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Cannabis Receptor Haplotype Associated With Fewer Cannabis Dependence Symptoms In Adolescents

Christian J. Hopfer[1], **Susan E. Young**[2], **Shaun Purcell**[4], **Thomas J. Crowley**[1], **Michael C. Stallings**[2], **Robin P. Corley**[2], **Soo Hyun Rhee**[2], **Andrew Smolen**[2], **Ken Krauter**[3], **John K. Hewitt**[2], and **Marissa A. Ehringer**[2,5]

[1] Department of Psychiatry, University of Colorado Health Sciences Center, Denver, CO

[2] Institute for Behavior Genetics, University of Colorado, Boulder, CO

[5] Department of Integrative Physiology, University of Colorado, Boulder, CO

[3] Department of Molecular Cellular and Developmental Biology, Boulder CO

[4] Massachusetts General Hospital, Boston, MA

Abstract

Cannabis is a major substance of abuse, and the gene encoding for the central cannabinoid receptor (CNR1) is a logical candidate gene for vulnerability toward developing symptoms of cannabis dependence. We studied four single-nucleotide polymorphisms (SNPs) in the CNR1 gene for association with having one or more symptoms of cannabis dependence in 541 adolescent subjects who had all tried cannabis five or more times. Cases (327) were defined as those who had tried marijuana and developed one or more symptoms, and controls (214) as those who had tried marijuana but developed no dependence symptoms. Cannabis dependence symptoms were assessed in these youth when they were 17 or older with the Composite International Diagnostic Interview- Substance Abuse Module. Univariate (single-marker) association tests demonstrated that SNP rs806380, located in intron 2 of the CNR1 gene, was significantly associated with developing one or more cannabis dependence symptoms, with the G allele having a protective effect ($p < 0.02$). This was consistent with the results of the global haplotype test ($p < 0.01$). One of the common haplotypes examined (present in 21% of the subjects) was significantly associated with a lower rate of having one or more cannabis dependence symptoms. Our findings provide evidence suggesting that a common CNR1 haplotype is associated with developing fewer cannabis dependence symptoms among adolescents who have experimented with cannabis.

Keywords

Cannabis; Adolescence; Genetics; CNR1

Introduction

Cannabis is the most commonly used illicit substance among adolescents and young adults. In 2004, 46% of 12th graders reported having tried cannabis at some point in their lifetime, 34% reported having used within the past month, and 5.6% reported having smoked cannabis daily (Johnston et al. 2005). Initiation into cannabis use typically begins in adolescence, as youths aged 12 to 17 constitute about two thirds of the new cannabis users

Address reprint requests to Dr. Christian J. Hopfer, 4200 E. Ninth Ave, Box C-268-35 University of Colorado Health Sciences Center, Denver, CO, 80262; Phone: 303-315-0798; Fax: 303-315-0394 Email: Christian.Hopfer@uchsc.edu.

(SAMHSA 2002). Approximately 14% of adolescent-onset cannabis users develop cannabis dependence, a rate roughly twice that reported for adult-onset user (Chen et al. 1997; Chen and Anthony 2003). Cannabis dependence is defined in the Diagnostic and Statistical Manual of Mental Disorder, 4th edition, text revision (DSM –IVTR) as having at least three out of seven symptoms within one year (APA, 2000). Symptoms of cannabis dependence include physiological symptoms, such as tolerance or withdrawal, cognitive symptoms such as being preoccupied with obtaining or using cannabis, and symptoms of psychosocial impairment such as continuing to use despite impairment in school or relationship functioning.

Twin studies have demonstrated genetic influences on the liability to develop cannabis dependence in both adults and adolescents (Kendler and Prescott 1998; Tsuang et al. 1998; Maes et al. 1999; Kendler et al. 2000; Miles et al. 2001; Lynskey et al. 2002; Rhee et al. 2003). A logical candidate gene that could influence the liability to develop cannabis dependence symptoms is CNR1, which codes for the cannabinoid receptor. The cannabinoid receptor is the principal site of action of delta-9-tetrahydrocannabinol (THC), the principal psychoactive ingredient in cannabis (as reviewed by Childers and Breivogel (1998) and Onaivi et al. (2002)). THC mimics the actions of endogenous cannabinoids, such as anandamide. The CNR1 receptor is found throughout the brain and is expressed at high levels (Childers and Breivogel 1998). Converging evidence from animal studies suggests that the endocannabinoid system is involved in the processing of rewarding stimuli (reviewed by Onaivi et al. (2002)) and substantial evidence now shows interactions with the opioid (Navarro et al. 2001) and dopaminergic systems (Chen et al. 1993).

