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SHORT COMMUNICATION

Strong association of common variants in the *CDKN2A/CDKN2B* region with type 2 diabetes in French Europids

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Abstract
Aims/hypothesis Genome-wide association studies (GWASs) recently identified common variants in the *CDKN2A/CDKN2B* region on chromosome 9p as being strongly associated with type 2 diabetes. Since these association signals were not picked up by the French-Canadian GWAS, we sought to replicate these findings in the French Europid population and to further characterise the susceptibility variants at this novel locus.
Methods We genotyped 20 single nucleotide polymorphisms (SNPs) spanning the *CDKN2A/CDKN2B* locus in our type 2 diabetes case-control cohort. The association between *CDKN2A/CDKN2B* SNPs and quantitative metabolic traits was also examined in the normoglycaemic participants comprising the control cohort.
Results We report replication of the strong association of rs10811661 with type 2 diabetes found in the GWASs ($p = 3.8 \times 10^{-7}$; OR 1.43 [95% CI 1.24–1.64]). The other *CDKN2A/CDKN2B* susceptibility variant, rs564398, did not attain statistical significance ($p=0.053$; OR 1.11 [95% CI 1.00–1.24]) in the present study. We also obtained several additional nominal association signals ($p<0.05$) at the *CDKN2A/CDKN2B* locus; however, only the rs3218018 result ($p=0.002$) survived Bonferroni correction for multiple testing (adjusted $p=0.04$).
Conclusions/interpretation Our comprehensive association study of common variation spanning the *CDKN2A/CDKN2B* locus confirms the strong association between the distal susceptibility variant rs10811661 and type 2 diabetes in the French population. Further genetic and functional studies

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are required to identify the aetiological variants at this locus and determine the cellular and physiological mechanisms by which they act to modulate type 2 diabetes susceptibility.

Keywords Association · CDKN2A/CDKN2B · Common variant · Replication · Single nucleotide polymorphism · SNP · tag SNP · Type 2 diabetes

Abbreviations

GWAS	genome-wide association study
LD	linkage disequilibrium
MAF	minor allele frequency
SNP	single nucleotide polymorphism

Introduction

CDKN2A and *CDKN2B* are adjacent cyclin-dependent kinase inhibitor genes on chromosome 9p. *CDKN2A* inhibits CDK4, a key regulator of pancreatic beta cell replication [1–3]. In mice, *Cdkn2a* overexpression leads to islet hypoplasia and diabetes [4]. Meta-analysis of genotype data from genome-wide association studies (GWASs) in northern Europeans have confirmed that single nucleotide polymorphisms (SNPs) rs10811661 and rs564398 in the *CDKN2A/CDKN2B* region are type 2 diabetes susceptibility variants, although the combined evidence for rs10811661 ($p = 7.8 \times 10^{-15}$) is far stronger than that for rs564398 ($p = 1.2 \times 10^{-7}$) [5–7].

The French-Canadian GWAS [8] obtained nominal association signals for proxies ($r^2 \geq 0.9$) of rs10811661 (rs2383208, $p = 2 \times 10^{-4}$) and rs564398 (rs1063192, $p = 0.02$), but they were above the stage 1 significance threshold ($p = 5 \times 10^{-5}$) and therefore were not reported (Sladek et al. [8]). We sought to confirm these findings in a larger study and to further characterise the susceptibility variants at this novel locus. To this end, we performed a detailed association study of HapMap Phase II tag SNPs spanning the *CDKN2A/CDKN2B* locus in 3,093 French Europids.

Methods

Case–control participants All participants were of French Europid ancestry. Individuals identified by Sladek et al. [8] to lie outside the HapMap northern and western Europe CEU ancestry cluster were excluded from the study. Type 2 diabetic participants were diabetic patients reporting a fasting blood glucose concentration ≥7.0mmol/l [9]. Normoglycaemic control participants were selected to have a fasting blood glucose concentration <7.0mmol/l [9]. The diabetic patient group was composed of: (1) 372 probands from diabetic families [10], recruited in Lille; and (2) 1,083 patients with a family history of type 2 diabetes mellitus, recruited at the Corbeil-Essonne Hospital. Control participants were composed of: (1) 353 normoglycaemic parents from type 2 diabetic families; (2) 543 participants from a prospective population-based cohort study [11]; and (3) 742 participants selected from the cohort of a large prospective study of insulin resistance in French participants [12]. In total, the case–control cohort comprised 1,455 type 2 diabetic patients (age 60±12 years; BMI 29.0±6.0 kg/m^2; 56% men, 44% women) and 1,638 normoglycaemic participants (age 54±13 years; BMI, 24.1±3.3 kg/m^2; 43% men, 57% women). Of these samples, 503 patients and 669 control participants were typed in common between the present study and stage 1 of an earlier GWAS [8], which comprised 694 patients and 669 control participants. Informed consent was obtained from all participants and the study was approved by the local ethics committees.

