ORIGINAL RESEARCH

Clinical significance and association of *RUNX3* hypermethylation frequency with colorectal cancer: a meta-analysis

Wei-Ping Mu
Jian Wang
Qiong Niu
Ning Shi
Hai-Feng Lian

Department of Gastroenterology,
Affiliated Hospital of Binzhou
Medical College, Binzhou,
People's Republic of China

Background: The RUNX family, which is composed of *RUNX1*, *RUNX2*, and *RUNX3*, is a sequence-specific transcription factor family and is closely involved in a variety of cellular processes including development, differentiation, participation in the regulation of *p53*-dependent DNA damage response and/or tumorigenesis. Emerging evidence indicates that RUNX3 is a candidate tumor suppressor in several types of human tumors including colorectal cancer (CRC). However, the correlation of RUNX3 inactivation with CRC remains unclear. In the study reported here, we conducted a systematic review and meta-analysis to quantitatively evaluate the effects of *RUNX3* hypermethylation/expression on the incidence of CRC.

Methods: A detailed search of the literature was made using Medline® and Web of Science for related research publications written in English. The methodological quality of the studies was also evaluated. The data were extracted and assessed by two reviewers independently. Analyses of the pooled data were performed. Odds ratios (ORs) and hazard ratios were calculated and summarized, respectively.

Results: A final analysis of 1,427 CRC patients from eleven eligible studies was performed. We observed that *RUNX3* hypermethylation was significantly higher in CRC than in normal colorectal mucosa. The pooled OR from six studies comprising 289 CRC and 188 normal colorectal mucosa was OR =0.07 (confidence interval [CI] =0.03–0.18, $P<0.00001$). Aberrant *RUNX3* hypermethylation/expression was significantly higher in advanced CRC than in early staged CRC (OR =0.54, CI =0.41–0.71, $P<0.0001$). Aberrant *RUNX3* hypermethylation/expression was also significantly higher in microsatellite instability (MSI)-positive CRC than in MSI-negative CRC (OR =0.44, CI =0.3–0.66, $P<0.0001$). In addition, CRC patients with *RUNX3* hypermethylation or lacking RUNX3 protein expression had a lower survival rate than those without *RUNX3* hypermethylation or those who did not express RUNX3 protein.

Conclusion: The results of this meta-analysis suggest that *RUNX3* hypermethylation is associated with an increased risk of CRC, increased risk of progression of CRC, and a poorer CRC survival rate. *RUNX3* hypermethylation, which induces the inactivation of *RUNX3* gene, plays an important role in colorectal carcinogenesis, high levels of MSI, as well as CRC progression and development.

Keywords: methylation, tumor-suppressor gene, odds ratio, hazard ratio, microsatellite instability

Correspondence: Wei-Ping Mu
Department of Gastroenterology,
Affiliated Hospital of Binzhou Medical
College, No 661, Yellow-River
Second Street, Binzhou 256600,
People's Republic of China
Tel +86 543 3256725
Email weipingmu@yeah.net

Introduction

Colorectal cancer (CRC) is one of the most malignant types of cancers. In the USA and Europe, CRC is the second most frequent cancer after lung cancer that leads to death.[1] The incidence of CRC in the People's Republic of China has been reported to increase annually and will continue to rise in the next few years.[2] Currently, there are

approximately 1.25 million patients diagnosed with CRC, and more than 600,000 patients will die from this disease every year worldwide.[3] Surgical resection can be performed to remove the tumor if no lymph-node or distant metastasis is present, but the recurrence rate after surgery remains high.[4,5] Therefore, investigation of the mechanism of initiation and progression and identification of prognostic markers are still needed in the treatment of CRC.

A proper DNA damage response has been considered a critical barrier to genetic alterations, preventing tumor initiation and progression.[6,7] The runt-domain-related (*RUNX*) family of genes, *RUNX1*, *RUNX2*, and *RUNX3*, have recently come to prominence because of their protein roles as essential regulators of cell fate in development and their paradoxical effects in cancer, as well as in the regulation of p53-dependent DNA damage response and/or tumorigenesis.[8–10] The *RUNX3* gene is one of the most critical members of the runt-domain family and has a crucial role in the regulation of cell proliferation and cell death by apoptosis, and in angiogenesis, cell adhesion, and invasion.[11,12] The *RUNX3* gene, localized to chromosome 1p36, a region that exhibits frequent loss-of-heterozygosity events in colon, gastric, breast, and ovarian cancers, is considered a tumor-suppressor gene involved in the transforming growth factor beta (TGF-β) signaling pathway.[13] Its precise function has been intensively studied in several tumors, with upregulation of the induction of cell-cycle arrest, apoptosis, and downregulation of cyclin D1 expression.[14–18] Inactivation of *RUNX3* by promoter methylation (hypermethylation) has been found to play an important role in colorectal epithelial tumorigenesis.[19–21] However, its role in CRC and its clinical significance have not been thoroughly investigated. In this study, we review and update the published clinical investigations regarding the effect of *RUNX3* on patients with CRC.

