Systematic Review and Meta-Analysis

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A systematic review and meta-analysis approach on diagnostic value of *MLH1* promoter methylation for head and neck squamous cell carcinoma

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Abstract

Background: Head and neck squamous cell carcinoma (HNSCC) is the leading histological type among head and neck cancers. Several studies have explored an association between aberrant methylation of MutL homolog-1 (*MLH1*) promoter and HNSCC risk. We aimed to explore the associations between *MLH1* promoter methylation and HNSCC by using a meta-analysis.

Methods: Systematic literature search was conducted among PubMed, Google Scholar, Web of Science, and China National Knowledge Infrastructure, and Wanfang databases to retrieve relevant articles published up to June 30, 2018. A total of 12 studies were included in this meta-analysis (including 717 HNSCC and 609 controls).

Results: The results demonstrated that *MLH1* promoter methylation was notably higher in patients with HNSCC than in controls (odds ratios [ORs] = 2.52, 95% confidence intervals [CIs] = 1.33–4.79). Besides, *MLH1* promoter methylation was not associated with tumor stage, lymph node status, smoking behavior, age, clinical stage, gender, and differentiation grade (all $P > .05$). The pooled sensitivity and specificity rates of *MLH1* methylation for HNSCC were 0.23 (95% CI = 0.12–0.38) and 0.95 (95% CI, 0.82–0.99), respectively. The area under the receiver operating characteristic (ROC) curve was presented as 0.64 (95% CI = 0.60-0.68).

Conclusion: The results of this meta-analysis suggested that hypermethylation of *MLH1* promoter was associated with HNSCC. Methylated *MLH1* could be a potential diagnostic biomarker for diagnose of HNSCC.

Abbreviations: AUC = area under the curve, CI = confidence interval, *DAP-K* = death-associated protein kinase, HNSCC = head and neck squamous cell carcinoma, HPV = human papillomavirus, HR = hazard ratio, *LRPPRC* = leucine-rich PPR-motif containing, LSCC = laryngeal squamous cell carcinoma, MMR = DNA mismatch repair, *MGMT* = O6-methylguanine-DNA methyltransferase, *MLH1* = MutL homolog-1, MSP = methylation-specific polymerase chain reaction, *MTHFR* = methylenetetrahydrofolate reductase, NOS = Newcastle–Ottawa Scale, OR = odds ratio, OSCC = oral squamous cell carcinoma, *RAB6C* = RAS oncogene family member-6C, ROC = receiver operating characteristic, RE-MSP = restriction enzyme MSP, SCCT = squamous cell carcinoma of the tongue, SROC = summary of receiver operation characteristic, TSGs = tumor-suppressor genes, *ZNF471* = zinc finger protein 471.

Keywords: diagnostic value, head and neck squamous cell carcinoma, methylation, *MLH1*

1. Introduction

Head and neck squamous cell carcinoma (HNSCC), noted as the sixth most common cancer,[1] is the leading histological type among head and neck cancers, accounting for up to 5% among the newly diagnosed malignancies throughout the world.[2] Tobacco, alcohol, and human papillomavirus (HPV) infection were identified as risk factors in head and neck tumorigenesis.[3] Despite the development in surgical and radiotherapy treatment,

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the survival rate of HNSCC patients is still relatively low, in which 5-year survival rate of patients was remained less than 50%.[4] The survival rate tends to be poorer in advanced stage of cancer due to lack of molecular biomarkers for early diagnosis. Accordingly, it is of great importance to find out early diagnostic biomarkers for HNSCC patients.

Epigenetic regulation of promoter hypermethylation in tumor-suppressor genes (TSGs) has been emerged as an important cause in human carcinogenesis. DNA hypermethylation of various genes were frequently involved in cancer progression and development in HNSCC patients. For example,[5] hypermethylation of O6-methylguanine-DNA methyltransferase (*MGMT*), death-associated protein kinase (*DAP-K*), and *E-cadherin* were detected in laryngeal and hypopharyngeal cancer.[6]*DAPK1*, leucine-rich PPR-motif containing (*LRPPRC*), RAS oncogene family member-6C (*RAB6C*), and zinc finger protein 471 (*ZNF471*) were confirmed in promoter regions of squamous cell carcinoma of the tongue.[7] Researchers have revealed that polymorphism of methylenetetrahydrofolate reductase (*MTHFR*) affected *p16* and *MGMT* methylation frequency in HNSCC.[8]

The MutL homolog-1 (*MLH1*), a member of DNA mismatch repair (MMR) gene, plays critical role in different types of cancer. Epigenetic silencing of *MLH1* promoter methylation can cause mismatch repair (MMR) deficiency, which may cause insertion or deletion mutations in repeated sequences.[9] The *MLH1* promoter methylation has been reported as a well-established biomarker in several types of cancer, such as esophageal cancer,[10] colorectal cancer,[11] non-small cell lung cancer,[12] gastric cancer,[13] papillary thyroid cancer,[14] and bladder cancer.[15]

To date, several studies have explored an association between aberrant methylation of *MLH1* promoter and HNSCC risk. However, using *MLH1* promoter methylation in the diagnosis of HNSCC has still remained inconclusive and inconsistent.

