



Association of the A1 allele of D2 dopamine Receptor gene Polymorphisms with Alcohol and Drug abuse among some of Iraqi Population

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Abstract: Dopamine is known to be involved in several essential brain functions, such as locomotor behavior, cognition, motivation and neuroendocrine secretion, with its actions mediated via dopamine receptors. In particular the dopamine D2 receptors has been implicated in reward mechanisms in the brain. Dysfunction of the D2 dopamine receptors lead to aberrant substance seeking behavior. A widely studied of single nucleotide polymorphism (SNP), which so-called *drd2/ANKK1 Taq1A* polymorphism (rs1800497, Glu713Lys) is located ~10 kb downstream from the *drd2* gene in the ankyrin repeat and kinase domain containing 1 (ANKK1) gene. The presence of A1 allele of the *drd2* gene has been associated with a predisposition for addiction although there are limited data about its phenotypic expression in addiction. Methods: The case control study was designed to determine the association of D2 dopamine receptors gene polymorphism and substances abused on 115 individuals (80 control and 35 person addicted with alcohol and drug substances) were collected from Al- Hilla Prison Reform Central in Babylon. DNA was extracted from the peripheral blood of all participants, and the abovementioned single-nucleotide polymorphisms (SNPs) were genotyped by RFLP –PCR (Restriction Fragment Length Polymorphism). The results were confirmed by using sequencing technique. Results: The result of the RFLP PCR and DNA sequencing methods revealed that the TaqI A1/A1 *drd2* genotype was significant associated with addiction (OR= 9.75 ; 95%, CI = 1.94-29.08, P= 0.003), among 80 control A1/A1 genotype was 2.5% (2/80), while in addicted individual was 20% (7/35) and the A1 allele frequency was related with 1.9 fold higher risk for addiction than the A2 allele (OR= 1.88;95%, CI= 1.04-3.4 , p= 0.028). In conclusion, the presence of the Taq A1 allele of the *drd2* gene results in a significant increase in the risk of developing addiction.

Key words: D2 dopamine receptors, Alcohol, drug substance, Addiction, *drd2* gene, A1allele

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Introduction

The dopaminergic system plays critical role in mediating behavior effects and artificial reward with brain stimulation reward and addictive drugs (1, 2). The deficiency in dopamine function result in abnormal drug and alcohol seeking behavior (3). The D2 dopamine receptor gene (*drd2*), localized in the q22-q23 segment of chromosome 11 and contains single

nucleotide polymorphism (SNP) located in 3'-noncoding region (*Taq1A* polymorphism). The polymorphism may serves as a biological marker of the expression level and function of dopamine receptor D2 (4). A strong correlation between variants of the dopamine D2 receptor gene and alcoholism and polysubstance abuse

(including crack/cocaine) has been reported by several Investigators (5, 6).

Addiction is a complex behavioral disorder which appeared to reflect the additive effects of genetic and risk environment factors. Since one of the neurotransmitters playing a major role in addiction is dopamine, reasonable candidates involved in alcohol and drug action are dopamine -related genes (7).

Several groups of genes that have been identified by researchers efforts and which have been implicated in affecting risk for dependence on both alcohol and illicit drugs include the genes that have been associated with alcohol dependence most consistently are those encoding the enzymes that metabolize alcohol. Genes encoding proteins that altering the transmission of signals among nerve cells (receptors) in the brain (8).

The evidence suggesting that certain genetic factors contribute to the development of Alcohol and Other Drug (AOD) lead to disorders, as well as to the development of a variety of forms of externalizing psychopathology that is psychiatric disorders characterized by disinhibited behavior such as antisocial personality disorder, attention deficit/hyperactivity disorder (ADHD) and conduct disorder. After summarizing the difficulties associated with, and recent progress made in the identification of specific genes associated with AOD dependence (9) The aim of this study was to determine the association between TaqI *drd2* gene polymorphism frequencies among alcohol and drug addictions.

