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Brain and Acute Leukemia Cytoplasmic (BAALC) Gene Expression in Acute Myeloid Leukemia: A Study of an Egyptian Cohort

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ABSTRACT

Background: Acute myeloid leukemia with normal cytogenetics (CN-AML) constitutes a heterogeneous group with variable prognosis. Among others, high BAALC gene expression is reported as an independent bad prognostic indicator in CN-AML. Egyptian studies on BAALC gene expression in AML are limited.

Objectives: We aimed to study the BAALC gene expression in an Egyptian cohort with AML and to verify its prognostic relevance on CNAML in the studied cohort.

Material and Methods: The cohort comprised 54 AML patients including 38 CNAML, 7 M3, 4 CML in blastic crisis, 2 on top of MDS, 2 (+8) and one AML case with hypodiploidy. Cases were diagnosed according to standard methods and FAB subtype specified. BAALC gene expression was evaluated by RT-PCR.

Results: High BAALC gene expression was detected in 19/38 (50%) of the CNAML cases. No statistically significant association of BAALC gene expression with Hb level, TLC, platelet count or percentage of BM blasts at first presentation, at day 14 or at day 28 post chemotherapy was encountered. High BAALC expression was significantly associated with FAB M0/M1 (p=0.01), lower CR rate (p=0.04), higher frequency of primary resistance to chemotherapy (p=0.02) and shorter OS (p=0.003).

Apart from the CNAML cases, high BAALC gene expression was encountered in 2/2 cases with trisomy 8, 1/1 with hypodiploidy, 2/4 CML in blastic crisis and 2/2 AML on top of MDS; none of the 7 M3 cases showed high BAALC expression.

Conclusion: Our study has further demonstrated, in an Egyptian cohort, that high BAALC gene expression is associated with lower incidence of CR, higher incidence of resistant disease and shorter OS in CNAML patients. This study would strongly recommend including BAALC gene evaluation in the initial molecular characterization of AML cases.

Key Words: *AML – CNAML – BAALC gene.*

INTRODUCTION

Chromosomal abnormalities provide a powerful tool to stratify acute myeloid leukemia (AML) patients into different prognostic risk groups. Patients lacking cytogenetic aberrations, accounting for approximately 45% of newly diagnosed de novo AML cases, were originally contained in an intermediate risk group **[1]**. However, this cytogenetically normal AML (CN-AML) actually constitutes a heterogeneous cohort of patients with favorable, intermediate, or relatively poor clinical outcome **[2]**. This was proved by molecular studies of CN-AML cases that revealed a striking heterogeneity with regards to the presence of acquired gene mutations and changes in gene expression **[3]**. Multiple submicroscopic genetic alterations with prognostic significance have been discovered, including internal tandem duplication of the FLT3 gene **[4]**, partial tandem duplication of the MLL gene **[5]**, mutations in the CEBPA gene **[6]**, NPM1 gene **[7,8,9]**, WT1 gene **[10]**, AML1 gene **[11]** and NRAS gene as well as high expression of ERG gene **[12]**, MN1 gene **[13]** and the BAALC gene **[14,15,16]**. BAALC (for brain and acute leukemia, cytoplasmic) is a relatively recently identified gene on chromosome 8q22.3 which is suspected in the pathogenesis of some AML cases **[14]**. BAALC expression in normal bone marrow is restricted to the compartment of progenitor cells and it shows high expression in a subset of leukemic blasts. It is absent in normal peripheral blood cells. The function of the BAALC protein in hematopoiesis and leu-

kemogenesis contributing to a more aggressive behavior of AML is obscure. Baldus et al., **[17]** suggested that BAALC may be seen as a stage-specific marker that maintains proliferative capacity and inhibits differentiation in a regulated way during hematopoiesis but if aberrantly expressed, this can lead to leukemogenesis. In AML patients with normal cytogenetics, high BAALC expression seems to predict a poor prognosis **[14,15,17]**.

Studies on Egyptian patients are limited. We aimed in this work to study BAALC gene expression in a cohort of Egyptian patients with myeloid malignancies and to verify its prognostic impact on prognosis of CN-AML cases in our cohort.

