Cellular Physiology and Biochemistry

Cell Physiol Biochem 2018;45:148-162
DOI: 10.1159/000486354
Published online: January 15, 2018
Accepted: December 01, 2017

© 2018 The Author(s)
Published by S. Karger AG, Basel
www.karger.com/cpb

Karger Open access

This article is licensed under the Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License (CC BY-NC-ND) (http://www.karger.com/Services/OpenAccessLicense). Usage and distribution for commercial purposes as well as any distribution of modified material requires written permission.

Original Paper

Epigenetic Silencing of the MLH1 Promoter in Relation to the Development of Gastric Cancer and its use as a Biomarker for Patients with Microsatellite Instability: a Systematic Analysis

Guimei Hu Lijun Qin Xinjun Zhang Guoliang Ye Tao Huang

The Affiliated Hospital of Medical School, Ningbo University, Zhejiang, People's Republic of China

Key Words
Mlh1 • Methylation • GC • Biomarker • Prognosis • Diagnosis

Abstract
Background/Aims: Human mutL homolog 1 (*MLH1*) promoter methylation was reported in gastric cancer (GC). This study determined the clinicopathological, prognostic, and diagnostic effects of *MLH1* promoter methylation in GC. ***Methods:*** The combined odds ratio (OR) or hazard ratio (HR) and their corresponding 95% confidence intervals (95% CI) were calculated. The pooled sensitivity, specificity, and area under the curve (AUC) were analyzed. ***Results:*** A total of 4654 GC patients and 3669 non-malignant controls were identified in this systematic analysis. *MLH1* promoter methylation was significantly higher in GC samples than in gastric adenomas, chronic gastritis, adjacent tissues, normal gastric mucosa, and normal healthy blood samples, but it exhibited a similar frequency in GC vs. intestinal metaplasia and dysplasia samples. *MLH1* promoter methylation correlated with age and microsatellite instability (MSI), but it was not associated with gender, *H. pylori* infection, smoking, drinking behaviors, pathological histology, tumor differentiation, clinical stage, lymph node status, distant metastasis, or overall survival of GC. *MLH1* promoter methylation exhibited a poor sensitivity value (< 0.5) in patients with GC compared with adjacent tissues, gastric adenomas, chronic gastritis, normal gastric mucosa, and normal healthy blood samples. The pooled sensitivity, specificity, and AUC of *MLH1* promoter methylation in GC with MSI vs. GC with microsatellite stability (MSS) samples were 0.64, 0.96, and 0.90, respectively. ***Conclusions:*** Our results suggest that the detection of *MLH1* promoter methylation may be a potential prognostic biomarker for GC patients with MSI.

© 2018 The Author(s)
Published by S. Karger AG, Basel

Tao Huang
and Guoliang Ye

The Affiliated Hospital of Ningbo University,
Ningbo, Zhejiang 315020 (China)
E-Mail huangtao334@163.com; ndfyygl@163.com

Introduction

Gastric cancer (GC) is one of the most common malignant tumors and the third leading cause of death from human cancers. An estimated 951, 600 new cases of GC were clinically diagnosed worldwide in 2012, which led to approximately 723, 100 deaths due to GC [1]. Lauren's classification identifies two main histotypes of GC, intestinal and diffuse [2]. GC patients remain a primary clinical challenge despite recent improvements in the diagnostic, surgical, and therapeutic opportunities for GC [3, 4].

Increasing evidence reveals that a common epigenetic modification, DNA methylation, plays a crucial role in cancer carcinogenesis, progression, and prognosis [5-7]. *Helicobacter pylori* (*H. pylori*) infection and microsatellite instability (MSI) are associated with an increased risk of developing GC [8, 9]. Human mutL homolog 1 (*MLH1*) gene is located on chromosome 3p21 and encodes a DNA mismatch repair (MMR) protein [10]. DNA MMR genes have important functions in the maintenance of genome stability. Therefore, loss of MMR function leads to MSI, which contributes to the development of gastric carcinoma [11, 12]. *MLH1* promoter methylation in sporadic tumors may primarily cause MSI [13]. MSI in GC is frequent because *MLH1* promoter methylation within CpG islands inactivates the *MLH1* gene [14, 15]. The presence of *MLH1* promoter methylation is widely reported in GC [16-19].

Numerous studies reported a relationship between *MLH1* promoter methylation and GC risk, but the results of these articles are inconsistent and conflicting. For example, Lee et al. reported that *MLH1* promoter methylation exhibited a similar frequency in GC and gastric adenomas [20], and Kang et al. demonstrated that *MLH1* promoter methylation exhibited a higher frequency in GC than in gastric adenomas [21]. Therefore, the current study evaluated the association between *MLH1* promoter methylation and the risk of GC in cancer vs. different control groups: gastric adenomas, intestinal metaplasia, chronic gastritis, dysplasia, adjacent to cancer, normal gastric mucosa, and normal healthy blood samples. We evaluated the correlation of *MLH1* promoter methylation with the clinicopathological characteristics of GC and its prognostic role. We also analyzed whether *MLH1* promoter methylation could be used as a biomarker for the diagnosis of GC.

Materials and Methods

Literature search

A comprehensive literature search was performed to identify eligible studies published before January 3, 2017, in the following online electronic databases: PubMed, Embase, Cochrane library, and EBSCO. We used the following combined key words and terms: (stomach OR gastric) AND (cancer OR tumor OR neoplasm OR carcinoma) AND (MLH1 OR hMLH1 OR mutL homolog 1 OR human mutL homolog 1) AND (methylation OR epigenetic silencing OR epigenetic inactivation OR hypermethylation). We also scanned the references of eligible articles for additional studies.

Inclusion criteria

The following inclusion criteria were used to select eligible studies for the meta-analysis: 1) all cancer samples were diagnosed as primary GC using histopathological identification; 2) studies included sufficient data on *MLH1* promoter methylation to assess the correlation between GC and non-malignant controls; 3) the control groups consisted of gastric adenomas, intestinal metaplasia, chronic gastritis, dysplasia, adjacent to cancer, normal gastric mucosa, and normal healthy blood samples; 4) studies provided sufficient information to evaluate the relationship between *MLH1* promoter methylation and the clinicopathological characteristics of GC patients; 5) studies provided prognostic analyses on overall survival (OS) or disease-free survival (DFS) if possible; and 6) studies were published in English. The more complete papers with more information were selected when authors published multiple papers using duplicated sample data.

Data extraction

Two authors independently extracted the following information from the included full-text studies: first author's surname; published year; country; ethnicity; age; clinical stage; detection method; types

of samples; the frequency of promoter methylation; the number of GC and control groups; prognostic information (OS or DFS); expression status; and clinicopathological parameters, such as age (≥ 60 years vs. < 60 years), gender (male vs. female), smoking behavior (yes vs. no), drinking behavior (yes vs. no), tumor differentiation (poor vs. well/moderate), tumor stage (stage 3-4 vs. stage 1-2), lymph node status (positive vs. negative), distant metastasis (yes vs. no), tumor histology (intestinal vs. diffuse), *H. pylori* infection (yes vs. no), and microsatellite status (microsatellite instability (MSI) vs. microsatellite stability (MSS)).

Statistical analysis

All data were analyzed using Stata 12.0 software (Stata Corporation, College Station, TX, USA). The combined odds ratio (OR) and corresponding 95% confidence interval (95% CI) were calculated to evaluate the relationship between *MLH1* promoter methylation and GC in cancer vs. different control groups and the correlation between *MLH1* promoter methylation and the clinicopathological parameters of GC. The pooled hazard ratio (HR) and the 95% CI were also calculated to analyze the clinical outcome of *MLH1* promoter methylation in GC patients where possible. Heterogeneity of this meta-analysis was detected using the Cochran's Q statistic [22, 23]. A random-effects model was used for the meta-analysis. Significant heterogeneity was considered for a *P* value less than 0.1 for the Q statistic. A sensitivity analysis for positive results was performed by omitting a single study to determine the stability of the pooled results [24, 25]. Publication bias was analyzed using the Egger linear regression test for results with greater than nine studies [26]. Pooled sensitivity, specificity, and area under the curve (AUC) were performed using bivariate analysis to evaluate the diagnostic role of *MLH1* promoter methylation in GC [27, 28].

