Cluster of Differentiation 96 in Children with Acute Leukemia: A Single Center Cohort Study

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Received: 27 March 2019 / Accepted: 3 June 2019 / Published online: 5 June 2019
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Abstract Cluster of differentiation 96 (CD96) is an important leukemic stem cells (LSCs) surface marker. We evaluated CD96 expression in children with acute leukemia (AL) and described its relation with treatment response. We conducted a prospective cohort study in Mansoura University Children's Hospital, Egypt during the period from 2014 to 2016. We studied 96 children with AL and 96 controls at clinical, laboratory and radiological levels. We assessed CD96% in LSCs using flow cytometry. AL group included 59 acute lymphoblastic leukemia (ALL) and 37 acute myeloid leukemia (AML) patients. ALL subgroup involved 44 B-ALL and 15 T-ALL patients while AML subgroup included 17 M2, 12 M4 and 8 M5 patients. CD96% was higher in AL group [57.63 (21.18–89.93)] than control [34.12 (16.15–39.51)] ($P < 0.001$). CD96% was higher in AML [68.25 (31.1–89.86)] than ALL [54.18 (21.18–89.93)] ($P < 0.001$). CD96% in AML was M4 > M2 > M5 ($P = 0.04$) while within ALL subgroup, no significant difference was found between B-ALL and T-ALL ($P = 0.807$). CD96% in patients with non-complete remission was higher than those with complete remission

($P = 0.004$). CD96 is a reliable diagnostic marker for AL mainly AML and could be used as a prognostic marker for treatment response.

Keywords Acute leukemia · Children · CD96 · Prognostic marker · Remission

Abbreviations

AL	Acute leukemia
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
BM	Bone marrow
CD	Cluster of differentiation
CR	Complete remission
DAPK	Death Associated Protein Kinase
EDTA	Ethylenediaminetetraacetic acid
FAB	French–American–British
FACS	Fluorescence-activated cell sorting
LSCs	Leukemic stem cells
MAL	Mixed lineage acute leukemia
NCR	Non-complete remission
NK	Natural killer
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
RBCs	Red blood cells
SE	Standard error

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Introduction

Identification of cell surface markers on leukemic stem cells (LSCs) has attracted much attention, allows better understanding of the molecular basis of acute leukemia

(AL) [1, 2] and aids in predicting prognosis [3]. Among these markers, cluster of differentiation (CD) 96 is expressed mainly on cells of hematopoietic origin, in particular to natural killer (NK) and T cells [4, 5].

CD96 was described initially as “T cell activation, increased late expression” [4]. It comprises three domains (V1, V2/C and C) in the extracellular region. CD96V1 domain contributes in cellular adhesion and NK cell-mediated killing activities [6]. It is considered a tumor marker for acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL) [7], and might assume a role in adhesion of LSCs to BM compartments [8].

Despite several studies were conducted about CD96 in AL, its clinical significance is not yet fully described in children. From this point, we investigated CD96 expression in LSCs of BM in children with newly diagnosed AL and described its expression among children who achieved complete remission (CR) and those with non-complete remission (NCR).

Materials and Methods

Design and Participants

We conducted a prospective cohort study from November 2014 to April 2016 enrolling 96 children with AL (AL group) and 96 normal BM samples as controls. Patients were recruited from inpatient wards and outpatient clinics of Mansoura University Children's Hospital, Egypt. We included children with newly diagnosed AL (BM blast > 25% for ALL and > 20% for AML). Patients subtyping was done according to French-American-British (FAB) classification and immune-phenotyping.

Patients were subjected to clinical and history evaluation by pediatric residents. We collected data about age, sex, fatigue (not improved with rest and interferes with daily activities), pallor, jaundice, bleeding tendency, unexplained weight loss ($\geq 5\%$ over 6–12 months), unexplained fever, bone aches, coughing or difficult breathing, lymphadenopathy, arthritis and hepatosplenomegaly. We accessed patients' files for radiological evidence of lung infiltrates, mediastinal masses, pleural effusion and para-aortic lymph nodes.

