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A targeted NGS approach to identify a c.352C>G variant in the TWIST1 gene in an Albanian family with Saethre–Chotzen syndrome

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ABSTRACT. A targeted next generation sequencing (NGS) approach analysing contemporaneously 20 different genes mainly involved in craniosynostosis was adopted to molecularly diagnose the family of a 2-years old girl affected by Saethre–Chotzen syndrome, a syndromic form of craniosynostosis. The identified pathogenic variant in the TWIST1 gene lies in a conserved residue (Arg118) that shifts from a positively charged amino acid to a non-

polar amino acid and segregates in all the examined affected familial members, although with variable phenotypic expression. An accurate molecular diagnosis is of obvious value for the clinical management of individuals with isolated or syndromic craniosynostosis to define their medical needs, the recurrence risk and allow the identification of available therapeutic opportunity. Given the high number of associated genes, an NGS approach is the election choice to increase the yield of genetic diagnosis, leading to an expansion of the genotypic and phenotypic spectrum of these rare syndromes.

KEY WORDS: craniosynostosis, variant, Saethre-Chotzen syndrome, TWIST1.

INTRODUCTION

Craniosynostosis is a fairly prevalent condition that affects ~1 in 2000 births,(Lajeunie et al. 1995) is defined as an early fusion of one or several cranial sutures, resulting in misshapen skull and facial bones.(Morriss-Kay and Wilkie 2005) It is characterized by either monogenic or polygenic transmission with environmental factors contributing to its complex etiology.(Johnson and Wilkie 2011) Usually, craniosynostosis occurs isolated, but in a minority of cases is associated with additional clinical features as part of a syndrome, such as Alpert, Crouzon, Pfeiffer and Saethre-Chotzen syndrome as the most common. Mutations in at least 57 genes have been associated with craniosynostosis, not all routinely included in laboratory genetic testing; and just six genes (*FGFR2*, *FGFR3*, *TWIST1*, *TCF12*, *ERF* and *EFNB1*) are found to be the underlying genetic cause identified in 1 every 4 cases.(Miller et al. 2017)

Genetic testing and an accurate molecular diagnosis and classification of diseases is usually suggested to all patients affected by craniosynostosis. A genetic diagnosis is of obvious value for patient's clinical management to define medical needs and recurrence risks, especially in syndromic cases that often require an early intervention to prevent significant co-morbidity. In addition, traditional phenotypically guided genetic testing may fail to identify rarer causes of disease as many conditions have a highly variable clinical presentation, and variants in different genes can present the same genetic defects.(de Heer et al. 2005; Lin et al. 2017; Xu et al. 2017)

Patient data

Here, we report on a 2-year-old Caucasian female patient (ID # IV 1, Figure 1A) with family history of dysmorphic features. She had multi-suture cranial involvement, facial asymmetry, unilateral ptosis (blue arrow in Figure 1B), low frontal hairline and a low nasal bridge. A narrow palate was absent. The ears were small, low-set and with prominent crus helicis. She presented bilateral partial syndactyly of the second and third toe, and the pectus was excavatum. Intelligence and hearing parameters were normal for her age.

Primary management concerns for this patient were the need to monitor for signs/symptoms of raised intracranial pressure. The pedigree analysis revealed that there were additional family members (ID# III 1, III 3; III4, II 4, II 5) with a similar phenotype (Figure 1A) however detailed clinical data and DNA were available for just the direct descendants of ID II 4 (Figure 1B). In Table 1 are reported signs and symptoms of the affected family members.

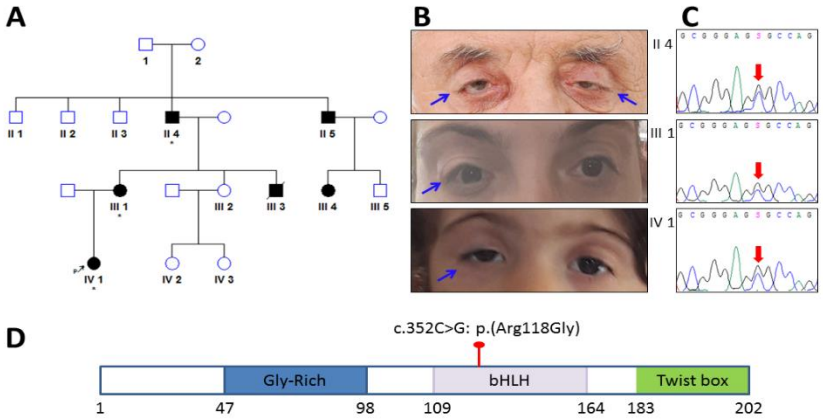

Figure 1. (A) Family pedigree (square represent male subject; circle, female subject. Affected individuals are in black, black arrow depicts the proband, * identify individual whose DNA was available for genetic testing and presented a *TWIST1* variant) (B) clinical photographs highlighting with blue arrows either bilateral ptosis (upper photo, ID II 4) or unilateral ptosis (middle and lower photo, ID III 1 and IV 1, respectively); *(C)* sequence chromatogram with c.352C>G variant identified in *TWIST1 and highlighted with the red arrow* for all family members (upper, ,middle and lower photo represent the sequence for ID II 4, III 1 and IV 1 respectively); (D) *TWIST1* picture highlighting the different domains of the protein (Gly-Rich, glycine rich region; bHLH, basic Helix Loop-Helix; Twist box) and indicating with the red lollipop the position of the identified missense variant.

