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ORIGINAL INVESTIGATION

Genetic association suggests that SMOC1 mediates between prenatal sex hormones and digit ratio

Adam J. Lawrance-Owen · Gary Bargary · Jenny M. Bosten · Patrick T. Goodbourn · Ruth E. Hogg · J. D. Mollon

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Abstract Men and women differ statistically in the relative lengths of their index and ring fingers; and the ratio of these lengths has been used as a biomarker for prenatal testosterone. The ratio has been correlated with a wide range of traits and conditions including prostate cancer, obesity, autism, ADHD, and sexual orientation. In a genome-wide association study of 979 healthy adults, we find that digit ratio is strongly associated with variation upstream of SMOC1 (rs4902759: $P = 1.41 \times 10^{-8}$) and a meta-analysis of this and an independent study shows a probability of $P = 1.5 \times 10^{-11}$. The protein encoded by SMOC1 has recently been shown to play a critical role in limb development; its expression in prostate tissue is dependent on sex hormones, and it has been implicated in the sexually dimorphic development of the gonads. We put forward the hypothesis that SMOC1 provides a link between prenatal hormone exposure and digit ratio.

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A. J. Lawrance-Owen (✉) · G. Bargary · J. M. Bosten · P. T. Goodbourn · R. E. Hogg · J. D. Mollon
Department of Experimental Psychology, University of Cambridge, Downing Street, Cambridge CB2 3EB, UK
e-mail: ajl69@cam.ac.uk

G. Bargary
Department of Optometry and Visual Science, City University London, Northampton Square, London EC1V 0HB, UK

R. E. Hogg
Centre for Vision Science and Vascular Biology, Queen's University Belfast, University Road, Belfast BT7 1NN, UK

Introduction

The ratio of the lengths of the second and fourth digits (2D:4D) is sexually dimorphic (Ecker 1875): statistically women tend to have a larger ratio than men but the distributions overlap and there is a large variation within each sex. It has been suggested that 2D:4D may be a biomarker for prenatal exposure to androgen (Manning et al. 1998; Wilson 1983): sex steroids, produced by the developing gonads, were proposed to modulate digit growth early in development and hence determine digit ratio in adults. Digit ratio would thus be an attractive measure because androgens have organising effects on the developing brain (MacLusky and Naftolin 1981) and digit ratio could serve as a retrospective, non-invasive measure of this early exposure to androgen.

Digit ratio in humans is established during the first trimester of development (Malas et al. 2006), coinciding with a period of high testosterone production in males (Abramovich 1974). Furthermore, it appears to remain stable across the lifespan (McIntyre et al. 2005). Until recently, evidence for Manning's biomarker hypothesis had come entirely from correlational studies (McIntyre 2006). For instance, males and females with congenital adrenal hyperplasia, a condition resulting in elevated androgen production, have been found to have lower, more masculinised, digit ratios (Brown et al. 2002; Okten et al. 2002). Also, men with complete androgen insensitivity syndrome, due to non-functional androgen receptors, show feminized digit ratios (Berenbaum et al. 2009). Furthermore, a significant relationship has been shown between digit ratio at age 2 and the ratio of testosterone to oestrogen, measured in amniotic fluid (Lutchmaya et al. 2004). However, the most convincing evidence comes from experimental animal studies, which now confirm that prenatal hormones

control 2D:4D development (Talarovicová et al. 2009; Zheng and Cohn 2011). Zheng and Cohn (2011) showed that digit ratio in mice is determined by the ratio of testosterone to oestrogen acting on the fourth digit in a narrow window early in development.

In the years since the biomarker hypothesis was proposed, over 400 published studies have used digit ratio to investigate the effect of prenatal sex hormones on human traits. Digit ratio has been reported to correlate with an impressive range of traits and conditions that seem to have in common a dependence on sex hormones. These include diseases such as prostate cancer (Jung et al. 2010; Rahman et al. 2011), breast cancer (Manning and Leinster 2001), osteoarthritis (Zhang et al. 2008) and obesity (Fink et al. 2006); psychological disorders such as autism (Hönekopp 2012), ADHD (Martel et al. 2008; Stevenson et al. 2007), eating disorders (Klump et al. 2006) and alcohol dependency (Kornhuber et al. 2011); features such as sperm count (Bang et al. 2005), age of menarche (Manning and Fink 2011), penis size (Choi et al. 2011) and facial shape (Fink et al. 2005); and behavioural traits such as aggression (Hönekopp 2011), visuo-spatial ability (Peters et al. 2007), handedness (Fink et al. 2004), sporting ability (Hönekopp and Schuster 2010), successful financial risk-taking (Coates et al. 2009) and sexual orientation (Grimbos et al. 2010). Analogous polymorphisms have been found in other species (Burley and Foster 2004; Forstmeier et al. 2010). Cross-species variation in digit ratio has even been used to predict social behaviour from hominoid fossils (Nelson et al. 2011).

