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Original Article

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Molecular dissection of two Pakistani families segregating Leukocyte Adhesion Deficiency Type-I

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ABSTRACT

BACKGROUND & OBJECTIVE: Leukocyte adhesion deficiency (LAD1) is an autosomal recessive type of inherited disorder caused by total or partial deficiency of CD18 expression. LAD1 is characterized by recurrent bacterial and fungal infections, in some cases delayed umbilical cord separation, delayed wound healing due to blockade in leukocyte migration to site of inflammation and infection. The present study involves genetic analysis of two unrelated families suffering from LAD1.

METHODOLOGY: In the present study, two separated and unrelated Pakistani families are included suffering from Leukocyte Adhesion Deficiency type -1 (PAD1). After detailed clinical evaluation, whole blood samples were collected from patients, parents and available healthy siblings. Genomic DNA was extracted from all the blood samples, and using a specific primer all the coding ITGB2 gene exons were PCR amplified.

RESULTS: The amplified products were sanger sequenced. DNA sequencing analysis revealed a nonsense mutation c.186C > A, p.(Cys62*) in exon four and a missense mutation c.382G>T, p.(Asp128Tyr) in exon five of the gene. The mutation is segregating in autosomal recessive pattern in the family.

CONCLUSION: Recurrent mutations on a specific locus (gene) changes the allele frequency from a healthy allele to a disease allele and hence play role in new genotype. This research study demonstrates the allelic heterogeneity of the ITGB2 gene in Pakistani patients diagnosed with LAD1. The research findings in the present study suggest that every population should develop national registry of patients suffering from primary immune deficiencies and a mutation database for rare genetic disorders. This will facilitate early diagnosis and genetic counseling to the patient family.

KEYWORDS: Leukocyte adhesion deficiency, Flowcytometry, DNA sequencing.

INTRODUCTION

Autosomal recessive type of leukocyte adhesion deficiency type I (LAD1) (OMIM #116920) is a rare autosomal recessive type of inherited disorder with an estimated prevalence of one out of 1 million births worldwide, without the involvement of ethnicity. The leukocytes (Neutrophils) roll in the along the walls of vessel with blood, adhere firmly to the endothelium. These leukocytes, one of the first immune cells, extravasate from blood vessels and travel to the damaged or infected tissue. Leukocyte adhesion with the endothelial cells is a multi-step process [1]. During an active inflammation process, endothelium gets activated, which results in a cascade of multi-step processes of capturing, rolling, chemokine signaling, slow rolling, arrested, and transmigration of neutrophils causing their accumulation to the site of injury [2].

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Three subtypes of LAD1 syndromes (LAD -I, LAD-II and LAD–III) have been documented in literature. Each subtype exhibits specific clinical, biochemical and genetic expressions [3]. LAD1 is characterized by a variety of recurrent infections (sepsis, periodontitis & otitis media), poor wound healing and skin ulcers owing to obstruction in leukocyte trafficking, migration to and accumulation at the site of inflammation. The poor immunological response against invading pathogens leads to infections starting from birth involving skin, bowel, respiratory tract & perirectal areas [3, 4]. These LAD1 related clinical manifestations are attributed to mutations in gene ITGB2 which is localized on chromosome 21 (21q22.3) which encodes β2 subunit of protein integrin (CD18) [5].

The ITGB2 gene mutations greatly affect the function of neutrophils and has less or no effect on immune cells such as lymphocyte, natural killer (NK) or T-cells [6]. Besides early-stage blood screening, DNA Sanger sequencing of ITGB2 genes is highly recommended to have a LAD1 diagnosis at a molecular level.

Till date different types of pathogenic variants including deletion, substitution, frameshift and few intronic mutation have been reported in CD18 gene. The present study aimed to investigate genetic etiology of two unrelated Pakistani families suffering from leukocyte adhesion deficiencies.

METHODOLOGY

After getting approval from institute review board (BCH/QAU-IRB. 2021) and detailed discussions with guardians of each case research written consents were collected from the parents/guardian of the patients to conduct the genetic analysis and to publish the findings. In the present study two male patients with age of under 6 months belonging from two unrelated families were examined by Child Specialist at Neonatal Department of Combined Military Hospital Rawalpindi (CMH).

