

Role of adenosine deaminase and purine nucleoside phosphorylase in severe combined immunodeficiency disease: A biochemical and molecular study

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(Received: March 22, 2007; Accepted: April 28, 2007)

ABSTRACT

Deficiencies of adenosine deaminase (ADA) and purine nucleoside phosphorylase (PNP) have been implicated in the pathogenesis of severe combined immunodeficiency disease (SCID). This study examined the activities of ADA and PNP enzymes in 450 school children and 20 suspected SCID patients. The results showed a significant increase in serum ADA of SCID patients as compared to controls whereas the activity of PNP did not differ significantly between the two groups. Single stranded conformational polymorphism (SSCP) analysis did not reveal any molecular defect in ADA and PNP genes of patients' suggesting that the suspected patients were not suffering from immunodeficiency due to ADA or PNP deficiency. Further studies on large number of SCID patients are needed to determine the involvement of allelic variants or novel mutations resulting ADA/PNP deficient immunodeficiency in Saudi population.

Keywords: Adenosine deaminase, Purine nucleoside, Immunodeficiency disease, Biochemical and molecular study.

INTRODUCTION

Adenosine deaminase (adenosine aminohydrolase, ADA, EC 3.5.4.4), an essential zinc metalloenzyme of purine salvage pathway catalyses the irreversible hydrolytic deamination of adenosine to inosine and ammonia. Although this enzyme activity is widely distributed in human tissues, it is highest in lymphoid cells such as those of the thymus, spleen, and lymph nodes^{1, 3}. Levels are notably high in immature T lymphocytes³⁻⁶. Genetically determined deficiency of adenosine deaminase activity in human is associated with an autosomal recessive form of severe combined immunodeficiency disease (SCID)⁷. Whereas, the inhibition of ADA in immature T lymphocytes with deoxycytosine renders these cells extremely sensitive to cytotoxic effects of deoxyadenosine; B-lymphocytes are not adversely affected⁷.

The ubiquitous purine nucleoside phosphorylase (PNP) plays a key role in purine salvage pathway, and PNP deficiency in humans leads to an impairment of T cell function, usually with no apparent effects on B-cell function. It has been considered as primary target for chemotherapeutic intervention since its discovery⁸ as the children suffering from lymphopenia, with severely defective T-cell, but normal B-cell immunity, exhibited a total lack of PNP activity. PNP deficiency is a rare disorder associated autosomal recessive form of cellular, but not humoral immunodeficiency and comprises about 4% of all cases of SCID.

Approximately 85% of ADA deficient infants present classical SCID. In these patients, cellular and humoral immune functions are both profoundly impaired, their illness begins during the

first few months of life, and they succumb rapidly to their multiple infections. About 15% of ADA deficient infants become ill at a later age and have more prolonged course, although they too eventually succumb to their disease within the few years of life. In vitro results of immune function in partial ADA deficient patients revealed a more profound defect of cellular than humoral immunity^{9, 10}. The clinical heterogeneity could result from either genetic heterogeneity or random differences in exposure to infectious agent. Genetic heterogeneity could appear in immunologically normal individual who was discovered during population studies of Kung tribe of Kalahari Desert¹¹. People of this tribe lacks erythrocyte's ADA but lymphocytes contain appreciable ADA activity¹². Small and large molecular forms of the enzymes are observed in different human tissue¹. The small molecular form of ADA is catalytically active single polypeptide chain of about 38,00 daltons which contains carbohydrate and additional post-translational modification^{13, 14}. The large molecular form exists as a complex of catalytic ADA subunit and a nonenzymatic binding protein¹⁵. Adenosine deaminase is coated on the large arm of chromosome 20^{16, 17}.

The present study was conducted to screen the Saudi population for the frequency of SCID due the deficiency of ADA activity and to characterize the molecular defects causing this syndrome.