Methods
Search strategy and selection criteria
We searched PubMed, Embase, and ISI Web of Knowledge to identify studies from January 1, 1998 to December 2013 using the search terms "colorectal" and "cancer" or "tumor" or "neoplasm" or "carcinoma", "methylation", and "RUNX3". We also manually searched the reference lists of the retrieved articles and reviews for additional articles. Although our search did not initially have language limits, for the full-text reading and final evaluation, we reviewed only studies published in English. Conference abstracts were not selected for our analysis due to their containing insufficient data. After the exclusion of irrelevant and/or redundant publications from

the different databases, the full-text versions of the remaining papers were evaluated against inclusion and exclusion criteria (see the following paragraph), their reference lists, and/or bibliographies searched for additional relevant articles. All searched data were retrieved. To avoid duplication, if the same patient population was reported in several publications, the most complete study was chosen for review.

The three inclusion criteria were as follows: 1) *RUNX3* methylation and/or expression evaluated in the circulation and/or primary CRC tissues, 2) research revealed the relationship between *RUNX3* methylation and/or expression and CRC clinicopathological parameters and prognosis, 3) *RUNX3* methylation and/or expression examined by polymerase chain reaction (PCR), 4) sufficient information provided to estimate hazard ratio (HR) concerning overall survival (OS) and 95% confidence interval (CI). The two exclusion criteria were: 1) letters, reviews, case reports, conference abstracts, editorials, expert opinion; and 2) all in vitro/ex vivo studies. Studies on cell lines and human xenografts were also excluded.

Data extraction and methodological assessment
Two authors (WM, JW) independently reviewed and extracted the data from eligible studies. Disagreements were resolved by discussion and consensus. Two authors (QN, NS) reviewed all of articles that fitted the inclusion criteria. The following information was recorded for each study: the first author's name, year of publication, sample source, number of cases, clinicopathological parameters, cancer tumor-node-metastasis stage, methylation detection method, methylation rate and/or expression, and follow-up. Data for study characteristics and clinical responses were summarized and transferred into table format. The heterogeneity of investigation was evaluated to determine whether the data from the various studies could be analyzed in a meta-analysis.

For the methodological evaluation of the studies, three investigators (WM, JW, and HL) read each publication independently, and assessed and scored them according to Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK) guidelines and the European Lung Cancer Working Party quality scale.[22,23] The three readers provided the quality scores and compared them, then reached a consensus value for each item.

Statistical analysis
Analysis was conducted using STATA (v 12.0; StataCorp LP, TX, USA) and Review Manager (v 5.2; The Cochrane

Collaboration, Oxford, UK) software. The pooled frequency of *RUNX3* hypermethylation and 95% CI were estimated. The frequency of *RUNX3* hypermethylation in different tumor characteristics was compared. Heterogeneity among studies was evaluated with Cochran's Q test[24] and the I^2 statistic.[25,26] When heterogeneity was not an issue (I^2 values <50%), a fixed-effects model was used to calculate parameters. If there was substantial heterogeneity (I^2 values ≥50%), a random-effects model was used to pool data and attempt to identify potential sources of heterogeneity based on subgroup analyses. The pooled OR was estimated for the association between *RUNX3* hypermethylation and clinicopathological features. *P*-values less than 0.05 were considered statistically significant.

Publication bias was assessed using a method reported by Egger et al.[27] We also explored reasons for statistical heterogeneity using meta-regression, subgroup analysis, and sensitivity analysis. The analysis of meta-regression and publication bias was performed using STATA (v 10.0; StataCorp LP).

Results
Identification of relevant studies
Sixty-seven publications were identified using the described search method. Of these, 56 were excluded due to being laboratory studies, non-original articles (ie, reviews), or irrelevant to the current analysis. Eventually, eleven studies were included in the final meta-analysis,[19–21,28–35] as shown in Figure 1.

