รายงานวิจัยฉบับสมบูรณ์

โครงการ การค้นหาชีวโมเลกุลที่จำเพาะต่อการวินิจฉัยและพยากรณ์โรคไตอักเสบลูปัส

(The molecular profiling of urine and kidney tissue: biomarkers discovery in lupus nephritis)

โดย รศ.นพ. ยิ่งยศ อวิหิงสานนท์

2553

สัญญาเลขที่ MRU5080076

รายงานวิจัยฉบับสมบูรณ์

โครงการ การค้นหาชีวโมเลกุลที่จำเพาะต่อการวินิจฉัยและพยากรณ์โรคไตอักเสบลูปัส
(The molecular profiling of urine and kidney tissue: biomarkers discovery in lupus nephritis)

โดย รศ.นพ. ยิ่งยศ อวิหิงสานนท์
คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

บทคัดย่อ

รหัสโครงการ :RMU5080076

ชื่อโครงการ : การค้นหาชีวโมเลกุลที่จำเพาะต่อการวินิจฉัยและพยากรณ์โรคไตอักเสบลูปัส

ชื่อนักวิจัย : รศ.นพ. ยิ่งยศ อวิหิงสานนท์

คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

E-mail Address: yingyos.a@gmail.com

ระยะเวลาโครงการ : 3 ปี (1 มิถุนายน 2550 - 31 พฤษภาคม 2553)

Systemic lupus erythematosus (SLE) เป็นโรคทาง autoimmune ที่เป็นปัญหาสำคัญและมีความชุกสูงในคนไทยและชาวเอเชีย โดยเฉพาะอาการไตอักเสบลูปัส (Lupus Nephritis; LN) นั้นสามารถนำผู้ป่วยไปสู่โรคไตวายเรื้อรังหรือเสียชีวิตได้ การศึกษาที่ผ่านมาผู้วิจัยพบการแสดงออกที่แตกต่างกันของยีนในกลุ่ม Chemokine และ Growth factor ในปัสสาวะของผู้ป่วย LN สามารถใช้ในการวินิจฉัยและพยากรณ์โรคได้ ดังนั้นการศึกษานี้มีวัตถุประสงค์เพื่อค้นหาชีวโมเลกุลอื่นๆภายในชิ้นเนื้อไต, เลือด และปัสสาวะของผู้ป่วย LN ซึ่งอาจมีความสัมพันธ์กับการเกิดพยาธิสภาพภายในไต, ความรุนแรงของโรค รวมทั้งสามารถใช้เป็นเครื่องหมายชีวภาพ (Biomarker) ในการทำนายการตอบสนองต่อการรักษาในช่วงเวลาสั้นๆ (short-term outcome) ได้ ผู้วิจัยแบ่งการศึกษาออกเป็น 3 ส่วน ได้แก่ การแสดงออกของยีน (mRNA expression), การแสดงออกของโมเลกุลบนผิวเซลล์ในปัสสาวะ (Urinary cells type specific) และการแสดงออกของโปรตีน (Proteomic) ผลการศึกษาพบว่าในผู้ป่วย LN มีการแสดงออกของยีน Vascular Endothelial growth Factor (VEGF), Heme oxygenase 1 (HO-1), Wilms' tumour suppressor gene (WT1), Forkhead box P3 (FOXP3) และ Notch-1 แตกต่างจากคนปกติอย่างมีนัยสำคัญทางสถิติ โดยเฉพาะการแสดงออกของยีน VEGF ในเนื้อไตซึ่งมีปริมาณลดลงในผู้ป่วยที่มีอาการรุนแรง (Class IV LN) และสัมพันธ์กับความรุนแรงของโรคและการเกิดไตวายเรื้อรังในระยะเวลาอันใกล้ด้วย ซึ่งผู้วิจัยพบว่าการลดลงดังกล่าวมีความสัมพัธ์กับการเพิ่มขึ้นของการแสดงออกของ VEGF และ WT-1 ในปัสสาวะ ดังนั้นผู้วิจัยได้มีสมมติฐานเพิ่มเติมว่าเซลล์ที่หลุดมาในปัสสาวะส่วนหนึ่งเป็นเซลล์ Podocytes ภายในไต จึงได้ตรวจวัดชนิดของเซลล์ในปัสสาวะด้วยเทคนิค Flow Cytometry และใช้โปรตีน WT-1 ซึ่งจำเพาะบนผิวเซลล์ของเซลล์ Podocytes พบว่าผู้ป่วยที่มีอาการกำเริบของโรค (active LN) มีเซลล์ podocytes หลุดออกมามากกว่าผู้ป่วยที่ไม่มีอาการกำเริบของโรค (inactive LN) อย่างมีนัยสำคัญทางสถิติ ซึ่งสอดคล้องกับการแสดงออกของยีนข้างต้น นอกจากนี้ผู้วิจัยได้ศึกษาการแสดงออกของโปรตีน (Proteomic) ในปัสสาวะด้วยวิธี 2-Dimension Gel พบว่าโปรตีน Prostaglandin H2 D-isomerase (PGD2) มีความแตกต่างกันในผู้ป่วย LN และคนปกติ นอกจากนี้เมื่อวัดปริมาณการแสดงออกของโปรตีน Interferon gamma-induced protein 10 kDa (IP-10) ก็พบว่าผู้ป่วย LN มีปริมาณโปรตีนดังกล่าวสูงกล่าวคนปกติ โดยเฉพาะผู้ป่วย active LN จะมีปริมาณโปรตีน

ดังกล่าวสูงกว่าผู้ป่วย inactive LN อีกด้วย ทั้งนี้การลดลงของปริมาณโปรตีน IP-10 ยังมีความสัมพันธ์กับตอบสนองต่อการรักษาด้วย จากการศึกษาทั้งหมดข้างต้นแสดงให้เห็นว่าการแสดงออกของยีน VEGF และ WT-1 (podocyte marker) และโปรตีน IP-10 และ PGD2 ในปัสสาวะสามารถใช้ประโยชน์เป็น non-invasive marker ในผู้ป่วย LN โดยที่การแสดงออกดังกล่าวสอดคล้องกับความรุนแรงและการตอบสนองต่อการรักษาในช่วงเวลาสั้น ๆ (short-term outcome) อีกด้วย

คำหลัก : โรคไตอักเสบลูปัส, Vascular Endothelial growth Factor, Forkhead box P3, Notch-1, Interferon gamma-induced protein 10 kDa, เซลล์ Podocyte, การแสดงออกของยีน , Real-Time PCR, Proteomic

Abstract

Project Code: RMU5080076

Project Title: The molecular profiling of urine and kidney tissue: biomarkers discovery in lupus nephritis

Investigator: Assoc. Prof. Yingyos Avihingsanon

Faculty of Medicine, Chulalongkorn University

E-mail Address: yingyos.a@gmail.com

Project Period: 3 years (1 June 2007 – 31 May 2010)

SLE is the most common autoimmune disease in Thailand and Asian countries. Lupus nephritis, the severe form of SLE, could lead to the end-stage kidney disease or death. We formerly studied urine mRNA expression of chemokines and growth factors as diagnostic / prognostic markers of lupus nephritis. In this project, we aimed to discover biomarkers in serum, urine and kidney tissue of patients with lupus nephritis. These markers might be associated with severity of renal histology, renal function or response to treatments. The project was divided into part 1. mRNA expression study, 2. Urinary cell-surface marker study, and 3. Proteomics study. We found Vascular endothelial growth factor (VEGF), Heme oxygenase-1 (HO-1), Wilm's tumor suppressor gene (WT-1), Fox-head box P3 (FoxP3) and Notch-1 associated with LN. In particular, VEGF was associated with disease severity and end-stage kidney disease. The reduction of VEGF expression was partly caused by podocytes loss into urine. The decreased VEGF levels were associated with poor renal outcomes. This may lead to the novel molecular classification which may be complement to the current diagnostic method of renal biopsy. In proteomics study, the 2-D PAGE method was used and discovered Prostaglandin-H2D isomerase. Furthermore, urine Interferon-inducible protein-10 (IP-10) was found to be useful urinary biomarkers for monitoring response to treatment.

In conclusion, this project discovered and validated a number of biomarkers for development of diagnostic / monitoring / prognostic tests.

Keywords: Vascular Endothelial growth Factor, Forkhead box P3, Notch-1, Interferon gamma-induced protein 10 kDa, Podocyte, gene expression, Real-Time PCR, Proteomics

<u>สรุปโครงการ</u> (Executive Summary)

1. ชื่อโครงการ การค้นหาชีวโมเลกุลที่จำเพาะต่อการวินิจฉัยและพยากรณ์โรคไตอักเสบลูปัส (The molecular profiling of urine and kidney tissue: biomarkers discovery in lupus nephritis)

2. ชื่อหัวหน้าโครงการ

ชื่อ-นามสกุล	ยิ่งยศ อวิหิงสานนท์
คุณวุฒิ	พ.บ.
ตำแหน่ง	รองศาสตราจารย์
โทรศัพท์	02-256-4251 ต่อ 204, E-mail: yingyos.a@gmail.com

3. ปัญหาที่ทำการวิจัย และความสำคัญของปัญหา

Systemic lupus erythematosus (SLE) เป็นปัญหาสาคัญที่พบได้บ่อยในประเทศไทย รายงานต่างๆ แสดงถึงบทบาทของความหลากหลายทางพันธุกรรมต่อการเกิดโรคและการดาเนินโรค โดยพบว่าผู้ป่วย SLE ที่เป็นชาวเอเชียมีโอกาสเกิดภาวะไตอักเสบจากลูปัสได้รวดเร็วและมีแนวโน้มที่รุนแรงกว่าชาวตะวันตก รายงานในประเทศไทยพบว่ามีอัตราการตายของผู้ป่วยลูปัส สูงกว่าประเทศทางตะวันตก ปัจจัยที่เชื่อว่าน่าจะมีบทบาทสาคัญ ได้แก่ ปัจจัยทางพันธุกรรมและเชื้อชาติ

ภาวะไตอักเสบจากลูปัสเป็นภาวะที่เกิดได้บ่อย และมักจะมีความรุนแรงมากในผู้ป่วยที่เป็นโรคลูปุส หนึ่งในสามของผู้ป่วยจะเกิดมีไตอักเสบซึ่งอาจนำไปสู่การเสียชีวิตหรือพัฒนาไปสู่ไตวายเรื้อรังระยะสุดท้าย ผู้ป่วยส่วนใหญ่มีการอาการกำเริบภายในห้าปีหลังจากได้รับการวินิจฉัยโรค ซึ่งเป็นสาเหตุของการนำไปสู่การเสียชีวิตนอกเหนือจากการติดเชื้อ ภาวะโลหิตเป็นพิษ และ ทางเดินหายใจล้มเหลว เป็นต้น

การเข้าใจถึงธรรมชาติหรือพยาธิสภาพทางไตในระยะต่างเป็นสิ่งที่สำคัญในการทำนายการพยากรณ์โรคของผู้ป่วย ยกตัวอย่าง เช่น ผู้ป่วยที่มีพยาธิสภาพอยู่ในกลุ่มสี่ โดยทั่วไปมีแนวโน้มที่จะนำไปสู่ไตวายเรื้อรังภายในไม่กี่เดือนในขณะที่ผู้ป่วยกลุ่มสอง หรือห้า อาจจะมีหน้าที่ของไตได้เหมือนกับคนปกติ ดังนั้นการตรวจเนื้อไตจึงเป็นสิ่งที่จำเป็นไม่เพียงแต่วินิจฉัยเท่านั้นแต่ยังบอกอาการกำเริบได้อีกด้วย ส่วนกลุ่มสี่เป็นกลุ่มที่พบได้บ่อยและรุนแรงมากซึ่งผู้ป่วยจะมีการอาการไตอักเสบพร้อมกับการรั่วของโปรตีนในปัสสาวะ ในปัจจุบันได้มีการหาตัวบ่งชี้ทางชีวโมเลกุลในเนื้อเยื่อของไตมาเป็นในการวินิจฉัยโรค

แม้ว่าการตรวจพยาธิสภาพของชิ้นเนื้อเป็นสิ่งที่จำเป็นที่สุดแต่การตรวจซ้ำเป็นเรื่องยากเนื่องจากผู้ป่วยจะได้บาดเจ็บจากการเจาะไต และอาจทำให้เกิดภาวะแทรกซ้อนได้ ดังนั้นการหาวิธีที่ทำให้ผู้ป่วยไม่เจ็บตัวเพื่อมารองรับการตรวจวัดอาการกำเริบเพื่อใช้ในการรักษา ในปัจจุบันได้มีความสนใจที่หาตัวดัชนีชี้วัดทางชีวภาพในปัสสาวะที่เกิดจากไรคไต กลุ่มผู้ทำการทดลอง

ของเราพบว่า ระดับ mRNA ในปัสสาวะสามารถใช้ในการวินิจฉัยของผู้ป่วยไตอักเสบลูปัสขั้นรุนแรงและมี chemokine และ growth factor ที่มีความสัมพันธ์กับการตอบสนองต่อการรักษาของผู้ป่วยอีกด้วย นอกจาก mRNA แล้วยังมีระดับโปรตีนของ chemokine จากการเก็บตัวอย่างในปัสสาวะ พบว่าผลการทดลองสนับสนุนว่า chemokine สามารถวินิจฉัยและพยากรณ์การเกิดโรคได้ จากที่กล่าวมารูปแบบทางโมเลกุลที่แสดงออกในปัสสาวะอาจจะมีความสัมพันธ์กับพยาธิสภาพของไต และหรือการตอบสนองต่อการรักษา ดังนั้นระดับของการแสดงของยีนสามารถถูกนำใช้ศึกษากลไกการเกิดโรคและยังอาจเป็นตัวชี้วัดของความรุนแรงหรือความเรื้อรังของโรคได้เช่นเดียวกัน

โครงการวิจัยนี้มีเป้าหมายที่จะศึกษาความสัมพันธ์ระหว่างการแสดงออกของยีนและโปรตีนในปัสสาวะ เลือด เม็ดเลือดขาว กับลักษณะความรุนแรงและพยากรณ์ของโรคภาวะไตอักเสบจากลูปัส โดยมีสมมุติฐานว่าการวัดระดับการแสดงออกของยีนและโปรตีนในเนื้อไตนอกเหนือจากการจัดแบ่งตามลักษณะทางพยาธิสภาพ (pathological classification) จะสามารถทาให้แพทย์จัดกลุ่มโรคเพิ่มเติมตามลักษณะทางอณูชีวโมเลกุล(molecular classification) นอกจากนี้ กลุ่มผู้วิจัยได้ยังทำการศึกษาเกี่ยวกับระดับยาที่เหมาะสมกับการตอบสนองต่อการรักษาอีก

4. วัตถุประสงค์

Primary Objectives

1. To determine whether urine mRNAs of selected genes could identify active nephritis (class IV versus Non-class IV LN by WHO criteria).
2. To determine whether serial measurement of urine mRNAs could early predict response to standard treatment.

Secondary Objectives

1. To determine whether urine mRNAs of selected genes could predict short-term clinical outcomes such as renal flare, ESRD or death.
2. To determine different pattern of gene expression between conventional treatment (cyclophosphamide) and novel treatment (MMF).

5. ระเบียบวิธีวิจัย

Study design

Prospective study

Patients

All SLE patients who were planned for kidney biopsy would be asked for urine samples

Sample collection

- Pre-biopsy samples

Two urine samples were collected from each individual patient approximately two weeks and one day before renal biopsy.

- Prospective samples in class IV LN

Urine samples were collected monthly from pre-treatment baseline to the 5th month of treatment.

Experimental methods

Gene expression: quantitative real-time PCR

Protein expression: Proteomics, ELISA

6. แผนการดำเนินงานวิจัยตลอดโครงการ

No	Scheduling plan	Fiscal year		
		1 st	2 nd	3 rd
1	Sample collection and patient consent	←	→	
2	Database collection	←	→	
3	Technical development	←	→	
4	Extraction RNA and realtime expression	←	→	
5	Proteomics and ELISA	←	→	
6	Therapeutic drug	←	→	
7	Data analysis	←	→	
8	Manuscript	←	→	

7. ผลงานที่คาดว่าจะตีพิมพ์ในวารสารวิชาการนานาชาติ

Two peer-reviewed journals

a. Title “gene expression profile predict renal outcome of lupus nephritis”

Journal. Kidney International Impact factor 4.9

b. Title “urine molecular profile predict renal flare in SLE”

Journal. J Am Soc Nephrol Impact factor 7.2

บทนำ

ภาวะไตอักเสบลูปัส (Lupus Nephritis; LN) เป็นภาวะที่พบเกือบร้อยละ 60 ของผู้ป่วยโรค Systemic lupus erythematosus (SLE) และสัมพันธ์กับอัตราการบาดเจ็บและเสียชีวิตของผู้ป่วย (morbidity and mortality) (1) จากการศึกษาพบความแตกต่างของอุบัติการณ์ในการเกิดโรคของชาว African-American สูงกว่าคนผิวขาว (Caucasian) (2) ในปัจจุบันยังไม่ทราบสาเหตุของโรคแน่ชัด แต่จากการศึกษาต่างๆเชื่อว่าเกิดจากความผิดปกติของระบบภูมิคุ้มกัน โดยที่ผู้ป่วยจะมีการสร้าง autoantibody ต่อ antigen ของตนเอง (self-antigen) และสร้างเป็น immune complex ไปสะสมในไตชักนำให้มีการเข้ามาของเซลล์ที่เกี่ยวกับระบบภูมิคุ้มกันและเกิดการอักเสบของไต (4) World Health Organization และ International Study of Kidney Disease in Childhood (ISKDC/WHO) ได้แบ่งระดับความรุนแรงของโรคออกเป็น 6 ระดับ (class I – class VI) ตามพยาธิสภาพของไต โดยผู้ป่วยที่ถูกจัดอยู่ในระดับที่ 4 (class IV) เป็นกลุ่มที่มีอาการของโรครุนแรงกว่าระดับอื่นๆและยังมีการพยากรณ์โรคที่ไม่ดีด้วย (5) สำหรับการศึกษาในประชากรไทยโดย P. Parichatikanond และคณะ ปี 1986 พบว่าผู้ป่วย SLE ของไทยส่วนใหญ่จะมีอาการไตอักเสบร่วมด้วย โดยมีความรุนแรงจัดอยู่ใน class IV และมีสัดส่วนของการเกิด class IV ต่อ class อื่นมากว่าชาวอเมริกาและอังกฤษ (Caucasians) อีกด้วย (6)

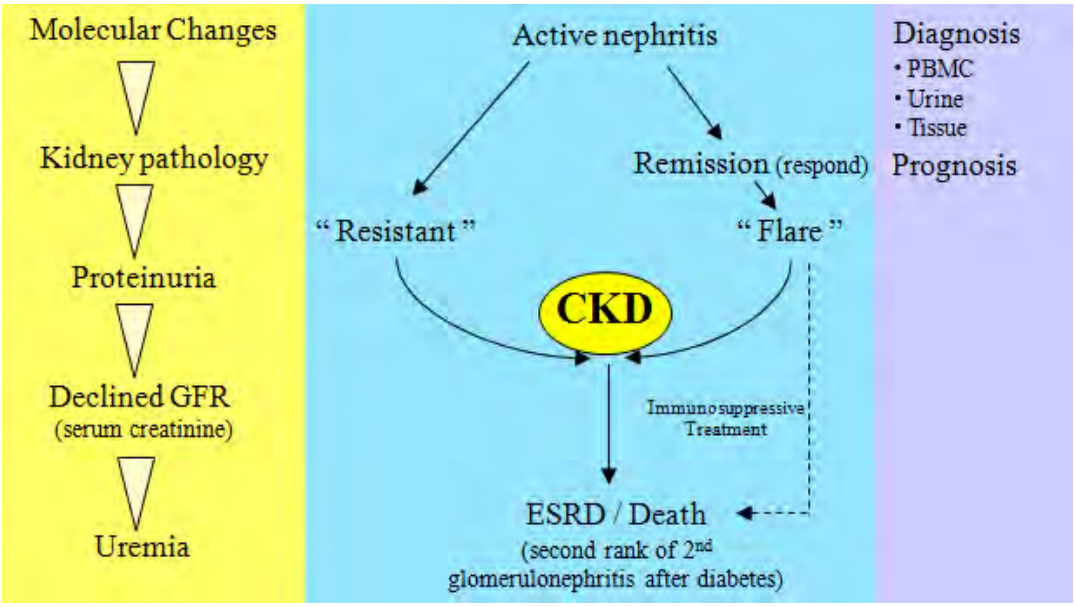
ในปัจจุบันวิธีมาตรฐาน (Gold standard) ในการตรวจวินิจฉัยโรคไตอักเสบลูปัสคือ การเจาะตรวจชิ้นเนื้อไต (Kidney Biopsy) เพื่อวินิจฉัยระดับความรุนแรงของโรค (class) ร่วมกับการตรวจการทำงานของไต (renal function) จากห้องปฏิบัติการ ซึ่งตาม American College of Rheumatology กำหนดเกณฑ์ในการระบุว่าผู้ป่วยมีอาการ LN เมื่อตรวจพบว่า 1) มีโปรตีนรั่วออกมาในปัสสาวะ (proteinuria) เกินกว่า 0.5 กรัมต่อวัน และ 2) ตรวจพบเซลล์ต่างๆหลุดปนออกมาในปัสสาวะ (cellular cast) โดยที่ผู้ป่วยแต่ละรายจะได้รับการรักษาตามความรุนแรงของโรคที่แพทย์วินิจฉัย ผู้ป่วยส่วนใหญ่จะได้รับยากดภูมิคุ้มกัน (immunosuppressive) เช่น steroids และอาจจำเป็นต้องใช้เวลา 5-20 ปี อาการของโรคจึงจะสงบลงและสามารถหยุดการให้ยาได้ หลังจากได้รับการรักษาแล้วในทางทฤษฎีจำเป็นต้องมีการทำ renal biopsy ซ้ำอีกครั้งเพื่อครั้งยืนยันว่าการรักษาได้ผล (14) แต่ในทางปฏิบัติกระทำได้ยากเนื่องจากการ biopsy เป็นวิธีที่ผู้ป่วยจะได้รับความเจ็บปวด (Invasive) แพทย์จึงจำเป็นต้องพิจารณาผลการรักษาจากผลตรวจการทำงานของไต แต่อย่างไรก็ตามมีผู้ป่วย LN บางรายที่ไม่ปรากฏความผิดปกติของการทำงานของไต (normal renal function) แต่ผลการตรวจชิ้นเนื้อไตพบการสะสมของ immune complex ใน glomerulus ซึ่งผู้ป่วยในกลุ่มนี้ถือว่าเป็น “silencing lupus nephritis” (13) แสดงให้เห็นว่าการกำเริบหรืออาการที่รุนแรงของโรคอาจจะไม่สามารถถูกตรวจพบและวินิจฉัยได้ด้วย

การตรวจทางห้องปฏิบัติการชีวเคมี และการเปลี่ยนแปลงพยาธิสภาพภายในไตจะเกิดขึ้นก่อนที่จะตรวจพบการทำงานที่แย่ลงของไต ดังนั้นการศึกษาหาตัวชี้วัดทางชีวภาพ (Biomarker) หรือวิธีการตรวจที่ไม่ทำให้ผู้ป่วยได้รับความเจ็บปวด (Non-invasive) จะสามารถเข้ามามีบทบาทช่วยในการวินิจฉัยโรคได้มากขึ้น

Eikmans M และคณะ พบว่าผู้ป่วยโรคไตที่มีการแสดงออกของยีน Transforming growth factor-β (TGF-β) และ Fibronectin ภายในเนื้อไตสูงขึ้นจะสัมพันธ์กับการทำงานที่ดีของไต (9) เช่นเดียวกับการศึกษาในหนูทดลองที่พบว่ายีน Vascular endothelial growth factor (VEGF) เป็นยีนที่ทำหน้าที่ในการรักษาสมดุลของการกรอง (permeability) ภายในไต เนื่องจากพบว่าหนูที่ขาด VEGF จะเกิด proteinuria และมีการเพิ่มจำนวน endothelial cell ที่ผิดปกติ (endotheliosis) ทำให้เกิดการเปลี่ยนแปลงพยาธิสภาพภายในไตจนหนูตายภายใน 2.5 สัปดาห์ (10,11) ตรงกันข้ามกับการศึกษาในผู้ป่วยโรคเบาหวานชนิดที่ 1 และ 2 ซึ่งพบว่ามีการแสดงออกของยีนและโปรตีน VEGF เพิ่มมากขึ้น(12) แสดงให้เห็นว่ายีน VEGF มีการทำหน้าที่ที่แตกต่างกันในโรคไตชนิดต่างๆ แสดงให้เห็นว่าการศึกษาการเปลี่ยนแปลงในระดับโมเลกุลสามารถนำมาใช้ประโยชน์สำหรับการทำนายโรคได้ ซึ่งในปี 2006 ผู้วิจัยและคณะได้ทำการศึกษาพบว่าในปัสสาวะของผู้ป่วย LN มีการแสดงออกของโมเลกุลที่ผ่านมาผู้วิจัยพบว่าระดับการแสดงออกที่เพิ่มขึ้นของยีนในกลุ่ม Growth Factor และ Chemokine ในปัสสาวะ (Urinary Chemokine) ของผู้ป่วยที่มีอาการรุนแรง (Class IV LN) สูงกว่า Class อื่นๆ และการแสดงออกของยีนจะลดลงเมื่อผู้ป่วยตอบสนองต่อการรักษาอีกด้วย (2006)

ในปัจจุบัน การศึกษาโปรตีนโดยใช้วิธีทาง proteomics กำลังได้รับความนิยมมากขึ้นเนื่องจากมีการพัฒนาเทคนิกทั้งในด้าน isoelectric focusing และ mass spectrometer รวมทั้งมีฐานข้อมูลที่แพร่หลายของทั้งทาง genomic และ โปรตีน ดังนั้นการใช้วิธี two-dimensional gel electrophoresis ร่วมกับ mass spectrometry จึงเป็นวิธีที่ใช้ในการศึกษาโปรตีนหลายๆชนิดพร้อมกัน แม้ปริมาณโปรตีนที่ศึกษาจะมีไม่มาก แต่มีความไว ความแม่นยำและความเที่ยงตรงสูง การศึกษาหา biomarker ในคนไข้ไตอักเสบลูปัส นั้นมีการศึกษามานานแล้ว แต่เป็นการศึกษาหาแต่ตัวละตัวโปรตีนในปัสสาวะ ดังนั้นวิธีการนี้จะเป็นการศึกษาหากลุ่มของโปรตีนที่แสดงออกมาแตกต่างกันในปัสสาวะได้

เนื่องจากผู้วิจัยมีสมมติฐานว่าการเปลี่ยนแปลงระดับโมเลกุล (Molecular change) นั้นมักเกิดขึ้นก่อนอาการแสดงทางคลินิกหรือแม้แต่การเปลี่ยนแปลงทางพยาธิวิทยาอีกด้วย (รูปที่ 1) ทำให้ในการศึกษานี้ผู้วิจัยสนใจที่จะศึกษาเพื่อค้นหาการเปลี่ยนแปลงการแสดงออกของระดับ mRNA และโปรตีนในผู้ป่วย LN เพื่อนำมาใช้เป็น Biomarkers ชนิดใหม่ๆ ซึ่งอาจจะมีประโยชน์ในการนำไปใช้เพื่อทำนายการเกิดโรคต่อไป



<u>รูปที่ 1</u> สมมติฐานในการวิจัย

วัตถุประสงค์ของโครงการวิจัย

การศึกษานี้มีวัตถุประสงค์หลัก เพื่อค้นหาชีวโมเลกุล (Biomarker) ที่จำเพาะต่อการวินิจฉัยและพยากรณ์โรคไตอักเสบลูปัส โดยจำแนกวัตถุประสงค์หลักและรองดังนี้

วัตถุประสงค์หลัก

1) เพื่อศึกษาหาปริมาณ urine mRNA ของยีนที่สนใจซึ่งจะสามารถใช้ในการจำแนกผู้ป่วย Class IV LN และ Non-class IV LN ได้
2) เพื่อศึกษาหาปริมาณ mRNA ในแต่ละช่วงเวลา (serial measurement) เพื่อใช้ในการทำนายการตอบสนองต่อการรักษาผู้ป่วยที่ได้รับการรักษาด้วยยามาตรฐาน

วัตถุประสงค์รอง

1) เพื่อศึกษาการใช้ปริมาณ urine mRNA ของยีนที่สนใจในการทำนายการเปลี่ยนแปลงทางคลินิกของผู้ป่วยในช่วงเวลาสั้นๆ (short-term clinical outcome) ได้แก่ renal flare, ESRD หรือ การตาย
2) เพื่อหารูปแบบการแสดงออกของยีนของผู้ป่วยที่ได้รับการรักษาด้วยยาดั้งเดิม (conventional treatment) เปรียบเทียบกับผู้ป่วยที่ได้รับการรักษาด้วยยาใหม่ (novel treatment)

ในระหว่างการดำเนินการวิจัยผู้วิจัยได้รวบรวมข้อมูลและทบทวนวรรณกรรมเพิ่มเติม จึงได้แบ่งการศึกษาตามหัวข้อต่อไปนี้

1) *mRNA Expression Study*:
   1.1) ผู้วิจัยได้ทำการศึกษาเพิ่มเติมจากข้อมูลที่รายงานไปก่อนหน้า ที่เน้นให้เห็นถึงบทบาทของยีน VEGF (MRG4880103) โดยในการศึกษานี้ได้วัดการแสดงออกของยีน Heme Oxygenase-1 (HO-1) ซึ่งจากการศึกษาก่อนหน้านี้แสดงให้เห็นว่า HO-1 เป็น cytoprotective gene และการแสดงออกของยีนนี้ถูกควบคุมด้วย transcription factor ชนิดเดียวกันกับยีน VEGF (Avihingsanon Y et al., 2002, Ferrara N, 2004) และศึกษาการแสดงออกของยีน Wilms' tumour suppressor gene (WT1) ซึ่งเป็น marker ของเซลล์ podocyte ที่ทำหน้าที่สร้าง VEGF ภายในไตและอาจจะหลุดปนมาในเซลล์ปัสสาวะของผู้ป่วย LN
2) *Urinary cells type specific:* จากผล mRNA Expression study ในตัวอย่างปัสสาวะของผู้ป่วย LN ทำให้ผู้วิจัยมีสมมติฐานว่าอาจมีเซลล์ภายในไตหลุดปนมาในปัสสาวะ ดังนั้นจึงต้องศึกโปรตีน Wilms' tumour suppressor gene (WT1) ซึ่งเป็น marker ของเซลล์ podocyte ที่ทำหน้าที่สร้าง VEGF ภายในไตและอาจจะหลุดปนมาในเซลล์ปัสสาวะของผู้ป่วย LN

