

CD44 Expression and Soluble CD44 as a Potential Marker of Tumor Load in Pediatric Acute Leukemia

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ABSTRACT

Background: CD44 standard transcript (CD44s) is known to be widely expressed on ANLL and in a high percentage of ALL where it is reported to correlate with tumor burden. Different isoforms have been reported to be expressed on ANLL and/or ALL with various impact on prognosis. In the present study the expression of CD44s and its variant isoforms CD44v4, v6 and v7/8 were analyzed in 76 newly diagnosed children with acute leukemia and correlated with immunophenotyping, TLC and tumor burden.

Patients and Methods: The cases comprised 62 ALL and 14 ANLL cases, ALL cases included 42 males and 20 females with an age range of 1 to 17 years, a mean of 3.6 years and a median of 4 years. ANLL cases included 4 males and 10 females with an age range of 2 to 16 years, a mean of 9 years and a median of 9.5 years.

Results: CD44s was detected on all ANLL and 98.3% (61/62) of ALL cases tested. CD44v4 was encountered, probably for the first time, in 7/52 ALL and 1/14 ANLL; no other variants were encountered. Serum CD44 (sCD44) level was elevated in 25/35 ALL and in 11/14 ANLL with no correlation to CD44s expression.

Conclusion: CD44 variant isoform expression could be used as a marker for monitoring minimal residual disease (MRD) in positive cases. Serum CD44s levels can also be used as a tumor marker for follow up of patients and monitoring of therapy in ALL and ANLL cases independent of CD44 expression.

Key Words: CD44 - CD44v - Adhesion molecules - Acute leukemia.

INTRODUCTION

The CD44 antigen is a highly glycosylated transmembrane protein that displays many variant isoforms (CD44v) generated by alternative splicing of exons 2v to 10v [1]. It is also named

H-CAM (Homing Associated Cell Adhesion Molecule). CD44 standard transcript known as CD44s is located on the short arm of human chromosome 11 [2]. It is formed of 50 to 60 kb of genomic DNA and contains at least 20 exons. CD44s is known to be expressed on all hematopoietic cell lineage and accordingly it is known as hematopoietic CD44 (CD44H) [3]. However, the level, type and ligand binding ability of CD44 normally varies according to the state of proliferation and/or activation of the cell [4].

Though leukemia is disseminated from the very beginning, yet expression of CD44 as an adhesion molecule may help the by passing leukemic cells to lodge and proliferate in different tissues. CD44 has been reported to be expressed on almost all ANLL and in about 77% of ALL [5-9]. Marked variability in the expression of isoforms was encountered by various authors. Upregulated CD44-10v is observed in ANLL [10]. Legras S. et al., 1998 [11], gave evidence that a strong expression of CD44-6v correlates with shorter survival of ANLL patients treated with conventional chemotherapy.

Upon interaction with its natural ligand, or when ligated by specific antibodies, CD44 sheds from the cell surface and is released as a soluble molecule that lacks the transmembrane portion and the cytoplasmic tail, thus displaying a molecular weight lower than that of the cell membrane molecule [12].

Soluble serum CD44 has been detected and measured in serum and other biological fluids, e.g. synovial fluid and its level increases in the

course of immune activation and inflammatory conditions [13]. Elevated serum concentrations of CD44s and CD44v have been demonstrated with gastric and colon cancers [14].

In the present study, we studied the expression of CD44s and its variants as well as soluble serum CD44 in pediatric acute leukemia. The aim was to study the pattern of expression of CD44s and its variants in ALL and ANLL and to study if its soluble serum level (sCD44) can serve as a potential tumor marker.

MATERIAL AND METHODS

This work was performed on 76 newly diagnosed children with acute leukemia, including 46 males and 30 females with an age range of 1 to 17 years. Sixty-two patients were diagnosed as ALL of which 42 were males and 20 were females. The ANLL cases were 14, 4 of them were males and 10 were females. Their age ranged from 2 to 16 years. All patients presented to the Pediatric Oncology Unit and the Clinical Pathology department, National Cancer Institute (NCI), Cairo University during the period between June 1999 and the end of February 2000. All patients were diagnosed according to standard methods including; detailed history, thorough clinical examination, complete blood picture, bone marrow examination and cytochemical stains. Patients were evaluated for the following:

- Symptoms of bone marrow infiltration (failure) including fatigue, pallor headache and leucopenia fever which result from infection and symptoms of thrombocytopenia including petechiae, purpura, epistaxis and easy bruising.
- Signs of lymphoid system invasion i.e. lymphadenopathy, hepatomegaly and splenomegaly and signs of extramedullary invasion i.e. CNS involvement, renal involvement and testicular involvement in males.

