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Association of the proprotein convertase subtilisin/kexin-type 2 (*PCSK2*) gene with type 2 diabetes in an African American population

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Abstract

In a genome-wide scan for type 2 diabetes (T2DM) in African American (AA) families, ordered subsets analysis (OSA) provided evidence for linkage to chromosome 20p in a subset with later age at diagnosis (max. LOD 2.57, $P = 0.008$). The proprotein convertase subtilisin/kexin-type 2 (*PCSK2*) gene is within the LOD-1 interval of this linkage peak. Twenty-nine single nucleotide polymorphisms (SNPs) were genotyped across this gene in 380 unrelated AA individuals with T2DM and end-stage renal disease (T2DM-ESRD), 278 AA controls, 96 European Americans (EA) and 120 Yoruba Nigerian (YRI) controls. In addition, 22 ancestry-informative markers (AIMs) were genotyped in all AA subjects, 120 YRI, and 96 EA controls. ADMIXMAP was used to model the distributions of admixture and generate score tests of allelic and haplotypic association. Association with T2DM was observed among 4 SNPs: rs2021785 (admixture-adjusted $P_a = 0.00014$), rs1609659 ($P_a = 0.028$), rs4814597 ($P_a = 0.039$) and rs2269023 ($P_a = 0.043$). None of the *PCSK2* SNPs were associated with age at T2DM diagnosis. A variant in the *PCSK2* gene, rs2021785, appears to play a role in susceptibility to T2DM in this AA population.

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Keywords

Proprotein convertase subtilisin/kexin 2; association; type 2 diabetes; haplotype; African American; admixture

Introduction

Ordered subset analysis (OSA) of a genome-wide scan for type 2 diabetes (T2DM) using 638 African American (AA) affected sibling pairs with T2DM (675 individuals from 247 families) provided evidence of linkage on chromosome 20p (max. LOD 2.57; $P = 0.008$) at 39 cM, closest to marker D20S470, in families with later pedigree mean age at diagnosis (13% of pedigrees) [1]. The mean age at T2DM diagnosis in linked pedigrees was 56.2 ± 4.5 years, while the mean age at diagnosis in unlinked pedigrees was 39.2 ± 7.3 years. Nonparametric linkage (NPL) regression interaction analysis also provided evidence of an interaction with later age at T2DM diagnosis ($P = 0.013$) at 43.5 cM on chromosome 20p, closest to marker D20S470.

The gene encoding the proprotein convertase subtilisin/kexin 2 (*PCSK2*) is located on chromosome 20p11.2. The cleavage of proinsulin is achieved by the concerted action of two specific endopeptidases; PCSK1/3 (EC 3.4.21.93) and *PCSK2* (EC 3.4.21.94) respectively. *PCSK2* is a type II proinsulin-processing enzyme involved in cleavage of des 31–32 proinsulin to insulin and C-peptide by the hydrolysis of the COOH-terminal side of Lys⁶⁴ and Arg⁶⁵ bond [2]. Defects affecting the catalytic activity of the prohormone-processing enzymes have recently been found to be associated with obesity and other metabolic disorders [3–4]. The etiology of hyperproinsulinemia is thought to be due to pancreatic β -cell dysfunction which is manifested in part by inadequate cleavage of proinsulin. Previous studies have shown that increased concentrations of proinsulin is a significant predictor of the development of T2DM in several ethnic groups [5–8]. In a report by Furuta *et al.* [9], it has been shown that increased levels of proinsulin and split proinsulin were detected in pancreatic islet cells isolated from homozygous *PCSK2* null mice.

The *PCSK2* gene consists of 12 exons and spans 257.6 kbp, encoding a 68kD protein product [10] that is expressed specifically in pancreatic islets, pituitary and brain [11–12]. Yoshida *et al.* [13] have reported that a microsatellite marker within the *PCSK2* gene is associated with T2DM in Japanese subjects.

We genotyped 29 SNPs at an average spacing of 8.7 kb in 380 AA T2DM cases and 278 AA controls to investigate the role of this positional candidate gene in susceptibility to T2DM and age at disease onset.

