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Human Twinning Is Not Linked to the Region of Chromosome 4 Syntenic With the Sheep Twinning Gene *FecB*

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The tendency to dizygotic (DZ) twinning is inherited in both humans and sheep, and a fecundity gene in sheep (*FecB*) maps to sheep chromosome 6, syntenic with human 4q21-25. Our aim was to see whether a gene predisposing to human DZ twinning mapped to this region. DNA was collected from 169 pairs and 17 sets of 3 sisters (trios) from Australia and New Zealand who had each had spontaneous DZ twins, mostly before the age of 35, and from a replication sample of 111 families (92 affected sister pairs) from The Netherlands. Exclusion mapping was carried out after typing 26 markers on chromosome 4, of which 8 spanned the region likely to contain the human homologue of the sheep *FecB* gene. We used nonparametric affected sib pair methods for linkage analysis [ASPEX 2.2, Hinds and Risch, 1999]. Complete exclusion of linkage (lod < -2) of a gene conferring a relative risk for sibs as low as 1.5 ($\lambda_s > 1.5$) was obtained for all but the p terminus region on chromosome 4. Exclusion in the syntenic region was stronger, down to $\lambda_s = 1.3$. We concluded that if there is a gene influencing DZ twinning on chromosome 4, its effect must be minor.

KEY WORDS: dizygotic twinning; genetic linkage; Booroola sheep

INTRODUCTION

Natural pregnancy in women leading to dizygotic (DZ) twins clusters within individuals and in families and is under genetic control. Bulmer [1970] estimated that the probability of a subsequent twin pregnancy was increased fourfold in mothers of twins. Mothers and daughters of women who have had DZ twins are 1.8 times more likely than average to have DZ twins themselves, and sisters of women who have had DZ twins are 2.6 times more likely to have DZ twins [Bulmer, 1970]. An analysis of data on twin relatives of 6,596 proband twin pairs in the Australian Twin Registry found similar relative risks [Lewis et al., 1996]. From either set of figures, the combined risk to first degree female relatives is in excess of 2 [comparable with breast cancer; Claus et al., 1991]. Using the relative risks quoted above, Bulmer [1970] postulated a recessive mode of inheritance with a gene frequency of 0.5 and a penetrance (by mother) of approximately 0.05. However, recent segregation analysis of a Belgian/Dutch population based study of twinning pedigrees suggests an alternative model [Meulemans et al., 1996]. The inheritance of DZ twinning was modeled in the maternal ancestors of 1,422 DZ probands. A dominant model was favored, with gene frequency 0.03 and penetrance 10%. Sporadic and polygenic models were also rejected.

Strong evidence for major genes influencing ovulation rate and multiple birth comes from several animal models. A locus on the sheep X chromosome (*FecXI*) increases the frequency of multiple ovulation in heterozygous female carriers [MIA number 000386; Davis et al., 1991]. Homozygous female carriers of the *FecXI* locus are infertile, with streak ovaries severely depleted of follicles with more than a single layer of

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granulosa cells [Davis et al., 1992]. A locus with codominant inheritance for increased multiple ovulation in Booroola Merino sheep (*FecB*) maps to sheep chromosome 6 [MIM number 134720; MIA number 000383; Montgomery et al., 1993, 1994]. Several studies provide evidence for a gene affecting ovulation rate on pig chromosome 8 [Rathje et al., 1997; Rohrer, 1999; Wilkie et al., 1999], a region syntenic with sheep chromosome 6. The gene responsible may be the porcine homologue of the *FecB* locus, but gene order is not conserved between pig chromosome 8 and sheep chromosome 6 [Johansson et al., 1992; Ellegren et al., 1993; Lord et al., 1996]. Further comparative mapping is required to define the region of synteny.