The aim of this meta-analysis was to clarify the diagnostic capability of *MLH1* methylation status in the assessment of HNSCC patients. We also checked the relationship between *MLH1* promoter methylation and the clinicopathological characteristics of HNSCC patients.

2. Materials and Methods

2.1. Literature search and selection criteria

Studies were gathered by thoroughly searching online through PubMed, Google Scholar, Web of Science, and China National Knowledge Infrastructure, and Wanfang databases. The search included all articles published before June 30, 2018. The following search terms and key words were used: "*MLH1*", "*hMLH1*", "MutL homolog-1", "methylation epigenetic", "head and neck cancer (HNSCC)", "laryngeal squamous cell carcinoma (LSCC)", "squamous cell carcinoma of the tongue (SCCT)", "oral squamous cell carcinoma (OSCC)", and "hypopharyngeal squamous cell carcinoma". Three reviewers consulted all candidate articles independently. We also scanned review articles to identify eligible studies. The reference lists of the identified articles were manually reviewed to find out relevant articles. Furthermore, title and abstract of each study were scanned to exclude any irrelevant publications. The references of the articles were also screened for additional applicable papers. The investigators reach a consensus in the case of a conflict views by discussion.

The exclusion criteria were as follows:

1. Irrelevant reviews, letters, personal opinions, book chapters, and meeting abstracts.
2. Language restrictions.
3. Full paper copy was not available.
4. The authors have published articles using the same data.
5. Newcastle–Ottawa Scale (NOS) criteria were used to assess the quality of studies. Studies that scored seven or more were included.

The literature selection process of the eligible studies is shown in Fig. 1. This study was approved by ethics committee of Lihuili Hospital of Ningbo University.

2.2. Data extraction

As mentioned before, 3 reviewers reviewed the eligible articles independently. The following data were extracted as follows: first author's full-name, year of publication, country, methods for detecting the methylation status, hazard ratio (HR) and the corresponding 95% confidence interval (CI), histology of the sample, sample type in case and control group, sample number of the total people (in the case and control groups), clinicopathological characteristics, and the tumor location. Discrepancy in the data was solved and discussed by other two reviewers to reach a mutual agreement.

2.3. Statistical analysis

The pooled odds ratios (ORs) with 95% CIs were calculated to assess the association between *MLH1* promoter methylation and risk of HNSCC, and the association of *MLH1* promoter methylation with other clinical features was investigated as well. Fixed-effects model was adopted when there was no evidence of significant heterogeneity ($P > .05$ and/or $I^2 < 50\%$). On the other hand, a random-effects model was employed to evaluate the potential sources of heterogeneity. Moreover, a meta-regression and subgroup analysis was undertaken to explore the potential sources of heterogeneity. Sensitivity analysis was performed to check the stability of the publications. Subgroup analysis stratified by year of publication, sample size, race, detection method, control type was carried out to detect the potential source of heterogeneity. Begg test and Egger test were performed to evaluate the publication bias of the included studies. A sensitivity analysis was conducted to assess the influence of individual study excluded in the combined OR. Receiver operating characteristic (ROC) curves were plotted to identify diagnose value. Post-test probability was calculated with a presumed pre-test probability of 25%, 50%, and 75%. Statistical analyses were conducted by STATA-12.0 software (Stata Corporation, College Station, TX).

3. Results

Fig. 1 illustrates the detailed selection process of articles. Here, 208 relevant articles were identified for initial review by literature search. After screening according to the inclusion and exclusion criteria, information obtained from 12 studies was finally included in the meta-analyses. All the eligible articles were published in English. The 12 studies with 717 cases and 609 controls encompassed the years of publication from 2003 to 2015. The detailed characteristics of the included studies are summarized in Table 1. All the sample types were tissue and half

