SOP TRANSACTIONS ON INHERITANCE AND GENETIC ENGINEERING

# Associations of Polymorphic Variants of CCK, CCKAR, and CCKBR Genes with Panic Disorder

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**Abstract:**

Panic disorder is a widespread socially significant disease which genetic nature is poorly known. Since panic-driving features of cholecystokinin had been discovered, the gene that encodes this polypeptide (*CCK*) and its receptors (*CCKAR*, *CCKBR*), as well as the mutations within, have been extensively studied. The aim of the present research was to assess frequencies of occurrence of seven single nucleotide substitutions in genes *CCK*, *CCKAR*, and *CCKBR* in the sample of patients with diagnosed panic disorder, and in the control sample of unexamined Moscow citizens. Reliable increase in occurrence frequency of rs1805000:T single nucleotide substitution in *CCKBR* gene was eventually found in the sample of panic disorder patients compared to the control, which allow us to suspect the involvement of this SNP into panic disorder aetiology. We also found the association of allele combination *CCK* rs11571842:A + *CCKAR* rs1800908:T with panic disorder development.

**Keywords:**

Panic Disorder; Cholecystokinin; Genes *CCK*; *CCKAR*; *CCKBR*; Single Nucleotide Polymorphism (SNP)

## 1. INTRODUCTION

Panic disorder (PD) is the disease characterized by spontaneous panic attacks which can occur from several times a year up to several times a day, and anticipatory anxiety in between [1, 2]. According to the statistics, 3 to 6 percent of individuals in a population suffer anxious disorders and panic attacks. The disease manifests mostly in young age (15 – 35 years) and thus affects most socially active part of a population [3].

The distinctive features of PD are: recurring paroxysms of anxiety (panic) – panic attacks; forthcoming of anticipatory anxiety in interictal periods and frequent emergence of agoraphobia; high hereditary risk of the disease; strong correlation with depression [2, 4].

Panic disorder has substantial genetic basis: the evidences of direct hereditary transmission of this disease was shown (15 – 17% relatives of grade 1 patients also suffer the disease), as well as high concordance of PD was shown in identical twins (80 – 90%). The heritability of PD is considered 48% [5, 6]. However, molecular genetics nature of this disease is still poorly known.

Currently, great importance is attached to cholecystokininergic system in regard to anxiety disorders. Cholecystokinin (CCK) has initially drawn interest to itself as the factor of anxiety in 1984, injections of this peptide to humans cause a distinct feeling of uneasiness and panic [7]. In 1989 De Montigny has confirmed that CCK participates in the development of panic attacks [8].

The biological effect of cholecystokinin consists of the modulation of a series of classical neurotransmitters, including GABA, endogenous opioids and dopamine. There are two CCK receptors: CCKAR and CCKBR. CCKBR agonists are a powerful anxiogens with the ability to cause agitation, fear and panic behaviour in humans [9], as well as in animals [10].

It is assumed that the changes in activity of cholecystokininergic system may be neuro-biological basis for panic disorders [9, 11]. One of the possible mechanisms of dysfunction of cholecystokinenergic system may be polymorphism in protein-coding and/or regulatory regions of *CCK*, *CCKAR* and *CCKBR* genes. SNPs, found in these genes, are still poorly understood, and the available literature on their impact on development of pathological anxiety states are contradictory [12–18]. Meanwhile, it is cholecystokinenergic system that can play a major role in the generation and development of the main clinical manifestations of panic disorder, panic attack.

The aim of this work is to evaluate the frequency of seven single-nucleotide substitutions in genes *CCK, CCKAR* and *CCKBR* in a sample of patients with panic disorder and a control sample of residents of Moscow, as well as a search for complex haplotypes associated with the disease.

