

Role of Bone Sialoprotein-1 and Its Receptor CD44 Variant 6 in Childhood Acute Lymphoblastic Leukemia

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ABSTRACT

Acute lymphoblastic leukemia (ALL) represent about 77% of leukemia in children and it is one of the most common malignancies in children. Bone Sialoprotein-1 is a tumor – associated antigen that is highly expressed in many malignancies in human. This study was aimed to study the role of Bone Sialoprotein-1 and its receptors CD44 variant 6 in children's with acute lymphoblastic Leukemia in Tanta University Hospital. This study was carried out in hematology unit, pediatric department and clinical pathology department, Tanta university hospital, Egypt. 40 children patients were included in this study and 10 healthy controls. The serum levels of Bone Sialoprotein-1 were measured in both healthy control and ALL children patients by ELISA and CD44 variant 6 by flowcytometry. Bone Sialoprotein-1 levels in patient group was 112.69 ± 11.54 ng/mL which was significantly higher than that of control group 8.58 ± 2.19 ng/mL (P value was 0.001), CD44 variant 6 level in patient group was 32.18 ± 15.26 which was significantly higher than that of control group 3.54 ± 1.40 (P value was 0.001) and there was significant difference between level of both Bone Sialoprotein-1 and CD44 variant 6 in cases of good and bad prognosis (P value was 0.001). As regard comparison between level of CD44 variant 6 and level of BSP-1 in cases of bad prognosis 22 (55%) of cases were positive for both BSP-1 and CD44 variant 6 and of bad prognosis (P value was 0.002). BSP-1 level and CD44v6 expression in childhood ALL patients may be used as prognostic markers to predict outcome as it has a tendency to be high with cases of bad prognosis.

Keywords

Acute lymphoblastic leukemia, Bone Sialoprotein-1, CD44 variant 6.

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Introduction

Acute lymphoblastic leukemia (ALL) was defined as a malignancy of lymphoid progenitor cells that proliferate and replace the normal hematopoietic cells of the bone marrow. Then the normal bone marrow elements replaced by these lymphoblasts thus the production of normal blood cells markedly decreased Gowda and Dovat, (2013). The prognosis and the response to

treatment of ALL were affected by many prognostic factors Amirghofran *et al.*, (2011). These prognostic factors include age, sex, count of bone marrow blasts, white blood cell (WBC) count and platelet count, cytogenetic abnormalities, extramedullary involvement (EMI), and immune phenotyping Fathi *et al.*, (2012). The different markers expression in childhood ALL were reported and can be used

as an important source of information for its classification, diagnosis and treatment Kamazani *et al.*, (2013). Immunophenotyping by flowcytometry is considered as one of the most important method used for the diagnosis, classification, staging and monitoring of childhood ALL Craig and Foon, (2008). CD44 is a glycoprotein adhesion molecule; it is expressed by B and T lymphocytes, that responsible for cell attachment to extracellular matrix components and adhesion to endothelial cells. Its level of expression change according to type of cell and their activation state Ulrike *et al.*, (2014). CD44 variants (CD44v) are mainly expressed on epithelial cells, There were about 10 types of CD44 variants which detected by PCR (Williams *et al.*, 2013). CD44v6 is reported to play a major role in hematopoietic malignancies pathophysiology and prognosis and to be related to tumor cells metastatic behavior. The increased expression of exons v6 and v9 genes is associated with poor prognosis in non-Hodgkin lymphoma and myeloma (Hao *et al.*, 2012). Bone Sialoprotein-1 (BSP-1) is an adhesive glycoprotein that is secreted and expressed by numerous human malignancies including ALL. It was first detected in bone and later in many other tissues including cartilage, kidney and vascular tissues. BSP-1 plays an important role in different biological processes including inflammation, wound healing and cell survival (Takahashi *et al.*, 2004).

In addition, BSP-1 also might play a protective role in interactions between the epithelial surfaces and the external environment (Rodrigues *et al.*, 2007). It functions in cell adhesion, chemotaxis, macrophage directed interleukin 10 suppression, stress dependent angiogenesis, prevention of apoptosis, anchorage independent growth of tumor cell matrix interactions and cellular signaling through

binding with integrin and CD44 receptors (Wai and Kuo, 2004). The various effects produced by BSP-1 is related to its different forms and its many receptors and binding sites, such as multiple integrins (avb3, a9b1, avb7) (Halock and Nilsson, 2006) and CD44 through the v6 and v7 variants (Katagiri *et al.*, 1999). Serum BSP-1 levels were increased in cases with lung, breast, prostate, liver and ovarian cancers, and have been recommended as a prognostic or diagnostic marker (Rittling and Chambers, 2004). In addition, the more increase in the levels of BSP-1 n ALL may give more information on prognosis of ALL in children, Elli *et al.*, (2012) also, monitoring of these levels along the course of the disease is needed to become sure whether this marker gives more information about bad prognosis of ALL. In our study, we aimed to evaluate the role of Bone Sialoprotein-1 and its receptors CD44 variant 6 in children's with acute lymphoblastic Leukemia.