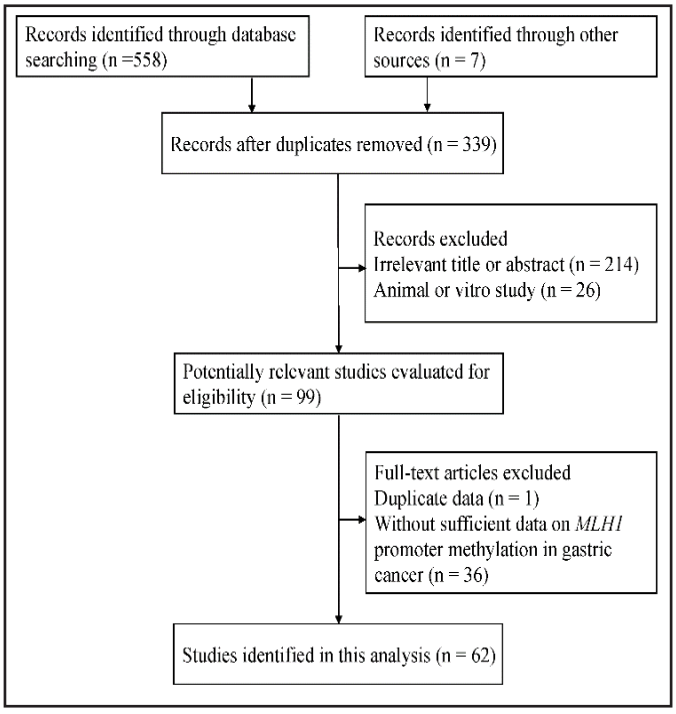

Fig. 1. Flow diagram of the relevant literature in this study.

Results

Study characteristics

Fig. 1 shows that careful scanning using the inclusion criteria yielded 62 studies published from 1999 to 2016 [15-21, 29-83], including 4654 patients with GC and 3669 non-malignant controls. Twenty-nine studies involving 2583 GC patients and 2396 adjacent tissue samples evaluated the association between *MLH1* promoter methylation and GC [18-20, 30-33, 36, 40, 41, 49, 50, 55, 56, 59, 61-63, 65, 67, 68, 70, 73, 74, 76, 78, 80-82]. Seven studies with 409 GC patients

Table 1. Subgroup analyses for *MLH1* promoter methylation in GC vs. adjacent tissues. OR: odds ratio; 95% CI: 95% confidence interval; GC: gastric cancer: mix: mixed population; MSP: methylation-specific polymerase chain reaction

Subgroups	OR (95% CI)	Heterogeneity: *P*	*P* value	Cases	Controls
Testing method					
MSP	5.38 (3.07-9.42)	< 0.001	< 0.001	2191	2092
Non-MSP	4.41 (2.29-8.47)	0.418	< 0.001	392	304
Ethnicity					
Caucasians	6.51 (2.68-15.79)	0.001	< 0.001	555	514
Asians	4.98 (2.69-9.21)	< 0.001	< 0.001	1885	1740
Mix	14.32 (1.79-114.60)	0.354	0.012	143	142

and 266 intestinal metaplasia cases assessed the correlation between *MLH1* promoter methylation and GC [17, 20, 21, 29, 32, 34, 38]. Four studies involving 214 GC patients and 203 adenomas evaluated the correlation between *MLH1* promoter methylation and GC [20, 21, 37, 38]. Four studies involving 246 patients with GC and 319 cases with chronic gastritis analyzed the correlation between *MLH1* promoter methylation and GC [16, 21, 38, 71]. Two studies involving 84 patients with GC and 96 cases with dysplasia analyzed the relationship between *MLH1* promoter methylation and GC [17, 20]. Five studies analyzed the correlation between *MLH1* promoter methylation and GC in cancer vs. normal gastric mucosa [29, 34, 47, 50, 54], including 333 GC patients and 285 normal gastric mucosa. Four studies assessed the association between *MLH1* promoter methylation and GC in cancer vs. healthy blood samples, including 270 GC blood samples and 104 healthy blood samples [17, 53, 75, 83]. Forty-eight studies evaluated the relationship between *MLH1* promoter methylation and the clinicopathological features of 3656 GC patients [15, 18, 19, 29-37, 39-48, 51, 52, 54-58, 60, 62-64, 66-70, 72, 73, 76-83]. Two studies reported the prognostic information on OS [53, 56]. For all online suppl. material, see www.karger.com/doi/10.1159/000486354, Table S1 lists the general information of the included studies.

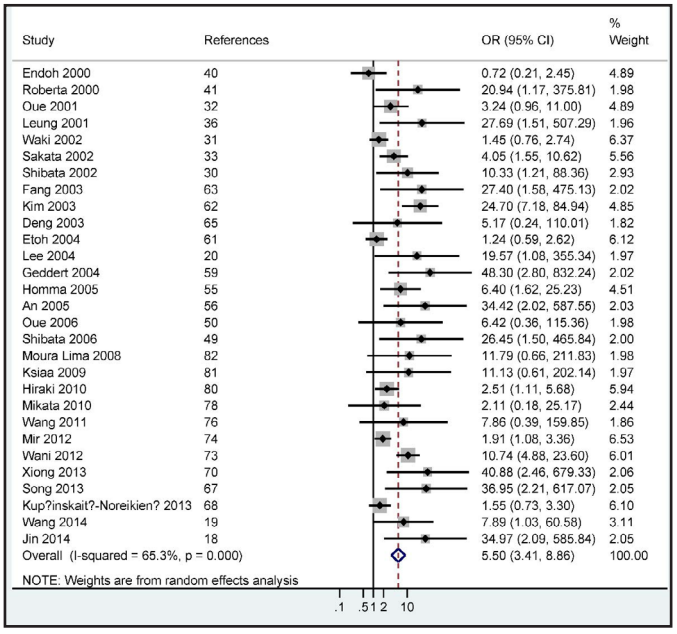

Fig. 2. Forest plot showing the correlation of MLH1 promoter methylation in GC vs. adjacent tissues, OR = 5.50, 95% CI = 3.41-8.86, P<0.001.

Association between MLH1 promoter methylation and GC in cancer vs. adjacent tissues

Fig. 2 shows that the level of *MLH1* promoter methylation was significantly increased in GC samples compared to adjacent tissue samples (OR = 5.50, 95% CI = 3.41-8.86, $P < 0.001$).

Subgroup analyses were performed based on the detection method ((methylation-specific polymerase chain reaction (MSP) and non-MSP)) and ethnicity (Asian, Caucasian, and mixed populations) to assess the strength of the associations between different subgroups (Table 1). Subgroup analysis based on ethnicity demonstrated that *MLH1* promoter methylation correlated with GC in Asian, Caucasian, and mixed populations (OR = 4.98, 95% CI = 2.69-9.21, $P < 0.001$; OR = 6.51, 95% CI = 2.68-15.79, $P < 0.001$; OR = 14.32, 95% CI = 1.79-114.60, $P = 0.012$; respectively).

Subgroup analysis by the detection method revealed that *MLH1* promoter methylation was associated with GC in the MSP and non-MSP methods (OR = 5.38, 95% CI = 3.07-9.42, $P < 0.001$; OR = 4.41, 95% CI = 2.29-8.47, $P < 0.001$; respectively).

Substantial heterogeneity was measured in the comparison of cancer and adjacent tissue samples ($P < 0.001$). Therefore, we successively removed seven studies ([31, 40, 61, 62, 68, 73, 74]). The recalculated OR was 7.02 (95% CI = 4.44-11.10, $P < 0.001$) with no heterogeneity ($P = 0.310$).

Association between MLH1 promoter methylation and GC in cancer vs. benign lesions

MLH1 promoter methylation in GC was notably higher than that in gastric adenoma or chronic gastritis (OR = 2.44, 95% CI = 1.36-4.39, $P = 0.003$; OR = 8.78, 95% CI = 4.52-17.05,

$P < 0.001$; respectively) (Fig. 3). There was no significant difference in *MLH1* promoter methylation between GC and intestinal metaplasia (OR = 2.15, 95% CI = 0.76-6.10, $P = 0.151$) or GC and dysplasia (OR = 1.27, 95% CI = 0.67-2.40, $P = 0.472$) (Fig. 3).

Association between MLH1 promoter methylation and GC in cancer vs. normal controls

MLH1 promoter methylation was notably higher in GC than in normal control samples (tissue samples: OR = 8.06, 95% CI = 1.63-39.93, $P = 0.011$; blood samples: OR = 5.87, 95% CI = 1.72-19.97, $P = 0.005$) (Fig. 4).

Association between MLH1 promoter methylation and gender

Data from 28 studies of 2576 GC patients demonstrated that *MLH1* promoter methylation did not correlate with the gender of GC patients (male vs. female: OR = 0.73, 95% CI = 0.51-1.06, $P = 0.097$) (Fig. 5).

Heterogeneity was high ($P = 0.001$), and three studies (47, 54, 73]) were successively removed. The overall OR was recalculated (OR = 0.76, 95% CI = 0.57-1.02, $P = 0.064$), and the P value of heterogeneity was 0.190.

