The prognostic value of CDKN2A hypermethylation in colorectal cancer: a meta-analysis

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Background: The prognostic value of *CDKN2A* promoter hypermethylation in colorectal cancer remains controversial. We systematically reviewed the evidence for assessment of *CDKN2A* methylation in colorectal cancer to elucidate this issue.

Methods: Pubmed, Embase and ISI web of knowledge were searched to identify eligible studies to evaluate the association of *CDKN2A* hypermethylation and overall survival and clinicopathological features of colorectal cancer patients. Combined hazard ratios (HRs) or odds ratios (ORs) with 95% confidence interval (95% CI) were pooled using a random-effects model.

Results: A total of 11 studies encompassing 3440 patients were included in the meta-analysis. *CDKN2A* hypermethylation had an unfavourable impact on OS of patients with colorectal cancer (HR 1.65, 95% CI 1.29–2.11). Subgroup analysis indicated that *CDKN2A* hypermethylation was significantly correlated with OS in Europe (HR 1.49; 95% CI 1.28–1.74) and Asia (HR 3.30; 95% CI 1.68–6.46). Furthermore, there was a significant association between *CDKN2A* hypermethylation and lymphovascular invasion (OR 1.68, 95% CI 1.15–2.47), lymph node metastasis (OR 1.68, 95% CI 1.09–2.59) and proximal tumour location (OR 2.09, 95% CI 1.34–3.26) of colorectal cancer.

Conclusion: This meta-analysis indicated that *CDKN2A* hypermethylation might be a predictive factor for unfavourable prognosis of colorectal cancer patients.

Colorectal cancer is the third most frequent malignancy worldwide, and represents the fourth most common cause of cancer-related deaths, leading to an estimated 608 000 deaths by 2008 (Ferlay *et al*, 2010). Despite recent advances in the management of colorectal cancer and new developments of cancer surveillance, the majority of colorectal cancer patients are still diagnosed at an advanced stage when the therapeutic options are limited, and the 5-year survival rate of colorectal cancer patients remains much lower than expected (Ferlay *et al*, 2010; Jemal *et al*, 2010).

Many efforts have been made to identify the molecular prognostic biomarkers including epigenetic markers for patients with colorectal cancer, in order to make a better selection of therapeutic approaches after surgery and improve patients' survival (Draht *et al*, 2012). *CDKN2A* gene functions as an important tumour suppressor in various human malignancies including colorectal cancer, and its activation prevents carcinogenesis via induction of cell growth arrest and senescence (Collado *et al*, 2007; Rayess *et al*, 2012). *CDKN2A* promoter methylation is a frequent epigenetic event and an important mechanism leading to silencing

and dysfunction of *CDKN2A* gene, which further results in uncontrolled cell proliferation and tumour development and progression (Samowitz *et al*, 2005). Ever since Liang *et al* (1999) reported that the presence of *CDKN2A* promoter hypermethylation predicted shorter survival in colorectal cancer patients, the prognostic value of *CDKN2A* hypermethylation in colorectal cancer have been widely investigated, owing to the fact that these results exhibited an increasing relevance with clinical practice. Although multiple studies were conducted on colorectal cancer patients, whether *CDKN2A* hypermethylation is a predictive factor for poor prognosis remains controversial, and several studies in this field possessed a small sample size. Therefore, we conducted the present meta-analysis to appraise the prognostic value of *CDKN2A* hypermethylation in colorectal cancer.

MATERIALS AND METHODS

Search strategy and selection criteria. We conducted and reported this systematic review and meta-analysis following the PRISMA statement (Moher *et al*, 2009). The following were the criteria for the eligibility of included studies: (1) the study assessed the association between *CDKN2A* methylation and overall survival (OS) of patients with colorectal cancer; (2) the study evaluated the methylation status of *CDKN2A* promoter in primary tumour tissues after surgical resection; (3) the study provided a hazard ratio (HR) and 95% confidence interval (CI) directly or gave the data, which allow for the estimation of the HR and 95% CI; (4) the sample size of the study is not <40 patients.

