J Med Biochem 2018; 37 (2) DOI: 10.1515/jomb-2017-0042

UDK 577.1 : 61 **ISSN 1452-8258**

J Med Biochem 37: 128–133, 2018

Original paper
Originalni naučni rad

ADENOSINE DEAMINASE 1 AS A BIOMARKER FOR DIAGNOSIS AND MONITORING OF PATIENTS WITH ACUTE LYMPHOBLASTIC LEUKEMIA

ADENOZIN DEAMINAZA 1 KAO BIOMARKER ZA DIJAGNOZU I PRAĆENJE PACIJENATA SA AKUTNOM LIMFNOBLASTNOM LEUKEMIJOM

Mina Ebrahimi-Rad[1], Shohreh Khatami[1], Shahla Ansari[2], Shohreh Jalylfar[1], Shirin Valadbeigi[1], Reza Saghiri[1]

[1]Biochemistry Department, Pasteur Institute of Iran, Tehran
[2]Department of Hematology-Oncology Ali Asghar Children's Hospital, School of Medicine, Iran University of Medical Sciences, Iran, Tehran

Summary

Background: Acute lymphoblastic leukemia (ALL) is known as the most prevalent pediatric malignancy all around the world. Identification of specific biomarker is necessary for early diagnosis and effective therapy. It is believed that Adenosine deaminase (ADA) as an enzyme involved in the purine salvage pathway increases in ALL patients. Herein, the quantity and pattern of ADA isoenzymes were surveyed among ALL patients in comparison to healthy subjects.
Methods: Serum and RBC samples of three different groups of ALL patients, including newly diagnosed cases without any drugs administration, subjects with the relapsed disease, patients in the remission stage after therapy, and the healthy subjects were enrolled in the study. Then, the activity and pattern of ADA1, ADA2 and ADA1+cp were determined using ADA kit and electrophoresis on SDS-PAGE, respectively. To confirm the presence of ADA enzyme, the fresh serums, extractions from erythrocytes, JM cell line as a human T lymphocyte line and J774 A.1 as mouse monocyte line were electrophoresed on 1.2% agarose gel and stained with the specific dye.
Results: The activities of ADA1 isoenzyme and total ADA in new cases and subjects with the relapsed disease were significantly higher than their activities in the patients in the remission stage and healthy controls ($p<0.001$). The unbounded ADA1 isoenzyme was found to exist in the erythrocyte, lymphocyte and monocyte. But in serum, all the ADA1 was bounded to the cp protein.

Kratak sadržaj

Uvod: Akutna limfnoblastna leukemija (ALL) je poznata kako najučestalija pedijatrijska maligna bolest širom sveta. Identifikacija specifičnih biomarkera je potrebna za ranu dijagnozu i efikasnu terapiju. Smatra se da je adenozin deaminaza (ADA) enzim koji je uključen u metabolizam purina povećana kod ALL pacijenata. U ovom radu izučavan je kvantitativni profil ADA izoenzima kod ALL pacijenata u odnosu na zdrave ispitanike.
Metode: Uzorci seruma i eritrocita kod tri različite grupe ALL pacijenata, uključujući novo dijagnostikovane slučajeva koji nisu primali lekove, pacijente sa povratkom bolesti i pacijente u remisiji posle terapije, kao i grupa zdravih ispitanika, su sakupljani i ispitivani na aktivnost i profil ADA1, ADA2 i ADA1+cp primenom ADA kita odnosno SDS-PAGE elektroforezom. Da bi se potvrdilo prisustvo ADA enzima, sveži serumi, hemolizati eritrocita, JM linija ćelija kao humana T limfocitna linija I J774 A.1 i mišija monocitna linija analizirana je elektroforetski na 1.2% agaroza gelu i bojena specifičnim bojama.
Rezultati: Aktivnost ADA1 izoenzima i ukupne ADA u novim slučajevima i kod pacijenata sa povratkom bolesti je bila značajno viša nego kod pacijenata u stanju remisije bolesti odnosno kod zdravih ispitanika ($p < 0.001$). Nesumnjivo da ADA1 enzim postoji u eritrocitima, limfocitima i monocitima. Međutim u serumu ADA1 je vezana za cp protein.

Address for correspondence:

Reza Saghiri
Biochemistry Department, Pasteur Institute of Iran, Tehran, Iran
e-mail: Saghiri@pasteur.ac.ir

Conclusions: ADA1 is the key isoenzyme elevating in ALL patients, therefore this isoenzyme could be a useful biomarker to diagnose ALL patients and monitor their therapies.

