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Frequency of *ANKK1*, *DRD2*, *DRD3* gene polymorphisms in refractory schizophrenia patients

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Genet. Mol. Res. 18 (4): gmr18389
Received May 29, 2019
Accepted July 24, 2019
Published November 30, 2019
DOI http://dx.doi.org/10.4238/gmr18389

ABSTRACT. Schizophrenia is considered one of the most severe and complex mental disorders; it affects both the quality of life of the patient and his family. The dopamine hypothesis is the main concept concerning antipsychotic activity. Patients with treatment-refractory schizophrenia have a lower capacity for dopamine synthesis than those with a good response to first-generation antipsychotics. The polymorphisms *rs1800497*, *rs1799732* and *rs6280*were chosen for evaluation because they are associated with decreased dopamine receptor expression and occur in genes encoding these receptors, namely, *ANKK1*, *DRD2* and *DRD3*, respectively. This effect caused by these polymorphisms enhances refractoriness to treatment. We investigated the frequency of these polymorphisms and evaluated their association with refractory schizophrenia. This was a case-control molecular genetic study, with patients who were divided into three groups of 72 participants each: patients with refractory schizophrenia, with schizophrenia and controls with no diagnosis of any type of mental disorder. All participants of the research were from the extended Midwest region of Minas Gerais. Polymorphisms were evaluated by PCR followed by RFLP. The allele and genotype

frequencies were determined, the association tests performed using Pearson's Chi Square, and Odds Ratio values were estimated. Genotypic models of dominance and heterosis were constructed. An association of the Del C allele of *rs1799732* polymorphism and schizophrenia (P = 0.03) was found. Further research on this subject is merited, since response to treatment is of utmost importance to the patient's quality of life.

Key words: Nursing; Schizophrenia; Genetic polymorphism; Frequency; Receptors; Dopamine; Case-control studies

INTRODUCTION

Schizophrenia (SCZ) is pathology that affects more than 21 million people worldwide (Patel et al., 2014). It is considered one of the most severe and complex mental disorders that affects both the quality of life of the individual living with the disease and that of the family (Freitas et al., 2016). It is mainly characterized by symptoms denominated positive (alteration in the thought process, perceptions and affection) and negative (affective-volitional blunting, cognitive losses and depressive symptoms) (Bongaarts, 2016). The risk factors for this disorder are epiphenomena of pathophysiological processes that result from a gene-environment interaction that is still poorly understood (Edwards et al., 2016).

Although typical (or first generation) antipsychotics are effective in the treatment of SCZ, they do not alleviate all symptoms and generally cause serious adverse effects (parkinsonism, muscle stiffness, dystonia, tardive dyskinesia), reducing drug efficacy due to poor adherence to the treatments (Lawford et al., 2013). The dopamine hypothesis is the main concept used to explain antipsychotic activity in SCZ, with positive symptoms related to elevated dopamine levels in the mesolimbic pathway, while negative and cognitive symptoms are associated with decreased levels of this neurotransmitter in the mesocortical pathway (Kunii et al., 2014).

About 40% of patients remain resistant (refractory) to treatment, even with regular use of antipsychotics. Thus, they have a lower capacity for dopamine synthesis than those with a good response to first generation antipsychotics (Demjaha et al., 2012); an adequate response to drug treatment coincided with a higher density of dopaminergic synapses, showing a biological basis for refractoriness (Utsunomiya et al., 2012).

The blocking action of dopamine D2 receptors (*DRD2*) by antipsychotics has been highlighted as a central element in response to treatment. However, the occurrence of refractoriness, even with complete blockade of these neuroreceptors, as well as the efficacy of clozapine, the standard medication for these cases, also demonstrates the involvement of other factors (Nkam et al., 2017).

To try to understand the refractoriness of several drugs a pharmacogenetic approach has been used. Pharmacogenetics place genetic variation as a possible reason for the absence or poor response to adequate medication (Cui et al., 2015). Large-scale genomic association studies of SCZ identified 108 genetic loci associated with this disorder. Among these 108 loci, 75% are genes encoding proteins. Notable associations relevant to the etiology and treatment of SCZ include dopamine receptor D2 (the target for all effective

antipsychotics) dopamine receptor D3 (*DRD3*) and ankyrin repeat and kinase domain containing 1 (*ANKK*) genes (Hwang et al., 2010; Bhathena et al., 2013; Lochman et al., 2013; Purcell et al., 2014).

