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Association of endothelin-converting enzyme-1b C-338A polymorphism with gastric cancer risk: A case–control study

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ABSTRACT

To investigate the association between endothelin-converting enzyme-1b (*ECE-1b*) C-338A polymorphism and gastric cancer risk, we conducted a hospital-based case–control study of 256 gastric cancer cases and 256 controls matched on age and gender. The genotypes were identified by polymerase chain reaction-restriction fragment length polymorphism. We found that the genotype frequencies were significantly different ($P = 0.005$) between cases and controls. Compared with the wild genotype CC, the variant genotypes (CA + AA) were associated with a 64% increased risk of gastric cancer [adjusted odds ratio (OR) = 1.64, 95% confidence interval (CI) 1.15–2.33]. Further stratification analyses indicated that the increased risk was especially noteworthy in older subjects (age $\geqslant$58) (adjusted OR = 1.91, 95% CI 1.18–3.09), women (adjusted OR = 2.30, 95% CI 1.11–4.79) and non-smokers (adjusted OR = 1.79, 95% CI 1.19–2.67). Our results suggest that the *ECE-1b* C-338A polymorphism may be associated with increased risk of gastric cancer.

1. Introduction

China is one of the highest gastric cancer risk areas in the world.[1] Almost 42% of the gastric cancer cases occur in China alone.[1] Gastric cancer is thought to result from a combination of multiple environmental factors and the accumulation of specific genetic alterations, including polymorphism.[2,3] Our previous epidemiological work also provided the evidence that the risk of gastric cancer was associated with *myeloperoxidase* G-463A polymorphism.[3]

Endothelin-1 (ET-1) is a 21 amino acid regulatory peptide with multiple biological actions.[4,5] It has been implicated as a causative factor in the pathogenesis of a wide variety of diseases,[4–7] including hypertension, coronary artery disease and cancer.[6,7] Furthermore, ET-1 has been reported to be produced by gastric cancer cell lines, indicating that it may be involved in the pathogenesis of gastric cancer.[8,9]

Active ET-1 is generated from its inactive precursor big-ET by endothelin-converting enzyme-1 (ECE-1).[4,5] Significantly elevated ECE-1 expression has been observed in several cultured tumour cell lines and human cancer specimens, suggesting its potential role in carcinogenesis and cancer development.[10–15]

Human ECE-1 protein exists as four distinct isoforms termed ECE-1a, 1b, 1c and 1d. These isoforms differ only in their N-terminal regions and are derived from a single gene through the use of alternative promoters.[16] Recently, a polymorphic C → A substitution has been described in the

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5′-regulatory region of the *ECE-1b* gene (338 bp upstream from the translation start site).[17] It has been shown to create a binding site for transcription factor E2F-2, thus resulting in significantly increased transcriptional activity.[17]

Some investigations also suggested that the variant A allele was associated with elevated *ECE-1b* promotor activity, thus higher ECE-1 enzymatic activity compared with the C allele.[17–20] One study on Alzheimer's disease demonstrated that A allele was associated with upregulation of the gene transcription via the E2F pathway.[18] Moreover, the A allele was associated with higher blood pressure in 101 untreated hypertensive women in Germany.[17] Another study in France also reported that blood pressure was higher in AA homozygote women.[19] In addition, our previous study observed an association between *ECE-1b* variant genotypes and increased to risk of coronary artery disease.[20] However, no significant influence of *ECE-1b* polymorphism on the progression of autosomal dominant polycystic kidney disease was found.[21]

Given the roles of ET-1 in to early development of gastric cancer, the role of ECE-1 in the biosynthesis of ET-1 as well as the polymorphism in *ECE-1* gene transcriptional activity, the variant A allele may be associated with the risk of gastric cancer. Therefore, to test the hypothesis, we conducted a hospital-based case–control study in the Chinese population.