MATERIALS AND METHODS

Venous blood (10ml) was collected from 450 healthy children attending various primary

school and 25 patients of SCID from various hospitals of Riyadh. Blood was aliquoted in two tubes, one was used for enzyme activity and other was used to extract RNA and DNA. Adenosine deaminase activity was assayed in the erythrocyte according to the method of Hopkinson et al²⁰. This method measure the increase in absorbance at 293 nm resulting from the conversion of adenosine to inosine by endogenous ADA then to uric acid by the sequential action of exogenous nucleoside phosphorylase and xanthine oxidase at 37 °C. The reaction tube contains 1.3mM adenosine, 1 µg/ ml nucleoside phosphorylase, and 50µg/ml xanthine oxidase in 0.05 M sodium phosphate buffer pH 7.5; 100 µl of hemolysate were added per milliliter of reaction mix. To distinguish between ADA1 and ADA2, the activity was measured using the procedure with or without erthro-9-(2-hydroxy-3-nonyl) adenine (EHNA). It is a potent inhibitor of only ADA1 isoenzyme and used in the concentration of 200mmol/L in the reaction mix¹⁷. The activity of ADA1 was then calculated from the total ADA activity. The normal inherited polymorphic form of ADA (ADA1, ADA2 and ADA 2-1) was also determined by electrophoresis in starch gel¹⁸. The coupled xanthine oxidase assay of Kalckar¹⁹ as modified by Hopkinson et al²⁰ was used: the assay mixture contained 50mM Phosphate buffer pH 7.5, 1 mM inosine and 0.025 units of xanthine oxidase per ml. The absorbance at 293 nm due to the formation of uric acid was monitored.

DNA was isolated from large series of blood samples by NaCl extraction method²¹. Isolation of total cellular RNA was carried out by guanidinium isothiocyanate method²². First-strand

Table - 1: Serum PNP, ADA and its isoenzymes in healthy controls and SCID patients.

Enzymes (U/L)	School Children	Patients
PNP	37.38 ± 2.12	33.73 ± 4.06
ADA	15.07 ± 1.12	26.32 ± 0.98*
ADA ₁	10.45 ± 1.02	23.01 ± 0.98*
ADA ₂	4.62 ± 0.95	03.31 ± 0.98

*P<0.05 using t-test. Values are means ± SEM.

cDNA Synthesis Kit (Amersham Pharmacia biotech) Amplification of full-length and partial coding regions of ADA and PNP genes was carried out using several sets of specific primers (unlabelled or Cyanine 5' labeled,) for single strand conformation polymorphism (SSCP) was used to synthesize full length and various fragments of coding regions of ADA and PNP. The amplified PCR products were visualized by loading 5µl of the PCR product by electrophoresis on 1.8% agarose gel and ethidium bromide staining. A DNA molecular weight marker, DNA VIII (Roche) was always included beside the PCR products on the agarose gel electrophoresis to confirm the specificity by size.

For the detection of point mutations in the ADA/PNP genes, Cyanine 5' labeled primers were used for PCR-SSCP analysis on the AlfExpress II by allele locator program. PCR-SSCP is a rapid and sensitive method to detect mutations or polymorphism in DNA. The products of PCR generated by Cyanine 5' labeled primers are denatured by heat to single stranded DNA (ssDNA), and the mutations or polymorphism can be detected electrophoretically as mobility shifts resulting from a change in the conformation of ssDNA. PCR

amplification of the coding and genomic DNA fragments of the ADA/PNP gene was performed using labeled primers. For PCR-SSCP analysis, 2 µl of each PCR product was supplemented with 2 µl of denaturing solution (10 ml formamide + 1 mg bromophenol blue), equilibrate for 4 minutes at 95°C. The samples were quickly placed on ice/water bath and allowed to cool for few minutes. Short electrophoresis cassette was used. Samples were applied to the gel and run immediately started.

