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Effects of *NRG1* and *DAOA* genetic variation on transition to psychosis in individuals at ultra-high risk for psychosis

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Prospective studies have suggested genetic variation in the neuregulin 1 (*NRG1*) and D-amino-acid oxidase activator (*DAOA*) genes may assist in differentiating high-risk individuals who will or will not transition to psychosis. In a prospective cohort (follow-up = 2.4–14.9 years) of 225 individuals at ultra-high risk (UHR) for psychosis, we assessed haplotype-tagging single-nucleotide polymorphisms (htSNPs) spanning *NRG1* and *DAOA* for their association with transition to psychosis, using Cox regression analysis. Two *NRG1* htSNPs (rs12155594 and rs4281084) predicted transition to psychosis. Carriers of the rs12155594 T/T or T/C genotype had a 2.34 (95% confidence interval (CI) = 1.37–4.00) times greater risk of transition compared with C/C carriers. For every rs4281084 A-allele the risk of transition increased by 1.55 (95% CI = 1.05–2.27). For every additional rs4281084-A and/or rs12155594-T allele carried the risk increased ~1.5-fold, with 71.4% of those carrying a combination of ⩾3 of these alleles transitioning to psychosis. None of the assessed *DAOA* htSNPs were associated with transition. Our findings suggest *NRG1* genetic variation may improve our ability to identify UHR individuals at risk for transition to psychosis.
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Introduction

Identification of individuals at high risk for psychotic disorder has proven challenging. The development and validation of standardized clinical criteria to detect individuals at ultra-high risk (UHR) of psychosis has improved our ability to identify individuals at the greatest risk for transition from an at-risk state to frank-level psychotic symptoms.[1–6] Transition rates to psychotic disorder were found to be 18% 6 months after identification in a high-risk clinic, 22% after 1 year, 29% after 2 years and 36% after 3 years in a meta-analysis of 27 studies including 2 500 patients.[7] Most (about 70%) of those who developed psychotic disorder had a diagnosis of schizophrenia-spectrum disorder.[8] However, the majority of individuals meeting UHR criteria do not develop psychosis.[9] Thus, use of secondary markers within the UHR group could further improve our ability to identify those most at risk.

Previous research has identified clinical features, such as severity of negative symptoms,[2,10–12] sub-threshold positive symptoms and poor life functioning,[5,13,14] as well as neuroimaging markers, such as insula and pituitary volumes,[15,16] thickness of anterior cingulate cortex and corpus callosum,[17,18] and whole-brain neuroanatomical abnormalities[19] as predictive markers of transition to psychosis in those at UHR. Neurocognitive markers have been less consistent, though recent meta-analyses[20–22] suggest verbal fluency, memory functioning and olfactory identification as potential promising predictors. Interestingly, a recent review of predictive models for psychosis transition has concluded that measures of psychopathology, life functioning and brain imaging are the strongest predictors of psychosis transition, regardless of the predictive algorithm used.[23] Although such markers are promising, they may represent 'state' rather than 'trait' markers, given the dynamic brain changes identified in these earliest stages of psychosis.[24] Markers that are not influenced by such brain changes would include DNA sequence variation.

Recently, DNA sequence variation in two promising candidate genes for schizophrenia,[25] neuregulin 1 (*NRG1*) and D-amino acid oxidase activator (*DAOA*), have been examined for their ability to differentiate high-risk individuals who will or will not transition to psychosis. Hall *et al.*[26] and Keri *et al.*[27] reported that the T/T genotype of the SNP8NRG243177 (rs6994992) located in the *NRG1* HapICE schizophrenia-risk haplotype[28] was associated with a 100% psychosis transition rate in two independent high-risk populations, the first a genetic high-risk (for example, family history of schizophrenia) sample and the second a clinical high-risk sample. Furthermore, Mossner *et al.*[29] examined variation in the *DAOA* gene and found 100% of UHR individuals carrying the rs1341402 T/T genotype and 50% of those carrying the rs778294 A/A genotype transitioned to psychosis.

