

Expression of Activation-Induced Cytidine Deaminase Gene in B Lymphocytes of Patients with Common Variable Immunodeficiency

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Abstract

Objective: Common variable immunodeficiency (CVID) is a heterogeneous disorder characterized by reduced serum level of IgG, IgA or IgM and recurrent bacterial infections. Class switch recombination (CSR) as a critical process in immunoglobulin production is defective in a group of CVID patients. Activation-induced cytidine deaminase (AID) protein is an important molecule involving CSR process. The aim of this study was to investigate the *AID* gene mRNA production in a group of CVID patients indicating possible role of this molecule in this disorder.

Methods: Peripheral blood mononuclear cells (PBMC) of 29 CVID patients and 21 healthy controls were isolated and stimulated by CD40L and IL-4 to induce *AID* gene expression. After 5 days *AID* gene mRNA production was investigated by real time polymerase chain reaction.

Findings: *AID* gene was expressed in all of the studied patients. However the mean density of extracted *AID* mRNA showed higher level in CVID patients (230.95±103.04 ng/ml) rather than controls (210.00±44.72 ng/ml; $P=0.5$). CVID cases with lower level of *AID* had decreased total level of IgE ($P=0.04$) and stimulated IgE production ($P=0.02$); while cases with increased level of *AID* presented higher level of IgA ($P=0.04$) and numbers of B cells ($P=0.02$) and autoimmune disease ($P=0.02$).

Conclusion: Different levels of *AID* gene expression may have important roles in dysregulation of immune system and final clinical presentation in CVID patients. Therefore investigating the expression of *AID* gene can help in classifying CVID patients.

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Key Words: Activation-Induced Cytidine Deaminase; AICDA Protein; Common Variable Immunodeficiency; Class Switching

Introduction

Common variable immunodeficiency (CVID) is a heterogeneous type of primary immunodeficiency characterized by hypogammaglobulinemia and increased susceptibility to recurrent infections^[1-3].

Involved molecular mechanisms in the pathogenesis of the CVID are not completely identified and its diagnose should be confirmed by exclusion of other antibody deficiencies^[4]. Despite numeral investigation to find the etiology of CVID and reports of various defects in B cells, T cells, or

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dendritic cells, the exact pathogenesis of this disease is yet unknown^[5-8].

There are several reported defects in the process of antibody production in CVID patients including impaired differentiation of B cells into plasma cells, impaired class switch recombination (CSR), impaired antibody affinity maturation and somatic hypermutation (SHM), and deficiency of memory B cells^[9-12]. In recent years, various gene defects (CD19, CD81, CD20, CD21, ICOS and LRBA) have been reported as an etiological mechanism of CVID patients and were classified as a separated disease^[13-16].

One of the most important and effective molecules in the germinal center reactions is activation-induced cytidine deaminase (AID) which has critical role in CSR and SHM processes^[17-20]. Despite the probable important role of AID in CSR process, there are few studies focusing on the investigation of *AID* gene and its expression in CVID patients^[21-22].

The aim of this study was to evaluate the expression of AID messenger RNA (mRNA) in peripheral B cells of a group of Iranian CVID patients.

Subjects and Methods

Patients' characteristics

Twenty-nine CVID patients and a control group of 21 age and sex matched healthy donors were enrolled in this study. The diagnosis of CVID was made in all of the patients by using standard criteria of the European Society for Immune Deficiency (ESID) and the Pan American Group for Immunodeficiency (PAGID)^[23]. All patients received monthly intravenous immunoglobulin (IVIg). The process of this study was approved by the ethics committee of Tehran University of Medical Sciences and informed consent was obtained from all of the subjects. The clinical phenotypes of patients were assigned based on our previous article^[24].

Preparation of blood culture

A five ml blood sample was taken from each patient or healthy donor. Peripheral blood mononuclear cells (PBMCs) were isolated from the

samples using Ficoll-Paque density gradient centrifugation. Samples were centrifuged at 400 g for 30 minutes to remove the culture. Separated PBMCs were then moved to another falcon tube, were washed for two times at 300 g for 5 minutes and at 150 g for 10 minutes with Phosphate-Buffered Saline (PBS) solution (Sigma Aldrich, Germany). Isolated PBMCs (1×10^5 cells) were added to culture to be stimulated by rh-IL4 and rh-CD40L (Invitrogen, Germany) and were evaluated for molecular analysis of class switching and IgE production at the end of 5th and 12th day, respectively.