Pubmed, Embase and ISI web of knowledge were searched by using the combinations of following terms: 'CDKN2A', 'p16', 'methylation', 'colon', 'rectum', 'colorectal', 'cancer', 'carcinoma', 'prognosis', 'prognostic' and 'survival'. The search concluded in February 2013, and no lower date limit was used. We only included the studies that were published in the English language, given the fact that other languages were often not available for both authors and readers.

The bibliographies in selected articles were also examined to identify other relevant studies. Conference abstracts were not in the scope of this analysis owing to the insufficient data provided by the authors. All studies were carefully examined to avoid the inclusion of the duplicate data. Two reviewers (XX and CW) assessed the eligibility of the screened studies independently. Agreement was reached for the discrepancies by discussion.

Data extraction and management. Data were extracted independently by two authors (XX and CW) from each eligible study. The predefined form used for data extraction documented the most relevant items including author's name, year of publication, study location, number of patients, methylation detection method, methylation rate and follow-up.

Methodological assessment. Methodological assessment for each of the included studies was performed by three investigators (XX, CW and CM) and scored them using REMARK guidelines and ELCWP quality scale (Steels *et al*, 2001; McShane *et al*, 2005). The three investigators reported the quality scores of reviewed studies independently, and reach a consensus value for each item.

Statistical analysis. For the quantitative aggregation of the survival data, HRs and their 95% CIs were used to assess the impact of *CDKN2A* hypermethylation on survival of colorectal cancer patients. Studies reporting results of multivariate or univariate analysis for survival were used for the aggregation of the survival data. If HRs and their corresponding SEs were not directly reported in the included studies, they were estimated according to the available survival data by using a method reported by Parmar *et al* (1998). The individual HR estimates were pooled

into a summary HR using the approach provided in a previous published study (Yusuf *et al*, 1985). For the analysis of the association of *CDKN2A* hypermethylation and clinicopathological features, odds ratios (ORs) and their 95% CIs were applied to estimate the effect. An observed HR or OR >1 implied a worse survival for the group with *CDKN2A* hypermethylation or the significant association between *CDKN2A* hypermethylation and clinicopathological characteristics, respectively. We pooled HRs and ORs of the studies by using Review Manager Version 5.1 (The Nordic Cochrane Centre, Copenhagen, Denmark). Publication bias was assessed by using a method reported by Egger *et al* (1997a). We also explored reasons for inter-study heterogeneity using meta-regression analysis and subgroup analysis by study location, publication year, number of patients, methylation rate and quality score. The analysis of publication bias and meta-regression was performed by using STATA version 10.0 (StataCorp, College Station, TX, USA). Sensitivity analysis was conducted by omission of each single study to investigate the stability of the results.

RESULTS

Study selection and characteristics. Through the database search, a total of 135 articles were identified for initial evaluation (Figure 1). After excluding the studies out of the scope of our meta-analysis, 46 studies evaluating the prognostic effect of *CDKN2A* hypermethylation in patients with colorectal cancer were considered for further assessment in detail. By further review, 28 were excluded because the estimation of HRs in these studies was not allowed because of the insufficient data provided by the authors, three were excluded because the authors determined the methylation status of *CDKN2A* promoter by using DNA from other than tumour tissues (e.g., serum), one was excluded because it had overlapped data with another study, two were excluded because the studies were not publications in English, and one was excluded because of the small sample size in this study. Therefore,
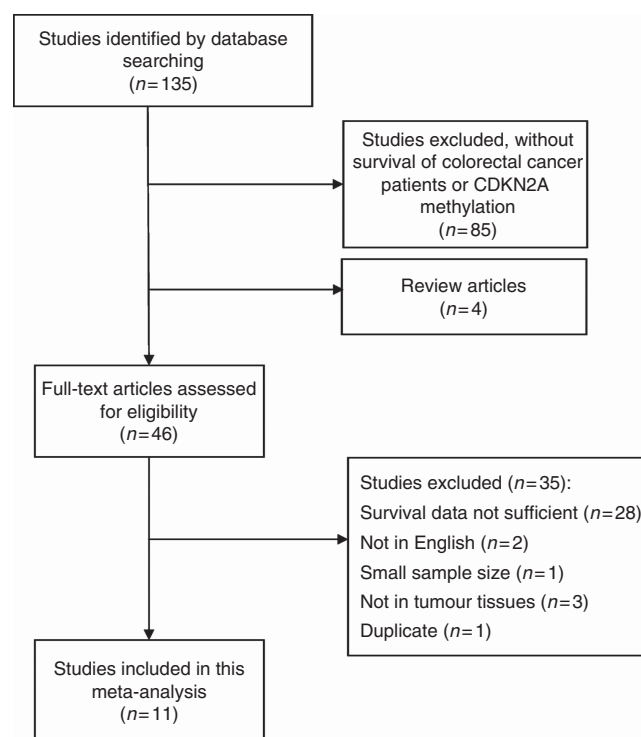

