Original Article

Toll-like Receptor Gene Polymorphisms and Susceptibility to Epstein–Barr Virus-associated and -Negative Gastric Carcinoma in Northern China

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ABSTRACT

Background/Aims: Various polymorphisms in toll-like receptor (TLR) genes have been identified and associated with susceptibility to various malignancies, such as gastric carcinoma (GC), breast cancer, and prostate cancer. However, little is known about the polymorphisms of TLR genes and the susceptibility to GC in Northern China, especially to Epstein–Barr virus-associated GC (EBVaGC). We focused on the association with susceptibility to GC, especially to EBVaGC. **Patients and Methods:** Polymorphisms of the *TLR2*, *3*, *4*, and *9* genes were measured in 52 cases of EBVaGC and 157 cases of EBV-negative GC (EBVnGC). Ninety-four peripheral blood samples from healthy individuals were also examined. **Results:** For the *TLR2* gene (196 to 174 del), there was no significant difference between the GC group and control group in genotype, but there was a significant difference in the del allele. As for the *TLR3* gene (c. 1377C/T), there were significant differences between the GC group and the control group in both genotype and allelic frequency. No SNPs single nucleotide polymorphisms (SNPs) were found in the *TLR4* gene at the sites Asp299Gly and Thr399Ile. As for *TLR9* 1486T/C (rs187084) and C2848T (rs352140), there was also no association between the GC group and control. In all of the indicators, there were no significant differences between EBVaGCs and EBVnGCs. **Conclusions:** The *TLR3* gene (c. 1377C/T) polymorphisms and the del allele of the *TLR2* gene (196 to 174) were both associated with susceptibility to GC in Shangdong Province of Northern China. There was no interaction between EBV and *TLR* gene polymorphisms in EBVaGC.

Key Words: Epstein–Barr virus-associated gastric carcinoma, Epstein–Barr virus-negative gastric carcinoma, toll-like receptor gene polymorphism

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Of all cancer types, gastric carcinoma (GC) ranks second in incidence and third in mortality. In China in 2010, the estimated incidence was approximately 400,000 new cases, and the national average cancer mortality rate was as high as 20/100,000.[1] It is widely accepted that the major etiological risk factor for GC is *Helicobacter pylori* bacteria, which lead to GC through a multistep process, developing from gastritis, to gastric atrophy, intestinal metaplasia, dysplasia, and finally to carcinoma.[2] It is well accepted that the gastric atrophy and hypochlorhydria are the precursors of all pathophysiological changes of gastric carcinogenesis, which are induced by chronic *H. pylori* infection.[3] However, infection with *H. pylori* can lead to different outcomes. Nearly all *H. pylori*-positive subjects suffer chronic gastritis, and only 1%–2% of infected cases develop GC.[4] Consequently, other factors are likely to be related to gastric tumorigenesis, such as host genetic factors, as well as other infections including EBV.

Recognition of bacteria or viruses by the diversification level of the cytokine response and immune system is closely related to polymorphisms in host inflammatory response genes.[5] It has been shown that the immune response against *H. pylori* infection is regulated by host factors such as cytokines, growth factors, and chemokines.[6,7] Analysis of the polymorphisms in genes related to the inflammatory response in the gastric mucosa and the associated risk for

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malignancy has been the central focus in many studies.[8,9] Other mediators, such as the toll-like receptors (TLRs), act as the first defense against pathogenic microorganisms. Many polymorphic variants exist among TLRs that regulate the pattern of innate immune response.[10]

TLRs play an essential role in innate immunity. TLRs are involved in the regulation of inflammatory reactions and activation of the adaptive immune response to eliminate pathogenic microorganisms. Many associations have been reported between *TLR* polymorphisms and infectious diseases or cancers. The interaction between the development of infection and chronic inflammation most likely mediates the increased risk of cancer. It has also been reported that *TLR* polymorphisms are closely connected with GC. However, the results vary in different parts of the world.[11-13] No associations of the *TLR2* -196 to -174 del polymorphism with the risk of *H. pylori* seropositivity, gastric atrophy, or gastric cancer were found in a Japanese population.[11] Nevertheless, polymorphisms of the *TLR2* and *TLR4* genes have been reported to be associated with the risk of gastric cancer in a Brazilian population.[13]

EBV is one member of the subfamily of γ-herpes viruses and was first identified in cultured lymphoma cells.[14] The global latent infection rate with EBV is 95% in adults.[15] EBV is associated not only with malignancies derived from B cells but also with epithelial malignancies, such as nasopharyngeal carcinoma[16] and EBVaGC.[17] The exact mechanism of EBV in these associated malignancies is still unclear, but the fact that gene products of EBV can induce cellular transformation supports a pathogenic role for EBV. Priming of protective T-cell responses is required to keep EBV in check in most infected individuals.[18] Antigen-presenting cells (APCs) carry TLRs for optimal T-cell priming, most likely through direct recognition of EBV-associated molecular patterns.[19,20] *TLR3* and *TLR9* are involved in the detection of EBV and might complement each other for the identification of this virus. Furthermore, monocytes, which detect EBV via *TLR2*, secrete cytokines, and chemokines.[21,22] Prominently, both *TLR3* and *TLR9* are expressed in B cells, which are the primary targets of EBV.[23] TLR3 recognizes dsRNA in the endosomal compartments of human dendritic cells (DCs). EBV-encoded small RNA (EBER) is the most abundant EBV viral transcript and has been described to form stem-loop structures, which can then be bound by TLR3.[24]

Although many studies on TLRs in GC have been carried out in the past years, few studies have examined *TLR* polymorphisms in GC, especially in EBVaGC in Shandong Province of Northern China. The purpose of the present study is to evaluate the influence of the -196 to –174 del allele in the *TLR2* gene, the 1377C/T polymorphism in the *TLR3* gene, the + 896A/G and + 1196C/T polymorphisms (Asp299Gly and Thr399Ile) in the *TLR4* gene, and the 1486T/C (rs187084) and C2848T (rs352140) polymorphisms in the *TLR9* gene on the risk of GC in Shandong Province of Northern China. We also investigated whether there is a relationship between the polymorphisms of *TLR* genes with EBV infection in GC.

