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Original Article

Association detection between genetic variants in the microRNA binding sites of toll-like receptors signaling pathway genes and bladder cancer susceptibility

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Abstract: Bladder cancer (BCa) is the second most common urological malignancy, and the incidence of BCa has dramatically increased recently. Various toll-like receptors (TLRs) signaling pathway proteins were proven to be associated with BCa susceptibility. However, the effect of genetic variants in TLRs signaling pathway genes on risk of BCa has not been elucidated clearly. Previous studies mainly focused on the coding region of target genes, while in this study, polymorphisms in the non-coding region, microRNA (miRNA) binding sites were investigated as potential targets. We used bioinformatics approach to screen 100 BCa related TLRs signaling pathway genes. Candidate polymorphisms were select in this region and 8 polymorphisms were confirmed. Rs72552316, located at the 3'UTR of the *TLR7* gene, exhibited significant association with risk of BCa, indicating a strong relationship with decreased risk of BCa ($P \leq 0.0001$). Furthermore, no association was detected between all the polymorphisms and recurrence-free survival time of overall study population or non-muscle invasive BCa subgroups. In conclusion, rs72552316 in the miRNA binding sites of *TLR7* might contribute to BCa susceptibility, and this finding provided new targets for high BCa risk population screening.

Keywords: Polymorphism, bladder cancer, TLRs signaling pathway genes, microRNA binding sites

Introduction

Bladder cancer (BCa) accounts for approximately 2% of all human malignancies and it has the highest incidence and mortality rate among urinary system tumors [1, 2]. In China, the occurrence of BCa has dramatically increased in recent years [3]. Various risk factors have been demonstrated to be closely associated with the development of BCa, including smoking, occupational and environmental exposures, and chronic irritation [4]. Recently, several studies have identified those polymorphisms in individuals' genetic susceptibility contributed to the susceptibility of BCa [5]. However, the precise gene interactions and signaling pathways in the development of BCa are still unclear.

Toll-like receptors (TLRs) belong to the family of pattern-recognition receptors (PRRs). Furthermore, TLRs play an important role in innate immune system as the most known pathogen sensors, and detect invariant pathogen molecules through pathogen-associated molecular patterns (PAMPs). Interestingly, various studies have shown that TLRs might influence tumor initiation and progression through regulating the activation of transcription factors such as NF-κB, interferon regulatory factors (IRFs) or AP-1 via mitogen-activated protein kinase (MAPKs) signaling integrators. As a consequence, inflammatory responses are affected by these transcription factors, leading to the change of inflammatory-related cytokines and type I interferon [6-8]. Recently, the development of several major cancers has been proven to be associated with TLRs, including BCa [9-15]. It has been shown that TLRs might facilitate bladder tumor development and progression by the induction of tumor-associated inflammatory responses and immune escape,

and the initiation of bladder tumor regrowth after local radiotherapy [15-18].

MicroRNAs (miRNAs) contain 21-25 nucleotides and belong to non-coding small RNA family. Gene expression was controlled by post-transcriptional regulation through the inhibition of specific mRNAs by strict matching between the seeding region, 2-7 nucleotides in the 5' miRNA sequence, and the non-coding region, 3' untranslated region (3'UTR) of the target genes [19, 20]. Recently, several studies identified that miRNAs could act as oncogenes or tumor suppressors by targeting 3'UTR of cancer-related genes [21, 22]. Thus, any disruption in the microRNA binding sites such as SNPs could disturb the binding process to cause cancer development and progression [23].

Previous studies have demonstrated that SNPs on coding regions of TLRs signaling pathway genes were associated with BCa susceptibility. Shen *et al.* [24] showed that *TLR4* + 3725GC polymorphism CC genotype was associated with increased risk of Bca initiation and progression. In addition, polymorphism rs4129009 in *TLR10* was found to play a role in modulating urothelial cancer risk and progression [25]. However, the association between SNPs in the miRNA binding sites of TLRs signaling pathway genes and BCa susceptibility has not been discussed before. So we conducted this hospital-based case-control study with an integrative bioinformatics SNPs selecting approach in order to provide data for screening high-risk individuals in Chinese Han population.

