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RESEARCH ARTICLE

EVALUATION OF SURVIVIN AND VEGF EXPRESSION AS INDEPENDENT PREDICTORS OF RELAPSE IN EGYPTIAN CHILDREN WITH ACUTE LYMPHOBLASTIC LEUKEMIA

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ABSTRACT

Acute lymphoblastic leukemia (ALL) is the most common form of childhood cancer. Overexpression 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 neovascularity. The aim of the current study was to evaluate the prognostic significance of expression of survivin and VEGF in pediatric patients with acute lymphoblastic leukemia at induction and their correlation with minimal residual disease (MRD) following chemotherapy. This study was conducted on 100 patients with de novo ALL. Survivin and VEGF expression were analyzed before and after chemotherapy. Patients were examined for MRD by flow cytometry to determine those prone to relapse. Using real time polymerase chain reaction, there was an over expression of both survivin and VEGF in patients before induction chemotherapy. After induction, survivin expression declined significantly, while VEGF expression significantly increased. Patients with MRD had significantly higher expression of both genes with a significant positive correlation before and after induction chemotherapy. In conclusion, these data support the association between a high coexpression of survivin and VEGF and disease activation as well as their correlation with a higher tendency to relapse in pediatric ALL.

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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 overall survival for patients has improved to approximately 80% for children (Pui *et al*., 2011). At relapse, risk stratification depends on the time from initial diagnosis to relapse, the anatomic site of relapse, and immunophenotype (Tallen *et al*., 2010). In addition, the most recent relapse protocols have integrated therapy response with minimal residual disease (MRD) for further treatment adjustments (Eckert *et al*., 2013). One of the regulators of both cell-cycle progression and apoptosis is survivin, which is overexpressed in practically all human cancers, but is low in most normal terminally differentiated tissues except for the liver, ovary, testes and hematopoietic progenitor cells (Knauer *et al*., 2007). Furthermore, survivin overexpression has been associated with resistant and refractory disease in many different malignancies including ALL (Pennati *et al*., 2007).

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Expression of survivin has been reported to be up to ten-fold higher in ALL blasts than in normal peripheral blood and bone marrow (Fukuda and Pelus, 2006). 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 (Bhojwani *et al*., 2006). Thus, survivin is an attractive target in cancer therapy and currently, several clinical trials employing different approaches including antisense oligonucleotides, small molecule inhibitors and immunotherapy are in progress (Troeger *et al*., 2007). Vascular endothelial growth factor (VEGF) is cytokine involved in angiogenesis (Ferrara, 1995). 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 (Zhu *et al*., 2003). Since VEGF and its receptor levels are lower in lymphoma I-II stages than in III-IV stages then VEGF expression has a certain correlation with the degree and the stage of tumor malignancy (Kessler *et al*., 2007). It also has been shown, that high VEGF secretion is necessary for the growth of leukemia cells, while VEGF inhibition led to

apoptosis (Broggini *et al*., 2003). In addition, VEGF expression levels were found higher during recurrences compared to the ones at the time of diagnosis, but without any effects on prognosis (Koomagi *et al*., 2001). Survivin and VEGF coexpression levels have been implicated to be associated with a wide range of clinical disorders, including cancer and cardiovascular diseases (Choi and Choi, 2012). The aim of the current study was to evaluate the prognostic significance of expression of the antiapoptotic factor, survivin and the angiogenic factor, VEGF as well as their quantitative analysis in pediatric patients with acute lymphoblastic leukemia at induction and their correlation with minimal residual disease (MRD) following chemotherapy.

MATERIALS AND METHODS

This study was conducted on 100 patients with de novo acute lymphoblastic leukemia (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 years (median age 4.25). Fifty age and sex matched patients with hematological diseases to whom bone marrow examination is one of the required investigations were selected as a control group. They were 10 males and 40 females with age range from 2 to 13 years (median age 3.5). 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^{TM} 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. Survivin and VEGF levels in sera were quantified using ELISA to investigate their possible correlation in the pathogenesis of pediatric ALL.