Study characteristics
The eleven studies eligible for meta-analysis were published between 2004 and 2013, and between them reported on a total of 1,427 CRC patients from Japan, Singapore, Brazil, Norway, and the USA. The basic characteristics of these patients are summarized in Table 1.

Correlation of *RUNX3* hypermethylation and expression with clinicopathological features
Inactivation of *RUNX3* through hypermethylation in CRC and adenoma
The loss of *RUNX3* messenger RNA (mRNA) and protein expression was found to be statistically correlated with the promoter hypermethylation in several types of cancer including CRC.[20,30,36–39] *RUNX3* hypermethylation was significantly higher in CRC than in normal colorectal mucosa. The pooled OR from six studies of a total of 289 CRC and 188 normal colorectal mucosa, as shown in Figure 2A (OR =0.07, CI =0.03–0.18, *P*<0.00001) indicates that *RUNX3*
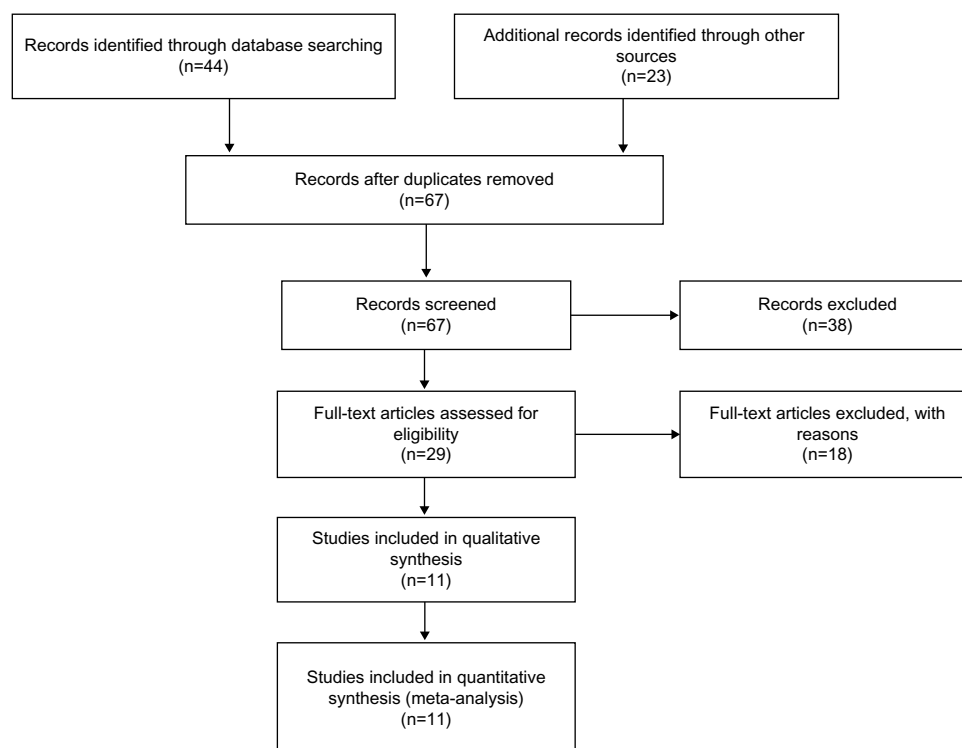

Figure 1 Flowchart of study selection.

Table 1 Basic characteristics of the included studies

Study	Patients, N	Method(s)	Primary aim	Methylation site	*RUNX3* expression
Imamura et al[19]	92	MSP	Determination of the methylation status of the *RUNX3* gene and correlation of the results with the clinicopathological features of affected patients	Promoter, CpG islands	–
Goel et al[20]	91	MSP, RT-PCR	Determination of *RUNX3* expression and the frequency of *RUNX3* promoter hypermethylation	Promoter, CpG islands	+
Ku al[21]	87	RT-PCR, MSP	Determination of whether there is promoter methylation of the *RUNX3* gene in CRC	Promoter, CpG islands	+
Ahlquist et al[28]	25	MSP	Pinpointing of the epigenetic markers that can discriminate between nonmalignant and malignant tissue from the large bowel	Promoter, CpG islands	–
Soong et al[29]	849	Immunohistochemistry	Determination of the clinical significance of *RUNX3* expression in a large series of CRC patients		+
Ito et al[30]	48	MSP, RT-PCR	Investigation of the possible roles of *RUNX3* in regulating the Wnt pathway and in intestinal tumorigenesis	Promoter, CpG islands	+
Hibi and Nakao[31]	59	MSP	Determination of whether *RUNX3* methylation status is correlated with the clinicopathological features of the affected patients	Promoter, CpG islands	–
Ogino et al[32]	30	MethyLight	Determination of whether CpG island methylation may predict poor survival in CRC	Promoter, CpG islands	+
Tan et al[33]	17	MSP	Determination of the promoter status of *RUNX3* in CRC	Promoter, CpG islands	–
Silva et al[34]	10	Methyl-Profiler™ PCR Array*	Identification of useful epigenetic markers for noninvasive CRC screening	Promoter, CpG islands	–
Nishio et al[35]	119	MSP	Analysis of *RUNX3* promoter hypermethylation as a novel tumor marker	Promoter, CpG islands	–

