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Research Article

A Novel Genotyping Method for Detection of Muscarinic Receptor M1 Gene rs2067477 Polymorphism and Its Genotype/Allele Frequencies in a Turkish Population

Muskarinik Reseptör M1 Geni rs2067477 Polimorfizminin Tespiti Icin Yeni Bir Genotipleme Metodu ve Türk Şizofreni Hastalarındaki Genotip/Allel Frekansları

Short Title: Novel Genotyping Assay for Muscarinic Receptor M1

Kısa Başlık: Muskarinik Reseptör M1 için Yeni Genotipleme Yöntemi

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ABSTRACT
Objective: Gene variation in the cholinergic muscarinic receptor 1 (CHRM1) has a potential to become one of candidate biomarker in the development of several disorders and also drug response. In this study, a novel PCR- RFLP assay was developed to determine the C to A single nucleotide polymorphism at position 267 in the *CHRM1* gene.
Materials & Methods: A new reverse primer and a mismatched forward primer were designed to obtain 125 bp PCR products. PCR products were then digested with the *Hae III* restriction enzyme to detect the rs2067477 polymorphism that comprises of C to A base change. The developed novel assay was tested in 51 Turkish schizophrenia patients.
Results: Genotyping assay was successfully performed in patients with schizophrenia in order to confirm the accuracy and validity of this method. The frequency of CC, CA and AA genotypes were found as 72.5%, 25.5% and 2%, respectively. On the basis of this data, the allele frequency of C was 0.85 and the allele frequency for A was 0.15.
Conclusion: The suggested genotyping assay is practical for screening the *CHRM1* C267A polymorphism in pharmacogenetic studies. The present polymorphism may use as a candidate biomarker to find out genetic susceptibility to related diseases' and may contribute to the implementation of individualized drug therapy for M1 related diseases.
Keywords: CHRM1, C267A, Turkish, Schizophrenia, PCR-RFLP

ÖZ
Amaç: Kolinerjik muskarinik reseptör 1'deki (*CHRM1*) gen varyasyonu, çeşitli bozuklukların ve ayrıca ilaç yanıtının gelişiminde aday biyogöstergelerden biri olma potansiyeline sahiptir. Bu çalışmada, *CHRM1* geninde 267. pozisyondaki C'den A'ya olan tek nükleotid polimorfizmini belirlemek için yeni bir PCR-RFLP analizi geliştirilmiştir.
Gereç ve Yöntemler: 125 bp PCR ürünlerini elde etmek için yeni bir reverse primer ve uyumsuz bir forward primer tasarlanmıştır. PCR ürünleri daha sonra C'den A'ya olan baz değişikliğini içeren rs2067477 polimorfizmini tespit etmek için *Hae III* restriksiyon enzimi ile kesilmiştir. Geliştirilen yeni analiz, 51 Türk Şizofreni hastasında test edilmiştir.

Bulgular: Bu yöntemin doğruluğunu ve geçerliliğini onaylamak için genotipleme analizi, şizofreni hastalarında başarıyla uygulanmıştır. CC, CA ve AA genotiplerinin sıklığı sırasıyla% 72.5,% 25.5 ve% 2 olarak bulundu. Bu verilere dayanarak, C alel frekansı 0.85 ve A alel için frekans 0.15 idi.
Sonuç: Önerilen genotipleme deneyi, farmakogenetik çalışmalarda *CHRM1* C267A polimorfizminin belirlenmesi için pratiktir. Bu polimorfizm, ilgili hastalıklara karşı genetik duyarlılığı göstermek için aday bir biyogösterge olarak kullanılabilir ve M1 ile ilgili hastalıklar için bireyselleştirilmiş ilaç tedavisinin uygulanmasına katkıda bulunabilir.
Anahtar Kelimeler: CHRM1, C267A, Türk, Şizofreni, PCR-RFLP

