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***Retrospective Cohort Study***

**Increased 5-hydroxymethylcytosine is a favorable prognostic factor of *Helicobacter pylori*-negative gastric cancer patients**

Fu YL *et al*. 5-hydroxymethylcytosine and gastric cancer

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**Abstract**

BACKGROUND

Most gastric cancer (GC) patients are diagnosed at middle or late stage because the symptoms in early stage are obscure, which causes higher mortality rates of GC. *Helicobacter pylori* (*H. pylori*) was identified as a class I carcinogen and leads to aberrant DNA methylation/hydroxymethylation. 5-hydroxymethylcytosine (5-hmC) plays complex roles in gene regulation of tumorigenesis and can be considered as an activating epigenetic mark of hydroxymethylation.

AIM

To explore the association between 5-hmC levels and the progression and prognosis of GC patients with or without *H. pylori* infection*.*

METHODS

A retrospective cohort study was conducted to estimate the predicted value of 5-hmC level in the progression and prognosis of GC patients with different *H. pylori* infection status. A total of 144 GC patients were recruited.

RESULTS

The levels of 5-hmC were significantly decreased in tumor tissues (0.076 ± 0.048) compared with the matched control tissues (0.110 ± 0.057, *P* = 0.001). A high level of 5-hmC was an independent significant favorable predictor of overall survival in GC patients (hazard ratio = 0.61, 95% confidence interval: 0.38-0.98, *P* = 0.040), the *H. pylori*-negative GC subgroup (hazard ratio = 0.30, 95% confidence interval: 0.13-0.68, *P* = 0.004) and the GC patients with TNM stage Ⅰ or Ⅱ (hazard ratio = 0.32, 95% confidence interval: 0.13-0.77, *P* = 0.011).

CONCLUSION

Increased 5-hmC is a favorable prognostic factor in GC, especially for*H. pylori*-negative subgroups.

**Key Words:** 5-hydroxymethylation; 5-hydroxymethylcytosine; *Helicobacter pylori*; Gastric cancer; Prognosis

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**Core Tip:** *Helicobacter pylori* (*H. pylori*) was identified as a class I carcinogen and leads to aberrant DNA methylation/hydroxymethylation. 5-hydroxymethylcytosine plays complex roles in the gene regulation of tumorigenesis and is considered an activating epigenetic mark of hydroxymethylation. We conducted a retrospective cohort study to estimate the predictive value of 5-hydroxymethylcytosine levels in the progression and prognosis of gastric cancer patients with different *H. pylori* infection statuses. The results indicated that increasing 5-hydroxymethylcytosine is a favorable prognostic factor in gastric cancer patients who were not infected with *H. pylori*, but no associations were observed in *H. pylori*-positive gastric cancer patients.

**INTRODUCTION**

Gastric cancer (GC) is a serious disease with over 1 million estimated new cases annually around the world, and it is the fifth most diagnosed malignancy worldwide[1]. Due to the symptoms in early stage being obscure, most GC patients are diagnosed at middle or late stage, which causes higher mortality rates, and accounted for 769000 deaths globally in 2020[1].

Recent comprehensive analyses showed that many GC-related pathways are more frequently altered by aberrant DNA methylation than by mutations[2], and the degree of accumulation of aberrant DNA methylation is highly correlated with GC risk[3,4].

*Helicobacter pylori* (*H. pylori*)was identified as a class I carcinogen leading to gastric adenocarcinoma by the World Health Organization[5]. *H. pylori*-induced chronic inflammation plays a direct role in the induction of aberrant DNA methylation. The methylation level in an *H. pylori*-positive group was 2.5-34.1 times higher than a negative group. *H. pylori* eradication leads to a decrease in DNA methylation levels[6,7].

A promising method to reverse the progression of GC is effective demethylation treatment. The passive demethylation agents (5-azacytidine/decitabine), which relies on DNA methyltransferase, are not effective in the treatment of solid tumors and have serious side effects. A newly proposed classical active demethylation process involves oxidizing 5-methylcytosine to 5-hydroxymethylcytosine (5-hmC) and further downstream products by the ten-eleven translocation (TET) family. The median product, 5-hmC, is considered an activating epigenetic marker, and it plays complex roles in gene regulation of tumorigenesis[8-10]. Significant reductions in 5-hmC levels have been found in hematological malignancies, such as breast cancer, colon cancer, prostate cancer and melanoma. A few small size studies analyzed the association between 5-hmC levels and GC, but the evidence is still lacking[11,12], especially for the *H. pylori*-induced GC.

