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GENETICS

Detecting the *OPRK1* rs963549 and rs997917 Polymorphisms by Novel PCR-RFLP Methods and Determining Their Genotype/Allele Frequencies in a Turkish Population

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Abstract—Opioid receptor kappa (KOR) is one of three endogenous opioid receptors which has critical roles in rewarding systems by promoting anti-reward effects and causes dysphoria and relapse. KOR is encoded by the *OPRK1* gene and the polymorphisms of *OPRK1* gene have been reported to be associated with various diseases. *OPRK1* rs963549 and rs997917 are two polymorphisms of the *OPRK1* gene. They are shown to have potential to affect addiction and alter the response to pharmacotherapy leading to individual differences, therefore they are accepted as marker polymorphisms. Hence, in the current study, it is aimed to develop PCR-RFLP assays to genotype *OPRK1* rs963549 and rs997917 polymorphisms. The PCR-RFLP methods were developed for the detection of *OPRK1* rs963549 and rs997917 polymorphisms and were tested in 100 healthy Turkish controls. Allele frequencies of rs963549 in the Turkish population were 0.81 for allele C and 0.19 for allele T, and the genotype frequencies were 66% for CC, 31% for CT, and 3% for TT. For rs997917 polymorphism, 32% of studied subjects had TT genotype, 56 and 12% of them had TC and CC genotypes, respectively and allele frequencies were 0.60 for allele T and 0.40 for allele C. Novel PCR-RFLP methods were successfully developed for the detection of *OPRK1* rs963549 and rs997917 polymorphisms, which could be preferred and easily applied in the laboratories aiming to genotype *OPRK1* rs963549 and rs997917 polymorphisms. Moreover, obtained allele frequencies may provide a perspective to pharmacogenetics and individualized therapy.

Keywords: *OPRK1* gene, polymorphism, PCR-RFLP, rs963549, rs997917, optimization

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INTRODUCTION

Endogenous opioid system of the brain is the main system responsible for regulating emotional behavior and nociception (Mysels, 2009; Lalanne et al., 2014; Karkhanis et al., 2017). This system also has a role in over-consumption diseases such as obesity and addiction (Lalanne et al., 2014; Karkhanis et al., 2017). Endogenous opioid system consists of three G-coupled nuclear receptors namely as mu (MOR), kappa (KOR) and delta (DOR), which are activated by endogenous ligands as beta-endorphin, dynorphin and enkephalin, respectively (Lalanne et al., 2014). Opioid receptors are responsible for addiction and drug response. It is reported that polymorphisms on opioidergic receptors genes are associated with more successful treatment of drug and alcohol addiction. The success of the treatment of substance use is also dependent on genes encoding the neurotransmitters which mediate reward function and substance abuse (Bauer et al., 2015). Amongst opioid receptors, KOR has a critical impact on rewarding systems by stimulating anti-reward effects and leads to dysphoria especially following prolonged drug exposure and therefore promoting relapse (Wang et al., 2019; Abijo et al., 2020). KORs are the most affluent opioid receptors present in the human brain regions of stress response. KORs are also involved in mood and pain modulation, memory and learning (Burns et al., 2019).

KOR is encoded by the *OPRK1* gene which is located in chromosome 8q11.2 in humans. It is composed of four exons and three introns (Yuferov et al., 2004). *OPRK1* is one of the candidate genes possessing a critical role in the reward pathway (Abijo et al., 2020). Several studies have shown the association between *OPRK1* gene polymorphisms and the risk of addiction particularly alcohol dependence and opioid withdrawal (Xuei et al., 2006; Gerra et al., 2007; Edenberg et al., 2008; Ashenhurst et al., 2012; Xu et al., 2013; Albonaim et al., 2017; Nagaya et al., 2018; Park et al., 2019; Al-Eitan et al., 2021). Moreover, *OPRK1* variants have been shown to be associated with

abnormal stress response and increased risk for substance use (Bauer et al., 2015).

