Genetic study of the CD36 gene
In a French diabetic population

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SUMMARY

Objectives: CD36 is a multifunctional membrane receptor widely expressed in different tissues which binds and internalizes oxidized low-density lipoprotein. In rodents, CD36 gene variations modulate glucose homeostasis and contribute to metabolic syndrome associated with type 2 diabetes but the effects in human are unknown.

Methods: We screened the entire coding sequence of the CD36 gene in 272 individuals and we genotyped both rare and frequent variants in 454 T2D subjects and 221 controls.

Results: We detected five mutations, P191P and N247S were only found each in one family and did not segregate with diabetes, the three others (A/C-178 in the promoter, A/G-10 in intron 3 and (GGGTTGAGA) insertion in intron 13) being equally frequent in diabetic subjects and in controls. However, adiponectin levels, a marker for insulin sensitivity, were significantly associated with the -178 A/C promoter variant allele (p = 0.003, p corrected for multiple testing = 0.036), possibly reflecting association with insulin-resistance in the French population.

Conclusion: Thus, the -178 A/C SNP promoter mutation in the CD36 gene represents a putative genetic marker for insulin-resistance in the French population, although it does not appear to contribute to the genetic risk for T2D.

Key-words: CD36 · Type 2 Diabetes · Insulin-resistance · Adiponectin · Genetics.

RÉSUMÉ

Étude génétique du gène CD36 dans une population de diabétiques français


Méthodes : Nous avons examiné la séquence entière du gène CD36 dans 272 individus.

Résultats : Nous avons détecté cinq mutations, deux rares (P191P et N247S) seulement retrouvées chacune dans une famille et ne s’associant ni avec le diabète, ni avec aucune donnée clinique, et trois fréquentes (A/C-178 dans le promoteur, A/G-10 dans l’intron 3 et une insertion (GGGTTGAGA) dans l’intron 13). Ces variants ont été retrouvés dans 454 sujets diabétiques et 221 contrôles et retrouvés à fréquences égales dans les deux groupes. Les taux d’adiponectine, un marqueur de sensibilité à l’insuline, ont été sensiblement associés à l’allèle variant du -178 A/C du promoteur (p = 0.003, p corrigé = 0.036), reflétant probablement une association avec l’insulino-résistance dans la population Française.

Conclusion : Ainsi, le polymorphisme -178 A/C du promoteur du gène CD36 est un marqueur génétique putatif pour l’insulino-résistance dans la population française.

Mots-clés : CD36 · Diabète de type 2 · Insulinorésistance · Adiponectine · Génétique.
CD36 is a multifunctional transmembrane receptor highly expressed in different tissues. CD36 primarily binds collagen, thrombospondin and long-chain fatty acids (FA). Higher long-chain FA levels contribute to T2D to worsen insulin-resistance by reducing muscle glucose uptake and favoring liver neoglucogenesis. CD36 also binds and internalizes oxidized low-density lipoprotein, a key event in macrophage foam cell development within atherosclerotic lesions, and its transcription is induced by the nuclear receptor peroxisome proliferator-activated receptor gamma, the target of insulin-sensitizing thiazolidinedione class of drugs used in T2D treatment. Moreover, in vitro overexpression of human CD36 gene revealed that CD36 mediates endocytic uptake and subsequent intracellular degradation of advanced glycation end proteins, well known products involved in diabetic vascular complications and atherosclerosis [1].

In the spontaneously hypertensive rat (SHR), an animal model of insulin-resistance, QTL for hypertension, hypertriglyceridaemia, reduced high density lipoprotein and metabolic defects in adipocytes revealed a single locus in the telomeric region of rat chromosome 4 [2] where CD36 was identified as a defective SHR gene [3]. In mice, muscle-specific overexpression of CD36 enhances FA oxidation, reduces plasma triglycerides, and increases plasma glucose and insulin [4]. CD36 null mice had reduced uptake of FA in heart, skeletal muscle and adipose tissue compared to wild type [5]. Transgenic expression of CD36 in SHR ameliorates insulin-resistance and lowers serum fatty acids [6]. Moreover, an insulin-resistance background was found in a small number of CD36-deficient patients [7].

Altogether, these data support CD36 as a plausible metabolic link between the various features of insulin-resistance, but its role in humans is mostly unknown. Previously, we reported evidence for familial linkage to T2D (MLS = 1.68, but its role in humans is mostly unknown. Previously, we reported evidence for familial linkage to T2D (MLS = 1.68, P = 0.00479) at the CD36 locus [8]. Thus, we considered CD36 as a plausible candidate gene for the common forms of polygenic T2D and therefore studied the contribution of CD36 genetic DNA variability in French T2D subjects.

