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Association of *OPRK1* rs963549 and rs997917 polymorphisms with opioid use disorder and related phenotypes

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Aim: To evaluate the association between *OPRK1* rs963549 and rs997917 and opioid use disorder (OUD) and related phenotypes. **Methods:** A sample of 208 individuals with (n = 100) and without (n = 108) OUD were enrolled. *OPRK1* rs963549 and rs997917 were analyzed by PCR–RFLP. Craving, opioid withdrawal and the intensity of depressive and anxiety symptoms were measured by the appropriate scales. **Results:** *OPRK1* rs963549 variation showed a trend of association with decreased opioid withdrawal. No significant associations were found between *OPRK1* rs963549 and rs997917 polymorphisms and craving, depression or anxiety symptoms. Neither single *OPRK1* SNPs nor *OPRK1* haplotypes were associated with OUD. **Conclusion:** Our results could be useful for treatment failures of individuals who experience greater opioid withdrawal due to their *OPRK1* rs963549 genotypes.

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Keywords: individual differences • κ-opioid receptor • opioid use disorder • opioid withdrawal • OPRK1 gene

Opioid use disorder (OUD), a major public health challenge all over the world, is a chronic, progressive and complex neurobiological disorder associated with changes in neuroadaptive mechanisms causing addiction, craving and relapse [1–4]. Opioids are the most abused drugs worldwide due to possessing inherent euphoric and rewarding properties and also extremely powerful pain-relieving effects [2]. Opioids, including heroin and morphine, exert their effects by binding to their specific opioid receptors such as μ-, κ- and δ-opioid receptors [5]. Opioid receptors are expressed in the nervous system including the dopaminergic, serotonin and noradrenaline systems that send axonal projections to various brain regions of the stress axis such as the amygdala, hippocampus and the prefrontal cortex. Opioid receptors affect the reward system, feelings and moods by modulating the functioning of monoamine systems in coordination with the hypothalamic–pituitary–adrenal (HPA) axis [6]. Among opioid receptors, κ-opioid receptors with their ligands, the dynorphins, are involved in the regulation of stress-related disorders that may increase the risk of addictive and relapse-like behaviors [7–9].

Genes encoding opioid receptors are good candidates in genetic association studies of OUD to understand the etiology of these brain disorders, which will help in the identification of individuals with OUD who experience treatment failures due to their genetic make-up [5]. Although genome-wide association studies have revealed the importance of a small number of candidate genes, the underlying molecular mechanisms of these genes on the development of OUD could not be well understood since their functional interpretation with molecular mechanisms are complicated. Therefore, case–control genetic association studies based on mechanistic hypotheses are still helpful to determine the effects of candidate genes on the risk of OUD and related phenotypes [7].

There are several studies showing the association of *OPRK1* gene polymorphisms with substance use disorders [5,10–16], treatment-related withdrawal [14,17], craving [18] and negative mood [6,7] in individuals from different

ethnic backgrounds. Gerra *et al.* reported a point-wise significant association of *OPRK1* rs1051660 (36G >T, exon 1) with heroin addiction [11]. Xuei *et al.* [10] and Levran *et al.* [12] found a significant relationship between *OPRK1* rs6473797 (intron 2) and opioid or alcohol use disorder. Jones *et al.* investigated the effect of some *OPRM1*, *OPRD1* and *OPRK1* polymorphisms on opioid withdrawal severity in opioid users who had an abstinence-induced withdrawal or who were subjected to a naloxone-precipitated withdrawal procedure, and they found that *OPRM1* rs6848893, *OPRD1* rs10753331 and rs678849 polymorphisms were associated with abstinence-induced withdrawal, whereas only *OPRK*1 rs6473797 was significantly associated with naloxone-precipitated withdrawal [17]. Karpyak *et al.* found no association with *OPRK1* polymorphisms and depression or negative craving measures in patients with alcohol use disorder [19]. However, Masih and Verbeke found that *OPRK1* rs16918875 and rs963549 SNPs are associated with depression and anxiety in individuals with OUD [6]. However, to the best of our knowledge, none of these studies examined the effects of *OPRK1* rs963549 and rs997917 gene polymorphisms on negative craving and opioid withdrawal in opioid users who had no abstinence and had not been subjected to any opioid maintenance treatment. Thus, we evaluated the association of each phenotype with individual *OPRK1* rs963549 and rs997917 polymorphisms due to both their ability to change gene expression and their population frequencies (>10%). It is believed that rs963549, an upstream variant of *OPRK1*, may cause alterations in gene expression by changing mRNA folding or stability [20,21]. *OPRK1* rs997917 is located in intron 2 of *OPRK1*, in which Lutz *et al.* identified an enhancer region; they suggested that *OPRK1* expression could be regulated by binding of the glucocorticoid receptor complex to this enhancer region [22]. All in all, we aimed to assess the influence of *OPRK1* rs963549 and rs997917 on OUD and related phenotypes in a population of Caucasian ancestry, hypothesizing that these two candidate SNPs would associate with negative mood disorders such as depression and anxiety, as well as with craving and opioid withdrawal that could be attenuated by negative mood in individuals with OUD.

