Association of an intronic variant of faciogenital dysplasia 1 (FGD1) gene with X-linked intellectual disability.

Yashvant Khimsuriya¹, Nikhil Kharod², Ghanshyam Padmani³, Jenabhai Chauhan¹, Nilanjan Roy¹
¹Department of Genetics, Ashok and Rita Patel Institute of Integrated Study & Research in Biotechnology and Allied Sciences (ARIBAS), New Vallabh Vidyanagar, Anand-388121, Gujarat, India.
²HM Patel Center for Medical Care and Education, Karamsad, Gujarat, India.
³Bachpan Children Hospital and Neonatal Care, Surat, Gujarat, India.

Abstract

Background: Faciogenital Dysplasia Gene (FGD1) mutations are seen to be underlined causes of the broad spectrum of Aarskog-Scott syndrome (ASS) as well as Non-Syndromic X-linked intellectual disability. The protein FGD1 is an important regulator of events that control extracellular matrix remodeling, bone development, cell migration and also involved in the regulation of few secretory proteins. The reported mutations suggest that non-coding variants are equally important for disease progression. The current study tests manifestations of intronic variant SNP rs2239809 of FGD1 in intellectually disabled children.

Methods: The selection of associated intronic variants of FGD1 gene was performed using GWAS (Genome Wide Association Study) central database and GWAS 3D web server. PCR-RFLP was performed for screening of selected intronic variants. Sanger sequencing was used to validate single nucleotide change in FGD1 gene. Clinical features were studied and compared; partial pedigree was recorded for affected patients.

Results: In this study, significant alteration was found in two affected children from different family harboring intronic variant NM_004463.2: c.659+27T>C (rs2239809) at intron 3 of FGD1 gene. Surprisingly, female and male showed different phenotypic appearance for the same variation.

Conclusion: The present study provides significant insight that intronic variant rs2239809 affects FGD1 gene functions and on the basis of computational study this variant active for transcription regulation process. The study need to translate the computational genetic signals into biological mechanism of Transcription Factor Binding Site (TFBS) affinity of this intronic variant.

Keywords: Faciogenital dysplasia 1 (FGD1), X-linked intellectual disability, Intronic variant, Skeletal dysmorphism, TFBS affinity, Regulatory variant.

Accepted October 25, 2016
acromelic short stature. The exact molecular mechanism of FGD1 mutations leading to skeletal deformations of faciogenital dysplasia remain unclear, but the pathogenicity and recent studies clearly show that the protein is involved in the regulation of bone development [3-5].

The FGD1 gene encodes Cdc42-specific GEF (Guanine nucleotide Exchange Factor) protein. Like most Rho GEFs, FGD1 contains a Dbl Homology Domain (DH) adjacent to a Pleckstrin Homology (PH) domain that catalyzes the exchange of GDP for GTP on Cdc42, an N-terminal Proline-Rich Domain (PRD), a cysteine-rich zinc-finger FYVE domain and a second COOH-terminal PH domain. The PRD negatively regulates enzymatic activity and contains two putative Src-Homology 3 (SH3)-binding domains, whose known interactors are cortactin and Actin-Binding Protein 1 (mAbp1) [5]. Biochemical studies show that FGD1 is a specific Cdc42 activator and this activation is due to the SH3 binding domain interaction with cortactin and mAbp1 [6]. By activating Cdc42, FGD1 stimulates fibroblasts to form filopodia, cytoskeletal elements involved in cellular signaling and controls transcriptional activation secretory membrane trafficking [3,7,8]. Through Cdc42, FGD1 also activates the c-Jun N-terminal kinase signaling cascade, a pathway that regulates cell growth and differentiation. These data indicate that FGD1 is a central regulator of the Cdc42 intracellular signaling pathway. FGD1 is an important regulator of events that control extracellular matrix remodeling, bone development, cell migration and also involved in the regulation of secretory protein export from the Golgi complex [3,9].

