Original Article
Association between *hMLH1* promoter methylation and gastric cancer in East Asians: a meta-analysis

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Abstract: *Background:* The *hMLH1* gene is a key member of DNA damage repair genes. The studies on the relationship between *hMLH1* promoter methylation and gastric cancer are frequently appearing in East Asians. However, the exact conclusion is not clear. To better understand the association between gastric cancer and *hMLH1* promoter methylation, we performed a comprehensive meta-analysis. *Methods:* We conducted a meta-analysis by systematically reviewing the related articles which were published in English or Chinese and concerned on *hMLH1* promoter methylation and gastric cancer in East Asians. Finally 17 studies with 1943 cases and 1575 controls were included in this meta-analysis. Odds ratios and their 95% confidence intervals were used to reveal the quantitative relationship between gastric cancer and *hMLH1* promoter methylation. *Results:* A strong significant association was observed between *hMLH1* promoter methylation and gastric cancer. The frequencies of *hMLH1* promoter methylation in gastric cancer tissues were higher than those of adjacent normal tissues (OR=15.73, 95% CI=8.05-30.75) and cancer-free subjects (OR=19.03, 95% CI=7.83-46.23). In addition, we observed significant association of *hMLH1* promoter methylation with age, gender, histological differentiation and lymph node metastasis (age: OR=0.53, 95% CI=0.36-0.79; gender: OR=0.71, 95% CI=0.52-0.96; histological differentiation: OR=2.26, 95% CI=1.06-4.81; lymph node metastasis: OR=0.59, 95% CI=0.39-0.88), but no association of *hMLH1* promoter methylation status with peritoneal or distant metastasis and *Helicobacter pylori* infection (peritoneal or distant metastasis: OR=0.56, 95% CI=0.28-1.15; *Helicobacter pylori* infection: OR=2.02, 95% CI=0.96-4.25). *Conclusion:* Our investigations demonstrated that strong associations exist between *hMLH1* gene methylation and gastric cancer in populations of East Asia.

Keywords: *hMLH1*, methylation, gastric cancer, meta-analysis

Introduction

Gastric cancer is one of the most common digestive track malignant tumors all over the world. East Asia is the region with the highest incidence rate of gastric cancer [1]. In China alone, 400000 new cases of gastric cancer were diagnosed each year which accounted for over 40% of the total number in the world, the large number, poor prognosis and limited treatment options have made gastric cancer become a major health burden in East Asia [2]. The progress of gastric cancer involves a complex process of multiple factors, multiple genes and multiple steps of common participation, the details of the process is not completely understood. Previous studies indicated that environmental factors such as eating habits, smoking, alcohol consumption and *Helicobacter pylori* infection are related to gastric cancer. With the development of tumor molecular biology, genetic factors which include a number of genetic and epigenetic alterations of tumor-related and tumor suppressor genes have also been confirmed to involved in the pathogenesis of gastric cancer [3-5]. Recent studies have shown that abnormal promoter methylation of some specific genes may play a critical role in gastric tumorigenesis [6-8].

DNA mismatch repair (MMR) genes play a very important role in keeping genetic stability to avoid the occurrence of gene mutation [9]. In gene replication stage, MMR defection caused the recognition and repair of DNA mismatches cannot be completed, which lead to a higher

