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ORIGINAL ARTICLE

More evidence supports the association of PPP3CC with schizophrenia

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Calcineurin is a calcium/calmodulin-dependent protein phosphatase composed of two subunits, a regulatory subunit of calcineurin B (CNB) and a catalytic subunit of calcineurin A (CNA). PPP3CC is the γ isoform of CNA located at the chromosome 8p21.3 region. To evaluate the association between PPP3CC and schizophrenia in the Taiwanese population, 10 single nucleotide polymorphism (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 was nominally associated with schizophrenia ($P = 0.024$) and significantly associated with the expression of PPP3CC in lymphoblast cell line; the TT and TG genotype had significantly higher relative expression levels than the GG genotype ($P = 0.0012$ and 0.015, respectively). In further endophenotype stratification, the single locus of rs2272080 and the haplotypes of both two-SNP haplotype (rs7833266–rs2272080) and seven-SNP haplotype (rs2461491–rs2469758–rs2461489–rs2469770–rs2449340–rs1482337–rs2252471) showed significant associations with the subgroup of schizophrenia with deficits of the sustained attention as tested by the continuous performance test (CPT, $P < 0.05$) and the executive functioning as tested by the Wisconsin Card Sorting Test (WCST, $P < 0.05$). The results suggest that PPP3CC gene may be a true susceptibility gene for schizophrenia.
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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.[1,2] This protein is a heterodimer which consists of a regulatory subunit, known as calcineurin B (CNB), and a catalytic subunit, known as calcineurin A (CNA).[3] At least three isoforms have been cloned for the catalytic subunit of CNA; CNA-α, CNA-β[4] and CNA-γ (also named PPP3CC).[5] Differential expressions of these three CNA isoforms have been demonstrated in various areas of the brain.[6,7]

The functional role of these CNA subunits in the brain is not clear; however, calcineurin may regulate the dopaminergic receptor signal transduction pathway[8] as well as synaptic efficiency through the NMDA receptors.[1] There are multiple behavior alterations mimicking the symptoms of schizophrenia in conditional CNB knockout mice.[9] When the knockout only occurred in the forebrain, working/episodic-like memory was impaired.[10]

The PPP3CC subunit of CNA located on chromosome 8p21.3 has been reported to be associated with schizophrenia in American samples.[7] However, this result was not confirmed in other ethnic groups.[11] To

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assess further the association between PPP3CC and schizophrenia, we genotyped 10 single-nucleotide polymorphisms (SNPs) across the gene in 218 Taiwanese schizophrenia families with at least two affected siblings. We also measured the expression levels of the gene and correlated it with the genotype of the SNP marker significantly associated with schizophrenia. The expression levels of the gene were then determined in Epstein–Barr virus (EBV)-transformed lymphoblasts from another 60 patients.

Using endophenotypes to refine the phenotype characterization of schizophrenia has been advocated,[12] we therefore tested two potential endophenotypes: sustained attention and executive dysfunction. Both endophenotypes have substantial empirical evidence to support them as endophenotypic markers for schizophrenia. Sustained attention deficits as measured on the continuous performance test (CPT)[13] 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.[14–19] The normalized *z* score for *d′*, which is the sensitivity measure of sustained attention assessed by CPT, has been frequently used as a schizophrenia endophenotype. When patients with schizophrenia having a *z* score below −2.5 were assigned to have deficit in sustained attention, the recurrence risk ratio for schizophrenia among parents or siblings in the subgroup of schizophrenia with this CPT endophenotype was higher than that in the whole group of schizophrenia probands.[20,21]

Executive functions as measured by the Wisconsin Card Sorting Test (WCST)[22] are known to be impaired in schizophrenic patients[23,24] and their first-degree relatives.[25] The WCST is a classic neuropsychological procedure used to evaluate cognitive-based executive function of the frontal lobe dysfunction. Among schizophrenic patients, impaired executive functioning has been related to hypofrontality.[26] Using the performance on the CPT and the WCST to define endophenotypes for schizophrenia might be helpful for addressing the heterogeneity issue of schizophrenia in association analysis.