Materials and methods

Study population

We recruited 317 patients from the Department of Urology, West China Hospital Sichuan University during the period from 2000 to 2012. All enrolled patients in this study were sporadic cases with pathological diagnosis (mean age 63.35 years; 242 males and 75 females). Those with history of other cancers were excluded. 268 healthy individuals with no evidence of cancer or immune disease were recruited in a routine check-up or health awareness campaigns as controls (mean age 64.13 years; 199 males and 69 females) from West China Hospital Sichuan University. All controls were matched to cases by gender and age (± 5 years) with the same ethnicity and had no evidence of immune disease. Clinical and epidemiology information were collected from all subjects, including tumor grade, tumor stage, smoking status and history, and event (recurrence/non recurrence, and death/live). At the end of the interview, 1 mL peripheral blood of all the subjects were collected and then frozen at -80°C. Informed consents were signed by all subjects after completely understanding the purpose of this case-control study.

The follow-up process was carried out by interviewing the patients via telephone or in the outpatient. The recurrence-free survival time was the specific period from the date of surgery to the date of recurrence or death. Malignant urothelial tumors that have not invaded the detrusor were defined as non-muscle invasive BCa, while the others were muscle invasive BCa.

SNP selection

In order to select potential SNPs within miRNAs binding sites, we initially used bioinformatics approach to select TLRs signaling pathway genes in the databases (http://www.biocarta.com, http://cgap.nci.nih.gov/Pathways) and a total of 100 genes were included in this study. Their names and designations were shown in Table S1. In addition, 90 cancer-related miRNAs which potentially target TLRs signaling pathway genes were found in Patrocles (http://www.patrocles.org) (Table S2). Then three different web databases, including TargetScanS (http://genes.mit.edu/tscan/targetscanS2005.html), PicTar (http://pictar.mdc-berlin.de), and PolymiRTS (http://compbio.uthsc.edu/miRSNP), were searched to identify the SNPs within miRNAs binding sites of the selected 100 genes based on these 90 miRNAs. After bioinformatics comparison, the searching results were intersected and 42 SNPs were chosen (Table S3). Furthermore, minor allele frequency (MAF) based on the frequencies in Asia population of these 42 SNPs (http://www.ncbi.nlm.nih.gov/snp/) were checked to exclude SNPs with the frequency of no more than 5%, and LDSelect program (http://droog.gs.washington.edu/ldSelect.html) was used to remove SNPs in high linkage disequilibrium. Finally, 24 SNPs in the potential miRNA binding sites were selected to accomplish the test.

DNA isolation and SNP genotyping

Genomic DNA was extracted from peripheral blood samples with QIAmp DNA extraction kit (Qiagen) according to the manufacturer's protocol. After extraction, DNA purity and concentration were determined by spectrophotometer. Genotyping of the selected microRNA binding site SNPs were done with Sequenom MassARRAY & iPLEX assay of Capitalbio Company. In brief, primers for PCR and iPLEX reaction were designed by Genotyping Tools & MassARRAY Assay Design software, and then synthesized. PCR amplification was performed in 384-well plate and the product was dealt with shrimp alkaline phosphatase in order to dephosphorylate unincorporated dNTPs in PCR system. Then the Mass ARRAY iPLEX reaction was performed. After the iPLEX reaction, each base of SNP sites will create iPLEX products with different molecular weight. At last, matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) can identify the different iPLEX product, and then we can get the genotyping information of each SNP site for every subject in the result of MALDI-TOF with TYPER 4.0 software.

Statistical analysis

In this study, Hardy-Weinberg equilibrium was detected in each SNP in controls. Pearson x^2 or Fisher's exact tests was carried out to analysis the potential difference in the SNP genotypes between patients and controls. Student's t test was used to explore the difference in the distribution of age. The odds ratio (OR) and 95% confidence interval (95% CI) was calculated with the risk option of crosstabs. We used Kaplan-Meier and Cox proportional hazard models to examine the correlation between the genotypes and recurrence-free survival time. All the analyses in this study were univariable analyses. Statistical analysis was done using the Statistical Package for Social Sciences software, v.13.0 (SPSS, Chicago, IL) and $P < 0.05$ was considered statistically significant with two-side tests.

Results

SNPs identification

According to previously described process, we obtained 24 SNPs in the 3'UTR of TLRs signaling pathway genes. However, 16 were removed for no proper PCR condition. Finally, 8 SNPs were selected for genotyping, including *TLR7* rs10127190, *TLR4* rs11536887, *MAP3K7* rs3734657, *TOLLIP* rs41314515, *TLR7* rs5743786, *TLR7* rs72552316, *TLR6* rs5743823, and *TLR4* rs7869402 (**Table 1**).

