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Variant screening of *PRKAB2*, a type 2 diabetes mellitus susceptibility candidate gene on 1q in Pima Indians

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The AMP-activated protein kinase (AMPK) is a key enzyme involved in the regulation of lipid and glucose metabolism. There are multiple isoforms of the three subunits of this enzymatic complex, each encoded by a different gene in humans. We have investigated the *PRKAB2* gene encoding the β2 subunit, which is located on chromosome 1q within a region linked with type 2 diabetes mellitus (T2DM) in the Pima Indians and four different Caucasian populations. The gene consists of eight exons spanning about 15 kb, and we detected nine variants in the introns and 3′ UTR, including eight informative single nucleotide polymorphisms (SNPs) and one rare 4 bp insertion/deletion. In an analysis of representative markers in selected Pima Indians including 149 diabetic cases (onset age <25 years) and 150 controls (at least 45 years old, with normal glucose tolerance), we found no evidence for association of this locus with T2DM. We conclude that variants in *PRKAB2* are unlikely to contribute to the disease susceptibility in Pima Indians.

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KEYWORDS: AMP-activated protein kinase, β2 subunit gene, chromosome 1q, association, type 2 diabetes mellitus, Pima Indians.

INTRODUCTION

The AMP-activated serine/threonine protein kinase (AMPK) is a multisubunit enzyme that plays a major role in fat and carbohydrate metabolism.[1] Furthermore, this enzyme is involved in the inhibition of expression of several glucose-activated genes[2] and has also been implicated in the modulation of glucose-stimulated insulin secretion.[3] AMPK is a ubiquitously expressed heterotrimeric complex composed of a catalytic α subunit combined with a β and γ regulatory subunits.[4] In human and rat, different isoforms of each subunit have been identified including two α, two β, and three γ subunits,[5,6] each encoded by a separate gene mapping to a different chromosomal location in human.[4–7] (http://www.ncbi.nlm.nih.gov; in OMIM database.)

Based on the significant role of AMPK in the regulation of energy fuel metabolism including glucose and lipids, it has been suggested[1] that defective function of this kinase might contribute to a predisposition to type 2 diabetes mellitus (T2DM). This view is supported by a recently reported functional interaction of AMPK with the hepatocyte nuclear factor 4α[8] which, when mutated, causes maturity onset diabetes of the young-1 (MODY1), an autosomal dominant form of T2DM.[9] In addition, a recent study in the rat reported that AMPK is activated by metformin, an anti-diabetic drug frequently used for treatment of T2DM. These

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investigators proposed that this enzyme is an important mediator of the therapeutic effects of metformin, including suppression of glucose production by hepatocytes and facilitation of glucose uptake in skeletal muscle.[10] The role of AMPK in glucose and glycogen metabolism is further evidenced by a discovery of a mutation in the γ3 subunit in the pig, leading to ~70% increase in glycogen content in skeletal muscle without any of the abnormalities that are characteristic for glycogen storage diseases.[7]

T2DM is a heterogeneous group of disorders of glucose homeostasis that are frequently characterized by a combination of an impaired glucose uptake and glycogen synthesis in skeletal muscle (insulin resistance) accompanied by an impaired insulin secretion.[11] Susceptibility to the common form of T2DM is familial, involving an unknown number of genes,[12] and we have recently described a linkage of the disease with markers on chromosome 1q21–q23 in the Pima Indians of Arizona, a population with the highest documented prevalence of this disease in the world.[13] Subsequently, linkage of this region with T2DM was replicated in four different Caucasian populations,[14–17] consistent with a shared diabetes susceptibility locus in this area.

