IL10 hypomethylation is associated with the risk of gastric cancer

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Abstract. Interleukin-10 (IL10), a pleiotropic cytokine secreted by type-2 helper (Th2) T cells, contributes to the oncogenic activation or inactivation of tumor-suppressor genes. The present study investigated whether hypomethylation of *IL10* CpG island (CGI) was associated with the risk of developing gastric cancer (GC) and the prognosis of patients with GC. A fragment (hg18, chr1: 206945638-206945774) at the CGI of IL10 was selected for the present methylation assay. Quantitative methylation-specific PCR was used to evaluate the methylation of *IL10* CGI in 117 tumor samples from patients with GC. The results demonstrated that *IL10* CGI methylation was significantly lower in the tumor tissues compared with that in the paired adjacent non-tumor tissues (median percentage of methylated reference, 29.16 vs. 42.82%, respectively; $P=4x10^{-8}$). Furthermore, results from receiver operating characteristic curve analysis identified a significant area under the curve of 0.706, with a sensitivity and a specificity of 77.8 and 58.1%, respectively, between cancer tissues and paired adjacent non-tumor tissues. Furthermore, the methylation of *IL10* CGI was significantly associated with patients' age at diagnosis (r=-0.201; P=0.03). Subgroup analyses demonstrated that the association between *IL10* CGI hypomethylation and the risk of GC was specific for patients with low differentiation ($P=1x10^{-7}$) and Borrmann types III+IV ($P=1x10^{-7}$). In addition, *IL10* CGI hypomethylation was significantly associated with the risk of GC for patients without smoking history ($P=3x10^{-7}$) or a family history of cancer ($P=2x10^{-7}$). The results from Kaplan-Meier survival analysis demonstrated that *IL10* CGI hypomethylation was associated with a significantly shorter overall survival of patients with GC (P=0.041). Similar results were identified for patients with GC who did not have smoking history (P=0.037) or a family history of cancer (P=0.049). The results from this study demonstrated that *IL10* CGI hypomethylation may be considered as a potential biomarker for the diagnosis and prognosis of patients with GC in the Chinese population.

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Introduction

Although the incidence of gastric cancer (GC) is declining, GC caused ~1 million new cases and 781,000 deaths in 2018 and remains the third leading cause of death in the world (1). Numerous types of cancer, including GC have a background of chronic inflammatory processes caused by infection or exposure to environmental factors (2). Understanding the molecular mechanisms of inflammation during GC is therefore crucial for the development of novel therapeutic strategies against GC (1). *H. pylori* infects >50% of the world population and may affect gastric physiology, via triggering gastritis and canceration processes (3). Treatment of *H. pylori* infection may therefore help prevent GC in the general population (4,5); however, this treatment appears to be unrelated to the prognosis of patients with early GC (5,6). Gastric biopsy of patients with gastrointestinal-related symptoms demonstrated that in addition to the common *H. pylori*-related gastritis, several other modes of mucosal damage are also increasing (7).

Interleukin-10 (IL10) is one of the most important anti-inflammatory cytokines (8). IL10 is associated with oncogenic activation or inactivation of tumor-suppressor genes (9). High levels of circulating IL10 were identified in

patients with digestive cancers (10-12). In addition, the persistence of circulating IL10 in patients with colorectal cancer following surgery could predict a high tendency to relapse in these patients (13). High levels of circulating IL10 were also reported to be associated with an unfavorable prognosis for patients with GC (14,15). In addition, a meta-analysis demonstrated that a high level of circulating IL10 resulted in poor survival in patients with various types of cancer (16).

IL10 tends to be hypomethylated in several cancer types (17), including pancreatic (18), breast (19) and cervical cancer (20), as well as chronic lymphocytic leukemia (12). TET-2 catalyzes the conversion of 5-methylcytosine to 5-hydroxymethylcytosine (21), which is an important intermediate in the process of active DNA demethylation in primary human monocytes (22). The expression of IL10 and TET-2 was found to increase in Vicenin-2 treatment (23), which indicated that IL10 expression is increased during active DNA demethylation. Demethylation with 5'-AZA-deoxycytidine (5-AZA) results in an upregulation of *IL10* expression in $CD4^+$ T cells, and CpG-DNA methylation of the DNA methyltransferase 3α gene was demonstrated to silence *IL10* transcription in macrophages, indicating the crucial role of CpG methylation in the regulation of *IL10* expression (24-26).

According to these previous findings, the present study determined the association between *IL10* CpG island (CGI) methylation and the risk of GC. This study aimed to evaluate the diagnostic and prognostic value of *IL10* CGI methylation for Chinese patients with GC.

