


# Peripheral Blood-Based DNA Methylation of Long Non-Coding RNA HI9 and Metastasis-Associated Lung Adenocarcinoma Transcript I Promoters are Potential Non-Invasive Biomarkers for Gastric Cancer Detection

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## Abstract

**Introduction.** The early diagnosis and detection could greatly improve the clinical outcome of gastric cancer (GC) patients. However, the non-invasive biomarkers for GC detection remain to be identified.

**Method.** We used online databases (GEPIA, UALCAN, Kaplan-Meier plotter, TIMER, and MEXPRESS) to explore the association between HI9 or metastasis-associated lung adenocarcinoma transcript I (MALAT1) expression in tissues and the occurrence, development, prognosis, the levels of immune cell infiltration, and methylation of GC; the correlation between mRNA expression and DNA methylation levels of genes were also examined. Methylation levels of HI9 or MALAT1 in peripheral blood were compared between 150 GC patients and 100 healthy controls (HCs). Predictive nomograms were constructed among female and male groups for GC diagnosis. The calibration curves, Hosmer–Lemeshow test, and decision curve analysis were also used to examine the nomograms' predictive ability and clinical values.

**Results.** Using multiple online databases, we found that the mRNA expressions of HI9 and MALAT1 in tissues were related to the occurrence of GC, and such expressions were associated with immune cell infiltration of GC and negatively correlated with DNA methylation levels of HI9 and MALAT1. HI9 gene, HI9C island, and MALAT1B island, as well as 20 CpG sites were hypermethylated in peripheral blood of GC patients compared with HCs; similar results were also found in female and male groups ( $P < .05$  for all). The combination of HI9c3, HI9c4, MALAT1b12, and age, as well as the combination of HI9b7, HI9c1, HI9c5, and age in the nomograms could distinguish GC patients from HCs in the female group and male group, respectively.

**Conclusion.** We found statistically significant hypermethylation of HI9 and MALAT1 promoters in GC patients, and meaningful sensitivity and specificity of MALAT1 and HI9 methylation in discriminating GC and HCs were observed in both female and male groups, which indicates that the peripheral blood-based DNA methylation of HI9 and MALAT1 could act as potential non-invasive biomarkers for the diagnosis of GC.

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## Keywords

DNA methylation, peripheral blood, H19, metastasis-associated lung adenocarcinoma transcript 1, gastric cancer

## Introduction

Following behind the malignancies of lung, breast, colorectum, and prostate, gastric cancer (GC) remains the fifth most common carcinoma and the third leading disease of tumor-related deaths globally.<sup>1,2</sup> The estimated new cases and deaths of GC were 1000 000 and 783 000 in 2018 worldwide.<sup>3</sup> It is well accepted that GC patients with early-stage have a great long-term outcome, with the reported 5-year overall survival (OS) between 84% and 97%.<sup>4,5</sup> Endoscopy and biopsy are known as the primary diagnostic method for GC; however, such procedures are invasive and expensive and are challenging to clinicians; few people take these examinations.<sup>6</sup> Therefore, nearly 3 quarters of GC patients are diagnosed at an advanced stage, missing the chance to get the gastrectomy and endoscopic resection treatment, which is limited to patients with early GC.<sup>7</sup> Although there is significant progress in understanding the molecular mechanisms of GC, the precise diagnostic biomarkers for early GC detection remain limited. In the last few decades, epigenetic and genetic alterations had been identified as several significant factors that could induce GC, including DNA methylation,<sup>8</sup> non-coding RNAs (lncRNAs),<sup>9</sup> histone modifications,<sup>10</sup> and microRNAs.<sup>9</sup> Aberrant DNA methylation, up to now, is the most well-studied deregulated molecular mechanism in the carcinogenesis of GC. Nevertheless, most of the studies on methylation related to GC's molecular markers were based on tissues; few of them focused on samples that can be collected non-invasively, such as blood and urine.<sup>10-13</sup>

Long lncRNAs are regulatory RNAs that exceed 200 nucleotides (nt) in length and cannot encode proteins.<sup>14</sup> Increasing evidence suggests that the expression of lncRNAs contribute to many types of human cancers,<sup>15-17</sup> and the regulation of DNA methylation by lncRNAs plays an important role in controlling gene expression in the process of tumor progression and prognosis.<sup>18</sup> As a highly conserved representative of lncRNA in mammals, metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) is an oncogene that is regulated by DNA methylation and is highly expressed in many carcinomas such as breast, prostate, and GC.<sup>19,20</sup> Growing evidence has shown that MALAT1 has the potential to be not only a diagnostic but also a prognostic biomarker in GC.<sup>21,22</sup> H19 is a transcript subjected to genomic imprinting and located on chromosome 11p15.5.<sup>23</sup> As an imprinted gene, lncRNAH19 has been widely studied and was found to participate in nearly all stages of tumorigenesis.<sup>24</sup> In GC, upregulated expression of H19 was observed, and higher expression was positively correlated with a worse prognosis of GC.<sup>23</sup> Although the role of MALAT1 and H19 expression in the occurrence and prognosis of GC had been studied, bioinformatic evidence that supported these findings is limited,

and the correlation between the expression and methylation of these genes had not been explored. It is also unclear whether the methylation levels of these 2 genes in peripheral blood can be used as non-invasive markers for the early diagnosis of GC. Therefore, we first applied a bioinformatic analysis to explore the role of H19 and MALAT1 expression in the tumorigenesis, progression, and prognosis of GC. As we hypothesize that methylation levels of H19 and MALAT1 in peripheral blood have the potential to be biomarkers for GC detection, we also evaluated the correlation between the methylation and expression levels of these 2 genes in the online database. We performed a case-control study to examine the relationship between carcinogenesis of GC and methylation levels of MALAT1 and H19 in peripheral blood, which are helpful to identify precise biomarkers for early diagnosis of GC.

