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Evaluation of SFRP1 as a candidate for human retinal dystrophies

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Purpose: Secreted Frizzled Related Proteins (SFRPs) are soluble molecules capable of modulating Wnt signalling. Different lines of evidence indicate that SFRP activity is related with the development and function of the retina photoreceptor cells as well as with their apoptotic degeneration associated with the onset of different cases of retinal dystrophy (RD). Because the genetic causes of many retinal dystrophies still need to be determined, we have asked whether mutations in the SFRP genes might be associated with retinal dystrophies.
Methods: Here we describe the genomic structure of SFRP1, SFRP2, and SFRP5 and a mutational screening of SFRP1 in 325 individuals affected by various non X-linked forms of inherited retinal disorders.
Results: Three polymorphic variants were identified.
Conclusions: Our data, so far, exclude SFRP1 as a molecular cause of RD, since two out of three genetic variants of the gene were present in both RD patients and normal population.

Inherited retinal dystrophies (RD), typified by retinitis pigmentosa (RP), macular degeneration, or Usher syndrome, comprise a wide variety of genetically determined conditions, which are the most prevalent cause of visual handicap in developed countries. To date, RDs are neither preventable nor curable and are characterized by the progressive degeneration of the retinal photoreceptors, with a consequent loss of visual acuity and reduction of the visual field [1].

The molecular defects responsible for these disorders are very heterogeneous. In fact, 153 different RD loci have been characterized so far and, among these, 106 responsible genes have been identified (RetNet [2]). These genes encode functionally diverse proteins, which, just considering RP, span from proteins implicated in visual transduction such as rhodopsin or the alpha and beta sub-units of the rod cyclic GMP phosphodiesterases or structural proteins like peripherin or transcription factors, which include CRX or NRL [3]. In spite of these advances, full understanding and treatment of RD still requires, among other approaches, the identification of the remaining responsible candidate genes and of molecular determinants that might predispose to the disease.

In search for such candidates, we have analyzed a new class of molecules: the Secreted Frizzled Related Proteins (SFRPs). These are secreted proteins of about 36 kD that receive their name by their structural homology with the extracellular cystein-rich domain (CRD) of Frizzled (Fz) [4-7], a family of developmentally important signaling molecules. Because of this structural similarity and their ability to bind to both Wnt and Fz proteins, SFRPs are though to be functional modulators of Wnt signalling, a role that has attracted the attention of many researchers given the multiple activities of Wnt proteins in the regulation of cell fate and behaviour during embryonic development, tumour formation and apoptotic events [8,9]. In humans, there are five SFRP genes which, according to phylogenetic analysis, are divided in two groups. The first comprises SFRP1, SFRP2 and SFRP5, and the second, SFRP3 and SFRP4 [10]. Expression analyzes in chick, mouse, and humans have shown that all members of the first class are expressed in both the developing and adult eye [11-15]. In particular, SFRP5 mRNA has been localized in the retinal pigmented epithelium in humans [12], while both SFRP1 and SFRP2 are expressed in the undifferentiated retinal neuroepithelium of chicks and mice [13,15]. As development proceeds, SFRP1 expression is specifically maintained in the inner nuclear and photoreceptor layers, at least in the mouse [15]. On the basis of this expression pattern, it has been proposed that SFRPs may be involved in determining photoreceptor cell polarity [12]. Furthermore, analysis of a few cases of RP has related photoreceptor degeneration with an up-regulation of SFRP1, SFRP2, and SFRP5 [16,17]. In contrast with these observations, functional analysis of SFRP1 activity in the embryonic chick retina has shown that this gene eases the differentiation of early born neurons, including the photoreceptors [18]. SFRP1, SFRP2 and SFRP5 map to human chromosomes 8p12-p11.1, 4q32.1 and 10q24.2, respectively, which are locations that might be related to different forms of RD, including [2], autosomal recessive cone rod dystrophy (CORD9; OMIM 608194) [19], atypical Vitelliform Macular Dystrophy (VMD1; OMIM 153840) [20], and autosomal recessive Retinitis Pigmentosa (RP29) [21].