OPRK1 rs963549 is a single nucleotide polymorphism located 3-UTR of the fourth exon of *OPRK1* gene (Kumar et al., 2012). The relationship between *OPRK1* rs963549 polymorphism and naltrexone treatment, an opioid-blocking drug used in the therapy of alcohol dependence, was investigated and no significant interaction was found in European and African Americans (Gelernter et al., 2007). A study investigating the effect of *OPRK1* rs963549 on alcohol and heroin dependence reported no association in the Indian population (Kumar et al., 2012). Jones and colleagues (2016) reported that there was no association between *OPRK1* rs963549 and opioid withdrawal. In a recent study investigating the role of *OPRK1* rs963549 polymorphism on the daily dose of methadone, a drug used in the pharmacotherapy of heroin use disorder, reported an association between *OPRK1* rs963549 genotypes and methadone dose in Chinese Han population (Zhang et al., 2020).

OPRK1 rs997917 polymorphism is located on the second intron of *OPRK1* gene (Albonaim et al., 2017). Intronic polymorphisms have potential to alter gene expression and mRNA stability in the cytoplasm (Shaul et al., 2017). Ashenhurst et al. (2012) reported a significant interaction between *OPRK1* rs997917 polymorphism and naltrexone. Albonaim and colleagues (2017) found rs997917 a risk factor for opioid use disorder in Iranian population but Zhang and colleagues (2020) showed no significant association in European Americans. Furthermore, a recent study stated the association of *OPRK1* rs997917 polymorphism with cocaine and opioid dependence in African Americans (Yuferov et al., 2022).

The results of the limited number of studies on *OPRK1* rs963549 and rs997917 polymorphisms can be defined as controversial. However, almost all studies mentioned here were done with a limited sample size, therefore there is a need to repeat these studies in larger populations with different ethnic backgrounds in order to reveal the potential association in independent populations. To work with the larger populations, methodology is an important factor due to the cost and a cost-effective way should be considered. Gene polymorphisms can be detected by several molecular methods including TaqMan assay, fluorescent labeled probing, DNA sequencing, molecular beacons, tetra-primer amplification refractory mutation system polymerase chain reaction (T-ARMS PCR) and restriction fragment length polymorphism (RFLP). Amongst these the latter is the most preferred mean to genotype single nucleotide gene polymorphisms (SNP) due to its features such as rapidness, sensitivity and cost-effectiveness (Feng et al., 2016). Here it is aimed to develop PCR-RFLP assays to detect rs963549 and rs997917 SNPs of *OPRK1* gene and also examine the application of this method.

MATERIALS AND METHODS

Study Population and DNA Isolation

A total of 100 healthy controls were recruited in this study and 2 mL whole blood samples were collected into ethylenediaminetetraacetic acid (EDTA) containing tubes from these individuals. Tubes were kept at −20°C until genomic DNA isolation which was done using QIAmp DNA Blood Mini Kit (Qiagen, Germany) according to the procedure recommended by the manufacturer. The quality and the quantity of the samples were measured by Nanodrop 2000 spectrophotometer (Thermo) and all samples were stored at −20°C until further analysis was performed. The study population consists of healthy individuals born in Turkey, living in Ankara and possessing native Turkish parents. The study design was approved by Ankara University Ethics Committee (Approval no. 07-536-19 in 2019). Samplings were performed in accordance with the principles of the Declaration of Helsinki.

Polymerase Chain Reaction for OPRK1 rs963549 and rs997917 Polymorphisms

The sequence information of rs963549 and rs997917 SNPs in the human *OPRK1* gene region was obtained from the NCBI database. In order to amplify the genomic regions containing these two SNPs, novel primers were designed using the NCBI Primer-Blast tool as below: rs963549 forward: 5'-GTTGCGTGGACCTTTTGTCC-3', rs963549 reverse: 5'-GGCTCCCGAGAGAAAGATCG-3', rs997917 forward: 5'-CTGAACACCAGAAGGAAAAA-3', rs997917 reverse: 5'-ATACTTGACCGTCCTCATCA-3'. PCR reactions consist of 5 μL of 5× standard buffer, 160 mM dNTP mix, 0.4 μM forward and reverse primers, 1.25 mM of $MgCl_2$, 1.25 units HotStart Taq polymerase (NEB, UK), 200 ng genomic DNA and sterile distilled water up to 25 μL. PCR conditions were as follows: initial denaturation at 95°C for 15 min, followed by 36 cycles of denaturation at 94°C for 60 s, annealing at 55°C for 90 s (rs997917)/60°C for 60 s (rs963549), elongation at 72°C for 60 s, and a final extension step at 72°C for 10 min. The expected sizes of the PCR products were 563 bp for rs963549 and 489 bp for rs997917 and these amplicons were confirmed by agarose gel electrophoresis.

