Original Article

Avicenna Journal of Medical Biotechnology
Volume 15, Issue 3, 167-172
https://doi.org/10.18502/ajmb.v15i3.12926

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Investigation of Expression Profile of Placenta-specific 1 (PLAC1) in Acute Myeloid and Lymphoid Leukemias

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Received: 24 Sept 2022
Accepted: 26 Apr 2023

Abstract

Background: Placenta-specific 1 (PLAC1) is one of the cancer-testis-placenta antigens that has no expression in normal tissue except placenta trophoblast and testicular germ cells, but is overexpressed in a variety of solid tumors. There is a lack of studies on the expression of PLAC1 in leukemia. We investigated expression of *PLAC1* in Acute Myeloid Leukemia (AML) and Acute Lymphoblastic Leukemia (ALL).

Methods: In this study, we investigated expression pattern of *PLAC1* gene in peripheral blood and bone marrow mononuclear cells of newly-diagnosed patients with AML (n=31) and ALL (n=31) using quantitative real-time PCR. Normal subjects (n=17) were considered as control. The PLAC1 protein expression in the samples were also detected using western blotting.

Results: Our data demonstrated that *PLAC1* transcripts had 2.7 and 2.9 fold-change increase in AML and ALL, respectively, compared to normal samples. *PLAC1* transcript expression was totally negative in all studied normal subjects. Level of *PLAC1* mRNA expression in ALL statistically increased compared to normal samples (p=0.038). However, relative mRNA expression of *PLAC1* in AML was not significant in comparison to normal subjects (p=0.848). Furthermore, relative mRNA expression of *PLAC1* in AML subtypes was not statistically significant (p=0.756). *PLAC1* gene expression showed no difference in demographical clinical and para-clinical parameters. Western blotting confirmed expression of PLAC1 in the ALL and AML samples.

Conclusion: Considering *PLAC1* expression profile in acute leukemia, PLAC1 could be a potential marker in leukemia which needs complementary studies in the future.

Keywords: Acute lymphoblastic leukemia, Acute Myeloid Leukemia, Biomarker, Expression profile, Leukemia, PLAC1

To cite this article: Gholami P, Asgarian-Omran H, Yaghmaie M, Mahmudian J, Kianersi Sh, Salari S, et al. Investigation of Expression Profile of Placenta-specific 1 (PLAC1) in Acute Myeloid and Lymphoid Leukemias. Avicenna J Med Biotech 2023;15(3):167-172.

Introduction

Acute Myeloid Leukemia (AML) is a heterogeneous disorder of hematopoietic stem cells that mainly affects middle-aged people (mean age of diagnosis is 69 years). AML is seen with uncontrolled clonal proliferation of myeloid progenitor cells leading to defects in normal hematopoiesis [1]. It is the most common acute leukemia that accounting for about 80% of cases in this group. In the United States, the incidence of AML is 3-5 cases per 100000 population and over 20000 per year [2]. Acute Lymphoblastic Leukemia (ALL) is a clonal malignancy characterized by the accumulation of lymphocyte blasts that do not differentiate into adult lymphocytes [3]. ALL is the most common cancer among children and the most important cause of cancer death

in people under 20 years of age [4]. The disease appears to begin with genetic mutations in hematopoietic stem cells. These mutations accumulate in stem cells to eventually lead to three functional disorders, including increased proliferation, cessation of blast differentiation, and decreased apoptotic death [5]. There is no tumor marker with a wide expression pattern in the acute leukemia that could be applicable in general diagnosis or targeting leukemic cells, thus scientists are now searching for such biomarker.

Placenta-specific 1 (*PLAC1)* is an X-linked (Xq26) gene with a conserved structure in mice and humans that is involved in placental evolution [6,7]. It has six exons located in an area of about 200 *Kbp*, with only one protein-containing transcript in humans (NP-068568.1, ENS T00000359237) [7]. *PLAC1* has two promoters, P1 and P2, which are about 100 *Kbp* apart, but all 639 *bp* that contain the gene coding sequence are in the exon 6. Therefore, the PLAC1 protein is the product of the last and the largest exon of *PLAC1* gene [8]. Keratinocyte Growth Factor (KGF) increases the expression of *PLAC1* transcript by 2-3 times. This factor plays a role in regulating placental function and differentiation in mammals [9]. In healthy individuals, the expression of *PLAC1* is mainly limited to placental tissue and its lower levels of mRNA expression have been reported in the testes [10,11] and cerebellum [11,12].

