Atypical haemochromatosis: phenotypic spectrum and \( \beta_2 \)-microglobulin candidate gene analysis

Ann P Walker, Daniel F Wallace, Jason Partridge, Adrian B Bomford, James S Dooley

Abstract

\( \beta_2 \)-microglobulin was investigated in atypical haemochromatosis patients not homozygous for the C282Y mutation of HFE (OMIM *235200), because the HFE protein binds \( \beta_2 \)-microglobulin, and in mice \( \beta_2 \)-microglobulin gene knockout causes hepatic iron overload. Six unrelated patients with atypical haemochromatosis were studied. Five patients had normal HFE coding sequence and the sixth was heterozygous for C282Y. We show that the spectrum of atypical haemochromatosis includes two distinct familial forms: juvenile haemochromatosis (OMIM *602390) and a novel form of familial iron overload, with apparently autosomal dominant inheritance, predominant Kupffer cell siderosis, and possible minimal dyserythropoiesis on bone marrow examination. Serial serum \( \beta_2 \)-microglobulin estimation showed normal levels in all patients. Southern blot analysis showed normal \( \beta_2 \)-microglobulin gene structure, excluding major gene rearrangement. Several corrections to the published \( \beta_2 \)-microglobulin sequence were identified, but all six patients had normal \( \beta_2 \)-microglobulin sequence. Western blot analysis of serum showed \( \beta_2 \)-microglobulin protein of normal size. In conclusion, we found no evidence to implicate \( \beta_2 \)-microglobulin mutation in atypical haemochromatosis. Two forms of familial iron overload appear unrelated to either HFE or \( \beta_2 \)-microglobulin. Linkage studies are required to identify the genes involved, which may encode novel proteins crucial to the regulation of iron metabolism. Identification of these loci will aid the diagnosis, counselling, and treatment of iron overload disorders.

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Genetic haemochromatosis (GH) is an autosomal recessive disorder of iron metabolism which affects 1 in 300 people in the UK. In GH, iron absorption from the upper small intestine continues, despite increasing body iron overload. The iron loading, if recognised, results in tissue damage, affecting the liver, pancreas, joints, heart, and anterior pituitary. Treatment is initially by repeated venesection to mobilise and remove iron stores, followed by periodic maintenance venesection. Early diagnosis and treatment restores normal life expectancy.

A candidate gene (HFE; originally named “HLA-H”) for GH was isolated and shown to have homology to the human leucocyte antigen (HLA) family of proteins of the major histocompatibility complex. In different samples of GH patients of European origin, homozygosity for the C282Y mutation has ranged from 64 to 100%. A second mutation of HFE was reported, H63D. The third missense variant reported, S65C, is of uncertain significance in the development of iron overload. However, a small percentage of GH patients are negative for both the C282Y and H63D mutations of HFE. Evidence of locus heterogeneity was supported by the demonstration of normal HFE coding sequence.

This group of iron overloaded patients negative for C282Y includes some with a distinct presentation, juvenile haemochromatosis (JH). JH typically has onset before the age of 20–30 years, sometimes in the first few years of life. JH has a high frequency of cardiomyopathy and hypogonadism and a male:female sex ratio of 1:1 was reported, compared to 9:1 in an adult GH group. Menstrual iron loss was proposed to explain the reduced penetrance of disease in adult females. In JH, normal HFE coding sequence and normal chromosomal organisation of the HFE region have been reported (unpublished observations).

Segregation of HFE region markers in well documented Italian consanguineous families has shown that JH is not linked to the HFE region on chromosome 6. As the \( \beta_2 \)-microglobulin gene is located on chromosome 15, it could therefore be a potential candidate locus for JH.

