ejpmr, 2020,7(8), 182-187

SJIF Impact Factor 6.222

EUROPEAN JOURNAL OF PHARMACEUTICAL AND MEDICAL RESEARCH

www.ejpmr.com

Research Article

ISSN 2394-3211

EJPMR

ECTOPIC PLACENTAL-LIKE ALKALINE PHOSPHATASE (PLAP) REPROGRAMMING BY VITAMIN E IN CML BLAST CRISIS LEUKEMIC K562 CELLS

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Article Received on 13/06/2020 **Article Revised on 03/07/2020** **Article Accepted on 23/07/2020**

ABSTRACT

Bagraund*:** Placental-like alkaline phosphatase **(**PLAP)-1ike enzymes are expressed by many tumors and can be detected in sera of patients with various cancers. Their ectopic expression has been considered to be potentially useful as tumor marker. Indeed, elevated levels of PLAP were found in 43% of seminomas and in 75% of recurrent metastatic tumors. However, the biological background of the role of this aberrant alkaline phosphatase in cancer progression is still unclear. We have focused on the study of the biological role of PLAP in leukemogenesis on the model of chronic myeloid leukemia (CML) K562 cells. ***Objective: The aim of the study was to attempt to use vitamin E for LSC reprogramming by inducing CEBP alpha (CCAAT/enhancer-binding protein α) and G-CSFR (granulocyte colony stimulating factor receptor) analyzing aberrant PLAP as biomarker of LSC phenotype. ***Methods*:** RNA extracted from K562 cells cultured with vitamin E was converted to for quantitative SYBR Green qRT-PCR analysis using primers to tissue nonspecific alkaline phosphatase (TNAP) and C/EBP alpha and G-CSFR respectively. Samples were cycled using the standard SYBR Green protocol on Q5 Bio-Rad StepOne qPCR instrument and analyzed using the comparative cycle threshold (*C*T) method to obtain relative mRNA expression. Amplicon (410 bp) was eluted from agarose gel (2.5%) and DNA- sequenced. ***Results*:** The progression of CML to blast crisis is correlated with both down-modulation of TNAP and C/EBP alpha as master regulator of granulocytic differentiation in myelopoesis. We have observed the ectopic expression of mRNA PLAP gene in K562 cells by real-time RT-PCR. We founded by sequencing that PLAP gene corresponds to embryonic type of placental alkaline phosphatase (EPLAP) and have no gene homology with tissue placental alkaline phosphatase (PAP) that was absent in K562. Vitamin E decreases the mRNA PLAP expression level and increases the mRNA TNAP gene expression. Moreover, along with down-regulation of aberrant PLAP and up-regulation of TNAP, vitamin E in a dose of 100 µM increases mRNA expression of transcription factor C/EBP alpha and G-CSFR. It was reported that osteoblast-expressed TNAP is a biomarker of HSCs and directly associated with HSC hematopoiesis in bone marrow (BM). We have proposed that loss of TNAP in BM HSC niche may contribute into CML pathogenesis, suggesting the impairment of bone marrow mesenchymal stem cell (BM MSC) – derived osteoblast differentiation. The aberrant PLAP in K562 cells seems to differ from tissue TNAP and tissue placental alkaline phosphatase. ***Conclusion.*** EPLAP may be considered as a putative target in differentiation therapies in myeloid leukemias. Our findings suggest the potential role of vitamin E in adjuvant therapy of lelukemia as the inducer of differentiation potential of K562 leukemic cells through decreasing ectopic EPLAP along with induction of granulopoiesis factors CEBP alpha/G-CSFR. We have declared that embryonic PLAP is pluripotent LSC-associated biomarker in contrast of TNAP as multipotent HSC-associated biomarker. Vitamin E may be putative modulator of LSCs reprogramming in biotherapies of CML.

KEYWORD: EPLAP may be considered as a putative target in differentiation therapies in myeloid leukemias.

