ORIGINAL ARTICLE

TGFB3 displays parent-of-origin effects among central Europeans with nonsyndromic cleft lip and palate

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Abstract Mice with a deletion of Tgf-β3 ($-/-$) and association studies in humans of different ethnicities support the involvement of *TGFB3* in the etiology of orofacial clefts. In this study, we investigated the relevance of *TGFB3* in the development of cleft lip and palate (CL/P) among 204 triads of central European origin. Transmission-disequilibrium test (TDT) analysis revealed no significant transmission distortions for each marker alone, and none for any possible haplotypes. However, we found strong evidence for parent-of-origin effects, with lower risk of maternal transmission compared with paternal transmission [$I_\mathrm{M} = 0.38$; confidence interval (CI): 0.17–0.86] of the risk allele T to an affected offspring at marker rs2300607. This is also expressed in an increased risk of heterozygous children having the T allele inherited from the father ($R_\mathrm{P} = 3.47$; CI: 1.32–9.11). Our data support the involvement of *TGFB3* in the development of oral clefts in patients of central European origin.

Keywords Nonsyndromic · Cleft lip and palate · *TGFB3* gene · Parent-of-origin effect

Introduction

The genetic background of nonsyndromic orofacial clefts is complex, and most causal genes remain to be identified. The majority of pertinent candidate genes have been deducted from murine knockout models. One of these genes is the transforming growth factor-beta 3 gene *TGFB3* (NM003239.1 NP003230.1), located on human chromosome 14q24. *TGFB3* is expressed in medial-edge epithelium cells of palatal shelves (Gato et al. 2002). Mice with a genetic

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deletion of Tgf-β3 ($-/-$) present with either complete cleft palate or severe partial cleft palate (Kaartinen et al. 1995; Proetzel et al. 1995). Genetic association studies in humans of different ethnicities suggest *TGFB3* may be involved in the formation of orofacial clefts (Maestri et al. 1997; Lidral et al. 1998; Romitti et al. 1999; Mitchell et al. 2001; Sato et al. 2001; Beaty et al. 2002; Scapoli et al. 2002; Jugessur et al. 2003; Kim et al. 2003; Slayton et al. 2003; Vieira et al. 2003; Suzuki et al. 2004), although negative studies have also been reported (Lidral et al. 1997; Tanabe et al. 2000; Beaty et al. 2001; Morkūnienė et al. 2007). Until recently, most studies used a CA repeat located 61.215 bp upstream from translation starting point of *TGFB3* as well as a VNTR marker in the 5′ untranslated region (UTR) (Δ at -21083 to -21086 (AGAGGG repeat)) and with X5.1 a T > C substitution [ref. single nucleotide polymorphism (SNP) rs3917200] at position -24 relative to intron 4/exon 5 junction to test for involvement of the gene (Lidral et al. 1998; Beaty et al. 2002). Recently, the study by Ichikawa et al. (2006) aimed at a comprehensive coverage of the gene and tested a total of 23 SNP in a Japanese cleft lip and palate (CL/P) population. These authors observed significant association, with $P < 0.01$ at four SNPs and $P < 0.05$ at an additional five SNPs. As this is the most convincing human study of *TGFB3* in CL/P published to date, we investigated the relevance of their findings for CL/P patients of a different ethnic background, namely, patients of central European origin. In addition, we searched for parent-of-origin effects, which have been previously described for candidate genes associated with nonsyndromic oral clefts (Scapoli et al. 2002; Rubini et al. 2005).

Materials and methods

Sample

In our study, we included 204 patients with nonsyndromic CL/P and their parents. Triads were recruited throughout Germany from hospitals and non-hospital-based institutions. Ethical approval was obtained from ethics committees of the relevant medical facilities. All individuals and, in cases of children younger than 18 years, parents gave written informed consent for participation. To rule out syndromic CL/Ps, each patient or the parents were asked to complete a detailed questionnaire on psychomotor development and general health status of the affected person. Furthermore, a thorough clinical examination was performed in each patient and his/her parents by one of two clinical geneticists (see also Birnbaum et al. 2007). The ethnic background of the patients was assessed by identifying the origin of their grandparents. Only patients whose four grandparents were born in central Europe were

classified as being of central European origin. Peripheral venous blood was taken for DNA extraction from all participants. The patient sample comprised 121 male subjects and 83 female subjects. In 176 families, both parents participated in the study (complete triads); in 26 family units, only the mother was available, and in two only the father (28 incomplete triads) was available. Of the 204 triads, 14 mothers and 21 fathers had a nonsyndromic CL/P themselves.