Materials and Methods

The present study included 40 children's patients admitted to pediatric department (hematology Unit), Tanta University Hospital and 10 healthy control children's, the duration of the study was from June 2016 till February 2017. All the studied patients were subjected to the following: Full history taking, complete clinical examination, Routine laboratory investigations (Complete blood count, Erythrocyte sedimentation rate, Serum Lactate dehydrogenase, Liver and kidney function tests, Bone marrow aspiration, cytochemistry and immunophenotyping for patients only), Detection of BSP-1 by ELISA and CD44V6 expression by flowcytometry. Inclusion criteria was newly diagnosed ALL children's patients selected from the pediatric Department (hematology Unit)-Tanta University Hospital and the exclusion criteria were Patients with other types of leukemia or other malignancies.

Blood Collection and Laboratory Assay: Specimens: Laboratory assays were performed on blood obtained at the time of diagnosis of ALL patients. Whole blood was collected by standard venipuncture in VACUETTE® Blood Collection Tubes (Greiner Bio-One, Austria) containing k2EDTA and containing clot activator/Sep.

Bone marrow aspiration and aspirate examination, cytochemistry and flow cytometry. CBC, liver functions, kidney functions and LDH were done by routine laboratory methods at hematology unit and clinical chemistry unit, clinical pathology department, Tanta University Hospital, Egypt. Serum samples for determination of BSP-1 were aliquoted and stored frozen at -20°C to avoid loss of human BSP-1 .

Laboratory assay of BSP-1 levels by ELISA

BSP-1 levels were measured by Human Osteopontin Platinum Enzyme Linked Immunosorbent Assay (ELISA) (BMS2066/BMS2066TEN, ebioscience). Briefly, BSP-1 present in the sample or standard bound to antibodies adsorbed to the microwells (first incubation). Following incubation unbound biological components were removed by washing and a biotin-conjugated anti-human BSP-1 antibody was added and bound to BSP-1 captured by the first antibody (second incubation). Following this step, unbound biotin conjugated anti-human BSP-1 antibody was removed by washing. Streptavidin-HRP was added and bound to the biotin-conjugated antihuman BSP-1 antibody (third incubation). Following this step, unbound Streptavidin-HRP was removed by washing and substrate solution reactive with HRP was added to the wells (fourth incubation). A colored product was formed in proportion to the amount of human BSP-1 present in the sample or standard. The reaction was terminated by

addition of acid and absorbance was measured at 450nm. A standard curve was prepared and human BSP-1 concentrations were determined.

Detection of surface CD44v6 expression by flowcytometry

For surface staining we first adjust the cell count of each tube to become 1×10^6 / ml, then the blood were mixed with 10ul of APC-conjugated antihuman CD44v6 moAb (R&D Systems Inc., Clone 2F10, Catalog Number: FAB3660A) and incubated for 25-30 minutes at room temperature in dark place (the same was done for tube of negative isotope control), followed by red cell lysis using B.D lysing solution (diluted 1:9 with D.W) as follow: mix 1ml of diluted lysing solution to the tested tubes and incubated for 15 minutes at room temperature in dark place, wash twice with PBS buffer, the tubes were centrifuged at 3000 rpm for 5 minutes and the supernatant was discarded, analysis was performed using cell quest software of FACS caliber cytometer.

The percent of cells positive for CD44v6 was determined relative to the negative isotype-matched control with cut off value for positivity 5%.

The considered negative (-) If less than 5% of the cells were labeled, positive (+) if 5 to less than 20% of cells were labeled, and strong positive (+ +) when 20% or more of the cells expressed CD44v6.

Results and Discussion

Our study was included 40 children patients and 10 healthy controls, their age was ranged from 3 to 12 years old, there were 25(62.5%) males and 15 (37.5%) females, as regard immunophenotyping 50% of patients were pre-B-ALL, 2.5% were mature B-ALL, early

pre B 2.5%, Common B 35% (total: 90%) and 10% were T-ALL, as regard results of measuring BSP-1 levels by ELISA in patient group was 112.69 ± 11.54 ng/mL which was significantly higher than that of control group 8.58 ± 2.19 ng/mL (P value was 0.001) as shown in table 1, as regard CD44 variant 6 level by flowcytometry in patient group was 32.18 ± 15.26 which was significantly higher than that of control group 3.54 ± 1.40 (P value was 0.001) as shown in table 2 and out of 40 cases 18 (45%) were high positive results, 16 (40%) were positive and 6 (15%) cases were

negative as shown in table 3, and there was significant difference between the level of both Bone Sialoprotein-1 and CD44 variant 6 in cases of good and bad prognosis (P value was 0.001) as shown in tables 4 and 5 (cases had good or bad prognosis results according to the response to chemotherapy) (Figs 1–3). As regard comparison between level of CD44 variant 6 and level of BSP-1 in cases of bad prognosis 22 (55%) of cases were positive for both BSP-1 and CD44 variant 6 and of bad prognosis (P value was 0.002) as shown in table 6.