Characteristics of the study objects

The demographic information of the study subjects and clinical characteristics of each patient are presented in **Table 2**. We found no significant difference in the distribution of age between the cases (63.35 ± 12.95) and the controls (64.13 ± 12.38) ($P = 0.443$). The percentage of patients in clinical grade I, II, and III was 13.6% (n = 43), 34.1% (n = 108), and 48.9% (n = 155), respectively. The rest 3.4% (n = 11) were mixed grade tumor. In addition, patients with superficial BCa accounted for 43.5% (n = 138), while the remaining 56.5% (n = 179) were invasive BCa patients.

Association of SNPs with BCa susceptibility

The genotype and allele frequencies of 8 selected SNPs in all subjects were listed in **Table 3**. All the SNPs were in Hardy-Weinberg equilibrium ($P > 0.05$). Genotype and allele distributions of *TLR7* gene polymorphism rs72552316 exhibited significant difference between cases and controls. The frequency of the TC genotype and C allele was significant higher in controls than in patients ($P \leq 0.0001$). However, for three polymorphisms (*MAP3K7* rs3734657, *TLR6* rs5743823, *TLR4* rs7869402), the genotype and allele frequencies did not differ significantly between the cases and the controls ($P > 0.05$). For the remaining 4 polymorphisms, no mutant was found.

No association between SNPs and BCa recurrence-free survival

The follow-up was conducted in 317 BCa patients, of which 125 patients completed with 71 patients suffering from BCa recurrence. The mean and median follow-up time in this study was 38.25 ± 3.62 months and 24 months respectively. And the mean and median recurrence-free survival time was 38.87 ± 2.79 months and 25 months respectively. No relationship was detected between the eight polymorphisms and BCa recurrence-free survival

Table 1. Information about predicted miRNAs binding sites SNPs

gene	*SNP*	*Variation*	*MAF*[a]	*PCR primers*	*Putative microRNAs*
TLR7	Rs10127190	[T/A]	NA	F 5'-ACGTTGGATGACTTGCCACTCTTTTACAGG-3' R 5'-ACGTTGGATGCTTTCTTATCTCTCTGTGTC-3'	hsa-miR-19a, hsa-miR-19b
TLR4	Rs11536887	[A/G]	0.016	F 5'-ACGTTGGATGCGAGTGACAAAGTGACAGAG-3' R 5'-ACGTTGGATGAAGACGTGCTTCAAATATCC-3'	-
MAP3K7	Rs3734657	[C/T]	0.016	F 5'-ACGTTGGATGAGAAGTCAGCAGCAGAAACG-3' R 5'-ACGTTGGATGGTCTTTCTTTGCATATTTC-3'	hsa-miR-194, hsa-miR-212, hsa-miR-132
TOLLIP	Rs41314515	[C/T]	0.0028	F 5'-ACGTTGGATGTGCACCCAAGAACAGGTGTG-3' R 5'-ACGTTGGATGGATTCCCGTGAAAGAGCACC-3'	hsa-miR-608
TLR7	Rs5743786	[T/C]	0.000	F 5'-ACGTTGGATGAGGAGGACTCCAAGAGTGTG-3' R 5'ACGTTGGATGAGGAATCCATATAATTGGC-3'	hsa-miR-548a-3p, hsa-miR-548e, hsa-miR-548f
TLR7	Rs72552316	[C/T]	NA	F 5'-ACGTTGGATGGTAGGTGGACCATATGCATT-3' R 5'-ACGTTGGATGTTGGGCCTGCTTCTGGGTT-3'	hsa-miR-1265, hsa-miR-4764-5p, hsa-miR-541-5p
TLR6	Rs5743823	[T/C]	0.005	F 5'-ACGTTGGATGGGAAATTCAACTTAAGAAACC-3' R 5'-ACGTTGGATGCCTCCAGACAGTTACTTACG-3'	hsa-miR-452
TLR4	Rs7869402	[C/T]	0.092	F 5'-ACGTTGGATGTTTAGGGAGACACAGATGGC-3' R 5'-ACGTTGGATGACCTTCACACGTAGTTCTCC-3'	hsa-miR-539

[a]MAF (Minor Allele Frequency) was cited from http://www.ncbi.nlm.nih.gov/SNP.

