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Original article

Utility of BMI-1 and NANOG expression levels in survival prediction of pediatric acute lymphoblastic leukemia

Sara Abdel-khalek[a], *Layla M. Saleh*[b,c,*], *Sherin Abdel-Aziz*[b,c], *Ayman Hyder*[a], *Hasan Abdel-ghaffar*[b,c]

[a] *Faculty of Science, Damietta University, Damietta, Egypt*
[b] *Oncology Center, Mansoura University, Mansoura, Egypt*
[c] *Faculty of Medicine, Mansoura University, Mansoura, Egypt*

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ABSTRACT

Background: Acute lymphoblastic leukemia (ALL) is the most common malignancy in children characterized by the overproduction and accumulation of immature lymphoid cells in the bone marrow and peripheral blood. The BMI-1 is an important component of the Polycomb Repressive Complex-1 (PRC1). It is an important molecule for the self-renewal of hematopoietic stem cells (HSCs). The BMI-1 expression is generally high in HSCs and decreases after cell differentiation. The BMI-1 is required for the maintenance of normal and cancer stem cells and has been reported as an oncogene in various tumors. The NANOG is a homeodomain transcription factor responsible for maintaining the stem cell compartment at the blastocyst stage of developing embryos. The NANOG gene has been proven to be transcribed in CD34+ cells and different leukemic cells.

Methods: The ribonucleic acid (RNA) was extracted from the peripheral blood mononuclear cells (PBMNCs) of 30 pediatric ALL patients (16 B-ALL and 14 T-ALL) and 14 healthy controls. The Bmi-1 and NANOG expression levels were determined using the quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR).

Results: Compared to normal controls, patients with ALL exhibited upregulated levels of Bmi-1 ($p = 0.03$). Patients who overexpressed Bmi-1 and NANOG displayed a significantly worse survival than low-expressing patients (hazard ratio (HR) 5.74, 95% confidence interval (CI):1.48–22, $p = 0.012$ and HR 3.8, 95% CI:1.009–14.3, $p = 0.048$, respectively).

Conclusions: Taken together, these data suggest that the Bmi-1 and NANOG might serve as a novel survival predictor in ALL patients. Our observation also suggests that the Bmi-1 and NANOG could serve as new therapeutic targets for treatment of pediatric ALL.

* *Corresponding author at*: Faculty of Medicine, Mansoura University, PO Box 35516, Mansoura, Egypt.
E-mail address: layla_sarwat@hotmail.com (L.M. Saleh).

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Introduction

Acute lymphoblastic leukemia (ALL) is a common pediatric malignant tumor which accounts for nearly 25% of all cancers among children.[1]

Although treatment options for ALL have significantly improved, as many as 15–20% of ALL patients cannot achieve long-term remission, and relapse remains a challenge in treating pediatric ALL. Therefore, identifying novel prognostic markers is an urgent issue in ALL.[2,3] Although there are reports studying stem cells in ALL, these are fewer than those on other cancers.[4,5]

The Bmi-1 gene is located at chr.10p13 and has been shown to undergo rearrangements in malignant T-cell lymphomas and chromosomal translocation in infant leukemia.[6] It is an important molecule for the self-renewal of hematopoietic stem cells (HSCs), is highly expressed in HSCs and decreases after cells differentiate.[7]

Bmi-1 has been the focus of significant clinical interest, as many studies have demonstrated its upregulation in various malignancies,[8–10] as well as hematological malignancies, including mantle cell lymphoma,[11] B-cell non-Hodgkin's lymphoma,[12] diffuse large B-cell lymphomas,[13] chronic myeloid leukemia[14,15] and acute myeloid leukemia (AML).[16,17]

Overexpression of Bmi-1 has been proposed to be involved in tumor invasion, metastasis, cancer therapy failure and poor prognosis, and its overexpression is correlated with the patient survival rate,[18] This high expression might be the reason that some cancers to become chemoresistant.[19,20] Hence, Bmi-1 is a suitable candidate for predicting outcomes.