The majority of associated variants have been found to localize outside of known protein coding sequencing [10]. There are growing number of examples of trait associated intronic variants that play a significant role in defining the contribution in gene expression [11]. Intronic sequences responsible for gene regulatory elements such as enhancers, silencers and insulators of transcriptional regulation process, where they beat binding sites of transcription factors or co-factors recruitment [12,13].

In this study, we present findings related to one FGD1 gene intronic variant NM_004463.2: c.659+27T>C (rs2239809) at the boundary of SH3 binding domain region. Other selected variants were not present in the selected patients so we were directly focused on this single variant which is found altered in the selected population.

Materials and Methods

Selection of Variants

GWAS Central database tool used for selection of disease associated variants of FGD1 gene [14]. Total 13 markers selected on the basis of non-coding variants and association with significant findings. Out of these, 6 variants were selected followed by GWAS 3D web server on the basis of their gene regulatory mechanisms [15].

Enrolment of Patients and Sample Collection

The selection of patients was carried out as per the criteria are given by Teebi et al. for faciogenital dysplasia [16]. All protocols used in this study were approved by Human Research Ethics Committee of HM Patel Centre for Medical Care and Education, Karamsad, Gujarat, India. Informed consent forms were signed by parents. Total 40 children with intellectual disability and 50 controls were included from Anand, Bhavnagar and Surat districts of Gujarat, India. The symptoms were evaluated through accurate clinical examination and anthropometric measurements.

Genomic DNA Isolation

Genomic DNAs from children were extracted from peripheral blood leukocytes by standard procedures. All samples were subjected to screen selected intronic variant rs2239809 using primer specific PCR analysis.

PCR Amplification

DNA samples were prepared for the PCR with the master mix (50 units/ml of Taq DNA Polymerase, 400 µM each of dNTPs and 3 mM MgCl2 with pH 8.3) from Xcleris Labs Limited, Ahmedabad. The primers for SNP rs2239089 were FGD1 forward TCC ACC ATC ACG CCC ACT (5’ CˇTAG 3’) and FGD1 reverse TCT CCT GAC TAT CCC TTC CTG (5’ CˇTGC 3’). UCSC genome browser and NEB Cutter2 tool used for the selection of RE (Bfa1) at position chrX: 54496989 which is an exact site for rs2239089. The reaction mixture for restriction digestion was prepared by mixing 10 µl of PCR product, 14.5 µl of 1X NEBSmart™ buffer and 0.5 µl (5 U) of Bfa1. The mixture was incubated at 37°C for 2 h in the water bath. The digested products were analyzed by 3% agarose gel electrophoresis prepared in 1X TAE buffer and the DNA band pattern was compared with 50 bp DNA ladder.

RFLP Analysis

Restriction digestion of the FGD1 PCR products was carried out by using Bfa1 restriction endonuclease (5’ TAG3’). UCSC genome browser and NEB Cutter2 tool used for the selection of RE (Bfa1) at position chrX: 54496989 which is an exact site for rs2239089. The reaction mixture for restriction digestion was prepared by mixing 10 µl of PCR product, 14.5 µl of 1X NEBSmart™ buffer and 0.5 µl (5 U) of Bfa1. The mixture was incubated at 37°C for 2 h in the water bath. The digested products were analyzed by 3% agarose gel electrophoresis prepared in 1X TAE buffer and the DNA band pattern was compared with 50 bp DNA ladder.