These seminal studies support the notion that genotypic-based tools could be used to identify UHR individuals who will or will not transition to psychosis. However, before these

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findings can become clinically useful, critical limitations related to the small number of individuals must be examined (*N* range: 67–82) as well as potential confounders (for example, duration of symptoms, cannabis use, medication, etc.) and biases (departures from Hardy–Weinberg equilibrium[30]) must be addressed.

In the present study, we sought to examine the effect of *NRG1* and *DAOA* gene variants on transition to psychosis within the largest and longest running UHR cohort assembled to date. We hypothesized that if genetic variation in *NRG1* and *DAOA* are independent markers for transition to psychosis, then they should be replicable and detectable after adjustment for possible confounders.

Materials and methods

Study population. Participants were recruited as part of a large follow-up study at the PACE (Personal Assessment and Crisis Evaluation) Clinic at Orygen Youth Health in Melbourne, Australia that aimed to locate and reassess all participants identified as UHR for psychosis between 1993 and 2006. At baseline, all participants met the PACE UHR criteria, rated on the CAARMS (Comprehensive Assessment of At-Risk Mental States).[2] These criteria are (1) attenuated psychotic symptoms, (2) brief limited intermittent psychotic symptoms, and/or (3) trait vulnerability for psychotic illness (schizotypal personality disorder or a history of psychosis in a first-degree relative) and deterioration in functioning or chronic low functioning. The age range accepted to PACE was 15–30 years from 1993 to 2006 and changed in 2006 to 15–25 years owing to service system changes. Exclusion criteria at baseline were current or past psychotic disorder (treated or untreated), known organic cause for presentation and past neuroleptic exposure equivalent to a total continuous haloperidol dose of >50 mg (as this could modify the risk of transition). Participants underwent a face-to-face follow-up interview, including assessment of psychopathology and functioning 2.4–14.9 years after baseline.

Phenotypic variables

Outcome. The main outcome of interest was transition to psychosis. This was defined as at least one fully positive psychotic symptom several times a week for over 1 week. From 1993 to 1995, this was assessed using a combination of the Brief Psychiatric Rating Scale (BPRS; to assess intensity of psychotic experience)[31] and the Comprehensive Assessment of Symptoms and History (to assess conviction).[32] Since 1996, psychosis status was determined by the Comprehensive Assessment of At Risk Mental States (CAARMS).[2] This allows intensity, conviction, frequency, recency and duration to be assessed comprehensively using one instrument that has well-defined anchor points. The CAARMS has good to excellent reliability.[2] If CAARMS data were not available (for example, because of not being able to locate the subject), the state public mental health records were consulted to determine whether the person had any contact with public psychiatric services since last contact with PACE and, if so, whether they had been diagnosed with a psychotic disorder.

Potential confounders. At baseline, negative symptoms were assessed using the Scale of Assessment for Negative Symptoms (SANS),[33] and positive symptoms with the BPRS, psychotic subscale.[31] Functioning and disability of participants was determined using the Quality of Life Scale[34] and the Global Assessment of Functioning Scale (GAF).[35] Lifetime alcohol, cannabis, opioid, sedative, stimulant, hallucinogen and inhalant use were determined using a substance use questionnaire. Any treatment (for example, medication) that participants received during their time at PACE was also recorded.

Psychopathological and clinical data were collected by trained research assistants. All procedures were conducted in accord with principles expressed in the Declaration of Helsinki and obtained approval from the Melbourne Health Human Research Ethics Committee.

