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Insights into Hemolytic Uremic Syndrome: Segregation of three independent predisposition factors in a large, multiple affected pedigree.

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Abbreviations:

MCP: Membrane Cofactor Protein or CD46; aHUS: atypical Hemolytic Uremic Syndrome; SNP: Single Nucleotide Polymorphism; CFH: Complement Factor H; IF: Factor I;

ABSTRACT

Mutations in the complement regulators factor H, Membrane Cofactor Protein (MCP), and factor I are associated with atypical Hemolytic Uremic Syndrome (aHUS, MIM 235400), suggesting that the disease develops as a consequence of the inefficient protection of the renal endothelium from damage by the complement system. Incomplete penetrance of the disease in individuals carrying these mutations is, however, relatively frequent. Here, we report the identification of a large, multiple affected aHUS pedigree in which there is independent segregation of three different aHUS risk factors: a *MCP* missense mutation (c.598C>T; Pro165Ser) that decreases MCP expression on the cell surface, a dinucleotide insertion in the coding sequence of factor I (c.1610insAT) that introduces a premature stop codon in the factor I protein, and the *MCP ggaac* SNP haplotype block that was previously shown to decrease the transcription activity from the *MCP* promoter. Interestingly, individuals affected by aHUS in the pedigree are only those who have inherited the three aHUS risk factors. These data show an additive effect for mutations in MCP and factor I and provide definitive support to the conclusion that aHUS results from a defective protection of cellular surfaces from complement activation. Furthermore they help to explain the incomplete penetrance of the disease, illustrating that concurrence of multiple hits in complement regulatory proteins may be necessary to significantly impair host tissue protection and to confer susceptibility to aHUS.

1. Introduction

Hemolytic Uremic Syndrome (HUS) is clinically defined by thrombocytopenia, Coomb's test negative microangiopathic haemolytic anemia and acute renal failure. Most HUS cases occur associated to *E.coli* infections leading to hemorragic diarrhea (Karmali, 2004). This typical form of HUS usually resolves satisfactorly and complete recover of the renal function is achieved. However, five to ten percent of HUS cases lack a particular relationship with infection and have a poorer prognosis (Moake, 2002). This idiopathic atypical form of HUS (aHUS) is frequently associated with immunosuppressive drugs, cancer therapies, oral contraceptives, pregnancy or postpartum. The molecular mechanisms underlying atypical HUS are not completely understood. The complement system has been implicated in the pathophysiology of this syndrome for many years (Thompson and Winterborn, 1981), but only recently mutations in the genes for the complement proteins factor H (*CFH*) (Warwicker et al. 1998, Pérez-Caballero et al. 2001, Caprioli et al. 2001, Richards et al. 2001, Sánchez-Corral et al. 2002, Manuelian et al. 2003; reviewed in Rodríguez de Córdoba et al. 2004), membrane cofactor protein (*MCP*) (Noris et al. 2003, Richards et al. 2003) and factor I (*IF*) (Fremeaux-Bacchi et al. 2004, Kavanagh et al. 2005) have been shown to predispose to aHUS. Functional characterization of several of these mutations suggest that the disease likely develops as a consequence of a defective protection of cellular surfaces from complement activation due to an improper function of complement regulatory proteins (Sánchez-Corral et al. 2002, Manuelian et al. 2003, Richards et al. 2003, Sánchez-Corral et al. 2004).

Despite these advances in our understanding of the molecular basis of aHUS,

incomplete penetrance of the disease in individuals carrying factor H, MCP or factor I mutations is relatively frequent, suggesting the existence of additional genetic factors contributing to aHUS. Recently, different groups have reported that relatively frequent *CHF* and *MCP* SNPs are strongly associated with aHUS (Caprioli et al. 2003, Esparza-Gordillo et al. 2005, Fremeaux-Bachi et al. 2005). Among these SNPs, the *MCP* haplotype block *MCPggaac* is particularly interesting because it includes two SNPs, c.-547G/C and c.-261G/C, that influence transcription from the *MCP* promoter in transient transfection experiments (Esparza-Gordillo et al. 2005). Moreover, the observation that *MCPggaac* was especially frequent among patients who carry mutations in *CFH*, *MCP* or *IF* suggested that the concurrence of different mutations and polymorphisms in the complement regulatory genes increases predisposition to aHUS (Esparza-Gordillo et al. 2005). Here we provide further support to this conclusion identifying a pedigree in which the affected individuals carry three different genetic susceptibility factors in two different complement regulatory genes (*IF* and *MCP*).