Figure 1. Flow diagram of study inclusion.

						Follow-up (months)		
Study	Study location	Number of patients	Methylation rate (%)	Methylation detection method	Stage	Median	Range	Survival analysis
Barault et al, 2008	France	582	26.3	MSP	I–IV	—	—	Multivariate
Bihl et al, 2012	Switzerland	422	20.6	Pyrosequencing and MSP	I–IV	60	46–74	Multivariate
Esteller et al, 2001	Spain	86	34.9	MSP	Dukes' A–C	68	16–89	Univariate
Ishiguro et al, 2006	Japan	88	22.7	MSP	I–IV	53.2	—	Univariate
Kim et al, 2010	Korea	131	10.7	MSP	I–IV	49	1–116	Univariate
Liang et al, 1999	China	84	28.6	MSP	Dukes' B_2	—	60–86	Univariate
Maeda et al, 2003	Japan	90	13.3	qMSP	II–IV	54.5	—	Univariate
Shen et al, 2007	USA	182	17.0	MSP	IV	14	—	Univariate
Shima et al, 2011a	USA	902	29.8	qMSP	I–IV	146	—	Multivariate
Veganzones-de-Castro et al, 2012	Spain	318	24.8	qMSP	Dukes' A–D	92	75–111	Univariate
Ward et al, 2003	Australia	555	23.1	MSP	I–IV	32	1–60	Univariate

Table 1. Major features of the included studies

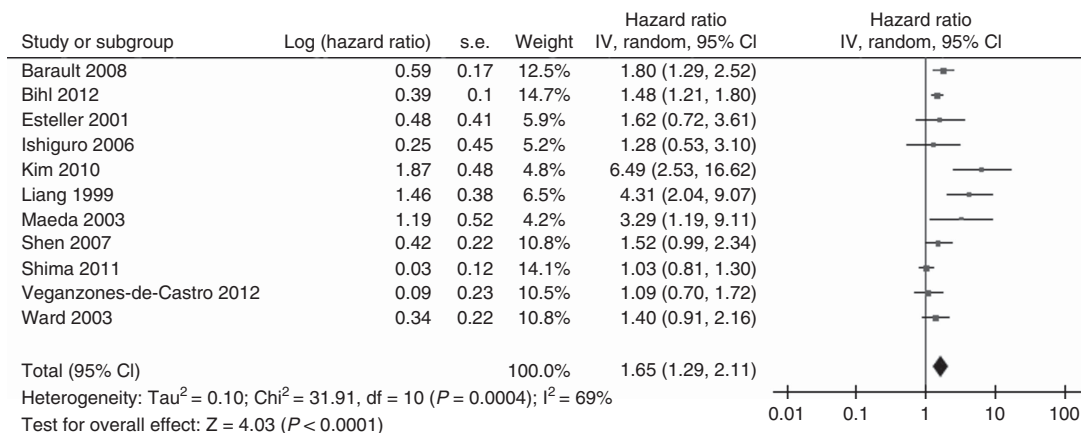Abbreviations: MSP = methylation-specific PCR; qMSP = quantitive MSP.