To create a suitable culture for IgE stimulation, 400 ng/ml rh-IL4 and 200 IU/ml rh-CD40L were added to a humidified atmosphere provided by 5% CO₂ in 1 ml of Iscove's Modified Dulbecco's medium (IMDM) at 37°C. Further supplements including 10% fetal calf serum, 1% sodium pyruvate and 1% non-specific amino acids were added to provide a better condition for cell proliferation. A mixed solution of 10.000 U/ml penicillin and 10.000 µg/ml of streptomycin were added to the culture in order to prevent bacterial contamination.

Evaluation of mRNA production

At the end of fifth day, PBMCs were removed from the culture using 0.5 ml Guanidinium thiocyanate-phenol-chloroform (TRIzol, Gibco-BRL Grand Island, NY, USA) at -20°C as was explained in the manufacturer's manual for mRNA extraction. Quantitative measurement of mRNA was performed using BioPhotometer (Roche, Germany) which evaluates peak optical density (OD) at 230, 260 and 280 nm. In addition to *AID*, mRNA production of β -actin gene was also measured as a control. We performed real time polymerase chain reaction (RT-PCR) method using cDNA Synthesis Kits (Fermentas, Canada); after mRNA extraction, cDNA (for *AID* and β -actin genes) were produced by Oligo (dT) primer and reverse transcriptase enzyme. Specific primers for each gene were designed by the information on GenBank nucleotide sequence database and their characteristics were evaluated using Oligo V6 software (Molecular Biology Insights, Inc., Cascade, Co.). Oligonucleotide sequences of these primers are shown in Table 1. At the end, PCR method was performed on produced cDNAs using Master Mix Kit (Fermentas, Canada).

Table 1: Primer sequences for β -actin and *AID* genes

Gene	Primer sequence
<i>Actin</i>	Forward: 5`-TACCACTGGCATCGTGATGGACT-3` Reverse : 5`-TCCTTCTGCATCCTGTGCGCAAT-3`
<i>AID</i>	Forward: 5`-GAGGCAAGAAGACACTCTGG-3` Reverse : 5`-GTGACATTCTGGAAGTTGC-3`

Aid: Activation-induced cytidine deaminase;

Statistical analysis

We classified patients based on the cut-off point of *AID* mRNA count calculating two standard deviations (2SD) below and over the mean of healthy controls. We generated three groups of patients including G1 (2SD below), G1 (2SD over) and normal *AID* patients based on this classification. Comparisons between groups were performed using Student t test (between two groups) and ANOVA test (Post Hoc analysis between three groups) in all continued numerical data and χ^2 test was performed when rates and ratios were compared between groups. Difference was considered as significant if the *P*-value was lower than 0.05.

Findings

Characteristics of Patients

Twenty-nine CVID patients (24 males and 5 females) were enrolled in this study. The mean age of the patients at the time of this study was 15.86 ± 9.42 years (median age, 14 years; ranged 3-51 years). The mean age of the controls was 18.23 ± 9.41 years (median age, 17 years; ranged 5-

51). The median age of the patients at the onset of disease was 24 months (ranged 1 month-15 years). The median age of the patients at the time of diagnosis was 6 years (ranged 4-43 years) and the median diagnosis delay was 4 years (ranged 1-336 months).

Serum levels of IgG, IgM and IgA of all patients were below the mean normal values for age and sex by at least 2SD or more. The median serum levels of IgG was 127 mg/dL (ranged 0-720 mg/dL). The median serum levels of IgM and IgA were 31 mg/dL (ranged 0-88 mg/dL) and 7 mg/dL (ranged 0-52 mg/dL) respectively.