**Material and methods**

**Subjects and phenotypes**

French DNA samples are part of a publicly advertised campaign for “200 families to overcome diabetes” and details of the recruitment have been described elsewhere [9].

**Mutation screening**

We screened coding exons of the gene including intron-exon boundaries, the novel 5’exon, alternative and proximal promoters, the 5’ untranslated region (UTR) up to 1 kb from the translational start codon and the 3’UTR up to +2.8 kb. Screening was conducted on 272 individuals, 41 unrelated patients extracted from families contributing to the described linkage [8], 39 unrelated patients extracted from families not contributing to the linkage, 96 unrelated T2D patients and 96 control subjects.

**Genotyping**

Apart from the 9 bp insertion genotyped on 4% endothium bromide stained agarose gels, the single nucleotide polymorphisms (SNP) were genotyped using the LightCycler™ technology (Roche) with probes provided by Tib-MolBiol. A set of 675 individuals, including 311 unrelated T2D subjects, 221 non diabetic subjects and one T2D patient from each of the 143 genome wide scan families [8], was genotyped for statistical analyses.

**Statistical analyses**

Genotype frequencies were compared through likelihood tests combined with determination of p-value via permutations for allelic associations. Adiponectin levels were compared using the Wilcoxon Kruskal Wallis test. Haplotypic frequencies and standardized linkage disequilibrium were determined with the PM+EH+ software [10]. HTR software was used with discrete and continuous trait for association of haplotype frequencies (http://statgen.ncsu.edu/zaykin/htr.html).

**Adiponectin levels**

Adiponectin levels were assessed by Radio-Immuno-Assays and confirmed by quantitative Western blotting.

**Expression and ligand binding analysis of the N247S protein**

Site directed mutagenesis. Cloning of the rat wild-type Cd36 has been described previously [11]. The oligonucleotide, 5’-GCGACATGATTAGTGGCACAGATG-3’, was used to mutate codon 247 in pAlterCd36 following the Altered sites mutagenesis protocol (Promega). The mutated gene, Cd36N247S, was introduced into pCIneo by replacing the wild-type Cd36 sequence to generate pciCd36N247S.

**Expression of wild type and mutant CD36 protein**

Whole cell lysates were prepared from tansiently-transfected HEK293T (human embryonic kidney cells) and analyzed by western blot, probed with mouse anti-human CD36 MCB131.4 (generously provided by Brian Curtis) and an HRP-conjugated, goat anti-mouse secondary antibody (DAKO). Bound HRP was detected by enhanced chemiluminescence (Amersham).
Bodipy-acetylated low density lipoprotein (Bodipy-acLDL) binding assay

Transiently-transfected HEK293T cells were incubated with Bodipy-acLDL (Molecular Probes) and analysed in a FACs Vantage flow cytometer (Beckton Dickinson), as described previously [16]. Fluorescence data from 10000 cells, gated for normal shape and morphology and shown to be intact by their failure to accumulate propidium iodide, were analysed using CELLQuest software (Becton Dickinson).

Results

Mutation screening revealed five SNP: -178 A/C in the promoter, -10 A/G in intron 3, a silent Pro191 (CCG/CCA) variant in exon 5, a missense Asn247Ser (AAT/AGT) mutation in exon 7 and a 9 bp (gggttgaga) insertion in intron 13, 410 bp before exon 14. Pro191 and Asn247Ser were both found heterozygous with the wild-type gene, each in a single subject (freq < 0.2%) while frequencies of the promoter SNP (SNP1), the intron 3 SNP (SNP2) and the 9 bp insertion (9 bp) were 54.1%, 5.4% and 44.3% respectively. Genotype frequencies of these SNP were in Hardy-Weinberg’s equilibrium and they were not in complete linkage disequilibrium (D’ = 0.6794, -0.5798, and -0.4832 respectively for SNP1-SNP2, SNP1-9bp and SNP2-9 bp couples).

Distribution of SNP genotype frequencies did not differ between the 454 T2D individuals and the 221 control subjects (p = 0.39; p = 0.69; p = 0.94, respectively; Tab I). As the power calculation with a fixed Odd-Ratio of 2 gave a power of, respectively, 0.9985, 0.15 and 0.9984 to detect association with the 3 detected SNPs, it is unlikely that these individual SNPs contribute to the genetic risk of T2D in our population. Furthermore, we analyzed the three SNP haplotype contribution to T2D. Four haplotypes had a frequency higher than 5%. None of them was associated with T2D (data not shown).

