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Dizygotic Twinning Is Not Linked to Variation at the α-Inhibin Locus on Human Chromosome 2*

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ABSTRACT

Natural multiple pregnancy in women leading to dizygotic (DZ) twins is familial and varies across racial groups, suggesting a genetic predisposition. Mothers of DZ twins have a higher incidence of spontaneous multiple ovulation and elevated FSH concentrations. FSH release is controlled by feedback of inhibin peptides from the ovary, and immunization against inhibin α-subunit results in an increased ovulation rate in animals. The inhibin α-subunit is therefore a candidate gene for mutations that may increase the frequency of DZ twinning. Restriction digests of a PCR product from exon 1 with the enzyme *Spe*I detects a C/T polymorphism at bp 128 with two alleles of 447 and 323/124 bp. The polymorphism was typed in 1125 individuals from 326 pedigrees with 717 mothers of spontaneous DZ twins. The α-inhibin locus mapped within 3 centimorgans of D2S164, and linkage with DZ twinning was excluded [decimal log odds ratio (LOD) score, −2.81 at $\theta = 0$]. There was complete exclusion of linkage (LOD, less than −2) of a gene conferring relative risk 1.8 (λs, >1.8) across the chromosome, except at the p-terminus region and a small peak (maximum LOD score, 0.6) in the region of D2S151-D2S326. Analysis using either recessive or dominant models excluded linkage with DZ twinning in this population (LOD score, less than −2.5) across chromosome 2. We conclude that dizygotic twinning is not linked to variation in the α-inhibin locus. The results also suggest that mutations in other candidates on chromosome 2, including the receptor for FSH and the β_B-inhibin subunit (INHBB) cannot be major contributors to risk for DZ twinning. (*J Clin Endocrinol Metab* **85:** 3391–3395, 2000)

NATURAL MULTIPLE pregnancy in women leading to dizygotic (DZ) twins is familial and varies across racial groups, suggesting a genetic predisposition. Female DZ twins and sisters of DZ twins have an increased frequency of twins compared with male DZ twins or brothers of DZ twins (1). Genetic effects on DZ twinning have been confirmed in subsequent investigations, but the estimated effects and modes of inheritance vary between studies (2–4). Analysis of families of 6596 twin pairs from the Australian Twin Registry found significantly higher frequencies of DZ twins in female relatives of DZ probands compared with MZ probands (4). The relative risks of DZ twin pregnancy for sisters of women with DZ twins and for female offspring of DZ probands were 1.7 and 2.5, respectively (4). Bulmer (2) postulated a recessive model of inheritance for DZ twinning, with a frequency of 0.5 and a penetrance of 5% in susceptible women, based on the data pooled from several studies. Segregation analysis of a Belgian/Dutch population-based study of twinning pedigrees suggests a dominant model of inheritance, with a gene frequency of 0.03 and a penetrance of 10% (3).

DZ twinning results from the release and fertilization of multiple oocytes, and mothers of DZ twins have a higher incidence of spontaneous multiple follicle growth and multiple ovulation (5, 6). Several studies report increased concentrations of FSH during the menstrual cycle in mothers of DZ twins (7–9). The higher FSH concentration appears to result from an increase in the number of spontaneous FSH pulses without concurrent LH pulses (10).

FSH release is controlled in part by feedback from inhibin peptides secreted from the ovary. Inhibin peptides are heterodimers of a common α-subunit and either β_A- or β_B-inhibin subunit, linked by disulfide bonds (11, 12). The two forms of inhibin (A and B, respectively) appear to have similar biological properties, but are secreted differently during the cycle (13). Inhibin B is secreted mainly during the early follicular phase, whereas inhibin A increases gradually to peak concentrations during the luteal phase. A related family of peptides, the activins, is formed from homo- and heterodimers of the β_A- or β_B-inhibin subunit (14, 15). Activin stimulates FSH secretion from cultured pituitary cells. However, most activin in human serum is bound to circulating proteins, particularly follistatin.