One of the most studied genetic variant associated with SCZ is the SNP *rs1800497 (TaqIA)*, which leads to the exchange of cytosine for thymine (C / T). This exchange of bases causes a replacement of the amino acid glutamine by lysine at position 713 *(Glu713Lys)* of exon 8 in the *ANKK1* gene, on chromosome 11; this would lead to over expression of *DRD2* (Ghosh et al., 2013).

The *DRD2* gene consists of eight exons and is located at position 11q23.2 on chromosome 11, adjacent to the *ANKK1* gene. It has approximately 270 kb, and about 250 kb of an intron separates the first from the second exon. Protein coding regions are absent in exon 1 but present in exons 2 to 8 (Grandy et al., 1989; Gandelman et al., 1991; Eubanks et al., 1992).

The polymorphism *rs1799732 (-141C Ins / Del)*, present in the *DRD2* gene seems to confer effects on the etiology and pathophysiology of this disorder according to studies of genomic association, but the results have been inconsistent (Saiz et al., 2010; Wang et al., 2016). The *-141 C Ins / Del* polymorphism is characterized by the presence (Insertion: Ins) or absence (Deletion: Del) of the cytosine base at position -141, located in the 5' promoter region of *DRD2*. This has resulted in the reduction of D2 neuroreceptors in the brain; however, the effect of this polymorphism in SCZ remains obscure (Saiz et al., 2010).

The *DRD3* gene is located at position 3q13.31 on chromosome 3. It is composed of seven exons and five introns between its coding regions and it differs from *DRD2* by encoding a receptor protein that acts on the post-synaptic signaling and as an autoreceptor (Sokoloff et al., 1990). The *DRD3* gene has a polymorphic site in the first exon that leads to an exchange of the cytosine (C) by thymine (T) in the extracellular N-terminal domain of the receptor which results in a substitution of amino acids serine for glycine in the residue 9 (*Ser 9 Gly*) (Le Coniat et al., 1991). This variant may influence the membrane expression of this receptor by modifying its intracellular maturation (Reynolds et al., 2005); this may be a reason why it has already been associated with the change in dopamine binding affinity.

Ankyrin repeat and kinase domain containing 1 gene (*ANKK1*) is located at position 11q23.2 on chromosome 11. It is a gene comprising 13 kb, has 12 exons encoding a 765 amino acid protein, which has 12 replication domains of ankyrin, belongs to the serine / threonine-specific protein kinase family and to the protein kinase superfamily involved in signal transduction pathways (Ponce et al., 2009; Suchanecka et al., 2011). Although the function of *ANKK1* is unknown, recently it has been shown that the *ANKK1* protein is activated by apomorphine, a dopaminergic agonist, indicating a potential link between *ANKK1* and the dopaminergic system. This relationship is also suggested by in silico analyzes of the *ANKK1* and *DRD2* genes (Hoenicka et al., 2010).

The genetic evaluation of patients with refractory SCZ can become a fundamental part of care, using genetic counseling, which could help guide drug treatment and provide a better quality of life for those individuals, especially the most serious cases of schizophrenia (Pinto et al., 2018). SNPs could be used as potential pharmacogenetic markers for the response to antipsychotic treatment and predict the occurrence of refractoriness in SCZ, considering that pharmacological treatment should be specifically tailored to each patient with SCZ. We investigate possible associations between polymorphisms *rs1800497*,

rs1799732 and *rs6280*, of the *ANKK1*, *DRD2* and *DRD3* genes, respectively, and the occurrence of refractory SCZ.

MATERIAL AND METHODS

It is a genetic-molecular study of the case-control type with patients who were divided into three groups: I: individuals with diagnosis of refractory SCZ. II: individuals diagnosed with other schizophrenias. III: controls, individuals without diagnosis of any type of mental disorder. All the participants of the research are part of the Midwest region of Minas Gerais.

In order for the participants to contribute to the research, the following inclusion criteria were adopted: Group I: medical diagnosis of refractory SCZ, 18 years of age or older, recorded in the Regional Health Management (RHM) registry located in Divinópolis - MG. Group II: medical diagnosis of other types of SCZ, being 18 years of age or older. Group III, controls - having no medical diagnosis of mental disorder, having an age equal to or greater than 18 years. The exclusion criteria were: Conditions that interfered with the collection of data, such as being in crisis due to the disease.

The sample size calculation was performed using the program OpenEpi version 3.03a, considering a population of 169 individuals to an expected 50% proportion of the event. The level of significance is of 5% and an error margin of 10%, in sample of about approximately 62 individuals with refractory SCZ. In the end they amounted to 72 users who attended the Centre for Psychosocial Care of Divinópolis, Minas Gerais.