PATIENTS AND METHODS

The study included 68 cases with AML presented to the NCI, Cairo University, during the period of March 2007 to June 2010.

Patients were evaluated according to standard methods including clinical, radiological and laboratory workup. Diagnosis was confirmed by complete blood picture, bone marrow (BM) examination, cytochemistry as indicated and immunophenotyping. Routine karyotyping and evaluation of t(8;21), t(15;17) and inv (16) were performed. BAALC gene expression was evaluated in all cases.

Of the 68 patients, data was available for 54. Among these 38 were CN-AML; the others included 7 M3 cases, 4 CML cases in blastic crisis, 2 AML cases on top of MDS, 2 AML +8 cases and one AML case with hypodiploidy. Statistical analysis was confined to the 38 CN-AML. Results of the other cases will be presented in a descriptive way.

Patients with normal cytogenetics (38) included 19 males and 19 females; they had an age range of 0.5-80 and a median of 32 years; 7 were children (≤16 years), 20 were adults (16-60) and 5 were elderly AML cases (≥60 years). Of these 26 were followed-up for a period of 11-26 months with a median of 17 months to detect the clinical outcome of treatment emphasizing on achievement of CR, failure to achieve CR at the end of induction therapy, incidence of relapse and OS.

BAALC gene expression was evaluated by reverse transcription-polymerase chain reaction (RT-PCR). RNA extraction was performed by QIAamp® RNA Blood Mini Kit Catalog no. 52304; USA, its concentration (ng/ul.) and purity were measured using The Thermo Scientific NanoDrop™ 1000 Spectrophotometer, Wilmington, DE 19810 USA. The ratio of the readings at 260 nm and 280 nm (A260/A280) provides an estimate of the purity of RNA with respect to contaminants that absorb in UV spectrum, such as protein; pure RNA has an A260/A280 ratio of 1.9-2.1.

The GeneAmp® Gold RNA PCR Reagent Kit (Applied Biosystems, 850 Lincoln Centre Drive, Foster City, California 94404) was used for the reverse transcription (RT) and polymerase chain reaction (PCR) amplification. First-strand cDNA was synthesized from 1µg RNA in a 20µL reaction mix containing 1X RT-PCR Buffer, 25mM $MgCl_2$, 10mM dNTP Blend, 0.5µL RNase Inhibitor 10 unit/20ul, 100 mM DTT, 0.5µL (1.25uM) random hexamers, 0.3µL MultiScribe Reverse Transcriptase (50 units/µL) in RNase-free water. Cycling parameters for the RT included hybridization for 10min at 25°C and reverse transcription for 45min at 42°C.

To check the integrity of the cDNA, the house keeping gene glucose phosphate isomerase (GPI) was amplified as a control gene by single-round PCR with the following primer pair (forward primer, TaqE1F: 5- CCCCCAGTTCCAGAAGCTG 3- and reverse primer: TaqE2R: 5- GCATCACGTCCTCCGTCAC 3-). A 63ng cDNA in 2.5µL was added to a final PCR reaction mixture of 25µL containing 5.0µL of 5 X PCR Buffer, 1.75µL of 10mM dNTPs, 1.9µL of 25mM $MgCl_2$ 0.25µL of 5U/µL AmpliTaq Gold DNA Polymerase and 1.0µL of each of the10 pmole/µL GPI primers. The reaction mixture was subjected to an initial step of denaturation at 95ºC for 10 minutes followed by 40 cycles of denaturation at 95ºC for 15 seconds, annealing at 60ºC for 15 seconds and extension at 72ºC for 1 minute followed by a terminal step of extension at 72ºC for 5.0 minutes. The BAALC cDNA was amplified in the same way using BAALC specific primers:

- Forward primer, 5- GGGAGACAGAATCCACCTG 3-

• Reverse primer: 5- TGGACTCTCTGCTAGT-TGAC 3-.

