High expression of LMO2 predicts a favorable outcome in adult patients with BCR/ABL negative B-cell acute lymphoblastic leukemia

RABAB M. ALY[1], MONA M. TAALAB[2], EMAN M. ABDSALAM[3], OMAR H. ELYAMANY[4] and OMAR E. HASAN[4]

[1]Department of Clinical Pathology; [2]Clinical Hematology Unit, Internal Medicine Department, Faculty of Medicine, Mansoura University, Mansoura 31115; [3]General Medicine Department, Faculty of Medicine For Girls, Alazhar University, Cairo 31991; [4]Department of General Medicine, Faculty of Medicine, Mansoura University, Mansoura 35516, Egypt

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Abstract. The LIM domain only protein 2 (LMO2) is a key regulator of hematopoietic stem cell development. Expression of LMO2 has been evaluated in B-cell lymphoma, T-cell acute lymphoblastic leukemia and acute myeloid leukemia; however, information concerning its role in breakpoint cluster region/Abelson murine leukemia viral oncogene homolog 1 (BCR/ABL) negative B-cell acute lymphoblastic leukemia (B-ALL) remains limited. The present study investigated LMO2 expression using quantitative polymerase chain reaction in 85 adult patients with BCR/ABL negative B-ALL, and associated the expression of LMO2 with established prognostic factors. LMO2 expression levels in patients with BCR/ABL negative B-ALL was not significantly different compared with control individuals (P=0.25). However, LMO2 expression levels were associated with the immunophenotypical features of the patients; a high LMO2 expression was associated with a higher incidence of complete remission (P=0.03) and lower rate of relapse (P=0.01), and patients with a high LMO2 expression had a significantly improved overall survival rate (P=0.01) and disease-free survival (P=0.01). The present results suggest that LMO2 expression is a favorable prognostic marker in adult patients with BCR/ABL negative B-ALL and may be used as a diagnostic marker and therapeutic target. However, additional studies regarding its prognostic role in patients with BCR/ABL negative B-ALL are required.

Introduction

LIM domain only protein (LMO)2 belongs to the LMO family of transcription factors that contain zinc binding finger-like motifs, termed LIM domains, which are required in protein-protein interactions (1). LMO2, also termed rhombotin-like 1, is a member of a multigene family that is extremely well-conserved (2). LMO2 encodes a cysteine-rich LIM domain containing transcription factor, which has been demonstrated to be required for complete hematopoiesis in mice (3), and has also been identified in key events in erythropoiesis, angiogenesis and embryogenesis (4,5). LMO2 is expressed in a number of tissues during fetal life, and its expression in hematopoietic cells is tightly regulated and varies at different stages of maturation. In normal B cell differentiation and maturation, the majority of LMO2 is expressed in the bone marrow during early lymphopoiesis and in the germinal centers (GC) of secondary lymphoid organs (6).

In lymphoid and myeloid leukemias there is a deregulated expression of LMO2 (7-9), and LMO2 is expressed in ~9% of pediatric patients with T-cell acute lymphoblastic leukemia (T-ALL) (10). LMO2 activation is a result of chromosomal translocations and cryptic deletions in the LMO2 negative regulatory domain (11,12). Previously, it has been demonstrated that LMO2 was insertionally mutated by retroviral gene therapy vectors in X-linked severe combined immunodeficiency (SCID-X1) and Wiskott-Aldrich syndrome (13-15). Notably, transgenic mouse models revealed that an ectopic expression of LMO2 in T cells leads to the development of a leukemogenic lesion (16). Overall, these previous studies indicate that LMO2 may have a role in leukemogenesis.