Restriction Fragment Length Polymorphism for OPRK1 rs963549 and rs997917 Polymorphisms

Restriction Fragment Length Polymorphism (RFLP) was performed in order to determine the genotypes. Enzymes digesting the PCR products for genotyping were determined using online tools such as restrictionmapper.org and NEBcutter V2.0. Restriction conditions and products were given in Table 1. The digested products were electrophoresed on 3%

Table 1. RFLP conditions and products of rs963549 and rs997917

Polymorphism	PCR product, bp	Restriction enzyme	Restriction products, bp	Reaction ingredients
OPRK1 *rs963549*	563	*AcuI* 37°C 1 hour incubation	CC: 281, 234, 48 CT: 281, 234, 140, 94, 48 TT: 281, 140, 94, 48	5 µL PCR product 1 µL G buffer 1 µL *AcuI* 0.2 µL SAM 2.8 µL nuclease-free water
OPRK1 *rs997917*	489	*EcoRV* 37°C 1 hour incubation	TT: 265, 224 TC: 489, 265, 224 CC: 489	5 µL PCR product 1 µL R buffer 0.5 µL *EcoRV* 3.5 µL of nuclease-free water

agarose gel stained with ethidium bromide (EtBr). The RFLP products of rs963549 were loaded using 6xLoading dye with 2% SDS in order to make the DNA bands sharper. Additionally, to confirm the RFLP results, three PCR products from each different genotype (wild type, heterozygous and variant genotypes) for both SNPs were sequenced with the same sets of primers (Applied Biosystems 3730xl DNA analyzer, USA).

Statistical Analysis

Allele and genotype frequencies of both SNPs were calculated by direct counting, and chi-square test was used to evaluate the departure from the Hardy–Weinberg equilibrium. Statistical analyses were performed using The Statistical Package for Social Sciences (SPSS) version 21.0 software for Windows. All categorical data were shown as numbers. $P < 0.05$ was considered as statistically significant.

RESULTS

Novel PCR-RFLP assays for genotyping rs997917 and rs963549 polymorphisms developed here were summarized in Fig. 1. With the new primers designed in this study, 563 and 489 bp regions of the *OPRK1* gene to detect rs963549 and rs997917 polymorphisms were amplified successfully (Fig. 2). The enzymes determined to digest these amplicons were *AcuI* and *EcoRV*, respectively. *AcuI* has two recognition sites to determine rs963549 C to T polymorphism and the expected restriction fragments were 281, 234 and 48 bp for the wild type as shown in Fig. 3a. *EcoRV* has one recognition site to determine rs997917 T to C polymorphism and does not cut the variant genotype. The expected restriction fragments were 265 and 224 bp for the wild type (Fig. 4a).

RFLP products of wild type, heterozygous and variant genotypes of both SNPs were verified by sequencing given below in Figs. 3b and 4b. The accuracy and reliability of the novel assays were confirmed