We defined the complete remission as $\leq 5\%$ BM blasts (irrespective of cellularity) without Auer rods, a platelet count of $\geq 100,000/\mu\text{l}$ and absolute neutrophil of $> 1000/\mu\text{l}$ without extra-medullary leukemia after induction chemotherapy. When the patients had 5–25% BM blasts with $\geq 50\%$ decrease in blasts and peripheral blood count recovery after induction chemotherapy or relapsed in 6 months after the first CR, they were labeled as NCR [9–11].

Flow Cytometry Analysis

In our genetic unit, 3 ml of BM sample (for each participant) was analyzed on the FACS Aria III flow cytometer (BD Biosciences, Sunnyvale, California, USA). Red blood cells (RBCs) were lysed on ice with 10 ml lysis buffer (10 mmol/l KHCO_3 , 155 mmol/l NH_4Cl , 0.1 mmol/l Na_2 and EDTA), and washed with the phosphate-buffered saline (PBS). We added 10 μl of CD96 phycoerythrin, and let the samples in the dark for 30 min at 4 °C. The samples were lysed with a FACS lysing solution for 10 min, washed with PBS for 5 min at 4 °C and finally, the cell pellets were re-suspended in 300 μl PBS. Histograms were used to show the results using the FACS Diva software [9].

Statistics

We used SPSS version 23. Kolmogorov–Smirnov test was used to test continuous data for normality. Non-parametric data were expressed as medians and ranges (minimum–maximum); and analysed using Mann–Whitney and Kruskal–Wallis tests. Categorical data were expressed as numbers and percent; and analysed using Chi square, Fisher's exact and Monte Carlo exact tests. $P \leq 0.05$ was considered significant.

Results

Table 1 shows classification of the study participants. AL and control groups were matched as regards age [6.5 years (2–17) and 5.5 years (2–17), respectively, $P = 0.081$], and sex ($P = 0.233$).

Unexplained fever, weight loss, bleeding tendency and lymphadenopathy were more frequent in ALL than AML

Table 1 Classification of the study participants

Study participants	<i>n</i> (%)	Gender M/F
Control group	96	56/40
AL	96 (100)	64/32
AML	37 (38.5)	25/12
M2	17 (17.7)	11/6
M4	12 (12.5)	8/4
M5	8 (8.3)	6/2
ALL	59 (61.5)	39/20
B-ALL	44 (45.8)	30/14
T-ALL	15 (15.6)	9/6

Data were expressed as numbers and percent

AL acute leukemia, ALL acute lymphoblastic leukemia, AML acute myeloid leukemia, F female, M male, *n* number

group ($P = 0.05$, 0.011 , 0.011 and ≤ 0.001 respectively, Table 2). Actually the smaller number of AML relative to ALL cases and absence of certain subsets of AML during the study period explain the more frequent bleeding tendency among ALL group.

CD96% was higher in AL group [57.63% (21.18–89.93)] than controls [34.12% (16.15–39.51)] ($P < 0.001$); and higher in AML [68.25% (31.1–89.86)] than ALL patients [54.18% (21.18–89.93)] ($P < 0.001$) (Mann–Whitney test).

Within AML subgroups, CD96% was higher in M4 [71.03% (37.85–82)] $>$ M2 [70.34% (60.59–89.86)] $>$ M5 [49.68% (31.1–68.25)] subtypes ($P = 0.04$, Kruskal–Wallis test). While within ALL subgroup, no statistically significant difference was found between B-ALL [54.18% (21.18–89.93)] and T-ALL patients [52.24% (50.37–59.64)] as regards CD96% ($P = 0.807$, Mann–Whitney test).

Performance analysis of CD96 within AL and control groups showed a sensitivity of 83.33% and a specificity of 100% for AL with a positive performance value of 100% and

a negative performance value of 85.7% (Fig. 1).

CD96% in patients with NCR (15 ALL and 29 AML) was higher than patients with CR (44 ALL and 8 AML) [59.64% (25.3–89.8) and 54.18% (21.18–89.93), respectively] ($P = 0.004$, Mann–Whitney test).

