

ABERRANT EXPRESSION OF PLACENTAL-LIKE ALKALINE PHOSPHATASE IN CHRONIC MYELOID LEUKEMIA CELLS *IN VITRO* AND ITS MODULATION BY VITAMIN E

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Background: Placental-like alkaline phosphatase (PLAP) is expressed by many tumors and can be detected in sera of patients with various cancers. Its aberrant expression has been considered to be potentially useful as tumor marker. However, the biological background of the role of this aberrant alkaline phosphatase (AP) in cancer is still unclear. The expression of various forms of AP in cells of chronic myeloid leukemia (CML) has not yet been studied. **Aim:** To analyze the expression patterns of various AP forms in cells originated from CML patients in blast crisis and to modify their expression by vitamin E. **Materials and Methods:** RNA extracted from leukemic cells was converted to cDNA and real-time reverse transcription polymerase chain reaction was performed using SYBR Green protocol with primers to tissue non-specific alkaline phosphatase (TNAP), intestinal alkaline phosphatase and CCAAT-enhancer-binding proteins alpha (C/EBP α). To analyze the modulation of expression of APs and C/EBP α , CML cells were incubated with 100 μ M vitamin E. **Results:** We have observed the aberrant expression of mRNA intestinal alkaline phosphatase in CML cells that upon sequencing demonstrated the significant alignment with PLAP sequence while no gene homology with tissue placental alkaline phosphatase (PAP) was revealed. Vitamin E decreases mRNA PLAP expression and increases mRNA TNAP expression. Moreover, along with down-regulation of aberrant PLAP and up-regulation of TNAP, vitamin E increases C/EBP α mRNA expression. **Conclusion:** The loss of TNAP in CML may contribute to pathogenesis of this disease. PLAP may be considered as a putative target in differentiation therapies in myeloid neoplasms. Our findings suggest the potential role of vitamin E as the inducer of differentiation potential of leukemic cells in CML.

Key Words: chronic myeloid leukemia, differentiation, alkaline phosphatase, vitamin E.

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Chronic myeloid leukemia (CML) is a clonal hematopoietic stem cell (HSC) disorder associated with the activity of BCR-ABL fusion oncogene due to the reciprocal translocation t(9;22)(q34;q11) that is characterized by an increased growth of abnormal myeloid progenitor cells within the bone marrow [1, 2]. The constitutively active P210 BCR-ABL tyrosine kinase is considered as a key player in the molecular pathogenesis of CML [3, 4]. CML treatment involves targeting BCR-ABL to prevent its tyrosine kinase activity by tyrosine kinase inhibitors (imatinib, nilotinib and dasatinib), which effectively target progenitor cells, however leukemic stem cells (LSCs) are less sensitive to such a treatment [5–8]. CML LSCs do not depend on BCR-ABL signaling for their survival [9, 10], and their persistence remains a major obstacle to curing CML [11, 12]. The search for new biological markers of LSC phenotype is still relevant today.

The activity of alkaline phosphatase (AP) in blood serum known as nonspecific marker of bone metastasis [13] is also of potential significance for the identification of stem cell phenotype [14]. Moreover,

AP activity is a widely accepted marker of stem cells associating with embryonic stem cell pluripotency [15]. AP (EC 3.1.3.1 orthophosphoric-monoesterase, alkaline optimum) is a membrane bound enzyme with commonly bone matrix mineralization function [16]. Four genes encode APs in humans: three genes, intestinal (IAP), placental (terminal) (PAP), and placental-like known also as germ cell AP (PLAP), display tissue-specific expression and are located near the end of long arm of chromosome 2, whereas the fourth AP is tissue non-specific (bone, kidney liver) (TNAP) and is located near the end of the short arm of chromosome 1. IAP, PAP and PLAP share more homology with each other compared to TNAP [17]. Recently, TNAP recognized ultimately as mesenchymal stromal cell antigen-1 [18] was described as a biomarker associated with normal hematopoiesis as well as with terminal myeloid differentiation [19]. The decreased TNAP synthesis is a classical feature of CML used as one of diagnostic cytochemical markers in differential diagnosis [20]. Nevertheless, in available literature we found no data on the expression of different AP forms in CML. We suggest that the analysis of AP expression may provide better understanding of CML biology. Therefore, the aim of our study was to analyze the expression patterns of various AP forms in CML cells and to explore the possibility of modifying their expression *in vitro* by vitamin E.

