The high frequency of Complement Factor H-Related CFHR1 Gene Deletion is restricted to specific subgroups of patients with atypical Haemolytic Uraemic Syndrome

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Abbreviations:

AHUS : atypical hemolytic uremic syndrome

CFHR : Complement Factor H Related

MLPA   :Multiplex Ligation-dependant Probe Amplification

FH : Factor H

CFH : Complement Factor H

CFI : Complement Factor I

MCP : Membrane Cofactor Protein

CFB : Complement Factor B

RCA : Regulators of Complement Activation

AMD : Age-related macular degeneration

IgG : Immunoglobulin G

ABSTRACT

Background: Deletion of the Complement Factor H Related 1 (*CFHR1*) gene is a consequence of non-allelic homologous recombination and has been reported to be more frequent in atypical hemolytic uremic syndrome (aHUS) patients than in the normal population. Therefore, it is considered a susceptibility factor for the disease. Atypical HUS is associated with hereditary or acquired abnormalities that lead to uncontrolled alternative pathway complement activation. We tested the *CFHR1* deletion for association with aHUS in a population of French aHUS cases and controls. Furthermore, we examined the effect of the deletion in the context of known aHUS risk factors.

Methodology and findings: 177 aHUS patients and 70 healthy donors were studied. The number of *CFHR1* alleles was quantified by Multiplex Ligation-dependant Probe Amplification (MLPA). The frequency of the deleted allele was significantly higher in aHUS patients than in controls (22.7% versus 8.2%, $P<0.001$). The highest frequency was in the subgroup of patients exhibiting anti-Factor H (FH) auto-antibodies (92.9%, $P<0.0001$ versus controls) and in the group of patients exhibiting a Factor I (*CFI*) gene mutation (31.8%, $P<0.001$ versus controls). The *CFHR1* deletion was not significantly more frequent in the cohort of aHUS patients when patients with anti-FH IgG or *CFI* mutation were excluded.

Conclusions: The high frequency of *CFHR1* deletion in aHUS patients is restricted to the subgroups of patients presenting with anti-FH auto-antibodies or, to a lesser degree, *CFI* mutation. These results suggest that the *CFHR1* deletion plays a secondary role in susceptibility to aHUS.

INTRODUCTION

Atypical, or non-Shigatoxin-associated haemolytic uremic syndrome (aHUS, OMIM#235400), is a rare form of thrombotic microangiopathy that principally affects the kidneys' microvasculature, leading to severe acute renal failure. Genetic susceptibility factors have been identified in almost 60% of aHUS cases. These factors are genetic abnormalities leading to loss-of-function proteins encoded by genes involved in the complement alternative pathway regulation: Factor H (*CFH*), Membrane Cofactor Protein (*MCP* or *CD46*) and Factor I (*CFI*) [1]. Recently, other mutations have been found that lead to gain-of-function proteins encoded by genes for the C3 convertase components, *C3* or factor B (*CFB*) [2, 3]. In addition, a genetic abnormality has been identified that is due to unequal recombination in the RCA (Regulators of Complement Activation) locus, which comprises several genes encoding proteins implicated in regulation of complement activation. *CFH* and the genes encoding the five Complement Factor H Related proteins (*CFHR1-CFHR5*) exhibit a high degree of sequence homology. There are two highly homologous sequences with long interspersed nuclear elements (retrotransposons) within this locus, which favours the occurrence of unequal recombinations [4]. This mechanism leads to the production of a hybrid protein secondary to the fusion of the *CFH* gene encoding factor H and *CFHR1* gene, leading to a Factor H functional deficiency [5].

Finally, aHUS may occur in the context of an auto-immune disease, with the development of anti-FH auto-antibodies leading to an acquired FH deficiency [6, 7]. All these abnormalities lead to a hyperactive alternative pathway C3 convertase.

In addition to these causative factors, several studies have identified additional susceptibility factors that consist of genetic polymorphisms more frequently observed in aHUS patients than in the control population. These include single nucleotide polymorphisms (*SNP*) identified in

the *CFH*, *MCP* or *C4BP* genes [8, 9] or a deletion of *CFHR1* and *CFHR3* genes resulting from an unequal recombination occurring outside the coding sequences of *CFH* and *CFHR1* [4]. This deletion of *CFHR1-CFHR3* has been reported to be associated with aHUS and also to be protective against age-related macular degeneration (AMD; OMIM#603075) [10, 11]. Recently, Jozsi et al. reported a high frequency of lack of circulating CFHR1 and CFHR3 proteins in a group of patients with the auto-immune form of aHUS [7], which could be due to the same genetic mechanism.

