

Vitamin D Receptor Gene Polymorphisms Have Negligible Effect on Human Height

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Human height is a highly heritable trait, with genetic factors explaining up to 90% of phenotypic variation. Vitamin D levels are known to influence several physiological processes, including skeletal growth. The vitamin D receptor (VDR) gene has been reported as contributing to variation in height. A meta-analysis of 13607 adult individuals found a small but significant association with the rs1544410 (BsmI) polymorphism. In contrast, the meta-analysis found no effect in a sample of 550 children. Two recent studies reported variants with large effect on height elsewhere in VDR (rs10735810 [FokI] and rs7139166 [-1521] polymorphisms). We genotyped large Caucasian samples from Australia ($N = 3906$) and the Netherlands ($N = 1689$) for polymorphisms in VDR. The Australian samples were twin families with height measures from 3 time points throughout adolescence. The Dutch samples were adult twins. We use the available family data to perform both within and between family tests of association. We found no significant associations for any of the genotyped variants after multiple testing correction. The (non-significant) effect of rs1544410 in the Australian adolescent cohort was in the same direction and of similar magnitude (additive effect 0.3cm) to the effect observed in the published adult meta-analysis. An effect of this size explains ~0.1% of the phenotypic variance in height — this implies that many, probably hundreds, of such variants are responsible for the observed genetic variation. Our results did not support any role for two other regions (rs10735810, rs7139166) of VDR in explaining variation in height.

Keywords: stature, Australian twins, Dutch twins, VDR, polygenic

Height is one of the most highly heritable human traits, with heritability estimates up to 90%, particularly when measurement error is minimized (Macgregor et al., 2006; Silventoinen et al., 2003). Height has long been the subject of genetic studies, beginning with

Galton in the 19th century (Galton, 1886). As a result of improved environment (primarily nutrition), mean height has increased over the second half of the 20th century (Vogel & Motulsky, 1997). Despite the increases in mean height, the mean difference between European countries has remained stable and trait variance and heritability estimates are broadly similar across countries (Silventoinen et al., 2003). In recent years the role of short stature as a risk factor for cardiovascular disease has been hotly debated, with a number of reports of an inverse correlation between stature and disease risk (Hebert et al., 1993; Kannam et al., 1994).

The effects of the vitamin D receptor (VDR) gene on height and on various bone measurements has been the subject of many studies. Linkage studies have indicated that several chromosomes are likely to influence height (Hirschhorn et al., 2001; Perola et al., 2001, 2007). The linkage peaks include chromosome 12q, the location of the VDR gene, with one study finding genome wide significant linkage (Hirschhorn et al., 2001) and others finding weaker (suggestive) evidence (Dempfle et al., 2006; Hirschhorn et al., 2001; Perola et al., 2001). In some cases, linkage was found by examining the extreme low end of the height range (Dempfle et al., 2006).

Several studies have examined the VDR gene for association with height. The most widely typed polymorphism examined, rs1544410 (BsmI), was the focus of a meta analysis (Fang et al., 2007) that concluded that this variant (or one in high linkage disequilibrium with it) had a significant effect on height (additive effect of each copy of B allele = 0.3cm). This effect was only significant in the adult samples examined, with child samples indicating a (nonsignificant) effect in the opposite direction to that seen in the adult

Received 17 April, 2008; accepted 28 April, 2008.