The P191P variant was found once at heterozygous state in a T2D proband showing normal clinical characteristics, and was not detected again in this family. The N247S mutation was found in three members of one family, two of them had T2D, the third sib showed atherogenic features but did not harbor T2D. This mutation was also detected in the next generation but no obvious phenotypic variation could be observed. N247 is located in the large extracellular domain of CD36 and has been reported to be glycosylated [12]. Ligand binding sites on the extracellular domain capture modified low density lipoproteins and long chain fatty acids which are then internalized by endocytosis. Deleterious amino acid substitutions in the extracellular domain are therefore most likely to affect ligand binding, either directly or allosterically. We tested for such an effect by substituting serine for asparagine at position 247 in the homologous rat CD36. The mutated protein was expressed transiently in cell culture and shown by western analysis (Fig 1A) to be the same size as the wild type CD36, indicating that the substitution does not affect the glycosylation status of the protein. The N247S mutant protein was also detected on the cell surface by antibody binding (data not presented) and could be shown to bind acetylated-low density lipoprotein (Fig 1B), suggesting that trafficking to the cell surface and ligand binding were similar in the wild-type and mutated CD36 N247S proteins. Taken together, the genetic data and functional analyses suggest that the N247S mutant does not have a significant effect on CD36 function and does not cause T2D.

As adiponectin is considered a good marker for insulin sensitivity [13-14] and also a protective factor against atherosclerosis [15], we then analyzed the potential effect of the 3 CD36 common SNPs in the 675 genotyped subjects for which plasma adiponectin was available. As expected, adiponectin levels were lower in T2D subjects compared to controls (12.91 ± 7.96 vs 18.05 ± 8.72, p < 0.0001). In the whole cohort, adiponectin levels were significantly associated with the -178 A/C promoter SNP (p = 0.003, p corrected for multiple testing = 0.036). Mean adiponectin levels were 14.92 ± 8.21, 14.95 ± 9.25 and 12.32 ± 6.53 for the subjects with the AA, AC and CC genotypes respectively (Tab II), suggesting that this SNP negatively associates with insulin sensitivity. Analysed separately, the association between this SNP and adiponectin levels remained significant in controls (p = 0.012) but only a trend for association was found in T2D subjects (p = 0.12). Analyses using adjusted adiponectin values (for age, sex, BMI and for T2D) gave similar results, especially in control subjects (p = 0.006).

Table I

Genotypes frequencies of the three SNP tested for association with T2D in the French population.

<table>
<thead>
<tr>
<th>SNP</th>
<th>Genotypes</th>
<th>Control subjects</th>
<th>T2D patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>-178 A/C</td>
<td>A/A</td>
<td>0.175</td>
<td>0.194</td>
</tr>
<tr>
<td></td>
<td>A/C</td>
<td>0.568</td>
<td>0.507</td>
</tr>
<tr>
<td></td>
<td>C/C</td>
<td>0.257</td>
<td>0.299</td>
</tr>
<tr>
<td>p = 0.39</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-10 A/G</td>
<td>A/A</td>
<td>0.941</td>
<td>0.950</td>
</tr>
<tr>
<td></td>
<td>A/G</td>
<td>0.590</td>
<td>0.500</td>
</tr>
<tr>
<td></td>
<td>G/G</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>p = 0.69</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intron 13 insertion</td>
<td>D/D</td>
<td>0.294</td>
<td>0.304</td>
</tr>
<tr>
<td></td>
<td>D/I</td>
<td>0.527</td>
<td>0.512</td>
</tr>
<tr>
<td></td>
<td>I/I</td>
<td>0.179</td>
<td>0.184</td>
</tr>
<tr>
<td>p = 0.94</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The intron 3 A/G SNP and the 9 bp insertion in the 3'UTR were not associated with differing adiponectin levels and none of the 4 frequent haplotypes involving the 3 SNPs was more strongly associated with adiponectin levels than the -178 A/C promoter SNP (data not shown). Other clinical values (insulin and glucose levels, lipid values, insulin indexes as HOMA) were not associated with any of these 3 SNP (data not shown).

**Discussion**

With regard to the CD36 polymorphisms found in the coding region and 5' and 3'UTR sequences, no association with T2D was detected in our French population. Recently, it was suggested that restricting variation discovery to coding regions and proximal promoter does not adequately describe all common haplotypes or the true haplotype block structure observed when all common variation is used to infer haplotypes [16]. Thus, it is still possible (but not very likely) that we have missed functional SNPs in intronic regions that may truly contribute to the genetic risk for T2D.