LMO2 expression has been observed in certain malignancies, including a subset of acute myeloid leukemia (AML) (17), and notably, LMO2 protein expression has been demonstrated to have an effect on the survival time of patients with chronic myeloid leukemia (CML) treated with imatinib mesylate (18). In addition, LMO2 has been suggested as a novel predictive marker for an improved prognosis in patients with pancreatic cancer (19). It was also clinically relevant in patients with diffuse large B-cell lymphoma (DLBCL), since its level of expression was a predictor of patient survival time, possibly since it identified cases of DLBCL with a germinal-center cell origin (20). Previously, it was reported that LMO2 was expressed in a significant proportion of patients with B-cell acute lymphoblastic leukemia (B-ALL), but was not

Correspondence to: Dr Rabab M. Aly, Department of Clinical Pathology, Faculty of Medicine, Mansoura University, 25 Salam Street, Mansoura 31115, Egypt
E-mail: rababzeadah@yahoo.com

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prognostic in acute lymphoid leukemia (21). However, a separate study revealed that LMO2 expression was associated with an improved overall survival time in patients with normal karyotype B-ALL (22). Therefore, the aim of the present study was to evaluate the prognostic role of LMO2 in adult patients with breakpoint cluster region/Abelson murine leukemia viral oncogene homolog 1 (BCR/ABL) negative B-ALL.

Patients and methods

Patients. LMO2 expression was assessed in a cohort of 85 adult patients, which consisted of 48 men and 37 women, with BCR/ABL negative B-ALL. The patients were enrolled between December 2010 and August 2013 at the Oncology Center of Mansoura University Hospital (Mansoura, Egypt). The present study was approved by the Institutional Review Board at the Mansoura University Hospital (approval number, H-01-R-009). Written informed consent was obtained from each patient. The age of the patients ranged between 19 and 62 years with a median age of 50 years. In total, 32 control individuals, which consisted of 18 men and 14 women (median age, 49 years; age range, 19-60 years) that were healthy blood donors and had normal laboratory results (white blood cells, normal range 4.5-11.0 cells/cmm; red blood cells, normal range 3.8-5.8 cells/ccm; hemoglobin, normal range 13-18 gm/dl; platelets, normal range 150-440 cells/cmm) and no history of malignancies were selected to closely match the age and gender of the patients with BCR/ABL negative B-ALL.

A diagnosis of B-ALL was based on morphological, cytogenetic and immunophenotypic criteria (23-25). Blood samples of the patients were collected prior to treatment. Briefly, treatment consisted of 6 weeks of induction, 2 weeks of consolidation and 120 weeks of continuation therapy. Induction therapy consisted of vincristine (VCR; 1.5 mg/m^2), daunomycin (45 mg/m^2), asparaginase (5000 U/m^2), etoposide (VP16; 300 mg/m^2), aracytin (Ara C; 300 mg/m^2) in addition to triple intrathecal (IT) therapy, which consisted of 2 courses of high-dose methotrexate (MTX; 15 mg), 6-mercaptopurine (6-MP; 40 mg) and dexamethasone (Dex; 4 mg). Consolidation therapy consisted of high-dose MTX (500 mg/m^2 over 1 h followed by 1500 mg/m^2 over 23 h) and triple IT therapy. Continuation therapy consisted of triple IT therapy and 15 cycles of an 8 week course of VP16 + cyclophosphamide (300 mg/m^2), 6-MP + MTX (75 and 40 mg/m^2), MTX + Ara C (40 and 300 mg/m^2), VP16 + Ara C (300 mg/m^2), 6-MP + high dose MTX (75 mg/m^2) and prednisone + VCR (40 mg/m^2). During continuation therapy re-induction was administered in the form of VCR, daunomycin, Dex, high dose MTX, 6-MP and triple IT therapy.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Patient bone marrow samples were collected in heparinized glass tubes and mononuclear cells were isolated by Ficoll gradient centrifugation using a High Pure RNA Isolation kit (Roche Diagnostics GmbH, Mannheim, Germany). Total RNA from the mononuclear bone marrow cells of the patients was extracted using the High Pure RNA Isolation kit (Roche Diagnostics GmbH), according to the manufacturer's protocol. RNA quantity and quality were assessed using spectrophotometric measurements at 260/280 nm (V1000 Visible Spectrophotometer; AOE Instruments (Shanghai) Co., Ltd., Shanghai, China). The starting concentration of the RNA was 30 μl. Reverse transcription was performed with 25.0 μl total RNA, 2.5 μl reverse transcriptase, 5.0 μl reverse transcriptase buffer, 4.0 μl deoxynucleotides, 5.0 μl random primers and 8.5 μl H_2O. The reaction was incubated at 42˚C for 1 h and inactivated by heating to 95˚C for 5 min. qPCR was performed using a MicroAmp Optical 96-well plate (Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10 μl cDNA, 1.0 μl of the forward and reverse primers, 10 μl distilled water, 0.5 μl Taqman probe (Sigma Scientific Services Co., Cairo, Egypt) and 25 μl universal master mix (Qiagen, Inc.). The expression levels of LMO2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; reference gene) were quantified using qPCR analysis on an ABI Prism 7700 sequence detection system (Thermo Fisher Scientific, Inc.). The PCR program was as follows: 50˚C for 2 min, 95˚C for 10 min, then 40 cycles of 95˚C for 15 sec and 60˚C for 1 min.