Note: *Qiagen NV, Venlo, the Netherlands.
Abbreviations: CRC, colorectal cancer; MSP, methylation-specific polymerase chain reaction; PCR, polymerase chain reaction; RT-PCR, reverse transcription followed by conventional polymerase chain reaction.

inactivation through hypermethylation plays an important role in the pathogenesis of CRC. In addition, *RUNX3* hypermethylation was found to occur in colorectal adenoma but slightly less so than in CRC, as shown in Figure 2B.

Role of *RUNX3* hypermethylation in CRC development

We analyzed 1,059 CRC patients pooled from three studies to assess whether the aberrant *RUNX3* hypermethylation/expression in CRC was associated with advanced stage. As shown in Figure 3, aberrant *RUNX3* hypermethylation/expression was significantly higher in advanced CRC compared with in early staged CRC (OR =0.54, CI =0.41–0.71, $P<0.0001$). These results suggest that epigenetic silencing of *RUNX3* gene expression by promoter hypermethylation

may play an important role in CRC progression and development.

Correlation of *RUNX3* hypermethylation/expression with microsatellite instability (MSI) in CRC

We also examined the data for 1,039 CRC patients pooled from four studies to assess whether the aberrant *RUNX3* hypermethylation/expression in CRC was associated with MSI. As shown in Figure 4, aberrant *RUNX3* hypermethylation/expression was significantly higher in MSI-positive CRC compared with in MSI-negative CRC (OR =0.44, CI =0.3–0.66, $P<0.0001$). These results suggest that epigenetic silencing of *RUNX3* gene expression by promoter hypermethylation may play an important role in high levels of MSI.
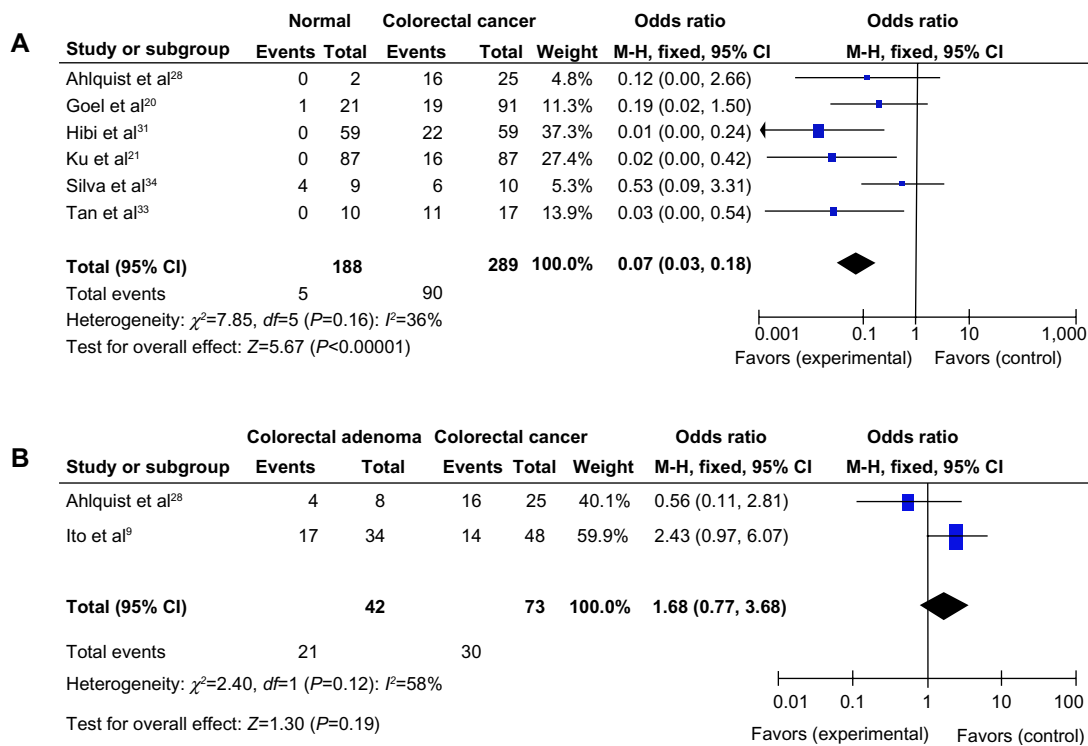
A