Materials and methods

Subjects. A total of 117 patients with GC (mean age, 56.40 years; age range, 21-83 years) treated at the Taihu Hospital of Wuxi, China, between January 2008 and February 2015 were included in the present study. Gastric tumor and adjacent non-tumor tissues (5 cm from the tumor) were collected. Patients were diagnosed by pathologists' histological diagnosis, and none had received radiation or chemotherapy prior to sample collection. The time between primary surgery and the death of the patient or the last follow-up was defined as the overall survival (OS) (27). This study was approved by the Ethics Committee of Taihu Hospital of Wuxi (approval number: 20160026) (Wuxi, China). All patients provided written informed consent.

Sanger sequencing, capillary electrophoresis and DNA methylation assay. Genomic DNA extraction (QIAamp DNA mini kit; Qiagen GmbH) and subsequent bisulfite conversion (EpiTech Bisulfite kits; Qiagen Benelux B.V.) were performed as previously described (28,29). The program of qMSP was as follows: Pre-denaturation at 95˚C for 10 min; 45 cycles of denaturation at 95˚C for 20 sec, annealing at 58˚C for 20 sec and extension at 72˚C for 30 sec. Sanger sequencing (BGI Biotech Co., Ltd) was used to determine randomly selected sodium bisulfite-modified DNA sequences, as previously described (30). A fully automated high-resolution capillary electrophoresis apparatus (Qsep100; BIOptic Inc.) was used to conduct the fragment size of the quantitative methylation-specific PCR (qMSP) product. This fragment size was compared with the theoretical fragment length to verify the uniqueness of *IL10*. The methylation level of *IL10* CGI was assessed by qMSP as previously described (12,31). The qMSP primer sequences of *IL10* were as follows: Forward 5'-AAG GTATTTCGGAGATTTC-3' and reverse 5'-AACTCAACA CTACTCTATTAC-3'. The qMSP primer sequences of actin β *(ACTB)* were as follows: Forward 5'-TGGTGATGGAGGAGG TTTAGTAAGT-3' and reverse 5'-AACCAATAAAACCTA CTCCTCCCTTAA-3'. The percentage of methylated reference (PMR) was applied to quantitatively represent the methylation level (28). The PMR in each sample was calculated by the $2^{-\Delta\Delta Cq}$ quantification method to represent the methylation level of *IL10* CGI, and the ΔΔCq equation was as follows: $\Delta\Delta Cq$=sample DNA (Cq_{IL10} -$Cq_{ACTB\ control}$)-fully methylated DNA (Cq_{IL10}-$Cq_{ACTB\ control}$) (32).

Data mining study. To evaluate the association between mRNA expression and *IL10* methylation, the gene expression and methylation data from 371 stomach adenocarcinoma samples were collected from the stomach adenocarcinoma dataset (TCGA, PanCancer Altas) (33) through cBioPortal (http://www.cbioportal.org/). Furthermore, the data of the relative mRNA expression of *IL10* in 4 oral squamous cell carcinoma cell lines (OC3, SAS, SCC and HSC3) before and after treatment with 10 μm 5-AZA for 4 days was retrieved from the Gene Expression Omnibus (GEO) database (GSE38823; https://www.ncbi.nlm.nih.gov/geo).

Statistical analysis. Wilcoxon rank sum test was used to compare the methylation level of *IL10* CGI obtained from Chinese cohort and TCGA cohort in tumor tissues with adjacent non-tumor tissues. A Spearman correlation test was used to evaluate the correlation between *IL10* methylation and patients' age, and between *IL10* methylation and *IL10* expression from TCGA dataset. A χ^2 test was used to investigate whether IL10 CGI methylation of cancer tissues was associated with other clinical parameters. Independent t-test was used to compare mRNA expression of *IL10* in 4 carcinoma cell lines before and after treatment with 10 μm 5-AZA for 4 days. Receiver operating characteristic (ROC) analysis was used to determine the diagnostic value of *IL10* CGI methylation in GC. With the mean methylation value (*PMR*=32.0%) as the cutoff value, the OS of TCGA patients with gastric adenocarcinoma was divided into 2 groups according to the *IL10* CGI methylation status. Subsequently, the Kaplan-Meier method and log-rank test was used to test whether *IL10* CGI methylation status could affect patients' OS. Two-sided $P<0.05$ was considered to indicate a statistically significant difference.

Results

DNA methylation analysis. As shown in Fig. 1A, the amplified fragment is located on chr11:206945637-206945773 based on the genomic region from the University of California Santa Cruz genome browser according to Human 2013 (GRCh38/hg38) assembly (34). The methylation of CpG sites on the amplification fragment of *IL10* CGI was measured (Fig. 1A). The results from capillary electrophoresis demonstrated that the qMSP product was unique and its length was 137 bp, as expected (Fig. 1B). In addition, the qMSP product was sequenced with the expected bisulphite-converted sequence according to the Sanger sequencing result (Fig. 1C).

