Identification of novel mutations in the *SEMA4A* gene associated with retinal degenerative diseases

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Semaphorins are a large family of transmembrane proteins. The gene for *SEMA4A* encodes a transmembrane protein comprising 760 amino acids. To investigate its association with human retinal degeneration, mutation screening of the *SEMA4A* gene was carried out on 190 unrelated patients suffering from a variety of eye diseases. We report the first observation of the involvement of *SEMA4A* gene mutations causing retinitis pigmentosa (RP) and cone rod dystrophy (CRD). We screened the DNA of 135 patients with RP, 25 patients with CRD, and 30 with LCA using SSCP and direct DNA sequencing for mutations in the *SEMA4A* gene. Two mutations, p.D345H and p.F350C, were observed only in affected patients; they were not observed in any of the normal members or the 100 control subjects. Both mutations identified occur in the conserved semaphorin domain. Multiple sequence alignments using Clustal analysis showed that R713Q is a conserved substitution and D345H is a semi-conserved substitution. We conclude that these mutations are a cause of various retinal degenerations.

During the development of the nervous system of multicellular organisms, several proteins play a role in communication between the cells and their local environment. Among those known to be important for axon guidance are ephrins, netrins, slits, and semaphorins.¹

Semaphorins are a large family of transmembrane proteins. The whole family shares a conserved domain (Sema) at the NH₂ terminal. The semaphorin family is further divided into seven subclasses based on their functional domains and sequence similarity. Classes 1 and 2 are found in invertebrates, and classes 3 to 7 are found in vertebrates.^{2–3} Semaphorin proteins are involved in a variety of biological mechanisms, such as organogenesis, angiogenesis, and neuronal development.^{4–7} It has been reported that the semaphorin subclasses, SEMA4 A and D, possess similar structures and are involved in cell to cell communication between T cells and antigen presenting cells in the immune response.^{8–9} SEMA4A provides a co-stimulatory signal for T cell priming and regulation.¹⁰

The gene for *SEMA4A* encodes a transmembrane protein comprising 760 amino acids. SEMA4A contains a signal peptide preceding a conserved semaphorin domain (amino acids 64–478), followed by a plexin/semaphorin/integrin domain (496–580), an Ig-like domain (570–630), a transmembrane domain (680–702), and a short (703–760) cytoplasmic tail. SEMA4A is highly expressed in the brain and eye, and during embryonic development, it is expressed in the ganglion cells, inner retinal neurones, and retinal pigment epithelial (RPE) cells, particularly at the time of contact between photoreceptors and RPE.¹¹

Recently, in a study on a mouse model, it was shown that disruption of the mouse *Sema4A* gene results in severe retinal

degeneration, attenuation of retinal blood vessels and depigmentation of the retinal pigment epithelium. The disruption also affects the physiological function of both rod and cone photoreceptor cells. Hence, it had been suggested that Sema4A functions as a transmembrane ligand for a receptor present on photoreceptor cells.¹¹

The gene for the human SEMA4A protein is present on chromosome 1q22 (<http://www.ensembl.org>). To date, no study has been published showing an association between SEMA4A gene mutations and human retinal degeneration. To investigate its association with human retinal degeneration, mutation screening of the SEMA4A gene was carried out on 190 unrelated patients suffering from a variety of eye diseases. We report the first observation of the involvement of SEMA4A gene mutations causing retinitis pigmentosa (RP) and cone rod dystrophy (CRD).

SUBJECTS AND METHODS

Blood samples were obtained with the informed consent of all subjects. Leukocyte DNA was extracted from peripheral blood of 190 unrelated patients suffering from various retinal diseases, including RP, CRD, and Leber congenital amaurosis (LCA). Blood samples were also collected from 100 ethnically matched control subjects.

All patients studied were of Pakistani origin belonging to various northern ethnic groups, such as Pathans, Punjabis, and Kashmiris. Diagnosis was made on the basis of the patient's previous history and clinical notes from childhood examinations, and on fundoscopy performed at the time of sample collection.

RP patients initially had night blindness followed by complete blindness. Fundoscopic examination revealed the clinical features of retinitis pigmentosa that includes typical bony corpuscle-type pigmentation, deposited mainly in the equatorial and peripheral regions. Attenuated blood vessels were also seen towards the periphery. The macula was clear in those patients who were in the early stages of the disease. Patients were diagnosed with CRD if they had progressive loss of visual acuity and colour vision followed by night blindness and loss of peripheral vision. Most of the CRD patients had severe photophobia and epiphora in bright light. Fundoscopic examination revealed a high degree of fundus granularity with marked macular degeneration and a significant level of peripheral retinal pigmentation. Patients who were blind at birth or during infancy were considered to have LCA.