Table 1. Basic characteristics of included studies

Author	Year	Country	Language	Gender (M/F)	Mean/median Age (range age)	Neoplasm	Adjacent	Normal	Method
Wang MM [28]	2014	China	English	109/25	59	134	46	-	MSP
Jin J [22]	2014	China	English	152/131	53.7±10.3	283	283	-	MSP
Li YZH [25]	2014	China	English	73/30	53 (26-76)	102	-	-	MSP
Song BB [30]	2013	China	English	173/149	54.5±8.5	322	322	-	MSP
Xiong HL [31]	2013	China	English	222/191	52.8±10.3	413	413	-	MSP
Zhang KL [33]	2008	China	English	34/13	-	47	-	31	MSP
Huang Q [19]	2007	China	English	29/20	51 (15-77)	49	-	-	MSP
Wai KL [23]	2001	China	English	14/12	65.1 (39-83)	26	25	-	MSP
Li DX [24]	2013	China	Chinese	30/15	57.5 (20-77)	45	21	-	MSP
Jiao HL [52]	2008	China	Chinese	23/18	54 (38-72)	41	-	38	MSP
Wu ACH [29]	2008	China	Chinese	42/18	62 (35-80)	60	60	-	MSP
RINTARO [20]	2010	Japan	English	14/7	70 (56-85)	21	21	-	MSP
Naohide O [27]	2006	Japan	English	52/23	68.6 (34-87)	75	25	-	MSP
Yasuhito Y [32]	2005	Japan	English	58/15	-	73	-	-	MSP
Naoyuki H [17]	2004	Japan	English	43/9	69.7 (49-83)	52	52	-	MSP
Tomoko N [26]	2001	Japan	English	63/37	69.5 (43-99)	100	100	0	COBRA
Su HH [18]	2005	Korea	English	64/36	62	100	-	238	MSP

mutation rate [10]. Mutation and promoter methylation of MMR have been confirmed to be related to human cancer [11, 12]. Human mutL homolog 1 gene (*hMLH1*) is a major part of MMR, aberrant DNA methylation of *hMLH1* gene promoter is an important epigenetic alteration involved in silencing of MMR gene. A lot of Studies have shown that aberrant DNA methylation of *hMLH1* promoter associated with many human cancer types [13-16].

Many studies have discussed about the relationship between aberrant methylation of *hMLH1* promoter and gastric cancer in East Asians, however the results of the related studies are unsatisfactory. Due to the limitations of small sample sizes, low statistical power, selection bias and other mistakes in a single study, a systemic meta-analysis combine all the available studies needs to be performed.

Materials and methods

Publication search

All studies published before January 1st, 2016 that investigated the association between the *hMLH1* promoter methylation and gastric cancer in populations of East Asia were considered in this meta-analysis. We conducted a systematic search via PubMed, Web of Science, China National Knowledge Infrastructrue (CNKI) using

the following keywords: ["methylation" OR "hypermethylation"] AND ["hMLH1"] AND ["gastric cancer" OR "gastric carcinoma" OR "gastric tumor" OR "gastric neoplasm" OR "stomach cancer"]. The articles included in the meta-analysis were limited to be published in only English and Chinese. More available articles were further collected by reviewing the bibliographies to make sure that all relevant articles were included.

Inclusion and exclusion criteria

Studies were included and excluded according to the following criteria: 1) Case-control and case-cohort studies which assessed the association of *hMLH1* methylation and gastric cancer. 2) The study population is East Asian (Chinese, Korean or Japanese). 3) All the included studies must provide original data about the frequency of *hMLH1* promoter methylation. 4) All patients must be histologically identified with gastric cancer. 5) If some studies may have overlapping data, we selected the study with the largest sample size.