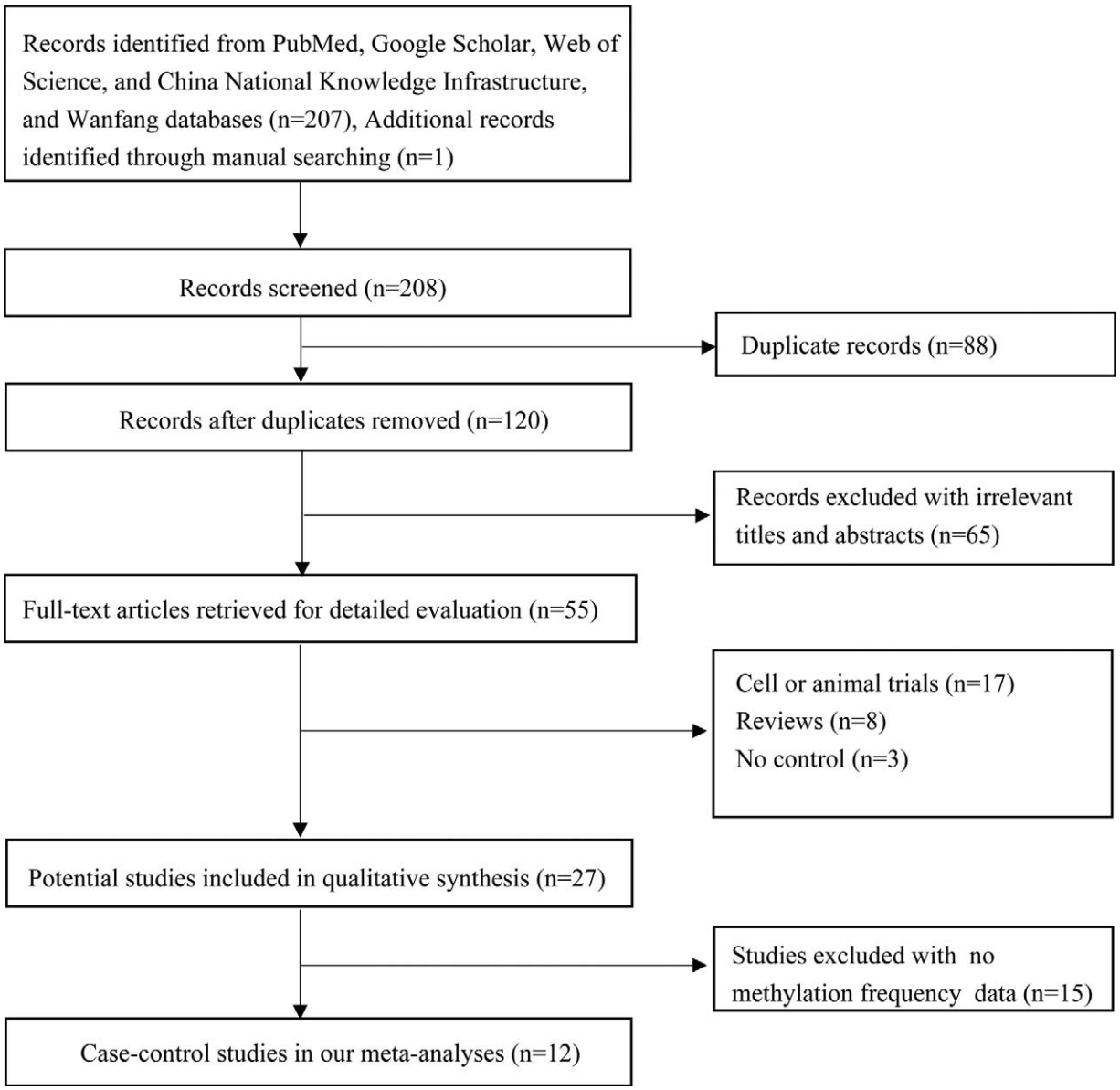

Figure 1. Flow-chart of study selection.

Table 1

General characteristics of the eligible articles.

The first author's name	Year	Country	Method	Histology	Sample type	Tumor		Normal		Control type
						M+	total	M+	total	
Tawfik[22]	2010	Egypt	MSP	HNSCC	Tissue	14	49	10	49	A
Zuo[16]	2009	USA	MSP	HNSCC	Tissue	39	120	13	52	A
Sengupta[20]	2007	India	RE-MSP	HNSCC	Tissue	45	123	27	123	A
Asokan[25]	2014	india	MSP	OSCC	Tissue	1	10	0	5	H
Supic[26]	2011	Serbia	MSP	OSCC	Tissue	12	47	6	47	A
Ramírez[27]	2011	Mexico	MSP	OSCC	Tissue	38	50	0	200	H
Czerninski[28]	2009	Israel	MSP	OSCC	Tissue	5	28	0	6	H
Viswanathan[29]	2003	Japan	RE-MSP	OSCC	Tissue	8	99	0	25	H
Choudhury[30]	2015	India	MSP	HNSCC	Tissue	4	71	1	45	A
Steinmann[31]	2009	Germany	MSP	HNSCC	Tissue	37	54	10	23	A
Weiss[32]	2011	Germany	MSP	HNSCC	Tissue	6	43	3	31	H
Koutsimpelas[19]	2012	Germany	MSP	HNSCC	Tissue	1	23	0	3	H

A = Autologous control, H = Heterogeneous control, HNSCC = head and neck squamous cell carcinoma, M+ = *MLH1* promoter methylated, MSP = methylation specific PCR, NA = not applicable, OSCC = oral squamous cell carcinoma, RE-MSP = restriction enzyme MSP.

