Holoprosencephaly due to mutations in *ZIC2*: alanine tract expansion mutations may be caused by parental somatic recombination

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We report on the prevalence of mutations in the zinc finger transcription factor gene, *ZIC2*, in a group of 509 unrelated individuals with isolated holoprosencephaly (HPE) and normal chromosomes. Overall, we encountered 16 HPE patients (from 15 unrelated families) with *ZIC2* mutations. Thus, *ZIC2* mutation was the apparent cause of HPE in 3–4% of cases. Seven mutations were frameshifts that were predicted to result in loss of function, further supporting the idea that *ZIC2* haploinsufficiency can result in HPE. One mutation, an alanine tract expansion which is caused by the expansion of an imperfect trinucleotide repeat, occurred in seven patients from six different families. In three of those families, the father was found to be apparently mosaic for the mutation. We hypothesize that this mutation can arise through errors in somatic recombination, an extremely unusual mutation mechanism. In addition, one mutation resulted in a single amino acid change and one mutation was an in-frame deletion of 12 amino acids. The central nervous system malformations seen in patients with *ZIC2* mutations ranged from alobar HPE (most common) to middle interhemispheric fusion defect (one case). Although severe facial anomalies are common in HPE, all of the patients with *ZIC2* mutations had relatively normal faces, suggesting that *ZIC2* mutations represent a large proportion of HPE cases without facial malformation.

INTRODUCTION

Holoprosencephaly (HPE) is a term that was first used in 1963 to describe a spectrum of malformations which have, as their common finding, failure of the proper formation of midline structures of the forebrain (1). In its most severe form (alobar HPE), the forebrain consists of a single ventricle, and midbrain structures may be malformed as well. In milder forms (semilobar and lobar HPE), rudimentary midline structures are present. Up to 85% of patients with the diagnosis of HPE have accompanying facial malformations which may include cyclopia and a proboscis-like structure in place of a nose (2). Minor facial malformations that have been associated with HPE include ocular hypotelorism and the presence of a single central incisor (3). There are many causes of HPE, including teratogens, chromosome abnormalities and single gene defects (4). The majority of HPE cases are sporadic, although families with both autosomal dominant and autosomal recessive HPE have been described. The association of HPE with recurrent cytogenetic abnormalities has led to the definition of at least twelve chromosomal regions that may contain genes involved in HPE (5). In the past few years, positional cloning efforts have succeeded in discovering several genes in which mutations result in HPE. These include *SHH* (which corresponds to HPE3) (6,7), *ZIC2* (HPE5) (8), *SIX3* (HPE2) (9) and TG interacting factor (*TGIF*) (HPE4) (10).

ZIC2 was identified as a candidate for HPE based on the observation that chromosome deletions, including the previously defined critical region in 13q32, result in brain malformations (11,12). Patients with deletions of this region typically have severe brain malformations which include HPE as well as encephalocele and others. *ZIC2* was cloned and sequenced during a search for genes within the 13q32 critical deletion region. Subsequently heterozygous loss-of-function mutations in *ZIC2* were encountered in patients with isolated HPE and normal chromosomes. These results established that *ZIC2* mutations cause HPE and suggested that *ZIC2* hemizygosity is at least part of the basis of the severe malformations seen in patients with del(13q) (8).