INTRODUCTION

Chronic myeloid leukemia (CML) is a clonal hematopoietic stem cell (HSC) disorder in bone marrow (BM) associated with the activity of BCR-ABL fusion oncogene during reciprocal translocation t(9;22)(q34;q11) and is characterised by an increased growth of abnormal myeloid progenitor cells within the bone marrow (BM).[1,2] The resultant constitutively active P210 BCR-ABL tyrosine kinase promptes the unraveling of the molecular pathogenesis of CML.[3,4] Chronic myeloid leukaemia (CML) treatment involves targeting BCR-ABL to prevent its tyrosine kinase activity by TKIs ((imatinib, nilotinib and dasatinib) which effectively target progenitor cells, however leukaemic stem cell (LSC) are less sensitive to such treatment.[5,8] CML LSCs don't depend on BCR-ABL signaling for their survival.[9,10] Therefore, leukaemic stem cell (LSC) persistence remains a major obstacle to curing chronic myeloid leukaemia (CML).[11,12] The search of new biological markers of leukemic stem cell

phenotype in myeloproliferative neoplasias does not lose its relevance today. In the present study we focus on the known facts of alkaline phosphatase **(**AP) not only as nonspecific marker of bone metastasis.[13] but on the known AP role in and potential significance for the identification of stem cell phenotype.[14] Moreover, AP activity is a widely accepted marker of pluripotent stem cells associating with embryonal stem cell pluripotency.[15] Therefore, alkaline phosphatese (AP) gene expression we used as possible tool in our study of leukemic stem cell phenotype in blast-crisis CML on the K562 CML blast crisis model leukemic cell line investigation.

Alkaline phosphatase (AP, EC 3.1.3.1 orthophosphoric-monoesterase, alkaline optimum) is a membrane bound enzyme with commonly bone matrix mineralization function.[16] There are four genes encoding APs in humans. Three genes, intestinal (IAP), placental (PAP), and germinal (GAP), display tissue-specific expression (TSAP proteins) 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.[17] In the present study we develop a deeper insight into the feature of the alkaline phosphatase gene expression that potentially provide a better understunding in CML leukemic stem cell (LCS) biology. Moreover, in the present study we develop the efforts to regulate CML leukemic stem cell phenotype through aberrant EPLAP reprogramming to differentiation by vitamin E treatment in K562 CML blast leukemic cell line.

THE METHODS

The samples of peripheral blood from the patients (n=9) with CML, AML, MPN (essential polycytemia) was obtained from the National Institute of Cancer, Ukraine, Kyiv.

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 NAS of Ukraine. The cells were grown in suspension in RPMI-1640 medium supplemented with 10% of fetal calf serum.

Total RNA was extracted using TRIzol (Invitrogen, Gaithersburg, MD) according to the manufacturer's instructions. RNA was converted to cDNA using the Qiagen's QuantiTect Rev. Transcription Kit (Qiagen, Hilden, Germany). Real-time RT-PCR was performed to ensure quantitative nature of mRNA gene expressions of C/EBPalpha and G-CSFR using SYBR Green protocol. RT-PCR reactions were carried out using HotStarTaq DNA polymerase (Qiagen), 50 ng of cDNA and SYBR Green in a 1:60,000 dilution in triplicate. PCR conditions were: a 95 °C initial activation for 15 min was followed by 45 cycles of 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 30 s on an Bio-Rad Real-time PCR Detection System IQ5, CA.

The primers using for real time RT-PCRassay:
TNAP (non specific, liver/bone/kidney): forward - 5`-TGGCCGGAAATACATGTACCC-3`; reverse - 5`-TTCCGTGCGGTTCCAGATG-3`; *PAP* (terminal placental): forward - 5`-CCAGACCATTGGCTTGAGT-3`; reverse - 5`- AGTACCAGTTGCGGTTCAC-3`; *IAP* (intestinal): forward - 5`-AAGGGCAGAAGAAGGACAAA; reverse — 5`-GTCGTGTTGCACTGGTTAAAG; *C/EBP alpha :* forward - 5`- CAA GAA CAG CAA CGA GTA CCG-3`; reverse - 5`- GTC ACT GGT CAA CTC CAG CAC-3`; *GAPDH:* forward - 5`- CGCTCTCTGCTCCTCCTGTT-3 `; reverse - 5`- CCATGGTGTCTGAGCGATGT- 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.