Materials and methods

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 siblings after written informed consents had been obtained. The subjects were recruited from two research programs: the Multidimensional Psychopathology Study of Schizophrenia (MPSS)[27] from 1993 to 2001 and the Taiwan Schizophrenia Linkage Study (TSLS)[28,29] from 1998 to 2002. The 86 families of the MPSS subjects were interviewed by the research psychiatrists using the Psychiatrist Diagnostic Assessment (PDA).[30] The 132 TSLS families were interviewed by well-trained assistants using the Mandarin Chinese version of the Diagnostic Interview for Genetic Studies (DIGS).[31] 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 218 schizophrenic nuclear families with at least two affected siblings, and had a total of 864 subjects participated in this genotyping study.

SNP genotyping

The SNP markers were selected across the whole gene to cover the complete linkage disequilibrium (LD) structure of the gene and its regulatory regions and to obtain an accurate estimate of disease association locus, which might vary among different ethnic groups. Failure to genotype the whole gene could result in a missed associated region and false negative results.[11] For this study, we selected two SNP markers (CC1a rs1049437 and CCS3 rs2461491) reported previously[7] with identification number released in NCBI and seven other SNPs across PPP3CC.

All SNP genotypings were performed by the method of matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS).[32] Primers and probes flanking the SNPs were designed using SpectroDESIGNER software (Sequenom, San Diego, CA, USA). A DNA fragment (100–300 bp) encompassing the SNP site was amplified using polymerase chain reaction (PCR) (GeneAmp 9700 thermocycler, Applied Biosystems, Branchburg, NJ, USA) according to the manufacturer's instructions.

After removing the un-incorporated deoxynucleotide triphosphate (dNTP) and inactivating the shrimp alkaline phosphatase (SAP) from the PCR product, primer extension was performed by adding the probe, Thermo Sequenase (Amersham Pharmacia, Piscataway, NJ, USA) and appropriate dideoxynucleotide triphosphate (ddNTP)/dNTP mixture, followed by 55 cycles of denaturing at 94°C for 5 s, annealing at 52°C for 5 s, and extension at 72°C for 5 s. The various extension products were differentiated by mass through MALDI-TOF. This genotyping method has been applied in a broad variety of clinical applications, as it fulfills criteria such as accuracy of SNP detection, sensitivity to score SNPs using a small amount of template throughput capacity, flexibility of the procedure and cost-effectiveness.[33]

Real-time reverse transcription polymerase chain reaction for the expression of PPP3CC gene

Lymphocytes from another 60 subjects (20 controls, 20 CPT non-deficit and 20 CPT deficit schizophrenia) were transformed by EBV and used to assess PPP3CC expression. The lymphocytes were harvested from the whole blood of these subjects and layered onto a Histopaque-1077 Hybrimax gradient (Sigma-Aldrich,

St Louis, MO, USA) and resuspended in culture medium containing EBV and the mitogen phytohemagglutinin (PHA; Sigma-Aldrich). The cultures were monitored for signs of transformation (increased cell growth, aggregation or clumping), generally apparent within 3–8 weeks in successful cultures. The successful lymphoblast transformations were washed once with 1× ice cold phosphate-buffered saline before total RNA extraction. RNA-Bee (Tel-Test, Friendswood, TX, USA) was used according to the manufacturer's guidelines to extract total RNA from the cultured EBV-transformed lymphoblasts.

To analyze the expression of PPP3CC, real-time RT-PCR was performed for PPP3CC and a housekeeping gene, TATA-box binding protein (TBP), using predesigned gene-specific TaqMan probes and primer sets (Hs00194467 m1 for PPP3CC and Hs00427620_m1 for TBP) purchased from Applied Biosystems. Real-time RT-PCR amplification was conducted using Taqman One-Step RT-PCR Master Mix Reagent (Applied Biosystems) on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems), according to the manufacturer's instructions. Gene expression was quantified relative to TBP expression using Sequence Detector Software (Applied Biosystems) and the relative quantification method. The relative expression level of PPP3CC compared with that of TBP was defined as $-\Delta CT = -[CT_{PPP3CC} - CT_{TBP}]$. The PPP3CC mRNA/TBP mRNA ratio was calculated from $2^{-\Delta CT} \times K$, in which K is a constant.

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.[34] Briefly, numbers from 0 to 9 were randomly presented for 50 m 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 min 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 min 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 non-target trials).[35] Sensitivity is an individual's ability to discriminate target stimuli from non-target stimuli. In a 1-week test-retest reliability study[34] 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.

