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Posterior Polymorphous Corneal Dystrophy Is Associated With *TCF8* Gene Mutations and Abdominal Hernia

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Mutations in the two-handed zinc-finger homeodomain transcription factor gene (*TCF8*) have been associated with posterior polymorphous corneal dystrophy (PPCD) and extraocular developmental abnormalities. We performed screening of *TCF8* in 32 affected, unrelated probands, affected and unaffected family members of probands identified with a *TCF8* mutation, and in 100 control individuals. Eight different pathogenic mutations were identified in eight probands: four frameshift (c.953_954insA, c.1506dupA, c.1592delA, and c.3012_3013delAG); three nonsense (Gln12X, Gln214X, Arg325X); and one missense (Met1Arg). Screening of *TCF8* in affected and unaffected family members in six families demonstrated that each identified mutation segregated with the disease phenotype in each family; two probands did not have additional family members available for analysis. None of the eight *TCF8* mutations was identified in 200 control chromosomes. The prevalence of hernias of the abdominal region in affected individuals with PPCD associated with *TCF8* mutations was significantly higher than the prevalence in both individuals with PPCD not associated with a *TCF8* mutation and in unaffected individuals. Therefore, PPCD is associated with *TCF8* mutations in one quarter of affected families in this study, or about one third of all PPCD families that have been screened thus far. In these families, the presence of apparently causative *TCF8* mutations is associated with abdominal and inguinal hernias. © 2007 Wiley-Liss, Inc.

Key words: posterior polymorphous corneal dystrophy; two-handed zinc-finger homeodomain transcription factor gene (*TCF8*); abdominal hernia; inguinal hernia

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INTRODUCTION

Posterior polymorphous corneal dystrophy (PPCD, OMIM #122000) is an autosomal dominant corneal disorder that demonstrates highly variable expressivity, ranging from an asymptomatic, isolated, unilateral corneal endothelial vesicle to bilateral congenital corneal edema and associated glaucoma. Locus heterogeneity has been demonstrated for PPCD, with three published reports of four large pedigrees in which linkage was demonstrated to the pericentromeric region of chromosome 20 (designated the PPCD1 locus) [Heon et al., 1995; Gwilliam et al., 2005; Yellore et al., 2007], and the identification of pathogenic mutations in *TCF8*, located on chromosome 10p11 (designated the PPCD3 locus) [Krafchak et al., 2005]. While Krafchak and colleagues provided convincing evidence that *TCF8* mutations cause PPCD, not all of the identified mutations segregated with the affected phenotype in the five families in which a *TCF8* mutation was identified. In the originally described pedigree, the investigators concluded that incomplete penetrance and parental germline mosaicism explained the *TCF8* mutation and affected phenotype cosegregation pattern [Krafchak et al., 2005]. Additionally, as two of the other four PPCD pedigrees they described with *TCF8* mutations demonstrated affected offspring of unaffected parents, the authors genotyped

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multiple regional microsatellites to confirm parentage prior to concluding that the observed *TCF8* sequence variants represented de novo mutations.

Other investigators have reported a mutation in the collagen, type VIII, alpha 2 gene (*COL8A2*, OMIM #120252), located on the short arm of chromosome 1(known as the PPCD2 locus), in two affected members of a single family with PPCD [Biswas et al., 2001]. However, as segregation of the mutation with the affected phenotype was not demonstrated in this family, and we and other investigators have not identified this or any other presumed pathogenic mutations in *COL8A2* in a large number of individuals with PPCD [Kobayashi et al., 2004; Krafchak et al., 2005; Yellore et al., 2005] there is no compelling evidence for a causative role for *COL8A2* mutations in PPCD. Similarly, although two mutations in the visual system homeobox 1 gene (*VSX1*, OMIM # 605020), localized to the originally described chromosome 20 candidate gene region, have been suggested to cause PPCD [Heon et al., 2002] the exclusion of the *VSX1* gene in the refined chromosome 20 candidate gene region and the subsequent demonstration that the identified sequence variants are likely non-pathogenic polymorphisms suggests that *VSX1* is not the gene within the PPCD1 candidate region that is associated with PPCD [Aldave et al., 2005; Gwilliam et al., 2005].

