行政院國家科學委員會專題研究計畫　成果報告

精神分裂症致病基因之定位研究（3/3）

計畫類別：個別型計畫
計畫編號：NSC93-3112-B-002-012-
執行期間：93 年 05 月 01 日至 94 年 04 月 30 日
執行單位：國立臺灣大學醫學院精神科

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報告類型：完整報告
報告附件：出席國際會議研究心得報告及發表論文
處理方式：本計畫可公開查詢

中　華　民　國 94 年 7 月 29 日

中文摘要

關鍵詞: 精神分裂症，致病基因，基因定位，亞型界定，內因性亞型，遺傳連鎖，候選基因

台大醫學院/臺大醫院的精神疾病基因體醫學研究群已系統性地進行精神分裂之異質性/亞型區分研究，也進行系統性之遺傳連鎖研究。目前可資後續研究參考之研究結果有：(1) 區分 2-3 類臨床亞型，(2) 發現注意力障礙為一遺傳生物指標，(3) 發現神經心理之執行功能障礙，神經生理之 p50 激發腦波抑制現象有障礙，(4) 5 個染色體上分別各有 1 個遺傳標記有遺傳連鎖現象，包括 D1S251[1q42.1]，D6S296[6p22], D8S1222(8p14)， D15S976(15q14)，D22S 278(22q12) ，(5) 發現候選基因 NOTCH4(6p22)與 CHNRA7(15q14)具有遺傳連鎖現象，(6) 成功蒐集全台灣 700 個有至少 2 個同胞患有精神分裂症之家庭，(7) 建立了 DNA 與腦細胞庫，以及臨床資料庫。此一系列之研究經驗、團隊默契與研究資料、成果，提供本致病基因定位研究之堅實基礎。

截至目前，全世界之遺傳連鎖研究仍未有一致之發現。究其原因，不是實驗室分子遺傳技術之問題，而是研究樣本之異質性過高或研究樣本數太少之故。為能在此領域有所突破，本研究乃利用突破性之設計，所根據之特點是下列三項要點：(1) 臨床亞型之區分，(2) 以注意力/執行功能障礙做內因型之定義， (3)以台灣漢民族單一種族之大樣本(700 個)家庭作為研究樣本。

本研究之主要研究工作有：(1) 以全基因體(500 指標)之多態基因型做內因型亞型之遺傳連鎖分析(與哈佛大學合作)，(2) 以高產能之基因型定位技術，在 700 個家屬樣本共約 3,000 個個案，分別依二個階段，各有 5 個有連鎖意義之基因指標附近 2cM 之處，做平均間隔長度約為 30kb 的 SNP 遺傳指標(含 SNP，dinucleotide repeats)[共約 300 個指標]之基因型定型，並進行遺傳連鎖分析與量性特徵基因座之分析。(3)以基因定序與多態型偵側法探索二個階段，分別各有 10 個具潛力之候選基因。包括神經發展相關基因，如 DISC1, NOTCH4, DTNBP1, NRG1；及與 NMDA 神經傳導相關之基因，如 DAAO, G72, RGS4, AKT1, ZDHHC, PPP3CC 等基因。

研究結果顯示在 1q 的細部定位研究中，發現 DISC1 與 GNPAT 基因顯著與精神分裂症有相關，且特別與精神分裂症的注意力缺損障礙有關。在 6p 的細部定位研究中發現以下候選基因：BMP6, TXNDC5。在 8p 的細部定位研究中發現以下候選基因：DPYSL2, TRIM35, PTK2B, CHRNA2。在 22q 的細部定位研究中發現以下候選基因：CACNG2。各基因與精神分裂症注意力，執行功能缺損的相關目前仍在進行分析中。

在候選基因的研究中，發現台灣的精神分裂症樣本在 NOTCH4, NRG1, DAAO, G72 基因上有顯著的相關。各基因與精神分裂症注意力，執行功能缺損的相關目前仍在進行分析中。

Abstract in English

Keywords: schizophrenia, vulnerability gene, positional cloning, subtype definition, endophenotype, genetic marker, genetic linkage, candidate genes

This Group of Genomic Research in Psychiatric Disorders (GENOP) located at the Department of Psychiatry, College of Medicine and National Taiwan University Hospital (NTUH) had completed a serial psychopathological study of schizophrenia (SCH) defined by DSM-IV criteria. The results of this GENOP included: (1) delineating 2 to 3 subtypes of schizophrenia with prospective follow-up validity; (2) finding a trait marker of impaired attention measured by continuous performance test (CPT); (3) impaired executive function assess by Wisconsin Card Sorting Test (WCST) and impaired inhibition of P50 evoked potential; (4) five dinucleotide repeat polymorphism (DRP) markers in 5 different chromosomes with significant linkage scores, including D1S251 at 1q42.1, D6S296 at 6p22 , D8S1222 at 8p14, and D15S976 at 15q14, and D22S278 at 22q12; (5) finding a significant linkage of polymorphism marker located in a neurodevelopmental gene NOTCH4 (6p22); and neurophysiological function related gene CHNRA7 (15q14); (6) successfully collected 700 multiplex families, collected by the collaboration between of NTUH, Taiwan – NIMH, USA in the Taiwan Schizophrenia Genetic Linkage Study (TSLS) project, with at least two siblings affected with schizophrenia in Taiwan. A genome-wide scan on this big sample will be completed recently in the laboratory of NIMH, U.S.A.. Around 300 families had also CPT data in the whole family. This is probably the biggest number of multiplex families of a single ethnicity all over the world; (7) successfully setting up DNA and cell banks as well as clinical data bank. This substantial long track of this GENOP provided convincing background for this Positional Cloning Study on Schizophrenia (POCOS).

Understanding the controversial results of current linkage study on SCH world-wide, this POCOS was designed to make a break through design in the study for locating and identifying the vulnerability genes of SCH by using (1) phenomenological subtypes; (2) endophenotype defined by impaired attention (CPT) and/or impaired executive function (WCST); (3) using large enough size of samples of a single ethnicity of Taiwanese family pedigrees.

Major research tasks include (1) Linkage analysis and quantitative trait loci analysis, in collaboration with the team of Harvard Medical School, on the endophenotype defined by impaired attention and impaired executive function in 300 families with at least two siblings affected with SCH; (2) Two stages of genotyping, using High Throughput technology, of dense SNP markers, around STRP markers with significant linkage scores in the NTUH and TSLS studies, with average marker interval of 30kb in 3000 subjects of 700 multiplex families (a total of 300 markers) for linkage and quantitative trait loci analysis; (3) Two stages of study on the polymorphisms and/or mutations of candidate genes using association study and TDT test. Each stage with 10 Candidate Genes in the NTUH and TSLS project, respectively. We tested the following candidate genes: DISC1, NOTCH4, DTNBP1, NRG1, DAAO, G72, RGS4, AKT1, ZDHHC, PPP3CC.

In the 1q fine-mapping study, we found significant association between schizophrenia with DISC1 and GNPAT gene, especially associated with the impairment of sustained attention of schizophrenia. In the 6p fine-mapping study, we found the following

significant associated genes: BMP6, TXNDC5. In the 8p fine-mapping study, we found the following significant associated genes: DPYSL2, TRIM35, PTK2B, CHRNA2. In the 22q fine-mapping study, we found significant association between CACNG2 and schizophrenia.

In the candidate gene approach, we found significant association between schizophrenia and NOTCH4, NRG1, DAAO, and G72. Further analysis using the impairment of sustained attention and executive function of schizophrenia for subtyping is on-going.

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報告內容

研究目的：

This component project of positional cloning study of schizophrenia (POCOS) has two specific aims: (1) To locate and identify the vulnerability genes of the phenomenological phenotype as well as the endophenotypes of schizophrenia (SCH) at specific chromosome regions; (2) To find specific polymorphism of candidate genes associated with endophenotypes and/or phenomenological phenotypes of schizophrenia. These results will lead this POCOS team: (1) to develop new clinical diagnostic method and new prevention program including pharmacological agent in early intervention treatment of SCH for public health purpose; (2) to do functional genomic study on SCH; (3) to study the pathogenetic process of abnormal genes in abnormal neuropsychological and neurobiological functions of SCH; (4) to delineate the nature and the effect of gene-environmental interaction in the etiology of SCH.

Up to the present time, all genome-wide scans for localization of vulnerability genes revealed no consistent results. The difficulty of molecular genetic study on schizophrenia is not the technology of molecular genetic study; It is the difficulty in clinical recruitment of adequate samples. Small sample size, diagnostic uncertainty, and multiple ethnicities of study samples were major reasons for this present unfruitful condition. This POCOS is designed with a remarkable strength of sample characteristics: (1) Using enough big sample size with DNA sample of around 700 families with at least two schizophrenic siblings. This family sample assures adequate power for linkage analysis and further positional cloning strategy, (2) Using standardized diagnostic assessment method for diagnosis assessment, including the Diagnostic Interview for Genetic Study (DIGS) and Medical Chart Records, (3) Using data of impaired attention and executive function for defining endophenotype of SCH in 300 families with at least two siblings affected with schizophrenia. These conditions make the design of this POCOS a break through in current molecular genetic study of SCH nowadays.

The hypotheses to be tested are: (1) There are 3 to 5 vulnerability genes responsible for phenomenological schizophrenia, defined by DSM-IV criteria, locating in chromosome 1q42, 6p22, 8p14, 15q14, 22q12 near markers DIS251, D6S296, D8S1222, D15S976, D22S278, respectively; (2) One vulnerability gene is responsible for endophenotype of schizophrenia, defined by impaired sustained attention assessed by continuous performance test (CPT) (3) Different phenomenological subtypes of schizophrenia, including negative subtype, and non-negative subtype may have different lod scores in linkage analysis with specific genetic markers proposed in this study; (4) There are mutations and/or polymorphisms in the introns and/or exons of the candidate genes associated with the occurrence and/or specific subtypes of SCH.

文獻探討：

Schizophrenia (SCH) is as devastating and stigmatized psychiatric disorder with brain pathology and high genetic loading. The patients usually become dependent on the family with great social cost. To locate and identify the vulnerability genes, to solve the social stigma, to design genetic counseling can bring a revolutionary development in psychiatry.

This Genomic Study on Schizophrenia(GEMS)comprises of two complimentary component projects to do the positional cloning study on SCH (POCOS) and to do the psychological and genetic counseling study (POGES) in the same pool of families recruited for study. This component project (NO.1), the POCOS is designed using a breakthrough approach to locate and identify vulnerability genes. The project of POGES (NO.2) is a humanity study complimentary to molecular genetic study and it is designed for exploring psychological issues related to stigma and genetic counseling of this devastating disease of Human Being.

1. Genetic Basis of Schizophrenia

Genetic epidemiological studies revealed that SCH is familial and the risk to first-degree relatives is approximately ten times the risk to relatives of controls (Tsuang et al., 1980, 1995; Guze et al., 1983; Kendler 1988). Monozygous twin pairs had concordance rates of 46%~53% and 14%~15% for dizygous twin pairs (Kendler KS 1983; Gottesman II 1993; Prescott and Gottesman II, 1993). The heritability was around 0.7. However, the concordance rate in MZ twins is far from 100%, the environmental factors should also be considered. The evidence of genetic contribution to the etiology of SCHwas further supported by adoption study (Heston 1966; Kety et al., 1968,1994; Kendler et al., 1994).

Segregation analyses indicate that the model of multiple genes better fit the observed patterns of schizophrenia in family studies than do single major locus model (Faraone and Tsuang, 1985; Risch and Baron, 1984; Vogler et al., 1990). It was suggested that several genes (3 to 5 in number) in epistasis might responsible for genetic etiology of schizophrenia (Risch, 1990).

2. Molecular Genetic Studies of Schizophrenia

For linkage analysis, except the sex chromosomes (Delisi and Crow, 1989), there is no a priori hypothesis to focus on any given chromosomal region. The whole genome needs to be systemically screened. A few genome-wide scans of SCH for the decade found that many chromosome regions had suggestive evidences for linkage, including chromosome 1q21-q22, 1q31-q42, 2p22-q21, 4q24-q32, 6p24-p22, 6q16-q23, 8p24-p21, 10p14-p13, 13q14-q32, 15q13-q14,

22q11-q13 (Coon et al., 1994; Shaw et al., 1998; Levinson et al.1998; Blouin et al., 1998; Kaufmann et al.1998; Faraone et al., 1998; Rees et al., 1999; Williams et al., 1999; Hovatta et al., 1999; Brzustowicz et al., 2000). However, only a few chromosome regions were ever reported to have genome-wide significant linkage evidences, including chromosome **1q21-q22** (Brzustowicz et al.,, 2000), **6p24-p22** (Wang et al., 1995), **8p21** (Blouin et al., 1998), and **13q32** (Blouin et al., 1998).

Another promising chromosome region is chromosome **1q 42**. A balance translocation (1; 11)(q42.1; q14.3) was associated with major mental illness including schizophrenia in a Scottish large family pedigree (St Clair et al., 1990). Two novel genes named DISC1 (Disrupted in Schizophrenia 1) and DISC2 (Disrupted in Schizophrenia 2) at chromosome 1q42.1 were disrupted at the breakpoint (Millar et al., 2000; 2001). This was confirmed in a Finnish family sample (Hovatta et al., 1999) and another study (Ekelund et al., 2001; Hwu et al, 2001).

All these studies have shown a replication and non-replication pattern (Riley 2000). For detection of genes of modest effect in complex disorders, inadequate sample size and mixed ethnicity were major methodological problems. It is argued at least 600 hundred affected sib-pairs may be required for adequate power (Hauser et al., 1996).

Candidate genes studies revealed inconsistent results in the past decade. Neurotransmitter related genes, such as dopamine (D1, D2, D3, D4, D5), serotonin, r-aminobutyric acid and Glutamate receptor genes had been studied using both association and linkage studies and no consistent results obtained (Asherson et al., 1995, Breyler et al, 1995; Hranilovic et al. 2000; Catalano et al., 1993, Serretti et al., 1999; Chen ACH, 1996,1997). Neuron growth related genes (Margolis et al., 1994), phospholipase genes (Peet, 1998, Wei 1998), and a potassium channel gene (hKCa3/KCNN3) (Dror et al., 1999) have been reported association with schizophrenia. In case-control design, many genes and phenotypes being evaluated and mixture of ethnicity in the sample may inflate the type I errors.

Positional candidate gene approach using linkage dysequilibrium strategy may resolve the above two problems raised by the approach of candidate gene association study basing upon the previous linkage results to increase the prior probability and using parent-offspring trios as internal control. This approach is more powerful than linkage study to locate the susceptibility genes of complex disorder as schizophrenia (Risch and Merikangas, 1996). With the fine mapping linkage evidences, the whole genome sequence and single nucleotide

polymorphism (SNP) map, and the advancing microarray technique available, this approach is more efficient to locate the susceptibility genes of schizophrenia, (Owen et al., 2000; Baron 2001). Recently, a study using above strategy has been reported significant linkage dysequilibrium evidence of schizophrenia to a microsatellite polymorphism and a SNP of a gene, NOTCH4 gene, at chromosome 6p21 (Wei and Hemmings, 2000).

Considering the importance of adequate power for linkage analysis and the potentials of positional candidate gene approach using linkage dysequilibrium strategies, we propose this project to do positional cloning of vulnerability genes of schizophrenia. In this stage, we have collected the DNA sample of around 700 families with at least two schizophrenic siblings by our own efforts as well as through collaboration with Harvard University in these four years, and this family sample assures adequate power for linkage analysis and further positional cloning strategy.

3. Neuropsychological Deficit in Schizophrenia

SCH was fond to have impairment in neuropsychological functions of executive function, sustained attention and working memory (Goldberg and Gold, 1995) and was due to frontostriatal dysfunction (Elliot et al., 1995). Multiple impairment may be best demonstrated by test batteries, rather than single, isolated test (Kremen et al., 1994) and the impairments strongly suggested dysfunction of frontal-temporal-limbic circuit (Gold and Harvey, 1993).

Visual sustained attention by the Continuous Performance Test (CPT) and executive function by the Wisconsin Card Sorting Test (WCST) were studied more thoroughly. The more difficult ones are stable vulnerability indicators, while the simpler ones might be mediating vulnerability indicators in schizophrenia (Chen and Faraone, 2000). CPT deficits were associated with negative symptoms (Nuechterlein et al., 1986; Hain et al., 1993; Johnstone and Frith, 1996; Liu et al., 1997) and with thought disorder (Nuechterlein et al., 1986; Strauss et al., 1993; Nelson et al., 1998) or disorganized symptoms (Liu et al., 1997).

Deficits in WCST performance were enduring and predicted long term disability, independent of other cognitive deficits (Weinberger et al., 1986; Goldberg et al., 1988). WCST deficits were found to be related to dorsolateral prefrontal cortex (Weinberger et al., 1986; Berman et al., 1995) and that the dopamimetic drugs improve its performance (Daniel et al., 1991; Mattay et al., 1996).

These deficits, being found to be specific to SCH and with genetic risk of SCH, can thus serve as endophenotypes in genetic analysis on SCH.

4. Endophenotype Approach in Molecular Genetic Studies of Schizophrenia

To resolve the insufficient power of analyses and genetic heterogeneity of SCH, an alternative strategy was to use of a specific neurobiological characteristic of the illness as an endophenotype reflecting the effect of a single genetic alteration (Lander, 1988).

The CPT deficit was a potential endophenotype of the genetic susceptibility to SCH (Chen and Faraone, 2000). It was present not only in SCH patients, but also in their non-psychotic relatives (Grove et al., 1991; Mirsky et al., 1995; Chen et al., 1998). Using data from 148 non-psychotic relatives and 345 community adults, Chen et al. (1998) found that the recurrence risk ratio λ was greater than 15 for the undegraded CPT and greater than 30 for the degraded CPT.

Thus, using CPT deficits as endophenotypes of SCH would provide a valuable measure of genetic risk, would improve the power of genetic analyses and may help identify susceptibility genes for schizophrenia. In our sample, around 220 families have received CPT and WCST assessment. We intent to add 80 families with CPT and WCST data, and to make a 300 of families with available data for endophenotype study. It is feasible to use these endophenotypes for further genetic analysis.

This endophenotype strategy has been successful in mapping of a neurophysiological deficit of schizophrenia, decrease of P50 inhibition, to loci at chromosome 15q13-14, recently. The genome-wide linkage analysis of the P50 inhibition deficit in nine multiplex SCH families found a significant lod score ($Z = 5.30$, $\theta = 0$) at a loci chromosome 15q14. When the clinical diagnosis of SCH was used as the affected phenotype, the maximum lod score at the same marker was not statistically significant (Freedman et al., 1997). The other neurobiological deficit, eye-tracking dysfunction of schizophrenia has been mapped to chromosome 6p23-21 with the maximum multipoint lod score of 4.02. Again, while the clinical diagnosis of schizophrenia was used as the affected phenotype, the linkage result was non-significant (Arolt et al., 1996). In summary, with the endophenotype approach using sustained attention deficits and the adequate power our sample provides, we have confidences in the breakthrough of the searching for vulnerability genes of SCH.

研究方法：

The basic design of this POCOS project includes : (1) using Taiwan Chinese families with at least 2 siblings affected (i.e. sib-pair family) with schizophrenia fulfilling DSM-IV criteria assessed by standardized diagnostic interview of Diagnostic interview for Genetic Study (DIGS) or Psychiatrist Diagnostic Assessment (PDA); (2) using fine mapping approach to map SNP genotypes with an average marker-marker interval of 30kb; (3)using subtypes and/or endophenotypes of schizophrenia for linkage analyses and/or quantitative trait loci analyses.

The methods of this POCOS are as follow.

A. Sampling Work:

1. DATA and DATA Bank Management

   a. NTUH (National Taiwan University Hospital) DATA BANK Management

   Sampling started in locating the available data and clinical data from the Molecular Genetic Data Bank located in Molecular Genetic Laboratory, Department of Psychiatry, College of Medicine and National Taiwan University Hospital.

   b. DNA Sample Management from Repository of Taiwan-U.S.A. TSLS Project

   DNA samples will be allocated from the repository laboratory of NIMH, U.S.A.. DNA Samples of this TSLS project will be sent to Taipei. The DNA allocation and cargo charge will be one part of the expense of this POCOS project.

2. Collection of Supplementary Data of Attention and Executive Function

   The attention and executive function are assessed by continuous performance test (CPT) and Wisconsin Card Sort Test (WCST), respectively. And we have 220 families with data of CPT and WCST. We plan to add 80 families with CPT and WCST. These families had DNA samples already.

3. Supplementary DNA Sample Collection

   In the process of genotyping, some DNA samples, which had been used in the other studies, may be inadequate. If there is transformed lymphoblastoid cell-line reserved, the DNA sample will be extracted from the re-growth lymphoblastoid cells. If there is no cell-line available, the study subjects will be called up for blood drawn and DNA extraction.

B. Laboratory Work:

The laboratory work is for localization and identification of the candidate genes by fine-mapping with single nucleotide polymorphisms (SNPs).

There are two main approaches to locate the vulnerability genes of schizophrenia are fine mapping and candidate gene detection.

1. Candidate Regions Scan with fine mapping: fine mapping at 4 genetic markers.

In the initial stage, these 5 dinucleotide repeats polymorphism markers in 5 different chromosome regions with significant linkage scores found in our NTUH sample, were used as the landmark for fine mapping. Since the regions in original linkage analysis are too large (about 5-10 megabases) to localize the candidate gene. To fine-map the regions, we intend to utilize SNPs in the data base (The SNP Consortium). The SNPs, which were localized near those 5 dinucleotide repeats polymorphism markers, were selected makers about every 30,000 nucleotides (30kb). We intend to select 60 SNPs markers for each original dinucleotisde repeat polymorphism marker interval. A total of 300 markers will be studied..

Since the SNPs in the SNP consortium were derived mainly from Caucasians, most of them might not show any polymorphism in Chinese. We need to choose 600 SNPs to do validation in 94 individuals by DNA pooling method and assuming half of them will show polymorphism in Chinese.

2. Candidate Gene Studies

There are two sets of candidate genes. The first set is the candidate genes near the genetic markers identified by our previous linkage study. These candidate genes are identified with neurobiological functions related to pathophysiological hypothesis of schizophrenia. The selected candidate genes are RGS4, NOTCH4, DTNBP1, AKT1, ZDHHC8, PPP3CC, DAAO, G72, TNF-alpha, MRDS1.

SNP genotyping

The selected SNPs were genotyped by the method of matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS). Primers and probes flanking the SNPs were designed by using SpectroDESIGNER software (Sequenom, San Diego, CA, USA). A DNA fragment (100-300 bp) encompassing the SNP site of was amplified using the polymerase chain reaction (PCR) (GeneAmp 9700 thermocycler, Applied Biosystems, USA) according to the manufacturer's instruction.

After PCR amplification, the un-incorporated deoxynunleotide triphosphate (dNTP) of the PCR reaction mixture were neutralized by treating with shrimp alkaline phosphatase (SAP) at 37℃ for 20 minutes. The reaction mixture were incubated at 85℃ for 5 minutes to inactivate the SAP activity. Primer extension was performed by

adding the probe, Thermo Sequenase (Amersham Pharmacia, Piscataway, NJ, USA) and appropriate dideoxynucleotide triphosphate (ddNTP)/dNTP mixture, and following 55 cycles of denaturing at 94℃ for 5 sec, annealing at 52℃ for 5 sec, and extension at 72℃ for 5 sec. Different extension products were differentiated by mass through MALDI-TOF as follows.

Cation exchange resin (SpectroCLEAN, Sequenom) was added to the above reaction mixture to get rid of salt, which would interfere with the mass reading in the MALDI-TOF analysis. After desalting, the reaction mixture was spotted onto the SpectroCHIP (using the SpoectroPOINT) where they were co-crystallized with the matrix. After introduced to the SpectroREADER, a nitrogen laser with nanosecond-wide pulses interrogated the samples on the SpectroCHIP in the high-vacuum environment of the time-of-flight mass spectrometer. Upon laser irradiation, the matrix crystals absorbed the laser's energy and the extension products were converted into gas phase ions, which upon acceleration undergo mass-dependent separation over approximately 1-meter flight path. Molecular masses were determined with 0.01 to 0.02% estimated accuracy. Acquired spectra were transferred to the MassARRAY Server (Sequenom, San Diego, CA) where they were automatically interpreted and corrected for their genotypes.

C. Statistical Analysis:

In order to verify the sample accuracy included family relationship and genotype, PEDCHECK version 1.1 (O'Connell and Weeks 1998) and UNKNOWN version 5.23 (Terwilliger 1994) were employed to check Mendelian inheritance and Procedure ALLELE in SAS/GENETICS release 8.2 (SAS 2002) was used to test for Hardy-Weinberg equilibrium. Linkage disequilibrium of inter-markers was measured using coefficient D´(Hedrick 1987) which was also used to define haplotype blocks. A graphic presentation of block pattern was completed using GOLD software (Abecasis and Cookson 2000).

Family-based transmission disequilibrium tests were applied to test linkage disequilibrium. Both of single-locus and haplotype-based association analyses were carried out simultaneously using two popular programs for the nuclear family data, haplotype FBAT version 1.4.1 (Horvath et al 2001; Horvath et al 2004; Laird et al 2000) and TRANSMIT version 2.5.4 (Clayton 1999) An individual's haplotype was inferred using SimWalk2 version 2.86 (Sobel and Lange 1996; Sobel et al 2002; Sobel et al 2001), which uses Markov Chain Monte Carlo algorithm. Moreover, GEE method (Liang 1986) was applied to test the interaction between haplotype blocks using the Proc GENMODE of the SAS (version 8.0 for Windows).

Besides the analysis of qualitative trait, quantitative analysis using highly

heritable quantitative trait was also considered. The analysis of heritability and quantitative-type transmission disequilibrium test based on variance component approach was applied using QTDT version 2.4.3 (Abecasis et al 2000a; Abecasis et al 2000b).

結果與討論：

I. Fine mapping studies of chromosome 1q42.1, 6p24-23, 8p21, 22q12

1. **A SNP fine mapping study of chromosome 1q42.1 reveals the vulnerability genes for schizophrenia, GNPAT and DISC1: association with impairment of sustained attention (submitted, see Appendix 1):**

   Background: On the basis of our previous finding that the marker D1S251 of chromosome 1q42.1 was significantly associated with schizophrenia, we adopted a two-stage approach for further fine mapping in this study.

   Methods: In the first stage, 120 single nucleotide polymorphisms (SNPs) around 1 Mb of D1S251 were selected from public database and genotyped in 95 individuals to validate if allele frequency above 10%. In the second stage, we genotyped the 47 validated SNPs out of 120 SNPs in 102 families with at least 2 siblings affected with schizophrenia. Two phenotype models (narrow: DSM-IV schizophrenia only; and broad: including schizophrenia, schizoaffective, and other non-affective psychotic disorders) were used to define the disease phenotype.

   Results: We found two SNPs blocks significantly associated with schizophrenia through family-based association analyses. Five SNPs in the block 1, which located between intron 2 and intron 13 of the GNPAT gene, showed significant associations either through single locus TDT ($p<0.05$) or haplotype association analysis ($p<0.05$). Two SNPs in the block 2, which located between intron 4 and intron 5 of the DISC1 gene, also showed significant results in both the single locus ($p<0.05$) and haplotype association analysis ($p<0.05$). The potential involvement of the GNPAT with schizophrenia is preliminary, while the finding of the DISC1 is a replication of previous studies.

   Conclusions: We further revealed that the association of the DISC1 gene with schizophrenia was mainly limited to those with sustained attention deficits assessed by the Continuous Performance Test and may indicate a potential biological pathway for future investigation.

2. SNP fine mapping of chromosome 22q12 reveals the novel vulnerability gene for schizophrenia, CACNG2: association with impairment of sustained attention and executive function **{prepared, see Appendix 2, to be presented in 13th World Congress of Psychiatric Genetics (WCPG), Oct. 2005, Boston USA}**

   The D22S278 marker of chromosome 22q12 had shown the highest linkage association with schizophrenia in the family samples of our previous study.

