Original Articles/นิพนธ์ต้นฉบับ

Renal Transforming Growth Factor Beta Gene Expression

Srimawong P, M.Sc.[1], Tantiwetrueangdet A, M.Sc.[1], Pinpradap K, MD.[2], Sumethkul V, MD.[3], Kochakarn W, MD.[4], Kijvikai K, MD.[4], Ratana-Olarn K, MD.[4], Chaimuangraj S, MD.[4], Leenanupunth C, MD.[4], Jirasiritham S, MD.[4], Kongchareonsombat W, MD.[4], Kitiyakara C, MD.[3]

[1] *Research Center, Faculty of Medicine Ramathibodi Hospital, Bangkok, Thailand*
[2] *Department of Pathology, Faculty of Medicine Ramathibodi Hospital, Bangkok, Thailand*
[3] *Department of Medicine, Faculty of Medicine Ramathibodi Hospital, Bangkok, Thailand*
[4] *Department of Surgery, Faculty of Medicine Ramathibodi Hospital, Bangkok, Thailand*

Abstract

Background: Fibrosis in the renal biopsy is associated with poor long term outcome in many kidney diseases. However, fibrosis occurs at a late stage when the kidney is irreversibly damaged. Molecular biology techniques are currently being investigated to identify early prognostic markers in kidney diseases. Reverse transcriptase real-time PCR (RT-qPCR) is a highly sensitive technique capable of detecting small changes in gene expression. Transforming growth factor-β1 (TGF-β1) is a key mediator of fibrosis and is expected to be increased in damaged tissues. This is a pilot study to investigate the feasibility of the using RT-qPCR to study gene expression of TGF-β1 in human kidney tissues.

Methods: RNA was extracted from normal and diseased human kidneys with fibrosis and reverse transcribed. TGF-β1 gene expression was studied by multiplex RT-qPCR using cyclophilin A as a housekeeping gene. Relative gene expression was calculated from 2-DDCT method.

Results: The expression TGF-β1 in three different areas of the same kidney were similar The expression of TGF-β1 was 4-5 fold higher in disease kidney tissues compared to normal ($p<0.001$).

Summary: TGF-β1 gene expression can be measured from human kidney using RT-qPCR. There are minimal effects of tissue sampling on gene expression levels, hence tissue obtained from a kidney biopsy should be representative of the whole kidney cortex. The expression of TGF-β1 is higher in fibrotic kidneys. Future studies are necessary to determine if the TGF-β1 gene expression markers can predict disease progression.

Keywords: fibrosis; kidney; renal failure; transforming growth factor beta, real time PCR

Corresponding author: Chagriya Kitiyakara MD. Renal Division, Department of Medicine, Ramathibodi Hospital, Mahidol Univeristy. Tel 02-201-1400, Fax 02-201-1400 Email: rackt@mahidol.ac.th

Introduction

Kidney diseases are increasingly common world wide.[1] The prognosis of patients with kidney diseases is variable. In some cases, patients respond to treatment and are able to maintain their kidney function or even recover. In many cases, however, there is progressive decline in kidney function leading to end-stage renal failure requiring life long dialysis. Treatments of kidney conditions often involve immunosuppressive therapy, which are themselves highly toxic. Renal biopsy is an essential diagnostic tool in managing patients with kidney diseases. Classical renal histopathological analysis provides descriptive information enabling the classification of diseases in native and allograft kidneys. Although current renal histology methods are capable of detecting moderate fibrosis indicative of chronic injury, such findings occur at a late stage when the kidney is irreversibly damaged. Markers that can predict disease progression or regression early and accurately would be extremely useful in selecting and evaluating therapeutic approaches in clinical practice.[2]

