

Original Article

Investigation of *ITGB2* Gene in 12 New Cases of Leukocyte Adhesion Deficiency-Type I Revealed Four Novel Mutations from Iran

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Abstract

Background: Leukocyte adhesion deficiency type I (LAD-I) is a rare, autosomal recessive inherited immunodeficiency disease. LAD-I is caused by mutations in the *ITGB2* gene and characterized by recurrent severe bacterial infections, as well as impaired wound healing with lack of pus formation.

Methods: In this study, we investigated *ITGB2* gene mutations in 12 patients and their parents. Genomic DNA was extracted from whole blood samples. All coding regions of the *ITGB2* gene were amplified using PCR and followed by direct sequencing.

Results: Genetic analysis revealed 12 different homozygous mutations, including six missense (c.382G>A, c.2146G>C, c.715G>A, c.691G>C, c.1777C and new c.1686C>A), two new nonsense (c.1336G>T and c.1821C>A), three-frame shift (c.1143delc, c.1907delA and new c.474dupC) and a splice site (c.1877+2T>C). Flow cytometry analysis of CD11/CD18 expression on neutrophils revealed defect in CD18 in all twelve cases (1.4% to 42%), CD11a in ten cases (0.1% to 26.7%), CD11b in nine cases (1.2% to 58.8%), and CD11c in all cases (0% to 18.1%). The patients' parents were both heterozygous carriers.

Conclusion: Our findings showed four new mutations in the *ITGB2* gene. These results can be used for decisive genetic diagnosis, genetic counseling, as well as prenatal diagnosis for all patients who are suspected to LAD I.

Keywords: Genetic analysis, immunodeficiency, leukocyte adhesion deficiency, nonsense, novel mutation

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Introduction

Leukocyte adhesion deficiency (LAD) is an autosomal recessive disease and a rare type of congenital immunodeficiency¹ that have been classified into three types: LAD I, II and III. Type I is the most common one with a prevalence of 1 in 1,000,000 live births.^{2,3} Clinically, LAD I is characterized by delayed umbilical cord separation, recurrent severe bacterial infections, absence of pus formation, periodontitis, delayed wound healing, and often persistent leukocytosis.⁴ Two clinical phenotypes of LAD I (severe and moderate) appear to be directly related

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to the level of CD18 expression on patients' leucocytes. Patients with severe form display less than 1% of CD18, and patients with moderate deficiency express 2.5% – 10% of CD18 on leucocytes.⁵

LAD I is the consequence of mutations in the *ITGB2* gene (CD18 protein) that is located on chromosome 21 (21q22.3) and encodes the $\beta 2$ subunit of the integrin molecule, CD18.^{3,6} B2 integrins family has critical roles on leukocyte adhesion, functions in immune and inflammatory reactions such as adhesion of leukocyte to endothelial cell, transendothelial migration and chemotaxis.⁷ Heterogeneity in the *ITGB2* mutations results in different clinical features of this disorder.⁸ These mutations influence on the function of neutrophils, and at lesser extent on the function of lymphocyte such as proliferation, cytotoxic T lymphocyte response, and natural killer cell (NK) activity.⁹ Different types of mutations have been reported in the CD18 gene, including deletion, truncation, substitution, frameshifts and intronic mutations.¹⁰ *ITGB2* gene mutations analyses in different ethnics help to clarify genetic basis of this disease. In this study, we performed DNA analysis of *ITGB2* genes on 12 unrelated Iranian patients with LAD I to clarify the genetic and clinical characteristics of Iranian patients with LAD I.

