UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE BIOCIÊNCIAS

O PAPEL DO GENE RECEPTOR DE SEROTONINA 2A NA DEPENDÊNCIA DE ÁLCOOL E TABAGISMO

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Orientador: Claiton H. Dotto Bau

Trabalho apresentado como um dos requisitos para obtenção do grau de Bacharel no Curso de Ciências Biológicas, Ênfase Molecular, Celular e Funcional.

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1. ARTIGO – revista *Drug and Alcohol Dependence*

1.1. Carta de aceitação

Sunday, November 02, 2008

Ref.: Ms. No. WB-08-0178R2
The Serotonin 2A Receptor Gene in Alcohol Dependence and Tobacco Smoking
Drug and Alcohol Dependence

Dear Dr. Claiton Bau,

I am pleased to inform you that your submission to Drug and Alcohol Dependence has now addressed all of the issues that were raised in the review process. Thank you for your careful responses to the questions raised by the reviewers and myself. Congratulations again on this interesting and important paper.

The manuscript is being forwarded to the Central Editorial Office with my recommendation that it be published. If there are no further changes that need your attention, the manuscript will promptly be sent on to Elsevier Science Ireland, Ltd. where it will be prepared for publication. You will be alerted by email about the availability of page proofs and about any other actions needed from you. Please respond to such emails as quickly as you can to avoid delays in the appearance of your paper in the journal. After you receive communication from Elsevier, you will be able to follow the status of your paper on the Internet by logging on and registering with the Elsevier (http://www.elsevier.com/authors) which provides additional services, such as Contents Direct that sends to you the tables of contents of your selected journals.

Thank you for submitting your work to this journal.

With kind regards,

Wim van den Brink, MD, PhD
Associate Editor
Drug and Alcohol Dependence

1.2. Normas para publicação

Drug and Alcohol Dependence is an international journal devoted to publishing original research, scholarly reviews, commentaries, and policy analyses in the area of drug, alcohol and tobacco use and dependence. It is sponsored by the

College on Problems of Drug Dependence (CPDD), the oldest scientific organization in the United States concerned with research on addiction. The goal of its editors is to promote mutual understanding of the many facets of drug abuse to the benefit of all investigators involved in drug and alcohol research, and to facilitate the transfer of scientific findings to successful treatment and prevention practices. Drug and Alcohol Dependence is currently being distributed to all the members of CPDD.

Short Communications reporting on research that has progressed to the stage where a preliminary publication is appropriate. The maximum length allowed will be 2000 words plus references and illustrations. There should be not more than 2 illustrations (figure or tables).

Manuscript submission requirements

1) There should be a **title page** which provides a title and addresses (including postal codes) for all of the authors as they should appear in the publication and full contact details for the corresponding author (address with postal codes and countries, phone, FAX and E-mail).

2) An **abstract** with a 200-word summary (250-word maximum). Abstracts can be either unstructured or structured with specific sections describing the background, methods, results and conclusions.

3) 3-6 **key words or phrases** for indexing placed on the bottom of the abstract page.

4) The body of research reports will generally include introduction, methods, results and discussion sections. Further subheadings are acceptable. Review papers should also use section headings and subheadings. Sections should be numbered using the 1., 1.1, 1.1.1, 2., 2.1 etc. system. Extensive use of footnoting is not encouraged.

5) References should be assembled beginning on a separate sheet. Within the text they should be referred to by author surname and year. When referring to a work by more than two authors, the name of the first author should be given followed by et al. Examples of the correct format for citation within the text are (Jessor and Jessor, 1977; Smith and Davis, 1975) and (Chutuape et al., 2001). Citations to organization reports should spell out the name of the organization (National Institute on Drug Abuse, 2005). Personal communications and papers submitted for publication should be so indicated and appear with the source or author's name(s) in the text in parentheses. In the References section of the manuscript, they should be listed alphabetically by first author surname and must consist of names and initials of all authors, year, title of paper, abbreviated title of journal, volume number and first and last page numbers of the paper.

Chutuape, M.A., Katz, E.C., Stitzer, M.L., 2001. Methods for enhancing transition of substance dependent patients from inpatient to outpatient treatment. Drug Alcohol Depend. 61, 137-143.