The patients with multiple episodes of pneumonia and other respiratory tract infections, GIT infections with multiple stools, bronchitis, laryngitis & omphalitis were admitted. These infections specially diarrhea and pneumonia were developed at the age of just 15 days after birth. Both the patients were thoroughly examined by a consultant immunologist at Department of Immunology CMH – Rawalpindi and flow-cytometry was performed to check status of the immune system.

Patient complete blood picture (CP) test were performed in KX21 hematology analyzer and flow-cytometry on automated machine Becton Dickinson (USA). At first the peripheral blood samples were incubated with fluorochromes labelled with [Phycoerythrin (PE)] antibodies, including a variety of cluster of differentiations like (Integrin beta chain-2 (CD18), Complement protein 11b (CD11b), and Complement protein 11 c (CD11C) (BD Biosciences, USA). According to International Society of Immunodeficiencies, patients with CD levels less than 2% are grouped into most severe LAD1, while patients with CD18% levels between (2-30) are grouped as moderate level with moderate level of skin and mucosal infections [7, 8].

In order to perform genetic analyses, whole blood samples were acquired (3–5CC) in K-EDTA tubes from all participants including patient, parents and available healthy siblings. DNA was extracted from the blood using the GenEluteTM blood genomic DNA kit (Sigma-Aldrich, St. Louis, MO, USA). The extracted DNA samples were quantified on Nanodrop1000 spectrophotometer (Thermal Scientific, Wilmington, MA, USA). PCR was performed to amplify ITGB2 exons using exon specific primers and PCR conditions according to our previous report [9]. The amplified PCR products of each exon was electrophoresed on 2% agarose gel and checked by using illumination. Prior to DNA sequencing the amplified PCR products were subjected to purification with commercially available kit (Axygen® AxyPrep PCR clean-up Kit). The amplified and purified samples were Sanger Sequenced using Beckman Coulter CEQ-8800 Analyzer (USA). DNA sequencing facility is available at the Department of Biochemistry Quaid-i-Azam University Islamabad. The data obtained through Sanger sequencing was matched with genomic sequences of corresponding exons which are available online (http://ensembl.org/index.html). In order to identify mutations in ITGB2 gene exons Bio Edit Sequence editors (6.0.7) was used. The pathogenicity of the identified variant were measured using Mutation Taster or Polyphene-2 software's.

RESULTS

Clinical findings: The first patient in the present study was a 3 months old male child belonging to a remote village of Punjab, Pakistan. The patient was a case of term pregnancy with Apgar score of 10 points. The second male child, age 4 months was presented in the same department of CMH Rawalpindi. Both the patients (P-V of family A & P-III of family B) after two weeks of their birth suffered from different infections including omphalitis, skin ulcers, poor wound healing and delayed umbilical cord separation time (20 and 25 days, respectively). Based on clinical presentations and laboratory findings the patients were diagnosed with LAD1 (see table-I).

Lab Findings: Inpatient one (P-V) of family A, hematological findings revealed elevated white blood cell (22.06 × 109 /L), platelets count (338 × 109 /L), lymphocytes at 4.23 × 109 /L, neutrophils (20.49× 109 /L), blood hemoglobin at 14.3 g/L, and CRP at 58.60 mg/L. Etiological tests including HIV, Mycoplasma and EB virus were found negative. The patient immunoglobulins levels revealed IgG at 800.95 mg/dL, IgM at 285.75 mg/dL, IgA at 440.50 mg/dL, and IgE at 67.48 mg/dL. Flow-cytometry was performed which clearly showed reduced level of CD18 expression.

The second patient (P-III) of family B in the present study revealed elevated WBC count of 30.23 × 109 /L, platelets 246 × 109 /L, lymphocytes at 6.72 × 109 /L, neutrophils at 22.68 × 109 /L, hemoglobin at 15.11 g/L, and CRP at 77 mg/L. EB virus, HIV and mycoplasma testing were negative. The immunoglobulins levels in the patient were; IgG at 1144 mg/dL, IgM at 206.34 mg/dL, IgA at 504.78 mg/dL, and IgE at 45.67 mg/dL. CD18 expression on leukocyte is the measure of disease severity[7]. Both the patients presented in the current study revealed < 2% CD18 level (Table-I).

Table-I: Patients CBC & Immunoglobulin Levels.