Materials and Methods

Patients

In this study, we investigated 12 patients (7 boys and 5 girls) with a clinical phenotype suggestive of LAD I at the Immunol-

Table 1. Clinical and laboratory findings in patients with LAD I

Patient No.	Sex	Age at onset of disease (month)	Clinical findings	Cord separation (day)	CD18* (% of normal range)	CD11a* (% of normal range)	CD11b* (% of normal range)	CD11c* (% of normal range)	Leukocyte* no/mm ³
P1	F	1	Omphalitis	15	2.9	26.7	1.2	4.9	55000
P2	F	1	Omphalitis	26	8.5	87	91.67	1.03	10000
P3	M	1	Otitis media	12	10	3.5	3	0	38800
P4	M	1	Osteomyelitis	25	8.3	11.2	58.8	18.1	21800
P5	F	84	Skin ulcers	10	17.5	99	33.7	10.77	12000
P6	F	1	Omphalitis	15	8.4	14.6	4.7	9.8	37000
P7	F	1	Fever, Infection	15	1.8	0.1	1.4	2	60000
P8	F	1	Omphalitis	16	42	2.4	15	1.9	21000
P9	F	1	Omphalitis	25	1.4	1	98	10	55000
P10	M	1	Omphalitis	15	24.10	3.76	28.86	5.38	10350
P11	M	1	Omphalitis	20	6	10	48	13.4	39000
P12	M	48	Skin ulcers	18	16.80	3.16	75.89	34.20	30000

*Normal range: WBC; 4000–19000 for 1 m–5 m, 6000–17000 for < 6 m 2 years, 5000–15.500 for 2–6 years and 4500–13500 for 6–12 years, CD18; > 90%, CD11a; > 90%, CD11b; > 90%, CD11c; > 90%. Normal Range from Chapter 14, Ahsan S, Noether J. Hematology. In: Tschudy MM, Arcara KM, editors. The Harriet Lane Handbook. USA: Elsevier Mosby, 2010: 322 – 353.

ogy, Asthma and Allergy Research Institute (IAARI) from Jun 2010 to Jun 2013. The diagnosis of LAD I was confirmed based on clinical and laboratory findings. Clinical findings consisted of recurrent severe infections, impaired pus formation, impaired wound healing, delay in umbilical cord separation, and omphalitis. Laboratory findings confirming LAD I included leukocytosis and defect in expression of CD18 and/or CD11 (CD11a, CD11b and CD11c) on leukocytes of patients in flow cytometry (Table 1). CD11/CD18 expression in the peripheral blood neutrophils was measured by flow cytometry and analysed by FACStar plus flow cytometer (Becton Dickinson, USA).

Ethics

This study was approved by the Research Committee and Ethics Committee of IAARI. Informed consent was taken from all participants (the patients, their parents).

DNA analysis of the ITGB2 gene

After obtaining 2 mL whole blood in sodium-EDTA from all the participants, DNA was extracted, using high pure extraction kit (Roche, Germany). Polymerase chain reaction (PCR) amplification of the 16 exons of the *ITGB2* (CD18) gene was performed using specific primers covering 16 coding regions and exon–intron boundaries of an *ITGB2* gene (Table 2). The PCR reaction contained 0.5 µL of both forward and reverse primers (10PM), 12.5 red master mix (pishgam), 1 µL of DNA (about 100ng) and 10 µL ddH₂O in a total volume of 25 µL. The PCR condition consisted of an initial denaturation at 95°C for 5 min, 30 cycles including 94°C for 30 second, 60°C for 40 second, and 72°C for 30 second, as well as a final extension at 72°C for 4 min.

PCR results of all exons and intron-exon boundaries of the *ITGB2* gene was analyzed by direct DNA sequencing in both orientations. DNA sequencing was done by ABI 3730XL genetic analyzer (Applied Biosystem, ABI, USA).

Results

Clinical and laboratory findings

Twelve patients with LAD I entered to this study (92 % of their parents had consanguineous marriages). The first and most common manifestation of our cases was omphalitis (~59%). Chronic skin ulcers, osteomyelitis, otitis media, and recurrent infection were other manifestations. Flow cytometric analysis of CD11/CD18 expression on neutrophils revealed defect in CD18 in all cases (1.4% to 42%), CD11a in ten cases (0.1% to 26.7%), CD11b in nine cases (1.2% to 58.8%), and CD11c in all cases (0% to 18.1%). All of the patients exhibited persistent leukocytosis between 10000 and 60000 n/mm³ (median 36085). Clinical and laboratory findings of the patients are summarized in Table 1.