Table 1. Clinical characteristic of affected family members.

Clinical characteristics	II 4	III 1	IV 1
Craniosynostosis	Yes	Yes	Yes
Low frontal hairline	Yes		Yes
Low nasal bridge	Yes	Yes	Yes
Ptosis	bilateral	unilateral	unliteral
Brachycephaly	Yes		
Small, low-set ears with prominent crus helicis	Yes	Yes	Yes
Hearing loss	Yes		
Pectus escavatum	Yes	Yes	Yes
Heart malformation		Yes	
Syndactyly	bilateral partial syndactyly of the second and third toe	bilateral partial syndactyly of the second and third toe	bilateral partial syndactyly of the second and third toe

METHODS

Genetic analysis

Genetic analysis was carried out after obtaining informed consent from the patients. Genetic testing was performed on DNA extracted from whole blood. A next generation sequencing (NGS) approach was preferred in order to analyze disease-associated genes in parallel, increasing substantially the detection rate with the most time and cost-effective choice. The targeted resequencing was performed using the Illumina commercial kit, TruSight One Sequencing Panel, on a Illumina MiSeq platform. This kit was designed for genomic analysis of the coding regions of 4,813 genes with associated clinical phenotypes (http://www.illumina.com/ products/TruSight-one-sequencing-panel.ilmn). The in-solution target enrichment was performed according to the manufacturer's protocol. Briefly, 50 ng of genomic DNA was simultaneously fragmented and tagged by Nextera transposon-based shearing technology. A 3-plex sample library pool were sequenced using a 150 bp paired-end reads protocol on MiSeq sequencer (Illumina, San Diego, CA). About 50 genes associated to craniosynostosis are available among the 4,813 genes in the TruSight One Sequencing Panel. We decided to focus at first to those 20 genes defined as associated to craniosynostosis accordingly to Orphanet (http://www.orpha.net/). The genes analyzed are *ALX4* (NM_021926; OMIM#605420), *CD96* (NM_198196; OMIM#606037), *CYP26B1* (NM_019885; OMIM#605207), *FBN1* (NM_000138; OMIM#134797), *FGFR1* (NM_015850; OMIM#136350), *FGFR2* (NM_001320658; OMIM#176943), *FGFR3* (NM_001163213; OMIM#134934), *FREM1* (NM_144966; OMIM#608944), *IFT122* (NM_052989; OMIM#606045), *IFT43* (NM_052873; OMIM#614068), *IL11RA* (NM_001142784; OMIM#600939), *LRP5* (NM_002335; OMIM#603506), *MSX2* (NM_002449;

OMIM#123101), *RAB23* (NM_001278668; OMIM#606144), *RECQL4* (NM_004260; OMIM#603780), *SKI* (NM_003036; OMIM#164780), *TWIST1* (NM_000474; OMIM#601622), *WDR19* (NM_001317924; OMIM#608151), *WDR35* (NM_020779; OMIM#613602), *ZIC1* (NM_003412; OMIM#600470). Bioinformatic analysis of the data was performed using an in-house pipeline to align the sequence reads with a reference genome, as well as variant calling, annotation and variant filtering to remove benign single nucleotide polymorphisms (variants with allele frequency $\leq$ 0.03). Public databases such as 1000 Genomes Project Database (http://www.internationalgenome.org/), Exome Variant Server (EVS) database (http://evs.gs.washington.edu/EVS/), Exome Aggregation Consortium (ExAC) database (exac.broadinstitute.org/) and the public database of single nucleotide variants (dbSNP, www.ncbi.nlm.nih.gov/SNP/) were used to filter and prioritize the variants and to check for allele frequencies, while the Human Gene Mutation Professional Database (HGMD) (http://www.biobase-international.com/product/hgmd) was used to identify genetic variations previously reported as pathogenic. *In silico* prediction software such as SIFT (Sorting Intolerant from Tolerant), PolyPhen-2 (Polymorphism Phenotyping v2), and Mutation Taster, Mutation Assessor and LRT (Likelihood Ratio Test) were used to assess the potential deleterious effect of missense variants. Wild-type amino acid properties were compared with the variations (http://www.russelllab.org/aas/aas.html)[8]. Criteria applied to evaluate the pathogenic nature of selected variant were accordingly to Richards *et al* (Richards et al. 2015). Literature and databases were carefully checked for previous description of selected candidate variants.

Genetic variation of just the pathogenic variant was confirmed by PCR coupled with direct Sanger sequencing of target regions on a CEQ8800 Sequencer (Beckman Coulter) according to the manufacturer's protocols by using the following *TWIST1* primer sequences AGAGATGATGCAGGACGTGT and GAGAGGGGAGGAAATCGAGG. Sanger sequencing, in search of the specific genetic variant detected by NGS sequencing were also programmed for all available relatives (indicated by * in the pedigree, Figure 1A).

