

# Screening for Complement System Abnormalities in Patients with Atypical Hemolytic Uremic Syndrome

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The past decade has seen the identification of mutations in the genes for complement factor H (CFH) (1–6), membrane co-factor protein (MCP) (7–12), and factor I (CFI) (9,13–15) as predisposing factors for the development of atypical hemolytic uremic syndrome (aHUS). With this increased understanding of the genetic basis of aHUS and its implications for patient treatment, particularly transplantation, has come an increasing demand for genetic screening. A full analysis for mutations in all three of these complement proteins is expensive and time-consuming. A rational system is required to optimize the timely delivery of results and to reduce the cost of screening. We suggest some proposals that are based on our own experience with aHUS and our review of the relevant literature.

The strategy that we propose (Figure 1) involves an initial screen that is based on protein levels (either serum levels or surface expression). This offers a rapid mechanism to identify the likely gene involved. In those in whom normal levels of complement regulators are found, we propose screening genes on the basis of their order of frequency of mutation detection (CFH, approximately 30%; MCP, approximately 10%; CFI, 2 to 5%) (15). Furthermore, mutations cluster in certain exons of the genes that code for the protein (60% of CFH mutations cluster in complement control protein modules 19 and 20; 90% of MCP mutations in control protein modules 1 to 4; 60% of CFI mutations in the serine protease domain) (15). By screening these regions first, cost and detection time can be minimized.

## Serum Measurement

When possible, blood should be taken for CFH or CFI measurement before plasma infusion/exchange is commenced. If

blood is taken in a convalescent period, then it should be at least 2 wk after the last infusion of plasma to ensure that the levels measured are from natively synthesized proteins.

Serum C3 and C4 levels generally will form part of a basic complement screen. In almost all cases of aHUS, C4 levels are normal (9). Low C3 levels are commonly seen in patients with mutations in CFH, CFI, and MCP (9). However, this is not a sensitive screening test, and normal C3 levels do not exclude the presence of mutations in complement regulatory proteins (15). In those with mutations in CFH, approximately 50% have normal C3 levels. For CFI, this figure is 40% and for MCP approximately 70% (15). There is also a group with low C3 levels (approximately 30%) and no mutations in MCP, CFH, or CFI (9). This group likely represents a cohort with mutations in an as-yet-unidentified complement gene.

## CFH and CFI Levels

Because most cases of CFH-associated HUS have a heterozygous mutation in CFH (15), patients in whom the mutant protein is not expressed would be expected to have a 50% level. Factor H serum levels, however, vary considerably, and the range changes with age (neonates 170 to 397  $\mu\text{g/ml}$ ; adults 242 to 759  $\mu\text{g/ml}$ ) (16). Most children reach adult levels by 1 yr of age. It therefore is essential that an age-matched control panel be established in a reference laboratory to give interpretable results. No significant difference in CFH levels has been shown between genders (16).

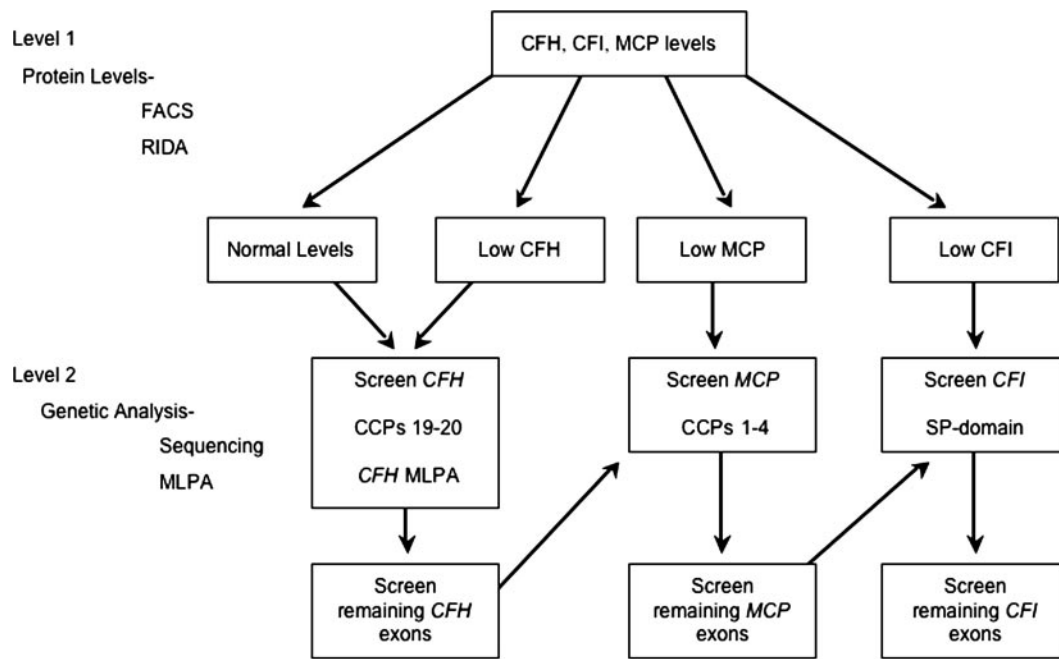
From the reports to date, serum measurement of CFH will detect approximately 25% of patients with mutations in CFH (15). As with CFH, there are differences in CFI concentration according to age (neonate 15 to 55  $\mu\text{g/ml}$ ; adults 39 to 100  $\mu\text{g/ml}$ ) (16). Again, most children reach adult levels by 1 yr of age. Serum measurement will be low in approximately 40% of mutations in CFI (15).