## Methods

### *Bioinformatic Analysis of H19 and MALAT1*

In the present study, bioinformatic analysis was utilized to verify the reported association between MALAT1 and H19 expression and the occurrence, development, and prognosis of GC. Database of GEPIA<sup>25</sup> (Student's *t*-test, cutoff *P*-value: .05) and UALCAN<sup>26</sup> (Student's *t*-test, cutoff *P*-value: .05) was first used to test the expression of H19 and MALAT1, comparing GC tissues and the relevant normal tissues. The associations between MALAT1 or H19 expression and the pathological stages were explored by GEPIA and UALCAN, while the relationship of these 2 genes and the survival status of GC patients was explored by Kaplan–Meier plotter and UALCAN, respectively (since the data of H19 expression and the survival of GC patients were not available on Kaplan–Meier plotter,<sup>27</sup> we thus performed the survival analysis on UALCAN database). As both MALAT1<sup>28</sup> and H19<sup>29</sup> were reported to be involved in immune cell infiltration, the correlations between gene expression and the levels of immune infiltration were also evaluated within the TIMER database.<sup>30</sup> Finally, we utilized the MEXPRESS database<sup>31</sup> to investigate the potential association between the methylation and expression levels of MALAT1 and H19. The methylation level of each probe was represented with a beta value, and the Pearson correlation coefficient value (R) and the adjusted *P*-value (Benjamini–Hochberg) were also obtained.

### *Patients and Samples*

Among the Chinese Han population, we carried out a case-control study that enrolled 150 GC patients from the First Affiliated Hospital of Anhui Medical University. Patients who were diagnosed by histopathology and had no previous history

of malignant tumors in any organs and healthy controls (HC) who were age- and gender-matched with GC patients were recruited to test the DNA methylation levels. Five milliliters of each participant's peripheral blood samples were obtained, and face-to-face interviews were performed by investigators with a comprehensive questionnaire among GC patients only. Clinical and demographic information such as age, gender, tumor site, TNM stage, tumor differentiation, smoking, drinking, family history, and the dynamic changes of the psychological situation during the treatment are contained in this questionnaire and were described previously. All procedures conducted by this study were approved by Anhui Medical University's ethical community and abide by the 1964 Helsinki Declaration and the later amendments. All patients had signed written informed consent.

### DNA Methylation Detection

The methods of DNA extraction and bisulfite conversion are described at length.<sup>32,33</sup> Briefly, we used the QIAGEN kit to extract the genomic DNA from peripheral blood leukocytes of GC and HCs following the manufacturer's protocols. According to previously reported criteria for CpG islands (CGIs) and CpG island shores (CGI shores) selection,<sup>34</sup> we finally selected for sequencing 60 CpG methylation sites on 3 CGIs (H19A, H19B, H19C) and 1 CGI shore (H19D) from H19 promoter, as well as 31 CpG methylation sites on 2 CGIs (MALAT1A, MALAT1B) and 1 CGI shore (MALAT1C) of MALAT1 promoter (the position and the primer sequences of the CGIs, CGI shores, and CpG sites are shown in [Supplementary Tables S1 and S2](#)).

After DNA extraction and CpG site selection, 400 ng genomic DNA was bisulfite-converted using the EZ DNA Methylation™-GOLD kit (Zymo Research) for methylation analysis. Utilizing indexed primers, we amplified the bisulfite-modified DNA sequence with multiplex PCR. We then utilized agarose electrophoresis to separate these PCR amplicons and purified them with a QIAquick Gel Extraction kit (QIAGEN). The methylation levels of H19 and MALAT1 were analyzed using an NGS-based multiple-targeted CpG methylation analysis method manufactured by Methyl-Target™ (Genesky Biotechnologies Inc, Shanghai, China), and the methylation detection was conducted on an Illumina HiSeq/Miseq 2000 following the manufacturer's instructions.

### Statistical Analysis

The skewed data in the current study were presented as median with interquartile range (IQR), while the normally distributed data were depicted as mean  $\pm$  standard deviation (SD). Gender and age differences between GC and HCs were compared by Student's *t*-test, and the Mann–Whitney U test was used to explore the differences of methylation levels between the 2 categories. Binary and Multinomial regression analyses were carried out to identify the associations of methylation levels

between GC and HCs and between various clinicopathological variables of GC. The OR (odds ratios) and 95% CI (confidence intervals) were then calculated. All statistical analysis was conducted using SPSS 24.0 and R software, and Graphpad Prism 8.0 was also used to generate the figures.