Association between MLH1 promoter methylation and age of GC patients

Data from 13 studies of 712 GC patients demonstrated that *MLH1* promoter methylation correlated with patient age (OR = 1.72, 95% CI = 1.14-2.60, $P = 0.01$) (Fig. 6).

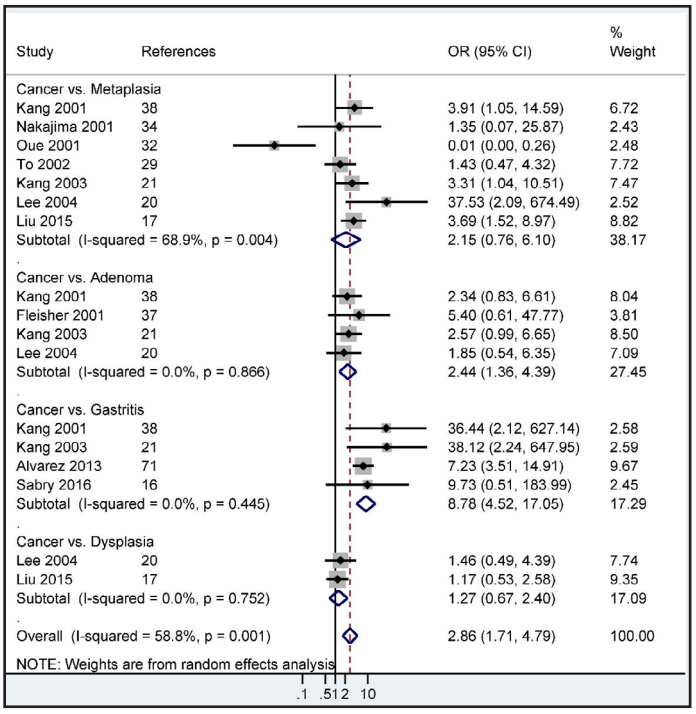

Fig. 3. Forest plot showing the correlation of MLH1 promoter methylation in GC vs. benign lesions, cancer vs. intestinal metaplasia: OR = 2.15, 95% CI = 0.76-6.10, P = 0.151; cancer vs. gastric adenoma: OR = 2.44, 95% CI = 1.36-4.39, P = 0.003; cancer vs. chronic gastritis: OR = 8.78, 95% CI = 4.52-17.05, P<0.001; cancer vs. dysplasia: OR = 1.27, 95% CI = 0.67-2.40, P = 0.472.

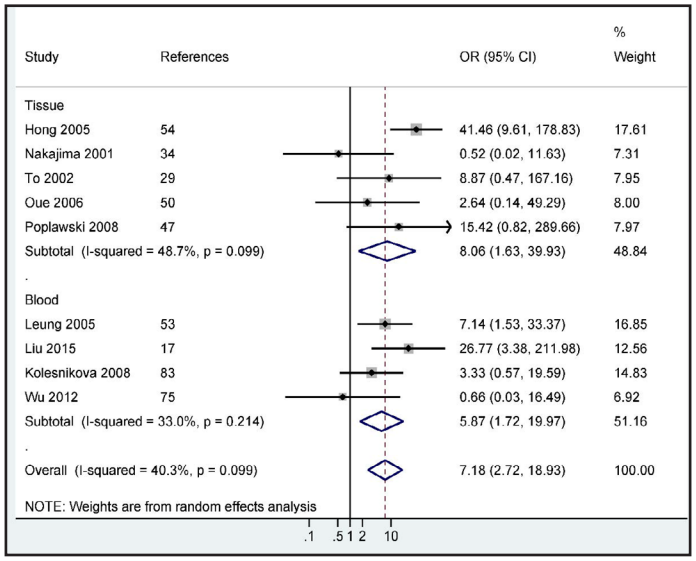

Fig. 4. Forest plot showing the correlation of MLH1 promoter methylation in GC vs. normal controls, tissue: OR = 8.06, 95% CI = 1.63-39.93, P = 0.011; blood: OR = 5.87, 95% CI = 1.72-19.97, P = 0.005.

Association between MLH1 promoter methylation and smoking or drinking behavior

The data included four studies of smoking behavior with 305 GC patients and two studies of drinking behavior with 206 GC patients. No significant correlation between *MLH1* promoter methylation and smoking or drinking behavior was observed (OR = 1.26, 95% CI = 0.44-3.59, *P* = 0.67; OR = 0.73, 95% CI = 0.39-1.35, *P* = 0.309; respectively) (Fig. 6).

Association between MLH1 promoter methylation and tumor differentiation or GC stage

No correlation was observed between *MLH1* promoter methylation and tumor differentiation or clinical stage (OR = 1.12, 95% CI = 0.67-1.89, *P* = 0.658; OR = 1.12, 95% CI = 0.68-1.82, *P* = 0.66; respectively) (Fig. 7), including eight studies of 561 GC patients and nine studies of 562 GC patients, respectively.

Association between MLH1 promoter methylation and lymph node status or distant metastasis of GC

MLH1 promoter methylation did not correlate with lymph node status or distant metastasis (OR = 1.04, 95% CI = 0.71-1.50, *P* = 0.852; OR = 1.49, 95% CI = 0.86-2.60, *P* = 0.157; respectively) (Fig. 8), including 18 studies with 1954 GCs and 10 studies with 1493 GCs, respectively.

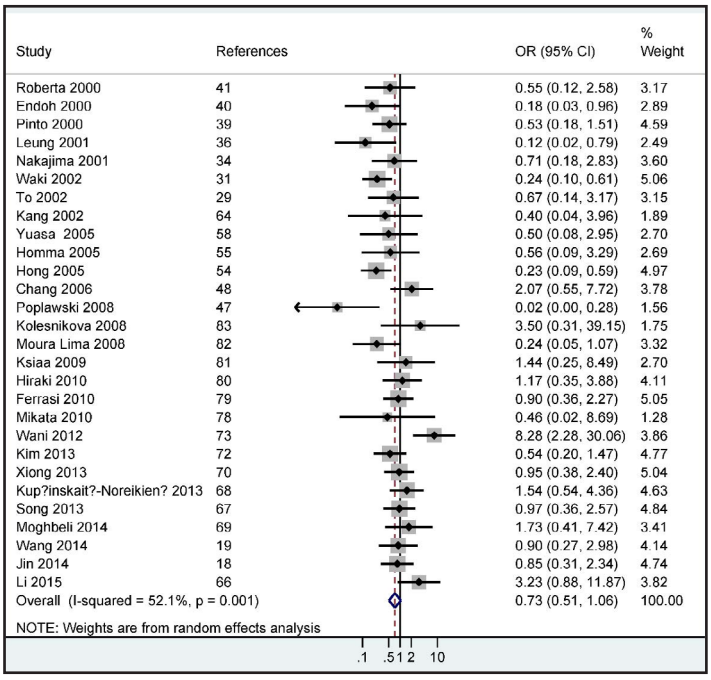

Fig. 5. Forest plot showing the association of MLH1 promoter methylation with gender, male vs. female: OR = 0.73, 95% CI = 0.51-1.06, P = 0.097.

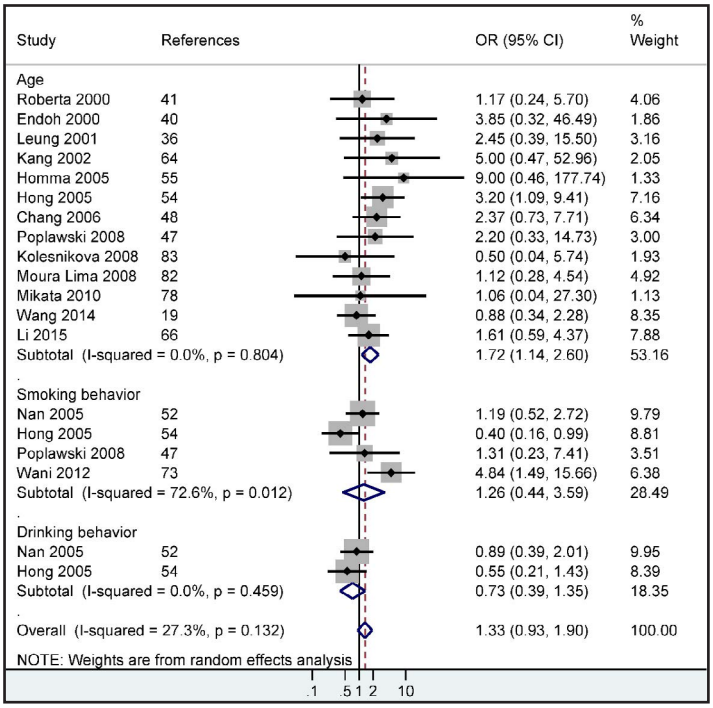

Fig. 6. Forest plot showing the association of MLH1 promoter methylation with smoking or drinking behavior (P > 0.1) and age factor, ≥ 60 years vs. < 60 years: OR = 1.72, 95% CI = 1.14-2.60, P = 0.01.