To investigate the possible role of *TCF8* mutations in the pathogenesis of PPCD, we performed screening of the *TCF8* coding region and splice sites in 32 unrelated probands with PPCD. For individuals in whom a sequence variant was identified, affected and unaffected family members were screened for the mutation, as were 100 control individuals. Additionally, as affected individuals have been reported to have a high rate of abdominal hernias and orthopedic anomalies, we determined the prevalence of these extraocular anomalies in affected individuals both with and without *TCF8* mutations as well as in their unaffected relatives.

MATERIALS AND METHODS

The researchers followed the tenets of the Declaration of Helsinki in the treatment of the subjects reported herein. Study approval was obtained from the institutional review board at The University of California, Los Angeles (UCLA IRB # 94-07-243-22, 02-10-092-01, and 02-10-092-04).

Patient Identification/DNA Collection and Preparation

After informed consent was obtained, affected patients were enrolled in the study. The diagnosis of PPCD was based upon the presence of one or more of the following characteristic clinical features in each cornea: a single or grouped endothelial vesicles surrounded by a gray halo; one or more endothelial bands associated with parallel borders; and/or geographic gray endothelial opacities between 0.5 and 2.0 mm in diameter. The diagnosis was confirmed by histopathologic examination of the excised corneal button, when available. Family members were also enrolled in the study after informed consent was obtained and a slit lamp examination was performed to confirm their affected status. Unrelated, unaffected, healthy volunteers were recruited to serve as controls. Blood was obtained from nearly all subjects, although buccal epithelial swabs (Cyto-Soft™ Cytology Brush; Medical Packaging Corporation, Camarillo, CA) and Oragene saliva collection kits (DNA Genotek, Inc., Ontario, Canada) were utilized for DNA collection from patients in whom phlebotomy could not be performed. Genomic DNA was prepared from the peripheral blood leukocytes and buccal epithelial cells using the FlexiGene DNA and QIAamp DNA Blood Mini Kits (Qiagen, Valencia, CA), respectively.

PCR Amplification

Each of the nine exons of TCF8 was amplified using the previously described primers and conditions, [Krafchak et al., 2005] with the exception of exon 1, which was amplified using custom-designed oligonucleotide primers (forward/reverse: AGCAGTGCCCACGGTTGC/GAGACATAACGGTTCAGGGAGAGC). The 25 μl reaction contained 50 mM Tris-HCl (pH 9.0, 25°C), 20 mM NH4Cl, 2.5 mM MgSO4, 200 μM each dNTP plus 20 μM 7-deaza-dGTP, 0.5M Betaine, 2.5 μl DMSO, 150 mM Trehalose, 0.002% Tween-20, 0.12 μM of each primer, 0.5 units of REDTaq Genomic DNA Polymerase (Sigma–Aldrich, St. Louis, MO), and approximately 60 ng of genomic DNA. Thermal cycling was performed in an iCycler Thermal Cycler (Bio-Rad, Hercules, CA).

DNA Sequencing

Purification of the PCR products was achieved by incubating 15–30 ng DNA with 5U Exonuclease I and 0.5U Shrimp Alkaline Phosphatase (USB Corp., Cleveland, OH) for 15 min at 37°C. After inactivation of the nucleases for 15 min at 80°C, sequencing reactions were performed by the addition of 2 μl BigDye Terminator Mix v3.1 (Applied Biosystems, Foster City, CA), 2 μl of SeqSaver (Sigma–Aldrich) and 0.2 μl primer (10 pM/μl). Samples were denatured at 96°C for 2 min, then cycled 25 times at 96°C for 10 sec, 50°C for 5 sec, and 60°C for 4 min. Unincorporated nucleotides were removed using the CleanSeq reagent and an SPRI plate (Agencourt Bioscience Corporation, Beverly, MA) following the manufacturer's instructions and then analyzed on an ABI-3100 Genetic Analyzer (Applied Biosystems)

after resuspension in 0.1 mM EDTA. Nucleotide sequences, read manually and with Mutation Surveyor v2.2 (Softgenetics, State College, PA), were compared with the published *TCF8* cDNA sequence (GenBank accession number NM_030751).

Evaluation of Extraocular Manifestations of PPCD

A brief questionnaire was mailed to each of the 32 affected probands, as well as 19 affected and 51 unaffected relatives who had previously undergone slit lamp examination and provided DNA for analysis. Each questionnaire asked whether the recipient had a history of an inguinal, abdominal or umbilical hernia, hydrocele (men only) or orthopedic abnormality, such as bone spurs, an abnormal bone growth or structure or Dupuytren contracture.

