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THE ROLE OF DOPAMINE D2 RECEPTOR POLYMORPHISM TOWARDS SUSCEPTIBILITY TO ALCOHOL DEPENDENCE AMONG MALE CASES OF BILASPUR DISTRICT: A PRELIMINARY STUDY

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ABSTRACT
Dopamine is a key substrate for alcohol and other drug related behaviours and plays an important role in the central reward process. The psychoactive substances and high levels of alcohol elevate the dopamine release in the NAc zone of the CNS and it is thought that this release is important for the addictive behaviour as it is associated with positive moods, pleasure and euphoria. The DRD2 gene has three most commonly investigated polymorphisms i.e. -141C ins/Del, TaqI B and TaqI A. As a result, we studied association of three single nucleotide polymorphisms (SNP) in DRD2 gene with alcohol dependence in the central Indian subjects using a case-control approach. Cases satisfied DSM-IV criteria for Alcohol Dependence was determined and total of 50 AD cases and 50 healthy unrelated age-matched control samples were included. To determine risk conferred by a predisposing allele/genotype/ haplotype Odds ratio and confidence interval was calculated. The investigation revealed a significant association of -141C Ins allele and a tendency of association of *TaqI* A1 allele of DRD2 with alcohol dependence. Haplotype with the influencing -141C Ins and *TaqI* A1 alleles (-141C Ins-A-A1) appears to bestow 3.19 times risk to acquire alcohol dependence. Present study imparts initial understanding towards genetic susceptibility to alcohol dependence in central Indian males. Polymorphisms namely, -141C Ins/Del and *TaqI* A at DRD2 gene could have proven influences among central Indian alcoholic cases.

KEYWORDS: Alcohol Dependence, DRD2, SNP, Genotype, Allele Frequency.

INTRODUCTION
Alcoholism is a complex illness associated with impairment involving physiological, psychological and social dysfunction. It is a combined effect of interaction of genes with the environment. Being a multi factorial disorder, in the study of alcoholism therefore the study of the genetic component amongst population becomes inevitable (Bhaskar et al.2011). Alcohol dependency is common but a complex trait and is prevalent among developing countries. The genetic influence of alcohol dependency can be significantly assessed from the family, twin and adoption studies. It has been well established from the studies that the genetic influence on alcoholism can account up to 50%. In recent years, the studies involving genes responsible for the alcohol metabolism and the alcohol related neurological studies have increased widely (Singh et al.2013). Particularly the specific focus is in the function of the pathways that involve dopamine receptors as these receptors are thought to be significantly associated with various disorders like Parkinson's disease, psychotic disorders like Schizophrenia and alcohol dependence. Dopamine is a key substrate for alcohol and other drug related behaviours and plays an important role in the central reward process. (Bhaskar et al.2011; Singh et al.2013) The dopaminergic pathway involves the VTA (Ventral tegmental area) and NAc (Nucleus accumbens). While the former is responsible for the thought, memory, emotion, planning and executing behaviour; the latter is associated with the motivation, learning and their assessment. The psychoactive substances and high levels of alcohol elevate the dopamine release in the NAc zone of the CNS and it is thought that this release is important for the addictive behaviour as it is associated with positive moods, pleasure and euphoria. The most commonly and frequently studied polymorphism is at the dopamine D2 receptor (DRD2 gene) on chromosome 11 (q22-23). The DRD2 gene has three most commonly investigated polymorphisms i.e. -141C ins/Del, TaqI B and TaqI A. While the promoter polymorphism (−141C

ins/Del, rs1799732) of the DRD2 gene involving the insertion (Ins) /deletion (Del) of a cytosine is related to receptor density; SNP TaqI B is closer to the regulatory and structural coding regions (5' region) of the gene and is considered to play important role in transcription regulation. However, the Taq I A site of the ANKK I gene is widely used in the studies related to substance abuse and addiction. The restriction of Taq I results in polymorphic fragments identifying the alleles namely A1 and A2 which have a difference in the number of their receptor sites. Earlier studies have shown that the people having A1 allele are more prone to alcohol dependency as the A1 allele decreases the expressivity of D2 dopamine receptors. However, variations in the results of such studies conducted worldwide may be seen due to variable AD cases, nature of control, the study design and the sampling methods. It also becomes very important to study and understand the ethnic and geographical background of the subjects chosen for the study because the frequency of the A1 allele may vary greatly among different populations. The current study is an attempt to investigate the association of -141 Ins/Del, TaqI B and TaqI A sites of the DRD2 gene for susceptibility towards alcohol dependency in a male population of Bilaspur district of Chhatisgarh, India.

