An Insertion/Deletion Polymorphism Within the Proximal Promoter of *EGLN2* Is Associated With Susceptibility for Gastric Cancer in the Chinese Population

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Gastric cancer (GC) is among the most common human malignancies and the second leading cause of cancer-related death worldwide. Accumulated evidence from molecular genetics indicates that an individual's genetic factors are involved in their susceptibility to GC. Hypoxia is a common feature of cancer and the hypoxia-inducible factor (HIF), a transcription factor that regulates oxygen homeostasis, plays key roles in the growth of solid tumors and regulating cellular responses to hypoxia. Prolyl hydroxylase (PHD1, also known as *EGLN2*) is one of the three enzymes capable of hydroxylating the α subunit of HIF and results in polyubiquitinylation and proteasomal degradation of HIF. A case-control study, including 415 GC patients and 830 healthy controls, was conducted to investigate the association between GC susceptibility with a 4-bp insertion/deletion polymorphism (rs10680577) in the proximal promoter of *EGLN2*. Logistic regression analysis showed that the heterozygote and the homozygote 4-bp del/del confer a significantly increased risk of GC after controlling for other covariates (adjusted odds ratio [OR] = 1.35, 95% confidence interval [CI] 1.05–1.75, $p = 0.017$; OR = 2.19, 95% CI 1.15–4.18, $p = 0.009$, respectively). Carriage of the 4-bp deletion allele was associated with a greatly increased risk of developing the disease (OR = 1.38, 95% CI 1.12–1.70, $p = 0.002$). Moreover, stratification analysis showed that the association was more prominent in smokers (adjusted OR = 2.09, 95% CI = 1.40–3.12, p for heterogeneity = 0.01). Our data suggested that common genetic polymorphisms in *EGLN2* may influence GC risk in the Chinese population. Considering the relative small sample size, replication in other populations with a larger sample size and further functional analysis are required for fully understanding the roles of *EGLN2* polymorphisms in predisposition for GC.

Introduction

GASTRIC CANCER (GC) is among the most common human malignancies and the second leading cause of cancer-related death worldwide; ~40% of all cases occurred in China (Krejs, 2010; Jemal *et al.*, 2011). GC is considered as a multifactorial disease caused by multiple risk factors, including genetic predisposition, environment, and viral/bacterial infections (Guggenheim and Shah, 2013). Accumulated evidence from molecular genetics indicates that an individual's genetic factors are involved in their susceptibility to GC (Persson *et al.*, 2011; Wang *et al.*, 2012). Recent genome-wide association studies have also revealed several susceptibility loci for GC (Yoshida *et al.*, 2010; Shi *et al.*, 2011), which are helpful to predict individual and population risk and clarify molecular mechanisms relevant to GC. However, to date, the carcinogenic mechanism of GC is still far from clear.

Hypoxia is a common feature of many types of cancer. It contributes to local and systemic tumor progression and potentially compromises radiotherapy and chemotherapy (Quintero *et al.*, 2004). The hypoxia-inducible factor (HIF), a transcription factor that regulates oxygen homeostasis, plays key roles in the growth of solid tumors and regulating cellular responses to hypoxia (Kaelin and Ratcliffe, 2008). HIF consists of α - and β -subunits, which are both helix-loop-helix transcription factors. During normoxia, HIF α is polyubiquitinated and subjected to proteasomal degradation by three prolyl hydroxylase domain enzymes capable of hydroxylating the α subunit of HIF. Under low oxygen conditions, however, HIF α would accumulate, dimerize with HIF β , and transcriptionally activate over 100 genes, including genes involved in angiogenesis, autophagy, and energy metabolism (Kaelin, 2005). There is some evidence that the HIF pathway might be involved in gastric carcinogenesis

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(Griffiths *et al.*, 2005). For example, *Helicobacter pylori* (*H. pylori*)-induced reactive oxygen species may lead to HIF-1 α expression under normal oxygen conditions (Park *et al.*, 2003). The hypoxia-dependent activation of the NF- κ B/HIF-1 α /VEGF pathway may contribute to GC promotion through enhancement of angiogenesis (Nam *et al.*, 2011). Meanwhile, inhibition of HIF-1 α activity impairs gastric tumor growth, angiogenesis, and vessel maturation (Stoeltzing *et al.*, 2004). Furthermore, genetic variations within the HIF pathway have been shown to be associated with incidence or clinical progression of multiple cancers, including GC (Tanimoto *et al.*, 2003; Chau *et al.*, 2005; Li *et al.*, 2009).

