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血清素傳遞系統基因與躁鬱症病因之功能性研究

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中文摘要

雙極性情緒障礙（又稱躁鬱症）是一種常見的多基因遺傳疾病，患者具有狂躁與憂鬱兩者相互循環的情緒周期。先前利用雙胞胎、家族史、和領養的研究顯示這種疾病具有強烈的遺傳性。但對於躁鬱症的致病基因及其在病源學上的影響仍所知有限。而大腦皮質上主要的神經傳導物質之一，血清素，已知對情緒狀況的調控有很大影響。本實驗室先前已利用遺傳學中的候選基因的分子遺傳分析法，來研究神經傳導物質血清素系統上的各基因與雙極性情感疾病 - 躁鬱症之間的關聯性。發現在合成酵素基因、血清素運轉基因以及血清素受體基因上都發現顯著的相關性存在。因此證明血清素在躁鬱症的病理成因中的確扮演了相當重要的角色。在本篇報告中我們已接續先前的遺傳分析工作，完成血清素代謝基因與躁鬱症之間的關連性。並開始進行功能性分析法來研究血清素傳遞系統基因與躁鬱症致病機轉之間的關係。另外針對目前治療躁鬱症最有效的藥劑—鋰鹽，在細胞體內對血清素傳遞系統基因及其訊息傳遞的影響，本報告中也開始進行相關的釐清工作。這些研究之目的是希望利用現代分子生物技術來瞭解血清素系統基因的生物功能進一步了解血清素系統與情感性疾病的關係，以期此研究能對躁鬱症的致病機轉和其治療有所貢獻。

關鍵字：躁鬱症，血清素，鋰鹽
Abstract

Bipolar affective disorder (BPD), also known as manic-depressive illness, is a severe mood disorder characterized by manic and depressive episodes. Serotonin (5-hydroxytryptamine; 5-HT) is a key neurotransmitter in the central nervous system, and dysfunction of the serotonergic system has been implicated in several psychiatric diseases. Little is known about the underlying causes of this common and severe illness which was estimated a lifetime prevalence of 0.5% to 1% in various populations. Among manic-depressive patients, if untreated approximate 20% risk of suicide was observed. The high prevalence, together with frequently occurrence of hospitalization, psychosocial impairment, substance abuse and suicide, has made bipolar illness a major public health concern. Although previous studies strongly suggested the involvement of genetic factors in BPD etiology, searching for predisposing genes using classical linkage analysis has been fraught with difficulty. Genes in serotonin transmission and metabolic pathway thus are good candidates for the involvement of BPD pathogenesis. Our previous studies using population-based association analysis also strongly suggested an important role of serotonin system genes in BPD etiology. To extend the previous works on characterizing genetic effect of serotonin system genes, we have performed the association of Monoamine oxidase A gene and BPD. We also started our further functional study on serotonin system genes, and to examine the biological effect of leading therapeutic drug, Lithium, on serotonin production and neurotransmission. Results of this study will help us to understand the serotonin effect on etiology of BPD in Taiwan, and thus provide knowledge for genetic diagnosis and gene therapy in the future.

Keyword : Bipolar affected disorder, serotonin, Lithium
Background and Specific Aims

Bipolar affected disorder is a chronic, severe mood disorder characterized by episodes of mania and depression, has an estimated lifetime prevalence of 0.1%-1% in various populations, including Taiwanese (Yeh 1994). Little is known about the underlying causes of these common and severe illnesses. Although previous studies strongly suggested the involvement of genetic factors in BPD etiology, the search for predisposing genes using classical linkage analysis has been fraught with difficulty. Current strategy suggests using the association analysis to investigate several genes that might be related to BPD pathogenesis. In particularly, genes that are implicated in the metabolism or kinetics of neurotransmitter and receptors in the central neurons system (CNS) are most likely the candidates for studying the association with BPD.