In this study, the z score of d' of CPT was used as the endophenotype indicator of schizophrenia: If one of the affected siblings in the family has CPT deficit, then the family is classified as a CPT deficit family. If all of the affected siblings in the family are CPT non-deficit, then the family is classified as CPT non-deficit. According to the above subgrouping principle, there were CPT deficit, defined by z score of d' value < -2.5 in 454 (96 families) subjects for undegraded group and 487 subjects (103 families) for degraded group. The CPT non-deficit, defined by z score of d' value $\geqslant -2.5$, included 345 (75 families) subjects for undegraded CPT group and 287 (62 families) subjects for degraded CPT group.

WCST. We employed a computerized version of the WCST[36] that had been used in a previous study of a Taiwanese population.[37] 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 four number keys (1–4) 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 are completed, the testing in this study continued until all 128 cards had been sorted. All of the indexes defined in the WCST manual,[38] except for Total Correct, were used for analysis. The Total Correct index was not included as it is complementary to Total Errors. In this study, the indexes of WCST for association analyses were (1) Perseverative Errors (PE): the number of errors that were perseverative, reflecting the tendency towards perseveration; and (2) categories achieved (CAT): the number of times that 10 consecutive correct responses were made, reflecting overall success.These two indicators were found to be impaired in schizophrenic probands[23,24] and in the first-degree relatives of schizophrenic probands.[25] The PE and CAT were used as the endophenotype indicators of WCST in this study. Based on the familial distributions of the z scores of PE and CAT indicators, schizophrenia patients with a z score of PE $\geqslant 1$ were assigned as having deficit in PE of WCST, and those with a z score of CAT < -2.5 were assigned as having deficit in CAT of WCST. Families where one of the affected siblings had a WCST deficit were classified in the WCST deficit group. Families where all affected siblings in the family were WCST non-deficit were classified as WCST non-deficit. The WCST deficit group was comprised of 453 subjects in 97 families when defined by z score of PE $\geqslant 1$, and 433 subject from 95 families when defined by z score of CAT < -2.5. The WCST non-deficit included subjects from 69 families when defined by z score of PE < 1, and 337 subjects from 70 families when defined by z score of CAT $\geqslant -2.5$.

Statistical analysis

Hardy–Weinberg equilibrium was assessed by using the ALLELE procedure in SAS/GENETICS release 8.2.[39] Family relationships were verified by PEDCHECK version 1.1[40] and UNKNOWN version 5.23[41] 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*.[42] Both single point and haplotype association analyses were carried out using TRANSMIT version 2.5.4[43] for parent to affected offspring association analyses and FBAT version 1.4.1[44–46] for affected offspring association analyses.

Multiple tests were considered to be necessary. However, the SNP markers used in this study were high density and the application of Bonferroni's procedure might yield too conservative results. In this study, we apply simulation study, using Merlin software[47] for simulating the pedigree for 1000 times assuming no linkage/no association on the interested SNP marker identified in TRANSMIT and FBAT programs. The empirical *P*-value was calculated as a false-positive rate in which on a given SNP, by use of TRANSMIT and FBAT over 1000 times, percentage of a *P*-value lower than its nominal *P*-value was counted. The final empirical *P*-values less than a nominal *P*-value ($P = 0.05$) could interpret that these identified interested SNP markers might not arise by chance.

The relative expression levels of PPP3CC between normal controls and schizophrenia were compared using a *t*-test for independent groups. The differences among genotypes were tested first by analysis of variance (ANOVA) and post-hoc comparisons between groups by *t*-test.

Results

A total of 10 SNP markers were genotyped to encompass the adjoining regions, 3′-untranslated SLC39A14, PPP3CC genetic and the SCAM-1 promoter (Table 1). All SNPs were validated and had minor allele frequencies above 10% and a missing genotyping rate below 5%. All the SNP markers were in Hardy–Weinberg equilibrium. Haplotype blocks with two-SNP and seven-SNP markers were constructed by Haploview,[42] which created a block when 95% of the informative (i.e., non-inconclusive) comparisons had strong linkage disequilibrium and spanned not more than 30 kb (Figure 1).

Table 1 shows the result of single locus association analyses using the TRANSMIT program version 2.5.4.[43] A significant association was demonstrated between schizophrenia and an SNP marker (rs2272080; primer ID 6577), located in the exon 1 of PPP3CC.