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INTRODUCTION

The prenatal and perinatal risks, negative early life events and genetic predisposition may cause neurodevelopmental alterations and sensitize the dopamine system in the brain; the presence of these factors may contribute to development of schizophrenia.[1,2] The prevalence of schizophrenia varies from 3 to 7 per 1000 in worldwide and the average lifetime prevalence is 4/1000 while the lifetime risk is 7.2 per 1000.[3,4] However, the studies about the prevalence of schizophrenia have shown that the disorder differs in all societies and that could vary according to the characteristics of the society.[5,6] A systematic review based on a limited number of general population survey conducted in Turkey showed that the prevalence of schizophrenia, was 8.9 in 1000.[7]

The risk of schizophrenia is 10% for first degree relative while 40% for child if both parents have schizophrenia.[8] In addition to heredity in the development of this disease, the gene differences involved in the pharmacokinetics and pharmacodynamics of the drugs which are used in the treatment of schizophrenia also play a major role in treatment, response and adverse drug reactions.

Antipsychotic drugs used in the treatment of schizophrenia such as clozapine (CLZ) and olanzapine have been found to be antagonistic to muscarinic receptors.[9] Clozapine is prescribed especially in treatment resistant schizophrenia patients and it is a weak muscarinic receptor 1 (M1) agonist while its active metabolite, N-desmethylclozapine (NCLZ), is a potent M1 agonist receptor.[9] In addition, M1 receptor agonist DCLZ plays an important role in determining the clinical effects and pharmacotherapy in the treatment of psychotic disorders. Studies have also pointed out that a decreased density of M1 receptors particularly in the neocortical regions was associated with schizophrenia.[10] Similarly, some studies showed that reduced M1 receptor mRNA level in brain samples of schizophrenia patients.[11] Considering all of these, M1 receptor is one of the important targets in the development and also treatment of schizophrenia.

There are five types of cholinergic muscarinic receptors designated as M1 to M5. Among these, the Muscarinic receptor 1 (M1) is mostly located in the nervous system. M1 is typically found in the parasympathetic ganglia, cortical and hippocampal regions of brain, less in airway epithelial cells and is involved in cognitive functions such as learning and memory, as well as regulation of cardiac contraction.[12,13] The M1 is encoded by the *CHRM1* gene located on chromosome 11q12.3. There are fifteen single nucleotide polymorphisms (SNPs) in *CHRM1* gene region, one of them is the C267A (rs2067477) base change. This polymorphism is a silent mutation which is a transversion of cytosine (C) to adenine (A) at position 267 on the *CHRM1* gene region. It is in wobble site of the codon (GG**C**→GG**A**) so the protein sequence is preserved.[13,14]

In short, the determination of the SNPs in the gene regions which are potentially involved in schizophrenia, are important because they could affect the disease susceptibility, cognitive performance, drug response or adverse drug reactions. The Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) assay is one of the most common, simple, effective, fast and inexpensive method used to determine the SNPs. Thus, our aim was to develop a novel PCR- RFLP method for genotyping *CHRM1* C267A polymorphism. Subsequently, the developed PCR-RFLP assay was performed for validation of the method and determination of genotype and allele frequencies in Turkish patients with schizophrenia.

MATERIALS & METHODS

Study Subjects and DNA Isolation

Whole blood samples were obtained from 51 Turkish schizophrenia consecutive outpatients who admitted to Ankara University Medical Faculty Psychiatry Department and diagnosed with Diagnostic and Statistical Manual of Mental Disorders, fourth edition[15] between October

2016- April 2018. Inclusion criteria were: age between 18- 65 and having signed the written informed consent. Patients with additional psychiatric diagnosis as well as general medical comorbidity were excluded. An informed consent was obtained from all subjects and the protocol was approved by the Research Ethics Committee of Medical Faculty, Ankara University. Genomic DNA was extracted with high salt method from peripheral blood of 51 subjects.[16] The absorbance level of DNA samples for 260 and 280 nm was detected with spectrophotometric analysis and the purity of the samples was between 1.7- 2.0.