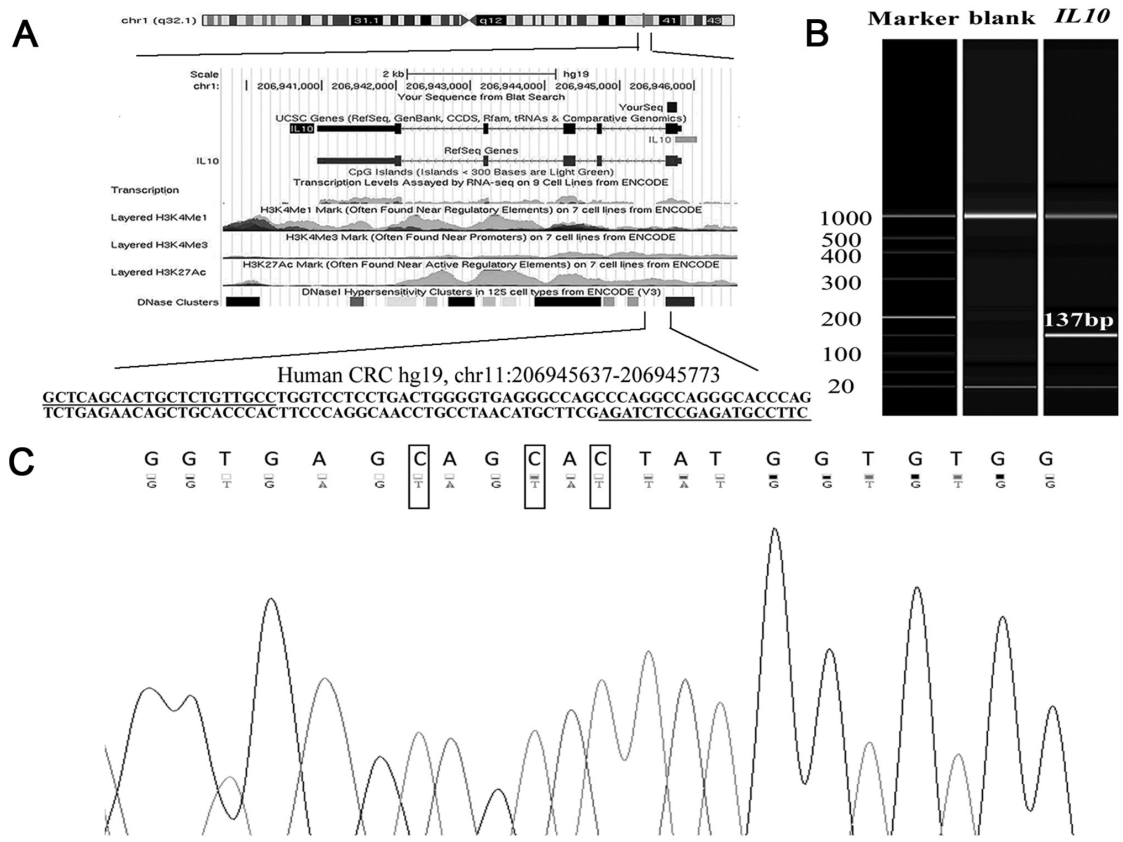


Figure 1. Target sequence on *IL10* promoter region. (A) Genomic position and functional annotation of the amplified fragment. The primers for the quantitative methylation-specific PCR were underlined, and five CpG sites were identified (in grey). (B) Capillary electrophoresis of the amplified fragment (137 bp). (C) Sanger sequencing results. Top row of the sequence represents the original sequence of the fragment. Second row of the sequence represents the converted sequences. C nucleobase with corresponding converted T were in black boxes. A, adenine; C, cytosine; F, forward; G, guanine; IL10, interleukin 10; R, reverse; T, thymine.

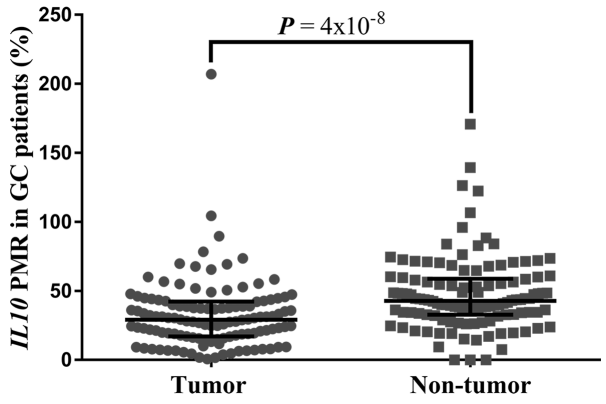


Figure 2. Comparisons of *IL10* CpG island methylation between tumor tissues and paired adjacent normal tissues. IL10, interleukin 10, PMR, percentage of methylated reference. GC, gastric cancer.