Jessor, R., Jessor, S.L., 1977. Problem Behaviour and Psychosocial Development: A Longitudinal Study of Youth. Academic Press, New York. National Institute on Drug Abuse, 2005. Epidemiologic Trends in Drug Abuse. Vol. 1: Proceedings of the Community Epidemiology Work Group. Highlights and Executive Summary. NIH Publication No. 07-5879A. U.S. Department of Health and Human Services, Washington, DC. Smith, S.G., Davis, W.M., 1975. A method for chronic intravenous drug administration in the rat. In: Ehrenpreis, S., Neidle, A. (Eds.), Methods in Narcotics Research. Marcel Dekker, New York, pp. 3-21.

6) Figure legends (descriptive captions) should be numbered consecutively and typed on a separate page as a text file and included as part of the manuscript, not placed within the graphics file of the illustration. If there is more than one figure, the legends should be placed together on one page (or more if necessary).

7) Tables should be prepared as text files and are to be numbered consecutively (Table 1, Table 2, etc.) and uploaded as a step in the submission process. The captions go above the body of the Table and are left justified; Tables are read from the top down, consistent with others in this journal.

1.3 ARTIGO

The Serotonin 2A Receptor Gene in Alcohol Dependence and Tobacco Smoking

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Abstract

Alcohol and nicotine abuse and dependence are multifactorial traits that frequently co-occur, where 80-90% of alcohol-dependent individuals who seek treatment smoke. Nicotine is the main compound of tobacco and one of its effects is to increase the release of serotonin. Variations in the serotonergic system may influence some aspects of smoking. The serotonin receptor gene HTR2A has been a candidate gene with some evidence for association with alcohol and nicotine dependencies. The polymorphism HTR2A A-1438G is a functional SNP, and the presence of the A allele increases the transcriptional activity of the gene. The aim of the present study was to test for possible associations between the A-1438G polymorphism in the serotonin receptor gene (HTR2A) with tobacco smoking combined or not with alcohol dependence. The polymorphic site was genotyped in three groups of European-derived Brazilians: individuals with co-occurrence of alcohol dependence and tobacco smoking (n=113), non-alcoholic smokers (n=120) and non-smoking controls (n=115). A higher frequency of the A allele was observed in the two groups of smokers than in the non-smoking controls (x^2= 6.53, p= 0.04). Combining these groups in comparison with the control group, the difference is more significant (x^2= 6.45, p= 0.01). These results support previous evidence for association between HTR2A polymorphisms and substance use disorders.

Keywords: serotonergic; nicotine; alcoholism; candidate gene; addiction

1. Introduction

The genetic contribution to smoking initiation and persistence was estimated to be approximately 40% and 60%, respectively (Li et al., 2003). Nicotine is the main compound of tobacco and is responsible for the development and maintenance of addiction (Henninfield et al., 1985). Smoking frequently co-occurs with alcohol dependence, where 80-90% of alcohol-dependent individuals who seek treatment smoke (Berggren et al., 2007). Several genes have been shown to be associated with both tobacco smoking and alcohol dependence. These include the dopamine D2 receptor (Freire et al., 2006), alpha2A-adrenergic receptor (Prestes et al., 2007), tryptophan hydroxilase (Lerman et al., 2001; Sullivan et al., 2001; Parsian & Cloninger, 2001; Ishiguro et al., 1999) and serotonin transporter (Ishikawa et al., 1999; Schuckit et al., 1999).

The release of serotonin may increase due to nicotine, suggesting that variations in the serotonergic system may influence some aspects of smoking, such as mood variations during the nicotine withdrawal (Tyndale, 2003). Serotonin release is increased in the brain cortical region of rats treated with nicotine, and nicotine withdrawal seems to be related to the subsequent serotonin decrease (Ribeiro et al., 1993). The exposure to tobacco decreases the monoamine oxidase A and B activity, promoting the availability of neurotransmitters including serotonin (Fowler et al., 2003). The availability of these neurotransmitters due to inhibition of monoamine oxidase can cause anxiety, change of mood and activate the reward system, (Brody, 2006) behaviors that may increase smoking risk.

The serotonin 2A receptor gene (HTR2A) is located in the chromosomal region 13q14 - q21. The transition A>G (A-1438G, rs6311) in the position -1438 of the HTR2A is a functional SNP that affects the promoter activity (Parsons et al., 2004). There is evidence that this polymorphism is associated with different psychiatric disorders that may in turn predispose to smoking. Such results include the association between the G allele with bulimia nervosa (Nishiguchi et al., 2001) and alcoholism in patients with the inactive aldehyde dehydrogenase-2 (Nakamura et al., 1999). The A allele was associated with anorexia nervosa (Collier et al., 1997) and the remission in attention deficit/hyperactivity disorder (Li et al., 2006).