Statistical power The case–control cohort comprised 1,455 type 2 diabetic participants and 1,638 normoglycaemic participants. At α=0.05, this sample size provided 87 and 67% power [13] respectively, to detect the variants rs10811661 and rs564398, assuming allele frequencies of 0.80 and 0.62, a disease prevalence of 0.1, heterozygote relative risks of 1.20 and 1.12 [5–7], and a multiplicative model.

CDKN2A/CDKN2B tag SNP selection The target region for tag SNP selection across the *CDKN2A/CDKN2B* locus was Chr9:21,947,752..22,009,312 (NCBI36). Using the Haploview [14] implementation of the Tagger algorithm [16], a total of 18 HapMap Phase II multimarker tagging SNPs (http://www.hapmap.org) were required to tag the region at r^2 and minor allele frequency (MAF) thresholds of 0.9 and 0.05, respectively. In addition, the GWAS-identified type 2 diabetes susceptibility SNPs rs10811661 and rs564398 [5–7] were added to the SNP set. Thus, 18 tagging SNPs, plus rs10811661 and rs564398, making a total of 20 SNPs, were tested for association with type 2 diabetes.

SNP genotyping Genotyping was performed with the Sequenom iPLEX system [15]. SNP genotype frequencies were tested for accordance with Hardy–Weinberg equilibrium using χ^2 analysis. SNPs with a call rate <90%, a Hardy–Weinberg p<0.05 or those that exhibited poorly defined genotype clusters were disqualified from association analysis.

Statistical analyses To test for association of *CDKN2A/CDKN2B* SNPs with type 2 diabetes, χ^2 analysis of allele and genotype counts was performed. Pairwise SNP linkage disequilibrium (LD) values were calculated from the genotype data of the control cohort using Haploview [14].

Quantitative phenotypes were available for 1,539 of the normoglycaemic participants in the control cohort and were log-transformed and adjusted for age, sex and BMI, as appropriate. SNPs were tested for association with adjusted quantitative traits (BMI, fasting glucose, fasting insulin) using SPSS 14.0 (SPSS, Chicago, IL, USA) with the ANOVA test under a codominant model. Quantitative trait *p* values are presented uncorrected for multiple testing.

Results and discussion

The first generation GWASs for type 2 diabetes [5–7], although successful in identifying susceptibility variants in the *CDKN2A/CDKN2B* region, provided incomplete coverage of common SNPs [16] spanning the gene locus. In this follow-up study, we set out to achieve an efficient and comprehensive coverage [17] of common variation at the

Table 1 Association of *CDKN2A/CDKN2B* SNPs with type 2 diabetes in French Europids

