



Mutation characterization and heterodimer analysis of patients with leukocyte adhesion deficiency: Including one novel mutation



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ABSTRACT

Background and aim: Leukocyte adhesion deficiency type 1 (LAD-I) is a rare, autosomal recessive disorder of neutrophil migration, characterized by severe, recurrent bacterial infections, inadequate pus formation and impaired wound healing. The *ITGB2* gene encodes the $\beta 2$ integrin subunit (CD18) of the leukocyte adhesion cell molecules, and mutations in this gene cause LAD-I. The aim of the current study was to investigate the mutations in patients diagnosed with LAD-I and functional studies of the impact of two previously reported and a novel mutation on the expression of the CD18/CD11a heterodimer.

Materials and methods: Blood samples were taken from three patients who had signed the consent form. Genomic DNA was extracted and *ITGB2* exons and flanking intronic regions were amplified by polymerase chain reaction. Mutation screening was performed after Sanger sequencing of PCR products. For functional studies, COS-7 cells were co-transfected with an expression vector containing cDNA encoding mutant CD18 proteins and normal CD11a. Flow cytometry analysis of CD18/CD11a expression was assessed by dimer-specific IB4 monoclonal antibody.

Results: Two previously reported mutations and one novel mutation, p. Cys562Tyr, were found. All mutations reduced CD18/CD11 heterodimer expression.

Conclusion: Our strategy recognized the p.Cys562Tyr mutation as a pathogenic alteration that does not support CD18 heterodimer formation. Therefore, it can be put into a panel of carrier and prenatal diagnosis programs.

1. Introduction

Integrins are transmembrane receptors that are involved in cell–cell and cell–extracellular matrix. Interactions, immunity, wound healing, hemostasis and the development throughout the body. These proteins are large, heterodimeric cell adhesion molecules composed of α and β subunits. The $\beta 2$ integrins (CD18) are β subunits in a family of heteromeric proteins: $\alpha L\beta 2$ (LFA-1, CD11a/CD18), $\alpha M\beta 2$ (Mac-1 or CR3, CD11b/CD18), $\alpha X\beta 2$ (p150,95, CD11c/CD18) and $\alpha D\beta 2$ (CR4, CD11d/CD18). These four proteins are expressed on leukocytes, except for $\alpha D\beta 2$ (CR4, CD11d/CD18), which is only expressed on macrophages. The integrin $\beta 2$ family has a crucial role in the immune system,

because they recruit and activate leukocytes during inflammation [1–6]. Integrins can bind to extracellular matrix (ECM) glycoproteins, including collagens, fibronectins, laminins, and cellular receptors such as vascular cell adhesion molecule-1 (VCAM-1) and the intercellular cell adhesion molecule (ICAM) family [7]. Genetic alterations in the beta2-integrin gene play an important role in the pathophysiology of several diseases and genetic syndromes, including Leukocyte Adhesion Deficiency (LAD-I) and Systemic Lupus Erythematosus (SLE) [3].

CD18 is encoded by a gene located on chromosome 21q22.3 known as *ITGB2*. CD18 deficiency leads to incomplete formation and/or dysfunction of $\beta 2$ integrins. More than 100 mutations have been reported in the *ITGB2* gene, including missense mutations (40%), splice

