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Genetic studies into inherited and sporadic hemolytic uremic syndrome

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Genetic studies into inherited and sporadic hemolytic uremic syndrome. Hemolytic uremic syndrome (HUS) in adults carries a high morbidity and mortality, and its cause remains unknown despite many theories. Although familial HUS is rare, it affords a unique opportunity to elucidate underlying mechanisms that may have relevance to acquired HUS. We have undertaken a genetic linkage study based on a candidate gene approach. A common area bounded by the markers D1S212 and D1S306, a distance of 26 cM located at 1q32 segregated with the disease (Z max 3.94). We demonstrate that the gene for factor H lies within the region. Subsequent mutation analysis of the factor H gene has revealed two mutations in patients with HUS. In an individual with the sporadic/relapsing form of the disease we have found a mutation comprising a deletion, subsequent frame shift and premature stop codon leading to half normal levels of serum factor H. In one of the three families there is a point mutation in exon 20 causing an arginine to glycine change, which is likely to alter structure and hence function of the factor H protein. Factor H is a major plasma protein that plays a critical regulatory role in the alternative pathway of complement activation. In light of these findings and previous reports of HUS in patients with factor H deficiency, we postulate that abnormalities of factor H may be involved in the etiology of HUS.

Hemolytic uremic syndrome (HUS) is characterized by the triad of the Coomb's test-negative microangiopathic hemolytic anemia, thrombocytopenia and acute renal failure. It exists in a diarrheal-associated epidemic form (classified as d+ HUS), and nondiarrheal (d− HUS) sporadic and familial forms.

The d+ HUS form usually affects young children and they recover spontaneously. Verocytotoxin is undoubtedly a primary factor in the pathogenesis of the disease, but it is unclear why only a percentage of children with the diarrhea (9 to 30% in the face of *E. coli* 1057:H7 [1]) develop the syndrome.

[1] See *Editorial* by Remuzzi, p 1085

Key words: hemolytic uremic syndrome, complement factor H, inherited renal disease, genetic linkage, human chromosome 1.

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Sporadic, noninfective HUS usually affects adults and generally carries a worse prognosis. Predisposing factors include the oral contraceptive, cyclosporine, quinine, pregnancy, tumors and connective tissue disorders, but it is often idiopathic.

The familial form of the disease is rare but affords a unique opportunity to elucidate underlying mechanisms that may have relevance to acquired and epidemic HUS. The majority of families reported are recessive, but dominant inheritance has also been reported [2, 3].

Since the first description by Gasser et al in 1955 [4], many theories have been suggested for the cause of nondiarrheal HUS. These have included altered prostacyclin metabolism [5], unusual von Willebrand factor multimers [6], increased levels of plasminogen-activator inhibitor 1 [7], the presence of platelet activating and agglutinating factors [8, 9], and increased complement activity [10–12]. Because the disease recurs in renal allografts and responds in some individuals to plasma exchange it is probable that a plasma factor is in part responsible. In this study we present compelling new evidence from three families with inherited HUS and one individual with sporadic HUS that the primary abnormality is overactivity of the alternative complement pathway.

Family pedigrees and clinical histories

Hemolytic uremic syndrome was defined in our families by the presence of acute renal failure and at least two of the following: characteristic histopathological findings on biopsy or at post mortem, acute anemia, thrombocytopenia, and fragmented red cells on blood film. Several individuals in each family exhibited all five features.

Families. Family 1 was first described in 1975 [13], and since then three further family members have been affected (Fig. 1). In addition to the classical hematological and histological features of HUS, severe hypertension and recurrence in renal allografts has occurred.

Family 2a has already been described in part [14]. Two other families with familial HUS were found to live within a few miles, and we have established a common lineage for one dating back to the start of the last century. The other, family 2b, probably has the same lineage but we have been unable to definitively establish this premise (Fig. 1). Clinical features are similar to family 1, including

