Aberrant methylation of the *GCK* gene body is associated with the risk of essential hypertension

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Abstract. Essential hypertension (EH) is commonly accompanied by a dysfunction of glucose metabolism. Glucokinase (GCK) is a key enzyme involved in glucose metabolism. The aim of the present study was to investigate whether *GCK* gene-body methylation contributed to the risk of EH. A total of 47 patients with EH and 47 age-matched controls were recruited for methylation research in the current study. *GCK* gene-body methylation was measured using bisulphite pyrosequencing technology. DNA methylation levels were closely correlated among CpG1, CpG2 and CpG3 ($r>0.70$; $P<0.001$), in contrast with a weaker correlation between CpG4 and the preceding three CpGs ($r<0.3$ or $r=1$; $P>0.05$). Significantly lower CpG1-3 methylation (cases vs. controls, 49.13±5.72 vs. 53.49±7.53%; adjusted $P=0.006$) and significantly higher CpG4 methylation (cases vs. controls, 46.34±6.48 vs. 34.74±12.73%; adjusted $P=0.002$) were observed in patients with EH. The present study indicated that aberrant methylation of the *GCK* gene body was significantly associated with the risk of EH in the population assessed. The discrepancies between CpG1-3 and CpG4 methylation may suggest distinct roles for each of them in the determination of the risk of EH.

Introduction

Hypertension is a major risk factor in cardiovascular disease and end-stage renal damage, and increases mortality rates worldwide (1). Essential hypertension (EH) is affected by genetic and environmental factors (2). Genetic factors are estimated to be responsible for 30~50% of variations in blood pressure (BP) levels (3). Previous studies have identified multiple genetic loci associated with BP or hypertension in various ethnic populations (4,5). Epidemiological studies have reported that multiple environmental factors are associated with risk of hypertension (6,7).

Aberrant epigenetic modifications, including DNA methylation, may bridge the environmental and genetic contributing factors. Gene-body methylation was found to be positively correlated with gene expression (8). Although the functions of the gene-body CpG island (CGI) remain to be elucidated, methylation of the gene-body is frequently associated with active transcription in humans and other animals (9). Aberrant DNA methylation has been extensively investigated in the context of the pathogenesis of multiple types of cancer, including colorectal cancer (10), lung cancer (11) and leukemia (12). However, little evidence has demonstrated an association between DNA methylation and the risk of EH. A significant decline in global DNA methylation levels are observed in patients with EH and the trend continues alongside the progression of hypertension (13). In addition, altered global DNA methylation in the placentas of patients with pre-eclampsia was demonstrated to be associated with maternal hypertension (14). Aberrant DNA methylation of the *11β-HSD2*, *Adrb1* and *ADD1* genes was demonstrated to be associated with EH (15,16).

Hypertension and diabetes are two closely associated, common diseases and their coexistence may increase the risk of cardiovascular disease (17). *GCK*, a candidate gene for type 2 diabetes (18), encodes glucokinase, which is a key enzyme involved in glucose metabolism (19,20). The association between EH and *GCK* polymorphisms was previously disclosed in several studies (21,22). *GCK* gene-body hypomethylation was associated with the risk of coronary heart disease (23), which is closely correlated with EH (24,25). The present study performed an association study of *GCK* gene-body methylation with the risk of EH, in order to assess whether *GCK* methylation is associated with EH, and to examine the interactions between *GCK* methylation and age, as well as clinical indicators.

