CHAPTER III

MATERIALS AND METHODS

1. Patients

Between February 2003 and July 2004, we enrolled forty-one patients who had been diagnosed lupus nephritis (LN) and had the criteria for diagnosis of SLE according to the American College of Rheumatology. Renal involvement was documented by having one of the following criteria: a total urinary protein level of more than 0.5 gram per day, an increment of serum creatinine levels of more than 0.5 mg/dl during one month period of follow up or presence of pyuria, hematuria or urinary cast by microscopic examination (see appendix A). All kidney biopsy were using the classification of the histological types of lupus nephritis by World Health Organization (WHO). The study has been approved by the Ethics Committee for Human Research of the Faculty of Medicine, Chulalongkorn University and written informed consents were obtained from all subjects. Most patients had received the same immunosuppressive treatment including prednisolone and cyclophosphamide or mycophenolate mofetil. In addition, six kidney sections from living donors for kidney transplant were used as controls.

2. Sample Size Calculation

Preliminary study showed the difference of VEGF mRNA and controls (n=10) and LN patients (n=10) as natural log mRNA levels (-0.39±0.73 and -1.00±0.62, respectively), afterward the sample size was calculated by

$$n/group = 2(Z_{\alpha/2}+Z_{\beta})^2\sigma^2 / (\overline{X}_1-\overline{X}_2)^2$$

When $\alpha = 0.05$

$\beta = 0.10$

$Z_{\alpha/2} = Z_{0.05/2} = 1.96$ (two-tail)

$Z_{\beta} = Z_{0.10} = 1.28$

$\overline{X_1}$ = mean of VEGF expression in healthy controls = (-0.39)

$\overline{X_2}$ = mean of VEGF expression in LN patients = (-1.00)

σ^2 = Pooled variance = / (n_1+n_2-2)

$= \{(10-1)(0.73)^2 + (10-1)(0.62)^2\} / (10+10-2)$

$= 0.46$

n/group $= 2(1.96+1.28)^2 (0.46) / \{(-0.39)-(-1.00)\}^2$

$= 26.10$

Sample size calculation showing to samples number in each group (LN patients n=27, healthy control n=27).

3. Definitions

Lupus nephritis was defined as the histological diagnosis of glomerulonephritis based on the WHO classification. The renal outcomes were defined as 1) A doubling of serum creatinine levels (final / baseline ratio of serum creatinine levels > 2 folds) or 2) the development of end-stage renal disease (ESRD). ESRD was defined by glomerular filtration rate (GFR) or creatinine clearance less than 15 ml/min/1.73 m^2 or a requirement of chronic dialysis or kidney transplantation.

4. Kidney Biopsy Collection

Kidney biopsies were performed in patients and controls and divided into three parts. Firstly, the section for RNA study was transferred into RNA stabilization solution (RNA*later*®, Ambion Inc., Austin, TX, USA) for preserve RNA and stored at -70°c until RNA extraction. Secondly, the section for immunohistochemistry study was

immersed in 10% formalin and embedded in paraffin block. And lastly, the sections for histological study was kept in dry ice and immediately transferred to histopathology laboratory.

5. Total RNA Extraction

Total RNA were extracted by RNeasy mini kit (Qiagen, Chatworth, CA, USA) according to the instruction of manufacturer. The kit combines the selective binding properties of a silica-based membrane with the speed of microspin technology. Briefly, kidney tissues were first lysed and homogenized in the presence of a highly denaturing guanidine-thiocyanate containing buffer, which immediately inactivates RNases to ensure purification of intact RNA. Ethanol was added to provide appropriate binding conditions, and the sample was then applied to an RNeasy Mini spin column, where the total RNA binds to the membrane and contaminants were efficiently washed away. High-quality RNA was then eluted in 35 ul water and stored at -70°c.