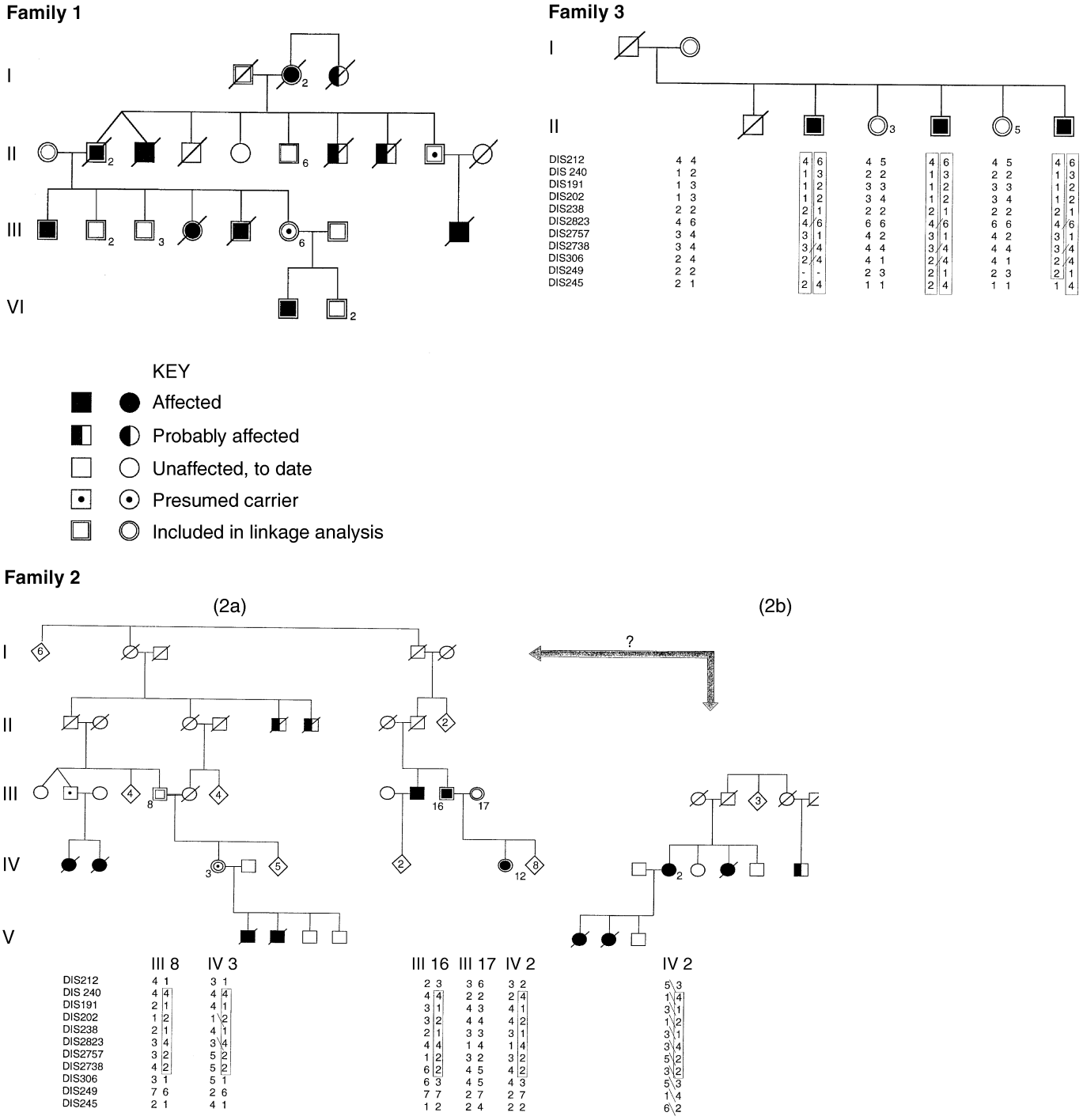

Fig. 1. Pedigrees of family 1, 2a and 2b, and 3. It is likely that families 2a and 2b are related to each other. The microsatellite results for eleven chromosome 1 loci are aligned beneath the pedigree symbols for the five individuals from family 2a included in the linkage analysis and for IV 2 of family 2b who was not included in the linkage analysis. A common haplotype extending from D1S240 to D1S2738 is boxed. The symbol/between alleles indicates that it is not clear which is the maternal allele and which is the paternal allele. In family 3 the affected individuals are haploidentical for the markers extending from D1S212 to D1S249.

some individuals with hypertension requiring bilateral nephrectomy. However, two family members have renal allografts functioning well after seven and eight years.

Family 3 (Fig. 1) has been described in detail [15]. Three male siblings were affected at ages 27, 31 and 35, and all three have had successful renal transplants with no recurrence of disease.

