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ORIGINAL ARTICLE – CANCER RESEARCH

Relationship between *DLEC1* and *PBX3* promoter methylation and the risk and prognosis of gastric cancer in peripheral blood leukocytes

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Abstract

Purpose Aberrant DNA methylation could regulate the expression of tumor suppressor gene *DLEC1* and oncogene *PBX3* and was related to the occurrence and prognosis of gastric cancer (GC). In this study, the associations between *DLEC1* and *PBX3* promoter methylation in peripheral blood leukocytes (PBLs) and the risk and prognosis of GC were investigated.

Methods The methylation status of *DLEC1* and *PBX3* promoter in PBLs of 368 GC cases and 382 controls was detected by the methylation-sensitive high-resolution melting (MS-HRM) method. Logistic and Cox regression were adopted to analyze the associations of *DLEC1* and *PBX3* methylation with GC risk and prognosis, respectively. Confounding biases were controlled by propensity score (PS).

Results Compared with negative methylation (Nm), *DLEC1*-positive methylation (Pm) was associated with increased GC risk in PS (OR 2.083, 95% CI 1.220–3.558, $P=0.007$), but *PBX3* Pm was not associated with GC risk. In the elderly group (≥ 60 years), *DLEC1* Pm was associated with increased GC risk (OR 2.951, 95% CI 1.426–6.104, $P=0.004$). The combined effects between *DLEC1* methylation and consumption of dairy products, fried food intake and *Helicobacter pylori* (*H. pylori*) infection on GC risk were discovered (OR_c 3.461, 95% CI 1.847–6.486, $P<0.001$, OR_c 3.246, 95% CI 1.708–6.170, $P<0.001$ and OR_c 2.964, 95% CI 1.690–5.197, $P<0.001$, respectively). Furthermore, *DLEC1* and *PBX3* methylation were not associated with GC prognosis.

Conclusion *DLEC1* methylation in PBLs and the combined effects of gene–environment can influence GC risk.

Keywords DNA methylation · Peripheral blood leukocytes · Gastric cancer · Propensity score

Introduction

Gastric cancer (GC) is a common malignant tumor and is responsible for 1,034,000 new cases and 783,000 deaths in 2018, making it the fifth most commonly diagnosed cancer and the third major reason of cancer death all over the world (Bray et al. 2018). China is a country with high incidence of GC, with estimates of 679,000 new cases and 498,000 deaths in 2015 according to the China Cancer Data Report (Chen et al. 2016).

Genetic and epigenetic alterations are considered to be the two main factors involved in gastric carcinogenesis. There is plenty of evidence to suggest that gene polymorphisms and mutations are associated with the occurrence of GC (He et al. 2012, 2018). Beside the genetic change, research has shown that epigenetic dysregulation plays a crucial role in GC development (Nakamura et al. 2014).

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Epigenetics is defined as heritable changes in gene expression that are not due to alterations in gene sequence (Guo and Yan 2015). As one of the most widely studied epigenetic modifications, DNA methylation is closely related to the occurrence and prognosis of GC. Abnormal DNA methylation often occurs at the promoter regions of genes, particularly tumor-suppressor genes, which are related to tumor cell cycle, apoptosis, proliferation, differentiation, and invasion (Li and Chen 2013). To date, more and more researches have revealed that dietary factors and lifestyle could contribute to cancer development by inducing both epigenetic and genetic changes (Herceg 2007).

Deleted in lung and esophageal cancer 1 (*DLEC1*), located at the commonly deleted locus 3p22.3, has been demonstrated to act as a tumor suppressor gene in multiple cancers (Kwong et al. 2006; Pastuszak-Lewandoska et al. 2016; Zhang et al. 2015) which can suppress tumor growth or reduce the invasiveness of cancer cells (Ye et al. 2014). It was reported that the promoter methylation of *DLEC1* could result in the downregulation or silence of its own expression in most gastric cell lines (Ying et al. 2009) and was associated with GC risk and prognosis in tissue samples (Ye et al. 2014; Zhang et al. 2010).

Pre-leukemia transcription factor 3 (*PBX3*) is a member of the PBX family that belongs to the three amino acid loop extension family with a highly conserved homologous domain (Han et al. 2014). As an oncogene, *PBX3* is overexpressed in GC and its overexpression can accelerate cell proliferation and colony formation. In addition, *PBX3* is closely associated with invasion depth, clinical stage, and differentiation of GC (Li et al. 2014; Wang et al. 2016a). A recent research reported that *PBX3* hypermethylation in PBLs was associated with prognosis in colorectal cancer (Sun et al. 2019). However, the relationship between *PBX3* methylation and GC is unclear.