The haplotype analyses (data not shown) for the two-SNP (rs7833266–rs2272080) haplotypes revealed no significant associations with schizophrenia. The seven-SNP (rs2461491–rs2469758–rs2461489–rs2469770–rs2449340–rs1482337–rs2252471) haplotype A-C-G-G-G-A-C (2-2-2-2-1-1-1), which was low frequency (0.0063) showed significant association with schizophrenia ($\chi^2 = 4.754$, $df = 1$, $P = 0.0292$). The other haplotypes in this seven-SNP block showed no significant associations with schizophrenia.

Table 2 shows that one SNP marker (rs2272080; primer ID 6577) was significantly associated with the subgroup of schizophrenia having deficits in sustained attention (degraded and undegraded CPT *z* score of *d*′ below −2.5) defined by undegraded and degraded CPT ($P = 0.008$ in degraded and $P = 0.026$ in undegraded deficit group) as analyzed by TRANSMIT. Even after correcting for multiple testing, the association remained significant ($P = 0.049$) as assessed by FBAT. For the schizophrenia endophenotypes defined by deficit in CAT (*z* score of CAT < -2.5) or deficit in PE (*z* score of PE $\geqslant 1$) of WCST, significant associations were demonstrated between the same SNP marker and the WCST deficit subgroup with both TRANSMIT ($P = 0.029$ for CAT deficit and $P = 0.015$

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

SNP_ID	*Primer ID*	*Chromosome*	*Genetic region*	*Allele type*	*MF*	*HW test*	*Schizophrenia*		
						P	N	χ	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

Abbreviations: Chi, Chi-square test; HW, Hardy-Weinberg's test; MF, minor allele frequency; N, number of families; P, *P*-value.
The significance of bold means *P*-value < 0.05.

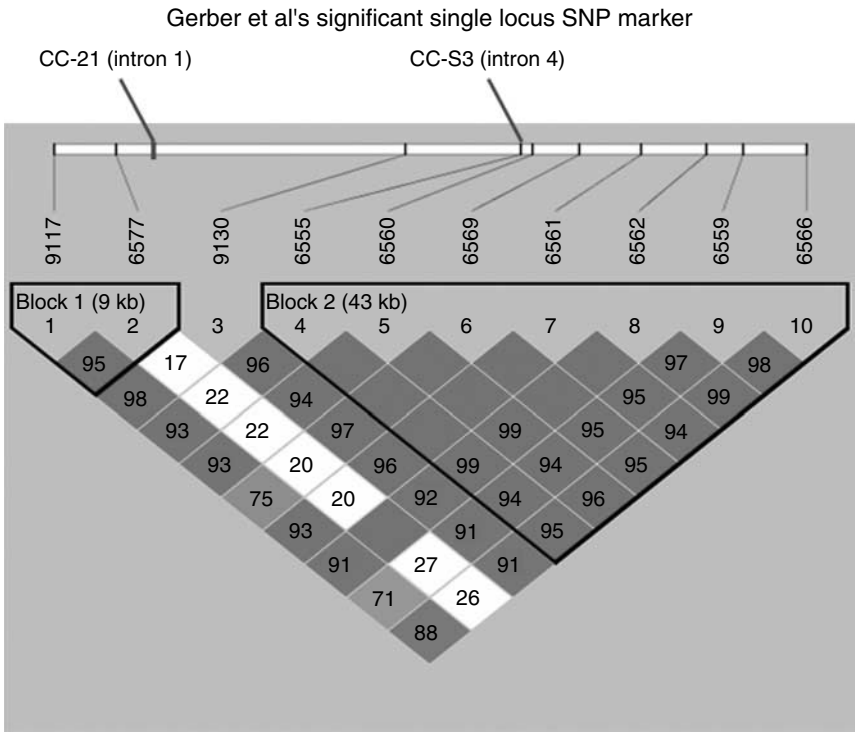

Figure 1 Haploview linkage disequilibrium displays two haplotype blocks within the 10 PPP3CC SNP markers (numbered by primer ID according to the physical length of each SNP). The number in each square is $D' \times 100$ between two SNPs, and the color indicates the significance of $D' > 0$. For squares without numbers, the pairwise D' is 1. The black or gray indicates log-of-the-odds (LOD) $\geqslant 2$ and white indicates LOD < 2. The significant SNPs of Gerber *et al*.'s study were compared with the SNPs of this study.

