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***ANKK1* and *DRD2* pharmacogenetics of disulfiram treatment for cocaine abuse**

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Abstract

Objective—Disulfiram is a potential cocaine addiction pharmacotherapy. Since dopamine deficiency has been found with cocaine addiction, our objective was to examine whether functional variants in the *ankyrin repeat and kinase domain-containing 1* gene (*ANKK1*) and/or the *dopamine receptor D2* gene (*DRD2*) interact with response to treatment with disulfiram.

Methods—Cocaine and opioid co-dependent (DSM-IV) patients were stabilized on methadone and subsequently randomized into treatment groups, disulfiram (250 mg/day, $N = 31$) or placebo ($N = 37$), genotyped for *ANKK1* (rs1800497) and *DRD2* (rs2283265) polymorphisms, and the data evaluated for association with cocaine-free urines in the presence or absence of disulfiram. Data was analyzed using repeated measures analysis of variance (ANOVA) corrected for population structure.

Results—Patients with CT or TT *ANKK1* genotypes dropped from 80% to 52% cocaine-positive urines on disulfiram ($N = 13$; $p = 0.0001$) while those on placebo ($N = 20$) showed no treatment effect. Patients carrying the CC *ANKK1* genotype showed no effect from disulfiram ($N = 18$) or placebo ($N = 17$). The GT/TT *DRD2* genotype group showed a significant decrease in cocaine-positive urines on disulfiram ($N = 9$; 67% – 48%; $p = 0.0001$), while the GG *DRD2* genotype group showed a marginal decrease ($N = 23$; 84% – 63%; $p = 0.04$). Genotype pattern analysis revealed that individuals carrying at least one minor allele in either gene responded better to disulfiram treatment ($N = 13$; $p = 0.0001$) than individuals carrying only the major alleles ($N = 17$).

Conclusions—A patient's genotype for *ANKK1*, *DRD2*, or both, may be used to identify individuals for whom disulfiram may be an effective pharmacotherapy for cocaine dependence.

Keywords

ANKK1; *DRD2*; disulfiram; cocaine; treatment; dependence

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Introduction

Cocaine is the third most commonly used drug of addiction with over 1.5 million addicts in 2010 [1]. Cocaine dependence (CD) has significant social and economic impact and is also currently without an FDA-approved pharmacotherapy. CD often occurs in tandem with alcohol dependence and frequently within methadone maintenance programs, where rates of cocaine use may range from 30% to 50%. CD may also lead to HIV risk behaviors, illicit opioid use, and overdoses requiring emergency department utilization [1–13]. Various pharmacological approaches have been evaluated to reduce CD, but with limited success [e.g. 14, 15]. Disulfiram has shown some initial promise in treating CD, including among opioid-dependent patients [16–20].

Disulfiram is a pharmacotherapy originally used to treat alcoholism and is also known as Antabuse. Disulfiram blocks the conversion of acetaldehyde (a metabolite of ethanol) into acetic acid, thereby inducing aversive physiological symptoms such as nausea and vomiting after alcohol consumption. The exact mechanism by which disulfiram exerts its effect to reduce cocaine cravings or use is not known, but may be via dopamine availability and signaling in the brain. Disulfiram inhibits dopamine β -hydroxylase (D β H), which converts dopamine (DA) to norepinephrine [21, 22]. Inhibiting D β H increases brain DA levels, which in turn increases stimulation of dopamine D2 receptors, part of the brain's 'reward circuitry' [23, 24].

Given that disulfiram influences dopamine levels in the brain, and given the strong genetic basis of CD with heritability estimated at up to 72% [25], we adopted a molecular genetic approach examining genes in, or peripheral to, the dopaminergic pathway to investigate disulfiram's efficacy in CD patients [26–29]. We recently showed that a polymorphism in the *dopamine β -hydroxylase* gene (*DBH*) that associates with normal D β H levels (rs1611115, T allele) identified CD patients who reduce their cocaine use with disulfiram [30]. This finding led us to examine other genes coding for proteins involved in dopamine signaling such as the *dopamine receptor D2* (*DRD2*) and the *ankyrin repeat and kinase domain-containing 1* (*ANKK1*) genes.