Materials & methods

Study population

A total of 100 individuals who had an OUD by the Diagnostic and Statistical Manual of Mental Disorders (DSM-5) criteria were enrolled in this study. Individuals with OUD were eligible for inclusion in the study if: they were at least 18 years of age; they had no clinically significant comorbid psychiatric illness, severe depression or other mood disorders; and they had no past or current substance use disorders other than heroin and nicotine dependence, as proved by the urine drug test performed by the routine laboratory at the AMATEM Clinic to ensure subjects with OUD only had heroin and nicotine use and not polysubstance abuse. Healthy control subjects (n = 108) who attended the Blood Donation Center were matched to individuals with OUD for gender and smoking habits. The control subjects were ≥18 years of age and had no diagnosis of past substance use disorder. Written informed consent was obtained from each subject who was eligible for the study. Sampling was performed in accordance with the principles of the Declaration of Helsinki. The study design was approved by the institutional ethics committee (approval no: I4-207-20) in 2020. A questionnaire regarding age, marital, education and employment status, duration of opioid use, age at first opioid use, quantity of heroin consumed (g/day) and family history of illicit substance use was given to the individuals.

Determination of the *OPRK1* rs963549 & rs997917 polymorphisms

For genotyping of *OPRK1* rs963549 and rs997917 SNPs, 2 ml of venous blood was taken from all subjects into tubes with ethylenediaminetetraacetic acid and used for PCR-RFLP testing. Firstly, DNA was isolated from the blood samples using the QIAamp DNA blood kit (Qiagen, Hilden, Germany) as recommended by the manufacturer. PCR amplification was conducted on a Techne Tc 512 PCR System (Keison Products, Essex, UK) in a 25 µl reaction mixture containing 0.16 µM of dNTPs, 0.4 pmol each of the forward and reverse primers, 1.25 U of HotStarTaq DNA polymerase (New England Biolabs, Hitchin, UK), 5× PCR buffer (New England Biolabs) and 50 ng of genomic DNA. Then the PCR products were digested in a reaction containing 5 U of restriction enzyme. The undigested and digested PCR products were separated by gel electrophoresis on a 3% agarose gel, visualized by ethidium bromide staining under an UV illuminator, and then scanned and photographed using the Syngene Monitoring System (SYNGENE, Cambridge, UK). Primer sequences, restriction enzymes, PCR conditions and restriction enzyme digestion conditions as well as the lengths of PCR products and restriction fragments are given in Table 1.

Table 1. *OPRK1* single-nucleotide polymorphisms: analysis conditions.

SNP (rs no.)	Primer sequences (5′→3′)	PCR conditions (Tm/Cy)	RE/digestion condition	PCR product and restriction fragment sizes (bp)
OPRK1 rs963549	F: GTTGCGTGGACCTTTTGTCC R: GGCTCCCGAGAGAAAGATCG	60/35	*AcuI* 37°C 1 h	PCR: 563 C: 281, 234, 48 T: 281, 140, 94, 48
OPRK1 rs997917	F: CTGAACACCAGAAGGAAAAA R: ATACTTGACCGTCCTCATCA	55/36	*EcoRV* 37°C 1 h	PCR: 489 T: 265, 224 C: 489

Cy: Cycle number; F: Forward; R: Reverse; RE: Restriction endonuclease; Tm: Melting temperature.