In this type of case-control study, experts in epidemiology suggest that the ratio of each group is at least 1: 1. Thus, since the sample of cases I consisted of 72 individuals, the other two groups also consisted of 72 participants each. Individuals who were part of the eligible population were previously invited to collect data by sending letters and conducting telephone contact when they received all the necessary guidelines for conducting the research. The period of data collection comprised the months of December 2014 to July 2016.

The data of the groups I and II were collected at the Center for Psychosocial Care of the municipality-polo of the Midwestregion. On the other hand, the participants of the "controls" group were recruited at the Municipal Health Support Center of Divinópolis, which is a reference for the Midwest region of Minas Gerais in biological and clinical analyses.

The sociodemographic profile of the subjects of this study was established through a structured questionnaire, elaborated by the authors, which addressed the following data: gender, age, labor status, alcoholism and smoking, and the variables gender and age range were paired. For genetic analysis, blood samples were collected. The analysis was done at the Laboratory of Molecular Biology of the Federal University of São João Del Rei / Midwest Campus Dona Lindu.

The genetic evaluation consisted of five steps: the first one was the extraction of the genomic DNA using the Mini Kit QIAamp DNA Blood (Qiagen) according to the protocol of the manufacturer; the second step was to amplify the DNA target sequences of genes included in the study using the Polymerase Chain Reaction (PCR): the third step meant that each sample of PCR product was subjected to electrophoresis to confirm the amplification of the regions of interest: for the fourth step the genotyping using Restriction Fragment

Length Polymorphism (RFLP) technique was performed and, finally, for the fifth step, the samples digested in the previous step were submitted in an 8% polyacrylamide gel for the separation of the DNA fragments, which were identified by their respective sizes.

Data processing and analysis were performed using the Statistical Package for Social Science (SPSS), version 20.0. To describe the results, frequency distribution tables were used. The Pearson's Chi-square test was used to compare the occurrence of the polymorphisms *rs1800497 (TaqIA)* of the *ANKK1* gene, *rs1799732 (-141C)* of the *DRD2* gene and *rs6280* of the *DRD3* gene among the three groups. In addition, Odds Ratio (OR) values were estimated and in all analyzes a significance level of 5% was considered. These estimates were made considering two different comparisons: the first compared the control group versus patients with other schizophrenias and the second compared the control group versus patients with refractory SCZ. Genotypic models of dominance and heterosis were constructed.

The Hardy-Weinberg equilibrium analysis was performed using the Pearson chi-square test, comparing the frequencies of the genotypes observed with those expected for this population. This calculation was performed using the Hardy-Weinberg equilibrium calculator software including analysis for ascertainment bias (Rodriguez et al. 2009). The study was approved by the Research Ethics Committee of the Federal University of São João Del Rei under Protocol No. 1.406.658, in compliance with the recommendations of Resolution 466/2012 of the National Health Council. All participants signed informed consent forms.

RESULTS

A total of 216 individuals were analyzed, being 72 in each group (patients with refractory SCZ, patients with SCZ and controls). The gender and age variables are shown below in Table 1 as the distribution of each group.

Table 1. Characterization of sociodemographic variables sex and age by group, control group, group of individuals with schizophrenia and group of individuals with refractory schizophrenia.

	Total sample (n=216)	**Control group (n=72)**	**SCZ group (n=72)**	**Refractory Schizophrenia group (n=72)**
Gender				
Female	53.7 %	55.6%	55.6%	50.0%
Male	46.3%	44.4%	44.4%	50.0%
Age Range				
Less than 40 years	29.8%	23.6%	25.0%	40.8%
40 to 50 years	24.7%	15.3%	26.4%	32.4%
50 years or more	45.6%	61.1%	48.6%	26.8%

Most refractory patients reported not to be working at the present time, being this rate 90.3%. As for the patients with SCZ 72.2% of them declared to be currently employed. Regarding the usetobacco, over 60% of individuals in both groups

(refractory SCZ and SCZ) make use of this chemical, while the alcohol consumption rates in both groups were lower, 2.8% for the refractory group, and 13,9% for patients with SCZ.

The frequencies of the polymorphisms investigated, their respective association tests and strength of association are presented in Table 2. It was observed that there was a significant association of the *Del C* allele in the group of individuals with SCZ (P = 0.03) and p value close to significant for the refractory group (P = 0.09).