3) *Proteomic Study:* นอกจากการศึกษาในระดับ mRNA แล้วผู้วิจัยได้พัฒนาเทคนิคและรวบรวมตัวอย่างปัสสาวะของผู้ป่วย LN เพิ่มเติม เพื่อทำการศึกษาการแสดงออกของโปรตีน Interferon-inducible protein-10 (IP-10) ในปัสสาวะ เนื่องจากการศึกษาก่อนหน้านี้ของผู้วิจัยพบว่าผู้ป่วยไตอักเสบลูปัสที่มีอาการของโรครุนแรงจะมีการแสดงออกของ IP-10 mRNA ในปัสสาวะเพิ่มสูงขึ้น (Avihingsanon Y et al., 2006) อีกทั้งเพื่อให้ได้ข้อมูลการแสดงออกของโปรตีนต่างๆในปัสสาวะมากขึ้น ผู้วิจัยได้เริ่มทำการศึกษาหาโปรตีนทั้งหมดในปัสสาวะ (Proteomic) ด้วยวิธี 2D-PAGE เพื่อใช้ประโยชน์จากข้อมูลที่ได้นี้ในการศึกษา Biomarker ชนิดอื่นต่อไป
4) *Therapeutic drug monitoring*: ในปัจจุบันการรักษาผู้ป่วย lupus nephritis ด้วยยา mycophenolate mofetil ซึ่งจะถูกเปลี่ยนแปลงเป็น mycophenolic acid (MPA) พบว่าสามารถลดอัตราการกำเริบของโรค แต่ในขณะเดียวกันพบว่าการตอบสนองต่อยาของผู้ป่วยแต่ละคนไม่เท่ากันเมื่อได้รับยาในขนาดที่เท่ากัน ระดับยาของ MPA ที่อยู่ในกระแสเลือดจึงมีความสำคัญในการตอบสนองต่อการรักษา

ระเบียบวิธีวิจัย

1. รวบรวมตัวอย่างและเก็บข้อมูลผู้ป่วย

<u>รวบรวมตัวอย่างทั้งชิ้นเนื้อและปัสสาวะ รวมทั้งตัวอย่างเลือดจากผู้ป่วยไตอักเสบลูปัส</u>

<u>(Sample collection and patients consent)</u>

ผู้วิจัยได้ดำเนินการรวบรวมอาสาสมัครผู้ป่วยโรคไตอักเสบลูปัสเพื่อเข้าร่วมโครงการวิจัย โดยได้ดำเนินการเก็บตัวอย่างปัสสาวะ, ตัวอย่างเลือด (EDTA blood) ของผู้ป่วยติดต่อกันทุกเดือนที่มาทำการรักษา และเก็บตัวอย่างชิ้นเนื้อไต (kidney biopsy) ของผู้ป่วยที่เข้ารับการเจาะไตด้วย โดยมีจำนวนตัวอย่างโดยสรุปดังนี้

โดยแบ่งผู้ป่วยออกเป็น 4 กลุ่ม มีรายละเอียดดังนี้

1. ผู้ป่วยกลุ่ม active lupus nephritis (ได้รับการวินิจฉัยว่ามีอาการรุนแรงอยู่ใน class III และ class IV)
2. ผู้ป่วยกลุ่ม inactive lupus nephritis (non-class III or IV)
3. กลุ่มควบคุมที่เป็นประชากรปกติ
4. กลุ่ม kidney tissue control คือไตจากผู้บริจาคที่บริจาคให้ใช้ในการปลูกถ่ายอวัยวะ เพื่อใช้เป็นกลุ่มชิ้นเนื้อไตควบคุม

<u>เก็บบันทึกประวัติและข้อมูลทางคลินิกจากกลุ่มตัวอย่าง (Database collection)</u>

ในการเก็บตัวอย่างผู้ป่วยแต่ละรายผู้วิจัยได้ทำการรวบรวมประวัติและข้อมูลทางคลินิกของผู้ป่วยทุกราย โดยข้อมูลจะถูกเก็บรวบรวมอย่างมีระบบ และมีการตรวจสอบความครบถ้วนโดยพยาบาลวิจัย จากนั้นจะบันทึกลงในฐานข้อมูล Online ที่ได้จัดทำขึ้น เพื่อความสะดวกในการใช้งานและสามารถนำมาใช้ในการวิเคราะห์ข้อมูลร่วมกันได้

ในการวิจัยนี้ผู้วิจัยได้แบ่งการศึกษาออกเป็น 4 หัวข้อ ตามที่กล่าวไว้ในวัตถุประสงค์ข้างต้น ซึ่งมีรายละเอียดดังนี้

2. วิธีทดลอง

Part 1: mRNA Expression Study

1) การศึกษาเปรียบเทียบการแสดงออกของยีนที่สนใจ (mRNA Expression) ในผู้ป่วยไตอักเสบลูปัส และคนปกติด้วยวิธี Real-time PCR

1.1) <u>สกัดแยก RNA จากตัวอย่างเลือด, ปัสสาวะ และชิ้นเนื้อของผู้ป่วย LN และคนปกติ</u>

เลือด (Whole Blood)

1. ตัวอย่าง Whole blood ที่ได้รับจากผู้ป่วยและคนปกติ จะถูกเก็บในหลอด EDTA ซึ่งสามารถป้องกันการแข็งตัวของเลือดได้
2. แบ่ง Whole blood ออกเป็นสองส่วน ส่วนที่หนึ่งนำไปแยกเพื่อเก็บเซลล์เม็ดเลือดขาวทั้งหมด (Total Leukocytes) โดยการใช้ Erythrocytes lysis buffer (QIAGEN, GmbH Hilden, Germany) ทำลายเม็ดเลือดแดง และแยกเฉพาะเม็ดเลือดขาวทั้งหมดออกมา และนำเม็ดเลือดขาวทั้งหมดที่ได้ไปสกัด total RNA ตามข้อ (4) สำหรับเลือดในส่วนที่สอง จะนำไปแยกเซลล์เม็ดเลือดขาวชนิดนิวเคลียสเดียว (Peripheral Blood Mononuclear Cells; PBMCs) โดยแยก PBMCs ด้วยการปั่นเหวี่ยงบน Ficoll–Hypaque reagent (Sigma,St Louis, Missouri, USA).ซึ่งอาศัยคุณสมบัติที่เซลล์ต่างชนิดมีความหนาแน่นแตกต่างกัน จึงสามารถแยก PBMCs ได้โดยการปั่นเหวี่ยงบน density gradient และนำไปศึกษาในขั้นตอนต่อๆไป
3. คัดเลือก T-cells : PBMCs ที่ได้ส่วนหนึ่งจะถูกนำมาคัดเลือกเฉพาะเซลล์เม็ดเลือดขาวชนิด T-cells โดยใช้ Magnetic Beads (Miltenyi Biotech, Bergisch Gladbach, Germany) ที่จำเพาะต่อโมเลกุลบนผิว T-cells หลังจากนั้นนำ T-cells ที่ได้มาเพาะเลี้ยง (culture) ด้วยอาหารเลี้ยงเซลล์ RPMI1640 ที่มี 10% fetal bovine serum (Hyclone, Logan, Utah, USA), penicillin และ streptomycin ที่อุณหภูมิ 37°C, 5% CO_2
4. สกัด RNA จาก total leukocytes, PBMCs และเซลล์ที่เราทำการศึกษาด้วยชุดสกัด QIAGEN RNA mini kit (QIAGEN, GmbH Hilden, Germany) โดย

ทำตามขั้นตอนของชุดสกัดซึ่งอาศัยหลักการของ Silica-gel-based membrane หลังนั้นนำ RNA ที่สกัดได้ไปวัดปริมาณความเข้มข้นและคุณภาพด้วยเครื่อง NanoDrop® ND-1000 UV-Vis Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA)

ปัสสาวะ (Urine)

1. เก็บตัวอย่างปัสสาวะของผู้ป่วยที่มีอาการรุนแรง (active LN) และผู้ป่วยที่มีอาการไม่รุนแรง (inactive LN) ปริมาตร 50 มิลลิลิตร
2. ปั่นเหวี่ยงปัสสาวะที่ได้ที่อุณหภูมิ 4 องศาเซลเซียส, ความเร็ว 1000g, 30 นาที แล้วเก็บตะกอนเซลล์ที่ได้นำมาปั่นล้างด้วยสารละลาย PBS 1x
3. นำตะกอนเซลล์ที่ได้มาสกัด RNA ด้วยชุดสกัด QIAGEN RNA mini kit (QIAGEN, GmbH Hilden, Germany) เช่นเดียวกับขั้นตอนการสกัด RNA จากเลือด

ชิ้นเนื้อไต (Kidney biopsy)

1. ทำการเก็บตัวอย่างชิ้นเนื้อไตหลังจากการทำการเจาะไต (renal biopsy) ในผู้ป่วยไตอักเสบลูปัส และชิ้นเนื้อไตจากไตของผู้บริจาคไต (Normal tissue control) ซึ่งได้รับการตัดชิ้นเนื้อไตก่อนการผ่าตัดปลูกถ่ายไต โดยชิ้นเนื้อที่ได้จะถูกแบ่งเป็น 3 ส่วน

   - ส่วนที่ 1 จะถูกนำมาเก็บไว้หลอดเก็บชิ้นเนื้อที่มีสารละลายรักษาสภาพ RNA (*RNAlater*) และถูกแช่ในภาชนะที่บรรจุน้ำแข็งแห้ง แล้วนำมาเก็บที่ตู้แช่ –70°c เพื่อใช้ในการสกัด RNA และศึกษาการแสดงออกของยีน (gene expression)

   - ส่วนที่สอง ถูกเก็บในน้ำยารักษาสภาพชิ้นเนื้อ (Fixative solution) และนำไปศึกษาปริมาณโปรตีนด้วยวิธีการย้อม Immunohistochemistry (IHC)

   - ส่วนที่ 3 ตัวอย่างชิ้นเนื้อจะถูกเก็บแช่แข็ง (frozen) และนำไปศึกษาลักษณะทางพยาธิสภาพ (pathology study) ของชิ้นเนื้อ
2. สกัด Total RNA จากชิ้นเนื้อไตที่ได้ด้วยชุดสกัด ด้วยชุดสกัด QIAGEN RNesy mini kit (QIAGEN, GmbH Hilden, Germany) โดยทำตามขั้นตอนของชุดสกัดซึ่งอาศัยหลักการของ Silica-gel-based membrane หลังนั้นนำ RNA ที่สกัดได้ไปวัดปริมาณความเข้มข้นและคุณภาพด้วยเครื่อง NanoDrop® ND-1000 UV-Vis Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA)

1.2) สร้าง complementary DNA (cDNA) โดยปฏิกิริยา reverse-transcription polymerase chain reaction (RT-PCR)

1. คำนวณปริมาณตัวอย่าง RNA ให้ได้ 0.5 ug โดยที่ปริมาตรต้อง ไม่เกิน 11.5 ul หากปริมาตรน้อยกว่านั้นให้ปรับปริมาตรให้เป็น 11.5 ul โดยใช้น้ำที่ปราศจากการปนเปื้อนของ RNase
2. เตรียม master mix ซึ่งใช้ random primer และเอนไซม์ Multiscribe® reverse transcriptase (TaqMan® RT-PCR, Applied Biosystem, CA., USA.) เพื่อสร้างสาย cDNA โดยใช้สาย RNA เป็นแม่แบบ เพื่อใช้ในการเพิ่มจำนวนในขั้นต่อไป (Condition: 25°c 10 min, 48°c 30 min, 95°c 5 min)

1.3) วัดปริมาณการแสดงออกของยีนที่สนใจด้วยเทคนิค Real-time polymerase chain reaction (real-time PCR)

1. เตรียม PCR master mix (QuantiTech Probe PCR, Qiagen, Canada) ซึ่งประกอบไปด้วย 2xQuantiTech probe PCR , primer forward, primer reverse, probe ที่จำเพาะต่อยีนที่สนใจศึกษา
2. เติม cDNA template ของแต่ละตัวอย่างลงใน PCR master mix นำไปวัดปริมาณการแสดงออกของยีนที่สนใจด้วยเครื่อง Real-time PCR machine

Part 2: Urinary cells type specific by Flow Cytometry

1. เก็บตัวอย่างปัสสาวะของผู้ป่วยที่มีอาการรุนแรง (active LN) และผู้ป่วยที่มีอาการไม่รุนแรง (inactive LN) ปริมาตร 100 มิลลิลิตร
2. ปั่นเหวี่ยงปัสสาวะที่ได้ที่อุณหภูมิ 4 องศาเซลเซียส, ความเร็ว 1000g, 30 นาที แล้วเก็บตะกอนเซลล์ที่ได้นำมาปั่นล้างด้วยสารละลาย PBS 1x
3. นำตะกอนเซลล์ที่ได้ไปศึกษาชนิดของเซลล์ซึ่งหลุดปนมาในปัสสาวะด้วยวิธี Flow cytometry โดยอาศัยโมเลกุลที่จำเพาะบนผิวเซลล์โดยเฉพาะ WT-1 ซึ่งจำเพาะต่อเซลล์ podocytes ของไต

Part 3: Proteomic Study

3.1 เก็บตัวอย่างคนไข้ที่แบ่งออกเป็นกลุ่มคือ กลุ่มคนไข้ active และ inactive lupus nephritis ตกตะกอนโปรตีนด้วย 75% ethanol

3.2 การแยกโปรตีนด้วย 2 มิติ (2 dimension electrophoresis)

ละลายตะกอนด้วย lysis solution แล้วนำไปวัดความเข้มข้นโปรตีนด้วยวิธี Bradford ใช้ความเข้มข้นโปรตีน 200 ug มาผ่านกระแสไฟฟ้าในเจลเพื่อแยกชนิดของโปรตีนต่างๆในปัสสาวะให้แยกออกจากกันตาม isoelectric point และตาม molecular weight แล้วมีการย้อมสีโปรตีนด้วย Coomassie Brilliant Blue R-250

3.3 วิธีวิเคราะห์ข้อมูล

ในการทำ two-dimensional gel electrophoresis จะมีการใช้กลุ่มตัวอย่างของคนไข้ 2 กลุ่ม เพื่อเป็นการเปรียบเทียบการเปลี่ยนแปลงของโปรตีน หลังจากการย้อมด้วย Coomassie Brilliant Blue แล้ว โปรตีนที่มีการเปลี่ยนแปลงเพิ่มขึ้นหรือลดลง จะถูกนำไปวิเคราะห์หาชนิดของโปรตีนดังกล่าวด้วยวิธี mass spectrometry ที่ ศูนย์เครื่องมือกลาง จุฬาลงกรณ์มหาวิทยาลัย และ/หรือ สถาบันวิจัยวิทยาศาสตร์และเทคโนโลยีแห่งชาติ

3.4 Candidate biomarkers validation

โปรตีนที่ได้ถูกนำมาวิเคราะห์เพื่อหาความแม่นยำในจำนวนคนไข้ที่มากขึ้น ด้วยวิธีELISA

Part 4: Therapeutic drug monitoring

1. การศึกษาเภสัชจลนศาสตร์ของยา mycophenolic acid

ในผู้ป่วยแต่ละคนที่ช่วงระยะเวลาระหว่าง 1-6 เดือน หลังได้รับยา mycophenolate จะมีการเจาะเลือดปริมาณ 3 ml.ใส่ในหลอดแก้วที่มี heparin เพื่อตรวจหาระดับของ mycophenolic acid ซึ่งเป็น active metabolite ของ mycohephenolate ที่ 0, 30 นาที, 1, 2, 3, 4, 8 และ 12 ชั่วโมงหลังรับประทานยา mycophenolate และวัดระดับของ mycophenolic acid ในพลาสม่าด้วยวิธี enzyme multiplied immunoassay technique (EMIT) แล้วนำค่าระดับยา 8 จุดดังกล่าวมา plot curve จะได้กราฟดังแสดงในรูปที่ 2

「

รูปที่ 2 กราฟแสดงความเข้มข้นของ MPA ในเลือด ณ จุดเวลาต่างๆ

แล้วคำนวณหาระดับของ mycophenolic acid โดยการคำนวณหาค่าพื้นที่ใต้กราฟได้เป็นค่าที่เรียกว่า MPA AUC_{0-12} โดยใช้สูตร

Area = ½ x (Cn + Cn-1) x (tn – tn-1)

C = concentration of mycophenolic acid (µg/ml)

t = time (hour)

2. การวัดระดับของ mycophenolic acid ในพลาสม่าด้วยวิธี enzyme multiplied immunoassay technique (EMIT)

ขั้นตอนการทำ

-การเตรียมตัวอย่าง

เก็บตัวอย่างเลือด 3 ml. บรรจุใน EDTA tube เพื่อทำการปั่นแยก plasma ที่ความเร็วรอบ 1200 g เป็นเวลา 15 นาที เก็บส่วนที่เป็น plasma ไว้ในหลอดพลาสติกไว้ที่ -80 ˚C จนกว่าจะนำมาวิเคราะห์ MPA โดยวิธี total mycophenolic acid

-การตรวจหา MPA

ชุดน้ำยาแบ่งออกเป็น 3 ชุด ประกอบด้วย

working solution ประกอบด้วย enzyme reagent และ substrate reagent

Total MPA calibrators ที่มีความเข้มข้น 0, 1, 3, 5, 10, และ 15 mcg/ml

Total MPA control

ทำการผสมสารระหว่าง enzyme reagent 185 mcl. กับ substrate reagent 19 mcl. และนำมาผสมกับตัวอย่าง 3 ul นำไปวิเคราะห์หา MPA ด้วยเครื่อง cobas integra 400 ที่ความยาวคลื่น 340 nm.

3. การประเมินผลการรักษา

-ผลการเปลี่ยนแปลงทางคลินิค โดยประเมินจากการเปลี่ยนแปลงของปริมาณโปรตีนในปัสสาวะที่ได้จากการเก็บปัสสาวะ 24 ชั่วโมง หน่วยเป็นกรัมต่อวัน, การเปลี่ยนแปลงของการทำงานของไตที่ก่อนและหลังการรักษานาน 6 เดือน, การประเมินค่าการทำงานของไตโดยเจาะเลือดวัดระดับ serum creatinine และคำนวณหาค่า estimated glomerular filtration rate (eGFR) โดยใช้สมการของ Modification of Diet in Renal Disease (MDRD) 4 variables และCockcroft-Gault formula และการตอบสนองต่อการรักษาภายหลังการได้รับยา mycophenolate นาน 6 เดือน

-ผลการเปลี่ยนแปลงทางพยาธิวิทยาของชิ้นเนื้อไต ประเมินโดยเจาะชิ้นเนื้อไตเพื่อตรวจหาความรุนแรงทางพยาธิวิทยาโดยใช้ activity index score ที่ก่อนและหลังการรักษานาน 6 เดือนและดูการเปลี่ยนแปลงของ activity index score

ผลการทดลอง

Part 1: mRNA Expression Study

1. การแสดงออกของยีน Notch1 ในเซลล์เม็ดเลือดขาวชนิด T-cells

ใน T-cells ของผู้ป่วย SLE ที่มีอาการกำเริบ (active SLE) มีการแสดงออกของ Notch1 mRNA ต่ำกว่ากลุ่มควบคุม (healthy control) อย่างมีนัยสำคัญ (p=0.025) และเมื่อศึกษาปริมาณโปรตีน Notch1 ก็พบว่ามีการแสดงออกลดลงในผู้ป่วย active SLE ด้วยเช่นกัน นอกจากนี้ยังพบว่าโมเลกุลที่เป็นเป้าหมายของ Notch (Notch targeted gene) ได้แก่ Hes1 และ deltex mRNA ก็มีการแสดงออกลดลงในผู้ป่วยด้วยเช่นกัน (รูปที่ 3) ผลการศึกษานี้ได้ถูกตีพิมพ์ในวารสาร Lupus ปี

2008, volume 17, หน้า 645–653. (Defects in Notch1 Upregulation upon Activation of T Cells from Patients with Systemic Lupus Erythematosus are related to Lupus Disease Activity, *Pimpayao Sodsaia, Nattiya Hirankarna, Yingyos Avihingsanon, and Tanapat Palaga*)

「

รูปที่ 3 การแสดงออกของ Notch1 และ Hes1 mRNA ใน T-cells

2. การแสดงออกของยีน WT-1 ในตะกอนเซลล์ที่หลุดปนมาในปัสสาวะ

เนื่องจากการทบทวนข้อมูลที่ผ่านๆมา podocytes เป็นเซลล์ภายในไตที่ทำหน้าที่สร้าง VEGF ดังนั้นเมื่อเซลล์ดังกล่าวหลุดมาในปัสสาวะเราจึงพบว่าการแสดงออกของ VEGF ในไตลดลง (MRG4880103) แต่การแสดงออกของ VEGF mRNA จะเพิ่มขึ้นในปัสสาวะ (Avihingsanon et al., 2006) ซึ่งจากข้อมูลดังกล่าวนี้ ทำให้กลุ่มผู้ทำวิจัยสนใจการแสดงออกของยีน WT1 ซึ่งเป็นตัวบ่งชี้ (cell surface marker) ของ podocytes กับการแสดงออกของ VEGF mRNA ในปัสสาวะ พบว่าในปัสสาวะของผู้ป่วย class IV LN มีการแสดงออกของ WT1 mNRA และ VEGF mRNA เพิ่มขึ้น โดยพบความสัมพันธ์ของการเพิ่มขึ้นของโมเลกุลทั้งสองในปัสสาวะอย่างมีนัยสำคัญทางสถิติที่ (Spearman's rho; p=0.002, r=0.51) (รูปที่ 4)ซึ่งผลการศึกษานี้ได้รับรางวัลที่ 1 ในการนำเสนอผลงานในการประชุมระดับนานาชาติ World Congresses of Nephrology **2009 และได้ตีพิมพ์เป็นส่วนหนึ่งใน Kidney International. 2009; 12: 1340-1348 (Decreased renal expression of vascular endothelial growth factor in lupus nephritis is associated with worse prognosis, *Avihingsanon Y, Benjachat T, Tassanarong A, Sodsai P, Kittikovit V, Hirankarn N*)**

「

รูปที่ 4 การความสัมพันธ์ของการแสดงออกของ WT-1 และ VEGF mRNA ในปัสสาวะและชิ้นเนื้อไตของผู้ป่วย class IV LN

3. **การแสดงออกของยีนที่สนใจในชิ้นเนื้อไต**

เนื่องจากในการศึกษาก่อนหน้านี้ (MRG4880103) ผู้วิจัยพบการลดลงของ VEGF mRNA ในเนื้อไตของผู้ป่วย class IV LN ดังนั้นในการศึกษานี้จึงสนใจศึกษายีนที่เกี่ยวข้องกับการทำงานของยีน VEGF เพิ่มเติม ทั้งนี้ยีน Heme Oxygenase-1 (HO-1) เป็นยีนที่ทำหน้าที่เกี่ยวข้องกับการแสดงออกของยีน VEGF ดังนั้นผู้วิจัยจึงสนใจศึกษาการแสดงออกของยีน HO-1 mRNA ในเนื้อไต (intra-renal HO-1) ซึ่งพบว่าผู้ป่วย LN ที่มีอาการรุนแรงจัดอยู่ใน class III และ class IV จะมี

ระดับการแสดงออกของยีน HO-1 ในเนื้อไตมีปริมาณต่ำกว่ากลุ่มควบคุม ทั้งนี้ผลที่ได้สอดคล้องกับระดับการแสดงออกของ intra-renal VEGF โดยการแสดงออกของยีนทั้งสองมีความสัมพันธ์กันอย่างมีนัยสำคัญ (Spearman's rho; R=0.65, P<0.001) นอกจากนี้ผู้วิจัยยังพบว่ากการแสดงออกของยีน TGF-β mRNA ในเนื้อไตของผู้ป่วย Class IV LN มีปริมาณต่ำกว่ากลุ่มควบคุม และมีความสัมพันธ์กับการแสดงออกของ VEGF mRNA ในเนื้อไตอย่างมีนัยสำคัญทางสถิติ (Spearman's rho; R=0.41, P=0.02) (รูปที่ 5)

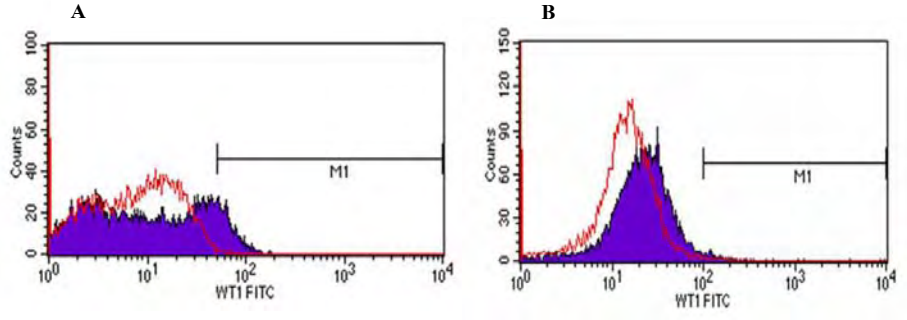
การศึกษานี้ได้ตีพิมพ์เป็นส่วนหนึ่งใน Kidney International. 2009; 12: 1340-1348 (Decreased renal expression of vascular endothelial growth factor in lupus nephritis is associated with worse prognosis, *Avihingsanon Y, Benjachat T, Tassanarong A, Sodsai P, Kittikovit V, Hirankarn N*)

「

รูปที่ 5 การความสัมพันธ์ของการแสดงออกของ TGF-β , AGPT-1 และ VEGF mRNA ในชิ้นเนื้อไตของผู้ป่วย class IV LN

Part 2: Urinary cells type specific

ผู้วิจัยได้เก็บตัวอย่างปัสสาวะของผู้ป่วยที่มีอาการรุนแรง (active LN) และผู้ป่วยที่มีอาการไม่รุนแรง (inactive LN) มาทำการศึกษาหาชนิดของเซลล์ที่สนใจซึ่งหลุดปนมาในปัสสาวะ ในที่นี้ผู้วิจัยมีสมมติฐานว่าเซลล์ที่หลุดมาในปัสสาวะส่วนหนึ่งน่าจะเป็นเซลล์ภายในไตที่ทำหน้าที่ในการสร้าง VEGF ได้แก่ podocyte cells เนื่องจากจากผลการศึกษาที่ผ่านมาของผู้วิจัยสนับสนุนสมติฐานดังกล่าว ดังนั้นผู้วิจัยจึงศึกษาหาปริมาณเซลล์ podocytes ในปัสสาวะของผู้ป่วยทั้งสองกลุ่มเปรียบเทียบกันโดยใช้เทคนิค Flow cytometry และใช้ Wilms' tumor suppressor gene-1 (WT-1) เป็น surface marker ของ podocyte cells พบว่าผู้ป่วย active LN มีจำนวนเซลล์ podocytes หลุดออกมามากกว่าผู้ป่วย inactive LN อย่างมีนัยสำคัญทางสถิติ (p=0.02) (รูปที่ 6)



รูปที่ 6 ก ...บผู้ป่วย inactive ... arker ของ pod ...

Part 3: ...

ผู้วิจัยได้ทำการเก็บปัสสาวะของกลุ่มคนไข้และกลุ่มควบคุมแล้วนำมาแยกโปรตีนด้วย 2D-PAGE และถูกย้อมสีด้วย Coomassie Brilliant Blue หลังจากนั้นทำการวิเคราะห์ spot ที่มีการแสดงออกที่แตกต่างระหว่างกลุ่มด้วยโปรแกรม image master 2D platinum พบว่ามีความแตกต่างทั้งหมด 11 spots ดังตารางที่ 1 เมื่อนำทั้งหมดไปวิเคราะห์ชนิดของโปรตีน คือ albumin, transferrin, immumoglobulin, kininogen, alpha-beta-glycoprotein, Zn-alpha-2-glycoprotein และ prostaglandin H2 D-isomerase ดังตารางที่ 1

Table 1. Spot intensity of differentially expressed proteins

Spot	Spot intensity (mean ± SEM)		
No	LN: Active	LN: Inactive	Normal
9744	53.405±12.145	63.944±9.276	34.478±14.075
9107	3.519±2.972	1.993±1.634	0.000±0.000
9105	13.841±9.472	11.978±3.614	2.714±1.387
9791	0.922±0.082	1.061±1.041	0.467±0.291
10265	1.20±.075	0.85±0.401	0.26±0.24
10331	0.7899±0.354	0.7375±1.026	0.1927±0.097
11572	0.4745±0.4697	0.3482±0.402	1.3191±0.7664
11750	0.0721±0.055	0.1451±0.1654	1.0255±0.360
11798	0.1634±0.080	0.0632±0.0145	1.0289±0.6413
11633	0.1219±0.1209	0.1694±0.079	1.6295±0.992
15338	0.2253±0.1451	0.043±0.038	0.063±0.053
15533	0.0819±0.076	0.029±0.014	0.052±0.017

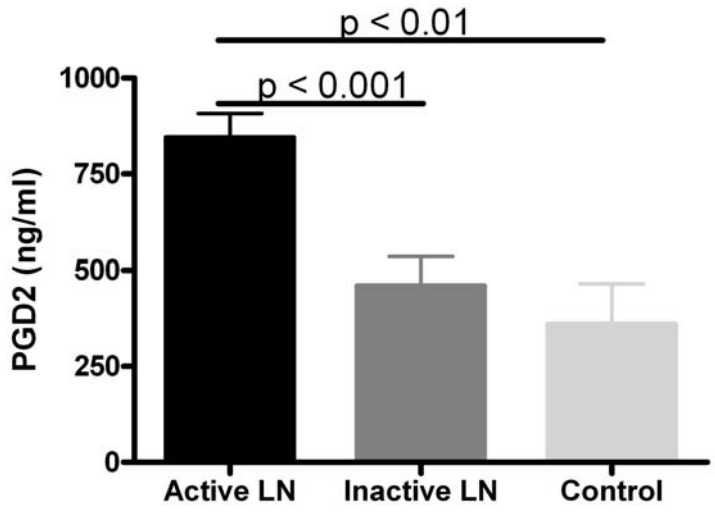
All of spots had $p > 0.05$ by ANOVA with T- test.

