Sequence Variants in Toll-Like Receptor 10 Are Associated with Nasopharyngeal Carcinoma Risk

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Abstract

Nasopharyngeal carcinoma (NPC) is a common malignancy in southern China and Southeast Asia. Genetic susceptibility is a major factor in determining the individual risk of NPC in these areas. To test the association between NPC and variants in *Toll-like receptor 10* (*TLR10*), we conducted a hospital-based case-control study in a Cantonese-speaking population in Guangdong province. Seven single nucleotide polymorphisms in *TLR10*, selected with a tagging algorithm, were genotyped. When assessing each unique haplotype compared with the most common haplotype, ''GAGTGAA,'' with the expectation-maximization algorithm in Haplo.stats, the risk of developing NPC was significantly elevated among men who carried the haplotype ''GCGTGGC'' (P = 0.005). After adjusting for age, gender, and VCA-IgA antibody titers, this association was more significant (P = 0.0007). To further assess the overall differences of haplotype frequency profiles between cases and healthy controls, the global score test, considering all haplotypes and adjusting for age, gender, and VCA-IgA antibody titers, gave a haplo score of 27.52 with P = 0.002. The haplotype specific odds ratio was 2.66 (confidence interval, 1.34-3.82) for GCGTGGC. We concluded that in this Cantonese population–based study, haplotype GCGTGGC with frequency of 11.4% in TLR10 was found to be associated with NPC and this association was statistically significant after adjusting for age, gender, and VCA-IgA antibody titers. It is possible that this is not a causal haplotype for NPC; rather, it is in strong linkage disequilibrium with a causal single nucleotide polymorphism in close proximity. (Cancer Epidemiol Biomarkers Prev 2006;15(5):862–6)

Introduction

Nasopharyngeal carcinoma (NPC) occurs sporadically in Western countries, but is endemic in southern China and Southeast Asia, with incidence rates from 15 to 50 per 100,000 (1, 2). Etiologic studies have shown that NPC is a complex disease involving genetic, EBV infection and environmental factors, and potential interactions between these factors (3-6). NPC is more prevalent in Cantonese-speaking Guangdong regions than in other regions of China. Evidence from family, twin, and segregation studies has indicated that genetic susceptibility is a major factor in determining the individual risk of NPC in areas with the highest incidences (7).

Both genetic and environmental factors have been implicated in the etiology of NPC. EBV, a member of the herpes virus family that plays a role in cancers such as Burkitt's lymphoma and Hodgkin's lymphoma, has been consistently identified as an important risk factor for NPC, with a dose-response relationship between EBV antibodies and NPC risk (8, 9). Cantonese-style salted fish that contains high levels of nitrosamines have been long been thought to be a risk factor for NPC (10, 11). A recent IARC monograph (2004) has established cigarette smoking as a risk factor for NPC based on consistent findings of increased risk in both high- and low-risk geographic regions for NPC (12). However, in recent years, great progress has been made in genetic research of familial and nonfamilial NPC cases. HLA Bw46 has been shown to be associated with increased risk of NPC (13, 14). Hildesheim et al. (15) reported an association between HLA class I and II alleles (HLA-A*0207 and HLA-B*4601) and extended haplotypes with NPC in a case-control study conducted in Taiwan. Further, a meta-analysis of 13 HLA association studies in southern China done by Goldsmith et al. (16) reported evidence for positive associations between NPC and the HLA alleles A2, B14, and B46 and negative associations for the alleles A11, B13, and B22. Other studies revealed associations between a few metabolic enzyme genes and NPC risk. These genes include Glutathione *S*-Transferase M1 (17), cytochrome P450 2E1 enzyme (18), and DNA repair genes *hOGG1* (codon 326) and *XRCC1* (codon 280; ref. 19). Apart from case-control studies, genetic linkage studies are also one of the most common approaches for identification of cancer susceptibility genes (20). A genome-wide search was conducted by our research group on high-risk NPC families from Cantonese-speaking populations in the Pearl River area, Guangdong Province. Both multipoint parametric and nonparametric linkage analyses supported evidence for linkage (21). We mapped a NPC susceptibility locus to chromosome 4p15.1-q12, and this region was further narrowed down from 14.21 to 8.29 cM using linkage disequilibrium mapping with newly collected trio families (22). Since then, we have taken a candidate gene approach for further fine mapping and to identify potentially causal genetic variants in this region.