Table 1. Summary of patients and mutations

Patient no.[a]	Mutation	Phenotype	Photographs	Reported
1	G deletion at amino acid 8, stop is at amino acid 41. *De novo*	HPE		No
2	56 bp insertion in exon 1. Frame shift begins at amino acid 61, stop is at amino acid 237. *De novo*	HPE		(8)
3	D153F (GAC→TTC). Inherited from mother who has hypotelorism	Lobar HPE	Figure 2A	No
4	Single G deletion. Frame shift at amino acid 312, stop is at amino acid 413. *De novo*	Semilobar HPE	Figure 2B	No
5	TC deletion at amino acid 344, stop is at amino acid 365	Semilobar HPE		No
6	7 bp deletion in zinc finger region, exon 2. Frame shift is at amino acid 349, stop is at amino acid 411	Alobar HPE	(8)	(8)
7	AG deletion at amino acid 365, creates stop at amino acid 366. *De novo*	Semilobar HPE	Figure 2C and D	No
8	Single G insertion in exon 3. Frame shift begins at amino acid 441, stop is at amino acid 530. *De novo*	HPE		(8)
9	36 bp deletion in exon 3 predicts deletion of amino acids 435–447. Mother is normal, father is unavailable	Middle interhemispheric fusion	Figure 2E. and F	No
10 and 11	Alanine tract expansion. Father is mosaic carrier	Alobar HPE in both sibs	(8)	(8)
12	Alanine tract expansion. Parents are unavailable	Alobar HPE	(6)	(6)
13	Alanine tract expansion. *De novo*	Semilobar HPE	Figure 2G.	No
14	Alanine tract expansion. Father is mosaic carrier, one sibling was affected with HPE and one sibling was anencephalic	Alobar HPE		No
15	Alanine tract expansion. Parents untested	HPE		No
16	Alanine tract expansion. Father is mosaic carrier. Sibling was affected with HPE	Semilobar HPE		No

[a]Rrefers to numbers in Figure 1.

The *Zic* genes are a family of zinc finger transcription factor genes that were originally defined by their similarity to the *Drosophila* pair ruled gene, odd-paired (*opa*) (13). In mice, *Zic2* is expressed beginning at ~7 days and continues to be expressed along the dorsal neural tube and hindbrain until birth (14). Mice with a targeted mutation that results in diminished expression of *Zic2* have a brain malformation which is similar to human HPE as well as lumbo-sacral neural tube defect (15). This result helps to confirm that *Zic2* is a dosage-sensitive gene with a critical role in central nervous system (CNS) development.

In this report, we present further evidence that haploinsufficiency for *ZIC2* can result in brain malformation. In addition, we present evidence that a recurrent alanine tract expansion may have arisen by somatic recombination in the fathers of affected individuals.

RESULTS

Mutations

We screened for *ZIC2* mutations in a total of 509 unrelated patients with HPE and normal chromosomes. From this screening, a total of 16 patients from 15 families were shown to have *ZIC2* mutations. The present study includes the four patients we originally reported as well as 12 additional patients. Phenotype and mutation data are summarized in Table 1. Each mutation with corresponding number is also
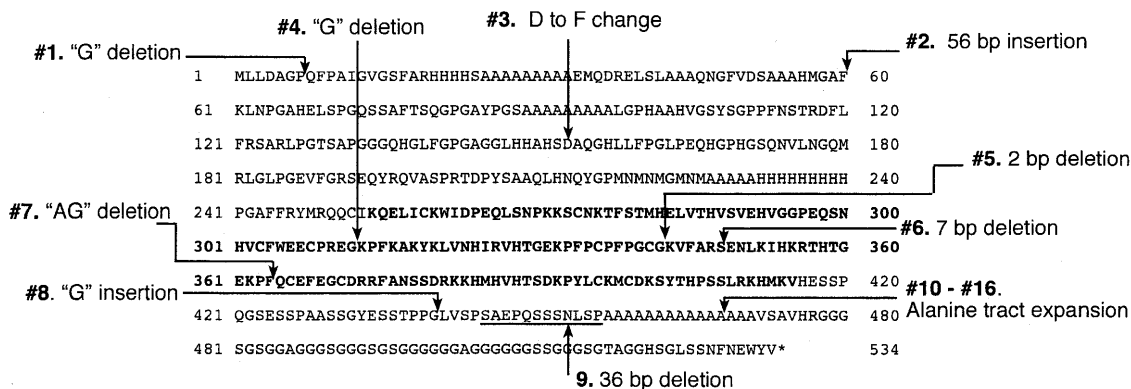