In recent years, increasing studies focused on the relationship between DNA methylation in tissues and the incidence and prognosis of tumors. Compared with the acquisition of tissues, blood sampling is convenient and minimally invasive, making it adaptive for the population-based study (Tahara and Arisawa 2015). Furthermore, DNA from peripheral blood can dynamically monitor tumors in real-time, so it can provide more information for the early detection and prognosis of tumors than tissues (Hu et al. 2019). Therefore, we conducted this case–control study to detect the methylation levels of *DLEC1* and *PBX3* in PBLs, explore the relationship between environmental factors and gene methylation and investigate the relationship between environmental factors, gene methylation and their interactions with GC risk. We also conducted a follow-up study of GC patients to assess the association of gene methylation in PBLs with GC prognosis.

Materials and methods

Study samples

368 GC cases and 382 controls were enrolled in a hospital-based case–control study. Patients of GC diagnosed by pathological examination of the Third Affiliated Hospital of Harbin Medical University in 2010 and 2012 were selected as cases. The vast majority of these patients (> 98%) had undergone surgery. Patients from the Department of Orthopaedics and Ophthalmology of the Second Affiliated Hospital and the Department of Neurology of the Fourth Affiliated Hospital of Harbin Medical University, and healthy people who participated in physical examination at the Harbin Xiangfang Center for Disease Control and Prevention between 2010 and 2013 were selected as controls. The control individuals with the history of malignant tumors such as gastric cancer and gastrointestinal diseases were excluded. After obtaining the patient's informed consent, face-to-face investigations were conducted in both cases and controls, and 5 ml blood samples were collected from each subject. The overall response rate for cases and controls were approximately 90%. All cases were included in the follow-up study. Finally, a total of 347 GC patients were included in the analysis, 21 cases were lost to follow-up due to death or withdrawal. Demographic, clinical, and treatment information of each patient was extracted from the electronic medical record system.

H. pylori serologic tests by ELISA

The enzyme immunoassay kit (IBL, German) was used to detect the infection status of *H. pylori*. The criterion is that lower than 8 units/ml was represented negative, from 8 to 12 units/ml was represented suspicious, upper to 12 units/ml was represented positive.

Methylation assay

The DNA was extracted using QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany), and then modified by bisulfite using EpiTect Fast DNA Bisulfite Kit (Qiagen). The procedure was referred to the kit instructions. Nanodrop 2000c (Thermo Scientific) was adopted to measure the DNA quantity. The bisulfite-modified DNA was stored at – 80 ℃. Primers of *DLEC1* and *PBX3* gene were designed using Primer Premier 5.0 software. PCR amplification and MS-HRM assay were implemented on the LightCycler480 (Roche Applied Science, Mannheim, Germany) equipped with Gene Scanning software (version 2.0) to identify and analyze the methylation status of genes. The 5 μl reaction system contained 2.5 μl LightCycler480 High-Resolution

Melting Master Mix (Roche), 0.5 µl sodium bisulfite-modified template DNA, 0.1 µl forward primer, 0.1 µl reverse primer, 0.6 µl $MgCl_2$ and 1.2 µl PCR-grade water. The primer sequences and reaction conditions are listed in Table S1.

Methylated DNA standards with different levels, including 100%, 5%, 2%, 1%, 0.5%, and 0% methylated DNA, were constructed by mixing 100% methylated and 0% methylated human whole genomic DNA (Zymo Research). Normalized melting curves and melting peaks of the MS-HRM assay for two genes are shown in Figs. 1 and 2. The methylation levels of genes in samples were confirmed by contrasting with the standard curves. In Figs. S1 and S2, the distribution of DNA methylation levels of samples was showed. Based on the area under the curve (AUC), 0% and 2% methylated DNA acted as the cut-off values to divide Nm and Pm of *DLEC1* and *PBX3*, respectively (Fig. S3). Replicate measurements of some samples were performed at different times for *DLEC1* and *PBX3*. The consistency rates of *DLEC1* and *PBX3* were 96.0% and 95.2%, respectively. Rank sum test of paired samples was used for consistency analysis, and the results showed no difference ($P > 0.05$) (Tables S2). PCR-grade water was employed as negative (no-template) control in each batch, and second experiments were performed for the equivocal results.