The aim of our study was to determine the frequency of *CFHR1* deletion in a French cohort of aHUS patients and to correlate the deleted allele and the deletion homozygosity frequencies with other susceptibility factors. We show that the high frequency of *CFHR1-CFHR3* deletion found in aHUS patients is due to a particularly high frequency of the homozygous deletion in the subgroup of patients with anti-FH auto-antibody – associated aHUS and, to a lesser degree, a *CFI* mutation.

MATERIALS AND METHODS

Participants

All patients of the French cohort of aHUS were studied between 2002 and 2007. The criteria for the diagnosis of HUS were thrombocytopenia (<150 G/l), renal dysfunction associated with acute anemia and fragmented red cells on blood film. None of the patients exhibited biological criteria of a shigatoxin productive bacteria infection. Patients who developed the illness in a context of infectious disease (HIV, *Mycoplasma pneumoniae*, *Borderellia pertussis*, *Varicellavirus*), hemopathy, solid neoplasia or organ transplantation (except renal graft) were excluded. The cohort consisted of 86 children and 91 adults. Informed consent was obtained from each patient (or parents in the case of children), and the study was

approved by the Ethics Committee of the Assistance Publique-Hôpitaux de Paris. The control group comprised 70 French healthy blood donors.

Complement assays

Measurement of CH50 activity in EDTA plasma samples was performed as previously described. Plasma concentrations of the complement components C4, C3 and Factor B (FB) antigens were measured by nephelometry (Dade Behring, Paris La Defense, France). Factor H and Factor I antigen concentrations were measured by sensitive ELISA methods and CD46 membrane expression was determined by flux cytometry as previously described [12]. Presence of anti-FH IgG was detected by using an ELISA method as previously described [6].

Genomic CFH, CFI and MCP DNA sequencing

For genetic analysis, genomic DNA was extracted from peripheral blood cells and amplified by Polymerase Chain Reaction (PCR) using oligonucleotides flanking each exon of the *CFH, FI* and *MCP* genes. Primer sequences, length of the PCR-amplified fragments and temperatures of hybridization used for each reaction and direct DNA sequencing procedure have been previously described [12]. Samples were run on the ABI PRISM 3730 Analyser Capillary electrophoresis system (Applied Biosystems) and sequence analyses were performed using the Sequencher® software.

Multiplex Ligation-dependant Probe Amplification (MLPA)

The MLPA reaction was performed as previously described [13]. Briefly, 50 ng DNA was incubated with 2 fmol of each set of 2 synthetic probes that hybridize immediately adjacent targets at the sequences of interest. Sequences of probes were designed to determine dosage for exon 5 of *CFHR1* and exon 23 of *CFH* along with control probe *C1INH* exon 8. In some patients, dosage for exon 3 of *CFHR3* was determined. Hybridization sequences were :

CFHR1a : 5'-GACTGACTGAGGACAGCCAAACAGAAGCTTTATTT-3', *CFHR1b* : 5'-GAGAACAGGTGAATCAGCTGAATTTG-3', *CFHex23a* : 5'-GGACAGCCAAACAGAAGCTTTATTC-3', *CFHex23b* : 5'-GAGAACAGGTGAATCAG TTGAATTTG-3', *C1Iex8a* : 5'-CTGAAGGGCTTCACGACCAAAGGTGT-3', *C1Iex8b* : 5'-CACCTCAGTCTCTCAGATCTTCCCAC-3', *CFHR3a* : 5'-GTTTGTACAGGGT AACTCTAC-3', *CFHR3b* : 5'-AGAAGTTGCCTGCCATCCTGGC-3'. Probes contained binding sites for primers used for MLPA and a stuffer sequence used to determine a unique length of each amplified probe product. Right-hand probes were 5' phosphorylated (MWG, Roissy, France). MLPA reagents were purchased from MRC Holland (MRC Holland, Amsterdam, The Netherlands), and the reaction was carried out according to the manufacturer's recommended protocol. Amplified products were diluted 1/10 in deionised formamide (Applied Biosystems, Courtaboeuf, France) with a ROX 400HD (Applied Biosystems, Courtaboeuf,France) internal size standard. Samples were run on the ABI PRISM 3730Analyser Capillary electrophoresis system (Applied Biosystems, Courtaboeuf, France). Peaks and areas for each sample were determined using Genemapper v4.0 Software (Applied Biosystems, Courtaboeuf, France) and dosage quotients were calculated.

Statistical analysis

The statistical analysis used the Chi square methods.

RESULTS

We used MLPA (Multiplex Ligation-dependant Probe Amplification) reaction to quantify the number of allele copies of *CFHR1* gene (Figure 1) in a French cohort of 177 patients with aHUS.