CNR1 has been examined for association with a range of substance abuse phenotypes, however, to our knowledge none have focused primarily on cannabis dependence symptoms. Opioid dependence (Li et al. 2000), drug dependence (Covault et al. 2001), extreme response to cannabis use (cannabis-induced psychosis) (Hoehe et al. 2000), alcohol withdrawal delirium (Schmidt et al. 2002), intravenous drug use (Comings et al. 1997), and P300 evoked potential have been examined for their association with CNR1. Covault et al. (2001) reported no association between a CNR1 microsatellite polymorphism and drug or alcohol dependence. Li et al. (2000) reported that in a sample of 375 Han Chinese, there was no evidence of an association between alleles of the same microsatellite repeat in the CNR1 gene and heroin dependence. Hoehe et al. (2000) reported no difference in allele frequencies of two silent mutations in the coding region of CNR1 between individuals exhibiting an extreme response to cannabis and a control group. Schmidt et al. (2002) reported a positive association between alcohol withdrawal delirium and a silent mutation in the CNR1 receptor (1359 G/A). Comings et al. (1997) reported a positive association between a CNR1 microsatellite polymorphism and intravenous drug use as well as cocaine, amphetamine, and cannabis dependence. Johnson et al. (1997) also showed an association between the polymorphism and lower P300 evoked related potential, which in turn has been associated with alcohol and drug dependence. Recently, Zhang et al. (2004) reported on the structure of the CNR1 gene, including findings of three novel exons, and an association between a CNR1 haplotype and polysubstance abuse. Of the reviewed studies, only two (Comings et al. 1997; Hoehe et al. 2000) report an association between CNR1 polymorphisms and cannabis-related phenotypes. Both studies examined only one or two polymorphisms in adults. The purpose of this study was to examine the association between Single Nucleotide Polymorphisms (SNPs) within the CNR1 gene and developing cannabis dependence symptoms in adolescents using both single-marker and haplotype analyses. We are not aware of any previous studies that have examined this association in adolescents.

Methods

Subjects

Subjects were participants in ongoing studies of the genetics of adolescent and young adult substance abuse in Colorado, funded by the National Institute of Drug Abuse. Youth who participated in these studies were recruited from treatment settings for youth with substance use disorders, criminal justice settings, and community-based twin, adoption, and family studies of adolescent substance use disorders. The consents for the original studies asked whether the information gathered could be used for analyses of "genetic tests related to substance use disorders." Only those subjects whose parents consented and themselves assented to this statement were included in these analyses.

The entire pool of potential subjects encompassed over 5000 youth and we selected for inclusion in this study those who met the following criteria: a) at least age 17 or older, b) had endorsed using marijuana at least 5 times, and c) at least one year between age of first marijuana use and age at testing. Additionally, we limited subject selection to one youth per family. Five hundred forty-one youth met the inclusion criteria and were selected for the present study.

Assessments

All youth were assessed with the Composite International Diagnostic Interview – Substance Abuse Module (CIDI-SAM). This instrument diagnoses DSM-IV Abuse and Dependence (APA 1994) for ten drug classes and assesses individual symptoms for each drug class. It primarily consists of a 30-to-60-minute interview that is designed for administration by trained, lay interviewers. It is a descendent of the NIMH Diagnostic Interview Schedule. The CIDI's reliability and validity (Cottler et al. 1989) made it the main assessment for DSM-IV Substance Field Trials. Its validity and reliability has been established in substance-dependent adolescents (Crowley et al. 2001).

Sample Description

Cases for this study were defined as those who had one or more dependence symptoms (i.e., endorsed at least one DSM-IV dependence symptom for cannabis), and controls as those who experimented with marijuana at least five times, but had no dependence symptoms. Table 1 describes the ethnic, age, and gender distribution of the cases and controls, as well as the percent meeting criteria for lifetime abuse or dependence on 7 classes of substances. We define "problem cannabis use" as meeting the criteria of "caseness" (i.e. one or more dependence symptoms).

