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Heritability and Genetics of Serum Dickkopf 1 Levels in African Ancestry Families

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Abstract

Purpose—To determine the heritability of serum dickkopf-1 (DKK1) and its association with DKK1 polymorphisms in African ancestry subjects.

Methods—Serum DKK1 was measured in 422 Afro-Caribbean men and women aged 18+ from 7 large, multigenerational families (mean family size: 60; 3215 relative pairs). Twenty-four common single nucleotide polymorphisms (SNP) were genotyped within an 80 kilobase pair region encompassing the *DKK1* gene. Heritability was estimated and SNPs were tested for association with serum DKK1 using variance components analysis. *DKK1* mRNA expression was tested in peripheral blood of 16 individuals from each of the rs7069912 genotypes.

Results—Mean serum DKK1 was 1724.1pg/mL and was significantly lower in women than men ($P=0.043$). Residual genetic heritability of serum DKK1 was 0.4460 ($P<0.0001$). Six SNPs reached nominal significance with DKK1, with rs7069912 being significant after adjustment for multiple comparisons. Two of these 6 SNPs represented independent association signals (rs7069912 and rs16928725), which accounted for 4.6% of the phenotypic variation in DKK1. Additionally, carriers of the rs7069912 variant had significantly greater *DKK1* expression than non-carriers ($P=0.036$).

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CONFLICTS OF INTEREST

Allison Kuipers: The author declares that they have no conflicts of interest.

Shibing Yu: The author declares that they have no conflicts of interest.

Candace Kammerer: The author declares that they have no conflicts of interest.

Cara Nestlerode: The author declares that they have no conflicts of interest.

Clareann Bunker: The author declares that they have no conflicts of interest.

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Conclusions—Serum DKK1 levels are highly heritable in African ancestry families. Two SNPs within the *DKK1* region accounted for nearly 5% of the variation in serum DKK1.

Keywords

Dickkopf-1; heritability; single nucleotide polymorphism; gene expression; African ancestry

INTRODUCTION

Wingless (Wnt) signaling is mediated by its membrane-bound co-receptors, frizzled and low-density lipoprotein receptor related protein (LRP). Dickkopf-1 (DKK1) is an antagonist of Wnt signaling that binds directly to LRP thereby inhibiting Wnt signal transduction[1]. Circulating DKK1 has been associated with bone mineral density (BMD)[2-5], vascular disease[2,6,3,7,8], and cancer[9,10] but little is known about its determinants. To our knowledge, there have been no studies of the heritability and genetics of circulating DKK1 concentrations. Therefore, we estimated the heritability of serum DKK1 levels and assessed the association of common genetic variants in the *DKK1* gene region with serum DKK1 protein levels in a sample of large, multi-generational African ancestry families.

METHODS

Tobago Family Health Study

Briefly, probands were recruited without regard to medical history from a population-based cohort study of body composition on the Caribbean island of Tobago[11]. Probands were eligible if they had a spouse willing to participate and had at least six living first-degree relatives aged ≥18 years residing in Tobago. All first-, second-, and third-degree relatives of the probands were invited to participate. The final sample consisted of 471 individuals belonging to 7 independent, large, multigenerational families. Serum DKK1 levels and *DKK1* genetic variants were measured in 422 individuals who form the basis of the current analyses (mean family size: 60; 3215 relative pairs). Tobago is a relatively small island population raising the potential for founder effects. However, we tested for inbreeding in our unrelated individuals using genome-wide SNP variation and have found no evidence of a founder effect (unpublished observations). Our findings are consistent with Tobago residents being descendants of ~10,000 West African slaves who settled on the island between 1765 and 1805[12]. The Tobago Division of Health and Social Services and the University of Pittsburgh Institutional Review Boards approved the study. Written informed consent was obtained from each participant.

Serum DKK1 Measurement

Blood samples were collected from participants in the morning after an overnight fast. Serum was separated and stored at –80°C until time of assay. Serum DKK1 levels were measured according to the manufacturer's protocol using a validated sandwich ELISA assay (R&D Systems, Minneapolis, MN, USA). Intra- and inter-assay coefficients of variation within our study were 6.9% and 8.5%, respectively.