What is not known is the mechanism by which sex hormones have their effect on digit ratio. Manning made the early suggestion that androgen may affect digit ratio via regulation of the HOX genes (Manning et al. 1998). Although the posterior HOX genes are known to be important in the formation of the digits (Kondo et al. 1997), little evidence has emerged to link them to variation in digit ratio and it is not known how their regulation or expression levels might affect digit ratio. Our present results lead us to an alternative hypothesis.

Digit ratio is known to be highly heritable: twin studies give an estimate of about 60 % (Medland and Loehlin 2008). To date, there has been one genome-wide association study (GWAS) that investigated digit ratio (Medland et al. 2010). The authors listed a suggestive association with a variant within the gene SMOC1 (MIM 608488, rs11158820: $P = 1.3 \times 10^{-6}$), but they made no comment on this particular finding. Here, in a GWAS using a genotyping array of higher density, we find a much stronger association with SMOC1. In our discussion, we assemble the evidence that this gene mediates between prenatal hormone exposure and digit ratio. We draw on recently accumulated evidence that SMOC1 has a critical role in limb development and that its expression is dependent on sex hormones.

Subjects and methods

Participants

Digit ratio was measured as part of the PERGENIC project, which tested 1,060 individuals on a 2.5-h battery of optometric, perceptual and oculomotor tests (Goodbourn et al. 2012). It included several biometric measurements. Participants were recruited from the Cambridge area by advertisements within the university and online. Participants were all of European descent, as established by the nationality of their four grandparents. Further checks on ancestry were made using their genotypes. They were aged from 16 to 40 years ($M = 22$ years, SD = 4 years). The study received approval from the Cambridge Psychology Research Ethics Committee. All participants gave written consent after having been given information about the study.

Digit ratio

A flatbed scanner (Cannon 8800F) was used to take an image of the left hand of each participant. The left hand was chosen as it is reported to have the larger genetic component (Medland and Loehlin 2008). Lengths of the second and fourth digits were derived by a computer-assisted measurement program, as this is the most reliable method (Allaway et al. 2009). Lengths were defined from the most proximal metacarpophalangeal flexion crease to the fingertip. The flexion crease develops around the ninth week of gestation and is a deep and permanent crease that forms over joints (Kimura et al. 1990). The length of the second digit was divided by the length of the fourth digit to give the 2D:4D ratio. For each image, the computer required the manual input of four points: the creases and the fingertips of the index and ring fingers. The program automated the zoom level to allow visualisation of the image features and automatically calculated the finger lengths and digit ratios from the input points. A second independent image was available for each of 103 randomly chosen individuals (66 females) who returned for a second visit a minimum of 1 week later; data from these participants were used to estimate reliability.

Genotyping

DNA from 1,008 individuals was collected from saliva samples taken during the participants' visit using Oragene OG-500 kits (DNA Genotek Inc., Ottawa, Canada). DNA extraction and microarray processing were performed by

Cambridge Genomic Services (University of Cambridge, UK) using standard manufacturers' protocols. Individuals were genotyped at 733,202 single nucleotide polymorphisms (SNPs) on the Illumina HumanOmniExpress BeadChip. Genotype calling was by custom clustering. Thirty individuals were excluded from the analysis, on the basis of genetic and phenotypic quality control. Criteria for exclusion were: inadequate image quality (10 individuals), genotypic sex anomalies (3 individuals), low (<0.97) genotyping call rate (1 individual), population outliers (1 individual) and duplicate or related samples (15 individuals removed in total). These were defined by inspecting the histogram of all identity-by-descent (IBD) estimates. It showed 4 pairs with an IBD close to 1 (duplicate samples or MZ twins), a cluster of 12 pairs with an IBD close to 0.5 (sibling or parent-offspring pairs) and 1 pair with an IBD close to 0.25 (half-sibling, avuncular, or grandparent-grandchild). Inspecting our participant registration databases, palm scans and iris photographs allowed us to conclude the identical pairs were duplicate samples, individuals who presented for testing on two separate occasions. The other related individuals were siblings. In each case, we excluded the sample with the lower call rate. This left 979 individuals in the analysis (599 females). Quality control was also conducted on individual SNPs. Markers with >2 % missing genotypes (12,706 SNPs) and markers with <1 % minor allele frequency (77,738 SNPs) were excluded, leaving 642,758 SNPs in the analysis.

