A *DRD2* and *ANKK1* haplotype is associated with nicotine dependence

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Abstract

To test the importance of the dopamine D2 receptor (DRD2) region in nicotine dependence, 150 smokers and 228 controls were genotyped for the *DRD2* C957T, -141delC and *ANKK1 Taq*IA polymorphisms (rs6277, rs1799732 and rs1800497, respectively). The -141delC SNP did not show any association but both the C957T and *Taq*IA SNPs showed association at the allele, genotype, haplotype and combined genotype levels. The 957C/*Taq*I A1 haplotype was more than 3.5 times as likely to be associated with nicotine dependence compared with the 957T/*Taq*I A1 haplotype (P = 0.003). Analysis of the combined genotypes of both SNPs revealed that individuals who were homozygous for the 957C-allele (CC) and had either one or two copies of the *Taq*I A1-allele were 3.3 times as likely to have nicotine dependence compared to all other genotype combinations (P = 0.0003) and that these genotypes accounted for approximately 13% of the susceptibility to nicotine addiction in our population. Our findings suggest that the *DRD2* C957T polymorphism and the *ANKK1 Taq*IA polymorphism are key contributors to the genetic susceptibility of nicotine dependence.

Keywords: genetic association; dopamine D2 receptor; ANKK1; addiction; haplotype

1. Introduction

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 stimulates the mesolimbic reward pathways of the brain (Corrigall et al., 1992; Corrigall et al., 1994; Cami et al., 2003). Various aspects of smoking such as initiation of smoking and cigarette consumption levels are also known to be highly heritable, as is the heritability of nicotine dependence, estimates of which range from 31% to 75% (Vink et al., 2005). Genes involved in dopamine metabolism within the reward pathways of the brain are excellent candidates for genes contributing to the genetic component of nicotine dependence.

Much interest has focussed on the dopamine receptor genes, which are expressed in these brain regions (Hurd et al., 2001), particularly the dopamine D2 receptor (*DRD2*) gene (Noble, 2000; 2003). The *DRD2* region of chromosome 11 (11q23) has also been linked with cigarette consumption in genome wide linkage analyses although not with nicotine dependence unless comorbid with alcohol dependence (Gelernter et al., 2004). A number of polymorphisms have been studied in *DRD2* but it is interesting that the one most commonly tested and referred to as a *DRD2* variant, known as the *Taq*I A (rs1800497) polymorphism, was subsequently shown to lie in an adjacent gene named the ankyrin repeat and kinase domain containing 1gene (*ANKK1*) (Dubertret et al., 2004; Neville et al., 2004). The *Taq*I A1 (T) allele has been associated with a variety of smoking measures in a number of different studies although such findings are not consistent (Ho et al., 2007). A recent meta-analysis also failed to observe association between smoking behaviour and the *Taq*I A polymorphism (Munafo et al., 2009) but reports of association continue to appear (Stapleton et al., 2011). Other polymorphisms within *DRD2* include *Taq*I B (rs1079597), *Taq*I C (simple tandem repeat), *Taq*I D

(rs1800498), -141delC (rs1799732), and C957T (rs6277), all of which have been reported to be associated with aspects of smoking behaviour or response to treatment in at least one study (Lerman et al., 2006; Ho et al., 2007; Vandenbergh et al., 2007). No associations have been reported, however, for the exon 8 G/A SNP, rs6276 (Johnstone et al., 2004) or SNPs rs6589377, rs4482060, or rs4938023 (Gelernter et al., 2006). Given the number of genetic variants found to be associated with nicotine dependence and other smoking related phenotypes in the *DRD2* region and the lack of consistency in replication of many of those associations between studies it is likely that the influence of this region is not simple. Rather than a single variant of substantial effect there may be two or more polymorphisms that contribute additively or epistatically, or are subject to genotype by environment interaction.

Here we describe an association study involving individual SNPs and specific haplotypes of SNP alleles for three variants spanning this region including -141delC (rs1799732) in the promoter of *DRD2*, C957T (rs6277) 62.8Kb down-stream in exon 7 of *DRD2*, and *Taq*I A (rs1800497) a further 12.6 Kb away in exon 8 of *ANKK1* and only about 9.5 Kb from the 3'-end of the *DRD2* mRNA.

2. Methods

2.1. Participants

A total of 150 smokers (Caucasian, age: 43.3±11.1; 68 male, 79 female) were recruited for this study through hospital and media advertisements. Participants were 18 years of age or older and had smoked for at least three years and were generally healthy despite currently smoking 15 cigarettes or more per day. All were motivated to reduce smoking and had the goal of eventual cessation. However, all participants had at least one serious but unsuccessful attempt at quitting in the previous 24 months. Patients were administered the Fagerstrom test for Nicotine Dependence (Heatherton et al., 1991).

