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Türk Hijyen ve Deneysel Biyoloji Dergisi

PCR-RFLP optimisation for *OPRM1* rs540825 and rs510769 gene polymorphisms and their allele/genotype frequencies in Turkish population

OPRM1 rs540825 ve rs510769 gen polimorfizmleri için PCR-RFLP yöntemi optimizasyonu ve Türk popülasyonundaki alel/genotip frekansları

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ABSTRACT

Objective: Opioid dependence, which has environmental and genetic components, is an important public health problem. *OPRM1* gene encodes mu opioid receptor (MOR) which is a primary target for opioids. Polymorphisms on the *OPRM1* gene have been shown to alter the properties and physiology of MOR and also may have impact on opioid dependence. The association between *OPRM1* rs540825 and rs510769 polymorphisms and substance dependence have been shown through various studies. The purpose of this study is to develop reliable, robust and easily applicable genotyping procedures for *OPRM1* rs540825 and rs510769 polymorphisms as they possess rising importance in the context of addiction and therapy success.

Methods: A novel and an improved method based on polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was employed to determine *OPRM1* gene polymorphisms at positions rs510769 and rs540825, respectively. *OPRM1* gene regions containing these two polymorphisms were amplified using PCR; then, RFLP method was performed using the restriction

ÖZET

Amaç: Opioid bağımlılığı, çevresel ve genetik bileşenleri olan önemli bir halk sağlığı problemidir. Opioidlerin başlıca hedefi olan mu opioid reseptörü (MOR) *OPRM1* geni tarafından kodlanmaktadır. *OPRM1* geninde bulunan polimorfizmlerin MOR'un fizyolojisini ve özelliklerini değiştirdiği gösterilmiştir ve bunun opioid bağımlılığında bir etkisi olabileceği düşünülmektedir. *OPRM1* polimorfizmlerinden rs540825 ve rs510769'un madde bağımlılığı ile ilişkisi yapılan çalışmalarda gösterilmiştir. Bu çalışmanın amacı bağımlılıkta ve bağımlılık tedavisinde önemi gittikçe artan *OPRM1* rs540825 ve rs510769 polimorfizmleri için güvenilir, güçlü ve her laboratuvarda uygulanabilecek bir genotiplendirme yöntemi geliştirmektir.

Yöntem: *OPRM1* gen polimorfizmlerinden rs510769 için yeni ve rs540825 içinse iyileştirilmiş polimeraz zincir reaksiyonu-restriksiyon parçacık uzunluk polimorfizmi (PCR-RFLP) tekniğine dayanan yöntemler geliştirilmiştir. *OPRM1* geninde bu iki polimorfizmin bulunduğu bölgeler PCR ile çoğaltılmış ve sonrasında da rs540825 için *NlaIII* enzimi ve rs510769 için *SspI* enzimi kullanılarak

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enzymes *NlaIII* and *SspI* for rs540825 and rs510769, respectively. A total of 70 healthy samples from the Turkish population were tested to evaluate these two developed PCR-RFLP methods.

Results: Amplicons were 276 base pair (bp) and 563 bp in length for rs540825 and rs510769, respectively. The length of the restriction products of *OPRM1* rs540825 were 276 bp for wild type AA genotype, 153 bp and 123 bp for polymorphic TT genotype. Digestion of PCR region including rs510769 polymorphism yielded 364 bp, 146 bp and 53 bp fragments for polymorphic TT genotype and 417 bp and 146 bp fragments for wild-type CC genotype. Allelic and genotypic frequencies of rs540825 in the Turkish population were calculated as 29 % for allele A and 71 % for allele T, 10 % for AA, 38.6 % for AT, and 51.4 % for TT. For rs510769 polymorphism, 60 % of the individuals had CC genotype, 24.3 % and 11.4 % of them had CT and TT, respectively. Allele frequencies of rs510769 polymorphism were 75 % for allele C and 25 % for allele T.

Conclusion: Novel, reliable and easy-to-apply PCR-RFLP technologies were developed to detect rs540825 and rs510769 polymorphism in the *OPRM1* gene. Moreover, genotype and allele frequencies of them were determined in the Turkish population.