PATIENTS AND METHODS

Ethics statement

This study was approved by the Medical Ethics Committee of the Medical College of Qingdao University and was performed after written informed consent was obtained from all subjects. This study was carried out in accordance with the guidelines of the 1975 Declaration of Helsinki.

Patients

The case groups comprised 209 individuals (111 men and 98 women) with a histopathologically confirmed diagnosis of gastric cancer (Lauren's classification), with a mean age of 59.2 ± 11.2 years (range 32-87 years). In case groups, the EBVaGC group comprised 52 cases (32 men and 20 women) with a mean age of 58.1 ± 13.2 years (range 34-79 years) and the EBVnGC group comprised 157 individuals (86 men and 71women) with a mean age of 56.4 ± 11.2 years (range 32-87 years). The control group was composed of 94 healthy individuals (49 men and 45 women) with no gastric disease history, mainly blood donors, with a mean age of 55.7 ± 17.3 years (range 20-83 years). All the individuals were ethnically classified as Chinese Han in the two groups. EBV positivity in GC tissues was determined by *in situ* hybridization of EBV-encoded small RNA 1, as previously described.[25]

DNA extraction

DNA was extracted from fresh tumor tissues and whole blood specimens by a standard method using proteinase K digestion and phenol–chloroform purification. A QIAamp DNA FFPE Tissue Kit (QIAGEN GmbH, Hilden, Hilden, Germany) was used to extract the DNA from paraffin-embedded tumor tissues. The extracted DNA was prepared for next polymerase chain reaction.

Polymerase chain reaction amplification

The polymerase chain reaction (PCR) technique was used to detect the polymorphism in the *TLR2* gene (-196 to -174 del), *TLR3* gene (c. 1377C/T), *TLR4* gene [Asp299Gly (rs4986790) and Thr399Ile (rs4986791)], and *TLR9* gene [1486T/C (rs187084) and C2848T (rs352140)], respectively. The primer sequences and the sizes of PCR products are shown in Table 1. PCR was performed with 1.5 μL of DNA extracts (100 ng/μL) in a 25-μL reaction mixture containing standard PCR buffer, 1.5 mM $MgCl_2$, 200 μM dNTP, 0.5 μM of each primer, and 1.0 U *Taq* DNA

polymerase. The DNA amplification protocol included 1 cycle at 94°C for 5 min, followed by 40 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 40 s. The program ended with 10 min at 72°C. The reaction was carried out in the GeneAmp PCR system 2700 (Applied Biosystems, Foster City, CA, USA). PCR products were analyzed via electrophoresis in a 2% agarose gel. After electrophoresis, the gels were stained with ethidium bromide and photographed under the UV light transilluminator. Sterile double distilled water was used as negative control in each PCR reaction. The representative PCR products were analyzed using an ABI 3730 DNA sequencer to confirm genotype identity.

Enzymatic digestion of PCR products

Polymorphisms in the *TLR3* gene (c. 1377C/T), *TLR4* gene [Asp299Gly (rs4986790) and Thr399Ile (rs4986791)], *TLR9* gene [1486T/C (rs187084), and C2848T (rs352140)] are based on digestion with TaqI, HinfI, NcoI, AflII, and BstUI restriction enzymes of each PCR product, respectively.[26-28] The PCR products were purified using a gel extraction kit (Qiaex II; Qiagen GmbH, Germany) according to the manufacturer's instructions. The enzymatic reactions were performed in a 20-μL reaction mixture containing 10 μL of the PCR products, 1 × reaction buffer and 10 units of TaqI, HinfI, NcoI, AflII, and BstUI, respectively. After incubation at 65°C, 37°C, 37°C, 37°C, and 60°C for 4 min, 1 h, 5 min, 1 h, and 1 h, respectively, the DNA products were analyzed on a 2% agarose gel and then visualized by ethidium bromide staining.

Statistical analysis

The Chi-square test was used to compare the differences in each group regarding genotype and allele frequencies. Nonconditional logistic regression was used to compare the odds ratio (OR) and *P* values to indicate the correlation between genotype and the risk of GC. Significance was set at $P < 0.05$. Statistical analyses were conducted using SPSS 18.0 statistical software (SPSS, Chicago, IL, USA).

RESULTS

Analysis of the TLR2 gene (-196 to -174 del) polymorphism

Determination of the *TLR2* genotype was based on the presence of the specific band. As indicated by the electrophoresis results, PCR products of 286 bp indicated homozygocity for the wild-type (ins/ins) allele, amplified bands of 264 bp and 63bp indicated the homozygous mutant (del/del) allele, whereas simultaneous presence of the 286 bp and 264 bp and 63bp amplified bands indicated heterozygous (ins/del) alleles [Figure 1]. Compared with EBVnGC, there was no association with the EBVaGC group in genotype and allelic frequency. The allelic frequency showed significant differences between GC patients and healthy donors ($\chi^2 = 5.62$, $P = 0.018$), which indicated that the del allele was a risk factor for GC. The distribution of *TLR2* (-196 to -174 del) genotypes and alleles in patients and controls are listed in Tables 2 and 3.