The patients then underwent the following chemotherapy: IV Vincistine VCR 1.5 mg$^{/m2}$on day 0,7,14 and 21; P.O Dexamethasone 6 mg$^{/m2}$ from day 0 – 27; IM L-asparaginase 6000 I.U$^{/m2}$ twice/week; IT Methotrexate MTX (8 mg from 1 to 1.99 years, 10 mg from 2 to 2.99 years, 12 mg > 3 years) on day 7 and 28; IT Cytosine arabinoside Ara-C (30 mg from 1 to 1.99 years, 50 mg from 2 to 2.99 years, 70 mg > 3 years) on day 0. The patients were followed up by bone marrow aspiration at the end of induction (day 28). Survivin and Vascular endothelial growth factor (VEGF) gene expression were analyzed before chemotherapy. After achieving remission, patients were examined for MRD by flowcytometry to determine those at increased risk of relapse and expression of survivin and VEGF was repeated. 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).

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 260 nm (A260) on a spectrophotometer (Nanodrop® ND-1000 spectro photometer). Purity of RNA was assessed using the ratio of the readings at 260 nm and 280 nm (A260/A280). Pure RNA has an A260/A280 ratio of 1.9-2.1. RT-PCR was performed 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/A280 ratio, between 1.7 and 2.0 generally represents a high quality DNA sample. The specific primer pairs are 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). 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).

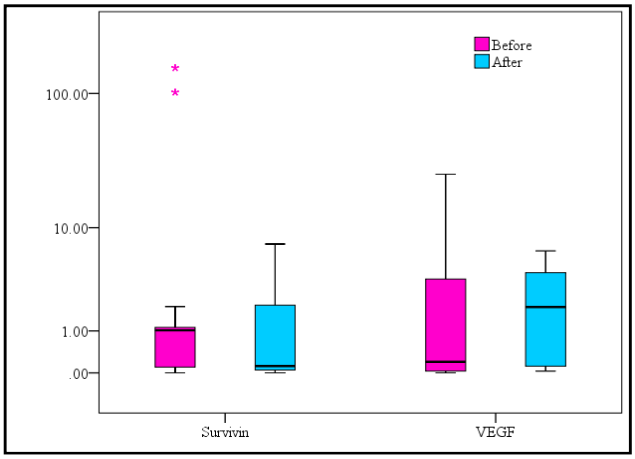

Figure 1. mRNA expression levels of Survivin and vascular endothelial growth factor (VEGF) in patients with acute lymphoblastic leukemia (ALL) before and after induction chemotherapy

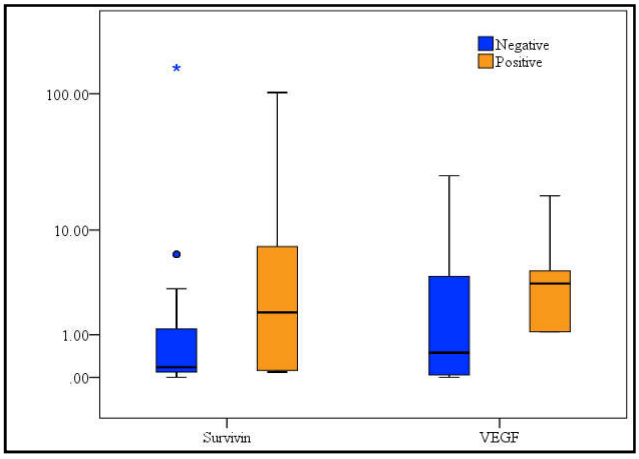

Figure 2. mRNA expression levels of Survivin and vascular endothelial growth factor (VEGF) in patients with acute lymphoblastic leukemia (ALL) with minimal residual disease (MRD positive) versus patients (MRD negative)

Table 1. Correlation analysis of mRNA expression levels of survivin and vascular endothelial growth factor (VEGF) in patients with with minimal residual disease (MRD positive) versus patients (MRD negative) before and after induction chemotherapy

	Survivin versus VEGF			
	Before		After	
MRD	r_s	p	r_s	p
Negative	0.248*	0.039	0.142	0.240
Positive	0.866*	<0.001	0.866*	<0.001

r_s: Spearman coefficient
*: Statistically significant at $p \leq 0.05$

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). Survivin expression was significantly lower in patients with B-ALL than those with T-ALL (P=0.015). On the contrary, patients with B-ALL showed a significantly higher expression level of VEGF than those with T-ALL (P<0.001). In addition, peripheral blood in patients with T-ALL showed a significantly higher WBC count as well as blasts (P=0.003, P=0.011 respectively). As shown in figure (1), in patients before induction chemotherapy, survivin expression level was significantly higher than after induction (P<0.001), while the level of VEGF expression before induction was significantly lower than after induction (P=0.004). Minimal residual disease was only detected in patients with B-ALL. In patients with positive MRD, both survivin and VEGF expression levels were significantly higher than those with negative MRD (P=0.015, P<0.001 respectively) as shown in Figure (2).