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Table 1

Previously Reported Results

| Authors | Age | Sample size | rs1544410 (BsmI) | rs731236 (TaqI) | rs10735810 (FokI) | rs7139166 (-1521) |
|--------------------------|-------------------|---------------|---------------------|--------------------|----------------------|----------------------|
| Xiong et al. | Adult | 1873 (406) | 0.05 | 0.04 | | |
| D'Alesio et al. | Teenage | 185 | 0.26 | | 0.05* | 0.05* |
| Terpstra et al. | Teenage | 255 | | | NS | |
| Handoko et al. | Adult | 368 | NS | NS | NS | |
| Dempfle et al. | Child | 184 | 0.7 (91) | 1 | 0.04 | |
| Fang et al. (Meta anal.) | Adult/child | 14157 | 0.006 | | | |
| Fang et al. (Meta anal.) | Child only subset | 550 | 0.95 | | | |

Note: Alternative marker names are in brackets after rs names. Unless otherwise stated results are *p* values for single SNP test under an additive model (trend test). Blank cells indicate that marker was not typed in that study. NS denotes case where association was reported to be nonsignificant ($p > .05$) but insufficient details were given to give an exact *p* value. * denotes cases where result was for haplotype based test. For family-based samples, the total number of individuals is given, followed by the number of families in brackets; for population-based samples the sample size column contains a single number (i.e., the total number of individuals). *P* values for family based analyses are for the within family test.

samples. Since the collation of the data for the meta-analysis, further studies have been published on the effects of rs1544410 (BsmI) on height. A number (d'Alesio et al., 2005; Dempfle et al., 2006; Handoko et al., 2006) reported no association between rs1544410 (BsmI) and height. Conversely, another study (Xiong et al., 2005) found evidence for association and for linkage (single marker *p* values in range .01 to .05). Some studies have examined regions of the VDR gene which are in linkage equilibrium with the region containing rs1544410 (BsmI). One (Dempfle et al., 2006) found weak ($p=0.04$) evidence for association with rs10735810 (FokI), whilst another did not ($p > .05$, Terpstra et al. (2006)). Another (d'Alesio et al., 2005), found similarly weak ($p = .05$) evidence for association with a different region rs7139166 (-1521). All these previous results are summarized in Table 1.

Here, we examine the relationship between height and the VDR polymorphisms in a large sample of Australian and Dutch twin families. The goal is to compare and add to the previous results, using family based tests of association, which have not been utilized in many of the earlier studies.

Material and Methods

Phenotypic Data

Australian Caucasian Sample

The Australian data used in this paper were derived from a study of adolescent twins recruited from the Australian Twin Registry. The zygosity of twin pairs was initially determined by twins' responses to standard items about physical similarity and the degree to which others confused them with one another. Many of the twins were subsequently included in studies in which they were typed for large numbers of genetic markers. As a result we expect misclassifications to be very rare in this sample (Cornes et al., 2005).

The data consisted of 1077 families. Each family had one twin pair, with 45% of families also having one or more additional siblings. 77% of parents, 83% of twins and 88% of additional siblings of individuals were genotyped (82% overall). The sample included both monozygotic and dizygotic twins. Twins and their siblings were measured for height at up to three age points; 12, 14 and 16 years. Height measurements were performed by an experienced research nurse.

Dutch Caucasian Sample

The Dutch data on body height were obtained from a longitudinal survey study on health, lifestyle and personality by the Netherlands Twin Register (NTR, for a detailed description see Boomsma et al. (2006)). Surveys were sent out every 2 to 3 years to twins and their family members. The twins were included all surveys (1991, 1993, 1995, 1997, 2000, 2002 and 2004); parents in 1991, 1993, 2000, 2002, 2004; siblings of twins were invited from 1995 onwards and spouses of twins were included from 2000 onwards. In each survey, individuals were asked to report on their body height in cm. In addition, measured height was available from several subsamples of the NTR which took part in laboratory studies of cardiovascular risk factors, brain function and cognition. All available measures on body height from age 18 years onwards were combined into one adult body height, with preference given to measured height (available for 15% of the sample). When measured height was unavailable, and self-reported height was present for multiple surveys, the average self-reported height was used. Differences in height across the questionnaires were checked and height data were discarded when there was no consistency across questionnaires and differences were larger than 5 cm ($N = 35$). The correspondence between measured height and average height based on self-reports is 0.93 (Silventoinen et al., 2003). As during the last century the average stature