Selection of SNPs and genotyping

Using the Celera Discovery System, we completed preliminary bioinformatics work examining the human CNR1 gene. The gene is located on chromosome 6q14 spanning 26085 nucleotides. There have been six possible transcripts predicted by Zhang et al. (2004), with the largest consisting of four exons which produces an mRNA of 5795 nucleotides. Forty-three different SNPs have been identified and are fairly evenly distributed throughout the gene. Of these, five were selected to be genotyped using Applied Biosystems TaqMan Assays-on-Demand™ (described below). These five SNPs (rs2273512, rs6454674, rs806380, rs806377, rs104935) were selected based on information available at the time. Criteria for selection included validation status of the SNP based on the public dbSNP database and from the Celera Discovery System, minor allele frequencies (MAF) greater than 0.10 (if known), and location in the gene such that the SNPs would be approximately evenly distributed throughout the gene. There are few SNPs located within the coding regions of the gene and none that predicted amino acid changes. Genomic DNA was isolated

from buccal cell swabs and preamplified using the method of Zheng et al. (2001). Data obtained using this DNA are high-quality; these methods have been shown to be reliable for genotyping (Anchordoquy et al. 2003). TaqMan® assays for allelic discrimination (Applied Biosystems) were used to determine SNP genotypes, per instructions of the manufacturer under standard conditions using ABI PRISM® 7000 and 7900 instruments. We genotyped 541 subjects for four of the SNPs (rs6454674, rs806380, rs806377, rs1049353). SNP rs2273512, located in the first intron, was genotyped in 100 subjects and we did not find it to be polymorphic. The recently reported estimated minor allele frequency for rs2273512 in dbSNP is 0.017, so it is a rare SNP. In addition, and according to the protocols of the ongoing studies, we genotyped at least one parent of 106 subjects in order to improve haplotype determination. These data are included in the analyses of the full sample.

Analytic Methods

Linkage Disequilibrium Calculations and Haplotype Estimates—Pairwise linkage disequilibrium (D′) was calculated using GOLD (Abecasis and Cookson 2000 and Haploview (Barrett et al. 2005). A logistic regression-based test of association predicting case/control status from the individual SNPs was conducted using WHAP (http://www.broad.mit.edu/personal/shaun/whap/). WHAP uses a weighted logistic regression based method based on estimated haplotypes to test for association with a phenotype. The haplotypes are estimated using SNPHAP (http://www-gene.cimr.cam.ac.uk/clayton/software/), which assigns weighted haplotypes to each individual (i.e., haplotypes are not known with certainty but must be estimated from the data). In order to confirm the accuracy of the haplotype assignments, we compared the haplotypes estimated by WHAP (SNPHAP) to those estimated by PHASE, v.2.0.2, which incorporates relative position and distance between SNPs in its algorithms (Stephens and Donnelly 2003; Stephens et al. 2001). Less than 1% of the haplotype assignments were different between PHASE and SNPHAP, and differing haplotype assignments occurred for those individuals who had multiple possible haplotype assignments. Therefore, all analyses were conducted using WHAP, because WHAP allows inclusion of the few parental genotypes we were able to obtain, which provided better estimates of subject haplotypes.

Test for association

A similar regression-based test of association for haplotypes of the four SNPs was conducted using WHAP to compare the cases and controls. The omnibus test can be used as an initial test of association with the overall gene locus. Each estimated haplotype is included in the model and regression weights (β coefficients) are calculated to provide the relative contribution of each haplotype. The effect of the most common haplotype is fixed to 0 and the effects all others are estimated relative to it. All haplotypes with frequencies less than 1% are excluded. In subsequent analyses, the haplotype specific option ("–hs") in WHAP can be used to test the effect of each haplotype individually against all other haplotypes by constraining all other haplotypes to have equal β weights. All analyses were first conducted on the entire sample. A secondary analysis was then conducted separately for the ethnic groups Hispanic and Caucasian.

Inclusion of covariates

Covariates were included in WHAP to control for other variables that may influence marijuana dependence. This is accomplished by adding the covariate in the .dat file, as described in the instructions for WHAP (http://www.broad.mit.edu/personal/shaun/whap/), and including the "—cov" option, which provides a joint test of haplotype effects while adjusting for the covariate. Age, sex, and group (i.e. ascertained from community or clinical

settings) were included in the analyses as covariates in the separate data sets of Caucasians or Hispanics.