### Diagnostic Nomogram for GC

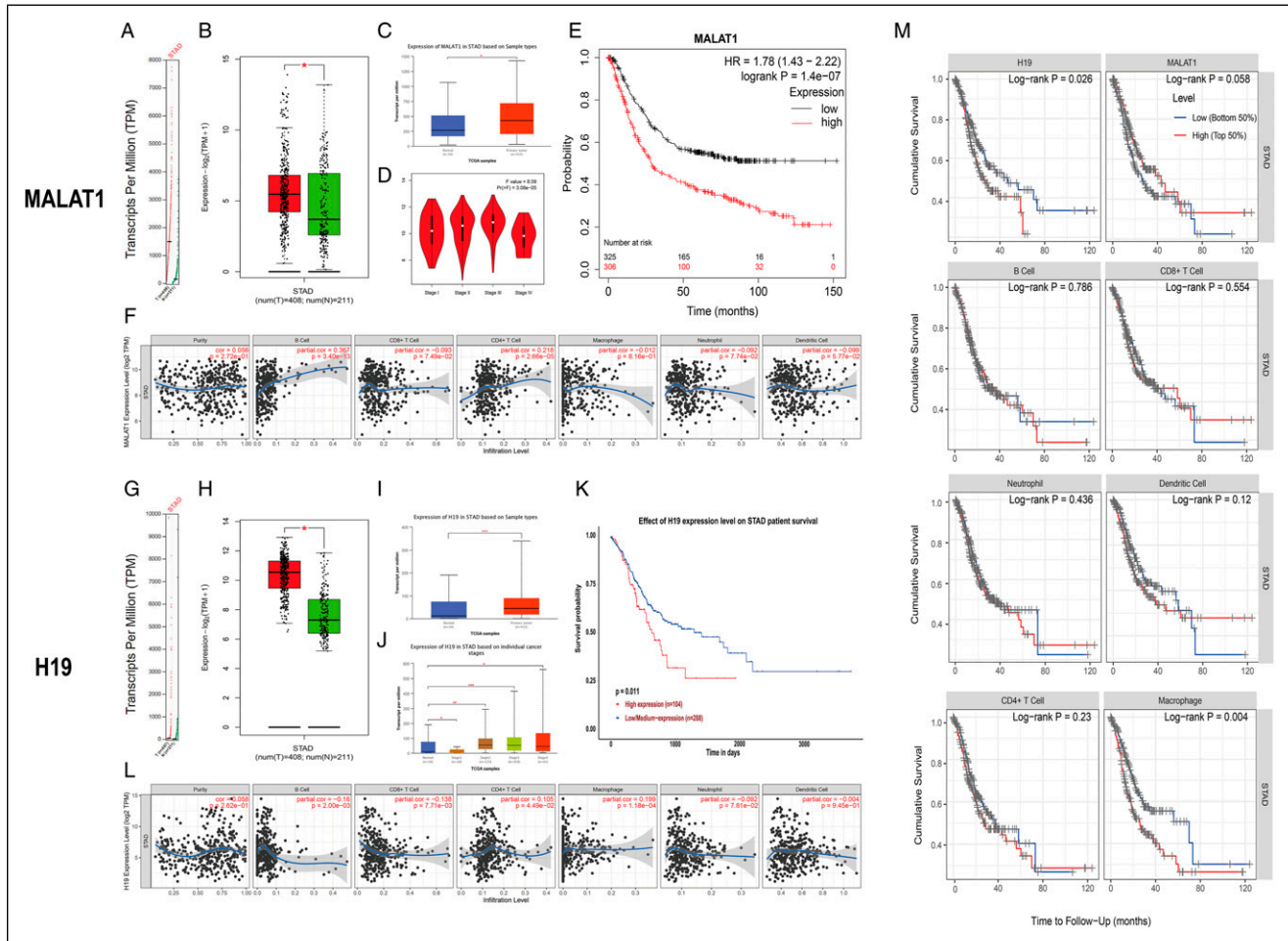
Among GC and HCs in female and male groups, a receiver operating curve (ROC) was implemented to evaluate the predictive value of CpG sites of H19 and MALAT1 promoters as a biomarker for the diagnosis of GC. The area under curve (AUC) was calculated. The first 3 CpG sites with the highest AUC values among female and male groups were combined with age to devise GC's diagnostic nomogram. We used the calibration curves and the Hosmer–Lemeshow test ( $P > .05$ ) to examine the calibration ability of the nomograms. The discriminative ability of the nomograms was evaluated by ROC.<sup>35</sup> We also carried out a decision curve analysis (DCA) to evaluate the nomograms' clinical values using the R library rmda package; the method for calculating the net benefit of the nomograms was described in detail elsewhere.<sup>36</sup>

## Results

### Bioinformatic Analysis of MALAT1 and H19 in GC

To evaluate the potential diagnostic and prognostic value of MALAT1 and H19 in GC, we assessed the expression levels of these 2 genes in GC tissues and normal tissues in GEPIA and UALCAN. As expected, the transcriptional levels of MALAT1 and H19 in GC tissues were significantly elevated ([Figures 1A-1C and 1G-1I](#); [Figures 1A-1C and 1G-1I](#)). Using GEPIA and UALCAN, we then explored the associations between the expression of variously expressed MALAT1 or H19 and the pathological stages of GC; significant correlations were observed in the databases. In general, GC tumors of more advanced stages expressed higher levels of MALAT1 and H19 ([Figures 1D and 1J](#)). The prognostic values of the expression of MALAT1 and H19 in GC patients were also explored. As shown in [Figures 1E and 1K](#), higher expressions of MALAT1 and H19 were significantly correlated with a shorter survival probability of GC (Kaplan–Meier plotter,  $P = 1.4 \times 10^{-7}$ ; UALCAN database,  $P = .011$ ).

As for the correlation between the immune cell infiltration and the expressions of MALAT1 and H19, we found the expression of MALAT1 was positively correlated with the infiltration of B cells (Cor = .367,  $P = 3.40 \times 10^{-13}$ ) and CD4<sup>+</sup> T cells (Cor = .218,  $P = 2.66 \times 10^{-5}$ ) ([Figure 1F](#)), while the expression of H19 and the infiltration of B cells (Cor =  $-.16$ ,  $P = 2.00 \times 10^{-3}$ ) and CD8<sup>+</sup> T cells (Cor =  $-.138$ ,  $P = 7.71 \times 10^{-3}$ ) ([Figure 1F](#)) were negatively correlated ([Figure 1L](#)). Interestingly, positive correlations between the expression of H19 and the infiltration of CD4<sup>+</sup> T cells (Cor = .105,  $P = 4.49 \times 10^{-2}$ ) and macrophages (Cor = .199,  $P = 1.18 \times 10^{-4}$ ) were also observed ([Figure 1L](#)). In addition, using the TIMER database, we also drew Kaplan–Meier plots for immune



**Figure 1.** Bioinformatic analysis of metastasis-associated lung adenocarcinoma transcript I and H19 in GC. (A-C, G-I) The expression of metastasis-associated lung adenocarcinoma transcript I and H19 in GC tissues and adjacent normal tissues: A and G, scatter diagram (GEPIA); B and H, box plot (GEPIA); C and I, box plot (UALCN). (D, J) Correlation between metastasis-associated lung adenocarcinoma transcript I and H19 expression and tumor stages in GC patients (D, GEPIA; J, UALCA). (E, K) Prognostic value of metastasis-associated lung adenocarcinoma transcript I and H19 expression in GC (E, Kaplan-Meier plotter; K, UALCA). (F, L) Correlation between metastasis-associated lung adenocarcinoma transcript I and H19 expression and immune cell infiltration of GC (TIMER). (M) Survival differences for immune infiltrates and the expression of H19 and MALAT1 (TIMER). GC: gastric cancer.

infiltrates and the expression of H19 and MALAT1 to visualize the survival differences, which are shown in Figure 1M. We observed that H19 ( $P = .026$ ) expression and macrophage ( $P = .04$ ) were significantly correlated with the clinical outcome of GC patients.

With respect to the correlation between methylation and expression levels of H19 and MALAT1, we observed negative statistical correlations between MALAT1 or H19 gene expression and DNA methylation at numerous probes, such as cg26489875 ( $R = -.407$ ,  $P < .001$ ) in MALAT1 and cg011716026 in H19 ( $R = -.427$ ,  $P < .001$ ) (Figure 2).

