รายงานวิจัยฉบับสมบูรณ์

โครงการ การศึกษาหาความสัมพันธ์ระหว่างยีนที่ควบคุมการสร้างกรดเรติโนอิก (retinoic acid) และ ทีจีเอฟเบต้า (transforming growth factor beta ในผู้ป่วยโรคไตเรื้อรัง

(Retinoic acid and extracellular matrix pathways in kidney diseases)

โดย นายแพทย์หม่อมหลวง ชาครีย์ กิติยากร

เสร็จโครงการ พฤษภาคม 2561

สัญญาเลขที่ RMU4880048

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัยและมหาวิทยาลัยมหิดล

(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกว. และ มหาวิทยาลัยมหิดล ไม่จำเป็นต้องเห็นด้วยเสมอไป)

Abstract

Background: Kidney disease progression is due to the activation of multiple signaling pathways, and altered extracellular matrix (ECM) deposition. Retinoic acid (RA) are natural derivatives of vitamin A. The enzyme retinaldehyde dehydrogenases (RALDHs) and cytochrome P26 (CYP26) are rate limiting enzymes responsible for synthesis and degradation of RA, respectively. Both RA and mediators ECM deposition such as transforming growth factor beta (TGF β) and connective tissue growth factor (CTGF), correlate with kidney disease in animals, but their role in human disease have yet to be fully evaluated.

Aims: This study aimed to explore the expression of genes linked to RA pathways, CTGF and TGF-β in different human kidney diseases, and relate these gene expression changes to renal histology and clinical disease progression.

Methods: We evaluated the renal mRNA expression by real-time reverse transcriptase polymerase chain reaction for genes linked to retinoic synthesis (RALDH1, RALDH2, RALDH3)) and degradation (CYP26A, CYP26B) and TGF β, CTGF in lupus nephritis (LN), diabetic nephropathy (DN), or chronic allograft nephropathy (CAN). Gene expression was compared to normal controls and correlated with renal histology or with glomerular filtration rate (GFR).

Results:

1. RA genes in LN and DN. Compared to controls, RALDH1 and CYP26A was lower in LN, but there were no differences between DN and controls.
2. RA genes and LN histology. There were no differences in RALDH or CYP26 isoforms between different LN histological categories. The ratio of RALDH2 to CYP26A and RALDH3 to CYP26A were increased in LN with crescents.
3. ECM genes in LN. CTGF gene was higher in LN patients who developed GFR decline
4. ECM genes in CAN. There was a correlation between changes in CTGF or TGF-β and change in GFR after modifications in therapy in patients with established CAN

Conclusions: Endogenous retinoic acid metabolic pathways do not appear to have a major role in in LN or DN, although a potential role in crescent formation may be possible in LN. CTGF is a predictor of decline in kidney function in LN and CAN.

บทคัดย่อ

บทนำ การเสื่อมสภาพของโรคไตเกิดจากการปลี่ยนแปลงสารเคมีหลาย ๆชนิด และการสะสมของเมทริกซ์นอกเซลล์ (ECM) กรด Retinoic (RA) เป็นอนุพันธ์ทางธรรมชาติของวิตามินเอ เอนไซม์ retinaldehyde dehydrogenases (RALDHs) และ cytochrome P26 (CYP26) เป็นเอนไซม์ที่จำกัดอัตราสำหรับการสังเคราะห์และการย่อยสลาย RA ตามลำดับ ทั้ง RA และโมเลกุลที่ควบคุม ECM deposition เช่น transform growth factor beta (TGF β) และ connective tissue growth factor growth factor (CTGF) มีความสัมพันธ์กับโรคไตในสัตว์ทดลอง แต่มีข้อมูลจำกัดในคนที่เป็นโรคไต

วัตถุประสงค์: การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อศึกษาการแสดงออกของยีนระบบ RA, CTGF และ TGF-βในโรคไต และศึกษาความสัมพันธ์การแสดงออกของยีนเหล่านี้ในเนื้อเยื่อไตกับพยาธิสภาพและการลดลงของการทำงานของไต

วิธีการ: การแสดงออกของ mRNA ที่สังเคราะห์ RA (RALDH1, RALDH2, RALDH3)) และการย่อยสลาย (CYP26A, CYP26B) และ TGF β, CTGF ในไตได้รับการประเมินโดย real time reverse transcriptase polymerase chain reaction นักวิจัยจะเปรียบเทียบการแสดงออกของยีนในโรคไตอักเสบลูปัส (LN) โรคเบาหวาน (DN) หรือ chronic allograft nephropathy (CAN) กับคนปกติและทดสอบความสัมพันธ์กับพยาธิสภาพหรืออัตราการกรอง (GFR)

ผล

1. <u>ยีน RA ใน LN และ DN</u> RALDH1 และ CYP26A ต่ำกว่าใน LN เมื่อเทียบกับคนปกติ แต่ไม่มีความแตกต่างระหว่าง DN และคนปกติ
2. <u>ยีน RA และ LN histology</u> ไม่พบความแตกต่าง RALDH หรือ CYP26 LN ในพยาธิสภาพที่แตกต่างกัน อัตราส่วนของ RALDH2 ต่อ CYP26A และ RALDH3 ต่อ CYP26A เพิ่มขึ้นใน LN มี crescent
3. <u>ยีน ECM ใน LN</u> ยีน CTGF สูงกว่าในกลุ่มผู้ป่วย LN ที่มี GFR ลดลง
4, <u>ยีน ECM ใน CAN</u> พบความสัมพันธ์ระหว่างการเปลี่ยนแปลง CTGF หรือ TGF-β และการเปลี่ยนแปลง GFR หลังการปรับเปลี่ยนการรักษาในผู้ป่วยที่มี CAN

สรุป: RA ไม่น่าจะมีบทบาทสำคัญใน LN หรือ DN แม้ว่าจะเป็นไปได้ที่มีบทบาท ใน crescent ใน LN CTGF เป็นตัวทำนายการลดลงของการทำงานของไตใน LN และ CAN

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Project Title : Retinoic acid and extracellular matrix pathways in kidney diseases
(ชื่อโครงการ) การศึกษาหาความสัมพันธ์ระหว่างยีนที่ควบคุมการสร้างกรดเรติโนอิก (retinoic acid) และ ทีจีเอฟเบต้า (transforming growth factor beta) ในผู้ป่วยโรคไตเรื้อรัง

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(คำหลัก)

INTRODUCTION

Kidney diseases are common in Thailand. In addition to causing significant morbidity, kidney diseases can progress to end-stage renal disease (ESRD), a condition associated with high mortality and high health care costs. Disease progression is the result of scarring of glomeruli (glomerulosclerosis) and fibrosis of the tubulointersitium due to the activation of multiple signaling pathways, and altered extracellular matrix (ECM) deposition. (1) (2, 3) The prognosis and responses of each patient to therapy vary even within the same disease category, and cannot be predicted accurately from clinical or renal histological findings alone. For many kidney diseases, treatment often requires prolonged exposure to corticosteroids or cytotoxics with significant risks of toxicities. Development of novel non-toxic therapies or the ability to accurately predict patients with the worse prognosis to target these patients to more aggressive therapy could lead to improved patient outcomes.

Retinoids are natural derivatives of vitamin A (retinol), and regulate a variety of important cellular functions including nephrogenesis and epithelial cell differentiation. (4, 5). The ability of retinoids to promote epithelial cell differentiation suggests that these agents might have therapeutic potential in kidney diseases characterized by glomerular (parietal cell or podocytes) or tubular epithelial cell injury. In vivo, retinol originates from the diet and absorbed as retinol. Retinol is converted to active retinoids of which, tretinoin (all-trans-retinoic acid), is the major biologically active metabolite via two steps. (4, 5) (6) First, retinol (vitamin A alcohol) is oxidized to retinaldehyde (retinal, vitamin A aldehyde). Second, the aldehyde form is irreversibly converted to retinoic acid (RA) by the enzyme retinaldehyde dehydrogenases (RALDHs), which are believed to be the major rate limiting step responsible for the regulation of RA synthesis under physiological conditions. The enzyme cytochrome P26 (CYP26A) converts RA into inactive metabolites. RA induces CYP26A1 expression. Thus CYP26A1 plays a key role in regulating cellular RA levels in a feedback loop controlled by RA. Previous studies have recently shown a role of retinoic acid (RA) in experimental renal diseases. (7), The role of RA in human kidney diseases has been poorly recognized.

Retinoids regulate RNA transcription via binding to two distinct families of nuclear receptors, retinoic acid receptors (RAR) and retinoid X receptors (RXR), that act as ligand-activated transcription factors. (5). The RAR and RXR families each contain three receptor isotypes (alpha, beta, and gamma) encoded by different genes. RARs heterodimerize with RXR, whereas RXR may homodimerize or form heterodimers with other nuclear receptors, such as the vitamin D receptor and the thyroid hormone receptor. Such heterodimers may facilitate cross-talk between various nuclear hormone signaling pathways. RA have both direct and indirect effects on gene transcription. The existence of multiple types of receptors, hormone response elements, mechanisms of action, and regulatory proteins suggest that RA action is mediated through a variety of pathways and results in the complex regulation of genes that may be involved in kidney injury or repair.

Interstitial fibrosis has been associated with poor clinical outcome in numerous renal diseases. (2, 8) Genes involved in matrix expansion and fibrosis have been shown to correlate with renal failure in a number of experimental models. Transforming growth factor beta (TGF β) has been

shown to be a key intermediary step in the pathogenesis of fibrosis in many forms of experimental kidney disease. However, TGF-β has other beneficial roles in immunosuppression, such that long-term suppression of TGF-β may be hyper-inflammatory and pro-carcinogenic, thus highlighting a need for alternate therapeutic targets for inhibition of fibrosis. Connective tissue growth factor (CTGF) is a cysteine-rich peptide in the CCN family of growth factors, which is partly a downstream mediator of the profibrogenic function of TGF-β. Experimental data shows that CTGF induces fibroblast proliferation, ECM synthesis and integrin expression and may therefore play significant roles in human renal diseases and fibrosis. (9)However, the role of CTGF levels in renal disease progression and the relationship of CTGF with TGF-β and collagen have yet to be fully evaluated in human kidney diseases including LN and chronic allograft nephropathy.

We hypothesized that genes regulating endogenous retinoic acid metabolic pathways and genes known to be involved in extracellular matrix (ECM) deposition and fibrosis, namely transforming growth factor beta (TGF-β) and connective tissue growth factor (CTGF) would be altered in human kidney diseases and that these changes would correlate with adverse pathological findings or predict decline in kidney function. This study aims to explore the gene expression profiles of several genes linked to RA pathways, and TGF-βin different human kidney diseases, and relate these gene expression changes to extent of renal histology and clinical disease progression.