RESULTS AND DISCUSSION

The mean values for ADA and PNP activity for healthy and suspected SCID patients are given in Table 1. Statistical analysis revealed significantly higher values for ADA and in suspected patients than healthy individuals. The activities for isoforms of ADA revealed significantly higher values of ADA₂ in the suspected patient than the values of the healthy individuals. However the values of ADA₁ did not differ significantly between controls and patient. ADA₁ isozyme is found in all the cells with highest concentration found in lymphocytes and monocyte²³. Higher levels of ADA₁ in the biological fluids are mainly due to cell necrosis²⁴. Whereas, ADA₂

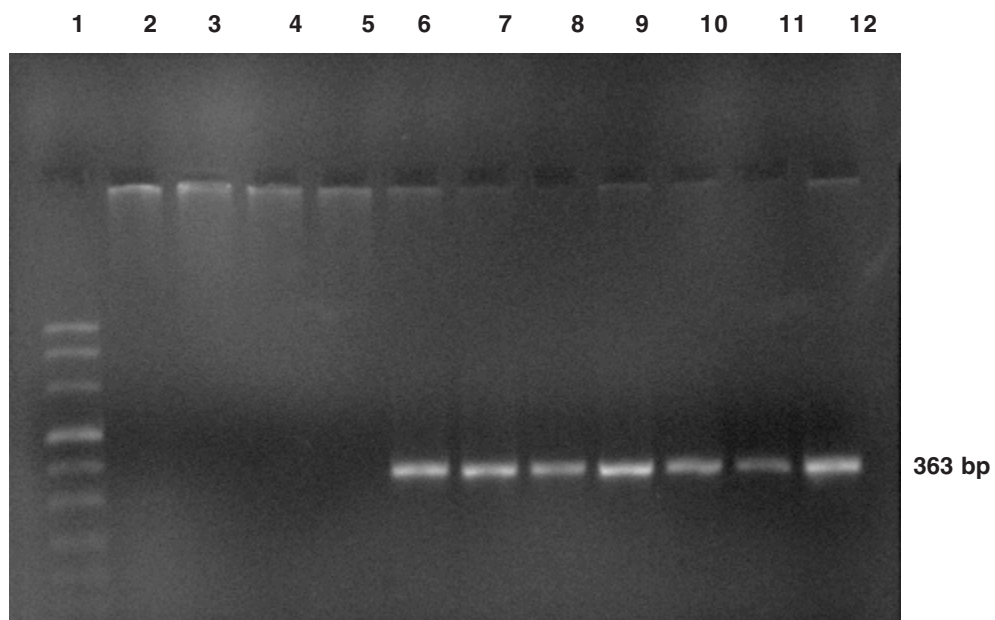


Fig. -1: A representative agarose gel electrophoresis of PCR amplified products from patients and control DNA samples (lanes 5-12; 363 bp) for exon 4 of the ADA gene using CY⁵ labeled primer sets 10a-10b SSCP by Alfexpress automated DNA analyzer. Lanes 1 represent DNA Marker VIII.

isoenzyme appears to be found only in monocyte macrophages from which it is released when they are stimulated by the presence of living organisms in their interior. This explains why ADA₂ increase in biological fluids in the course of infectious diseases causing the immunodeficiency like tuberculosis, HIV, HCV etc²⁵.

Our results of enzyme activity revealed the lower chances of the SCID due ADA deficiency. However, one patient's serum showed lower levels of total ADA activity and the activities of this sample's isoforms also revealed the lower values for ADA₁ and ADA₂. Zymography also confirmed the lower

activity of isoforms ADA where as it was normal for PNP. Since there are reports, which indicated the higher values of ADA during the childhood and later, they developed the late onset the disease²⁶. Some previous reports indicated that the heterozygous/ carriers for SCID retained the activities of the enzymes in normal range²⁷ or even little higher than the normal individuals. Full-length cDNA was amplified for both PNP and ADA gene by reverse transcriptase polymerase chain reaction (RT-PCR) for all patients and 15 selected healthy individuals. Manual and automated screening for ADA and PNP gene by SSCP revealed no molecular defects in suspected or normal individuals.

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