Thrombotic microangiopathy in *INF2* mediated renal disease.

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Abstract

The demonstration of impaired complement regulation in the thrombotic microangiopathy, atypical haemolytic uraemic syndrome (aHUS) has resulted in the successful introduction of the complement inhibitor eculizumab into clinical practice. Complement abnormalities account for approximately 50% of aHUS cases however recently mutations in the non-complement gene *DGKE* have been described in individuals not responsive to eculizumab.

We report here a family where the proposita presented with aHUS but did not respond to Eculizumab. Her mother had previously presented with a post renal transplant thrombotic microangiopathy (TMA). Both also had Charcot Marie Tooth (CMT). Using whole exome sequencing we identified a mutation in *INF2* in the mutational hotspot for focal segmental glomerulosclerosis (FSGS). Subsequent analysis of the Newcastle aHUS cohort identified another family with a functionally significant mutation in *INF2*. In this family renal transplantation was associated with post transplant TMA. In all individuals with *INF2* mutations presenting with a TMA, aHUS risk haplotypes were also present, potentially accounting for the genetic pleiotropy.

Identifying individuals with TMAs who may not respond to eculizumab will avoid prolonged exposure of such individuals to the infectious complications of terminal pathway complement blockade.

Key words

Inverted formin 2, Charcot Marie Tooth. Atypical Haemolytic Uraemic Syndrome, Focal Segmental Glomerulosclerosis

Genetic abnormalities in the alternative pathway of complement have been demonstrated to account for many cases of the thrombotic microangiopathy (TMA) atypical haemolytic uraemic syndrome (aHUS)(MIM 235400)[1]. Understanding the role of complement in the pathogenesis of aHUS has resulted in the successful introduction of the complement inhibitor eculizumab into clinical practice[2].

Recently mutations in the non-complement gene *DGKE* have been demonstrated to be associated with aHUS (MIM615008)[3]. Individuals with *DGKE* mutations display phenotypic variability with some patients presenting with membranoproliferative glomerulonephritis[4]. As might be expected with a genetic cause which does not appear to be complement mediated, individuals have not responded to treatment with eculizumab[3].

Despite recent advances, the genetic basis of many cases of familial aHUS remain unsolved. In this study we describe the finding of *INF2* mutations in two families with TMA.

Our index case, patient III:2 presented aged 7 with pex cavus and difficulty walking and was diagnosed with Charcot-Marie-Tooth (CMT) (figure 1). Aged 15 she presented 5 days after a sore throat with microangiopathic haemolytic anaemia on blood film (Hb, 9.4 g/dl), thrombocytopenia (platelets 119 $\times 10^9$/L) and renal failure (creatinine 879 µmol/l). Haptoglobins were undetectable, LDH was 844 U/L and there was proteinuria (4.8g/l). Blood pressure on admission was 185/110 mm Hg. Haemodialysis was commenced at presentation and plasma exchange was undertaken prior to commencement of eculizumab. Initially there was an improvement in the platelet count to 173 $\times 10^9$/L but subsequently this fell to 100 $\times 10^9$/L. A bone marrow biopsy was unremarkable and trough eculizumab concentration was adequate with a completely suppressed CH50. Three months after presentation a renal biopsy

was undertaken demonstrating characteristic changes of a thrombotic microangiopathy (figure 2a). There were also features of a distinct glomerulosclerosis with small glomeruli and arteriosclerosis (figure 2b). After 9 months with no renal recovery eculizumab was withdrawn.

Screening for known inherited and acquired causes of aHUS did not reveal any abnormality[1]. The *C5* variant c.2654G>A, (p.R885H) which impairs eculizumab efficacy was not present[5].

Family history revealed that the proposita's mother, patient II:2 (figure 1), also had CMT, had presented with ESRF aged 17 and had a post transplant TMA (figures 2d,e) (case history supplementary data).

Due to the absence of an abnormality in a known aHUS associated gene we sequenced the exomes of the two affected individuals in this family.

This revealed a rare variant in *INF2*, c.305T>A (p.V102D) (figure 1). Mutations in *INF2* are the commonest cause of familial autosomal dominant nephrotic syndrome[6-8]. In a minority of these cases the mutations cause a syndromic form of Focal Segmental Glomerulosclerosis (FSGS) associated with the demyelinating peripheral neuropathy, CMT [9, 10] (figure 3).

To assess whether mutations in *INF2* account for other cases of familial or sporadic TMA previously referred to the Newcastle aHUS centre[11] we analysed 28 familial cases by exome sequencing and undertook Sanger sequencing on an additional 161 sporadic aHUS cases. In one family, in which no known genetic risk factors had been found, we identified a functionally significant mutation in *INF2*, c.530G>A (p.R177H), which segregated with the disease (supplementary data, Figures 1,2). No *INF2* variants were identified in sporadic aHUS cases.

