Original Report: Patient-Oriented, Translational Research

American Journal of Nephrology

Am J Nephrol 2014;40:131–139
DOI: 10.1159/000365934

Received: April 2, 2014
Accepted: July 13, 2014
Published online: August 23, 2014

A Functional Polymorphism in the Promoter Region of TLR3 Is Associated with Susceptibility to End-Stage Renal Disease

Hsin-Yi Yang[a] Shih-Ming Huang[b] Kuo-Cheng Lu[f] Chia-Chao Wu[c] Chi-Yu Kang[d] Yuh-Feng Lin[c, g] Chin Lin[e] Fu-Huang Lin[a] Sen-Yeong Kao[a] Sui-Lung Su[a]

[a]School of Public Health, [b]Department of Biochemistry, National Defense Medical Center, [c]Division of Nephrology, Department of Medicine, Tri-Service General Hospital, National Defense Medical Center, [d]Department of Aviation Medicine and Physical examination, National Defense Medical Center Tri-Service General Hospital Songshan Branch, [e]Graduate Institute of Life Sciences, National Defense Medical Center, Taipei, [f]Division of Nephrology, Department of Medicine, Cardinal Tien Hospital, School of Medicine, Fu Jen Catholic University, and [g]Division of Nephrology, Department of Medicine, Shuang Ho Hospital, Graduate Institute of Clinical Medicine, Taipei Medical University, New Taipei City, Taiwan

Key Words
End-stage renal disease · Toll-like receptor 3 · Single-nucleotide polymorphism

Abstract
Background/Aims: End-stage renal disease (ESRD) is simultaneously associated with immune activation, systemic inflammation and immune deficiency. Toll-like receptor 3 (TLR3), a receptor for viral double-stranded RNA, is involved in immune cell activation in renal diseases and may contribute to chronic inflammatory disease progression. To date, effects of *TLR3* polymorphisms on ESRD remain unknown. Therefore, we determined the predictive value of *TLR3* polymorphisms and further functionally studied ESRD. ***Methods:*** We performed a case-control association study and genotyped 616 ESRD patients and 813 healthy controls. Patients were genotyped for –7C/A, 1377C/T and 1234C/T polymorphisms of *TLR3* using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis. The Haplotype association analysis was performed using the Haploview package. A luciferase reporter assay and real-time PCR were used to test the function of the –7C/A promoter polymorphism in TLR3 expression in human embryonic kidney 293 (HEK293) cells. ***Results:*** Genotype distributions of –7C/A and 1377C/T in TLR3 were significantly different in ESRD patients and healthy controls. The ATC haplotype of TLR3 was associated with a decreased risk of ESRD. We also found significant differences in TLR3 expression by dexamethasone treatment between various genotypes of –7C/A ($p = 0.02$). TLR3 transcriptional activity of the variant –7 C allele was higher than that of the –7 A allele after dexamethasone treatment. ***Conclusion:*** Results indicate that, in our population, the presence of the C allele of –7C/A in TLR3 increases the susceptibility to ESRD. In vitro studies demonstrated that –7C/A may be involved in ESRD development through transcriptional modulation of TLR3.

Introduction

Taiwan has the third highest incidence and highest prevalence rates of end-stage renal disease (ESRD) worldwide. These high rates not only burden healthcare re-

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Dr. Sui-Lung Su
School of Public Health
National Defense Medical Center
No.161, Min-Chun E. Rd., Sec. 6, Neihu, Taipei 114 (Taiwan)
E-Mail a131419@gmail.com

sources but also majorly impact patients and their families [1]. ESRD is simultaneously associated with immune activation, marked by systemic inflammation and immune deficiency.

Recent studies have indicated the involvement of the innate immune system [2], including members of the toll-like receptor (TLR) family, in renal disease development and progression. Expression of TLR, constitutively expressed in renal epithelial cells [3], is upregulated in response to inflammation [4]; various potential roles have been proposed for TLR in renal inflammation [5].

The TLR family comprises at least 11 mammalian receptors (currently 10 in humans) that bind a restricted repertoire of ligands and recruit common adaptor molecules to induce cell signalling [6]. Among TLR subtypes, TLR3 has been reportedly associated with renal diseases [7]. TLR3 is a pathogen recognition receptor for viral double-stranded RNA. TLR3 signalling is important in antiviral responses; however, inappropriate TLR3 signalling may be related with inflammatory renal diseases [8, 9].