Methodology

Before initiation of the study, ethical committee clearance from the Chhattisgarh Institute of Medical Sciences (CIMS), Bilaspur (C.G.) was obtained. With prior individual written informed consent recruitment of case and control subjects were done. The clinical assessment process of was carried out by qualified and experienced psychiatrist. Fifty male alcohol dependent subjects attending the outpatient department (OPD) at Chhattisgarh Institute of Medical Sciences (CIMS) Bilaspur (C.G.) were screened fall in the age range of 18-60 years, were enrolled as cases (AD). A total of 50 unrelated healthy male of Bilaspur district of Chhattisgarh, without any history of alcohol or any other substance use (except nicotine) were included as controls (C). Individuals considered themselves to be of central Indian origin. Experienced psychiatrists by using the DSM-IV criteria (American Psychiatric Association, 1994 diagnosed the alcohol dependence (DSM, 1994). For alcohol use parameters all patients were assessed by using AUDIT (Saunders et al. 1993), and a semi-structured questionnaire. Clinical details like ethnicity, family history, age at first use of alcohol, quantity of alcohol consumption (g/day), duration of alcohol use, duration of alcohol dependence, age at onset of dependence, and any other physical illness were included in the semi-structured questionnaire. The control population were assessed by using the same semi-structured questionnaire.

Genetic Analysis: DNA was isolated from the 5ml blood by using the conventional salting-in salting-out technique (Miller et al. 1988) and used for genetic analysis. Based on prior published association reports, Minor Allele Frequency (MAF), Linkage Disequilibrium (LD) structure, information content, and supporting evidence three SNPs were selected from the DRD2 locus. SNPs were genotyped by using polymerase chain reaction (PCR)-restriction fragment length polymorphisms (RFLP) on the basis of standardised protocol (Itokawa et al.1993; Grandy et al.1993). The PCR products digested with RE were separated on 2-3% of agarose gel stained with ethidium bromide. The SNPs studied in DRD2 their position along with their identification number is represented as -141C Ins/Del, promoter (rs1799732); G>A, TaqI B, 1 kb upstream from exon 2 (rs17294542); and T>C, TaqI A, 10 kb downstream from exon 8 (rs1800497) (Table1).

Statistical Analysis: All clinical variables between alcohol dependent (AD) and controls (C) were compared by performing $\chi 2$ test for nominal variables or student's t-test for continuous variables. For each of the genetic marker Hardy-Weinberg equilibrium (HWE) was performed. Allelic and genotypic associations of SNPs were evaluated by Pearson's $\chi 2$ test followed by Odds Ratio and 95% CI calculation. Based on the frequency of each haplotype versus all others between the AD and the controls, chi-square values were determined from a series of 2 × 2 contingency tables.

Table 1. showing detailed information of genotyped SNPs, annealing temp and PCR-RFLP product size

SNP	Primers	Annealing temp.	R.E./fragment size
-141C Ins/De	F:5'-GACCCAGCCTGCAATCAC-3' R:5'-AGGAGCTGTACCTCCTCGG-3'	57*C	*Bst I* Ins C = 124, 32 Del C = 156
TaqI B	F:5'-GATGTGTAGGAATTAGCCAGG-3' R:5'-GATACCCAGTTCAGGAAGTC-3'	56*C	*Taq I* G = 459 A = 267,192
TaqI A	F:5'-CCGTCGACGGCTGGCCAAGTTGTCCA- 3' R:5'-CCGTCGACCCTTCCTGAGTGTCATCA-3'	58*C	*Taq I* T (A1) = 310 C (A2) = 180,130

RESULTS

The demographic and other alcohol dependent details of the cases and controls are presented in Table 1- 6. A total of 100 cases and controls were recruited into the study. The study group consisted of 50 alcohol dependent cases, aged 18 to 70 years, and the control group consisted of 50 non- alcohol dependent men (Table1). Maximum frequency (24%) were observed for age group between

42-52 years among cases and among controls high frequency (48%) was observed from age group of 18-30 years. Occupation wise distribution of cases and controls reported higher frequency of government servants (36%) in cases and higher frequency of unemployed (32%) among controls (Table2). Category wise distribution of cases and controls were represented in Table 3. OBC was represented in higher frequency (44%) among cases and General reported higher frequency (72%) among controls. Age of initiation for alcohol intake among cases were higher (48%) in 10-20 years (Table4). The frequency of alcohol intake among cases was higher (52%) on regular basis (Table5) and the type of alcoholic beverage most frequently (56%) consumed by cases were Indian liquors (Table6).