Analysis of TLR3 gene (c. 1377C/T) polymorphism

The length of the specifically amplified band of the *TLR3* gene (c. 1377C/T) was 337 bp. All 52 cases of EBVaGC, 157 cases of EBVnGC, and 94 cases of blood donors were positive for this band [Figure 2A]. After digestion with *TaqI*, the homozygous wild-type genotype CC showed two bands, namely, 274 bp and 63 bp. The homozygous mutant genotype TT remained as only one 337 bp band. The heterozygous genotype CT showed three bands, 274 bp,

Table 1: List of primers used in TLRs genotypes

Name of primers	Sequence (5'-3')	Size of PCR products
TLR2 (−196 to −174 del)	CACGGAGGCAGCGAGAAA	286 bp=Wild type (ins/ins)
	CTGGGCCGTGCAAAGAAG	264 bp (del/del)
		286 and 264 bp (ins/del)
TLR3 (c. 1377C/T)	CCAGGCATAAAAAGCAATATG	337 bp
	GGACCAAGGCAAAGGAGTTC	
TLR4 (Asp299Gly)	CTGCTCTAGAGGGCCTGTG	140 bp
	TTCAATAGTCACACTCACCAG	
TLR4 (Thr399Ile)	CTACCAAGCCTTGAGTTTCTAG	110 bp
	AAGCTCAGATCTAAATACCT	
TLR9 (1486T/C)	TTCATTCATTCAGCCTTCACTCA	565 bp
	GAGTCAAAGCCACAGTCCACA	
TLR9 (C2848T)	GCAGCACCCTCAACTTCACC	360 bp
	GGCTGTGGATGTTGTTGTGG	

TLRs: Toll-like receptors, PCR: Polymerase chain reaction

Table 2: Genotype and allele frequencies of TLR2 (−196-174) between EBVaGCs and EBVnGCs

Genotype/allele	EBVaGC *n*=52 (%)	EBVnGC *n*=157(%)	OR (95%CI)	*P*
TLR2 (−196 to −174)				
Genotypic frequencies				
ins/ins	17 (32.69)	56 (35.67)	1.00	
ins/del	23 (44.23)	63 (40.13)	1.20 (0.58-2.48)	0.62
del/del	12 (23.08)	38 (24.20)	1.04 (0.45-2.42)	0.93
Recessive model				
Others	40 (76.92)	119 (75.80)	1.00	
del/del	12 (23.08)	38 (24.20)	0.94 (0.45-1.97)	0.87
Dominant model				
ins/ins	17 (32.69)	56 (35.67)	1.00	
others	35 (67.31)	101 (64.33)	1.14 (0.59-2.22)	0.70
Allelic frequencies				
ins allele	56 (53.85)	170 (54.14)	1.00	
del allele	48 (46.15)	144 (45.86)	1.01 (0.65-1.58)	0.97

TLRs: Toll-like receptors, OR: Odds ratio

63 bp, and 337 bp [Figure 2B]. There was no significant difference between EBVaGC and EBVnGC. The differences in the distribution of genotype and allele frequency between the GC patients and the blood donors were statistically significant. The distributions of *TLR3* (c. 1377C/T) genotypes and alleles in patients and controls are listed in Tables 4 and 5. Analysis of the representative sequence is demonstrated in Figure 2C.

Analysis of the TLR4 gene [Asp299Gly (rs4986790) and Thr399Ile (rs4986791)]

The expected amplification products of the *TLR4* gene Asp299Gly (rs4986790) and Thr399Ile (rs4986791) polymorphisms were 140 bp and 110 bp, respectively. All of the samples from patients and healthy donors were positive for these two amplification bands [Figure 3a and 3b]. After the digestion with HinfI and NcoI, all bands remained at 140 bp or 110 bp, respectively, in all samples, indicating that there was no mutation at these two sites.

Table 3: Genotype and allele frequencies of TLR2 (1196-174) between GCs and controls

Genotype/allele	GC *n*=209 (%)	Control *n*=94 (%)	OR (95%CI)	*P*
TLR2 (−196 to −174)				
Genotypic frequencies				
ins/ins	73 (34.93)	42 (44.68)	1.00	0.11
Ins/del	86 (41.51)	37 (39.36)	1.34 (0.78-2.30)	0.29
del/del	50 (23.92)	15 (15.96)	1.92 (0.96-3.83)	0.06
Recessive model				
Others	159 (76.08)	79 (84.04)	1.00	
del/del	50 (23.92)	15 (15.96)	1.66 (0.88-3.13)	0.12
Dominant model				
ins/ins	73 (34.93)	42 (44.68)	1.00	
Others	136 (65.07)	52 (55.52)	1.51 (0.92-2.47)	0.11
Allelic frequencies				
ins allele	226 (54.07)	121 (64.36)	1.00	
del allele	192 (45.93)	67 (35.64)	1.53 (1.08-2.19)	0.018

TLRs: Toll-like receptors, OR: Odds ratio

Table 4: Genotype and allele frequencies of TLR3 (c. 1377C/T) between EBVaGCs and EBVnGCs

Genotype/allele	EBVaGC *n*=52 (%)	EBVnGC *n*=157(%)	OR (95%CI)	*P*
TLR3 (c. 1377C/T)				
Genotypic frequencies				
CC	16 (30.77)	59 (37.58)	1.00	
CT	23 (44.23)	61 (38.85)	1.40 (0.67-2.89)	0.38
TT	13 (25.00)	37 (23.57)	1.30 (0.56-3.00)	0.55
Recessive model				
Others	39 (75.00)	120 (76.43)	1.00	
TT	13 (25.00)	37 (23.57)	1.08 (0.52-2.24)	0.83
Dominant model				
CC	16 (30.77)	59 (37.58)	1.00	
Others	36 (69.23)	98 (62.42)	1.36 (0.69-2.65)	0.38
Allelic frequencies				
C allele	55 (52.89)	179 (57.01)	1.00	
T allele	49 (47.11)	135 (42.99)	1.18 (0.76-1.84)	0.46

