Endocannabinoid receptor 1 gene variations increase risk for obesity and modulate body mass index in European populations

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The therapeutic effects of cannabinoid receptor blockade on obesity-associated phenotypes underline the importance of the endocannabinoid pathway on the energy balance. Using a staged-approach, we examined the contribution of the endocannabinoid receptor 1 gene (CNR1) on obesity and body mass index (BMI) in the European population. With the input of CNR1 exons and 3′ and 5′ regions sequencing and HapMap database, we selected and genotyped 26 tagging single-nucleotide polymorphisms (SNPs) in 1932 obese cases and 1173 non-obese controls of French European origin. Variants that showed significant associations (P < 0.05) with obesity after correction for multiple testing were further tested in two additional European cohorts including 2645 individuals. For the identification of the potential causal variant(s), we further genotyped SNPs in high linkage disequilibrium (LD) with the obesity-associated variants. Of the 25 successfully genotyped CNR1 SNPs, 12 showed nominal evidence of association with childhood obesity, class I and II and/or class III adult obesity (1.16 < OR < 1.40, 0.00003 < P < 0.04). Intronic SNPs rs806381 and rs2023239, which resisted correction for multiple testing were further associated with higher BMI in both Swiss obese subjects and Danish individuals. The genotyping of all known variants in partial LD (r² > 0.5) with these two SNPs in the initial case–control study, identified two better associated SNPs (rs6454674 and rs10485170). Our study of 5750 subjects shows that CNR1 variations increase the risk for obesity and modulate BMI in our European population. As CB1 is a drug target for obesity, a pharmacogenetic analysis of the endocannabinoid blockade obesity treatment may be of interest to identify best responders.

INTRODUCTION

The endocannabinoid pathway is mediated by the binding of 2-arachidonyl glycerol (2-AG) and anandamide (AEA) with the cannabinoid receptor (CB1) (1). Animal studies have identified its physiological role in the regulation of energy metabolism (2–5), as well as in food intake and addictive behaviours (6–8). These discoveries have led to the development of CB1 antagonists as potential therapeutic agents for treating obesity (9–12) and metabolic complications (12,13). In humans, CB1 antagonists induce weight loss, reduce food intake (10–13), improve glucose metabolism (10–13) and modulate lipid levels (10–13). These findings show the importance of CB1 in human physiology and qualify CNR1 as a biological candidate for human obesity.

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Previously, a study showed modest evidence of a modulating effect of the rs12720071 CNR1 variant on body fat mass and distribution in white European adult men (14). Other studies have also tested the contribution of the CNR1 rs1049353 single-nucleotide polymorphism (SNP) on obesity but found inconsistent results (15–17). To analyze whether CNR1 variations are implicated in human obesity, and in view of the weak linkage disequilibrium (LD) coverage of the previous studies, we chose to investigate the possible role of 26 tagging SNPs, representing 100% of the common variation in a 37.4 kb region encompassing the CNR1 locus (http://www.hapmap.org) in 1932 obese cases and 1173 controls of French origin. We then selected two SNPs displaying P-values that resisted permutation-based correction for multiple testing and analyzed them in two independent European cohorts. Finally, in an attempt to identify putative functional SNP(s), we genotyped in the initial case–control study, all SNPs having a minor allele frequency (MAF) > 5% and in LD (r^2 > 0.5) with the two obesity-associated tagging SNPs.

RESULTS

SNP identification and selection

Over 21 kb, including the four known exons described by Zhang et al. (18), the exon–intron boundaries, the intronic SNP-rich regions as well as 5.3 kb upstream and 5.9 kb downstream of CNR1, were sequenced in 24 adult controls and 48 obese children (Supplementary Material, Fig. S1). Thirty-six SNPs with a MAF ≥ 5% were identified and used to establish an LD map using Haploview. In addition, we ran ‘Tagger’ of the Haploview program with the HapMap phase II data in a 37.4 kb region defined by LD blocks and encompassing the CNR1 locus (chr6:88904301–88941649 on NCBI Build 36.1). Combining the sequencing results to the HapMap phase II data (http://www.hapmap.org) and for an r^2 > 0.8, we selected and genotyped for the case–control association studies, 26 tagging SNPs with a MAF ≥ 5% that ensure complete coverage of the known haplotype diversity of the locus according to the HapMap II.

