Confirmation of the recurrent ACVR1 617G>A mutation in South Africans with Fibrodysplasia Ossificans Progressiva: narrowing targets for molecular therapeutic intervention

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Running title: ACVR1 617G>A SNP is associated with FOP in South Africans
Abstract

Background

Fibrodysplasia Ossificans Progressiva (FOP) is a rare autosomal dominant genetic condition in which the main feature is progressive ossification of fibrous tissue, tendons and ligaments, which leads to severe disability. World-wide, most affected individuals who have been studied have a recurrent 617G>A mutation, the gene that codes for activin A type 1 receptor/activin-like kinase 2 (ACVR1/ALK2). Recently, however, other mutations in the same gene that are associated with FOP have been identified. The majority of publications on the genetics of FOP are based on patients of either Caucasian or Asian extraction and no genetic information is available concerning indigenous African populations. In order to address this situation, we have undertaken genotyping of the ACVR1 receptor gene for 617G>A mutation which results in histidine replacing arginine at position 206 (R206) using PCR/RFLP and sequencing in six affected South Africans of whom 4 were of indigenous African stock.

Results

They were all heterozygous for the ACVR1/ALK2 617G>A mutation. The confirmation of this recurrent mutation in 100% of indigenous African patients studied in South Africa has relevance in the genetic diagnosis of affected persons in this country, indicating the need for genetic analysis in suspected cases to compliment diagnosis.

Conclusion

World-wide the presence of ACVR1/ALK2 617G>A in the majority of affected persons narrows down the search for molecular targets for rational intervention to this specific ACVR1/ALK2 domain.
Key words: activin A type 1 receptor; African; Fibrodysplasia Ossificans Progressiva; ossification; single nucleotide polymorphism
Introduction

Fibrodysplasia Ossificans Progressiva (FOP) [OMIM 135100] is an uncommon genetic disorder in which ossification of connective tissue leads to severe disability. Interplay between genetic determinants and environmental factors influences the phenotype during prenatal development and postnatal progression to ossification [1, 2]. The condition is an autosomal dominant trait and affected persons have mutations in the activin A type I receptor gene (ACVR1), chromosomal locus 2q23-24 [3]. ACVR1 is one of the four type I receptors that mediate in the highly conserved bone morphogenetic protein (BMP) signalling pathway, through a domain rich in glycine and serine (GS-domain) residues [2, 4,5]. BMP type I receptors transmit downstream signals through the BMP pathway-specific Smads and the MAPK signalling pathway to regulate transcription of target genes [1, 2, 4, 6].

The diagnosis of FOP has depended on recognition of characteristic clinical and radiological features, and until recently the causative mechanism has remained elusive [1, 4]. Sequence analysis in FOP patients worldwide has now revealed that the majority of affected individuals in whom mutational analysis has been undertaken have the same single nucleotide change 617G>A in the ACVR1 gene [1, 3, 4, 7]. The base change is a missense mutation which leads to the substitution of arginine with histidine (R206H). The point mutation 617G>A in ACVR1 which occurs in the GS-domain alters the ligand-dependent sensitivity for BMP signalling in connective tissue progenitor cells [1, 8, 9]. Under normal circumstances, type I receptors such as ACVR1 are inactive until stimulated by extracellular BMPs through phosphorylation.

The substitution of arginine which is positively charged by neutrally charged histidine affects electrostatic properties of the GS-domain, destabilising the binding of FKBP12 to ACVR1 receptor and resulting in increased basal activity of the BMP signalling in the absence of
ligand or hyper responsiveness of the BMP signalling in the presence of ligand (Kaplan et al., 2008a, Shen et al., 2009). Under normal circumstances, FKBP12 binds and stabilises the inactive conformation of ACVR1 receptor, preventing leaky activation of ACVR1 in the absence of ligand [4, 10].

The constitutive activation of ACVR1 observed in the presence of the 206 histidine variant induces downstream processes such as up regulation of BMP4, down regulation of BMP antagonists and induces ectopic chondrogenesis leading to joint fusions in FOP. Although most of the documented persons with FOP have been shown to exhibit the R206H mutation in ACVR1, it is now clear that FOP and related disorders such as some forms of myositis could be due to the involvement of the ACVR1 receptor [2, 4, 8]. This concept is supported by the demonstration of other mutations in the ACVR1 gene, Arg202Ile, Gly328Glu, Arg258Ser, L1996P and G356D among a few persons with FOP [5, 10-12].