Figure 1. Amino acid sequence of the *ZIC2* gene with the location and type of mutations indicated. Numbers refer to patient numbers in Table 1.
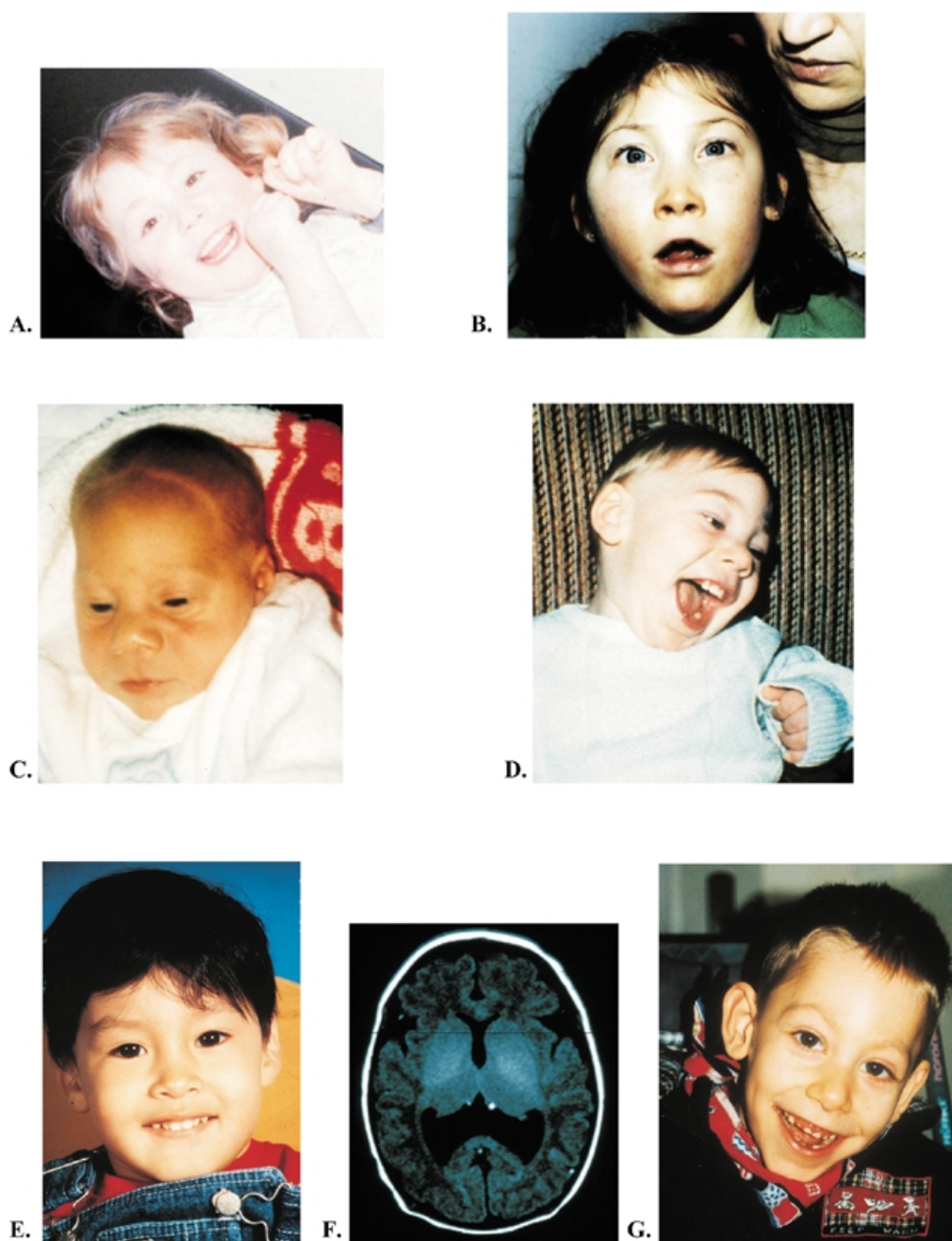
Figure 2. Facial photographs of HPE patients showing lack of severe facial malformations. (**A**) Patient 3; lobar HPE. (**B**) Patient 4; semi-lobar HPE. (**C**) Patient 7 at birth; semi-lobar HPE. (**D**) Patient 7 at 21 months. (**E**) Patient 9; middle interhemispheric fusion defect. (**F**) Patient 9; MRI showing middle interhemispheric fusion defect. (**G**) Patient 13; semi-lobar HPE.

shown in Figure 1. Facial photographs of several of the patients are included in Figure 2.

