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Comparison of mutation findings in *ZIC2* between microform and classical holoprosencephaly in a Brazilian cohort

Lucilene A. Ribeiro^{1, #}, Erich Roessler^{2, #}, Ping Hu², Daniel E. Pineda-Alvarez², Nan Zhou², MaryPat Jones³, Settara Chandrasekharappa³, Antonio Richieri-Costa¹, and Maximilian Muenke^{2, *}

¹Molecular Genetics Laboratory and Clinical Genetics Service, Hospital for Rehabilitation and Craniofacial Anomalies, USP, Bauru, SP, Brazil

²Medical Genetics Branch, National Human Genome Research Institute (NHGRI), National Institutes of Health, Bethesda, Maryland, USA

³Genomics Core, Genome Technology Branch, National Human Genome Research Institute (NHGRI), National Institutes of Health, Bethesda, Maryland, USA

Abstract

BACKGROUND—Holoprosencephaly is the most frequent congenital malformation of the forebrain in humans. It is anatomically classified by the relative degree of abnormal formation and separation of the developing midline central nervous system. Mutations of *ZIC2* are the second most common heterozygous variations detected in holoprosencephaly (HPE) patients. Mutations in most known HPE genes typically result in variable phenotypes that range from classical alobar HPE to microforms represented by hypotelorism, solitary central maxillary incisor (SCMI), cleft lip/palate, among others. Here we explore the molecular diagnostic yield of *ZIC2* testing in subjects seen at a Brazilian craniofacial center (subjects with or without brain findings) and compare our results with other centers.

METHODS—We used Sanger bidirectional DNA sequencing in a cohort of 105 Brazilian patients within the clinical spectrum of HPE, including classical and microform groups. We also illustrate the use of capillary array genotyping of a common histidine tract expansion c.716_718dup (p.His239dup) on our cases and over 500 control individuals to document a wide extent of population variation.

RESULTS—We detected a total of five variants in the *ZIC2* gene: an unusually common histidine tract expansion c.716_718dup (p.His239dup), a rare c.1377_1391del_homozygous (p.Ala466_470del, or Ala 15 to 10 contraction), a novel intronic c.1239+18G>A variant, a novel frameshift c.1215dupC (p.Ser406Glnfs*11), and a c.1401_1406dup (p.Ala469_470dup, or alanine tract expansion to 17 residues).

CONCLUSIONS—From these patients, we concluded that only the latter two mutations found in classical HPE are likely medically significant. In contrast, variants detected in the microform group are not likely pathogenic. In particular, we confirm that the extremely common histidine tract expansion is indeed a polymorphic alteration which demonstrates considerable differences in allele frequencies across different ethnic groups. Therefore, careful population studies of rare variants can improve genotype-phenotype correlations when used in conjunction with ethnically matched controls.

*Corresponding author: Maximilian Muenke, M.D., National Institutes of Health, 35 Convent Drive, Building 35, room 1B403, Bethesda, Maryland, USA, 20892-3717, Phone: +1-(301) 402-8167, Fax: +1-(301) 480-7876, mamuenke@mail.nih.gov.

#In the authors opinion, these individuals contributed equally to this work.

Keywords

ZIC2; mutation; alanine tract expansion or contraction; poly-histidine tract expansion; holoprosencephaly; polymorphism

INTRODUCTION

Holoprosencephaly (MIM #236100, <http://www.omim.org>) is the most common disorder of the developing forebrain in humans, and is characterized by the lack of separation of the cerebral hemispheres into two distinct halves; this frequent malformation has been classified within a spectrum of severity, with alobar HPE at the most severe end of the spectrum, and semilobar, lobar, and, middle interhemispheric variant (MIHF) or syntelencephaly at the milder end of the spectrum (Barkovich and Quint, 1993; Cohen, 2006; Dubourg et al., 2007). HPE is often associated with a spectrum of craniofacial anomalies, including cyclopia or synophthalmia, proboscis and cleft lip and palate in the most severely affected patients, and slit hypotelorism, sharp nasal bridge and subtle ocular anomalies in mildly affected individuals (Solomon et al., 2010a; Pineda-Alvarez et al., 2011).

