Linkage and Association Studies of Schizophrenia

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Recent twin studies confirm that schizophrenia is highly heritable, but attempts to locate and identify genes have proved to be difficult. This is largely because major genes appear to be rare or nonexistent. Instead, genetic liability almost certainly results from the combined effects of multiple susceptibility loci and most studies have been underequipped to detect such effects. Nevertheless, several regions of the genome have been implicated by more than one linkage study and chromosome 22q has been implicated by linkage and by studies of patients with microdeletions. Recent work attempting to refine regions of interest using linkage dysequilibrium mapping has identified four promising and novel "positional candidates;" they are *neuregulin-1* on chromosome 8p-p21, *G72* located at chromosome 13q34, dysbindin at 6p22.3, and proline dehydrogenase, which is a gene that maps to chromosome 22q11. In addition, there is renewed interest in a fifth gene, catechol-O-methyltransferase, also on chromosome 22q11.

Introduction

Although once hotly debated, the notion that there is a substantial genetic contribution to the etiology of schizophrenia is now generally accepted as a fact. A meta-analysis and review of five recent twin studies by Cardno and Gottesman [1] found that the estimated heritability, or proportion of variance in liability to schizophrenia explained by additive genetic effects, was in excess of 80%, which confirmed the results of earlier twin analyses based on modern explicit diagnostic criteria [2].

Given that there is consistent evidence of strong genetic effects, it might be expected that locating and identifying the genes involved in liability to schizophrenia would be straightforward. However, as most readers will be aware, this has not been the case. Although the quantitative genetic research looks compelling, coherent attempts to apply molecular methods have, until recently, yielded confusing and inconsistent results. But just within the past few months, there have been strong claims concerning the identification of five different susceptibility genes that even some former skeptics have found convincing. The authors will outline some of the major background issues and recent studies in linkage and association before dealing in a little more detail with these promising "positional candidates."

Attempts to map genes involved in schizophrenia date back nearly 50 years, and there were several attempts to discover schizophrenia associated genes in the "pre-molecular" era using what are now called "classical" genetic markers, such as blood groups and human leukocyte antigen types [3]. Such attempts might now be seen as valiantly over-optimistic, because before the advent of DNA-based markers, the proportion of the genome (*ie,* the 23 pairs of chromosomes that carry our genetic materials) that could be thoroughly searched was in the region of 6%. The situation since the introduction of the first DNA based markers in the early 1980s has dramatically changed, and, in early 2001 [4], the first (nearly) complete annotated draft of the human genome DNA was published. One of the major spin-offs from the completion of the genome sequence has been a hastening of the production of highly detailed single-nucleotide polymorphism (SNP) maps of every human chromosome. Single-nucleotide polymorphisms are single-base variants that occur roughly once every kilo base (1000 bases) throughout the genome. Millions of SNPs have now been identified and matched to particular chromosome locations (http://snp.cshl.org/linkage_maps/). This information, together with genome maps based on another type of marker, the so-called microsatellites (*see* http://www.cidr.jhmi.edu/markerset.html), means that the would-be mappers of disease genes have very powerful set of tools at their disposal. What, then, are the obstacles in tracking down the genes involved in schizophrenia?

The Problems of Linkage in Complex Traits

Classic linkage analysis is designed to examine the segregation of a trait of interest, such as disease, and one or more marker traits; the aim is detecting departure from the Mendelian independent assortment. Linkage is considered to be present if recombination between a marker and the disease occurs significantly less than 50% of the time. The aim is also to estimate the recombination rate or fraction, because this is related to the distance between the marker and the disease on the same chromosome. The classic

approach involves a likelihood method called logarithm of odds (LOD) score analysis [5], which requires that the mode of transmission of the disease is known. Unfortunately, for complex diseases, such as schizophrenia, the mode of transmission is unknown. Furthermore, locus heterogeneity may exist; some forms of the disease may be linked to a particular locus, whereas others are not. Finally, although it may be easy to classify clear-cut cases and definitely healthy members within the families, multiply affected pedigrees often contain mild cases or relatives with "spectrum" disorders who are difficult to classify as clearly affected or unaffected.