TLRs: Toll-like receptors, OR: Odds ratio

Analysis of TLR9 [1486T/C (rs187084) and C2848T (rs352140)]

The length of the specific amplification band of the *TLR9* gene (1486T/C) was 565 bp [Figure 4a]. All of the samples were positive for this band. The PCR products were digested by Afl II. After electrophoresis, the CC genotype showed two bands, 416 bp and 149 bp. The homozygous mutant TT genotype band remained at 565 bp band. The heterozygous CT genotype showed three bands, 565 bp, 416 bp, and 149 bp [Figure 4b]. The associations between the EBVaGC and EBVnGC groups, the GC group, and the control group were not statistically significant both in genotype and allelic frequencies. Analysis of the representative sequence is demonstrated in Figure 4C.

The length of specific amplification band of the *TLR9* gene (C2848T) was 360 bp. All of the samples were positive for this band [Figure 5A]. The PCR products were digested with BstUI. The CC genotype showed a 360 bp band. The TT genotype was two bands, 227 bp and 123 bp. The CT genotype showed three bands, 360 bp, 227 bp, and 123 bp [Figure 5B]. The associations between the EBVaGC and EBVnGC groups, the GC group, and the control group were not statistically significant in both genotype and allelic frequency. The distributions of the *TLR9* genotypes and alleles in patients and controls are listed in Tables 6 and 7. Analysis of the representative sequence is demonstrated in Figure 5C.

DISCUSSION

We investigated whether *TLR2* -196 to –174 del, *TLR3* (c. 1377C/T), *TLR4* [Asp299Gly (rs4986790) and Thr399Ile (rs4986791)], and *TLR9* [1486T/C (rs187084) and

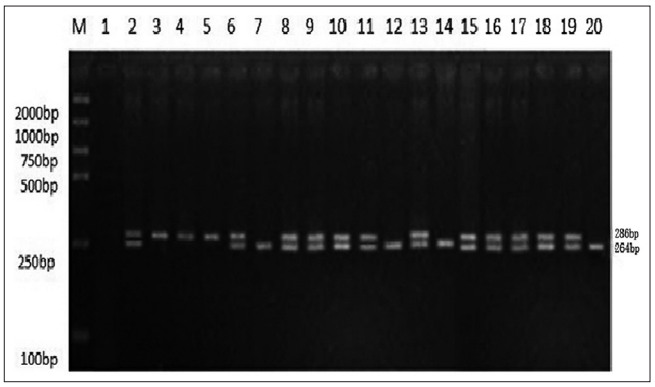

Figure 1: PCR analysis for TLR2 genotyping. Lane M, DL 2000 DNA Marker; lane 1, negative control; lane 3–5 ins/ins genotype (homozygous wild type); lane 2, 6, 8–11, 13, 15–19 ins/del genotype (heterozygous mutated type); lane 7, 12, 14, 20 del/del genotype (homozygous mutated type).