Key Words: *OPRM1* gene, single nucleotide polymorphism, PCR-RFLP, optimisation, genotyping

RFLP yöntemi uygulanmıştır. Sağlıklı bireylerden oluşan 70 kişilik bir Türk popülasyonu örneklemi üzerinde geliştirilen yöntemler test edilmiştir.

Bulgular: *OPRM1* rs540825 ve rs510769 polimorfizmleri için PCR ürünleri sırasıyla 276 baz çifti (bç) ve 563 bç uzunluğundadır. *OPRM1* rs540825 için kesim ürünleri yabanıl AA genotipi için 276 bç iken polimorfik TT genotipi için 153 bç ve 123 bç boyutundadır. *OPRM1* rs510769 polimorfizmini içeren PCR ürününün kesimi sonucunda polimorfik TT genotipi için 364 bç, 146 bç ve 53 bç'lik ürünler elde edilirken, yabanıl CC genotipi için 417 bç ve 146 bç boyutunda kesim ürünleri elde edilmiştir. *OPRM1* rs540825 polimorfizminin alel ve genotip frekansları A aleli için % 29 ve T aleli için % 71 olarak, AA genotipi için % 10, AT genotipi için % 38,6 ve TT genotipi için de % 51,4 olarak hesaplanmıştır. *OPRM1* rs510769 polimorfizmi için, bireylerin %60'ının CC genotipine, sırasıyla % 24,3 ve % 11,4' ünün CT ve TT genotiplerine sahip olduğu gösterilmiştir. *OPRM1* rs510769 polimorfizminin alel frekansları C aleli için % 75 ve T aleli için % 25 olarak belirlenmiştir.

Sonuç: Yeni, güvenilir ve uygulaması kolay PCR-RFLP yöntemleri *OPRM1* geninde bulunan rs540825 ve rs510769 polimorfizmlerinin genotiplendirilmesi için geliştirilmiştir. Ayrıca bu polimorfizmlerin alel ve genotip frekansları Türk popülasyonunda belirlenmiştir.

Anahtar Kelimeler: *OPRM1* geni, tek nükleotit polimorfizmi, PCR-RFLP, optimizasyon, genotiplendirme

INTRODUCTION

Addiction is described as a multifactorial disease and the mechanism underneath the susceptibility differences between individuals still remains unclear. Addiction arises with the effects of cultural, environmental, developmental, neurobiological and also genetic factors (1). As an important public health problem, opioid abuse also has both environmental and genetic components (2,3).

Opioids are one of the most effective analgesics in medical use, however, they may easily lead to addiction due to their powerful rewarding features (4). When opioids enter the body and reach the nervous

system, they exert their function via binding opioid receptors (5). Opioid receptors are the members of G-protein superfamily which is a cell-surface receptor class where many drugs show their action by binding them (6). Opioid receptors are present in the brain, spinal cord, gastrointestinal system and skin. These receptors have critical roles in substance addiction, mood swings and pain management and following the stimulation of them euphoria, sedation, analgesia and respiratory depression occurs (7). Mu, kappa and delta opioid receptors are the three major subtypes having diverse impacts where analgesia is the common one for three of them. Mu opioid receptors (MORs) are critical for the reward system and stimulation of them leads to euphoria, physical dependence and hypoventilation (respiratory depression). MORs regulate the response to pain treatment, stress and rewarding impacts of numerous drugs via mesolimbic dopamine system (1). As MORs are the main receptor activated by opioids, studies on opioid use disorder have focused on them as they play a critical role in substance dependence and tolerance (8). MORs are crucial mediators of rewarding features of various drugs used for addiction therapy and therefore stand as important targets of them (9).

The mu-opioid receptor is encoded by *OPRM1* gene which possess various isoforms and splice variants including pharmacologically important ones. *OPRM1* gene is a complex gene with 12 exons and two independent promoters. MOR-1 is a member of the G-protein coupled receptor family and composed of seven transmembrane domains (10). Various variations on the *OPRM1* gene have been reported to change the properties and physiology of MOR and be clinically relevant. Moreover, significant variations have been reported in the *OPRM1* gene amongst different human populations (11).