Fig. 1 Roc curve illustrates performance analysis of CD96% within children with acute leukemia and controls

Discussion

Increasing attention has been paid for LSCs biomarkers in AL [9, 12–14]. In the current study, the higher CD96 expression among AL patients is consistent with Du et al. [9] and Wu et al. [15] (Table 3). In our study, the relatively

Table 2 Descriptive data of acute leukemia (AL) subgroups

Clinical and radiological data ^d	AL patients (total = 96)		<i>P</i>
	ALL (<i>n</i> = 59) <i>n</i> (%)	AML (<i>n</i> = 37) <i>n</i> (%)	
Unexplained fever	28 (47.5)	25 (67.6)	0.05 ^a
Bone aches	28 (47.5)	12 (32.4)	0.146 ^a
Pallor	44 (74.6)	24 (64.9)	0.308 ^a
Unexplained weight loss	20 (33.9)	4 (10.8)	0.011 ^a
Bleeding tendency	20 (33.9)	4 (10.8)	0.011 ^a
Significant lymphadenopathy	35 (59.3)	8 (21.6)	$\leq 0.001^a$
Fatigue	43 (72.9)	28 (75.7)	0.761 ^a
Jaundice	4 (6.8)	4 (10.8)	0.707 ^b
Arthritis	4 (6.8)	0	0.157 ^b
Pleural effusion (chest X-ray)	3 (5.1)	0	0.282 ^b
Hepatosplenomegaly	36 (61)	16 (43.2)	0.208 ^c
Para-aortic LNs (abdominal ultrasonography)	12 (20.3)	8 (21.6)	0.88 ^a
Mediastina mass (chest X-ray)	15 (25.4)	9 (24.3)	0.904 ^a
Lung infiltration (chest X-ray)	4 (6.8)	4 (10.8)	0.707 ^b

AL acute leukemia, ALL acute lymphoblastic leukemia, AML acute myeloid leukemia, *n* number

^aData were expressed as numbers and percent; and analyzed by Chi square test

^bFisher's exact test

^cMonte Carlo exact test

^dCategories are not mutually exclusive

Table 3 Various studies about CD96 expression in acute leukemia

References	Studied groups	Methodology for detection of CD96 expression	Study outcomes
Du et al. [9]	105 AL (87 AML, 15 ALL and 3 MAL) and 15 healthy control	CD34/CD38/CD96 expression in LSCs of AL patients using FCM	CD96 expression was higher in CD34 ⁺ CD38 ⁺ LSCs of AL patients than healthy control ($P < 0.01$); higher in AML than ALL ($P < 0.05$) with insignificant differences among AML subtypes ($P > 0.05$); and inversely associated with chemotherapy response
Wu et al. [15]	91 AL (59AML, 21 B-ALL, 11 T-ALL) and 15 healthy control	CD96 expression on CD45 ⁺ CD34 ⁺ CD19 ⁺ CD7 ⁺ CD38 ⁺ in bone marrow mononuclear cells using FCM	CD96 expression was higher in AL patients than control ($P < 0.05$); higher in T-ALL than B-ALL ($46.98 \pm 45.55\%$ vs. $17.41 \pm 27.97\%$); and also associated with chemotherapy resistance, relapse and progression
Wang et al. [12]	69 AL children	CD96 expression on CD34 ⁺ CD38 ⁺ CD123 ⁺ LSCs of AL patients using FCM	CD96 expression was higher in AML than ALL ($P < 0.05$). CD96 ⁺ children had lower CR; higher infection and relapse rates ($P < 0.05$)
Jiang et al. [14]	156 AML (27 M1, 35M2, 25 M3, 40 M4 and 29 M5)	CD33, CD96, and DAPK expression was analyzed in CD34 ⁺ CD38 ⁺ CD123 ⁺ LSC using FCM	CD96 was expressed in 50.6% of AML cases (79/156) (M5 > M4 > M2 > M3 > M1). High CD96 expression was associated with higher relapse, lower response to chemotherapy and a lower survival rate ($P = 0.001$, < 0.001 and 0.03 , respectively)
Hosen et al. [17]	29 AML and 3 control	CD96 expression was analyzed by the signal sequence trap PCR screening method and FCM in Lin/CD34/CD38/CD90 BM cells	CD96 was expressed on the majority of Lin ⁺ CD34 ⁺ CD38 ⁺ CD90 ⁺ AML blasts; expressed in AML M2 subtype more than other subtypes; and represented a candidate therapeutic target
Hailiqiguli and Yan [18]	13 AML, 13 B-ALL and 9 T-ALL	The immuno-phenotype of LSCs was described in vitro in culture by FCM	12 patients died (9 CD96 positive and 3 CD96 negative patients) ($P < 0.05$)
Yabushita et al. [3]	80 de novo AML	CD25, CD96, and CD123 expression in LSCs (historical cohort study)	Multiple LSC marker expression was significantly associated with shorter survival compared with a single marker expression ($P < 0.001$)

