Cohort of Iranian Patients with Congenital Agammaglobulinemia: Mutation Analysis and Novel Gene Defects


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Cohort of Iranian Patients with Congenital Agammaglobulinemia: Mutation Analysis and Novel Gene Defects

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ABSTRACT

Objectives: Impairment in early B-cell development can cause a predominantly antibody deficiency with severe depletion of peripheral B-cells. Mutations in the gene encoding for Bruton’s-tyrosine-kinase (BTK) and the components of the pre-B-cell receptor complex or downstream signaling molecules have been related to this defect in patients with agammaglobulinemia.

Methods: Iranian patients with congenital agammaglobulinemia were included and the correlation between disease-causing mutations and parameters such as clinical and immunologic phenotypes were evaluated in available patients.

Results: Out of 87 patients, a molecular investigation was performed on 51 patients leading to the identification of 39 cases with BTK (1 novel mutation), 5 cases of μ-heavy chain (3 novel mutations) and 1 case of Igα-deficiencies.

Conclusion: Although there is no comprehensive correlation between type of responsible BTK mutation and severity of clinical phenotype, our data suggest that BTK-deficient and autosomal recessive agammaglobulinemia patients differ significantly regarding clinical/immunologic characteristics.

Introduction

Predominantly antibody deficiencies (PADs) are the most frequent primary immunodeficiency diseases. The first described PAD was X-linked agammaglobulinemia (XLA) in which affected patients have an early block in B-cell development resulting in a severe reduction in the numbers of peripheral B and very low serum levels of all immunoglobulin classes [1,2]. Patients affected with XLA typically present an increased susceptibility to infections especially of the respiratory and gastrointestinal tracts and the central nervous system (CNS). The introduction of immunoglobulin replacement treatment has significantly reduced the infectious complications and improved the survival rates of affected patients [3–5]. Mutations in the gene encoding for Bruton’s tyrosine kinase (BTK), a cytoplasmatic kinase essential for B-cell receptor (BCR) downstream signaling, have been established as the causative element of XLA. More than 1100 mutations in BTK gene have to date been reported in affected males [5–8].

Besides the X-recessive form of agammaglobulinemia caused by BTK deficiency, autosomal recessive forms (ARA) have also been reported in both males and females. The genes so far linked to the autosomal recessive forms of agammaglobulinemia include the μ-heavy chain (IGHM) [9,10], B-cell linker adaptor protein (BLNK) [11], the immunoglobulin λ-like polypeptide1 (IGHL1) [12], leucine-rich repeat-containing 8 (LRRC8A) [13], as well as Igα(CD79A)-Igβ(CD79B) signaling molecules [14–19]. Recently, variations in the E47 protein (dominant negative mutations in TCF3) and PI3 kinase p85α subunit (autosomal recessive mutations in PIK3R1) have also in a few cases been reported to be causative of agammaglobulinemia [20,21].

The presented study aims to characterize the genetic causes of agammaglobulinemia in affected Iranian patients and correlate the nature of the genetic defects with the severity of clinical and immunological symptoms.

Materials and methods

Patients

All Iranian patients for whom a definitive diagnosis of primary agammaglobulinemia according to the criteria of the Expert Committee of International Union of Immunological Societies...
on Primary Immunodeficiency [22] was made between March 1998 and December 2012 including previously reported cases [15,23–30] were enrolled in this study.

Informed consent for the performed studies was obtained from the patients or their parents, in accordance with the principles of the ethics committee of the Tehran University of Medical Sciences, Iran. An evaluation sheet was used to compile demographic information and clinical records as well as laboratory and molecular data [31].

Complete blood count was evaluated by automated cell counting and the Westergren method, using anticoagulated whole blood. At the time of the diagnosis serum levels of IgG, IgA, and IgM were measured by nephelometry (Behring Nephelometer, Behringwerke, Marburg, Germany) and lymphocyte subpopulations (CD3, CD4, CD8, and CD19) were quantified by flow cytometry (Partec PAS, Münster, Germany). Likewise, immunoglobulin E levels were measured, using an enzyme-linked immunosorbent assay (ELISA, Neuss, Germany).