Molecular approaches are now included in the initial diagnosis, subgroup classification, therapy selection, and monitoring of outcome in many areas of medicine. In kidney diseases, the use of molecular biology techniques to evaluate gene expression as new diagnostic and prognostic markers are currently being investigated.[3,4] Reverse transcription (RT) followed by real-time polymerase chain reaction (PCR) is the technique of choice to analyze mRNA expression.[5] This new technique is a refinement of the original polymerase chain reaction (PCR) developed in the 80's.[6] PCR is performed on a DNA template and requires two oligonucleotide primers that flank the DNA sequence to be amplified.[5] The reaction is performed by temperature cycling during which the nucleic acid sequence between the primers is amplified to generate a large number of identical copies. The original PCR assay analyzes the product at the end of the reaction by agrose gel electrophoresis, and is not very accurate for DNA quantification. In real-time PCR, a reporter dye linked to an oligonucleotide probe with sequences corresponding to the amplified DNA product is added to the reaction. As the DNA product is formed, the reporter dye is released from the probe. The amount of product generated after each cycle of amplification can be monitored by the increasing fluorescence during the course of the reaction (i.e. in real time). In applying real time PCR to gene expression measurements, the mRNA in the sample must first be copied to complementary DNA (cDNA) by reverse transcription (RT) before the PCR reaction. The amounts of cDNAs produced reflect the input amounts of the mRNAs in the initial sample. Reverse transcriptase real-time PCR (RT-qPCR) is highly sensitive allowing quantification of rare transcripts and detection of small changes in gene expression. Furthermore, it is easy to perform, provides accurate as well as rapid quantification results. Thus gene expression in small amounts of tissues such as a kidney biopsy may be quantified.[3,4]

Transforming growth factor-β1 (TGF-β1) is involved in tissue repair and tissue scarring via the induction of extracellular matrix gene (ECM) expression.[7] TGF-β is a key mediator of glomerular and tubulointerstitial fibrosis in chronic kidney diseases. In animal models, mRNA levels for TGF-β and ECM components are upregulated early after the induction of renal disease, and they precede the development of glomerulosclerosis and interstitial fibrosis.[8] In human patients, mRNA levels of genes linked to ECM deposition may provide useful prognostic information, but the studies in different kidney diseases remain limited up to now[3,4]

This is a pilot study to investigate the feasibility of the using RT-qPCR to study gene expression of TGF-β1 in human kidney tissues. It is hoped that methods developed in this study can be used to investigate gene expression in the kidney and other organs.

Materials and Methods

Tissues

Samples of human kidney cortex were obtained from operation rooms of Ramathibodi Hospital. Normal tissue was obtained from normal portions of the kidney after total nephectomy for renal cell carcinoma. Diseased kidney was obtained from nephrectomy from patients with advanced allograft nephopathy and from patients with renal stones. Kidney tissues were harvested in RNA later (Promega, USA) and immediately transfer to -80°C. All steps were carried out under RNase-free conditions. Ethics committee approval was obtained (ID 05-48-09).

RNA extraction and cDNA synthesis

Total RNA was isolated from kidney core in RNA later (Ambion, Texas, USA) using RNeasy Mini Kit (Qiagen, Courtaboeuf, France) according to the manufacture's protocol. The disruption and homogenization of the kidney core samples were performed using a Rotor-Stator Homogenizer (Ultra-Turrax T8, IKA Labortechnik Staufen, Janke & Kunkel QmbH & Co. KQ, Germany). The amount of extracted RNA was quantified by measuring the absorbance at 260 nm. The purity of the RNA was checked by measuring the ratio of the absorbance at 260 and 280 nm, where a ratio ranging from 1.9 - 2.1 was taken to be pure. The quality and approximate yield of RNA was confirmed by RNA 6000 LabChip on an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc.) using 1 ul of the RNA sample.

All the individual mRNA (genes) expressed in the kidney tissues are changed to cDNA by reverse transcription in a proportional manner based on the level of expression of the mRNA in the initial sample. 800 ng of total RNA from each kidney core was reverse transcribed using oligo dT from iScript cDNA synthesis kit (Biorad Laboratories, Hercules, CA, U.S.A) according to the manufacture's protocol and stored at -20°C. The first-strand complementary DNA was used as a template for PCR.