The following primers were used for qPCR at a concentration of 1.0 μl: LMO2, forward 5'GGCGGCGCCTCTACT ACA-3', reverse 5'-CCAAAAAGCCTGAGATAGTCTCT-3' and probe 5'-CTGGGCCGGAAGCTCTGCC-3'; GAPDH, forward 5'-GAAGGTGAAGGTCGGAGTC-3', reverse 5'-GAAGATGGTGATGGGATTTC-3' and probe VIC-CAA GCTTCCCGTTCTCAGCC-TAMRA. The relative expression level of LMO2 was measured using the comparative cycle threshold method (26). The experiment was performed twice.

Statistical analysis. SPSS software version 15.0 (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. To compare LMO2 expression between various groups, the Mann-Whitney U-test and the Kruskal-Wallis H-test were used. Overall survival (OS) time was defined as the time interval between the date of diagnosis and the date of that the patient succumbed to the disease or the date of the last follow-up. Disease-free survival (DFS) was measured between the date of complete remission to the date of relapse or the date the patient succumbed to the disease. Cox regression analysis was performed for all patient variables. All descriptive statistics and tests were calculated using SPSS. $P<0.05$ was considered to indicate a statistically significant difference.

Results

LMO2 expression in BCR/ABL negative B-ALL patients and control individuals. LMO2 expression was decreased in control individuals (mean relative expression, 1.75; range, 0.05-8.0) compared with B-ALL patients (mean, 1.82; range, 0.07-8.2). The difference was not statistically significant (P=0.25; Fig. 1).

LMO2 expression levels in association with clinical and molecular variables of patients with B-ALL. The present study divided the patients into two subgroups with high or low levels of LMO2 expression using the median LMO2 expression (median, 1.82) value of the whole cohort as a cut-off value. Table I reveals that among subgroups, pro-B exhibited the highest LMO2 expression while B-ALL with common-cluster of differentiation (CD)10$^+$ immunophenotype, pre-B-ALL

Table I. Clinical and molecular characteristics at diagnosis in adult patients with B-cell acute lymphoblastic leukemia, according to LMO2 expression.

Characteristic	Total, value	High LMO2 expression, value	Low LMO2 expression, value	P-value
Total, n (%)	85 (100.0)	46 (46.0)	39 (39.0)	
Age, years				
Median	52	50	52	0.64
Range	19-62	19-60	19-61	
Gender, n (%)				
Male	48	26 (56.5)	22 (56.4)	0.60
Female	37	20 (43.5)	17 (43.6)	
TWBC, n (%)				
>50x10^9 cells/l	34	18 (39.1)	16 (41.0)	0.32
<50x10^9 cells/l	51	29 (63.0)	22 (56.4)	
Immunophenotype, n (%)				
Pro-B	25	17 (37.0)	6 (15.4)	0.01
Common (CD10$^+$)	51	27 (58.7)	26 (66.6)	
Pre-B	8	2 (4.3)	6 (15.4)	
Mature	1	0 (0.0)	1 (2.6)	

LMO2, LIM domain only protein 2; TWBC, total white blood cell count; CD, cluster of differentiation.

