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ScienceDirect
Molecular Genetics and Metabolism 93 (2008) 200–209
Molecular Genetics and Metabolism
www.elsevier.com/locate/ymgme

Single nucleotide polymorphisms in genes encoding LKB1 (*STK11*), TORC2 (*CRTC2*) and AMPK α2-subunit (*PRKAA2*) and risk of type 2 diabetes

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Received 18 June 2007; received in revised form 30 August 2007; accepted 31 August 2007
Available online 18 October 2007

Abstract

The LKB1–AMPK–TORC2 signaling pathway controls glucose homeostasis in the liver, and mediates therapeutic effects of insulin-sensitizing antidiabetic agents. To examine whether genetic variations in genes encoding components of this signaling pathway contribute to increased susceptibility to type 2 diabetes, we screened *STK11* (LKB1) and *CRTC2* (TORC2) genes for genetic variants and conducted a case-control study in 1787 unrelated Japanese individuals. Additionally, the previously described association between the *PRKAA2* (AMPK α2-subunit) haplotype and type 2 diabetes was tested for replication. We observed associations of nominal significance with two SNPs, an intronic SNP in the *STK11* (rs741765; OR 1.33, 95% CI 1.05–1.67, $p = 0.017$, under a recessive genetic model), and a non-synonymous SNP in the *CRTC2* (6909C > T: Arg379Cys; OR 3.01, 95% CI 1.18–7.66, $p = 0.016$, under a dominant model), although neither withstood correction for multiple testing. We were unable to replicate the association between the *PRKAA2* haplotype and type 2 diabetes: however, in the single SNP evaluation, an intronic *PRKAA2* SNP (rs1418442) that had previously been reported to be associated with serum cholesterol levels in Caucasian females showed a weak association (OR 0.62, 95% CI 0.40–0.96, $p = 0.030$, under a recessive model). Among the three genes investigated herein, gene–gene (SNP–SNP) interaction studies provided evidence for an interaction between *STK11* and *CRTC2* influencing susceptibility to type 2 diabetes. Our findings suggest that genetic variants of LKB1–AMPK–TORC2 pathway components may exert a weak influence on the occurrence of type 2 diabetes in Japanese.

Keywords: Single nucleotide polymorphism; Type 2 diabetes; Adenosine 5′-monophosphate-activated protein kinase; *PRKAA2*; *STK11*; *CRTC2*; Case-control association study

Introduction

AMP-activated protein kinase (AMPK) has recently received considerable attention because: (i) AMPK activation is a major regulator of both glucose and lipid metabolism connected with cellular energy status, and (ii) the antidiabetic drugs metformin and rosiglitazone, as well as antidiabetic adipokines such as adiponectin, improve insulin sensitivity by activating AMPK [1,2]. AMPK is present in various tissues, including the liver and skeletal muscle, and exists as a heterotrimer composed of a catalytic α-subunit and two regulatory β- and γ-subunits. In humans, two isoforms each of the α- and β-subunits (α1 and α2, and β1 and β2) and three isoforms of the γ-subunit (γ1, γ2 and γ3), all encoded by separate genes, have been identified [3]. It has been described that AMPK α2-knockout mice exhibit increased sensitivity to diet-induced obesity and insulin resistance, whereas α1-knockout mice have no apparent

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doi:10.1016/j.ymgme.2007.08.125

metabolic defects [4,5]. Both sequential phosphorylation events and transcriptional regulation of key enzymes or transcription factors are involved in AMPK-mediated regulation [3]; AMPK is known to be activated not only via an allosteric mechanism by AMP, but also through phosphorylation of a key threonine residue (Thr172) on the α-catalytic subunit. In the liver, this is catalyzed by LKB1, a tumor suppressor gene responsible for the Peutz–Jeghers syndrome [6], and deletion of hepatic LKB1 in mice has been shown to result in a near complete loss of AMPK activity, leading to increased gluconeogenesis and lipogenic gene expression [7]. In the hepatocytes of these mice, TORC2, a transcriptional coactivator of CREB [8], is dephosphorylated and translocates from the cytoplasm to the nucleus, which in turn stimulates PGC-1α expression, which is a strong transcription coactivator of the genes encoding key gluconeogenic enzymes, such as PEPCK and G6Pase. Hence, adenovirus-mediated inhibition of hepatic TORC2 reduced PGC-1α expression and normalized blood glucose levels, indicating that TORC2 is a critical target of LKB1/AMPK signals in the regulation of gluconeogenesis [7].

It has been suggested that early metabolic changes in peripheral tissues such as the liver, skeletal muscle and adipose could initiate the subsequent development of insulin resistance, obesity and type 2 diabetes (T2D) [9]. Therefore, genes that encode components of the AMPK signaling pathway, including various AMPK subunits, LKB1 and TORC2, are intriguing candidates which might explain the inherited basis of T2D. In addition to being strong functional candidates, some of these genes map to chromosomal regions previously implicated in T2D and related traits: e.g. the *PRKAA2* (AMPK α2-subunit gene) locus on 1p36-32 (Japanese [10], Northern Chinese [11]), *STK11* (LKB1 gene) on 19p13.3 (GENNID study [12]) and *CRTC2* (TORC2 gene) on 1q21 (at least eight different populations; for review, see [13,14]). Moreover, genetic variants in *PRKAA2* were previously examined for associations with T2D in at least two ethnic groups. Although Sun et al. [15] failed to detect a significant association in their large Caucasian populations, Horikoshi et al. [16] genotyped *PRKAA2* SNPs and found a significant association between one SNP haplotype (haplotype "AGTAAT") and T2D in multiple independent Japanese sample sets. They further demonstrated the SNP rs2051040 to be associated with decreased insulin sensitivity in non-diabetic subjects as assessed by the homeostasis model assessment of insulin resistance (HOMA-IR) index.