In humans, PLAC1 protein is found in placenta with relatively constant expression during pregnancy [9,13]. The PLAC1 protein is found in the apical syncytiotrophoblast [7,13] and has been shown to bind to F-actin at the membrane and apical syncytial trophoblast levels. Studies have shown that PLAC1 protein is associated with membranes and is predominantly found in microvillous brush border membranes [7]. *In vitro* studies have shown that silencing *PLAC1* gene using siRNA in MCF-7 and BT-544 cell lines reduces motility, migration, and invasion, as well cell cycle arrest leading to a reduction of about 80-90% in cell proliferation. Decreased *PLAC1* expression was associated with decreased in cyclin D1 expression and AKT kinase phosphorylation [14]. Importantly, *PLAC1* expression in both mRNA and protein levels were detected in a verity of malignancies including hepatocellular carcinoma [15], ovarian cancer [16], prostate cancer [17], melanoma [18], gastric adenocarcinoma [19] and breast cancer [20].There is a lack of study on the expression of *PLAC1* in leukemia such as AML and ALL. Based on popularity of this type of cancers, here we investigated the expression pattern of *PLAC1* in the newly-diagnosed patients with AML and ALL.

Materials and Methods

Samples collection

Sixty two EDTA-anticoagulated Peripheral Blood (PB) and Bone Marrow (BM) samples were obtained from AML (BM=13, and PB=18) and ALL (BM=16, and PB=15) patients that had refreed to Blood and Oncology Department of Imam Khomeini Hospital (Mazandaran University of Medical Sciences), Hematology Research Center of Oncology and Stem Cell Transplantation (Tehran University of Medical Sciences) or Taleghani Hospital (Shahid Beheshti University of Medical Sciences) during October 2018 to September 2019 (Table 1). Blood samples were also taken from 17 normal healthy individuals (the mean age: 35.6 years; SD: 12.9 and 64.7% male) for the control group. A consent letter was taken from all patients and control group. This study was approved by the Ethical Committee of Shahid Beheshti University of Medical Sciences (IR.SBMU.MSP.REC.1398.328).

RNA Extraction and cDNA preparation

Mononuclear cells were immediately isolated by Ficoll density centrifugation, and washed twice with Phosphate Buffered Saline (PBS, pH=7.2, 0.15 *M*). The separated cells were lysed in Trizol reagent (Yekta Tajhiz Azma, Iran). Total RNA extraction was performed following the manufacturer's instructions. Single-stranded cDNA preparation was performed from 1 μg total RNA of each sample using reverse transcriptase (Thermo Scientific, Lithuania) and random primer (Cybergene, Sweden).

Quantitative RT-PCR

We used the SYBR green (Yekta Tajhiz Azma, Iran) to determine *PLAC1* expression levels. The specific primers were used as follows: *PLAC1* sense, 5'-GTGAGCACAAAGCCACATTTC-3' and antisense, 5'-GCAGCCAATCAGATAATGAACC-3'; *GAPDH* sense, 5'- GAAGGTGAAGGTCGGAGTC -3' and antisense, 5'-GAAGATGGTGATGGGATTTC-3' (Bioneer,

Table 1. Demographic and laboratory findings of studied acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL)

Characteristic	Results	
	ALL group	AML group
Age, mean+SD	20.8+16.7	48.7+19.1
Male	19 (73.1%)	17 (54.8%)
Female	7 (26.9%)	14 (45.2%)
Sample	BM: 16 PB: 15	BM: 13 PB: 18
WBC ($\times 10^3/\mu l$), mean+SD	46.2+62.6	38.6+55.6
PLT ($\times 103/\mu l$), mean+SD	347+23.6	545+32.9
Hb (*gr/dl*), mean+SD	8.8+2.0	8.1+1.3
PB Blast (%)	26.3	36.4
BM Blast (%)	67.3	58.2
Subtype (FAB/ immunophenotype)	B-ALL: 26 (83.9%) T-ALL: 5 (16.1%)	M0: 2 (6.5%) M1: 7 (22.6%) M2: 8 (25.8%) M3: 3 (9.7%) M4: 5 (16.1%) M5: 4 (12.9%) M6: 1 (3.2%)