Single-nucleotide polymorphisms (SNPs) in TLR3 have been correlated with diseases such as osteoarthritis (OA) [10], type 1 diabetes mellitus (T1DM) [11], asthma [12] and bladder cancer [13]. Previous studies have indicated that the presence of TLR3 SNPs influences promoter activity (gene expression), mRNA conformation (stability) and subcellular localisation of mRNAs and/or protein structure and function. TLR3 rs121434431 (Pro554Ser) within LRR20 is linked with partial penetrance of herpes simplex virus-associated encephalitis in children [14]. TLR3 rs3775291 (Leu412Phe) may protect against geographic atrophy in macular degeneration by reduced binding capacity of TLR3 to dsRNA [15].

We have previously demonstrated that –1237T/C may be involved in ESRD development through transcriptional modulation of TLR9 [16]. TLR9 is expressed within endosomal compartments where it binds to CpG motif-rich microbial DNA. TLR3 is also present on the endosomal surface and primarily responds to nucleic acid–based, pathogen-associated molecular patterns (PAMPs) from viral double-stranded RNA.

However, the role of TLR3 expression in ESRD and effects of TLR3 polymorphisms on ESRD remain largely unknown. Therefore, here, we investigated the predictive value of TLR3 polymorphisms and further functionally studied ESRD in a Han Chinese population.

Methods

Subjects

This case-control study included 616 ESRD patients (326 females and 290 males; age, 64.20 ± 14.79 years) receiving care at the Tri-Service General Hospital and Cardinal Tien Hospital in Taipei, Taiwan. ESRD was defined as an estimated glomerular filtration rate (eGFR) <15 ml/min/1.73 m^2 and associated with clinical signs of uremic syndrome requiring dialysis [17]. All patients undergoing hemodialysis (HD) for >6 months with stable condition (without clinical complications) were enrolled. Patients with autoimmune disease [systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), sjogren syndrome, scleroderma, etc.], malignancy and acute or chronic infection were also excluded. Causes of ESRD were diabetes mellitus (244 patients; 39.6%), chronic glomerulonephritis (192 patients; 31.2%), hypertensive nephropathy (78 patients; 12.7%), systemic nephropathy (42 patients; 6.8%) and unknown (60 patients; 9.7%). The systemic nephropathy means systemic disorders causing nephropathy other than hypertension and diabetes mellitus such as analgesic nephropathy, aristolochic nephropathy, et al. The 831 healthy controls (488 females and 343 males; age, 70.08 ± 9.68 years) had eGFRs of at least 60 ml/min/1.73 m^2, with no proteinuria, and were recruited from the Center of Physical Examination at Cardinal Tien Hospital. Healthy controls showed no microalbuminuria, proteinuria or hematuria and had normal abdominal and renal ultrasonography. Clinical variables were collected from medical records.

Ethics Statement

Institutional ethics committees of Tri-Service General Hospital (TSGH-100–05–249) and Cardinal Tien Hospital (CTH-100–3-5–025) reviewed and approved this study. Following committee approval, all clinical and biological samples were collected, and DNA genotyped. After fully explaining the study, written informed consent was obtained from all participants.

Genomic DNA Extraction and Genotyping

Genomic DNA was extracted from peripheral blood samples using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). The –7C/A (rs3775296), 1377C/T (Phe459Phe, rs3775290) and 1234C/T (Leu412Phe, rs3775291) polymorphisms of TLR3 were screened using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Primers were designed based on published sequences [18, 19] or using the primer Z software (http://genepipe.ngc.sinica.edu.tw/primerz/beginDesign.do) (online suppl. table 1; for all online suppl. material, see www.karger.com/doi/10.1159/000365934). PCR program comprised the following steps. Cycling conditions involved initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 45 s, extension at 72°C for 30 s and final extension at 72°C for 7 min. PCR products were digested with the respective restriction endonucleases (New England Biolabs, Inc., Ipswich, Mass., USA); resulting fragments were separated in 2.5% agarose gel containing 0.5 μg/ml ethidium bromide by electrophoresis at 100 V and visualized under ultraviolet light. Genotyping was performed by blinding the case or control status. To validate genotyping results, at least 10% of samples were randomly selected for repeat genotyping.

Peripheral Blood Mononuclear Cell Culture

Peripheral blood mononuclear cells (PBMCs) were prepared from the venous blood of ESRD patients (n = 42) using Ficoll-Hypaque density gradient centrifugation (Amersham Pharmacia Biotech, Little Chalfont, UK). PBMCs were plated at a density of 1×10^6 cells/ml in 12-well cell culture plates with RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (FCS) and 100 μg/ml streptomycin.