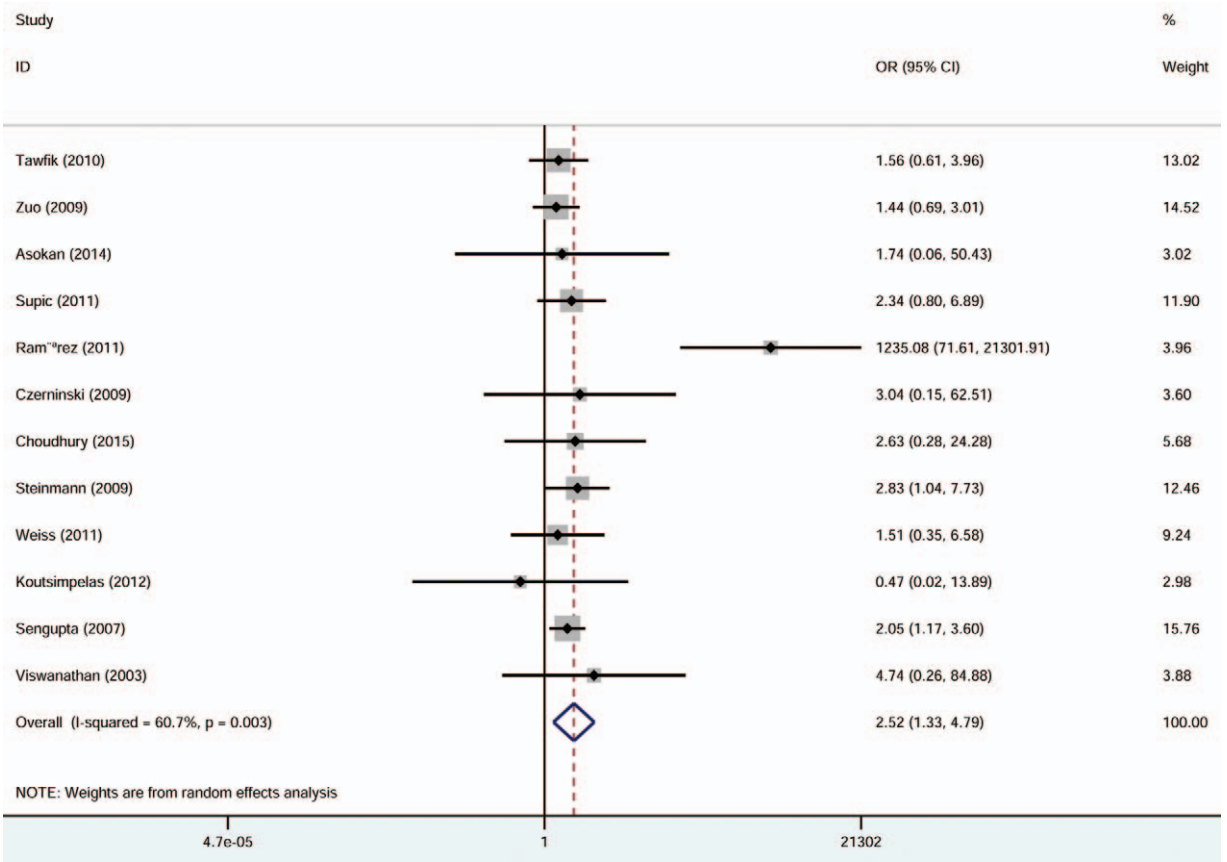

Figure 2. The pooled frequency of *MLH1* promoter methylation in HNSCC patients. The estimates for *MLH1* methylation frequency were associated with HNSCC in the meta-analysis.

of the studies chose heterogeneous tissues as control source. A total of 10 studies conducted methylation-specific polymerase chain reaction (MSP) to assess the gene methylation status, while other 2 studies used restriction enzyme MSP (RE-MSP) to evaluate the *MLH1* methylation status.

3.1. An association between MLH1 promoter methylation and HNSCC carcinogenesis

Overall, there were 12 studies with 280 patients, demonstrating *MLH1* promoter methylation. There was a significant heterogeneity among the studies ($I^2 = 60.7\%$, $P = .003$). Thus, we used a random-effect model to evaluate the association between *MLH1* promoter methylation and HNSCC patients. The methylation status of the *MLH1* in HNSCC varied from 4.3% to 76.0% in the literatures. The frequency of *MLH1* promoter methylation in HNSCC was 29.3%, which was significantly higher than that in normal controls (OR = 2.521, 95% CI = 1.327–4.788). The pooled ORs and 95% CIs are shown in Fig. 2.

Meta-regression analysis was employed to identify the source of heterogeneity. However, the results suggested that the accuracy required for detecting *MLH1* was not significantly affected by the covariates of year of publication, sample size, race, detection method, and control type (see Table 2). On the other hand, we performed a subgroup meta-analysis by published year of studies, sample size, detected method, ethnicity

Table 2

Meta-regression analysis with the aid of year of publication, sample size, race, detection method, and control type.