The *FecB* locus is an important candidate for a DZT gene in humans. In sheep, the *FecB* locus maps to chromosome 6 between the alcohol dehydrogenase cluster and secreted phosphoprotein 1 [SPP1; Montgomery et al., 1993; Lord et al., 1996; Lumsden et al., 1999]. Comparative mapping has established that human chromosome 4 from 4p16 to 4q26 is syntenic with sheep chromosome 6 [Lord et al., 1996], although gene order is not conserved. On sheep chromosome 6, the region from platelet derived growth factor receptor, alpha polypeptide (PDGFRA), to bone morphogenetic protein 3 (BMP3) appears to be inverted with respect to SPP1 on human chromosome 4 [Lord et al., 1996]. Dentin-specific acidic phosphoprotein (DMP1) has recently been mapped in sheep and is located approximately 70 cM distal to SPP1 [Lumsden et al., 1999]. In contrast, *DMP1* maps close to *SPP1* in the human, within the same yeast artificial chromosome approximately 470 kb from *SPP1* [Aplin et al., 1995]. The location of DMP1 in the two species defines the inversion breakpoint close to SPP1 and just outside the critical region for the *FecB* locus [Lumsden et al., 1999]. Comparing maps from the human and the sheep, the region most likely to contain the human homologue for the *FecB* locus is a 16 cM region from D4S231 to D4S411 (Fig. 1).

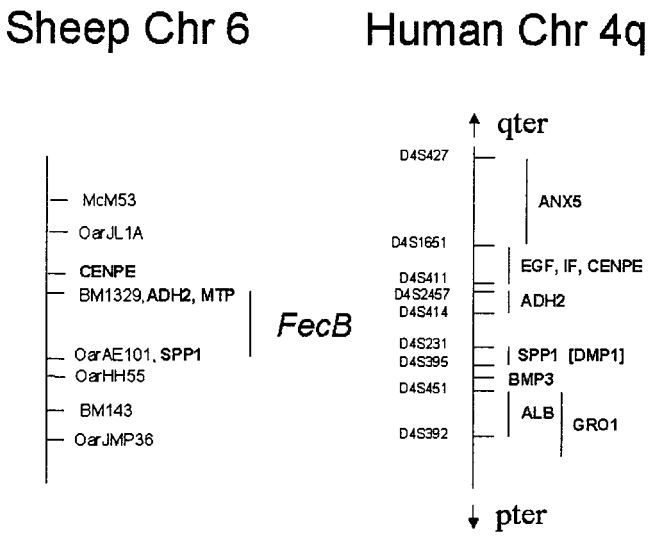

Fig. 1. A comparison of the genetic maps for sheep chromosome 6 and human chromosome 4 in the region of the sheep fecundity gene *FecB*. The map for sheep chromosome 6 is redrawn from published data [Lord et al., 1996; Lumsden et al., 1999] and data from the sheep mapping database (http://www.ri.bbsrc.ac.uk/cgi-bin/arkdb/browsers/browser.sh?species=sheep). The linkage map for human chromosome 4 is inverted and drawn from the CHLC chromosome 4 version 3.0 sex averaged recombination minimization map (http://lpg.nci.nih.gov/CHLC/). Gene locations were taken from the Genetic Location Database (http://cedar.genetics.soton.ac.uk/public_html/ldb.html). There is an inversion of gene order between the species with the breakpoint between SPP1 and DMP1 in sheep just outside the critical region containing the *FecB* locus [Lumsden et al., 1999].

DZ twinning presents difficulties for traditional linkage analysis in extended pedigrees because the penetrance is low and gene frequencies are unknown. Males and nulliparous females cannot be scored for the trait. Multiparous females may fail to express the trait because multiple ovulation only occurs in some cycles, or because of partial failure of multiple pregnancy. In such circumstances, the affected relative pair method is particularly attractive since it makes no assumptions about mode of inheritance, merely expecting that affected relatives will share linked marker loci more often than Mendelian expectation would allow. The aims of the present study were to genotype markers from chromosome 4 and analyze linkage to DZ twinning in families with affected sister pairs.