Measurements

Individuals with OUD were assessed by the Substance Craving Scale (SCS), Beck Depression Inventory-II (BDI-II), Beck Anxiety Inventory (BAI) and Clinical Opiate Withdrawal Scale (COWS) to determine the effects of *OPRK1* rs963549 and rs997917 polymorphisms on the intensity of craving, depression, anxiety and withdrawal, respectively, 2–3 h after the last heroin use. SCS, a version of the Penn Alcohol Craving Scale for substances other than alcohol, was used to evaluate the craving. The validities and reliabilities of Turkish versions of these four measurements have been demonstrated [23–26].

Statistical analyses

SPSS v. 21.0 software for Windows (IBM Corp., NY, USA) was used for the statistical analyses. The normality of numerical variables was examined using the Kolmogorov–Smirnov test. Data were presented as the mean and standard deviation or median and the interquartile range (IQR) according to the normality of the data. Numbers and percentages were given for categorical data. The frequencies of the *OPRK1* rs963549 and rs997917 alleles and genotypes were obtained by direct count, and the departure from the Hardy–Weinberg equilibrium ($p2 + 2pq + q2 = 1$) was evaluated by the χ-square test. For the *OPRK1* rs997917 polymorphism, genotypes were subdivided into three groups (homozygote wild-type, heterozygote and homozygote variant type) and statistically compared according to *OPRK1* rs997917 codominant, T-dominant and T-recessive models. All parameters were also compared according to the *OPRK1* rs963549 C-dominant model, but not for codominant and C-recessive models due to the low frequency of the TT genotype, as shown in Table 4. Genotypes were compared using independent t-test, one-way analysis of variance, Mann–Whitney test or Kruskal–Wallis test, as appropriate. The relationship between the *OPRK1* polymorphisms and OUD was modeled by binary logistic regression analysis. Linkage disequilibrium and haplotype-based case–control analyses were performed using SHEsis-Plus software to evaluate the combined effect of both polymorphisms [27]. The correlations between scores of measures and clinical parameters were analyzed by the Spearman correlation test. $p < 0.05$ was considered statistically significant.

Results

Characteristics of subjects

There was a total of 208 Caucasian subjects. Of them, 100 were individuals with an OUD diagnosis by DSM-5 and 108 were control subjects. All the individuals were Turkish. The median age of the individuals with OUD (n = 91 male; n = 9 female) was 27.0 years (IQR: 25.0–30.0). The median age of the control subjects (n = 98 male; n = 10 female) was 39.0 years (IQR: 30.25–47.0). The median ages of the study groups at the time of ascertainment were significantly different (p = 0.001). However, there was no statistically significant difference in age between the *OPRK1* rs963549 and rs997917 genotype subgroups ($p > 0.05$).

Genotype distribution of the *OPRK1* rs963549 & rs997917 polymorphisms

The genotype and allele frequencies of *OPRK1* rs963549 and rs997917 polymorphisms in individuals with OUD (n = 100) and control subjects (n = 108) are presented in Table 2. For individuals with OUD, the genotype and allele frequencies of *OPRK1* rs963549 and rs997917 polymorphisms were consistent with Hardy–Weinberg equilibrium ($\chi^2 = 0.62$; p = 0.43 and $\chi^2 = 0.31$; p = 0.58, respectively). As for the control subjects, the genotype and allele frequencies of both polymorphisms were also consistent with Hardy–Weinberg equilibrium ($p > 0.05$). The relationship between the *OPRK1* rs963549 and rs997917 polymorphisms and OUD was examined by logistic regression analysis and neither of the *OPRK1* polymorphisms were found to be associated with OUD (Table 2).

Table 2. Comparison of individuals with opioid use disorder: scores for Substance Craving Scale, Beck Depression Inventory-II, Beck Anxiety Inventory and Clinical Opiate Withdrawal Scale according to *OPRK1* rs963549 and rs997917 genotypes.