Heterogeneity sources	Coefficient	95% CI		*P*-value
		Lower	Upper	
Year of publication	0.026	−0.284	0.337	.851
Case sample size	−0.004	−0.019	0.011	.58
Race				
No Caucasian	−0.198	−1.527	1.13	.744
Detection method				
RE-MSP	−0.048	−1.748	1.652	.951
Control type				
Autologous	−0.919	−2.778	0.939	.292

CI = confidence interval, RE-MSP = restriction enzyme methylation specific PCR.

Table 3

Subgroup analyses of MLH1 promoter methylation in HNSCC patients.

	Case		Control				Heterogeneity	
Subgroup	M+	M−	M+	M−	Pooled OR (95%CI)	*P* value	I^2 (%)	*P* value
Published year								
≥2010	76	141	20	340	3.505 (0.811-15.142)	.093	79	**<.001**
<2010	134	290	50	179	2.012 (1.348-3.001)	**=.001**	0	0.802
Case sample size								
≥60	96	317	41	204	1.897 (1.232-2.921)	**.004**	0	0.784
<60	114	190	29	335	3.320 (1.025-10.749)	**.045**	75	**<.001**
Methods								
MSP	157	181	43	375	2.728 (1.151-6.464)	**.023**	68.9	**=.001**
RE-MSP	53	24	27	332	2.161 (1.247-3.745)	**.006**	0	.573
Control source								
Heterogeneous	59	194	3	267	5.349 (0.481-59.496)	.172	79.3	**<.001**
Autologous	151	313	67	272	1.932 (1.363-2.739)	**< .001**	0	.901
Ethnicity								
Caucasian	138	227	32	330	3.426 (1.013-11.59)	.048	79.2	< .001
No Caucasian	72	280	38	209	2.011 (1.273-3.179)	.003	0	9.952

CI = confidence interval, M+ = positive for *MLH1* methylation, MSP = methylation specific PCR, OR = odds ratio, RE-MSP = restriction enzyme methylation specific PCR.

and control source to investigate the potential reasons of heterogeneity. As shown in Table 3, in the control source analyses, the pooled OR for *MLH1* methylation in HNSCC compared with heterogeneous controls was 5.349 (95% CI: 0.481–59.496, $P = .172$), while that was 1.932 (95% CI: 1.363–2.739, $P < .001$) in the autologous control group. Furthermore, subgroup meta-analysis by studies published after 2010 indicated no significant association between *MLH1* methylation and risk of HNSCC with pooled OR = 3.505 (95% CI: 0.811–15.142, $P = .093$). The results of this subgroup analysis showed that *MLH1* hypermethylation was significantly associated with other subgroups ($P < .05$).

The sensitivity analysis was carried out to assess the stability of the conclusions. As illustrated in Fig. 3, the omission of individual studies did not significantly change the pooled OR, which demonstrated the stability of our analyses.