## 2. PATIENTS AND METHODS

### 2.1 Patients

Samples of DNA were extracted from whole blood of patients with the panic disorder diagnosis under DSM-IV criteria. The study included only those patients that were subject to frequent panic attacks (at least one per week). The sample size was 63 people. All the patients live in Moscow region. All the patients gave their informed consent to the participation in the study. The study is approved by the Local ethical committee of Vavilov Institute of General Genetics of Russian Academy of Sciences. DNA samples extracted from the whole blood of unscreened volunteers residing in Moscow were used as the control. DNA samples were provided to the Laboratory of functional genomics of Vavilov Institute of General Genetics of Russian Academy of Sciences. The size of control group was 170 people.

### 2.2 Molecular Genetic Analysis

DNA was extracted according to protocol to commercial DNA Magna$^{TM}$ DNA Prep 200 kit (Isogen Lab Ltd., Moscow, Russia). Genotypes were identified by PCR-RFLP method. The PCR was conducted according to protocol of commercial kit GenePak TM PCR Core (Isogen Lab Ltd., Moscow, Russia). Primers were designed in Primer 5.0 program. Primers were synthesized by DNA-Synthesis Ltd. (Moscow, Russia). The restriction endonucleases produced by SibEnzyme Ltd. (Novosibirsk, Russia) were used for digestion; reactions were carried out in conditions recommended by producer. Primer sequences together with restriction endonucleases are listed in **Table 1**. Electrophoretic analysis of PCR products was performed in 2% agarose gel. Gels were analysed using ViTran utility and software (Biokom Ltd, Russia).

Standard approaches were used to carry out the statistical data processing. Allele frequencies and studied loci genotypes, correspondence of the distribution of genotype frequencies to Hardy-Weinberg equilibrium, comparison of allele frequencies in the two samples of the study were determined by the standard method $\chi^2$ with Yates's continuity

correction. Analysis of disequilibrium of linkage between pairs of DNA markers was performed using Arlequin 3.5 and Haploview 4.0. The search for combinations of alleles (haplotype) associated with PD was performed using APSampler 3.6 software package [19]. For visualisation of the data in the Venn diagram Google Chart API, available through https://code.google.com/p/vienna5/ was used. DNA variant rs1800857 was analysed with the splicing prediction program Human Splicing Finder [20].

## 3. RESULTS AND DISCURSIONS

For the study, we selected two SNPs in promoter region of *CCK* gene, three SNPs in *CCKAR* gene (two in promoter region and one at the border of intron 1 / exon 2), and two SNPs in coding sequence of *CCKBR* gene (**Table 1**).

Table 1. Structure of primers and parameters of PCR-RFLP.

| Gene | SNP | Location in gene | Primers | Annealing temperature, °C | Restriction enzyme | RFLP product length, bp |
|---|---|---|---|---|---|---|
| *CCK* | rs11571842 (c.-213-47G>A) | promoter | F:5'-CCAACGCTGACGCAGACTG-3' R:5'-GAAGCTTCTCGGATCCAGA-3' | 64 | BslI | GG – 124, 24, 20; GA – 148, 124, 24, 20; AA – 148, 20. |
| *CCK* | rs1799923 (c.-108T>C) | promoter | F:5'-GCTCTACCCACCCAGACCTC-3' R:5'-GAAGCTTCTCGGACCCAGA-3' | 65 | FauI | CC – 193, 174, 90, 41; CT – 234, 193, 174, 90, 41; TT – 234, 174, 90. |
| *CCKAR* | rs1799723 (c.-286A>G) rs1800908 (c.-333G>T) | promoter promoter | F:5'-GCATATGTACACATGTGTGT AAAAAGCAGCCAGAC-3' R:5'-GCCCTTTCCTGGGCCAGACT-3' | 65 | HinfI | Only 4 genotypes was found: I - AA, GG; II - AG, GG; III - AG, GT; VI - AA,GT. Genotype I – 103; genotype II – 103, 83, 20; genotype III – 103, 50, 33, 20; genotype VI – 103, 70, 33. |
| *CCKAR* | rs1800857 (c.113-5T>C) | intron 1 | F:5'-ATCGTGGGTCCAGTGATGT-3' R:5'-GGCTCCTTTGCTGTGATTGT-3' | 63 | PstI | TT – 350, 122; TC – 472, 350, 122; CC – 472. |
| *CCKBR* | rs1805000 (c.109C>T) | exon 1 | F:5'-CATGGAGCTGCTAAAGCTGAAC-3' R:5'-CTGGGGTACAGTGAGAAATAGC-3' | 60 | BstDEI | CC – 110, 55, 38; CT – 165, 110, 55, 38; TT –165, 38. |
| *CCKBR* | rs1805002 (c.373G>A) | exon 2 | F:5'-CTGGCAGTCAGCGACCTCCT-3' R:5'-ACAAGCATCAGTGGGACTTC-3' | 62 | Bst4CI | GG – 150, 87; GA – 237, 150, 87; AA – 237. |