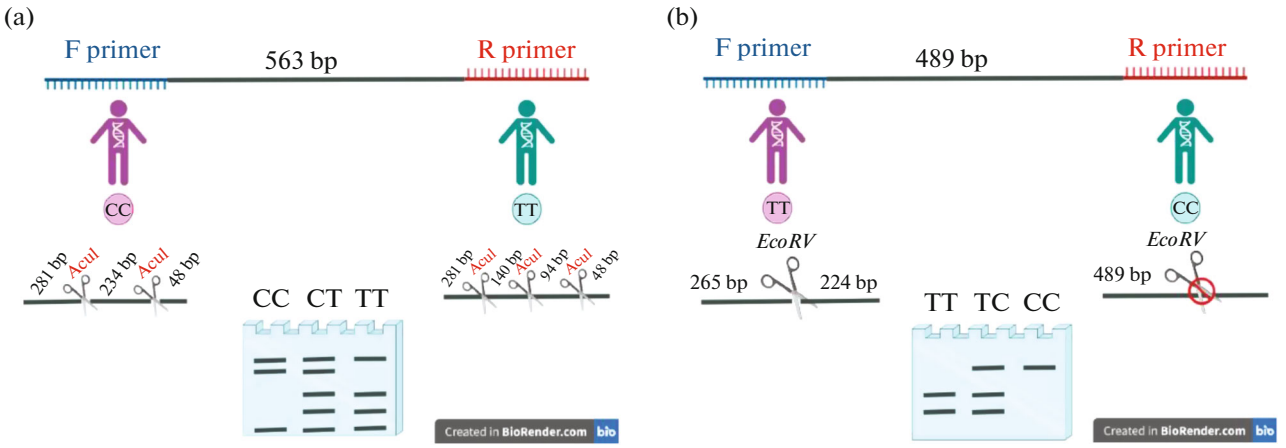

Fig. 1. Schematic illustrations of PCR-RFLP method and restriction fragments for each genotype of *OPRK1* rs963549 (a) and *OPRK1* rs997917 (b).

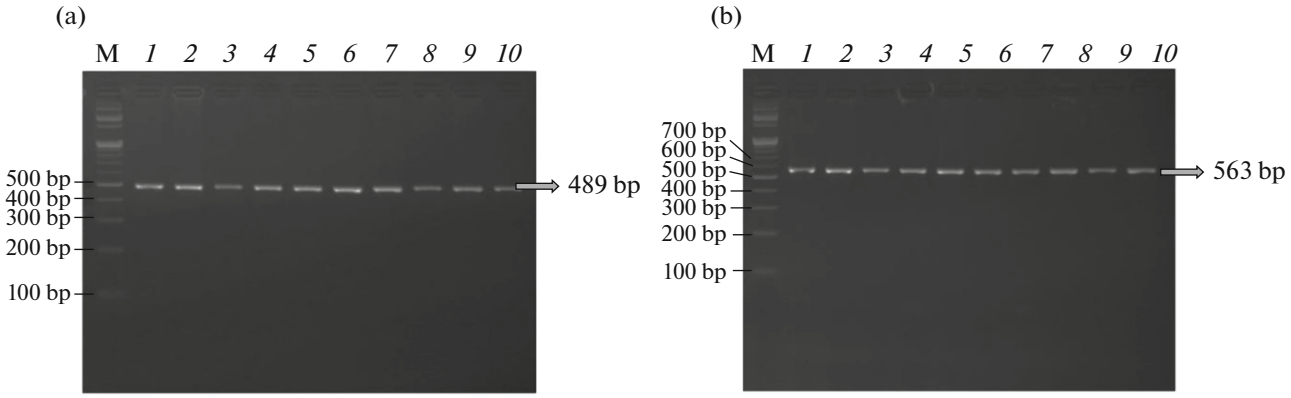

Fig. 2. Agarose gel electrophoresis showing the sizes of PCR products containing *OPRK1* rs963549 (a) and *OPRK1* rs997917 (b) SNPs. Lane M represents 100 bp ladder.