According to this result, we conducted further fine mapping by using the criterion of minor allele frequency above 10%. We selected 47 single nucleotide polymorphisms (SNPs) from 94 potential markers around 1 Mb of D22S278 in 95 individuals. We then genotyped these 47 validated SNPs in 218 pedigrees with at least 2 siblings affected with schizophrenia. We found three genes; MCM5 (rs875400), RASD2 (rs736212), and CACNG2 (rs2283986 and rs2092662) significantly associated with schizophrenia in single locus association analyses ($p<0.05$). There were five haplotypes in four genes; APOL5 (rs1540297-rs2899256-rs2076671- rs879680), MYH-9 (rs2481-rs875726-rs1009150) and (rs3752463-rs1557540-rs713839-rs739097), EIF3S7 (rs140002-rs2142824), and CACNG2 (rs2267360-rs140526-rs1883987- rs916269), showed significantly associations with schizophrenia ($P<0.05$). When correlate these five haplotypes with sustained attention deficits assessed by Continuous Performance Test and executive functions assessed by Wisconsin Card Sort Test, the haplotypes of G-T-G-A and G-T-A-G of CACNG2 gene showed significantly association with both neuropsychological assessments ($P<0.05$). These results suggest that the CACNG2 gene may be a novel susceptibility gene of schizophrenia in the 22q12 region.

3. SNP fine mapping of chromosome 6p24-23 reveals two potential candidate genes for schizophrenia, BMP6 and TXNDC5 (manuscript under preparation, to be presented in 13th WCPG Oct. 2005, Boston USA)

There was suggestive linkage evidence of chromosome 6p24-23 to schizophrenia in our previous linkage study. In this study, we conducted a two-stage approach for further fine mapping. In the first stage, 30 single nucleotide polymorphisms (SNPs) around D6S296 with average intermarker distance of 24 kbp, and 69 SNPs around D6S274 with average intermarker distance of 23 kbp, were selected from the public database for SNP validation in a subset of our sample. Of the 99 SNPs selected, 68 SNPs met the validation criterion of minor allele frequency above 10%. In the second stage, we genotyped the 68 validated SNPs in 216 families with at least 2 siblings affected with schizophrenia. We found the SNP (rs270413) of the gene, Bone morphogenetic protein 6 (BMP6), and the SNP (rs13873) of thioredoxin domain containing 5 (TXNDC5) were significantly associated with schizophrenia ($p<0.05$) through single locus association analysis using either Transmit or FBAT program. The haplotype block of rs270413-rs270393 in the genomic region of BMP6 and the haplotype block of rs1225934-rs13873 in the genomic region of TXNDC5 were significantly associated with schizophrenia ($p<0.05$) through haplotype association analysis. These results suggest that BMP6 and TXNDC5 may

be potential candidate genes of schizophrenia in the 6p24-23 region and replication in another sample is further warranted.

4. SNP fine mapping of chromosome 8p21-12 reveals four potential candidate genes for schizophrenia, DPYSL2, TRIM35, PTK2B, and CHRNA2 (manuscript under preparation, to be presented in 13th WCPG Oct. 2005, Boston USA)

There was suggestive linkage evidence of chromosome 8p21-12 to schizophrenia in our previous linkage study. In this study, we conducted a two-stage approach for further fine mapping. In the first stage, 171 single nucleotide polymorphisms (SNPs) around D8S1222 with average intermarker distance of 27 kbp were selected from the public database for SNP validation in a subset of our sample. Of the 171 SNPs selected, 101 SNPs met the validation criterion of minor allele frequency above 10%. In the second stage, we genotyped the 101 validated SNPs in 216 families with at least 2 siblings affected with schizophrenia. We found the SNP (rs4733044) of dihydropyrimidinase-like 2 (DPYSL2), the SNP (rs2322604) of tripartite motif-containing 35 (TRIM35), and the SNP (rs7007145) of cholinergic receptor, nicotinic, alpha polypeptide 2 (neuronal) (CHRNA2) were significantly associated with schizophrenia ($p<0.05$) through single locus association analysis using either Transmit or FBAT program. The haplotype block of two SNPs in the genomic region of DPYSL2 and the haplotype block of six SNPs across the genomic regions of TRIM35 and protein tyrosine kinase 2 beta (PTK2B), and the haplotype block of 3 SNPs across the genomic regions of PTK2B and CHRNA2 were significantly associated with schizophrenia ($p<0.05$) through haplotype association analysis. These results suggest that DPYSL3, TRIM35, PTK2B and CHRNA2 may be potential candidate genes of schizophrenia in the 8p21-12 region and replication in another sample is further warranted.

II. Candidate gene association studies

1. Haplotypes and Interaction Analyses Support G72 as a Regulator and D-Amino Acid Oxidase as a Susceptibility Gene for Schizophrenia (submitted, see Appendix 3)

The D-amino acid oxidase (DAAO) activator (G72; 13q34) and DAAO (12q24) have been suggested as candidate genes and involved in the N-methyl-D-aspartate receptor regulation pathway for schizophrenia. To evaluate if these two genes were associated with schizophrenia in a Taiwanese sample, three single nucleotide polymorphisms (SNPs) for DAAO (rs2111902, rs3918346, rs3741775) and eleven SNPs for G72 (rs3916965, rs3916966, rs3916967, rs2391191, rs3916968,

rs947267, rs778294, rs3916970, rs3916971, rs778293, rs3918342) were genotyped by the MALDI-TOF MS method in 218 schizophrenic affected sib-pair families (864 individuals). In haplotype-based association analyses, two protective G-G-A and T-A-C, and one risk T-G-C haplotypes for DAAO (rs2111902–rs3918346– rs3741775) showed significant associations with schizophrenia in both a narrow model (DSM-IV schizophrenia only; p=0.017 and p=0.0024 for protective, p=0.0144 for risk, respectively) and in a broad model (including schizophrenia, schizoaffective disorder, and other non-affective psychotic disorders; p=0.0171 and p=0.0024 for protective, p=0.0165 for risk, respectively). The G72 gene only showed significant interactions with DAAO (p<0.02). These results suggest that DAAO may be a susceptibility gene for schizophrenia and that G72 is likely to be a regulator for DAAO.

2. **Failure to support RGS4 as a candidate gene for schizophrenia (submitted, see Appendix 4)**

   Several studies have been suggested that the regulator of G-protein signaling 4 (RGS4) may be a positional and functional candidate gene for schizophrenia. Three single nucleotide polymorphisms (SNP) located at the promoter region (SNP4, and SNP7) and the intron 1 (SNP18) of RGS4 have been verified constantly in different ethnic groups. Positive results have been reported in these SNPs with different numbers of SNP combinatory haplotypes. In this study, these four SNP markers were genotyped in 218 schizophrenia pedigrees of Taiwan (864 individuals) for linkage association analysis. Among these three SNPs, neither SNP4, SNP7, or SNP18 has shown significant association with schizophrenia in single locus association analysis, nor any compositions of the three SNP haplotypes has significantly associated with the phenotypic narrow model (DSM-IV schizophrenia only) of schizophrenia. Our results fail to support the RGS4 as a positional candidate gene for schizophrenia when evaluated from these three SNP markers.

3. **Association analysis between schizophrenia and the candidate genes on chromosome 6p: DTNBP1 and NOTCH4 (manuscript prepared, see Appendix 5)**

   Several linkage studies have linked schizophrenia to chromosome 6p24-21 region and the positional candidate genes, DTNBP1 and NOTCH4, have been reported associated with schizophrenia. However, the association evidence seemed inconsistent across studies. We aimed to test the association of the two candidate genes by a two-stage fine-mapping approach in 218 co-affected sib pair

families of schizophrenia in Taiwan. First, we selected 18 single nucleotide polymorphisms (SNPs) of DTNBP1 and 14 SNPs of NOTCH4 from public SNP database, which distributed across the entire gene region from promoter to 3' untranslated region. We genotyped these SNPs for validation in a sample subset. Nine SNPs of DTNBP1 with average intermarker distance of 17 kb, and seven SNPs of NOTCH4 with average intermarker distance of 5.3 kb met the validation criterion of minor allele frequency above 10%. Secondly, the validated SNPs were genotyped in 218 families with at least 2 siblings affected with schizophrenia. Intermarker linkage disequilibrium was calculated by Gold program. Single locus and haplotype association analysis were performed with Transmit v2.5.4 program. We found the T allele of the SNP rs2071285 (p=0.035) and the G allele of the SNP rs204993 (p=0.0097) were significantly preferentially transmitted to the affected individuals in the single locus association analysis. The two SNPs were in high linkage dysequilibrium (D' > 0.8). The T-G haplotype of the two SNPs showed marginally significantly over-transmitted (p=0.053) and the A-A haplotype was significantly under-transmitted to affected individuals (p=0.034). The associated region distributed across the distal portion of NOTCH4 gene and overlapped with the genomic region of P78548, GPSM3, and PBX2. There was no significant association evidence with DTNBP1 either through single locus or haplotype association analysis. In summary, our results showed there was significant association with the distal genomic region of NOTCH4, though the association with the genomic region of P78548, GPSM3, and PBX2 cannot be ruled out, and no significant association with DTNBP1 in Taiwanese co-affected sib pair families of schizophrenia.

4. Absence of Significant Associations between Four AKT1 SNP Markers and Schizophrenia in Taiwanese (submitted, see Appendix 6)

AKT1 (V-akt murine thymoma viral oncogene homolog 1) is a protein kinase isoform of AKT. Five single nucleotide polymorphisms (SNPs); rs3803300, rs1130214, rs3730358, rs2498799 and rs2494732, at the genomic region of AKT1 have been reported to be significantly associated with schizophrenia. We tested for the presence of these five SNPs in Taiwanese by genotyping 218 co-affected schizophrenia families. Both single locus and haplotypes analyses showed no association of these SNPs with schizophrenia. These findings fail to support AKT1 as a candidate gene for schizophrenia susceptibility in the Taiwanese population.

5. Evaluation of the ZDHHC8 as a candidate gene for schizophrenia (submitted, see Appendix 7)

ZDHHC8 (Zinc finger, DHHC domain containing 8; also annotated as KIAA1292) was reported as a schizophrenia candidate gene located around the chromosome region of 22q11.21. As this newly discovered gene renders further support from other ethnic group, we selected two single nucleotide polymorphisms (SNP) located before and within the locus of ZDHHC8 gene to test if it is susceptible to schizophrenia in a population sample of Taiwan. There were 218 schizophrenia families with at least two affected siblings participated in this study. These two SNPs were genotyped by the method of MALDI-TOF mass spectrometry. The SNP of rs175174 locating in the intron 4 of ZDHHC8 which might affect the splicing process and its 27 kb nearby SNP (rs1633445) did not show significant association with schizophrenia. Base on these two markers, we can not confirm the ZDHHC8 gene as susceptible gene for schizophrenia in the cohort of Taiwan.

6. Exon 1 region of PPP3CC may be associated with schizophrenia (submitted, see Appendix 8)

Calcineurin is a calcium/calmodulin-dependent protein phosphatase composed by two subunits; a regulatory subunit of calcineurin B (CNB), and a catalytic subunit of calcineurin A (CNA). PPP3CC is the gamma isoform of the CNA located at the chromosome 8p21.3 region. In prior work, PPP3CC, among the other calcineurin subunits of CNB, CNA-alpha, and CNA-beta, showed the only significant associations with schizophrenia for the population of Caucasian and South Africa in either single locus and haplotype analyses. To evaluate if PPP3CC is a candidate gene for schizophrenia in the population of Taiwan, ten SNP markers across the gene were genotyped by the method of MALDI-TOF in 218 schizophrenia families with at least two affected siblings. One SNP (rs2272080) located around the exon 1 untranslated region of PPP3CC was nominally associated with schizophrenia ($p=0.024$). This result suggests the PPP3CC gene may be a candidate gene for schizophrenia in the population of Taiwan.

7. Significant association between schizophrenia and Neuregulin 1 Gene (manuscript under preparation)

Several linkage studies have linked schizophrenia to chromosome 8p21-12 region and the positional candidate gene, Neuregulin 1 (NRG1), have been reported associated with schizophrenia in several ethnic samples. However, the

regions reported association seemed inconsistent across studies. We aimed to test the association of NRG1 by a two-stage fine-mapping approach in 218 co-affected sib pair families of schizophrenia in Taiwan. Firstly, we selected 36 single nucleotide polymorphisms (SNPs) of NRG1from public SNP database, which distributed across the entire gene region from promoter to 3' untranslated region. We genotyped these SNPs for validation in a sample subset. 16 SNPs with average intermarker distance of 50 kbp, met the validation criterion of minor allele frequency above 0.1. Secondly, the validated SNPs were genotyped in 218 families with at least 2 siblings affected with schizophrenia. Intermarker linkage disequilibrium was calculated by Gold program. Single locus and haplotype association analysis were performed with Transmit v2.5.4 program. We found the SNP rs3735776 was significantly associated with the narrow (p=0.04) and the broad (p=0.03) models of schizophrenia in single locus association analysis. The NRG1 haplotype (SNP8NRG221-rs4452759-rs4476964- SNP8NRG243) of T-T-C-T is significantly associated with the narrow (p=0.02) and the broad (p=0.03) models of schizophrenia, haplotype (rs2439325-rs2954041) of A-G is significantly associated with the narrow (p=0.04) and the broad (p=0.04) models of schizophrenia, and the haplotype of (rs3735775-rs3735776) of G-C and A-A was significantly associated with the broad (p=0.04 and 0.05, respectively) model of schizophrenia. In summary, our results showed there was significant association with the three regions of NRG1 in Taiwanese co-affected sib pair families of schizophrenia. The true associated functional variants associated with schizophrenia in our sample remained to be further clarified.

8. **Neuregulin 1 gene is associated with variations in perceptual aberration of schizotypal personality in adolescents (accepted by Psychological Medicine, see Appendix 9)**

Background: We test the hypothesis that the *neuregulin 1* (*NRG1*) gene at chromosome 8p22-p12, which has been implicated as a susceptibility gene to schizophrenia, is associated with variations in schizotypal personality in non-clinical populations.

Methods: A randomly selected sample of 905 adolescents were assessed for their personality features using the Perceptual Aberration Scale (PAS) and the Schizotypal Personality Questionnaire (SPQ) and genotyped for three single nucleotide polymorphisms (SNP8NRG221533, rs3924999, and rs2954041) at the *NRG1* gene.

Results: Single locus analysis showed that the A allele of rs3924999, a functional polymorphism in exon 2, had the largest effect size and exhibited a prominent

allele-dose trend effect for the PAS score. Haplotype analyses using the haplotype trend regression test indicated that the A allele of rs3924999 was mainly responsible for the association with the PAS but not with the SPQ, and the magnitude of significance was not strengthened by the combination of this allele with adjacent locus.

Conclusions: Our study provides the first evidence for the association of the *NRG1* with schizotypal personality and indicates a possible role of *NRG1* in the genetic etiology of schizophrenia through perceptual aberrations.

Summary:

We have identified nine positional candidate genes on the linked chromosome regions, including DISC1(1q42.1), GNPAT(1q42.1), CACNG2(22q12), BMP6(6p24), TXNDC5(6p24), DPYSL2(8p21), TRIM35(8p21), PTK2B(8p21), and CHRNA2(8p21) and 4 significant associated genes, including NOTCH4, NRG1, DAAO, G72 in our sample. DISC1 and GNPAT were shown to significantly associate with the impairment of sustained attention assessed by CPT, and CACNG2 was shown to associate with the impairment of executive function assessed by WCST. Further analysis using the impairment of sustained attention and executive function of schizophrenia for subtyping is on-going.

We need to interpret the result with caution and cannot exclude the possibility of false positive. Further replication and fine mapping study to delineate the true associated genomic region is warranted. The on-going project is to clarify the risk polymorphisms of the potential candidate genes and their correlated functional expression. We hope it will be fruitful to identify the role of the candidate genes playing in the pathophysiological mechanism of schizophrenia.

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計畫成果自評：

本研究計劃是一個三年計劃，運用各種研究技術，整合不同研究專業，進行精神分裂症致病性基因之標認研究。本研究有下列幾點成果，值得討論：

1. 本研究之研究過程，整合臨床精神科醫師、生物統計、遺傳統計、分子生物學、遺傳流行病等不同專業，統合多機構，含台大醫學院、台大公衛學院流病所、中研院生物醫學研究所、統計所，為一個精神分裂症致病基因研究團隊，此研究團隊之成員，互相學習，有專業成長之目的，且培養碩士班與博士班學生，且可為下階段研究精神分裂症致病基因功能變異之基礎。
2. 本研究呈現 13 個候選之致病性基因，分佈在 6 個染色體上。本研究結果印證“多基因致病”之假說。本研究結果呈現所謂的多基因是在 13 個基因，或許比 13 個基因為少，此有待下階段基因功能研究予以檢驗。此研究結果具體呈現精神分裂症可能致病基因在 10 個基因左右。此可以為下一步，研究計劃鋪路，例如可以研究各個基因多態性或病理性突變在精神分裂症之病理基礎。
3. 本研究結果將在世界精神醫學遺傳學大會發表研究結果，且將陸續寫成論文發。目前有一篇論文被 Psychological Medical 接受，6 篇論文正在投稿中，有 3 篇將在 2000 年 10 月世界精神遺傳學大會中發表，有二篇正在準備論文之中。
4. 本研究之結果，最後雖然將可以 10 篇左右論文發表，但仍未能在最領先之雜誌上被刊登。未來之研究，將結合更多專業，進行致病性基因之功能研究，進而探討基因與環境互動之研究。更多專業參與此研究團隊，一起合作，為提昇本研究團隊具國際競爭力的最好方法。本研究團隊，正在下一個研究計劃中，朝此方向努力。羅馬不是一天完成，本研究計劃正努力，步步為營，朝更具國際競爭力之路發展。

Appendix 1

ORIGINAL ARTICLES

A SNP Fine Mapping Study of Chromosome 1q42.1 Reveals the Vulnerability Genes for Schizophrenia, GNPAT and DISC1: Association with Impairment of Sustained Attention

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Background: On the basis of our previous finding that the marker D1S251 of chromosome 1q42.1 was significantly associated with schizophrenia, we adopted a two-stage approach for further fine mapping in this study.
Methods: In the first stage, 120 single nucleotide polymorphisms (SNPs) around 1 Mb of D1S251 were selected from public database and genotyped in 95 individuals to validate if allele frequency above 10%. In the second stage, we genotyped the 47 validated SNPs out of 120 SNPs in 102 families with at least 2 siblings affected with schizophrenia. Two phenotype models (narrow: DSM-IV schizophrenia only; and broad: including schizophrenia, schizoaffective, and other non-affective psychotic disorders) were used to define the disease phenotype.
Results: We found two SNPs blocks significantly associated with schizophrenia through family-based association analyses. Five SNPs in the block 1, which located between intron 2 and intron 13 of the GNPAT gene, showed significant associations either through single locus TDT ($p<0.05$) or haplotype association analysis ($p<0.05$). Two SNPs in the block 2, which located between intron 4 and intron 5 of the DISC1 gene, also showed significant results in both the single locus ($p<0.05$) and haplotype association analysis ($p<0.05$). The potential involvement of the GNPAT with schizophrenia is preliminary, while the finding of the DISC1 is a replication of previous studies.
Conclusions: We further revealed that the association of the DISC1 gene with schizophrenia was mainly limited to those with sustained attention deficits assessed by the Continuous Performance Test and may indicate a potential biological pathway for future investigation.
Key Words: schizophrenia; sustained attention; DISC1; GNPAT; haplotype association; quantitative TDT

Schizophrenia is a familial complex disorder and the risk to first-degree relatives is approximately ten times the risk to relatives of controls (Guze et al 1983; Tsuang et al 1980). The concordance of schizophrenia for monozygous twin pairs (46%~53%) is higher than that for dizygotic twin pairs (14%~15%) with a heritability estimated to be around 0.7 (Kendler 1983; Prescott and Gottesman 1993). The evidence of genetic contribution to the etiology of schizophrenia was further supported by adoption studies (Heston 1966; Kendler et al 1994; Kety et al 1994). Segregation analyses indicated that models involving multiple genes better fit the observed patterns of schizophrenia in family members than do the single major locus model (Faraone and Tsuang 1985; Risch and Baron 1984). It was suggested that several genes in epistasis might be responsible for the genetic etiology of schizophrenia (Risch 1990).

One of the promising chromosome region possibly hosting the candidate vulnerability genes of schizophrenia was 1q 42, where a balanced translocation (1; 11)(q42.1; q14.3) disrupted two genes, i.e., Disrupted-in-Schizophrenia 1 (DISC1) and DISC2, has been found to be associated with major mental illnesses including schizophrenia in a large Scottish family pedigree (Blackwood et al 2001; Devon et al 2001; Millar et al 2001; Millar et al 2000). This region of linkage evidence has also been independently confirmed in a Finnish family sample (Hovatta et al 1999) and a Taiwan family sample (Hwu et al 2003). However, the linkage evidence has not been consistently supported in other ethnic groups (Bassett et al 2002; Levinson et al 2002; Macgregor et al 2002).

Because of the concern about the heterogeneity and inconsistent findings regarding linkage studies of schizophrenia, using certain endophenotype to refine the phenotype characterization has been advocated (Gottesman and Gould 2003). Both sustained attention deficit and executive dysfunction have substantial empirical evidence to support them as potential candidates for such endophenotypic markers. First, sustained attention deficits as measured on the Continuous Performance Test (CPT) (Rosvold et al 1956) have been shown to be presented not only in schizophrenic patients, but also in subjects with schizotypal personality disorder and in nonpsychotic relatives of schizophrenic patients (Chen and Faraone 2000; Cornblatt and Keilp 1994). Using 2.5 standard deviations or more below the population mean as threshold, the recurrence risk ratio for CPT performance among parents or siblings was higher than that of schizophrenia alone (Chen et al 2004; Chen et al 1998b). Second, executive functions as measured by the Wisconsin Card Sorting Test (WCST) (Robinson et al 1980) are known to be impaired in schizophrenic patients (Goldberg et al 1987; Koren et al 1998) and their first degree relatives (Wolf et al 2002). Among schizophrenic patients, impaired executive functioning has been related to hypofrontality (Weinberger et al 1988).

Using both the CPT and WCST to define endophenotypes for schizophrenia might be helpful for addressing the heterogeneity and variable expression of schizophrenia in linkage analyses. On the basis of a previous finding that the short tandem repeat marker D1S251 located within the gap between the TRAX and DISC1 genes had significant linkage with schizophrenia in a Taiwanese sample (Hwu et al 2003), we aimed in this study to pursue the possible significant association of SNP markers located nearby the D1S251 region using the SNP fine mapping. Our first hypothesis is that if there are some SNP markers nearby the D1S251 marker found to be significantly associated with schizophrenia, these SNP markers would belong to a significant haplotype located in some functional genes expressed in the brain. In addition to the clinical diagnosis of schizophrenia, we intend to examine the relations between the CPT and WCST performance and the SNPs. If there is association

between schizophrenia and some SNPs, our second hypothesis is that the association would become more significant in the subgroup of patients with a certain endophenotype whereas become non-significant in the subgroup without the endophenotype.

Methods and Materials

Subjects

Schizophrenic probands who had at least two affected siblings were identified from the Department of Psychiatry, National Taiwan University Hospital and the University-affiliated Taoyuan Psychiatric Center. Data collection was initiated after informed consents were obtained from the identified study subjects and their families. All of the family members were personally interviewed by the research psychiatrists using the Psychiatrist Diagnostic Assessment (PDA) (Hwu 1999). The final diagnostic assessment was formulated by integrating the PDA data and clinical information of medical chart records. The final diagnosis was done following the DSM-IV criteria for schizophrenia, schizoaffective disorder, and other non-affective psychoses. Clinical data of age at onset of initial symptoms, negative symptoms, and positive symptoms were collected. The negative and positive symptoms were assessed using schedule for assessment of negative symptoms (SANS) (Andreasen 1983) and schedule for assessment of positive symptoms (SAPS) (Andreasen 1984) with satisfactory reliability. Negative symptom score was the sum of all global scores of five negative symptom dimensions, including affective flattening, alogia, avolition/apathy, anhedonia/asociality, and impaired attention. Positive symptom score was the sum of all global scores of 4 positive symptom dimensions, including hallucination, delusion, excitements, and thought derailment.

In total, 102 schizophrenic nuclear families with at least two affected siblings were recruited in this study. Among the 399 individuals, 231 individuals underwent an undegraded CPT test and 225 individuals underwent a degraded CPT test. Meanwhile, 164 subjects underwent WCST assessment.

Neuropsychological assessment

CPT A CPT machine from Sunrise System, v. 2.20 (Pembroke, MA, USA), was used to assess sustained attention. The procedure has been described in detail elsewhere (Chen et al 1998a). Briefly, numbers from 0 to 9 were randomly presented for 50 msec each, at a rate of one per second. Each subject undertook two CPT sessions: the undegraded 1-9 task and the 25% degraded 1-9 task. Subjects were asked to respond whenever the number “9” preceded by the number “1” appeared on the screen. A total of 331 trials, 34 (10%) of which were target stimuli, were presented over 5 minutes for each session. During the 25% degraded session, a pattern of snow was used to toggle background and foreground so that the image was visually distorted. Each test session began with 2 minutes of practice (repeated if subjects required). One signal-detection index of performance on the test, sensitivity (d´), was derived from the hit rate (probability of response to target trials) and false-alarm rate (probability of response to nontarget trials) (Nuechterlein 1991). Sensitivity is an individual’s ability to discriminate target stimuli from nontarget stimuli. In a 1-week test-retest reliability study (Chen et al 1998a) of the CPT versions used in this study, the intraclass correlation coefficients or reliability of d′ were 0.83 and 0.82 for the undegraded and the 25% degraded 1-9 task, respectively.

WCST We employed a computerized version of the WCST (Tien et al 1996) that

had been applied in a previous study in Taiwanese population (Lin et al 2000). During the WCST, subjects were required to match response cards to the four stimulus cards along one of three dimensions (color, form, or number) by pressing one of the 1 to 4 number keys on the computer keyboard. Subjects were not informed of the correct sorting principle, nor were they told when the principle would shift during the test, but they were given feedback ("Right" or "Wrong") on the screen after each trial. Unlike one common form of the traditional WCST in which the test ends after six correct categories achieved, the testing in this study continued until all 128 cards were sorted. All of the indexes defined in the WCST manual (Heaton et al 1993) except for Total Correct were used for analysis. The Total Correct index was not included since it is complementary to Total Errors. The indexes used were (1) Total Errors: total number of perseverative and nonperseverative errors; (2) Nonperseverative Errors: number of errors that were not perseverative; (3) Perseverative Errors: number of errors that were perseverative, reflecting tendency towards perseveration; (4) Perseverative Responses: number of responses that were perseverative, regardless of whether they were correct or not; (5) Categories Achieved: number of times 10 correct responses in a row were made, reflecting overall success; (6) Trials to Complete First Category: number of trials to successfully complete the first category (counted as 129 if no category was completed), reflecting initial conceptual ability; (7) Conceptual Level Response: proportion of consecutive correct responses occurring in runs of 3 or more, reflecting insight into the correct sorting principles; (8) Failure to Maintain Set: number of times subject makes between 5 and 9 correct responses in a row, reflecting efficiency of sorting; and (9) Learning to Learn: average difference in percent errors between successive categories, reflecting the average change in conceptual efficiency during the test (Heaton et al 1993). The last index can be calculated only for whom the total numbers of Categories Achieved and categories attempted are larger than 3.

SNP selection criteria and validation

Fine mapping studies were using SNP dense markers spreading upstream and down stream of the dinucleotide marker D1S251 located at 1q42.1. In the defined region of 2cM around DIS251, a total of 120 SNPs were selected. The inter SNP marker distance ranged from 5 kb to 60 kb, with an average of 32.5 kb. According to the location of these SNPs relative to the position of the functional genes, the SNPs were selected based upon the following priority of exon, 5'-untranslated region, 3-untranslated region, introns, promoter (CpG island), and gaps between functional genes, which expressed in the central nervous system. We used 31 trios and 2 independent individuals of a totally 95 individuals to validate the 120 SNPs from public database (http://www.ensembl.org/Homo_sapiens/martview).

SNP genotyping

All SNP genotypings were performed by the method of matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS). Primers and probes flanking the SNPs were designed by using SpectroDESIGNER software (Sequenom, San Diego, CA, USA). A DNA fragment (100-300 bp) encompassing the SNP site was amplified using the polymerase chain reaction (PCR) (GeneAmp 9700 thermocycler, Applied Biosystems, USA) according to the manufacturer's instruction.