Table 2

Allele Frequency Estimates

| | Australia | Netherlands |
|-------------------|------------------|------------------|
| rs7139166 (-1521) | 0.43 (G/C) | 0.43 (G/C) |
| rs10735810 (FokI) | not typed | 0.35 (T/C) |
| rs1544410 (BsmI) | 0.41 (G/A) (b/B) | 0.43 (G/A) (b/B) |
| rs731236 (TaqI) | 0.41 (T/C) (T/t) | 0.41 (T/C) (T/t) |

Note: Markers are in order from 5' end of gene. Alleles are coded in brackets after the frequencies.

of the Dutch population has increased rapidly with every generation (Fredriks et al., 2000), we used birth year as a covariate. Stature and genotypic information were available for 1687 individuals from 493 families. For 485 families, data on 487 parents were also available. The sample included both monozygotic and dizygotic twins. The average age of the Dutch population at measurement(s) was 39 years.

Genotype Data

SNPs were selected in the VDR gene based on previous studies (Table 2). We also typed rs4516035 (= -1012 from d'Alesio et al. [2005]) in the Australian sample; this was in very high r^2 ($r^2 = 0.95$) with rs7139166 and hence association results are very similar (data not shown). Assays were designed using the Sequenom MassARRAY Assay Design (version 3.1) software (Sequenom Inc., San Diego, CA).

For the Australian samples, forward and reverse PCR primers and primer extension probes were purchased from Bioneer Corporation (Daejeon, Korea). Genotyping was carried out in standard 384-well plates with 12.5 ng genomic DNA used per sample. We used a modified Sequenom MassARRAY protocol where half reaction volumes were used in each of the PCR, SAP and iPLEX stages giving a total reaction volume of 5.5 μ L. The iPLEX reaction products were desalted by diluting samples with 18 μ L of water and 3 μ L SpectroCLEAN resin (Sequenom) and then spotted on a SpectroChip (Sequenom), processed and analyzed on a Compact matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) Mass Spectrometer by MassARRAY Workstation software (version 3.3; Sequenom). Allele calls for each 384-well plate were reviewed using the cluster tool in the MassARRAY TyperAnalyzer 3.4 software (Sequenom) to evaluate assay quality. Call rates of $\geq 98\%$ were achieved for all SNPs. Discordant genotypes between MZ twins and mendelian errors were identified using PEDSTATS (Wigginton and Abecasis, 2005) and made up 0.07% of the data.

For the Dutch samples, DNA was extracted from whole blood or buccal swab (Miller et al., 1988; Meulenbelt et al., 1995; Min et al., 2006). To assay these polymorphisms, we used a modified MassARRAY protocol where the polymerase chain reaction (PCR) cycling protocol was performed as a 'touchdown' pro-

tol (i.e., 15 min at $95 \pm C$, followed by 4 cycles of 20 s at $95 \pm C$, 30 s at $65 \pm C$ and 1 min at $72 \pm C$, then followed by 4 cycles of 20 s at $95 \pm C$, 30 s at $58 \pm C$ and 1 min at $72 \pm C$, and subsequently followed by 38 cycles of 20 s at $95 \pm C$, 30 s at $53 \pm C$ and 1 min at $72 \pm C$. Final extension was carried out at $72 \pm C$ for 3 min, and the sample was cooled to $15 \pm C$). Reaction products were desalted, processed and analyzed on an Autoflex (Bruker, Wormer, Netherlands) MALDI-TOF Mass Spectrometer and genotypes were assigned real-time using MassARRAY Typer3.4 software (Sequenom). As quality controls, 5 to 10% of the Dutch samples were genotyped in duplicate. No inconsistencies were observed. Positive and negative controls uniquely distributed in each 384 wells plate were also consistent. Cluster plots were made of the signals from the low and the high mass allele. Two independent researchers carried out scoring. Disagreements or vaguely positioned dots produced by Typer Analyzer 3.4 (Sequenom) in addition to all wells that had 50% or more failed SNPs were excluded from analysis.