Based on the key role of AMPK in glucose homeostasis, it is conceivable that alterations in any of the genes encoding the subunits may contribute to the features underlying T2DM, including insulin resistance and/or impaired insulin secretion. The gene coding for the human AMPK β2 subunit (*PRKAB2*) has been assigned to chromosome 1q12–q21 (http://www.ensembl.org/Homo_sapiens;/www.ncbi.nlm.nih.gov/LocusLink/), within an interval overlapping the T2DM-linked segment. Furthermore, this isoform is highly expressed in skeletal muscle,[18,19] which is the most important insulin-responsive tissue involved in whole-body glucose disposal. Because of the significance of AMPK in the metabolism of glucose and the location of the β2 subunit gene at 1q, we have investigated *PRKAB2* as a positional and biologically relevant candidate for T2DM. We report here its exon-intron organization, as well as the results of variant screening and association analysis in relation to diabetes in Pima Indians.

MATERIALS AND METHODS

Subjects and genomic DNA

Subjects selected for genomic screening are participants of ongoing prospective studies of T2DM conducted among members of the Gila River Indian Community since 1965.[11] Diabetes was diagnosed by WHO criteria as described.[13] Association studies were performed by comparing allele and genotype frequencies at individual SNPs in 299 Pimas, representing 149 subjects diagnosed with type 2 diabetes mellitus before the age of 25 years and 150 unaffected subjects, who were at least 45 years of age and had normal glucose tolerance. None of the individuals were first-degree relatives among themselves. The studies were approved by the Institutional Review Board of the National Institutes of Health and by the Gila River Tribal Council, and signed informed consent was obtained from all participants.

Pima genomic DNA samples were described previously,[13] and DNA samples from somatic cell hybrids containing either human chromosome 1 or human chromosome 2 (NA13139 and NA10826B, respectively) were obtained from the NIGMS Human Genetic Mutant Cell Repository (Coriell Institute, Camden, NJ, USA).

Variant detection, genotyping, and statistical analysis

SNP detection was performed by PCR and denaturing HPLC analysis of genomic products amplified from two separate DNA pools as described in detail previously.[20] Each pool consisted of five affected and five control individuals, thus representing a total of 20 different subjects. Primers for PCR, sequencing and variant genotyping of the exons were designed at appropriate positions to include up to 100 bp of adjacent intronic sequences. Primer sets for the 5′ and 3′ flanking regions were designed to amplify overlapping segments, thus assuring a complete coverage of each region (Table 1). Amplifications were performed in the GeneAmp System 9600 thermocycler (Applied Biosystems, Foster City, CA, USA) using the Expand PCR System kit (Roche Diagnostics Corp., Indianapolis, IN, USA), which in our experience consistently yields robust products regardless of the sequence composition. A typical 50 µl reaction consisted of 1× buffer no. 2 (containing 2·25 mM $MgCl_2$) and 0·5 µl of enzyme mix (both provided with the kit), 350 µM of each dNTP, 8 pmol of each primer, 0·5 µl of TaqStart antibody (Clontech, Palo Alto, CA, USA) and 500 ng genomic DNA. Amplifications were initiated by denaturation at 96°C for 1 min, followed by 30 cycles of 96°C for 20 s, 57°C for 30 s, 68°C for 45 s, and final extension at 68°C for 5 min.

The size of the amplicons ranged between 350 and 500 bp and the products were screened for variants by dHPLC using the WAVE DNA fragment analysis system according to the manufacturer's protocol