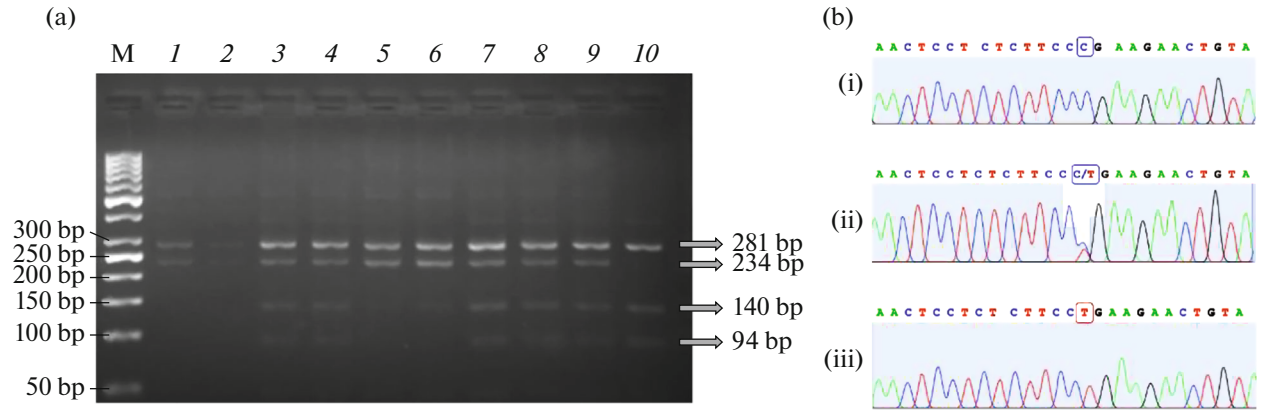

Fig. 3. Agarose gel electrophoresis (a) and DNA sequencing (b) results of *OPRK1* rs963549. In the agarose gel image (a), Lane M represents 50 bp ladder; Lanes *1*, *2*, *5* and *6* represent homozygous wild-type genotype (CC); Lanes *3*, *4*, *7*, *8* and *9* represent heterozygous genotype (CT) and Lane *10* represents homozygous polymorphic genotype (TT). In the DNA sequencing histograms (b), (i), (ii) and (iii) panels represent *OPRK1* rs963549 CC, CT and TT genotypes, respectively.

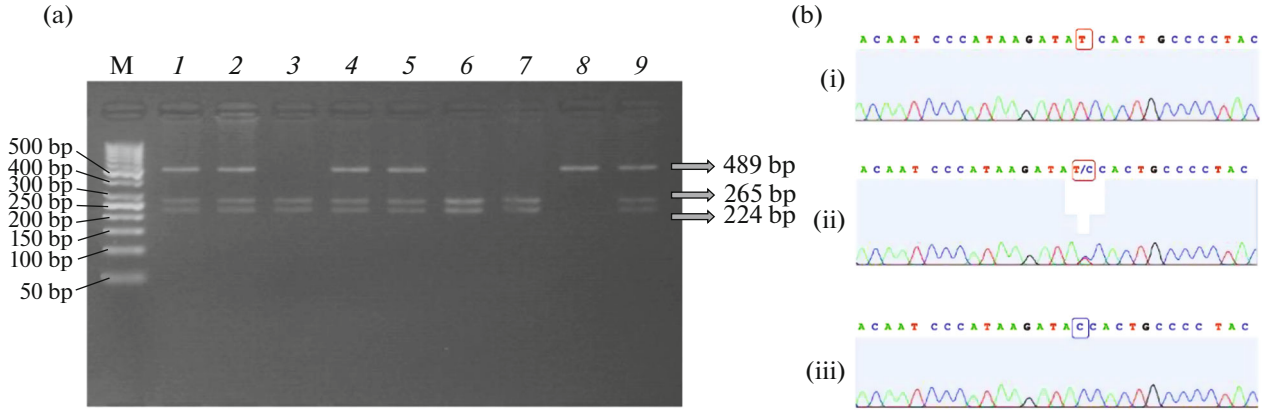

Fig. 4. Agarose gel electrophoresis (a) and DNA sequencing (b) results of *OPRK1* rs997917. In the agarose gel image (a), Lane M represents 50 bp ladder; Lanes *3*, *6* and *7* represent homozygous wild-type genotype (TT); Lanes *1*, *2*, *4*, *5* and *9* represent heterozygous genotype (TC) and Lane *8* represents homozygous polymorphic genotype (CC). In the DNA sequencing histograms (b), (i), (ii) and (iii) panels represent *OPRK1* rs997917 TT, TC and CC genotypes, respectively.

Table 2. Genotype and allele frequencies of *OPRK1* rs963549 and rs997917 polymorphisms

SNP	Genotype	Expected	Observed	Allele	Chi-square, χ^2	*P*-value
OPRK1 rs963549 C>T	CC	66.4	66	C: 0.81 T: 0.19	0.078	0.78
	CT	30.2	31			
	TT	3.4	3			
OPRK1 rs997917 T>C	TT	36.0	32	T: 0.60 C: 0.40	2.7	0.09
	TC	48.0	56			
	CC	16.0	12			

as the sequencing results revealed 100% concordance with them.

Allele frequencies of rs963549 in the Turkish population were 0.81 for allele C and 0.19 for allele T, and the genotype frequencies were 66% for CC, 31% for CT, and 3% for TT (Table 2). For rs997917 polymorphism, 32% of studied subjects had TT genotype, 56% and 12% of them had TC and CC, respectively and the allele frequencies were 0.60 for allele T and 0.40 for allele C (Table 2).