Mutation analysis

For all available patients, genomic DNA was extracted from peripheral blood mononuclear cells by the following method. Peripheral blood mononuclear cells from 5 ml of blood were isolated and were diluted in 1 ml of lysis buffer (100 mm NaCl, 10 mm Tris–HCl pH 7.4, 25 mm ethylene diamine tetraacetic acid pH 8.0 and 0.5% sodium dodecyl sulfate). Thereafter, 20 µg/ml proteinase K was added and the samples were incubated for 15 h at 50°C. The samples were extracted once with phenol–chloroform followed by two times extraction with chloroform. The DNA was precipitated by addition of (5 ml volume) ethanol and the pellet washed twice in 75% ethanol and finally dissolved in 80–150 µl Tris-EDTA (TE) buffer. Mutational analysis was performed in two steps. First, DNA samples from male patients were screened for mutations in BTK gene. In a subsequent step, DNA samples from female patients and males (negative for mutations in BTK) were analyzed for mutations in genes causing autosomal recessive agammaglobulinemia (CD79A, CD79B, IGHM, and IGLL1).

DNA fragments were amplified by the polymerase chain reaction (PCR) using the GoTaq Hot Start Polymerase (Promega Corporation, Madison, WI, USA) under the following conditions: a denaturation step at 95°C for 2 min, followed by 38 cycles (95°C/30 s, Tm°C/30 s, 72°C/45 s), and a final elongation step of 72°C for 7 min. The PCR primers were placed on the intronic sequences flanking exons, with amplicons including the splice donor and acceptor recognition sites. Each amplicon contains at-most two exons. The reference sequences used for the CD79A, CD79B, IGHM, IGLL1, and BTK genes were obtained from the Ensembl Genome Browser (www.ensembl.org) by the following accession numbers ENSG00000105369, ENSG0000007312, ENSG00000211899, ENSG00000128322, and ENST00000308731, respectively. The complete primer sequences, relative melting temperatures, and the amplified fragments sizes for the CD79A, CD79B, IGHM, and IGLL1 genes are available in Table S1. Molecular mutation analysis of the BTK gene was performed by Denaturing High Pressure Liquid Chromatography followed by sequencing (manuscript in preparation, conditions available upon request).

The amplified PCR products were purified with ExoSAP-IT kit (GE HealthCare Life Science, Buckinghamshire, UK) and the DNA fragments sequenced using the BigDye Terminator v1.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) with primers identical to those used for the amplification. The DNA sequences were determined with the ABI Prism™ 310 genetic analyzer using the SeqScape v2.5 software (Applied Biosystems).

Genotype–phenotype correlation (BTK)

The potential consequences of mutations in the BTK gene in XLA patients were analyzed based on predicted alteration of protein structure and the genotypes/variants classified into severe or mild as described previously [1,2]. Briefly, the following mutations were considered as severe/damaging: out-of-frame variation, termination codon change, variation attributed to invariant regions within 1–2 base pair at the beginning and end of an intron, and missense variation located in conserved subdomains as well as those at structurally or functionally important sites.

Moreover, domain analysis was used for comparison of defects in the different BTK protein domains. The severe medical phenotype was defined having two of three following criteria: early age at onset of symptoms (less or equal 6 months), frequent infectious symptoms (according to the 10 warning signs of primary immunodeficiency), and development of severe infectious complications during the course of the disease (bloodstream, CNS, and deep-seated infections like osteomyelitis and arthritis). Finally, the predicted severity of mutations was correlated with the medical severity, immunologic phenotype, expression of BTK [23,28], and survival rate of the patients.

Comparison of XLA and ARA

Patients with autosomal recessive agammaglobulinemia and XLA were categorized as two separate groups for further analysis. Clinical records and survival analysis were used as basis for comparison between the patients classified in either the ARA or XLA group.

Statistical analysis

Data analysis was conducted using the SPSS software package version 20.0 (IBM, New York, NY, USA). The age at which the patient was identified as being immunodeficient and started receiving treatment was considered the age at diagnosis. The time between onset of symptoms and the diagnosis was considered as diagnostic delay. The follow-up period was defined as the time between the diagnosis of disease and either the time of death or last recorded visit of patients. The Kolmogorov–Smirnov test was performed to evaluate the normality of data distribution. Data are presented as mean ± SD if normally distributed and otherwise as median (range). Correlation between variations assigned to the five different domains of the BTK protein and clinical/immunologic parameters were tested by the one-way ANOVA test and post-
Results