Association between MLH1 promoter methylation and tumor histology or H. pylori status

Data from 19 studies of tumor histology with 1148 GCs and four studies of *H. pylori* status with 236 GCs revealed no association between *MLH1* promoter methylation and these two clinicopathological features (tumor histology: OR = 1.00, 95% CI = 0.73-1.38, *P* = 0.98; *H. pylori* status: OR = 1.50, 95% CI = 0.58-3.87, *P* = 0.397) (Fig. 9).

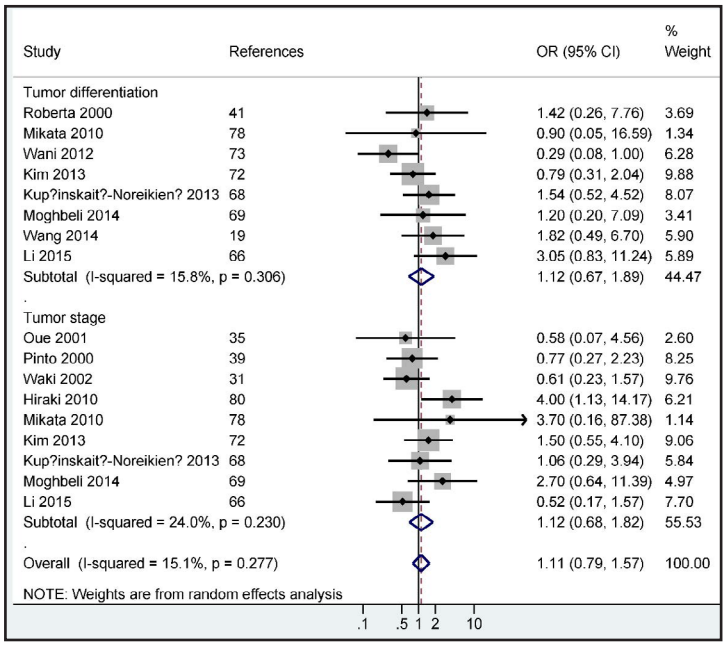

Fig. 7. Forest plot showing the association of MLH1 promoter methylation with tumor differentiation or clinical stage (P > 0.1).

Association between MLH1 promoter methylation and microsatellite status of GC

Data from 23 studies of 1294 patients with GC revealed that *MLH1* promoter methylation was closely linked to microsatellite status (MSI vs. MSS: OR = 21.52, 95% CI = 12.93-35.82, *P* < 0.001) (Fig. 10).

Prognostic effect of MLH1 promoter methylation

Only two studies (143 GCs) investigated *MLH1* promoter methylation and prognosis and reported that *MLH1* promoter methylation did not correlate with patient prognosis of OS (data not shown) [53, 56].

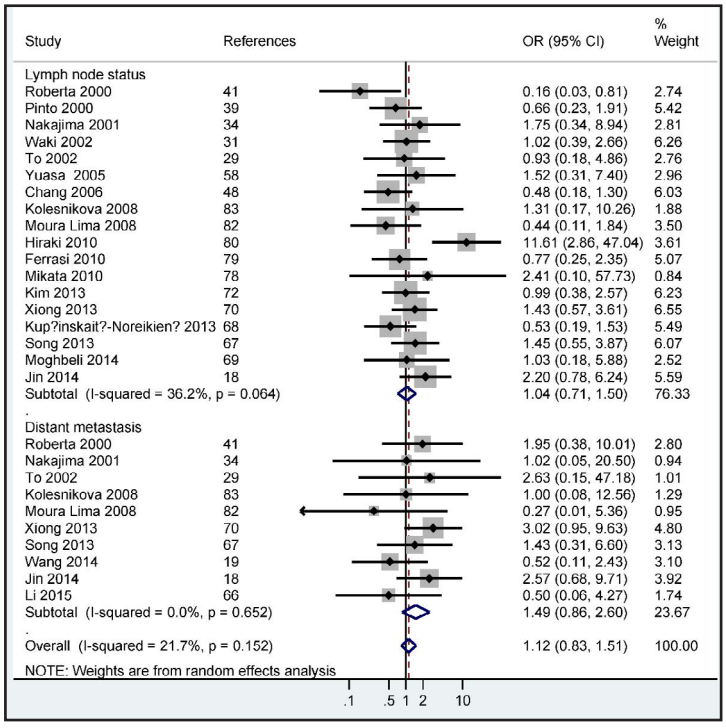

Fig. 8. Forest plot showing the association of MLH1 promoter methylation with lymph node status or distant metastasis (P > 0.1).

Publication bias

The potential publication bias was measured in the comparison of GC and adjacent tissues, in gender, age factor, lymph node status, distant metastasis, tumor histology, and microsatellite status (see online suppl. material, Fig. S1). There was evidence of publication bias in GC vs. adjacent tissue samples and tumor histology (*P* < 0.05). No publication bias was found between *MLH1* promoter methylation and other clinicopathological features (*P* > 0.1).

Diagnostic effect of MLH1 promoter methylation in GC

We further assessed the diagnostic effect of *MLH1* promoter methylation. The data demonstrated that *MLH1* promoter methylation exhibited a low sensitivity value (< 0.5) in patients with GC vs. adjacent tissues, gastric adenomas, chronic gastritis, normal gastric mucosa, and normal healthy blood samples (data not shown), which suggests that *MLH1* promoter methylation does not well distinguish between GC and different types of non-malignant control groups. Comparison of GC patients with MSI with GC patients with MSS revealed that the pooled sensitivity, specificity, and AUC of *MLH1* promoter methylation were 0.64 (95% CI = 0.53-0.75), 0.96 (95% CI = 0.91-0.98), and 0.90 (95% CI = 0.87-0.93), respectively (Fig. 11). These values of sensitivity, specificity, and AUC (sensitivity = 0.64 > 0.5, specificity = 0.96 > 0.9, and AUC = 0.90 ≥ 0.9) suggest that *MLH1* promoter methylation may be a useful non-invasive biomarker for GC patients with MSI.

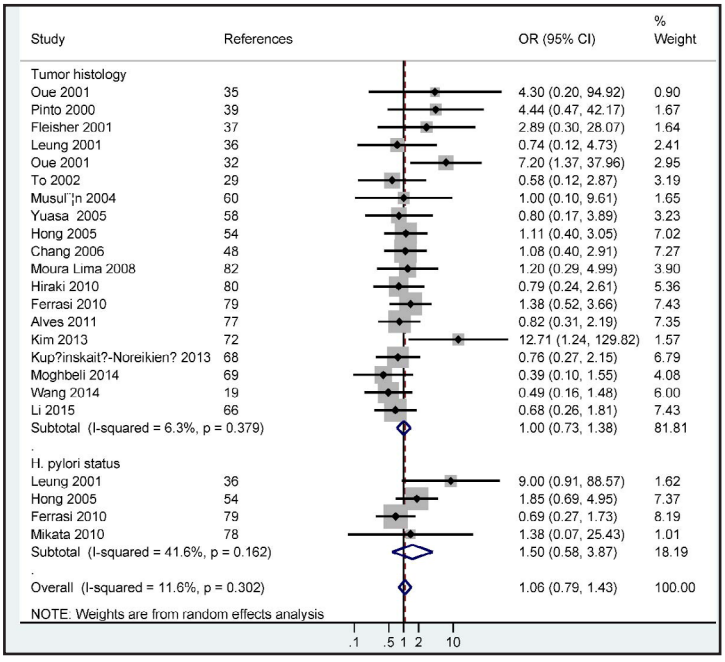

Fig. 9. Forest plot showing the association of MLH1 promoter methylation with tumor histology or H. pylori status (P > 0.1).