DISCUSSION

Opioids are the key regulating elements of brain functions including mood and reward. Among opioid receptors, KOR has particular importance in addiction as it involves in the rewarding system and dysphoria. *OPRK1* gene encoding KOR has become a potential biomarker for addiction due to its genetic variations. Therefore, it is critical to investigate the role of its polymorphisms have been found to be associated with a wide range of disorders (Kumar et al., 2012).

Single nucleotide polymorphisms can be detected by several methods such as TaqMan genotyping, tetra-ARMS PCR, DNA sequencing and PCR-RFLP. Amongst these methods, PCR-RFLP is the most preferred one as it provides several advantages including accuracy, sensitivity, rapidness, ease and being low-cost (Feng et al., 2016).

Limited number of studies have focused on genotyping *OPRK1* rs963549 polymorphism and none of them has utilized the PCR-RFLP method (Kumar et al., 2012; Jones et al., 2016; Zhang et al., 2020). Previously, Kumar and colleagues (2012) investigated the role of *OPRK1* rs963549 polymorphism in the susceptibility to addiction where the genotyping was performed by sequencing following PCR. Jones et al. (2016) and Zhang et al. (2020) used matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) and SNaPshot SNP technology for genotyping, respectively. The genotype frequencies of rs963549 were found as 81% for CC and 19% for CT+TT in the Indian population (Kumar et al., 2012). In a Chinese Han population, genotype frequencies were 86.9, 12.6, 0.5% for CC, CT and TT, respectively and allele frequencies were 0.93 for C allele and 0.07 for T allele (Zhang et al., 2020). Subjects carrying CC genotype of *OPRK1* rs963549 polymorphism were more frequent in the Chinese and Indian populations when compared with the Turkish population. In the current study, the frequency of the polymorphic T allele was 0.19 in the Turkish population, whereas it was found to be very low in the Chinese population as 0.07 (Kumar et al., 2012; Zhang et al., 2020).

OPRK1 rs997917 polymorphism has been investigated in a few studies and those studies utilized TaqMan assays and amplification refractory mutation system-polymerase chain reaction (ARMS-PCR) for genotyping (Ashenhurst et al., 2012; Albonaim et al., 2017; Yuferov et al., 2022). In one of these studies with a mixed population where the majority has White background, genotype frequencies were detected as 46% for TT, 48% for TC and 5% for CC and allele frequencies were as 0.70 for T allele and 0.30 for C allele (Ashenhurst et al., 2012). Data obtained from an Iranian population showed that the frequencies of T and C alleles were 0.30 and 0.70, respectively and genotype frequencies were detected as 15% for TT, 30% for TC and 55% for CC (Albonaim et al., 2017). In the current study, the frequency of the polymorphic C allele was 0.40 in the Turkish population, which was similar with White-American population whereas it was reported to be very high in Iranian population as 0.70 (Ashenhurst et al., 2012; Albonaim et al., 2017). The results of the present study were in accordance with the ones obtained from White-Americans as both ethnicities belong to Caucasian ethnicity.

As a first study, genotype and allele frequencies were obtained from a small Turkish population. With the help of developed PCR-RFLP assays in the current study, more information could be gathered for these two polymorphisms in the future with the larger sample size in Turkish population and other nationwide studies.

CONCLUSIONS

In conclusion, novel and low-cost PCR-assays to genotype rs963549 and rs997917 SNPs of *OPRK1* gene

were developed. The validity and accuracy check of the developed methods were also done. To our knowledge, the current study is the first one documenting genotype and allele frequencies of rs963549 and rs997917 in the Turkish population. The developed genotyping assays and determined allele frequencies could be considered as useful contributions to the literature and future studies. These novel assays may be preferred in the studies investigating biological basis of other diseases related to neuroscience.

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AUTHOR CONTRIBUTIONS

S.Ö.K. developed the study concept, conducted the genetic analysis and wrote the manuscript.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interests. Author has no competing interests to declare.

Statement of compliance with standards of research involving humans as subjects. The study design was approved by Ankara University Ethics Committee (Approval no. 07-536-19 in 2019). Samplings were performed in accordance with the principles of the Declaration of Helsinki.

