

# Reproductive hormone genes in mothers of spontaneous dizygotic twins: an association study

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**Abstract.** There are important genetic influences on the tendency to dizygotic (DZ) twinning and it is a plausible hypothesis that these reside in one or more of the genes coding for the major reproductive hormones. We used Southern analysis of DNA from 50 young (<32) mothers of DZ twins, who also had a family history of DZ twinning, and 50 controls, to examine allele frequencies of five restriction fragment length polymorphisms (RFLPs) in four hormone genes coding for follicle stimulating hormone  $\beta$  (FSH $\beta$ ), chorionic gonadotropin  $\beta$  (CG $\beta$ ), inhibin  $\beta_B$  and gonadotropin releasing hormone (GnRH). Comparison of allele frequencies revealed no significant differences between DZ twin mothers and controls. However this does not rule out the role of these genes in the hereditary tendency of multiple ovulation in humans, since absence of linkage disequilibrium does not imply absence of linkage.

## Introduction

There appear to be important genetic influences on the tendency to dizygotic (DZ) twinning. DZ twins often seem to run in families although the pattern of inheritance is far from clear (Parisi et al. 1983; Philippe 1985). Recurrence risks predict correlations in liability to DZ twinning of approximately 0.3 for mothers and daughters and 0.5 for sisters, which are consistent with a high heritability and a large amount of nonadditive genetic variance (Bulmer 1970).

Striking racial differences exist in DZ twinning rates: in Europeans, DZ twins are born in about 8/1,000 confinements but in Africans the rate is doubled and in Orientals it is less than half. There are also maternal effects, since DZ twinning increases with maternal age and parity (Bulmer 1970).

The tendency to DZ twinning in humans may result from alterations in the copy number or structure of one of the genes involved in ovulation. The recruitment, growth, ovulation and luteinisation of ovarian follicles are coordinated by

regulatory signals including steroid and peptide hormones from the brain, anterior pituitary, adrenals and ovaries. The two primary regulators of ovarian function are the pituitary gonadotropins, follicle stimulating hormone (FSH) and luteinising hormone (LH). FSH and LH are heterodimers and share the same  $\alpha$  subunit [as also do chorionic gonadotropin (CG) and thyroid stimulating hormone (TSH)] but have different  $\beta$  subunits. They are usually secreted in tandem under the influence of the hypothalamic decapeptide, gonadotropin releasing hormone (GnRH, also known as LHRH) (Hall et al. 1990; Mercer 1990). FSH release is also modified by secretions from the granulosa cells of the ovary. These secretions include inhibin, a heterodimer comprising an  $\alpha$  chain and either a  $\beta_A$  or  $\beta_B$  chain, activin, a dimer of two  $\beta$  chains (de Kretser 1990), and follistatin, which consists of two glycosylated single chain proteins unrelated to the inhibins (Shimasaki et al. 1988), although binding to both activin and inhibin by the common  $\beta$ -subunit (Shimonaka et al. 1991).

Martin et al. (1984, 1991) have shown that mothers of DZ twins have higher early follicular levels of LH, FSH and inhibin than controls. We therefore focussed initially on the structural genes for these hormones, and also other genes involved in the bioregulation of FSH and LH levels. Southern analysis was used to compare allele frequencies for five different hormone gene polymorphisms in mothers of DZ twins and controls.

## Materials and methods

### Subjects

Mothers of DZ twins (MODZTs) were recruited from the Australian Multiple Birth Association and the Australian National Health and Medical Research Council Twin Registry. Because many DZ twins appear to be a 'sporadic' occurrence, being born to mothers with no previous family history, and because the incidence of DZ twinning rises sharply with maternal age and parity, peaking at about age 37 (Bulmer 1970), it is important to select MODZTs who are more likely to be genetically predisposed than 'phenocopies'.

Forty-nine of the mothers were between 22 and 31 years (average 26.7 years) when the twins were conceived spontaneously. The remaining mother had her first pregnancy at 32.5 years but then produced four

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**Table 1.** Genotype and allelic frequencies for polymorphic hormone genes in mothers of dizygotic twins (MODZT) and controls. \* Fischer's exact test (2 tail)

Polymorphism	No. scored	1,1	1,2	2,2	Frequency allele 1	Frequency allele 2	P*
Inhibin $\beta$ B- <i>Bam</i> HI RFLP	MODZT (50)	2	3	45	0.07	0.93	0.33
A1 4.4-kb	Controls (50)	0	3	47	0.03	0.97	
A2 3.4-kb							
FSH $\beta$ chain- <i>Hind</i> III RFLP	MODZT (50)	16	26	8	0.58	0.42	0.56
A1 15-kb	Controls (50)	21	21	8	0.63	0.37	
A2 10-kb							
FSH $\beta$ chain- <i>Msp</i> I RFLP	MODZT (50)	9	32	9	0.50	0.50	0.57
B1 10.6-kb	Controls (50)	11	33	6	0.55	0.45	
B2 1.8-kb							
Gonadotropin releasing hormone			1,2 or 2,2		*	*	1.0
<i>Nco</i> I RFLP	MODZT (50)	45	5				
A1 5.5-kb absent	Controls (50)	45	5				
A2 5.5-kb present							
Chorionic gonadotropin $\beta$ chain- <i>Pst</i> I RFLP	MODZT (49)	14	35		*	*	0.64
A1 3.8-kb absent	Controls (49) <sup>b</sup>	11	38				
A2 3.8-kb present							