Table 2. Physical Properties of the Differentially expressed proteins

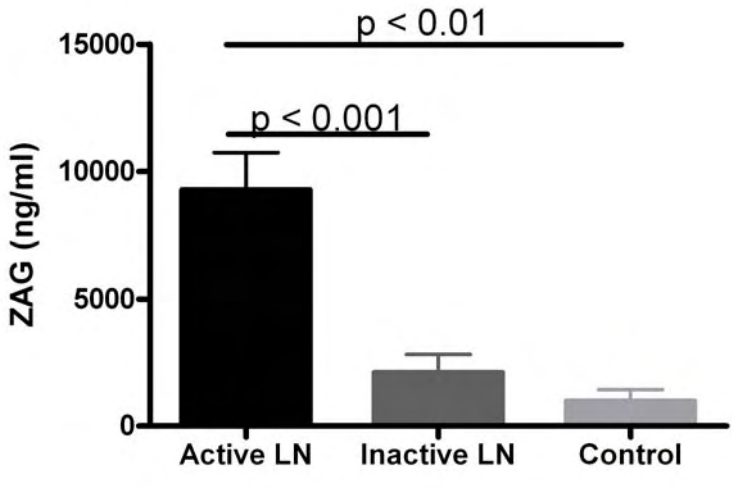
Spot	Protein Number	Accession no.	Mw	pI	Protein score
9744	Serum albumin	gi|28592	71361	6.05	2802
9107	serotransferrin precursor	gi|4557871	79280	6.81	1119
9105	Orosomucoid	gi|219519923	23817	4.93	904
9791	Kininogen-1 isofrom 2	gi|4504893	48936	6.29	460
10265	Zn-alpha2-glycoprotein	gi|38026	34946	5.71	696
10331	Zn-alpha2-glycoprotein	gi|38026	34946	5.71	806
11572	Immumoglobulin light chain	gi|218783338	24162	5.95	736
11750	IgG kappa chain	gi|4176418	23690	6.92	460
11633	Prostaglandin-H2 D-isomerase	gi|3217249	21243	7.99	109
15497	Chain A, The X-ray crystal structure Refinements of normal human transthyretin	gi|44329	13810	5.35	530
15533	Serum albumin variant	gi|456784	2975	5.61	85

prostaglandin H2 D-isomerase เป็นตัวที่ทำหน้าที่สร้าง cyclooxugenase-1 และ 2 (COX-1 และ COX-2) ซึ่ง COX-2 ทำหน้าที่เกี่ยวข้องกับการอักเสบ ดังนั้นผู้วิจัยจึงทำการ validate ของโปรตีน prostaglandin H2 D-isomerase ด้วยวิธี ELASA ในกลุ่มตัวอย่างที่มากขึ้น พบว่าทั้งสามกลุ่มผู้ป่วยที่ active มีปริมาณมากกว่าผู้ป่วย inactive กับคนปกติอย่างมีนัยสำคัญดังรูปที่ 7

Zn-alpha-2-glycoprotein (ZAG) โครงสร้างของ ZAG มีขนาดและน้ำหนักน้อยกว่า albumin ดังนั้นโปรตีน ZAG อาจจะเป็นโปรตีนที่หลั่งออกมาในปัสสาวะก่อนโปรตีน albumin นอกจากนั้นผู้วิจัยได้ทำการตรวจสอบในกลุ่มประชากรที่มากขึ้นพบว่ามีการแสดงของ ZAG ในปัสสาวะของกลุ่มคนไข้ active มากกว่ากลุ่มคนไข้ inactive และกลุ่มคนปกติ อย่างมีนัยสำคัญ ดังรูปที่ 8



รูปที่ 7 ระดับการแสดงออกของ PGD_2 ในปัสสาวะ ของผู้ป่วยเทียบกับกลุ่มคนปกติ



รูปที่ 8 ระดับการแสดงออกของ ZAG ในปัสสาวะ ของผู้ป่วยเทียบกับกลุ่มคนปกติ

นอกจากนั้นกลุ่มผู้วิจัยยังได้ทบทวนวรรณกรรมพบว่ามีการแสดงออกของโปรตีน **Interferon-inducible protein-10 (IP-10)** จากการศึกษา มีผู้เข้าร่วมการศึกษาทั้งหมด **28** ราย แบ่งเป็นผู้ป่วยกลุ่มไตอักเสบจากโรคลูปัสที่มีอาการกำเริบที่ได้รับการตรวจวินิจฉัยโดยการตรวจชิ้นเนื้อไตทุกรายจานวน **18** ราย และผู้ป่วยกลุ่มไตอักเสบจากโรคลูปัสอยู่ในระยะสงบ **10** ราย และกลุ่มควบคุม **10** ราย ข้อมูลในผู้ป่วยกลุ่มไตอักเสบจากโรคลูปัสอาการกำเริบพบว่า ผู้ป่วย ช่วงอายุระหว่าง 23.75-33.50 ปี มีค่ามัธยฐานของคะแนน renal SLEDAI อยู่ที่ 15 โดยช่วงคะแนนอยู่ระหว่าง 11-16 คะแนน ข้อมูลในผู้ป่วยกลุ่มไตอักเสบจากโรคลูปัสที่

อยู่ในระยะสงบ พบว่า ผู้ป่วยอายุเฉลี่ยช่วงอายุระหว่าง 25.50-34.75 ปี ค่ามัธยฐานของคะแนน renal SLEDAI อยู่ที่ 0 ข้อมูลพื้นฐานโดยละเอียดแสดงดังตารางที่ 3

ตารางที่ 3 ข้อมูลพื้นฐานของคนไข้ทั้งหมด

	Active LN	Inactive LN	Healthy control	*P*-value
Number	**18**	**10**	**10**	
Urine IP-10 (pg/mg Cr)	**0.49 (0.31-1.16)**	**0.13 (0.07-0.16)**	**0.05 (0.01-0.09)**	**<0.001**
Class of LN	**IV(15); III(3)**	**Not done**	**-**	
Gender (F/M)	**17/1**	**10/0**	**10/0**	
Age (Year)	**29.5 (23.75-**	**28.5 (25.50-**	**24.5 (22.75-**	**0.140**
SLEDAI score*	**33.50)**	**34.75)**	**27.50)**	**<0.0001**
Urine erythrocyte count* (per	**15 (11-16)**	**0**	**-**	**<0.0001**
HPF)	**12 (6.50-32.50)**	**2 (0-2.50)**	**0 (0-2.25)**	**<0.0001**
Proteinuria* (g/day)	**3.17 (2.16-5.02)**	**0.12 (0.04-0.49)**	**1.5 (0-3)**	**<0.0001**
Serum creatinine (mg/dL)	**0.9 (0.71-1.23)**	**0.65 (0.60-0.80)**	**0.6 (0.52-0.68)**	
Drug treatment, No. (%) of patients	**18 (100%)**	**10 (100%)**	**-**	**<0.0001**
Prednisolone Dose (mg/day)	**22.5 (7.50-50)**	**2.5 (2.50-3.13)**	**-**	**0.626**
Azathioprine	**4 (22%)**	**1 (10%)**	**-**	**0.364**
Mycophenolate mofetil	**6 (33%)**	**1 (10%)**	**-**	**0.025**
Enteric-coated mycophenolate	**8 (44%)**	**0**	**-**	
sodium				

ผลการศึกษาพบว่าผู้ป่วยกลุ่มไตอักเสบจากโรคลูปัสที่มีอาการกำเริบมีค่ามัธยฐานระดับโปรตีน IP-10 ในปัสสาวะในช่วงระหว่าง 0.49(0.31-1.16) สูงกว่ากลุ่มผู้ป่วยที่อยู่ในระยะสงบซึ่งมีค่าระหว่าง 0.13(0.07-0.16) และกลุ่มควบคุม0.05(0.01-0.09) โดยผู้ป่วยกลุ่มไตอักเสบจากโรคลูปัสที่มีอาการกำเริบมีการแสดงที่สูงกว่ากลุ่มที่มีอาการสงบและควมคุมแตกต่างกันอย่างมีนัยสำคัญทางสถิติ (P<0.001) นอกจากนั้นพบว่ามีความสัมพันธ์กับระดับ IP-10 ในปัสสาวะซึ่งแสดง ROC curve พบว่าสามารถพยากรณ์ความล้มเหลวระหว่างกลุ่มผู้ป่วยทั้งสองกลุ่มได้ โดยค่า cut off อยู่ที่ 0.20 pg/ml ซึ่งค่าดังกล่าวจะมีความไวประมาณร้อยละ 94 และความจำเพาะประมาณร้อยละ 100

นอกจากนี้ผู้วิจัยได้ศึกษาเพิ่มเติมโดยการหาความสัมพันธ์ระหว่าง lupus activity parameters ต่าง ๆทั้งจากลักษณะทางคลินิกกับระดับโปรตีนของ IP-10 ในปัสสาวะ พบว่า SLEDAI (r = 0.87; p-Value <0.001) และค่าย่อยของ renal SLEDAI ทุกตัว (SCr r = 0.6; p-Value < 0.001, Urine RBC r = 0.82; p-Value = 0.001, urine protein r = 0.76; p-Value <

0.001) นอกจากนั้นยังพบว่าเมื่อติดตามผู้ป่วยไปทุกๆสองเดือน ผู้ป่วยทที่มีการตอบสนองต่อการรักษาได้ดีในช่วงเวลา 6 เดือนจะมีระดับ urine IP-10 ลดลงเมื่อเทียบกับผู้ป่วยที่ไม่มีการตอบสนองต่อการรักษา (p=0.004) (รูปที่ 9)

รูปที่ 9 ระดับการแสดงออกของ IP-10 protein ในปัสสาวะของผู้ป่วย lupus nephritis หลังจากได้รับการรักษาด้วย immunosuppressive treatment เป็นระยะเวลา 6 เดือน

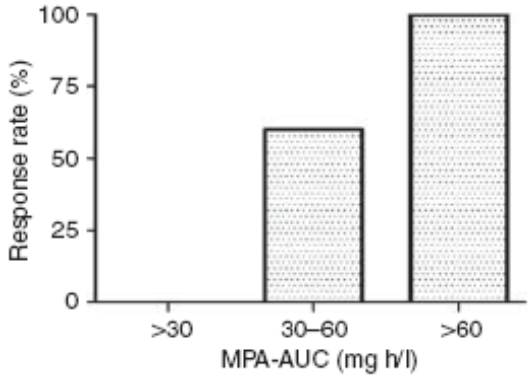
Part 4: ***Therapeutic drug monitoring***

ผลการทดลองพบว่ามีผู้ป่วยทั้งหมด 18 ราย ผู้ป่วยทั้งหมดมีพยาธิสภาพ class III, IV และ IV+V ผู้ป่วย 12 ได้รับการรักษาด้วยยา MMF อีก 6 รายได้รับการรักษาด้วย EC-MPS ซึ่งผู้ป่วยจะถูกเจาะเลือดในเวลา 0, 0.5, 1, 2, 3, 4, 8 และ 12 ชั่วโมง นำค่าที่ได้ไปคำนวณหาพื้นที่ใต้ระดับยาทุกจุดเวลาต่างๆ ภายหลังการให้ยาในช่วงเวลา 12 ชั่วโมง (MPA AUC $_{1\text{-}12}$) ดังตารางที่ 4 นอกจากนั้นยังพบว่า $AUC_{0\text{-}12}$ ของ MPA ในกลุ่มผู้ป่วยที่มีค่าของ MPA AUC $_{0\text{-}12}$ สูงมีเปอร์เซ็นต์การตอบสนองต่อการรักษาสูงกว่าผู้ป่วยที่มีค่า MPA AUC $_{0\text{-}12}$ ที่ต่ำกว่าอย่างมีนัยสำคัญทางสถิติ ผู้ป่วยที่มีค่า MPA -AUC $_{0\text{-}12}$ น้อยกว่า 30 h*mg/L มีเปอร์เซ็นต์การตอบสนองต่อการรักษา 0% เมื่อเทียบกับผู้ป่วยมีค่า MPA AUC $_{0\text{-}12}$ อยู่ระหว่าง 30-60 และ มากกว่า 60 h*mg/L ผู้ป่วยมีเปอร์เซ็นต์การตอบสนองต่อการรักษาเพิ่มเป็น 60% และ 100% ตามลำดับ ดังรูปที่ 10 นอกจากนั้นกลุ่มผู้วิจัยพบว่าค่า $AUC_{1\text{-}12}$ ของ MPA ไม่มีความสัมพันธ์กับขนาดของยา ซึ่งระยะเวลาที่ระดับ MPA จะสูงสุดหลังจากรับประทาน 1 ชั่วโมง ($r = 0.92$, $P < 0.001$) และมีความสัมพันธ์กับกลุ่มที่ตอบสนองต่อการรักษา (24.48 ± 11.82 mg/L VS. 5.96 ± 3.93 mg/L; $p=0.01$) กลุ่มผู้วิจัยยังได้หาความสัมพันธ์ระหว่าง plasma MPA concentration ที่จุดเวลาต่างๆโดยเฉพาะ C_0, C_1, C_4 และ C_8 กับ MPA AUC $_{0\text{-}12}$ในผู้ป่วยที่ได้รับยา MMF เพื่อสร้างเป็น model ต่างๆเพื่อใช้ทำนายค่า MPA AUC $_{0\text{-}12}$ ในผู้ป่วยที่ได้รับยา MMF และแสดงค่า correlation coefficient ดังภาพที่ 11 พบว่ะดับยาของ , C_1, C_4 และ C_8 มีความสัมพันธ์กับ MPA AUC $_{0\text{-}12}$อย่างมีนัยสำคัญทางสถิติในผู้ป่วยที่ได้รับยา MMF ทำให้กลุ่มผู้วิจัยพบว่า ค่า $AUC_{1\text{-}12}$ ของ MPA มีความสัมพันธ์กับการตอบสนองต่อการรักษา การควบคุมความเข้มข้นของระดับยา MPA มีผลต่อการประสิทธิภาพในการรักษาของผู้ป่วยไตอักเสบลูปัส

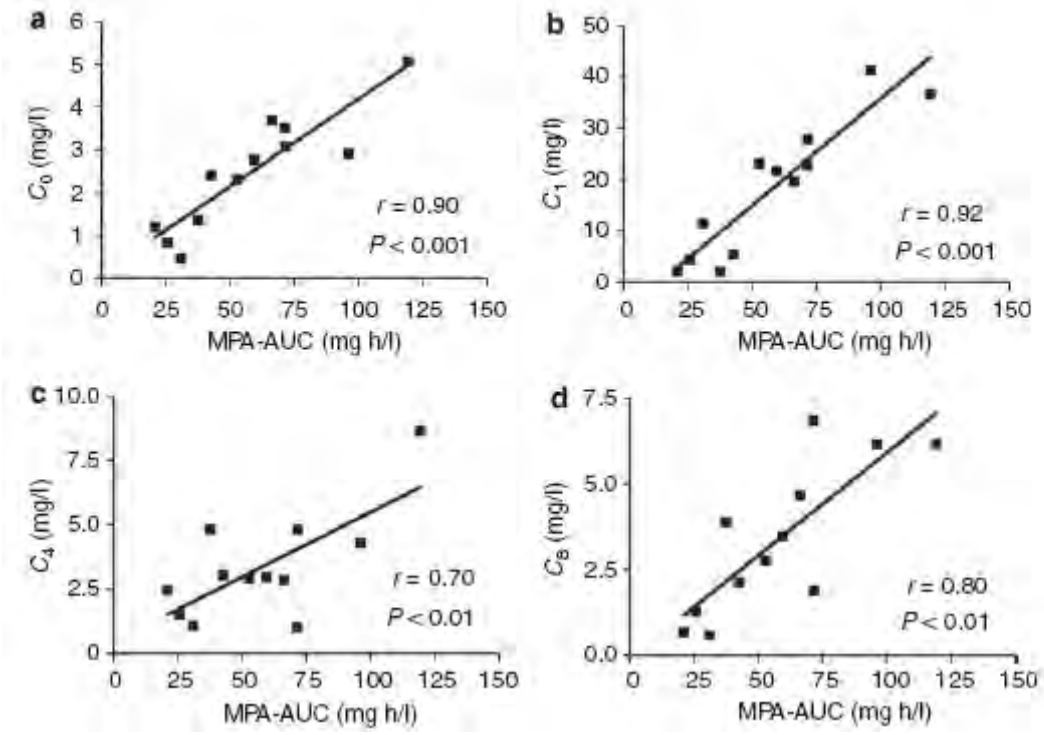
Table 4: Pharmacokinetic parameters of mycophenolic acid (MPA) after oral administration of 1-1.5 g/day MMF and 1,080-1,440 mg/day EC-MPS in patients with class III or IV lupus nephritis

Parameters	MMF (n=12) Means ± SD.	EC-MPS (n=6) Means ± SD.
C_0 (mg/L)	2.47 ± 1.33	2.73 ± 1.46
$C_{0.5}$ (mg/L)	7.08 ± 7.16	2.68 ± 1.46
C_1 (mg/L)	18.3 ± 13.28	2.02 ± 0.7
C_2 (mg/L)	6.72 ± 2.26	7.48 ± 5.83
C_3 (mg/L)	4.05 ± 2.64	6.23 ± 4.85
C_4 (mg/L)	3.37 ± 2.11	3.59 ± 1.99
C_8 (mg/L)	3.38 ± 2.19	2.92 ± 2.04
C_{12} (mg/L)	2.59 ± 1.9	2.14 ± 1.54
AUC_{0-12} (h*mg/L)	57.97 ± 29.38	42.44 ± 9.86
C_{max} (mg/L)	19.43 ± 12.01	10.12 ± 5.13
T_{max} (hours)	1.54 ± 1.03	3.25 ± 2.52
Dosage (mg./day)	1,416.67 ± 194.63	1,260 ± 197.18
Dosage/ IBW (kg.)	28.07 ± 5.39	25.37 ± 7.19

MMF: mycophenolate mofetil; EC-MPS: enteric-coated mycophenolate sodium; AUC_{0-12}: area under the concentration-time curve for 0 to 12 hours; C_{max}: maximum concentration of drug; T_{max}: maximum drug concentration; C_0: trough plasma concentration of mycophenolic acid; $C_{0.5}$, C_1, C_2, C_3, C_4, C_8, and C_{12}: plasma concentrations of mycophenolic acid at 0.5, 1, 2, 3, 4, 8 and 12 hours post-dose, respectively; IBW: ideal body weight.



รูปที่ 10 แสดงค่าระดับค่า MPA – AUC กับเปอร์เซ็นต์การตอบสนองต่อการรักษา



รูปที่ 11 แสดงความสัมพันธ์ของระดับยา MPA-AUC ในแต่ละจุดเวลา

การศึกษานี้ได้ตีพิมพ์เป็นส่วนหนึ่งใน Kidney International. 2010; 12: 1340-1348 (Paungpaga Lertdumrongluk, Poorichaya Somparn, Wonngarm Kittanamongkolchai, Opas Traitanon, Somratai Vadcharavivad, Yingyos Avihingsanon. Pharmacokinetics of mycophenolic acid in severe lupus nephritis. Kidney International (2010) 78, 389–395.)

Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

1. Avihingsanon Y, Hirankarn N. Major lupus organ involvement: severe lupus nephritis. *Lupus* 2010 19: 1391.

2. Paungpaga Lertdumrongluk, Poorichaya Somparn, Wonngarm Kittanamongkolchai, Opas Traitanon, Somratai Vadcharavivad, Yingyos Avihingsanon. Pharmacokinetics of mycophenolic acid in severe lupus nephritis. Kidney International (2010) 78, 389–395.

3. Yingyos Avihingsanon, Thitima Benjachat, Adis Tassanarong, Pimpayao Sodsai1, Vipawee Kittikovit, Nattiya Hirankarn. Decreased renal expression of vascular endothelial growth factor in lupus nephritis is associated with worse prognosis. Kidney International (2009) 75, 1340–13483)

4. Pimpayao Sodsaia, Nattiya Hirankarna, Yingyos Avihingsanon, and Tanapat Palaga. Defects in Notch1 Upregulation upon Activation of T Cells from Patients with Systemic Lupus Erythematosus are Related to Lupus Disease Activity. Lupus (2008) 17, 645–653.

5. Avihingsanon Y, Lekprasert P, Benjachat T, Kongpunvijit J, Traitanon O, Hirankarn N. Urinary IP-10 Protein Is Associated with Active Lupus Nephritis. J Am Soc Nephrol 2007; 18: 779A. (Abstract) (ตามเอกสารแนบ)

การไปเสนอผลงานทั้งในประเทศและต่างประเทศ

1) **Yingyos Avihingsanon, Patamaporn Lekprasert, Thitima Benjachat, Janejira Kongpunvijit, Opas Traitanon, and Nattiya Hirankarn. Title "Urinary IP-10 protein Is Associated with Active Lupus Nephritis" The American Society of Nephrology Meeting 2007, San Francisco, CA, USA. Nov 4, 2007 (Poster presentation)**
2) **Avihingsanon Y, Benjachat T, Tassanarong A, Kittikowit V, Hirankarn N and Eiam-Ong S. Expression of Vascular Endothelial Growth Factor (VEGF) is a Molecular Prognostic Marker in Proliferative Lupus Nephritis. 7th The Meeting of Young and Senior TFR Researchers, Oct 11-13, 2007. Ambassador City Jomtien Hotel, Chonburi, Thailand. (Oral presentation)**
3) **Avihingsanon Y, Benjachat T, Townumchai N, Tungsanga K, Eiam-org S, Hirankarn N. Urinary podocyte loss: Biomarker of active lupus nephritis. The WCN in Milan, May 22-26, 2009.**

ภาคผนวก

http://www.kidney-international.org

see commentary on page 1251

Decreased renal expression of vascular endothelial growth factor in lupus nephritis is associated with worse prognosis

Yingyos Avihingsanon[1,2], Thitima Benjachat[1], Adis Tassanarong[3], Pimpayao Sodsai[1], Vipawee Kittikovit[1,4] and Nattiya Hirankarn[1,5]

[1]Renal Division, Lupus Research Unit, Department of Medicine, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand; [2]Department of Medicine, Chulalongkorn University, Bangkok, Thailand; [3]Department of Medicine, Faculty of Medicine, Thammasart University, Pathumthani, Thailand; [4]Department of Pathology, Chulalongkorn University, Bangkok, Thailand and [5]Department of Microbiology, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand

Recent studies suggest that vascular endothelial growth factor (VEGF) plays a crucial role in the preservation of renal function and may also serve as a useful biomarker in monitoring the progression of lupus nephritis (LN). Here we sought to correlate intrarenal VEGF expression with renal histopathology and prognosis of LN. Biopsy specimens from 35 patients with Class III or IV LN (ISN/RPS categorization) were found to have lower levels of intrarenal VEGF than those found in biopsy tissue taken from 10 donor kidneys sampled at the time of allograft reperfusion. This reduced amount of VEGF mRNA in the patients with LN negatively correlated with glomerular endocapillary proliferation, crescent formation, and a high histologic activity index but was positively associated with increased numbers of urinary podocytes. The level of intrarenal VEGF mRNA accurately predicted the deterioration of renal function in these patients within 12 months. Our study shows that expression of VEGF in renal tissue may serve as a molecular marker of renal damage and may be a predictive factor for short-term loss of kidney function in patients with LN.

Kidney International (2009) **75,** 1340–1348; doi:10.1038/ki.2009.75; published online 18 March 2009

KEYWORDS: biomarker; lupus nephritis; molecular diagnosis; podocyte; VEGF; WT-1

Correspondence: *Yingyos Avihingsanon, Renal Division, Lupus Research Unit, Department of Medicine, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand. E-mail: yingyos.a@gmail.com*

Received 2 July 2008; revised 23 January 2009; accepted 27 January 2009; published online 18 March 2009

Proliferative lupus nephritis (LN) is the most common and severe histology of LN.[1] Steroids and cytotoxic drugs remain the most commonly used treatments despite the many associated adverse events.[2] Renal histology is essential for the selection of proper treatment and prognostication of the disease.[3] Specific histological findings, such as crescent formation, may determine prognosis.[3] However, immunosuppressive therapy could mitigate such pathology and therefore improve renal prognosis.[4] Since the discovery of the molecular mechanisms of systemic lupus erythematosus (SLE), novel immunosuppressive therapies have been introduced.[5] Nevertheless, the mechanism of the loss of renal function remains unknown.[6] Studies of intrarenal molecular signatures could reveal the molecular mechanism of the disease and predict renal prognosis.[7–9] Finally, molecular classification may be integrated into the histological classification of LN.

The integrity of the glomerular and peritubular capillaries is vital for renal function. Progressive capillary loss, with obliteration of the microvasculature, frequently accompanies fibrosis, which is a characteristic feature of progressive renal disease.[10,11] Progression of glomerulopathy is, at least in part, due to loss of glomerular integrity. Vascular endothelial growth factor (VEGF) promotes survival, proliferation, and differentiation of glomerular endothelial cells.[12] Decreased expression of VEGF has been associated with various glomerulopathies such as crescentic glomerulonephritis, focal glomerulosclerosis, IgA nephropathy, pre-eclampsia, and aging kidneys.[10,13,14] Administration of VEGF has been shown to stabilize kidney function in many models including the remnant model, thrombotic microangiopathy, and chronic cyclosporine nephropathy.[15–18] The protective actions were principally mediated through preservation of glomerular and peritubular capillary structures.[18] A recent study in human diabetic nephropathy has confirmed the role of VEGF in maintaining renal vasculature and identified it as a novel biomarker.[19] The growing evidence supports the potential role of VEGF in SLE and nephritis, but the studies remain inconclusive.[13,20–23]

Intrarenal quantitative gene expression may be used for grading of disease severity.[8] For instance, intrarenal transforming growth factor-β (TGF-β) gene expression could determine progression of chronic kidney disease.[9] In LN, it is difficult to determine prognosis at the time of renal flare. In an earlier study, we suggested a serial non-invasive measurement of urinary mRNA for chemokines and growth factors that could predict the prognosis of this disease.[24] In this study, we aim to determine an association between intrarenal molecular signatures and renal histology. Furthermore, we determined whether the molecular signature in the kidney could predict the progression of LN.

RESULTS

Patients

Fifty-one biopsy samples were obtained during a diagnostic process of clinically active LN. Ten samples were excluded due to an inadequate number of glomeruli (less than five) or chronic scarring glomeruli. Six samples from patients with LN class V were excluded. Thirty-four samples were from female patients. Mean (s.e.) age was 31 (1.27) years. The mean (s.e.) SLE disease activity index (SLEDAI)[25] and level of serum creatinine, 24 h urine protein, and erythrocyturia were 10.58 (0.91), 1.74 (0.26) mg per 100 ml, 3.53 (0.47) g/day, and 32.70 (15.47) cells per high power field, respectively (Table 1).

Ten samples of implantation biopsies from donor kidneys were used as controls. Six kidney samples were from living donors, and four were from deceased donors (Table 1). Histological examination of the wedge-biopsies showed unremarkable findings except for minimal tubular injury.

Renal histology of LN

Eight samples were class III and 27 were class IV by the ISN/RPS classification of LN, respectively. More details of renal histology are shown in Table 1. The mean (s.e.) of renal activity and chronicity indices were 7.49 (0.85) and 4.06 (0.53), respectively. Crescent formation was observed in 12 samples (34%). Other pathologies observed in the samples were endocapillary proliferation (82%), fibrinoid necrosis (34%), glomerular neutrophil infiltration (65%), and thrombotic microangiopathy (16%).

Intrarenal expression of VEGF in LN

The levels of VEGF mRNA in the kidneys of LN patients ($n=35$) were decreased as compared with the implantation biopsies of kidney donors ($n=10$) (-0.64 ± 0.05 vs -0.08 ± 0.13 log copies; $P<0.001$) (Table 1). The levels of heme-oxygenase-1 (HO-1) were decreased in LN (-0.45 ± 0.06 vs 0.30 ± 0.26 log copies; $P=0.002$), whereas the levels of TGF-β and angiopoeitin-1 (ANGPT-1) were not different between patients and controls (-0.13 ± 0.06 vs -0.03 ± 0.15 log copies; $P=0.22$ and (0.60 ± 0.08 vs 0.16 ± 0.38 log copies; $P=0.44$).

Intrarenal VEGF mRNA levels were lower in samples with crescent formation (-0.98 ± 0.03 vs -0.62 ± 0.05 log copies;

Table 1 | Clinical and histological variables at the time of biopsy[a]

	Controls[b]	Patients	*P*-value
Number	10	35	
Gender (female/male)	2/8	34/1	
Age (years)	33.40 ± 3.76	31.74 ± 1.27	0.74
Clinical parameters			
Serum creatinine (mg per 100 ml)	1.18 ± 0.13	1.74 ± 0.26	0.93
Proteinuria (g/day)	0	3.53 ± 0.47	<0.001
Urinary erythrocyte count (per high power)	0	32.70 ± 15.47	<0.001
MDRD-GFR (ml/min per 1.73 m^2)[c]	81.10 ± 9.80	66.36 ± 6.84	0.49
SLEDAI[d]	NA	10.58 ± 0.91	
Steroid dose (mg/day)	0	32.20 ± 6.64	<0.001
Activity index	NA	7.49 ± 0.85	
Chronicity index	NA	4.06 ± 0.53	
Renal histology			
III (S), (G)	NA	7, 1	
IV (S), (G)	NA	10, 17	
III (A), (A/C)	NA	4, 4	
IV (A), (A/C)	NA	16, 11	
Intrarenal mRNA levels			
VEGF	−0.08 ± 0.13	−0.64 ± 0.05	<0.001
HO-1	0.30 ± 0.26	−0.45 ± 0.06	0.002
TGF-β	−0.03 ± 0.15	−0.13 ± 0.06	0.22
Angiopoeitin-1	0.16 ± 0.38	0.60 ± 0.08	0.44

[a]Data are expressed as mean ± s.e.
[b]Control: implantation biopsy from six living donors and four deceased donors.
[c]MDRD-GFR, glomerular filtration rate at the time of biopsy.
[d]SLEDAI, Systemic Lupus Erythematosus Disease Activity Index.[25]
A, active; C, chronic; G, global; HO-1, heme-oxygenase-1; NA, not applicable; S, segmental; TGF-β, transforming growth factor-β; VGEF, vascular endothelial growth factor.

$P = 0.04$). The biopsy samples with diffuse endocapillary proliferation ($\geqslant 25\%$ of glomeruli) expressed lower VEGF levels (-0.72 ± 0.05 vs -0.44 ± 0.06 log copies; $P = 0.003$). Samples with a high activity score (score $\geqslant 3$ of total 24) expressed lower VEGF levels (-0.70 ± 0.05 vs -0.40 ± 0.07 log copies; $P = 0.009$). However, samples with glomerular neutrophil infiltration ($\geqslant 25\%$ of glomeruli) showed no difference in VEGF levels (-0.67 ± 0.08 vs -0.62 ± 0.05 log copies; $P = 0.09$). Figure 1a–d shows that the presence of crescent formation, endocapillary proliferation, and a high activity index were associated with decreased VEGF mRNA levels.

Correlation between VEGF, TGF-β, AGPT-1, and HO-1

As there is a functional relationship among genes in the hypoxia-inducible pathway, such as VEGF, TGF-β, AGPT-1, and HO-1, we attempted to determine the association between each mRNA level in the biopsy tissues. Figure 2a–c shows that mRNA levels of VEGF were associated with TGF-β ($R = 0.41$, $P = 0.02$) and HO-1 ($R = 0.65$, $P < 0.0001$), but not ANGPT-1 ($R = 0.15$, $P = 0.41$) mRNA levels.

Immunohistochemistry localization of VEGF within renal biopsies

In kidney donors, the VEGF protein was expressed on podocytes and markedly expressed on tubular epithelial cells (Figure 3c and e). In LN, weak VEGF staining was observed in all samples (Figure 3b, d and f). The VEGF protein was scarcely seen on glomeruli and tubular epithelial cells of samples with diffuse endocapillary proliferation (Figure 3b) or crescent formation (Figure 3d).