After PCR amplification, the un-incorporated deoxynunleotide triphosphate (dNTP) of the PCR reaction mixture was neutralized by treating with shrimp alkaline phosphatase (SAP) at 37 °C for 20 minutes. The reaction mixture was incubated at 85

°C for 5 minutes to inactivate the SAP activity. Primer extension was performed by adding the probe, Thermo Sequenase (Amersham Pharmacia, Piscataway, NJ, USA) and appropriate dideoxynucleotide triphosphate (ddNTP)/dNTP mixture, and following 55 cycles of denaturing at 94 °C for 5 sec, annealing at 52 °C for 5 sec, and extension at 72 °C for 5 sec. Different extension products were differentiated by mass through MALDI-TOF as follows.

Cation exchange resin (SpectroCLEAN, Sequenom) was added to the above reaction mixture to get rid of salt, which would interfere with the mass reading in the MALDI-TOF analysis. After desalting, the reaction mixture was spotted onto the SpectroCHIP (using the SpoectroPOINT) where they were co-crystallized with the matrix. After introduced to the SpectroREADER, a nitrogen laser with nanosecond-wide pulses interrogated the samples on the SpectroCHIP in the high-vacuum environment of the time-of-flight mass spectrometer. Upon laser irradiation, the matrix crystals absorbed the laser's energy and the extension products were converted into gas phase ions, which upon acceleration undergo mass-dependent separation over approximately 1-meter flight path. Molecular masses were determined with 0.01 to 0.02% estimated accuracy. Acquired spectra were transferred to the MassARRAY Server (Sequenom, San Diego, CA) where they were automatically interpreted and corrected for their genotypes.

Statistical analysis

In order to verify the sample accuracy included family relationship and genotype, PEDCHECK version 1.1 (O'Connell and Weeks 1998) and UNKNOWN version 5.23 (Terwilliger 1994) were employed to check Mendelian inheritance and Procedure ALLELE in SAS/GENETICS release 8.2 (SAS 2002) was used to test for Hardy-Weinberg equilibrium. Linkage disequilibrium of inter-markers was measured using coefficient D´(Hedrick 1987) which was also used to define haplotype blocks. A graphic presentation of block pattern was completed using GOLD software (Abecasis and Cookson 2000).

Family-based transmission disequilibrium tests were applied to test linkage disequilibrium. Both of single-locus and haplotype-based association analyses were carried out simultaneously using two popular programs for the nuclear family data, haplotype FBAT version 1.4.1 (Horvath et al 2001; Horvath et al 2004; Laird et al 2000) and TRANSMIT version 2.5.4 (Clayton 1999) An individual's haplotype was inferred using SimWalk2 version 2.86 (Sobel and Lange 1996; Sobel et al 2002; Sobel et al 2001), which uses Markov Chain Monte Carlo algorithm. Moreover, GEE method (Liang 1986) was applied to test the interaction between haplotype blocks using the Proc GENMODE of the SAS (version 8.0 for Windows).

Besides the analysis of qualitative trait, quantitative analysis using highly heritable quantitative trait was also considered. The analysis of heritability and quantitative-type transmission disequilibrium test based on variance component approach was applied using QTDT version 2.4.3 (Abecasis et al 2000a; Abecasis et al 2000b).

Results

SNP validation

A SNP was considered valid if the frequency of minor allele was larger than 10% and genotyping missing rate was smaller than 30%. Forty-seven out of 120 SNPs met the validity criteria. The 47 SNPs span across 1591 kb around D1S251 marker (Table

1) and cover 11 known functional genes of COG2, AGT, CAPN9, FLJ14525, FLJ 22584, ARV1, GNPAT, DKFZP547NO43, EGLN1, TRAX, and DISC1. Except five SNPs (SNP495, 506, 513, 527, 581), the other 42 SNP markers are compatible with the Hardy-Weinberg equilibrium.

Construction of SNP block

In order to perform haplotype analysis, haplotype block was evaluated using inter-marker linkage disequilibrium coefficient. Two SNP blocks were identified by using two criteria: 1) a significant inter-marker association based on the chi-squared test, and 2) coefficient D′ was higher than 0.8. The locations of these two SNP blocks are shown in Figure 1. The first block covers the SNP markers of 482 (intron 2), 485 (intron 2), 479 (intron 5), 488 (intron 11) and 489 (intron 13) in the GNPAT gene region, and the second block covers the markers of 517 (intron 4) and 518 (intron 5) in the DISC1 gene region (Figure 2).

Single-locus association analysis

Preliminary analyses were conducted in nuclear families to evaluate the potential association between each SNP and phenotype, defined in either a narrow model (DSM-IV schizophrenia only) or broad model (DSM-IV schizophrenia, schizoaffective disorder, and other nonaffective psychotic disorders). From the result of single-locus association analyses using computer program FBAT version 1.4.1 (Horvath et al 2001; Laird et al 2000), which is robust to population admixture, we found that some SNP variants exhibited a significant association with schizophrenia (Table 2). For the broad model, the significant SNP marker on GNPAT gene was SNP 485 (rs508908), and that on DISC1 gene were SNP517 (rs2793092) and SNP518 (rs2793091). For the narrow model, SNP marker 485 (rs508908) on GNPAT gene showed a borderline effect, whereas SNP 517 (rs2793092) and 518 (rs2793091) on DISC1 gene exhibited highly significant associations. For comparison, similar analyses using computer program TRANSMIT version 2.5.4 (Clayton 1999), which can utilize data from all families even when parental genotypes are unknown, yielded similar results but with more significance, especially for the SNPs on GNPAT gene under the narrow model of phenotype, i.e., 485 (rs508908) ($p=0.019$) and 479 (rs538643) ($p=0.049$).

Haplotype-based association analysis

Since there were SNPs in each block that exhibited single-locus association with schizophrenia, either narrowly or broadly defined, haplotype-based association analysis was further pursued. The results from haplotype FBAT program version 1.4.1 (Horvath et al 2004) showed that haplotype GATTT in the GNPAT gene SNP block (block 1) is only slightly significant for the broad model of schizophrenia phenotype ($p=0.0461$) (Table 2). The haplotypes AG and GA in the DISC1 gene SNP block (block 2) exhibited a significant association for both of the narrow model and the broad model of schizophrenia. It seemed that haplotype GA was a risk haplotype (negative Z statistic), whereas AG possessed a protection effect. Analyses carried out by TRANSMIT program version 2.5.4 (Clayton 1999) yielded similar pattern and the results are not shown here. The interaction of two haplotype blocks was investigated by using the GEE method (Liang 1986). However, no significant interaction effect between the two was found.

Quantitative TDT for phenotypic indicators

In exploring the relationship between the SNP genotypes and potential endophenotypes, we examined four types of traits: the age of onset of the initial symptom, clinical symptoms (PANSS Negative Scale scores, PANSS Positive Scale scores), CPT scores (undegraded CPT d′, degraded CPT d′), and WCST scores (total errors, nonperseverative errors, perseverative errors, perseverative responses, categories achieved, conceptual level responses, trials to complete first category, learning to learn, and failure to maintain set). The heritability analysis using QTDT version 2.4.3 (Abecasis et al 2000a; Abecasis et al 2000b) was conducted among the schizophrenic families to screen for important quantitative phenotypes, in which the variance components were decomposed into those due to environment (σ_e^2) and genetic effect (σ_g^2). Heritability h^2 is defined as the explained proportion of the variance due to genetic component, i.e., $\sigma_g^2/(\sigma_g^2+\sigma_e^2)$. Among the 14 definitions examined, only two traits were found to have a significant heritability on the basis of likelihood ratio test: the undegraded CPT d′ (h^2 =0.831, p = 8x10^{-7}) and the degraded CPT d′ (h^2 =0.751, p = 2x10^{-7}).

Quantitative haplotype analysis that detects unbalanced allelic transmission and important variance components was then conducted using these two highly heritable traits. Among different phenotypic mean and variance component models, we applied the Akaike information criterion (AIC) to select the optimum genetic model underlying the linkage and association with schizophrenia. Of note, the response criterion lnβ of the CPT was regarded as a covariate to be adjusted for in the model fitting because it reflected an individual motivation for the test. The fitted models showed that the first haplotype block was linked and associated the degraded CPT d′ (log likelihood =-105.14, AIC=222.28), and the second haplotype block was linked and associated with the undegraded CPT d′ (log likelihood =-164.57, AIC=343.14).

To further examine the relationship between the SNPs and CPT performance, we next treated schizophrenic patients' CPT performance as a covariate on the association between schizophrenia diagnosis and the SNPs. We first inferred each individual's haplotype using the SimWalk2 and then subject the individual's clinical diagnosis (schizophrenic or not) to logistic regression on the haplotypes with the deficit status in CPT d′ (with an adjusted z score ≤-2.5 or not) as a covariate using the PROC GENMOD. The reason for choosing -2.5 was based on a previous finding that a recurrence risk ratio analysis of the CPT among the non-psychotic relatives of schizophrenic patients indicated that a criterion more stringent than 2 standard deviations below the population mean led to a risk ratio higher than schizophrenia itself (Chen et al 2004).

Because the effect of CPT d′ was significant in these logistic regression analyses, we then stratified the sample into those without CPT deficit and those with CPT deficit and conducted the logistic regression analysis separately for these two subgroups. It turned out the haplotypes of the second block, i.e., SNP517-518, tended to exhibit signification association with schizophrenia in those with CPT deficit but not in those without CPT deficit (Table 3). For patients with deficit on the undegraded CPT, a single copy of haplotype GA had an increased risk for schizophrenia (those with genotype GA/GA or GA/others had a OR > 1.0, regardless of narrow or broad model being used for analysis), whereas two copies of haplotype AG had a protective effect for schizophrenia (those with genotype AG/AG had a OR < 1.0, regardless of narrow or broad model being used for analysis). However, for patients without deficit on the undegraded CPT, the pattern was less consistent (only those with genotype

GA/others had a decreased risk for schizophrenia). Intriguingly, no association between SNP517-518 haplotypes and schizophrenia were found when the sample was stratified by the degraded CPT. When similar stratified analyses were conducted for the haplotypes of the first block, i.e., SNP482, 485, 479, 488, and 489, none of the haplotypes exhibited a significant association with schizophrenia in either those with or without CPT deficit (data not shown).

Discussion

Several genomewide searches have shown that the chromosome 1q42 region may harbor candidate genes for schizophrenia (Curtis et al 2003; Hovatta et al 1999; Hwu et al 2003). On the basis of a previous finding that the chromosome sequence tagged site D1S251 had the highest NPL score of 1.73-2.18 (p=0.01-0.03) for the linkage with schizophrenia in Taiwanese population (Hwu et al 2003), we carried out this SNP fine mapping study in the 2 cM area surrounding D1S251 to search for specific vulnerable genes of schizophrenia in this population. Two blocks were found to be significantly associated with schizophrenia and their implications are examined in turn.

The first block covers the genetic region of GNPAT (or DHAPAT) gene, which encodes the dihydroxyacetone-phosphate acyltransferase enzyme that is located within peroxisomes and catalyzes the biosynthesis of ether phospholipids. This enzyme was found to be in deficiency of varying degrees in patients with congenital peroxisomal disorders than normal controls (Hajra 1997; Schutgens et al 1984). A girl with deficient DHAPAT was reported to have severe mental retardation, developmental delay, and growth failure (Elias et al 1998). Our result suggests that the GNPAT may also be involved in the broad definition of schizophrenia (p=0.046), though may not play a major role due to its weak association. Since this is the first report of the potential involvement of GNPAT gene in schizophrenia, further work examining the SNP markers located within the exons of the gene is warranted.

The second block that was significantly associated with schizophrenia is located within the DISC1 gene, and consistently exhibited significant associations in both single locus and haplotype-based association analysis. The DISC1 gene was found to be closer to the peak NPL score of D1S251 marker than to the adjacent lowest NPL score of the D1S404 marker in the previous Taiwan study (Hwu et al 2003). Furthermore, the D1S251 marker also showed a highest LOD scores of 2.5 (p=0.002) in the populations of Britain and Iceland (Curtis et al 2003). Thus, this study in an expanded sample of affected sib-pair families provides a replication of the linkage of DISC1 to schizophrenia.

In terms of haplotype analysis, our results suggest that the haplotypes consisting of SNPs in introns 4 and 5 of the DISC1 gene were significantly associated with schizophrenia. In contrast, the significant haplotype constituted by two SNPs for the Finnish population is located between the intron 1 and exon 2 (Hennah et al 2003). The discrepancy in the composition of the associated haplotype between the two studies could have two explanations. First, these reported regions may be in linkage disequilibrium and the associations of the haplotypes merely suggest that the DISC1 gene is nearby a true susceptibility gene for schizophrenia. Second, both regions may be truly involved in the genetic susceptibility to schizophrenia. However, both regions of the DISC1 gene may interact with different cytoskeletal proteins. The exons 1 and 2 of the DISC1 gene encode the putative globular domains that bind strongly with the cytoplasmic microtubules of α-tubulin, while the exons 3-13 encode the putative helical tail (coiled-coil motif) that is essential for interaction with neurodevelopmental

protein NUDEL (Brandon et al 2004; Ozeki et al 2003). If the second explanation holds, it implies different pathological pathways in different ethnic groups. Further investigation to see whether there are risk mutations in the exon 5 of DISC1 in our study population is warranted to distinguish these two possibilities.

There are two more differences in the results between the Finnish study (Hennah et al 2003) and ours. Neither the gender difference in the under-transmission of the DISC1 gene nor the association of the TRAX gene located in the upstream of the DISC1 gene with schizophrenia in the Finnish sample was found in our study. Nevertheless, the two studies did have a consistent finding that the DISC1 gene was not associated with the age onset of schizophrenia. Since we have ruled out the possible existence of the balanced translocation (q42.1; q14.3) in our schizophrenia DNA samples (Liu et al., unpublished results), the involvement of the DISC1 gene in our schizophrenic patients may be exerted through mechanisms other than genomic disruption.

One unique feature of this study is that we further revealed that the association of the DISC1 with schizophrenia was limited to those with CPT deficits. The CPT is a measure of sustained attention and has been demonstrated to be highly sensitive to brain damage or dysfunction (Riccio et al 2002). Previous studies in Taiwanese population indicated that the heritability for the CPT performance in the nonpsychotic first-degree relatives of schizophrenic patients ranged from 0.48 to 0.62 (Chen et al 1998a), and the CPT deficits in schizophrenic patients were not amenable to 3-month neuroleptic treatment (Chen and Faraone 2000). In this study, the heritability for the CPT in the families of the affected sibpairs was even higher (0.751 to 0.831). These consistent results indicate that the CPT deficits are likely to be a useful indicator for the genetic susceptibility to schizophrenia.

Under the AIC test for the optimum genetic model, the GNPAT genetic haplotype was significantly associated with the degraded CPT scores, whereas the DISC1 genetic haplotype with the undegraded CPT scores. When further stratifying the CPT performance into deficit or non-deficit according to the threshold of an adjusted z score of –2.5, the GA haplotype of DISC1 gene was found to be the dominant susceptible indicator for the undegraded CPT deficit, yet the AG haplotype was the dominant protective indicators for the CPT deficit. In contrast, the GNPAT did not show such an association with schizophrenia in the similar stratification analysis. The differential association of both versions of the CPT with the two blocks might be due to chance finding, since the significance level of some association was borderline only (e.g., the GNPAT block with the degraded CPT). Another possibility is that the two versions of the CPT tap different aspects of neuropsychological functioning: the undegraded session involved mainly a working memory component, whereas the degraded session included a sensory-perceptual component as well (Chen and Faraone 2000). Our results suggest that the DISC1 gene, especially the intron 4-intron 5 region, may be involved in regulating the sustained attention per se, i.e., working memory, of schizophrenia. The opposite direction in predicting the risk to sustained attention deficit for the GA and AG haplotypes might be accounted for by binding affinities with different regulators.

Unlike the results of the CPT, the WCST scores were associated with neither the DISC1 nor the GNPAT genes. It appears that the 1q42.1 region may have little contribution to the pathological process of executive functioning in schizophrenia.

In summary, a 2 cM region surrounding the D1S251 marker was screened with SNPs for genetic association with schizophrenia. Two blocks of SNP haplotypes in this region showed significant associations with schizophrenia. These two blocks

were located within the regions of GNPAT (a five-SNP block) and DISC1 (a two-SNP block) genes, respectively. The potential involvement of the GNPAT with schizophrenia is preliminary, while the finding of the DISC1 is a replication of previous studies. We further revealed that the association of the DISC1 with schizophrenia was limited to those with CPT deficits and may indicate a potential biological pathway for future investigation.

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Table 1. Description of 47 SNP markers

SNP Order Number	SNP Study Number	SNP ID	Number of families	Allele Type	Minor allele freq.	Chromosomal Position
1	565	rs917384	101	G/ C	0.5652	chr1:227185526
2	472	rs1887492	101	G/ A	0.1585	chr1:227252942
3	471	rs699	102	G/ A	0.2000	chr1:227318982
4	478	rs1202566	100	G/ A	0.3648	chr1:227461322
5	484	rs2275333	101	A/ T	0.4024	chr1:227530435
6	475	rs2153051	101	T/ C	0.4187	chr1:227549768
7	576	rs2024816	98	C/ T	0.1250	chr1:227707934
8	569	rs765265	91	G/ A	0.385	chr1:227749195
9	482	rs487047	101	G/ A	0.3871	chr1:227850811
10	485	rs508908	100	A/ T	0.3843	chr1:227859800
11	479	rs538643	102	T/ C	0.388	chr1:227873448
12	488	rs539699	102	T/ C	0.384	chr1:227880149
13	489	rs578945	102	T/ C	0.388	chr1:227883303
14	483	rs1435167	102	A/ T	0.468	chr1:227975433
15	494	rs1621135	102	A/ T	0.504	chr1:228137941
16	501	rs1655290	102	C/ T	0.504	chr1:228143015
17	491	rs1615409	101	A/ C	0.4797	chr1:228156605
18	496	rs766288	101	G/ T	0.3252	chr1:228166876
19	581	rs892356	102	C/ T	0.2114	chr1:228220138
20	6055	rs1030711	85	A/ T	0.1316	chr1:228228649
21	6054	rs1865226	94	C/ T	0.2714	chr1:228231070
22	502	rs1865225	101	A/ G	0.4065	chr1:228236192
23	498	rs2082552	101	T/ C	0.4836	chr1:228241078
24	495	rs1094658	102	C/ G	0.18	chr1:228246585
25	499	rs980394	102	A/ G	0.184	chr1:228252695
26	506	rs1417585	79	T/ C	0.1822	chr1:228276792
27	513	rs1417584	100	C/ T	0.1736	chr1:228292238
28	524	rs1977797	92	T/ A	0.4363	chr1:228298262
29	514	rs1954175	92	T/ C	0.4451	chr1:228328598
30	517	rs2793092	102	A/ G	0.449	chr1:228353528
31	518	rs2793091	101	G/ A	0.4569	chr1:228367796
32	590	rs2812393	100	C/ G	0.3941	chr1:228386861
33	526	rs1407598	99	T/ G	0.3621	chr1:228420013
34	529	rs1000730	85	C/ T	0.396	chr1:228436789
35	527	rs734551	85	G/ A	0.4624	chr1:228467621
36	531	rs999710	89	A/ G	0.4485	chr1:228484131
37	544	rs999709	100	A/ G	0.4487	chr1:228484218
38	535	rs967433	101	T/ C	0.3554	chr1:228505003
39	541	rs2038636	101	G/ A	0.1992	chr1:228528437
40	548	rs928100	102	G/ C	0.3525	chr1:228543084
41	543	rs1417866	91	A/ G	0.3333	chr1:228560101
42	547	rs701160	101	C/ T	0.2438	chr1:228565669
43	552	rs701161	98	A/ G	0.4397	chr1:228577302
44	557	rs821664	102	C/ T	0.2917	chr1:228595110
45	6056	rs821616	102	A/ T	0.127	chr1:228617786
46	582	rs1338302	102	C/ T	0.2927	chr1:228751787
47	583	rs1766982	101	A/ G	0.3571	chr1:228847915

Table 2. Haplotype analysis of all families using FBAT program

Block 1		Frequency	Narrow Model N^*	Z^{**}	P-value	Broad Model N	Z	P-value
SNP	482		58	-1.133	0.2573	32	-1.462	0.1437
	485		52	-1.811	0.0701	31	-2.072	0.0383
	479		59	-1.316	0.1882	33	-1.623	0.1045
	488		55	-1.065	0.2870	31	-1.401	0.1611
	489		59	-1.316	0.1882	33	-1.623	0.1045
Haplotype	GATTT	0.608	28	-1.721	0.0853	31	-1.994	0.0461
	ATCCC	0.381	26	1.453	0.1463	29	1.761	0.0782
Block 2								
SNP	517		28	-3.215	0.0013	26	-2.786	0.0053
	518		39	-2.694	0.0071	37	-2.67	0.0076
Haplotype	AG	0.505	33	-2.608	0.0091	31	-2.177	0.0295
	GA	0.416	31	3.347	0.0008	30	3.146	0.0017

**N*: the number of informative family

** *Z*: the test statistic

Table 3. Logistic regression analysis of schizophrenia on haplotypes of SNP517-SNP518 stratified by CPT deficit status

		Without CPT deficit[a]					With CPT deficit				
			Narrow model		Broad model			Narrow model		Broad model	
CPT version	Haplotypes	N	OR	P-value	OR	P-value	N	OR	P-value	OR	P-value
Undegraded CPT	GA/GA	20	1.67	0.342	1.04	0.941	15	3.84	0.074	5.48	0.003
	GA/others	50	0.51	0.123	0.38	0.016	26	3.86	0.047	5.72	0.001
	Others/others	40	1.00		1.00		17	1.00		1.00	
Degraded CPT	GA/GA	18	2.98	0.059	2.40	0.111	16	1.69	0.307	0.98	0.976
	GA/others	52	0.92	0.856	0.81	0.558	23	1.10	0.882	0.82	0.796
	Others/others	39	1.00		1.00		22	1.00		1.00	
Undegraded CPT	AG/AG	33	0.78	0.595	1.44	0.426	17	0.19	0.038	0.13	0.001
	AG/others	54	0.83	0.647	0.97	0.931	23	0.63	0.562	0.58	0.398
	Others/others	27	1.00		1.00		18	1.00		1.00	
Degraded CPT	AG/AG	28	0.38	0.066	0.52	0.156	22	0.66	0.371	1.18	0.814
	AG/others	56	0.75	0.461	0.81	0.594	20	0.77	0.684	1.07	0.913
	Others/others	25	1.00		1.00		19	1.00		1.00	

[a]CPT deficit was defined as an adjusted z score of ≤ -2.5.

Figure legends

Figure 1. Linkage disequilibrium of all SNP markers showed two significant SNP blocks located within a block of 482, 485, 479, 488, and 489, and a block of 517 and 518.

Figure 2. Blocks of SNPs located within genes of GNPAT and DISC1 showed significant linkage disequilibrium and associations among 9 other functional genes in the 2 cM ranges around D1S251 marker.

Fig. 1

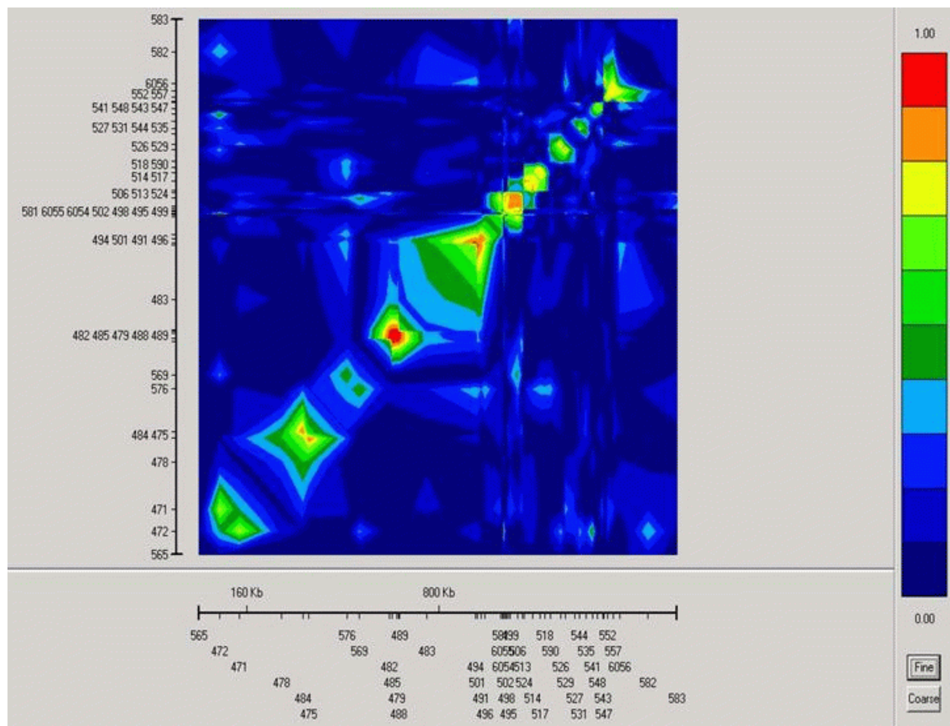
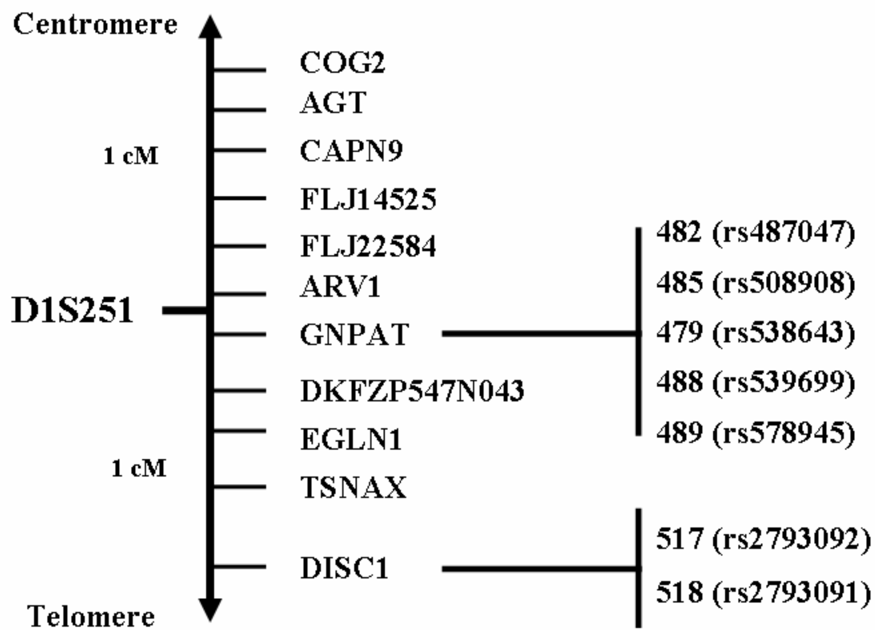


Fig. 2



Appendix 2

SNP Fine Mapping of Chromosome 22q12 Reveals the Novel Vulnerability Gene for Schizophrenia, CACNG2: Association with Impairment of Sustained Attention and Executive Function

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ABSTRACT

The D22S278 marker of chromosome 22q12 had shown the highest linkage association with schizophrenia in the family samples of our previous study. According to this result, we conducted further fine mapping by using the criterion of minor allele frequency above 10%. We selected 47 single nucleotide polymorphisms (SNPs) from 94 potential markers around 1 Mb of D22S278 in 95 individuals. We then genotyped these 47 validated SNPs in 218 pedigrees with at least 2 siblings affected with schizophrenia. We found three genes; MCM5 (rs875400), RASD2 (rs736212), and CACNG2 (rs2283986 and rs2092662) significantly associated with schizophrenia in single locus association analyses ($p<0.05$). There were five haplotypes in four genes; APOL5 (rs1540297-rs2899256-rs2076671- rs879680), MYH-9 (rs2481-rs875726-rs1009150) and (rs3752463-rs1557540-rs713839-rs739097), EIF3S7 (rs140002-rs2142824), and CACNG2 (rs2267360-rs140526-rs1883987- rs916269), showed significantly associations with schizophrenia ($P<0.05$). When correlate these five haplotypes with sustained attention deficits assessed by Continuous Performance Test and executive functions assessed by Wisconsin Card Sort Test, the haplotypes of G-T-G-A and G-T-A-G of CACNG2 gene showed significantly association with both neuropsychological assessments ($P<0.05$). These results suggest that the CACNG2 gene may be a novel susceptibility gene of schizophrenia in the 22q12 region.