6. Measurement Total RNA Concentration

Each RNA sample was aliquoted 5 ul for measuring total RNA concentration. Aliquot RNA had diluted into 20 times (fill up RNase-free water to 100 ul), total RNA measured by spectrophotometer at optical density 260 nm (OD_{260}) and 280 nm (OD_{280}). Calculation of total RNA was necessary for processing in reverse transcription polymerase chain reaction (RT-PCR). One unit of optical density at 260 nm equal 40 ug of total RNA per ml. Pure RNA had an OD_{260}/OD_{280} ratio of 1.6-1.9.

7. Complementary DNA (cDNA) synthesis (RT-PCR)

Synthesis of single-strand cDNA was carried out, as used total RNA 0.25 ug (maximum volume of RNA template not exceed 11.5 ul) for reverse-transcribed into cDNA by Taqman™ Reverse Transcriptase Reagent (Applied Biosystems, Roch

Molecular Biochemical, NJ, USA). Eighteen microliters of reverse transcription mastermix containing 3 ul of 10xRT buffer, 6.6 ul of 25mM $MgCl_2$, 6.0 ul of 10mM 4dNTP, 1.5 ul of 50uM Random primer, 12 U of RNase inhibitor and 37.5 U of MutiScribe™ Reverse Transcriptase, 18 ul of mastermix was added into 0.25 ug (11.5 ul) RNA template and transcribe at 25°c for 10 minutes, 48°c for 30 minutes and 95°c for 5 minutes. Complementary DNA was kept at -20°c until used for measuring gene expression by real-time PCR.

8. Gene Expression Study

The mRNA levels of VEGF and the housekeeping gene 18s rRNA were measured by LightCycler® machine (Roche Molecular Biochemicals, Indianapolis, IN, USA). The principle of real-time PCR using TaqMan probe was described previously. Table 6 illustrates the sequences of primers and fluorescence probes for VEGF and 18s rRNA. The probes were labeled with 6-carboxy-fluorescein (FAM) at the 5 end and with 6-carboxytetramethylrodamine (TAMRA) at the 3 end. FAM serves as the reporter dye, and TAMRA serves as the quencher dye. All primer pairs were designed to span across an intron-exon boundary to distinguish an amplification of genomic DNA. Each PCR reaction was set up for 20 ul reaction volume comprising of 18 ul of real-time PCR mastermix that containing 10 ul of 2xQuantiTech Probe mastermix (Qiagen, Chatworth, CA, USA), 0.5 ul of 20uM forward primer, 0.5 ul of 20uM reverse primer and 0.2 ul of 20uM probe and than add 2 ul of cDNA template, PCR amplification included an initial denature at 95°C for 15 minutes then followed amplification step by heating at 95°C and immediately cool down to 60°C for 60 seconds repeated for 40 cycles. After the processes were completed, the real-time PCR results were automatically reported by LightCycler® software version 4.05 and analyzed by relative quantification method (comparative Ct method) **(70)**.

Table 6. The sequences of primers and fluorescence probes for VEGF and 18s rRNA

Gene	Primer sequences	Product size
VEGF	Forward: 5 cct aca gca caa caa atg tga atg3 Reverse: 5 caa atg ctt tct ccg ctc tga3 Probe: 5 FAM-caa gac aag aaa atc cct gtg ggc ct-TAMRA3	94 bp
18s rRNA	Forward: 5 gcc cga agc gtt tac ttt ga3 Reverse: 5 tcc att att cct agc tgc ggt atc3 Probe: 5 FAM-aaa gca ggc ccg agc cgc c-TAMRA3	81 bp

9. Immunohistochemistry Study

Immunohistochemical study for VEGF protein was performed in paraffin embedded kidney tissue sample. The reagents included polyclonal antibody of VEGF (Santa Cruz Biotechnology Inc.) and Envision reagent (Dako, Mississauga, Ontario) which base on peroxidase activity. Laboratory procedures followed by 8 steps.