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Materials and methods

Sample collection. Samples from a total of 47 patients with EH and 47 age-matched control individuals were collected from the community residents in Ningbo Baizhang Street Community Health Service Center (Zhejiang, China) and the Seventh Hospital of Ningbo (Zhejiang, China). The samples were collected from Han Chinese individuals, who had been living in Ningbo for a minimum of three generations. Hypertensive patients were defined according to the 'gold standard' (26). All the hypertensive patients had received antihypertensive medication for >3 months, or had at least three consecutive records of systolic blood pressure (SBP) >140 mmHg and/or diastolic blood pressure (DBP) >90 mmHg (26). Those patients exhibiting SBP <120 mm Hg and DBP <80 mmHg, with no family history of hypertension in their first degree relatives were recruited as control individuals. The control individuals had not received antihypertensive therapy. The ages of the control individuals were matched with those of the patients with EH. None of the control or hypertensive individuals had a history of diabetes mellitus, secondary hypertension, myocardial infarction, stroke, renal failure, drug abuse or other serious diseases. A calibrated mercury sphygmomanometer with an adult-sized cuff was used to measure blood pressure according to the standard instructions recommended by the American Heart Association (27). Blood pressure was measured in supine position by two trained observers, with an interval of ≥10 min. Following a 12 h overnight fast, blood samples were obtained from the antecubital vein using vacutainer tubes containing EDTA (Hebei Chaoran Medical Instrument Company, Baoding, China) and were stored at -80˚C for DNA extraction. All experiments were approved by the Ethics Committee of Ningbo University (Ningbo, China) and written informed consent was obtained from all subjects.

Biochemical analyses. A nucleic acid extraction analyzer (Lab-Aid 820; Zeesan Biotech, Xiamen, China) was used to extract genomic DNA from the peripheral blood samples. The concentration of extracted DNA was measured using an ultramicro nucleic acid ultraviolet tester (NanoDrop 1000; Thermo Fisher Scientific, Wilmington, DE, USA). Plasma levels of cholesterol, triglyceride (TG), alanine transaminase (ALT), aspartate transaminase, uric acid and glucose concentrations were enzymatically measured using a CX7 biochemical analyzer (Beckman Coulter, Brea, CA, USA). DNA methylation was measured using sodium bisulphite DNA conversion coupled with pyrosequencing (28). Briefly, genomic DNA was chemically modified by sodium bisulphite (EpiTech Bisulphite kit; Qiagen, Hilden, Germany) to convert all the unmethylated cytosines into uracils, while the methylated cytosines remained unchanged. The converted DNA were selected and the polymerase chain reaction (PCR) primers were designed with PyroMark Assay Design software, version 2.0.1.15 (Qiagen). The PCR products were subsequently degenerated using denaturation solution (Qiagen) and released to single strand products for pyrosequencing (29). PCR amplification was performed in reaction mixtures containing 10 μl ZymoTaq™ Premix (Zymo Research Corporation, Irvine, California, USA), 1.5 μl forward primer, 1.5 μl reverse primer, 2 μl converted DNA and 5 μl DNAase/RNAase free water. PCR amplification was conducted using an Eppendorf Mastercycler Nexus Gradient (Eppendorf, Hamburg, Germany) under the following conditions: 95˚C for 10 min, then 40 cycles of 95˚C for 30 sec, 54.5˚C for 40 sec and 72˚C for 50 sec, followed by one cycle of 72˚C for 7 min. The primer sequences were as follows: Forward, 5'-TGGATGGTTTAGTGTATAAGTTGTATT-3'; reverse, 5'-Biotin-CACCTCATCCTCC ACATTCAT-3' and sequencing primer, 5'-AAGTGGGGTTTAAAAAG-3'.

Statistical analyses. Statistical analyses were performed to investigate the association of *GCK* methylation with metabolic profile and EH. A two sample t-test was performed to determine the association of EH with continuous variables, including age, body mass index, cholesterol, TG, glucose, ALT and uric acid. Pearson's correlation was used to determine the association between *GCK* methylation and the biochemical indicators. A more conservative non-parametric approach was used for data which were unable to be normalized. Pearson's χ^2 or Fisher's exact test was used to evaluate the association between EH and categorical variables, including gender, smoking and drinking. A receiver operating characteristic curve was used to examine the sensitivity of *GCK* methylation in EH diagnosis. In addition, logistic regression was implemented to investigate the interactions between *GCK* methylation and age. $P<0.05$ was considered to indicate a statistically significant difference. The aforementioned statistical analyses were performed using PASW Statistics 19.0 software [SPSS (Honk Kong) Ltd., Hong Kong, China].