Table 2. Demographic characteristics in the cases and controls

	Variables	*Cases (n = 317) (%)*	*Controls (n = 268) (%)*	*P*
Sex	Male	242 (76.3)	199 (74.3)	
	Female	75 (23.7)	69 (25.7)	
	Age (Mean age ± SD)	63.35 ± 12.95	64.13 ± 12.38	0.443
Smoking	Non smokers	168 (53.0)		
	Smokers	149 (47.0)		
Grade*	I	43 (13.6)		
	II	108 (34.1)		
	III	155 (48.9)		
	Mixed*	11 (3.4)		
Stage	Superficial	138 (43.5)		
	Invasive	179 (56.5)		
Event	Recurrence/non recurrence	71/54		
	Death/live	38/87		

time among the 125 patients (**Table 4**). In the subgroup of non-muscle invasive BCa cases, no correlation was found either (**Table 5**).

Discussion

Previous studies mostly focused on the association between polymorphisms in the coding regions of TLRs signaling pathway genes and BCa susceptibility. In this study, polymorphism rs72552316 in miRNA binding sites of *TLR7* gene was found to be related with the occurrence and progression of BCa. In addition, TC carriers of this polymorphism were associated with a decreased risk of BCa, indicating that rs72552316 might be used as a potential predicted target for the evaluation of BCa susceptibility.

TLR7 have been reported to affect various bladder tumor biological behaviors. TLR7 is a receptor that can recognize nucleic acid ligand, expressed on endosomal membranes of antigen presenting cells and leukocytes as dimers structurally. TLR7 activation could induce viability decline, proliferation suppression, and apoptosis of tumor cells, with immunogenic effects on tumors. A number of studies have reported the critical role of TLR7 in tumor development and progression, including melanoma, basal cell carcinoma and BCa [26, 27]. Activation of TLR7 in bladder tumor cells was associated with decreased proliferation and apoptosis induction, which was mediated by down-regulation of a suppressive apoptotic molecule Bcl-2. In addition, increased proinflammatory cytokine, such as IL-6 and TNF-α, contributed to the immune mediated BCa cell cytotoxicity through MyD88/NF-κB pathway [28-30]. Moreover, Smith and his colleagues observed growth inhibition of bladder tumor in vivo after TLR7 stimulation, which suggested a promising target for BCa therapy. Given the multifaceted role of TLR7 in the

Table 3. The polymorphisms of the *TLR7* gene and the risk of BCa

Gene name	*Polymorphism*		*Patients (n = 317)*		*Controls (n = 317)*		*OR (95%)*	*P value*[a]
			n	*%*	*n*	*%*		
TLR7	rs10127190	TT (584)	316	100.00	268	100.00	1 ref	-
		TA (0)	0	0.00	0	0.00	-	-
		AA (0)	0	0.00	0	0.00	-	-
		T (1168)	632	100.00	536	100.00	1 ref	-
		A (0)	0	0.00	0	0.00	-	-
TLR4	rs11536887	AA (583)	317	100.00	268	100.00	1 ref	-
		AG (0)	0	0.00	0	0.00	-	-
		GG (0)	0	0.00	0	0.00	-	-
		A (1170)	634	100.00	536	100.00	1 ref	-
		G (0)	0	0.00	0	0.00	-	-
MAP3K7	rs3734657	CC (537)	289	91.17	248	92.54	1 ref	-
		CT (47)	28	8.83	19	7.09	0.791(0.431-1.451)	0.447
		TT (1)	0	0.00	1	0.37	-	-
		C (1121)	606	95.58	515	96.08	1 ref	
		T (49)	28	4.42	21	3.92	0.883(0.495-1.573)	0.671
TOLLIP	rs41314515	CC (584)	317	100.00	267	99.63	1 ref	-
		TC (1)	0	0.00	1	0.37	-	-
		TT (0)	0	0.00	0	0.00	-	-
		C (1169)	634	100.00	535	99.81	1 ref	-
		T (1)	0	0.00	1	0.19	-	-
TLR7	rs5743786	TT (585)	317	100.00	268	100.00	1 ref	-
		TC (0)	0	0.00	0	0.00	-	-
		CC (0)	0	0.00	0	0.00	-	-
		T (1170)	634	100.00	536	100.00	1 ref	-
		C (0)	0	0.00	0	0.00	-	-
TLR7	rs72552316	TT (367)	317	100.00	50	18.66	1 ref	-
		TC (218)	0	0.00	218	81.34	-	0.000
		CC (0)	0	0.00	0	0.00	-	-
		T (952)	634	100.00	318	59.33	1 ref	-
		C (218)	0	0.00	218	40.67	-	0.000
TLR6	rs5743823	TT (580)	313	99.05	267	99.63	1 ref	
		TC (4)	3	0.95	1	0.37	0.391(0.040-3.779)	0.735
		CC (0)	0	0.00	0	0.00	-	-
		C (1164)	629	99.53	535	99.81	1 ref	-
		T (4)	3	0.47	1	0.19	0.392(0.041-3.779)	0.736
TLR4	rs7869402	CC (492)	267	84.49	225	83.96	1 ref	-
		CT (86)	46	14.56	40	14.93	1.032(0.652-1.634)	0.893
		TT (6)	3	0.95	3	1.11	1.187(0.237-5.973)	1.000
		C (1070)	580	91.77	490	91.42	1 ref	-
		T (98)	52	8.23	46	8.58	1.047(0.692-1.585)	0.828