In total, 87 agammaglobulinemic patients including 81 males (93%) and 6 females (6.9%) were enrolled in this study (Table 1). The mean ± SD patient’s age at the time of study was 16.5 ± 10.3 years with a range from 1 to 43 years. The mean ± SD age of the patients at the onset of disease was 2.5 ± 2.2 years. The mean ± SD of diagnostic delay was 3.6 ± 3.1 years and patients were followed up for a mean ± SD period of 6.5 ± 6.2 years. A family history of either PAD or recurrent infections was found for 34 (39%) of the patients and parental consanguinity was recorded in 33 (37.9%) patients. The most common presenting symptom at the time of onset was mucosal infections with pneumonia, otitis media, and infectious diarrhea being the most frequent. The most common occurring infections before and after diagnosis were pneumonia (51 patients, 58.6%), sinusitis (42 patients, 48.2%), and otitis media (39 patients, 44.8%). Bronchiectasis was documented in 16 patients (from 69 investigated cases, 23%) by high-resolution-computed tomography of the lungs by the time of study. Interestingly, the majority of patients presented bronchiectasis at diagnosis (9 of 16, 56.2%). Eighteen patients (20.6%) died during the follow-up period mostly due to respiratory failure and meningitis. The age of onset and delayed diagnosis did not differ significantly between this group and alive patients, but the rates of bronchiectasis before diagnosis were higher in deceased patients (55% vs. 13%, p = 0.02). Forty-six patients (52.8%) were known to be alive and 24 patients (27.5%) could not be located during the last 6 months of follow-up period (Figure 1).

Mutational analysis performed in 51 patients leading to identification of 45 mutations including BTK gene variations in 39 patients (30 different families, 1 novel variation, Table 2), IGHM variations in 5 cases (5 different families, 3 novel variations, Table 3), and CD79A variation in 1 case (Table 3). From these individuals, the genetic defects were reported in 23 XLA [23,25–28], 1 µ-heavy chain deficient [24], and 1 iga-deficient [32] patients previously. Detailed clinical and immunological features of patients carrying novel mutations are summarized in Table 4. Six out of 51 patients (11.7%) did not show any variation in the investigated genes and were enrolled for exome sequencing analysis (data not shown in this paper).

Table 1. Clinical and immunologic features of 87 Iranian patients with congenital agammaglobulinemia.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>All</th>
<th>XLA</th>
<th>ARA</th>
<th>p-value</th>
<th>Mild BTK mutation</th>
<th>Severe BTK mutation*</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients</td>
<td>87 (51 studied genetically)</td>
<td>39</td>
<td>6 (1 Iga–5 µ-heavy)</td>
<td>–</td>
<td>21</td>
<td>18</td>
<td>–</td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>81/6</td>
<td>39/0</td>
<td>4/2</td>
<td>0.13</td>
<td>21/0</td>
<td>18/0</td>
<td>–</td>
</tr>
<tr>
<td>Mean age ± SD (years)</td>
<td>16.5 ± 10.3</td>
<td>18.2 ± 10.1</td>
<td>6.9 ± 5.7</td>
<td>0.54</td>
<td>15.5 ± 9.8</td>
<td>21.8 ± 9.7</td>
<td>0.93</td>
</tr>
<tr>
<td>Mean onset age ± SD (years)</td>
<td>2.5 ± 2.2</td>
<td>2.7 ± 2.3</td>
<td>0.4 ± 0.3</td>
<td>0.019*</td>
<td>1.9 ± 1.3</td>
<td>2.8 ± 2.4</td>
<td>0.50</td>
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<tr>
<td>Mean age at diagnosis ± SD (years)</td>
<td>6.1 ± 5.3</td>
<td>6.6 ± 5.6</td>
<td>1.18 ± 0.4</td>
<td>0.06</td>
<td>6.9 ± 5.8</td>
<td>6.3 ± 3.2</td>
<td>0.06</td>
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<tr>
<td>Mean diagnostic delay ± SD (years)</td>
<td>3.6 ± 3.1</td>
<td>4.4 ± 4.0</td>
<td>0.7 ± 0.5</td>
<td>0.08</td>
<td>5.0 ± 3.8</td>
<td>3.8 ± 3.0</td>
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<td>Positive family history (%)</td>
<td>34 (39.0)</td>
<td>18 (46.1)</td>
<td>3 (50.0)</td>
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<td>11 (50.0)</td>
<td>6 (46.1)</td>
<td>0.19</td>
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<td>Number of hospitalizations, mean ± SD</td>
<td>4.2 ± 2.9</td>
<td>4.5 ± 2.9</td>
<td>2.7 ± 2.3</td>
<td>0.37</td>
<td>4.0 ± 3.2</td>
<td>5.2 ± 2.5</td>
<td>0.12</td>
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<tr>
<td>Severe infections (%)</td>
<td>31 (36.4)</td>
<td>18 (46.2)</td>
<td>4 (66.6)</td>
<td>0.31</td>
<td>6 (28.5)</td>
<td>12 (66.6)</td>
<td>0.019*</td>
</tr>
<tr>
<td>Immunoglobulin G (mg/dl), mean ± SD</td>
<td>152.1 ± 19.6</td>
<td>142.6 ± 115.9</td>
<td>90.1 ± 29.2</td>
<td>0.51</td>
<td>140.0 ± 115.9</td>
<td>146.0 ± 119.7</td>
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<td>Immunoglobulin M (mg/dl), mean ± SD</td>
<td>20.9 ± 13.2</td>
<td>17.1 ± 12.4</td>
<td>2.8 ± 2.4</td>
<td>0.02*</td>
<td>19.2 ± 18.6</td>
<td>14.3 ± 12.0</td>
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<td>Immunoglobulin A (mg/dl), mean ± SD</td>
<td>16.1 ± 14.3</td>
<td>7.6 ± 5.9</td>
<td>0.8 ± 0.3</td>
<td>0.015*</td>
<td>6.6 ± 5.8</td>
<td>8.8 ± 7.9</td>
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<td>CD3 (%), mean ± SD</td>
<td>85.2 ± 9.5</td>
<td>88.6 ± 17.5</td>
<td>86.3 ± 6.0</td>
<td>0.85</td>
<td>87.5 ± 18.8</td>
<td>90.1 ± 25.2</td>
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<td>CD4 (%), mean ± SD</td>
<td>40.6 ± 15.3</td>
<td>44.4 ± 14.2</td>
<td>42.8 ± 16.7</td>
<td>0.79</td>
<td>43.8 ± 14.7</td>
<td>45.3 ± 19.1</td>
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<td>CD8 (%), mean ± SD</td>
<td>40.5 ± 15.9</td>
<td>37.4 ± 15.5</td>
<td>43.7 ± 19.0</td>
<td>0.98</td>
<td>36.7 ± 16.3</td>
<td>38.3 ± 13.5</td>
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<td>CD19 (%), mean ± SD</td>
<td>0.9 ± 0.7</td>
<td>0.87 ± 0.84</td>
<td>0.5 ± 0.4</td>
<td>0.58</td>
<td>0.9 ± 0.8</td>
<td>0.7 ± 0.4</td>
<td>0.12</td>
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</tbody>
</table>

