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Association between Toll-like receptor 2 (*TLR2*) polymorphisms and asymptomatic bancroftian filariasis

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Abstract Lymphatic filariasis is mainly caused by the filarial nematodes *Wuchereria bancrofti* and *Brugia malayi*. *Wolbachia*, intracellular symbiotic bacteria in filarial parasite, is known to induce immune response predominantly through Toll-like receptor 2 (*TLR2*). This study was performed to investigate the association between polymorphisms of the *TLR2* gene and susceptibility to asymptomatic bancroftian filariasis. A total of 142 unrelated asymptomatic bancroftian filariasis patients and 151 endemic normal controls in Tak province, Thailand were recruited into this study. The −196 to −173 deletion (del) polymorphism in the 5′ untranslated region was investigated by allele-specific polymerase chain reaction. Two single nucleotide polymorphisms, +597 T>C and +1350 T>C, in exon 3 were identified by polymerase chain reaction–restriction fragment length polymorphism analysis. Furthermore, we analyzed the functional difference between the *TLR2* −196 to −173 del and wild-type (wt) alleles using the luciferase reporter assay. All three polymorphisms were associated with a higher risk of asymptomatic bancroftian filariasis and were in strong linkage disequilibrium with each other. The *TLR2* haplotype −196 to −173del/+597C/+1350C was strongly associated with an increased risk of asymptomatic bancroftian filariasis. The *TLR2* −196 to −173 del allele had a significantly lower transcriptional activity than wt allele. The results of our study indicate that *TLR2* −196 to −173 del, +597 T>C and +1350 T>C polymorphisms are associated with asymptomatic bancroftian filariasis in Thailand. Our functional study also supports this finding with respect to differential *TLR2* gene expression by −196 to −173 del polymorphism.

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Introduction

Lymphatic filariasis is caused by mosquito-transmitted lymphatic filarial parasites, including *Wuchereria bancrofti*, *Brugia malayi*, and *Brugia timori* (Guilbert 2003). More than 120 million people in 83 countries in the tropics and subtropics, including Thailand, are infected with filarial nematodes. The disease is ranked by the World Health Organization (1995) as the second leading cause of permanent and long-term disability, and it has been targeted by the International Task Force for Disease Eradication to be eliminated by the year 2020 (Behbehani 1998). In Thailand, lymphatic filariasis caused by *W. bancrofti* and *B. malayi* is endemic in the Thai–Myanmar and Thai–Malaysia borders, respectively (Triteeraprapab et al. 2000, 2001; Nuchprayoon et al. 2005).

Differential susceptibility to filarial infection within a population and within families has been documented (Subrahmanyam et al. 1978; Das et al. 1990, Tisch et al. 2001). The prevalence of lymphatic filariasis is affected by local socioeconomic status, most frequently by activities

that provide mosquito breeding sites (Haddix and Kestler 2000). The frequency of lymphatic filariasis infection increased with increasing age, consistent with the notion that the parasite burden increases with cumulative exposure to mosquito-borne larvae (Tisch et al. 2001). In addition, males are more susceptible to lymphatic filariasis infection than females (Brabin 1990). Previous studies have shown the association between polymorphisms in various host genes [e.g., human leukocyte antigen (*HLA*), Chitotriosidase (*CHIT1*), mannose-binding lectin (*MBL*), and vascular endothelial growth factor A (*VEGF-A*)] and susceptibility as well as clinical types of lymphatic filariasis (Chan et al. 1984; Yazdanbakhsh et al. 1997; Chanock and Foster 1999; Choi et al. 2001; Debrah et al. 2007; Meyrowitsch et al. 2010). However, its actual cause has not yet been elucidated to date.

Toll-like receptors (*TLRs*), a family of pattern recognition receptors, function as important sensors of infection in the innate immune system that, in turn, directs the responses of the adaptive immune system (Medzhitov 2001; Takeda et al. 2002; Venugopal et al. 2009). Mutations and polymorphisms in the *TLR2* gene that affect host susceptibility to infectious diseases (e.g., leprosy, tuberculosis, and Lyme disease) have been identified (Ben-Ali et al. 2004; Ogus et al. 2004; Schroder and Schumann 2005; Thuong et al. 2007; Velez et al. 2010).