\* The restriction fragment length polymorphism (RFLP) demonstrates presence or absence of a band, hence allele frequencies cannot be calculated

<sup>b</sup> Insufficient DNA to type all individuals reliably

sets of DZ twins; her mother, sister, niece and daughter also had DZ twins. All MODZTs had at least one female blood relative (up to 3rd degree) who also had DZ twins. The control group was unselected for twinning status, with the exception of ten mothers of MZ twins. All members of the sample were unrelated and of Caucasian origin.

### Zygosity determination

In cases where the zygosity of the twins was questionable DNA fingerprints were obtained (Chen et al. 1990). We detected 20–30 well resolved bands greater than 6-kb, which appeared highly polymorphic. The probability of dizygosity given concordance for all bands has not been calculated for the M13 system, but Hill and Jeffreys (1985) have estimated that for double stranded minisatellite probes this probability is less than  $10^{-4}$ .

### Southern blot analyses

Genomic DNA was isolated from peripheral blood lymphocytes or lymphoblastoid cell lines by the 'salting out' method (adapted from Miller et al. 1988). DNA (10  $\mu$ g) was digested with restriction enzymes in the presence of 4 mM spermidine prior to Southern transfer (Reed and Mann 1985). Plasmids were radioactively labelled by random priming and hybridized to Southern filters at 65°C (except for FSH $\beta$  for which 68°C was used). The plasmids used were pBTA528, pBTA904, pBTA531 (Mason et al. 1986; Stewart et al. 1986),  $\lambda$ 15B (Watkins et al. 1987). *Eco*RI-*Sac*I fragment encoding exon IV (GnRH) (Adelman et al. 1986), pHTF309R (Shimasaki et al. 1988), pCG $\alpha$  (Boothby et al. 1981),  $\beta$ LH (Talmadge et al. 1983) and pCG $\beta$ 474 (Policastro et al. 1986).

### Results

We searched for polymorphisms in nine hormone genes in MODZTs and controls using between 11 and 25 different restriction enzymes, selected mainly on the basis of their efficiency in detecting restriction fragment length polymor-

phisms (RFLPs) (Devor 1988). We initially digested DNA from five to nine MODZTs in order to have a reasonable chance of detecting common polymorphisms. Sampling ten alleles gives a 95% chance of detecting an allele whose frequency is at least 0.26 and an 80% chance of finding an allele of frequency 0.15. It is possible, therefore that we have missed less common polymorphisms but, if they exist, they could only be found in a minority of MODZTs. As a result of this RFLP search we found four new polymorphisms in inhibin  $\beta_B$ , FSH $\beta$ , GnRH and CG $\beta$  (Southall and Chenevix-Trench 1990; Chenevix-Trench et al. 1991; Healey et al. 1992a, b). No structural rearrangements were found in any of the genes during this RFLP search.

Allele and genotype frequencies for five RFLPs associated with these hormone genes are shown in Table 1. There were no significant differences in the frequencies of any of the alleles in the MODZTs compared with the controls (Fisher's exact test).

### Discussion

The aims of this study were twofold. Firstly, we looked for previously unpublished hormone gene RFLPs and were successful in finding four (Chenevix-Trench et al. 1991; Southall and Chenevix-Trench 1990; Healey et al. 1992a, b). No RFLPs were detected for INHA, INH $\beta_A$  or follistatin.

Secondly, we wished to calculate allele frequencies in rigorously selected "familial" MODZTs and unselected controls for these hormone gene RFLPs, to determine whether there is an association between any allelic forms and the tendency to DZ twinning. There were no major differences in the allele frequencies in these two groups (Table 1).

The absence of linkage disequilibrium between a marker and a twinning candidate gene does not rule out its role in the inheritance of a multiple ovulation trait. It is possible that expression of the dizygotic twinning trait in a mother is dependent on the cumulative effect of several genes (polygenic), or that there are several major genes that can produce the same effect (heterogeneity). But in either case, the relatively high frequency of the trait in the population, the fact that inheritance appears to be recessive, and the high recurrence rate in some pedigrees suggest that the predisposing allele(s) are likely to be fairly common (Bulmer 1970). Elsewhere we have shown, in two populations, that the S allele of the Protease Inhibitor (Pi) locus (which codes for alpha-1-antitrypsin) is twice as frequent in mothers of DZ twins as in controls (Clark and Martin 1982; Boomsma et al. 1992).

Our understanding of the endocrine control of ovulation is ever more complex and hormone receptor genes are among new candidates needing to be examined (Loosfelt et al. 1989; Laws et al. 1990). Alternatively, more subtle alterations than our techniques allow us to detect may exist in the genes we have already examined.

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