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Materials and Methods

Sample Collection

The study samples consisted of 35 person addicted with alcohol and drug substances. They were collected from Al- Hilla Prison Reform Central in Babylon. In addition 80 healthy individuals were enrolled in the study (controls). Blood samples (3-5ml) were collected using EDTA tubes from each subject and stored frozen at -20°C until analysis. Each frozen blood specimen was thawed and genomic DNA was then extracted directly using tissue genomic DNA extraction kit (FAVORGEN - Taiwan). DNA purity and concentration were determined using a spectrophotometer (Nanodrop).

Genotyping of the *drd2* Taq I A polymorphism

Genotyping was performed using Restriction Fragment Length Polymorphism (RFLP - PCR) of the D2 receptor gene and DNA sequencing method was used for polymorphism analysis. The polymorphism was detected by PCR using primers forward: 5'CCGTCGACGGCTGGCCAAGTTG TCTA-3' and reverse: 'CGTCGACCCTTCCTGAGTG TCATCA-3' (10). The components of PCR working solution were mentioned (Table 1).

Table (1): The Master Mix components of PCR.

Component	Amount (µl)	Concentration
Master Mix	12.5	1X
DNA	5	25 ng/µl
<i>drd2</i> primers	2	10pmol
DNAs free water	Up to 25µl	-
Total volume	25µl	-

PCR Protocol

The PCR was performed in a thermo cycler under the following conditions adopted (10) (Table2).

Table (2): The PCR protocol for *drd2* Taq I A polymorphism detection.

No	Steps	Temperature °C	Time	Cycle	Product size (bp)
1.	Initial denaturation	94	10 min.	1	310
2.	Denaturation	94	1 min.	35	
3.	Annealing	68	1 min.		
4.	Extension	72	1 min.		
5.	Final extension	72	5 min.	1	
6.	Hold	4	5 min.	1	

The PCR products were loaded to electrophoresis with gel electrophoreses in 2 % agarose gels stained with ethidium bromide (10 mg/ml), photographed and analyzed using gel documentation system (Harvard/UK) to check the amplification of the desire piece of gene which is approximately 310 bp A 50 bp DNA ladder (Bioneer-south Korea) were used as a size marker.

RFLP – PCR Protocol

The RFLP analysis of Taq 1A *drd2* gene was accomplished according to New BioLabs England company protocol with some modifications. The digested amplified DNA fragments were electrophoresed on 2% agarose for 2 hour at (5-7 V/cm). The bands were visualized after staining with ethidium bromide under UV light. A 50 and 100 bp DNA ladder (promega /USA) were used as a size marker for estimation of the molecular size of the amplified bands (Table 3).

Table (3) Component volume of RFLP Taq 1A SNP digested by restriction enzyme Taq^o1

Materials	Volume µl
PCR product	10
Enzyme	0.25 (5 unit)
Cut smart buffer 1X	5
dH2O	To 30
Incubation at 2-4 hour	65° C

DNA Sequencing (PCR-Sequences) PCR- Sequences of *drd2* was performed by MACROGEN Company / Korea, for confirming the presence of polymorphisms. Analysis of sequence

results for both strand (forward and reverse) were accomplished by (NCBI BLAST) online at (www.ncbi.nlm.nih.gov) Statistical Analysis

Statistical analysis was carried out using SPSS version 23. Categorical variables were presented as frequencies and percentage. Chi-square test and Fisher exact test were used to compare between percentages (frequencies) in this study. The odds ratios (ORs) and 95% confidence intervals (95% CIs) were used to evaluate the potential associations between genetic variants dopaminergic genes and the risk of

violent criminal behaviour in this study. *P* value for all tests was considered significant if ≤ 0.05 .

Results and Discussion

Concentration and Purity of DNA
Based on nanodrop device, the concentration and purity of DNA extracted from blood samples range from 23 to 186 $\mu\text{g/ml}$ and 1.5 to 2.0 respectively (Figure 1).