Genetic analysis all the three SNPs analyzed in the study were not in HWE in the base line control population. Allele and genotype frequencies of SNPs in DRD2 and their association status with AD are presented in Table 3. Of the three polymorphisms analyzed in DRD2 gene, significant allelic and genotypic association of -141C Ins/Del SNP was observed with alcohol dependence. SNP TaqI B did not show any allelic or genotypic association. For *Taq* I A SNP, genotype *Taq* A1A1 was present in less frequency. Categorical analysis showed non-significant association of *Taq* A1/A2 genotype (Chi square Test, P=0.05) with alcohol dependence. In addition, a trend towards association of allele *TaqI* A1 with AD was also found (Table 7).

Significantly associated haplotypes of DRD2 gene present in AD subjects are shown in Table 4. Out of eight possible haplotypes two haplotypes (-141C Ins-A-A1, and -141C Del-A-A2) in these cases were significantly associated with AD, former being influencing and latter provide protection to AD (Table 8).

Table. 1: Age Wise Distribution of Cases and Controls.

S. No.	Age group	Case		Control	
		Frequency	%	Frequency	%
1	**18-30**	10	20	24	48
2	**30-42**	10	20	10	20
3	**42-52**	12	24	10	20
4	**52-60**	10	20	4	8
5	**60 Above**	8	16	2	4
Total		**50**	**100**	**50**	**100**

Table. 2: Occupation Wise Distribution of Cases and Controls.

S. No.	Occupations	Case		Control	
		Frequency	%	Frequency	%
1	**Government service**	18	36	12	24
2	**Private service**	14	28	6	12
3	**Agriculture**	12	24	4	8
4	**Retired**	2	4	2	4
5	**Businessman**	4	8	10	20
6	**Unemployed**	0	0	16	32
Total		**50**	**100**	**50**	**100**

Table. 3: Category wise Distribution of Cases and Controls.

S. No.	Caste	Case		Control	
		Frequency	%	Frequency	%
1	**General**	8	16	36	72
2	**OBC**	22	44	14	28
3	**SC**	6	12	0	0
4	**ST**	14	28	0	0
Total		**50**	**100**	**50**	**100**

Table. 4: Age of Initiation of Alcohol in Cases.

	Age at initiation	Cases	
S. No.		Frequency	%
1	10 -20	24	48
2	20 -30	22	44
3	30-40	2	4
4	40-50	2	4
Total		**50**	**100**

Table. 5: Frequency of Intake of Alcohol.

S. No.	Frequency of Intake	Cases	
		Frequency	%
1	Regular	26	52
2	Occasional	6	12
3	Rare	18	36
Total		50	100

Table. 6: Distribution of Cases on the Basis of Type of Alcoholic Beverage Consumed by Cases.

S. No.	Type of Liquor	Cases	
		Frequency	%
1	Indian liquor	28	56
2	Foreign liquor	20	40
3	Both	2	4
		50	100

Table. 7: Genotype and Allele Distribution of Dopamine Receptor D2 SNPs in Cases with Alcohol Dependents and Controls.

SNP	Genotype	Cases (N=50)	Controls (N=50)	Allele Frequency	Cases (N=50)	Controls (N=50)
-141C Ins/De	Ins/Ins	24 (0.48)	18 (0.36)	Ins C	0.66	0.45
	Ins/Del	18 (0.36)	9 (0.18)	Del C	0.34	0.55
	Del/Del	8 (0.16)	23 (0.46)			
		$\chi 2$= 11.12, P< 0.05 P < 0.05; OR 1.19, 95%, CI 0.10-2.36				
TaqI B	GG	6 (0.12)	10 (0.2)	G	0.32	0.38
	GA	20 (0.40)	18 (0.36)	A	0.68	0.62
	AA	24 (0.48)	22 (0.44)			
		$\chi 2$= 1.19, P> 0.05				
TaqI A	A1A1	4 (0.8)	7 (0.14)	A1	0.29	0.34
	A1A2	21 (0.42)	20 (0.40)	A2	0.71	0.36
	A2A2	25 (0.50)	23 (0.46)			
		$\chi 2$= 2.93, P< 0.05				

Table. 8: Significantly associated SNP haplotypes in DRD2 gene.