Figure 2. Meta-analysis of impact of *CDKN2A* hypermethylation on overall survival of patients with colorectal cancer. Results are presented as individual and pooled HR, and 95% CI.

11 studies meeting the inclusion criteria were finally enrolled in this systematic review and meta-analysis (Liang *et al*, 1999; Esteller *et al*, 2001; Maeda *et al*, 2003; Ward *et al*, 2003; Ishiguro *et al*, 2006; Shen *et al*, 2007; Barault *et al*, 2008; Kim *et al*, 2010; Shima *et al*, 2011a; Bihl *et al*, 2012; Veganzones-de-Castro *et al*, 2012; Figure 1).

The characteristics of the 11 eligible studies are extracted and summarised in Table 1. One study assessed the patients from China, one from Korea, two from Japan, two from Spain, one from France, one from Switzerland, two from USA and one from Australia. The total study sample size was 3440 with a mean of 313 (range, 84–902 patients). Four of these studies included <100 patients and 5 studies enrolled >200 patients (Table 1). These eligible studies were published from 1999 to 2012.

Study results report and meta-analysis. *CDKN2A* gene was found to be methylated in 23% of total colorectal cancer patients. The survival data by multivariate analysis can be obtained from three studies (27.3; Table 1). Five of 11 studies (45.5%) revealed

that *CDKN2A* hypermethylation was a poor prognostic factor for survival of patients, and 6 (54.5%) reported that *CDKN2A* hypermethylation did not have an unfavourable impact on survival.

The individual HRs of the 11 included studies were estimated using the methods reported by Parmar *et al* (1998). Five of these 11 studies directly provided their HRs. Two studies reported the total number of events and the log-rank statistic or its *P*-value according to which HRs can be approximated. In the four remaining studies, HRs had to be extrapolated from the graphical representations of the survival distributions.

Figure 2 demonstrates a forest plot of the individual HRs and results from the meta-analysis. Overall, *CDKN2A* hypermethylation in the primary tumour had significant association with an enhanced mortality risk of colorectal cancer patients in the random-effects model (combined HR 1.65, 95% CI 1.29–2.11), despite the exhibition of heterogeneity among studies (I^2 69%, $P = 0.0004$). For the exploration of the source of heterogeneity,

Table 2. Meta-regression and subgroup analysis of the studies reporting the association of *CDKN2A* hypermethylation and overall survival of cancer patients

Stratified analysis	No. of studies	No. of patients	Pooled HRs (95% CI)		Meta-regression *P*-value	Heterogeneity	
			Fixed	Random		I^2(%)	*P*-value
Study location					0.008		
Europe	4	1408	1.49 (1.28, 1.74)	1.49 (1.27, 1.76)		4	0.37
Asia	4	393	3.32 (2.14, 5.15)	3.30 (1.68, 6.46)		56	0.08
USA	2	1084	1.13 (0.92, 1.39)	1.20 (0.83, 1.74)		59	0.12
Oceania	1	555	1.40 (0.91, 2.16)	1.40 (0.91, 2.16)		–	–
Publication year					0.510		
≥2007	6	1085	1.73 (1.35, 2.21)	1.85 (1.29, 2.65)		44	0.11
>2007	5	2355	1.37 (1.20, 1.56)	1.52 (1.09, 2.13)		80	0.0004
No. of patients					0.226		
<200	6	661	2.14 (1.61, 2.85)	2.45 (1.46, 4.10)		63	0.02
>200	5	2779	1.33 (1.18, 1.51)	1.34 (1.08, 1.65)		58	0.05
Methylation rate (%)					0.592		
<24	6	1468	1.56 (1.33, 1.82)	1.76 (1.27, 2.43)		57	0.04
>24	5	1972	1.31 (1.11, 1.56)	1.57 (1.03, 2.39)		78	0.001
REMARK score (%)					0.770		
<12	5	1001	1.54 (1.19, 2.00)	1.54 (1.19, 2.00)		0	0.65
>12	6	2439	1.42 (1.25, 1.61)	1.75 (1.22, 2.51)		83	0.0001
ELCWP score (%)					0.279		
<66	6	1399	1.64 (1.40, 1.92)	1.92 (1.36, 2.70)		57	0.04
>66	5	2041	1.24 (1.05, 1.47)	1.45 (1.01, 2.08)		73	0.006