INF2 is a ubiquitously expressed formin protein[12] which accelerates actin polymerization and depolymerisation, thus regulating a range of cytoskeleton dependent cellular functions including the secretory pathway [13, 14]. INF2 comprises formin homology 1 and 2 domains (FH1 and FH2); an N-terminal diaphanous inhibitory domain (DID) and a C-terminal diaphanous autoregulatory domain(DAD)[15]. Mutations in *INF2* predominate in the DID domain[6, 7, 10]. Functional analysis of *INF2* mutations in disease has demonstrated disorganised cytoskeletal functions[7, 9] although the precise mechanism of disease remains elusive.

The two *INF2* variants we describe here reside in the mutational hotspot for disease [6-10]. The c.305T>A (p.V102D) variant resides in exon 2 while c.530G>A (p.R177H) resides in exon 4 (figure 3, 4). Structural modelling reveals that the p.V102D variant is in close proximity to the DAD binding region. Modelling does not predict a surface exposed residue but instead the variant may be expected to disrupt the architecture of the 8th α-helix of the DID domain (Figure 4). The p.R177H variant resides before the 13th α-helix of the DID domain and is surface exposed (figure 4). Amino acids at both these positions are conserved across species with GERP++ scores of 4.76 (p.V102D) and 4.48 (p.R177H) (figure 1).

Disease-causing mutations in FSGS, have mainly been found to occur in the DID domain, in exons 2-4, with only one report of a mutation in exon 6 (figure 3)[6-8, 16]. Within this hot-spot there is a cluster between nucleotides 300 and 500 which accounts for those with FSGS and CMT[9, 10]. The p.V102D mutation resides in this region and this family have CMT while the p.R177H mutation resides downstream of this region and this family has no neurological phenotype. The p.R177H mutation has previously been reported in 3 unrelated pedigrees and in all cases had the non-syndromic form of

FSGS[6, 8]. Functional analysis of this mutation demonstrated altered INF2 localisation and disruption of the actin cytoskeleton[9].

It is well reported that even in individual families with the same *INF2* mutation there is phenotypic variability. Most commonly individuals present with disease in adolescence with mild proteinuria, developing ESRF in the 3rd or 4th decade, although individuals have been reported to be unaffected into their 6th or 7th decade[16]. Variable intrafamilial penetrance has also been reported for the neurological phenotype[16]. The clinical and pathological disease pleiotropism we describe with *INF2* mutations is also seen in individuals with recessive *DGKE* mutations where some individuals present with proteinuria and progressive renal failure while others present with aHUS. Likewise the biopsy findings in *DGKE* associated disease are also heterogenous ranging from a membranoproliferative pattern to a TMA. These findings can vary according to the time of presentation. It is only with genetic analysis that the underlying pathological process can be identified and a therapeutic intervention sought. It has been suggested that genetic background or environmental factors modify the penetrance and phenotype of disease[16, 17]. It is interesting to note that in family 1 both affected members, II:2 and III:2, were both homozygous for the aHUS at-risk *CFH-H3* haplotype[18], and both carried one risk *CD46* allele[19]. In family 2, patient III:1 was homozygous for the $CD46_{GGAAC}$ risk haplotype while III:2 was heterozygous. III:1 also carried one copy of the *CFH-H3* haplotype (figure 1).

Although *INF2* mutations have not previously been associated with a renal thrombotic microangiopathy, aHUS has been reported in patients with primary FSGS[20-23] and FSGS is a frequent pathological sequalae of sporadic Stx-HUS[24]. A thrombotic microangiopathy has also been associated with other causes of nephrotic

syndrome[25, 26] and primary glomerulonephritis including IgA nephropathy[27-29]; Henoch Schonlein Purpura[23]; ANCA associated vasculitis[22, 23]; and anti-GBM nephropathy[23].

It has been hypothesised that either direct or indirect (via impaired VEGF secretion from podocytes) endothelial injury leads to a constricted microvasculature with perturbed haemodynamic flow leading to the formation of platelet microthrombi and a thrombotic microangiopathy. Loss of coagulation regulators with upregulation of procoagulation factors has also been suggested as a contributory factor in those individuals with co-existent nephrotic syndrome [30-33].