Intrarenal VEGF mRNA levels predict a loss of renal function within 12 months

Active LN patients were treated with standard therapy (see Materials and Methods) and were followed for 12 months after kidney biopsy. Of all 35 patients, 10 patients experienced a loss of their renal function within 12 months (doubling serum creatinine levels or end-stage renal disease (ESRD)). We performed receiver operating characteristic analysis to determine the best cutoff that had the maximal sensitivity and specificity based on a loss of renal function. The mRNA cutoff level of -0.63 log copies could predict a loss of renal function with negative and positive predictive values of 100 and 53%, respectively. We observed that 53% of patients with low VEGF mRNA levels, but none of the patients with high levels, experienced loss of their renal function within 12 months. Patients with low VEGF mRNA levels had a significantly increased risk of loss of renal function, including doubling serum creatinine levels, ESRD, or both events combined ($P < 0.001$ by log-rank test) (Figure 4a–c). In contrast, crescent formation and a high renal activity index were not associated with ESRD (data not shown).

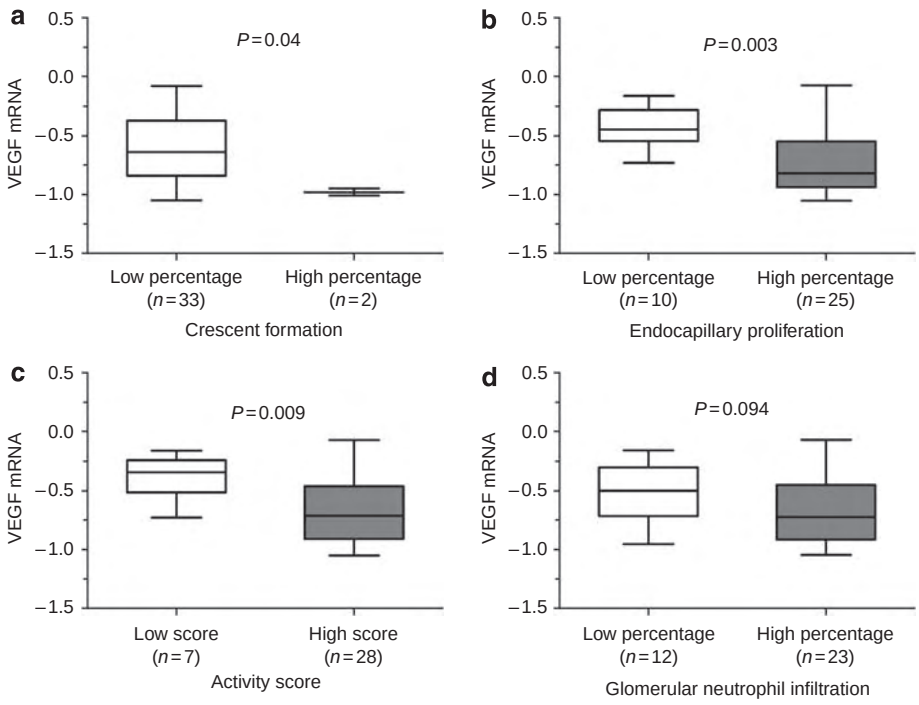


Figure 1 | Intrarenal vascular endothelial growth factor (VEGF) mRNA and renal pathology. Box and whisker plots show the 10th, 25th, 50th (median), 75th, and 90th percentiles of values (log) for VEGF mRNA levels in the kidney tissue of patients with class III/IV lupus nephritis. The levels of VEGF mRNA were significantly lower in samples with the presence of crescentic formation (**a**), endocapillary proliferation (>25% of glomeruli) (**b**), and high activity index (AI >3) (**c**), but not glomerular neutrophil infiltration (>25% of glomeruli) (**d**). (*P*-value by Mann–Whitney test.) Numbers in the parentheses indicate the number of biopsy samples.

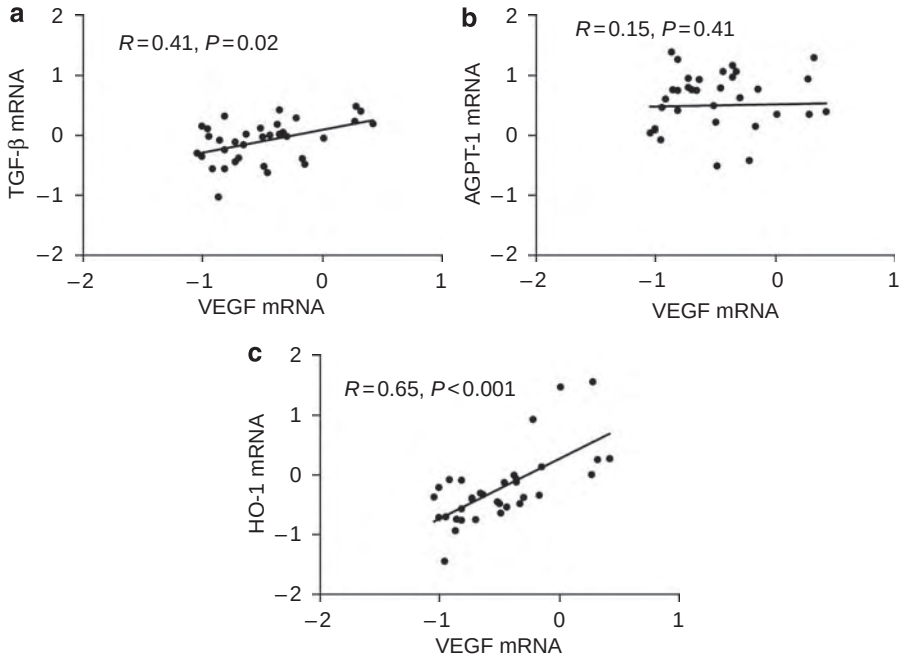


Figure 2 | Coordinated expression of intrarenal VEGF and related genes. (**a**) The relationship between the levels of VEGF and TGF-β was significant at $P = 0.02$ ($R = 0.41$). (**b**) The relationship between the levels of VEGF and ANGPT-1 was not significant ($P = 0.41$, $R = 0.15$). (**c**) The relationship between the levels of VEGF and HO-1 was significant at $P < 0.0001$ ($R = 0.65$).

Urinary loss of podocytes is associated with active LN

In an earlier study, we reported an increase in urinary VEGF levels in patients with biopsy-proven proliferative LN.[24] Although tubular epithelial cells are the main source of VEGF in the kidney, we hypothesized that the reduction of intrarenal VEGF in this study may partly be explained by a loss of podocyte cells into the urine. We therefore analyzed the relationship between mRNA levels from urine cells and renal tissue during active LN. To perform a proper comparison, urine samples were collected from patients ($n = 21$) on the day of renal biopsy. There was a significant association between urinary WT-1 (podocyte marker) and VEGF mRNA levels (Figure 5a) ($R = 0.51$; $P = 0.02$). Urine WT-1 and VEGF mRNA levels were increased in active LN (urine WT-1 $= 2.88 \pm 0.25$ and VEGF $= 2.14 \pm 0.23$ log copies). In contrast, intrarenal WT-1 and VEGF mRNA levels were decreased in active LN (renal WT-1 $= -0.56 \pm 0.08$ and VEGF $= -0.73 \pm 0.14$ log copies) (Figure 5b).

DISCUSSION

Renal histology study is essential for guidance of patient management and for predicting prognosis of renal disease.[26] Patients with proliferative LN (ISN/RPS class III or IV) are inevitably destined for chronic or end-stage kidney disease.[27,28] The use of currently available immunosuppressive treatment has significantly improved renal prognosis.[29] However, physicians have been unable to readily predict individual responses until patients finish a 6-month course of immunosuppressive treatment.[28] In general, a loss of renal function within 6 months after induction of treatment could determine long-term prognosis.[3] In this retrospective study, intrarenal VEGF expression was decreased in severe LN. Moreover, VEGF expression at the time of renal flare may be a useful predictor of poor renal function within 12 months. Molecular biomarkers may be useful in the diagnosis and prognosis of LN in the future.

Several studies have suggested that VEGF plays a key role in endothelial cell proliferation and capillary repair.[16,18,30] In the model of membranoproliferative GN, blockade of the $VEGF_{165}$ protein could lead to progressive renal damage.[31] In the remnant kidney model, VEGF is reduced in both glomeruli and tubular cells, which could be corrected by VEGF replacement.[17] VEGF could enhance endothelial cell repair as well as increase angiogenic response of peritubular capillaries.[32] It is known that VEGF can alter endothelial cell growth, integrity, and function and may eventually contribute to glomerulopathy.[12,18,30] It is expected for reduced endocapillary proliferation to be associated with decreased VEGF. This study could not determine such an association.

Vascular endothelial growth factor has been shown to stabilize kidney function in animal models of thrombotic microangiopathy[16] and chronic cyclosporine nephropathy.[17] The protective actions were principally mediated through preserved glomerular and peritubular capillary structures.[18] This may help to preserve glomerular filtration rate by maintaining glomerular capillary filtration surface area as well as preventing tubulointerstitial fibrosis.[18] This vascular protective action of VEGF could explain, in this study, an association between intrarenal VEGF and a loss of renal function in 12 months. Furthermore, histological evidence in this study showed expression of VEGF in both the glomeruli and the tubular cells of kidney donors, whereas there was a

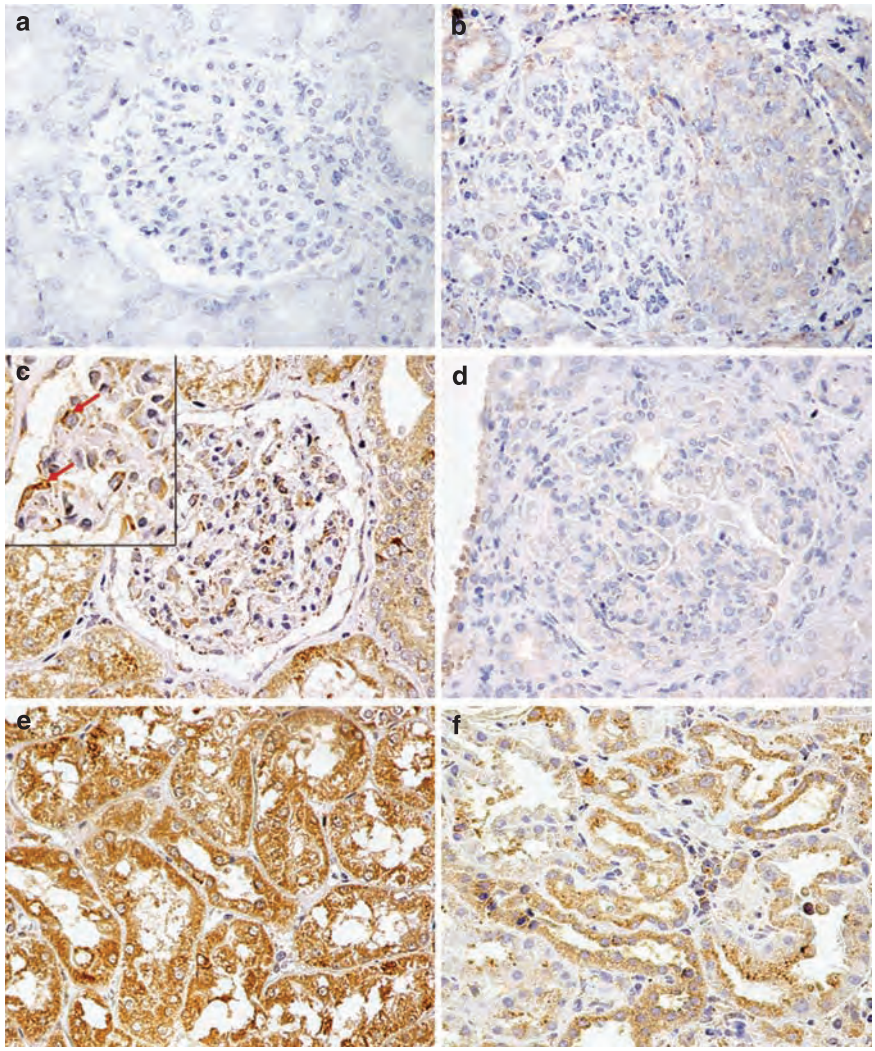


Figure 3 | Immunohistochemistry localization of VEGF within renal biopsies of patients with lupus nephritis or kidney donors (implantation biopsy). (**a**) Sections were not stained with control monoclonal antibodies. (**b**) VEGF protein was absent in crescentic glomeruli of patients with lupus nephritis but markedly expressed in glomeruli of implantation biopsies of kidney donors (**c**); inset shows VEGF ± podocyte cells (arrows). (**d**) VEGF was scarcely seen in glomeruli with endocapillary proliferation of lupus nephritis. (**e**) VEGF was ubiquitously expressed on tubular epithelial cells of donor kidney tissues but was reduced in the tubulointerstitium of kidneys of patients with lupus nephritis (**f**). (Original magnification × 400, except inset × 600).

marked reduction of VEGF in both structures of kidneys with active LN.[13,33] A study of renal tubular cell lines found that VEGF acts as a survival factor by induction of cell proliferation and antiapoptotic responses.[34] The expression of VEGF in renal tubules may result to protect against injury such as hypoxia, ischemia/reperfusion, hypokalemia, or oxidative stress.[34–36]

In this study, intrarenal HO-1 expression was well correlated with VEGF expression. The mRNA levels of both genes were decreased in LN as compared with kidney donors. In human kidney transplantation, both HO-1 and VEGF mRNA levels have been shown to be decreased in deceased donors (prolonged ischemia time) as compared with living donors (short ischemia time).[33] In this study, the levels of VEGF decreased as the severity increased; therefore, further studies are needed to clarify the role of hypoxic injury in LN.

Studies of serum VEGF in SLE patients have shown higher levels of VEGF in patients with active SLE than in patients with inactive SLE or healthy individuals.[20–22] It is difficult to compare VEGF expression among the studies, as the quantification methods and studied samples (serum versus tissue) were different.[23] The local effects of VEGF may be different from the systemic responses. The variation of patients' characteristics and immunosuppressive treatments may be considered to be confounding factors. It should be noted that patients received a moderately high doses of steroids in this study, although we did not find a relationship between steroid dose and molecular profiling. Furthermore, serum VEGF levels may not be associated with intrarenal VEGF levels, as we may detect VEGF expression from different cell sources. For instance, the serum VEGF may originate from vascular endothelial cells, whereas renal VEGF,

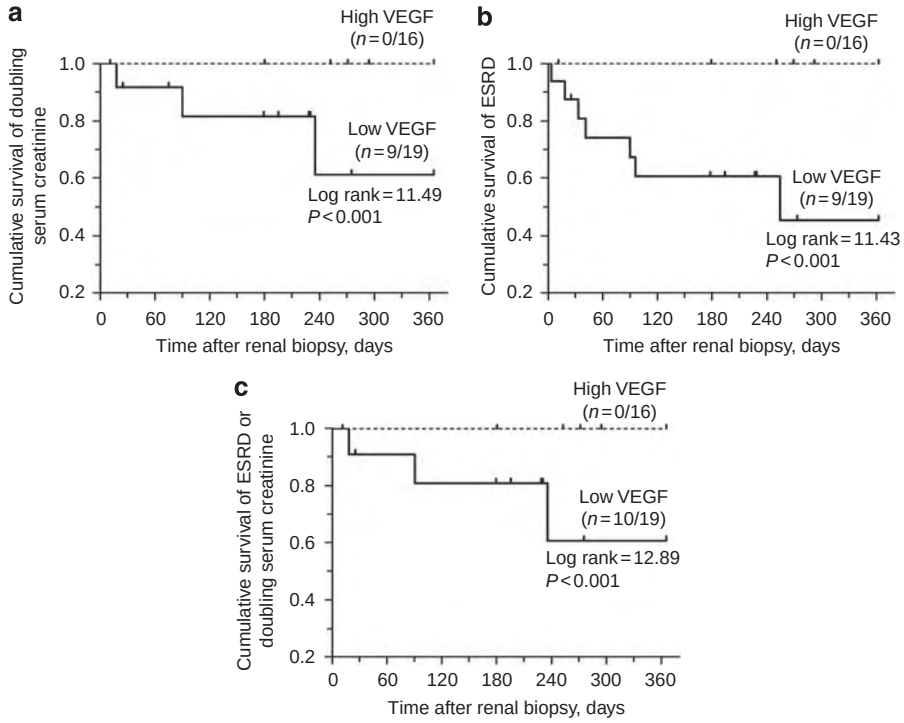


Figure 4 | Analysis of the relationship between VEGF mRNA levels and a loss of renal function in 12 months. (**a**) Shows Kaplan–Meier estimates of the time to doubling serum creatinine in all patients with class III/IV LN, as defined by two groups of VEGF mRNA levels (the best cutoff mRNA level identified by ROC analysis). (**b**) Shows Kaplan–Meier estimates of the time to end-stage renal disease (ESRD). (**c**) Shows Kaplan–Meier estimates of the time to both outcomes combined (doubling serum creatinine or ESRD). According to log-rank analysis, *P*-values were <0.001 for the model based on the two groups shown in all panels.

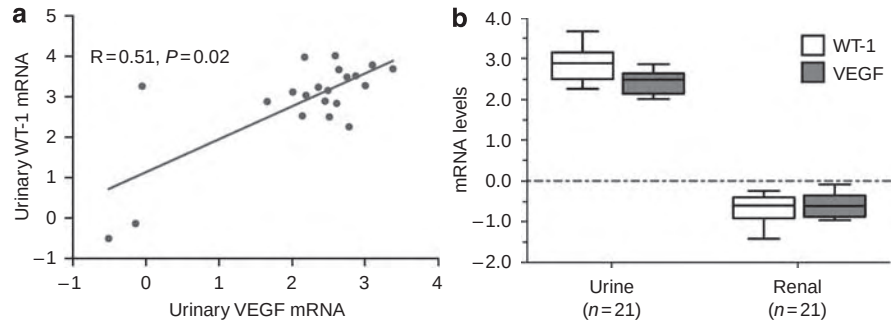


Figure 5 | Study of a podocyte marker (WT-1) and VEGF from urine samples of lupus nephritis patients. (**a**) The relationship between the levels of VEGF and WT-1 was significant at $P=0.02$ ($R=0.51$). (**b**) Box and whisker plots show the 10th, 25th, 50th (median), 75th, and 90th percentiles of values (log) for WT-1 and VEGF mRNA levels in the urine as compared with their levels in the kidney tissue of patients with class III/IV lupus nephritis ($n=21$). The dotted line represents normal values.

in this study, originates from tubular epithelial cells and podocytes.[34,37] Finally, patients with different stages and levels of severity of SLE may show different patterns of VEGF expression.[13,22]

We previously reported an association between urinary VEGF expression and active proliferative LN,[24] whereas in this study, an inverse relationship between intrarenal VEGF and histological activity was found. This has been validated by performing mRNA studies from same-day collection of urine and renal tissue. Figure 5 shows an inverse relationship between urine and intrarenal VEGF mRNA levels. We hypothesize that there may be a significant urinary podocyte loss in the active LN.[37] Although tubular epithelial cells are the main source of VEGF in the kidney, urinary podocyte loss may partly contribute to a reduction of VEGF in crescent formation.[37] Selective knockout of VEGF in the podocyte showed impaired glomerular capillary formation due to a loss of endothelial cells, supporting the important role of VEGF in podocytes in maintaining capillary integrity.[38] Yu *et al.*[39] showed that urinary podocyte loss is associated with glomerular damage in both primary and secondary glomerulonephritis in mice. We confirmed that there was urinary podocyte loss in LN by demonstrating an increase in urinary WT-1 mRNA levels of patients with active nephritis

(Figure 5). It would be interesting to further determine whether urinary podocyte numbers might be a functional biomarker for disease activity.

In conclusion, this study shows the pivotal role of renal VEGF expression in human LN. Patients with proliferative LN who had decreased intrarenal VEGF expression are at risk for a rapid decline of renal function. At the time of renal flare, the combination of renal pathology such as class III/IV LN and reduced VEGF expression could predict poor renal survival. Intrarenal VEGF may become a candidate surrogate marker for targeting therapy and in development of clinical trials.

MATERIALS AND METHODS

Patients

A total of 51 patients underwent renal biopsy for diagnostic evaluation of active LN between 2002 and 2005. All patients had been diagnosed with SLE according to the 1997 American College of Rheumatology criteria. All biopsies were examined by one pathologist (VK) who was not aware of the results of the molecular study. The samples were classified according to the histological types of LN using the ISN/RPS classification.[40] Six samples from patients with LN class V were excluded from the study. Ten samples were excluded because of inadequate number of glomeruli (less than five) or chronic scarring glomeruli. In class III or IV LN, patients were treated with oral prednisolone plus a 6-month course of intravenous cyclophosphamide[41] or oral mycophenolate mofetil.[42] In rapidly progressive renal failure from crescentic LN, three consecutive doses of intravenous methylprednisolone were given and three sessions of plasmapheresis were performed. The patients then received oral prednisolone plus a 6-month course of intravenous cyclophosphamide.[43]

The control group consisted of kidney samples from implantation biopsies after reperfusion of kidney allografts. Inclusion criteria were living or deceased donors with normal serum creatinine levels and donor age of less than 55 years. The kidney samples from patients with delayed graft function or prolonged ischemia time were excluded.

The study was approved by the Ethics Committee for Human Research of the Faculty of Medicine, Chulalongkorn University, and written informed consent was obtained from all patients.

Renal histology

Paraffin sections were stained with hematoxylin and eosin, periodic acid-Schiff, trichrome, and silver for light microscopy. The specimens were scored for activity and chronicity indices as described earlier.[1] The maximum scores of the activity index and chronicity index were 24 and 12, respectively. The activity index was the sum of semiquantitative scores of the following parameters: endocapillary proliferation, fibrinoid necrosis, cellular crescents, leukocyte infiltration, hyaline thrombi, and interstitial infiltration. The chronicity index was the sum of semiquantitative scores of the following parameters: glomerular sclerosis, fibrous crescents, interstitial fibrosis, and tubular atrophy. A percentage of each parameter was calculated by the equation:

$$\text{Percentage of each pathology} = (\text{Number of involved glomeruli}/\text{Total number of obtained glomeruli}) \times 100\%$$

RNA isolation from renal biopsy samples

Diagnostic renal biopsy specimens from LN patients were obtained and stored at -80°C. RNA isolation, quantification, and reverse transcription into complementary DNA were performed as described earlier.[8]

Quantification of mRNA

The mRNA of 18s rRNA, VEGF, ANGPT-1, TGF-β, and HO-1 were measured using a Light Cycler machine (Roche Molecular Biochemicals, Indianapolis, IN, USA). The sequences of primers and fluorescence probes are as follows: 18s rRNA sense, 5′-gcccgaagcgtttactttga-3′; 18s rRNA antisense, 5′-tccattattcctagctgcggtatc-3′; 18s rRNA probe, 5′FAM-aaagcaggcccgagccgcc-TAMRA3′; VEGF sense, 5′-cctacagcacaacaaatgtgaatg-3′; VEGF antisense, 5′-caaatgctttctccgctctga-3′; VEGF probe, 5′FAM-caagacaagaaaatccctgtgggcct-TAMRA3′; ANGPT-1, sense 5′-tgcaaatgtgccctcatgtta-3′; ANGPT-1 antisense, 5′-tcccgcagtatagaacattcca-3′; TGF-β sense, 5′-ccctgcccctacatttggag-3′; TGF-β antisense, 5′-ccgggttatgctggttgtaca-3′; TGF-β probe, 5′FAM-cacgcagtacagcaaggtcctggcc-TAMRA3′; HO-1 sense, 5′-gcccttcagcatcctcagttc-3′; HO-1 antisense, 5′-ggtttgagacagctgccacat-3′; HO-1 probe, 5′FAM-tgcagcagagcctggaagacaccc-TAMRA3′. All primer pairs were designed to span across an intron–exon boundary to distinguish amplification of genomic DNA. Each PCR was carried out in a 20 μl reaction volume composed of 2 μl of cDNA template and 18 μl of a real-time PCR mastermix that contained 10 μl of 2 × QuantiTech Probe Mastermix (Qiagen Inc., Chatworth, CA, USA), 0.5 μM forward primer, 0.5 μM reverse primer, and 0.2 μM probe. No fluorescent signal was generated by these assays when genomic DNA was used as a substrate, which confirmed that the assays measured only mRNA. The levels of mRNA were analyzed by a comparative method.[44] The reference RNA was a pool of RNA from implantation kidney biopsies of live-donors. To control possible variation among PCR runs, VEGF and an 18s rRNA plasmid (housekeeping gene) were used as calibrators. The PCR amplicon for 18s rRNA was used for developing standard curves. The standard curves were based on the principle that a plot of the log of the initial target copy of a standard versus threshold cycles results in a straight line. The levels of mRNA were expressed as the number of copies per microgram of total RNA isolated from renal biopsy tissues.

Immunohistochemistry

For immunohistochemistry, tissue samples were fixed in 4% buffered paraformaldehyde and embedded in paraffin. Deparaffinized sections were heated in a microwave oven with sodium citrate buffer. The rabbit anti-VEGF-A antibody

sc152 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA; dilution of 1:100) and an Envision reagent kit (Dako, Carpinteria, CA, USA) were used. The color product of peroxidase was developed by the 3,5 diaminobenzidine substrate and counterstaining with hematoxylin. Negative controls included normal rabbit IgG.

Quantitation of the urine podocyte marker and VEGF

To properly compare intrarenal and urinary gene expression, a 50 ml urine sample was collected on the day of kidney biopsy. The urine sample was immediately centrifuged after collection at 1000 *g* for 30 min at 4°C. Total RNA was isolated from the cell pellets using an RNA blood mini kit (Qiagen, Chatworth, CA, USA), measured for concentration, and reverse transcribed into complementary DNA as described earlier.[24,45] The mRNA levels of VEGF, WT-1 (podocyte marker), and 18s rRNA (housekeeping gene) were measured as described above.

Statistical analysis

Statistical analysis was performed using the SPSS software (version 11.5, SPSS Inc., Chicago, IL, USA). The levels of mRNA deviated significantly from the normal distribution ($P<0.001$) and were reduced by log-transformation. All data are given as mean and s.e. The Mann–Whitney test was used for comparison between the two groups. The relationship between the mRNA levels of each group was estimated with Spearman's ρ correlation. A loss of renal function was determined by a doubling of serum creatinine calculated from the renal biopsy date or ESRD. The criteria of ESRD included having a calculated MDRD-GFR below 15 ml/min or initiation of renal replacement therapy (dialysis or transplantation). To distinguish patients who had a loss of renal function, a receiver operating characteristic curve of mRNA levels was used to determine the cutoff levels that maximized the combined sensitivity and specificity. We estimated the probabilities of a loss of renal function using the Kaplan–Meier method and compared them using a log-rank test. All *P*-values below 0.05 were considered statistically significant.

DISCLOSURE

All the authors declared no competing interests.

ACKNOWLEDGMENTS

Parts of this study were presented at the American Society of Nephrology meeting in November 2006 as a free communication and at the Lupus meeting in May 2007 as a poster discussion. We thank Chutima Rukrung, RN and Sujinda Pipopsuthipibul, RN for management of the clinical database. We also thank Supranee Buranapraditkun, MSc. and Preecha Raungwetworachai for excellent technical assistance and Panita Lertwiriyananthakul for the pathology database. This work was supported by the National Research Council of Thailand. YA is a recipient of a grant from the Thailand Research Fund (RMU 5080076).

REFERENCES

1. Austin III HA, Muenz LR, Joyce KM *et al.* Diffuse proliferative lupus nephritis: identification of specific pathologic features affecting renal outcome. *Kidney Int* 1984; **25**: 689–695.
2. Shayakul C, Ong-aj-yooth L, Chirawong P *et al.* Lupus nephritis in Thailand: clinicopathologic findings and outcome in 569 patients. *Am J Kidney Dis* 1995; **26**: 300–307.
3. Austin III HA, Boumpas DT, Vaughan EM *et al.* Predicting renal outcomes in severe lupus nephritis: contributions of clinical and histologic data. *Kidney Int* 1994; **45**: 544–550.
4. Traitanon O, Avihingsanon Y, Kittikovit V *et al.* Efficacy of enteric-coated mycophenolate sodium in patients with resistant-type lupus nephritis: a prospective study. *Lupus* 2008; **17**: 744–751.
5. Ronnblom L, Alm GV. Systemic lupus erythematosus and the type I interferon system. *Arthritis Res Ther* 2003; **5**: 68–75.
6. Tang S, Lui SL, Lai KN. Pathogenesis of lupus nephritis: an update. *Nephrology (Carlton)* 2005; **10**: 174–179.
7. Avihingsanon Y, Ma N, Csizmadia E *et al.* Expression of protective genes in human renal allografts: a regulatory response to injury associated with graft rejection. *Transplantation* 2002; **73**: 1079–1085.
8. Avihingsanon Y, Ma N, Pavlakis M *et al.* On the intraoperative molecular status of renal allografts after vascular reperfusion and clinical outcomes. *J Am Soc Nephrol* 2005; **16**: 1542–1548.
9. Eikmans M, Baelde HJ, Hagen EC *et al.* Renal mRNA levels as prognostic tools in kidney diseases. *J Am Soc Nephrol* 2003; **14**: 899–907.
10. Kang DH, Anderson S, Kim YG *et al.* Impaired angiogenesis in the aging kidney: vascular endothelial growth factor and thrombospondin-1 in renal disease. *Am J Kidney Dis* 2001; **37**: 601–611.
11. Nangaku M, Nishi H, Miyata T. Role of chronic hypoxia and hypoxia inducible factor in kidney disease. *Chin Med J (Engl)* 2008; **121**: 257–264.
12. Schrijvers BF, Flyvbjerg A, De Vriese AS. The role of vascular endothelial growth factor (VEGF) in renal pathophysiology. *Kidney Int* 2004; **65**: 2003–2017.
13. Shulman K, Rosen S, Tognazzi K *et al.* Expression of vascular permeability factor (VPF/VEGF) is altered in many glomerular diseases. *J Am Soc Nephrol* 1996; **7**: 661–666.
14. Maynard SE, Min JY, Merchan J *et al.* Excess placental soluble fms-like tyrosine kinase 1 (sFlt1) may contribute to endothelial dysfunction, hypertension, and proteinuria in preeclampsia. *J Clin Invest* 2003; **111**: 649–658.
15. Kang DH, Hughes J, Mazzali M *et al.* Impaired angiogenesis in the remnant kidney model: II. Vascular endothelial growth factor administration reduces renal fibrosis and stabilizes renal function. *J Am Soc Nephrol* 2001; **12**: 1448–1457.
16. Kim YG, Suga SI, Kang DH *et al.* Vascular endothelial growth factor accelerates renal recovery in experimental thrombotic microangiopathy. *Kidney Int* 2000; **58**: 2390–2399.
17. Kang DH, Kim YG, Andoh TF *et al.* Post-cyclosporine-mediated hypertension and nephropathy: amelioration by vascular endothelial growth factor. *Am J Physiol Renal Physiol* 2001; **280**: F727–F736.
18. Kang DH, Johnson RJ. Vascular endothelial growth factor: a new player in the pathogenesis of renal fibrosis. *Curr Opin Nephrol Hypertens* 2003; **12**: 43–49.
19. Lindenmeyer MT, Kretzler M, Boucherot A *et al.* Interstitial vascular rarefaction and reduced VEGF-A expression in human diabetic nephropathy. *J Am Soc Nephrol* 2007; **18**: 1765–1776.
20. Robak E, Sysa-Jedrzejewska A, Robak T. Vascular endothelial growth factor and its soluble receptors VEGFR-1 and VEGFR-2 in the serum of patients with systemic lupus erythematosus. *Mediators Inflamm* 2003; **12**: 293–298.
21. Heshmat NM, El-Kerdany TH. Serum levels of vascular endothelial growth factor in children and adolescents with systemic lupus erythematosus. *Pediatr Allergy Immunol* 2007; **18**: 346–353.
22. Navarro C, Candia-Zuniga L, Silveira LH *et al.* Vascular endothelial growth factor plasma levels in patients with systemic lupus erythematosus and primary antiphospholipid syndrome. *Lupus* 2002; **11**: 21–24.
23. Aringer M, Smolen JS. Cytokine expression in lupus kidneys. *Lupus* 2005; **14**: 13–18.
24. Avihingsanon Y, Phumesin P, Benjachat T *et al.* Measurement of urinary chemokine and growth factor messenger RNAs: a noninvasive monitoring in lupus nephritis. *Kidney Int* 2006; **69**: 747–753.
25. Gladman DD, Ibanez D, Urowitz MB. Systemic lupus erythematosus disease activity index 2000. *J Rheumatol* 2002; **29**: 288–291.
26. Schwartz MM. The pathology of lupus nephritis. *Semin Nephrol* 2007; **27**: 22–34.
27. Schwartz MM. The pathological classification of lupus nephritis, Chap 5. In: Lewis EJ, Schwartz MM, Korbet SM (eds). *Lupus Nephritis*. Oxford University Press: New York, 1999, pp 126–158.
28. Cameron JS. Lupus nephritis. *J Am Soc Nephrol* 1999; **10**: 413–424.
29. Austin HA, Balow JE. Natural history and treatment of lupus nephritis. *Semin Nephrol* 1999; **19**: 2–11.