RESULTS AND DISCUSSION

Recently, TNAP (tissue non specific alkalin phosphatase) ultimatelly recognised as mesenchymal stromal cell antigen-1 (MSCA-1)[18] was descreabed as biomarker associated with normal HSCs hematopoiesis as well with terminal myeloid differentiation.[19]

We detected that TNAP gene expression was lossing on the level of mRNA expression by RT-PCR assay in CML blast phase patient sample as we investigation (Fig. 1). Along with TNAP we not detected also (terminal) placental alkaline phosphatase (PAP) and intestinal alkaline phosthatase (IAP) (Fig. 1). However, in CML blast phase patient sample we have revealed mRNA expression of aberrant alkaline phosphatase gene resulted from using the oligodeoxy-nucleotide primers to tissue intestinal alkaline phosphatase (IAP) gene as 410 bp product (Fig.1)

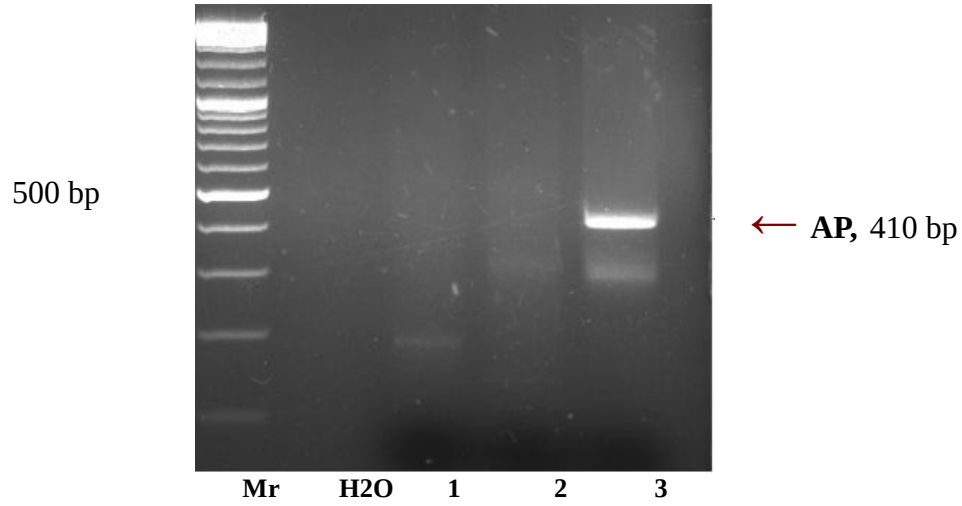


Fig. 1. The aberrant alkaline phosphatase gene expression on the level of mRNA by RT-PCR in CML blast phase patient sample. The amplification products of alkaline phosphatase (AP) genes was: 1 - PAP (tissue (terminal) placental alkaline phosphatase; 2 — TNAP (tissue non-specific, bone, kidney, liver, alkaline phosphatase; 3- IAP (tissue intestinal alkaline phosphatase (IAP).

The abberant alkaline phosphatase amplification product was elueted from agarose gel and was performed DNA-sequencing data base analysis with oligodeoxy-primers to IAP gene. On the data obtained in DNA-sequencing analysis this abberant alkaline phosphatase gene was determined as placental-like alkaline phosphatase (PLAP) gene. It was reffered that placental-like alkaline phosphatase (PLAP) and placental (terminal) alkaline phosphatase PAP are encoded by different genes.[20] Indeed, we have suggested this fact, because PAP was do not expressed in CML patient as we shown (Fig. 1). Moreover, it was reffered the immunological cross-reactivity between human PLAP, IAP (intestinal), and GAP (germinal), but no cross-reactivity between any of these and the TNAP (tumor non-specific: bone, kidney, liver).[21] Indeed, we have also suggested this fact because aberrant PLAP mRNA was expressed using the oligodeoxy-nucleotide primers to intestinal alkaline phosphatase (IAP) gene as we shown by RT-PCR (Fig. 1). Therefore, on the data obtained we propose that revealed aberrant PLAP that associated with CML blast crisis progression has not tissue origin but has embryonal-like origin as embryonal placental like alkaline phosphatase (EPLAP). Moreover, we have shown the ectopic expression of EPLAP in progression of myeloproliferation neoplasms as the polycytemia vera (A), the blast-crisis CML (B), and the acute myeloid leukemia (C) (Fig. 2).