Keywords: Acute Lymphoblastic Leukemia (ALL), Adenosine Deaminase (ADA), ADA isoenzymes, electrophoresis, agarose gel

Zaključak: ADA1 je ključni enzim povećan kod ALL pacijenata tako da ovaj izoenzim može da bude značajan marker za dijagnostikovanje ALL pacijenata i praćenje terapije.

Ključne reči: akutna limfnoblastna leukemija (ALL), adenozin deaminaza (ADA), ADA izoenzim, elektroforeza, agaroza gel

Introduction

Acute lymphoblastic leukemia (ALL), the most prevalent malignancy among children includes approximately 25% of pediatric cancers in developed countries (1). Abnormal transformation, epigenetic and genetic aberrations in the progenitor cells of T and B cell lineages results in ALL. Additionally, in young cases, recurred ALL originated from B cell lineage causes a high rate of cancer death (2–4).

Despite the aggressive nature of ALL, it has been shown that individual therapy in developed health centers, nowadays, leads to high rate remission and the long–lasting survival time in patients. Therefore, rapid and facile detection of ALL is necessary to save the lives in all countries (5, 6).

Adenosine deaminase (ADA) (adenosine aminohydrolase, EC 3.5.4.4) is a polymorphic enzyme involved in the purine salvage pathway. It converts adenosine and deoxyadenosine to inosine and deoxyinosine, respectively. The ADA activity in lymphocytes, especially, T cells, is higher than erythrocytes. Moreover, the activity of this enzyme has some variations during differentiation phases of T-cells and is greater among immature T-cells (7).

In physiological conditions, there are three discrepant isoenzymes in the human tissues, including ADA1, ADA1+cp and ADA2 (8). ADA1 is the monomeric protein that is distributed in all tissues throughout the human body such as lymphocytes and monocytes. ADA2 is another isoenzyme expressed in the monocytes and macrophages and secreted to the serum by both of the cells (9). ADA1+cp consists of two ADA1 molecules linked through 280 kDa binding protein (6).

In addition to developing the immune system, ADA plays a momentous role in proliferation, differentiation, and maturation of lymphocytes (10). Since the quantity of adenosine in inflammatory responses is controlled via ADA activity, alteration of ADA level in plasma is seen in a wide variety of diseases especially those related to the immune system and hematologic malignancies. The previous findings reported that the ADA quantity and activity increase in serum of children and young patients with ALL. Therefore, this enzyme could be a suitable marker to detect and monitor ALL, together with other alternatives (11, 12).

In this study, we analyzed the activity and isoenzyme patterns of ADA among four different groups of ALL patients including newly diagnosed cases without any drugs administrations, subjects with the relapsed disease, and patients in the remission stage after therapy and compared their outcomes with those of the healthy control subjects.

Materials and Methods

Patients and serum samples

This study was a cross-sectional research, been performed in Tehran, Iran. Fifty-four patients suffered from ALL who referred to Ali Asghar children's hospital from April 2014 to March 2015 were included in the study. All the patients were 6–12 year old children without the other hematological disease. Twenty-two, 18 and 14 out of 54 patients were newly diagnosed cases without administration of any drugs (known as new cases in this text), subjects with the relapsed disease, and patients in the remission stage after therapy, respectively. ALL patients were diagnosed based on the bone marrow examination, lung radiography, and complete blood cell analysis. Thirty healthy subjects without leukocytosis and lymphocytosis were tested as controls. The consent forms were completed and signed by the children's parents. This study was designed and approved by the Ethics Committee of Pasture Institute of Iran and performed in accordance with its guideline's.

Samples collection

To provide the RBC and serum samples, the blood specimen was collected from each patient and centrifuged at 3000 rpm for 15 min. The RBC fraction and plasma were separated and transferred to fresh tubes and used for further investigations.

ADA enzyme assay

The ADA enzyme activity was determined by ADA kit (DIAZYME Company) according to the manufacturer's guideline. The kit contains glutamate dehydrogenase, which consumes the $NH4^+$ produced by ADA in step one along with NADPH, leading to the release of $NADP^+$. Reduction of NADPH results in decreased absorbance at 340 nm that was an indication of total ADA quantity in the serum sample.