Immunophenotyping was carried out on mononuclear cells from peripheral blood or bone marrow using fluorescently labeled monoclonal antibodies (Mo Abs). Double marker labeling was performed, including proper isotype controls. The Mo Abs included: CD1a, CD2, CD3, CD4, CD5, CD7, CD8, as T markers, CD19, CD20, CD22, CD24, Cytoplasmic μ , kappa and Lambda as B markers, CD13, CD14, CD33 and myeloperoxidase as myeloid markers, CD16 & CD56 as natural killer markers and other markers as CD10, CD34 & HLA DR. Fluorescently labeled

Mo Abs against standard human H-CAM (CD44), CD44v4, CD44v6 & CD44v7/8 adhesion molecule were included in the Mo Abs panel. All Mo Abs and isotypic controls were obtained from Serotec (UK). For detection of surface markers, the whole blood staining method was performed using Coulter Epics system (Coulter Corporation, Hialeah). In short 10 μ l whole blood, incubated in the dark for 20 minutes, processed by the Q prep system where immunoprep reagent A for lysing, B as stabilizer and C as fixative were consecutively added. The samples were then analyzed on the flow cytometer and cases were assigned to different immunophenotypes as previously described (Kamel et al., 1989) [15]. The reactivity was detected using XL-MCL flow cytometer (Coulter Corporation, Hialeah). For CD44 markers expression an arbitrary cut off value of 20% expression was used to categorize the patients into a group with low expression (< 20%) and a group with high expression (> 20%). Assay of serum CD44 was done using Quantitative Diaclone Elisa Kit (solid phase sandwich Enzyme Linked Immunosorbent Assay). Fifteen healthy people of matched age and sex were included as control for serum CD44.

Statistical analysis: Chi-square and fisher exact tests were used to test proportion independence. Kruskal Wallis ANOVA was used for comparison of means of independent groups.

RESULTS

This work was carried out on 76 newly diagnosed patients including 46 males and 30 females with an age range of 1 to 17 years a mean of 6.4 years and a median of 5.5 years. Sixty-two patients (81.5%) were diagnosed as ALL of which 42 were males (60.5%) and 20 were females (26.3%). The age range of the ALL cases was from 1 to 17 years with a mean of 3.6 years and a median of 4 years. The ANLL cases were 14, 4 of them were males (28.6%) and 10 were females (71.4%). Their age ranged from 2 to 16 years with a mean of 9 years and a median of 9.5 years.

Acute lymphoblastic leukemia:

This group included 62 patients. Lymphadenopathy was encountered in 43/62 (71.6%), huge generalized lymph nodes in 11/62 (17.7%), hepatomegaly in 52/62 (83.8%), splenomegaly in 45/62 (72.5%) and massive tumor burden

(expressed as mediastinal mass, organomegally and CNS infiltration) was detected in 29/62 (46.7%) ALL patients. Fever was recorded in 43/59 (72.9%) at presentation and bleeding tendency was observed in 25/62 (40.3%) cases. Eleven of 54 (20.4%) patients had CNS involvement and 13/50 (26%) patients showed mediastinal involvement.

The TLC ranged from 1.0×10^9 to $603.2 \times 10^9/l$. Twenty one of 62 cases (33.3%) had $TLC \leq 10 \times 10^9/l$, 20/62 (32.2%) cases had TLC ranging from $10 \times 10^9/l$ to $50 \times 10^9/l$ and 22/62 (35.5%) cases had a $TLC \geq 50 \times 10^9/l$. Four patients out of 62 (6.5%) had hemoglobin level > 10 g/dl and 34/62 (54.5%) had hemoglobin level < 7 g/dl. According to FAB classification the cases included 9 L1, 47 L2, 2 L3 and 4 cases remained uncharacterized. Table (1) shows the immunophenotypic classification of the studied ALL cases.

CD44s expression was encountered on all 22 cases (100%) with $TLC \geq 50 \times 10^9/l$ and on 34/40 (85%) patients with $TLC < 50 \times 10^9/l$ (Table 2). No significant association between tumor burden, immunophenotype and mediastinal involvement on one side and CD44s expression on the other side was encountered.