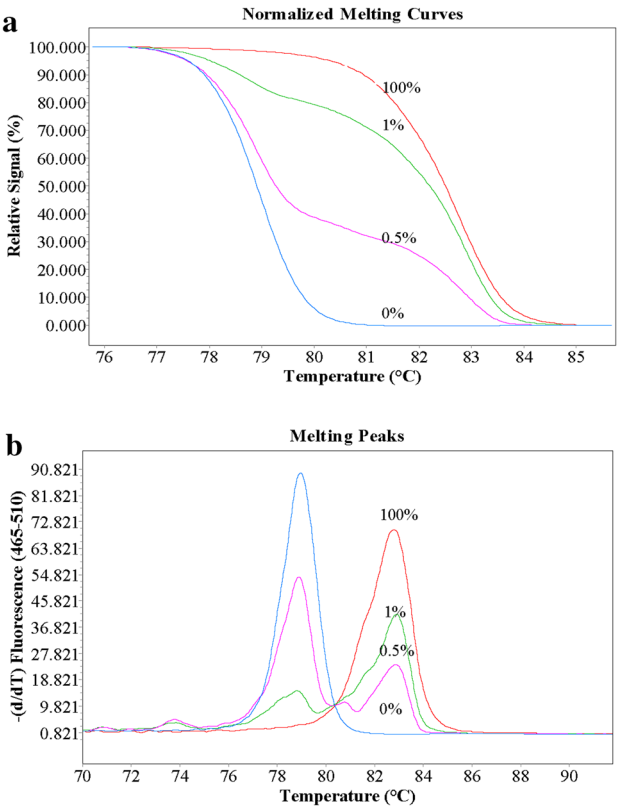

Fig. 1 A series of methylated DNA standards (100%, 1%, 0.5% and 0% methylated DNA) was used for *DLEC1*. **a** Normalized melting curves of the MS-HRM assay for *DLEC1*. **b** Melting peaks of the MS-HRM assay for *DLEC1*

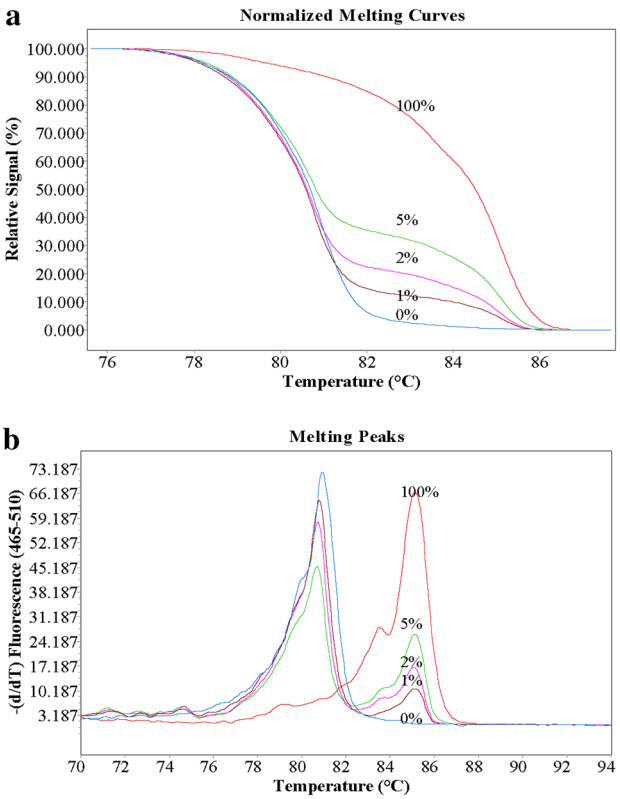

Fig. 2 A series of methylated DNA standards (100%, 5%, 2%, 1% and 0% methylated DNA) was used for *PBX3*. **a** Normalized melting curves of the MS-HRM assay for *PBX3*. **b** Melting peaks of the MS-HRM assay for *PBX3*

Statistical analysis

The Chi-square (χ^2) test was used for categorical variables and *t* test was used for continuous variables. Multiple imputation method was adopted for variables with less than 30%. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated by logistic regression and adjusted by propensity score (PS). Univariate and multivariate logistic regression analyses were adopted to evaluate the relationships between gene methylation, environmental factors and GC risk, as well as the relationships between gene methylation and environmental factors. The interactions of gene methylation and environmental factors on GC risk were estimated on a multiplicative scale with a product-term coefficient using multivariate logistic regression. The combined effects of gene methylation and environmental factors on GC risk were calculated by crossover analysis. Kaplan–Meier analysis was adopted to acquire the survival curve of GC patients. Univariate and multivariate Cox regression analyses were

adopted to calculate hazard ratios (HRs) and 95% CIs for the relationship between gene methylation and clinical characteristics and prognosis of GC patients, and these results were adjusted by PS. All statistical analyses were performed using SPSS software version 23.0. PS was executed using R-3.1.3 for Windows with PS matching 3.04 software packages. *P* values < 0.05 were considered statistically significant.