INTRODUCTION

The long arm of chromosome 22 has been reported of harboring several susceptible regions to the disease of schizophrenia [1-5]. The regions studied by the sequence tagged site (STS) linking to schizophrenia include of D22S278 (22q13.1)[6-8], D22S283 (22q13.1)[9, 10], D22S279 (22q13) -276 (22q13.2) [11], and D22S683 (22q12.3-13.1) [4]. However, the STS marker at the D22S278 and D22S283 were not fully supported in the ethnic groups of Caucasian [12] and Palestinian Arab population [13]. The family sample we collected previously had joined an international collaboration study organized by Gill et al (1996) entitled as "Schizophrenia Collaborative Linkage Group", which reported suggestive linkage evidence to D22S278 (p=0.001) on chromosome 22q12.{Gill, 1996 #14} This raised up the probabilities that there are schizophrenia candidate genes harbored in this 22q13.1 region in the cohort of Taiwan.

Several genes located in the 22q chromosome regions have been studied and suggested as candidate genes for schizophrenia; the synaptogyrin 1 (SYNGR1; 22q13.1) [2], the phosphatidylinositol 4-kinase, catalytic, alpha polypeptide (PIK4CA; 22q11.21) [14], the synaptosomal-associated protein, 29kDa (SNAP29; 22q11.21) [15], adenosine A2a receptor gene (ADORA2A; 22q11.23) [16], the catechol-O-methyltransferase (COMT; 22q11.21) [17], . This region of linkage evidence has also been independently confirmed in a Finnish family sample [18] and a Taiwan family sample [19]. However, the linkage evidence has not been consistently supported in other ethnic groups [20-22].

As the heterogeneity and inconsistent findings regarding linkage studies of schizophrenia, using certain endophenotype to refine the phenotype characterization has been advocated [23]. Both sustained attention deficit and executive dysfunction have substantial empirical evidence to support them as potential candidates for such endophenotypic markers. First, sustained attention deficits as measured on the Continuous Performance Test (CPT) [24] have been shown to be presented not only in schizophrenic patients, but also in subjects with schizotypal personality disorder and in nonpsychotic relatives of schizophrenic patients [25, 26]. Using 2.5 standard deviations or more below the population mean as threshold, the recurrence risk ratio for CPT performance among parents or siblings was higher than that of schizophrenia alone [27, 28]. Second, executive functions as measured by the Wisconsin Card Sorting Test (WCST) [29] are known to be impaired in schizophrenic patients [30, 31] and their first degree relatives [32]. Among schizophrenic patients, impaired executive functioning has been related to hypofrontality [33].

Using both the CPT and WCST to define endophenotypes for schizophrenia might be helpful for addressing the heterogeneity and variable expression of schizophrenia in linkage analyses. On the basis of a previous finding that the short tandem repeat marker D1S251 located within the gap between the TRAX and DISC1 genes had significant linkage with schizophrenia in a Taiwanese sample [19], we aimed in this study to pursue the possible significant association of SNP markers located nearby the D1S251 region using the SNP fine mapping. Our first hypothesis is that if there are some SNP markers nearby the D1S251 marker found to be significantly associated with schizophrenia, these SNP markers would belong to a significant haplotype located in some functional genes expressed in the brain. In addition to the clinical diagnosis of schizophrenia, we intend to examine the relations between the CPT and WCST performance and the SNPs. If there is association between schizophrenia and some SNPs, our second hypothesis is that the association would become more significant in the subgroup of patients with a certain endophenotype

whereas become non-significant in the subgroup without the endophenotype.

RESULTS

SNP Validation

A SNP was considered valid if the frequency of minor allele was larger than 10% and genotyping missing rate was smaller than 10%. Forty-seven out of 94 SNPs met the validity criteria. The 47 SNPs span across 1747 kb around D22S278 marker (Table I) and cover 20 known functional genes of HMG2L1, TOM1, HMOX1, MCM5, RASD2, APOL6, APOL5, RBM9, APOL2, APOL1, MYH-9, TXN2, FLJ23322, EIF3S7, CACNG2, RABL4, PVALB, NCF4, CSF2RB, and TST. Except two SNPs (SNP 7217, 7300), the other 45 SNP markers are compatible with the Hardy-Weinberg equilibrium.

Construction of SNP block

In order to perform haplotype analysis, haplotype block was evaluated using inter-marker linkage disequilibrium coefficient. Nine SNP blocks were identified by using two criteria: 1) a significant inter-marker association based on the chi-squared test, and 2) coefficient D′ was higher than 0.8. The locations of these nine SNP blocks are shown in Figure 1. The nine blocks covers the SNP markers of 7209-7210-7221-7211, 7214-7222-7225, 7212-7217-7223, 7231-7228-7243-7233, 7256-7265-7266, 7260-7264-7269-7277, 7270-7271-7268, 7279-7275, and 7286-7287-7276-7280. There are five blocks showed significant associations with schizophrenia; the 7231-7228-7243-7233 in the APOL5 gene, the 7256-7265-7266 and the 7260-7264-7269-7277 in the MYH-9 gene, the 7279-7275 in the EIF3S7, and the 7286-7287-7276-7280 in the CACNG2 gene (Fig. 2).

Single-locus association analysis

Preliminary analyses were conducted in nuclear families to evaluate the potential association between each SNP and phenotype, defined in either a narrow model (DSM-IV schizophrenia only) or broad model (DSM-IV schizophrenia, schizoaffective disorder, and other nonaffective psychotic disorders). From the result of single-locus association analyses using computer program FBAT version 1.4.1 [34, 35], which is robust to population admixture, we found that some SNP variants exhibited a significant association with schizophrenia. For the broad model, the significant SNP marker on MCM5 gene was SNP 7217 (rs875400; p=0.0311), on RASD2 gene was SNP 7216 (rs736212; p=0.0481) and that on CACNG2 gene were SNP 7285 (rs2283986; p=0.0458) and SNP 7284 (rs2092662; p=0.0257). For the narrow model, SNP marker 7217 (rs875400; p=0.0143) on MCM5 gene, marker 7216 (rs736212; p=0.0276) on RASD2 gene and SNP 7285 (rs2283986; p=0.0486) on CACNG2 gene exhibited significant associations. For comparison, similar analyses using computer program TRANSMIT version 2.5.4 [36], which can utilize data from all families even when parental genotypes are unknown, yielded similar results but with more significance, especially for the SNP on RASD2 gene, i.e., 7216 (rs736212) p=0.0067 for the narrow model and p= 0.0097 for the broad model of schizophrenia.

Haplotype-based association analysis

Since there were SNPs in each block that exhibited single-locus association with schizophrenia, either narrowly or broadly defined, haplotype-based association analysis was further pursued. The results from haplotype FBAT program version 1.4.1 [37] showed that haplotype CG in the EIF3S7 gene is significant for both the narrow (p=0.0102) and the broad model (p=0.0122) of schizophrenia phenotype. The

haplotypes GTAG in the CACNG2 gene exhibited a significant association in the narrow model (p=0.0262) of schizophrenia. Analyses carried out by TRANSMIT program version 2.5.4 [36] yielded the haplotypes TCTG and CTTT in the APOL5 significant for the narrow model of schizophrenia (p=0.0478 and p=0.0424, respectively). The three-SNP haplotype AAT and the four-SNP haplotype GCAA in the MYH-9 gene showed significant association in the narrow (p=0.0316 and p=0.0428, respectively) model, and only the AAT significant in the broad model (p=0.0258) of schizophrenia. The interaction of two haplotype blocks was investigated by using the GEE method [38]. However, no significant interaction effect between the two was found.

Quantitative TDT for phenotypic indicators

In exploring the relationship between the SNP genotypes and potential endophenotypes, we examined four types of traits: the age of onset of the initial symptom, clinical symptoms (PANSS Negative Scale scores, PANSS Positive Scale scores), CPT scores (undegraded CPT d′, degraded CPT d′), and WCST scores (total errors, nonperseverative errors, perseverative errors, perseverative responses, categories achieved, conceptual level responses, trials to complete first category, learning to learn, and failure to maintain set). The heritability analysis using QTDT version 2.4.3 [39, 40] was conducted among the schizophrenic families to screen for important quantitative phenotypes, in which the variance components were decomposed into those due to environment (σ_e^2) and genetic effect (σ_g^2). Heritability h^2 is defined as the explained proportion of the variance due to genetic component, i.e., $\sigma_g^2/(\sigma_g^2+\sigma_e^2)$. Among the 14 definitions examined, only two traits were found to have a significant heritability on the basis of likelihood ratio test: the undegraded CPT d′ (h^2 =0.831, p = $8x10^{-7}$) and the degraded CPT d′ (h^2 =0.751, p = $2x10^{-7}$).

Quantitative haplotype analysis that detects unbalanced allelic transmission and important variance components was then conducted using these two highly heritable traits. Among different phenotypic mean and variance component models, we applied the Akaike information criterion (AIC) to select the optimum genetic model underlying the linkage and association with schizophrenia. Of note, the response criterion lnβ of the CPT was regarded as a covariate to be adjusted for in the model fitting because it reflected an individual motivation for the test. The fitted models showed that the first haplotype block was linked and associated the degraded CPT d′ (log likelihood =-105.14, AIC=222.28), and the second haplotype block was linked and associated with the undegraded CPT d′ (log likelihood =-164.57, AIC=343.14).

To further examine the relationship between the SNPs and CPT performance, we next treated schizophrenic patients' CPT performance as a covariate on the association between schizophrenia diagnosis and the SNPs. We first inferred each individual's haplotype using the SimWalk2 and then subject the individual's clinical diagnosis (schizophrenic or not) to logistic regression on the haplotypes with the deficit status in CPT d′ (with an adjusted z score ≤-2.5 or not) as a covariate using the PROC GENMOD. The reason for choosing -2.5 was based on a previous finding that a recurrence risk ratio analysis of the CPT among the non-psychotic relatives of schizophrenic patients indicated that a criterion more stringent than 2 standard deviations below the population mean led to a risk ratio higher than schizophrenia itself [28].

Because the effect of CPT d′ was significant in these logistic regression analyses,

we then stratified the sample into those without CPT deficit and those with CPT deficit and conducted the logistic regression analysis separately for these two subgroups. It turned out the haplotypes of the second block, i.e., SNP517-518, tended to exhibit signification association with schizophrenia in those with CPT deficit but not in those without CPT deficit (Table 3). For patients with deficit on the undegraded CPT, a single copy of haplotype GA had an increased risk for schizophrenia (those with genotype GA/GA or GA/others had a OR > 1.0, regardless of narrow or broad model being used for analysis), whereas two copies of haplotype AG had a protective effect for schizophrenia (those with genotype AG/AG had a OR < 1.0, regardless of narrow or broad model being used for analysis). However, for patients without deficit on the undegraded CPT, the pattern was less consistent (only those with genotype GA/others had a decreased risk for schizophrenia). Intriguingly, no association between SNP517-518 haplotypes and schizophrenia were found when the sample was stratified by the degraded CPT. When similar stratified analyses were conducted for the haplotypes of the first block, i.e., SNP482, 485, 479, 488, and 489, none of the haplotypes exhibited a significant association with schizophrenia in either those with or without CPT deficit (data not shown).

DISCUSSION

Several genomewide searches have shown that the chromosome 1q42 region may harbor candidate genes for schizophrenia [18, 19, 41]. On the basis of a previous finding that the chromosome sequence tagged site D1S251 had the highest NPL score of 1.73-2.18 (p=0.01-0.03) for the linkage with schizophrenia in Taiwanese population [19], we carried out this SNP fine mapping study in the 2 cM area surrounding D1S251 to search for specific vulnerable genes of schizophrenia in this population. Two blocks were found to be significantly associated with schizophrenia and their implications are examined in turn.

The first block covers the genetic region of GNPAT (or DHAPAT) gene, which encodes the dihydroxyacetone-phosphate acyltransferase enzyme that is located within peroxisomes and catalyzes the biosynthesis of ether phospholipids. This enzyme was found to be in deficiency of varying degrees in patients with congenital peroxisomal disorders than normal controls [42, 43]. A girl with deficient DHAPAT was reported to have severe mental retardation, developmental delay, and growth failure [44]. Our result suggests that the GNPAT may also be involved in the broad definition of schizophrenia (p=0.046), though may not play a major role due to its weak association. Since this is the first report of the potential involvement of GNPAT gene in schizophrenia, further work examining the SNP markers located within the exons of the gene is warranted.

The second block that was significantly associated with schizophrenia is located within the DISC1 gene, and consistently exhibited significant associations in both single locus and haplotype-based association analysis. The DISC1 gene was found to be closer to the peak NPL score of D1S251 marker than to the adjacent lowest NPL score of the D1S404 marker in the previous Taiwan study [19]. Furthermore, the D1S251 marker also showed a highest LOD scores of 2.5 (p=0.002) in the populations of Britain and Iceland [41]. Thus, this study in an expanded sample of affected sib-pair families provides a replication of the linkage of DISC1 to schizophrenia.

In terms of haplotype analysis, our results suggest that the haplotypes consisting of SNPs in introns 4 and 5 of the DISC1 gene were significantly associated with schizophrenia. In contrast, the significant haplotype constituted by two SNPs for the

Finnish population is located between the intron 1 and exon 2 [45]. The discrepancy in the composition of the associated haplotype between the two studies could have two explanations. First, these reported regions may be in linkage disequilibrium and the associations of the haplotypes merely suggest that the DISC1 gene is nearby a true susceptibility gene for schizophrenia. Second, both regions may be truly involved in the genetic susceptibility to schizophrenia. However, both regions of the DISC1 gene may interact with different cytoskeletal proteins. The exons 1 and 2 of the DISC1 gene encode the putative globular domains that bind strongly with the cytoplasmic microtubules of α-tubulin, while the exons 3-13 encode the putative helical tail (coiled-coil motif) that is essential for interaction with neurodevelopmental protein NUDEL [46, 47]. If the second explanation holds, it implies different pathological pathways in different ethnic groups. Further investigation to see whether there are risk mutations in the exon 5 of DISC1 in our study population is warranted to distinguish these two possibilities.

There are two more differences in the results between the Finnish study [45] and ours. Neither the gender difference in the under-transmission of the DISC1 gene nor the association of the TRAX gene located in the upstream of the DISC1 gene with schizophrenia in the Finnish sample was found in our study. Nevertheless, the two studies did have a consistent finding that the DISC1 gene was not associated with the age onset of schizophrenia. Since we have ruled out the possible existence of the balanced translocation (q42.1; q14.3) in our schizophrenia DNA samples (Liu et al., unpublished results), the involvement of the DISC1 gene in our schizophrenic patients may be exerted through mechanisms other than genomic disruption.

One unique feature of this study is that we further revealed that the association of the DISC1 with schizophrenia was limited to those with CPT deficits. The CPT is a measure of sustained attention and has been demonstrated to be highly sensitive to brain damage or dysfunction [48]. Previous studies in Taiwanese population indicated that the heritability for the CPT performance in the nonpsychotic first-degree relatives of schizophrenic patients ranged from 0.48 to 0.62 [49], and the CPT deficits in schizophrenic patients were not amenable to 3-month neuroleptic treatment [26]. In this study, the heritability for the CPT in the families of the affected sibpairs was even higher (0.751 to 0.831). These consistent results indicate that the CPT deficits are likely to be a useful indicator for the genetic susceptibility to schizophrenia.

Under the AIC test for the optimum genetic model, the GNPAT genetic haplotype was significantly associated with the degraded CPT scores, whereas the DISC1 genetic haplotype with the undegraded CPT scores. When further stratifying the CPT performance into deficit or non-deficit according to the threshold of an adjusted z score of –2.5, the GA haplotype of DISC1 gene was found to be the dominant susceptible indicator for the undegraded CPT deficit, yet the AG haplotype was the dominant protective indicators for the CPT deficit. In contrast, the GNPAT did not show such an association with schizophrenia in the similar stratification analysis. The differential association of both versions of the CPT with the two blocks might be due to chance finding, since the significance level of some association was borderline only (e.g., the GNPAT block with the degraded CPT). Another possibility is that the two versions of the CPT tap different aspects of neuropsychological functioning: the undegraded session involved mainly a working memory component, whereas the degraded session included a sensory-perceptual component as well [26]. Our results suggest that the DISC1 gene, especially the intron 4-intron 5 region, may be involved in regulating the sustained attention per se, i.e., working memory, of schizophrenia. The opposite direction in predicting the risk to sustained attention deficit for the GA

and AG haplotypes might be accounted for by binding affinities with different regulators.

Unlike the results of the CPT, the WCST scores were associated wtih neither the DISC1 nor the GNPAT genes. It appears that the 1q42.1 region may have little contribution to the pathological process of executive functioning in schizophrenia.

In summary, a 2 cM region surrounding the D1S251 marker was screened with SNPs for genetic association with schizophrenia. Two blocks of SNP haplotypes in this region showed significant associations with schizophrenia. These two blocks were located within the regions of GNPAT (a five-SNP block) and DISC1 (a two-SNP block) genes, respectively. The potential involvement of the GNPAT with schizophrenia is preliminary, while the finding of the DISC1 is a replication of previous studies. We further revealed that the association of the DISC1 with schizophrenia was limited to those with CPT deficits and may indicate a potential biological pathway for future investigation.

MATERIALS AND METHODS

Subjects

Schizophrenic probands who had at least two affected siblings were identified from the Department of Psychiatry, National Taiwan University Hospital and the University-affiliated Taoyuan Psychiatric Center. Data collection was initiated after informed consents were obtained from the identified study subjects and their families. All of the family members were personally interviewed by the research psychiatrists using the Psychiatrist Diagnostic Assessment (PDA) [50]. The final diagnostic assessment was formulated by integrating the PDA data and clinical information of medical chart records. The final diagnosis was done following the DSM-IV criteria for schizophrenia, schizoaffective disorder, and other non-affective psychoses. Clinical data of age at onset of initial symptoms, negative symptoms, and positive symptoms were collected. The negative and positive symptoms were assessed using schedule for assessment of negative symptoms (SANS) [51] and schedule for assessment of positive symptoms (SAPS) [52] with satisfactory reliability. Negative symptom score was the sum of all global scores of five negative symptom dimensions, including affective flattening, alogia, avolition/apathy, anhedonia/asociality, and impaired attention. Positive symptom score was the sum of all global scores of 4 positive symptom dimensions, including hallucination, delusion, excitements, and thought derailment.

In total, 102 schizophrenic nuclear families with at least two affected siblings were recruited in this study. Among the 399 individuals, 231 individuals underwent an undegraded CPT test and 225 individuals underwent a degraded CPT test. Meanwhile, 164 subjects underwent WCST assessment.

Neuropsychological assessment

CPT. A CPT machine from Sunrise System, v. 2.20 (Pembroke, MA, USA), was used to assess sustained attention. The procedure has been described in detail elsewhere [49]. Briefly, numbers from 0 to 9 were randomly presented for 50 msec each, at a rate of one per second. Each subject undertook two CPT sessions: the undegraded 1-9 task and the 25% degraded 1-9 task. Subjects were asked to respond whenever the number “9” preceded by the number “1” appeared on the screen. A total of 331 trials, 34 (10%) of which were target stimuli, were presented over 5 minutes for each session. During the 25% degraded session, a pattern of snow was used to toggle background and foreground so that the image was visually distorted. Each test

session began with 2 minutes of practice (repeated if subjects required). One signal-detection index of performance on the test, sensitivity (d′), was derived from the hit rate (probability of response to target trials) and false-alarm rate (probability of response to nontarget trials)[53]. Sensitivity is an individual's ability to discriminate target stimuli from nontarget stimuli. In a 1-week test-retest reliability study [49] of the CPT versions used in this study, the intraclass correlation coefficients or reliability of d′ were 0.83 and 0.82 for the undegraded and the 25% degraded 1-9 task, respectively.

WCST. We employed a computerized version of the WCST [54] that had been applied in a previous study in Taiwanese population [55]. During the WCST, subjects were required to match response cards to the four stimulus cards along one of three dimensions (color, form, or number) by pressing one of the 1 to 4 number keys on the computer keyboard. Subjects were not informed of the correct sorting principle, nor were they told when the principle would shift during the test, but they were given feedback ("Right" or "Wrong") on the screen after each trial. Unlike one common form of the traditional WCST in which the test ends after six correct categories achieved, the testing in this study continued until all 128 cards were sorted. All of the indexes defined in the WCST manual [56] except for Total Correct were used for analysis. The Total Correct index was not included since it is complementary to Total Errors. The indexes used were (1) Total Errors: total number of perseverative and nonperseverative errors; (2) Nonperseverative Errors: number of errors that were not perseverative; (3) Perseverative Errors: number of errors that were perseverative, reflecting tendency towards perseveration; (4) Perseverative Responses: number of responses that were perseverative, regardless of whether they were correct or not; (5) Categories Achieved: number of times 10 correct responses in a row were made, reflecting overall success; (6) Trials to Complete First Category: number of trials to successfully complete the first category (counted as 129 if no category was completed), reflecting initial conceptual ability; (7) Conceptual Level Response: proportion of consecutive correct responses occurring in runs of 3 or more, reflecting insight into the correct sorting principles; (8) Failure to Maintain Set: number of times subject makes between 5 and 9 correct responses in a row, reflecting efficiency of sorting; and (9) Learning to Learn: average difference in percent errors between successive categories, reflecting the average change in conceptual efficiency during the test [56]. The last index can be calculated only for whom the total numbers of Categories Achieved and categories attempted are larger than 3.

SNP selection criteria and validation

Fine mapping studies were using SNP dense markers spreading upstream and down stream of the dinucleotide marker D1S251 located at 1q42.1. In the defined region of 2cM around DIS251, a total of 120 SNPs were selected. The inter SNP marker distance ranged from 5 kb to 60 kb, with an average of 32.5 kb. According to the location of these SNPs relative to the position of the functional genes, the SNPs were selected based upon the following priority of exon, 5'-untranslated region, 3-untranslated region, introns, promoter (CpG island), and gaps between functional genes, which expressed in the central nervous system. We used 31 trios and 2 independent individuals of a totally 95 individuals to validate the 120 SNPs from public database (http://www.ensembl.org/Homo_sapiens/martview).

SNP genotyping

All SNP genotypings were performed by the method of matrix-assisted laser

desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS). Primers and probes flanking the SNPs were designed by using SpectroDESIGNER software (Sequenom, San Diego, CA, USA). A DNA fragment (100-300 bp) encompassing the SNP site was amplified using the polymerase chain reaction (PCR) (GeneAmp 9700 thermocycler (Applied Biosystems, USA) according to the manufacturer's instruction.

After PCR amplification, the un-incorporated deoxynunleotide triphosphate (dNTP) of the PCR reaction mixture was neutralized by treating with shrimp alkaline phosphatase (SAP) at 37 °C for 20 minutes. The reaction mixture was incubated at 85 °C for 5 minutes to inactivate the SAP activity. Primer extension was performed by adding the probe, Thermo Sequenase (Amersham Pharmacia, Piscataway, NJ, USA) and appropriate dideoxynucleotide triphosphate (ddNTP)/dNTP mixture, and following 55 cycles of denaturing at 94 °C for 5 sec, annealing at 52 °C for 5 sec, and extension at 72 °C for 5 sec. Different extension products were differentiated by mass through MALDI-TOF as follows.

Cation exchange resin (SpectroCLEAN, Sequenom) was added to the above reaction mixture to get rid of salt, which would interfere with the mass reading in the MALDI-TOF analysis. After desalting, the reaction mixture was spotted onto the SpectroCHIP (using the SpoectroPOINT) where they were co-crystallized with the matrix. After introduced to the SpectroREADER, a nitrogen laser with nanosecond-wide pulses interrogated the samples on the SpectroCHIP in the high-vacuum environment of the time-of-flight mass spectrometer. Upon laser irradiation, the matrix crystals absorbed the laser's energy and the extension products were converted into gas phase ions, which upon acceleration undergo mass-dependent separation over approximately 1-meter flight path. Molecular masses were determined with 0.01 to 0.02% estimated accuracy. Acquired spectra were transferred to the MassARRAY Server (Sequenom, San Diego, CA) where they were automatically interpreted and corrected for their genotypes.

Statistics analysis

In order to verify the sample accuracy included family relationship and genotype, PEDCHECK version 1.1 [57] and UNKNOWN version 5.23 [58] were employed to check Mendelian inheritance and Procedure ALLELE in SAS/GENETICS release 8.2 [59] was used to test for Hardy-Weinberg equilibrium. Linkage disequilibrium of inter-markers was measured using coefficient D´ [60] which was also used to define haplotype blocks. A graphic presentation of block pattern was completed using GOLD software [61].

Family-based transmission disequilibrium tests were applied to test linkage disequilibrium. Both of single-locus and haplotype-based association analyses were carried out simultaneously using two popular programs for the nuclear family data, haplotype FBAT version 1.4.1 [34, 35, 37] and TRANSMIT version 2.5.4 [36]. An individual's haplotype was inferred using SimWalk2 version 2.86 [62-64], which uses Markov Chain Monte Carlo algorithm. Moreover, GEE method [38] was applied to test the interaction between haplotype blocks using the Proc GENMODE of the SAS (version 8.0 for Windows).

Besides the analysis of qualitative trait, quantitative analysis using highly heritable quantitative trait was also considered. The analysis of heritability and quantitative-type transmission disequilibrium test based on variance component approach was applied using QTDT version 2.4.3 [39, 40].

Acknowledgement

This study was supported by the grants from NRPGM, NSC, Taiwan (NSC-91-3112-B-002-011; NSC-92-3112-B-002-019; NSC-93-3112-B-002-012), from NHRI, Taiwan (NHRI-90-8825PP; NHRI-EX91, 92, 93-9113PP) and from NIMH, USA (IRO1 MH59624-01). We acknowledge the SNP genotyping work done by the National Genotyping Center (NGC), NSC, Taiwan.

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FIGURE LEGENDS

Fig. 1. Linkage disequilibrium of all SNP markers showed five significant SNP blocks located within blocks of SNP study number 7231-7228-7243-7233, 7256-7265-7266, 7260-7264-7269-7277, 7279-7275, and 7286-7287-7276-7280.

Fig. 2. Blocks of SNPs located within genes of APOL5, MYH-9, EIF3S and CACNG2 showed significant linkage disequilibrium and associations among 16 other genes in the 2 cM ranges around D22S278 marker.

Appendix 3

Haplotypes and Interaction Analyses Support G72 as a Regulator and D-Amino Acid Oxidase as a Susceptibility Gene for Schizophrenia

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Running title: DAAO is a schizophrenia susceptibility gene

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Abstract

The D-amino acid oxidase (DAAO) activator (G72; 13q34) and DAAO (12q24) have been suggested as candidate genes and involved in the N-methyl-D-aspartate receptor regulation pathway for schizophrenia. To evaluate if these two genes were associated with schizophrenia in a Taiwanese sample, three single nucleotide polymorphisms (SNPs) for DAAO (rs2111902, rs3918346, rs3741775) and eleven SNPs for G72 (rs3916965, rs3916966, rs3916967, rs2391191, rs3916968, rs947267, rs778294, rs3916970, rs3916971, rs778293, rs3918342) were genotyped by the MALDI-TOF MS method in 218 schizophrenic affected sib-pair families (864 individuals). In haplotype-based association analyses, two protective G-G-A and T-A-C, and one risk T-G-C haplotypes for DAAO (rs2111902– rs3918346– rs3741775) showed significant associations with schizophrenia in both a narrow model (DSM-IV schizophrenia only; p=0.017 and p=0.0024 for protective, p=0.0144 for risk, respectively) and in a broad model (including schizophrenia, schizoaffective disorder, and other non-affective psychotic disorders; p=0.0171 and p=0.0024 for protective, p=0.0165 for risk, respectively). The G72 gene only showed significant interactions with DAAO (p<0.02). These results suggest that DAAO may be a susceptibility gene for schizophrenia and that G72 is likely to be a regulator for DAAO.