	SCS	BDI-II	BAI	COWS
	Mean ± SD (min–max)	Mean ± SD (min–max)	Mean ± SD (min–max)	Median (IQR)
***OPRK1* rs963549 genotype**				
CC (n = 70)	17.91 ± 8.74 (0.0–32.0)	26.13 ± 12.74 (0.0–58.0)	25.69 ± 15.96 (0.0–63.0)	4.0 (0.75–7.0)
CT + TT (n = 30)	15.93 ± 8.16 (0.0–29.0)	23.7 ± 14.9 (0.0–63.0)	23.47 ± 13.33 (2.0–55.0)	2.00 (0.0–4.0)
Statistical test†	t = 1.059 p = 0.292	t = 0.829 p = 0.409	t = 0.667 p = 0.506	U = 797.5 Z = -1.924 p = 0.05
***OPRK1* rs997917 genotype (codominant model)**				
TT (n = 35)	17.94 ± 8.2 (0.0–32.0)	26.5 ± 12.49 (7.0–58.0)	25.97 ± 16.04 (2.0–60.0)	4.0 (1.0–6.0)
TC (n = 46)	16.89 ± 8.48 (0.0–32.0)	24.39 ± 14.5 (0.0–63.0)	24.04 ± 15.37 (0.0–63.0)	3.0 (0.0–6.25)
CC (n = 19)	17.21 ± 9.82 (0.0–32.0)	25.79 ± 12.79 (0.0–47.0)	25.68 ± 13.7 (6.0–55.0)	2.0 (0.0–4.0)
Statistical test†	F = 0.149 p = 0.862	F = 0.255 p = 0.775	F = 0.175 p = 0.840	$\chi 2$ = 2.167 p = 0.338
***OPRK1* rs997917 genotype (T-dominant model)**				
TT + TC (n = 81)	17.35 ± 8.32 (0.0–32.0)	25.31 ± 13.6 (0.0–63.0)	24.85 ± 15.58 (0.0–63.0)	4.0 (0.0–6.0)
Statistical test	t = 0.062 p = 0.951	t = -0.140 p = 0.889	t = -0.215 p = 0.831	U = 620.0 Z = -1.331 p = 0.183
***OPRK1* rs997917 genotype (T-recessive model)**				
TC + CC (n = 65)	16.98 ± 8.82 (0.0–32.0)	24.8 ± 13.93 (0.0–63.0)	24.52 ± 14.82 (0.0–63.0)	3.0 (0.0–6.0)
Statistical test†	t = 0.531 p = 0.597	t = 0.608 p = 0.545	t = 0.444 p = 0.658	U = 992.5 Z = -1.061 p = 0.289

†*OPRK1* rs963549 and rs997917 genotypes were compared using the independent t-test, one-way analysis of variance, Mann–Whitney test or Kruskal–Wallis test, as appropriate.
BAI: Beck Anxiety Inventory; BDI-II: Beck Depression Inventory-II; COWS: Clinical Opiate Withdrawal Scale; IQR: Interquartile range; SCS: Substance Craving Scale; SD: Standard deviation.

The linkage disequilibrium test using SHEsis-Plus showed weak linkage disequilibrium (r^2 <0.8) for rs963549 and rs997917 polymorphisms. Haplotypes with frequencies >3% in both individuals with OUD and control subjects were CT, TC and CC. The haplotypes CT (odds ratio [OR]: 0.882; 95% CI: 0.596–1.304; p > 0.05), TC (OR: 0.94; 95% CI: 0.566–1.559; p > 0.05) and CC (OR: 1.246; 95% CI: 0.789–1.967; p > 0.05) showed no significant association with the risk for OUD (Table 3).

The total scores of SCS, BDI-II, BAI & COWS in OUD across *OPRK1* rs963549 genotypes

Table 4 shows the SCS, BDI-II, BAI and COWS scores of individuals with OUD according to their *OPRK1* rs963549 genotypes. OUD patients with the TT genotype were merged with those with CT genotype due to low frequency and were compared statistically with those with the CC genotype. It was found that the COWS scores of individuals with the *OPRK1* rs963549 CC genotype were significantly higher (4.0; IQR: 0.75–7.0) than those of individuals with the CT + TT genotypes (2.0; IQR: 0.0–4.0) (U: 797.5; p = 0.05). Comparison of the SCS, BDI-II and BAI total scores of individuals with the *OPRK1* rs963549 CC and CT + TT genotypes showed no statistically significant differences between genotypes (p > 0.05), although individuals with OUD with the CC genotype had higher SCS, BDI-II and BAI total scores (17.91 ± 8.74, 26.13 ± 12.74 and 25.69 ± 15.96, respectively) than those with the CT + TT genotypes (15.93 ± 8.16, 23.7 ± 14.9 and 23.47 ± 13.33, respectively).

Table 3. Genotype frequencies of *OPRK1* rs963549 and rs997917 polymorphisms in individuals with opioid use disorder and healthy subjects.

