No association of the polyhistidine tract polymorphism of the *ZIC2* gene with neural tube defects in a South American (ECLAMC) population

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Abstract. The ZIC genes comprise a family of transcriptional factors associated with neural tube defects (NTDs) in mice and with holoprosencephaly in humans. An allelic variant of *ZIC2*, a CAC repeat within the first exon, was reported in association with an increased risk of non-syndromic NTDs in patients with a Hispanic ethnic background. We investigated whether this 10-residue histidine tract polymorphism of the *ZIC2* gene (c.718_720dupCAC) was associated with the risk of NTDs in a sample of 138 patients and their parents from the Latin American Collaborative Study of Congenital Malformations (ECLAMC) hospital network. Analysis with log-linear models of 138 family triads of mother, father and affected child did not provide evidence to support the notion that case (or maternal) 10H/10H or -/10H genotypes were associated with NTDs in this South American population sample, where the 10H variant occurred in 5% of newborns affected with NTDs. We also described the first example of the homozygous state of the 10H allele in a patient with cephalocele, holoprosencephaly and microphthalmia, but did not ascertain whether this polymorphism is associated with the increased risk of a specific subgroup of NTDs, as a normal father of a patient with anencephaly presented the same genotype.

Introduction

Failure of the neural tube to close results in anencephaly, spina bifida or cephalocele, severe malformations known as neural tube defects (NTDs) with an estimated frequency of 1-2/1,000 births. NTD etiology is complex, involving both genetic and environmental factors (1). Even though several genes associated with NTDs have been identified in murine models (2), no individual gene has been identified to play a major role in the development of the neural tube in humans.

NTDs are commonly found in the 13q deletion syndrome. The minimal deletion critical region includes 13q32, which is where the *ZIC2* gene is located (3). Reduced or absent expression of *Zic2* in mice is associated with holoprosencephaly (HPE), spina bifida, exencephaly, anencephaly and skeletal abnormalities (4); in humans, mutations in *ZIC2* lead to HPE (5,6) and NTDs can be present in the patient or in the family (7-11).

ZIC2 is consequently a candidate gene for NTDs. To date, at least three mutations in this gene have been found in NTD patients. The silent polymorphism c.1059C>T (H353) was studied in child-parent triads, but a transmission disequilibrium test showed no significant association with NTDs (10). An insertion of 70 bp within the first intron of *ZIC2* was found in one NTD patient in a series of 192 cases, but the same mutation was found in the unaffected mother and sister of the proband (9). As well, a deletion of an alanine codon (c.94-96delGCG) in a polyalanine (pA) stretch inside the first exon of the *ZIC2* gene was described in one male spina bifida patient out of 117 NTD cases from The Netherlands (10). The mutant allele was not present in 364 control samples, but was found in heterozygosity in both the patient's unaffected mother and grandmother.

The possible association of another variant, the CAC repeat within the first exon of *ZIC2*, and an increased risk of non-syndromic NTDs was proposed for patients with Hispanic ethnicity (9). However, this association was not observed in another Hispanic population (12), and the variant was not found in a population of Caucasian NTD cases (10). The polymorphic tract analyzed codes for a 10-residue histidine (10H) instead of the nine (9H) usually found in the normal alleles.

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We analyzed the possible association of the polyhistidine 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.

Materials and methods

Latin American Collaborative Study of Congenital Malformations. Data were derived from the Latin American Collaborative Study of Congenital Malformations (ECLAMC), a hospital-based registry that has been examining births in several South American countries since 1967. Operational protocol followed by ECLAMC has been described elsewhere (13). Briefly, all consecutive live and still births at participating hospitals with a birth weight of ≥500 g were examined for major and minor congenital anomalies. Anencephaly, spina bifida and cephalocele have specific detailed guides for their identification. Information regarding ancestry was registered for each newborn in eight non-exclusive categories: Latin-European, non-Latin European, Jewish, Native, Arab, African-American, Oriental and other. In South America, 'Native' most likely refers to people with mixed Latin European and Amerindian ancestry. A newborn with two checked ethnic categories, i.e., Native and Latin-European, indicates that he/she also has more recent (19th and 20th century) ancestors who had immigrated from Latin European countries, mainly Spain, Portugal and Italy. Since the year 2000, blood spot samples were collected from babies with major defects, including NTDs, and from mothers and fathers whenever possible, following informed consent and protocol approved by the ethics committees at ECLAMC and at the reporting hospitals.

