SHORT REPORT

The development of atypical haemolytic-uraemic syndrome is influenced by susceptibility factors in factor H and membrane cofactor protein: evidence from two independent cohorts

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Background: In both familial and sporadic atypical haemolytic-uraemic syndrome (aHUS), mutations have been reported in regulators of the alternative complement pathway including factor H (CFH), membrane cofactor protein (MCP), and the serine protease factor I (IF). A characteristic feature of both MCP and CFH associated HUS is reduced penetrance and variable inheritance; one possible explanation for this is that functional changes in complement proteins act as modifiers.

Objective: To examine single nucleotide polymorphisms in both *CFH* and *MCP* genes in two large cohorts of HUS patients (Newcastle and Paris).

Results: In both cohorts there was an association with HUS for both *CFH* and *MCP* alleles. *CFH* and *MCP* haplotypes were also significantly different in HUS patients compared with controls.

Conclusions: This study suggests that there are naturally occurring susceptibility factors in *CFH* and *MCP* for the development of atypical HUS.

Haemolytic-uraemic syndrome (HUS) is characterised by the triad of thrombocytopenia, Coomb's test negative microangiopathic haemolytic anaemia, and acute renal failure.[1 2] HUS is classified as either (D+) when it is associated with a preceding diarrhoeal illness which in most is cause by infection with *E coli* O157 or less commonly as non-diarrhoeal associated (D−) (also called "atypical", hence aHUS). aHUS may be sporadic or familial. In both types, mutations have been reported in regulators of the complement pathway including factor H (CFH), membrane cofactor protein (MCP, CD46),[3–11] and the serine protease factor I (IF).[12] Such mutations result in impaired protection of host surfaces against complement activation[13 14] and it is likely that they predispose to rather than directly cause a thrombotic microangiopathy. In this situation endothelial activation secondary to injury is maintained by excessive complement activation.[15] A characteristic feature of both MCP and CFH associated HUS is variable penetrance and inheritance. The penetrance of the disease phenotype in our panels of families is approximately 50%. One possible explanation for this is that functional variants in complement proteins act as modifiers. In support of this is the finding of an association between *CFH* single nucleotide polymorphisms (SNPs) and aHUS,[16] and a recent study which identified a specific SNP haplotype block spanning *MCP* which is over-represented in aHUS patients.[17] To extend these observations we have examined *CFH* and *MCP* SNPs in two cohorts of aHUS patients.

METHODS

A clinical diagnosis of aHUS was made in all the patients included in this study. Appropriate ethics approval was given for both cohorts to be studied and all subjects gave informed consent. There were 75 patients in the Newcastle cohort and 77 in the Paris cohort, some of whom have been reported previously.[3 5 6 9 11 12 18–24] Of the 75 patients in the Newcastle cohort there were four families in which more than one family member was included in the cohort. In three families there were two siblings included and in one there were three siblings. Of the 77 patients in the Paris cohort there were again four families in which more than one family member was included in the cohort. In three families there were two siblings included and in one there were three siblings.

In the Newcastle cohort 15 had a *CFH* mutation, four had an *MCP* mutation, and three had an *IF* mutation; in the Paris cohort 21 patients had a *CFH* mutation, eight had an *IF* mutation, 10 had an *MCP* mutation, and one had anti-CFH antibodies. Of the 21 patients with a *CFH* mutation, eight had ~50% and three had no detectable antigenic level. Eight patients had a mutation located in the C-terminal region, with normal CFH levels. While the functional significance of each of these mutations has not been examined, their location would suggest that they act in a way similar to those previously studied in detail.[13 14] Of the 18 patients with either an *FI* or *MCP* mutation, in ~60% there was evidence of a decreased antigenic level of the protein.

One hundred locally recruited normal controls were studied for the Newcastle cohort and 84 for the Paris cohort. Both cohorts and their respective control populations were predominantly white (Newcastle cohort 65/75; Paris cohort 74/77).