REFERENCES

Abijo, T., Blum, K., and Gondre-Lewis, M.C., Neuropharmacological and Neurogenetic correlates of opioid use disorder (OUD) as a function of ethnicity: relevance to precision addiction medicine, *Curr. Neuropharmacol.,* 2020, vol. 18, pp. 578–595.
https://doi.org/10.2174/1570159X17666191118125702

Albonaim, A., Fazel, H., Sharafshah, A., Omarmeli, V., Rezaei, S., Ajamian, F., and Keshavarz, P., Association of *OPRK1* gene polymorphisms with opioid dependence in addicted men undergoing methadone treatment in an Iranian population, *J. Addict. Dis.,* 2017, vol. 36, p. 227–235.
https://doi.org/10.1080(10550887.2017.1361724

Al-Eitan, L.N., Rababa'h, D.M., and Alghamdi, M.A., Genetic susceptibility of opioid receptor genes polymorphism to drug addiction: a candidate-gene association study, *BMC Psychiatry*, 2021, vol. 21, pp. 5.
https://doi.org/10.1186/s12888-020-03006-z

Ashenhurst, J.R., Bujarski, S., and Ray, L.A., Delta and kappa opioid receptor polymorphisms influence the effects of naltrexone on subjective responses to alcohol, *Pharmacol. Biochem. Behav.*, 2012, vol. 103, pp. 253–259.
https://doi.org/10.1016/j.pbb.2012.08.019

Bauer, I.E., Soares, J.C., and Nielsen, D.A., The role of opioidergic genes in the treatment outcome of drug addiction pharmacotherapy: a systemic review, *Am. J. Addict.,* 2015, vol. 24, pp. 15–23.
https://doi.org/10.1111/ajad.12172

Burns, J.A., Kroll, D.S., Feldman, D.E., Liu, C.K., Manza, P., Wiers, C.E., Volkow, N.D., and Wang, G.-J., Molecular imaging of opioid and dopamine systems: insights into the pharmacogenetics of opioid use disorders, *Front. Psychiatry,* 2019, vol. 20, p. 626.
https://doi.org/10.3389/fpsyt.2019.00626

Edenberg, H.J., Wang, J., Tian, H., Pochareddy, S., Xuei, X., Wetherill, L., Goate, A., Hinrichs, T., Kuperman, S., Nurnberfer, J.I., Jr., Schuckit, M., Tischfield, J.A., and Foroud, T., A regulatory variation in *OPRK1*, the gene encoding the k-opioid receptor, is associated with alcohol dependence, *Hum. Mol. Genet.,* 2008, vol. 17, pp. 1783–1789.
https://doi.org/10.1093/hmg/ddn068

Feng, X., Wang, S., Duan, X., Li, C., Yan, Z., Feng, F., Yu, S., Wu, Y., and Wang, W., An improved PCR-RFLP assay for the detection of a polymorphism rs2289487 of *PLIN1* gene, *J. Clin. Lab. Anal.,* 2016, vol. 30, pp. 986–989.
https://doi.org/10.1002/jcla.21968

Gelernter, J., Gueorguieva, R., Kranzler, H.R., Zhang, H., Cramer, H., Rosenheck, R., and Krystal, J.H., Opioid receptor gene (*OPRM1, OPRK1*, and *OPRD1*) variants and response to naltrexone treatment for alcohol dependence: results from the VA cooperative study, *Alcohol Clin. Exp. Res.,* 2007, vol. 31, pp. 555–563.
https://doi.org/10.1111/j.1530-0277.200700339.x

Gerra, G., Leonardi, C., Cortese, E., D'Amore, A., Lucchini, A., Strepparola, G., Serio, G., Farina, G., Magnelli, F., Zaimovic, A., Mancini, A., Turci, M., Manfredini, M., and Donnini, C., Human kappa opioid receptor gene (*OPRK1*) polymorphism is associated with opiate addiction, *Am. J. Med. Genet. B: Neuropsychiatr. Genet.*, 2007, vol. 144, pp. 771–775.
https://doi.org/10.1002/ajmg.b.30510

Jones, J.D., Luba, R.R., Vogelman, J.S., and Comer, S.D., Searching for evidence of genetic mediation of opioid withdrawal by opioid receptor gene polymorphisms, *Am. J. Addict.,* 2016, vol. 25, pp. 41–48.
https://doi.org/10.1111/ajad.12316

Karkhanis, A., Holleran, K.M., and Jones, S.R., Dynorphin/kappa opioid receptor signaling in preclinical models of alcohol, drug, and food addiction, *Int. Rev. Neurobiol.,* 2017, vol. 136, pp. 53–88.
https://doi.org/10.1016/bs.irn.2017.08.001