2. Materials and methods

2.1. Study population

Between March 2005 and January 2007, unrelated patients with newly diagnosed gastric cancer who attended the First Affiliated Hospital of Nanjing Medical University were consecutively collected in the study. Gastric cancer was histopathologically confirmed by endoscopic biopsy or by surgical specimen. Six cases with secondary or recurrent tumours were excluded. During the time of case collection, cancer-free controls were selected amongst inpatients from the same hospital. They were matched to the cases on gender and age (within 5 years). The most common reasons for hospitalisation for control subjects were hernias, appendicitis, hydrocele, cholecystitis and cataract. Four controls were not matched to any case and were excluded from the analysis. After exclusions, the present hospital-based case–control study consisted of 256 gastric cancer cases and 256 controls. All eligible subjects were unrelated Han nationality and from Jiangsu Province or its surrounding regions. Information on age, gender, residence, personal medical history and smoking status was collected by questionnaire. Individuals who formerly or currently smoked $\geqslant$10 cigarettes per day on average were defined as smokers. In addition, the following parameters were obtained from the pathological reports of the gastric cancer patients studied: tumour, node, metastasis (TNM) staging, differentiation grade (World Health Organisation classification) and tumour location.[22] Informed consent was obtained from each subject and the study protocol was approved by the Ethics Committee of Nanjing Medical University First Affiliated Hospital.

2.2. Genotyping

The protocol for genomic DNA extraction was described in our previous study.[3] The polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was used to determine the *ECE-1b* C-338A polymorphism. Primers and PCR conditions were as described.[20] The 446 bp PCR products were digested for 3 h at 65 °C, using restriction enzyme *Tsp*509I (New England BioLabs, Waltham, MA) and separated on a 3% ethidium bromide-stained agarose gel. The wild-type homozygotes (CC) produced two bands at 247 and 199 bp, and the variant homozygotes (AA) produced three bands at 247, 178 and 21 bp, and the heterozygous CA produced four bands at 247, 199, 178 and 21 bp. All assays were conducted by laboratory personnel unaware of case–control status. About 10% of cases and controls were randomly selected and retested, and the reproducibility was 100%.

2.3. Statistical analysis

We performed statistical analyses using Stata Version 8.0 (STATA Corporation, College Station, TX). The Shapiro–Wilk statistic was used to test for normality of the distribution. Quantitative variables departing from the normal distribution were summarised as median and analysed by Mann–Whitney rank sum test. Pearson χ^2-test was used to compare the distribution of categorical variables and genotype frequencies between the cases and controls. Hardy–Weinberg equilibrium was tested for controls by the goodness-of-fit χ^2 test. Odds ratio (OR) and 95% confidence interval (CI) were calculated to estimate the association between the polymorphism and the risk of gastric cancer. The homozygotes of the common allele were used as the reference. Crude OR was calculated using the Woolf approximation method, and the adjusted OR was calculated using unconditional logistic regression to control for age, gender, smoking status, hypertension and diabetes. All statistical tests were two-sided and considered statistically significant at $P < 0.05$.

3. Results

Selected characteristics of the 256 gastric cancer cases and 256 cancer-free controls are summarised in Table 1. Cases and controls were well matched and similar with regard to gender, age, smoking status, history of hypertension and diabetes. Most of the cases were adenocarcinoma (97.66%). For those 96.09% ($n = 246$) of cancers with clinical data, 31, 22,

Table 1 – Distribution of selected characteristics of cases and controls

Characteristics	Cases, *n* (%)	Controls, *n* (%)	*P*-value
Overall	256	256	
Gender (male)	192 (75)	192 (75)	1.000
Age[a] (years)	59 (51–66)	58 (51–66)	0.956
Hypertension	39 (15.23)	49 (19.14)	0.241
Diabetes	15 (5.86)	21 (8.20)	0.300
Smoking	56 (21.88)	59 (23.05)	0.751

a Median (25–75th percentiles).