2. Patients and methods

2.1. Family RCO.

This study focuses on the characterization of aHUS genetic predisposition factors in a large, multiple affected Spanish pedigree, referred to as family RCO. This pedigree was initially selected because we found that two patients in the Spanish HUS registry, HUS 68 and HUS 84, were first cousins and the only affected members in their family. HUS 68 has been reported earlier (Esparza-Gordillo et al. 2005). All protocols included in these studies have been approved by national and/or local institutional review boards, and all subjects gave their informed consent.

2.2. Case reports.

HUS 68 (Figure 1, III-5) presented with acute renal failure, thrombocytopenia and Coomb's test negative microangiopathic haemolytic anemia at the age of 57. Plasma creatinine was 405 µmol/L. Renal biopsy was not performed due to the small size of her kidneys. Because of the rapid and progressive loss of renal function, hemodialysis was commenced and plasma exchange was undertaken which controlled the microangiopathic anemia and stabilized the creatinine levels around 300 µmol/L. Fifteen months later she presented again with microangiopathic anemia and thrombocytopenia, showing alternative pathway complement activation. Despite repeated plasma exchanges and fresh plasma infusions, she rapidly lost renal function and since 1994 she is being treated with hemodialysis. She is currently 68 years old.

HUS 84 (Figure 1, III-8) presented with HUS at the age of 41 after an acute catarrh episode. She was under periodic hemodialysis for six months and recovered a limited renal function that allowed her to abandon the hemodialysis

program. However, progressive loss of renal function took her back to hemodialysis in 2004, 17 years later. HUS 84 is currently 59 years old.

2.3. The relatives of HUS 68 and HUS 84.

Individuals II-5, III-1, III-3, III-4, III-6, III-7, III-9, III-10, III-11, IV-1, IV-2 and IV-3 (Figure 1) are all alive and healthy with no history of renal disease. Current age for each of these indivuduals is depicted in Figure 1. All other members of the pedigree RCO are deceased. Relatives I-1, I-2, II-2, II-3, II-4 and II-7 died at advanced age, most of them over 80 years old, with no record of renal disease. II-1 and II-8 died in their 50s from heart disease. II-6 is the only member of the RCO pedigree who died in her 30s and the only one who may have had an hemolytic anemia related disorder. No samples were, however, available for analyses from this individual or from any of the deceased relatives of HUS 68 and HUS 84.

2.4. Complement analyses

C3, C4, factor H and factor I levels were measured in serum or plasma samples as previously described (Pérez-Caballero et al. 2001, Esparza-Gordillo et al. 2004, Gonzalez-Rubio et al. 2001). The standard hemolytic assays CH50 and AP50, and a recently described hemolytic assay to test factor H function (Sánchez-Corral et al. 2004) were performed to assess complement function and regulation. Expression levels of the membrane regulators MCP (CD46) and Decay Accelerating Factor (DAF or CD55) in peripheral blood lymphocytes (PBLs) were analyzed by flow cytometry in whole blood samples drawn in the presence of EDTA. Cells were assessed using three-colour acquisition on a FACSCalibur (Becton Dickinson, CA, USA). The monoclonal antibodies PE-anti-CD45, FITC-anti-CD46 and PerCP-anti-CD55, and the appropiate isotypic

controls were purchased from BD Biosciences (Pharmingen, San Diego, CA, USA). PBLs were examined using side-scatter versus CD45 gating, and data analysed using CELLQUEST software (Becton Dickinson).

