospital between 2014 and 2016. The patients were 50 males and 50 females with age range from 1.5 to 15 (median 4.25) years. Fifty age and gender matched normal bone marrow transplantation (BMT) donors were selected as a control group. The selection of these patients was based on the following criteria: Full history taking; thorough clinical examination; peripheral blood and bone marrow diagnosis of ALL which was established by immunophenotyping using Miltenyi Biotec MACS Quant™ flowcytometry analyzer equipped with MACS Quantify software version 2.4. After diagnosis, cases were further subdivided into 85 patients with B-lineage ALL and 15 cases with T-ALL. Patients received the following chemotherapy: IV Vincistine VCR 1.5mg/m^2 on day 0,7,14 and 21; P.O Dexamethasone 6mg/m^2 from day 0-27; IM L-asparaginase 6000 I.U/m2 twice/week; IT Methotrexate MTX (8 mg from 1 to 1.99 years, 10mg from 2 to 2.99 years, 12mg > 3 years) on day 7 and 28; IT Cytosine arabinoside Ara-C (30mg from 1 to 1.99 years, 50mg from 2 to 2.99 years, 70mg > 3 years) on day 0.

Patients were followed-up by bone marrow examination at the end of induction at 1 and 3 months time point. *Survivin* and *VEGF* gene expression were analyzed before chemotherapy. Only those with remission were examined for MRD by flowcytometry at 1 and 3 months time point. Expression of *survivin* and *VEGF* was repeated at 3 months time point. The panel used for detection of MRD included; CD19, CD34, CD10, CD20, CD22, CD33, CD38, CD45, CD58, TdT and CD3. Expression of these profile markers in patients with remission is sensitive to identify one leukemic cell among 10^{-4} cells ($\leq 10^{-4}$) which cannot be evident by morphological analysis. The cutoff level for MRD negative patients is $<5 \times \leq 10^{-4}$, while MRD positive is $\geq 5 \times 10^{-4}$ at 1 and 3 months time point.

Expression of survivin and VEGF by Reverse transcription (RT)-PCR:

Purification of total cellular RNA from human whole blood was done using QIAamp® RNA blood Mini kit (Qiagen Inc., Valencia, CA, USA). The concentration of RNA was determined by measuring the absorbance at 260nm (A260) on a spectrophotometer (Nanodrop® ND-1000 spectrophotometer). Purity of RNA was assessed using the ratio of the readings at 260nm and 280nm (A260/A280). Pure RNA has an A260/A280 ratio of 1.9-2.1. cDNA was prepared using high capacity cDNA reverse transcription kit (Applied Biosystems, USA) according to the manufacturer's instructions.DNA purity was estimated using the ratio of the readings at A260 and A280; a ratio between 1.7 and 2.0 generally represents a high quality DNA sample. A relative quantitation of *survivin* and *VEGF* gene expression; normalized to the endogenous gene Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was performed by real-time RT-PCR, using real-time cycler Rotor gene Q® (Qiagen) and ready to use QuantiFast® probe two-step RT-PCR assay (Qiagen, USA). The specific primer pairs were as follows: for survivin, 5'-GGA CCA CCG CAT CTC TAC ATT-3' (forward) and 5'-AGA AGA AAC ACT GGG CCA AGT C-3' (reverse); for *VEGF*, 5'-TTG CTG CTC TAC CTC CAC-3' (forward) and 5'-AAT GCT TTC TCC GCT CTG-3' (reverse); and for β-actin, 5'-GCT CAC

CAT GGA TGA TGA TAT C-3' (forward) and 5'-GCC AGA TTT TCT CCA TGT CGT C-3' (reverse).

The study was approved by the medical ethics committee and informed consents were obtained from the patients' parents to participate in the study. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008 **[5]**.

Statistical analysis:

Data were fed to the computer and analyzed using IBM SPSS software package version 20.0 (SPSS Inc., Chicago, IL, USA). Comparisons between groups for categorical variables were assessed using Chi-square test (χ^2). Student *t*-test was used to compare two groups for normally distributed quantitative variables. Mann-Whitney test was used to compare two groups for abnormally distributed quantitative variables. Paired *t*-test and Wilcoxon signed ranks test were assessed for comparison between different periods. Spearman coefficient was used to correlate between quantitative variables. Significance of the obtained results was judged at the 5% level.