ARA: autosomal recessive agammaglobulinemia; BTK: Bruton’s tyrosine kinase; XLA: X-linked agammaglobulinemia.

* Statistically significant.

**Severe BTK mutation: out-of-frame variation, termination codon change, variation attributed to invariant regions within 1–2 base pair at the beginning and end of an intron and missense variation located in conserved subdomains as well as those at structurally or functionally important sites.
<table>
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<tr>
<th>ID</th>
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<th>History of other autoimmune diseases</th>
<th>Medical history</th>
<th>BTK domain</th>
<th>Protein variation</th>
<th>Variation type</th>
<th>Predicted severity of mutation</th>
<th>Expression severity</th>
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<td>B-cells (%)</td>
<td>BTK domain</td>
<td>RNA variation</td>
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<tr>
<td>F25</td>
<td>6 m</td>
<td>24</td>
<td>22</td>
<td>IgA 3 (mg/dl)</td>
<td>0</td>
<td>SH2</td>
<td>Premature stop codon</td>
<td>Nonframeshift nonsense</td>
<td>Mild</td>
</tr>
<tr>
<td>F26</td>
<td>6 m</td>
<td>25</td>
<td>22</td>
<td>IgM 3 (mg/dl)</td>
<td>0</td>
<td>PH</td>
<td>Premature stop codon</td>
<td>Nonframeshift nonsense</td>
<td>Mild</td>
</tr>
<tr>
<td>F27</td>
<td>6 m</td>
<td>26</td>
<td>22</td>
<td>IgG 3 (mg/dl)</td>
<td>0</td>
<td>SH1/TK</td>
<td>Premature stop codon</td>
<td>Nonframeshift nonsense</td>
<td>Mild</td>
</tr>
<tr>
<td>F28</td>
<td>1 y</td>
<td>27</td>
<td>22</td>
<td>IgM 3 (mg/dl)</td>
<td>0</td>
<td>PH</td>
<td>Premature stop codon</td>
<td>Nonframeshift nonsense</td>
<td>Mild</td>
</tr>
<tr>
<td>F29</td>
<td>1 y</td>
<td>28</td>
<td>22</td>
<td>IgG 3 (mg/dl)</td>
<td>0</td>
<td>SH2</td>
<td>Premature stop codon</td>
<td>Nonframeshift nonsense</td>
<td>Mild</td>
</tr>
<tr>
<td>F30</td>
<td>1 y</td>
<td>29</td>
<td>22</td>
<td>IgM 3 (mg/dl)</td>
<td>0</td>
<td>PH</td>
<td>Premature stop codon</td>
<td>Nonframeshift nonsense</td>
<td>Mild</td>
</tr>
</tbody>
</table>