Results

Analysis of Individual SNPs

There was evidence for an association by likelihood ratio test (LRT) with SNP rs806380 (LRT=4.48, p=0.03). The results for the other SNPs did not approach statistical significance (shown in Table II). The estimated proportions of cases for each rs806380 SNP genotype are presented in Figure 2 with error bars showing 95% confidence intervals. For example, approximately 60% of individuals with the AA or AG genotype met our criteria for caseness, while only 40% of individuals with the GG genotype were cases.

Analysis of Haplotypes

Pairwise linkage disequilibrium (LD) estimates (r^2) obtained from Haploview (Barrett et al. 2005) are shown in Figure 3. One haplotype block consisting of "strong LD" between SNPs rs806380 and rs806377 was determined using the Haploview default algorithm based on Gabriel et al. (2002), using the 95% confidence D' rule. The estimated r^2 between these two markers was a modest 0.42. There was also noteworthy LD between the first (rs6454674) and second (rs806380) markers (r^2=0.63) and between the first (rs6454674) and third (rs806377) markers (r^2=0.40). The fourth marker (rs1049353) exhibited the lowest LD with the others.

Using the omnibus test in WHAP, which tests each estimated haplotype while controlling for all others, provided evidence for an association between problem cannabis use (cases) and the CNR1 gene locus (LRT=16.47, p=0.021, shown in Table III). The regression weights (β coefficients) indicate the relative contribution of each haplotype; the effect of the most common haplotype is fixed to 0 and the effects of all others are estimated relative to it. By default, all haplotypes with frequencies less than 1% are excluded. The "–hs" option in WHAP was used to test the effect of each haplotype individually against all other haplotypes (i.e. constraining all other haplotypes to have equal β weights). Three haplotypes (GGCC, TACC, and GACC) were significantly associated when tested individually against all others. GGCC (LRT=5.984, p=0.0144) was associated with a protective effect (β= -0.366), while haplotypes TACC (LRT=5.847, p=0.0156) and GACC (LRT=6.475, p=0.0109) were associated with increased risk for dependence symptoms (β=0.585 and β=0.951, respectively). The proportion of cases for the GGCC haplotype are presented in Figure 4.

Secondary Analyses by Ethnicity

Because there is some ethnic diversity within the sample, we conducted post-hoc analyses to examine two ethnic groups Caucasians and Hispanics separately. There were significant differences in allele frequency for three of the four SNPs between Caucasians and Hispanics (rs806380, χ^2=10.25, p=0.006; rs806377, χ^2=7.84, p=0.02; rs1049353, χ^2=12.14, p=0.002), so this was an important consideration.

Secondary analysis of the individual SNPs by ethnic group are shown in Table IV. For Caucasians (N=386), there was a significant association between rs806380 and problem cannabis use ($p < 0.04$), as we found in the full sample. Although there was no evidence for significant association with this SNP and problem cannabis use in the Hispanic sample, this may be the result of reduced power, as the size of the Hispanic sample is low (N = 104). The pattern of estimated proportions of cases by SNP rs806380 genotype within each ethnic group is similar to that of the full sample. In the Caucasians, approximately 60% of subjects with genotypes AA and AG are estimated to be cases, while only 40% with the GG

genotype are. In Hispanics, the effect is more modest; approximately 60% of AA and AG individuals are cases and approximately 50% of GG subjects are cases.

When the sample is divided into ethnic groups for the haplotype analyses, the omnibus test is no longer significant in either group (Table V). However, within both groups, the overall directions of the β coefficients are largely comparable in direction and strength, particularly for the three significant haplotypes observed in the full sample when using the "—largest" option. In Caucasians, the GGCC haplotype was statistically significant (β=-0.402, LRT=5.230, p=0.0222), and results for the TACC and GACC haplotypes were suggestive (β=0.563, LRT=3.078, p=0.0794 and β=1.121, LRT=3.943, p=0.0471). In Hispanics, the β coefficient is -0.374 (LRT=1.173, p=0.279) for the GGCC haplotype, -0.004 (LRT=0.000, p=0.995) for the TACC haplotype, and 0.289 (LRT=0.179, p=0.672) for the GACC haplotype. To further illustrate this point, the proportion of cases for the GGCC haplotype are presented in Figure 4 for the combined sample.

Covariate Analyses

In the Caucasian sample, we included the covariates age, sex, and group status (clinical or community) in the omnibus test and in a test of the individual SNP rs806380. When all three covariates were included the likelihood ratio test actually improved, resulting in a suggestive p-value (LRT=13.13, p=0.069). Similarly, the association with SNP rs806380 became significant once the covariates were included in the model (LRT=7.045, p=0.0079). However, in the Hispanic sample, neither the omnibus test nor the individual SNP were significant (LRT=2.937, p=0.94 omnibus; LRT=0.602, p=0.44 SNP rs806380).