[a]The bold numbers mean the *P* value is < 0.05.

occurrence and progression of BCa, it is not surprising that rs72552316 in miRNA-binding sites of *TLR7* could affect BCa susceptibility and progression in Chinese Han population. However, due to the limited validated studies investigating the association between different genotypes of this polymorphism and diverse TLR7 mRNA/protein expression, it is too early

Table 4. Recurrence-free survival analysis of 125 bladder cancer patients

polymorphisms	*genotype*	*N (all, n = 125)*	*N (reoccurence, n = 71)*	*HR (95% CI)*
rs10127190	TT	125 (100.0%)	71 (56.8%)	1
	TA	0	0	-
	AA	0	0	-
rs11536887	AA	125 (100.0%)	71 (56.8%)	1
	AG	0	0	-
	GG	0	0	-
rs3734657	CC	117 (93.6%)	66 (56.4%)	1
	CT	8 (6.4%)	5 (62.5%)	1.244 (0.501, 3.090)
	TT	0	0	-
rs41314515	CC	125 (100.0%)	71 (56.8%)	1
	TC	0	0	-
	TT	0	0	-
rs5743786	TT	125 (100.0%)	71 (56.8%)	1
	TC	0	0	-
	CC	0	0	-
rs72552316	TT	125 (100.0%)	71 (56.8%)	1
	TC	0	0	-
	CC	0	0	-
rs5743823	TT	123 (98.4%)	70 (56.9)	1
	TC	2 (1.6%)	1 (50.0%)	0.946 (0.131, 6.816)
	CC	0	0	-
rs7869402	CC	103 (82.4%)	56 (54.4%)	1
	CT	19 (15.2%)	13 (68.4%)	1.278 (0.698, 2.339)
	TT	3 (2.4%)	2 (66.7%)	1.041 (0.514, 2.107)

to define the feasibility of rs72552316 to predict BCa risk.

In rs72552316 (*TLR7*), the base transition from T to C may affect the binding process between miRNAs and the target region in 3'UTR of *TLR7*. If miRNA loss combination to the corresponding miRNA binding site, the downstream process such as mRNA synthesis, might return to normal level or even increase. Thus, expression level of TLR7 in TC carriers could be higher than in cases. Due to the potential activation of TLR7, tumor growth would be suppressed to a certain extent. However, it is still under exploring about the exact regulation mechanisms between these miRNAs and BCa. Furthermore, whether this predicted target is the exact miRNA binding site needs further study.

No relationship was detected between the other seven polymorphisms and the risk of BCa in this study. The number of previous studies regarding to the relationship between these polymorphisms and BCa was extremely limited, which suggested that BCa susceptibility might be independent of the seven polymorphisms.

Up to date, several studies have investigated the prognostic role of TLRs signaling pathway genes in predicting survival outcome of BCa patients. In this study, no significant association was found between the eight polymorphisms and recurrence-free survival time of 125 patients who completed the follow-up. However, treatment and prognosis for muscle invasive bladder cancer (MIBC) and non-muscle invasive bladder cancer (NMIBC) are quite different. Radical cystectomy or radiotherapy was the most commonly used procedure in MIBC, and approximately 50% of patients ultimately die of distant metastases [31]. NMIBC is currently treated using a combination of transurethral resection (TURBT) and intravesical therapy, and 50-70% of patients will develop disease recurrence within two years of their initial diagnosis [32, 33]. The recurrence rate is high in both low-and high-grade disease, and the degree of malignancy of NMIBC increases