**Table 2. Clinical and immunologic features and location of Bruton's tyrosine kinase (BTK) mutations in 39 Iranian patients with X-linked agammaglobulinemia.**

***Severe medical phenotype defied by having two of three criteria: early age at onset of symptoms (less or equal 6 months), frequent infectious symptoms (according the 10 warning signs of primary immunodeficiency), and development of severe infectious complications during the course of the disease (bloodstream, CNS, and deep-seated infections like osteomyelitis and arthritis).***
Table 3. Clinical and immunologic features and mutations in six Iranian patients with autosomal recessive agammaglobulinemia.

<table>
<thead>
<tr>
<th>ID</th>
<th>Age at onset</th>
<th>Gender</th>
<th>Family history</th>
<th>IgG (mg/dl)</th>
<th>IgA (mg/dl)</th>
<th>IgM (mg/dl)</th>
<th>B-cells (%)</th>
<th>Gene</th>
<th>RNA variation</th>
<th>Protein variation</th>
<th>Variation type</th>
</tr>
</thead>
<tbody>
<tr>
<td>F31</td>
<td>2 m</td>
<td>Male</td>
<td>–</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>&lt;1</td>
<td>IGHM</td>
<td>Homozygous c.56C &gt; A</td>
<td>p.S19X</td>
<td>Nonframeshift nonsense, Premature stop codon</td>
</tr>
<tr>
<td>F32</td>
<td>1 y</td>
<td>Male</td>
<td>–</td>
<td>60</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>9.6</td>
<td>IGHM</td>
<td>Homozygous c.208C &gt; T</td>
<td>p.Q70X</td>
<td>Nonframeshift nonsense, Premature stop codon</td>
</tr>
<tr>
<td>F33</td>
<td>1 y</td>
<td>Male</td>
<td>–</td>
<td>18</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>IGHM</td>
<td>Homozygous c.525–526 ins.C</td>
<td>p. Y176Lfs.87X*</td>
<td>Frameshift nonsense, Premature stop codon</td>
</tr>
<tr>
<td>F34</td>
<td>3 m</td>
<td>Male</td>
<td>–</td>
<td>30</td>
<td>0</td>
<td>0</td>
<td>&lt;1</td>
<td>IGHM</td>
<td>Loss of PCR</td>
<td>frameshift nonsense, Gross deletion</td>
<td></td>
</tr>
<tr>
<td>F35</td>
<td>7 m</td>
<td>Female</td>
<td>–</td>
<td>54</td>
<td>9</td>
<td>0</td>
<td>3</td>
<td>IGHM</td>
<td>Homozygous c.68 dup.C</td>
<td>p. W24Vfs.452X</td>
<td>Nonframeshift nonsense, Premature stop codon</td>
</tr>
<tr>
<td>F36</td>
<td>6 m</td>
<td>Female</td>
<td>–</td>
<td>30</td>
<td>0</td>
<td>0</td>
<td>0.28</td>
<td>CD79a</td>
<td>Homozygous c.157C &gt; T</td>
<td>p.Q53X*</td>
<td>Nonframeshift nonsense, Premature stop codon</td>
</tr>
</tbody>
</table>

* Previously published variations.