DRD2 and *ANKK1* are both located on chromosome 11q22-23 in human. The genes are approximately ten-thousand nucleotides apart and variants in these genes frequently have some degree of linkage disequilibrium with each other [e.g. 31, 32]. *DRD2* encodes the G-protein coupled dopamine D2 receptor. Given the involvement of dopamine in several psychiatric disorders it is not surprising that variants in *DRD2* have been found to be associated with several psychiatric conditions, including alcohol and nicotine addiction, schizophrenia, and migraine [33–43]. The T allele of the rs2283265 variant in the *DRD2* gene has been shown to be overrepresented in cocaine addicts as well as associated with decreased expression of the D2S form of the D2 receptor [44, 45].

The *ANKK1* gene, which encodes the ankyrin repeat and kinase domain-containing 1 protein, has a functional variant, rs1800497 (C→T, also known as *TaqIA*) that encodes a glutamate to lysine substitution at amino acid position 713. ANKK1 alters D2 receptor

stimulation via D2 receptor density, with the T allele found to be associated with lower receptor density than with the C allele [46]. *ANKK1* spans about thirteen-thousand nucleotides and is expressed in astroglial cells [47]. The T allele of rs1800497 has been shown to be associated with alcohol addiction, several psychiatric disorders [e.g. 39, 41, 48], as well as with cocaine addiction [49]. Because disulfiram increases dopamine concentration in the synapse via inhibition of D β H, and because variants in *ANKK1* and *DRD2* have been found to be in association with cocaine addiction, dopamine signaling, and D2 dopamine receptor density in the brain, we hypothesized that *ANKK1* rs1800497 and *DRD2* rs2283265 may mediate response to disulfiram pharmacotherapy in CD individuals.

Materials and methods

Participants

Ninety-three cocaine and opioid-dependent patients were stabilized on methadone maintenance during a two-week screening period at Yale University ($N=40$) and Baylor College of Medicine ($N=53$) from 2004 to 2008 [30]. Patients were selected based on the presence of at least one cocaine-positive urine sample. Eleven patients were excluded for lack of positive urine samples. Eight patients dropped out during this two week screening, and six others were eliminated due to indeterminate genotyping for a final cohort of 68 subjects. A psychiatric interview, which included the administration of the ASI [50] and MINI (English Version 5.0.0, July 1, 2006) [51], confirmed DSM-IV opioid and cocaine dependence. Exclusion criteria included a current diagnosis of other drug or alcohol physical dependence (other than tobacco), current major medical illness unstabilized on medications, a history of major psychiatric disorder (e.g. psychosis, schizophrenia, or bipolar), current suicidality, and an inability to read and understand the consent form. Women of childbearing age required an initial negative urine pregnancy test, use of adequate contraception to prevent pregnancy during the study, and monthly pregnancy tests throughout the study. All signed an informed consent approved by Yale University and the Baylor College of Medicine Institutional Review Boards that gave specific consent for genetic studies. Ethnicity was based on self-report of ethnic/cultural background of the patients.

The 68 patients were stabilized on methadone maintenance at 60 mg daily and were assigned randomly to placebo or disulfiram (250 mg daily, which was dissolved into their daily methadone dose to assure medication compliance) groups. Methadone dose increased 5 mg per day from an initial 25 mg/day until patients reached a 60 mg/day maintenance dose. Individual manual-driven cognitive behavioral therapy [52] was provided weekly to all patients. Supervised urine samples were obtained thrice weekly and tested for the presence of the cocaine metabolite benzoylecgonine using an Olympus AU 640 Emit system (Olympus America Inc., Melville, NY) with a cut-off concentration of 300 ng/ml. We obtained saliva samples for DNA isolation.

Genotyping and statistical analyses

DNA purification, genotyping, and statistical analyses were performed as previously described [30, 53]. Briefly, DNA was isolated using the Gentra Puregene Buccal Cell Kit (Qiagen, Valencia, CA) following the manufacturer's recommendations from pelleted buccal

cells that were obtained by the centrifugation of 10 ml Scope® mouthwash that was used to rinse the subject's mouth for 60 seconds.

