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Additional *EFNB1* Mutations in Craniofrontonasal Syndrome

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To the Editor

Craniofrontonasal syndrome (CFNS) was first described by Cohen in 1979, and is a distinctive genetic disorder whose main clinical manifestations include coronal synostosis, hypertelorism, clefting of the nasal tip and various skeletal anomalies. As there are no substantiated instances of male-to-male transmission and all daughters of affected males are affected, X-linked inheritance was suggested. However, expression of CFNS is more severe in females than males, a phenotypic pattern not usually seen in this mode of inheritance. In addition, there is a paucity of the number of affected males in comparison to the number of affected females.

Based on a female with CFNS and a chromosomal deletion involving the terminal region of Xp, the gene map locus for CFNS was initially assigned to Xp22 [McPherson et al., 1991]. Previously, we used 12 unrelated CFNS Families to perform linkage analysis with 14 microsatellite markers on Xp and placed the CFNS gene to Xp22 with a maximum two-point lod score of 3.9 ($\alpha = 0$) and a multipoint lod score of 5.08 [Feldman et al., 1997].

More recently, several independent groups have published that loss-of-function mutations in *EFNB1* located in Xq13.1 cause CFNS [Twigg et al., 2004, 2006; Wieland et al., 2004, 2005, 2007; Shotelersuk et al., 2006; Vasudevan et al., 2006; Torii et al., 2007]. In these reports, 129 unrelated CFNS patients (both familial and sporadic) were tested for mutations in *EFNB1* and

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69 different small *EFNB1* mutations throughout the gene and 7 gene deletions were identified in about 87% of cases (112 of 129). Based on these data, mutations in *EFNB1* are the cause of CFNS in the majority of patients.

Ephrin B1 is a member of the ephrin family of transmembrane ligands for Eph receptor tyrosine kinases. These proteins play a crucial role in cell migration and pattern formation during embryonic development [Klein, 2004]. In mice, *Efnb1* is expressed in the frontonasal neural crest and demarcates the position of the future coronal suture. The mouse expression data correlate well with the CFNS phenotype [Twigg et al., 2004]. *EFNB1* is X-inactivated, and it has been proposed that in heterozygous females, patchy loss of ephrin B1 disturbs tissue boundary formation at the developing coronal suture. This process has been described as “cellular interference” [Wieland et al., 2004, 2005]. At the same time, males deficient for ephrin B1 maintain a normal boundary through an alternative mechanism (presumably due to ephrin redundancy) and thus are phenotypically less affected than females. In fact this effect is mimicked in mice deficient for *Efnb1*. *Efnb1* null mice have shortened skulls, but *Efnb1* heterozygous female mice are more severely affected and have additional limb anomalies [Compagni et al., 2003; Davy et al., 2004].

Due to these recent findings, we screened our cohort of 35 unrelated CFNS patients (both familial [19] and sporadic [16]) for mutations in *EFNB1*. Genomic DNA was obtained from lymphoblastoid cell lines. *EFNB1* primers and standard PCR conditions were used as previously described [Twigg et al., 2004] and products were subjected to direct cycle sequencing. All five coding exons and adjoining splice acceptor and donor sites of *EFNB1* were sequenced in all 35 unrelated patients. All mutations were confirmed at least once by reamplification and resequencing the mutation in each patient. When available an additional affected family member was also used to confirm the mutation. In the case of heterozygous frameshift mutations, PCR products were cloned and inserts of single colonies were sequenced. When no mutation was identified, the entire gene was reamplified and resequenced. If a second affected family member was available, they were also sequenced to again confirm that no mutation was identified. Furthermore, female patients with no identifiable mutation were subjected to multiplex ligation-dependent probe amplification (MLPA) P080 Craniofacial kit (MRC Holland) to test for deletions within *EFNB1*.

We found *EFNB1* mutations in 19 of our 35 patients (Table I and Fig. 1); 9 of which are novel. The mutation spectrum includes one gene deletion, three splice site, three frameshift, four nonsense, seven missense, and one in frame deletion. Of these 19 mutations, 10 are recurrent both in either our sample, in the literature, or in both. We found one patient with exon 1–5 deleted (seven previously published), one patient with V51del (one previously published) one with the C64Y mutation (one previously published), three patients with the R66X mutation (nine previously published), one patient with P119A (one previously published), one patient with 406 + 2T > G (one previously published), and two patients with the G151S mutation (five previously published) [Twigg et al., 2004, 2006; Wieland et al., 2004, 2005, 2007; Vasudevan et al., 2006]. (The potential effects of these mutations have already been discussed in the original manuscripts.)

Our novel mutations are predicted to cause disruption of ephrin B1 function and thus are likely disease-causing. The nonsense mutation E33X as well as the splice site mutations lead to premature termination codons and complete loss of ephrin B1 function likely due to nonsense-mediated mRNA decay [Maquat, 2004]. While we have not pursued functional studies for these specific mutations, another group has provided experimental evidence for this process in CFNS patients with *EFNB1* mutations and found severe depletion of transcripts for mutant alleles harboring either splice site mutation c.407–2A > T at the exon 2/3 boundary or frameshift mutation c.377_384delTCAAGAAG in exon 2 [Wieland et al., 2008]. The remaining missense