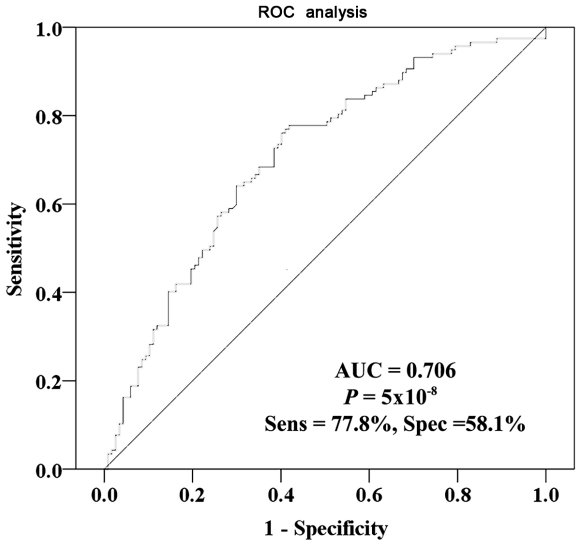


Figure 3. Diagnostic value of *IL10* CGI hypomethylation between gastric cancer tissues and paired para-tumor tissues. ROC curve analysis confirmed the potential diagnostic value of *IL10* CGI hypomethylation, and yielded a significant AUC of 0.706 with a sensitivity of 77.8% and a specificity of 58.1% between cancer tissues and para-tumor tissues. AUC, area under the curve; CGI, CpG island; IL10, interleukin 10; ROC, receiver operating characteristic; Sens, sensitivity; Spe, specificity.

Association between IL10 CGI hypomethylation and the risk of GC and patients' clinicopathological characteristics. The results demonstrated that *IL10* CGI methylation was significantly lower in tumor tissues compared with that in adjacent non-tumor tissues (median PMR, 29.16 vs. 42.82%; P=4x10^{-8}; Fig. 2). Furthermore, results from ROC curve analysis confirmed that *IL10* CGI hypomethylation may be a potential marker of GC diagnosis. In particular, a significant AUC of 0.706 with a sensitivity of 77.8% and a specificity of 58.1% was identified between cancer tissues and para-tumor tissues (Fig. 3). In addition, *IL10* CGI methylation in tumor tissues was significantly associated with patient's age at

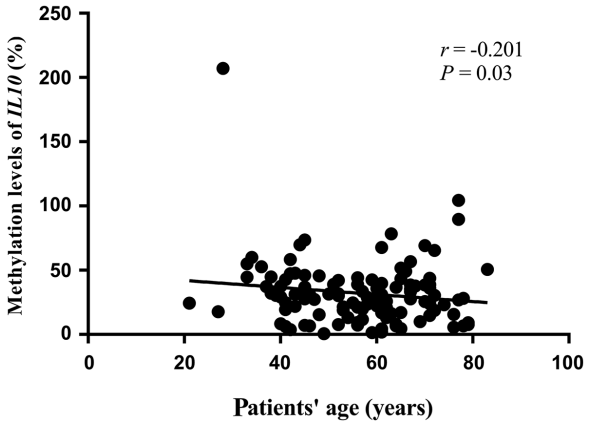


Figure 4. Association of *IL10* CGI methylation with age of patients with GC. An inverse correlation was found between *IL10* methylation in tumor and age of patients with GC. The P-value was calculated by Spearman correlation test. GC, gastric cancer.

diagnosis (r=-0.201; P=0.03; Fig. 4). Using the average *IL10* CGI methylation level as the cutoff value, 117 patients were divided into either *IL10* CGI hypermethylated patients (n=48) or *IL10* CGI hypomethylated patients (n=69). Next, c^2 test was used to investigate whether IL10 CGI methylation of cancer tissues was associated with other clinical parameters. It was found that *IL10* CGI methylation was not associated with other parameters, including gender, surgical procedures, differentiation, lymph node metastasis, TNM stage, Borrmann type, disease recurrence, nerve invasion, family history of cancer, drinking history or smoking history (P>0.05; Table SI).