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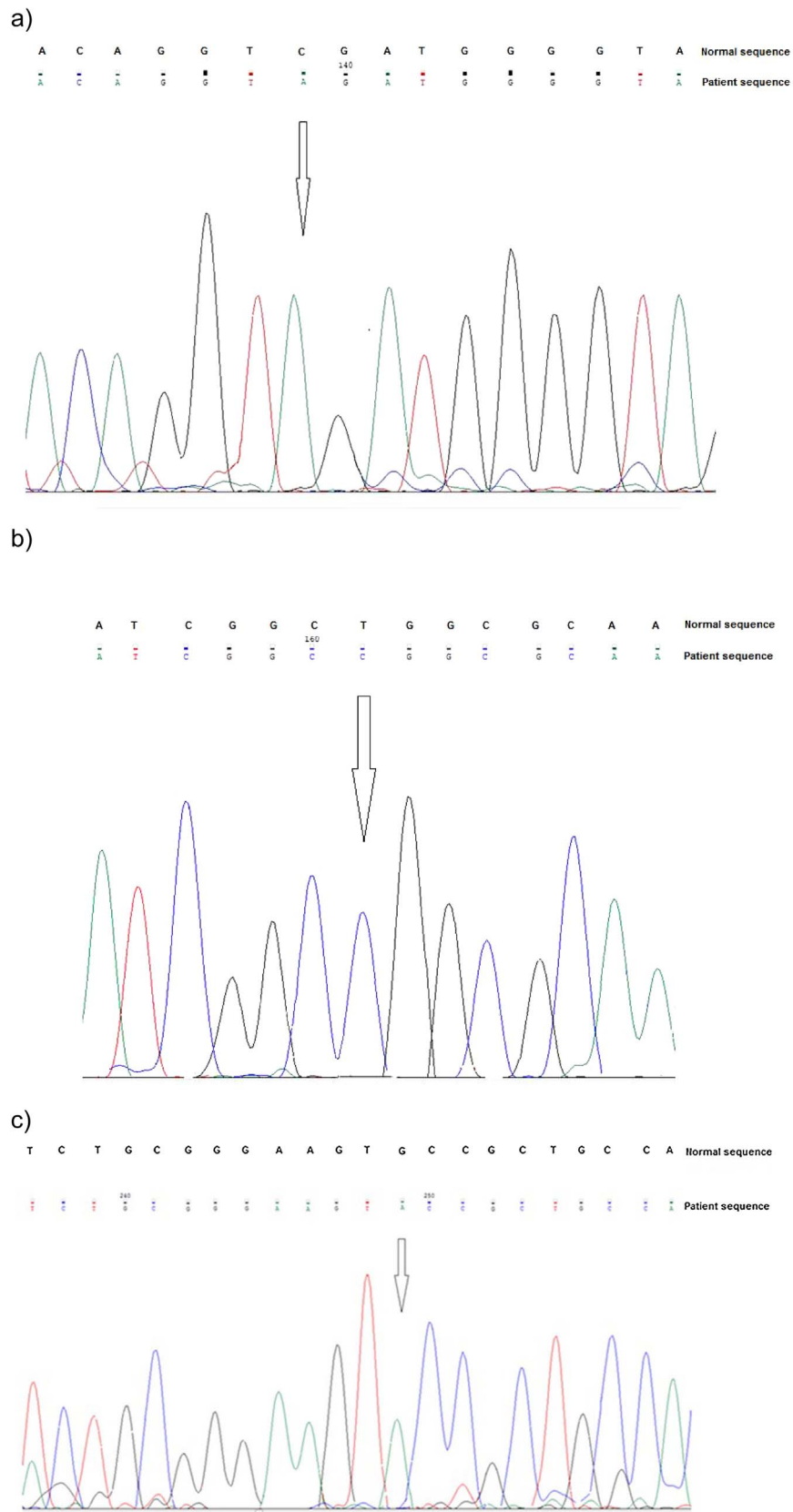


Fig. 1. Electropherogram of mutations.a) c.382 C > A on (-) strand leading to; p. Asp128Tyr b) c.754 T > C on (+) strand leading to; p.Trp282 Arg, and finally c) c.1885 G > A on (+) strand leading to; p. Cys563Tyr.Normal sequence is shown above each panel.

Table 1
Clinical and molecular features of patients with LAD.

