

## **Association of an intronic variant of facio-genital dysplasia 1 (FGD1) gene with X-linked intellectual disability.**

**Yashvant Khimsuriya<sup>1</sup>, Nikhil Kharod<sup>2</sup>, Ghanshyam Padmani<sup>3</sup>, Jenabhai Chauhan<sup>1</sup>, Nilanjan Roy<sup>1</sup>**

<sup>1</sup>Department of Genetics, Ashok and Rita Patel Institute of Integrated Study & Research in Biotechnology and Allied Sciences (ARIBAS), New Vallabh Vidyanagar, Anand-388121, Gujarat, India.

<sup>2</sup>HM Patel Center for Medical Care and Education, Karamsad, Gujarat, India.

<sup>3</sup>Bachpan Children Hospital and Neonatal Care, Surat, Gujarat, India.

### **Abstract**

**Background:** Facio-genital 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:** Facio-genital dysplasia 1 (FGD1), X-linked intellectual disability, Intronic variant, Skeletal dysmorphism, TFBS affinity, Regulatory variant.

*Accepted October 25, 2016*

### **Abbreviations**

ASS: Aarskog-Scott Syndrome; Cdc42: Cell Division Cycle 42; DH: Dbl Homology; FGD1: Facio-genital Dysplasia; GWAS: Genome Wide Association Study; GEF: Guanine Exchange Factor;  $\mu$ L: Microliter;  $\mu$ M: Micromolar; ml: Milliliter; mM: Millimolar; min: Minute; ng: Nanogram; PH: Pleckstrin Homology; PCR: Polymerase Chain Reaction; RDS: Respiratory Distress Syndrome; RFLP: Restriction Fragment Length Polymorphism; sec: Seconds; SNP: Single Nucleotide Polymorphism; TFBS: Transcription Factor Binding Site

### **Introduction**

The Mutations in facio-genital dysplasia gene are seen to be underlined causes of the broad spectrum of Aarskog-Scott Syndrome (ASS) as well as Non-syndromic X-linked intellectual disability [1,2]. These mutations affect the size and shape of a specific set of skeletal elements resulting abnormal functions of affected organs and tissues. The cardinal features of this disease include hypertelorism, ptosis, down slanting palpebral fissures, broad nasal bridge, anteverted nostrils, low set and protuberant ears, abnormally formed teeth, maxillary hypoplasia and transverse crease below the lower lip and disproportionate

acromelic short stature. The exact molecular mechanism of FGD1 mutations leading to skeletal deformations of facio-genital 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 facio-genital 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 leucocytes 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 MgCl<sub>2</sub> with pH 8.3) from Xcleris Labs Limited, Ahmedabad. The primers for SNP rs2239809 were FGD1 forward TCC ACC ATC ACG CCC ACT and FGD1 reverse TCT CCT GAC TAT CCC TTC CTG [17]. Aliquot of the DNA sample, 5 µL of 20 ng/µL were transferred to the tubes. The tubes containing mixture were vortex-mixed and transferred to a thermal cycler. Samples were amplified with an initial denaturation step of 95°C for 5 min; 30 cycles of 94°C for 30 s, 58°C for 10 s and 72°C for 30 s and a final extension at 72°C for 5 min. After the PCR amplification, samples were stored protected from light at -15°C to -20°C before RFLP analysis and DNA sequencing.

### RFLP Analysis

Restriction digestion of the FGD1 PCR products was carried out by using *Bfal* restriction endonuclease (5'C~TAG3'). UCSC genome browser and NEB Cutter2 tool used for the selection of RE (*Bfal*) at position chrX: 54496989 which is an exact site for rs2239809. 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 *Bfal*. 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 1.** Non-coding variants affecting FGD1 gene regulation

SNP ID	Position	GWAS3D status				
		LV	TF	DI	PE	CE
rs17002528	X:54520257	√	√	√	√	√
rs2284710	X:54510913	√	√	√	√	-
rs2239809	X:54496989	-	√	√	√	√
rs3213533	X:54482508	-	√	-	√	√
rs5915127	X:54506066	√	√	√	√	√
rs3830137	X:54481833	√	√	-	√	√

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 23.7 Kg. Her head circumference was 48 cm. She has short stature and small feet. She showed maxillary hypoplasia with small teeth 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 was found in this variant. *Bfal* 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 NM\_004463.2: c.659+27T>C variant position, two bands (128 bp and 56 bp) obtained after *Bfal* 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 2.** Clinical features of patients