OPRM1 rs540825 polymorphism is a missense variant located in the final exon and consists of a histidine to glutamine substitution in the C-terminal of the receptor (12). The association of this polymorphism with depression, pain and substance dependence have been examined by many studies (8; 12-17).

OPRM1 rs510769 is located at the first intron of the *OPRM1* gene and involves a cytosine to thymine substitution (8,18). It is one of the most studied polymorphisms of this gene as its potential to influence alternative splicing and by means of this alters the gene function. It was also found to be associated with alcohol and substance dependence including heroin, amphetamine (18-21).

PCR-RFLP is a valuable tool for molecular biology due to being easy and cheap to be applied in a basic laboratory (22). Therefore, it is important to develop this method as a reliable and rapid option for the detection of gene variants.

To our knowledge this study presents a novel PCR-RFLP method to genotype *OPRM1* rs510769 polymorphisms and an improved PCR option for *OPRM1* rs540825. In the literature a study by Smith and colleagues (13) reporting PCR-RFLP method for rs540825 polymorphism however with currently improved method serves better discrimination of the bands after restriction digestion. Moreover, a total of 70 healthy samples from the Turkish population were tested to evaluate these two developed PCR-RFLP methods and allele and genotype frequencies of these SNPs were determined for the first time.

MATERIAL and METHOD

Study sampling and genomic DNA isolation

Whole blood in EDTA was collected from healthy volunteers (n=70) who were admitted to Blood Donation Center of Ankara University. They were given informed consent by following the principles outlined in the Declaration of Helsinki. All participants were born in Turkey and their parents were native Turkish. Blood samples in EDTA tubes were kept at -20 ^{0}C until genomic DNA isolation was performed using QIAamp DNA blood kit (Qiagen, Germany) according to the manufacturer's recommendations.

Polymerase chain reactions for *OPRM1* rs540825 and rs510769 polymorphisms

The sequence of *OPRM1* gene containing the regions of rs540825 and rs510769 polymorphisms was obtained from NCBI website (http://www.ncbi.nlm.nih.gov). Novel primers were designed using the NCBI primer design tool as below: rs540825 forward 5'-CTTAAAATGCCTAGTCCTCAGCTA-3', rs540825 reverse 5'-GAAATGCTCCACCAGACGGG-3', rs510769 forward 5'-GCCTAGACCAGTTTGCCGTTA-3', rs510769 reverse 5'-AGAGCTCCGCTGAAACCTG-3'. PCR amplification was conducted on a Techne Tc 512 PCR System in a 25 µL reaction mixture which consists of 50 ng of genomic DNA, 4mM of each dNTPs, 10 pmol each of forward and reverse primers, 1.25 mM MgCl2, 1.25 U of Taq DNA polymerase (Amplicon, Denmark) and 10x Ammonium Buffer (Ampliqon, Denmark). PCR conditions were as follows: initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, elongation at 72 °C for 1 min, and a final extension step at 72 °C for 10 min. The expected sizes of the PCR products were 276 bp for rs540825 and 563 bp for rs510769 and these amplification products were confirmed by agarose gel electrophoresis.

Restriction fragment length polymorphism (RFLP) methods to detect *OPRM1* rs540825 and rs510769 genotypes

Restriction fragment length polymorphism (RFLP) was used to genotype *OPRM1* rs540825 and rs510769 polymorphisms. Enzyme (*SspI*) digesting the PCR product for genotyping *OPRM1* rs510769 was determined using online tools such as RestrictionMapper version 3 and NEBcutter V2.0. following accession of the sequence region containing rs510769 SNP from dbSNP database. The enzyme used to detect the PCR product of *OPRM1* rs540825 was *NlaIII* which had been published by Smith and colleagues (13). Conditions and products of restriction reactions were given in Table 1. The digested products were electrophoresed on 3 % agarose gel stained with ethidium bromide (EtBr).