Patient ID	Sex	Main clinical presentation	Region of born	Separation of umbilical cord	Other clinical presentation	WBC/ μ l $\times 10^3$	Isolated organism	Outcome	Level of CD18(%) at diagnosis	Nucleotide change	Amino acid change	Type of mutation	Novelty
1	F	Skin ulcer	South-west	15 days	pneumonia, skin ulcer, omphalitis, sepsis	58	<i>P. aeruginosa</i> , <i>S. aureus</i>	alive	1.05	c.382 G > T	p.Asp128Tyr	Missense mutation	Known
2	F	Skin ulcer	East	45 days	Otitis media,	54	<i>P. aeruginus</i>	died	1	c.754 T > C	p.Trp252Arg	Missense mutation	Known
3	F	Skin ulcer	North-west	30 days	Osteomyelitis periodontitis, diarrhea, LAP	72	<i>P. aeruginosa</i> , <i>E. coli</i>	alive	2.26	c.1885 G > A	p.Cys562Tyr	Missense mutation	Novel

Table 2

In silico prediction of p.Asp128Tyr, p.Trp252Arg, and the novel mutation of p.Cys562Tyr on CD18 structure and stability. These residues are conserved in: *Mus musculus* (Mouse), *sus scrofa* (Pig), *Bos Taurus* (Cow), *Rattus norvegicus* (Rat), and equine (horse).

Bioinformatics prediction tools	p.Asp128Tyr	p.Trp252Arg	p.Cys562Tyr novel
SIFT	DAMAGING	DAMAGING	DAMAGING
PolyPhen-2	Probably damaging	Probably damaging	Probably damaging
MUpro	DECREASE of stability of protein structure	DECREASE of stability of protein structure	DECREASE of stability of protein structure
Project HOPE	Disturbed	Disturbed	Disturbed

site mutations (14%), small deletions (15%), large deletions (11%) and other mutations (10%) [1,8]. These mutations are mostly located in a conserved domain of CD18 known as Von Willebrand Factor type A (VWFA) [1,9]. Most missense mutations interfere with integrin heterodimer expression, whereas other missense mutations allow normal expression of integrin heterodimers but without adhesive capacity. Some mutations support subnormal expression of integrin heterodimers with adhesion activity, while a few do not affect the expression of integrins [2].