Statistical analysis

Association analysis was conducted assuming an additive genetic effect using PLINK (Purcell et al. 2007). To control for any residual population stratification resulting from multiple genetic subgroups or genetic admixture in our population, we used Eigensoft (Price et al. 2006) to extract the top three principal components (PCA) of variation in the sample. The three PCA axes were entered together with phenotypic sex as covariates in the regression model. The phenotype was normally distributed (Online Resource Fig. 1). At any suggestive ($P < 1 \times 10^{-5}$) loci, 2.5 Mb regions centred on these locations were defined for imputation. These regions were imputed using IMPUTE2 (Howie et al. 2009, 2011) with the phased haplotypes of the 1000 genomes project (1000 Genomes Project Consortium 2010). Association analysis of these high-density regions was then carried out on the genotype probabilities using the dosage association feature of PLINK. The four covariates were added to the regression model as before. Finally, regions corresponding to the association signal were defined. These regions are blocks that are in linkage disequilibrium with the most strongly associated marker and contain other "clumped" SNPs that are associated with the phenotype with a specified P value. The range, therefore, defines the region likely to contain the gene of interest, where the causal polymorphism associated with the phenotype lies. We used PLINK's clumping function to define the regions, using a significance threshold of index SNPs of 0.00001, a significance threshold for clumped SNPs of 0.01, an LD threshold for clumping of 0.1 and a physical distance threshold for clumping of 1,250 Kb. For all significant or suggestive SNPs, cluster plots were inspected manually and genotype distributions were evaluated for deviation from Hardy–Weinberg equilibrium. All genomic references are based on NCBI Build 37. Analyses were performed using Matlab (2011b) and PLINK (v1.07) software.

Results

The measurements of digit ratio were highly reliable, as established by the correlation between measurements taken on the first and second visits from a randomly chosen subsample of 103 participants who participated twice (2D: Pearson's $r = 0.98$, 4D: $r = 0.99$, 2D:4D: $r = 0.92$). We confirm the classical finding that 2D:4D is higher in females ($M = 0.979$ SD $= 0.030$) than in males ($M = 0.964$, SD $= 0.031$); this difference was highly significant [$t(977) = -7.3$, $P = 6.48 \times 10^{-13}$].

Inspection of the QQ plot resulting from the association (Online Resource Fig. 2) and the value of the genomic inflation factor ($\lambda = 1.00$) showed no evidence of increased signals due to technical error or to population stratification. Table 1 details the genotyped and imputed SNPs within one region of chromosome 14 with $P < 5 \times 10^{-7}$. The strongest signal was at rs4902759 ($P = 1.41 \times 10^{-8}$). Each additional copy of the C allele was associated with a 0.0076 decrease in 2D:4D. The power to detect this effect was 78 % (Online Resource Fig. 3).

The region giving rise to the association signal included the gene SMOC1 (Fig. 1; Online Resource Fig. 4). Although there were associations within the coding region, the strongest signal originated from a region upstream of the gene, and so may reflect variation in a regulatory element. Post-association quality control showed no evidence of departure from Hardy–Weinberg equilibrium (Table 1) and inspection of the signal intensity plots showed that the SNPs were well called (Online Resource Fig. 5). Two additional regions were suggestive of association (Online Resource Table 1).

We examined the association of digit ratio with LIN28B, identified by Medland et al. (2010). Our strongest association within the gene was with the same SNP (rs314277) and the direction of the effect was the same as reported in the previous study (our study $\beta = 0.0034$, SD $= 0.002$) but the association was not significant