Commercially available radioimmunoassay kits for CFI and CFH are available and give reliable results on serum or plasma. Serum generally is preferred, and it should be collected and stored frozen as the laboratory does for C3 and C4 assays.

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*Figure 1.* Flow diagram demonstrating screening strategy in atypical hemolytic uremic syndrome (aHUS). Our suggested screening strategy in aHUS involves a three-tier approach. Level 1 investigations provide a quantitative assessment of protein concentrations by radioimmunoassay (RIDA) or FACS. Level 2 investigations provide a genetic assessment of complement genes using direct fluorescence sequencing and mixed ligation-dependent probe amplification (MLPA). The level 1 screen may demonstrate low levels of a protein and thereby suggest the mutated gene. Confirmatory level 2 genetic testing of the corresponding gene can then be undertaken. If this testing is negative at level 2, then a guide to further evaluation is presented in level 3, which is based largely on the frequency of mutations in each of the three genes in patients with aHUS. In those with normal protein expression, we suggest screening genes on the basis of frequency of mutation detection and on the clustering of mutations in certain exons of the genes (as is shown for level 2). Because of the high frequency of genomic rearrangements in the gene for complement factor H (*CFH*), we favor performing MLPA in addition to sequencing of control protein modules 19 and 20 initially. A possible alternative in patients with normal protein expression is to screen for the gene for factor I (*CFI*) after *CFH*, because of their greater relevance of a mutation to transplantation than is the case for the gene for membrane co-factor protein (*MCP*). However, mutations in *CFI* are much rarer than mutations in *MCP*. SP, serine protease domain.

## MCP Levels

FACS analysis of peripheral blood mononuclear cells provides a quick and relatively cheap screening option for MCP mutations. From currently available data, screening in this manner will detect approximately 75% of mutations (12). The mean fluorescence intensity in patients and family carriers of a heterozygous mutation should be approximately 50% of the normal range. Rare carriers of either compound heterozygous or homozygous MCP mutations show almost absent MCP signal by FACS (9,10,12).

There is a modest (approximately 20%) variation in MCP expression level on peripheral blood mononuclear cells using serial measurements up to 5 d after venipuncture (unpublished observations). When possible, blood should be examined at a standard number of days after collection and be compared with wild-type controls that were collected and stored under similar conditions. We recommend that blood be stored for no more than 5 d at 4°C before FACS analysis.

## Genetic Analysis of the Genes for CFH, CFI, and MCP

Screening for mutations by serum levels or cell surface expression will fail to detect low protein levels in 25 to 75% of mutations. Genetic analysis therefore also is required.

Genetic screening for mutations has most frequently been performed using either denaturing HPLC or single-strand conformation polymorphism. Both of these options are relatively inexpensive but are not sensitive. In addition, these techniques do not reveal the position of a mutation, and further confirmatory direct sequencing is required (17).

Sequencing PCR amplicons is now becoming the method of choice for mutation screening. Although it can be expensive, it is the most sensitive method available (17).