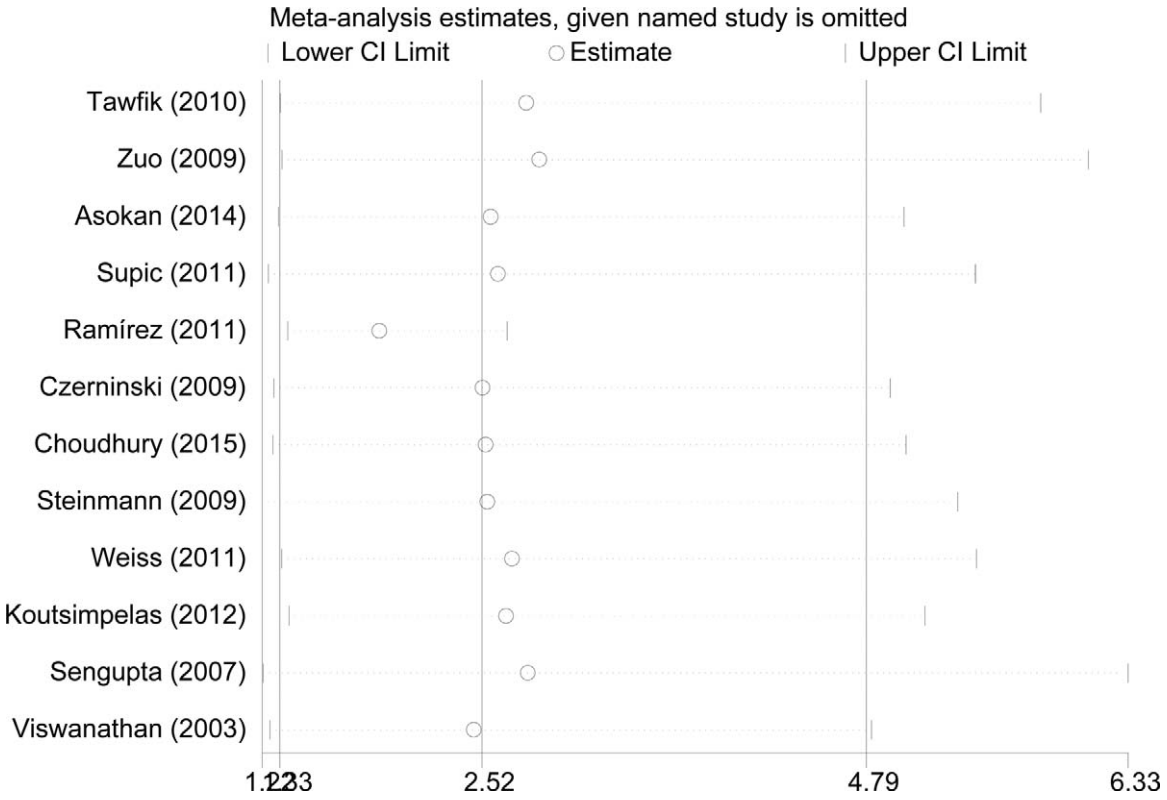

Figure 3. Begg funnel plot and Egger plot of publication bias for *MLH1* in HNSCC patients.

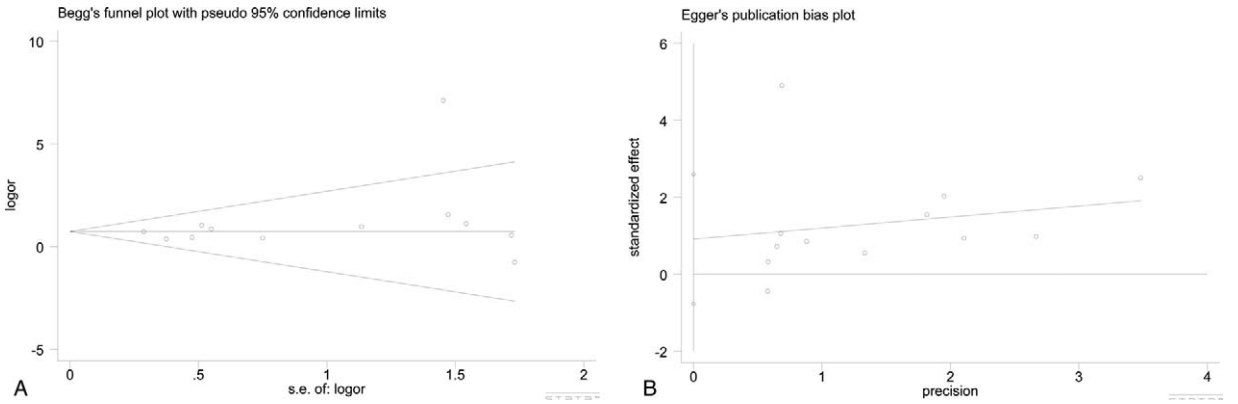

Figure 4. Sensitivity analysis of pooled ORs for investigating the association between *MLH1* methylation and HNSCC.

3.2. Publication bias

We used Begg test and Egger test to assess the publication bias of the 12 studies (Fig. 4). The publication bias was not significantly associated with Begg test ($P=.732$) and Egger test ($P=.254$).

3.3. Correlation of MLH1 promoter methylation with clinicopathological features of HNSCC

Further analysis of tumor stage, lymph node metastasis, smoking behavior, age, clinical stage, gender, and differentiation grade was conducted. Table 4 demonstrates that no significant association was observed in relation to tumor stage, lymph node metastasis, smoking behavior, age, clinical stage, gender, and differentiation grade in HNSCC patients (OR=0.484, 95%CI=0.151–1.556, $P=.223$; OR=1.053, 95% CI=0.587–1.89, $P=.863$; OR=1.483, 95% CI=0.615–3.574, $P=.38$, OR=0.471, 95%CI=0.154–1.434, $P=.185$; OR=2.501, 95% CI=0.368–17.012, $P=.349$; OR=0.607, 95% CI=0.201–1.833, $P=.376$, OR=0.90, 95% CI=0.352–2.299, $P=.825$; respectively).

3.4. ROC

Subsequently, we estimated the diagnostic values of *MLH1* promoter methylation in HNSCC patients. Summary of receiver operation characteristic (SROC) curve was plotted. The summery sensitivity, summery specificity, and area under the curve (AUC) value of *MLH1* promoter methylation in HNSCC patients versus healthy individuals were 0.23, 0.95, and 0.64, respectively.

3.5. Fagan plot analysis

We performed Fagan plot analysis from 12 case-control studies to evaluate the clinical utility of *MLH1* promoter methylation in HNSCC. As illustrated in Fig. 6, when pre-test probabilities of 25%, 50%, and 75% were assumed, the positive post-probabilities were 54%, 81%, and 91%, and the negative post-probabilities were 22%, 45%, and 72%, respectively.