Expression Analysis of TLR3 by Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction

PBMCs (1×10^6), cultured as described above, were serum starved and treated with 100 pg/ml dexamethasone for 24 h. Total RNA was isolated using TriZOL reagent (Invitrogen Corp., Carlsbad, Calif., USA). Total RNA (2 μg) was reverse transcribed using the High Capacity Complementary DNA (cDNA) Reverse Transcription Kit (Applied Biosystems, Foster City, Calif., USA) into cDNA. Real-time PCR was performed using the Maxima® SYBR green qPCR master mix (Fermentas, Glen Burnie, Md., USA) with an ABI 7500 real-time PCR system (Applied Biosystems). Primer sequences were as follows: TLR3, forward 5′-AGCCTTCAACGACTGATGCT-3′ and reverse 5′-TTTC CAGAGCCGTGCTAAGT-3′; β-actin, forward 5′-AGTTGC GTTACACCCTTTCTTG-3′ and reverse 5′-TCACCTTCACC GTTCCAGTTT-3′. Thermocycling was performed at 95°C for 10 min, 40 cycles at 95°C for 15 s and 60°C for 60 s to measure the fluorescence signal. Dissociation stages, melting curves and quantitative analyses of data were performed using 7500 system software v1.2.3 (Applied Biosystems). β-actin expression was used as an internal control. TLR3 expression normalized by β-actin was calculated using the $2^{-\Delta\Delta Ct}$ method.

Transient Transfection and Luciferase Assay

TLR3 promoter reporters (from chr4: 186997707–186997985, 279 bp), TLR3 –7CC-LUC and TLR3 –7AA-LUC, were amplified from human genomic DNA, including the SNP of interest (–7C/A), using PCR and subcloned into a pGL3 basal reporter cut at *Xho*I and *Hind*III sites. The 5′ and 3′ primers used for PCR were 5′-AAGTTGGCGGCTGGTAATCT-3′ and 5′-GCATTTG AAAGCCATCTGCT-3′. Human embryonic kidney 293 (HEK293) cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% charcoal/dextran-treated fetal bovine serum. Cells in each well (24-well plate) were transfected with jetPEI (PolyPlus-transfection, Illkirch, France) according to the manufacturer's protocol; total DNA was adjusted to 1.0 μg by addition of the pGL3 reporter. Post transient transfection (24 h), HEK293 cells were treated with dexamethasone (100 nM) for an additional 18 h. A luciferase assay was performed using the Promega Luciferase Assay Kit; measurement was expressed numerically in relative light units. Luciferase activity was shown as the mean and deviation from mean of the two transfected sets. Results shown are representative of at least three independent experiments.

In Silico Analysis

To investigate the possibility of the direct effect of TLR3 –7C/A on transcription factor binding, we used the prediction web software Transcription Element Search System (TESS) to search for promoter elements (http://www.cbil.upenn.edu/tess).

Statistical Analysis

For each SNP, deviation from the Hardy-Weinberg equilibrium (HWE) was assessed in controls using the standard χ^2 test. Genotypes and allelic frequencies were compared between ESRD patients and healthy controls using χ^2 or Fisher exact tests when appropriate. Demographics were evaluated by the Student t test or Mann-Whitney U test for continuous variables and expressed as mean ± standard deviation (SD). Logistic regression was used to estimate crude and adjusted odd ratios (ORs) and 95% confidence intervals (CIs) for age, gender, body mass index and smoking status, as measures of association with the risk of ESRD. Linkage disequilibrium (LD) and haplotype analyses were performed using Haploview software (http://www.broad.mit.edu/mpg/haploview/) [20]. The association of the TLR3 promoter polymorphism with TLR3 mRNA expression was tested using the Kruskal-Wallis (KW) test. The significance level was determined using the Bonferroni method for correcting multiple testing errors. For the three selected SNPs, $p < 0.0167$ (0.05 divided by 3) was considered statistically significant. All statistical analyses were performed using SPSS 18.0 for Windows (SPSS, Inc., Chicago, Ill., USA). Since the frequency distributions of demographic characteristics and the genotype and allelic distributions of the 3 SNPs at TLR3 are similar in study 1 cohort and study 2 cohort. The data from the two independent studies were simply pooled to analyze the association between SNPs and ESRD. In the absence of interstudy heterogeneity within samples, we also constructed a Mantel-Haenszel meta-analysis of data from the samples to assess the overall evidence of association. The Mantel-Haenszel χ^2 test and estimate of the OR were computed with or without the inclusion of covariates using the R, version 3.0.2, using the 'metafor' and 'meta' packages. The assumption of heterogeneity for each analysis was tested using the DerSimonian-Laird method. The level of significance was determined by the Bonferroni's method for correcting multiple testing errors.