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

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,

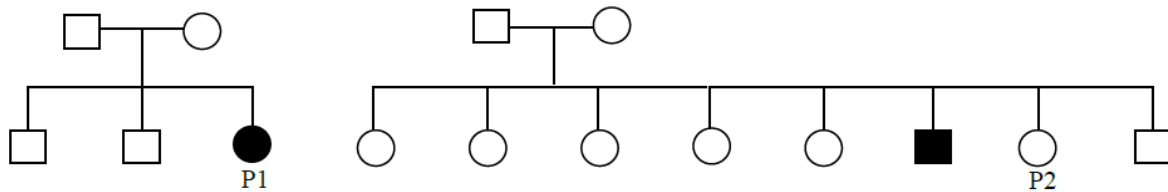
cytoskeleton remodeling and other signaling pathways [4]. FGD1 was notified for spontaneous mutational effects. This spontaneous mutation may lead to forming abnormal domain structure that may be responsible for the abnormal bone development and other symptoms of the faciogenital dysplasia.

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].

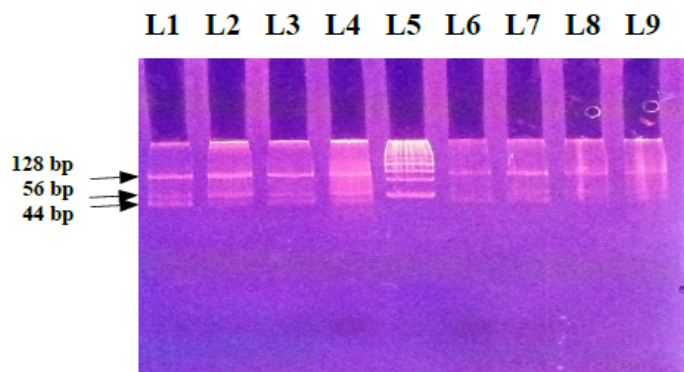
The further *in silico* analysis of the prediction related to the pathogenicity of this variant (rs2239089) using GWAS 3D web server and F-SNP database revealed that the variant responsible for transcriptional regulation processes. The



**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 (Hidden), b/c. Broad ears, d. Abnormal hands, e. Everted feet]



**Figure 2.** Partial pedigree of families of P1 and P2



**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)

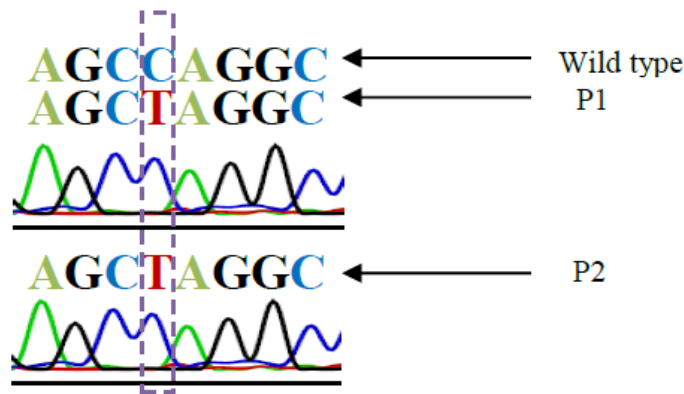


Figure 4. DNA sequencing for rs2239809 screening showing C>T transition

predicted transcription 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), Vidyanagar 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

1. Lebel RR, May M, Pouls S, et al. Non-syndromic X-linked mental retardation associated with a missense mutation (P312L) in the FGD1 gene. 2002; 61: 139-145.
2. Desai VD, Maheshwari S, Kumar MVS, et al. Scott syndrome : A rare case with review of literature. 2016; 6: 41-44.
3. Genot E, Daubon T, Sorrentino V, et al. FGD1 as a central regulator of extracellular matrix remodelling - lessons from faciogenital dysplasia. J Cell Sci 2012; 125: 3265-3270.
4. Daubon T, Buccione R, Genot E. The Aarskog-Scott syndrome protein Fgd1 regulates podosome formation and extracellular matrix remodeling in transforming growth factor stimulated aortic endothelial cells. Mol Cell Biol 2011; 31: 4430-4441.
5. Pedigo NG, Van Delden D, Walters L, et al. Mini review: Role of genetic changes of faciogenital dysplasia protein 1 in human disease. Physiol Genomics 2016; 48: 446-454.
6. Hou P, Estrada L, Kinley AW, et al. Fgd1, the Cdc42 GEF responsible for faciogenital dysplasia, directly interacts with cortactin and mAbp1 to modulate cell shape. Hum Mol Genet 2003; 12: 1981-1993.
7. Pariltay E, Hazan F, Ataman E, et al. A novel splice site mutation of FGD1 gene in an Aarskog-Scott syndrome patient with a large anterior fontanel. J Pediatr Endocrinol Metab 2016; 29: 1111-1114.
8. Whitehead IP, Abe K, Gorski JL, et al. CDC42 and FGD1 cause distinct signaling and transforming activities. Mol Cell Biol 1998; 18: 4689-4697.
9. Ayala I, Giacchetti G, Caldieri G, et al. Faciogenital dysplasia protein Fgd1 regulates invadopodia biogenesis and extracellular matrix degradation and is up-regulated in prostate and breast cancer. Cancer Res 2009; 69: 747-752.
10. Maurano M, Humbert R, Rynes E, et al. Systematic localization of common disease-associated variation in regulatory DNA. Science 2012; 337: 1190-1195.