Table.1 Measurement of bone sialoprotein-1 by ELISA

	Cases (n=40)	Control (n=10)	T test	P value
BSP-1 level by ELISA	112.69 ± 11.54 ng/mL	8.58 ± 2.19 ng/mL	28.192	0.001*

Table.2 Measurement of CD44 variant 6 by flowcytometry

	Cases (n=40)	Control (n=10)	T test	P value
CD 44 V 6 level by flowcytometry	32.18 ± 15.26	3.54 ± 1.40	8.645	0.001*

Table.3 Numbers and percentage of different levels of CD44 variant 6

CD 44 V 6	Cases (n=40)	Control (n=10)	X ²	P value
High positive (%)	18 (45%)	0 (0%)	26.562	0.001*
Positive (%)	16 (40%)	0 (0%)		
Negative (%)	6 (15%)	10 (100%)		

Table.4 Comparison between levels of bone sialoprotein-1 by ELISA in cases of good and bad prognosis

BSP-1 level by ELISA	Cases (n=40)	Control (n=10)	X ²	P value
Cases of good prognosis (%)	16 (40%)	10 (100%)	11.542	0.001*
Cases of bad prognosis (%)	24 (60%)	0 (0%)		

Table.5 comparison between level of CD44 variant 6 in cases of good and bad prognosis

CD 44 V 6 level by flowcytometry	Cases (n=40)	Control (n=10)	X ²	P value
Cases of good prognosis (%)	13 (32.5%)	10 (100%)	14.672	0.001*
Cases of bad prognosis (%)	27 (67.5%)	0 (0%)		

Table.6 Comparison between level of CD44 variant 6 and level of bone sialoprotein-1 in cases of bad prognosis

Cases of bad prognosis	Cases (n=40)	Control (n=10)	X ²	P value
Positive cases for CD 44 V 6	27 (67.5%)	0 (0%)	14.672	0.001*
Positive cases for BSP-1	24 (60%)	0 (0%)	11.542	0.001*
Positive cases for both BSP-1 & CD 44 V 6	22 (55%)	0 (0%)	9.821	0.002*