In this study, power estimation was performed using CaTS (http://www.sph.umich.edu/csg/abecasis/CaTS/) and is summarized in the online supplementary table 2.

Results

Basic Characteristics of the Study Population

The frequency distributions of demographic characteristics between the overall 826 ESRD cases and 1,119 healthy controls in the two independent studies are shown in table 1. No significant differences were observed in diastolic blood pressure between ESRD patients and healthy controls. However, significant differences were observed in other variables between ESRD patients and controls ($p < 0.05$).

Association Analyses of TLR3 Polymorphisms with Susceptibility to ESRD

Table 2 shows genotype distributions and allelic frequencies of TLR3 polymorphisms in ESRD and control patients from the study 1 and study 2 populations re-

Table 1. Characteristics of ESRD and control patients

Variables	Study 1		Study 2		Study 1 + study 2		p value
	case (%)	control (%)	case (%)	control (%)	case (%)	control (%)	
Number	616	831	210	288	826	1,119	
Male (%)	290 (47.1%)	343 (41.3%)	95 (45.2%)	103 (35.8%)	385 (46.6%)	446 (39.9%)	0.003
Age, years	64.20±14.79	70.08±9.68	64.50±15.90	69.73±9.73	64.28±15.07	69.99±9.69	<0.0001
Body mass index, kg/m^2	22.40±3.88	24.60±3.54	22.42±4.26	24.54±3.60	22.40±3.98	24.58±3.55	<0.0001
Current or former smoker	130 (21.1%)	100 (12.0%)	35 (20.0%)	25 (8.7%)	143 (20.8%)	125 (11.2%)	<0.0001
Systolic blood pressure, mm Hg	140.47±33.67	127.03±16.11	144.67±49.78	126.52±15.73	141.54±38.42	126.90±16.00	<0.0001
Diastolic blood pressure, mm Hg	75.36±10.96	75.17±11.42	76.16±10.79	75.43±11.37	75.56±10.91	75.24±11.40	0.527
Fasting plasma glucose, mg/dl	153.04±61.38	100.78±23.97	153.12±58.93	101.41±26.16	153.06±60.73	100.94±24.55	<0.0001
eGFR, ml/min/1.73 m^2	5.52±2.10	84.27±15.70	5.65±2.23	84.63±16.31	5.55±2.08	84.36±15.86	<0.0001
BUN, mg/dl	61.51±18.90	16.55±6.62	60.55±18.62	17.15±9.14	61.26±18.82	16.70±7.35	<0.0001
Uric acid, mg/dl	7.25±1.34	5.56±1.41	7.01±1.28	5.66±1.83	7.19±1.33	5.59±1.53	<0.0001
Serum creatinine, mg/dl	9.58±2.54	0.91±0.73	9.50±2.75	0.91±0.89	9.56±2.59	0.91±0.77	<0.0001
Serum total cholesterol, mg/dl	168.08±186.27	34.31±35.67	163.77±34.61	185.81±35.84	166.98±34.42	186.15±35.70	<0.0001
Serum triglyceride, mg/dl	160.53±109.41	125.54±92.05	148.58±88.16	126.07±100.81	157.49±104.50	125.68±94.34	<0.0001

Table 2. Genotype distributions and allelic frequencies of TLR3 in ESRD

SNP	Study 1			Study 2		
	case	control	adjusted OR (95% CI)[1]	case	control	adjusted OR (95% CI)[1]
–7C/A						
CC	391	496	2.19 (1.23–3.90)	124	168	6.79 (1.53–30.11)
CA	204	272	2.14 (1.18–3.86)	84	98	7.62 (1.69–34.30)
AA	21	63	1	2	22	1
C allele	0.80	0.76	1.20 (0.97–1.48)	0.79	0.75	1.29 (0.91–1.82)
A allele	0.20	0.24	1	0.21	0.25	1
1377C/T						
CC	290	380	1.64 (1.07–2.50)	96	133	2.15 (0.98–4.72)
CT	275	336	1.64 (1.07–2.52)	102	116	2.56 (1.17–5.61)
TT	51	115	1	12	39	1
C allele	0.69	0.66	1.15 (0.96–1.38)	0.70	0.66	1.17 (0.86–1.60)
T allele	0.31	0.34	1	0.30	0.34	1
1234C/T						
CC	231	352	0.77 (0.52–1.15)	83	109	0.94 (0.47–1.89)
CT	301	386	0.95 (0.65–1.39)	102	148	0.91 (0.47–1.80)
TT	84	93	1	25	31	1
C allele	0.62	0.66	0.84 (0.71–1.01)	0.64	0.64	0.99 (0.73–1.33)
T allele	0.38	0.34	1	0.36	0.36	1