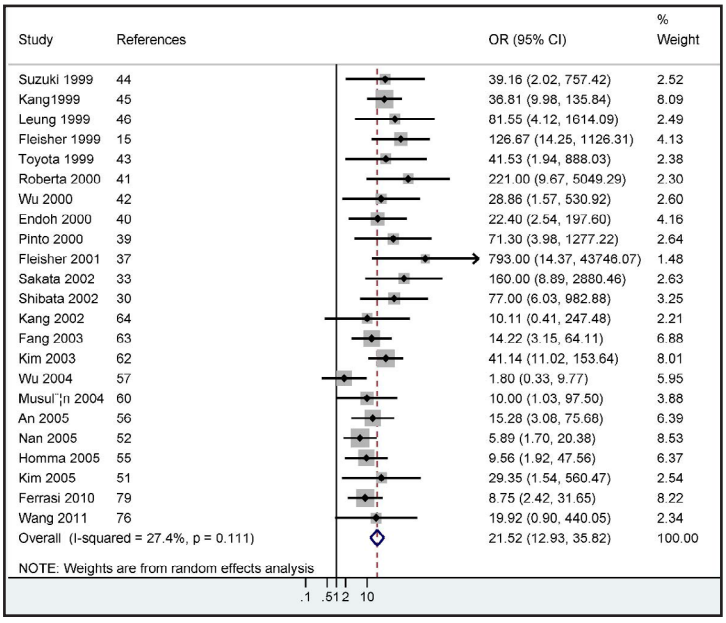

Fig. 10. Forest plot showing the correlation of MLH1 promoter methylation with microsatellite status, MSI vs. MSS: OR = 21.52, 95% CI = 12.93-35.82, P<0.001.

Discussion

GC remains a notable clinical challenge with an unfavorable prognosis. Cancer-related genes, such as tumor suppressor genes (TSGs), or DNA repair genes are commonly methylated in the promoter regions of CpG islands, which leads to the dysfunction or loss of gene expression, cancer initiation and progression [84, 85]. The absence or downregulation of *MLH1* gene expression via promoter methylation was reported in GC [15, 32-34, 36, 37, 39, 42, 44-46, 50, 56, 59, 60, 62, 64, 72, 76, 77]. *MLH1* promoter methylation is detected in some cancers, such as bladder cancer [86], colorectal

cancer [87] and GC [18]. However, some studies reported that *MLH1* promoter methylation exhibited a low frequency in GC [18, 34, 43, 44, 57, 65, 67, 70, 75, 78, 81]. Other studies reported that *MLH1* promoter methylation exhibited a high frequency in GC [16, 33, 40, 62, 72-74, 80]. Therefore, we performed an integrated analysis to investigate whether *MLH1* promoter methylation was a non-invasive biomarker that provided valuable insight for GC diagnosis and clinical outcome and a novel therapeutic target for GC.

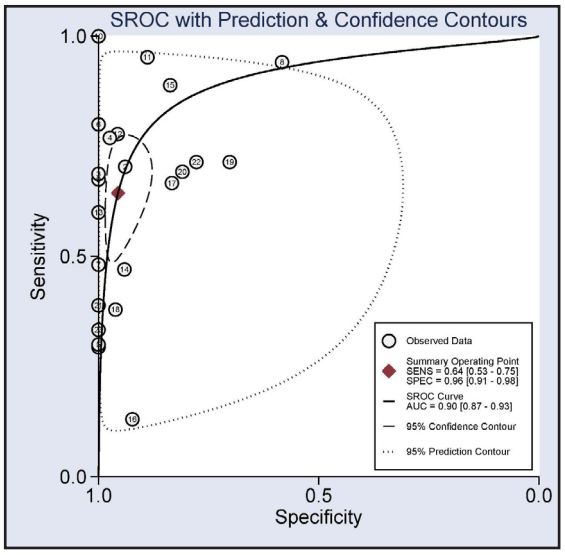

Fig. 11. Summary receiver operating characteristics (SROC) estimation of MLH1 promoter methylation in patients with MSI vs. patients with MSS, sensitivity = 0.64, specificity = 0.96, and AUC = 0.90.

Our results from the data of more articles with larger study populations suggest that *MLH1* promoter methylation is notably higher in GC than in gastric adenomas (OR = 2.44, $P = 0.003$), chronic gastritis (OR = 8.78, $P < 0.001$), adjacent (OR = 5.50, $P < 0.001$) and normal tissue samples (OR = 8.06, $P = 0.011$). *MLH1* promoter methylation exhibited similar levels in GC vs. intestinal metaplasia and dysplasia. We demonstrated a specific methylation profile of the *MLH1* gene during gastric carcinogenesis, from adenoma/chronic gastritis to GC. *MLH1* promoter methylation may play a role in the malignant transformation of gastric precancerous lesions (adenoma and chronic gastritis).

Eligible studies with larger sample sizes revealed that *MLH1* promoter methylation was not associated with tumor histology, gender, *H. pylori* infection, smoking, or drinking behaviors of GC patients. *MLH1* promoter methylation was also not associated with tumor differentiation, clinical stage, lymph node status, distant metastasis, or OS, which indicated that *MLH1* promoter methylation did not play a key role in the progression, metastasis, or prognosis of GC. Hong et al. [54]. observed an association between *MLH1* promoter methylation and age, but other studies reported no correlation [19, 36, 40, 41, 47, 48, 55, 64, 66, 78, 82, 83]. Twenty studies reported that *MLH1* promoter methylation significantly correlated with microsatellite status [15, 30, 33, 37, 39-46, 51, 52, 55, 56, 60, 62, 63, 79], but three studies demonstrated no association [57, 64, 76]. Our study revealed that *MLH1* promoter methylation correlated with age and microsatellite status, and it was notably higher in patients 60 years of age or older than in patients younger than 60 years and higher in patients with MSI than in patients with MSS. These results suggest that *MLH1* promoter methylation plays a more important role in elderly GC patients and GC patients with MSI.

Some studies suggested DNA methylation as a promising tool for the diagnosis of cancer [88-91]. We analyzed the diagnostic effect of *MLH1* promoter methylation in GC for the results with significant OR values and found that *MLH1* promoter methylation could not distinguish GC from adjacent tissues, gastric adenomas, chronic gastritis, normal gastric mucosa, or normal healthy blood samples (i.e., the poor sensitivity value of < 0.5). The existence of cell-free circulating tumor DNA (ctDNA) was found in blood samples, and the presence of promoter methylation of tumor-related genes was examined in the cirDNA in many cancers [92]. Only Kolesnikova et al. reported the existence of ctDNA and *MLH1* promoter methylation in blood samples of GC, with a frequency of 25% in GCs and a frequency of 9% in healthy subjects [83]. The combination of *p15* and *MLH1* promoter methylation in ctDNA exhibited a sensitivity of 65% and specificity of 72% [83], which suggests that the combination of these two genes may significantly contribute to the diagnosis of GC. More studies are needed to analyze the diagnostic effect of tumor DNA circulating in the blood of GC patients to improve

clinical practice. Notably, comparison of GCs with MSI with GCs with MSS demonstrated that *MLH1* promoter methylation exhibited a sensitivity value of 0.64, a specificity value of 0.96, and an AUC value of 0.90. The relevant values of *MLH1* promoter methylation were good in GC with MSI vs. GC with MSS. We also found that the *MLH1* promoter in patients with MSI exhibited a significantly higher mean methylation level than that in patients with MSS (0.622 vs. 0.104). *MLH1* dysfunction via methylation of the promoter likely leads to MSI. Therefore, the above analyses suggest that *MLH1* promoter methylation may be a prognostic marker for GC patients with MSI.

Several limitations should be considered in this study. First, there was substantial heterogeneity in GC vs. adjacent tissues and gender, and seven studies [31, 40, 61, 62, 68, 73, 74] were removed in the comparison of GC and adjacent tissue samples. Three studies [47, 54, 73] were excluded in relation to gender. The pooled results were not significantly changed, with no evidence of heterogeneity, which indicates the stability of our analyses. Second, publication bias was measured in GC vs. adjacent tissue samples and tumor histology. We searched the relevant databases to minimize the possible publication bias as completely as possible, but positive results are more easily published than negative results. Only articles published in the English language were selected, and articles in languages other than English were excluded. Third, sample sizes for the comparison between GC and benign lesions and GC and normal controls were not very large. Finally, sample sizes of subgroup analyses of mixed populations and non-MSP method were small.

Conclusion

Our results suggest that *MLH1* promoter methylation exhibits a significantly higher frequency in GC than gastric adenoma, chronic gastritis, adjacent tissues, normal gastric mucosa, and normal healthy blood samples but a similar rate in GC and intestinal metaplasia and dysplasia. *MLH1* promoter methylation correlated with age and the MSI of GC patients, but it was not associated with *H. pylori* infection, gender, smoking, drinking behaviors, tumor histology, tumor differentiation, clinical stage, lymph node status, distant metastasis, or the OS of GC patients. The use of *MLH1* promoter methylation may be a potential prognostic biomarker for GC patients with MSI. More well-designed prospective trials are necessary to further validate our findings.