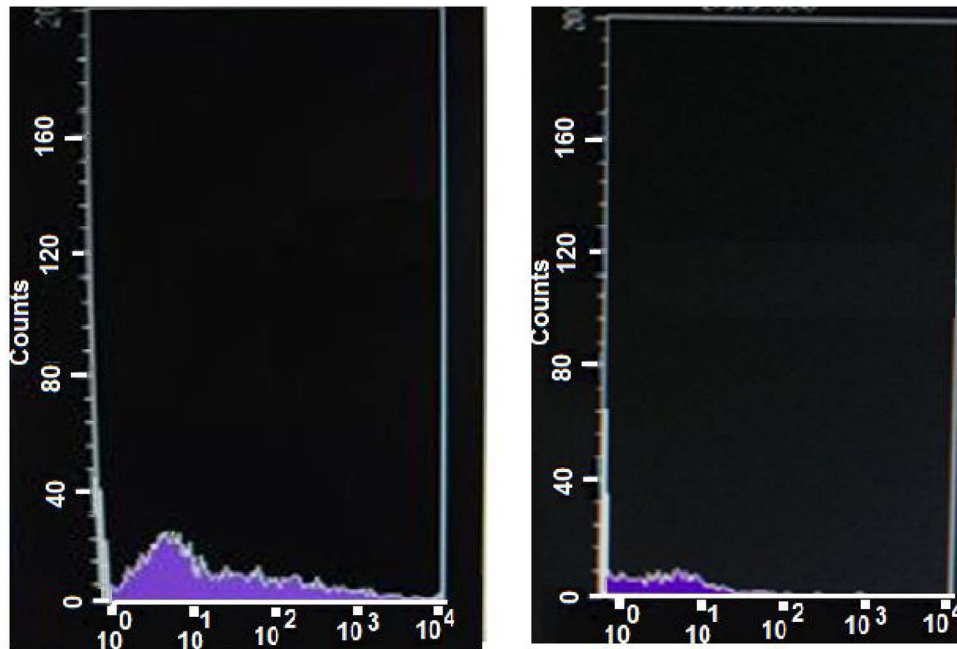
Inflammation due to infection or tissue injury induces a cascade of cellular and microvascular reactions that allow the removal of pathogens or cell debris, and finally give rise to wound healing, repair and homeostasis [10,11]. The process of inflammation includes recruitment (migration) of immune cells such as polymorphonuclear neutrophils (PMN) and monocytes/macrophages to the site of infection. Essential steps during leukocyte recruitment include tethering and rolling over the vasculature, activation, firm adhesion, intraluminal crawling, and extravasation. Firm adhesion and crawling are largely mediated by $\beta 2$ integrins [12,13]. Therefore, the clinical picture of LAD-I is characterized by marked leukocytosis, neutrophilia and localized bacterial infections that are difficult to detect until they have progressed to an extensive level secondary to the lack of leukocyte recruitment at the site of infection [14]. The level of expression of $\beta 2$ integrins has a direct relationship with two relatively distinct clinical phenotypes: Expression of less than 1% of normal CD18/CD11 is seen in the severe form of LAD-I, whereas 3–10% of expression is related with a moderate phenotype [15]. Leukocyte adhesion deficiency (LAD) is a type of autosomal recessive immunodeficiency disorder which is divided into three subgroups, including LAD-I, LAD-II and LAD-III [16]. LAD-I is a rare disease (1 in every 100,000 live births) [17]. As migration capacity of neutrophils is eliminated in LAD-I, these patients consequently suffer from serious bacterial and fungal infection since their neonatal period [18]. Delayed separation of the umbilical cord (after 19 ± 1 day), omphalitis, sepsis, impaired wound healing and impaired pus formation, periodontitis, otitis media, cellulitis, pneumonia, colitis, sepsis, ophth, cervical lymphadenopathy, osteomyelitis are other classical clinical characteristics of LAD-I patients [1].

To answer the question how genetic mutations exactly affect the expression of the encoded protein we investigated the genetic mutations in three patients with LAD-I by PCR sequencing, and then analyzed the effect of the mutations on expression and heterodimer formation of CD18 and the CD11a subunit *in vitro*.

2. Materials and methods

2.1. Patients

In the current study, three patients diagnosed with LAD-I were referred to a children's medical center in Iran in 2016. These patients were from consanguineous marriage. No positive family history was detected in their pedigrees. The disease was confirmed according to



A Normal
Control

B c.382G>T Asp128Tyr
Patient 1



C c.754 T>C Trp252Arg
Patient 2



D c.1885 G>A Cys562Tyr (Novel)
Patient 3

Fig. 2. Expression of LAF-1 on CD11a/CD18 transfected COS-7 cells. Subtracted profiles; those obtained with transfectants of CD11a/CD18 minus profiles of the transfectants with CD18 alone are shown, obtained with CellQuest software, **A:** Cotransfection of wild-type CD18 with wild-type CD11a, **B:** Cotransfection of CD18^{Asp128Tyr} with wild-type CD11a, **C:** co-transfection of CD18^{Trp252Arg} with wild-type CD11a, and **D:** co-transfection of CD18^{Cys562Tyr} with wild-type CD11a.

