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BRIEF REPORT

Novel Variants in Growth Differentiation Factor 9 in Mothers of Dizygotic Twins

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Context: Genes from the ovarian bone morphogenetic signaling pathway (GDF9 and BMP15) are critical for normal human fertility. We previously identified a deletion mutation in GDF9 in sisters with spontaneous dizygotic (DZ) twins, but the prevalence of rare GDF9 variants in twinning families is unknown.

Objective: The objective was to evaluate the frequency of rare variants in GDF9 in families with a history of DZ twinning.

Design and Subjects: We recruited 3450 individuals from 915 DZ twinning families (1693 mothers of twins) and 1512 controls of Caucasian origin. One mother of DZ twins was selected from 279 of the 915 families, and a DNA sample was screened for rare variants in GDF9 using denaturant HPLC. Variants were confirmed by DNA sequencing and genotyped in the entire sample by matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry.

Results: We found two novel insertion/deletions (c.392-393insT, c.1268-1269delAA) and four missense alterations in the GDF9 sequence in mothers of twins. Two of the missense variants (c.307C>T, p.Pro103Ser and c.362C>T, p.Thr121Leu) were located in the pro-region of GDF9 and two (c.1121C>T, p.Pro374Leu and c.1360C>T, p.Arg454Cys) in the mature protein region. For each variant, the frequencies were higher in cases compared with controls. The proportion of mothers of DZ twins carrying any variant (4.12%) was significantly higher ($P < 0.0001$) than the proportion of carriers in controls (2.29%).

Conclusion: We describe new variants in the GDF9 gene that are significantly more common in mothers of DZ twins than controls, suggesting that rare GDF9 variants contribute to the likelihood of DZ twinning. (***J Clin Endocrinol Metab* 91: 4713–4716, 2006**)

VARIATION IN DIZYGOTIC (DZ) twinning has a genetic component (1–3) with family studies showing significantly higher frequencies of DZ twins in female relatives of mothers of DZ twins compared with mothers of monozygotic (MZ) twins (2, 3). Women with a history of DZ twinning have an increased incidence of multiple follicle growth (4, 5), and in other mammals, members of the TGFβ superfamily, growth differentiation factor-9 (GDF9), and bone morphogenetic protein-15 (BMP15) play crucial roles in determining follicle growth and ovulation rate (6).

These genes are critical for human fertility. A dominant-negative mutation in BMP15 in Italian sisters resulted in ovarian dysgenesis (7), and rare mutations in both GDF9 and BMP15 contribute to premature ovarian failure (POF) (8–10). We previously reported a heterozygous loss-of-function mutation in the pro-region of GDF9 in two sisters with spontaneous DZ twins (11), and rare mutations in GDF9 may contribute to an increased frequency of twins in some families. We therefore conducted a screen for GDF9 variants in mothers of spontaneous DZ twins and assessed the frequency of the variants in DZ twinning families.

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Abbreviations: BMP15, Bone morphogenetic protein-15; DZ, dizygotic; GDF9, growth differentiation factor-9; MZ, monozygotic; POF, premature ovarian failure.

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Subjects and Methods

Study subjects were recruited from 915 families with a history of DZ twinning [755 families from Australia and New Zealand and 160 families from The Netherlands] with 3450 individuals available for genotyping. We recruited families with two or more sisters who gave birth to spontaneous DZ twins (12) and also families with a single case where at least one third-degree female relative had DZ twins. Samples were also obtained from parents of mothers of twins where available and from additional sibs. Mothers of DZ twins and their families were identified through records from our genetic epidemiology studies using twins and their families in Australia (2), through organizations for mothers of twins in Australia and New Zealand, and through appeals in the media in both countries. In The Netherlands, ascertainment was population-based through community records as part of a systematic recruitment to The Netherlands Twin Register (3, 13). Mothers were explicitly asked about fertility treatments, and all such cases were excluded.

Genetic investigation was extended to a population-based control group of 1512 Caucasian individuals (unselected for twinning history). Individuals were selected at random from the electoral role in Australia and enrolled in the study. Study protocols were reviewed and approved by the QIMR Human Research Ethics Committee and the Ethics Committee of the Vrije Universiteit Hospital. Participation was voluntary, and each participant gave written informed consent.

Genetic analysis

The entire coding sequence and intron-exon junctions of the GDF9 gene were analyzed in 279 mothers of DZ twins (one woman from 279

of our 915 sister pair families). Samples were screened by denaturant HPLC of PCR-amplified fragments on a Varian Helix System (Varian, Walnut Creek, CA). Variants were confirmed by DNA sequencing from new PCR products (Applied Biosystems, Foster City, CA).