The problems are usually dealt with in schizophrenia, as in other complex diseases, by assuming that, even though the mode of transmission is not known, it can be inferred by examining pedigrees and that, even though there may be heterogeneity in the disorder as a whole, affected family members will tend to have the same genetic condition. The problem of phenotypic definition is typically dealt with by analyzing the data under two or more diagnostic models that range from narrow and restricted to broad and inclusive. Such simplifying assumptions have been a great success in detecting linkage and identifying genes in dementing disorders, such as Alzheimer's disease [6], where some comparatively rare single gene forms can be identified. However, linkage has generally been much less successful in most other common diseases [7], including schizophrenia.

Linkage Findings

There have now been many linkage studies on schizophrenia, and Riley and McGuffin [8] have comprehensively reviewed the recent findings. They concluded that, although there have been reports of linkage between schizophrenia and genetic markers on 14 different chromosomes, attempted replications of positive findings have been difficult to interpret and have mainly been negative. Where positive data were found, in replication of these, the positioning over the locus was unreliable. Once correction for multiple models was taken into account, no studies fulfilled the criteria proposed by Lander and Kruglyak [9] for "significant" results (a LOD of 3.3), but many of the findings were "suggestive," with a LOD of 1.9 or more.

However, shortly after this review was published, there was a report of linkage at chromosome 1q21-q22 by Brzustowicz *et al.* [10] that clearly appeared to be significant with a maximum LOD score, allowing for heterogeneity, of 6.5. This was followed by a genome-wide linkage scan, which offered support for a schizophrenia susceptibility locus on chromosome 1q [11], but the locus was somewhat distal to that reported by Brzustowicz *et al.* [10]. Subsequently, Levinson *et al.* [12] performed re-analysis of a combination of data sets consisting of 779 families from eight different centers and found no evidence for linkage on chromosome 1q. This appears to rule out the existence of a 1q locus in a great majority of families in which schizophrenia is segregating, but nevertheless it remains a possibility that there is a gene on chromosome 1q that causes susceptibility in some families. In particular, a single large Scottish pedigree [13] gives strong evidence for non-independent assortment of a broad spectrum of the disorders that include unipolar and bipolar affective disorder, as well as schizophrenia, with a balanced translocation involving chromosomes 1 and 11. This anomaly disrupts genes at the distal end of chromosome 1, one of which has been cloned and named *disrupted in schizophrenia-1* [13]. Two Finnish studies [14,15], one of which focuses on an isolated sub-population from Northern Finland [14], also support the existence of a schizophrenia locus in this region.

Other recent genome scans have also reported positive results in regions near or overlapping previously reported regions of interest, and a recent review of the data [16] suggests that best support is provided for regions of interest on 5q21-q31, 6p24-p22, a fairly broad region of 6q, 8p22-p21, 10p15-p11, 13q14.1-q32, and 20q11-q22. Interest is also focused on chromosome 22q, because a high proportion (20% to 30%) of patients with velocardiofacial syndrome (VCFS) have a schizophrenic-like syndrome [17], and VCFS results from a micro-deletion in the 22q11 region. Although most of the linkage studies appear to map outside of this region, the power to resolve precise locations in complex diseases using linkage is low, and, therefore, it is possible that the VCFS findings and the linkage study results are compatible.

Given the complexity of linkage study results and the difficulty in separating out what is signal and what is noise, one might be asked why not simply put all the published data together and perform a meta-analysis? The problem here is that, in addition to using some what different diagnostic methods, the various groups performing linkage analysis have selected their families in different ways and, more problematic still, have used different sets of genetic markers in performing their genome scans. This means that special methods need to be devised to combine the data. One such method has been devised by Badner and Gershon [18], and combines reported *P* values from individual studies, after correcting each value from the size of the region containing a minimum *P* value. Badner and Gershon [18] found that the strongest evidence for linkage with susceptibility loci for schizophrenia was on chromosomes 8p, 13q, and 22q. Only published data were analyzed, and the results may be influenced by publication bias, but the authors point out that whole genome scans involving hundreds of markers are inherently likely to produce at least one positive result (even if it is false-positive). There are likely to be few unpublished positive studies.

