**Mutational screening of the CD40 ligand (CD40L) gene in patients with X linked hyper-IgM syndrome (XHIM) and determination of carrier status in female relatives**

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**Aims:** To analyse the gene encoding the CD40 ligand (CD40L) in 11 Australian patients from 10 unrelated families with the X linked hyper-IgM (XHIM) phenotype.

**Methods:** The CD40L gene was screened for mutations using direct sequencing of exon specific polymerase chain reaction (PCR) products.

**Results:** Ten mutations were identified. Seven of these mutations have been described previously, whereas three new nonsense mutations were identified, namely: E108X (c.322G>T), G167X (c.499G>T), and C218X (c.654C>A). Ten of 15 female family members revealed both a mutated allele and a normal allele, indicating that they were XHIM carriers.

**Conclusion:** The 10 mutations (including the three new ones) identified in this study reflect the heterogeneity of the CD40L gene, and indicate the need for accurate and reliable molecular testing of those patients suspected of XHIM.

**X** linked hyper-IgM syndrome (XHIM; MIM 308230) is a severe primary immunodeficiency caused by mutations in the gene encoding the CD40 ligand (CD40L, also called CD154). CD40L is a surface molecule present on activated T cells, which interacts with CD40 on the surface of B cells to provide an essential signal for B cell proliferation and immunoglobulin class switching. As a consequence of CD40L deficiency, patients with XHIM are characterised by very low amounts or the absence of IgG, IgA, and IgE, with normal to raised serum IgM concentrations, although, more recently, panhypogammaglobulinaemia of IgA, IgG, and IgM was seen in 26% of these patients. This defective production of most isotypes of immunoglobulin renders affected individuals extremely susceptible to recurrent bacterial infections, starting in the 1st year of life.

“The mutations are heterogeneous, with functional deficiency ranging from a complete lack of CD40L protein expression to missense mutations that interfere with its interaction with CD40 on the surface of B cells.”

The gene that encodes CD40L has been mapped to Xq26.3–27.1 and is a member of the tumour necrosis factor α (TNFα) superfamily. It consists of four introns and five exons, encoding a type II membrane glycoprotein of 261 amino acids, with a short intracytoplasmic tail (22 amino acids), a transmembrane region (24 amino acids), and an extracellular TNFα homologous domain (215 amino acids).

To date, over 130 unique CD40L mutations have been identified in patients with XHIM, which are spread throughout the entire length of the gene. The mutations are heterogeneous, with functional deficiency ranging from a complete lack of CD40L protein expression to missense mutations that interfere with its interaction with CD40 on the surface of B cells. They are recorded in a CD40L mutation database (found at http://bioinf.uta.fi/base_root/).

In our study, we report the identification of mutations in the CD40L gene in 11 Australian patients from 10 unrelated families with XHIM phenotype.

**MATERIAL AND METHODS**

**Clinical samples**
Eleven patients with a clinical XHIM phenotype and greatly reduced serum concentrations of IgG and IgA, but normal or increased serum concentrations of IgM, and with a history of an increased susceptibility to infections, were referred to our laboratory by clinicians around Australia for the confirmation of diagnosis. The patients were all male, aged between 1 and 27 years, and were unrelated, with the exception of two individuals who were first cousins (patients 8 and 9; table 1).

Table 1 summarises the patients’ data. First degree female relatives of a proband were also assessed as requested. Thirty healthy individuals were investigated as controls. Informed consent for the CD40L mutational analysis was obtained from the patients or their respective guardians.

**Molecular analysis**
Genomic DNA was obtained from whole blood. All five exons of the CD40L gene, including the exon–intron boundaries, were individually amplified with primers described previously. Sequencing reactions were performed using the dye deoxyterminator cycle sequencing kit (Applied Biosystems, Foster City, California, USA) with an automated ABI373 DNA sequencer. Detected mutations were confirmed by sequencing in the opposite direction from an independent PCR product.

**RESULTS**
Using direct sequence analysis of exon specific PCR products, we were able to detect 10 mutations in 11 patients from 10 unrelated families. Mutations were observed in both coding and non-coding regions of the entire CD40L gene. Seven have been described previously, and three were new mutations, which were not on the CD40L database or in the literature. Table 1 summarises the mutations for individual patients.

No correlation was seen between the clinical phenotype and the site of the mutation, which is in agreement with the previously published data.

We also tested the XHIM carrier status in female members of seven families, after identifying mutations in the patients (table 1). Healthy female carriers were identified by direct determination of carrier status in female relatives.

