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Exploring Subclinical Phenotypic Features in Twin Pairs Discordant for Cleft Lip and Palate

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Abstract

Objective—Monozygotic twins of an individual with an orofacial cleft have a significantly elevated risk for orofacial cleft compared with the general population, but still the concordance rate for orofacial cleft in monozygotic twins is about 40% to 50%. The goal of this study was to determine whether unaffected cotwins have an increased frequency of orbicularis oris muscle defects, a subclinical form of orofacial cleft. The presence of such defects may reduce the overall rate of discordance.

Method—A total of 63 discordant monozygotic and dizygotic twin pairs, 262 unaffected nontwin siblings, and 543 controls with no history of orofacial clefts were assessed for orbicularis oris defects by high-resolution ultrasound. Frequencies were compared by the Fisher exact test.

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Results—Unaffected cotwins from discordant monozygotic pairs had a higher frequency of defects (12.5%) than the other test groups (6.38% to 6.99%), but the difference was not statistically significant ($P = .74$).

Conclusions—In this study, orbicularis oris defects were not statistically significantly more common among the unaffected twins from orofacial cleft discordant twin pairs. The trends in the results warrant future studies with larger sample sizes and additional subclinical phenotypes.

Keywords

discordant; orofacial cleft; subclinical phenotypes; twin pairs

Nonsyndromic orofacial clefts (OFCs) are common birth defects occurring in approximately 1 in 700 births worldwide. The OFCs have considerable phenotypic heterogeneity and are generally divided into three categories: cleft lip (CL), cleft palate (CP), and cleft lip with cleft palate (CLP). Within these categories there is a wide range of severity because clefts can range from microform defects of the upper lip resembling scars to complete bilateral clefts of the lip and palate. Recent evidence suggests that the phenotypic range of OFCs extends beyond these visible, overt phenotypes to include a range of subclinical features, which may be present in the “unaffected” relatives of individuals with OFCs. These phenotypes include subepithelial discontinuities of the orbicularis oris (OO) muscle, dental anomalies, face shape differences, and dermatoglyphic lip-print whorls (Weinberg et al., 2006).

A genetic basis of OFCs was first proposed by Fogh-Andersen (1942) from observations of increased frequency of clefting in relatives of patients with an OFC. In addition, the concordance rate of OFCs in monozygotic (MZ) twins (40% to 60%) is higher than the rate in dizygotic (DZ) twins (3% to 5%), suggesting a strong genetic etiology (Grosen et al., 2011). Although the incomplete concordance for OFCs in MZ twins is attributed to differential environmental exposures, there are other possible molecular mechanisms including somatic *de novo* mutations, chromosomal abnormalities, and skewed X-chromosome inactivation. These mechanisms have been explored in twin pairs discordant for OFCs without much success (Mansilla et al., 2005; Kimani et al., 2007; Kimani et al., 2009).

Recently, a Danish study (Grosen et al., 2010) of twin pairs discordant for OFCs reported that the recurrence risks for the offspring of unaffected cotwins was not significantly different from the recurrence risk for offspring of affected cotwins (2.3% versus 1.8%). Furthermore, the recurrence risks for both groups were significantly increased compared with the background risk of 0.18%. The effect was most notable in the unaffected cotwins of discordant MZ pairs, where the recurrence risk (7.7%) was significantly increased over the background risk. This suggests that both twins carry the susceptibility alleles for OFCs, providing a possible explanation for the largely negative results of genetic studies of discordant twin pairs. Although this does not rule out the possibility of differential environmental exposures or additional genetic risk factors in the affected twin or affected offspring, another possible explanation is that the unaffected cotwins are in fact “affected” but with an extremely subtle expression of the cleft phenotype. In other words, the

recurrence risk is the same among these unaffected cotwins because they share an affection status resulting from shared susceptibility alleles. In this study, we focused on discontinuities of the OO muscle, a subclinical phenotype that could be considered the mildest expression of a cleft lip (Martin et al., 1993; Neiswanger et al., 2007). A high frequency of OO defects has been reported in the clinically unaffected relatives of individuals with OFCs (Neiswanger et al., 2007). In this study, we hypothesized that OO discontinuities will be increased in the unaffected cotwins of discordant MZ twin pairs.