Table 2 – Distributions of the ECE-1b C-338A genotype in cases and controls and risk estimates for the variant ECE-1b genotype

ECE-1b Genotype	Cases	Controls[a]	Crude OR[b] (95% confidence interval (CI)[b])	P-value	Adjusted OR[c] (95% CI[b])	P-value
CC	108 (42.19)	138 (53.91)	1.00		1.00	
CA	111 (43.36)	93 (36.33)	1.53 (1.03–2.25)	0.026	1.53 (1.05–2.23)	0.027
AA	37 (14.45)	25 (9.77)	1.89 (1.04–3.49)	0.026	2.04 (1.14–3.66)	0.017
AA + CA	148 (57.81)	118 (46.09)	1.60 (1.11– 2.31)	0.008	1.64 (1.15–2.33)	0.006
C-allele	327 (63.87)	369 (72.07)				
A-allele	185 (36.13)	133 (27.93)				

a Distribution of the *ECE-1b* C-338A genotypes amongst the control group was in Hardy–Weinberg equilibrium ($\chi^2 = 2.439$, $P = 0.118$).
b OR, odds ratio; CI, confidence interval.
c Adjusted for age, sex, smoking status, hypertension and diabetes.

Table 3 – Stratified analyses for variant ECE-1b genotypes in cases and controls

Variable	(AA + CA)/CC		Crude OR[a] (95% CI[a])	P-value	Adjusted OR[ab] (95% CI[a])	P-value
	Cases	Controls				
Age (median)						
<58	72/47	63/55	1.34 (0.77–2.31)	0.269	1.33 (0.79–2.24)	0.287
⩾58	76/61	55/83	1.88 (1.13–3.12)	0.010	1.91 (1.18–3.09)	0.009
Sex						
Females	41/23	29/35	2.15 (1.00–4.65)	0.033	2.30 (1.11–4.79)	0.025
Males	107/85	89/103	1.46 (0.96–2.22)	0.066	1.48 (0.99–2.22)	0.058
Smoking status						
Smokers	30/26	29/30	1.19 (0.54–2.65)	0.636	1.19 (0.57–2.48)	0.645
Non-smokers	118/82	89/108	1.75 (1.15–2.65)	0.006	1.79 (1.19–2.67)	0.005

a OR, odds ratio; CI, confidence interval.
b Adjusted for age, sex, smoking status, hypertension and diabetes.

96 and 42 were T1, T_2, T_3 and T_4, respectively; 32, 134 and 80 were well, moderate and poor differentiation, respectively. Positive lymph nodes were noted in 155 cases. Patients with adenocarcinoma of the gastric cardia and non-cardia were 64 and 182, respectively.

As shown in Table 2, the frequency of variant A alleles in cases was 36.13%, which was higher than in the controls (27.93%) ($\chi^2 = 7.91$, $P = 0.005$). The difference in *ECE-1b* genotype distributions between the cases and controls was statistically significant ($\chi^2 = 7.57$, $P = 0.023$). In addition, the distribution of the genotypes in controls did not deviate from that expected by Hardy–Weinberg equilibrium ($\chi^2 = 2.439$, $P = 0.118$). When the wild genotype CC was used as the reference group, the adjusted OR associated with the risk of gastric cancer was 1.53 (95% CI 1.05–2.23) for CA genotype and 2.04 (95% CI 1.14–3.66) for AA genotype. Moreover, individuals with the variant genotypes (CA + AA) had a 1.64-fold increased risk of developing gastric cancer (95% CI 1.15–2.33, $P = 0.006$) (Table 2).