Recently, we have investigated a few candidate genes on chromosome 4 related to immune response, with a strong focus on the variants in Toll-like receptor (*TLR*) genes. TLRs play an essential role in initiating the immune response against pathogens and can recognize a wide variety of pathogen-associated molecular patterns from bacteria, viruses, and fungi (23-25). Chronic infection and inflammatory processes related

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to carcinogenesis are also mediated, in part, through recognition of various stimuli by TLRs (26). Our previous research has detected associations between two single nucleotide polymorphisms (SNP) in coding regions (*TLR1* 130T/C, $P = 0.044$; *TLR6* 359T/C, $P = 0.024$) and NPC,[4] separately. Because phylogenetic analysis indicates the amino acid sequence of *TLR10* is similar to that of *TLR1* and *TLR6* (27), and *TLR1*, *TLR6*, *TLR10* are actually in the same chromosome 4p15.1-q12 we have identified, it is important to investigate *TLR10* in the same study population. Interestingly, a recent report has shown associations between sequence variants in *TLR6*, *TLR1*, and *TLR10* and prostate cancer risk and this finding suggested a role of the *TLR6-TRL1-TLR10* gene cluster in the development of cancer via inflammation process (28, 29), thus further justifying testing our hypothesis. In this study, we hypothesize that sequence variants in *TLR10* that regulate inflammation may modify individual risk to NPC.

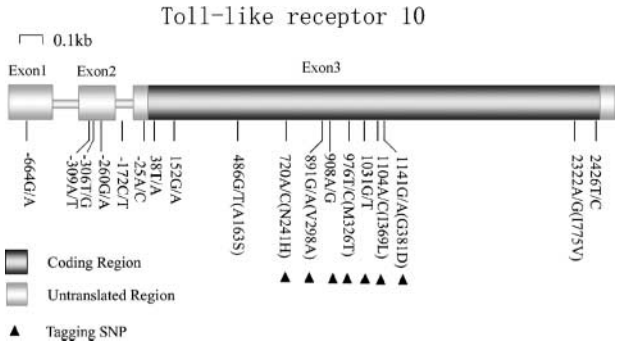

Figure 1. Schematic of SNPs discovered in *TLR10. Dark gray bar,* coding region. The location of 18 SNPs with nucleotide substitutions were shown (seven nonsynonymous SNPs are shown in bracket). *TLR10* is located in a single haplotype block. ▲, seven tagging SNPs selected to represent at least 95% of the haplotype information.

Materials and Methods

Study Population. All subjects were unrelated ethnic Han Chinese from the Cantonese-speaking population in Guangdong Province, China. Cases were recruited consecutively from December 2003 to October 2004 with pathologically confirmed diagnosis of NPC at the Cancer Center, Sun Yat-sen University, Guangzhou, China.

Of 487 recruited cases, 111 cases (22.79%) had been diagnosed before 2003 and returned to the NPC clinic during the period of December 2003 to October 2004. The remaining 376 cases were incident cases (77.21%). The average age at diagnosis of NPC for all patients was <50 years. Population controls were cancer-free individuals, randomly selected from individuals who attend annual community-based physical examinations during the same time period. The selection criteria for control subjects included no individual history of cancer and matched to NPC cases by age (±5 years), residential region, and the time period for blood sample collection. This study was approved by the Human Ethics Approval Committee, Cancer Center, Sat Yat-sen University.

Variation Screening. We first obtained the *TLR10* genetic sequence from the published database of the National Center for Biotechnology Information. We located all known exons and untranslated regions of the *TLR10* gene, located on chromosome 4p14 using the National Center for Biotechnology Information database. The full-length transcripts are 3,270 bases. The *TLR10* gene contains three exons. A standard PCR method routinely used in our laboratory was used for various exons and exon-intron boundary regions of *TLR10*. The primers for the target region were designed with the web-based software, Primer 3.0. In preliminary tests, we amplified and purified the DNA samples from 24 sporadic NPC cases. These samples included 48 chromosomes, providing a 95% confidence level to detect alleles with frequencies >5% (Fig. 1).