Stratified analyses by clinical phenotypes. Stratified analyses demonstrated that the association between *IL10* CGI hypomethylation and the risk of GC was specific for patients with low differentiation (P=1x10^{-7}; Fig. 5) and III+IV Borrmann types (P=1x10^{-7}; Fig. 5), but not in high and medium differentiations (P=0.12) or in I+II Borrmann types (P=0.35). In addition, *IL10* CGI hypomethylation was significantly associated with the risk of GC for patients without smoking history (P=3x10^{-7}; Fig. 5) or a family history of cancer (P=2x10^{-7}; Fig. 5).

IL10 CGI hypomethylation is correlated with poor prognosis in patients with GC. OS analysis was performed among the 117 patients. As presented in Fig. 6A, the 5-year OS rate and the median survival time were 25.0% and 36.0 months for the these patients, respectively (Fig. 6A). By using the average *IL10* CGI methylation level as a cut-off value (*PMR*=32.0%), the 117 patients were divided into patients with *IL10* CGI hypermethylation (n=48) or patients with *IL10* CGI hypomethylation (n=69). The results from Kaplan-Meier survival analysis demonstrated that *IL10* CGI hypomethylation was associated with a significantly shorter OS of patients with GC (P=0.041; Fig. 6B). Furthermore, Kaplan-Meier survival analysis of patients with GC and no and low differentiation demonstrated that *IL10* CGI hypomethylation was associated with a significantly worse

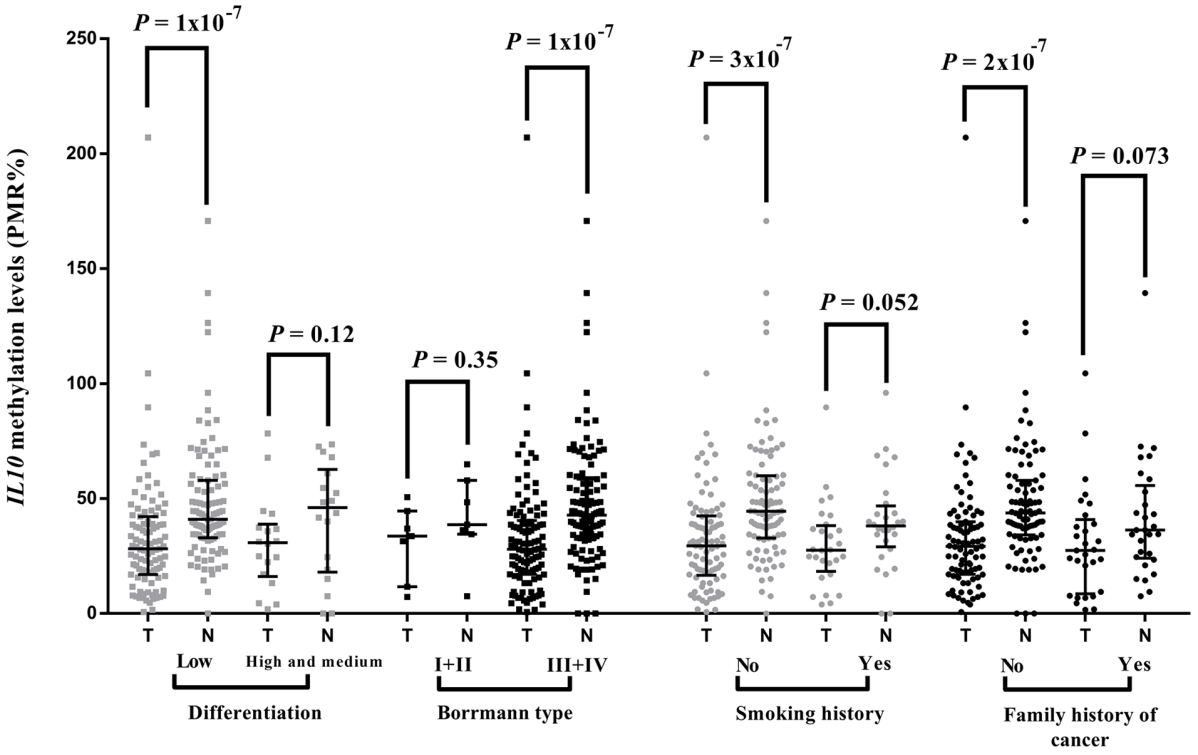


Figure 5. Stratified comparisons of *IL10* CpG island methylation between tumor tissues and paired para-tumor tissues. P-values were calculated using Wilcoxon rank sum test. Stratified tests were performed according to tumor differentiation, Borrmann type, smoking history and family history of cancer. Plots are presented as median with interquartile range. A significant P-value was identified in the following subgroups: Low differentiation, III+IV Borrmann types, non-smoker patients and patients with no family history of cancer. IL10, interleukin 10; N, normal adjacent tissue; PMR, percentage of methylated reference; T, tumor tissue.