DNA Sequencing

The amplified PCR product was purified using Qiagen Mini elute Gel extraction kit according to the manufacturer’s protocol. The concentration of the purified DNA was determined and was subjected to automated DNA sequencing on ABI 3730xl Genetic Analyzer (Applied Biosystems, USA). Sequencing was carried out using BigDye® Terminator v3.1 Cycle sequencing kit following manufacturer’s instructions. The reaction was carried out in a final reaction volume of 20 µl using 200 µl capacity
thin walls PCR tube. The cycling protocol (Denaturation at 96°C for 10 s, annealing 52°C for 5 s and extension at 60°C) was designed for 25 cycles with the thermal ramp rate of 1°C per s. After cycling, the extension products were purified and mixed well in 10 μl of Hi-Di formamide. The contents were mixed on the shaker for 30 mins at 300x g. Eluted PCR products were placed in a sample plate and covered with the septa. Sample plate was heated at 95°C for 5 min, snap chilled and loaded into Autosampler of the instrument. Electrophoresis and data analysis were carried out on the ABI 3730xl Genetic Analyzer using the appropriate Module, Basecaller, Dyeset/Primer and Matrix files.

Results

Bioinformatics Approach to Select Variant Affecting Functions

According to GWAS Central, 13 SNP markers of FGD1 gene were selected on the basis of intronic variants and genetic association. All selected SNPs were analyzed using GWAS3D and evaluated on the basis of non-coding genetic variant affecting the gene regulation (Table 1). There are six SNPs found having significant Transcription Factor Binding Sites Affinity (TF) and showing mapping on Putative Enhancer Region (PE). Except for rs2284710, the other shows mapping on CTCF binding region (CE). Leading Variant (LV) and mapping on Distal Interaction (DI) negatively resulted in two SNPs as described in Table 1.

The SNP rs2239809 may functionally active in transcriptional regulation and having significant transcription factors binding site affinity (TFs: AP-2 known2, NF-kappaB known3, CEBPB known4, SRF known3, NF-kappaB known4). These results were predicted by GWAS 3D. Alterations in this position may affect the binding affinity of the transcription factors and transcription regulation. When splicing variant analysis was performed using Alamut® Visual 2.4, no significant association found for this intronic variant.

Clinical Evaluation

The clinical evaluations of both the patients out of 40 screened patients are summarized in Table 2. Both patients are the children of non-consanguineous parents of two different families. The study is carried out on two patients after confirming mutation through DNA analysis.

<table>
<thead>
<tr>
<th>SNP ID</th>
<th>Position</th>
<th>GWAS3D status</th>
<th>LV</th>
<th>TF</th>
<th>DI</th>
<th>PE</th>
<th>CE</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs17002528</td>
<td>X:54520257</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>√</td>
</tr>
<tr>
<td>rs2284710</td>
<td>X:54510913</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>rs2239809</td>
<td>X:54496989</td>
<td>-</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>√</td>
</tr>
<tr>
<td>rs3213533</td>
<td>X:54482508</td>
<td>-</td>
<td>√</td>
<td>-</td>
<td>√</td>
<td>√</td>
<td>√</td>
</tr>
<tr>
<td>rs5915127</td>
<td>X:54506066</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>√</td>
</tr>
<tr>
<td>rs3830137</td>
<td>X:54481833</td>
<td>√</td>
<td>√</td>
<td>-</td>
<td>√</td>
<td>√</td>
<td>√</td>
</tr>
</tbody>
</table>

LV: Leading Variant; TF: Transcription Factor Binding Sites Affinity; DI: Mapping on Distal Interaction; PE: Putative Enhancer Region; CE: CTCF Binding Region

Patient 1 was 12 year old girl at the time of evaluation. She was born with 1.5 month post gestational period. About 1 hour delayed birth cry noted and she suffered from pneumonia after 15 days of birth. We evaluated her at 12 years of age. Her height was 121 cm and weight was 52.3 Kg. Her head circumference was 48 cm. She has short stature and small feet. She showed maxillary hypoplasia with small teethes and small oral cavity. She also showed flat forehead and broad nose. Carrying angle and tong out observed and also no speech achieved till to date. Additionally, brittle hair and non-paralytic fish shaped eye observed. She showed separator anxiety behavior problem. The clinical evaluation of other family members was observed normal with these findings.