The filarial parasites, *W. bancrofti*, *B. malayi*, and *Onchocerca volvulus*, harbor an intracellular symbiotic bacterium, *Wolbachia* (Bazzocchi et al. 2000; Hoerauf et al. 2000; Bandi et al. 2001). Many studies have shown that filarial parasites, through products of their *Wolbachia* bacteria, can elicit an immune response predominantly via TLR2 (Brattig et al. 2001, 2004; Punkosdy et al. 2003). Brattig et al. (2004) showed that the *Wolbachia* surface protein (WSP) can induce an inflammatory response, measured by pro-inflammatory cytokines, in murine macrophages and dendritic cells through both TLR2 and TLR4. Further studies have demonstrated that the inflammatory pathways activated by endosymbiotic *Wolbachia* in *B. malayi* and *O. volvulus* filaria are dependent on TLR2/6 recognition and signaling through the myeloid differentiation primary response gene 88 (MyD88) and the MyD88 adaptor-like protein pathway (Hise et al. 2007). A recent study has provided evidence that *B. malayi Wolbachia* lipoprotein, peptidoglycan-associated protein (wBmPAL), stimulates innate and adaptive immunity through the activation of TLR2/6 receptors and, to a minor extent, TLR2/1 heterodimers (Turner et al. 2009).

As TLR2 plays an important role in the immune response against *Wolbachia* (Hise et al. 2007), polymorphisms of the *TLR2* gene may be relevant to susceptibility to *W. bancrofti* infection. By unrelated case–control association and functional studies, we investigated the association between *TLR2* gene polymorphisms and susceptibility to asymptomatic bancroftian filariasis.

Materials and methods

Study areas and population

This study was approved by the Ethics Committee of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand. The objective of the study was disclosed to the subjects, and their written informed consent was obtained before drawing any blood samples. Every infected individual was given the standard treatment (diethylcarbamazine).

In cooperation with the Vector Born Disease Control Center (VBDC) 9.3 (Tak Province), Department of Disease Control, Ministry of Public Health, Thailand, blood samples were collected from the study populations in Tak Province in the west of Thailand between 1998 and 2006. The study area is known for its endemicity for lymphatic filariasis caused by *W. bancrofti*, which is mainly transmitted by *Ochlerotatus* (*Aedes*) *niveus* group. Criteria for selecting the villages for this study were that they had not previously received antifilarial treatment. All of the study populations are in rural areas in nature. They mainly depend on subsistence agriculture and related activities, such as agricultural labor. Bed nets had been used habitually by residents of study area for several years prior to this study. All individuals in the villages were asked to come for a blood test. Pregnant women, babies <2 years of age, and sick individuals were excluded.

We recruited 142 unrelated clinically asymptomatic bancroftian filariasis patients who had antigenemia [positive for NOW® ICT Filariasis Test (Binax, Portland, ME) or the Og4C3 ELISA (TropBio Pty Ltd., Townsville, Queensland, Australia)]. We also recruited 151 unrelated normal controls who fulfilled the following criteria: negative for circulating filarial antigens, had not experienced filariasis-specific symptoms or signs, the same ethnic group as the bancroftian filariasis patients, and had been living for more than 10 years in the same household as the bancroftian filariasis patients. Among the 142 unrelated bancroftian filariasis patients, 75 (52.82%) were male and 67 (47.18%) were female. Of the 151 endemic normal controls, 79 (52.32%) were male and 72 (47.68%) were female. The mean age (±SD) in the bancroftian filariasis patients and endemic normals was 29.9±12.5 and 30.8±12.9 years, respectively. The bancroftian filariasis patients and normal controls were not significantly different in terms of gender, age, and bed net usage (*P*>0.05, data not shown). Characteristics of the study population are shown in Table 1.

Table 1 Characteristics of the study population

Characteristics	Bancroftian filariasis patients	Normal controls
Gender (M/F)	75/67	79/72
Median age (range)	30.6 (16–61)	31.8 (18–68)
Mean age (±SD)	29.9±12.5	30.8±12.9
Treatment	No	No
Bed net usage	Yes	Yes
Pathology	None	None
Ag Test (ICT card test and Og4C3 ELISA)	All positive	All negative
W. bancrofti circulating antigen levels (U/ml, range)	29,986 (133–71,339)	<32 (<32)

Selection of *TLR2* polymorphisms

TLR2 polymorphisms were selected from a literature review and the public database of TLR SNPs (Innate Immunity Program in Genomic Applications database; available at http://innateimmunity.net/). The −196 to −174 deletion (del) polymorphism in the 5′ untranslated region (UTR) of the *TLR2* gene displays reduced transcriptional activity (Noguchi et al. 2004). Thus, this polymorphism was selected for our study. From the public database of TLR SNPs, SNPs in the coding region of *TLR2* that induce amino acid changes as well as synonymous SNPs with a frequency of >5% were selected for genotyping. On the basis of these criteria, four SNPs were selected [+597 T>C, Asn199Asn, rs3804099; +1350 T>C, Ser450Ser, rs3804100; +2029 C>T, Arg677Trp (no rs designation available); and +2258 G>A, Arg753Gln, rs5743708]. However, only two synonymous SNPs (+597 T>C and +1350 T>C) in exon 3 have been detected in the Asian population and thus chosen for this study.