HPE has a worldwide distribution with a live birth prevalence of 1/16,000 and incidence as high as 1:250 in conceptuses (Roach et al., 1975; Matsunaga and Shiota, 1977). However, with recent advances in neuroimaging, we can anticipate that children with milder forms of HPE, who were previously undiagnosed, should now be identified leading to an increasing prevalence of the disease. Thorough epidemiologic studies on microforms of HPE have not been systematically performed; hence, this lack of precise information represents a challenge in several clinical spheres including: the establishment of an accurate diagnosis, differential diagnoses, a proper genetic diagnostic approach, and ultimate genetic counseling.

The etiology and pathogenesis of the HPE phenotypic spectrum is complex and heterogeneous. Several genetic and environmental factors have been implicated, such as microscopically visible cytogenetic rearrangements, submicroscopic deletions and duplications of known HPE loci, mutations in known HPE-associated genes (*SHH*, *ZIC2*, *SIX3*, *TGIF*, *GLI2*, among others), and exposure to teratogens including maternal diabetes, retinoic acid, and alcohol, among others. Despite immense efforts to identify all the etiologic factors of the HPE spectrum, the cause of this disorder in a significant number of patients remains unclear (reviewed in Roessler and Muenke, 2010).

The diagnostic approach to evaluate patients with HPE and a normal karyotype typically includes mutation analysis of four genes: *SHH* (MIM #600725, Roessler et al., 1996), *ZIC2* (MIM #603073, Brown et al., 1998), *SIX3* (MIM #603714, Wallis et al., 1999), and *TGIF* (MIM #602630, Gripp et al., 2000). Mutations in these genes can occur *de novo* or may be inherited and identified among multiple members of large families segregating anomalies typically associated with HPE. Analyses of large kindred segregating the aforementioned mutations, have demonstrated extreme phenotypic variability, which implies both incomplete penetrance and variable expressivity (Cohen, 2006; Dubourg et al., 2007; Solomon et al., 2010b).

The gene *ZIC2*, located at chromosome 13q32, encodes a Zinc-finger transcription factor that plays several roles in neurological development (Aruga et al., 2004; 2006). Early in development, *ZIC2* is predicted to play a role in axial midline establishment (Beddington and Robertson, 1999; Warr et al., 2008). At later stages, *ZIC2* appears to affect the development of the dorsal telencephalon (Nagai et al., 2000; Wilson and Houart, 2004; Cheng et al., 2006; Monuki, 2007; Hébert and Fischell, 2008). *ZIC2* was first identified as

an HPE candidate gene in individuals with brain anomalies who had deletions involving the long arm of chromosome 13 (Brown et al., 1993). Subsequently, mutation analyses in patients with HPE lead to the identification of several pathogenic variants in *ZIC2* (Brown et al., 1998). Studies have reported that mutations in *ZIC2* gene occur in 6–8% of individuals with non-syndromic and non-chromosomal HPE, making this gene the second most common molecular cause of holoprosencephaly (Brown et al., 1998; Kauvar and Muenke, 2010). Interestingly, patients with mutations in *ZIC2* display distinctive phenotypic characteristics including bitemporal narrowing, upslanting palpebral fissures, short nose with anteverted nares, broad and well demarcated philtrum, and large ears (Solomon et al., 2010a).

Those craniofacial anomalies that occur without structural brain anomalies have been described as “microforms of HPE” and yet are more difficult to classify by dysmorphological criteria alone. In addition, “HPE-like features” as described by Roessler et al., 2003 have been attributed to mutations in *GLI2* with major craniofacial and pituitary anomalies without conspicuous structural brain findings. A further potential discordance between brain and facial findings described as “HPE-like” has also been documented (Richieri-Costa and Ribeiro, 2006); however, in some individuals this particular phenotype can be super imposed with characteristics observed in patients with lobar HPE. Although similar developmental pathways, such as hedgehog signaling, are implicated in both brain and face development, the reasons for both the concordances and discordances of these malformations are presently poorly understood.

