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Dopamine receptor gene DRD1 (*A-48G)* polymorphism and smoking related behavior among smokers of Punjab (North West India)

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ABSTRACT

Addictive behaviors exert an enormous cost on society. Cigarette smoking, like other substances of abuse is a public health problem associated with considerable morbidity, mortality, personal and public cost. In the present study, we have attempted to investigate possible links between the dopamine receptor gene DRD1 and smoking behavior in smokers inhabiting the North West Indian region. A total of 361 subjects (173 smokers with a mean age 35.37±14.29 years and 188 healthy age/ethnicity matched non-smokers with a mean age of 35.79±13.37 years) participated in the study. The degree of nicotine dependence was ascertained by commonly used measure: The Fagerstrom Test for Nicotine Dependence (FTND). Measures such as cigarettes per day, smoking history, pack years smoked and age at initiation of smoking were also used as predictive measures of nicotine dependence. On categorization of smokers into low nicotine dependence (LND) and high nicotine dependence (HND), significantly high differences were seen between HND vs. LND for all the smoking related variables except age at initiation of smoking. However, our study could not find any significant associations between various smoking variables and DRD1 genotypes. Also, no significant differences were seen between smokers and non-smokers at both genotypic and allelic level.

Key words: DRD1, FTND, Genetic polymorphism, nicotine dependence, North West Indian region.

INTRODUCTION

One of the leading causes of preventable deaths in the developed countries is tobacco use (Benowitz, 2008, Mathers and Loncar, 2006; Peto et al. 1996), and the main addictive component is nicotine (Benowitz, 2009; Dani et al. 2009; Mansvelder and McGehee 2002; Dani and Heinemann, 1996). Smoking is known to cause an increase in dopamine release in the left ventral caudate/nucleus accumbens and putamen in humans (Brody et al 2004), which stimulate the mesolimbic reward pathways of the brain (Cami and Farre, 2003; Corrigall et al. 1994, 1992). Various smoking aspects such as initiation of smoking and cigarette consumption levels are known to be highly heritable, like the heritability of nicotine dependence and the estimates ranges from 31% to 75% (Vink et al, 2005). Genes involved in dopamine metabolism within the reward pathways of the brain are excellent candidates in contributing to the genetic component of nicotine dependence. Dopaminergic neurons of the mesolimbic reward pathways have frequently been implicated in the etiology of alcoholism, drug abuse and other addictive behaviors (Koob, 1992; Kuhar et al. 1991; Wise and Rompre, 1989; DiChiara and Imperato, 1988; Dackis and Gold, 1985; Routtenberg, 1978). G-protein-coupled dopamine receptors consist of D1-like (D1 and D5) receptors, which stimulate cyclic adenosine monophosphate (AMP) production, and D2-like (D2, D3 and D4), which inhibit cyclic AMP production (Civelli, 1991). Evidences suggest that D1-like dopamine receptors (DRD1 and DRD5) are important components of mesolimbic reward-related pathway (Missale et al., 1998; Beninger and Miller, 1998).

Numerous groups have studied the individual effects of dopamine candidate genes with various substance dependent disorders and neuropsychiatric disorders from India and worldwide. The present study was planned with an aim to investigate possible links between the dopamine receptor gene DRD1 and smoking behavior in smokers inhabiting the North West Indian region. Gene *DRD1* is located at chromosome 5q35.1 and contains two exons separated by a small intron in the 5'-untranslated region (UTR). A region near *DRD1* has shown a significant linkage to cigarette consumption and smoking initiation in a genome-wide linkage scans (Vink et al. 2006; Saccone et al. 2003; Duggirala et al. 1999), and has been suggested as a potential target for nicotine dependence by Vink et al. (2006) and Duggirala et al. (1999). The *Dde*I polymorphism is an A>G transition in the 5′-UTR, A-48G (Cichon et al. 1994). Polymorphisms within or near *DRD1*, especially the *Dde*I polymorphism (rs4532), has been widely studied for genetic association with other neuropsychiatric diseases (Wong et al. 2000).