Single-nucleotide polymorphism selection. For both the *NRG1* and *DAOA* genes, 17 haplotype-tagging single-nucleotide polymorphisms (htSNPs) spanning each gene and $\sim$500 kbp of the 5′ flanking region were selected using the International Haplotype Map (HapMap) Project (release 27) and Tagger.[36] The minimum pairwise linkage disequilibrium rate and minor allele frequency were set at 0.80 and 0.20, respectively. In addition, the three single-nucleotide polymorphisms (SNPs; *NRG1*: rs6994992, *DAOA*: rs1341402 and rs778294) previously associated with transition were included.[26,27,29] Where applicable, the traditional M nomenclature[37] for *DAOA* htSNPs was used. To determine the presence of population stratification, 60 unlinked ancestry informative markers (AIMs; Supplementary Table S1) representing three HapMap phase III populations (Northern/Western European, Han Chinese, and Yoruba in Nigeria) were also genotyped.[38]

DNA isolation and genotyping. DNA was isolated from Oragene DNA sample collection kits (DNA Genotek, Kanata, ON, Canada) using the Autopure LS nucleic-acid purification system and Qiagen Puregene salting out methodology (Qiagen, Valencia, CA, USA) at Genetic Repositories Australia. Extracted DNA was quantified fluorometrically using Quant-iT *PicoGreen* dsDNA reagent (Invitrogen, Mulgrave, Victoria, Australia) on an Eppendorf Mastercycler RealPlex (Eppendorf South Pacific, North Ryde, New South Wales, Australia) and stored in 1.0$\times$ tris-EDTA buffer at $-80\,^{\circ}$C until required.

htSNPs were genotyped with the Sequenom MassARRAY MALDI-TOF genotyping system using Sequenom iPLEX Gold chemistries, according to manufacturer's instructions. The 60 AIMs and 37 htSNPs were multiplexed into four separate assays, using the Assay Design v3.1 software (Sequenom, San Diego, CA, USA). Two htSNPs (rs6994992 and rs1341402) were unable to be multiplexed using the assay design program; these assays were designed manually. PCR and extension primers for each genotyped SNP are listed in Supplementary Table S2. A random selection of 10% of samples was genotyped twice for all candidate htSNPs. Data analysis was performed in a semiautomated manner using the Typer 4.0 Analyser Software (Sequenom). All genotype calls not assessed as 'conservative' by the analysis program

were manually checked by the operator and discarded if a clear call could not be made. Genotyping accuracy was assessed by checking concordance among the 10% of samples genotyped twice.

Statistical analysis. The CubeX program[39] was applied to detect departures from Hardy–Weinberg equilibrium, determine minor allele frequency, and estimate pairwise linkage disequilibrium measures r^2 and D′. SNPs with Hardy–Weinberg equilibrium <0.01 or minor allele frequency <0.10 were excluded from the analysis. Utilizing the 60 AIMs, participants were assigned to the HapMap ancestral group (Northern/Western European, Han Chinese and Yoruba in Nigeria) for which they carried the greatest proportion of that population's AIMs. Cox proportional hazard regression (that is, time-to-event) analysis was used to estimate univariate hazard ratios for each htSNP on transition to psychosis. Where applicable, multivariate Cox regressions were also conducted, which adjusted for factors previously shown to predict transition to psychosis (that is, age, sex, GAF score, BPRS total and psychotic subscale scores, SANS attention subscale, lifetime cannabis use and duration of symptoms).[5,13] A Bonferroni correction was applied to adjust for multiple comparisons and guard against reporting false-positive findings. Raw *P*-values for the *DAOA* and *NRG1* analyses were multiplied by the number of htSNPs examined to derive an adjusted *P*-value.

Results

Of the 416 individuals enrolled in the PACE UHR cohort, 247 (59.3%) underwent a face-to-face interview that included assessment of psychopathology and DNA collection at follow-up. We excluded 13 individuals that were of non-European ancestry based on the 60 unlinked AIMs, as well as nine individuals missing a majority of phenotypic data. This resulted in 225 individuals included in the analysis.

Over one-quarter (26.6%, $n=60$) of participants transitioned over the follow-up period. The mean number of days to transition was 572 (s.d. = 710; median = 281, range = 4–3537) and the mean age of those who transitioned was 20.2 (s.d. = 4.1; median = 19.2, range = 14–31). Table 1 provides characteristics of all participants at baseline and follow-up assessments.