PCR Primers and Conditions

The sequence data of C267A (rs2067477) polymorphism in human *CHRM1* gene region was obtained from NCBI website (http://www.ncbi.nlm.nih.gov) and the new primers were designed as follows based on the published sequence: forward primer: 5'-TACTTCCTGCTGAGCCTAGCC-3', reverse pimer: 5'-GCCAGCCAGAGGTCACAAGCC-3'. The PCR reaction was carried out in a volume of 25 µl, which was containing 10x PCR buffer (Amplicon, Denmark; containing 10 X Ammonium and 15mM Mg), 1.1 mM $MgCl_2$, 0.1 mM dNTP, 10 pmol from each primers, 1.5 µl DMSO, 0.45 U of *Taq* DNA polymerase (Amplicon, Denmark), approximately 100 ng of genomic DNA and distilled water to complete final volume to 25 µl. One hundred twenty five (125) bp PCR product was obtained by using the following PCR cycling conditions; the initial denaturation at 94 °C for 3 min, followed by 3 graded 30 cycles were performed which are denaturation at 94 °C for 30 sec, annealing for 30 sec at 59 °C and elongation at 72 °C for 45 sec. In the end, final extension for 5 min at 72 °C was carried out. The PCR products (125 bp) were visualized under ultraviolet illuminator on a 1% agarose gel which was stained with ethidium bromide.

RFLP Conditions

The RFLP was carried out in a 20 µl volume mixture consisting of 2 µl 10x Buffer, 10 U *Hae III* enzyme (New England Biolabs, USA), 10 µl PCR product and 7 µl distilled H_2O. The reactions were incubated at 37 ºC overnight and the digested products were visualized under an UV transilluminator after they were electrophoresed on 3% agarose gel containing ethidium bromide for 1 hour. The digested RFLP products were obtained for wild-type genotype while there were undigested RFLP products for mutant genotype on the agarose gel. To further assess the reliability of the presented assay, PCR product of each different genotype was verified by direct sequencing using the same set of primers.

Statistical Analysis

Allele and genotype frequencies were calculated by genotype counting method. The observed genotype frequencies of *CHRM1* C267A were compared with the expected frequencies according to Hardy–Weinberg equilibrium. Obtained data were compared with previously reported representative data in other ethnic groups. Differences in allele frequencies between schizophrenic groups were tested by Pearson's Chi-square Test and while a p value < 0.05 was considered statistically significant.

RESULTS

A novel PCR-RFLP assay was designed to detect C267A SNP in *CHRM1* gene region in schizophrenic patients with schizophrenia. We also evaluated the accuracy and validity of this novel method. New primers were designed and the PCR products were digested with *Hae III* restriction enzyme for determination of the variant genotypes. Schematic illustration of the assay is given in Figure 1.

