Characterization of allelic variants at chromosome 15q14 in schizophrenia

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Evidence of genetic linkage for schizophrenia at chromosome 15q14 has been reported in nine independent studies, but the molecular variants responsible for transmission of genetic risk are unknown. National Institute of Mental Health Schizophrenia Genetics Initiative families were genotyped for single nucleotide polymorphisms (SNPs) and dinucleotide repeat markers in the 15q14 linkage region and analyzed based on the presence of particular alleles of the dinucleotide repeat marker D15S165 in the 15q14 region. Two alleles showed both familial transmission disequilibrium and population-wide association with schizophrenia. The two groups identified by these two D15S165 alleles differ in age of onset, number of hospitalizations and intensity of nicotine abuse, as well as in predominant ethnicity. Variations in the frequency of SNPs in CHRNA7, the α-7-nicotinic acetylcholine receptor subunit gene at 15q14, were found in each group. Further sequencing in these two groups may yield more definitive identification of the molecular pathology.

Keywords: Genetic analysis, haplotype, human chromosome 15, nicotinic receptor, polymorphism, schizophrenia

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Strategies to find molecular abnormalities in common disorders are based on extensive genotyping in regions that have previously shown evidence for linkage (Hoehe et al. 2000; Theuns et al. 2000; Sun et al. 2002). The intent is to capture molecular changes in the region and then to determine which of these are likely to be responsible for the transmission of the illness. This strategy has been used most often with isolated populations (Horikawa et al. 2000). The rationale is that the pathogenic change in molecular sequence may well have occurred initially in a single founder individual with the result that most affected persons in the population share this allele. This study investigates the applicability of this strategy to one of the many genetic loci that have been identified in schizophrenia (Harrison & Weinberger 2004). Rather than an isolated population, the study population is African American and European American families, ascertained by the National Institute of Mental Health (NIMH) Schizophrenia Genetics Initiative.

Linkage at chromosome 15q14 was first observed using a physiological endophenotype, autosomal dominant transmission of diminished inhibition of the P50-evoked response to repeated auditory stimuli, in European-American families selected for multiple cases of schizophrenia. The maximum linkage, using both parametric and non-parametric multipoint analyses, occurred at D15S1360, a dinucleotide repeat isolated from a yeast artificial chromosome that contained a candidate gene, CHRNA7 (Z = 5.30, P = 8.05 × 10−7; Freedman et al. 1997). CHRNA7 codes for the subunit in the homomeric α-7-nicotinic acetylcholine receptor. The α-7-receptor had been previously shown in electrophysiological studies to be critical for inhibition of the P50-evoked response. Furthermore, expression of the receptor is reduced in post-mortem brain tissue from individuals who had schizophrenia (Court et al. 1999; Freedman et al. 1995; Guan et al. 1999). Linkage to schizophrenia itself has been demonstrated in this region in eight other studies, with European Americans (Gejman et al. 2001; Tsuang et al. 2001), African Americans (Kaufmann et al. 1998), Azorean Portuguese (Xu et al. 2001), Germans (Stassen et al. 2000; Stöber et al. 2000), Bantu-speaking Africans (Riley et al. 2000) and Han Chinese (Liu et al. 2001). Various analytic strategies were used, which yielded significance levels from P = 0.047–5.12 × 10−5. Several studies have also shown linkage to bipolar disorder (Edenberg et al. 1997; Faraone et al. 2004), including a subtype characterized by psychotic symptoms and resistance to lithium treatment (Z = 3.55, P = 6.68 × 10−5; Turecki et al. 2001). Negative studies have also been reported. One found only negative results in five Canadian families (Neves-Pereira et al. 1998). That group has more recently reported linkage to a haplotype including CHRNA7 (De Luca et al. 2004a). Another negative study, in a combined group of African Americans and European Americans, reached a significance level of only P = 0.07 for allele sharing at a flanking marker (Curtis et al. 1999).