Sporadic case. A male with sporadic/relapsing HUS presented at the age of 36 with a one week history of malaise, nausea, loin

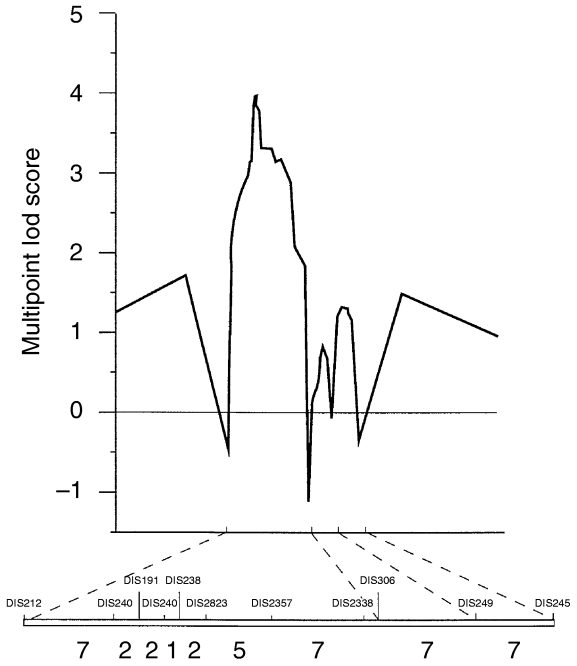

Fig. 2. Multipoint linkage map of the results from the three families. Chromosome 1q loci and the sex averaged distances (cM) between them are shown beneath

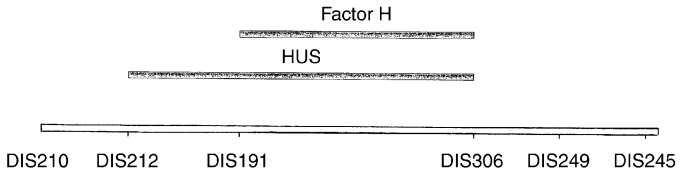

Fig. 3. This Figure shows the position of the gene for factor H relative to the region of chromosome 1 segregating with HUS in these three families.

ache and anorexia. He was profoundly anemic (Hb 6.9 g/dl), thombocytopenic (79×10^9/liter), hypertensive, and in advanced acute renal failure. Renal biopsy revealed a thrombotic microangiopathy consistent with HUS, and immunohistochemistry showed deposition of C3 in vessel walls. C3 levels were decreased (0.59 g/liter, NR 0.65 to 1.7) with normal levels of C4, consistent with activation of the alternative pathway. Despite treatment with a short course of plasma exchange and angiotensin II converting enzyme inhibitors he failed to regain renal function.

Fifteen months post-presentation he received a cadaveric renal allograft, under cover of anti-thymocyte globulin and subsequently cyclosporine based immunosuppression. Despite a good early urine output the graft function deteriorated acutely on day 24 post-transplantation, and allograft biopsy showed recurrent HUS. There was an improvement with plasma exchange, but this was not sustained and the graft eventually failed, requiring nephrectomy three months later. He is currently being treated with hospital based hemodialysis.

His mother has no relevant medical history and his father is deceased (CVA).

METHODS

The study was approved by the Joint Ethics Committee, University of Newcastle upon Tyne and Newcastle and North Tyneside Health Authority.

DNA extraction

Genomic DNA was isolated from whole blood using the nucleon 2 DNA extraction kit (Scotlab Bioscience, UK). Archival tissue was obtained from paraffin-embedded microscope slides and tissue blocks with the consent of the nearest family members. Twenty micromolar sections of tissue blocks and scrapings from microscope slides were deparaffinized with xylene and washed in ethanol. These were then digested overnight with proteinase K. The proteinase K was then heat inactivated at 95°C for 10 minutes, the sample centrifuged, and aliquots of supernatant used directly for polymerase chain reaction (PCR) amplification.

Microsatellite polymorphism genotyping

Microsatellite polymorphism primers were obtained from the 'Human Screening Panel' (Research Genetics) or synthesized in the University Facility for Molecular Biology, University of Newcastle upon Tyne. Microsatellite polymorphisms flanking or within the genes for prostaglandin synthase 2 (PTGS 2 or COX 2, chromosome 1q25.2–25.3) the inducible gene involved in prostacyclin synthesis, von Willebrands Factor (vWF, chromosome 12p 13.2–13.3), tissue factor (TF, chromosome 1p21 to 22), plasminogen activator inhibitor 1 (PAI 1, chromosome 7q 22.1–22.3), the selectin gene cluster (SEL, chromosome 1q22 to 25) and complement factor H (HF, chromosome 1q32) were studied in a candidate gene approach to linkage analysis in the 3 families.

100 ng of genomic DNA template (previously aliquoted and dried at 65°C onto 96 well microtiter plates) was amplified in total volumes of 10 μl containing 1.3 pmol ^{32}P end-labeled 3′ primer (0.3 μCi gamma ^{32}P ATP (Amersham) and T4 polynucleotide kinase/buffer (New England Biolabs)), 1.3 pmol 5′ primer, 0.15 u *Taq* polymerase and 1.5 mM Mg^{2+} buffer (Promega) and 2 pmol dNTPs overlaid with 1 drop of mineral oil. The standard programme profile was denaturation 94°C for four minutes, followed by 30 cycles of 94°C denaturation -one minute, 55°C annealing - one minute, 72°C extension- one minute and a final extension step of 72°C for 10 minutes. The standard annealing temperature was 55°C, although a range of temperatures from 50 to 60°C were required to optimize the reaction. PCR of template obtained from paraffin embedded tissue usually required either more PCR cycles (40 to 60) or a double PCR technique using an aliquot of first round product. PCR fragments were resolved on a 6% denaturing polyacrylamide gel.

