

Hypomethylation of the Toll-like receptor-2 gene increases the risk of essential hypertension

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Abstract. Studies on the etiology of essential hypertension (EH) have demonstrated that chronic inflammation contributes to the onset and development of elevated blood pressure. Toll-like receptors (TLRs), important immune receptors, serve a role in chronic inflammation and are associated with EH. In the present study, 96 patients with EH, and 96 age- and sex-matched healthy controls were recruited, and eight cytosine-phosphate-guanine (CpG) dinucleotides (CpG1-8) were analyzed using bisulfite pyrosequencing technology. It was observed that the methylation levels of all of the eight CpG dinucleotides were decreased in the EH group compared with the control group; however, only CpG1 (2.83 ± 1.34 vs. 3.44 ± 1.75 ; $P=0.009$), CpG6 (3.58 ± 3.64 vs. 8.30 ± 4.13 ; $P<0.001$) and CpG8 (8.91 ± 5.32 vs. 11.33 ± 3.87 ; $P<0.001$) were significantly different, as demonstrated by paired t-test analysis. In addition, logistic regression analysis demonstrated that CpG6 hypomethylation was a risk factor of EH (odds ratio=1.10; adjusted $P=0.009$), and CpG6 methylation level was observed to be negatively correlated with systolic blood pressure ($r=-0.304$; $P<0.001$) and diastolic blood pressure ($r=-0.329$; $P<0.001$). Additionally, receiver operating characteristic curve analysis demonstrated that a methylation level of 7.5% for CpG6 (area under the curve,

0.834; $P<0.001$) was an appropriate threshold value to predict the risk of EH. With generalized multifactor dimensionality reduction, a potential gene-gene interaction between CpG6 and CpG8 ($P=0.001$), and gene-environment interactions between smoking, alcohol consumption, CpG6, CpG7 and CpG8 ($P=0.011$), were observed. In conclusion, the results of the present study demonstrated that hypomethylation of the *TLR2* promoter, particularly CpG6, was associated with the risk of EH in this population. Additionally, a gene-gene interaction between CpG6 and CpG8, and interactions between environmental factors, including smoking and alcohol consumption, and CpG6, CpG7 and CpG8, may be associated with the risk of EH.

Introduction

Essential hypertension (EH) is a risk factor for cardiovascular diseases and organ damage, which has become a major cause of morbidity and mortality worldwide (1). The World Health Organization (Geneva, Switzerland) has reported that 29.2% of the global population will develop hypertension by 2025, and the prevalence in China was observed to be 26.7% in 2010 (2). Although the etiology of EH remains unclear, previous studies have indicated that low-grade chronic inflammation is a hallmark of EH and contributes to target organ damage and the development of atherosclerosis, which is a process mediated by circulating immune cells, particularly leucocytes (3-5).

Toll-like receptors (TLRs), members of the interleukin (IL) 1R superfamily, are transmembrane receptors with extracellular leucine-rich repeats and an important intracellular signaling domain. TLRs are expressed in monocytes, macrophages and neutrophils, and recognize pathogen-associated molecular patterns to initiate an innate immune response. TLR2 serves a role in endothelial cell activation, macrophage recruitment and pro-inflammatory cytokine production. In TLR2 signaling, TLR2 dimerizes with TLR1 or TLR6. The heterodimers recruit and activate interleukin-1 receptor-associated kinase 4 via a myeloid differentiation primary response protein MyD88 (MyD88)/MyD88 adaptor-like (Mal)-dependent mechanism, and therefore facilitate the induction of cytokines (6-8).

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Previously, elevated circulating pro-inflammatory cytokine markers related to the TLR signaling pathway, including tumor necrosis factor (TNF)- α , C-reactive protein (CRP) and IL-6, were hypothesized to be important risk factors for EH (4,9). TLRs have been suggested to serve a role in the pathogenesis of EH (10,11) and atherosclerotic diseases (12-14). However, the underlying mechanisms that regulate this response remain unclear.

An important mechanism of epigenetic regulation, DNA methylation is reversible and primarily occurs at cytosine residues in cytosine-phosphate-guanine (CpG) dinucleotides, in mammalian cells (15). Gene promoter hypermethylation silences gene expression, while promoter hypomethylation promotes active transcription (16). Previous studies into the etiology of EH have focused on DNA methylation. *ADD1*, *AGTR1* and *GCK* gene methylation have been demonstrated to be associated with EH (17-19). Alexeeff *et al* (9) reported that the aberrant methylation of *TLR2*, inducible nitric oxide synthase and interferon- γ was associated with blood pressure.

