國立臺灣大學醫學院物理治療學系暨研究所

博士論文

School and Graduate Institute of Physical Therapy
College of Medicine
National Taiwan University
Doctoral Dissertation

臺灣早產兒罹患肺支氣管發育不全症的潛在生物標記及學齡前行為與情緒評估常模之初探

Investigation of Potential Biomarkers for the Susceptibility of Bronchopulmonary Dysplasia and Normative Data for a Preschool Behavioral and Emotional Assessment in Taiwanese Preterm Children

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中華民國一百年一月

January, 2011

國立臺灣大學博士學位論文
口試委員會審定書

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本論文係吳晏慈君（D94428002）在國立臺灣大學物理治療學系暨研究所完成之博士學位論文，於民國一百年一月二十四日承下列考試委員審查通過及口試及格，特此證明

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誌　謝

時光飛逝如梭，轉眼間自大學畢業後踏入台大的學術殿堂已經快九個年頭，回憶這些年來的點點滴滴，我的心情充滿了無法言語的感謝，要感謝的人真的太多太多了！多麼想把這些年曾經幫助我的師長、同學、朋友與曾經給予我鼓勵的人們一一列舉以表達我的感謝之意，但在這短短的謝辭中，還是必須忍痛取捨。今日能夠順利畢業，這份成果要獻給我最愛的家人、師長和所有認識的朋友，沒有你們沒有今日的我！

此篇論文的誕生最感謝的是我的指導老師鄭素芳教授，這些年來因為有著老師在早產兒研究上的不遺餘力和提攜後進，我就像一株小幼苗在多元的研究主題裡成長茁壯，從繕寫計畫書、實際執行計劃、分析資料到完成論文，這些年老師給了我寬廣的學習機會了解與研究有關的每一個環節，也啟發我的博士論文能夠初次伸出觸角，探究與早產兒肺部疾病有關的遺傳生物標誌，這是當時剛走進學術殿堂時的我想像不到的，因此我深切期盼自己能透過此篇論文的初探，為將來物理治療在流行病學與預防醫學領域上開闢一條研究道路，我也會在未來的研究生涯中繼續努力下去，以報答老師多年的培育之恩。

此篇論文要感謝許多的師長和醫療人員的大力協助，其中非常感謝台大流行病學與預防醫學研究所陳為堅教授，陳老師是我走進遺傳學領域的啟蒙老師，也是帶領我探究微小核醣核酸奧秘的師長，回憶與陳老師討論論文主題、實驗方法、繕寫計畫書及資料分析的過程，陳老師總是在我頭腦思考不清的時候適時的指點我，不厭其煩幫我導向問題的核心，以及提供我在基因實驗上的強力後盾。透過陳老師的引薦，我得以向陳老師的博士班學生賴季侑同學請教基因實驗的相關內容，一步一步的提升自己在遺傳知識上的不足，由衷的感謝陳老師和季侑的協助。

此外，更感謝協助研究收案的台大醫院新生兒科團隊：謝武勳主任、張睿真醫師及新生兒加護病房的醫師及護士們，因為有你們隨時的電話告知抽血的時間和立即將檢體冰存起來，我才能順利取得血液檢體進行研究。另外，更要感謝口試委員曹伯年醫師與我討論有關基因標誌的分析及論文的書寫，曹醫師指導我如何從眾多的基因資料訊息裡尋找可行的分析方式及如何解讀，幫助我對研究結果逐步的抽絲剝繭。此外我也十分感謝台大基因體中心俞松良老師在研究期間提供微小核醣核酸定量分析的服務，讓我能夠在很短的時間內取得微小核醣核酸的表現結果，以及口試委員李文宗教授給予論文精闢的建議。

這些年來在求學過程中的點點滴滴，其中有歡笑也有淚水，父母家人一直是我心靈上的最大支助，感謝你們對我的愛和包容，才能讓我不斷的堅持下去！我也感謝實驗室的伙伴們(盈瑾、硯婷、智航、珮姍、含芳、郁文、昱志、昕如等學弟妹)及博士班的同學及學長姐們(秀娟、郁秀、貞儀、榮娟、書旋、鴻基、靄雯等)在生活上對我的體貼，你們是我歡樂與信心的泉源。更感謝男友曜任的支持及其家人對我無限的關懷和鼓勵。因為有你們的體諒，常常鞭策著我要努力的學習，並在生活中時時的反省與改變自己成為更好的人，由衷的謝謝你們！

最後我想將此論文獻給所有參加此研究的早產兒及家長們，謝謝你們的參與，我永遠感激你們！

晏慈

謹誌於台大物理治療學系暨研究所 民國一百年一月

Preface

Preterm birth (gestational age < 37 weeks) is a significant public health issue worldwide. Despite the advance in perinatal and neonatal medicine over the past three decades has substantially lowered the neonatal mortality associated with preterm birth, preterm infants with very low birth weight (VLBW, birth weight < 1,500 grams) are more likely to survive into childhood.[1] However, their occurrences of neonatal morbidity and long-term neurodevelopmental sequelae remain essentially unchanged.[1, 2] Research on the mechanisms for such morbidities and the psychometric study of neurodevelopmental assessments associated with preterm birth has become the focus for prevention and management of adverse outcome.

Recent data have indicated that bronchopulmonary dysplasia (BPD) is a major neonatal disease among VLBW preterm infants.[3, 4] Although the recently wide use of antenatal steroid, exogenous surfactant therapy and gentle ventilator strategies has led to pathological changes of BPD to milder clinical manifestation in the neonatal lung,[4] the incidence of BPD among VLBW preterm infants remains high in the US (3%-43%)[5] and Taiwan (19%-41%).[6] Furthermore, preterm infants surviving from BPD, particularly those with severe form of respiratory illness, are more likely to show poor respiratory and developmental outcomes.[3, 7] Therefore, exploration of potential biomarkers for the susceptibility of BPD in VLBW preterm infants is crucial for early diagnosis and management.

In addition to the risk of neonatal diseases, several follow-up studies showed that the incidence of major developmental disabilities (i.e., severe mental retardation, hearing loss, blindness and cerebral palsy) has remained relatively constant at around 8% to 12% in VLBW preterm children over the past decade.[1] Furthermore, the prevalence estimates of low severity dysfunctions in the aspects of behavioral and

emotional development (e.g., learning disabilities, attention-deficit hyperactivity disorder and behavior problems) appear to increase to as high as 50% to 70% of VLBW infants.[1, 2, 8] There is a need for early detection and intervention of preterm children who may later develop behavioral and emotional problems at preschool age.

Although few epidemiological studies of behavioral and emotional problems in Taiwanese preschoolers existed, they rarely established local norms and did not make multi-cultural comparison of the results.[9, 10] Establishment of normative data is important to serve as the basis for understanding the prevalence estimate of behavioral and emotional problems in general population and for assessing the behavioral and emotional problems of at-risk populations such as preterm children. Furthermore, multi-cultural comparisons will help determine whether the prevalences of behavioral problems are similar across societies.

To fulfill the aforementioned purposes, this dissertation has composed of two parts. The first part was to explore potential biomarkers for the susceptibility of BPD in VLBW preterm infants, in search of genetic biomarkers of microRNA (Chapter 1, pp. 11-43). The second part was to investigate the behavioral and emotional problems in a full-term population of Taiwanese preschoolers using a commonly used behavioral and emotional assessment- the Child Behavior Checklist for Ages 1½-5 (Chapter 2, pp.44-72). The behavioral problems scores and prevalence of Taiwanese preschoolers were compared with those of American and Dutch preschoolers.

The results of this study showed that a 6-miRNA signature was highly predictive of the occurrence of BPD in VLBW preterm infants. The target genes associated with the 6-miRNA signature revealed a functional relevance to developmental-related functions and diseases which might serve as genetic biomarkers of BPD. Our findings further showed that the normative behavioral scores

of Taiwanese preschoolers were higher, particularly in the internalizing-related syndromes, when compared to those of American and Dutch preschoolers. These results may assist in clinical diagnosis of BPD in VLBW preterm infants at early stage of life and will assist in assessment of behavioral and emotional problems in preterm preschooler.

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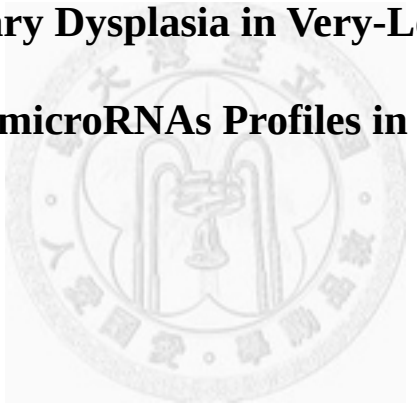
Chapter 1

Exploration of Potential Biomarkers for Susceptibility to Bronchopulmonary Dysplasia in Very-Low-Birth-Weight Preterm Infants: microRNAs Profiles in Peripheral Blood



Abstract

Background: Genetic factors have been shown to account for more than 50% of the variation in bronchopulmonary dysplasia (BPD) in preterm infants. However, human genetic association studies have failed to show consistent associations, and altered expression of transcription factors has only been observed in animal studies. An emerging focus in the search for biomarkers of BPD has been on post-transcriptional regulator of messenger RNA translation such as microRNAs (miRNAs). **Objective:** This study aimed to identify differentially expressed miRNAs in the peripheral blood of very low birth weight (VLBW, birth weight < 1,500 g) preterm infants with and without BPD. **Methods:** Blood samples from 15 VLBW preterm infants with BPD and from 15 sex-matched controls were collected in early postnatal life, and 365 miRNA expression profiles were assessed using a quantitative real-time polymerase chain reaction system. **Results:** The BPD group was more likely than the non-BPD group to show increased expression of *miR-133b* and *miR-7* and decreased expression of *miR-152*, *miR-30a-3p*, *miR-650* and *miR-624* (all $p < 0.2$). This 6-miRNA signature was highly predictive of the occurrence of BPD after controlling for the effects of confounders (accuracy = 0.995). Forty-one shared target genes were associated with the 6-miRNA signature, and seventeen of them were relevant to development-related functions and diseases. The Wnt/β-catenin and axon guidance signaling pathways were associated with the target genes ($p < 0.05$ for both). **Conclusions:** This 6-miRNA signature of peripheral blood might serve as a biomarker predictive of the occurrence of BPD in VLBW preterm infants.

