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

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Key Words: Semaphorins, SEMA4A, retinal degeneration, retinitis pigmentosa, cone rod dystrophy,

ABSTRACT:
INTRODUCTION: SEMA4A is a member of a large transmembrane protein family known as Semaphorins. Disruption in SEMA4A gene has been reported to be associated with severe retinal degeneration in a mouse model. To date no study has been published showing an association between SEMA4A gene mutations and human retinal degeneration. This study was carried out to investigate the association of SEMA4A gene with the human retinal degenerations.
METHODS: A panel of 190 unrelated patients suffering from various retinal diseases, including RP, CRD and LCA was collected. To identify disease-associated mutations in the SEMA4A gene, DNA samples of patients were screened by SSCP, followed by direct DNA sequencing.
RESULTS: We found a heterozygous G to C change (D345H) and a heterozygous T to G change (F350C), co-segregating in two RP and two CRD patients. Another heterozygous G to A mutation (R713Q) was found in three RP patients and a patient with congenital blindness. Besides these disease-causing mutations several non-pathogenic changes were also found, including a 2 base pair deletion at 26bp down stream of exon 10 and three isocoding single base pair substitution.
DISCUSSION: Identification of four heterozygous mutations including a compound heterozygous mutation suggest an autosomal dominant type of retinal degeneration associated with the mutations in SEMA4A gene. This is the first report of the involvement of the SEMA4A gene mutations causing retinitis pigmentosa (RP) and cone rod dystrophy (CRD) in humans. Further biochemical studies are needed to be done to elucidate the role of this gene in the disease mechanism.

Introduction:
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 semaphorins1.
Semaphorins are a large family of trans-membrane 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 whereas classes 3 to 7 are found in vertebrates. Semaphorin proteins are involved in a variety of biological mechanisms, such as organogenesis, angiogenesis, and neuronal development. It has been reported that semaphorin subclasses, Sema4A and D possess similar structures and are involved in cell-cell communication between T cells and antigen-presenting cells in the immune response. Sema4a provides a co-stimulatory signal for T cell priming and regulation. The gene for Sema4A encodes a transmembrane protein comprising 760 amino acids. Sema4A contains a signal peptide preceding a conserved semaphorin domain (aa 64-478), followed by a PSI domain (aa 496-580), an Ig-like domain (aa 570-630), a transmembrane domain (aa 680-702), and a short (AA 703-760) cytoplasmic tail. Sema4A is highly expressed in the brain and eye. During embryonic development SEMA4A is expressed in the ganglion cells, inner retinal neurons and retinal pigment epithelial (RPE) cells, particularly at the time of contact between photoreceptors and RPE.

Recently in a study on a mouse model, it was shown that disruption of the 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.

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 SEMA4A gene was carried out on 190 unrelated patients suffering from a variety of eye diseases. Here we report the first observation of the involvement of the 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 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 previous history of patients and clinical notes from their childhood examinations and fundoscopy, done 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 corpuscles-type pigmentation, deposited mainly in the equatorial and peripheral region. Attenuated blood vessels were also seen towards the periphery. The macula was clear in those patients who were at early stages of the disease. Patients were labelled 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 high degree of fundus granularity with marked macular degeneration and significant level of
peripheral retinal pigmentation. Patients presented blind by birth or during infancy were considered to have LCA.

To identify disease-associated mutations in the SEMA4A gene, DNA samples of patients were screened by SSCP, followed by direct DNA sequencing. From the SEMA4A gene sequence (accession number, NM_022367) exon-specific intronic primers were designed covering the splice sites on both ends of the exons (Table 1). The SEMA4A gene consists of 15 exons. Each exon was individually amplified from genomic DNA samples by PCR in a 50µl reaction volume under standard PCR conditions or as specified otherwise (Table 1).

Table 1. SEMA4A gene primers and PCR conditions.