Statistical Analyses

The Fisher exact test was used to evaluate the statistical significance of the differential distribution of extra-ocular manifestations between those with and without PPCD and between PPCD patients with and without *TCF8* mutations. To verify that the independence assumption of the Fisher's exact test was valid in these analyses that included family data, we explored the correlations among family members with respect to extra-ocular manifestations. Based on logistic regression models with generalized estimating equation of compound symmetry covariance structure to account for correlations among multiple family members within any given family, there appeared to be no significant correlations of extraocular manifestations within families ($N = 73$, or $N = 30$ for hydrocele [male only]), indicating that there was no major violation of the independence assumption in the Fisher exact test.

RESULTS

TCF8 Screening

Presumed pathogenic mutations were identified in eight of the 32 (25%) probands affected with PPCD. Three of the eight probands represented isolated cases in that additional family members were either not available for examination (two probands) or were examined and found to be unaffected (one proband). Four frameshift mutations were identified: c.953_954insA (Gln310fsX26), c.1506dupA (Glu495fsX10), c.1592delA (Glu523fsX4), and c.3012_3013delAG (Thr996fsX8), each in the heterozygous state in one of four affected probands. Each of these mutations produces an alteration of the reading frame 3′ of the nucleotide insertion or deletion, and is predicted to produce a stop codon, resulting in a truncated protein product, 4–26 codons downstream. The predicted effect on the encoded protein domains would be loss of the homeodomain and the third through the eighth (Gln310fsX26) or the fifth through the eighth (Glu495fsX10 and Glu523fsX4) zinc finger domains (Fig. 1). The Thr996fsX8 mutation would not be predicted to result in a loss of either the homeodomain or any of the zinc finger domains, but may result in an unstable mRNA product that would be susceptible to undergoing nonsense-mediated mRNA decay. While the Gln310fsX26, Glu495fsX10, and Glu523fsX4 mutations segregated with the affected phenotype in the families in which they were identified, (Fig. 2) family members of the proband in whom the Thr996fsX8 mutation was identified were not available for DNA collection. Interestingly, the proband with the Glu523fsX4 mutation was the subject of a previous report on the histopathologic features of PPCD [Anderson et al., 2001].

Three nonsense mutations were identified, each in a different proband: Gln12X (c.58C > T), Gln214X (c.664C > T), and Arg325X (c.997C > T). Two of these mutations, Gln12X, and Gln214X, are predicted to result in a truncated protein product, with loss of the homeodomain, and each of the zinc finger domains. The location of the Arg325X mutation would be predicted to result in elimination of the homeodomain and six of the zinc finger domains. While the Gln12X and the Gln214X mutations segregated with the affected phenotype in the families in which they were identified, no unaffected individuals were available for screening in the family

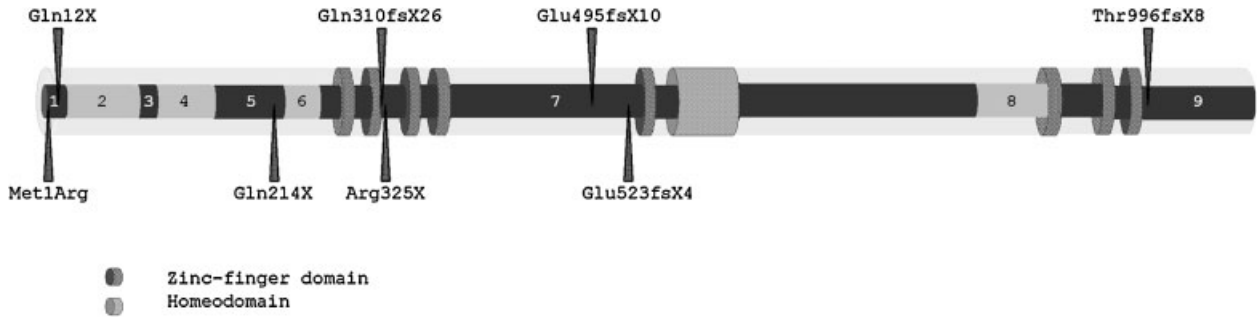

FIG. 1. Depiction of the *TCF8* transcript, demonstrating the location of each of the eight identified mutations, as well as the homeodomain and the zinc-finger domains.