Table 2. Frequency, Chi Square and Odds Ratio of genotypes and alleles of the Simple Nucleotide Polymorphisms rs1800497 of the ANKK1 gene, rs1799732 of the DRD2 gene, rs6280 of the DRD3 gene by group, control group (n = 72), group of individuals with schizophrenia (n = 72) and group of individuals with refractory schizophrenia (n = 72).

	Refractory Schizophrenia group %	**SCZ group %**	**Control group %**	**P-Value 1**	**P-Value 2**	**OR 1**	**OR 2**
SNP*rs1800497*of gene *ANKK1*							
Genotypes							
CC	51.4	52.8	48.6	0.682	0.291	1.69	2.89
CT	41.7	43.0	40.3			1.65	2.85
TT	6.9	4.2	11.1			1.00	1.00
Alelles							
C	72.0	74.3	68.8	0.605	0.360	1.18	1.31
T	28.0	25.7	31.2			1.00	1.00
rs1799732DRD2							
Genotypes							
Del Del	68.0	69.4	55.6	0.264	0.161	1.00	1.00
Del Ins	25.0	25.0	31.9			0.64	0.63
InsIns	7.0	5.6	12.5			0.45	0.36
Alelles							
Del C	80.6	82.0	71.5	0.097	*0.036	1.00	1.00
Ins C	19.4	18.0	28.5			0.17	0.55
SNP *rs6280* of gene *DRD3*							
Genotypes							
TT	34.7	31.0	23.6	0.315	0.203	1.00	1.00
TC	41.7	51.0	45.8			0.62	0.87
CC	23.6	18.0	30.6			0.52	0.46
Alelles							
T	55.6	56.0	46.5	0.157	0.098	1.00	1.00
C	44.4	44.0	53.5			0.70	0.68

*P-Value < 0,05; P-Value 1: patients with ER vs. controls; P-Value 2: patients with EQZ vs. controls; OR 1: patients with ER vs. controls; OR 2: patients with EQZ vs. controls.

In the same table it is still observed that Odds Ratio values were calculated using the models: 1-Control x patients with refractory SCZ; 2-Control x patients with SCZ. According to these results, no significant differences (confidence intervals are close to rate 1) were observed for the analysis of the *rs1799732* polymorphism genotypes of the *DRD2* gene, both in the comparison between controls and SCZ, and in the comparison between controls and refractory SCZ patients.

In the analysis of the Hardy-Weinberg equilibrium in both groups of cases and controls, the genotype frequencies were in Hardy-Weinberg equilibrium.

Genotypic models of dominance and heterosis were constructed as described in Table 3. Among the analyzes of these genotypic models, no association was found, only a P value (0.056) borderline for the dominant *Ins C* allele in the *-141C* polymorphism

Table 3. Genotypic models of dominance and heterosis for the three simple nucleotide polymorphisms in the group of patients with refractory schizophrenia.

Polymorphisms	Models	Genotypes	P-Value*
rs1800497	C dominant	*C/C e C/T vs. TT*	0.141
	T dominant	*T/T e C/T vs. CC*	0.630
	Heterosis	*C/C e T/T vs. C/T*	0.883
rs1799732	Del C dominant	*Del/Del e Del/Ins vs. Ins/Ins*	0.117
	Insc C dominant	*Ins/Ins e Ins/Del vs. Del/Del*	0.056
	Heterosis	*Del/Del e Ins/Ins vs. Del/Ins*	0.280
rs6280			
	T dominant	*T/T e T/C vs. CC*	0.115
	C dominant	*C/C e C/T vs. TT*	0.170
	Heterosis	*T/T e C/C vs. T/C*	0.923

*Pearson's chi-square test. The table shows the generated genotypic models. For the polymorphisms *rs1800497* and *rs6280* in the dominant model *C*, the *C / C* and *C / T* genotypes were grouped. In the dominant T model, the T / T and T / C genotypes weregrouped and the *C / C* genotypes were grouped in the heterosis model. T / T. For the polymorphism *rs1799732* in the *Del C* dominant model, the *Del / Del* and *Del / Ins* genotypes were grouped in the dominant Ins model, the Ins / Ins and Ins / Del genotypes were grouped and the *Del / Del* and *Ins / Ins*.