It is intriguing that all 3 patients who had renal transplants had biopsy proven evidence of a thrombotic microangiopathy in their renal allografts. The risk of FSGS recurrence post transplant in those with genetic defects of the glomerular filtration barrier is low due to correction of the underlying defect[34-36]. An exception to this is in those individuals with complete deficiency of NPHS1 due to the presumed generation of antibodies against this immunologically novel protein in the allograft. Such a scenario would not be expected in a dominantly inherited condition.

Currently, there is little information available as to the recurrence of FSGS post transplantation in individuals carrying *INF2* mutations. However, in one small study recurrence was seen in one of three individuals[8]. INF2 is expressed ubiquitously[12] and recurrence of FSGS in an allograft suggests that a circulating factor or cell type is predisposing to recurrent disease. Such a factor may account for post transplant TMA. It should be noted that INF2 has been demonstrated to complex with and alter the intracellular transport of the complement regulators CD55 and CD59 which are present on all circulating cells including platelets. We cannot however rule out the possibility that the TMA was a consequence of the post-transplant milieu (eg, viral diseases, ischemia reperfusion injury, donor-specific antibodies, immunosuppressive drugs).

In summary we describe two families with mutations in *INF2* in addition to common aHUS risk haplotypes who present with aHUS or a post transplant TMA. Eculizumab was unsuccessful in preventing either ongoing TMA or ESRF as is seen with other non-complement mediated causes of aHUS. Indentifying individuals who will not respond to eculizumab will avoid exposing these individuals to the infectious risks of terminal pathway complement blockade. This study represents an initial application of whole-exome sequencing in personalised management of TMA.

Concise Methods

The study was approved by Newcastle and North Tyneside 1 Research Ethics Committee, and informed consent was obtained in accordance with the Declaration of Helsinki.

Complement Assays

C3 and C4 levels were measured by rate nephelometry (Beckman Coulter Array 360). FH levels were measured by radial immunodiffusion (Binding Site). Screening for complement autoantibodies were undertaken using ELISA as described previously[37, 38].

Genetic Analysis and Multiplex ligation-dependent probe amplification

Mutation screening of *CFH*[39], *CFI*[40], *CFB*[41], *MCP*[42], *C3*[43], and *DGKE*[3] was undertaken using Sanger sequencing as previously described. Screening for genomic disorders affecting *CFH*, *CFHR1*, *CFHR2*, *CFHR3*, *CFHR5, CFI* and *CD46* was undertaken using multiplex ligation-dependent probe amplification (MLPA)[44, 45]. Mutation screening of *INF2* was undertaken using Sanger sequencing using the primer conditions in supplemental table 5.

Whole exome sequencing

Enrichment from isolated DNA was performed using either Illumina Nextera Rapid Capture Exome by AROS AB (family 1) or Agilent SureSelect^{XT} Human All Exon V5 by GATC Biotech, Konstanz (family 2, III:1) as described previously[46]. Library preparation was performed post-capture, with adaptor sequences and indexing incorporated using proprietary methods of AROS AB and GATC Biotech, compatible for Illumina sequencing technology. Illumina sequencing was performed on the HiSeq2000 instrument (v3 chemistry)(Supplemental table 2).

The quality of sequencing reads was firstly checked with FastQC [47]. Duplicated reads were removed with FastUniq [48]. The remaining reads were mapped to the human reference genome GRCh37 with BWA [49]. The alignments were refined with tools of the GATK suite [50]. Variants were called according to GATK Best Practice recommendations [51, 52], including recalibration. Freebayes was also used to call variants from the same set of samples [53]. The variants called by Freebayes with total coverage ≥ 5, minor allele coverage ≥ 5 and variants call quality ≥ 20 were added to those identified by GATK. Annovar was used for annotations and prediction of functional consequences [54]. Variants identified in family 1 were filtered as detailed (Supplemental table 2). First, we selected for variants in high impact regions and selected variants at a minor allele frequency (MAF) <5% in 1000G and ESP6500. We then selected those variants segregating in a dominant fashion with disease. Variants predicted to be deleterious by Polyphen-2 HDIV and HVAR, Mutation Taster, Mutation Assessor, FATHMM or RadialSVM were selected for further analysis. A more stringent MAF cut-off of <0.1% in 1000G and ESP6500 was applied and non-conserved variants (<2 by GERP++ and <0.5 by PhyloP) were discarded (Supplemental table 3).

Phenotypic data was then used to interrogate the remaining 34 genes providing only one candidate gene known to have both renal and neurological conditions inherited in an autosomal dominant pattern (Supplemental table 4).