Study or subgroup	Normal Events	Total	Colorectal cancer Events	Total	Weight	Odds ratio M-H, fixed, 95% CI	Odds ratio M-H, fixed, 95% CI
Ahlquist et al[28]	0	2	16	25	4.8%	0.12 (0.00, 2.66)	
Goel et al[20]	1	21	19	91	11.3%	0.19 (0.02, 1.50)	
Hibi et al[31]	0	59	22	59	37.3%	0.01 (0.00, 0.24)	
Ku et al[21]	0	87	16	87	27.4%	0.02 (0.00, 0.42)	
Silva et al[34]	4	9	6	10	5.3%	0.53 (0.09, 3.31)	
Tan et al[33]	0	10	11	17	13.9%	0.03 (0.00, 0.54)	
Total (95% CI)		**188**		**289**	**100.0%**	**0.07 (0.03, 0.18)**	
Total events	5		90				

Heterogeneity: χ^2=7.85, *df*=5 (*P*=0.16): I^2=36%
Test for overall effect: *Z*=5.67 (*P*<0.00001)

B

Study or subgroup	Colorectal adenoma Events	Total	Colorectal cancer Events	Total	Weight	Odds ratio M-H, fixed, 95% CI	Odds ratio M-H, fixed, 95% CI
Ahlquist et al[28]	4	8	16	25	40.1%	0.56 (0.11, 2.81)	
Ito et al[9]	17	34	14	48	59.9%	2.43 (0.97, 6.07)	
Total (95% CI)		**42**		**73**	**100.0%**	**1.68 (0.77, 3.68)**	
Total events	21		30				

Heterogeneity: χ^2=2.40, *df*=1 (*P*=0.12): I^2=58%
Test for overall effect: *Z*=1.30 (*P*=0.19)

Figure 2 (**A**) Pooled odds ratios (ORs) from six studies of 289 colorectal cancer (CRC) and 188 normal colorectal mucosa.[a] (**B**) The pooled OR from two studies including 73 CRC and 42 colorectal adenoma.[b]
Notes: [a]OR =0.07, CI =0.00–0.54, *P*<0.00001; [b]OR =1.68, CI =0.77–3.68, *P*=0.19.
Abbreviations: CI, confidence interval; M-H, Mantel-Haenszel; OR, odds ratio; *df*, degrees of freedom.

RUNX3 hypermethylation/expression as a prognostic factor for CRC

Only two studies estimated the relationship between OS and *RUNX3* hypermethylation/expression in CRC. Ogino et al showed that CRC patients with *RUNX3* hypermethylation had a lower 1-year survival than those without *RUNX3* hypermethylation (*P*<0.001).[32] Soong et al observed that nuclear RUNX3 expression was associated with significantly better patient survival than no nuclear RUNX3 expression (*P*=0.025).[29] The pooled HR for OS showed that *RUNX3* hypermethylation or decreased RUNX3 expression was associated with worse survival in CRC patients, as shown in Figure 5 (HR =0.44, 95% CI =0.27–0.70, *P*=0.0006).
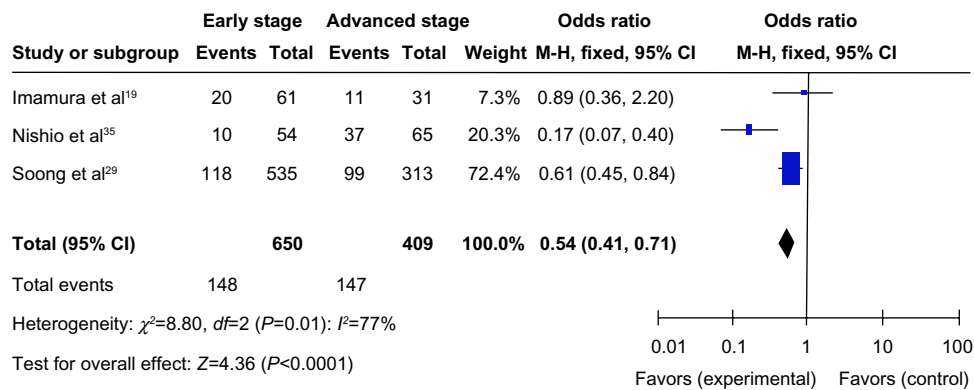
Study or subgroup	Early stage Events	Total	Advanced stage Events	Total	Weight	Odds ratio M-H, fixed, 95% CI	Odds ratio M-H, fixed, 95% CI
Imamura et al[19]	20	61	11	31	7.3%	0.89 (0.36, 2.20)	
Nishio et al[35]	10	54	37	65	20.3%	0.17 (0.07, 0.40)	
Soong et al[29]	118	535	99	313	72.4%	0.61 (0.45, 0.84)	
Total (95% CI)		**650**		**409**	**100.0%**	**0.54 (0.41, 0.71)**	
Total events	148		147				