Recently, a novel 4-bp insertion/deletion (indel) polymorphism (rs10680577) has proved to be associated with hepatocellular carcinoma risk, possibly through regulating expression of prolyl hydroxylase 1 (EGLN2) (Zhu *et al.*, 2012). Thus, we hypothesized that rs10680577 was associated with altered EGLN2 expression and GC risk. To test this hypothesis, we conducted a case-control study in a Chinese population to investigate the associations between the indel polymorphism and GC risk.

Materials and Methods

Study participants

The case series comprised 415 GC patients diagnosed, hospitalized, and treated in our department from 2010 to 2012. The diagnosis of these patients was confirmed by both gastric endoscopy and pathological examination. Staging of GC was accessed by two pathologists according to the

World Health Organization classification and the tumor-node-metastasis classification issued in 2006. All GC patients were tested for *H. pylori* infection using *H. pylori*-specific immunoglobulin G by enzyme linked immunosorbent assay and by the urea breath test. Individuals who tested positive by either of these methods were considered as positive for *H. pylori*. Patients who were excluded were those suffering from rheumatoid arthritis, diabetes, thrombosis, metabolic disorders, severe cardiovascular diseases, recurrence of GC, or malignancies other than GC. The age (± 5 years) and sex matched control group consisted of 830 participants representing clinically healthy controls recruited during regular medical examination at our hospital. All subjects recruited were unrelated ethnic Han Chinese. The subjects who smoked more than one cigarette per day for more than 1 year were classified as smokers. Others were defined as non-smokers. Subjects were considered as alcohol drinkers if they drank at least once per week. After obtaining informed consent, genomic DNA was extracted from the peripheral blood of control subjects and GC patients before any clinical intervention, such as surgery or chemotherapy. The design of the study was approved by the Ethics Committee of Suzhou Municipal Hospital.

DNA extraction and genotyping

Genomic DNA of blood samples and tissues was isolated using a genomic DNA purification kit (Qiagen). The genotyping was performed using a polymerase chain reaction (PCR) method as previously described (Tanimoto *et al.*, 2003). Briefly, DNA fragments containing rs10680577 were

TABLE 1. CLINICAL CHARACTERISTICS OF GASTRIC CANCER CASES AND CONTROLS ENROLLED IN CURRENT STUDY

Characteristics	Case		Control		p
	n (= 415)	Frequencies (%)	n (= 830)	Frequencies (%)	
Age (year, mean $\pm$ SD)		58.7 $\pm$ 11.5		58.1 $\pm$ 11.9	
Gender					
Male	253	61.0	506	61.0	1.00
Female	162	39.0	324	39.0	
Smoking status					
Smokers	178	42.9	291	35.0	0.007
Nonsmokers	237	57.1	539	65.0	
Drinking status					
Drinker	160	38.6	248	29.9	0.002
Nondrinker	255	61.4	582	70.1	
Histological type					
Intestinal	285	68.7			
Diffuse	94	22.7			
Mixed	21	5.1			
Unclassified	15	3.6			
Tumor stages					
I	184	44.3			
II	85	20.5			
III	72	17.3			
IV	64	15.4			
Unclassified	10	2.4			
<i>Helicobacter pylori</i>					
Positive	323	77.8			
Negative	92	22.2			

SD, standard deviation.