Abbreviations

*MLH1 (*human mutL homolog 1*);* GC (gastric cancer); MSI (microsatellite instability); MSS (microsatellite stability); AUC (area under the curve); *H. pylori (Helicobacter pylori);* MMR (mismatch repair); OS (overall survival); OR (odds ratio); 95% CI (95% confidence interval); HR (hazard ratio); MSP (methylation-specific PCR); TSG (tumor suppressor gene);

Acknowledgements

The research was supported by a grant from the Natural Science Foundation of Ningbo City (2006A610122).

Tao Huang and Guoliang Ye contributed to the conception, design and final approval of the submitted manuscript version. Guimei Hu, Lijun Qin, Xinjun Zhang, Guoliang Ye, and Tao Huang contributed to data interpretation and the completion of Figs. and tables. All authors read and approved the final manuscript.

Disclosure Statement

The authors declare no competing financial interests.

References

1 Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A: Global cancer statistics, 2012. CA Cancer J Clin 2015;65:87-108.
2 Yuasa Y: Control of gut differentiation and intestinal-type gastric carcinogenesis. Nat Rev Cancer 2003;3:592-600.
3 Veitch AM, Uedo N, Yao K, East JE: Optimizing early upper gastrointestinal cancer detection at endoscopy. Nat Rev Gastroenterol Hepatol 2015;12:660-667.
4 Thrumurthy SG, Chaudry MA, Chau I, Allum W: Does surgery have a role in managing incurable gastric cancer? Nat Rev Clin Oncol 2015;12:676-682.
5 Ye M, Huang T, Ying Y, Li J, Yang P, Ni C, Zhou C, Chen S: Detection of 14-3-3 sigma (sigma) promoter methylation as a noninvasive biomarker using blood samples for breast cancer diagnosis. Oncotarget 2017;8:9230-9242.
6 Reid T, Oronsky B, Scicinski J, Scribner CL, Knox SJ, Ning S, Peehl DM, Korn R, Stirn M, Carter CA, Oronsky A, Taylor MJ, Fitch WL, Cabrales P, Kim MM, Burris HA, 3rd, Lao CD, Abrouk NE, Fanger GR, Infante JR: Safety and activity of RRx-001 in patients with advanced cancer: a first-in-human, open-label, dose-escalation phase 1 study. Lancet Oncol 2015;16:1133-1142.
7 Villanueva A, Portela A, Sayols S, Battiston C, Hoshida Y, Mendez-Gonzalez J, Imbeaud S, Letouze E, Hernandez-Gea V, Cornella H, Pinyol R, Sole M, Fuster J, Zucman-Rossi J, Mazzaferro V, Esteller M, Llovet JM, Consortium H: DNA methylation-based prognosis and epidrivers in hepatocellular carcinoma. Hepatology 2015;61:1945-1956.
8 Graham DY: Helicobacter pylori update: gastric cancer, reliable therapy, and possible benefits. Gastroenterology 2015;148:719-731 e713.
9 McLean MH, El-Omar EM: Genetics of gastric cancer. Nat Rev Gastroenterol Hepatol 2014;11:664-674.
10 Papadopoulos N, Nicolaides NC, Wei YF, Ruben SM, Carter KC, Rosen CA, Haseltine WA, Fleischmann RD, Fraser CM, Adams MD, et al.: Mutation of a mutL homolog in hereditary colon cancer. Science 1994;263:1625-1629.
11 Yoon K, Lee S, Han TS, Moon SY, Yun SM, Kong SH, Jho S, Choe J, Yu J, Lee HJ, Park JH, Kim HM, Lee SY, Park J, Kim WH, Bhak J, Yang HK, Kim SJ: Comprehensive genome- and transcriptome-wide analyses of mutations associated with microsatellite instability in Korean gastric cancers. Genome Res 2013;23:1109-1117.
12 Ottini L, Falchetti M, Lupi R, Rizzolo P, Agnese V, Colucci G, Bazan V, Russo A: Patterns of genomic instability in gastric cancer: clinical implications and perspectives. Ann Oncol 2006;17 Suppl 7:vii97-102.
13 Parsons MT, Buchanan DD, Thompson B, Young JP, Spurdle AB: Correlation of tumour BRAF mutations and MLH1 methylation with germline mismatch repair (MMR) gene mutation status: a literature review assessing utility of tumour features for MMR variant classification. J Med Genet 2012;49:151-157.
14 Yamamoto H, Imai K: Microsatellite instability: an update. Arch Toxicol 2015;89:899-921.
15 Fleisher AS, Esteller M, Wang S, Tamura G, Suzuki H, Yin J, Zou TT, Abraham JM, Kong D, Smolinski KN, Shi YQ, Rhyu MG, Powell SM, James SP, Wilson KT, Herman JG, Meltzer SJ: Hypermethylation of the hMLH1 gene promoter in human gastric cancers with microsatellite instability. Cancer Res 1999;59:1090-1095.
16 Sabry D, Ahmed R, Abdalla S, Fathy W, Eldemery A, Elamir A: Braf, Kras and Helicobacter pylori epigenetic changes-associated chronic gastritis in Egyptian patients with and without gastric cancer. World J Microbiol Biotechnol 2016;32:92.
17 Liu L, Yang X: Implication of Reprimo and hMLH1 gene methylation in early diagnosis of gastric carcinoma. Int J Clin Exp Pathol 2015;8:14977-14982.
18 Jin J, Xie L, Xie CH, Zhou YF: Aberrant DNA methylation of MGMT and hMLH1 genes in prediction of gastric cancer. Genet Mol Res 2014;13:4140-4145.
19 Wang M, Li Y, Gao J, Li Y, Zhou J, Gu L, Shen L, Deng D: p16 Methylation is associated with chemosensitivity to fluorouracil in patients with advanced gastric cancer. Med Oncol 2014;31:988.

20 Lee JH, Park SJ, Abraham SC, Seo JS, Nam JH, Choi C, Juhng SW, Rashid A, Hamilton SR, Wu TT: Frequent CpG island methylation in precursor lesions and early gastric adenocarcinomas. Oncogene 2004;23:4646-4654.
21 Kang GH, Lee S, Kim JS, Jung HY: Profile of aberrant CpG island methylation along multistep gastric carcinogenesis. Lab Invest 2003;83:519-526.
22 Zintzaras E, Ioannidis JP: HEGESMA: genome search meta-analysis and heterogeneity testing. Bioinformatics 2005;21:3672-3673.
23 Han S, Zong S, Shi Q, Li H, Liu S, Yang W, Li W, Hou F: Is Ep-CAM Expression a Diagnostic and Prognostic Biomarker for Colorectal Cancer? A Systematic Meta-Analysis. EBioMedicine 2017;20:61-69.
24 Zintzaras E, Ioannidis JP: Heterogeneity testing in meta-analysis of genome searches. Genet Epidemiol 2005;28:123-137.
25 Lau J, Ioannidis JP, Schmid CH: Quantitative synthesis in systematic reviews. Ann Intern Med 1997;127:820-826.
26 Peters JL, Sutton AJ, Jones DR, Abrams KR, Rushton L: Comparison of two methods to detect publication bias in meta-analysis. JAMA 2006;295:676-680.
27 Reitsma JB, Glas AS, Rutjes AW, Scholten RJ, Bossuyt PM, Zwinderman AH: Bivariate analysis of sensitivity and specificity produces informative summary measures in diagnostic reviews. J Clin Epidemiol 2005;58:982-990.
28 Jones CM, Athanasiou T: Summary receiver operating characteristic curve analysis techniques in the evaluation of diagnostic tests. Ann Thorac Surg 2005;79:16-20.
29 To KF, Leung WK, Lee TL, Yu J, Tong JH, Chan MW, Ng EK, Chung SC, Sung JJ: Promoter hypermethylation of tumor-related genes in gastric intestinal metaplasia of patients with and without gastric cancer. Int J Cancer 2002;102:623-628.
30 Shibata DM, Sato F, Mori Y, Perry K, Yin J, Wang S, Xu Y, Olaru A, Selaru F, Spring K, Young J, Abraham JM, Meltzer SJ: Hypermethylation of HPP1 is associated with hMLH1 hypermethylation in gastric adenocarcinomas. Cancer Res 2002;62:5637-5640.
31 Waki T, Tamura G, Tsuchiya T, Sato K, Nishizuka S, Motoyama T: Promoter methylation status of E-cadherin, hMLH1, and p16 genes in nonneoplastic gastric epithelia. Am J Pathol 2002;161:399-403.
32 Oue N, Sentani K, Yokozaki H, Kitadai Y, Ito R, Yasui W: Promoter methylation status of the DNA repair genes hMLH1 and MGMT in gastric carcinoma and metaplastic mucosa. Pathobiology 2001;69:143-149.
33 Sakata K, Tamura G, Endoh Y, Ohmura K, Ogata S, Motoyama T: Hypermethylation of the hMLH1 gene promoter in solitary and multiple gastric cancers with microsatellite instability. Br J Cancer 2002;86:564-567.
34 Nakajima T, Akiyama Y, Shiraishi J, Arai T, Yanagisawa Y, Ara M, Fukuda Y, Sawabe M, Saitoh K, Kamiyama R, Hirokawa K, Yuasa Y: Age-related hypermethylation of the hMLH1 promoter in gastric cancers. Int J Cancer 2001;94:208-211.
35 Oue N, Kuraoka K, Kuniyasu H, Yokozaki H, Wakikawa A, Matsusaki K, Yasui W: DNA methylation status of hMLH1, p16(INK4a), and CDH1 is not associated with mRNA expression levels of DNA methyltransferase and DNA demethylase in gastric carcinomas. Oncol Rep 2001;8:1085-1089.
36 Leung WK, Yu J, Ng EK, To KF, Ma PK, Lee TL, Go MY, Chung SC, Sung JJ: Concurrent hypermethylation of multiple tumor-related genes in gastric carcinoma and adjacent normal tissues. Cancer 2001;91:2294-2301.
37 Fleisher AS, Esteller M, Tamura G, Rashid A, Stine OC, Yin J, Zou TT, Abraham JM, Kong D, Nishizuka S, James SP, Wilson KT, Herman JG, Meltzer SJ: Hypermethylation of the hMLH1 gene promoter is associated with microsatellite instability in early human gastric neoplasia. Oncogene 2001;20:329-335.
38 Kang GH, Shim YH, Jung HY, Kim WH, Ro JY, Rhyu MG: CpG island methylation in premalignant stages of gastric carcinoma. Cancer Res 2001;61:2847-2851.
39 Pinto M, Oliveira C, Machado JC, Cirnes L, Tavares J, Carneiro F, Hamelin R, Hofstra R, Seruca R, Sobrinho-Simoes M: MSI-L gastric carcinomas share the hMLH1 methylation status of MSI-H carcinomas but not their clinicopathological profile. Lab Invest 2000;80:1915-1923.
40 Endoh Y, Tamura G, Ajioka Y, Watanabe H, Motoyama T: Frequent hypermethylation of the hMLH1 gene promoter in differentiated-type tumors of the stomach with the gastric foveolar phenotype. Am J Pathol 2000;157:717-722.
41 Bevilacqua RA, Simpson AJ: Methylation of the hMLH1 promoter but no hMLH1 mutations in sporadic gastric carcinomas with high-level microsatellite instability. Int J Cancer 2000;87:200-203.