Data extraction

Two investigators independently extracted the following information from each included article: language of publication, first author's name, publication year, design of study, num-
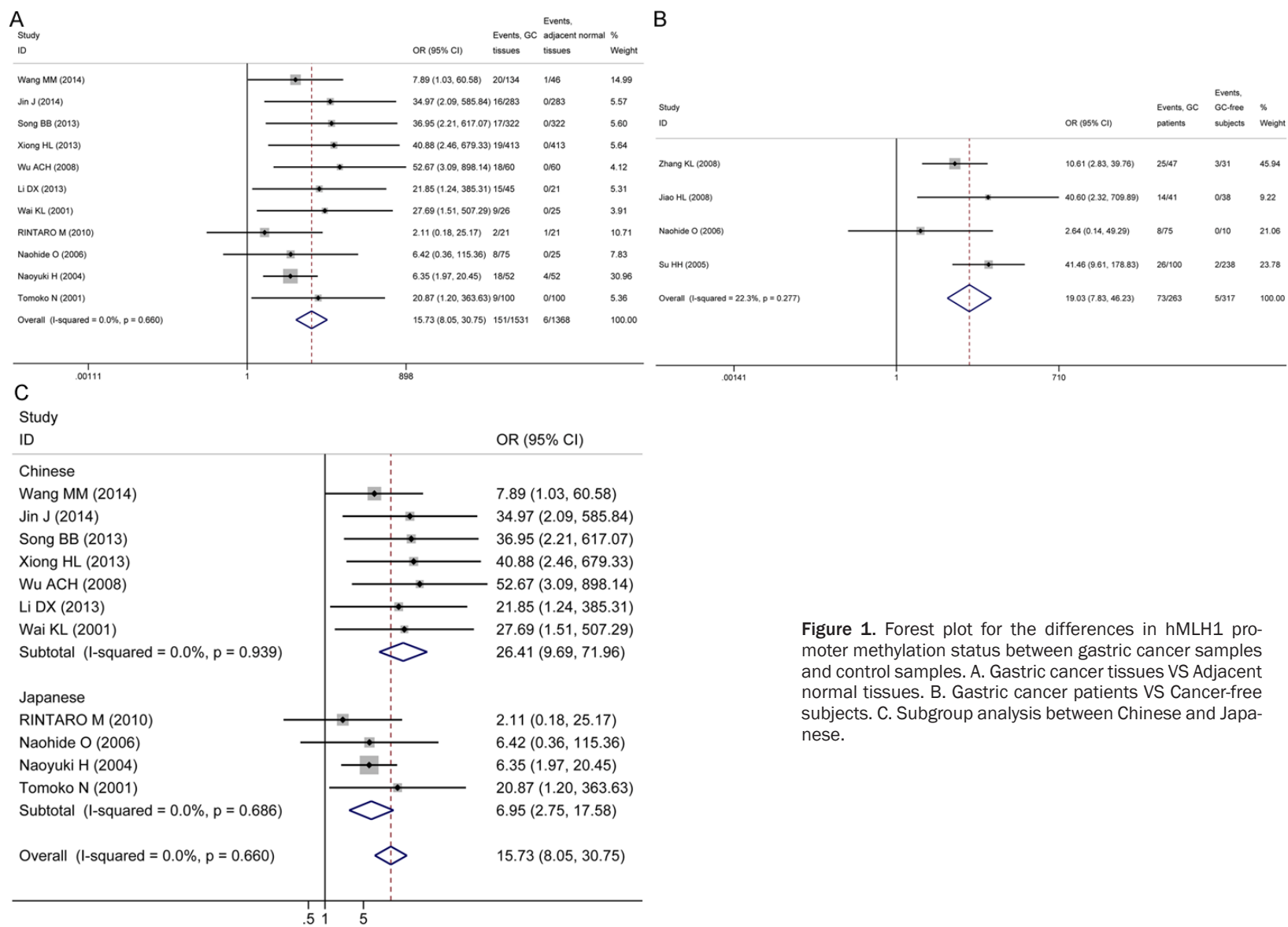
Figure 1. Forest plot for the differences in hMLH1 promoter methylation status between gastric cancer samples and control samples. A. Gastric cancer tissues VS Adjacent normal tissues. B. Gastric cancer patients VS Cancer-free subjects. C. Subgroup analysis between Chinese and Japanese.

Table 2. Summary of relationships between clinicopathological characteristics and hMLH1 methylation status

Clinicopathological characteristics	Pooled OR	95% CI	P value
Age (<60 VS ≥60)	0.530	0.357-0.788	0.002
Gender (Male VS Female)	0.708	0.522-0.960	0.026
Histological differentiation (Poor VS Good)	2.262	1.064-4.809	0.034
Peritoneal or distant metastasis (M0 VS M1)	0.564	0.278-1.145	0.113
Helicobacter pylori infection (HP+ VS HP-)	2.020	0.961-4.247	0.064
lymph node metastasis (N0 VS N1)	0.586	0.389-0.882	0.011

bers of cases and controls, the source of samples, methylation frequencies, detection methods of methylation analysis, etc. If the two investigators have inconsistent conclusions which cannot be unified until after discussion, another investigator would participate in the discussion and give a final vote to make the conclusion.