The aim of the present study was to test for possible associations between the HTR2A A-1438G polymorphism with tobacco smoking combined or not with alcohol dependence.

2. Material and methods

2.1 Subjects

A clinical sample with co-occurrence of alcohol dependence and tobacco smoking (n=113) was interviewed in an alcoholism treatment ward. The diagnosis of alcohol dependence followed the DSM–IV criteria (American Psychiatric Association, 2000), and the interviews were performed with the Semi-Structured Assessment for the Genetics in Alcoholism (Bucholz et al., 1994).

A group of 235 individuals (120 non-alcoholic smokers and 115 non-smoking controls) was assessed in a blood bank close to the hospital. This sample was designed to be non-screened, representative of the gene

frequencies of individuals from European descent in Porto Alegre. These individuals are replacement donors, that is, they are people that replaced the blood used by a hospitalized family member or friend. For this reason, a behavior-related bias is not likely. Exposure to alcohol was measured in the individuals assessed in the blood bank by the CAGE questionnaire (Ewing, 1984) and by inquiring about the type, quantity and frequency of alcoholic beverage consumption. None of the individuals in this group was a likely alcohol dependent. While individuals in the alcohol dependence sample consumed on average 32 drinks/day, the comparison between non-alcoholic smokers and non-smoking controls revealed that smokers were more frequently (33.4%) regular drinkers (at least two occasions in a week) than non-smokers (14.8%), but most individuals in these samples drank less than once in a week. Smokers gave 2 or three positive answers in CAGE more frequently (11.7%) than non-smokers (2.6%). None of the individuals reported 4 positive answers.

All individuals included in this study are therefore males of European descent ascertained in Porto Alegre, the capital of Rio Grande do Sul, the southernmost state of Brazil. The mean age (±SD) of individuals with co-occurrence of alcohol dependence and smoking was 41 (±10) years old, non-alcoholic smokers were on average 37 (±10) and non-smoking controls 31 (±9). This population is mainly of European descent (Salzano & Bortolini, 2002), and no significant population structure was found in the European derived population of Porto Alegre (Zembrzuski et al., 2006), making errors due to population stratification unlikely to occur in this kind of situation (Hutchison et al., 2004). The degree of African admixture in this European-derived population has been estimated as approximately 6% (Zembrzuski et al, 2006). In Brazil

there might be a cultural bias toward claiming European ancestry, therefore we prefer to use morphological classification based on skin color and morphological traits instead of self-classification for ethnicity. In addition, we did not include in the sample individuals that informed to have grandparents with non European origin.

The criterion for smoking in the alcoholic and non-alcoholic samples was current or past daily use of tobacco for at least one month. Daily smoking is strongly related to nicotine dependence, since it usually starts when dependence is already established (Mayhew et al., 2000; Wellman et al., 2004).

All individuals sampled signed an informed consent approved by the Ethics Committee of Federal University of Rio Grande do Sul.

2.2 Laboratory methods

DNA was extracted following the method described by Lahiri and Nurnberger (1991). The polymorphic region A-1438G in the HTR2A gene was amplified using the Polymerase Chain Reaction (PCR) as described by Nishiguchi and co-workers, (2001). PCR products were digested with the *MspI* restriction enzyme and revealed an undigested band of 468bp (A allele) and two bands of 244bp and 224bp (G allele). Genotyping was blind to the diagnosis of smoking and was assessed by two observers. Gel resolution was insufficient in 6% of the samples. These samples were re-genotyped.

2.3 Statistical analysis

Allele frequencies were estimated by direct counting. The analyses of Hardy-Weinberg equilibrium and comparisons among individuals with combined

alcohol dependence and tobacco smoking, non-alcoholic smokers and non-smoking controls were performed using the chi-square test.

3. Results

The samples were in Hardy-Weinberg equilibrium. Genotype and allele frequencies as well as the average number of cigarettes smoked per day among individuals with co-occurrence of alcohol dependence and smoking, non-alcoholic smokers and non-smoking controls are presented in Table 1.