Haplotype	AD (50)	Controls (50)	$\chi 2$	P	OR (95% CI)
-141C Ins A-A1	16 (0.32)	9 (0.18)	4.67	0.03	3.19 (2.19-5.14)
-141C Del A-A2	11 (0.22)	8 (0.16)	4.39	0.02b	0.62 (0.56-0.97)

a Order of SNPs in the DRD2 haplotypes: -141C Ins/Del - G>A (Intron 1) - TaqI A (Intron 7)
b Significant after Bonferroni correction a = 0.01

DISCUSSION

In the context of alcohol dependence (AD), the studies provide explicit indication that polymorphisms at genes show a significant role in its expansion (Dick et al.2006). Alcohol rises synaptic dopamine, which strengthens self-giving out. The dopamine D2 receptors down regulation in the ventral striatum and particularly in the nucleus accumbens of the brain have been linked with alcohol desiring and an increment in the administering of alcohol- associated stimuli in the medial prefrontal cortex (Kienast and Heinz,2006). Probable link between genetic polymorphisms at the DRD2 and alcohol dependence (AD) has been widely studied in 1990 (Blum et al.1990). The present analysis verified association of three SNPs -141C Ins/Del, *Taq*I B and *Taq*I A, at DRD2 gene locus with alcohol dependence (AD) in central Indian male cases. To the best of our knowledge, this report represents the first association study in context to these three SNPs (-141C Ins/Del, TaqI B and TaqI A in DRD2 gene) with alcohol dependence from central India. A study on south Indian population reported no association between TaqI A and alcohol dependence (Shaikh et al.2001). In several studies, the -141C Ins/Del polymorphism has been studied for its association with alcohol dependence across different populations (Florez et al. 2008; Konishi et al. 2004; Wiesbeck et al. 2006), though, the findings are varying. In D2 receptor expression this polymorphism at promoter performs a significant role. An in vitro investigation put forward that SNP -141C Ins/Del of DRD2 modifies its activity of transcription and thus controls the DRD2 receptor expression (Arinami et al.1992). Samochowiec et al. (2000) in a genetic association study, reported a probable function of SNP -141C Ins/Del (in particular haplotypic

combination, Ins/ G/A2) in German alcohol dependent (Samochowiec et al.2000). Then again, in a case-control family based study of a Polish population, they come to nothing in order to reproduce this observation (Samochowiec et al.2006). Johann et al. (2005) investigated the association of a -141delC of the DRD2 gene in German descent suitably - distinguished and initial chronic alcoholics observed with an excess of the -141delC alleles in alcoholics and also having paternal and grand-paternal account of alcohol dependence. They accomplished that although the -141delC alternate of DRD2 could be a defensive element contrary to the expansion of withdrawal symptoms, it might also be a influencing factor in a highly saddled subcategory of alcohol dependents with a prior history of paternal and grand-paternal alcoholism (Johann et al.2005). Significant association of SNP -141C Ins/Del ($P < 0.05$; OR 1.19, 95% CI= 0.10-2.36) in DRD2 gene with alcohol dependence go along by categorical study signifying -141C Ins allele to be influencing to alcohol dependence in the population studied and is consistency with observations of Konishi et al.(2004), which suggested that -141C Ins allele causes genetic susceptibility for alcohol dependence in Mexican-Americans (Konishi et al.2004). In this way, a meaningful frequency of -141C Ins allele in alcohol dependent cases in the present study may be associated with lessened DRD2 receptor concreteness in AD cases, which consecutively encourages desire - compensate pathway – thus stimulating alcohol dependence. Additionally, our findings also indicate that presence of genotype -141C Del/Del deliberates defence from Alcohol Dependence (AD) and allele -141C Ins carriers are more susceptible for acquiring AD in contrast to -141C Del carriers. SNP *TaqI* B is confine occurrence near the regulatory and structural coding regions (5' region) of the DRD2 and consequently assumed to play a significant role in functioning of gene (Itokawa et al.1993). It has been infrequently studied for its correlation with Alcohol Dependence (AD). Two studies (Konishi et al.2004a; Konishi et al. 2004b) conducted out in population of Mexican-American and described inconsistent findings in context to association of this polymorphism with Alcohol Dependence (AD). Our findings suggest no allelic or genotypic correlation of genetic polymorphism *TaqI* B at DRD2 with AD in central Indians go along with Konishi et al. (2004) stating no assured association of *TaqI* B with AD in population of Mexican-Americans. Though in a successive study, on the same population suggested a correlation of *TaqI* B polymorphism with early age of onset for alcohol drinking in Mexican-Americans (Konishi et al.2004b). "DRD2 TaqI A" is currently known as ANKK1 (rs1800497), and is one of the utmost commonly investigated polymorphisms in genetic studies on DRD2. Neville et al. (2004) revealed that the DRD2 *TaqI* A polymorphism is in fact placed not within DRD2, but somewhat within exon 8 a protein-coding region adjacent to ANKK1 gene. Blum et al. (1993) revealed an association of A1 allele with alcoholism as the existence of A1 allele of DRD D2 receptor gene categorize 77% of alcohol dependents. Due to contradictory findings association investigations of the DRD2 *TaqI* A1 allele and alcohol dependence have stayed uncertain and debateable (Lucht et al. 2007). Though the DRD2 *TaqI* A polymorphism and alcohol dependence have been researched expansively, it appeared that this polymorphism possibly will assume substrate binding specificity. It continues to be revealed whether the polymorphism *TaqI* A has associations with AD entitled to its own functional properties or linkage disequilibrium it carries with a further variation of DRD2 or with the ANKK1 gene (Luchi et al.2007). Pohjalainen et al. (1998) suggested a correlation between low D2 receptor accessibility and A1 allele in controls indicating that A1 allele of the *TaqI* A polymorphism could be in linkage disequilibrium with a mutation in the promoter region of the gene that influences the expression of dopamine D2 receptor. This was expanding to confer risk association with A1 allele in later association studies in various populations like Han Chinese, Caucasians and Europeans (Shaikh et al.2001). In spite of this, some investigations from East Asian, European and Caucasian populations revealed negative association of *TaqI* A1 allele with alcohol dependence (AD) (Noble, 1998). In recent times, Samochowiec et al. (2006) also did not discover any correlation of *TaqI* A1 with Alcohol Dependence in population of Polish. Though, they revealed a statistically significant favoured A2 allele transmission of genetic polymorphism at DRD2 *TaqI* A in smaller group of cases with initial commencement and withdrawal difficulties. Results of above mentioned studies also recommended several subcategories of alcohol dependence (AD), which varies and relies on their genetic environment and could mix up gene association findings (Samochowiec et al. 2006). Aditionally, Laruelle et al. (1998) in his outcomes shows association between A1 allele and lower D2 receptor expression and suggested that D2 receptors binding capacity is not influenced by *TaqI* A polymorphism at the Dopamine D2 receptor gene. In the present study observations on correlation of *TaqI* A is entitled to vitally added heterozygote of *TaqI* A1/A2 genotype in the Alcohol Dependence (AD) cases from central India which might be recognised to so far anonymous selection pressure. Though, a negligible correlation of allele *TaqI* A1 is in accordance with the meta-analysis conceded out by Munafo et al. (2007) where A1 allele confers modest increase in risk of alcoholism. Taking into consideration, the association of *TaqI* A1 allele with Alcohol Dependence (AD) in the present investigation might be due to the modified expression of D2 receptor and compensate system in alcohol dependent cases. Our finding suggests A2/A2 genotype has the most common occurrence in central Indian population which is in consensus with Caucasian population where an additional of A2 homozygotes was observed (Kidd et al.1998). In populations with European ancestry the A1 allele reported frequency between 0.06 and 0.18, whereas knowingly a higher frequency (0.7) was