Other Characteristics

Lifestyle and medical history related information was documented in participants by trained clinic staff through interview-administrated questionnaires. The Tobago population is predominately of West African origin with <6% admixture as assessed by ancestry informative markers[13]. Diabetes was defined as a fasting serum glucose level ≥ 126 mg/dl or current use of diabetes medication. Blood pressure was measured three times and was recorded as the average of the 2nd and 3rd reading. Hypertension was defined as a SBP ≥ 140 mmHg, DBP ≥ 90 mmHg and/or current use of antihypertensive medication. Smoking status was classified as either current or not, and participants reporting smoking <100 cigarettes in their lifetime were considered non-smokers. Alcohol consumption was coded as having ≥ 4 drinks per week (yes/no). Physical activity was assessed by the number of minutes walked per week and participants were dichotomized <60 minutes versus ≥ 60 minutes walked/week.

Genotyping

We used a single nucleotide polymorphism (SNP) tagging procedure to identify common variation 50KB up- and 30KB down-stream of the *DKK1* gene. We identified all common variation in this region with $\geq 10\%$ minor allele frequency (MAF) in the Yoruban population using the International HapMap database (Release 27, Phase II+III found at <http://hapmap.ncbi.nlm.nih.gov/>). Next, we used HaploView[14] to select the subset of SNPs that predicted the remainder of SNPs in this region with $r^2 \geq 0.80$. There were 25 SNPs that tagged the 42 common SNPs in the *DKK1* region with a mean r^2 of 0.974.

Genomic DNA was isolated from whole blood extracted by the salting out method or by Qiagen column procedure (Qiagen, Santa Clara, CA). Genotyping was completed by iPLEX mass spectroscopy (Sequenom Inc., San Diego, CA). Related individuals have genotype distributions that may not conform to Hardy Weinberg Equilibrium (HWE) and result in an inflated type I error[15]. To account for this bias, SNPs were tested for deviation from HWE using a more-stringent P-value threshold of <0.0001. All successfully genotyped SNPs met this criterion. Out of the 25 attempted SNPs only 1 failed to be genotyped (rs7916242). The average genotyping completeness rate was 95.0% and the average genotyping consensus rate among 35 blind replicate samples was 99.2%.

DKK1 mRNA Expression

We selected 16 individuals from each rs7069912 genotype matched on age and sex for mRNA analysis. Using 16 individuals per group and an alpha of 0.05 gave us 80% power to detect a 0.9 SD difference and 90% power to detect a 1 SD difference in *DKK1* mRNA levels between rs7069912 carriers and non-carriers. Total RNA was extracted from whole blood collected in PAXGene tubes that were stored at -80°C using PAXgene blood RNA kit (Qiagen, Valencia, CA). The RNA was quantified using nanoQuant (Tecan, Switzerland). Real time PCR with SYBR-green labeled probes was performed. *DKK1* expression levels were adjusted using beta-actin as an internal control ($CT = CT^{DKK1} - CT^{actin}$). The relative expression level was calculated using the mean expression level of the wild type (GG) genotype group ($CT = CT - \text{mean } CT^{GG}$) and the relative fold change was calculated using 2^{-CT} .

Statistical Analysis

Serum DKK1 concentrations were assessed for normality and log transformed prior to statistical analysis. Outliers, defined as ± 4 SD from the mean, were removed ($N=2$). Maximum likelihood methods were used to simultaneously model the effect of additive genetics, or heritability (h^2), fixed covariate effects (age, age² and sex), and error for serum DKK1 using the variance components framework implemented in the Sequential Oligogenic Linkage Analysis Routines (SOLAR) program[16,17]. SOLAR estimates the relationship between each relative pair and uses the covariance matrix to directly adjust phenotypic and genetic analyses for the non-independence of observations. We used SOLAR to estimate the residual heritability and the variance attributable to the fixed covariate effects of serum DKK1, as well as, each genotyped SNP.