The NANOG is a divergent homeobox domain protein, first discovered in embryonic stem cells (ESCs), with canonical functions in the transcriptional regulation of self-renewal and pluripotency. It is highly expressed in pluripotent cells, such as ESCs, and Embryonic Germ (EG) and Embryonal Carcinoma (EC) cells and its expression is downregulated upon differentiation.[21,22]

Aberrant expression of the NANOG has been reported in many types of human cancers.[23] The expression levels of the NANOG are often positively correlated with the treatment resistance and poor survival of cancer patients. Various studies have shown that upregulation of the NANOG expression enhances the tumorigenicity, both in vivo and in vitro, whereas repression of the NANOG inhibits tumor initiation. Thus, the NANOG expression is linked to the tumor progression, therapeutic resistance, relapse and metastasis.[24]

Together with the SOX2 and OCT4, the NANOG plays a key role in maintaining the properties of the ESCs, Through forming a transcriptional network, these three key factors generally function to control the expression of a whole set of pluripotent-related genes and establish the pluripotency of the ESCs.[22]

In consideration of the important role of the Bmi-1 and NANOG expression in tumorigenesis, the expression and prognostic value of the Bmi-1 and NANOG in pediatric ALL was evaluated in this study.

Table 1 – Demographic characteristics of pediatric ALL patient.

	Number	%
Median age (years)		
Median	6.5	
(IQR)	(5–16.25)	
Gender		
Male	19	63
Female	11	37
White blood cell count (10^3*/uL)*		
Median	7.8	
(IQR)	(3.6–35.6)	
HB (g/dL)		
Median	9	
(IQR)	(7.4–11)	
Platelets count (10^3*/uL)*		
Median	54.7	
(IQR)	(30–69.7)	
ALL type immunophenotype		
B-ALL	16	53
T-ALL	14	47
Blast % in BM		
Median	90%	
(IQR)	(72–90%)	
LDH (U/L)		
Median	865	
(IQR)	(490–1858)	

Materials and methods

Patient samples

This study involved 30 newly diagnosed pediatric ALL patients attending the Oncology Center Mansoura University. The ALL diagnosis was performed according to standard cytomorphology and immunophenotypic criteria. All patient samples were obtained in accordance with the Declaration of Helsinki, with informed consent from the patients, parents or guardians and approval from the faculty of medicine Mansoura University institutional review board. The peripheral blood (PB) samples were obtained from the 30 ALL patients (19 (63%) males and 11 (37%) females, with a median age of 6.5 years) and healthy control PB samples were obtained from 14 healthy donors (with a median age of 17 years). Additional clinical data is included in (Table 1).

Methods

PB mononuclear cells isolation

Mononuclear cells were isolated by the density gradient centrifugation, using the lymphocyte separation medium (Lonza, Walkersville, MD); the RNA was isolated from mononuclear cells, using the miRNeasy Mini kit (Qiagen, Germantown, MD). The RNA concentration and purity were determined by the NanoDrop.

Quantitative real-time PCR (qRT-PCR)

The complementary deoxyribonucleic acid (cDNA) was synthesized from 2ug of RNA, using the high capacity reverse transcription kit (Applied Biosystems) a 20ul reaction was prepared as follows: 2 ul 10× RT buffer, 0.8 ul 25× dntps 100 mM, 2 ul 10× random primers, 1ul Multiscribe reverse transcriptase enzyme (50 U/ul), 1ul RNase inhibitor and 13.2 ul of nuclease-free water and extracted RNA.

Then sample wells were incubated in the thermal cycler at 25 °C for 10 min, 37 °C for 120 min, 85 °C for 5 min and then maintained at 4 °C. The quantitative real-time PCR was performed on the StepOne™, using TaqMan gene expression assays for the BMI-1, NANOG, SOX2 and OCT4 (Life Technologies, Grand Island, NY), and the real-time PCR was performed using Applied Biosystems TaqMan Gene Expression Assays. The house-keeping gene GAPDH was used as an internal control. The relative gene expression level was calculated as ($2^{-\Delta\Delta Ct}$).