FIG. 1. Example sequencing and genotyping output for rs10680577 polymorphism. **(A)** Displays an example of the genotyping assay results. Lanes 1, 3, 8, 10, 11, and 12, ins/ins genotype; lane 9, del/del genotype; lanes 2, 4, 5, 6, and 7, ins/del genotype. **(B, C)** Show the sequencing results of deletion and insertion allele, respectively. The underlined base pairs in C panel indicate the inserted (TACT) sequences.

amplified with a pair of genotyping primers (forward primer: 5'-TGATTCCCACAATCGTCC-3', reverse primer: 5'-ATGGCTACGCAGTTAGTTGA-3'). Subsequently, the PCR products were separated using 7% nondenaturing polyacrylamide gel electrophoresis and visualized by silver staining (Allen *et al.*, 1989). The genotypes were determined by the numbers and the length of the band(s) in the gels. Genotyping was performed without knowledge of case or control status. To validate the genotyping method, we also analyzed 20 randomly selected DNA samples by direct sequencing. The coincidence rate of these two methods was 100%, suggesting that the PCR-based method was reliable. Approximately 10% of the samples were randomly selected and examined in duplicates by independent researchers, and the reproducibility was 100%.

Statistical analysis

The genotype distribution was analyzed by the Hardy-Weinberg equilibrium using the χ^2 test. Logistic regression was used to analyze the association between rs10680577 and GC risk, adjusted by sex and age. In addition, stratified an-

alyses by these common confounders were performed using the binary logistic regression model. Due to the limited number of del/del genotype, they were integrated with the ins/del group when appropriate. These statistical analyses were implemented in the Statistic Analysis System software (version 8.0, SAS Institute). $p < 0.05$ was used as the criterion of statistical significance. All statistical tests were two sided.

Results

The distribution of selected characteristics of the 415 GC patients and 830 controls included in the analysis are summarized in Table 1. There were no significant differences between cases and controls in the distribution of sex and age. However, the percentages of smokers and drinkers in GC cases were significantly higher compared with controls ($p = 0.007$ and 0.002 , respectively). Among the 415 GC cases, 285 (68.7%) were classified as the intestinal type, 94 (22.7%) as the diffuse type, 21 (5.1%) as mixed, and 15 (3.6%) as unclassified. The example output from sequencing and genotyping assays of rs10680577 is shown in Figure 1.

TABLE 2. ASSOCIATIONS BETWEEN RS10680577 AND GASTRIC CANCER SUSCEPTIBILITY

Genetic model	Genotype	Cases (%)	Control (%)	OR (95% CI) ^a	p
Codominant model	ins/ins	235 (56.6)	541 (65.2)	1.00 (Reference)	
	ins/del	159 (38.3)	266 (32.0)	1.35 (1.05–1.75)	0.017
	del/del	21 (5.1)	23 (2.8)	2.19 (1.15–4.18)	0.009
Dominant model	ins/ins	235 (56.6)	541 (65.2)	1.00 (Reference)	
	ins/del + del/del	180 (43.4)	289 (34.8)	1.42 (1.11–1.82)	0.004
Recessive model	ins/ins + ins/del	394 (94.9)	807 (97.2)	1.00 (Reference)	
	del/del	21 (5.1)	23 (2.8)	1.96 (1.04–3.71)	0.024
Additive model	ins allele	629 (75.8)	1348 (81.2)		
	del allele	201 (24.2)	312 (18.8)	1.38 (1.12–1.70)	0.002

^aAdjusted for sex, age, smoking, and drinking status.
OR, odds ratio; CI, confidence interval.

TABLE 3. STRATIFIED ANALYSIS OF rs10680577 GENOTYPES ASSOCIATED WITH GASTRIC CANCER SUSCEPTIBILITY

Variables	Genotypes (cases/controls)		OR (95% CI)	Heterogeneity p
	ins/ins	ins/del + del/del		
Gender				
Male	139/324	114/182	1.46 (1.06–2.01)	0.86
Female	96/217	66/107	1.39 (0.93–2.10)	
Age (median)				
<45 years	112/261	95/154	1.44 (1.01–2.05)	0.99
≥45 years	123/280	85/135	1.43 (1.00–2.05)	
Smoking status				
Nonsmokers	150/350	87/189	1.07 (0.77–1.49)	0.01
Smokers	85/191	93/100	2.09 (1.40–3.12)	
Drinking status				
Nondrinkers	141/384	114/198	1.57 (1.15–2.14)	0.32
Drinkers	94/157	66/91	1.21 (0.79–1.86)	

Bold value indicates the heterogeneity between two groups is significant ($p < 0.05$).