Mutation analysis

We amplified all exons and intron-exon boundaries of the *ITGB2* gene in patients with LAD I, and their parents who were referred to our center during the 3 years. Direct sequencing of the PCR products revealed twelve different mutations in these patients (Table 3). Six patients (P1, P2, P3, P4, P5 and P8) showed missense mutations. Five of them c.382G>A (Asp128Asn), c.2146G>C (Gly716Arg), c.715G>A (Ala239Thr), c.1777C>T (Arg593Cys) and c.691G>C (Asp231His) have previously been reported.^{11–13} P2 showed new missense mutation c.1686C>A that create an amino acid change (Cys562Tyr) in the cystein domain 3 (CYS3). P10 and P11 showed a known c.1907delA (Lys636fsX22) in exon 14 and a known c.1143delC (Tyr382fsX9) in exon 10 in the extracellular domain (EC), respectively. P7 showed a new c.474dupC mutation in exon 6 leading to p.E159fsx27 in the von Willebrand factor type A (VWFA) domain. In the studied patients, P12 showed a known c.1877+2T>C splice donor defect in intron 13. In this survey, we also found two new nonsense mutations; c.1336G>T (Glu446stop) in exon 11 and c.1821C>A (Tyr607stop) in exon 13 leading to a premature stop codon in the EC and cystein domain 4 (CYS4), respectively. All parents were heterozygous for these mutations.

Table 2. PCR primer sequence and PCR product size

Exon	Primer sequence	PCR product (bp)
Exon 2	F2: CCTCTCCAAGTGTAACTCG R2: ATGTGGCTCTGCTCTTGGT	518
Exon 3/4	F3/4: CTCCTCCCTGGCTGTTG R3/4: AGTTGTGGTCCCTGGGGTGC	779
Exon 5	F5: GTCCTGGGAGGCTGAAGGAG R5: CCAGAGCCAATAACCAGCACAG	407
Exon 6	F6: TGTGAGATGTGAGGTGTGGCT R6: CTGAGTGCAGGAGTTGTGTG	306
Exon 7	F7: TAGGAGAGAGGAAACAGGCTTG R7: TTCCTCCTGCCCTTTCCTG	520
Exon 8	F8: AGCTCTGCATCGTCTCCTCT R8: GGGGTGGGGACTTACGAAT	255
Exon 9	F9: CTCTCCAGCCTTCCCCAG R9: CCAACAGCACTCACCTCGTAG	240
Exon 10	F10: CGTCCCAGCCATTTGACAG R10: TGCGGGAGACTCACATTGTA	211
Exon 11	F11: GCCAGGACAACAGAAACACTC R11: CTCAGTGTGCTGGGATGG	285
Exon 12	F12: CTCTTCTGCTCCTGGTAC R12: CTCGGGGATGGTTCAACAGG	366
Exon 13	F13: ATGTGCCATAGGGGTGTTCTC R13: CCCCTTCTGTTCCACTCGTTG	458
Exon 14	F14: GTGCTGCCCGTCTTCCA R14: CAGACGCACCCGCAGAGAAC	367
Exon 15	F15: ATCAGGCTTCCAGTTCACCCG R15: ATCCTCCTCCGCACCACC	466
Exon 16	F16: ATGGTGATGGATGGTGAAGC R16: GACGCCAGAGGACAAGC	444
Exon 17	F17: GCTCTGAAAACCTCCCACACT R17: GGGAATACAGCGGACACAGT	554