Discussion

Taken together, these data support the hypothesis that variation within the CNR1 gene may be associated with developing one or more symptoms of cannabis dependence in adolescents who have experimented with cannabis. The results support findings by Zhang et al. (2004), who reported an association between a haplotype between three SNPs (hCV1652584, hCV8943758, and hCV11600616) in intron 2 of the CNR1 gene and polysubstance abuse in adults. Of the SNPs studied here, rs806380, which was significantly associated with cannabis problem use in the current sample, is most proximal to the three SNPs examined by Zhang et al. (2004). However, given the relatively low linkage disequilibrium between the SNPs in this study, it is possible that more genetic diversity within the gene could be captured by examining additional SNPs in this sample.

The main limitation of this study was the difficulty in controlling for population stratification. Although we did not find a statistically significant association when analyzing the data separately for Caucasians and Hispanics, the sample sizes were substantially smaller. Also, the direction of effect was similar in the Caucasians and Hispanics, and in the larger ethnic group, Caucasians, we did find a significant result for the rs806380 SNP and a trend toward significance for the omnibus test. In addition, once the covariate age, sex, and group status were included in the model, the omnibus test in Caucasians approached significance and the individual SNP rs806380 association became highly significant.

Another potential limitation is that cases and controls were drawn from three groups: youth in treatment for substance dependence, adjudicated youth, and youth who were subjects of general-population twin and family studies. All of the studies of these groups used common assessment batteries and were designed to be able to compare across groups. In order to increase power, cases and controls were drawn from any of the groups if they met the criteria of having experimented with marijuana. Recently, Hartman et al. (in press)

demonstrated that the patterns of endorsement of marijuana abuse and dependence were similar across all three groups.

Despite this limitation, the study has a number of strengths. First, to our knowledge, it is the first report that has specifically examined symptoms of cannabis dependence as a phenotype for association with CNR1. Second, by focusing on adolescents, we are examining the association during a time where initiation and progression toward developing dependence symptoms frequently occurs. Previous studies of CNR1 and its association with a range of drug abuse phenotypes have examined only one or two polymorphisms and have typically examined alcohol or "polysubstance abuse" phenotypes in adults. In addition, to our knowledge, it is the largest study to examine an association between CNR1 and any drug dependence phenotype.

In addition to having one or more cannabis dependence symptoms, many of the cases in this study met full criteria for cannabis dependence, as well as abuse or dependence on other substances. Thus, CNR1 polymorphisms may be associated with a broader range of substance abuse phenotypes. This would be consistent with results by Zhang et al. (2004) as well as twin studies that have reported that the genetic risk for developing marijuana dependence overlaps with the genetic risk for other substance dependence diagnoses (Kendler et al. (2003), Young et al. (in press)), although other authors (Tsuang et al. 1998) have reported that there are some marijuana –specific genetic influences.

Future work should focus on careful definition and examination of quantitative marijuana- and drug-related phenotypes to further clarify this possibility. In addition, future studies should include dense SNP mapping as such data become available through the HapMap project (Gibbs et al., 2003), combined with resequencing in select samples for SNP discovery. Bioinformatics and functional molecular approaches will be necessary to understand the possible functional roles of associated SNPs.

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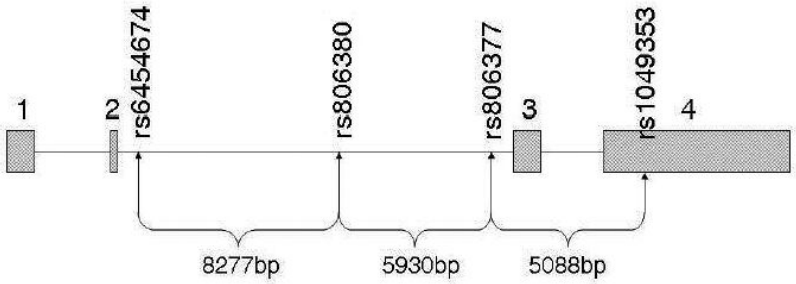

Figure 1.
Diagram of CNR1 gene and locations of SNPs examined. Exons are shown as boxes with cross-hatched fill. The rs numbers indicate the SNPs genotyped with the base pair (bp) distances between them.