To assess ADA2 activity, the erytro-9-(2-hydroxy-3-nonyl) adenine (EHNA) (Sigma, Germany) was

used to inhibit ADA1 activity, specifically. The ADA2 isoenzyme activity was measured in the presence of 0.1 mmol/L EHNA after 20 min at 340 nm using Automatic Analyzer (Technicon RA-1000). Finally, the ADA1 isoenzyme activity was assayed by subtracting the activity of ADA2 from that of the total ADA.

Furthermore, to identify the pattern of ADA1, ADA2, and ADA1+cp isoenzymes, electrophoresis on the SDS-PAGE (10, 8, and 6%) was done, respectively. To visualize the protein bands, the SDS-PAGE was stained with Coomassie blue.

Enzyme assay of ADA of RBCs

Because of the binding of ADA1 to cp protein in the serums of ALL patients, RBC samples were applied to assess the free ADA1 isoenzyme. To protect the enzyme activity, the RBCs were washed 3 times with normal saline. An aliquot of washed erythrocytes was lysed with toluene and cold water (1 mL RBC + 0.5 mL toluene + 1 mL cold water) and centrifuged at 15000 × g for 40 minutes. After then, the lysed RBC samples were analyzed on 6–10% SDS-PAGE to separate the ADA isoenzymes.

Specific ADA staining

The presence of ADA enzyme was confirmed by electrophoresis of the fresh serums collected from patients and healthy subjects on 1.2% agarose gel at 4 °C for 3 h.Then stained with the specific dye containing sodium phosphate (0.1 mol/L, pH=7), adenosine, phenazinemetosulphate, di-methyl tiazolyl tetrazolium bromide (MTT), nucleoside phosphorylase (1 mg/mL), gezantin oxidase (10 mg/mL) for 45 min at 37 °C. Standard ADA was examined along with the samples as a control (13).

Furthermore, to determine the pattern of ADA isoenzymes in the lymphocytes and monocytes, the cell extraction of JM cell line as a human T lymphocyte line, and J774 A.1, as a mouse monocyte line were prepared using freezing-thawing method (12). The cell extracts were applied to 1.2% agarose gel and after electrophoresis, stained using the specific dye as described above.

Statistical analysis

To compute the difference between the different patient and control groups, the t-test and ANOVA were done by using SPSS 17 software (Chicago, USA). The p-value less than 0.05 ($p<0.05$) was considered as a significant difference.

Results

In the current study, the enzymatic activity of ADA and its isoenzymes was analyzed among patients with ALL and healthy subjects. The mean value of ADA1 activity was found to be 22, 23.2, 5.07 and 5.83 IU/L in the new cases, subjects with the relapsed disease, those in the remission stage and