Functional evidence also suggests \( \beta_2 \)-microglobulin as a potential candidate locus for atypical iron overload with normal HFE sequence. A protein dimer of \( \beta_2 \)-microglobulin and HFE is proposed to regulate iron absorption. Mutation of either subunit could thus influence this regulation. Furthermore, \( \beta_2 \)-microglobulin gene knockout in mice results in hepatocellular siderosis, exacerbated by a high iron diet, as in human GH. Liver tumours and hyperglycaemia may develop in the \( \beta_2 \)-microglobulin knockout mouse, similar to the development of hepatocellular carcinoma and diabetes mellitus in GH. There is also a lack of CD4+ from the CD8+ T lymphocytes in the \( \beta_2 \)-microglobulin knockout mouse. These cells are also deficient in the human disorder, where the CD4+/CD8+ ratio was found to be positively correlated with the degree of iron overload.
Patient 5 presented with joint pains and high serum transferrin saturation and ferritin concentration, but had borderline iron overload (table 1). He is currently undergoing maintenance venesection every three months and is in good health. Patient 6 appeared to have “classical” GH. He was treated with venesection, insulin, and testosterone. Following removal of excess iron, he is receiving maintenance venesection every 10 weeks. He has arthropathy, particularly affecting the first and second metacarpophalangeal joints of the right hand, unresponsive to non-steroidal anti-inflammatory agents.

With respect to HFE analysis, patient 6 was heterozygous for C282Y. The entire HFE coding sequence (223-1265 of GenBank accession number U60319), 149 bp of the 5' untranslated region (UTR; 73-222 of U60319), and 53 bp of the 3' UTR (1266-1319 of U60319) were otherwise normal in all six patients (DFW, unpublished observations). Patients 1, 2, 5, and 6 had normal chromosomal arrangement of the HFE region.11 Where available, liver biopsy was examined for histology and grade of siderosis, and the hepatic iron index was determined.22 Serum iron, total iron binding capacity, transferrin saturation, ferritin, and $\beta_2$-microglobulin levels were measured; normal ranges are given in table 1. Healthy controls for DNA and protein analyses had normal serum iron indices.

SOUTHERN BLOT ANALYSIS OF $\beta_2$-MICROGLOBULIN GENE STRUCTURE

Genomic DNA from the six patients, the brother and father of patient 3 (both affected), and six normal controls was digested with HinmIII, TaqI, and PvuI (New England Biolabs) for preparation of Southern blots and hybridisation. $\beta_2$-Microglobulin PCR probes were as

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Table 1  Clinical details of patients with atypical haemochromatosis

<table>
<thead>
<tr>
<th>Patient</th>
<th>Genotype*</th>
<th>FH‡</th>
<th>Age at onset (y)</th>
<th>Clinical features</th>
<th>Liver histology/grade hepatocyte siderosis/Kupffer siderosis HH</th>
<th>Serum Fe/TIBC* (µmol/l) (11-36/53-85)**</th>
<th>Transferrin saturation (%) 20-40**</th>
<th>Serum ferritin (µg/l) (2-10)</th>
<th>$\beta_2$-microglobulin (mg/dl)/date</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (M)</td>
<td>1</td>
<td>N††</td>
<td>24</td>
<td>Cardiomyopathy,</td>
<td>14</td>
<td>No biopsy††</td>
<td>4579</td>
<td>2.7/080497</td>
<td>1.3/190598</td>
</tr>
<tr>
<td>2 (M)</td>
<td>1</td>
<td>Y</td>
<td>16§§</td>
<td>FH cardiomyopathy</td>
<td>10</td>
<td>F4/K—</td>
<td>14/45</td>
<td>1/210197</td>
<td>1/210197</td>
</tr>
<tr>
<td>3 (M)</td>
<td>1</td>
<td>Y</td>
<td>36</td>
<td>Apparent autosomal dominant inheritance</td>
<td>12</td>
<td>Minimal F2/K</td>
<td>42/26</td>
<td>2082/1</td>
<td>2082/1</td>
</tr>
<tr>
<td>4 (F)</td>
<td>1</td>
<td>Y</td>
<td>34</td>
<td>Apparent aut dom iner, 7 minimal dyserythropoiesis</td>
<td>8</td>
<td>No F2/K-predominant s</td>
<td></td>
<td>1/210197</td>
<td>1.2/070197</td>
</tr>
<tr>
<td>5 (M)</td>
<td>1</td>
<td>N</td>
<td>66</td>
<td>Arthralgia, colonic polyposis</td>
<td>5</td>
<td>K—III=1.7</td>
<td>14/57</td>
<td>1/210197</td>
<td>1.2/070197</td>
</tr>
<tr>
<td>6 (M)</td>
<td>2</td>
<td>N</td>
<td>38</td>
<td>Diabetes mellitus, hypogonadism, arthropathy</td>
<td>14</td>
<td>Minimal F2/3/—</td>
<td>14/57</td>
<td>1.3/190598</td>
<td>1.3/190598</td>
</tr>
</tbody>
</table>