In the current study, we explored the level of 5-hmC and *H. pylori* infection in a relatively large scale GC patient cohort to assess the association between 5-hmC level and the malignant progression of the tumor and the overall survival of GC patients with different *H. pylori* infection status.

**MATERIALS AND METHODS**

***Ethics statement***

This study was approved by the Institutional Review Board of the First Hospital of Jilin University. All participants provided written informed consent prior to joining the study.

***Study population***

A total of 158 patients with histologically diagnosed GC who underwent radical gastrectomy at the Department of Gastric and Colorectal Surgery in the First Hospital of Jilin University (Changchun, China) during 2007 to 2017 were recruited in this cohort study. For each patient, 5 mL of peripheral blood before surgery and 0.5 cm3 of tumor tissue were collected. Among the patients, 38 specimens of 0.5 cm3 adjacent tissue were collected during the operation. All patients did not undergo chemotherapy or radiotherapy before surgery. Demographic information (sex, age) and principal clinical pathological information (histological grade, TNM stage, tumor size, neural invasion, vascular invasion, *etc*) were collected. The tumor histological grade was evaluated by the World Health Organization criteria. TNM stages were classified according to the 8th edition of the TNM staging system of the Union for International Cancer Control/American Joint Committee on Cancer (2017). Patients with the following conditions were excluded from this study: (1) patients with distant metastasis or a positive surgical margin; (2) patients who died due to complications of the surgical procedure during the perioperative period; and (3) patients who were lost at the first time of interview.

***Follow-up***

Follow-up for all patients was implemented at 3 mo, 6 mo, 12 mo and annually afterwards until death or the end of the follow-up. Information on general status and postoperative chemotherapy were collected during each follow-up. If the patients had died, the date of death and potential cause were recorded. The duration from the date of surgery to the date of death or the last successful interview date was defined as the survival time. If the patient was lost to follow-up, survival time was defined as the duration from the date of surgery to the date of the last successful interview.

***5-hmC quantification and test for H. pylori infection***

The genomic DNA from primary tumors and paired noncancerous mucosa tissues were extracted using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. The 5-hmC content of genomic DNA was determined with a Quest 5-hmC DNA enzyme-linked immunosorbent assay (ELISA) Kit (Zymo Research, Irvine, CA, United States) according to the manufacturer’s instructions. Assays were performed using 4 μg/mL anti-5-hmC polyclonal antibodies, loading 200 ng of DNA per well. Absorbance at 405 nm was evaluated using a SynergyH1 microplate reader and Gen5 software (BioTek, Winooski, VT, United States). The amount of 5-hmC was calculated as a percentage based on a standard curve generated using kit controls and the median value was used as the cutoff of 5-hmC level category. Values above the median value were considered to be the 5-hmC high group (≥ 0.106%), and those below the median value were considered the 5-hmC low group (< 0.106%).

A commercial ELISA kit for *H. pylori* immunoglobulin G (Biohit, Helsinki, Finland) was used to detect the serum *H. pylori* immunoglobulin G antibodies. The antibody titers were quantified by optical density readings according to the manufacturer’s protocol, and titers higher than the threshold value of 30 EIU were considered as positive for *H*. *pylori* infection.

***Statistical analysis***

Continuous variables that followed a normal distribution were shown as the mean ± standard deviation. Independent samples were compared by two-sample *t*-test, and matched-paired samples were compared by paired *t*-test. Categorical variables were presented as frequencies with percentages and were compared with the *χ*2 test or Fisher’s exact test when appropriate.

Survival curves within each stratification of variables were plotted by the Kaplan-Meier method and compared by log-rank test. The forward stepwise multivariate Cox proportional hazard model was used to evaluate the prognostic role of clinical characteristics and 5-hmC level. Hazard ratios (HRs) with their 95% confidence intervals (Cis) were calculated. All analyses were conducted with the SPSS program (version 21.0; IBM Corp., Armonk, NY, United States) or GraphPad Prism 5.0 (La Jolla, CA, United States). A two-tailed *P* value < 0.05 indicated statistical significance.

**RESULTS**

In the present study, 144 GC patients were involved for the final prognostic analysisand were followed up until August 2021. The median survival time was 73.59 mo. During the follow-up period, 75 (52.1%) patients died, 68 (47.2%) patients remained alive, and 1 (0.7%) patient was lost to follow-up (Figure 1).

Among the 38 paired tissues, the 5-hmC levels were significantly reduced in tumor tissues (0.076 ± 0.048) compared with the matched control tissues (0.110 ± 0.057, *P* = 0.001) (Figure 2A).