No significant correlation was detected between CD44s expression and other prognostic factors: Hb ($p = 0.65$), CSF involvement ($p = 0.68$), bone marrow (BM) at day 15 ($p = 0.85$) and CD34 expression ($p = 0.14$).

On evaluating the expression of CD44 variant isoforms (v4, v6, v7/v8) on ALL cases, CD44v4 was expressed on 7/49 (14.3%) of the cases (Fig. 1) and no expression of the other isoforms was detected. Significant association of CD44v4 expression was encountered with tumor burden ($p = 0.013$), TLC and immunophenotype but not with mediastinal involvement (Table 3). Cases with $TLC \geq 50 \times 10^9/l$ showed expression of CD44v4 in 5/19 (26.3%) as compared to 2/33 (6.1%) in patients with $TLC < 50 \times 10^9/l$ (p value = 0.05). Significant correlation between CD44v4 and immunophenotyping was encountered ($p = 0.002$), as patients with T-cell phenotype showed high expression of CD44v4.

No significant correlation was detected between CD44v4 and sex ($p = 0.4$), Hb ($p = 0.35$), CSF involvement ($p = 0.9$), BM day 15 ($p = 0.48$) and CD34 expression ($p = 0.12$).

Acute myeloid leukemia:

This group comprised 14 patients including 4 M2, 3 M4, 2 M5, 1 M6 and 4 uncharacterized. Eight patients showed hepatosplenomegaly, two of them showed lymphadenopathy. Fever was recorded in 9/13 (69.2%) and bleeding tendency was observed in 7/14 (50%) ANLL patients. Two patients of twelve (16.7%) had CNS involvement. Mediastinal involvement was detected in 1/12 (8.3%). TLC ranged from 6.7 to $160.7 \times 10^9/L$.

CD44s was highly expressed on all AML cases, but there was no significant association with any of the prognostic parameters such as sex, age, organomegally, TLC, FAB classification, CNS involvement, response to treatment or CD34 expression. Considering the expression of CD44 variant isoforms, none of the ANLL cases examined revealed the expression of CD44v6, v7/v8 isoforms.

CD44v4 was expressed in one of twelve cases tested. The patient was a 12 years old female, presented with liver of 6 cm in mid clavicular line (MCL), spleen of 8 cm. She was classified as M4. The patient did not show remission after first course of induction chemotherapy.

Serum CD44:

Serum level of CD44 was evaluated in 35 ALL cases and 14 AML cases as well as 15 healthy controls. The level in the control group ranged from 8.3 to 12.6 ng/ml with a mean of 10.24 ± 1.39 . In ANLL it was elevated in 11/14 patients (78.5%) with a range of 8.84 to 21.7 ng/ml and a mean of 15.57 ± 4.47 and was significant as compared to the control ($p = 0.045$). In ALL cases it was elevated in 25/35 patients (71.4%) and ranged from 5.95-26.56 ng/ml with a mean of 16.31 ± 5.99 and was significant as compared to the control ($p = 0.005$).

Table (1): Immunophenotype of ALL cases.

IPT	Frequency	Percentage
T-cell	18	29
<i>Precursor B:</i>	42	67.6
Pre B	23	37
Common ALL	14	22.5
Pro B	5	8.1
Mature B	1	1.6
Mixed*	1	1.6

* Expressing both T (CD7 and CD2) and B (CD19 and CD10) associated markers.

Table (2): CD44s expression in ALL in relation to some prognostic parameters.

Parameter	No.	High expression of CD44s	Low expression of CD44s	p-value
<i>Tumor burden:</i>				
High	29	28 (96.6%)	1 (3.4%)	0.13
Low	33	28 (84.8%)	5 (15.2%)	
<i>TLC:</i>				
≥ 50.000	22	22 (100%)	—	0.06
< 50.000	40	34 (85%)	6 (15%)	
<i>Immunophenotyping*:</i>				
T cell	18	18 (100%)	—	0.13
Precursor B	38	33 (86.8%)	5 (13.2%)	
<i>Mediastinal involvement**:</i>				
Involved	13	13 (100%)	—	0.14
No involvement	36	30 (83.3%)	6 (16.7%)	

p value is significant if < 0.05.

* Association of immunophenotyping with high expression of CD44s (> 20%) and low CD44s expression (< 20%) was done on 56 cases which were positive for CD44s expression.

** Mediastinal involvement in association with high & low expression was done on only 49 patients who were examined by chest X-ray.