Research Design and Methods

Subjects

This study was conducted under Institutional Review Board approval from Wake Forest University School of Medicine and adhered to the tenets of the Declaration of Helsinki. We recruited 380 unrelated AA individuals with T2DM and end-stage renal disease (T2DM-ESRD). The clinical characteristics and recruitment of AA patients and controls have been previously described in detail [14]. The case population was recruited independently of the families used for linkage analyses [1]. We also recruited 278 unrelated AA controls, who denied personal knowledge of renal disease, diabetes, or the presence of first-degree relatives with ESRD. For admixture analyses, 282 unrelated European American (EA) controls were recruited using the same criteria. DNA extraction was performed using the PureGene system (Gentra Systems, Minneapolis, MN).

SNP selection and genotyping

Genetically, the AA populations in the US are mosaic admixtures of ancestral European and African chromosomal segments. In the absence of YRI and CEPH HapMap genotype data at the time of SNP selection we utilized several SNP databases such as Applied Biosystem's SNPbrowser Software (Foster City, CA), the SNP database (dbSNP), and SNPper. Only SNPs with a minor allele frequency (MAF) $\geq 10\%$ in AA were chosen. This yielded 29 SNPs at an average density of 1 SNP every 8.7 kb, with the largest gap 30kb and the smallest 4.1 kb.

Subsequent assessment of *PCSK2* coverage using data from the International HapMap Consortium was made. Considering only the Yoruba Nigerian dataset (YRI), 23 of the 29 *PCSK2* SNPs had been genotyped by the HapMap project (HapMap Data Release #20). Using the aggressive 2- and 3-marker tagging option implemented in the program Tagger [15], these 23 SNPs captured 82.1% of 280 HapMap *PCSK2* SNPs with MAF $> 10\%$ with $r^2 > 0.2$; 44.3% of SNPs with $r^2 > 0.5$; and 21.8% of SNPs with $r^2 > 0.8$.

Genotyping of SNPs was performed using a MassARRAY genotyping system (Sequenom Inc., San Diego, CA) [16]. Primer sequences are available on request. Fragment sizes were determined by mass spectrometry (Bruker Daltonics, Billerica, MA), and the genotypes were viewed and analyzed using SpectroTyper software (Sequenom Inc., San Diego, CA). Genotyping success rates for *PCSK2* SNPs were $\geq 95\%$ in cases and $\geq 93\%$ in controls.

Twenty-two admixture-informative markers (AIMs) were selected [17] and also genotyped using a MassARRAY genotyping system (Sequenom Inc., San Diego, CA) [16] in the AA sample population, 120 Yoruba Nigerians (YRI) from the Coriell Institute for Medical Research (Coriell Cell Repositories, Camden, NJ) and 282 EA. In addition, singly-associated SNPs and SNPs within the associated *PCSK2* haplotype blocks were genotyped in the same 120 YRI and 96 EA samples.

Direct Sequencing

Fisher's exact test was used to test each SNP for departure from Hardy-Weinberg equilibrium (HWE) in cases and controls separately. All SNPs that were inconsistent with Hardy-Weinberg proportions were sequenced in a subset of samples to ascertain the accuracy of genotype results. Six SNPs: rs890614, rs761017, rs6044702, rs934010, rs2859875 and rs2269002, were sequenced in 12 AA T2DM cases and in 12 AA controls using BigDye Terminator v.1.1 Cycle Sequencing Kits (Applied Biosystems, Foster City, CA) and an Applied Biosystems 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA), and data was viewed using Sequencher 4.1.4 software (Gene Codes Corporation, Ann Arbor, MI). For these 6 SNPs, sequences showed 100% concordance with genotype calls.

SNP rs2021785 was inconsistent with Hardy-Weinberg proportions in AA controls due to an excess of homozygotes. A 400bp amplification product containing rs2021785 was bi-directionally sequenced, and heterozygote calls found to be underestimated from Sequenom genotyping. Consequently, this SNP was sequenced in all individuals. Genotype calls from sequence data were consistent with Hardy-Weinberg proportions, and only genotypes obtained via sequencing were used for rs2021785 analyses presented.