Keywords: Bronchopulmonary dysplasia, microRNA, Very low birth weight, Preterm

中文摘要

研究背景：過去的研究顯示早產兒罹患肺支氣管發育不全症具有 50%以上的變異度能夠被遺傳因素解釋，然而，過去的人類基因關聯性研究無一致的結論，並且在肺支氣管發育不全症內會受到改變的轉譯因子，仍然在動物模型的實驗階段。有鑑於過去研究的不足，探索與基因調控有關的遺傳因子，例如微小核醣核酸，作為肺支氣管發育不全症的生物標記，是值得嘗試的研究方向。**目的：**本研究的目的為探索罹患肺支氣管發育不全症之極低出生體重早產兒(出生體重低於 1,500 公克)，其周邊血液中的特異微小核醣核酸。**方法：**本研究搜集 15 位罹患肺支氣管不全症的極低出生體重早產兒及另 15 位性別配對之未罹病早產兒的周邊血液檢體，使用同步定量聚合鏈反應系統檢驗人類共 365 個微小核糖核酸於周邊血液的表現剪影。**結果：**研究結果顯示罹病早產兒在 *miR-133b* 及 *miR-7* 的表現比例上較未罹病早產兒多，然而在 *miR-152*, *miR-30a-3p*, *miR-650* 及 *miR-624* 則呈現較低比例的表現(所有 $p < 0.2$)。此六個特異微小核醣核酸所構成的生物標誌對於肺支氣管不全症的發生具有高預測性，在調控與疾病相關的干擾因子後，仍可以高度區分肺支氣管發育不全症之罹病早產兒與未罹病早產兒(準確率 $= 0.995$)。由微小核醣核酸的基因預測資料庫內發現此六個特異微小核醣核酸共同協調 41 個基因標的，功能路徑分析結果發現其中 17 個基因標的互相作用於一個網絡內，並且與發育相關的功能與疾病有關，並可能參與 Wnt/β-catenin 與 axon guidance 的訊號路徑(兩者 $p < 0.05$)。**結論：**研究結果顯示周邊血液中的六個特異微小核醣核酸之表現剪影對於肺支氣管發育不全症的發生是可行的生物標誌。

關鍵詞：肺支氣管發育不全症、微小核醣核酸、極低出生體重、早產

1. Introduction

1.1 Background and Purpose

Bronchopulmonary dysplasia (BPD) is a major chronic lung disease in preterm infants with very low birth weight (VLBW, birth weight < 1,500 g).[1, 2] Recent advances in the prevention and management of this respiratory illness such as antenatal steroid treatment, exogenous surfactant therapy and gentle ventilator strategies have caused the pathology of BPD to change from severe structural disruption to developmental arrest of the neonatal lung.[3-5] This new form of developmental disorder in lung frequently occurs in extremely small preterm infants, with pathological features such as impaired alveolarization, vascular growth abnormalities and mild inflammation.[5, 6] Little is known about the etiology of the developmental abnormalities in BPD.

A role for genetic factors has been increasingly recognized in pathogenetic studies of BPD in preterm infants, with twin studies indicating that the contribution of genetic factors is more than 50%.[7, 8] Several genetic association studies have examined the roles of genetic variants in inflammatory regulation and surfactant synthesis in BPD in VLBW preterm infants, but they failed to find consistent associations (results shown in appendix Table 1).[9-18]Although altered expression of certain transcription factors for protein-coding RNA has been linked to alveolarization, angiogenesis and remodeling of the lung extracellular matrix during lung development, such findings are limited to animal models of BPD.[19-23]

Instead of targeting DNA or protein-coding RNA in BPD, recent advances in molecular genetics have provided a powerful new strategy for understanding the regulation of gene expression via non-coding RNA, in particular microRNAs (miRNAs). miRNAs are a small class of non-coding RNAs (about 22 nucleotides) that

have a regulatory effect on messenger RNA (mRNA) translation and protein production.[24] Furthermore, miRNAs are closer temporally to the occurrence of disease than is information encoded in the genome; thus, understanding these RNAs is an important step towards understanding pathogenesis. To date, over 700 miRNAs have been identified and sequenced in humans, and approximately 30% of mRNAs are targeted by miRNAs.[25, 26] Some miRNAs may be involved in the pathogenesis of adult lung cancer and could be useful for classification, prediction and prognosis.[27-30] Whether altered expression of miRNAs is also related to susceptibility to BPD remains unknown.

To determine whether a global miRNA expression profile could be used to predict the occurrence of BPD, we surveyed 365 miRNAs for differential expression in the peripheral blood of VLBW preterm infants with and without BPD in this study.

2. Methods

2.1 Participants

This study prospectively enrolled VLBW preterm infants who were admitted to the neonatal intensive care unit (NICU) at the National Taiwan University Hospital (NTUH) in Taipei, Taiwan in 2009. The inclusion criteria included: gestational age ≤ 33 weeks, birth weight < 1,500 g, absence of congenital and chromosomal abnormalities, born in or admitted to the NTUH within 7 days of birth and alive at 36 weeks postmenstrual age (PMA). The exclusion criteria were: parents of non-Taiwanese ethnicity, maternal age of ≤ 18 years, and maternal history of substance abuse or a psychiatric disorder. The presence of BPD was defined as infants showing mild to severe BPD at 36 weeks PMA: “no BPD” as a need of supplemental oxygen for < 28 days, “mild BPD” as oxygen use for ≥ 28 days but not at 36 weeks’ PMA or discharge, “moderate BPD” as oxygen use for ≥ 28 days plus treatment with

< 30% oxygen at 36 weeks' PMA, and "severe BPD" as oxygen use for ≥ 28 days plus ≥ 30% oxygen use and/or positive pressure at 36 weeks' PMA (severity defined by the National Institute of Health consensus definition of BPD).[3] The study was approved by the Ethics Committee of NTUH (ID: 200805034R). Written informed consent was obtained from the parents after a complete description of the study.

The perinatal and demographic data of the VLBW preterm infants were obtained from the infants' medical charts or via parental interview during hospitalization. Perinatal data included birth variables, respiratory variables and neonatal morbidities, which were recorded prior to 36 week' PMA. Birth variables included child's sex, gestational age, birth weight, birth set (singleton, twin or multiple births), 1- and 5-min Apgar scores and intrauterine growth status. Intrauterine growth status was classified as small (SGA), appropriate (AGA) or large for gestational age (LGA), with each defined as birth weight below the 10^{th} percentile, within the 10^{th} and 90^{th} percentiles, and above the 90^{th} percentile, respectively, according to the corresponding gestational age of the normative intrauterine growth data for Taiwanese infants.[31] Respiratory variables included duration of ventilation, continuous positive airway pressure (CPAP), oxygen therapy and respiratory medications (antenatal steroid use and postnatal surfactant therapy). The presence and/or severity of neonatal morbidities (i.e., respiratory distress syndrome [RDS], intraventricular hemorrhage [IVH], periventricular leukomalacia [PVL], patent ductus arteriosus [PDA], retinopathy of prematurity [ROP], necrotizing enterocolitis [NEC] and sepsis) that commonly occur in preterm infants were also noted from the medical record. Demographic data included the infant's birth order and parental education and occupation.

From September, 2008 to December, 2009, 46 VLBW preterm infants were

eligible for the study, of whom 1 (2%) declined consent and 45 (98%) participated. During the study period, 3 participating infants (7%) succumbed to the illness and 42 (93%) survived to 36 weeks PMA (mild to severe BPD in 19 and no BPD in 23). Four infants with BPD and 8 infants without BPD were excluded because their twin siblings were enrolled in the BPD or non-BPD group (N=4) or the quantity of blood was insufficient for analysis (N=8). The study infants included 15 VLBW preterm infants with BPD and 15 sex-matched infants without BPD.

2.2 Blood Sampling and RNA Extraction

Approximately 500 μl of arterial blood was collected from the infants during the 31-36 week PMA period. Blood samples were collected by a pediatric resident in the NICU in conjunction with routine blood tests during the day. An infant's blood was not collected for the study if systematic inflammation, infection, sepsis, hypoglycemia, blood transfusion or surgery occurred within 7 days of the blood collection. The RiboPure^{TM}-Blood Kit (Applied Biosystems, CA, USA) was used to extract 2-4 μg of RNA from each specimen.

2.3 miRNA Expression Profiling

Expression profiling of 365 human miRNAs was performed using the ABI PRISM 7900 Real Time Polymerase Chain Reaction (PCR) System and the TaqMan® Low Density Array Human microRNA Panel v1.0 (Applied Biosystems, Foster City, CA, USA). The corresponding cDNAs were made using TaqMan MicroRNA RT reagents and primers specific for the miRNAs. The miRNA expression profiles were quantified using the normalized threshold cycle number (△Ct), with RNU48 as the internal control.

2.4 Statistical Analysis

The perinatal and demographic variables of the BPD and control groups were

compared using Fisher's exact test for categorical variables and the Wilcoxon signed rank test for continuous variables. Variables that were significantly different between groups were considered as potential confounders for subsequent analysis of miRNA expression relative to BPD occurrence.

The expression of individual miRNAs was first categorized into expressed (with miRNA levels detectable in 2 or more infants) or non-expressed (with miRNA levels detectable in less than 2 infants). For the miRNAs expressed to detectable levels in all of the infants, the Wilcoxon signed rank test was used to compare the expression level ($\triangle$Ct) between groups. For the miRNAs expressed to detectable levels in only some of the infants, Fisher's exact test was used to compare the proportion of infants with miRNA expression between groups. Initially, univariable logistic regression was used to select individual miRNAs for differential expression analysis with a liberal threshold *p* value of 0.2. Then multivariable logistic regression analysis was used to construct a miRNA signature for the prediction of BPD occurrence with a leave-one-out cross-validation procedure. Because the number of subjects was small in this study, the odds ratios of potential confounders were estimated from the logistic regression and were then combined into a single confounding score for each infant.[32] The logistic model provides information concerning the area under the receiver operating characteristics (ROC) curve (AUC) for the prediction of BPD by the miRNA signature.

Information regarding chromosomal locations and the genes relevant to the differentially expressed miRNAs was obtained from miRNAMAP (www.mirnamap.mbc.nctu.edu.tw/), and the target genes were determined from miRWALK (www.ma.uni-heidelberg.de/apps/zmf/mirwalk/) that the database involves 5 algorithms of miRNA targets (miRanda, miRDB, miRWalk, RNA22, and

TargetScan). The data for shared target genes with the miRNA signature were uploaded into Ingenuity Pathways Analysis (IPA) (Ingenuity Systems®, www.ingenuity.com) to generate network information for candidate genes. IPA contains global molecular network data from the literature that reports connections between networks and molecular relationships. Functional pathway analysis was later performed in IPA to identify the biological functions, diseases and pathways significantly related to the candidate genes in the network. All statistical analyses in this study were performed using the Statistical Analysis Software program (version 9.1, SAS Institute, Cary, NC) with a p value of < 0.05 considered to be statistically significant.

3. Results

3.1 Sample Characteristics

Patients in the BPD group showed significantly lower gestational ages, birth weights and 5-min Apgar scores and were more likely to be AGA than subjects in the control group (all $p < 0.05$) (Table 1-1). Furthermore, the BPD group required ventilation, CPAP and oxygen therapy for a longer duration of time and more often had RDS and PDA than the control group (all $p < 0.05$) (Table 1-1).

3.2 miRNA Expression Profiles

Although the collection of blood samples tended to be earlier for the BPD group than for the control group (postnatal age 37 ± 11 days vs. 47 ± 12 days), the difference did not reach statistical significance ($p = 0.09$) (Table 1-2). Of the 365 human miRNAs examined, 210 (58%) were expressed and 155 (42%) were not expressed. Among those expressed, only 61 miRNAs showed detectable expression in all infants (Figure 1-1), whereas 149 exhibited detectable expression in at least 2 infants. Wilcoxon signed rank tests of the expressed miRNAs revealed no difference in the

expression levels between groups. However, Fisher's exact tests showed a difference in the proportion of infants that expressed or did not express in 6 miRNAs between the groups (Table 1-3). More infants in the BPD group than in the non-BPD group expressed *miR-133b* and *miR-7*, whereas more infants in the BPD group than in the non-BPD group did not express *miR-152*, *miR-30a-3p*, *miR-650* or *miR-624* (all $p < 0.2$).

3.3 Prediction of BPD Based on Six Differentially Expressed miRNAs

Multivariable logistic regression analysis with a leave-one-out cross-validation procedure showed that the 6-miRNA signature as a set had the greatest predictive value (AUC = 0.951) (Figure 1-2A). Positive expression status of *miR-133b* and *miR-7* together with a lack of expression of *miR-152*, *miR-30a-3p*, *miR-650* and *miR-624* corresponded to the most accurate prediction of BPD (sensitivity = 0.867 and specificity = 0.733). Exploration of perinatal variables showed that gestational age, birth weight, SGA, 5-min Apgar score, surfactant therapy and PDA were confounders for the association of these miRNAs with BPD (effect of confounders shown in Table 1-4) (all $p < 0.05$). The predictive value of the 6-miRNA signature for BPD was improved after adjustment for the effects of the confounders (AUC = 0.995) (Figure 1-2B).