Of the total of 16 mutations, seven result in frameshifts and are predicted to radically alter and prematurely truncate the protein and, most likely, are null alleles. One mutation (patient 9) is an in-frame deletion predicted to result in the loss of 12 amino acids in the third exon. Interestingly, only one missense mutation was encountered (patient 3), which is predicted to change a highly conserved D to an F. This mutation is also present in the mother of the proband, who is phenotypically normal except for hypotelorism.

In six unrelated families (seven patients) there was an identical expansion of an alanine tract which is located near the C-terminus of the protein. In normal individuals there are 15 alanines, which are coded for by an imperfect trinucleotide repeat, and in the HPE patients this is expanded to 25 alanines as a result of a partial duplication of the trinucleotide repeat (Fig. 3). It is interesting that in all six cases the mutations were identical. In four of the six alanine expansion cases, we were able to test both parents of the affected individuals. In one previously reported family, two siblings had HPE and their father was found to be an apparent mosaic for the mutation
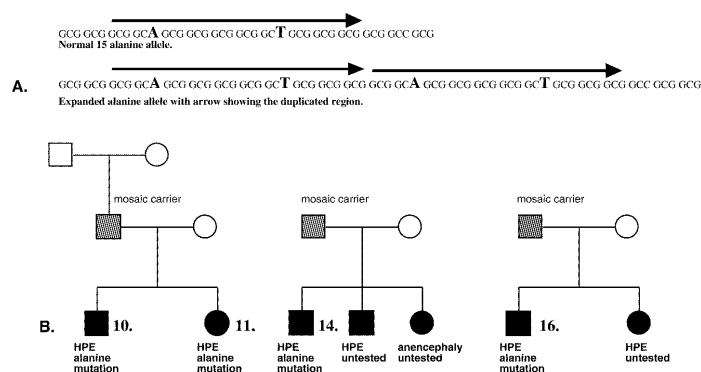
Figure 3. (**A**) Sequence of the normal alanine tract and the expanded alanine tract. The arrow indicates the duplicated region and bold type indicates the alternate alanine codons. (**B**) Pedigrees of the three families in which the father was found to be a mosaic carrier of the alanine expansion mutation. Numbers refer to patient numbers in Table 1 and Figure 1.

(patients 10 and 11). We have now encountered two additional cases in which the father of a child with a *ZIC2* alanine tract expansion weakly amplified the expanded allele (patients 14 and 16). In two of the three cases, we have been able to exclude the possibility that the father's DNA sample was contaminated by repeating the analysis on a newly obtained sample. In all three families, two children were affected with HPE, and in one family a third child was born with anencephaly (Fig. 3B). Although no DNA samples were available for confirmation, it is likely that the other affected children in these families also inherited the mutant *ZIC2* allele from their carrier father. Interestingly, in the course of screening 300 normal unrelated controls (600 chromosomes) we did not encounter any polymorphic variants of the alanine tract.

Polymorphisms

In addition to the alanine tract in the third exon, the *ZIC2* gene contains a tract of histidines in exon 1. In 99.5% of chromosomes ($n = 400$), this tract consists of nine histidines. In the other 0.5% there are 10 histidines and, in the course of screening 200 normal controls and 450 HPE patients, we encountered one example of 11 histidines and one example of 12. In addition, we encountered one HPE patient with an eight-histidine allele. Although this was never encountered in a control sample, it was inherited from a normal parent. We have not reported this as a mutation since we are unsure of its significance. Interestingly, the mouse *ZIC2* gene has eight histidines.