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Altogether these observations point to a strong relationship between SFRPs and photoreceptor generation and function. However, they do not establish a clear link between them. To address this issue, we have begun a study aimed at determining whether alteration in the SFRP genes might be directly related to the onset of a specific type of RD. Here, we report a mutational screening of Sfrp1 in 325 individuals affected by various non X-linked forms of inherited retinal disorders. So far, our data excludes SFRP1 as a molecular cause of RD but confirms and describes the presence of specific new sequence variants, which may be potentially related to disease susceptibility.

Table 1. Cases of retinal degeneration collected for this study

Clinical diagnosis	Number of analyzed individuals
Autosomal Dominant Macular Dystrophy	11
Autosomal Recessive Macular Dystrophy	11
Sporadic Macular Dystrophy	2
Autosomal Dominant Retinitis Pigmentosa	84
Autosomal Recessive Retinitis Pigmentosa	81
Sporadic Retinitis Pigmentosa	65
Unclassified Retinitis Pigmentosa	3
Various Syndromic Retinitis Pigmentosa	14
Usher type I	12
Usher type II	33
Optic Atrophy	7
Leber Congenital Amaurosis	2
TOTAL	325

The table provides a list of the different cases of retinal degenerations (RDs) collected for this study, specifying the number of individuals analyzed in each case.

Table 2. Primers for the amplification of SFRP1

Fragment	Size (bp)	Primers
1out	1176	F: 5' GGGTGTTGAGCCGCGTCTGGTTCTA 3'
		R: 5' ACGCGCTTAGGAATCACGTGCACAG 3'
1A	288	F: 5' CCGGGAGCTGATTGGCTGCG 3'
		R: 5' GACCTCCGGGGACAAAAGGC 3'
1B	245	F: 5' GCCTTTTGTCCCCGGAGGTC 3'
		R: 5' GCTCTGGTACGGGCCGATG 3'
1C	292	F: 5' GACTACGTGAGCTTCCAGTCGGAC 3'
		R: 5' GCCAGCGACACGGGTAGATG 3'
1D	281	F: 5' GCTCTTCGCGCCCGTCTGCCTG 3'
		R: 5' CGTAGGGTGGCGCGGGTTCTCC 3'
2	285	F: 5' CCAGAAGACCTTTAGCCACT 3'
		R: 5' GCTGATGTATCTCGTTGCA 3'
3A	251	F: 5' CCCTCTTTTCTCTTCTTCTGTCCTC 3'
		R: 5' GTACTGGCTCTTCACCTTGCG 3'
3B	251	F: 5' GTACTTGCTGACGGCCATCC 3'
		R: 5' GCAAGAACAAGCCGACTGGA 3'

Primers for the amplification of SFRP1 gene fragments. Fragment 1out contains the whole exon 1, and is the first step of a nested PCR, needed to amplify fragment 1A. "F" refers to a forward primer; "R" refers to a reverse primer.