Linkage analysis

Those included in the linkage calculation are indicated in Figure 1, including in family 1 individuals designated I-2 and II-2, in whom genotype could be inferred although DNA was not available. Multipoint linkage analysis was carried out using the FASTLINK program of the LINKAGE package [16].

For the purposes of the analysis HUS was assumed to be inherited as an autosomal dominant trait with incomplete penetrance, and a gene frequency in the general population of 0.0001.

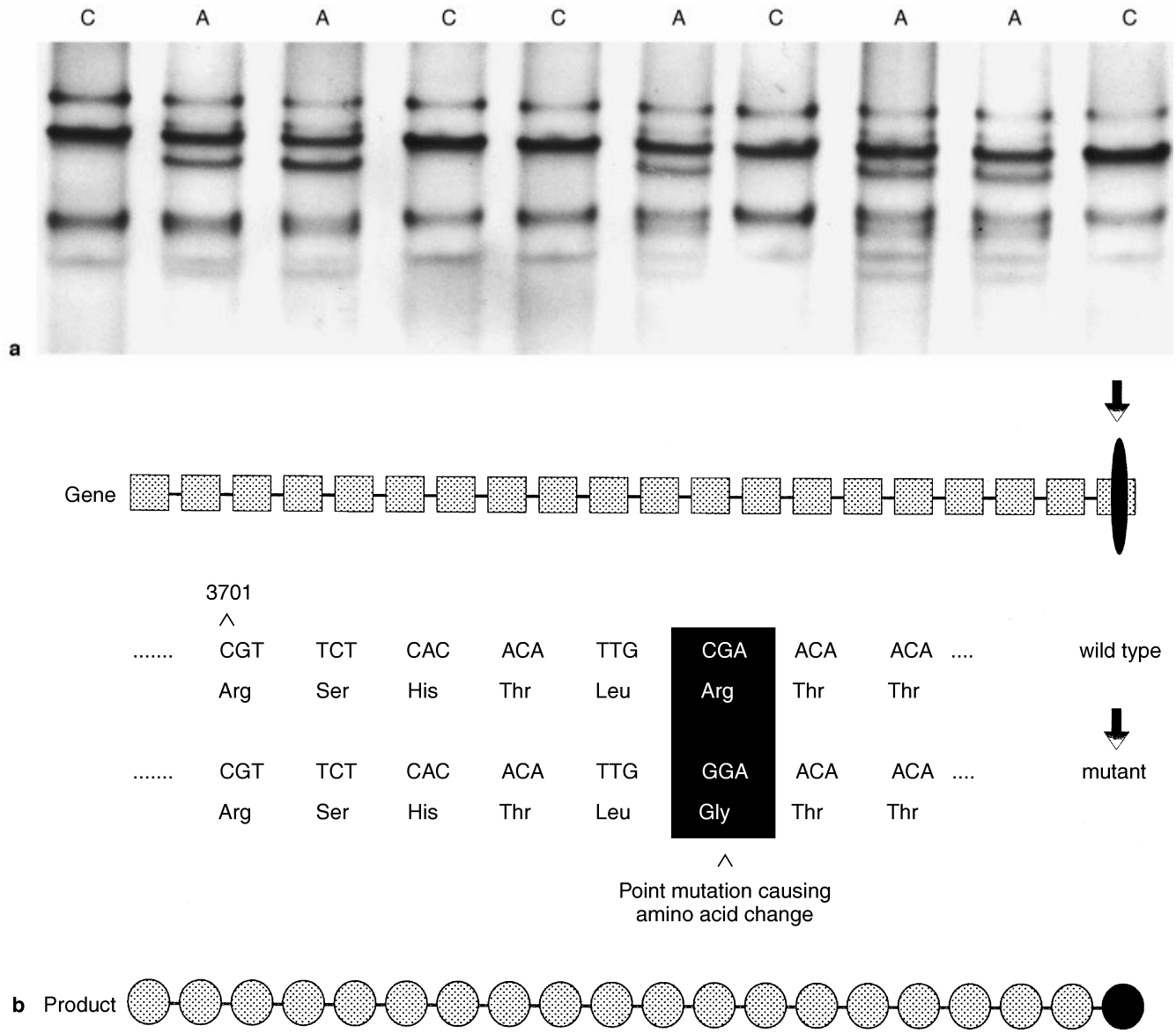

Fig. 4. (*a*) **SSCP (single stranded conformational polymorphism) appearance of exon 20 amplicon in affected individuals of family 2, and controls (C).** Note the band shifts representing a mutation in the factor H gene. (*b*) Part of exon 20 codon sequence (numbered according to [32]), demonstrating the mutation in affected members of families 2a and 2b, with corresponding amino acid product, and mutated polypeptide.