2.5. Genomic analyses of complement regulatory genes

Patients and their relatives were screened for mutations and polymorphisms in *CFH, MCP* and *IF* genes. DNA from these individuals was extracted from PBLs or from buccal mucosa cells collected with cheek brushes (MasterAmp buccal swab brush, Epicentre Technologies) using standard procedures. Each exon of the *CFH, MCP* and *IF* genes was amplified from genomic DNA of patients using specific primers derived from the 5' and 3' intronic sequences. The sequence of these primers and the PCR conditions used for the amplifications are described elsewhere (Pérez-Caballero et al. 2001, Richards et al. 2003, Fremeaux-Bacchi et al. 2004). Direct sequencing of PCR products was performed automatically in an ABI 3730 sequencer using a dye terminator cycle sequencing kit (Applied Biosystems, NJ). *MCP* c.2181T>C SNP (NCBI id rs7144) was used to identify the presence of the *MCPggaac* SNP haplotype block. Genotyping of the *MCP* c.2181T>C and the *CFH* -257C>T SNPs was performed by allelic discrimination on 10 ng of genomic DNA using TaqMan probes (MCP assay id: 2784726; CFH assay id: 2530387; Applied Biosystems; Foster city, CA) and real time PCR equipment (PE7700; Applied Biosystems; Foster city, CA) following the manufacturer specifications.

3. Results

Blood samples obtained from patients HUS 68 and HUS 84 showed normal complement activity through the classical (CH50) and the alternative (AP50) pathways. Similarly, plasma levels of C3, C4 and factor H also were within the normal reference range of variation (Table 1). However, both patients presented half-normal plasma levels of factor I, and also showed a 50% reduction in the MCP expression levels in PBLs (Table1). These observations prompted us to search for mutations in the *MCP* and *IF* genes in both patients.

MCP sequencing revealed a novel heterozygous mutation (c.598C>T; Pro165Ser) in exon 5, encoding Short Consensus Repeat 3 (SCR3), in both aHUS patients (Figure 2a). This missense mutation shows a perfect segregation with the 50% reduction in the MCP expression levels in PBLs that present different members of the RCO pedigree (Table 1 and figure 1). Pro165Ser results in a partial MCP deficiency because it affects a fully conserved proline residue that is characteristic of the SCR consensus sequence (Law and Reid. 1995) and likely interferes with the proper folding and transport of MCP to the cell membrane (Figure 2b and 2c). The 50% reduction in MCP levels place the carriers of the Pro165Ser MCP mutation outside the normal range of MCP levels (Table 1). The pathogenic consequences of a 50% reduced MCP expression in the context of aHUS have been demonstrated in earlier work (Noris et al. 2003, Richards et al. 2003).

Sequencing of the *IF* gene led to the identification of an heterozygous c.1610insAT mutation in both patients and some of their relatives (Figures 1 and 3b). This dinucleotide insertion, located in *IF* exon 13, causes a frameshift that generates a truncated factor I protein at position 538 that lacks most of the

fuctional serine protease region. In addition, as illustrated in Figure 3b, this truncated form of factor I is likely not secreted to the plasma, as it cannot be detected in western blots of whole plasma from the patients using anti factor I polyclonal antibodies. A total of five *IF* mutations leading to factor I deficiency have been reported thus far in aHUS patients (Fremeaux-Bacchi et al. 2004, Kavanagh et al. 2005). The factor I mutation described here is a novel mutation located in the C-terminal region of the protein, 8 residues upstream of another HUS-associated mutation (c.1637G>A; Trp546Stop) that also leads to partial factor I deficiency (Fremeaux-Bacchi et al. 2004).

Since HUS 68 and HUS 84 are first cousins, the presence of identical *MCP* (1q32) and *IF* (4q25) mutations in both patients clearly indicate a germline transmission from a common ancestor. Analysis of all available relatives in family RCO showed segregation of the *MCP* and *IF* mutations, supporting this conclusion (Figure 1). Furthermore, as expected, we observed that members of the RCO pedigree carrying the Pro165Ser *MCP* mutation have reduced MCP levels on their lymphocytes and that carriers of the c.1610insAT *IF* mutation, with the exception of IV-2, also had decreased levels of factor I in plasma (Table 1). Together, these findings strongly support that the identified *IF* and *MCP* mutations lead to partial factor I and MCP deficiencies, respectively. Both, factor I and MCP partial deficiencies, have been reported previously to confer separately susceptibility to aHUS (Noris et al. 2003, Richards et al.2003, Fremeaux-Bacchi et al. 2004, Kavanagh et al. 2005).