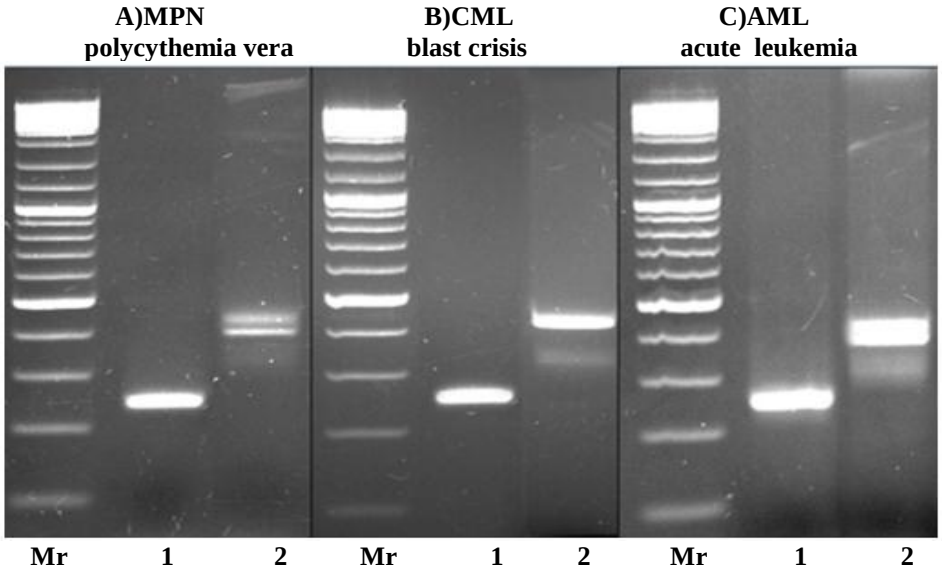


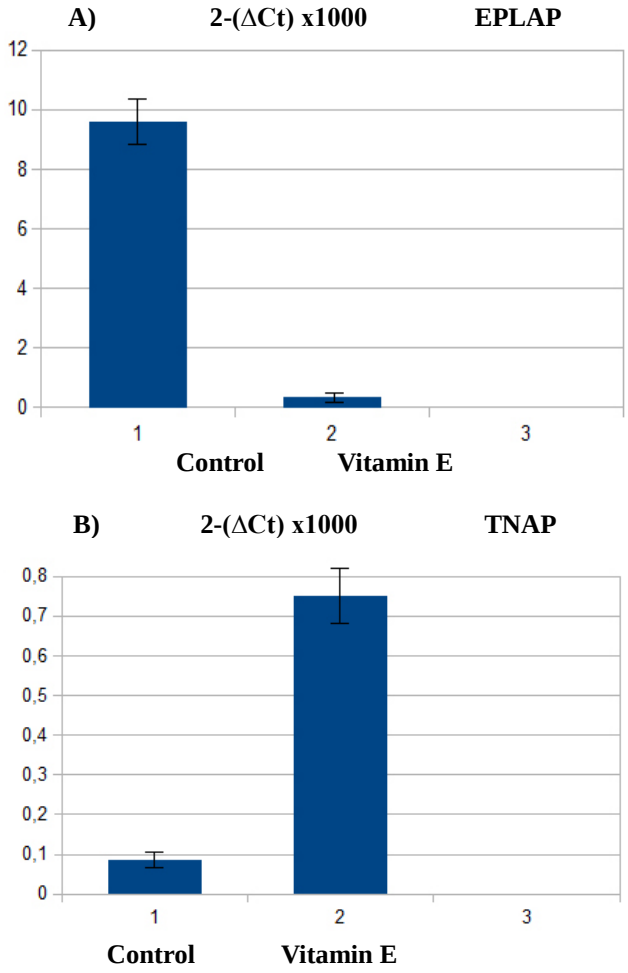
Fig. 2. Ectopic gene expression of embryonal placental-like AP (EPLAP) on the mRNA level under myeloproliferative leukemia progression. 1 — GAPDH, refferent gene; 2 — aberrant EPLAP; A) — polycythemia vera, myeloproliferative neoplasm (MPN); B) — blast-crisis CML, chronic myeloid leukemia blast stem-like phase; C) — AML, acute myeloid leukemia.

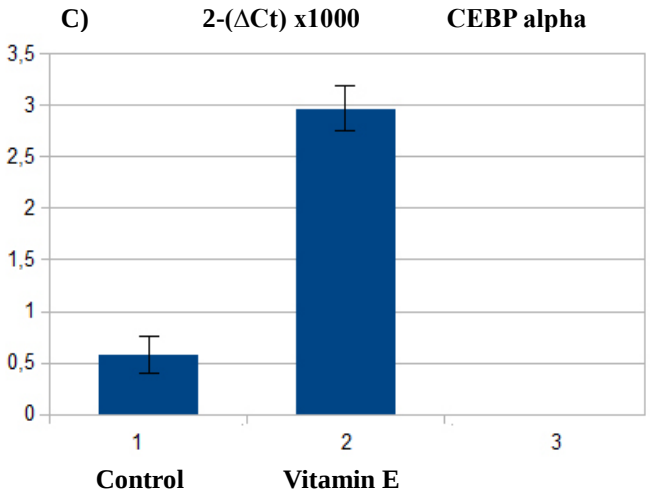
Our results have elucidated that revealed ectopic expression of embryonal-origin placental like alkaline phosphatase (EPLAP) associates only with leukemic stem cell (LSC) phenotyphe in CML blast crisis and AML progression in contrast to MPN as polycytemia vera (Fig. 2).