Given the strong functional and positional candidacy of genes involved in the AMPK signaling cascade, and the lack of any previous analysis of *STK11* and *CRTC2* genes in T2D, in this study, we first systematically screened all exons including adjacent splice sites of the two genes. We then tested SNPs and haplotypes for associations with T2D in a case-control population consisting of 911 Japanese T2D patients and 876 control subjects. Additionally, SNPs in the *PRKAA2* gene were analyzed for replication of previous genetic findings.

Subjects and methods

Study subjects

This study was conducted in accordance with the tenets of the Declaration of Helsinki. All subjects consented to participate in the process approved by the Ethics Committee for Human Genome/Gene Research at the University of Tokushima. The study population consisted of 911 unrelated Japanese patients with T2D (50.2% females; age at recruitment 63.5 ± 9.9 [SD] years) and 876 unrelated control subjects (50.9% females; 37.3 ± 11.7 years). Subject recruitment and clinical characteristics were previously described in detail [17,18]. Genomic DNAs were extracted from peripheral blood leukocytes or Epstein–Barr virus immortalized B-lymphoblasts by standard techniques.

Identification and genotyping of SNPs

For the *STK11* and *CRTC2* genes, all exons (10 and 14 exons, respectively) and relevant intron–exon boundaries were screened by PCR direct-sequencing. The screening DNA panel consisted of 32 unrelated T2D subjects randomly selected from among the 911 patients. PCR was carried out using AmpliTaq Gold DNA polymerase (Perkin-Elmer, Norwalk, CT, USA), followed by purification of the product with ExoSAP-IT (USB, Cleveland, OH, USA) treatment to remove the residual primers. All primer sequences and PCR conditions are available upon request. Sequencing reactions were conducted on both strands using an ABI PRISM BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) and an ABI 3730XL automated sequencer (Applied Biosystems). Additional intergenic and intronic SNPs in the *STK11* and *CRTC2* gene regions were selected either from dbSNP (http://www.ncbi.nlm.nih.gov/SNP/) or HapMap (http://www.hapmap.org/) database. Those were validated by direct-sequencing in the DNA screening panel for their marker informativity in the Japanese population. For the *PRKAA2* gene, six SNPs (rs2051040, rs2796495, rs2143754, rs1418442, rs932447, rs3738568) were selected based on a report from Horikoshi et al. [16], and were validated as well.

All SNPs except for the three *CRTC2* SNPs were genotyped using TaqMan SNP genotyping assays and an ABI 7900HT sequence detection system (Applied Biosystems) as previously described [17]. All TaqMan primers, probes, and conditions for genotyping are available upon request. For the three *CRTC2* SNPs, 6909C > T, 7042C > G and 9093C > T, genotyping was carried out by direct-sequencing, because of problems with TaqMan assay development.

Statistical analysis

All data management and statistical computations were performed using SPSS for Windows (Version 12.0; SPSS Japan Inc., Tokyo, Japan), SNPAlyze Pro software (Version 5.1; Dynacom, Yokohama, Japan) and GOLD software (http://www.sph.umich.edu/csg/abecasis/GOLD/docs/ldmax.html). Power calculations were conducted with the PS power and sample-size program (http://biostat.mc.vanderbilt.edu/twiki/bin/view/Main/PowerSampleSize [19]). Deviations from Hardy–Weinberg equilibrium (HWE) were tested using a χ^2 goodness-of-fit test. The χ^2 test was used to compare SNP frequencies between patients and controls. Analyses were also performed assuming dominant, co-dominant and recessive genetic models, and the *p*-values, crude odds ratios (ORs) and their 95% confidence interval (CI) ranges were calculated. Logistic regression was used to estimate both unadjusted and age-, gender- and body mass index (BMI)-adjusted ORs. Correction for multiple testing was performed using Storey's *q*-value method [20] employing the QVALUE program, in which *p*-values were adjusted according to the experimental false discovery rate (FDR). If *q*-values $\leqslant 0.05$ are taken as significant, there is an FDR of 5% among the significant associations. The SNP haplotypes and their

frequencies were estimated by the maximum-likelihood method with an expectation–maximization algorithm. Permutation *p*-values were calculated by comparing haplotype frequencies between cases and controls on the basis of 10,000 replications. Logistic regression models were used to test for gene–gene (locus–locus) interactions for both "additive only" and "additive and dominant" models using Genetic Association Interaction Analysis (GAIA) software [21], a web-based application (http://u004.pc.uwcm.ac.uk/biostat/stuart/gaia.html), in which correction for multiple testing was performed by permuting the interaction model terms.

Results

Identification of SNPs in the STK11 and CRTC2 genes

Detailed descriptions of sequence variations of the *STK11* and *CRTC2* genes have not been reported previously. We therefore screened the entire coding portions of the two genes by direct-sequencing for putative SNPs in a DNA panel prepared from 32 unrelated Japanese T2D patients. We discovered a total of 25 SNPs, 18 of which were in the *STK11* and 7 in the *CRTC2*. The locations of SNPs on the map of the corresponding genes are presented in Fig. 1 (boxed letters). These included five non-synonymous SNPs: *STK11*;Phe354Leu (16212C > G), *CRTC2*;Met147Val (rs11264680), *CRTC2*;Arg379Cys (6909C > T), *CRTC2*;Ala423Gly (7042C > G) and *CRTC2*;Arg482Trp (9093C > T), and two SNPs in 3′-untranslated regions: *STK11*;21347C > A and *CRTC2*; rs8450. Additionally, to provide dense coverage across the genes and their putative regulatory regions, we searched for additional SNPs in the public databases, and validated them through sequencing of the same DNA panel (9 and 7 SNPs for the *STK11* and *CRTC2* genes, respectively). Furthermore, we selected the six *PRKAA2* SNPs that form the previously reported T2D-susceptibility haplotype [16]. Information on all of the SNPs studied in the initial DNA panel is summarized in Table 1.