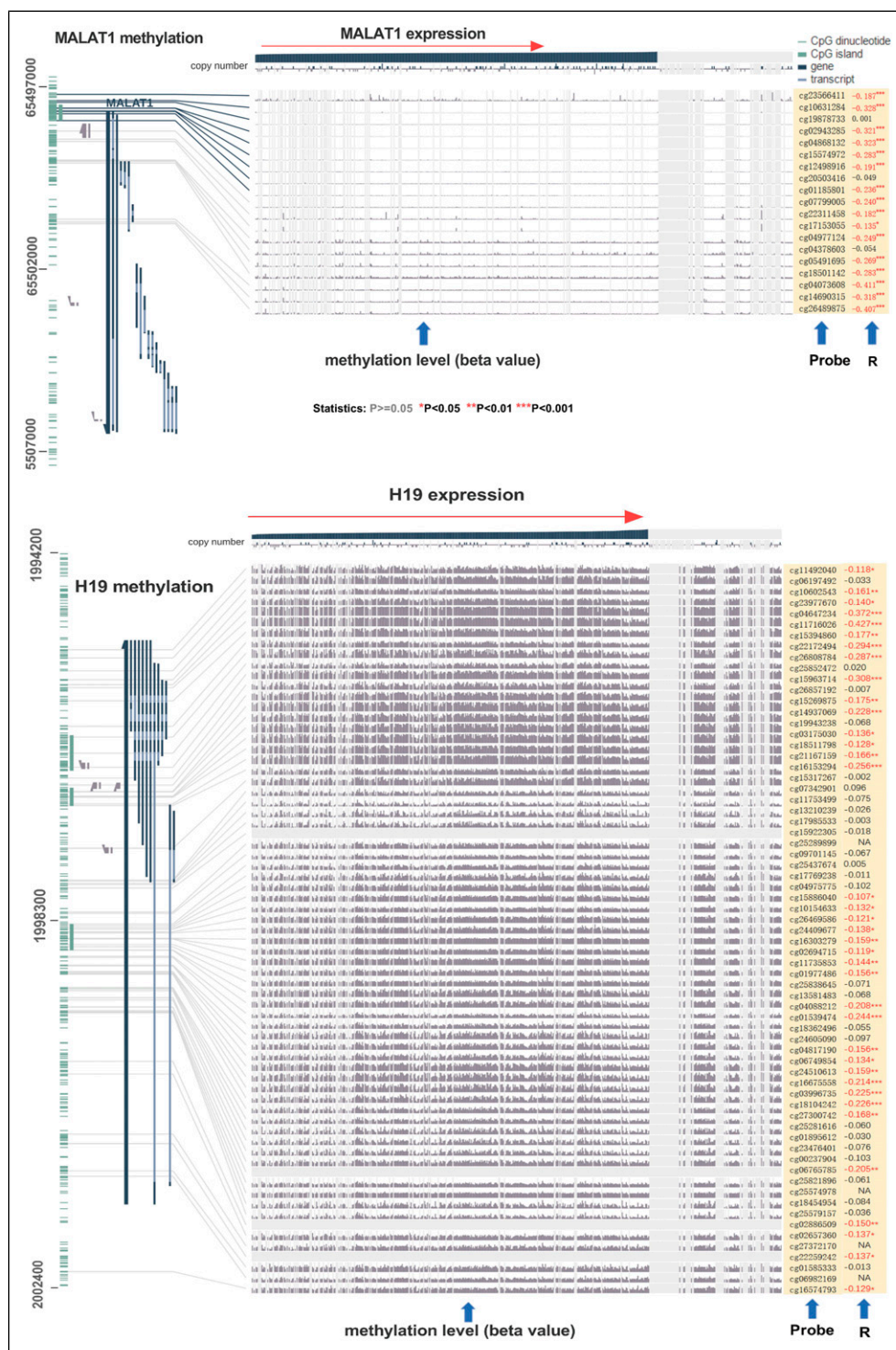
### Characteristics of Participants in the Study

The demographic information (gender, age) of 150 GC patients and 100 controls and the clinical and pathological characteristics of GC patients were presented in our previous study.<sup>37</sup> No significant difference was detected in gender and age between GC patients

and HCs ( $P = .592$ ,  $P = .413$ , respectively). We redivided the GC patients according to the various metastasis status. Among 150 patients, lymph node or visceral metastasis was found in 68 patients (45.33%), 53 patients did not metastasize (35.33%), and 29 patients (19.33%) had both lymph node and visceral metastasis. Body mass index (BMI) and HER-2 status were also included in the current study. Underweight to normal patients ( $BMI < 25$ ) accounted for 74% of all patients, and only 39 patients had tested their HER-2 status (positive: 9 cases, negative: 30 cases).

### Association Between Methylation Levels of H19, MALAT1 Promoters in Peripheral Blood, and GC Risk Among all Participants

A total of 91 CpG sites of H19 and MALAT1 promoters were detected for the differential methylation analysis. Differences in the methylation levels of H19 and MALAT1 promoters



**Figure 2.** Correlation between metastasis-associated lung adenocarcinoma transcript I and H19 DNA methylation and gene expression in MEXPRESS.

between GC and HCs are shown in [Supplementary Table S3](#). The gene/CpG regions/CpG sites with statistical significance (all  $P < .05$ ) are shown in [Table 1](#). Among all the CpG sites, 20 CpG sites in the H19B island, H19C island, H19D island

shore, and MALAT1B island were hypermethylated in GC patients compared with HCs ( $OR > 1$ ,  $P < .05$  for all), while 1 CpG site (MALAT1a5) in MALAT1A island was hypermethylated in HCs conversely ( $OR = .584$ ,  $P = 3.11 \times 10^{-4}$ )

**Table 1.** Association between methylation levels of H19, MALAT1 promoters in peripheral blood and GC risk\* ( $P < .05$ ).