Passive and active immunizations against inhibin

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α-subunit in animals result in increased ovulation rate (16–19). Immunization against inhibin-α in ewes increases the ovulation rate and the concentration of FSH (17, 18); similar increases are observed in cattle (20). A rise in FSH concentration does not always accompany the increase in ovulation rate seen after immunization against inhibin-α, and the mechanism for increased ovulation rate could also involve changes in the concentrations of activins within the follicle (21).

Genes in the pathway controlling the synthesis and release of FSH may be candidates for increased twinning frequency. Mutations in the gene for inhibin α-subunit (INHA) that decrease the expression or activity of inhibin-α may increase the frequency of DZ twinning in women. The aim of these studies was to identify a polymorphism in the human INHA locus and analyze linkage to DZ twinning in women.

Subjects and Methods

Study subjects

The study protocol was reviewed and approved by the Bancroft Center research ethics committee established under guidelines from the Australian National Health and Medical Research Committee. Participation was voluntary, and each patient gave informed consent. Study subjects were pairs of sisters who had each given birth to spontaneous DZ twins. These were ascertained through records from our genetic epidemiology studies using twins and their families in Australia (4), through organizations for mothers of twins in Australia and New Zealand, and through appeals in the media in both countries. In Holland, ascertainment was population based through community records as part of a systematic recruitment to the Netherlands Twin Register (3, 22). Mothers were explicitly asked about fertility treatments, and all cases involving fertility treatment were excluded. Women were also excluded if the zygosity of the twins could not be confirmed based on a difference in sex or clear phenotypic differences in coloring or appearance. In some equivocal cases, zygosity was confirmed by genetic marker analysis of buccal or lymphocyte DNA.

Laboratory methods

Genomic DNA was extracted (23) from peripheral venous blood samples obtained from consenting family members. Primer sequences were designed to amplify exon 1 of the INHA locus (primer F, 5′-GGAAGACTGGATGAGAAGG-3′; primer R, 5′-GCTTTTTCT-CAAAGTCATCC-3′). PCR reactions were carried out in a 25-μL volume containing 1.5 mmol/L $MgCl_2$, 200 μmol/L deoxy-NTPs, and 25 pmol of each primer. PCR amplification was performed in a Selby thermocycler (Selby-Bioloab, Clayton, Australia). There was an initial denaturation step of 5 min at 95 C, followed by 35 cycles: 45-s denaturation at 95 C, 45-s annealing at 60 C, and 90-s extension at 72 C. The final extension step was carried out for 5 min at 72 C. Ten microliters of PCR product were digested with the restriction enzyme *Spe*I (New England Biolabs, Beverly, MA) at 37 C for 2 h. The products were separated on a 2% agarose gel, and bands were visualized on a UV transilluminator.

Genomic DNA was typed for 25 highly polymorphic fluorescent microsatellite markers across chromosome 2 (see Fig. 3), as described by Hall and Nanthakumar (24). In brief, individual DNA samples were arrayed in 96-well microtiter plates, and samples were amplified by PCR with primers for individual markers. The amplified products were pooled, heat denatured, and subjected to electrophoresis on denaturing polyacrylamide gels. Data were collected using ABI 377 automated DNA sequencers (Applied Biosystems, Inc., Foster City, CA), with data analysis performed using GENESCAN (version 2.1) and GENOTYPER (version 1.1.1) software. Allele analysis and allele calling were performed and genotype errors as a result of non-Mendelian segregation in pedigrees were detected and corrected as described in Hall and Nanthakumar (24).

Data analysis

The dataset comprised 416 sibships from 326 families for which genotype data were available for at least 1 marker. The number of families from Australia, New Zealand, and The Netherlands were 155, 59, and 112, respectively. Genotype data for the INHA locus were obtained from 717 mothers of DZ twins. There were 267 sibships with 2 sisters who had given birth to DZ twins and 17 sibships with 3 affected sisters. In addition, data were obtained from 132 sibships with a single individual drawn from families in which at least one third degree relative had given birth to a set of DZ twins. Among the sibships there were 106 and 105, respectively, with 1 or both parents typed for the INHA locus. Genotype data for the 25 microsatellite markers were obtained from 383 mothers of DZ twins, 166 sister pairs, and 17 sets in which 3 sisters had DZ twins.