Statistical analysis

We used STATA version 12.0 software for the meta-analysis. The strength of the association between the *hMLH1* promoter methylation and gastric cancer was measured by pooled odds ratios (ORs) and their 95% confidence intervals (CIs). The significance of pooled ORs was determined by Z test. Q test and I^2 were used to assess the heterogeneity among the studies. If the values of Q test <0.05 or I^2>50% which indicated the existence of significant heterogeneity, the random effects model would be used, otherwise we selected the fixed effects model. Sensitivity analysis was carried out to test a single study on the influence of overall results. Publication bias was estimated by funnel plot and Egger's liner regression, of which P<0.05 was considered to be a statistically significant publication bias.

Results

Characteristics of studies

By searching the keywords, 344 English articles and 60 Chinese articles were retrieved, and 261 of them were excluded by reviewed the title and abstract. After the step of full text reviewing, 108 of these articles were further excluded. However there were 18 articles existing overlapped data. Finally 17 studies were included in this meta-analysis [17-33].

Among the 17 studies, 15 of them were case-control study and the other 2 were clinical cohort study, which all together contain 1943 cases and 1575 controls. All included studies contain 14 English and 3 Chinese articles, 11 of which take Chinese as their study subjects, and Japanese 5, South Korean 1. All their publication years range from 2001 to 2014. Only one study used combined bisulfite restriction analysis (COBRA) method, all the other 16 studies used methylation-specific polymerase chain reaction (MSP) method to detect *hMLH1* promoter methylation status in samples. The basic information of the included studies was summarized in **Table 1**.

Quantitative data synthesis

Since the results of heterogeneity test showed that there was no remarkable heterogeneity within the included studies, the fixed effects model was used to explore the association between *hMLH1* promoter methylation and gastric cancer. The results overall showed that the frequencies of *hMLH1* promoter methylation in gastric cancer tissues were significant higher than those adjacent normal tissues (OR=15.73, 95% CI=8.05-30.75, P<0.01) (**Figure 1A**) and cancer-free subjects (OR=19.03, 95% CI=7.83-46.23, *P*<0.01) (**Figure 1B**). In the subgroup analysis by study population, the same strong association was observed in both Chinese (OR=26.41, 95% CI=9.69-71.96, *P*<0.01) and Japanese (OR=6.95, 95% CI=2.75-17.58, *P*<0.01) (**Figure 1C**).