3.4 Target Genes and Functional Relevance of the Six-miRNA Signature

A total of 6124 target genes were identified as relevant to at least one of the 6 miRNAs using the miRWALK algorithm, with 41 of them being shared target genes (Table 1-5 & Table 1-6). Uploading the target gene information to IPA showed that 17 of them could be mapped to a network (Table 1-5 & Figure 1-3). Subsequent functional pathway analysis of these 17 target genes showed relevance to organ and embryonic development and to developmental disorders (all $p < 0.05$) (Table 1-7).

Furthermore, the Wnt/β-catenin and axon guidance signaling pathways were significantly associated with the target genes ($p = 0.04$ for both).

4. Discussion

Analysis of 365 miRNA expression profiles showed differences in the expression pattern of 6 miRNAs (i.e., *miR-152*, *miR-30a-3p*, *miR-650*, *miR-133b*, *miR-7* and *miR-624*) between VLBW preterm infants with BPD and those without BPD in the neonatal period. The 6-miRNA signature had high predictive value for BPD occurrence at 36 weeks' PMA. Furthermore, algorithm-based functional analysis revealed that target genes shared among the six miRNAs of the signature are relevant to development-related functions and diseases. These findings suggest that the 6-miRNA signature may play an important role in the pathogenesis of BPD in VLBW preterm infants.

The 6-miRNA signature was highly predictive of susceptibility to BPD in VLBW preterm infants after controlling for the effects of perinatal confounders (accuracy = 0.995). Specifically, the BPD group was more likely to show expression of *miR-133b* and *miR-7* and to not express *miR-152*, *miR-30a-3p*, *miR-650* and *miR-624* than the non-BPD group. These data suggest that the expression status of the 6-miRNA signature could distinguish VLBW preterm infants with BPD from those without BPD. Four of these miRNAs (*miR-30a-3p*, *miR-133b*, *miR-7* and *miR-152*) have previously been reported as associated with childhood or adult lung diseases. Down-regulated expression of *miR-30a-3p* and *miR-133b* and up-regulated expression of *miR-7* were found in lung tumor tissue of adults with adenocarcinoma.[28-30] In addition, genetic polymorphisms of histocompatibility antigen, class I, G (HLA-G) were reported to affect the targeting of *miR-152* in children with asthma.[33] Thus, two-thirds of the 6 miRNAs have established associations with neonatal, childhood

and adult lung diseases.

BPD in premature infants has been proposed to be a lung injury related to prolonged use of ventilation and oxygen therapy in the neonatal period.[3, 5, 6] Our BPD group infants were subjected to ventilation (median 3 days vs. 0 day), CPAP (median 43 days vs. 4 days) and oxygen therapy (median 51 days vs. 7 days) for a longer duration than the control group infants. Furthermore, the 6 miRNAs were found to be differentially expressed in VLBW preterm infants with BPD at or near a postnatal age of 37 days, when respiratory support was in use. It is possible that the aberrant expression levels of miRNAs could be related to hypoxic or hyperoxic insults from ventilator and oxygen use. One prior study has shown that hypoxic events induced altered expression of several miRNAs (e.g., *miR-152*) in human colonic cells.[34] Further studies should focus on collecting blood samples prior to oxygen use to determine how the use of respiratory support might alter expression of the 6 miRNAs in VLBW preterm infants.

In addition to the lung injury caused by oxidative stress, developmental disorders of alveolarization and dysmorphic pulmonary vasculature have recently been recognized as pathological hallmarks of the new form of BPD.[6, 35] In our study, a total of 41 shared target genes were identified as related to the 6-miRNA signature, and 17 of them were linked to development-related functions and diseases. Several of the target genes have been previously reported as related to lung development in animal studies. For example, the gene families of *Fam123b* (wtx/amer) and the transcription factors encoded by *NFIB* genes were shown to be highly expressed in the embryonic mouse lung and might be involved in fetal lung maturation.[36, 37] *GLI3*, a transcription factor of the Sonic hedgehog cascade, was reported to be critical for the patterning of early lung morphogenesis in mice.[38, 39] Moreover, *NFIB*-deficient mice

have been found to die quickly postnatally, and their severe lung hypoplasia resembled the phenotype of BPD.[37, 40] These data provide evidence that supports the relationship of the target genes of the 6-miRNA signature to lung development and developmental disorders in the pathogenesis of BPD. Furthermore, the identified Wnt/β-catenin signaling pathway has been found to regulate airway epithelial differentiation and vascular smooth muscle proliferation in the developing lung.[41] Disruption of the Wnt/β-catenin signaling pathway in the primordial mouse lung could result in a failure to form lung buds and thereby arrest proliferation in the vascular smooth muscle of the lungs.[42, 43] Further investigation should examine how the target genes and the 6-miRNA signature modulate the Wnt/β-catenin signaling pathway in an animal or cell line model of BPD.

The results of this study should be interpreted with some limitations in mind. First, the sample size was relatively small, so validation of our results in another independent sample is necessary. Second, although all infants' blood samples were collected prior to 36 weeks' PMA, individual difference in the age of blood collection was not accounted for in this study. Third, despite the utility of blood-based miRNA signatures in predicting the occurrence of BPD in VLBW preterm infants, these miRNAs might not represent the whole spectrum of regulatory changes involved in the pathophysiology of BPD in the developing lung. Direct examination of the lung tissue is needed to clarify the underlying mechanisms.

In summary, this study was the first to investigate the miRNA expression profiles of the peripheral blood of VLBW preterm infants with and without BPD. The present report contributes to the growing understanding of the role of miRNAs in BPD, and the high predictive value of the 6-miRNA signature may provide clinicians with a useful biomarker for early prediction of this disease. More importantly, the link

between the target genes of the 6-miRNA signature and development-related functions and diseases suggests that aberrant expression of the 6-miRNA signature in early postnatal life may be involved in subsequent development of BPD. However, these preliminary data should be validated in other populations of VLBW preterm infants.

5. Acknowledgments

This study was supported by grants from National Taiwan University Hospital (NTUH-98-S-1085) and the National Health Research Institute (NHRI-EX98-9519PI) in Taiwan. We thank the infants and their parents for participating in the study and the medical and nursing staff of the Neonatal Intensive Care Unit at National Taiwan University Hospital for their assistance in patient recruitment and data collection.

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Figure Legends

Figure 1-1. The expression levels of expressed miRNAs, sixty-one were fully expressed to detectable levels in both VLBW infants with BPD (red points) and without BPD (blue points).

Figure 1-2. Receiver operating characteristics (ROC) curve for the prediction of BPD occurrence by (A) the 6-miRNA signature model and (B) the adjusted 6-miRNA signature model.

Figure 1-3. The seventeen target genes mapped to a network in 6-miRNA signature. Network of genes were generated using IPA (Ingenuity®Systems, www.ingenuity.com). Genes are represented as nodes, and the biological relationship between two nodes is represented as an edge (line). The nodes of seventeen target genes of six-miRNA signature are red. Solid edges represent a direct relationship and dashed edges represent an indirect relationship. The shape of each node represents the functional class of the gene product, as shown in the left side.

Table 1-1. Perinatal and demographic characteristics of VLBW preterm infants with and without BPD

Characteristic	BPD (N=15)	No BPD (N=15)
Perinatal		
Gestational age (wk)*	28 (24-30)	31 (27-33)
Birth weight (g)*	1060 (506-1440)	1314 (1040-1478)
Intrauterine growth status as SGA*	1 (7)	7 (47)
Birthset as twin or multiple births	5 (33)	6 (40)
1-min Apgar score (points)	5 (2-7)	7 (4-8)
5-min Apgar score (points)*	8 (6-9)	8 (7-9)
Ventilation use*	11 (73)	4 (27)
Duration of ventilation (days)*	3 (0-71)	0 (0-3)
Duration of CPAP (days)*	43 (33-275)	4 (0-17)
Duration of oxygen therapy (days)*	51 (33-343)	7 (0-22)
Antenatal steroid use	10 (67)	11 (73)
Surfactant therapy*	8 (53)	0 (0)
RDS*	14 (93)	9 (60)
IVH		
Normal	10 (66)	9 (60)
Grade I-II	4 (27)	6 (40)
Grade III-IV	1 (7)	0 (0)
PVL	5 (33)	2 (13)
PDA*	6 (40)	1 (7)
NEC	1 (7)	0 (0)
ROP		
Normal	12 (80)	13 (87)
Stage I-II	2 (13)	2 (13)
Stage III-IV	1 (7)	0 (0)
Sepsis	1 (7)	2 (13)
Demographic		
First birth order	8 (53)	10 (67)
Maternal education (years)	14 (12-16)	16 (12-18)
Paternal education (years)	14 (9-18)	16 (12-18)
Maternal occupation		
Unemployed or unskilled labor	8 (53)	7 (47)
Technician	4 (27)	6 (40)
Professional	3 (20)	2 (13)
Paternal occupation		
Unemployed or unskilled labor	6 (40)	1 (7)
Technician	5 (33)	11 (73)
Professional	4 (27)	3 (20)

Data are presented as median (range) or N (%).
BPD = bronchopulmonary dysplasia; CPAP = continuous positive airway pressure; RDS = respiratory distress syndrome; IVH = intraventricular hemorrhage; PVL = periventricular leukomalacia; PDA = patent ductus arteriosus; NEC = necrotizing enterocolitis; ROP = retinopathy of prematurity.
* $p < 0.05$.

Table 1-2. Age of blood sampling in each study infant

Participants	No.	Gender	GA (week)	Age of blood sampling	
				Postnatal day	PMA (week)
VLBW	1	F	24	44	31
infants	2	F	27	67	36
with BPD	3	F	28	56	36
	4	F	28	33	32
	5	F	28	65	36
	6	F	29	37	34
	7	M	25	35	31
	8	M	26	59	34
	9	M	26	47	32
	10	M	26	63	35
	11	M	28	44	34
	12	M	28	62	36
	13	M	29	43	35
	14	M	29	34	33
	15	M	30	30	34
VLBW	1	F	27	44	33
infants	2	F	29	48	35
without	3	F	30	36	35
BPD	4	F	31	39	36
	5	F	31	31	35
	6	F	32	20	35
	7	M	28	30	32
	8	M	29	34	33
	9	M	31	43	36
	10	M	31	52	36
	11	M	32	47	36
	12	M	32	40	36
	13	M	32	39	36
	14	M	33	32	36
	15	M	33	3	33

F= female; M= male; GA= gestational age; PMA= postmenstrual age

Table 1-3. Six miRNAs differentially expressed in blood samples of VLBW preterm infants with and without BPD

miRNA*	Infants with Detectable Expression†			Chromosome (Region)
	BPD (N=15)	No BPD (N=15)	*P* Value	
miR-152	1 (7)	8 (53)	0.01	17q21.32 (Intron)
miR-30a-3p	0 (0)	5 (33)	0.04	6q13 (Intron)
miR-650	11 (73)	15 (100)	0.09	22q11.23 (Intergenic)
miR-133b	12 (80)	7 (47)	0.12	6p12.2 (Intron)
miR-7	10 (67)	5 (33)	0.14	9q21.32 (Intron and Exon)
miR-624	1 (7)	5 (33)	0.16	14q12 (Intron)

* MiRNAs listed in ascending order of *p* value.

