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The T-1237C Polymorphism of the Toll-like Receptor-9 Gene Is Associated with Chronic Kidney Disease in a Han Chinese Population

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Chronic kidney disease (CKD) is increasingly recognized as a global public health problem. As inflammatory processes and genetic factors are involved in the pathogenesis of CKD, we have investigated the potential genetic contribution of Toll-like receptor (TLR) gene polymorphisms in CKD. In a case-control association study, 149 CKD patients and 429 healthy controls were genotyped by real-time polymerase chain reaction. CKD patients were defined as kidney damage (albuminuria, proteinuria or hematuria) or glomerular filtration rate < 60 ml/min/1.73 m^2 for 3 months or more. Single nucleotide polymorphisms (SNPs) at TLR-2 G2408A, TLR-4 A12874G and C13174T, and TLR-9 T-1237C, T-1486C, and G1635A were assessed, and linkage disequilibrium calculations and haplotype association analysis were undertaken. The functions of TLR-9 have been documented to recognize the viral and bacterial CpG DNA sequences, whereas detects microbe-derived peptidoglycan and lipopeptides and TLR-4 binds lipopolysaccharides. SNPs within the TLR genes may influence promoter activity, mRNA conformation and subcellular localization, and/or protein structure and function. Our results show that only the TLR-9 T-1237C and G1635A gene polymorphisms demonstrate an association with CKD (p = 0.002 and p = 0.04, respectively). The TLR-9 TCA haplotype at T-1237C, T-1486C, and G1635A was associated with a lower risk of CKD, whereas the TTA haplotype was associated with a higher risk of CKD. In the Han Chinese population, those who carry the C and A alleles at SNPs T-1237C and G1635A in the TLR-9 gene appear to be more susceptible to the development of CKD.

Keywords: chronic kidney disease; single nucleotide polymorphism; Toll-like receptor 2; Toll-like receptor 4; Toll-like receptor 9
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In Taiwan, 11.9% of the general population is estimated to have chronic kidney disease (CKD). The rate of CKD is projected to increase with an associated impact on public health costs (Hwang et al. 2010). The most common causes of CKD are diabetes, hypertension and glomerulonephritis, whilst other etiologies include inherited polycystic kidney disease, reflux nephropathy, kidney stones, infection and regular use of Chinese herbal medicines (McClellan and Flanders 2003; Wen et al. 2008). The importance of genetic factors on development and progression of CKD is increasingly recognized (Luttropp et al. 2009). CKD displays familial clustering that may be explained by the dual effects of genetic susceptibility and exposure to environmental agents (Satko et al. 2007). The interactions between genetic susceptibility, environment and CKD are likely to be complex but there is evolving evidence for involvement of the innate immune system, including members of the Toll-like receptor (TLR) family in kidney disease (Gluba et al. 2010).

Members of the TLR family play an important role in both innate and adaptive immune responses. To date 11 TLRs, expressed by different cells of the immune system,

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have been described in humans (Lien and Ingalls 2002). TLRs are germline encoded, evolutionarily conserved, pattern recognition receptors (PRRs) that recognize pathogen associated molecular patterns (PAMPs) (Akira et al. 2001). TLRs are activated by both exogenous ligands such as bacterial cell wall proteins (Werts et al. 2001; Hajjar et al. 2002) and unmethylated CpG motifs prevalent in bacterial and viral DNA (Schwartz and Cook 2005) or non-infectious molecules including cellular fibronectin, heat shock protein and endogenous peptides that are produced in response to tissue injury (Beutler and Rehli 2002). Activation of TLRs may stimulate production of inflammatory cytokines or induce apoptosis and contribute to tissue damage and disease progression.

TLR genes have been shown to be polymorphic (Schwartz and Cook 2005). Single nucleotide polymorphisms (SNPs) within the TLR genes influence promoter activity (gene expression), mRNA conformation (stability), and subcellular localization of mRNAs and/or protein structure and function. As such they may influence the risk of bacterial and viral infections (Papadopoulos et al. 2010) and susceptibility to, or severity of, a range of conditions, including cardiovascular, autoimmune and malignant diseases (Misch and Hawn 2008; Shastry 2009; Drexler and Foxwell 2010).