Two-point decimal log odds ratio (LOD) scores and multipoint analysis for linkage between INHA and microsatellite markers from chromosome 2 were calculated using the VITESSE program (25). Linkage between INHA and DZ twinning was tested using VITESSE, and multipoint analysis was carried out with GENEHUNTER (26). Genetic models tested for linkage to DZ twinning were a recessive model with a gene frequency of 0.5 and a penetrance of 5% (2) and a dominant model with a gene frequency of 0.04 and a penetrance of 10% (3). Allele sharing and linkage mapping with affected sister pairs were analyzed using the ASPEX package (27).

Results

Two polymorphisms were identified within the coding region of the INHA gene. Both polymorphisms were C to T transitions and were located at position 129 in exon 1 and position 675 in exon 2. PCR products for exons 1 and 2 were amplified and sequenced from samples of 21 individuals. Within this group, all samples with the single base change at position 675 in exon 2 also contained the single base change at position 129 in exon 1. PCR for exon 1 generates a product of 447 bp. The C to T transition at position 129 in the exon 1 fragment generates a restriction enzyme site for the enzyme *Spe*I. Chromosomes with a C at position 129 remain uncut, with a single band of 447 bp. Chromosomes with a T at position 129 are cut with *Spe*I and produce two bands of 323 and 124 bp (Fig. 1).

The polymorphism was typed in 1125 individuals from 326 pedigrees. Allele frequencies for the 447- and 323/124-bp alleles were 0.802 and 0.198, respectively, and the heterozygosity was 0.318. The allele frequencies in Australian and New Zealand families were similar to frequencies in the Dutch families.

The location of INHA was determined by linkage mapping. Based on the two-point LOD scores, INHA was closely linked to D2S164 [maximum LOD score, 6.03 at a recombination fraction (θ) of 0.01], and preliminary analysis placed INHA between markers D2S325 and D2S126. Multipoint mapping with markers D2S117, D2S325, D2S164, and D2S126 confirmed the location of INHA close to D2S164 (Fig. 2). The maximum LOD score was 8.93, and the one LOD support interval placed INHA within 3 centimorgans (cM) of D2S164.

Linkage between the INHA locus and DZ twinning was excluded in this population (LOD score, -2.81 at $\theta = 0$). Multipoint analysis excluded linkage in the region of marker D2S164 (Fig. 3), the region containing the INHA locus. Using the affected sibpair approach, there was complete exclusion of linkage (LOD, less than -2) of a gene conferring relative risk of 1.8 (λs, >1.8) across the chromosome, except at the p-terminus region and a small peak (maximum LOD score, 0.6) in the region of D2S151-D2S326 (Fig. 3). Under genetic

