A mutation in the pleckstrin homology (PH) domain of the FGD1 gene in an Italian family with faciogenital dysplasia (Aarskog–Scott syndrome)

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Abstract Aarskog–Scott Syndrome (AAS) is an X-linked disorder characterised by short stature and multiple facial, limb and genital abnormalities. A gene, FGD1, altered in a patient with AAS phenotype, has been identified and found to encode a protein with homology to Rho/Rac guanine nucleotide exchange factors (Rho/Rac GEF). However, since this original report on identification of a mutated FGD1 gene in an AAS patient, no additional mutations in the FGD1 gene have been described. We analysed 13 independent patients with clinical diagnosis of AAS. One patient presented a mutation that results in a nucleotide change in exon 10 of the FGD1 gene (G2559 > A) substituting a Gln for Arg in position 610. The mutation was found to segregate with the AAS phenotype in affected males and carrier females in the family of this patient. Interestingly, Arg-610 is located within one of the two pleckstrin homology (PH) domains of the FGD1 gene and it corresponds to a highly conserved residue which has been involved in InsP binding in PH domains of other proteins. The same residue is often mutated in the Bruton's tyrosine kinase (Btk) gene in patients with an X-linked agammaglobulinemia. The Arg610Gln mutation represents the first case of a mutation in the PH domain of the FGD1 gene and additional evidence that mutations in PH domains can be associated to human diseases.

Key words: Aarskog–Scott disease; FGD1 gene; Pleckstrin homology domain; Missense mutation

1. Introduction

Aarskog–Scott Syndrome (AAS), also referred as faciogenital syndrome or faciodigitogenital syndrome (MIM # 305400), is an X-linked disorder characterised by multiple limb and genital abnormalities with short stature, ocular hypertelorism, downslanting palpebral fissures, anteverted nostrils, shawl scrotum, broad and short hands, hyperextensibility of the proximal interphalangeal joints and, occasionally, slight mental retardation [1–3]. Establishing the carrier status in females is difficult since they are largely asymptomatic, although they can occasionally present a partial expression of the syndrome with short stature, small hands and feet and some facial features. In 1993, a locus for the AAS was mapped to the Xp11.12 region by studies on a family in which a balanced X-autosome translocation segregated with Aarskog Syndrome [4] and a gene, termed FGD1, was identified by Pasteris et al. [5]. The human FGD1 gene is subdivided into 18 exons spanning over 51 kb of genomic DNA from which a 4.4 kb mRNA is transcribed which codes for a protein of 961 amino acids [6]. The predicted FGD1 protein contains domains normally involved in signal transduction pathways, including two pleckstrin homology (PH) domains, a putative Src homology 3 (SH3)-binding region and a DBL homology (DH) domain [5]. The presence of PH and DH domains suggests that FGD1 may act as a guanine nucleotide exchange factor (GEF) for members of the Rho/Rac family of small GTP-binding proteins, binding specifically to the Rho GTPase cdc42 and stimulating the GDP–GTP exchange of the isoprenylated form of cdc42 [6,7]. In their initial report, Pasteris et al. [5] demonstrated that an insertion of a guanine residue after nucleotide 2122 resulted in a frameshift predicted to cause premature translation termination at codon 469. Despite the relative frequency of the clinically diagnosed AAS patients [3,8] and the availability of a DNA analysis, however, additional mutations in the FGD1 gene have not been further reported, leading to the suspicion that the syndrome could encompass a group of genetically heterogeneous disorders resulting from mutations of different genes.

In the course of a study on AAS, DNA samples from 13 unrelated patients were screened for mutations in the FGD1 gene. In one of these patients, a point mutation (G2559A) was identified in exon 10, changing a Gln for Arg in position 610. Extension of the molecular characterisation to the entire family revealed that the mutation was associated to the typical AAS phenotype in all the affected members.