Then, the PCR products were sequenced using ABI PRISM Dye Terminator Sequencing kits and loading the samples onto an ABI 377 Automatic Sequencer (Applied Biosystems, Foster City, CA). We used the Polyphred/phredphrap/consed software package to identified SNP candidates that were confirmed by two independent observers. These SNP positions and individual genotypes were further confirmed by reamplifying and sequencing from the 5′ or 3′ end.

Selection of Haplotype Tagging SNPs. The linkage disequilibrium structure of 18 SNPs in *TLR10* was estimated and haplotype tagging SNPs (htSNP) were then defined using the Dynamic Programming Algorithm (30). Twenty-four unrelated individuals were resequenced for screening variants in TLR10, and then HapBlock 3.0 program was done on the genotype data set for Tag SNPs selection. We used one of the dynamic programming algorithms to minimize the total number of tagging SNPs in a region of interest. Haplotype blocks were defined by the empirical linkage disequilibrium measure D'. The threshold for strong linkage disequilibrium was set to be 0.8. The minimum set of SNPs that can distinguish 95% common haplotypes within a block is defined as a set of tagging SNPs (the threshold for common haplotypes frequency is 0.05). Nonsynonymous SNPs located in exon 3 of TLR10 were preselected as tagging SNPs for their potential important biological function. All seven htSNPs were then genotyped in 487 cases and 580 healthy controls (Table 2).

Genotyping Methods. Genotype variations were determined by PCR-based DNA direct sequencing. Genomic DNA was extracted from peripheral blood samples of all case and control subjects using the DNAzol kit following the protocol of the manufacturer (Life Technologies, Shanghai, China). A two-step strategy of sequence screening was used in this study. The first step was to find htSNPs in 24 NPC cases, as described above. Second, we genotyped these htSNPs in all 487 NPC cases and 580 Cantonese-speaking controls. PCR amplification was done on the GeneAmp 9700 PCR system (Applied Biosystems). The PCR for amplification refractory mutation system was accomplished with a 25 μL reaction mixture containing 20 ng DNA, 0.2 μmol/L of each primer, 200 μmol/L of each deoxynucleotide triphosphate, 1.5 mmol/L $MgCl_2$, 0.4 units Hot-Star Taq DNA polymerase with 1× buffer, and 1× Q-solution (Qiagen, Chatsworth, CA). The reaction was carried out under the following conditions: an initial melting step of 15 minutes at 95°C; followed by 30 cycles at which each one consisted of 30 seconds at 94°C, 30 seconds at 60°C, and 45 seconds at 72°C, respectively; and a final elongation step of 5 minutes at 72°C. Automatic DNA sequencing was conducted on the ABI 377 Automatic Sequencer (Applied Biosystems) using direct PCR products of the samples according to the protocol of the manufacturer. The raw data was collected using ABI PrismTM 377-96 Collection software and analyzed with the Sequencing Analysis software V3.3 on a MAC operating system V9.1, Polyphred/phredphrap/consed software package, DNAStar/Taqman software package and CHROMAS. All genotyping was done blind to case/control status, and blind quality control samples were inserted to validate genotyping. A random sample of 10% of the cases and controls were

[4] Submitted for publication.

sequenced twice by different investigators to confirm genotyping and reproducibility was 100%.

Statistical Analysis. For each polymorphism, deviation of genotype frequencies in control subjects from those expected under Hardy-Weinberg equilibrium was assessed using the Pearson χ^2 test (Table 2) and pairwise linkage disequilibrium tests for all pairs of sequence variants were done with the Fisher probability test statistic (data not shown). For each SNP polymorphism, genotype frequency differences between cases and controls were tested by conditional logistic regression. All analyses were done using Statistical Analysis System software (version 8.0, SAS Institute, Cary, NC). All statistical tests were two-sided, and a probability level below 0.05 was used as the criterion for a significant difference. Haplotype analyses were conducted using Haplo.stats.[5] Haplo.stats is a score test from generalized linear models that tests for associations between haplotypes and disease under the null hypothesis of no haplotype effect without any assumption about mode of inheritance. The algorithm implemented in Haplo.stats is an iterative process of regression substitution by which the posterior probabilities of subject-specific haplotype pairs (generated from the expectation-maximization algorithm) are used as weights to update the regression coefficients whereas the regression coefficients are simultaneously used to update the posterior probabilities. This software provides several different global and haplotype-specific tests for association and allows the possibility to include several nongenetic covariates. More importantly, it also allows computation of permutation *P* values, which helps resolve potential problems in dealing with sparse data.