Among the 144 subjects, there were 99 (68.7%) males, and the median age was 62.82 (range 39–90) years old. The mean 5-hmC level of the 144 GC patients was 0.104 ± 0.062. We investigated possible correlations between 5-hmC levels and general demographic characteristics/routine clinicopathological parameters in the GC patients. The TNM stages (*P* = 0.012), neural invasion (*P* = 0.008), age (*P* = 0.008) and *H. pylori* infection (*P* = 0.049) were associated with 5-hmC level. Details were shown in Table 1.

For the 144 GC patients, theresults of *H. pylori* infection examination showed that 89 (61.8%) subjects were positive and 55 (38.2%) subjects were negative. We compared the 5-hmC level between *H. pylori*-positive infection and *H. pylori*-negative infection groups. It showed that the 5-hmC level was reduced in the *H. pylori*-positive group, but the *P* value was at the boundary of significance (*P* = 0.067, Figure 2B).

We further investigated the association between 5-hmC level and characteristics stratified by *H. pylori* infection status. We found that 5-hmC levels were higher in patients aged more than 60 years (*P* = 0.009), with neural invasion positive (*P* = 0.002), with low histological grade (*P* = 0.042) or with later TNM stage (*P* = 0.012) in the *H. pylori*-positive subset, but no significant associations were observed in the *H. pylori*-negative subset except sex (Table 2).

Overall survival analyses were performed in total patients and patient stratification by *H. pylori* infection status or TNM stage. The results of the Kaplan-Meier analysis showed that the 5-hmC level was not associated with overall survival in total patients or *H. pylori*-negative or positive groups (log rank *P* valueswere 0.406, 0.094 and 0.763, respectively, Figure 3A-C). Furthermore, the5hmC high level was associated with longer overall survival time compared with the 5hmC low group in the TNM stage Ⅰ and Ⅱ subgroup, and log rank test showed the survival curves were significantly different (log rank *P* = 0.037, Figure 3D), but the association was not significant in the TNM stageⅢ subgroup (log rank *P* = 0.547, Figure 3E).

In the full patient set, 5-hmC high level was a significant favorable predictor of overall survival in multivariate Cox regression analysis (HR = 0.61, 95%CI: 0.38-0.98, *P* = 0.040) after adjustment for tumor size, histological grade and TNM stage (Table 3).

Multivariate Cox regression analysis for overall survival was also performed in GC patients stratified by *H. pylori* infection status or TNM stage. In the *H. pylori-*negative GC subgroup, increased 5-hmC level was a favorable prognostic factor in the multivariate Cox regression analysis (HR = 0.30, 95%CI: 0.13-0.68, *P* = 0.004) (Table 4), which indicated that higher 5-hmC level was an independent significant protective factor of overall survival time in patients without *H. pylori* infection. However, within the *H. pylori*-positive group, we did not observe any significant association between 5-hmC level and GC patient prognosis.

Among patients with TNM stage Ⅰ or Ⅱ,increased 5-hmC level was associated with favorable prognosis after adjustment for sex in the multivariate Cox regression analysis (HR = 0.32, 95%CI: 0.13-0.77, *P* = 0.011). However, no significant association was observed between 5-hmC level and the prognosis in patients with TNM stage Ⅲ (Table 5).

**DISCUSSION**

Long-time *H. pylori* infection leads to chronic inflammation and further aberrant DNA methylation, which plays an important role in tumorigenesis of GC. The global prevalence of *H. pylori* reported by a meta-analysis across individual countries varied from 18.9% to 87.7%, and the prevalence in China was 55.8% (95%CI: 51.8%-59.9%)[13]. Among our 144 GC patients, 89 (61.8%) patients were defined as *H. pylori*-positive by ELISA. The infection rate was slightly higher than the prevalence in the general Chinese population but was similar to the previously reported prevalence in GC patients[14,15], indicating that our study cohort was representative.

DNA methylation/hydroxymethylation is one of the most widely studied epigenetic modifications and has been shown to play significant roles in tumorigenesis and prognosis[16]. Previous studies have shown that aberrant DNA methylation is a common event and a strong candidate mechanism for early nongenetic alterations in GC[17]. Nevertheless, the reports of DNA hydroxymethylation and GC are limited to several small studies.

We estimated the 5-hmC level with an absolutely quantitative method ELISA, which is more objective than the semi-quantitative evaluation system of immunohistochemistry. The results showed that the 5-hmC level was downregulated in GC tissues compared with matched control tissues, which revealed that it was associated with the occurrence of GC and is consistent with previous reports[18]. Although some evidence has emerged about the potential progression and prognostic implications of 5-hmC level in GC[12], very few studies have evaluated the association stratified by *H. pylori* infection status. The present study was performed on a well-characterized cohort to simultaneously evaluate the level of 5-hmC in GC patients and subsets stratified by *H. pylori* infection to assess the association between 5-hmC levels and the susceptibility or prognosis of GC in order to provide more evidence for the effect of *H. pylori*-infection DNA hydroxymethylation on GC.