The NIMH Genetics Initiative Schizophrenia families have contributed to the replication and extension of these
findings. The families were ascertained by the presence of an affected sibpair, diagnosed with schizophrenia or schizoaffective disorder, depressed type, by DSM-IIIIR criteria. They were initially genotyped with simple sequence repeat markers at approximately 10-cM intervals by Millenium Pharmaceuticals. This initial genotyping showed significant non-parametric evidence for linkage at 15q14 in African Americans (Z = 1.96, P = 0.027; Kaufmann et al. 1998). Further analyses using a parametric model of autosomal dominant transmission showed a significant genome-wide lod score (Z = 3.97, P = 1.95 × 10^{-5}) in the entire population at the 15q14 location of CHRNA7 (Freedman et al. 2001b). Fine mapping of the area using D15S1360 and two flanking markers, D15S165 and D15S144, showed significant allele identity by descent between affected siblings at D15S1360 (P = 0.0024; Leonard et al. 1998). Transmission disequilibrium was particularly significant for D15S165, which is the most polymorphic of the three markers (P = 0.0069); alleles 6 (194 bp) and 13 (208 bp) showed significant individual allelic disequilibrium (Freedman et al. 2001a).

Molecular studies of the 10 exons of CHRNA7 have revealed no changes in the coding region that are generally associated with schizophrenia. A variant in intron 9 (G→A, 37 bp from the preceding exon junction) was associated with schizophrenia in African Americans (P = 0.0016) but not in European Americans (Gault et al. 2003). The core promoter region of CHRNA7 contains a number of polymorphisms, one of which was significantly associated with risk of schizophrenia in a mixed ethnic group (−86 bp from exon 1, C→T, P = 0.041) and others which were rarer but uniquely found in schizophrenic individuals (Leonard et al. 2002). However, these variants do not account for transmission of risk in the majority of families. A major concern is the presence of duplicated genomic sequences, which can result in several possible pathogenic mechanisms (Iafrate et al. 2004; Sebat et al. 2004). The genomic structure of the CHRNA7 gene is complicated by a duplication of exons 5–10 approximately one 1-Mb centromeric to CHRNA7 (Gault et al. 1998; Riley et al. 2002). The duplicated sequence (CHRFAM7A) is expressed as mRNA, but its functional role is uncertain. It often contains a two base pair deletion leading to premature termination in both schizophrenic individuals and controls (Gault et al. 1998; Thibaut et al. 2001).

The goal of the present study was to determine to what extent the significant transmission disequilibrium of D15S165 alleles might help elucidate specific subpopulations in which the molecular basis of transmission of illness at 15q14 can be further examined.

Materials and methods

Sample population

The ascertainment, clinical phenotyping and genotyping of the NIMH Genetics Initiative Schizophrenia families have been described in previous publications (Cloninger 1994). Informed consent for the study, including inclusion of anonymous clinical information and genetic material in a national database, was obtained for each subject through the Institutional Review Boards at Harvard University, Washington University and Columbia University. The families have a minimum of two siblings with schizophrenia or schizoaffective disorder, depressed type, by DSMIII-R criteria. The Diagnostic Instruments for Genetic Studies were created to acquire the data to make these diagnoses, as well as other clinical information (Nurnberger et al. 1994). Ethnicity was determined by asking probands to describe the ethnicity of their parents; the primary ethnicity was used to classify families for this study.

Genotyping

An initial genome scan was performed by Millenium Pharmaceuticals (Kaufmann et al. 1998), with further genotyping in the chromosome 15q14 region (Leonard et al. 1998). D15S165 was typed in 111 European American and 88 African American schizophrenics. Genotypes and phenotypes are available through the NIMH Genetics Initiative. The distribution of alleles of D15S165 in the U.S. population was obtained through the National Cancer Institute (Smith et al. 2001). The database has allele frequencies from 1094 African Americans and 1862 European Americans. One of the alleles of interest, allele 13 (208 bp) did not have detectable prevalence (0.001) in the European Americans; however, this allele had been reported in Europeans in the CEPH database. Therefore, its frequency in the CEPH European database (0.018) was used for calculations of association. The allele frequencies for D15S1360, which do not appear in public databases, were estimated from genotypes of unaffected individuals who had married into the NIMH Genetics Initiative families (Leonard et al. 1998).