MATERIALS AND METHODS

Subjects

Study subjects were pairs of sisters who had each given birth to spontaneous DZ twins. These were ascertained through records from our genetic epidemiological studies using twins and their families in Australia [Lewis et al., 1996], through organizations for mothers of twins in Australia and New Zealand (ANZ), and through appeals in the media in both countries. In Holland (NL), ascertainment was population based through community records as part of a systematic recruitment to the Netherlands Twin Register [Meulemans et al., 1996]. 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 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.

Individuals were recruited from 165 pairs and 17 sets of 3 sisters (trios) from ANZ who had each had spontaneous DZ twins, mostly before the age of 35, and from a replication sample of 110 families (92 affected sister pairs) from The Netherlands. Blood samples were obtained from all subjects and from available parents or unaffected sibs for future association analyses.

Genotyping

Genomic DNA was extracted [Miller et al., 1988] from peripheral venous blood samples obtained from consenting family members. DNA samples were genotyped for 18 highly polymorphic fluorescent microsatellite markers across chromosome 4 (see Fig. 2), as described by Hall and Nanthakumar [1997]. In brief, individual

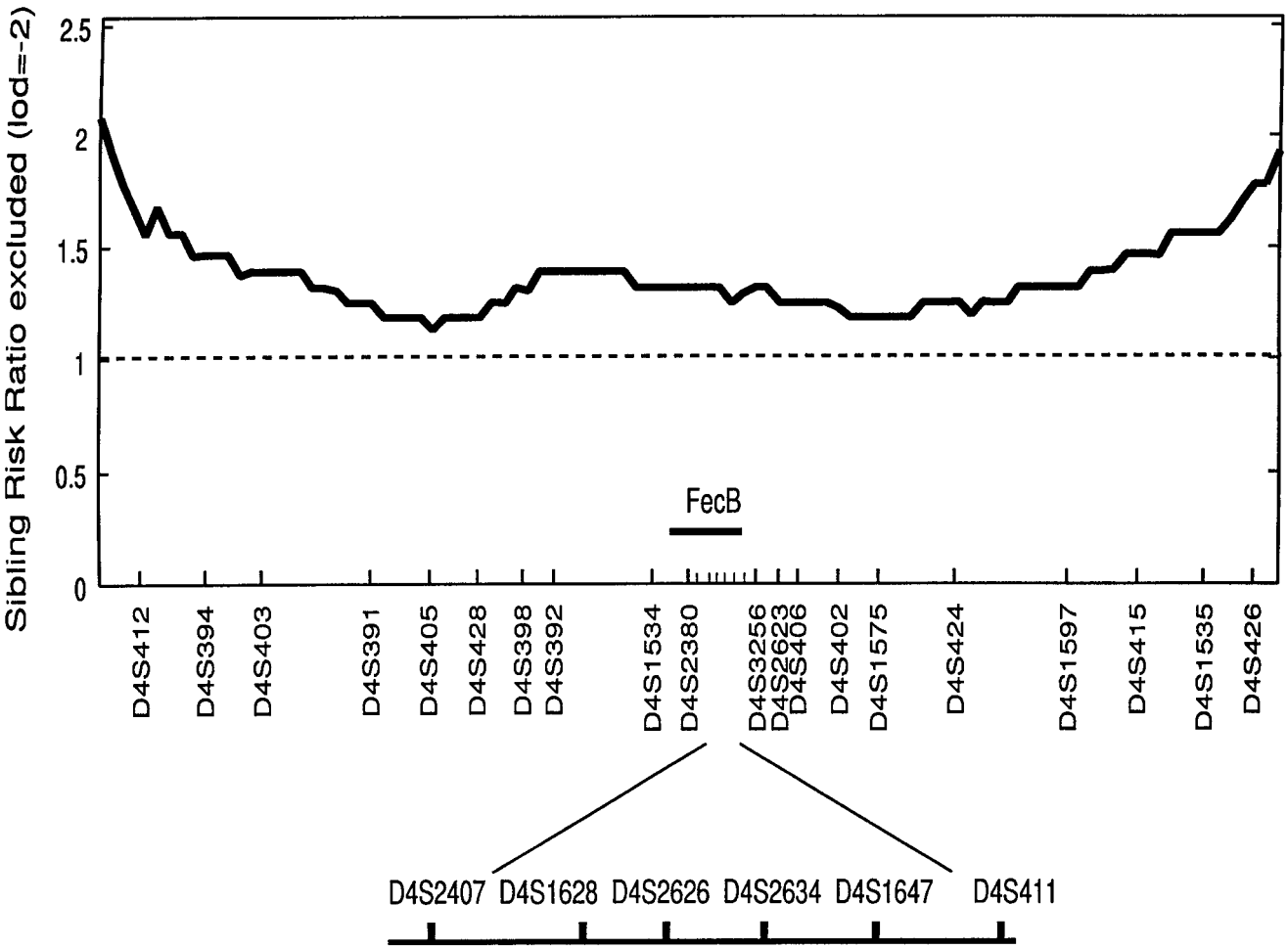

Fig. 2. Multipoint exclusion map of sibling risk ratio (λ_s) of DZ twinning due to loci on chromosome 4.

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 electophoresis on denaturing polyacrylamide gels. Data were collected using ABI 377 automated DNA sequencers, 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 previously [Hall and Nanthakumar, 1997].

Additional microsatellite markers were identified in the critical region of chromosome 4 and 8 markers (D4S2380, D4S2407, D4S1628, D4S2626, D4S2634, D4S1647, D4S411, and D4S3256) were typed in a larger set of pedigrees. DNA 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 was collected using ABI 373 automated DNA sequencer, 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.