Table (3): CD44v4 expression in relation to some prognostic parameters.

Parameter	No.	Positive expression of CD44v4	Negative expression of CD44v4	p-value
<i>Tumor burden:</i>				
High	21	6 (28.6%)	15 (71.4%)	0.013
Low	31	1 (3.2%)	30 (96.8%)	
<i>TLC:</i>				
≥ 50.000	19	5 (26.3%)	14 (73.7%)	0.05
< 50.000	33	2 (6.1%)	31 (93.9%)	
<i>Immunophenotyping*:</i>				
T cell	14	6 (42.9%)	8 (57.1%)	0.002
Precursor B	33	1 (2.7%)	32 (88.9%)	
<i>Mediastinal involvement:</i>				
Involved	11	3 (27.3%)	8 (72.7%)	0.1
No involvement	31	2 (6.5%)	29 (93.5%)	

p value is significant if < 0.05.

* Two patients, one with a mature B phenotype and the second with mixed B and T phenotype, did not demonstrate CD44v4 expression and were not included in the statistical evaluation.

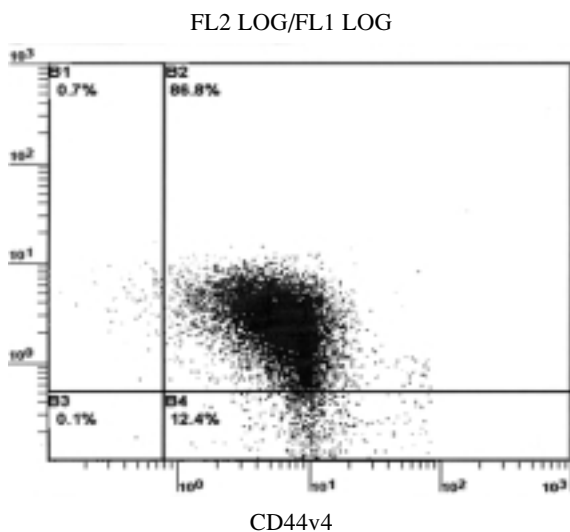


Fig. (1): An ALL case coexpressing CD44s and CD44v4.

DISCUSSION

Acute leukemia constitutes 97% of all childhood leukemias. Acute lymphoblastic leukemia is the most common malignancy in children. In the present study sixty-two out of 76 patients (78.9%) were diagnosed as acute lymphoblastic leukemia (ALL) and fourteen (18.4%) as acute myeloid leukemia (AML). All of them were newly diagnosed cases with no prior therapy.

In the present study, different clinical and laboratory findings are in concordance with other studies conducted on Egyptian children with ALL. Hepatomegaly was encountered in 52/62 (83.9%); splenomegaly in 45/62 (75.6%); mediastinal involvement in 13/20 (26%) and TLC ≥ 50x10⁹/L in 22/62 (35.5%). On the other hand,

there was a difference in the incidence of lymphadenopathy (43/62; 69.3%) as compared to 85% in other studies [16-20]. However, our study was done on a limited number of cases and large number of patients are needed to be compared with previous studies. As previously reported [21-23], the clinical findings in the Egyptian population are different from those reported in Western countries, where a lower incidence of hepatosplenomegaly (60%), lymphadenopathy (50%) [21] and mediastinal involvement (10% and 7.9%) [22,23] as well as a lower percentage of patients with TLC $\geq 50 \times 10^9/L$ (17%) [24] were encountered.

This high TLC was thought to be attributed to the higher incidence of T-cell phenotype among Egyptian patients which was reported earlier [15] and/or very late presentation. However, this high total leukocytic count was not confined to T-cell phenotype in our patients and it occurred in association with all other phenotypes even common ALL [25]. This signifies that a higher number of Egyptian patients present with TLC of poor prognostic implication.

In the present study, immunophenotyping showed that T-cell was encountered in 30.5% of cases while precursor B constituted 66.1%. These figures are consistent with the current pattern of immunophenotypes recorded at NCI and previously documented [25]. Original reports on Egyptian children showed a high incidence of T phenotype ($> 50\%$) [15,26,19]. An appreciable shift in the phenotype towards a relatively higher incidence of precursor B lineage has apparently occurred in our population as it was previously reported in other populations [27]. However, our current figures are still not comparable to western countries where the precursor B phenotype constitutes $> 80\%$ of cases especially in children [22,28].