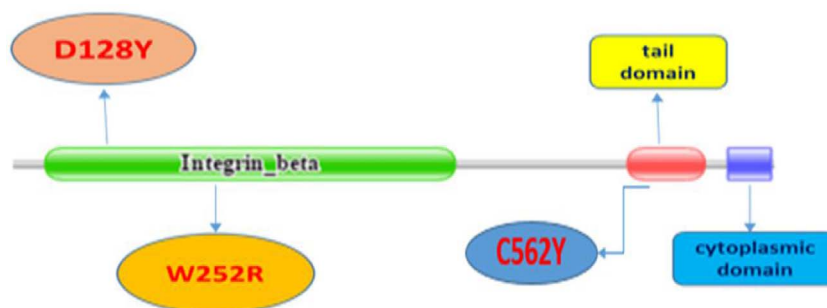


Fig. 3. Mutations in CD18 found in LAD patients. The novel mutation found in this study is shown. Here, it is clear that the C562Y mutation has occurred in the tail domain or Cysteine-rich region that is very conserved.

clinical diagnosis and laboratory findings. This included recurrent severe infections, impaired pus formation, impaired wound healing, delay in umbilical cord separation and omphalitis as clinical features, and flow cytometry study of CD18 and/or CD11b expression as laboratory data. Consent forms were signed by the parents of all patients to guarantee that they understood and participated voluntarily. The ethical committee of the Iran University of Medical Sciences (Tehran) approved the current study.

2.2. cDNA amplification and sequencing

RNA extraction from mononuclear leukocytes and cDNA synthesis were performed according to the manufacturer's instructions (High Pure RNA Isolation Kit, Roche). As reported before [15], the coding region of CD18 cDNA was amplified by polymerase chain reaction (PCR). The PCR products were run on agarose gels to ensure the size of amplified targets. Finally, PCR amplified products were sequenced by Sanger sequencing in both directions.

2.3. Sequencing of genomic DNA

Blood samples were taken from all participants, and DNA was extracted by salting-out [19]. Altogether, sixteen coding exons and their flanking intronic sequences were PCR amplified in the ABI 9700 thermal cycler. The PCR reaction took place in a volume of 25 μ l, including 12.5 μ l of Taq DNA Pol. 2X Master mix Red (Ampliqon, Denmark), 1 μ l of each primer (15 pmol/ μ l), 1.5 μ l of genomic DNA (100 ng/ μ l) and 9 μ l of distilled water. DNA was amplified according to the following program: initial denaturation at 95 $^{\circ}$ C for 4 min, and 30 cycles of denaturation at 95 $^{\circ}$ C for 32 s, primer annealing at 52–62 $^{\circ}$ C for 30 s and DNA amplification at 72 $^{\circ}$ C for 32–80 s. These steps were followed by a final DNA amplification at 72 $^{\circ}$ C for 4 min. The accuracy of the target amplification was analyzed by electrophoresis of PCR products on 2% agarose gel. Primer sequences of target regions have been mentioned previously [15].

PCR products were cleaned up with PCR purification kit (High Pure PCR Product Purification Kit – Roche Life Science). The purified samples together with 10 μ l of related primers used to obtain these PCR products were used for sequencing. Sequencing of samples was performed in an ABI 3130 XL DNA Analyzer (Applied Biosystems, Foster City, CA, USA). Sequencing results were analyzed by Chromas software version 2.33. Alterations were found by alignment of sequencing results against a reference sequence database. Numbering of DNA and protein sequence was performed based on GenBank entry NM_000211.4 and NP_000202.3 as reference. Pathological impact of found variants was predicted by tools consisting of PolyPhen-2 and SIFT.

2.4. Functional heterodimer formation study

2.4.1. Mutation insertion in cDNA and construction of expression plasmid

cDNA encoding the three mutant CD18 or normal CD18 proteins

together with the CD11a subunit were cloned in the expression vector of pvtiro 2 neo. The ability of the three CD18 mutant proteins to form a stable heterodimer with CD11a was investigated with flow cytometry. The introduction of mutations in the putative pvtiro 2 neo was performed with the QuikChange II XL site-directed mutagenesis kit (Stratagene) with the wild-type vector as a template. Briefly, mutant strand synthesis was performed by initial denaturation at 95 $^{\circ}$ C for 1 min, followed by 18 thermal cycles at 95 $^{\circ}$ C for 50 s, 60 $^{\circ}$ C for 50 s, and 68 $^{\circ}$ C for 9 min 24 s, with a final extension at 68 $^{\circ}$ C for 7 min. The construct was sequenced to check that only the desired mutations had been introduced. The expression vector has two cloning sites: one for cloning of nucleic acids encoding mutant CD18 and another for cloning of normal CD11a subunit.