There is increasing evidence for involvement of TLRs in the pathogenesis of renal disease (Anders and Schlondorff 2007; Smith 2008; Robson 2009). Expression of TLRs-2, 4 and 9 is altered in a range of human renal diseases (Batsford et al. 2011) and TLR-9 gene polymorphisms have been shown to be associated with progression of IgA nephropathy (Suzuki et al. 2008). As yet there have been no reports on the relationship of TLR gene polymorphisms with CKD. The present study therefore aims to identify whether there are associations between TLR-2, TLR-4 and TLR-9 gene polymorphisms and CKD in a Han Chinese population.

Subjects and Methods

Patients and controls

This case-control study included 149 (66 females and 83 males; age 69.26 ± 12.04) CKD patients with stages 3 to 5 disease receiving nephrology care at Cardinal Tien Hospital, Taipei, Taiwan. Kidney function was calculated using the four-variable equation of the Modification of Diet in Renal Diseases (MDRD) Study for estimating GFR (Levey et al. 2000). CKD was defined and classified according to the Kidney Disease Quality Outcome Initiative (Levey et al. 2005) where CKD is defined as kidney damage or glomerular filtration rate (GFR) < 60 ml/min/1.73 m^2 for 3 months or more. The 429 healthy control subjects (227 females and 202 males; age 71.78 ± 8.82) whose eGFR was ≥ 60 ml/min/1.73 m^2 with no albuminuria, proteinuria or haematuria were recruited from the Center of Physical Examination

Table 1. Primer sequences for genotyping of TLR polymorphism.

Gene	SNP ID	SNP	Primer
TLR-2	rs5743708	G2408A	Sense
			5′-AGTGAGCGGGATGCCTACT-3′
			Antisense
			5′-GACTTTATCGCAGCTCTCAGATTTAC-3′
TLR-4	rs4986790	A12874G	Sense
			5′-ATTTAAAGAAATTAGGCTTCATAAGCT-3′
			Antisense
			5′-CCAAGAAGTTTGAACTCATGGTAA-3′
	rs4987233	C13174T	Sense
			5′-ATTTAAAGAAATTAGGCTTCATAAGCT-3′
			Antisense
			5′-CCAAGAAGTTTGAACTCATGGTAA-3′
TLR-9	rs5743836	T-1237C	Sense
			5′-TCCCAGCAGCAACAATTCATTA-3′
			Antisense
			5′-CTGCTTGCAGTTGACTGTGT-3′
	rs187084	T-1486C	Sense
			5′-ATGGGAGCAGAGACATAATGGA-3′
			Antisense
			5′-CTGCTTGCAGTTGACTGTGT-3′
	rs352140	G1635A	Sense
			5′-CAAGTCCAGCCAGATCAAA-3′
			Antisense
			5′-GCTAGACCTGTCCCACAATAA-3′

at Cardinal Tien Hospital. Clinical information was collected from the medical records. The relevant institutional committee has given approval for this study and participants gave their written informed consent to participate.

Genomic DNA extraction

Venous blood (5 ml) was collected in tubes containing ethylenediaminetetraacetic acid (EDTA) (50 mM of disodium salt) and genomic DNA isolated from the peripheral blood leucocyte cell pellet with the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) and stored at −80°C before testing.

Genotyping of the TLR gene polymorphisms

Six SNPs, TLR-2 G2408A (rs5743708), TLR-4 A12874G (rs4986790) and C13174T (rs4987233), TLR-9 SNPs T-1237C (rs5743836), T-1486C (rs187084) and G1635A (rs352140) were assessed by real-time polymerase chain reaction (RT PCR). Genotypes were determined using the LightCycler 1.2 System (Roche Diagnostics, Salt Lake City, UT, USA). Primer and detection probes for each polymorphism were based on Hamann et al. (2004) and Soriano-Sarabia et al. (2008). The primers and probes used in this study were shown in Tables 1 and 2, respectively. Melting curve analyses for TLR genes were performed using 2.5 mM of each detection probe. After an initial denaturation at 95°C for 10 minutes at a ramp rate of 4.4°C /s, the temperature was dropped to 45°C at a ramp rate of 1°C /s and then raised to 80°C with one acquisition per degree Celsius. Genotyping was performed by laboratory personnel blinded to case status, and 10% of the samples were randomly selected for repeated testing to validate genotyping procedures.