[1] Data have been adjusted by age, gender, BMI and smoking status.

spectively. No deviation from the HWE was observed in this study ($p > 0.05$). In the study 1, the Genotype distributions of –7C/A and 1377C/T in TLR3 were significantly different between ESRD patients and healthy controls ($p < 0.05$). When the TLR3 –7AA genotype was used as the reference group, –7CA and -7CC genotypes appeared to have a higher risk of ESRD (adjusted OR = 2.14, 95% CI = 1.18–3.86; adjusted OR = 2.19, 95% CI = 1.23–3.90, respectively). To see whether the results could be replicated in a different population we undertook another study for genotyping of the 3 SNPs. –7C/A was also significantly different in their genotype distribu-

Table 3. Association analyses for TLR3 SNPs with ESRD in the combined analyses of study 1 and study 2

SNP	Case	Control	Simply pooled OR (95% CI)[1]	p value	OR_{Meta} (95% CI)[1]	I^2
–7C/A						
CC	518	664	3.08 (1.78–5.34)	<0.001	3.10 (1.11–8.64)	48.09
CA	288	370	3.09 (1.76–5.42)	<0.001	3.32 (1.02–10.84)	57.67
AA	85	85	1			
C allele	0.80	0.20	1.25 (1.04–1.49)	0.015	1.22 (1.02–1.47)	0
A allele	0.76	0.24	1			
1377C/T						
CC	386	513	1.74 (1.20–2.52)	0.004	1.74 (1.20–2.54)	0
CT	377	452	1.83 (1.26–2.65)	0.002	1.82 (1.25–2.64)	0
TT	63	154	1			
C allele	0.70	0.30	1.17 (1.00–1.37)	0.048	1.16 (0.99–1.35)	0
T allele	0.66	0.34	1			
1234C/T						
CC	314	461	0.81 (0.58–1.14)	0.231	0.81 (0.57–1.14)	0
CT	403	534	0.94 (0.67–1.30)	0.691	0.94 (0.68–1.31)	0
TT	109	124	1			
C allele	0.62	0.38	0.90 (0.77–1.05)	0.166	0.87 (1.02–10.84)	0
T allele	0.65	0.35	1			

[1] Data have been adjusted by gender, age and BMI.

tions between the ESRD cases and healthy controls in this second study group (adjusted OR = 7.62, 95% CI = 1.69–34.30; adjusted OR = 6.79, 95% CI = 1.53–30.11, respectively). The 1234C/T SNP demonstrated no significant genotypic and allelic association between ESRD cases and healthy controls in either the study 1 and 2 study groups (table 2).

Association Analyses for the TLR3 Gene Polymorphisms in the Combined Two Independent Studies

When the data from the two independent studies were combined, the association of the –7C/A polymorphism with ESRD is maintained (adjusted OR = 3.09, 95% CI = 1.76–5.42; adjusted OR = 3.08, 95% CI = 1.78–5.34, respectively). In addition, the minor allele of –7C/A appeared now to be associated with a statistically significantly higher risk of ESRD (adjusted OR = 1.25, 95% CI = 1.04–1.49) (table 3). After correction for multiple comparisons, TLR3 –7CA and TLR3 –7CC genotypes appeared to have a higher risk of ESRD, but TLR3 1377C/T appeared to have no statistically significant association. No significant differences were observed in genotypic and allelic frequencies of TLR3 1234C/T between ESRD patients and healthy controls. In dominant genetic models, results show that –7C/A is a risk factor of ESRD (adjusted OR = 2.17, 95% CI = 1.23–3.86, p = 0.008). However, no significant differences were observed in the three TLR3 SNPs between ESRD patients and controls in the recessive model (data not shown).

TLR3 –7C/A Gene Polymorphism Associated with Risk of Different ESRD Causes

We analyzed for possible associations between TLR3 –7C/A gene polymorphisms and underlying ESRD etiology. After stratifying the ESRD patients according to different underlying causes for renal disease, a marginal associations with genotype and hypertension (adjusted OR = 1.78, 95% CI = 1.01–3.13) and glomerulonephritis (adjusted OR = 3.29, 95% CI = 1.17–9.20) but not with diabetic, systemic nephropathy or nephropathy arising from other or unknown (table 4).

