Identification of Nine Novel Mutations in the Bruton’s Tyrosine Kinase Gene in X-linked Agammaglobulinaemia Patients

Paola Orlandi1*, Kostas Ritis2, Viviana Moschese1, Federica Angelini1, Konstantinos Arvanitidis3, Matthaios Speletas2, Paschalis Sideras3, Alessandro Plebani4, and Paolo Rossi1, in collaboration with the Italian XLA Collaborative Group

1Dept. of Pediatrics, Division of Immunology and Infectious Diseases, Children’s Hospital “Bambino Gesu”, University of Rome Tor Vergata, Rome, Italy; 2Dept. of Haematology, Democritus University of Thrace, Alexandroupolis, Greece; 3Department of Applied Cell and Molecular Biology, Umea University, Umea, Sweden; 4Dept. of Pediatrics, University of Brescia, Brescia, Italy

*Correspondence to: Dr. Paola Orlandi, Dept. of Pediatrics, University of Rome “Tor Vergata”, Via di Tor Vergata, 135, 00133 Rome, Italy; Tel: 06/72596492; Fax: 06/72596824; E-mail: Paros@Med.UniRoma2.it

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Mutations in the Bruton’s tyrosine kinase (BTK) gene are responsible for X-linked Agammaglobulinemia (XLA), an immunodeficiency caused by a block in B cell differentiation. Non Isotopic RNAs Cleavage Assay (NIRCA), followed by sequencing was used to screen for BTK mutations in 11 Italian XLA patients. Nine novel mutations were identified: 6 missense (Y39S, L512P, L512Q, R544G, S578Y, E589K), one non-sense (Q260X), one frameshift (1599-1602del GCGC) and one in-frame insertion (2037-2038insTTTTAG), that represents the first case of premature stop codon introduction in the BTK coding frame. These data support the high molecular heterogeneity of BTK gene in XLA disease and provide new insight to the diagnosis and to the role of BTK domain in XLA and in B cell signal transduction and development. © 1999 Wiley-Liss, Inc.

KEY WORDS: Bruton’s tyrosine kinase, BTK, X-linked Agammaglobulinemia

INTRODUCTION

X-linked agammaglobulinemia (XLA; MIM# 300300) is an immunodeficiency characterized by a paucity of circulating B cells and a marked reduction in serum levels of all Ig isotypes, which causes susceptibility to recurrent and severe bacterial infections in affected males. This disorders is caused by a lymphocyte differentiation block affecting the transition of B cell progenitors into mature B lymphocytes (Sideras et al., 1995). The affected protein is a cytoplasmic tyrosine kinase, Bruton's agammaglobulinemia tyrosine kinase, BTK, consisting of five

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distinct structural domains, each of them with a specific function: pleckstrin homology (PH), tec homology (TH), Src homology 3 (SH3), SH2 and the Kinase domain (Vetrie et al, 1993, Tsukada et al, 1993). BTK gene is localized at Xq21.3-Xq22 and encompasses 37.5 Kb containing 19 exons (Sideras et al, 1994). Mutations in the BTK gene associated with the XLA phenotype have been characterized in a large number of patients and collected in a database called BTKbase (http://www.uta.fi/laitokset/imt/bioinfo/BTKbase/) (Vihinen et al, 1998).

Despite the large number of BTK mutations reported, no clear correlation between the clinical phenotype and the gene defect has been established so far (Vorechovsky et al, 1997; Holinski-Feder et al, 1998). Furthermore, the demonstration of BTK integrity in a certain number of XLA patients suggests that this disorder is genetically heterogeneous (Bradley et al, 1994; Hagemann et al, 1994; Hashimoto et al, 1996). To provide a definitive diagnosis of XLA and estimate the frequency of genetic heterogeneity within this gene in Italy, we analyzed at molecular level 11 patients enrolled in the XLA Italian Multicenter Clinical Study. We report the identification of 9 novel BTK mutations in subjects with X-linked agammaglobulinemia.

METHODS

Patients

11 patients from 9 unrelated families, enrolled in the XLA Italian Multicenter Clinical Study, were selected and included in this study (Table 1). XLA was diagnosed according to following criteria: low levels of circulating B cells (measured by levels of CD19-positive cells), decreased or absent immunoglobulins in serum, typical clinical history with recurrent bacterial infections and/or a positive family history. In some cases diagnosis was confirmed by X-inactivation studies in carrier mothers.

cDNA preparation

RNA for PCR amplification was prepared directly from uncultured patients' PBMC according to common procedures. Total RNA was extracted by TRizol® (Gibco-BRL/Life Technologies) and cDNA synthesis was performed using Promega M-MLV reverse transcriptase, according to manufacturer's instructions.