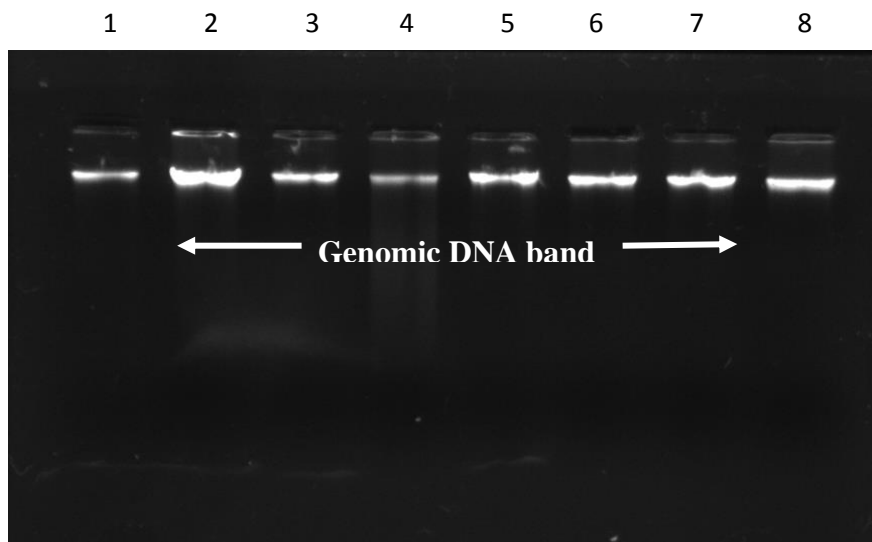


Figure (1): Gel electrophoresis for DNA human blood samples on 1% agarose gel at 70 volt for 1 hour.

Genotypes and Allele Frequencies for *drd2* TaqI A polymorphism Analysis of *drd2* Taq1A Polymorphism by RFLP-PCR

The TaqI allele is the most frequently studied polymorphism of the *drd2* gene that has a point mutation (C \rightarrow T) to produce two alleles, minor (A1) allele that removes the TaqI site and major (A2) allele has the TaqI site intact (11). Using the restriction

enzyme Taq⁰1 for the digestion of PCR product of *drd2* gene, the genotypes of studied subject were distributed into three groups based on the presence or absence of TaqI A *drd2* polymorphism: A2/A2 (CC) homozygous, demonstrated both 180 and 130 bp fragments; A1/A1(TT) homozygous, presented with the expected 310 bp fragment; and A1/A2 (CT) heterozygous state exhibited 310, 180 and 130 fragments (Figure 2, 3).

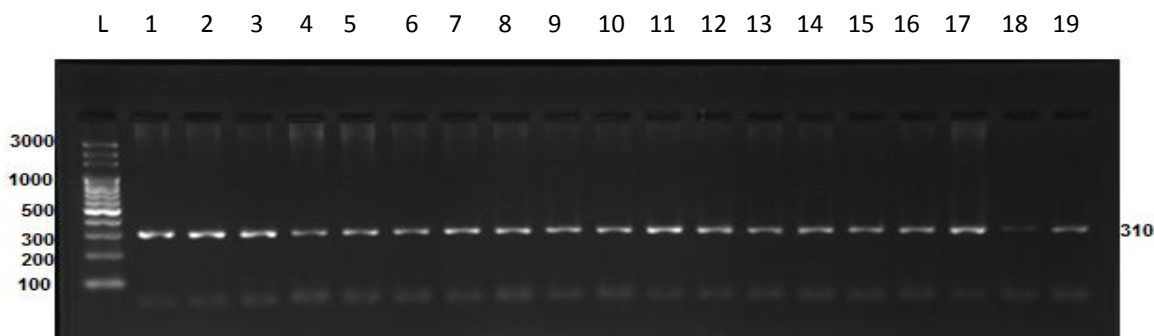


Figure (2): Electrophoresis pattern of PCR product for *drd2* gene (310bp, 2% agarose, 6 V/cm, for 2 h, Lane L: DNA ladder (bp), lane 1-19: PCR product).