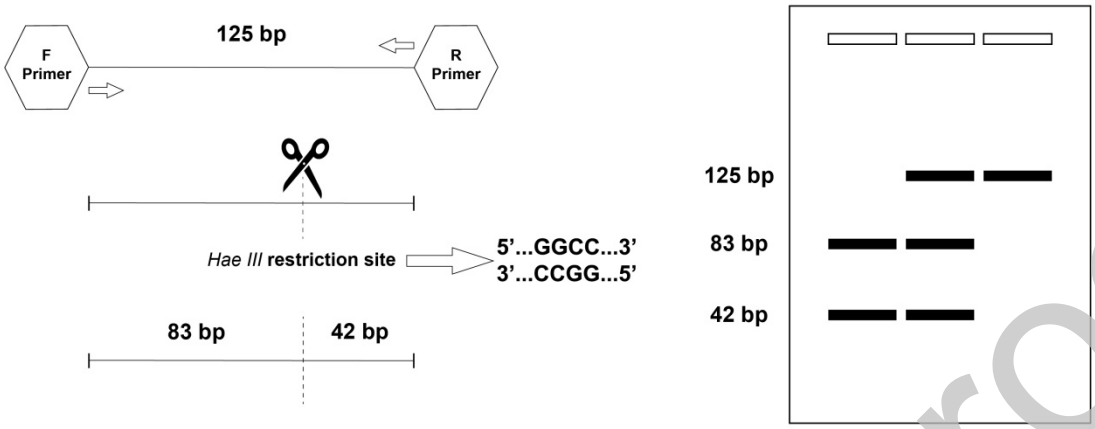

Figure 1. Diagrammatic representations of recognition sites of *Hae III* enzyme and the schematic illustration of the restriction fragments for each genotype of *CHRM1* C267A SNP. The previous genotyping method for rs2067477 by Liao et al., could not perfectly apply to analyze this SNP due to the difficulties in the finding primer sites. This method did also not include any information about PCR product fragments, PCR conditions and base pairs of the restriction fragments for genotyping.[17] In the present study, a novel genotyping assay was developed and successfully performed by utilizing a reverse primer and mismatch forward primer, which are explained above. As shown in Figure 2, the underlined A (adenine base) is the mismatched base in the forward primer which was replaced with the ancestral base G (Guanine base) to eliminate the recognition site of *Hae III* restriction enzyme (GG▼CC) in the primer binding site. This was also confirmed by sequencing (data not shown).