MATERIAL AND METHODS

Subjects

The present case-control study was carried out in the North-West region of India. We recruited a total of 361 participants in this study (173 smokers with a mean age 35.37±14.29 years and 188 apparently healthy age/ethnicity matched non-smokers with a mean age 35.79±13.37 years). Participants were 17 years of age or older (smokers and non-smokers). Cases consisted of those who had smoked for at least last 12 months. They were generally healthy despite smoking 5-10 cigarettes per day. Written pre-informed consent was obtained from all the subjects and the study protocol, forms/procedures were approved by the Institutional Ethical Committee. Subjects were interviewed using a pre-tested and modified schedule to identify cases and controls. Information was also collected regarding name, gender, date of birth, caste/religion, occupation, cigarette/ bidi smoker, cigarettes smoked per day (CPD), smoking history (duration), age at initiation of smoking (AIS) and pack years smoked (average packs per day smoked, multiplied by number of years smoked).

Assessment of nicotine dependence

Assessment of dependence on nicotine has become an important objective in tobacco related studies. For each smoker, the degree of nicotine dependence was ascertained by commonly used measure: The Fagerstrom Test for Nicotine Dependence (FTND; 0–10 scale and consists of six items), Heatherton et al. 1991. The six items include, (1) time to take up the first cigarette/bidi after awakening in the morning (2) difficulty in refraining from smoking in places where it is forbidden (3) unwillingness to give up the first cigarette in the morning (4) number of cigarettes/bidis smoked per day (5) intensity of smoking during the morning hours and (6) smoking even when bedridden due to illness. A score of 5 or more indicates a significant dependence while a score of 4 or less presents a low to moderate dependence (Heatherton et al. 1991). FTND is the widely accepted, reliable and valid evaluation instrument used to establish and quantify nicotine dependence (Dijkstra and Tromp, 2002; Etter, 2005). The scores of FTND ≥6 were determined as high nicotine dependence (HND) and the scores ≤5 were determined as low nicotine dependence (LND) (Fagerstrom et al, 1996; Gallus and La 2004; Diaz et al. 2005; Kozlowski et al. 1994; Moolchann et al. 2002; De Leon et al. 2003; Fagerstrom et al. 1990; De Leon et al. 2002 and John et al. 2004).

DNA extraction, SNP selection, and genotyping

Each genomic DNA sample was extracted from peripheral blood samples using salting out method of Miller et al. (1988) with some modifications. The *A-48G* (rs4532) polymorphism was genotyped in cases and controls. SNP selection was done on the basis of information available in

the literature for DRD1 gene variation, NCBI dbSNP database and published reports on DRD1polymorphism. The oligonucleotide primers 5′-GGCTTTCTGGTGCCCAAGACAGTG-3′ and 5′-AGCACAGACCAGCGTGTTCCCCA-3′ were used to amplify a 405 base-pair (bp) fragment spanning the polymorphic *A-48G* (rs4532) site of the DRD1 gene at an annealing temperature of 63ºC for 30 sec. The PCR cycling conditions were set according to the technique mentioned by Comings et al.1997.

The PCR products were digested using *DdeI* restriction enzyme (New England Bio labs), Comingset al.1997. For restriction fragment length polymorphism (RFLP), 2% agarose gel was prepared and run for 45 min. Digestion resulted in three fragments (146bp, 42bp, 217 bp) in *-48A* homozygous subjects, and 146bp and 259 bp in *-48G* homozygous subjects and in combination of the two profiles in the heterozygous subjects *-48A/G* (146bp, 42bp, 217bp, 259bp).

Statistical analysis

Allele frequencies were calculated by gene counting method (Mourant et al. 1976). Data were analyzed using SPSS 16.0 for windows (SPSS Inc., Chicago, IL, USA). Hardy-Weinberg equilibrium was tested to check significant departure if any, by using χ^2 goodness-of-fit test. Contingent χ^2 test was used to test for differences in genotypic (2 x 3 chi square contingency table) and allelic (2 x 2 chi square contingency table) distribution between the smokers and the non-smokers. A p-value ≤ 0.05 was considered to be statistically significant. Sample size was determined by calculating power of the study at 80% (significance level $\alpha = 0.05$, GE_{COR} statistical software, (Murphy and Kraft, http://www.hsph.harvard.edu/faculty/kraft/soft.htm).