Keywords: DAAO, G72, schizophrenia, Taiwanese family, SNP, haplotype

1. Introduction

D-amino acid oxidase (DAAO) is a flavoenzyme of peroxisomes presented in the brain, kidney and liver of mammals (Fukui and Miyake, 1992). The physiological role of DAAO is not clear, and the DAAO gene is composed of 11 exons distributed in 25 kbs at chromosome 12q24.11. The interest in D-amino acid oxidase (DAAO) and its activator G72 as susceptibility genes for schizophrenia was initiated by several linkage reports on the findings of G72 at the chromosome 13q22-q34 region (Blouin et al., 1998; Brzustowicz et al., 1999; Riley et al., 1998; Shaw et al., 2003). In spite of several negative linkage study results (DeLisi et al., 2002; Fallin et al., 2003), the G72 locus was shown to have significant association with schizophrenia using single nucleotide polymorphism (SNP) fine mapping studies following the linkage study in two ethnic groups; the French Canadian population and the Russian population (Chumakov et al., 2002). The search for schizophrenia susceptibility genes related to G72 was continued by a yeast two-hybrid experiment, which identified that the enzyme DAAO may interact with G72 on the protein level. The role of DAAO as a susceptibility gene was further confirmed by showing it to be associated with schizophrenia (Chumakov et al., 2002).

These two susceptibility genes were later also confirmed in the Chinese (Liu et al., 2004; Wang et al., 2004), German (Schumacher et al., 2004), and Ashkenazi populations (Korostishevsky et al., 2004) using SNP fine mapping of case-control studies. We intended to evaluate the association findings of these two genes among the Taiwan Chinese population using the data gathered from 218 affected sib-pair schizophrenia families.

2. Method

2.1. Subjects

This research project was approved by the Institutional Review Board of the National Taiwan University Hospital. After obtaining the signed up inform consents, the study subjects were recruited for diagnostic assessment and collection of the genomic DNA samples. The subjects were recruited from two sample-collecting programs. These were the multidimensional psychopathology study of schizophrenia (MPSS) (Hwu et al., 2002) from 1993 to 2001 and the Taiwan schizophrenia linkage study (TSLS) (Hwu et al., 2005) from 1998 to 2002. The 86 families of the MPSS subjects were interviewed by the research psychiatrists using the Psychiatrist Diagnostic Assessment (PDA) (Hwu, 1999). The 132 families of the TSLS subjects were interviewed by well-trained assistants using the Mandarin Chinese version of the Diagnostic Interview for Genetic Studies (DIGS) (Chen, 1999). The final diagnostic assessment of both MPSS and TSLS were formulated by integrating either the PDA or the DIGS

data and the clinical information of medical chart records using the Specialist Diagnostic Assessment Sheet (SDAS), based upon the criteria of the Diagnostic and Statistical Manual of Mental Disorder, 4th edition (DSM-IV). The study samples included two hundred and eighteen schizophrenic nuclear families with at least two affected siblings, which had a total of 864 subjects been genotyped (Table 1). Within these 218 families, there were 216 families having at least two sibs genotyped, 103 families having one parent, and 96 families having both parents genotyped in this study.

2.2. SNP genotyping

All SNP markers were genotyped by the method of matrix-assisted laser desorption/ionization-times of flight mass spectrometry (MALDI-TOF MS). A DNA fragment (100-300 bp) encompassing the SNP site was amplified using the polymerase chain reaction (PCR) GeneAmp 9700 thermocycler (Applied Biosystems, USA) according to the manufacturer’s instructions. After the PCR amplification and neutralization of the deoxynucleotide triphosphate (dNTP) were performed, the primer extension was done by adding the probe, Thermo Sequenase (Amersham Pharmacia, Piscataway, NJ, USA) and the appropriate dideoxynucleotide triphosphate (ddNTP)/dNTP mixture. Different extension products were differentiated by mass through the MALDI-TOF.

2.3. Statistical analyses

The quality of genotyping data was checked on the indicators of Hardy-Weinberg equilibrium, the lineage of the study families, and the deviation from Mendelian inheritance by using the statistical programs of the ALLELE procedure in the SAS/GENETICS (SAS Institute, 2002), the PEDCHECK (O'Connell and Weeks, 1998), and the UNKNOWN (Terwilliger and Ott, 1994), respectively. The haplotype blocks was defined using the linkage disequilibrium of inter-markers measured by coefficient D’ (Lewontine, 1964), and the haplotype blocks were demonstrated using a graphic presentation by GOLD software (Abecasis and Cookson, 2000). The phenotype of schizophrenia was divided into narrow models (DSM-IV defined schizophrenia only) and broad models (including schizophrenia, schizoaffective, and other non-affective psychotic disorders defined by DSM-IV) for the association study. Both single point and haplotype association analyses were carried out simultaneously using the TRANSMIT (Clayton, 1999) and the FBAT (Horvath et al., 2004) programs. Individual haplotypes were inferred by using the SIMWALK2 program (Sobel and Lange, 1996). Based on the reality of physiological interaction of these two genes of DAAO and G72 (Chumakov et al., 2002), we tested the interaction effect of these two

genes using logistical regression based on Generalized Estimating Equation (GEE) model (Liang, 1986), where family members were treated as repeated measures.

3. Results

Three SNPs of DAAO and eleven SNPs of G72 were first validated on a small scale of 92 biologically independent individuals to ensure the existence them in the Taiwan ethnic group. A SNP was considered valid if the minor allele frequency was larger than 10% and the genotype missing rate was smaller than 30%. All the SNP markers were compatible with the Hardy-Weinberg equilibrium distribution, except rs3918346 (MDAAO-5) of DAAO, and rs947267 and rs3918342 of G72 (Table 2). The haplotype block structure was evaluated by the inter-marker linkage disequilibrium coefficient. A SNP block with three SNP markers (MDAAO-4 -MDAAO-5 -MDAAO-6) for DAAO and two SNP blocks; one with four SNP markers (rs3916965-rs3916966-rs3916967-rs2391191) and the other with two SNP markers (rs947267-rs778294), for G72 were further identified with a coefficient D' higher than 0.7.

In single SNP locus association analyses using the TRANSMIT (Clayton, 1999) and FBAT (Horvath et al., 2004) programs, there were no significant associations found in G72 and DAAO in both the narrow and broad phenotype models of schizophrenia, respectively (Table 2). Haplotype analyses of G72 found no significant associations in any compositions of either two-SNP block or four-SNP block. For DAAO, there were significant associations for the three-SNP (MDAAO-4 -MDAAO-5 -MDAAO-6) haplotypes. The G-G-A and T-A-C haplotypes showed a protective effect for both the narrow and broad models of schizophrenia, and the T-G-C haplotype showed a risk effect for both the narrow and broad models (Table 3).

To detect the interaction effects between DAAO and G72, we used the two most frequent haplotypes (T-C-C-A 63% and T-C 62%) of G72 and three haplotypes (G-G-A, T-A-C, and T-G-C) of DAAO that were significant in the transmission tests. In total, six logistic regression models were performed. Each model contained one haplotype of G72 and DAAO respectively. The coefficients for main effects and interaction terms were all significant when the model included G-G-A of DAAO and T-C-C-A of G72. The coefficients for the haplotype of DAAO and the interaction term were significant when the model included T-A-C or T-G-C of DAAO and T-C of G72 (Table 4). The conditional odds ratios of the DAAO haplotypes given the G72 haplotype and their 95% confidence intervals are shown in Table 4-1. For example, the conditional odds ratio for haplotype G-G-A of DAAO is not significant given that the G72 haplotype is T-C-C-A, however given that the haplotype of G72 is not T-C-C-A, the odds ratio for DAAO of G-G-A is 0.63 suggesting a protective effect.

4. Discussion

In this study, we have evaluated the association of two candidate genes, G72 (located on 13q33.2) and DAAO (located on 12q24.11), using a single locus and haplotype-based association analyses. The selected SNPs on G72 are not fully consistent with the SNPs reported by Chumakov et al (Chumakov et al., 2002), but were exactly the same SNPs reported by Korostishevsky et al (Korostishevsky et al., 2004). Both these reports found significant associations between G72 and schizophrenia. However, our results for G72 did not replicate the strong significant associations reported by these two groups. None of the single locus or the haplotype association analyses were significant at this region. This suggests that the association of G72 with schizophrenia is, at best, weak in our Taiwanese sample.

The SNPs for DAAO were selected from three of the SNPs significantly associated with schizophrenia reported by Chumakov et al (Chumakov et al., 2002). These three SNPs (MDAAO-4 in intron 1, MDAAO-5 in intron 3 and MDAAO-6 in intron 4) did not show a significant association in our single locus association analyses, which is different from the results reported from a German sample (Schumacher et al., 2004). There were significant associations with all three SNPs, and the results in the Chinese population, which found a significant association with MDAAO-6 (Liu et al., 2004). As our study was the only one using the family-based association design, and all the others used the case-control design, it is possible that the case-control study is more sensitive with regard to single SNP allele-type association analysis (Zhang et al., 2002). It is also possible that the stratification artifacts have given false positive results in the case-control studies or that these results are due to the differential effects of genotyping errors found in family-based and case-control studies (Mitchell et al., 2003).

Our population has agreeable risk haplotypes as compared to the population in the German study of DAAO. Schumacher et al reported that A-C-G was the risk haplotype for schizophrenia (Schumacher et al., 2004). This is in an opposite direction to the risk haplotype T-G-C shown in this study. Our T-G-C risk haplotype had a slightly lower frequency (0.29) and slightly higher significance level ($p=0.014$ to 0.017) than the frequencies (0.34 for control and 0.4 for schizophrenia) and the significance level ($p=0.045$, odds ratio = 1.29) reported by Schumacher et al (Schumacher et al., 2004). Our protective haplotypes, G-G-A and T-A-C, were different from their protective haplotype C-T-T (opposite to G-A-A). Moreover, our haplotype of G-G-A had a very low frequency of 0.001 and the T-A-C was significantly under transmitted in patients ($p=0.0004$ for the narrow model and $p=0.0006$ for broad model; data not shown). These might be the reasons which account for the differences between these two studies and which merit further

investigation.

The physiological interactions between DAAO and G72 have been proved by Chumakov et al (Chumakov et al., 2002). In this study, the DAAO-G72 interaction was first time tested by a gene-gene interaction statistical model and showed significance in both the narrow and the broad models of schizophrenia. In this logistic analysis, the haplotypes of DAAO and G72 were further evaluated and revealed that G72 is not a major susceptibility gene, because the single locus and the haplotype association analyses failed to show a significant association. G72 may be just a regulator for DAAO because the haplotype of G72 was shown to have a p-value less than 0.0001 as it was analyzed with DAAO in their interaction term by the logistic regression. The interaction regions in each gene could be expected from the haplotype covered exon regions. The DAAO gene had significant haplotypes in rs2111902-rs3918346- and rs3741775 covering the genetic regions from exon 2 to exon 4. The G72 gene had two significant haplotypes of rs3916965- rs3916966- rs3916967-rs2391191 which covered the range from exon 1 to exon 3 and the rs947267 -rs778294 possibly in exon 5. This interaction effect was strongly associated with the broad model of schizophrenia.

In summary, we have genotyped three SNPs of DAAO and eleven SNPs of G72 in a relatively large sample size of 218 families comprised of 864 subjects. The G72 gene alone had no significant association with schizophrenia. The DAAO gene was significantly associated with schizophrenia for the rs2111902-rs3918346-rs3741775 haplotype. T-G-C was a risk haplotype, and G-G-A and T-A-C were protective haplotypes for schizophrenia. The G72 haplotypes (rs3916965- rs3916966-rs3916967- rs2391191) and (rs947267-rs778294) may interact with the DAAO (rs2111902-rs3918346-rs3741775) haplotype in both the broad and the narrow phenotype models of schizophrenia.

Acknowledgements

We acknowledge the SNP genotyping works done by the National Genotyping Center (NGC) and the help by the Department of Medical Research in NTUH. This study was supported by the grants from the NRPGM, National Science Council, Taiwan (NSC91-2314-B-002-216; NSC91-3112-B-002-011; NSC 92-3112-B-002-019; NSC93-3112-B-002-012), National Health Research Institute, Taiwan (NHRI-EX-91,92,93-9113PP), and National Institute of Health, U.S.A. (IR01 MH 59624-01).

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Table 1. Distribution of families by number of siblings and parents genotyped

	Parents Genotyped per Family			
Sibs Genotyped per Family	0	1	2	Total Families
1	0	0	2	2
2	9	15	74	98
3	8	78	17	103
4	2	9	2	13
5	0	1	1	2
Total Families	19	103	96	218

Table 2
Minor allele frequencies of the single nucleotide polymorphisms of the DAAO and G72 genes and association with narrow and broad phenotype models of schizophrenia

Genes	SNP_ID	Chromosome	Allele Type	MF	HW Test	Narrow Model			Broad Model		
					P	N	Chi	P	N	Chi	P
DAAO	**MDAAO-4** (rs2111902)	chr12:109211693	G/T	0.48	0.9248	216	0.43	0.5144	218	0.17	0.6831
	MDAAO-5 (rs3918346)	chr12:109214830	A/G	0.39	**0.0000**	213	0.64	0.4245	215	0.25	0.6170
	MDAAO-6 (rs3741775)	chr12:109216549	A/C	0.40	0.9814	215	0.71	0.4000	217	0.42	0.5163
G30/G72	**G30** (rs3916965)	chr13:104939998	T/C	0.36	0.1826	216	0.56	0.4548	217	0.41	0.5200
	G72 (rs3916966)	chr13:104947533	C/A	0.38	0.1388	216	0.41	0.5205	218	0.34	0.5583
	G72 (rs3916967)	chr13:104953986	C/T	0.37	0.1362	214	0.84	0.3596	216	0.82	0.3661
	G72 (M15; rs2391191)	chr13:104956084	A/G	0.37	0.1362	214	0.92	0.3381	216	0.80	0.3702
	G72 (rs3916968)	chr13:104957057	C/T	0.18	0.9268	215	1.10	0.2936	217	1.50	0.2213
	G72 (rs947267)	chr13:104998639	T/G	0.38	**0.0054**	213	0.15	0.7021	215	0.16	0.6918
	G72 (rs778294)	chr13:104964167	C/T	0.15	0.1182	216	0.00	0.9532	218	0.00	0.9853
	G30 (rs3916970)	chr13:104976300	T/C	0.43	0.1366	216	0.74	0.3903	218	0.23	0.6335
	G30 (rs3916971)	chr13:104978873	T/C	0.47	0.0608	215	1.59	0.2079	217	1.47	0.2260
	G30 (M19; rs778293)	chr13:105005837	T/C	0.33	0.8671	216	3.41	0.0650	218	3.53	0.0603
	G30 (rs3918342)	chr13:105022387	T/C	0.46	**0.0399**	216	2.44	0.1181	218	2.64	0.1041

MF: minor allele frequency
HW: Hardy-Weinberg's test
P: p-value
Narrow model: only subjects of schizophrenia fulfilling criteria of Diagnostic and Statistical Manual IV (DSM-IV)
Broad model: composed of DSM-IV schizophrenia, schizoaffective disorder and non-affective psychotic disorders
N: number of families
Chi: chi-square test

Table 3

Haplotype frequencies (HF) of DAAO gene and association with narrow and broad phenotype models of schizophrenia

Haplotype (**MDAAO-4-MDAAO-5-MDAAO-6**) (**Intron 1-Intron 3-Intron 4**)	**HF**	**Narrow Model** (N=197)		**Broad Model** (N=198)	
		Chi	P	Chi	P
G-A-A	0.5329	0.49	0.4834	0.66	0.4177
T-A-A	0.0205	3.20	0.0736	3.25	0.0713
G-G-A[a]	0.0097	5.69	***0.0170-**	5.69	***0.0171-**
T-G-A	0.0576	0.03	0.8637	0.02	0.8863
G-A-C	0.0027	0.83	0.3612	0.83	0.3616
T-A-C	0.0833	9.19	**#0.0024-**	9.23	**#0.0024-**
G-G-C	0.0068	0.01	0.9061	0.01	0.9069
T-G-C	0.2865	5.99	***0.0144**+	5.75	***0.0165**+

+: risky

-: protective

[a] : observation number less than 5.

N: Number of families

* : P-value <0.05 from FBAT v1.4.1 output.

\# : P-value <0.01 from FBAT v1.4.1 output.

Table 4

Interaction effect of the haplotypes of DAAO and G72 genes in two phenotype models of schizophrenia

Effect	DAAO Haplotype	G72 Haplotype		Narrow Model			Broad Model		
				Estimate	C.I.	P-value	Estimate	C.I.	P-value
DAAO	G-G-A		$\hat{\beta}_1$	-0.4597	(-0.8755, -0.0439)	**0.0302**	-0.5852	(-0.9948, -0.1757)	**0.0051**
G72		T-C-C-A	$\hat{\beta}_2$	-0.3713	(-0.5828, -0.1599)	**0.0006**	-0.4761	(-0.6816, -0.2705)	**<0.0001**
DAAO * G72	G-G-A	T-C-C-A	$\hat{\beta}_3$	0.8507	(0.1663, 1.5351)	**0.0148**	1.1137	(0.3717, 1.8557)	**0.0033**
DAAO	T-A-C		$\hat{\beta}_1$	-0.3875	(-0.806, 0.0311)	0.0696	-0.5311	(-0.9358, -0.1265)	**0.0101**
G72		T-C-C-A	$\hat{\beta}_2$	-0.3692	(-0.5778, -0.1606)	**0.0005**	-0.4905	(-0.6827, -0.2983)	**<0.0001**
DAAO * G72	T-A-C	T-C-C-A	$\hat{\beta}_3$	0.8624	(0.3606, 1.3644)	**0.0008**	1.0912	(0.5851, 1.5973)	**<0.0001**
DAAO	T-G-C		$\hat{\beta}_1$	0.6741	(0.2123, 1.1359)	**0.0042**	0.9706	(0.4498,1.4915)	**0.0003**
G72		T-C	$\hat{\beta}_2$	0.2470	(-0.1374, 0.6315)	0.2079	0.3134	(-0.127,0.7538)	0.1631
DAAO * G72	T-G-C	T-C	$\hat{\beta}_3$	-0.7221	(-1.294, -0.2502)	**0.0037**	-1.1013	(-1.6832,-0.5193)	**0.0002**

*: Genetic interaction

C.I.: 95% confidence interval

$\hat{\beta}_1$, $\hat{\beta}_2$, $\hat{\beta}_3$: the coefficients of the DAAO, G72 and their interaction term

Table 4-1

Conditional odds ratio of the haplotypes of DAAO and G72 genes in two phenotype models of schizophrenia

	Narrow Model			Broad Model		
	OR	**C.I.**	**P-value**	**OR**	**C.I.**	**P-value**
$OR(DAAO=GGA\,|\,G72=TCCA)=e^{\hat{\beta}_1+\hat{\beta}_3}$	1.48	(0.871, 2.5099)	0.1475	1.70	(0.9524, 3.0213)	0.0727
$OR(DAAO=GGA\,|\,G72=other)=e^{\hat{\beta}_1}$	0.63	(0.4167,0.9570)	**0.0302**	0.56	(0.3698, 0.8389)	**0.0051**
$OR(DAAO=TAC\,|\,G72=TCCA)=e^{\hat{\beta}_1+\hat{\beta}_3}$	1.61	(1.2524, 2.0643)	**0.0002**	1.35	(1.3519, 2.2674)	**<.0001**
$OR(DAAO=TAC\,|\,G72=other)=e^{\hat{\beta}_1}$	0.68	(0.4466, 1.0316)	0.0696	0.59	(0.3923, 0.8812)	**0.0101**
$OR(DAAO=TGC\,|\,G72=TC)=e^{\hat{\beta}_1+\hat{\beta}_3}$	0.73	(0.7338, 1.1203)	0.364	0.88	(0.7006, 1.0992)	0.2555
$OR(DAAO=TGC\,|\,G72=other)=e^{\hat{\beta}_1}$	1.96	(1.2365, 3.1140)	**0.0042**	2.64	(1.5680, 4.4438)	**0.0003**

| : Genetic interaction

C.I.: 95% confidence interval

Appendix 4

Failure to Support RGS4 as a Candidate Gene for Schizophrenia

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Abstract

Several studies have been suggested that the regulator of G-protein signaling 4 (RGS4) may be a positional and functional candidate gene for schizophrenia. Three single nucleotide polymorphisms (SNP) located at the promoter region (SNP4, and SNP7) and the intron 1 (SNP18) of RGS4 have been verified constantly in different ethnic groups. Positive results have been reported in these SNPs with different numbers of SNP combinatory haplotypes. In this study, these four SNP markers were genotyped in 218 schizophrenia pedigrees of Taiwan (864 individuals) for linkage association analysis. Among these three SNPs, neither SNP4, SNP7, or SNP18 has shown significant association with schizophrenia in single locus association analysis, nor any compositions of the three SNP haplotypes has significantly associated with the phenotypic narrow model (DSM-IV schizophrenia only) of schizophrenia. Our results fail to support the RGS4 as a positional candidate gene for schizophrenia when evaluated from these three SNP markers.

Keywords: schizophrenia, RGS4, haplotype, linkage analysis, Taiwan families

Introduction

Regulator of G-protein signaling (RGS) is a family of proteins modulating G-protein signaling pathways (Hollinger and Hepler, 2002; Ishii and Kurachi, 2003). RGS proteins have one major role as GTPase-activating proteins (GAP) which accelerate Gα-catalyzed GTP hydrolysis and shorten the G protein mediated intracellular signaling (Dohlman and Thorner, 1997). There are at least 20 RGS family members identified; the subtypes expressed in human brain include of RGS4, RGS7, RGS8, RGS11 and RGS17 (Erdely *et al.*, 2004; Larminie *et al.*, 2004). The roles of RGS proteins in the central nervous system have not been extensively characterized, the RGS4 gene has been suggested as a candidate gene for schizophrenia (Chowdari *et al.*, 2002).

RGS4 was supported as a candidate gene in schizophrenia from several studies. In global gene expression microarray analysis, RGS4 expression was decreased across the cerebral cortex of patients with schizophrenia (Mirnics *et al.*, 2001a; Mirnics *et al.*, 2001b). In the functional study, RGS4 regulates the G-protein signaling pathways in several schizophrenia-associated receptors; such as dopaminergic receptors and glutamate receptors (Saugstad *et al.*, 1998; Taymans *et al.*, 2004; Taymans *et al.*, 2003). In the genomic positional mapping study, RGS4 was suggested to map in the genomic region of 1q21–q22 where has previously been implicated by linkage studies in schizophrenia (Brzustowicz *et al.*, 2002; Hwu *et al.*, 2003; Owen *et al.*, 2004). Furthermore, two proband–parent trio samples from the US, and from India, and a third small sample recruited by the NIMH Collaborative Genetics Initiative had obtained significant associations independently in each of the US samples for haplotypes encompassing four SNPs in the 5' flanking sequence (SNP1, SNP4, and SNP7) and first intron (SNP18) of RGS4 (Chowdari *et al.*, 2002). Besides different risk haplotypes showed across different ethnic groups, there is no significant association obtained from the larger Indian sample (Chowdari *et al.*, 2002). In this study, the four SNP markers of RGS4 gene were genotyped in the Taiwan schizophrenia family members and verified if the RGS4 is a potential candidate gene for schizophrenia.

Subjects and methods

This research project was approved by the Institutional Review Board of National Taiwan University Hospital. All genomic DNA samples were collected from co-affected sib-pair family subjects with written inform consents. The subjects were recruited from two sample-collecting programs; the multidimensional psychopathology study of schizophrenia (MPSS) (Hwu *et al.*, 2002) from 1993 to

2001 and the Taiwan schizophrenia linkage study (TSLS) (Hwu *et al.*, 2005) from 1998 to 2002. The 86 families of MPSS subjects were interviewed by the research psychiatrists using the Psychiatrist Diagnostic Assessment (PDA) (Hwu, 1999). The 132 families of TSLS subjects were interviewed by well-trained assistants using the Mandarin Chinese version of the Diagnostic Interview for Genetic Studies (DIGS) (Chen, 1999). The final diagnostic assessment of both MPSS and TSLS was formulated by integrating either the PDA or the DIGS data and clinical information of medical chart records using the Specialist Diagnostic Assessment Sheet (SDAS), based upon the criteria of Diagnostic and Statistical Manual of Mental Disorder, 4th edition (DSM-IV). This study sample included two hundred and eighteen schizophrenic nuclear families with at least two affected siblings, and had a total of 864 subjects participated in this genotyping study. Genomic DNA samples of 864 subjects were genotyped by the matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS).

In order to verify the sample accuracy included family relationship and genotype, PEDCHECK version 1.1 (O'Connell and Weeks, 1998) and UNKNOWN version 5.23 (Terwilliger and Ott, 1994) were employed to check Mendelian inheritance and Procedure ALLELE in SAS/GENETICS release 8.2 (Chowdari *et al.*, 2002) was used to test for Hardy-Weinberg equilibrium. Linkage disequilibrium of inter-markers was measured using coefficient D’ (Hedrick, 1987) which was also used to define haplotype blocks. A graphic presentation of block pattern was completed using GOLD software (Abecasis *et al.*, 2000). Both of single point and haplotype association analyses were carried out simultaneously using the program of TRANSMIT version 2.5.4 (Clayton, 1999).

Results

Four RGS4 SNP markers (SNP1, SNP4, SNP7, and SNP18) were first validated in a small independent 92 individuals to ensure the existence of these SNPs in the Taiwan ethnic group before typing the rest of the genomic samples. A SNP was considered valid if the frequency of minor allele was larger than 10% and genotyping missing rate was smaller than 30%. All three SNP markers were compatible with the Hardy-Weinberg equilibrium distribution, except SNP1 (Table I). Each SNP of RGS4 was analyzed with the single locus association analyses using the TRANSMIT program version 2.5.4 (Clayton, 1999) which can utilize data from all families even when parental genotypes are unknown. No significant associations were found in the narrow phenotype model of schizophrenia.

The haplotype block was evaluated by inter-marker linkage disequilibrium coefficient D', and the three-SNP (SNP 4-7-18) block has the coefficient D' higher than 0.79. In haplotypes association analyses, there were no significant associations between the narrow model of schizophrenia and all the compositions of the SNP 4-7-18 haplotypes when analyzed with the TRANSMIT program version 2.5.4 (Clayton, 1999).

Discussion

RGS4 is a gene composed of 5 exons with a transcript position spanning for 7228 bps around the chromosome 1q23.3 (http://snpper.chip.org/bio/find-gene). This position has a short distance from the schizophrenia susceptible locations of 1q21-22 (Brzustowicz *et al.*, 2002) (Hwu *et al.*, 2003; Owen *et al.*, 2004). Most of the studies suggesting the significant association between the RGS4 and schizophrenia is located within the promoter regions (SNP1, SNP4, SNP7) and intron 1 (SNP18) (Chowdari *et al.*, 2002), a position matches the declining region of the 1q21-22 of genome wide mapping, which may affect the expression of RGS4 as reported by the microarray expression analysis (Mirnics *et al.*, 2001a; Mirnics *et al.*, 2001b). Our result in the SNP1 violates the Hardy-Weinberg's equilibrium distribution, which is a place located closest to the 1q22 with the highest linkage disequilibrium with schizophrenia. We suspect that the RGS4 gene may be not the major gene in this 1q21-22 region as a positional candidate gene for schizophrenia.

In order to replicate the linkage findings reported by Chowdari et al, we used 218 schizophrenia families for the four SNP markers (Chowdari *et al.*, 2002). This sample size has far beyond the 80% of statistic power suggested by their TDT-trio estimation. They suggested with 69 parent-child trios in their NIMH sample or 130 trios from their Pittsburg samples (Chowdari *et al.*, 2002). If it takes more than estimated amount of families to prove that the RGS4 gene as a candidate gene in schizophrenia, we would suspect that the RGS4 may be having less impact to the heterogeneous disease of schizophrenia. Further stratifications according to the disease endophenotypes will be necessary to examine if there would have a cluster of patients significantly relating to RGS4.

The haplotype linkage study of Chowdari et al did not show significant result in all ethnic groups (Chowdari *et al.*, 2002). The Caucasian ethnic group; the Pittsburgh and NIMH samples, has shown significant RGS4 haplotypes linkage to schizophrenia, but not in the Indian ethnic group. It is possible that the RGS4 has a different mechanism involved in the pathogenesis of schizophrenia in Asian population, because no postmortem brain tissues had demonstrated decreased expression in the patients of

Asian. Besides the difference in ethnic group, the SNP structure in this gene may be different in the population of Taiwan. We had selected a SNP (rs14665) located in the exon 5 of RGS4 in addition to these four reported SNPs of RGS4, there was no heterozygosity existed in the validation samples and was therefore excluded from our further typing. These results suggest that if RGS4 is a positional candidate gene for schizophrenia, it seems having less influence at these SNP markers on the ethnic groups of Taiwan.