*Genotype 1 is negative for the H63D, S65C, and C282Y mutations; genotype 2 is negative for H63D and S65C and heterozygous for C282Y; the remainder of the HFE coding sequence was normal in all cases. ‡FH denotes family history. Iron removed by venesection (and desferrioxamine therapy in patient 1). F, female; M, male; NK, not known.


One study of $\beta_2$-microglobulin in seven patients not homozygous for the C282Y mutation of HFE has reported normal $\beta_2$-microglobulin coding sequence.21 This study did not investigate gene structure and protein expression. In the present report, we describe the phenotypic spectrum of six patients with atypical haemochromatosis, including a novel phenotype of familial iron overload, and investigate the $\beta_2$-microglobulin gene structure, sequence, and protein expression.

Patients and methods

PATIENTS

All six patients had a hepatic iron index of greater than 1.9 µmol iron/g dry weight/year22 or $\geq$5 g of iron removed by phlebotomy, in the absence of either a diagnosis of haematological disease, iron supplementation, or other known cause of iron loading (table 1). Hepatitis B and C serology was negative. Serum copper and caeruloplasmin were normal. Patients 1 and 2 had JH.3 Following removal of excess iron, they are treated with maintenance venesection, have no cardiac problems, and are in good health. Patient 1 is also treated with testosterone. Patients 3 and 4 had raised serum ferritin but normal or only mildly raised transferrin, and iron in Kupffer cells as well as hepatocytes (table 1). Both had a family history of disease. Patient 3 had one brother, also affected; their father and two of his six surviving sibs were affected. The HLA serotypes for patient 3 and his affected brother were different: A28, −; B14, 44(12); Bw4, Bw6, and A11, 28; B44(12), B22; Bw4, Bw6, respectively. Patient 4 had one sister, also affected; in total seven family members were affected in three generations, with apparently autosomal dominant inheritance. Two aunts of patient 4 had identical HLA serotypes (HLA A2, 32; B15, −; DR11, −; DQ7, −). However, one had a hepatic iron index of 2.5 and a ferritin of 1345 µg/l, and the other was apparently unaffected, with a serum ferritin of 33 µg/l. Bone marrow examinations of patient 4 and her sister were not diagnostic for a haematological condition, although both indicated the possibility of minimal dyserythropoiesis. Patients 3 and 4 had no clinical evidence of an associated metabolic syndrome (obesity, hyperlipidaemia, abnormal glucose metabolism, or hypertension).23 Following removal of excess iron, patients 3 and 4 are currently in good health.


Figure 1  Exclusion of $\beta$-microglobulin gene rearrangement. The map of control genomic DNA shows the exons as black boxes, and restriction enzyme sites as vertical lines: H, HindIII; P, PstI; T, TaqI. The scale bar indicates 1 kb. All six patients and the brother and father of patient 3 (both affected) had a normal gene map, excluding major intragenic $\beta$-microglobulin rearrangement as the cause of their iron overload.

Described for direct sequencing, with an additional exon 4 probe amplified using primers E4F, CCTTTAAAAGGCTGCTTACAC, E4R, GTGAGATATAAGAGATTACAC (666 bp).