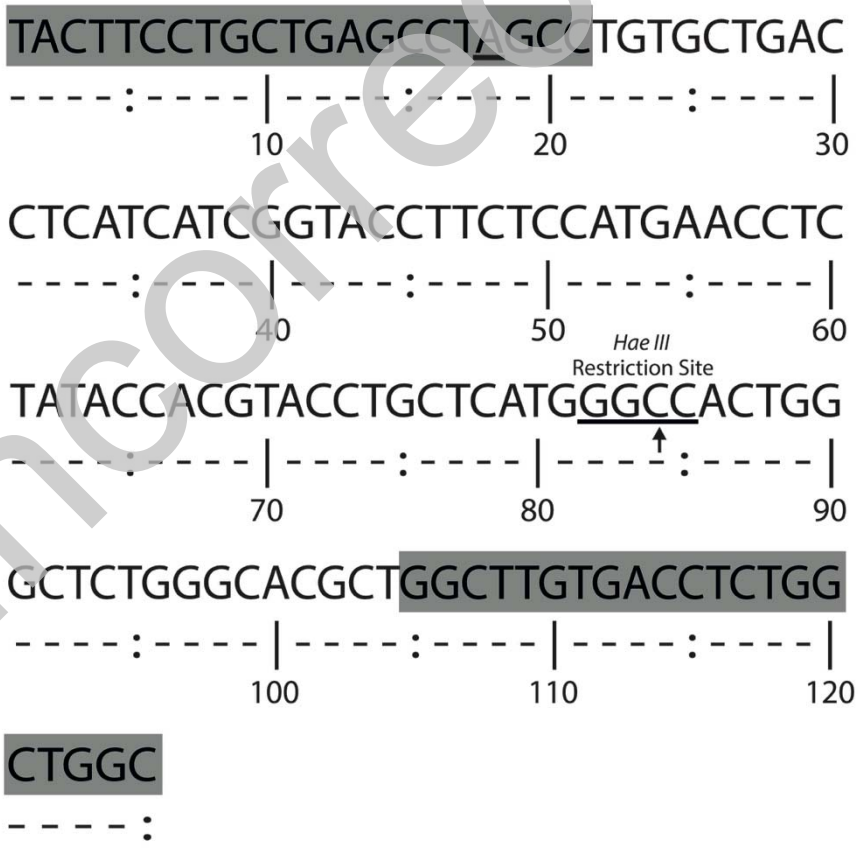

Figure 2. Restriction analysis of *CHRM1* with *Hae III* endonuclease. Forward and reverse primers are highlighted as grey. The mismatch base (A), which is used to eliminate the recognition site of *Hae III* in forward primer, is underlined. *Hae III* recognition site is depicted by underline in the middle of the *CHRM1* sequence. This recognition site also includes rs2067477 SNP, which is depicted with capital and bold letter in recognition site (C). In case of ancestral C allele at position 267 of *CHRM1* gene 83 bp and 42 bp DNA fragments obtained, after *Hae III* digestion. Conversely, no digestion site for *Hae III* endonuclease is found, when C allele is replaced by A allele at position 267 which giving one fragment of 125 bp.

The individuals with CC genotype (wild-type) yielded two bands of 83 bp and 42 bp, while the individuals with AA genotype (mutant type) gave undigested band (125 bp) on the 4% agarose gel. Agarose gel electrophoresis results of the RFLP products on 3% agarose gel are given in Figure 3.

Figure 3. Agarose gel electrophoresis demonstrated the expected RFLP product sizes. The results shown in 1,2,3 were in the same order as in Figure 1. (M: Thermo Fisher Scientific GeneRuler Ultra Low Range DNA Ladder Marker (10 -300 bp, SM1211). 1: CC genotype, 2: CA genotype and 3: AA genotype).

One sample of each different genotype PCR products were sequenced for confirm the expected sequence of each genotype and the obtained data was consistent with our findings. The sequencing results of three genotypes are given below in Figure 4. PCR products of each different genotype sequencing result precisely demonstrated that confirmation the reliability of our novel assay.