Protein modelling

Phyre2 was used to generate an approximate protein structure using the inputted amino acid sequence of INF2 (NP 071934.3, amino acids 1-250) using the intensive modelling mode. Protein domain boundaries for INF2 were taken from Pfam[55].Three-dimensional protein structures were manipulated using PyMOL [56]

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Disclosure

T.H.J.G., and D.K. have received honoraria for consultancy work from Alexion Pharmaceuticals. D.K. is scientific advisor to Gyroscope therapeutics.

Figure Legends

Figure 1 Pedigrees of families with Inverted formin 2 *(INF2)* genetic variants. The pedigrees demonstrate the segregation of the renal / neurological phenotype with the rare genetic variant, c.305T>A (p.V102D) in family 1 (a) and c.530G>A (p.R177H) in family 2 (d). Individuals tested but not carrying the mutation are shown – nmd (no mutation detected). The number of alleles carrying the aHUS risk haplotype *CFH-H3* (CFH_{risk}) and $CD46_{GGAAC}$ ($CD46_{risk}$) are shown on the pedigree. Sanger sequencing trace of wild type (WT) and mutant (Mut) for c.305T>A (p.V102D) (b) and c.530G>A (p.R177H) (e). Alignment of human, chimpanzee, orangutan, mouse, rat, dog, opossum, platypus and zebrafish *INF2* demonstrating amino acid conservation (c, f) (performed using http://genome.ucsc.edu/cgibin/hgTrackUi?hgsid=309786867&c=chr21&g=cons46way#a_cfg_phyloP).

Figure 2 Renal biopsies. Native renal biopsy from family 1, patient III:2. (a) Two arterioles (right) showing features of active thrombosis and a small artery (left) with relatively slight intimal oedema with fibrosis, Hematoxylin and eosin stain (H&E). (b) Glomerulus (left) showing global sclerosis and occluded arteriole (right), Periodic acid–Schiff (PAS). Native renal biopsy from family 1, patient II:2 demonstrating end-stage changes with diffuse global sclerosis (c). Renal transplant biopsy from family 1 patient II:2 demonstrating (d) an occluded arteriole (PAS) and (e) a capillary loop with abundant subendothelial fluffy material (Electron microscopy). Native renal biopsy from family 2, patient III:2 demonstrating (f) a sclerosed glomeruli and (g) a segmental sclerosing lesion. Renal transplant biopsy from family 2 patient III:1. Mucoid intimal thickening is seen in an interlobular artery with red cell fragmentation in the wall and

luminal thrombus (h). Mesangiolysis is also seen 3-6 o'clock (i) (silver stain). Native renal biopsy from family 2, patient III:2 showing a subacute/chronic arterial TMA with fibroproliferative obliteration of small arteries and arterioles (J) (H&E) and (K) (trichrome). Renal transplant biopsy from family 2, patient III:2 demonstrating end stage change with fibrous obliteration of arteries. (l)(H&E)

Figure 3 Inverted formin 2 *(INF2)* variants in FSGS, CMT and aHUS. A representation of the domain structure of INF2 showing the diaphanous inhibitory domain (DID), formin homology domains (FH1, FH2) and the diaphanous autoregulatory domain (DAD). Genetic variants associated with isolated FSGS are below the domain structure. Genetic variants with a combined FSGS/CMT phenotype are shown above the domain structure. Variants from Mademan *et al*[57]. The locations of the aHUS associated mutations demonstrated in the study are shown in red.

Figure 4 Predicted structure of diaphanous inhibitory domain (DID) of Inverse Formin 2 (INF2). A structural model of the DID domain (amino acids 1-234) was generated using Phyre2 (a)[58]. Blue spheres represent the amino acids involved in binding to DAD domain: (R106, N110, A149, I152). The position of the p.V102D and R177H mutations are highlighted in red. The p.V102D lies in the 8th α-helix of the DID (amino acids) domain. (b) Surface representation of the modelled structure highlighting the surface exposed amino acids responsible for DAD binding (R106, N110, A149, I152). The mutant p.V102D is not buried but may be expected to disrupt the architecture of the 8th α-helix. The p.R177H variant resides before the 13th α-helix of the DID domain and is surface exposed .