DNA extraction from blood samples

Genomic DNA was extracted using a modified salting out procedure (Miller et al. 1988). Briefly, buffy coats of nucleated cells obtained from EDTA-treated blood were resuspended in nuclei lysis buffer (10 mM Tris–HCl, 400 mM NaCl, and 2 mM EDTA, pH 8.2). The cell lysates were digested overnight at 37°C with 0.5 mg/ml proteinase K and 1% SDS. Proteins were then precipitated with a solution of saturated NaCl and removed by centrifugation. Finally, genomic DNA was ethanol-precipitated, washed with 70% ethanol, and resuspended in 100 μl TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.5).

Genotyping for *TLR2* polymorphisms

To genotype −196 to −173 del polymorphism, primers flanking the region containing the 23-bp del were designed as previously described (Noguchi et al. 2004). Polymerase chain reaction (PCR) was carried out using the Perkin Elmer thermal cycler Gene Amp #2400 in a reaction volume of 25 μl containing PCR buffer (10 mM Tris–HCl, pH 9.0, 1.5 mM MgCl$_2$, 50 mM KCl; MBI Fermentas, Ontario, Canada), 200 μM dNTP mix (MBI Fermentas), 1.0 U of *Taq* DNA polymerase (MBI Fermentas), 10 pmol of each primer, and 50–100 ng of genomic DNA. After incubation at 94°C for 5 min, amplification was carried out for 35 cycles with the following temperature cycling parameters: denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s with a final extension at 72°C for 10 min. PCR products were distinguished with 3% agarose gel, stained with ethidium bromide, and visualized with UV transilluminator.

The +597 T>C polymorphism was identified using the PCR–restriction fragment length polymorphism (PCR-RFLP) analysis. The polymorphic region containing the *Mae*II restriction site at position +597 (T>C) was amplified using primer +597-F primer (5′-CCTGAGAGTGGGAAA TATGGAC-3′) and +597-R primer (5′-CTCCATTAAGGG TACAGTCATC-3′). The PCR was carried out in a reaction volume of 50 μl containing PCR buffer, 200 μM dNTP mix, 1.0 U of *Taq* DNA polymerase, 10 pmol of each primer, and 75–100 ng of genomic DNA as the target. After incubation at 94°C for 5 min, the 35 cycles of amplification were carried out as follows: denaturation at 94°C for 30 s, annealing at 52°C for 30 s, and extension at 72°C for 30 s with final extension at 72°C for 10 min. The 10 μl aliquot of PCR products were digested with 5.0 U of *Mae*II restriction enzyme according to the manufacturer's protocols (MBI Fermentas). Analysis of DNA fragments was performed by electrophoresis on a 2.5% agarose gel.

The +1350 T>C polymorphism was identified by PCR-RFLP technique. The polymorphic region containing the *Mwo*I restriction site at position +1350 (T>C) was amplified using +1350-F primer (5′-AGCCTGTGAG GATGCCTG-3′) and +1350-R primer (5′-AACATGGG TAAGAGGGAGGC-3′). PCR amplification was performed under the same conditions as applied for *TLR2* +597 T>C genotyping. The PCR products were digested with 5.0 U of *Mwo*I restriction enzyme according to the manufacturer's protocols (MBI Fermentas). Analysis of DNA fragments was performed by electrophoresis on a 2.5% agarose gel.

Sequencing of PCR products

The accuracy of genotyping was confirmed by direct sequencing of samples obtained from five randomly selected bancroftian filariasis patients and normal controls. The regions of interest were amplified and then subjected to direct sequencing with the Big Dye Terminator kit according to the manufacturer's protocol (Applied Biosystems, Foster City, CA, USA) on an ABI PRISM 3100 DNA Sequencer (Applied Biosystems).

Linkage disequilibrium

Pairwise linkage disequilibrium (LD) among the three polymorphisms was calculated using the Java LINkage Disequilibrium Plotter (JLIN; Carter et al. 2006).

Haplotype analysis

Haplotype phases (computed using expectation–maximization algorithm) and haplotype associations were both determined using PLINK v1.06 software (Purcell et al. 2007).

Statistical analysis

Statistical analysis was carried out using PLINK v1.06 software (Purcell et al. 2007). The goodness of fit to Hardy–Weinberg Equilibrium (HWE) was performed using a chi-square (χ^2) test. Allele and genotype frequencies were determined by direct counting and then divided by the number of chromosomes to produce an allele frequency or by the number of subjects to produce the genotype frequency.