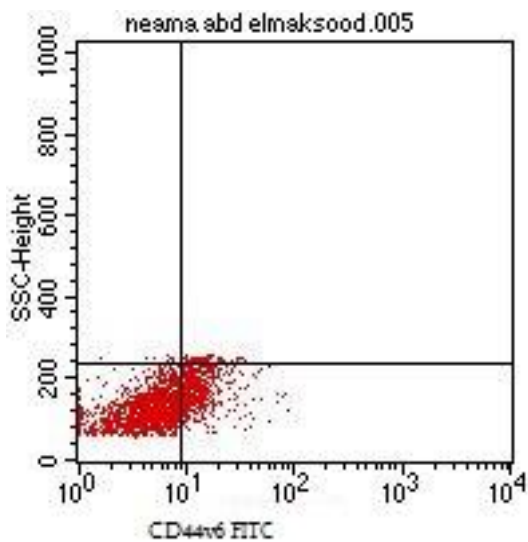


Fig.1 High positive cases for CD44 variant 6

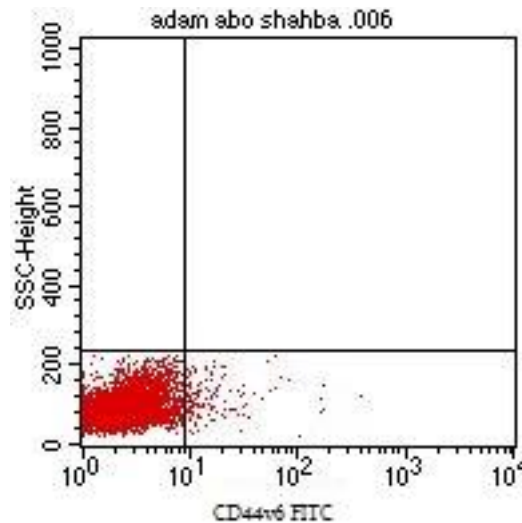


Fig.2 Negative cases for CD44 variant 6

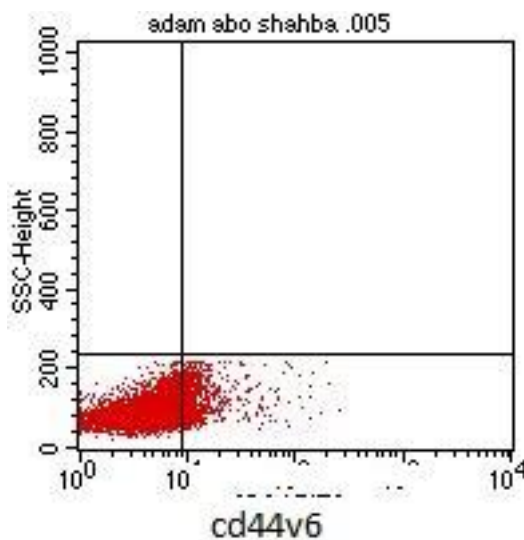


Fig.3 Positive cases for CD44 variant 6

One of the commonest childhood malignancy is Acute leukemia, with childhood acute lymphoblastic leukemia (ALL) being more common than childhood acute myeloid leukemia (AML). Usually the prognosis of childhood ALL is good, with about 80% cure rates. Despite this high cure rate relapse still occurs in 20-25 % of the cases (Khan *et al.*, 2008). These cases of relapse cannot be sufficiently assessed for their chance of experiencing relapse at diagnosis. For this assessment we need Immunophenotypic and cytogenetic prognostic markers for these patients and these markers also help us to divide cases into high and low risk patients Khan *et al.*, (2008). In our study there were 25 (62.5%) males and 15 (37.5%) females, these results were similar to the result of Wu *et al.*, (2010), Reichard *et al.*, (2011) and Stacy and Patrick (2015) who reported increase percentage of male than female in childhood ALL patients. The majority of patients with ALL were of B-lymphoid origin. The B lymphoblastic leukemia is classified as precursor B leukemia (B-ALL) since the blast cells are neoplastic counterparts of normal B-cell precursors Scott (2013). The classification into B- and T-ALL is important for risk stratification and treatment Abdollah *et al.*, (2014).

Immunophenotyping in our study were 50% of pre-B-ALL, 2.5% were mature B-ALL, early pre B 2.5%, Common B 35% (total: 90%) and 10% were T-ALL. This was agreed with the study done by Zhang *et al.*, (2012). In our study, as regard results of measuring BSP-1 levels by ELISA in patient group was 112.69 ± 11.54 ng/mL which was significantly higher than that of control group 8.58 ± 2.19 ng/mL (P value was 0.001) these results agreed with Wang *et al.*, (2011). Many studies reported that increased BSP-1 levels in a variety of malignancies suggested that it might be used as a prognostic marker in these patients as reported by Ang *et al.*, (2005). The

increased BSP-1 levels in such malignancies may be attributed to increased expression and secretion by leukemic blast cells Chagan-Yasutan *et al.*, (2011). These studies suggested that the expression of BSP-1 was related to the occurrence and development of acute leukemia. Cao *et al.*, (2012) reported that BSP-1 prevent cellular apoptosis and thus play an important role in growth, progression and metastasis of malignancies. In our study there was significant difference between level of both BSP-1 in cases of good and bad prognosis (P value was 0.001). This was agreed with Elli *et al.*, (2012) who found that BSP-1 level at the diagnosis may give more information on prognosis in childhood leukemias and lymphoma. As regard CD44 variant 6 level by flowcytometry in patient group was 32.18 ± 15.26 which was significantly higher than that of control group 3.54 ± 1.40 (P value was 0.001) and there was significant difference between level CD44 variant 6 in cases of good and bad prognosis (P value was 0.001). This was in agreement with Bendall *et al.*, (2004), Kyueng *et al.*, (2011) and Todaro *et al.*, (2014) who reported That CD44v6 expression is correlated directly with bad prognosis. As regard comparison between level of CD44 variant 6 and level of BSP-1 in cases of bad prognosis 22 (55%) of cases were positive for both BSP-1 and CD44 variant 6 and of bad prognosis (P value was significant equal to 0.002) and these results were in agreement with Liu *et al.*, (2006) who reported that the level of both of BSP-1 and its receptor CD44 variant 6 were significantly related to cases of bad prognosis, tend to be positive in these cases and thus can predict the bad outcome of cases of childhood ALL.

BSP-1 level and CD44v6 expression in childhood ALL patients may be used as prognostic markers to predict outcome as it has a tendency to be high with cases of bad prognosis.

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