**Table II**

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Whole sample</th>
<th>Controls</th>
<th>T2D</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$p = 0.003$</td>
<td>$p = 0.012$</td>
<td>$p = 0.12$</td>
</tr>
<tr>
<td></td>
<td>$b = 0.081$</td>
<td>$p = 0.006$</td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>$14.92 \pm 8.21$</td>
<td>$17.69 \pm 8.15$</td>
<td>$13.63 \pm 8$</td>
</tr>
<tr>
<td></td>
<td>$0.01759 \pm 0.4890$</td>
<td>$0.0404 \pm 0.4358$</td>
<td>$0.04377 \pm 0.5116$</td>
</tr>
<tr>
<td>AC</td>
<td>$14.95 \pm 9.25$</td>
<td>$19.79 \pm 9.29$</td>
<td>$13.28 \pm 8.65$</td>
</tr>
<tr>
<td></td>
<td>$0.03286 \pm 0.5199$</td>
<td>$0.1031 \pm 0.4302$</td>
<td>$0.0102 \pm 0.5445$</td>
</tr>
<tr>
<td>CC</td>
<td>$12.32 \pm 6.53$</td>
<td>$14.62 \pm 7.23$</td>
<td>$11.72 \pm 6.24$</td>
</tr>
<tr>
<td></td>
<td>$-0.0753 \pm 0.5098$</td>
<td>$-0.1929 \pm 0.5099$</td>
<td>$-0.0470 \pm 0.5077$</td>
</tr>
</tbody>
</table>

However, the -178 A/C promoter SNP in homozygous state was significantly associated with lowered adiponectin levels (even after correction for multiple testing), suggesting a recessive effect of this SNP on the adiponectin phenotype. Although association (or a trend toward association) between the CD36 promoter SNP and adiponectin was found in the whole population, the genetic association was stronger in normoglycaemic subjects than in diabetics. This is not surprising, as chronic hyperglycaemia per se decreases adiponectin levels making the correlation between adiponectin and insulin sensitivity less robust. In non diabetics, adiponectin strongly correlates with insulin sensitivity and may be a better marker of insulin-resistance than indirect indexes based on fasting glucose and insulin [13-14]. Furthermore, adiponectin decreases in parallel with insulin sensitivity during the progression to T2D in monkey [17]. Thus our data indicate that CD36 gene variation may be associated with adiponectin levels in humans suggesting a modulation of insulin sensitivity. Further studies in larger sample sets will be necessary to confirm this hypothesis.

Computational analysis revealed a nucleotide sequence [agttca], corresponding to a Glucocorticoid Receptor Binding site (GRE) around position -178 in the CD36 promoter. The glucocorticoid receptor and its binding sites regulates expression and activity of key genes involved in glucose and fatty acids homeostasis, and thus have been implicated in the pathogenesis of T2D and insulin-resistance syndrome. This SNP could therefore modify the binding activity of this site. It is also possible that this SNP is in linkage disequilibrium with an undetected functional SNP in the CD36 locus as discussed above. More systematic SNP analysis of the gene are required to clarify this issue.

Genetic variations in the CD36 gene modulating the uptake of circulating fatty acids may indirectly modify insulin sensitivity displayed by variations of serum adiponectin levels, even if this effect may be not sufficient to increase the risk for T2D. Interestingly, the -178 A/C promoter SNP is not associated with an atherogenic lipid profile in our French population (data not shown) as it is in the MONICA North of France general reference population [18], high-

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**Figure 1**

(A) Western blot analysis of whole cell lysates of HEK293T cells transfected transiently with pCIneo vector-only control (left lane), pcICD36N247S (middle) or pcICD36 (right), probed with an anti-CD36 antibody. (B) Flow cytometric dot plots of Bodipy-ac-LDL binding to cells transfected transiently with vector alone (left panel), pcICD36N247S (middle) or pcICD36 (right). Cell size is described by the abscissae and bound fluorescent acetylated-LDL in arbitrary units by the ordinate.
lighting the necessity to test multiple populations in genetic studies. On the other hand, we recently identified a CD36 L360X nonsense mutation which co-segregated in a Mendelian fashion with insulin resistance and type 2 diabetes in a large pedigree [19]. From all these data, one may speculate that although rare mutations responsible for severe CD36 impairment are associated with severe insulin resistance and T2D in rats and in humans, more frequent CD36 SNP may modulate gene expression with a mild effect on lipids and/or insulin sensitivity, contributing to modulate the metabolic syndrome rather than predispose to overt T2D.

Acknowledgement – We are indebted to all families who participated to this study. We thank Valérie Delannoy, Sophie Gallina and Stéphane Gaget for their help with bioinformatics networks, and Ollert Landt (Tib-MolBiol) for his help in probes design. This work was supported by grants from the Nord-Pas-de-Calais Region administration and by the Centre National de Recherche Scientifique (CNRS).

References


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