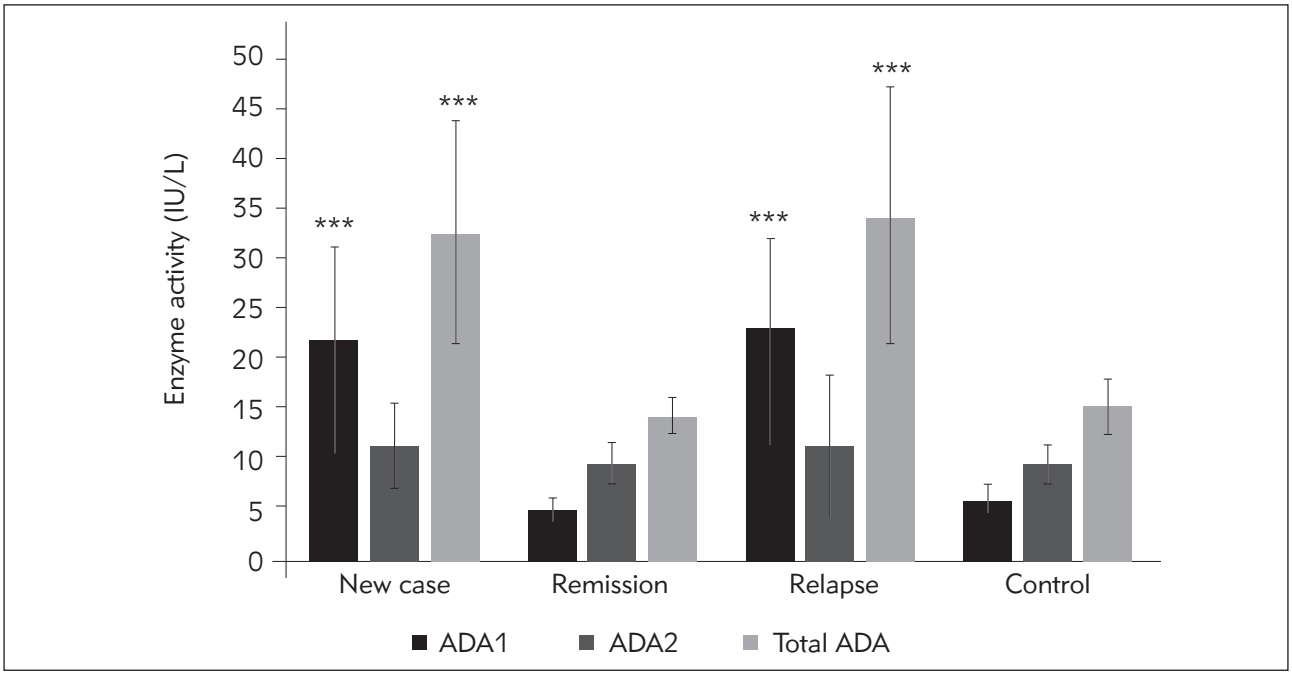

Figure 1 Enzymatic activity of adenosine deaminase isoenzymes in serums of ALL patients and control groups.
"***" indicates $p<0.001$.
The activity of ADA1 isoenzyme and total ADA in the new cases and subjects with relapsed disease were found to be significantly higher than those of the patients at remission stage and the healthy controls.

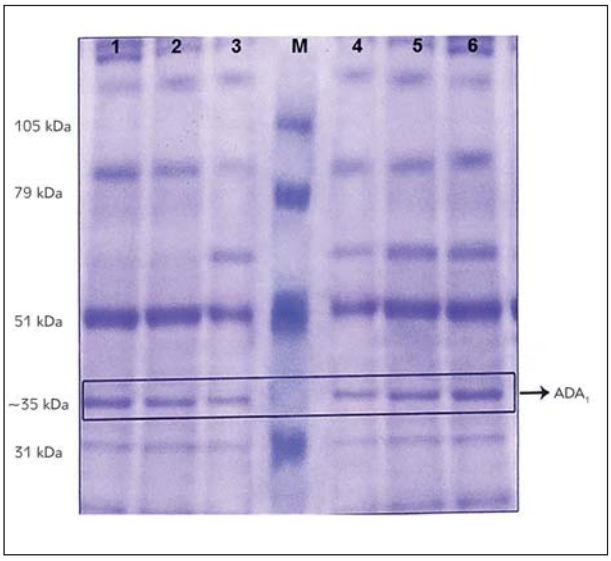

Figure 2 Illustration of ADA1 isoenzyme within RBC on 10% SDS-PAGE. The molecular weight of ADA1 isoenzyme is approximately 35 KDa. Lanes 1 and 2 is related to the healthy subjects; lanes 3, 4, 5 and 6 are the samples of the ALL patients; M indicates the low molecular weight protein marker.

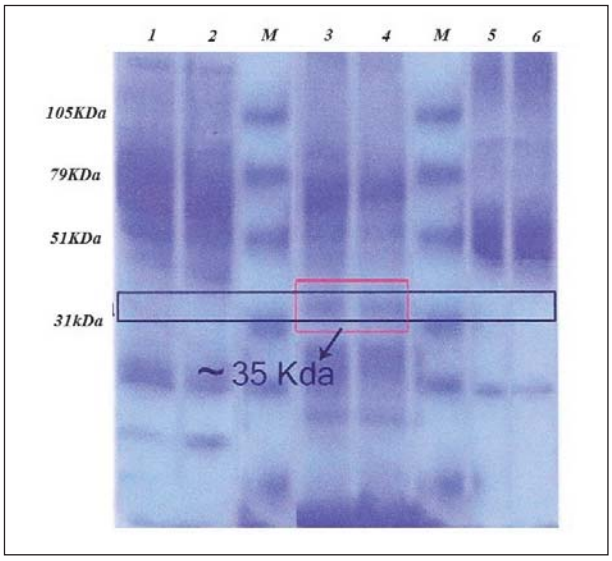

Figure 3 Comparison of the status of ADA1 isoenzyme of intact serum, heated serum, serum without SDS or 2-ME, and RBC. Lane1 is heated serum of a healthy case; lane 2 is related to the heated serum of ALL patient; lanes 3, and 4 show erythrocyte ADA1 isoenzyme; lanes 5 and 6 show the serum of ALL patients without SDS and 2-ME; M indicates the low molecular weight protein marker.