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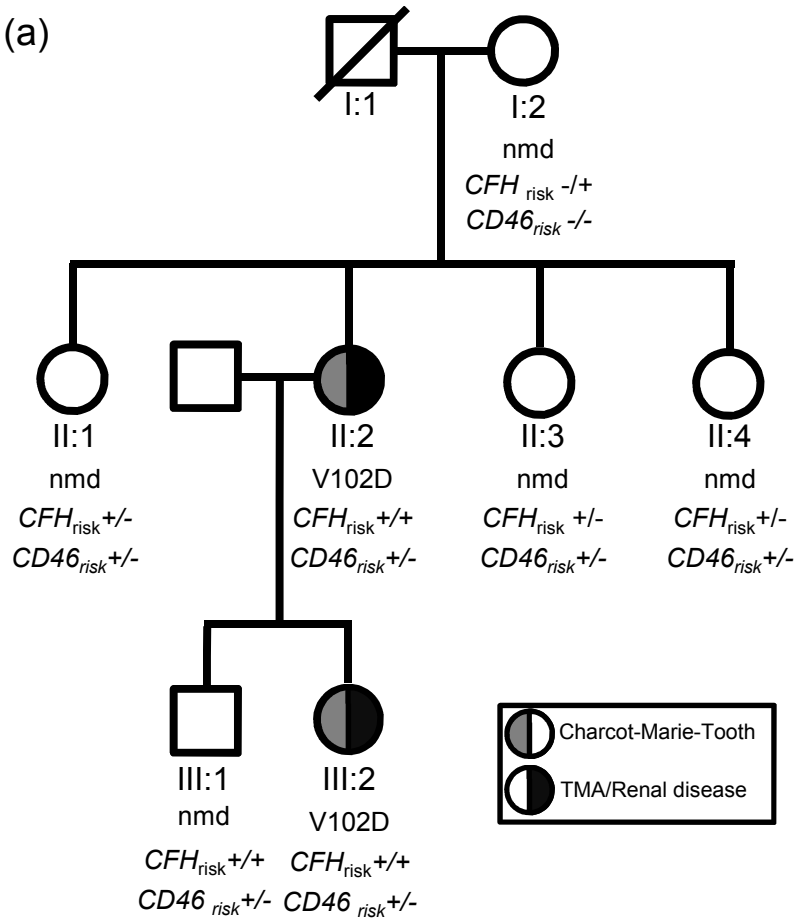
Figure 1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nmd
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CD46risk -/-
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nmd
CFHrisk+/-
CD46risk+/-
II:2
V102D
CFHrisk+/+
CD46risk+/-
II:3
nmd
CFHrisk +/-
CD46risk+/-
II:4
nmd
CFHrisk+/-
CD46risk+/-
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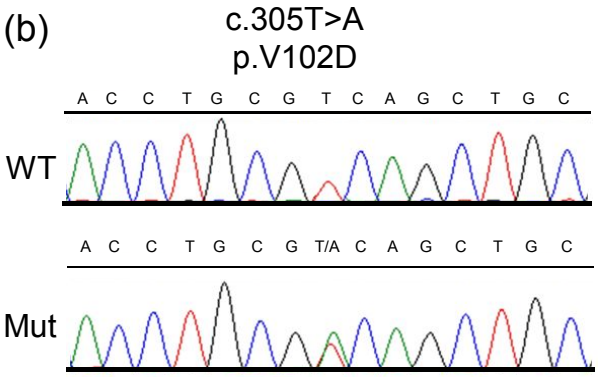
CD46 risk+/-
Charcot-Marie-Tooth
TMA/Renal disease


(b)
c.305T>A
p.V102D
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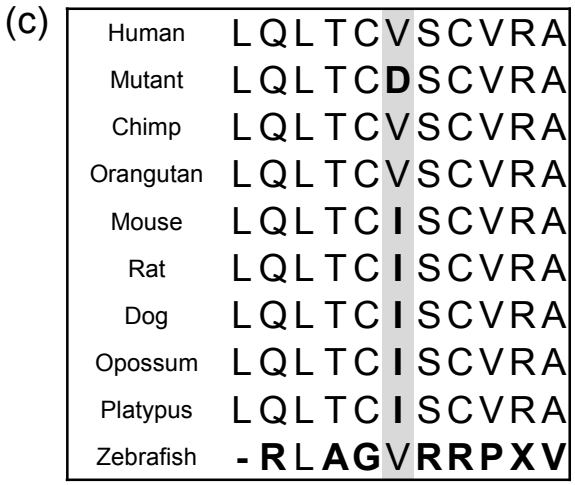
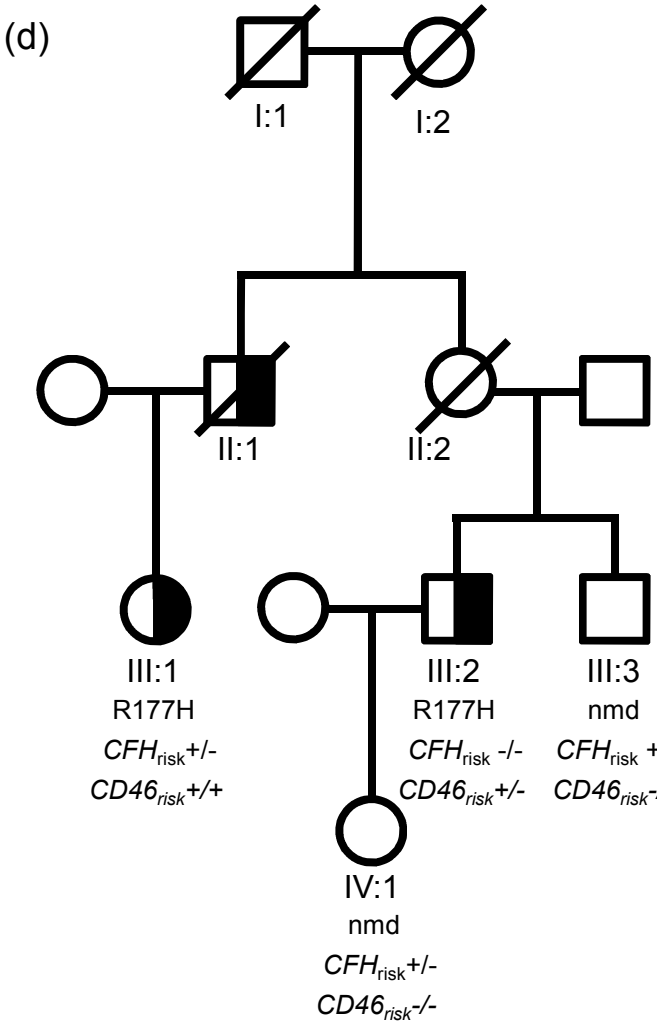
WT
A C C T G C G T/A C A G C T G C
Mut