**DIRECT SEQUENCE ANALYSIS OF THE $\beta$-MICROGLOBULIN GENE**

PCR primers were designed from the human $\beta$-microglobulin genomic sequences (GenBank accession numbers M17986, M17987): exon 1, E1F, GGCCTTTGTTCCTGATTGGCTGGG, E1R, GCGAGAGAGCATCAGCGAC (237 bp); exon 2, E2F, GATATATATATGATGATGGG (486 bp); E3F, GAAACAGCAGCCTATTCTGCC, E3R, TAGAGCTGTCTATAATAGTCC (212 bp). The reverse primer of each pair was 5' biotinylated. The “hot start” PCR reactions (212 bp). The reverse primer of each pair was 5' biotinylated. The “hot start” PCR reactions (1.5 mmol/l MgCl$_2$) used annealing temperatures of 55°C (E1), 50°C (E2, E4), or 55°C (E3). Direct sequence analysis was performed as previously described.$^{24}$

**WESTERN BLOT ANALYSIS OF $\beta$-MICROGLOBULIN**

Serum samples (1 µl) and $\beta$-microglobulin standard (10 ng, Chemicon International) were analysed. $\beta$-microglobulin was visualised with the amplified alkaline phosphatase goat antirabbit Immun-Blot assay kit (BioRad), using a 1/500 dilution of a polyclonal rabbit antihuman $\beta$-microglobulin (Dako) as first antibody.

**Results**

**SERUM $\beta$-MICROGLOBULIN**

Serum was available for $\beta$-microglobulin estimation in five of the six patients (table 1). Repeated estimation showed normal $\beta$-microglobulin levels, although these tended to be clustered around the lower limit of normal (1.3-2.4 mg/l). Patient 6 had a serum concentration of $\beta$-microglobulin slightly above the normal range on two occasions (2.7, 2.6 mg/l), although a third sample gave a normal result (1.4 mg/l).

**SOUTHERN BLOT ANALYSIS OF $\beta$-MICROGLOBULIN GENE STRUCTURE**

Hybridisation of probes for exons 1-4, corresponding to the entire coding region of $\beta$-microglobulin, to restriction digests of leucocyte DNA allowed the construction of a map of genomic DNA spanning the $\beta$-microglobulin gene (fig 1). The maximum size of the human genomic $\beta$-microglobulin locus is 9 kb. No polymorphism of the $\beta$-microglobulin gene was detected in the six controls, or the six patients, or the brother and father of patient 3 (both affected).

**DIRECT SEQUENCE ANALYSIS OF THE $\beta$-MICROGLOBULIN GENE**

Several differences from the published $\beta$-microglobulin sequence were observed, but these were detected uniformly in controls as well as in all patients. All samples were sequenced directly from two independent PCR reactions on both strands. In the exon 1 PCR fragment, the 5' untranslated region sequence around the TATA box and transcriptional start was as published (GenBank M17986), except that G837 was not present.$^{25}$ The sequence encoding the last amino acid of the signal peptide was found to be GCT in all subjects, in place of the published GGC (nucleotides 940-942 of M17986).$^{25}$ This would predict alanine rather than glycine (fig 2). We did not observe the previously reported change involving only nucleotide 942 in any of the patients or controls.$^{25}$ Further sequence differences were observed in intron 1: we detected an additional “C” after nucleotide 983 of M17986 and an additional 21 bp (GCTCTGACCCCTCTTGTGGGCC) after nucleotide 989 of M17986. Also in intron 1, we observed a T rather than a C at nucleotide 107 of GenBank M17987. In exon 2, at nucleotide 210 of M17987, a C was observed rather than an A, predicting a proline rather than a glutamine residue. We did not

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**Figure 2**  $\beta$-microglobulin sequence of exon 1-intron 1 detected in all patients and controls. The nucleotide sequence detected in the present report and that of GenBank M17986 are shown; the amino acid one letter code is given in lower case for the signal peptide and in upper case for the mature protein. The splice site is indicated by the large Y. The positions of nucleotides not reported in M17986 are indicated by a hyphen, to maintain collinearity of the two sequences. Three differences between the sequences are highlighted in bold and underlined in the sequence of the present report.
observe a similar DNA change previously reported at nucleotide 200. Apart from the above homozygous sequence changes seen in all subjects, the six patients had normal \( \beta \)-microglobulin coding sequence, normal 5'-untranslated sequence spanning the TATA box, and normal splice sites.