Characterization of the linkage disequilibrium (LD) structure

SNP genotype data from the initial DNA panel ($n = 32$) was used to roughly characterize the extent and pattern of LD for each gene locus. All SNPs that were in HWE and having a minor allele frequency (MAF) of >0.05 were analyzed using the two common measures of LD, $|D'|$ and r^2

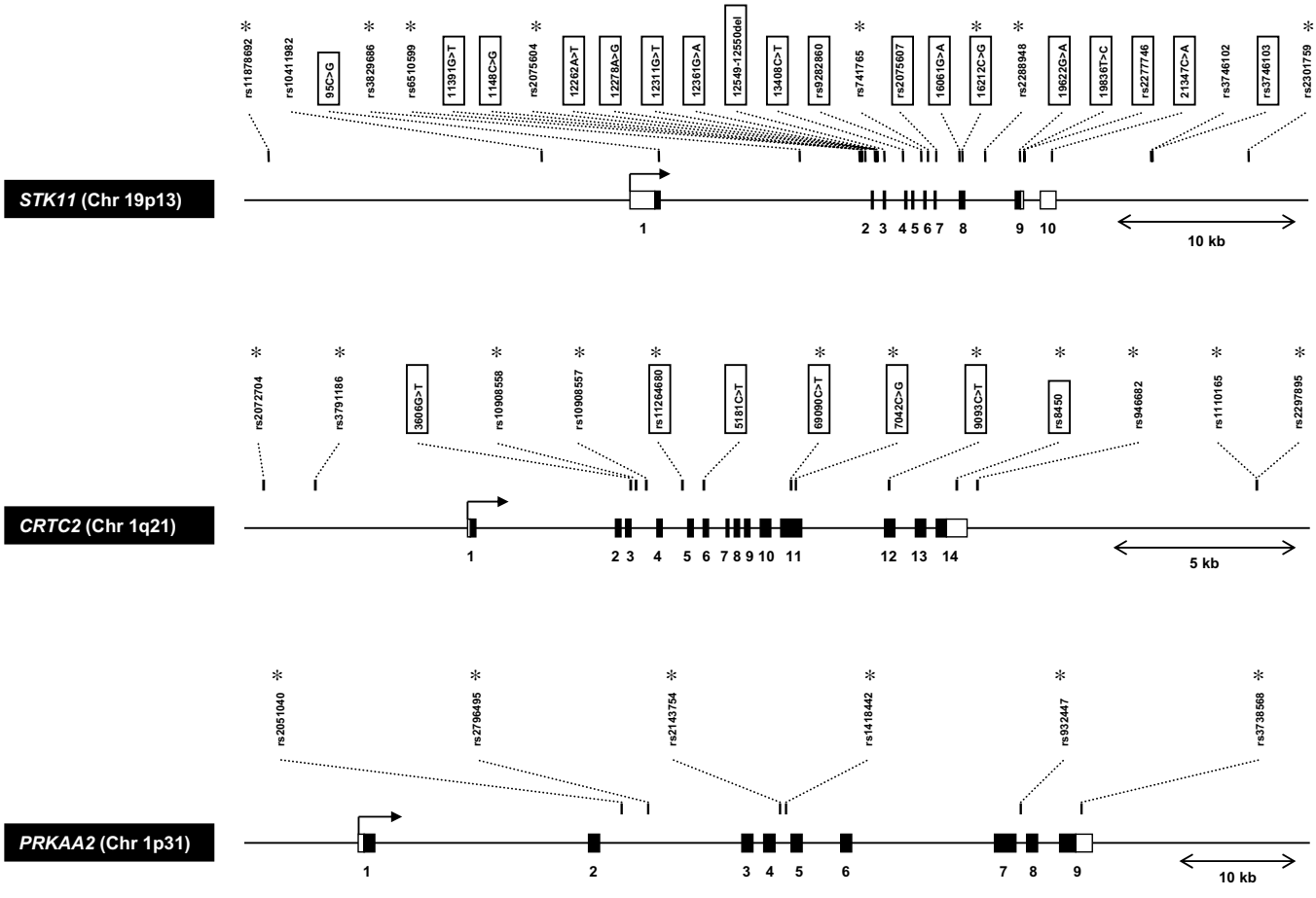

Fig. 1. Genomic structures and locations of SNP sites for human *STK11*, *CRTC2* and *PRKAA2* genes. The arrow indicates the direction of transcription and the transcription start site of each gene. Exons are denoted by boxes, with untranslated regions in white and translated regions in black. The exon number is shown below each box. The locations of SNPs used in this study are shown above, and their IDs correspond to those given in Table 1. The SNPs identified by direct-sequencing of exons are shown as boxed letters, and those used in the association analysis are marked with asterisks.