Frequencies of single nucleotide polymorphisms (SNPs) in CHRNA7 and CHRFAM7A were determined in 79 European Americans and 54 African Americans. Twenty six of the samples were taken from post-mortem brain tissue; their clinical histories and medical records were reviewed for absence of psychotic illnesses. The remainder came from peripheral blood lymphocytes from living individuals, who were screened by SCID-NP to have no personal or family history of psychotic illness (First et al. 1986).

CHRFAM7A contains (5′ to 3′) novel exons D through A and then CHRNA7 exons 5–10. The methods for detecting SNPs in CHRNA7 and CHRFAM7A and localizing them to the full-length gene or the partial duplication are described in Gault et al. (1998). These methods rely on the polymerase chain reaction to amplify cDNA from exon 1 through exon 10 for CHRNA7 and cDNA from exon D to exon 10 for CHRFAM7A. The cDNA is either derived from mRNA extracted from brain tissue or from low copy transcription in lymphoblastoid cell lines prepared from the peripheral
blood specimens. Intronic variants could not generally be mapped. An exception was intron 9 G→A, +37 bp. Exon 9 G→A 933 bp was found to be in linkage disequilibrium with intron 9 G→A, +37 bp. If the exon 9 933-bp polymorphism was not present, an alternative change at exon 9 966 C→T was nearly always present. The intronic mutations are likely to be in the full-length CHRNA7, as the exonic mutations were mapped there. D' for exon 9 933 bp and intron 9 bp +37 in European Americans is 0.944. The variants at exon 9 bp 933 and exon 9 966 were never observed together (D' = 1.000).

Clinical measures
Three clinical measures were chosen initially for analysis: age of onset, number of hospitalizations and number of packs of cigarettes per day. Age of onset and number of hospitalizations are objective measures of severity of illness; the number of packs smoked per day indicates a possible role of CHRNA7 in nicotine’s effects in schizophrenia. Analyses with other clinical variables were subjected to Bonferroni correction for multiple testing.

Statistical analyses
Probabilities of genetic disequilibrium in the subgroups with various alleles of D15S165 were calculated by the binomial distribution, using allele frequencies established in normal populations as described above. This method was used to characterize more accurately the significance of findings in the small populations in the NIMH sample, compared with the allele frequencies reported in much larger population databases. Because of the partial admixture of ethnic groups in the NIMH sample, the final probability for each allele of D15S165 is the product of the probabilities determined separately for each ethnic group. Bonferroni corrections for multiple testing were used for the multiple alleles of D15S165 and D15S1360. Comparisons of clinical features between different allelic groups were made using t-tests and χ². Variance in the frequency of SNPs was also calculated using the binomial theorem, with allele frequencies established from control populations from the same ethnic groups. These probabilities were not corrected for multiple testing, because the entire haplotype itself was subsequently examined as a whole.

Results

Transmission of alleles of D15S165 and flanking markers
The previous study of transmission disequilibrium at chromosome 15q14 had shown significant genotype disequilibrium for D15S165 and specific significant allelic transmission for two of its alleles. Population-wide association with schizophrenia was also highly significant for both allele 6 (P = 2.40 × 10⁻⁹) and allele 13 (P = 1.90 × 10⁻⁶; Table 1). The markers centromeric and telomeric to D15S165 showed decreasing disequilibrium (Fig. 1). Recombinations in the pedigrees were consistent with the region of interest being located near D15S165, i.e. excluding the more telomeric D15S144 and the more centromeric D15S128 but retaining D15S165 and D15S1360 (Fig. 2).