Case–control study

Twenty-five of the 26 tagging SNPs that best represent the known haplotype diversity of CNR1 were successfully genotyped in 1932 obese cases and 1173 controls. Results of the case–control analyses are given in Table 1 and in Supplementary Material, Table S1. All SNPs were in Hardy–Weinberg equilibrium (P > 0.05) in the normal, glucose-tolerant non-obese French Caucasian control collection. Twelve SNPs showed nominal evidence of association with childhood obesity, class I and II and/or class III adult obesity [odds ratios (ORs) between 1.16 and 1.40; P = 0.04 to P = 0.00003]. Using spectral decomposition, we estimated the total number of efficient tests at 16 (19,20). The number of SNPs with a P-value < 0.05 accounted for nine tests (according to the same method). As an estimate of the concentration of high P-values, we estimated that the probability of 10 significant tests (P < 0.05 level) out of 16 trials was 5.91 × 10^-10.

The haplotypes showing the lowest Akaike Information Criterion (AICmin) were tested for association with obesity. None of the major haplotypes (frequency > 5%) showed a stronger association with obesity than the single SNPs (Supplementary Material, Table S2).

For the replication study, we selected SNPs with P-values that survived permutation-based correction for multiple testing in the case–control analysis. After this test, the rs806381 SNP (−10908 A>G) was associated with childhood obesity (experiment-wide P-value, eP-values = 0.002) and the rs2023239 SNP (−5489 T>C) with class I and II adult obesity (eP-values = 0.016) (Table 1). To further account for multiple testing, we also performed a False discovery rate (FDR) analysis for the 25 SNPs: the tests that displayed q-value < 0.05 accounted for the two variants described above (Table 1).

BMI analysis

The potential effect of rs806381 and rs2023239 on the continuous obesity-related trait, body mass index (BMI), was then tested in two additional independent European cohorts (Table 2). In a Swiss cohort of 865 obese adults, both rs806381 obesity G at-risk allele and rs2023239 obesity T at-risk allele were associated with increased BMI. For the

Table 1. Association study of two CNR1 variants showing evidence of association with obesity after multiple testing corrections

<table>
<thead>
<tr>
<th>Tagging SNPs</th>
<th>&gt;Subjects</th>
<th>&gt;MAF</th>
<th>&gt;At-risk allele</th>
<th>&gt;Odds ratio (Allelic)</th>
<th>&gt;P-value (Allelic)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs806381 (−10908 A&gt;G) Chr6:88922620</td>
<td>Controls 0.29 — — —</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Obese children 0.36 G</td>
<td>1.39 [1.19–1.63]</td>
<td>0.0003x,b</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Class I and II obese adults 0.35 G</td>
<td>1.31 [1.13–1.53]</td>
<td>0.0006b</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Class III obese adults 0.33 G</td>
<td>1.22 [1.05–1.42]</td>
<td>0.01</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls 0.20 — — —</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>Obese children 0.18 — — —</td>
<td>1.14 [0.95–1.37]</td>
<td>0.15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Class I and II obese adults 0.15 T</td>
<td>1.40 [1.16–1.69]</td>
<td>0.0004b</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Class III obese adults 0.18 — — —</td>
<td>1.19 [0.99–1.44]</td>
<td>0.07</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Nucleotide changes are given with the most frequent allele first.

xSignificant P-values after permutation-based analyses (based on 25 SNPs and three cohorts).

