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A common variation in the cannabinoid 1 receptor (CNR1) gene is associated with pre-eclampsia in the Central European population

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ABSTRACT

Objective: Recently it has been proposed that tightly regulated levels of endogenous cannabinoids play a fundamental role in early placental development. The aim of this study was to investigate associations of three single-nucleotide polymorphisms (SNPs) in the cannabinoid 1 receptor (CNR1) gene (rs1049353, rs12720071 and rs806368) and their inferred haplotypes with pre-eclampsia, a severe pregnancy-associated condition characterized by abnormal development and remodeling of spiral decidual arteries.
Study design: The case-control study comprised a total of 115 pre-eclamptic women and 145 healthy pregnant controls, all originating from the Central-European Czech population. Using PCR-based methods, we tested rs1049353, rs12720071 and rs806368 in the CNR1 gene and haplotypes were constructed.
Results: Statistically significant difference in genotype distributions of rs806368 ($p_g < 10^{-3}$) was observed when comparing the cases and the controls; the cases presenting with significantly lower proportion of CC homozygotes. In multivariate modeling, the rs806368 served as a predictor for pre-eclampsia development ($\beta = 0.15$; $p = 0.04$). Haplotype analysis revealed presence of four common haplotypes; the CAA haplotype being less frequent in pre-eclamptic cases compared to the controls ($p < 0.008$). Analysis of regression models confirmed the independent prediction role of AAC haplotype for pre-eclampsia onset ($\beta = -0.18$; $p = 0.03$).
Conclusion: This is the first study focusing on the relationship between SNPs in the CNR1 gene and pre-eclampsia risk. Although limited by a relatively small sample size, the study indicates that rs806368 in the CNR1 gene may act as a susceptibility marker for pre-eclampsia in humans.

1. Introduction

The endocannabinoid system (ECS), including cannabinoid receptors 1 and 2 (CB1 and CB2), endogenous ligands (“endocannabinoids”), regulating enzymes and transporter molecules, has been detected from the earliest embryonal stages, implantation and throughout pre- and postnatal development [1,2]. The ECS seems to play an essential role not only during the crucial developmental stages, i.e. early embryonal development, the early phase of fetal brain development, and regulation of suckling in newborns and infants, but also in adulthood, where it has pleiotropic modulatory and regulatory effects on various neuro-endocrinological pathways.

Successful implantation and pregnancy progression are characterized by low plasma levels of anandamide (AEA), the crucial endocannabinoid [3,4], while in labour in humans the levels of this molecule dramatically rise [3]. It has been suggested that for successful pregnancy progression, a higher plasma AEA level at ovulation and a significantly lower level during implantation are required [5]. On the contrary, high levels of anandamide in early pregnancy [6] were reported to be associated with spontaneous miscarriage.

Following implantation, the uterus undergoes continuous extensive remodeling in order to provide an adaptive environment for the successful development of the embryo [7]. The ongoing process of tissue remodeling of the uterus represents a crucial condition for successful pregnancy, and its failure might result in development of pre-eclampsia, but very little is known about the molecular mechanisms underlying this process.

The recent study by Fonseca et al. [7] indicates that a tightly regulated level of endocannabinoids might play a crucial role not only in the early implantation period but also in the subsequent

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spatio-temporal placental development. The results of immunohistochemical analysis indicated that CB1 is expressed mainly in decidual cells throughout gestation, suggesting that functioning of CB1 is related to the further phases of pregnancy following implantation. The differential spatio-temporal expression patterns of CB1 and CB2 in the pregnant uterus suggest that there is an uncoupling of these two types of anandamide binding receptors in mid/late gestation that are related to changes in cell phenotype.

Pre-eclampsia is a severe pregnancy-associated condition with relatively high morbidity and mortality that is characterized by abnormal development and remodeling of spiral decidual arteries, resulting later in pregnancy in impaired blood supply to the fetus and maladaptive maternal systemic response. Since the ECS plays an important role during the early stages of pregnancy which are crucial for optimum decidual spiral artery development, dysregulation of this system could be involved in the pathogenesis of pre-eclampsia.

The aim of the study therefore was to investigate possible associations of three common polymorphisms in the cannabinoid 1 receptor (CNR1) gene, 1359 A/G (rs1049353), 3813 A/G (rs12720071) and 4895 C/T (rs806368), and their inferred haplotypes with pre-eclampsia risk and its related risk factors and fetal parameters.

2. Materials and methods

2.1. Subjects

At the Clinic of Gynaecology and Obstetrics of the University Affiliated Hospital Brno, a total of 115 pre-eclamptic women were enrolled into this study, together with 145 healthy pregnant women of similar parity whose pregnancies were physiological, uncomplicated and spontaneous. The controls were recruited after the index cases in order to reduce possible bias. Gestational age was confirmed by first-trimester ultrasound in all subjects; gestational age at recruitment to the study was 27–40 weeks for the cases and 38–41 weeks for the controls.