Production *AID* and β -actin mRNA

The result of RT-PCR for *AID* gene showed the presence of *AID* mRNA expression in all subjects in both patient and control groups. A pair of primers for β -actin gene was also used as ladder control to evaluate the accuracy of mRNA production in the assessment of *AID* gene. All patients and their controls were also able to produce normal values of β -actin mRNA. Since β -actin mRNA expression occurs in all cells, the result of this test indicates that the process of mRNA production was performed correctly. Sample results of electrophoresis for the mRNA production of *AID* and β -actin genes are shown in Fig. 1 and 2 respectively.

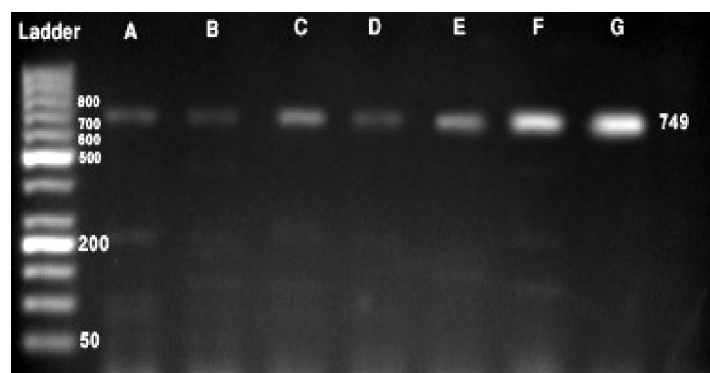


Fig. 1: Electrophoresis of *AID* gene RT-PCR products (749 base pairs). PBMCs of both patient and controls were studied for mRNA expression after being simulated by CD40L and IL-4. A-D (patients) and E-G (control) samples show normal expression of mRNA

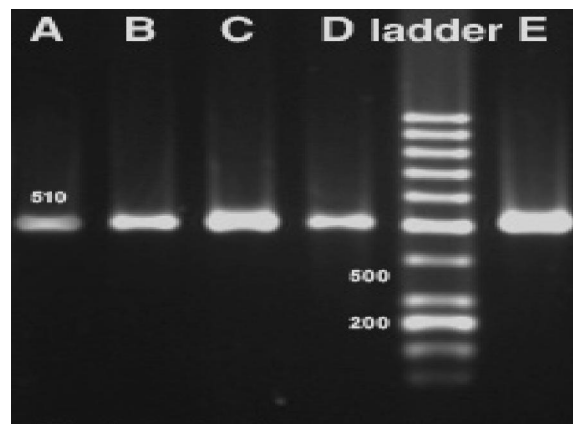


Fig. 2: Electrophoresis of β -actin gene RT-PCR products (510 base pairs). β -actin gene was used as an internal control to evaluate the synthesis and production of cDNA. A and B are patients' samples while C, D and E are samples from the control groups

The qualitative assessment of the extracted mRNA was checked by evaluation of the presence of respective bands of 18s and 23s ribosomal RNA as controls in the electrophoresis which was normal in all samples. In addition in quantitative measurement, the mean density of extracted AID mRNA was 230.95 ± 103.04 ng/ml in CVID patients and 210.00 ± 44.72 ng/ml in controls ($P=0.5$). Therefore these findings showed non-significant increased level of produced mRNA by PBMCs samples in cases compared with controls. According to the results of healthy controls two cut-off points were measured (2SD below: 120 ng/ml and 2SD over: 300 ng/ml) regarding the statistical method of comparison. By current method of analysis four (G1) and five patients (G2) had the density below and over the cut-off point, respectively.

All patients in G1 suffered from infections only clinically. The means of the density AID mRNA were significantly different between G1 and other cases (100.0 ± 50.2 vs. 261.7 ± 89.3 ng/ml; $P=0.002$). Surprisingly comparing these 4 patients with lower level of AID mRNA to other CVID patients, the total level of IgE (1.09 ± 0.42 vs. 4.02 ± 0.53 IU/ml; $P=0.04$) and stimulated IgE production (0.60 ± 0.31 vs. 3.97 ± 0.53 IU/ml; $P=0.02$) were significantly reduced.