Patient 2 was 16 years old boy at the time of evaluation. He was born at preterm gestational age (7 Month) with birth weight 1.3 Kg. He had respiratory distress syndrome (RDS) and kept in the special care newborn unit (SCNU) for 25 days. We evaluated him at 16 years of age. He had difficulties in walking and aged 2.5 years. Delayed development observed. His height was 160.1 cm and weight 57.3 Kg. His head circumference was 52.3 cm. The other abnormal physical features include squint eyes with mild ptosis, everted feet and lordotic spine was observed (Figure 1). Partial pedigree was recorded for both families (Figure 2).

Genetic Analysis

Genetic analysis of both affected children revealed significant alteration in rs2239809 (NM_004463.2: c.659+27T>C) at FGD1 gene intron 3. The base substitution significant alteration in rs2239809 (NM_004463.2: c.659+27T>C) at FGD1 gene intron 3. The base substitution was found in this variant. Bfa1 restriction endonuclease cuts at two different positions within PCR amplified product of 184 bp and revealed 3 different bands of 128 bp, 44 bp and 12 bp in the normal case. If alteration found according to Bfa1 restriction digestion and incase of heterozygous three bands (128 bp and 56 bp) obtained after Bfa1 restriction digestion and Incase of heterozygous three bands (128 bp, 56 bp and 44 bp) were observed (Figure 3). 12 bp DNA band is invisible in this image due to its smaller size.

<table>
<thead>
<tr>
<th>Features</th>
<th>Patient 1</th>
<th>Patient 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>Female</td>
<td>Male</td>
</tr>
<tr>
<td>Age at examination (years)</td>
<td>12</td>
<td>16</td>
</tr>
<tr>
<td>Birth weight (kg)</td>
<td>3</td>
<td>1.3</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>121</td>
<td>160.1</td>
</tr>
<tr>
<td>Head circumference (cm)</td>
<td>48</td>
<td>52.3</td>
</tr>
<tr>
<td>Open mouth</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Ptosis</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Wide foot</td>
<td>-</td>
<td>+ (Everted)</td>
</tr>
<tr>
<td>Dysmorphic ears</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Abnormal spine</td>
<td>+</td>
<td>(Lordotic)</td>
</tr>
<tr>
<td>Broad nose</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Short stature</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Speech delay</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Behavior problems</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Mental impairments</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>WISC IQ</td>
<td>56</td>
<td>64</td>
</tr>
</tbody>
</table>

Table 2. Clinical features of patients
Association of an intronic variant of faciogenital dysplasia 1 (FGD1) gene with X-linked intellectual disability.

This alteration was found only in two children with intellectual disability and absent in normal individuals (50 controls). This intronic variant alteration further confirmed through DNA sequencing of PCR products (Figure 4).

**Discussion**

In this study, several primary characteristics were observed, namely, short stature, dysmorphic face, ptosis, abnormal feet and lordotic spine. This is in agreement with reports related to the Mutational etiology of Faciogenital dysplasia [18-20]. Various types of FGD1 mutations have been identified in coding and non-coding regions of the gene [21-23]. There are also evidence in the literature that intronic variants are also found to cause alterations in protein expression in genetic disorders [24].

The variant affects functions (rs2239809) found in the children is lying within the boundary sequences of the SH3 binding domain of FGD1 gene. It was reported that SH3 binding domain of FGD1 is interact with cortactin and mAbp1 to activate Cdc42 for ECM remodeling, cytoskeleton remodeling and other singling pathways [4]. FGD1 was notified for spontaneous mutational effects. This spontaneous mutation may lead to forming abnormal domain structure that may be responsible for the abnormal bone development and other symptoms of the faciogenital dysplasia.

On the basis of clinical features screening of patient 1, there were clearly seen the short stature, microcephaly, maxillary hypoplasia with hyperactivity symptoms and mild intellectual disability. These features were reported in heterozygous female with FGD1 mutations. Whereas in the case of patient 2 major skeletal deformities with the mild intellectual disability noted. It includes facial asymmetry, squints eyes with mild ptosis, lordotic spine structure and everted feet. These symptoms were reported in diverse cases of faciogenital dysplasia [19,20].