Statistical analysis

For this case-control association study demographic and clinical data between groups were compared by the Student's *t* test or Mann-Whitney *U* test. The allele and genotype frequencies of the control and patient groups were compared using χ^2 statistics or Fisher's exact test when the expected count was less than five in > 33% of the input cells of the contingency table. The odds ratios (ORs) and corresponding 95% confidence intervals (CIs) for assessing the effect of the genotype distribution and allele frequencies on CKD were calculated by multiple logistic regression analysis with adjustment for relevant significant variables. $P < 0.05$ was considered statistically significant for SNP association. Linkage disequilibrium (LD) and haplotype analyses were performed using Haploview software (Barrett et al. 2005) (http://www.broad.mit.edu/mpg/haploview/) and WHAP (Purcell et al. 2007) (http://pngu.mgh.harvard.edu/~purcell/whap/), respectively. Using Quanto software (http://hydra.usc.edu/GxE/) (Gauderman 2002) for a power of 0.8 at a significance level of $p < 0.05$ ninety one cases and 91 controls are required. Data were analyzed using SPSS

Table 2. Detection probes and melting temperatures.

Gene	Probe	WT (°C)	MT (°C)
TLR-2	Sensor	61.8	66.3
G2408A	5′-CAAGCTGCAGAAGATAATGAACACCAAG-3′FL		
	Anchor		
	LC Red640-5′-CCTACCTGGAGTGGCCCATGGACG-3′		
TLR-4	Sensor	62.0	58.0
A12874G	5′-CTACTACCTCGATGATATTATTGACTTATT-3′-FL		
	Anchor LC		
	Red640-5′-AATTGTTTGACAAATGTTTCTTCATTTTCC-3′		
TLR-4	Sensor	67.0	60.0
C13174T	5′-CTTGAGTTTCAAAGGTTGCTGTTCTCAAAGT-3′-FL		
	Anchor		
	LC Red705-5′-ATTTTGGGACAACCAGCCTAAAGTAT-3′		
TLR-9	Sensor	52.3	61.6
T-1237C	5′-GGAGTTTCCAGGCAGAGG-3′-FL		
	Anchor		
	LC Red705-5′-ACAGCACATCCCAAGGCCCT-3′		
TLR-9	Sensor	51.4	60.6
T-1486C	5′-ATCACTGCCCTCAAGAAGCT-3′-FL		
	Anchor LC		
	Red705-5′-ACAGCACATCCCAAGGCCCT-3′		
TLR-9	Sensor	56.0	65.0
G1635 A	5′-TCCAGTCGCGGTAGCTCC-3′		
	Anchor LC		
	Red640-5′-GCTGTTGTAGCTGAGGTCCAGGG-FL		

WT, wild-type allele; MT, mutated allele; FL, Fluorescein; LC Red640, LightCyler Red-640; LC Red705, LightCycler Red-705.

statistical software 17.0 (SPSS Inc., Chicago, IL, USA).

Results

Basic characteristics of the study population

Participant baseline characteristics are presented in Table 3. Of the patients with CKD 65 patients (43.6%) had stage 3 disease, 47 (31.6%) stage 4 disease and 37 (24.8%) stage 5 disease. The cause of the CKD in this patient group was predominantly diabetic nephropathy (n = 75) and glomerulonephritis (n = 49) whilst the remainder (n = 25) had a variety of other etiologies. There was no significant difference in gender, age and BMI between the two groups. Patients with CKD had higher blood pressure and were more likely to be current or ex-smokers. In addition to having higher serum creatinine levels and increased GFR the patients with CKD had higher fasting blood glucose levels and higher levels of serum cholesterol and serum triglycerides.