The control group consisted of 95 females and 133 males, with a mean age of 36.8 years (s.d. $\pm$ 12.8 years) and were recruited from volunteers from the general public and staff and students of the Royal Brisbane Hospital and the Queensland University of Technology. Formal screening for nicotine dependence was not undertaken as this control group represents a random sample of the populations tested and comprised non-smokers and smokers different categories, including nicotine dependent, in ratios approximating that of the whole population.

2.2. Genotyping

*Taq*I A and -141delC genotyping was performed by restriction fragment length polymorphism (RFLP) analysis of PCR products. A genomic sequence of 500 bp of the coding region of *ANKK1* was amplified by PCR using the forward primer 5′-GCACGTGCCACCATACCC-3′ and the reverse primer 5′-TGCAGAGCAGTCAGGCTG -3′. A genomic sequence including 284 bp from the 5′-flanking region and 274 bp from exon 1 of *DRD2* was amplified by PCR using forward primer 5′-ACTGGCGAGCAGACGGTGAGGACCC-3′ and reverse primer 5′-TGCGCGCGTGAGGCTGCCGGTTCGG-3′. A total of 5-10 ng of genomic DNA was amplified in a PCR master mix containing 0.2 μM of forward primer and 0.2 μM of reverse primer, 1x PCR buffer, 1.5 mM $MgCl_2$, 200 μM dNTPs and 1 unit of Platinum *Taq* DNA Polymerase (Invitrogen) in a 25 μL volume. Amplification conditions for the *Taq*I A polymorphism were: Step 1: 94°C for 4 min, Step 2: 94°C for 30 s, Step 3: 68°C for 30 s, Step 4: 72°C for 30 s, Steps 2-4 were repeated by 40 cycles followed by 72°C for 3 min. Amplification conditions for the -141delC polymorphism were: Step 1: 95°C for 3 min, Step 2: 95°C for 30 s, Step 3: 68°C for 30 s, Step 4: 72°C for 30 s, Steps 2-4 were repeated 40 times followed by a final 72°C step for 2 min. Amplified PCR

fragments were digested with *Taq*I restriction enzyme or *Bst*NI (New England Biolabs) (*Taq*I A and -141delC, respectively) and digested fragments were visualized via agarose gel electrophoresis.

The DRD2 C957T polymorphism was genotyped as previously described (Lawford et al., 2005).

2.3. Statistical analysis

For each polymorphism, a homogeneity χ^2 analysis was employed to test the hypothesis of homogeneity of allele frequency distributions between nicotine dependent and control populations. An odds ratio with 95% confidence interval (CI) was also calculated. Genotype frequencies were similarly compared between nicotine dependent and control populations. However, where an allele was found to be associated with nicotine dependence, an extended Mantel-Haenszel test was performed to test for a trend to nicotine dependence with increasing copy number (0, 1, or 2) of the allele. Odds ratios were calculated relative to the homozygote for the non-associated allele and population attributable fractions calculated. Genotype data were entered into JLIN v1.6.0 (Carter et al., 2006) to estimate haplotype frequencies. Haplotype frequencies were compared between nicotine dependent and control groups using a homogeneity χ^2 analysis. Each haplotype with a significant association was analysed in isolation by partitioning the χ^2 accordingly. All statistics were carried out using the epidemiological software Compare2 v2.17 (Abramson, 2004). Generated haplotypes were analysed for linkage disequilibrium (LD) measures (D' and r^2) using the JLIN (Carter et al., 2006).

3. Results

3.1. C957T genotyping

In order to find genetic associations between nicotine dependence and SNPs in the *DRD2* region the C957T polymorphism was genotyped in 228 controls and 150 nicotine dependent subjects. The C957T polymorphism showed association with nicotine dependence at the allele level ($P = 0.022$, Table 1). The genotypes of both groups are displayed in Table 2. A significant trend in susceptibility to nicotine susceptibility was found when analysed under a partially dominant model for C957T ($P = 0.024$; Mantel-Haenszel test for trend). The attributable fraction of the C957T polymorphism was calculated using genotypes counting the presence of the C-allele as a risk factor. The attributable fraction, which is the proportion of cases in the study population that would not have occurred had the risk factor (risk allele) not have been present, is a useful measure to quantify the impact of the allele on the development of nicotine dependence (Lappalainen et al., 2002). The analysis revealed that 14.0% (95% CI = 3.18 to 24.71%) of the susceptibility to nicotine dependence could be attributed to having the CC genotype of the C957T *DRD2* polymorphism. Examination of the ORs for the 3 genotypes indicates that a C-allele recessive pattern of inheritance exists, i.e. the CT and TT genotype ORs are both approximately 1. Under a C-allele recessive model, i.e. CC vs CT plus TT genotypes, the CC genotype was more strongly associated with nicotine dependence ($\chi^2 = 5.716$, $P = 0.017$, OR = 1.82, 95% CI = 1.08 to 3.07).