### 3.1 SNPs in CCK Gene

The results of identification of alleles and genotypes frequencies in *CCK* gene in the sample of patients and the control are presented in **Table 2**. Genotype frequencies in samples of patients and controls corresponded to Hardy-Weinberg equilibrium for both substitutions ($p>0.1$).

SNP rs1799923 (c.-108T>C, also known as -36C>T) causes a change in the sequence of the binding site for transcription factor Sp1, which in turn entails a reduction of transcription activity of CCK gene [21]. Previously it was found that the allele frequency rs1799923:T is significantly higher with people suffering from panic disorders when compared to the healthy volunteers [12]. However, several studies have found no such association [15, 22]. Moreover, Danish researchers have found a protectoral role of polymorphism rs1799923:T [23]. Our data shows that allele frequencies of patients and of control group show no significant difference, which allows to hypothesise the

lack of significant input into the development of disease from this polymorphism.

SNP rs11571842 (c.-213-47G>A) is also located in the promoter region of the gene *CCK,* but is less studied (only in association with weight change [24]). Frequencies of alleles rs11571842 obtained by us in the control sample and the sample of patients with panic disorder are also not significantly different (**Table 2**).

Table 2. The distribution of genotypes and alleles frequencies of SNPs in *CCK*, *CCKAR* and *CCKBR* genes. Patients with PD (n=63), control (n=170).

| Gene | SNP / group | Genotypes frequency, % | | | Alleles frequency, % | |
|---|---|---|---|---|---|---|
| *CCK* | rs11571842 (c.-213-47G>A) | GG | GA | AA | G | A |
| | Patients | 22 | 48 | 30 | 46 | 54 |
| | Control | 19 | 45 | 36 | 42 | 58 |
| | | $\chi$2=0.64; d.f.=2; p=0.425 | | | $\chi$2=0.68; d.f.=1; p=0.408 | |
| | rs1799923 (c.-108T>C) | CC | CT | TT | C | T |
| | Patients | 81 | 19 | 0 | 90 | 10 |
| | Control | 82 | 16 | 2 | 90 | 10 |
| | | $\chi$2=0.02; d.f.=2; p=0.88 | | | $\chi$2=0.02; d.f.=1; p=0.878 | |
| *CCKAR* | rs1799723 (c.-286A>G) | AA | AG | GG | A | G |
| | Patients | 87 | 13 | 0 | 94 | 6 |
| | Control | 91 | 9 | 0 | 95 | 5 |
| | | $\chi$2=0.54; d.f.=2; p=0.465 | | | $\chi$2=0.51; d.f.=1; p=0.476 | |
| | rs1800908 (c.-333G>T) | GG | GT | TT | G | T |
| | Patients | 89 | 11 | 0 | 94 | 6 |
| | Control | 95 | 5 | 0 | 98 | 2 |
| | | $\chi$2=3.13; d.f.=2; p=0.077 | | | $\chi$2=3.03; d.f.=1; p=0.135 | |
| | rs1800857 (c.113-5T>C) | TT | TC | CC | T | C |
| | Patients | 73 | 22 | 5 | 86 | 14 |
| | Control | 72 | 26 | 2 | 85 | 15 |
| | | $\chi$2=0.05; d.f.=2; p=0.818 | | | $\chi$2=0.05; d.f.=1; p=0.816 | |
| *CCKBR* | rs1805000 (c.109C>T) | CC | CT | TT | C | T |
| | Patients | 90 | 10 | 0 | 95 | 5 |
| | Control | 100 | 0 | 0 | 100 | 0 |
| | | $\chi$2=16.62; d.f.=2; p=0.00005 | | | $\chi$2=16.40; d.f.=1; p=0,00074; OR=36.734; 95% C.I.=[ 2.054-656.977] | |
| | rs1805002 (c.373G>A) | GG | GA | AA | G | A |
| | Patients | 75 | 22 | 3 | 86 | 14 |
| | Control | 82 | 16 | 2 | 90 | 10 |
| | | $\chi$2=1.29; d.f.=2; p=0.256 | | | $\chi$2=1.45; d.f.=1; p=0.228 | |