4. Discussion

MLH1 was reported as a TSG in various cancers.[16] Increasing lines of evidence suggested that *MLH1* plays a critical role in genome stability system by correcting replicative DNA polymerase errors or mismatched genes. Inactivation of *MLH1* increased microsatellite instability.[17] It has been reported by several scholars that aberrant *MLH1* methylation appears as a major mechanism in HNSCC.[18,19] Promoter methylation is a well-known epigenetic process that has been implicated in various human cancers, that may affect apoptosis, proliferation, and cell adhesion process.[20] It has been reported that MLH1 protein expression was decreased in HNSCC patients compared with normal squamous epithelium due to promoter hypermethylation of *MLH1* gene.[21] Promoter hypermethylation of *MLH1* was found to be associated with cancer regional lymph node invasion, in laryngeal squamous cell carcinoma.[20] However, the relationship between *MLH1* promoter methylation and HNSCC has still remained inconsistent. The goal of this article was to assess the relationship between the methylation status and HNSCC, in addition to explore the diagnostic value of *MLH1* methylation status in HNSCC patients.

Table 4

***MLH1* promoter methylation and clinicopathological features of HNSCC patients.**

Characteristics	No	Case/control	Pooled OR (95% CI)	Heterogeneity		*P* value
				I^2%	*P* value	
Tumor stage	4	T_{1-2}/T_{3-4}	0.484 (0.151-1.556)	63.6	**.041**	.223
Lymph node metastasis	4	Yes/No	0.945 (0.373-2.397)	54.5	.086	.905
Smoking behavior	2	Yes/No	1.483 (0.615-3.574)	0	.618	.38
Age	2	<60/≥60	0.471 (0.154-1.434)	38.8	.201	.185
Clinical stage	3	III, IV/I, II	2.501 (0.368-17.012)	65.6	.055	.349
Gender	3	Male/female	0.607 (0.201-1.833)	0	.53	.376
Differentiation grade	3	Poor/Well and moderate	0.810 (0.109-6.011)	4.93	.085	.837

CI=confidence interval, OR=odds ratio.

After careful screening, the remaining 12 articles were allocated to be analyzed in this study. In our study, 717 cases and 609 controls were ultimately used for pooled analysis. Our results indicated that the frequency of methylation of *MLH1* promoter was significantly higher in HNSCC patients than in normal controls, which supported hypermethylation of *MLH1* associated with an increased risk of HNSCC. Due to significant heterogeneity among the included studies, we performed meta-regression and subgroup analysis to investigate the potential sources of heterogeneity. Meta-regression analysis revealed year of publication, sample size, race, detection method, and control type contributed to the heterogeneity. However, in subgroup analysis of studies used for autologous controls, a more significant association was found between *MLH1* methylation and risk of HNSCC with a lower heterogeneity compared with heterogeneous control studies. On the other hand, the subgroup meta-analysis based on the year of publication revealed that no significant association of *MLH1* methylation was available in studies published after 2010, however, those studies published before 2010 presented a significant correlation between hyper-methylated *MLH1* and HNSCC. We noted that for studies published after 2010, MSP was mainly used to check the methylation frequency. MSP is known as a high sensitive technique for assessment of methylation accompanied with high false positive results because of incomplete bisulfite conversion. In addition, in our subgroup analysis, detection performed by MSP method revealed a higher OR with a higher heterogeneity than other methods. The subgroup analysis by ethnicity showed that Caucasian population had a higher OR than the other population, which suggested that Caucasian population may be more susceptible to *MLH1* promoter methylation. Additionally, in subgroup analysis grouped by sample size, studies with sample size less than 60 were significant in terms of heterogeneity, suggesting that the studies with small sample size might be a potential resource for heterogeneity. According to the results of sensitivity analysis, we found our meta-analysis as stable and reliable.

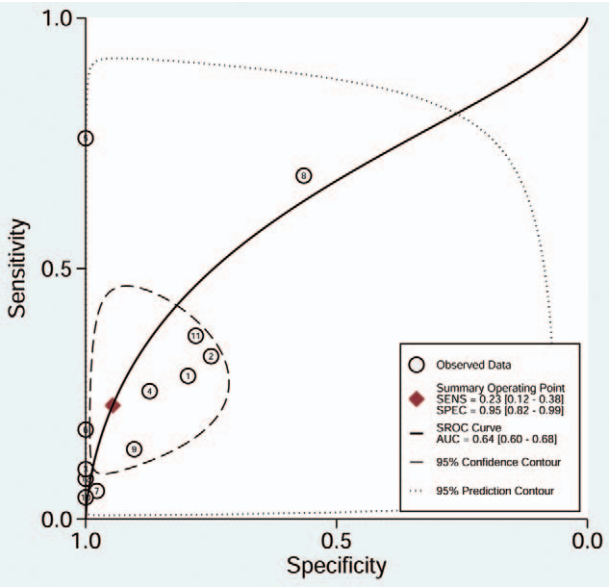

Figure 5. SROC curve.