Statistical Methods

We estimated allele frequencies for each SNP using a maximum likelihood procedure in SOLAR (<http://www.sfbr.org/solar>). Markers were tested for deviation from Hardy-Weinberg equilibrium using pedstats (www.sph.umich.edu/csg/abecasis/PedStats). Tests for association were performed using QTDT (www.sph.umich.edu/csg/abecasis/QTDT/). In both samples sex, age, age squared and a sex-age interaction were fitted as covariates (in the Australian data there was still some age variation between individuals within the 12, 14 and 16 age groups). Tests were done for both within family association (which is unaffected by any population stratification) and total association (which can be affected by population stratification). The within family test uses information from transmissions from parents to offspring (typically the deviation of coded offspring genotypes from the expected coded genotypes given the parental genotypes) and relates this information to the observed phenotypes in a variance components framework. The total association test uses the information from transmissions but also uses between family information (in the simplest case where parents are genotyped, this means the coded parental genotypes are used in the model in addition to the coded offspring genotype deviations from the coded parental genotypes, see Abecasis et al. (2000) for full details). In each case association was modeled using variance terms for linkage; this maximizes power and maintains type I error at the expected level (Abecasis et al., 2000).

Results

All markers showed no deviations from Hardy-Weinberg equilibrium ($p > .05$). The allele frequencies of each SNP are given in Table 2. These frequencies are similar to those observed in other Caucasian popu-

Table 3

Association Results

| Age | Australia | | | | | | Netherlands | |
|-------------------|---------------|------|------|----------------|------|------|---------------|----------------|
| | Within family | | | Between family | | | Within family | Between family |
| | 12 | 14 | 16 | 12 | 14 | 16 | Adult | Adult |
| rs7139166 (-1521) | 0.74 | 0.47 | 0.47 | 0.64 | 1 | 0.5 | 0.62 | 0.99 |
| rs10735810 (FokI) | NA | NA | NA | NA | NA | NA | 0.02 | 0.26 |
| rs1544410 (BsmI) | 0.41 | 0.08 | 0.28 | 0.2 | 0.14 | 0.26 | 0.91 | 0.38 |
| rs731236 (TaqI) | 0.51 | 0.15 | 0.38 | 0.19 | 0.17 | 0.3 | 0.7 | 0.48 |

Note: Results are *p* values

lations (Fang et al., 2007). As expected, rs1544410 (BsmI) was in high linkage disequilibrium (LD) with rs731236 (TaqI); the observed LD (Australian and Dutch; $r^2 > 0.9$) is stronger than reported elsewhere (Xiong et al., 2005).

Association test results are given in Table 3. There is little evidence for association to any of the genotyped variants for the Australian sample (smallest *p* value .08 without correction for multiple testing). For the Dutch data there is slight evidence for within family association for marker rs10735810 (FokI), *p* = .02. However, no evidence for association was found using the total (i.e., between + within) association tests. The within-between discrepancy is also observed in the QTDT test for population stratification (*p* = .03 for rs10735810). The other three markers, rs7139166 (-1521), rs731236 (TaqI) and rs1544410 (BsmI) showed no evidence for association. After correction for multiple testing, all total and within association results are not significant (*p* > .05). The nominally significant population stratification test result (*p* = .03 before correction for multiple testing) for rs10735810 is likely to be a multiple testing artefact.

Discussion

In two previous reports (d'Alesio et al., 2005; Dempfle et al., 2006), the rs7139166 (-1521) and rs10735810 (FokI) polymorphisms were reported to significantly influence variation in height. In two large samples from Australia and the Netherlands, we found little support for the role of the rs7139166 (-1521) polymorphism in determining height. For rs10735810 (FokI) the Dutch data showed some evidence of within family association, but no between family association. Here, in addition to the possibility of this being a chance finding due to multiple testing, population substructure, generational height differences, as well as an increased sample size in the between family analysis could have played a role. After correction for multiple testing, the rs10735810 (FokI) result is nonsignificant. The samples typed are more than an order of magnitude larger than previous reports and would hence have excellent power to replicate the previously reported (large) effect sizes.