Genotype analysis

To test for association, we used three SNPs (IVS1 +2118, IVS1 +5321, and IVS1 −1572), which were significantly associated with CL/P in the study by Ichikawa et al. (2006) and which had minor allele frequencies >0.05 in Japanese and central Europeans (see NCBI: http://www.ncbi.nlm.nih.gov/SNP). In Ichikawa et al.'s study (2006), markers IVS1 +2118 and IVS1 +5321 displayed the most significant association in their single-marker analysis. In the two marker haplotype analysis, the combination of IVS1 +5321 and IVS1 −1572 showed the most significant result. Genotyping of SNP rs2268626 (IVS1 +2118) and rs2268625 (IVS1 −1572) was accomplished using TaqMan assay on an ABI 7500 (Applied Biosystems) according to predesigned conditions. Because SNP rs2300607 (IVS1 +5321) was located in a highly polymorphic region, allele screening was performed with a polymerase chain reaction (PCR)-based restriction assay. PCR was performed using the FastStart Taq DNA Polymerase kit (Roche, Applied Science, Indianapolis, IN, USA) following standard conditions with the use of modified primers (4F: 5′-TCAGCCTGGACAACATAGG GAGGACC-3′; 4R: 5′-TATGTACATTTTCTTTGATCTC CCAGG-3′). If the minor allele T was present in the amplified product (405 bp), two *Apo*I (Fermentas ER1382) restriction sites resulted in fragments of 51, 140, and 214 bp in size. The digested PCR products were separated on 3% agarose gels.

Statistical analysis

Genotype distribution obtained by combining the two non-transmitted parental alleles in each family was tested for deviations from Hardy–Weinberg equilibrium by Weir's exact test (Weir 1996). We used the transmission disequilibrium test (TDT) to check for distortion of the inheritance of alleles from parents to affected offspring (Spielman et al. 1993). Haplotype analysis was performed with the software FAMHAP (Becker und Knapp 2004). A log-linear model (Weinberg et al. 1998; Weinberg 1999) allows screening for genotype effects of child and mother as well as parent-of-origin effects. The program LEM (http://www.uvt.nl/faculteiten/fsw/organisatie/departementen/mto/software2.html) (Vermunt 1997; van den

Oord and Vermunt 2000) was applied to estimate the relative risks (RR) of Weinberg's model and to obtain corresponding 95% confidence intervals (CI). CI not covering the value of 1 provide evidence of a significant effect at $\alpha = 5\%$.

Results

For all SNPs, the distribution of genotypes obtained by combining the two nontransmitted parental alleles was consistent with Hardy–Weinberg equilibrium (data not shown). TDT analysis revealed no significant transmission distortions for each marker alone and none for any of the possible haplotypes (Tables 1 and 2). Our sample had power of approximately 80% to detect (at $\alpha = 5\%$) a transmission distortion associated with a RR $\Psi_2 = 1.8$ for the homozygous genotype and $\Psi_1 = 1.0$ for the heterozygous genotype (recessive mode of inheritance) and for RR of $\Psi_2 = 2.56$ and $\Psi_1 = 1.6$ (multiplicative model).

Analysis according to Weinberg's log-linear model (Weinberg 1999) revealed that a paternally derived copy of allele T at marker rs2300607 (IVS1 +5321) induced a greater increase in risk than did a maternally derived copy. Table 3 presents estimates of RR and corresponding 95% CI for these parameters. Here, parameters S_1 or S_2, respectively, means RR of one copy or two copies, respectively, against no copy of the variant allele in the mother. Two copies of a variant in a child increased the RR by a factor of R_2. When considering parent-of-origin effects, R_P is the relative risk associated with a single copy inherited from the father, and $R_P \times I_M$ represents the risk associated with a single copy inherited from the mother, i.e., $I_M = 0.38 < 1$ (CI: 0.17–0.86, $P = 0.019$), which reflects a decreased probability of a maternally derived copy compared with a paternally derived copy. In addition, our analysis demonstrated an elevated $R_P = 3.47$ (CI: 1.32–9.11), i.e., an increased risk for children with only one paternally inherited allele.

Table 1 Results of single-marker analysis transmission disequilibrium test (TDT)

Marker	Allele	Transmissions	Nontransmissions	P values
rs2268626 (IVS1 +2118)	T	56	68	0.28
rs2300607 (IVS1 +5321)	T	70	70	1.00
rs2268625 (IVS1 −1572)	T	54	57	0.78

Table 2 Results of haplotype analysis

Combination of markers[a]	Haplotypes	Transmissions	Nontransmissions	P values
2–3	1 1	71.99	69.99	0.72
	1 2	1.01	3.01	
	2 1	21.01	26.01	
	2 2	55.99	50.99	
1–2	1 1	73.99	81.00	0.49
	1 2	21.01	26.00	
	2 1	15.01	8.00	
	2 2	54.99	50.00	
1–3	1 1	59.00	69.00	0.35
	1 2	1.00	3.00	
	2 1	14.00	7.00	
	2 2	56.00	51.00	
1–2–3	1 1 1	74.99	81.00	0.15
	1 1 2	0.00	1.00	
	1 2 1	21.01	25.00	
	1 2 2	1.00	2.00	
	2 1 1	14.00	6.00	
	2 1 2	1.01	2.01	
	2 2 1	0.00	1.00	
	2 2 2	54.99	48.99	
Global P value				0.36

[a] Markers used to construct haplotypes 1 = rs2268626 (IVS1 +2118), 2 = rs2300607 (IVS1 +5321), 3 = rs2268625 (IVS1 −1572)