Heterogeneity: χ^2=8.80, *df*=2 (*P*=0.01): I^2=77%
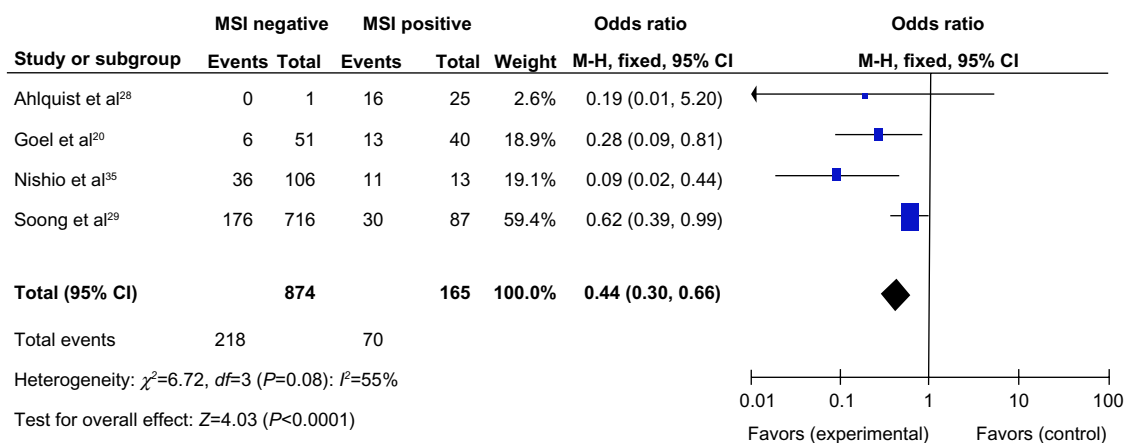Test for overall effect: *Z*=4.36 (*P*<0.0001)

Figure 3 A total of 1,059 colorectal cancer (CRC) patients pooled in three studies. Aberrant *RUNX3* hypermethylation/expression was significantly higher in advanced CRC than in early staged CRC.*
Notes: *OR =0.54, CI =0.41–0.71, *P*<0.0001.
Abbreviations: CI, confidence interval; M-H, Mantel-Haenszel; OR, odds ratio; *df*, degrees of freedom.

| Study or subgroup | MSI negative | | MSI positive | | | Odds ratio | Odds ratio |
	Events	Total	Events	Total	Weight	M-H, fixed, 95% CI	M-H, fixed, 95% CI
Ahlquist et al[28]	0	1	16	25	2.6%	0.19 (0.01, 5.20)	
Goel et al[20]	6	51	13	40	18.9%	0.28 (0.09, 0.81)	
Nishio et al[35]	36	106	11	13	19.1%	0.09 (0.02, 0.44)	
Soong et al[29]	176	716	30	87	59.4%	0.62 (0.39, 0.99)	
Total (95% CI)		874		165	100.0%	**0.44 (0.30, 0.66)**	
Total events	218		70				

Heterogeneity: χ^2=6.72, df=3 (P=0.08): I^2=55%

Test for overall effect: Z=4.03 (P<0.0001)

0.01 0.1 1 10 100
Favors (experimental)   Favors (control)

Figure 4 A total of 1,039 colorectal cancer (CRC) patients pooled in four studies. Aberrant *RUNX3* hypermethylation/expression was significantly higher in microsatellite instability (MSI)-positive CRC than in MSI-negative CRC.*
Notes: *OR =0.44, CI =0.30–0.66, P<0.0001.
Abbreviations: CI, confidence interval; M-H, Mantel-Haenszel; OR, odds ratio; *df*, degrees of freedom.