Abbreviations: CI = confidence interval; HR = hazard ratio.

meta-regression and subgroup analysis were conducted by study location, publication year, number of patients and methylation rate, REMARK score and ELCWP score. Study location was found to be significantly correlated with the inter-study heterogeneity ($P = 0.008$) while other covariates was not (Table 2). Furthermore, subgroup analysis revealed that the significant correlation between *CDKN2A* hypermethylation and OS was present in Europe (HR 1.49; 95% CI 1.28–1.74) and Asia (HR 3.30; 95% CI 1.68–6.46). We also observed the significant association of *CDKN2A* hypermethylation and OS of patients in studies with REMARK score > 12 (HR 1.75, 1.22–2.51), further confirming the prognostic role of *CDKN2A* hypermethylation in colorectal cancer. Of note, the pooled HR estimate by multivariate analysis was 1.38 (95% CI: 1.02–1.87), suggesting that *CDKN2A* hypermethylation might be an independent prognostic factor for patients with colorectal cancer.

In addition, we assessed the association between *CDKN2A* hypermethylation and clinicopathological features of colorectal cancer patients. As indicated in Table 3, *CDKN2A* hypermetylation had a significant association with lymphovascular invasion (positive *vs* negative: OR 1.68, 95% CI 1.15–2.47), lymph node metastasis (positive *vs* negative: OR 1.68, 95% CI 1.09–2.59) and tumour location (proximal *vs* distal: OR 2.09, 95% CI 1.34–3.26). The meta-analysis of the correlation between *CDKN2A* hypermethylation and lymphovascular invasion, lymph node metastasis, Duke's stage and tumour size did not exhibit inter-study heterogeneity (I^2 0%), whereas the analysis of other clinicopathological parameters exhibited heterogeneity (I^2 51–92%).

Sensitivity analysis revealed that omitting any single study did not affect the pooled HR significantly. The evaluation of

publication bias indicated that the Egger test reached the significance ($P = 0.063 < 0.1$) for studies involved in the analysis of OS. The funnel plots for publication bias also demonstrated a certain degree of asymmetry (Figure 3).

DISCUSSION

Although promoter methylation of a number of tumour suppressors (e.g., *RASSF1A*, *MGMT*) have been studied for their prognostic value in colorectal cancer patients recently, the identification of an epigenetic marker with established predictive value for survival of colorectal cancer patients remains a issue that needs to be addressed (Shima *et al*, 2011b; Chen *et al*, 2012). *CDKN2A* promoter hypermethylation been found to lead to silencing of the tumour-suppressor *CDKN2A* gene, which might further result in the development, progression and invasion of colorectal cancer, and could be associated with poor prognosis of patients (Tada *et al*, 2003; Rayess *et al*, 2012). Despite an increasing number of studies performed on promoter status of *CDKN2A* gene, there is still controversy over the prognostic value of *CDKN2A* hypermethylation in colorectal cancer. We thus conducted this systematic review and meta-analysis to assess whether *CDKN2A* hypermethylation could predict the prognosis of colorectal cancer patients.

This study aggregated the outcomes of 3440 colorectal cancer patients from 11 individual trials, revealing that *CDKN2A* hypermethylation is a significant predictor for poor OS of colorectal cancer patients (HR 1.65, 95% CI 1.29–2.11).