Genotypes were determined using 5'-fluorogenic exonuclease assays (TaqMan®, Applied Biosystems, Foster City, CA). The *ANKK1* rs1800497 variant was genotyped using the TaqMan® primer-probe sets (Applied Biosystems) assay ID C_7486676_10, and the *DRD2* rs2283265 variant with assay ID C__16070796_10. PCR amplifications were performed using Platinum® quantitative PCR SuperMix-UDG (Invitrogen, Carlsbad, CA) on a GeneAmp® PCR system 9700 (Applied Biosystems).

A repeated measures analysis of variance (ANOVA) was used to analyze the number of cocaine positive urines over the total number of samples (six) for each two week period. Data from the disulfiram treatment group was compared to the placebo group over time to determine if the effect of disulfiram is modulated by the *ANKK1* locus, the *DRD2* locus, or both, using R version 2.9.1 [54]. We compared condition (disulfiram or placebo), *ANKK1* genotype (0 = CC genotype, 1 = all other genotypes), *DRD2* genotype (0 = GG genotype, 1 = all other genotypes), *ANKK1* plus *DRD2* genotype (0 = CC/GG genotype, 1 = all other genotypes), time (each two week period), and interactions between condition and time, and between condition and genotypes. We analyzed all individuals who had complete data ($N = 55$) and unbalanced repeated measures ANOVA for all individuals ($N = 68$). The two analyses yielded similar results. ANOVA was also used to analyze demographic and MINI/ASI data.

To determine population structure, our cohort was compared against CEPH-HGDP samples (1,035 subjects of 51 populations) as previously described [30, 53]. Gender, recruitment site, and population structure were run as covariates in the statistical model. We calculated effect size as a partial eta-squared statistic using condition or SNP variance over residual variance. The three general cut-offs for effect size are the following: a large effect is 0.14, a medium effect is 0.06, and a small effect is 0.01. Hardy-Weinberg equilibrium was calculated for each genetic variant and resulted in non-significant p -values indicating that the genotypes were observed in the expected ratios (*ANKK1*, rs1800497; $p = 0.567976$ and *DRD2*, rs2283265; $p = 0.3588037$).

Results

Baseline characteristics by treatment and *ANKK1/DRD2* genetics

Genotyping failed for six patients thereby providing a sample of 68 patients; 31 in the disulfiram group and 37 in the placebo group. The patients included thirty-five CC; twenty-four CT; and nine TT genotypes for *ANKK1* and twenty-three GG; thirty GT; and fifteen TT genotypes for *DRD2* (See Table 1). The patients were mostly Caucasian males with a mean age of 39 years. Forty (54%) patients previously had been treated with methadone maintenance. On average, participants used cocaine for 13 years, and for 19 days in the month prior to entering this study. Only 30 patients (40%) reported any alcohol abuse history reflecting our exclusion criteria, and 39 patients (53%) reported marijuana use. As shown in Table 1, we found no significant baseline differences among the four treatment by genotype groups in any clinical characteristics ($p > .05$).

Treatment outcomes by *ANKK1* genotype

To examine whether *ANKK1* genotypes would be found to associate with response to disulfiram, we divided the 68 patients who had been genotyped for the *ANKK1* rs1800497 variant into two groups based on presence of the minor T allele. The CT/TT genotype group showed significantly fewer cocaine-positive urines during the twelve weeks of disulfiram treatment ($F = 21.69$; $df = 1,175$; $p < 0.0001$, with an effect size of .0365) compared to placebo, while the CC genotype group showed no difference between disulfiram and placebo ($F = 2.09$; $df = 1,176$; $p = 0.15$, with an effect size of .0117). As shown in Figure 1A, cocaine-positive urines for the CT/TT genotype group during the two baseline weeks were 74% and 82% for disulfiram and placebo, respectively. These percentages dropped to 52% for the disulfiram group during the last two weeks of treatment and were unchanged for placebo. In contrast, cocaine-positive urines for the CC genotype group during the two baseline weeks were 88% for disulfiram and 80% for placebo. These rates were relatively unchanged during the last two weeks of treatment (71% for disulfiram and to 76% for placebo) (see Figure 1B). When only the fifty-five subjects who completed the study were included in the analyses, the disulfiram treatment effect remained highly significant in the CT/TT genotype group ($F = 17.46$; $df = 1,186$; $p < 0.0001$, with an effect size of .0995).