Table 1. Conditions and products of digestion reactions to determine *OPRM1* rs540825 and rs510769 genotypes

SNP	PCR product size (bp)	Restriction enzyme and digestion conditions	Digestion products (bp)	Ingredients of digestion reaction
***OPRM1* rs540825**	276	*NlaIII* 37 °C 1 hour	AA: 276 AT: 276 + 153 + 123 TT: 153 + 123	5.0 µl PCR product 1.0 µl G buffer (10X) 1.0 µl *NlaIII* enzyme 3.0 µl sdH_2O
***OPRM1* rs510769**	573	*SspI* 37 °C 1 hour	CC: 417 + 146 CT: 417 + 364 + 146 + 53 TT: 364 + 146 + 53	5.0 µl PCR product 1.0 µl G buffer (10X) 0.5 µl *SspI* enzyme 3.5 µl sdH_2O

Statistical analysis

Direct counting was used to calculate allele and genotype frequencies of both SNPs and the departure from the Hardy-Weinberg equilibrium (HWE) was evaluated by chi-square test. 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.

The study was approved by the Ankara Universtiy Clinical Research Ethics Committee (Date: 08.04.2019 and Number: 07-536-19).

RESULTS

PCR-RFLP assays for genotyping *OPRM1* rs540825 and rs510769 polymorphisms were developed and summarised in Figure 1. New primers were designed and 276 bp and 563 bp regions of *OPRM1* gene to detect rs540825 and rs510769 polymorphisms were amplified successfully (Figure 2). *OPRM1* rs540825 polymorphism was determined by digesting with *NlaIII* enzyme as described by Smith and colleagues (13). SspI restriction enzyme was used to detect *OPRM1* rs510769 polymorphism. This enzyme has two recognition sites in the presence of polymorphic allele. Restriction products of wild type, heterozygous and variant genotypes were given in Figure 3.

Figure 1. The design of PCR and RFLP for the detection of *OPRM1* rs540825 (A) and rs510769 (B) polymorphisms. Forward and reverse primers were highlighted in green and pink, respectively on the sequence. Polymorphic nucleotide was highlighted in red and recognition regions of restriction enzymes were indicated as underlined text

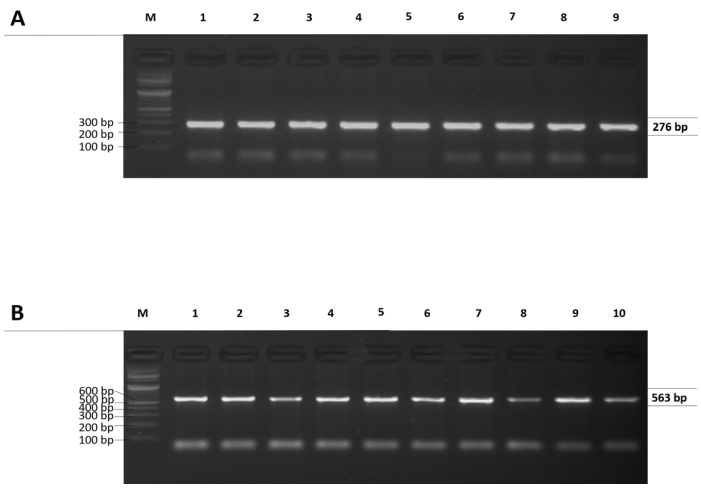

Figure 2. PCR amplicons containing *OPRM1* rs540825 (A) and rs510769 (B) polymorphisms run on agarose gel electrophoresis. Lane M represents a 100 bp marker