The chromosome 15q14 locus is one of several that show significant evidence for linkage in the NIMH Genetics Initiative pedigrees (Freedman et al. 2001b). Selection of families segregating D15S165 allele 6 or 13 did not alter the proportion of families that show evidence for linkage at other loci. At the chromosome 15q14 locus, the two groups of families, representing 20% of the sample, had a combined lod score of 1.20, accounting for 30% of the total multipoint lod score previously reported in this region (Z = 3.97; Freedman et al. 2001b).

<table>
<thead>
<tr>
<th>Allele</th>
<th>European</th>
<th>African-American</th>
<th>European</th>
<th>African-American</th>
<th>Significance* (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>0.063</td>
<td>0.049</td>
<td>0.063</td>
<td>0.256</td>
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</tr>
<tr>
<td>13</td>
<td>0.018</td>
<td>0.020</td>
<td>0.090</td>
<td>0.047</td>
<td>1.90 × 10⁻⁶</td>
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<table>
<thead>
<tr>
<th>Allele</th>
<th>Transmissions</th>
<th>Number possible</th>
<th>Significance* (P)</th>
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<tr>
<td>6</td>
<td>29</td>
<td>37</td>
<td>2.44 × 10⁻³</td>
</tr>
<tr>
<td>13</td>
<td>14</td>
<td>15</td>
<td>6.87 × 10⁻³</td>
</tr>
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*Binomial probabilities are Bonferroni-corrected for multiple testing, based upon 16 alleles of D15S165 present in the National Institute of Mental Health Schizophrenia Genetics Initiative sample. Of the 111 European American persons with schizophrenia, seven had allele 6 and 11 had allele 13. Of the 86 African American persons with schizophrenia, 22 had allele 6 and four had allele 13.
Clinical characterization of D15S165 haplotypes

Neither allele 13 nor 6 was invariably associated with schizophrenia. Schizophrenia spectrum disorders (schizotypal personality disorder, schizoid personality disorder, paranoid personality disorder and other psychoses) were found in five additional persons with allele 6 and in three with allele 13 in the NIMH families. Furthermore, for allele 6, four had other mental disorders, two were classified as unknown and two as having no mental disorder. For allele 13, four were classified as unknown and two as having no mental disorder. Thus, 71% of subjects with allele 6 or 13 had schizophrenia and an additional 12% had schizophrenia spectrum disorders.

Persons with schizophrenia with allele 6 or allele 13 were compared with the persons with schizophrenia from families that do not have either allele and have negative lod scores at chromosome 15q14 markers (Table 2). There was no significant difference in age or sex between the three groups. The allele 6 group had a higher proportion of African Americans (0.75) than the comparison group (0.42; \(P = 0.0027\)). The proportion of African Americans in the allele 13 group (0.29) was not significantly different from the comparison group. Schizophrenics with allele 13 sought help significantly earlier in life than those with allele 6 (\(P = 0.023\)), but neither group was significantly different from the comparison group. Schizophrenics with allele 6 were hospitalized fewer times than the group with allele 13 (\(P = 0.028\)) and the comparison group (\(P = 0.020\)). The allele 13 group smoked more heavily than the comparison group (\(P = 0.011\)), while the allele 6 group did not. There were no significant differences between the European Americans and African Americans in the comparison group for the age at which they sought help (21.2 ± 5.1 vs. 22.5 ± 9.4 years, respectively), number of times hospitalized (9.3 ± 10.4 vs. 10.9 ± 15.4) or packs per day smoked (1.2 ± 0.7 and 1.0 ± 0.5).

The NIMH Genetics database contains several hundred clinical variables, including the individual responses to the Diagnostic Interview for Genetic Studies and the Family Interview for Genetic Studies for each subject. None of these showed significant differences between the groups after Bonferroni correction for multiple testing.