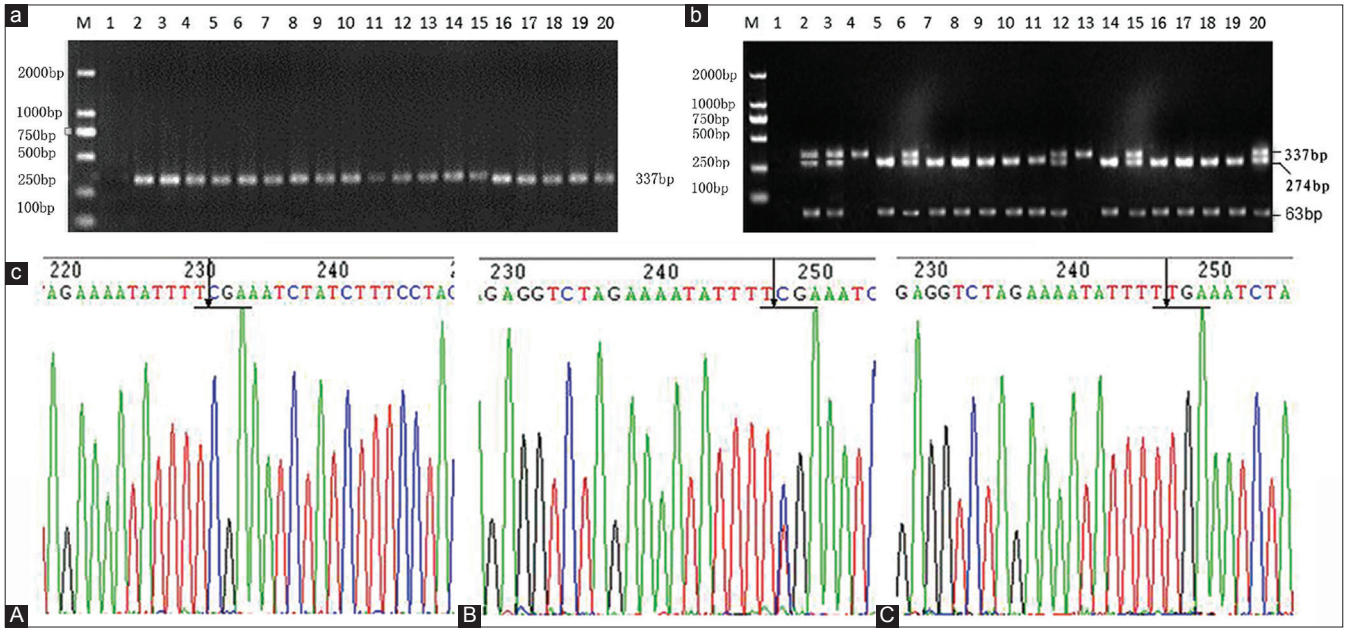

Figure 2: (a) Gel electrophoresis of the TLR3 (c.1377C/T) PCR product. Lane M, DL 2000 DNA Marker; lane 1, negative control; lane 2–20,TLR3 (c.1377C/T) PCR product of gastric carcinoma patients and healthy controls. (b) RFLP analysis with TaqI restriction enzyme digestion after PCR amplification for TLR3 (c.1377C/T) genotyping. Lane M, DL 2000 DNA Marker; lane 1, negative control; lane 5, 7–11, 14, 16–19 CC genotype (homozygous wild type); lane 4, 13 TT genotype (homozygous mutated type); lane 2, 3, 6, 12, 15, 20 CT genotype (heterozygous type).(c) Sequence analysis of the representative PCR products for TLR3 (c.1377C/T). A, representative samples (Type CC) that possess the TaqI restriction site; B, representative samples (Type CT) that possess the TaqI restriction site in the corresponding region; C, representative samples (Type TT) that lack the TaqI restriction site. Arrow indicates the TaqI restriction site

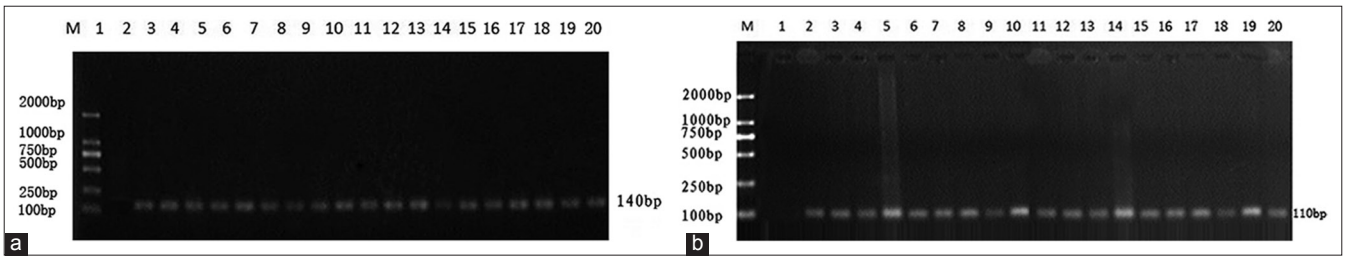

Figure 3: (a) Gel electrophoresis of the TLR4 Asp299Gly PCR product. Lane M, DL 2000 DNA Marker; lane 1, negative control; lane 2–20, TLR4 Asp299Gly PCR product of GC patients and healthy controls. (b) Gel electrophoresis of the TLR4 Thr399Ile PCR product. Lane M, DL 2000 DNA Marker; lane 1, negative control; lane 2–20TLR4 Thr399Ile PCR product of GC patients and healthy controls

C2848T (rs352140)] polymorphisms affect the risk of developing GC as well as the possible interaction between EBV and *TLR* gene polymorphisms in Shandong Province of Northern China. Our results indicated an association of the *TLR2* -196 to -174 del and the *TLR3* [c. 1377C/T (rs 3775290)] polymorphisms with susceptibility for GC in this population. The polymorphisms of *TLR9* [1486T/C (rs187084) and C2848T (rs352140)] were not related to the risk of GC, and the polymorphisms of *TLR4* were not observed in the studied population. Meanwhile, it appeared that there was no interaction between EBV and *TLR* polymorphisms.

The *TLR2* gene is located on chromosome 4; the -196 to -174 del polymorphism changes the promoter activity of this gene. It has been reported that the *TLR2* del/del genotype decreased the transcriptional activity of this gene.[29] One study conducted by de Oliveira and Silva showed that the frequency of *TLR2* -196 to -174 ins/del + del/del was significantly different between GC patients and healthy blood donors in Brazil.[13] However, the findings of our study were different. Our results are consistent with the conclusions of Hishida *et al.* in Japan.[11] Perhaps a district or ethnic difference exists between these two populations. Higher frequencies of the *TLR2* del allele were only observed in the GC group compared with the blood donors, emphasizing the role of this polymorphism in gastric tumorigenesis.

TLR3 specifically recognizes dsRNA, which activates NF-kB and interferon IFN-β precursors. There is already evidence

Table 5: Genotype and allele frequencies of TLR3 (c. 1377C/T) between GCs and controls

Genotype/allele	GC *n*=209 (%)	Control *n*=94 (%)	OR (95%CI)	*P*
TLR3 (c. 1377C/T)				
Genotypic frequencies				
CC	75 (35.89)	53 (56.38)	1.00	
CT	84 (40.19)	30 (31.91)	1.98 (1.15-3.41)	0.014
TT	50 (23.92)	11 (11.71)	3.21 (1.53-6.74)	0.002
Recessive model				
Others	159 (76.08)	83 (88.29)	1.00	
TT	50 (23.92)	11 (11.71)	2.37 (1.17-4.80)	0.014
Dominant model				
CC	75 (35.89)	53 (56.38)	1.00	
others	134 (64.11)	41 (43.62)	2.31 (1.41-3.79)	0.001
Allelic frequencies				
C allele	234 (55.98)	136 (72.34)	1.00	
T allele	184 (44.02)	52 (27.66)	2.06 (1.42-2.99)	0.000

