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Association of Toll-Like Receptor 2 Polymorphisms with Papillary Thyroid Cancer and Clinicopathologic Features in a Korean Population

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Toll-like receptors (TLRs) single nucleotide polymorphisms (SNPs) were analyzed in patients with papillary thyroid cancer (PTC; n = 133) and their clinicopathologic features and age-matched controls (n = 321) using direct sequencing. PTC patients were divided into subgroups according to size, number, location, extrathyroidal invasion and lymph node metastasis. The two SNPs of *TLR2* gene were not associated with the development of PTC. In clinical analysis, two SNPs were associated with location of cancer (rs3804099, $P = 0.032$, OR, 0.52; 95% CI, 0.28-0.96 in log-additive model; rs3804100, $P = 0.039$, OR, 0.46, 95% CI, 0.22-0.96 in codominant1 model; $P = 0.018$, OR, 0.42, 95% CI, 0.21-0.87 in dominant model; $P = 0.011$, OR, 0.46, 95% CI, 0.25-0.85 in log-additive model). The allele frequencies of two SNPs also showed significant associations with location of cancer (rs3804099, $P = 0.046$, OR, 0.57, 95% CI, 0.33-0.99 and rs3804100, $P = 0.019$, OR = 0.52, 95% CI = 0.30-0.90). However, two SNPs were not associated with the clinicopathologic features of PTC. It is suggested that *TLR2* polymorphisms may contribute to the clinicopathologic features of PTC, especially the PTC in both lobes.

Key Words: Papillary Thyroid Cancer; Single Nucleotide Polymorphisms; Clinicopathologic Characteristics; Toll-Like Receptors

INTRODUTION

Thyroid cancer is characterized by the most common endocrine malignant carcinoma and is one of the fastest growing cancer diagnoses worldwide. There are four major types of thyroid cancers that are clinically significant: papillary, follicular, anaplastic, and medullary thyroid cancers. Of these, papillary thyroid cancer (PTC) is the most common type, showed about 80% of all cases of thyroid cancer. It is well-known that ionizing radiation exposure is a main risk factor (1). However, the exact etiology of PTC has been still unknown.

The Toll-like receptors (TLRs) family of transmembrane receptors plays a fundamental role in pathogen recognition and activation of innate immunity. Ligation of TLRs results in substantial immune responses that may be directed against tumor-associated antigens (2). A latent role for radiation in signaling 'danger' and, perhaps, in the activation of antigen presenting cells on immune system has been suggested (3-6). Radiation may also activate effectors of innate immunity through TLR-dependent mechanisms, thereby up-regulate the adaptive immune response to cancer (2). The *TLR2* gene is located in the chromosome 4p32 protein coding gene, and is expressed most abundantly in peripheral blood leukocytes, and mediates host response (http://www.ncbi.nlm.nih.gov/gene).

Hashimoto's thyroiditis is the most common gradual autoimmune-mediated thyroid failure and it is developed goiter at times. Some studies have found an association between Hashimoto's thyroiditis and PTC (7). Previous study was demonstrated that Hashimoto's thyroiditis was associated with an increased risk of developing PTC (7). Also, genetic factors seem to be involved as a risk factor for PTC (8-12). Indeed, several single nucleotide polymorphisms (SNPs) are thought to contribute to susceptibility to PTC (13-15).

In this study, we investigated whether synonymous SNPs in

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TLR2 contribute to the development of PTC. We also assessed the relationships between *TLR2* SNPs and the clinicopathologic characteristics of PTC.

MATERIALS AND METHODS

Patients and controls

Study subjects consisted of PTC patients (n = 133, 48 males and 85 females) and controls (n = 321, 129 males and 192 females). The mean ages of the PTC and control groups were 54.7 ± 12.3 yr (mean ± SD) and 56.3 ± 11.9, respectively (Table 1). PTC patients were recruited among participants visiting Kyung Hee University Medical Center, Seoul, Republic of Korea. Control subjects were enrolled from healthy participants examined in a general health check-up program. Participants with cancers, thyroid diseases, or any other severe diseases were excluded. PTC diagnosis was confirmed by pathologic examination. Patients with anaplastic carcinoma, follicular carcinoma, double primary of PTC and follicular carcinoma, follicular variant of PTC, or nodular hyperplasia were excluded.

Patient subgroups

To assess the relationship between *TLR2* SNPs and the clinical pathologic characteristics of PTC, patients were divided into subgroups according to the size (< 1 cm and ≥ 1 cm), number (unifocality and multifocality), location (one lobe and both lobes), extrathyroidal invasion (present and absent), and lymph node metastasis (present and absent). Demographic features of PTC patients are summarized in Table 1.