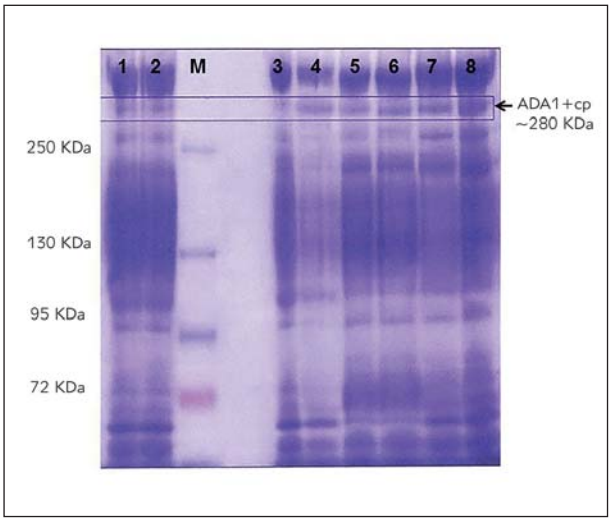

Figure 4 Determination of molecular weight of serum ADA1+cp complex on 6% SDS-PAGE. Lanes 1 to 6 show serum of ALL patients; Lanes 7 and 8 are related to serum of healthy subjects; M indicates the high molecular weight protein marker.

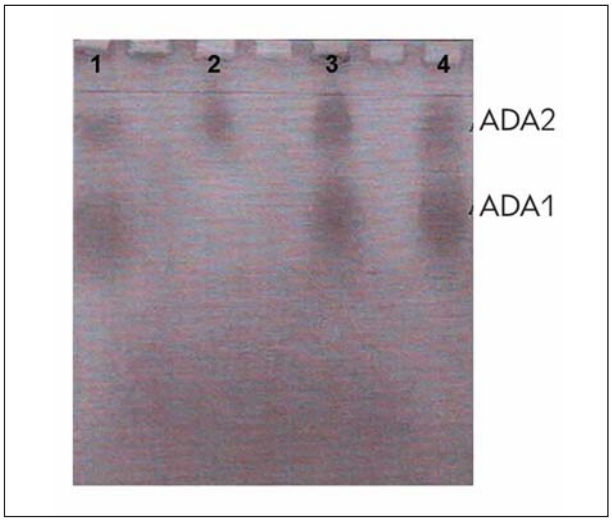

Figure 5 Illustration of ADA isoenzymes on 1.2% agarose gel stained using the specific dye. Lane 1, 2, 3 and 4 are related to the serum of healthy subject, patient serum containing the erytro-9-(2-hydroxy-3-nonyl) adenine, serum of ALL and ALL patient serum, respectively.

healthy controls, respectively. However, the activity of ADA2 was 11.5, 11.3, 9.7 and 4.57 IU/L in new cases, subjects with the relapsed disease, and patients in the remission stage, and healthy controls, respectively. No significant difference was seen in the ADA2 activity between the tested groups. The activity of total ADA was 32.7, 34.5, 14.5 and 15.4 IU/L in the new cases, subjects with the relapsed disease, and patients in the remission stage, and healthy controls, respectively. As illustrated in *Figure 1*, the activity of ADA1 isoenzyme and total ADA in the new cases and subjects with the relapsed disease were significantly higher than those of patients in the remission stage and the healthy controls ($p<0.001$). Nevertheless, there was not a significant difference, either between new cases and those at relapsed step or patients in the remission stage and healthy controls.

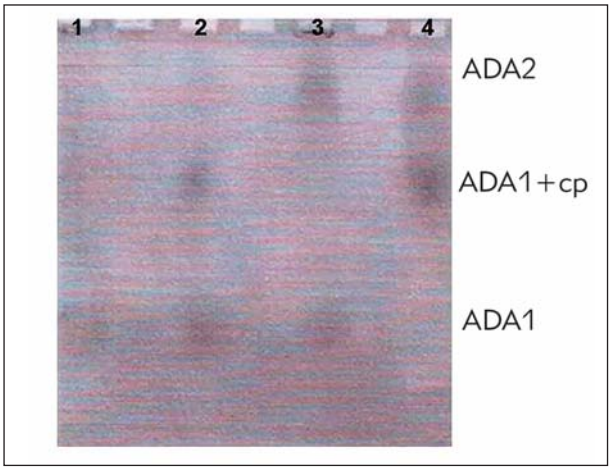

Figure 6 The pattern of ADA isoenzymes in blood cells and serum on 1.2% agarose gel. Lane 1, 2, 3, and 4 are related to erythrocyte extract, JM cell line extract (as T cell lymphocytes), J774 A.1 cell line extract (as a monocyte) and serum, respectively.