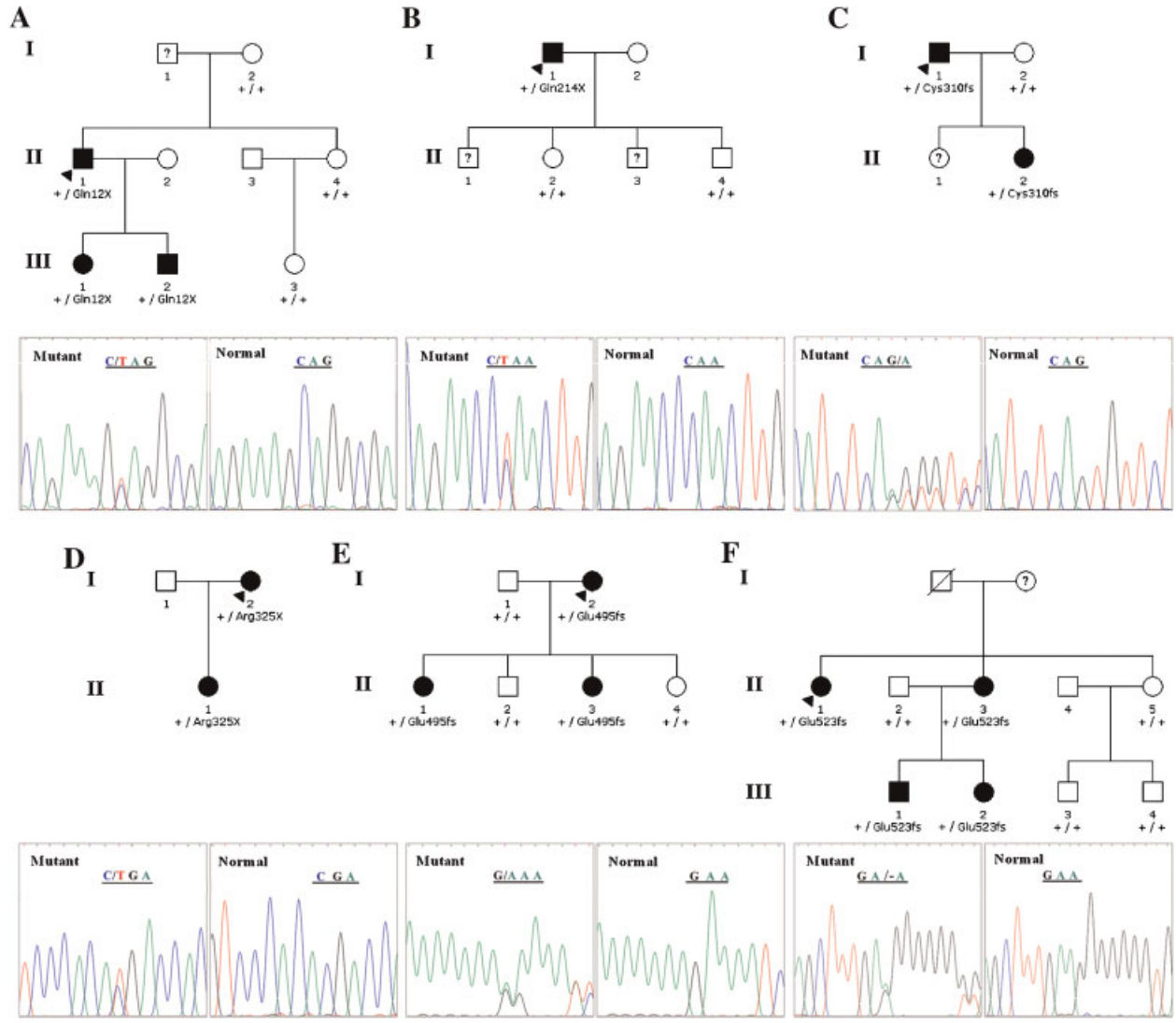

FIG. 2. Six of eight pedigrees in which *TCF8* mutations were identified and family members were available for screening. In each pedigree, filled symbols represent affected individuals, and open symbols represent unaffected individuals. Question marks indicate individuals of undetermined affected status. Below the symbol for each individual in whom DNA collection and *TCF8* screening was performed, the presence of the wild type allele (designated by the + symbol) or the mutant allele is indicated. Beneath each pedigree, chromatograms demonstrating the identified mutation, and the wild-type DNA sequence are shown. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

in which the Arg325X mutation was identified in the proband and her affected mother (Fig. 2).