Sensitivity analyses and publication bias

A "sensitivity analysis", in which one study was removed at a time, was conducted to assess the stability of the results. The pooled ORs and HRs were not significantly changed, indicating the stability of our analyses. The funnel plots were largely symmetrical (Figure 6), suggesting there were no publication biases in our meta-analysis of *RUNX3* methylation/expression and clinicopathological features.

Discussion

"Epigenetics" is the study of heritable and age-related modifications of the genome that occur without a change in the primary DNA sequence. Epigenetic alterations, particularly aberrant DNA methylation, one of the best-characterized epigenetic modifications, contribute to tumor initiation and progression.[40,41] RUNX3 belongs to the runt-domain family of transcription factors, and its inactivation by promoter hypermethylation plays an important role in the normal development of, and tumorigenesis in, several types of tumors

including CRC.[14,42–53] To date, there have been some studies describing the precise expression, prognostic impact, and methylation status of *RUNX3* in CRC; however, the role of *RUNX3* inactivation in CRC and *RUNX3* inactivation have not been thoroughly investigated. We conducted the meta-analysis to evaluate the correlation of *RUNX3* hypermethylation/low expression with CRC. Analysis of the pooled data showed that: CRC had a higher hypermethylation OR than normal colorectal mucosa; aberrant *RUNX3* hypermethylation/expression was significantly higher in advanced CRC compared with in early staged CRC, indicating that epigenetic silencing of *RUNX3* gene expression by promoter hypermethylation may play an important role in CRC progression and development; and aberrant *RUNX3* hypermethylation/expression was significantly higher in MSI-positive CRC compared with in MSI-negative CRC. In addition, CRC patients with *RUNX3* hypermethylation or lacking RUNX3-protein expression had a lower survival rate than those without *RUNX3* hypermethylation or with RUNX3-protein

Study or subgroup	Log (hazard ratio)	SE	Weight	Hazard ratio IV, fixed, 95% CI	Hazard ratio IV, fixed, 95% CI
Ogino et al[32]	0.274	1.205	4.0%	1.32 (0.12, 13.95)	
Soong et al[29]	−0.876	0.246	96.0%	0.42 (0.26, 0.67)	
Total(95% CI)			100.0%	**0.44 (0.27, 0.70)**	

Heterogeneity: χ^2=0.87, df=1 (P=0.35); I^2=0%

Test for overall effect: Z=3.44 (P=0.0006)

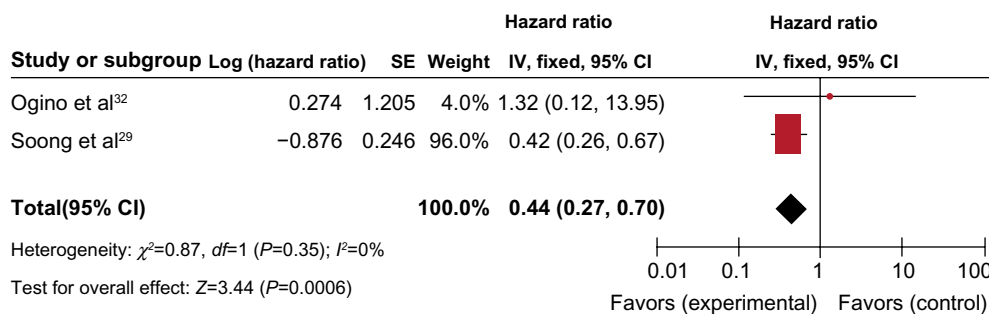0.01 0.1 1 10 100
Favors (experimental)   Favors (control)