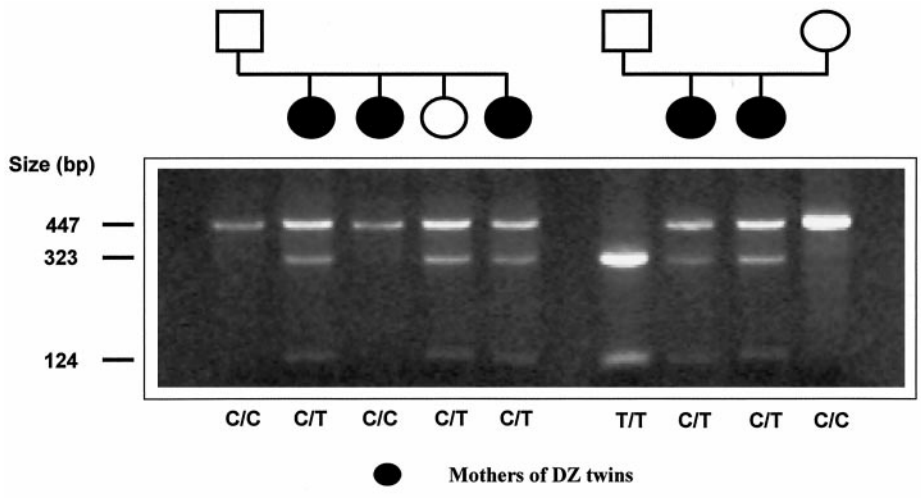

FIG. 1. Segregation of a PCR restriction fragment length polymorphism at the INHA locus. The C to T transition at position 129 in exon 1 generates a restriction enzyme site for the enzyme *Spe*I. Chromosomes with a C at position 129 remain uncut, with a single band of 447 bp. Chromosomes with a T at position 129 are cut with *Spe*I and produce two bands of 323 and 124 bp.

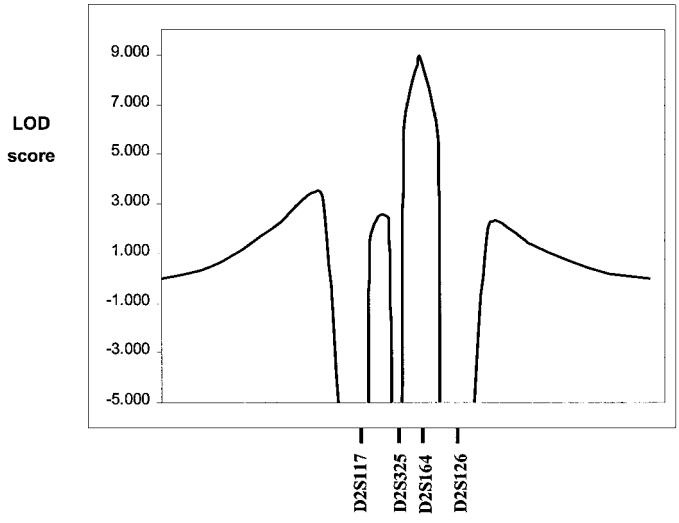

FIG. 2. Multipoint mapping of the INHA polymorphism with markers D2S117, D2S325, D2S164, and D2S126 from chromosome 2.

models of recessive (2) or dominant (3) inheritance of a gene for DZ twinning, there was complete exclusion of linkage (LOD, less than −2.5) across chromosome 2.

Discussion

The number of follicles that ovulate during reproductive cycles is characteristic for each mammalian species. The dominant follicle(s) that subsequently ovulates is selected at a time of rising concentrations of FSH around luteal regression (28, 29). Current views suggest that concentrations of FSH exceeding some threshold around the time of follicle selection lead to multiple follicle growth and multiple ovulation (28–30). Strong evidence for major genes influencing the ovulation rate and multiple birth comes from animal models (31–35). These models also suggest that intraovarian factors play a role in control of the ovulation rate (36, 37).

The α-subunit of inhibin has important roles in the control of ovarian function through feedback mechanisms on pituitary FSH release and through the effects of an altered balance between activins and inhibin in intraovarian regulation (15, 38). Immunization against inhibin α-subunit in animals results in an increased ovulation rate (16–19). We identified 2 polymorphisms in the coding region of INHA. The C to T transitions (positions 129 and 675) were in complete linkage disequilibrium in the 21 mothers of DZ twins analyzed. The 2 variants were silent mutations that did not alter the protein sequence for inhibin-α. The C to T transition at position 129 generates a *Spe*I restriction polymorphism and was relatively common in our families. Physical mapping data place the INHA locus at chromosome 2q33-q34 (39, 40). In the present study, linkage analysis with chromosome 2 markers located INHA close to D2S164 between markers D2S325 and D2S126.