To assess associations of interest, allele or genotype frequencies were compared between bancroftian filariasis patients and normal controls in a different model. The models were the allelic model, the genotypic model, the additive model (Cochran–Armitage trend test), dominant model and recessive model. All test statistics were distributed as χ^2 or Fisher's exact test with 1 *df* under the null, with the exception of the genotypic test which has 2 *df*. Logistic regression analyses were used to compute the odds ratios (OR) and 95% confidence interval (CI). A *P* value of <0.05 was considered significant.

Cell culture

The human acute monocytic leukemia cell line THP-1 was kindly provided by Assoc. Prof. Dr. Sukathida Ubol (Faculty of Science, Mahidol University, Bangkok, Thailand). The cells were cultured in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO, USA) with 10% heat-inactivated fetal calf serum (FCS; Sigma-Aldrich). Cells were grown at 37°C in a 5% carbon dioxide atmosphere.

Plasmid construction

A fragment of *TLR2* from nucleotides −281 to +108 relative to the transcription initiation site, containing promoter and 5′ UTR, was amplified with *Bg*/II-TLR2F primer (5′-GAAGATCTTCCTTCTTCGCAGCCTTG-3′) and *Bg*/II-TLR2R primer (5′-GAAGATCTTCCTGGGAGAACTCC GAGCAG-3′) from the genomic DNA of a subject homozygous for −196 to −173del allele and homozygous for wild-type (wt) allele. Following digestion with *Bgl*II, the PCR product was cloned into the *Bgl*II-digested pGL3-Basic Vector (Promega, Madison, WI, USA). All plasmids were purified using the endo-free plasmid purification kit from Qiagen (Valencia, CA, USA). All constructs were sequence-verified. The correct orientation of all constructs was confirmed.

Transient transfection and luciferase assay

The THP-1 cells were washed twice and resuspended in culture medium lacking FCS. The THP-1 cells (1×10^7 cells in 800 μl) were co-transfected with 10 μg of the experimental construct and 0.1 μg of pRL-CMV as a control to monitor the transfection efficiency by electroporation at 400 V for 252 ms using the EC100 electroporator (E-C apparatus Corporation, Holbrook, NY, USA). After incubation for 24 h, the cells were analyzed for the firefly and *Renilla* luciferase activities using Dual-Glo™ luciferase assay system according to the manufacturer's instructions (Promega) on a VICTOR3 luminometer (PerkinElmer, Waltham, MA, USA). In each transfection experiment, untransfected cells were included as controls for a background level of firefly and *Renilla* luciferase. The firefly luciferase activities were normalized to *Renilla* luciferase activities. Luciferase levels were reported as fold elevation in activity over that seen in transfections with the promoterless pGL3-Basic vector.
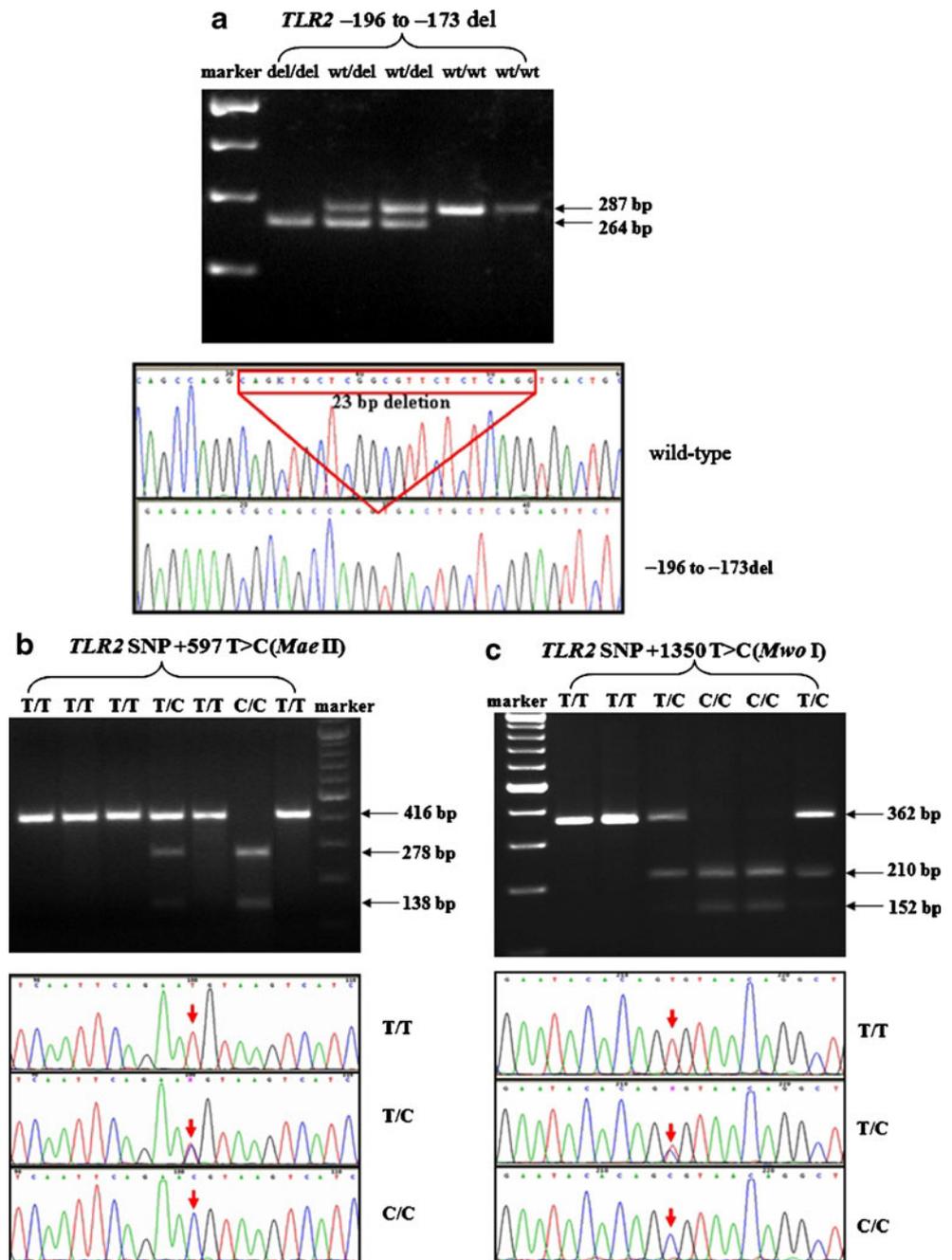
Results