mutations occur at positions critical to the function of ephrin B1 [Himanen et al., 2001]. The missense mutation W37R occurs at a position that is identical in the 3 human B-type ephrins, the mouse, chick, zebrafish, and *Xenopus* Ephrin B1, and the *C. elegans* Ephrin A homologue VAB-2. L80 is conserved in all but *Xenopus*, *C. elegans*, and Ephrin B2. R109 is conserved in all but zebrafish, *C. elegans*, and Ephrin B2. The amino acids L80 and R109 occur within a β -sheet. Additional evidence that they are loss of function mutations is suggested by mutations in *C. elegans* of the ephrin A homologue VAB-2. VAB-2 W30 is analogous to W37 and when mutated to an opal codon results in significant embryonic and larval lethality [Chin-Sang et al., 1999]. It is interesting to note that in this study and others [Wieland et al., 2005; Twigg et al., 2006] only frameshift mutations have been identified in exon 5. It is likely that these frameshift mutations interfere with ephrin B1 reverse signaling and contribute to the CFNS phenotype. In agreement with other studies we have been unable to make phenotype genotype correlations either between mutations or between individuals with and without mutations.

Furthermore, we did not detect mutations in 16 unrelated individuals with CFNS. Thus, at least 33 unrelated patients published to date have no identifiable mutation in *EFNB1* (16 from this study and 17 from previous reports [Wieland et al., 2005; Twigg et al., 2006]). This accounts for 20% of the total CFNS cases screened (35 in this study and 129 from previous reports.) Several factors could lead to nondetectable mutations. (1) CFNS has been mis-diagnosed in some of the patients studied. In fact, the diagnosis of CFNS has been called into question regarding one of our previously published families [Morris et al., 1987; Gorlin et al., 2001]. To decrease the risk of misdiagnosis, the patients in this study were diagnosed by experienced dysmorphologists/clinical geneticists and on multiple occasions, the same clinicians have referred both mutation-positive and mutation-negative cases with approximate equal frequency. (2) Females may have a large heterozygous deletion not detected by PCR and sequencing; however, MLPA analysis should be able to detect these. In fact, seven large deletions have already been described [Wieland et al., 2004, 2007; Twigg et al., 2006]. (3) Patients with CFNS may have large rearrangements involving *EFNB1*. (4) Or, patients may have mutations involving *EFNB1* outside the coding region. (5) Lastly, patients may have postzygotic mutations that occurred in cell compartments not including blood precursors. Mosaicism has been described in 6 (of 59) cases [Twigg et al., 2006] by using DNA from hair root and buccal samples in addition to blood samples. Unfortunately, a similar type study was not possible for patients in our study as alternative DNA sources were not available and DNA utilized in these specific studies was derived from lymphoblastoid cell lines. (6) Alternatively, there may be one or more additional CFNS loci.

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Fig. 1. Schematic diagram of the *EFNB1* mutation spectrum in patients with CFNS. Mutations identified in the present study are listed below the putative *EFNB1* protein structure, those published previously are above.

TABLE I

Molecular Analysis of *EFNB1*

Proband ID	Familial sporadic	Sex of affecteds	Nucleotide change	Exon/intron	Predicted protein	Structure
2462	S	1F	97G > T	1	E33X	Truncation
2510	S	1F	109T > C	1	W37R ^d	Completely conserved; b-sheet
3275 ^a	F	1M/3F	129-1G > A	(1)	Splice site	
2022	F ^c	1F	149_151del TGG	2	V51del	Conserved vertebrates; b-sheet
2669	S	1F	191G > A	2	C64Y ^e	Completely conserved; b-sheet
1629	S	1F	196C > T	2	R66X ^e	Truncation
1851	S	1F	196C > T	2	R66X ^e	Truncation
1995 ^{b11}	F	1M/2F	196C > T	2	R66X ^e	Truncation
2512	S	1F	239T > C	2	L80P	Conserved in mouse, chick, zebrafish; b-sheet
2287	F	1M/1F	325C > T	2	R109C	Conserved in mouse, chick, Xenopus; part of dimerization interface; b-sheet
1547 ^{b7}	F	2M/4F	355C > G	2	P119A ^{ed}	Completely conserved, part of dimerization interface
1426	F	1M/1F	406 + 2T > G	(2)	Splice site	
1428 ^{a4}	F	2F	406 + 2T > C	(2)	Splice site	
1544	S	1F	451G > A	3	G151S ^e	Completely conserved; a-helix
2507	S	1F	451G > A	3	G151S ^e	Completely conserved; a-helix
1493 ^{b6}	F	1M/3F	649_660del AAGAGTGGGCCCA/insT	5	K217fsX10	
3975	S	1F	689delC	5	P230fsX28	
2282	F	1M/1F	729delT	5	V244fsX14	
2373	S	1M	Deletion exon 1-5			
1397 ^{b1}	F	1M/2F	None			
1400 ^{b2}	F	4F	None			
1437	S	1M	None			
1444 ^{b5}	F	2M/3F	None			
1570 ^{b8}	F	1M/1F	None			
1621 ^{b10}	F	4M/2F	None			
2458 ^{b15}	F	3F	None			
2627	S	1M	None			
2980	F ^c	1M	None			
3286	S	1F	None			
3558	S	1F	None			
3947	S	1F	None			
3951	F	2F	None			
3953	F	1M/1F	None			
4496	S	1M	None			
1856	F ^c	1F	None			

^aFamily previously published [Kere et al., 1990].

^bFamily previously published [Morris et al., 1987; Feldman et al., 1997].

^cFamilial based on history and clinical exam of individuals who did not participate in the research study.

^d*C. elegans* has mutation in analogous codon W30opal or P108L.

^ePreviously published mutation.