Table 2 Association analysis of PPP3CC single locus and the subgroup of schizophrenia defined by neuropsychological assessment of CPT and WCST by TRANSMIT and FBAT programs

CPT/WCST	*SNP_ID*	*TRANSMIT*			*FBAT*		
		N	*Chi*	P	N	*Z*	P
CPT Degradeda d' *z* score < -2.5	rs2272080	101	7.069	**0.008 (0.005)**[b]	28	2.203	**0.028 (0.022)**[b]
CPT Undegraded[c] d' *z* score < -2.5	rs2272080	92	4.988	**0.026 (0.034)**[b]	17	1.610	0.107
WCST CAT[d] *z* score < -2.5	rs2272080	93	4.753	**0.029 (0.04)**[b]	26	1.976	**0.048 (0.048)**[b]
WCST PER[e] *z* score $\geqslant 1$	rs2272080	94	5.913	**0.015 (0.002)**[b]	26	2.229	**0.026 (0.025)**[b]
	rs2449340	95	0.511	0.475	26	−1.992	**0.046 (0.044)**[b]

Abbreviations: Chi = Chi-square test; CPT, continuous performance test; N, number of families; P, *P*-value; WCST, wisconsin card sorting test.
[a]The subgroup of schizophrenia defined by *z* score of $d' < -2.5$ for deficit in sustained attention assessed by degraded CPT.
[b]*P*-value of simulation test representing a false-positive rate.
[c]The subgroup of schizophrenia defined by *z* score of $d' < -2.5$ for deficit in sustained attention assessed by undegraded CPT.
[d]The subgroup of schizophrenia defined by *z* score of categories achieved < -2.5 for deficit in CAT of WCST.
[e]The subgroup of schizophrenia defined by *z* score of perseverative error γ 1 for deficit in PE of WCST.
The significance of bold means *P*-value < 0.05.

for PE deficit) and FBAT programs ($P = 0.048$ in CAT deficit and $P = 0.026$ in PE deficit).

Table 3 shows the association results for the two-SNP and seven-SNP haplotypes of the PPP3CC gene. The two-SNP haplotype was significantly associated with the subgroups of schizophrenia defined by the sustained attention deficit endophenotype ($P = 0.027$ for degraded CPT and $P = 0.048$ for undegraded CPT, respectively). For the schizophrenia endophenotype defined by the WCST, the two-SNP haplotype showed a significant association ($P = 0.019$) with the PE deficit subgroup of schizophrenia when analyzed by FBAT. By contrast, significance was demonstrated for the seven-SNP haplotype ($P = 0.008$–0.041) and the schizophrenia CAT non-deficit subgroup using TRANSMIT.

Table 3 Results of association analyses for PPP3CC haplotype and subgroup of schizophrenia defined by neuropsychological assessment of CPT and WCST by TRANSMIT and FBAT programs

CPT/WCST	*SNP-bloc*	*Haplotype*	*TRANSMIT*			*FBAT*			
			HF	χ	P	*HF*	N	Z	P
CPT Degraded d' z score < -2.5	2-SNP	2.2	0.169	4.923	**0.027**−	0.166	26.5	−1.910	0.056
	7-SNP	2.2.2.2.2.1.2	0.014	4.170	**0.041**−	0.014	2.0	NA	NA
CPT Undegraded d' z score < -2.5	2-SNP	2.2	0.150	3.910	**0.048**−	0.148	17.9	−1.495	0.135
	7-SNP	2.2.2.2.2.1.2	0.014	3.734	0.053−	0.015	2.0	NA	NA
WCST CAT z score $\geqslant -2.5$	7-SNP	1.1.1.1.1.1.1	0.543	6.925	**0.008**+	0.577	14.0	1.416	0.157
	7-SNP	2.2.2.2.2.2.2	0.290	4.194	**0.041**−	0.228	14.0	−1.329	0.184
WCST PER z score $\geqslant 1$	2-SNP	2.1	0.298	2.037	0.154	0.302	30.0	2.353	**0.019**+

Abbreviations: Chi = Chi-square test; CPT, continuous performance test; N, number of families; NA, not analyzed; P, *P*-value; WCST, wisconsin card sorting test.
+: Risk effect; −: protective effect.
The definitions of CPT and WCST are the same as in Table 2.
The significance of bold means *P*-value < 0.05.