MATERIALS AND METHODS

The samples of peripheral blood from the patients diagnosed with CML in blast crisis (n = 3), acute mye-

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Abbreviations used: AML – acute myeloid leukemia; AP – alkaline phosphatase; C/EBP α – CCAAT-enhancer binding protein alpha; CML – chronic myeloid leukemia; HSC – hematopoietic stem cell; IAP – intestinal AP; LSC – leukemic stem cell; PAP – placental AP; PLAP – placental-like AP; PV – polycythemia vera; RT-PCR – reverse transcription polymerase chain reaction; TNAP – tissue non-specific AP.

loid leukemia (AML) (n = 2), and polycythemia vera (PV) (n = 1), who were under the treatment at the National Institute of Cancer, Kyiv, Ukraine, were analyzed in the study. The informed consent of all patients for taking blood samples for investigational purpose was provided.

K562 cell line originated from a CML patient in blast crisis was obtained from Depository of Cell Lines and Tumor Strains of the R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, the National Academy of Sciences of Ukraine. The cells were grown in suspension in RPMI- 1640 medium supplemented with 10% of fetal calf serum. Vitamin E (Technolog, Ltd., Ukraine) was added to culture medium in a final concentration of 100 μ M.

Total RNA was extracted using TRIzol (Invitrogen, USA) according to the manufacturer’s instructions. RNA was converted to cDNA using QuantiTect Reverse Transcription Kit (Qiagen, Germany). Real-time reverse transcription polymerase chain reaction (RT-PCR) was performed using SYBR Green protocol. RT-PCR reactions were carried out using HotStarTaq DNA polymerase (Qiagen, Germany), 50 ng of cDNA and SYBR Green in a 1:60,000 dilution in triplicate. PCR conditions were as follows: 95 $^{\circ}$ C initial activation for 15 min was followed by 45 cycles of 95 $^{\circ}$ C for 15 s, 60 $^{\circ}$ C for 15 s, and 72 $^{\circ}$ C for 30 s on iQ5 Real-time PCR Detection System (Bio-Rad, USA).

The primers used for real time RT-PCR assay: TNAP: forward — 5’-TGGCCGGAAATACATGTACCC-3’; reverse — 5’-TTCCGTGCGGTTCCAGATG-3’; PAP: forward — 5’-CCAGACCATTGGCTTGAGT-3’; reverse — 5’-AGTACCAGTTGCGGTTTCAC-3’; IAP: forward — 5’-AAGGGCAGAAGAAGGACAAA; reverse — 5’-GTC-GTGTGCACTGGTTAAAG; CCAAT-enhancer binding protein alpha (C/EBP α): forward — 5’-CAAGAA-CAGCAACGAGTACCG-3’; reverse — 5’-GTCACGTG-TCAACTCCAGCAC-3’; GAPDH: forward — 5’-CGCTCTCTGCTCCTCTGTT-3’; reverse — 5’-CCATG-GTGTCTGAGCGATGT-3’. The gene expression was quantified using 2^{- Δ Ct} method with normalization to mRNA expression of GAPDH. Statistical significance of differences was evaluated by Student’s *t*-test.

DNA sequencing analysis was performed on the automatic 3130 Genetic Analyzer (Applied Biosystems, USA) with computer Program Sequencing Analysis software V5.2. NCBI (American National Center for Bioinformation) for gene homology search strategies.