The overall conclusion that must be reached as a result of the genome scans in schizophrenia conducted so far is that genes of large effect are rare or perhaps nonexistent. That is, the data are most compatible with the existence of multiple genes, each of which on their own confer a

relative risk of less than 2. This has the consequence that linkage studies require large samples in order to detect such small effects and even larger samples to replicate positive findings [19].

Association Studies

Compared with linkage studies, where the aim is to detect non-independent assortment between susceptibility genes and a locus, the aim of association is to detect a significant relationship between the disease and a particular allele or haplotype (*ie*, a set of two or more alleles carried on the same chromosome). Association is most commonly detected by comparing the frequency of genetic marker alleles or halpotypes in unrelated cases and controls. However, because of the potential problems of hidden population substructures resulting from mixing of ethnic groups that may differ in the frequency of the disease under study and marker alleles, methods of analysis that derive "internal controls" by studying family members have become popular [5]. A major attraction of association analysis is that it is capable of detecting genes of very small effect, for example, genes conferring an odds ratio (OR) of less than 2 or as little as 1% of variance in liability in the disorder. The downside of association analysis is that it can only detect polymorphisms that cause susceptibility to a disease or markers that are in linkage dysequilibrium with them. The phenomenon of linkage dysequilibrium operates only over very small distances, so that a whole genome scan by association will require many thousands of markers (this is compared with linkage where a genome scan can be achieved with a few hundred markers, but where only comparatively large effects can be detected with feasible samples sizes). Therefore, although genome scans using association are theoretically feasible, they are not really practicable. Consequently, association studies either focus on polymorphisms in or near genes that are "functional candidates" or are used to scan a region of interest identified by linkage studies, with the aim of narrowing this down and focusing on so-called "positional candidates."

Functional Candidate Genes

The traditional approach to selecting candidate genes for study has been to focus on neurochemical pathways thought to be involved in the pathogenesis of schizophrenia. For example, this would include genes involved in serotonin and dopamine pathways. Only two such genes, the serotonin receptor 5HT2A and the dopamine receptor DRD3, have received reasonable support for a role in schizophrenia from multiple studies [20]. The most frequently studied variant in the 5HT2A receptor gene involves a single nucleotide polymorphism in the first exon termed *T102C*. This is classified as a "silent" polymorphism, because it does not result in an amino acid change. However, a recent report [21] described the finding of lower expression of the C allele in the temporal cortex of normal individuals and schizophrenia patients, which suggests that the T102C polymorphism or another variant in linkage dysequilibrium does indeed have a functional effect. An over-representation of the C allele in schizophrenia patients compared with control individuals has been found in several, but not all studies, and a meta-analysis based on over 3000 subjects [22] showed a small, but highly significant, effect with no evidence of publication bias. Nevertheless, the two most recent attempts to replicate the T102C association with schizophrenia have been negative [23,24]. The larger of the two studies [23] contained just under 1000 subjects and reduced confidence in the T102C association. Nevertheless, the study cannot be seen as an absolute refutation, because the small effect size calculated by Williams *et al.* [22], an odds ratio of 1.2, would require 2000 subjects (1000 patients and 1000 control individuals) to have 80% power of being detected.

A meta-analysis containing over 5000 individuals from case-control studies [25] supported an association between homozygosity at the DRD3 Ser9Gly polymorphism and schizophrenia. The effect size was small (odds ratio of 1.2), but the nominal significance was high. However, the most recent report from the same group that conducted this meta-analysis and contributed to the original report on the DRD3 association, is essentially negative. Anney *et al.* [26] examined 10 novel SNPs, including some in the promoter region of the gene, but found no evidence for associations between schizophrenia and any of these or with the Ser9Gly variant.