Statistical Methods

Allelic and haplotypic association analyses—Haplotype block structure was established using Haploview 3.2 [18], extending the block if pairwise $D' \geq 0.7$ using the Solid Spine of LD option. Unadjusted allelic and genotypic association tests were assessed by a χ^2 statistic using 2×2 and 2×3 contingency tables. Haplotype frequencies were estimated using

the EM algorithm implemented in the software program Dandelion 1.26 using a permutation test of the multinomial likelihood ratio statistic using 1000 permutations [19].

Admixture adjustment of association results—The program ADMIXMAP 1.6.0 [20] was used to model the distributions of admixture and generate score tests of allelic and haplotypic association, adjusted for individual estimates of admixture. Estimates of ancestral allele frequencies were obtained from the genotyped YRI and EA control populations.

Age at diagnosis analyses—Genotypic association with age at T2DM and ESRD onset was assessed using analysis of variance (ANOVA), comparing the mean age at diagnosis across the three genotypic groups.

Results

Population characteristics

Characteristics of the AA participants are shown in Table 1. The T2DM-ESRD subjects had a mean age at diabetes diagnosis of 42.8 ± 11.8 years and a mean BMI of 29.3 ± 7.3 kg/m². The proportion of females in cases and controls was similar (60% and 59% respectively), and probably reflects a combination of participation and survival bias.

SNP and haplotype association analyses

Of the 29 SNPs, 24 were consistent with HWE. SNPs that deviated from HWE proportions in both controls and cases included rs890614 ($P = 0.020$; $P = 0.036$) and rs761017 ($P = 0.00002$; $P = 0.00018$), with P -values given for cases and controls respectively. Deviations from HWE only in controls were observed for rs6044702 ($P = 0.0003$) and rs934010 ($P = 0.0079$), and in cases only for rs2859875 ($P = 0.047$) and rs2269002 ($P = 0.023$). The concordance rate between sequencing genotype calls and Sequenom genotype calls for these SNPs in 12 AA cases and 12 AA controls was 100%.

ADMIXMAP [21] analysis indicated the mean proportions of African ancestry were 0.82 (95% credible interval: 0.79–0.84) for AA controls and 0.80 (95% credible interval: 0.78–0.83) in AA cases.

Allele frequencies, χ^2 and admixture-adjusted (P_a) P -values for all 29 SNPs from the single SNP association analysis are shown in Table 2. Four SNPs were significantly associated with T2DM-ESRD: rs4814597 ($P = 0.042$; $P_a = 0.038$), rs1609659 ($P = 0.032$; $P_a = 0.028$), rs2021785 ($P = <0.001$; $P_a = 0.00014$) and rs2269023 ($P = 0.037$; $P_a = 0.047$).

Single-SNP genotypic association results are presented in Table 3. Genotype frequencies and counts for each SNP are shown in Supplementary Table 1. Genotypic frequencies and counts for all 29 *PCSK2* SNPs are shown in Supplementary Table 1. Single marker tests of unadjusted genotypic association yielded significant results under all genotypic models for SNP rs2021785: Dominant $P = 0.0006$, Additive $P = 0.0003$, Recessive $P = 0.028$.

Haplotype block structure using the Solid Spine of LD option of Haploview, revealed seven blocks of high LD (Figure 1). The association observed in this block appears to be driven by a risk haplotype and a protective haplotype, with SNP rs2021785 distinguishing the two. The risk haplotype, CAAGCC, has a frequency of 7% in cases and 2.8% in controls (OR 2.53; $P = 0.017$; $P_a = 0.0027$). The second haplotype, CAAGTC, has a frequency of 14.7% in cases and 22.8% in controls (OR 0.59; $P = 0.008$; $P_a = 0.0003$). It therefore appears that the haplotypic association with this block, and any subhaplotypes that contain rs2021785 ($P < 0.001$ – $P = 0.003$ for 2-SNP to 6-SNP haplotypes), is driven by the association between this SNP and T2DM-ESRD (Supplementary Table 2).

Association analyses with age at diagnosis

Using ANOVA, none of the SNPs were significantly associated with age at T2DM diagnosis or ESRD onset ($P > 0.05$; data not shown).