Assays for all confirmed variants plus two missense mutations c.199A.C (p.Lys67Glu) and c.646G.A (p.Val216Mat) reported by Dixit *et al.* (9) were designed and genotyped in all study subjects using a compact matrix-assisted laser desorption ionization time of flight (MALDI-TOF) Mass Spectrometer (Sequenom Inc., San Diego). The potential functional significance of missense mutations was predicted using the Align-GVGD program (14) (available at http://agvgd.iarc.fr) with a multiple sequence alignment for mammalian GDF9 protein sequences. Frequency differences were tested by contingency χ^2 testing with χ^2 and asymptotic P values estimated, allowing for the family nature of the data as implemented in MENDEL 6.0 (15). To assess the significance of frequency differences between cases and controls, the likelihood for a family containing an individual who was sequenced was corrected by dividing by the likelihood of observing the variant in the discovery phase.

Results

We identified six new variants within the coding region of GDF9. One mutation (c.1268-1269delAA) resulted in a 2-bp deletion in exon 2 at position 1268. The deletion introduced a frame shift resulting in a premature stop codon 10 amino acids downstream of the deletion and truncation of the mature protein in the putative translation product. A second mutation (c.392-393insT) resulted in a single base insertion in exon 1 at position 393 in the pro-region. The insertion also introduced a frame shift resulting in a premature stop codon at amino acid position 143. We detected four missense alterations in the GDF9 sequence in mothers of twins. Two variants (c.307C>T, p.Pro103Ser and c.362C>T, p.Thr121Leu) were located in the pro-region of GDF9, and two variants (c.1121C>T, p.Pro374Leu and c.1360C>T, p.Arg454Cys) were located in the mature protein region.

We typed five of these variants (c.1268-1269delAA and the missense mutations) in 915 families with a history of DZ twinning including 1693 mothers of DZ twins with genotype data (Table 1). The c.1268-1269delAA deletion mutation was detected in two families and in each family was carried by both sisters with DZ twins. One individual carrying the mutation had three sets of DZ twins (Fig. 1). The allele frequencies of the five variants were also compared with frequencies in the control group. For each variant, the frequency in cases was greater than the frequency in controls (Table 1). The variants c.1268-1269delAA and p.Pro374Leu were not observed in control samples, and the difference between cases and controls was significant for c.1268-1269delAA, p.Pro103Ser, and p.Pro374Leu. Considered together, the frequency of any of the five variants in GDF9 in mothers of twins was twice the frequency in the controls ($P = 0.0001$). The difference was still significant ($P < 0.0026$) if only Australia and New Zealand families were compared with controls. The relative risk for carrying a GDF9 variant was 2.15 (95% confidence interval, 1.42–3.24). Because these variants were carried by 2.2% of controls, the attributable risk for twinning due to these five variants was 2.4% (95% confidence interval, 0.9–4.7). No mothers of twins in our sample carried the missense mutations in GDF9 reported in Indian POF patients.

Discussion

GDF9 is an oocyte-derived growth factor essential for follicle growth. We previously reported a heterozygous loss-of-function mutation in GDF9 in two sisters with spontaneous DZ twins (11). We have now identified six additional rare variants in GDF9 in mothers of DZ twins. After ascertainment correction, equivalent to the classic correction of removing the proband variants from the counts for cases before doing the comparison, the frequency of all novel variants was significantly higher in mothers of DZ twins compared with a population-based control group. Frequencies for three individual variants (c.1268-1269delAA, p.Pro103Ser, and p.Pro374Leu) were significantly higher in cases compared with controls.

We observed a low frequency of functional variants within our control group, but this might be expected. Functional variants in GDF9 are unlikely to cause twin ovulations at all cycles, and not all twin ovulations will result in twin pregnancies due to partial failure of such pregnancies ("vanishing twins") (16). The penetrance (defined as the chance of having twins when carrying a functional variant) is expected to be low, as suggested by observations of low concordance rates of female MZ twins giving birth to DZ twins (2). The controls comprised both sexes, and females were unselected with respect to reproductive history.

The significantly higher frequency of GDF9 variants in mothers of twins suggests that some variants may be functional. Two of the novel variants were insertion/deletion mutations that introduce stop codons immediately downstream, increasing the number of truncating mutations in GDF9 observed in sister pairs with spontaneous DZ twins to

TABLE 1. Number of heterozygous carriers (Het) and minor allele frequencies (Freq) of variants identified in GDF9 in 915 DZ twinning families (with 1693 mothers of DZ twins) and in 1512 control samples

GDF9 variant	Protein variant	Cases		Controls		*P* value
		Het[a]	Freq[b]	Het	Freq	
c.307C>T	Pro103Ser	30	0.0119	13	0.0048	0.02886
c.362C>T	Thr121Leu	4	0.0010	1	0.0004	0.20362
c.1121C>T	Pro374Leu	4	0.0019	0	0	0.00842
c.1268–1269delAA	Frame shift	4	0.0020	0	0	0.03574
c.1360C>T	Arg454Cys	22	0.0067	12	0.0044	0.12986
Any of the above			0.0229		0.0111	0.00015

P values were calculated for the likelihood ratio test (MENDEL binomial link measured genotype model), testing for effect of the variant on the likelihood of having twins under a multiplicative model.