Table 4. Clinical and immunologic features of 5 Iranian agammaglobulinemic patients with novel mutations.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>F23-1</th>
<th>F23-2</th>
<th>F31</th>
<th>F32</th>
<th>F35</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>Male</td>
<td>Male</td>
<td>Male</td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>Gene</td>
<td>BTK</td>
<td>BTK</td>
<td>IGHM</td>
<td>IGHM</td>
<td>IGHM</td>
</tr>
<tr>
<td>Protein variation</td>
<td>p.HS50D</td>
<td>p.HS50D</td>
<td>Homozygous</td>
<td>Homozygous</td>
<td>Homozygous</td>
</tr>
<tr>
<td>Clinical manifestation</td>
<td>Recurrent pneumonia</td>
<td>Recurrent pneumonia, septic arthritis, bronchiectasis</td>
<td>Recurrent URI, paralysis after OPV vaccination</td>
<td>Recurrent URI, paralysis after OPV vaccination</td>
<td>Omphalitis, paralysis after OPV vaccination</td>
</tr>
<tr>
<td>Age at onset of disease</td>
<td>1 y</td>
<td>8 y</td>
<td>2 m</td>
<td>1 y</td>
<td>7 m</td>
</tr>
<tr>
<td>Age at diagnosis</td>
<td>13 y</td>
<td>8 y</td>
<td>1.5 y</td>
<td>1.5 y</td>
<td>1 y</td>
</tr>
<tr>
<td>Consanguinity</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Family history of PID</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Family history of early age of death</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Immunoglobulins</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG (mg/dl)</td>
<td>49</td>
<td>342</td>
<td>0</td>
<td>60</td>
<td>54</td>
</tr>
<tr>
<td>IgM (mg/dl)</td>
<td>29</td>
<td>36</td>
<td>5</td>
<td>&lt;5</td>
<td>0</td>
</tr>
<tr>
<td>IgA (mg/dl)</td>
<td>0</td>
<td>30</td>
<td>0</td>
<td>&lt;5</td>
<td>9</td>
</tr>
<tr>
<td>IgE (mg/dl)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0</td>
</tr>
<tr>
<td>Specific antibodies</td>
<td>–</td>
<td>–</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Anti-diphtheria antibody (IU/ml)</td>
<td>–</td>
<td>–</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Anti-tetanus antibody (IU/ml)</td>
<td>–</td>
<td>–</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Complete blood count</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White blood cells (cells/ml)</td>
<td>11,000</td>
<td>9000</td>
<td>14,600</td>
<td>14,630</td>
<td>10,500</td>
</tr>
<tr>
<td>Neutrophils, cells/ml (% of WBC)</td>
<td>19</td>
<td>32</td>
<td>40</td>
<td>18.6</td>
<td>30.4</td>
</tr>
<tr>
<td>Lymphocytes, cells/ml (% of WBC)</td>
<td>64</td>
<td>59</td>
<td>40</td>
<td>70.3</td>
<td>62.7</td>
</tr>
<tr>
<td>Lymphocyte subsets</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD19 (%)</td>
<td>0.33</td>
<td>0.5</td>
<td>0.7</td>
<td>1.1</td>
<td>3</td>
</tr>
<tr>
<td>CD3 (%)</td>
<td>86</td>
<td>80</td>
<td>91.9</td>
<td>87.4</td>
<td>85.2</td>
</tr>
<tr>
<td>CD4 (%)</td>
<td>23</td>
<td>22</td>
<td>43.7</td>
<td>46.1</td>
<td>44.6</td>
</tr>
<tr>
<td>CD8 (%)</td>
<td>43.8</td>
<td>45</td>
<td>44.4</td>
<td>41.7</td>
<td>38.9</td>
</tr>
</tbody>
</table>

PID: primary immunodeficiency; URI: upper respiratory tract infection; OPV: oral polio virus.

More in detail, among the whole cohort BTK gene variations were found in 39 out of the 51 patients analyzed (76.4%, 86.6% of males), 21 were associated with missense variations, 9 nonsense variations, and 9 with effect on RNA splicing. These variants were localized on the Src homology 1/tyrosine kinase (SH1/TK) catalytic domain of the protein in 19 out of 39 cases (48.7%), in the pleckstrin homology (PH) domain in 11 of cases (28.6%), in the Src homology 2 (SH2) domain in 4 of cases (10.2%), in the Src homology 3 (SH3) in 3 cases (7.6%), and in the Tec homology (TH)/cys-rich domain in 2 cases (5.1%). The novel c.1048C>G missense variation (p.HS50D) was identified in two brothers (F23-1 and F23-2) (for detailed clinical and immunological features see Tables 2 and 4 and Supplementary Material).

Genotype–phenotype correlation

So far, no specific genotype–phenotype correlation has been found in XLA. We decided to evaluate this correlation in our cohort of patients especially for affected individuals within the same family. BTK gene mutations were predicted and assigned to mild (n = 21) or severe (n = 18) for all patients (Table 2). The level of protein expression was evaluated in 27 patients; among those only 5 (18.5%) patients has a normal level of
BTK comparable with wild type controls. Thereafter, correlation between mutation severity, protein expression, and disease severity was investigated. All five patients with normal expression level of BTK had mild mutations and all severe predicted mutations had reduction in or absence of protein production. Eight families were found with more than one patient with the same mutation and obvious different clinical or immunologic phenotypes were recorded in these cases (Supplementary Material).