All common SNPs were tested for Mendelian inconsistencies and any inconsistent genotypes were excluded. Between 0 and 10 genotypes were excluded for each SNP. Genetic association analyses of common variants with serum DKK1 levels were tested assuming an additive inheritance model by regressing DKK1 levels on the minor allele count (0, 1 or 2). All genetic association tests were adjusted for age, age² and sex. Menopausal status was not associated with serum DKK1 levels and was not included as a potential confounder in subsequent genetic association tests. To determine significant, statistically independent SNPs, we used backwards and forwards selection on all nominally significant ($P<0.05$) SNPs to determine the SNP set that explained the greatest variance in serum DKK1. Significance levels for individual SNPs were adjusted for multiple comparisons using a Bonferroni correction alpha level of 0.002 ($0.05/24$ SNPs= 0.002).

Gene expression levels were calculated as fold-difference in expression versus individual levels of actin expression. The expression data were then log transformed for analysis. Differences in mean expression by rs7069912 carrier status was tested using a T-test in individuals matched on age and sex.

RESULTS

Population Characteristics

The African ancestry family members were 61% female and on average aged 42.7 years (Table 1). Approximately 8% of the family members were diabetic and 28% were hypertensive, with women being more likely to be diabetic than men ($P<0.05$). There was a relatively low prevalence of smoking, alcohol intake and physical activity; however, men were more likely to smoke, drink alcohol and walk for ≥ 60 minutes per week than women (all $P<0.05$). Median serum DKK1 was 1724.1pg/mL and was significantly lower in women than men ($P=0.043$). There were no other correlates of serum DKK1 identified in our sample.

Genetic Determinants of Serum DKK1

The heritability of unadjusted serum DKK1 was 0.4569 ($P<0.0001$). Residual heritability after adjusting for age, age² and sex was 0.4460 ($P<0.0001$). Of the 24 successfully genotyped SNPs, 6 showed nominal significance ($P<0.05$) with serum DKK1 levels (Table

2). Each of the six nominally significant SNPs explained between 0.2-2.5% of the phenotypic variation in serum DKK1. One SNP, rs7069912 ($P=0.0013$), remained significantly associated with serum DKK1 levels after applying a Bonferroni adjustment for multiple comparisons. Individuals with two copies of the minor (A) allele of this SNP had 33% greater serum DKK1 levels than individuals with zero copies of this allele. Furthermore, additional analysis showed that rs7069912 and rs16928725 represented independent associations with serum DKK1 and collectively accounted for 4.63% of its phenotypic variance. Residual heritability after accounting for the effects of these two SNPs remained statistically significant at 0.4134 ($P<0.0001$). Additional adjustment for menopausal status did not impact our results (data not shown).

DKK1 Expression Levels

DKK1 expression in peripheral blood was significantly greater in carriers (genotype GA or AA) of the rs7069912 variant ($P=0.036$) than non-carriers (genotype GG). Median *DKK1* expression was about equivalent (1.0-fold) to the expression of actin in wild-type individuals. However, in carriers of the rs7069912 variant, median *DKK1* expression was 2.09-fold greater than that of actin (data not shown).

DISCUSSION

We found that serum DKK1 levels are highly heritable in African ancestry families. Of 24 SNPs genotyped in an 80KB region encompassing the *DKK1* gene, we found 6 with nominal significance ($P<0.05$) and rs7069912 in the 5' region remained statistically significant after adjusting for multiple comparisons. Carriers of the rs7069912 variant also had significantly greater *DKK1* mRNA expression in peripheral blood. This is the first study to investigate if genetic variation, in general or within the *DKK1* region specifically, is associated with serum DKK1 levels.

Participants who were homozygous for the minor allele (AA) of rs7069912 had 33%, or 0.5 SD, greater serum DKK1 than individuals with the wildtype genotype (GG). This SNP explained 2.5% of the overall variance in serum DKK1 and was associated with 2.1-fold greater *DKK1* mRNA expression in peripheral blood. This SNP is located about 2.5KB upstream of the *DKK1* gene transcription start site. The rs7069912 SNP is polymorphic in African ancestry populations (MAF: 0.25-0.34) but is monomorphic in European ancestry samples in public databases. Thus, this variant may represent an ethnicity-specific regulator of serum DKK1 variation. To our knowledge, rs7069912 has not been previously reported to be associated with any phenotype and there are no known functional consequences of this SNP. However, rs7069912 lies in a genomic region with several putative transcription factor-binding sites (UCSC Genome Browser; genome.ucsc.edu). For instance, the minor (A) allele is predicted to generate new AP1 and HNF transcription factor binding sites, as determined by EIDorado (Genomatix Software GmbH, Munich, Germany). Further studies will be required to determine the molecular mechanisms by which rs7069912 might influence *DKK1* mRNA expression and protein levels.