A total of five individuals in our pedigree carry mutations in both factor I and MCP (HUS 68, HUS 84, III-11, IV-1 and IV-2) and four additional ones carry mutations in either factor I or MCP (III-3, III-9, III-10 and IV-3), however, only two

of them (HUS 68 and HUS 84) have developed aHUS thus far. To identify additional aHUS-susceptibility factors in the RCO pedigree that may explain the differences between HUS 68, HUS 84, III-11, IV-1 and IV-2, we analyzed the levels of expression of decay accelerating factor (DAF; CD55), we functionally characterized factor H in the serum from affected and non-affected members (Sánchez-Corral et al. 2004), and we genotyped all members of the pedigree for the presence of *CFH* and *MCP* aHUS-associated SNPs (Caprioli et al. 2003, Esparza-Gordillo et al. 2005, Fremeaux-Bacchi et al. 2005). DAF levels and factor H activity were normal and all individuals in the pedigree carry the *CFH*-257T risk allele (not shown). Therefore differences among carriers of the *MCP* and *IF* mutations cannot be atributed to these parametres. Interestingly, the *MCPggaac* risk allele was present in family RCO and segregated independently of the Pro165Ser *MCP* mutation, demonstrating that these two genetic traits are carried by different *MCP* alleles. Segregation analyses in family RCO also revealed that the patients HUS 68 and HUS 84, but not III-11, IV-1 and IV-2 carried the *MCPggaac* risk allele, strongly suggesting that concurrence of the three aHUS susceptibility factors greatly influences the disease manifestation in this family.

4. Discussion

We report here the identification of three independent aHUS risk factors in a large Spanish pedigree with two members affected. These factors are: a *MCP* missense mutation (c.598C>T; Pro165Ser) that decreases MCP expression on the cell surface, a dinucleotide insertion in the coding sequence of factor I (c.1610insAT) that introduces a premature stop codon in the factor I protein, and the *MCPggaac* SNP haplotype block that was previously shown to influence the transcription activity from the *MCP* promoter (Esparza-Gordillo et al. 2005). Segregation analysis demonstrated that the three aHUS risk factors segregate independently and, most important, that individuals, thus far affected from aHUS in this pedigree are only those who inherited the three risk factors (Figure 1). The HUS pedigree reported here is exceptional because mutations in *MCP* and *IF* are very rare. Together, our findings provide further support for the concept that aHUS results from a defective protection of cellular surfaces from complement activation and help to understand the incomplete penetrance of the disease in carriers of mutations in genes encoding complement regulatory proteins.

The efficiency of the complement system as an innate defense mechanism against microbial infections depends on a fine control that avoids the wasteful consumption of its components and restricts its activation to the surface of microorganisms, thus preventing non-specific damage to host tissues. Control of the complement system is performed by a set of plasma and membrane-associated regulatory molecules acting as a protein network (Law and Reid, 1995). Genetic and functional analyses have shown that this critical control of complement activation may be impaired in aHUS patients. Accordingly, it is

generally accepted that the mutations in *CFH*, *MCP* or *IF* found in many patients predispose to aHUS because they generate a situation unable to provide efficient protection to the host cellular surfaces in the case of complement activation (Warwicker et al. 1998, Pérez-Caballero et al. 2001, Caprioli et al. 2001, Richards et al. 2001, Sánchez-Corral et al. 2002, Manuelian et al. 2003, Noris et al. 2003, Richards et al. 2003, Fremeaux-Bacchi et al. 2004, Kavanagh et al. 2005, Sánchez-Corral et al. 2004).