bSignificant P-values after the FDR test for a q-value < 0.05.
r806381, the GC heterozygous carriers were 1 BMI unit heavier and the GG homozygous carriers ~3 BMI units heavier ($P = 0.015$) than the individuals with rs806381 CC genotypes. A similar trend was observed for the rs2023239 variant ($P = 0.02$). Additionally, we studied the effect of two $CNR1$ SNPs on BMI in a general population of 1780 Danish adults. Data were in agreement with the Swiss results, showing a significant additive effect of the rs806381 G at-risk allele: heterozygous carriers had a higher BMI than the CC homozygotes and a lower BMI when compared with the GG homozygotes ($P = 0.0023$). For the rs2023239 variant, the obesity T at-risk allele carriers had a significantly higher BMI in the Danish cohort ($P = 0.021$). We further tested for association of rs806381 and rs2023239 on BMI in the French obese children cohort and in a pooled cohort of French obese adults (class I, II and III obese individuals). While no significant result of the Z-score of BMI was observed in the children cohort, we found that the rs806381 obesity G at-risk allele carriers had a significantly higher BMI when compared with the non carriers in the obese adult cohort ($P = 0.016$).

**Meta-analysis test**

Overall significance was assessed using Fisher’s method, which combines $P$-values of independent analyses. We used the number of effective tests (19) at each step to correct for multiple testing while accounting for the between SNPs correlations. The meta-analysis combining evidence of association for obesity and higher BMI found for SNP rs806381 and rs2023239, $P$ ($df = 6$) = $1.1 \times 10^{-6}$ and $P = 4.7 \times 10^{-4}$, respectively.

**Fine mapping of rs2023239 and rs806381 LD blocks**

In an attempt to identify the potential causal variant(s), we increased the SNP density of rs2023239 and rs806381 LD blocks. Using phase II genotyping data from the HapMap project (http://www.hapmap.org) in the 37.4 kb region defining the $CNR1$ locus, we selected 17 SNPs in LD ($r^2 > 0.5$) with rs806381 and rs2023239. The comparison of the fine mapped SNP to the initial LD block representative SNP (rs806381 and rs2023239), (for the same subjects) found one additional variant for each block with increased significance of association with obesity (Fig. 1 and Supplementary Material, Table S3). In the rs2023239 block, in the class I and II obese adult group, the rs10485170 obesity A at-risk allele carriers are at substantially increased risk of being obese [$OR = 1.85$ ($1.41–2.44$), $P = 0.000008$] compared with the rs2023239 T at-risk allele carriers [$OR = 1.40$ ($1.16–1.69$), $P = 0.0004$]. In the rs806381 block, in the childhood obesity group, the risk was also increased for one of the SNPs: rs6454674 OR = 1.52 ($1.26–1.84$), $P = 0.00001$ compared with rs806381 OR = 1.39 ($1.19–1.63$), $P = 0.00003$ (Fig. 1 and Supplementary Material, Table S3).

To test if these two new SNPs were the best associated SNPs in their respective blocks, we performed a logistic regression analysis including in the model the initial LD block representative (i.e. rs2023239) and the fine mapping SNP (i.e. rs10485170). Interestingly, the rs10485170 was found to be significantly associated with obesity independently of the rs2023239 ($P = 0.015$), whereas rs2023239 no longer remained associated with obesity with the rs10485170 in the model ($P = 0.23$). In the logistic regression analysis of the other LD block, we could not discriminate the effect of rs806381 and rs6454674 on obesity.