Table 1. Clinical characteristics of the study population

Parameters	PTC (No. = 133)	Control (No. = 321)
Age (mean ± SD)	55.7 ± 12.8	56.3 ± 11.9
Sex (male/female, No.)	48/85	129/192
Size of cancer (No.)		
< 1 cm	61	
≥ 1 cm	70	
Number of cancer (No.)		
One	83	
Multiple	45	
Location of cancer (No.)		
One lobe	63	
Both lobes	65	
Extrathyrodial invasion (No.)		
Absent	67	
Present	62	
Cervical lymph node metastasis (No.)		
Absent	84	
Present	40	

PTC patients having inappropriate clinical data were excluded. Because of PTC patients having inappropriate clinical data, the total number of PTC phenotype are different. PTC, papillary thyroid cancer; No., number of subjects; SD, standard deviation.

SNP selection and genotyping

For the selection among *TLR2* SNPs, we searched the synonymous SNPs of the *TLR2* gene in the SNP database of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/SNP, BUILD132). Because 16 missense SNPs of the *TLR2* gene had an unknown heterozygosity or minor allele frequency (MAF) below 0.05, all missense SNPs were excluded. Out of 13 synonymous SNPs of the *TLR2* gene, there were 11 SNPs with MAF below 0.05. Finally, two SNPs (rs3804099, Asn199Asn; rs3804100, Ser132Ser) were selected. Blood samples for DNA extraction from all subjects were collected. Genomic DNA was extracted with 200 μL whole blood using DNA Isolation Kit for Cells and Tissues (Roche, Indianapolis, IN, USA) and stored at -20°C before use. SNP genotyping was determined by direct sequencing. Polymerase chain reactions (PCRs) were performed using the primers for two synonymous SNPs (Table 2). PCR comprised 40 cycles at 94°C for 30 sec, 58°C for 30 sec, 72°C for 30 sec, and 1 cycle at 72°C for 5 min for the final reaction. The PCR products were sequenced by an ABI PRISM 3730XL analyzer (PE Applied Biosystems, Foster City, CA, USA). Sequencing data were analyzed using SeqManII software (DNASTAR, Madison, WI, USA).

Statistical analysis

Hardy–Weinberg equilibrium (HWE) was estimated using SNPStats (http://bioinfo.iconcologia.net/index.php?module = Snpstats) in both the patient and control groups. Multiple logistic regression models were applied to obtain odds ratios (ORs), 95% confidence intervals (CIs), and *P* values (codominant1, codominant2, dominant, recessive, and log-additive models). When the numbers of genotype and allele were less than 5, Fisher's exact test was performed. Data analysis was performed using SPSS 18.0 (SPSS, Chicago, IL, USA) and SNPstats (http://bioinfo.iconcologia.net/index.php?module = Snpstats). Linkage disequilibrium (LD) block and haplotypes were evaluated using Haploview version 4.2 (Daly Lab Inc., Cambridge, MA, USA). $P < 0.05$ was considered significant.

Ethics statement

This study was approved by the institutional review board of the Medical Research Institute, Kyung Hee University Medical Cen-

Table 2. Primer sequences for each SNP

SNP	Forward (5′-3′)	Reverse (5′-3′)	Size (bp)
rs3804099	ATTGCAAATCCTGAGAGTGGGA	GTTTCACCAGTGGATAGTTCTG	291
rs3804100	GAAAAATTCAGCCTGTGAGGAT	GGAGGCATCTGGTAGAGTCATC	358

SNP, single nucleotide polymorphism; bp, base pair.

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ter, Seoul, Korea (20040915). Written informed consent was obtained from all subjects.

RESULTS

We genotyped two synonymous SNPs in the *TLR2* gene. The genotype and allele frequencies of two synonymous SNPs are presented in Table 3. Multiple logistic regression analysis with adjustment for age and gender was performed: codominant1 (major allele homozygotes vs heterozygotes), codominant2 (major allele homozygotes vs minor allele homozygotes), dominant (major allele homozygotes vs heterozygotes + minor allele homozygotes), recessive (major allele homozygotes + heterozygotes vs minor allele homozygotes), and log-additive (major allele homozygotes vs heterozygotes vs minor allele homozygotes). The two SNPs were in Hardy-Weinberg equilibrium in the control group (rs3804099, $P = 0.30$; rs3804100, $P = 0.11$). Our two synonymous SNP in Table 3 (rs3804099, Asn199Asn; rs3804100, Ser132Ser) of *TLR2* was not associated between control and PTC patients (rs3804099, $P = 0.680$ in allele [reference T vs C]; rs3804100, $P = 0.850$ in allele [reference T vs C]).