Subjects. Blood spots for 515 of 2079 NTD cases from 2000 to 2005 were available . We analyzed 138 NTD cases for which blood samples from both the mother and father were available. These 138 triads were from six countries: Argentina (90), Brazil (17), Chile (23), Ecuador (2), Uruguay (4) and Venezuela (2). There were 42 cases of anencephaly (30.5%), 78 cases of spina bifida (56.5%) and 18 cases of cephalocele (13%), which did not differ from the relative frequency of each neural tube defect type in the cases not studied molecularly: 35, 51 and 14% (χ^2=1.68, DF=2, P=0.43) respectively. Of the NTD cases, 22 also presented malformations independent of the NTD sequence, 5 with Meckel-Gruber syndrome, 3 with early amnion rupture sequence, 3 with exstrophy of the cloaca/bladder sequence, 2 with the holoprosencephaly sequence (besides parieto-occipital cephalocele in one case and lumbar mielomeningocele in another), and 9 multiply malformed infants without a recognizable syndrome, sequence or association at birth. The proportion of studied associated cases (22/138) did not differ from the proportion of the not molecularly studied associated cases (310/1564) (χ^2=0.98, P=0.32, DF=1).

Clinical NTD ascertainment was complete for live and still births at the collaborating hospitals (14), in contrast to the blood samples. These were not available for all cases, mainly due to patient death, parental refusal to participate, or because the hospital was new in the ECLAMC network. New hospitals begin the blood collection routine only after one year of experience in the program.

Genotyping. Genomic DNA was extracted from filter card blood spots using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's specifications. For samples collected on Isocode™ paper, extraction was performed directly by boiling the spot in water. PCR amplification of a *ZIC2* fragment containing the polymorphic histidine tract was performed as described elsewhere (6), with minor modifications. Reactions were performed in an MJ Research thermocycler in a final volume of 35 μl with annealing steps at 55˚C. Amplified fragments were diluted with sterile distilled water, subjected to dHPLC, and analyzed using the WAVE system (Transgenomics, Ohama, NE). Triads of which any member showed an altered migration pattern were sequenced using DYEnamic ET Terminator Cycle Sequencing Kit (Amersham-Pharmacia) according to the manufacturer's specifications in an ABI 377 automated DNA sequencer (Applied Biosystems, Foster City, CA). This strategy was adopted in light of the low probability of both parents being 'not 9H' homozygotes. Sixty-six triads were also directly sequenced after PCR amplification.

Statistical analysis. All statistical tests were performed with subroutines of the statistics package STATA version 8.0 (Statacorp, TX). In the NTD cases and their parents, Hardy-Weinberg equilibrium for genotype frequencies of the 3 identified alleles was tested . The birth prevalence rates of '10H' alleles with a 95% confidence interval (CI) were calculated after the 8H and the 9H alleles had been grouped together as 'not 10H' alleles .

To avoid the risk of bias from the population stratification of the case control studies, we used the transmission disequilibrium test (15), where the frequency of transmission of '10H' or 'not 10H' alleles to newborns with NTDs from heterozygous mothers and fathers was compared using the McNemar test. Log-linear models were adjusted using the Poisson command of STATA 8.0 in accordance with the Weinberg method (16), which assesses both the fetal and maternal effects of the 10H allele.

Results

The ethnic background of the studied newborn sample included Natives (mixed Amerindian and Latin-European; 33.6%), Natives and Latin-Europeans (42.7%) or Natives with other groups (16.0%), with only 7.6% of the sample not including Natives. As only 3 cases presented mixed Native and African-American backgrounds, we can consider our sample to be similar to the ethnic background described as Hispanic in North American studies (9,12).