Table 5. Recurrence-free survival analysis of 50 non-muscle invasive bladder cancer patients

polymorphisms	*genotype*	*N (all, n = 50)*	*N (reoccurence, n = 29)*	*HR (95% CI)*
rs10127190	TT	50 (100.0%)	29 (58.0%)	1
	TA	0	0	-
	AA	0	0	-
rs11536887	AA	50 (100.0%)	29 (58.0%)	1
	AG	0	0	-
	GG	0	0	-
rs3734657	CC	46 (92.0%)	27 (58.7%)	1
	CT	4 (8.0%)	2 (50.0%)	0.671 (0.159, 2.824)
	TT	0	0	-
rs41314515	CC	50 (100.0%)	29 (58.0%)	1
	TC	0	0	-
	TT	0	0	-
rs5743786	TT	50 (100.0%)	29 (58.0%)	1
	TC	0	0	-
	CC	0	0	-
rs72552316	TT	50 (100.0%)	29 (58.0%)	1
	TC	0	0	-
	CC	0	0	-
rs5743823	TT	50 (100.0%)	29 (58.0%)	1
	TC	0	0	-
	CC	0	0	-
rs7869402	CC	40 (80.0%)	22 (55.0%)	1
	CT	9 (18.0%)	6 (66.7%)	1.106 (0.448, 2.734)
	TT	1 (2.0%)	1 (100.0%)	1.167 (0.427, 3.185)

with the increase of recurrence. Until now, no certain factors have been proven to be sufficient to predict the recurrence of NMIBC. In order to verify the prognostic role of these polymorphisms, subgroup survival analysis for patients with NMIBC was conducted in this study. However, no significant relationship was found either. Since the index for the evaluation of survival outcome varied, significant association might be found between these polymorphisms and other indicators, such as overall-survival time. In addition, the number of patients who completed the follow-up was insufficient, which might affect the outcome of survival analysis. Sample size expansion should be adopted in the future for more accurate detection.

In conclusion, our data demonstrated the potential relationship between rs72552316 in *TLR7* and BCa susceptibility. This finding might be helpful for the development of better tools to screen high BCa risk population and predict prognosis of BCa patients. However, recruitment of regional population, limitation of SNPs selection, and deficiency of functional analysis should not be ignored when we explain the significance of this study. In order to confirm our results, prospective, large scale and long-time follow-up studies needed to be conducted in the future.

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Disclosure of conflict of interest

None.

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Table S1. Candidate genes in the SNPs selection flow

Gene Full Name	Gene ID
similar to calmodulin 3 (phosphorylase kinase, delta) (H. sapiens)	147908
similar to calmodulin 2	124827
cytochrome c, somatic pseudogene 35	121916
toll-interleukin 1 receptor (TIR) domain containing adaptor protein	114609
toll-like receptor 10	81793
toll interacting protein	54472
toll-like receptor 9	54106
ECSIT homolog (Drosophila)	51295
toll-like receptor 7	51284
lymphocyte antigen 96	23643
mitogen-activated protein kinase kinase kinase 7 interacting protein 2	23118
mitogen-activated protein kinase kinase kinase 7 interacting protein 1	10454
toll-like receptor 6	10333
ribosomal protein S6 kinase, 90kDa, polypeptide 5	9252
mitogen-activated protein kinase kinase kinase 14	9020
peptidoglycan recognition protein 1	8993
Fas (TNFRSF6) -associated via death domain	8772
receptor (TNFRSF) -interacting serine-threonine kinase 1	8737
TNFRSF1A-associated via death domain	8717
MAP kinase interacting serine/threonine kinase 1	8569
inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase gamma	8517
TNF receptor-associated factor 6	7189
tumor necrosis factor receptor superfamily, member 1B	7133
tumor necrosis factor receptor superfamily, member 1A	7132
tumor necrosis factor, alpha-induced protein 3	7128
tumor necrosis factor (TNF superfamily, member 2)	7124
toll-like receptor 4	7099
toll-like receptor 3	7098
toll-like receptor 2	7097
mitogen-activated protein kinase kinase kinase 7	6885
signal transducer and activator of transcription 3 (acute-phase response factor)	6774
v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian)	6714
son of sevenless homolog 1 (Drosophila)	6654
SHC (Src homology 2 domain containing) transforming protein 1	6464
mitogen-activated protein kinase kinase 4	6416
ribosomal protein S6 kinase, 90kDa, polypeptide 1	6195
v-rel reticuloendotheliosis viral oncogene homolog A, nuclear factor of kappa light polypeptide gene enhancer in B-cells 3, p65 (avian)	5970
v-raf-1 murine leukemia viral oncogene homolog 1	5894
protein tyrosine phosphatase, receptor type, R	5801
eukaryotic translation initiation factor 2-alpha kinase 2	5610
mitogen-activated protein kinase kinase 6	5608
mitogen-activated protein kinase kinase 3	5606
mitogen-activated protein kinase kinase 2	5605
mitogen-activated protein kinase kinase 1	5604
mitogen-activated protein kinase 8	5599
mitogen-activated protein kinase 3	5595