The PCR products were resolved on ethidium bromide-stained 1.5% agarose gel and UV photographed. BAALC gene yields products of 221 and 388bp according to different splicing patterns 1, 8 or 1, 6, 8 respectively, and the GPI control primers result in an amplified fragment of 176bp (Fig. 1).

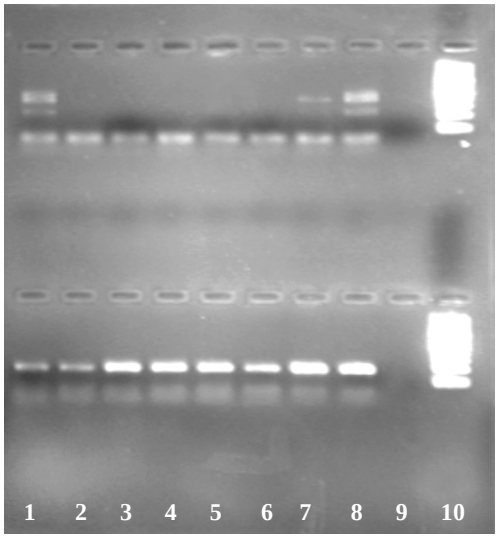

Fig. (1): Gel documentation of BAALC and GPI gene expression.

Lane 10 : 100bp ladder, Lane 9: Empty.
Upper panel : BAALC expression: 221 and 338bp bands are detectable in lanes 1, 7 and 8 and undetectable in lanes 2-6.
Lower panel: GPI gene expression: Lanes 1-8 show a 176 bp band.

Interpretation of BAALC gene expression was done according to Baldus et al. **[17]**. The authors evaluated BAALC gene expression by both real-time PCR and conventional PCR. BAALC gene is expressed in all cases and controls. However, only in cases with high expression levels an apparent band is detected on gel upon electrophoresis of the amplified product. On the other hand, when the expression of the gene is low by real time technique, no band could be detected on the gel. Accordingly, we considered cases with an apparent band as high expressers and those with undetectable bands as low expressers.

Statistical analysis was performed using SPSS version 17 & EpiInfo 6 (WHO) software. Normality of distribution was computed by W Shapiro-Wilk's test. The comparison of quantitative data was performed by independent *t*-test or Man Whitney test for 2 groups and by ANOVA test or Kruskal Wallis test for more than 2 groups. Comparison of qualitative variables were done using X^2 test or Fisher exact test where appropriate. Statistical significance was set at a level of 0.05. Survival curves according to type of gene expression whether high or low were derived using the Kaplan-Meier method and compared using log-rank tests.

RESULTS

BAALC gene expression in CN-AML:

High and low BAALC gene expression was encountered each in 50% of the studied cases. No statistically significant differences were encountered between both groups with regards to age, gender, organomegaly, lymphadenopathy, or occurrence of infection (Table 1). Extramedullary disease was not detected in any of the studied cases.

Table (1): Demographic and clinical features of 38 CN-AML patients in relation to BAALC gene expression.

Parameter	BAALC gene expression			*p* value
	High (N. 19)	Low (N. 19)	Total (N. 38)	
Age: Years	35.3±16.8* (2-61)	30.7±27 (0.5-80)	–	0.79
Gender:				
Male	7 (36.8)**	12 (63.2)	19 (50)	0.1
Female	12 (63.2)	7 (36.8)	19 (50)	
Hepatomegaly	18 (94.7)	14 (73.7)	32 (84.2)	0.18
Splenomegaly	12 (63.2)	15 (78.9)	27 (71.1)	0.28
Lymphadenopathy	3 (15.8)	2 (10.5)	5 (13.2)	1
Infection	10 (52.6)	6 (33.3)	16 (43.2)	0.244

* Mean ± SD (range).
** No (%).

No statistically significant differences were encountered between high and low BAALC expressers with regards to Hb level, TLC, platelet count or percentage of BM blasts at first presentation, at day 14 or at day 28 post chemotherapy (Table 2).

Patients with high BAALC expression tended to have higher blast percentage in peripheral blood at presentation than those with low BAALC expression; the difference is near significance (*p*-value 0.08).