Results

Demographic characteristics of all subjects

368 cases and 382 controls were carried out in our study. The PS value calculated by all 28 covariates was an adjustment factor. The demographic characteristics of all subjects are listed in Table 1.

The results showed no difference between cases and controls in the aspect of sex and age ($P = 0.878$ and $P = 0.301$, respectively). The distribution of body mass index (BMI) and monthly income between GC cases and controls were statistically different ($P < 0.05$). Compared with controls (3.1%), the percentage of cases with GC family history (13.9%) was higher ($P < 0.001$).

Table 1 The basic demographic characteristics of GC cases and controls

Variables	Cases (%) $n = 368$	Controls (%) $n = 382$	*P*
Sex			
Male	278 (75.5)	290 (75.9)	0.878
Female	90 (24.5)	92 (24.1)	
Age (mean ± SD)	58.20 ± 11.064	59.02 ± 10.480	0.301
< 60	197 (53.5)	190 (49.7)	0.289
≥ 60	171 (46.5)	192 (50.3)	
BMI (kg/m^2)			
< 23.00	224 (60.9)	161 (42.1)	< 0.001
≥ 23.00	144 (39.1)	221 (57.9)	
Monthly income (RMB/per capita)			
< 1000	129 (35.1)	173 (45.3)	0.006
≥ 1000	239 (64.9)	209 (54.7)	
Family history of gastric cancer			
No	317 (86.1)	370 (96.9)	< 0.001
Yes	51 (13.9)	12 (3.1)	

Associations between environmental factors and GC risk

Multivariate logistic regression analysis was adopted to assess associations between environmental factors and GC risk, and the results are shown in Table S3. Then backward conditional selection method was adopted for multivariate analysis. Finally, thirteen environmental factors were integrated into the regression model. As shown in Table S4, alcohol consumption, *H. pylori* infection, salty food intake, food left overnight intake, dairy products intake, eat fried food and freshwater fish intake significantly increased GC risk (adjusted by PS, $P < 0.05$). On the contrary, regular diet, drinking tap water and mineral water, refrigerated food, beef and mutton intake, garlic intake and green vegetable intake significantly decreased GC risk ($P < 0.05$).

Associations between the methylation of *DLEC1* and *PBX3* and GC risk

As shown in Table 2, *DLEC1* Pm was associated with increased GC risk compared with Nm (OR 2.083, 95% CI 1.220–3.558, $P = 0.007$). However, *PBX3* methylation was not associated with GC risk.

Table 2 Associations between methylation of *DLEC1* and *PBX3* and GC risks

Methylation status	Case (%)	Control (%)	Crude OR (95% CI)	*P*	OR[a] (95% CI)	*P*	OR[b] (95% CI)	*P*
DLEC1								
Nm	273 (77.3)	299 (86.7)	1.000		1.000		1.000	
Pm	80 (22.7)	46 (13.3)	1.905 (1.279–2.836)	0.002	1.930 (1.145–3.255)	0.014	2.083 (1.220–3.558)	0.007
PBX3								
Nm	164 (47.4)	159 (44.9)	1.000		1.000		1.000	
Pm	182 (52.6)	195 (55.1)	0.905 (0.672–1.218)	0.510	0.770 (0.506–1.173)	0.224	0.804 (0.526–1.229)	0.313

Nm negative methylation, *Pm* positive methylation, *CI* confidence interval, *OR* odds ratio

[a]Adjusted for all variables of the regression model

[b]Adjusted for propensity score of all variables

Associations between the methylation of *DLEC1* and *PBX3* and GC risk by stratified analysis

In the elderly group (≥ 60 years), the results displayed that *DLEC1* Pm was associated with increased GC risk (OR 2.951, 95% CI 1.426–6.104, $P = 0.004$), but no correlation was found in the younger group (< 60 years). When the individuals were classified by *H. pylori* infection, *DLEC1* Pm was marginally associated with increased GC risk in *H. pylori*-positive and -negative individuals (OR 1.875, 95% CI 0.944–3.727, $P = 0.073$ and OR 2.179, 95% CI 0.935–5.080, $P = 0.071$, respectively). But *PBX3* Pm was still not associated with GC risk by stratified analysis (Tables 3, 4).