RESULTS

Survivin and VEGF gene expression in patients with ALL:

Using an RT-PCR approach, we detected the expression levels of *survivin* and *VEGF* in patients with ALL before induction chemotherapy. Both *survivin* and *VEGF* expression levels were significantly higher in cases than controls ($p<0.001$). As shown in Table (1), in patients before induction chemotherapy, *survivin* expression level was significantly higher than after induction ($p<0.001$), and the level of *VEGF* expression before induction was significantly higher than after induction ($p=0.010$). In patients with positive MRD, both *survivin* and *VEGF* expression levels were significantly higher than those with negative MRD ($p=0.015$, $p=0.039$ respectively) as shown in Table (2).

Table (1): Hematological parameters and expression of survivin and *VEGF* in acute lymphoblastic leukemia patients before and after induction chemotherapy.

Parameter	Acute lymphoblastic leukemia (100 cases)		*p*
	Before Induction	After Induction	
WBC: (x 10^9/L)	13.56 (1.66 – 366.0)*	3.45 (0.50 – 15.50)	<0.001
Hb: g/dl (mean ± SD)	8.33±1.59	9.13±1.73	<0.001
PLT: (x 10^9/L)	41.0 (9.0 – 174.0)	110.0 (27.0 – 867.0)	<0.001
Bone marrow blasts: %	94.50 (73.0 – 99.0)	2.0 (1.0 – 50.0)	<0.001
Survivin expression	1.11 (0.0 – 153.27)	0.12 (0.0 – 7.40)	<0.001
VEGF expression	2.02 (0.03 – 6.49)	0.98 (0.0 – 25.60)	0.010

*Median (range). WBC: White cell count. Hb: Hemoglobin. PLT: Platelets. VEGF: Vascular endothelial growth factor.

Table (2): Comparison between ALL patients with negative versus positive MRD as regards the different parameters before induction chemotherapy.

Parameter	MRD		*p*
	Negative (n=85)	Positive (n=15)	
Gender:			
Male (No 50)	35 (70%)	15 (30%)	<0.001*
Female (No 50)	50 (100%)	0 (0.0%)	
Age (years)	4.0 (1.50 – 13.0)*	6.0 (4.0 – 15.0)	0.069
WBC (10^9/l)	15.1 (5.50 – 366.0)	7.24 (1.66 – 303.0)	0.003*
Hb (g/dl)	8.25±1.58	8.80±1.61	0.076
PLT (10^9/l)	36.0 (9.0 – 174.0)	68.0 (21.0 – 174.0)	0.333
Peripheral blood blasts (%)	65.0 (3.0 – 98.0)	91.0 (5.0 – 95.0)	0.011*
Bone marrow blasts (%)	89.41±7.97	93.67±2.72	0.147
Survivin expression	1.10 (0.0 – 153.27)	1.71 (0.12 – 102.0)	0.015*
VEGF expression	0.86 (0.0 – 25.60)	1.10 (1.10 – 18.20)	0.039*

*Median (range). WBC: White cell count. Hb: Hemoglobin. PLT: Platelets. VEGF: Vascular endothelial growth factor.

Correlation between survivin and VEGF expression:

A significant positive correlation was found between *survivin* and *VEGF* expression levels in patients with positive MRD before and after induction chemotherapy (r_s=0.866 in both cases) as shown in Figs. (1,2). Meanwhile, in patients with negative MRD, there was a significant positive correlation only before induction (r_s=0.248) as shown in Table (3).

Table (3): Correlation between *survivin* and *VEGF* expression in minimal residual disease (MRD) positive versus MRD negative patients before and after induction chemotherapy.

Parameter MRD	Before induction		After induction	
	r_s	*p*	r_s	*p*
Negative	0.248*	0.039	0.142	0.240
Positive	0.866*	<0.001	0.866*	<0.001

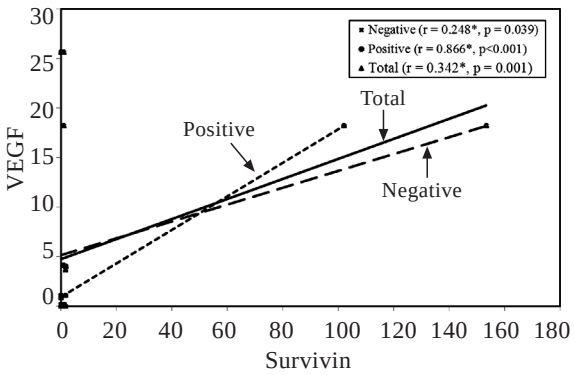

Fig. (1): Correlation between *Survivin* and *VEGF* in ALL patients before induction.