Thus, according to accumulated data, SNPs in promoter of *CCK* gene are not significant for the risk of development PD in Moscow region population.

Also, during the analysis of disequilibrium of linkage between two polymorphisms in *CCK* gene (hypothesis about the non-dependent inheritance of markers was tested with the use of criterion $\chi^2$), we have found that genetic variants - rs11571842 and rs1799923 in gene *CCK* are predominantly inherited together ($\chi^2$ = 20.44, p = 0.00001, D' = 0.79, LOD = 4.44, $R^2$ = 0.09).

## 3.2 SNPs in CCKAR Gene

As the part of the study of polymorphic variants of promoter region of *CCKAR* gene two invariant positions: rs1799723 (c.-286A>G) and rs1800908 (c.-333G>T) were discovered, as well as a significant increase in allele frequency rs1799723:G and rs1800908:T in patients suffering from panic disorders compared with control in a sample from Japan [25, 26]. It is known that these two polymorphisms are predominantly inherited together [25, 27]. Our data also show an increase in the frequency of alleles rs1799723:G and rs1800908:T in a sample of patients with panic disorder compared with controls, but after statistical processing, the correlation is deemed unreliable (**Table 2**).

In *CCKAR* gene SNP rs1800857 (c.113-5T>C) was detected, which is located at the border of the intron 1 and exon 2 (fifth nucleotide from the end of intron 1 is changed). This substitution does not alter splicing, but results in generation of two new exonic splicing enhancer sites and exonic splicing silencer site, as well as disruption of exon-identity elements, intron-identity elements, and exonic splicing regulatory sequences. This may affect splicing efficiency. There exists a large amount of information about the association of such polymorphism with the development of schizophrenia [28–31], but not of panic disorder [16]. The allele frequency of rs1800857 in the Moscow patients with panic disorder is similar to that in the control samples (**Table 2**), which may indicate a lack of effect of the genetic variant on the development of the disease.

Genotype frequencies for all substitutions in CCKAR gene in samples of patients and controls corresponded to Hardy-Weinberg equilibrium ($p>0.1$).

## 3.3 SNPs in CCKBR Gene

In CCK type 2 receptor gene (*CCKBR*) two SNPs were examined: rs1805000 (c.109C>T) and rs1805002 (c.373G>A), these are located in the coding region of the gene and lead to amino acid polymorphisms (respectively p.L37F and p.V125I) in the sequence of receptor [32]. Frequencies of alleles and genotypes of these SPNs are presented in **Table 2**.

Differences in frequencies of alleles and genotypes of SPN rs1805002 in a sample of patients with panic disorders and in control are not significant (**Table 2**). Genotype frequencies for rs1805002 substitution in samples of patients and controls corresponded to Hardy-Weinberg equilibrium ($p>0.1$).