Next, we assessed the relationship between *TLR2* SNPs and the clinical pathologic features of PTC. In the location of cancer (one lobe vs both lobes), the SNP (rs3804099) was related to location of PTC in genotype (rs3804099, $P = 0.032$, OR, 0.52; 95% CI ,0.28-0.96 in log-additive model [reference T/T vs T/C vs C/C]) and allele distributions ($P = 0.046$, OR, 0.57; 95% CI = 0.33-0.99, reference T vs C). Another SNP (rs3804100) was significantly associated with location of PTC in genotype (rs3804100, $P = 0.039$, OR, 0.46, 95% CI, 0.22-0.96 in codominant1 model

Table 3. Frequencies of genotype and allele of *TLR2* in control and patients with papillary thyroid cancer (PTC)

SNP		Type	Control	PTC	Model	OR (95% CI)	*P* value
			n (%)	n (%)			
rs3804099	Genotype	T/T	157 (48.9)	61 (46.9)	Codominant1	1.23 (0.80-1.88)	0.350
Asn199Asn		T/C	129 (40.2)	61 (46.9)	Codominant2	0.56 (0.24-1.28)	0.170
		C/C	35 (10.9)	8 (6.2)	Dominant	1.08 (0.72-1.62)	0.710
					Recessive	0.51 (0.23-1.13)	0.080
					Log-additive	0.93 (0.68-1.27)	0.630
	Allele	T	443 (69.0)	183 (70.4)		1	0.680
		C	199 (31.0)	77 (29.6)		0.94 (0.68-1.28)	
rs3804100	Genotype	T/T	165 (51.4)	64 (48.1)	Codominant1	1.30 (0.85-1.99)	0.220
Ser450Ser		T/C	122 (38.0)	61 (45.9)	Codominant2	0.58 (0.25-1.33)	0.200
		C/C	34 (10.6)	8 (6.0)	Dominant	1.14 (0.76-1.71)	0.530
					Recessive	0.089	
					Log-additive	0.96 (0.71-1.31)	0.810
	Allele	T	452 (70.4)	189 (71.1)		1	
		C	190 (29.6)	77 (28.9)		0.97 (0.71-1.33)	0.850

The *P* values were calculated from logistic regression analyses adjusting sex and age (codominant1, A/A vs A/B; codominant2, A/A vs B/B; dominant A/A vs A/B+B/B; recessive, A/A+A/B vs B/B; log-additive, A/A vs A/B vs B/B). Missing data of genotype were excluded for exact analysis. PTC, papillary thyroid cancer; SNP, single nucleotide polymorphism; OR, odds ratio; CI, confidence interval.

Table 4. Genotype and allele frequencies of SNPs of *TLR2* gene in PTC patients with one lobe and PTC patients with both lobes

SNP		Type	One lobe	Both lobes	Model	OR (95% CI)	*P* value
			n (%)	n (%)			
rs3804099	Genotype	T/T	23 (37.1)	34 (54.0)	Codominant1	0.53 (0.25-1.13)	0.100
Asn199Asn		T/C	33 (53.2)	27 (42.9)	Codominant2	0.24 (0.04-1.34)	0.100
		C/C	6 (9.7)	2 (3.2)	Dominant	0.49 (0.24-1.02)	0.054
					Recessive	0.34 (0.06-1.78)	0.170
					Log-additive	0.52 (0.28-0.96)	**0.032**
	Allele	T	79 (63.7)	95 (75.4)		1	
		C	45 (36.3)	31 (24.6)		0.57 (0.33-0.99)	**0.046**
rs3804100	Genotype	T/T	23 (36.5)	37 (56.9)	Codominant1	0.46 (0.22-0.96)	**0.039**
Ser450Ser		T/C	34 (54.0)	26 (40.0)	Codominant2	0.21 (0.04-1.18)	0.080
		C/C	6 (9.5)	2 (3.1)	Dominant	0.42 (0.21-0.87)	**0.018**
					Recessive	0.32 (0.06-1.68)	0.150
					Log-additive	0.46 (0.25-0.85)	**0.011**
	Allele	T	80 (63.5)	100 (76.9)		1	
		C	46 (36.5)	30 (23.1)		0.52 (0.30-0.90)	**0.019**

The *P* values were calculated from logistic regression analyses adjusting sex and age (codominant1, A/A vs A/B; codominant2, A/A vs B/B; dominant A/A vs A/B+B/B; recessive, A/A+A/B vs B/B; log-additive, A/A vs A/B vs B/B). Bold numbers mean significance association. Missing data of genotype were excluded for exact analysis. PTC, papillary thyroid cancer; SNP, single nucleotide polymorphism; OR, odds ratio; CI, confidence interval.