Results

A summary of selected characteristics of cases and controls is shown in Table 1. No statistical differences were observed between cases and controls in the distribution of age ($P = 0.83$) and gender ($P = 0.69$). Table 1 also described the distribution of titer category of the cases and controls. In this study, the positive rates detected by VCA-IgA antibody titer are 18.79% in controls and 94.46% in cases.

We genotyped 487 NPC case and 580 control subjects for these seven htSNPs in *TLR10* (Table 2). We would like to note that some genotypes were missing for both cases and controls for all seven SNPs due to insufficient amount of DNAs in some samples and the details are given in Table 2. Genotype distributions in the control subjects did not differ significantly from Hardy-Weinberg equilibrium (Table 2).

Single SNP Association. When analyzed separately using conditional logistic regression, no statistically significant associations between NPC and any of the seven htSNPs in *TLR10* were found. Detailed odds ratios (OR) are given in Table 3.

Association with Haplotypes. We omitted haplotypes with a frequency <0.006. The remaining seven haplotypes were analyzed with Haplo.stats. The risk of developing NPC was significantly elevated among men who carried the haplotype ''GCGTGGC'' [$P = 0.005$; OR, 1.415; 95% confidence interval (95% CI), 1.02-1.94], compared with men who carried the most common haplotype ''GAGTGAA.'' This significant association between this haplotype and NPC was consistent even when adjusting for age, gender, and VCA-IgA antibody titers ($P = 0.002$). However, when adjusting for age and gender separately, the results were similar to the results without any covariates (data not shown). Further, global score tests were also done using Haplo.stats. When no covariates were included, the global haplo score was 26.58 ($df = 7$) with an asymptotic *P* value of 0.008 and a simulation-based *P* value of 0.0008 (10,000 permutations). When adjusting for VCA-IgA, age, and gender, the global haplo score was 23.78 with an asymptotic *P* value of 0.003 and a simulation-based *P* value of 0.006 (10,000 permutations). In addition, we did Haplo.GLM to evaluate the effect for each haplotype. Results are displayed in Table 4. The haplotype-specific *P* value for GCGTGGC was 0.002 (OR, 2.66; 95% CI, 1.34-3.82), when adjusted for age, gender, and VCA-IgA antibody titers.

Table 1. Distribution of characteristics of study subjects

Characteristic	Cases ($n = 487$)	Controls ($n = 580$)	*P**	log *P*
Gender, *n* (%)				
Male	358 (73.5%)	420 (72.4%)		
Female	129 (26.5%)	160 (27.6%)	0.69	6.9e−1
Age, y, *n* (%)				
≤30	104 (21.4%)	131 (22.6%)		
30-40	188 (38.6%)	208 (35.9%)		
40-50	137 (28.1%)	171 (29.5%)		
≥50	58 (11.9%)	70 (12.1%)	0.81	8.1e−1
VCA-IgA(%) †				
0 (<1:10)	27 (5.54%)	471 (81.21%)		
1 (1:10-1:20)	66 (13.55%)	87 (15%)		
2 (1:40)	89 (18.28%)	18 (3.1%)		
3 (≥1:80)	305 (62.63%)	4 (0.69%)	0.000	5.03e−161

*P values obtained from χ^2 tests.

†Subjects were divided into four titer categories: 0, subjects with VCA-IgA antibody titers <1:10; 1, subjects with VCA-IgA antibody titers equal to 1:10 or 1:20; 2, subjects with VCA-IgA antibody titers equal to 1:40; 3, subjects with VCA-IgA antibody titers >1:80.