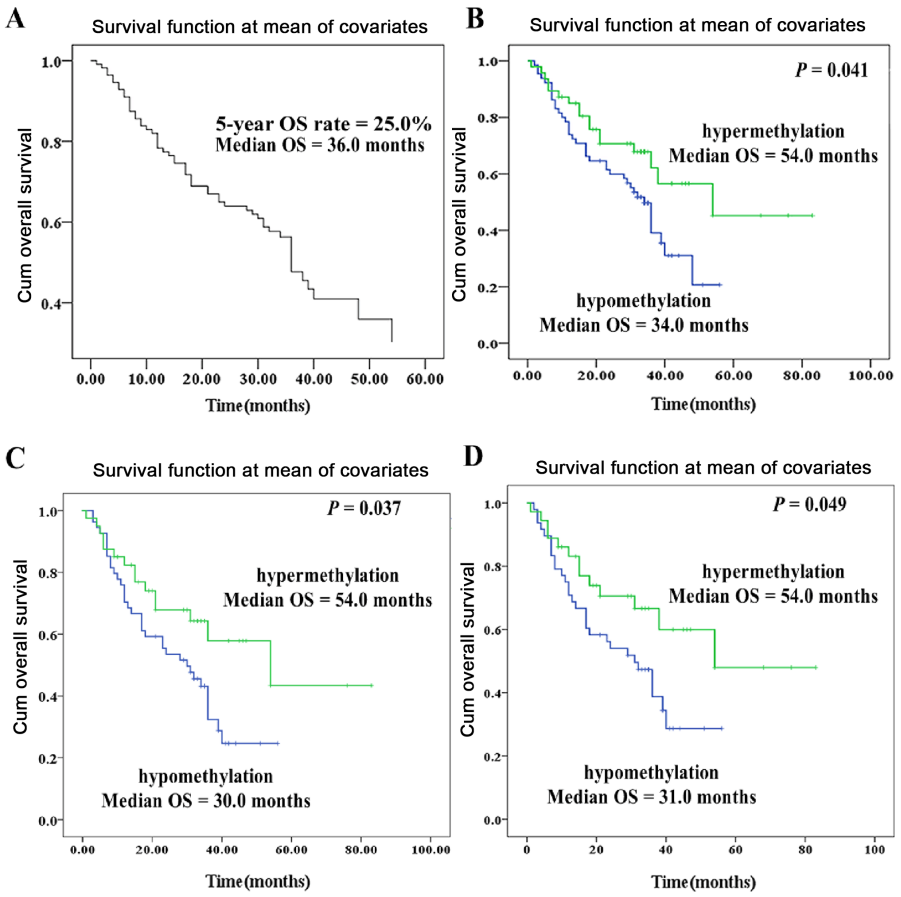


Figure 6. Association between aberrant *IL10* CGI methylation and the prognosis of patients with GC. (A) Cox regression model of OS analysis demonstrated that the median OS time was 36.0 months. (B) Kaplan-Meier and log-rank analysis of the OS of patients with GC. (C) Kaplan-Meier and log-rank analysis of the survival in patients with GC with no and low differentiation. (D) Kaplan-Meier and log-rank analysis of the survival in non-smoker patients with GC. The cut-off value of *IL10* CGI hypermethylation was set at 32.0%. GC, gastric cancer; OS, overall survival; CGI, CpG island; *IL10*, interleukin 10.

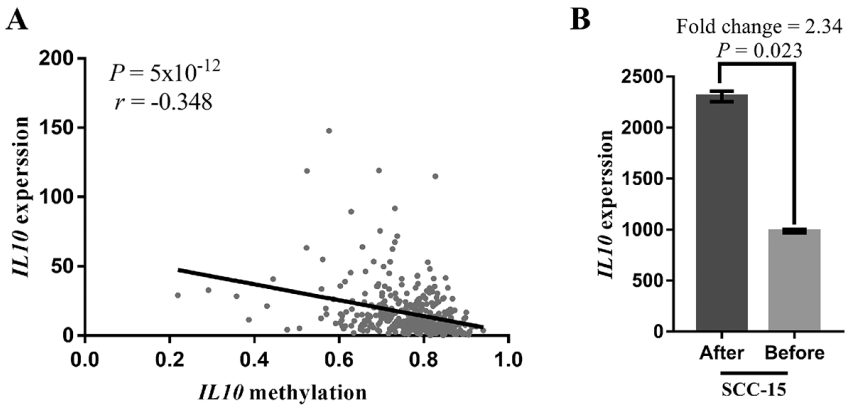
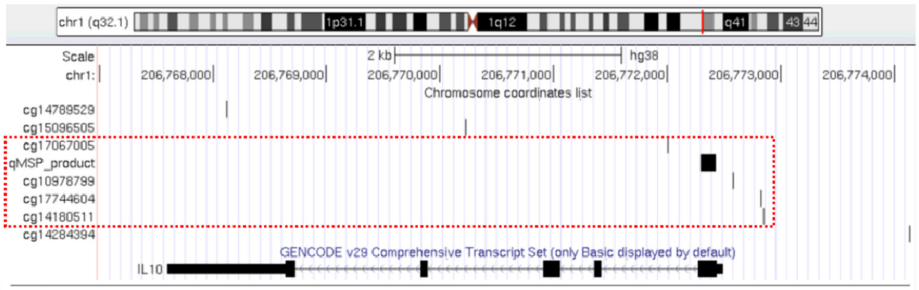