RESULTS

Table 1: Clinical Characteristics of Smokers in the present study

Smoking Variables	LND (n= 62)	HND (n =111)	t	p (LND vs. HND)
Age at Initiation (yrs)	18.34 ± 3.76	16.99 ± 6.64	1.471	0.143
Smoking History (yrs)	7.61 ± 8.80	23.64 ± 15.35	7.554*	0.000
Cigarette per day	9.29 ± 5.53	20.28 ± 11.36	7.147*	0.000
Pack year Smoked (yrs)	4.75 ± 7.69	26.69 ± 26.47	6.372*	0.000
FTND score	3.13 ± 1.55	7.77 ± 1.31	20.893*	0.000

FTND-=Fagerstrom Test for Nicotine Dependence, LND= Low Nicotine Dependence, HND-=High Nicotine Dependence

*Statistically significant ($p \leq 0.05$)

We analyzed Dopamine Receptor gene DRD1 (*A-48G*, rs4532) polymorphism in a sample of 173 smokers and 188 non-smokers from north-west region of India. Table 1 represents the clinical characteristics of smokers in the present study. Significantly high differences were seen between LND vs. HND for almost all variables ($0.000<p<0.05$), viz., cigarettes per day (9.29±5.53 vs. 20.28±11.36), smoking history (7.61± 8.80 vs. 23.64 ± 15.35), number of pack

years smoked, (4.75±7.69 vs. 26.69±26.47) and Fagerstrom Test for Nicotine Dependence (FTND) (3.13±1.55 vs. 7.77±1.31) except at age of initiation of smoking.

Table 2: Distribution of smoking variables by genotypes among Low nicotine dependence and High nicotine dependence cases in the present study.

CHARACTERS	LND (0-5 FTND SCORE)		t	p	HND (6-10 FTND SCORE)		t	p
	AA	*GG*			*AA*	*GG*		
Age at Initiation (yrs)	18.22±3.43	18.38±3.92	0.155	0.470	16.72±6.09	17.15±6.98	0.328	0.780
Smoking History (yrs)	6.44±6.22	8.09±9.68	0.666	0.256	23.30±16.91	23.84 ±14.47	0.175	0.385
Cigarette per day	7.92±5.30	9.86±5.58	1.264	0.843	20.44±11.86	20.19±11.14	0.113	0.811
Pack year Smoked (yrs)	4.67±8.45	4.78±7.44	0.057	0.953	27.05±29.37	26.48±24.82	0.108	0.526
FTND (score)	2.78±1.56	3.27±1.55	1.143	0.900	7.76±1.32	7.79±1.32	0.114	0.842

FTND- Fagerstrom Test for Nicotine Dependence,LND- Low Nicotine Dependence, HND- High Nicotine Dependence

*Statistically significant ($p \leq 0.05$)

Table 3. Observed and expected genotype number and allele frequency distribution of dopamine receptor D1 (*DRD1 A-48G*) SNP in Smokers and Non-smokers

SNP (rs ID)	Study Group	Number	Genotype			$\chi^2_{H\text{-}W}$	Allele frequency	
			AA	*AG*	*GG*		*A*	*G*
DRD1 (*A-48G*) (rs4532)	Cases (n=173)	Observed Expected	59 58.38	83 84.23	31 30.38	0.037	0.581	0.419
	Controls (n=188)	Observed Expected	62 58.08	85 92.83	41 37.09	1.340	0.556	0.444

*Statistically significant ($p \leq 0.05$)

Table 4.Comparison between Smokers and Non-smokers inhabiting North-West Indian region for dopamine receptor D1 (*DRD1A-48G*) SNP using contingent χ^2 test

SNP (rs ID)	Study group	Genotype			χ^2 (d.f=2)	p	Allele Number		χ^2 (d.f=1)	p
DRD1 (*A-48G*) (rs4532)	Cases (n=173)	*AA*	*AG*	*GG*	0.865	0.649	*A*	*G*	0.462	0.497
		59 (34.10)	83 (47.98)	31 (17.92)			201 (116.18)	145 (83.82)		
	Controls (n=188)	62 (32.98)	85 (45.21)	41 (21.81)			209 (111.17)	167 (88.83)		

*Statistically significant ($p \leq 0.05$)
Figures in parentheses are percentages.

