嘉南藥理科技大學專題研究計畫成果報告

先天智能障礙患者之小胖威利症候群（Prader-Willi Syndrome）基因篩檢及相關研究

計畫類別：■個別型計畫　　□整合型計畫

計畫編號：CNEN92-04

執行期間：92 年 1 月 1 日至 92 年 12 月 31 日

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中華民國 93 年 2 月 20 日

先天智能障礙患者之小胖威利症候群基因篩檢及相關研究

Molecular diagnosis of Prader-Willi Syndrome and relative study

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中文摘要

小胖威利症候群是一種罕見的神經行為上的遺傳疾病，症狀包括輕度到中度智能障礙、嬰兒時期肌肉低張力、外陰部發育不良、發育遲緩、身材矮小、手掌及腳掌偏小、嬰兒期後因過於旺盛的食慾導致肥胖並伴有早發性糖尿病的發生。目前已知其發病原因在於第十五號染色體上的小核內核糖核蛋白(*SNRPN*)異常所導致。此基因受基因印記的遺傳機制所調控，因此百分之七十的 PWS 病患起因來自於父方的第十五號染色體缺失，百分之廿五是由於母源單親二體症導致，即兩條十五號染色體皆源自母方。本研究嘗試以甲基聚合酵素連鎖反應作為第一線分子診斷的工具，針對 200 位 PWS 可能患者的檢體進行篩檢，發現其中有 30 位為 PWS 患者，與臨床表徵吻合。結果顯示此方法能準確診斷 PWS 發病成因，若能以螢光原位雜交及小微星標竿多形性基因標記作為母源單親二體症的檢查，更能使 PWS 的判定更加精確。此技術可使得家長能夠快速的得到結果，也能提早對於低張力的狀況做復健，並控制病童飲食避免過胖，同時也使得病童免於因肌肉低張力所進行的穿刺性檢查，在遺傳諮詢上提供有效協助。

關鍵字：小胖威利症候群、甲基聚合酵素連鎖反應

Abstract

Prader-Willi syndrome (PWS) is associated with distinct phenotypes which include mental retardation and caused by loss of function of genes, located in chromosome 15q11-q13, an area subjected to genomic imprinting. Methylation-specific polymerase chain reaction (M-PCR) based on parents of origin specific DNA methylation at the promoter region of small nuclear ribonucleoprotein polypeptide N gene (SNRPN) can provide accurate and rapid diagnosis for nearly all PWS patients. We report on the development of a referral system for molecular diagnosis of PWS based on M-PCR. Pediatric geneticists, psychiatrists or neurologists were asked to evaluate phenotypes of patients with PWS and complete a questionnaire designed according to the consensus criteria to diagnose these conditions. Molecular analysis based on M-PCR was performed for patients with a score of at least two. A total of 200 patients with suspected PWS were referred for diagnostic testing. PWS was diagnosed in 30 of these patients. M-PCR is a cost-effective method for the diagnosis of PWS.

Key words: Prader-Willi syndrome, methylation-specific PCR

Introduction

Prader-Willi syndrome (PWS [MIM 176270]) was first described in the medical literature in 1956 [1]. Subsequently, several cases were reported but it was not until 1981 that diagnostic criteria were proposed [2]. PWS occurs at a frequency of 1 in approximately 15,000 births. The major neonatal characteristics are hypotonia with failure to thrive. Hyperphagia and severe obesity typically develop

during early childhood. Other manifestations include hypogonadism, short stature, small hands and feet, mental retardation with learning disabilities, and obsessive-compulsive disorder [3].

PWS is caused by loss of function of genes, located in chromosome 15q11-q13, an area subjected to genomic imprinting. The most common genetic mechanism in PWS is a large (3-4 Mb) chromosomal deletion that is the same size in the majority of deletions in this syndromes. However, all deletions in PWS are paternal in origin. Another common mechanism that leads to PWS is maternal uniparental disomy (UPD). About 2-8% of PWS patients inherit a copy of chromosome 15 from each parent , but they have abnormal gene expression pattern typical for the syndrome throughout the imprinted 15q11-q13, suggesting that such patients have a mutation in the imprinting process (imprinting mutation) [4,5].