The majority of genetic analyses have been carried out in populations of Caucasian and Asian origin and the question arises as to whether or not this specific mutation (617G>A) is associated with FOP in all populations in the world [2, 3, 7]. No molecular studies interrogating the genetics of ACVR1/ALK2 and its association with FOP have been reported from sub-Saharan Africa. For this reason, we have undertaken an investigation in order to determine if the commonly reported ACVR1/ALK2 617G>A recurrent mutation, is also the main cause of FOP in indigenous South Africans. Our findings are presented and discussed in this article.

Materials and Methods

Subjects
A total of six persons (n=6), 4 of indigenous African origin from the Xhosa group and one each from the South African Mixed Ancestry and Caucasian populations with the characteristic clinical and radiological manifestations of FOP were available for molecular investigation. In order to provide a perspective on the features and the natural history of FOP, pertinent details of the 6 affected persons are summarised in Table I.

The first three cases designated FOP1.1, 1.2 and 1.3 were the subject of earlier reports on the management of FOP in the South African context [13], and the dental implications of the disorder [14].

The features of FOP in the affected South Africans were entirely consistent with the descriptions of the disorder in the literature. The severity and rate of progression of FOP is variable, but the condition often presents at birth with shortening and deviation of the great toes. General health remains good and early development is normal. In mid-childhood tender subcutaneous lumps may appear, most frequently on the upper region of the back. Thereafter, these masses become ossified and widespread ectopic ossification develops in the connective tissues. The limbs, neck and jaw become tethered by bands of ossification and affected persons may eventually be completely immobile. In the final stages, movements are limited to the external muscle of the eye and the diaphragm. Demise usually occurs in middle age from respiratory insufficiency.

**Genetic and sequence analysis of ACVR1/ALK2 gene**

Blood was obtained for molecular genetic analysis from the six FOP patients and six ethnic matched controls after informed consent was obtained in each case in accordance with the requirements of Human Research Ethics Committee, University of Cape Town. Genomic DNA was extracted from peripheral blood leukocytes using a Qiagen DNA extraction kit.
(Valencia, USA). The single nucleotide polymorphism 617G>A in ACVR1 was analyzed according the method of Shore et al. [3] using PCR and restriction fragment length polymorphism (RFLP). The method involved amplification of a part of exon 4 of the ACVR1 gene using antisense). The forward and reverse primers, 5'-CCA GTC CTT CTT CCT TCT TCC-3 and 5'-AGC AGA TTT TCC AAG TTC CAT C-3, respectively, were used to amplify a 350 bp fragment using the following conditions; denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 30s, annealing at 59°C for 30s and extension at 72°C for 30s. A final extension step at 72°C for 7 minutes completed the reaction.

Each PCR reaction contained 100 ng of genomic DNA in 1X PCR buffer, 1.5mM MgCl₂, 0.2mM dNTP mixture, 0.2µM of each primer and 1U of GoTaq Flexi DNA polymerase (Promega, USA) in a final volume of 25 µl. Five µl of the amplified PCR product were electrophoresed on 1.8% agarose to confirm successful amplification of the 350 bp fragment. The remaining PCR product for each subject was divided into two aliquots of 10 µl for digestion with \textit{HphI} and \textit{Cac8I} (New England Biolabs, Beverley, MA, USA), respectively. The digested PCR products were electrophoresed on 2.6%. The 617G>A point mutation in \textit{ACVR1} eliminates a \textit{Cac8I} site and forms a new \textit{HphI} site. The genotyping results are shown in Figure 1. In addition, an aliquot of the PCR product for each of the samples (6 patients and 6 controls) was subjected to sequence analysis and representative sequence chromatograms (pherograms) are shown in Figure 2.

**Results**

The 350 bp PCR product from the normal allele (617G) after digestion with \textit{Cac8I} showed the three bands (139, 114, and 97 bp) while the mutant allele (617A) appeared as two bands (253 and 97 bp) in persons with FOP. For \textit{HphI}, PCR products the 617G allele (normal) were not digested but PCR products of the 617A allele showed bands of 228 and 122 bp in the FOP patients. A representative picture showing the results of PCR product digestion of each
of the samples with *Hph* I and *Cac8I*, respectively is shown in Figure 1. All six FOP patients in our study were heterozygous for the 617G>A mutation, while in all six controls, the mutation was absent. Sequence analysis showed the heterozygous genotype for all the patients while all the controls showed the homozygous 617G/G genotype (shown in Figure 2).