The incomplete penetrance of aHUS often found in carriers of the *CFH*, *MCP* and *IF* mutations could be explained by the existence of genetic modifiers of these mutations. Individual IV-2 (Figure 1) could be an example of this situation. Despite carrying the c.1610insAT *IF* mutation, IV-2 shows levels of factor I in plasma that are comparable to those found in the reference control sample (Table 1). Like many other complement components, factor I shows a relatively large (two-fold) normal range of variation (Gonzalez-Rubio et al. 2001; Fremeaux-Bacchi et al. 2004; Kavanagh et al. 2005). IV-2 could have inherited a paternal *IF* allele with high expression and, thus, compensate the effect of the c.1610insAT *IF* mutation. Similar quantitative variations have been observed in carriers of *CFH* mutations in other aHUS pedigrees with incomplete penetrance of the disease (Fremeaux-Bacchi et al. 2002).

The case of HUS68 and HUS84 in family RCO and previous findings describing concurrence of different predisposition factors in HUS patients provides an explanation for the incomplete penetrance of the disease in carriers of *CFH*, *MCP* and *IF* mutations. They suggest that multiple hits, probably involving plasma and membrane-associated complement regulatory proteins, are required to impair protection to host tissues significantly. Factor I and MCP are

proteins acting in the same complement regulatory pathway (Law and Reid, 1995). Therefore, it is likely that genetic variants decreasing the levels of MCP and factor I have an additive effect in the context of complement regulation and aHUS susceptibility. Similarly, concurrence of the Pro165Ser mutation with the risk allele *MCPggaac* may have an additive effect reducing further the expression of MCP in carriers of the *MCP* mutation. Although we have not been able to document a further decrease of MCP levels in PBLs in members of the RCO pedigree carrying both the Pro165Ser mutation and the risk allele *MCPggaac* (Table 1), our earlier observation that two SNPs, c.-547G/C and c.-261G/C, included in the *MCPggaac* haplotype block decreases transcription from the *MCP* promoter in transient transfection experiments (Esparza-Gordillo et al. 2005) supports this conclusion. *MCPggaac* is an important HUS risk factor that has been found strongly associated with the disease in three different HUS cohorts (Esparza-Gordillo et al. 2005, Fremeaux-Bacchi et al. 2005). Future experiments should address whether carriers of the *MCPggaac* allele show reduced expression of MCP locally in the kidneys or in conditions of infection or inflammation.

We found remarkable that in family RCO the individuals affected from aHUS are only those who inherited the three risk factors segregating in this pedigree (Figure 1). We are aware that HUS68 and HUS84 presented with the disease at the ages of 57 and 41, relatively late for aHUS, and that there is always the possibility that those in the kindred with one or two risk alleles may get disease in the future. However, considering that many of the individuals carrying one or two risk alleles are at present over the age of onset of HUS 68 and HUS84, we suggest that the concurrence of different genetic risk factors influences the onset of the disease and the penetrance of aHUS in carriers of *MCP* and *IF*

mutations (Figure 1).

We believe that these findings provide clues to understand the incomplete penetrance of the disease in other aHUS pedigrees and, therefore, we suggest that all genes currently known to be aHUS risk factors should be analyzed routinely in aHUS patients. Furthermore, analysis of additional proteins involved in complement activation, such as C3, factor B or properdin, may unravel novel aHUS risk factors and provide further insights into genetic factors predisposing to aHUS in the still high percentage of aHUS patients in whom mutations in *CFH*, *MCP* or *IF* have been excluded.

In conclusion, our data indicate that concurrence of different susceptibility alleles affecting complement regulator expression greatly influences predisposition to aHUS and provides an explanation for the incomplete penetrance of aHUS in carriers of mutations in the complement regulatory genes. They also consolidate the hypothesis that an inefficient protection of the cellular surfaces from complement activation is a general feature of patients with aHUS. As a whole our current knowledge of the molecular mechanisms underlying aHUS reinforce the concept that complement inhibition therapies to prevent or reduce tissue damage by complement activation is indicated for the treatment of aHUS patients.

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FOOTNOTES FOR THE FIGURES

Figure 1. Pedigree of family RCO.