OPRK1 rs963549 genotype	OUD (n = 100)		Control subjects (n = 108)		p-value	Odds ratio (95% CI)
	n	%	n	%		
CC	70	70	72	66.7	$p > 0.05$	Reference
CT	26	26	33	30.6		1.371 (0.296–6.35)
TT	4	4	3	2.8		1.692 (0.348–8.238)
Variant allele freq.	17%		18%			
HWE p-value	$\chi 2 = 0.62$; $p = 0.43$		$\chi 2 = 0.11$; $p = 0.73$			
***OPRK1* rs997917 genotype**	**OUD (n = 100)**		**Control subjects (n = 108)**		**p-value**	**Odds ratio (95% CI)**
	n	**%**	**n**	**%**		
TT	35	35	35	32.4	$p > 0.05$	Reference
TC	46	46	61	56.5		0.632 (0.267–1.494)
CC	19	19	12	11.1		1.326 (0.724–2.428)
Variant allele freq.	42%		39%			
HWE p-value	$\chi^2 = 0.31$; $p = 0.58$		$\chi^2 = 3.63$; $p = 0.06$			

HWE: Hardy–Weinberg equilibrium; OUD: Opioid use disorder.

Table 4. Haplotype associations of *OPRK1* rs963549 and rs997917 polymorphisms according to SHEsis-Plus software.

OPRK1 haplotype		OUD n (freq.)	Controls n (freq.)	χ^2	Fisher's p-value	Pearson's p-value	Odds ratio (95% CI)
rs963549	rs997917						
C	T	116 (0.058)	133 (0.61)	0.392	0.55	0.531	0.882 (0.596–1.304)
T	C	34 (0.17)	39 (0.178)	0.057	0.897	0.81	0.94 (0.566–1.559)
C	C	50 (0.25)	46 (0.211)	0.896	0.354	0.343	1.246 (0.789–1.967)

OUD: Opioid use disorder.

The total scores of SCS, BDI-II, BAI & COWS in OUD across *OPRK1* rs997917 genotypes

The SCS, BDI-II, BAI and COWS scores of individuals with OUD were also compared according to codominant, T-dominant and T-recessive models of the *OPRK1* rs997917 polymorphism (Table 4). Although the mean SCS, BDI-II and BAI scores were not statistically different ($p > 0.05$), the mean SCS, BDI-II and BAI scores of individuals with the *OPRK1* rs997917 TT genotype were higher (17.94 ± 8.2, 17.94 ± 8.2 and 25.97 ± 16.04, respectively) than those of individuals with the TC + CC genotypes (16.98 ± 8.82, 24.8 ± 13.93 and 24.52 ± 14.82, respectively). When the median COWS scores and *OPRK1* rs997917 genotypes were compared, the COWS scores of individuals with the TT genotype (4.0; IQR: 1.0–6.0) were higher than those of individuals with the TC + CC genotypes (3.0; IQR: 0.0–6.0); however, this difference was not statistically significant ($p > 0.05$).

Drug use-related phenotypes across *OPRK1* rs963549 & rs997917 genotypes

Analysis of the individuals with OUD according to their *OPRK1* rs963549 and rs997917 genotypes revealed no differences between individuals with the homozygous wild-type genotype and those with heterozygote and/or homozygote variant genotypes in regard to age at onset of first heroin use, opioid addiction (years), daily amount of opioid consumed (g/day) or the longest time of abstinence (months) ($p > 0.05$). Individuals with OUD with the *OPRK1* rs963549 CC genotype (7 years; IQR: 4.4–8.5 and 7.5 months; IQR: 1.0–12.5) had a longer duration of opioid use and time of abstinence than those with the CT + TT genotypes (6 years; IQR: 3.0–7.0 and 6 months; IQR: 1.0–12.0). On the other hand, individuals with OUD with the *OPRK1* rs997917 TT genotype had a shorter duration of opioid use (6 years; IQR: 5.0–8.0) and consumed a higher amount of heroin per day (3 g/day; IQR: 1.0–5.0) than those with the TC + CC genotypes (7 years; IQR: 3.5–8.0 and 2 g/day; IQR: 1.0–4.0). Furthermore,

individuals with OUD with th*; OPRK1* rs997917 CC genotype had a longer time of abstinence (8 months, IQR: 1.0–12.0) than those with the CT + TT genotypes (6 months; IQR: 1.0–12.0).