DISCUSSION

It was concluded that when comparing the frequency among the three groups was no statistically significant difference between any of the genotype frequencies, which implies no association of these variants with refractory SCZ. A similar study was carried out and no associations were found between genotypes for *rs1800497* and *rs6280* polymorphisms, clinical findings and the occurrence of refractory SCZ (Patel et al., 2014). Already meta-analysis revealed that polymorphism rs1800497 showed protective effect in all populations of the 17 studies included in the meta-analysis (González-Castro et al., 2016). Another meta-analysis showed that polymorphism *rs1800497* was associated with schizophrenia in East Asians, and in the others populations as a whole this was not identified (Yao et al., 2014). It should be noted that the divergence of results in these meta-analysis with the present study can be due to the smaller sample used here and the different environmental exposure in which each population is submitted (González-Castro et al., 2016).

The results related to allele and genotype frequencies of *rs1800497* and *rs6280* variant in individuals with SCZ are quite controversial. While some authors found association (Giegling et al., 2013; Kohlrausch, 2013; Lochman et al., 2013), others do not (Zheng et al., 2012; Cordeiro and Vallada, 2014).

Another study related the T allele of polymorphism *rs1800497* to EQZ and demonstrated that individuals homozygous for this allele had higher scores in the overall clinical evaluation of akathisia (one of the extrapyramidal effects of atypical antipsychotic use) than homozygous subjects for the *C allele* (Lawford et al., 2012). In a recent meta-analysis it was identified that this SNP would be associated with changes in striatal dopaminergic neurotransmission (Kunii et al., 2014). However, in the analyzes of both

allele frequencies and dominance and heterosis of the present study no association was identified.

Another investigation that looked at attention and cognitive function in individuals with SCZ showed that patients and healthy controls with the C allele of *rs1800497* performed better than those without this allele (Nkam et al., 2017). Although studies have shown that polymorphism *rs1800497* increases susceptibility to SCZ, or is associated with its refractoriness, other studies have shown contrary evidence. In this way, the investigations must be continued in order to clarify the controversies.

Regarding *rs6280* polymorphism alone, a recent study demonstrated that levels of *DRD3* mRNA on T lymphocytes were significantly different between controls and individuals with unspecified psychotic disorder and SCZ (Cui et al., 2015). Another study showed that there is an excess of the *C* allele and homozygotes for this allele in patients with tardive dyskinesia (an irreversible adverse effect of the antipsychotic medication) (Utsunomiya et al., 2012). A more recent meta-analysis with a much larger sample size (n = 758) reported a negative but consistent trend for the *DRD3T* allele and a poor response to clozapine (Nkam et al., 2017). However, in the present study both in the allele e genotype frequency and in the dominance and heterosis analyzes, no association was found.

Regarding polymorphism *rs1799732*, a greater strength of association of the *Del C* allele was found for patients with refractory SCZ and those with other types of SCZ. Arinami et al. verified that the different alleles differ in relation to luciferase activity in vitro in Y-79 and 293 cells, indicating a functional importance of this genetic variation. Deletion of the C nucleotide (*Del* allele) reduced gene transcription at an average rate of 68%. Already another association study identified that the group with the *Ins C* allele presented a score for symptoms and a significantly higher excitation score than the group with the *Del C* allele (Xiao et al., 2013). In the present study it was possible to identify association between the Del C allele and SCZ (P = 0.03).The variant *rs1799732*, for which an association of the Del C allele was identified comparing the groups of individuals with SCZ with the controls, as well as other researches (Miura et al., 2015; Zhao et al., 2016).

It is also important to note that Table 2 shows that EQZ patients are 0.55 times less likely to have the Ins C allele than controls compared to Del C allele, and this chance may vary in the 95% confidence population between 0.32 and 0.97 being statistically significant. While refractory patients are 0.17 times less likely to have the Ins C allele than controls, compared to the Del C allele, and this chance may vary in the population with 95% confidence between 0.08 and 0.39 too being statistically significant.

Other recent research has shown a significant relationship between the *Ins / Del* genotype (heterozygotes) and severe positive symptoms (Miura et al., 2015), but did not correlate the variant with the occurrence of EQZ refractoriness. Another study found that*rs1799732* polymorphism implied a variability in response to haloperidol pharmacotherapy (Giegling et al., 2013), and a recent meta-analysis has shown that the efficacy of treatment with this medication is associated with this polymorphism (Kohlrausch, 2013).

CONCLUSIONS

We examined the frequencies of the genetic polymorphisms *rs1800497*, *rs1799732*, *rs6280* and their association with SCZ. A significant association of the Del C allele of *rs1799732*polimorphismand SCZ was found.

ACKNOWLEDGMENTS

Authors are grateful to the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for encouragement and support during the study and the molecular biology laboratory of the Federal University of São João Del Rei for providing facilities and support.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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