Haplotype Analysis of TLR3

Haplotype analysis of TLR3 polymorphisms in ESRD patients and healthy controls is shown in table 5. The frequency of haplotype 'ATC' was 18.1% in the ESRD patients compared with 20.7% in the controls (OR = 0.85, 95% CI = 0.72–0.99). ACT haplotype frequency was 0.9% among ESRD patients compared with 1.7% in controls (OR = 0.54, 95% CI = 0.30–0.97). Other haplotypes showed no significant change.

Table 4. TLR3 –7C/A polymorphism with risk of different cause of ESRD

Cause of ESRD	Model	Adjusted OR (95% CI)[1]	p value
Diabetic nephropathy (n = 244)	CC/AA	1.47 (0.72–3.00)	0.296
	CA/AA	1.22 (0.58–2.59)	0.598
Glomerulonephritis (n = 192)	CC/AA	2.54 (0.92–7.02)	0.073
	CA/AA	3.29 (1.17–9.20)	0.024
Hypertensive nephropathy (n = 78)	CC/AA + CA	1.78 (1.01–3.13)	0.047
Systemic nephropathy (n = 42)	CC/AA	1.98 (0.25–15.69)	0.517
	CA/AA	2.51 (0.31–20.32)	0.390
Nephropathy with other causes or unknown[2] (n = 60)	CC/AA	4.01 (0.53–30.22)	0.177
	CA/AA	3.23 (0.41–25.26)	0.264

[1] Adjusted for gender, age, BMI, smoking status. [2] Others: e.g. kidney stone, polycystic kidney disease etc.

Table 5. Haplotype frequencies of TLR3 between ESRD and control patients (study 1 and study 2)

–7C/A	1377C/T	1234C/T	Freq. (case)	Freq. (control)	OR (95% CI)
C	C	T	0.351	0.321	1.14 (1.00–1.31)
C	C	C	0.327	0.308	1.09 (0.95–1.25)
A	T	C	0.181	0.207	0.85 (0.72–0.99)*
C	T	C	0.107	0.121	0.87 (0.71–1.07)
A	C	T	0.009	0.017	0.54 (0.30–0.97)*
A	C	C	0.009	0.014	0.63 (0.33–1.19)

* p value <0.05.

Association of TLR3 –7C/A Genotype with TLR3 mRNA Expression

In silico analysis revealed that the T allele of –7C/A generated several novel binding sites for different transcription factors; the potential regulatory transcription factor-binding motif with the highest score corresponded to the glucocorticoid receptor (GR) response element (fig. 1). Primary PBMC cultures were incubated with 100 nM dexamethasone for 24 h; we found significant differences in TLR3 expression between different genotypes [mean ± standard error of the mean (SEM); relative mRNA expression, 1.07 ± 0.12 (n = 23) and 0.75 ± 0.09 (n = 19) in CC and CA + AA, respectively (p = 0.043 by Mann-Whitney U test)] (fig. 2). Using wild-type CC as a reference group, the CA + AA TLR3 mRNA expression was reduced approximately by 30.39%. Under basal conditions, no significant difference was observed in TLR3 mRNA expression between CC (n = 23) and CA + AA (n = 19) genotypes (basal condition, CC = 1.38 ± 0.12, CA + AA = 1.15 ± 0.14, mean ± SEM relative mRNA expression).

Comparison of Promoter Activity Between CC and AA Alleles of the TLR3 Promoter –7C/A

To establish whether TLR3 SNPs were functionally important, we investigated whether dexamethasone influenced TLR3 promoter activity using the luciferase reporter assay in HEK293 cells. Luciferase activity of the C allele was significantly higher in the absence (11,580 ± 360) and presence of dexamethasone (5,770 ± 260) in comparison with that of the A allele (basal condition, 5,910 ± 450; following dexamethasone treatment, 3,280 ± 40) (p < 0.01 and 0.02, respectively) (fig. 3).

Discussion

Here, we investigated the association of TLR3 polymorphisms with ESRD and showed that the TLR3 –7C/A polymorphism was significantly associated with ESRD. The C risk allele is a common allele with comparable frequencies among Asian, European and African popula-