Table II. Clinical outcome in adult patients with B-cell acute lymphoblastic leukemia, according to LMO2 expression.

Clinical outcome	Total, n (%)	High LMO2 expression, n (%)	Low LMO2 expression, n (%)	P-value
Total	85 (100.0)	46 (46.0)	39 (39.0)	
Complete remission	62 (72.9)	38 (82.6)	24 (61.5)	0.03
Refractory disease	12 (14.1)	6 (13.0)	6 (15.3)	0.87
Mortality induction	11 (12.9)	2 (4.3)	9 (23.0)	0.03
Relapse risk	25 (29.4)	8 (17.3)	17 (43.5)	0.01

LMO2, LIM domain only protein 2.

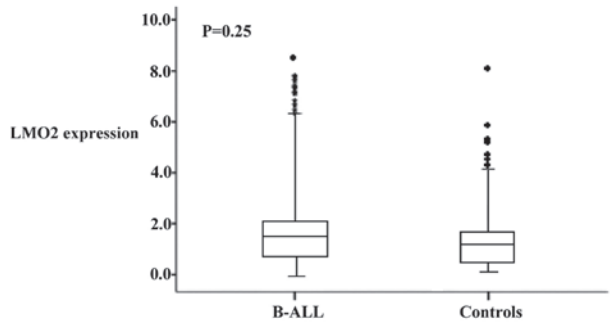

Figure 1. Expression levels of LMO2 in patients with B-ALL and control individuals. LMO2, LIM domain only protein 2; B-ALL, B-cell acute lymphoblastic leukemia.

and mature B-ALL had a lower expression (P=0.01). Pro-B immunophenotype occurred more commonly in patients with a high LMO2 expression compared with patients with low LMO2 expression (36.9 vs. 15.3%). Common CD10$^+$ and pre-B immunophenotypes were more commonly observed in patients with low LMO2 expression levels. However, no significant differences were observed between patients with high and low LMO2 expression levels for age, gender and total white blood cell count (TWBC).

Prognostic significance of LMO2 expression in adult patients with BCR/ABL negative B-ALL. Table II showed that the complete remission (CR) rate of patients following induction therapy was 72.9%. The CR rate was 82.6% in patients with high LMO2 expression and 61.5% in patients with low LMO2 expression (P=0.03). The relapse rate was significantly different between the high and low LMO2 expression groups (high vs. low, 17.3 vs. 43.5%; P=0.01). Patients with high LMO2 expression demonstrated a lower induction mortality rate compared with patients with low LMO2 expression (high vs. low, 4.3% vs. 23.0%; P=0.03).

Table III. Multivariate analysis of LMO2 expression for clinical outcome of adult patients with B-cell acute lymphoblastic leukemia.

	Overall survival time			Disease-free survival		
Variable	HR	95% CI for relative risk	P-value	HR	95% CI for relative risk	P-value
LMO2 expression	0.60	0.32-0.85	0.010	0.56	0.24-0.90	0.020
Age	0.80	0.55-0.95	0.425	0.85	0.49-0.98	0.456
Gender	0.78	0.32-1.05	0.892	0.94	0.60-1.05	0.680
Immunophenotype	1.00	0.87-1.24	0.576	1.02	0.76-1.24	0.605
WBC >50x10^9 cells/l	0.93	0.69-1.06	0.357	0.82	0.62-1.18	0.822

LMO2, LIM domain only protein 2; HR, hazards ratio; CI, confidence interval; WBC, white blood cells.