Family members who were asymptomatic at the time of analysis (family 1 - II-6, III-2 and III-3, family 3 - II-3 and II-5) were given an age related liability score based on age at onset in affected members in family 1. Such an assumption was justified in the absence of any obvious peaks in the age of patients at onset of disease. The parents in family 3 (I-1 and I-2) were assigned liability scores of 0.5.

As we were unable to confirm the relationship of family 2b to family 2a, it was not included in the linkage analysis.

mRNA Extraction and cDNA Synthesis

Messenger RNA was extracted from peripheral blood leukocytes (Quickprep microRNA purification kit - Pharmacia Biotech). Reverse transcription was performed on oligo (dT) pooled RNA (Reverse transcription system- Promega) in a total volume of 40 μl for one hour, as described. Product was heated to 95°C for five minutes followed by incubation at 0°C for five minutes to inactivate the reverse transcriptase.

RT-PCR and SSCP (single strand conformational polymorphism) analysis of Factor H polymorphisms

Sections of the HF gene were amplified from 2 μl of cDNA product in a total volume of 100 μl containing 100 pmol each of forward and reverse primers (sequences available on request), 20 pmol dNTPs and 2.5 u *Taq* polymerase and 1.5 mM Mg^{2+} buffer (Promega). Reaction conditions consisted of four minutes denaturation, 30 cycles of one minute denaturation, one minute

annealing and one minute extension (annealing temperature as listed) and a final 10 minutes extension step. A 'touchdown' PCR technique was used to reduce spurious priming. Individual amplicons were amplified via further rounds of PCR using nested primers and 2 μl of the first round product.

SSCP was performed using 4 μl of final product, added to 6 μl of formamide loading buffer and denatured for three minutes before cooling on ice. This final mixture was electrophoresed in a 1X Hydrolink™ gel (J. T. Baker) with 2.5–5.0% glycerol at 10 to 25 mA for 18 to 24 hours. Gels were then silver stained.

We have previously discovered a novel polymorphism in the gene for factor H, located in exon 5 [17]. This was studied by SSCP analysis in the 3 families. Intraexonic primers for exons 1 and 20 were designed to facilitate screening of 60 healthy volunteers using genomic DNA as template.

Sequencing

PCR products containing an aberrant SSCP band were subcloned into pTAg plasmid (Ligator, Ingenius) and DNA was extracted using a commercial kit (Wizard minipreps, Promega). Using the same primers and conditions, PCR and SSCP were repeated alongside parent PCR product to ensure integrity of inserts. 3 separate clones containing inserts of the aberrant band were sequenced on an ABI Prism 377 DNA sequencer using dye-labeled M13 forward and reverse primers.

Complement studies. C3 and C4 were measured by nephelometry using a Beckman Array 360 immunochemistry system. Factors B and H were measured by single radial immunodiffusion (Binding Site Ltd, Birmingham). CH100 and AP 100 were measured using gel haemolytic assays according to standard protocols [18].

RESULTS

Linkage data

The multipoint linkage analysis between disease and markers on chromosome 1q is shown in Figure 2. No recombinations were seen between the disease and a haplotype extending from D1S212 to D1S306 in all three families. The maximum lod score is 3.94. The data from family 2 is a significant underestimation of the 'true' lod score as we were unable to include family 2b which was subsequently found to have the same mutation.

The three affected members of family 3 share an identical genotype profile across the region, compatible with either a dominant or recessive form of inheritance (Fig. 1). Analyzing the data from this family on a dominant model gave a conservative estimate of the lod score; for example for D1S2757 the 2 point lod score for this family is 0.75 assuming dominant inheritance and 1.27 assuming recessive inheritance.

A novel polymorphism in the factor H gene was informative in family 1 and allowed us to confirm that factor H maps within the region (Fig. 3).

Mutation analysis

Mutations were sought in the coding region of factor H by single strand conformational polymorphism (SSCP) and heteroduplex analysis of RT-PCR amplified fragments, using mRNA extracted from peripheral blood leukocytes as template and a multiple PCR technique with nested primers.