PCR and NIRCA (Non Isotopic RNase Cleavage Assay)

cDNA samples were amplified by PCR as previously described (Ritis et al, 1998). NIRCA was based on the previously described protocols (Goldrik et al, 1996). The optimal hybridization and cleavage conditions for BTK mutation analysis have been also recently described. (Ritis et al, 1998). Briefly, 1-4 µl of inner PCR product was used as template for 10 µl transcription reaction, performed at 37°C for 90 min. Hybridation reactions were carried out according to manufacturer instructions. Any mismatches in RNA-RNA hybrids were revealed by RNase digestion and separated by 1.5 % agarose gel electrophoresis.

Sequencing

Mutated fragment was amplified with Pfu Polymerase and cloned into PCR-Script Amp Cloning Kit (Stratagene). Positive clones were then amplified for direct solid-phase DNA sequencing according to standard procedures (Hultman et al, 1989) and sequenced by an automated laser fluorescent (ALFExpress) sequencing apparatus (Pharmacia LKB) using the Autoread Sequencing Kit (Pharmacia LKB).

RESULTS

The BTK gene has been analyzed in 11 XLA patients from 9 unrelated families enrolled in the XLA Italian Multicenter Clinical Study and diagnosed as classical XLA according to peripheral B cells and serum Ig levels. Five patients were affected by a familiar XLA, whereas 6 were sporadic cases. The results of mutation analysis are summarized in Table 1. In particular, patient 1 showed a 248A>C mutation, that replaced a tyrosine with a serine at residue 39 in the PH domain. This substitution of a hydrophobic residue with a polar amino acid occurred in a cleft formed by hydrophobic and aromatic residues. In siblings 2 and 3, a 1678T>C substitution altered residue 512 in the kinase domain from a leucine to a proline. The same codon was involved in patient 4, where a 1678T>A change replaced the L512 with a polar glutamine. Siblings 5 and 6 showed a 1763A>G change which results in a R544G substitution. A 1865C>A substitution was detected in patient 7, causing the replacement
Three novel mutations lead to a premature termination, introducing either a non-sense substitution or a frameshift. A 910C>T substitution introduced a premature termination signal at codon 260 in patient 9. In patient 10, a four base pair deletion was found, 1599-1602delGCGC, that involved codons R490/H491 in the kinase domain and caused premature stop codon 9 residues downstream. A six base pair insertion, 2037-2038insTTTTAG, introducing in frame a F residue and a termination signal after codon 635, in the C-terminus of the kinase domain was detected in patient 11. This insertion represents the first case of premature stop codon introduction in the BTK coding frame. No correlation between mutation type and location and clinical phenotype was also observed in our cohort. The polymorphism 2031C/T has been observed in patients 5 and 6.

DISCUSSION

We have reported here the identification of 9 novel BTK mutations in patients with X-linked agammaglobulinemia. While location and preponderance of BTK mutations presented in this study mirror previous reports, the type of mutation reported here showed a different feature. In fact, 6 out of 9 (67%) of the novel mutations were missense mutations, compared to 32.7% of the mutations reported in the BTK base mutation database (http://www.uta.fi/laitokset/imt/bioinfo/BTKbase/). This might be due to the small size of the analyzed cohort.

Previous studies showed that missense mutations are not equally distributed over the coding region. On larger cohorts of XLA patients, a tendency for the PH and KIN domains has been reported (Hashimoto et al., 1996; Holinski-Feder et al., 1998). Mutations that enable the PH domain to bind PI(3,4,5)P$_3$ (phosphatidylinositol-3,4,5-trisphosphate) have been shown to compromise BCR-mediated B cell activation (Bradley et al., 1994; de Weers et al., 1994; Salim et al., 1996; Hyvonen and Saraste, 1997). Conversely, substitutions at codon 41 of the PH domain has been demonstrated to be associated to an increased phosphorylation of tyrosine residues and membrane targeting, suggesting an unregulated BTK activation (Li et al., 1995; Li et al., 1997).

In our study, patient n.1 showed a single amino acid substitution in the third sheet of the PH domain at codon 39. The involved amino acid was a tyrosine that represents a conserved aromatic residue in PH domains. Moreover, the corresponding position in pleckstrin and dynamin PH domains shows a chemical shift upon binding to Ins (1,4,5)P$_3$ (Hyvonen and Saraste, 1997).

It can be assumed that this site could be involved in phosphorylation linked to membrane interaction and in binding of inositol phosphate ligands. Almost half of the XLA-causing mutations are in the kinase domain, which is the largest of the BTK domains. Although a large number of involved residues directly affect cofactor or substrate binding, many mutations are likely to have structural consequences (Vihinen et al., 1998).

We have identified seven novel mutations affecting the kinase domain. The L512 residue is close to the conserved catalytic region. Therefore, the substitutions found in these patients could have structural implications. Residue R544 is conserved in protein tyrosine kinases, and a basic amino acid is present at the corresponding position in protein serine kinases, suggesting that it might play a critical role in the catalytic activity (Vihinen et al., 1994). Y578 is located in a region typical for PTKs and could be involved in catalysis or substrate binding. E589 residue locates in a cleft corresponding to the substrate binding region. Charge modification at this position might prevent the ionic interaction with the substrate.