Characterization of the htSNPs in *NRG1* and *DAOA* (Supplementary Tables S3 and S4) revealed all htSNPs in both genes were in Hardy–Weinberg equilibrium ($P>0.01$). One *NRG1* htSNP (rs62497784) had a minor allele frequency $<10\%$ and was removed from further analyses. In addition, three pairs of htSNPs (rs7320588-rs1335075; rs2391191-rs3918341; rs3918342-rs1421292) in the *DAOA* gene were in strong LD ($r^2>0.80$); arbitrarily we retained the htSNP closest to the 5′ end of the gene from each pair for further analysis. Thus, 16 *DAOA* and 17 *NRG1* htSNPs were included in hypothesis testing. Genotyping accuracy based on re-genotyping a random 10% of the sample revealed 100% concordance for all *NRG1* htSNPs, and 96–100% for *DAOA* htSNPs (Supplementary Table S5).

None of the examined *DAOA* htSNPs reached statistical significance (Supplementary Table S6). However, two (rs12155594 and rs4281084) *NRG1* htSNPs were shown to predict transition to psychosis (Supplementary Table S7). Carriers of the rs12155594 T/T or T/C genotype had a 2.34 (95% confidence interval (CI) = 1.37–4.00, raw $P=0.001$) greater risk of transition compared with C/C carriers. Similarly, for every rs4281084 A-allele the risk for transition increased by a factor of 1.55 (95% CI = 1.05–2.27, raw $P=0.027$). For every additional rs4281084 A-allele and/or rs12155594 T-allele carried, the risk for transition increased 1.56 times (95% CI = 1.20–2.04, raw $P=0.004$) (Figure 1). However, only rs12155594 remained significant after adjustment for multiple comparisons (Bonferroni $P=0.033$; Supplementary Table S7) and adjustment for factors previously shown to predict transition to psychosis[5,13] (Table 2). Examination of baseline and follow-up clinical characteristics by rs12155594 genotype (Supplementary Table S8) showed no significant differences, with the exception of T-allele (T/T or T/C) carriers meeting PACE entry criteria for attenuated psychotic symptoms and trait vulnerability for psychosis in greater proportion compared with C/C carriers (29.5% versus 9.8%; $\chi^2=11.5$, df = 1, $P=0.001$).

Table 1 Participant characteristics at baseline and follow-up ($n=225$)

Baseline	
PACE entry criteria[a]*, % (n)*	
APS only	61.0 (133)
BLIPS only	5.0 (11)
Vulnerability only	14.7 (32)
APS and BLIPS	7.8 (17)
APS and vulnerability	13.8 (30)
BLIPS and vulnerability	3.2 (7)
All three criteria	2.8 (6)
Age, mean (s.d.) years	18.6 (3.3)
Gender, % (n) female	58.2 (132)
Time from Sx onset to study enroll, mean (s.d.) days	407 (488)
BPRS total, mean (s.d.)	47.4 (10.0)
BPRS psychosis subscale, mean (s.d.)	9.5 (3.0)
SANS total, mean (s.d.)	19.7 (13.3)
GAF, mean (s.d.)	58.7 (11.4)
QLS total score, mean (s.d.)	76.1 (23.2)
Substance use[b]*, % (n) ever used*	
Alcohol	88.2 (186)
Cannabis	50.0 (107)
Opioids	2.8 (6)
Sedatives	10.3 (22)
Stimulants	22.4 (48)
Hallucinogens	13.6 (29)
Inhalants	9.3 (20)
Follow-up	
Time from baseline to follow-up, mean (s.d.) days	2698 (1189)
Age of censored/psychosis onset, mean (s.d.) years	24.0 (5.2)
Time to psychosis transition, mean (s.d.) days	552 (710)
Received trail treatment at PACE, % (n) yes	
Case management	100 (225)
Risperdone	11.1 (25)
Olanzapine	0.4 (1)
Lithium	5.8 (13)
Cognitive behavioral therapy	94.2 (212)

Abbreviations: APS, attenuated psychotic symptoms; BLIPS, brief limited intermittent psychotic symptoms; BPRS, Brief Psychiatric Rating Scale; GAF, Global Assessment of Functioning; QLS, Quality of Life Scale; SANS, Scale of Assessment for Negative Symptoms; Sx, symptoms.
[a]PACE entry criteria was unavailable for five participants. [b]Substance use information was unavailable for six participants.