Table I

Sample characteristics of cases and controls

	% Cases (N = 327)	% Controls (N = 214)
Race/Ethnicity		
Caucasian	67.9	72.9
Hispanic	19.3	18.7
African American	6.4	5.1
Other	6.4	3.3
Gender		
Male	74.3	49.5
Female	25.7	50.5
Age	18.1+1.4	18.2+1.5
Lifetime DSM-IV Substance Abuse or Dependence Diagnoses		
Cannabis Abuse	35.2	14.0
Cannabis Dependence	44.6	0
Tobacco Dependence	57.0	25.7
Alcohol Abuse	39.1	27.1
Alcohol Dependence	28.4	3.3
Amphetamine Abuse	4.0	1.0
Amphetamine Dependence	11.6	0.5
Cocaine Abuse	4.9	1.4
Cocaine Dependence	9.2	0.5
Hallucinogen Abuse	10.1	2.3
Hallucingogen Dependence	13.2	0.5
Opioid Abuse	3.7	1.0
Opioid Dependence	1.8	0

Table II

WHAP analyses for Association with CNR1 SNPs and Cannabis Dependence Symptoms. Full Sample N=541 young adults,

SNP	Variant	MAF	LRT	p
rs6454674 (hCV11418433)	T/G	0.31	1.793	0.181
rs806380 (hCV1652583)	A/G	0.31	4.482	0.034*
rs806377 (hCV1652585)	C/T	0.49	0.072	0.789
rs1049353 (hCV1652590)	C/T	0.24	0.002	0.962

*Note. MAF-Minor Allele Frequency, LRT-Likelihood Ratio Test, p-Statistical significance

Table III

WHAP Haplotype Analyses Omnibus Test for Association

	Full Sample N=541 young adults, 106 with at least one parent	
Haplotype	**Frequency**	**β**
TATC	0.380	0.000
GGCC	0.210	-0.259
TATT	0.115	0.027
TACC	0.087	0.571
TACT	0.068	-0.105
GGCT	0.053	-0.076
TGCC	0.051	0.127
GACC	0.037	0.941
LRT	16.474	
p	0.0211	
Empirical p	0.0339	

*Note. Frequency column shows the frequency of each estimated haplotype in the sample. β column provides the β coefficient estimate derived from the logistic regression test for association. LRT – Likelihood Ratio Test, p – statistical significance, Empirical p-determined after permutation testing

Table IV

Secondary WHAP analyses for Association with CNR1 SNPs and Problem Marijuana Use in Caucasian and Hispanic Sub-Groups

Caucasian sample N=386 young adults				**Hispanic Sample N=104 young adults**		
SNP Variant	**MAF**	**LRT**	**p**	**MAF**	**LRT**	**p**
rs6454674 T/G	0.32	2.406	0.121	0.26	0.871	0.351 *(hCV11418433)*
rs806380 A/G	0.34	4.262	0.039*	0.24	0.257	0.612 *(hCV1652583)*
rs806377 C/T	0.46	0.033	0.857	0.42	0.018	0.894 *(hCV1652585)*
rs1049353 C/T	0.29	0.052	0.820	0.17	3.971	0.046 *(hCV1652590)*

*Note. MAF-Minor Allele Frequency, LRT-Likelihood Ratio Test, p-Statistical significance

Table V

WHAP haplotype analyses Omnibus Test for Association in Caucasian and Hispanic Sub-Groups

	Caucasian sample N=386 young adults		Hispanic Sample N=104 young adults	
Haplotype	**Frequency**	**β**	**Frequency**	**β**
TATC	0.331	0.000	0.488	0.000
GGCC	0.236	-0.384	0.176	-0.116
TATT	0.130	-0.139	0.096	1.137
TACC	0.078	0.449	0.077	0.152
TACT	0.095	-0.162	0.041	0.610
GGCT	0.064	-0.127	0.020	-0.048
TGCC	0.046	0.106	0.033	0.874
GACC	0.020	1.001	0.057	0.409
GATC	n.o.	n.o.	0.012	-0.259
LRT	11.239		5.268	
p	0.129		0.729	
Empirical p	0.179		0.778	

*Note. Frequency column shows the frequency of each estimated haplotype in the sample. β column provides the β coefficient estimate derived from the logistic regression test for association. LRT – Likelihood Ratio Test, p – statistical significance, Empirical p-determined after permutation testing. N.o. – not observed.