The polyhistidine tract within the first exon of *ZIC2* is codified by a CAC pure trinucleotide repeat. We analyzed 414 samples (138 parent-child triads) by means of dHPLC and/or direct sequencing, and identified three alleles harboring eight (8H), nine (9H) or ten (10H) histidine residues (Table I). Most of the samples were 9H/9H homozygous with a frequency of 0.88 in the NTD cases, 0.88 in the mothers and 0.92 in the fathers. We found 2 10H/10H homozygotes, one among the

Table I. Crude and combined genotype and allele frequencies of the polyhistidine tract for NTD cases and their parents.

Allele	No. cases (%)	No. mothers (%)	No. fathers (%)
9H/9H	122 (0.88)	122 (0.88)	127 (0.92)
9H/10H	13 (0.09)	14 (0.10)	8 (0.06)
9H/8H	2 (0.01)	2 (0.01)	2 (0.01)
10H/10H	1 (0.01)	0 (0.00)	1 (0.01)
9H	259 (0.94)	260 (0.94)	264 (0.96)
10H	15 (0.05)	14 (0.05)	10 (0.04)
8H	2 (0.01)	2 (0.01)	2 (0.01)
10H/10H	1 (0.01)	0 (0.00)	1 (0.01)
10H/-	13 (0.09)	14 (0.10)	8 (0.06)
-/-	124 (0.90)	124 (0.90)	129 (0.93)
10H	15 (0.05)	14 (0.05)	10 (0.04)
-	261 (0.95)	262 (0.95)	266 (0.96)

-, not 10H, i.e., 9H or 8H.

Table II. Maternal, paternal and NTD case genotype combinations in all triads considering 10H but not 10H (-) alleles.

		NTD cases			
Maternal	Paternal	-/-	-/10H	10H/10H	Total
-/-	-/-	114	-	-	114
-/-	-/10H	4	1	-	5
-/10H	-/-	6	4	-	10
-/10H	-/10H	0	3	0	3
-/-	10H/10H	-	1	-	1
10H/10H	-/-	-	0	-	0
10H/10H	-/10H	-	0	0	0
-/10H	10H/10H	-	0	0	0
10H/10H	10H/10H	-	-	0	0
Total		124	9	0	133[a]
Percentage		93.23	6.77	0	100.00

[a]Four triads (mother, father, child) presented genotypes -/-, -/-, -/10H and one triad genotypes -/10H, -/-, 10H/10H. These five triads are examples of misattributed paternity or parental gonadal mosaicism.

cases (0.01) and the other among the fathers (0.01). We found 6 8H/9H heterozygotes; 2 from the cases (0.01), 2 from the mothers (0.01) and 2 from the fathers (0.01). We also identified 35 9H/10H heterozygotes, of which 13 were cases (0.09), 14 were mothers (0.1) and 8 were fathers (0.06). Alleles with 10H were found in 5% and alleles with 8H in 1% of children with NTDs, with the three alleles in Hardy-Weinberg equilibrium (χ^2=1.07, DF=3, P>0.05) (Table I).

When distributed by type of defect, the 13 9H/10H heterozygotes were found in 5 cases of isolated spina bifida, 5 cases of isolated anencephaly, 1 case of isolated cephalocele, 1 case of multiple malformed anencephaly and 1 case associated with spina bifida with exstrophy of the cloaca. The 2 9H/8H heterozygotes were found in an isolated case of anencephaly and another of spina bifida. The 10H/10H homozygous case was female with a birth weight of 3270 g born after a 38-week gestation, presenting parieto-occipital cephalocele, severe hydrocephalus, CC 40 cm, bilateral enophthalmia with blepharophimosis and left microphthalmia with coloboma of the choroid and retina; cerebral CT showed corpus callosum hypoplasia and semilobar holoprosencephaly. She had a normal brother, a female maternal cousin with congenital hip dislocation and two female cousins (sisters) with equinovarus feet.