A genetic susceptibility factor was identified in 117 (66%) of patients. A genetic abnormality implicating *CFH* (comprising one *CFH/CFHR1* hybrid gene), *CFI* or *MCP* was found in

21.5%, 12.4% and 9.6% of patients, respectively. In addition, sixteen (9%) patients had a *C3* mutation and seven (4%) patients had combined mutations implicating two or three genes. Some of the mutations identified have been reported previously [2, 12, 14, 15, 16, 17]. Finally, fourteen (7.9%) patients presented with an auto-immune form of aHUS with anti-Factor H auto-antibodies.

A genomic deletion of *CFHR1* was found in 56 (31.6%) patients. This deletion was homozygous in 12.4 % (n=22) or heterozygous in 19.8% (n=35) of patients, as determined by the number of *CFHR1* copies (Table 1).

Table 1 : Determination of the number of CFHR1 alleles according the previously identified susceptibility factor in the French aHUS cohort (n=177 patients).

% (n)	0 allele	1 allele	2 alleles
Total cohort	12.4 (22)	19.8 (35)	68 (120)
No factor identified	4.7 (3)	26.6 (17)	68.8 (43)
CFH **mutation**	2.6 (1)	10.5 (4)	86.8 (33)
CFI **mutation**	18.2 (4)	27.3 (6)	54.5 (12)
MCP **mutation**	0	6.25 (1)	94 (16)
C3 **mutation**	6.3 (1)	31 (5)	62.5 (10)
Combined mutations	0	29 (2)	71 (5)
Anti-FH IgG	93 (13)	0	7 (1)
Normal Population	*2.8 (2)*	*11.4 (8)*	*85.7 (60)*

Thus, the calculated frequency of the deleted allele was 22.7%. In the control population, a genomic deletion was found in ten controls (14.3%, homozygous in two and heterozygous in

eight), giving an allele frequency of 8.2%. Thus, the *CFHR1* deleted allele frequency was significantly higher in aHUS patients as compared to controls (22.7% versus 8.2%, *P*<0.001). The distribution of the deleted allele in our cohort of aHUS patients, according to the susceptibility factor(s) identified in each patient, is depicted in Table 2.

Table 2 : Frequency of a CFHR1 deleted allele according to the susceptibility factor and comparison of each subgroup with the control population using a χ^2 test.

	Frequency of CFHR1 deleted allele (%) in each subgroup	Chi 2	*P*
Total cohort	22.3	12.6	*P= 0.0003*
No factor identified	18.3	3.04	*p = 0.08*
CFH mutation	7.9	0.03	*P = 0.9*
FI mutation	31.8	14.9	*P = 0.0001*
MCP mutation	2.9	1.25	*P=0.3*
C3 mutation	21.9	4.69	*P=0.03*
Combined mutations	14.3	0.5	*P=0.5*
Anti-FH IgG	92.9	94.7	*P<0.0001*
Cohort without anti-FH IgG cases	16.3	4.8	*P = 0.02*
Cohort without anti-FH IgG nor FI cases	13.8	2.43	*P = 0.12*
Control Population	8.6	-	

The frequency of *CFHR1* deletion was particularly high in the subgroup of patients presenting with an auto-immune form of aHUS, in which the allele frequency was 92.9% ($\chi^2 = 94.7$, *P*<0.0001). Among the patients with a genetic abnormality of the complement genes, only

those exhibiting a *CFI* mutation (31.8 %, *P*<0.001) or a *C3* mutation (21.9%, *P*=0.03) showed a significantly higher frequency of the deleted allele compared to controls. In the other subgroups, no significant difference was observed compared to controls. In patients in whom no genetic factor was identified, the frequency of the deleted allele was significantly higher than in the control population (18.3%, *P*=0.02). On the contrary, the frequency of the deleted allele was lower in subgroups of patients exhibiting a genetic abnormality in the *CFH* and *MCP* genes than in the control group (7.9% and 2.9% respectively, versus 8.6% in controls) but did not confer a significant protective status (OR=0.75, 95% CI= 0.25-2.21, *P*>0.1) (Figure 2). After exclusion of patients with anti-FH IgG and *CFI* mutation, the frequency of the *CFHR1* deletion in the cohort of aHUS patients was not significantly different from the frequency in the control group.

We then hypothesized that *CFHR1* deletion homozygosity alone could be a susceptibility factor. Therefore, we studied the distribution of homozygous *CFHR1* deletion in the different subgroups of patients according to susceptibility factor(s) (Table 3).

Table 3 : Frequency of a homozygous CFHR1 deletion according to the susceptibility factor and comparison of each subgroup with the control population using a χ^2 test.