Table 1
SNPs in *STK11*, *CRTC2* and *PRKAA2* genes

Locus/gene		SNP ID	Contig position[a]	Location	Coding SNP[b]	Allele	MAF[c]	Information source[d]
Chr.19p13	Intergenic	rs11878692	1,125,924	—		A/G	0.17	db
		rs10411982	1,140,713	—		C/A	0.20	db
	STK11	95C > G	1,147,008	Exon 1	Thr32Thr	C/G	0.016	seq
		rs3829686	1,154,657	Intron 1		G/A	0.20	db
		rs6510599	1,158,219	Intron 1		C/T	0.41	db
		11391G > T	1,158,304	Intron 1		G/T	0.031	seq
		1148C > G	1,158,393	Intron 1		C/G	0.016	seq
		rs2075604	1,158,523	Intron 2		G/T	0.41	db
		12262A > T	1,159,175	Intron 2		A/T	0.65	seq
		12278A > G	1,159,191	Intron 2		A/G	0.34	seq
		12311G > T	1,159,224	Intron 2		G/T	0.13	seq
		12361G > A	1,159,274	Intron 2		G/A	0.77	seq
		12549-12550:del	1,159,462	Intron 3		CA/-	0.24	seq
		13408C > T	1,160,321	Intron 3		T/C	0.55	seq
		rs9282860	1,161,161	Intron 5		C/T	0.20	seq
		rs741765	1,161,545	Intron 6		C/T	0.55	db
		rs2075607	1,162,012	Intron 7		G/C	0.22	seq
		16061G > A	1,162,974	Intron 7		G/A	0.016	seq
		16212C > G	1,163,125	Exon 8	Phe354Leu	C/G	0.07	seq
		rs2288948	1,164,627	Intron 8		A/G	0.23	db
		19622G > A	1,166,535	Exon 9	Ala397Ala	G/A	0.016	seq
		19836T > C	1,166,749	Intron 9		T/C	0.016	seq
		rs2277746	1,166,772	Intron 9		C/T	0.42	seq
		21347C > A	1,168,260	Exon 10	3′ UTR	C/A	0.13	seq
	C19ORF26	rs3746102	1,173,597	Intron 2		G/A	0.047	db
		rs3746103	1,173,681	Intron 2		G/A	0.13	seq
	Intergenic	rs2301759	1,178,899	—		T/C	0.42	db
Chr. 1q21	DENND4B	rs2297895	4,404,039	Intron 7		G/T	0.41	db
		rs1110165	4,404,054	Intron 7		T/G	0.38	db
	Intergenic	rs946682	4,410,111	—		T/G	0.38	db
	CRTC2	rs8450	4,410,641	Exon 14	3′ UTR	C/T	0.36	seq
		9093C > T	4,412,176	Exon 12	Arg482Trp	C/T	0.016	seq
		7042C > G	4,414,227	Exon 11	Ala423Gly	C/G	0.016	seq
		6909C > T	4,414,360	Exon 11	Arg379Cys	C/T	0.016	seq
		5181C > T	4,416,088	Intron 6		C/T	0.016	seq
		rs11264680	4,416,433	Exon 5	Met147Val	T/C	0.38	seq
		rs10908557	4,417,407	Intron 3		C/G	0.38	db
		rs10908558	4,417,625	Intron 3		T/C	0.38	db
		3606G > T	4,417,663	Intron 3		G/T	0.016	seq
	SLC39A1	rs3791186	4,424,658	Intron 4		A/G	0.22	db
		rs2072704	4,425,898	Intron 2		C/T	0.22	db
Chr. 1p31	*PRKAA2*	rs2051040	27,114,674	Intron 2		G/A	0.45	ref
		rs2796495	27,114,898	Intron 2		G/A	0.63	ref
		rs2143754	27,130,780	Intron 4		T/C	0.42	ref
		rs1418442	27,131,055	Intron 4		A/G	0.20	ref
		rs932447	27,142,148	Intron 7		A/G	0.22	ref
		rs3738568	27,145,510	Exon 9	3′ UTR	T/C	0.14	ref

[a] SNP locations are based on NCBI Chr 19 contig NT_011255.14 (*STK11*), Chr 1 contig NT_004487.18 (*CRTC2*) and Chr 1 contig NT_032977.8 (*PRKAA2*).
[b] 3′ UTR, 3′-untranslated region.
[c] MAF, minor allele frequencies in 32 subjects with type 2 diabetes.
[d] db, SNPs from public SNP database; seq, direct-sequencing analysis in this study; ref, Horikoshi et al. [16].

[22]. The results indicated that, for the *STK11* and *CRTC2* genes, the entire gene regions were characterized by significant LD and that this was particularly evident with $|D'|$-based analysis (Fig. 2). When LD was assessed by the more stringent measure of r^2, which also accounts for differences in allele frequencies, the LD structure for *STK11* appeared to be more fragmented, while six *CRTC2* SNPs covering a 13.6 kb genomic region that contained about two-thirds of the gene again showed a high degree of LD ($r^2 > 0.8$). The LD structure of the *PRKAA2* gene showed essentially the same pattern as that previously obtained in a Japanese population by Horikoshi et al. [16], but notable differences were observed in the extent of LD between rs1418442 and rs932447, or rs2796495 and rs2143754 (Fig. 2): i.e. our