Discussion

NPC constitutes an endemic multifactorial genetic disease (31). Whereas the disease is rare in the Western world, it occurs with high frequencies in Southern China, Southeast Asia, and among the Greenland Inuit. The highest age-adjusted incidence has been reported in southern China to be 30 to 50 per 100,000 (2). Epidemiologic investigation shows that NPC has a striking geographic and ethnic distribution, with particularly high rates observed among southeast Chinese and other individuals of Chinese descent (32). Genetic susceptibility plays a role in determining the individual risk of NPC (7). Although we have identified a susceptibility locus in an 8.29 cM segment on 4p11-1p14 with genome-wide scan and haplotype analyses (21, 22), no major susceptibility locus has been identified for NPC.

EBV is a ubiquitous herpes virus that infects >90% of the human population and establishes a lifelong viral persistence in the host. It has been consistently identified as an important risk factor for NPC, with a dose-response relationship between EBV antibodies and NPC risk. The single clonally derived viral genome can be found in all endemic NPC cells (33-35). The first contact of an individual with EBV typically happens in the first decade of life and results in an asymptomatic latent infection. The virus establishes a persistent infection in healthy virus carriers characterized by expression of EBV-encoded genes, such as *EBERs, EBNA1, LMP1*, and *LMP2A*, which can be detected by PCR after reverse transcription of RNA (reverse transcription-PCR) in infected cells (36). High EBV antibody titers indicate reactivation of EBV and lytic infection, which could be detected by serologic method. High VCA-IgA antibody titer (≥1:10) is an index of reactivated status of EBV, and the level of VCA-IgA antibody titer represents the replicating level of the virus. In this study, the positive rates detected by VCA-IgA antibody titer are 18.79% in control subjects and 94.46% in case subjects (Table 1).

[5] http://www.mayoresearch.mayo.edu/mayo/research/biostat/schaid.cfm.

Table 2. Hardy-Weinberg equilibrium tests and minor allele frequencies

Genotype	Position*	Accid	HW-pval	%Genotyped †	MAF
720A/C	5925134	rs10856837	1	98.6	0.473
891G/A	5924963	rs11466651	0.285	98.8	0.095
908A/G	5924946	rs11466652	0.793	98.6	0.137
976T/C	5924878	rs11466653	0.791	99	0.095
1031G/T	5924823	rs11096956	0.805	95.7	0.341
1104A/C	5924750	rs11096955	0.708	96.7	0.471
1141G/A	5924713	rs11466655	0.795	96.5	0.253

Abbreviations: Accid, accession ID; HW-pval, *P* value of Hardy-Weinberg equilibrium tests for control subjects; MAF, minor allele frequency.
*Contig position.
†Percentage of samples successfully genotyped.

An association between NPC and a risk haplotype in TLR10 may indicate that a mutation in a causal SNP or haplotype modifies immune response to EBV and thus increases the individual risk for NPC, although this speculation is not warranted. The fact that all seven individual SNPs gave negative results may also suggest that none of these particular SNPs are causal variants but they may be in linkage disequilibrium with a causal SNP that is physically close. Further research is needed to verify this finding.

The other limitations of this study includes the following: (*a*) Limited power: Although we conducted statistical tests to examine interaction between the haplotype GCGTGGC and VCA-IgA and obtained negative results (data not show), we need to point out that the power to detect gene-EBV interaction is limited. (*b*) Lack of epidemiologic investigation: We have not obtained information on other environmental exposure, such as consumption of salted fish and preserved vegetables, and fruits, smoking/drinking status, and occupational history. This may bias our results and reduce the precision of the estimated effect size of the putative risk haplotype. (*c*) Only *TLR10* was examined using tagging SNPs, whereas we have not studied other TLRs in a similar manner. Because *TLR6-TRL1-TLR10* gene cluster locates in the linkage region of NPC discovered earlier, we plan to continue this study by including tagging SNPs in *TLR 1* and *TLR6* in future analysis. (*d*) Multiple testing was not adjusted.