Affected individuals are indicated with an arrow. Deceased individuals are crossed. Carriers of the *MCP* c.598C>T mutation, the *IF* c.1610insAT mutation and the *MCPggaac* risk haplotype are indicated by a code described at the bottom of the pedigree. Current age of individuals in the pedigree is indicated (for deceased members, age at death was indicated in Patients and Methods).

Figure 2. Mutation in Membrane Cofactor Protein.

a) Identification of MCP mutations in patients HUS 68 and HUS 84. The chromatogram corresponding to the DNA sequence surrounding the mutated nucleotide in *MCP* exon 5 is shown for the HUS68/HUS84 patients and for a control sample. The corresponding aminoacid sequences for the wild type and the mutated alleles are shown at the right. The amino acid numbering is referred to the translation start site (Met +1) and the nucleotide nomenclature is refered to the transcription start site described by Cui et al. (1993). **b)** Diagram of the MCP molecule with four extracellular SCR domains, a transmembrane region and an intracytoplasmic tail. The location of the Pro165Ser mutation in the consensus sequence of a prototypic SCR domain (circled amino acids) (Law and Reid, 1995) is indicated by an arrow. **c)** Flow cytometry analysis of peripheral blood lymphocytes from the HUS 68/HUS 84 patients (red) and from normal control samples (blue). Isotypic control is shown in black.

Figure 3. Mutation in factor I.

a) Identification of factor I mutations in patients HUS 68 and HUS 84. The chromatogram corresponding to the DNA sequence surrounding the mutated

nucleotide in *IF* exon 13 is shown for the HUS 68/HUS 84 patients and for a control sample. The corresponding aminoacid sequences for the wild type and the mutated alleles are shown at the right. The amino acid and nucleotide numbering is referred to the translation start site (Met +1). Nucleotide 1 is the adenine of the start (ATG) codon. **b)** Diagram of the factor I molecule with the protein domains that organize the heavy and light chains indicated. I/C_{67}, Factor I/membrane attack complex C6/7 module; SCAr, Scavenger receptor; LDRr, Low density lipoprotein receptor; SP, serine protease domain. The location of the stop codon generated by the c.1610insAT mutation is indicated by an arrow. **c)** Western blot of unreduced IF in human serum. Arrrow indicate the position of the factor I protein as detected by a goat anti human factor I polyclonal antibody (Quidel. San Diego. CA)

Table1

TABLE 1. Complement profiles in members of family RCO.

	C3[1, 2] (77-210mg/dl)	C4[2] (14-47mg/dl)	Factor H[3] (12-56mg/dl)	Factor I[4] (75-115 %)	MCP[5] MFI (38-55) (% of control)	Factor H Hemolytic assay[5] (2-25% of total lysis)
II-5	102	21	34	106	44.5 (99)	17
III-5 (HUS68)	96	42	30	53	28 (62)	5
III-8 (HUS84)	84	27	30	46	25.4 (56)	2
III-9	128	32	39	53	42.9 (95)	3
III-10	112	25	24	101	27.6 (61)	4
IV-1	102	24	30	50	24.9 (55)	1
IV-2	128	16	31	95	25.1 (56)	3
IV-3	124	26	32	100	24.2 (54)	3

1) Normal range of variation in controls is shown between brackets for each variable.

2) C3 and C4 were determined by nephelometry.

3) Factor H and Factor I plasma levels were determined by ELISA. Factor I levels are referred to a reference serum; each value corresponds to the mean of three independent determinations.

4) Levels of MCP in PBLs (MFI, mean fluorescence intensity) were determined by flow cytometry and referred to a control sample drawn and analysed the same day as indicated in Patients and Methods.

5) Lysis observed in samples from control individuals varies from 2 to 25% of total lysis (Sánchez-Corral et al. 2004).

