74658_Auto_Edited.docx

WORD COUNT 2793
TIME SUBMITTED 29-APR-2022 04:51PM
PAPER ID 85371067

Name of Journal: *World Journal of Gastrointestinal Oncology*
Manuscript NO: 74658
Manuscript Type: ORIGINAL ARTICLE

Retrospective Cohort Study

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

Ying-Li Fu, Yan-Hua Wu, Dong-Hui Cao, Zhi-Fang Jia, Ao Shen, Jing Jiang, Xue-Yuan Cao

Abstract

BACKGROUND

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

AIM

To explore the association between 5-hmC level and the progression and prognosis of gastric cancer patients who with or without *H.pylori* infection.

METHODS

A retrospective cohort study were conducted to estimate the predicted value of 5-hmC level in the progress and prognosis of gastric cancer patients with different *H.pylori* infection status. A total of 144 gastric cancer 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). High level of 5-hmC was an independent significant favorable predictor of overall survival in the total gastric cancer patients (HR=0.61, 95%CI=0.38-0.98, *P*=0.040), the *H.pylori* negative gastric cancer subgroup (HR=0.30, 95%CI=0.13-0.68, *P*=0.004) and the gastric cancer patients with TNM stageI orII(HR=0.32, 95%CI=0.13-0.77, *P*=0.011).

CONCLUSION

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

Key Words: 5-hydroxymethylation; 5-hydroxymethyl-cytosine(5-hmC); Helicobacter pylori; Gastric cancer; Prognosis

Fu Y, Wu Y, Cao D, Jia Z, Shen A, Jiang J, Cao X. Increased 5-hydroxymethylation is a favorable prognostic factor of Helicobacter pylori negative gastric cancer patients. *World J Gastrointest Oncol* 2022; In press

Core Tip: Helicobacter pylori (H. pylori, Hp) was identified as a class I carcinogen and leads to aberrant DNA methylation/hydroxymethylation. 5-Hydroxymethylcytosine (5-hmC) 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 the 5-hmC level in the progression and prognosis of gastric cancer patients with different H. pylori infection statuses. The results indicating that increasing 5-hmC is a favourable prognostic factor in gastric cancer who were without H.pylori infection but no association were observed in H. pylori infection 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 are obscure, most gastric cancer patients were diagnosed at middle or late stage because ,which causing higher mortality rates, and with 769,000 deaths globally in 2020 [1].

Recent comprehensive analyses showed that many gastric cancer-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 gastric cancer risk[3,4].

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

A promising method to reverse the progression of gastric cancer is effective demetylation treatment. The Passive demethylation agents (5-AZa-Cr/decitabine), which is relied on DNA methyltransferase (DNMT) is not effective in the treatment of solid tumors and has serious side effects. A newly proposed classical active demethylation progress was oxidizing 5-methylcytosine (5mC) to 5-hydroxymethyl - cytosine (5-hmC) and further downstream products by the Ten-eleven translocation (TET) family. The median product 5-hmC is considered as 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, breast cancer, colon cancer, prostate cancer and melanoma. A few small size studies analyzed the association between 5-hmC levels and gastric cancer, but the evidence was far from enough [11,12], especially for the *H.pylori* induced gastric cancer.

In current study, we explored the level of 5-hmC and *H.pylori* infection in a relative large scale GC patient cohort to assess the association between 5-hmC level and the malignant progression of tumor and the overall survival of GC patients under different *H.pylori* infectious 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 155 patients with histologically diagnosed GC and 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, 5 mL peripheral blood before surgery and 0.5cm^3 tumor tissue were collected from each patient, 38 patients among them were together collected 0.5cm^3 adjacent tissue specimens during the operation. All patients didn't undergone chemotherapy or radiotherapy before surgery. Demographical information (gender, age) and principal clinical pathological information (histological grade, TNM stage, tumor size, neural invasion, vascular invasion *et al*) 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 (UJCC/AJCC, 2017). Patients with the following conditions were excluded from this study: ① patients with malignancy distant metastasis or a positive surgical margin, ②patients who died due to complications of the surgical procedure during the perioperative period ③patients who were lost at the first time of interview.

Follow-up

Follow-up for all patients were implemented at the 3th month, 6th month, 12th month and annually afterwards until interviewees 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 followup,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 tumor 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, USA) 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, USA). 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 5-hmC high group（≥0.106%）and those below the median value were considered as 5-hmC low group（<0.106%）.

A commercial ELISA kit for *H. pylori*-(Ig) G (Biohit, Helsink, Finland) was used to detect the Serum *H. pylori* immunoglobulin (Ig) G antibodies of the whole subjects. The antibody titers were quantified by optical density (OD) readings according to the manufacturer's protocol and titres higher than the threshold value of 30EIU were considered as positive for *H. pylori* infection.

Statistical analysis

Continuous variables which follows a normal distribution are shown as the mean ± standard deviation (SD), independent samples were compared by two-sample *t*-test

and matched-paired samples were compared by paired *t*-test. Categorical variables are 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%CIs were calculated. All analyses were conducted with the SPSS program (version 21.0, Chicago, IL, USA) or GraphPad Prism 5.0. A two-tailed P-value < 0.05 indicated statistical significance.

RESULTS

In present study, 144 GC patients were involved for the final prognostic analysis and 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 2-A).

Among the 144 subjects, males were 99 (68.7%) and the median age was 62.82 (range 39–90) years old. The mean 5-hmC level of the total 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 total 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 showed in Table 1.