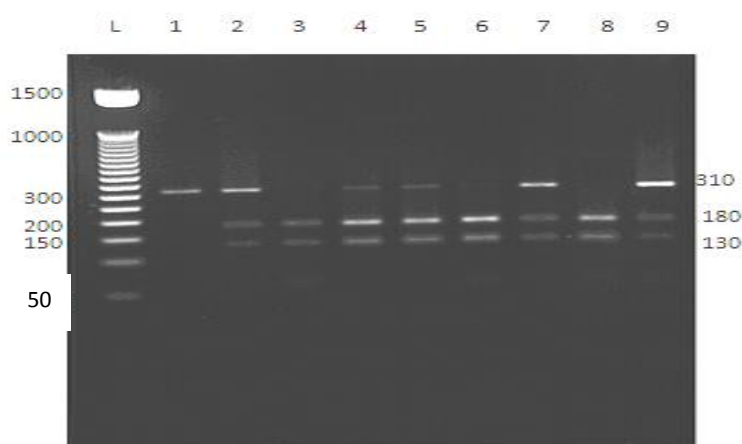


Figure (3): Electrophoresis pattern of RFLP- PCR for PCR product (310bp) with restriction enzyme (*Taq^I*), 2% agarose, 6 V/cm for 2 h (10 μ l in each well). Lane L: DNA ladder 50 bp. Lane 3, 6, 8: showing bands 180 and 130 bp, (A2/A2) genotype, (homozygotes state). Lane 1: showing band 310 bp (A1/A1) genotype (homozygotes state), Lane 2, 4, 5, 7, 9: showing bands 310,180 and 130 bp (A1/A2) Genotype (heterozygotes state).

Analysis of *drd2* TaqI A Polymorphism by DNA Sequencing Method

Samples related to A2/A2, A2/A1, and A1/A1 genotypes, were sequenced to confirm and determine the nucleotide changes responsible for antisocial behavior by Company Macrogen / Korea. Results analysis of the sequencing of samples with genotype A2/A2 were 100% compatible with the sequence of the gene bank of the NCBI (<http://www.ncbi.nlm.nih.gov/nuccore/L22303.1>). Whereas the sequencing analysis of the samples with variability regions revealed 98%-99%

compatibility through substitution of one SNP. PCR-Sequences of 3'UTR was done for sequencing of products by applied forward and reverse primer to detect variant region of *drd2* gene. The results were compared with data obtained from Gene Bank published which is available at the National Center Biotechnology Information (NCBI) online at (www.ncbi.nlm.nih.gov) and aligned by using BLAST program with a comparative by BioEdit program. The results of alignment showed that 100% similarity or homology of normal genotype sample with sequence from the Gene Bank is shown in (Figure 4).