**WESTERN BLOTTING**

\( \beta \)-microglobulin was detected as a 12 kDa band in the protein standard and in sera from normal controls (fig 3). In serum samples, two additional bands were also detected at approximately 40 and 52 kDa, possibly representing cross reaction of the polyclonal antiserum against other serum proteins, or detection of residual undenatured \( \beta \)-microglobulin protein complexes, or both. \( \beta \)-microglobulin is detected as a 12 kDa band.

**Discussion**

The serum \( \beta \)-microglobulin levels were mildly abnormal in several of the atypical haemochromatosis patients, but these initially suggestive results were not reproduced upon serial serum assay. Raised serum \( \beta \)-microglobulin levels, as observed in patient 6, can result from reduced renal clearance. However, at the times of raised \( \beta \)-microglobulin levels, indicators of renal function (creatinine and urea) were normal. Stimulation of the immune response can also cause raised serum \( \beta \)-microglobulin levels, but the white cell count was normal. The cause of the observed minor fluctuations of \( \beta \)-microglobulin levels within and around the normal range is not known.

Southern blot analysis allowed the construction of a genomic DNA map of the \( \beta \)-microglobulin gene in control and patient DNA. This showed a good agreement with partial data from analysis of cloned DNA (GenBank M17986; M17987). The finding of a normal gene map for all patients has excluded intragenic rearrangement of \( \beta \)-microglobulin as the causative mutation for their iron overload. Although allelic variation of the \( \beta \)-microglobulin gene has been described in the mouse, there have been no reports of germ-line mutations in man. However, somatic mutation of the \( \beta \)-microglobulin gene has been reported in some human colonic tumours.28 This is predicted to cause defective MHC class I antigen presentation, allowing the tumour cells to escape immune recognition by cytotoxic T cells. In the present study, sequence analysis of the \( \beta \)-microglobulin gene did show several differences from published sequences, two of which would influence the predicted protein sequence. However, these differences were observed in all patients and controls and were therefore not related to iron overload or, indeed, to the development of colonic polyps in patient 5. It is nevertheless important that these corrections are considered when preparing native \( \beta \)-microglobulin constructs for protein expression and functional studies.

Western blot analysis of serum showed \( \beta \)-microglobulin protein of normal size. This excludes the possibility of non-coding mutation of a regulatory sequence affecting splicing or gene expression. It remains formally possible that post-translational modification or tissue specific effects may be involved, or that \( \beta \)-microglobulin may contribute to iron overload in a group of patients that has not yet been studied.

The aetiology of iron overload in the patients with neither family history nor apparent contributing factors remains unclear. In patient 5, dietetic iron supplementation, alcohol, and hepatitis B and C serology were negative. Copper and caeruloplasmin were normal, arguing against iron accumulation owing to acaeruloplasminaemia.23 24 In the absence of any identified mutations of HFE, involvement of this locus is unlikely. For patient 6 with a classical phenotype of genetic haemochromatosis but only heterozygous for C282Y, iron overload could potentially result from either compound heterozygosity with a rare non-coding mutation of HFE, or from the combined effects of heterozygosity for C282Y together with functional polymorphisms of other genes influencing iron metabolism, or unidentified environmental factors.

Two different forms of familial atypical haemochromatosis appear to be associated with normal HFE and \( \beta \)-microglobulin genes. The first of these is JH (patients 1 and 2). In consanguineous Italian families, JH has been shown to be unlinked to the HFE region, and thus distinct from GH.10 The second (patients 3 and 4) is characterised by apparently autosomal dominant inheritance, relatively early onset, high serum ferritin often with normal or mildly raised transferrin saturation, predominate Kupffer cell siderosis, and absence of metabolic disease. Familial iron overload with possible autosomal dominant inheritance has been reported once previously, in an extended Melanesian kindred.25 Genetic analysis of
families with atypical forms of iron overload may identify further genes crucial to the regulation and metabolism of iron. These studies may ultimately yield further important clues to elucidate the regulation of iron metabolism and to aid the diagnosis, counselling, and treatment of iron overload disorders.

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