The relative expression levels of PPP3CC for normal controls and schizophrenic patients were 0.032 and 0.026, respectively. No significant differences were demonstrated comparing the controls, CPT-deficit and CPT non-deficit groups of 60 EBV-transformed lymphoblasts. Analyzing the relative expression differences among the genotypes of SNP marker rs2272080 (primer ID 6577), a significant difference was found between the genotypes of either TT or TG and GG ($P = 0.0012$ and 0.015, respectively; Figure 2).

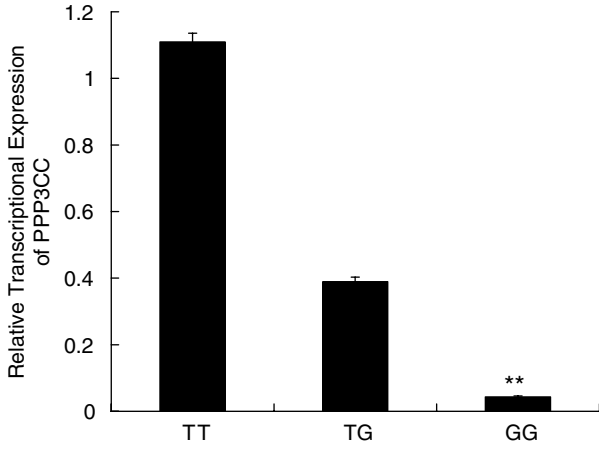

Figure 2 Differential PPP3CC expression for various genotypes of SNP 6577 (rs2272080). The transcriptional PPP3CC expression is measured by real-time RT-PCR in the EBV-transformed lymphoblasts of 60 subjects. The relative PPP3CC expressions for subjects with TT ($n = 36$) and TG ($n = 16$) genotypes are significantly higher comparing to those GG genotype ($n = 3$) ($P = 0.0012$ and 0.015, respectively). Data are presented as mean ± s.d. The '**' represent significant differences between the genotypes of TT or TG and the genotype of GG.

Discussion

In this study, we found an SNP (rs2272080; primer ID 6577) located at the 5′-untranslated region (5′-UTR) of exon 1 of the PPP3CC gene to be significantly associated with schizophrenia ($P = 0.024$). When the schizophrenic patients were further stratified according to the neuropsychological functions of sustained attention and executive function, even stronger associations were demonstrated between this significant SNP and the sustained attention deficit ($P = 0.008$) and executive function deficit schizophrenia subgroups ($P = 0.015$). When measuring the relative PPP3CC expression levels, the genotype of the same SNP marker was significantly associated with the level of the gene's expression in the EBV-transformed lymphoblasts.

Further, haplotype analysis failed to reveal a significant association for both the two-SNP or seven-SNP haplotype and schizophrenia; however, significant associations were demonstrated between these haplotypes and the schizophrenia subgroups defined by neuropsychological functioning. This result suggests that the power of statistical association analyses can be improved by the use of endophenotype subgrouping when searching for vulnerability genes for schizophrenia.

The results of a number of genomewide scans suggested a linkage between chromosome 8p21–22 region and schizophrenia.[48,49] Additionally, our previous linkage study yield evidence of a linkage between the D8S1222 marker located at 8p21 and schizophrenia (NPL score 2.45; $P = 0.008$).[50] The PPP3CC gene is not located in 8p21 region with the highest NPL score region but at 8p21.3 about 5 cM away from a marker located around regions with an NPL score of 2.0.

Comparing our SNP markers with the significant SNPs reported by Gerber's *et al.*,[7] we had a wider range (from the promoter region to intron 12) of coverage in PPP3CC gene than did Gerber's SNPs (from exon 1 to intron 4). Distal to exon 1, the SNP of rs2272080 (primer ID 6577) showed statistical

significant association with schizophrenia; two additional SNPs (9117 and 9130) were further genotyped on all subjects. The SNP 9117 is 9.7 kb proximal to the significant SNP 6577 and the SNP 9130 is 44.7 kb distal to SNP 6577, both SNPs did not reach the statistical significant level in our study. Gerber *et al.* demonstrated significant SNP CC21 (hCV1341817), which is 21.6 kb distal to SNP 6577. Consider the distance between both significant SNPs, the association region for PPP3CC gene may be very narrow. The significant level for either CC21 or SNP 6577 is not high, and we consider that Bonferroni's correction for multiple test is too conservative to identify both loci.[51] In order to avoid the possibility of false negative results, we, therefore, applied an alternative empirical *P*-value derived from simulation method for the adjustment of multiple testing. Under this operation, we found that the association between the SNP marker (rs2272080; primer ID 6577) and the attention deficit subgroup of schizophrenia was still significant.