Regarding binding of ADA1 to cp protein in serum, the patterns of ADA1 isoenzyme in RBC and serum samples were assessed by the electrophoresis on 10% SDS-PAGE (*Figures 2* and *3*, respectively). The molecular weight of the unbounded ADA1 isoenzyme was estimated to be approximately 35 KDa. According to the *Figures 2* and *3*, RBC has a free ADA1 isoenzyme while, intact serums, heated, and SDS or 2-mercaptoethanol treated serum samples were lack of free ADA1 in both, ALL patients and healthy controls.

Figure 4, depicts the pattern of the ADA1+cp protein in serum samples of ALL patients and healthy controls. The band with a molecular weight of 280 KDa is a representative of the ADA1+cp protein. This data showed that only bounded ADA, as ADA1+cp form; exists in the serums of both, ALL patients and the healthy controls.

Figures 5 and *6* show that unbounded ADA1 isoenzyme exists in the erythrocytes, lymphocytes, and monocytes. However, in serum, ADA1 isoenzyme is fully bounded to the cp protein. Furthermore, unlike monocytes, lymphocytes and erythrocytes lack ADA2.

Discussion

It is believed that ADA has a momentous role in the developing of the immune system and different growth stages of T cell and B cell lymphocytes. The increased quantity and activity of ADA is an accepted indicator for lymphocytes immaturity (10). Similar to the abnormalities in the immune system, several malignancies such as breast, colorectal, lung, and ovarian cancer and etc. are accompanied with augmentation of ADA activity.

In ALL patients, the presence of genetic and epigenetic abnormality in the stem cells of T and B cell lineages located in bone marrow results in over-proliferation of progenitors with high speed and release of numerous immature and undifferentiated lymphocytes in the peripheral blood (8, 14). Increased proliferation leads to salvage of nucleoside, therefore, ADA quantity and activity enhance to rectify the purine limitation.

To our knowledge, there are few studies working out on the ADA status in ALL patients. In the research performed by Mishra et al. (8), the significant higher ADA activity was reported among ALL patients compared to that of the control group. However, they found no different ADA activities between ALL patients and those with either leukocytosis and lymphocytosis (8). In addition, in another study (9) it is observed the higher level of serum ADA but reduced lymphocytes count among patients suffered from ALL comparing with the normal individuals. Our findings viewed the significantly higher activity of ADA in ALL patients in comparison to the healthy subjects. In this study, data revealed that active ALL groups, new cases, subjects with the relapsed disease have a higher ADA1 activity while there was no increased ADA activity among patients in the remission stage and those in the healthy control group. The previous researchers have assessed the activity of the total ADA. In the present study, we have analyzed the activity of both ADA1 and ADA2 isoenzymes in the patients with ALL and the healthy control group. Our results revealed that ADA1 is a key isoenzyme, considerably over-expressed in ALL malignancy. However, we identified no significant alterations in the activity and quantity of ADA2 in the patients studied.

Although the present study was done on three different groups of patients related to ALL, two ADA isoenzymes, and total ADA, there are few limitations in it. The number of patients in the current study is few and to confirm the findings, it is necessary to perform the study in the larger sample sizes. In addition, the genetic and epigenetic profiles of the patients in each tested groups could be supporting to decide if the application of ADA assay might be considered for ALL monitoring and detection.

Conclusion

Overall, ADA activity and quantity increase in ALL patients and relapsed stage of the disease. In addition, ADA1 is the key isoenzyme elevating in ALL patients, therefore, it might be a useful biomarker together with other approaches to diagnose ALL patients and monitor their therapies.

Acknowledgments. This study has been granted by Pasteur Institute of Iran. We would like to thank the staff of Biochemistry Department, Pasteur Institute of Iran, for their helpful assistance.

Conflict of interest statement

The authors stated that they have no conflicts of interest regarding the publication of this article.

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Received: May 22, 2017
Accepted: August 7, 2017