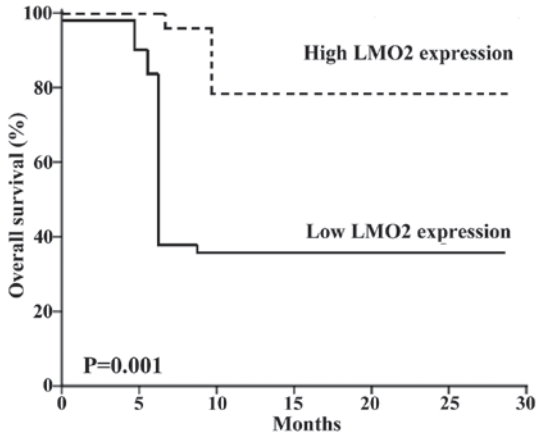

Figure 2. Overall survival rate of patients with breakpoint cluster region/Abelson murine leukemia viral oncogene homolog 1 negative B-cell acute lymphoblastic leukemia, according to LMO2 expression. LMO2, LIM domain only protein 2.

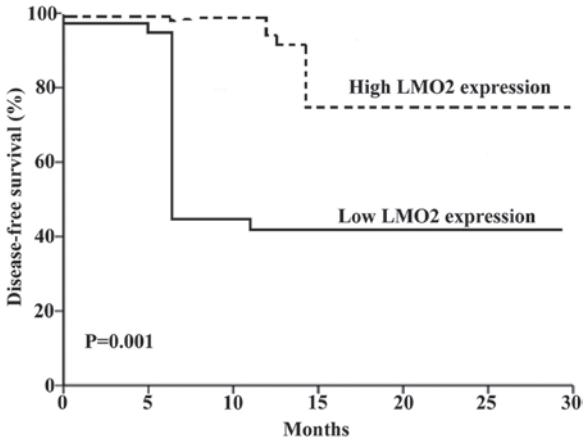

Figure 3. Disease-free survival of patients with breakpoint cluster region/Abelson murine leukemia viral oncogene homolog 1 negative B-cell acute lymphoblastic leukemia, according to LMO2 expression. LMO2, LIM domain only protein 2.

Multivariate analysis of LMO2 expression is associated with the clinical outcome of patients. Multivariate analyses demonstrated that in patients with BCR/ABL negative B-ALL, an increased expression of LMO2 was an independent predictor for an improved OS rate [P=0.010; hazards ratio (HR), 0.60; 95% confidence interval (CI), 0.32-0.85] and DFS (P=0.020; HR, 0.56; 95% CI, 0.24-0.90) (Table III).

Patient outcome is associated with LMO2 expression. The overall survival rate was significantly increased in BCR/ABL negative B-ALL patients that were identified as having high levels of LMO2 expression in comparison to those patients with low levels of LMO2 expression (OS rate high LMO2, 78.6%; OS rate low LMO2, 37.2%; P=0.001; Fig. 2). In addition, DFS was improved in patients with high LMO2 expression compared with patients with low LMO2 expression (high vs. low, 72.4 vs. 41.1%; P=0.001; Fig. 3).

Discussion

The identification of prognostic markers in precursor B-ALL is required for the development of novel molecular therapies that may improve risk-adapted treatment stratification for patients with B-ALL. LMO2 is a key regulator of hematopoiesis and vascular development (27). In hematopoietic cells, it is observed to have a role as an intranuclear bridging molecule, which aids in the formation of protein-protein interactions in multiprotein complexes that are required for the specification of cell lineage and differentiation (28). It has been previously reported that increased levels of LMO2 transcripts were observed in CD34$^+$ stem cells, and LMO2 protein expression was clearly observed in endothelia, GC B cells and B-cell lymphoma (29,30). In addition, activation of LMO2 has been demonstrated in T-ALL, as a result of gene translocations and deletions (31,32), and LMO1/2 is transcriptionally deregulated in the majority of patients with acute T-ALL (33).