Linkage and association studies provide a valuable approach to understanding the contributions of individual genes to variation in twinning frequency. Results from the present study exclude linkage between the INHA locus and DZ twinning in our families. A LOD score of −2 is considered significant evidence against linkage [odds of 100:1 against (41)]. LOD scores of less than −2 were observed for linkage between DZ twinning and the INHA locus for all genetic models tested in our families. The exclusion with the INHA locus was supported from multipoint linkage analysis with markers from distal end of chromosome 2 including D2S164, which maps close to INHA. Although our studies exclude a direct role for mutations in the INHA locus influencing variation in DZ twinning, genetic variation in DZ twinning could act through pathways that include the inhibin-α protein. For example, mutations in genes located elsewhere in the genome could influence the synthesis or stability of inhibin-α (messenger ribonucleic acid or protein), alter relative concentrations of inhibins and activins, or modify physiological actions of inhibin proteins.

The 25 microsatellite markers provided adequate coverage across chromosome 2, with an average distance between markers of 9.4 cM and the largest gap being 20.1 cM. Multipoint analysis excluded linkage to a twinning gene conferring a relative risk of 1.8 across the whole chromosome except for the p-terminus and a small peak in the region of 2q24 (D2S151-D2S326). Across the chromosome, there was complete exclusion of linkage to DZ twinning at odds of 300:1 under genetic models of recessive or dominant inheritance. Two additional candidate genes on chromosome 2 that could

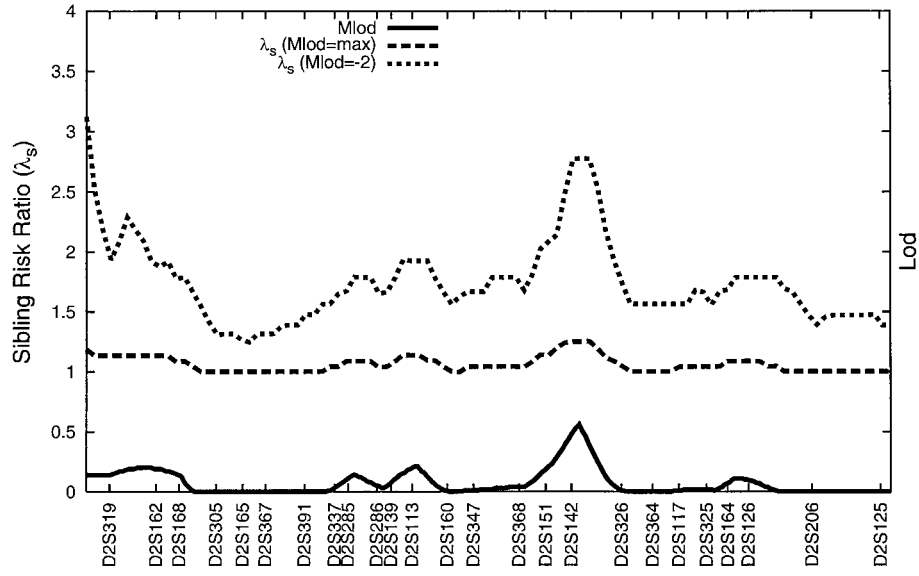

FIG. 3. Multipoint analysis of DZ twinning with markers on chromosome 2.

influence multiple ovulation and twinning frequency are the receptor for FSH (FSHR) and the β_B-inhibin subunit (INHBB). FSHR is located at 2p21-p16 (42, 43), and INHBB is located at 2cen-q13 (39). The locations of both loci lie outside the small peak between D2S142 and D2S326.

A linkage study of this sort cannot exclude mutations with small effects, but does exclude mutations of moderate effect present in the majority of our families. Gain of function mutations in the human LH receptor gene have been found to be the basis of familial male-limited precocious puberty (44). Gain of function mutations in the FSH receptor gene, like the loss of function mutants in the inhibin subunit genes, could be associated with increased incidence of dizygotic twinning. However, exon 10 of the FSH receptor gene was sequenced in 21 mothers of DZ twins studied here, including individuals from families in which DZ twins were born to three sisters. No sequence differences in the transmembrane region of the FSH receptor gene were found (Kudo. M., and A. Hsueh, unpublished observations). Variation in twinning frequency could still act through pathways of FSH action.

We conclude that dizygotic twinning is not linked to variation in the α-inhibin locus and that it is unlikely that there are genes with a large effect on twinning present on chromosome 2.

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