TLRs: Toll-like receptors, OR: Odds ratio

Table 6: Genotype and allele frequencies of TLR9 between EBVaGCs and EBVnGCs

Genotype/allele	EBVaGC *n*=52 (%)	EBVnGC *n*=157 (%)	OR (95%CI)	*P*
TLR9 (-1486T/C)				
Genotypic frequencies				
TT	14 (26.92)	44 (28.02)	1.00	
CT	31 (59.62)	80 (50.96)	1.22 (0.59-2.53)	0.60
CC	7 (13.46)	33 (21.02)	0.67 (0.24-1.84)	0.43
Recessive model				
Others	45 (86.54)	124 (78.98)	1.00	
CC	7 (13.46)	33 (21.02)	0.59 (0.24-1.42)	0.23
Dominant model				
TT	14 (26.92)	44 (28.03)	1.00	
Others	38 (73.08)	113 (71.97)	1.06 (0.52-2.14)	0.88
Allelic frequencies				
T allele	59 (56.73)	168 (53.50)	1.00	
C allele	45 (43.27)	146 (46.50)	0.88 (0.56-1.37)	0.57
TLR9 (C2848T)				
Genotypic frequencies				
CC	18 (34.62)	41 (26.12)	1.00	
CT	22 (42.31)	84 (53.50)	0.60 (0.29-1.23)	0.16
TT	12 (23.07)	32 (20.38)	0.85 (0.36-2.03)	0.72
Recessive model				
Others	40 (76.93)	125 (79.62)	1.00	
TT	12 (23.07)	32 (20.38)	1.17 (0.55-2.49)	0.68
Dominant model				
CC	18 (34.62)	41 (26.12)	1.00	
Others	34 (65.38)	116 (73.88)	0.67 (0.34-1.31)	0.24
Allelic frequencies				
C allele	58 (55.77)	166 (52.87)	1.00	
T allele	46 (44.23)	148 (47.13)	0.89 (0.57-1.39)	0.61

TLRs: Toll-like receptors, OR: Odds ratio,

Table 7: Genotype and allele frequencies of TLR9 between GCs and controls

Genotype/allele	GC *n*=209 (%)	Control *n*=94 (%)	OR (95%CI)	*P*
TLR9 (1486T/C)				
Genotypic frequencies				
TT	58 (27.75)	29 (30.85)	1.00	
CT	111 (53.11)	55 (58.51)	1.01 (0.58-1.75)	0.97
CC	40 (19.14)	10 (10.64)	2.00 (0.88-4.56)	0.10
Recessive model				
Others	169 (80.86)	84 (89.36)	1.00	
CC	40 (19.14)	10 (10.64)	1.99 (0.95-4.17)	0.07
Dominant model				
TT	58 (27.75)	29 (30.85)	1.00	
Others	151 (82.25)	65 (69.15)	1.16 (0.68-1.98)	0.58
Allelic frequencies				
T allele	227 (54.31)	113 (60.11)	1.00	
C allele	191 (45.69)	75 (39.89)	1.27 (0.89-1.80)	0.18
TLR9 (C2848T)				
Genotypic frequencies				
CC	59 (28.23)	27 (28.72)	1.00	
CT	106 (50.72)	55 (58.51)	0.88 (0.50-1.54)	0.66
TT	44 (21.05)	12 (12.77)	1.68 (0.77-3.68)	0.19
Recessive model				
Others	165 (79.95)	82 (87.23)	1.00	
TT	44 (21.05)	12 (12.77)	1.82 (0.91-3.64)	0.09
Dominant model				
CC	59 (28.23)	27 (28.72)	1.00	
Others	150 (71.77)	77 (71.28)	0.89 (0.52-1.52)	0.67
Allelic frequencies				
C allele	224 (53.59)	109 (57.98)	1.00	
T allele	194 (46.41)	79 (42.02)	1.19 (0.84-1.69)	0.32

TLRs: Toll-like receptors, OR: Odds ratio

that *TLR3* is closely related to tumor occurrence. *TLR3* mRNA expression levels were significantly increased in breast cancer. However, studies reporting on *TLR3* gene polymorphisms and their association with GC are rare. A study in 2012 by Mandal *et al.* reported that *TLR3* [c. 1377C/T (rs3775290)] polymorphisms were not associated with prostate cancer risk in a North Indian population.[30] Our results, for the first time, show a significant difference between the GC group and healthy blood donors in *TLR3* genotype and allele frequency.

The *TLR4* gene consists of three exons and is situated on chromosome 9. There are two single nucleotide polymorphisms (SNPs), *TLR4* + 896A/G and + 1196C/T, in exon 3, which lead to the substitutions Asp299Gly and Thr399Ile, respectively.[13] Haploview analyzed that there were higher frequencies of *TLR4* G-C (299Gly-399Thr) and G alleles in GC patients, demonstrating an association with the increased risk of GC for carriers of these SNPs. In TLR4, the normal extracellular structure can be disrupted by the amino acids substitution Asp299Gly, which may cause a reduced ability to recognize ligands, interact with proteins,