Statistical analysis

The statistical analyses were performed using the GraphPad Prism software (GraphPad Software, La Jolla, CA). The Shapiro–Wilk test, *t*-test, Mann–Whitney and/or Wilcoxon tests were used when indicated. The continuous variables, including age and peripheral blood count, were summarized by median and range and analyzed using the Mann–Whitney test. The overall survival (OS) analysis was considered from the date of diagnosis to the date of death or last contact and prepared by the Kaplan–Meier method. The *p*-values of less than 0.05 were considered significant.

Results

Bmi-1 and NANOG expression levels in ALL patients

To determine the expression pattern of the Bmi-1 and NANOG in ALL, an analysis by qRT-PCR was performed on 30 PB mononuclear cells from pediatric ALL patients and 14 from normal subjects. Thirty patients (19 males, 11 females), with a median age of 6.5 years (range 5–16.25 years), comprising T-ALL ($n = 14$) and B-ALL ($n = 16$), were analyzed. The Bmi-1 expression was significantly higher in the ALL samples, compared to that in the samples from healthy donors (**$p = 0.04$**) (Figure 1).

On analyzing the NANOG expression results, they were not significantly differentially expressed between healthy controls and ALL samples ($p > 0.05$); the same was found in the SOX2 and OCT4 expression (data not shown).

Relationship between Bmi-1 and NANOG expression and the clinicopathological characteristics of pediatric ALL patients

To determine whether the Bmi-1 and NANOG expression levels correlate with the clinicopathological characteristics of pediatric ALL patients, we divided the patients into high and low groups, based on the median expression value of the Bmi-1 and NANOG.

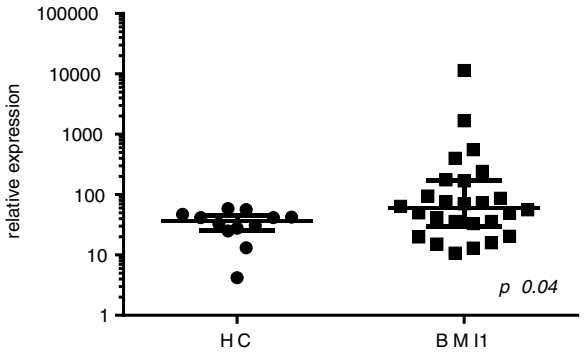

Figure 1 – BMI-1 expression levels relative to healthy controls, in comparison to ALL samples.

Among the study cohort, no significant difference in the BMI-1 and NANOG expression levels were observed, with respect to age, gender hemoglobin level, platelet count or bone marrow blasts. The ALL patients with a high BMI-1 expression exhibited a significantly high median white blood cell (WBC) count (19.6×10^3/uL vs. 4.9×10^3/uL, **$p = 0.017$**) than those with a low BMI-1 expression, as well as higher absolute lymphocytic count (ALC) (10.8×10^3/uL vs. 3.3×10^3/uL, **$p = 0.031$**), compared to patients with a low BMI-1 expression. Moreover, ALL patients with high NANOG expression exhibited a trend toward a higher median WBC count (12.3×10^3/uL vs. 5.8×10^3/uL, $p = 0.17$), as well as a higher absolute lymphocytic count (10.5×10^3/uL vs. 3.9×10^3/uL, $p = 0.15$) than those with a low NANOG expression (Tables 2 and 3). The median follow-up period was 22 months (range: 11–24 months). The Event-Free Survival (EFS) and Overall Survival (OS) of the whole cohort were 73.3% and 70%, respectively.

The high BMI-1 patients displayed a significantlyworse survival (**$p = 0.012$**) than the low BMI-1 patients (HR 5.74, 95% CI:1.48–22). Similarly, the high NANOG patients showed a significantly worse survival (**$p = 0.048$**) than the low NANOG patients (HR 3.8, 95% CI:1.009–14.3) (Figures 2 and 3).