AL acute leukemia, ALL acute lymphoblastic leukemia, AML acute myeloid leukemia, B-ALL acute B lymphoblastic leukemia, CR complete remission, DAPK Death Associated Protein Kinase, FCM flow cytometry, LSCs leukemic stem cells, MAL mixed lineage acute leukemia, PCR polymerase chain reaction, T-ALL acute T lymphoblastic leukemia

larger number of healthy controls together with the high specificity of CD96 for AL give a stronger evidence about the association of CD96 with AL. Assuming CD96 role in adhesion of malignant cells to BM compartments, targeted elimination of CD96⁺ LSCs via monoclonal antibodies may be a promising therapeutic approach [16].

Moreover, our study proved a higher CD96% in AML than ALL confirming the clinical implication of CD96 as a tumor marker in AML [9, 12]. Its expression among AML subtypes was M4 > M2 > M5. On reviewing the literature, the results were conflicting [9, 14, 17]. While in ALL patients, although CD96 expression was higher in B-ALL than T-ALL, yet no statistically significant difference was detected. This was in contrast to Wu et al. [15] (Table 3). These discrepancies may be explained by the differences in the study design and sample size.

Also, the higher CD96% among NCR patients supports the association between high levels of this surface marker and poor prognosis in AL patients. This copes with Du

et al. [9], Wang et al. [12], Jiang et al. [14], Wu et al. [15] and Hailiqiguli and Yan [18] (Table 3).

Interestingly, we reported a case with symptoms of AL (fever, fatigue, weight loss, lymphadenopathy and splenomegaly) with initial BM blasts of 2% and high CD96 (77.61%). Eight months later, this patient developed pleural effusion, re-evaluated and finally diagnosed as AML (M2). The patient died shortly after starting chemotherapy. This case report opens the gate for a potential early predictor value of CD96 in AL.

Conclusions

CD96 is a specific diagnostic marker for pediatric AL (mainly AML) and has a prognostic role. The role of CD96 as an early predictor for AL needs more attention. We recommend large scale studies using an extended panel of LSCs surface markers taking in consideration minimal residual disease.

Study Limitations A relatively small sample size, lack of correlations between CD96 and neither immuno-phenotypic markers nor cytogenetic/molecular abnormalities of AL, limited studied risk factors for treatment response and finally, a short follow-up period.

Acknowledgements We greatly acknowledge the patients and their families for their patience and help.

Authors' Contributions Hanan Mohammad: Collection of clinical data, revising the article draft and final approval of the version to be submitted. Yahya Wahba: Idea of the research, conception and design of the study, interpretation of clinical and laboratory data, drafting the article and final approval of the version to be submitted. Mona Gouda: Analysis of the laboratory data, revising the article draft and final approval of the version to be submitted. Ali Shaltout: Analysis of clinical and laboratory data, conception of the study, drafting the article and final approval of the version to be submitted.

Compliance with Ethical Standards

Conflict of interest Authors declared that they have no conflict of interest.

Ethical Standards All procedures performed in this study were in accordance with the ethical standards of the institutional research committee of Faculty of Medicine, Mansoura University, Egypt (Code No: MS/804) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed Consent Informed consents were obtained from all individual participants included in the study.