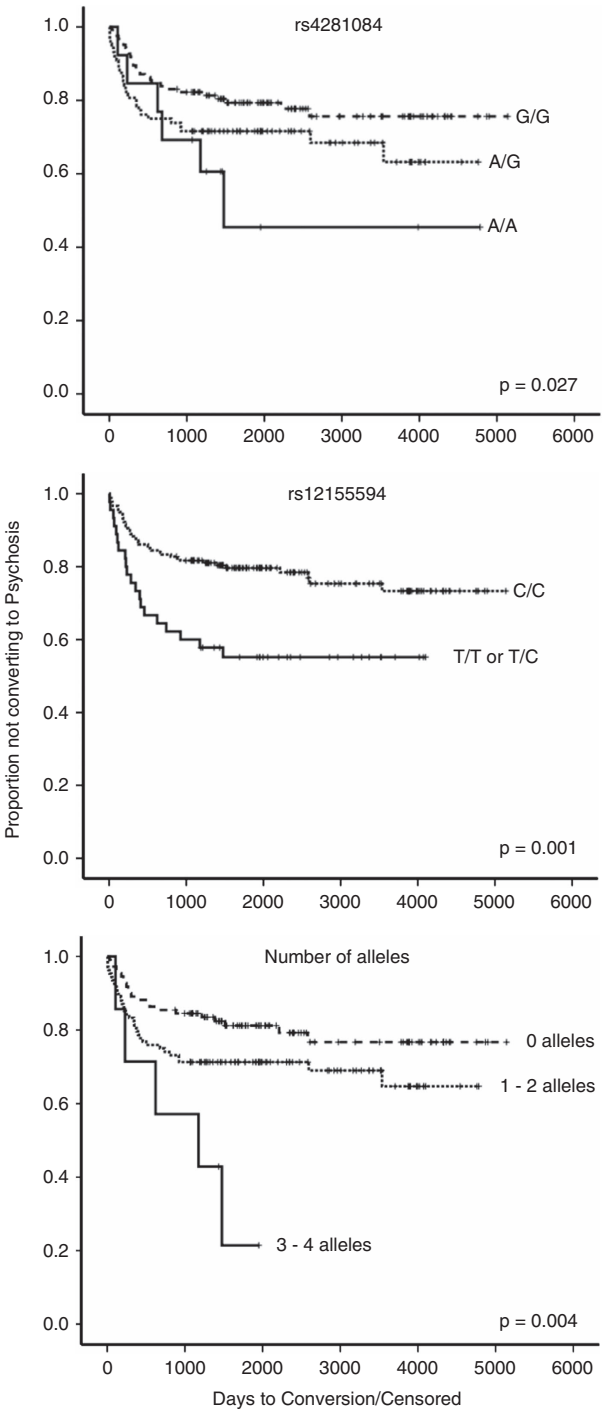

Figure 1 Kaplan–Meier estimates of the rate of psychosis transition in ultra-high risk (UHR) individuals by *NRG1* rs4281084 (top), rs12155594 (middle), rs4281084 A-alleles and rs1255594 T-alleles (bottom). The Cox proportional hazards model was used.

Discussion

Our findings suggest that *NRG1* genetic variation may improve our ability to identify UHR individuals at risk for transition to frank psychosis. We found in the largest and longest running cohort of UHR individuals reported to date, that nearly half (46.2%) of the *NRG1* rs4281084 AA genotype carriers and 44% of those carrying a rs12155594 T-allele transitioned to psychosis, a 1.55 and 2.29 increase in risk compared with those carrying the G-allele and CC genotype, respectively. For every additional rs4281084-A and/or rs12155594-T allele carried, the risk increased $\sim$1.5-fold, with 71.4% of those carrying a combination of $\geqslant$3 of these alleles transitioning to psychosis. Notably, the rs4281084 association did not survive correction for multiple comparisons and did not remain significant after adjusting for factors previously shown to predict transition to psychosis,[5,13] suggesting a potential false-positive finding.