Furthermore, we found that cases with high BMI-1 and NANOG expression levels showed a shorter EFS than the cases with low BMI-1 and NANOG expression levels (12 & 18 months vs. 20 & 21.5 months, $p = 0.12$ and 0.096, respectively), but significant levels were not reached (Figures 2 and 3). Notably, the highly expressed Bmi-1 and NANOG were observed to be closely correlated with poor survival.

Discussion

Previous reports have shown an association between high Bmi-1 expression and unfavorable prognosis in AML, chronic myeloid leukemia (CML) and myelodysplastic syndrome (MDS)[14–16,25] and its potential as a target therapy.[17] However, few reports have demonstrated its impact in ALL.[26,27]

Our study on PBMNCs from pediatric ALL patients confirmed what was previously demonstrated by Peng et al.,[26] showing a significantly elevated Bmi-1 expression level in

Table 2 – Clinical characteristics of BMI-1 high- and low-expressing ALL patients.

	High BMI-1	Low BMI-1	*p*-Value
Age years Median (IQR)	8.5 (5–15.5)	6 (3–12)	0.43
Gender Male/female	9/4	10/7	0.71
ALL type B-ALL/T-ALL	7/6	9/8	0.99
White blood cell count (*10^3/uL) Median (IQR)	19.6 (5.9–75)	4.9 (3.3–15)	**0.017**
ALC (*10^3/uL) Median (IQR)	10.8 (3.5–48.4)	3.3 (1–12)	**0.031**
Hb (g/dL) Median (IQR)	8.4 (6.8–11.2)	9.5 (8.1–10.7)	0.54
Platelets count (*10^3/uL) Median (IQR)	53.5 (19.4–133.8)	68 (49–99.5)	0.68
Blast % in BM Median (IQR)	90% (80–92%)	85% (70–90%)	0.89
Event-free Survival months Median (IQR)	12 (2.9–22)	20 (13.5–25)	0.12
Overall Survival months Median (IQR)	20 (3.5–22.5)	22 (20–27.5)	**0.012**

Table 3 – Clinical characteristics of NANOG high- and low-expressing ALL patients.

	High NANOG	Low NANOG	*p*-Value
Age years Median (IQR)	8 (5–12)	6 (3–13)	0.47
Gender Male/female	10/5	9/6	0.99
ALL type B-ALL/T-ALL	7/8	9/6	0.72
White blood cell count (*10^3/uL) Median (IQR)	12.3 (5–43)	5.8 (3.5–28.4)	0.17
ALC (*10^3/uL) Median (IQR)	10.5 (2.8–41.4)	3.9 (1.3–17.3)	0.15
Hb (g/dL) Median (IQR)	8.7 (7.1–11)	9.4 (7.9–10.8)	0.69
Platelets count (*10^3/uL) Median (IQR)	56 (21–148)	53 (48–87)	0.69
Blast % in BM Median (IQR)	90% (80–90%)	85% (70–90%)	0.13
Event-free Survival months Median (IQR)	18 (4.7–22)	21.5 (14–26)	0.096
Survival months Median (IQR)	20 (6–23)	22 (20–26)	**0.048**

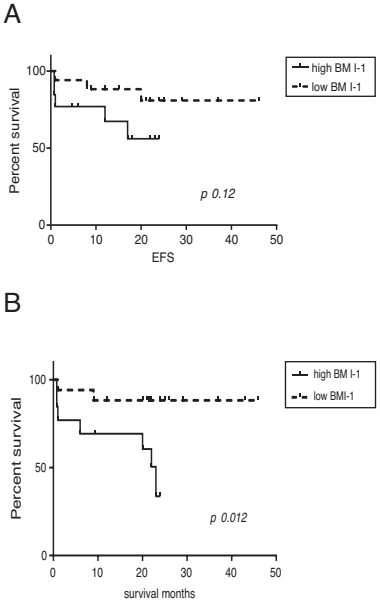

Figure 2 – Correlation of BMI-1 expression levels and survival in ALL. (A) Event-free survival (EFS) and (B) Overall survival (OS).