Genotyping for *TLR2* polymorphisms

The genotype frequencies of three polymorphisms in *TLR2* gene, −196 to −173 del, +597 T>C, and +1350 T>C, displayed HWE (*P*>0.05, data not shown). Representative results of gel electrophoresis patterns and chromatogram of DNA sequences of *TLR2* polymorphisms are shown in Fig. 1.

The *TLR2* −196 to −173 wt/wt homozygote showed a single band of 287 bp, whereas the *TLR2* −196 to −173 del/del homozygote displayed a single band of 264 bp. The

Fig. 1 Sequence analysis and gel electrophoresis patterns of *TLR2* polymorphisms: **a** −196 to −173 del, **b** +597 T>C, and **c** +1350 T>C. *Black arrows to the right of the gel electrophoresis photos* indicate the possible PCR products and their expected sizes. The 23-bp deletion is shown by a *rectangle* in the truncated sequence, while the positions of the SNPs are indicated by the *red arrows* in the DNA sequencing chromatograms

TLR2 −196 to −173 wt/del heterozygote resulted in two bands of 287 and 264 bp. Interestingly, 23-bp deletion polymorphism was found in the Thai population, while only 22-bp deletion polymorphism was found in the Japanese population (Noguchi et al. 2004; Wang et al. 2007). The −196 to −173 del was previously reported as −196 to −174 del in the Japanese population (Noguchi et al. 2004; Wang et al. 2007).

The *TLR2* +597 T/T homozygote PCR products do not contain the *Mae*II restriction site and remained undigested, showing a single band of 416 bp. The *TLR2* +597 C/C homozygote PCR products contained the *Mae*II restriction site, showing two bands of 138 and 278 bp. The *TLR2* +597 T/C heterozygote showed three bands of 138, 278, and 416 bp.

The *TLR2* +1350 T/T homozygote PCR products do not contain the *Mwo*I restriction site and remained undigested, showing a single band of 362 bp. *TLR2* +1350 C/C homozygote PCR products contained the *Mwo*I restriction site, showing two bands of 152 and 210 bp. The *TLR2* +1350 T/C heterozygote showed three bands of 152, 210, and 362 bp.

Genotype distribution and allele frequencies

Genotype distribution and allele frequencies of *TLR2* polymorphisms in bancroftian filariasis patients and normal controls were compared to determine susceptibility to *W. bancrofti* infection. Genotype and allele frequencies of *TLR2* polymorphisms, −196 to −173 del, +597 T>C, and +1350 T>C, in bancroftian filariasis patients and normal controls are shown in Tables 2 and 3. All three *TLR2* polymorphisms, −196 to −173 del, +597 T>C, and +1350 T>C, were associated with asymptomatic bancroftian filariasis in both allelic and genotypic comparisons.