Table 3. Genetic mutations of the *ITGB2* gene in patients with LAD I

NO	Location	cDNA numbering	Mutation type	Amino acid change	Affected domain	References
P1	E6	c.382G>A	Missense	p.Asp128Asn	VWFA	(11)
P2	E13	c.1686G>A	Missense	p.Cys562Tyr	CYS3	this study
P3	E15	c.2146G>C	Missense	p.Gly716Arg	- - -	(11)
P4	E6	c.715G>A	Missense	p.Ala239Thr	VWFA	(12)
P5	E13	c.1777C>T	Missense	p.Arg593Cys	CYS4	(13)
P6	E11	c.1336G>T	Nonsense	p.Glu446stop	EC	this study
P7	E6	c.474dupC	Frameshift	p.E159fsx27	VWFA	this study
P8	E6	c.691G>C	Missense	p.Asp231His	VWFA	(13)
P9	E13	c.1821C>A	Nonsense	p.Tyr607stop	CYS4	this study
P10	E14	c.1907delA	Frameshift	p.Lys636fsx22	EC	(12)
P11	E10	c.1143delC	Frameshift	p.Tyr382fsx9	EC	(12)
P12	In13	c.1877+2T>C	splice donor site	- - -	EC	(11)

Discussion

LAD I is caused by mutation in the $\beta 2$ integrin gene (*ITGB2*) at chromosome 21q22.3. Up to now, over 60 different mutations in the *ITGB2* gene have been identified (bioinf.uta.fi/*ITGB2*base). Regarding the high rate of consanguineous marriages in Iran,¹⁴ there is an increasing risk of autosomal recessive disorders such as LADs.^{15,16} In Iran, several reports have been published regarding

clinical features^{5,17-20} and *ITGB2* mutations analyses^{11,12} of LAD I and also about Hematopoietic stem cell transplantation (HSCT) in these patients.^{21,22} In this study, twelve different mutations were detected; in which four were new (Table 2). From 8 previously reported mutations, 6 were first reported from Iran^{11,12} and 2 from United States of America.¹³ Mutation analysis in P2 showed a missense mutation, c.1686G>A, in exon 13 in the CYS3 domain leading to substitution of cysteine for a tyrosine at position 562.

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..PQCECQCRDASRDGSIICGGGRSMECGVCRCDAGYIGKNCECQTQGRSSQLELGSCRKDNS 502 Cow
..PQCECQCRDQSRQSLCGGKGVMECGICRCESGYIGKNCECQTQGRSSQELERNCRKDNS 502 Mouse
..PQCECQCRDTSFGRSLCRDKGFMECGICRCDTGYIGKNCECQTQGRSSQLELGSCRKDN 502 Horse
..PLCEECRCRDQQRERGLCGGKGSMECGVCRCDAGYTGNKCECQTQGRSSQLELGSCRKDN 502 Rabbit
..PQCECQCRDQSRDRSLCHGKGFLECGICRCDTGYIGKNCECQTQGRSSQLELGSCRKDN 502 Gorilla
..PQCECQCRDVGQDHGLCSGKGSLECGICRCEAGYIGKNCECLTHGRSSQLELGSCRKDNS 502 Dog

..RCHPGFEGSACQCERTTEGCLNPRRVECSGRGRRCRCNVCECHSGYQLPLCQCEPCGCPSPC 622 Human
..RCDEQYEGSACQCLKSTQGCLNLDGVECSGRGRRCRCNVQCDFGYQPFLCSEPCGCPVPC 622 Cow
..SCKPGYEGSACQCQRSTTGCLNARLVECSGRGRHCQCNCRCICDEGYQPPMCEDCPSCGSHC 622 Mouse
..QCCEGFEFSACQCPRSTDGCLNQRGTECSGRGRRCNVCECDDGYQPFLCQDCPCGCPSPC 622 Hours
..HCNDGFEFSACQCCKKSTDGCLDAQGVECSGRGRRCRCNVCVCKKYQPPMCQECLGCTSPC 622 Rabbit
..HCRPGFDGSACQCERTTEGCLNPRRVECSGRGRRCRCNVCECHSGYQLPLCQCEPCGCPSPC 622 Gorilla
..QCEQNYEGSACQCCKSTQGCLSTEGIECNGRGRRCRCNVCECDGGYQPFLCGDCLGCPSPC 622 Dog