Glutamate Receptors

There is growing support for the hypothesis of glutamate dysfunction in the etiology of schizophrenia. Phencyclidine, a non-competitive inhibitor of the *N*-methyl-D-aspartate (NMDA) receptor can replicate the positive and negative symptoms of schizophrenia in healthy humans, and results in a deterioration of symptoms in patients with schizophrenia. Glutamate concentrations in the cerebrospinal fluid of schizophrenia patients have been decreased, and alterations in glutamate receptor subunit messenger RNA levels have been found in postmortem brains of schizophrenia patients. There have been a number of allelic association studies performed involving NMDA receptor subunits and metabotropic glutamate receptors. A study performed by Williams *et al.* [27] reconstructed the genomic structure of the five genes encoding the NMDA receptor in silico and performed mutation screening. However, they found no evidence for association in their case-control study that included 184 white patients with schizophrenia and control individuals with the polymorphisms they identified. This does not exclude a possible role for NMDA receptor in the etiology of schizophrenia, because there may be as yet unidentified polymorphisms, and the sample size is too small to detect a gene of small effect.

The ionotropic glutamate receptor kainate-3 gene (*GRIK3*) has been found to contain a functional polymorphism (T928G) leading to a Ser310Ala amino substitution, and the T allele has been associated with lower levels of expression compared with the G allele. Begni *et al.* [28] found that there was a significant excess of the Ala allele in schizophrenia patients in their sample of 99 patients of 116 control individuals, and when the Ala allele was considered as dominant, the significance level was P=0.0105. Again, this finding does not reach genome-wide significance and needs to be replicated.

There have also been allelic association studies performed with the metabotropic glutamate receptor subunit genes (*GRM2*, *GRM5*, *GRM7*, and *GRM8*). A positive finding was found with the *GRM5* gene; *GRM5* knockout mice have sensorimotor deficits characteristic of schizophrenia, and the gene is also involved in synaptic plasticity. *GRM5* levels are also increased in certain pyramidal call neurons in schizophrenia patients compared with control individuals. The gene also neighbors a translocation that segregates with schizophrenia in a large Scottish family. Devon *et al.* [29] discovered an intragenic microsatellite polymorphism, and performed a case-control association study with 231 schizophrenia patients and 421 control individuals. They found a total of 11 different sized alleles, and found a significant difference in the distribution of allele frequencies between schizophrenia patients and control individuals, with the greatest contribution from 197bp allele, which was only found in schizophrenic patients, although at low frequency (1.3%).

Neurodevelopmental Candidate Genes

There has also been growing interest in performing allelic association studies using neurodevelopmental genes as candidates, given the neuropathologic findings from postmortem brains of schizophrenic patients and results from scanning studies showing changes in brain morphology (*eg,* ventricular enlargement). The *Notch* gene family encodes transmembrane receptors that may regulate embryonic cell migration. The *NOTCH4* gene is located at 6p23, a chromosomal region implicated by linkage studies. Wei and Hemmings [30] found an association between schizophrenia and *NOTCH4* in a comparatively small sample of 80 parent offspring trios. However, there have been negative results from larger studies attempting to replicate the finding [31–33].

Positional Candidates

Positional candidates genes are putative susceptibility loci that are implicated by their location within a region of interest identified by linkage studies and by there being at least a prima facie case for their being involved in neurochemical pathways or neurodevelopmental processes relevant to the pathogenesis of schizophrenia (Fig. 1). Four such genes identified in this way have recently provoked a high level of interest. These are *neuregulin-1 (NRG1)*, a gene that maps to the 8p-p21 region identified by linkage studies; a novel gene called *G72*, located at the chromosome 13q34 linkage region; dysbindin, a gene that is found at the chromosome 6p22.3 linkage region; and proline dehydrogenase (*PRODH2*), a gene that maps to the chromosome 22q11 region that is deleted in VCFS. In addition, there is renewed interest in a fifth gene. The gene encoding for catechol-O-methyltransferase (COMT) has received considerable past attention as a functional candidate, but there is also positional evidence that it maps to the VCFS microdeletion region.

Neuregulin-1

Stefansson *et al.* [34••] carried out a genome-wide linkage scan in 33 Icelandic families multiply affected by schizophrenia, and they were able to confirm previous findings of linkage on chromosome 8p. They then performed haplotype association analysis, genotyping 373 additional patients in an attempt to narrow down the region. This identified *NRG1* as the prime candidate. Neuregulin-1 has a role in expression and activation of glutamate and other neurotransmitter receptors. Furthermore, mice in which one copy of *NRG1* or its receptor, *ErbB4*, had been "knocked out" showed behaviors, including abnormality of prepulse inhibition, that occur in schizophrenia patients. Such abnormalities could be partially reversed with clozapine. The mutant mice also had fewer functional NMDA glutamate receptors compared with wild-type mice. The *NRG1* association with schizophrenia has been replicated in a sample of over 600 Scottish patients and a similar number of controls [35].