The 10 SNP markers used in this study covered a wide-enough genomic regions, ranging from the SLC39A14 gene close to the PPP3CC promoter region to the promoter region of the next SCAM-1 gene, with an average SNP marker distance of about 12.9 kb. One SNP, CCS3 (equal to primer ID 6555), was also used by Gerber *et al.*[7] Significant associations had been demonstrated between SNP CCS3 and schizophrenia in the latter investigation ($P = 0.041$) but not in the present study ($P = 0.33$). Of the five PPP3CC SNPs screened by Gerber *et al.*,[7] which covered the genomic regions from exon 1 (rs1049437) to intron 4 (rs2461491), their most significant SNP locus is at the intron 1 of CC21 ($P = 0.038$) close to our significant SNP 6577. The significant SNP variant in our study is different from the Gerber's findings. A possible explanation could be due to the different ethnic origins of genomic structure. The available data from International HapMap Project website (http://www.hapmap.org/index.html.en) including African (YRI), European (CEU) and Chinese (CHB) were retrieved for analyzing the LD structure around exon 1 of PPP3CC. This region was about 40 kb and the data contained these two SNPs of both CC21 and SNP 6577 in CEU and CHB, respectively. The LD structure (data not shown) was not exactly the same, and did differ mildly between the CHB and CEU samples, and the LD score was higher in the CEU and CHB than that in the YRI samples.

Besides, both SNPs are common variants, and this phenomenon may also be due to various factors, such as the population history, expansions of population size, founder effect, admixture between populations and patterns (demographic and social factors). In addition, the degree of contribution of these two SNPs may also vary according to common disease/common-variant hypothesis.

Because the SNP CC21 and the SNP 6577 are close proximity, the risk allele in PPP3CC might be near the 5′-UTR of exon 1 (SNP 6577) and intron 1 (CC21 region) close to the promoter region. As the 5′-UTR may play a role in modulation of the stability of PPP3CC mRNA, this speculation is supported by the expression differences between the SNP 6577 genotypes and is consistent with reports of a significant reduction of PPP3CC expression in the hippocampus of schizophrenic patients.[52]

In haplotype association analyses, the A-C-G-G-G-A-C seven-SNP haplotype, ranging from intron 4 (rs2461491) to the SCAM-1 promoter region (rs2252471), was the only haplotype that showed a nominally significant association with schizophrenia ($P = 0.0292$). This result differs from that of Gerber *et al.*, with these investigators demonstrating a significant association between schizophrenia and two-, three- and four-SNP haplotypes covering the region from intron1 (CC20) to intron 4 (CCS3). In the present study, however, we were unable to identify similar haplotypes in the region bounded by exon 1 (SNP 6577) and intron 4 (SNP 6555 or SNP 6560). This may be due to the heterogeneous spectrum of schizophrenia, further stratification of the schizophrenia according to the assumed endophenotypes defined by deficits in sustained attention and executive function as determined by CPT and WCST, respectively, produced significant association with schizophrenia subgroups for the two-SNP haplotype and the seven-SNP haplotype.

After subgrouping the schizophrenia subjects according to neuropsychological subgroups, we found that there were even higher significant associations with schizophrenia in both the single locus and the haplotypes analyses of PPP3CC. However, differences in relative expression levels of PPP3CC were not demonstrated comparing the normal controls and schizophrenic patients, or among the normal controls, and the neuropsychological groups. This may be due to the small sample sizes in this study, which may have resulted in the failure to identify modest PPP3CC expression difference in this complex disease. However, we did find that the schizophrenia-associated SNP 6577 (rs2272080) was associated with the relative expression levels of PPP3CC. Cell lines having the TT risk genotype had higher levels of PPP3CC expression. This provides further support for the idea that PPP3CC is a susceptibility gene for schizophrenia.

In summary, PPP3CC may be a susceptibility gene for schizophrenia. The significant association region may be near SNP rs2272080; it was associated with schizophrenia and its genotypes were related to the expression levels of PPP3CC. Further stratification of schizophrenic patients into the groups with deficit in sustained attention and deficit in executive function group; PPP3CC showed even greater significant association with schizophrenia in both the single locus and haplotype analyses.

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