Results

In the present study, a fragment in the *GCK* gene-body CGI, spanning exon 9 and a section of exon 10 (hg19, chr7: 44184771-44185695) was selected automatically by PyroMark Assay Design software for analysis. The results demonstrated that DNA methylation levels were closely correlated between CpG1, CpG2 and CpG3 (Fig. 1; $r>0.70$; $P<0.001$). By contrast a markedly weaker correlation was observed between CpG4 and the preceding three CpGs ($r<0.3$ or $r=1$; $P>0.05$).

As shown in Table I and Fig. 2, the results demonstrated that CpG1-3 methylation levels were significantly lower in samples from patients with EH (cases vs. controls, 49.13±5.72 vs. 53.49±7.53%; adjusted $P=0.006$). By contrast, the CpG4 methylation levels were significantly higher in the samples from patients with EH (cases vs. controls,46.34±6.48 vs. 34.74±12.73%; adjusted $P=0.002$). No correlation was detected between *GCK* DNA methylation and age amongst the patients or controls (data not shown), and no significant interactions of *GCK* methylation and age were revealed to affect EH susceptibility ($P>0.05$). Additionally, CpG4 methylation level was demonstrated to be an effective predictor of EH (Fig. 3; area under curve=0.769; $P=6.82\text{x}10^{-6}$), however, CpG1-3 methylation was not a successful diagnostic marker of EH risk.

As shown in Table I, significant differences were observed in glucose ($P=0.001$), TG ($P=0.006$), ALT ($P=0.007$) and uric acid ($P=0.001$) between EH cases and controls. In addition, gender-stratified correlation assessments were performed to assess the association between *GCK* DNA methylation levels and metabolic phenotypes, including glucose, triglycerides, cholesterol, uric acid and ALT, in the control samples. A

Table I. Characteristics of subjects.

Characteristic	EH (Mean ± SD)	Non-EH (Mean ± SD)	P-value
Age (years)	59.34±7.20	59.21±7.62	0.934
BMI (kg/m^2)[a]	24.02±2.62	22.52±4.08	0.103
Smoking (Y/N)	7/40	8/27	0.356
Alcoholic intake (Y/N)	16/31	5/30	0.043
Cholesterol (mmol/l)	5.28±0.92	5.04±1.07	0.238
TG (mmol/l)	1.87±1.40	1.31±0.83	0.006
Glucose (mmol/l)	5.43±0.52	5.23±0.92	0.001
ALT (IU/l)	26.26±18.58	18.17±12.14	0.007
Uric acid (mmol/l)	363.71±84.82	302.00±91.30	0.001
CpG1-3 methylation (%)	49.13±5.72	53.49±7.53	0.002
CpG4 methylation (%)	46.34±6.48	34.74±12.73	4.78x10^{-7}

[a]n=94 (47 EH vs. 47 Non-EH); EH, essential hypertension; SD, standard deviation; BMI, body mass index; Y, yes; N, no; TG, triglyceride; ALT, alanine transaminase.

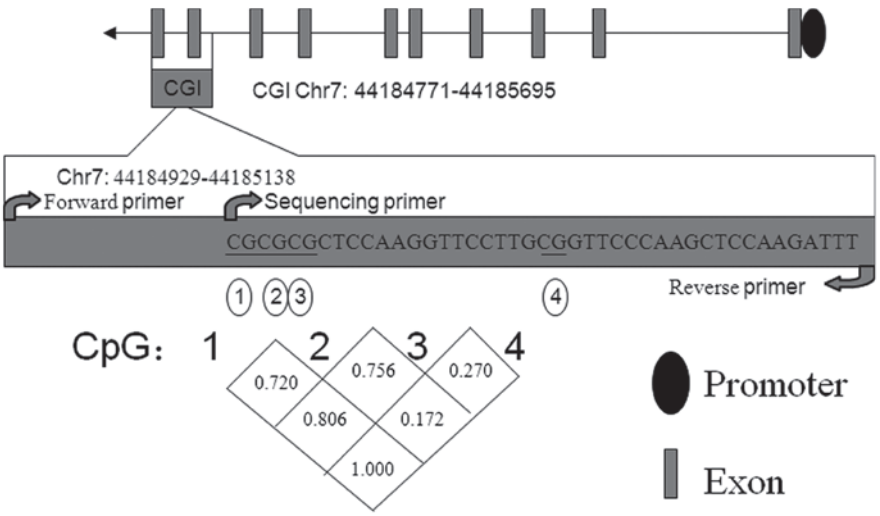

Figure 1. CpG sites assessed in the *GCK* gene.