The *CFH* gene consists of 23 exons. Primers for mutation screening of *CFH* using single-strand conformation polymorphism, denaturing HPLC, and direct sequencing have been reported (3–6,18). The primers chosen will reflect whichever technology is available and optimized in the laboratory of choice. Furthermore, it is vital that the primers chosen be able to differentiate between *CFH* and the factor H-related proteins, especially CFHL1 (19). Errors have been made as a result of use of nonspecific primers, and this is a vital component of the screening process because the majority of *CFH* mutants cluster in the region of maximal CFHL1 homology (19,20). For this reason, it may be prudent to adopt the primers that already are used and verified by one of the recognized groups in this area.

*CFI* has 13 exons, and *MCP* has 14 exons. Primers for mutation screening for *CFI* and for *MCP* using direct sequencing again have been reported by several groups who are experienced in this field (9,10,13,14).

## Genomic Rearrangements

The genes for *CFH* and *MCP*, as well as many other complement regulatory genes, all are tightly linked in a region that is known as the regulators of complement activation (RCA) cluster on chromosome 1q32. The RCA cluster arose by several large genomic duplications. These duplications have resulted in a high degree of sequence identity between the gene for *CFH* and the genes for the five factor H-related proteins (*CFHL1* through 5) (21,22). These large genomic duplications predispose to gene conversion events and genomic rearrangements (23).

Unambiguous evidence of a role for gene conversion in generation of *CFH* mutants in aHUS was provided by Heinen *et al.* (24). In that study, the Goodship group described two patients with conclusive evidence of gene conversion and another nine patients in whom a *CFH* mutation could have arisen by gene conversion. This type of mutation can be detected by genomic sequencing.

The Goodship group also described a large genomic rearrangement in *CFH* that resulted in a hybrid gene in which exons 1 to 21 are derived from *CFH* and exons 22 and 23 are derived from *CFHL1* (25). Subsequent analysis of a large panel of patients with aHUS revealed similar deletions of exons 22 and 23 with formation of a hybrid gene in an additional three of 80 patients. Such a change accounts for approximately 5% of mutations in aHUS in this series. This is of particular importance when screening patients for mutations in *CFH*, because large genomic rearrangements of this type would not be detected by genomic sequencing using *CFH*-specific primers. Because these individuals lacked *CFH* exons 22 and 23 on one allele, the specific primers would amplify only exons 22 and 23 of *CFH* on the normal allele. The sequencing therefore would seem normal.

Given the high frequency of genomic rearrangements in *CFH*, it is recommended that all patients be screened with multiplex ligation-dependent probe amplification. No data yet exist for genomic rearrangements in *MCP* and *CFI*, however, multiplex ligation-dependent probe amplification of these genes should be considered in those in whom a mutation has not been discovered (17).

## Combined Mutations

When screening for mutations in complement regulators, it also should be noted that in several cases, individuals have had mutations in two complement regulators (9,11,26). This raises the question of whether after the identification of a mutation in one complement regulator, efforts should be made to seek out another predisposing gene. The small number of cases with multiple mutations discovered so far makes genotype-phenotype correlations difficult to derive. We believe that when a mutation has been identified in either *CFH* or *CFI*, which are poor prognostic indicators for successful renal transplantation,

there is no clinical utility in searching for another mutation. In those in whom an *MCP* mutation has been identified, which has a better prognosis for renal transplantation, there may be an argument for screening *CFH* and/or *CFI* to exclude mutations in these genes that would adversely affect transplant outcome.

## Autoantibodies

In those without detectable mutations in *CFH*, *CFI*, or *MCP*, autoantibodies to *CFH* have been identified in a small percentage of cases (approximately 6%) (27). An ELISA with purified human factor H-coated plates to capture anti-*CFH* antibodies is used.

## Interpreting Results

### Functional Significance of Mutations

Mutation screening of *CFH*, *MCP*, and *CFI* is challenging because many distinct disease-associated mutations are individually rare. In most cases, interpreting the functional significance of nonsense mutations, large gene rearrangements, frameshift mutations, and obvious splice junction mutations is clear-cut. However, a significant proportion of variants consist of missense mutations of unknown functional significance.

Functional studies have been performed for all *MCP* mutations that are associated with aHUS (reviewed in reference [12]) and for many *CFH* mutations (reviewed in reference [15]). Functional analysis of *CFI* mutations are awaited. These functional analyses have only been undertaken in a research situation and are too costly and time-consuming to be used in a diagnostic screening service.