Table 1. *PRKAB2* primers for variant screening

Designation[a]	Region	Sequence (5′-3′)
PROM1	Promoter	CTCCTTCCTTGTCGCTC
PROM2		GCAGAAGGAAGCAGTGGTC
PROM3	Promoter +	AGAACAGTTTTAACCTCCAGC
PROM4B	Exon 1	GATGGTGGGCAGTCGCAC
1F	Exon 1	CCAAGCGCTCCTATTATCC
1R		CGGGAACGCCTGCAAATAC
2F	Exon 2	GGAATCCCTTGCCCTGTC
2R		CCCGATAAATCCCTTTAATTC
3F	Exon 3	GTTCAAACTCTGATTTAGTTGC
3R		GGTAAAACATCTCTGACTCAC
4F	Exon 4	ATATGATACTATGATTGCCAC
4R		GCTGCCCATCAGTCTTGAC
5F	Exon 5	TGGAGGTGCTGAGAACAATC
5R		TGTATCATGAGACTTGTGTTAC
6F	Exon 6	GTTACCTTACAGCTGTAGATC
6R		CTGAAAAAGCTGTGACTCACC
7F	Exon 7	AGCTCTGAGGAAGAGTAAGC
7R		TTACCCTTGACCTCTCATTC
8F	Exon 8	GACTGTGAAGGACTGATGAC
8R		ACTCATAAAGCTCTCATCTGC
UTR1F	3′ UTR	GACTGAACCAGTCTTACCTG
UTR1R		AATGAGGCAGCTGTTAAGTAG
UTR2F	3′ UTR	GGTAAATGGAAGCCTCCTAG
UTR2R		AGGTATGGGATCTGAAGGAG
UTR3F	3′ UTR	CCAGAGTATGTGGCACAATC
UTR3R		CCTGCCTTCAGAACTGTTTAC
UTR4F	3′ UTR	AAGGCAGAATGCAGAACACC
UTR4R		GGTTGGGAATACTAGGAATAC
UTR5F	3′ UTR	CCCTGTACACATCACTACAC
UTR5R		CTAGGGTCATCTGAGCAGTC
UTR6F	3′ UTR	ACACTAAAGGAGGCTAAACTG
UTR6R		CTGAAGGCTGTAAATCCTGAG
UTR7F	3′ UTR	TACTTTAGCCATCCAAGGCC
UTR7R		GATCTGAAGATCACTAGCATC
UTR8F	3′ UTR	CCACACAGGTGTTTCAGTAG
UTR8R		CCTTTAACTAATCAATATACAATG
UTR9F	3′ UTR	GCTGATACATCCCATGGTTC
UTR9R		GTGAGATCCAAGGACACCAC
UTR10F	3′ UTR	AGAGTGACTGAGCTGAGAAG
UTR10R		GCCAACACTATTGAGGTTAGG
UTR11F	3′ UTR	CCATGCTGCTGGTATGGAG
UTR11R		AGCAAGGAGTTCTATTAGTATG
UTR12F	3′ UTR	CAATATTCCATGTATGCTGCTG
UTR12R		GTGATTGGCACTTAAGGAGAG
UTR13F	3 UTR	GGTAGCCATTAGGGTCTTTC
UTR13R		GGTATCCCTTGACCATTTAAC
UTR14F	3′ UTR	GTGTGAAATACTAAACACTATGG
UTR14R		AAAGCAGCTCTGTACCTCAG
UTR15F	3′ UTR	CATTTCGAATGAGTGCAGCTC
UTR15R		TTGCAGTTTACATTCTTTAGGC

[a] F = forward, R = reverse orientation.

Table 2. *PRKAB2* variants and association analysis of representative SNPs

Marker	Variant[a]	Frequency	Primers[b] (5′-3′)	Assay[c]	*P*-value[d]
SNP-1	T/C	0·67/0·33	6F + 6R	Seq.	
SNP-2	C/T	0·67/0·33	2F: GGAATCCCTTGCCCTGTC 2RB: CTATTTTGAGGCGGCACTTC	*Nla* III	0·7 (LD cluster I)
SNP-3	C/G	0·84/0·16	Ex1F: AGCAGAACAGCCATCACATC Ex2R: TGGTGGTGTTTCCCATGGC	*Bgl* I	0·94
SNP-UTR1	A/G	0·59/0·41	UTR1F + UTR1R	Seq.	
SNP-UTR5	A/G	0·67/0·33	UTR5F + UTR5R	Seq.	
SNP-UTR6	-/G	0·67/0·33	UTR6F + UTR6R	Seq.	
SNP-UTR13	C/G	0·67/0·33	UTR13F + UTR13R	Seq.	
SNP-UTR14	A/G	0·59/0·41	UTR14FB: TTGGTACATTGGAGCATGTTG UTR14R	*Tsp* 509 I	0·65 (LD cluster II)
Insertion/deletion	AGAC/-	0·97/0·03	5F + 5R	Seq.	N/A