Figure 5 Two studies estimated the relationship between overall survival (OS) and *RUNX3* hypermethylation/expression in colorectal cancer (CRC). The pooled hazard ratios (HRs) for OS showed that *RUNX3* hypermethylation or decreased RUNX3 expression was associated with worse survival in CRC patients.*
Notes: *HR =0.44, 95% CI =0.27–0.70, P=0.0006.
Abbreviations: CI, confidence interval; M-H, Mantel-Haenszel; HR, hazards ratio; *df*, degrees of freedom.
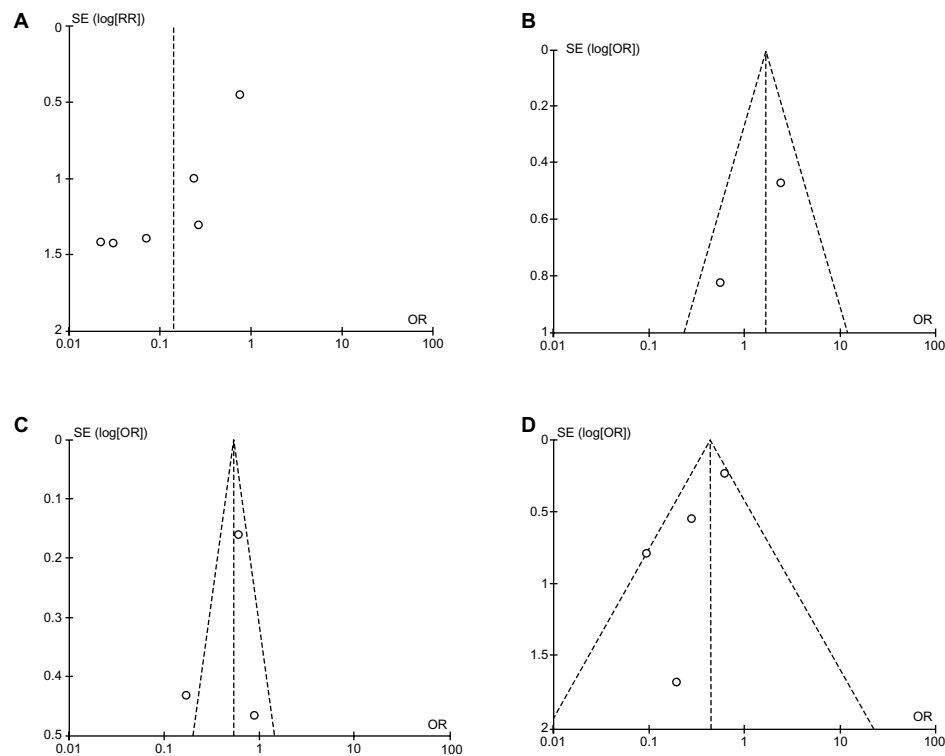
Figure 6 Funnel plots, which are largely symmetrical, suggesting there were no publication biases in the meta-analysis of *RUNX3* hypermethylation/expression and clinicopathological features. (**A**) Funnel plot from six studies comparing colorectal cancer (CRC) with normal colorectal mucosa. (**B**) Funnel plot from two studies comparing CRC and colorectal adenoma. (**C**) Funnel plot from three studies comparing different stages of CRC. (**D**) Funnel plot from four studies comparing microsatellite instability (MSI)-positive and MSI-negative CRC.
Notes: X axis, value of OR; Y axis, SEs multiplied by log scale of OR.
Abbreviations: OR, odds ratio; SE, standard error.

expression. Progression from colorectal adenoma to CRC appears to mirror the accumulation of genetic abnormalities, suggesting a stepwise progression of genetic changes. In fact, the hypermethylation of several tumor suppressors has been reported in colorectal adenoma.[54–56] Combining *RUNX3* with other genes to develop several gene markers will be a good strategy for risk stratification to predict initial carcinogenesis and neoplastic progression, including in CRC.[54]

RUNX3 exerts pleiotropic effects during tumor suppression. RUNX3 inhibits the oncogenic Wnt signaling pathway via the formation of a complex with the TCF4-β-catenin complex and hampers it from binding to target genes such as *c-myc* and *cyclin D1*.[42,57] RUNX3 interacts with SMAD3/SMAD4 to activate TGF-β-dependent proliferation inhibition and apoptosis by activation of p21 and Bim. Although only two studies estimated the relationship between OS and *RUNX3* hypermethylation/expression in CRC, both showed very similar results. Ogino et al showed that CRC patients with *RUNX3* hypermethylation had lower 1-year survival than those without *RUNX3* hypermethylation ($P<0.001$).[32] Soong et al observed that nuclear RUNX3 expression was associated with significantly better patient

survival than no nuclear RUNX3 expression ($P=0.025$).[29] In addition, detection of *RUNX3* methylation status may predict treatment effects among CRC patients who receive fluorouracil-based and irinotecan-based chemotherapy.[32]

Consistent results were shown in sensitivity analyses, and no evidence of publication bias was found. This study has several potential limitations. First, the possibility of information and selection biases and unidentified confounders could not be completely excluded, because all of the included studies were observational. Second, the search strategy was restricted to articles published in English. Articles with potentially high-quality data published in other languages were not included because of anticipated difficulties in obtaining accurate medical translation. Hence, caution should be taken in interpreting our findings among the general population.

Disclosure

The authors declare no conflicts of interest in this work.

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