Subsequently we analyzed the relationship between gender, age, histological differentiation, peritoneal or distant metastasis, *Helicobacter pylori* infection, lymph node metastasis and *hMLH1* methylation status, among them, age, gender, histological differentiation and lymph node metastasis showed a statistical differences. The detailed results showed in **Table 2** and **Figure 2**. The pooled data indicated that the younger patients with a lower methylation rate of the pooled OR is 0.53 (95% CI=0.357-0.788, P=0.002).
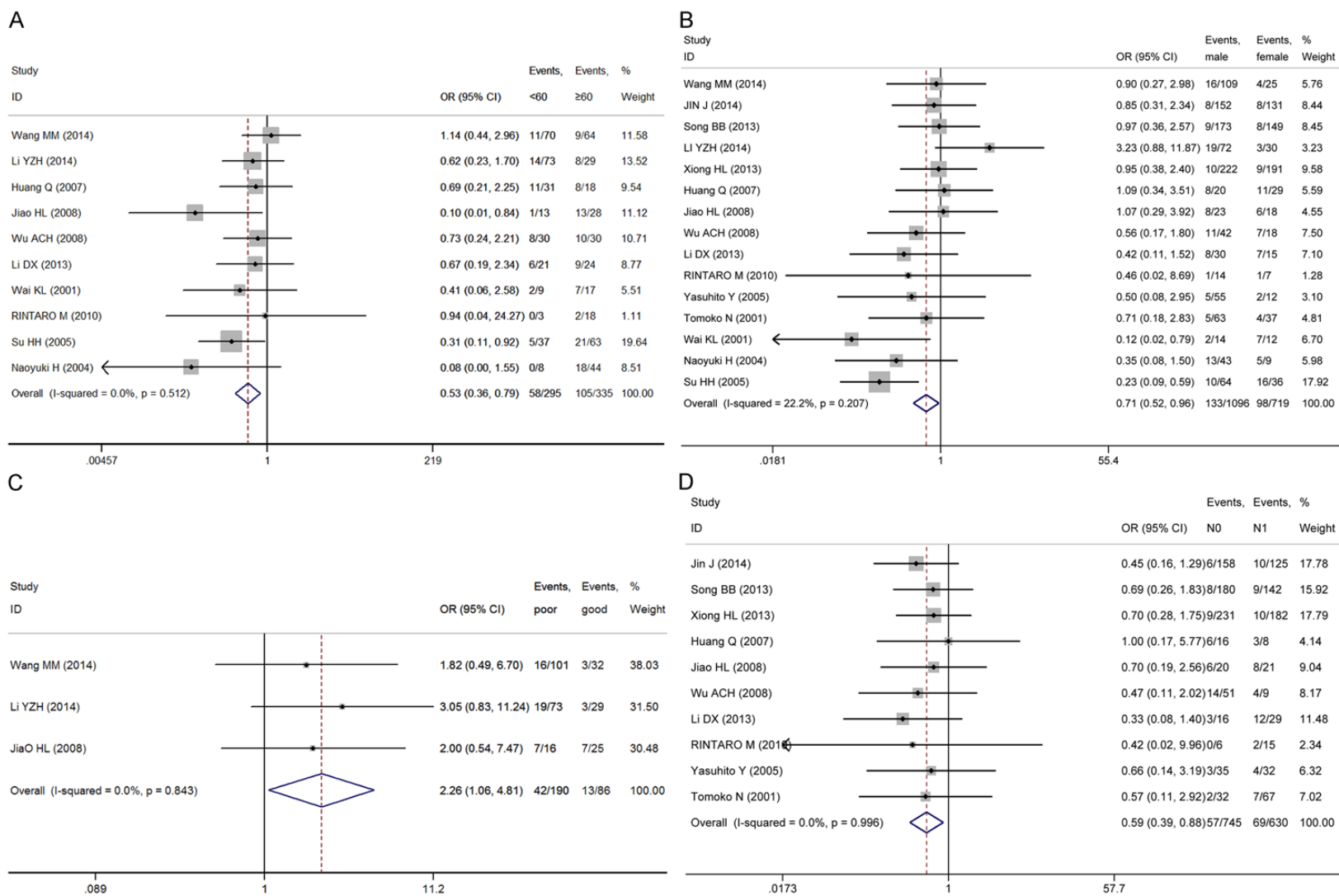
Figure 2. Forest plot for the differences of hMLH1 promoter methylation status between different clinicopathological characteristics. A. Age (<60 VS ≥60). B. Gender (Male VS Female). C. Histological differentiation (Poor VS Good). D. Lymph node metastasis (N0 VS N1).
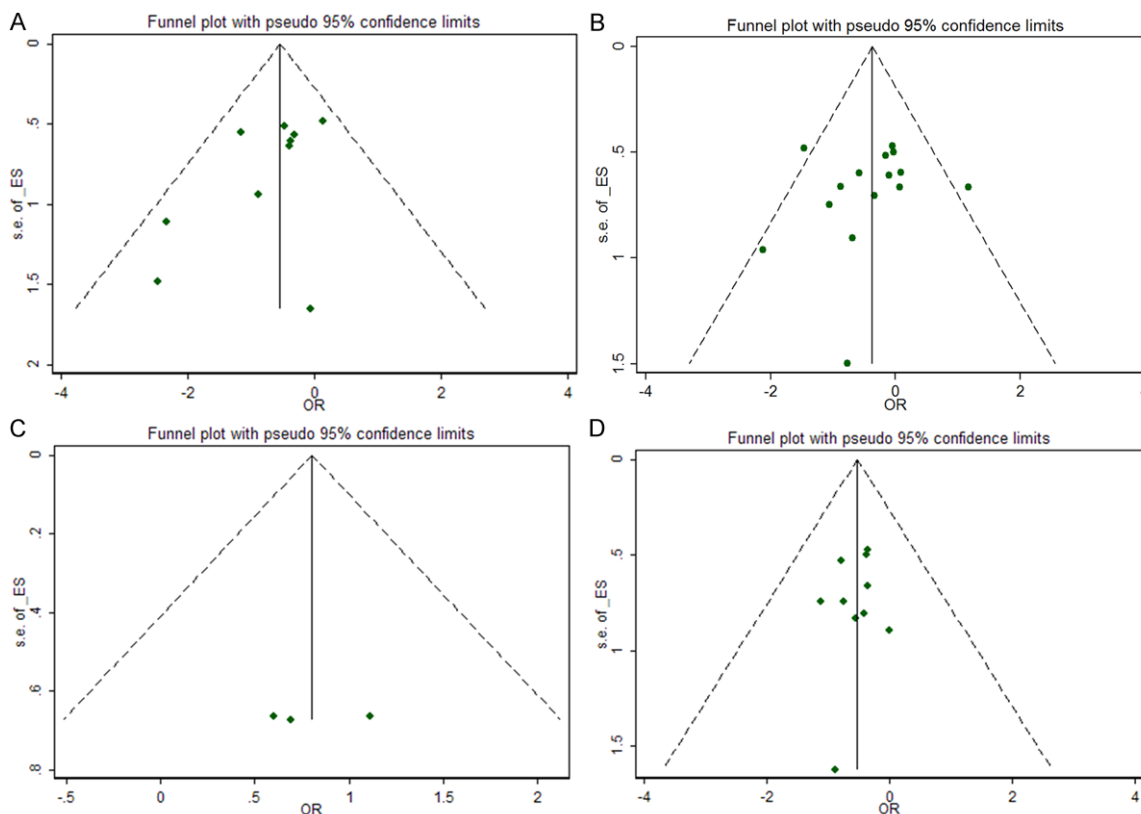
Figure 3. Funnel plot analysis to detect publication bias. A. AGE. B. Gender. C. Histological differentiation. D. Lymph node metastasis.