Treatment outcomes by *DRD2* genotype

We next examined response to disulfiram in relation to *DRD2* genotype. Patients genotyped for the *DRD2* rs2283265 variant were separated into two groups based on presence of the minor T allele. Individuals with GT/TT genotypes showed a decreased number of cocaine-positive urines during the twelve week treatment with disulfiram while the GG genotype group showed only a marginal decrease. Specifically, cocaine-positive urines dropped from 67% to 48% in the GT/TT genotype group with disulfiram, compared to the GT/TT genotype group under placebo conditions who showed no significant decrease in cocaine positive urines (80% to 79%; $F = 26.12$; $df = 1,121$; $p < .0001$, with an effect size of .1775) (see Figure 3A). Within the GG genotype group there was only a small difference between treatment groups; at baseline the placebo GG group had 80% cocaine positive urines, which dropped to 75% at 12 weeks, while the disulfiram group had 84% positive urines which dropped to 63% at 12 weeks ($F = 4.16$; $df = 1,234$; $p = 0.04$, with an effect size of .0174) (see Figure 3B). When only the individuals with complete urine toxicology data were analyzed, the effect remained highly significant for the GT/TT genotype group ($F = 23.23$; $df = 1,121$; $p < 0.0001$, with an effect size of .1775) and less so for the GG genotype group ($F = 8.26$; $df = 1,110$; $p = 0.004$, with an effect size of .1743).

Treatment outcomes in Caucasians by *ANKK1* and *DRD2* genotype

To examine if there was a treatment effect in individual ethnic groups without correcting for population structure, we examined the data by self-reported ethnicity. The only group with enough individuals to make analysis possible was the Caucasian group ($N = 50$ for both genes). The analyses showed results similar to the previous analyses including all individuals that were corrected for population structure. Specifically, the analyses within the Caucasians for *ANKK1* remained significant ($F = 9.499$ (1,262), $p = 0.00228$) and trended towards significance for *DRD2* ($F = 3.383$, (1,266), $p = 0.0670$) indicating that the CT/TT or

GT/TT genotypes in *ANKK1* and *DRD2*, respectively, showed fewer cocaine-positive urines when treated with disulfiram. Hence, the results with Caucasians only demonstrate that it is unlikely that the results with the total cohort were due to population stratification.

Treatment outcomes by *ANKK1/DRD2* genotype pattern

We went on to analyze disulfiram response in relation to genotype patterns of the *ANKK1* and *DRD2* genotypes combined. We divided the patients into two genotype pattern groups: individuals carrying the genotype homozygous for the major alleles only (*ANKK1* CC/*DRD2* GG), versus all other possible genotype patterns. Individuals in the latter group who carried at least one minor allele in *ANKK1*, *DRD2*, or both genes showed significantly reduced cocaine-positive urines as compared to individuals with a *ANKK1* CC/*DRD2* GG genotype pattern (see Figure 1) when treated with disulfiram ($F = 20.72$; $df = 1,173$; $p < 0.0001$, with an effect size of 0.1187). Linkage disequilibrium analysis showed that the two loci (rs2283265 and rs1800497) had medium linkage disequilibrium in our cohort ($r^2 = 0.57$).

Discussion

In this study, we found that the T allele of rs1800497 (also known as the *TaqIA*1 allele) in the *ANKK1* gene, the T allele of rs2283265 in the *DRD2* gene, and the genotype patterns including at least one minor allele from either gene, were found to be associated with significant reduction in cocaine-positive urines when in combination with disulfiram treatment for CD. We speculate that the increased dopamine levels as a result of disulfiram therapy more easily saturate the lower density of D2 receptors in individuals carrying the minor T allele of rs1800497 and/or rs2283265, therefore eliciting a better response compared to individuals carrying only the major alleles who would have a higher density of D2 receptors.