Further haplotype characterization

D15S165 is physically located between CHRNA7 and CHRFAMA7. It is found in a 1.03-Mb YAC (969bll) that includes both genes (Fig. 3). D15S1360 has been localized to either chromosome 15q14 markers (Table 2). There was no SNP or SNP haplotype that was invariantly associated with schizophrenia. Schizophrenia spectrum disorders (schizotypal personality disorder, schizoid personality disorder, paranoid personality disorder and other psychoses) were found in five additional persons with allele 6 and in three with allele 13 in the NIMH families. Furthermore, for allele 6, four had other mental disorders, two were classified as unknown and two as having no mental disorder. For allele 13, four were classified as unknown and two as having no mental disorder. Thus, 71% of subjects with allele 6 or 13 had schizophrenia and an additional 12% had schizophrenia spectrum disorders.

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to have the exon 9 933 bp variant than the normal population ($P = 0.039$).

A number of SNPs in the core promoter of CHRNA7 have been reported (Leonard et al. 2002). There was no difference in the frequency of these polymorphisms between the two allelic groups.

**Discussion**

The aim of this study was to determine whether alleles of a highly polymorphic marker identify specific subgroups of persons with schizophrenia within a heterogeneous population. The marker, D15S165, had prior evidence of significant genotype-wise transmission disequilibrium in this population. The results of the present investigation identified two alleles, the 194-bp allele 6 and the 208-bp allele 13; each show both significant co-transmission with schizophrenia within families and significant population-wide association with schizophrenia. Alleles 6 and 13 appear to identify groups that differ in severity of illness, as indicated by the earlier need for treatment for patients with allele 13, compared with allele 6 patients. There are also differences in the frequency of polymorphisms in at least one of the genes in the region, CHRNA7.

There are several obvious limitations to these results. The two alleles of D15S165 together comprise just over 20% of the persons with schizophrenia in the NIMH families, accounting for 30% of the lod score at the 15q14 locus. Thus, the majority of patients with the illness, and even the majority with inheritance of risk at this locus, are not accounted for in this analysis. Within families in which alleles

**Figure 2: Recombination in two pedigrees from the National Institute of Mental Health Schizophrenia Genetics Initiative.** Affected status is indicated by darkened symbols; subject numbers are shown below each affected individual with genotypes. The unaffected parents were not available for genotyping. In family 30116, subjects 001, 002, 003 had DSMIIIIR criteria for schizoaffective disorder, depressed type; subject 004 had schizophrenia. In family 31113, subject 001 had schizoaffective disorder, depressed type; subject 002 had psychosis not otherwise specified and subject 003 had schizophrenia. The bold numerals in each box are the alleles of the probable founder haplotype associated with transmission of schizophrenia; truncation of the box indicates a possible genetic recombination in that subject. Recombination in 002 in 30116 excludes D15S144 and more telomeric regions; there are several possible reconstructions of the recombination in 31113 but all exclude D15S128 and more centromeric regions. Family 30116 is also shown in Leonard & Freedman (2003).
6 and 13 are present, only 83% of affected individuals receive these alleles. Schizophrenia is thought to be a multigenic and heterogeneous illness; thus, this limitation is not unexpected. Conversely, although we did not calculate the penetrance of either allele, because the families were non-randomly selected for the presence of multiple affected cases, inheritance of either of these alleles does not invariably result in schizophrenia. These findings are consistent with the conceptualization of schizophrenia as a complex illness, in which multiple genetic and non-genetic factors interact to produce illness.