However, the association of *TLR2* methylation with EH remains unclear. The present study aimed to investigate whether *TLR2* promoter methylation was associated with EH and to assess the association of *TLR2* promoter methylation with age, blood pressure and other risk factors of EH.

Materials and methods

Sample collection. A total of 192 individuals, including 96 healthy controls, and 96 newly-diagnosed patients with EH who had not received anti-hypertensive therapy, were recruited at Ningbo Seventh Hospital (Ningbo, China). Patients were defined as hypertensive according to the 'gold standard' diagnostic criteria, and exhibited ≥ 3 consecutive measurements of systolic blood pressure (SBP) >140 mmHg and/or diastolic blood pressure (DBP) >90 mmHg (20). Controls exhibited SBP <120 mmHg and DBP <80 mmHg, and reported no family history of hypertension in first degree relatives. All of the participants were from Han Chinese families who had been residing in Ningbo for ≥ 3 generations, with no history of secondary hypertension, diabetes mellitus, stroke, renal failure, myocardial infarction, drug abuse, or other serious diseases.

A calibrated mercury sphygmomanometer with an adult-sized cuff was used to measure blood pressure based on the standard protocols of the American Heart Association (21). Blood pressure was measured twice in the supine position, ≥ 10 min apart by trained technicians. Blood samples were drawn from the antecubital vein using vacutainer tubes containing EDTA, and stored at -80°C for DNA extraction. The protocol was approved by the ethics committee of Ningbo Seventh Hospital, and written informed consent was obtained.

Biochemical analyses. Plasma levels of triglyceride, total cholesterol, uric acid, high-density lipoprotein (HDL), low-density lipoprotein (LDL), alanine aminotransferase, serum creatinine and leucocytes were measured enzymatically using an AU2700 automatic analyzer (Olympus Corporation, Tokyo, Japan). A Lab-Aid 820 nucleic acid

Table I. Primers of the Toll-like receptor-2 cytosine-phosphate-guanine island sequence.

Primers	Sequence
Forward	5'-Biotin-GGTAGTTGTAGGGGTAGGAT-3'
Reverse	5'-ACCCAAAAAACTCTAAACCTC-3'
Sequence	5'-TTCCAAACAAATAACC-3'

extraction analyzer (Xiamen Zeesan Biotech Co., Ltd., Xiamen, China) was used to extract genomic DNA from peripheral blood samples. DNA concentration was measured using a NanoDrop 2000 ultra-micro nucleic acid ultraviolet tester (Thermo Fisher Scientific, Inc., Wilmington, DE, USA).

The sequencing-by-synthesis technique of pyrosequencing was used to measure methylation levels. DNA sequences were reacted with sodium bisulfite (EpiTech Bisulfite kit; Qiagen GmbH, Hilden, Germany) to convert unmethylated cytosine residues to thymine, and subsequently amplified by polymerase chain reaction (PCR) prior to being 'sequenced by synthesis' (Pyromark Gold Q96, Qiagen GmbH) (22). The CpG sites of target gene sequences and PCR primers were chosen according to the scores automatically calculated by the PyroMark Assay Design (version 2.0.1.15; Qiagen GmbH) using previously established protocols for primer design (23). CpG island sequences were amplified using a Mastercycler Nexus Gradient (Eppendorf, Hamburg, Germany) in reactions containing 12 μl ZymoTaq™ Premix (Zymo Research Corporation, Irvine, CA, USA), 8 μl DNase/RNase-free H_2O , 1.5 μl each of forward and reverse primer and 2 μl bisulfite-converted DNA. Reactions were first denatured at 95°C for 10 min; amplified over 40 cycles at 95°C for 30 sec, 54.1°C for 40 sec and 72°C for 50 sec; and extended at 72°C for 7 min. *TLR2* CpG island sequences were amplified with the primers presented in Table I.