Korea). The quantitative PCR (qPCR) assays were conducted on a Corbett rotor gene 6000 (QIAGEN, Germany). qPCR mainly included enzyme activation (15 *min* at 95°*C*), followed by denaturation (15 *s*, 95°*C*), annealing, extension and florescence acquiring (60 *s*, 60°*C*) for 40 cycles. The amplicons' size of *PLAC1* and *GAPDH* genes were 118 *bp* and 226 *bp*, respectively. qPCR results were analyzed and expressed relative to threshold cycle (CT) values and then converted to fold-change. The *PLAC1* gene expression was normalized using *GAPDH* as house-keeping internal control and relative gene expression data presented as fold-change compared to normal group based on $2^{-\Delta\Delta CT}$ method.

Western blotting

Separated mononuclear cells (1×10^6) were lysed in 50 *µl* sample buffer containing 150 *mM* Tris Ph=6.8, 1.2% SDS, 200 *mM* 2-ME, 0.2% bromophenol blue and 33% glycerol on ice. The lysates were boiled for 10 *min* and then centrifuged at 17700×g for 20 *min* at 4°*C*. Thirty *µl* of cell lysates and 35 *ng* recombinant human PLAC1 (rhPLAC1) (produced in Dr. Zarnani's lab) were run on a 14% SDS-PAGE gel at 100 *V* for 2 *hrs* and then transferred to PVDF membrane at 200 *mA* for one *hr*. The existence of protein bands was visualized by Ponceau S staining. The membrane was blocked with PBS-0.1% Tween 20 (PBS-T) containing 5% skimmed milk solution overnight without shaking at 4°*C*. The membrane was then immunoprobed with rabbit anti-rhPLAC1 antibody (produced in Dr. Zarnani's lab) at 2 *µg/ml* in PBS-T containing 1% skimmed milk solution for 1.5 *hr* with shaking at RT followed by goat anti-rabbit IgG-HRP (1:6000) (Bio-Rad, Hercules, CA, USA) in PBS-T containing 1% skimmed milk solution for one *hr* with shaking at RT. After extensive washing, membrane was developed with ECL. Anti-β-actin rabbit monoclonal antibody (Sigma, St. Louis, USA) was used for visualization of β-actin after re-probing of the membrane.

Statistical analysis

IBM SPSS statistics 26 (SPSS Inc., Chicago, IL, USA), and the REST software was used for the statistical analysis. The results of data analysis are reported based on p value and in the form of tables and graphs. In a quantitative study, the Kolmogrov-Smirnov test was used to determine whether the data distribution was normal or abnormal. Because the distribution of our study data was abnormal, non-parametric statistical tests such as Kruskal Wallis and Man-Whitney U tests were used to compare the means between several groups and the two groups, respectively, and to determine the correlation between quantitative parameters performed by Spearman Correlation Test. The results with $p<0.05$ were considered statistically significant.

Results

Relative expression analysis of PLAC1 by real time PCR

Specific amplification of the *GAPDH* and *PLAC1* genes were confirmed by agarose gel electrophoresis. The amplicons' size of *PLAC1* and *GAPDH* genes were compatible with the expected sizes as 118 *bp* and 226 *bp*, respectively (Figures 1A and B). The amplicon and melting curves of the *GAPDH* and *PLAC1* was also confirmed with their specific amplification (Figure 2). Furthermore, *PLAC1* PCR product of one AML sample was confirmed by Sanger sequencing (ABI 3500 genetic analyzer, Thermofisher Scientific, Waltham, MA, USA; data not shown). In addition, the efficiency of *PLAC1* (96%) and *GAPDH* (98%) amplification was validated using MDA-MB-231 cells, as positive control.