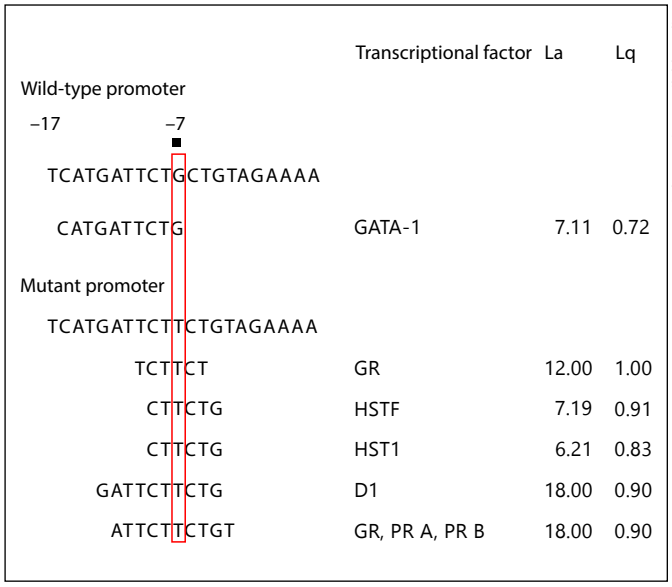

Fig. 1. In silico analysis of the *TLR3* promoter fragment containing the G/T substitution using the TESS interface. Parameters used in motif prediction included La (log-likelihood score) and Lq (measure of the goodness-of-fit of the DNA sequence to the consensus binding motif); the best possible Lq value was 1.000.

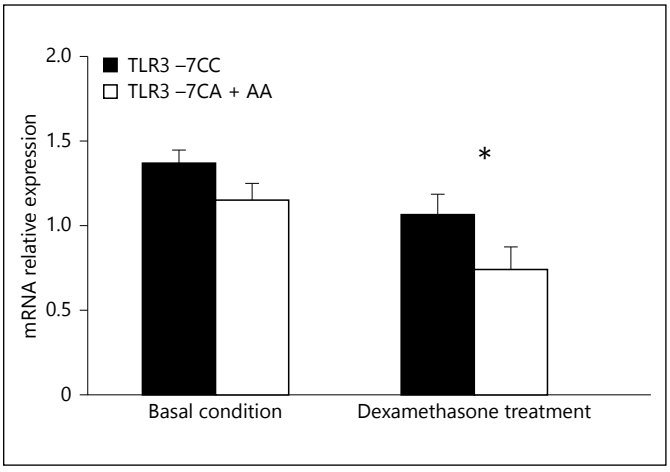

Fig. 2. Comparison of TLR3 promoter transcriptional level among different genotypes of rs3775296. β-actin was used as an internal control gene. TLR3 mRNA expression in CC (n = 23) or CA + AA (n = 19) PBMC cells following dexamethasone (100 nM) treatment. Values are mean ± SEM. Experiments were performed in triplicate.

tions, indicating that it may be an ancestral allele. Our study design was confined to a single ethnic group (Han Chinese); therefore, conclusions may not be applicable to other ethnic groups.

How polymorphisms in this study functionally influence renal disease onset or progression is not clear, but the role of TLR3 in renal disease development is supported by several studies. It has been reported that TLR3 signalling in human glomerular mesangial cells induces chemokine ligand 5 (CCL5) and C-X-C motif chemokine 10 (CXCL10) via the TLR3-interferon-β (IFN-β)-retinoic acid-inducible gene-I (RIG-I)-CCL5 [21] and TLR3-IFN-β-melanoma differentiation-associated protein 5 (MDA5)-CXCL10 axes [22], respectively.

In animal studies, it has been demonstrated that TLR3 activation induces renal disease progression in MRL-Fas (lpr) mice [7, 23]. Findings from another study suggest that TLR3, TLR4, TLR7 and TLR9 may play roles in the modulation of inflammatory processes in lupus nephritis [5]. TLR3 has been shown to be involved in antigen-induced immune complex glomerulonephritis [7], pathogenesis of lupus nephritis [8] and hepatitis C-associated glomerulonephritis [24, 25].

A number of studies have revealed that some SNPs in TLR3 are associated with several human diseases, such as