2. Materials and methods

2.1. Patients

We included in this study 13 independent patients who were clinically examined and fitted a clinical diagnosis of AAS in accord with the primary and secondary diagnostic criteria for AAS as derived by Teebi et al. [9]. The family, in which the Arg610 > Gln mutation was identified, was investigated following genetic counselling for a clinical diagnosis of the proband (IV9), examined when he was 2 years old. On physical examination, he fitted the clinical description of classical

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AAS as he was a subject of short stature (87 cm, −2 S.D.) and normal intelligence with round faces, prominent foreheads with widow's peak, hypertelorism, antimongoloid slant of palpebral fessures, bilateral ptosis, hypoplasia of the midface, short and broad nose with anteverted nostrils and long broad philtrum, small hands and feet. The typical genital appearance reported in AAS (shawl scrotum) was not evident, while bilateral criptorchidism had been surgically corrected. Within the family the trait segregated with a typical X-linked pattern of inheritance (Fig. 1) as there were six males in four generations, of which three examined, which presented a phenotype compatible with AAS. Some of the females (including the mother of patient IV9) presented a milder expression of the clinical manifestations. Molecular analysis of the FGD1 gene started from IV9 and additional members were recruited subsequently when molecular genetic testing was offered to all the subjects at risk. Overall, 13 members of the family were clinically evaluated and their DNA collected. The male subjects II1 and IV10 were of short stature and showed features similar to those of patient IV9. Partial expression of the syndrome seemed to be present in four examined females (III1, III4, IV8 and IV11) who had short stature, small hands and feet, short neck, and a round face with widow's peak. Other females of the family (III2, III3, and IV7) presented similar facial and digital features without short stature (Table 1).

2.2. Mutational analysis

Genomic DNA was extracted from blood leukocytes using a standard protocol [10]. Exons 1–18 of the FGD1 gene were amplified by PCR to give products in the size range 150 to 250 bp suitable for the subsequent single strand conformational polymorphism (SSCP) analysis. Primer sequences were designed to span the intron/exon boundary regions of the published sequence of the gene (GeneBank sequence: U11690) and in part suggested by Dr. Schwartz CE (personal communication). PCR reactions were performed in 25 μl containing 100 ng of genomic DNA, 1×PCR buffer (Perkin Elmer), 0.5 pmol of each primers, 180 μM of each dNTP and 1 U of Taq DNA polymerase (Perkin Elmer). Amplified samples were diluted 1:1 in formamide buffer (95% formamide, 10 mM NaOH, 0.025% bromophenol blue and 0.025% xylene cyanol), denatured at 95°C for 5 min and cooled on ice. 4–8 μl of PCR products were loaded on non-denaturating polyacrylamide gel containing 6% acrylamide prepared with a 99:1 ratio between acrylamide and bis-acrylamide. Gels were run in a cold room for 1–4 h at 30 W. DNA bands were visualised by silver staining. PCR products that revealed an aberrant conformer were first reconfirmed by an independent PCR and then purified using Qiagen purification columns according to manufacturing instructions and both strands were sequenced using the Sequenase 2.0 kit (USB).

3. Results

Based on the known structure of the FGD1 gene, DNA primers were designed to cover all 18 exons of the FGD1 gene. Single strand conformation polymorphism (SSCP) was performed on all 18 exons of the FGD1 gene in 13 independent patients who fitted a clinical diagnosis of AAS according to the primary and secondary diagnostic criteria for AAS as derived by Teebi et al. [9]. In one patient we found a base change (G > C) in position +24 in intron 12. Since mutations at this position cannot cause splicing defects and given that it was found in eight of 56 (14.3%) normal chromosomes, we consider this base substitution as a common polymorphism.

In a second patient, the subject IV9 of the family in Fig. 1, we found a mutation in exon 10 of the FGD1 gene (G2559 > A) which substitutes a Gln for Arg in position 610. This mutation was absent in 106 normal X chromosomes. SSCP and sequence analysis of exon 10 was extended to the family of this patient, allowing the assessment of cosegregation of the mutated allele with the AAS phenotype in other affected males and in carrier females.

The pedigree of this family (Fig. 1) showed an X-linked pattern of inheritance with six affected males in four generations (three examined) and some females with a milder expression of the trait. Among the examined members, the male subjects II1 (66 years old) and IV10 (19 years old) were found to carry the G2559 > A mutation. They were of short stature (157 cm and 156 cm respectively) and showed similar facial, limb and genital features (Table 1). In addition, by medical records and photographs obtained by family members who underwent genetic counselling, strikingly similar features were detected in three other subjects of the family (II4, II7, III5) who were not available for further investigation since individual III5 had died, and II4 and II7 could not be traced.