42 Wu MS, Lee CW, Shun CT, Wang HP, Lee WJ, Chang MC, Sheu JC, Lin JT: Distinct clinicopathologic and genetic profiles in sporadic gastric cancer with different mutator phenotypes. Genes Chromosomes Cancer 2000;27:403-411.
43 Toyota M, Ahuja N, Suzuki H, Itoh F, Ohe-Toyota M, Imai K, Baylin SB, Issa JP: Aberrant methylation in gastric cancer associated with the CpG island methylator phenotype. Cancer Res 1999;59:5438-5442.
44 Suzuki H, Itoh F, Toyota M, Kikuchi T, Kakiuchi H, Hinoda Y, Imai K: Distinct methylation pattern and microsatellite instability in sporadic gastric cancer. Int J Cancer 1999;83:309-313.
45 Kang GH, Shim YH, Ro JY: Correlation of methylation of the hMLH1 promoter with lack of expression of hMLH1 in sporadic gastric carcinomas with replication error. Lab Invest 1999;79:903-909.
46 Leung SY, Yuen ST, Chung LP, Chu KM, Chan AS, Ho JC: hMLH1 promoter methylation and lack of hMLH1 expression in sporadic gastric carcinomas with high-frequency microsatellite instability. Cancer Res 1999;59:159-164.
47 Poplawski T, Tomaszewska K, Galicki M, Morawiec Z, Blasiak J: Promoter methylation of cancer-related genes in gastric carcinoma. Exp Oncol 2008;30:112-116.
48 Chang MS, Uozaki H, Chong JM, Ushiku T, Sakuma K, Ishikawa S, Hino R, Barua RR, Iwasaki Y, Arai K, Fujii H, Nagai H, Fukayama M: CpG island methylation status in gastric carcinoma with and without infection of Epstein-Barr virus. Clin Cancer Res 2006;12:2995-3002.
49 Shibata D, Mori Y, Cai K, Zhang L, Yin J, Elahi A, Hamelin R, Wong YF, Lo WK, Chung TK, Sato F, Karpeh MS Jr, Meltzer SJ: RAB32 hypermethylation and microsatellite instability in gastric and endometrial adenocarcinomas. Int J Cancer 2006;119:801-806.
50 Oue N, Mitani Y, Motoshita J, Matsumura S, Yoshida K, Kuniyasu H, Nakayama H, Yasui W: Accumulation of DNA methylation is associated with tumor stage in gastric cancer. Cancer 2006;106:1250-1259.
51 Kim HC, Kim JC, Roh SA, Yu CS, Yook JH, Oh ST, Kim BS, Park KC, Chang R: Aberrant CpG island methylation in early-onset sporadic gastric carcinoma. J Cancer Res Clin Oncol 2005;131:733-740.
52 Nan HM, Song YJ, Yun HY, Park JS, Kim H: Effects of dietary intake and genetic factors on hypermethylation of the hMLH1 gene promoter in gastric cancer. World J Gastroenterol 2005;11:3834-3841.
53 Leung WK, To KF, Chu ES, Chan MW, Bai AH, Ng EK, Chan FK, Sung JJ: Potential diagnostic and prognostic values of detecting promoter hypermethylation in the serum of patients with gastric cancer. Br J Cancer 2005;92:2190-2194.
54 Hong SH, Kim HG, Chung WB, Kim EY, Lee JY, Yoon SM, Kwon JG, Sohn YK, Kwak EK, Kim JW: DNA hypermethylation of tumor-related genes in gastric carcinoma. J Korean Med Sci 2005;20:236-241.
55 Homma N, Tamura G, Honda T, Jin Z, Ohmura K, Kawata S, Motoyama T: Hypermethylation of Chfr and hMLH1 in gastric noninvasive and early invasive neoplasias. Virchows Arch 2005;446:120-126.
56 An C, Choi IS, Yao JC, Worah S, Xie K, Mansfield PF, Ajani JA, Rashid A, Hamilton SR, Wu TT: Prognostic significance of CpG island methylator phenotype and microsatellite instability in gastric carcinoma. Clin Cancer Res 2005;11:656-663.
57 Wu M, Semba S, Oue N, Ikehara N, Yasui W, Yokozaki H: BRAF/K-ras mutation, microsatellite instability, and promoter hypermethylation of hMLH1/MGMT in human gastric carcinomas. Gastric Cancer 2004;7:246-253.
58 Yuasa Y, Nagasaki H, Akiyama Y, Sakai H, Nakajima T, Ohkura Y, Takizawa T, Koike M, Tani M, Iwai T, Sugihara K, Imai K, Nakachi K: Relationship between CDX2 gene methylation and dietary factors in gastric cancer patients. Carcinogenesis 2005;26:193-200.
59 Geddert H, Kiel S, Iskender E, Florl AR, Krieg T, Vossen S, Gabbert HE, Sarbia M: Correlation of hMLH1 and HPP1 hypermethylation in gastric, but not in esophageal and cardiac adenocarcinoma. Int J Cancer 2004;110:208-211.
60 Musulen E, Moreno V, Reyes G, Sancho FJ, Peinado MA, Esteller M, Herman JG, Combalia N, Rey M, Capella G: Standardized approach for microsatellite instability detection in gastric carcinomas. Hum Pathol 2004;35:335-342.
61 Etoh T, Kanai Y, Ushijima S, Nakagawa T, Nakanishi Y, Sasako M, Kitano S, Hirohashi S: Increased DNA methyltransferase 1 (DNMT1) protein expression correlates significantly with poorer tumor differentiation and frequent DNA hypermethylation of multiple CpG islands in gastric cancers. Am J Pathol 2004;164:689-699.
62 Kim H, Kim YH, Kim SE, Kim NG, Noh SH, Kim H: Concerted promoter hypermethylation of hMLH1, p16INK4A, and E-cadherin in gastric carcinomas with microsatellite instability. J Pathol 2003;200:23-31.