† Number and % of infants showing detectable expression of miRNA.

Table 1-4. Univariable logistic regression for the relations of perinatal variables with BPD.

Variable	OR (95% CI)	P Value
Gestational age (per wk decrease)	3.04 (0.15 - 0.71)	0.00
Birth weight (per g decrease)	1.01 (0.98 – 0.99)	0.02
Intrauterine growth status as SGA (vs. AGA)	0.08 (0.01 - 0.79)	0.03
5-min Apgar score (per score decrease)	5.52 (0.04 - 0.77)	0.02
Surfactant therapy (vs. no surfactant therapy)	35.1 (1.5 – 823.2)	0.00
PDA (vs. no PDA)	38.5 (3.75 - 395.4)	0.00
PMA of blood sampling (per wk decrease)	1.62 (0.40 – 0.95)	0.03

OR= odds ratio; 95%CI= 95% Confidence Interval; PDA=patent ductus arteriosus

Table 1-5. Shared target genes of the 6-miRNA signature and genes mapped to the network

Six-miRNA Signature	Shared Target Genes[*, ‡]	Genes Mapped to the Network[†, ‡]
miR-152 *miR-30a-3p* *miR-650* *miR-624* *miR-133b* *miR-7*	*AAK1, ANKRD52, AP4E1, ATP7A, BCL11B, BNC2, CSNK1G1, EIF2C1, ENAH, FAM123B, FRS2, GCNT2, GLI3, JPH4, JUNDM2, KIAA1024, KIAA1632, KLF12, LEPROTL1, MAFG, MTF1, NAV1, NFAT5, NFIB, NTRK2, OCRL, ONECUT2, PITPNM2, RALGPS1, SELI, SH3PXD2A, SH3TC2, SLC1A2, SLC24A4, SOX11, SPOPL, STX6, TNKS, TULP4, VANGL1, ZSCAN22*	*AAK1, ATP7A, BCL11B, BNC2, CSNK1G1, EIF2C1, ENAH, GLI3, KLF12, MTF1, NFIB, OCRL, SH3PXD2A, SLC1A2, SOX11, TULP4, VANGL1*

[*] Forty-one shared target genes were identified using miRWALK at www.ma.uni-heidelberg.de/apps/zmf/mirwalk/.

[†] Seventeen out of 41 shared target genes were mapped to a network in the library of Ingenuity Pathways Analysis.

[‡] The gene names are available at www.ncbi.nlm.nih.gov/entrez.

Table 1-6. Gene symbols of the target genes with 6-miRNA signature.

Gene Symbol	Full Name
AAK1	Adaptor-associated kinase 1
ANKRD52	Ankyrin repeat domain 52
AP4E1	Adaptor-related protein complex 4, epsilon 1 subunit
ATP7A	ATPase, Cu++ transporting, alpha polypeptide
BCL11B	B-cell CLL/lymphoma 11B (zinc finger protein)
BNC2	Basonuclin 2
CSNK1G1	Casein kinase 1, gamma 1
EIF2C1	Eukaryotic translation initiation factor 2C, 1
ENAH	Enabled homolog (Drosophila)
FAM123B	Family with sequence similarity 123B
FRS2	Fibroblast growth factor receptor substrate 2
GCNT2	Glucosaminyl (N-acetyl) transferase 2, I-branching enzyme
GLI3	GLI family zinc finger 3
JPH4	Junctophilin 4
JUNDM2	Jun dimerization protein 2
KIAA1024	KIAA1024
KIAA1632	KIAA1632
KLF12	Kruppel-like factor 12
LEPROTL1	Leptin receptor overlapping transcript-like 1
MAFG	V-maf musculoaponeurotic fibrosarcoma oncogene homolog G
MTF1	Metal-regulatory transcription factor 1
NAV1	Neuron navigator 1
NFAT5	Nuclear factor of activated T-cells 5, tonicity-responsive
NFIB	Nuclear factor I/B
NTRK2	Neurotrophic tyrosine kinase, receptor, type 2
OCRL	Oculocerebrorenal syndrome of Lowe
ONECUT2	One cut homeobox 2
PITPNM2	Phosphatidylinositol transfer protein, membrane-associated 2
RALGPS1	Ral GEF with PH domain and SH3 binding motif 1
SELI	Selenoprotein I
SH3PXD2A	SH3 and PX domains 2A
SH3TC2	SH3 domain and tetratricopeptide repeats 2
SLC1A2	Solute carrier family 1 (glial high affinity glutamate transporter), member 2
SLC24A4	Solute carrier family 24 (sodium/potassium/calcium exchanger), member 4
SOX11	SRY (sex determining region Y)-box 11
SPOPL	Speckle-type POZ protein-like
STX6	Syntaxin 6
TNKS	Tankyrase, TRF1-interacting ankyrin-related ADP-ribose
TULP4	Tubby like protein 4
VANGL1	Vang-like 1 (van gogh, Drosophila)
ZSCAN22	Zinc finger and SCAN domain containing 22

Information obtained from the PubMed (www.ncbi.nlm.nih.gov/entrez).

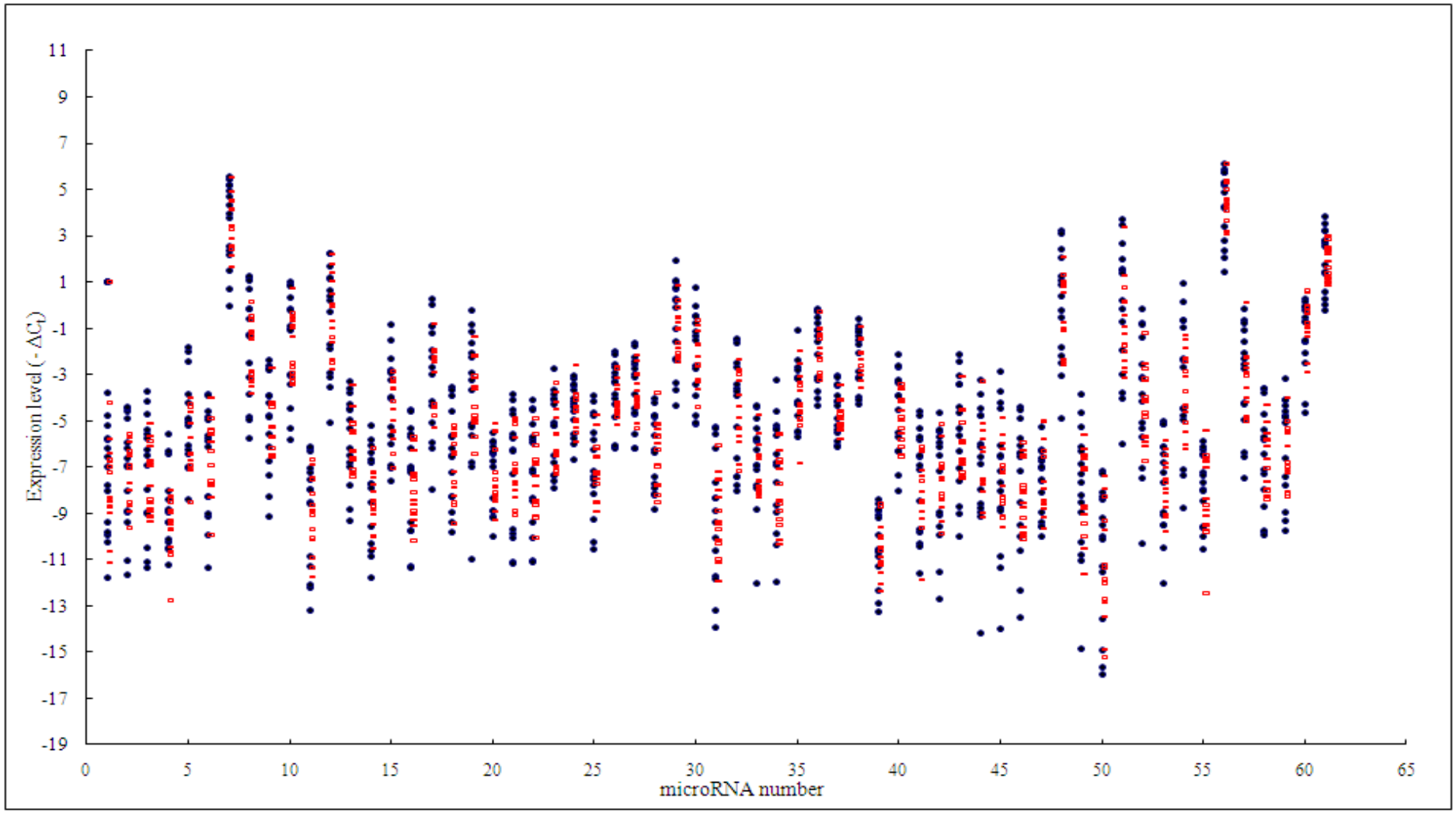
Table 1-7. Biological functions and diseases related to the target genes in the 6-miRNA signature

Category	Function and Disease*	Involved Target Gene†
Organ development	Organ development	*APTA7A, BCL11B, GLI3, NFIB, SLC1A2*
	Lung development	*GLI3, NFIB*
	Forebrain development	*GLI3, NFIB*
	Brain development	*GLI3, NFIB, SLC1A2*
Embryonic development	Neural tube development	*ENAH, GLI3*
	Embryonic tissue development	*ENAH, GLI3*
	Cell death of embryonic cell lines	*AAK1, MTF1*
	Neurological process of embryonic tissue	*ENAH, GLI3*
Developmental disorder	Developmental disorder	*ATP7A, GLI3, NFIB, SLC1A2, VANGL1*

* The listed functions and diseases were determined by the Ingenuity Pathways Analysis.

† Target genes of the 6-miRNA signature involved in the biological function or disease.

All $p < 0.05$.


11
9
7
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Expression level (- ΔCt)
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microRNA number

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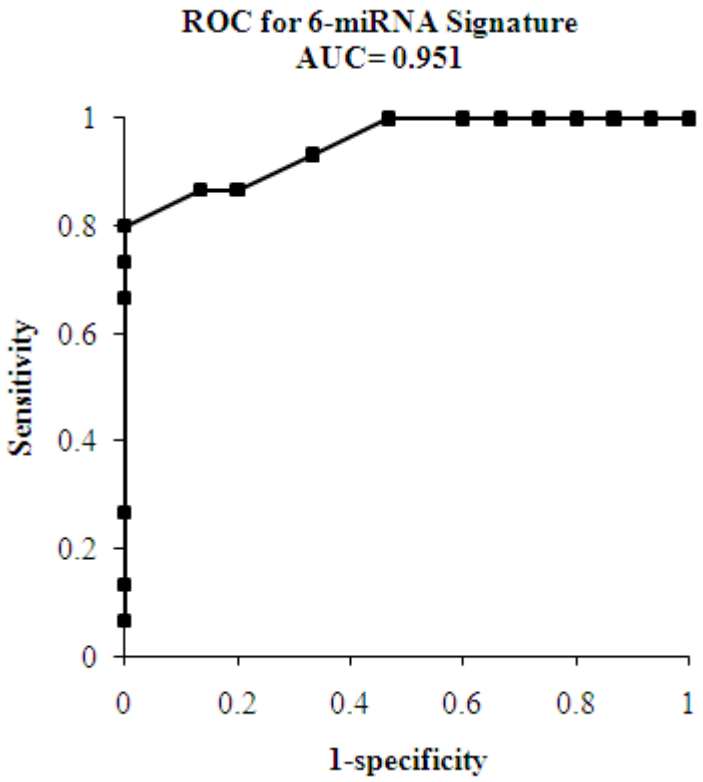
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AUC= 0.951
Sensitivity
1-specificity
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0.2
0.4
0.6
0.8
1