mitogen-activated protein kinase 1	5594
protein kinase C, beta 1	5579
protein kinase C, alpha	5578
protein kinase, cAMP-dependent, regulatory, type II, beta	5577
protein kinase, cAMP-dependent, regulatory, type II, alpha	5576
protein kinase, cAMP-dependent, regulatory, type I, beta	5575
protein kinase, cAMP-dependent, regulatory, type I, alpha (tissue specific extinguisher 1)	5573
protein kinase, cAMP-dependent, catalytic, gamma	5568
protein kinase, cAMP-dependent, catalytic, beta	5567
protein phosphatase 3 (formerly 2B), catalytic subunit, gamma isoform	5533
protein phosphatase 3 (formerly 2B), catalytic subunit, beta isoform	5532
protein phosphatase 3 (formerly 2B), catalytic subunit, alpha isoform	5530
protein phosphatase 2 (formerly 2A), catalytic subunit, alpha isoform	5515
peroxisome proliferator-activated receptor alpha	5465
platelet-derived growth factor receptor, alpha polypeptide	5156
nitric oxide synthase 1 (neuronal)	4842
nerve growth factor receptor (TNFR superfamily, member 16)	4804
nerve growth factor, beta polypeptide	4803
nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	4792
nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105)	4790
myeloid differentiation primary response gene (88)	4615
v-myc myelocytomatosis viral oncogene homolog (avian)	4609
mitogen-activated protein kinase kinase kinase 1	4214
jun oncogene	3725
integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)	3688
interleukin-1 receptor-associated kinase 1	3654
interleukin 1 receptor, type I	3554
interleukin 1, alpha	3552
inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta	3551
inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta	3551
insulin-like growth factor 1 receptor	3480
v-Ha-ras Harvey rat sarcoma viral oncogene homolog	3265
glutamate receptor, ionotropic, N-methyl D-aspartate 2D	2906
glutamate receptor, ionotropic, N-methyl D-aspartate 2C	2905
glutamate receptor, ionotropic, N-methyl D-aspartate 2B	2904
glutamate receptor, ionotropic, N-methyl D-aspartate 2A	2903
glutamate receptor, ionotropic, N-methyl D-aspartate 1	2902
growth factor receptor-bound protein 2	2885
MAP kinase interacting serine/threonine kinase 2	2872
guanine nucleotide binding protein (G protein), gamma transducing activity polypeptide 1	2792
guanine nucleotide binding protein (G protein), beta polypeptide 1	2782
GNAS complex locus	2778
v-fos FBJ murine osteosarcoma viral oncogene homolog	2353
ELK1, member of ETS oncogene family	2002
ELK1, member of ETS oncogene family	2002
epidermal growth factor receptor (erythroblastic leukemia viral (v-erb-b) oncogene homolog, avian)	1956
discs, large homolog 4 (Drosophila)	1742
mitogen-activated protein kinase 14	1432
conserved helix-loop-helix ubiquitous kinase	1147

CD14 molecule	929
calmodulin 3 (phosphorylase kinase, delta)	808
calmodulin 2 (phosphorylase kinase, delta)	805
calmodulin 1 (phosphorylase kinase, delta)	801

Table S2. MiRNAs associated with TLRs signaling pathway genes

miR-939	miR-520a-5p	miR-202
miR-920	miR-519-e	miR-194
miR-768	miR-515-5p	miR-19
miR-653	miR-509-3p	miR-186
miR-646	miR-505	miR-185
miR-644	miR-499b	miR-185-5p
miR-635	miR-499a	miR-181d
miR-633b	miR-491-5p	miR-181c
miR-632	miR-483-3p	miR-181b
miR-626	miR-455-3p	miR-181a
miR-626	miR-452	miR-147
miR-608	miR-433	miR-145
miR-607	miR-381	miR-143
miR-593	miR-370	miR-138
miR-587	miR-37	miR-138
miR-584d-5p	miR-34a	miR-1323
miR-584c-5p	miR-342-3p	miR-130b
miR-584b-5p	miR-340	miR-1298
miR-584a-5p	miR-340	miR-1297
miR-559	miR-30b	miR-1294
miR-548f	miR-302d	miR-1288
miR-548e	miR-302C	miR-1274a
miR-548b-3p	miR-302b	miR-1271
miR-548-3p	miR-300	miR-1256
miR-548	miR-297	miR-1237
miR-548	miR-297	miR-1228
miR-541	miR-26b	miR-1201
miR-539	miR-26a	miR-615-5p
miR-520q	miR-207-3p	let-7f-2
miR-520h	miR-205	let-7b