SNP	Chr Pos (bp) NCBI36	Gene region	Allele	Allele count		*p* value	OR (95% CI)
				T2D (%)	NG (%)		
rs3731257	21,956,221	*CDKN2A* 3′ downstream (+1530 bp)	G	2,000 (72)	2,258 (72)	0.818	1.01 (0.90–1.14)
			A	772 (28)	860 (28)		
rs3731239	21,964,218	*CDKN2A* intron 2	A	1,739 (67)	2,039 (66)	0.505	1.04 (0.93–1.16)
			G	865 (33)	1,053 (34)		
rs4074785	21,971,583	*CDKN2A* intron 2	G	2,509 (93)	2,821 (92)	0.027	1.25 (1.03–1.52)
			A	183 (7)	257 (8)		
rs3731222	21,973,914	*CDKN2A* intron 2	T	2,258 (85)	2,698 (86)	0.462	1.06 (0.91–1.23)
			C	384 (15)	434 (14)		
rs3731201	21,978,896	*CDKN2A* intron 2	C	2,273 (84)	2,661 (87)	0.007	1.12 (1.06–1.42)
			T	427 (16)	409 (13)		
rs3731198	21,979,477	*CDKN2A* intron 2	T	2,255 (85)	2,692 (86)	0.485	1.05 (0.91–1.22)
			C	385 (15)	436 (14)		
rs7036656	21,980,457	*CDKN2A* intron 2	T	1,813 (69)	2,240 (72)	0.007	1.17 (1.04–1.31)
			C	825 (31)	872 (28)		
rs3218020	21,987,872	*CDKN2A/2B* intergenic	G	1,657 (60)	1,871 (61)	0.449	1.04 (0.94–1.16)
			A	1,115 (40)	1,209 (39)		
rs3218018	21,988,139	*CDKN2A/2B* intergenic	T	2,472 (89)	2,867 (92)	0.002	1.31(1.10–1.56)
			G	298 (11)	263 (8)		
rs3218009	21,988,757	*CDKN2A/2B* intergenic	C	2,486 (91)	2,809 (90)	0.768	1.03 (0.86–1.22)
			G	256 (9)	297 (10)		
rs3217992	21,993,223	*CDKN2B* exon 2	C	1,535 (58)	1,767 (58)	0.896	1.00 (0.91–1.12)
			T	1,105 (42)	1,281 (42)		
rs1063192	21,993,367	*CDKN2B* exon 2	G	1,645 (62)	1,824 (60)	0.224	1.07 (0.96–1.19)
			A	1,011 (38)	1,198 (40)		
rs2069422	21,998,026	*CDKN2B* exon 1	G	2,370 (89)	2,812 (91)	0.006	1.27 (1.07–1.51)
			T	296 (11)	276 (9)		
rs495490	22,000,412	*CDKN2B* 5′ upstream (−1100 bp)	A	2,464 (91)	2,786 (90)	0.524	1.06 (0.89–1.26)
			G	254 (9)	304 (10)		
rs573687	22,001,642	*CDKN2B* 5′ upstream (−2,330 bp)	G	1,782 (68)	2,063 (67)	0.190	1.08 (0.96–1.20)
			A	830 (32)	1,035 (33)		
rs13298881	22,002,051	*CDKN2B* 5′ upstream (−2,739 bp)	T	2,349 (87)	2,744 (89)	0.109	1.14 (0.97–1.33)
			C	343 (13)	352 (11)		
rs10811640	22,003,411	*CDKN2B* 5′ upstream (−4,099 bp)	G	1,307 (51)	1,533 (51)	0.919	1.01 (090–1.12)
			T	1,249 (49)	1,457 (49)		
rs523096	22,009,129	*CDKN2B* 5′ upstream (−9,817 bp)	A	1,635 (61)	1,793 (59)	0.080	1.10 (0.99–1.22)
			G	1,029 (39)	1,241 (41)		
rs564398	22,019,547	*CDKN2B* 5′ upstream (−20,225 bp)	T	1,764 (64)	1,916 (62)	0.053	1.11 (1.00–1.24)
			C	978 (36)	1,180 (38)		
rs10811661	22,124,094	*CDKN2B* 5′ upstream (−124,782 bp)	T	2,342 (85)	2,473 (80)	3.8×10^{-7}	1.43 (1.24–1.64)
			C	410 (15)	617 (20)		

p values are for χ^2 analysis

Chr Pos, chromosome position (bp); NG, normoglycaemic control participants; T2D, type 2 diabetic patients

CDKN2A/CDKN2B locus, by using a multimarker tagging approach at $r^2 \geq 0.9$. A total of 18 HapMap Phase II tag SNPs spanning the *CDKN2A/CDKN2B* locus, as well as the distal susceptibility variants rs10811661 and rs564398, were tested for association with type 2 diabetes (Table 1). The genotype counts for all SNPs are presented in the Electronic supplementary material (ESM) Table 1.

We report replication of the strong association between the major allele of rs10811661 and type 2 diabetes ($p = 3.8 \times 10^{-7}$; OR 1.43 [95% CI 1.24–1.64]), previously found in the GWASs [5–7]. In agreement with the GWASs [5–7], our data indicate that rs10811661, which is 124kb upstream of the *CDKN2A/CDKN2B* locus, is not in LD with any of the tag SNPs spanning the locus (Fig. 1).