30. Ferrara N. Vascular endothelial growth factor: basic science and clinical progress. *Endocr Rev* 2004; **25**: 581–611.
31. Ostendorf T, Kunter U, Eitner F *et al.* VEGF(165) mediates glomerular endothelial repair. *J Clin Invest* 1999; **104**: 913–923.
32. Kang DH, Joly AH, Oh SW *et al.* Impaired angiogenesis in the remnant kidney model: I. Potential role of vascular endothelial growth factor and thrombospondin-1. *Am Soc Nephrol* 2001; **12**: 1434–1447.
33. Lemos FB, Ijzermans JN, Zondervan PE *et al.* Differential expression of heme oxygenase-1 and vascular endothelial growth factor in cadaveric and living donor kidneys after ischemia-reperfusion. *J Am Soc Nephrol* 2003; **14**: 3278–3287.
34. Kanellis J, Fraser S, Katerelos M *et al.* Vascular endothelial growth factor is a survival factor for renal tubular epithelial cells. *Am J Physiol Renal Physiol* 2000; **278**: F905–F915.
35. Rosenberger C, Griethe W, Gruber G *et al.* Cellular responses to hypoxia after renal segmental infarction. *Kidney Int* 2003; **64**: 874–886.
36. Reungjui S, Roncal CA, Sato W *et al.* Hypokalemic nephropathy is associated with impaired angiogenesis. *J Am Soc Nephrol* 2008; **19**: 125–134.
37. Nakamura T, Ushiyama C, Suzuki S *et al.* Urinary podocytes for the assessment of disease activity in lupus nephritis. *Am J Med Sci* 2000; **320**: 112–116.
38. Eremina V, Wong MA, Cui S *et al.* Glomerular-specific gene excision *in vivo*. *J Am Soc Nephrol* 2002; **13**: 788–793.
39. Yu D, Petermann A, Kunter U *et al.* Urinary podocyte loss is a more specific marker of ongoing glomerular damage than proteinuria. *J Am Soc Nephrol* 2005; **16**: 1733–1741.
40. Weening JJ, D'Agati VD, Schwartz MM *et al.* The classification of glomerulonephritis in systemic lupus erythematosus revisited. *Kidney Int* 2004; **65**: 521–530.
41. Boumpas DT, Austin III HA, Vaughn EM *et al.* Controlled trial of pulse methylprednisolone versus two regimens of pulse cyclophosphamide in severe lupus nephritis. *Lancet* 1992; **340**: 741–745.
42. Chan TM, Li FK, Tang CS *et al.* Efficacy of mycophenolate mofetil in patients with diffuse proliferative lupus nephritis. Hong Kong-Guangzhou Nephrology Study Group. *N Engl J Med* 2000; **343**: 1156–1162.
43. Austin III HA, Klippel JH, Balow JE *et al.* Therapy of lupus nephritis. Controlled trial of prednisone and cytotoxic drugs. *N Engl J Med* 1986; **314**: 614–619.
44. Livak KJ. User Bulletin #2 ABI PRISM 7700 Sequence Detection System. 2001; **2**: 1–36.
45. Li B, Hartono C, Ding R *et al.* Noninvasive diagnosis of renal-allograft rejection by measurement of messenger RNA for perforin and granzyme B in urine. *N Engl J Med* 2001; **344**: 947–954.

Defects in Notch1 upregulation upon activation of T Cells from patients with systemic lupus erythematosus are related to lupus disease activity

P Sodsai, N Hirankarn, Y Avihingsanon and T Palaga

Lupus 2008 17: 645
DOI: 10.1177/0961203308089406

Lupus (2008) 17, 645–653
http://lup.sagepub.com

PAPER

Defects in Notch1 upregulation upon activation of T Cells from patients with systemic lupus erythematosus are related to lupus disease activity

P Sodsai[1,2], N Hirankarn[1,2,3], Y Avihingsanon[1,4] and T Palaga[2,5]
[1]Lupus Research Unit, Chulalongkorn University, Bangkok, Thailand; [2]Inter-Department of Medical Microbiology, Graduate School, Chulalongkorn University, Bangkok, Thailand; [3]Department of Microbiology, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand; [4]Department of Medicine, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand; and [5]Department of Microbiology, Faculty of Science, Chulalongkorn University, Bangkok, Thailand

Systemic lupus erythematosus (SLE) is an autoimmune disorder characterized by the production of autoantibodies and deposition of immune complexes in various organs. T cells play a central role in driving disease progression, and multiple defects in T cells from patients with SLE have been uncovered. Notch signalling is an evolutionarily well-conserved signalling cascade involved in the proliferation, differentiation and apoptosis of T lymphocytes during development and peripheral effector functions. In this study, we investigated the correlation between expression of Notch receptor and the severity of SLE disease. On the contrary to T lymphocytes from healthy controls ($n = 11$), T lymphocytes from patients with active SLE ($n = 12$) failed to upregulate *Notch1* upon in-vitro stimulation as quantified by quantitative real time RT-PCR ($P \leq 0.025$). Among patients with inactive SLE ($n = 10$), those with late onset of flare exhibited significantly less *Notch1* upregulation compared with SLE patients with remission. Expression of the Notch target genes, *Hes1* and *deltex*, was also lower in patients with active SLE. The decrease in *Notch1* mRNA expression was consistent with less Notch1 protein expression in patients with active SLE. The defects in Notch1 upregulation correlated with decreased proliferation, CD25 and *Foxp3* expression upon stimulation *in vitro*. Taken together, the failure of T cells to upregulate Notch1 upon activation may be a key feature of active SLE and a potential therapeutic target. *Lupus* (2008) **17,** 645–653.

Key words: activation; disease activity; notch receptor; SLE; T cells

Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disorder characterized by a wide spectrum of clinical manifestations. It is prominently characterized by abundant production of autoantibodies and the deposition of immune complexes in various tissues such as the kidney, central nervous system, and skin.[1] Because of the nature of autoantibody production in this disease, hyperactivation of B lymphocytes are considered to play a central role in disease onset.[2] In addition to B lymphocytes, evidence suggesting the involvement of T lymphocytes in pathogenic events leading to disease flares have been reported.[3] Cytokines derived from T-helper lymphocytes have been implied in driving affinity maturation of immunoglobulin present in high levels of serum from patients with SLE.[4] Furthermore, defects in signalling molecules of the T-cell receptor (TCR) complex and co-stimulatory molecules have been reported in SLE.[5] Although still controversial, the abnormalities in regulatory T cells with essential immune regulatory functions have also been suggested in SLE.[6–8] Therefore, T lymphocytes are considered to play an important role in the aetiology of SLE and knowledge of the T-cell defects may lead to a novel therapeutic intervention of autoimmune disorders, including SLE.[9]

Notch signalling is an evolutionarily well-conserved signalling pathway. It was first discovered

Correspondence to: Tanapat Palaga, Department of Microbiology, Faculty of Science, Chulalongkorn University, Payathai Road, Pathumwan, Bangkok, Thailand 10330. Email: tanapat.p@chula.ac.th
Received 30 August 2007; accepted 13 December 2007

10.1177/0961203308089406

and studied in *Drosophila melanogaster* as a neurogenic gene involved in cell fate decision during neuronal development.[10] Extensive studies have uncovered and broadened its role in regulating differentiation, proliferation and apoptosis in a wide spectrum of tissues, including haematopoietic cells. Various target genes of Notch have been identified such as *Hes1* and *Deltex*. Mammals have four Notch receptors, Notch1–4, and five distinct Notch ligands, Jagged1-2 and δ-like 1, 3 and 4. During the development and differentiation of T lymphocytes, Notch signalling plays an essential role in governing T/B lineage decision and cell proliferation, as well as influencing positive and negative selection and CD4/CD8 lineage choices.[11] Notch signalling also regulates the development and functions of T lymphocytes in the periphery during Th1/Th2 differentiation.[12] In addition, Notch has also been implied in regulating development of naturally occurring regulatory T cells as overexpression of the activated form of Notch3 enhances the generation of regulatory T cells.[13] Furthermore, overexpression of Notch ligands in APC leads to the development of antigen-specific regulatory T cells and the Notch/Hes axis is reported to be essential for the suppressive functions of TGFβ+ regulatory T cells.[14,15]

Defects in Notch signalling have been linked to several models of autoimmune disorder. Decreasing Notch signalling with pharmacological approaches protects animals from experimental autoimmune encephalomyelitis through down-regulation of *T-bet* expression.[16] Partial loss of presenilins, components of the enzyme γ-secretase, which is responsible for Notch receptor processing upon ligand engagement, results in severe autoimmune disease phenotypes.[17] In addition, presenilin2 is differentially expressed in human SLE T lymphocytes.[18] A direct link between defects in Notch signalling and SLE, however, is still missing. In this study, we investigated the expression of Notch1 and one of its target genes, *Hes1*, in in-vitro-activated T lymphocytes from patients with SLE. Upregulation of Notch1 was significantly decreased in patients with active SLE, suggesting a role for Notch1 in the disease activity of SLE.

Materials and methods

Patients and controls

Twenty-two patients with SLE, diagnosed based on the American College of Rheumatology classification criteria, were included in this study. According to the MEX-SLEDAI (Mexican-SLE disease activity index), 12 of these patients were classified as active SLE (MEX-SLEDAI ≥ 3) and 10 patients were in an inactive stage (MEX-SLEDAI < 3).[19,20] Patients with inactive SLE received low doses of prednisolone at less than 10 mg/day within at least 1 month and patients with active SLE received low to intermediate doses of corticosteroids (2.5–25 mg/day of prednisolone with or without mycophenolate, mofetil or azathioprine). In the inactive group, remission was defined as those who experienced inactive disease MEX-SLEDAI < 3 for at least 2 years before and more than 6 months after sample collection with low doses of steroid and normal urine analysis and stable renal function, whereas patients with late onset of flare were defined as those who experienced inactive disease within 3 months before or after blood collections. Twenty-one patients with SLE were women and the ages of all patients were in the range of 16–41 years old. Eleven sex and age-matched healthy volunteers were included as controls. This study was approved by the Ethics Committee for Human Research of the Faculty of Medicine, Chulalongkorn University, and written informed consents were obtained from all subjects.

Cells and cell cultures

Peripheral blood mononuclear cells (PBMC) were isolated using Ficoll–Hypaque reagent (Sigma, St Louis, Missouri, USA). T lymphocytes were purified using a positive selection strategy with magnetic beads (Miltenyi Biotech, Bergisch Gladbach, Germany). Cells were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum (Hyclone, Logan, Utah, USA) and penicillin and streptomycin. For stimulation experiments, cells were stimulated with either phytohemagglutinin (PHA) (10 μg/μL) or plate bound anti-human CD3 (clone UCHT1, 2.5 μg/μL) for indicated durations at 37 °C, 5% CO_2.

Quantitative real time RT-PCR

A quantitative real time RT-PCR assay was developed for the detection and quantification of *Notch1* and *Hes-1* transcripts using *β-actin* as an endogenous control. Total RNA was isolated from treated cells using Trizol reagent (Invitrogen Life Technologies, Carlsbad, California, USA). To avoid genomic DNA amplification, primers used in this study were designed to span intron–exon boundaries as follows: Notch1 forward 5′-CAG CCTGCACAACCAGACAGA-3′; Notch1 reverse 5′-TGAGTTGATGAGGTCCTCCAG-3′; Hes1 forward 5′-ACCAACTGGGACGACATGGAGAA-3′; Hes1 reverse 5′-GTGGTGGTGAAGCTGTAG

CC-3′; Foxp3 forward 5′-TCACCTACGCCACGC TCAT-3′; Foxp3 reverse 5′-ACTCAGGTTGTGG CGGATG-3′; β-actin forward 5′-ACCAACTGGG ACGACATGGAGAA-3′; β-actin reverse 5′-GTG GTGGTGAAGCTGTAGCC-3′. PCR amplification was performed with 2× QuantiTect SYBR Green PCR Master Mix with 0.5 μM primers, 16 ng cDNA, and nuclease-free water according to the manufacturer's protocol (Qiagen, Hilden, Germany). The PCR conditions were as follows: 95 °C for 15 min, followed by 40 cycles of 95 °C for 15 s, 55 °C (*Notch1*) or 57 °C (*Hes-1* and *Foxp3*) for 30 s, and 60 °C for 30 s. Levels of mRNA were measured by a Light Cycler (Roche Molecular Biochemicals, Indianapolis, Indiana, USA). Levels of mRNA were expressed as threshold cycle (C_T) and the comparative C_T method was used for analysis. For relative quantification, expressions of *Notch1*, *Hes1* and *Foxp3* were normalized to the expression of *β-actin*. The amount of target was calculated by $2^{-\Delta\Delta CT}$.

Expression of *Notch1* was confirmed by conventional RT-PCR. The PCR conditions were as follows: hot start 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 55 °C (for *Notch1* gene) or 60 °C (for *GAPDH*) for 30 s, 72 °C for 30 s and a final extension at 72 °C for 10 min. Amplification was performed in a Perkin Elmer/GeneAmp PCR system 2400 (Perkin Elmer, Waltham, Massachusetts, USA). The PCR products were analysed in 1.5% agarose gels.

Cell proliferation assay

PBMCs were stimulated with various concentrations of PHA for the indicated times at 37 °C in 5% CO_2 conditions. Cells were pulsed with 1 μCi/mL of ^{3}H-methyl-thymidine (3H-TdR) during the last 6 h of incubation. After incubation, cells were harvested and ^{3}H-TdR incorporation was measured with a scintillation counter (Packard Instruments, Downers Grove, Illinois, USA).

Western blot

Treated cells were harvested and cell lysates were prepared as described previously.[21] The total amounts of protein were measured using BCA protein assay kit (Pierce; Rockford, Illinois, USA). Cell lysates (30 μg) were separated by 8% sodium dodecyl sulphate polyacrylamide gel electrophoresis using the Protein III system (Bio-Rad, Hercules, California, USA). After gel separation, proteins were transferred to PVDF membranes (Amersham Biosciences, Piscataway, New Jersey, USA) and blocked in PBS containing 3% nonfat dry milk and 0.05% Tween 20. Blots were probed with rabbit anti-Notch1 Ab (Santa Cruz Biotech, Santa Cruz, California, USA) at a 1:1000 dilution, or anti-βactin mAb (Chemicon, Billerica, Massachusetts, USA) at a 1:5000 dilution, followed by washing and probing with HRP-conjugated donkey anti-rabbit IgG Ab or sheep anti-mouse IgG Ab at a 1:4000 dilution. After washing, signals were detected using the ECL Western blotting analysis system (Amersham Biosciences).

Flow cytometric analysis

T lymphocytes stimulated with PHA as described above were harvested. Cells were stained with cocktail containing FITC conjugated anti-CD3 Ab (clone S4.1), PE conjugated anti-CD4 Ab (clone S3.5), PE-Cy5.5 conjugated anti-CD8 Ab (clone 3B5) and APC conjugated anti-CD25 Ab (clone CD25-3G10) (Caltag, Burlingame, California, USA) for 20 min at room temperature. After washing, cells were fixed with 1% paraformaldehyde in PBS followed by analysis on a FACSCalibur flow cytometer using Cell Quest software (Becton Dickinson, Franklin Lakes, New Jersey, USA).

Statistical analysis

Mean ± SD of independent experiments was analysed. Inter-group comparisons in all experiments were analysed using independent *t* tests with SPSS software (version 11.5). A P value of < 0.05 was considered statistically significant.

Results

Notch1 has been shown to be upregulated in T lymphocytes upon stimulation *in vitro*.[21] To investigate the expression of Notch1 in T lymphocytes from patients with SLE upon activation *in vitro*, we stimulated purified T lymphocytes using the mitogenic stimulus PHA for 72 h, and the level of expression was measured by quantitative real time RT-PCR. As shown in Figure 1A, activated T lymphocytes from patients with active SLE showed a significant decrease in *Notch1* expression, compared with those from controls ($P \leq 0.025$). On the contrary, T lymphocytes from patients with inactive SLE did not show a significant decrease in *Notch1* expression compared with controls. When all SLE samples were combined regardless of disease stage, the level of *Notch1* was significantly lower in patients with SLE compared with controls ($P \leq 0.05$). Among patients with active

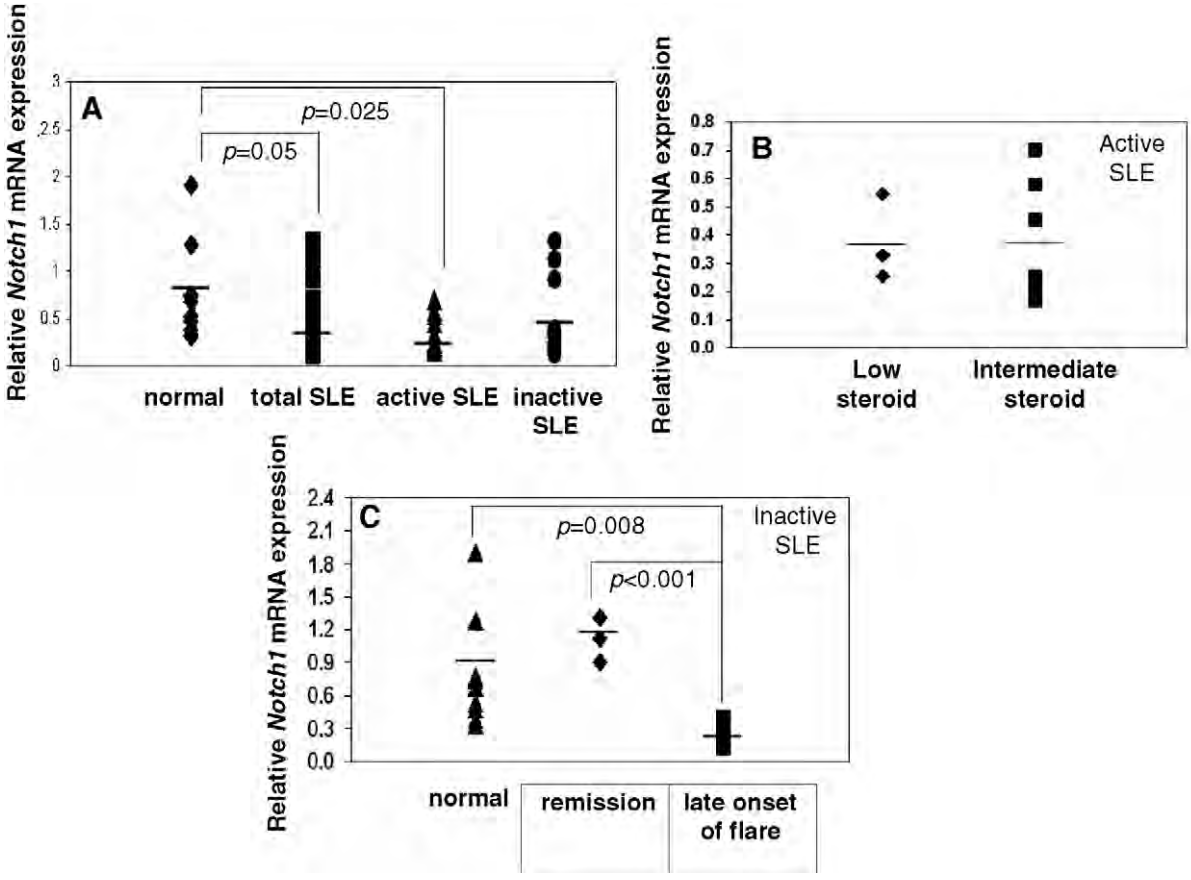


Figure 1 Decreased *Notch1* expression in activated T lymphocytes from patients with SLE. A) Purified PHA-stimulated T lymphocytes from healthy controls ($n = 11$), inactive SLE ($n = 10$) and active SLE ($n = 12$) patients were subjected to real time RT-PCR analysis for expression of *Notch1* and *β actin* as described in the 'Materials and methods'. The results shown are relative expression of *Notch1* normalized to *β actin*. B) Patients with active SLE were divided into those who received low doses ($n = 3$) or moderate doses of steroids ($n = 9$). Expression of *Notch1* was examined in PHA-stimulated T lymphocytes as described in 'a'. C) Patients with inactive SLE were grouped based on disease flare as remission ($n = 3$) and late onset of flare ($n = 7$). Expression of *Notch1* was examined in PHA-stimulated T lymphocytes as described in 'a'.

SLE included in this study, no significant differences in *Notch1* expression were observed between patients ($n = 3$) who took low doses of steroids (less than 10 mg/day of prednisolone) and those who took moderate doses of steroids (10–30 mg/day of prednisolone) ($n = 9$) (Figure 1B). Interestingly, among the patients with inactive SLE, T lymphocytes from remission SLE patients, as defined in 'Materials and methods' showed comparable levels of *Notch1* upregulation as controls (Figure 1C). On the contrary, patients with late onset of flare, as defined in 'Materials and methods', exhibited a significant decrease in *Notch1* expression as compared with healthy controls and remission patients with inactive SLE ($P \leq 0.008$ and $P < 0.001$, respectively). Expression of *Notch2* in stimulated T lymphocytes between patients with SLE and healthy controls was similar, whereas the level of Notch3 was significantly decreased in patients with active SLE ($P < 0.05$) (data not shown).

Because Notch expression can be regulated post-transcriptionally, we examined the expression patterns of Notch1 protein in activated T lymphocytes. The Notch receptor exists on the cell surface as a heterodimer of an extracellular domain and an intracellular plus transmembrane domain (ICT). The ICT domain is detectable using antibodies specific for the intracellular domain of Notch, which has a molecular weight of approximately 110 kDa. Consistent with previous reports, Notch1 protein was similarly upregulated upon stimulation of T lymphocytes from healthy controls and patients with inactive SLE. Lymphocytes of patients with active SLE, however, failed to upregulate Notch1 (Figure 2A). When the level of expression was quantified, lymphocytes from patients with active SLE showed a significant decrease in Notch1 expression upon stimulation compared with those from controls ($P = 0.001$) (Figure 2B). Interestingly, the level of Notch1 in unstimulated cells showed an inverse rela-

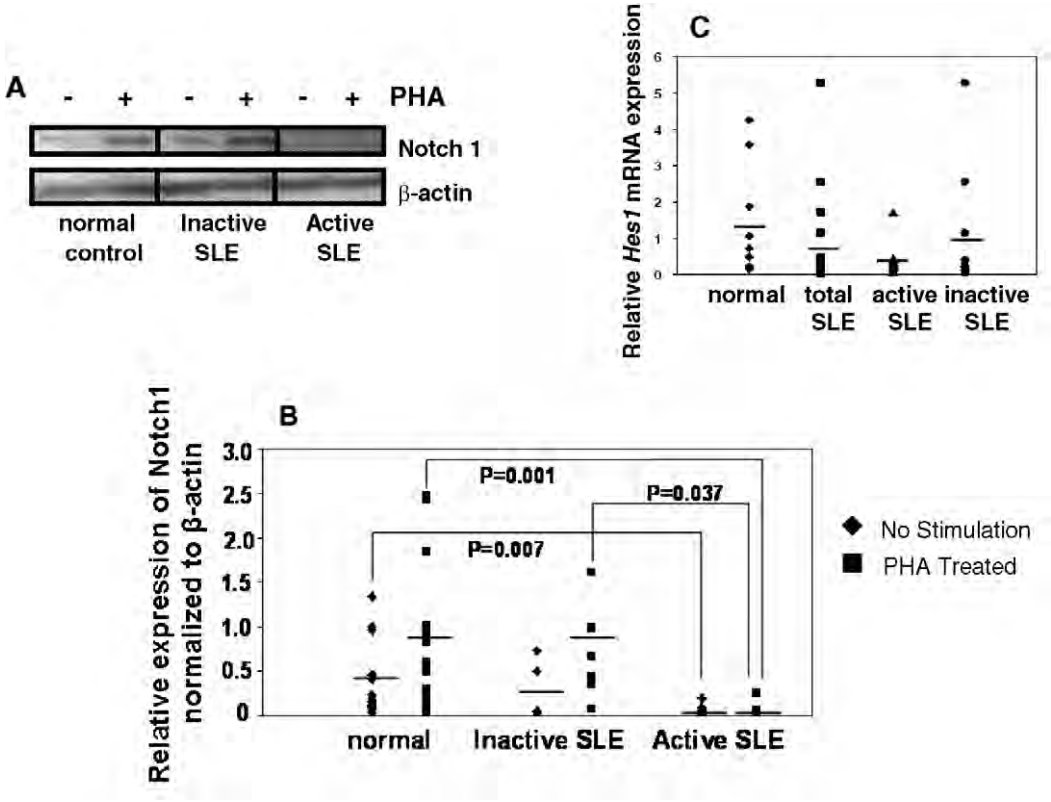


Figure 2 Decreased Notch1 protein expression in activated lymphocytes from patients with SLE. A) Peripheral blood mononuclear cells from patients with SLE or healthy controls were stimulated with PHA for 72 h and cell lysates were analysed for Notch1 expression by Western blot. β actin was used as a loading control. A representative Western blot is shown. B) The quantification of Notch1 protein in lymphocytes with or without PHA stimulation was measured. The level of Notch1 protein was normalized to the level of β-actin and measured as the density of Notch1 protein bands. C) *Hes1* expression in activated T lymphocytes was analysed using real time RT-PCR as described in the 'Materials and methods'.

tionship with disease severity, where patients with active SLE showed the lowest expression and healthy controls showed the highest expression. The lymphocytes from patients with active SLE exhibited a significant decrease in Notch1 expression compared with those from controls ($P = 0.007$).

Upregulation of Notch1 by T lymphocytes is coupled with proliferation and the upregulation of target genes of Notch signalling including *Hes1* and *deltex*.[21,22] Therefore, we examined *Hes1* and *deltex* expression and the proliferation of T lymphocytes from patients with SLE and healthy controls. Expression of one of the Notch target genes, *Hes1*, showed a tendency to decrease in patients with SLE, but the differences between controls and SLE groups were not statistically significant (Figure 2C). In addition, a lower, but not significant, expression profile of *deltex* was observed in patients with SLE compared with controls (data not shown). When proliferation assays were performed with T lymphocytes, as shown in Figure 3A, PBMC from patients with active SLE showed significantly decreased proliferative responses upon stimulation with PHA compared with control and inactive SLE PBMC, confirming previous reports.[23] Stimulation with plate-bound anti-CD3 Ab did not yield any differences in T-cell proliferation between controls and patients with SLE (data not shown). Therefore, defects seen in proliferation of T cells in PBMC may be the results of misregulation of co-stimulatory molecules on accessory cells such as dendritic cells from patients with SLE. Because Notch signalling has a role in augmenting CD25 expression in stimulated T cells, we examined the expression of cell surface CD25. As shown in Figure 3B, T lymphocytes from patients with SLE showed decreased CD25 expression. Finally, we determined cell populations based on the cell surface markers CD4 and CD8 after stimulation. T lymphocytes from patients with SLE showed decreased $CD4^+$ population and increased $CD8^+$ population profiles compared with healthy controls (Figure 3C). This result is consistent with a previous report showing abnormalities of $CD4^+$ and $CD8^+$ populations in patients with active SLE.[24] Because abnormalities in $Foxp3^+$ regulatory

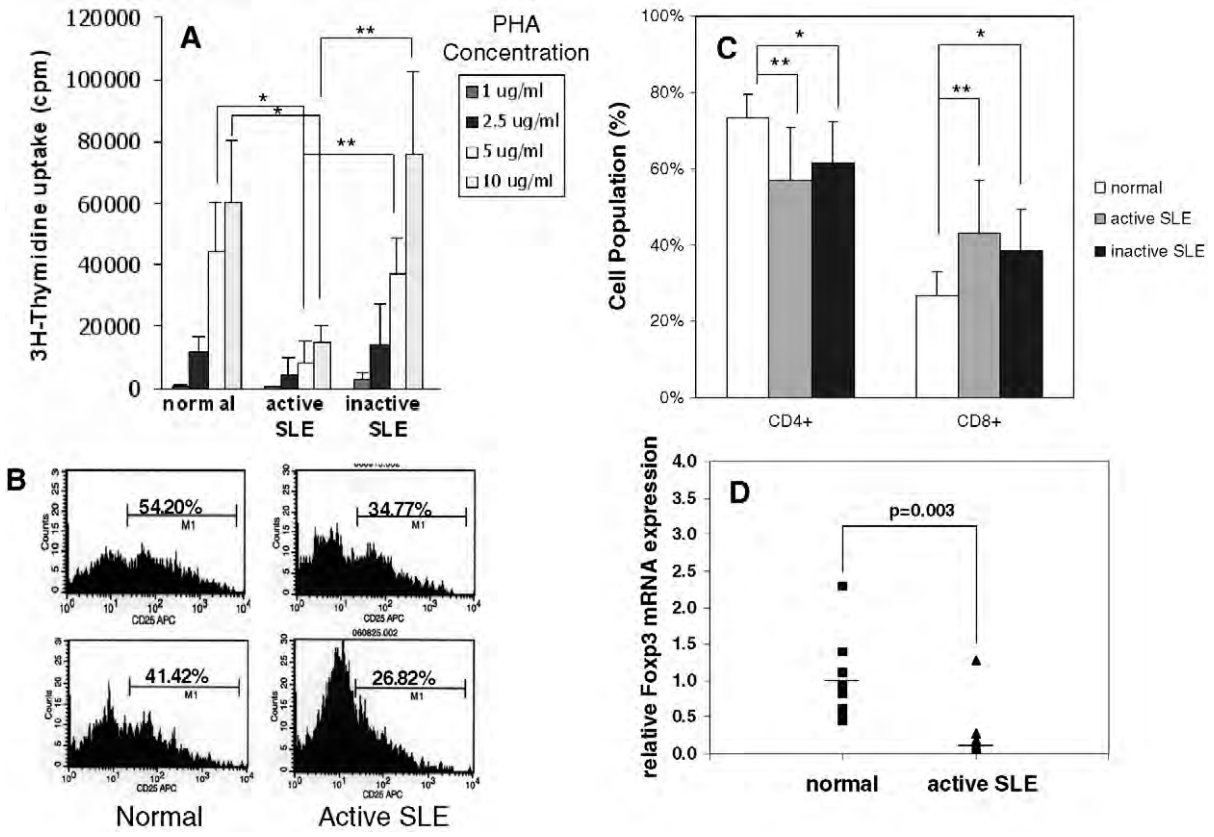


Figure 3 Proliferation and expression of CD25 and *Foxp3* in T lymphocytes from patients with SLE. A) PBMC from healthy controls or patients with SLE were stimulated with PHA at 1, 2.5, 5 and 10 μg/mL for 72 h before subjecting to TdR incorporation assay as described in the 'Materials and methods'. The results shown are mean ± SD of triplicates of all tested subjects. * and ** indicate statistical significance where $P \leq 0.5$ and 0.25, respectively. B) Purified T lymphocytes were stimulated by PHA and subjected to cell surface staining of CD4/CD25 for FACS analysis. The results shown are representative of two normal controls and two patients with active SLE. C) PBMC from healthy controls or patients with SLE were stimulated with PHA at 10 μg/mL for 72 h and CD4/CD8 cell population analyses were carried out by FACS. The results shown are mean ± SD all tested subjects. * and ** indicate statistical significance where $P \leq 0.01$ and 0.002, respectively. D) Purified PHA-stimulated T lymphocytes from healthy controls ($n = 10$) and active SLE ($n = 10$) patients were subjected to real time RT-PCR analysis for expression of *Foxp3* and *β actin* as described in the 'Materials and methods'. The results shown are relative expression of *Foxp3* normalized to *β actin*.