Associations between the methylation of *DLEC1* and *PBX3* and environmental factors

As shown in Table S5, consumption of garlic was associated with decreased risk of *DLEC1* methylation (OR 0.615, 95% CI 0.397–0.953, $P = 0.030$), and refrigerated food was marginally associated with *DLEC1* methylation (OR 0.664, 95% CI 0.441–1.000, $P = 0.050$). Alcohol consumption was associated with decreased risk of *PBX3* methylation (OR 0.728, 95% CI 0.539–0.984, $P = 0.039$) (Table S6).

The interactions between the methylation of *DLEC1* and *PBX3* and their interactions with environmental factors on GC risk

The results showed that the combined effects between *DLEC1* methylation and *H. pylori* infection, consumption of dairy products (≥ 1 times/week) and fried food intake (≥ 1 times/month) on GC risk existed (OR_c 2.964, 95% CI 1.690–5.197, $P < 0.001$, OR_c 3.461, 95% CI 1.847–6.486, $P < 0.001$ and OR_c 3.246, 95% CI 1.708–6.170, $P < 0.001$, respectively), whereas no interactions between *DLEC1* methylation and environmental factors on the GC risk were found (Table S7). As for *PBX3*, its methylation and some environmental factors have both interactions and combined effects on GC risk ($P < 0.05$) (Table S8). In addition, the results in Table S9 showed that *DLEC1* methylation did not interact with *PBX3* methylation on GC risk.

Demographic characteristics of GC patients

A total of 347 GC patients were included in this 5-year follow-up study. The association between demographic characteristics and prognosis of GC patients was analyzed, as shown in Table S10.

Although the relationship between each demographic characteristic and GC prognosis was not statistically significant, age, gender and BMI were still used as the

Table 3 Association between methylation of *DLEC1* and *PBX3* and GC risks by age

Gene	< 60 years			≥ 60 years		
	OR[a]	95% CI	*P*	OR[a]	95% CI	*P*
DLEC1						
Nm	1.000			1.000		
Pm	1.322	0.591–2.955	0.497	2.951	1.426–6.104	0.004
PBX3						
Nm	1.000			1.000		
Pm	0.607	0.311–1.182	0.142	0.986	0.561–1.736	0.962

Nm negative methylation, *Pm* positive methylation, *CI* confidence interval, *OR* odds ratio

[a]Adjusted for propensity score of all variables except age

Table 4 Association between methylation of *DLEC1* and *PBX3* and GC risks by *H. pylori* infection

Gene	*H. pylori* negative			*H. pylori* positive		
	OR[a]	95% CI	*P*	OR[a]	95% CI	*P*
DLEC1						
Nm	1.000			1.000		
Pm	2.179	0.935–5.080	0.071	1.875	0.944–3.727	0.073
PBX3						
Nm	1.000			1.000		
Pm	0.700	0.355–1.381	0.303	0.897	0.517–1.557	0.700

Nm negative methylation, *Pm* positive methylation, *CI* confidence interval, *OR* odds ratio

[a]Adjusted for propensity score of all variables except *H. pylori* infection

adjustment factors in analyzing the relationship between clinical characteristics and GC prognosis since they were common confounding factors. The results of multivariate Cox analysis revealed that tumor–node–metastasis (TNM) stage, differentiation, tumor size, carbohydrate antigen 19–9 (CA 19–9) level and carcinoembryonic antigen (CEA) level were significantly associated with GC prognosis (all P values < 0.05) (Table 5).

Backward conditional selection results showed that GC patients with TNM stage III had marginally poorer prognosis (HR 1.887, 95% CI 0.959–3.715, $P = 0.066$). GC patients with stage IV had obviously poorer prognosis (HR 4.178, 95% CI 2.296–7.603, $P < 0.001$). In addition, tumor size was also associated with poorer GC prognosis (HR 1.689, 95% CI 1.229–2.320, $P = 0.001$) (Table S11).

Associations between methylation of *DLEC1* and *PBX3* and GC prognosis

As shown in Table 6, compared with Nm, *PBX3* Pm was marginally associated with GC prognosis only by multivariate adjustment (HR 1.349, 95% CI 0.981–1.856, $P = 0.065$). *DLEC1* Pm had no association with GC prognosis by both multivariate and PS adjustment. The Kaplan–Meier survival curves for the relationships between methylation of *DLEC1* and *PBX3* and GC prognosis are shown in Fig. 3.