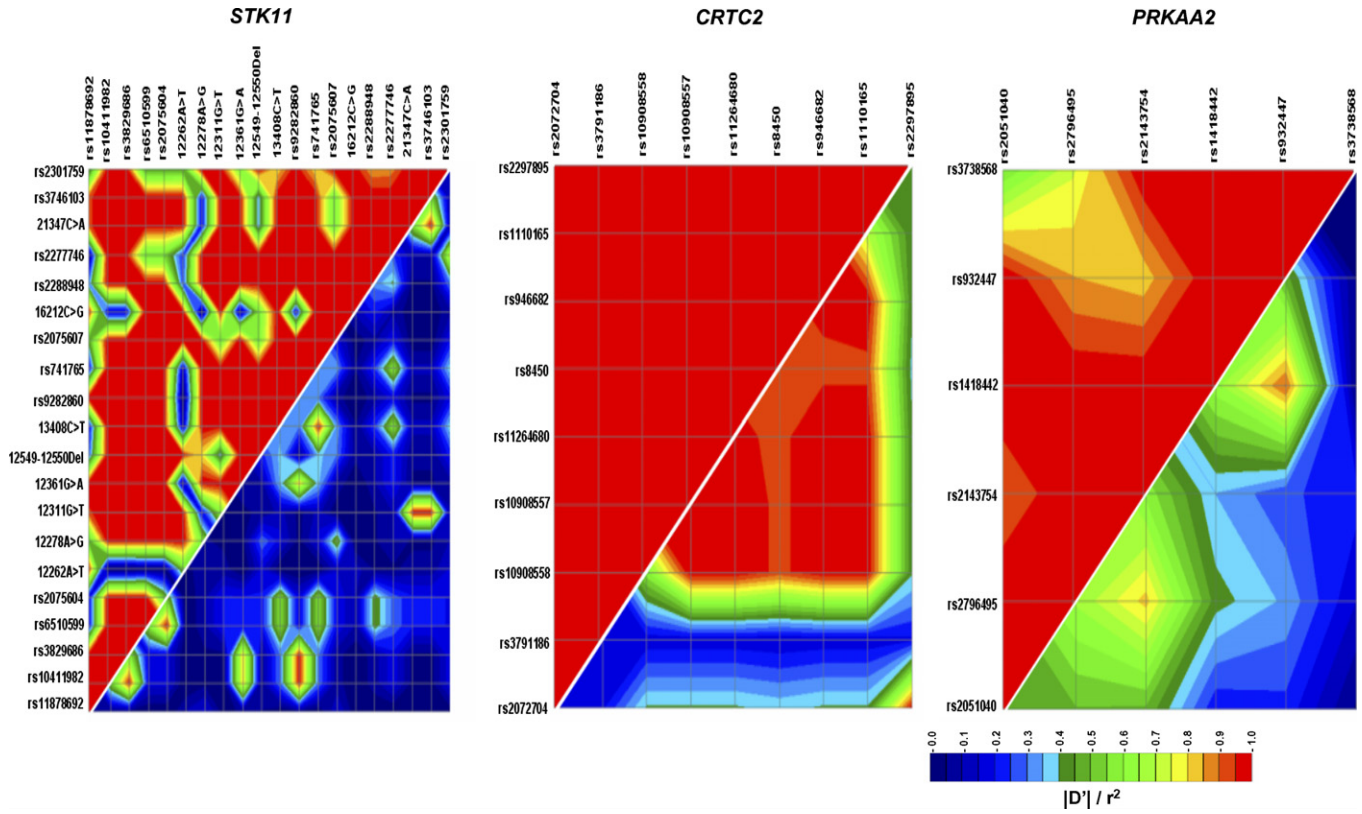

Fig. 2. Pairwise linkage disequilibrium (LD) between SNPs of *STK11*, *CRTC2* and *PRKAA2* in an initial DNA panel ($n = 32$). Pairwise LDs were measured by $|D'|$ (upper left) and r^2 (lower right) using the GOLD program. The different colors are used to represent the ranges of LD values from 1 (red) to 0 (dark blue). Red indicates that the corresponding SNPs are in perfect LD.

analysis revealed a very high r^2 between these SNP pairs (>0.8), while Horikoshi et al. reported a relatively low value (<0.8). These differences did not reflect the small sample sizes of our initial data set, because the high r^2 values between the two pairs of SNPs were still obtained when larger samples (911 type 2 diabetic subjects or 876 controls) were genotyped (Supplementary Fig. S1C).

Single SNP association analysis

It is generally accepted that, in a gene association study, genotyping all the SNPs identified within a candidate gene, which substantially increases the research costs and also necessitates a large consumption of DNA and multiple-testing problems [23], is unnecessary. On the other hand, the optimal method for selecting the most appropriate subset of SNPs to be tested for association has not yet been established. In this study, to develop a high-throughput genotyping assay, we selected 8 and 12 representative SNPs for the *STK11* and *CRTC2* gene regions, respectively, based on their locations, allelic frequencies, levels of LD with neighboring SNPs and their potential(s) to alter protein function or expression (Table 2; SNPs marked with an asterisk in Fig. 1). TaqMan SNP genotyping assays were successfully developed for all but three *CRTC2* non-synonymous SNPs (6909C > T, 7042C > G and 9093C > T), which we genotyped by direct-sequencing in a limited number of samples. In addition, we developed and validated TaqMan assays for all six SNPs in the *PRKAA2*. Genotyping accuracy and reproducibility of the TaqMan assay were demonstrated by comparing results from the direct-sequencing analysis ($n = 32$), which provided 100% concordance of genotype data.

The genotype and allele frequencies of the SNPs (a total of 26 SNPs) in the patient and control groups are shown in Table 2. Our study population consisted of 911 T2D patients and 876 controls, and had a statistical power of 60–80% to detect an association for ORs of 1.2–1.5, depending on the predisposing allele frequency, with a type 1 error rate of $\alpha = 0.05$ [18]. We detected possible evidence for associations with T2D at the nominal significance level ($p < 0.05$) in (i) an intronic SNP in the *STK11* (rs741765; OR 1.33, 95% CI 1.05–1.67, $p = 0.017$, $q = 0.079$, under a recessive genetic model), (ii) a non-synonymous SNP in the *CRTC2* (6909C > T: Arg379Cys; OR 3.01, 95% CI 1.18–7.66, $p = 0.016$, $q = 0.35$, under a dominant model) and (iii) two linked SNPs in high LD in the *PRKAA2* (rs1418442 and rs932447; OR 0.62, 95% CI 0.40–0.96, $p = 0.030$, $q = 0.079$, and OR 0.62, 95% CI 0.40–0.96, $p = 0.033$, $q = 0.079$, under a recessive model for both). It should be noted that these associations were insignificant after correction for multiple comparisons (FDR $q > 0.05$; Bonferroni significance threshold $p = 0.0019$ [0.05/26 tests]). On the other hand, these were significant even after being adjusted for age, sex and BMI (rs741765 and 6909C > T; adjusted $p < 0.05$), or at least the magnitude