Pre-eclampsia was defined according to the recent criteria of the International Society for the Study of Hypertension in Pregnancy [8]. These criteria include no previous history of hypertension, cardiovascular, or renal disease before pregnancy and blood pressure values exceeding 140/90 mmHg after the 20th week of gestation, confirmed by two consecutive readings at least six hours apart, with blood pressure reverting to normal within two months after delivery. The subjects were considered to have a physiological pregnancy if they did not have obstetrical, medical, or surgical complications of pregnancy, and delivered a term (>38 weeks) neonate with a birth weight above the 10th percentile for gestational age [9].

Intrauterine growth restriction (IUGR) was defined as an estimated birth weight below the 10th percentile of a reference group. Doppler velocimetry of material uterine arteries, umbilical cord, and middle cerebral arteries of the fetus was performed on a routine basis in the pre-eclamptic cohort. Pregnancy weight gain, pre-conception body mass index (BMI) and BMI at the time of delivery were calculated based on information obtained from the study subjects.

Women with pre-eclampsia superimposed on chronic hypertension preceding pregnancy were included in the study; on the contrary, subjects who had chronic hypertension of any etiology before pregnancy without superimposed consequent pre-eclampsia were not included in the study, as they might carry such genetic variants that are protective against pre-clampsia development.

This study was approved by the Committee for Ethics of Medical Experiments on Human Subjects, Faculty of Medicine, Masaryk University, Brno, and was performed in adherence to the Declaration of Helsinki Guidelines. Each participant gave her written informed consent, which has been archived.

2.2. Genotyping

DNA for analyses was extracted from 5 mL of the patient's peripheral blood using the standard protocol based on proteinase K. Genotyping of the polymorphisms was performed as described previously [10,11] using a standard PCR-based methodology with following restriction fragment length polymorphism (RFLP). Restricted fragments were separated by electrophoresis on 2% agarosis gels with ethidium bromide staining. To assess genotyping reliability, we performed double sampling in more than 20% of the samples and found no differences. We always used quality control, and negative controls were used to identify possible false-positives. The genotyping success was 100% for all included single-nucleotide polymorphisms (SNPs).

2.3. Statistics

The genotype distributions were tested for Hardy–Weinberg equilibrium by a set of chi-square tests. Allelic frequencies were estimated by "counting method" and differences in allele frequencies between case and control subjects were tested by likelihood ratio χ^2 tests for 2×2 tables (two alleles, case vs. control subjects). Where applicable, it was first determined whether the variable presented a normal distribution using the Kolmogorov–Smirnov test, and in cases of skewed variables, logarithmic transformation was performed. To identify genetic as well as non-genetic variables that may contribute to predicting the specific phenotype, we carried out a forward stepwise logistic regression, a sequential procedure of adding one input variable at a time to build up a regression model in which the dependent variable (i.e. probability of presence or absence of pre-eclampsia) is represented as the logistic function of linear variables (anthropometric and clinical data and genotypes of three investigated SNPs). Odds ratios were calculated for the multiple logistic regression analysis models; we adjusted for covariates including age (continuous), BMI ($<$23, 23–24.9, 25–29.9, 30–34.9, or $\geq$35 kg/m^2), smoking (never, past, and current) and parity.

The data analysis was performed using Statistica v. 8.0 (Statsoft Inc., Tulsa, OK, USA) program package at the significance level defined as $p < 5\%$. Analysis of haplotypes was performed using the Haploview program package [http://www.broad.mit.edu/haploview/].

3. Results

The clinical characteristics of the study population are shown in Table 1. Maternal age, parity, maternal pre-conceptional as well as end-pregnancy BMI, gestational age at delivery, birth weight and smoking status were found to be significantly different between the two cohorts ($p < 0.05$). In addition, 22 (19%) of the 115 preeclamptic patients delivered a fetus with IUGR, defined as birth weight below the 10th percentile for gestational age.

Table 2 shows the allele and genotype distribution of CNR1. For all of the investigated polymorphisms except for 4895 C/T (rs806368) in the case cohort, genotype distributions conformed to Hardy–Weinberg equilibrium. The determination of the pairwise linkage disequilibrium (LD) indicated that there was a strong LD between the polymorphisms rs806368 and rs12720071 ($D' > 0.8$) in the cases as well as in the controls.

3.1. 1359 A/G (rs1049353)

No significant differences in allele or genotype frequencies were observed between the cases and the controls. As only a few