Reverse Transcriptase real time polymerase chain reaction (RT-qPCR)

Comparing gene expression between different samples requires normalization to compensate for differences in the amount of biological materials in different test samples. A popular method is to compare the target gene expression to that of endogenous housekeeping gene, which is expected to be expressed at a constant level in different disease conditions.[5] The level of expression of housekeeping gene can be used to correct for variations in total RNA quantity, which may be induced by pipetting errors. Cyclophilin A, a member of a highly conserved, ubiquitous protein family and originally isolated as the main cyclosporin A-binding protein was selected as the housekeeping gene or endogenous control.[9] The expression of cyclophilin is not restricted to a specific cell type and is therefore useful as a housekeeper gene for comparative quantification of mRNA by RT-PCR.[10,11] Previously, cyclophilin has been shown to be a useful housekeeping gene in the kidney.[12] In this study, we used multiplex RT-qPCR, in which the expression of two genes is quantitated in a single sample in the same reaction using PCR probes labeled with different color dye for each gene.[5] In this case, the probe for the target gene, TGF-β1 is labeled with FAM (green) and the housekeeping gene, cyclophilin probe is labeled with VIC (yellow). Thus changes is relative expression of TGF-β1 to cyclophilin is expected to represent true changes in TGF-β1 expression induced by disease process.

The mulitiplex reaction contained 10 ul of 2xTaqman Universal PCR master mix, 1 ul of control primer and probe as well as Cyclophilin, 0.5 ul of 10 uM forward primer TGF-β1, 0.5 ul of 10 uM reverse primer TGF-β1, 0.5 ul of 10 uM TGF-β1 probe and 2 ul of cDNA template. Final volume was adjusted to 20 ul by nuclease-free water. Triplicate samples of each reaction were added to individual well of a 96 well plate. Reactions were run on an ABI PRISM 7500 Real Time PCR machine (Applied Biosystems) using

the default cycling conditions of 2 mins at 50°C, 10 mins at 95°C followed by 40 cycles of 15 sec at 95°C and 60 sec at 60°C. A non-template control was included in each experiment. The predesigned primers and probe for cyclophilin was purchased from Applied Biosystems. The sequences of TGF-β1 primers and probes have been previously published[13]: Forward: 5'CCA GCA TCT GCA AAG CTC 3', Reverse 5' GTC AAT GTA CAG CTG CCG CA 3', probe 5' (Fam) ACACCAACTATTGCTTCAGCTCCACGGA 3' (Tamra).

The threshold cycle (Ct) is defined as the cycle number at which the fluorescence intensity generated by the tracer dye, which is released from the probe during DNA amplification reaches a fixed limit of detection.[5] This threshold limit of detection was obtained automatically using the machine settings. The threshold cycle is determined at the most exponential phase of the reaction and is more reliable than end-point measurements of accumulated PCR products used by traditional PCR methods. Ct is inversely proportional to the copy numbers of the target template in the sample at the start of the reaction. The higher the initial template concentration, the earlier the DNA product can be amplified to a fixed limit of detection, and the lower the threshold cycle measured. A higher Ct means lower gene expression whereas a lower Ct means higher gene expression.

Efficiency

Initial studies were performed by varying the primers and probe concentrations for TGF-β1 to obtain the best combination to provide the most efficient amplification (close to 2).[14] The efficiency of the PCR assay is estimated from a standard curve based on serial dilution of RNA from a normal kidney.[5] The Ct values of the diluted standards are plotted against the logarithm of the samples' concentrations

The PCR efficiency (E) is calculated from the slope as:

$E=10^{-1/k}-1$

Effects of tissue sampling and disease on TGF-β1 expression.

To examine the effects of tissue sampling on gene expression, different cortical areas of the same kidney were studied. Different areas of the same kidney were dissected randomly. Three areas each were selected from of normal kidney (NK4) and disease kidney (DK1); NK4area1, NK4area2, NK4area3, DK1area1, DK1area2, and DK1area3. NK1 area 1 is selected as the reference sample.

To examine gene expression differences between normal and diseased kidneys, one area of the cortex from disease (n=2) and normal kidneys (n=7) were compared with NK10 used as the reference sample.