	Name	Methylation level <sup>a</sup>		Logistic regression analysis		
		GC	HCs	Odds ratio	95%CI	P-value
<b>Genes</b>	H19	76.61 (75.71,77.44)	76.10 (75.22,76.98)	1.224	1.029–1.455	<b>.022</b>
<b>Regions</b>	H19C	95.72 (95.38,96.03)	95.36 (94.95,95.72)	3.085	1.868–5.094	<b><math>1.10 \times 10^{-5}</math></b>
	MALAT1B	.75 (.69,0.80)	.72 (.68,0.77)	94.912	3.629–2482.615	<b>.006</b>
	H19b2	97.41 (96.99,97.85)	97.20 (96.85,97.58)	2.000	1.275–3.137	<b>.003</b>
	H19b5	93.68 (92.74,94.30)	93.36 (92.42,94.03)	1.374	1.085–1.740	<b>.008</b>
	H19b6	97.97 (97.62,98.32)	97.87 (97.49,98.09)	1.801	1.069–3.033	<b>.027</b>
	H19b7	92.45 (91.29,93.30)	91.77 (90.48,92.49)	1.496	1.238–1.807	<b><math>3.00 \times 10^{-5}</math></b>
	H19b8	96.22 (95.75,96.67)	95.86 (95.35,96.42)	1.487	1.099–2.012	<b>.010</b>
	H19b9	94.60 (93.77,95.54)	94.18 (92.82,95.04)	1.428	1.185–1.721	<b><math>1.84 \times 10^{-4}</math></b>
	H19b11	90.66 (89.71,91.52)	90.14 (89.28,91.24)	1.203	1.019–1.419	<b>.029</b>
<b>Sites</b>	H19b12	97.13 (96.61,97.48)	96.74 (96.36,97.15)	2.001	1.336–2.998	<b>.001</b>
	H19b14	95.30 (94.67,95.91)	94.93 (94.35,95.46)	1.324	1.058–1.658	<b>.014</b>
	H19c1	93.74 (92.81,94.67)	92.68 (91.9,93.68)	1.503	1.255–1.800	<b><math>9.00 \times 10^{-6}</math></b>
	H19c2	86.69 (84.89,88.78)	85.27 (83.62,87.49)	1.184	1.073–1.306	<b>.001</b>
	H19c3	95.85 (94.94,96.54)	94.96 (93.97,95.9)	1.453	1.201–1.757	<b><math>1.20 \times 10^{-4}</math></b>
	H19c4	95.63 (94.78,96.43)	94.95 (94.02,95.69)	1.399	1.145–1.710	<b>.001</b>
	H19c5	96.34 (95.55,96.94)	95.81 (95.18,96.36)	1.535	1.186–1.987	<b>.001</b>
	H19c8	96.99 (96.50,97.67)	96.57 (96.14,97.05)	1.355	1.069–1.719	<b>.012</b>
	H19c10	96.80 (96.22,97.44)	96.61 (96.04,97.09)	1.300	1.001–1.688	<b>.049</b>
	H19d6	82.48 (78.51,87.10)	80.37 (76.76,84.83)	1.049	1.007–1.094	<b>.022</b>
	H19d8	70.66 (65.50,76.87)	68.63 (63.94,73.20)	1.037	1.004–1.072	<b>.029</b>
	H19d9	76.82 (71.17,82.47)	74.51 (70.21,79.43)	1.040	1.004–1.078	<b>.031</b>
	MALAT1a5	4.94 (4.46,5.51)	5.45 (4.82,6.06)	.584	.436–.782	<b><math>3.11 \times 10^{-4}</math></b>
	MALAT1b9	.76 (.54,0.92)	.66 (.52,0.82)	3.210	1.250–8.244	<b>.015</b>

\*Adjusted for age and gender.

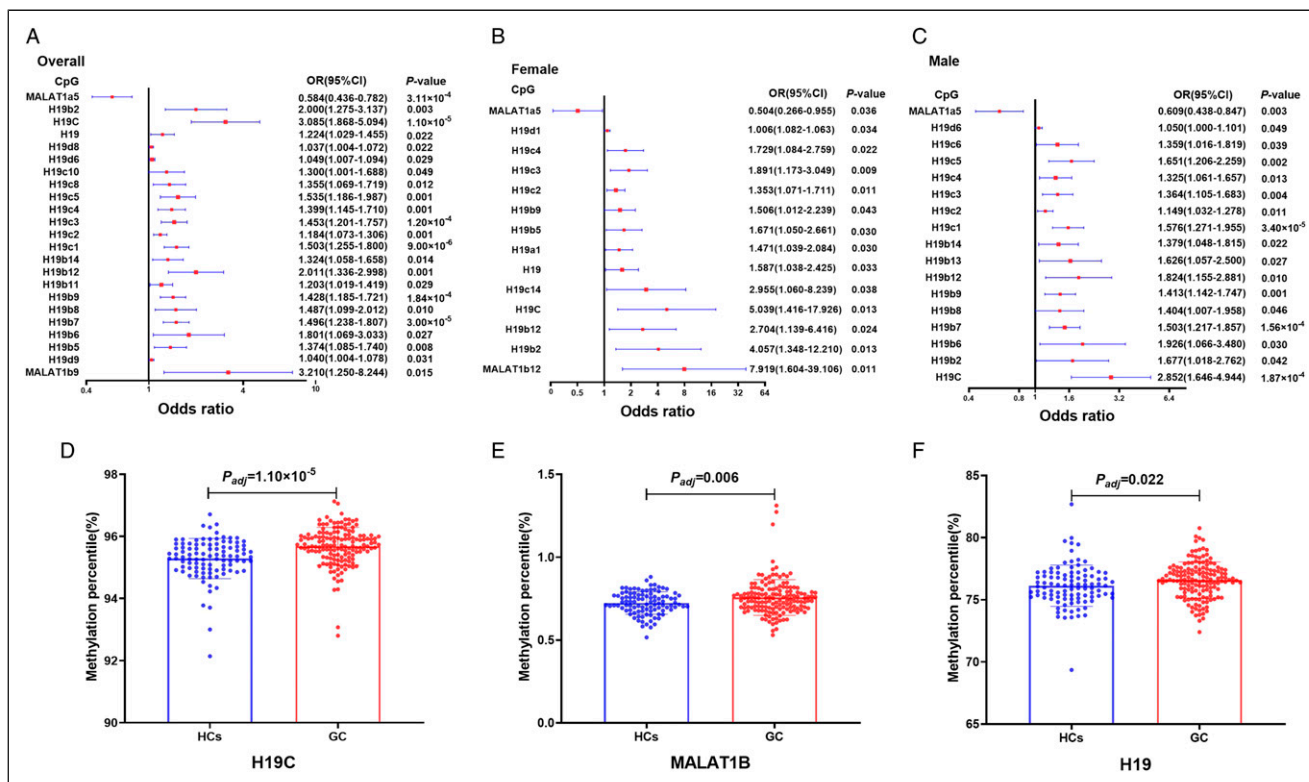
<sup>a</sup>Methylation level is expressed as a percentage. Data was expressed as median ( $P_{25}$ ,  $P_{75}$ ). GC: gastric cancer, HCs: health controls, MALAT1: metastasis-associated lung adenocarcinoma transcript 1.