RESULTS

The mean coverage of the NGS analysis was 60x; 89.0% and 96% of regions had a coverage of at least 25X and 10X, respectively. The NGS analysis detected as pathogenic just the variant c.352C>G or p.(Arg118Gly) in the *TWIST1* gene on 7p21 (Figure 1C and 1D, ClinVar Submission# SCV000599450). The variation lies on the DNA binding domain on a conserved amino acid and is defined as disease causing determining the change from a positively charged amino acid to a non-polar amino acid. The same variant was detected in all affected family members examined by Sanger sequencing confirmation (Figure 1C).

DISCUSSION

Mutations in at least 57 genes have been associated to craniosynostosis, a disease that occurs either in an isolated form or as part of a syndrome when coupled with additional clinical features. Given the high number of associated genes and the highly variable clinical presentation of craniosynostosis, an NGS approach that examine contemporaneously many genes associated to a disease, compared to Sanger sequencing that permit a phenotypically guided genetic testing, is suggested in order to increase the success rate of the genetic diagnosis especially for those rarer forms.

In this report, we adopted a protocol that screened 4,813 genes, among which there are about 50 genes described in the literature as related to craniosynostosis. We focused at first to 20 different genes more commonly associated to craniosynostosis, and found that the affected patients presented a c.352C>G nucleotide change in the *TWIST1* gene that translates in a residue substitution of the Arginine 118 with a Glycine in the helix-loop-helix domain of the protein. The identified variant falls in an hot spot that in the same syndrome has been found substituted also to cysteine, histidine, proline and serine with pathogenic implications.(Rose et al. 1997; Yousfi et al. 2002; Kress et al. 2005; Foo et al. 2009) Variants in *TWIST1* are associated to Saethre-Chotzen syndrome (OMIM#101400), to Robinow-Sorauf syndrome (OMIM#180750) and to Craniosynostosis type 1 (OMIM#123100); all condition inherited with an autosomal dominant pattern. *TWIST1* is a transcription factors, members of the basic helix-loop-helix family, playing a crucial roles in mesoderm development in all animals. The gene is constituted by two exons with only the first exon translated into a 201 amino acid residues protein. In humans two paralogous genes, *TWIST1* and *TWIST2*, have been associated to craniofacial disorders. The child phenotype highlighted other classical features of Saethre-Chotzen syndrome in addition to craniosynostosis and ptosis such as small ears, low-set and prominent crus helicis, low frontal hairline and a low nasal bridge, confirming the molecular diagnosis result. To date, in the HGMD database have been reported to cause Saethre-Chotzen syndrome 155 variants in the *TWIST1* gene, the majority of them are missense and reported in the DNA interacting domain of the protein

(BIOBASE, 2017). It is widely accepted that variants in *TWIST1* interfere with osteoblast differentiation, at least partially by disrupting the RUNX2 pathway (Lenton et al. 2005) and decreasing the expression of the notch ligand Jagged1.(Yen et al. 2010) Saethre-Chotzen syndrome has an estimated prevalence of 1 in 25,000 to 50,000 people. The signs of Saethre-Chotzen syndrome can fluctuate widely, even among affected individuals in the same family. Incomplete penetrance is also described in the literature.(Howard et al. 1997; Bourgeois et al. 1998; Chun et al. 2002) Mutations in *TWIST1* affects the development and maturation of cells in the skull, face, and limbs.

The segregation analysis confirmed that all the examined family subjects harbored the same variant supporting its pathogenic role although with variable phenotypic expression: while ID II 4 has a bilateral ptosis, both ID III 1 and IV 1 have a unilateral ptosis (Figure 1B, blue arrows). In addition, it has been reported in the American Society of Human Genetics 2013 meeting abstract by Castro *et al* that a patient affected by Saethre-Chotzen syndrome with the same substitution presented a clinical phenotype slightly different from the one reported in our family; in particular our patients did not present sensorineural hearing loss and speech delay. Common signs between the two reported cases were high forehead, low frontal hairline, ptosis, a broad depressed nasal bridge and prominent crus helicis.(Castro et al. 2013)

Multiple complications can arise in patients affected by craniosynostosis especially in syndromic forms: raised intracranial pressure, facial deformities affecting vision and breathing and other features such as intellectual defect for examples; therefore, patients have to be monitored to avoid criticality. (Johnson and Wilkie 2011) Regular follow-up throughout childhood is advisable, particularly to monitor for symptoms progressions. The identification of the specific variant not only is of prognostic value(Johnson and Wilkie 2011) but raises also the possibility of the use of adjuvant medical therapies in the future. For examples, potential therapeutic approaches for *TWIST*-associated craniosynostosis may target delivery of the normal TWIST1 or Jagged1 protein to the cranial sutures.(Kosty and Vogel 2015) The advent of therapeutic drugs aiming at preventing the premature suture re-fusion could be extremely valuable to avoid surgical intervention, the advent of neurological comorbidities prompting a surgical revision or the occurrence of suboptimal cosmetic results.

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