RESULTS

We have not detected *TNAP* mRNA expression by RT-PCR assay in peripheral blood cells of CML patients in blast crisis. The representative electrophoretic patterns of amplified products are given in Fig. 1. We also failed to detect *PAP* expression. However, in the blood sample of the CML patients in blast phase, we have revealed mRNA expression with primers to *IAP* gene, although the size of amplified product (410 bp) differed from that predicted for true *IAP* (see Fig. 1).

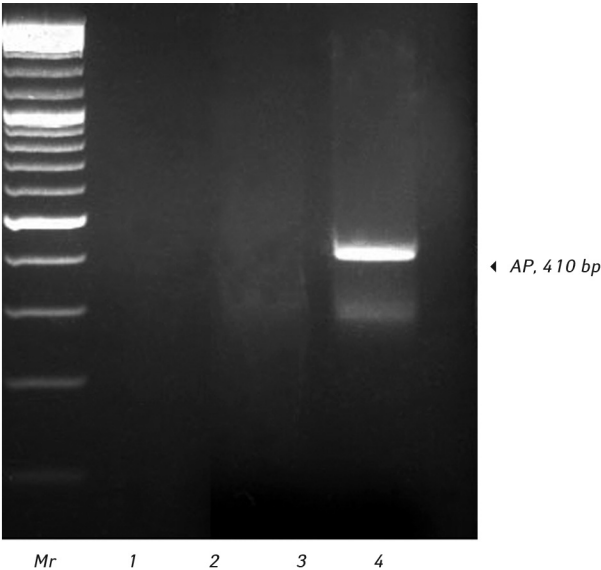


Fig. 1. The aberrant *AP* mRNA detected by RT-PCR in CML blast phase: 1 — control without primers; 2 — primers to *PAP*; 3 — primers to *TNAP*; 4 — primers to *IAP*

The aberrant *AP* amplification product was eluted from agarose gel and DNA-sequencing analysis was performed. According to the data obtained in DNA-sequencing analysis using GeneBank Program search, the aberrant *AP* gene matches more closely to *PLAP* (Table).

The forward primer for *IAP* gene as 5’-AAGGGCAGAAGAAGGACAAA-3’ was used for the amplicon 410 bp sequence analysis with following GeneBank (NCB) complete coding sequence homology search (see Table). Sequence alignment analysis of the amplified product (see Table) demonstrates the undoubted homology (89–92%) of the examined aberrant *AP* with human *PLAP* gene. Moreover, *PLAP* and *PAP* are known to be encoded by different genes [21]. Furthermore, the immunological cross-reactivity between human *PLAP*, *IAP*, and *PLAP* is known, but no cross-reactivity between any of these *AP* and

Table. Sequences of amplified product producing the most significant alignments

Description	Max score	Total score	Query cover, %	E value	Ident, %	Accession
Homo sapiens alkaline phosphatase, placental, RefSeqGene on chromosome 2	183	183	86	6e-43	92	NG_012189.1
	183	351	86	6e-43	92	AC068134.5
	183	345	86	6e-43	92	AF307337.1
Human placental heat-stable alkaline phosphatase (PLAP-1) gene, complete coding sequence	183	183	86	6e-43	92	M19159.1
Human alkaline phosphatase gene, complete coding sequence	167	167	86	6e-38	90	J03252.1
Human placental alkaline phosphatase-like gene 5’region	161	161	86	3e-36	89	X07247.1

the TNAP has been demonstrated [17]. The analysis of the alignment of the amplified sequences and the sequences from GeneBank (see Table), as well as the RT-PCR analysis of amplification products with primers to different APs (see Fig. 1), suggest the aberrant expression of *PLAP* mRNA in leukemic cells of CML patients in blast crisis. The embryonic origin of this AP should be mentioned.