(Figure 3A, Table 1). To further explore the methylation situations of H19 and MALAT1 in GC patients, the methylation levels of the CpG region (CGI or CGI shore) were calculated by averaging the methylation levels of all nearby CpG sites of H19 and MALAT1 promoters (since the range of CI of MALAT1B island was too broad, we did not include this site in Figure 3A. The CI values of MALAT1B island can be found in Table 1). Similarly, the hypermethylation of H19 C island and MALAT1B island was observed in the GC group (Mann–Whitney  $U$  test:  $P = 1.10 \times 10^{-5}$ ,  $P = .006$ , respectively) (Figures 3D and 3E, Table 1). Using the same way, we calculated the overall methylation level of H19 and MALAT1 genes by averaging the methylation levels of all nearby CpG regions of H19 and MALAT1, and only hypermethylation of the H19 gene in the GC group was observed (Mann–Whitney  $U$  test:  $P = .022$ ) (Figure 3F, Table 1).

#### Association Between Methylation Levels of H19, MALAT1 Promoters in Peripheral Blood, and GC Risk in Female and Male Groups

Gender variations in the occurrence and mortality of GC had been reported, with larger, higher stage, and worse OS of GC occurring in the male group.<sup>38</sup> We thus performed subgroup

analysis to evaluate the differences of H19 or MALAT1 methylation levels between GC and HCs in female and male groups. In the female group, 1 CpG site in the H19A island, 4 CpG sites in the H19B island, 4 CpG sites in the H19C island, 1 CpG site in the H19D island shore, and 1 CpG site in the MALAT1B island were hypermethylated in GC patients compared with that in HCs ( $OR > 1$ ,  $P < .05$  for all) (Figure 3B, Supplementary Table S4). Interestingly, hypermethylation of MALAT1a5 was also observed in the HCs group ( $OR = .504$ ,  $P = .036$ ). In the male group, similar results were observed compared with those in the female group. Hypermethylation of 8 CpG sites in the H19B island, 6 CpG sites in the H19C island, 1 CpG site in the H19D island shore, and 1 CpG site in the MALAT1B island were found in GC ( $OR > 1$ ,  $P < .05$  for all), while MALAT1a5 in the MALAT1A island was hypomethylated ( $OR = .609$ ,  $P = .003$ ) (Figure 3C, Supplementary Table S5). Identically, overall methylation levels of H19 and MALAT1 CpG regions and genes were calculated. We found hypermethylation of H19C island, MALAT1B island, and H19 gene in GC patients in the female group. While in the male group, such hypermethylation was observed in the H19C island ( $OR > 1$ ,  $P < .05$  for all) (since the range of CI of MALAT1B island and MALAT1b5 site was too wide, we did not include them in Figure 3. The CI values of



**Figure 3.** Correlation analysis of H19 and metastasis-associated lung adenocarcinoma transcript I methylation of peripheral blood between GC patients and HCs. (A-C) Methylation of H19 and metastasis-associated lung adenocarcinoma transcript I (including gene, CpG regions, and CpG sites) in the whole group of participants, in the female group, and in the male group. The squares and horizontal lines correspond to the odds ratio (OR) and 95% confidence interval (CI), respectively. (D-F) Comparisons of the methylation levels of H19 gene, H19C island, and MALAT1B island between GC patients and HCs. GC: gastric cancer, HCs: healthy controls.

MALAT1B island and MALAT1b5 site can be found in [Supplementary Tables S4 and S5](#) ([Figures 3B and 3C](#), [Supplementary Tables S4 and S5](#)).

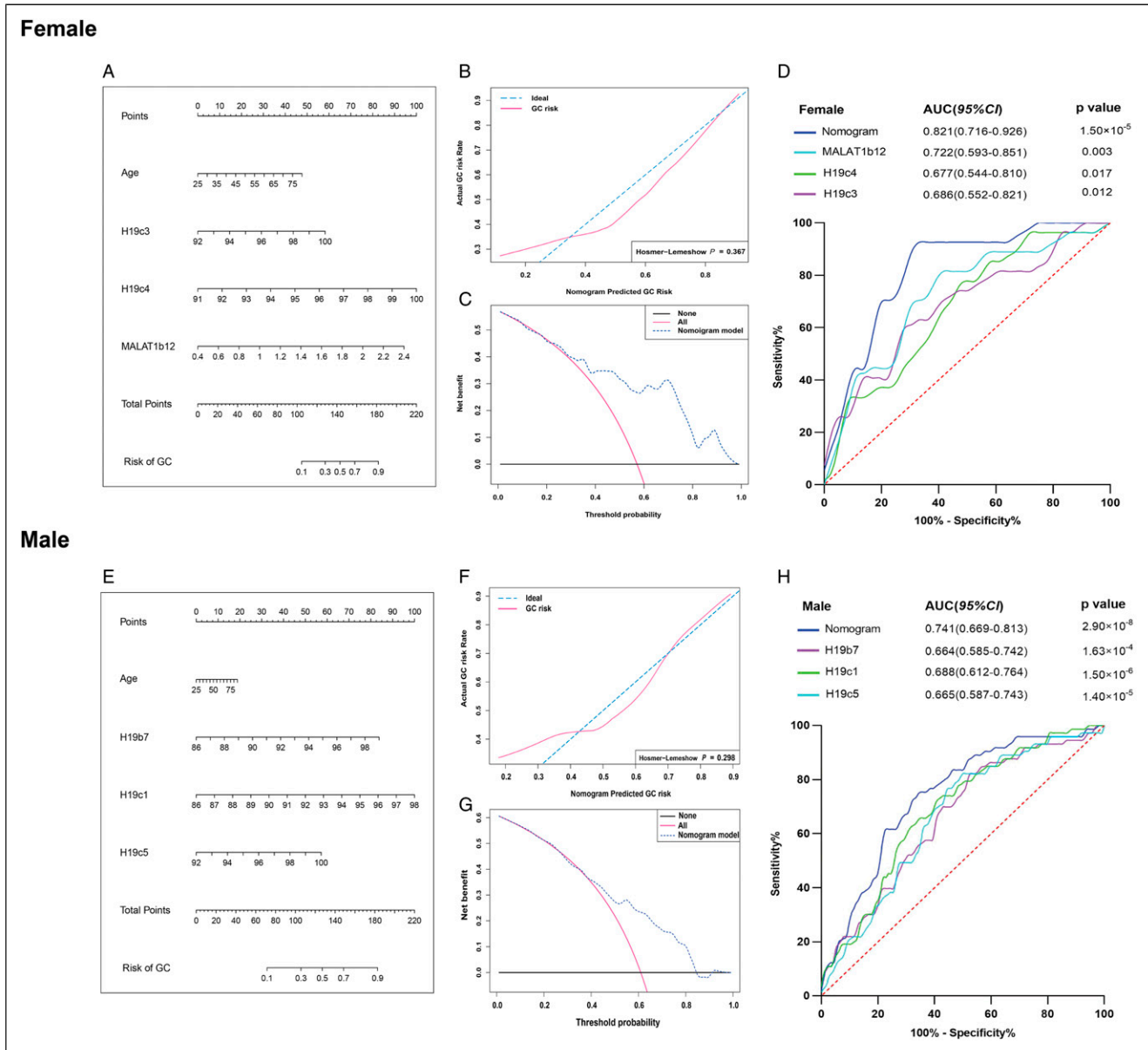
### Differences of Methylation Levels of H19, MALAT1 Promoters in Peripheral Blood Between GC Patients With Different Clinical Characteristics