Acknowledgements

We acknowledge the support from department of Medical Research and the SNP genotyping works done by the National Genotyping Center (NGC). This study was supported by the grants from the National Science Council, Taiwan (NSC-91-3112-B-002-011; NSC-92-3112-B-002-019; NSC-93-3112-B-002-012), and National Health Research Institute, Taiwan (NHRI-90-8825PP; NHRI-EX91, 92, 93-9113PP; IRO1 MH59624-01).

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Table I. Frequencies of the single nucleotide polymorphisms and haplotypes of the RGS4 gene and association with narrow phenotype model of schizophrenia

Name dSNP ID (Allele type)	Location (Inter-SNP distance from SNP1)	LD D'	Minor allele frequency (Hardy-Weinberg Test P-value)	Narrow Model			Haplotype (**SNP4-SNP7-SNP18**)	Haplotype Frequency	Haplotype Narrow Model N=216	
				N	Chi	P-value			Chi	P-value
SNP 4 rs951436 (A/C)	Ch1:160220719 (498 bp)	0.929	0.50 (0.5845)	215	1.40	0.2369	A-G-A	0.0573	0.10	0.7467
							C-G-A	0.0560	0.05	0.8283
SNP 7 rs951439 (G/A)	Ch1:160221068 (349 bp)	0.824	0.44 (0.5114)	216	0.85	0.3560	A-A-A	0.4273	1.18	0.2773
							C-G-G	0.4409	0.74	0.3911
SNP18 rs2661319 (A/G)	Ch1:160227154 (6086 bp)	0.791	0.47 (0.8659)	215	0.72	0.3966	A-G-A	0.0573	0.10	0.7467

LD: linkage disequilibrium of adjacent SNPs represented by D'

N: number of families with transmissions to affected offspring

Chi: the test statistics

Appendix 5

Association analysis between schizophrenia and the candidate genes on chromosome 6p: DTNBP1 and NOTCH4

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Abstract

Several linkage studies have linked schizophrenia to chromosome 6p24-21 region and the positional candidate genes, DTNBP1 and NOTCH4, have been reported associated with schizophrenia. However, the association evidence seemed inconsistent across studies. We aimed to test the association of the two candidate genes by a two-stage fine-mapping approach in 218 co-affected sib pair families of schizophrenia in Taiwan. First, we selected 18 single nucleotide polymorphisms (SNPs) of DTNBP1 and 14 SNPs of NOTCH4 from public SNP database, which distributed across the entire gene region from promoter to 3' untranslated region. We genotyped these SNPs for validation in a sample subset. Nine SNPs of DTNBP1 with average intermarker distance of 17 kb, and seven SNPs of NOTCH4 with average intermarker distance of 5.3 kb met the validation criterion of minor allele frequency above 10%. Secondly, the validated SNPs were genotyped in 218 families with at least 2 siblings affected with schizophrenia. Intermarker linkage disequilibrium was calculated by Gold program. Single locus and haplotype association analysis were performed with Transmit v2.5.4 program. We found the T allele of the SNP rs2071285 (p=0.035) and the G allele of the SNP rs204993 (p=0.0097) were significantly preferentially transmitted to the affected individuals in the single locus association analysis. The two SNPs were in high linkage dysequilibrium (D' > 0.8). The T-G haplotype of the two SNPs showed marginally significantly over-transmitted (p=0.053) and the A-A haplotype was significantly under-transmitted to affected individuals (p=0.034). The associated region distributed across the distal portion of NOTCH4 gene and overlapped with the genomic region of P78548, GPSM3, and PBX2. There was no significant association evidence with DTNBP1 either through single locus or haplotype association analysis. In summary, our results showed there was significant association with the distal genomic region of NOTCH4, though the association with the genomic region of P78548, GPSM3, and PBX2 cannot be ruled out, and no significant association with DTNBP1 in Taiwanese co-affected sib pair families of schizophrenia.

Key words: schizophrenia, NOTCH4, DTNBP1, chromosome 6p, family-based association study

Introduction

Schizophrenia is a serious neuropsychiatric illness affecting about 1% of the general population. Family, twin, and adoption studies have demonstrated that schizophrenia is predominantly genetically determined and has high heritability (McGuffin et al., 1994). The mode of transmission is still not clear, though a multilocus model is favored in which several genes each having a small effect and

acting in epistasis lead to schizophrenia according to the pattern of risk to families (Risch 1990). A number of positive linkage findings to schizophrenia have been reported on chromosome 6p (Straub et al., 1995; Wang et al., 1995; Schwab et al., 1995; Antonarakis et al., 1995; Schizophrenia Linkage Collaborative Group, 1996). There was also suggestive linkage evidence to chromosome 6p in our family sample (Hwu et al., 2000).

(Wei and Hemmings 2000) observed that NOTCH4 was strongly associated with schizophrenia in 80 British parent-offspring trios. This work has been replicated by several independent studies (Skol, Young et al. 2003; Luo, Klempan et al. 2004; Prasad, Chowdari et al. 2004; Zhang, Wei et al. 2004), but others failed to replicate(Imai, Harada et al. 2001; McGinnis, Fox et al. 2001; Sklar, Schwab et al. 2001; Ujike, Takehisa et al. 2001; Fan, Tang et al. 2002; Kaneko, Muratake et al. 2004; Tochigi, Zhang et al. 2004). Considering clinical heterogeneity of schizophrenia, though not associated with the phenotype of schizophrenia, two studies reported the NOTCH4 locus were associated with the age of onset (Anttila, Kampman et al. 2003; Takahashi, Cui et al. 2003) and one study reported association with frontal lobe function in schizophrenia (Wassink, Nopoulos et al. 2003). All the studies focus upon the polymorphisms the initial study reported, which located at the 5' genomic region of the gene.

There have been several reports implicating the DTNBP1 gene in the etiology of schizophrenia. (Straub, Jiang et al. 2002) reported the original evidence for a significant association between schizophrenia and SNPs within DTNBP1 in a sample of 270 multiply affected Irish pedigrees. In that study, eight SNPs across the gene, as well as several three-marker haplotypes, showed significant over-transmission to affected offspring across a range of different diagnostic categories. Several studies also showed significant genetic association evidence in different ethnic samples using different study designs(Schwab, Knapp et al. 2003; Tang, Zhou et al. 2003; van den Oord, Sullivan et al. 2003; Funke, Finn et al. 2004; Kirov, Ivanov et al. 2004; Numakawa, Yagasaki et al. 2004; Williams, Preece et al. 2004). One study reported the gene was significantly associated with schizophrenic patients with family history (Van Den Bogaert, Schumacher et al. 2003). Two studies failed to replicate the result (Morris, McGhee et al. 2003; Hall, Gogos et al. 2004).

Considering the inconsistency of the replication studies, we aimed to study the association between schizophrenia and DTNBP1, NOTCH4 using a systemic approach, that is, whole gene scan using evenly dispersing SNPs of the genes, in our co-affected sib pair families of schizophrenia.

Materials and Method

Subjects

The subjects were recruited from two sample-collecting programs, multidimensional psychopathology study of schizophrenia (MPSS) from 1993 to 2001 and Taiwan schizophrenia linkage study (TSLS) from 1998 to 2002. A total of 218 co-affected sib pair families were used for this study, of which 86 families from MPSS and 132 from TSLS.

The MPSS families were recruited mainly from the Department of Psychiatry, National Taiwan University Hospital and the University-affiliated Taoyuan Psychiatric Center. Data collection was initiated after informed consents were obtained from the identified study subjects and their families. All of the family members were personally interviewed by the research psychiatrists using the Psychiatrist Diagnostic Assessment (PDA) (Hwu et al., 1999). The final diagnostic assessment was formulated by integrating the PDA data and clinical information of medical chart records. The final diagnosis was done following DSM-IV criteria.

The TSLS families were recruited from the hospitals all over Taiwan except the above two hospitals. Data collection was initiated after informed consents were obtained from the identified study subjects and their families. All of the family members were interviewed by well-trained assistants using the Mandarin Chinese version of the Diagnostic Interview for Genetic Studies (DIGS) (Chen et al., 1998). The final diagnostic assessment was formulated by integrating the DIGS data and clinical information of medical chart records by two board certified research psychiatrists independently. Research diagnosis was made based on DSM-IV criteria. All data schedules and medical records of the subjects who had inconsistent diagnosis from these two independent research diagnosticians would be evaluated further by the senior researcher (H-G Hwu) to achieve final diagnosis.

A total of two hundred and eighteen schizophrenic nuclear families with at least two affected siblings, with 864 individuals' DNA available, were included in this study. 461 individuals were diagnosed as schizophrenia, of which 62.5% were male and the mean age was 34.5 ($\pm$ 9.4) years of age. The mean age at onset was 22.2 ($\pm$ 6.2) years. The unaffected subjects were 47.6% male and 52.6 ($\pm$ 15.3) years of age.

SNP selection and validation

For a systemic approach, we selected evenly dispersed SNPs of the two genes from public database (http://www.ensembl.org/Homo_sapiens/martview). These SNPs distributed across the entire genomic region from promoter to 3' untranslated region. A total of 18 SNPs of DTNBP1 and 14 SNPs of NOTCH4 were selected for further

validation. We used a sample subset of 31 trios and 1 independent individual to validate the 32 selected SNPs. Considering the power of further linkage disequilibrium test, we set the validation criteria of minor allele frequency above 10%.

SNP genotyping

The selected SNPs were genotyped by the method of matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS). Primers and probes flanking the SNPs were designed by using SpectroDESIGNER software (Sequenom, San Diego, CA, USA). A DNA fragment (100-300 bp) encompassing the SNP site of was amplified using the polymerase chain reaction (PCR) (GeneAmp 9700 thermocycler, Applied Biosystems, USA) according to the manufacturer's instruction.

After PCR amplification, the un-incorporated deoxynunleotide triphosphate (dNTP) of the PCR reaction mixture were neutralized by treating with shrimp alkaline phosphatase (SAP) at 37℃ for 20 minutes. The reaction mixture were incubated at 85℃ for 5 minutes to inactivate the SAP activity. Primer extension was performed by adding the probe, Thermo Sequenase (Amersham Pharmacia, Piscataway, NJ, USA) and appropriate dideoxynucleotide triphosphate (ddNTP)/dNTP mixture, and following 55 cycles of denaturing at 94℃ for 5 sec, annealing at 52℃ for 5 sec, and extension at 72℃ for 5 sec. Different extension products were differentiated by mass through MALDI-TOF as follows.

Cation exchange resin (SpectroCLEAN, Sequenom) was added to the above reaction mixture to get rid of salt, which would interfere with the mass reading in the MALDI-TOF analysis. After desalting, the reaction mixture was spotted onto the SpectroCHIP (using the SpoectroPOINT) where they were co-crystallized with the matrix. After introduced to the SpectroREADER, a nitrogen laser with nanosecond-wide pulses interrogated the samples on the SpectroCHIP in the high-vacuum environment of the time-of-flight mass spectrometer. Upon laser irradiation, the matrix crystals absorbed the laser's energy and the extension products were converted into gas phase ions, which upon acceleration undergo mass-dependent separation over approximately 1-meter flight path. Molecular masses were determined with 0.01 to 0.02% estimated accuracy. Acquired spectra were transferred to the MassARRAY Server (Sequenom, San Diego, CA) where they were automatically interpreted and corrected for their genotypes.

Statistical analysis

In order to verify the sample accuracy included family relationship and genotype,

PEDCHECK version 1.1 (O'Connell and Weeks, 1998) and UNKNOWN version 5.23 (Terwilliger 1994) were employed to check Mendelian inheritance and Procedure ALLELE in SAS/GENETICS release 8.2 (Institute, 2002) was used to test for Hardy-Weinberg equilibrium. Linkage disequilibrium (LD) of inter-markers was measured using coefficient D’ (Hedrick 1987) which was also used to define haplotype blocks. A graphic presentation of block pattern was completed using GOLD software (Abecasis et al., 2000; Abecasis and Cookson, 2000). Both of single point and haplotype association analyses were carried out simultaneously using the program of TRANSMIT version 2.5.4 (Clayton 1999). The SNPtagger program (Ke and Cardon, 2003) was used to screen for minimal sets of SNPs (haplotype tagging SNPs, htSNPs) to represent given haplotypes.

Result

In the stage of SNP validation, nine SNPs of DTNBP1 with average intermarker distance of 17 kb and seven SNPs of NOTCH4 with average intermarker distance of 5.3 kb met the validation criterion of minor allele frequency above 10%. Two SNPs (rs915895 and rs397081) of NOTCH4 were not compatible with the Hardy Weinberg equilibrium. The detail description of the validated SNPs was listed in Table 1. The detail association analysis results were listed as the following:

DTNBP1

The single locus association analysis showed no significant evidence with the SNPs of DTNBP1. The detail was listed in Table 1. The intermarker LD assessed by coefficient D’ was presented in Table 2. The eight SNPs from rs909706 to rs3829893 showed intermarker D’ above 0.7 and constituted a haplotype block. Three SNPs, rs909706, rs1011313, and rs3829893, were selected as htSNPs through the SNPtagger program . The haplotype association analysis using the three htSNPs showed no significantly preferential transmission of any four haplotypes. The detail haplotype frequency and analysis result was showed in Table 4. We also analyzed the 2-SNP, 3-SNP haplotype association of the 8-SNP haplotype block using moving window strategy and the results were all negative (data not shown).

NOTCH4

The single locus association analysis showed the T allele of rs2071285 and the G allele of rs204993 significantly preferentially transmitted to affected individuals (p=0.035, 0.0097, respectively). The detail was listed in Table 1. The intermarker LD assessed by coefficient D’ was presented in Table 3. The LD pattern showed two haplotype blocks, one was rs2071285-rs204993 (D’ >0.8), and the other

rs915894-rs915895 (D'>0.9). The haplotype association analysis revealed the T-G haplotype of rs2071285-rs204993 was marginally significantly over-transmitted to affected individuals (p=0.053) and the A-A haplotype was significantly under-transmitted to affected individuals (p=0.034). The haplotype association analysis of the rs915894-rs915895 block showed no significantly preferential transmission of any four haplotypes. The detail haplotype frequency and analysis result was showed in Table 2. The intermarker D' of rs397081-rs915894-rs915895 was above 0.7. The haplotype association analysis of the rs397081-rs915894-rs915895 block revealed negative result (data not shown).

Discussion

Our study revealed that there was significant association with the distal genomic region of NOTCH4 and no significant association with DTNBP1 in Taiwanese co-affected sib pair families of schizophrenia. The associated region spans about 25kb, from the distal genomic region of NOTCH4 (rs2071285) to the genomic region of PBX2 (rs204993), within which also includes the genomic region of P78548, GPSM3. Hence, the association with the genomic region of P78548, GPSM3, and PBX2 cannot be ruled out from this study.

We took a systemic approach, that is, whole gene scan using dispersing SNPs of the genes, to test the association between schizophrenia and NOTCH4. We selected different markers from previous studies. To compare with previous studies of NOTCH4, the relative locations of the markers genotyped in our study and previous studies were plotted in Fig.1.

The first study reported highly significant association with the markers on the 5' region of NOTCH4 (Wei and Hemmings 2000). The follow-up studies also focused upon the markers locating at the proximal part of NOTCH4 (Imai, Harada et al. 2001; McGinnis, Fox et al. 2001; Sklar, Schwab et al. 2001; Ujike, Takehisa et al. 2001; Fan, Tang et al. 2002; Anttila, Kampman et al. 2003; Skol, Young et al. 2003; Takahashi, Cui et al. 2003; Wassink, Nopoulos et al. 2003; Kaneko, Muratake et al. 2004; Luo, Klempan et al. 2004; Prasad, Chowdari et al. 2004; Tochigi, Zhang et al. 2004; Zhang, Wei et al. 2004). The meta-analysis of NOTCH4 studies revealed no significant association was detected between schizophrenia and the four polymorphisms, (TAA)n, SNP1, SNP2, and (CTG)n, on the 5' region of the gene (Glatt, Wang et al. 2005). Our study also revealed no significant association evidence with the SNPs on the 5' region. However, we found significant association evidence with the SNPs on the distal portion of the gene. Four studies (Wei and Hemmings 2000; Fan, Tang et al. 2002; Skol, Young et al. 2003; Kaneko, Muratake et al. 2004) genotyped the marker (TTAT)n in the intron 17, 2.7 kb distal to the SNP rs2071285, which was significant

association in our study, and found no significant association with schizophrenia. However, (TTAT)n was reported an unstable marker (Zhang, Wei et al. 2004)(ref). Therefore, our study is the first one showing probable association evidence between schizophrenia and the distal genomic region of NOTCH4.

The meta-analysis of NOTCH4 studies also found that heterogeneity and stronger evidence of association with the four polymorphisms on the 5' region of the gene in family-based studies than in case-control studies, suggesting that these polymorphisms may reliably influence risk for schizophrenia under certain circumstances (Glatt, Wang et al. 2005). Considering clinical and genetic heterogeneity of schizophrenia, though not associated with the phenotype of schizophrenia, two studies reported the NOTCH4 locus was associated with the age of onset (Anttila, Kampman et al. 2003; Takahashi, Cui et al. 2003) and one study reported association with frontal lobe function in schizophrenia (Wassink, Nopoulos et al. 2003). Our study revealed the phenomenon of either allelic heterogeneity (association with different genomic region of the gene) or locus heterogeneity (association with neighboring tightly linked loci) in the association between schizophrenia and NOTCH4 in an ethnic-distinct sample.

As for the negative result of DTNBP1 in our sample, we considered the following reasons. First, the ethnic difference might contribute to the replication failure. Though we reported suggestive linkage evidence to 6p22.3 (Hwu et al. 2000) in a sub-sample of this study, the peak of non-parametric linkage score was on the marker D6S285, as distant as 3.5 Mb from DTNBP1. The allele frequencies of the SNPs were also different from those of the initial report (Straub, Jiang et al. 2002). A recent study cannot identify any mutation at the 5' promoter region and in the protein-coding sequences of DTNBP1 in the Taiwanese Han sample, the same ethnic sample as ours (Liao and Chen 2004). Secondly, the power to detect association was above 80% in our sample while the minor allele frequencies of SNPs were above 0.1 and the intermarker D' above 0.7 under the family structure of our sample, considering the same genetic parameters as our previous linkage study of chromosome 6p (Hwu et al., 2000), that is, the disease gene frequencies (%) in the dominant and recessive models were 0.005 and 0.10, respectively, the penetrance was 0.5 and the probability of phenocopy was 0.005. Thirdly, we used the moving window strategy instead of SNPtagger program to detect all the possible significant associated haplotypes and the results were still not significant. Fourthly, we genotyped only one SNP in the distal genomic region of the gene; therefore, we cannot exclude completely the possibility of association with the distal genomic region of DTNBP1 in our sample.

Our analyses suggest association at nominal levels of significance with the

distal portion of NOTCH4. None of these results remained significant following corrections for multiple comparisons. Therefore, we need to interpret the result with caution and cannot exclude the possibility of false positive. Further replication and fine mapping study to delineate the true associated genomic region is warranted.

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Table 1. The detail description of the validated SNPs and single locus association analysis of DTNBP1 and NOTCH4 using Transmit program

Gene	dbSNP ID. (alternative SNP name)[a]	Location[b]	Intermarker Distance (kb)[c]	Polymorphism[d]	Minor allele frequency[e]	Single locus association	
						Chi-square	p-value
DTNBP1	rs909706 (P1583)	Intron 1	-	T/C	0.40	0.01	0.91
	rs1018381 (P1578)	Intron 1	3.8	G/A	0.08	0.60	0.44
	rs2619522 (P1763)	Intron 1	3.4	A/C	0.09	0.10	0.75
	rs2005976 (P1757)	Intron 3	2.8	C/T	0.09	0.05	0.82
	rs2619528 (P1765)	Intron 3	1.0	C/T	0.09	0.10	0.75
	rs1011313 (P1325)	Intron 4	16.4	C/T	0.19	0.08	0.78
	rs2619539 (P1655)	Intron 5	12.6	C/G	0.39	0.03	0.87
	rs3829893	Intron 5	5.2	G/A	0.31	0.20	0.67
	rs742106 (P1328)	Intron 9	91.2	A/G	0.43	1.18	0.28
NOTCH4	rs397081	Promoter	-	T/C	0.16	2.25	0.13
	rs915894	Intron 2	2.2	T/G	0.46	0.31	0.58
	rs915895	Intron 3	0.2	T/C	0.49	0.02	0.90
	rs415929	Intron 4	1.2	T/C	0.17	0.38	0.54
	rs3131290	Intron 11	5.9	G/A	0.13	1.26	0.26
	rs2071285	Intron 17	2.7	A/T	0.18	4.46	**0.035***
	rs204993	3'-UTR	24.9	A/G	0.39	6.69	**0.0097***

[a]: The SNP number in the study of Straub et al., 2002.

[b]: The SNP location of DTNBP1 was determined based upon the m-RNA accession NM_032122; The SNP location of NOTCH4 was determined based upon the m-RNA accession NM_004557.

[c]: The intermarker distance was determined based upon the genomic contig accession NT_007592.

[d]: Second allele is the minor allele.

[e]: Two SNPs, rs915895 and rs397081, are incompatible with Hardy Weinberg equilibrium (p=0.005, 0.002, respectively).

[*]: The over-transmitted alleles of the two SNPs, rs2071285 and rs204993 are T allele and G allele, respectively.

Table 2. Intermarker D' value of the nine SNPs of DTNBP1 calculated by Gold program

	rs909706	rs1018381	rs2619522	rs2005976	rs2619528	rs1011313	rs2619539	rs3829893	rs742106
rs909706	…								
rs1018381	0.87	…							
rs2619522	0.89	1.00	…						
rs2005976	0.89	0.97	0.96	…					
rs2619528	0.89	1.00	1.00	0.96	…				
rs1011313	0.89	0.84	0.86	0.86	0.86	…			
rs2619539	0.94	0.93	0.76	0.78	0.76	0.88	…		
rs3829893	0.95	0.95	0.91	0.87	0.91	0.88	0.94	…	
rs742106	0.16	0.37	0.34	0.36	0.34	0.53	0.17	0.11	…

Table 3. Intermarker D' value of the seven SNPs of NOTCH4 calculated by Gold program

	rs397081	rs915894	rs915895	rs415929	rs3131290	rs2071285	rs204993
rs397081	…						
rs915894	0.71	…					
rs915895	0.76	0.91	…				
rs415929	0.78	0.08	0.06	…			
rs3131290	0.90	0.18	0.32	0.27	…		
rs2071285	0.87	0.87	0.92	0.96	0.91	…	
rs204993	0.80	0.33	0.32	0.10	0.46	0.86	…

Table 4. Haplotype association analysis using Transmit empirical variance in 218 co-affected sib pair families

Gene	Haplotype	Haplotype frequency	Chi-square	p-value
DTNBP1	rs909706-rs1011313-rs3829893			
	C-C-G	0.42	0.19	0.66
	C-T-G	0.19	0.03	0.86
	T-C-G	0.09	0.44	0.51
	T-C-A	0.30	0.04	0.85
NOTCH4	rs915894-rs915895			
	T-C	0.04	1.25	0.26
	T-T	0.50	0.01	0.91
	G-C	0.46	0.17	0.68
	G-T	0.006	0.12	0.72
	rs2071285-rs204993			
	A-A	0.59	4.49	**0.034 -**
	A-G	0.23	0.66	0.42
	T-A	0.001	0.88	0.35
	T-G	0.18	3.76	**0.053** +

-: under-transmit to affected individuals

+: over-transmit to affected individuals

Appendix 6

Brief report

Absence of Significant Associations between Four AKT1 SNP Markers and Schizophrenia in Taiwanese

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Abstract

AKT1 (V-akt murine thymoma viral oncogene homolog 1) is a protein kinase isoform of AKT. Five single nucleotide polymorphisms (SNPs); rs3803300, rs1130214, rs3730358, rs2498799 and rs2494732, at the genomic region of AKT1 have been reported to be significantly associated with schizophrenia. We tested for the presence of these five SNPs in Taiwanese by genotyping 218 co-affected schizophrenia families. Both single locus and haplotypes analyses showed no association of these SNPs with schizophrenia. These findings fail to support AKT1 as a candidate gene for schizophrenia susceptibility in the Taiwanese population.

Keywords: AKT1, protein kinase, Taiwanese families, schizophrenia.

Introduction

AKT1 (V-akt murine thymoma viral oncogene homolog 1) is an isoform of serine/threonine protein kinase AKT (also known as protein kinase B) which was isolated from an AKR mouse thymoma cell line transforming with murine retrovirus AKT8 (Staal, 1987).

The AKT1 gene was first reported as a susceptibility gene in schizophrenia by Emamian et al (Emamian *et al.*, 2004), who found significant reduction of AKT1 in the viral-transformed peripheral lymphocytes of schizophrenic patients, and also confirmed this result in postmortem studies of human brain frontal cortex and hippocampus. Besides the proteomic findings, five single nucleotide polymorphisms (SNPs) of AKT1 markers located at the chromosome 14q32.32 spanning from the promoter to the intron 11 were genotyped in 265 schizophrenia proband families. Their study revealed significant haplotype associations close to the promoter region of the AKT1 gene. The substrate of AKT1, glycogen synthase kinase-3β (GSK-3β), decreased its phosphorylation of the serine 9 residue. The mRNA levels of AKT1 at dorsolateral prefrontal cortex were also decreased in the schizophrenia patients (Kozlovsky *et al.*, 2004). However, two separate genotyping studies of these five SNPs of AKT1 in the Japanese population revealed contradictory results (Ikeda *et al.*, 2004; Ohtsuki *et al.*, 2004). In order to determine whether the AKT1 gene is a schizophrenia susceptibility gene in the Taiwanese population, we genotyped these five SNPs in 218 affected sib-pair schizophrenia families.

Subjects and methods

This research project was approved by the Institutional Review Board of National Taiwan University Hospital. All genomic DNA samples were collected from the family subjects with at least two affected sibling after obtaining written informed consent. The subjects were recruited from two research programs; the multidimensional psychopathology study of schizophrenia (MPSS) (Hwu *et al.*, 2002) from 1993 to 2001 and the Taiwan schizophrenia linkage study (TSLS) (Hwu *et al.*, 2005) from 1998 to 2002. The 86 families of MPSS subjects were interviewed by the research psychiatrists using the Psychiatrist Diagnostic Assessment (PDA) (Hwu, 1999). The 132 TSLS families were interviewed by well-trained assistants using the Mandarin Chinese version of the Diagnostic Interview for Genetic Studies (DIGS) (Chen, 1999). For both studies, the final diagnostic assessment was formulated by integrating either the PDA or the DIGS data with clinical information from medical records using the Specialist Diagnostic Assessment Sheet (SDAS), based upon the criteria of the Diagnostic and Statistical Manual of Mental Disorders, 4th edition (DSM-IV). This study sample included two hundred and eighteen schizophrenic

nuclear families with at least two affected siblings, and had a total of 864 subjects participated in this genotyping study.

All SNP markers were genotyped by the method of matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS). A DNA fragment (100-300 bp) encompassing the SNP site was amplified using the polymerase chain reaction (PCR) GeneAmp 9700 thermocycler (Applied Biosystems, USA) according to the manufacturer's instruction. After PCR amplification and neutralization of the deoxynucleotide triphosphate (dNTP), the primer extension was performed by adding the probe, Thermo Sequenase (Amersham Pharmacia, Piscataway, NJ, USA) and appropriate dideoxynucleotide triphosphate (ddNTP)/dNTP mixture. Different extension products were differentiated by mass through MALDI-TOF.

We used the procedure ALLELE in SAS/GENETICS release 8.2 (SAS Institute, 2002) to assess Hardy-Weinberg equilibrium. Family relationships were verified by PEDCHECK version 1.1 (O'Connell and Weeks, 1998) and UNKNOWN version 5.23 (Terwilliger and Ott, 1994) to detect deviations from Mendelian inheritance. Both single point and haplotype association analyses were carried out using TRANSMIT version 2.5.4 (Clayton, 1999).

Results

All of the family relationships and genotypes were verified by checking Mendelian inheritance and using the Procedure ALLELE in SAS/GENETICS release 8.2 for Hardy-Weinberg equilibrium. The SNP markers were all compatible with the Hardy-Weinberg equilibrium distribution, except for SNP1 (rs3803300). The other four SNPs (SNP 2 -SNP 5) were validated and showed a minor allele frequency greater than 10% and a missing rate of less than 2% in this Taiwanese cohort (Table 1).