TLR-2, 4 and 9 allele and genotype frequencies

The observed genotype distribution for the six SNPs was consistent with the Hardy-Weinberg equilibrium. There was no significant difference in the genotype or allele frequencies of the TLR-2 (G2408A), and TLR-4 (A12874G and C13174T) polymorphisms between the patient and control groups (data not shown). The distributions of genotype and allele frequencies of the TLR-9 gene in the patient and control groups are shown in Table 4. There was no difference in the distribution of the TLR-9 genotypes between the groups of patients whose CKD developed subsequent to diabetic nephropathy, glomerulonephritis or other causes (data not shown). There was a significant association, $p < 0.002$, with T-1237C and CKD. When the TLR-9 -1237TT genotype was used as the reference group, the -1237TC heterozygotes appeared to have a higher risk for CKD (adjusted OR = 8.87, 95% CI = 2.31-34.06; $p = 0.001$).

There were no significant differences in genotype or allele frequencies in TLR-9 T-1486C between CKD patients and controls ($p = 0.82$). Analysis of TLR-9 T-1486C and G1635A in both dominant and recessive modes demonstrated no significant difference in frequency of TLR-9 T-1486C in the CKD patients as compared with the control group whilst TLR-9 G1635A in the dominant mode showed an increase (adjusted OR = 1.95, 95% CI = 1.07-3.58; $p = 0.03$) in the CKD patients as compared with the control group. A case only analysis showed no association between TLR-9 gene polymorphisms and the clinical stages of CKD (Table 5).

Linkage disequilibrium and haplotype analysis of TLR-9

Linkage disequilibrium analysis confirmed a strong disequilibrium level between the TLR-9 SNPs with r^2 = 0.80, 0.89 and 0.84 for T-1237C and T-1486C, T-1486C and G1635A, T-1237C and G1635A, respectively. The haplotype analysis of TLR-9 polymorphisms in CKD patients and control subjects is shown in Table 6. Only four of the 8 possible haplotypes were observed in both CKD patients and control subjects. The common alleles in the TLR-9 haplotypes included TTG, TCA, TTA and TCG (frequency > 0.01). The frequency of haplotype TCA was 26.0% in the CKD patients compared with 34.0% in the controls (OR = 0.86, 95% CI = 0.50-0.92, $p = 0.01$). In contrast haplotype TTA was more common in the CKD cases (10.0%) than in the controls (3.0%) (OR = 4.30, 95% CI = 2.44-7.57, $p < 0.0001$).

Discussion

In this study we have looked at the association of TLR gene polymorphisms in Han Chinese patients with CKD and identified a significant association with TLR-9 T-1237C. A weaker association between TLR-9 G1635A and CKD was seen when the effects of other significant covariates (age, BMI, gender and smoking status) were adjusted for. No associations with gene polymorphisms at

Table 3. Characteristics of subjects with chronic kidney disease (CKD) and control subjects.

	CKD n = 149	Control n = 429	p-value
Male (%)	55.7%	47.0%	0.07
Age (yrs)	69.26 ± 12.04	71.78 ± 8.82	0.06
Body mass index (kg/m^2)	25.39 ± 3.58	24.87 ± 6.23	0.33
Current or former smoker (%)	44.3%	13.5%	< 0.0001
Systolic blood pressure (mmHg)	134.82 ± 18.56	129.09 ± 13.14	0.001
Diastolic blood pressure (mmHg)	76.22 ± 11.55	75.22 ± 10.28	0.01
Fasting plasma glucose (mol/L)	118.74 ± 42.93	93.58 ± 11.68	< 0.0001
GFR	29.21 ± 16.42	84.43 ± 16.54	< 0.0001
BUN	41.72 ± 23.66	16.28 ± 6.10	< 0.0001
Serum creatinine (mol/L)	3.03 ± 2.15	0.85 ± 0.19	< 0.0001
Serum total cholesterol (mol/L)	186.09 ± 39.80	173.71 ± 24.79	< 0.0001
Serum triglycerides (mol/L)	145.58 ± 74.31	106.26 ± 42.43	0.03

Quantitative data were means ± S.D.

Table 4. Genotype distributions and allele frequencies for the TLR-9 gene in control subjects and in patients with CKD.