To see if there exists any association between DRD1 genotypes and smoking related variables, the genotypes were classified as ancestral '*A'* allele carrier (homozygous *AA*) or variant '*G'* allele carrier (*AG* and *GG*) for statistical purposes. However, we observed no significant differences between *DRD1* genotypes and various smoking related variables (Table 2).

Table 3 presents observed and expected genotype numbers and allele frequency distribution of dopamine receptor DRD1 (*A-48G*) in the smokers and non-smokers of North-West Indian region. Frequency range of the rare allele *G* was found between 0.419 in cases to 0.444 in the controls, while the ancestral allele *A* ranged from 0.581 in cases to 0.556 in controls. Both the cases and the controls were found to be in Hardy-Weinberg equilibrium. This demonstrated that the studied DNA marker is independently distributed in the samples studied and there were negligible chances of genotyping error, population stratification, migration and inbreeding.

Table 4 summarizes the comparison between smokers and non-smokers inhabiting North-West Indian region for dopamine receptor gene DRD1 (*A-48G*) SNP using contingent chi-square test. No significant associations were seen between smokers and non-smokers for nicotine dependence at both allelic and genotypic level.

DISCUSSION

The dopamine receptor D1 is one of the major receptors in the brain that mediate the actions of the neurotransmitter dopamine in a variety of psychomotor functions. *DRD1* has been extensively investigated in neuropsychiatric disorders because of its suggestive evidence in dopaminergic dysfunction and its role in the pathogenesis.

In the present study, no significant association between the DRD1 (*A-48G*, rs4532) polymorphism and the nicotine dependence was found in population of north west Indian region unlike other recently reported studies on association of DRD1 with bipolar disorder (Del Zompo et al. 2007; Severino et al. 2005), ADHD (Bobb et al. 2005; Misener et al. 2004), nicotine dependence (Lee et al. 2012; Huang et al. 2008 and Comings et al. 1997) and alcohol dependence (Kim et al. 2007; Limosin et al. 2003) as shown in table 5.. This finding agrees with other association studies suggesting no role of *A-48G* polymorphism in conferring susceptibility to nicotine dependence and some other neuropsychiatric disorders such as alcohol dependence (Singh et al. 2014; Zhu et al. 2011; Batel et al. 2008; Kim et al. 2007 and Szczepankiewicz et al. 2007) and methamphetamine abuse (Cheng et al. 2006). Such differences could be attributed to differences in genetic structure of the populations, study designs, variation in sampling like selection of nicotine dependent cases and nature of comparable controls. The *A-48G* polymorphism is localized in the 5′-UTR region of DRD1 gene and is unlikely to affect translation of the receptor (Cichon et al. 1996), where the nucleotide sequence is involved in transcription but is not being translated into amino acids. Due to *G>A* substitution, rs4532 may act as a short open

reading frame preceding the receptor cistron and encode a small peptide that inhibits receptor translation (Fung et al. 2009).

Table 5: Allele frequency distribution of dopamine receptor D1 (rs4532) polymorphism in various behavioral disorders and diseases in the Indian and world populations

Area/Population	Disorder	Study group	Number tested	Allele frequency		P (Case vs Control)	Reference
				A	*G*		
INDIAN							
India	Type 2-Diabetes	Cases	196	0.820	0.180	0.330	Prasad et al. (2008)
		Controls	225	0.550	0.450		
North India	Alcohol Dependence	Cases	90	0.380	0.620	0.040*	Prasad et al. (2013)
		Controls	122	0.520	0.480		
Manipur	Alcohol Dependence	Cases	143	0.780	0.220	0.270	Singh et al (2014)
		Controls	318	0.780	0.220		
North India	Nicotine Dependence	Cases	173	0.580	0.420	0.497	Present Study
		Controls	188	0.560	0.440		
WORLD							
Non-Hispanic Caucasians,USA	Tourette Syndrome	Cases	63	0.370	0.630	0.756	Comings et al. (1997)
		Controls	227	0.340	0.660		
	Smokers	Cases	61	0.340	0.660	0.876	
		Controls	177	0.350	0.650		
	Gamblers	Cases	163	0.350	0.650	0.860	
		Controls	124	0.360	0.640		
Japan	Schizophrenia	Cases	48	0.896	0.104	0.264	Iwata et al. (2003)
		Controls	145	0.931	0.069		
Poland	Alcohol Abuse in Bipolar patients	Cases	42	0.286	0.714	0.110	Szczepankiewicz et al. (2007)
		Controls	350	0.379	0.621		
China	Schizophrenia	Cases	373	0.868	0.132	0.138	Zhang et al. (2010)
		Controls	379	0.842	0.158		
China	Schizophrenia	Cases	385	0.882	0.118	0.086	Zhu et al. (2011)
		Controls	350	0.851	0.149		
Brazil	Schizophrenia	Cases	59	0.640	0.360	0.001*	Ota et al. 2012
		Controls	65	0.800	0.200		
North India	Nicotine Dependence	Cases	173	0.580	0.420	0.497	Present Study
		Controls	188	0.560	0.440		