The rs1544410 (BsmI) polymorphism (and the correlated rs731236 [TaqI] polymorphism) was found to have a nonsignificant effect on height in both our samples. In previous studies, the B allele (as defined in Fang et al. (2007)) was associated with increased height. In a large meta-analysis which considered only population based samples (Fang et al., 2007), the B allele had an additive effect of +0.3cm on height. In the Australian samples here, the nonsignificant effect of the B allele was of similar magnitude to this for both total and within association models. In the Dutch samples the B allele additive effect was larger for the total model (+0.6cm) and in the opposite direction for the within model (-0.2cm). Even if the Australian and Dutch samples were analyzed together, the effect of the B allele would still not be significant (*p* > .05). The fact that our large sample was unable to generate a significant result for this VDR variant raises questions about the overall importance of this locus to variation in height. In Caucasian populations, the rs1544410 (BsmI) variant typically has minor allele frequency close to the frequency observed here, 0.41. This frequency and effect size (additive effect ~0.3cm) implies that the rs1544410 (BsmI) variant explains ~0.1% of the variance in height. In Asian populations the frequency is ~7%, implying the rs1544410 (BsmI) variant explains ~0.03% of the variance. The effect size in terms of proportion of variance is one of the primary determinants of the power of genetic association studies of quantitative traits. The sample size required to detect, with 80% power, a QTL explaining 0.1% of the variance at the alpha = 5% level is 7845 (assuming a population based sample and assuming complete LD between variant and marker). For a genome wide association study (alpha = 0.0000001), 38030 samples are required.

In the light of the likely small effect size at rs1544410 (BsmI), the failure of most of the studies performed since the meta-analysis to find any significant results is not surprising (d'Alesio et al., 2005; Dempfle et al., 2006; Handoko et al., 2006) find no association, Xiong et al. (2005) find nominally significant single SNP *p* values). One study (Xiong et al., 2005) increased their evidence for association by performing haplotype based analysis but in our data the

haplotype based results are similar to the (non-significant) single SNP results. The similarity between single SNP and haplotype results was because our sample showed high LD between rs1544410 (BsmI) and rs731236 (TaqI). The observed r^2 here was higher than reported by Xiong et al. (2005) and is interesting because it impacts upon the haplotype frequencies. Xiong et al. (2005) report that the 4 haplotype frequencies for rs731236, rs1544410 are 0.05, 0.37, 0.53, 0.05. In contrast, due to the high LD that we observe, there are only 2 haplotypes at appreciable frequency in the Australian or Dutch data (combined haplotype frequencies of 2 most frequent haplotypes > 0.98). This of course means that the 2 locus haplotype based association results are rather similar to the single locus results for either rs1544410 (BsmI) or rs731236 (TaqI) (data not shown). This result contrasts with the results in Xiong et al. (2005), where the most significant results are for haplotypes (in particular for haplotypes we only find at a very low frequencies in our data).

SNPs within VDR have been examined in two publicly available genome-wide association studies. Association results from British 1958 Birth Cohort (1958BC) have been made available (<http://www.b58cgenegene.sgul.ac.uk/>) and the rs10735810 (FokI) and rs1544410 (BsmI) SNPs appear in their set of ~1500 (unrelated) genotyped individuals. Neither show any evidence of association ($p > .5$) — this is likely to also be true for rs731236 (TaqI) because both the Australian and Dutch samples found substantial linkage disequilibrium between rs1544410 and rs731236. rs7139166 (–1521) is not typed but it has $r^2 = 1$ with rs4516035 which is genotyped in 1958BC — there is nominal evidence for an association with height for this SNP in 1958BC ($p = .03$). In total 43 SNPs are typed in the 1958BC; aside from rs4516035 (and the adjacent SNP), there are no SNPs which show evidence for association ($p > .10$). Association results from the Framingham Heart Study (FHS) data (Cupples et al., 2007; <http://www.ncbi.nlm.nih.gov/projects/gap/cgi-bin/analysis.cgi?id=pha000136>) are available (1380 genotyped individuals in families) but presently only 4 SNPs in the VDR gene are available that pass quality control. None of the 4 FHS typed SNPs are the same as those typed here although one of the SNPs typed in the FHS, rs3890734, is in high linkage disequilibrium ($r^2 = 0.66$) with rs7139166. None of the 4 SNPs show evidence for association in the FHS (all $p > .30$).