Table 1 Association results for the SNPs with $P < 5 \times 10^{-7}$

SNP	Location	LD	Allele 1	Allele 2	MAF	HWE	β	SE	P value
Genotyped									
rs2332175	70345411	0.885	G	A	0.461	0.482	−0.0074	0.0013	3.44×10^{-8}
rs1952198	70339755	0.901	C	T	0.451	0.140	−0.0070	0.0013	1.57×10^{-7}
Imputed									
rs4902759	70328953	1.00	C	T	0.475	0.274	−0.0076	0.0013	1.41×10^{-8}
rs11158817	70331469	0.998	T	C	0.476	0.303	−0.0076	0.0013	1.50×10^{-8}
rs4902758	70328107	0.905	A	T	0.447	0.070	−0.0070	0.0013	1.90×10^{-7}
rs4902760	70329198	0.905	A	C	0.447	0.070	−0.0070	0.0013	1.90×10^{-7}
rs9323530	70332304	0.896	T	C	0.445	0.060	−0.0070	0.0013	2.08×10^{-7}
rs1318485	70333355	0.900	G	A	0.447	0.060	−0.0070	0.0013	1.69×10^{-7}
rs958056	70333576	0.900	A	G	0.447	0.060	−0.0070	0.0013	1.68×10^{-7}
rs4899317	70335347	0.903	C	T	0.449	0.070	−0.0070	0.0013	1.79×10^{-7}
rs4899318	70335429	0.903	T	G	0.448	0.070	−0.0070	0.0013	1.84×10^{-7}
rs12431570	70336961	0.903	C	A	0.449	0.070	−0.0070	0.0013	1.72×10^{-7}
rs12435823	70340544	0.904	C	T	0.450	0.106	−0.0070	0.0013	1.49×10^{-7}
rs4899319	70342363	0.907	C	T	0.450	0.119	−0.0070	0.0013	1.55×10^{-7}

All SNPs are located on chromosome 14. Locations are GRCh37 coordinates. LD is the r^2 linkage disequilibrium between each SNP and rs4902759. Allele 1 is the minor allele. All imputed SNPs have an IMPUTE2 quality score ≥1

MAF Minor allele frequency, β change in 2D:4D per additional minor allele, *SE* standard error of beta, *HWE* Hardy–Weinberg equilibrium *P* value

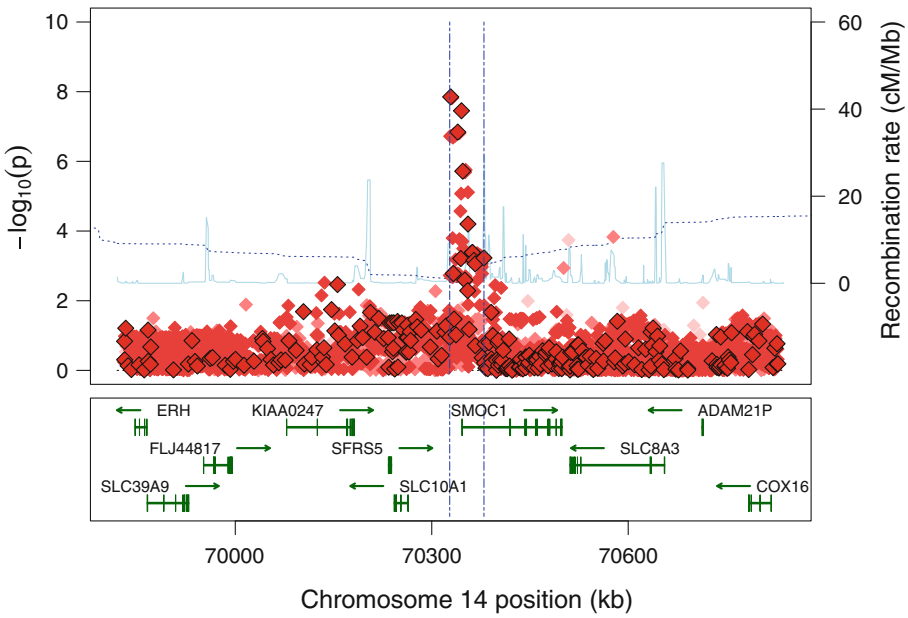

Fig. 1 Manhattan plot of the SMOC1 region. (*Top*) Association results for the genotyped SNPs (*red diamonds with black borders*) and imputed SNPs (*red diamonds*; saturation corresponds to imputation quality). Recombination rate is plotted with a *solid blue line*; genetic distance from rs4902759 is plotted with a *dotted blue line*. (*Bottom*) The genomic context of the region. *Vertical rectangles* indicate exons. (*Both*) *Vertical blue dashed lines* illustrate the region giving rise to the association signal (colour figure online)

($P = 0.084$). However, given our smaller sample size compared with the previous study, the power to detect the effect was only 3 %. We also tested digit ratio for association with several other candidate genes. None of these associations reached significance at a genome-wide level (Online Resource Table 2).

We performed a meta-analysis of the SMOC1 region using the results from the Medland et al. study and our own. They list results for rs11158820 and rs11621436 where they report association signals of $P = 1.3 \times 10^{-6}$ and $P = 3.3 \times 10^{-6}$, respectively. Our association at these loci was $P = 1.7 \times 10^{-6}$ and $P = 7.7 \times 10^{-6}$. An inverse variance meta-analysis of the two studies produced associations of $P = 1.5 \times 10^{-11}$ and $P = 2.7 \times 10^{-10}$.