Despite these limitations, several findings seem worthy of further investigation. The relationship between inheritance at 15q14, severity of illness and cigarette consumption is one such finding. The allele 13 subjects have both an earlier onset and more severe illness, relative to allele 6 subjects, and increased use of cigarettes. Nicotine normalizes the deficit in inhibition of the P50 auditory-evoked response that is linked to the chromosome 15q14 region in schizophrenia (Adler et al. 1993). The deficit is reproduced in animal models by blockade of the α-7-nicotinic receptor, whose gene, CHRNA7, is also located in this region (Luntz-Leybman et al. 1992). Whether or not the increased nicotine use in allele 13 subjects reflects greater differences in the expression of the α-7 receptor remains to be determined. However, the study shows that an allelic difference at 15q14 is related to both increased cigarette consumption and earlier onset of illness. A relationship between heavy nicotine use and severe illness has been previously noted (Goff et al. 1992). The comparison patients with no evidence for linkage at CHRNA7 were hospitalized more times than the allele 13 or allele 6 patients, but they have the lowest cigarette consumption. Thus, in the absence of linkage to the CHRNA7 region, severity of illness itself has less relationship to cigarette consumption. Other investigators have also reported an association between severity of smoking and specific CHRNA7 genotypes (De Luca et al. 2004).

The ultimate goal of linkage disequilibrium analyses is to identify allelic variants that could be pathogenic mutations.

### Table 2: Clinical characteristics of persons with schizophrenia with different D15S165 alleles

<table>
<thead>
<tr>
<th></th>
<th>Allele 6 (194 bp)</th>
<th>Allele 13 (208 bp)</th>
<th>Comparison</th>
</tr>
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<tbody>
<tr>
<td><strong>n</strong></td>
<td>29</td>
<td>14</td>
<td>43*</td>
</tr>
<tr>
<td><strong>Demographics</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Age at study</td>
<td>43.4 ± 13.7</td>
<td>41.1 ± 13.1</td>
<td>38.6 ± 11.0</td>
</tr>
<tr>
<td>African-American proportion</td>
<td>0.76†</td>
<td>0.27</td>
<td>0.42</td>
</tr>
<tr>
<td><strong>Onset and severity of illness</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age sought help</td>
<td>23.8 ± 6.5</td>
<td>19.0 ± 5.7</td>
<td>20.9 ± 6.8</td>
</tr>
<tr>
<td>Times hospitalized</td>
<td>4.5 ± 3.6§</td>
<td>7.7 ± 5.6*</td>
<td>10.1 ± 12.3</td>
</tr>
<tr>
<td>Nicotine abuse</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cigarette packs per day</td>
<td>1.3 ± 0.8</td>
<td>1.9 ± 1.6**</td>
<td>1.1 ± 0.7</td>
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</tbody>
</table>

*These are persons with schizophrenia from National Institute of Mental Health families who do not segregate either allele 6 or 13 and have negative lod scores at 15q14.
†Significantly different from comparison group, X² = 9.21, df: 1, P = 0.0027.
‡Significantly different from allele 6, t = 2.35, df: 42, P = 0.023.
§Significantly different from comparison group, t = 2.38, df: 71, P = 0.020.
**Significantly different from allele 6, t = 2.27, df: 42, P = 0.028.

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**Figure 3:** Map of 15q14 showing the location of D15S165 relative to CHRFAM7A, CHRNA7 and their associated single nucleotide polymorphisms. The distances shown are from physical maps prepared by Gault et al. (1998) and Riley et al. (2001).
by characterizing the complete genetic variance in the region. For allele 6 subjects, CHRNA7 showed moderately significant association across the entire haplotype. For allele 13, there was a smaller sample with accordingly less power; only one SNP showed significant association. For both groups, most of the association was with the exon 9 at 933 bp, exon 9 at 966 bp and intron 9 at + 37-bp haplotype, which is the most informative haplotype currently available.

Recombination mapping in this study is also consistent with the identification of CHRNA7 and CHRFAM7A as candidate genes in this region. The presence or absence of founder mutations in the D15S165 allele 6 and 13 groups is not resolved. The evidence presented in this paper suggests that each allelic group may have distinct features consistent with such a possibility. However, the complete genetic variance in the region must be analyzed to identify such mutations and to determine the contribution of the chromosome 15q14 region to the transmission of genetic risk for schizophrenia.

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