Our findings are in contrast with the two previous studies that examined the association between *NRG1* variation and transition to psychosis,[26,27] in which a significant risk-conferring effect for the T/T genotype of the rs6994992 (SNP8NRG243177) located in the HapICE schizophrenia-risk haplotype[28] was observed. The risk-conferring rs4281084 SNP in the current study is positioned 207 base pairs upstream from rs6994992 and was in moderate linkage disequilibrium (D' = 1.0, $r^2 = 0.23$), whereas the rs12155594 SNP is located in intron 1 between two HapICE microsatellite repeats (478B14–848 and 420M9–1395). Thus, our findings point to a potentially generalized HapICE haplotype region effect for transition to psychosis, in which any number of polymorphisms in this region may confer risk for transition. This interpretation is supported by the absence of any significant main effects in the current study for *NRG1* htSNPs located outside the boundaries of the HapICE risk haplotype region, albeit htSNP coverage outside this region may not have been dense enough to detect such an effect. Our results also align with the notion that discrepancies among identified risk-conferring SNPs between studies are the expectation rather than the exception. In fact, meta-analyses[40–43] suggest that specific *NRG1* risk alleles are likely to vary between populations, and more recently Weickert *et al.*[44] suggested that the risk conveyed by sequence variation within *NRG1* is likely not to be driven by one SNP, but a diverse accumulation of nucleotide changes.

The mechanism by which the *NRG1* genotypic variation observed might confer risk for transition to psychosis is not clear. However, Law *et al.*[45] have suggested *NRG1* genotypic variation, particularly in the HapICE region of the gene, in post-mortem brain is associated with *NRG1* type IV isoform expression. More recently, an Australian post-mortem brain study[44] has shown that the risk-conferring 5-SNP HapICE haplotype[28] is associated with increased *NRG1* type III (that is, sensory and motor neuron-derived factor) isoform expression and an additional cluster of SNPs in intron 1, including the 478B14-848 microsatellite, was associated with increased type II (that is, glial growth factor) isoform expression. Increased *NRG1* type III isoform expression was also associated with earlier age of onset. Interestingly, in the human brain, *NRG1* type III and II isoforms are the most abundant (73% and 21%, respectively).[46] Type III isoform expression has been associated with lateral ventricle enlargement, axonal myelination, and reduced prefrontal cortex and hippocampus function in animal models,[47,48] whereas type II *NRG1* has been linked to Schwann cell development, myelination and ensheathment of primary nerves[49,50] as well as protection of dopaminergic neurons.[51] Given that the two

Table 2 Univariate and multivariate Cox regression models for progression to psychosis ($N = 225$)

Variables	*Model 1*		*Model 2*		*Model 3*	
	HR (95% CI)	P	*HR (95% CI)*	P	*HR (95% CI)*	P
Univariate models						
rs4281084 (NRG1)	1.55 (1.05–2.27)	0.027				
rs12155594 (NRG1)			2.29 (1.39–3.78)	0.001		
Number of risk alleles[a]					1.56 (1.20–2.04)	0.001
Multivariate models						
rs4281084	1.48 (0.97–2.25)	0.071				
rs12155594			2.06 (1.21–3.49)	0.008		
Number of risk alleles[a]					1.47 (1.11–1.95)	0.007
Age	0.97 (0.89–1.05)	0.428	0.97 (0.89–1.05)	0.410	0.97 (0.88–1.05)	0.415
Sex	1.00 (0.57–1.76)	0.999	1.04 (0.59–1.82)	0.907	1.00 (0.57–1.76)	0.999
Duration of symptoms[b]	1.00 (1.00–1.01)	0.105	1.00 (1.00–1.01)	0.076	1.00 (1.00–1.01)	0.112
GAF score	0.95 (0.92–0.98)	0.002	0.95 (0.92–0.98)	0.003	0.95 (0.92–0.98)	0.002
BPRS total score	0.95 (0.90–0.99)	0.042	0.95 (0.91–1.01)	0.078	0.95 (0.90–0.99)	0.048
BPRS psychotic subscale	1.07 (0.93–1.24)	0.340	1.08 (0.92–1.24)	0.340	1.08 (0.93–1.25)	0.319
SANS attention subscale	1.10 (0.95–1.28)	0.195	1.09 (0.94–1.27)	0.239	1.10 (0.94–1.27)	0.237
Cannabis use (yes)	0.34 (0.10–1.31)	0.078	0.31 (0.09–1.03)	0.060	0.31 (0.09–1.07)	0.064