<table>
<thead>
<tr>
<th>Exon</th>
<th>Primer sequence (5’&gt; 3’)</th>
<th>PCR product size (bp)</th>
<th>PCR conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1F 1R</td>
<td>CCACCAACTTCCGCCCAAGCC CCGGCTCCAGCTTGCAGC</td>
<td>372</td>
<td>1.5M MgCl₂ @60°C</td>
</tr>
<tr>
<td>2F 2R</td>
<td>CCTGCCACCAATACACACGC GTCCCTGTTTCTCCATATCC</td>
<td>389</td>
<td>1.5M MgCl₂ @60°C</td>
</tr>
<tr>
<td>3F 3R</td>
<td>TGCATCTAGCTGCCAAGGCC TTATTCATGCACAGGCAGCAC</td>
<td>375</td>
<td>1.5M MgCl₂ @67°C</td>
</tr>
<tr>
<td>4F 4R</td>
<td>ATCAGCATGTCATAACCACC AACATAATCCAGGAGATAACC</td>
<td>320</td>
<td>1.5M MgCl₂ @57°C</td>
</tr>
<tr>
<td>5F 5R</td>
<td>AGCATTACCTAGCCITTCTCC CATCTGGAAGGCAGAGTTT</td>
<td>299</td>
<td>1.5M MgCl₂ @58°C</td>
</tr>
<tr>
<td>6F 6R</td>
<td>AGACCAATTTCCTCTATGTCCC AGCATCCTTCAACCTCAGCTT</td>
<td>368</td>
<td>1.5M MgCl₂ @59°C</td>
</tr>
<tr>
<td>7F 7R</td>
<td>GGTGCAGAAACTGATGCAGG GAAGCATTTCAAGAGAAAAGGAAG</td>
<td>402</td>
<td>1.5M MgCl₂ @60°C</td>
</tr>
<tr>
<td>8F 8R</td>
<td>GAGGAAGCCTGTGTCTCTGG TATTGGTCTCTCCAGTTTAG</td>
<td>283</td>
<td>1.5M MgCl₂ @62°C</td>
</tr>
<tr>
<td>9F 9R</td>
<td>GGGTGGCTAGAGTCCTGCAAGG GAAAGTGCTGAGTGAGTTGCTTGG</td>
<td>623</td>
<td>1.5M MgCl₂ @60°C</td>
</tr>
<tr>
<td>10F 10R</td>
<td>CTCCTTTGGCCCTTTACACAC GCCATAGTAAATGCCCAATAAA</td>
<td>409</td>
<td>1.5M MgCl₂ @60°C</td>
</tr>
<tr>
<td>11F 11R</td>
<td>CCACCCCTGAATAGGACTGCCC CCAGGCACATAGCAAGTGCTC</td>
<td>434</td>
<td>1.5M MgCl₂ @65°C</td>
</tr>
<tr>
<td>12F 12R</td>
<td>CCCGCGGCAGTACATATCACCC CCCAGCAGCTGGTGACGCTC</td>
<td>335</td>
<td>1.5M MgCl₂ @68°C</td>
</tr>
<tr>
<td>13F 13R</td>
<td>CCTGACCATCAAATGGGCTTCC CATATCCTGAGGAAACACCCACTGC</td>
<td>441</td>
<td>1.5M MgCl₂ @60°C</td>
</tr>
<tr>
<td>14F 14R</td>
<td>TTCCCGCTTCTCTCTCTCTGCC TCGGTTGTAAGATCTCGAGGGG</td>
<td>361</td>
<td>1.5M MgCl₂ @55°C</td>
</tr>
<tr>
<td>15.1F 15.1R</td>
<td>GGGCTGGCTCCTACAGGATAAG GACTGCTGCGGCAAGCCAGGCG</td>
<td>437</td>
<td>1.5M MgCl₂ @71°C</td>
</tr>
<tr>
<td>15.2F 15.2R</td>
<td>CTTTTCATACCCCTGTGATCTCC CAGAGTACGAGGCTCTCTCTC</td>
<td>531</td>
<td>2.5M MgCl₂ @65°C</td>
</tr>
</tbody>
</table>

F = Forward, R= Reverse
For SSCP, PCR products were electrophoresed on a 12% non-denaturing resolving gels in Tris-glycine buffer at 70-80V, overnight. The 12% resolving gel was prepared by adding 12ml of a 30% polyacrylamide stock solution (30:1, acrylamide: bisacrylamide), 6ml of 5xTGB (125mM Tris pH8.0, 0.96M glycine) and the volume adjusted to 30ml with deionized water. The bands were visualized by silver staining.