9.1. Preparation of Tissue Sections

Kidney tissues were fixed in 10% formaldehyde for tissue preservative before tissues were trimmed to approximately 5 micrometer. Then, tissue sections were fixed in glass slide.

9.2. Tissue deparaffinization and rehydration

Kidney tissue sections were deparafinized in 2 changes of xylene for 5 minutes each and were rehydrated in 2 changes of absolute ethanol for 3 minutes each. Then rinse in distilled water for 5 minutes.

9.3. Antigen Retrieval

For retrieved antigen, tissue sections were heated in microwave oven with sodium citrate buffer (10mM sodium citrate, 0.05% Tween 20, pH 6.0) for 20 minutes. After that tissue sections were still immersing in sodium citrate buffer and allow the slides to cool for 20 minutes, at room temperature. Sections were rinse with phosphate-buffered saline pH 7.4 (PBS, pH 7.4) for 5 minutes. Tissue sections were circled with DAKO pen.

9.4. Endogenous Peroxidase Blocked

To eliminate fault positive from endogenous peroxidase that found in tissue sections, endogenous peroxidase blocked should perform. The endogenous peroxidase activity was eliminated by the pretreatment of tissue sections with 3% hydrogen peroxide (H_2O_2) in phosphate-buffered saline (PBS) (pH 7.4) for 20 minutes at room temperature.

9.5. Non-Specific Antibody Binding Blocked

The main cause of non-specific background staining is non-immunological binding of the specific immune sera by hydrophobic and electrostatic forces to certain sites within tissue sections. The tissue sections were reduced background by blocking those sites with 20% normal goat serum in phosphate-buffered saline (PBS) (pH 7.4) for 20 minutes at room temperature.

9.6. Antigen-Antibody complexes formation

The staining performed with indirect method by used Envision system, base on dextran polymer technology. The tissue sections were reacted with primary antibody, anti-VEGF polyclonal antibody, for 1 hr which diluted 10^4 times with PBS pH7.4. Bound primary antibodies were detected with the Envision reagent (Dako, Mississauga, Ontario) using anti-rabbit secondary antibodies for 30 min.

9.7. Color Development

The color product of enzyme was developed by the 3,5 diaminobenzidine (DAB) substrate. DAB substrate solution was contained with in Envision system kit. Tissue sections were dropped with DAB solution and incubated for 10 minutes.

9.8. Counterstain and Mounting

All tissue sections were counterstained with hematoxylin for 2 min. Finally, slides were dehydrated and mounted with permount solution.

10. Statistical Analysis

10.1. Gene Expression Analysis

Gene expression data were calculated by relative quantitation method (comparative Ct method) **(70)**. The statistical analysis was performed by using SPSS software version 11.5 (SPSS Inc, Chicago, IL). The VEGF gene expression levels were shown as natural log mRNA levels and tested for normal distribution with Shapiro-Wilk test. The mRNA levels deviated significantly from normal distribution ($P<0.05$). The non parametric test (Mann-Whitney U test) was used to compare data between groups. To distinguish lupus nephritis severity, receiver operator characteristic (ROC) curve analysis of mRNA levels was used to determine the cutoff levels that maximized the combined sensitivity and specificity. All statistic results were presented with mean and standard deviation or median and inter-quartile range, as appropriate. A p-value of less than 0.05 was considered significant.

10.2. An Analysis of Renal Outcomes

All patients were followed a standard treatment protocol for 6 months. The renal outcomes were defined as 1) a doubling of serum creatinine levels (final / baseline ratio of serum creatinine levels > 2 folds) or 2) the development of end-stage renal disease; ESRD). ESRD was defined by glomerular filtration rate (GFR) or creatinine

clearance less than 15 ml/min/1.73 m^2 or a requirement of chronic dialysis or kidney transplantation. The cumulative survival curve was derived by the Kaplan-Meier method and tested by log-rank test. A p-value of less than 0.05 was considered significant.