Relative gene expression

Relative gene expression was calculate from $2^{-\Delta\Delta CT}$ method.[14] (REF) such that

The amount of target relative to housekeeping $= 2^{-(CTB2-CTB1)-(CTC2-CTC1)}$

Where CTB represent Ct value for target gene TGF-B and CTC present Ct value for housekeeping gene, cyclophilin. 2 denotes test sample, 1 denotes sample set as reference, which is NK10 in this case.

Statistics

The Efficiency and relative gene expression analysis was performed using software from ABI PRISM 7500 Real Time PCR machine. Mann-Whitney test was used to compare gene expression between different samples.

Results

Relative Efficiency of Target and housekeeping gene in multiplex RT-PCR

The analysis of an intact total RNA sample from kidney tissue by Agilent 2100 Bioanalyzer software is shown in figure 1. The 18S and 28S ribosomal RNA peaks are well preserved indicating good quality RNA that have not degraded.

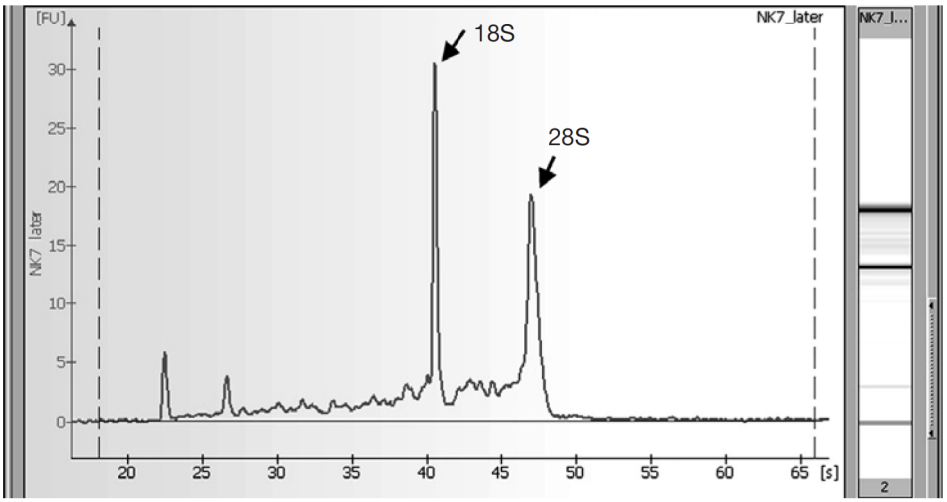


Figure 1. *Total RNA from kidney tissue was isolated and analyzed using the RNA 6000 LabChip kit. Intact 18S and 28S ribosomal RNA peaks are shown indicating intact RNA.*

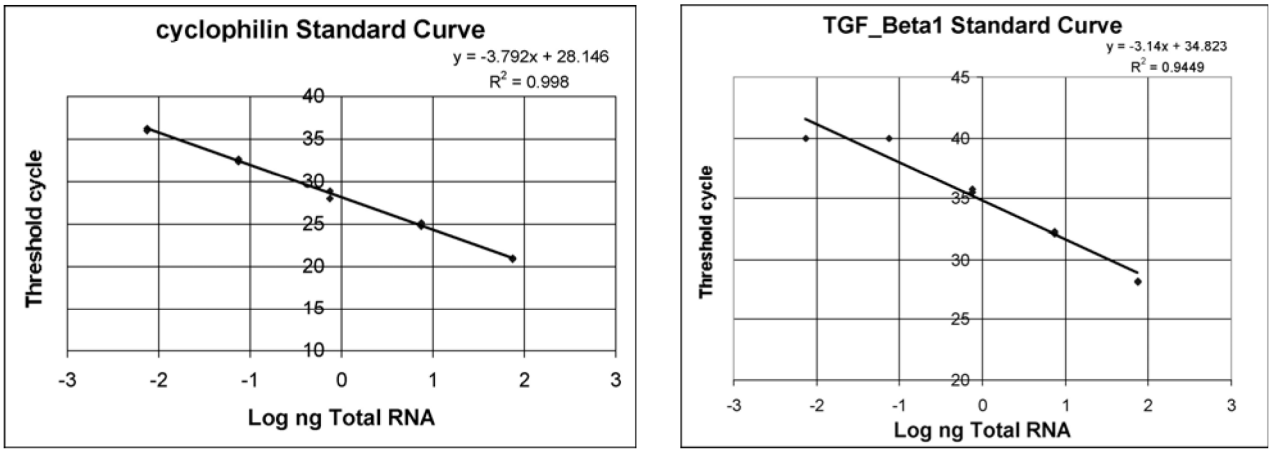


Figure 2. *Standard curves generated by normal kidney cDNA for A/Cyclophilin and B/TGF-beta1.*