Table 2
Single SNP association results

Gene	SNP ID	Genotype			MAF[b]		Association test		Model specific association		
							Allele	Genotype			
		MM/Mm/mm[a]	Case	Control	Case	Control	*p*-value	*p*-value	Model	*p*-value	OR (95% CI)[c]
STK11	rs11878692	AA/AG/GG	607/264/31	570/257/37	0.18	0.19	0.41	0.62			
	rs3829686	GG/GA/AA	717/162/16	683/171/13	0.11	0.11	0.62	0.63			
	rs6510599	CC/CT/TT	320/413/166	291/423/157	0.41	0.42	0.60	0.52			
	rs2075604	GG/GT/TT	320/411/168	291/419/156	0.41	0.42	0.69	0.53			
	rs741765	CC/CT/TT	294/402/207	265/444/159	0.45	0.44	0.44	**0.010**	Recessive	**0.017**	**1.33 (1.05, 1.67)**
	16212C > G	CC/CG/GG	839/67/3	790/79/1	0.040	0.047	0.35	0.27			
	rs2288948	AA/AG/GG	518/310/69	506/298/63	0.25	0.25	0.72	0.93			
	rs2301759	TT/TC/CC	291/411/200	279/419/171	0.45	0.44	0.48	0.37			
CRTC2	rs2072704	CC/CT/TT	300/417/190	297/408/164	0.44	0.42	0.34	0.55			
	rs3791186	AA/AG/GG	510/332/68	470/340/57	0.26	0.26	0.75	0.44			
	rs10908558	TT/TC/CC	441/385/83	406/375/87	0.30	0.32	0.40	0.70			
	rs10908557	CC/CG/GG	453/376/74	418/372/83	0.29	0.31	0.24	0.49			
	rs11264680	TT/TC/CC	441/384/83	408/378/87	0.30	0.32	0.39	0.69			
	6909C > T	CC/CT/TT	207/20/0	187/6/0	0.044	0.015	**0.017**	ND	Dominant	**0.016**	**3.01 (1.18, 7.66)**
	7042C > G	CC/CG/GG	227/1/0	194/1/0	0.002	0.003	0.91	ND			
	9093C > T	CC/CG/GG	131/1/0	96/0/0	0.004	0.000	0.39	ND			
	rs8450	CC/CT/TT	438/374/82	402/368/86	0.30	0.32	0.35	0.65			
	rs946682	TT/TG/GG	438/378/83	408/376/87	0.30	0.32	0.40	0.70			
	rs1110165	TT/TG/GG	440/383/83	410/375/87	0.30	0.32	0.45	0.75			
	rs2297895	GG/GT/TT	296/414/190	299/404/159	0.44	0.42	0.18	0.36			
PRKAA2	rs2051040	GG/GA/AA	299/430/165	307/404/154	0.43	0.41	0.42	0.66			
	rs2796495	GG/GA/AA	325/428/146	333/379/154	0.40	0.40	0.82	0.26			
	rs2143754	TT/TC/CC	294/434/168	292/409/168	0.43	0.43	0.95	0.85			
	rs1418442	AA/AG/GG	544/321/34	517/298/52	0.22	0.23	0.27	0.094	Recessive	**0.030**	**0.62 (0.40, 0.96)**
	rs932447	AA/AG/GG	539/325/34	518/301/52	0.22	0.23	0.33	0.096	Recessive	**0.033**	**0.62 (0.40, 0.96)**
	rs3738568	TT/TC/CC	678/199/19	655/199/11	0.13	0.13	0.69	0.37			

Significant results (*p*-value <0.05) are shown in bold type.

[a] MM, major/major allele; Mm, major/minor allele; mm minor/minor.

[b] Minor allele frequency.

[c] Crude odds ratios (ORs) are given with 95% confidence intervals (CI).

of the association was similar to that obtained in the unadjusted analysis (rs1418442 and rs932447; adjusted $p = 0.09$). We also conducted a subanalysis to test possible associations between the genotypes and various clinical data, such as BMI, blood pressure and HbA1c levels: however, no consistent genotype-dependent effects were observed (data not shown).

Haplotype association analysis

To perform haplotype-based association analyses, we reexamined LD structures within each of three genes employing all genotype data used for a single-locus association analysis. The extent and patterns of LD essentially did not differ from those obtained from genotyping data of 32 individuals, although we observed possible effects of sample size on the pairwise $|D'|$ values between some pairs of SNPs: *STK11*; rs11878692–rs3829686, rs11878692-16212C > G, rs11878692–rs2288948, rs3829686-16212C > G (Supplementary Fig. S1A), and *PRKAA2*; rs2051040–rs3738568 (Supplementary Fig. S1C). To infer haplotypes, we used SNPs with MAF > 0.05, since rarer SNPs have little power for detection of LD and the addition of such SNPs increases the number of lower-frequency haplotypes [24]. These results revealed major haplotypes of six (Hap-S1 to -S6), three (Hap-C1 to -C3) and six (Hap-P1 to -P6) for *STK11*, *CRTC2* and *PRKAA2* genes, respectively, which covered more than 97% of all haplotypes in each gene. Table 3 shows the haplotype composition of each gene using selected sets of haplotype-tagging SNPs (ht-SNPs), which are sufficient to capture all haplotype patterns. When estimated haplotype frequencies were compared between patient and control groups, only the Hap-S6 in *STK11* showed a nominal association with T2D ($p = 0.040$, Permutation-$p = 0.037$; Table 3A). No association between the *PRKAA2* haplotypes and T2D in Japanese were observed in our population (Table 3C). We thus failed to replicate the reported positive association between T2D and the haplotype "AGTAAT" [16] (Hap-P1 in the present study) in our sample population.