Correlation analysis

The Spearman correlation test was used due to the non-normal distribution of numerical data such as the age at onset of first heroin use, opioid addiction, daily amount of opioid consumed and the longest time of abstinence as well as the total COWS score. It was found that there were significant and positive correlations between the COWS score and the scores of SCS and BDI-II ($r = +0.296$; $p < 0.001$ and $r = +0.204$; $p < 0.05$, respectively). There were also significant and positive correlations between the duration of opioid use and the scores of SCS and BDI-II ($r = +0.247$; $r = +0.204$; $p < 0.05$, respectively), and between the daily amount of opioid consumed and the SCS score ($r = +0.233$; $p < 0.05$) and the longest time of abstinence ($r = +0.356$; $p < 0.001$). In addition, there was a significant negative correlation between the duration of opioid use and the longest time of abstinence ($r = -0.243$; $p = 0.05$).

Discussion

Existing studies have mostly reported the association of *OPRK1* gene polymorphisms with substance use disorders [5,10–16]. There have also been a few studies regarding the effects of *OPRK1* polymorphisms on negative mood [6,7]. To date, only Jones *et al.* have reported an association of *OPRK1* rs6473797 polymorphism with naloxone-precipitated withdrawal, but not with abstinence-induced withdrawal. The authors did not mention the ethnic background of the participants, who were admitted to their study from the New York State Psychiatric Institute [17]. The current study included individuals with OUD who were not subject to any opioid maintenance treatment, such as buprenorphine/naloxone combination or naltrexone. To the best of our knowledge, this is the first report showing a trend association of *OPRK1* rs963549 variation with opioid withdrawal (reflected by an elevated COWS score) in Caucasian individuals with OUD.

Opioid withdrawal can occur when opioids are stopped or reduced and/or when a maintenance treatment with opioid agonist or antagonists is started [28]. Although it is thought that the etiology of opioid withdrawal is complicated, *in vivo* and *in vitro* studies with animals have shown that opioid withdrawal is associated with hyperactivity of the noradrenergic neurons (due to the loss of opioid feedback inhibition), having an increased number of opioid receptors in the locus coeruleus [29,30]. Glutamate, one of the excitatory amino acids, participates in this increase in neuronal activity [30,31]. In a rodent model, the κ-opioid receptor was shown to be an important regulator of glutamate increase in the locus coeruleus [32]. Additionally, gray matter and nucleus raphe magnus are also implicated in opioid withdrawal [28]. Furthermore, an increased activity of the HPA axis is associated with opioid withdrawal; this increase in the HPA axis is characterized by an increase in corticotropin, β-endorphin and cortisol plasma levels [33]. Consistent with a previous study showing a decrease in naloxone-precipitated withdrawal signs when the κ-opioid receptors were masked with norBNI (nor-binaltorphimine) during the development of opioid dependence on butorphanol [34], in the current study, opioid withdrawal was lower in *OPRK1* rs963549 variant individuals who take heroin daily as compared with individuals with wild-type allele. The *OPRK1* rs963549 SNP is an upstream variant on chromosome 8 [6]. The functional effect of this *OPRK1* polymorphism on behavior and changes in neurochemistry remains unclear due to the fact that the vast number of noncoding or intronic polymorphisms in the opioid receptors are far less studied compared with coding polymorphisms [35]. However, it has been suggested that *OPRK1* rs963549 synonymous variants that do not alter the protein sequence may cause changes in gene expression and translation efficiency; there have been studies demonstrating alterations in mRNA folding or stability associated with synonymous variants in humans [20,21]. *OPRK1* contains mainly silent polymorphisms [35]. In experimental animals, different post-transcriptional mechanisms have been demonstrated due to these silent variants [36,37]. Furthermore, Mayer and Höllt hypothesized that silent variations may induce significant changes in the structure and function of κ-opioid receptors [35]. Additionally, it has been reported that *OPRK1* rs963549 has undergone selection due to its high F_{ST} (a measure of population differentiation) [38]. Consistent with these previous studies, our results regarding the effect of *OPRK1* rs963549 polymorphism on opioid withdrawal indicate that this polymorphism seems to have a functional importance.