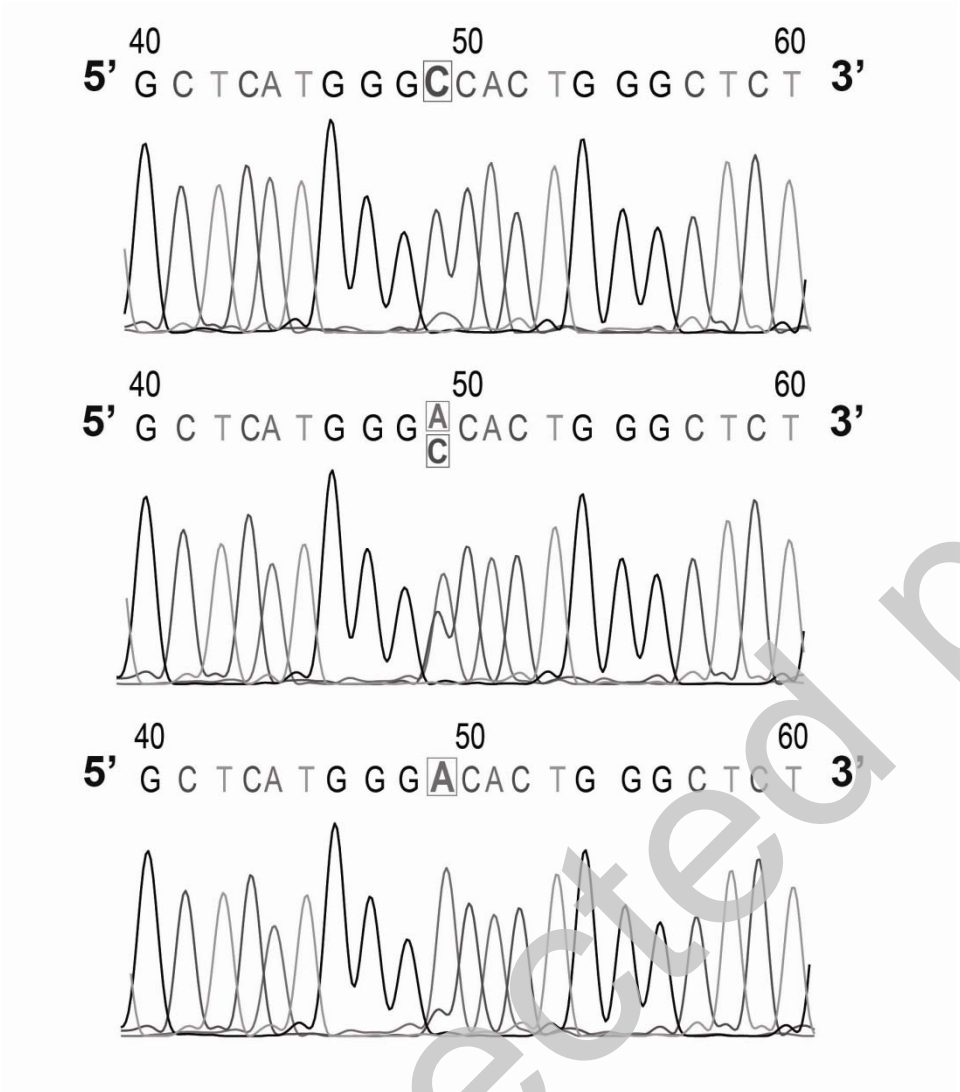

Figure 4. Examples of DNA sequencing of the polymerase chain reaction product of the *CHRM1* gene. From top the bottom three figures represent the genotype of CC, CA and AA respectively and the sequenced result of the heterozygote genotype have C and A alleles in the same position.

The allele and genotype frequencies in 51 Caucasian Turkish schizophrenic patients are shown in Table 1 for C267A polymorphism in *CHRM1* gene. This is the first study to document the frequencies and genotypes of *CHRM1* C267A alleles in Turkish patients with schizophrenia. Molecular analyses revealed that, among the 51 patients tested for the C267A genotype, 37 (72.5%) were CC, 13 (25.5%) were CA and 1 (2%) were AA. On the basis of this data, the allele frequency of C was 0.85 and the frequency for A was 0.15. The distribution of *CHRM1* genotypes in our samples is presented in Table 1. The *p* value of present results was $p>0.05$ and it was in good accordance with expected genotype distributions, calculated using the Hardy–Weinberg equilibrium. (χ^2*: 0.013; p= 0.9*).

Table 1. The distribution of the *CHRM1* gene polymorphism in Turkish patients with schizophrenia.

Gene	Genotype	Observed Frequency	Expected Frequency	Allele Frequencies
CHRM1	CC	37	37.1	C:0.85 A:0.15
	CA	13	12.8	
	AA	1	1.1	

Total		51	51	1.00

DISCUSSION

Due to several gene variations that are potentially involved in physiopathology of mental disorders, *CHRM1* C267A polymorphism has a probability to become a genetic biomarker. On the other hand this variation might have a role in psychopharmacotherapy since muscarinic M1 receptor is a prominent target for a considerable number of medications. Three primary objectives were aimed in this study. The main purpose of this study was to develop a novel genotyping assay for *CHRM1* C267 polymorphism and to test the accuracy and validity of the developed method. The other two aims were to draw attention to the importance of *CHRM1* gene in the pathology of schizophrenia and to determine genotype and allele frequencies of *CHRM1* C267 polymorphism in Turkish patients with schizophrenia.

M1 receptors could be important for neuronal disorder and cognitive function in the pathophysiology of schizophrenia due to the location in the medial prefrontal cortex and hippocampus.[18,19] Lower levels of muscarinic receptors in the CNS of people with schizophrenia have been found in some studies.[18,20] Scarr et al., showed that decreased M1 levels in cortical region of brain could contribute to the pathophysiology of schizophrenia. Thus, a brain imaging test before treatment could be useful in identifying the patients with low M1 levels who could be treatment resistant.[21] Another neuroimaging study also showed that muscarinic receptors were extensively decreased in schizophrenia patients under treatment during neuroimaging.[20]