Moreover, we have shown the aberrant *PLAP* expression in AML cells and a weak expression in cells of the peripheral blood of the patient with PV originating from HSC with limited lineage potential (Fig. 2). Therefore, presumable *PLAP* expression does not seem to be a characteristic feature of CML only. The ectopic *PLAP* expression in leukemic cells of different

myeloid neoplasms suggests its importance in biology of these malignancies.

To analyze further whether ectopic *PLAP* expression in CML cells *in vitro* may be modulated, we studied *PLAP* and *TNAP* expression in K562 cells incubated with vitamin E for 48 h. In fact, such treatment affects the expression of *PLAP* and *TNAP* in different ways. Namely, *PLAP* expression decreased significantly while *TNAP* expression increased (Fig. 3, a, b). Increase in *TNAP* expression was paralleled with increased *CEBPa* expression (Fig. 3, c).

Our data suggest that vitamin E represses the gene expression of aberrant *PLAP* and consequently restores the expression of two key factors of hematopoietic differentiation, namely *TNAP* and *C/EBPa* mRNA

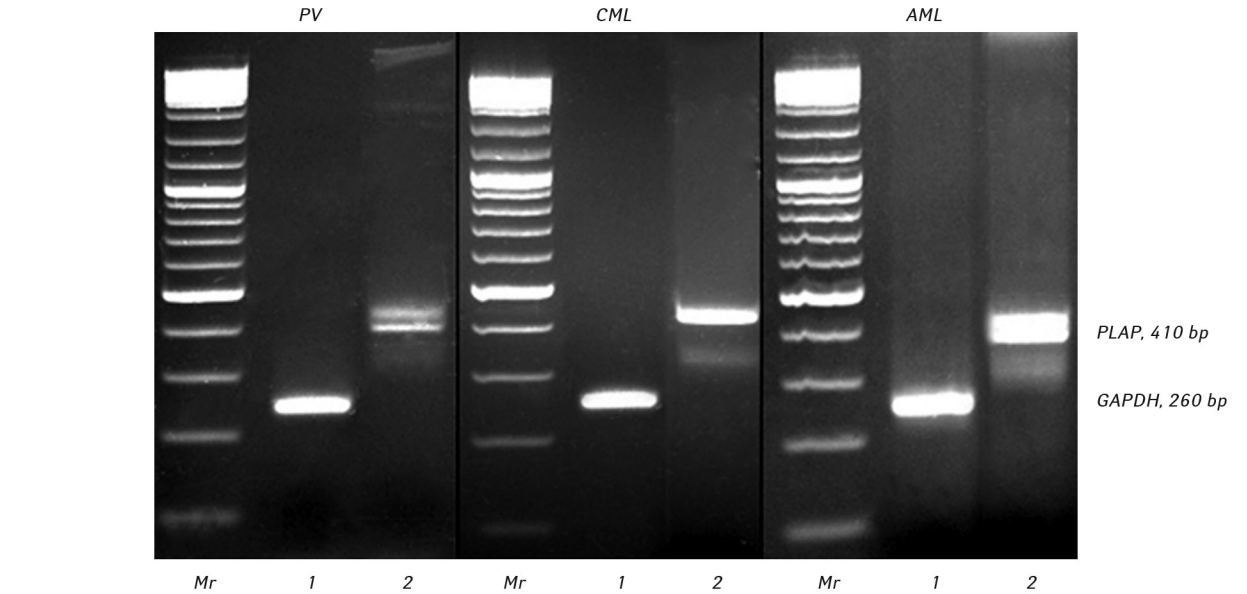


Fig. 2. Ectopic gene expression of embryonic *PLAP* mRNA in peripheral blood cells of the patients with CML, AML, and PV: 1 — *GAPDH*, reference gene; 2 — aberrant *PLAP*