Regarding severity of genotype and disease within all enrolled patients in the cohort, no significant correlation was observed. Discrepancies between predicted damage in protein and clinical severity were observed in 11 patients; mostly, severe damaging mutations were presented by mild clinical phenotype (9 out of 11, 81.8%). Interestingly, five cases (45.4%) harboring variations with effect on RNA splicing (particularly in exons encoding the PH domain) belong to this group with discordance of severity of mutation and clinical severity (Table 2). Age at onset of symptoms was not influenced by the type of BTK gene variation (p = 0.6). No association was observed between the percentage of peripheral B-cells and plasma IgM levels with type of BTK gene variation (p = 0.12 and p = 0.81, respectively). Severe infections developed in 66.6% (12/18) of patients with severe mutations and 28.5% (6/21) of patients with mild mutations (p = 0.019, Table 1).

Patients with variations in BTK gene were then divided into five groups depending on the domain of localization on the protein. The domain of localization in the BTK gene product did not influence the clinical and immunologic phenotype of patients. However, the survival rate of XLA patients with nonsense variation was significantly lower when compared to XLA patients with missense variation (p = 0.02 value log rank Mantel–Cox, Figure 2). Comparison between patients affected with XLA and ARA showed that age at onset was significantly lower in patients with ARA compared to XLA patients (0.4 ± 0.3 vs. 2.7 ± 2.3 years, p = 0.019). Statistical analysis revealed significant difference between two groups regarding serum IgM levels (17.1 ± 2.4 vs. 2.8 ± 2.4 mg/dl, p = 0.02), serum IgG levels (7.6 ± 5.9 vs. 0.8 ± 0.3 mg/dl, p = 0.015), and survival rate (p = 0.04, Table 1 and Figure 1). In order to define the clinical manifestation of ARA comparing to early stages of XLA, A group of XLA patients with matched ages was subtracted. Intriguingly the significant difference in the survival rate of these two groups was changed to non-significant value (p = 0.17).

Two patients with autoimmunity (F15-2 with Kawasaki disease and F29 with vitiligo) and one patient with lymphadenopathy (F15-2) were reported in the group of XLA patients, while no similar findings were observed in the ARA group.

Agammaglobulinemic patients are known to have an increased susceptibility to entero viral infections including echo, coxsackie, and both wild type and vaccine-associated polio infections [33,34]. Five patients from our initial 87 patients’ cohort were diagnosed with agammaglobulinemia after developing vaccine-associated polio infection. Four of these patients underwent mutation analysis: One was mutated in BTK gene (mild mutation), while the other three patients were mutated in the IGHM gene (F31, F32, and F35) (Tables 2 and 3). Overall, severe infections occurred in 18 out of 39 patients (46.1%) with XLA and 4 out of 6 patients (66.6%) with ARA (p = 0.31).

Discussion

Agammaglobulinemia is typically characterized by very low immunoglobulin serum levels and the absence of peripheral B-cells. The majority of affected patients are males (85–90%) and the disease is caused by variations in BTK gene transmitted in an X-linked recessive pattern. The remaining 10–15% of the patients are predominantly affected by ARA due to mutations in components of the pre-BCR complex or in downstream signaling components [3,5,20,21].

In this study, we have evaluated 87 Iranian patients affected with agammaglobulinemia. A genetic analysis was performed on 51 patients from 42 unrelated families. Variations in BTK gene were responsible for the disease in approximately 76% of the studied cases (86.6% of studied all male cases), while variations causing ARA was confirmed in almost 12% of cases, in line with previously published data. However, the pattern of ARA is prominent in Iranian cohort probably due to a higher rate of consanguinity in families of patients (38%).

The majority of the variations in the BTK gene found in the present study were both in type and pattern of distribution similar to those already identified as disease causing in the BTK database (http://bioinf.uta.fi/BTKbase). The main difference was noted for variations in the TK and PH domains of BTK. In our cohort, the frequency of variations in these domains was similar, while previously published data report a higher frequency of variations in the TK domain when compared to the PH domain.