In contrast to G1, cases of G2 with higher amount of AID expression, presented autoimmune manifestation as a most frequent clinical phenotype (2 cases of idiopathic thrombocytopenic purpura and 2 cases of autoimmune hemolytic anemia; 0% vs. 80%; $P=0.02$). This group of cases had significantly increased level of IgA comparing with other CVID patients

(19.42 ± 3.1 vs 9.0 ± 4.2 ; $P=0.04$). The cell count of CD 19+ lymphocytes in G2 (520.7 ± 144.2 cell/ml) was significantly higher than in G1 (396.8 ± 98.1 cell/ml; $P=0.02$). Gender ratio and parental consanguinity did not differ between groups. ANOVA test and comparison between groups is summarized in Table 2.

Discussion

Definitive single gene defect responsible in the pathogenesis of CVID is not identified^[25]. However, heterogeneous clinical symptoms and laboratory findings suggest various types of genetic defects in the pathogenesis of this disorder. Defective antibody production in CVID may be resulted from a failure in differentiation of B cells into plasma cells^[26]. The current study is the first study that investigates the AID gene expression in CVID cases as a probable reason for defect in CSR and production of class switched plasma cells.

AID is a RNA-editing deaminase that can change mRNAs through base substitution and generate mRNAs with various functions. AID has a critical role in late differentiation of B cells^[27]. This enzyme is induced following CD40 and CD40L interaction^[28]. In most cases, CSR in B cells initiates by binding of CD40 and CD40L that are located on the surface of B cells and T cells respectively^[29]. CD40L and IL-4 could be used for in vitro stimulation of B cells similar to current study which clarified the critical role of AID in late

Table 2: Demographic and immunologic data comparing between three groups of Common variable immunodeficiency cases classified based on activation-induced cytidine deaminase mRNA expression

Parameters	Reduced AID mRNA G1 (N=4) Mean (SD)	Normal AID mRNA (N=20) Mean (SD)	Increased AID mRNA G2 (N=5) Mean (SD)	total	P-value *		
					G1 and G2 †	G1 and Normal group †	G2 and Normal group †
Age at onset, year	3.0 (2.82)	3.38 (0.44)	2.7 (0.41)	0.3	1	0.9	0.9
Age at Diagnosis, year	6.25 (3.0)	9.26 (1.1)	6.6 (0.92)	0.7	1	0.6	0.8
Diagnostic delay, year	3.2 (2.6)	5.8 (0.7)	3.8 (0.53)	0.5	1	0.5	0.7
Current age, year	11.0 (3.16)	19.6 (10.3)	16.4 (9.09)	0.2	0.8	0.1	0.7
WBC, cell/ml	9900.0 (1979.8)	7526.6 (4534.4)	5152.0 (4195.7)	0.3	0.4	0.3	0.4
Lymphocyte, cell/ml	4550.9 (1202.1)	2920.45 (1507.9)	2268.8 (1385.0)	0.6	1	1	0.7
CD3+ lymphocyte, cell/ml	3731.5 (2372.8)	1927.07 (1233.4)	1270.8 (476.1)	0.1	0.06	0.1	0.5
CD4+ T cells, cell/ml	2053.9 (1480.0)	1139.2 (452.7)	680.4 (557.2)	1	0.2	0.3	0.5
CD8+ T cells, cell/ml	1417.2 (899.2)	751.5 (372.1)	577.0 (238.5)	0.9	0.4	0.7	0.8
CD19+ lymphocyte, cell/ml	396.8 (98.1)	403.2 (146.7)	520.7 (144.2)	0.4	0.02	0.5	0.07
CD 19+ CD27+ B cells, cell/ml	7.6 (3.0)	13.5 (4.9)	10.9 (2.4)	0.6	0.6	0.3	0.7
CD19+CD21+ memory Bcell, cell/ml	14.2 (10.4)	11.4 (9.1)	5.9 (1.8)	0.3	0.4	0.8	0.2
IgG, mg/dl	189.3 (38.6)	321.5 (256.6)	245.7 (200.9)	0.5	0.9	0.4	0.8
IgA, mg/dl	8.5 (6.3)	9.0 (4.2)	19.42 (3.1)	0.5	0.01	0.5	0.04
IgM, mg/dl	23.0 (12.7)	41.3 (22.2)	43.2 (14.9)	0.5	0.6	0.3	1
IgE, IU/ml	1.09 (0.42)	4.02 (0.53)	6.47 (3.0)	0.2	0.02	0.04	0.4
IgE stimulated, IU/ml	0.60 (0.31)	3.97 (0.53)	6.4 (5.7)	0.2	0.01	0.02	0.08

* P-value of ANOVA test ; † P. value of Post Hoc analysis

differentiation of these cells^[30].