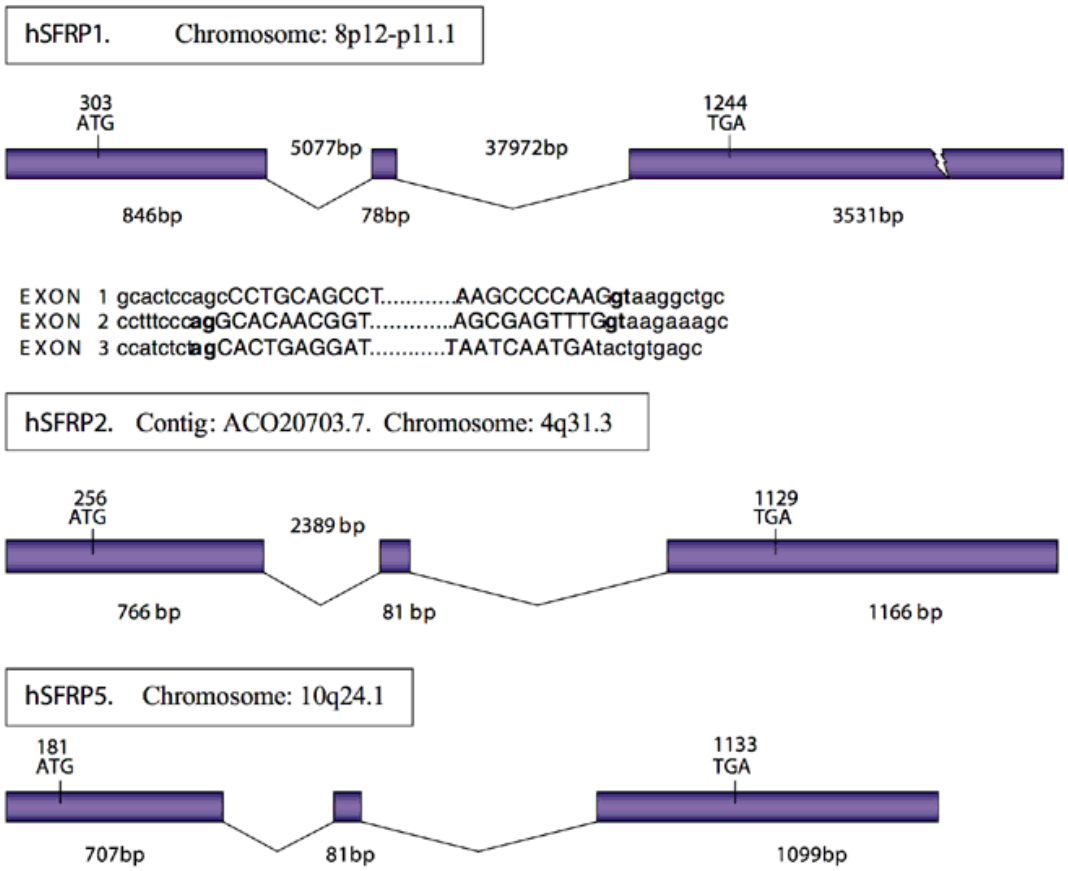

Figure 1. Human SFRP1, SFRP2 and SFRP5 genes. Diagram representing the structural organization of the human SFRP1, SFRP2 and SFRP5 genes. The structures were obtained with searches in the Ensembl compilation of the human genome and with PCR amplifications and sequencing of the intron-exon regions of each gene.

METHODS

Patients: A group of 325 unrelated families with various non X-linked forms of inherited retinal disorders was collected for this study (Table 1). Only one affected individual from each family, was analyzed. As a control group, 95 DNA samples derived from non-related individuals from the general population were analyzed.

Methods: Mutational screening of SFRP1 was performed using SSCP [22]. The screening included all three exons and the flanking intronic regions to account for possible splice site mutations. DNA extracted from peripheral blood samples (15-20 ml) by means of the "salting out" method [23] was used as template for PCR reactions. Primers are listed in Table 2. PCR conditions used for the amplification of SSCP fragments are as follows: almost every fragment is amplified with an initial denaturation step of 95 °C for 5 min; 35 cycles of 94 °C for 45 s, 60 °C for 30 s, 72 °C for 30 s; and a final elongation step of 72 °C for 7 min. Exceptions are fragment 1out, with an annealing temperature of 57 °C and a longer cycle elongation step of 45 s, and fragment 2, with an annealing temperature of 56 °C. All PCR reactions contain 20% GC Rich Solution (Roche Diagnostics) except for fragments 2 and 3A.

For SSCP analysis, amplified PCR samples were run on a SSCP poly-acrylamide gel, whose composition and electrophoretic conditions varied for each of the different fragments analyzed. Fragment 1A: 12% gel (49:1), run at 10 W for 12 h; fragment 1B: 11% gel (29:1), run at 18 W for 22 h; fragment 1C: 10% gel (49:1), run at 16 W for 16 h; fragment 1D: 10% gel (49:1), run at 16 W for 16 h; fragment 2: 10% gel (49:1), run at 10 W for 22 h; fragment 3A: 11% gel (29:1), run at 10 W for 20 h; and fragment 3B: 11% gel (29:1), run at 10 W for 20 h. All gels contained 10% glycerol, and all samples were electrophoresed in duplicate, at 25 °C and 4 °C.