*Statistically significant ($p \leq 0.05$)

The present study fails to provide a hypothesized link between the dopamine receptor gene D1 and selected smoking variables unlike other studies, reporting a strong association between smoking variables and DRD1 genotypes (Novak et al. 2010; Huang et al. 2008 and Comings et al. 1997), alcohol dependent phenotypes (Kim et al. 2007). On comparison with other studies from India (Table 5) the minor allele (*G*) frequency of the present study (0.440 in controls) was found to be close to that reported by Prasad et al. (2013) for alcohol dependence (0.480) and Prasad et al (2008) for type 2 diabetes (0.450). Association studies done worldwide involving DRD1*A-48G* polymorphism with other behavioral disorders found only two significant associations for alcohol

dependence in a north Indian population by Prasad et al (2013) and for schizophrenia in a Brazilian population by Ota et al (2012).

Categorization of smokers into low nicotine dependence (LND) and high nicotine dependence (HND), depicted statistically significant differences were for almost all smoking related variables except age at initiation. High nicotine dependence smokers have shown higher values for cigarettes per day, smoking history i.e. number of years they have been smoking, pack years smoked and FTND (Fagerstrom Test for Nicotine Dependence). Our findings suggest that high nicotine dependence smokers are susceptible to risk of nicotine dependence and various smoking related disorders as compare to low nicotine dependence smokers. Similar results have been reported by Wei et al. (2012) on a Chinese population with LND vs. HND values depicting age of smoking onset (25.0±7.7 vs. 20.8±6.3), years of smoking (24.4±11.5 vs. 24.7±12.3), FTND score (1.4±1.0 vs. 7.6±1.0), other studies (Perez et al., 2009; Diaz et al., 2005; De leon et al., 2003 and De leon et al 2002).

In particular, high nicotine dependence smokers exhibited a greater smoking intensity (evaluated from cigarettes per day, years of smoking history, pack years smoked and FTND score) and were younger at the age of initiation of smoking. On comparing the distribution of smoking variables by genotypes among low nicotine dependence and high nicotine dependence, individuals carrying rare homozygote *GG* exhibited greater smoking intensity and nicotine dependence as compared to individuals carrying common homozygote *AA.* HND smokers were more vulnerable to nicotine dependence and exhibited higher smoking intensity as compare to low nicotine dependence smokers. However, there were no significant differences found for genotypic distribution in both the groups (low nicotine dependence and high nicotine dependence).

CONCLUSION

In the present investigation, DRD1 (*A-48G*, rs4532) polymorphism did not show any significant associations with risk of nicotine dependence among smokers of North West Indian region. It could be attributed to the small sample size of the present study. It has to be viewed in perspective of potential limitation and needs to be confirmed by replication in large sample sets. In addition to the sample size, examination of other polymorphic loci of DRD1 gene is necessary to elucidate the significance of genetic variations and may improve understanding of the pathophysiology of nicotine dependence.

An emphasis on more comprehensive approach to the genetic determinants of smoking, improved measures of dependence, biological measures of nicotine pharmacokinetics, receptor activity, identification of functional consequences at the RNA and protein level are vital in future research. It would be helpful to identify the individuals at risk of nicotine dependence and to distil the cause of craving for smoking and its consequences on health of individual.

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