The 5-hmC level was slightly decreased in the *H. pylori*-positive subset compared to the *H. pylori*-negativegroup in our study. It is hypothesized that *H. pylori* infection affects *TET1* expression in normal gastric epithelial cells and reduces the genome hydroxymethylation level[19]. Interestingly, higher global 5-hmC levels were associated with GC progression in the *H. pylori*-positive subset. A similar phenomenon was reported that the 5-hmC level in *ERG-* prostate cancer patients was lower than *ERG*+ patients, but a higher 5hmC level was associated with tumor progression in *ERG*- prostate cancer patients[20]. This could be explained by cells responding to hypoxia inducing a transcriptional program regulated by the *TET* family. Hypoxia together with reactive oxygen species increase global 5-hmC levels by transcriptional activation of *TET1*[21,22]. *H. pylori* infection induced the expression of hypoxia-inducible factor[23],which is required for hypoxic induction of *TET1* and global increase of 5-hmC. The proliferation rate of *H. pylori* under aerobic conditions was 3-fold higher than under microaerophilic conditions, and the bacterial growth was more dependent on carbon dioxidethan on oxygen[24].

This interesting phenomenon and potential mechanism suggested to us that the 5-hmC level changed due to *H. pylori* infection and was not simply one direction but complicated. Therefore, it was essential to assess the association between 5-hmC and the prognosis of GC patients in negative or positive *H. pylori* infection. Our results first showed that reduced 5-hmC was associated with poor prognosis in all GC patients, which was consistent with previous studies[11,12]. Furthermore, in *H. pylori*-negative GC patients, the 5-hmC level was a significant predictor of prognosis, independent of routine clinicopathological factors. But in contrast, 5-hmC had no prediction value of prognosis in *H. pylori*-positive GC patients. These results highlight the importance of *H. pylori* stratification in GC biomarker studies. Similarly to our results, the study conducted in prostate cancer patients also showed that the prognostic predictor value of 5-hmC was discrepant in *ERG-* and *ERG+* prostate cancer patients[20]. Together with our results, it supports potential prognostic implications of 5-hmC as cancer subtype-specific.

In this study, the association between 5-hmC level and the prognosis of GC patients was not significant in the Kaplan-Meier analysis, which could not be adjusted for potential confounders. However, it showed significant association in the multivariate Cox regression after the confounders such as TNM stage were adjusted. This indicated that the clinical characteristics such as TNM stage (which is strongly associated with the prognosis of GC patients) confused the relationship between 5-hmC level and the prognosis. This conclusion was further supported by the Cox regression results of TNM stage stratified analysis.

Several limitations should be mentioned of the present study. First, our study was based at a single center. The prognostic value of 5-hmC in *H. pylori*-negative but not positive GC patients’ needs to be validated in larger and multicenter GC patient cohorts. Another limitation of our study is the lack of data of 5-methylcytosine and enzymes related to 5-hmC regulation for our sample set. Thus, we have not investigated the correlation between them, which should be investigated in future studies.

**CONCLUSION**

5-hmC level was a significant predictor of the prognosis of GC patients without *H. pylori* infection, independent of routine clinicopathological factors.

**ARTICLE HIGHLIGHTS**

***Research background***

Most gastric cancer (GC) patients are diagnosed at middle or late stage because the symptoms in early stage are obscure, which causes higher mortality rates of GC. Analyses show that aberrant DNA methylation is highly correlated with GC risk. *Helicobacter pylori* (*H. pylori*)was identified as a class I carcinogen leading to gastric adenocarcinoma, and *H. pylori*-induced chronic inflammation plays a direct role in the induction of aberrant DNA methylation. The median demethylation product 5-hydroxymethylcytosine (5-hmC) is considered as an activating epigenetic marker, and it plays complex roles in gene regulation of tumorigenesis.

***Research motivation***

A few small studies analyzed the association between 5-hmC levels and GC, but the evidence is lacking, especially for *H. pylori*-induced GC.

***Research objectives***

Exploring the association between 5-hmC level and the progression and prognosis of GC patients with or without *H. pylori* infection*.*

***Research methods***

This was a retrospective cohort study to estimate the predicted value of 5-hmC level in the progression and prognosis of GC patients with different *H. pylori* infection status.