3.2. TaqIA genotyping

The *Taq*IA polymorphism was also found to be significantly associated with nicotine dependence at the allele level ($P = 0.017$, Table 1). The genotypes of both groups are displayed in Table 2. A significant trend in susceptibility to nicotine dependence was

found when analysed under a partially dominant model ($P = 0.018$). Examination of the ORs for the 3 genotypes indicates that a A1-allele dominant pattern of inheritance exists, i.e. the A1A1 and A1A2 genotypes both have similar elevated ORs relative to the A2A2 genotype. When analysed under an A1-dominant model, the A1-containing genotypes (A1/A1 and A1/A2) were strongly associated with nicotine dependence ($\chi^2 = 6.598$, $P = 0.010$). Those individuals with an A1-containing genotype were almost twice as likely to have nicotine dependence compared to those with the A2A2 genotype (OR = 1.75, 95% CI = 1.11 to 2.75). Calculation of the attributable fraction revealed that 18.6% (95% CI = 4.1 to 30.9%) of the susceptibility to nicotine dependence could be attributed to having the A1/A1 or A1/A2 genotype of the *Taq*IA polymorphism.

3.3. -141delC genotyping

No association with nicotine dependence was observed for the -141delC polymorphism at the allele (Table 1) or genotype level (Table 2).

3.4. *Haplotype analysis*

As both polymorphisms showed association with nicotine dependence, haplotype analysis was also performed for the C957T and *Taq*IA polymorphisms. Haplotype frequencies for the two SNPs are shown in Table 3. Overall haplotype frequency was highly associated with nicotine dependence ($P = 0.0009$). This association was due to the over representation of the 957C/*Taq*I A1 haplotype in nicotine dependent patients. The 957C/*Taq*I A2, 957T/*Taq*I A2, and the 957T/*Taq*I A1 haplotypes were not significantly associated with nicotine dependence. The 957C/*Taq*I A1 haplotype was more than three times as likely to be associated with nicotine dependence compared to the 957T/*Taq*I A1 haplotype. As the 957T/*Taq*I A1 haplotype is quite rare (6.6% of

controls), it is probably more realistic to consider the 957T/*Taq*I A2 and 957C/*Taq*I A2 haplotypes as "normal" risk and the 957T/*Taq*I A1 haplotype as a "low risk" protective haplotype, although it did not reach significance ($P = 0.079$).

3.5. Linkage disequilibrium

The *Taq*I A1 and the 957C-alleles were in partial linkage disequilibrium (LD) in both the control population $D' = 0.321$ and $r^2 = 0.029$ and the nicotine dependent population $D' = 0.676$ and $r^2 = 0.139$. While LD exists in the control population it is significantly larger in the nicotine group (Goodness-of-fit $\chi^2 = 11.61$, $P = 0.003$) suggesting a functional relationship between alleles not just LD as a consequence of proximity alone. Both nicotine dependent and control groups were determined to be in Hardy-Weinberg equilibrium based on the respective genotype frequencies of each group.

3.6. Combined C957T and TaqIA genotypes

The combined genotypes of both the C957T SNP and the *Taq*IA SNP were scored for each individual. All nicotine dependent patients genotyped for both SNPs so 150 combined genotypes were obtained, however one control sample failed to genotype for C957T and a different sample failed to genotype for *Taq*IA so only 226 combined control genotypes were obtained (Table 4). As there are 9 possible combined genotypes, the counts for each genotype are quite low and none reach significance except the CC12 genotype. People with the CC12 genotype are almost six times as likely to have nicotine dependence than those with the TT12 genotype ($P = 0.016$). Examination of the odds ratios in Table 4 reveals that there are two high risk genotypes (CC11 and CC12) compared to the remaining low risk genotypes. When the CC11 and CC12genoypes were compared with the rest, people with these genotypes were more

than 3 times as likely to have nicotine dependence (χ^2 = 12.933, *P* = 0.0003, OR = 3.32, 95% CI = 1.61 to 7.11) and the attributable fraction in the population was 12.6% (95% C.I. = 5.1% to 19.5%).