To explore the association between H19 or MALAT1 methylation in GC with different clinical characteristics, GC patients were divided into the following groups: poorly differentiated group and well/moderately differentiated group; stage I–III group and stage IV group; patients without metastasis and patients with metastasis (including patients with lymph node or visceral metastases and patients with both lymph node and visceral metastases); underweight to normal group (BMI < 25) and overweight/obese group (BMI ≥ 25); upper third (tumor site) group, middle third group, lower third group, and diffused type group; patients with age ≤ mean group and patients with age > mean group; patients with HER-2 negative group and positive group (patients who had not tested their HER-2 status were not included). Significant differences were observed for the methylation levels of H19

and MALAT1 between patients with various differentiations, TNM stages, with and without metastasis, BMI levels, tumor sites, and age groups ([Supplementary Tables S6–11](#)), while no significant difference was observed for the methylation levels of H19 and MALAT1 between patients with HER-2 negative and positive ([Supplementary Table S12](#)).

### Diagnostic Nomograms and Model Performance of GC in Female and Male Groups

To examine the potential diagnostic value of H19 and MALAT1 methylation as non-invasive biomarkers for GC, ROC curve analysis was performed. Taking age and the first 3 CpG sites with the highest AUC values among the female (H19c3, H19c4, MALAT1b12) and male groups (H19b1, H19c1, H19c5) into consideration, we generated 2 diagnostic nomograms ([Figures 4A and 4E](#)). Based on the sum of the nomograms, the higher the total points, the higher the GC risk. The calibration curve of the nomograms displayed accurate predictive ability for the diagnosis of GC both in the female and male groups, and no significant differences exist between the predictive GC risk calibration curve and the ideal curve for GC from the Hosmer–Lemeshow test ( $P = .367$ ,  $P = .298$ ,



**Figure 4.** The logistic regression-based nomogram model for predicting the risk of GC in the female and male groups. The methylation levels of H19c3, H19c4, MALAT1b12, and age were used to construct the nomogram for predicting the risk of GC in female group (A-D), while the methylation levels of H19b7, H19c1, and H19c5 were used to construct the nomogram for predicting the risk of GC in male group (E-H). (A, D) The construction of the nomogram. The value of each variable was given a score on the point scale axis. A total score could be calculated by adding each single score, and by projecting the total score to the lower total point scale, we were able to estimate the probability of GC in female and male groups. (B, E) The calibration curve and the Hosmer–Lemeshow (H-L) test of the nomogram. The calibration curve with 1000 bootstrap resampling was made for an assessment of the nomogram-predicted probabilities of GC in female and male groups. The H-L test was used to examine how well the percentage of the actual probability of the GC matched the percentage of the nomogram-predicted probability of GC over deciles of predicted risk in the female and male groups. (C, F) Decision curve analysis of the nomogram. The decision curve analysis was performed to compare the standardized net benefit of the nomogram at the different threshold probabilities. The ranges of the standardized net benefit of the nomogram were very wide and practical. (D, H) Receiver operating characteristic curve. The area under receiver (AUC) operating characteristic curve values for the constructed nomogram of GC in female and male groups was .821 and .741, respectively. GC: gastric cancer.

respectively) (Figures 4B and 4F). The clinical values of the nomograms were evaluated by DCA; the results demonstrated wide ranges of the threshold and probabilities of the nomograms in the diagnosis of GC in both female (Figure 4C) and

male (Figure 4G) groups. The AUC of the nomogram was .821 (95% CI: .716 - .926,  $P = 1.50 \times 10^{-5}$ ), with the sensitivity and specificity as 69.4% and 92.6% in the female group and .741 (95% CI: .669 - .813,  $P = 2.90 \times 10^{-8}$ ), with



the sensitivity and specificity as 65.8% and 75.3% in the male group, respectively (Figures 4D and 4H, Supplementary Table S13). We found the nomograms generated in the female and male groups had higher AUC, indicating more potential in GC diagnosis than that of each CpG site alone. The AUC of MALAT1b12, H19c4, H19c3 in the female group were .722, .677, .686, respectively, while AUC of H19b7, H19c1, H19c5 in the male group were .664, .688, .665, respectively (Figures 4D and 4H).