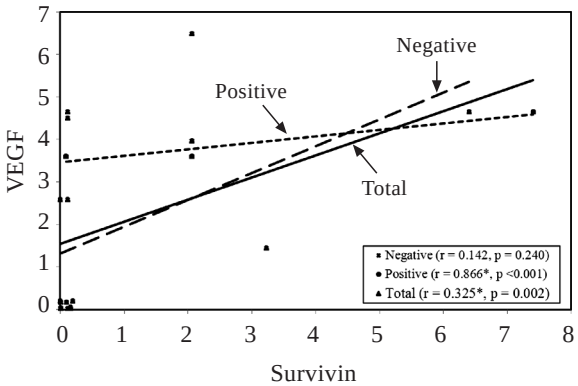

Fig. (2): Correlation between *Survivin* and *VEGF* in 100 acute lymphoblastic leukemia patients after induction.

DISCUSSION

Survivin expression has been studied in a number of hematologic neoplasms **[15]**; it was reported in 83.8% of acute leukemia patients and its expression was associated with bad prognosis **[16]**. In the present study, *survivin* expression level was significantly higher in cases than controls. There was a significantly higher level of expression of *survivin* in ALL patients before induction chemotherapy than after induction. In addition, before and after induction, there was a significant positive correlation between *survivin* and WBC count. Moreover, after induction a significantly negative correlation was evident between survivin and bone marrow blasts. *Survivin* overexpression was found to be linked to tumor aggressiveness **[17]** and chemoresistance in adult acute lymphoblastic leukemia **[18]**. It was demonstrated that knockdown of *survivin* improved the chemotherapeutic response in ALL models **[18]**. In acute myeloid leukemia, *survivin* expression levels were found to be significantly predictive of shorter overall and event-free survival **[19]**.

Patients with positive MRD had a significantly higher expression of *survivin* than those with negative MRD. Similarly, it has been found that overexpression of survivin in precursor B-ALL identifies patients with a high risk of early relapse **[6]**. A report from the Children's Oncology Group has shown a differential expression profile of relapsed ALL compared with initial diagnosis **[7]**. One of the genes showing a marked increase in expression in patients with recurrent disease was *survivin*. Furthermore, *survivin* overexpression has correlated with resistant and refractory disease in many different malignancies including ALL **[8]**.

Different studies have given variable results regarding *VEGF* expression in various hematological malignancies with some showing increased expression while others concluding that no difference exists in *VEGF* expression between hematological malignancies and controls **[20]**. These contradictory results may suggest that a complex regulation of the cytokine system exists during the angiogenesis process in hematological malignancies including VEGF and other proangiogenic cytokines **[21]**.

In the present study, we demonstrated that *VEGF* expression level was significantly higher

in cases than in controls. In addition, its expression before induction was significantly higher than after induction chemotherapy. This may be explained by a study that has reported a relation between VEGF levels and disease activation **[13]**. This also agrees with the current results where patients with positive MRD had significantly higher *VEGF* expression levels than those with negative MRD. Interestingly, serum VEGF level was found to be significantly lower in ALL patients than healthy controls **[22]**. As an explanation to this controversy, it has been suggested that with active angiogenesis, cell-associated *VEGF* may have an elevated expression, which was observed in ALL cell lines, but as a result of incremented consumption of VEGF by the increased angiogenesis, the locally present VEGF may not be reflected in the bone marrow **[23]**.

The apparent correlation between *VEGF* and survivin expression in cancer can be explained by the fact that *VEGF* induces survivin transcription. Extracellular stimuli that activate transcription pathways include VEGF, EGF, and cytokines **[24]**. Additionally, it has been demonstrated that *survivin* overexpression activates *PI3K/AKT* signaling and subsequent β-catenin/Tcf-Lef-dependent transcription, which elevates *VEGF* expression, among other transcriptional target genes **[25]**. Furthermore, downregulation of *survivin* correlates with lower levels of *VEGF* and reduced angiogenesis in cancer cells **[26]**.

In the current study, there was an overexpression of both *survivin* and *VEGF* in pediatric patients with ALL before induction chemotherapy than in controls. After induction, survivin as well as *VEGF* expression levels declined significantly. Moreover, patients with MRD following chemotherapy had significantly higher levels of expression of both genes than those with negative MRD. A significant positive correlation was found in our study between *survivin* and *VEGF* expression levels in patients with positive MRD before and after induction chemotherapy. Meanwhile, patients with negative MRD showed a significant positive correlation only before induction.

In conclusion, these data suggest the association between a high co-expression of *survivin* and *VEGF* and disease activation as well as their correlation with a higher tendency to relapse in pediatric ALL which supports their role as candidate therapeutic targets.

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