Abbreviations: BPRS, Brief Psychiatric Rating Scale; CI, confidence interval; GAF, Global Assessment of Functioning; HR, hazard ratio; SANS, Scale for the Assessment of Negative Symptoms.
[a]rs4281084 A-alleles + rs1255594 T-alleles. [b]Time between onset of symptoms and first contact with PACE Clinic.

risk-conferring SNPs (rs42810894 and rs12155594) in the current study are located within this region, it would seem possible that these htSNPs are also associated with expression and age of onset or are successfully 'tagging' for the polymorphisms that have been previously implicated in schizophrenia. Additional research is needed to determine the biological and clinical effects of increased *NRG1* gene expression, particularly during the transition period. An example is a recent study[52] of 97 UHR individuals and 50 controls, which reported a decrease in peripheral blood expression of combined *NRG1* type I and II among those who transitioned to psychosis compared with those who did not transition and controls. Unfortunately, *NRG1* genetic variation of these individuals was not reported, and the concordance between blood and brain *NRG1* expression is not yet clear. Further work examining the effects of *NRG1* genetic variation on peripheral expression of *NRG1* is needed to determine whether peripheral blood may serve as an appropriate surrogate for the *NRG1* genotype expression relationship in the brain.

Although the *DAOA* locus has been associated with schizophrenia in two meta-analyses[53,54] and has been shown to be upregulated in the dorsolateral prefrontal cortex of those with schizophrenia,[55] we did not observe significant effects for any of the examined *DAOA* htSNPs or previously reported risk-conferring SNPs in our sample of newly transitioned individuals. This finding diverges from the only other study to examine the ability of *DAOA* genetic variation to predict transition to psychosis. In that study Mossner *et al.*[29] reported 100% and 50% of UHR individuals carrying the rs1341402-CC and rs778294-AA transitioned to psychosis, respectively. In contrast, we observed a non-significant 37.5% of rs1341402-CC and 29.4% of rs778294-AA carriers transitioned to psychosis, despite a study sample size that was more than two times that used by Mossner *et al.*[29] The conflicting results suggest the initial finding may have been a false-positive, the current finding is a false-negative, or sample heterogeneity between the two studies resulted in findings that are not comparable. Further examination of the link between *DAOA* genetic variation and psychosis transition among UHR individuals is required as the collective number of individuals examined to date ($n = 307$) is not large enough for firm conclusions to be made.

In summary, our findings provide evidence supporting *NRG1* genetic variation as a promising factor for differentiating UHR individuals who will or will not transition to psychosis. Current tools used for early identification of individuals at risk for transition, although standardized, are dependent on subjective measures (for example, clinical rating scales) that relative to genotyping are more susceptible to measurement errors and consequently reduced prediction accuracy. Our findings in combination with previous studies provide support for the addition of measures that are not dependent on state-related changes that have the potential to further improve upon current levels of risk prediction.[9] However, we caution against the temptation to include *NRG1* genetic variation in psychosis risk prediction until replication has been undertaken in much larger samples, and the true effect size of any replicated association can be more accurately determined.[56,57] Additional investigation may then determine which genetic markers, in what contexts, and at what costs will provide the most clinically useful genetically informed risk prediction among at-risk populations.

Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Translational Psychiatry website (http://www.nature.com/tp)