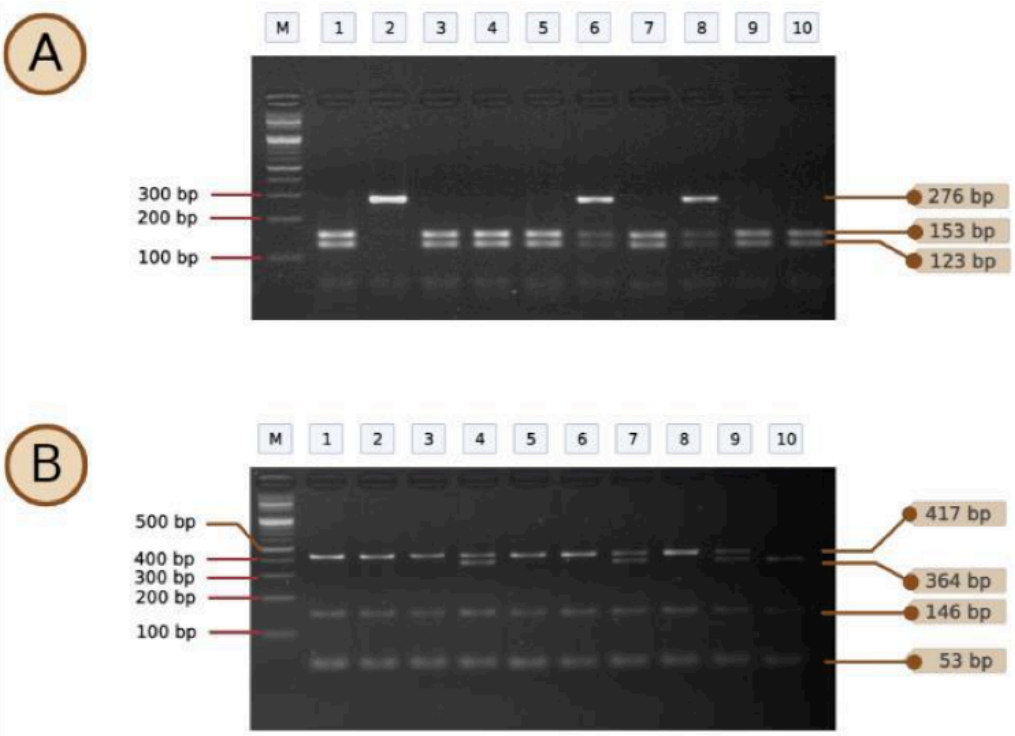

Figure 3. Restriction products of *OPRM1* rs540825 (A) and rs510769 (B) polymorphisms run on agarose gel electrophoresis. In the image (A), Lane M represents 100 bp ladder; Lanes 1, 3, 4, 5, 7, 9 and 10 represent homozygous polymorphic genotype (TT) of *OPRM1* rs540825; Lanes 6 and 8 represent heterozygous genotype (AT) and Lane 2 represents homozygous wild-type genotype (AA). In the image (B), Lane M represents a 100 bp ladder; Lanes 1, 2, 3, 5, 6 and 8 represent homozygous wild-type genotype (CC) of *OPRM1* rs510769; Lanes 4, 7 and 9 represent heterozygous genotype (CT) and Lane 10 represents homozygous polymorphic genotype (TT)

In the whole sample, while the genotype frequencies of the *OPRM1* rs540825 (x^2= 0.33; *p*= 0.56) were within the expected HWE, those of rs510769 (x^2= 5.34; p= 0.02) were in Hardy-Weinberg disequilibrium. Allelic and genotypic frequencies of rs540825 in the Turkish population were calculated as 29% for allele A and 71% for allele T, 10 % for AA, 38.6% for AT, and 51.4% for TT as summarised in Table 2. For rs510769 polymorphism, 60 % of the individuals had CC genotype, 24.3% and 11.4% of them had CT and TT, respectively. Allele frequencies of rs510769 polymorphism were 75% for allele C and 25% for allele T (Table 2).

DISCUSSION

Opioid system, serves a central role in analgesia and nociception, regulates mood, well-being and also addictive behaviours. This system consists of kappa, delta and mu opioid receptors and among them, mu opioid receptor has a key role in dopamine release and reward and is encoded by *OPRM1* gene (23). Polymorphisms in *OPRM1* gene have shown to affect the function and expression of mu opioid receptor resulting in alterations in the reward pathway and therefore may contribute to addiction and have pharmacological importance (2,3,10,24).