Table (2): Hematological parameters in 38 CN-AML patients in relation to BAALC gene expression.

Parameter	BAALC gene expression		*p* value
	High expressers (N. 19)	Low expressers (N. 19)	
Hb.: (g/dl)	7.6±2.6* (3.6-11.6)	8±1.6 (4.4-11.8)	0.96
TLC: x 10^9/L	52±57 (1.7-374)	57.8±68 (2.7-240)	0.71
Platelets: x 10^9/L	88.9±75.9 (13-280)	67.5±51.9 (18-181)	0.45
PB blast. At presentation: %	61.4±30 (2-90)	44.6±24 (5-90)	0.08
BM blast At presentation: %	60.4±23.6 (24-98)	58.8±18 (31-95)	0.82
On day 14: %	25.4±30.7 (0-75)	8.4±13.7 (0-50)	0.24
On day 28: %	15±23 (0-64)	12±26.5 (0-90)	0.79

* Mean ± SD (range).

FAB classification was available for 36 cases. They included 11M0, 22 M1, 3 M4; no M5, M6 or M7 were encountered in our cohort.

High BAALC expression was encountered in 81.8% of both M0/M1 cases as compared to 55.9% of the M2 and 66.6% of the M4 cases. The difference is statistically significant (*p*=0.01).

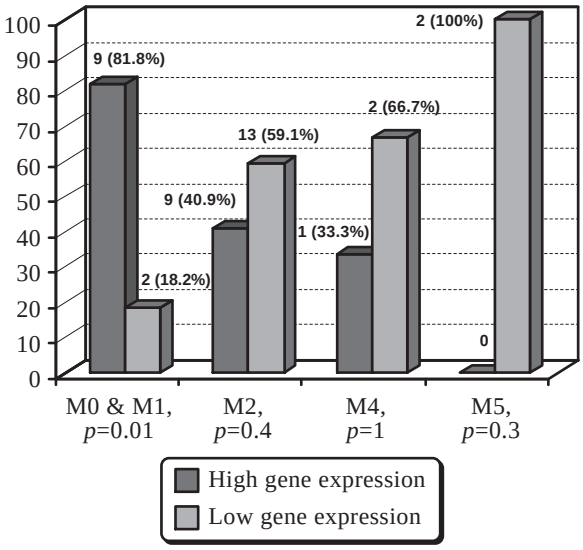

Fig. (2): Distribution of high and low BAALC gene expressers among the different FAB subgroups in 38* AML patients in relation to BAALC gene expression.

No statistically significant association was encountered between high and low BAALC expressers and any of the surface markers expressed by the leukemic cells.

The clinical outcome of treatment in 26 CN-AML patients in relation to BAALC gene expression is presented in Table (3). High BAALC gene expressers had significantly lower CR rate (*p*=0.04) and higher frequency of primary resistance to chemotherapy (*p*-value 0.02).

There was a trend for high expressers to have relapsed after remission (66.7% vs. 27.3%). However the difference did no achieve statistical significance.

Table (3): Clinical outcome of treatment in 26 CN-AML patients in relation to BAALC gene expression.

Outcome	BAALC gene expression			*p* value
	High No. (13)	Low No. (13)	Total No. (26)	
Complete remission	6 (46.2)	11 (84.6)	17 (65.4)	0.04
Primary resistance	9 (69.2)	3 (23.1)	12 (46.2)	0.02
Relapse after remission	4 (66.7	3 (27.3)	7 (41.2)	0.29

The overall survival in 26 AML patients with normal cytogenetics in relation to BAALC gene expression is presented in Table (4) and Fig. (3). Patients with high BAALC gene expression had a median OS of 1.5 with a range of 0-4 months as compared to a median of 10.5 and a range of 1.5-16.5 months in patients with low BAALC gene expression (*p*=0.003).