Publication bias and sensitivity analysis

Funnel plot and Egger's test were used to detect the presence of publication bias. The funnel plots showed no obviously asymmetry by visual observation (**Figure 3**). Then Egger's test also provided statistical evidence to support that no publication bias overall the analysis. We also conducted a sensitivity analysis to assess the impact on the overall results by one single study. There was no single study that could make a qualitative difference on the overall pooled estimates, which indicating that our results were robust and reliable.

Discussion

The damaged and inappropriate base pairs will also appear in the process of normal DNA metabolism, and body have some special system to repair these genetic damages, including nucleotide excision repair (NER), base excision repair (BER) and mismatch repair (MMR). MMR is a very critical genome caretaker system,

which can repair DNA mismatches, reduce the spontaneous mutation, make trigger apoptosis of large amounts DNA damage cell to achieve the purpose of maintaining genomic stability and preventing cell excess growth such as tumor [12, 34]. Defects in MMR lead to a mutator cellular phenotype, which is treated as hallmarks of high spontaneous mutation rate and increased microsatellite instability (MSI) [11]. In human MMR pathway mainly included the participation of MutS, MutL, exonuclease (ExoI), and their function is DNA mismatch/ damage recognition (MutS), molecular match-maker/chaperone (MutL), and removing mispaired base (ExoI) respectively. So far four kinds of human MutL homologs have been found, *hMLH1*, *hMLH3*, *hPMS1* and *hPMS2*. The effect of *hMLH1* is particularly critical, because it plays a role in MMR pathway by forming three kinds of heterodimeric complexes with *hMLH3*, *hPMS1* or *hPMS2* [12]. Existing studies have shown that *hMLH1* germline mutation is mainly involved in the hereditary tumors, *hMLH1* gene inactivation in sporadic

tumors displaying MSI is mainly due to the *hMLH1* promoter methylation, not mutation in coding sequence [14, 35-37]. The following vitro experiments have confirmed that in tumor cell line lake of *hMLH1* expression by hypermethylation in *hMLH1*, after being treated with 5-aza-deoxycytidine (a kind of demethylation agent), *hMLH1* protein expression was restored, and the expression level is associated with drug does [38, 39].