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 the Big Dye Terminator cycle sequencing Kit (ABI). The products were separated by electrophoresis and analysed using an ABI 377 automated DNA sequencer. A missense, 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 through SSCP and direct DNA sequencing for mutations in the SEMA4A gene. The results are summarised in table 2.
Table 2. Summary of Sema4A gene sequencing results.

<table>
<thead>
<tr>
<th>Exon</th>
<th>Base Change</th>
<th>Codon</th>
<th>Amino Acid</th>
<th>No of patients/controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pathogenic changes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>1033G→C</td>
<td>GAC-CAC</td>
<td>D345H</td>
<td>4 patients</td>
</tr>
<tr>
<td></td>
<td>1049T→G</td>
<td>TTT-TGT</td>
<td>F350C</td>
<td>4 patients</td>
</tr>
<tr>
<td>15</td>
<td>2138G→A</td>
<td>CGG-CAG</td>
<td>R713Q</td>
<td>4 patients</td>
</tr>
<tr>
<td>Nonpathogenic changes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>39C→A</td>
<td>CTC-CTA</td>
<td>L13L</td>
<td>6 patients</td>
</tr>
<tr>
<td>8</td>
<td>762T→C</td>
<td>TTT-TTC</td>
<td>F254F</td>
<td>11 patients</td>
</tr>
<tr>
<td>Intron 10</td>
<td>CAdel, 26bp downstream of exon 10</td>
<td></td>
<td></td>
<td>13 patients &amp; 11 controls</td>
</tr>
<tr>
<td>15</td>
<td>1716C→T</td>
<td>CCC-CCT</td>
<td>P572P</td>
<td>9 patients</td>
</tr>
</tbody>
</table>

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 to 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 to G substitution in codon 350 (c.350TTT→TGT; phenylalanine→cysteine) results in a non-conservative p.F350C mutation (Fig 1A). Both p.D345H & p.F350C mutations were identified in four patients (RODS002, 006,067,119). Of these, two were diagnosed to have RP and other two had 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 the parents appeared to be normal. None of the normal controls had either one of these mutations. It can therefore, be inferred that compound heterozygous mutations cause this disease phenotype.

The remaining 16 patients (out of the 20 individuals that showed a mobility shift in SSCP analysis) had a 2 base pair deletion in intron 10, 26bp down stream of exon 10. In addition a large number of samples from the normal controls were also identified as having the above-mentioned 2 base pair deletion. This polymorphic deletion was heterozygous in all the samples that were examined. This deletion was considered nonpathogenic because it was found in both the patients and the control subjects. 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 to A transition mutation was identified. Due to this mutation, codon 713 for arginine (CGG) is replaced by a codon for 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 members of an RP patient (RODS52) confirmed that 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.

Among the 190 patients analysed, three novel point mutations were found in the SEMA4A gene. These mutations could be considered pathogenic for two reasons. First, these mutations were not observed in any of the normal or the 100 control subjects. Second, the p.D345H & 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. 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 mutation is a conserved substitution and D345H mutation 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 system and the role that it plays in the signalling mechanism to control the development of the outer retina.

Acknowledgments:
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Figure Legend

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).

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.
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Figure 1

A

Reverse

Forward

Reverse

B

Forward

Reverse

A C C T T G C C C N G A G C C C C G G A G

C T T N A G A C A C G T T C A A T G T N C A A

T T G N A C A T T G A A C G T G T C T N T A A G

C T T N A G A C A C G T T C A A T G T N C A A

260

180

190

210

260

250

250
Figure 2A

Figure 2B
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