DISCUSSION

Prevalence of *ZIC2* mutations in HPE patients

One goal in the present study was to determine the overall frequency of *ZIC2* mutations in HPE patients. In total, 509 unrelated HPE patients were screened. Of these, 460 were sporadic and 49 were probands from families in which there was more than one sib affected with HPE. *ZIC2* mutations were encountered in three of the 49 probands from families with more than one affected (6%), and in 14 of 460 sporadic cases of HPE (3%). Thus, ~3–4% of HPE patients overall were found to have *ZIC2* mutations and ~1.5% of all HPE is explained by the recurring alanine tract mutation. This is a

conservative estimate since single strand conformation polymorphism (SSCP) (the only method used in the majority of cases) is an imperfect screening tool and is insensitive to deletions of the entire gene. In addition, it was technically impossible to design PCR primers which allowed screening of two of the four intron/exon boundaries, further diminishing the sensitivity of the screening procedure. An exhaustive search for mutations and complete gene deletions may somewhat increase the proportion of HPE cases due to *ZIC2* mutation.

ZIC2 and facial anomalies

HPE patients frequently have varying degrees of facial malformation, ranging from severe (cyclopia and the presence of a proboscis-like structure instead of a nose) to mild (hypotelorism, single central incisor). In the original description of HPE, it was stated that HPE is always associated with severe facial malformation (1,16); however, subsequent publications have noted that up to 17% of HPE patients do not have diagnostic faces (2,17). In particular, Cohen (2) made the observation that patients with HPE associated with chromosome 13q deletion (as opposed to 13 trisomy) typically have only mild facial dysmorphism. In this series of 16 patients with HPE and documented *ZIC2* mutations, there were no examples of cyclopia, clefting or other major facial malformation, despite the fact that the underlying brain malformations ranged from severe to mild. Thus, it appears that we have identified a subset of HPE patients in whom the face does not predict the brain. In contrast, at least some of the patients with mutations in *SHH*, *TGIF* and *SIX3* have had severe facial malformation (7,10,18). This observation implies that the face and underlying brain must, at least to some extent, develop independently of each other, an idea that is supported by experiments in which the expression of *Shh* is specifically disrupted in the developing face (19).

Spectrum of brain abnormalities associated with *ZIC2* mutation

Of the 16 patients, we report that all except one had either alobar, semi-lobar or lobar HPE. The remaining patient had middle interhemispheric fusion on MRI (patient 9, Table 1 and Fig. 1; Fig. 2E and F). Middle interhemispheric fusion abnormalities were first described and proposed to be part of the

spectrum of HPE in 1993 (20). In this malformation, patients have fusion of the middle portions of the cerebral hemispheres with near normal anterior interhemispheric fissures. The finding of a middle interhemispheric fusion defect in a patient with a *ZIC2* mutation argues that this malformation is indeed a variant of HPE. Interestingly, the mutation in this patient is an in-frame deletion of 12 amino acids. The finding of a relatively mild malformation in a patient with a small in-frame deletion raises the possibility that this deletion results in a partially functional allele and that the degree of malformation depends on the amount of functional protein.

Molecular effects of *ZIC2* mutations

Several of the mutations we have encountered are very likely to represent loss-of-function or null alleles. This, taken together with the findings in 13q deletion patients, has led us to conclude that brain development is exquisitely sensitive to the level of ZIC2 protein present. In keeping with this, mice with a targeted mutation that results in an 80% diminution of ZIC2 expression have a variety of malformations including HPE and spina bifida. There are very few other examples in either humans or mice of such extreme sensitivity to gene dosage and it will be of great interest to understand the molecular details of this sensitivity to the level of *ZIC2* expression.

The alanine tract expansion we have described has the potential to result in a protein which either has a novel function or antagonizes the function of the normal protein. At this time, there are four other human genetic conditions which can be caused by alanine tract expansions, namely oculopharyngeal muscular dystrophy (21), cleidocranial dysplasia (22), synpolydactyly caused by HOXD13 mutations (23,24) and hand–foot–genital syndrome (25). In all four of these conditions, there is evidence that the alanine expansion results in a functional protein that can act dominantly. Therefore, we consider it possible, if not likely, that molecular mechanisms other than diminished dosage of *ZIC2* can cause HPE.