Gastric tumorigenesis involves composite effect of genetic and environmental factors. Early genetic factors studies mainly concentrated on the genetic alterations, in the past several decades, the relationship between epigenetics and tumorigenesis has been paid more attention. Aberrant promoter methylation cause of cancer-related gene inactivation is widely considered the most relevant epigenetic mechanism in gastric cancer [40]. In the study of epigenetics in gastric cancer, *RUNX3, P16, DAPK, RASSF1A, hMLH1* promoter methylation statuses were frequently concerned [7, 41, 42].

Our study is the first meta-analysis focus on *hMLH1* promoter methylation and gastric cancer. Finally the study included 17 related articles to quantify the exact role of *hMLH1* methylation playing in gastric cancer. The overall OR for methylation status in gastric cancer tissues vs adjacent normal tissues was 15. 73, gastric cancer patients vs cancer free subjects was 19.03, which demonstrated a strong association between gastric cancer and *hMLH1* promoter methylation status.

Our results also showed that a significant association between *hMLH1* methylation status and age, *hMLH1* promoter methylation is frequently found in older people. Nan et al [43] carried out a study in South Korea which showed that cigarette smoking and alcohol consumption were associated with increasing likelihood of gastric cancer with hypermethylation of *hMLH1* gene promoter. In Kashmiris, a significant association between aberrant methylation of *hMLH1* gene promoter and smoking, consumption of local hot salted tea, intake of sundried vegetables was existed too [44, 45]. These results indicate that methylation status of *hMLH1* gene may be affected by environmental risk factors, with the growth of the age, the methylation status of age-related methylation gene changes, and the effect of cellular-environmental interactions steady accumulation.

Then, we found that there's a statistical association between *hMLH1* promoter methylation status and histological differentiation, lymph node metastasis. This result suggests that promoter methylation of *hMLH1* gene may involve in the progression of gastric cancer and appears a poor prognosis. And the relationship between the defects of *hMLH1* protein expression and drug resistance has been confirmed in several types of cancer, such as ovarian cancer and breast cancer [46, 47]. These promptings it have a potential clinical application value of being a biomarker for prognostic evaluation in gastric cancer; therefore, further studies should be performed.

In this study, no statistically significant association between *Helicobacter pylori* infection and *hMLH1* promoter methylation status was found. As early as 1994, International Agency for Research on Cancer (IARC) recognized *Helicobacter pylori* infection as a certain cause of gastric cancer, the epidemiological investigation showed that the occurrence of gastric cancer is closely related to the *Helicobacter pylori* infection [48]. The relationship between *Helicobacter pylori* infection and DNA methylation remains controversial, but a further study found that tumors with methylated *hMLH1* gene was associated with *Helicobacter pylori* vacA s1 [49-51]. Studies suggest that missing the *Helicobacter pylori* typing step may be the cause of the conflicts between the related studies, further study should verify this reason.

Some limitations of this meta-analysis should be realized. First of all, most of the studies didn't get the original database, and part of them didn't show the detailed information such as gender, age, and clinical pathological features. What's more, meta-analysis may cause the selection bias inevitable. However, many advantages exist in our meta-analysis. Firstly, it's the first meta-analysis about the relationship between *hMLH1* promoter methylation status and gastric cancer. Secondly, no obvious publication bias was found in the included studies. Thirdly, no heterogeneity was observed in the study. Consequently, the results of this study are reliable and stable.

Conclusions

In summary, this meta-analysis provides evidence to prove that there's a strong association of *hMLH1* promoter methylation status with the

risk and prognosis of gastric cancer How-ever, due to the mentioned disadvantages, fur-ther studies with a larger sample size are required to confirm the exact participatory pro-cess of *hMLH1* promoter methylation in gastric tumorigenesis.

Disclosure of conflict of interest

None.

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