References

- Laranjeira ABA, Yang SX (2016) Therapeutic target discovery and drug development in cancer stem cells for leukemia and lymphoma: from bench to the clinic. *Expert Opin Drug Discov* 11:1071–1080
- Pollyea DA, Gutman JA, Gore L, Smith CA, Jordan CT (2014) Targeting acute myeloid leukemia stem cells: a review and principles for the development of clinical trials. *Haematologica* 99:1277–1284
- Yabushita T, Satake H, Maruoka H et al (2018) Expression of multiple leukemic stem cell markers is associated with poor prognosis in de novo acute myeloid leukemia. *Leuk Lymphoma* 59:2144–2151
- Wang PL, O'Farrell S, Clayberger C, Krensky AM (1992) Identification and molecular cloning of tactile. A novel human T cell activation antigen that is a member of the Ig gene superfamily. *J Immunol* 148:2600–2608
- Fuchs A, Cella M, Giurisato E, Shaw AS, Colonna M (2004) Cutting edge: CD96 (tactile) promotes NK cell-target cell adhesion by interacting with the poliovirus receptor (CD155). *J Immunol* 172:3994–3998
- Jiang W, Zhang S, Yan J, Guo N (2013) Expression, crystallization and crystallographic study of the 1st IgV domain of human CD96. *Chin J Biotechnol* 29:657–663
- Meyer D, Seth S, Albrecht J et al (2009) CD96 interaction with CD155 via its first Ig-like domain is modulated by alternative splicing or mutations in distal Ig-like domains. *J Biol Chem* 284:2235–2244
- Nodehi SM, Repp R, Kellner C et al (2012) Enhanced ADCC activity of affinity matured and Fc-engineered mini-antibodies directed against the AML stem cell antigen CD96. *PLoS ONE* 7:e42426
- Du W, Hu Y, Lu C et al (2015) Cluster of differentiation 96 as a leukemia stem cell-specific marker and a factor for prognosis evaluation in leukemia. *Mol Clin Oncol* 3:833–838
- Cheson BD, Bennett JM, Kopecky KJ et al (2003) Revised recommendations of the international working group for diagnosis, standardization of response criteria, treatment outcomes, and reporting standards for therapeutic trials in acute myeloid leukemia. *J Clin Oncol* 21:4642–4649
- Fleming S, Ong DM, Jackson K et al (2017) Partial response after induction chemotherapy has clinical relevance in acute myeloid leukaemia. *Br J Haematol* 177:328–330
- Wang XM, Yan M, Liu Y, Hailiqiguli NE (2013) CD96 expression on leukemia stem cells in 69 children with acute leukemia. *Chin J Contemp Pediatr* 15:633–637
- Ding Y, Gao H, Zhang Q (2017) The biomarkers of leukemia stem cells in acute myeloid leukemia. *Stem Cell Investig* 4:19
- Jiang Y, Xu P, Yao D, Chen X, Dai H (2017) CD33, CD96 and death associated protein kinase (DAK) expression are associated with the survival rate and/or response to the chemotherapy in the patients with acute myeloid leukemia (AML). *Med Sci Monit Int Med J Exp Clin Res* 23:1725–1732
- Wu Y, Xiao M, Zhu L et al (2011) CD96 expression on bone marrow mononuclear cells in 91 patients with acute leukemia. *Zhongguo shi yan xue ye xue za zhi* 19:585–588
- Staudinger M, Humpe A, Gramatzki M (2013) Strategies for purging CD96⁺ stem cells in vitro and in vivo: new avenues for autologous stem cell transplantation in acute myeloid leukemia. *Oncoimmunology* 2:e24500
- Hosen N, Park CY, Tatsumi N et al (2007) CD96 is a leukemic stem cell-specific marker in human acute myeloid leukemia. *Proc Natl Acad Sci* 104:11008–11013
- Hailiqiguli N, Yan M (2013) Immunophenotyping of leukemic stem cells and chromosome karyotype characteristics in Uyghur leukemia pediatric patients. *Chin J Oncol* 35:501–504

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