The frequency of the A allele was higher in both groups that include smokers than in the group of non-smoking controls (x^2= 6.53, p= 0.04). In the joint analysis of both groups of smokers compared to the control group, the difference was more significant than with the three groups analyzed separately (x^2= 6.45, p= 0.01). The average number of cigarettes smoked per day was higher in the individuals with co-occurrence of alcohol dependence and smoking than in the non-alcoholic smokers (F= 7.52; P= 0.007). However, there was no association between the HTR2A A-1438G polymorphism and the number of cigarettes smoked per day among alcoholic smokers (F= 0.33, p= 0.72) or among non-alcoholic smokers (F= 0.00, p= 1.00).

4. Discussion

The results of the present study suggest a contribution of the serotonin 2A receptor gene in tobacco smoking. The A-1438G polymorphism presented a higher frequency of the A allele in groups of smokers with or without alcohol dependence, compared to the control group.

According to these findings, the A-1438G polymorphism seems to influence both problems by the same mechanism, since the groups containing alcohol dependents and smokers and only smokers had similar allelic and genotypic frequencies. Unfortunately, we do not have access to a sample of patients with alcohol dependence without smoking. Such data could help to solve the doubt whether the A allele is also a risk factor for alcoholism independently of smoking.

Interestingly, similar results were obtained by our group in the ANKK1/DRD2 TaqI A allele (Freire et al., 2006) and C-1291G ADRA2A polymorphism (Prestes et al., 2007). These data are consistent with the hypothesis that tobacco smoking may be a gateway to many cases of alcohol dependence (Biederman et al., 2006), and possibly with the common genetic vulnerability to nicotine and alcohol dependence in men (True et al., 1999).

Our findings differ from the study by Terayama et al. (2004) that found no association between the A-1438G polymorphism and smoking in the Japanese population. Interestingly, Nakamura et al. (1999) verified an association between the G allele and alcoholism, also in a Japanese sample. A possible explanation for these controversies could be the between-population differences in allelic frequencies and linkage disequilibrium patterns. The A allele frequency is around 36-45% in European (Campbell et al., 1998; Collier et al., 1997; Hinney et al., 1997; Nacmias et al., 1999; Saiz et al., 2008; Sorli et al., 2008) or European-descent populations (Enoch et al., 1999), and 48-54% in Asians (Chee et al., 2001; Nakamura et al., 1999; Nishiguchi et al., 2001; Terayama et al., 2004; Zoroglu et al., 2003).

Alcohol and nicotine dependences are complex, multifactorial traits that depend on many genes and environmental factors, and their interactions. Unfortunately, the present sample size does not allow tests for gene X gene or gene X environment interactions. Another limitation is that this association may only be valid for the population studied. The strength of the finding is that there are previous neurobiological evidences that the serotonergic system is involved in nicotine dependence (Brody, 2006; Fowler et al., 2003; Ribeiro et al., 1993).

The accumulating classical and molecular genetics findings in substance dependencies have reinforced the role of genetics in tobacco smoking and the role of smoking as a risk factor to alcohol dependence. There is a clear need for further clarify these associations since they may have important prevention and treatment implications.

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Table 1: Genotype and allele frequencies of the HTR2A A-1438G polymorphism in individuals with tobacco smoking and alcohol dependence, tobacco smoking and controls.

	Tobacco smoking and alcohol dependence (TA)		Tobacco smoking (T)		Controls
	N (%)	Mean (SD)[1]	N (%)	Mean (SD)[1]	N (%)
Genotypes					
GG	29 (25%)	24 (13)	32 (24%)	18 (12)	38 (36%)
GA	56 (50%)	22 (15)	54 (50%)	18 (13)	62 (48%)
AA	28 (25%)	24 (11)	34 (26%)	18 (14)	15 (16%)
Total	113 (100%)	23 (13)	120 (100%)	18 (13)	115 (100%)
Alleles					
G	114 (50%)		118 (49%)		138 (60%)
A	112 (50%)		122 (51%)		92 (40%)

[1]Number of cigarettes smoked per day. None of the comparisons between genotypes resulted in significant differences. TA x T: F= 7.52; P= 0.007.

Genotype frequencies
TA x T x Controls: x^2= 8.98, p= 0.06
TA + T x Controls: x^2= 8.38, p= 0.01
Allele frequencies
TA x T x Controls: x^2= 6.53, p= 0.04
TA + T x Controls: x^2= 6.45, p= 0.01