Figure 7. Association between *IL10* methylation and *IL10* expression. (A) An inverse correlation was found between *IL10* methylation and mRNA expression in 372 samples of the stomach adenocarcinoma dataset (TCGA, PanCancer Altas) (r=-0.348, P=$5x10^{-12}$). (B) Gene Expression Omnibus data mining results showed that the relative expression level of *IL10* increased when OC3, SAS, SCC and HSC3 (oral squamous cell carcinoma cell lines) cells were treated with the demethylation agent 5'-AZA.

prognosis (P=0.037; Fig. 6C). Kaplan-Meier survival analysis of non-smoker patients with GC demonstrated that *IL10* CGI hypomethylation was associated with a significantly worse prognosis (P=0.049; Fig. 6D).

Data mining of public databases. The analysis of data from 372 GC tissues from TCGA dataset demonstrated a significantly negative correlation between *IL10* methylation and gene expression (r=-0.348; P=$5x10^{-12}$; Fig. 7A), suggesting a pivotal role of *IL10* methylation in the regulation of *IL10* gene function. In addition, the results from GEO data analysis demonstrated that the relative expression level of *IL10* significantly increased in cell lines treated with the demethylation agent 5-AZA (fold-change=2.34; P=0.023; Fig. 7B). As shown

A



B

r value	cg14789529	cg15096505	cg17067005	cg10978799	cg17744604	cg14180511	cg14284394
cg14789529	1	0.47	0.14	0.42	0.42	0.47	0.24
cg15096505	0.47	1	0.23	0.47	0.38	0.42	0.28
cg17067005	0.14	0.23	1	0.24	0.22	0.16	0.14
cg10978799	0.42	0.47	0.24	1	0.54	0.59	0.29
cg17744604	0.42	0.38	0.22	0.54	1	0.73	0.15
cg14180511	0.47	0.42	0.16	0.59	0.73	1	0.27
cg14284394	0.24	0.28	0.14	0.29	0.15	0.27	1

P-value	cg14789529	cg15096505	cg17067005	cg10978799	cg17744604	cg14180511	cg14284394
cg14789529		0	0.0055	0	0	0	0
cg15096505	0		0	0	0	0	0
cg17067005	0.0055	0		0	0	0.0012	0.0052
cg10978799	0	0	0		0	0	0
cg17744604	0	0	0	0		0	0.0026
cg14180511	0	0	0.0012	0	0		0
cg14284394	0	0	0.0052	0	0.0026	0	

Figure 8. Association of CGI methylation with other 7 CpG sites at the *IL10* locus based on bioinformatics analysis. (A) The genomic positions (GRCh38/hg38 version) of 7 CpG sites at the IL10 locus. (B) The table shows the association analysis of 7 CpG loci on the IL10 locus. The top table represents the correlation coefficients between 7 CpG sites at the IL10 locus, while the bottom table represents the P-values between 7 CpG sites at the IL10 locus.

in Fig. 8, the analysis of the TCGA dataset demonstrated that the methylation levels of the seven CpG sites among 372 TCGA GC tissue samples were positively correlated with each other ($r>0.13$; $P<0.006$) especially for the two CpG sites cg17067005 and cg10978799 that are near the two ends of the qMSP product ($r=0.33$; $P<0.0001$).

Discussion

The present study demonstrated for the first time that *IL10* CGI hypomethylation was associated with the risk of GC and with worse OS in patients with GC. *IL10* CGI methylation in tumor tissues was significantly associated with patient's age at diagnosis Furthermore, the results from this study demonstrated that the contribution of *IL10* CGI hypomethylation to GC was specific to patients with low differentiation, patients with Borrmann types III and IV, non-smoker patients or patients with no family history of cancer.