	Frequency of homozygous CFHR1deletion (%) in each subgroup	Chi 2	*P*
Total cohort	12.4	5.24	*P= 0.022*
No factor identified	4.8	0.33	*NS*
CFH mutation	2.6	0.002	*NS*
FI mutation	15	6.45	*P = 0.011*
MCP mutation	0	0.50	*NS*
C3 mutation	6.3	0.31	*NS*

Combined mutations	0	0.20	*NS*
Anti-FH IgG	92.9	64.45	*P<0.0001*
Cohort without anti-FH IgG cases	5.5	0.77	*NS*
Cohort without anti-FH IgG nor FI cases	3.5	0.07	*NS*
Control Population	2.9	-	

The frequency of homozygous *CFHR1* deletion was higher than in the control population only in the subgroup of patients with *CFI* mutation (15% versus 2.9% in controls, *P* = 0.011) and in patients exhibiting anti-FH auto-antibodies (92.9% versus 2.9% in controls, *P*<0.0001). In this last subgroup, 13 of 14 patients exhibited homozygous *CFHR1* deletion. The frequency of the deletion homozygosity in the subgroup of patients with no known susceptibility factor was not significantly higher than in controls (5.5% versus 2.9%, OR = 1.96, 95% CI 0.32-11.8, *P*>0.1) (Figure 2).

We then performed *CFHR3* allele quantification by MPLA in the subgroup of patients with anti-FH IgG auto-antibodies. With one exception, a complete absence of the *CFHR3* gene was observed in all *CFHR1* deletion patients.

DISCUSSION

In our study, the deleted allele's frequency is 8.2% and 22.7% in the normal and the study population, respectively, with a deletion homozygosity 's frequencies of 2.9% and 12.4%. These results are in accordance with the previously reported frequencies in healthy controls and in aHUS patients. The frequency of the *CFHR1* homozygous deletion in different control populations has been estimated at 2% to 5.7% [4, 10, 18]. Zipfel et al. have reported a

frequency of homozygous deletion of *CFHR1* in 16% and 10.6% of aHUS patients from the Jena and Newcastle cohorts, respectively [4]. In addition, Hughes et al. have reported the presence of the deleted allele in 20% of chromosomes in a normal but elderly population (n=170). The frequency was significantly lower (8%) in an age-matched population (n=173) affected by AMD, suggesting that the *CFHR1* deletion confers protection against AMD (OR 0.4 (95%CI,0.3-0.5) [11]

The fact that the *CFHR1* deletion occurred at a higher frequency in the subgroups of patients exhibiting a mutation in a gene located outside the RCA locus (*CFI*, *C3*) suggests that a defect affecting one gene in the RCA locus might be necessary for the development of the disease or play a role for its severity. These roles remain to be elucidated.

The deletion homozygosity is particularly high in the subgroup of patients exhibiting anti-FH auto-antibodies. This group comprises eleven children (age at disease onset : 7 months –13 years, median : 9 years old, five males and six females) and three adults (median age at disease onset : 28 years old, all males). The deletion affects the *CFHR1* and *CFHR3* genes. These results confirm that the homozygous deletion of *CFHR1* and *CFHR3* genes is the genetic mechanism responsible for the absence of circulating CFHR1 and CFHR3 observed in the particular group of patients exhibiting an auto-immune form of aHUS [7].

The particular correlation that exists between the presence of a homozygous deletion of *CFHR1* and *CFHR3* genes and the development of anti-FH auto-antibodies reveals that most, if not all, patients with an "acquired" form of aHUS share the same homozygous genetic polymorphism. This genetic predisposition seems to be necessary but not sufficient for developing the disease, as this homozygous deletion is also observed in controls. The mechanisms responsible for development of auto-antibodies against FH are not yet understood. Our results suggest a particular role of CFHR1 and CFHR3 proteins in the development of this auto-immunity.

In the group of patients without genetic or acquired abnormality affecting the alternative pathway (n = 55), the frequency of the deletion homozygosity is not different than in the control population conferring a non significant risk for the disease. These results suggest that in our study population, the *CFHR1* deletion does not represent a susceptibility factor for aHUS by itself.

In conclusion, the high frequency of *CFHR1* deletion observed in our aHUS patients is due to the presence of *CFHR1* homozygous deletion in only two categories of patients: those with anti-FH auto-antibodies associated HUS and, to a lesser degree, those with a *CFI* mutation. The results highlight the link between the *CFHR1* and *CFHR3* homozygous deletion and the development of anti-FH auto-antibodies.

FIGURE LEGEND:

Figure 1 : Risk for developing aHUS associated with CFHR1 deletion homozygosity.
The Odds Ratio values are represented by circles. The risk was significant in the subgroups of patients with anti-FH auto-antibodies and CFI mutation. In 2 groups of patients, no Odds Ratio may be calculated (N/A: no available) as no CFHR1 homozygous deletion was found in these groups.

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Total cohort: 4.49

MCP mutation: N/A

Combined mutations: N/A

C3 mutation

CFH mutation

No factor identified

CFI mutation: 7.56

Anti-FH auto-antibodies: 442

-10   1   10   20   30   40

OR, 95% CI