The allele frequency of the −196 to −173del was significantly higher in bancroftian filariasis patients (23.24%) compared with normal controls (12.91%; OR = 2.04, 95%CI = 1.32–3.15, χ^2 = 10.61, P = 0.0011). The genotype frequencies of this polymorphism were also significantly different in bancroftian filariasis patients when compared to normal controls (χ^2 = 9.48, P = 0.0087). The association of the −196 to −173 del with asymptomatic bancroftian filariasis was confirmed in both the additive (χ^2 = 9.20, P = 0.0024) and dominant models (χ^2 = 9.01, P = 0.0026).

The allele frequency of +597C was significantly higher in bancroftian filariasis patients (20.77%) compared with normal controls (10.93%; OR = 2.19, 95%CI = 1.35–3.39, χ^2 = 10.72, P = 0.0011). Analysis using Fisher's exact test showed a significant difference between bancroftian filariasis patients and normal controls for genotype frequencies of +597 T>C (P = 0.0027). Moreover, +597 T>C SNP was significantly associated with the susceptibility to asymptomatic bancroftian filariasis in both

additive (χ^2 = 11.07, P = 0.0008) and dominant models (χ^2 = 10.00, P = 0.0016).

The allele frequency of the +1350C was significantly higher in bancroftian filariasis patients (14.08%) compared with normal controls (7.62%; OR = 1.90, 95%CI = 1.16–3.42, χ^2 = 6.38, P = 0.0115). The genotype frequencies of 1350 T>C SNP were also significantly different in bancroftian filariasis patients when compared to normal controls (Fisher's exact test, P = 0.0324). In addition, the genotype distributions of this SNP were significantly different between bancroftian filariasis patients and normal controls in both the additive (χ^2 = 6.25, P = 0.0124) and dominant models (χ^2 = 6.00, P = 0.0143).

Linkage disequilibrium

To examine the LD among the three *TLR2* polymorphisms, pairwise LD coefficients |D′| were calculated using the JLIN program (Table 4). The −196 to −173 del polymorphism was in LD with +597 T>C and +1350 T>C (|D′| = 0.8018 and 0.8322, respectively). The +597 T>C was in strong LD with +1350 T>C (|D′| = 0.9402). Finding significant LD among three *TLR2* polymorphisms indicated that these polymorphisms were strongly associated and frequently inherited together.

Association between *TLR2* haplotypes and asymptomatic bancroftian filariasis

To determine whether coinheritance of three *TLR2* polymorphisms, −196 to −173 del, +597 T>C, and +1350 T>C,

Table 2 Allele frequencies of *TLR2* polymorphisms in bancroftian filariasis patients and normal controls

TLR2 polymorphisms	Bancroftian filariasis patients (n = 142)	Normal controls (n = 151)
−196 to −173 del (no rs designation available)		
wt	218 (76.76%)	263 (87.09%)
del	66 (23.24%)	39 (12.91%)
χ^2(1 *df*)	10.61	
P value	0.0011*	
OR (95%CI)	2.04 (1.32–3.15)	
+597 T>C (rs3804099)		
T	225 (79.23%)	269 (89.07%)
C	59 (20.77%)	33 (10.93%)
χ^2 (1 *df*)	10.72	
P value	0.0011*	
OR (95%CI)	2.19 (1.35–3.39)	
+1350 T>C (rs3804100)		
T	244 (85.92%)	279 (92.38%)
C	40 (14.08%)	23 (7.62%)
χ^2 (1 *df*)	6.38	
P value	0.0115*	
OR (95%CI)	1.90 (1.16–3.42)	

*P<0.05

Table 3 Genotype frequencies of *TLR2* polymorphisms in bancroftian filariasis patients and normal controls

Polymorphisms	Bancroftian filariasis patients ($n = 142$)	Normal controls ($n = 151$)	Genotypic model		Additive model		Dominant model		Recessive model	
			χ^2 (2 df)	P value	χ^2 (1 df)	P value[b]	χ^2 (1 df)	P value	χ^2 (1 df)	P value
−196 to −173 del										
wt/wt	87 (61.27%)	117 (77.48%)	9.48	0.0087*	9.20	0.0024*	9.01	0.0026*	2.79	0.0949
wt/del	44 (30.98%)	29 (19.21%)								
del/del	11 (7.75%)	5 (3.31%)								
+597 T>C (rs3804099)										
T/T	88 (61.97%)	119 (78.81%)		0.0027[a],*	11.07	0.0008*	10.00	0.0016*		0.0933[a]
T/C	49 (34.51%)	31 (20.53%)								
C/C	5 (3.52%)	1 (0.66%)								
+1350 T>C (rs3804100)										
T/T	105 (73.94%)	129 (87.68%)		0.0324[a],*	6.25	0.0124*	6.00	0.0143*		0.2885[a]
T/C	34 (23.95%)	21 (11.59%)								
C/C	3 (2.11%)	1 (0.73%)								

*$P<0.05$

[a] P value for Fisher's exact test

[b] P for trend (Armitage's trend test)

increased the risk of asymptomatic bancroftian filariasis, we examined the association between *TLR2* haplotypes and bancroftian filariasis (Table 5).