Standard curves were generated by plotting the amount of total RNA from normal kidney against respective threshold cycles (C_T) (Figure 2). The standard curves showed good linearity with correlation coefficient of 0.998 for Cyclophilin (efficiency = 1.83) and 0.945 for TGF-beta1 (efficiency = 2.07).

Before using the $\Delta\Delta C_T$ method for quantitation of fold differences between different samples, the amplification efficiencies of the target gene and the housekeeping gene must be approximately equal, and the relative expression of target and housekeeping gene should be constant over a wide range of amount of input RNA in the sample.[5,15] The absolute value of the log RNA input amount and ΔCt should be close to zero and less than 0.1. Figure 3 shows the absolute value of the slope of log input RNA amount versus ΔC_T were -0.07 for normal kidney cDNA and 0.08 for disease kidney cDNA. Mean coefficient of variation for ΔCt for each sample were 9.0% and 11.7% from normal and disease kidney, respectively.

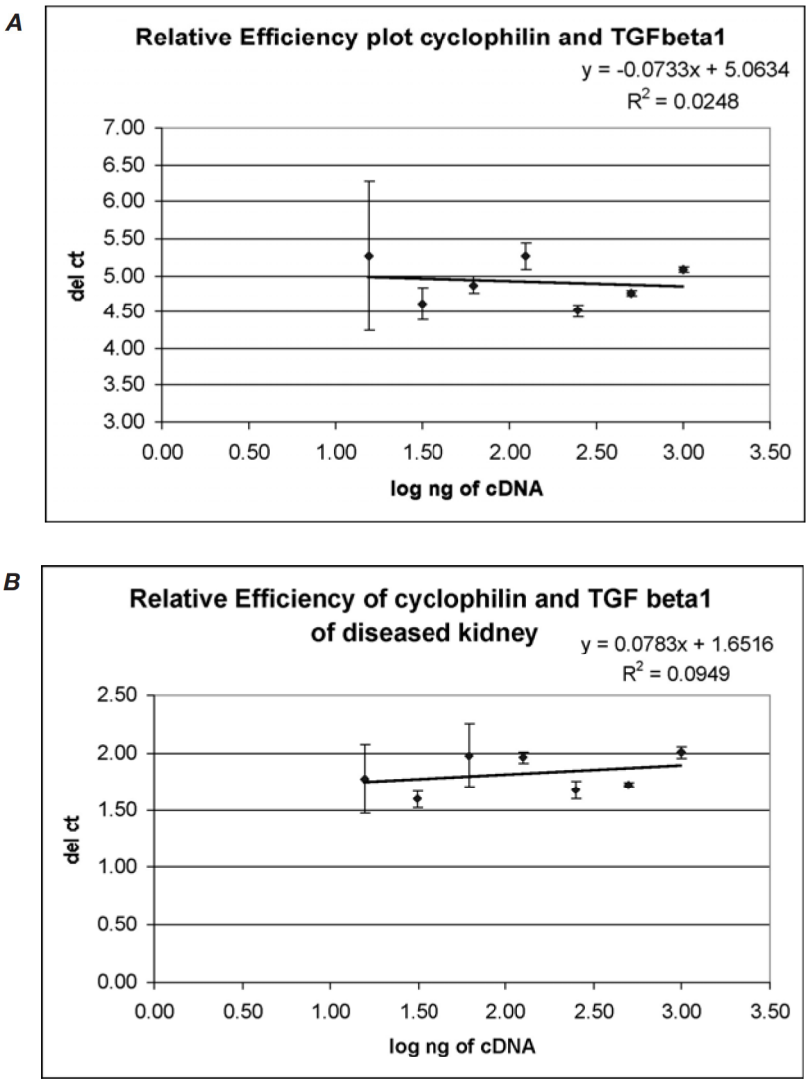


Figure 3. *A plot of log input template amount versus the difference between threshold (ΔC_T) for TGF-β1 and Cyclophilin in both A:normal and B: diseased kidneys. The relative expression for TGF-β1 and Cyclophilin is stable (slope < 0.1) over 2 log concentrations.*