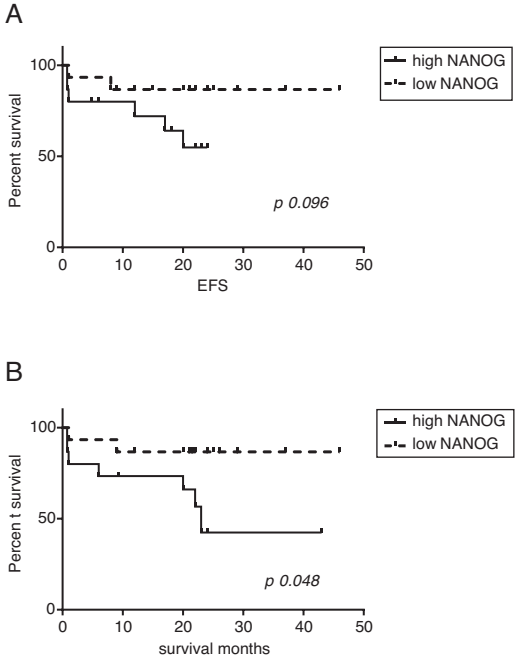

Figure 3 – Correlation of NANOG expression levels and survival in ALL. (A) Event-free survival (EFS) and (B) Overall survival (OS).

mononuclear cells from the bone marrow of pediatric ALL patients.

Furthermore, they demonstrated that a higher expression of Bmi-1 was associated with a significantly lower OS[26] and this was also confirmed in our study, as high BMI-1 patients displayed a significantly worse survival (***p* = 0.012**) than low BMI-1 patients (HR 5.74, 95% CI: 1.48–22).

However, Kajiume et al.,[27] investigating different subsets of sorted cells by FACS, reported that the Bmi-1 expression was lower in pediatric acute lymphoblastic leukemia cells, in comparison to normal B-cells and there were no significant correlations between the Bmi-1 gene expression and clinical characteristics, such as patient prognosis and survival,

assuming that pediatric acute lymphoblastic leukemia cells are no longer in immature form. Moreover, they speculated that cancer stem cells in pediatric acute lymphoblastic leukemia might not exist. However, these results were inconsistent with those of our study.

The NANOG expression has been reported to be elevated in a variety of cancers, and its expression levels seem to positively correlate with patient survival, implicating the NANOG as an oncogenic factor in cancer development.[24] The NANOG2 gene was shown to be transcribed in the CD34+ cells, indicating that the hematopoietic stem cell compartment may use the NANOG system to gain stem cell-like properties.[28]

These results were inconsistent with those of our study, as the NANOG and the other pluripotent-related genes SOX2 and OCT4 expressions were not found significantly differentially expressed among the ALL samples, indicating the possibility of a differentiated stage and loss of self-renewal of isolated cells from our patients; this may also be due to the different leukemia subtype and the limited number of samples.

Interestingly, while the expression level of the NANOG was not significant, the high NANOG-expressing patients showed a significantly worse survival (HR 4.2, $p = 0.048$) than low NANOG-expressing patients. This finding raises our interest in considering a future study on a large number of patient samples.

In conclusion, our study confirmed that the Bmi-1 was significantly upregulated in pediatric ALL. A significantly poorer survival was observed in patients with a high Bmi-1 expression. These findings suggest that the Bmi-1 could be a biomarker for predicting the outcome of patients with pediatric ALL. Although no difference was observed in the NANOG expression, the significant association of its high level with survival leads us to consider a future study on a large number of ALL patient samples and use of sorted cells. Thus, we highlight that the Bmi-1 and NANOG could be considered as potential prognostic markers in pediatric ALL patients.

Author contributions

Hasan Abdel-ghaffar, Layla M. Saleh and Sherin Abd EL Aziz designed the study. Layla M. Saleh and Sara Abdel-khalek performed experiments. Layla M. Saleh, Sherin Abdel-Aziz, Hasan Abdel-ghaffar analyzed the data and wrote the manuscript. Sara Abdel-khalek, Ayman Hyder and Hasan Abdel-ghaffar assisted in editing the manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

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