***Research results***

A high level of 5-hmC was an independent significant favorable predictor of overall survival in the entire GC patient cohort (hazard ratio = 0.61, 95% confidence interval: 0.38-0.98, *P* = 0.040), the *H. pylori*-negative GC subgroup (hazard ratio = 0.30, 95% confidence interval: 0.13-0.68, *P* = 0.004) and GC patients with early TNM stage (hazard ratio = 0.32, 95% confidence interval: 0.13-0.77, *P* = 0.011).

***Research conclusions***

5-hmC level was a significant predictor of the prognosis of GC patients without *H. pylori* infection.

***Research perspectives***

A large-scale GC patient cohort to assess the association between the level of 5-hmC and the prognosis of GC patients, especially for different *H. pylori* infection status, should be conducted.

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**Footnotes**

**Institutional review board statement:** This study was approved by the First Hospital of Jilin University Institutional Review Board (Approval No.2021-493).

**Informed consent statement:** Patients were not required to give informed consent to the study because the analysis used anonymous clinical data that were obtained after each patient agreed to treatment by written consent.

**Conflict-of-interest statement:** All authors declared no competing financial interests.

**Data sharing statement:** Technical appendix, statistical code, and dataset available from the corresponding author at email jd3d2ub@jlu.edu.cn.

**STROBE statement:** The authors have read the STROBE Statement—checklist of items, and the manuscript was prepared and revised according to the STROBE Statement—checklist of items.

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**Provenance and peer review:** Unsolicited article; Externally peer reviewed.

**Peer-review model:** Single blind

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**Peer-review report’s scientific quality classification**