Our data demonstrated that *PLAC1* transcripts had 2.9 fold-change increase in ALL compared to normal samples. Further analysis pointed out that the level of *PLAC1* mRNA expression in both PB and BM mononuclear cells of ALL patients was significantly higher compared to normal samples (p=0.038) (Figure 3). Interestingly, *PLAC1* transcript expression compared to normal control group was statistically significant just in B-ALL but not in T-ALL subtype (p=0.029) (Figure 3). Furthermore, *PLAC1* expression was significantly different just in BM and PB samples of B-ALL (p=0.04), but not in T-ALL.

Furthermore, *PLAC1* transcripts had 2.7 fold-change increase in AML compared to normal samples. But despite of ALL samples, relative mRNA expression of *PLAC1* in AML samples was not significantly different compared to normal samples (p=0.848) (Figure 3). Furthermore, relative mRNA expression of *PLAC1* in AML subtypes was not statistically significant (p= 0.756). In comparison to normal PB samples, *PLAC1* expression was significantly higher in PB (p=0.011)

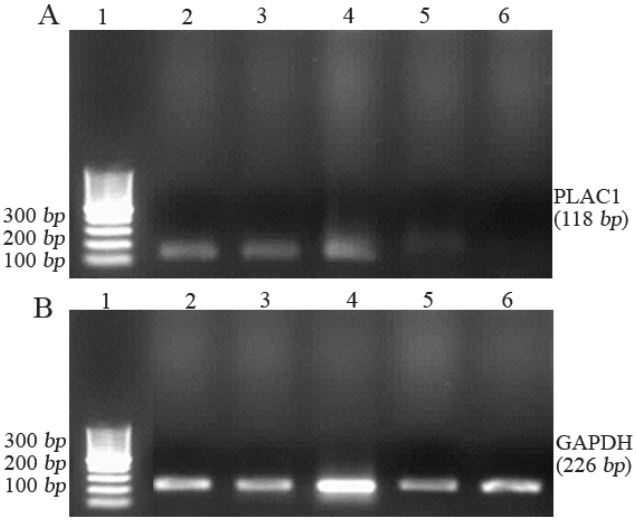

Figure 1. Agarose gel electrophoresis of real-time PCR products of PLAC1 (A) and GAPDH (B).
Lanes 1: marker 100 *bp*, 2: AML12-BM, 3: AML7-PB, 4: ALL1-BM, 5: ALL23-PB, 6: Normal 4.

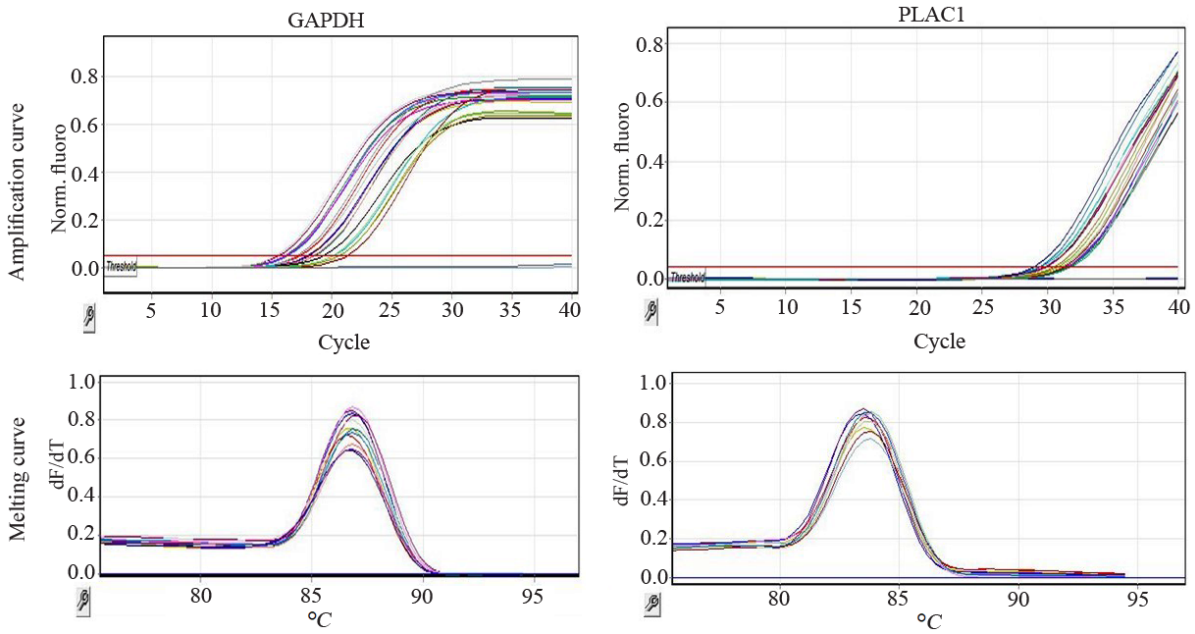

Figure 2. The real time PCR amplification curve and melting curve of *PLAC1* and *GAPDH.*