We used haploview software to construct haplotype blocks constituted by "strong LD" markers according to the criteria proposed by Gabriel et al (Gabriel *et al.*, 2002). The results show that only one haplotype block formed by SNP4 and SNP5 (D'=0.96) was found among the four markers. The TRANSMIT program version 2.5.4 (Clayton, 1999), which can utilize data from all families even when parental genotypes are unknown, was used to analyze the associations between single SNPs, the haplotypes and schizophrenia. No significant association was found between either a single locus or haplotype and schizophrenia (Table 1). Similar results were also obtained from single locus; SNP 2 (N=35, Z=-0.277, p-value =0.782), SNP 3 (N=21, Z=-0.14, p-value =0.889), SNP 4 (N=81, Z=0.32, p-value =0.748), and SNP 5 (N=74, Z=0.37, p-value =0.714), and from haplotypes; C-T (N=85, Z=0.8, p-value =0.422), T-C

(N=79, Z =-0.45, p-value =0.65), C-C (N=50, Z=-0.449, p-value =0.65), and T-T (N=1) association analyses performed by family-based tests, using the FBAT program (Horvath *et al.*, 2004).

Discussion

Although chromosome 14q has been reported in a genomewide scan as a susceptible locus for schizophrenia (Bailer *et al.*, 2000), AKT1 was the first candidate gene for schizophrenia in the chromosome 14q32.32 region reported from SNP2-SNP3-SNP4 (T-C-G) haplotype association analysis (Emamian *et al.*, 2004). The single locus result was similar to the result obtained from this Taiwanese sample. While Emamian et al (Emamian *et al.*, 2004) reported the risk haplotypes in the SNP2-SNP3 had linkage disequilibrium of around 0.67, this study found an SNP4-SNP5 block with D' of 0.98. This result suggests inheritance differences between different ethnic groups.

If the AKT1 gene has a major role in schizophrenia susceptibility, we would expect studies to consistently demonstrate its association with the disease development among members of an ethnic group. However, in the Japanese population, inconsistent results for this association were reported (Ikeda *et al.*, 2004; Ohtsuki *et al.*, 2004). The present study also could not confirm the significance of Emamian et al's finding of an association between AKT1 haplotypes and schizophrenia (Emamian *et al.*, 2004). This suggests that AKT1 does not have an important role in the predisposition to schizophrenia in Taiwanese.

Acknowledgements

We acknowledge the help from the Department of Medical Research in National Taiwan University Hospital and the SNP genotyping work done by the National Genotyping Center (NGC). This study was supported by grants from the National Science Council, Taiwan (NSC-91-3112-B-002-011; NSC-92-3112-B-002-019; NSC-93-3112-B-002-012), and the National Health Research Institute, Taiwan (NHRI-90-8825PP; NHRI-EX91, 92, 93-9113PP; IRO1 MH59624-01).

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Table 1. Frequencies of the single nucleotide polymorphisms and haplotypes of the AKT1 gene and associations (analyzed by Transmit program 2.5.4.) with schizophrenia

SNP_ID	**Position**	**Genetic Region**	**Allele Type**	**MF**	**HW Test**	**Schizophrenia N=214**		**Haplotypes**		**Schizophrenia N=214**	
					P	Chi	P	Haplotype (rs2498799-rs2494732)	Haplotype Frequency	Chi	P
SNP2 rs1130214	10323141	AKT1 (Promoter)	C/A	0.1098	0.6157	0.177	0.674	C-T	0.5594	1.635	0.201
SNP3 rs3730358	10321808	AKT1 (intron 3)	G/A	0.0538	0.2056	0.267	0.605	T-C	0.2755	0.124	0.724
SNP4 rs2498799	10321157	AKT1 (exon 9)	T/C	0.4414	0.6591	0.829	0.363	C-C	0.1573	1.283	0.257
SNP5 rs2494732	10321086	AKT1 (Intron 11)	C/T	0.2966	0.9365	0.464	0.496	T-T	0.0077	2.834	0.092

MF: Minor allele frequency

HW: Hardy-Weinberg's test

P: p-value

N: Number of families

Chi: Chi-square

Appendix 7

SCIENTIFIC CORRESPONDENCE

Evaluation of the ZDHHC8 as a Candidate Gene for Schizophrenia

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Abstract

ZDHHC8 (Zinc finger, DHHC domain containing 8; also annotated as KIAA1292) was reported as a schizophrenia candidate gene located around the chromosome region of 22q11.21. As this newly discovered gene renders further support from other ethnic group, we selected two single nucleotide polymorphisms (SNP) located before and within the locus of ZDHHC8 gene to test if it is susceptible to schizophrenia in a population sample of Taiwan. There were 218 schizophrenia families with at least two affected siblings participated in this study. These two SNPs were genotyped by the method of MALDI-TOF mass spectrometry. The SNP of rs175174 locating in the intron 4 of ZDHHC8 which might affect the splicing process and its 27 kb nearby SNP (rs1633445) did not show significant association with schizophrenia. Base on these two markers, we can not confirm the ZDHHC8 gene as susceptible gene for schizophrenia in the cohort of Taiwan.

Keywords: ZDHHC8, KIAA1292, schizophrenia, Taiwan, 22q11.21

Chromosome 22q11.21 region had previously been reported with microdeletions and were significantly associated with schizophrenia.[1, 2] The discovery of ZDHHC8 (Zinc finger, DHHC domain containing 8) gene in this region as a candidate for schizophrenia is inferred by the finding of a single nucleotide polymorphism (SNP) rs175174 located at intron 4,[3] which may alter the genetic splicing and modulate the levels of ZDHHC8 expression at different allele types. In ZDHHC8 knock out mice, Mukai et al observed alterations in the sensorimotor gating of prepulse inhibition, and exploratory activity of locomotor response to novelty. As this gene has only been genotyped in two independent family samples, it warrants substantial supports from other ethnic groups to prove that the ZDHHC8 gene is a true susceptibility gene for schizophrenia.

The functional role of ZDHHC8 in the brain is not clear. ZDHHC8 is found widely expressed in adult human brain.[3] In different species of homology search, there are two yeast ZDHHC proteins demonstrating as the enzyme of palmitoyltransferase.[4,5] Palmitoylation was known to involve in neuronal development and neuronal function.[6] This suggests that the ZDHHC8 may have involved in the pathological process of schizophrenia.

The studies of ZDHHC8 as a candidate gene of schizophrenia has been studied by case-control design in three ethnic groups; the population of European,[7] the population of Japan[8] and the population of Han Chinese.[9] Contradictory results were obtained from these two studies. It is promising to test if ZDHHC8 is a susceptibility gene in a study sample of Taiwan, and we genotyped two SNPs in 218 families with at least two siblings affected with schizophrenia.

This research project was approved by the Institutional Review Board of the National Taiwan University Hospital. After obtaining the signed up inform consents, the study subjects were recruited for diagnostic assessment and collection of the genomic DNA samples. The subjects were recruited from two sample-collecting programs. These were the multidimensional psychopathology study of schizophrenia (MPSS)[10] from 1993 to 2001 and the Taiwan schizophrenia linkage study (TSLS)[11] from 1998 to 2002. The 86 families of the MPSS subjects were interviewed by the research psychiatrists using the Psychiatrist Diagnostic Assessment (PDA).[12] The 132 families of the TSLS subjects were interviewed by well-trained assistants using the Mandarin Chinese version of the Diagnostic Interview for Genetic Studies (DIGS).[13] The final diagnostic assessment of both MPSS and TSLS were formulated by integrating either the PDA or the DIGS data and the clinical information of medical chart records using the Specialist Diagnostic Assessment Sheet (SDAS), based upon the criteria of the Diagnostic and Statistical Manual of Mental Disorder, 4th edition (DSM-IV). The study samples included two hundred and eighteen schizophrenic

nuclear families with at least two affected siblings, which had a total of 864 subjects been genotyped (Table 1). Within these 218 families, there were 216 families having at least two sibs genotyped, 103 families having one parent, and 96 families having both parents genotyped in this study.

The SNP markers were genotyped by the method of matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS). A DNA fragment (100-300 bp) encompassing the SNP site was amplified using the polymerase chain reaction (PCR) GeneAmp 9700 thermocycler (Applied Biosystems, USA) according to the manufacturer's instruction. After PCR amplification and neutralization of the deoxynucleotide triphosphate (dNTP), the primer extension was performed by adding the probe, Thermo Sequenase (Amersham Pharmacia, Piscataway, NJ, USA) and appropriate dideoxynucleotide triphosphate (ddNTP)/dNTP mixture. Different extension products were differentiated by mass through MALDI-TOF.

The quality of genotyping data was checked on the indicators of Hardy-Weinberg equilibrium, the lineage of the study families, and the deviation from Mendelian inheritance by using the statistical programs of the ALLELE procedure in the SAS/GENETICS, the PEDCHECK,[14] and the UNKNOWN,[15] respectively. The haplotype blocks was constructed by the Haploview software for haplotype blocks constituted by "strong LD" markers according to the criteria proposed by Gabriel et al.[16] The phenotype of schizophrenia was divided into narrow models (DSM-IV defined schizophrenia only) and broad models (including schizophrenia, schizoaffective, and other non-affective psychotic disorders defined by DSM-IV) for the association study. Both single point and haplotype association analyses were carried out simultaneously using the TRANSMIT[17] and the FBAT[18] programs.

We selected two SNP markers located at the intron 4 locus of ZDHHC8 gene and a nearby SNP marker (rs1633445) located at the intron 10 of HpaII tiny fragments locus 9C (HTF9C) gene to evaluate if this region is important in schizophrenia (Table 2). These SNPs were validated and showed the minor allele frequency larger than 15% and the genotyping missing rate was smaller than 2%. These two SNP markers were compatible with the Hardy-Weinberg equilibrium distribution. A haplotype block with two SNP markers was generated by Haploview[16] which created a block when 95% of informative comparisons are strongly linked and to span no more than 30kb. The linkage disequilibrium (D') is equal to 0.24 between the two markers.

There was no significant association found in any single SNP marker of ZDHHC8 or HTF9C gene with both the narrow and broad phenotype models of schizophrenia (Table 2). No transmission distortions were discovered between genders in schizophrenia. The transmission to non-transmission ratios in each SNP

are 1.0124 (in broad model) and 1.0161 (in narrow model) for rs1633445, and 1.0138 (in broad model) and 1.0107 (in narrow model) for rs175174, respectively. The transmission to non-transmission ratios of SNP (rs175174) are 1.0762 (p=0.3172) in the narrow model and 1.0909 (p=0.2175) in the broad model of schizophrenia, respectively in female, and the ratios are 0.9714 (p=0.5902) in the narrow model and 0.9667 (p=0.5219) in the broad model of schizophrenia, respectively in male.

Several schizophrenia candidate genes have been reported around the 22q11 region.[3, 19, 20] These candidate genes include catechol-o-methyltransferase (COMT),[19] armadillo repeat gene deletes in velocardiofacial syndrome (ARVCF),[20] and ZDHHC8.[3] The COMT was shown to have significant association with schizophrenia in the Caucasian subjects, but we found no association in the study subjects of Taiwan.[21, 22] We suspect that the ZDHHC8 may have the same situations as COMT and may not be a major susceptible gene for schizophrenia in the study sample of Taiwan.

The possible significant association region on chromosome 22q in the population of Taiwan may be located at the 22q12 regions, which had shown a p-value of 0.001 to a sequence tag site of D22S278 in an international collaboration study organized by Gill et al.[23] ZDHHC8 and COMT are located about 16 cM away from D22S278. We suspect that the negative result may be obtained due to its location being far outside the 95% association confidence interval from the linkage region.

The result supporting ZDHHC8 as a susceptibility gene for schizophrenia also showed the transmission distortions in female for the SNP marker of rs175174.[3] The transmitted to nontransmitted ratio was about 1.91 (p=0.0005) in female, but was about 0.98 (p=0.88) in male. Our result did not show transmission distortions for this SNP marker in both female and male.

Until now, there were at least four studies to test the association of the SNP marker (rs175174) of ZDHHC8 in case-control studies. Our study is the first one to examine its association status with schizophrenia using families with at least two affected siblings. Base on this result, our study failed to confirm the association of the rs175174 marker of ZDHHC8 with schizophrenia in the sample of Taiwan.

Acknowledgements

We acknowledge the help from the Department of Medical Research in National Taiwan University Hospital and the SNP genotyping work done by the National Genotyping Center (NGC). This study was supported by grants from the National Science Council, Taiwan (NSC-91-3112-B-002-011; NSC-92-3112-B-002-019; NSC-93-3112-B-002-012), and the National Health Research Institute, Taiwan (NHRI-90-8825PP; NHRI-EX91, 92, 93-9113PP; IRO1 MH59624-01).

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Table 1. Distribution of families by number of siblings and parents genotyped

Sibs Genotyped per Family	Parents Genotyped per Family			
	0	1	2	Total Families
1	0	0	2	2
2	9	15	74	98
3	8	78	17	103
4	2	9	2	13
5	0	1	1	2
Total Families	19	103	96	218

Table 2. Frequencies of the single nucleotide polymorphisms of the ZDHHC8 gene and association with two phenotype models of schizophrenia

SNP_ID	**Chromosome**	**Genetic Region**	**Allele Type**	**MF**	**HW Test**	**Narrow Model**			**Broad Model**		
					P	N	Chi	P	N	Chi	P
rs1633445	18475150	HTF9C (Intron 10)	T=1 C=2	0.1667	0.4143	214	0.975	0.324	216	0.429	0.512
rs175174	18502108	ZDHHC8 (intron 4)	G=1 A=2	0.3328	0.2938	214	1.053	0.305	216	1.223	0.269

MF: Minor allele frequency

HW: Hardy-Weinberg's test

P: p-value

Narrow model: only subjects of schizophrenia fulfilling criteria of Diagnostic and Statistical Manual IV (DSM-IV)

Broad model: Composed of DSM-IV schizophrenia, schizoaffective disorder and non-affective psychotic disorders

N: Number of families

Appendix 8

ORIGINAL ARTICLE

Exon 1 Region of PPP3CC May be Associated with Schizophrenia

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Abstract

Calcineurin is a calcium/calmodulin-dependent protein phosphatase composed by two subunits; a regulatory subunit of calcineurin B (CNB), and a catalytic subunit of calcineurin A (CNA). PPP3CC is the gamma isoform of the CNA located at the chromosome 8p21.3 region. In prior work, PPP3CC, among the other calcineurin subunits of CNB, CNA-alpha, and CNA-beta, showed the only significant associations with schizophrenia for the population of Caucasian and South Africa in either single locus and haplotype analyses. To evaluate if PPP3CC is a candidate gene for schizophrenia in the population of Taiwan, ten SNP markers across the gene were genotyped by the method of MALDI-TOF in 218 schizophrenia families with at least two affected siblings. One SNP (rs2272080) located around the exon 1 untranslated region of PPP3CC was nominally associated with schizophrenia (p=0.024). This result suggests the PPP3CC gene may be a candidate gene for schizophrenia in the population of Taiwan.

Keywords: Calcineurin, PPP3CC, Calcineurin A gamma, 8p21.3, SNP

1. Introduction

Calcineurin (protein phosphatase 2B) is a calcium/calmodulin-dependent serine/threonine protein phosphatase acting as a calcium-dependent modulator of phosphorylation status for a variety of cellular activities (Gooch et al., 2004; Groth et al., 2003). Calcineurin is a heterodimer which consists of a regulatory subunit, known as calcineurin B (CNB), and a catalytic subunit, known as calcineurin A (CNA) (Guerini, 1997). There are at least three isoforms cloned for the catalytic subunit of CNA; CNA-alpha, CNA-beta (Guerini et al., 1989), and CNA-gamma (also named PPP3CC) (Muramatsu et al., 1992). All these CNA isoforms have been found differentially expressed in different areas of the brain (Gerber et al., 2003; Yokoyama et al., 1994).

The functional role of these CNA subunits in the brain is not clear. Calcineurin may regulate the dopaminergic receptor signal transduction pathway (Greengard, 2001) and synaptic efficiency through NMDA receptors (Groth et al., 2003). There are multiple behavior alterations mimicking the symptoms of schizophrenia in conditional CNB knock out mice (Miyakawa et al., 2003). When the knock out only occurred in the forebrain, working/episodic-like memory was impaired (Zeng et al., 2001).

The PPP3CC subunit of CNA located on chromosome 8p21.3 has been reported to be associated with schizophrenia in American samples (Gerber et al., 2003). However, this result was not confirmed in other ethnic groups (Kinoshita et al., 2005). To further assess the association between PPP3CC and schizophrenia, we genotyped ten single nucleotide polymorphisms across the gene in 218 schizophrenia families with at least two affected siblings from the population of Taiwan.

2. Methods and materials

2.1 Subjects

This research project was approved by the Institutional Review Board of National Taiwan University Hospital. All genomic DNA samples were collected from the family subjects with at least two affected sibling after obtaining written informed consent. The subjects were recruited from two research programs; the multidimensional psychopathology study of schizophrenia (MPSS) (Hwu et al., 2002) from 1993 to 2001 and the Taiwan schizophrenia linkage study (TSLS) (Hwu et al., 2005) from 1998 to 2002. The 86 families of MPSS subjects were interviewed by the research psychiatrists using the Psychiatrist Diagnostic Assessment (PDA) (Hwu, 1999). The 132 TSLS families were interviewed by well-trained assistants using the Mandarin Chinese version of the Diagnostic Interview for Genetic Studies (DIGS) (Chen, 1999). For both studies, the final diagnostic assessment was formulated by integrating either the PDA or the DIGS data with clinical information from medical

records using the Specialist Diagnostic Assessment Sheet (SDAS), based upon the criteria of the Diagnostic and Statistical Manual of Mental Disorders, 4th edition (DSM-IV). This study sample included two hundred and eighteen schizophrenic nuclear families with at least two affected siblings, and had a total of 864 subjects participated in this genotyping study.

2.2 SNP genotyping

All SNP markers were genotyped by the method of matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS). A DNA fragment (100-300 bp) encompassing the SNP site was amplified using the polymerase chain reaction (PCR) GeneAmp 9700 thermocycler (Applied Biosystems, USA) according to the manufacturer's instruction. After PCR amplification and neutralization of the deoxynucleotide triphosphate (dNTP), the primer extension was performed by adding the probe, Thermo Sequenase (Amersham Pharmacia, Piscataway, NJ, USA) and appropriate dideoxynucleotide triphosphate (ddNTP)/dNTP mixture. Different extension products were differentiated by mass through MALDI-TOF.

2.3 Statistical analysis

We used the procedure ALLELE in SAS/GENETICS release 8.2 (SAS Institute, 2002) to assess Hardy-Weinberg equilibrium. Family relationships were verified by PEDCHECK version 1.1 (O'Connell et al., 1998) and UNKNOWN version 5.23 (Terwilliger and Ott, 1994) to detect deviations from Mendelian inheritance. We used Haploview software to construct haplotype blocks constituted by "strong LD" markers according to the criteria proposed by Gabriel et al (Gabriel et al., 2002). Both single point and haplotype association analyses were carried out using TRANSMIT version 2.5.4 (Clayton, 1999).

3. Results

There were ten SNPs markers selected to cover the region running from the 3'-untranslated region of SLC39A14 across the PPP3CC genetic region up to the promoter region of SCAM-1 (Table 1). All SNPs were validated and had minor allele frequencies larger than 10% and a genotyping missing rate smaller than 5%. All the SNP markers were in Hardy-Weinberg equilibrium. Haplotype blocks with two-SNP and seven-SNP markers were generated by Haploview (Gabriel et al., 2002), which created a block when 95% of informative (i.e. non-inconclusive) comparisons had strong linkage disequilibrium and spanned no more than 30kb (Fig. 1).

In single SNP association analyses using the TRANSMIT program version 2.5.4

(Clayton, 1999), there was significant association of schizophrenia with one SNP marker (rs2272080), located in the exon 1 of PPP3CC (Table 1).

The haplotype analyses for the two-SNP (rs7833266- rs2272080) haplotypes showed no significant associations with schizophrenia. The seven-SNP (rs2461491- rs2469758- rs2461489- rs2469770- rs2449340- rs1482337- rs2252471) haplotype A-C-G-G-G-A-C, which had a very low frequency of 0.0063 showed significant association with schizophrenia (Chi =4.754, df =1, p=0.0292). The other haplotypes in this seven-SNP block showed no significant associations with schizophrenia.

4. Discussion

The chromosome 8p21-22 region has been suggested by genomewide scans to be linked to schizophrenia (Blouin et al., 1998; Gurling et al., 2001). In our previous linkage study, we found that the D8S1222 marker located at 8p21 showed strong evidence of linkage to schizophrenia (NPL score of 2.45, p-value =0.008) (Liu et al., 2005). The NPL score around 2.0 of flanking region, which covers the markers D8S1820 and D8S1810, is located more close to the 8p21.1-8p12. PPP3CC is at 8p21.3 about 5 cM away from D8S1820 and the linkage NPL score is around one, without significant linkage with schizophrenia.

The SNP markers should be selected across the whole gene in order to obtain an accurate estimation of disease association locus, which might vary among different ethnic groups. Failure to genotype the whole gene could obtain negative results in a study (Kinoshita et al., 2005). In this study, we have selected 10 SNP markers covering a wide-enough genomic regions ranged from SLC39A14 gene close to the promoter region of PPP3CC to the promoter region of next SCAM-1 gene, with an average SNP marker distance of about 12.9 kb. The selected ten SNP markers were not exactly the same as the SNP markers reported by Gerber et al (Gerber et al., 2003). They screened five SNPs of PPP3CC covering the genomic regions from exon 1 (rs1049437) to intron 4 (rs2461491) and found intron 1 SNP (hCV1341817 or rs10108011) had significant association with schizophrenia. This intron 1 SNP was close to our significant SNP at a distance of 21.6 kb.

When correlating the genomic region to the role of PPP3CC with schizophrenia, the only one SNP marker (rs2272080) having significant association with schizophrenia was in the promoter region close to exon 1 of an untranslated region of PPP3CC. This result suggests that a genetic expression alteration may have occurred in the schizophrenic patients. This speculation could be supported by the evidence of a significant decrease of PPP3CC expression in the hippocampus of schizophrenia (Eastwood et al., 2005).

In haplotype association analyses, the A-C-G-G-G-A-C seven-SNP haplotype,

ranging from the intron 4 of rs2461491 to the SCAM-1 promoter region of rs2252471 was the only haplotype that showed a nominally significant association with schizophrenia (p=0.0292).

In summary, PPP3CC may be a candidate gene of schizophrenia in the population of Taiwan. The significant association region is located at the exon 1 untranslated SNP rs2272080. However, our result might be a false positive. We tested many SNPs and haplotypes and, although we had two nominally significant findings, these would not be significant after correction for multiple testing.

Acknowledgements

We acknowledge the SNP genotyping works done by the National Genotyping Center (NGC) and the help by the Department of Medical Research in NTUH. This study was supported by the grants from the NRPGM, National Science Council, Taiwan (NSC91-2314-B-002-216; NSC91-3112-B-002-011; NSC 92-3112-B-002-019; NSC93-3112-B-002-012), National Health Research Institute, Taiwan (NHRI-EX-91,92,93-9113PP), and National Institute of Health, U.S.A. (IR01 MH 59624-01).

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Table 1. Frequencies of the single nucleotide polymorphisms of the PPP3CC gene and its association with schizophrenia

SNP_ID	Primer ID	Chromosome	Genetic Region	Allele Type	MF	HW Test	Schizophrenia		
						P	N	Chi	P
rs7833266	9117	chr8:22344940	SLC39A14 (3'-UTR)	G/A	0.488	0.8831	214	0.002	0.964
rs2272080	6577	chr8:22354671	PPP3CC (exon 1)	T/G	0.1685	0.2981	210	5.133	**0.024***
rs2469745	9130	chr8:22399409	PPP3CC (intron 3)	T/C	0.3997	0.9518	214	0.017	0.896
rs2461491	6555	chr8:22417197	PPP3CC (intron 4)	G/A	0.4137	0.8003	214	0.949	0.330
rs2469758	6560	chr8:22418977	PPP3CC (intron 4)	T/C	0.4103	0.8876	214	0.356	0.551
rs2461489	6569	chr8:22426198	PPP3CC (intron 5)	A/G	0.4667	0.8164	214	0.052	0.819
rs2469770	6561	chr8:22435801	PPP3CC (intron 6)	A/G	0.4164	0.989	214	0.254	0.615
rs2449340	6562	chr8:22445845	PPP3CC (intron 11)	G/T	0.3131	0.3123	213	0.015	0.904
rs1482337	6559	chr8:22451454	PPP3CC (intron 12)	A/G	0.4629	0.9826	209	0.186	0.667
rs2252471	6566	chr8:22461173	SCAM-1 (Promoter)	C/G	0.4199	0.6151	214	0.156	0.693

MF: Minor allele frequency

HW: Hardy-Weinberg's test

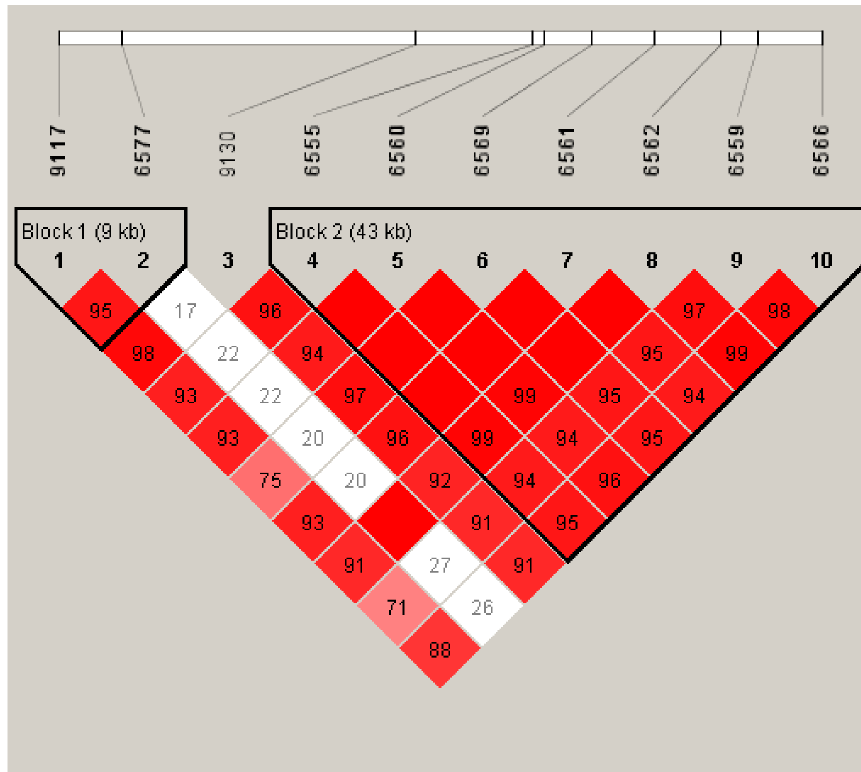
P: p-value

N: Number of families

Chi: Chi-square test

Figure legend:

Fig. 1. Haploview linkage disequilibrium displays two haplotype blocks within the ten SNP markers (labeled with their primer ID) of PPP3CC.



Appendix 9

Word count of text: 3927 (excluding abstract, references, and 3 tables)

Neuregulin 1 Gene and Variations in Perceptual Aberration of Schizotypal Personality in Adolescents

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Running title: NRG1 and schizotypal personality in adolescents

ABSTRACT

Background. We test the hypothesis that the *neuregulin 1* (*NRG1*) gene at chromosome 8p22-p12, which has been implicated as a susceptibility gene to schizophrenia, is associated with variations in schizotypal personality in non-clinical populations.

Methods. A randomly selected sample of 905 adolescents were assessed for their personality features using the Perceptual Aberration Scale (PAS) and the Schizotypal Personality Questionnaire (SPQ) and genotyped for three single nucleotide polymorphisms (SNP8NRG221533, rs3924999, and rs2954041) at the *NRG1* gene. Relations between the three genetic variants and continuous schizotypal personality scores were evaluated using ANOVA for single-locus analyses and haplotype trend regression test for multi-locus analyses.

Results. Single locus analysis showed that the A allele of rs3924999, a functional polymorphism in exon 2, had the largest effect size and exhibited a prominent allele-dose trend effect for the PAS score. Haplotype analyses using the haplotype trend regression test indicated that the A allele of rs3924999 was mainly responsible for the association with the PAS but not with the SPQ or its three factors, and the magnitude of significance was not strengthened by the combination of this allele with adjacent locus.