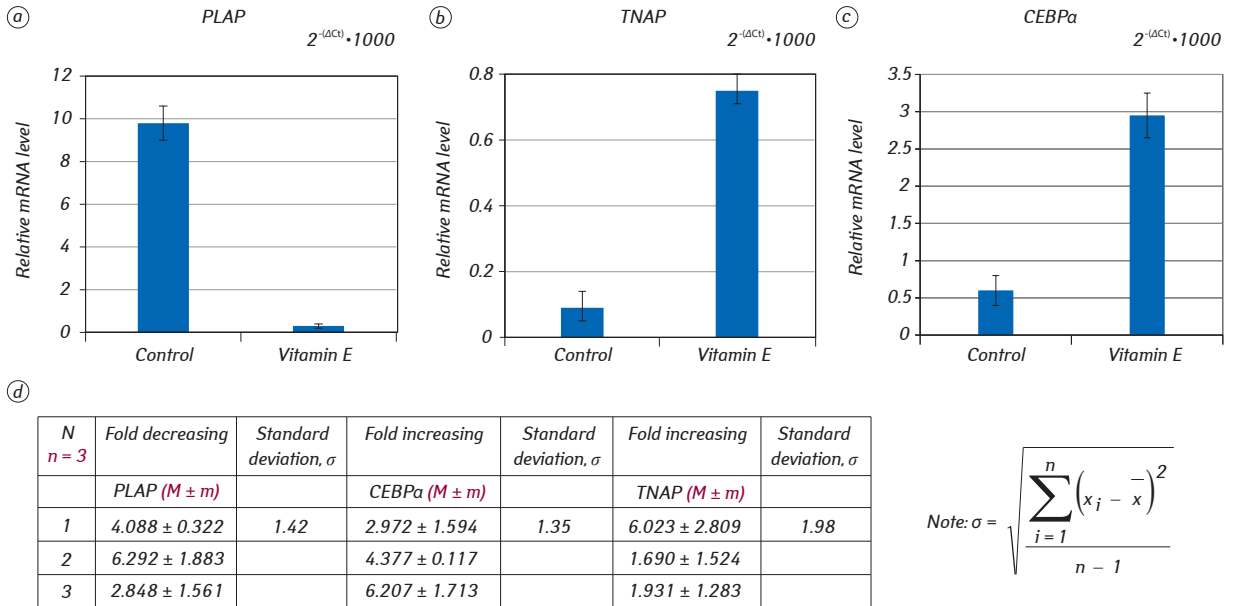


Fig. 3. The relative mRNA expression levels of *PLAP* (a), *TNAP* (b), and *C/EBPa* (c) in K562 cells exposed to vitamin E (100 μ M) for 48 h by real time RT-PCR $2^{-(\Delta Ct)}$ method. The relative fold decrease in *PLAP* and relative fold increase in *CEBPa* and *TNAP* are calculated based on the data from three independent experiments (d) by $2^{-(\Delta Ct)}$ method

in K562 cells. These results revealed the tight regulation between decreased aberrant PLAP expression and increased expression of both differentiation markers, namely TNAP as a niche regulator for HSCs function and CEBP alpha as a master regulator for HSCs myelopoiesis [22]. Earlier, we have demonstrated the role of vitamin E as a differentiation-like factor inducing G-CSFR (granulocyte-colony-stimulating factor receptor), whose transcription is directly activated by C/EBP alpha during common myeloid progenitor lineage committing activation in K562 cells [23].

Fig. 3 demonstrates that vitamin E targeted PLAP expression is closely related to restoring expression of C/EBPα and TNAP, both being tightly associated with possible reactivation of myeloid differentiation potential. Therefore vitamin E seems to be able to affect remodeling of leukemic blast stem cell phenotype.

To sum up, we have demonstrated increased PLAP expression in leukemic cells of myeloid origin in the setting of decreased TNAP expression. Aberrant expression of embryonic PLAP may be considered as one of the putative markers of myeloid cell undifferentiated state, which deserves further study. On the other hand, the potential of PLAP as one of the possible targets for controlling LSC phenotype should be further explored. More attention is needed to explore the potential of the bioactive molecules such as vitamin E that may induce reprogramming of the profiles of granulopoiesis.

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