The *TLR2* haplotype −196 to −173del/+597C/+1350C was found more frequently in bancroftian filariasis patients (0.1162) than in normal controls (0.0464; $\chi^2 = 9.21$, $P = 0.0024$). Thus, individuals carrying the haplotype −196 to −173del/+597C/+1350C had a higher risk of asymptomatic bancroftian filariasis. In contrast, the *TLR2* haplotype −196 to −173wt/+597 T/+1350 T was more frequent in normal controls (0.8411) than in bancroftian filariasis patients (0.7504; $\chi^2 = 8.35$, $P = 0.0039$). This finding suggested the possibility of a protective effect. A combination of three polymorphisms (−196 to −173 del, +597 T>C, and +1350 T>C) appears to affect susceptibility to asymptomatic bancroftian filariasis.

Reporter assay

The effect of the −196 to −173 del polymorphism on the level of promoter activity is shown in Fig. 2. The wt

Table 4 Linkage disequilibrium coefficients ($|D'|$) among *TLR2* polymorphisms

$|D'|$	−196 to −173 del	+597 T>C	+1350 T>C
−196 to −173 del	–	0.8018	0.8322
+597 T>C		–	0.9402
+1350 T>C			–

sequence resulted in luciferase activity that was more than 51-fold higher than that from vector alone (pGL3-Basic vector), indicating that this region encompasses strong promoter activity. The *TLR2* −196 to −173del allele significantly reduced promoter activity to 27% that of the wt allele ($P = 0.005$).

Discussion

Wolbachia is known to induce immune response predominantly through TLR2 (Brattig et al. 2004; Hise et al. 2007; Turner et al. 2009). This interaction could be an important mechanism of host inflammatory response to different stages of the filarial parasites. We found that the individual *TLR2* polymorphisms, −196 to −173 del, +597 T>C, as well as +1350 T>C, were associated with asymptomatic bancroftian filariasis. In addition, haplotypes that have all three polymorphisms (−196 to −173del/+597C/+1350C) were more likely to present bancroftian filariasis infection.

The *TLR2* +597 T>C (Asn199Asn) and +1350 T>C (Ser450Ser) SNPs described here did not induce any amino acid change. The molecular mechanism by which this synonymous polymorphism might affect susceptibility to *W. bancrofti* infection is not fully understood. Many studies have provided evidence that synonymous SNPs lead to change in protein amount, structure, and/or function via alterations in the mRNA structure and stability, kinetic of translation, and alternative splicing (Duan et al. 2003;

Table 5 Haplotype frequencies of the *TLR2* polymorphisms between bancroftian filariasis patients and normal controls

Haplotype with allele frequency >0.05 are shown

*P<0.05

Haplotype	Haplotype frequency		χ^2	P value
	Bancroftian filariasis patients	Normal controls		
−196 to −173wt/+597T/+1350T	0.7504	0.8411	8.35	0.0039*
−196 to −173del/+597C/+1350C	0.1162	0.0464	9.21	0.0024*
−196 to −173del/+597T/+1350T	0.0563	0.0464	0.29	0.5908
−196 to −173del/+597C/+1350T	0.0458	0.0298	1.45	0.2298
−196 to −173 wt/+597C/+1350T	0.0211	0.0132	0.88	0.3491

Capon et al. 2004; Sauna et al. 2007). Moreover, these synonymous SNPs might be proxies for other polymorphisms that have not been examined. The +597 T>C has been previously reported in connection with increased susceptibility to tuberculous meningitis (Thuong et al. 2007). The *in vitro* phenotypes of synonymous SNPs need to be investigated more thoroughly to determine their effect on TLR2 expression. However, the strongest evidence for explaining the association between these synonymous SNPs and asymptomatic bancroftian filariasis is that +597C allele and +1350C allele are in linkage disequilibrium with a functional polymorphism in the 5′ UTR, −196 to −173del allele.