63 Fang DC, Wang RQ, Yang SM, Yang JM, Liu HF, Peng GY, Xiao TL, Luo YH: Mutation and methylation of hMLH1 in gastric carcinomas with microsatellite instability. World J Gastroenterol 2003;9:655-659.
64 Kang GH, Lee S, Shim YH, Kim JC, Ro JY: Profile of methylated CpG sites of hMLH1 promoter in primary gastric carcinoma with microsatellite instability. Pathol Int 2002;52:764-768.
65 Deng DJ, Zhou J, Zhu BD, Ji JF, Harper JC, Powell SM: Silencing-specific methylation and single nucleotide polymorphism of hMLH1 promoter in gastric carcinomas. World J Gastroenterol 2003;9:26-29.
66 Li Y, Yang Y, Lu Y, Herman JG, Brock MV, Zhao P, Guo M: Predictive value of CHFR and MLH1 methylation in human gastric cancer. Gastric Cancer 2015;18:280-287.
67 Song B, Ai J, Kong X, Liu D, Li J: Aberrant DNA Methylation of P16, MGMT, and hMLH1 Genes in Combination with MTHFR C677T Genetic Polymorphism in gastric cancer. Pak J Med Sci 2013;29:1338-1343.
68 Kupcinskaite-Noreikiene R, Skieceviciene J, Jonaitis L, Ugenskiene R, Kupcinskas J, Markelis R, Baltrenas V, Sakavicius L, Semakina I, Grizas S, Juozaityte E: CpG island methylation of the MLH1, MGMT, DAPK, and CASP8 genes in cancerous and adjacent noncancerous stomach tissues. Medicina (Kaunas) 2013;49:361-366.
69 Moghbeli M, Moaven O, Memar B, Raziei HR, Aarabi A, Dadkhah E, Forghanifard MM, Manzari F, Abbaszadegan MR: Role of hMLH1 and E-cadherin promoter methylation in gastric cancer progression. J Gastrointest Cancer 2014;45:40-47.
70 Xiong HL, Liu XQ, Sun AH, He Y, Li J, Xia Y: Aberrant DNA methylation of P16, MGMT, hMLH1 and hMSH2 genes in combination with the MTHFR C677T genetic polymorphism in gastric cancer. Asian Pac J Cancer Prev 2013;14:3139-3142.
71 Alvarez MC, Santos JC, Maniezzo N, Ladeira MS, da Silva AL, Scaletsky IC, Pedrazzoli J, Jr., Ribeiro ML: MGMT and MLH1 methylation in Helicobacter pylori-infected children and adults. World J Gastroenterol 2013;19:3043-3051.
72 Kim KJ, Lee TH, Cho NY, Yang HK, Kim WH, Kang GH: Differential clinicopathologic features in microsatellite-unstable gastric cancers with and without MLH1 methylation. Hum Pathol 2013;44:1055-1064.
73 Wani M, Afroze D, Makhdoomi M, Hamid I, Wani B, Bhat G, Wani R, Wani K: Promoter methylation status of DNA repair gene (hMLH1) in gastric carcinoma patients of the Kashmir valley. Asian Pac J Cancer Prev 2012;13:4177-4181.
74 Mir MR, Shabir N, Wani KA, Shaff S, Hussain I, Banday MA, Chikan NA, Bilal S, Aejaz S: Association between p16, hMLH1 and E-cadherin promoter hypermethylation and intake of local hot salted tea and sun-dried foods in Kashmiris with gastric tumors. Asian Pac J Cancer Prev 2012;13:181-186.
75 Wu PY, Zhang Z, Wang JM, Guo WW, Xiao N, He Q, Wang YP, Fan YM: Germline promoter hypermethylation of tumor suppressor genes in gastric cancer. World J Gastroenterol 2012;18:70-78.
76 Wang X, Fan J, Liu D, Fu S, Ingvarsson S, Chen H: Spreading of Alu methylation to the promoter of the MLH1 gene in gastrointestinal cancer. PLoS One 2011;6:e25913.
77 Alves MK, Ferrasi AC, Lima VP, Ferreira MV, de Moura Campos Pardini MI, Rabenhorst SH: Inactivation of COX-2, HMLH1 and CDKN2A gene by promoter methylation in gastric cancer: relationship with histological subtype, tumor location and Helicobacter pylori genotype. Pathobiology 2011;78:266-276.
78 Mikata R, Fukai K, Imazeki F, Arai M, Fujiwara K, Yonemitsu Y, Zhang K, Nabeya Y, Ochiai T, Yokosuka O: BCL2L10 is frequently silenced by promoter hypermethylation in gastric cancer. Oncol Rep 2010;23:1701-1708.
79 Ferrasi AC, Pinheiro NA, Rabenhorst SH, Caballero OL, Rodrigues MA, de Carvalho F, Leite CV, Ferreira MV, Barros MA, Pardini MI: Helicobacter pylori and EBV in gastric carcinomas: methylation status and microsatellite instability. World J Gastroenterol 2010;16:312-319.
80 Hiraki M, Kitajima Y, Sato S, Mitsuno M, Koga Y, Nakamura J, Hashiguchi K, Noshiro H, Miyazaki K: Aberrant gene methylation in the lymph nodes provides a possible marker for diagnosing micrometastasis in gastric cancer. Ann Surg Oncol 2010;17:1177-1186.
81 Ksiaa F, Ziadi S, Amara K, Korbi S, Trimeche M: Biological significance of promoter hypermethylation of tumor-related genes in patients with gastric carcinoma. Clin Chim Acta 2009;404:128-133.
82 Moura Lima E, Ferreira Leal M, Cardoso Smith Mde A, Rodriguez Burbano R, Pimentel de Assumpcao P, Bello MJ, Rey JA, Ferreira de Lima F, Casartelli C: DNA mismatch repair gene methylation in gastric cancer in individuals from northern Brazil. Biocell 2008;32:237-243.

83 Kolesnikova EV, Tamkovich SN, Bryzgunova OE, Shelestyuk PI, Permyakova VI, Vlassov VV, Tuzikov AS, Laktionov PP, Rykova EY: Circulating DNA in the blood of gastric cancer patients. Ann N Y Acad Sci 2008;1137:226-231.

84 Tamura G: Promoter methylation status of tumor suppressor and tumor-related genes in neoplastic and non-neoplastic gastric epithelia. Histol Histopathol 2004;19:221-228.

85 Herman JG, Baylin SB: Gene silencing in cancer in association with promoter hypermethylation. N Engl J Med 2003;349:2042-2054.

86 Wojtczyk-Miaskowska A, Presler M, Michajlowski J, Matuszewski M, Schlichtholz B: Gene Expression, DNA Methylation and Prognostic Significance of DNA Repair Genes in Human Bladder Cancer. Cell Physiol Biochem 2017;42:2404-2417.

87 Li X, Yao X, Wang Y, Hu F, Wang F, Jiang L, Liu Y, Wang D, Sun G, Zhao Y: MLH1 promoter methylation frequency in colorectal cancer patients and related clinicopathological and molecular features. PLoS One 2013;8:e59064.

88 Liu Y, Wu H, Zhou Q, Song Q, Rui J, Zou B, Zhou G: Digital quantification of gene methylation in stool DNA by emulsion-PCR coupled with hydrogel immobilized bead-array. Biosens Bioelectron 2017;92:596-601.

89 Yoshida W, Yoshioka H, Bay DH, Iida K, Ikebukuro K, Nagasawa K, Karube I: Detection of DNA Methylation of G-Quadruplex and i-Motif-Forming Sequences by Measuring the Initial Elongation Efficiency of Polymerase Chain Reaction. Anal Chem 2016;88:7101-7107.

90 Diaz-Lagares A, Mendez-Gonzalez J, Hervas D, Saigi M, Pajares MJ, Garcia D, Crujerias AB, Pio R, Montuenga LM, Zulueta J, Nadal E, Rosell A, Esteller M, Sandoval J: A Novel Epigenetic Signature for Early Diagnosis in Lung Cancer. Clin Cancer Res 2016;22:3361-3371.

91 Ye M, Huang T, Ni C, Yang P, Chen S: Diagnostic Capacity of RASSF1A Promoter Methylation as a Biomarker in Tissue, Brushing, and Blood Samples of Nasopharyngeal Carcinoma. EBioMedicine 2017;18:32-40.

92 Salvianti F, Orlando C, Massi D, De Giorgi V, Grazzini M, Pazzagli M, Pinzani P: Tumor-Related Methylated Cell-Free DNA and Circulating Tumor Cells in Melanoma. Front Mol Biosci 2015;2:76.