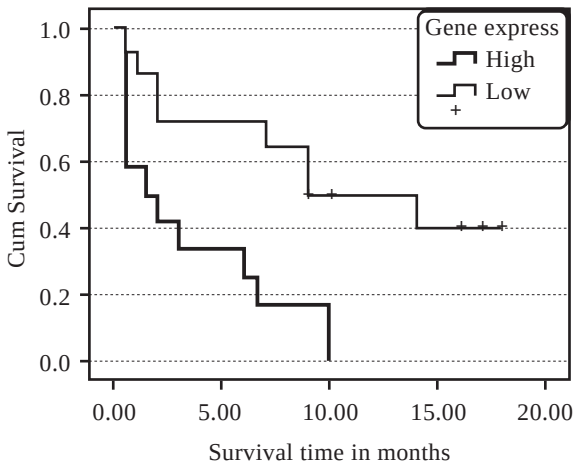

Fig. (3): Relation between overall survival in months and BAALC gene expression in 26 cytogenetically normal AML patients.

Apart from the CN-AML cases, high BAALC gene expression was found in 2/2 AML cases with trisomy 8, 1/1 AML case with hypodiploidy, 2/4 CML cases in blastic crisis and 2/2 secondary AML cases on top of MDS. On the other hand, none of the 7 M3 cases showed high BAALC expression.

DISCUSSION

In this work we have evaluated the BAALC gene expression in 68 AML cases. Patients were judged as high or low expressers according to the detect ability of the amplified band on gel electrophoresis. Most of the previous studies evaluated BAALC expression by real-time PCR considering the median level obtained in each cohort as the discriminator between high and low expression. Accordingly the cutoffs are different according to the studied cohort and comparison between studies is difficult. This issue was objectively addressed in the study of Baldus et al., **[17]** and Tanner et al. **[18]**. The authors evaluated the expression level by both real-time and conventional PCR. In high expressers, as judged by real-time, the amplicon gave a detectable band on gel electrophoresis while no band could be visualized in low expressers. Thus conventional electrophoresis could be considered as simple relatively objective method for categorization of cases into high and low BAALC gene expression which was adopted in the current study.

In the present work, no statistically significant differences were encountered between high and low BAALC gene expresser CN-AML patients with regards to age, gender, organomegaly, lymphadenopathy, or infection. This is in agreement with Baldus et al., **[15,17]**. One previous study reported male predominance **[19]** and another reported female predominance **[20]**. Both studies used the median level obtained by real-time PCR as the discriminating level which makes comparison difficult.

In the current study, no statistically significant differences were encountered between the high and low BAALC gene expresser CN-AML patients with regards to Hb level, TLC, platelet count or number of blasts in bone marrow at presentation. This is in agreement with previous studies **[15,17,21]**. However, we encountered a higher PB blast% at presentation in the high expresser group with a near significant difference (p=0.08). This is supported by the findings of Baldus et al. **[15]** who reported that high BAALC expressers had a 56% PB blasts at presentation vs. 31%, in low BAALC expressers, (p-value 0.004). Failure of achieving statistical significance in our cohort may be attributed to our small sample size. The association of high BAALC expression with higher PB blasts may be explained by the effect of BAALC gene on hematopoietic progenitors; it inhibits differentiation of the progenitors and favors their proliferation **[18]**. In contrast, Baldus et al. **[17]** reported the association of low BAALC expression with a significantly higher white blood count. The discrepancy is difficult to explain and it may be just a coincidence rather than a real association.

In the current study, high BAALC gene expression was found to be associated with the primitive FAB subgroup (M0 and M1). Similar findings were reported by Bienz et al., **[14]** and Tanner et al. **[18]**. The higher frequency of high BAALC gene expression among M0 (Minimally differentiated AML) and M1 (Acute myeloid leukemia without maturation) FAB subgroups can be attributed to the effect of BAALC gene on differentiation and proliferation, inhibiting differentiation and promoting proliferation **[17]**.

In the current study, no significant differences were reported between high BAALC- and low BAALC-expresser CN-AML patients with respect to the marker expression by the leukemic blasts including CD34, as well as different myeloid, monocytic and aberrant lymphoid markers. In contrast significant association between CD34 and high BAALC expression was previously reported **[14]**. The finding of the latter authors is in harmony with the assumed role of BAALC gene in inhibiting differentiation and favoring proliferation of hematopoietic progenitors **[17]**. We have to study a larger cohort to verify this potential association in our series.