2.5. Cell culture and transfection

COS-7 cells were purchased from the Pasteur Institute of Iran. Cells were cultured in DMEM (Invitrogen) supplemented with 10% (v/v) fetal bovine serum (Invitrogen), 100 IU/ml penicillin and 100 μ g/ml streptomycin (Invitrogen) at 37 $^{\circ}$ C in a 5% CO₂ humidified incubator. COS-7 cells were transfected with expression plasmids by means of Lipofectamin 2000 (Invitrogen) according to the manufacturer's instruction.

2.6. Flow cytometry

Surface expression of the β 2 integrins was determined by flow cytometry on a FACScan flow cytometer and analyzed by Cell Quest software (Becton Dickinson, Mountain View, CA, USA). The expression of heterodimers was determined from 1×10^4 cells for each group. Transfected COS-7 cells stained with integrin β 2 antibody (IB4), (Integrin b2 (IB4): sc-65254, Santa Cruz) followed by PE-conjugated goat anti-mouse IgG: sc-3738. Isotype control was normal mouse IgG2a: sc-3878.

2.7. Statistical analysis

Every assay was performed independently with duplicate samples and was repeated three times. Statistical analysis was performed with the Mann–Whitney *U* test. The statistical significance of the difference between the control and other groups was evaluated with a one-way ANOVA. The criterion for statistical significance was taken as $P < 0.05$.

3. Results

3.1. Clinical and laboratory finding

In this study, the most common clinical presentation among the patients were recurrent bacterial infection, diarrhea and skin ulcers. The other common clinical presentation were omphalitis, delayed

umbilical cord separation (after 19 ± 1 day), periodontitis, otitis media, cellulitis, pneumonia, sepsis, ophth, cervical lymphadenopathy, osteomyelitis. In most patients, skin ulcers were first clinical presentation. The patients were presented with leukocytosis and neutrophilia.

3.2. Mutation analysis

Beside clinical features including infection, omphalitis and delayed umbilical cord separation, flow cytometry study of patients confirmed the defect in CD18 expression, indicating that mutations in the *ITGB2* gene may confer this effect on its expression.

Genomic DNA taken from each subject was successfully PCR-amplified for target exons and flanking sequences. The amplified targets were sequenced and sequence analysis was performed for identifying any possible variant in *ITGB2*, (Fig. 1). Mutations and variants found in CD18 are summarized in Table 1. In addition to previously reported mutations, we found one novel mutation NM_000211.4 (*ITGB2*): c.1685G > A, predicting p.Cys562Tyr. In silico prediction of the effect of these mutations on protein structure and stability is shown in Table 2.

3.3. Expression of heterodimer of mutant CD18/normal CD11a subunit

Flow cytometric analysis of cloned cDNA encoding normal CD18 and normal CD11a subunit indicated effective expression of CD11a/CD18 (Fig. 2-A). Our flow cytometry data disclosed that cDNA containing CD18^{Asp128Tyr} can noticeably reduce LFA-1 heterodimers on the surface of transfected COS-7 cells, (Fig. 2-B). Co-transfection of COS-7 cells with mutant CD18^{Trp252Arg} with wild-type CD11a also revealed that the substitution of tryptophan with arginine at the amino acid position 252 leads to a defect in surface expression of the CD18/CD11a heterodimer (Fig. 2-C), $P < 0.005$. Finally, expression analysis of COS-7 cells transfected with cDNA carrying CD18^{Cys562Tyr} together with wild-type CD11a disclosed that the substitution of Cysteine with Tyrosine dramatically decreases the formation of CD18/CD11a complex, $P < 0.005$. (Fig. 2-D).