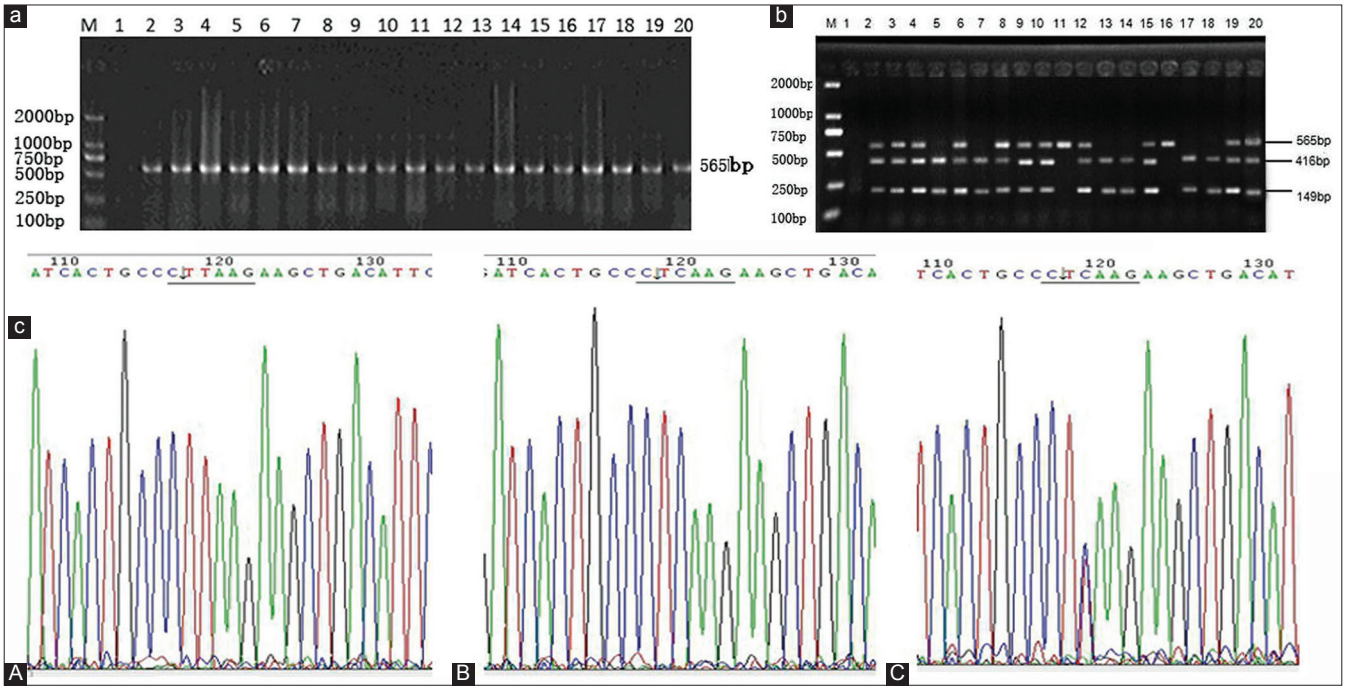

Figure 4: Gel electrophoresis of the TLR9 1486T/C PCR product. Lane M, DL 2000 DNA Marker; lane 1, negative control; lane 2–20TLR9 1486T/C PCR product of GC patients and healthy controls. (b) RFLP analysis with Afl II restriction enzyme digestion after PCR amplification for TLR9 1486T/C genotyping. Lane M, DL 2000 DNA Marker; lane 1, negative control; lane 11, 16 TT genotype (homozygous wild type); lanes 5, 7, 13, 14, 17, 18 CC genotype (homozygous mutated type); lanes 2–4, 6, 8–10, 12, 15, 19, 20 CT genotype (heterozygous type). (c) Sequence analysis of the representative PCR products for TLR9 (1486T/C). A, representative samples (Type TT) that possess the Afl II restriction site; B representative samples (Type CC) that lack the Afl II restriction site; C, representative samples (Type CT) that possess the Afl II restriction site in the corresponding region. Arrow indicates the Afl II restriction site