AIMS

1. To study the renal expression of genes of the retinoic acid pathways in different kidney diseases
2. To study the renal expression of ECM-related genes in different kidney diseases
3. To relate the expression of ECM-related genes and genes in the retinoic acid pathways to clinicopathologic findings

METHODS

The overall plan consisted of initial method development for gene expression studies in whole kidney cortex obtained from frozen and or paraffin fixed tissues and relating the gene expression to clinical or histological grade. In addition, the invetigators also attempted to evaluate gene expression in laser-microdissected glomeruli. We studied gene expression in normal controls and three kidney diseases (lupus nephritis, diabetic nephropathy, and chronic allograft nephropathy).

Patients' baseline data and management

Patients with a formal diagnosis of various types of kidney diseases undergoing a kidney biopsy at Ramathibodi Hospital for clinical indications between 1995 and 2015 were included in this study. This study was approved by the Ethical Committee of Ramathibodi Hospital, Faculty of Medicine and all participants gave written informed consent. Patients were managed at the physicians' discretion based on standard International guidelines. In general, patients received antihypertensive therapy if their blood pressure exceeded 130/80 mmHg, and received immunosuppressive treatment according to the type and severity of kidney diseases. Routine clinical characteristics for each patient were recorded at baseline (time of kidney biopsy) and at follow-up.

Laboratory monitoring

Urinalysis was performed at presentation to assess disease activity and at follow up to assess remission or relapse. Urinalysis involves examining number of red blood cells. Significant microscopic hematuria (>5 Red blood cell (RBC) / high power field) represent the presence of nephritis.

Proteinuria was quantitated by either randomly performed spot urine protein/creatinine ratio or 24 hour urine collection. The numeric ratio of protein and creatinine concentrations approximates the number of grams per day of proteinuria. This method has steadily gained wider application due to its simplicity and convenience.

Glomerular filtration rate (GFR) was estimated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) formula as follows: (10).

$$GFR = 141 \times \min(Scr/\kappa,1)^{\alpha} \times \max(Scr/\kappa,1)^{-1.209} \times 0.993^{Age} \times 1.018 \text{ [if female]} \times 1.159 \text{ [if black]}$$

Where Scr is serum creatinine (mg/dL), κ is 0.7 for females and 0.9 for males, α is –0.329 for females and –0.411 for males, min indicates the minimum of Scr/κ or 1, and max indicates the maximum of Scr/κ or 1

or by MDRD equation according to KDIGO recommendations

Patient groups

a) <u>Lupus nephritis</u>

Patients with systemic lupus erythematosus with nephritis by American Rheumatology Association criteria were included. Kidney biopsies were classified according to the International Society of Nephrology/Renal Pathology Society Class (RPS/ISN) criteria (Table1).

Table 1: Abbreviated International Society of Nephrology/Renal Pathology Society (ISN/RPS) classification of lupus nephritis (2003)

Class I	Minimal mesangial lupus nephritis
Class II	Mesangial proliferative lupus nephritis
Class III	Focal lupus nephritis[a]
Class IV	Diffuse segmental (IV-S) or global (IV-G) lupus nephritis[b]
Class V	Membranous lupus nephritis[c]
Class VI	Advanced sclerosing lupus nephritis
Indicate and grade (mild, moderate, severe) tubular atrophy, interstitial inflammation and fibrosis, severity of arteriosclerosis or other vascular lesions.	

a Indicate the proportion of glomeruli with active and with sclerotic lesions.

b Indicate the proportion of glomeruli with fibrinoid necrosis and cellular crescents.

c Class V may occur in combination with class III or IV, in which case both will be diagnosed.

In addition, the Activity Index (AI) and Chronicity Index (CI) (below) were also assessed (Table 2).

Table 2: Activity and Chronicity lesions

Activity index
Glomerular abnormalities
Cellular proliferation
Leukocyte infiltration
Fibrinoid necrosis or karyorrhexis†
Cellular crescents†
Hyaline thrombi, wire loops
Tubulointerstitial abnormalities
Mononuclear cell infiltration
Chronicity index
Glomerular abnormalities
Glomerular sclerosis
Fibrous crescents
Tubulointerstitial abnormalities
Interstitial fibrosis
Tubular atrophy

* Each component is scored on a 0 to 3 scale (*see* text).
† Weighted by a factor of 2.

The patients were also divided into categories: high or low AI (AI >7 or ≤7) and high or low CI (CI >or ≤ 3), based on a previous study showing these values to be predictive of adverse outcome

The percent of glomeruli with crescents as a proportion to the total glomeruli obtained in a biopsy was also assessed.

b) Diabetic nephropathy

Patients with type 2 diabetes with proteinuria diabetic nephropathy were included. The biopsies were classified according to the Renal Pathology Society classification as follows:

Class I: Isolated glomerular basement membrane thickening. Basement membranes are greater than 430 nm in males older than age 9 and 395 nm in females. There is no evidence of mesangial expansion, increased mesangial matrix, or global glomerulosclerosis involving >50 percent of glomeruli.

Class II: Mild (class IIa) or severe (class IIb) mesangial expansion. A lesion is considered severe if areas of expansion larger than the mean area of a capillary lumen are present in >25 percent of the total mesangium.

Class III: At least one Kimmelstiel-Wilson lesion (nodular intercapillary glomerulosclerosis) is observed on biopsy and there is <50 percent global glomerulosclerosis.

Class IV: Advanced diabetic sclerosis. There is >50 percent global glomerulosclerosis that attributable to diabetic nephropathy.

c) Chronic renal allograft nephropathy or IFTA

Kidney transplant patients with chronic renal allograft nephropathy were included. The clinical diagnosis is usually suggested by gradual deterioration of graft function, as manifested by slowly rising plasma creatinine concentration, increasing proteinuria, and worsening hypertension. The pathology of patients were classified according to Baanf criteria including the following features:

- Interstitial fibrosis with tubular atrophy and the presence of this type of double-contour appearance are considered the most characteristic findings for chronic nephropathy within the Banff classification system
- The vessel walls are thickened by the subintimal accumulation of loose and then organized connective tissue, variable mononuclear cellular infiltration, proliferation of myofibroblasts, and disruption and duplication of the internal elastic lamina

In the absence of features of cyclosporine toxicity or c4d deposition.

Normal controls

Control subjects with preserved renal function (serum creatinine < 1.2 mg/dl) were recruited from patients undergoing nephrectomy for renal cell carcinoma. A tissue core was obtained from the renal cortex of the non-involved pole of the kidney, snapped frozen and stored in liquid nitrogen or fixed in paraffin.

Kidney tissues processing

Depending on the disease, two or three kidney biopsy tissue cores were obtained. In all cases, one core was were fixed in gliofixx and used for routine histology (Hematoxyln and Eosin), Silver, PAS, and another core was stored at -20 C for routine immunofluorescence studies. The fibrosis score of interstitial fibrosis (IF) was evaluated by Masson's Trichrome stain. IF was scored zero if none of the tubulointerstitial area was involved in blue/green staining, 1 if 1-25% of the area was involved and 2 if 26-50% of the area was involved. In transplant patients with CAN and selected patients with LN, an extra third core was obtained and immediately frozen in liquid nitrogen for gene expression studies.

The assessment of tissue was performed by a specialist nephropathologist, who did not have knowledge of the clinical outcome.

Kidney *sample preparations*

Paraffin embedded samples

Samples left over from routine was histological analysis was obtained. RNA was extracted using Quiagen kit according to manufacturer's instructions.

Frozen tissue samples

Frozen tissue stored at -80C from left over specimen from routing immunofluorescence was used for RNA extraction.

Laser catapult microdissection

LCM was performed with Laser microdissection with PALM MicroBeam, Zeiss.

Positive RNA control samples

To act as positive controls and to generate dose response calibration curve for RNA expression, RNA was obtained from cells and tissues likely to have high expression of gene products including PBMC and cancer cells.

PBMC

Peripheral blood mononuclear (PBMC) were obtained from forty milliliters of heparinized peripheral blood by density gradient centrifugation.

Malignant tissues

Frozen Tissues from renal cell carcinoma and nasopharyngeal carcinoma were obtained and tissue extracted for RNA.

RNA extraction and cDNA synthesis

Total RNA was isolated from the whole kidney biopsy core, kidney tissues from operating room, laser capture kidney tissues, malignant tumors or PBMC by silica gel-based membrane spin technology with DNase I treatment (RNeasy micro kit: Qiagen, Chatsworth, CA). cDNA was synthesized by iScripts cDNA synthesis Kit using MMLV-derived reverse transcriptase pre-mixed with an RNase inhibitor (Biorad, Philadelphia, USA) using a blend of oligo (dT) and random hexamer primers.

RNA extraction and cDNA synthesis. Total RNA was isolated from kidney tissue by silica gel-based membrane spin technology with DNase I treatment (RNeasy micro kit: Qiagen, Chatsworth, CA, USA). cDNA was synthesized with an iScripts cDNA synthesis

kit using MMLV-derived reverse transcriptase pre-mixed with an RNase inhibitor (Bio-Rad, Hercules, CA, USA) using a blend of oligo (dT) and random hexamer primers.

Quantification of renal gene expression

Different strategies were used for renal gene expression.

a) Retinoic acid genes

The expression levels of retinoic-related genes were determined by SYBR Green-based real time quantitative PCR (RT-QPCR). Quantitative RT-PCR was performed using the SYBR Green Master kit (SigmaAldrich) according to the manufacturer's instructions [31]. By this method, the threshold cycle (Ct) of target genes in each sample was normalized with housekeeping to account for variation in RNA amount. Ct is defined as the cycle number at which the fluorescent intensity generated by the tracer dye, which is released from the probe during DNA amplification, reaches a fixed limit of detection. Ct was determined at the exponential phase, and is inversely related to the initial mRNA amount. The mean Ct of triplicates was used.

The primers and probes were tested without reverse transcriptase to ensure that they did not amplify genomic DNA. A standard curve was generated for each gene using pooled cDNA. Conditions of the PCR reaction were optimized so that the amplification efficiency of the target genes and the endogenous reference gene were comparable across 3 log dilutions of pooled cDNA (11). The PCR conditions were individualized for each gene but were approximately: 50^{o}C for 2 min, 95^{o}C for 10 min, followed by 40 cycles at 95^{o}C for 0.15 min, and 60 ^{o}C for 1 min. The corresponding ΔCt was plotted against the log concentrations of template cDNA. The slope of the linear regression of 0.1 indicated that the amplification efficiencies were comparable.

