X-LINKED AGAMMAGLOBULINEMIA: MOLECULAR GENETIC ASSESSMENT OF THE BRU...
We present the molecular genetic findings in three unrelated patients and their mothers; this is the first report of genetic assessment of XLA patients in Romania.

MATERIAL AND METHODS

XLA was diagnosed according to the following criteria: low levels of circulating B-cells – measured by levels of CD19/CD20 positive cells in plasma samples, decreased or absent immunoglobulins (Ig) in serum, and a typical clinical history, with recurrent bacterial infections or a positive family history.

Genomic DNA was obtained from whole blood lysis, using standard methods. The coding segments of Btk gene were amplified by PCR (Polimerase Chain Reaction) using a set of primer pairs flanking all exons, designed according to the published cDNA sequence. The amplified segments were analyzed by the single strand conformation polymorphism (SSCP) method. Exons demonstrating altered migration pattern were regarded as indicative of altered sequence (i.e. mutation) and sequenced. The DNA of the patients’ mothers were then tested for the same mutation by SSCP.

RESULTS

Patient 1 was diagnosed at the age of nine months. He has a positive family history with two affected brothers, who died before the age of 1 year. Serum immunoglobulin levels at diagnosis were as follows: IgG = 1.59 g/l, IgA <0.06 g/l, IgM = 0.19 g/l; CD19+B-cells<10/mm$^3$ (0.8%), CD3+T-cells 2240/mm$^3$ (94%).

Patient 2 is a sporadic case, aged nine years at the time of diagnosis. He presented with a milder clinical form of the disease. Ig levels were: IgG = 2.68 g/l, IgA = 0.29 g/l, IgM = 0.14 g/l; CD19+B-cells<10/mm$^3$ (0.10%), CD3+T-cell 1650/mm$^3$ (90%).

Patient 3 is an 8 years old boy was treated for recurrent, severe respiratory tract infections, pyogenic arthritis and sepsis. Agammaglobulemia was diagnosed at two and a half years of age, based on clinical and immunological phenotypes: IgG<0.05 g/l, IgA = 0.1 g/l, IgM = 0; CD19+B cells not detectable, CD3+T cells 3400/mm$^3$ (94%). (Table 1)

In patient 1 a 4 base pair deletion in exon 16 was found, involving TTTG at positions 257-258 resulting in a frameshift, the substitution of cysteine for tryptophan in position 257 and a termination codon in position 528 (c1581-1584delTTTG). The patients’ mother was found to carry the same mutation.

The Btk gene of patient 2 showed a nonsense mutation (c1558C>T, pArg520Stop) in exon 15, a C to T transition that converts the codon for arginine (CGA) into a stop codon (TGA) at position 520. His mother was also found to be a carrier of the mutation.

Both mutations are located in the middle of the kinase domain; they predicted absent BTK protein synthesis, as mRNA molecules carrying a premature termination codon at least 50 nucleotides upstream of the last splice junction are usually rapidly degraded by nonsense-mediated mRNA decay, a mechanism that prevents the production of a truncated polypeptide.$^{12,13}$

In patient 3 mutational analysis disclosed a c.29T>A missense mutation in exon 2 leading to a pF10Y amino acid change. (Fig. 1)

The types of mutations and their localizations in Btk domains PH, TH, SH3, SH2 or SH1 are presented in Figure 2.

DISCUSSION

XLA is caused by a B-lymphocyte developmental defect, with a partial block between the pro- and pre-B cell stage and the marked decrease in the number of circulating B lymphocytes, that result in a pronounced reduction of serum Ig of all classes, hence affected

<table>
<thead>
<tr>
<th>No.</th>
<th>Patient</th>
<th>Age (years)</th>
<th>Age at diagnosis</th>
<th>Immunoglobulin Levels (g/l)</th>
<th>B cells (nr/mm$^3$)</th>
<th>Clinical Symptoms</th>
<th>XLA in the family</th>
<th>Substitution (since year)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>IgG</td>
<td>IgA</td>
<td>IgM</td>
<td>&lt; 10</td>
<td>Recurrent respiratory infection</td>
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<td>1</td>
<td>A.C.</td>
<td>16</td>
<td>9 months</td>
<td>1.59</td>
<td>0.06</td>
<td>0.19</td>
<td>&lt; 10</td>
<td>Idem, milder form</td>
</tr>
<tr>
<td>2</td>
<td>V.D.</td>
<td>14</td>
<td>9 years</td>
<td>2.68</td>
<td>0.29</td>
<td>0.14</td>
<td>&lt; 10</td>
<td>Recurrent, severe and systemic infection</td>
</tr>
<tr>
<td>3</td>
<td>R.M.</td>
<td>8</td>
<td>2.5 years</td>
<td>&lt;0.05</td>
<td>0.1</td>
<td>Not detectable</td>
<td>Not detectable</td>
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</tr>
</tbody>
</table>

Table 1. Clinical data for 3 XLA patients.
individuals suffer from recurrent bacterial and enteroviral infections. Bruton tyrosine kinase (BTK), the defective protein in XLA, is a member of the Tec family of cytoplasmic tyrosine kinases, expressed primarily in hemopoietic cells. BTK is a signal transduction molecule, expressed at all stages of B cell differentiation except plasma cells, that interacts with a variety of cell-surface receptors, of which most important are the pre-B cell and B cell antigen receptor complex.  