Discussion

PCSK2, a strong functional candidate for T2DM, appears to play a role in susceptibility to T2DM-ESRD in an AA population. Of the SNPs analyzed we have identified an allelic variant – rs2021785, located in intron 8 – that shows evidence for association with T2DM-ESRD.

Marker rs2021785 is located 4.5kb from exon 8 and 12kb from exon 9. Haplotype frequencies and odds ratio results were similar across the associated 2- to 6-SNP haplotypes containing rs2021785 (Supplementary Table 2). Significant risk and protective haplotypes in this region were distinguished by rs2021785, suggesting this SNP is “driving” the positive haplotypic results. The relationship between the intronic rs2021785 T allele and T2DM susceptibility is unknown. The slightly more common “risk” C allele appears to be ancestral since it is present in the chimpanzee sequence. There were no splice sites predicted by the program SPLICEPREDICTOR [22] for either of the rs2021785 alleles, although a putative splice site is predicted 19 bp distal from this SNP. It is possible that this intronic polymorphism is in LD with the true casual SNP located some distance away. This hypothesis will need to be explored with denser SNP genotyping of this region and possibly sequencing.

Our initial interest in *PCSK2* was as a positional candidate for later age at T2DM diagnosis. We found no evidence for association between the 29 *PCSK2* SNPs genotyped and age at T2DM diagnosis. It is possible that a moderating protective variant at this locus is responsible for tempering the effect of other variants in the genome, or that older age is needed because the variant results in phenotype expression only in the presence of physiological conditions associated with older age (such as prior insulin resistance or higher BMI). These factors cannot be explored using our case-control population. Additionally, the mean age at T2DM diagnosis in cases is 42 years, so this population may not be representative of the linked families with an older mean age at diagnosis. However, the lack of association with age of diagnosis in cases is not surprising since only 13% of families were linked. This suggests the presence of a low frequency variant with quite strong effect, which is similar to what we observed with rs2021785.

Yoshida *et al.* [6] genotyped and identified an association between a microsatellite marker located in intron 2 of the *PCSK2* gene and T2DM in Japanese patients. This microsatellite was not genotyped in our population, although genotyped SNPs located in intron 2 (rs1340932, rs1340933, rs6080618, rs11696277, rs6136064, rs727472, rs2424079, rs981662 and rs6075196) were not associated with T2DM in our study population.

One limitation of this study is that the ascertainment scheme means that we cannot distinguish whether associations are with T2DM and/or nephropathy. However, given the growing prevalence and public health importance of both diseases, any insight into susceptibility to either disease is of value. Polymorphisms of *PCSK2* have previously been associated with T2DM [13]. However additional studies will be needed to determine the trait responsible for these associations. After Bonferroni correction for the 225 (Allelic: 29; 2-SNP moving Window: 28 tests, 3-SNP: 27; 4-SNP: 26; 5-SNP: 3; 6-SNP: 2; Allelic Admixture Adjustment: 23; Additive: 29, Dominant: 29 and Recessive: 29 tests) tests performed, association with SNP rs2021785 would remain significant ($P_a = 0.032$). The sample sizes of this study are relatively modest, although a significant impact (OR 1.72) on T2DM-ESRD risk has been detected. These results will need to be confirmed in larger, independent AA populations.

In light of modest allele frequency differences between populations of European and African ancestry, it is possible that population stratification could influence the results by creating false associations. The associated SNP rs2021785 had similar frequencies in AA controls (minor allele frequency (MAF) = 0.26) and the YRI control population (MAF = 0.28 data not shown), and a higher frequency in EA controls (0.35). While twenty-two AIMS were genotyped to adjust for estimates of admixture, it is unclear how accurately estimates of ancestry were captured and subsequently accounted for in adjusted analyses. There is little empiric data on this issue, and investigations of greater numbers of AIMS are underway in this population. AA cases and controls were recruited from the same geographic locations and the control population was not tested for diabetes in an attempt to reduce potential population stratification due to ascertainment biases. Although this approach may increase the likelihood of type 2 error, we were able to detect a positive association.