Methods

Subjects

The subjects for this study were part of the Pittsburgh Orofacial Cleft Study, a multinational research effort that collects extensive phenotype data and DNA from families with OFCs and from controls. Individuals with a clinical diagnosis of nonsyndromic cleft lip with or without cleft palate were recruited from cleft or surgical clinics in the United States (Pittsburgh, PA, St. Louis, MO, and Denver, CO), Hungary (Budapest), and Spain (Madrid). Families from Denmark were identified from the Danish Facial Cleft Database, which contains 10,025 individuals born with OFCs in Denmark between 1936 and 2005 (Grosen et al., 2010). Informed consent was obtained for all participants, and approval for all research work was obtained from the institutional review boards of participating institutions. We restricted our analysis to Europeans and North Americans who self-reported their race as white.

This study used four different analysis groups: (1) unaffected cotwins from discordant MZ twins (n = 16 pairs), (2) unaffected cotwins from discordant DZ twins (n = 47 pairs), (3) unaffected siblings of OFC probands (n = 262), and (4) controls with no family history of OFCs (n = 543). Because cleft lip and/or palate (CL/P) and CP can be observed within the same pedigree and due to our previous observations of OO discontinuities in 15% of isolated CP cases (Weinberg et al., 2008), unaffected cotwins and siblings of probands with all three subtypes of OFCs (i.e., CL, CLP, and CP) were included in this study. For each family, we assigned a family cleft group based on all affected family members. For example, if all affected family members had CL, they were assigned to “CL only”; a family with one individual with CL and one with CLP was assigned to the “CL + CLP” group. We compared the frequency of family cleft groups in each analysis group and found no significant difference ($P = .30$), indicating that cleft type would be unlikely to be a confounding factor in our analysis of OO muscle defects.

Subclinical Phenotyping of OO Muscle

High-resolution ultrasound of the upper lip was used to see the OO muscle, as previously described (Neiswanger et al., 2007). Videos of the ultrasound were rated independently by three raters trained to recognize discontinuities in the OO muscle. The ultrasounds were scored as *having no discontinuity* (unaffected), *having a clear discontinuity* (affected), or *unrateable*. All raters were blinded to the OFC affection status of the participants. Full details of the scoring and rating procedure have been described in greater detail previously (Neiswanger et al., 2007; Weinberg et al., 2008). The level of interrater agreement was

previously shown to be good (mean kappa = .64). In calibration studies conducted since 2007, interrater and intrarater agreements have been consistently good (kappa > .6).

Data Analysis

An overall affection status was derived from the ratings to indicate whether a participant had an OO defect. The rate of affection was tested for homogeneity across the four analysis groups. Fisher exact tests were used to test whether the rate of affectedness depended on the analysis groups under consideration. Analyses were conducted using R (version 3.0.2).

Results

The goal of this study was to determine whether the frequency of OO defects is increased in unaffected cotwins from discordant MZ twin pairs compared with DZ twins, nontwin siblings, and controls. We analyzed a total of 63 discordant MZ and DZ twin pairs, 262 unaffected nontwin siblings, and 543 controls with no history of OFCs (Table 1). The OO muscle defects were nearly twice as frequent in the unaffected MZ cotwins (12.5%) as in the other groups, which ranged from 6.38% to 6.99%. Overall, however, these differences were not statistically significant ($P = .74$). We performed pairwise comparisons between MZ cotwins and all other groups; these differences were also not significant ($P > .3$). When each study group was stratified according to the types of OFCs in the family, no clear trends arose; however, the sample sizes were very small.

Discussion

We studied 63 twin pairs discordant for OFCs who had also been assessed for OO muscle defects. We observed a trend toward higher frequencies of OO defects in unaffected MZ cotwins versus unaffected DZ cotwins, nontwin siblings, and controls, suggesting that this general approach may prove fruitful in larger samples. Small sample size is a limitation of this study, despite the fact that this is the largest group of discordant OFC twins studied to date. Here we focused on white twins pairs because subclinical phenotypes have been better characterized in white populations. Future studies should include twin pairs from diverse ethnic groups.