Very recently, three height genome wide association studies have been published (Gudbjartsson et al., 2008; Lettre et al., 2008; Weedon et al., 2008). In each of these studies, the authors report the top 20-60 SNPs identified from a genome-wide scan followed by individual genotyping. None of the associated SNPs are near VDR (all chromosome 12 SNPs >20 megabases away). The closest gene with associated SNPs was the

previously reported HMGA2 gene (Sanna et al., 2008; Weedon et al., 2007), ~21 megabases from VDR.

We have not examined as many SNPs as in the study by Fang et al. (2007). In particular, since the 3 groups of SNPs (rs7139166, rs10735810 and rs1544410/ rs731236) are in linkage equilibrium, we have not reported the results of haplotype analysis. In Fang et al. (2007), a combination of variants that increased body height was assembled into a haplotype genotype, which increased height with an additive effect of 0.7cm (reported as difference between homozygotes = 1.4cm in the Rotterdam Study). Examining the composition of the particular combination, it is clear that much of this effect comes from variation in the rs1544410 (BsmI) region (Block 5 Hap 2 in Fang et al. [2007], additive effect in Rotterdam Study = 0.45cm). Although we have not typed all the relevant SNPs to enable us to replicate the haplotype genotype result in Fang et al. (2007), since we find little evidence for association near rs1544410 (BsmI), our data suggest the haplotype genotype will not have a large effect upon height.

The effect of rs1544410 (BsmI) variation in determining height at different ages is of interest. The opposite effect direction to that seen in adults was seen in a small ($n = 550$) meta-analysis of younger individuals, although in this case the effect of rs1544410 (BsmI) on height was nonsignificant (Fang et al., 2007). For the Australian adolescent sample, the (nonsignificant) effect of rs1544410 (BsmI) was in the same direction as in found in adults. Overall, it appears that the effects of rs1544410 (BsmI) on height are likely to be small, in both adult and child groups.

In summary, our results provide no evidence to support the claimed large effect sizes of the rs10735810 (FokI) and rs7139166 (–1521) polymorphisms in VDR for height (d'Alesio et al., 2005; Dempfle et al., 2006). The effect of the previously reported rs1544410 (BsmI) polymorphism on height is also likely to be small. More generally, very recent studies (Gudbjartsson et al., 2008; Lettre et al., 2008; Visscher et al., 2007; Weedon et al., 2008) underline the fact that height is a polygenic trait, whose variation is underpinned by a great many different loci of small effect.

Acknowledgments

We would like to thank the twins and their parents for their co-operation; Anjali Henders and Megan Campbell for managing sample processing and DNA extraction; Alison Mackenzie for coordinating the mail-out; Marlene Grace, Ann Eldridge and the many research interviewers for data collection.

This research was supported in part by grants from the National Health and Medical Research Council of Australia (NHMRC Grant 389892), by the Dutch Research Council (NWO and ZonMW) and by EU/QLRT-2001-01254. SM is the recipient of an NHMRC Career Development Award (496674).

We acknowledge use of genotype data from the British 1958 Birth Cohort DNA collection, funded by the Medical Research Council grant G0000934 and the Wellcome Trust grant 068545/Z/02.

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