Whenever a change in the SSCP pattern was observed, automatic sequencing analysis was carried out in an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA). The different variants are described taking as control the sequence deposited in Genbank with accession number BC036503, where +1 has been considered the putative transcriptional initiation start site of the sequence.

RESULTS

Genomic structure of SFRP1, SFRP2, and SFRP5: A search of the Ensembl compilation of the human genome with the SFRP1, SFRP2, and SFRP5 cDNAs was used to establish the intron-exon genomic organization of the three genes. This organization was confirmed by sequencing the PCR fragments amplified from genomic DNA, using primers designed at the intron and exon sequences of each region to determine the precise splice sites in each case.

Figure 1 shows a schematic representation of the information deposited in the EntrezGene. SFRP1 spans about 47 Kb of genomic DNA and it is split in three exons of variable size separated by two introns, one 5077 bp and the other 37972 bp long. It is transcribed to produce a 4455 nucleotide long transcript with an open reading frame of 942 nucleotides that encodes a polypeptide of 314 amino acids. SFRP2 and SFRP5 have a genomic organization similar to that of SFRP1, comprising three exons, each of which has a length comparable to its equivalent in SFRP1 (Figure 1).

The structural similarity among SFRP1, SFRP2, and SFRP5, together with their sequence homology is consistent with a close relationship of the three genes, which is supported by their phylogenetic analysis [10]. Interestingly, the structural organization of SFRP1, SFRP2, and SFRP5 is different from that predicted for the remaining members of the family, SFRP3 and SFRP4, which are composed by six exons [10].

SFRP1 analysis in RP patients: The information obtained by the analysis of the genomic organization of the SFRP genes was used to design the tools for screening a collection of patients affected by various non X-linked forms of inherited retinal disorders and determine whether any alteration in the SFRP genes, specifically in SFRP1, might be directly related to the onset of a specific type of RD.

To identify possible genetic defects in the affected subjects, genomic DNA was isolated from blood samples of 325 individuals. Ninety-five individuals from the general population were used as controls in the mutational screening. The entire encoding region of SFRP1 was amplified by PCR using specific primers (Table 2). Using SSCP analysis, all three exons of SFRP1 were screened for changes in electrophoretic mobility. If a difference in band migration was found by SSCP, then sequencing of the DNA fragment was carried out.

SSCP analysis highlighted four different electrophoretic mobility patterns that we noted as gene variants V1 through

Table 3. Nucleotide changes and their descriptive statistics

		Patient Group				Control Group			
Fragment	Change	Neg	Het	Hom	Freq	Neg	Het	Hom	Freq
1B	c.357delAGC	130	136	28	33%	47	39	9	30%
2	IVS2+60C>T	295	6	1	1.32%	92	2	0	1.06%
3B	c.1352G>A	302	1	-	0.003%	60	-	-	0%

Nucleotide changes and their descriptive statistics. The table uses the following abbreviations: Neg (Negative), Het (Heterozygote), Hom (Homozygote), and Freq (Frequency).

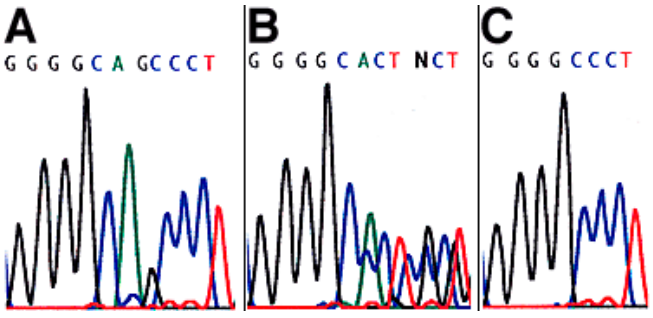

Figure 2. Sequence of the exon 1 genetic variant. Automatic sequencing of fragment 1B of exon 1 showing the genetic variant c.357delAGC that was found in heterozygosis (**B**) and homozygosis (**C**) in both control and affected populations, as well as the wild type genotype (**A**).