Genotype distributions had no deviation from the Hardy–Weinberg equilibrium in both case and control groups ($p > 0.05$). Genotype frequencies and odds ratio (OR) and 95% confidence interval (CI) for cases and controls are presented in Table 2. Under the codominant model, compared with the ins/ins wild genotype, subjects with the heterozygous ins/del or homozygous del/del variants of rs10680577 had a significantly increased risk of GC in a dose-dependent manner (adjusted OR = 1.35, 95% CI = 1.05–1.75; adjusted OR = 2.19, 95% CI = 1.15–4.18, respectively). Similar trends were observed in other genetic models. Each additional copy of the deletion allele was associated with a 38% increased risk of GC (OR = 1.38, 95% CI = 1.12–1.70, $p = 0.002$). In the stratification analysis, the association was more prominent in smokers (adjusted OR = 2.09, 95% CI = 1.40–3.12, p for heterogeneity = 0.01). However, there was no significant difference of the association for rs10680577 among other subgroups (Table 3). We also performed interaction analyses between rs10680577 and smoking, drinking, or *H. pylori* infection and did not observe any significant interaction effect on GC risk (data not shown).

Discussion

To our knowledge, this is the first study investigating the association of rs10680577 polymorphism with the risk of GC. In a case–control study using 415 GC cases and 830 controls, we found that the variant deletion allele was significantly associated with an increased risk of GC, particularly of those smokers in the Chinese population.

Over the past decade, it has become clear that overexpression of HIF-1 α in GC may upregulate its downstream gene products leading to VEGF-mediated angiogenesis and resulting in a poor prognosis for patients (Mizokami *et al.*, 2006; Urano *et al.*, 2006; Oh *et al.*, 2008). EGLN2 is capable of hydroxylating the α subunit of the heterodimeric HIF-1 α . Subsequently, prolyl hydroxylated HIF-1 α is recognized by a ubiquitin ligase complex containing the pVHL tumor suppressor protein, leading to its polyubiquitinylation and subsequent proteasomal degradation. Therefore, genetic variations within the regulatory regions of the *EGLN2* gene may influence its expression, contributing to GC tumorigenesis by affecting HIF-1 α . The rs10680577 is located at

–1641 bp upstream of the transcription start site of *EGLN2*. According to the study of Zhu *et al.* (2012), rs10680577 is associated with EGLN2 expression. However, the genotype–phenotype correlation is not likely mediated by a differential promoter polymorphism-associated regulatory mechanism. Instead, rs10680577 may affect EGLN2 expression through regulating a long noncoding RNA named RERT-lncRNA. There is a possibility that the same regulatory mechanism may exist in GC, further studies still need to be fully elucidated both at the genetic and functional levels.

Several limitations in our study need to be addressed. First, in the current sample size, the statistical power is about 58% when detecting an effect size of 1.42 with an α -level of 0.05 for the association of rs10680577 with GC risk. Further studies with larger sample sizes are needed to evaluate our findings. Second, there is no prognosis data for the current sample, which would prevent us to effectively detect the association between GC prognosis and rs10680577 indel polymorphism.

Taken together, our data suggest that common genetic polymorphisms in *EGLN2* may influence GC risk in the Chinese population. However, the presence of this GC-associated polymorphism does not immediately imply that this polymorphism is causative since other genetic variations linked to rs10680577 may be the true causal mutation driving the association. Replication in other populations with larger sample sizes and further functional analysis are required for fully understanding the roles of *EGLN2* polymorphisms in predisposition for GC.

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Author Disclosure Statement

No competing financial interests exist.

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