Serotonin (also known as 5-HT) is a major neurotransmitter in the CNS and involved in various physiological events such as mood control, sleep, thermoregulation, learning and memory, and etc. Disruption of serotonergic function has been implicated in the pathogenesis of many psychiatric disorders including BPD (Brunner, Nelen et al. 1993; Bellivier, Leboyer et al. 1998). In addition, previous report indicates the treatment of some agonist of serotonin receptors (HTRs) can affect neurotransmitter release and cause behavioral change (Tsuji, Takeda et al. 2001). Nevertheless, an altered numbers of 5-HT receptors and components have been observed in the brains of bipolar and suicidal patients (Pandey, Pandey et al. 1995). And higher number of platelet 5-HT2A receptors in suicidal patients, and significant decrease of 5-hydroxyindoleacetic acid (5-HIAA; 5-HT metabolite) in the brain of BPD patients (Young, Warsh et al. 1994; Pandey, Pandey et al. 1995), were also report. These finding indicate that genes involved in serotonin transmission and its metabolic pathway are good candidates for involvement in BPD pathogenesis. During the past few years, we have studied the genetic association of serotonin system genes and BPD in Taiwan. Our studies successfully identified polymorphisms in the serotonin system genes that were significantly associated BPD(Lai, Wu et al. 2002; Lin, Yang et al. 2003; Sun, Tsai et al. 2004; Lai, Wu et al. 2005) thus suggested the serotonin system is indeed involved in the etiology of BPD.

The overall objective of this study is to resolve the in depth cellular and molecular mechanisms responsible for pathological process of BPD and the potential therapeutic effect of lithium. It is part of our serial studies on unraveling the etiology of BPD and is a natural extension of our previous works on characterizing genetic association of serotonin system and BPD. The specific aims of this study are:

1. To study the association of Monoamine oxidase A gene and BPD.
2. To study the effects of sequence polymorphisms of the serotonin receptor genes on receptor function and serotonin transmission.
3. To examine the effect of lithium treatment on serotonin gene expression and serotonin production.
Progress Summary

I. Association study of monoamine oxidase A with BPD

MAOA is a mitochondrial enzyme that involved in degradation of several different biological amines including the serotonin, norepinephrine and dopamine (Hsu YP 1989). Several evidences indicated that MAOA play an important role in etiology of BPD (Brunner HG 1993a; Brunner HG 1993b). And the MAOA inhibitors have been effectively used in clinical trials to treat BPD (Amrein R 1999). There are studies claiming both positive and negative association for these markers with BPD, and therefore not conclusive (Craddock N 1995).

This study was carried out to find the association of MAOA with BPD cases from Taiwan population. A total of 117 BPD patients, 108 age-matched normal controls from the same ethnic group were involved in the study. All the samples were typed for two markers, MAOA-CA dinucleotide polymorphism in exon 2 and 30 bp functional MAOA-VNTR on exon 1 of MAOA gene. The allele frequencies and haplotype analysis of MAOA-VNTR and MAOA-CA in the general population and the control group were determined. Strong linkage disequilibrium was observed between the two markers. In male patients a highly significant association was found between MAOA-CA and MAOA-VNTR haplotype ($\chi^2=349$, $p<0.001$). In female patients and in the overall population, there was no significant association. This study depicts that the haplotype markers in males showed highly significant association for BPD when compared to the matched controls (Davamani, Yung et al. 2005).

II. The effect of sequence polymorphisms of the serotonin receptor genes on receptor function and serotonin transmission

There is increasing evidence that abnormalities in various second messenger systems is involved in the pathophysiology of affected disorder(Gould and Manji 2002; Chang, Li et al. 2003). These signaling networks rely on membrane receptors to communicate information from extracellular environment to the interior of the cell. Most members of the serotonin receptors (HTRs) family are belonged to G-protein coupled receptors except the HTR3, who is a ligand-gated ion channel receptor. The extracellular signals induce changes in gene and protein expression of the cell through binding of HTRs and G-proteins. Our previous study suggested that HTR2C and HTR7 play important roles in predisposition to BPD (Lin, Yang et al. 2003). We hypothesize that SNPs identified in these two receptors will alter the ligand binding affinity or amounts of receptor expression thus leading to altered gene expression patterns and induction of BPD. To illustrate our hypothesis on the effect of sequence polymorphisms on receptor function and serotonin transmission, we want to employ the functional assay of these two polymorphisms.
Receptor binding assay of HTR2C with different alleles: We want to use the receptor binding assay to determine the effects of SNP on serotonin binding capacity. The full length HTR2C CDS have amplified from the brain cDNA of CLONTECH cDNA panels (BD Biosciences, CA, USA) with the flanking primers: HTR2C_mRNAf: 5’-ACG CCA TCC TTC AAA AAC AA-3’ and HTR2C_mRNAr: 5’-CCG TAG GAA AAG ACT GTG CTG-3’. Secondary PCR was performed using a pair of primer that introduced restriction cloning sites PacI and SacI: HTR2C_PacI: 5’-TTA ATT AAT GCC GCC ACC ATG GTG AAC CTG-3’ and HTR2C_SacI: 5’-GAG CTC CTT TCT CAC ACA CTG CTA AT-3’. The PCR product was cloned into pCFB-EGSH vector (Stratagene, CA, USA). The HTR2C isoform with different allele was prepared by the Quickchange site-directed mutagenesis kit (Stratagene, CA, USA). The HTR2C expression vector will transfect into specific cell lines using the Retroviral Inducible Mammalian Expression System followed the manufacture protocols (Stratagene, CA, USA). After transfection, we will perform western blotting to confirm the expression status of HTR2C in target cell line. The HTR2C Ab was purchased from commercial company (Santa Cruz Inc, Santa Cruz, CA, USA) and used for western blot. The optimal condition for western blot analysis is testing (data not shown).