The clinical features of PWS are non-specific in infants and young children and early diagnosis is difficult on clinical grounds [3]. The parental copies of 15q11-q13 differ by DNA replication timing and DNA methylation. Most pairs of this region replicate early on the paternal copy and late on the maternal copy [4,5]. Differential DNA methylation has been detected by many CpG dinucleotides, including the ZNF127 locus , the D16S63 (PW71) locus, and SNRPN CpG island [4,5]. Using the bisulphite protocol of genomic sequencing, Zeschnigk et al performed a detailed methylation study around D15S63 and SNRPN exon 1. They found that all surveyed CpG dinucleotides around SNRPN exon 1 are methylated on the maternal chromosome and unmethylated on the paternal chromosome [6]. Methylation analysis is now widely used for testing patients suspected of having PWS [7,8]. This report describes the development of a referral system for molecular diagnosis of PWS and the results of correlation of clinical data with the findings of molecular analysis in a series of patients with these conditions.

Materials and Methods

Patients and record of phenotypes

Members of the Taiwanese Pediatric Neurology Association and Taiwan Human Genetic Society, and directors of Identification Centers for Mental Deficits sponsored by Department of Health were asked to join the multi-center study protocol. The participating physicians were pediatric neurologists, psychiatrists and geneticists working at 16 different hospitals throughout Taiwan. Of the ten Identification Centers, eight also joined the study. Patients with a score of at least two were referred for molecular analysis. The referring physicians were asked to evaluate phenotypes of patients and complete a questionnaire modified from consensus criteria for PWS (Fig. 1) [2,3,6]. From June 1999 to February 2001, a total of 200 patients (120 males and 80 females) with clinical suspicion of PWS were recruited. The ages of the patients ranged from 1 month to 23 years for the patients suspected of having PWS. All patients had undergone chromosome analyses and none of them were found to have balanced chromosomal rearrangements (including translocations and inversion).

Methylation-specific polymerase chain reaction (M-PCR)

Methylation-specific PCR was performed according to the methods described by Kubota et al [12]. In brief, the DNA was treated with sodium bisulphite, which converts cytosine to uracil except when cytosine is methylated. 5-methylcytosine is resistant to bisulphite and remains unchanged. The CpG islands of small nuclear ribonucleoprotein-

associated polypeptide N (SNRPN) gene contain a potential imprinting center (IC) for a chromosome domain at 15q11-q13. It has been shown that nearly all CpG dinucleotides are methylated on the maternal chromosome, whereas none are methylated on the paternal chromosome. Two sets of primers were designed for the maternal-specific (methylated) and paternal-specific (unmethylated) versions of the CpG island of SNRPN gene. The maternal-specific primer sequences are 5'-TAAATAAGTACGTTTGCGCGGTC-3' (SNRPN-M, forward) and 5'-AACCTTACCCGCTCCATCGCT-3' (SNRPN-M, reverse). The paternal-specific primer sequences are 5'-GTAGGTTGGTGTGTATGTTTAGGT-3' (SNRPN-P, forward) and 5'-ACATCAAACATCTCCAACAACCA-3' (SNRPN-P, reverse). For sodium bisulphite treatment, 1.0 μg of genomic DNA was denatured by sodium hydroxide, and incubated at 55^{o}C overnight with hydroquinone and sodium bisulphite (Sigma), and purified using the Wizard DNA clean-up system (Promega, Madison, WI , USA). Modification was completed by sodium hydroxide treatment, followed by ethanol precipitation. DNA was resuspended in 50 μl of TE. PCR reactions were carried out in a 30 μl volume containing 1 X PCR Buffer II (Perkin-Elmer), 200 μM dNTP, 2.0 mM $MgCl_2$, 1.2 μM SNRPN-M primers and 0.4 μM SNRPN-P primers, 0.5 U of AmpliTaq Gold (Perkin-Elmer) and bisulphite-modified DNA (~30 ng). Thermocycling (OmniGene Thermal Cycler, Hybaid Ltd., Ashford Middlesex, UK) consisted of 10 min at 95°C for initial denaturation, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 62°C for 1 min, extension at 72°C for 1 min, and a final extension at 72°C for 10 min. The reaction products were fractionated on 3% agarose gels, stained with ethidium bromide, and visualized under UV illumination. For each assay, we incorporated three samples as controls: a genomic DNA sample from a normal man, a genomic DNA sample of a PWS patient. The PWS control patients had been diagnosed using Southern blot analysis for methylation status of SNRPN gene promoter region, fluorescence in situ hybridization (FISH) for deletion of SNRPN gene and PCR-based microsatellite analysis for UPD [8,10].