Quantitative PCR was normalized using a housekeeping gene. The mRNA expression of target genes were calculated by using the ΔCt procedure with GAPDH as housekeeping gene for normalization where ΔCt values were calculated from:

$$\Delta Ct = Ct_{\text{target gene}} - Ct_{\text{housekeeping gene}}.$$

The expression of target genes in disease tissues were expressed as fold change of the value of a control subject, which were arbitrary set as 1

Relative expression to control tissues = $2^{-\Delta\Delta CT}$

Where $\Delta Ct = Ct_{\text{target gene}} - Ct_{\text{housekeeping gene}}$;

$\Delta\Delta Ct = \Delta Ct$ disease tissues – ΔCt control normal subject

b) Extracellular matrix genes (Transforming growth factor beta or connective tissue growth factor).

Instead of the SYBR green method used for retionoic metabolizing genes expression, Messenger RNA expression of renal fibrogenic gene expression was quantified by multiplex real-time quantitative polymerase chain reaction (RT-PCR) using Applied Biosystems to enable more accurate quantification although this was more costly. Briefly, a fast Start Universal Probe Master kit (Applied Biosystems, Foster City, CA, USA). cDNA was amplified using an iScripts cDNA synthesis kit (Bio-Rad) in a 96-well plate. Multiplex quantitative PCR was performed using target and housekeeping genes in the same well. Primer and probe sequences for CTGF, TGF-β1 and collagen I are shown in Table I. The mRNA expression of the target genes was calculated by using the ΔCt procedure with VIC-TAMRA-labeled GAPDH or CIC-TAMRA-labeled cyclophilin (Applied Biosystems) used as a housekeeping gene for normalization as in the above method for SYBR green. Conditions of the PCR reaction were optimized so that the amplification efficiency of target genes and the endogenous reference genes were comparable across 3 log dilutions of pooled cDNA for each pair of target and housekeeping genes and results calculated by $\Delta\Delta$CT method as above.

Primers

a) Retinoic acid related genes

Table 3: Retinoic acid related genes- primer sequences

	ACCEssion	5-3 sequence	Tm
RALDH2#1 (122 bp)	NM_003888	F-CACTGAGCAGGGTCCCCAGATTG R- ACCCCTTTCGGCCCAGTCCT	58.9 59.4
RALDH1#5 (78bp)	NM_000689.4	F-GCTTCCGAGAGGGGGCGACTA R- CTCCATTGTCGCCAGCAGCAGA	59.5 59.5
RALDH3#1 (117bp)	NM_000693	F- ACCGGCTCCACAGAGGTTGGA R- GTCAGCGTCCGCACACACGA	59.5 60
CYP26A1#1 (75 bp)	NM_000783	F- CGGCTGGACATGCAGGCACTA R- TGCACTGGCCGTGGTTTCGT	59.2 59
CYP26B1#2 (76bp)	NM_019885	F- TGGCTACCGGCGGGGCATTC R- CTGCAGCTTCTCCCGGATGGC	61.5 59
RARA1#5 (71bp)	NM_000964	F- AGAGGGCTTCCCCGGTTCTCC R- CCTGTGATGCTGCTCAGGTGTGT	59.6 59.1
RARA2#1 (114 bp)	NM_001024809	F- GAACCGGGCCTGTTTGCTCCC R- CTGCTCTGGGTCTCAATGGAGTGG	60.2 58.8
RARB1#2 (108 bp)	NM_000965.3	F- CGGGCCTGTTTGCTCCCAGAG R- GCTCTGGGTCTCAATGGAGTGG	59.7 56.7
RARB2#1 (140bp)	NM_016152.3	F- TCTGGGCACCGTCGGGGTAG R- TCAATTGCATTTTCCAGGCTTGCTC	60 57
RARy#1 (76 bp)	NM_000966.5	F- TGCGGAGACCGCATGGACCTG R- GCCTCAGGGCTTCCAGCAGTG	61.1 59.7
RXRa#1 (122bp)	NM_002957.4	F- TCCTGCCGCTCGATTTCTCCAC R- CTGTCCCGGGGAGCCGATGC	59 61.6
RXRb#1 (150 bp)	NM_021976.3	F- GGTAGCGGCAGCAGCCCAAA R- CCAGCCTGCCCGCAGCAATA	60 60
RXRc#1	NM_006917.4	F- GGGCCTGTCCAACCCCTCTGA R-TTGGCAAACCTGCCTGGCTGT	59.8 60.1

b) Extracellular matrix genes

Table 4: Primer and probe sequences for ECM genes

Genes	Accession no.	Primer (Forward/Reverse)	Product length (bp)
TGF-β1	NM_000660.4	F: 5'-CCAGCATCTGCAAAGCTC R: 5'-GTCAATGTACAGCTGCCGCA Probe: 5'- (6-FAM)ACACCAACTATTGCTTCAGCTCCACGGA (Tamra)	100
COL1	NM_000088.3	F: 5'-CCTCAA GGCTCCAACGAG R: 5'-TCAATCACTGTCTTGCCCCA Probe: 5'- (6-FAM)ATGGCTGCACGAGTCACACCGGA (Tamra)	117
CTGF	NM_001901.2	Assay-by-design (Applied Biosystems: ABI) Assay ID: Hs00170014m1 Probe: 5'-(6-FAM)AGAACATTAAGAAGGGCAAAAAGTG (Tamra)	60

RESULTS

A) Laser catapult microdissection

Study 1: Gene expression in Laser catapult microdissected glomeruli

Because of potential variations in gene expression within different parts of the kidney, we wanted to develop a method for quantifying gene expression in the glomeruli separately from the tubulonintersitial compartment,

First we tested different staining protocols that would allow us to distinguish glomeruli and tubules while at the same time was able to preserve RNA integrity in frozen kidney tissues obtained in the OR. We were successful in separating glomeruli from the rest of the kidney biopsy tissues using laser catapult microdissection (Figure 1).

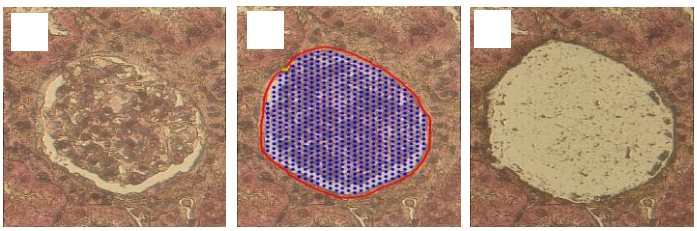


Figure 1: Laser catapult microdissection (LCM) procedure.

A glomerulus (A) is selected bay drawing on the perimeter (B). Activation of the laser pulse beams the selected glomerulus into the receptacle for RNA collection. In this way, the glomerulus can be separated from the tubulointersitial compartments.

We tested RNA extraction methods initially on frozen cancer tissues obtained from patients. This was used because of greater availability of tissues. Once we had developed the ability to extract RNA from histologically-stained tissues, we tested the methods on normal kidney controls. We were able to extract RNA from kidney tissues cortex. The integrity of RNA was tested using Agilent 'Lab on a chip'method (nano-chip) (Figure 2). The RNA obtained from kidney whole cortex from the operating room appeared to be intact, but the amounts were reduced by histological staining and LCM.

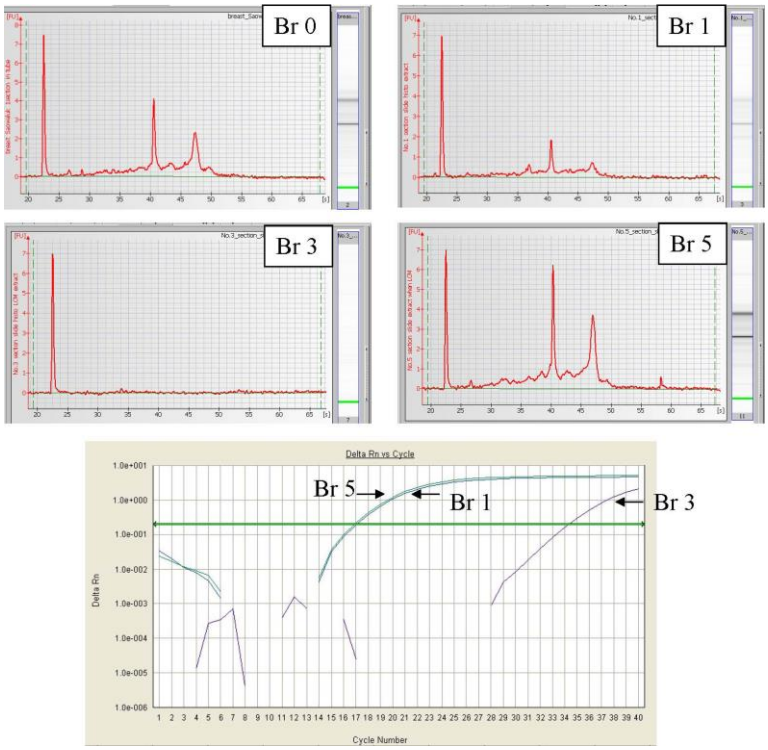
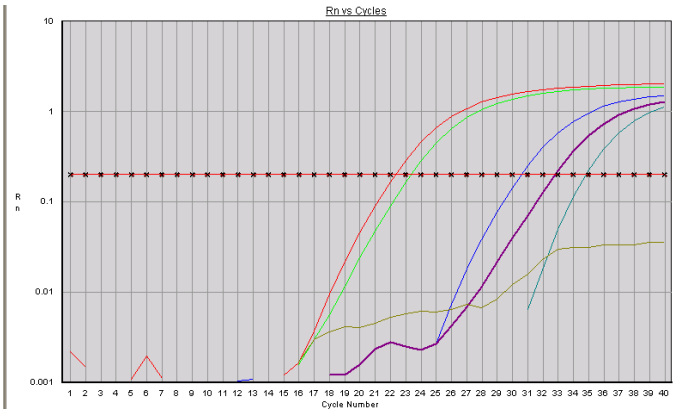


Figure 2: RNA from kidney cortex unstained and after staining with Histogene LCM frozen Section Staining kit. RNA analyzed with Eukaryote total RNA nano LabChip Kit (Agilent), and real-time RT-PCR for housekeeping gene, GAPDH. Br 0, Unstained, unsectioned frozen kidney cortex; Br 1, whole section scraped from slide after staining; Br 3, An small area of slide obtained by LCM after staining; and Br 5: whole section on slide scraped from unstained slide. The amount of RNA could not be measured by the nono Labchip in the LCM sample, but gene expression for GAPDH was still quantifiable.