The SNPs analysed in the Newcastle cohort were *CFH* (c.−257C→T, promoter; c.2089A→G, synonymous; c.2881G→T, E963D) and *MCP* (IVS12+638A→G; c.2232T→C, 3′ UTR). DNA was prepared from peripheral blood according to standard procedures. Regions of DNA containing the SNPs of interest were amplified by polymerase chain reaction (PCR). Genotyping was carried out through a primer extension reaction, using the ABI PRISM SNaPshot ddNTP primer extension kit (Applied Biosystems, Courtaboeuf, France) and for subsequent detection we employed an ABI PRISM 3100 genetic analyser (Applied

Abbreviations: aHUS, atypical haemolytic-uraemic syndrome; CFH, complement factor H; HUS, haemolytic-uraemic syndrome; MCP, membrane cofactor protein; SNP, single nucleotide polymorphism

Table 1 Primer sequences and conditions for SnaPshot reactions in the Newcastle cohort

SNP	PCR			SNaPshot reaction			
	Primer sequence (5′→3′)						
	Forward	Reverse		Annealing temp (°C)	Primer sequence (5′→3′)	Pooled primer conc (μM)	Length (bp)
CFH c. −257 C→T	TCTTTACCTTCTCAATATCCAGC	ACTCCTGTGAAAAGCATCATTAG		60	(6A)GGGTTTATGAAATCCAGAGGATAT	0.3	30
CFH c.2089 A→G	TATATTGTAAAACAGACAATTTAACC	ATACAAAATACAAAAGTTTTGACAAG		57	CTAATGAAGGGACCTAATAAAATTCA	0.67	26
CFH c.2881 G→T	TAAATTTATGAGTTAGTGAAACCTG	TGGTACCACTTACACTTTGAATG		57	TTCCTTGTAAATCTCCACCTGA	0.04	22
MCP c. IVS12+638 A→G	CCCAATTTGGTGTCTTTTCA	TCTGTGTTCCAGGATTCATTC		54	(19A)GCTGAGAGGGGTTAGATCT	0.1	38
MCP c.2232 T→C	TAATTTCAGAATCAGATGCATCC	TCAAAACCACCTACTTTTAGAGG		59	(13A)CGGAGTTCTCTTTGGGAAAAC	0.2	34

CFH, complement factor H; conc, concentration; MCP, membrane cofactor protein; PCR, polymerase chain reaction; SNP, single nucleotide polymorphism; temp, temperature.

Biosystems). SNaPshot reactions were carried out on pooled PCR products according to the manufacturer's instructions, using pooled SNaPshot primers at the concentrations described in table 1. Primer sequences and conditions for PCR and SNaPshot analysis are also shown in table 1. In addition, ambiguous SNPs were verified by direct sequencing using DYEnamic ET dye terminator cycle sequencing (Amersham, UK), as described by the manufacturer.

The SNPs analysed in the Paris cohort were *CFH* (c.−257C→T, promoter; c.2089A→G, synonymous; c.2881G→T, E963D) and *MCP* (c.−547A→G, promoter; c.−261A→G, promoter; IVS8+23T→G; IVS9-78G→A; c.2232T→C, 3′ UTR). DNA was extracted from whole blood using the proteinase K/phenol method.[25] Genomic DNA was amplified using the primer sequences and conditions shown in table 2.[11] PCR products were purified using Multiscreen plates according to the manufacturer's instructions (Millipore, Molsheim, France). Direct DNA sequencing of the purified PCR products was then carried out by the Dye terminator cycle sequencing method (Applied Biosystems, Courtaboeuf, France) using a 96 capillary Sequencer 3700. Sequence analyses were done using Sequencher software (Gene Codes Corporation, Ann Arbor, Michigan, USA).

The NCBI SNP ID numbers, in parentheses, are *CFH* −257C→T (rs3753394), *CFH* c.2089A→G (rs3753396), CFH c.2881G→T (rs1065489), MCP-547A→G (rs2796267), MCP c.IVS8+23T→G (rs2724374), MCP c.IVS9-78G→A (rs1962149), MCP c.IVS12+638 (rs859705), and MCP c.2232T→C (rs7144).