A variant of the *PCKS2* gene, rs2021785, appears to play a role in susceptibility to T2DM and/or nephropathy in this AA population. This may not represent the casual variant but may be in LD with a functional SNP. Functional studies investigating this region of the *PCKS2* gene will be necessary to explain the etiology of T2DM-ESRD risk.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

LD structure of the *PCSK2* gene in AA controls ($n = 278$), based on 29 genotyped SNPs, with blocks determined using the solid spine of LD option of Haploview. D' values are displayed in the squares: Empty squares represent a pairwise $D' = 1$; red squares represent high pairwise LD, slowly coloring down to white squares of low pairwise LD.

Table 1

Demographic data for cases and controls

a) AA T2DM-ESRD cases (n=380)			
Trait	Mean	Median	SD
Female (%)	60%	-	-
BMI (kg/m ²)	29.3	28.2	7.3
Age (years)	63.4	64	10.8
Age at T2DM diagnosis (years)	42.8	40	11.8
Age at ESRD diagnosis (years)	60.1	60	10.2
b) AA Controls (n=278) *			
Female (%)	59%		

* BMI and age data were unavailable for controls.

Table 2

Single-SNP allelic association analyses for PCSK2

SNP	Alleles (Major/Minor)	AA controls (n=278) MAF	AA cases (n=380) MAF	Allelic Association P-value	Admixture-adjusted P-value
rs4814597	G/A	0.348	0.406	0.047	0.039
rs1609659	A/G	0.363	0.423	0.041	0.028
rs1578405	G/A	0.486	0.482	0.269	0.29
rs6044702	T/A	0.487	0.438	-	-
rs1340932	C/T	0.148	0.182	0.118	0.12
rs1340933	C/T	0.158	0.181	0.312	0.3
rs6080618	G/A	0.138	0.137	0.961	0.98
rs11696277	G/A	0.09	0.123	0.054	0.059
rs6136064	A/G	0.285	0.288	0.927	0.84
rs727472	A/T	0.298	0.293	0.814	0.94
rs2424079	G/A	0.425	0.434	0.734	0.77
rs981662	A/G	0.333	0.355	0.439	0.43
rs6075196	C/T	0.193	0.19	0.922	0.922
rs890614	G/A	0.144	0.139	-	-
rs6044764	T/C	0.264	0.277	0.599	-
rs6044767	T/C	0.276	0.272	0.865	-
rs934010	A/T	0.417	0.395	-	-
rs6080678	C/T	0.429	0.425	0.877	0.92
rs2859875	C/T	0.228	0.237	-	-
rs2269002	G/A	0.391	0.383	-	-
rs742424	G/A	0.41	0.422	0.672	0.67
rs2281204	A/G	0.323	0.288	0.189	0.18
rs2021785	C/T	0.256	0.167	<0.001	0.00014
rs1010291	T/C	0.291	0.244	0.064	0.71
rs2269023	T/C	0.488	0.453	0.04	0.043
rs2284911	G/C	0.295	0.332	0.183	0.18
rs2284916	G/A	0.396	0.394	0.927	-
rs2269034	A/G	0.422	0.405	0.524	-

SNP	Alleles (Major/Minor)	AA controls (n=278) MAF	AA cases (n=380) MAF	Allelic Association P=	Admixture-adjusted P=
rs761017	G/C	0.138	0.175	-	-
MAF: Minor allele frequency. Bold: P value <0.05.					