Associations between methylation of *DLEC1* and *PBX3* and GC prognosis by stratified analysis

Stratified analyses were conducted in prognostic analysis by age, gender, *H. pylori* infection, TNM stage, and tumor size. The results indicated *PBX3* Pm was associated with poorer GC prognosis only in the elderly group (HR 1.678, 95% CI

Table 5 Association between clinical characteristics and GC prognosis

Clinical characteristics	Cases (%)	HR (95% CI)	P	HR[a] (95% CI)	P
Tumor site					
Distal stomach	214 (61.7)	1.000		1.000	
Others	133 (38.3)	1.323 (0.973–1.798)	0.074	1.340 (0.982–1.828)	0.065
Tumor size					
< 5 cm	174 (50.1)	1.000		1.000	
≥ 5 cm	173 (49.9)	2.275 (1.664–3.111)	< 0.001	2.279 (1.658–3.133)	< 0.001
CA19-9					
< 37 μ/ml	277 (79.8)	1.000		1.000	
≥ 37 μ/ml	70 (20.2)	1.610 (1.128–2.298)	0.009	1.707 (1.196–2.435)	0.003
CEA					
< 5 ng/ml	273 (78.7)	1.000		1.000	
≥ 5 ng/ml	74 (21.3)	1.640 (1.142–2.354)	0.007	1.682 (1.174–2.409)	0.005
Histological type					
Adenocarcinoma	184 (53.0)	1.000		1.000	
Mixed carcinoma	59 (17.0)	0.781 (0.512–1.191)	0.252	0.770 (0.504–1.178)	0.229
Others	104 (30.0)	0.652 (0.458–0.929)	0.018	0.667 (0.466–0.954)	0.027
Pathological type					
Polypoid type	30 (8.6)	1.000		1.000	
Ulcer type	54 (15.6)	0.666 (0.291–1.523)	0.332	0.675 (0.294–1.550)	0.350
Infiltrating ulcer type	196 (56.5)	1.145 (0.620–2.115)	0.664	1.147 (0.618–2.128)	0.662
Infiltrating type	60 (17.3)	1.586 (0.791–3.182)	0.192	1.572 (0.777–3.181)	0.206
Other type	7 (2.0)	0.318 (0.041–2.438)	0.269	0.332 (0.043–2.571)	0.290
TNM stage					
I	52 (15.0)	1.000		1.000	
II	18 (5.2)	1.393 (0.342–5.675)	0.641	1.359 (0.333–5.552)	0.667
III	76 (21.9)	2.294 (0.963–5.467)	0.061	2.227 (0.929–5.340)	0.072
IV	201 (57.9)	6.165 (2.799–13.576)	< 0.001	5.942 (2.687–13.141)	< 0.001
Differentiation					
Low	208 (59.9)	1.000		1.000	
Middle to high	139 (40.1)	0.697 (0.486–0.998)	0.049	0.695 (0.484–0.997)	0.048

[a]Adjusted for age, sex, BMI

Table 6 Association between methylation of *DLEC1* and *PBX3* and GC prognosis

Methylation status	Case (%)	HR (95% CI)	*P*	HR[a] (95% CI)	*P*	HR[b] (95% CI)	*P*
DLEC1							
Nm	256 (76.9)	1.000		1.000		1.000	
Pm	77 (23.1)	1.072 (0.757–1.517)	0.695	1.261 (0.878–1.811)	0.210	1.245 (0.862–1.799)	0.242
PBX3							
Nm	156 (47.7)	1.000		1.000		1.000	
Pm	171 (52.3)	1.295 (0.951–1.762)	0.101	1.349 (0.981–1.856)	0.065	1.260 (0.907–1.749)	0.169

Nm negative methylation, *Pm* positive methylation, *CI* confidence interval, *HR* hazard ratio