At the molecular level, Mancama et al., demonstrated that the levels of *CHRM1* cDNA was 28% lower than control group in schizophrenia patients.[22] Moreover, the studies suggested that there could be a relationship between rs2067477 SNP and reduction in grey matter volume in patients with schizophrenia.[23] The studies have shown that rs2067477 might be associated with in cognitive performance. In these studies the Wisconsin Card Sorting Test performance which is a measure of prefrontal and executive functions was better in heterozygous individuals than the homozygous wild-type carriers.[17,24] In one of these studies, 243 schizophrenic patients were assessed according to the rs2067477 genotype and the genotypes have differed in responses in the Wisconsin Card Sorting Test but not in other parameters including age of onset, chlorpromazine equivalents, and Brief Psychiatric Rating Scale.[17] Contrary to these, Cropley et al., indicated that homozygous CC genotype did not have impact on attention, visuospatial-construction, verbal fluency or working memory and they did not assess the patients with Wisconsin Card Sorting Test.[23] All of these studies showed the importance of the determination of *CHRM1* C267A alleles in schizophrenic patients. To the best of our knowledge, this is the first study to document the frequencies of *CHRM1* C267A alleles and its genotype distribution in Turkish schizophrenia patients.

In this study, genotype distribution and allele frequencies of *CHRM1* C267A polymorphism were obtained from 51 Turkish schizophrenia patients. Obtained data were compared with previously reported representative data in other schizophrenia patients as shown in Table 2. Present results showed that the C and A allele frequencies in Turkish patients with schizophrenia was 0.85 and 0.15, respectively. The C267A variant frequency ranges between 0.07- 0.11 in Australian patients with schizophrenia or schizoaffective disorder, while it was found as 0.09 in Chinese schizophrenia patients.[17,23-26] The difference in frequency of C267A SNP between Turkish schizophrenia patients and other populations patients was not statistically significant ($p>0.05$).

Table 2. Genotypes and allele frequencies of C267A SNP in *CHRM1* in this study and the other populations.

Study population	**n**	**Genotype Frequency n (%)**	**Allele frequency**	**Reference**

		CC	CA	AA	C	A	
Turkish patients with schizophrenia	51	37(72.5)	13(25.5)	1(2)	0.85	0.15	Present study
Chinese patients with schizophrenia	243	202(83.1)	40(16.5)	1(0.4)	0.91	0.09	Liao,2003
Australian patients with schizophrenia and schizoaffective disorder	97	83 (86)	14(14)	-	0.93	0.07	Scarr et al.,2012
Australian patients with schizophrenia or schizoaffective disorder	267	191(84.1)	35(15.4)	1(0.4)	0.92	0.08	(Cropley et al., 2015
Australian patients with schizophrenia and schizoaffective disorder	176	147(83.5)	29(16.5)	-	0.92	0.08	Carruthers, 2018
Australian patients with schizophrenia and schizoaffective disorder	147	114(77.6)	33(22.4)	-	0.89	0.11	Carruthers, 2019

In summary, a novel, practical, low-cost and reproducible PCR- RFLP method was developed for genotyping the *CHRM1* C267A polymorphism. The developed method is based on the elimination of recognition site of *Hae III* in the forward primer binding site by utilizing a mismatch base in the forward primer. As a result of this study, the validity and accuracy of the present novel method has been proven. Thus, the genotype and allele frequency of *CHRM1* C267 polymorphism in Turkish patients with schizophrenia has been determined for the first time. The number of samples should be increased in further studies for more certain and reliable results. Additionally, the effect of *CHRM1* gene in the pathology and treatment of schizophrenia is explained with the data in the literature. The developed genotyping assay and results could be useful and provide a perspective for future studies.

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Ethical conduct of research: All authors state that the appropriate institutional review board approval had obtained and the informed consent has been obtained from the participants involved study. The authors state that all experiments had followed the principles outlined in the Declaration of Helsinki.

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