## Discussion

Previous studies have shown that aberrant DNA methylation plays an important role in the occurrence and development of GC; however, most of these studies conducted their research on tumor tissues.<sup>10-13</sup> Non-invasive diagnostic biomarkers for GC are urgently needed. In the current study, we found that the expressions of MALAT1 and H19 were associated with the tumorigenic and prognostic of GC and may also regulate the immune cell infiltration in GC. Moreover, H19 and MALAT1 expression were found significantly negatively correlated with their methylation levels, which indicates a significant role of the DNA methylation of H19 and MALAT1 in GC's occurrence and development. Our case-control study results show significant differences in the peripheral blood-based DNA methylation levels of MALAT1 and H19 between GC patients and HCs, and such methylation is also associated with the characteristics of GC. Meaningful sensitivity and specificity of MALAT1 and H19 methylation in discriminating GC and HCs were observed in both female and male groups.

The alteration of DNA methylation may activate oncogenes, suppress suppressor genes, and induce chromosome instabilities, which initiates tumorigenesis and stimulates cancer development.<sup>39</sup> During cancer progression, an important mechanism for controlling gene expression is regulating DNA methylation by lncRNAs.<sup>18</sup> Accumulating evidence has shown that DNA methylation in peripheral blood can be used as biomarkers for early diagnosis of several tumor types, such as malignant pleural mesothelioma, breast cancer, and GC.<sup>40-42</sup>

Abnormal expression of the MALAT1 oncogene was first reported in metastatic non-small cell lung carcinoma.<sup>19</sup> Higher MALAT1 expression was also observed in liver, breast, pancreatic, colorectal, and prostate cancer.<sup>43-45</sup> Consistent with our results, a similar higher expression of MALAT1 in gastric carcinoma tissues and cell lines was previously reported.<sup>18</sup> Downregulated expression of UPF1, negatively correlated with MALAT1, was found in GC, and the downregulation may be due to the hypermethylation in the promoter region.<sup>46</sup> Like MALAT1, aberrant expression of H19 was detected in various mammalian tumors,<sup>47,48</sup> and the upregulated expression of H19 in GC is positively correlated with the worse clinical outcome of GC patients.<sup>23</sup> As an imprinted gene, H19 exhibits a dynamic expression pattern during human embryonic development, and the methylation levels of

the imprinted control regions (ICRs) of the H19 promoter are considered the main regulator of its expression during mammalian development.<sup>49,50</sup> Therefore, it is reasonable to speculate that the higher methylation of MALAT1 and H19 promoters in GC and the associations of their methylation with GC's clinical characteristics (eg, tumor differentiation, TNM stages) may associate with their aberrant expressions. In the current study, we found the H19 gene, H19 C island, and MALAT1B island, as well as 20 CpG sites in the H19B island, H19C island, H19D island shore, and MALAT1B island were hypermethylated in GC patients compared with HCs; similar results were also found in the female and the male groups. Moreover, evidence from nomogram analysis suggests that combined detection of multiple CpG sites of MALAT1 and H19 genes in peripheral blood are helpful for diagnosing GC. Particularly in the female group, the combination of H19c3, H19c4, MALAT1b12, and age could be used to predict GC's occurrence, with high sensitivity and specificity, which suggests the peripheral blood-based DNA methylation of H19 and MALAT1 have the potential to serve as non-invasive biomarkers for GC diagnosis.

Previous studies reported that one of the crucial determinant factors that influence immunotherapy and clinical prognosis of cancer is immune cell infiltration, which is associated with tumor recurrence and progression.<sup>51</sup> In the current study, we observed significant correlations between H19 or MALAT1 expression and macrophages, B cells, CD8<sup>+</sup> T cells, and CD4<sup>+</sup> T cells infiltration, which implies that the expression of H19 and MALAT1 may also affect the immune status in GC. As one of the crucial forms of epigenetic modifications, DNA methylation is tightly involved in regulating the development, function, and differentiation of the immune system.<sup>52</sup> Recently, a divergent application of DNA methylation between immune cells was detected by investigators.<sup>53</sup> Hence, we hypothesize that the methylation of H19 and MALAT1 promoter in peripheral blood may be associated not only with H19 and MALAT1 expression but also with immune cell infiltration of GC. However, further experiments in vivo and in vitro are needed to clarify the correlation between the methylation and expression of H19 and MALAT1 and GC's immune cell infiltration.

## Strengths and Limitations

This is the first study utilizing the quantitative method to detect the methylation levels of H19 and MALAT1 in peripheral blood, which covered all CpG sites of all CpG regions, and the diagnostic value of each CpG site and regions could thereby be evaluated. However, there are several potential limitations needed to be recognized. First, we did not detect the expressions of H19 and MALAT1 in the involved participants, and the immune status of the GC patients was not examined. Second, we did not perform sample size estimations, despite the great potential of H19 and MALAT1 DNA methylation in peripheral blood to be non-invasive biomarkers

for GC diagnosis; further larger-multicenter systematic, unbiased prospective studies are required to validate our observed results and develop a novel feasible peripheral blood-based assay for clinical application.

## Conclusion

In the current study, we found a hyperexpression of H19 and MALAT1 in GC tissues, and such expression is correlated with DNA methylation and immune cell infiltration of GC. Moreover, we discovered that H19 and MALAT1 are significantly hypermethylated in the peripheral blood of GC patients, and the combination of multiple CpG sites of H19 and MALAT1 promoters could be used to distinguish GC patients from HCs, especially in the female group. This suggests that the peripheral blood-based DNA methylation of H19 and MALAT1 could act as potential non-invasive biomarkers for GC diagnosis.

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## Author Contributions

Fang Wang conceived the study idea. Dingtao Hu collected the data. Xiaoqi Lou, Nana Meng, Zhen Li, Ying Teng, Yanfeng Zou, and Fang Wang contributed to the analysis of the data. Dingtao Hu, and Xiaoqi Lou wrote the initial draft with all authors providing critical feedback and edits to subsequent revisions. All authors approved the final draft of the article. All authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Fang Wang is the guarantor. The corresponding author attests that all listed authors meet authorship criteria and that no others meeting the criteria have been omitted.

## Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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## Ethical Statement

Our study was approved by The Ethics Committee of Anhui Medical University (approval no. PJ2019-01-12). All patients provided written informed consent prior to enrollment in the study.

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## Supplemental Material

Supplemental material for this article is available online.

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