In our previous work demonstrating a high frequency of OO defects in unaffected relatives of individuals with OFCs, we focused on multiplex families (Neiswanger et al., 2007). In the current study, there was no difference in frequency of OO defects between the twins or siblings and controls. We were unable to stratify our sample of twin pairs into multiplex and simplex families because most of our twin pairs were from simplex families (12/16MZ pairs, 26/47 DZ pairs). However, in the unaffected nontwin siblings we observed OO defects in 4.7% of multiplex siblings (n = 127) and 8.9%of simplex families (n = 135). It is unclear why the frequency among siblings from multiplex families is lower than in our previous report; although, it may be due to population differences, cleft type differences, or that the present study includes only one unaffected family member.

Given that none of the studies investigating discordance in MZ pairs has identified a major underlying contributor, it is likely that there are multiple factors at work. We studied one

possible factor: unrecognized affection in the form of OO defects. By including this one subclinical phenotype, we reduced the number of discordant MZ pairs in our sample by 12.5%. Other subclinical phenotypes and traits currently associated with OFCs include velopharyngeal insufficiency, bifid uvula, dental anomalies, structural brain defects, and altered dermatoglyphic patterns or other minor physical traits (Weinberg et al., 2006). Inclusion of such additional phenotypes in future studies may allow additional discordant twin pairs to be reclassified as concordant.

Other factors contributing to the discordance in MZ twin pairs include postzygotic twinning mutations, environmental differences, and X-chromosome inactivation or imprinting. These possibilities have been previously explored, but none have produced positive results. Screening for mutations was previously performed in 15 candidate genes, which was a fairly comprehensive study at the time of publication (Mansilla et al., 2005). Given the advances in sequencing technology, a broad study using whole exome or whole genome sequencing in discordant twin pairs may identify causal mutations. Other complementary approaches, such as high-resolution array comparative genomic hybridization and genomewide methylation sequencing may also reveal causal genetic variants. However, the underlying assumption required for these approaches is that a genetic or epigenetic difference exists between the affected and unaffected cotwins of a discordant pair. We will illustrate some possible consequences of this assumption with a hypothetical example drawn from previous work demonstrating that rare variants in *BMP4* were associated with overt and microform CL and OO muscle defects (Suzuki et al., 2009). In this simplistic example, MZ twin A has CL and MZ twin B has an OO defect, and they share a *BMP4* mutation causing both phenotypes. False negatives could result by assuming the causal mutation cannot be shared because twin B is not overtly affected, thereby excluding the *BMP4* mutation. On the other hand, this same assumption could lead to falsely assigning causality to an unrelated *de novo* somatic mutation found in twin A.

Our study suggests that subtle, even subclinical, phenotypes are present in apparently unaffected MZ cotwins. We hypothesize this could explain the increased recurrence risks for offspring of these individuals. Although this should be explored in larger sample sizes and with additional phenotypes and populations, unappreciated affection statuses should be considered in studies of discordant twin pairs. In some families, inclusion of one or more subclinical phenotypes could clarify the nonpenetrance seen in complex traits and identify genetic modifiers of the clefting phenotype.

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TABLE 1

Counts of OO Muscle Subclinical Phenotypes Among Study Subjects With No Overt OFC*

Study Group	Family Cleft Group†	OO Muscle Defect	No Defect	Frequency	Pairwise Comparison With MZ‡
Monozygotic cotwins (n = 16)	All groups	2	14	12.50%	–
	CL only	0	4		
	CLP only	0	4		
	CP only	1	4		
	CP + CL/P	1	1		
	CL + CLP	0	1		
Dizygotic cotwins (n = 47)	All groups	3	44	6.38%	*P* = .59, OR = 2.06 (0.15 to 20.03)
	CL only	0	9		
	CLP only	1	14		
	CP only	1	8		
	CP + CL/P	1	5		
	CL + CLP	0	8		
Siblings (n = 262)	All groups	18	245	6.87%	*P* = .32, OR = 1.93 (0.19 to 9.49)
	CL only	4	38		
	CLP only	8	112		
	CP only	3	30		
	CP + CL/P	1	26		
	CL + CLP	2	38		
Controls (n = 543)	NA	38	505	6.99%	*P* = .32, OR = 1.89 (0.2 to 8.73)

*OO = orbicularis oris; OFC = orofacial cleft; MZ = monozygotic; OR = odds ratio.

†Family cleft group is based on the cleft types of all affected members of a family.

‡Odds ratios of OO muscle defect for MZ versus DZ, siblings, or controls.