Grade A (Excellent): A

Grade B (Very good): B

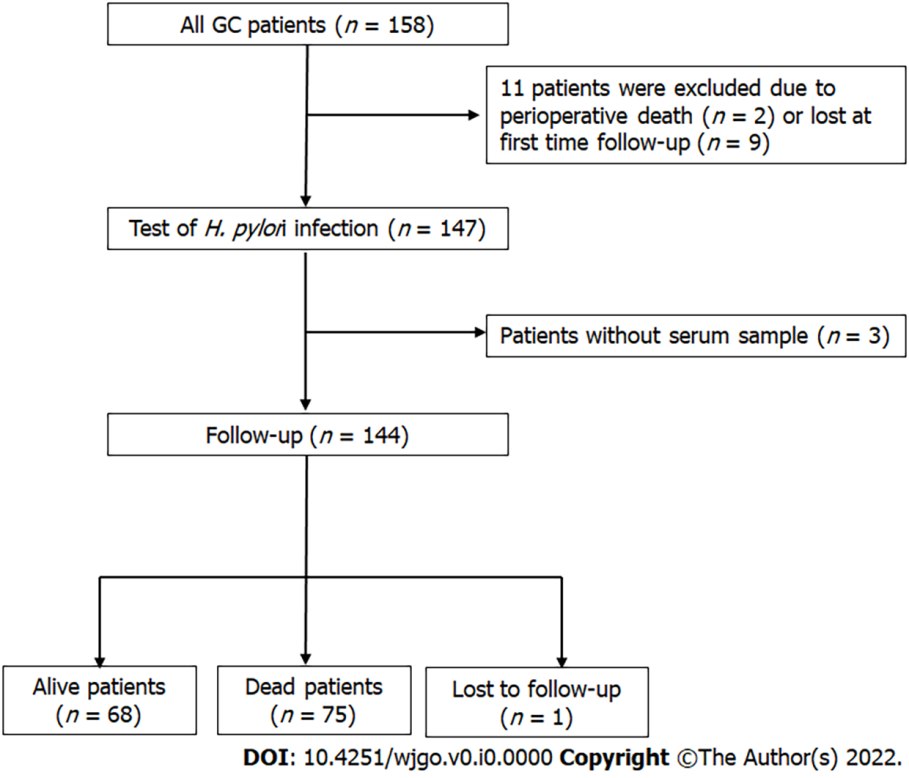
Grade C (Good): 0

Grade D (Fair): 0

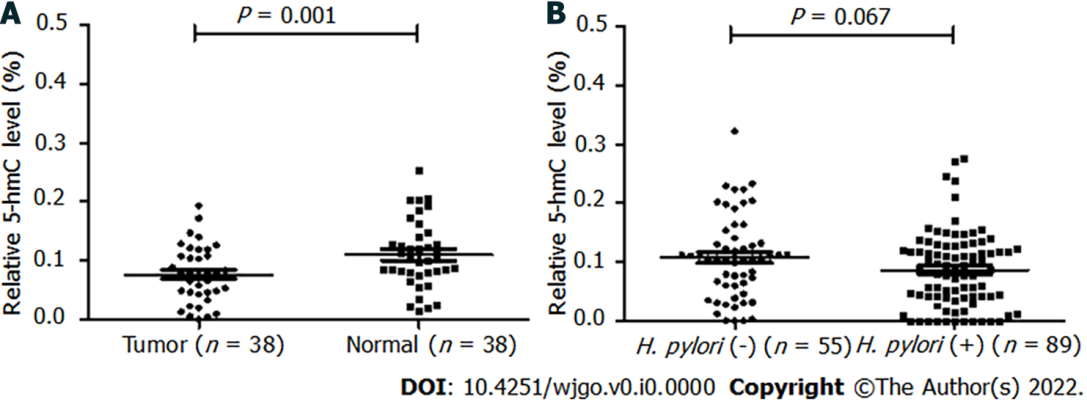
Grade E (Poor): 0

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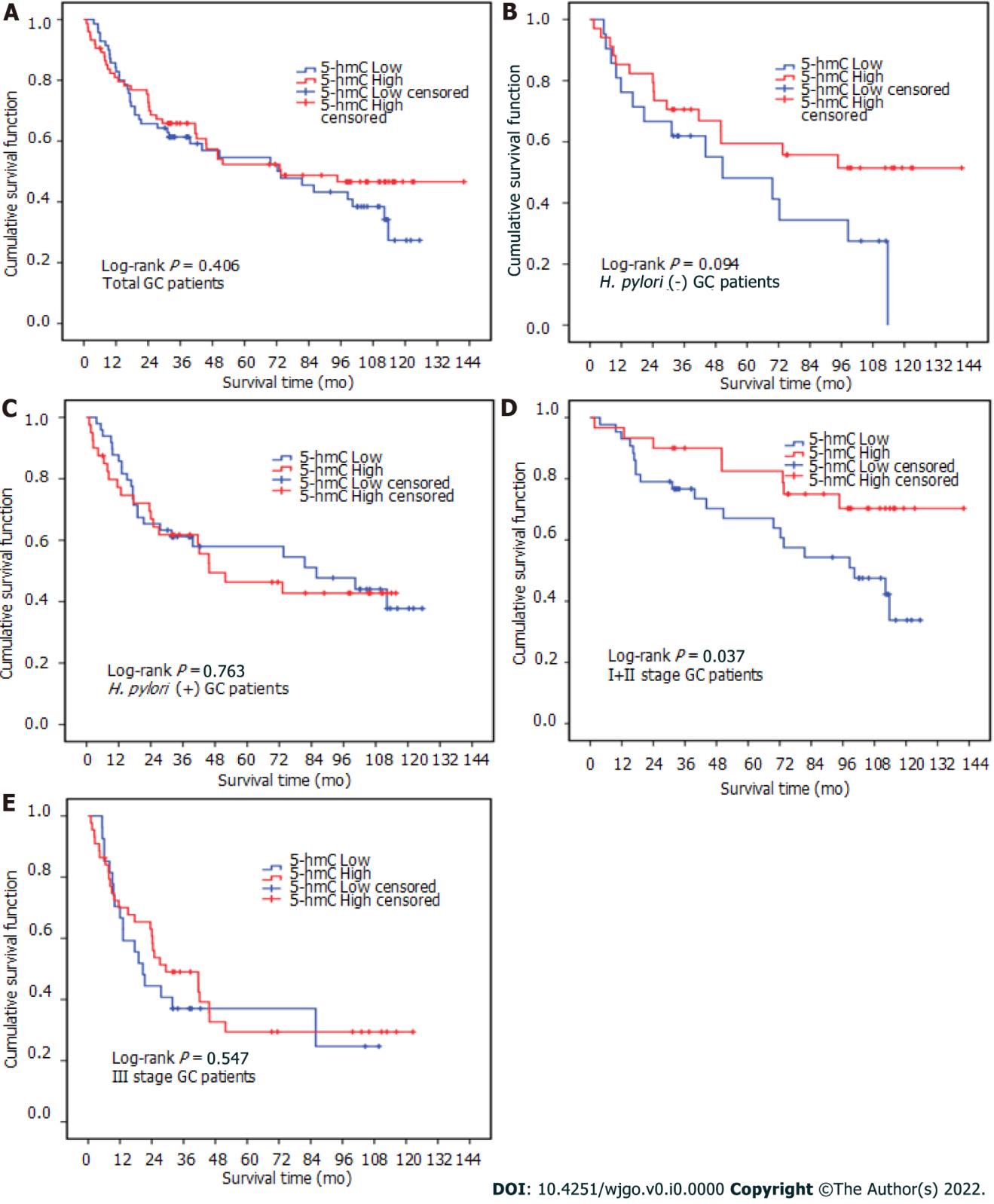
**Figure Legends**



**Figure 1 Flow chart of the subjects enrolled.** GC: gastric cancer; *H. pylori*: *Helicobacter pylori*.



**Figure 2 5-hydroxymethylcytosine level measurement compared between tumor *vs* control tissues and *Helicobacter pylori*-negative *vs* positive subgroups.** A: tumor *vs* control tissues; B: *Helicobacter pylori* -negative *vs* positive subgroups. 5-hmC: 5-hydroxymethylcytosine.