G72

Taking a broadly similar approach, Chumakov *et al.* [36••] constructed a map of 191 SNPs across the linkage region on chromosome 13q34. The original linkage region was very large, five million bases (Mb), but these authors were able to narrow the possibilities down by genotyping 213 schizophrenia patients and 241 control individuals from Canada to two subregions, a 1.4 Mb and one of just 65 thousand bases (65 kb). The 65-kb region contained markers that could also be shown to have different allele frequencies in Russian cases and control individuals. Potential genes in the region were identified by searching computer databases after experimental annotation using a method called reverse transcription polymerase chain reaction. Two genes were identified, one of which, *G72*, was expressed in the human brain, including the caudate nucleus and amygdala. The longest transcript of the gene in chimpanzee brain was only half the length of its human counterpart, perhaps suggesting rapid evolutionary change. Chumakov *et al.* [36••] went on to perform two hybrid experiments as a way of identifying interacting proteins, and they found in vitro interaction between *G72*

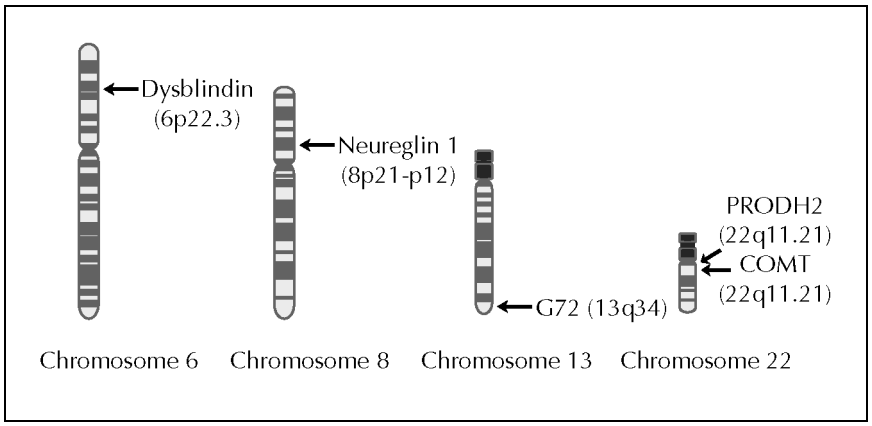

Figure 1. Chromosome ideograms showing positional candidates in schizophrenia. COMT—catechol-O-methyltransferase.

and D-amino acid oxidase (DAAO). This is expressed in the brain and oxidizes D-serine, which, in turn, activates NMDA receptors. Single nucleotide polymorphism markers from DAAO were associated with schizophrenia in the Canadian sample, and there appeared to be an interaction between the most significant *G72* (OR=1.89) and DAAO (OR=1.04) haplotypes, giving an OR of 5.02, which was much larger than the expectation for an additive effect.

Dysbindin

Straub *et al.* [37•] explored the region originally identified by themselves in 270 multiply affected Irish families. In the same material that showed the linkage in the region 6p24-21, family based association analysis was performed by constructing a map across the region consisting of 36 microsatellite markers and 17 SNPs. Straub *et al.* [37•] were able to identify a subregion, in which SNPs were associated with schizophrenia, that contained a 140 kb gene, dystrobrevin-binding protein-1, or dysbindin (the authors described the association as "strong," but it is difficult to be sure of the effect size from their paper). The dysbindin protein is expressed widely in mouse brain, and, by binding to dystrobrevin, it probably plays a role in synaptic formation and maintenance and possibly in NMDA-receptor clustering.