Table 3. Meta-analysis of the association between *CDKN2A* hypermethylation and clinicopathological features of colorectal cancer

Stratification of CRC	No. of studies	No. of patients	Pooled OR (95%CI)		P-value	Heterogeneity	
			Fixed	Random	P-value	I^2(%)	P-value
Gender	6	2369	1.31 (1.09, 1.58)	1.27 (0.94, 1.71)	0.12	51	0.07
Stage of disease	3	1168	1.24 (0.95, 1.62)	1.63 (0.84, 3.16)	0.14	65	0.06
Dukes' stage	2	406	1.49 (0.94, 2.35)	1.49 (0.94, 2.35)	0.09	0	0.51
Lymphovascular invasion	3	784	1.68 (1.15, 2.47)	1.68 (1.15, 2.47)	0.008	0	0.89
Lymph node metastasis	2	496	1.68 (1.09, 2.59)	1.68 (1.09, 2.59)	0.02	0	0.99
Grade of differentiation	7	2597	0.55 (0.42, 0.72)	1.23 (0.39, 3.83)	0.72	92	<0.00001
Mucinous type	4	1379	1.36 (0.94, 1.94)	0.98 (0.35, 2.71)	0.97	82	0.001
Tumour location	7	2616	2.68 (2.23, 3.23)	2.09 (1.34, 3.26)	0.001	77	0.0002
Tumour size	2	369	1.08 (0.56, 2.09)	1.07 (0.54, 2.10)	0.81	0	0.32

Abbreviations: CRC=colorectal cancer; CI = confidence interval; OR = odds ratio.
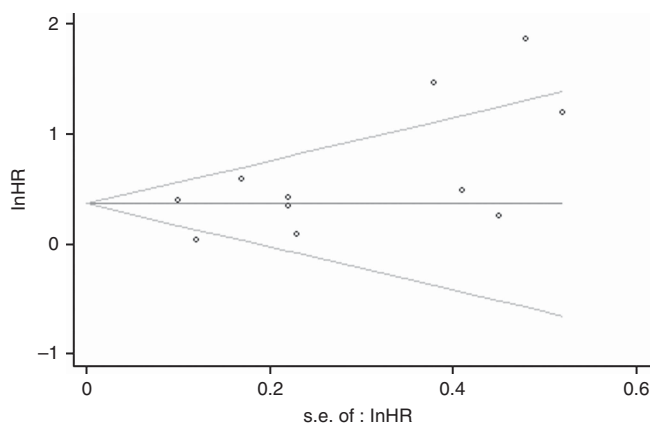

Figure 3. Funnel plot for the evaluation of potential publication bias in the impact of *CDKN2A* hypermethylation on overall survival of colorectal cancer patients. The funnel graph plots the log of HR against the standard error of the log of the HR (an indicator of sample size). The circles indicate the individual studies in the meta-analysis. The line in the centre represents the pooled HR. The Egger test for publication bias was significant ($P = 0.063 < 0.1$).

The significant association between *CDKN2A* hypermethylation and OS of patients was also present in the analysis of studies following REMARK guidelines more rigorously (HR 1.75, 95% CI 1.22–2.51), further confirming the prognostic role of *CDKN2A* hypermethylation in colorectal cancer. Moreover, the significant associations were exhibited between *CDKN2A* hypermethylation and clinicopathological parameters such as lymphovascular invasion and lymph node metastasis.

In addition, *CDKN2A* promoter hypermethylation was also found to have a significant correlation with proximal tumour location. Various environmental and genetic factors contribute to carcinogenesis and progression of colorectal cancer while their roles are different for proximal and distal tumours. Increasing evidence has shown that CpG island methylator phenotypes (CIMPs) and microsatellite instability are associated with proximal tumours while chromosomal instability with distal tumours (Iacopetta, 2002). This distinction might lead to the different sensitivities of proximal and distal tumours to fluorouracil-based chemotherapy and thus different management options for the tumours in the two locations (Elsaleh *et al*, 2000).