[a]Adjusted for age, sex, BMI, tumor size, TNM stage

[b]Adjusted for propensity score of all variables

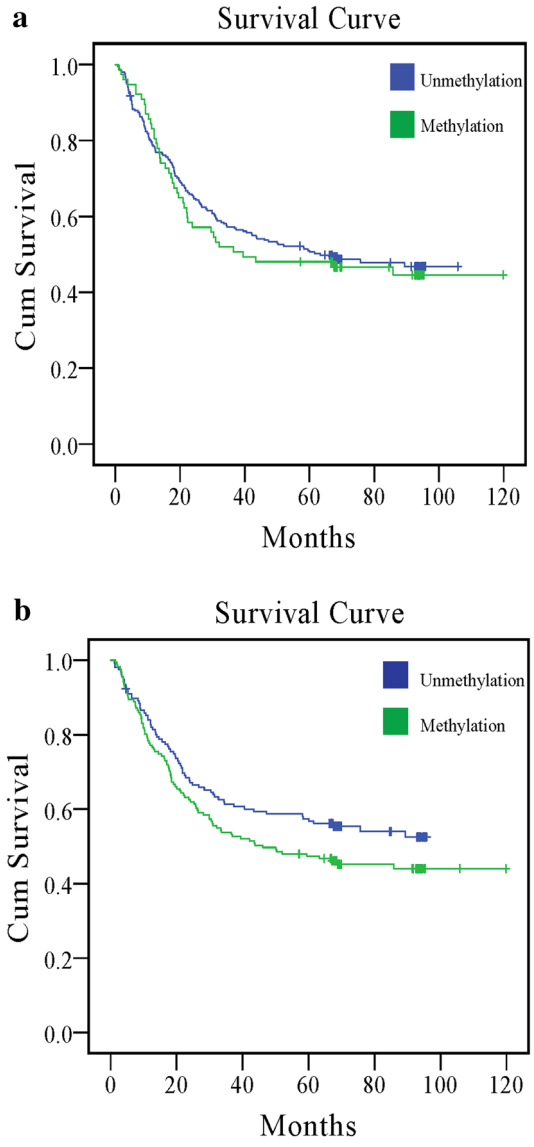

Fig. 3 Survival curves of for the associations between methylation status of *DLEC1* (**a**) and *PBX3* (**b**) and GC prognosis

1.046–2.693, $P=0.032$) and female group (HR 2.058, 95% CI 1.024–4.137, $P=0.043$), but *DLEC1* Pm was not associated with GC prognosis in each age and gender subgroup (Tables S12 and S13). In addition, stratified analysis by *H. pylori* infection indicated that *DLEC1* Pm was associated with poorer GC prognosis in *H. pylori*-negative individuals (HR 2.040, 95% CI 1.104–3.769, $P=0.023$), but *PBX3* Pm was not associated with GC prognosis in both *H. pylori*-positive and -negative individuals (Table S14). No significant relationships of *DLEC1* Pm and *PBX3* Pm with GC prognosis were found in each TNM stage and tumor size subgroup (Tables S15 and S16).

Discussion

Gastric cancer is a common malignant tumor of the gastrointestinal tract. In recent decades, more and more evidence indicates that epigenetics plays an important role during cancer progression, including GC (Heyn and Esteller 2012). Abnormal DNA methylation, a common event of epigenetics, could regulate the expression of tumor suppressor genes and oncogenes (Puneet et al. 2018; Qu et al. 2013). It is reported that tumors do not develop as an isolated phenomenon in their target tissue; other organ systems including the immune system (such as PBLs) also participate in tumor initiation and prognosis (Marsit and Christensen 2013). Moreover, since peripheral blood is relatively easy to obtain, PBLs are the most commonly used alternative to studying the risk of epigenome induction and the epigenetic response to disease-associated stress (Hohos et al. 2016). Therefore, this study was performed to evaluate the effect of the promoter methylation of genes derived from PBLs on the risk and prognosis of GC.

Compared with Nm, *DLEC1* Pm increased GC risk (OR 2.083). Wang et al. found that the hypermethylation levels of *DLEC1* were significantly associated with GC risk by quantitative methylation-specific PCR in the serum of 82

GC patients, 46 chronic atrophic gastritis subjects, and 40 healthy controls (Wang et al. 2015). Our finding verified above result in larger populations by MS-HRM.

It is reported that DNA methylation changes during aging are closely correlated to the occurrence of cancer (Wang et al. 2016b). The result of age-stratified analysis declared that *DLEC1* Pm individuals had a higher GC risk than Nm individuals in the elderly group. Fuke et al. measured 5-methyldeoxycytidine ((met)C) content by HPLC in PBLs obtained from 76 healthy individuals and found that the age-dependent decrease of (met)C was statistically highly significant in the aged group compared with the young group (Fuke et al. 2004), which indicated that methylation differences were remarkable in older individuals. Previous studies had revealed that *H. pylori* infection could cause an intensive inflammatory response in the gastric mucosa, leading to upregulation of certain inflammatory cytokines such as IL-1β which in turn caused abnormal levels of DNA methylation (Lamb and Chen 2013). The result of *H. pylori* infection-stratified analysis declared that *DLEC1* Pm was marginally associated with GC risk in both *H. pylori*-positive and -negative individuals. Considering *DLEC1* Pm was significantly associated with GC in unstratified analyses, so more researches were needed to determine marginal associations in *H. pylori* infection-stratified analysis.