It is noteworthy that we detected two mutations, in unrelated patients, affecting residue L512 replaced with different amino acids. Moreover, sites R 544 and E589 have been found to be involved in substitutions different from those previously described (Vihinen et al., 1994; Zhu et al., 1994; Conley and Rohrer, 1995; Kobayashi et al., 1996). None of those base replacements affect a CpG doublet, that represents a well-known mutation hot spot. Therefore, the fact that independent mutations occurring at the same residue suggests a crucial role for these codons.

Mutations leading to premature termination (non-sense, deletion and insertion) detected in this cohort have predictable consequence on BTK expression. However, amplification of selected region on cDNA indicates the presence of stable BTK transcripts, suggesting an impairment at protein level (data not shown).

Previous studies have shown that the majority of identified mutations, including missense mutations, lead to no detectable protein or kinase activity (Hashimoto et al., 1996; Holinski-Feder et al., 1998). Furthermore,
mutations causing premature stop codons and frameshifts have been reported to affect essential BTK domains in patients with clinically mild phenotypes. These data suggest that the level of protein expression and kinase activity as well as the severity of the disease, can not be predicted on the basis of the nature and location of the mutation. Recently, autosomal recessive disorders have been shown to result in a phenotype indistinguishable from XLA, suggesting that B cell differentiation is under multiple genes control. (Yel et al, 1996; Minegishi et al, 1998; Fruman et al, 1999).

BTK mutation analysis represents an important tool to provide a definitive and early diagnosis of XLA and to further define the role of BTK domains in signal transduction and B cell development.

Table 1. Clinical data of XLA analyzed patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age at diagnosis( yr)</th>
<th>B cells (%)</th>
<th>IgG levels (mg/dL)</th>
<th>Inheritance</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>8</td>
<td>&lt; 1</td>
<td>523</td>
<td>Sporadic</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>0</td>
<td>280</td>
<td>Familial</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>0</td>
<td>250</td>
<td>Familial</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>2</td>
<td>188</td>
<td>Sporadic</td>
</tr>
<tr>
<td>5</td>
<td>7</td>
<td>0.5</td>
<td>118</td>
<td>Familial</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>0.6</td>
<td>224</td>
<td>Familial</td>
</tr>
<tr>
<td>7</td>
<td>1</td>
<td>1</td>
<td>63</td>
<td>Sporadic</td>
</tr>
<tr>
<td>8</td>
<td>1</td>
<td>4</td>
<td>123</td>
<td>Sporadic</td>
</tr>
<tr>
<td>9</td>
<td>2</td>
<td>2</td>
<td>179</td>
<td>Sporadic</td>
</tr>
<tr>
<td>10</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>Familial</td>
</tr>
<tr>
<td>11</td>
<td>4</td>
<td>0</td>
<td>17</td>
<td>Sporadic</td>
</tr>
</tbody>
</table>

Table 2. BTK mutations in Patients with X-linked Agammaglobulinemia

<table>
<thead>
<tr>
<th>Patient</th>
<th>Protein domain *</th>
<th>Nucleotide Change</th>
<th>Effect on Coding Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PH</td>
<td>248A&gt;C</td>
<td>39 Y&gt;S</td>
</tr>
<tr>
<td>2</td>
<td>KIN</td>
<td>1668T&gt;C</td>
<td>512 L&gt;P</td>
</tr>
<tr>
<td>3</td>
<td>KIN</td>
<td>1668T&gt;C</td>
<td>512 L&gt;P</td>
</tr>
<tr>
<td>4</td>
<td>KIN</td>
<td>1668T&gt;A</td>
<td>512 L&gt;Q</td>
</tr>
<tr>
<td>5</td>
<td>KIN</td>
<td>1763A&gt;G</td>
<td>544 R&gt;G</td>
</tr>
<tr>
<td>6</td>
<td>KIN</td>
<td>1763A&gt;G</td>
<td>544 R&gt;G</td>
</tr>
<tr>
<td>7</td>
<td>KIN</td>
<td>1865C&gt;A</td>
<td>578 S&gt;Y</td>
</tr>
<tr>
<td>8</td>
<td>KIN</td>
<td>1897G&gt;A</td>
<td>589 E&gt;K</td>
</tr>
<tr>
<td>9</td>
<td>SH3</td>
<td>910C&gt;T</td>
<td>260 Q&gt;X</td>
</tr>
<tr>
<td>10</td>
<td>KIN</td>
<td>1599-1602del GCGC</td>
<td>Frameshift</td>
</tr>
<tr>
<td>11</td>
<td>KIN</td>
<td>2037-2038ins TTTTAG</td>
<td>FX in frame insertion</td>
</tr>
</tbody>
</table>

*: PH=pleckstrin homology domain; SH3=Src homology domain; KIN=kinase domain.
9 Novel BTK Mutations in XLA Patients

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REFERENCES