We reasoned that several potential sources of ascertainment bias are inherent in the mutational spectrum of HPE genes that are reported by diagnostic centers that define HPE exclusively on the basis of overt brain findings (or a family history of HPE compatible brain findings), or depend on passive referral, or depend on testing for maternal-fetal medicine diagnostic indications. Any, or all, of these factors could distort the molecular findings. Hence, we felt that our Brazilian subject group uniquely permits a direct side-by-side comparison of the types of variations typically seen in microform vs. classical HPE groups, and also provides an opportunity to describe those variants prevalent in an ethnic group under-represented in public databases (e.g. length variants of the poly-histidine and poly-alanine tracts) as detailed below.

MATERIALS AND METHODS

This study was approved by the Ethics Committee of the Hospital de Reabilitação de Anomalias Craniofaciais (HRAC-USP) after obtaining written informed consent from participants. The clinical sample set consisted of 105 patients from our center with the classical HPE phenotype (43 individuals) and with microforms of HPE (62 individuals) as previously reported in the American Journal of Medical Genetics, Part C, 2010 (Richieri-Costa and Ribeiro, 2010). The DNA samples were analyzed at the National Human Genome Research Institute, Bethesda, USA. These samples were treated as anonymous coded clinical samples according to our NHGRI IRB approved research protocols. All patients included in this study were assessed in person by clinical dysmorphologists. All HPE types were identified by neuroimaging performed by Computerized Tomography (CT), or Magnetic Resonance Imaging (MRI). Mutational analysis was performed on an ABI 3100 genetic analyzer (Applied Biosystems, Foster City, CA) using the strategy outlined in Supplementary Table 1. Well-established polymorphisms, known to be present in healthy subjects based on our experience running a CLIA laboratory at the NIH, are not reported as meaningful alterations. Length variations of the poly-histidine and poly-alanine tract formed a separate group for further consideration (see below). Mutalyzer software was used to confirm that mutation descriptions were consistent with approved nomenclature (<http://www.mutalyzer.nl/2.0/>). The uniqueness of these mutations was determined by comparisons

to dbSNP (<http://www.ncbi.nlm.nih.gov/projects/SNP>) and the 1000genomes databases (<http://www.1000genomes.org>). All non-synonymous variants ascribed to each subject are shown in Table 1. Non-coding sequence variations were not analyzed beyond noting that they were outside recognized consensus splice junctions.

In order to establish the allelic frequency of length variants of the poly-histidine tract of *ZIC2* (NM_007129.3), several panels of commercially available human controls were interrogated (Genomics Core, NHGRI). As described in Table 2, these human controls included: 200 Caucasians, 99 African-Americans, 100 Mexican-Americans, 45 Han Chinese, 45 Japanese, and 58 Brazilian individuals (this study). Oligonucleotide primers (forward 5'-CAA CTC CAC AAC CAG TAC G -3', and reverse 5'-CAG ATT AGC TCC TGC TTG AT-3') were designed using Primer3 (<http://primer3.sourceforge.net/>) to span the genomic segment including the poly-histidine tract (c.691_717; p.His231_His239) of *ZIC2* based on the genomic assembly hg18 (<http://www.genome.ucsc.edu>). A M13 tail (5'-TGT AAA ACG ACG GCC AGT-3') and a polishing tail (5'-GTG TCT T-3') was added to the forward and reverse primer respectively for labeling purposes. The expected fragment sizes (including the primer tails) were 174 base-pairs (bp) for the wild-type allele (nine residue His tract = H9) and 177 bp for the p.His239dup (H10) allele. Subsequently, the fragments were amplified and labeled under the following amplification conditions: 12 min denaturation at 95°C; 10 cycles of 94°C for 45 sec, 55°C for 1 min, and 72°C for 1 min; 20 cycles of 89°C for 1 min, 55°C for 1 min, and 72°C for 1 min; 10 min final extension at 72°C. Fragment separation was achieved by capillary electrophoresis on a Genetic Analyzer 3130 using a 36cm capillary array and POP-7 polymer. The ROX400 size standard (Applied Biosystems) was run as an internal size-standard. Allele size was calculated using the local Southern algorithm available in the GENESCAN software program (Applied Biosystems). Allele calling and binning was done using the GENOTYPER software. (GeneScan and Genotyper are no longer commercially available and have been combined in GeneMapper, Applied Biosystems). All genotyping was done including a CEPH control individual #134702 (Applied Biosystems) for quality control purposes.