As an essential anti-inflammatory cytokine, IL10 can be produced in response to pro-inflammatory signals in most immune cells, including macrophages, and T, B and dendritic cells (35). IL10 expression is highly dynamic and requires strict regulation (36). High level of IL10 is observed in the human monocytic cell line following stimulation by coagulation factors, leading to GC cell migration and invasion via transformation of macrophages into tumor-associated macrophage-like cells (37). It was also reported that IL10 can stimulate MCF-7 breast cancer cell proliferation by activating the IL10-signal transducer and activator of transcription 3-neutrophil gelatinase-associated lipocalin axis, which may contribute to tumor progression (38).

A previous study demonstrated that increased IL10 production in chronic lymphocytic leukemia cells was associated with decreased DNA methylation at the *IL10* locus (12). Another study also reported that *IL10* hypomethylation in cancerous tissues from patients with breast cancer can activate *IL10* gene expression (19). It was demonstrated that DNA methylation of the *IL10* promoter can inhibit IL10 expression in Th1 cells (39), CD^{4+} T lymphocytes, macrophages (39), blood cells and gingival tissues (25). In addition, transfection of 1 and 0.6-kb non-CpG methylated *IL10* proximal promoter fragments into HeLa cells cannot increase the reporter gene expression, which can be reversed by cassette methylation of these promoter fragments (20). Furthermore, upregulation of *IL10* expression in tumor tissues is associated with poor prognosis in patients with breast cancer (40) and laryngeal squamous cell carcinoma (41).

IL10 acts as a cellular molecule with broad-spectrum antiinflammatory activity (42). IL10 overexpression inhibits the phagocytosis of effector cells by macrophages, therefore promoting cancer progression (43). Due to the limited sample size, the present study did not perform correlation analysis between *IL10* CGI methylation and IL10 expression level. Therefore, the data from a TCGA dataset were analyzed to determine the association between *IL10* methylation and expression level. The data mining in 372 GC tissues from TCGA confirmed that *IL10* methylation was negatively correlated with *IL10* expression. *IL10* hypomethylation may therefore serve a crucial role in the development and progression of GC by upregulating *IL10* expression level. The present study demonstrated that *IL10* hypomethylation was associated with poor OS in patients with GC, in particular in non-smoker patients and those with poorly

differentiated tumor tissues. This study demonstrated that *IL10* CGI hypomethylation was associated with the risk of developing GC and the worse prognosis of GC, suggesting its potential role as a diagnostic and prognostic biomarker.

It was previously reported that smoking was associated with increased methylation of microRNA-124a-3 in the gastric mucosa of healthy Japanese volunteers (44), and with increased methylation of transmembrane protein 106A gene (45), mutL homolog 1 gene (46,47), O-6-methylguanine-DNA methyltransferase gene (47), methylated in tumors 25 (MINT25) CGI (47), tumor-suppressor candidate 3 gene (48) and cadherin 1 gene (49) in patients with GC. The present study demonstrated that *IL10* CGI hypomethylation was associated with the risk of GC and worse OS in non-smoker patients with GC. The findings from the present study suggested that *IL10* CGI methylation may be considered as a biomarker independent of smoking. In this study, 87 patients had no family history of cancer. The results demonstrated that the association between *IL10* CGI methylation and the risk of GC was specific to patients with no family history of cancer. These findings suggested that epigenetics may serve a crucial role in the development of cancer for individuals with no family history of cancer (50).

The present study only examined the CpG site within the qMSP product of *IL10*. Further investigation is therefore required to confirm whether the results from this study corresponded to the methylation of the CpG sites in other *IL10* regions. There are seven CpG sites at the *IL10* locus in the Infinium Human Methylation 450K BeadChip (Illumina, Inc.). The analysis of the TCGA dataset further demonstrated that the methylation levels of the seven CpG sites among 372 TCGA GC tissue samples were positively correlated with each other, especially for the two CpG sites cg17067005 and cg10978799 that are near the two ends of the qMSP product. However, further investigation is required to verify whether the *IL10* CGI methylation corresponds to the promoter methylation of *IL10*.

In conclusion, the present study provided a potential epigenetic cause for the contribution of *IL10* to the risk of developing GC and the OS of patients with GC. Future work may confirm that *IL10* CGI hypomethylation is specific to patients with low differentiation, Borrmann types III and IV, a family history of cancer, and non-smokers.

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Availability of data and materials

The *IL10* methylation data generated and/or analyzed during the current study are not publicly available due to patient privacy restrictions on this joint project but are available from the corresponding author on reasonable request.

Authors' contributions

JW and SD conceived and designed the study. JT, RP, LX, QM, XY, JZ, HZ, LM and YX performed the experiments. JT, RP and LX analyzed the data. JT, RP, JW and SD wrote and revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Zhejiang Province Cancer Hospital. Written consent was provided by all patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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