Conclusions. Our study provides the first evidence for the association of the *NRG1* with schizotypal personality and indicates a possible role of *NRG1* in the genetic etiology of schizophrenia through perceptual aberrations.

INTRODUCTION

The concept of schizotypal personality was first postulated as a preclinical genetic expression for schizophrenia (Rado, 1953; Meehl, 1962). Family, adoption, and twin studies in both categorical (Kendler et al., 1993; Torgersen et al., 1993; Kety et al., 1994) and dimensional (Lenzenweger & Loranger, 1989; Kendler & Hewitt, 1992; Chapman *et al.*, 1994) approaches have indicated that schizotypal personality is genetically related to schizophrenia and may reflect some aspects of the genetic predisposition to schizophrenia. The heritability for schizotypal personality was found to be substantial (Lyons et al., 1995). Factor-analytic studies have revealed that schizotypal personality may consist of three factors, i.e., positive (psychotic-like cognitive and perceptual experiences), negative (anhedonia and interpersonal dysfunction), and disorganization (Vollema & van den Bosch, 1995; Venables & Rector, 2000), each with differential associations with biobehavioral traits (Chen et al., 1997; Barrantes-Vidal et al., 2003).

Taken together, schizotypal personality has been proposed as an endophenotype for schizophrenia and may be closer to the expressions of schizophrenia genes (Gottesman & Gould, 2003). Assessing the relation of the candidate genes for schizophrenia to schizotypal personality may help shed light on the etiological pathway for the genes. Recent studies for identifying susceptibility genes for schizophrenia seemed to have accumulated more replicable findings with biologically plausible mechanism in pathogenesis being postulated (Owen et al., 2004). One of these findings is the newly discovered gene *Neuregulin 1* (*NRG1*), which involves regulation of N-methyl-D-aspartate (NMDA) receptors and has role in neurodevelopment and synaptic plasticity, on chromosome 8p22-p12 in a study in Iceland (Stefansson et al., 2002). An appealing feature for the association of *NRG1* with schizophrenia is its replications in a variety of populations, including independent studies in Scotland (Stefansson et al., 2003), England (Williams et al., 2003), China (Yang et al., 2003; Zhao et al., 2004), and Ireland (Corvin et al., 2004), though not in a high density family study of schizophrenia in Ireland (Thiselton et al., 2004). However, the first three studies (Stefansson et al., 2002; Stefansson et al., 2003; Williams et al., 2003) did not find a clear pathogenic mutation and all the markers of a 7-marker haplotype were within the 5′-promoter to the first intron. Yang et al. (2003) extended the covering range of single nucleotide polymorphisms (SNPs) by randomly selected two more SNPs, rs3924999 in the second exon (a functional polymorphism with Arg 38 Gln) and rs2954041 in the fifth intron, in addition to the most significant SNP (SNP8NRG221533) found by Stefansson et al. (2002). All the three SNPs showed linkage disequilibrium (LD) with schizophrenia, and so did the haplotypes of the three SNPs (Yang et al., 2003).

So far there has been no study examining the role of *NRG1* gene in schizotypal personality. Our objective was to investigate associations between polymorphisms in *NRG1* and variations in schizotypal personality features in adolescents randomly selected from the school population. Our rationale was twofold. First, because features of schizotypal personality diminish with age (Chen et al., 1997), young people might be better than older ones to obtain consistent schizotypal personality scores. Second, adolescents with schizotypal personality usually have not yet been diagnosed with a personality disorder or influenced by diagnosis-related intervention or social stigma than their adult counterparts. Hence, adolescents with schizotypal personality might be relatively conducive to identifying the effect of *NRG1* gene without being confounded by age or therapeutic interventions if it indeed exists.

METHODS

Subjects

The subjects in this study were 905 (459 girls and 446 boys) junior high school students in Taipei City selected through a multiple-stage sampling procedure. Because

junior high school education is compulsory basic education in Taiwan, this student body represents an unselective population, especially for those in public schools. The sampling of the participants has been described in detail elsewhere (Lin et al., 2000). Briefly, we stratified the 71 public junior high schools in Taipei City in 1996 by educational levels of the residents into the three groups, and randomly selected one school from each group. Then, three classes were randomly selected from each grade (1 to 3) in each of the three participating schools. In total, there were 971 students from 27 classes eligible for this study. After informed written consent was obtained from the students and their parents, 905 (93%) junior high school students completed the self-report questionnaire and 878 (90%) did a mouth rinse with 4% sucrose to offer buccal cells. The study was approved by the institutional review board of the College of Public Health, National Taiwan University.

Measurement instruments

The participants were asked to complete a questionnaire inquiring demographic features and schizotypal personality features, including the Perceptual Aberration Scale (PAS) (Chapman et al., 1978), Schizotypal Personality Questionnaire (SPQ) (Raine, 1991), and Junior Eysenck Personality Questionnaire (JEPQ) (Eysenck, 1975). The PAS contains 35 true-false items inquiring about the experience of body-image distortions, while the SPQ contains 74 true-false items that were grouped into nine subscales corresponding to all 9 criteria of the schizotypal personality disorder in the DSM-III-R. The two parts were intermingled to minimize the potential offensiveness of some questions regarding aberrations in bodily perception, as suggested by Chapman et al (1978). The reliability and validity of both scales in the Taiwanese population have been reported in detail elsewhere (Chen et al., 1997; Chen et al., 1998). Briefly, both the SPQ and PAS were translated from English to Chinese by means of two-stage translation. The 1-week test-retest reliability coefficient (0.86 for the SPQ and 0.80 for the PAS) and the internal consistency alpha (0.95 for the SPQ and 0.84 for the PAS) in a sample of 30 subjects were satisfactory. Furthermore, a combination of the PAS and SPQ has been found to have good validity in screening for schizophrenia-related personality disorders in the community adults (Chen et al. in press).

In the JEPQ, which contains 81 “yes-no” self-report items, there is an L scale consisting of 20 items that was presumed to measure the “desirability response.” The establishment and psychometric properties of the Chinese version of the JEPQ was reported previously (Kuo et al., 2004). For this study, we used the score on L as a control for potential unreliable responders.

Genomic DNA preparation

Buccal mucosa cells were collected from each participant by mouthwashing for at least 10 sec with a 10 ml solution of 4% sucrose (Lench et al., 1988; Hayney et al., 1996). The procedure of obtaining DNA from the mouthwashing sample has been reported elsewhere (Chen et al., 1999). Briefly, by a series of centrifuging and discarding the supernatant, the sediment was stored at -70°C until used for DNA extraction. The genomic DNA was extracted by following the standard protocol of a commercial kit, QIAamp Mini kit (Qiagen, Chatsworth, CA, USA).

SNP Genotyping

Genotyping of the three SNPs of NRG1 selected for this study, SNP8NRG221355, rs3924999, and rs2954041, was conducted by using the method of matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF MS). The specific PCR primers and extension primers were designed by using SpectroDesigner software (Sequenom, San Diego) according to the sequences available in the GeneBank

(http://www.ncbi.nlm.nih.gov/GeneBank, AF491780). Genomic DNA of 2.5 ng was used as template and a DNA fragment (100-300 bp) encompassing the SNP site was amplified by the polymerase chain reaction (PCR) according to the manufacturer's instruction. After PCR amplification, the MassEXTEND reaction was performed by incubating the termination mixtures, i.e., dideoxynucleotide triphosphate (ddNTP) and deoxynunleotide triphosphate (dNTP). A MassEXTEND probe which sat just next to the SNP site, Thermosequenase (Amersham Pharmacia Biotech, Buckinghamshire, UK), and appropriate ddNTP/dNTP mixture were added to the above reaction mixture. Allele-specific extended products were obtained by the GeneAmp 9700 thermocycler (ABI, U.S.A.) under the conditions of 55 cycles of denaturing at 94°C for 5 sec, annealing at 52°C for 5 sec, and extension at 72°C for 5 sec.

After desalting the reaction product with SpectroCLEAN, approximately 15 nl were spotted onto the SpectroCHIP using the SpectroPOINT (Sequenom, San Diego). These primer extension products were then analyzed by the MALDI-TOF MS in the fully automated mode. After acquired spectra were transferred to the MassARRAY system (Sequenom, San Diego), genotype calling was performed by applying the SpectroTYPER software (Sequenom, San Diego) and a set of digital filters were optimized automatically for mass spectra of DNA.

Statistical Analysis

A confirmatory factor analysis of the SPQ was conducted using the Proc CALIS of the software package SAS (version 8.2 for Windows). We adopted the three-factor model of Raine et al. (1994) as the following: (a) Cognitive-Perceptual Dysfunction (ideas of reference, magical thinking, unusual perceptual experience and paranoid ideation); (b) Interpersonal Dysfunction (social anxiety, no close friends, constricted affect, and paranoid ideation); and (c) Disorganization (odd behavior and odd speech). Following two previous studies on the confirmatory factor analysis of the SPQ (Raine et al., 1994; Chen et al., 1997), the fitness of the model was evaluated by three indices: the goodness of fit, the adjusted goodness of fit, and the normed fit index. Values of goodness of fit (Cole, 1987) and normed fit index (Bentler and Bonett, 1980) greater than 0.9 and an adjusted goodness of fit value greater than 0.8 (Cole, 1987) indicate a good fit.

Because the distribution of the PAS and the SPQ scores were skewed, these personality scores were first normalized using the PROC RANK and then standardized using the PROC STANDARD of the SAS. The transformation reduced non-normality and standardized scores to unity variance and zero mean. The relations between genotypes in each SNP marker and the standardized normal scores of schizotypal personality were assessed using ANOVA (one-way or two-way, depending on whether to adjust for sex effect) or ANCOVA (to adjust for age). For those with a significant effect, effect sizes were calculated: a value of 0.2 was considered a small effect and that of 0.5 a medium effect (Cohen, 1987). Possible dose-response effect of an allele was further evaluated by regressing the schizotypal scores on the number the allele in each genotype with sex and age as covariates. In each marker, three comparisons between the individual factors of the SPQ were dealt with Bonferroni correction by controlling for the testing of multiple phenotypes.

We assessed the Hardy-Weinberg equilibrium by the χ^2 test for each marker. Haplotype frequencies were estimated using the EM algorithm and whether there was linkage disequilibrium (LD) was examined using the χ^2 test or exact test based on 10000 permutations, if some haplotype frequency was rare. Potential associations between the set of haplotypes and schizotypal personality features were examined via haplotype trend regression with permutation-based hypothesis testing procedures as described by Zaykin et al. (2002), which yields an *F*-test for the significance and a mean trait value for each haplotype in relation to the trait under test. Following the suggestion in a recent review

(Wall & Pritchard, 2003), two important pairwise measures of LD, |D′| and r^2, were then calculated. High LD was defined as a r^2 of >1/3 as suggested by Ardlie et al. (2002) or a |D′| of > 0.7 as suggested by Gabriel et al. (2002). All these genetic analyses were performed by using the computer program PowerMarker V3.09 (Liu & Muse, 2004). To examine whether there was population stratification in our sample, which might lead to a false positive results, a Z statistics using a panel of SNP (Lee, 2003) was calculated.

RESULTS

The confirmatory factor analysis revealed that the three-factor model of the SPQ had a good fit to the data as indicated by the high values of relevant indices, i.e., 0.92 for the goodness of fit index, 0.85 for the adjusted goodness of fit index, and 0.89 for the normed fit index. There were moderate but significant correlations between the PAS and the SPQ (r=0.57), Cognitive-Perceptual Dysfunction (r=0.55), Interpersonal Dysfunction (r=0.37), and Disorganization (r=0.53). The mean age of the participating students was 14.0 (SD=0.9), ranging from 12.0 to 16.9 years. Mean scores for these schizotypal personality features were 5.4 (SD=4.4), 23.8 (SD=11.9), 11.5 (SD=6.2), 10.4 (SD=6.4) and 4.9 (SD=3.5) for the PAS, SPQ, Cognitive-Perceptual Dysfunction, Interpersonal Dysfunction, and Disorganization, respectively. There were no differences between boys and girls except that girls had a higher mean SPQ total score ($F_{1, 902}$ = 8.62; p = 0.0034) and Cognitive-Perceptual Dysfunction ($p \leq 0.05$ for the t-test) than boys. Besides, the SPQ total score increased with age ($F_{1, 902}$ = 3.83; p = 0.05).

To examine the potential impact of unreliable responders on the results, we conducted a separate analysis by deleting those individuals with an extreme score on the L scale of the JEPQ. The mean score of L scale in the sample was 8.8 (SD=3.6) for boys and 9.6 (SD=3.7) for girls; hence 11 boys and 19 girls were deleted because of a score > 2 SDs. However, the distribution of the PAS, SPQ, Cognitive-Perceptual Dysfunction, Interpersonal Dysfunction, and Disorganization remained almost unchanged for each sex, and the pattern and magnitude of their associations with the three SNPs of NRG1 were similar to those of the total sample. Thus, only the results for the total sample were presented for the subsequent analyses.

The number of successful genotyping for each of the three SNP markers among the 878 students' DNA and the corresponding genotype counts and allele frequencies were as follows: 867 for SNP8NRG221533 (Counts: CC=254, CT=455, TT=158; frequencies: C=0.56, T=0.44), 870 for rs3924999 (Counts: AA=538, AG=295, GG=37; frequencies: A=0.79, G=0.21), and 871 for rs2954041 (Counts: GG=298, GT=419, TT=154; frequencies: G=0.58, T=0.42). All the distributions of the three markers did not deviate from the Hardy-Weinberg Equilibrium. In the ANOVA testing for the personality score difference among the three genotypes for each marker, only the marker on the second exon, rs3924999, revealed a significant association with the PAS and the SPQ total scores (Table 1). All those three comparisons between separate factors of the SPQ in each marker were dealt with Bonferroni correction. In terms of the effect size, the genotype AA of rs3924999 had a greatest effect on the PAS as compared with that of the genotype GG, and that of the genotype AG in between. In contrast, the effect size of rs3924999 genotypes on the SPQ total scores was less linear, with the effect of heterozygous being the lowest. In the testing for trend, only the genotypes of rs3924999 had a significant trend effect on both the PAS and the SPQ total scores.

In terms of the LD between the three SNPs, the pairwise |D′| between marker 2 (rs3924999) and marker 3 (rs2954041) was moderately high, that between marker 1 (SNP8NRG221533) and marker 2 modest, and almost not existing between marker 1 and marker 3 (Table 2). Despite the significant LDs between adjacent markers, all the r^2s and the differences between estimated haplotype frequency and expected haplotype frequency

were minimal.

In examining the relationship between individual haplotypes and each of the schizotypal personality features, the software program PowerMarker 3.0 did not allow for the adjustment of other covariates. Thus, in addition to the standardized normal scores for the personality scales, z scores adjusted for correlated demographic features were also used for analysis. The adjusted z score was calculated using the method as described in Chen et al. (1998) for the SPQ total score (adjusted for age and sex) and the Cognitive-Perceptual Dysfunction score (adjusted for sex). Because the results of both unadjusted z scores and adjusted z scores were very similar, only the results of unadjusted scores are presented in Table 2. According to haplotype trend regression, only the PAS scores were related to the haplotypes consisting of SNP8NRG221533 and rs3924999 ($p = 0.01$) as well as the haplotypes of rs3924999 and rs2954041 ($p = 0.03$). All the other schizotypal traits were not associated with the two-locus haplotypes.

For the three-locus haplotype analysis, LD was significant, with the four haplotypes that contain the allele A of rs3924999 having a frequency of > 20% and the remaining four a frequency of < 10% (Table 3). Comparing the mean scores of each schizotypal trait for the haplotypes, only the PAS scores were associated with the haplotypes, in which the haplotypes with the A allele of rs3924999 had higher PAS scores than those without. Similar results were obtained when adjusted z scores were used for the analysis and hence were not shown in the table.

In terms of the detection for hidden population stratification, the Z statistics on the basis of the three SNPs was only 1.00, which did not reach statistical significance ($p = 0.159$, one-sided).

DISCUSSION

Among the three SNPs examined in this study, it was the A allele of rs3924999 that had the largest effect size and exhibited a prominent allele-dose trend effect on both the PAS and the SPQ total scores. However, in terms of the trend effect of A allele, the pattern for the PAS was more consistent (the mean score for heterozygote AG was in-between of the two homozygotes) than that for the SPQ total (heterozygote AG had the lowest mean score). Furthermore, the two-locus haplotypes that contained rs3924999, either SNP8NRG221533-rs3924999 or rs3924999-rs2954041, were significantly associated with scores on the PAS. In contrast, there was no such association between the haplotypes and the SPQ total scores. In the three-locus haplotype analysis, it turned out that only the PAS was associated with the SNPs. The inconsistency in the association between the rs3924999 and the SPQ total score was further indicated by the lack of any association between the SNP and the three factors of the SPQ. Thus, it appears that the rs3924999 is consistently associated with the schizotypal personality features measured by the PAS but not by the SPQ or its three factors.

Both the PAS and the Cognitive-Perceptual Dysfunction of the SPQ are thought to be representative of so-called 'positive schizotypy.' However, the correlation between the PAS and the Cognitive-Perceptual Dysfunction scores in this study was not high, which is consistent with a previous study (Chen et al., 1997). Furthermore, subscales constituting the Cognitive-Perceptual Dysfunction were not limited to the unusual perceptual experience. Based on the three-factor model (Raine et al., 1994), some other parts, such as ideas of reference, magical thinking, and paranoid ideation, were also included. Thus, the PAS and the Cognitive-Perceptual Dysfunction are likely to represent two different schizotypal personality traits. It might then lead to their differential relations to the genetic variation in *NRG1* gene. As indicated by the non-differential correlations between the PAS and the three-factors of the SPQ, the finding also calls into question whether perceptual aberration should be pooled with other aspects of positive schizotypy to form a single factor. Further research in other samples is warranted to clarify this issue.

Overall, our results seem to indicate that the association of *NRG1* with the PAS was mainly due to the effect of rs3924999, whereas the effects of the other two SNPs were negligible. If all the three SNPs are not the true susceptibility locus for schizotypal personality but are merely nearby the true one, then a multipoint haplotype formed by these SNPs would likely lead to a more significant association with the schizotypal personality than the individual SNP because of inclusion of all pairwise and higher order disequilibria terms (Zaykin et al., 2002). However, the multi-locus haplotype analyses in this study did not lead to an increase in the significance of the associations as compared with that of the single-locus analysis. This is further supported by the findings of low values of r^2 between the adjacent SNPs.

The SNP rs3924999 is in the 12 position within the 2nd exon of *NRG1* gene (Yang et al., 2003). Its G to A base change causes amino acid transfer from arginine to glutamine, although the change is conservative in terms of the isoforms of NRG1. Whether the functional polymorphism of rs3924999 in protein level is pathogenic requires further study. Several lines of research have provided circumstantial evidence for the involvement of *NRG1* in psychopathology. First, NRG1 affects expression and function of several central nervous system (CNS) neurotransmitter receptors. The *NRG1*-knock-out mice were found to have deficiency in glutamatergic innervation and hyperactivity in behavioral tests, which was partially reversed with clozapine (Stefansson et al., 2002). The pattern was similar to the defects in glutamatergic neurotransmission caused by N-methyl-D-aspartate receptor hypofunction, which was associated with psychosis in schizophrenic patients and positive schizotypy in normal individuals (Krystal et al., 1999). Second, NRG1 regulates radial glial cells, whose abnormality may lead to wrong placement and impaired connectivity of neurons (Schmid et al., 2003). The deficiency in glial growth factors caused by NRG1 may lead to synaptic destabilization, which has been hypothesized to be involved in the etiology of schizophrenia (Moises et al., 2002). Moreover, other NRG1 signaling in the CNS suppresses the induction of long-term potentiation, a form of synaptic plasticity (Huang et al., 2000). Intriguingly, higher scores on items pooled from different scales pertaining to perceptual unreality in college students were found to be related to extreme puberty timing (Gruzelier & Kaiser, 1996), which was postulated to be related to abnormal synaptic plasticity. Taken together, our finding of the association between the perceptual aberration aspect of schizotypy and *NRG1* gene is compatible with the possible biological function of the *NRG1* gene.

Some other features of this study are worthy of note. First, we adopted a quantitative approach for the analysis of schizotypal personality features. This may provide more statistical power than a categorical approach relying on an arbitrarily defined cut-off point. Second, our sample was free from population stratification bias since the detection for the population stratification turned out to be not significant. Nevertheless, it should also be kept in mind that the allele associated with an increased PAS score is very common (a frequency of 0.79) and the effect size is modest at best (0.1 to 0.27). Given the lack of proved functional meaning of the polymorphism, the association might not be necessarily etiological.

Some limitations in this study should be kept in mind in interpreting our results. First, because the age range of this study was small, we do not know whether the association between the SNP marker rs3924999 and the scores on the PAS will stay the same in adulthood when both the PAS and the SPQ scores decreased with age (Chen et al., 1997). Future investigation in adults is warranted. Second, our sample was limited to non-clinical population and whether the finding extends to people with more extreme schizotypy needs replications.

In conclusion, among the three SNPs of *NRG1* examined in this study, the SNP marker on exon 2, rs3924999, was consistently associated with the scores on the PAS but not with the SPQ in a randomly selected sample of adolescents. Single locus as well as

multi-locus haplotype analyses indicate that the A allele of rs3924999 was mainly responsible for the association with the PAS, and the magnitude of significance was not strengthened by the combination of this allele with adjacent locus. Possible biological function of the *NRG1* gene is compatible with the association of the rs3924999 with the perceptual aberrations measured by the PAS. We therefore conclude that *NRG1* may be a candidate gene for variations in perceptual aberration of schizotypal personality and may have a role in the genetic etiology of schizophrenia through perceptual aberrations.

Acknowledgements

This study was supported by grants from the National Science Council, Taiwan (NSC86-2314-B002-328, NSC87-2314-B002-290, NSC 91-2314-B-002-216; NSC91-3112-B-002-011), National Health Research Institute, Taiwan (NHRI-EX-92-9113PP). The authors thank the assistance of the Core Laboratory of Population Genetic Polymorphisms at the NTU Center for Genomic Medicine for DNA extraction, Mr. SY Yang for his supervision in laboratory procedures, the National Genotyping Center at Academia Sinica for SNP genotyping, and the principals and teachers of the participating schools for their help in recruiting students.

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Table 1. The distributions of the PAS and SPQ scores by the genotype of each of the three SNPs of *NRG1*

Genotype	N	PAS		SPQ		Cognitive-perceptual dysfunction	Interpersonal dysfunction	Disorganization
		Mean (SD)	Effect size	Mean (SD)	Effect size	Mean (SD)	Mean (SD)	Mean (SD)
SNP8NRG221533								
CC	250	5.2 (4.0)		24.9 (12.2)		12.0 (6.0)	11.0 (6.6)	5.0 (3.5)
CT	453	5.3 (4.3)		23.2 (11.8)		11.1 (6.3)	10.3 (6.3)	4.8 (3.5)
TT	157	5.5 (5.0)		23.1 (11.7)		11.5 (6.2)	9.7 (6.1)	4.9 (3.5)
Trend test[a]		P = 0.84		P = 0.10[b]		P = 0.30[c]	P = 0.07	P = 0.64
Rs3924999								
AA	533	5.6 (4.5)†	0.27	24.5 (12.3)‡	0.13	11.7 (6.4)	10.8 (6.5)	5.1 (3.6)
AG	293	4.9 (3.9)†	0.10	22.4 (11.4)‡	-0.07	11.1 (6.0)	9.8 (6.2)	4.4 (3.2)
GG	37	4.4 (4.4)†		23.1 (10.3)‡		10.7 (5.6)	10.2 (5.9)	5.0 (3.6)
Trend test[a]		P = 0.004		P = 0.03[b]		P = 0.11[c]	P = 0.08	P = 0.05
Rs2954041								
GG	295	5.4 (4.3)		23.4 (11.6)		11.3 (6.1)	10.1 (6.1)	4.9 (3.5)
GT	417	5.3 (4.1)		24.1 (12.0)		11.5 (6.2)	10.9 (6.6)	4.9 (3.5)
TT	153	5.6 (5.1)		23.8 (12.3)		11.8 (6.4)	9.9 (6.3)	5.1 (3.5)
Trend test[a]		P = 0.92		P = 0.52[b]		P = 0.43[c]	P = 0.96	P = 0.53

[a]based on the standardized normal scores of each personality scale

[b]further adjusted for age and sex

[c]further adjusted for sex

†p = 0.01 for the one-way ANOVA ($F_{2,\ 867;\ 0.05} = 4.30$)

‡p = 0.05 for the ANCOVA with sex and age adjustment ($F_{2,\ 858;\ 0.05} = 3.01$)

Table 2. Estimated haplotype frequency and mean schizotypal scores in each of two-locus haplotypes

Two-locus Haplotype		Haplotype Frequency	Mean score				
			PAS	SPQ	Factor 1	Factor 2	Factor 3
Marker 1	Marker 2	(n=857)					
C	A	0.45	5.5	24.5	11.7	10.8	5.1
T	A	0.34	5.6	23.4	11.4	10.1	4.9
T	G	0.11	5.1	22.7	11.2	9.8	4.7
C	G	0.10	4.6	22.6	10.9	10.0	4.4
|D′|		0.1292					
r^2		0.0056					
χ^2 (p-value)[a]		9.6 (0.002)					
HTR[b]			P = 0.01	P = 0.07	P = 0.20	P = 0.11	P = 0.14
Marker 2	Marker 3	(n=858)					
A	G	0.41	5.5	24.1	11.6	10.6	5.0
A	T	0.38	5.5	24.0	11.6	10.4	5.0
G	T	0.04	4.8	23.1	11.3	10.3	4.5
G	G	0.17	4.9	22.6	11.0	9.8	4.6
|D′|		0.5611					
r^2		0.0608					
χ^2 (p-value)[a]		104.4 (<.001)					
HTR[b]			P = 0.03	P = 0.29	P = 0.40	P = 0.32	P = 0.16
Marker 1	Marker 3	(n=855)					
C	T	0.23	5.2	24.6	11.9	10.7	5.1
T	G	0.26	5.6	23.4	11.4	10.0	4.9
C	G	0.32	5.2	23.8	11.4	10.6	4.8
T	T	0.19	5.3	23.1	11.2	10.1	4.8
|D′|		0.0037					
r^2		0					
χ^2 (p-value)[a]		0.02 (0.89)					
HTR[b]			P = 0.13	P = 0.19	P = 0.19	P = 0.42	P = 0.31

Note. Marker1=SNP8NRG221533; Marker2=rs3924999; Marker3=rs2954941; Factor 1=perceptual-cognitive dysfunction; Factor 2=Interpersonal dysfunction; Factor 3=Disorganization

[a] Chi-square test statistic for the null hypothesis that the pairwise LDs are zero, df =1.

[b] Haplotype trend regression test for the standardized normal scores

Table 3. Estimated haplotype frequency and mean schizotypal scores in each of three-locus haplotypes (n = 852)

Haplotype[a]	Haplotype Frequency		Mean Score				
	Estimated frequency	Expected frequency	PAS	SPQ	Factor 1	Factor 2	Factor 3
C-G-T	0.02	0.05	5.1	23.6	11.4	10.7	4.5
C-G-G	0.09	0.07	4.4	22.4	10.8	9.9	4.4
T-A-T	0.16	0.15	5.3	23.2	11.2	10.1	4.8
T-A-G	0.17	0.20	5.7	23.7	11.5	10.2	5.0
T-G-T	0.02	0.04	4.6	22.7	11.2	10.1	4.4
T-G-G	0.09	0.05	5.2	22.8	11.2	9.7	4.7
C-A-T	0.22	0.19	5.5	24.6	11.9	10.7	5.1
C-A-G	0.24	0.26	5.4	24.4	11.6	11.0	5.0
Exact p value[b]	$p < 0.001$						
HTR[c]			$P = 0.02$	$P = 0.30$	$P = 0.45$	$P = 0.41$	$P = 0.48$

Note. Factor 1=perceptual-cognitive dysfunction; Factor 2=Interpersonal dysfunction; Factor 3=Disorganization

[a] Allele of haplotype presented in order of SNP8NRG221533, rs3924999, and rs2954941

[b] Testing for multi-locus association by the exact test based on 10000 permutations (p-value cutoff = 0.05)

[c] Haplotype trend regression test for the standardized normal scores