[a] The precise position of individual SNPs can be found in the annotation of GenBank entries encompassing the corresponding gene segments (AF504538–AF504543).
[b] F = forward, R = reverse orientation. See Table 1 for primer sequences not given here.
[c] Restriction endonuclease used for genotyping. Seq. = determined by sequencing.
[d] Significance values based on association analysis in 149 Pima diabetic cases and 150 controls. SNP-2 represents cluster I of markers in linkage disequilibrium (SNP-1, SNP-2, UTR5, UTR6, UTR13); SNP-UTR14 represents cluster II (SNP-UTR1, SNP-UTR14); SNP-3 has a genotype distribution different from cluster I and cluster II. N/A = marker not analyzed because of a low frequency of the deletion allele.

(Transgenomics, Omaha, NE, USA) as described in detail previously.[20] Putative variants detected by dHPLC were validated by direct sequencing in individuals using ABI Prism BigDye Terminator Cycle Sequencing kit and the ABI 377 or 3700 Sequencers (ABI, Foster City, CA, USA). Characterization of the rare 4 bp insertion/deletion variant in intron 5 was performed after subcloning the PCR product from a heterozygous individual into the pGEM-T Easy Vector (Promega, Madison, WI, USA), and sequencing recombinant clones representing the alternate alleles.

Genotypings of representative markers were performed by PCR-RFLP using primers listed in Table 2 and the appropriate restriction endonucleases (SNP-2 digested with *Nla* III; SNP-3 with *Bgl* I; SNP UTR14 with *Tsp* 509 I) according to manufacturer's recommendations (New England Biolabs, Beverly, MA, USA). Two investigators scored the results and 31 encrypted duplicate samples were included as internal controls to check the consistency of the genotypings. Only data showing none or one genotype discrepancy (corresponding to 0–3% error rate) were allowed for further analysis. Differences of allelic frequencies between the diabetic and control groups were analyzed by the Chi-square test.

RESULTS AND DISCUSSION

The *PRKAB2* gene maps to human chromosome 1q12–q21 (www.ncbi.nlm.nih.gov/LocusLink; Locus ID 5565; http://www.ensembl.org/Homo_sapiens) and using a BLAST search of the public databases with the cDNA sequence (Genbank no. NM_005399) we identified two BAC clones (337C18 and 325P15; Genbank no. AL356378 and AC015706, respectively) carrying the entire gene. Based on their annotation, 337C18 was assigned to chromosome 1, whereas 325P15 to chromosome 2. The *PRKAB2* sequences (including exons and introns) were identical in both clones, indicating either the possibility of an incorrect assignment of one of the clones or a duplication of this genomic segment. To address this question, we performed separate PCR with *PRKAB2*-specific primers using DNA from a chromosome 1- as well as chromosome 2-specific somatic cell hybrid (NA13139 and NA10826B, respectively). In this experiment, only NA13139 (as well as a human total genomic DNA control) yielded the expected PCR band (not shown). Furthermore, both BACs share anchor markers within their overlapping part and we conclude that the correct position of clone 325P15 is on chromosome 1q.

The published cDNA has an open reading frame of 816 bases encoding a protein of 272 amino acid residues.[19] By alignments of the BAC sequences with the cDNA and by long distance PCR we have determined that *PRKAB2* consists of eight exons and the entire locus spans approximately 15 kb (Fig. 1). The first exon and the initial 23 bases of the second exon are non-coding, and all exon-intron boundaries conform to the consensus GT-AG motifs for splice donor and acceptor sites in eukaryotic genes.[21]

Based on published northern blot data,[19] we estimated that the human *PRKAB2* transcript is at least