We also found that lymph node metastasis, tumor stage, differentiation grade, and clinical stage were closely associated with the outcomes of patients with HNSCC.[22] We sought to determine the associations of methylation with those clinical parameters. However, no significant association was observed between *MLH1* promoter methylation and those clinicopathological features, in which it may be related to the small sample size. Therefore, further studies with larger sample size need to be conducted.

It is also essential to know the diagnostic value of the *MLH1* methylation status in HNSCC. Hence, in the present study, the ROC curves were plotted, and Fagan plot analysis was performed. The pooled ROC curve (Fig. 5) indicated that *MLH1* hypermethylation yielded an AUC value of 0.64 (95% CI: 0.60–

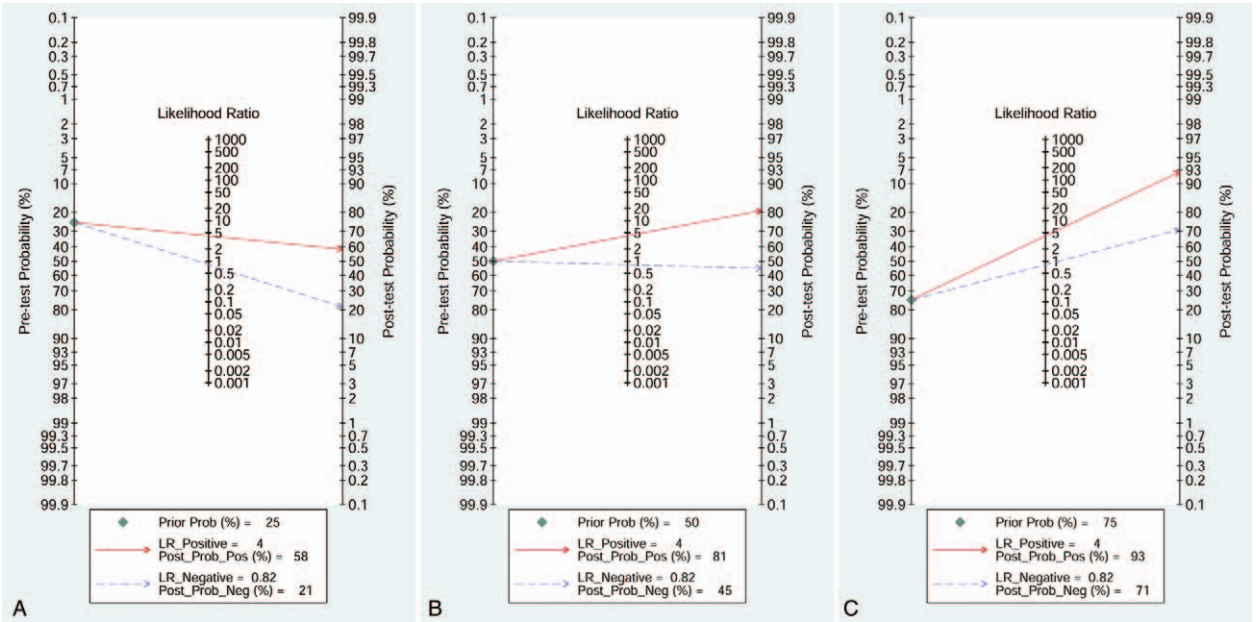

Figure 6. Fagan plot analysis to evaluate the clinical utility of *MLH1* promoter methylation in HNSCC.

0.68) in distinguishing HNSCC from normal control with a sensitivity of 0.23 and a specificity of 0.95. Besides, Fagan plots analysis indicated that the probability of HNSCC diagnosis was significantly elevated by the detection of hypermethylation of *MLH1*. These results indicated that hypermethylation of *MLH1* could be a potential biomarker with low sensitivity for diagnosis of HNSCC. However, we concluded that *MLH1* hypermethylation may be a specific method in diagnosis of HNSCC, which could be used as combined with other methods to improve the diagnostic value of a disease.

This meta-analysis has some limitations that must be taken into consideration. First, the studies were from different countries, and population differences were observed regarding the status of *MLH1* methylation. Second, MSP and RE-MSP methods were employed to detect the methylation status in the studies. However, MSP has been commonly considered as non-quantitative method that may increase false positive results.[23] Third, due to lack of sufficient information, we did not check the methylation status in other clinical features, such as smoking history, alcohol history, and HPV infection. Lack of original data also limited our further evaluation of clinicopathological features and methylation status of *MLH1* promoter.

5. Conclusions

This research indicated that aberrant methylation of *MLH1* promoter was significantly associated with tumor progression in HNSCC patients, and it could be a potential tumor-specific biomarker for diagnose of HNSCC. However, further verification is required by providing large sample studies.

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Project administration: Zhenhua Wu.
Software: Yi Shen.
Writing – original draft: Qun Li.
Writing – review & editing: Qun Li, Jinjiong Hong, Chongchang Zhou.

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