4. Discussion

Analysis of three SNPs in the *DRD2* region, including the *ANKK1* gene revealed associations with nicotine dependence at the allele level for the 957C>T and the *Taq*I A polymorphisms. At the genotype level, the best significance was reached under a recessive model for C957T and a dominant model for *Taq*I A. These results are consistent with other studies that report the A1 allele of the *Taq*IA polymorphism and the C-allele of the *DRD2* C957T to be associated with a range of behavioural and brain responses to nicotine in humans (Jacobsen et al., 2006; Lerman et al., 2006; Ho et al., 2007; Vandenbergh et al., 2007; Perkins et al., 2008a; Perkins et al., 2008b; Wang et al., 2008). We did not observe any association with the -141delC polymorphism and nicotine dependence in our Australian population. This is also consistent with other studies that have not reported association with -141delC and smoking behaviour (Yoshida et al., 2001; Styn et al., 2009). However, the -141delC polymorphism has been associated with response to pharmacotherapy for tobacco dependence (Lerman et al., 2006).

In this study, along with individual associations we also found a haplotype including the 957C and *Taq*IA1 alleles to be significantly associated with nicotine dependence.

In a recent study a protective haplotype including the C957T polymorphism was found to be associated with reduced risk to become a smoker (Wernicke et al., 2009).

Earlier studies have reported that dopamine release in the ventral striatum is highly sensitive to nicotine (Brody et al., 2004). Our results support the hypothesis that a defect in the central dopaminergic pathway contributes to nicotine dependence (Grieder

et al., 2010) by stimulation of the dopamine reward pathway. It has long been known that Nicotine preferentially stimulates dopamine release in the limbic system of rats (Imperato et al., 1986) and it is believed that smoking stimulates the mesocorticolimbic circuits of the brain, which is thought to be central in behavioural reward and reinforcement (Noble, 2000). Using DRD2 knockout mice it has been shown that signalling at DRD2 is critical in mediating withdrawal aversions in nicotine-dependent animals. One or more polymorphisms in the DRD2 receptor and nearby genes which are central to the dopamine system could result in increased production of dopamine thus increasing the risk of nicotine dependence.

Examination of the odds ratios for the C957T polymorphism suggests it follows a recessive pattern of inheritance with respect to nicotine dependence. We describe the pattern as recessive because the odds ratio of the heterozygote did not differ from the odds ratio of the homozygote for the allele that was not associated with nicotine dependence. In contrast the *Taq*IA polymorphism appears to follow a dominant mode of inheritance for nicotine dependence. Both observations are consistent with inheritance patterns that have previously been observed for these two polymorphisms (Lawford et al., 2005; Connor et al., 2007). Also consistent with this pattern of inheritance is the observation that there were two high-risk double genotypes (CC11 and CC12), i.e. those people who are homozygous for the C957T C-allele and also have one or two *Taq*IA A1-alleles have increased risk of nicotine dependence.

It is known that these *DRD2*-associated SNPs are involved in other disorders involving dopamine perturbation like schizophrenia, PTSD and alcohol dependence (Lawford et al., 2005; Connor et al., 2008; Voisey et al., 2009). The C957T polymorphism has been shown to affect mRNA stability and protein translation of the receptor (Duan et al., 2003) and affects striatal dopamine D2 binding in healthy subjects (Hirvonen et al.,

2004). A common molecular defect in the dopamine pathway is likely to explain some of the genetic susceptibility to mental illness and substance misuse.

Using a general population as controls we were able to calculate attributable risk and conclude that 14% of the susceptibility to nicotine dependence could be attributed to having the CC genotype of the C957T polymorphism and 18.6% of the susceptibility to nicotine dependence could be attributed to having the A1/A1 or A1/A2 genotype of the *Taq*I A polymorphism. Using combined genotype data we were able to identify two high-risk genotypes that are more than 3 times as likely to have nicotine dependence and account for almost 13% of the population risk for nicotine dependence. This information provides a good starting point for the development of a test that can discriminate those at high and low risk of developing alcohol dependence.

The sample size for the nicotine dependent group in our study was small and the associations will need to be confirmed independently, preferably using a larger sample size of nicotine dependent individuals. However, our significant results are encouraging using unselected controls as a more significant association is likely to be found using controls screened for smoking behaviours. In this study, we used an unselected control population of unknown smoking status. Further studies would also benefit from having a control group who have never smoked as well as a control group that have been casual smokers but who do not have nicotine dependence.

Considerable evidence suggests the importance of dopamine D2 receptor function in nicotine dependence. This study supports these findings by confirming the associations with the C957T (rs6277) and the *Taq*I A (rs1800497) polymorphisms with nicotine dependence. Further investigation of the *DRD2* region may improve understanding of the pathophysiology of nicotine dependence which may lead to targeted

pharmacogenomic treatment strategies and the ability to discriminate those people who are likely to develop nicotine dependence from those who are not.