The differences in genotype frequency between HUS patients in the two cohorts and the respective control individuals were tested using either χ^2, or if there were less than five observations in a cell, Fisher's exact test. As the SNPs are not assorting independently it would not be appropriate to apply a Bonferroni correction equal to the total number of SNPs studied in each cohort. Haplotype analysis (FUGUE) showed that there were three *CFH* and two *MCP* haplotypes with a frequency greater than 10% in both cohorts. We therefore applied a Bonferroni correction of (0.05/5) giving a significance value of 0.01.

Comparison of haplotypes was undertaken using FUGUE-CC (Goncalo Abecasis, Center for Statistical Genetics, University of Michigan, USA). This allows significance to be computed by analysis of random permutations of the data.

RESULTS

The genotype and allele frequency for the two cohorts is shown in table 3. In both cohorts there was an association between *CFH* alleles and HUS. The same *CFH* SNPs were analysed in both cohorts. There was also an association between *MCP* alleles and HUS in both cohorts. The results for the one SNP (c.2232T→C) analysed in both cohorts showed a strong association ($p<0.001$) in the Paris cohort but failed to reach a significance value of <0.01 in the Newcastle cohort ($p = 0.012$).

The allele frequency in those with and without known mutations in *CFH*, *MCP*, and *IF* is shown in table 4. For *CFH* in the Newcastle cohort c.−257T c.2089G, c.2881T were associated with atypical HUS in those not known to have a mutation but not in those known to have a mutation. For *CFH* in the Paris cohort c.−257T c.2089G, c.2881T were associated with atypical HUS only in those known to have a mutation. For *MCP* the association was present in both those with and those without an identified mutation for all the SNPs in the Paris cohort. In the Newcastle cohort c.IVS12+638A was associated with atypical HUS in those without a mutation.

The haplotypes generated by FUGUE are shown in tables 5–7. For both the Newcastle and the Paris cohort there was a

Table 2 Primer sequences and conditions for direct sequencing (polymerase chain reaction; Paris cohort)

SNP	Primer sequence (5′–3′) Forward	Reverse	Annealing temp (°C)
CFH −257 C→T	GGGGTTTTCTGGGATGTAATA	GTGATTAGTGCAGGAAAGAAC	60
CFH c.2089 A→G	TTGATCAAATGCTTGCCTCAG	TATATCTCCACAGGTACTCTC	60
CFH c.2881 G→T	TAGACAGACAGACACCAGAA	ACCACTTACACTTTGAATGA	57
MCP −547A→G	GCAAAGGGCAAATTACCTTAG	ACCCCTCAGGGTTAGTTTTAT	62
MCP −261A→G	ATAAAACTAACCCTGAGGGGT	CCTTTTTTCTTGCTAAGCCCT	60
MCP c.IVS8+23 T→G	CCAAGTGGTTGATCTTCTAAC	ATGGCTATACAAATGTCCTCC	60
MCP c.IVS9−78 G→A	GGGGAGGAAGAAGAAAGATTA	CTATGTTTGGGCACCTCATAA	60
MCP c. 2232 T→C	GTGTTCGGTGATTTCAGAAAG	TAAGGAGGGAGAGAAAAACAC	55

CFH, complement factor H; MCP, membrane cofactor protein; PCR, polymerase chain reaction; SNP, single nucleotide polymorphism; temp, temperature.

significant difference for both *CFH* and *MCP* haplotype frequency in the HUS patients and controls.

The genotype and haplotype results for both cohorts are internally consistent in that an increase in frequency of the rarer allele and haplotype for both *MCP* and *CFH* was associated with HUS.