One missense mutation was identified, Met1Arg (c.26T > G), which resulted in a substitution of the initiating amino acid methionine. It remains to be seen whether loss of the initiating methionine would be lead to use of a later methionine for initiation, with resulting loss of amino terminal domains of the protein, or lack of translation product altogether. Segregation analysis of the mutation in the proband's family was not possible as he was adopted and did not know the identity of his biologic family. The other sequence variant that was identified, Asp64Asp (c.216C > T), is a previously identified polymorphism that was typed in HapMap and found to have an average heterozygosity of 0.12 (refSNP ID: rs7918614).

None of the identified sequence variants in *TCF8* was identified in 200 control chromosomes.

Extraocular Manifestations of PPCD

Surveys regarding extraocular manifestations of PPCD were received from 73 of the 102 individuals to whom they were sent, 34 of whom were affected and 39 of whom were unaffected family members. Ten of the 34 affected individuals (29%) harbored a *TCF8* mutation (*TCF8*+): four men, each a proband in a different family, and six women from two families, three of whom are from each of the families shown in

Figure 2E and F. Of the 24 affected individuals who did not demonstrate a *TCF8* mutation, 10 were men from seven different families, and 14 were women from nine different families.

Hernias and hydroceles. Each of the four affected men with a *TCF8* mutation had a history of a hernia (two inguinal, one abdominal and one unknown), and three of the four had a history of a hydrocele. Two of the six women with a *TCF8* mutation had a history of a hernia (one inguinal and one abdominal). Two of the 10 affected men without a *TCF8* mutation reported an inguinal hernia, one of whom was a member of a large family that has been linked to the PPCD1 locus [Yellore et al., 2007]. This individual, as well as one of the other three affected male respondents from this family, also reported a history of a hydrocele, which was not reported in any of the 16 unaffected males. One of the 14 affected women without a *TCF8* mutation reported a history of a hernia (umbilical); this individual was also a member of the aforementioned large family linked to the PPCD1 locus [Yellore et al., 2007]. None of the 39 unaffected individuals had a history of a hernia or hydrocele.

The prevalence of hernias in affected individuals was 26% (9/34), as compared to a prevalence of 0% (0/39) in unaffected individuals, a difference that was statistically significant (Fisher exact test $P < 0.001$). When the groups were divided by sex, the difference in prevalence of hernias between affected men (43% (6/14)) and unaffected men (0% (0/16)) was statistically significant (Fisher exact test $P = 0.005$), but the difference between affected women (15% (3/20)), and unaffected women (0% (0/23)) was not (Fisher exact test $P = 0.092$).

When the prevalence of hernias in *TCF8*+, and *TCF8*− affected individuals was compared, 60% (6/10) of *TCF8*+ affected individuals versus 13% (3/24) of *TCF8*− affected individuals had a history of either an inguinal, umbilical or abdominal hernia, which was a statistically significant difference (Fisher exact test $P = 0.009$). When the groups were divided by sex, the difference in prevalence of hernias between *TCF8*+ affected men (100% (4/4)), and *TCF8*− affected men (20% (2/10)) was also statistically significant (Fisher exact test $P = 0.015$), but the difference between *TCF8*+ affected women (33% (2/6)), and *TCF8*− affected women (7% (1/14)) was not (Fisher exact test $P = 0.202$).

The prevalence of hydroceles in affected men was 36% (5/14), as compared to a prevalence of 0% (0/16) in unaffected men, a difference that was found to be statistically significant (Fisher exact test $P = 0.014$). When the prevalence of hydroceles in *TCF8*+ and *TCF8*− affected men was compared, 75% (3/4) of *TCF8*+ affected men versus 20% (2/10) of *TCF8*− affected men had a history of a hydrocele, which was not a statistically significant difference (Fisher exact test $P = 0.095$).

When the groups were compared in regards to the percentage of men with a history of a hernia, hydrocele or both, a statistically significant difference was identified in the prevalence among affected men (50% (7/14)) compared with unaffected men (0/16; Fisher exact test $P = 0.002$). However, when the percentage of *TCF8*+ affected men with a history of a hernia, and/or hydrocele (100% (4/4)) was compared to the percentage in *TCF8*− affected men (30% (3/10)), the difference was not statistically significant (Fisher exact test $P = 0.070$).

