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Original Article

Association of *toll-like* receptors polymorphisms and the risk of colorectal cancer in Chinese Han population

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Abstract: Background: Toll-like receptor 2 (TLR2) has been proven to play an important role in Colorectal cancer (CRC) in previous studies. Polymorphic variants of *TLR2* are thought to be predisposing factors for various cancers. A number of studies have tried to determine whether the polymorphism of *TLR* influences its expression, susceptibility to CRC, but no accordant result was obtained due to the heterogeneity of the genetic background among populations. The aim of this retrospective study was to evaluate variants affecting CRC in a Chinese Han population. Methods: We chose 4 tg SNPs from Hap Map data on *TLR2*. They were genotyped on a total of 248 CRC patients and 226 healthy controls. We identified the correlation between the polymorphisms and the risk of CRC. Single nucleotide polymorphism locus was genotyped using PCR-RFLP. Results: The association of rs11938228 genotype CA was significantly different (P=0.010, OR=1.933) between CRC patients and healthy controls. Furthermore, one haplotype GCAA) had a significantly higher frequency in patients (11/496=2.21%) than in controls (1/452=0.21%) (*P*=0.003; OR=11.387, 95% CI=1.465-88.524). Conclusions: The current study suggests that genetic variants rs11938228 in *TLR2* might serve as candidate markers for susceptibility to CRC and haplotype GCAA of *TLR2* gene is a risk factor for CRC patients in Chinese Han populations.

Keywords: Colorectal cancer (CRC), genetic polymorphism, toll-like receptor 2 (TLR2)

Introduction

Chronic inflammation has become one major cancer risk factors and characteristics of cancer. It can effect possessive phase of tumorigenesis, generating a microenvironment which is beneficial to cancer development and progression, and promoting the survival, proliferation and migration with cancer cells [1-3]. Thus, many cancers can arise from local stimulation, chronic infection and inflammation. Colorectal cancer (CRC) is one of the main examples of the inflammation-cancer association and a malignant neoplasm which remains the third most commonly diagnosed cancer and the fourth leading cause of cancer mortality worldwide [4]. With the progressive Westernized lifestyles, the incidence of CRC have increased rapidly in Asian, especially fifth highest mortality rate in China [5]. Furthermore, evidence that innate immune system chemical mediators and bacterial toxins play key roles in CRC development have been reported in experimental models [6].

Because of the intestine with a constant inflammation process and presence of microorganisms, their pathogen-associated molecular patterns changes in proteins or receptors are involved in inflammation and immune responses may facilitate an increased risk of developing cancer. In this respect, the toll-like receptor (TLR) family that encodes type I transmembrane proteins plays an essential role in pathogen recognition by the extracellular matrix, leading to activation of innate and adaptive immune responses and to a process of controlled inflammation [7, 8]. TLRs activate the nuclear factor kappa B (NF-κB) pathway, the main regulatory inflammation signaling pathway, and this activation is involved in the pathogenesis of CRC. TLR2 is known to recognize

peptidoglycan and lipopolysaccharide (LPS) from bacteria. Stimulation of TLR2 could stimulated immune responses of Th2, Treg, or Th17, and TLR2 also plays an important role in the tumor tolerance, progression and metastasis [9-11]. Stimulation of TLR2 by Listeria monocytogenes promoted growth of tumor [12]. Blocking TLR2 could significantly weaken pulmonary metastases of tumor and increased the survival of tumor-bearing mice [13].

Genes TLR2 (4q32) is highly polymorphic, which may cause changes in protein expression or function, resulting in a differentiated inflammatory response that in turn can influence the progression of several cancer types, such as with cancers (breast cancer, gastric cancer, lymphoma, acute myeloid leukemia and hepatocellular carcinoma) [14-18]. A number of studies have tried to determine whether the polymorphism of *TLR* influences its expression, susceptibility to CRC, but no accordant result was obtained due to the heterogeneity of the genetic background among populations [6]. Whether genetic variations of the TLR conferred susceptibility to CRC patients in Chinese was puzzled.

Thus, we selected four tagging SNPs in the *TLR2* gene to evaluate whether common gene variants involved in the inflammatory response and whether these functional polymorphisms were associated with the risk of CRC in Chinese Han population.

Materials and methods

Ethics statement

This study was approved by the Medical Ethics Committee of the Affiliated Hospital of Hangzhou Normal University. Written informed consents conforming to the tenets of the Declaration of Helsinki were obtained from each participant prior to the study. The epidemiological data on the study subjects were collected using a standard interviewer-administered questionnaire, with questions on current and past occupation, smoking habits, alcohol intake and family history of cancer or adenomatous polyps and lesions.