To identify disease associated mutations in SEMA4A, patients' DNA samples were screened by single stranded conformational polymorphism (SSCP), followed by direct DNA sequencing. From the SEMA4A gene sequence (accession number, NM_022367), exon specific intronic primers

Abbreviations: CRD, cone rod dystrophy; LCA, Leber congenital amaurosis; RPE, retinal pigment epithelial; RP, retinitis pigmentosa; SSCP, single stranded conformational polymorphism

were designed, covering the splice sites on both ends of the exons (table 1). *SEMA4A* consists of 15 exons. Each exon was individually amplified from genomic DNA samples by PCR in a 50 µl reaction volume under standard PCR conditions or as otherwise specified (table 1). For SSCP, PCR products were electrophoresed on a 12% non-denaturing resolving gels in Tris-glycine buffer at 70–80 V overnight. The 12% resolving gel was prepared by adding 12 ml of a 30% polyacrylamide stock solution (30:1, acrylamide: bisacrylamide), 6 ml of 5×TGB buffer (125 mmol/l Tris pH 8.0, 0.96mol/l glycine) and the volume adjusted to 30 ml with deionised water. The bands were visualised by silver staining.¹²

Samples that showed a mobility shift in SSCP analysis were sequenced. Genomic DNA fragments containing the coding sequence and the flanking splice junction consensus sequences of each exon were amplified by PCR. The amplified fragments were purified on QIAquick[®] spin columns (Qiagen) and subjected to sequence analysis in both forward and reverse directions. For each sample, the sequencing reaction was set up using a commercial kit (Big Dye Terminator cycle sequencing kit; Applied Biosystems). The products were separated by electrophoresis and analysed using an ABI 377 automated DNA sequencer. A missense mutation, in frame change, or compound heterozygous mutation (see below) was considered pathogenic if found only in the patients and not in any of the 100 normal controls.

RESULTS AND DISCUSSION

We screened the DNA of 135 patients with RP, 25 patients with CRD, and 30 with LCA using SSCP and direct DNA sequencing for mutations in the *SEMA4A* gene. The results are summarised in table 2. During SSCP analysis for exon 10, two types of variant bands were seen in 20 different samples. The variant bands were further subjected to genomic DNA

sequencing. Sequencing analysis revealed two heterozygous mutations in codon 345 and 350. A heterozygous G→C substitution (c.345GAC→CAC; aspartic acid→histidine) in codon 345 results in a p.D345H mutation that is conservative in nature. The second T→G substitution in codon 350 (c.350TTT→TGT; phenylalanine→cysteine) results in a non-conservative p.F350C mutation (fig 1A). Both the p.D345H and p.F350C mutations were identified in four patients (RODS002, 006, 067, and 119). Of these, two were diagnosed with RP and two with CRD. It is noteworthy that all the patients had both mutations and none had only one. Subsequently the sequencing analysis of the parents of one of the CRD patients (RODS006) revealed that he inherited the p.D345H mutation from his father while the p.F350C mutation came from his mother (fig 2A). Upon clinical examination, both parents appeared to be normal. None of the normal controls had either of these mutations. It can therefore be inferred that compound heterozygous mutations cause this disease phenotype.

The remaining 16 patients (of the 20 who showed a mobility shift in SSCP analysis) had a 2 bp deletion in intron 10, 26 bp downstream of exon 10. In addition, a large number of samples from the normal controls were also identified as having the aforementioned 2 bp deletion. This polymorphic deletion was heterozygous in all the samples that were examined. It was considered nonpathogenic because it was found in both patients and controls. In addition, three isocoding substitutions (C→A, T→C, and C→T) were also identified in exon 2, 8, and 15, respectively.

In exon 15, a heterozygous G→A transition mutation was identified. This mutation causes a change in codon 713, whereby arginine (CGG) is replaced by glutamine (CAG) (fig 1B). This R713Q mutation was found in four patients. Of these, one patient had congenital blindness while the remaining three had RP. Sequence analysis of the family