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Table 1. Allele frequencies of C957T, *Taq*I A, and –141delC polymorphisms of the *DRD2* region in patients with nicotine dependence compared to controls

Group	***n***	**Allele frequency**		χ^2	***P***	**Odds Ratio (95% CI[a])**
C957T		**C (%)**	**T (%)**			
Control	454	193 (42.5)	261 (57.5)	5.242	0.022	1.41 (1.04 to 1.91)
Nicotine	300	153 (51.0)	147 (49.0)			
***Taq*I A**		**A1 (%)**	**A2 (%)**			
Control	454	77 (17.0)	377 (83.0)	5.646	0.017	1.55 (1.06 to 2.25)
Nicotine	300	72 (24.0)	228 (76.0)			
–141delC		***Del* (%)**	***Ins* (%)**			
Control	424	48 (11.3)	376 (88.7)	0.231	0.631	1.13 (0.67 to 1.94)
Nicotine	266	27 (10.2)	239 (89.8)			

[a] Fisher's exact confidence interval.

Table2. Individual genotype frequencies of C957T, *Taq*I A, and –141delC polymorphisms of the *DRD2* region in patients with nicotine dependence compared to controls

Group	***n***	**Genotype frequency**			**χ^2 (*P*)**
C957T		**C/C (%)**	**C/T (%)**	**T/T (%)**	
Control	227	40 (17.6)	113 (49.8)	74 (32.6)	6.053* (0.048)
Nicotine	150	42 (28.0)	69 (46.0)	39 (26.0)	5.086[a] (0.024)
Odds Ratio[b]		1.992 *P* = 0.039	1.159 *P* = 1.000	1.000	
***Taq*I A**		**A1/A1 (%)**	**A1/A2 (%)**	**A2/A2 (%)**	
Control	227	8 (3.5)	61 (26.9)	158 (69.6)	6.622* (0.036)
Nicotine	150	7 (4.7)	58 (38.7)	85 (56.7)	5.605[a] (0.018)
Odds Ratio[b]		1.626 *P* = 0.734	1.767 *P* = 0.025	1.000	
–141delC		***Del/Del* (%)**	***Del/Ins* (%)**	***Ins/Ins* (%)**	
Control	212	4 (1.9)	40 (18.9)	168 (79.2)	0.813 (0.666)[c]
Nicotine	133	1 (0.008)	25 (18.8)	107 (80.5)	0.224[a] (0.636)
Odds Ratio[b]		0.393 *P* = 0.735	0.981 *P* = 0.999		

* Likelihood-ratio χ^2 test .
[a] Mantel-Haenszel χ^2 test for trend.
[b] With respect to homozygous genotype of allele not associated with nicotine dependence
[c] Likelihood-ratio χ^2 test .At least one cell has an expected frequency of < 5

Table 3. Haplotype analysis of C957T and *Taq*I A polymorphisms in patients with nicotine dependence

Haplotype	**Controls** [c]	**Nicotine** [c]	**Odds Ratio**[a] **(*P*)**	χ^{2} [b]	***P***
957T/*Taq*I A1	30	11	1	3.087	0.079
957T/*Taq*I A2	230	136	1.613 (0.548)	2.225	0.136
957C/*Taq*I A2	145	92	1.730 (0.403)	0.167	0.683
957C/*Taq*I A1	47	61	3.540 (0.003)	14.471	0.0001
TOTAL	452	300		16.57	0.0009

[a] Odds ratio with respect to 957T/*Taq*I A1 haplotype which is more commonly associated with a normal phenotype.

[b] Likelihood ratio χ^{2} test comparing haplotype frequency between groups when all other haplotypes were pooled.

[c] Haplotypes frequencies were estimated using JLIN. Note, haplotypes with a missing genotype were omitted from the analysis (2 control and no nicotine dependence samples).

Table 4. Combined genotype frequencies of the C957T and *Taq*I A polymorphisms in patients with nicotine dependence

Genotype	Control	Smokers	OR	χ^2	*P*
CC11	4	5	3.333	2.177	0.981**
CC12	10	22	5.867	9.254	0.016
CC22	26	15	1.538	0.568	1.000
CT11	4	2	1.333	0.083	1.000**
CT12	35	30	2.286	2.502	0.796
CT22	73	37	1.352	0.347	1.000
TT11	0	0			
TT12	16	6	1		
TT22	58	33	1.517	0.653	1.000
Total				17.148	0.016

** At least one cell has an expected frequency < 5