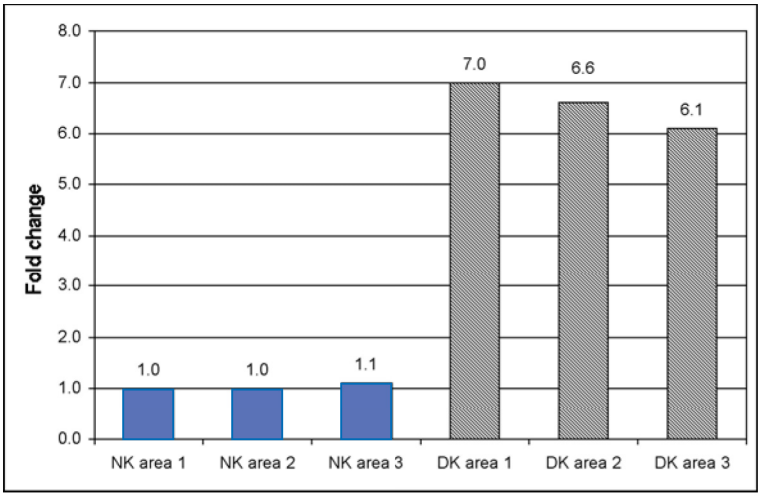


Figure 4. *TGF-β1 gene expression in different areas from the same kidney. NK: normal kidney and DK: diseased kidney. NK1 area is designated calibrator*

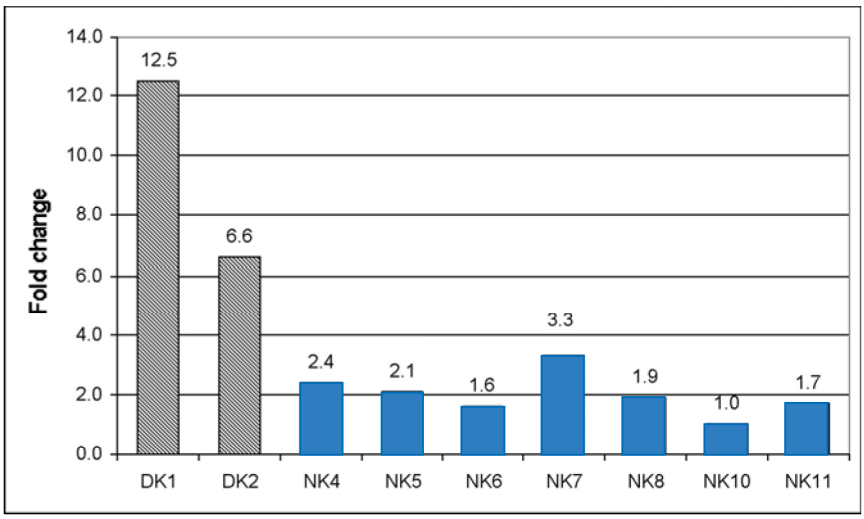


Figure 5. *Relative expression of TGF-β1 in normal kidneys (n=7), and diseased kidney (n=2). The NK10 is designated as the calibrator.*

Expression of Cyclophilin and TGF-beta1 in human kidney tissues

To examine the effects of tissue sampling on gene expression, TGF-β1 expression in three areas of the same kidney: normal kidney (NK4) or disease kidney (DK1) were measured using multiplex real-time PCR with the expression of NK4 area1 set arbitrarily as 1. Figure 4 shows a comparison of the relative expression of TGF-β1 from different areas of NK4 or DK1. The expressions of TGF-β1 from were similar in different areas of the same kidney in both disease and normal kidneys.