(c)
Human L Q L T C V S C V R A
Mutant L Q L T C D S C V R A
Chimp L Q L T C V S C V R A
Orangutan L Q L T C V S C V R A
Mouse L Q L T C I S C V R A
Rat L Q L T C I S C V R A
Dog L Q L T C I S C V R A
Opossum L Q L T C I S C V R A
Platypus L Q L T C I S C V R A
Zebrafish - R L A G V R R P X V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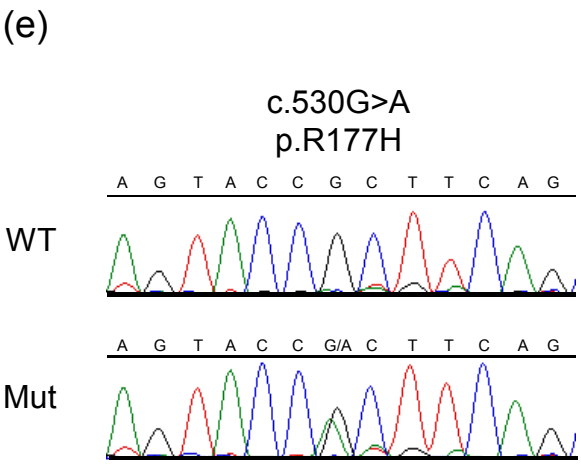
I:2
II:1
II:2
III:1
R177H
CFHrisk+/-
CD46risk+/+
III:2
R177H
CFHrisk -/-
CD46risk+/-
III:3
nmd
CFHrisk +/-
CD46risk-/-
IV:1
nmd
CFHrisk+/-
CD46risk-/-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p.R177H
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WT
A G T A C C G/A C T T C A G
Mut

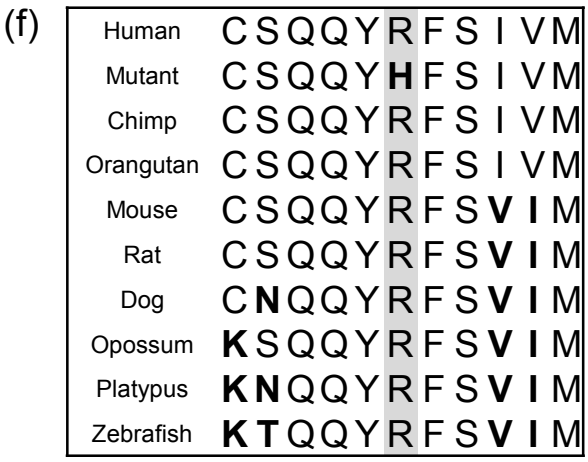
(f)
Human C S Q Q Y R F S I V M
Mutant C S Q Q Y H F S I V M
Chimp C S Q Q Y R F S I V M
Orangutan C S Q Q Y R F S I V M
Mouse C S Q Q Y R F S V I M
Rat C S Q Q Y R F S V I M
Dog C N Q Q Y R F S V I M
Opossum K S Q Q Y R F S V I M
Platypus K N Q Q Y R F S V I M
Zebrafish K T Q Q Y R F S V I M