Once we had developed the ability to extract RNA from histologically-stained tissues, we tested the methods on frozen normal kidney controls. We were able to extract RNA from kidney tissues. The integrity of RNA was tested using Agilent 'Lab on a chip'method. We are successful in separating glomeruli from the rest of the kidney biopsy tissues using laser catapult microdissection. Because the amount of RNA from kidney tissues obtained by LCM was too small to be quantitated using the nano-chip, we proceeded to identify optimal conditions for the kidneys using real time RT-PCR for

house keeping genes, GAPDH or cyclophilin A (Figure 3). Thus we were able to obtain quantifiable gene expression from areas as small as a cross-section from 3 glomeruli.



F*igure 3. Real time RT-PCR showing GAPDH mRNA expression from normal kidney frozen section obtained by scraping or after LCM using different staining protocols. CT threshold cycle. (1) Scraped whole section after H&E (with xylene) CT 22.28; (2) Scraped whole section after H&E (without xylene) CT 23.30 cycles; (3) LCM of 300,000 uM^2 area after H&E (with xylene)CT 30.54 cycles; (4) LCM of 300,000 uM^2 area after H&E (without xylene) CT 32.78 cycles; (5) 3 glomeruli from one section after H&E (with xylene) CT 34.79 cycles*

Laser catapult microdissection in archived patient samples

Initially, we also tried to separate glomeruli from tubulointersitium using laser catapult microdissection. 5 x 10um thick sections of Glomeruli and tubulointersitial area were separated into separate aliquots. However, the retinoic acid genes were expressed in low quantities and the archived sample RNA quality was of low to medium quality and expression levels were too low for accurate quantification. After repeated modifications in protocol, we abandoned use of laser captured glomeruli and focused on whole tissue samples.

Conclusions: Although LCM and staining procedures were effective in evaluating separate glomerular gene expression of GAPDH, the gene expression levels of retinoic acid genes were too low to be quantified. The LCM technique was abandoned. All future studies were then performed in whole renal cortex tissues.

B) Retinoic acid studies

Study 2: Primer testing and expression

All primers were tested and developed using cDNA derived from PBMC or malignant tissues using SYBR green. Amplification Conditions/time were adjusted to obtain optimal amplification. No RT was performed to exclude genomic DNA amplification. Melt curve analysis and template variation was performed to obtain amplification efficiency of 0.9-1.3 as in example below (Figure 4)

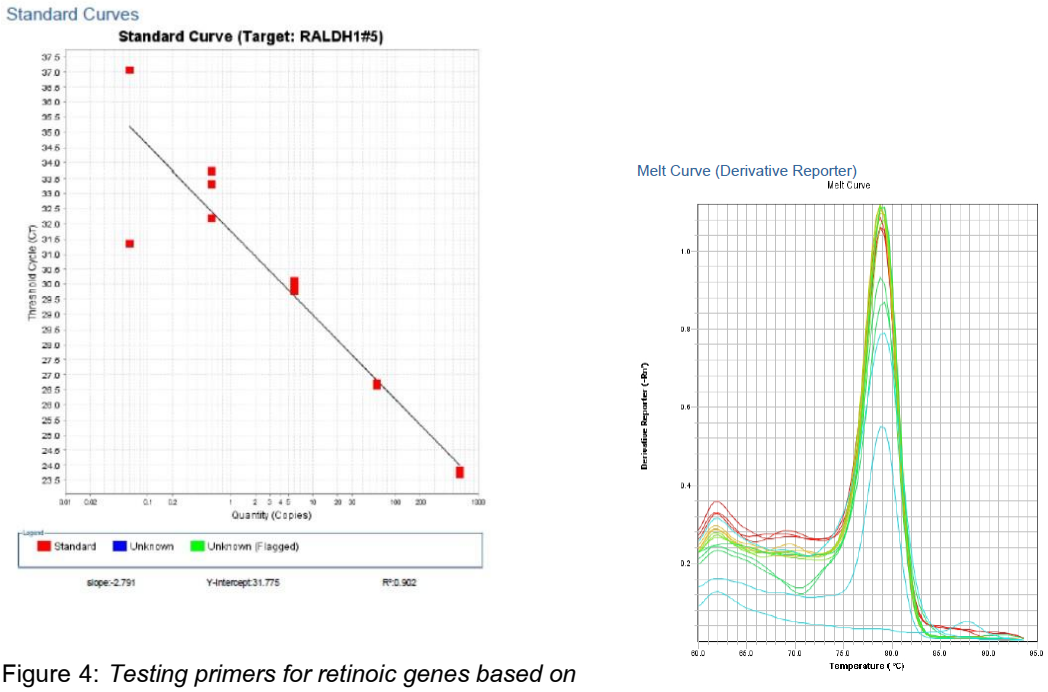


Figure 4: *Testing primers for retinoic genes based on SYBR green method. A) Standard curve and B) melt curve*

Similar experiments were performed for other primer pairs in Table 1. For primers that demonstrated multiple bands or could not achieve appropriate efficiency, new primers were designed. The final primers used are shown in Table 1.

Conclusions: Primers based on SBR green method were developed for retinoic acid related genes and house-keeping genes.

Study 3: Endogenous Retinoic acid metabolizing pathway in lupus nephritis and diabetic nephropathy

The gene expression of endogenous retinoic acid synthetic enzymes (RALDH1, 2, 3) and degradation enzyme (CYP26A, CYP26B) were studied. Expression was compared between control subjects and lupus nephritis and between control and patients with moderate to advanced diabetic nephropathy.

Patient characteristics

Controls

Table 5: Characteristics of controls

ID	Age	sex	eGFR
NKOR_7	30	F	117
NKOR_8	54	M	89
NKOR_9	63	M	73
NKOR_13	66	F	102
NKOR_16	55	F	66
NKOR_17	66	F	69
median	59		81
(25,75)	(54,65)		(70,99)

Diabetic nephropathy

Table 6: Characteristics of patients with diabetic nephropathy

ID	Age	Sex	eGFR	Proteinuria	Class
1	43	M	5.9	2600	4
2	50	M	30	1500	4
3	52	M	45	1200	3
4	29	F	35.6	2995	3
	33	M	42	3200	3
median	43				3
(25,75)	33,50				3,4

SLE patients

Table 7: Characteristics of patients with lupus nephritis

ID	Age	Sex	eGFR	Proteinuria	Class	AI	CI
1	17	M	103.1	3000	4	17	2
2	32	F	100	2500	4	10	2
3	10	F	159.9	3010	3	5	3
4	47	F	61.1	504	4	8	4
5	22	F	161.3	1500	4	10	0
6	20	F	73.4	3000	3	9	1
7	47	F	70	2500	3	6	3
8	32	F	128.5	1400	3	4	2
9	13	F	77.6	11000	4	11	0
10	29	F	124.8	2210	4	8	5
11	42	M	42	2100	4	9	3
12	15	F	89	2500	5	0	2
13	18	M	70	2800	5	0	3
14	31	F	126.14	2700	5	0	2
median	25.5				4	8	2
(25,75)	(17,32)					(4.,10)	(2,3)

Gene expression of retinoic genes between Control vs Lupus

Initial comparisons in controls (n=6) vs lupus nephritis (class 3-5) (n=14). The relative expression are shown in table 8 and figure 5

Table 8: Relative expression of retinoic genes in LN vs controls.

	LN (n=14)	Control (n=6)	P=
RALDH1	.1093 (.0938, .1244)	.8392 (.5396, 1.1321)	**0.000**
RALDH2	.6030 (.2994, 1.0481)	1.4727 (.6964, 3.0704)	0.051
RALDH3	1.2482 (.3433, 1.9312)	3.3100 (.9800, 6.6596)	0.062
CYP26A	.2313 (.1014, .3202)	1.0905 (.8541, 1.7597)	**0.00**
CYP26B	.8082 (.1435, 2.0236)	1.6408 (.8946, 6.9450)	0.151

The expression of RALDH1, and CYP26A was lower in LN vs control and RALDH2 and RALDH3 tended to be lower.

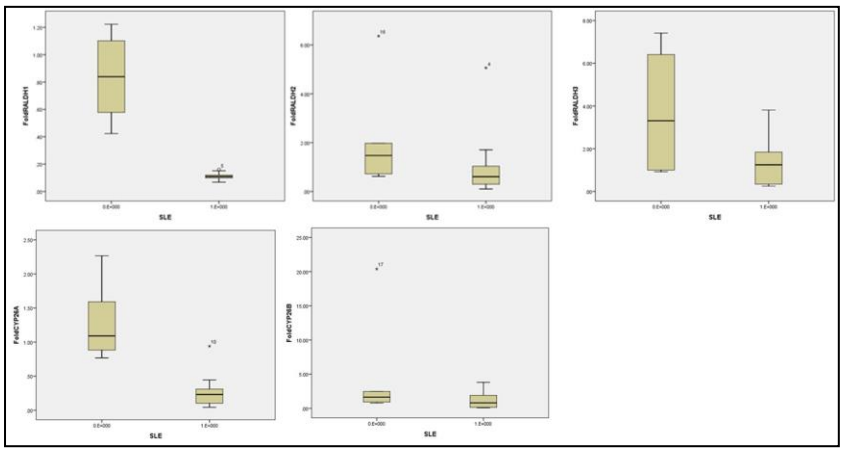


Figure 5 Relative expression of endogenous retinoic acid gene in LN vs Control

Ratio of renal synthetic (RALDH1,2,or 3) to Renal metabolizing (CYP26A or CYP26B) were not altered between LN compared to controls.

Retinoic acid gene expression between Control vs Diabetic nephropathy

Gene expression was evaluated in patients with advanced class 3 or 4 diabetic nephropathy.