Statistical Methods of Linkage Analysis

The dataset comprised 421 sibships from 292 families where genotype data were available for at least one marker. Genotype data for the 18 microsatellite markers were obtained from 165 sister pairs and 17 sets where three sisters had DZ twins. Both nonparametric and parametric linkage analyses were performed. The nonparametric multipoint affected sib pair linkage analysis was carried out using the program SIB_PHASE from the ASPEX 2.2 suite of programs [Hinds and Risch, 1999]. We present both the maximum lod score results, and an exclusion map expressed as the sibling relative risk (λ_s) associated with a lod score of -2 under an additive model for the ibd probabilities. The multipoint parametric lod scores were calculated using Genehunter 2.0r2 beta [Kruglyak et al., 1996; Kruglyak and Lander, 1998] under the two segregation models described earlier. As a final check, a multiallelic transmission-disequilibrium test

using the program Sib-pair [Duffy, 1999] was also performed for each marker through the critical region.

RESULTS

There was no evidence for linkage to a gene affecting DZ twinning in the likely region for the human homologue of the sheep fecundity locus *FecB*. Multipoint analysis with 26 microsatellite markers from chromosome 4 excluded linkage to a locus with a relative risk of 1.5 ($\lambda_s > 1.5$) across the chromosome except at the p terminus region (Fig. 2). Eight markers were typed in the critical region expected to contain the human homologue of the *FecB* locus in sheep (D4S1534–D4S3256). There was complete exclusion of linkage (lod < -2) of a gene conferring relative risk 1.3 ($\lambda_s > 1.3$) in this region (Fig. 2). The lod scores in the critical region with the two models never exceeded -4.0 (data not shown).

DISCUSSION

The human homologue of the *FecB* locus in sheep is a strong candidate for a DZ twinning gene in humans. In sheep, the *FecB* locus is autosomal with codominant expression, leading to increased multiple ovulation and litter size. The phenotype of carriers of the *FecB* locus has similarities to physiological changes reported in mothers of DZ twins.