The expression of TGF-β1 in diseased kidney is compared to normal kidney by setting NK10 as having arbitrarily as 1. Figure 5 shows the expression of TGF-β1 was 4-5 folds higher among disease kidney tissues (Fold expression: DK 9.45±4.1, NK 2.0±0.72, respectively ($p<0.001$)).

Discussion

This study has demonstrated that TGF-β1 gene expression can be measured from human kidney cortex using reverse-transcriptase real-time PCR. The relative expression of TGF-β1 from different regions of the cortex in the same kidney is similar. The expression of TGF-β1 is about 4-5 folder higher in diseased compared to normal kidneys.

Gene expression of the kidney can be studied by various methods.[3,4] Northern blot was one of the earliest methods used, but requires large quantity of input mRNA and tissue. In the past, standard PCR is performed by gel electrophoresis, which has low sensitivity and is imprecise as a quantification method. Competitive real time PCR and RNAase protection assays are more sensitive, but can be laborious to set up. Reverse-transcriptase real-time PCR (RT-qPCR) is highly sensitive method that capable of detecting minute quantities of mRNA and has a large dynamic range[5] and is capable of rapid and reliable mRNA measurement in small tissue specimens such as kidney biopsy. The multiplex RT-qPCR can be performed as in this study to standardize gene expression data to a housekeeping gene to limit sample to sample variations in tissue amount or due to limit pipetting errors.

Initial experiments are necessary to optimize condition for the multiplex RT-qPCR, but these are relatively straight forward.[14] Using sequences from published articles or from publicly available database such as NCBI, it is relatively simple to design specific

primers and probes for most genes.[5] The choice of genes to be studied depends on the design of individual studies. Numerous biotec companies can now synthesize primers at affordable prices. By designing specific primers and probes, the potential role of a virtually any gene can studied in many disease conditions.

The finding that the expression of TGF-β1 from different regions of the same kidney is similar suggests that sampling bias is not a major problem for this gene. Thus the level of TGF-β1 in a kidney biopsy of a random region in the kidney is likely to be representative of the whole kidney. We have found that the expression of TGF-β1 is higher in the diseased kidney, which has greater degree of fibrosis. This is consistent with previous studies of native kidney diseases[8] and in chronic renal allograft nephropathy.[13]

In conclusion, this study has shown that the expression of genes involved with tissue fibrosis can be accurately and reliably assessed in human kidney using real time RT-PCR. Future studies are necessary to determine if fibrogenic gene expression can used to predict response to therapy or long term disease outcome.

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Original Articles/นิพนธ์ต้นฉบับ

Renal Transforming Growth Factor Beta Gene Expression

ประไพพิศ ศรีมาวงศ์, วท.บ.[1], อัญชลี ตันติเวทเรืองเดช, วท.บ.[1], โกเศศ ปิ่นประดับ, วท.บ.[2], วสันต์ สุเมธกุล, พบ.[3], วชิร คชการ, พบ.[4], กิตติณัฐ กิจวิกัย, พบ.[4], กฤษฎา รัตนโอฬาร, พบ.[4], สุชาติ ไชยเมืองราช, พบ.[4], เจริญ ลีนานุพันธุ์, พบ.[4], โสภณ จิรสิริธรรม, พบ.[4], วิสูตร คงเจริญสมบัติ, พบ.[4], ชาครีย์ กิติยากร, พบ.[3]

[1] สำนักงานวิจัย, [2] ภาควิชาพยาธิวิทยา, [3] ภาควิชาอายุรศาสตร์, [4] ภาควิชาศัลยศาสตร์,
คณะแพทยศาสตร์โรงพยาบาลรามาธิบดี มหาวิทยาลัยมหิดล กรุงเทพฯ ประเทศไทย