Genotypes	Patients (n = 149)	Control (n = 429)	Crude OR (95% CI)	p-value	[+]Adjusted OR (95% CI)	p-value
	n or (%)	n or (%)				
T-1237C						
TT	139	425	1		1	
TC	10	4	7.64 (2.36-24.76)	< 0.0001*	8.87 (2.31-34.06)	0.001*
Alleles						
T-allele	96.6%	99.5%	1		1	
C-allele	3.4%	0.5%	7.41 (2.31-23.82)	< 0.0001*	8.48 (2.55-31.91)	0.002*
T-1486C						
TT	71	172	1		1	
TC	65	205	0.77 (0.52-1.14)	0.19	1.00 (0.61-1.63)	1.00
CC	13	52	0.61 (0.31-1.20)	0.15	1.21 (0.54-2.36)	0.76
Alleles						
T-allele	69.4%	64.0%	1		1	
C-allele	30.6%	36.0%	0.78 (0.59-1.04)	0.10	1.04 (0.74-1.46)	0.82
TT/TC+CC	71/78	172/257	0.74 (0.51-1.0)	0.12	1.02 (0.64-1.63)	0.92
TT+TC/CC	136/13	377/52	0.70 (0.37-1.33)	0.28	1.13 (0.56-2.26)	0.74
G1635A						
GG	56	166	1		1	
GA	69	211	0.97 (0.65-1.46)	0.90	1.18 (0.71-1.97)	0.52
AA	24	52	1.37 (0.77-2.42)	0.28	2.22 (1.13-4.34)	0.02
Alleles						
G-allele	60.7%	63.3%	1		1	
A-allele	39.3%	36.7%	1.12 (0.85-1.46)	0.45	1.41 (1.02-1.96)	0.04
GG/GA+AA	56/93	166/262	1.05 (0.72-1.55)	0.85	1.35 (0.84-2.18)	0.22
GG+GA/AA	125/24	376/52	1.39 (0.82-2.35)	0.26	1.95 (1.07-3.58)	0.03

[+]Data were expressed as n or (%) and have been adjusted by gender, age, BMI, and smoking status. *$p < 0.01$.

Table 5. Genotype distribution of CKD subgroups stratified by CKD stage.

Stage	No.	T-1237C			
		TT	TC	CC	p-value*
3	65	60	5	–	0.90
4	47	44	3	–	
5	37	35	2	–	
Stage	No.	T-1486C			
		TT	TC	CC	p-value
3	65	29	27	7	0.56
4	47	26	19	2	
5	37	15	8	4	
Stage	No.	G1635A			
		GG	GA	AA	p-value
3	65	25	32	8	0.92
4	47	20	17	10	
5	37	11	20	6	

*The p-value was calculated using 2 (3) × 3 χ^2 test.

Table 6. Haplotype frequencies in TLR-9 between CKD patients and control subjects.

Haplotype			Frequencies		*p*-value	95% CI
T-1237C	T-1486C	G1635A	CKD	Control		
T	T	G	0.58	0.61	0.26	0.86 (0.66-1.12)
T	C	A	0.26	0.34	0.01	0.86 (0.50-0.92)
T	T	A	0.10	0.03	< 0.0001	4.30 (2.44-7.57)
T	C	G	0.03	0.02	0.26	1.58 (0.71-3.53)

TLR-9 1486C, TLR-2 G2408A or TLR-4 A12874G and C13174T were identified.

The frequencies of the three TLR-9 SNPs rare alleles, -1237C, -1486C, and 1635A were 0.5%, 36.0% and 36.7%, respectively. Similar to other studies of TLR in Chinese populations (Cheng et al. 2007) our Han Chinese population displayed different allele frequencies of TLR-9 to Caucasians and African Americans. The frequency of TLR-2 2408A and TLR-4 12874G and 13174T variants in Caucasian populations ranges from 5% to 10% (Lorenz et al. 2000) but in the current study no gene variants at TLR-2 G2408A, TLR-4 A12874G or C13174T could be detected. Similar observations have been made in Japanese and Chinese populations previously suggesting that Asian populations also differ from Caucasian and African Americans in their TLR 2 and 4 gene properties (Noguchi et al. 2004). These geographic/ethnic-specific differences in TLR-2, TLR-4 and TLR-9 gene polymorphisms may influence development and progression of CKD and other diseases in different populations.