CBC Profile	Results	Immunoglobulin Levels	Results
		Patient – I (P- V)	
WBC Total	22.06 × 109 /L	IgG	800.95 mg/dL
Platelet Count	338 × 109 /L	IgM	285.75 mg/dL
Hemoglobin	14.3 g/L	IgA	440.50 mg/dL
RBC Total	3.79m/uL	IgE	67. 48 mg/dL
CRP	58.60 mg/L	-	-
CD18	0.5%	-	-
		Patient – II (P- III)	
WBC Total	30.23 × 109 /L	IgG	1144 mg/dL
Platelet Count	246 × 109 /L	IgM	206.34 mg/dL
Hemoglobin	15.11 g/L	IgA	504.78 mg/dL
RBC Total	4.23m/uL	IgE	45.67 mg/dL
CRP	77 mg/L	-	-
CD18	0.9%	-	-

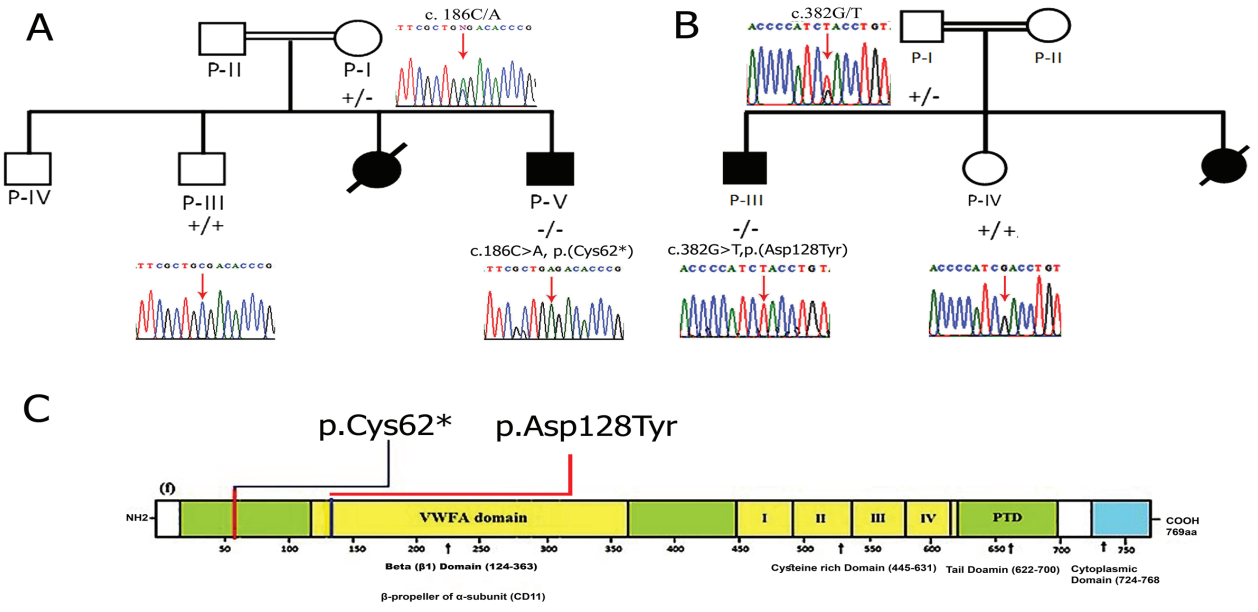

Figure-I: Pedigree & Mutation analysis of ITGB2.

A. Family pedigree showing autosomal recessive mode of inheritance of LAD1. Arrowhead in patient (P-V) showing the point of variant c. 186C>A, p.(Cys62*), arrowhead in mother (P-I) showing carrier peaks while arrowhead in healthy sibling (P-III) showing normal sequencing results B. Pedigree of family B showing autosomal recessive mode of inheritance of LAD1. Arrowhead in patient (P-III) indicating a missense variant c.382G>T, p.(Asp128Tyr) while arrow head in father (P-I) showing carrier peaks whereas arrow in healthy sibling (P-IV) showing normal peaks C. Structure of the β2 integrin protein showing the location of the variants.

DISCUSSION

Primary immunodeficiency disorders, also termed as primary immunodeficiency (PIDs) are variety of heterogeneous rare genetic disorders which badly deteriorate the immune system which lead to variety of different infections and related health anomalies to occur more easily. In the subcontinent, LAD1 is relatively common type of primary immunodeficiency.