T cells have been reported, we examined the expression of *Foxp3* in stimulated T lymphocytes from patients with SLE.[6,8] As shown in Figure 3D, the expression of *Foxp3* in T lymphocytes from patients with SLE is significantly decreased in comparison with normal controls. Therefore, defects in Notch1 upregulation in T lymphocytes from patients with SLE correlated with decreased CD25 and *Foxp3* expression and coupled with defects in proliferation, but it did not significantly affect *Hes1* and *Deltex* expression.

Discussion

The rationale behind this study is based on the mounting evidence suggesting crucial roles for Notch signalling during T-cell activation, its involvement in autoimmune disorders and the central role of T cell-driven hyperactivation of immune responses in SLE. First, Notch signalling governs multiple choices during thymic development.[11] Therefore, anomalies in Notch signalling, by way of abnormal expression of Notch receptors and ligands, are expected to influence the ontogeny of T lymphocytes. The link between anomalies in Notch signalling during T-cell development and autoimmune disorders, however, is still missing. Second, Notch signalling is involved in helper T-cell development, particularly Th1/Th2 lineage choice.[12] Conflicting evidences have been reported on this issue. Some studies suggest that Notch signalling favours Th2 development by directly regulating *IL-4* expression and conditionally targeted deletion of a Notch-specific scaffold protein encoding gene, *MAML*, results in compromised Th2-type immune

response against helminthic infection.[25,26] However, several studies suggested that Notch signalling regulates Th1-type cytokine IFNγ via direct regulation of the Th1 master regulator, T-bet.[16,21,27] The questions addressed by these conflicting reports remain unresolved. SLE is generally considered a Th2-driven disorder through the hyperactivation of B cells, but studies of cytokine patterns in patients with SLE were not conclusive in determining if it is a Th1 or Th2 type immune response.[28,29] Third, Notch signalling is reported to be involved in the generation and suppressive functionality of regulatory T cells.[13,15,30] There has been a recent resurging interest in these regulatory T cells in the pathogenesis of autoimmune disorders, including SLE. Multiple studies suggest that there are defects in the frequency of regulatory T cells and their suppressive functions in patients with SLE.[6,8,17] Fourth, differential expression of presinilin2, one of the components of the Notch receptor processing enzyme γ-secretase, is reported in SLE, and partial loss of presenilin1 in a *presenilin2* null background results in systemic autoimmune phenotypes similar to human SLE.[17,18]

In this study, we investigated the expression of *Notch1* in T lymphocytes of patients with SLE upon activation and discovered that decreases in *Notch1* upregulation correlated well with disease activity. These defects are intrinsic to T cells, because both PBMC and purified T lymphocytes from patients with active SLE showed similar phenotypes. Multiple signalling pathways, including Notch signalling, and cell cycle regulators govern proliferation in stimulated T cells.[21,31,32] T lymphocytes from patients with active SLE exhibited decreased CD25 expression, confirming previous reports.[17] Overexpression of activated Notch is reported to enhance CD25 expression in primary T cells, linking Notch signalling and CD25 expression in T lymphocytes.[22] In line with CD25 expression in T lymphocytes from patients with SLE, PBMC from patients with SLE showed a significant decrease in proliferative responses upon in-vitro stimulation. Proliferation of T lymphocytes have been reported to be defective in SLE through multiple signalling pathways, including co-stimulatory molecules on antigen presenting cells;[23,33] however, in some instances, normal T-cell proliferation was observed.[34,35] In our study, defects in Notch1 upregulation were coupled with decreased proliferation. Notch signalling is involved in the proliferation of various cell types including activated T lymphocytes through the regulation of cyclin D and c-myc, and defects in Notch1 expression may impinge upon cell cycle entry.[21,32,36–38] Defects in Notch1 upregulation may contribute to disease activity in SLE partly through dysregulation of CD25 expression. It is currently unclear whether impaired Notch signalling in T cells from active SLE directly affects CD25 expression and cell proliferation.

Defects in Notch1 upregulation led to decreased *Hes1* expression but were not statistically significant different from controls. The normal expression of *Hes1* in stimulated T cells from SLE, despite the marked decrease in Notch1 expression, may be the result of functional redundancy among Notch receptors or through *Hes1* activation by other signalling cascade.[39] Notch1, 2 and 3 have been shown to be upregulated in activated T lymphocytes.[21,22,27] We observed decreases in *Notch1* and *3* expression, but expression of *Notch2* remained intact in T lymphocytes of patients with SLE.

On the basis of our observations and the reported roles of Notch signalling in regulating the effector function of T lymphocyte, there are potential links that need to be further investigated. Notch signalling may regulate Th1/Th2 differentiation and skew cytokine profiles to be pathogenic in patients with SLE. The generation and suppressive function of regulatory T cells also involve Notch signalling, a process that needs to be further explored. Interestingly, Notch1 is upregulated in antigen-tolerized T lymphocytes upon activation, whereas T cells from airway inflammation animal models fail to do so.[15] This is intriguing in light of the observation that the regulatory function of regulatory T cells is mediated through Notch1 and the membrane form of TGFβ. Therefore, it may be that T lymphocytes from active SLE escape regulatory mechanisms of regulatory T cells by down-regulating Notch1. In this study, we observed a correlation between *Notch1* and *Foxp3* expression in patients with SLE. In addition, Notch signalling interacts with various signalling pathways, notably NF-κB.[40] In fact, defects in NF-κB signalling have been reported in SLE.[41] Therefore, defects in Notch signalling may affect the NF-κB activation status in T lymphocytes from patients with SLE.

In conclusion, we provide evidence in this report for the first time that Notch1 upregulation in stimulated T lymphocytes is significantly defective in patients with active SLE.

Although T lymphocytes from patients with active SLE completely failed to upregulate Notch1 upon in-vitro stimulation, those from inactive SLE showed intermediate phenotypes. T lymphocytes from active SLE displayed decreased CD25 expression and decreased proliferation. Therefore, defects in Notch1 upregulation in T lymphocytes may indicate SLE disease activity and are likely to be involved in immune dysregulation in SLE. The consequence of

this defect in T-cell functions remains to be determined. The uncovered link between Notch signalling and SLE may lead to a new therapeutic intervention for correcting T-cell functions.

Competing Interests

The authors declare that they have no competing interests.

Acknowledgements

This work is supported in part by a research grant from Lupus Research Unit, the 90th Year Anniversary of Chulalongkorn University (Ratchadphiseksomphot) Endowment Fund, the Thai Government Research Grant 2006 and the Thailand Research Fund (RMU5080076). The authors would like to thank Shayanit Singaselit, RN for collecting patient database and blood samples. The authors also thank Noppadol Sa-Ard-Iam for his help in FACS analysis.

References

1 Mills, JA. Systemic lupus erythematosus. *N Engl J Med* 1994; **26**: 1871–1879.
2 Lipsky, PE. Systemic lupus erythematosus: an autoimmune disease of B cell hyperactivity. *Nat Immunol* 2001; **9**: 764–766.
3 Hoffman, RW. T cells in the pathogenesis of systemic lupus erythematosus. *Clin Immunol* 2004; **1**: 4–13.
4 Maddison, PJ, Reichlin, M. Quantitation of precipitating antibodies to certain soluble nuclear antigens in SLE. *Arthritis Rheum* 1977; **3**: 819–824.
5 Takeuchi, T, Tsuzaka, K, Abe, T, *et al.* T cell abnormalities in systemic lupus erythematosus. *Autoimmunity* 2005; **5**: 339–346.
6 Crispin, JC, Martinez, A. Alcocer-Varela, J. Quantification of regulatory T cells in patients with systemic lupus erythematosus. *J Autoimmun* 2003; **3**: 273–276.
7 Liu, MF, Wang, CR, Fung, LL, Wu, CR. Decreased CD4+CD25+ T cells in peripheral blood of patients with systemic lupus erythematosus. *Scand J Immunol* 2004; **2**: 198–202.
8 Alvarado-Sanchez, B, Hernandez-Castro, B, Portales-Perez, D, *et al.* Regulatory T cells in patients with systemic lupus erythematosus. *J Autoimmun* 2006; **2**: 110–118.
9 Takeuchi, T, Tsuzaka, K, Kameda, H, Amano, K. Therapeutic targets of misguided T cells in systemic lupus erythematosus. *Curr Drug Targets Inflamm Allergy* 2005; **3**: 295–298.
10 Artavanis-Tsakonas, S, Rand, MD, Lake, RJ. Notch signaling: cell fate control and signal integration in development. *Science* 1999; **5415**: 770–776.
11 Radtke, F, Wilson, A, Mancini, S, MacDonald, H. Notch regulation of lymphocyte development and function. *Nat Immunol* 2004; 247–253.
12 Osborne, BA, Minter, LM. Notch signalling during peripheral T-cell activation and differentiation. *Nat Rev Immunol* 2007; **1**: 64–75.
13 Anastasi, E, Campese, AF, Bellavia, D, *et al.* Expression of activated Notch3 in transgenic mice enhances generation of T regulatory cells and protects against experimental autoimmune diabetes. *J Immunol* 2003; **9**: 4504–4511.
14 Hoyne, GF, Le Roux, I, Corsin-Jimenez, M, *et al.* Serrate1-induced notch signalling regulates the decision between immunity and tolerance made by peripheral CD4(+) T cells. *Int Immunol* 2000; **2**: 177–185.
15 Ostroukhova, M, Qi, Z, Oriss, TB, Dixon-McCarthy, B, Ray, P, Ray, A. Treg-mediated immunosuppression involves activation of the Notch-HES1 axis by membrane-bound TGF-beta. *J Clin Invest* 2006; **4**: 996–1004.
16 Minter, LM, Turley, DM, Das, P, *et al.* Inhibitors of gamma-secretase block in vivo and in vitro T helper type 1 polarization by preventing Notch upregulation of Tbx21. *Nat Immunol* 2005; **7**: 680–688.
17 Tournoy, J, Bossuyt, X, Snellinx, A, *et al.* Partial loss of presenilins causes seborrheic keratosis and autoimmune disease in mice. *Hum Mol Genet* 2004; **13**: 1321–1331.
18 Xu, L, Zhang, L, Yi, Y, Kang, HK, Datta, SK. Human lupus T cells resist inactivation and escape death by upregulating COX-2. *Nat Med* 2004; **4**: 411–415.
19 Khanna, S, Pal, H, Pandey, RM, Handa, R. The relationship between disease activity and quality of life in systemic lupus erythematosus. *Rheumatology (Oxford)* 2004; **12**: 1536–1540.
20 Arce-Salinas, A, Cardiel, MH, Guzman, J, Alcocer-Varela, J. Validity of retrospective disease activity assessment in systemic lupus erythematosus. *J Rheumatol* 1996; **5**: 846–849.
21 Palaga, T, Miele, L, Golde, TE, Osborne, BA. TCR-mediated Notch signaling regulates proliferation and IFN-gamma production in peripheral T cells. *J Immunol* 2003; **6**: 3019–3024.
22 Adler, SH, Chiffoleau, E, Xu, L, *et al.* Notch signaling augments T cell responsiveness by enhancing CD25 expression. *J Immunol* 2003; **6**: 2896–2903.
23 Garcia-Cozar, FJ, Molina, IJ, Cuadrado, MJ, Marubayashi, M, Pena, J, Santamaria, M. Defective B7 expression on antigen-presenting cells underlying T cell activation abnormalities in systemic lupus erythematosus (SLE) patients. *Clin Exp Immunol* 1996; **1**: 72–79.
24 Wouters, CH, Diegenant, C, Ceuppens, JL, Degreef, H, Stevens, EA. The circulating lymphocyte profiles in patients with discoid lupus erythematosus and systemic lupus erythematosus suggest a pathogenetic relationship. *Br J Dermatol* 2004; **4**: 693–700.
25 Ansel, KM, Djuretic, I, Tanasa, B, Rao, A. Regulation of Th2 differentiation and Il4 locus accessibility. *Annu Rev Immunol* 2006; 607–656.
26 McKenzie, G, Ward, G, Stallwood, Y, *et al.* Cellular Notch responsiveness is defined by phosphoinositide 3-kinase-dependent signals. *BMC Cell Biol* 2006; 10.
27 Maekawa, Y, Tsukumo, S, Chiba, S, *et al.* Delta1-Notch3 interactions bias the functional differentiation of activated CD4+ T cells. *Immunity* 2003; **4**: 549–559.
28 Lit, LC, Wong, CK, Li, EK, Tam, LS, Lam, CW, Lo, YM. Elevated Gene Expression of ThTh2 Associated Transcription Factors Is Correlated with Disease Activity in Patients with Systemic Lupus Erythematosus. *J Rheumatol* 2007; **1**: 89–96.
29 Mok, CC, Lau, CS. Pathogenesis of systemic lupus erythematosus. *J Clin Pathol* 2003; **7**: 481–490.
30 Kared, H, Adle-Biassette, H, Fois, E, *et al.* Jagged2-expressing hematopoietic progenitors promote regulatory T cell expansion in the periphery through notch signaling. *Immunity* 2006; **5**: 823–834.
31 Qi, R, An, H, Yu, Y, *et al.* Notch1 signaling inhibits growth of human hepatocellular carcinoma through induction of cell cycle arrest and apoptosis. *Cancer Res* 2003; **23**: 8323–8329.
32 Eagar, TN, Tang, Q, Wolfe, M, He, Y, Pear, WS, Bluestone, JA. Notch 1 signaling regulates peripheral T cell activation. *Immunity* 2004; **4**: 407–415.
33 Gottlieb, AB, Lahita, RG, Chiorazzi, N, Kunkel, HG. Immune function in systemic lupus erythematosus. Impairment of in vitro T-cell proliferation and in vivo antibody response to exogenous antigen. *J Clin Invest* 1979; **5**: 885–892.
34 Stohl, W. Impaired generation of polyclonal T cell-mediated cytolytic activity despite normal polyclonal T cell proliferation in systemic lupus erythematosus. *Clin Immunol Immunopathol* 1992; **2**: 163–172.
35 Tada, Y, Nagasawa, K, Yamauchi, Y, Tsukamoto, H, Niho, Y. A defect in the protein kinase C system in T cells from patients with systemic lupus erythematosus. *Clin Immunol Immunopathol* 1991; **2**: 220–231.

36 Stahl, M, Ge, C, Shi, S, Pestell, RG, Stanley, P. Notch1-induced transformation of RKE-1 cells requires up-regulation of cyclin D1. *Cancer Res* 2006; **15**: 7562–7570.
37 Sarmento, LM, Huang, H, Limon, A, *et al*. Notch1 modulates timing of G1-S progression by inducing SKP2 transcription and p27 Kip1 degradation. *J Exp Med* 2005; **1**: 157–168.
38 Weng, AP, Millholland, JM, Yashiro-Ohtani, Y, *et al*. c-Myc is an important direct target of Notch1 in T-cell acute lymphoblastic leukemia/lymphoma. *Genes Dev* 2006; **15**: 2096–2109.
39 Curry, CL, Reed, LL, Nickoloff, BJ, Miele, L, Foreman, KE. Notch-independent regulation of Hes-1 expression by c-Jun N-terminal kinase signaling in human endothelial cells. *Lab Invest* 2006; **8**: 842–852.
40 Shin, HM, Minter, LM, Cho, OH, *et al*. Notch1 augments NF-kappaB activity by facilitating its nuclear retention. *Embo J* 2006; **1**: 129–138.
41 Wong, HK, Kammer, GM, Dennis, G, Tsokos, GC. Abnormal NF-kappaB activity in T lymphocytes from patients with systemic lupus erythematosus is associated with decreased p65-RelA protein expression. *J Immunol* 1999; **3**: 1682–1689.

Major lupus organ involvement: severe lupus nephritis

Y. Avihingsanon and N. Hirankarn

Lupus 2010 19: 1391
DOI: 10.1177/0961203310376522

Lupus (2010) 19, 1391–1398
http://lup.sagepub.com

REVIEW

Major lupus organ involvement: severe lupus nephritis

Y Avihingsanon[1,2] and N Hirankarn[1,3]
[1]Lupus Research Unit, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand; [2]Department of Medicine, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand; and [3]Department of Microbiology, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand

Lupus nephritis is a common and severe complication of systemic lupus erythematosus. A number of patients have nephritis as a presenting feature that, in its severe form, can shortly lead to end-stage renal disease and/or death. Renal flare usually occurs a few years after the first episode and is remarkably predominant in the Asian population. Frequent monitoring for renal flare enhances early recognition and timely treatment. The mainstay therapy continues to be the prolonged use of cytotoxic/immunosuppressive drugs that have a number of undesirable effects, particularly ovarian failure and development of opportunistic infections. This review will focus on the pathogenesis and the unique genetic factors found in Asian patients with lupus nephritis. Here, we propose an appropriate management scheme for the treatment of lupus nephritis in Asian patients. *Lupus* (2010) **19,** 1391–1398.

Key words: Asia; genetic; immunosuppressive therapy; lupus nephritis

Introduction

Renal disease is a common and serious manifestation of systemic lupus erythematosus (SLE). The presentation can range from asymptomatic urinary abnormalities to rapidly progressive renal failure leading to end-stage renal disease (ESRD). Renal failure remains an independent risk factor for death in patients with lupus nephritis (LN). Currently available immunosuppressive regimens are toxic due to their broad immunosuppressive effects. Cyclophosphamide (CY) plus prednisolone is the standard regimen for the severe form of LN. However, this regimen has several side effects and, as SLE usually affects women of child-bearing age, one of its serious adverse effects, gonadal toxicity, is of concern. Nevertheless, evidence from randomized controlled trials supports the use of CY as a remission induction therapy as it appears to have advantages in early therapeutic response and dramatically improves long-term outcomes.[1]

Unfortunately, most of the therapeutic data on LN and SLE are not applicable to the Asian population because they were conducted in African-Americans and Caucasians. There is a need to assess the appropriate dosage and genetic variations in Asian patients to avoid unnecessary toxicities and financial expenditure. Even though the mortality rate of LN in Asia has improved since the 1980s[2] with a 5-year survival rate of 76.5%, which is similar to that of Western countries,[3–5] there can be room for improvement.

In order to improve the supportive care and kidney specific therapy for LN and SLE, it is important to first understand the pathogenesis of the disease. Hopefully this information will help us direct treatment to specific and appropriate areas of the body. Recent progress in LN treatment includes the use of monoclonal antibodies, anti-cytokine therapies and other components of the complement-system blockade.[6] An example of this treatment is mycophenolate mofetil (MMF) and its potential use as an alternative agent. Unlike CY, MMF has a more specific immunosuppressive action and, therefore, causes fewer adverse events than CY. However, MMF is expensive and, in the long run, may not be feasible in resource-limited settings. Therefore this paper will review the immunopathogenesis of LN and the appropriate treatment from an Asian perspective.

Epidemiological and clinical outcomes in Asia

In Asia, the prevalence rate of SLE (per 100,000) varies from 19.3 to 60.[7] A study conducted in the

Correspondence to: Yingyos Avihingsanon, Lupus Research Unit, Renal Division, Department of Medicine, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand.
Email: yingyos.a@gmail.com

10.1177/0961203310376522

USA looked at the rate of nephritis among heterogeneous populations of SLE patients, which showed a prevalence of 17% in Caucasians and 50% in Asian-Americans.[8] However, this number is higher in Southeast Asian countries ranging between 70–80%.[9] Shayakul *et al.* retrospectively reviewed 569 cases of LN at a tertiary care hospital in Thailand and showed that 60% of the cases had a creatinine clearance <50mL/min. From the study, the 5-year patient survival was calculated to be 76%. The most common causes of death were opportunistic infections (OI) and uremia.

Although in the last decade, the overall mortality rate of SLE patients has decreased in most parts of the world with a 5-year patient survival of over 90%,[10,11] yet patients with severe LN have a poorer 5-year survival outcome of 70–80%.[3] Factors associated with poor outcome were: co-existence of cardiopulmonary involvement, anti-phospholipid syndrome nephropathy,[12] male gender, impaired renal function, and poor socioeconomic status.[10]

Pathogenesis of LN

The pathogenesis of LN has been elucidated either by studying the immune effector mechanism or by genetic approach. The immune effector that causes damage to the kidney is mediated mainly by autoantibodies that are nephritogenic. In addition to immune complexes that filter through a glomerulus and deposit there non-specifically, autoantibodies against the nucleosome are also deposited in the kidney through the absorption of the nucleosome to certain proteins in the glomerular basement membrane (e.g., heparin sulfate, collagen type IV, fibronectin and laminin). It is these autoantibodies, not the immune complexes, that can directly cross-react with kidney antigen[13] and induce the inflammatory responses by activating neutrophils and macrophages. This deposition will ultimately result in activation of the complement system resulting in tissue destruction. However, not all SLE patients with anti-dsDNA will develop LN and the level of this autoantibody does not always correlate with the severity of LN. It is hypothesized that the quality of the antibodies[14] as well as chemokines and cytokines such as interferon-inducible protein 10 (IP-10) and its receptor chemokine (C–X–C motif; CXCR3), vascular endothelial growth factor (VEGF) and transforming growth factor beta (TGF-β)[15] are important in the pathogenesis of LN. Identification of infiltrated T cells and macrophages in the kidney suggests that cellular immunity is also involved in the pathogenesis of LN.[16]

Other factors responsible for initiating lupus are driven by an interaction between the environment and certain genes. Genetic predisposition is firmly established as a key element in disease susceptibility.[17] Three defective pathways have been proposed. First, a defect in the apoptosis pathway will increase the apoptosis of immune and renal cells and can serve as a source of nuclear antigen in situ that traps autoantibodies in the kidney.[18] It has been shown that mutations or susceptible polymorphisms of genes can contribute to this defect, i.e. tumor necrosis factor receptor superfamily, member 6 (*TNFRSF6* or *FAS*), Fas ligand (*FASL*), deoxyribonuclease I (*DNASE1*), three prime repair exonuclease 1(*TREX1*), and programmed cell death 1 (*PDCD1*) genes. On the other hand, environmental factors such as ultraviolet (UV) light can also increase the apoptosis rate in lupus patients. It has been suggested that drugs and UV light may effect the methylation profiles of certain genes in T cells, resulting in a defective T-cell control.[19] The second defect causes the body to lose its self tolerance to widely available nuclear antigen. There are a number of abnormal immune responses due to overstimulation of the innate immunity involving interferon (IFN) type I, regulatory T cells, and overactivity of T and B cells. Other factors such as gene polymorphisms of human leukocyte antigen (*HLA*), interferon regulatory factor 5 (*IRF5*), signal transducer and activator of transcription 4 (*STAT4*), protein tyrosine phosphatase non-receptor type 22 (*PTPN22*), B lymphoid tyrosine kinase (*BLK*) and B-cell scaffold protein with ankyrin repeats 1 (*BANK1*) and viral infections may contribute to an overstimulation of IFN type I response.[17,20] Moreover, in the later stages of the disease, epitope spreading exacerbates autoantibody production against various self antigens. Third, the body is unable to clear the formation of immune complexes. In fact, this defect has been attributed to a link found between genetic mutation in the early component of complement genes and the immune complex diseases such as *C1q*, *C3*, *C4*, *FCGR* (Fc fragment of IgG low affinity receptor) and *MBL* (mannose-binding lectin).[17] Several genetic studies have shown an association between polymorphism within *FCGR* gene and LN. Interestingly, it was suggested that copy number variation in inhibitory *FCGR3B* gene may contribute to LN.[21]

Another novel gene discovered by a recent lupus genomewide association study (GWAS) showed

that integrin-α_M (*ITGAM*) was associated with the susceptibility to LN.[22,23]

Along the same lines, a genetic study conducted in Hong Kong Chinese and Thai confirmed the significance of this gene in causing LN.[24] Other genes identified to increase the risk of acquiring LN included angiotensinogen (*AGT*), interleukin 8 (*IL8*), plasminogen activator inhibitor-1 (*PAI-1*), endothelial nitric oxide synthase (*eNOS*), monocyte chemotactic protein 1 (*MCP-1*), angiotensin-converting enzyme (*ACE*), and endothelial protein C receptor (*EPCR*).[25] However, these last groups of LN-specific genes need to be validated in a bigger study with a larger sample size. Recently, GWAS in two Asian populations reported some novel genes contributing to lupus pathogenesis including: (1) genes involved in signal transduction of lymphocytes (V-ets erythroblastosis virus E26 oncogene homolog 1 (*ETS1*), Ikaros family zinc finger 1 (*IKZF1*), RAS guanyl releasing protein 3 (*RASGRP3*)); (2) the solute carrier family 14 (*SLC15A4*) gene, which is involved in immune complex processing; and (3) tumor necrosis factor alpha induced protein 3 interacting protein 1 (*TNIP1*) gene which involved with toll-like receptor and the production of interferon type I.[26,27] However, the GWAS studies declared that information on genes contributing to LN pathogenesis in patients with severe LN was lacking. A genetic study that compares mild and severe cases of LN is needed to accomplish this search.

Renal histology as a diagnostic tool

Aside from clinical manifestations, renal histology is important in diagnosing LN because it has been shown that glomerular lesions, i.e. crescentic formations or fibrinoid necrosis, are associated with poor renal outcomes. Even the World Health Organization (WHO) pathologic classification of LN is based on the extent and severity of glomerular inflammation, which has been the cornerstone of diagnosing and treating LN.[28,29]

Several previous studies have shown semiquantitative analysis to be helpful in assessing the activity and chronicity of nephritis.[29,30] The maximum activity score is 24 and the maximum chronicity score is 12. The score may be used as a prognostic index as well as for research purposes. Even though there are inter- and intra-observer variations in the scoring, certain pathologic findings, such as crescentic formation and/or thrombotic microangiopathy, are useful prognostic indicators that can help reduce this variability. Another prognostic factor that has been associated with the worsening of LN is anti-phospholipid nephropathy.[12,31] Despite these established and well accepted classifications, recently, a newer classification, modified from WHO classification, was proposed.[32] It remains unclear whether this new classification is correlated with long-term outcome. Perhaps in the future, molecular classification will be incorporated in the diagnostic process of renal biopsy. A perfect example of this proposal can be seen through the use of intra-renal gene expression to determine if the patients are at risk of a declining renal function within 12 months.[33]

Aside from the above, clinicopathologic diagnosis of LN is essential for selecting the appropriate treatment protocol. Severe histologic forms of nephritis are usually correlated with worse clinical manifestations. However, so-called silent nephritis was clinically undetectable and had a number of abnormal renal histology results[34] while, on the other hand, non-specific findings of mild proteinuria, hematuria and proteinuria with bland urine sediment can be found in focal or diffuse proliferative lesions or membranous type. Hence, renal biopsy is recommended in most SLE patients with the first episode of renal involvement regardless of clinical symptoms. As for late progressors, repeated biopsy may be required to distinguish between active lupus and scars of previous inflammatory injury. This differentiation is important in the treatment of LN because patients with active lupus would require an immunosuppressive therapy, whereas patients with scarring may use antihypertensive therapy like angiotensin converting enzyme inhibitor.

An appropriate treatment for severe LN

In this review, severe LN is defined as diffused or severe focal proliferative glomerulonephritis (WHO type III or IV). The treatment for severe LN focuses on: (1) achieving immediate renal remission; (2) preventing renal flares; (3) avoiding chronic renal impairment; and (4) fulfilling these objectives with minimal drug toxicities. The treatment for severe LN is divided into two distinct parts. The first part of the induction treatment relies heavily on immunosuppressive drugs whereas, in the second part, the treatment focuses on preventing relapse and minimizing the side effects of treatment as much as possible. It should be noted that many studies looking at treatment for

LN were conducted in the USA whose study populations were consisted mostly of African-Americans. In contrast, studies from Europe included more Caucasians. Hence, it should be warned that utilizing such data in Asian patients may not be suitable or applicable.