Table 3
Single-SNP genotypic tests of association with T2DM-ESRD

SNP	Major/Minor alleles	Dominant P-value	OR (95% CI)	Additive P-value	OR (95% CI)	Recessive P-value	OR (95% CI)
rs4814597	G/A	0.019	1.48 (1.06–2.06)	0.042	1.27 (1.01–1.61)	0.436	1.2 (0.76–1.88)
rs1609659	A/G	0.007	1.58 (1.13–2.19)	0.030	1.29 (1.02–1.63)	0.609	1.12 (0.72–1.74)
rs1578405	G/A	0.922	0.98 (0.68–1.41)	0.284	0.89 (0.71–1.11)	0.095	0.73 (0.51–1.06)
rs6044702	T/A	0.006	0.59 (0.41–0.86)	0.069	0.8 (0.63–1.02)	0.979	1.01 (0.67–1.52)
rs1340932	C/T	0.156	1.29 (0.91–1.82)	-	-	-	$\frac{+}{-}$
rs1340933	C/T	0.337	1.19 (0.84–1.69)	-	-	-	$\frac{+}{-}$
rs6080618	G/A	0.907	1.02 (0.71–1.47)	-	-	-	$\frac{+}{-}$
rs11696277	G/A	0.046	1.51 (1.01–2.28)	-	-	-	$\frac{+}{-}$
rs6136064	A/G	0.883	0.98 (0.71–1.35)	0.905	1.02 (0.79–1.31)	0.574	1.19 (0.64–2.21)
rs727472	A/T	0.879	1.03 (0.74–1.42)	0.840	0.97 (0.75–1.26)	0.436	0.78 (0.42–1.45)
rs2424079	G/A	0.821	1.04 (0.74–1.46)	0.766	1.03 (0.83–1.3)	0.792	1.06 (0.7–1.59)
rs981662	A/G	0.455	1.13 (0.82–1.57)	0.433	1.1 (0.87–1.4)	0.620	1.13 (0.69–1.87)
rs6075196	C/T	0.914	1.02 (0.73–1.42)	0.925	0.99 (0.74–1.31)	0.585	0.8 (0.35–1.81)
rs890614	G/A	0.929	0.98 (0.68–1.42)	0.831	0.97 (0.71–1.31)	0.692	0.84 (0.36–1.98)
rs6044764	T/C	0.590	1.09 (0.79–1.51)	0.604	1.07 (0.82–1.4)	0.846	1.07 (0.55–2.09)
rs6044767	T/C	0.763	1.05 (0.76–1.45)	0.853	0.98 (0.76–1.26)	0.307	0.73 (0.4–1.34)
rs934010	A/T	0.984	1 (0.72–1.4)	0.475	0.92 (0.74–1.15)	0.175	0.76 (0.5–1.13)
rs6080678	C/T	0.695	1.07 (0.76–1.51)	0.876	0.98 (0.78–1.24)	0.442	0.85 (0.56–1.29)
rs2859875	C/T	0.638	1.08 (0.78–1.49)	0.701	1.06 (0.8–1.4)	0.939	0.97 (0.42–2.22)
rs2269002	G/A	0.743	1.06 (0.76–1.48)	0.775	0.96 (0.76–1.23)	0.304	0.78 (0.48–1.25)
rs742424	G/A	0.958	1.01 (0.72–1.41)	0.680	1.05 (0.84–1.32)	0.489	1.16 (0.76–1.77)
rs2281204	A/G	0.173	0.8 (0.58–1.1)	0.173	0.84 (0.66–1.08)	0.494	0.82 (0.47–1.45)
rs2021785	C/T	0.001	0.56 (0.4–0.78)	0.0003	0.6 (0.45–0.79)	0.028	0.42 (0.19–0.93)
rs1010291	T/C	0.139	0.78 (0.57–1.08)	0.065	0.78 (0.6–1.02)	0.107	0.58 (0.3–1.13)
rs2269023	T/C	0.029	0.66 (0.46–0.96)	0.039	0.78 (0.62–0.99)	0.257	0.8 (0.55–1.18)
rs2284911	G/C	0.185	1.25 (0.9–1.72)	0.178	1.18 (0.93–1.51)	0.445	1.23 (0.72–2.11)
rs2284916	G/A	0.803	0.96 (0.69–1.34)	0.944	0.99 (0.79–1.25)	0.847	1.04 (0.68–1.61)
rs2269034	A/G	0.198	0.8 (0.57–1.12)	0.537	0.93 (0.74–1.17)	0.624	1.11 (0.72–1.72)
rs761017	G/C	0.073	1.41 (0.97–2.04)	0.124	1.25 (0.94–1.67)	0.681	1.16 (0.57–2.36)

SNP	Major [†] /Minor alleles	Dominant* P-value	OR (95% CI)	Additive P-value	OR (95% CI)	Recessive P-value	OR (95% CI)
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* Only the dominant model was considered where the minor allele homozygote count for either cases or controls was <10. Test models refer to the minor allele.

[†] Dominant model. **Bold**: Only significant SNPs presented P-values ≤0.05. Test models refer to the minor allele.