Table S3. Candidate SNPs in the SNPs selection flow

Gene	SNP	Chromosome	Position	Major/Minor Allele	Putative microRNAs
CD14	rs3776138	5	140011321	C/G	hsa-miR-302b, hsa-miR-302d
IRAK1	rs11556423	X	152929358	G/T	hsa-miR-587
IRAK1	rs3027901	X	153277280	A/G	hsa-miR-488-5p
MAP3K7	rs2131906	6	91282763	A/G	hsa-miR-297, hsa-miR-548e, hsa-miR-548f
MAP3K7	rs9451441	6	91282764	A/T	hsa-miR-297
MAP3K7	rs3734657	6	91282929	C/T	hsa-miR-194, hsa-miR-212, hsa-miR-132
MAP3K7	rs34631230	6	91226286	C/T	hsa-miR-138
MAPK1	rs3810611	22	22115553	A/G	hsa-miR-138-5p
MAPK1	rs58437134	22	22117065	A/G	hsa-miR-5581-3p
MAPK1	rs61757989	22	22112868	G/A	hsa-miR-632
MAPK1	rs2276008	22	21759747	C/G	hsa-miR-300, hsa-miR-381-3p, hsa-miR-624-3p
MAPK1	rs2276007	22	21759742	C/T	hsa-miR-300
MAPK1	rs1803546	22	21764121	G/T	hsa-miR-1323
MAPK1	rs1048752	22	21764144	A/G	hsa-miR-520h
MAPK14	rs1803337	6	36185978	C/T	hsa-miR-539
MAPK14	rs8510	6	36186158	C/T	hsa-miR-541
MAPK14	rs1803334	6	36109525	C/T	hsa-miR-6758-5p, hsa-miR-6856-5p
MAPK3	rs11865228	16	30033161	G/T	hsa-miR-143
MAPK3	rs3751867	16	30033353	G/A	hsa-miR-491-5p
MAPK3	rs113204102	16	30128114	G/C	hsa-miR-608
MAPK3	rs1062543	16	30114441	C/T	hsa-miR-520a-5p, hsa-miR-1323
MAPK3	rs41280866	16	30114549	A/G	hsa-miR-491-5p, hsa-miR-185-5p
MAPK3	rs41280860	16	30114536	A/G	hsa-miR-491-5p, hsa-miR-185-5p
MAPK3	rs41280864	16	30114548	A/G	hsa-miR-491-5p
MYD88	rs6853	3	38184370	G/A	hsa-miR-143
TAB2	rs35859918	6	149731659	C/A	hsa-miR-124-3p, hsa-miR-3714, hsa-miR-506-3p
TAB2	rs34532338	6	149731141	A/C	hsa-miR-302c
TLR4	rs1057313	9	119517680	G/T	hsa-miR-202
TLR4	rs7869402	9	120478032	C/T	hsa-miR-539
TLR6	rs5743823	4	38828649	T/C	hsa-miR-452
TLR6	rs5743829	4	38827455	C/T	hsa-miR-4436b-3p
TLR7	rs10127190	X	12816916	T/A	hsa-miR-19a, hsa-miR-19b
TLR7	rs5743786	X	12817917	T/C	hsa-miR-548a-3p, hsa-miR-548e, hsa-miR-548f
TLR7	rs80280330	X	12907534	A/C	hsa-miR-147
TLR7	rs72552316	X	12889591	C/T	hsa-miR-1265, hsa-miR-4764-5p, hsa-miR-541-5p
TNF	rs3093667	6	31653746	G/T	hsa-miR-570
TNF	rs3093666	6	31545733	C/T	hsa-miR-4721, hsa-miR-4446-3p
TNF	rs28501663	6	31545828	G/T	hsa-miR-150-5p
TOLLIP	rs41314515	11	1298137	C/A	hsa-miR-608
TRAF6	rs5030486	11	36509146	A/G	hsa-miR-138-5p
TRAF6	rs11033658	11	36509510	C/T	hsa-miR-130b
TRAF6	rs56289909	11	36511067	C/T	hsa-miR-1237