V4. The electrophoretic mobility variants were observed in the PCR-amplified fragments 1B, 2, and 3B. These fragments were therefore sequenced. Variation in fragment 1B is due to a deletion of three base pairs (c.357delAGC), which affects both codon 13 and 14, normally encoding two consecutive alanine residues. The resulting codon also codes for alanine and was detected in 30% of the normal population and with a slightly higher frequency (33%) in RD affected individuals (Table 3). Figure 2 shows the automatic sequencing analysis of this variant in individuals that were either wild type, homozygote, or heterozygote for the variation. In fragment 2, a single nucleotide polymorphism (SNP) was found in intron 2 (IVS2+60C>T) in 1% of both control and affected population (Table 3). These variants correspond to those already noted in the databases. An additional, novel, and rare SNP (c.1352G>A) was found in exon 3 within the non-coding region. This SNP was observed in one of the 325 RD cases analyzed (Figure 3; Table 3). No changes were observed in the remaining fragments.

The genetic variants described above were detected with no significant differences in both the control and the affected population. Furthermore, mutations in SFRP1 never co-segregated with the disease (Figure 3), indicating that, at least in the cases we analyzed, the sequence variants of SFRP1 are not responsible for the disease.

DISCUSSION

SFRPs are widely expressed molecules (developing and adult limbs, heart, kidney, ovary, gut, CNS) that seem to have opposite functions in many of the biological processes in which they have been implicated. Thus, SFRPs appear to act as tumour suppressor genes and their expression is silenced by an abnormal hypermethylation of their promoter regions at least in cases of colorectal cancer [24], but opposite results have been observed in malignant glioma cell lines [25]. Dual activity of SFRPs has also been reported in relation to cell death, suggesting that these molecules may either favour or prevent apoptosis [5]. Their function seems complex also in the retina. Indeed, and possibly in line with their involvement in apoptotic events, SFRP mRNA levels are up-regulated in individuals affected by RP but analyzes in humans and other vertebrates indicate that SFRPs might contribute to the development and function of the retina photoreceptor cells [12,18]. Because SFRP chromosomal localisations are related to different forms