In the present study all acute lymphoblastic leukemia cases expressed CD44s, with 56/62 (90.3%) cases showing high expression and 6/62 (9.7%) cases showing low expression. Our figures are slightly higher than Cavalcanti et al. (1994) [8] and Geraldo et al. (1997) [9] who encountered CD44s expression in 77% and 76.8% of ALL cases respectively.

In this work, the high expression of CD44s was associated with high tumour burden (i.e. mediastinal mass, organomegaly and CNS in-

filtration) but not with other bad prognostic parameters (high TLC, T cell phenotype, +ve BM at day 15 and CD34 expression) A finding that may recommend CD44 expression to be considered as a marker for tumor burden. CD44 being an adhesion molecule, may be involved in the pathogenesis of a high tumor burden, where it might facilitate lodgment of passing by leukemic cells in different tissues, allowing them to proliferate.

Contrary to the wide expression of CD44s in ALL, the variant isoforms CD44v6, CD44v7/8 were not expressed on any of our cases with the exception of CD44v4 which was expressed on 7/49 of the cases (14.3%). Different workers reported that CD44v isoforms are not expressed on normal resting PBLs or BM cells and are upregulated only on stimulation [7,29,30,31]. Therefore, CD44v4 detection could be used as a marker of malignancy as well as, for monitoring minimal residual disease (MRD) in positive cases. In our series, CD44v4 isoform expression on ALL cases was significantly associated with high tumor burden, TLC $\geq 50,000/mm^3$ and T-cell immunophenotype ($p = 0.01, 0.05$ and 0.05 respectively). The association of CD44v4 isoform expression with high tumor burden may be explained by the findings that CD44 variant isoforms expression is known to confer an *in vivo* metastatic ability in experimental models [32].

In the present study, CD44s was highly expressed on all ANLL cases (14 cases) tested, similar findings were previously reported [5-7]. On the other hand, the expression of CD44 variants were different in our series; CD44v4 was detected in only 1/14 (7.1%) of these cases, none of the ANLL cases examined revealed the expression of CD44v6, v7/8 isoforms. Yokota et al. (1998) [6] reported the expression of different isoforms, namely CD44v6, v7/8, v9 and v10, but neither v4 nor v5. Bendall et al. (2000) [7] working on 30 ANLL patients, reported expression of v3, v6, v7, v8, v9 and v10 in 62, 70, 21, 83, 71 and 92% respectively; v4 and v5 were not detected. This shows agreement on the expression of CD44s in ANLL, yet a clear difference can be observed in the pattern of CD44 variant isoform expression which might be attributed to a biologically different disease entities in different patients or to the small number of cases studied in different series. Different techniques used to detect CD44 variants may also

contribute to such discrepancy; as in Yokota et al., they examined the expression of CD44v mRNA by semiquantitative RT-PCR and in Bendall et al., they used RT-PCR and Southern blotting while surface expression was evaluated in the present work.

In this work serum level of CD44 was elevated in 25/35 ALL patients (71.4%) compared to the control group, with no significant correlation between its level and any of the standard prognostic parameters. This finding is in concordance with Takeuchi et al. (1999) [33], Yokota et al. (1999) [34] and Tacyildiz et al. (2001) [35] who reported that the mean serum concentration of sCD44 in patients with ALL at presentation was significantly higher than in control subjects. They also reported that after treatment the serum CD44 levels significantly decreased, but was still higher than in normal subjects. Yokota et al. [34] also reported that serum CD44 levels increased in patients during relapse which makes it a useful marker to monitor disease progression.

In our ANLL patients, 11/14 patients (78.5%) showed increased level of sCD44 as compared to the control group. Nasu et al. (1998) [36] reported that sCD44 level was significantly elevated in patients with acute myeloid leukemia as well as in ANLL on top of myelodysplastic syndrome (MDS). On the other hand, Tacyildiz et al. (2001) [35] reported that there was no significant difference between ANLL and control group, but they concluded that a high serum level at diagnosis in acute leukemias and malignant lymphoma is associated with worse prognostic criteria and/or unfavourable outcome.

In conclusion, CD44s may be considered as a marker for tumor burden. CD44v4 was encountered, probably for the first time, in 7/52 ALL and 1/14 ANLL, so CD44v4 isoform expression could be used as a marker for monitoring minimal residual disease (MRD) in positive cases. Serum CD44 levels may also be used as a tumor marker for follow up of patients and monitoring of therapy in ALL and ANLL cases independent of CD44 expression.

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