In order to perform a transmission disequilibrium test, we selected 21 heterozygous parents, as one heterozygous mother from a triad with misattributed paternity or parental mosaicism was excluded (Table II). Although the sample was insufficiently large to provide enough power to the test, 8 cases were observed to transmit the 10H allele, while 10.5 were expected. This did not deviate from the one-to-one expected ratio in transmission.

Excluding the 5 triads where misattributed paternity or parental mosaicism was thought to exist (Table II), we tested allele 10H transmission by log-linear models using the remaining 133 triads, assuming Hardy-Weinberg equilibrium. No significant risk of NTDs was conferred by the homozygous or heterozygous case genotypes (relative risk 0.65, 95% CI 0.27-1.54, P=0.33), and no risk of NTDs was conferred by the homozygous or heterozygous maternal genotypes (relative risk 1.40, 95% CI 0.59-3.31, P=0.45).

Ancestral information was unspecified in the case of 7 newborns only. Most of the 131 newborns with specified information had Native ancestry (n=44) or Native ancestry mixed with another ethnicity (n=77). All 17 variants of the 9H allele (two alleles with 8H and fifteen alleles with 10H) were found among pure or mixed Natives.

Discussion

The histidine tract in exon 1 of the *ZIC2* gene has been described as having 8 (c.718_720delCAC), 9 (more common), 10 (c.718_720dupCAC), 11 (c.718_720dupCACCAC) and 12 histidines (c.718_720dupCACCACCAC) (6,9,12,17). Brown *et al* (9) suggested a possible association betwen NTDs and the 10H allele in the *ZIC2* gene. This was not confirmed by Zhu *et al* (12).

In the mixed Hispanic and Caucasian sample of NTD cases (n=192) studied by Brown *et al* (9) in the US, the 10H allele had a frequency of 0.03 (0.01-0.05). In the Texas-Mexican sample of NTD patients (n=28), the frequency was 0.04 (0-0.08) (12). The expanded allele was not found in a sample of 117 NTD cases from a Dutch population (10). In our NTD sample (n=138), we observed a frequency of 0.05 (0.03-0.08), with no distortion of 10H segregation from parents to children. The differences observed by Brown *et al* (9) and Zhu *et al* (12) in the 10H allele frequency in North American 'Hispanic' controls (2/100 and 7/69, respectively) could have been the

result of the different techniques used, small sample sizes, or because the ethnic self-classification of the controls was, as suggested by the authors and in combination with other facts, inappropriate (9). Zhu *et al* (12) and our present negative results using more reliable genotyping techniques (18) than Brown *et al* (9) indicate 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. We cannot definitively exclude a small risk of NTDs, since low frequency alleles conferring small risks would need huge sample sizes to be implicated. Considering that the frequency of the 10H allele in control populations in the literature is 0.03, and having analyzed 133 complete triads, we have excluded it as a factor responsible for 3-fold increased risk of NTDs among children with this *ZIC2* variant, assuming a power test of 0.80.

It is noteworthy that the homozygous 10H/10H child presented occipital cephalocele, microphthalmia and holoprosencephaly, suggesting that the holoprosencephaly in this case may be attributed to the homozygous state of the 10H allele of the *ZIC2* gene. Neural tube defects are not more associated with holoprosencephaly than what is to be randomly expected from the ECLAMC data (19). Nevertheless, instances of this association, particularly for cephalocele in the same patient or in the same family, have been described by several authors (reviewed in ref. 20) as being relatively common in Meckel-Gruber and 13 trisomy syndromes. Thus, it can be hypothesized that the 10H/10H genotype could be the cause of our patient phenotype, and/or be a factor co-acting with another unknown gene mutation, or be a chance finding in a carrier for a specific syndrome, such as Meckel-Gruber, which can present cephalocele, holoprosencephaly and microphthalmia as in our patient. The presence of this same genotype 10H/10H in the father of an anencephaly case does not exclude a pathological role for this mutation, since the finding of the same mutation occurring in a holoprosencephaly case and in normal relatives is a common finding in holoprosencephaly families (17). Although we cannot test these hypotheses due to the lack of patient material, future *ZIC2* gene case studies can help to elucidate this question.

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