**Discussion**

FOP usually presents in childhood with swellings on the dorsum of the trunk and microdactyly of the great toes is a useful diagnostic marker at this stage. Progressive heterotopic ossification replaces fibrous tissue, tendons, muscles and ligaments. This process leads to increasing immobility and affected persons become severely handicapped [1,4]. The majority of individuals with FOP are sporadic, representing new mutations for the determinant gene. Nevertheless, typical AD transmission has been documented in a few mildly affected families. It is relevant that the specific R206H mutation in the ACVR1 gene is consistent in these kindreds and in sporadic persons, thus confirming the molecular homogeneity of the disorder. The mutation rate in FOP in the UK has been calculated as 1.8 million gametes per generation, with a point prevalence of 0.61 per million [15]. On this basis, it can be estimated that there should be about 25-30 persons with FOP in South Africa. In 1982, it was possible to document 6 affected South Africans [16]. This number was subsequently increased to 9, with the inclusion of additional cases from the radiological archives [17]. Two other adult males of Afrikaner stock with FOP were also documented under the non-specific designation “Myositis Ossificans Progressiva” [18, 19].

Although FOP has a worldwide distribution, there are few reports of affected persons of indigenous African stock. Four of the six persons documented in Cape Town had indigenous African ancestry and from the literature only three more individuals of African stock can be identified in other countries, notably “Africa” [20], Dominica [21] and Nigeria [22]. It is
probable that this paucity of reports reflects difficulty with diagnosis and ascertainment together with compromised survival in disadvantaged circumstances rather than any actual discrepancy in mutation rate.

The determinant gene for FOP, ACVR1/ALK2, was localised to chromosome 2q23-q24 and thereafter a specific mutation 617G>A (resulting in a R206H substitution) was identified [3]. Although most of the documented persons with FOP have been shown to exhibit the recurrent 617G>A mutation in ACVR1/ALK2, recently, other mutations in the same gene have been shown to associate with FOP in the absence of the classical 617G>A mutation [11, 23-25]. In addition, there has been considerable controversy as to whether mutations in the NOG gene at 17q21-q22 can also cause FOP. This issue has now been settled in favour of the ACVR1/ALK2 gene [6, 25-27].

In addition to Europe and North America, reports from several other parts of the world have confirmed the presence of the 617G>A specific mutation in persons with FOP. These include Japan, Korea, China and France [2, 7, 27-29]. However, the concept of one specific mutation in the ACVR1 is no longer tenable as evidenced by the reports associating 605G>T, 983G>A, 774G>C and 1067G>A with FOP in the absence of the recurrent 617G>A [2, 11, 24, 25]. Nevertheless, the important feature of these mutations is that they result in amino acid changes and mostly occur in the Glycine/Serine (GS) or kinase coding domains (reviewed by [25]). This observation makes molecular sense in that different mutations in the ACVR1/ALK2 receptor gene could also lead to different clinical presentations of FOP; this point is well illustrated in the report by Gregson et al. [25] where the 587T>C mutation is associated with comparatively delayed FOP onset. By extrapolation, mutations within the ACVR1/ALK2 receptor gene which cause variable ACVR1/ALK2 receptor activity could
also lead to different phenotypes, depending on the sensitivities of the domains in which they occur [2, 12, 25]. This concept could also argue for the involvement of mutations in ACVR1 receptor gene in other related disorders such as myositis [2, 25, 27]. The identification of the recurrent 617G>A mutation in almost all FOP patients world-wide together with the narrowing of all reported mutations to the GS and kinase domains of ACVR1/ALK2 gene, provides a specific target of intervention in a critical signalling pathway. The prime target intervention would be inhibition of the BMP signalling pathway in FOP patients, using RNA technology, or monoclonal antibodies [3, 4, 9, 30]. In our own circumstances in South Africa, the confirmation of the recurrent 617G>A mutation in all the six patients means that genetic analysis can be used to aid in the diagnosis of suspected FOP thereby negating the need for invasive procedures that can lead to increased inflammation and faster progression in FOP.

The demonstration that 6 South Africans with FOP have the same mutation (ACVR1 R206) that has been documented worldwide provides a firm starting point for the establishment of diagnostic molecular testing for FOP in sub-Saharan Africa. In turn, awareness of the disorder and the feasibility of diagnostic confirmation by the molecular laboratory will have a positive impact upon the medical management of affected persons in Africa.