[a] Numbers of mothers of twins carrying heterozygous GDF9 variants.

[b] Minor allele frequencies of GDF9 variants.

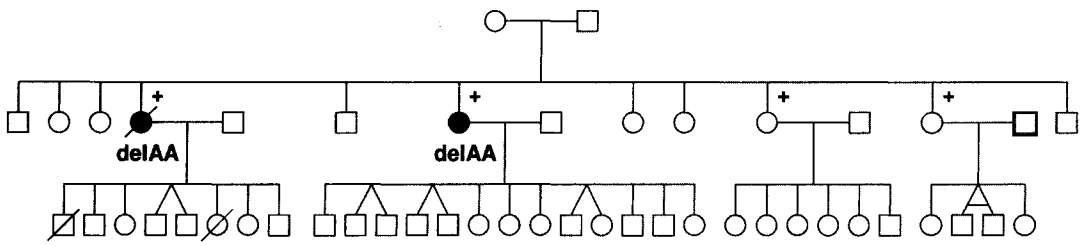

FIG. 1. Family of sisters with spontaneous DZ twins. Both sisters carry a heterozygous deletion mutation (c.1268-1269delAA) within the coding region of GDF9.

three (11). The c.1268-1269delAA heterozygous deletion was present in two pairs of sisters with DZ twins. One of the women had three sets of twins, supporting the view that the deletion mutation increases the frequency of twinning. The other women all had a single set of twins as well as other singleton pregnancies, consistent with the expectation that functional variants in genes influencing twinning will have low penetrance. Overall, the increase in twinning frequency is lower than the effect of loss-of-function mutations in GDF9 in sheep (17) and probably reflects species differences in ovulation rate and twinning frequency (6).

The four other novel variants detected were all missense mutations. GDF9, like other members of the TGFβ family, is synthesized as a preproprotein. After cleavage of the signal peptide, the GDF9 proprotein forms homodimers and heterodimers with BMP15. Naturally occurring variants in BMP15 and GDF9 affect normal processing of the proprotein dimers (18). Amino acid substitutions are more likely to influence protein function when differences in the biochemical properties are large and the amino acids are located at residues highly conserved between species. Prediction of the likely effects of the mutations (14) suggests that three of the variants (p.Pro103Ser, p.Pro374Leu, and p.Arg454Cys) are important amino acid substitutions at positions conserved in all mammalian GDF9 proteins and are expected to be deleterious. The proline at position 374 and the arginine at position 454 are also conserved in chicken and zebrafish sequences, providing further evidence that these substitutions are likely to have functional consequences. The p.Thr121Leu substitution is also predicted to be deleterious, but with weaker evidence because this amino acid position is not invariant in all mammalian species. Both changes affecting the proline residues were significantly higher in mothers of DZ twins, but not the p.Arg454Cys change.

The demonstration of rare alleles contributing to DZ twinning provides an example of rare variants contributing to a common phenotype. We have previously shown no association between common variation in GDF9 and DZ twinning (11). Recent papers describe rare variants in both GDF9 and BMP15 contributing to POF (7–10, 19) showing that rare variants in these two genes influence human fertility. The attributable risk for twinning associated with rare variants in GDF9 is small (2.4%), and it is estimated that several hundred similar variants would be required to explain the observed level of familial aggregation for DZ twinning. It is possible that rare variants from other genes in pathways regulating ovarian follicle growth and maturation (including BMP15) also contribute to variation in twinning.

The results also imply a more direct relationship between twinning and POF. Mothers of DZ twins reach menopause significantly earlier than mothers of MZ twins with the difference occurring before age 40 (20). The small increase in the frequency of POF in mothers of DZ twins could be explained by mutations in GDF9 and BMP15 influencing both aspects of ovarian function, although none of our mothers of twins carried the missense mutations reported in Indian POF patients (9). Future studies should examine whether the same variants in GDF9 and BMP15 affect both twinning and POF via common mechanisms or whether different variants affect these two phenotypes.

In conclusion, we identified six novel variants in GDF9 in mothers of DZ twins. Two of the variants were insertion/deletion mutations, with one mutation detected in a woman with three sets of twins. GDF9 gene variants were significantly more prevalent among mothers of DZ twins than controls. Additional studies will be required to determine the effect of these variants on GDF9 function.

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