**Figure 3 Kaplan–Meier estimates of overall survival in gastric cancer patients.** A: total gastric cancer (GC) patients; B: GC patients with negative *Helicobacter pylori* (*H. pylori*) infection; C: GC patients with positive *H. pylori* infection; D: GC patients with Ⅰ or Ⅱ TNM stage; E: GC patients with Ⅲ TNM stage. 5-hmC: 5-hydroxymethylation.

**Table 1 5-hydroxymethylcytosine levels between different characteristics, *n* = 144**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Characteristics** | **Total, *n* (%)** | **5-hmC low, *n* (%)** | **5-hmC high, *n* (%)** | ***P* value** |
| Sex |  |  |  |  |
| Male | 45 (31.3) | 17 (24.3) | 28 (37.8) | 0.079 |
| Female | 99 (68.7) | 53 (75.7) | 46 (62.2) |  |
| Age in yr |  |  |  |  |
| < 60 | 56 (38.9) | 35 (50.0) | 21 (28.4) | 0.008 |
| ≥ 60 | 88 (61.1) | 35 (50.0) | 53 (71.6) |  |
| Histological grade |  |  |  |  |
| Low | 106 (73.6) | 47 (67.1) | 59 (79.7) | 0.087 |
| High | 38 (26.4) | 23 (32.9) | 15 (20.3) |  |
| WHO Classification |  |  |  |  |
| Tubular adenocarcinoma | 115 (79.9) | 55 (78.6) | 60 (81.1) | 0.707 |
| Others | 29 (20.1) | 15 (21.4) | 14 (18.9) |  |
| Tumor size in cm |  |  |  |  |
| < 5 | 62 (43.1) | 32 (45.7) | 30 (40.5) | 0.531 |
| ≥ 5 | 82 (56.9) | 38 (54.3) | 44 (59.5) |  |
| Vascular invasion |  |  |  |  |
| Negative | 39 (27.1) | 23 (32.9) | 16 (21.6) | 0.129 |
| Positive | 105 (72.9) | 47 (67.1) | 58 (78.4) |  |
| Neural invasion |  |  |  |  |
| Negative | 64 (44.4) | 39 (55.7) | 25 (33.8) | 0.008 |
| Positive | 80 (55.6) | 31 (44.3) | 49 (66.2) |  |
| Depth of invasion |  |  |  |  |
| T1/T2 | 54 (37.5) | 30 (42.9) | 24 (32.4) | 0.197 |
| T3/T4 | 90 (62.5) | 40 (57.1) | 50 (67.6) |  |
| Lymph metastasis |  |  |  |  |
| N0 | 41 (28.5) | 24 (34.3) | 17 (23.0) | 0.133 |
| N1/N2/N3 | 103 (71.5) | 46 (65.7) | 57 (77.0) |  |
| TNM stage |  |  |  |  |
| Ⅰ + Ⅱ | 73 (50.7) | 43 (61.4) | 30 (40.5) | 0.012 |
| Ⅲ | 71 (49.3) | 27 (38.6) | 44 (59.5) |  |
| Chemotherapy |  |  |  |  |
| No | 75 (52.1) | 33 (47.1) | 42 (56.8) | 0.248 |
| Yes | 69 (47.9) | 37 (52.9) | 32 (43.2) |  |
| *H. pylori* |  |  |  |  |
| Negative | 55 (38.2) | 21 (30.0) | 34 (45.9) | 0.049 |
| Positive | 89 (61.8) | 49 (70.0) | 40 (54.1) |  |

5-hmC: 5-hydroxymethylcytosine; *H. pylori*: *Helicobacter pylori*; WHO: World Health Organization.