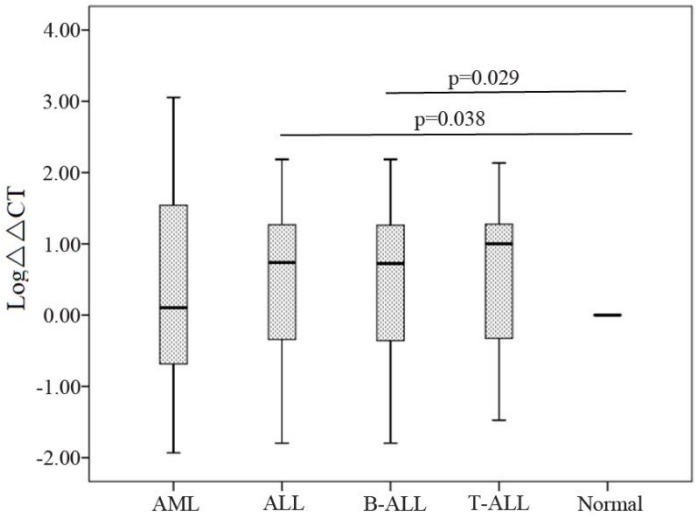

Figure 3. Comparison of *PLAC1* mRNA expression in leukemic and normal groups.

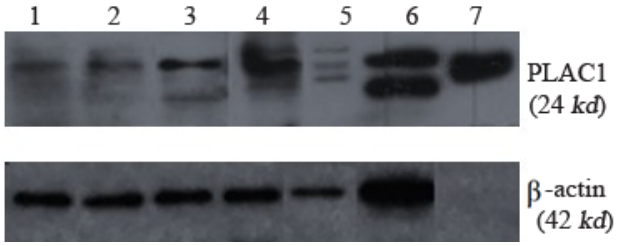

Figure 4. Representative data on PLAC1 protein expression pattern in AML and ALL patients. PLAC1 and β-actin proteins bands laid on 21 and 42 *kDa* in SDS-PAGE 10%.
Lanes 1: AML21-PB, 2: AML23-PB, 3: ALL25-PB, 4: ALL27-PB, 5: ALL19-BM, 6: human placenta, 7: PLAC1 recombinant protein.

and BM blasts (p=0.029) of AML and ALL patients. Analysis of *PLAC1* expression showed that *PLAC1* had no difference in demographical clinical and paraclinical subgroups.

PLAC1 ***protein expression by Western blotting***

PLAC1 expression at protein level in 5 samples was checked in immunoblotting using a rabbit polyclonal antibody specific to human PLAC1 recombinant protein (produced in Dr. Zarnani's laboratory). Based on the immunoblotting findings, the expression of PLAC1 protein was consistent with the expression of *PLAC1* gene, and samples with positive *PLAC1* transcript had PLAC1 protein expression, too. Representative Western blot results with anti-PLAC1 and β-actin antibodies are presented in figure 4, as expected PLAC1 and β-actin showed 24 and 42 *kDa* sizes, respectively.