Results of stratified analyses by the median age of controls, gender and smoking status for individuals with *ECE-1b* variant genotypes are presented in Table 3. The elevated risk of gastric cancer associated with variant genotypes was evident in older subjects (age ⩾58) (adjusted OR = 1.90, 95% CI 1.18–3.09), but not in younger subjects (age <58) (adjusted OR = 1.43, 95% CI 0.85–2.42). When stratified by gender, the risk was observed in women (adjusted OR = 2.30, 95% CI 1.11–4.79), but not in men (adjusted OR = 1.48, 95% CI 0.99–2.22). Stratification by smoking status revealed a significant association of *ECE-1b* C-338A with an increased gastric cancer risk for non-smokers (adjusted OR = 1.79, 95% CI 1.19–2.67), but not in smokers (adjusted OR = 1.19, 95% CI 0.57–2.48) (Table 3). However, no significant association was observed between the variant genotypes and clinicopathological features of gastric cancer, including tumour differentiation, depth of tumour infiltration, lymph node status and tumour location (data not shown).

4. Discussion

In the present study, we for the first time found that the C to A variant in the *ECE-1* gene promoter conferred an increased risk of gastric cancer in the Chinese population.

ECE-1b C-338A is a functional genetic variant located in the 5′-regulatory region of the *ECE-1* gene.[17] It was reported that *ECE-1b* promoter with variant A allele was associated with an increased binding affinity to transcription factor E2F-2, and the A allele was responsible for increased transcriptional activity in promoter-reporter gene assay.[17] Moreover, Funalot

and colleagues found that A allele was associated with increased ECE-1 mRNA expression in human neocortex via the E2F pathway.[18] In their study, homozygous carriers of the A allele who had higher levels of ECE-1 expression and enhancing ECE-1 activity could protect against late-onset of Alzheimer's disease (adjusted OR = 0.47, 95% CI 0.25–0.88).[18] In addition, the variant A allele was also considered to be associated with enhanced expression of ECE-1 in three recent studies.[17,19,20] One in Germany suggested an association between A allele and higher blood pressure levels in untreated hypertensive women.[17] Another in France reported that female AA homozygotes had significantly higher systolic, diastolic and mean blood pressure levels ($P = 0.01$, 0.02, 0.006, respectively).[19] Significant association between the polymorphism and a 58% increased risk of coronary artery disease was also demonstrated in our previous study (adjusted OR = 1.58, 95% CI 1.07–2.32).[20]

Multiple lines of evidence have indicated that ECE-1 is involved in the pathogenesis of cancer.[10–15] It was observed that ECE-1 expression levels were increased in numerous cancer types, including breast cancer,[10] lung cancer,[11] prostate cancer,[12] astrocytomas,[13] thyroid carcinoma[14] and oral carcinoma.[15] Reported by Bronislaw and colleagues, gastric mucosal expression of ECE-1 activity was up-regulated in response to *Helicobacter pylori* infection, which led to the enhancement of ET-1 production, induction of TNF-α, and triggering the apoptotic events that exacerbated the mucosal inflammatory process.[23] *Helicobacter pylori* has been clearly accepted as a causative agent in gastric cancer.[1,2] In addition, inflammation is a risk or prerequisite factor for the development of gastric cancer.[2] Therefore, we speculate that the elevated levels of ECE-1 are associated with an increased risk of gastric cancer.

Furthermore, elevated levels of ECE-1 induced by the polymorphic C → A substitution increased the turnover of ET-1 precursors, thereby leading to increased expression of ET-1.[4,5] Growing evidence has emerged showing that ET-1 stimulates cancer growth by promoting angiogenesis, inhibiting apoptosis and stimulating mitogenesis.[6,7] Moreover, the expression of ET-1 has been identified in numerous cancer types,[6,9] including gastric cancer.[8,9] Elevated plasma levels of ET-1 were also detected in gastric cancer patients.[24] Additionally, the ET-1 is now recognised to play a major role in the pathogenesis of gastric mucosal injury.[25] In brief, we hypothesise that the elevated ECE-1 activity and increased levels of ET-1 in the presence of variant A allele may be a possible mechanism for our observation that the *ECE-1-1b* C-338A polymorphism is associated with the increased risk of gastric cancer.