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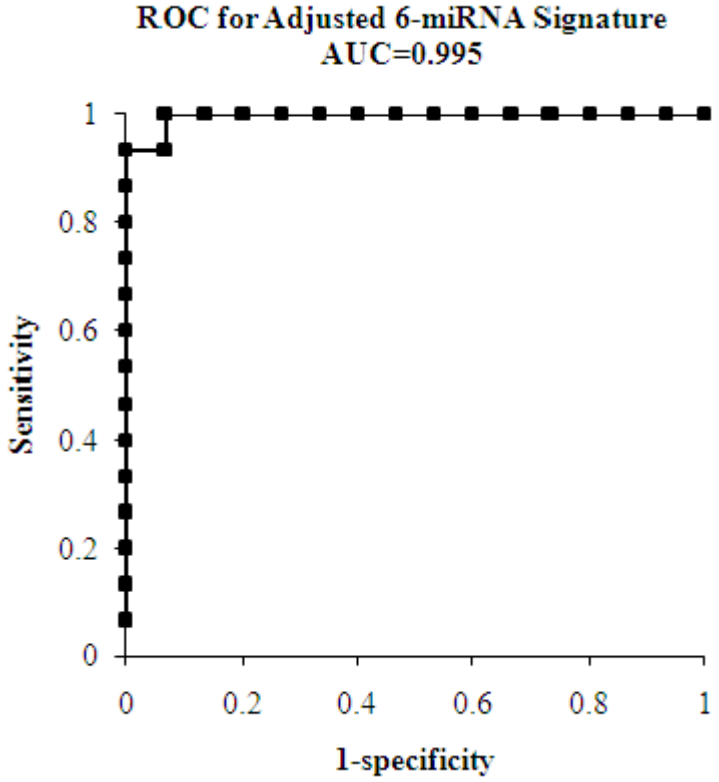
ROC for Adjusted 6-miRNA Signature
AUC=0.995
Sensitivity
1-specificity
0
0.2
0.4
0.6
0.8
1

Path Designer Network 1

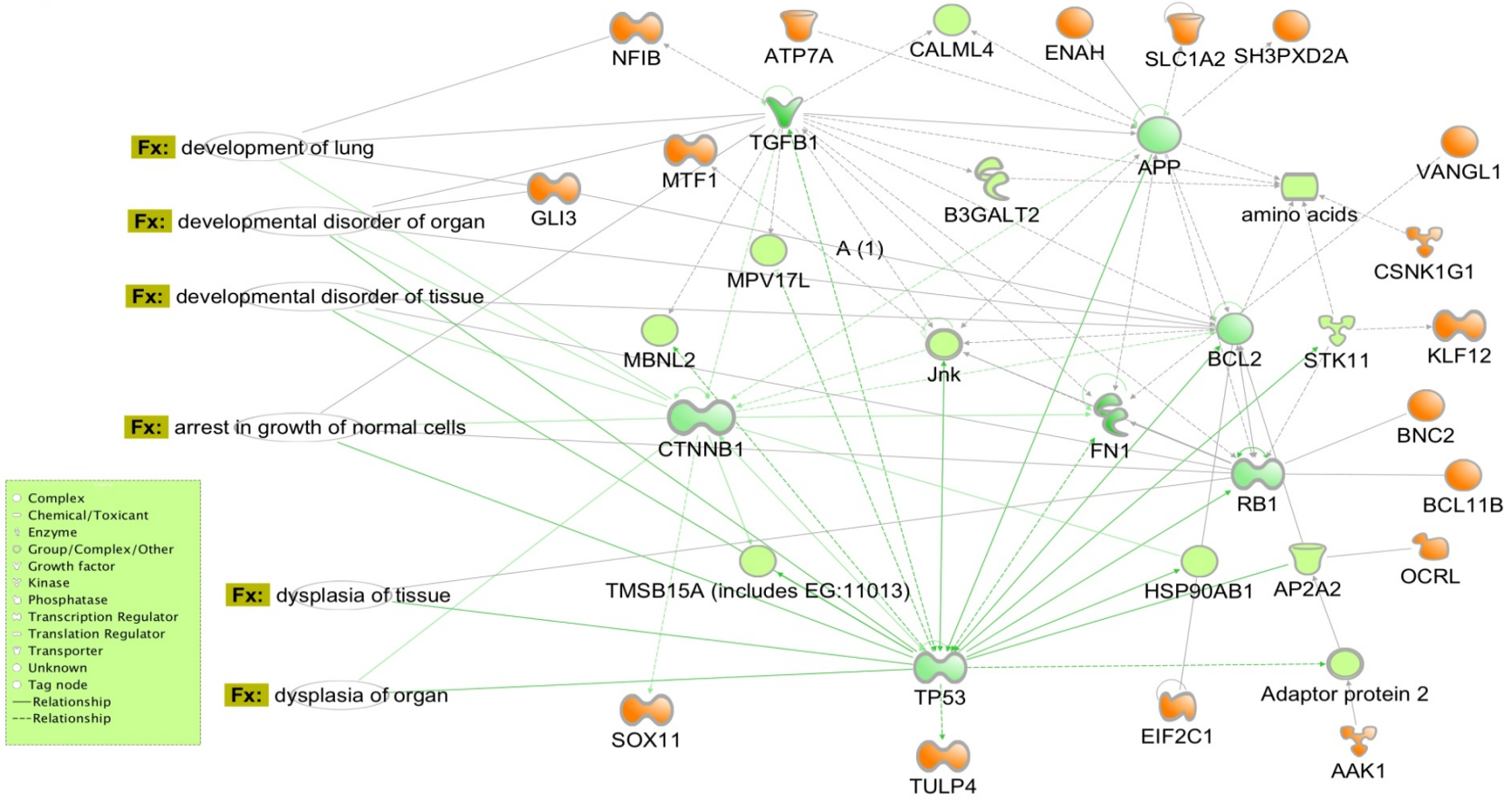

Table 1 (appendix). Genetic association studies for BPD in preterm infants.

Author (year of publication)	Subject	Race	Definition of BPD	Gene	Genetic Variant	Result
Adcock et al. (2003)	178 VLBW preterm infants	American sample	Oxgen use ≥ 28 days or at 36 week' PMA	Tumor necrosis factor-α (TNF-α) Transforming growth factor-β (TGF-β) Monocyte chemoattractant protein-1 (MCP-1)	TNF-α-308 A to G TGF-β1-915 G to C MCP-1-2518 A to G	No association of genetic variants and development of BPD or death
Kazzi et al. (2004)	154 preterm infants with body weight ≤ 1250g	American sample: White (20%), African Americans (78%), Hispanic (2%)	Oxygen use at 36 week' PMA and radiographic evidence of parenchymal lung disease	Tumor necrosis factor-α (TNF-α) Lymphotoxin-α (LT-α)	TNF-α-308 A to G TNF-α-238 A to G LT-α-250 A to G	1. The TNF-α-238 AA and GA genotypes were less likely to occur among infants with BPD than those without. 2. The number of infants with TNF-α-238 AA genotype was correlated inversely with the severity of BPD (r = -0.341)
Kazzi et al. (2005)	154 preterm infants with body weight ≤1250g	American sample: White (20%), African Americans (78%) and Hispanic (2%)	Severity defined by the NIH consensus definition of BPD	Angiotensin-converting enzyme (ACE)	ACE D (delection) and I (insertion) alleles	1. Infants with DD/DI genotype of ACE were more likely to have BPD than infants with II genotype (47% vs 22%). 2. ACE DD/DI genotype was more common among infants with moderate or severe BPD compared with infants with mild BPD (74% vs 26%).
Lin et al. (2005)	224 preterm infants with GA < 30 weeks	Taiwanese sample	Oxygen use at 36 week' PMA	Tumor necrosis factor-α (TNF-α) Interleukin 1β (IL-1β) Interleukin 1 receptor antagonist (IL1RA)	TNF-α-308 A to G IL-1β exon5: E1 homozygote and E2 heterozygote IL1RA: I homozygote and II heterozygote	No association of genetic variants and development of BPD or duration of intermittent mandatory ventilation supplement
Lin et al. (2005)	224 preterm	Taiwanese sample	Oxygen use at	The transporter	TAP1 A to G	No differences in genotype

	infants with GA < 30 weeks		36 week' PMA	associated with antigen processing 1 genes (TAP1)		distribution or allele frequency of the TAP1 polymorphisms between BPD and their respective control infants.
Yanamandra et al. (2005)	294 VLBW preterm infants	American sample: African (80%), Caucasian (19%) and Hispanic (1%)	Oxgen use ≥ 28 days or at 36 week' PMA	Interleukin 10	IL-10-1082 A to G	No association of genetic variants and the development of BPD.
Derzbach et al. (2006)	125 low-birth weight singleton infants with GA 24-36 weeks	Hungarian	Oxgen use ≥ 28 days or at 36 week' PMA	E- selectin P-selectin L-selectin	E-selectin Ser128Arg P-selectin Thr715Pro L-selectin Pro213Ser	No association between genetic variants and development of BPD or early postnatal sepsis or BPD.
Pavlovic et al. (2006)	71 preterm infants with GA < 30 weeks	American sample	Oxgen use ≥ 28 days or at 36 week' PMA	Surfactant proteins: SPs-A, B, C, and D	SP-A1 A to G SP-A2 A to G SP-B micosatellite markers SP-B A to G SP-C A to G SP-D A to G	Significant associations were observed for alleles of SP-B and SP-B microsatellite marker AAGG 6 and development of BPD.
Strassberg et al. 2007	105 preterm infants with birth weight < 1000g	American sample: Whites (32%), African (37%), Hispanics (20%) and other (11%)	Severity defined by the NIH consensus definition of BPD	Tumor necrosis factor-α (TNF-α)	TNF-α hyplotypes: -1031, -863, -857, -308, and -238	Haplotype-specific analysis revealed no significant association between the hyplotypes and the severity of BPD.
Bokodi et al. (2007)	153 VLBW preterm infants	Hungarian	Oxgen use ≥ 28 days or at 36 week' PMA	Interferon-γ (IFN-γ) Interleukin-12 (IL-12)	IFNc +874 A to T IL12 p40 promoter CTCTAA/GC genotypes	Carriers of the IFNc +874 T allele were protected against BPD (odds ratio = 0.35)

VLBW = very low birth weight; GA = gestational age; PMA = postmenstrual age; BPD = bronchopulmonary dysplasia.

Chapter 2

Behavioral and Emotional Problems in Taiwanese Preschool Children

Abstract

Background: There has been limited epidemiologic information concerning non-Western preschoolers' behavioral and emotional problems, and the data in Taiwanese preschoolers is lacking. **Objective:** This study aimed to investigate behavioral and emotional problems in a full-term population of Taiwanese preschoolers using the Child Behavior Checklist for Ages 1½-5 (CBCL/1½-5). The behavioral problems scores and prevalence of Taiwanese preschoolers were compared with those of American and Dutch preschoolers. The correlates of behavioral problems were also examined. **Methods:** Mothers of 306 Taiwanese preschoolers aged 20-60 months completed the CBCL/1½-5. The data of American and Dutch preschoolers were extracted from the previous studies and to compare with Taiwanese data. **Results:** The CBCL/1½-5 had satisfactory reliability and factorial validity for assessing Taiwanese preschoolers. Results of cross-cultural comparisons revealed that Taiwanese preschoolers exhibited higher behavioral problem scores on most of the behavioral syndromes and increased prevalence rates of internalizing-related syndromes and Sleep Problems. Younger age, male gender, and first birth order were unfavorable for behavioral performance. Girls' behavioral problems decreased with age, whereas boys' behavioral problems persistently high across ages. **Conclusions:** Results of cross-cultural comparisons and behavioral correlates are important to assist in clinical assessment and management of preschoolers' behavioral and emotional problems.