Statistical analyses. Experimental data were analyzed using PASW statistics software (version 19.0; IBM SPSS, Armonk, NY, USA). Results are presented as the mean \pm standard deviation or number (percentage) of patients. Continuous variables, including DNA methylation, age, body mass index (BMI), total cholesterol, triglycerides, uric acid, HDL, LDL, serum creatinine and leucocyte count, were compared by paired t-test or nonparametric test. The Pearson χ^2 or Fisher's exact test was used to analyze the association between categorical variables (sex, smoking and alcohol consumption) and essential hypertension. Pearson's correlation analysis was used to investigate interactions among the eight CpG sites in the *TLR2* promoter sequence. Receiver operating characteristic (ROC) curves were used to determine the sensitivity of *TLR2* promoter methylation as a predictor of EH. Logistic regression was implemented to adjust for confounding factors. Generalized multifactor dimensionality reduction (GMDR) was applied to investigate underlying high-order interactions between *TLR2* promoter methylation and risk factors of EH. $P < 0.05$ was considered to indicate a statistically significant difference.

Table II. Comparison of characteristics between controls and EH group.

Characteristics	Controls	EH	$t(Z)/\chi^2$	P-value
Age (mean \pm SD)	56.3 \pm 8.2	56.7 \pm 8.7	0.32	0.747
Sex, male/female	38/58	38/58	n/a	n/a
Smoking, yes/no	79/17	69/27	2.95	0.086
Alcohol consumption, yes/no	65/31	56/40	1.81	0.178
BMI (mean \pm SD)	22.16 \pm 2.30	23.6 \pm 3.09	4.09	9.1 $\times 10^{-5}$
HDL, mg/dl (mean \pm SD)	7.99 \pm 6.32	2.07 \pm 5.58	6.57	2.6 $\times 10^{-9}$
LDL, mg/dl (mean \pm SD)	3.21 \pm 0.87	3.31 \pm 0.68	0.90	0.370
ALT, IU/l (mean \pm SD)	26.41 \pm 16.1	28.27 \pm 12	0.89	0.370
Triglyceride, mmol/l (mean \pm SD)	1.21 \pm 0.68	1.43 \pm 0.72	2.33	0.022
Total cholesterol, mmol/l (mean \pm SD)	5.19 \pm 0.89	5.38 \pm 0.61	1.71	0.091
Urea, mmol/l (mean \pm SD)	4.96 \pm 1.07	5.03 \pm 1.11	0.52	0.607
Uric acid, μ mol/l (mean \pm SD)	300.32 \pm 73.15	325.75 \pm 82.63	2.75	0.007
Serum creatinine, μ mol/l (mean \pm SD)	82.68 \pm 12.28	83.46 \pm 11.04	0.53	0.600
WBC count (mean \pm SD)	5.59 \pm 0.93	6.17 \pm 0.98	4.84	1.30 $\times 10^{-6}$
Lymphocyte count (mean \pm SD)	1.98 \pm 0.61	2.1 \pm 0.55	2.05	0.040
Monocyte count (mean \pm SD)	0.30 \pm 0.18	0.31 \pm 0.17	0.49	0.622
Neutrophil granulocyte count (mean \pm SD)	3.05 \pm 0.97	3.35 \pm 0.92	2.55	0.011
Eosinophil granulocyte count (mean \pm SD)	0.13 \pm 0.13	0.129 \pm 0.10	0.75	0.451
Basophil granulocyte count (mean \pm SD)	0.15 \pm 0.64	0.02 \pm 0.02	0.66	0.508

BMI, body mass index; HDL, high-density lipoprotein; LDL, low-density lipoprotein; ALT, alanine aminotransferase; EH, essential hypertension; SD, standard deviation; WBC, white blood cell; n/a, not applicable.

Table III. Logistic regression analysis of the methylation levels of the eight CpG sites.