Table 9: Retinoic gene expression in controls vs diabetic

	DM (n=5)	Control (n=6)	P=
RALDH1	.6820 (.4148, .9022)	.8376 (.5398, 1.1335)	NS
RALDH2	1.1981 (.9267, 1.7917)	1.4761 (.6986, 3.0722)	NS
RALDH3	3.7599 (2.5301, 5.7371)	3.3107 (.9800, 6.6812)	NS
CYP26A	1.2406 (.8509, 1.4299)	1.0903 (.8537, 1.7605)	NS
CYP26B	1.1827 (.5060, 7.2469)	1.6408 (.8982, 6.9545)	NS

In contrast to patients with LN, the gene expression level of diabetic kidneys and controls were not different (Table 9 and Figure 6)

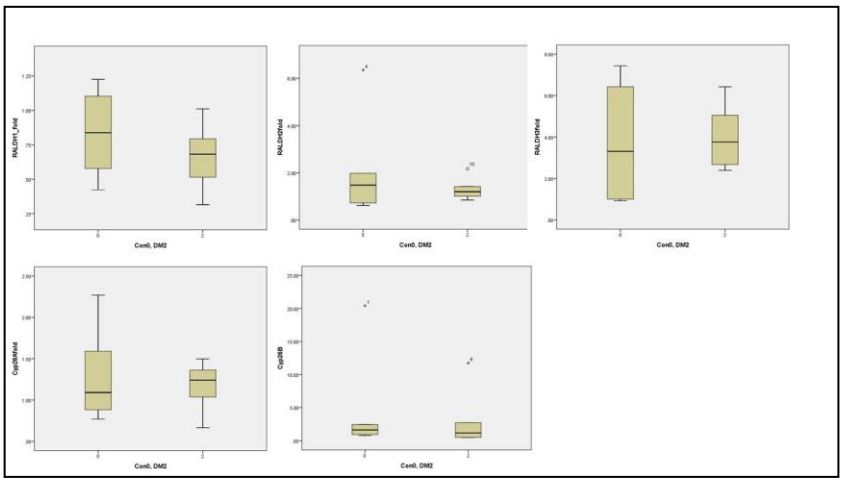


Figure 6: Relative expression of retinoic acid metabolizing genes in DM compared to controls (all p=NS)

Ratio renal synthetic (RALDH1,2,or 3) to Renal metabolizing (CYP26A or CYP26B) were not altered between DM vs control.

Conclusions: RALDH1, CYP26A were decreased in LN compared to controls whereas no differences were observed in DN. The ratio of renal synthetic genes and renal metabolic genes were unaltered in both LN and DN compared to controls.

Study 4: Correlations between retinoic gene expression and renal histology in LN

We evaluated the expression of retinoic metabolizing genes and correlated to renal pathology in patients with lupus nephritis.

Table 10: Histology and relative gene expression values for LN subjects (n=22)

	AI	CI	RALDH1	RALDH2	RALDH3	CYP26A	CYP26B	TotGlom	%Cresc
Median	4.5 (0,9)	2.5 (2-4)	0.9 (0.8-1.0)	3.5 (1.4-5.3)	2.4 (1.0-4.6)	2.5 (1.1-3.4)	7.0 (1.2-17.5)	12 (8-21)	0 (0-9)

Class

i) We compared gene expression for different classes (class 3+4 vs class 5 only) or classes with 5 (5 alone or 3 +4+5 vs 3+4 only) Figure 7

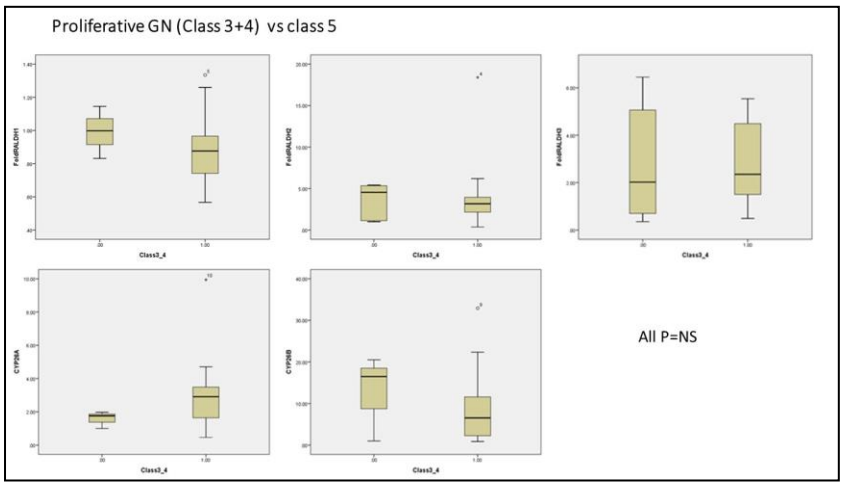


Figure 7. Renal Retinoic acid genes expression in proliferative (class 3 or class4) vs class 5

ii) Any class 5 (class 5 and class3+5 or class 4+5 vs Class 3or 4)

We compared patients with any membranous change (class 5) to patients with pure proliferative (Class 3 or 4)

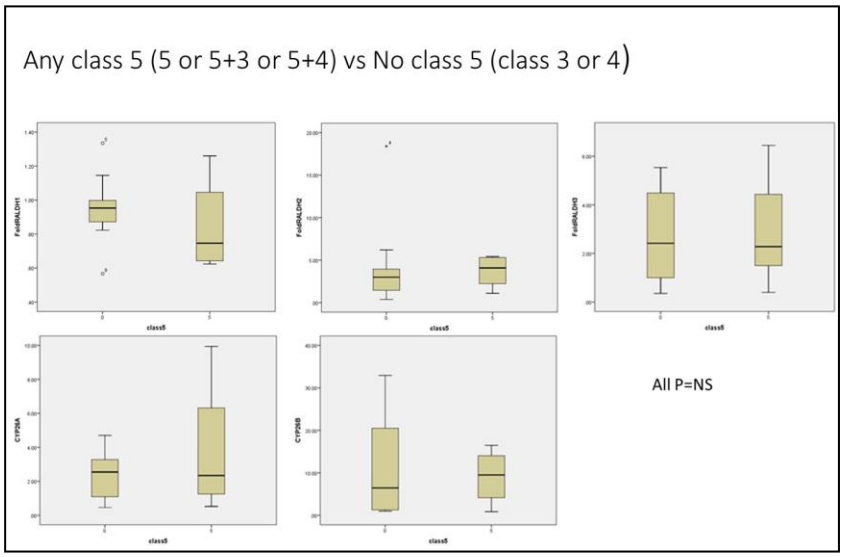


Figure 8. Renal Retinoic acid genes expression in patients with any membranous component (class 5 with or without class 3 or 4) vs pure proliferative (class 3 or class4)

Activity or chronicity index

i) Activity index

We compared patients high activity index (High>7) to patients with lower activity disease

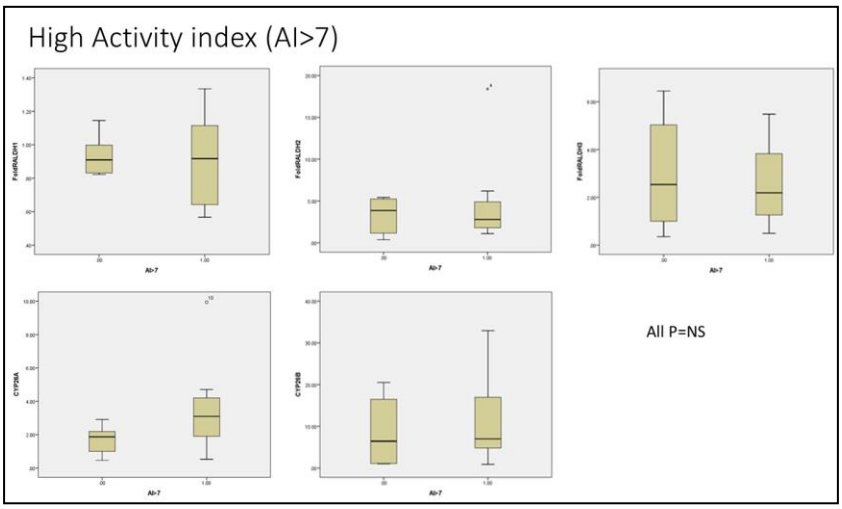


Figure 9 Renal Retinoic acid genes expression in patients with high vs low activity index

ii) Chronicity index

We compared patients high chronicity index (CI>2) to patients with lower chronicity disease

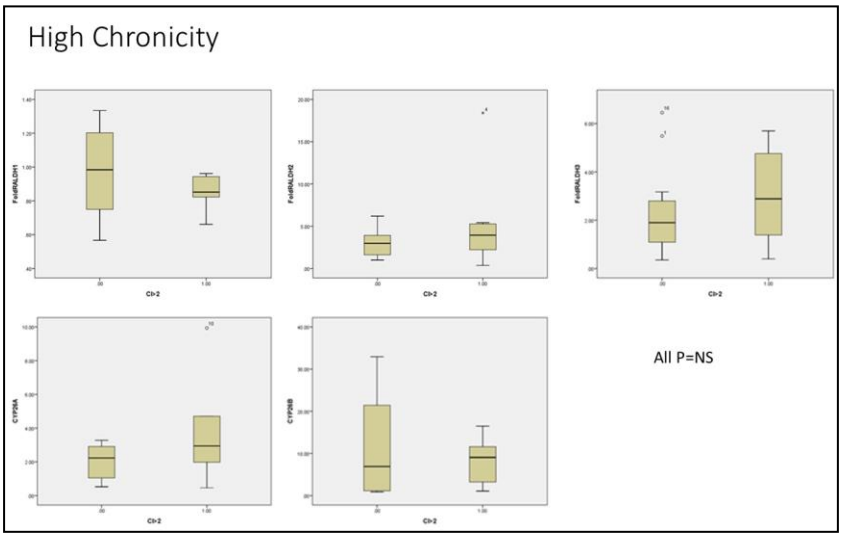


Figure 10 Renal Retinoic acid genes expression in patients with high vs low chronicity index

iii) Any crescent

We compared patients with crescents to those without crescents

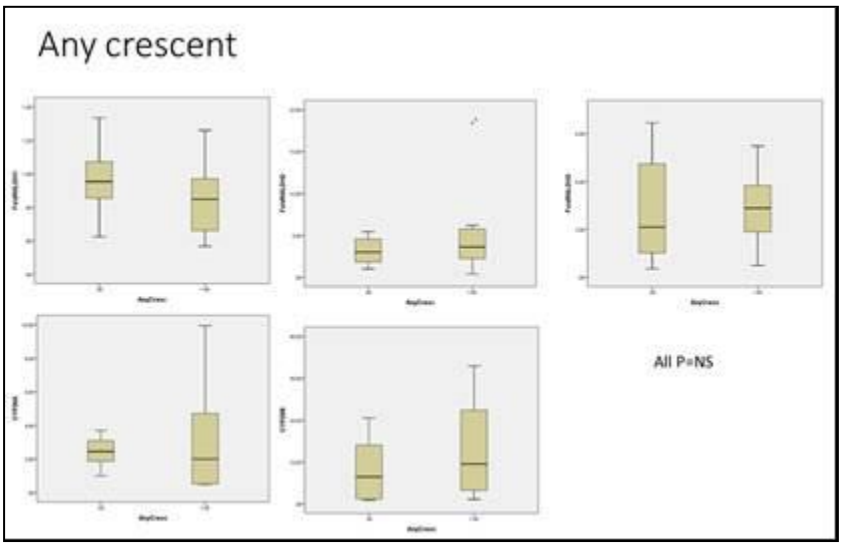


Figure 11. Renal Retinoic acid genes expression in patients with crescents vs those without chronicity index

When 10% crescent cut off is used, results are the same.(p=NS)

Ratio

The ratio of retionoic synthesizing enzymes (RALDH1, RALDH2, or RALDH3) to metabolizing enzymes (CYP26A or CYP26B) were correlated with renal pathology parameters. The ratios of RALDH2 or RALDH3 to CYP26a were significantly elevated in patients with 10% crescents. There were no other differences for other gene ratios or other pathological parameters, (data not shown).