The other independently significant SNP (rs16928725) did not surpass multiple comparisons adjustment. Nonetheless, rs16928725 explained 2.25% of the phenotypic variance in serum

DKK1 concentrations. This SNP is located approximately 43KB upstream of *DKK1* in an intron of a neighboring gene, protein kinase, cGMP-dependent, type I (PRKG1). Individuals in our study with two copies of the minor (TT) allele had 45% lower DKK1 than individuals with the wildtype genotype (CC). Neither published literature nor *in silico* analysis suggests that there are functional consequences of this SNP, but it may be in linkage disequilibrium with a functional SNP not genotyped in our study. Even though rs7069912 and rs16928725 collectively accounted for ~5% of the phenotypic variation in serum DKK1 levels, the residual heritability after including these SNPs from the model was still highly significant at 0.4134 ($P < 0.0001$). This suggests that additional genetic variants contribute to serum DKK1 levels.

In conclusion, we have shown for the first time that serum DKK1 levels are highly heritable in men and women of African ancestry. We have also identified two common SNPs near the *Dickkopf-1* gene that explain ~5% of the phenotypic variance in serum DKK1 levels. One of these SNPs, rs7069912, is predicted to have functional consequences and is also associated with *DKK1* mRNA expression levels in peripheral blood. Taken together, these findings illuminate a strong genetic basis for this circulating Wnt antagonist.

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Table 1

Characteristics of the African Ancestry Family Members

Characteristic	All (N=422)	Men (N=166)	Women (N=256)
Age (years)	42.66±16.49	43.2±16.9	42.3±16.3
BMI (kg/m ²)	28.20±6.45	26.6±4.9*	29.2±7.1*
Diabetes (%)	8.7	7.1	9.8
Hypertension (%)	28.0	30.3	26.5
Current smoking (%)	4.8	11.7*	0.4*
>3 Alcoholic drinks/week (%)	13.3	30.3*	2.4*
Walk 60 min/week (%)	18.2	24.2*	14.3*
Serum DKK1 (pg/mL)	1724.1(1257.3-2808.0)	1916.9(1310.7-2884.9)*	1665.8(1223.4-2633.6)*

All values shown as mean±SD or %, as appropriate, except Serum DKK1 which is shown as median(IQR),.

* Characteristic significantly different between men and women, P<0.05

Table 2

Association of Common Single Nucleotide Variants in the *DKK1* gene region with Serum Dickkopf 1 Levels in African Ancestry Families

SNP	Alleles	MAF	Genome Position [#]	Position Relative <i>DKK1</i> [#]	Adjusted [*] Mean(SE) by Genotype			P-Value [§]	Covariance Explained
					1/1 [§]	1/2 [§]	2/2 [§]		
rs16928725	C/T	0.13	54031957	43 KB 5'	2114 (62)	2014 (113)	1156 (391)	0.0255	2.25%
rs11001452	A/C	0.28	54045395	29 KB 5'	2044 (77)	2108 (87)	2362 (205)	0.0478	0.56%
rs7069912	G/A	0.38	54071719	3 KB 5'	1890 (90)	2108 (78)	2512 (151)	0.0013	2.50%
rs10824351	T/C	0.36	54080807	3 KB 3'	2194 (85)	1957 (90)	1944 (141)	0.0391	0.19%
rs1590105	A/C	0.35	54082535	5 KB 3'	2233 (85)	2043 (83)	1855 (153)	0.0238	1.44%
rs10824381	G/A	0.34	54098259	20 KB 3'	2206 (87)	2026 (83)	1836 (174)	0.0389	1.78%

SNPs in **bold** face font were independently associated with serum DKK1 and collectively explained 4.63% of the phenotypic variance.

[#] Based on human genome build 19 (GRCh37)

^{*} Adjusted for age, age² and sex

[§] Where 1 and 2 represent the major and minor allele of each SNP, respectively

[§] Significance was assessed using and additive genetic model wherein the value of 0, 1 or 2 copies of the minor allele was regressed on serum DKK1.