Discussion

Due to the lack of access to a suitable biomarker that can be used as a diagnostic marker in all AML or ALL patients, we investigated the expression of *PLAC1* transcript in these leukemias. In this study the expression of *PLAC1* transcript is detected in ALL and AML to a significant extent, which requires more examination on a larger number of samples. In addition to quantitative analysis of *PLAC1* real-time PCR, we arbitrary considered samples with CT ≤34 as positive and the cases with CT ≥35 as negative for *PLAC1* mRNA

expression. According to this criterion, *PLAC1* expression was positive in 67.7% of ALL and 48.3% of AML patients. In this regard, *PLAC1* gene expression was negative in all included normal samples. Furthermore, *PLAC1* expression was detected in 69.2 and 60% of B-ALL and T-ALL, respectively. Although, the sample size of different subgroups of B-ALL was very small, interestingly Pre-B ALL subtype had the highest *PLAC1* expression (6 out of 9; 65.2%). This finding was also found in AML-M1 with 71.4% (5 out of 7 cases) as the highest frequency of *PLAC1* positive samples among AML FAB subtypes. In addition, the expression of *PLAC1* transcript was examined in normal healthy individuals and none of the normal individuals had *PLAC1* transcript in PB samples, which is a confirmation of the lack of expression of *PLAC1* molecule in normal tissues except placenta and testis [10].

Identification of genes that are expressed in tumors and have no expression in normal tissues is a prerequisite for the study of cancer immunotherapy. Identification of these genes will also help advance diagnosis and treatment, including tumor immunotherapy. PLAC1 is a molecule of the cancer testis family of antigens that is expressed in placental and testicular tissue but is not expressed in normal tissues, according to published studies [10]. Xue-Yuan Dong *et al* showed that *PLAC1* is highly expressed in hepatocellular carcinoma tissue and anti-PLAC1 antibodies have been found it in the serum of patients which indicated its immunogenicity [15]. Nana E. Tchabo *et al* showed that *PLAC1* and DPPA2 were among the testicular cancer antigens (CTA) that were expressed on 21 and 31% ovarian epithelial cancer, respectively [16].

In another study Roya Ghods *et al* examined the pattern of expression of the *PLAC1* gene in advanced prostate adenocarcinoma. They reported that in prostate cancer, as the expression of *PLAC1* increased, the expression of (prostate specific antigen) PSA decreased [18]. In another study by Roya Ghods *et al* on melanoma, 100% of PLAC1 expression on melanoma tissues was observed [19]. Fangfang Liu *et al* proposed PLAC1 as a potential biomarker in the diagnosis and treatment of gastric adenocarcinoma [20]. In a study on breast cancer by Hongyan Yuan *et al*, PLAC1 was detected as a biomarker in the serum of 40-60% of breast cancer patients [21]. Yongfei Li and colleagues showed that following the interaction of *PLAC1* with Furin, the intracellular domain of Notch1 transcription factor, called NICD, is activated and inhibits PTEN suppressor tumor expression, a pathway leads to tumor progression, proliferation and metastasis. Therefore, high expression of *PLAC1* in breast cancer is associated with poor prognosis, migration and tumor invasion [22]. Qiongshu Li and colleagues investigated the targeting of PLAC1 in breast cancer using genetically engineered T cells against PLAC1 (CAR-T-PLAC1 cells) [23]. All reported studies indicated the importance of PLAC1 in biology of tumors and the potential applications of this molecule in diagnosis and therapy of PLAC1 positive cancers.

Conclusion

In conclusion, due to the lack of *PLAC1* expression in normal samples and its expression in ALL and AML patient samples, PLAC1 can be considered as a tumor-specific antigen or tumor-dependent antigen, which seems to be expressed in some ALL and AML patients. Additional studies are suggested in more samples in each leukemia group and with better homogeneity, for stronger results.

Statement of Ethics

The Shahid Beheshti University of Medical Sciences research committee approved the study (IR.SBMU.MSP.REC.1398.328). All experiments or tests performed involving human subjects were conducted according to the institute ethical standards and the Declaration of Helsinki. Samples of 1-2 *ml* of aspirated bone marrow and 3-5 *ml* of peripheral blood were collected in vials containing EDTA after obtaining the signature of the patient or the patient's guardian on an informed consent form as approved by Shahid Beheshti University of Medical Sciences Research Committee.

Data Availability Statement

The dataset used and analyzed during the current study are available from the corresponding author upon reasonable request.

Acknowledgement

This work is financially supported by Shahid Beheshti University of Medical Sciences (grant number: 15082). This work is part of Parastou Gholami's thesis. We appreciate Ms. Sedighe Vafaei for letting us have her valuable experience on setting up Western blot.

Conflict of Interest

All authors declare that there is no conflict of interest.

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