Interestingly, the elevated risk associated with the *ECE-1b* variant genotypes was significant in the subgroup of older subjects (age ⩾58), but not in the younger group. The results were consistent with our previous findings.[20] Carcinogenesis is considered as accumulation of genetic events during ageing, and gastric cancer has a steep slope for age-specific increase in incidence.[26] The increased risk observed in older subjects implies that gene–environment interaction may be involved in carcinogenesis and the *ECE-1b* genotype effects tend to be age specific. The *ECE-1b* polymorphism may contribute to elevated ECE-1 levels beyond the age of 58, and thus represents a significant risk factor in this age group. However, this is just a hypothesis to interpret the results of our study, and further research is warranted to clarify the mechanism underlying the interaction between the polymorphism and age.

We also found different effects of the *ECE-1b* variant genotypes on gastric cancer risk in men and women. Epidemiological studies,[17,19] including our previous research,[20] also noted the gender-specific associations between the polymorphism and phenotypes. It has been indicated that female hormones lower plasma ET levels, whereas male hormones raise them.[27] As a result, women exhibit lower plasma ET-1 levels than men. Because of the gender-specific modulation of the endothelin system, the androgen-stimulated ET-1 activity could mask the effect of the genetic variants in men.[17,19,20] Similarly, the *ECE-1b* C-338A polymorphism and, therefore increased ECE-1 expression, might have a greater impact on gastric cancer risk in women than men.[17,19,20]

We noted that increased risk of gastric cancer associated with the polymorphism was pronounced in non-smokers, but not in smokers. Exposure to tobacco smoke has been clearly accepted as a major risk factor for gastric cancer.[1] In addition, it was reported that the salivary immunoreactive-ET levels in the smokers were significantly higher than those of the non-smokers ($P < 0.01$).[28] Therefore, the smokers are at a higher risk of gastric cancer development than non-smokers. It is biologically possible that the effect from tobacco smoking is so great that differences in efficiencies by *ECE-1b* C-338A polymorphism may not be so apparent. In other words, the association between the polymorphism and gastric cancer risk could be masked by the effect of smoking.[29] On the other hand, gastric cancer development in non-smokers is more likely due to direct genetic effects other than smoke exposure.[30] This might explain the positive association of the polymorphism in non-smokers rather than in smokers with the risk of gastric cancer.

In the present study, no significant association was observed between the variant genotypes and clinicopathological parameters of gastric cancer. Gastric cancer progression has been considered as a complex, multistage event. Although our data did not support the association between the polymorphism and gastric cancer progression, we could not exclude the possibility that other factors might impact the association between them. Moreover, because our data of clinicopathological parameters were obtained at the time of diagnosis and the number of cases in the subgroups was relatively small; our findings from the stratified analyses should be interpreted with caution before being confirmed in further studies. Therefore, more studies of larger case series with prospectively followed-up clinical outcome, especially the survival rate, may be required to elucidate the association between the polymorphism and gastric cancer progression as well as prognosis.

Potential limitations of the present study should be considered. First, the design was a hospital-based case–control study, and the controls were recruited amongst patients with non-malignant diseases. Therefore, we could not rule out the possibility of selection bias. Nonetheless, the genotype frequency amongst controls was in Hardy–Weinberg equilibrium and was comparable with those in other studies.[17–21] Second,

our sample size was relatively small, especially for stratified analyses to explore the gene–environment interactions. However, our results provided valuable insights and interesting information and may serve to guide future studies in this area. Third, our data were obtained at the time of diagnosis. Nevertheless, to confirm the role of this *ECE-1b* C-338A polymorphism in cancer risk requires further, larger studies in different populations and with other types of tumours.

In conclusion, our study provides evidence that variant genotypes of *ECE-1b* C-338A are significantly associated with an increased risk of gastric cancer in a Chinese population. Furthermore, the association is especially noteworthy in older individuals, women and non-smokers.

Conflict of interest statement

The corresponding author has specifically obtained the approval of all other co-authors to submit the article to EUROPEAN JOURNAL OF CANCER in its present form. There are no financial or personal relationships of authors with other people or organisations that could inappropriate by influence their work.

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