The other 'confirmed' susceptibility variant, rs564398, did not quite attain statistical significance ($p=0.053$; OR 1.11 [95% CI 1.00–1.24]) in the present study. This was not unexpected as our study was underpowered to detect this variant (see Methods section). The status of rs564398 as a bona fide susceptibility variant appears to be largely driven by the Wellcome Trust Case Control Consortium study (13,965 participants, $p=1.13 \times 10^{-6}$) since the Finland–United States Investigation of NIDDM Genetics result (4,808 participants, $p=0.039$) was similar to ours, while the meta-analysed Diabetes Genetics Initiative data (13,781 participants, $p=0.5$) showed up negative for this variant [5–7].

We also obtained several additional nominal association signals ($p<0.05$) at the *CDKN2A/CDKN2B* locus (Table 1). However, only the rs3218018 result ($p=0.002$) survived Bonferroni correction for multiple testing (adjusted $p=0.04$). This SNP (MAF=8%) is correlated ($r^2=0.83$) with the *CDKN2B* exon 1 SNP rs2069422 (MAF=9%), which also exhibited nominal association ($p=0.006$).

The six SNPs nominally associated with type 2 diabetes were also tested for association with the quantitative traits

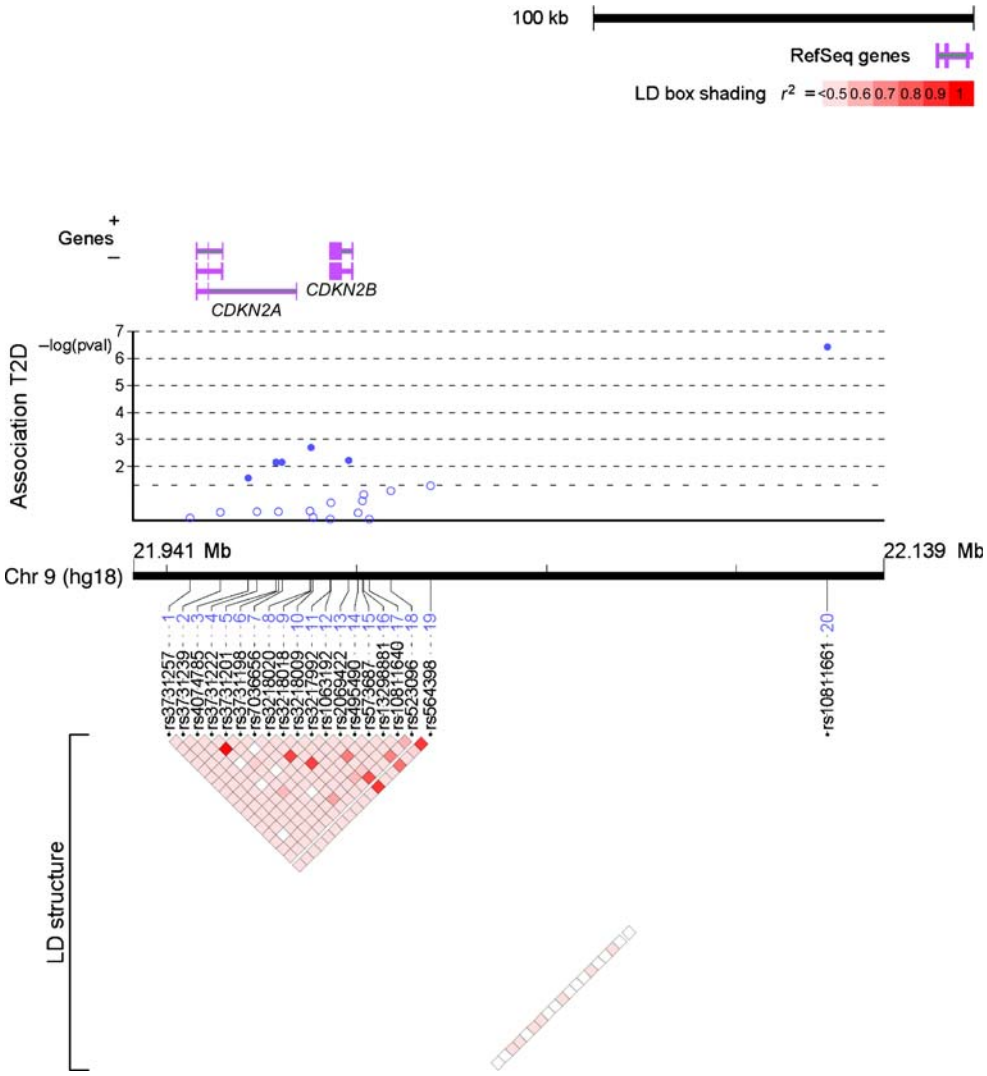

Fig. 1 Type 2 diabetes association signals and pattern of linkage disequilibrium across the *CDKN2A/CDKN2B* region. Plot of pairwise SNP r^2 values calculated from control genotype data. Association results ($-\log_{10}$ p value) and RefSeq genes are shown. The plot was drawn to scale using LocusView (T. Petryshen, A. Kirby, M. Ainscow, unpublished software). T2D, type 2 diabetes mellitus