Orthopedic anomalies. Three affected individuals, two men and one woman without a *TCF8* mutation, had a history of Dupuytren contracture, while none of the 14 affected individuals with a *TCF8* mutation or any of the 39 unaffected individuals had a history of Dupuytren contracture. One affected (*TCF8*+) and one unaffected woman had a history of bone spurs and one unaffected woman had a history of abnormal bone growth or structure.

No statistically significant difference was noted in the prevalence of orthopedic anomalies in affected individuals (9% (3/34)) when compared to that in unaffected individuals (5% (2/39); Fisher exact test $P = 0.659$). Additionally, when stratified by sex, no statistically significant difference was identified in the prevalence between affected and unaffected individuals.

When the prevalence of orthopedic abnormalities in *TCF8*+ and *TCF8*− affected individuals was compared, 0% (0/10) of *TCF8*+ affected individuals had a history of either a bone spur, Dupuytren contracture, or abnormal bone growth or structure versus 13% (3/24) of *TCF8*− affected individuals, which was not a statistically significant difference (Fisher exact test $P = 0.539$). When the groups were divided by sex, the difference in prevalence of orthopedic abnormalities between *TCF8*+ and *TCF8*− affected individuals was also not statistically significant.

DISCUSSION

The identification of *TCF8* mutations in 8 of the 32 PPCD families that were screened confirms the previous report by Krafchak et al. [2005] that mutations in *TCF8* are associated with PPCD3. *TCF8* is the first gene to be convincingly implicated in the pathogenesis of PPCD, with the identification of causative mutations in five of 11 probands previously screened [Krafchak et al., 2005] and eight of 32 probands that we report, giving a cumulative 30% (13/43) of affected probands with PPCD demonstrating *TCF8* mutations. The majority of mutations are frameshift (7) and nonsense (5), with only a single missense mutation identified to date. Given the large size of exons 7 and 9, it is not surprising that 10 of the 13 identified mutations have been located in these exons (Fig. 1).

As initially reported by Krafchak et al. [2005] each mutation identified to date has been observed in only a single affected individual or family. This is not unprecedented among the corneal dystrophies, as over 100 mutations have been identified in the *CHST6* gene in affected individuals with macular corneal dystrophy, many of which have been identified in only a single pedigree [Aldave and Sonmez, 2007]. This is in contrast to the *TGFBI* dystrophies, in which codons 124 and 555 are mutation hot spots, with the majority of affected individuals demonstrating a mutation in one of these loci [Aldave and Sonmez, 2007].

With very small numbers of affected individuals within each family, and no recurrence of mutations between families, a phenotype–genotype correlation has not been established for the various *TCF8* mutations. Additionally, we have not been able to appreciate a difference in the clinical manifestations of PPCD in affected individuals with and without *TCF8* mutations. Given this inability to distinguish PPCD1 and PPCD3 based on clinical features, and the demonstrated allelic heterogeneity in *TCF8* that Krafchak et al. [2005], and we report, the clinical utility of *TCF8* screening in affected individuals will likely be limited.

Although haplotype and *TCF8* mutation analysis demonstrated inconsistencies attributed to incomplete penetrance, age-related penetrance, and de novo mutations in the PPCD pedigrees reported by Krafchak et al. [2005], we did not find evidence of incomplete segregation or spontaneous mutations in the families that we report. As three of the five mutations described by Krafchak et al. [2005] were considered de novo mutations, they questioned whether this was a chance association or indicative of reduced reproductive fitness of the mutant alleles. While the development of PPCD is not likely to affect the reproductive fitness of the affected individual, the association of *TCF8* mutations with extraocular developmental abnormalities, such as inguinal hernia, does make this a reasonable consideration. Only three of the eight probands in whom *TCF8* mutations were identified represented isolated cases, in which spontaneous mutations are easier to identify (by simply screening the proband's parents) than in families with affected individuals in multiple generations, in which the parents of the oldest affected individual, who would be less likely to be available for analysis, would need to be screened. As the parents of the eldest affected individual in the multigenerational families of the other five probands in whom *TCF8* mutations were identified were not available for analysis, and we did not identify any pedigrees with an affected offspring of unaffected parents, we are able to state only that there is no evidence to suggest that any of the eight mutations we report arose as a de novo mutation. Additionally, such de novo mutations have not been identified at a sufficiently high rate to infer reduced reproductive fitness of the mutant alleles.