Induction treatment

It is important to note that the 5-year patient survival for Asians is still 75–80%.[3] Hence, there is a great deal of room for improvement. The morbid events in these patients are OI and severe impairment of a vital organ, which usually appears early in the course of the disease. It has been shown that inducing remission early can improve the long-term outcome in these patients.[1,35]

CY has remained the standard of treatment for LN since 1960. Its alkylating property inhibits DNA replication and transcription and is thus a good immunosuppressive agent, especially when used with high-dose prednisolone (1–2 mg/kg/day).[35] The intermittent use of pulse intravenous cyclophosphamide (IVCY) seems to have the best therapeutic effect in reducing mortality and risk of ESRD. Two prospective, randomized controlled trials performed by the National Institutes of Health (NIH), as well as a recent meta-analysis, support the use of IVCY over that of pulse methylprednisolone or prednisolone alone.[36,37] Two other reports showed that a combination of pulse methylprednisolone and IVCY was associated with a better outcome[38,39] (Table 1). Unfortunately, as a result of using this technique, there is an increased cost of patient care due to the adverse effects of suppressing the immune system and the need to treat acquired OIs. Aside from the NIH cohort, it has been shown that IVCY is very effective in Asian patients with severe LN.[3,40]

Needless to say, this indiscriminate use of the so-called 'NIH regimen' to treat all LN patients has recently raised some concerns within the scientific community.[41] First, high-dose IVCY treatment is extremely toxic, causing up to 25% patients to develop herpes zoster; up to 26% experience a severe infection; and up to 52% of women are at risk of developing ovarian failure.[35] Second, because of prompt assessment of early renal involvement, aggressive treatment for clinical mild cases of biopsy-proven proliferative nephritis is no longer justifiable (Table 1). This situation is further supported by the results obtained from a multicenter, prospective, randomized study known as the Euro-Lupus Nephritis Trial (ELNT),[42] which showed no difference in remission and flare between low-dose IVCY regimen (six fortnightly pulses of 500 mg; cumulative dose 3 g) and high-dose IVCY treatment therapy for proliferative lupus glomerulonephritis. In both treatment arms, azathioprine (AZA) was used as long-term immunosuppressive therapy. The severity of acquired OIs was less common in the low-dose group. This finding therefore calls into question the current practice of treating all LN patients with an extended course of IVCY.[43] Furthermore, the current doses used may not be applicable in the Asian population with a smaller build and lower body weight. Therefore, it

Table 1 Proposed therapeutic regimen for different categories of severe lupus nephritis

Clinical categories	*Definition*	*Preferable regimen*	*Alternative*
RPGN and/or severe extra-renal manifestation	Rapid decline in renal function and crescentic formation more than 30% of involved glomeruli or thrombotic microangiopathy	IV Pulse methylprednisolone plus IV pulse cyclophosphamide and/or plasmapheresis	IVIG
First episode of severe LN, intact GFR	Classical nephritic-nephrotic syndrome and newly diagnosed lupus nephritis	MMF plus steroid[a] or IV pulse cyclophosphamide[b]	IV low-dose CY
Relapse or refractory case	Multiple episodes of renal flares and/or cumulative high doses of CY and/or failure to respond to IV pulse CY	Combined FK-506 plus MMF and steroid (multi-target) or MMF plus steroid or EC-MPS plus steroid	Should consider participating in clinical trials

CY: cyclophosphamide; EC-MPS: enteric-coated mycophenolate sodium; GFR: glomerular filtration rate; IV: intravenous; IVIG: intravenous immunoglobulin; MMF: mycophenolate mofetil; RPGN: rapidly progressive glomerulonephritis.
[a]Females of child-bearing age can avoid ovarian failure by using this regimen.
[b]This regimen is preferred in resource-limited settings.

is essential that a low-dose regimen and its efficacy be explored.

On the other hand, an alternative treatment for severe LN is needed. The reason for this need is the side effects of the current treatment of CY in patients of reproductive age. A newer, alternative immunosuppressive agent such as MMF has been shown to selectively inhibit activated lymphocytes and renal mesangial cells.[44] Its safe profile in solid-organ transplantation and superiority to AZA in the prevention of acute graft rejection make MMF an ideal candidate for alternative therapy. Data from experimental models of immune-mediated glomerulonephritis, particularly LN, have shown that MMF ameliorates autoimmune phenomena, retards renal damage, and improves outcome.[45] Controlled studies further show that MMF is as effective as CY in the induction of renal remission in the short term.[46] Current dosages of MMF also appear to be well tolerated with no serious toxicities reported. MMF significantly causes less ovarian toxicity compared with CY and has made this agent particularly attractive for the treatment of LN.[46] Unfortunately, the costs of long-term MMF therapy (Table 1) may not make it the treatment of choice for severe LN in a resource-limited country. As a result of this situation, we will assess the efficacy, safety, affordability and quality of life in patients using mycophenolate sodium (MPS) versus pulse IVCY in a multi-center study known as the CONTROL lupus trial (ClinicalTrials.gov NCT# 01015456).

As for those patients with mixed class IV and V histology patterns, it was shown that a combination of FK-506 (Prograf) and MMF had better results compared with CY when used to treat Chinese patients with LN, yielding high rate of remission.[47] In patients refractory to treatment with IVCY, our preliminary data found enteric-coated MPS effectively controlled the activity of the disease.[48] (Table 1) This finding will aim to be validated in a large-scale, randomized-controlled study that begins in 2010 (CONTROL lupus study; ClinicalTrials.gov NCT# 01015456).

Maintenance treatment

One of the most perplexing aspects of the natural history of LN is its remitting and relapsing course. Modern treatment neither cures lupus nor completely prevents exacerbations. Approximately one-third to one-half of patients have a relapse of nephritis after achieving partial or complete remission of proliferative nephritis.[49] In our tertiary care center, the relapse rate of LN is close to 50% with a median time to relapse of 30 months (unpublished data). Several studies have shown that CY, AZA or even MMF are more effective than steroid alone in preventing relapse.[3,6,35,50] However, the risk–benefit of prolonged treatment, particularly with CY, remains controversial. At the present time, one can justifiably choose between quarterly pulses of IVCY, AZA or MMF. Although maintenance therapy with MMF appears to be more efficient and safer than IVCY,[51,52] but its high cost limits its use in many resource-limited settings.

How to monitor for relapse

The clinical course of LN usually consists of recurrent episodes of relapses and remissions. The cumulative rate of relapse of LN is approximately 25% at 5 years.[50,53] In contrast, Asian patients have a higher relapse rate of 30% at 2 years.[8,52] In our center, the relapse rate was as high as 50% at 3 years. The relapse was significantly associated with subsequent renal function deterioration.[54] Follow-up renal biopsy studies have shown that patients who experience multiple episodes of active nephritis are at risk of progressing to ESRD, because after each episode of nephritis there is a residual and cumulative irreversible parenchymal damage.[53]

It is imperative to recognize an early manifestation of relapse and provide an appropriate treatment. Unfortunately, there are no clinical or laboratory tools that can precisely predict a nephritic flare. However, identification of cellular casts or an increase in proteinuria may be useful. Other predictors include serological markers such as complement C3 or C4 components and anti-dsDNA levels, which are typically associated with the activity of the disease. At present, strict monitoring of urinary findings and serological activities is recommended.[55]

Fortunately, recent advances in molecular diagnostic technologies have provided new tools in diagnosis and prognosis of disease. The study of molecular signals from urinary cellular components is a logical approach in diagnosing an early relapse of nephritis and monitoring response to therapy.[15,56] It has been shown that urinary cytokines, like IP-10 and its receptor, and growth factors, like VEGF and TGF-β, in LN are associated with the severity of renal pathology.[15] Furthermore, the cytokine mRNAs of urine cells has been shown to be associated with response to therapy. It is likely that early treatment based on urinary molecular signals may alter the course of the disease.

Clinical trials in Asian patients with LN

Genetic and demographic differences may influence patients' responses to treatment. Several factors can be associated and influenced by ethnicity such as the severity of the disease, pharmacokinetics and pharmacodynamics of medicines, adverse events and socio-economic background. Asia has the most number of lupus patients, it is therefore good to know that clinical trials are now acknowledging the need to incorporate more Asian patients into their studies.

The first randomized controlled study of MMF treatment was conducted in Chinese patients with LN and successfully demonstrated the benefit of MMF as an induction to remission therapy.[46] Following this elegant trial, many studies on MMF treatment for lupus nephritis started to report long-term outcomes.[51,52] Contreras *et al*. demonstrated a significant advantage MMF use in reducing death and chronic renal failure.[51] On the other hand, a comparable study conducted in Asian patients showed no difference between sequential use of cyclophosphamide–azathioprine and that of MMF therapy.[52] Interestingly enough, in another multinational, randomized controlled trial, known as the Aspreva study, a high number of Asian patients receiving MMF therapy were reported to have died from acquired infections.[57] The highest dose of MMF used was 3 g/day, which may have been too high for the Asian population. Despite this factor, this trial also supported the previous finding that there was no difference in efficacy and safety between CY and MMF therapy in Asian patients. From an Asian perspective, MMF should be judiciously used in severe LN. A continued use of high dose MMF and steroids can contribute to overwhelming infections. At the moment, pharmacokinetics study of mycophenolic acid (MPA), the active metabolite of MMF, is warranted in this subgroup of patients. It is imperative to find the appropriate dose that will yield maximum efficacy with very little toxicity associated with the therapy. A prospective study in Thai patients with severe LN demonstrated, for the first time, the benefit of monitoring the 12-h area under the time–concentration curve for MPA (MPA-AUC). It is likely that therapeutic drug monitoring of MMF would improve its therapeutic efficacy.[58]

Conclusion

In order to provide optimal therapy for patients with severe LN, patients at risk are recommended to have renal histology. SLE patients with confirmed biopsy of the proliferative lesion (WHO type III or IV) should be considered for treatment with a combination of corticosteroids and immunosuppressive therapy. Monotherapy on corticosteroids is not recommended because it requires high doses that can disfigure and disable the patient and contribute to other life-threatening side-effects. Prolonged use of CY, given either intravenously or orally, may result in ovarian failure, bone marrow toxicity, bladder toxicity, alopecia, OIs and cancer. MMF has been suggested as an alternative agent but its cost is so staggering that its affordability in resource-limited settings remains questionable. Even though there are several options available for the treatment of severe LN, the most important thing is to make sure that the initial disease is treated aggressively and for a short period of time to prevent flares, and that maintenance therapy is administered at low doses to reduce severe iatrogenic morbidity. This strategy will protect the kidneys and other organs from the deleterious effects of SLE. Clinical trials in Asia are the most valuable resources for developing the appropriate therapy for Asian patients suffering from LN.

Acknowledgements

The authors sincerely thank the members of the Lupus Research Unit and Renal Division, Department of Medicine, Faculty of Medicine, Chulalongkorn University, Thailand.

Funding

This work has been supported by the CU Cluster Ratchadaphisek Somphot Endowment Fund, the National Research Council of Thailand and the Thailand Research Fund [grant number RMU 5080076].

References

1 Boumpas DT, Austin 3rd HA, Vaughn EM, *et al*. Controlled trial of pulse methylprednisolone versus two regimens of pulse cyclophosphamide in severe lupus nephritis. *Lancet* 1992; 340: 741–745.
2 Parichatikanond P, Francis ND, Malasit P, *et al*. Lupus nephritis: clinicopathological study of 162 cases in Thailand. *J Clin Pathol* 1986; 39: 160–166.
3 Shayakul C, Ong-aj-yooth L, Chirawong P, *et al*. Lupus nephritis in Thailand: clinicopathologic findings and outcome in 569 patients. *Am J Kidney Dis* 1995; 26: 300–307.

4 Cameron JS, Turner DR, Ogg CS, *et al*. Systemic lupus with nephritis: a long-term study. *Q J Med* 1979; 48: 1–24.
5 Heller T, Ahmed M, Siddiqi A, Wallrauch C, Bahlas S. Systemic lupus erythematosus in Saudi Arabia: morbidity and mortality in a multiethnic population. *Lupus* 2007; 16: 908–914.
6 Kuiper-Geertsma DG, Derksen RH. Newer drugs for the treatment of lupus nephritis. *Drugs* 2003; 63: 167–180.
7 Tikly M, Navarra SV. Lupus in the developing world—is it any different? *Best Pract Res Clin Rheumatol* 2008; 22: 643–655.
8 Seligman VA, Lum RF, Olson JL, Li H, Criswell LA. Demographic differences in the development of lupus nephritis: a retrospective analysis. *Am J Med* 2002; 112: 726–729.
9 Parichatikanond P, Chawanasuntorapoj R, Shayakul C, *et al*. An analysis of 3,555 cases of renal biopsy in Thailand. *J Med Assoc Thai* 2006; 89: S106–S111.
10 Lee BS, Cho HY, Kim EJ, *et al*. Clinical outcomes of childhood lupus nephritis: a single center's experience. *Pediatr Nephrol* 2007; 22: 222–231.
11 Al Arfaj AS, Khalil N. Clinical and immunological manifestations in 624 SLE patients in Saudi Arabia. *Lupus* 2009; 18: 465–473.
12 Cheunsuchon B, Rungkaew P, Chawanasuntorapoj R, Pattaragarn A, Parichatikanond P. Prevalence and clinicopathologic findings of antiphospholipid syndrome nephropathy in Thai systemic lupus erythematosus patients who underwent renal biopsies. *Nephrology* 2007; 12: 474–480.
13 Yung S, Chan TM. Anti-DNA antibodies in the pathogenesis of lupus nephritis—the emerging mechanisms. *Autoimmun Rev* 2008; 7: 317–321.
14 Tsirogianni A, Pipi E, Soufleros K. Relevance of anti-C1q autoantibodies to lupus nephritis. *Ann N Y Acad Sci* 2009; 1173: 243–251.
15 Avihingsanon Y, Phumesin P, Benjachat T, *et al*. Measurement of urinary chemokine and growth factor messenger RNAs: a noninvasive monitoring in lupus nephritis. *Kidney Int* 2006; 69: 747–753.
16 Foster MH. T cells and B cells in lupus nephritis. *Semin Nephrol* 2007; 27: 47–58.
17 Moser KL, Kelly JA, Lessard CJ, Harley JB. Recent insights into the genetic basis of systemic lupus erythematosus. *Genes Immun* 2009; 10(5): 373–379.
18 Zheng L, Sinniah R, Hsu SI. Renal cell apoptosis and proliferation may be linked to nuclear factor-kappaB activation and expression of inducible nitric oxide synthase in patients with lupus nephritis. *Human Pathol* 2006; 37: 637–647.
19 Pan Y, Sawalha AH. Epigenetic regulation and the pathogenesis of systemic lupus erythematosus. *Transl Res* 2009; 153: 4–10.
20 Allam R, Pawar RD, Kulkarni OP, *et al*. Viral 5′-triphosphate RNA and non-CpG DNA aggravate autoimmunity and lupus nephritis via distinct TLR-independent immune responses. *Eur J Immunol* 2008; 38: 3487–3498.
21 Willcocks LC, Lyons PA, Clatworthy MR, *et al*. Copy number of FCGR3B, which is associated with systemic lupus erythematosus, correlates with protein expression and immune complex uptake. *J Exp Med* 2008; 205: 1573–1582.
22 Nath SK, Han S, Kim-Howard X, *et al*. A nonsynonymous functional variant in integrin-α_M (encoded by ITGAM) is associated with systemic lupus erythematosus. *Nat Genet* 2008; 40: 152–154.
23 Hom G, Graham RR, Modrek B, *et al*. Association of systemic lupus erythematosus with C8orf13-BLK and ITGAM-ITGAX. *N Engl J Med* 2008; 358: 900–909.
24 Yang W, Zhao M, Hirankarn N, *et al*. ITGAM is associated with disease susceptibility and renal nephritis of systemic lupus erythematosus in Hong Kong Chinese and Thai. *Hum Mol Genet* 2009; 18: 2063–2070.
25 Morel L. Genetics of human lupus nephritis. *Semin Nephrol* 2007; 27: 2–11.
26 Han JW, Zheng HF, Cui Y, *et al*. Genome-wide association study in a Chinese Han population identifies nine new susceptibility loci for systemic lupus erythematosus. *Nat Genet* 2009; 41: 1234–1237.
27 Yang W, Shen N, Ye DQ, *et al*. Genome-wide association study in Asian populations identifies variants in ETS1 and WDFY4 associated with systemic lupus erythematosus. *PLoS Genet* 2010; 6: e1000841.
28 Appel GB, Cohen DJ, Pirani CL, Meltzer JI, Estes D. Long-term follow-up of patients with lupus nephritis. A study based on the classification of the World Health Organization. *Am J Med* 1987; 83: 877–885.
29 Banfi G, Mazzucco G, Barbiano di Belgiojoso G, *et al*. Morphological parameters in lupus nephritis: their relevance for classification and relationship with clinical and histological findings and outcome. *Q J Med* 1985; 55: 153–168.
30 Austin 3rd HA, Boumpas DT, Vaughan EM, Balow JE. Predicting renal outcomes in severe lupus nephritis: contributions of clinical and histologic data. *Kidney Int* 1994; 45: 544–550.
31 D'Agati V, Kunis C, Williams G, Appel GB. Anti-cardiolipin antibody and renal disease: a report three cases. *J Am Soc Nephrol* 1990; 1: 777–784.
32 Weening JJ, D'Agati VD, Schwartz MM, Seshan SV, Alpers CE, Appel GB. The classification of glomerulonephritis in systemic lupus erythematosus revisited. *Kidney Int* 2004; 65: 521–530.
33 Avihingsanon Y, Benjachat T, Tassanarong A, Sodsai P, Kittikovit V, Hirankarn N. Decreased renal expression of vascular endothelial growth factor in lupus nephritis is associated with worse prognosis. *Kidney Int* 2009; 75: 1340–1348.
34 Zabaleta-Lanz M, Vargas-Arenas RE, Tapanes F, Daboin I, Atahualpa Pinto J, Bianco NE. Silent nephritis in systemic lupus erythematosus. *Lupus* 2003; 12: 26–30.
35 Austin 3rd HA, Klippel JH, Balow JE, *et al*. Therapy of lupus nephritis. Controlled trial of prednisone and cytotoxic drugs. *N Engl J Med* 1986; 314: 614–619.
36 Austin HA, Boumpas DT. Treatment of lupus nephritis. *Semin Nephrol* 1996; 16: 527–535.
37 Flanc RS, Roberts MA, Strippoli GF, Chadban SJ, Kerr PG, Atkins RC. Treatment of diffuse proliferative lupus nephritis: a meta-analysis of randomized controlled trials. *Am J Kidney Dis* 2004; 43: 197–208.
38 Gourley MF, Austin 3rd HA, Scott D, *et al*. Methylprednisolone and cyclophosphamide, alone or in combination, in patients with lupus nephritis: a randomized, controlled trial. *Ann Intern Med* 1996; 125: 549–557.
39 Illei GG, Austin HA, Crane M, *et al*. Combination therapy with pulse cyclophosphamide plus pulse methylprednisolone improves long-term renal outcome without adding toxicity in patients with lupus nephritis. *Ann Intern Med* 2001; 135: 248–257.
40 Ong LM, Hooi LS, Lim TO, *et al*. Randomized controlled trial of pulse intravenous cyclophosphamide versus mycophenolate mofetil in the induction therapy of proliferative lupus nephritis. *Nephrology* 2005; 10: 504–510.
41 Ponticelli C, Zucchelli P, Moroni G, Cagnoli L, Banfi G, Pasquali S. Long-term prognosis of diffuse lupus nephritis. *Clin Nephrol* 1987; 28: 263–271.
42 Houssiau FA, Vasconcelos C, D'Cruz D, *et al*. Immunosuppressive therapy in lupus nephritis: the Euro-Lupus Nephritis Trial, a randomized trial of low-dose versus high-dose intravenous cyclophosphamide. *Arthritis Rheum* 2002; 46: 2121–2131.
43 Balow JE. Choosing treatment for proliferative lupus nephritis. *Arthritis Rheum* 2002; 46: 1981–1983.
44 Anthony C. Allison EME. Mycophenolate mofetil and its mechanisms of action. *Immunopharmacology* 2000; 47: 85–118.
45 van Bruggen MC, Walgreen B, Rijke TP, Berden JH. Attenuation of murine lupus nephritis by mycophenolate mofetil. *J Am Soc Nephrol* 1998; 9: 1407–1415.
46 Chan TM, Li FK, Tang CS, *et al*. Efficacy of mycophenolate mofetil in patients with diffuse proliferative lupus nephritis. Hong Kong-Guangzhou Nephrology Study Group. *N Engl J Med* 2000; 343: 1156–1162.
47 Bao H, Liu ZH, Xie HL, Hu WX, Zhang HT, Li LS. Successful treatment of class V+IV lupus nephritis with multitarget therapy. *J Am Soc Nephrol* 2008; 19: 2001–2010.
48 Traitanon O, Avihingsanon Y, Kittikovit V, *et al*. Efficacy of enteric-coated mycophenolate sodium in patients with resistant-type lupus nephritis: a prospective study. *Lupus* 2008; 17: 744–751.
49 Austin HA, Balow JE. Natural history and treatment of lupus nephritis. *Semin Nephrol* 1999; 19: 2–11.
50 Donadio Jr JV, Holley KE, Ferguson RH, Ilstrup DM. Treatment of diffuse proliferative lupus nephritis with prednisone and

combined prednisone and cyclophosphamide. *N Engl J Med* 1978; 299: 1151–1155.

51 Contreras G, Pardo V, Leclercq B, *et al*. Sequential therapies for proliferative lupus nephritis. *N Engl J Med* 2004; 350: 971–980.

52 Chan TM, Tse KC, Tang CS, Mok MY, Li FK. Long-term study of mycophenolate mofetil as continuous induction and maintenance treatment for diffuse proliferative lupus nephritis. *J Am Soc Nephrol* 2005; 16: 1076–1084.

53 Ponticelli C, Moroni G. Flares in lupus nephritis: incidence, impact on renal survival and management. *Lupus* 1998; 7: 635–638.

54 Moroni G, Quaglini S, Maccario M, Banfi G, Ponticelli C. ''Nephritic flares'' are predictors of bad long-term renal outcome in lupus nephritis. *Kidney Int* 1996; 50: 2047–2053.

55 Austin HA. Clinical evaluation and monitoring of lupus kidney disease. *Lupus* 1998; 7: 618–621.

56 Chan RW, Tam LS, Li EK, *et al*. Inflammatory cytokine gene expression in the urinary sediment of patients with lupus nephritis. *Arthritis Rheum* 2003; 48: 1326–1331.

57 Appel GB, Contreras G, Dooley MA, *et al*. Mycophenolate mofetil versus cyclophosphamide for induction treatment of lupus nephritis. *J Am Soc Nephrol* 2009; 20: 1103–1112.

58 Lertdumrongluk P, Somparn P, Kittanamongkolchai W, Traitanon O, Vadcharavivad S, Avihingsanon Y. Pharmacokinetics of mycophenolic acid in severe lupus nephritis. Kidney Int. 2010, Jun 9. [Epub ahead of print].

see commentary on page 335

Pharmacokinetics of mycophenolic acid in severe lupus nephritis

Paungpaga Lertdumrongluk[1], Poorichaya Somparn[1], Wonngarm Kittanamongkolchai[1], Opas Traitanon[2], Somratai Vadcharavivad[3] and Yingyos Avihingsanon[1]

[1]*Renal Division, Lupus Research Unit, Faculty of Medicine, Department of Medicine, Chulalongkorn University, Bangkok, Thailand;* [2]*Faculty of Medicine, Thammasart University, Bangkok, Thailand and* [3]*Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand*

Mycophenolic acid (MPA) is an effective treatment for active lupus nephritis despite its variable efficacy in different ethnic groups. Here we tested whether pharmacokinetic monitoring may help to optimize dosing of MPA in an Asian population. Patients with biopsy-proven class III or IV lupus nephritis (ISN/RPS category) were treated with mycophenolate mofetil or enteric-coated mycophenolate sodium. One month after initiating treatment we measured plasma MPA levels in eight samples taken over a 12-h period after drug administration. The mean area under the time-dependent curve for MPA of responding patients was significantly higher than those not responding. Successful treatment was seen in patients with areas >45 mg h/l. The dosage of the drug was not related to MPA pharmacokinetics. In the mycophenolate mofetil group, however, MPA-area under the curve was positively, and significantly, correlated with trough or 1 h after dose concentrations and associated with a therapeutic response. Thus, our study shows that MPA pharmacokinetics were positively correlated with therapeutic responses of mycophenolate, suggesting that controlling the concentrations may improve its therapeutic efficacy in lupus nephritis. As the absorption and pharmacokinetic peak of enteric-coated tablets is slower, it is important to take different formulations into account when determining optimal MPA concentrations.

Kidney International (2010) **78,** 389–395; doi:10.1038/ki.2010.170; published online 9 June 2010

KEYWORDS: glomerulonephritis; immunosuppression; lupus nephritis; mycophenolate mofetil; pharmacokinetics

Correspondence: *Yingyos Avihingsanon, Lupus Research Unit, Renal Division, Department of Medicine, Faculty of Medicine, Chulalongkorn University, Rama 4 Road, Bangkok 10330, Thailand. E-mail: Yingyos.a@gmail.com*

Received 7 January 2010; revised 8 April 2010; accepted 14 April 2010; published online 9 June 2010

Proliferative lupus nephritis (International Society of Nephrology/Renal Pathology Society class III/IV categorization) is the most common and serious complications of systemic lupus erythematosus (SLE). Without the appropriate treatment, this complication can turn into an end-stage kidney disease within a few months. The standard of care for lupus nephritis is cyclophosphamide and steroids, even though it has many drug-related adverse events.[1] The most common causes of death for SLE patients are fatal infections acquired in consequence of using immunosuppressive therapy.[2] Therefore, a more effective and safer treatment for lupus nephritis is needed.

Recently, the first-line treatment for proliferative lupus nephritis cyclophosphamide was replaced by mycophenolate mofetil (MMF), because its efficacy was comparable and less toxic.[3–10] Furthermore, results from a recent multicenter, randomized-controlled study showed that African Americans responded poorly to cyclophosphamide.[6] In contrast, the results from the Aspreva Lupus Management Study showed that Asians responded equally well to both MMF and cyclophosphamide, even though there were more deaths reported in the MMF group.[11] It should be noted that the Aspreva Lupus Management Study trial was the largest multinational study in lupus nephritis, which increased target MMF dose to 3 g per day. Although most studies used 2 g per day of MMF,[1,3,4] the high-targeted MMF dose in this Aspreva Lupus Management Study trial may be too much, particularly for Asians. It has been pointed out that ethnicity may partly explain the different pharmacokinetic profiles seen in MMF and variation in the treatment responses.[6,11] Therefore, therapeutic drug monitoring (TDM) study may help to optimize dosing of MMF in different ethnicities.

It has been shown that TDM of mycophenolic acid (MPA) can improve clinical outcomes in organ transplant recipients.[12] Acute rejection rate was reduced in kidney allograft recipients, who had achieved MPA therapeutic levels consistently and early after transplantation. Similar to that observed in organ transplant recipients, severe lupus nephritis also requires early and maximal therapeutic efficacy to stop the inflammation process. As MMF became the mainstay immunosuppressive drug with steroid minimization, thus we determined the correlation

Table 1 | Comparisons between responder and nonresponder groups in demographics, laboratory results, and treatments received during the study

Patient characteristics	All (*N*=18)	Responders (*n*=11)	Nonresponders (*n*=7)
Sex, female: male	16:2	10:1	6:1
Age, years	33 ± 7.76	34 ± 9.42	31 ± 4.1
Body mass index	22.2 ± 4.04	20.6 ± 1.4	24.5 ± 5.49
Duration of disease, years	7.5 ± 4.68	7.5 ± 4.79	7.4 ± 4.89
SLEDAI-2K score	15 ± 4.01	14.4 ± 4.4	17 ± 3.26
Serum creatinine (mg/dl)	1.26 ± 0.56	1.37 ± 0.66	1.09 ± 0.33
eGFR (CG) (ml/min)	70.71 ± 42.91	62.14 ± 35.67	84.18 ± 52.45
eGFR (MDRD) (ml/min)	69.94 ± 42.09	65.02 ± 39.23	77.66 ± 48.38
Serum albumin (g/dl)	2.6 ± 0.70	2.8 ± 0.73	2.3 ± 0.58
24-h urine protein (g/day)	6.3 ± 4.42	4.8 ± 3.38	8.7 ± 5.0
Hemoglobin level (g/dl)	10.5 ± 1.86	10.3 ± 1.77	10.7 ± 2.13
White blood cell (cell/mm^3)	9144 ± 4703	10,443 ± 4665	7102 ± 4296
Serum total bilirubin (mg/dl)	0.29 ± 0.07	0.33 ± 0.05	0.25 ± 0.06
ISN/RPS classification			
Class III, *n*	4	2	2
Class IV, *n*	9	6	3
Class IV+V, *n*	5	3	2
Activity index (0–24)	12 ± 4.84	12 ± 4.95	11 ± 4.96
Chronicity index (0–12)	3 ± 2.07	2 ± 1.95	3 ± 2.06
Dosage of steroid at baseline (mg/d)	39 ± 19.67	43 ± 21.5	32 ± 15.61
Dosage of steroid at 6 months (mg/d)	9 ± 1.61	9 ± 2.24	9 ± 2.48
Cumulative dosage of steroid (mg)	3400 ± 461	3175 ± 646	3738 ± 668
Number of patients on MMF/EC-MPS	12/6	8/3	4/3
Dosage of MMF (mg)	1416 ± 194.63	1375 ± 231.45	1500 ± 0.0
Dosage of EC-MPS (mg)	1260 ± 197.18	1080 ± 0	1440 ± 0
Dosage of MMF (mg)/IBW (kg)	28.07 ± 5.39	27.69 ± 5.85	28.84 ± 5.05
Dosage of EC-MPS (mg)/IBW (kg)	25.37 ± 7.19	19.24 ± 3.4	31.51 ± 2.17
Patients formerly received CY (*n*)	13	7	6
Number of patients on ACEI/ARB	13	8	5

Abbreviations: ACEI/ARB, angiotensin-converting enzyme inhibitor/angiotensin II receptor blocker; AUC, area under the concentration–time curve; CG, Cockcroft–Gault; CY, cyclophosphamide; EC, enteric coated; eGFR, estimated glomerular filtration rate; IBW, ideal body weight; ISN/RPS, International Society of Nephrology/Renal Pathology Society; MDRD, Modification of Diet in Renal Disease; MMF, mycophenolate mofetil; MPA, mycophenolic acid; SLEDAI, SLE disease activity index.
All *P*-values for comparison (responder vs nonresponder) were >0.05.

between the MPA levels of MMF or enteric-coated (EC)-mycophenolate sodium (MPS) and its therapeutic response in patients with proliferative lupus nephritis.

RESULTS

Patient characteristics

Out of a total of 18 patients, 2 patients were recently diagnosed with proliferative lupus nephritis, whereas the rest had renal relapse. In all, 15 patients had received cyclophosphamide therapy in the previous episodes of renal flare (Table 1). All patients had either class III or IV lupus nephritis at the time of enrollment. In addition, five patients had class IV + V lupus nephritis. A total of 12 patients were treated with MMF and 6 patients received EC-MPS.

After 6 months of mycophenolate therapy, 11 patients responded to treatment (responders) and 7 patients did not (nonresponders). The responder and nonresponder groups were generally comparable in age, body mass index, duration of lupus nephritis, disease activity, serum creatinine, estimated glomerular filtration rates, serum albumin, 24-h urinary protein, exposure to steroid, cyclophosphamide or ACEI/ARB, and renal pathological indices (Table 1).