Figure1

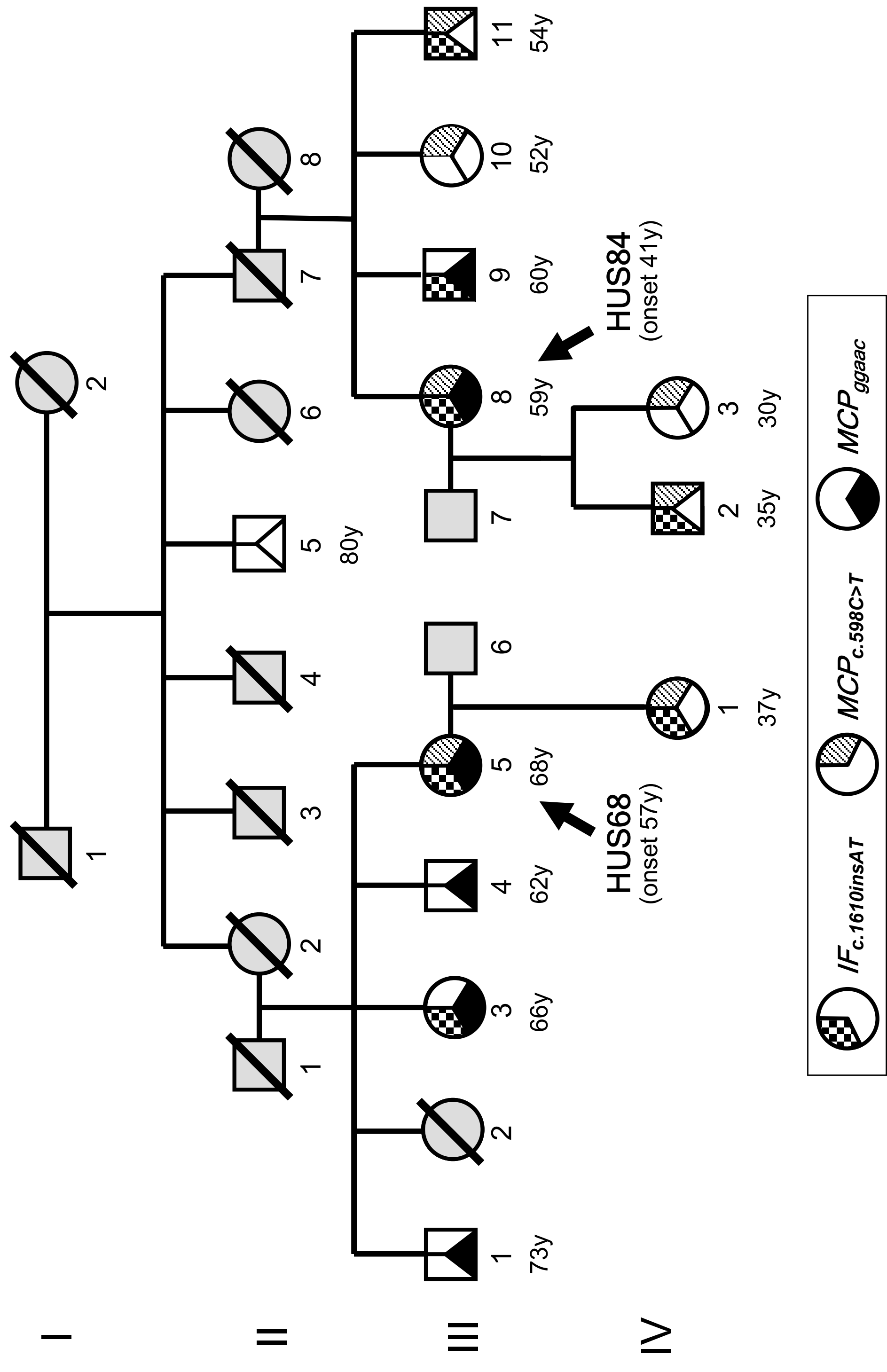
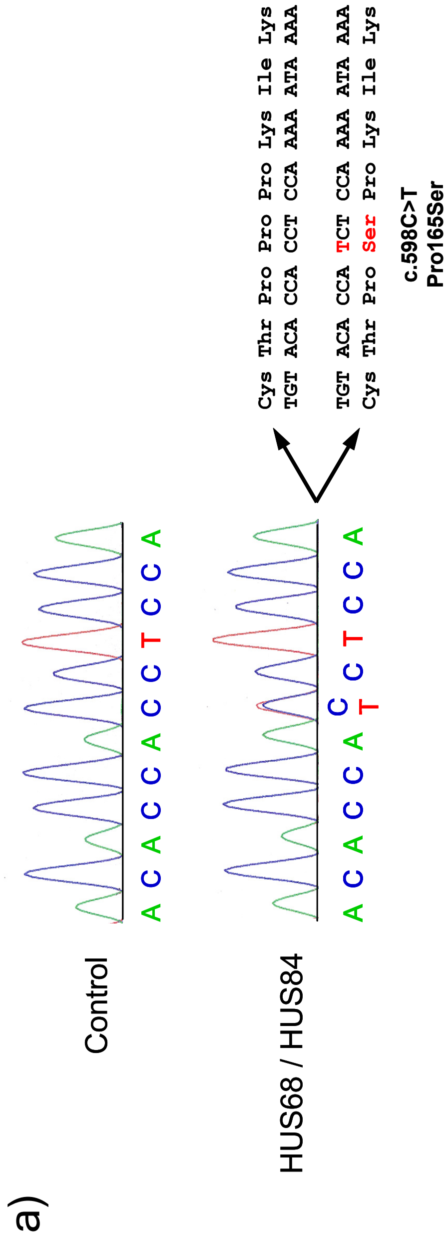