observed in South American native populations (Shaikh et al.2001). Prasad et al. (2010) reported A1 allele frequency of (0.22) in north Indian population. The present observation, reports allele frequency of (0.29) for A1 allele which is nearer to European population however in the south Indian population the A1 allele frequency was observed to (0.42) and is related to findings on south East Asian populations with allele frequency of 0.45 (Shaikh et al.2001). The noted variance in allele frequencies between population of central Indian and south Indian is because of many different waves of migrations that have induced the population genetic structure of India. To the development of Indian culture and society, the Indo-European (IE) and Dravidian (DR) speaking groups are considered as the major contributors. Population from south India belongs to Dravidian language family and those from northern India belongs to Indo-European (Caucasoid) background and the central Indians belongs to three language families- Dravidian, Indo-European and Austro-Asiatic language families (Prasad and Thelma, 2007). Findings of nonaligned association of SNPs were reproduced in haplotypic association. As assumed haplotype containing -141C Ins and *TaqI* A1 alleles was observed to be influencing to Alcohol Dependence and bestowed ≈ 3.2 times risk to cases with this combination of haplotype (-141C Ins-A-A1). Instead haplotypic combination -141C Del and *TaqI* A2 alleles (-141C Del-A-A2) appears to give resistance against alcoholism. Therefore, our findings of haplotype association (-141C Ins-A-A1) is in restricted consent with German population where haplotype Ins/A2 influences towards severe alcoholism [36]. Inconsistency between our and the previous report (Konishi et al.2004a) could be assigned to population and geographic specific differences in genomic structure.

CONCLUSIONS

In DRD2, two polymorphisms namely, -141C Ins/Del and *TaqI* A appear to have proven influences in the occurrence of alcoholism. Although, our findings have to be observed in the assessment of prospective drawback caused by small sample size and it demands reproduction in larger set of samples.

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