DISCUSSION

In this study we showed a significant difference in two independent cohorts of atypical HUS patients in both allele frequency and haplotypes for two complement regulatory genes, *CFH* and *MCP*. Mutations in both these genes have been described in aHUS patients. However, the inheritance

Table 3 Genotype and allele frequency

	Controls						HUS						
	Genotypes				Allele frequency		Genotypes				Allele frequency		
	1/1	1/2	2/2	n	1	2	1/1	1/2	2/2	n	1	2	p Value
Newcastle cohort													
CFH −257C→T	44	43	5	92	71	29	19	36	12	67	55	45	0.003
CFH c.2089A→G	72	25	1	98	87	13	30	38	6	74	66	34	<0.001
CFH c.2881G→T	62	32	2	96	81	19	32	30	7	69	68	32	0.006
MCP c.IVS12+638G→A	41	38	17	96	62	38	18	28	27	73	44	56	0.001
MCP c.2232T→C	36	41	21	98	58	42	17	32	26	75	44	56	0.012
Paris cohort													
CFH −257C→T	57	20	7	84	80	20	29	27	21	77	55	45	<0.001
CFH c.2089A→G	58	18	4	80	84	16	42	20	14	76	70	30	<0.001
CFH c.2881G→T	61	18	4	83	84	16	39	20	15	74	66	34	<0.001
MCP −547A→G	30	34	12	76	62	38	18	23	33	74	39	61	<0.001
MCP-261A→G	33	35	12	80	63	37	16	30	31	77	40	60	<0.001
MCP c.IVS8+23T→G	47	23	3	73	80	20	27	29	21	77	54	46	<0.001
MCP c.IVS9-78G→A	34	35	9	78	66	34	18	29	29	76	42	58	<0.001
MCP c.2232T→C	29	32	9	70	64	36	18	30	29	77	43	57	<0.001

CFH, complement factor H; HUS, haemolytic-uraemic syndrome; MCP, membrane cofactor protein.

Table 4 Allele frequency in patients with and without known mutations in *CFH*, *MCP* and *IF*

	Control allele frequency		HUS – mutation negative allele frequency			HUS – mutation positive allele frequency		
	1	2	1	2	p Value	1	2	p Value
Newcastle cohort (n = 22 mutation positive)								
CFH −257C→T	71	29	52	48	0.001	64	36	0.38
CFH c.2089A→G	87	13	64	36	<0.001	71	29	0.019
CFH c.2881G→T	81	19	65	35	0.002	76	24	0.52
MCP c.IVS12+638G→A	62	38	43	57	0.001	45	55	0.038
MCP c.2232T→C	58	42	43	57	0.018	45	55	0.14
Paris cohort (n = 39 mutation positive)								
CFH −257C→T	80	20	59	41	0.012	51	49	<0.001
CFH c.2089A→G	84	16	76	24	0.182	64	36	0.001
CFH c.2881G→T	84	16	76	24	0.143	57	43	<0.001
MCP −547A→G	62	38	42	58	0.005	39	61	0.001
MCP-261A→G	63	37	42	58	0.005	39	61	<0.001
MCP c.IVS8+23T→G	80	20	55	45	<0.001	53	47	<0.001
MCP c.IVS9-78G→A	66	34	43	57	0.001	43	57	0.001
MCP c.2232T→C	64	36	45	55	0.006	41	59	0.001

CFH, complement factor H; HUS, haemolytic-uraemic syndrome; MCP, membrane cofactor protein.

Table 5 Factor H haplotypes for both cohorts

			Newcastle		Paris	
−257C→T	c.2089A→G	c.2881G→T	HUS (%)	Controls (%)	HUS (%)	Controls (%)
1	1	1	47.98	60.21	53.09	78.52
2	1	1	17.06	15.27	11.83	5.41
1	2	1	2.72	5.65	0	0
2	2	1	0.25	0.26	0	0.60
1	1	2	0	4.41	0.48	0
2	1	2	1.60	6.34	4.16	0
1	2	2	4.78	0.94	1.62	1.24
2	2	2	25.59	6.93	28.81	14.23

Newcastle: log likelihood ratio = 19.65. Permutations with higher ratio 0/1000.
Paris: log likelihood ratio = 15.64. Permutations with higher ratio 779/10 000.