11. Laurent GS, Vyatkin Y, Kapranov P. Dark matter RNA illuminates the puzzle of genome-wide association studies 2014; 12: 1-8.
12. Sacchetti P, Mitchell T, Granneman J, et al. Nurr1 enhances transcription of the human dopamine transporter gene through a novel mechanism. *J Neurochem.* 2001; 76: 1565-1572.
13. Shihab HA, Rogers MF, Gough J, et al. An integrative approach to predicting the functional effects of non-coding and coding sequence variation. *Bioinformatics* 2015; 31: 1536-1543.
14. Beck T, Hastings RK, Gollapudi S, et al. GWAS Central: A comprehensive resource for the comparison and interrogation of genome-wide association studies. *Eur J Hum Genet* 2014; 22: 949-952.
15. Li MJ, Wang LY, Xia Z, et al. GWAS3D: Detecting human regulatory variants by integrative analysis of genome-wide associations, chromosome interactions and histone modifications. *Nucleic Acids Res* 2013; 41: W150-158.
16. Teebi AS, Rucquoi JK, Meyn MS. Aarskog syndrome : Report of a family with review and discussion of nosology. 1993: 509.
17. Li JL, Li YJ, Zhang KJ, et al. No association between FGD1 gene polymorphisms and intellectual developmental disability in the Qinba mountain area. *Genet Mol Res.* 2014; 13: 127-133.
18. Orrico A, Galli L, Faivre L, et al. Aarskog-Scott syndrome: Clinical update and report of nine novel mutations of the FGD1 gene. *Am J Med Genet Part A* 2010; 152: 313-318.
19. Al-Semari A, Wakil SM, Al-Muhaizea M, et al. Novel FGD1 mutation underlying Aarskog-Scott syndrome with myopathy and distal arthropathy. *Clin Dysmorphol* 2013; 22: 13-17.
20. Perez-Coria M, Lugo-Trampe JJ, Zamudio-Osuna M, et al. Identification of novel mutations in Mexican patients with Aarskog-Scott syndrome. *Mol Genet Genomic Med* 2015; 3: 197-202.
21. Zhang F, Lupski J. Non-coding genetic variants in human disease. *Hum Mol Genet* 2015; 24: 2-10.
22. Orrico A, Galli L, Clayton-Smith J, et al. Clinical utility gene card for: Aarskog-Scott syndrome (faciogenital dysplasia). *Eur J Hum Genet* 2015; 23: e1-4.
23. Völter C, Martínez R, Hagen R. Aarskog-Scott syndrome : A novel mutation in the FGD1 gene associated with severe craniofacial dysplasia. *Eur J Pediatr* 2014; 173: 1373-1376.
24. Kilic U, Gok O, Bacaksiz A, et al. SIRT1 gene polymorphisms affect the protein expression in cardiovascular diseases. *PLoS One* 2014; 9: 1-9.
25. Me S. NF- $\kappa$ B functions in the nervous system : From development to disease. 2006; 72: 1180-1195.
26. Nanes M. Tumor necrosis factor- $\alpha$ : molecular and cellular mechanisms in skeletal pathology. *Gene* 2003; 321: 1-15.
27. <http://exac.broadinstitute.org>

**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](mailto:jenabhaichauhan@aribas.edu.in)

**Special issue:** Pediatric Research

**Editor:** Abdulla A Alharthi, Department of pediatric nephrology, Taif University, Saudi Arabia