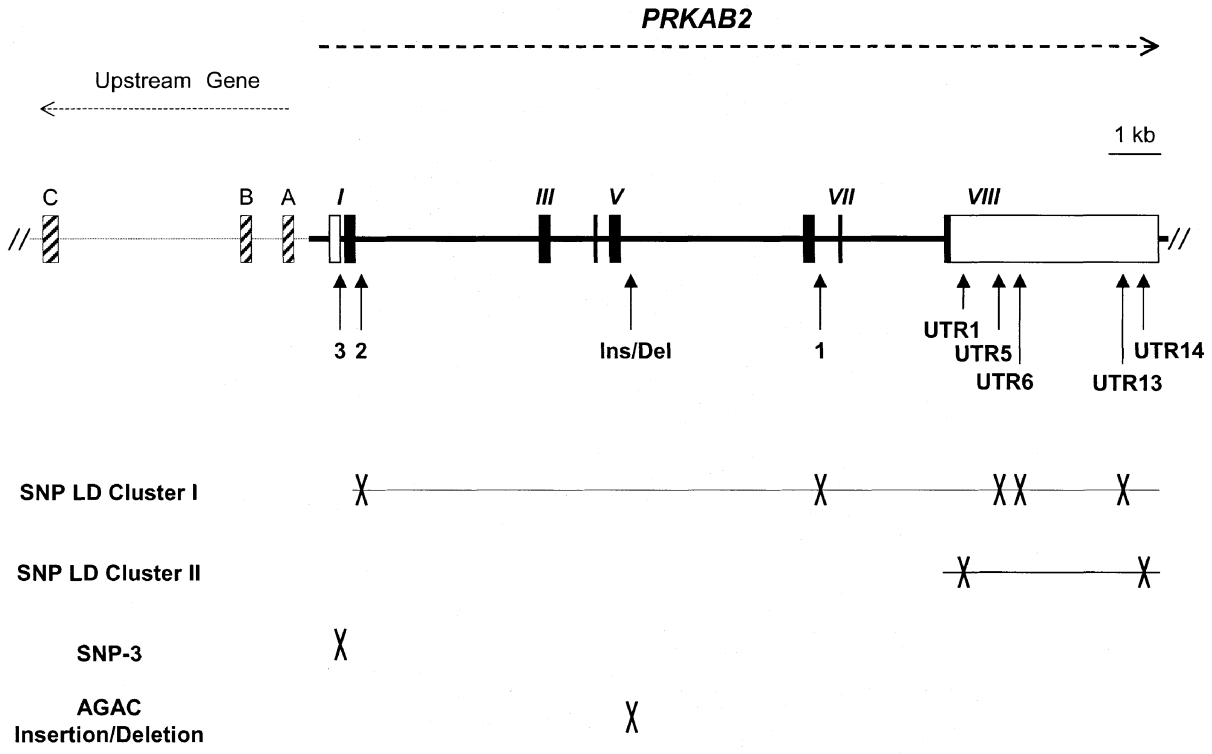

Fig. 1. Genomic organization and variants of the *PRKAB2* locus. The coding and non-coding *PRKAB2* sequences are shown as filled and open boxes, respectively, and the odd exons plus exon 8 are indicated in Roman numerals; the three deduced exons of the upstream gene (labeled A, B, and C) are shown as shaded boxes. Individual variants with their corresponding designations underneath are indicated by vertical arrows and with X below the gene. Unique markers (including clusters of SNPs in linkage disequilibrium, LD) are indicated to the left. Dotted horizontal arrows above the diagram indicate the opposite transcriptional orientation of both genes.

5 kb long. Because the coding sequence is only 816 bp in length, we searched public databases with genomic sequences flanking *PRKAB2* for additional 5′ and 3′ sequences to account for the reported size of the transcript. We identified a set of overlapping EST entries extending approximately 4·5 kb downstream from the stop codon, indicating that this entire segment represents the 3′ UTR. By performing the same analysis with the 5′ region, we discovered that a part of the flanking genomic sequence beginning approximately 800 bp upstream from the end of the first *PRKAB2* exon is represented in three overlapping EST entries (AV759949: 659 bp; AL516944: 834 bp; AW665927: 462 bp), which are not contiguous with any of the *PRKAB2* transcribed sequences. Furthermore, we have determined that the combined consensus cDNA sequence derived from these ESTs is a composite of three separate genomic segments located within the BAC 337C18 upstream of *PRKAB2*. With the exception of the proximal end of the first segment (oriented towards *PRKAB2*), each is flanked at their respective ends by the splice donor (GT) and acceptor (AG) site motifs, and we conclude that these ESTs represent the spliced 5′ part of an adjacent (upstream) gene transcribed in the opposite direction (Fig. 1).