The protein consists of five domains, each with a distinctive function: the plecstrin homology (PH), Tec homology (TH), src homology 3 (SH3), SH2 and the catalytic SH1 or kinase domain. The PH domain is the site of activation by PIP2 and G protein βλ subunits and inhibition by protein kinase C. The TH, SH3 and SH2 domains interact with distinct functional domains of other signaling molecules, important in BTK activation and signal transduction. Some of the functions of the BTK protein have been shown to be independent of its kinase activity. 

Mutations in all five domains of the Btk gene have been found to cause XLA. By now, more than 550 unique mutations, distributed almost uniformly along the coding sequence, have been identified; thus, almost half of the XLA-causing mutations are in the kinase domain, which forms more than 40% of BTK. The majority of Btk mutations result in deficient expression of the BTK protein, presumably due to a reduction in Btk mRNA or instability of the protein product. 

The first two mutations detected in our patients are placed in the 3’ terminal part of the Btk gene, coding for the kinase domain of the protein. The 4 bp deletion at codons 527-528, found in patient 1 has been reported before in patients with classical XLA. 

Our first patient, diagnosed at an early age (nine months), exhibited a severe clinical picture, and his family history includes two similarly severely affected brothers deceased before the age of one year. This is consistent with previous reports of the phenotype caused by this 4 bp deletion: the patient described by Conley et al. in 1994, carrying this mutation died at 11 months of age of bacterial sepsis, all the five patients listed in BTKbase (a database of Btk mutations: http://www.uta.fi/imt/bioinfo/BTKbase/) with this mutation presented with the classical form of the disease. These data are concordant with the observation that mutations that generate premature stop codons are generally associated with no detectable levels of the protein product, and therefore, a more severe disease phenotype. 

The mutation detected in patient 2 is a nonsense mutation, a C to T transition in exon 15, that results in a stop codon at position 520. This is also a recurrent mutation, that affects a codon for arginine, containing the CpG dinucleotide. CpG dinucleotides are considered mutational hotspots, as methylated cytosine residues by spontaneous deamination may lead to the formation of thymine, thus a C to T transition (when the sequence change goes undetected by endogenous repair mechanisms). 

The R520X mutation is one of the most frequent mutations in the Btk gene. As it also leads to a premature termination codon, it has been found in patients with severe XLA, that have no detectable protein product in their cells. Interestingly, our patient, diagnosed at age nine years, had a milder disease course, in contrast to most patients carrying this mutation. In addition to our patient, there has been one more patient reported with this mutation that presented with a milder phenotype. The phenotypic variability is well recognized in XLA, individuals (and even members of the same family) carrying the same mutation may show a highly variable degree of disease severity. Besides the specific mutation in Btk, factors that influence disease severity are thought to include polymorphic variants in proteins that could partially substitute for Btk (like Tec), polymorphic variants in other components of the Btk mediated signaling pathway (µ heavy chain, λ5) and variants in proteins that function as part of the innate immune system.
It was the phenotypic heterogeneity in XLA that made researchers declare the lack of genotype/phenotype correlations in the disease. Earlier studies failed to detect such correlations, however most of the times these included only severely affected patients, that carried substitutions at functionally significant conserved residues leading to nonfunctional or unstable protein, or frameshift and nonsense mutations producing premature termination codons and a subsequent lack of protein product.\textsuperscript{22,29,30}

Nevertheless, recent studies have demonstrated that certain substitutions at less conserved residues or splice defects that allow for the production of small amounts of functional protein, result in a milder phenotype, with higher immunoglobulin concentrations and slightly more circulating B cells.\textsuperscript{29,30}

Mutation analysis is the only means to establish a definitive diagnosis in male patients with no family history of immunodeficiency, as mutations in the heavy chain and $\lambda_5$ gene result in primary immunodeficiency diseases clinically indistinguishable from XLA, but with autosomal recessive inheritance.\textsuperscript{31,32} Although these are much rarer than XLA, the mutation analysis of the Btk gene is important in order to set an accurate diagnosis and provide appropriate genetic counseling.\textsuperscript{33}

The mother of patient 2 was also found to have the R520X mutation, therefore she should be counseled with regard to the 50 % likelihood of transmitting the disease-causing mutation to her offspring and the possibility of prenatal diagnosis: she has a 50 % risk of having an affected son or a carrier daughter at each pregnancy; however, in view of phenotypic heterogeneity, disease severity in future offspring cannot be predicted. Her sisters may also have inherited the mutation and be at risk of giving birth to affected sons; they should be offered carrier testing.

**CONCLUSIONS**

The genetic assessment of the patients with XLA, revealing chain-terminating mutations in the kinase domains at nearby sites (positions 520 and 528, respectively) and PH domain, points to the phenotypic heterogeneity generated by Btk mutations, and highlights the importance of molecular genetic testing in order to establish a definitive diagnosis of XLA, which is a prerequisite of genetic counseling, carrier testing and prenatal diagnosis.

Clarification of the role of the Btk domains in cell functioning may help to decipher the complex role that tyrosine kinase play in health and disease.