The dysfunction of κ-opioid receptors could underlie the association of vulnerability to mood disorders related to the risk of substance use disorders, because they are widely distributed in the brain regions implicated in the regulation of stress-related disorders such as depression and anxiety [6,11]. It is also hypothesized that κ-opioid receptor antagonists produce antidepressant-like effects based on studies in laboratory animals [6,39]. A

sequence of intracellular events is triggered by stress or chronic drugs abuse in the nucleus accumbens (NAc; a part of the mesolimbic system related to motivation) and the ventral tegmental area (VTA) of the midbrain [40]. The involvement of the VTA–NAc pathway in depression-like behaviors was first suggested in studies with dopamine receptor antagonists [41]. Later studies proposed that stress induces VTA dopamine neurons and stimulates dopaminergic transmission to the NAc [42,43]. Additionally, CREB protein in the NAc could mediate alterations in the function of some genes such as opioid peptide dynorphin (*DYN*), which produces depressive symptoms by acting at κ-opioid receptors [44]. Few studies have examined the effects of *OPRK1* polymorphisms on stress-related disorders, personality or behavior, in spite of the fact that preclinical and clinical data show that the κ-opioid system modulates stress response [45]. Thus, the effect of *OPRK1* rs963549 polymorphisms on the intensity of anxiety and depression as well as opioid craving was investigated in Turkish OUD patients. In the current analysis, although the difference was not statistically significant ($p > 0.05$), individuals with the *OPRK1* rs963549 CC genotype had higher SCS, BDI-II and BAI total scores than those with the CT + TT genotypes, suggesting the association of opiate negative craving as well as the intensity of depressive and anxiety symptoms with the *OPRK1* rs963549 variant in individuals with OUD. This is consistent with the previous investigation by Masih and Verbeke on healthy subjects, which demonstrated an association of the variation with anxiety and with acute and chronic depression [6]. In contrast with these reports, Karpyak *et al.* found no association of *OPRK1* rs963549 with negative craving in alcohol-dependent subjects, although the inconsistency could be due to sample heterogeneity [19]. Future studies are needed to clarify the association between *OPRK1* and personality.

The human *OPRK1* gene, located on chromosome 8, contains three introns and four exons [46]. Lutz *et al.* identified an enhancer region in intron 2 of *OPRK1*, where *OPRK1* gene expression is regulated by binding of the glucocorticoid receptor complex [22]. Thus, the *OPRK1* rs997917 polymorphism that is located in the second intron of *OPRK1* was also analyzed in the present study. Although the difference was not statistically significant ($p > 0.05$), individuals with the *OPRK1* rs997917 TT genotype had higher SCS, BDI-II, BAI and COWS total scores than those with the TC + CC genotypes, indicating that *OPRK1* rs997917 may be associated with individual differences in negative mood and addiction-related phenotypes. According to our results, it is also plausible to suggest that the *OPRK1* rs997917 C allele may have a protective effect against depression and anxiety, and hence against craving and opioid withdrawal. Consistent with our hypothesis, Zhang *et al.* found that the minor C allele of *OPRK1* rs997917 might play a protective role for alcohol use disorder [5]. On the other hand, Karpyak *et al.* found no association of *OPRK1* rs997917 with negative craving or the risk of alcohol use disorder [19]. Furthermore, reduced feelings of alcohol sedation on naltrexone were reported in patients with *OPRK1* rs997917 homozygous TT as compared with C allele carriers. This study consisted of individuals with alcohol use disorders and with different ethnic backgrounds, such as Asian and Latino individuals [47]. These few inconsistent results indicate that future studies are needed to understand the exact role of *OPRK1* rs997917 polymorphism on negative mood and substance use disorders.

In the present study, significant and positive correlations between the COWS score and the scores of SCS and BDI-II were found ($r = +0.296$; $p < 0.001$ and $r = +0.204$; $p < 0.05$, respectively). Gong *et al.* showed that there is a positive relationship between craving and withdrawal symptoms and negative emotions in a study conducted among individuals with OUD receiving methadone treatment [48]. On the other hand, Yen *et al.* suggested that craving was not associated with withdrawal symptoms in heroin addicts receiving methadone treatment, but rather with depression [49]. Given that craving, withdrawal symptoms, anxiety and depression are factors that may increase the risk of relapse in individuals with OUD, it is very important to elucidate the mechanism underlying the relationship between them in terms of understanding the onset, maintenance and relapse processes of addiction.