Results

M-PCR

In duplex PCR reaction, normal individuals showed both a 174-bp product from the methylated maternal chromosome and a 100-bp product from the unmethylated paternal chromosome. PWS patients showed only the 174-bp PCR product (Fig. 2). Untreated DNA did not produce a PCR product (data not shown).

PWS

Among the 200 patients with suspected PWS, 30 (15%) had a classical PWS methylation pattern (Fig. 2). These 30 PWS patients included 16 males and 14 females. The mean score for PWS patients was 4.12 and with a standard deviation (SD) of 1.40. The mean score for non-PWS patients was 2.94 with a SD of 1.89. Among the 7 major diagnostic criteria for PWS, three (excessive weight gain, developmental delay, and hyperphagia) were found to be more prevalent in the group of PWS patients of age $\geq$ 1 year than in those of age less than 1 year (by Fisher's exact test) (Table 1).

Discussion

Approximately 70% of PWS patients have 15q11-q13 deletions, 28% have maternal UPD, and 2% have imprinting mutations [4,5]. A variety of

complementary cytogenetic and molecular techniques are used to investigate PWS, including high resolution cytogenetic analysis, fluorescence in situ hybridization (FISH), deletion/UPD analysis by microsatellite typing, and methylation analysis [10]. Two different approaches have been described by the American Society of Human Genetics /American College of Medical Genetics Test and Technology Transfer Committee (ASHG/ACMG) regarding the molecular diagnosis of PWS [10]. Approach I begins with methylation studies. Normal methylation results are particularly valuable for ruling out PWS. If methylation results are positive, FISH and PCR can be used to determine whether deletion, UPD, or imprinting mutation is present. Approach II begins with high- resolution cytogenetic analysis and FISH for deletions. Negative cytogenetic and FISH study are followed by methylation analysis. If methylation analysis is abnormal, and there is no deletion by FISH, UPD studies are performed using PCR. Whatever the mechanism, methylation studies will detect nearly 100% of PWS patients.

Traditionally, methylation tests for PWS and AS are based on Southern blot analysis of DNA cleaved with methylation sensitive enzymes such as *Hpa*II or *Cfo*I for the PW71B (D53S61) locus or *Not*I for the SNRPN locus [7,8]. D15S61 (PW71) cannot be used for prenatal diagnosis because extraembryonic tissues are hypomethylated at this locus [11]. Meanwhile, a polymorphic variant of D15S63 was identified recently [12]. Therefore, methylation analysis at D15S63 locus may lead to misdiagnosis. The tests based on SNRPN locus are reliable, but Southern blot analysis has inherent disadvantages. Rare restriction fragment length variants and partial cleavage may complicate interpretation. Partial cleavage is especially a problem with *Not*I, which is used for SNRPN and can lead to false-positive diagnosis. Methylation-specific PCR (M-PCR) takes advantage of the differentially methylated pattern of the paternal and maternal alleles and analyzes the CpG island of promoter/exon 1 region of the *SNRPN* gene [6]. The major advantage of M-PCR is the rapidity of a PCR-based assay compared with a Southern blot assay. Misdiagnosis can only occur when treatment of bisulphate is incomplete. To eliminate misdiagnosis caused by incomplete treatment with bisulphite, we always incorporated three controls in each experiment: a DNA sample of a normal subject, a DNA sample of a patient with PWS diagnosed by FISH. In this study, M-PCR, a more cost effective technique, achieved the same diagnostic efficiency as the combination of FISH test and microsatellite analysis followed with methylation analysis (approach II recommended by ASHG/ACMG).