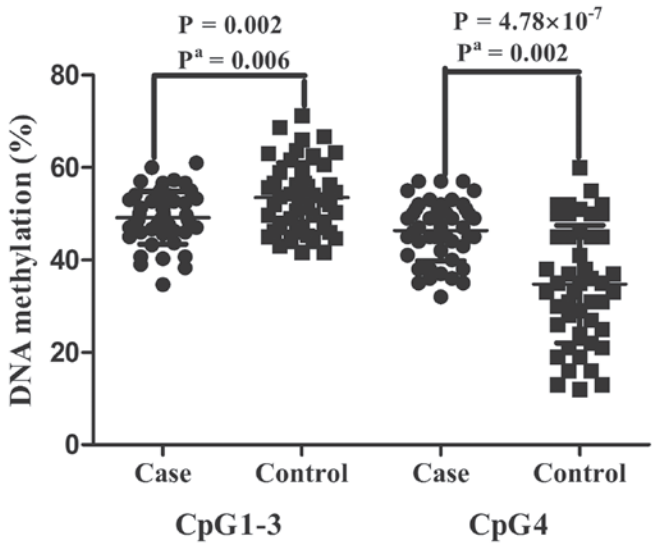

Figure 2. Differences in the DNA methylation of the G*CK* gene between case (n=47) and control (n=47) subjects.

significant correlation between the levels of CpG1-3 methylation and ALT (Fig. 4; r=-0.424; P=0.044) was observed in females, while no significant results were obtained for the remaining tests.

Discussion

Previous studies have reported that *GCK* polymorphisms were associated with hypertension (21,22). The present study hypothesized that the aberrant *GCK* gene-body methylation may also induce EH. The results revealed that patients with EH exhibited hypomethylation of CpG1-3 and hypermethylation of CpG4 in the *GCK* gene-body, compared with those of the control individuals. The results of the present study regarding the role of *GCK* methylation in the risk of EH may provide novel hints to aid the clarification of the pathogenesis of EH in future.

In the present study, no specific CGI was observed in the human *GCK* promoter. Gene-body methylation identified

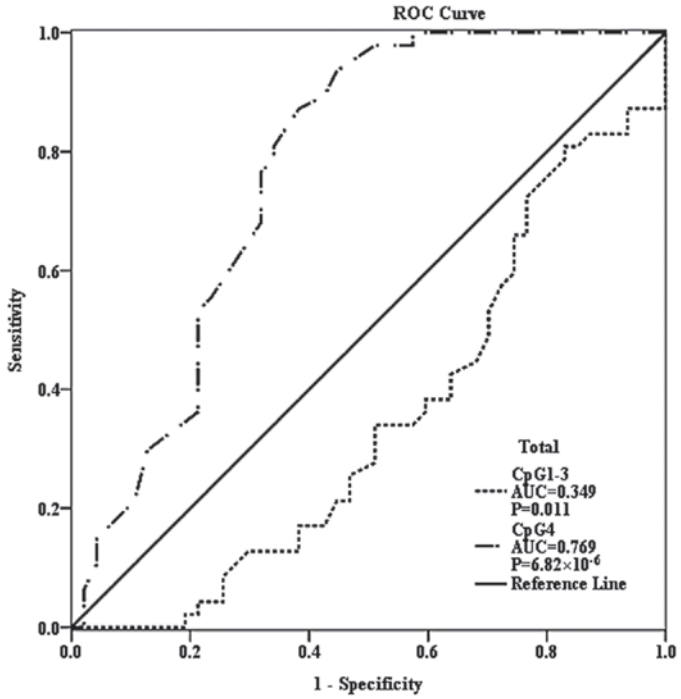

Figure 3. ROC curve demonstrating the DNA methylation of the *GCK* gene in essential hypertension. ROC, receiver operating characteristic.