Chronic exposure to several drugs of abuse such as opioids, cocaine and alcohol has been shown to alter (by either activation or sentitization) the dynorphin/κ-opioid receptor, which contributes to the development of drug seeking [50,51]. Opioids and cocaine act on neurons in the VTA, affecting reward pathways directly but with different effects [40]. Opioids excite the principal cells of the VTA, which hyperpolarize in response to dopamine *via* D2 receptors, by inhibition; this in turn hyperpolarizes the GABA-containing secondary cells of the VTA, and then the principal cells take an GABA-A inhibitory input. On the other hand, cocaine inhibits dopamine reuptake. Additionally, Cameron *et al.* identified 'tertiary cells' in the neurons of the VTA, where 5-hydroxytryptamine-mediated inhibitory effect was determined; thus, they showed the role of 5-hydroxytryptamine as a modulator of neuronal activity in the VTA [52].

In summary, we examined the association between OUD and *OPRK1* gene polymorphisms in the present study. Our findings showed that the rs963549 and rs997917 variants of the *OPRK1* gene were not associated with OUD in

Turkish individuals. To date, an association between *OPRK1* rs963549 and rs997917 polymorphisms and OUD in Iranian [15] and African–American [7] populations has been shown, but not in European–American [5] or Indian [13] populations. Among these studies, Albonaim *et al.* reported that *OPRK1* rs997917 and rs6985606 SNPs were significantly associated with opioid addiction under both codominant and recessive inheritance models among Iranians [15]. Additionally, rs997917 was found to be significantly associated with insomnia in individuals receiving methadone maintenance treatment. Similarly, Yuferov *et al.* provided additional support for the importance of rs997917 and rs10111937 SNPs in intron 2 of *OPRK1* in the development of opioid and cocaine addiction in a African–American population [7]. On the other hand, Zhang *et al.* and Kumar *et al.* could not find a significant association between rs997917 and rs963549 and OUD in European Americans and Indians, respectively [5,13], and our results are consistent with their studies. It can thus be suggested that the effects of *OPRK1* rs963549 and rs997917 polymorphisms may be ethnicity-dependent. We further investigated the interaction between rs963549 in 3′UTR and rs997917 in the second intron and observed no linkage disequilibrium, which is consistent with a previous study [5]. Like single-SNP associations, haplotype analysis revealed that the different *OPRK1* rs963549 and rs997917 haplotypes (CT, TC and CC) were not associated with OUD.

Conclusion

Neither single *OPRK1* rs963549 and rs997917 polymorphisms nor *OPRK1* haplotypes composed of these polymorphisms were found to be associated with OUD. However, our results show for the first time a trend association of *OPRK1* rs963549 polymorphism with opioid withdrawal in individuals with OUD. Further studies are needed to clarify the *OPRK1* genotype variables and their association with opioid withdrawal symptomatology, which could be useful for treatment failures of individuals who experience greater opioid withdrawal due to their *OPRK1* rs963549 genotypes.

Summary points

- κ-opioid receptors with their endogenous ligand, dynorphins, are involved in the regulation of negative mood.
- Negative mood could increase the risk of addictive and relapse-like behaviors.
- This study aimed to determine the effects of *OPRK1* rs963549 and rs997917 polymorphisms on opioid use disorder (OUD) and related phenotypes such as depression, anxiety, craving and opioid withdrawal in individuals with OUD.
- A sample of 100 individuals with OUD and 108 control subjects were enrolled in this study.
- *OPRK1* rs963549 and rs997917 SNPs were analyzed by PCR-RFLP.
- Craving, opioid withdrawal and the intensity of depressive and anxiety symptoms were measured by the Substance Craving Scale, Clinical Opiate Withdrawal Scale, Beck Depression Inventory-II and Beck Anxiety Inventory, respectively.
- A trend association between opioid withdrawal and the *OPRK1* rs963549 polymorphism was detected ($p = 0.05$), but no association was found with *OPRK1* rs997917.
- No significant associations were found between *OPRK1* rs963549 and rs997917 polymorphisms and craving and the intensity of depression and anxiety symptoms.
- Neither single *OPRK1* SNPs nor *OPRK1* haplotypes were associated with OUD ($p > 0.05$).
- Our results showed for the first time a trend association of *OPRK1* rs963549 polymorphism with opioid withdrawal in individuals with OUD, which could be useful for treatment failures of individuals who experience greater opioid withdrawal due to their *OPRK1* rs963549 genotypes.

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