We did, however, confirm another association made by Krafchak et al. [2005], the presence of a nonocular developmental abnormality associated with PPCD. They reported that 11 of the 12 affected men with identified *TCF8* mutations for whom information was available had a history of an inguinal hernia and/or hydrocele. We also found a significantly increased prevalence of inguinal hernias (43%), and hydroceles (36%) in affected men when compared with unaffected male relatives (0%), and a statistically significant greater prevalence of hernias in affected men with *TCF8* mutations (100%) compared with affected men without *TCF8* mutations (20%). As the estimated prevalence of inguinal hernias in male newborns and boys is estimated to be between 1% and 2.4% [Choi et al., 1989; Yucesan et al., 1993; Kapur et al., 1998; Chandrakala and Vijayashankara, 2005; Yegane et al., 2005] the prevalence rates observed in men affected with PPCD, both with and without *TCF8* mutations, are significantly increased.

When one combines the data from this study and the previous study by Krafchak et al. [2005], 69% (18/26) of men with PPCD have a history of a hernia and/or hydrocele, significantly greater than the estimated prevalence in the general population, and in the unaffected male relatives of the patients that we report (0%; Fisher exact test $P < 0.001$). When the percentage of *TCF8*+ affected men in the two studies with a history of a hernia and/or hydrocele (94% (15/16)) is compared to the percentage in *TCF8*− affected men that we report (30% (3/10)), the difference is once again statistically significant (Fisher exact test $P = 0.001$).

As the transcription factor *TCF8* regulates the expression of genes involved in the synthesis of several forms of collagen in a number of cell types, it is likely that the increased prevalence of inguinal hernias in patients with PPCD is secondary to aberrant collagen expression in multiple tissues. Given the increased prevalence of inguinal hernias in patients with systemic collagen disorders such as Ehlers–Danlos syndrome [Liem et al., 1997] and the decreased ratio of type I to type III collagen in the skin and peritoneal tissues of affected patients [Klinge et al., 1999a,b; Rosch et al., 2002; Taniguchi et al., 2006], inguinal hernia is commonly considered a localized manifestation of a systemic disorder of collagen metabolism [Klinge et al., 1999a,b; Ozdogan et al., 2006]. *TCF8* has been shown to regulate the expression of the *COL1A1* gene, which codes for the two alpha 1 chains of type I collagen, and the *COL1A2* gene, which codes for the single alpha 2 chain of type I collagen, in several cell types, including vascular smooth muscle cells [Ponticos et al., 2004] and osteoblasts [Terraz et al., 2001]. Therefore, it is quite feasible that haploinsufficiency

due to mutations in *TCF8* results in the observed collagen abnormalities, and altered ratio of collagen types in the skin, rectus sheath, and peritoneum in patients with inguinal hernias [Klinge et al., 1999a,b; Rosch et al., 2002; Ozdogan et al., 2006; Szczesny et al., 2006; Taniguchi et al., 2006].

Several other genes encoding different types of collagen also contain upstream regulatory regions to which *TCF8* has been shown to bind, and alter transcription, including the *COL2A1* gene, which encodes type II collagen, and the *COL4A3* gene, which encodes the alpha-3 chain of type IV collagen [Murray et al., 2000; Krafchak et al., 2005]. Krafchak and colleagues have shown that the promoter of *COL4A3* contains both a core and a secondary binding site for *TCF8*, and demonstrated the ectopic expression of *COL4A3* in the corneal endothelium of the proband of the large family in which the first *TCF8* mutation was identified. As *COL4A3* expression was not demonstrated in the control cornea, the authors concluded that aberrant regulation of collagen synthesis was the mechanism through which *TCF8* mutations led to the disordered formation of Descemet's membrane that characterizes PPCD [Krafchak et al., 2005]. We propose that *TCF8* mutations may lead to similar altered regulation of *COL1A1* and *COL1A2* expression, resulting in the increased prevalence of hernias of the abdominal wall in patients with PPCD. Additionally, the increased prevalence of inguinal hernias among PPCD cases that lack *TCF8* mutations raises the question of whether other PPCD loci may also affect the expression of collagen genes, either directly or indirectly.

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