A)) Score Expect Identities Gaps Strand

547 bits(296) 1e-160 307/312(98%) 4/312(1%) Plus/Plus

Query 1
CCGTCGACGGCTGGCCAAGTTGTCTAAATTTCCATCTCGGCTCCTGGCTTAGAACCACCC 60

|||||

Sbjct 1
CCGTCGACGGCTGGCCAAGTTGTCTAAATTTCCATCTCGGCTCCTGGCTTAGAACCACCC 60

Query 61
AGAGTGGCCACTGACGGCTCCTTGCCCTCTAGGAAGGACATGATGCCCTGCTTTCGGCTG 120

|||||

Sbjct 61
AGAGTGGCCACTGACGGCTCCTTGCCCTCTAGGAAGGACATGATGCCCTGCTTTCGGCTG 120

Query 121
CGGAGGGCCAGTTGCAGGGGTGTGCAGCTCACTCCATCCTGGACGTCCAGCTGGGCGCCT 180

|||||

Sbjct 121
CGGAGGGCCAGTTGCAGGGGTGTGCAGCTCACTCCATCCTGGACGTCCAGCTGGGCGCCT 180



Query 181
GCCTGACCAGCACTTTGAGGATGGCTGTGTTGCCCTTGAGGNCNGGNCCAGGTGGGCGG 240

|||||

Sbjct 181 GCCTGACCAGCACTTTGAGGATGGCTGTGTTGCCCTTGAGGGC-GG-
CCAGGTGGGCGG 238

Query 241 GTGTC-AGCCCA-
CTTGTTGCGGGCGTGGACATTTGCGTGATGTTCTAGGAGGTTGATGA 298

|||||

Sbjct 239
GTGTCCAGCCACCTTGTGCGGGCGTGGACATTTGCGTGATGTTCTAGGAGGTTGATGA 298

Query 299 CACTCAGGAAGG 310

|||||

Sbjct 299 CACTCAGGAAGG 310

The distribution of genotype and allele frequencies among groups studied compared with controls for the *drd2* gene is shown in (Table 4). The genotypic frequencies of A2/A2, A2/A1 and A1/A1 in addictive individuals were 42.9% and 37.1% and 20 %, respectively. The corresponding frequencies in control were 52.5%, 45% and 2.5% .A significant increase

in the genotype frequency of A1/A1 genotype was observed in addictive individual ($p = 0.003$; OR= 9.75)(Table 4). There were no significant different between the observed and expected genotype frequencies in patients or controls; therefore, the results were in a good agreement with H. W. Equilibrium.

Table (4): Comparison of the genotype and allele frequencies of *drd2* Taq1A polymorphism between control and addictive subjects

<i>drd2</i> TaqA1 Polymorphism	Frequencies (%)		P value	Odd ratio (95% CI)
	Controls (No=80)	Addictive subject (No=35)		
Genotype				
A2/A2(CC)	42(52.5%)	15(42.9%)	NS	0.68 (0.31-1.50)
A1/A2(TC)	36(45%)	13(37.1%)	NS	0.72 (0.32-1.62)
A1/A1 (TT)	2(2.5%)	7(20 %)	0.003	9.75 (1.94-49.08)
Allele				
A2(C)	120(75.0%)	43(61.4%)	0.028	0.53 (0.29-0.96)
A1(T)	40(25 %)	27(38.6%)	0.028	1.88 (1.04-3.42)

NS: Non-significant

The frequency of the A1 allele in addictive individuals and controls were 27(38.6%) and 40(25 %) respectively. The A1 allele was associated with 1.88 fold higher risk for addiction than the A2 allele (95% CI=1.0-3.6; $p= 0.028$). These results are in agreement with Berggren and his colleagues (11) found that TaqI A1 allele frequency was significantly over represented in alcohol dependent subjects compared with control (OR=1.34, 95% CI= 1.08-1.67, $P=0.007$). The observed association with alcohol consumption is most easily explained by the proposed association between TaqI A1 genotypes and D2 receptors availability. Non-alcohol individuals with low density of DRD2 may not gain much pleasure from drinking and the extra dopamine released, because they lack dopamine binding site which mediate reward (12). Ponce *et al.*(7) revealed that in their

study on sample of 103 alcohol – dependent male, approximately 39% of the substitution of C to T in sample carried the A1 allele, that the presence of A1 was the main factor to explain the diagnosis of antisocial personality disorder (13).

Molecular geneticists have also searched for how carriers of the A1 allele of the *drd2* expressed gene may be differentially predisposed to develop neuropsychiatric disorders and debilitating diseases (14).

These findings suggest that the DRD2 gene may also contribute to the development of aberrant behavior, such as addiction and impulsive behaviors (15). In conclusion, this study finding evidence suggestion that the Taq IA *drd2* gene polymorphism coincides with sensitivity to alcohol and drug abuse in addictive sample.

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