Variables	Controls (mean \pm SD)	EH (mean \pm SD)	t	P-value	Controls vs. EH	
					OR (95% CI)	P-value ^a
CpG1	3.44 \pm 1.75	2.83 \pm 1.34	2.69	0.009	0.99 (0.838~1.173)	0.921
CpG2	1.93 \pm 1.84	1.71 \pm 1.57	0.97	0.336	0.91 (0.715~1.146)	0.408
CpG3	0.69 \pm 1.60	0.58 \pm 1.80	0.43	0.665	0.96 (0.743~1.248)	0.776
CpG4	1.65 \pm 2.08	1.65 \pm 2.06	0.00	1.000	1.07 (0.883~1.305)	0.478
CpG5	0.68 \pm 1.53	0.66 \pm 1.80	0.09	0.926	1.03 (0.805~1.327)	0.798
CpG6	8.30 \pm 4.13	3.58 \pm 3.64	8.31	6.5 $\times 10^{-13}$	1.10 (1.021~1.161)	0.009
CpG7	3.51 \pm 3.90	3.25 \pm 3.51	0.51	0.613	0.99 (0.893~1.103)	0.889
CpG8	11.33 \pm 3.87	8.91 \pm 5.32	3.82	2.4 $\times 10^{-4}$	0.98 (0.939~1.025)	0.389

^aAdjusted for age, gender, smoking, alcohol consumption, uric acid, serum creatinine, triglyceride, HDL (mg/dl) and BMI. CpG, cytosine-phosphate-guanine; EH, essential hypertension; SD, standard deviation; OR, odds ratio; CI, confidence interval.

Results

Characteristics of the 96 healthy controls and 96 patients with EH are presented in Table II. The age (± 3 years) and sex ratio was matched in the participants between the two groups. In addition, as presented in Table II, BMI ($t=4.09$; $P=9.1\times 10^{-5}$), HDL ($t=6.57$; $P=2.6\times 10^{-9}$), triglyceride ($t=2.33$; $P=0.022$) and uric acid ($t=2.75$; $P=0.007$) were significantly different between the two groups.

In the present study, eight CpG sites were selected to investigate the association between methylation and EH in the

CpG island of the *TLR2* gene promoter. Details of the eight CpG sites are presented in Fig. 1 and Table III. As presented in Table III and Fig. 2, eight CpG sites of the EH group exhibited decreased methylation levels compared with healthy controls; however, only CpG1 (2.83 \pm 1.34 vs. 3.44 \pm 1.75; $P=0.009$), CpG6 (3.58 \pm 3.64 vs. 8.30 \pm 4.13; $P<0.001$) and CpG8 (8.91 \pm 5.32 vs. 11.33 \pm 3.87; $P<0.001$) were significantly different.

In order to adjust for confounding factors, logistic regression was applied to obtain the odds ratio (OR) of CpG1-8. As presented in Table III, when adjusted for age, gender, smoking,