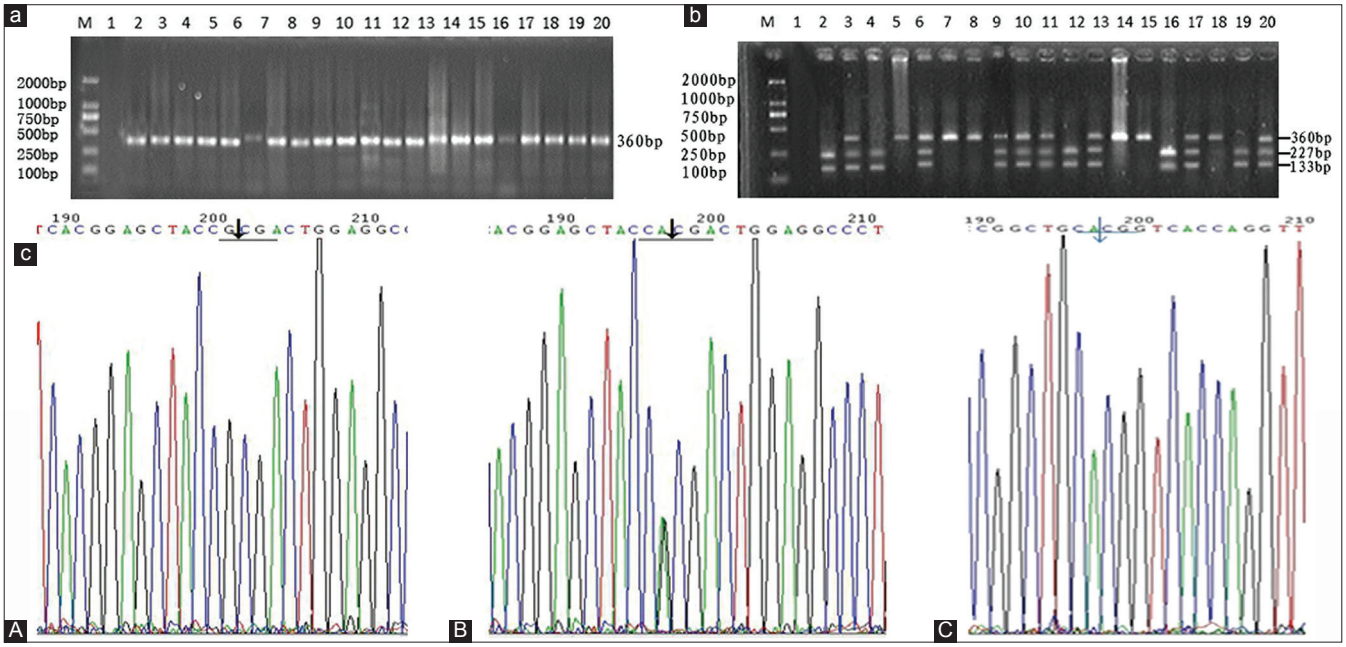

Figure 5: (a) Gel electrophoresis of the TLR9 (C2848T) PCR product. Lane M, DL 2000 DNA Marker; lane 1, negative control; lane 2–20TLR9 (C2848T) PCR product of GC patients and healthy controls. (b) RFLP analysis with BstUI restriction enzyme digestion after PCR amplification for TLR9 C2848T genotyping. Lane M, DL 2000 DNA Marker; lane 1, negative control; lanes 5, 7, 8, 14, 15, 18 CC genotype (homozygous wild type); lanes 2, 4, 12, 16, 19 TT genotype (homozygous mutated type); lane 3, 6, 9–11, 13, 17, 20 CT genotype (heterozygous mutated type). (c) Sequence analysis of the representative PCR products for TLR9 (C2848T). A, representative samples (Type TT) that lack the BstUI restriction site; B representative samples (Type CC) that possess the BstUI restriction site; C, representative samples (Type CT) that possess the BstUI restriction site in the corresponding region. Arrow indicates the BstUI restriction site

and react to lipopolysaccharide. As a result, TLR4 cannot be transported to the cell membrane.[31,32] This alteration causes an increased inflammatory reaction, likely due to the loss of ability to stimulate regulatory cells and produce IL-10.[33]

Approximately 10% of Caucasian and African populations carry both SNPs in *TLR4*. It has been reported that various infectious diseases are closely associated with these two SNPs. However, these polymorphisms were not found in the Asian population.[34,35] In our study, no polymorphisms were observed in these Northern Chinese populations, consistent with the results from Cheng *et al*.[35] These results may indicate that the Asp299Gly and Thr399Ile *TLR4* gene polymorphisms may be distributed differently among different regions and ethnicities.

Several SNPs have been identified within the *TLR9* gene. It was reported that individuals with the CC genotype of *TLR9* (T1486C) and those expressing the T allele of *TLR9* (T1486C) (CT and TT) were also significantly differently distributed between groups.[36] Compared with the TT genotype, the *TLR9* (T1486C) TC heterozygote was reported to be associated with a significantly increased risk of cervical cancer. Although the homozygous variant was associated with an insignificant increase in the risk of cervical cancer, the TC/CC genotypes contributed to the risk of cervical cancer in the dominant genetic model.[37] Wang *et al*. showed that, compared with the TT homozygote, patients with both the TC variant and the CC variant had a higher risk of gastric cancer.[38] In the present study, we found no significant association between the GC group and the healthy blood donors in either genotype or allelic frequency of *TLR9* (T1486C), a finding that is inconsistent with the former conclusion. Further investigation is needed to demonstrate this phenomenon. Recently, *TLR9* (C2848T) was reported to be associated with various diseases. There were no significant differences in the prevalence of the *TLR9* C > T (rs352140) genotype and alleles between patients with SLE and controls in a Polish population. However, there was a contribution of the T/T and T/C genotypes to renal and immunologic disorders in SLE patients.[39] Furthermore, there was no association between the *TLR9* gene (rs352140) polymorphism and SLE in Asian populations.[40] In northern India, there was no association between prostate cancer and controls in the *TLR9* (rs352140) polymorphism.[30] In the present study, no significant difference was observed between the GC group and healthy blood donors in *TLR9* (C2848T) polymorphisms. Functional studies in ethnically diverse populations are required to render a more comprehensive engagement of innate immunity in discovering the disease-related variants for specific disease etiology.

EBVaGC is distributed worldwide, with an annual incidence of more than 90,000 patients (10% of total GC).[41] Following infection, EBV remains in a latent state in EBVaGC, a period classified as latency I. Compared with EBVnGC, EBVaGC has unique clinical and pathological features, indicating a unique oncogenic mechanism. Many studies have investigated the interaction between TLRs and EBV in various diseases. It has reported that the EBV latent membrane protein 1 (LMP1) may inhibit transcription of the TLR9 gene. Overexpression of LMP1 in B cells reduced TLR9 promoter activity, mRNA, and protein levels. LMP1 mutants altered in their ability to activate the NF-κB pathway prevented *TLR9* promoter deregulation.[42] Furthermore, EBERs induce IL-10 through IRF3 but not NF-κB activation in BL cells, indicating that regulation of innate immune signaling by EBERs contributes to EBV-mediated oncogenesis. Most recently, it was reported that EBERs are secreted from EBV-infected cells and are recognized by *TLR3*, leading to the induction of type-I IFNs and inflammatory cytokines, as well as subsequent immune activation.[43] The interaction between TLR polymorphisms and EBV was seldom discussed in former studies. In the present research, we focused on the possible existence of such an interaction. To date, we have not found evidence of an interaction between these polymorphisms and EBV. Many more SNPs in the *TLR* genes require further investigation in order to elucidate this interaction.

In conclusion, in this study, we found a significant difference between the GC group and healthy blood donors with regard to the presence of the *TLR3* (c. 1377C/T) gene polymorphism, both in genotype and allele frequency in Northern China. Additionally, we found an association between the GC group and healthy blood donors in the del allele of *TLR2* (-196 to -174 del). Moreover, we found no interaction between EBV and TLR polymorphisms in EBVaGC in the Shandong Province of Northern China. The present study may help us to understand the relationship between *TLR* gene polymorphisms and GC (including EBVaGC and EBVnGC) deeply. However, the size of the samples in our research is small; large number of EBVaGC patients and more research are still required to reveal the association.

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