While M-PCR is a reliable way for rapid screening of PWS, the pathological mechanisms responsible for development of both disorders could not be detected by M-PCR alone. In this study, some patients also underwent PCR-based microsatellite and FISH analyses after positive methylation study, but the number of patients receiving comprehensive surveillance was limited. For couples who have delivered a child with PWS caused by deletions/UPD, the recurrent risk is very low. Couples who have delivered a child with imprinting defects with microdeletions in imprinting center (IC) have a recurrence risk of 50% [4]. Direct detection of the imprinting mutation can be used for prenatal diagnosis [4,5]. In some imprinting-defect families, the underlying defect is unknown. The families have a variable recurrence risk and the accurate risk assessment is

impossible [13,14]. In these families, prenatal diagnosis may be based on methylation analysis of SNRPN locus. A second-phase screening protocol has been launched recently. In the new protocol, a comprehensive surveillance (including FISH and microsatellite analysis) for cases with positive methylation studies is recommended and prenatal diagnosis will be offered to families with imprinting-defects.

In conclusion, we have developed a system for referral diagnosis of PWS. The list of participants was comprised mostly of geneticists, pediatric neurologists, pediatric psychiatrists and Identification Centers in Taiwan. Patients with developmental delay or mental deficits are referred to geneticists, pediatric neurologists, pediatric psychiatrists or to Identification Center for further evaluation. Samples of suspicious patients are then sent for DNA diagnosis.

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Table 1. The incidence of individual criterion in older (≥ 1 y/o) and younger (< 1 y/o) PWS patients

Diagnostic criteria	Results	Case number (%) of Groups		
		≥ 1 y/o	< 1 y/o	p*
Cerebral hypotonia	yes	14 (87.5%)	9 (90.9%)	1
	no	2 (12.5%)	1 (9.1%)	
Feeding problem	yes	11 (68.8%)	9 (90.9%)	0.35
	no	5 (31.3%)	1 (9.1%)	
Excessive weight gain	yes	8 (50.0%)	0	0.008
	no	8 (50.0%)	10 (100.0%)	
Facial features	yes	10 (62.5%)	3 (36.4%)	0.182
	no	6 (37.5%)	7 (63.6%)	
Hypogonadism	yes	9 (56.3%)	4 (45.5%)	0.581
	no	7 (43.8%)	6 (54.5%)	
Developmental delay	yes	10 (62.5%)	1 (9.1%)	0.008
	no	6 (37.5%)	9 (90.9%)	
Hyperphagia	yes	15 (93.8%)	4 (45.5%)	0.009
	no	1 (6.3%)	6 (54.5%)	

* Fisher's exact test

Figure 1.Prader-Willi Syndrome / Angelman Syndrome Checklist

Name : _____ Chart number：_____

Sex：□male □female Date of birth：_____

Family history of mental retardation ? □yes □no □unknown

Pedigree:

Suspicious Prader-Willi Syndrome

□ (1) general and infantile cerebral hypotonia

□ (2) infantile feeding problems / failure to thrive

□ (3) excessive weight gain

□ (4) facial features (≥ 3 items)

( ) dolichocephaly; ( ) narrow face; ( ) almond-shaped eye

( ) small mouth; ( ) thin upper lips; ( ) down-turned corners of mouth

□ (5) hypogonadism (any one; depend on age)

( ) genital hypoplasia; ( ) delayed or incomplete gonadal maturation

□ (6) developmental delay in child＜6 y/o

□ (7) hyperphagia, obsession with food

* Scoring: Each criterion is weighted as one point. For simplicity, minor criteria for PWS are omitted. At least two points are required for diagnosis.

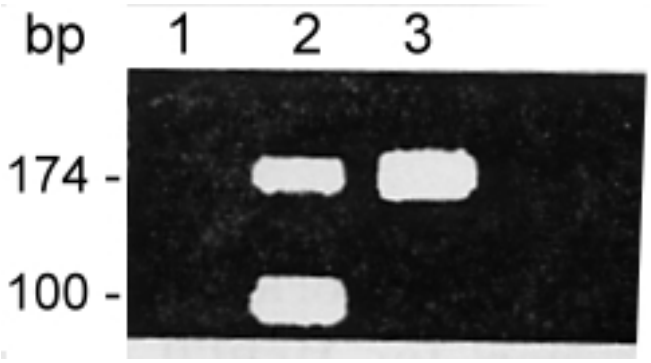

Figure 2. Methylation-specific PCR assay of a PWS patient (lane 3) one normal subject (lanes 2), and the blank control containing ddH_2O and PCR mixture (lane 1). The PCR products were separated on 3% agarose gel.