Keywords: behavioral/emotional problems, preschool, CBCL/1½-5, prevalence rate, behavioral correlates.

中文摘要

研究背景：現行對於非西方國家之學齡前兒童的行為與情緒問題流行病學資料仍顯不足，並且國內於學齡前兒童的行為與情緒問題的資料闕如。**目的：**本研究使用一歲半至五歲版本之兒童行為檢核量表做為研究工具，目的為探討臺灣足月兒於學齡前期的行為與情緒問題，並且與美國及荷蘭兒童的行為問題之分數及盛行率做比較，以及檢驗行為問題的關聯因子。**方法：**三百零六位臺灣學齡前兒童(年齡介於二十至六十個月間)的母親填寫兒童行為檢核量表一歲半至五歲版本。此研究擷取過去研究中美國與荷蘭學齡前兒童的行為問題數據與台灣的數據做比較。**結果：**使用行為檢核量表一歲半至五歲版本測量台灣學齡前兒童的行為問題呈現良好的信度及因素效度。相較於美國與荷蘭兒童的行為問題資料，臺灣學齡前兒童於多數的問題行為特徵具有較高的行為問題分數，並且在內顯相關的行為問題及睡眠問題上有較高的盛行率。許多社會人口學因子，例如較年幼的兒童、男性及第一出生序較不利於問題行為的表現。女孩的行為問題分數會隨著年齡的增長而下降，然而男孩問題行為分數則隨著年齡增加而持續維持較高的分數表現。**結論：**本研究對於臺灣學齡前兒童的行為與情緒問題提供具參考價值的流行病學資料，有關跨文化比較及行為關聯因子的結果將有助於臨床上評估與處置具行為問題之學齡前兒童。

關鍵詞：行為/情緒問題、學齡前、兒童行為檢核量表一歲半至五歲版本、盛行率、行為關聯因子

1. Introduction

1.1 Background and Purposes

Epidemiological studies in the US have revealed an increase in the prevalence of developmental or behavioral/emotional disorders in children and adolescents in the past decades, from 6% in 1979 to 18% in 1996.[1, 2] A recent national survey of American child's emotional, developmental and behavioral health has documented behavioral problems as one of the most commonly diagnosed problems among 6- to 17-year-old children.[3] Since behavioral and emotional problems at preschool ages may persist into preadolescence or adolescence, identification of such problems at preschool ages is important for early diagnosis and intervention efforts.[4] In this aspect, a dimensional approach that employs checklist measures and cut-off points for 'clinically significant' symptoms is a feasible way of assessing preschoolers' behavioral problems. Among these instruments, the Child Behavior Checklist for Ages 1½-5 (CBCL/1½-5),[5] expanded from the CBCL/2-3,[6] is a commonly used one with acceptable levels of test-retest reliability as well as construct and concurrent validity in American preschool children.[5]

Although the CBCL/2-3 or CBCL/1½-5 has been extensively used in many Western societies, its cross-cultural application to preschoolers in non-Western societies remains rare. One previous study has measured behavioral and emotional problems in the preschoolers of Mainland China using the CBCL/1½-5,[7] and two previous studies have examined the factorial structure of the CBCL/1½-5 among the preschoolers of 24 societies[8] and a sample of China girls adopted from Mainland China to the United States (U.S.).[9] Because cultural or social environments may be varied in diverse societies, there is a need to investigate behavioral and emotional problems in Taiwanese preschool children. Previous studies have found that

Taiwanese or Taiwanese-immigrant parents expressed an authoritarian style and a high discipline control of parenting to their children.[10-12] One previous study has reported higher degrees of behavioral problems on the CBCL/4-18 in Taiwanese adolescents than in American adolescents, which might be accounted for by the prevailing social emphasis on academic achievement in Taiwanese society.[13] In addition, Taiwan's fertility rate has been declining rapidly since 1960s and has reached the lowest level worldwide recently.[14, 15] Whether such a dramatic socio-behavioral shift can affect parental ratings of preschoolers' behavioral or emotional problems warrants further investigation.

There have been limited studies concerning cross-cultural comparisons on the taxonomy of preschool psychopathology. Cross-cultural comparisons for the data of behavioral problems in preschoolers may help understand whether behavioral problems are similar across societies and which levels of problems that is deviant from the preschoolers warrants clinical attention. To our knowledge, one prior study has reported multicultural comparisons of epidemiological data for the preschoolers in 24 societies, which included the data of Taiwanese preschoolers.[16] The results indicated that the mean scale scores of the CBCL/1½-5 varied across societies with the differences ranged from small to medium.[16] There is a need to further compare behavioral problem scores and the prevalence of Taiwanese preschoolers with other preschool samples.

Given the potential importance of cross-cultural psychopathology in preschoolers, it is important to examine the behavioral correlates of behavioral problems in Taiwanese preschoolers that may help early identification of at-risk children of behavioral disorders in Taiwanese society. In terms of correlates of preschoolers' behavioral problems, the number of studies to date is limited. Lower

parental education was consistently associated with more behavioral problems among 1- to 2-year-old in the US [17], 3-year-old in Finland[18] and 5- to 6-year-old in the Netherlands.[19] Parental unemployment or occupations of less prestige was associated with more behavioral problems in preschoolers of both Finland[18] and the Netherlands.[19] Some correlates were reported in single study, such as the association of elder birth order with more behavioral problems.[17] However, the relations of age and gender to preschoolers' behavioral problems remain conflicting. A study among 2- to 5-year-old American preschoolers found that boys had higher proportions of elevated Externalizing Problems or Total Problems than girls, whereas the proportion of preschoolers with elevated Internalizing Problems or Total Problems increased with age, as rated using the CBCL/2-3 or the CBCL/4-18 for appropriate age groups.[20] In contrast, there were no significant associations of gender and age with these behavioral problems among 2- to 3-year-old children in Finland,[18] Turkey[21] and Iceland[22] using the CBCL/2-3. Whether the inconsistency in the relations of age and gender to preschoolers' behavioral problems may be attributed to different instruments (CBCL/2-3 vs. CBCL/4-18) or some interactive effect between age and gender on behavioral problems requires investigation.

To fulfill the aforementioned gaps, this study aimed to investigate behavioral and emotional problems in a preschooler sample of ages covering the applicable range of the latest version of CBCL/1½-5 in Taiwan. The specific aims of this study were: (1) to investigate the reliability and construct validity of the CBCL/1½-5 when used with Taiwanese preschoolers; (2) to compare the behavioral problems of Taiwanese sample with the American and Dutch counterparts; and (3) to examine the relations of socio-demographic variables to behavioral problems in Taiwanese preschoolers.

2. Methods

2.1 Participants

The inclusion criteria for participating children were: gestational age within 37 to 42 weeks and no prenatal or perinatal complications. The study children consisted of two parts. Children in the first part were drawn from a multi-hospital based birth cohort study that longitudinally examined the development of full-term children from birth to 36 months of age during the years of 2004-2005.[23, 24] Children were recruited at neonatal stage from one medical center, one regional hospital and two local obstetric clinics in northern Taiwan. A copy of the CBCL/1½-5 was sent to mothers when the children were at 24-36 months of age. The second part of the sample was to supplement the first part for the age range applicable for the CBCL/1½-5 by means of recruiting children from four kindergartens in central Taiwan at around 18-24 or 36-60 months of age in year 2009. The study was approved by the institutional review boards of the participating hospitals. Written informed consent was obtained from parents after a complete description of the study.

Child's perinatal and demographic data were abstracted from medical charts and via parental interview. Perinatal variables included gestational age, birth weight, birth set, and intrauterine growth status. Demographic variables included child's age, gender, birth order, parental education and occupation and marital status. Parental education was stratified into ≤ 12 years and > 12 years, and parental occupation was stratified into professional/technician and unskilled labor/unemployed.

Out of the 444 eligible children in the first part, aged 24-36 months, 249 (56%) had their mothers completing the CBCL/1½-5. Meanwhile, out of 108 invited to participate in the second part, the mothers of 16 children aged 20-24 months and 41 children aged 36-60 months completed the questionnaire, with a participation rate of 53%. Of the 306 participating children, mean child age was 29.6 months (SD = 9.4)

with their mean gestational age of 39 weeks (SD = 1 week) and mean birth weight of 3,259 g (SD = 407 g). Most of the children were singletons, with only 1% (N = 4) as twin birth. Boys accounted for 54% of the total sample and almost half of the children (46%) were first-born. Only 2% of children were from single parent family. Approximately three-fourths of mothers (77%) and fathers (78%) had an educational level of >12 years, which was comparable to the live-birth census of northern and central Taiwan in the year of 2008.[25] The proportions of parental occupation as professional/technician were 70% in mothers and 88% in fathers.

2.2 Measurement

The CBCL/1½-5, a parental report of child behavioral, emotional and social functioning at ages 1.5 to 5 years, consists of 100 items, in which 99 items assess specific behavior problems and one item is blank for parents to note child behavior problems not listed in the questionnaire.[5] Each item is scored on a 3-point scale: 0 as "not true of the child," 1 as "somewhat or sometimes true," and 2 as "very true or often true." The sum of the 100 items is counted as the Total Problems score, whereas 67 of them were scored specifically to seven narrow-band behavioral syndromes, including Emotionally Reactive (9 items), Anxious/Depressed (8 items), Somatic Complaints (11 items), Withdrawn (8 items), Sleep Problems (7 items), Attention Problems (5 items), and Aggressive Behavior (19 items). In addition, two broad-band behavioral syndromes are derived, with the former four syndromes constituting the Internalizing syndrome and the latter two syndromes constituting the Externalizing syndrome.

For the categorization of individual narrow-band syndrome into either borderline or clinical range, the 93rd and 97th percentiles were chosen as the cut-off points, respectively. Similarly, the 83rd and 90th percentiles were chosen as the cut-off

points for the broad-band syndrome and the Total Problems. A normative data set for the CBCL/1½-5 in the US sample of 18- and 71-month-old children (n = 700) has been established, with thorough evaluations of internal consistency, 8-day test-retest reliability, factorial structure, and predictive validity [5].

On the basis of a Hong-Kong adaptation of the CBCL/1½-5 for Chinese language (Dr. Patrick Leung, personal communication), ten items were modified in this study for the accommodation of the terms used in Taiwan. The scale was mailed to mothers to score their children's behavioral performance and then was returned via mail. In a subsample of 25 families, both parents were asked to re-score the CBCL/1½-5 two months later, with one father failing to return the questionnaire. The data from mothers (n = 25) were used for test-retest reliability while those of fathers (n = 24) were used for inter-parent agreement evaluation.