Gene–gene interaction analysis

Given the putative biological interactions among LKB1, AMPK α2-subnit and TORC2 proteins, we tested for statistical interactions among SNPs in the three genes with respect to T2D susceptibility. To limit multiple testing, we used ht-SNP data sets (as indicated in Table 3) to represent each gene or locus, and the pairwise gene–gene (locus–locus) interactions were analyzed by GAIA (Genetic Association Interaction Analysis) [21], a recently developed statistical method for evaluating gene–gene interactions. As

Table 3
Haplotype-based association analysis

(A) *STK11*	ht-SNPs						Frequency		χ^2	*p*-value	Permutation-p
	rs11878692	rs3829686	rs6510599	rs741765	rs2288948	rs2301759	Case	Control			
Hap-S1	A	G	C	T	A	T	0.232	0.245	0.763	0.382	0.398
Hap-S2	A	G	T	C	G	C	0.220	0.218	0.014	0.907	0.936
Hap-S3	A	G	C	C	A	C	0.113	0.107	0.181	0.670	0.700
Hap-S4	A	A	C	T	A	T	0.091	0.096	0.218	0.641	0.676
Hap-S5	A	G	T	C	A	T	0.083	0.082	0.033	0.856	0.896
Hap-S6	G	G	C	T	A	T	0.088	0.069	**4.229**	**0.040**	**0.037**

(B) *CRTC2*	ht-SNPs			Frequency		χ^2	*p*-value	Permutation-p
	rs2072704	rs3791186	rs11264680	Case	Control			
Hap-C1	T	A	T	0.439	0.423	0.858	0.354	0.360
Hap-C2	C	A	C	0.303	0.315	0.577	0.448	0.463
Hap-C3	T	G	C	0.258	0.261	0.027	0.868	0.878

(C) *PRKAA2*	ht-SNPs[a]						Frequency		χ^2	*p*-value	Permutation-p
	rs2051040	rs2796495	rs2143754	rs1418442	rs932447	rs3738568	Case	Control			
Hap-P1	A	G	T	A	A	T	0.418	0.400	1.057	0.304	0.312
Hap-P2	G	A	C	G	G	T	0.214	0.229	1.055	0.304	0.326
Hap-P3	G	G	T	A	A	T	0.147	0.164	1.899	0.168	0.172
Hap-P4	G	A	C	A	A	C	0.128	0.116	1.005	0.316	0.312
Hap-P5	G	A	C	A	A	T	0.052	0.045	0.991	0.320	0.333
Hap-P6	G	G	c	A	A	T	0.025	0.024	0.019	0.889	0.906

For each gene locus, all common haplotypes (>2%) are shown, with a minimum set of SNPs that can distinguish each haplotype (haplotype-tagging SNPs: ht-SNPs).
The haplotype frequencies are estimated independently for the case ($n = 911$) and control ($n = 876$) groups.
The differences in the haplotype frequencies were analyzed by $\chi 2$, *p*-value and permutation tests (10,000 replicates), using SNPAlyze Version 5.0 Pro software.
Significant results (*p*-value < 0.05) are shown in bold type.

Table 4
Results of pairwise interaction tests between SNPs of *STK11* and *CRTC2*

SNPs		Interaction model	
Locus 1; *STK11*	Locus 2; *CRTC2*	Additive only	Additive and dominant
		p-value	*p*-value
rs11878692	rs2072704	0.556	0.782
rs11878692	rs3791186	0.063	—
rs11878692	rs11264680	0.243	0.808
rs3829686	rs2072704	**0.010**	**0.049**
rs3829686	rs3791186	0.243	—
rs3829686	rs11264680	**0.041**	—
rs6510599	rs2072704	**0.001**	**0.025**
rs6510599	rs3791186	0.173	0.691
rs6510599	rs11264680	**0.031**	0.212
rs741765	rs2072704	**0.006**	**0.043**
rs741765	rs3791186	0.058	0.150
rs741765	rs11264680	0.209	0.482
rs2288948	rs2072704	**0.030**	0.255
rs2288948	rs3791186	0.447	0.131
rs2288948	rs11264680	0.188	0.687
rs2301759	rs2072704	0.058	0.375
rs2301759	rs3791186	0.161	0.107
rs2301759	rs11264680	0.539	0.088

The interaction analysis was carried out using GAIA software [21].
Significant results (p-value < 0.05) are shown in bold type.

shown in Table 4, we observed significant and specific interactions between SNPs in *STK11* and *CRTC2* genes, while no interactions were found with either *STK11–PRKAA2* or *CRTC2–PRKAA2* SNPs (Supplementary Tables S1A and S1B). Between the *STK11* and *CRTC2* SNPs, at least three pairs of SNPs showed a statistically significant interaction with respect to T2D risk (rs3829686–rs2072704, rs6510599–rs2072704 and rs741765–rs2072704; Table 4), with the most significant pair (rs6510599–rs2072704) reaching a p-value of 0.0010 (Permutation-$p = 0.019$, under an "additive only" model). Interestingly, individual SNPs that were found to interact did not necessarily show significant single-locus associations, except for rs741765 (Table 2).

Discussion

Our goal is to identify novel genes that account for genetic susceptibility to T2D. In this study, we examined two new candidate susceptibility genes, *STK11* and *CRTC2*, which encode proteins involved in the AMPK-mediated signal pathway. Two SNPs, the rs741765 in intron 6 of the *STK11* and a non-synonymous SNP, 6909C > T (Arg379Cys) in exon 11 of *CRTC2* showed weak but possible associations with T2D in the Japanese population. Although significance disappeared with correction for multiple testing, we consider the present study to be explorative and hypothesis-generating and thus find the results intriguing. A coding variation might at least theoretically have functional consequences, while recent evidence suggests that common, non-coding genetic variants will explain at least some of the inherited variation in susceptibility to a common disease such as T2D [25]. Other than the SNP *CRTC2*:6909C > T, we identified four additional non-synonymous SNPs (one in *STK11* and three in *CRTC2* genes; Table 1) with relatively low allelic frequencies (i.e. <0.05). Obviously, the statistical power of the present analysis is insufficient to identify a possible disease contribution of such rare variants. It is therefore possible that non-synonymous SNPs showing no association in this study may confer risks of developing T2D in selected populations, and future investigations including studies using independent populations with larger sample sizes, analyses in other ethnic groups that differ in population allele frequencies, or family-based tests of association are warranted. In addition, an *in vitro* analysis investigating functional properties of these variant proteins may provide valuable clues to understanding their functional alternations.