**DISCUSSION**

Although little is known on how the endocannabinoid system contributes to human energy homeostasis (21), data derived from phase III trials of the CB1-receptor inhibitor rimonabant indicate clinical benefits of this antagonist on obesity and associated phenotypes (10–13). Based on these findings, we have tested the contribution of the endocannabinoid receptor 1 gene ($CNR1$) frequent genetic variation on the risk of obesity and increased BMI. An initial French case–control analysis of 25 tagging SNPs representative of the known haplotype diversity of the $CNR1$ locus, found a significantly higher number of associated SNPs than expected by chance ($5.91 \times 10^{-10}$). In an attempt to minimize false positives that could result from the number of variants tested, we performed a multiple testing permutation-based correction and selected two variants that were not in LD ($r^2 < 0.05$) for replication. The first replication sample was a Swiss cohort with patients with severe affection status (BMI > 35 kg/m$^2$). With the initial case–control study, the Swiss cohort included a large proportion of cases enriched...
for family history leading to an overestimation of the general population risk. For this reason, we chose to further test this variant in a general population-based Danish cohort. Despite the dissimilarity of these two independent replication cohorts, we found that both rs2023239 and the rs806381 at-risk allele carriers had a significantly higher BMI. Although the pooled analysis of the three studies remain slightly over the genome-wide level of significance ($P = 5.10^{-7}$), the consistent associations provide encouraging prospects for subsequent studies.

Recently a group found no evidence of an association of the rs2023239 in German obese children and adolescents (17). Interestingly, this variant that was strongly associated with obesity and BMI in our obese adult case–control and adult replication cohorts (overall $P < 10^{-5}$), the consistent associations provide encouraging prospects for subsequent studies. Presently, there are no features to qualify rs806381 and rs2023239 as functional SNPs, as there are no clear genetic mechanisms to explain how they may alter the function or expression of $CNR1$. For this reason, we chose to genotype all SNPs in partial LD with these two variants for the identification of potential causal variants. The genotyping of 17 additional variants in our initial case–control groups identified two variants showing increased significance of association with obesity. The comparison of these variants to the two LD block representatives found that the rs10485170 had a significantly stronger effect on obesity than the rs2023239, while we could not discriminate between the effect of the rs806381 and rs6454674 on obesity.

In conclusion, we suggest that the consecutive replications and overall significance of associations with obesity and BMI found for frequent $CNR1$ variants, in a total of 5750 white European participants, of all ages, in the most complete analysis of the $CNR1$ locus with obese children and adults, strongly support the contribution of the $CNR1$ gene to polygenic obesity in European populations. Furthermore, our results raise the important question of whether the pharmacological effect of CNR1 antagonists could differ between patients with different $CNR1$ genotypes.

![Figure 1. Structure of the $CNR1$ gene with the location of 26 common polymorphisms and the LD map. (a) Schematic representation of human $CNR1$ transcript isoforms (CB1A–E) as described by Zhang PW et al. (18); blue thin lines and bars represent introns and exons (ex1, ex1a, ex2, ex3a, ex3 and ex4) respectively; green CDS box indicates coding region. (b) Bar charts show sequenced regions in the 37.4 kb studied interval (c) 17 tagging SNPs found using the pairwise method ($r^2 > 0.8$) based on the sequencing data and 9 additional tagging SNPs (*) from HapMap II; SNPs highlighted in bold have a $P$-value that resisted multiple testing corrections; positions of new markers with no rs number are given relative to first base of ATG start codon. (d) LD plot indicating $r^2$ between the 26 SNPs with a MAF $\geq 5\%$ found by sequencing. Shades of grey indicate $0 < r < 1$. $r^2 = 0$ being white and $r^2 = 1$ being black.](image-url)
**MATERIALS AND METHODS**

**Subjects**

The study protocols were approved by all local ethics committees and informed consent was obtained from each subject before participation in the studies.

**Case–control study.** Subjects in the case–control association studies were all French Caucasians recruited through a multimedia campaign run by the Centre National de la Recherche Scientifique (CNRS), Hotel Dieu Hospital, the Pasteur Institute, Lille and the Department of Paediatric Endocrinology of Jeanne de Flandres Hospital.