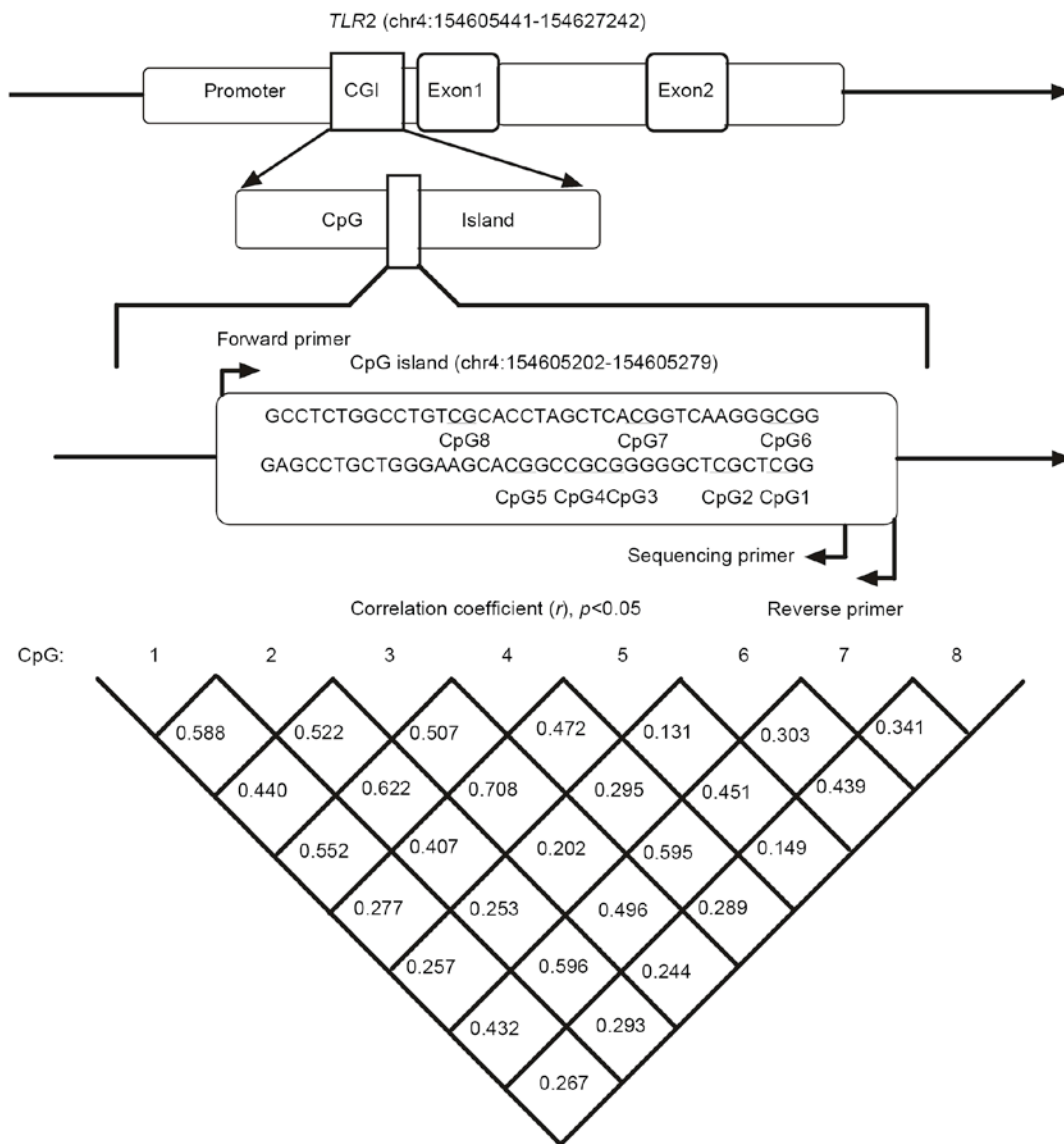


Figure 1. Correlation analysis between eight CpG sites in *TLR2* promoter region. *TLR2*, Toll-like receptor-2; CpG, cytosine-phosphate-guanine; CGI, CpG island.

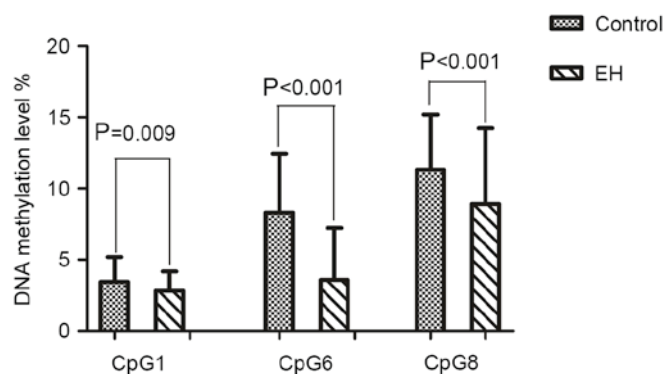


Figure 2. Analysis of CpG1, CpG6 and CpG8 site methylation. CpG, cytosine-phosphate-guanine; EH, essential hypertension.

alcohol consumption, uric acid, serum creatinine, triglyceride, HDL and BMI, the results indicated that the methylation level of CpG6 was an important risk factor for EH (OR=1.10;

adjusted $P=0.009$). Pearson correlation analysis demonstrated that the methylation level of CpG6 was negatively correlated with SBP ($r=-0.304$; $P<0.001$) and DBP ($r=-0.329$; $P<0.001$) (Fig. 3).

ROC curve analysis was used to analyze the diagnostic value of CpG6 methylation to EH. The results presented in Fig. 4 indicated that a methylation level of 7.5% for CpG6 (area under the curve, 0.834; $P<0.001$) was an appropriate threshold value to predict the risk of EH.

GMDR was used to investigate high-order interactions between the methylation of CpG sites of the *TLR2* promoter and other risk factors. The best models at various orders are summarized in Table IV. The two-order model between CpG6 and CpG8 is the best model of gene-gene interaction (testing balanced accuracy, 0.874; cross-validation consistency, 10/10; $P=0.01$), and the five-order model among smoking, alcohol consumption, CpG6, CpG7 and CpG8 is the best model of gene-environment interaction (testing balanced accuracy, 0.985; cross-validation consistency, 8/10; $P=0.01$).