Both *DRD2* and *ANKK1* gene products function in the dopaminergic pathway of the brain and studies have linked variants in these genes to several addictive disorders relating to alcohol, heroin, nicotine, cocaine, opioids, gambling, methamphetamine, and polysubstance addiction [39, 42, 55–59], as well as to psychiatric disorders such as eating disorders, schizophrenia, obsessive-compulsive disorder (OCD), attention-deficit hyperactivity disorder (ADHD), and others [35, 36, 60–62].

The *TaqIA* variant is one of the most frequently studied variants in relation to psychiatric conditions since its initial association to alcoholism in 1990 [63]. Initially thought to be a promoter variant of *DRD2*, *TaqIA* has subsequently been shown to reside in the last exon of the upstream *ANKK1* gene [64]. This variant codes for an amino acid substitution (Glu713Lys) in the 11th of 12 ankyrin repeats, but the functional consequences of this substitution have yet to be identified. Similarly, the *DRD2* gene is one of the most frequently examined genes in relation to psychiatric disorders. The rs2283265 variant examined in this study is a G→T transversion in intron 5 of the *DRD2* gene. There are two forms of the D2 dopamine receptor that result from alternative splicing of intron 6, known as D2S and D2L, for the short and long forms, respectively. The D2S protein is thought to be a presynaptic autoreceptor and may be involved in dopamine release and D2 receptor regulation [65]. The

rs2283265 variant probably alters the ratio of long (D2L) to short (D2S) D2 receptor isoforms [44], the function of which is still unclear.

The gene product of *DRD2*, the D2 dopamine receptor, is central to the ‘reward circuitry’ in the brain and is therefore integral to addictive and psychiatric conditions. Low D2 receptor availability has been shown to predict increased cocaine self-administration in primates [66], and the *ANKK1* rs1800497 and *DRD2* rs2283265 T allele have both been shown to associate with lower DA receptor density [67–69]. Given these data, the response of patients carrying these alleles may be related to the interaction of disulfiram and D2 receptor availability [16–20].

Given the close proximity of *ANKK1* to the *DRD2* gene, one might surmise that the association with the *ANKK1* rs1800497 variant may simply be due to linkage disequilibrium with the *DRD2* rs2283265 variant. Studies that have examined haplotypes in and across both genes sometimes show linkage between the two [32], but statistical associations of phenotypes relating to these genes are often distinct as well [e.g. 32–34, 70, 71]. Our data show a similar phenotype association, but with a low LD of $r^2 = 0.57$, therefore the two variants appear to have independent genetic influences. However, the two variants may affect response to disulfiram treatment via moderating the same pathway (e.g. dopamine signaling).

We have recently published two studies showing that associations exist between response to disulfiram and genes in the serotonergic pathway as well. Variants in the *dopamine-β hydroxylase (DBH)* [30], the *serotonin transporter (5-HTTLPR)* and the *tryptophan hydroxylase (TPH2)* [53] genes all were shown to be associated with response to disulfiram treatment for CD. Specifically, the first study found that the carrier group with only the major C allele of *DBH* showed decreased cocaine-positive urines in treatment with disulfiram, while the minor T allele showed no change. The second study, Nielsen et.al. 2012, showed that subjects with at least one copy of the minor allele of *5-HTTLPR* or *TPH2*, or both, responded to disulfiram therapy, while the genotype groups with the major alleles of both genes did not show reduced cocaine-positive urines. The same clinical cohort was used for these studies as well as the current study. We anticipate that data from studies such as these, taken together will ultimately lead us to define ‘responder’ or ‘non-responder’ genotype patterns and may allow for the more accurate prediction of individuals for whom disulfiram may be an effective therapy.