Table 3 Results of the analysis for single nucleotide polymorphism (SNP) rs2300607 (IVS1 +5321) (log-linear model, van den Oord and Vermunt 2000)

Parameters[a]	Description of RR and effect	RR	95% CI
R_P	RR associated with one copy of paternally inherited T allele	**3.47**	**1.32–9.11**
$I_M \times R_P$	RR associated with maternally inherited T allele	1.31	0.46–3.75
R_2	RR of children homozygous for the T allele	1.98	0.76–5.21
S_1	RR associated with T allele heterozygous versus A allele homozygous mothers	1.71	0.57–5.13
S_2	RR associated with T allele homozygous versus A allele homozygous mothers	1.68	0.46–6.19
I_M	Imprinting	**0.38**	**0.17–0.86**

[a] R_P is the relative risk associated with a single copy of allele T inherited from the father, and $I_M \times R_P$ is the relative risk associated with a single copy inherited from the mother. Thus, $I_M = 1$ if and only if there is no parent-of-origin effect. Two inherited copies of allele T increase the child's risk by a factor of R_2. The risk could also depend on the number of copies carried by the mother, through prenatal effects, and the maternally mediated relative risks will be denoted as S_1 and S_2. For more detailed description of these parameters, see Weinberg (1999)

Discussion

The process of palatal fusion is controlled by interactive signaling from the mesenchyme to the epithelium, which is mediated by growth factors and extracellular matrix (ECM) proteins (Baroni et al. 2003). One of the key components in palatal fusion is transforming growth factor $\beta 3$ (Tgf-$\beta 3$) (Nogai et al. 2008). Tgf-$\beta 3$ expression, which is coordinated temporally during palatogenesis (Britto et al. 2002), determines ECM macromolecule overaccumulation by stimulating ECM neosynthesis and by inhibiting many enzymes implicated in ECM degradation (Blavier et al. 2001). Consequently, reduced activity of Tgf-$\beta 3$ may increase the risk for oral clefting. Consistent with this hypothesis, Tgf-$\beta 3$ $-/-$ knockout mice present with a developmental defect of the secondary palate (Proetzel et al. 1995). In humans, however, evidence for any involvement of the *TGFB3* gene in development of oral clefts has remained inconclusive, with reports of significant (Maestri et al. 1997; Lidral et al. 1998; Romitti et al. 1999; Mitchell et al. 2001; Sato et al. 2001; Beaty et al. 2002; Scapoli et al. 2002; Jugessur et al. 2003; Kim et al. 2003; Slayton et al. 2003; Vieira et al. 2003; Suzuki et al. 2004) as well as negative (Lidral et al. 1997; Tanabe et al. 2000; Beaty et al. 2001; Morkūnienė et al. 2007) associations among different populations with nonsyndromic orofacial clefts. In this study, we investigated whether the results of Ichikawa et al. (2006), who conducted the most comprehensive study of *TGFB3* to date, were also apparent in CL/P families of central European descent. We used a case–parent triad design to avoid undetected ethnic stratification as a cause of false positive results.

In our sample of 204 families, we observed no significant transmission distortion, neither at the level of individual markers nor at the level of haplotypes (Tables 1 and 2).

Furthermore, because parent-of-origin effects have been previously described for oral-cleft candidate genes (Scapoli et al. 2002; Rubini et al. 2005), we tested *TGFB3* variants for evidence of genomic imprinting. Interestingly, however, we found evidence of a lower risk of maternal transmission compared with paternal transmission ($I_M = 0.38$; CI: 0.17–0.86) of allele T to the affected offspring at marker rs2300607 (IVS1 +5321) (Table 3). This is also expressed as an increased risk of heterozygous children having the T allele inherited from the father ($R_P = 3.47$; CI: 1.32–9.11 and $R_2 = 1.98$; CI: 0.76–5.21). According to our data, epigenetic modulation of the paternal inherited allele could lead to disturbance of palatal fusion during embryogenesis resulting in greater susceptibility to CL/P in the offspring.

Genomic imprinting modulates gene expression by turning specific alleles of a gene on or off depending on which parent transmits them. Both, genetic and environmental factors can affect the imprinting process and alter the level of expression of imprinted genes (Jirtle et al. 2000).

In general, epigenetic effects are increasingly recognized as an important source of variation in complex traits. Principle among these effects is genomic imprinting, which has generally been examined in analyses of complex traits by testing for parent-of-origin-dependent effects of alleles. However, we acknowledge that in most of these analyses, maternal effects are confounded with genomic imprinting because they can produce the same patterns of phenotypic variation expected for various forms of imprinting (Hager et al. 2008).

In summary, our analysis suggests a role for *TGFB3* in the etiology of CL/P among the central European population if the risk allele is inherited from the father. Although these data suggest an intriguing role of *TGFB3* in the development of oral clefts, the hypothesis still requires a convincing degree of support from independent studies. Also, functional studies are warranted to show any effect of allelic variants on activation of Tgf-$\beta 3$ and to provide experimental evidence for *TGFB3* being subjected to genomic imprinting.

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