Figure 2

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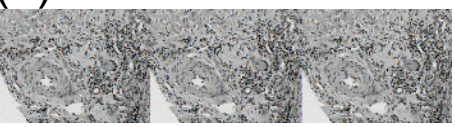

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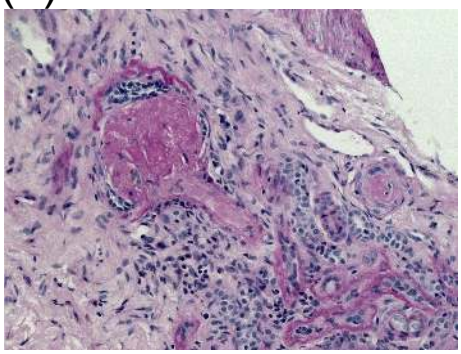

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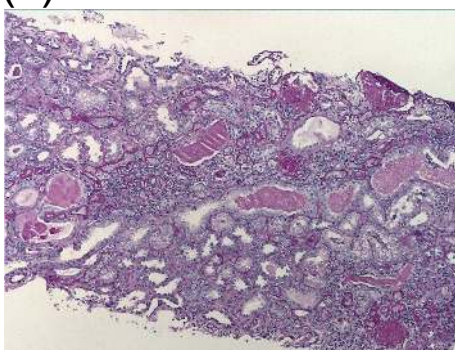
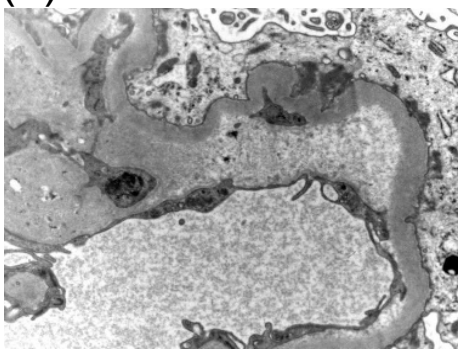


(d)

(e)



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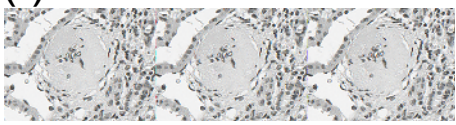
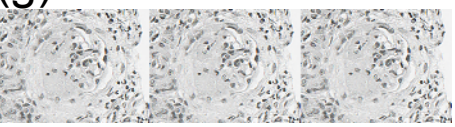


(g)



(h)

(i)

(j)

(k)

(l)