Table IV. Generalized multifactor dimensionality reduction models of essential hypertension and high-order interactions among CpG sites in the Toll-like receptor-2 promoter.

Model	Testing balanced accuracy	Cross-validation consistency	Sign test (P-value)
CpG8	0.762	10/10	10 (0.001)
CpG6-CpG8	0.874	10/10	10 (0.001)
CpG1-CpG6-CpG8	0.917	8/10	10 (0.001)
Alcohol consumption-CpG6-CpG7-CpG8	0.945	7/10	9 (0.011)
Smoking-alcohol consumption-CpG6-CpG7-CpG8	0.985	8/10	9 (0.011)

CpG, cytosine-phosphate-guanine.

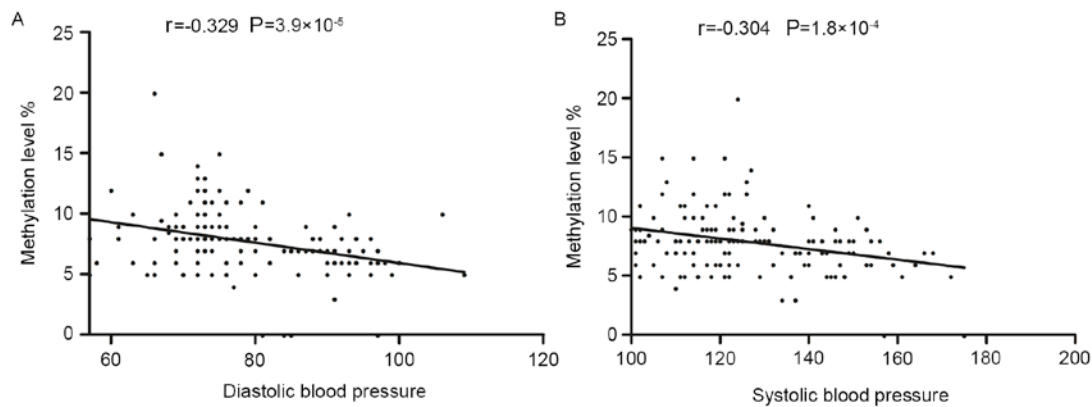


Figure 3. Pearson correlation analysis between cytosine-phosphate-guanine 6 site methylation and (A) diastolic and (B) systolic blood pressure.

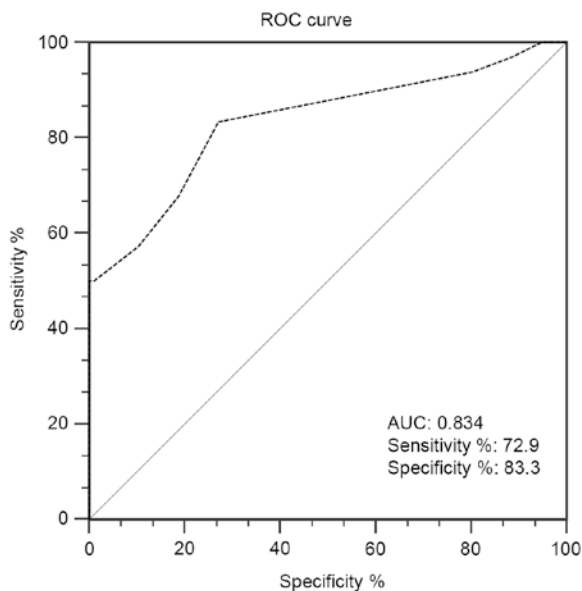


Figure 4. ROC analysis of the cytosine-phosphate-guanine 6 site in the diagnosis of essential hypertension. ROC, receiver operating curve; AUC, area under the curve.

Discussion

Previous studies have demonstrated that TLR2 activation may induce nicotinamide-adenine dinucleotide phosphate

oxidase to produce reactive oxygen species in monocytes and macrophages (24-26), and endothelial TLR2 signaling may result in inhibition of endothelial NO bioavailability (27). TLR2 dimers with TLR1 or TLR6 may lead to the upregulation of cytokines by a MyD88/Mal and nuclear factor (NF)- κ B pathway-dependent mechanism. One such control may be at the level of TLR expression itself. However, the underlying molecular mechanisms remain unknown.