A potential limitation of this study was the small sample size of the patient cohort. Additionally, since patients in the cohort were opioid co-dependent these findings may or may not translate to cocaine-dependent individuals who are not opioid co-dependent. Further studies will be required to confirm these findings.

In summary, this study identifies the rs1800497 T allele in the *ANKK1* gene, the rs2283265 T allele in the *DRD2* gene, and the genotype patterns including at least one of these two alleles as increasing the pharmacotherapeutic response to disulfiram pharmacotherapy for CD. This finding may be useful in personalizing treatment of CD to an individual based on

their genetic makeup. Again, this ‘personalized medicine’ strategy may help increase the efficacy of CD pharmacotherapy.

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Figure 1. Response of *ANKK1* or *DRD2* genotype groups to disulfiram pharmacotherapy ($N = 68$)
Cocaine-positive urine toxicology data are shown for each two-week time period across the 12-week trial in percent-positive urines. Time points for the placebo group are represented by the solid line, and time points for the disulfiram treatment group (250 mg/day) are represented by the dashed line. Each panel shows data for specific genotype groups: A – *DRD2* GT/TT genotypes, B – *DRD2* GG genotype, C – *ANKK1* CT/TT genotypes, D – *ANKK1* CC genotype, E – genotypes including a minor allele from *ANKK1*, *DRD2*, or

both, F – genotypes with only the major allele from *DRD2* and *ANKK1* (GG/CC). Standard error bars are shown for each time point.

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Table 1

Demographic and clinical characteristics by *ANKK1* and *DRD2* genotype

Gene	<i>ANKK1</i>				<i>DRD2</i>				<i>ANKK1/DRD2</i>			
	Placebo		Disulfiram		Placebo		Disulfiram		Placebo		Disulfiram	
Treatment	CT/TT	CC	CT/TT	CC	GT/TT	GG	GT/TT	GG	CC/GG	others	CC/GG	others
Characteristic												
N	20	17	13	18	14	22	9	23	17	19	17	13
% Male	65.0	70.6	61.5	61.1	71.4	68.2	44.4	69.5	70.6	61.5	58.8	68.4
%Caucasian	65.0	76.5	69.2	83.3	64.3	72.7	66.7	82.6	76.5	63.2	82.4	69.2
%African American	23.5	10.5	0.0	12.5	21.4	22.7	0.0	8.7	17.7	26.3	5.9	7.7
%Hispanic	11.8	0.0	18.8	16.7	14.3	4.5	33.3	8.7	5.9	5.9	28.6	23.1
% Employed	45.0	71.0	69.0	67.0	42.9	63.6	66.6	69.6	70.6	42.1	64.7	69.2
Average age (s.d)	42.7 (10.6)	36.7 (10.5)	39.7 (9.5)	37.8 (11.0)	42.7 (12.0)	37.7 (9.9)	38.9 (9.1)	38.2 (10.7)	36.6 (10.5)	42.4 (10.8)	38.5 (10.9)	39.7 (9.5)
Cocaine use, past 30 days (s.d.)	20.3 (8.2)	18.6 (7.4)	17.9 (8.2)	18.6 (9.2)	19.7 (9.9)	18.7 (8.8)	17.9 (9.4)	16.3 (10.9)	17.1 (8.3)	20.8 (9.6)	18.5 (10.8)	16.8 (9.1)
Cocaine use, years (s.d.)	13.7 (8.5)	12.7 (9.3)	14.3 (6.8)	10.4 (7.6)	14.6 (9.4)	14.4 (8.0)	11.6 (6.6)	8.8 (7.3)	14.1 (8.8)	14.9 (8.4)	9.1 (7.3)	11.5 (6.6)
% any alcohol use in lifetime	25.0	48.8	38.5	38.9	28.6	50.0	44.4	34.8	58.8	26.3	41.2	38.5
% any marijuana use in lifetime	30.0	64.7	61.5	52.9	45.8	46.2	66.7	52.2	64.7	31.6	58.8	53.9

No significant baseline differences among the two treatment groups by genotype in any clinical characteristics after adjusting for multiple testing ($p > 0.05$).