Table 2 Association of *CDKN2A/CDKN2B* SNPs with quantitative traits BMI, fasting glucose and fasting insulin

Parameter	Genotype						*p* value
rs4074785	GG	*n*	GA	*n*	AA	*n*	
BMI (kg/m^2)	24.40±3.32	1,155	24.29±3.38	204	23.15±2.64	15	0.313
Glucose (mmol/l)	5.21±0.57	1,041	5.23±0.61	182	5.06±0.43	15	0.427
Insulin (pmol/l)	49.28±33.88	602	53.59±36.15	110	37.37±20.69	9	0.080
rs3731201	TT	*n*	TC	*n*	CC	*n*	
BMI (kg/m^2)	24.24±3.17	1,034	24.83±3.73	305	23.74±4.38	29	0.017
Glucose (mmol/l)	5.21±0.58	927	5.24±0.55	278	4.97±0.40	28	0.058
Insulin (pmol/l)	49.49±36.55	528	50.16±25.06	171	49.78±39.11	20	0.889
rs7036656	TT	*n*	TC	*n*	CC	*n*	
BMI (kg/m^2)	24.32±3.20	732	24.46±3.48	523	24.15±3.47	121	0.788
Glucose (mmol/l)	5.22±0.58	663	5.17±0.57	475	5.14±0.52	106	0.248
Insulin (pmol/l)	50.81±40.30	369	49.51±25.81	293	46.26±25.42	74	0.728
rs3218018	TT	*n*	TG	*n*	GG	*n*	
BMI (kg/m^2)	24.31±3.25	1,167	24.63±3.75	217	23.81±3.64	8	0.489
Glucose (mmol/l)	5.21±0.58	1,054	5.21±0.54	194	4.90±0.21	8	0.462
Insulin (pmol/l)	50.30±35.44	604	47.15±25.90	118	48.71±38.36	6	0.288
rs2069422	TT	*n*	TG	*n*	GG	*n*	
BMI (kg/m^2)	24.33±3.28	1,140	24.59±3.55	228	23.83±3.41	10	0.603
Glucose (mmol/l)	5.22±0.58	1,029	5.20±0.54	204	4.91±0.20	9	0.402
Insulin (pmol/l)	50.13±35.65	589	47.05±23.99	124	65.56±56.70	7	0.592
rs10811661	TT	*n*	TC	*n*	CC	*n*	
BMI (kg/m^2)	24.25±3.20	875	24.55±3.51	438	23.83±3.17	59	0.148
Glucose (mmol/l)	5.21±0.57	794	5.23±0.59	393	5.14±0.55	50	0.721
Insulin (pmol/l)	49.30±28.36	456	51.46±44.74	235	47.41±22.39	30	0.697

Data are presented as mean±SD
Quantitative traits were ln-transformed and adjusted for the covariates sex, age and BMI, as appropriate
SNPs were tested for association with adjusted quantitative traits using the ANOVA test under a codominant model

BMI, fasting glucose and fasting insulin. The only association found was with rs3731201 (Table 2). The minor allele of this variant was weakly associated with lower BMI (p=0.017) and also showed a trend towards lower glucose levels (p=0.06). However, it was also the minor allele that was associated with type 2 diabetes (p=0.007). Thus, these associations are physiologically inconsistent and probably indicate that these qualitative and/or quantitative trait associations are false positives.

In conclusion, our comprehensive association study of common variation spanning the *CDKN2A/CDKN2B* locus provides further confirmation of the strong association between the distal susceptibility variant rs10811661 and type 2 diabetes in the French population. Further genetic and functional studies are required to identify the aetiological variants at this locus and determine the cellular and physiological mechanisms by which they act to modulate type 2 diabetes susceptibility.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

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