Two previous studies have investigated the role of the *PRKAA2* gene in T2D [15,16]. In an earlier study, Sun et al. [15] tested nine *PRKAA2* SNPs for associations with T2D in five different Caucasian samples, the largest of which comprised of 1189 discordant sibs, and found no significant associations. They also assessed the effects of genotypes on plasma glucose levels and insulin sensitivity in 756 control individuals and found no significant differences. In a second study, Horikoshi et al. [16] tested associations of *PRKAA2* SNPs, which differed from those used by Sun et al., in 192 Japanese patients with T2D and 272 control individuals and found no independent SNP associations, but they did identify an associated haplotype (haplotype "AGTAAT"), which was replicated in two other case-control sample sets. In addition, rs2051040 in intron 2 of *PRKAA2* was found to be associated with insulin sensitivity in their non-diabetic subjects. Based on these prior observations, we set out to examine whether positive associations of the *PRKAA2* haplotype with T2D could be replicated in an independent Japanese population. Although the individual allele frequencies of the six *PRKAA2* SNPs obtained in this study were comparable to the results of Horikoshi et al., our statistical analysis of data on haplotype frequencies failed to detect any significant differences (Table 3). This discrepancy between our results and those of Horikoshi et al. appear, at least in part, to be attributable to the observed differences in the extent of LD between a particular pair of SNPs: i.e. the pairwise r^2 between rs1418442 and rs932447 was very high ($r^2 > 0.9$) in our samples, whereas Horikoshi et al. reported a relatively low value ($r^2 = 0.57$). The high r^2 value we obtained between the two SNPs was supported by the analysis of publicly available Phase II HapMap SNPs data set for 45 Japanese individuals (Supplementary Fig. S1C-4): however, the precise factors accounting for the apparent differences are currently unknown.

Interestingly, we observed weak associations between the two linked *PRKAA2* SNPs (rs1418442 and rs932447; the pairwise r^2 for these two SNPs was >0.9) and T2D

($p = 0.03$ for individual SNPs, under a recessive model), findings contradicted by those of Horikoshi et al. [16]. These associations were not confirmed in pooled analyses combining our data with the data published by Horikoshi et al., in which a total of 2100 Japanese subjects with T2D and 1695 controls were assessed (Supplementary Table S2). However, in a recent study using a large sample of 2777 normal Caucasian females, Spencer-Jones et al. [26] showed significant and consistent associations of five *PRKAA2* tag SNPs, which included rs1418442 but not rs932447, with LDL- and total-cholesterol levels on both single-locus and haplotype analyses. As increased LDL- and total-cholesterol levels have been well established as T2D-related traits, and AMPK activation also plays a major role in regulating lipid metabolism as well as glucose metabolism, these observations are consistent with a putative causal role of rs1418442 in the pathogenesis of metabolic syndrome. Unfortunately, it remains unknown whether rs1418442 is a functional SNP that affects mRNA expression or stability, for example, or alternatively, is in LD with an unknown causative variant. In our sample population, clinical measures, such as plasma glucose, insulin and lipid levels were not fully available, and thus we were unable to assess the relationship between rs1418442 and such metabolic parameters in detail.

It is anticipated that genetic epistasis (gene–gene or locus–locus interactions) plays an important role in the etiology of complex traits including T2D. As we assessed multiple genes involved in a specific signaling pathway, assuming their putative biological interactions, we conducted a systematic search for gene–gene (SNP–SNP) interactions using the GAIA method [21], a novel logistic regression-based approach to evaluating gene–gene interactions. We detected a statistically significant gene–gene interaction between the *STK11* and *CRTC2* SNPs (Table 4) for T2D susceptibility. The interactions between the two genes involved multiple pairs of SNPs (*STK11–CRTC2*; rs3829686–rs2072704, rs6510599–rs2072704 and rs741765–rs2072704), most of which were insignificant in a single-locus analysis, and remained significant after permutation and correction for multiple testing (rs6510599–rs2072704). It is also noteworthy that the interaction effects were specific between the *STK11* and *CRTC2* SNPs and were not observed between the *PRKAA2–STK11* and the *PRKAA2–CRTC2* SNPs, indicating that the interactions are not likely to be false-positive results. We also found a significant interaction between the *STK11* and *CRTC2* genes using the multifactor-dimensionality reduction (MDR) method [27], which has been previously introduced as a non-parametric, model free method for detecting gene–gene and gene–environment interactions, thus supporting our GAIA results (P.K., unpublished observation).

In summary, the present results suggest a possible association between SNPs in the genes involved in the AMPK-mediated signaling cascade and the risk of T2D in a Japanese population. The magnitude of individual associations appears to be only modest: however, a potential interaction between *STK11* and *CRTC2* genes that might influence T2D susceptibility was also detected. Further investigations, including large-scale replication studies in other independent populations, family-based tests of association, and detailed assessment of genotype–phenotype relationships, are needed to confirm our findings and to clarify the underlying genetic and molecular mechanisms.

Acknowledgments

We thank our patients and volunteer blood donors for participating in this study. This study was partially funded by grants from the Ministry of Education, Science and Technology (Knowledge Cluster Initiative).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ymgme.2007.08.125.

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