Table 5. Genotype and allele frequencies of SNPs of *TLR2* gene in PTC patients with tumor size < 1 cm and PTC patients with tumor size ≥ 1 cm

SNP		Type	< 1 cm (Tumor size)	≥ 1 cm (Tumor size)	Model	OR (95% CI)	*P* value
			n (%)	n (%)			
rs3804099	Genotype	T/T	25 (41.7)	35 (51.5)	Codominant1	0.59 (0.28-1.25)	0.170
Asn199Asn		T/C	31 (51.7)	29 (42.6)	Codominant2	0.68 (0.15-3.18)	0.630
		C/C	4 (6.7)	4 (5.9)	Dominant	0.60 (0.29-1.24)	0.170
					Recessive	0.89 (0.20-3.94)	0.880
					Log-additive	0.69 (0.38-1.26)	0.230
	Allele	T	81 (67.5)	99 (72.8)		1	
		C	39 (32.5)	37 (27.2)		0.78 (0.45-1.33)	0.360
rs3804100	Genotype	T/T	27 (44.3)	36 (51.4)	Codominant1	0.62 (0.30-1.30)	0.200
Ser450Ser		T/C	31 (50.8)	29 (41.4)	Codominant2	1.18 (0.25-5.59)	0.840
		C/C	3 (4.9)	5 (7.1)	Dominant	0.67 (0.33-1.36)	0.270
					Recessive	1.49 (0.33-6.74)	0.600
					Log-additive	0.81 (0.45-1.45)	0.480
	Allele	T	85 (69.7)	101 (72.1)		1	
		C	37 (30.3)	39 (27.9)		0.89 (0.52-1.51)	0.660

The *P* values were calculated from logistic regression analyses adjusting sex and age (codominant1, A/A vs A/B; codominant2, A/A vs B/B; dominant A/A vs A/B+B/B; recessive, A/A+A/B vs B/B; log-additive, A/A vs A/B vs B/B). Missing data of genotype were excluded for exact analysis. PTC, papillary thyroid cancer; SNP, single nucleotide polymorphism; OR, odds ratio; CI, confidence interval.

Table 6. Genotype and allele frequencies of SNPs of *TLR2* gene in PTC patients with unifocality and PTC patients with multifocality

SNP		Type	Unifocality	Multifocality	Model	OR (95% CI)	*P* value
			n (%)	n (%)			
rs3804099	Genotype	T/T	34 (42.5)	23 (51.1)	Codominant1	0.75 (0.35-1.61)	0.470
Asn199Asn		T/C	40 (50.0)	20 (44.4)	Codominant2	0.45 (0.08-2.49)	0.360
		C/C	6 (7.5)	2 (4.4)	Dominant	0.71 (0.34-1.50)	0.370
					Recessive	0.51 (0.10-2.76)	0.420
					Log-additive	0.71 (0.38-1.33)	0.290
	Allele	T	108 (67.5)	66 (73.3)		1	
		C	52 (32.5)	24 (26.7)		0.76 (0.43-1.34)	0.340
rs3804100	Genotype	T/T	36 (43.4)	24 (53.3)	Codominant1	0.71 (0.33-1.52)	0.380
Ser450Ser		T/C	41 (49.4)	19 (42.2)	Codominant2	0.47 (0.09-2.60)	0.400
		C/C	6 (7.2)	2 (4.4)	Dominant	0.68 (0.33-1.42)	0.310
					Recessive	0.56 (0.11-2.96)	0.480
					Log-additive	0.70 (0.38-1.31)	0.260
	Allele	T	113 (68.1)	67 (74.4)		1	
		C	53 (31.9)	23 (25.6)		0.73 (0.41-1.30)	0.290

The *P* values were calculated from logistic regression analyses adjusting sex and age (codominant1, A/A vs A/B; codominant2, A/A vs B/B; dominant A/A vs A/B+B/B; recessive, A/A+A/B vs B/B; log-additive, A/A vs A/B vs B/B). Missing data of genotype were excluded for exact analysis. PTC, papillary thyroid cancer; SNP, single nucleotide polymorphism; OR, odds ratio; CI, confidence interval.