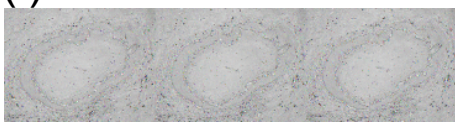
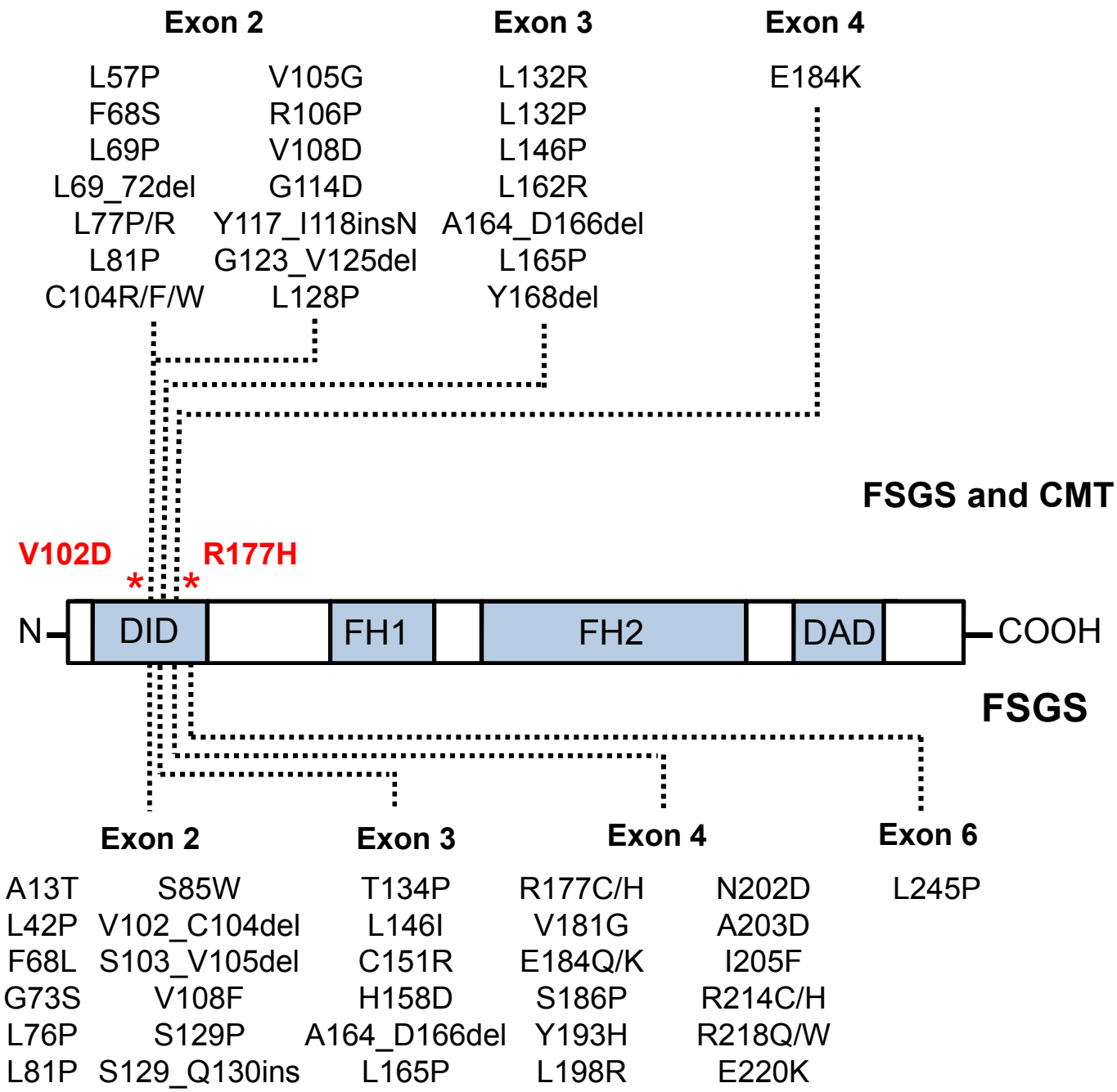


Figure 3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R106P
V108D
G114D
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G123_V125del
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L132R
L132P
L146P
L162R
A164_D166del
L165P
Y168del
E184K
FSGS and CMT
V102D
R177H
N
DID
FH1
FH2
DAD
COOH
FSGS
Exon 2
Exon 3
Exon 4
Exon 6
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F68L
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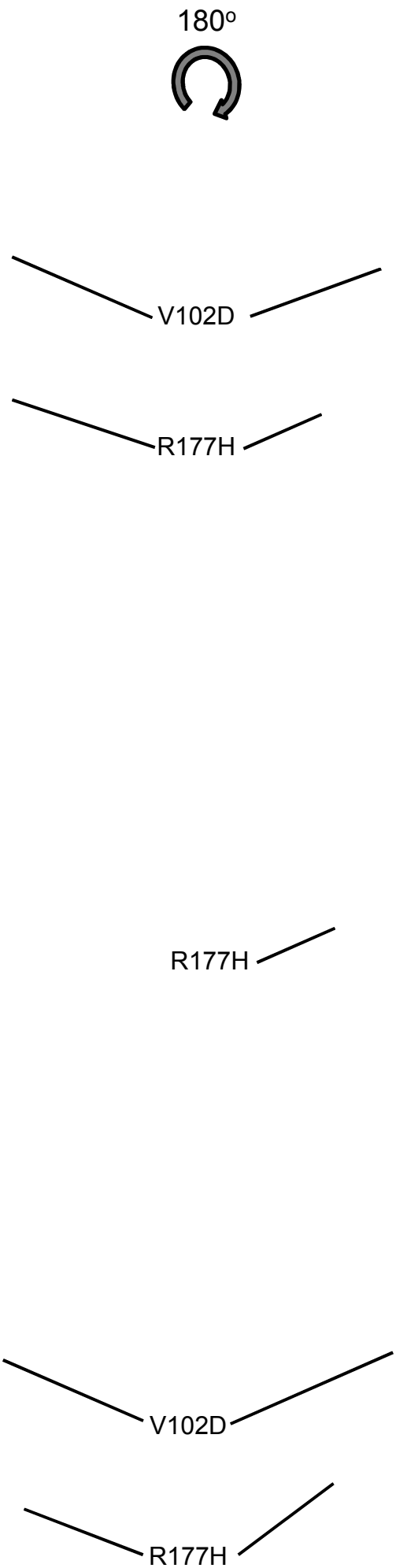

Figure 4