Ratio RALDH2:CYP26a: Cresecent >10% 2.71(2.09-3.76) vs Crescent <10% 0.73(0.54-1.07), p= 0.002

Ratio RALDH3:CYP26a: Cresecent >10% 2.3687 (2.1025-3.6023) vs Crescent <10% .7311 (0.2019-1.0000), p=0.014

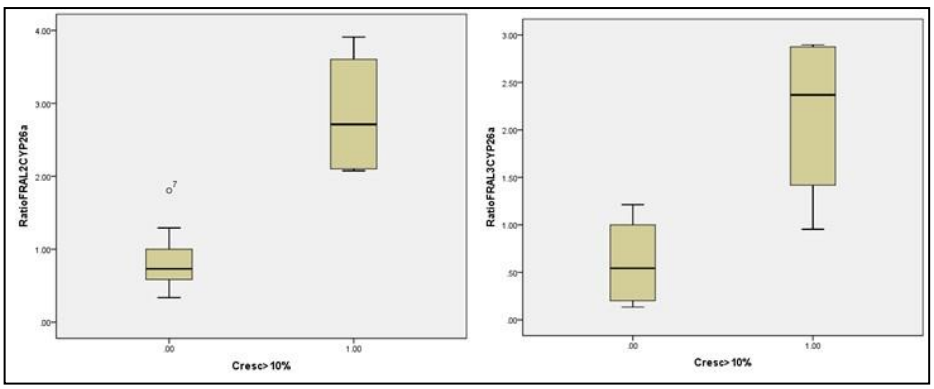


Figure 12: The ratio between RALDH2 or RALDH3 and cyp26 in patients with >10% vs those with fewer crescents

Conclusions: There were no differences in gene expression of retinoic synthesizing or metabolizing enzymes among different pathological sub-groups. The ratios of

RALDH2:CYP26a or RALDH3:CYP26b were higher in patients with high number of crescents. This suggests that endogenous retinoic availability might be increased in crescentic patients.

Study 5. Retinoic receptor genes

Since the effects of kidney diseases on endogenous retinoic metabolism appear to be minor, we decided to evaluate the modifications of retinoic pathway function by evaluating renal retinoic acid receptor expression by real time RT-PCR expression analysis.

First we designed the primers as shown in Table similar to study 4. Each primer pair for each receptor gene was tested for amplification efficiency. However, because of a problem with a PhD student responsible for the project, all primers, and reagents were lost before studies could be performed on kidney disease tissues. Data from the initial evaluation phase of the antibodies were also lost.

Study 6: Retinoic acid synthesis antibodies immunostaining.

To further assess the renal expression of retinoic acid synthesis, we also attempted to evaluate renal gene expression of RALDH1, RALDH2, RALDH3, and CYP26A by immunohistochemistry in paraffin-fixed tissue. After initial studies in developing the conditions for immunohistochemistry, we were unable to complete this part of the project due lack of co-investigator time due to reassignment of nephropathologist time to hospital management and change of research policy within the Department of Pathology. Problem with PhD student responsible for the project also led to degradation of all antibodies.

B. ECM-related gene expression in kidney diseases

In this series of studies, we evaluated the relationship of renal ECM-related genes (Transforming growth factor beta and connective tissue growth factor) and kidney function at baseline and subsequent function decline in LN and in CAN. We also evaluated the relationship of change in renal gene expression and change in renal function decline in kidney diseases.

Study 7: Extracellular matrix genes in LN

Background

In this study, we set out to evaluate whether CTGF mRNA expression correlates with baseline renal function, and whether it is able to predict loss of renal excretory

function in patients with LN. Furthermore, we examined whether renal CTGF mRNA correlates with TGF-β1 and collagen I expression in vivo in patients with LN. In LN, the presence of established interstitial fibrosis often implies poor outcome, but when such processes are finally demonstrable on routine histology, the process is already largely irreversible. Findings that renal CTGF expression predicts renal outcome may pave a way for therapeutic interventions aimed at decreasing CTGF and preventing renal failure.

Methods

We evaluated expression of renal CTGF and TGF-β1 mRNA in patients with LN using real-time multiplex RT-PCR by ABI probe and primer system using a separate core of biopsy stored at -80C to obtain accurate quantification of gene expression since SYBR green technology may not be as accurate.

Patients were also divided by severity into those with high GFR and low GFR using a cut-off eGFR of <60 ml/ min/1.73 m2 according to the K/DOQI guidelines in which GFR <60 ml/min/1.73 m2 constitutes those with moderate to severe CKD.

Rate of GFR change (ΔGFR). This was calculated by the slope of the GFR plot from biopsy to one year following biopsy. We divided patients into those with a decline in GFR (negative values) and those with no decline (positive values).

Outcomes

The levels of CTGF gene expression were correlated with baseline GFR and other clinical parameters, and with ΔGFR. In order to examine the potential mechanisms for CTGF actions, the levels of CTGF expression were also correlated with the expression of collagen I and TGF-β1.

Results

Patient characteristics

Patient characteristics are summarized in Table11. Of the 39 patients included in the study, the majority were women (n=38).

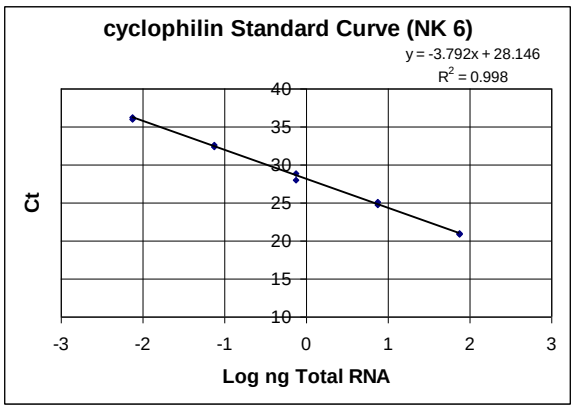
Table 11: Patient characteristics

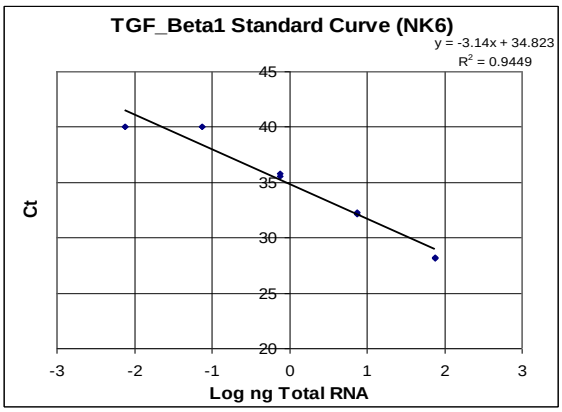
Age at initial biopsy (years)	31.4±10.6
Time from disease onset to biopsy (years)	4.6±4.9
Female patients (%)	97.4
Prior immunosuppressive agent (%)	87.2
Systolic BP (mmHg)	130.3±13.5
Diastolic BP (mmHg)	80.5±10.2
Hypertensive (BP >140/90) (%)	53.9
Serum albumin (g/l)	29.7±7.4
Creatinine (mg/dl)	1.7±2.3
eGFR (ml/min/1.73 m^2)	89.2±39.2
eGFR <60 ml/min/1.73 m^2 (%)	20.5
Urine protein (g/day)	2.5±4.1
Kidney biopsy	
RPS/ISN class n (%)	
II	7 (17.9)
III	3 (7.7)
IV	18 (46.2)
V	9 (23.1)
VI	2 (9.0)
Activity index	4.2±5.3
Chronicity index	2.0±1.7
Outcome	
GFR at 1 year	81.3±38.0
Rate of change of GFR	-7.94±31.13

Of the 39 patients, 8 (20.5%) had low GFR (GFR <60ml/ min/1.73 m2) and 31 (79.5%) had high GFR (GFR ≥60 ml/ min/1.73 m2). Proteinuria was 2.5±4.1 g/24 h. Urine RBC was 2.7±2.3/HPF.

Primer and probe performance

The primer and probes (Table2) development and efficiency are shown in figure 13





(c)

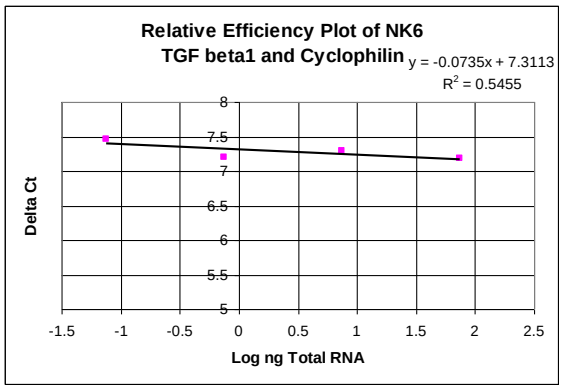


Figure 13. Relationship between template total RNA from kidney tissue and real time RT-PCR threshold cycle (CT) for (a) cyclophilin and (b) TGF_Beta1; and (c) relative efficiency plot (threshold cycle difference- delta CT) for multiplex RT-PCR for cyclophilin and TGF_Beta1.

i) CTGF gene expression and correlations with TGF-β1 and collagen I

In LN subjects, the mRNA expression of CTGF and COL1 in LN tissues were positively correlated (R=0.370, p=0.022) (Fig. 1A). In addition, the mRNA expression of CTGF and TGF-β1 in LN tissues were positively correlated (R=0.363, p=0.025) (Fig. 14).