TLRs play a central role in innate immunity and a number of genetic association studies suggest that TLR gene polymorphisms may be associated with susceptibility to different diseases. Analysis of potentially functional polymorphisms in candidate genes such as TLRs provides an opportunity to increase our understanding of the relationship between genotype and phenotype in complex diseases. TLR-9 has been mapped to chromosome 3p21.3. It spans approximately 5 kb and has two exons, the second of which is the major coding region. TLR-9 SNPs have been associated with susceptibility and progression of a number of clinical diseases. In particular the T-1237C SNP is associated with an increased risk for asthma in European and American populations (Lazarus et al. 2003) and Crohn's disease in a German population (Torok et al. 2009). TLR-9 G1635A SNP appears to play a role in susceptibility to SLE (Xu et al. 2009) and in relation to renal disease, TLR-9 gene polymorphisms are associated and the histologic severity of disease in Japanese patients with IgA nephropathy (Suzuki et al. 2008).

In the current study TLR-9-1237TT appeared to be associated with a higher risk for CKD in comparison with TLR-9-1237TC (adjusted OR = 8.87, 95% CI = 2.31-34.06; $p = 0.001$). The wide range of the 95% CI however suggests that the sample size was possibly too small to provide definitive results and additional work will need to be undertaken to confirm this observation. Similarly, analysis with adjustment for the effects of other significant covariates (age, BMI, gender and smoking status) showed a weak association of CKD with the TLR-9 G1635A, which will need to be substantiated with a larger patient cohort. In the present study, the CKD patients carrying the TCA haplotype had a lower risk of CKD than those not carrying the TCA haplotype, and the patients carrying TTA haplotype had a higher risk of CKD than those not carrying the TTA haplotype. Interestingly, Holla et al. (2010) demonstrated that the TLR-9 TTA haplotype may increase susceptibility to chronic periodontitis whereas the TLR-9 TCG haplotype has a protective effect in a Czech population.

The potential role of TLR-9 polymorphisms in CKD is not currently clear. TLRs have been shown to be expressed by both immune cells and renal cells (Kazimierczak et al. 2007). TLR signaling may contribute to infection-associated initiation of kidney disease or infection-associated flares of established kidney disease. Unlike TLR-2 and 4, which are expressed predominantly at the cell membrane, TLR-9 is expressed mostly on endosome membranes (Barton and kagan 2009) and is involved in the recognisition of self and foreign nucleic acids (Hemmi et al. 2000). TLR-2 and TLR-4 expression, but not TLR-9 expression, is increased in leucocytes of patients with end stage renal disease (Gollapudi et al. 2010) suggesting that involvement of TLR-9 in CKD may be related to expression by renal epithelial and interstitial cells. Indeed increased expression of TLR-9 within the glomeruli is seen in patients with lupus nephritis (Papadimitraki et al. 2009). Furthermore, activation of TLR-9 by unmethylated CpG-oligodeoxynucleotide enhances the progression of renal disease in MRL-Fas (lpr) mice by increasing renal chemokine and chemokine receptor expression (Anders et al. 2004). Functionally characterized SNPs of TLR-9 in the HEK293 model system have been identified (Kubarenko et al. 2010). Interestingly for the most functionally impaired variant, Pro99Leu (rs5743844), the ability to respond to physiological and therapeutic TLR-9 ligands is severely compromised. Specific haplotypes in the TLR-9 gene might influence the ability of TLR-9 to arouse a defense mechanism by affecting susceptibility or resistance to infections, but most of the functions of TLR-9 haplotypes are still largely unknown.

In conclusion, our present results reveal that in the Han Chinese of Taiwan, there is an association with individuals who carry the C and A alleles of SNP T-1237C and

G1635A in the TLR-9 gene and development of CKD. Increased understanding of how TLR-9 gene polymorphisms affect development or progression of CKD may allow targeting of susceptible populations with novel therapies in the early stage of disease to improve clinical outcomes.

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Conflict of Interest

All authors have no conflict of interest.

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