AID deficiency is known to be responsible in the pathogenesis of the autosomal recessive form of hyper-IgM syndrome (HIgM type 2) and causes impaired CSR in the patients^[18]. Possibly the role of AID in CSR process is after the generation of germ line transcripts because of normal induced Iε-Cε transcript by rh-CD40L and rh-IL-4 in HIgM type 2 patients^[18]. Defect in AID can possibly be found in a group of CVID patients that have normal level of IgM and reduced SHM^[9].

In this study, AID mRNA production was produced in all of the 29 CVID patients. In accordance with our findings, Minegishi et al^[21] studied *AID* mutation in HIgM patients comparing with CVID cases which showed defects of *AID* gene in 18 out of 27 (66.7%) HIgM patients while none of 23 CVID cases had defective *AID* gene expression. Also in a study performed by Ohm-Laursen et al^[22] investigating 34 CVID patients for *AID* gene, no mutation was found. These two above mentioned studies were performed at the stage of genome of DNA to investigate the incidence of mutation or deletion in *AID* gene but did not evaluate AID mRNA transcription in CVID

patients^[21,22].

In most CVID patients germinal center reactions occur following interaction of an antigen with B cells defectively leading to impaired CSR and reduced level of switched memory B cells^[31]. In addition, defect in SHM and impaired antibody affinity maturation has been reported in a group of CVID patients^[9,10,32-34]. Therefore one of the possible causes of CVID could be defects in molecules involving in germinal center reactions especially AID. CVID patients are classified into two subgroups based on the number of class switched memory B cells. The first group has reduced amount of class switched memory cells indicating the presence of a defective CSR in B cells, and the second one has normal numbers of these cells^[35]. Defect in AID besides profound CSR impairment, can also cause defects in generation of somatic point mutations in variable regions of *Ig* gene, leading to impaired antibody affinity maturation^[18].

Findings of this study showed for the first time non-significant over expression of AID in CVID cases compared to controls. This over expression may reflect any blocks in terminal plasma cell

differentiation of CVID patients which lead to decreased negative feedback for suppression of AID expression in most of cases. AID is a member of molecular group associated with proliferation, B cell receptor signaling, CSR or cell cycle as well as BCL-6, c-myc, BTK. The genes of these molecules are repressed by Blimp-1^[36,37].

A proposed model for double-negative feedback loop in lymph nodes of CVID patients was explained by Blimp-1 and Syndecan-1 expression. This study defined that plasma cells of CVID patients could divide into three subsets according to their differential expression of Blimp-1 and Syndecan-1^[31]. Based on these subsets we can interpret our findings with three categories of cases including decreased levels of AID, normal levels of AID and over expressed levels of AID.

Different levels of AID gene expression may have important role in dysregulation of immune system and final clinical presentation in CVID patients. Autoimmune disorder in G2 with elevated expression of AID could be associated with increased activity of germinal center and have undergone further SHM. This over activity is associated with pathogenic multireactive autoantibodies production^[38]. Although there was no patient with clinical phenotype of malignancy in G2, coincidence of malignancies especially lymphoma and over expression of AID was reported previously^[39-41]. We propose future AID expression study on selected groups of CVID cases including autoimmune and cancer phenotypes. Moreover mutational analysis and direct sequencing of AID gene in these selected cases of CVID may be useful for further clarification.

Conclusion

Based on the result of this study that shows normal AID mRNA transcription in most (69% quantitatively and qualitatively) of CVID patients, it has been suggested that the causing mechanism of this disorder can be the defects in other molecules involving the stages of CSR. However it should be noticed that CVID is a very heterogeneous disorder and there is still a possibility in the presence of defect in function of AID in this group of patients.

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Conflict of Interest: None

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