RESULTS

In this study, we report the *ZIC2* molecular findings in a cohort of Brazilian patients within the HPE phenotypic spectrum, who had been previously screened for sequence variants in *SHH*, *SIX3*, *TGIF*, *PTCH*, and *GLI2* genes. We analyzed a cohort of 105 patients consisting of 43 with HPE and 62 with microforms of HPE. Among the patients with HPE, 30.5% had alobar, 45% semilobar, and 22.5% had lobar forms of HPE. One patient presented a middle hemispheric fusion variant (MIHF) form of HPE.

Overall, five variants in *ZIC2* were found: c.1377_1391del_homozygous, c.1239+18G>A, c.1215dupC, c.1401_1406dup and several examples of a histidine tract expansion c.716_718dup (see Table 1). Although there are no routine functional tests of *ZIC2* mutations, the general observation is that the meaningful changes are frequently *de novo*, and almost always family-specific (Solomon et al., 2010a; Roessler et al., 2012). Among our patients, only 2 likely medically significant mutations (based on virtual translation of the protein product and functional data of Ala expansion alleles) were found (see Table 1: subjects #3 and #4; Brown et al., 2005). Both occurred in individuals with HPE brain findings. For the classical HPE group the mutations were very similar in nature to those reported in other case series (Mercier et al., 2011; Paulussen et al. 2011). The single case compatible with digenic inheritance (Table 1, subject #9) is best interpreted as an example of a microform single central incisor subject associated with a mutation in *SHH* (p.Leu219Pro) and who also has a length polymorphism in *ZIC2* (c.716_718dup, see discussion below) of neutral effect. The two cases with likely molecular pathogenicity, c.1215dupC and the Ala tract expansion to 17

repeats in the *ZIC2* gene (2/43 individuals = 4.6%), are typical for molecular findings seen in classical HPE. However, the cohort with microform findings, despite being of larger size, did not display a similar range of medically significant findings (0/62 individuals, or none). While this trend is not statistically significant, it does point to the difficulties in trying to recognize the causative gene(s) based on dysmorphological criteria alone. Certainly, phenocopies generated by non-HPE genes could confound any genotype-phenotype correlation by their inclusion in the microform HPE group. Further analysis will be needed to confirm this trend. In contrast, polymorphisms are the most prevalent findings from this study and are readily detectable in all groups investigated, including ethnically-matched controls (see Table 2).

Interestingly, the observed detection of the c.716_718dup was 14X the allele frequency first reported for the *ZIC2* gene in patients of Northern European ancestry (Brown et al., 2001, 2002). Population frequency differences had previously been described in self described Hispanic populations, but not in Brazilian cases or controls. In order to rule in or out a role for length variation of the poly-histidine tract in either brain or craniofacial features of HPE we performed an extensive population analysis. After analyzing over 500 individuals from different ethnic groups, we were able to identify three different alleles. The most common allele, was the wild-type allele with nine histidine repeats (H9 = fragment 174 bp), followed by an expansion of one additional histidine (c.716_718dup; p.His239dup, fragment 177 bp) commonly found in African-Americans, Mexican-Americans, and Asians; and finally a contraction of 3 histidine codons (c.709_717del; p.His237_His239del, a fragment 165 bp long) found in one individual from both the Caucasian and Brazilian cohorts. Although the p.His239dup allele was also seen in the Caucasian and Brazilian cohorts, the allelic frequency was not nearly as common as in other populations (see Table 2). This helps to explain why it was initially regarded as a medically relevant molecular finding.