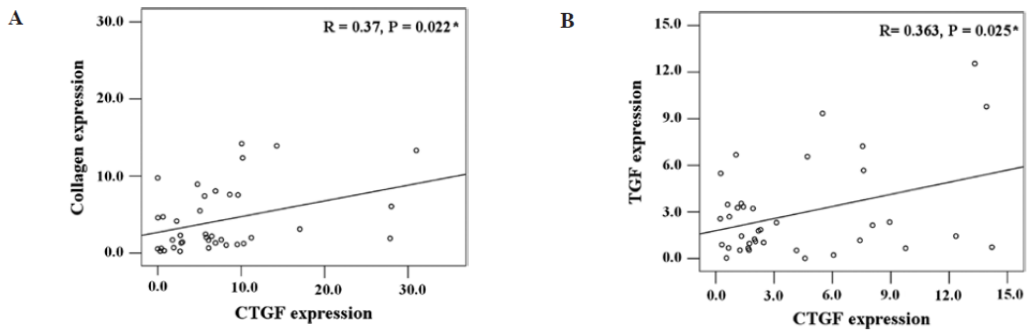


Figure 14: Correlations between renal CTGF mRNA expression and other fibrogenic genes in lupus nephritis (n=39). mRNA expression of (A) collagen I and (B) transforming growth factor (TGF)-β.

ii) Renal CTGF mRNA expression and baseline clinicopathological parameters

There were no significant correlations between CTGF and the degree of proteinuria or hematuria. There were no significant differences in CTGF expression between classes and no correlations with AI or CI.

CTGF expression correlated inversely with GFR at baseline (R=−0.41, p=0.009) (Fig. 15).

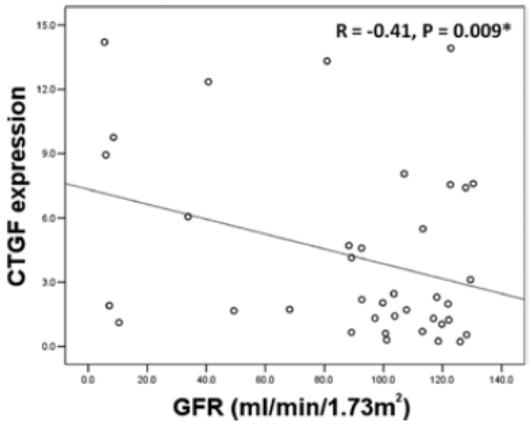


Figure 15: Correlations between renal CTGF mRNA expression and glomerular filtration rate in lupus nephritis (n=39).

When patients were divided into severity groups, CTGF expression was significantly higher in patients with moderate to severe CKD compared to those with milder forms of CKD (low GFR 4.92±4.34 vs. high GFR 1.52±1.94, p=0.005) (Fig.16).

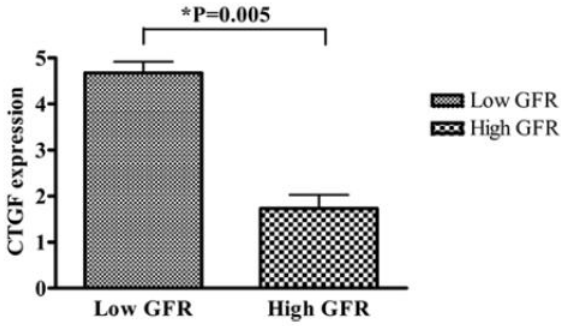


Figure 16. Renal CTGF mRNA expression in patients with mild chronic kidney disease (GFR ≥60 ml/min/1.73 m2) vs. moderate or severe chronic kidney disease (GFR <60 ml/min/1.73 m2).

iii) Renal CTGF mRNA expression and rate of decline in GFR

In patients with LN, the rate of change in GFR was −7.94±31.13 ml/ min/1.73 m2 per year. Overall, 29 cases exhibited a decline in GFR. CTGF expression was significantly higher in patients with a decline in GFR (decline 5.19±4.46 vs. no decline 1.79±1.97, p=0.010)

Conclusions: CTGF is tended to be increased in LN and correlated with baseline GFR and decline in GFR.

Study 8: Extracellular matrix genes in chronic allograft nephropathy

Background

Chronic allograft nephropathy characterized by interstitial fibrosis and tubular atrophy (IF/TA) is a leading cause of graft loss. Current recommendations for the treatment of IF/TA include reduction of calcinerin inhibitors and substitution of immunosuppressive therapy with mycohphenolate mofetil (MMF) or with sirolimus (SIR), an mTOR inhibitors. Previous animal studies have shown that MMF and SIR can cause decrease expression of TGF in cultured tissues, but the data in human is limited. This study examine the relationship between changes in drug regimen in patients with IF/TA and relates the changes in gene expression for CTGF and TGF-B and subsequent changes in kidney function.

Methods

CTGF or TGF mRNA expression were quantitated in biopsies at baseline and at 6 months after change in drug regimen to either MMF or SIR. Gene expression was evaluated by multiplex qRT-PCR using ABI system. Gene expression at first biopsy was related to baseline kidney function, pre-existing rate of decline in kidney function. A change in gene expression in individual patients were related to and subsequent decline.

Results

Patients

Fifteen patients with CAN were studied. After diagnosis of CAN biopsy, patients had drug regimen changed to either MMF or SIR. Follow up time before treatment was 80.7 ± 47.2 months. Follow up time after treatment was 18.3 ± 4.9 (SIR vs MMF, p =NS).

i) Kidney function

Table 12: Kidney function in CAN patients at first and second biopsies (n=15)

	All	MMF	SIR	p MMF vs SIR
GFR1	38.5 ± 10.6	42.4 ± 12.7	35.1 ± 7.5	NS
GFR2	42.9 ± 16.1	47.9 ± 20.5	38.5 ± 10.7	NS
Absolute change in GFR	3.27 ± 0.63	+ 5.52 ± 11.37	+3.39 ± 7.0	NS
Rate of decline in GFR before intervention	-0.21 ± 0.13	-0.2025 ± 0.08	-0.221 ± 0.17	NS
Rate of decline in GFR after intervention	+0.056 ± 0.36a	+0.160 ± 0.37a	-0.03 ± 0.35a	NS
Change in rate of decline of GFR	0.2689 ± 0.37	+ 0.363 ± 0.379	+0.187 ± 0.373	NS

a = p<0.05 comparing before to after

GFR was 38.5 ± 10.6 at hospital discharge and 38.5 ± 16.1 at 3 months. Serum creatinine at biopsy was 1.83 ± 0.39 mg/dl and at last follow-up was 1.85 ± 0.51. GFR1 (before intervention) tended to increase from 38.5 ± 10.6 to GFR 2 (after intervention): 42.9 ± 16.1 (p= 0.080) (Figure 17). Between SIR and MMF, GFR1 and GFR2 or absolute change in GFR were not significantly different.

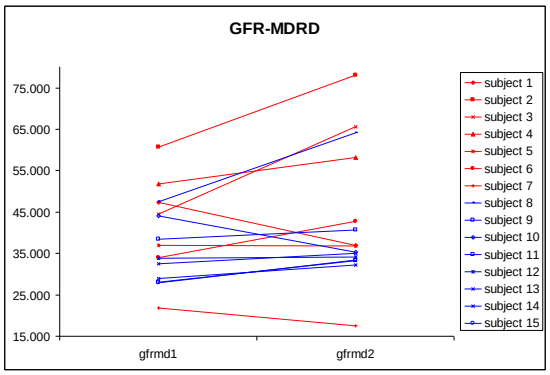


Figure 17. GFR of subjects before and after treatment

The rate of change in GFR (figure 18) before intervention was -0.21 ± 0.13 (ml/min/month) whereas the rate of change of GFR after intervention +0.056 ± 0.36 (p= 0.014). Overall, the rate of change of GFR improved + 0.2689 ± 0.37 ml/min/per year. The rates of change in GFR before or after intervention were not different in MMF vs SIR (p= NS).

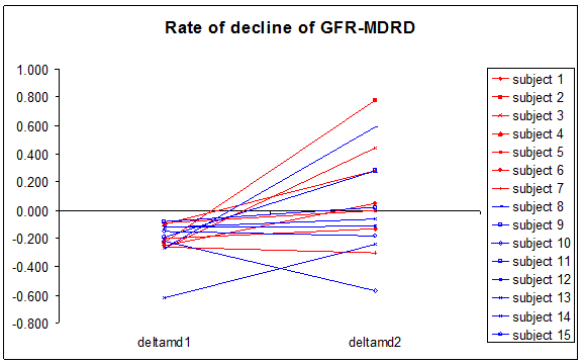


Figure 18. Rate of decline of subjects before and after treatment

ii) Gene expression

CTGF

Figure 19a shows individual CTGF gene expression values. Using 1st Bx sample ID 1 as 1, the relative expression of CTGF of Bx1 for the whole group was 2.26 ± 1.55 whereas the relative expression of CTGF of Bx2 was 1.70 ± 0.96 (p =NS). For both Bx1 or Bx2, the relative expression of CTGF for the MMF group was not different compared to SIR group (p=NS).

TGF-β

Figure 19b shows individual gene expression. Using 1st Bx sample 1 as 1, the relative expression of TGFb of Bx1 for the whole group was 3.25± 2.32 whereas the relative expression of TGFb of Bx2 was 2.63 ± 2.49 (p =0.609). The relative expression of TGF in BX1 or Bx2 were not different for the MMF group vs SIR group (p= NS).

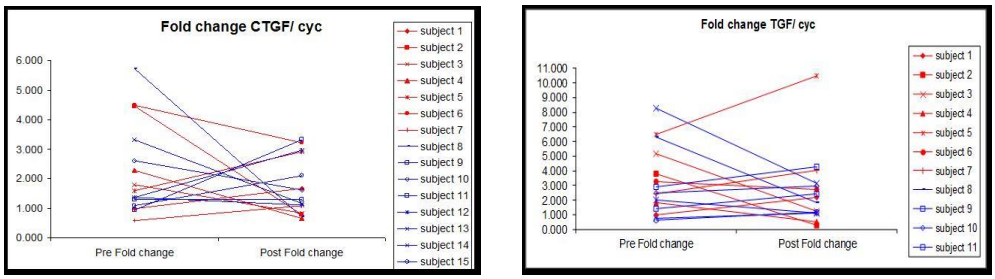


Figure19. Fold change of CTGF and TGF b gene expression before and after treatment

With Biopsy 1, sample 1 set as 1, when all biopsies are considered, there is good correlation between mRNA expression of CTGF and TGF (SL +0.256, R2 19.2, p= 0.009 at baseline.

iii) Change in gene expression in Individuals from biopsy 1 to biopsy 2

To compare the effects of therapy on gene expression in each individual, the gene expression of Bx1 was set as reference for each individual.