2.3 Statistical Analysis

The internal consistency of the CBCL/1½-5 was examined using Cronbach's alpha coefficient, following conventional interpretations that a value of ≥ 0.8 represents an excellent one and a value within 0.6-0.8 as a good one.[26] Test-retest reliability and inter-parent agreement were examined using the intra-class correlation coefficient (ICC) with 95% confidence interval (CI), with a value of ≥ 0.75 as excellent, a value between 0.40 and 0.75 as fair to good, and a value of < 0.40 as poor.[27]

The construct validity of the scale was examined using the confirmatory factor analysis (CFA) to determine the goodness of fit of seven-factor structure on 67 behavioral problem items as defined by Achenbach and Rescorla.[5] Each item was assigned to only one factor in the correlated seven-syndrome model. Tetrachoric correlations between items scored 0 vs. 1 or 2 were used to avoid statistical risks

associated with low frequency cells of scoring 2 in the sample. The degree of model fit was assessed using both the Comparative Fit Index (CFI), measuring the proportionate improvement in fit by comparing a target model with a restricted baseline model, and the Root Mean Square Error of Approximation (RMSEA), taking into account the error of approximation in the population correlation matrix that addresses how well a baseline model reproduces the sample data.[28] A good fit is indicated by a CFI of > 0.9 and a RMSEA of ≤ 0.06.[28] Standardized loading coefficient of each item loaded on specific factor was also estimated in the correlated model. The CFA procedure was conducted using the Mplus program (version 4.2, Muthén & Muthén, Los Angeles, CA, USA).

For cross-cultural comparison, the mean and standard deviation (SD) of the syndrome scores on the CBCL/1½-5 in Taiwanese sample were compared with those of American[5] and Dutch children[29] using Student's *t*-test. As the SD estimates were not reported in the Dutch study, those of the American sample were used as their proxies for the comparison analysis. Effect size (Cohen's *d*) was calculated for the differences in mean scores using the pooled SD of Taiwanese and American samples, with an effect size of < 0.5 being considered as small, 0.5–0.8 as median, and 0.8 as large.[30] Meanwhile, the prevalence rates of problem behaviors above the borderline and clinical cutoff points were estimated for the Taiwanese preschoolers based on the American normative data.[5] For cross-cultural comparisons in the magnitude of prevalence between studies, odds ratios (ORs) were used.

Socio-demographic variables were examined for their relations with Internalizing, Externalizing, and Total Problems scores, respectively, using univariable linear regression analysis. For any variables that showed potential association with any one of the three behavioral problem scales at a less stringent

criterion ($p < 0.10$), a multivariable analysis including all of these variables along with their pairwise interactions was further conducted. All statistical analyses in this study were performed using the software SAS program, version 9.1 (SAS Institute, Cary, NC). A p value of < 0.05 was considered statistically significant.

3. Results

3.1 Reliability and Construct Validity

Good to excellent internal consistency ($\alpha = 0.60$-0.95) and fair to excellent test-retest reliability were found for the syndromes and Total Problems scores (ICCs = 0.52-0.84) of the CBCL/1½-5 in the Taiwanese sample (Table 1). Inter-parent agreement was fair to excellent for eight out of nine syndromes and Total Problem scores (ICCs = 0.4-0.84), except for poor agreement on Sleep Problems (ICC = 0.25).

The CFA revealed that the CFI and RMSEA for the seven-factor model was 0.914 and 0.051, respectively, indicating a good model fit. All 67 items loaded significantly on their predicted factors, with a mean item loading of 0.59 (range 0.37-0.91, all $p < 0.05$). The mean factor loading by syndrome ranged from 0.55 (Aggressive Behavior) to 0.68 (Anxious/Depressed).

3.2 Cross-Cultural Comparisons in Behavioral Problems

Compared with the American or Dutch counterparts, Taiwanese preschoolers exhibited higher scores on all seven narrow-band syndromes except for Attention Problems, which did not reach statistical significance when compared with the American sample. The effect size tended to be larger, many reaching moderate or even large, for those syndromes belonging to Internalizing than those of Externalizing, whereas the effect sizes of Sleep Problems were moderate in the comparisons. This was consistent with the pattern for the broad-band syndromes, with the Internalizing syndrome having a two-fold greater effect size than the Externalizing syndrome.

When all items were counted, the increased scores on Total Problems for Taiwanese preschoolers had a moderate effect size versus the American (d = 0.52) or Dutch counterparts (d = 0.64).

Similar to the pattern in raw scores, the prevalence rate of the behavioral problems above the borderline cutoffs for Taiwanese preschoolers were significantly higher than the American or Dutch sample in those narrow-band syndromes belonging to Internalizing as well as Sleep Problems (ORs ranging from 1.9 to 5.5), except Emotionally Reactive versus the Dutch sample and Anxious/Depressed versus the American sample (Table 3). In contrast, there were no significant differences in Attention Problems or Aggressive Behavior in the comparisons with the American or Dutch sample. In terms of broad-band syndrome or Total Problems, Taiwanese preschoolers had higher prevalence of both Internalizing syndrome and Total Problems, with an OR ranging from 2.2 to 4.5, whereas Externalizing syndrome failed to show significant difference.

As for the prevalence rate above the clinical cutoffs in Taiwanese preschoolers, approximately one-fifth of children (22.9%) showed the Total Problems score in the clinical range. The prevalence rate of Internalizing syndrome (23.2%) was two times higher than that of Externalizing syndrome (10.8%). The three most frequently reported syndromes were Somatic Complaints (15.4%), Withdrawn (12.4%), and Sleep Problems (8.5%).

3.3 Socio-Demographic Correlates of Behavioral Problems

Univariable regression analyses revealed that there were several potential correlates of behavioral problems, including male gender for the Internalizing, age and gender for the Externalizing, as well as age, gender and birth order for the Total Problems. For the Externalizing and the Total Problems, their potential correlates

were then subjected to multivariable regression analysis (Table 4). The results indicated that age and age-gender interaction had significant associations with the Externalizing (all $p < 0.05$). Meanwhile, age, birth order, and age-gender interaction were significantly associated with the Total Problems (all $p < 0.05$).

To illustrate the effect of the interaction terms on the Externalizing or the Total Problems, these two behavioral problem scores are plotted against age separately for girls and boys (Figure 1). Boys exhibited persistently high Externalizing score from 20 (mean score = 16.1) to 60 months of age (mean score = 15.8), whereas girls showed a gradual decline in Externalizing score from 20 (mean score = 16.5) to 60 months of age (mean score = 8.5) (Figure 1A). Similarly, boys manifested persistently high Total Problems score from 20 (mean score = 47.2) to 60 months of age (mean score = 44.3), whereas girls exhibited a gradual decline in Total Problems score from 20 (mean score = 45.5) to 60 months of age (mean score = 21.5) (Figure 1B).

4. Discussion

This study demonstrated that the CBCL/1½-5 had acceptable reliability and construct validity in Taiwanese preschoolers, and cross-cultural comparisons revealed that Taiwanese preschoolers manifested higher behavioral problems and prevalence rates of behavioral problems, particularly in the Internalizing-related syndromes. Furthermore, girls showed a gradual decline in behavioral problems with age, whereas boys exhibited persistently high behavioral problems over age. Our results may provide new insights into parental perception of child's behavioral problems and the assessment and management of these problems in non-Western society.

For the results of reliability and construct validity of the CBCL/1½-5, our internal consistency values were similar to those of American counterparts and model fit of the seven-factor structure resembled those of American preschoolers [5]

and Chinese preschool girls adopted by Western parents.[9]. However, our 2-month test-retest reliability coefficients (0.52-0.84) were lower than those of 8-day test-retest reliability in American children (0.68-0.92). [5] The discrepancy might be due to our longer interval between the two measures. In addition, unlike the consistent parental rating on Sleep Problems (Pearson $r = 0.7$) in Dutch data,[31] poor inter-parent agreement was noted in Sleep Problems, with 75% of Taiwanese mothers reporting a higher score than fathers did. One possibility is that Taiwanese fathers may have less chance to note child's sleeping problems as Taiwanese mothers remained as the main caregiver of the infants. These results provide some support to the cross-cultural reliability and factorial validity of the CBCL/1½-5.

Taiwanese preschoolers exhibited high raw scores and prevalence rate on most CBCL/1½-5 scales. Our children's Total Problems scores (43.6) were higher than those of the preschoolers in Canada,[32] Netherlands,[29, 33] Finland,[18] Italy,[34] Iceland[22] and Turkey (27.5-39.5).[21] The discrepancy may be attributed in part to differences in the sampling, with our sample being birth cohort and regional school-based, whereas others being regional household-based. Moreover, social and cultural differences may also influence maternal perception and expectation of child behavior.[35, 36]

It is intriguing to find that the higher scores and prevalence rates were mostly on the Internalizing-related syndromes. This finding has contrasted the data of 2-year-old American children that showed a lower prevalence of Internalizing score (6.7%) than of Externalizing score (9.8%)[17]. The results might be due to the more emphasis of physical growth and health status by Taiwanese parents who have considered child's somatic problems as the major concern over the other behavior problems.[37] Several cross-cultural studies showed that Taiwanese parents and Taiwanese immigrant parents more often adopt a higher control and an authoritarian

style of parenting than Caucasian-American parents do.[10, 12] Some parents would like to coach their child appropriate behaviors when their child misbehaves or shows bad manners.[11, 38] Children may therefore be reluctant to express their emotion or thoughts under strict discipline. Yang et al. (2000) have observed higher Internalizing and Somatic Complaints scores in Taiwanese adolescents than in American adolescents. Moreover, Taiwanese adolescent girls exhibited higher Withdrawn syndrome scores than American adolescent girls did.[13] A longitudinal follow-up of our sample is necessary to investigate if the Internalizing problems persist into adolescence. Besides, higher Sleep Problems scores and prevalence rate were found in Taiwanese preschoolers. It may be explained by the fact that most Taiwanese children have co-slept with their families since birth. A Finish study demonstrated that 3-year-old children who co-slept with their parents had higher Sleeping Problems scores than those who did not.[18] The lack of independent sleeping experience may lead to difficulty of sleeping alone in Taiwanese preschoolers.

Child's gender, age and birth order were identified as the socio-demographic correlates for the behavioral problems in Taiwanese preschoolers. Boys exhibited persistently high Internalizing score from 20 to 60 months of age, whereas girls displayed a gradual decline in Externalizing and Total Problems score over age. Previous studies on the age and gender effects on preschooler's behavioral problems have revealed conflicting results. There was no age- or gender-related difference among 2- to 3-year-old children,[18, 21, 22] whereas one prior study has reported unfavorable effect on 2- to 3-year-old boys and elevated behavioral problems with ages as rated using the CBCL/2-3 or the CBCL/4-18 for appropriate age groups.[20] Our finding concerning an interactive effect between age and gender on preschooler's behavioral problems has important implications for clinical assessment and

management of behavioral problems. In Taiwan, the recent decline of fertility rate in women (1.03 in 2009)[25] have great impacts on family structure and school environment.[13, 39] Whether such social changes could result in unexpected discipline problems or alter maternal perception of behavior toward children of different gender is worth further investigation. Yang et al. (2000) reported that Taiwanese adolescent boys obtained higher Externalizing and Total Problems scores but lower Internalizing scores than girls. Furthermore, Wang et al.[40] found the prevalence estimate of attention deficit hyperactivity disorder in Taiwanese school boys to be three times higher than girls' (14.9% vs. 4.5%). A continued study will help understand the developmental trajectories of behavioral and emotional problems in Taiwanese boys and girls.

First-born children were more likely to have higher Total Problems scores than those of middle or younger children. Our observation was similar to the data of 1- to 2-year-old American children.[17] One prior study has found a smaller family size may increase parental alertness to detect children's behavior problems.[20] The association of first birth order with adverse behavioral performance may relate to the overly protective parenting or more attention toward the first child. Besides, no association was found for parental education and occupation with behavioral problems. This result was incongruent with the prior findings that parental education below high school) or occupation as low-skilled level or unemployment had adverse effects on behavioral outcome among the preschoolers in Finland, Netherlands and America (OR = 2-4).[17-19] The discrepancy may be attributed to our sample was mostly drawn from the urban cities, greater social and educational resources in the urban area may decrease the adverse effect of low parental education or occupation on child behavioral outcome. In addition, a prior study has found that 6- to 24-months-old

Taiwanese infants in northern city had superior mental development than those in the southern rural area, which may be accounted for by authoritative and permissive parenting in mothers of urban area.[37] Whether the sampling region may have impact on child behavioral outcome warrants further investigation.