The further *in silico* analysis of the prediction related to the pathogenicity of this variant (rs2239089) using GWAS 3D web server and F-SNP database revealed that the variant responsible for transcriptional regulation processes. The

![Figure 1. Clinical features (Patient 1 (P1): a. Broad lower lip, b. Maxillary hypoplasia, c. Microcephaly, d & e. Short stature; Patient 2 (P2): a. Facial asymmetry, Mild ptosis and squint eyes (Hided), b/c. Broad ears, d. Abnormal hands, e. Everted feet)](image)

![Figure 2. Partial pedigree of families of P1 and P2)](image)

![Figure 3. Comparative digestion pattern of FGD1 PCR products on 20% DNA-PAGE; L1- P1 (128 bp, 56 bp, 44 bp); L2- P2 (128 bp, 56 bp); L3- Control 1 (128 bp, 56 bp, 44 bp); L4- Control 2 (128 bp, 44 bp); L5- 50 bp range DNA ladder; L6-Father of P1 (128 bp, 44 bp); L7-Mother of P1 (128 bp, 56 bp, 44 bp); L8-Father of P2 (128 bp, 44 bp); L9- Mother of P2 (128 bp, 56 bp, 44 bp)](image)
predicted transcriptions factors are already known to cause relative pathogenicity are well documented in the literature [25,26]. The SNP rs2239809 was previously screened in Chinese population (East Asian Population) and revealed no association with faciogenital dysplasia in the Qinba mountain area of China [17]. The allele frequency of East Asian and South Asian populations is 0.5257 and 0.1605, respectively [27]. Interestingly, the variant affects functions were identified in patient 1 (female) and patient 2 (male) in Gujarati Indian population (South Asian Population). There were no phenotypic alterations in the parents of affected children, though they exhibit same base substitution. This may be due to reduced expressivity or incomplete penetrance. A study regarding X-inactivation process in case of the female and molecular mechanism for non-coding regions with potential regulatory effects would be studied for further approach.

Our comparative results of genetic analysis, in silico analysis and phenotypic features in the children clearly showed that FGD1 singling may play a critical role in ossification and skeletal development. The present findings on intronic variant and disease phenotype may play a significant role to develop a new hypothesis for research on intronic mutations and biological mechanisms of complex traits and diseases.

**Conclusion**

The present study provides significant insight that intronic variant rs2239809 affects FGD1 gene functions. Further study needed for confirmation of inheritance pattern and variability in phenotypic appearance. Regulatory mechanism of non-coding variants should be studied in depth to find their role in disease uncertainty and development of complex traits.

**Acknowledgement**

We thank all the children and the parents who participated in this study. We also thank every administrator of Mamta Hygienic Clinic, Surat, Gurukrupa Residential Special School, Anand, Mitra Rehabilitation Centre, Anand and Deep Special Education for Mental Retardation, Surat for providing samples. We also thank Charutar Vidya Mandal (CVM), Vidyamandir and Sophisticated Instrument Centre for Applied Research & Testing (SICART) for providing lab facilities. Mr. Khimsuriya is a recipient of University Grant Commission, Rajiv Gandhi National Fellowship for the PhD program.

**References**


Association of an intronic variant of faciogenital dysplasia 1 (FGD1) gene with X-linked intellectual disability.


Correspondence to:
Jenabhai B Chauhan, Department of Genetics, Ashok and Rita Patel Institute of Integrated Study and Research in Biotechnology and Allied Sciences (ARIBAS), New Vallabh Vidyanagar, Anand-388121, Gujarat, India.
Tel: +912692 229 189
Fax: +912692-229189
E-mail: jenabhaichauhan@aribas.edu.in

Special issue: Pediatric Research
Editor: Abdulla A Alharthi, Department of pediatric nephrology, Taif University, Saudi Arabia