Fig. 5A. SSCP [1] and Heteroduplex [2] appearance of exon 1 amplicon in affected individual with relapsing HUS. Note the band shifts representing a mutation in the factor H gene. (*B*) Part of exon 1 codon sequence in the individual with sporadic/relapsing HUS (numbered according to [32]), demonstrating the deletion, frame shift and premature stop codon with corresponding amino acid product, and truncated polypeptide.

A band shift was located in an amplicon containing part of exon 19 and exon 20 in affected members and obligate carriers of family 2a and 2b. Subcloning and sequencing of the mutant band revealed a point mutation (C→G transversion) causing an arginine → glycine change in exon 20 (*R*1197*G*) (Fig. 4). We have screened 60 healthy controls by PCR/SSCP with intraexonic exon 20 primers using conditions which clearly demonstrate the above transversion and no band shifts were seen. No band shifts were

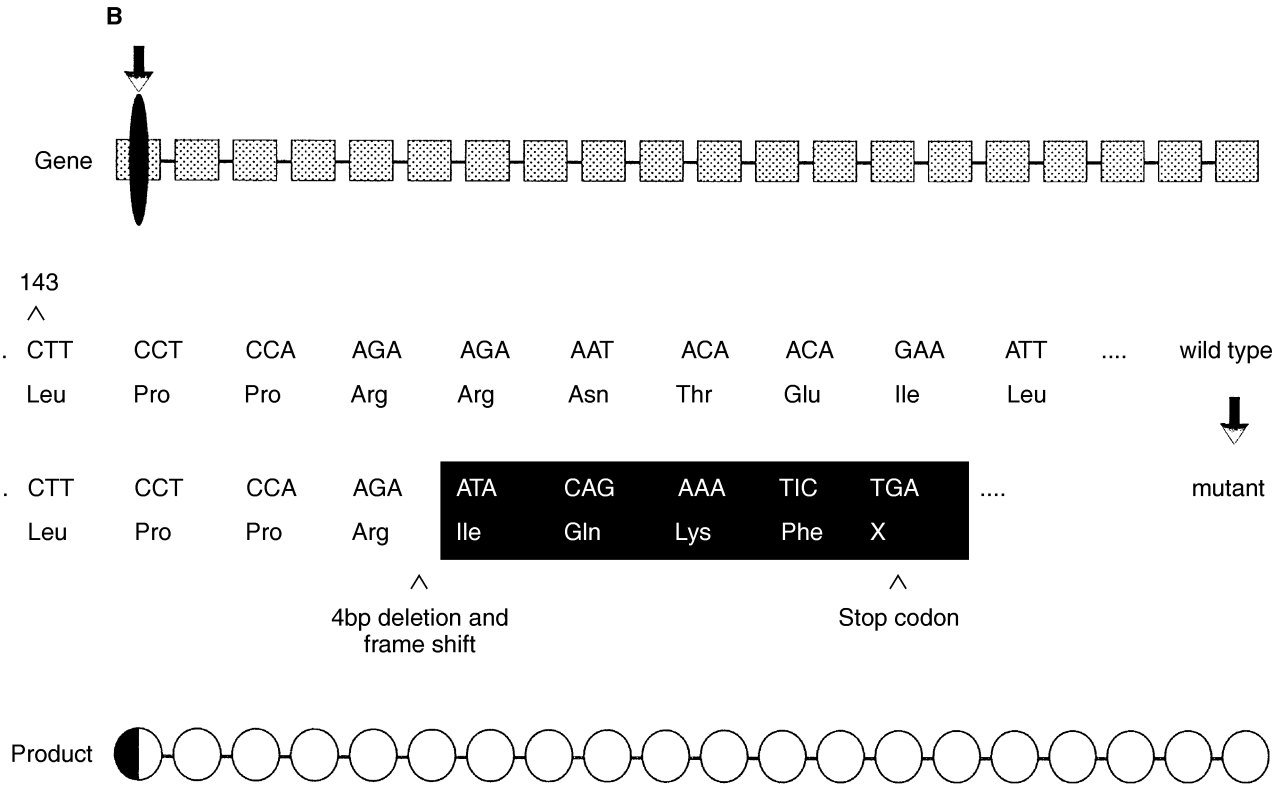

Fig. 5. Continued.

detected in the coding region of the gene in patients from families 1 and 3.

A band shift was located in an amplicon containing exon 1 and part of exon 2 in the sporadic case. Sequencing of the mutant band revealed a 4 base pair deletion in exon 1 causing a frame shift and subsequent premature termination codon (Fig. 5). The mutation is most clearly demonstrated using intraexonic primers and genomic DNA as substrate, and was not found in 60 healthy controls under these conditions. Microsatellite polymorphism profiles and mutation analysis from surviving family members suggest that the mutation was either inherited from the patient's father (who died of a cerebrovascular accident - no other information available, and no further paternal family members available to study) or represents a new mutation.