SIICSGLDGDCVCGQCLCHTSDVPGKLIYGQYCECDTINCERYNGQVCGGPGRGLCFGCKRC 562 Human
SIICSGLDGDCICGQCVCHTSDVFNKIIYGQFCECDNVNCERYDGVCGGKRGKLCFCGTGRC 562 Cow
SIVCSGLGDCICGQCVCHTSDVFNKEIFGQYCECDNVNCERYNSQVCGGSDRGSCNCGKCS 562 Mouse
SLVCSGLGDCVCGQCICHKSDVFNKEIFGQFCECDNVNCERYDGVCGGKRGKTCNCGKQC 562 Horse
SIVCSGLGDCICGQCVCHTSDVFNKQIFGRYCECDNMNCERYDGVCGGKRGKSCSCGQCH 562 Rabbit
SIICSGLDGDCVCGQCLCHTSDVPGKEIYGQYCECDTINCERYNGQVCGGPGRGLCFGCKHC 562 Gorilla
SLICSGLDGDCICGQCVCHRSDVFNKNIIFGRYCECDNVNCERYDGVCGGKVRGSCNCGKQC 562 Dog

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Figure 1. *ITGB2* gene regions in human and six other species that conserved. The amino acids Glu446 (red), Tyr607 (blue) and Cys562 (green)

The effect of this missense mutation (Cys562Tyr) on $\beta 2$ integrin function was evaluated using online prediction tools including PolyPhen-2,²³ Uniprot and HOPE. PolyPhen-2 predicted that this missense mutation affects $\beta 2$ integrin function. This mutation is located within cysteine-rich tandem repeats. Cysteine is involved in a cysteine bridge that is important for stability of the protein. Cysteine and tyrosine differ in size and hydrophobicity. The mutant amino acid (tyrosine) is bigger than cysteine, which might cause bump and incorrect conformation. The differences in amino acid properties can disturb this repeat and its function. In p6 and p9, we also found two new nonsense mutations; c.1336G>T (Glu446stop) in exon 11 and c.1821C>A (Tyr607stop) in exon 13 leading to a premature stop codon in the EC and CYS4 domain, respectively. CD18 residues and regions among other $\beta 2$ integrins have a high degree of conservation and these regions are critical for heterodimer formation. Mutations within the rigid cysteine-rich region may impair heterodimer formation.²⁴ Also, many studies have shown that this region was important for gene expression.²⁵ Sequence alignment of *ITGB2* gene (Ensemble, Blast/Blat) revealed that amino acids Glu446, Tyr607 and Cys562 are highly conserved (Figure1). Also, P7 had a new c.474dupC mutation in exon 6 leading to p.E159fsx27 in the VWFA domain. This domain is highly conserved and coded by exons 5 – 9 of the gene.^{13,26} Our results showed, 4 out of 12 (33.3%) mutations being occurred in the VWFA region of *ITGB2* gene. Most of the mutations in previous studies were found in exons 5 – 9 of *ITGB2* gene which is highly conserved.¹³ Shaw, et al. found that those mutations in this region impact on CD11/CD18 expression and function.⁸ Also, frameshift mutations are anticipated to change the reading frame or lead to a premature termination of the protein.

In conclusion, our findings showed four new mutations in the *ITGB2* gene and confirmed the allelic heterogeneity of the mutations in this gene. According to the results of this study, exons 6 and 13 appeared to be the most common regions for *ITGB2* gene mutation. Genetic analysis can be useful for exact diagnosis

and early effective treatment of LAD I. Also results of this study can be used for decisive genetic diagnosis, genetic counseling and prenatal diagnosis.

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Conflict of Interest: The authors declare no conflict of interest.

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