The interval between *PRKAB2* exon 1 and the first exon of the upstream gene does not contain a TATA box and has a relatively high GC content (64%). Using the TESS program (Transcription Element Search System; http://www.cbil.upenn.edu/tess/), we discovered that it comprises clusters of numerous consensus transcription factor binding site motifs, such as SP1, AP-2, and Adf1 (not shown; Genbank accession no. AF504538). Because of the proximity between the first exons of *PRKAB2* and the upstream gene, respectively, we are assuming that this area encompasses a promoter shared by both genes. Further studies will be required to determine which part(s) of this region has a functional significance for their transcription.

To search for variants, we analyzed all exons plus adjacent intronic segments of approximately 100 bp including splice sites, and also screened the sequence between the first exons of *PRKAB2* and the upstream gene, as well as the entire 3′ UTR. The screening was performed in two separate DNA pools (each consisting

of an equal mix of five diabetic and five non-diabetic Pima subjects) using dHPLC technology. We identified eight informative SNPs (frequency of the less common allele > 0·1) in the non-coding sequences, including one in intron 1, 2, and 6, respectively, and five in the 3′ UTR (Fig. 1; Table 2). Several of these variants correspond to entries in the dbSNP database (www.mcbi.nlm.nih.gov/SNP/; SNP-1 = rs1036852; SNP-2 = rs1348316; SNP-UTR1 = rs2304983; SNP-UTR13 = rs1047140; SNP-UTR14 = rs6937), thus supporting their validation. Seven of the SNPs are base substitutions, whereas SNP-UTR6 represented a single nucleotide (G) deletion. In addition, we detected a rare 4 bp deletion (AGAC; frequency 0·03) in intron 5, whereas no variants were detected in the coding sequences (Fig. 1; Table 2). By an initial analysis of each variant in 20–40 subjects we found that genotypes at SNP-1, -2, -UTR5, -UTR6 and -UTR13 were in complete concordance, consistent with linkage disequilibrium among these markers (LD cluster I in Fig. 1; allelic frequency 0·67/0·33). Similarly, genotypes at SNP-UTR1 and SNP-UTR14 appeared to be shared among these two markers but with a different distribution than cluster I, thus representing a second linkage disequilibrium group (LD cluster II in Fig. 1; allelic frequency 0·59/0·41). SNP-3 had a unique genotype distribution different from LD cluster I and II, thus constituting an additional, separate marker (allelic frequency 0·84/0·16). To assess the relationships of the variants with T2DM, we genotyped SNP-2 (representing LD cluster I), SNP-UTR14 (LD cluster II), and SNP-3 in 149 diabetic cases and 150 non-diabetic controls. The AGAC insertion/deletion marker was not analyzed because of its low frequency (0·97/0·03). The genotypic frequencies at each representative marker did not differ significantly from the Hardy–Weinberg equilibrium (not shown), and we did not detect any statistically significant associations with T2DM in the cases and controls (*P*-values = 0·65–0·94; Table 2).

In conclusion, we have screened the *PRKAB2* gene for variants in selected diabetic and non-diabetic Pima Indians and found no evidence that alterations in this gene contribute to T2DM in this population. However, elucidation of the genomic organization of *PRKAB2* and the variants detected in our study should help in evaluating this gene as a candidate for T2DM in other populations. As AMPK is an intriguing target for therapeutic interventions in diabetes[22] and disturbances of lipid metabolism,[23] the structural information for the β2 subunit may contribute to gene-based, pharmacogenetic efforts to advance the development of specific therapeutic treatments for disorders affecting glucose and lipid homeostasis.

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