DISCUSSION

Mutations in *ZIC2* are among the most common heterozygous variations detected in patients within the holoprosencephaly (HPE) phenotypic spectrum. *ZIC2* accounts for at least 3–8% of all detectable holoprosencephaly (HPE) associated-genes variation and is one of four genes, including *SHH* (Roessler et al., 1996), *SIX3* (Wallis et al., 1999) and *TGIF* (Gripp et al., 2000), commonly screened in clinical laboratories as part of the molecular evaluation of new sporadic or familial cases (Cohen, 2006; Dubourg et al., 2007; Krauss, 2007; Pineda-Alvarez et al., 2010; Roessler and Muenke, 2010; Roessler et al., 2012). The types of mutations observed in *ZIC2* are mostly private or novel truncating mutations, major alterations of the coding region of the gene, gross cytogenetic rearrangements, and recently described, microdeletions of one copy of the gene; moreover, mutations on this gene are frequently *de novo* (~70%), implying haploinsufficiency of this transcription factor as most likely mechanism in the causality of HPE (Brown et al., 1998, 2001, 2002, 2005; Bendavid et al., 2006a, b; Solomon et al., 2010).

Many patients with mutations in *ZIC2* display a distinctive phenotype with bitemporal narrowing, upslanting palpebral fissures, a short nose with anteverted nares, a broad and well demarcated philtrum, and large ears (Solomon et al., 2010a). In this study, we report a patient with an in-frame alanine tract contraction in *ZIC2* (c. 1377_1391del) that is homozygous. Genotyping for rs13542 ruled out the possibility of hemizygosity. Interestingly, heterozygosity for this variant has been recently described as possibly pathogenic in an HPE cohort (Mercier et al., 2011). This case presents a different facial phenotype that is more within the HPE-L phenotype (Richieri-Costa and Ribeiro, 2010). The brain MRI of this patient is normal. Interestingly, there are no reports of two deleterious alterations in the same gene among a decade of investigation in multiple diagnostic centers

(see Roessler et al., 2012 [in press, and references therein]; Mercier et al., 2011). Therefore, we recommend caution in inferring a significant pathological effect to this Ala tract contraction. There is excellent evidence for *de novo* occurrence of Ala tract expansion to 25 residues (Brown et al. 1998, 2001; Solomon et al., 2010; Paulussen et al., 2010; Mercier et al., 2011) and functional proof of loss of activity. However, there is little correlation between the length of the Ala tract and measurable function (Brown et al., 2005). Therefore, we should also consider an analogous genotyping experiment to determine the allele frequencies of Ala tract expansion/contraction alleles in patients and controls. Functional studies should also be performed.

Seven unrelated patients were observed to have the poly-histidine tract expansion to H10 (c. 716_718dup). The possible association of the variant within the first exon of *ZIC2*, and an increased risk of non-syndromic neural tube defects (NTDs) was first proposed for patients with Hispanic ethnicity (Brown et al., 2002). However, this association was not observed in another Hispanic population (Zhu et al., 2003), and the variant was not found in a population of Caucasian NTD cases (Klootwijk et al, 2004). Costa-Lima et al., 2008 analyzed the possible association of the poly-histidine tract polymorphism of the *ZIC2* gene in a large sample of NTD cases and their parents, of mainly mixed Amerindian and Latin-European ancestry, from six countries of South America. The negative results indicated that the 10H allele is found in a low frequency in 'Hispanic' populations, and that it is not associated with the increased risk of NTDs.