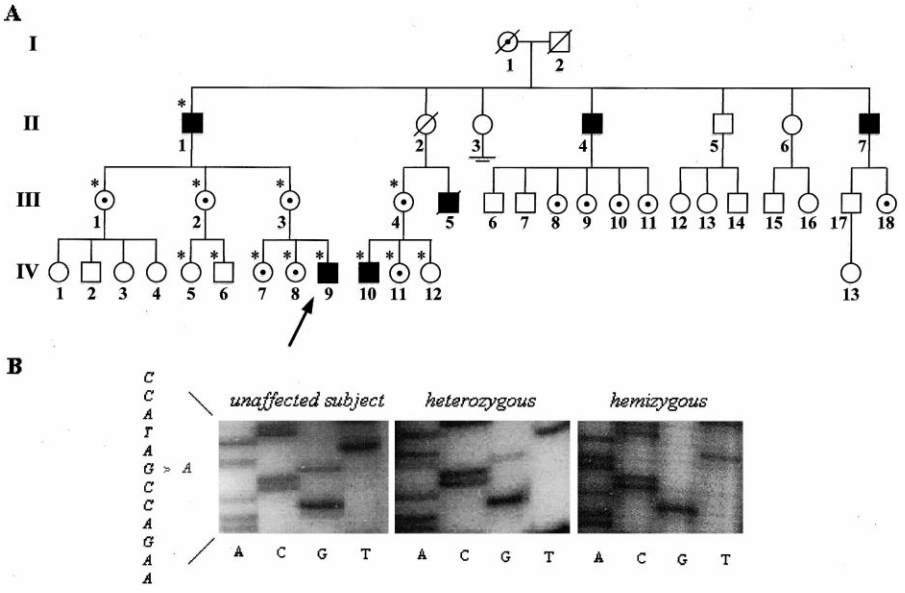

Fig. 1. A: Pedigree of the AAS family carrying the Arg610Gln mutation. Black symbols denote affected individuals; white symbols refer to unaffected individuals; central dot symbols indicate carrier females. Clinical examination and molecular analysis were carried out for the individuals referred to by asterisks. B: Direct sequencing of the exon 10 in different subjects of the family.

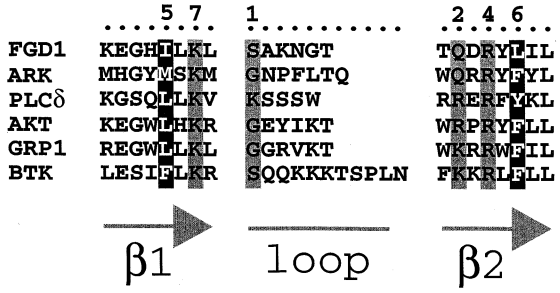

Fig. 2. Multiple sequence alignment of the β1–β2 region of PH domains involved in phosphoinositide binding. The FGD1 sequence shown here corresponds to residues 593–614 of human FGD1 (GenBank accession code GI4758358). Residue numbering refers to positions within each specific secondary structure elements, β1-strand, β1–β2 loop, and β2-strand. Two conserved hydrophobic residues at positions 5 and 6 of β1 and β2 respectively are important for the structural stability of the domain (dark grey columns with white type). Positions implicated in phosphoinositide binding are greyed. The Arg610Gln mutation affects an arginine residue (position 4 of β2) that is conserved in high-affinity phosphoinositide-binding PH domains such as the ones shown in this alignment.

Partial expression of the AAS phenotype was observed in four examined females (III1, III4, IV8 and IV11) who had short stature, small hands and feet, short neck, and a round face with widow's peak. Other females of the family (III2, III3, and IV7) presented similar facial and digital features without short stature (Table 1). All these seven females were found to carry the mutated allele in the heterozygote state (Fig. 1). Subjects IV5, IV6 and IV12, essentially free of clinical manifestations, were found to carry the normal allele.