In this study, a combined effect between methylation status of *DLEC1* and *H. pylori* infection on GC risk was found. *Helicobacter pylori* infection could contribute to GC due to its role in increasing chronic inflammation and cell proliferation (Yousefi et al. 2019). Cell proliferation has been recognized as a contributing factor for de novo DNA methylation (Issa et al. 2001; Velicescu et al. 2002). On the other hand, expression of many genes is inhibited in the processes of inflammatory and low expression of these genes can promote de novo methylation (De Smet et al. 2004; Song et al. 2002; Ushijima and Okochi-Takada 2005). Research showed that *H. pylori* infection enhances abnormal DNA methylation in the gastric mucosa, which further promotes GC by inducing abnormal methylation of gene promoters (Xie et al. 2017). After eradication of *H. pylori*, the methylation levels of genes were reduced, and *H. pylori*-mediated gastric tumorigenesis could be postponed or even reversed (Perri et al. 2007; Zhou et al. 2019). In this study, no interaction was found between *H. pylori* infection and *DLEC1* methylation.

Our results also observed that there was a combined effect between *DLEC1* methylation and consumption of dairy products on GC risk. Dairy products contain high amounts of methionine (Finkelstein 1990), which serves as the precursor of *S*-adenosylmethionine, the universal methyl donor for DNA methylation in the hepatic one-carbon metabolism (Zhang 2018). Moreover, we found that *DLEC1* methylation also showed the combined effect with fried food intake on GC risk. During the frying process, protein-rich foods produce heterocyclic amine carcinogens, and starchy foods produce acrylamide carcinogen, which causes human tumors to occur. Acrylamide is metabolized to glycidyl amide in the human body, and it can directly bind to hydrazine, altering DNA structure and causing DNA methylation (de Conti et al. 2019; Guo et al. 2018).

It is well known that dietary and lifestyle are convertible factors and profoundly influence the occurrence and development of GC. Among these influence factors, regular diet, refrigerated food, consumption of vegetables, garlic and beef and mutton and drinking tap and mineral water were recognized as protective factors for GC; however, alcohol consumption, salty food intake and consumption of food left overnight, dairy products and fried food were identified as risk factors for GC (den Hoed and Kuipers 2016; Ghaffari et al. 2019; Rastaghi et al. 2019; Zhao et al. 2011), and our results were consistent with these findings. In this study, we also found that consumption of freshwater fish was recognized as risk factor for GC, which was somewhat unexpected. Freshwater fish may increase tumor susceptibility. The reason may be that large amounts of heavy metals and toxic chemicals accumulate in freshwater fish (Hou et al. 1988). In addition, due to the habit of high-temperature cooking in China, cooking freshwater fish could produce high levels of carcinogenic compounds, which may be a major reason of the increased GC risk (Felton et al. 1997; Sugimura and Terada 1998).

The relationship between methylation status of *DLEC1* and *PBX3* and GC prognosis was also explored in this study. The results found that TNM stage and tumor size were factors influencing the GC prognosis. The previous research published by our department had been reported that *PBX3* hypermethylation in PBLs was associated with better prognosis in colorectal cancer (Sun et al. 2019). This was the first time to explore the association between *PBX3* methylation and GC prognosis. Compared with Nm, *PBX3* Pm was marginally associated with worse prognosis of GC only in multivariate Cox regression analysis. To be conservative, the result of this study preferred to prompt that *PBX3* Pm was not associated with GC prognosis. However, *PBX3* Pm subjects had a worse GC prognosis than Nm subjects in elder and female groups.

There were three limitations to this study. First, recall bias might remain unavoidable at the time of gathering information on environmental factor although we attempted to reduce this bias. Second, the causality between gene methylation and GC risk remains unclear, and prospective cohort studies are needed to determine in the future. Third, the intake of dietary was not explicitly quantified, which might affect the results of the gene–environment interaction analysis.

Conclusions

In conclusion, this study indicated that *DLEC1* methylation and the combined effects between environmental factors and its methylation in PBLs were associated with the GC risk. As a new biomarker, *DLEC1* methylation can predict the risk of GC.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The study was approved by the Human Research and Ethics Committee of Harbin Medical University.

Informed consent Informed consent was obtained from all individual participants included in the study.

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