Several studies have attempted to establish whether a genotype–phenotype correlation in patients with BTK variations exists, without a conclusive result so far. In a study by Holinski-Feder et al. on 56 European patients with XLA, the
correlation between genotype and clinical course was analyzed in 18 patients with no significant result [8].

The genotype–phenotype correlation was demonstrated in a genetic study on 54 Spanish patients [7]. Less severe mutations were associated with diagnosis being made at an older age of the patients and the patients having higher levels of immunoglobulins, a higher percentage of B-cells, and an increased number of hospitalizations before starting intravenous immunoglobulin replacement therapy.

Likewise, a study by Broides et al. on 110 XLA patients found that mild mutations were associated with an older age at diagnosis, a higher percentage of peripheral B-cells, and a higher IgM concentration [2]. In contrast to this, several studies have reported mild phenotypes in patients with severe mutations as termination codons or initiation codon changes [32,35] or discordant phenotype in the patients from the same family with identical mutations [15,20,21,32,36]. The current study does not show significant correlation between severity of BTK variations and immunologic phenotype of the patients. Moreover, patients of our study who were members of the same family and shared the same variations in BTK showed discordant clinical phenotypes. However, a likely correlation was observed in terms of severity of infections and on-time diagnosis with the predicted severity of the mutation. A selective correlation has also been reported in the large cohort of XLA patients (554 mutations/823 families) extracted from the BTK base [37]. Based on this survey, most of the recorded BTK variations are located in functionally significant conserved residues (overrepresentation of missense variations in the coding regions for the proline residue 407 and C-terminal of SH1 domain and absence in lower proline-rich lobe of TH and entire SH3 domains), in structural elements than coil structure (in core of the protein structure or in interactive residues with secondary structures), in identical residues (between human BTK and other human TK with TH), and in CpG dinucleotides (CpG-arginine codons except residue 12 and 255). They reported that nonsense variations are equally distributed through all coding regions of the protein domains but variations in the core regions are more severe than those found in the loops of the protein. The analysis of the mutations contained in the database was though not directly linked to the clinical data from the patients complicating a direct comparison with the previously described studies.

Interestingly, based on the above-mentioned molecular prediction from the database analysis, the new missense variation (c.1048C>G) found in our study is located in a coil structure of domain SH2 of BTK, and this histidine is a conserved residue predicted to form a trans-peptide. This variant changes the basic charged side chains of the histidine to the acidic aspartic acid giving a predicted change in the conformational folding of the domain and alteration of the trans-peptide bands formed (Figure S1).

In the presented study, variations in genes causing ARA were identified in six patients including five patients with μ-heavy chain deficiency and one patient with Igα deficiency. For those patients, the age at onset of diseases, serum levels of IgA and IgM and the severity of infections were significantly different from patients with XLA. This is in accordance with previously published data indicating the essential role of proteins involved in ARA for the development of progenitor relative to precursor B-cells [38].

Regarding the influence of the period of diagnosis and the medication available at this time on our findings, it should be noted that only few patients (<20 patients) were diagnosed before the year 2000. Most of these patients are either unavailable or deceased preventing genetic studies and are therefore not included in the analysis of the correlation between mutations and phenotypic effects. However, a similar analysis restricted only to patients diagnosed after 2000 showed identical results (data not shown).

The selection of the genes screened in the mutational analysis in the current study is based on a cost-effective evaluation of the expected probability for involvement in the disease and identified causative mutations in 88.2% of the screened patients. For future studies, the inclusion of BLNK, TCF3, PIK3R1, and LRRC8 in the screen or even further, whole exome sequencing, could help elucidate the causative mutations for the remaining patients.

In conclusion, our study broadens the mutational spectrum of agammaglobulinemia, for both XLA and ARA. The findings confirm the correlation between clinical phenotype and the category of agammaglobulinemia (XLA or ARA); they also suggest that the nature of the BTK variations does not conclusively correlate with the clinical phenotype observed. Furthermore, it appears that the severity of the mutation does not necessarily lead to corresponding severity of disease in all XLA patients. This may be due to the effects of modifier genes, epigenetic, environmental, or other factors that still need to be determined.

Financial & competing interests disclosure
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Key issues
- Although there is no clear genotype–phenotype in XLA yet, severe mutations in BTK may present with more severe infections leading to reduction in the delay of diagnosis.
- Patients affected with autosomal recessive agammaglobulinemia usually present with a more severe clinical and immunological phenotype compared to XLA leading to an earlier diagnosis.
- Patients harboring BTK mutations may present with unusual manifestations such as autoimmunity and lymphadenopathy.
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