Therefore, the pathogenicity of poly-histidine expansion in *ZIC2* has been questioned on the basis of multiple lines of evidence. In this study, we have screened large cohorts of human random controls, and are able to demonstrate that poly-histidine expansions are commonly found (~10%) in individuals of Asian and Hispanic (Central America in this particular study) descent, implying its benign or polymorphic character. Nevertheless, this variation was not common among Caucasians, and was only present in 4% of our Brazilian control cohort, which is similar to the frequency in our patient cohort, 7%. The higher allelic frequency comparing Brazilians to Caucasians may be explained due to higher population admixture among different ethnic groups in Brazil.

The phenotypic features of the patients with poly-histidine expansions and contractions are variable in our cohort and other unpublished cohorts, and the phenotypic characteristics are not typical of those found in patients with mutations in *ZIC2*, but rather of other genes patterning the midline structures, including *SHH*, other known, and new candidate genes along that signaling pathway (Solomon et al., 2009; Lacbawan et al., 2009; Pineda-Alvarez et al. 2011; Bae et al., 2001). Therefore, the clinical and molecular data presented here supports previous work concluding that variations in the poly-histidine tract are benign. Taken together, we conclude that 1) pathogenic mutations in *ZIC2* continue to be novel mutations generated by fundamental mistakes in DNA replication; 2) they are very similar in type (*de novo* or family-specific) across multiple ethnic groups; 3) although we can observe tremendous differences in the allele frequencies among different ethnic groups, our best evidence suggests that they are neutral in biological effect; and 4) nevertheless, these interpretations await confirmation by functional studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1