By investigating *TLR2* gene promoter methylation, the present study demonstrated that *TLR2* gene promoter methylation levels were decreased in patients with EH compared with healthy controls, particularly the CpG1, CpG6 and CpG8 sites. The hypomethylation of CpG6 hypomethylation was demonstrated to be a risk factor of EH. Previous studies have demonstrated that the methylation of gene promoters silences the transcription of the genes (28-30), and that an alteration in CpG methylation may influence gene expression via directly interfering with transcription factor-binding complexes or by histone modifications mediated by methyl-CpG-binding proteins (31,32). Therefore, the results of the present study suggest that hypomethylation of the *TLR2* gene promoter is likely to increase the expression of the *TLR2* gene and enhance pro-inflammatory responses in EH. Shuto *et al* (33) have reported that promoter hypomethylation of the *TLR2* gene is associated with an increased pro-inflammatory response and that *TLR2* expression is epigenetically upregulated in cystic fibrosis bronchial epithelial cells. In a

study of periodontitis, Benakanakere *et al* (34) observed that hypermethylation of the *TLR2* promoter was able to diminish *TLR2* and pro-inflammatory cytokine expression in response to infection with *Porphyromonas gingivalis*. Previous results from DNA methylation profiles of patients with Keshan disease compared with normal individuals demonstrated that selenium deficiency led to decreased methylation of CpG islands in the promoter region of *TLR2*, and upregulated mRNA and protein levels of *TLR2* (35). These previous results demonstrated that hypomethylation of *TLR2* promoter CpG islands was able to increase the expression of *TLR2* mRNA and protein. The expression of the *TLR2* gene will impair vascular endothelial cell repair and release pro-inflammatory cytokines via MyD88/Mal and NF- κ B pathways, including CRP, IL6 and TNF- α ; these pro-inflammatory cytokines have been reported to be associated with blood pressure (36-39). Therefore, the hypomethylation of the *TLR2* promoter may serve a role in the development of EH by activating pro-inflammatory responses.

In addition, Pearson correlation analysis in the present study suggested that blood pressure was negatively correlated with the methylation level of CpG6. The present results further indicate that *TLR2* gene promoter methylation serves a role in the development of EH. However, the present results were in contrast to those from a previous study (9), which reported a positive association between *TLR2* gene methylation and DBP. The disparity may be due to different CpG sites being analyzed in the different studies. In addition, different age ranges, race and inclusion criteria of the samples may have led to the above discrepancy. DNA methylation has been demonstrated to be a possible biomarker of cancer (40,41), and the present study observed that CpG6 methylation exhibited an appropriate threshold value to predict the risk of EH according to the results of ROC curve analysis. Therefore, the results of the present study may aid the clinical diagnosis and prediction of EH.

EH is a multifactorial chronic disease; gene-gene and gene-environment interactions contribute to its onset and progression. GMDR is a nonparametric and genetic model-free alternative to linear or logistic regression for detecting and characterizing nonlinear interactions among discrete genetic and environmental factors. GMDR is able to accommodate qualitative and quantitative phenotypes, enhance prediction accuracy, and adjust for discrete and continuous covariates (42); this increases the accuracy of the analysis and means that a more meaningful conclusion may be drawn. In the present study, a significant two-order gene-gene interaction between CpG6 and CpG8 was observed, in addition to a significant five-order gene-environment interaction among smoking, alcohol consumption, CpG6, CpG7 and CpG8, which may contribute to the risk of EH. However, the biological roles of these interactions are unclear, and further investigation is required in future studies.

There are certain limitations to the present study. mRNA and protein expression were not investigated, therefore transcriptomic regulation was not able to be demonstrated. In addition, the analysis of eight CpG sites may not be representative of the whole gene.

In conclusion, hypomethylation of the *TLR2* gene promoter, particularly CpG6, is associated with the risk of EH. In addition,

the CpG6 site of *TLR2* gene promoter exhibits utility in the diagnosis of EH. The two-order interaction between CpG6 and CpG8, and the five-order interaction among smoking, drinking, CpG6, CpG7 and CpG8, may be associated with EH risk. The present study may provide novel insights into the pathogenesis of EH from an epigenetic aspect.

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