In the current study, the response to treatment was impacted by the level of BAALC gene expression. The BM% blasts tended to be higher in CN-AML patients with high BAALC gene expression both on the 14th and 28th days after the start of chemotherapy though statistical significance was not achieved. Blasts on day 14 post chemotherapy is an early indicator of the prognosis; data from UK MRC AML10 study as well as from German Cooperative

Group showed that if patients failed to adequately clear their blast count, they will do badly even if CR is subsequently achieved **[22]**.

In the current study, failure of remission induction either due to death in induction or failure to reduce the blast count to less than 5% of nucleated marrow cells, at the end of 2nd induction cycle, was significantly higher in high than in low BAALC gene expressers. This is in agreement with previous studies that reported a higher rate of primary resistance and refractoriness to chemotherapy among high BAALC gene expressers **[15,21,23]**. Also, the current study showed a statistically significant association between low BAALC-expression and achievement of CR. Though this association was denied in earlier studies **[14,17]**; yet it was documented in more recent ones **[15,21]**.

Despite being statistically insignificant; that might be due to the small sample size, the present work showed a high tendency for relapse in the high BAALC gene expressers. 66.7% of high BAALC gene expression patients had relapses vs. 27.3% of low BAALC gene expression patients. This is in harmony with previous studies **[15,17,21]**.

In the present study, OS time was longer among the low BAALC gene expression patients, mean 10.8 (Range 7.1, 14.5) with a median of 9 months compared to a mean of 3.5 (Range 1.4, 5.6) with a median of 1.5 months in the high BAALC gene expression group (p=0.003). This is in agreement with several reports **[14,15,17,21]**. Baldus et al. **[17]** reported that high BAALC expression remained a significant adverse prognostic factor for FLT3ITD/WT and FLT3WT/WT patients, compared to those with low BAALC expression (OS, p=.002; hazard ratio [HR]=2.7; DFS, p=.04, HR=2.2). The emphasized that high BAALC expression is an independent adverse prognostic factor for EFS regardless of the FLT3 status (p=.008, HR=2.2). The adverse effects observed in high BAALC expresser CNAML patients was attributed, at least partly, to the highly up-regulated multidrug resistance genes MDR1 and ABCB1 **[24]**.

In the current study, apart from the CN-AML cases, high BAALC gene expression was detected in 2/2 AML cases with trisomy 8, 1/1 AML case with hypodiploidy, 2/4 CML cases in blastic crisis and 2/2 secondary AML cases on top of MDS but none of the 7 M3 cases. This is because BAALC gene expression in hematopoietic cells is restricted to the compartment of progenitor cells, whereas no expression could be detected in mature bone marrow or circulating normal white blood cells **[25]**. High BAALC gene expression levels were first identified in a study of AML patients with trisomy 8 as a sole abnormality **[25]**. Subsequent studies also suggested a trend towards higher BAALC in patients with trisomy 8 as compared to CN-AML patients **[21]**. This is reasonable because BAALC gene is located on chromosome 8q22.3 **[18]**. Trisomy 8 is known to have a negative prognostic relevance in AML and it might be hypothesized that over expression of BAALC might be the molecular basis of such outcome even in AML patients lacking this cytogenetic abnormalities **[25]**. High BAALC gene expression was previously reported in CML in blastic crisis while none of the chronic phase CML cases showed high BAALC gene expression **[17,18]**.

In conclusion, our study has proved, in an Egyptian cohort of CN-AML patients, that high-level expression of, BAALC gene, can predispose to an adverse outcome including lower incidence of CR, higher incidence of resistant disease, shorter OS and a trend to a higher incidence of relapse. Currently, in Egypt, evaluation of BAALC gene is not included in the routine workup of AML patients. This study would strongly recommend its inclusion in the initial molecular characterization of newly diagnosed cases.

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