บทคัดย่อ

บทนำ การตรวจพบเนื้อเยื่อชนิดที่เป็นเส้นใย (Fibrosis) ในชิ้นเนื้อที่ได้จากการเจาะไต สามารถใช้เป็นปัจจัยทำนายว่าผู้ป่วยจะมีการพยากรณ์โรคไม่ดีในโรคไตหลายชนิด อย่างไรก็ตาม การเกิดเนื้อเยื่อชนิดเส้นใย มักจะพบในระยะท้ายของโรค ซึ่งมักจะเป็นช่วงเวลาที่ไตถูกทำลายไปมากและไม่สามารถรักษาให้กลับมาเป็นปกติได้ การตรวจโดยใช้เทคนิคทางอณูชีววิทยา เป็นวิธีหนึ่งที่สามารถใช้สืบค้นหาปัจจัยที่มีประโยชน์ในการพยากรณ์โรคในระยะต้นของโรคไตหลายชนิด

วัตถุประสงค์ การศึกษานี้เป็นการศึกษานำร่อง เพื่อประเมินความเป็นไปได้ในการนำเทคนิค (RT-qPCR) มาใช้ในการตรวจหาหน่วยพันธุกรรมของ TGF-β1 ในชิ้นเนื้อที่ได้จากการเจาะไตของผู้ป่วย ทั้งนี้ มีแนวคิดว่าการตรวจด้วยวิธี Reverse Transcriptase realtime PCR (RT-qPCR) เป็นการตรวจที่มีความไวและสามารถนำมาตรวจหาการเปลี่ยนแปลงของการแสดงออกของหน่วยพันธุกรรม (gene) ในปริมาณน้อยๆ ได้ โดยมีข้อมูลว่า Transforming Growth Factor Beta (TGF-β1) เป็นกลไกหลักของการเกิดเนื้อเยื่อชนิดที่เป็นเส้นใยในระยะต้น และเป็นที่คาดหมายว่าสามารถตรวจพบได้ในชิ้นเนื้อไตที่เจาะจากผู้ป่วยโรคไตเรื้อรังระยะต้น

วิธีการวิจัย ได้ทำการสกัด RNA จากชิ้นเนื้อไตปกติ และชิ้นเนื้อไตที่เจาะจากผู้ป่วยที่เป็นโรคไตเรื้อรังระยะต้น และทำการแปลงกลับเป็น DNA ด้วยวิธี Reverese transcription ได้ทำการตรวจค้นหายีนของ TGF-β1 โดยใช้ Cyclophilin เป็นยีนที่ทำหน้าที่ช่วยในการแปลผล (house keeping gene) การวัดการแสดงออกของยีน ใช้วิธีคำนวนด้วยวิธี 2-ΔΔCT ทั้งนี้ในชิ้นเนื้อไต 1 ชิ้น จะได้รับการสุ่มให้วัดการแสดงออกของยีน TGF-β1 3 ตำแหน่ง

ผลการศึกษา การแสดงออกของยีน TGF-β1 ในชิ้นเนื้อไตเดียวกันจะมีค่าใกล้เคียงกัน แม้ว่าจะได้รับการสุ่มให้วัดในตำแหน่งที่ต่างกันเป็นจำนวน 3 ตำแหน่ง การแสดงออกของยีน TGF-β1 ในชิ้นเนื้อไตของผู้ป่วยโรคไตเรื้อรังระยะต้นสูงกว่าชิ้นเนื้อไตปกติ 3-4 เท่า ($P < 0.001$)

สรุปผลการศึกษา การใช้เทคนิค RT-qPCR สามารถนำมาใช้ตรวจหาการแสดงออกของยีน TGF-β1 จากชิ้นเนื้อไตของผู้ป่วยโรคไตเรื้อรัง ความแตกต่างของค่าที่วัดได้ในแต่ละตำแหน่งของการสุ่มวัดมีน้อยมาก ซึ่งทำให้สามารถนำผลที่ได้มาทำนายการเปลี่ยนแปลงของไตในภาพรวมได้ การแสดงออกของยีน TGF-β1 จะมีมากขึ้นในชิ้นเนื้อไตของผู้ป่วยโรคไตเรื้อรัง เมื่อเทียบกับชิ้นเนื้อไตปกติอย่างมีนัยสำคัญ ทั้งนี้ควรมีการศึกษาเพิ่มเติมว่าการตรวจพบการแสดงออกของยีน TGF-β1 ที่สูงขึ้นจะมีความสัมพันธ์กับการกำเริบของโรคไตในระยะยาว