Kumar, D., Chakraborty, J., and Das, S., Epistatic effects between variants of kappa-opioid receptor gene and A118G of mu-opioid receptor gene increase susceptibility to addiction in Indian population, *Prog. Neuropsychopharmacol. Biol. Psychiatry,* 2012, vol. 36, pp. 225–230.
https://doi.org/10.1016/j.pnpbp.2011.10.018

Lalanne, L., Ayranci, G., Kieffer, B.L., and Lutz, P.E., The kappa opioid receptor: from addiction to depression, and back, *Front. Psychiatry,* 2014, vol. 5, p. 170.
https://doi.org/10.3389/fpsyt.2014.00170

Mysels, D., The kappa-opiate receptor impacts the pathophysiology and behavior of substance use, *Am. J. Addict.,*

2009, vol. 18, pp. 272–276.
https://doi.org/10.1080/10550490902925862

Nagaya, D., Zahari, Z., Saleem, M., Yahaya, B.H., Tan, S.C., and Yusoff, N.M., An analysis of genetic association in opioid dependence susceptibility, *J. Clin. Pharm. Ther.,* 2018, vol. 43, pp. 80–86.
https://doi.org/10.1111/jcpt.12585

Park, C., II, Hwang, S.S., Kim, H.W., Kang, J.I., Lee, S.H., and Kim, S.J., Association of opioid receptor gene polymorphism with drinking severity and impulsivity related to alcohol use disorder in a Korean population, *CNS Neurosci. Ther.,* 2020, vol. 26, pp. 30–38.
https://doi.org/10.1111/cns.13138

Shaul, O., How introns enhance gene expression, *Int. J. Biochem. Cell Biol.,* 2017, vol. 91, pp. 145–155.
https://doi.org/10.1016/j.biocel.2017.06.016

Wang, S.C., Chen, Y.C., Lee, C.H., and Cheng, C.M., Opioid addiction, genetic susceptibility, and medical treatments: a review, *Int. J. Mol. Sci.,* 2019, vol. 20, p. 4294.
https://doi.org/10.3390/ijms20174294

Xu, K., Seo, D., Hodgkinson, C., Hu, Y., Goldman, D., and Sinha, R., A variant on the kappa opioid receptor gene (*OPRK1*) is associated with stress response and related drug craving, limbic brain activation and cocaine relapse risk, *Transl. Psychiatry,* 2013, vol. 3, p. e292.
https://doi.org/10.1038/tp.2013.62

Xuie, X., Dick, D., Flury-Wetherill, L., Tian, H-J., Agrawal, A., Bierut, L., Goate, A., Bucholz, K., Schuckit, M., Nurnberger, J., Tischfield, J., Kuperman, S., Porjesz, B., Begleiter, H., Foroud, T., and Edenberg, H.J., Association of the k-opioid system with alcohol dependence, *Mol. Psychiatry,* 2006, vol. 11, pp. 1016–1024.
https://doi.org/10.1038/sj.mp.4001882

Yuferov, V., Fussell, D., LaForge, K.S., Nielsen, D.A., Gordon, D., Ho, A., Leal, S.M., Ott, J., and Kreek, M.J., Redefinition of the human kappa opioid receptor gene (*OPRK1*) structure and association of haplotypes with opiate addiction, *Pharmacogenetics,* 2004, vol. 14, pp. 793–804.
https://doi.org/10.1097/00008571-200412000-00002

Yuferov, V., Butelman, E.R., Randesi, M., Ott, J., and Kreek, M.J., Analyses of polymorphisms of intron 2 of *OPRK1* (kappa-opioid receptor gene) in association with opioid and cocaine dependence diagnoses in an African-American population, *Neurosci. Lett.,* 2022, vol. 768, p. 136364.
https://doi.org/10.1016/j.neulet.2021.136364

Zhang, Q., Shi, M., Tang, H., Zhong, H., and Lu, X., k Opioid receptor 1 single nucleotide polymorphisms were associated with the methadone dosage, *Genet. Test. Mol. Biomarkers,* 2020, vol. 24, pp. 17–23.
https://doi.org/10.1089/gtmb.2019.0159