Pakistan with common cousin marriages (above 71%), LAD1 is the leading type of PID [10]. Till date more than 25 different cases have been reported [9-12].

Leukocyte adhesion and migration is a sequence of a highly dynamic processes which is orchestrated by detailed adhesion molecules such as selectins and integrin's and specific receptors and counter receptors, which are localized on leukocyte and endothelial cells surfaces. At least three different classes of adhesion molecules contribute in leukocyte margination, adhesion and transmigration to the site of inflammation [10,12, 13,14,15].

Leukocyte adhesion deficiency is diagnosed with recurrent skin infections, Omphalitis, poor wound healing and neutrophilia. In the present study conducted in the year 2020, we registered two unrelated highly consanguine Pakistani families with LAD1 patients, segregating in an autosomal recessive pattern. Patients' clinical history revealed skin and respiratory tract infections raised levels of neutrophils and leukocytes, and a delay of umbilical cord separation. Pus formation was not observed at the site of infections or umbilical cord separation which is considered as hall mark of leukocyte adhesion deficiency [3, 16].

It was suspected a case of LAD1 due to leukocyte adhesion deficiency. All these clinical manifestations observed in patients (P-V of family A & P-III of family B) in the present study were coherent to LAD1 cases reported earlier [9-12]. Different clinical and immunological laboratory tests such as blood complete picture (CP), and serum immunoglobulin levels were measure but LAD1 specific diagnosis was made through flow-cytometry, which revealed low level expressions of CD18 on the leukocyte surface. Severity of the LAD1 depends upon the level of CD18 expression [3]. The key function of CD18 is to guide neutrophils to the site of infection by adhering to different ligands. The LAD1 cases with mild symptoms and better survival rate exhibit mild reduction of CD18 levels [9].

Previous study reported that 12 unrelated Pakistani cases causing LAD-1 with novel and recurrent mutations in the gene ITGB2. DNA sequencing analysis in the present study revealed an already reported a non-sense mutation [c.186C > A, p.(Cys62*)] in Patient (P-V) of family A and a nucleotide transition is observed [c.382G>T, p.(Asp128Tyr)] in patient (P-III) of family B. The nonsense mutation resulted appearance of premature stop codon at position 62 of the protein will probably result into nonfunctional truncated protein. The nonsense mutation is located in N-terminal extracellular domain of the integrin β-2 subunit which is folded and is involved in mediating the interaction between the two integrin subunits α & β [17, 18].

ITGB2 sequencing in patient (P-III) of family B revealed ITGB2 sequencing in patient (P-III) of family B revealed a transition of G to T at position 382 which results into change of negatively charged polar amino acid aspartic acid to an uncharged tyrosine at position 128. The heterogeneous missense mutation identified in family B lies in a highly conserved (AA acid) domain called Von Willebrand Factor type A (VWFA) of CD18 [19-20]. Missense 3D model predicted that the p. (Asp128Tyr) is disrupting the salt bridge between Asp128 and Arg257. This change could be responsible for the protein (CD18) dysfunction and hence causing LAD1.

CONCLUSION

The present study describes clinical and molecular findings in two unrelated Pakistani family suffering from LAD1. In the last one year (2021) we could enroll two more separate Pakistani families suffering from LAD-1. The clinical findings are coherent to those presented earlier in different studies. The nonsense mutation [c.186C > A, p.(Cys62*)] found in exon 4 in N-terminal extracellular domain while the missense mutation [c.382G>T, p.(Asp128Tyr)] is found in exon 5 of the ITGB2 gene lies in the hotspot region. The recurrent mutations are highly significant in population genetics which cause disease or reduce the fitness of an individual in a given population. The clinical and lab findings presented in the current study will support in screening of LAD1 patients. The present research findings will facilitate clinicians for the better management of the LAD1 patients and also will help in rapid neonatal genetic testing and genetic counselling.

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Saadia Moazzam: Design of work and reviewing.
Muhammad Zeeshan Anwar: Drafting and reviewing.
Muhammad Akram Shahzad: Interpretation of data and reviewing.
Momin Iqbal: Final approval of the version for submission, revising critically for important intellectual content.
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