Study populations

In total, 248 CRC patients and 226 matched cancer-free controls were enrolled in this study. Blood samples were obtained between January 2009 and April 2015 in the Department of Surgical Oncology of the First Hospital of Wenzhou Medical University before any anticancer treatment. CRC patients were defined by histopathological examination of the biopsy. The inclusion criterion was as follows: patients with sporadic cancer and the exclusion criterion were patients with hereditary cancer. Healthy controls were defined with no previous history of colorectal disease.

Tagging SNP selection

4 SNPs (from *TLR2* gene were included and based on the International HapMap Project, release27 (http://hapmap.ncbi.nlm.nih.gov). The capture criterion: an r^2 cut off value of 0.8, MAF>0.05 and in the CHB population. SNPs that are mentioned in the literature which might to be a functional or non-synonymous SNP according to NCBI were selected firstly. We genotyped these four SNPs by using the PCR and restriction fragment length polymorphism and verified the genotyping results in 10% of total samples by sequencing. Haplotypes, which were consisted of four SNPs, were constructed by Phase software.

DNA extraction and SNP analysis

Genomic DNA was obtained from peripheral blood leukocytes by using the Wizard® Genomic DNA Purification Kit according to instructions (Promega, USA). DNA samples were measured and diluted to 40 ng/μl, and stored at -80°C for genotype analysis. Genotyping for the selected SNP polymorphisms in genomic DNA was performed using the PCR and restriction fragment length polymorphism (RFLP). Polymerase chain reaction products were generated in a 10 μL reaction volume containing 50 ng of genomic DNA, 1×PCR buffer, 2 mmol/L $MgCl_2$, 0.2 mmol/L of each dNTP, 1 μmol/L of each primer, and 0.25 U of Taq DNA polymerase (Invitrogen Corporation, Carlsbad, CA). Cycling conditions consisted of an initial denaturation step at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds and a final elongation step at 72°C for 1 minute. Polymerase chain reaction products were digested with 2 U of NcoI restriction enzyme at 37°C, according to the manufacturer's instructions (New England BioLabs, Ipswich, MA).

Table 1. Main demographic and clinical characteristics of the studied population

Characteristics	CRC patients N=248 (%)	Healthy control N=226 (%)	*P*-value
Age			
<50 year	114 (46.1)	130 (57.3)	0.028
>50 year	124 (53.9)	96 (42.7)	
Mean ± SD	52±16	49±12	
Sex			
Male	130 (52.3)	110 (48.4)	0.445
Female	118 (47.7)	116 (51.6)	
Alcohol			
Yes	141 (57.0)	146 (64.6)	0.0127
No	107 (43.0)	80 (35.4)	
Smoking			
Yes	132 (53.3)	132 (58.3)	0.327
No	116 (46.7)	94 (41.7)	

Abbreviation: CRC, colorectal cancer.

Statistical analysis

We evaluated the risk of patients according to genotypes and alleles in comparison between the CRC patients and healthy control. HWE software was used to test the Hardy-Weinberg equilibrium in healthy control (P<0.001 were discarded). Clinical Characteristics of patients were analyzed with χ^2 test. The relative risks of SNPs were assessed by binary logistic regression, adjusted for age and gender. Dominant and recessive genetic models were conducted to estimate each genotype. We used haploview software (v4.2) to identified haplotype frequencies. *P*-values <0.05 were thought to be statistical significance. SPSS 19.0 (SPSS Inc., Chicago, IL, USA) was carried out for statistical calculations.

Results

Selected SNP

The data for total 20 TLR2 SNPs genotyped in Chinese (CHD) people population from the database of HapMap project http://hapmap.ncbi.nlm.nih.gov/index.html. Fifteen TLR2 SNPs were of minor allele frequencies (MAF) <5% and were eliminated from subsequent analysis. The remaining four TLR2 SNPs were evaluated: two synonymous SNPs in the single exon of the gene (rs3804099, rs3804100) and two SNPs in intron (rs11938228, and rs1898830).

Clinical characteristics of patients

In this study, 248 CRC patients and 226 matched cancer-free controls were screened for *TLR2* rs1898830, rs11938228, rs3804099 and rs3804100 polymorphisms using PCR-RFLP methods. The mean age of asthmatic patients was 52 years, and mean age of matched controls was 49 years. There were no significant differences between two groups with regarded to gender and age distribution. **Table 1** showed the general characteristics of the studied subjects. Whereas 53.3% of patients were current smokers, cigarette smoking showed no significant association with increased risk of CRC compared with healthy individuals (OR=2.47, 95% CI=1.66-3.65, *P*=0.028). Moreover, the incidence of alcohol drinker also was higher in bladder cancer patients (44.35% Vs 25.67%). Alcoholic has shown significant association with increased risk of CRC (OR=2.8, 95% CI=1.56-3.73, P=0.0127).