Complement profiles. Complement studies of both affected and unaffected family members taken during disease free periods are shown in Table 1. In family 1 it is of note that both affected individuals and a disease free obligate gene carrier demonstrate relatively lower C3 levels than non gene carriers. This was not seen in family 2. Factor H levels are not significantly depressed in any of the families.

A full complement profile from the individual with sporadic HUS, taken five years after initial presentation and in the absence of evidence of active disease, is shown in Table 1. As well as low levels of factor H, both C3 and factor B are markedly reduced in keeping with activation of the alternative pathway.

DISCUSSION

To gain insight into the etiology of HUS we have undertaken a linkage study using a candidate gene approach to the familial form of HUS. In the 3 families HUS maps to a region of one of our candidate genes - that of complement factor H. In one of these families we found a mutation in this candidate gene. We have also found a mutation in a sporadic case. These findings represent the first reported molecular evidence of the involvement of factor H in the disease.

In a review of the literature, factor H deficiency in HUS has been recorded in 3 previous reports. The first reported deficiency, by Thompson and Winterborn in 1981 [19], was of an eight months old boy who presented with HUS and his healthy three years old brother who both had 5 to 10% of the normal level of factor H, inherited in an autosomal recessive fashion from consanguineous parents, each of whom had approximately 50% normal levels. Roodhooft et al in 1990 [20] described a female infant who suffered 3 recurrent episodes of HUS (including an ultimately fatal recurrence following renal transplantation) and had 48% of normal levels of factor H apparently inherited from her unaffected father who had 34% of normal levels.

Thirdly, Pichette et al in 1994 [21] reported a family in whom two siblings suffered HUS: one of whom died at an early age from diarrhoeal associated HUS (*E. coli* serotype 0119B14) and a female sibling who had suffered 3 episodes of HUS since the age of 19 and was also shown to have an autosomal recessive deficiency of factor H (5% of normal levels). An asymptomatic sister was found to have only 5% levels and several members of the extended family had approximately 50% levels. It is therefore possible that both autosomal recessive and autosomal dominant HUS are associated with abnormalities of factor H.

Normal levels of factor H do not exclude an underlying functional abnormality and the finding of a mutation in factor H

Table 1. Serum complement profiles of family members

Family	Subject	Status	C3 0.68–1.8 g/liter	C4 0.18–0.60 g/liter	Factor B 191–382 mg/liter	Factor H 345–590 mg/liter	CH100	AP100
1	II-1	Unaffected	1.23	0.34	256	665	N	N
1	II-2	Unaffected	1.00	0.27	247	648	N	N
1	II-3	Unaffected	1.51	0.42	N/A	N/A	N/A	N/A
1	III-1	Affected	0.55	0.22	265	532	N	N
1	IV-1	Affected	0.67	0.22	256	548	N	N
1	III-6	Carrier	0.72	0.22	265	532	N	N
2a	IV-11	Unaffected	0.92	0.22	372	517	N	N/L
2a	IV-23	Unaffected	1.27	0.24	293	564	N	N
2a	IV-12	Affected	0.75	0.22	165	486	N	N/L
2b	IV-1	Unaffected	1.27	0.24	293	564	N	N
2b	IV-2	Affected	1.16	0.27	247	648	N	N
2a	IV-3	Carrier	0.98	0.23	274	548	N	N
3	II-2	Affected	80%	N/A	N/A	N/A	N/A	N/A
3	II-4	Affected	51%	N/A	N/A	68%	N/A	N/A
3	II-6	Affected	76%	N/A	N/A	80%	N/A	N/A
Individual		Affected	0.4	0.25	157	222	N	N

'Carrier' refers to individuals who are obligate gene carriers but have not suffered HUS; 'N' and 'N/L' are normal and at the lower end of normal range; N/A is not available. Levels of C3 and H from family 3 are expressed as a percentage (of a standard level obtained from a panel of unaffected control sera). All samples were taken during periods of good health, and in the absence of evidence of active HUS.

in only one of the three families (family 2) may reflect the relative insensitivity of SSCP in detecting mutations (sensitivity approximately 65 to 75%). Certainly the finding of persistently lower C3 levels in both previously affected individuals and an unaffected obligate gene carrier in family 1 is interesting in this context. Recently, a family of human plasma proteins has been identified which are related structurally and immunologically to factor H and which map to the same area of chromosome 1 [22], and these genes would be candidates if mutations are not identified in families 1 and 3 after completing exhaustive mutation analysis.