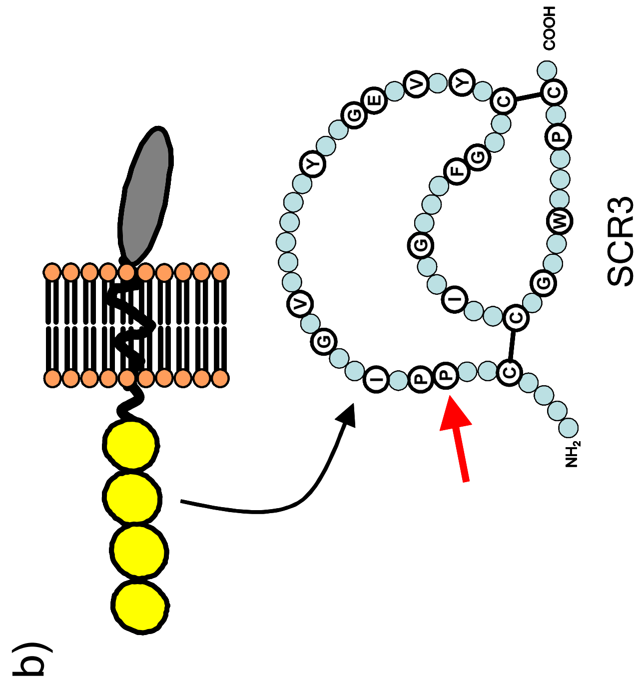


Figure2





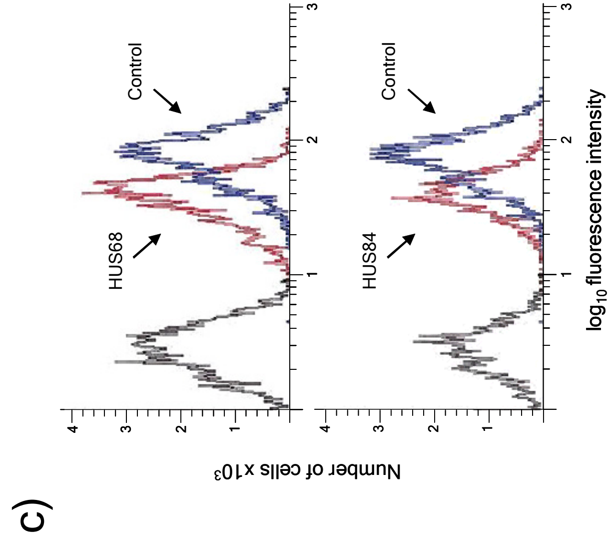


Figure3