Table 2. Genotype and allele frequencies of *OPRM1* rs540825 and rs510769 polymorphisms

SNP	Genotype	Expected	Observed	Allele	Chi-square (X^2)	P-value
OPRK1 **rs540825 A>T**	AA	6.0	7	A: 0.29 T: 0.71	0.33	0.56
	AT	29.0	27			
	TT	35.0	36			
OPRK1 **rs510769 C>T**	CC	39.4	43	T: 0.75 C: 0.25	5.34	0.02
	CT	26.3	19			
	TT	4.4	8			

OPRM1 variations rs540825 and rs510769 have been investigated in studies related to depression, pain and addictive diseases (3,16,17,18,28). Those results were contradictory amongst populations with different ethnic backgrounds. Therefore, it is important to evaluate their frequencies in various populations to reveal their roles in diseases. PCR-RFLP technique is a cheap and reliable fundamental method serving a useful option to screen large populations as cost is a limiting factor (22).

In the literature, genotyping of *OPRM1* rs540825 polymorphism was done by means of DNA sequencing, Taqman assay, Sequenom MassARRAYsystem and also PCR-RFLP (8,13,16,17). PCR-RFLP method has already been developed for *OPRM1* rs540825 however here we developed an improved version by designing new primers to amplify smaller portions of the gene including one restriction site of the enzyme in order to discriminate RFLP products easily and so genotypes better. Among previous studies only one employed PCR-RFLP technique for genotyping and allele frequencies were found as 0.25 for allele A and 0.75 for allele T in a European American population. In that study, the primers used to amplify *OPRM1* gene produces an amplicon in 362 bp size and this amplicon had two restriction sites of *NlaIII* enzyme. So the sizes of the restriction products were close to each other such as 139 bp and 128 bp which made them uneasy to distinguish on an agarose gel (13). In order to overcome this hurdle, we designed new primers to amplify the gene portion containing only one restriction site of the *NlaIII* enzyme. So with this improved PCR-RFLP method an easier genotyping by using agarose gel electrophoresis can be done and used in any laboratory. In a study searching the role of genetic variants on naltrexone treatment response, minor allele frequency of *OPRM1* rs540825 polymorphism was 0.24 in Caucasians (15). Shabalina and colleagues determined the frequency of allele T as 0.24 in European Americans (29). In a recent study investigating the impacts of *OPRM1* SNPs in pain used Taqman genotyping assay and determined the allele frequencies in African population as 0.22 and 0.78 for A and T alleles, respectively (17). In our study, the frequency of allele A was found as 0.29 and allele T was found as 0.71. These values were in accordance with the previous studies held on populations with discrete backgrounds.

An intronic common polymorphism rs510769 was found to be associated with opioid dependence

in Europeans (20). Moreover, it was reported to be involved in cannabis use (9). *OPRM1* rs510769 polymorphism has been detected by means of various methods including Matrix-assisted laser desorption/ ionisation mass spectrometry (MALDI-MS), TaqMan genotyping, gene chip and array technologies however no study has utilised PCR-RFLP method (19,21,25,26,27). So, a method which may be preferred in the laboratories with low-budget has been developed for the first time. In a study assessing the influence of *OPRM1* polymorphisms on alcohol and tobacco use in Spanish population, the genotype frequencies of *OPRM1* rs510769 have been reported as 60.9% for CC, 33.8% for CT and 5.3% for TT in control subjects (27). Kojevod and colleagues reported the allele frequencies of rs510769 as 72% for C and 28% for T allele in Caucasians (18). Our results reflecting the Turkish population are in accordance with the previous studies and global allele frequencies (obtained from dbSNP database) for rs510769 polymorphism.

The methods presented here provide clear detection of *OPRM1* rs540825 and rs510769 polymorphisms and contribute to the literature by serving cheap and easy to apply options. It is crucial to reveal differences between populations as *OPRM1* variations have potential to affect various responses in pain, stress and reward systems and might be useful for applications in hospitals as tailored therapies may only be accomplished by genotyping prior to treatment. Furthermore, if the allele frequency of a SNP is similar amongst populations and this polymorphism affects the metabolism of the drug, then this impact might be added to prospectus of the medicine. In summary, an improved rapid PCR for rs540825 and a novel PCR-RFLP method for rs510769 were developed for genotyping which can be routinely preferred in research laboratories.

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ETHICS COMMITTEE APPROVAL

* The study was approved by the Ankara Universtiy Clinical Research Ethics Committee (Date: 08.04.2019 and Number: 07-536-19).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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