Proline dehydrogenase

Liu *et al.* [38] studied a set of 18 SNPs across a fairly large (1.5 Mb) region on chromosome 22q11 in 107 parent offspring trios, and they found modest evidence for association between schizophrenia and a marker in the *PRODH2* gene. They then identified additional SNPs in this gene and attempted replication in two further small samples. One of these, consisting of childhood-onset schizophrenia patients, provided support. The authors reported that this is of particular interest because of a previous report of a high rate of 22q11 microdeletions in patients with childhood-onset disorder. However, a third sample consisting of adult schizophrenia patients showed only a trend in the same direction, but somewhat stronger support when early onset (18 years or earlier) cases were studied. The authors also identified several non-functional copies (pseudogenes) of *PRODH2* in their subjects, which provides new evidence on the genomic instability of this region of chromosome 22q. Jacquet *et al.* [39••] screened 63 unrelated French schizophrenia patients, and identified a family in which there was heterozygous deletion of the entire *PRODH2* gene and heterozygous *PRODH2* missense mutations in two other patients. These mutations were associated with hyperprolinaemia. They concluded that hyperprolinaemia may be a feature of a subset of schizophrenia patients. The mechanisms involved are speculative, but Liu *et al.* [38] and Jacquet *et al.* [39••] point out that a mouse mutant that is homozygous for a truncated, low activity form of *PRODH* shows the same abnormal pattern of prepulse inhibition that is seen in schizophrenia patients. There is some evidence that proline is a modulator of glutamatergic transmission in the brain.

Catechol-O-methyltransferase

Catechol-O-methyltransferase inactivates catecholamines, including dopamine, by methylating their m-hydroxy group. A functional *COMT* gene polymorphism influencing the enzyme activities, the high activity allele (*val*-108) and the low activity allele (*met*-108), has been described [40]. Li *et al.* [41] genotyped the *val*-108-*met* polymorphism in 178 Chinese trios consisting of affected probands and their parents, and found that the high activity *val* allele was significantly more likely to be transmitted to affected offspring. These results were combined with results from Kunugi *et al.* [42] who also performed a similar analysis on 22 multiply affected Japanese and white families, and this resulted in significant evidence for linkage. Another family based study by Egan *et al.* [43•] provided a more moderate level of significance for association between the *val* allele and schizophrenia in their family based study of 104 European trios. In their case-control analysis of their results, there was no evidence for association.

Other case-control association studies have also produced negative results [44–47], and Ohmori *et al.* [48] found an association with the low-activity *met* allele in a comparison of 150 Japanese patients with schizophrenia patients and control individuals. This suggests that

if *COMT* is truly associated with susceptibility to schizophrenia, then it is not the *val/met* polymorphism that is important, but some other variation nearby.

Strong evidence comes from a large case-control study from Israel where the subjects were ethnically homogenous [48]. The *val/met* polymorphism was found to have modest or no effect. However, several SNPs compared using DNA pools were found to differ between cases and control individuals. This was followed-up by individual genotyping and haplotype analysis, which revealed a highly significant association (P=9.5x10^{-8}) between schizophrenia and a *COMT* haplotype. As with the other recently reported positional candidates, the size of the effect was small (OR=1.46), and the authors have calculated that approximately 600 patients and the same number of controls would be required for 80% power to replicate the finding even at the modest P value of 0.05.

Conclusions

After a long period of uncertainty over what regions of the genome are involved in susceptibility to schizophrenia, can researchers now say that they are nearly at the end of the tunnel? Certainly, a focus on positional candidates is the logical approach if it is assumed that at least some of the stronger linkage findings are true signals. Mechanisms, however, remain obscure. The ways in which *COMT* variations might affect susceptibility to schizophrenia have been the subject of the closest investigation [43•], but the most convincing *COMT* association is with a haplotype where functional effects are as yet uncertain [49••]. This is true of all of the recently reported candidates. Moreover, the mechanisms by which *NRG1*, dysbindin, *G72* and *PRODH* may exert effects in schizophrenia are completely unknown, although all have been implicated in modulating glutamatergic transmission. A further caveat is that all five associations appear to have small effect sizes that might not fully explain how these regions could have been detected by linkage. This means that attempts at replication will require large samples. Nevertheless, is there any coherent theme that emerges? Moises *et al.* [50••] produced a broad, provocative hypothesis that many of the putative linkage regions for schizophrenia contain genes that can affect synaptic stabilization. They suggest that the common set of mechanisms may involve functional deficiency of glial growth factors or growth factors produced by glial cells.

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