The relative fold change was 1.18 ± 0.97 for CTGF and 1.05 ± 0.70 TGFb for all subjects (p= 0.058). The relative change for CTGF or TGFb were not different for MMF vs SIR (p= NS). Individually, there was a strong correlation between changes in TGF and CTGf expression (p = 0.005) Figure 20

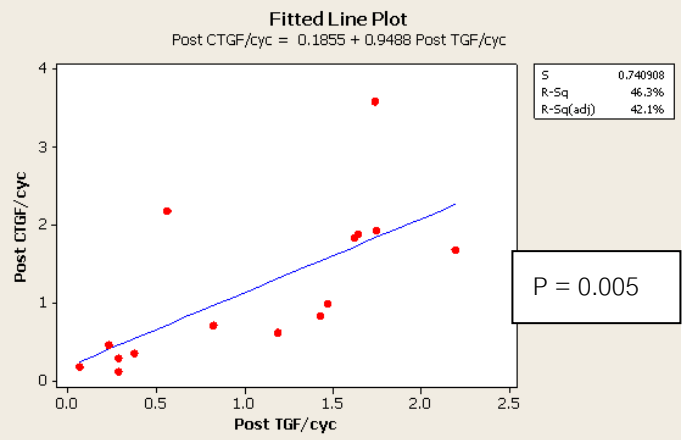


Figure 20. Correlations between change in CTGF and change in TGF-beta

iv) Gene and kidney function correlation

Gene expression at biopsy *1*

CTGF gene expression at Bx 1 tended to correlate positively with GFR1 (+0.06 , R2= 16.6 p = 0.074), but, there is no correlation between TGFb gene expression at Bx 1

Gene expression at Biopsy 2

There was no correlation between CTGF gene expression at Bx 2 and GFR2 or rate of decline of GFR2.

v) Changes in gene expression from biopsy 1 to biopsy 2 and changes in kidney function

Change in CTGF gene expression from Bx 1 to Bx 2 correlated negatively with the change in rate of change of GFR after intervention (SL=-1.36, R2 = 27 p = 0.046). Figure 21.

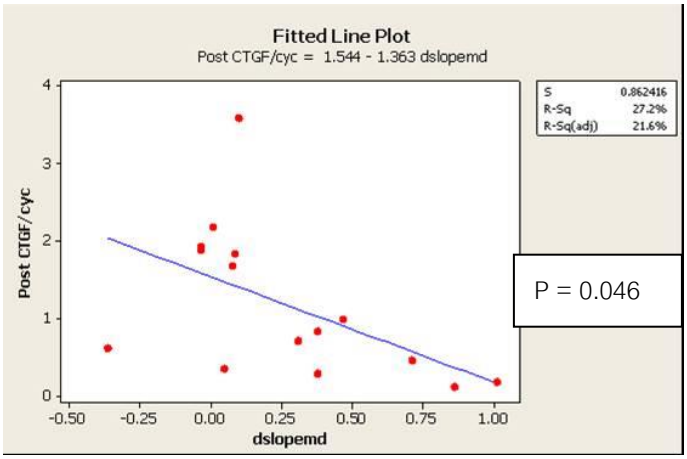


Figure 21. The correlation between change in renal CTGF expression and change in GFR slope

Change in TGFb gene expression from Bx 1 to Bx 2 also correlated negatively with the change in rate of change of GFR after intervention(SL= -1.100, R2 = 0.35, p = 0.021). Figure 22

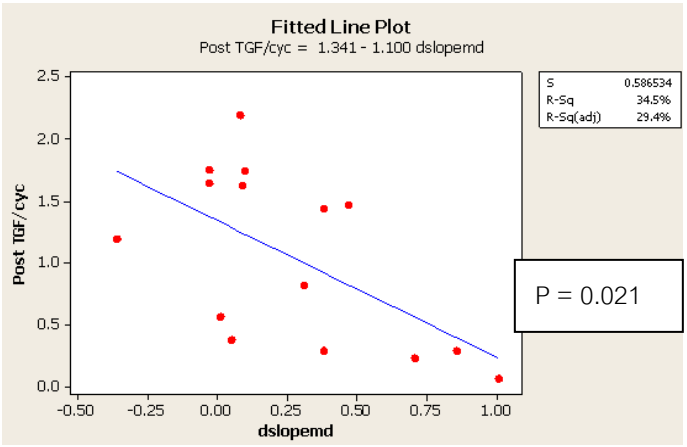


Figure 22. The correlation between renal TGF expression and GFR after treatment

Conclusions: In CAN, CTGF correlates with TGF-beta expression. After therapy, change of TGF or CTGF correlates with change in GFR.

DISCUSSIONS

In this study, we tested the hypothesis that genes regulating endogenous retinoic acid metabolic pathways and genes involved in extracellular matrix (ECM) production would be altered in kidney diseases and that these changes would correlate with adverse pathological findings or predict decline in kidney function.

We found that lupus nephritis was associated with significantly decreased renal expression of both genes for retinoic acid synthesis enzyme RALDH1 and genes for retinoic acid degradation, CYP26A compared to controls. The decrease appear to be specific to LN as patients with moderately advanced diabetic nephropathy did not show any alterations in gene expression compared to controls. Of interest, the ratio of retinoic synthetic enzymes to retinoic acid metabolism enzymes were unaltered in both LN and diabetic nephropathy compared to controls. This might suggest that despite changes in gene expression of retinoic metabolic enzymes, the balance of synthesis to degradation might not be altered in LN overall when compared to controls. The mechanisms underlying the changes in retinoic pathways in LN are unknown and could reflect a response to either a more generalized change in systemic retinoic metabolism or to an increased immune infiltration in class 3-5 LN.

The clinical significance of changes in retinoic acid metabolic pathways in LN is unclear. We did not find any correlations between expression of both retinoic acid synthetic and degradation enzymes with parameters of disease severity such as proliferation (Class 3 or 4), GBM membrane changes (class 5), activity or chronicity indices. It is of interest however, that the ratio of retinoic acid synthesis enzymes (RALDH2, and RALDH3) to degradation enzyme (CYP26A) were decreased in patients with greater than 10% crescents on the kidney biopsy. Crescentic change reflect de-dedifferention of podocytes and parietal epithelial cells and is often associated with more severe disease and adverse outcomes.(12) It might be hypothesized that increased RALDH2 or RALDH3 to CYP26A ratio might lead to increased retinoic acid availability in patients with crescents. Since all-trans retinoic acid, a product of RALDH typically promotes epithelial cell differentiation, it is possible that increased retinoic acid availability might be a response to protect against glomerular cell de-differentiation. This is consistent with previous studies in which RALDH2 is upregulated in the podocyte in animal models of nephrosis where increased retinoic acid synthesis was found to promote recovery from proteinuria. (13). (14).

We also evaluated the expression of ECM genes in kidney diseases. In particular, we evaluated the potential value of CTGF in renal prognosis and its

relationship with TGF-beta. In LN, CTGF correlated with TGF beta and collagen I expression. Connective tissue growth factor expression correlated inversely with GFR at baseline and was higher in patients with subsequent decline. We also evaluated the changes in ECM gene expression before and after changing treatment in patients with chronic allograft nephropathy. We showed that the change in CTGF correlated with changes in kidney function and changes in TGF gene expression after modifications of therapy. Taken together these findings suggest that CTGF might be a useful marker to predict decline in kidney function in different kidney diseases. This data is generally consistent with other experimental studies of kidney diseases. (9)

Thus from this research, we have found that endogenous retinoic acid metabolic pathways might be altered in LN, but not DN. The significance of this altered retinoic pathway in LN is uncertain as there was no correlations between individual genes and pathological severity and the ratio of degradation and synthetic pathway was unaltered overall. The lack of alterations of retinoic pathways in advanced DN suggest that these pathways may not have an important role in CKD and renal fibrosis. The finding that the ratio of RALDH2 and RALDH3 to CYP2626A is increased might suggest a potential role of retinoic pathway in crescent formation and should be explored further. We attempted to explore the significance of other aspects of retinoic pathways by studying changes in retinoic acid receptor expression and protein expression. Unfortunately, we were not able to pursue these results due to personnel problems.

Several limitations affected this research. RNA quality from routine left-over clinical specimen (both paraffin and frozen, left-over immunofluorescent specimen was quite poor. Since the mRNA expression of genes for retinoic synthetic enzymes was also quite low, we could only study all the retinoic genes and could not evaluate the ECM genes in the same biopsy sample. As such, we were unable study direct correlations between ECM and retinoic acid genes. However, we expect that there would be limited correlations as the levels of retinoic metabolizing pathways were unaltered in advanced DN where both CTGF and TGF-beta are both expected to be highly elevated. Other potential confounder to the expression studies could be the variable mix of glomerular and tubulointerstitium in different samples. Unfortunately, our attempt at LCM to separately study glomeruli and tubules was not successful because of low abundance of retinoic genes and low quality RNA in clinical samples.

CONCLUSIONS

There is limited evidence to support an important role for modulations in endogenous retinoic acid metabolic pathways in lupus nephritis or diabetic nephropathy, although a potential role in crescent formation may be possible in lupus nephritis. Connective tissue factor is a predictor of decline in kidney function in lupus nephritis and chronic allograft nephropathy. The role of CTGF or TGF-beta in predicting renal response in the long term should be evaluated.

FUTURE STUDIES

To explore the potential role of the balance of retinoic synthesis and degradation in crescentic glomerular disease, a larger study using ABI probe primer system for RALDH2 and CYP26a could be considered.

Larger studies to evaluate long term predictive value of renal CTGF or TGF beta expression should be performed in LN.

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Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

1. **ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ:** 3 Planned (1 accepted)

   i) Tachaudomdach C, Kantachuvesiri S, Changsirikulchai S, Wimolluck S, Pinpradap K, Kitiyakara C. Connective tissue growth factor gene expression and decline in renal function in lupus nephritis. Exp Ther Med. 2012 Apr;3(4):713-718. Epub 2012 Feb 3. PubMed PMID: 22969957

   ii) Endogenouc retinoic metabolic enzyme genes in lupus nephritis (Manuscript in preparation)

   iii) Change in CTGF and TGF beta mRNA and change in GFR in chronic allograft nephropathy (Manuscript in preparation)

2. **การนำผลงานวิจัยไปใช้ประโยชน์**

   PhD student trained: 2 students (1 completed, 1 terminated without completion)

   i) Chiraporn Tachaudomdach PhD Molecular Medicine, Faculty of Science, Mahidol University. Graduated 2012