It is known, *BCR-ABL1*-negative myeloproliferative neoplasms (MPNs), including three main diseases as polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF) are a group of closely related blood cancers as "clonal" overproduction of one or more blood cell lines resulted from one or more mutations (as examples Jak 2, Stat 3/5 , Tet 2, IDH1, NF1) in a single cell.[22] Therefore, MPNs are have not truly leukemic stem cell phenotype but carry an activating number mutations in the blood cell lines in the blood which are the offspring of that one mutant cell.[22]

As result we have postulated that revealed ectopic expression EPLAP in CML blast crisis and AML elusidates only the LSC phenotype and it is very important the pluripotent features of LSC phenotype in CML and AML biology. Consequently, we point if TNAP associates with normal multipotent HSCs biology than EPLAP associates with pluripotent reprogramming LSCs biology.

Moreover, we have revealed that vitamin E able to modulate LSC phenotype as we study in vitamin E-treatment of K562 CML blast crisis stem cell line under 48 h exposure. The results obtained are presented at Figure 3. We shown that vitamin E target decreased of EPLAP gene expression (A) along with target increased TNAP (B) and CEBP alpha (C) gene expression on the mRNA levels by real time RT-PCR assay (Fig. 3).





D)

N n=3	Fold decreasing	Standard deviation, σ	Fold increasing	Standard deviation, σ	Fold increasing	Standard deviation, σ
	PLAP(M±m)		CEBPα(M±m)		TNAP (M±m)	
1	4.088 ±0.322	1.42	2.972 ± 1.594	1.35	6.023 ± 2.809	1.98
2	6.292±1.883	1.42	4.377 ± 0.117	1.35	1.690 ± 1.524	1.98
3	2.848 ±1.561	1.42	6.207 ± 1.713	1.35	1.931 ± 1.283	1.98

Note: $\sigma = \sqrt{\frac{\sum_{i=1}^{n}(x_i - \bar{x})^2}{n-1}}$

Fig. 3. The relative mRNA expression levels of PLAP (A), TNAP (B), and C/EBPα (C) in K562 cells exposed to vitamin E (100 µM) for 48 h by real time RT-PCR $2^{-(\Delta Ct)}$ method. The relative fold decreasing PLAP corresponding to fold increasing CEBP α and TNAP, accordingly (D) by $2^{-(\Delta Ct)}$ method.

Our resuts suggest that vitamin E is capable to remodeling aberrant PLAP gene expression and consequently to restore the myelopoietic differentiation factors TNAP and C/EBPα gene expression in CML blast crisis K562 lineage cells. These results revealed the tightly regulation between decreasing aberrant EPLAP gene expression and increasing the both HSC - myelopoietic master regulators TNAP as HSC-niche regulator[23] and CEBP alpha as muster regulator of granulopoiesis.[24] Earlier, we first elucidated the role of vitamin E as potential differentiation-like factor inducing C/EBP alpha and concequently G-CSFR (granulocyt-colony-stimulativg factor receptor) directly activated by C/EBP alpha during common myeloid progenitor lineage committing activation in K562 cells.[25] In the present study we emphasise the role of vitamin E as contributing factor in remodeling aberrant EPLAP resulting in restored TNAP and C/EBP alpha expression potential in CML blast crisis K562 cells.

CONCLUSION

Firstly, we have proposed that aberrant placental-like alkaline phosphatase (PLAP) is not tissue (terminal) placental alkaline phosphatase (PAP) but is embryonal - like origin PLAP as biomarker of pluripotency LSC phenotype in K562 CML blast crisis leukemic cell line was study. Therefore, EPLAP could implicate as highly interesting target for control of LSC phenotype pluripotency in treatment of CML malignancies.

Secondly, we have proposed the role of vitamin E in reprogramming ectopic EPLAP with consequent restored TNAP and C/EBP alpha master regulators of myelopoiesis potential in CML blast crisis leukemic K562 cells exposured (48h) by vitamin E (100µM) was study. Therefore, vitamin E can be proposed as potential natural agent in the differentiation therapies of myelogenic leukemias.

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