[reference T/T vs T/C]; $P = 0.018$, OR, 0.42, 95% CI, 0.21-0.87 in dominant model [reference T/T vs T/C + C/C]; $P = 0.011$, OR, 0.46; 95% CI, 0.25-0.85 in log-additive model [reference T/T vs T/C vs C/C], respectively) and allele distributions ($P = 0.019$, OR, 0.52; 95% CI, 0.30-0.90, reference T vs C) (Table 4). However, no association was evident with clinical pathologic features of PTC (Tables 5, 6).

Linkage disequilibrium (LD) block between rs3804099 and rs3804100 was determined using Haploview version 4.2. Since the LD block was made ($D' = 1.00$ and $r^2 = 0.94$, data not shown), the analysis of haplotypes was performed. The frequency of TT was 0.69, CC was 0.297 and CT was 0.013, however, the association was not observed (data not shown). We calculated sample power to verify our data (http://pngu.mgh.harvard.edu/~purcell/gpc/cc2.html). In this study, the sample power of each SNP was 0.745 (rs3804099, alpha level = 0.05, the number of cases for 70% power = 91) and 0.730 (rs3804100, alpha level = 0.05, the number of cases for 70% power = 95). These sample powers were sufficient to detect positive associations between SNPs and PTC.

DISCUSSION

The functions of TLRs have provided potential insight into the cancer development. TLR-dependent mechanisms contribute to activate effectors of innate, thereby augmenting the adaptive immune response to cancer (2, 16, 17).

Numerous studies have investigated *TLR2* polymorphisms in different populations and different diseases with conflicting results. The two presently-studied *TLR2* SNPs, rs3804099 and rs3804100, were reportedly associated with sarcoidosis with

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cutaneous manifestations in a Japanese population (18), and rs3804099 was suggested as a relevant risk estimate for the development of sepsis and multiple organ dysfunction in Chinese patients with major trauma (19). In addition, *TLR2* -196 to -173 del, +597 T > C and +1350 T > C polymorphisms have been associated with asymptomatic bancroftian filariasis in Thailand (20). In a TLR2-related cancer study, SNPs of *TLR2* were not associated with colorectal cancer in individuals of three races (20 Malays, 20 Chinese and 18 Indians) (21). On the contrary, the association with microsatellite GT polymorphisms of *TLR2* gene and sporadic colorectal cancer among Croatians has been reported (22).

Here, we sought to determine the relationships between the *TLR2* SNPs and PTC in a Korean population. We also assessed the relationships between *TLR2* SNPs and the clinicopathologic characteristics of PTC. The findings of our study suggest that no significant differences exist in the frequency of *TLR2* genotypes and alleles in the PTC cases comparison with the controls. However, *TLR2* was associated with clinicopathologic features, especially in the locations of PTC. The T/T frequency of rs3804099 was different between the one lobe and both lobe groups (37.1% vs 54.0%, respectively). The T allele frequencies of rs3804099 in PTC patients with involvement of both lobes (75.4%) were higher than those in PTC patients with one lobe involvement (63.7%). The T allele frequencies of rs3804100 in PTC patients with involvement of both lobes (76.9%) were higher than those in PTC patients with involvement of one lobe (63.5%). Therefore, the T allele of rs3804099 and rs3804100 may be correlated with the location of PTC in the Korean population. Thyroid lobectomy alone may be sufficient treatment for small (< 1 cm), low-risk, unifocal, intrathyroidal papillary carcinomas in the absence of prior head and neck irradiation or radiologically or clinically involved cervical nodal metastases. But patients who have bilateral PTC should undergo total thyroidectomy. Therefore, tumor bilaterality can be used to determine surgical extent (23). The relationship between TLR2 polymorphisms and cancer location is significant.

This study has some limitations. The sample size of patients is small and control subjects did not receive the thyroid ultra sonography. Considering that the incidence of thyroid cancer (0.5-10/100,000 persons) is relatively low, more studies with larger numbers of patients are needed to verify our results.

In conclusion, based on our case-control association study of SNPs in *TLR2* genes in patients with PTC and control subjects, significant associations are reported between polymorphisms of the *TLR2* gene and PTC in both lobes. The results suggest that the *TLR2* polymorphisms may be associated with the clinicopathologic features of PTC in the Korean population, especially concerning the location of cancer.

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