4. Discussion

In the current study, we describe the clinical, laboratory and molecular characteristics of three patients with LAD-I. Genetic alterations in *ITGB2* were investigated by PCR sequencing of genomic DNA from patients. We introduced two previously reported mutations p.Asp128Tyr [20] and p.Trp252Arg [15] and the novel mutation p.Cys562Tyr into synthetic cDNA encoding CD18, transfected COS-7 cells with these constructs together with wild-type CD11a and measured by flow cytometry their ability to form heterodimers.

The $\beta 2$ subunit of integrins has several domains, which are shown in Fig. 3. Asp128 is located in the $\beta 1$ domain of the integrin beta-2 chain (Fig. 3). Aspartic acid is a negatively charged amino acid. According to Pfam database of protein families (Pfam: PF08441), aspartic acid of codon 128 is one of the most conserved positions in CD18. This region is believed to have a crucial role in the formation of integrin alpha-beta heterodimers. According to bioinformatic analysis, substitution of this conserved amino acid with tyrosine seems to be damaging and to decrease the stability of protein structure (Table 2). Therefore, the substitution of aspartic acid with the neutral amino acid tyrosine suggests a pathogenic role of this mutation in LAD-I. We analyzed the effect of p.Asp128Tyr missense mutation on membrane heterodimer formation with the CD11a subunit (Fig. 2-B). We found that this genetic alteration caused reduction of heterodimer formation of CD18 and CD11a subunit in COS-7 cells.

The second mutation studied was the substitution of tryptophan with arginine located in codon 252 (p.Trp252Arg). In silico analysis of this mutation disclosed that it can be pathogenic by decreasing the stability of CD18 protein structure (Table 2). Investigation of COS cells

transfected with vector containing mutant cDNA showed that this alteration exerts a deficiency in the complex formation of CD18 and CD11b molecules on the cell surface (Fig. 2-C). The wild-type tryptophan is located in a position that is highly conserved in all other integrin $\beta 2$ subunits and critical for the maintenance of the $\alpha\beta$ heterodimer configuration [15]. Thus, the p.Trp252Arg mutation seems to affect protein structure and consequently CD18 function.

Cysteine-562 is located in a major cysteine-rich region at the C-terminal side of the extracellular domain of the CD18 protein. This cysteine-rich region is very conserved in human β -integrin molecules. This region is believed to be crucial for the formation of the tertiary structure of the aminoterminal and extracellular region of the CD18 protein, because disulfide bonds play an important role in the folding and stability of proteins. Disulfide bonds in proteins are formed between the thiol groups of cysteine residues by the process of oxidative folding, so cysteine is an essential amino acid that participates in disulfide bonding [21]. Deletion of a cysteine residue is thought to change the normal pattern of disulfide bonding in this region. In previous reports, co-transfection of COS cells with CD11 subunits and CD18 mutant in the cysteine-rich region showed low expression of LFA-1 and Mo-1 [15]. In the current study, we found a novel mutation in codon 562, where cysteine is substituted with tyrosine. Co-transfection of COS cells with cDNA containing CD11a and CD18^{Cys562Tyr} revealed decreased expression of CD11a/CD18 complex (Fig. 2-D). According to clinical, molecular, and functional results, CD18^{Cys562Tyr} seems to have a pathogenic impact by interfering with the formation of CD11/CD18 complexes and protein structure and thereby its function. The *in silico* analysis of the novel mutation (Table 2) confirmed our experimental findings. Based on these bioinformatics tools, this substitution is damaging and possibly decreases the stability of protein structure.

We recommend that the CD18 p.Cys562Tyr mutation is included in the panel of carrier and prenatal diagnosis programs for LAD-I.

Conflict of interest

There is no conflict of interest.

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