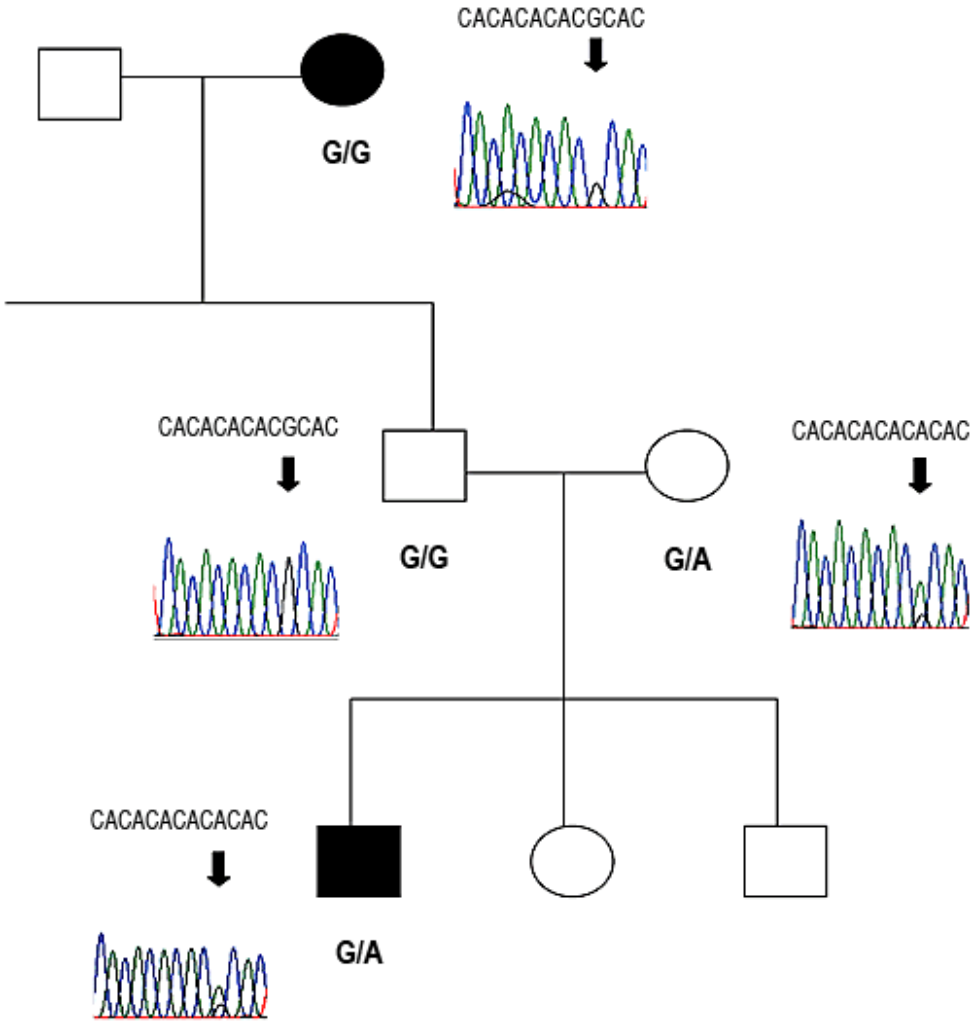

Figure 3. c.1352G>A change: sequence and familial segregation. Pedigree of a family with an affected individual that carries a c.1352G>A in exon 3. Note how the genetic variant is present in heterozygosis in both the unaffected mother and in the affected child.

of RD, we hypothesized that SFRPs could be candidate genes for those forms of RD, whose genetic causes are still unknown.

To begin this analysis, we have screened a relatively large group of individuals affected by different forms of RD for mutations in the SFRP1 gene. We have found four different genetic variants (including the wild type form) of this gene in the screened populations. It is worth noticing however, that the SSCP screening technique is not 100% sensitive, and the possibility of missing mutations, albeit small, does exist. None of the three genetic changes found in the study co-segregated with the disease or were present with a significant higher frequency in the affected population, excluding SFRP1 as a genetic cause of the different, non-X-linked forms of inherited RD we have analyzed. Nonetheless, our study describes the presence of these three sequence variants in the SFRP1 gene. Perhaps the most interesting one is the deletion observed in exon 1, which affects two triplets coding for two consecutive alanines located in the first α-helix of the protein. However, because the new codon resulting from the deletion also codes for alanine, it is expected that the resulting polypeptide will not suffer dramatic structural or functional changes. The similarity of the frequencies in both the normal and affected populations (30 and 33 percent), supports this idea. The slightly higher frequency of the affected group is not statistically significant (chi squared test with p=0.361). The remaining two genetic variants (c.1352G>A and IVS2+60C>T) are characterized by two different SNPs. Both of them are located in the non-coding portion of the gene: the former in the second intron and the latter in the non-coding region of exon 3. Segregation analyzes of the involved families confirmed the non-pathogenic nature of these three changes. It is unlikely that these changes may have any functional significance. However, at least in the case of the SNP found in exon 3, we cannot exclude that this might affect mRNA stability and therefore alter the protein levels, possibly modulating the clinical course of the disease, since the SNP was found in one affected and one unaffected individual within the same family.

Although we have found no evidence of a causative relationship between the SFRP1 gene and RD, it is interesting to note that other molecules implicated in Wnt signalling are responsible for retinal pathologies. This is the case of Mfrp, a splice variant of the human membrane-type frizzled-related protein in the mouse, normally expressed in the retinal pigmented epithelium. Mutations in this gene seem responsible for the photoreceptor degeneration present in the autosomal recessive retinal degeneration mouse line, known as rd6 [26], one of the models used to study the onset of RP in humans [27]. Moreover, mutations in Fz-4, encoding the putative Wnt receptor frizzled-4, segregate completely with individuals affected by familial exudative vitreo-retinopathy, a hereditary ocular disorder characterized by a failure of peripheral retinal angiogenesis [28].

In conclusion, our results, based on a rather large SSCP mutational screening, indicate that genetic alterations of SFRP1 are not involved in different inherited retinal degenerations in humans, at least in a causative manner. It is entirely possible however, that sequence variants of this gene could modulate certain retinal conditions. This will be the subject of future analysis.

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