MPA–area under the concentration–time curve (AUC) predicted the response to the therapy

The mean (s.d.) MPA–AUC of the responders was significantly higher than the nonresponders (65.98 (23.77) versus 32.08 (7.97); $P=0.002$) (Figure 1). The MPA–AUC level above 45 mg h/l can precisely predict the patient's response to therapy. With respect to treatment response, the receiver–operator characteristic curve (ROC) depicted the true-positive fractions (sensitivity) and false-positive fractions (1–specificity) at various cut points for MPA–AUC levels (Figure 2a). The calculated area under the ROC curve was 0.96 (95% confidence interval = 0.87–1.05). On the other hand, the therapeutic responses can not be predicted on the basis of daily doses of MMF or EC-MPS or other clinical parameters such as renal histology indices, pretreatment levels of urinary protein, serum complement, or estimated renal function (Figure 2b). The ROC–AUC and 95% confidence interval were 0.20(0.05–0.45), 0.25(0.01–0.51), 0.66 (0.38–0.94), 0.40(0.10–0.71), 0.65(0.37–0.93), 0.53(0.23–0.84), and 0.63(0.33–0.92) for urine protein, SLE disease activity index (SLEDAI), systolic blood pressure, estimated glomerular filtration rate, serum albumin, complement level, and activity

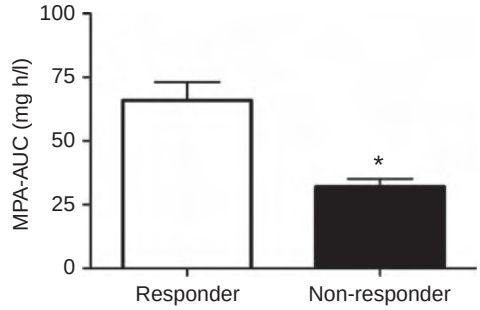


Figure 1 | Mycophenolic acid (MPA)–area under the concentration–time curve (AUC) levels and the response to therapy. Bars and lines represent the means and s.e. of the MPA–AUC levels. The responder (white bar) had significantly higher MPA–AUC levels than the nonresponder (black bar). AUC of MPA (0–12 h).

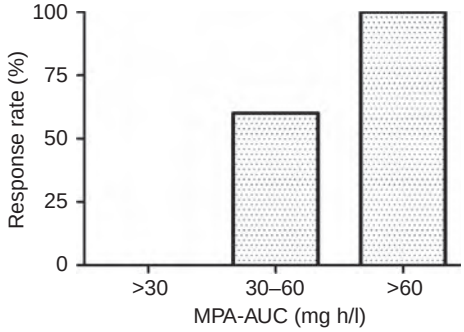


Figure 3 | Patients with high mycophenolic acid (MPA)–area under the concentration–time curve (AUC) levels have better therapeutic response rates. Patients were divided into three groups on the basis of their MPA–AUC levels of <30, 30–60, and >60 mg h/l.

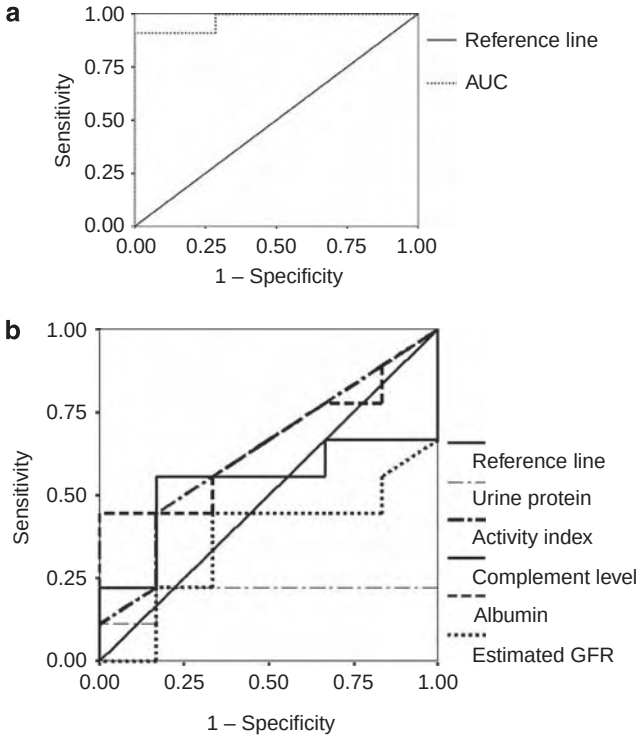


Figure 2 | Receiver-operator characteristic curve (ROC) curves of mycophenolic acid (MPA)–area under the concentration –time curve (AUC) levels to predict the response to therapy. The fraction of true-positive (sensitivity) and that of false-positive results (1−specificity) for (**a**) MPA–AUC and (**b**) other parameters as predictors of the response to therapy are shown. The area under the curve of ROC curve was 0.96 for MPA–AUC. The area under the curves of ROC curves were 0.20, 0.40, 0.65, 0.53, and 0.63 for urine protein, estimated glomerular filtration rate (GFR), serum albumin, complement level, and activity index.
A value of 0.5 (reference line) is no better than by chance and a value of 1.0 reflects a perfect indicator.

index, respectively. A value of 0.5 (reference line) is no better than by chance and a value of 1.0 reflects a perfect indicator. According to the MPA–AUC target levels used in organ transplantation, patients with MPA–AUC levels of >60, between 30 and 60 and <30 mg h/l had response rates of 100, 60, and 0%, respectively, ($P=0.02$) (Figure 3).

Association between plasma MPA concentrations at trough (C_0) or 1 h after dose (C_1) and response to therapy with MMF

In the MMF group, the plasma concentration of MPA at trough, 1, 4, and 8 h after dose were significantly correlated with MPA–AUC ($r=0.90$, 0.92, 0.70, and 0.80, respectively; all $P<0.05$) (Table 2, Figure 4). On the other hand, there was no correlation between each single-time point of MPA levels and MPA–AUC in the EC-MPS group.

Interestingly, the plasma MPA concentrations at trough and 1 h after dose (C_1) in the MMF group were associated with the response to MMF therapy. The mean C_0 and C_1 of the responders were significantly greater than the nonresponders (3.09 ± 1.07 versus 1.23 ± 0.85; $P=0.01$ and 24.48 ± 11.82 versus 5.96 ± 3.93 mg/l; $P=0.01$). However, there were overlapping data of individual C_0 and C_1 levels between both the groups (Figure 5).

Adverse events

Majority of the patients reported one or more adverse events. Common events were gastrointestinal events, hematotoxicity (anemia or leucopenia), and infection, which occurred in 50, 28, and 11% of the patients, respectively. There were no life-threatening adverse events. MPA–AUC was not associated with infectious episodes, gastrointestinal, or hematological adverse events (Table 3).

DISCUSSION

This study showed, for the first time, the association between MPA exposure and its therapeutic efficacy for active lupus nephritis. Favorable treatment response rates, without any additional adverse events, were associated with MPA–AUC above 45 mg h/l. This study showed a pivotal role of TDM of MPA for the treatment of lupus nephritis. ROC analysis (Figure 2) showed that the MPA–AUC was far superior as compared with other clinical or laboratory profiles in

predicting therapeutic responses. Furthermore, MPA–AUC was not associated with dosages of MMF or EC-MPS. Therefore, a direct measurement of blood MPA levels in each patient is required to determine MPA exposure.

On the basis of results obtained from transplant recipients, it has been suggested that TDM of MPA should be carried out to minimize the risk of allograft rejection.[13] In a randomized controlled trial known as APOMYGRE, it was shown that TDM of MPA can reduce risks of allograft rejection and treatment failure in renal transplant recipients.[12] In organ transplantation, the target therapeutic levels of MPA–AUC was above 45 mg h/l.[12] When we used this value for our study, we noticed that it can precisely predict a good response to therapy as well. Similarly, MPA–AUC of 30–60 mg h/l and >60 mg h/l that were used for transplantation,[13] when used in this study, were associated with increased response rates (60 and 100%, respectively) as well.

Table 2 | Pharmacokinetic parameters of mycophenolic acid (MPA) after oral administration of 1–1.5 g/day MMF and 1080–1440 mg/day EC-MPS in patients with class III or IV lupus nephritis

Parameters	MMF (n=12) Means ± s.d.	EC-MPS (n=6) Means ± s.d.
C_0 (mg/l)	2.47 ± 1.33	2.73 ± 1.46
$C_{0.5}$ (mg/l)	7.08 ± 7.16	2.68 ± 1.46
C_1 (mg/l)	18.3 ± 13.28	2.02 ± 0.7
C_2 (mg/l)	6.72 ± 2.26	7.48 ± 5.83
C_3 (mg/l)	4.05 ± 2.64	6.23 ± 4.85
C_4 (mg/l)	3.37 ± 2.11	3.59 ± 1.99
C_8 (mg/l)	3.38 ± 2.19	2.92 ± 2.04
C_{12} (mg/l)	2.59 ± 1.9	2.14 ± 1.54
AUC_{0-12} (mg h/l)	57.97 ± 29.38	42.44 ± 9.86
C_{max} (mg/l)	19.43 ± 12.01	10.12 ± 5.13
T_{max} (h)	1.54 ± 1.03	3.25 ± 2.52
Dosage (mg/day)	1416.67 ± 194.63	1260 ± 197.18
Dosage/IBW (kg)	28.07 ± 5.39	25.37 ± 7.19

Abbreviations: AUC_{0-12}, area under the concentration–time curve for 0–12 h; C_{max}, maximum concentration of drug; C_0, trough plasma concentration of mycophenolic acid; $C_{0.5}$, C_1, C_2, C_3, C_4, C_8, and C_{12}, plasma concentrations of mycophenolic acid at 0.5, 1, 2, 3, 4, 8 and 12 h post-dose, respectively; EC-MPS, enteric-coated mycophenolate sodium; IBW, ideal body weight; MMF, mycophenolate mofetil; T_{max}, maximum drug concentration.

It should be noted that reports from transplant recipients recommended having TDM of MPA carried out at several time points because of its large inter-subject variability in MPA exposure.[14] There are many factors that may influence the large inter-subject variability seen in MPA pharmacokinetics such as renal impairment, liver dysfunction, hypoalbuminemia, genetic factors, and concurrent use of calcineurin inhibitors.[14–16] As we did not aim to test influences of these factors, therefore in our study, our sample size was too small to answer this type of question.

Nevertheless, our results showed MPA at trough levels and 1 h after dose were closely associated with MPA–AUC in patients receiving MMF. This finding is consistent with studies conducted in patients with autoimmune diseases and glomerulopathy.[17–19] Neumann *et al.*[20] conducted a pharmacokinetic study comparing autoimmune disease with kidney transplantation, which showed a better correlation at a single-time point and full AUC for autoimmune disease. Along the same lines, another study showed that high MPA trough levels (C_0) in SLE patients receiving MMF treatment had lower recurrent rates.[19]

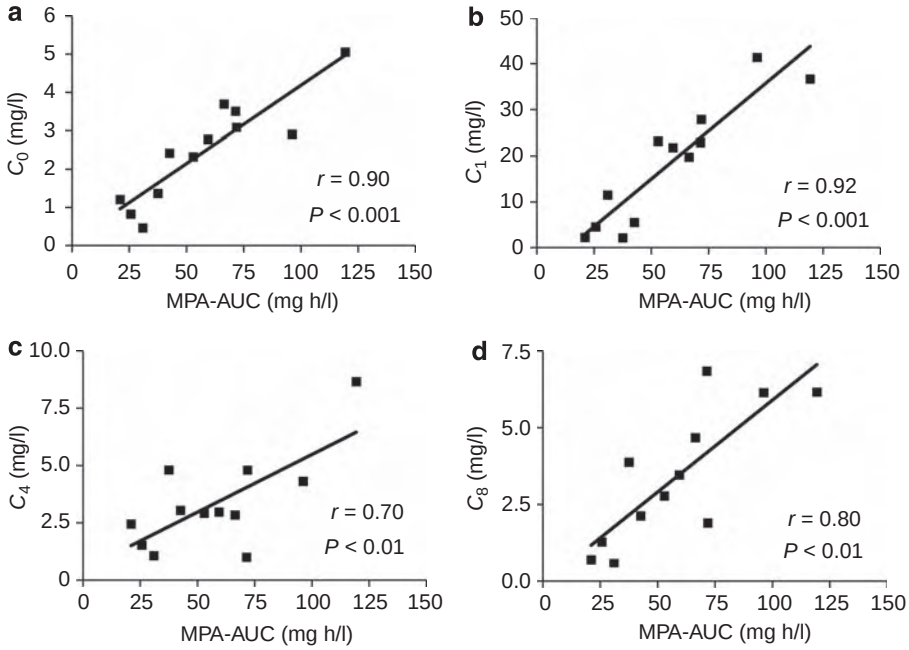


Figure 4 | Correlation between area under the concentration–time curve (AUC) and single-time point of plasma mycophenolic acid (MPA) levels from patients taking mycophenolate mofetil (MMF, $n = 12$). (**a**) The relationship between the AUC and C_0 was significant at $P<0.001$ ($r=0.90$). (**b**) The relationship between the AUC and C_1 was significant at $P<0.001$ ($r=0.92$). (**c**) The relationship between the AUC and C_4 was significant at $P<0.01$ ($r=0.70$). (**d**) The relationship between the AUC and C_8 was significant at $P<0.01$ ($r=0.80$). C_0, C_1, C_4, C_8: plasma concentrations of MPA at trough, 1, 4 and 8 h after dose, respectively.

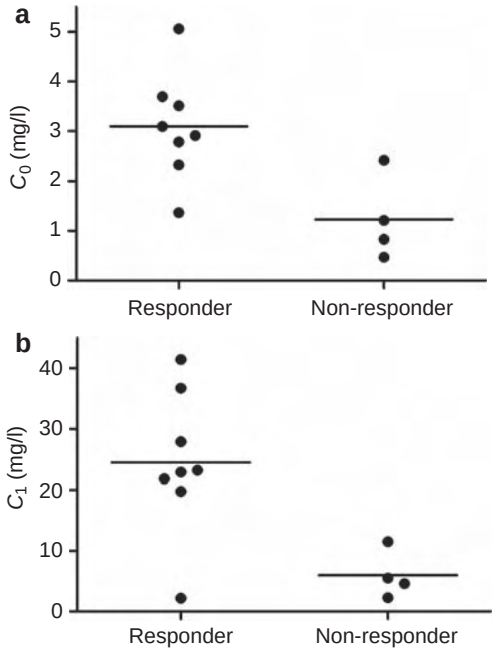


Figure 5 | Plasma mycophenolic acid (MPA) concentrations at trough (C_0) or 1 h after dose (C_1) and the response to therapy with mycophenolate mofetil (MMF). Dots and lines represent the individual and means of the MPA levels. (**a**) The responder ($n = 8$) had significantly higher MPA trough levels than the nonresponder ($n = 4$). Likewise, (**b**) the mean MPA level at 1 h after dose was higher in the responder.

In addition, our study confirmed that pharmacokinetic profiles of MPA were different between MMF and EC-MPS. The maximum MPA level had an earlier peak for MMF. These results indicated that the drug absorption of EC-MPS was slower and its pharmacokinetic peak was consistent with that of EC-formulated tablets. Therefore, it is important to take different tablet formulations in to account when determining optimal TDM of MPA.[21]

Aside from that, we demonstrated that there was a positive correlation between MPA trough levels or at 1 h after dose and the therapeutic response to MMF, even though there was an overlapping data for the MPA levels (Figure 5). This finding is in contrast to the results obtained from the renal transplant recipients, which showed weak correlations between MPA–AUC and trough level of MPA.[22,23] It was also pointed out that MPA trough levels obtained from renal transplant recipients were not specific and sensitive in predicting acute rejection compared with MPA–AUC.[24–28] This difference may be because of the fact that our lupus nephritis patients were only on MPA therapy without concomitant use of other immunosuppressive drugs. Other differences such as degree of renal impairment and serum albumin levels may have contributed to this discrepancy. Taking in to account our participants' mild degree of renal impairment and almost normal serum albumin levels, we noticed that these different factors did not significantly influence the pharmacokinetics of MPA.

Table 3 | Number of MPA-related adverse events for three different MPA–AUC levels

		MPA–AUC (mg h/l)		
Adverse events	Patients *N* (%)	<30	30–60	>60
GI symptoms	9 (50%)	2	4	3
Hematological symptoms	5 (28%)	1	1	3
Infections	2 (11%)	1	0	1
Total	18 (100%)	3	10	5

Abbreviations: AUC, area under the concentration–time curve for 0–12 h; GI symptoms, gastrointestinal symptoms such as diarrhea, nausea, vomiting; hematological symptoms, anemia (Hb < 11 g/dl) and leucopenia (white blood cell count $< 4000/mm^3$); infection, Herpes infection; MPA, mycophenolic acid.

The limitation of this study is our small sample size. Although this study may not be applicable to majority of the lupus patients, however, these results are relevant to those patients with class III or IV lupus nephritis. The immunosuppressive use in this study was relatively low dose of MPA with corticosteroids. In order to validate the benefit of MPA exposure in an immunosuppressive regimen, we had to use high doses of MPA without steroid. Another limitation of the study is that the pharmacokinetic profiles of MPA were assessed only once during the 6-month period. These results could not determine within-patient variability. However, it has been shown that there was minimal within-patient variability in patients taking MMF after transplantation.[14] On the other hand, multiple blood samplings of MPA–AUC may maximize the efficacy and minimize its toxicity but repeated measurements of MPA levels in real life is not practical and feasible. Therefore, single point or limited sampling strategies have been proposed to monitor MPA in the blood.

Despite these limitations, our study showed that MPA exposure is a good predictor for early therapeutic responses in patients who have biopsy-proven class III or IV lupus nephritis. Thus, we recommend maintaining MPA–AUC levels above 45 mg h/l in severe lupus nephritis patients taking mycophenolate treatment. It is important that MPA monitoring is carried out to ensure optimal dosing of the immunosuppressive drug in lupus patients.

MATERIALS AND METHODS

Patients and study design

This 6-month prospective study enrolled Thai patients with SLE as defined by the criteria of American College of Rheumatology.[29] Renal biopsy showed proliferative lupus nephritis class III or IV according to the 2003 International Society of Nephrology/Renal Pathology Society classification.[30] All participants were required to have any combinations of the two clinical criteria present: (1) 24-h urine protein >2 g/day, (2) active-urine sediments (red blood cells >5 or white blood cells >5 per high-power field) or estimated glomerular filtration rate <60 ml/min, and/or (3) a biopsy-confirmed diagnosis of ISN/RPS class III/IV LN within 12 weeks of study.

Patients were treated with a fixed dose of 1.0–1.5 g per day of MMF or 1080–1440 mg per day of EC-MPS for 6 months based on

the physicians' prescription. The highest dose was given within 2 weeks and then no dose change was allowed unless there was intolerance. All patients received oral prednisolone; starting dose of prednisolone was 0.7 mg per kg of body weight per day and this dose was reduced every 2 weeks by 5 mg/day until the dose was 5 mg/day. A short course (<2 weeks) of moderate steroid dose (<30 mg/d) was allowed in one patient (responder group) because of presentation of hemolytic anemia. The pharmacokinetic study was performed after 1 month of treatment. We excluded patients who were pregnant or had serum creatinine >3 mg/dl. Those who were nonadherent to the protocol or had a history of mycophenolate treatment, gastrointestinal disorders, or concurrent administration of medications that disturbed the MPA pharmacokinetics such as antacids, cholestyramine, acyclovir, and rifampin, were excluded from the study as well. The participants were asked to take the medicine on time with a 12 h interval and at least 1 h apart from meals. Drug pill count was carried out by the study team at every clinic visit. The follow-up period was for 6 months.

The study was approved by the Ethics Committee for Human Research, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand, and written informed consents were obtained from all the participants.

The pharmacokinetic study of MPA

We measured MPA plasma levels 1 month after initiating mycophenolate treatment. Venous blood samples were drawn at baseline, 30 min, 1, 2, 3, 4, 8, and 12 h after oral administration of mycophenolate. A volume of 3 ml of blood was collected in an ethylenediaminetetraacetic acid-treated vaccutainers and was centrifuged at 1200 *g* for 15 min. All plasma samples were stored at -80°C until further analysis was carried out.

MPA plasma concentration was measured by the new enzymatic method (Roche Total MPA Assay, Hoffmann-La Roche, Basel, Switzerland) on the basis of inhibition of inosine monophosphate dehydrogenase, the *in vivo* target of MPA. It is a rapid and reliable assay for determining plasma MPA concentrations when compared with the standard assays of LC–MS/MS. The method can be performed using fully automated COBAS INTEGRA analyzers and using the Cobas modular platform (Roche Diagnostics GmbH) in combination with routine clinical chemistry. Results can be obtained in 20 min.[31,32] The area under the plasma concentration–time curve (MPA–AUC) was calculated by using the linear trapezoidal rule. We assessed whether there was any relationship between single-time point and MPA–AUC.

Patient outcome assessment

The primary outcome of the study was to assess the correlation between MPA–AUC and treatment response. This was defined by the following criteria: (1) have a normal or increased estimated glomerular filtration rate by 25% if the baseline estimated glomerular filtration rate was abnormal, (2) have inactive-urine sediments (red blood cells <5 cells per high-power field and white blood cells <5 cells per high-power field), and (3) have a 50% reduction of 24-h urine protein resulting in a level <2 g/day.[33] The secondary objectives assessed the number of adverse events and their association with the drugs studied. Serum creatinine, complete blood count, serum albumin, liver function test, fasting blood sugar, lipid profiles, anti-nuclear antibodies, anti-double-stranded DNA, complement levels, and 24-h urine protein were assessed at baseline and the 6th month of treatment. Glomerular filtration rates were estimated using the Modification of Diet in Renal Disease study equations[34] and the Cockcroft–Gault formula.[35] All patients underwent medical history and physical examination, which included measurements of systolic and diastolic pressure, body weights, and vital signs. All adverse effects of mycophenolate were recorded.

Statistical analyses

This study had an 80% power to detect a 40% difference in mean MPA–AUC between responders and nonresponders with an α error of 0.05 (two-sided). According to the power or sample-size calculations, 18 patients were needed. We enrolled a total of 20 patients into the study, among whom 2 were excluded at the screening visit. Statistical analyses were performed by using the SPSS software version 16 (SPSS Inc., Chicago, IL, USA). Continuous data were tested for normal distribution by using Kolmogorov–Smirnov and Shapiro–Wilks tests. The normally distributed continuous data were expressed as mean and standard deviation (s.d.) unless otherwise specified. Differences in mean MPA–AUC in responders and nonresponders were analyzed by an independent sample *t*-test. To predict therapeutic response, ROC of MPA–AUC levels was analyzed. The area under the curve was calculated. After the therapeutic ranges in kidney transplantation,[13] MPA–AUC was categorized into three ranges (<30, 30–60 mg h/l, and >60 mg h/l), and the response rates of the patients from the three different groups were compared by using Chi-square test. Pearson's correlation analysis was used to determine whether there was any correlation between MPA–AUC and each single-point level of MPA plasma concentration. α was set at 0.05 and all tests were two-sided.

DISCLOSURE

All the authors declared no competing interests.

ACKNOWLEDGMENTS

This study is supported by Rajadapiseksompotch Funds and the National Research Council of Thailand. YA is the recipient of Thailand Research Fund (RMU 5080076). We thank our nurses, Sujinda Pipopsuttipaiboon and Chutima Rukrung, for collecting the blood samples from the patients. We also thank the staff from the Division of Nephrology for referring the patients to our study and Pisut Katavetin for critical reading the paper.

REFERENCES

1. Chan TM. Lupus nephritis: induction therapy. *Lupus* 2005; **14**(Suppl 1): S27–S32.
2. Shayakul C, Ong-aj-yooth L, Chirawong P *et al.* Lupus nephritis in Thailand: clinicopathologic findings and outcome in 569 patients. *Am J Kidney Dis* 1995; **26**: 300–307.
3. Chan TM, Tse KC, Tang CS *et al.* Long-term study of mycophenolate mofetil as continuous induction and maintenance treatment for diffuse proliferative lupus nephritis. *J Am Soc Nephrol* 2005; **16**: 1076–1084.
4. Contreras G, Tozman E, Nahar N *et al.* Maintenance therapies for proliferative lupus nephritis: mycophenolate mofetil, azathioprine and intravenous cyclophosphamide. *Lupus* 2005; **14**(Suppl 1): S33–S38.
5. Faurschou M, Sorensen IJ, Mellemkjaer L *et al.* Malignancies in Wegener's granulomatosis: incidence and relation to cyclophosphamide therapy in a cohort of 293 patients. *J Rheumatol* 2008; **35**: 100–105.
6. Ginzler EM, Dooley MA, Aranow C *et al.* Mycophenolate mofetil or intravenous cyclophosphamide for lupus nephritis. *N Engl J Med* 2005; **353**: 2219–2228.
7. Tse KC, Tang CS, Lio WI *et al.* Quality of life comparison between corticosteroid- and-mycofenolate mofetil and corticosteroid- and-oral cyclophosphamide in the treatment of severe lupus nephritis. *Lupus* 2006; **15**: 371–379.
8. Walsh M, James M, Jayne D *et al.* Mycophenolate mofetil for induction therapy of lupus nephritis: a systematic review and meta-analysis. *Clin J Am Soc Nephrol* 2007; **2**: 968–975.
9. Wetzels JF. Cyclophosphamide-induced gonadal toxicity: a treatment dilemma in patients with lupus nephritis? *Neth J Med* 2004; **62**: 347–352.

10. Zhu B, Chen N, Lin Y *et al.* Mycophenolate mofetil in induction and maintenance therapy of severe lupus nephritis: a meta-analysis of randomized controlled trials. *Nephrol Dial Transplant* 2007; **22**: 1933–1942.
11. Appel GB, Contreras G, Dooley MA *et al.* Mycophenolate mofetil versus cyclophosphamide for induction treatment of lupus nephritis. *J Am Soc Nephrol* 2009; **20**: 1103–1112.
12. Le Meur Y, Buchler M, Thierry A *et al.* Individualized mycophenolate mofetil dosing based on drug exposure significantly improves patient outcomes after renal transplantation. *Am J Transplant* 2007; **7**: 2496–2503.
13. van Gelder T, Hilbrands LB, Vanrenterghem Y *et al.* A randomized double-blind, multicenter plasma concentration controlled study of the safety and efficacy of oral mycophenolate mofetil for the prevention of acute rejection after kidney transplantation. *Transplantation* 1999; **68**: 261–266.
14. van Hest RM, Mathot RA, Pescovitz MD *et al.* Explaining variability in mycophenolic acid exposure to optimize mycophenolate mofetil dosing: a population pharmacokinetic meta-analysis of mycophenolic acid in renal transplant recipients. *J Am Soc Nephrol* 2006; **17**: 871–880.
15. Knight SR, Morris PJ. Does the evidence support the use of mycophenolate mofetil therapeutic drug monitoring in clinical practice? A systematic review. *Transplantation* 2008; **85**: 1675–1685.
16. Shaw LM, Figurski M, Milone MC *et al.* Therapeutic drug monitoring of mycophenolic acid. *Clin J Am Soc Nephrol* 2007; **2**: 1062–1072.
17. Czock D, Rasche FM, Carius A *et al.* Pharmacokinetics and pharmacodynamics of mycophenolic acid after enteric-coated mycophenolate versus mycophenolate mofetil in patients with progressive IgA nephritis. *J Clin Pharmacol* 2007; **47**: 850–859.
18. Filler G, Hansen M, LeBlanc C *et al.* Pharmacokinetics of mycophenolate mofetil for autoimmune disease in children. *Pediatr Nephrol* 2003; **18**: 445–449.
19. Neumann I, Fuhrmann H, Fang IF *et al.* Association between mycophenolic acid 12-h trough levels and clinical endpoints in patients with autoimmune disease on mycophenolate mofetil. *Nephrol Dial Transplant* 2008; **23**: 3514–3520.
20. Neumann I, Haidinger M, Jager H *et al.* Pharmacokinetics of mycophenolate mofetil in patients with autoimmune diseases compared renal transplant recipients. *J Am Soc Nephrol* 2003; **14**: 721–727.
21. Cattaneo D, Cortinovis M, Baldelli S *et al.* Pharmacokinetics of mycophenolate sodium and comparison with the mofetil formulation in stable kidney transplant recipients. *Clin J Am Soc Nephrol* 2007; **2**: 1147–1155.
22. Kuypers DR, Claes K, Evenepoel P *et al.* Long-term changes in mycophenolic acid exposure in combination with tacrolimus and corticosteroids are dose dependent and not reflected by trough plasma concentration: a prospective study in 100 *de novo* renal allograft recipients. *J Clin Pharmacol* 2003; **43**: 866–880.
23. Okamoto M, Wakabayashi Y, Higuchi A *et al.* Therapeutic drug monitoring of mycophenolic acid in renal transplant recipients. *Transplantation Proceedings* 2005; **37**: 859–860.
24. Barbari A, Stephan A, Masri MA *et al.* Mycophenolic acid plasma trough level: correlation with clinical outcome. *Exp Clin Transplant* 2005; **3**: 355–360.
25. Hale MD, Nicholls AJ, Bullingham RE *et al.* The pharmacokinetic-pharmacodynamic relationship for mycophenolate mofetil in renal transplantation. *Clin Pharmacol Ther* 1998; **64**: 672–683.
26. Pawinski T, Durlik M, Szlaska I *et al.* Comparison of mycophenolic acid pharmacokinetic parameters in kidney transplant patients within the first 3 months post-transplant. *J Clin Pharm Ther* 2006; **31**: 27–34.
27. Pawinski T, Durlik M, Szlaska I *et al.* The weight of pharmacokinetic parameters for mycophenolic acid in prediction of rejection outcome: the receiver operating characteristic curve analysis. *Transplantation Proceedings* 2006; **38**: 86–89.
28. Pillans PI, Rigby RJ, Kubler P *et al.* A retrospective analysis of mycophenolic acid and cyclosporin concentrations with acute rejection in renal transplant recipients. *Clinical Biochemistry* 2001; **34**: 77–81.
29. Tan EM, Cohen AS, Fries JF *et al.* The 1982 revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum* 1982; **25**: 1271–1277.
30. Weening JJ, D'Agati VD, Schwartz MM *et al.* The classification of glomerulonephritis in systemic lupus erythematosus revisited. *Kidney International* 2004; **65**: 521–530.
31. Bunch DR, Wang S. Measurement of mycophenolic acid in plasma or serum by a commercial enzyme inhibition technique in comparison with a high performance liquid chromatography method. *Clin Chem Lab Med* 2008; **46**: 1281–1284.
32. van Gelder T, Domke I, Engelmayer J *et al.* Clinical utility of a new enzymatic assay for determination of mycophenolic acid in comparison with an optimized LC-MS/MS method. *Therapeutic Drug Monitoring* 2009; **31**: 218–223.
33. Renal Disease Subcommittee of the American College of Rheumatology Ad Hoc Committee on Systemic Lupus Erythematosus Response Criteria. The American College of Rheumatology response criteria for proliferative and membranous renal disease in systemic lupus erythematosus clinical trials. *Arthritis Rheum* 2006; **54**: 421–432.
34. Levey AS, Greene T, Kusek J *et al.* A simplified equation to predict glomerular filtration rate from serum creatinine [abstract]. *J Am Soc Nephrol* 2000; **11**: 155A.
35. Cockcroft DW, Gault MH. Prediction of creatinine clearance from serum creatinine. *Nephron* 1976; **16**: 31–41.