Factor H is a major plasma protein, being present at a concentration of approximately 300 to 600 mg/l. It is synthesized mainly in the liver, but smaller amounts are generated by endothelium and macrophages. It is found in platelets and is membrane bound on some leukocytes [23]. Factor H has several important regulatory roles in the activity of the alternative pathway [23]; firstly, by binding with C3b, preventing the formation of the C3bBb (C3 convertase) complex and accelerating the dissociation of Bb from the active C3 convertase (so called decay-accelerating activity) [24, 25]; secondly, by acting as a cofactor for factor I, a serine protease, which degrades C3b by cleaving the alpha chain of C3, converting it into the major opsonic form, iC3b [25]; and thirdly, by distinguishing between activator and non-activator surfaces [26].

The single polypeptide chain of factor H is composed of 20 small independently folding units known as short consensus repeats (SCRs), each approximately 60 amino acids in size and generally coded for by single exons. Correct folding of SCRs is crucial to function (for example breakage of disulphide bonds of factor H results in complete loss of cofactor activity [27]) and even single amino acid substitutions can significantly alter function (for example His→Asp at position 1106 in the C4b molecule [28], which is also composed of SCRs).

We describe the finding of two mutations in the gene for factor H. The first, in family 2 comprises a single base substitution in exon 20 causing an arginine to glycine change in SCR20. Recently a domain encompassing SCRs 16 to 20 has been shown to bind C3b [29] and it is of note that SCRs with structural homology to 19 and 20 are located at the C-terminal end of all human and the majority of mouse factor H and H-related proteins [22]. This arginine shows positional conservation in 3 of the 5 human and 3 of the 4 murine terminal SCRs. Arginine is a basic amino acid with a polar side chain and its substitution by the uncharged glycine, often a pivotal point in polypeptides, may significantly affect structure and thus function. The second mutation in an individual with relapsing HUS comprises a deletion causing a frame shift and subsequent premature termination codon which leads to approximately 50% levels of serum factor H.

In order to explain disease associated with haploinsufficiency for factor H - as in our sporadic case one would have to postulate a 'dose effect' which possibly becomes important following an environmental trigger such as a drug or an infection. Precedent exists within the complement pathway. Haploinsufficiency for C1 esterase inhibitor predisposes to angioneurotic edema. In this condition there is a high rate of *de novo* mutations, demonstrating that a negative family history does not necessarily exclude a genetic cause. This may have relevance to sporadic HUS in general and our patient in particular. However in this context it is interesting that in the previously described families with homozygous factor H deficiency HUS is not seen in heterozygotes. Perhaps the nature of the genetic mutation causing the deficiency of factor H may be relevant or the activity of the remaining factor H may vary (there are known to be several polymorphic forms of the factor H protein and we have identified several genetic polymorphisms within the gene [17]). Deficiency of factor H has also been associated with SLE like collagen vascular disease, increased susceptibility to infection and glomerulonephritis [23].

In a pig model of inherited factor H deficiency homozygotes develop a lethal mesangioproliferative glomerulonephritis [30].

In two of the three families complement profiles were normal. It is possible that the response to activation of the alternative pathway could be exaggerated in the presence of factor H mutations despite normal basal activity.

Factor H would fit the profile of an agent involved in the etiology of HUS. Recurrence of disease in renal allografts, especially early recurrence, suggests a plasma factor, as does improvement following plasma exchange. Abnormal levels of complement factors, particularly factors B and C3 are often found in active d- HUS suggesting overactivity of the alternative pathway. Although it is abnormalities of factor H that we have shown to be associated with HUS, it is possible that abnormalities of other regulators of the alternative pathway, such as factor I, could also be implicated.

Are these findings of any relevance to the pathogenesis of the more commonly seen but histologically distinct d+ HUS ? There is evidence to suggest that there is increased complement activation in this condition. Two studies of children with d+ HUS show that low C3 levels are associated with a poor prognosis [11, 31]. Moreover, in another study increased breakdown products of C3 and factor B were found suggesting activation of the alternative complement pathway [12]. It is of interest that in the factor H deficient sibling pair who suffered with HUS described by Pichette, one presented with d+ HUS and the other with d-HUS. It is known that there are polymorphisms of the factor H protein and we have previously described a polymorphism in an NF kappa B responsive element in the promoter region of the gene [17]. It is intriguing to speculate that some of these polymorphic variants confer a genetic predisposition to d+HUS.

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NOTE ADDED IN PROOF

Mutations in the factor H gene have recently been described in a factor H deficient child with membranoproliferative glomerulonephritis [33].

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