It has been well documented that 5′ UTR is important in transcription, mRNA stability, and translation initiation (Derrigo et al. 2000; van der Stoep et al. 2002; Chen et al. 2008). The NF-κB and Sp1 family transcription factors play an important role in regulating the expression of *TLR2* (Haehnel et al. 2002; Johnson and Tapping 2007). Interestingly, the −196 to −173 del polymorphism is

Fig. 2 Relative luciferase activities of pGL3 vectors containing *TLR2* −196 to −173 deletion or wt sequences in THP-1 cells. The firefly luciferase activities were normalized to *Renilla* luciferase activity from co-transfected pRL-CMV plasmids. The normalized luciferase data (firefly/*renilla*) was reported as fold elevation in activity over the pGL3-Basic vector. *Bars* indicate the mean value of three independent experiments, and standard deviation is indicated by *error bars*. *P = 0.005 by Student's *t* test

located in the 5′ UTR of *TLR2*, approximately 110 and 60 bp downstream of the SP1 and NF-κB binding site, respectively. These 23 nucleotide deletion might affect chromatin remodeling and positioning of the two transcription factors in relation to the coding region (Johnson and Tapping 2007). In addition, the *TLR2* −196 to −173del allele, in concordance with the data presented by Noguchi et al. (2004), showed a significantly reduced transcriptional activity. Based on our functional assay data, it is likely that the −196 to −173 del polymorphism presumably affect *TLR2* gene expression at transcriptional levels. A recent study showed that the −196 to −174 del/del genotype of *TLR2* may increase the risk of gastric cancer in the Japanese population (Tahara et al. 2007). Another study demonstrated that the −196 to −174 del was present at a significantly higher frequency in steroid-dependent ulcerative colitis patients relative to controls (Wang et al. 2007).

Data from human and murine studies have shown that antibody responses against the WSP are made during natural filarial infection and the L3s are the major source of immunogenic material from *Wolbachia* (Lamb et al. 2004). As the initiation of infection occurs through the skin after a mosquito bite and conditions the subsequent immune response, interaction between *Wolbachia* from L3s and the relevant antigen presenting cell (APC) in the skin (e.g., dendritic cells, Langerhans cells and macrophage) through *TLR2* is important to initiate host defense response to filarial infection. We suggest that the −196 to −173 del polymorphisms are associated with diminished TLR2 expression. Moreover, impaired expression and function of TLR2 have been reported in APC and T cells of filarial-infected individuals (Babu et al. 2005, 2006, 2009). Diminished TLR2 expression may cause filarial parasites which evade the first line of defense without being fully recognized by the host cells at the entry level, resulting in quantitatively decreased *Wolbachia*-mediated signaling which in turn leads to manipulating the host environment to prevent a hostile pro-inflammatory response and enhanced susceptibility to *W. bancrofti* infection. Further investigations are needed to clarify whether these *TLR2* polymorphisms have an impact on dendritic cells and
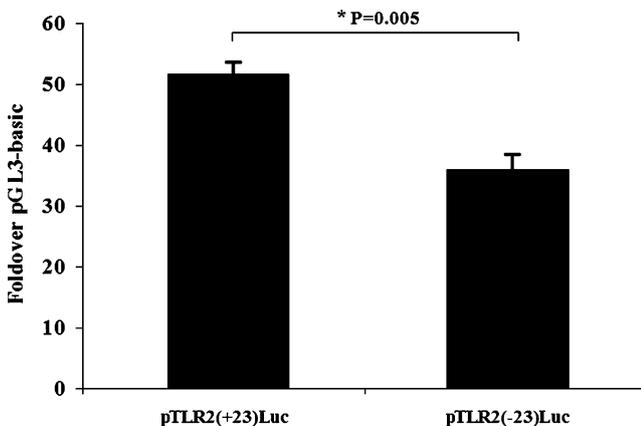
macrophage activation by its ligands to prove the hypothesis of a potential role of these SNPs in susceptibility to asymptomatic bancroftian filariasis.

In conclusion, the *TLR2* −196 to −173 del, +597 T>C, and +1350 T>C polymorphisms present an interesting target for investigating the genetic susceptibility to asymptomatic bancroftian filariasis. However, it is important to note that other polymorphisms/SNPs are involved since only about 58% of the bancroftian filariasis patient group has one of the polymorphisms/SNPs, meaning that the rest of this group (approximately 42%) are susceptible because of another reason. Additional association studies of TLR6, a co-receptor of TLR2, and other candidate genes involved in immune response will be required for determining their contribution to susceptibility to lymphatic filariasis. In addition, these polymorphisms in *TLR2* gene may influence the immune response to other pathogens that are recognized by TLR2 and may have important implications for understanding the pathogenesis of a wide range of infections. This study is the first presenting an association of human genetic polymorphism with asymptomatic bancroftian filariasis in a Thai population. The identification of *TLR2* polymorphisms as genetic risk factors for *W. bancrofti* infection may be beneficial to the lymphatic control program. Those who inherited higher-risk genes for bancroftian filariasis infection would probably need improved preventive measures, screened for *W. bancrofti* infection, and treated accordingly in the future. Moreover, knowledge regarding how the high-risk groups conduce to the overall transmission dynamics in endemic communities may be beneficial to designing appropriate strategies for the control of lymphatic filariasis.

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