**Table 2 5-hydroxymethylcytosine level stratified by different characteristics according to *Helicobacter pylori* infection condition**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Characteristics** | ***H. pylori* (-), *n* = 55** | | ***H. pylori* (+), *n* = 89** | |
| **5-hmC high, *n* (%)** | ***p* value** | **5-hmC high, *n* (%)** | ***P* value** |
| Sex |  |  |  |  |
| Male | 18 (50.0) | 0.013 | 28 (44.4) | 0.883 |
| Female | 16 (84.2) |  | 12 (46.2) |  |
| Age in yr |  |  |  |  |
| < 60 | 10 (55.6) | 0.505 | 11 (28.9) | 0.009 |
| ≥ 60 | 24 (64.9) |  | 29 (56.9) |  |
| Histological grade |  |  |  |  |
| High | 10 (55.6) | 0.505 | 5 (25.0) | 0.042 |
| Low | 24 (64.9) |  | 35 (50.7) |  |
| WHO classification |  |  |  |  |
| Tubular adenocarcinoma | 31 (60.8) | 11 | 29 (45.3) | 0.911 |
| Others | 3 (75.0) |  | 11 (44.0) |  |
| Tumor size in cm |  |  |  |  |
| < 5 | 13 (61.9) | 0.992 | 17 (41.5) | 0.542 |
| ≥ 5 | 21 (61.8) |  | 23 (47.9) |  |
| Vascular invasion |  |  |  |  |
| Negative | 10 (52.6) | 0.308 | 6 (30.0) | 0.127 |
| Positive | 24 (66.7) |  | 34 (49.3) |  |
| Neural invasion |  |  |  |  |
| Negative | 16 (57.1) | 0.467 | 9 (25.0) | 0.002 |
| Positive | 18 (66.7) |  | 31 (58.5) |  |
| Depth of invasion |  |  |  |  |
| T1/T2 | 12 (50.0) | 0.112 | 12 (40.0) | 0.504 |
| T3/T4 | 22 (71.0) |  | 28 (47.5) |  |
| Lymph metastasis |  |  |  |  |
| N0 | 9 (50.0) | 0.208 | 8 (34.8) | 0.255 |
| N1/N2/N3 | 25 (67.6) |  | 32 (48.5) |  |
| TNM stage |  |  |  |  |
| Ⅰ + Ⅱ | 17 (54.8) | 0.226 | 13 (31.0) | 0.012 |
| Ⅲ | 17 (70.8) |  | 27 (57.4) |  |
| Chemotherapy |  |  |  |  |
| No | 19 (61.3) | 0.927 | 23 (52.3) | 0.169 |
| Yes | 15 (62.5) |  | 17 (37.8) |  |

1*P* value of Fisher’ exact test.5-hmC: 5-hydroxymethylcytosine; *H. pylori*: *Helicobacter pylori*; WHO: World Health Organization.

**Table 3 Multivariate analyses of risk factors affecting overall survival in total patients, *n* = 144**

|  |  |  |  |
| --- | --- | --- | --- |
| **Characteristics** |  | **HR (95%CI)** | ***P* value** |
| Tumor size in cm | < 5 | 1 | 0.027 |
|  | ≥ 5 | 1.78 (1.07-2.95) |  |
| Histological grade | High | 1 | 0.011 |
|  | Low | 2.25 (1.21-4.18) |  |
| TNM stage | Ⅰ + Ⅱ | 1 | < 0.001 |
|  | Ⅲ | 2.84 (1.72-4.70) |  |
| 5-hmC | Low | 1 | 0.040 |
|  | High | 0.61 (0.38-0.98) |  |

5-hmC: 5-hydroxymethylcytosine; CI: Confidence interval; HR: Hazard ratio.

**Table 4 Multivariate analysis of risk factors affecting overall survival in the *Helicobacter pylori*-negative subgroup, *n* = 55**

|  |  |  |  |
| --- | --- | --- | --- |
| **Characteristics** |  | **HR (95%CI)** | ***P* value** |
| Neural invasion | Negative | 1 | < 0.001 |
|  | Positive | 5.45 (2.28-13.07) |  |
| Tumor size in cm | < 5 | 1 | 0.031 |
|  | ≥ 5 | 2.63 (1.09-6.32) |  |
| 5-hmC | Low | 1 | 0.004 |
|  | High | 0.30 (0.13-0.68) |  |

5-hmC: 5-hydroxymethylcytosine; CI: Confidence interval; HR: Hazard ratio.

**Table 5 Multivariate analyses of risk factors affecting overall survival in the TNM stage Ⅰ + Ⅱ subgroup, *n* = 73**

|  |  |  |  |
| --- | --- | --- | --- |
| **Characteristics** |  | **HR (95%CI)** | ***P* value** |
| Sex | Female | 1 | 0.047 |
|  | Male | 0.43 (0.19-0.99) |  |
| 5-hmC | Low | 1 | 0.011 |
|  | High | 0.32 (0.13-0.77) |  |

5-hmC: 5-hydroxymethylcytosine; CI: Confidence interval; HR: Hazard ratio.