4. Discussion

Overlapping phenotypes resulting from genetic heterogeneity represent a great limitation toward the identification of mutations causing human disease. Faciogenital or Aarskog–Scott Syndrome is frequently diagnosed after clinical evaluation of X-linked traits with facial dysmorphic features and short stature. Nevertheless, after the first reported mutation in the FGD1 gene in an AAS family by Pasteris et al. in 1994 [5], there have been no additional reports of mutations in the FGD1 gene, contravening the expectation of a univocal genotype–phenotype correlation. This has led to the suspicion that the AAS syndrome may encompass a group of genetically heterogeneous disorders, resulting from mutations of different genes. Analysis of all exons and of their flanking consensus splicing sites of the FGD1 gene was carried out in 13 independent patients fitting the clinical criteria for the diagnosis of AAS. In one of these patients we identified a base change (G > C) at position +24 in intron 12. Since mutations at this residue cannot cause splicing defects, and since the mutation was found in eight of 56 (14.3%) normal X chromosomes, we consider this base substitution as a common polymorphism.

A second patient was found to carry a mutation in exon 10 of the FGD1 gene (G2559 > A) which substitutes a Gln for Arg in position 610. When the analysis of the FGD1 gene was extended to members of the family of this patient, the mutated allele was found to segregate with the AAS phenotype in affected males and carrier females. Overall, in this family we found three affected males and seven carrier females out of 13 members examined. Affected males had the clinical features typical of the AAS and are phenotypically similar to previously reported affected subjects, but shawl scrotum, reported as one of the typical signs was barely detectable in our male patients. Furthermore, the phenotype of carrier females was very variable, ranging from near normal to clear facial and digital abnormalities. In particular, we found that short stature is not a constant feature in carrier females, as it is present in four out of seven carrier females, while it is present in all affected males of the family. This seems to be a distinguishing character between carrier females and affected males, as short stature is considered a primary criterion for diagnosis. Other features (digital anomalies, widow's peak, and shape of the mouth) are completely fulfilled and therefore seem more specific and thus critical for the identification of the carrier female at the clinical level at least in this family.

The Arg-610 to Gln mutation in the FGD1 gene is the second mutation found in patients with AAS following identification of the FGD1 gene. The mutation alters the coding sequence of the FGD1 PH domain (aa 590–689), a 100-resi-

Table 1
Summary and comparison of the clinical findings for the ASS subjects of the reported family

Primary criteria	Females							Males		
	III1	III2	III3	III4	IV7	IV8	IV11	II1	IV9	IV10
Hypertelorism	−	+	+	−	+	−	−	+	+	+
Short nose/Ant. nares	+	−	−	−	−	−	−	+	+	+
Crese below lower lip	+	−	−	+	−	+	−	+	+	+
Short broad hands	+	+	+	+	−	+	+	+	+	+
Interdigital webbing	+	+	+	+	+	+	+	+	+	+
Shawl scrotum								+/−	+/−	+/−
Short fifth finger/Clinod.	+	+	+	+	+	+	+	+	+	+
Camptodactily	−	−	+	−	−	−	−	+	−	−
Short stature	+	−	−	+	−	+	+	+	+	+
Secondary criteria										
Widow's peak	+	+	+	+	−	+	+	+	+	+
Ptosis	−	+	−	−	−	−	−	+	+	+
Downward slant palp	+	+	−	−	−	−	−	+	+	+
Abnormal auricles	−	−	−	−	−	+	−	−	+	−
Joint hyperextension	−	−	−	−	−	−	−	−	+	+
Broad feet	−	−	−	−	−	−	−	−	+	−
Cryptorchidism/ingui. umbilical hernia								+	+	+

The ASS diagnostic criteria (primary and secondary criteria) were derived from Teebi et al. [9].

due module that has been identified in over one hundred protein sequences [11,12]. Although there may be some functional variability within the PH domain family, it is now widely accepted that the majority of the members of the family are involved in binding of the phosphorylated head of inositol phospholipids (inositol phosphate, InsP) [12,13].

Phosphoinositides play a central role in a number of cellular processes, ranging from the intracellular transduction of signals originating at the plasma membrane, to the organisation of the actin cytoskeleton, to the prevention of apoptosis, to the regulation of vesicular endocytosis [13,14]. The absolute relevance of phosphoinositide binding by PH domain for proper cellular metabolism is demonstrated by the existence of disease-causing mutations affecting PH domain–lipid interaction in humans. In fact, critical missense mutations within the PH domain of the Btk gene result in a severe human immunodeficiency known as X-linked agammaglobulinemia (XLA) [15].