**Abbreviations:** XHIM, X linked hyper-IgM; CD40L, CD40 ligand; TNFα, tumour necrosis factor α; PCR, polymerase chain reaction
CD40L mutations in 11 Australian patients

<table>
<thead>
<tr>
<th>Patient no/age at diagnosis</th>
<th>CD40L expression</th>
<th>Exon/intron</th>
<th>Nucleotide change</th>
<th>AA change</th>
<th>Effect</th>
<th>CD40L database</th>
<th>Carrier status</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/1 year Absent</td>
<td>Exon 3</td>
<td>c.322G&gt;T*</td>
<td>E108X</td>
<td>Not reported</td>
<td>M+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2/1 year Absent</td>
<td>Intron 3</td>
<td>c.346+2T&gt;C</td>
<td>A123E</td>
<td>Reported*</td>
<td>M+, GM+, S+, S--</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3/2 years Absent</td>
<td>Exon 4</td>
<td>c.368C&gt;A</td>
<td>AA change in TNFα domain</td>
<td>Reported*</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4/12 years Absent</td>
<td>Intron 4</td>
<td>c.409+1G&gt;A</td>
<td>AA change in TNFα domain</td>
<td>Reported*</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5/6 years Absent</td>
<td>Exon 5</td>
<td>c.420G&gt;T</td>
<td>WI40C</td>
<td>Reported*</td>
<td>M+, GM+, S+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6/20 years Present</td>
<td>Exon 5</td>
<td>c.431G&gt;A</td>
<td>AA change in TNFα domain</td>
<td>Reported*</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7/27 years Absent</td>
<td>Exon 5</td>
<td>c.464T&gt;C</td>
<td>L155P</td>
<td>Reported*</td>
<td>M --, S --, S --</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8/13 years, cousin of 9</td>
<td>Exon 5</td>
<td>c.499G&gt;T*</td>
<td>G167X</td>
<td>Not reported</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9/14 years, cousin of 8</td>
<td>Exon 5</td>
<td>c.499G&gt;T*</td>
<td>G167X</td>
<td>Not reported</td>
<td>M+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10/1 year Absent</td>
<td>Exon 5</td>
<td>c.521A&gt;G</td>
<td>Q174R</td>
<td>Not reported</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11/5 years Absent</td>
<td>Exon 5</td>
<td>c.654C&gt;A*</td>
<td>C218X</td>
<td>Not reported</td>
<td>S--</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The mutation nomenclature follows the guidelines of den Dunnen and Antonarakis and HUGO (www.hgvs.org/mutnomen/). The numbering of nucleotide and amino acid positions refers to the cDNA sequence (GeneBank accession number L07414.1), where the A of the ATG translation initiation start site represents nucleotide +1. The reported age is the age of the patient when flow cytometry and molecular analysis were performed.

*New mutation.
A, aunt; AA, amino acid; FC, flow cytometry; GM, grandmother; M, mother; ND, not done; S, sister.

Take home messages

- We identified 10 mutations (including three new ones) in the CD40L gene in 11 patients with X linked hypogammaglobulinaemia (XHIM), reflecting the heterogeneity of this gene.
- Accurate and reliable molecular testing of patients with suspected XHIM is required.
- Such testing also provides valuable information for counselling female family members at risk for being XHIM carriers, in addition to offering the possibility of prenatal testing.

DISCUSSION

Eleven patients across Australia with a clinical XHIM phenotype were analysed using direct sequencing and were found to carry mutations in the CD40L gene. It is notable that six of 10 mutations, two of which are new, were found in exon 5 (patients 8, 9, and 11). It has been found previously that most of the CD40L gene mutations are located within exon 5, a region with the greatest homology to TNFα.5 To date, the CD40L database lists eight families with mutations occurring at this codon. Four families in the CD40L database have a WI40X substitution—two families have the WI40R substitution and one family each has the WI40G or WI40C substitution; the WI40C substitution was also found in our present study (table 1, patient 5).

“It is notable that six of 10 mutations, two of which are new, were found in exon 5”

The accurate diagnosis of XHIM is facilitated by reliable molecular testing, not only in patients with the typical phenotype or a positive family history, but also in previously undefined hypogammaglobulinaemia. It has been reported that men previously diagnosed with common variable immunodeficiency were subsequently diagnosed with XHIM. Although these patients may not develop the life threatening complications of XHIM, other affected family members could have a more severe phenotype.14 Furthermore, testing provides valuable information for counselling female family members at risk for being XHIM carriers, in addition to offering the possibility of prenatal testing.

This is the first Australian study to describe mutations in the CD40L gene in patients with XHIM. Three mutations identified in our study were new, not being present in the CD40L database or having been reported in the literature, and three had previously been described as playing an important role in CD40L and CD40 interactions.

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