Discussion

The gene SMOC1 encodes the protein SMOC1 (secreted modular calcium-binding protein 1) (Vannahme et al.

2002). SMOC1 is secreted into the cellular matrix during osteoblast differentiation (Bradshaw 2012; Choi et al. 2010) and is an antagonist of bone morphogenetic proteins (BMPs) (Thomas et al. 2009), which control the formation of cartilage in the digits (Stricker and Mundlos 2011).

Polymorphisms in or close to the SMOC1 gene have now emerged as associates of 2D:4D. There are firm independent grounds suggesting that SMOC1 controls the development of the digits. Three recent pedigree studies have shown that mutations of the SMOC1 gene are associated with Waardenburg anophthalmia (OMIM 206920) in humans (Abouzeid et al. 2011; Okada et al. 2011; Rainger et al. 2011). A common feature of this syndrome is abnormality of the digits, such as syndactyly, oligodactyly, or clinodactyly. Furthermore, knockout mice display a similar phenotype (Okada et al. 2011; Rainger et al. 2011). In addition, SMOC1 is expressed in mice in forelimb buds at E9.5, and in developing limbs between E10.5 and E11.5. Expression coincides with chondrogenic condensation at E12.5 (Okada et al. 2011; Rainger et al. 2011).

Given the clear role of SMOC1 in limb development and given that it is associated with variation in digit ratio in the normal population, what could be the mechanism by which it controls the phenotype? How should we reconcile our finding with the prenatal sex hormone theory? Zheng et al. showed that digit ratio is determined by the ratio of testosterone to oestrogen acting on the fourth digit, which is rich in androgen and oestrogen receptors. They found that activity of these receptors regulated the expression of skeletogenic genes that control chondrocyte proliferation, and that this happened differentially in the second and fourth digits (Zheng and Cohn 2011). The androgen and oestrogen receptors regulate gene expression by acting as nuclear receptors: activation by steroids causes the receptor to enter the nucleus and bind to its target DNA as a transcription factor. Of special interest, therefore, is the finding that SMOC1 is up-regulated by androgen (Love et al. 2009; Schaeffer et al. 2008) and down-regulated by oestrogen (Coleman et al. 2006) in prostate tissue. SMOC1 has also been shown to have a role in the sexually dimorphic development of the gonads (Pazin and Albrecht 2009). It is thus plausible that prenatal testosterone and oestrogen affect the expression of SMOC1, thus controlling digit ratio.

This would be consistent with our finding that our largest association with digit ratio arises at a polymorphism upstream of the gene, near to several dense clusters of transcription factor (TF) binding sites (Online Resource Fig. 6). We identified TF binding sites using the ChIP-seq data from the ENCODE project and the SABiosciences DECODE database. Notable are three CEBPB-beta binding sites and two SRY binding sites. CEBP-beta is known to associate with steroid hormone receptors including the oestrogen receptor (Boruk 1998; Stein and Yang 1995). SRY is the sex-determining gene and is known to interact with the androgen receptor (Yuan et al. 2001). It is also worth noting that according to the recent GENCODE (v12) annotations, rs4902759 actually falls within a processed transcript: a long non-coding RNA. These are thought to play an important role in gene regulation (Derrien et al. 2012).

It should be noted that we are not here claiming that polymorphism of SMOC1 itself explains a large proportion of the variance in digit ratio. Rather we suggest that the strong association found in the present study identifies the potential role of SMOC1 as an intermediate between prenatal sex hormones and digit ratio, much of the actual variance in digit ratio may well derive from prenatal sex hormone levels.

There are ethnic differences in digit ratio: populations of African origin have lower digit ratios than Caucasians, whereas Chinese populations have higher ratios than Caucasians (Manning et al. 2004). It is therefore interesting that the polymorphism in SMOC1 parallels ethnic differences in digit ratio. Although the frequency of the C allele at rs4902759 is 0.46 in the European population of the 1000 Genomes project and 0.48 in our own European sample, its frequency is 0.85 in African populations and 0.19 in Asians (1000 Genomes Project Consortium 2010). The difference in digit ratio between Africans and Chinese is of a similar order to the effect size for the two alleles at rs4902759. Thus, the ethnic differences in digit ratio could be derived from the distribution of the SMOC1 polymorphism between populations.

In conclusion, we have identified several polymorphisms within, and upstream of, the SMOC1 gene that are associated with digit ratio. The gene is known to play a critical role in limb development and there is evidence that it is regulated by sex hormones. Thus, we put forward the hypothesis that SMOC1 mediates between prenatal hormone exposure and digit ratio.

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