Table 1 SEMA4A gene primers and PCR conditions

Exon	Primer sequence (5'→3')	PCR product size (bp)	MgCl ₂ (mmol/l)	Temp (°C)
1F	CCACCAACTCCGCCAAGCC	372	1.5	60
1R	CCGGCTCCCAGCCTCTGCAGC			
2F	CCTGCCACCAATACACACGC	389	1.5	60
2R	TGTCCTGTTTCTCCATATCC			
3F	TGCATCTAGCTGCCAAGGCC	375	1.5	67
3R	TTATTCATGCACAGGCAGCC			
4F	ATCAGCATGTCACTAACCACC	320	1.5	57
4R	AACATAATCCAGGAGATAACC			
5F	AGCATTACCTAGCCTTTCTCC	299	1.5	58
5R	CATCTGGAAGGCCAGAGTTTTC			
6F	AGACCAATTTCCCTCTATGTCCC	368	1.5	59
6R	AGCATCCTTCAACTTCAGCTTCC			
7F	GGTGCAGAACTGATGCAGGG	402	1.5	60
7R	GAAAGCTTCAAGAGAACCAGGAG			
8F	GAGGAAGCCTGTGTCTCTGG	283	1.5	62
8R	TATTGGTCTCCTCCAGTTAG			
9F	GGGTGGCTAGAGTCTGCAAGG	623	1.5	60
9R	GAAAGTGCTGAGTAGGTGCTGG			
10F	CTCCCTTGCCCTTATCAACAC	409	1.5	60
10R	GGCATTAGTAAATGCCAATAAA			
11F	CCACCTGAAATGAGGACTGCC	434	1.5	65
11R	CCAGGCACATAGCAGGTGCTC			
12F	CCGCGCCAGTACATATCACCC	335	1.5	68
12R	CCCAGCACGCTGGTGCAGGCC			
13F	CCTGACCATCAAATGGCCTTCC	441	1.5	60
13R	CCATCCCTGGAGGAACCCACTGC			
14F	TTCCGCTTCTCTCTGTCTCC	361	1.5	55
14R	TCGGGTGTTGATCTCAAGCC			
15.1F	GGGCTGGGTCCAAAGATAGG	437	1.5	71
15.1R	GACTGCTGGGCAGCCAGGGCG			
15.2F	CTTTTCATACCCTGTGATCTCC	531	2.5	65
15.2R	CAGAGTAGCAGAAGCTCCTCTC			

F, Forward; R, Reverse.

Figure 1 Selected electropherograms (forward and reverse) of the patients identified with SEMA4A gene mutations. (A) Heterozygous G→C and T→G substitutions in exon 10. (B) Heterozygous G→A substitution in exon 15. Arrows indicate the site of mutation (double peak).

members of an RP patient (RODS52) confirmed that the R713Q mutation was segregating with the disease phenotype (fig 2B), with an autosomal dominant mode of inheritance. This mutation was not present in the 100 ethnically matched control subjects.

Of the 190 patients analysed, three novel point mutations were found in SEMA4A. These mutations could be considered

pathogenic for two reasons. Firstly, they were not observed in any of the normal members or the 100 control subjects. Secondly, the p.D345H and p.F350C mutations identified in this human study occur in the conserved semaphorin domain. In the mouse model, it has been shown that disruption in this domain causes severe retinal degeneration, including attenuated retinal blood vessels and depigmentation.¹¹ However, the R713Q mutation, found in the RP and congenitally blind patients, occurs in the cytoplasmic tail. This mutation probably disrupts the signal that activates the biochemical pathways required for the normal function of the cell. Multiple sequence alignments using Clustal analysis showed that R713Q is a conserved substitution and D345H is a semi-conserved substitution in which an acidic amino acid is changed into a basic amino acid (<http://www.ebi.ac.uk/clustalw/index.html>).

The novel identification of these mutations in patients as a cause of various retinal degenerations could be helpful to further understand the function of SEMA4A in the visual

Figure 2 Pedigrees of families of (A) a CRD patient with compound heterozygosity for the D345H, & F350C (G→C and T→G) mutations and (B) of an autosomal dominant RP patient in which sequencing analysis showed the segregation of the R713Q (G→A) mutation in the patients. Arrows indicate index cases.

Table 2 Summary of Sema4A gene sequencing results				
Exon	Base change	Codon	Amino acid	No of patients/controls
Pathogenic changes				
10	1033G→C	GAC-CAC	D345H	4 patients
	1049T→G	TTT-TGT	F350C	
15	2138G→A	CGG-CAG	R713Q	4 patients
Nonpathogenic changes				
2	39C→A	CTC-CTA	L13L	6 patients
8	762T→C	TTT-TTC	F254F	11 patients
Intron 10	CA del, 26bp of exon 10	downstream		13 patients, 11 controls
15	1716C→T	CCC-CCT	P572P	9 patients

system and the role that it plays in the signalling mechanism to control the development of the outer retina.

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