The case–control association study included three case groups: (i) 615 obese children (BMI above the 97th percentile), (ii) 625 class I and II obese adults (30 kg/m² ≤ BMI ≤ 40 kg/m²) (22), (iii) 692 class III obese adults (BMI > 40 kg/m²) (22). The case groups were compared to a total of 1173 controls. The control subjects were unrelated adult non-obese French Caucasians pooled from three separate studies: a set of (i) 266 individuals (mean BMI = 23.1 ± 2.16 kg/m²; mean age 42.5 ± 4.5 years; men/women 105/161), (ii) 171 controls (mean BMI = 22.9 ± 2.3 kg/m²; mean age 60.7 ± 11.5 years; men/women 71/100), (iii) 736 individuals (mean BMI = 23.8 ± 1.8 kg/m²; mean age 53.5 ± 5.6 years; men/women 293/443) recruited, respectively, at the CNRS Lille, the ‘Fleuroix-Laventie Ville-Sante’ study (23) and the Epidemiologic Data on the Insulin Resistance Syndrome (DESIR) Study (24). Before being pooled, the three control sets were compared with each other. The Woolf test was applied to assess the genotypic homogeneity among the control studied groups on 2 × 2 × k tables over strata (25,26). This test identified no genotypic heterogeneity between the different groups (Supplementary Material, Table S4).

**Replication cohorts.** Associations with BMI have been tested in 865 class II and III (BMI ≥ 35 kg/m²) unrelated Caucasian subjects from Switzerland (27) (mean BMI = 43.4 ± 7.1 kg/m²; mean age 42.8 ± 10.7 years; men/women 667/198) and in a random sample of 1780 middle-aged individuals recruited from the Danish general population (Inter99 cohort) (mean BMI = 26.4 ± 4.4 kg/m²; mean age 45.9 ± 7.9 years; men/women 914/866) (Supplementary Material, Table S5).

**Sequencing and SNP genotyping**

Sequencing was performed using the automated ABI Prism 3730 DNA sequencer in combination with the Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). Tagging SNPs were genotyped with SNplex, Taqman (Applied Biosystems) or Lightyper (Roche Diagnostics) technologies. As a standard laboratory quality control measure, a random 10% of DNA samples were systematically re-genotyped to ensure minimal genotyping error. Concordance rate was comprised between 99 and 100% for the 25 SNPs.

**Statistical analysis**

**Case–control analyses.** Allele frequencies in cases and controls were compared using the χ² test integrated in Cocaphase (28). ORs and P-values were from logistic regression, adjusted for age and gender, in the class I and II and class III adult obesity case–control study. For the childhood obesity case–control, the test was adjusted for gender. To test the association between case–control data and the combination of CNR1 SNPs, we selected haplotypes with frequencies of at least 5% and showing the lowest AICmin. These models were tested using Cocaphase.

**BMI analysis.** Associations between BMI and the SNPs were analyzed using SPSS software (version 14.0.2, SPSS Inc., Chicago, IL, USA). For the BMI analysis, we used a general linear model taking into account gender and age.

**Fisher’s test.** To produce an overall significance of increased allelic frequency in obese individuals, we combined the P-value from the case–control study (all obese versus controls) with the P-values of the replication studies using Fisher’s method in which the twice the negative sum of the natural log of n P-values follows χ² distribution with 2n degrees of freedom.

**Multiple testing.** Multiple testing issues in the case–control comparisons were addressed using permutation analysis that takes into consideration the inter-SNPs correlations. For the study of 25 SNPs in three cohorts, we performed 1000 permutations of case labels for each case–control analysis, obtaining three empirical P-values per SNP. These P-values were then corrected for the number of cohorts to obtain an eP-value for each SNP.

FDR analysis was also performed for the case–control comparisons: in this study the cut-off for significance was the P-value = 0.0006 with an FDR q-value < 0.05.

For the BMI analysis, the uncorrected P-values are presented and discussed in the main text. In consideration of the number of statistical tests carried out, a Bonferroni correction was applied and the significant corrected P-values (P ≤ 0.05) in the Table 2 are identified by an asterisk (*).

**Fine mapping.** To determine the best associated SNPs in each respective block, we performed a logistic regression test including in the model, the gender, the initial LD block representative and the ‘fine mapping’ SNP.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG Online.

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