Previous studies have reported the potential role for increased LMO2 expression in leukemia besides T-ALL. A previous study demonstrated that LMO2 upregulation had a role in the development of leukemia stem cell activity and disease in a mouse model of AML (34). Furthermore, studies revealed that there was an association between increased LMO2 levels at diagnosis with patient outcome (35). LMO2 was also demonstrated to be overexpressed in GC-type DLBCL (36), and an association between LMO2 expression

and tumor progression was reported in patients with prostatic carcinoma (37).

Other studies have revealed that LMO2 was a favorable prognostic factor in patients with CML and pancreatic cancer (18,19), and its expression in patients with DLBCL has been associated with a favorable outcome (38). The present study evaluated the prognostic role of the LMO2 gene in adult patients with BCR/ABL negative B-ALL and demonstrated that LMO2 was expressed in patients with B-ALL. This has also been demonstrated by previous studies (6,22).

Previously it was observed in SCID-X1 patients that the introduction of a retrovirus vector into the LMO2 gene promoter induced aberrant LMO2 expression, which resulted in the development of T-ALL (39). In addition, LMO2 was demonstrated to act as an oncogene in patients with T-ALL. However, this possibly does not occur in patients with B-ALL. Previous studies have reported that no LMO2 gene translocations have been identified in B-ALL, and other genetic alterations, which involve the LMO2 locus, are extremely rare among patients with B-ALL. Furthermore, in a genome-wide analysis only 1 out of 192 B-ALL samples revealed a 155 kb deletion upstream of the LMO2 gene locus; by contrast, 8% of T-ALL samples exhibited this alteration (40). In addition, it was demonstrated that B cells induce LMO2 expression during the immune response in the GC reaction, and this expression reaches levels similar to that exhibited by pro-B cells (22). Therefore, these results suggest that LMO2 does not act as an oncogene in B-ALL in contrast to T-ALL. In the present study, pro-B-ALL was more common in patients with a high LMO2 expression, which is in agreement with a previous study that revealed that there was an association between the pro-B immunophenotype and a high LMO2 expression (22).

The present study demonstrated that high LMO2 expression independently predicts an increased OS time in patients with BCR/ABL negative B-ALL in comparison to patients with low LMO2 expression. This was in agreement with Malumbres *et al* (22), who demonstrated that there was an improved OS rate of patients with normal karyotype B-ALL that had a high LMO2 transcript expression. This observation was also observed in patients with CML (18) and DLBCL (38). The present study observed that a high LMO2 expression was associated with improved DFS in patients with BCR/ABL negative B-ALL; however, Malumbres et al (22) did not identify any association between LMO2 expression and DFS in the subgroup of patients with a normal karyotype B-ALL, possibly due to the low number of relapses in this subgroup. The association between high LMO2 expression and increased OS time and improved DFS was additionally confirmed in multivariable analyses performed by the present study that adjusted for the most important clinical and molecular prognosis characteristics in ALL, including age, gender, immunophenotype and TWBC of >50,000/mm^3. The present results were in agreement with a previous study that demonstrated that a high LMO2 expression was as an independent favorable prognostic factor for OS rate in patients with normal karyotype B-ALL (22).

Previously LMO2 protein expression did not predict patient survival in 22 patients with B-ALL, which was evaluated using immunohistochemistry (21). This may be due to the low number of patients with B-ALL enrolled in that study, as well as the lack of immunophenotype stratification. Therefore, LMO2 expression may be used to additionally refine risk stratification for patients with BCR/ABL negative B-ALL. However, additional studies with large and molecularly well-characterized cohorts are required to develop and confirm this improved risk classification system for B-ALL.

In summary, the present study provides evidence that a high LMO2 expression is associated with favorable outcomes in adult patients with B-ALL, even following adjustment for known clinical and molecular risk factors.

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