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 (Rs=0.866 in both cases) as shown in table (1). Meanwhile, in patients with negative MRD, there was a significant positive correlation only before induction (Rs=0.248). Likewise, another positive correlation was found after induction chemotherapy in MRD negative patients, although it was not significant (Rs=0.142).

DISCUSSION

Survivin expression has been studied in a number of hematologic neoplasias (Carter *et al*., 2006). It was reported that survivin was expressed in 83.8 % of 74 patients with acute leukemia and concluded that its expression is a bad prognostic indicator while its negativity shows good clinical outcome in acute leukemia (Oto *et al*., 2007). In the present study, survivin expression level was significantly higher in cases than controls. In patients with B-ALL, survivin expression was significantly lower than those with T-ALL. 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 (Tyner *et al*., 2012) and chemoresistance in adult acute lymphoblastic leukemia (Morrison *et al*., 2012). It was demonstrated that knockdown of survivin improved the chemotherapeutic response in ALL models (Morrison *et al*., 2012). In acute myeloid leukemia, survivin expression levels were found to be significantly predictive of shorter overall and event-free survival (Carter *et al*., 2012). In the current study, minimal residual disease was only detected in patients with B-ALL. 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 (Fukuda and Pelus, 2006). A report from the Children's Oncology Group has shown a differential expression profile of relapsed ALL compared with initial diagnosis (Bhojwani *et al*., 2006). 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 (Troeger *et al*., 2007). 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 (Gianelli *et al*., 2007). It was indicated that polymorphisms in VEGF are associated with high relapse risk in ALL (Demacq *et al*., 2010). In the present study, we demonstrated that VEGF expression level was significantly higher in cases than controls. In patients with B-ALL, VEGF expression was significantly higher than those with T-ALL. The level of VEGF expression before induction was significantly lower than after induction chemotherapy. It has been found that serum VEGF level was lower in ALL patients than healthy controls with a statistical significance (Yetgin *et al*., 2001). In the current study, as regards patients with positive MRD, VEGF expression levels were significantly higher than those with negative MRD. Similarly, studying VEGF expression by real time PCR in ALL patients at diagnosis and recurrences has concluded that VEGF expression levels during relapses were significantly higher than those at diagnosis (Koomagi *et al*., 2001). It has been found that there was a relation between VEGF levels and disease activation. It has been reported that with the 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 and VEGF expression may also be minimal in the lymphoid system (Hacıhanefioglu *et al*., 2011).

Moreover, 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 in the present study showed a significant positive correlation only before induction. In addition, another positive correlation was found after induction chemotherapy in MRD negative patients, yet it was not significant. 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 (Kumar *et al*., 2007). 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 (Fernandez *et al*., 2014). Moreover, down-regulation of survivin correlates with lower levels of VEGF and reduced angiogenesis in cancer cells (Shen *et al*., 2014). In B- lineage ALL patients, survivin showed a lower expression whereas VEGF was significantly higher than those with T-ALL. This coincides with the worse prognosis usually prevalent in T-ALL than B-ALL (Ma *et al*., 2012). To summarize, there was an overexpression of both survivin and VEGF in pediatric patients with ALL before induction chemotherapy. After induction, survivin expression level declined significantly, while VEGF expression significantly increased. Therefore, survivin appears to be a candidate predictive biomarker of poor prognosis in pediatric ALL patients. A significant positive correlation was found between survivin and VEGF measured at the mRNA level in patients with positive MRD before and after induction chemotherapy. This could be explained by the fact that tumor cells overexpressing survivin protein induce VEGF synthesis/release in a β-catenin signaling-dependent manner (Fernandez *et al*., 2014). Moreover, patients with minimal residual disease following chemotherapy had significantly higher levels of expression of both genes than those with negative MRD.

In conclusion, these data support the association between a high coexpression of survivin and VEGF and disease activation as well as their correlation with a higher tendency to relapse in pediatric ALL.

Statistical notes

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.

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Declaration of Interest

The authors declare that they have no conflict of interest.

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