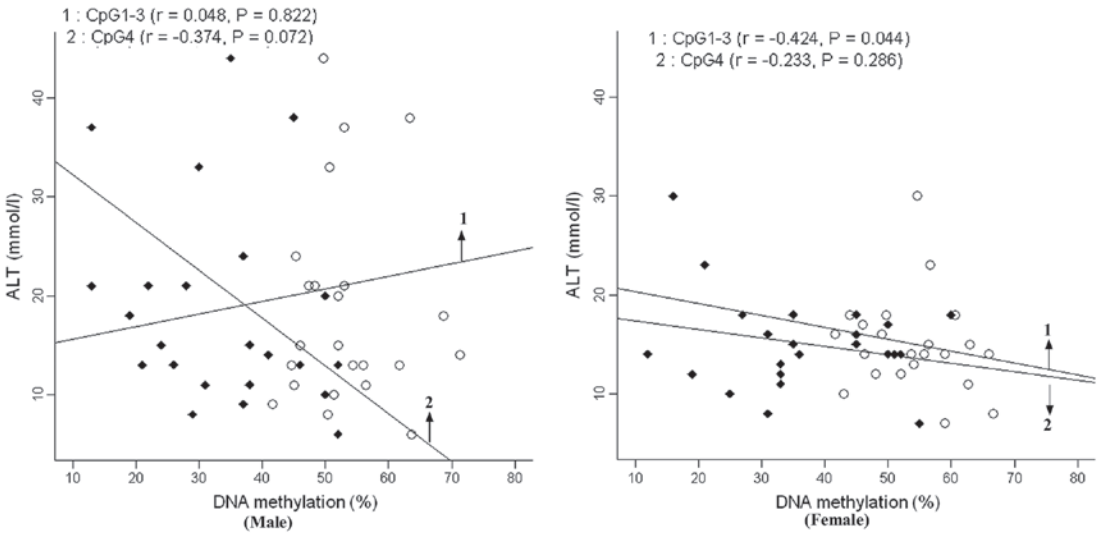

Figure 4. Pearson correlation between the DNA methylation of the *GCK* gene and ALT in 24 male and 23 female controls. White dots refer to line 1, and black dots refer to line 2. ALT, alanine transaminase.

'orphan promoters', which may be used in the early stages of development (30). Hypermethylation of gene-bodies frequently indicates higher levels of gene expression in human tissues and cell types (8,31). Aberrant methylation of the gene body was observed to contribute to the risk of heart failure (32) and coronary heart disease (23). DNA methylation of gene bodies has major significance in the regulation of gene expression (33). In certain types of human tissue, positive correlation between gene-body methylation and gene expression has been identified (34,35). Gene-body methylation was reported to be significant in regulating cell context-specific alternative promoters in human and mouse tissues (28).

Associations between DNA methylation and age were demonstrated to affect the EH status (16). This observation indicated that EH was affected by genetic and environmental factors, including DNA methylation status and age. Age was previously revealed to be positively correlated with *GCK* methylation levels (23), however the present study failed to demonstrate this association. No association between age and CpG1-3 or CpG4 methylation was observed (data not shown).

A positive correlation between plasma glucose levels and blood pressure has been previously reported (36). Increased levels of plasma ALT (37) and uric acid (38) were revealed to be associated with hypertension. In the present study, the association of uric acid and plasma glucose with *GCK*

DNA methylation levels was not observed, whereas CpG1-3 methylation levels were positively associated with ALT in the female control samples. These results suggested that *GCK* CpG1-3 methylation may exert its effects on the risk of EH via regulation of the levels of ALT in females. Ongoing research is required to investigate the specific mechanisms underlying this association.

In conclusion, the results of the present study demonstrated that a hypomethylation level of CpG1-3 and a hypermethylation level of CpG4 in the *GCK* gene-body increased the risk of EH. *GCK* CpG4 methylation may have potential in aiding the prediction of EH risk. The findings provide novel clues to facilitate the elucidation of the pathogenesis of EH.

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