Somatic recombination as a mechanism of mutation

The alanine tract expansion we describe is a partial direct repeat of an imperfect trinucleotide repeat, which suggests that the mechanism giving rise to this mutation is likely to be an unequal crossover between mispaired normal alleles, a mechanism which has been speculated to be the cause of the alanine tract expansion which occurs in HOXD13 (26). Although we were not able to directly prove it (due to lack of patient material), based on our findings in leucocyte DNA it is likely that the three fathers shown in Figure 3 are mosaic carriers of the alanine tract expansion. This implies that the mutation must have occurred as a consequence of a somatic (mitotic) recombination event in the father. Although somatic recombination has never (to the best of our knowledge) been described as a mechanism of disease-causing gene mutations, there is precedent for both somatic recombination and somatic gene conversion in humans (27,28). Although it remains unproven, these may be the first examples in which somatic recombination has resulted in human genetic disease. Given the frequency of finding mosaicism for this mutation in parents of affected children, the recurrence risk could be as high as 50% in a couple with a child with HPE due to an alanine expansion in *ZIC2*.

Role of *ZIC2* in CNS development and possible interaction with *SHH* signaling

In humans and mice, diminished levels of ZIC2 result in the failure to form midline CNS structures (15). Over-expression experiments in *Xenopus* embryos suggest that *Zic2* plays a role in determining the fate of pre-neural cells (29). *Zic2*-injected embryos showed an increase in the number of cells expressing neural crest markers and a decrease in those destined to become neurons. *Shh* over-expression in *Xenopus* results in upregulation of *Zic2* (30), which suggests that in very early neurogenesis, *Shh* and *Zic2* signaling may interact; an intriguing finding in light of the fact that mutations in both genes result in human HPE. Overall it appears that the *Zic* genes, including *Zic2*, act very early in CNS development, and that work in animal systems such as *Xenopus* and chick, where early events in nervous system development can be visualized, is likely to lead to a better understanding of their roles.

MATERIALS AND METHODS

Patient samples

Blood or DNA samples from HPE patients were submitted by a large number of clinical geneticists and/or neurologists from several countries. Patients were included in the study when the diagnosis of HPE was made by neuroimaging (magnetic resonance imaging or computerized tomography). All clinical information was reviewed by one of the authors (M.M.). Genomic DNA from HPE patients and family members (when available) was extracted from lymphocytes or lymphoblastoid cell lines by routine methods. All samples were obtained according to the guidelines of the various institutional review boards involved.

Mutation screening

SSCP analysis was performed for all 509 cases according to our previously published protocol (8). In addition, 59 cases were screened by heteroduplex analysis as well as by SSCP. In those cases screened by both methods, one case (patient 16) was detected only by heteroduplex analysis. PCR products that were abnormal by SSCP were sequenced directly with an ABI 310 automated sequencer. In all cases in which a sequence variation was apparent, the PCR products were cloned and individual clones were sequenced to confirm the mutation. Whenever possible, the parents of children with *ZIC2* mutations were tested for the presence of the same mutation.

Approximately 140 of the DNA samples were screened for mutations within the first intron of the gene. A total of three primer pairs were used for this analysis and are as follows:
(i) forward, 5′-CGCACCTCTAAATGACTCCAAGC-3′;
     reverse, 5′-GGGCATTATCAAAATCACACAGGC-3′;
(ii) forward, 5′ATTTACTCCGAAGTGGGGATGC3′;
     reverse, 5′-GAGGTGCGAGAACTCAAACGTC-3′;
(iii) forward, 5′-CCTGTGTGATTTTGATAATGCCCC-3′ ;
     reverse, 5′-GTCCCTCAAACCAGTCCGATCC-3′.

NOTE ADDED IN PROOF

Since the time of submission of this manuscript, we have obtained DNA samples from three other children from Family

3 in Figure 3B. Of these three, one (labeled 'untested') has HPE and an alanine tract expansion while the other two are normal and do not have the alanine tract expansion. This finding helps to confirm that the father in Family 3 is indeed a mosaic carrier of the alanine tract expansion.

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