The number of follicles that ovulate during reproductive cycles is characteristic for each mammalian species. Selection of the dominant follicle that will subsequently ovulate occurs at the time of luteal regression and is related to rising concentrations of follicle-stimulating hormone (FSH) around this time [Baird, 1983; Campbell et al., 1995]. Concentrations of FSH that exceed a threshold concentration for an extended time during the period of follicle selection can lead to growth of multiple follicles [Baird, 1983; Campbell et al., 1995]. Ewes carrying the *FecB* mutation have striking differences in the size and number of ovulatory follicles compared with the background breed [McNatty and Henderson, 1987; Montgomery et al., 1992]. Extensive studies by McNatty and colleagues have demonstrated small but consistent increases in FSH concentrations in homozygous carriers of the *FecB* mutation compared with non-carriers (++) ewes [McNatty and Henderson, 1987; Montgomery et al., 1992]. FSH concentrations in heterozygous carrier (B+) ewes are intermediate. The increase in FSH concentrations has not been recorded in all studies [Baird and Campbell, 1998] and variable results in different studies may be related to study design and the genetic background of the animals. Alternatively, the *FecB* gene product may act directly in the ovary, leading to secondary changes in gonadotrophin concentrations.

Growth and ovulation of multiple follicles has been shown to be more frequent in mothers of DZ twins [Martin et al., 1991b; Gilfillan et al., 1996]. In ovarian ultrasound scans taken over a number of cycles in 21 mothers of DZ twins and 18 controls, multiple large follicles were significantly more frequent in mothers of DZ twins. Multiple large follicles were observed in one individual in 7 out of 10 cycles in which she was scanned [Gilfillan et al., 1996]. Increased concentrations of FSH have been reported in mothers of DZ twins [Nylander, 1973; Martin et al., 1991a; Martin et al., 1991b]. In the case of hereditary DZ twinning, it appears that elevated FSH concentrations are associated with the number of FSH pulses in the early follicular phase of the menstrual cycle [Lambalk et al., 1998]. However, similar to studies in sheep carrying the *FecB* mutation, significant increases in FSH concentrations have not been recorded in all studies [Gilfillan et al., 1996].

The results from the present linkage study on chromosome 4 exclude the human homologue of the *FecB* locus from a major role in the genetic contribution to DZ twinning in women. The critical region for the FecB locus covers a 10 cM region from the ADH gene cluster to SPP1 [Lord et al., 1998]. This region lies outside an inversion breakpoint between sheep chromosome 6 and human chromosome 4, and the order of genes within the critical region appears to be conserved between the human and the sheep [Lumsden et al., 1999]. Comparative mapping (Fig. 2) demonstrates that the critical region spans a region on human chromosome 4q including 7 markers (D4S2380 to D4S411) typed in our families. If mutations in the human homologue of the FecB locus are present in the human population, the effect of any mutations on DZ twinning must be small or contribute only to a small number of families.

Multipoint analysis with 26 markers across chromosome 4 excluded linkage to a twinning gene, conferring a relative risk of 1.5 across the whole chromosome except for the telomeric regions. One additional candidate gene on chromosome 4 that could influence multiple ovulation and twinning frequency is the receptor for gonadotrophin releasing hormone receptor *(GnRHR)*. The ligand for GnRHR (gonadotrophin releasing hormone) is released from the hypothalamus in discrete pulses and transported to the pituitary gland via the hypothalamic-hypophyseal portal system where it acts through the receptor to stimulate the synthesis and release of FSH and luteinizing hormone (LH). Variations in FSH concentration may play a major role in the mechanism of DZ twinning [Baird and Campbell, 1998; Lambalk et al., 1998], and genes controlling FSH synthesis and release are candidates for a DZ twinning locus. *GnRHR* is located at 4p21.2 [Kottler et al., 1995] and was not linked to DZ twinning in the present study. In other work [Montgomery et al., 2000], we have excluded plausible candidate loci acting in this pathway found on chromosome 2, notably the FSH receptor (FSHR), α-inhibin (INHA) and the β_B-inhibit subunit (INHAB).

We conclude that if there is a gene influencing DZ twinning on chromosome 4, its effect must be minor.

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