Early structural analyses revealed that the PH domain folds into a seven-stranded β-barrell [16]. Successive structural studies of the PLC∂ PH domain complexed with inositol (1,4,5) triphosphate (Ins(1,4,5)P_3), and of the Btk PH domain in complex with Ins(1,3,4,5)P_4, revealed that the region located near the N-terminal end of the domain, comprising β-strands β1 and β2, and the loop connecting them (β1–β2 loop), is critically involved in substrate binding. The combination of structural information with data gathered from studies measuring the binding of several PH domains to differentially phosphorylated inositols, has allowed a preliminary definition of the minimal sequence requirements for selective InsP binding [16]. These studies seem to conclude that despite the direct involvement of other regions of the domain in substrate binding, most and probably all sequence information necessary to encode selective phosphoinositide recognition is contained within the β1–β2 region of the domain. A sequence alignment of this region is shown in Fig. 2. The reliability of the alignment is guaranteed by the presence of two very well conserved hydrophobic residues in positions flanking the β1–β2 region, which are essential determinants of the structural stability of the domain (at position +5 of the β1-strand and position +6 of the β2-strand). Residues implicated in InsP binding by other PH domains are conserved in the FGD1 domain, strongly suggesting that this domain is also involved in InsP binding. In particular, the alignment reveals a very strong similarity between the FGD1 and ARK PH domain. The latter has been shown to be involved in binding PtdIns-4,5-P_2 and PtdIns-3,4,5-P_3 [17]. The FGD1 mutation falls at position +4 from the start of the β2-strand. Within phosphoinositide-binding PH domains, the +4 position is occupied by an Arg residue, which the aforementioned crystal structures revealed to be critically involved in InsP binding [18–20]. In fact, mutations affecting the same position in the Btk PH domain (Arg28 in the Btk sequence) result in X-linked agammaglobulinemia [15,21–23]. Thus, it appears that the deleterious effect of the Arg610Gln mutation described in this study may result from the inability of the mutated PH domain to interact with a lipid functional modulator. Based on the functions of other PH domain containing proteins [24] we speculate that the mutation may hamper the localisation of FGD1 to specific cellular compartments, thus altering the GDP–GTP exchange function of the associated DH- and SH3-binding motifs.

Another example of disease with mutations affecting the function of a PH domain has been suggested to be represented by the X-linked Wiskott–Aldrich Syndrome (WAS) and its allelic variant X-linked thrombocytopenia (XLT) [25–30]. Nevertheless, the presence of a genuine PH domain in the WAS protein has been more recently questioned as the WAS protein sequence aligns very poorly with available multiple sequence alignments of bona fide PH domains. On the other hand, this sequence can be productively aligned to the so-called Ena-Vasp homology 1 (EVH1) domain [31]. Interestingly, the determination of the crystal structure of the EVH1 domain revealed a three-dimensional fold similar to that of the PH domain [32,33]. Despite structural similarity, however, the function of the EVH1 is distinct from that of the PH domain, and consists in polyproline type II (PPII) helix recognition. Indeed, several domain families have been described that share the PH fold while displaying distinct functional properties [12]. It is important to note that the alignment of the WAS protein sequence to the PH domain proposed [29] was largely offset with respect to the PH-like fold of the EVH1 domain [33], and thus did not reflect the proper alignment of very divergent sequences sharing a common fold. Altogether these observations definitively establish that the WAS protein does not contain a PH domain but rather an EVH1 domain.

In conclusion, we have found a novel mutation in a family with the classical manifestation of the AAS, allowing us to make diagnoses in affected patients and to detect the carrier females leading to better genetic counselling with respect to future risks of recurrence in the family. The clinical phenotype observed in this family may help to elaborate a more specific definition of the genotype–phenotype correlation in Aarskog–Scott Syndrome, especially with regard to phenotype of the carrier females. Furthermore, the present study is the first to demonstrate a causative mutation at a sensitive site of the PH domain of FGD1 protein in AAS, adding this disorder to the previously reported human disease XLA in which this domain has been found to be involved. Detection of a novel mutation in the FGD1 gene confirms the pathogenetic role of this gene in AAS, although the low frequency of mutations so far detected in AAS patients still leaves open the possibility that other genes may be involved.

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