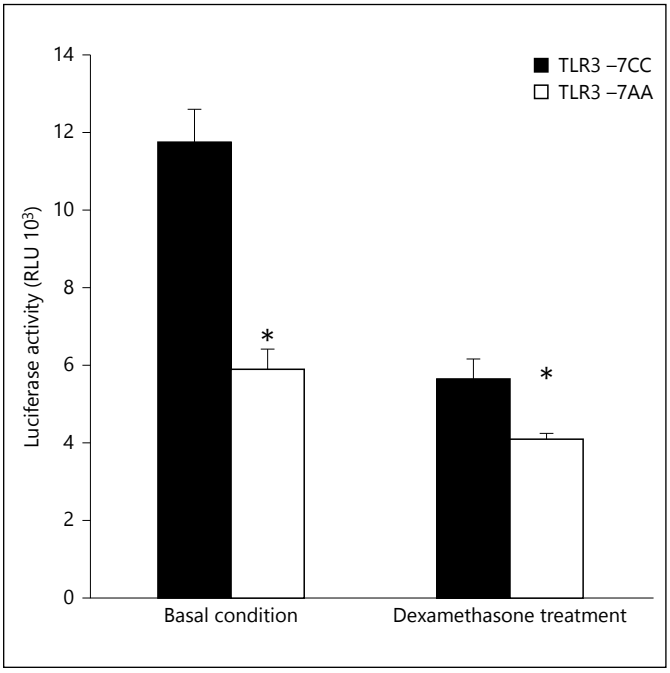

Fig. 3. Effects of the *TLR3* –7C/A genotype on luciferase activity in cultured HEK293cells. HEK293 cells were transfected with 0.4 μg pGL3 basic-LUC luciferase reporter recombinant plasmids containing an *TLR3* promoter sequence with the wild-type C allele or A allele at the –7C/A SNP. Transfected cells were grown with 100 nM dexamethasone for 24 h. Luciferase activity in cell extracts was expressed in relative light units (RLU). Mean ± SEM is given for each construct from three experiments.

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DOI: 10.1159/000365934

OA [10], nasopharyngeal carcinoma [26] and systemic lupus erythematosus (SLE) [27]. Here, the –7C/A polymorphism of TLR3 was strongly associated with ESRD. However, no significant differences were observed in TLR3 two SNPs (1377C/T, 1234C/T) between ESRD patients and controls after correction for multiple comparisons.

Haplotype analysis demonstrated an association between TLR3 and ESRD. ESRD patients carrying the ATC haplotype had a lower risk of ESRD. Our previous study also indicated that TLR3 haplotypes are associated with OA. Noguchi et al. indicated that no common TLR3 haplotype was associated with susceptibility to asthma or total serum immunoglobulin E levels [19]. Another study indicated that a TLR3 haplotype was associated with decreased susceptibility to melanoma (OR = 0.57, 95% CI = 0.35–0.95) compared with the most frequent haplotype [28]. To test the potential of genes highlighted by haplotype research fully, a follow-up study exploring the role of haplotypes in genetic susceptibility to ESRD may be desirable.

Specificity protein-1 (Sp-1), signal transducer and activator of transcription (STAT), GATA, interferon-stimulated response element (ISRE) and other transcription factors regulate TLR3 expression [29]. A previous study has found that the human TLR3 promoter contains functional ISRE or interferon regulatory factor elements [30]. Taura et al. have demonstrated that p53 activates TLR3 transcription by binding to the p53 consensus site in the TLR3 promoter [31]. Rb could modulate E2F transcription factor 1, which directly binds to the proximal promoter of TLR3 [32]. Regarding in silico analysis, TESS results also reveal several potential transcription factor binding motifs such as GR. Glucocorticoids and progesterone have been shown to influence TLR3 activity in dendritic cells [33]. However, the association between the transcription factor GR and TLR3 promoter transcriptional activity is unknown currently.

Our study is the first to analyze the distribution of polymorphisms of TLR3 gene and to assess the functionality of the TLR3 –7C/A polymorphism for transcriptional activity. The data suggest that TLR3 transcriptional activity of the variant C allele is higher than that of the mutant-type A allele. To determine whether the promoter polymorphism may modulate TLR3 expression, real-time PCR analysis was performed; significant differences were found in TLR3 expression among various genotypes following dexamethasone stimulation. Further investigation of the regulation of TLR3 expression by GR or dexamethasone in ESRD is needed.

Our study had some limitations. First limitation is that our patient group is not phenotypically homogenous (different primary renal diseases) and that might affect the results. Second, TLR3 is known to play a role in virus-associated glomerulonephritis. In our study, we have excluded patients with acute or chronic infection including viral infection such as HBV, HCV or HIV et al. Some patients with chronic glomerulonephritis still did not have definite diagnosis and were classified as unspecified. We cannot exclude the possibility that it is account for the TLR3 association. However, we thought the possibility is very rare. The TLR3 association is ESRD dependent dominantly.

Conclusion

In conclusion, we found that TLR3 SNPs, particularly –7C/A, were significantly associated with ESRD in the Chinese population. The functional study showed that –7C/A may be involved in ESRD development through transcriptional modulation of TLR3. It could provide a new insight into the role of TLR3 in this disease and has the potential to provide new treatment avenues and to identify individuals at risk.

Acknowledgements

This study was supported by grants from the Cardinal Tien Hospital and the Bureau of Health Promotion, Taiwan, ROC (CTH-102–1-2C07, CTH-101–1-2C03, DOH102-HP-1103, DOH101-HP-1103).

Disclosure Statement

No author has any interest that is potentially in conflict with this work.

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