Association between selected SNPs and colorectal cancer

Firstly, the frequencies of genotypes and alleles of *TLR2* Polymorphic loci rs1898830, rs11938228, rs3804099 and rs3804100 were detected in case and control groups. HWE of rs1898830, rs11938228, rs3804099 and rs3804100 in patients and controls were listed in **Table 2**, and the results showed allelic distribution of detected SNP were not deviated from HWE in both case and control populations. *TLR2* rs1898830 in the study population were as follows: 7.4% AA, 33.5% AG and 59.1% GG for the case study group and 4.9% AA, 30.4% AG, and 64.7% GG for the controls, indicating that the genotypes distributions were similar between the cases and the control groups. Also, genomic analysis did not reveal a difference between CRC patients and healthy controls in allelic frequency at rs3804099. Similarly, the genotypic and allelic frequency of rs3804100 did not show significant difference between asthmatic patients and normal controls. Whereas, the frequency of wild (AA) and homozygous mutant (CC) genotype rs11938228 genotypes in cases and controls was found more in controls (43% and 5.7% respectively), but that of the heterozygous genotype was higher (60.15%) in cases with CRC. Significant risk of CRC was observed for AC (OR=1.719

Table 2. Genotype and allele frequency of *TLR2* rs1898830, rs11938228, rs3804099 and rs3804100 and Pearson's chi-square test in CRC patients and normal controls

Genotype/Allele	Patients (n=248)	Controls (n=226)	*P*-value	OR (95% CI)
rs1898830	HWE *P=0.21	HWE P=0.25		
GG	148	145	0.260	0.935 (0.621-1.320)
AG	83	69	0.177	0.832 (0.682-1.484)
AA	17	12	0.221	4.562 (0.551-37.412)
G	379	359	0.314	0.851 (0.492-1.192)
A	117	93		
rs11938228	HWE *P=0.23	HWE P=0.74		
AA	106	81	0.0521	0.815 (0.526-1.220)
AC	127	140	0.024	1.719 (1.104-2.417)
CC	15	5	0.0519	0.912 (0.653-1.521)
A	339	302	0.275	0.511 (0.321-0.981)
C	157	150		
rs3804099	HWE *P=0.48	HWE P=0.56		
GG	172	169	0.559	0.819 (0.541-1.392)
GA	68	51	0.921	1.218 (0.781-2.010)
AA	8	6	0.350	3.318 (0.341-32.543)
G	412	389	0.356	0.813 (0.521-1.267)
A	84	63		
rs3804100	HWE *P=0.41	HWE P=0.51		
GG	171	170	0.559	0.819 (0.541-1.392)
GA	68	51	0.921	1.218 (0.781-2.010)
AA	9	5	0.350	3.318 (0.341-32.543)
G	410	391	0.356	0.813 (0.521-1.267)
A	86	61		

*Chi-square test for deviation from the Hardy-Weinberg equilibrium (a value of P<0.001 was regarded as a deviation from the HWE).

95% CI=1.104-2.417, P=0.024) genotype of rs11938228. The genomic analysis did not reveal differences in allelic frequencies of the rs11938228 between CRC patients and healthy controls.

Haplotype and CRC patients

A total of 16 and 15 haplotypes were constructed in patients with CRC patients and normal controls, respectively. Among these haplotypes, only 3 existed in both patients and controls (**Table 3**). The total P value of haplotype distributions between patients and controls showed no statistical difference (P=0.060). When we compared the difference of each haplotype between the two groups, one haplotype GCAA) had a significantly higher frequency in patients (11/496=2.21%) than in controls (1/452=0.21%) (P=0.003; OR=11.387, 95% CI=1.465-88.524).

Discussion

Colorectal cancer (also known as colon cancer, rectal cancer, or bowel cancer) is the development of cancer in the colon or rectum (parts of the large intestine). It is due to the abnormal growth of cells that have the ability to invade or spread to other parts of the body [19-22]. Signs and symptoms may include blood in the stool, a change in bowel movements, weight loss, and feeling tired all the time. Risk factors for colorectal cancer include lifestyle, older age, and inherited genetic disorders. Other risk factors include diet, smoking, alcohol, lack of physical activity, family history of colon cancer and colon polyps, presence of colon polyps, race, exposure to radiation, and even other diseases such as diabetes and obesity [23-26]. Genetic disorders only occur in a small fraction of the population. A diet high in red, processed meat, while low in fiber increases the risk of colorectal cancer.