In a diagnostic screening service, where functional analysis is not practical, a probability of significance is assigned to a missense mutant. This can be done by comparison with orthologous complement sequences, examination of the site of the mutation within the functional domains of the protein, and examination of the chemical difference in amino acid changes and how they would fit in available protein structures. This assignment often is far from absolute. Consequently, for many missense mutations, laboratories that specialize in these tests will need to be contacted so that they can assist in defining the functional significance of the change.

### Predisposing Genes

When interpreting the results of screening, it also should be noted that the mutations reported in the genes for *CFH*, *MCP*, and *CFI* in aHUS are increasingly believed to be predisposing rather than directly causal (*i.e.*, this is not a predictable single gene disorder, *e.g.*, adult polycystic kidney disease I and II).

Testing for predisposing mutations is a challenging issue. The general problem with predictive testing on the basis of a predisposing factor (as opposed to a causative mutation) is that, by definition, it is one of only several risk factors required for disease penetrance. Predictions that are based on a single risk factor in unaffected individuals are hazardous. From currently available data, the penetrance of disease for all mutations is approximately 50%. The degree of penetrance is thought to be determined by risk haplotypes in the RCA cluster (11,28,29) and by exposure to environmental triggers (*e.g.*, infection,

drugs) (9); consequently, the risk cannot be quantified for a given individual. In an unaffected family member with a mutation in a complement regulatory protein, screening may allow monitoring during periods of increased risk (*e.g.*, pregnancy, infections) and will allow avoidance of known precipitants of disease, such as the oral contraceptive pill.

### Management of aHUS

In those who have mutations and have developed aHUS, genotype–phenotype correlations can be seen. Those with *MCP* mutations have milder disease than those with either *CFH* or *CFI* mutations, and many recover without plasma therapy (9). This information is of limited use clinically because, at first presentation, the predisposing mutant will be unknown and will take many weeks to identify. Plasma therapy therefore will have been instigated long before a mutation has been identified. As treatment strategies to inhibit complement activation or enhance complement regulation come into clinical use, screening for mutations in patients with relapsing disease may become indicated.

### Renal Transplantation

The real utility of genetic screening is in those who already are on renal replacement as a result of aHUS and wish to be considered for a renal transplant. Renal transplantation is complicated by recurrence of aHUS in the allograft in >50% of cases. Historically, it was not possible to identify the subgroup of patients who did not have recurrent aHUS after transplantation. The characterization of the underlying molecular defect in aHUS has now revealed two distinct subgroups. For those with mutations in serum complement regulators (*CFH/CFI*), recurrence is very common. There have been 28 recurrences in 36 patients who had *CFH* mutations and received a renal transplant, and all six patients who had *CFI* mutations and received renal transplants have experienced recurrence. However, for those with mutations in the membrane-bound regulator *MCP*, recurrence was very rare, with only one recurrence in 10 transplants (30,31).

### Living-Related Renal Transplantation

Live-donor renal transplantation also carries a poor prognosis; moreover, in four reported cases, the donors themselves have gone on to develop HUS within 1 yr of donation (32). Many centers do not recommend live-related transplantation because of the risk for recurrence in the recipient and of *de novo* disease in the donor.

Genotyping of the donor and recipient should be undertaken when live donation is to be considered. This will not, of course, prevent the risk for the donor in those with an unknown genetic basis.

### Combined Liver/Kidney Transplantation

Genotyping also is important when considering aHUS patients for combined liver and renal transplants. Because *CFH* and *CFI* both are synthesized in the liver, combined liver and renal transplantation seems to be a logical form of treatment. Although the initial three reports (33–35) that described this for

*CFH*-associated HUS had a poor outcome, with two of three dying, a more recent report using plasma exchange preoperatively was successful (36). Unless functioning *CFH* is already present in the recipient, complement activation will result in rapid failure of the graft (36).

### Conclusion

We propose a method for screening for mutations in complement regulatory proteins in patients with aHUS. We believe that the evidence suggests that all patients who have aHUS and are being considered for renal transplantation should undergo screening for mutations in complement regulators. This will allow patients and clinicians to make informed decisions regarding listing for transplantation, on the basis of the risk for recurrence and its not inconsiderable consequent morbidity and mortality. Screening of unaffected family members has limited clinical utility unless they are being considered as living-related kidney donors. A useful source of guidance for genetic screening in aHUS is GeneTests, an National Institutes of Health–funded Web site that provides information on commercial and research-based testing for various genetic disorders (<http://www.genetests.org>).

### Disclosures

None.

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