Table 6 MCP haplotypes for the Newcastle cohort

c.IVS12+638G→A	c.2232T→C	HUS (%)	Controls (%)
1	1	41.90	53.05
2	1	2.10	4.52
1	2	2.09	8.91
2	2	53.91	33.52

Log likelihood ratio = 9.252. Permutations with higher ratio 7947/100 000.

and penetrance seen with mutations in both these is variable. For instance in two families with the same *MCP* mutation (S206P), only homozygotes are affected in one whereas in the other heterozygotes are affected.[9] Moreover, the series reported to date show that the penetrance of CFH associated HUS is approximately 50%. This suggests that other factors are modifying the inheritance and penetrance. Both *CFH* and *MCP* belong to a cluster of genes located at 1q32 which are involved in complement regulation, the so called RCA (regulators of complement activation) cluster. Other members of this group include decay accelerating factor (DAF), complement receptor 1 (CR1), C4 binding protein (C4BP), and five factor H related proteins (FHR1–5). To date mutations have only been found in *CFH* and *MCP* but it is possible that genetic variability in these other regulators could be acting as modifiers for the development of HUS.

Caprioli *et al* have previously reported that the *CFH* alleles −257T, c. 2089G, and c. 2881T are significantly more common in patients with atypical HUS. The results from both the Newcastle and Paris cohorts support this observation.[16] They found that this was true both for patients with a *CFH* mutation and for those without. In the Newcastle cohort we found that −257T c.2089G , and c.2881T were more frequent in those without known mutations in *CFH*, *MCP*, and *IF*. In the Paris cohort, the reverse was seen in that −257T, c. 2089G, and c. 2881T were more frequent in those with mutations. Caution must therefore be exercised in interpreting subgroup analysis such as this where numbers may be inadequate. It is not yet known whether c.−257C→T or c.2881G→T are functionally significant. c.2089A→G is a synonymous change. c.−257T is located in a putative NF −κB binding sequence of the *CFH* promoter[26] and it is known that CFH expression is upregulated by interferon γ, providing a possible link. c.2881G→T changes a glutamate to an aspartate in CCP16 of CFH. We have now not only confirmed Caprioli's observation but also extended it in two independent cohorts to show that genetic variability in *MCP* is also associated with atypical HUS. This confirms the recent finding by Esparza-Godilla *et al* that a specific SNP haplotype block which includes *MCP* was associated with aHUS.[17] In the Paris cohort this strong association was present in both those with and those without known mutations in *CFH*, *MCP*, and *IF*. In contrast, the association was only seen in those with known mutations in the study of Esparza-Godilla. This discrepancy emphasises the need to be cautious in interpreting such data. In all, six *MCP* SNPs were examined in the two

Table 7 MCP haplotypes for the Paris cohort

−547A→G	−261A→G	c.IVS8+23T→G	c.IVS9-78G→A	c.2232T→C	HUS (%)	Controls (%)
1	1	1	1	1	30.38	50.85
1	1	1	1	2	1.97	0.00
1	1	1	2	2	0.00	2.02
1	1	2	1	1	0.68	0.89
1	1	2	2	1	0.65	0.00
1	2	1	1	2	0.67	1.28
1	2	1	1	1	2.58	3.16
1	2	1	2	2	2.68	4.96
1	2	1	2	1	0.00	0.83
2	1	1	1	1	6.58	8.82
2	1	2	1	1	0.00	0.77
2	2	1	2	2	8.37	8.65
2	2	1	2	1	0.67	0.00
2	2	2	2	2	43.45	17.13
2	2	2	2	1	1.32	0.60

Log likelihood ratio = 20.28. Permutations with higher ratio 7/1000.

cohorts; of these three are intronic, two are in the promoter, and one is in the 3′ UTR. It is possible that the promoter SNPs or the 3′ UTR SNP are functionally significant. In support of this is the recent study from Esparza-Gordillo which showed that *MCP* −261G disrupts a potentially functional CBF-1/RBP-Jk binding site. Transient transfection showed that this was associated with a 25% lower transcriptional activity.[17] It is also possible that a combination of factors within the haplotype block results in a functional effect. Alternatively, these markers may simply be surrogates for another untested SNP in the vicinity.

Conclusion

This study emphasises the importance of variability in *CFH* and *MCP* as a modifier for the development of atypical HUS. The results suggest that complement regulatory genes in the RCA cluster are acting in a coordinated manner to prevent host cell damage and that perturbations of this network in the face of endothelial injury will lead to a thrombotic microangiopathy.

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