III. The effect of lithium treatment on serotonin gene expression and serotonin production

Lithium remains as one of the most effective drugs for treatment of bipolar affective disorder. Lithium ions (Li) are the drug of choice for BPD. Yet, 20–40% of patients fail to respond to Li. Although several biochemical and cellular effects have been attributed to Li, its therapeutic mechanism of action has not been elucidated. To determine whether the effect of Lithium treatment is mediated through the regulation of brain serotonin production and formulate the regulating mechanism, the following experiments will be executed.

The cell culture and Lithium treatment: Human neuroblastoma cell line SH-SY5Y was purchased from the American Type Culture Collection (ATCC, Manassas, VA). The cell cultures also followed the suggested condition of ATCC. Lithium Chloride was dissolved in the cell-specific medium for treatment. The CellTiter-Glo™ Luminescent Cell Viability Assay (Promega, Madison, Wisconsin, USA) was already used to quantify the changes in the viability of cells cultured under drug treatment (Figure 1). It is recommended that the biological range for lithium treatment is between 0.4-1.2 mM. And the concentration of lithium at 1mM does not affect the viability of cells thus it was applied in followed experiments.
The alteration of serotonin production level under Lithium treatment: We treat the neuroblastoma cell line with Lithium followed the above condition, and measured the serotonin production level among culture medium by serotonin ELISA (IBL, Hamburg, Germany). The culture medium of treated cells were harvested from duplicate wells, and the harvested time course is 0, 2, 6, 12, 24, 48 and 72 hours after drug treatment, and this experiment was performed three times. The measurement results of serotonin ELISA analysis of the Lithium- or un-treated cells, shown in Figure 2, indicates that 5-HT dynamic produced levels of cells treated with Lithium ahead the time than untreated. Further quantitative Real-time PCR analysis was used to verify and obtain mRNA expression data about the drugs effect on serotonin system genes. To prove the altered 5-HT level under Lithium treatments results from the regulation of serotonin system genes.

**Figure 1. Drug toxicity assay.** SH-SY5Y was treated with Lithium at various concentrations. No different in cell survival rates were observed in this test. A concentration of 1mM was determined to be used in the future treatment.

**Figure 2. Serotonin ELISA :** Time course of serotonin production in untreated, and Lithium-treated cells. The bars, from left to right in each group, represent 0, 2, 6, 12, 24, 48, and 72 hr after drugs treatment. Data are mean ± SD.
The alteration of serotonin system genes mRNA level under Lithium treatment: We treat the neuroblastoma cell line with Lithium followed the above condition, and the concentration of mRNA encoding for TPH1, TPH2, serotonin transporter, and MAOA were quantified by real-time RT-PCR. The total RNA of treated cells were isolated with REzol™ C&T reagent (PROtech Technology, Taipei, Taiwan) from duplicate wells, and the harvested time course is 2, 6, 12, 24, 48 and 72 hours after drug treatment. The RNA has processed with the DNA-free™ kit (Ambion, TX, USA) to avoid the DNA contamination. The samples were subjected for reverse transcription by adding 500ng total RNA, 5x First-Strand Buffer, 1µl 0.1M DTT, 40 units of Recombination RNASin® Ribonuclease inhibitor (Promega, Madison, USA), 200 units of Superscript™ III Rnase H reverse transcriptase (Invitrogen, Carlsbad, California.), 150ng oligo(dT)15 and 150ng random hexamer in 20ul cDNA synthesis mixture. Quantitative real-time PCR was performed using the ABI PRISM® 7900HT Sequence Detection System with Taqman system (Applied Biosystems, Forster City, CA, USA). The quantitative real-time PCR specific primers and probes of each gene were purchased from commercial company (Applied Biosystems, Forster City, CA, USA). The optimal condition for real-time quantitative PCR is testing (data not shown).
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