This study has two limitations of note. First, our response rate (53%-56%) was lower than those of prior studies (70%-84%). The exact prevalence rate of behavioral problems may differ slightly from the reported data. Second, this study included only parental report of child behavior. The lack of teacher's observation may limit the information concerning child's behavioral performance in the school context.

We conclude that the CBCL/1½-5 is a reliable and valid instrument for assessment of preschoolers' behavioral and emotional problems in Taiwan. Taiwanese preschoolers exhibited high behavioral problem scores particularly in Internalizing-related syndromes that warrant continued follow-up. Furthermore, the identified socio-demographic correlates for the behavioral problems may assist in clinical assessment and management of preschooler's behavioral and emotional problems.

5. Acknowledgement

This study was supported by a grant from the National Health Research Institute (NHRI-EX98-9519PI) and a grant from the Department of Health (DOH93-HP-1702) in Taiwan. We thank the infants and parents for their participation in this study, and the medical staff of National Taiwan University Hospital, En Chu Kong Hospital, Bobson obstetric clinic and Yuoson obstetric clinic for their assistance in data collection.

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Figure Legends

Figure 1. Illustration of the relations of (A) Externalizing and (B) Total Problems scores to age for girls and boys separately.

Table 2-1. Reliability data for the CBCL 1½-5 scales in Taiwanese children

Scales	Internal Consistency (n = 306)	Test-Retest Reliability (n = 25)	Inter-Parent Agreement (n = 24)
	Cronbach's α	ICC (95% CI)	ICC (95% CI)
Narrow-band Syndrome			
Emotionally Reactive	0.71	0.77 (0.54-0.89)	0.67 (0.38-0.84)
Anxious/Depressed	0.68	0.70 (0.42-0.86)	0.40 (-0.001-0.68)
Somatic Complaints	0.60	0.70 (0.41-0.86)	0.58 (0.24-0.80)
Withdrawn	0.72	0.52 (0.16-0.76)	0.52 (0.15-0.76)
Sleep Problems	0.65	0.72 (0.46-0.87)	0.25 (-0.17-0.59)
Attention Problems	0.66	0.54 (0.18-0.77)	0.66 (0.35-0.84)
Aggressive Behavior	0.88	0.79 (0.57-0.90)	0.82 (0.63-0.92)
Broad-band syndrome			
Internalizing	0.88	0.76 (0.51-0.89)	0.56 (0.22-0.79)
Externalizing	0.90	0.84 (0.67-0.93)	0.84 (0.66-0.93)
Total Problems	0.95	0.80 (0.60-0.91)	0.67 (0.38-0.85)

ICC = intraclass correlation coefficient

Table 2-2. Comparison of raw score of the CBCL 1½-5 Scales in Taiwanese children with American and Dutch children.

Scales	Raw Score, Mean (SD)			Cross-Cultural Comparison, Effect Size (Cohen's d)	
	Taiwan (n = 306)	US (n = 700)	Netherlands (n = 279)	Taiwan vs. US	Taiwan vs. Netherlands
Narrow-band Syndrome					
Emotionally Reactive	2.9 (2.3)	2.4 (2.2)	2.15	0.22 [b]	0.33 [c]
Anxious/Depressed	3.5 (2.2)	3.0 (2.3)	1.59	0.22 [b]	0.87 [c]
Somatic Complaints	2.8 (2.3)	1.9 (1.9)	1.86	0.43 [c]	0.41 [c]
Withdrawn	2.2 (2.1)	1.7 (1.7)	1.19	0.26 [b]	0.48 [c]
Sleep Problems	4.0 (2.3)	2.9 (2.4)	2.41	0.47 [c]	0.69 [c]
Attention Problems	2.8 (1.8)	2.6 (1.9)	2.41	0.11	0.22 [a]
Aggressive Behavior	12.0 (6.1)	10.5 (6.4)	10.84	0.24 [c]	0.19 [a]
Broad-band syndrome					
Internalizing	11.4 (7.1)	8.7 (6.3)	6.80	0.40 [b]	0.65 [c]
Externalizing	14.8 (7.4)	13.1 (7.8)	13.25	0.22 [b]	0.21 [a]
Total Problems	43.6 (20.5)	33.4 (18.8)	30.52	0.52 [c]	0.64 [c]

Note: The raw scores of the US and Dutch samples were from Achenbach and Rescorla (2000) and Tick et al. (2007) respectively.

[a] $p < 0.05$, [b] $p < 0.005$, [c] $p < 0.0005$

Table 2-3. Comparison of the prevalence rates of the CBCL 1½-5 Scales in Taiwanese children with the Dutch children.

Scales	Prevalence Rate (Above Borderline Cutoff) (%)			Cross-Cultural comparison, Odds Ratio (95%CI)	
	Taiwan (n = 306)	US (n = 700)	Netherlands (n = 279)	Taiwan vs. US	Taiwan vs. Netherlands
Narrow-band Syndrome					
Emotionally Reactive	12.4	7	12.6	1.9 (1.4-2.3) [a]	1.0 (0.5-1.5)
Anxious/Depressed	8.8	7	2.5	1.3 (0.8-1.8)	3.8 (2.9-4.6) [a]
Somatic Complaints	21.6	7	10.8	3.7 (3.3-4.1) [c]	2.3 (1.8-2.7) [b]
Withdrawn	12.4	7	2.5	1.9 (1.4-2.3) [a]	5.5 (4.7-6.3) [c]
Sleep Problems	15.7	7	3.6	2.5 (2.1-2.9) [c]	5.0 (4.3-5.7) [c]
Attention Problems	7.5	7	5.8	1.1 (0.6-1.6)	1.3 (0.7-2.0)
Aggressive Behavior	10.8	7	7.6	1.6 (1.1-2.1)	1.5 (0.9-2.0)
Broad-band syndrome					
Internalizing	31.0	17	12.9	2.2 (1.9-2.5) [c]	3.0 (2.6-3.5) [c]
Externalizing	21.9	17	15.8	1.4 (1.0-1.7)	1.5 (1.1-1.9)
Total Problems	36.0	17	11.2	2.8 (2.4-3.1) [c]	4.5 (4.0-4.9) [c]

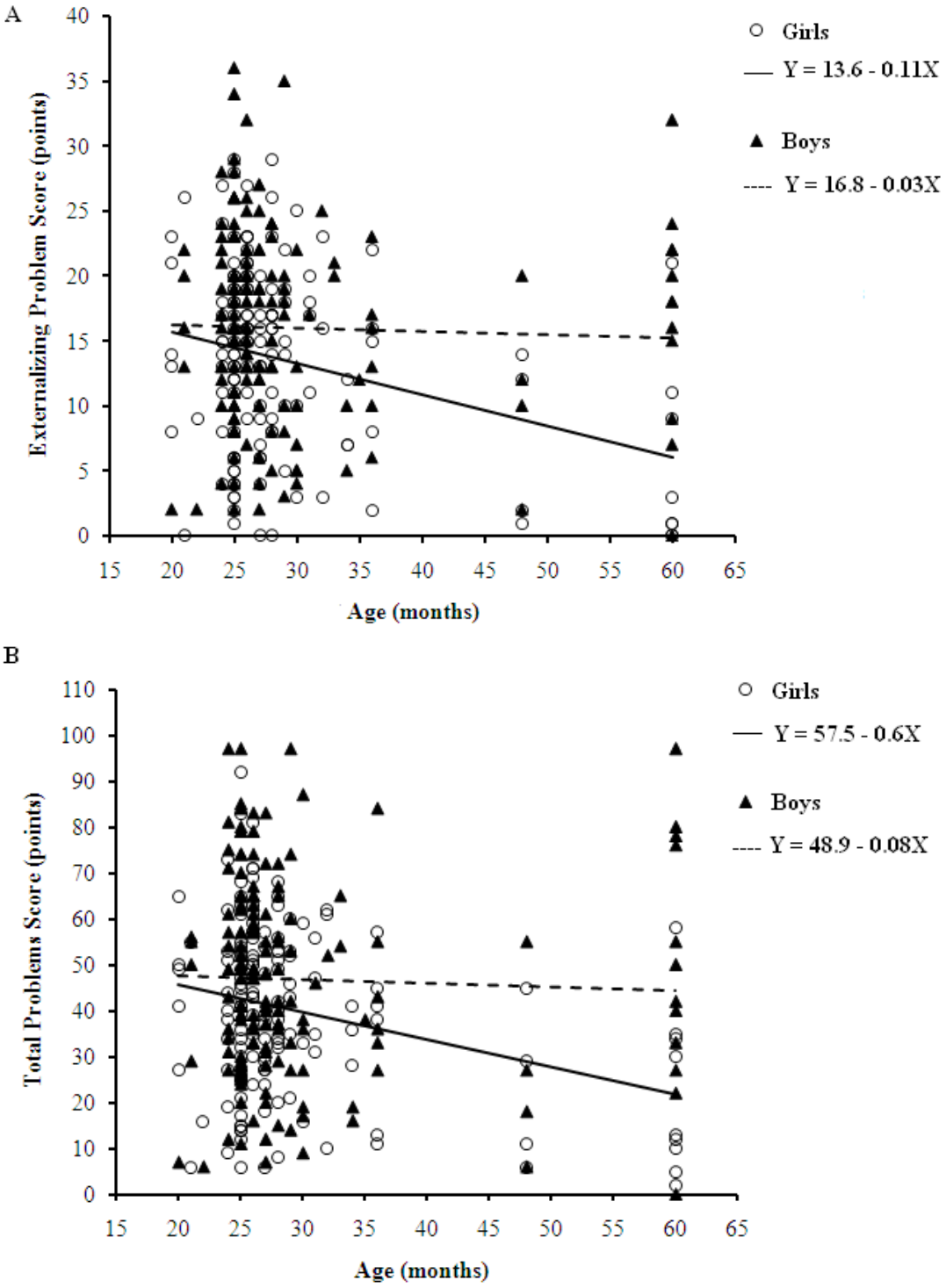
Note: The prevalence rates US sample were based on the borderline definition of the CBCL 1½-5 (Achenbach & Rescorla, 2000). The prevalence rates of Dutch sample were from Tick et al. (2007)

[a] $p < 0.05$, [b] $p < 0.005$, [c] $p < 0.0005$

Table 2-4. Multivariable regression analysis for the relations of socio-demographic variables and the Internalizing, Externalizing and Total Problems scores

Variable	Internalizing Score			Externalizing Score			Total Problems Score		
	β	SE	*P*	β	SE	*P*	β	SE	*P*
Boy (vs. girl)	2.1	0.8	0.01	3.7	2.7	0.17	10.2	10.4	0.32
Age (per month increase)				-0.5	0.1	0.002	-1.8	0.6	0.001
First-born child (vs. middle or younger)							17.0	7.9	0.03
Age×gender				0.2	0.1	0.01	0.6	0.2	0.02
Age×birth order							0.4	0.2	0.08
Gender×birth order							0.3	4.6	0.95

SE = standard error.


A
Externalizing Problem Score (points)
Age (months)
Girls
Y = 13.6 - 0.11X
Boys
Y = 16.8 - 0.03X
B
Total Problems Score (points)
Age (months)
Girls
Y = 57.5 - 0.6X
Boys
Y = 48.9 - 0.08X

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