We have shown a significant increase in frequency of an rs1805000:T allele in a sample of patients compared with control ($\chi^2=16.40$; $p=0.00074$). For this substitution, genotype frequencies in control sample did not correspond to Hardy-Weinberg equilibrium ($\chi^2=15,88$; $p=0,00048$) due to the fact that in the control sample of conditionally healthy residents of Moscow, T allele was not found, whereas the frequency of this allele in a sample of patients was 4.76% (six of 63 patients). The literature does not present studies on the role of single nucleotide polymorphism c.109C>T in aetiology of PD. Our results do not allow us to confidently define the role of T allele in PD pathogenesis, unless we increase samples of both patients and controls. Meanwhile, it is known that the most powerful panicogenic result can be attributed to selective agonists especially of CCK type 2 receptor, suggesting their key position in pathological anxiety, and genetically determined changes in the sensitivity of these receptors may play a role in the genesis of panic disorder. CCK receptors are metabotropic, their structure is a seven trans-membrane $\alpha$-spirals, connected by intra- and extracellular loops. Investigated polymorphism of amino acid p.Leu37Phe is positioned in the extracellular loop of the receptor, which is involved in direct interaction with ligand, and may affect the performance of ligand-receptor binding sites, which in turn affects the functioning of the whole CCK system [33].

### 3.4 Search for the association of complex genotypes of *CCK*, *CCKAR* and *CCKBR* genes with frequently repeated panic attacks

We have carried out a search for combinations of alleles (haplotype) on seven studied loci with the development of panic disorder.

We have found one combination of alleles (*CCK* rs11571842:A + *CCKAR* rs1800908:*T*) that was significantly more frequent with patients with PD compared to the control group (p (Fisher's exact p-value) = 0.0156255, OR=3.67164, C.I. (95%)=[1.25766-10.71910]). An interesting fact is that the significant association with PD is only discovered by us through the locus rs1800908:*T* ($\chi$2=4.01, p=0.045; p (Fisher's exact p-value) = 0.04227282), whereas the frequency of polymorphism rs11571842 does not significantly differ between the PD patient sample and the control group ($\chi$2=0.12, p=0.731; p (Fisher's exact p-value) = 0.42365). Apparently this can be explained by the presence of epistatic interactions between studied genes (*CCK* and *CCKAR*). This interaction is schematically shown by the Venn diagram in **Figure 1**.

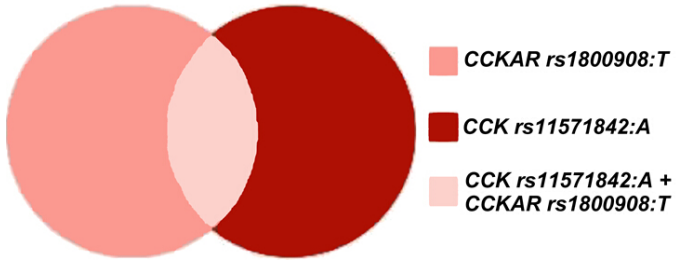


Figure 1. Venn diagram. Schematic representation of epistatic interaction of genes CCK and *CCKAR*, as the intersection of circles, combination of these alleles (shown on the Figure). Intensity of the colour of circles and their intersections is proportional to Fisher's exact p-value ($p$=0.424 for allele rs11571842:A *CCK*, $p$=0.042 for allele rs1800908:*T CCKAR*, $p$=0.016 for combination of alleles rs11571842:A *CCK* and rs1800908:*T CCKAR*).

Thus, these data indicate a much higher level of association of combination of alleles *CCK* rs11571842:A + rs1800908:*TCCKAR* with the development of panic disorder compared with the contribution of each of these SNPs individually.

## 4. CONCLUSION

Thus, our findings confirm the possible contribution of polymorphism of CCK system genes in the development of panic disorders. We have found a significant increase in the frequency of allele rs1805000 in gene *CCKBR*, and also have noted the tendency to increase the frequency of SNP rs1800908 in gene *CCKAR* in patients. Results of the analysis of complex haplotypes associated with the disease have pointed to the participation of combination of alleles *CCK* rs11571842:A + *CCKAR* rs1800908:*T* in the development of panic disorder.

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