Table 3. TLR10 genotypes and NPC risk

Genotype	Cases (n = 487)	Controls (n = 580)	OR* (95% CI*)
720A/C			
AA	136 (28.51)	154 (27.16)	
AC	235 (49.27)	286 (50.44)	0.930 (0.698-1.241)
CC	106 (22.22)	127 (22.40)	0.945 (0.669-1.336)
Missing	10	13	
891G/A			
GG	395 (82.81)	457 (80.18)	
GA	81 (16.98)	108 (18.95)	0.868 (0.631-1.193)
AA	1 (0.21)	5 (0.88)	0.231 (0.027-1.989)
Missing	10	10	
908A/G			
AA	345 (72.48)	431 (75.88)	
AG	121 (25.42)	129 (22.71)	1.172 (0.881-1.559)
GG	10 (2.10)	8 (1.41)	1.562 (0.610-4.000)
Missing	11	12	
976T/C			
TT	396 (82.67)	461 (81.02)	
TC	81 (16.91)	102 (17.93)	0.924 (0.671-1.275)
CC	2 (0.42)	6 (1.05)	0.388 (0.078-1.933)
Missing	8	11	
1031G/T			
GG	214 (45.44)	223 (41.30)	
GT	210 (44.59)	249 (46.11)	0.879 (0.676-1.143)
TT	47 (9.98)	68 (12.59)	0.720 (0.475-1.092)
Missing	16	40	
1104A/C			
AA	137 (28.84)	145 (26.51)	
AC	230 (48.42)	287 (52.47)	0.848 (0.634-1.135)
CC	108 (22.74)	115 (21.02)	0.994 (0.699-1.413)
Missing	12	33	
1141G/A			
GG	269 (57.23)	303 (55.09)	
GA	163 (34.68)	218 (39.64)	0.842 (0.649-1.093)
AA	38 (8.09)	29 (8.09)	1.476 (0.886-2.459)
Missing	17	30	

*ORs and 95% CIs estimated by conditional logistic regression.

Table 4. Haplotype frequencies of the *TLR10* and effect of environmental factors revealed by Haplo.GLM

Model	Frequencies		*P**	OR (95% CI)*
	Cases	Controls		
Covariates				
Sex			0.193	0.71 (0.42-1.19)
Age			0.595	0.99 (0.97-1.01)
VCA-IgA †			0.000	10.60 (8.00-14.05)
Haplotypes				
GCGTAGA	0.004	0.017	0.0376	0.52 (0.28-0.96)
GCTTAAA	0.214	0.229	0.522	0.86 (0.54-1.36)
GCTCGAA	0.075	0.079	0.763	0.90 (0.46-1.76)
GAGTGAA	0.269	0.267	Not applicable	Reference
GCGTAAA	0.010	0.007	0.302	1.91 (0.56-6.49)
AAGTGAA	0.237	0.217	0.134	1.40 (0.90-2.19)
GCGTGGC	0.138	0.092	0.002	2.66 (1.34-3.82)

**P* values and ORs for haplotypes were adjusted by sex, age, and VCA-IgA.
†*P* values and OR for VCA-IgA was based on ''per titer category'' as indicated in Table 1.

Further, we would like to ask the question of whether the increase in OR (1.41-2.66) for GCGTGGC when adjusting for VCA-IgA was caused by a possible role of VCA-IgA as a negative confounder if *TLR10* allelic variants happen to determine infectivity of EBV status. For this reason, we also evaluated whether there is association between EBV status (positive versus negative) and halotypes constructed by seven SNPs. No positive associations were detected using Haplo.stat (data not shown). Therefore, no evidence indicated VCA-IgA as a negative confounder in the current study.

In light of our findings, we will continue to investigate the role of sequence variants in a variety of TLRs. We suspect we will detect more associations between variances in TLRs and NPC via either haplotype-based or gene-gene interaction–based approaches. Malfunction or improper regulation of TLRs may lead to an unbalanced ratio of proinflammatory to anti-inflammatory cytokines and chemokines in the host, contributing to the onset of a number of inflammatory diseases and cancer (37, 38).

We recognize that results from association studies of candidate genes can sometimes be false-positive findings due to population stratification. Although the Cantonese population is considered ethnically homogeneous, the existence of population admixture needs more scrutiny. We plan to genotype 500 previously collected trio families to validate this finding by performing transmission disequilibrium test analysis. To conclude, in this Cantonese population-based case-control study, a relatively common variant in *TLR10* was likely associated with NPC and this association was significant even after adjusting for age, gender, and VCA-IgA antibody titers. Although we cannot conclude with confidence that this variant is a causal SNP for NPC, the significant global and haplotype-specific *P* values support the potential role of *TLR10* in the genetic etiology of NPC.

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