Clinical findings of mutation carriers

Subject	Clinical information	Inheritance
1	A rare sequence variant c.1377_1391del (p.Ala466_Ala470del) was detected in ZIC2. We report a girl, born in 1991 to a G4P4A1 mother, birth weight was normal at 2800 grams, who presented with a left cleft lip/palate with no familial history of such anomalies. Examination at age 16 years old showed OFC at centile 10 th , inner canthal distance and outer canthal distance < 25 th centile, flat face with markedly hypoplastic malar region. Developmental milestones were normal and now she is an intelligent girl attending regular school. Routine laboratory blood workup and karyotype were normal. However, we detect only the Ala 10 length and no evidence of the normal Ala 15. Heterozygosity for rs13542 in the <i>ZIC2</i> 3' untranslated region (data not shown) makes hemizygosity unlikely. A recent NIH CLIA case also had inherited this change from a clinically normal parent, was heterozygous for the alteration, and also had microcephaly, agenesis of the corpus callosum, cleft lip and palate and hypopituitarism. The evidence suggests that this is a variant of a low probability of being clinically relevant. DNA from the parents was not available for study.	UKN
2	Sequence analysis of ZIC2 showed a novel intronic variant of unknown significance (c.1239+18G>A). Here we report a girl, born in 2001 from a G1P1A0 mother, birth weight was normal for gestational age at 2950 grams, she was diagnosed at birth as having holoprosencephaly sequence associated to bilateral coronal craniosynostosis. Since birth she presented complex tonic seizures and febrile bursts non responsive to usual pharmacologic therapy. At 5 years of age she was again seen in our center, and presented with severe cognitive impairment, was wheelchair bound, non-verbal, and had craniofacial features characteristic of HPE. Brain MRI showed alobar holoprosencephaly. DNA from the parents was not available for study.	UKN
3	Mutational analysis of ZIC2 showed a novel frameshift mutation c.1215dupC (p. Ser406Glnfs*11). This was a girl referred to our Hospital to perform screening of HPE-associated genes. She was referred with the diagnosis of microcephaly, severe developmental delay. A brain MRI showed semilobar HPE. Facial gestalt showed no classic signs of HPE. DNA from the parents was not available for testing.	UKN
4	Sequence analysis of ZIC2 showed novel alanine tract expansion variant c.1401_1406dup (p.Ala469_Ala470dup) and a histidine tract expansion c.716_718dup (p.His239dup). This is a girl, born in 1988 from a G4P4A0 mother, birth weight was 2300 grams, and crown-heel length was 47 cm. At birth she was diagnosed with an isolated bilateral cleft lip/palate. Acquisition of developmental milestones was delayed. At 7 years of age she presented with moderate cognitive impairment, attending special school. Craniofacial features identified high forehead, large eyes, and bilateral scars from surgical correction for cleft lip/palate. MRI showed semilobar HPE. DNA from the parents was not available for testing.	UKN
5	Sequencing analysis of ZIC2 showed a histidine tract expansion c.716_718dup (p.His239dup). This is a male that presented with semilobar holoprosencephaly, ectrodactyly, and bilateral cleft of lip and palate was previously reported [Zechi-Ceide et al., 2009]. The mother was asymptomatic and also carried the expansion.	MAT
6	This patient also has a histidine tract expansion c.716_718dup (p.His239dup). She is a female, born in 1965, and presented with bilateral cleft lip/palate. She attended elementary school but with learning disabilities. At age 39 years old, she is shy woman presenting with microcephaly (OFC of 49 cm), hypotelorism (inner canthal distance –ICD-of 2.9 cm). DNA of the parents was not available for study.	UKN
7	A histidine tract expansion c.716_718dup (p.His239dup) was detected in ZIC2. This is a male, born in 1994 that presented with right cleft lip/palate, large eyes, and depressed midface. Examination at age 7 years of age showed small head for his age (OFC of 48 cm), short stature (height of 111 cm), hypotelorism (ICD of 2.2 cm and outer canthal distance –OCD-of 7.5 cm). At a later examination at age 14 years of age, he yet presented with short stature but he attends regular school with good progress. Brain MRI was normal. Mother was not a carrier of this variant and father was not available for study.	UKN
8	A histidine tract expansion c.716_718dup (p.His239dup) was detected in ZIC2. This is a female, born in 2001; last child of a G9P7A2 mother. Birth weight was 3100 grams, and length was 49 cm. At birth she presented hypotelorism, midline cleft lip and palate, and pre maxillary agenesis. At age 5 years she presented with seizures, severe cognitive impairment, no language acquisition, microcephaly, craniosynostosis, and hypotelorism. A brain MRI showed semilobar holoprosencephaly. Her mother was not a carrier of this variant and father was not available for study.	UKN
9	This patient presented with the histidine expansion c.716_718dup (p.His239dup) in ZIC2, and also a previously reported mutation in SHH (p.Leu218Pro). This is a female, born in 2000 from a G1P1A0 mother, and the father had a single central maxillary incisor. First examination at age 19 days of age showed rounded face, microcephaly, shallow supra-orbital ridges, depressed nasal bridge, right cleft lip and palate. Evaluation at age 10 years showed normal development for age. Brain imaging (MRI) was normal. Both variants in <i>ZIC2</i> and <i>SHH</i> were inherited from her father.	PAT

UKN = unknown; MAT = maternal; PAT = paternal

Table 2

Genotype results from multiple populations tested for c.716_718dup

	African-American N=99		Caucasian N=200		Han Chinese N=45		Japanese N=45		Mexican-American N=100		Brazilian Controls N=58	
	Count	Frequency	Count	Frequency	Count	Frequency	Count	Frequency	Count	Frequency	Count	Frequency
His 10 Minor Allele Frequency	19	0.096	1	0.003	9	0.100	9	0.100	23	0.115	4	0.034
His 6 Minor allele Frequency	0	0.000	1	0.003	0	0.000	0	0.000	0	0.000	1	0.009
Het H9/H10 (2pq)	17	0.172	1	0.005	9	0.200	9	0.200	21	0.210	4	0.070
Hom H9 p ²	81	0.818	198	0.995	36	0.800	36	0.800	78	0.780	53	0.930
Hom H10 q ²	1	0.010	0	0.000	0	0.000	0	0.000	1	0.010	0	0.000