Conclusions

Mutations in the ACVR1/ALK2 receptor gene are associated with FOP in individuals across the world. Although other mutations have been documented, this report confirms that in the South African Black population the 617G>A mutation accounts for all the genetically analysed FOP patients. Furthermore, in the cases worldwide where other mutations have been reported, they seem to fall within ACVR1/ALK2 functional domains such as the GS and
kinase domains. This finding could facilitate research on identifying molecular intervention strategies that may be applied in FOP patients to slow the progression of the disorder, particularly by targeting the BMP signalling pathway.

**Authors’ contribution**

CD, CS and PB conceptualised the study. CD designed the experiments, conducted the data analysis and wrote the manuscript. PB, KF, MU, RA, CS helped to write the manuscript. All authors have read and approved the final manuscript for publication.

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References


intracellular signaling equivalent to a typical mutation, R206H. *Biochemical and Biophysical Research Communications* 2011, 407:213–218.


Captions

Table I
Manifestations of FOP in the six affected persons.

**Figure 1:** Mutation analysis of the ACVR1 in South African FOP patients. Patient samples were number 1.1; 2.1; 3.1 and 4.1, respectively. UD represents undigested 350 bp PCR product while MM is the molecular weight marker. Samples 18 and 40 are healthy controls with no clinical manifestation of the disease. Each sample was digested with HphI and Cac8I in separate reactions and each reaction identified with either H for HphI or C for Cac8I respectively. The products for the separate digestions for each enzyme were run alongside each other, for example, 1.1H and 1.1C, respectively. After HphI digestion of the 350 bp PCR product, the 617G allele (normal) were not digested but PCR products of the 617A allele showed bands of 228 and 122 bp in the FOP patients only. The 350 bp PCR product from the normal allele (617G) after digestion with Cac8I showed the three bands (139, 114, and 97 bp) while the mutant allele (617A) appeared as two bands (253 and 97 bp) in FOP patients.

**Figure 2:** DNA Sequence analysis of a section of the ACVR1/ALK2 gene in South African FOP patients. The arrow indicates position of the gene where the mutation has been reported (c.617G>A; R206H). The numbering FOP1.1 to FOP6 refer to the different patients with GA representing the heterozygous genotype at this position.
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<table>
<thead>
<tr>
<th>Patient</th>
<th>Gender</th>
<th>Year of birth</th>
<th>Ancestry</th>
<th>Disability</th>
<th>Additional Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>FOP 1.1</td>
<td>F</td>
<td>2005</td>
<td>Xhosa</td>
<td>Extensive heterotopic ossification over back and spine. Arms and neck immobile and arms tethered to thorax.</td>
<td>Legs spared at this point.</td>
</tr>
<tr>
<td>FOP 2.1</td>
<td>M</td>
<td>1965</td>
<td>Xhosa</td>
<td>Spine and arms completely immobile. Able to walk unaided</td>
<td>No clinical features of thoracic insufficiency syndrome. Gainfully employed.</td>
</tr>
<tr>
<td>FOP 3.1</td>
<td>F</td>
<td>1964</td>
<td>Xhosa</td>
<td>Completely immobile. Only able to move eyes and tongue.</td>
<td>Profound iron deficiency anemia due to diet of tea and bread as unable to open mouth and chew. Thoracic insufficiency syndrome and pulmonary hypertension</td>
</tr>
<tr>
<td>FOP 4.1</td>
<td>F</td>
<td>2003</td>
<td>Mixed Ancestry</td>
<td>Heterotopic ossification over back and spine. Arms and neck immobile and arms tethered to thorax.</td>
<td>Presented after injury at school. Has had serious fall due to inability to protect with arms and developed large swelling over right eye.</td>
</tr>
<tr>
<td>FOP 5.1</td>
<td>F</td>
<td>2009</td>
<td>Xhosa</td>
<td>Hard mass between scapulae. Rigid cervical spine and limited abduction of arms</td>
<td>Fitted with a helmet to protect against head injuries.</td>
</tr>
<tr>
<td>FOP 6.1</td>
<td>F</td>
<td>1997</td>
<td>Caucasian</td>
<td>Spinal immobility noted only months before diagnosis.</td>
<td>Had surgical correction of toes at 5 months of age.</td>
</tr>
</tbody>
</table>