For the 144 GC patients, the results 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* infection positive [*Hp*(+)] and negative [*Hp*(-)] groups,

it showed that the 5-hmC level reduced in *H.pylori* positive group, but the *P*-value was at the boundary of significance (*P*=0.067, Figure2-B).

We further investigated the association between 5-hmC level and characteristics stratified by *H.pylori* infectious status, we found that 5-hmC levels were higher in patients aged more than 60 years old (*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 association were observed in the *H.pylori* negative subset except sex (Table 2).

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

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

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

Among patients with TNM stage I or II, increased 5-hmC level was associated with favorable prognosis after adjusted for the sex in the multivariate Cox regression analysis (HR=0.32, 95%CI=0.13-0.77, *P*=0.011) , but 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 gastric cancer. The global prevalence of *H.pylori* reported by a meta-analysis across individual countries was 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* infection positive by ELISA, the infection rate was slightly higher than the prevalence in general Chinese people, but was similar to the previous reported prevalence in GC patients[14,15], indicating that our study cohort was representative.

DNA methylation/hydroxymethylation as one of the most widely studied epigenetic modifications 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 strong candidate mechanism for early nongenetic alterations in gastric cancer [17], nevertheless, the reports of DNA hydroxymethylation and gastric cancer were limited to several small size researches. We estimated the 5-hmC level with an absolutely quantitative method ELISA which is more objective than the semi-quantitative evaluation system IHC. The results showed that the 5-hmC level was down-regulated in GC tissues compared with matched control tissues revealed that it was associated with the occurrence of gastric cancer, which is consistent with previous reports [18]. Although a few evidence has been emerged about the potential progress and prognostic implication of 5-hmC level in gastric cancer [12], but very few study evaluated the association stratified by *H.pylori* infection condition. The present study was performed on a well-characterized cohort to simultaneously evaluate the level of 5-

hmC in total GC patients and subsets stratified by *H.pylori* infection condition to assess the association between 5-hmC levels and the susceptibility or prognosis of gastric cancer, in order to provide more evidence for the effect of *H.pylori* infection DNA hydroxymethylation on gastric cancer.

The 5-hmC level was slightly decreased in *H.pylori* positive subset compared to *H.pylori* negative group in our study, it is complied with the mechanism that *H.pylori* infection affects *TET1* expression in normal gastric epithelial cells and reduces the genome hydroxymethylation level [19]. Interestingly, higher global 5-hmC level retaining was associated with GC progression in *H.pylori* positive subset. The similar phenomenon was reported that the 5-hmC level in *ERG*- prostate cancer patients was lower than *ERG*+ patients, but higher 5hmC level was associated with tumor progression in *ERG*- prostate cancer patients [20]. This could be explained by cells respond to hypoxia inducing a transcriptional program regulated by *TET*s, hypoxia together with reactive oxygen species (ROS) increase global 5-hmC levels by transcriptional activation of *TET1* [21,22]. *H.pylori* infection induced the expression hypoxia-induced factor (HIF)[23] which is required for hypoxic induction of *TET1* and global increases in 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 CO^2 than on oxygen [24].

This interesting phenomenon and potential mechanism hinted us that the 5-hmC level changed due to *H.pylori* infection was not simply one direction but complicated, so it is essential to assess the association between 5-hmC and prognosis of GC patients in negative or positive *H.pylori* infection, respectively. Our results firstly showed that reduced 5-hmC was associated with poor prognosis of total gastric cancer patients, which is consistent with previous studies [11,12]. Furthermore, in *H.pylori* negative GC patients, 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 to support that potential prognostic implications of 5-hmC are 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 adjusted for the potential confounders, but it showed significant association in the multivariate Cox regression after the confounders such as TNM stage were adjusted, indicated that the clinical characteristics such as TNM stage which 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 present study. Firstly, our study was based at a single site, the prognostic value of 5-hmC in *H.pylori* negative but not positive GC patients needed to be validated in larger scale and multi-center GC patient cohorts. Another limitation of our study is the lack of data of 5mC 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* infectious, independent of routine clinicopathological factors.

74658_Auto_Edited.docx

ORIGINALITY REPORT

4%
SIMILARITY INDEX

PRIMARY SOURCES

1	Masahiro Maeda, Hiroshi Moro, Toshikazu Ushijima. "Mechanisms for the induction of gastric cancer by Helicobacter pylori infection: aberrant DNA methylation pathway", Gastric Cancer, 2016 Crossref	30 words —	1%
2	Adiza Abass, Tokuju Okano, Kotchakorn Boonyaleka, Ryo Kinoshita-Daitoku, Shoji Yamaoka, Hiroshi Ashida, Toshihiko Suzuki. "Effect of low oxygen concentration on activation of inflammation by Helicobacter pylori", Biochemical and Biophysical Research Communications, 2021 Crossref	28 words —	1%
3	cyberleninka.org Internet	23 words —	1%
4	bmccancer.biomedcentral.com Internet	17 words —	1%
5	Menghui Wu, Yuchen Pan, Zhifang Jia, Yueqi Wang, Na Yang, Jianfeng Mu, Tianyu Zhou, Yaohua Guo, Jing Jiang, Xueyuan Cao. "Preoperative Plasma Fibrinogen and Serum Albumin Score Is an Independent Prognostic Factor for Resectable Stage II-III Gastric Cancer", Disease Markers, 2019 Crossref	16 words —	1%

EXCLUDE QUOTES	ON	EXCLUDE SOURCES	< 1%
EXCLUDE BIBLIOGRAPHY	ON	EXCLUDE MATCHES	< 10 WORDS