A significant heterogeneity of the included studies was observed in this systematic review except for the analysis of studies on OS in Europe. In sensitivity analysis, omission of any individual study did not reduce the heterogeneity or help to elucidate the source of heterogeneity. Meta-regression and subgroup analysis indicated that study location might account for part of inter-study heterogeneity. Moreover, subgroup analysis indicated that *CDKN2A* hypermethylation was significantly correlated with poor prognosis of patients in Europe (HR 1.49; 95% CI 1.28–1.74) and Asia (HR 3.30, 95% CI 1.68–6.46) while not in other study locations. As the characteristics of colorectal cancer in different regions might differ because of diverse environmental factors and genetic and epigenetic background of human races, the prognostic value of biomarkers such as *CDKN2A* promoter hypermethylation in colorectal cancer might be different among different study locations. However, the pooled analysis of all included studies from different regions in this meta-analysis indicated an overall predictive role of *CDKN2A* hypermethylation for poor survival in colorectal cancer patients. More multicenter studies might further clarify whether *CDKN2A* serves as a prognostic factor for colorectal cancer patients worldwide.

As for the detection of methylation status of *CDKN2A* promoter in tumour tissues, the studies included in this meta-analysis used either MSP that is a semi-quantitative approach or qMSP that is a quantitative approach. Moreover, the studies did not apply the same PCR primers and the *CDKN2A* promoter regions examined for methylation status was also not always uniform, leading to a potential bias because the sensitivity and specificity of MSP or qMSP might depend on the primers used, regions detected and other PCR conditions. Subgroup analysis was not possible to be conducted to address this technical issue, because the small groups of studies used the same primers and other PCR conditions. Furthermore, owing to the fact that an optimal threshold has not been defined for qMSP or pyrosequencing, the cutoff defining a colorectal cancer with *CDKN2A* promoter hypermethylation is not identical, which might also potentially generate a certain degree of heterogeneity.

The approach of extrapolating the HRs from studies was also a potential factor that might lead to bias. If HRs were not directly provided by the studies, we calculated them according to the data reported in the publications or estimated them by extrapolating the information from the survival curves. Although two of the investigators (XX and CW) extracted the survival rates according to the graphical representation of the survival curves, this did not completely rule out inaccuracy during reading the survival rates.

The estimated HRs might thus be not as reliable as those retrieved directly from reported statistics. However, we compared our estimated HRs and 95% CIs with the results reported in papers and did not identify any major deviation of our results from the results available in the publications.

Egger tests and funnel plots exhibited a certain degree of publication bias for the analysis of association between CDKN2A methylation and OS. As known, studies that did not possess statistically significant results are less frequently published, and even if these results are published, they are more frequently reported in a brief way and not easily available for analysis. It should be also noted that the positive studies are more frequently published in English language, while those negative ones tend to be more often published in native languages (Egger et al, 1997b). This analysis only included fully published studies in English, whereas unpublished studies and conference abstracts were not enrolled, because the data that can be used for methodological evaluation and meta-analysis were only available in full published studies. Moreover, studies that did not report sufficient data for estimation of HRs were ruled out from this systematic review, and this might potentially generate bias. However, we conducted a complete literature search through the above databases for the eligible studies to minimise the possible bias, and the large sample of colorectal cancer patients ($n = 3440$) enrolled in this analysis ensures the reliability and stability of the results.

In addition, CDKN2A promoter hypermethylation is a frequent epigenetic event, and is often included in the panel of genes for the assessment of the CIMP in colorectal cancer. Several studies in this meta-analysis included the analysis on the prognostic value of CIMP, which included CDKN2A, hypermethylation and other gene methylations (Ward et al, 2003; Shen et al, 2007; Barault et al, 2008; Kim et al, 2010; Shima et al, 2011a). Whereas, these studies usually applied the CIMPs comprising various gene patterns different among studies, which might lead to the difficulty of direct comparison and could generate more heterogeneity if the data were pooled. Therefore, more homogenous studies on the association between identical CIMP and cancers are needed in the future to allow for the meta-analysis on the prognostic value of CIMP in colorectal cancer.

To sum up, this meta-analysis indicated that CDKN2A hypermethylation was significantly associated with poor OS as well as lymphovascular invasion, lymph node metastasis, and proximal tumour location in colorectal cancer. CDKN2A hypermethylation might be a predictive factor of poor prognosis in patients with colorectal cancer particularly in Europe and Asia, and might predict invasion and metastasis. However, more prospective studies with homogeneity are needed to further confirm the results in this study.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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