TLRs are a type of pattern recognition receptor (PRR) and recognize molecules that are broadly shared by pathogens but distinguishable from host molecules, collectively referred to as pathogen-associated molecular patterns (PAMPs) [27-32]. TLRs together with the Interleukin-1 receptors form a receptor superfamily, known as the "interleukin-1 receptor/toll-like receptor superfamily"; all members of this family have in common a so-called TIR (toll-IL-1 receptor) domain [33-35].

TLRs are present in vertebrates, as well as in invertebrates. Molecular building blocks of the

Table 3. Haplotype Associations Analysis in TLR2 (rs3804100-rs3804099)

Haplotype	Patients	Controls	*P*-value*	OR	95% CI
GAGG	31	33	0.898	0.951	0.573-1.577
ACAA	29	36	0.443	0.807	0.487-1.337
GCAA	11	1	0.003	11.387	1.465-88.524

P-value* Fisher's exact test was used in this comparison because the number of some haplotypes are less than five.

TLRs are represented in bacteria and in plants, and plant pattern recognition receptors are well known to be required for host defense against infection [36-38]. The TLRs thus appear to be one of the most ancient, conserved components of the immune system. Recently, research based on case-control have been reported the polymorphism of *TLR* influences its expression, susceptibility to CRC, but no accordant result was obtained due to the heterogeneity of the genetic background among populations [39, 40]. Whether genetic variations of the TLR conferred susceptibility to CRC patients in Chinese was puzzled. Thus, we selected four tagging SNPs in the *TLR2* gene to evaluate whether common gene variants involved in the inflammatory response and whether these functional polymorphisms were associated with the risk of CRC in Chinese Han population.

In the current study, we selected four SNPs of *TLR2* on the risk of CRC in Chinese Han population. We found that only rs11938228 genotype CA increased CRC risk compared to individuals with the genotype CC. *TLR2* is one of the TLRs family members, and is proved to be associated with multiple cancers including CRC [7, 8, 14-18]. We found that rs11938228 genotype CA were significantly different between the CRC patients and healthy group. To the best of our known, this is the first study to evaluate the polymorphism of *TLR2* on the risk of CRC in Chinese Han population. The relationship between TLR2 polymorphisms and the progresses of tumors have been explored and reported by some researchers. Jelavić et al. reported an association of TLR2 GT microsatellite alleles with 20 and 21 GT repeats with sporadic colorectal cancer among Croatians. In the study by Srivastava et al del allele carriers of TLR2 (Delta22) polymorphism were associated with a 1.54-fold increased risk for gallbladder cancer. In another study by Pandey et al. TLR2 gene polymorphisms (-196 to -174del) showed significant association (OR 1.6, 95% CI 1.00-2.51) with cervical cancer susceptibility.

Previous study showed that rs11938228 might be a functional polymorphisms that mediate the NFκB-mediated inflammatory response [41], and associated with pelvic inflammatory disease and chronic obstructive pulmonary disease [42, 43]. *TLR2-196* to *-174del* was demonstrated to be associated with increased CRC risk, and individuals who carried the *TLR2-196* to *-174del* variant showed higher mRNA and protein relative expression than wild-genotype carriers in a population of Brazilian individuals [6]. In previous study, TLR2 agonist stimulated the production of tumor necrosis factor α (TNFα) and activated the expression of inducible nitric oxide synthase (iNOS), resulting in apoptosis of the tumor cells [44]. Stimulation of TLR2 could stimulate immune responses of Th2, Treg, or Th17. In the regulation of tumor tolerance, progression, and metastasis, TLR2 still plays a vital role [9-11]. Agonists of parenteral TLR2 have also been identified to increased survival in relapsed cancer patients after chemotherapy [45]. Therefore, we speculated that rs11938228 may be a functional SNP and could effect on the expression of TLR2, resulting in the stimulated of TLR2, and finally disrupting the production of TNFα and the inducing of the immune response cells (TH2, Treg, or TH17) in tumor.

In conclusion, the current study suggests that genetic variants rs11938228 in *TLR2* might serve as a candidate marker for susceptibility to CRC in Chinese Han patients. Our study has a relatively small sample size, and lack of a validation trial. In the future, it is necessary to generate more prospective longitudinal studies in larger populations to replicate and confirm the findings.

In summary, we identified rs11938228 genotype CA confer genetically susceptibility to CRC in Chinese Han population. Furthermore, we firstly confirmed one haplotype GCAA of *TLR2* gene is a risk factor for CRC patients.

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Disclosure of conflict of interest

None.

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