ORIGINAL RESEARCH

Clinicopathological significance and potential drug target of *RUNX3* in breast cancer

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Background: Previous reports indicate that RUNX3 is a tumor suppressor in several types of human tumors, including breast cancer (BC). However, the correlation between *RUNX3* hypermethylation and the incidence of BC remains unclear. In this study, we conducted a systematic review and meta-analysis aiming to comprehensively assess the potential role of *RUNX3* hypermethylation in the pathogenesis of BC.

Methods: A detailed literature search was made to identify studies for related research publications. Methodological quality of the studies was evaluated. Analysis of pooled data was performed. Odds ratio (OR) was calculated and summarized respectively.

Results: Final analysis of 565 BC patients from eleven eligible studies was performed. The results showed that *RUNX3* hypermethylation was significantly higher in BC than in normal breast tissue, the pooled OR from nine studies including 339 BC and 248 normal breast tissue (OR =24.12, 95% confidence interval [CI] =13.50–43.11, Z=10.75, P<0.00001). Further analysis also showed significantly increased OR of *RUNX3* hypermethylation in estrogen receptor (ER)-positive than in ER-negative BC patients (OR =5.67, 95% CI =2.69–11.95, Z=4.57, P<0.00001). In addition, *RUNX3* messenger RNA (mRNA) high expression was found to be correlated to better overall survival in 3,455 cases of BC patients that were followed up for 20 years (hazard ratio [HR] 0.79, P=8.8×10^{-5}). Interestingly, *RUNX3* mRNA overexpression was found to be correlated to better overall survival in only 668 cases of ER-negative patients (HR 0.72, P=0.01), but not in 1,767 cases of ER-positive patients (HR 0.87, P=0.13).

Conclusion: The results of this meta-analysis suggest that *RUNX3* hypermethylation may be implicated in the pathogenesis of BC. Detection of *RUNX3* mRNA may be a helpful and valuable biomarker for diagnosis of BC, especially in ER-negative BC. We also discussed the significance of RUNX3 as a potential drug target.

Keywords: methylation, tumor suppressor gene, meta-analysis, odds ratio

Introduction

Breast cancer (BC), with approximately 232,340 new cases every year, is the most common malignancy among women and is also the leading cancer-related death for women worldwide.[1] Although early diagnostic methods, surgical techniques, and molecular target therapy have undergone considerable advancements, the incidence of BC is still increasing, and the prognosis of BC patients remains discouraging due to the high postoperative recurrence rate and metastasis.[2,3] Therefore, investigations on the mechanism of incidence and progression of BC are still needed and will help to select patients with higher chances of BC recurrence and provide better prognostic prediction and individualized treatments.

The *runt*-domain related (*RUNX*) family genes are essential regulator genes of cell fate in development and regulation of p53-dependent DNA damage response and/or tumorigenesis.[4–6] *RUNX* family genes are composed of *RUNX1*, *RUNX2*, and *RUNX3*.

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Among the three members, *RUNX3* gene plays a critical role in the regulation of cell proliferation, apoptosis, angiogenesis, as well as cell adhesion and invasion.[7,8] *RUNX3* gene is considered as a tumor suppressor gene involved in the TGF-β signaling pathway because it is localized in chromosome 1p36, a region that exhibits frequent loss of heterozygosity events in breast, colon, gastric, and ovarian cancers.[9] RUNX3 is one of principal responders of the p14(ARF)-MDM2 cell surveillance pathway, and it is able to prevent pathologic consequences of abnormal oncogene activation.[10] The molecular and biological functions of RUNX3 have been intensively studied in several types of tumors through upregulation of inducing cell cycle arrest, apoptosis, and downregulation of cyclin D1 expression.[11–15] Downregulation of RUNX3 protein by promoter methylation (hypermethylation) has been found to play an important role in epithelial tumorigenesis and epithelial-mesenchymal transition of several malignancies including BC.[11,15–21] RUNX3 also inhibits the estrogen-dependent proliferation, transformation, and the tumorigenicity of BC cells in severe combined immunodeficiency mice.[22] Of female RUNX3 (+/−) mice, 20% spontaneously developed ductal carcinoma at an average age of 14.5 months, indicating that RUNX3 acts as a novel tumor suppressor in BC.[22]

Although previous studies have indicated that inactivation of the *RUNX3* gene is mainly induced by its promoter hypermethylation and is one of the important epigenetic alterations in BC, the reported rates of *RUNX3* hypermethylation in BC are remarkably diverse. Moreover, whether or not *RUNX3* gene hypermethylation is associated with the incidence of BC remains unclear. The various results of these studies underpin the need for assessing the evidence of the relationship between *RUNX3* inactivation and causes of BC. In the current study, we performed a systematic review and meta-analysis to quantitatively evaluate the effects of *RUNX3* hypermethylation on the incidence of BC. In addition, we evaluated *RUNX3* messenger RNA (mRNA) as a prognostic marker in BC patients and discussed RUNX3 as a potential drug target.

Material and methods
Search strategy and selection criteria
We searched PubMed, Embase, and ISI web of knowledge to identify studies from January 1, 2000 to July, 2014 using the search terms: "breast" 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.

After excluding the nonrelevant and/or redundant publications from the different databases, the remaining papers were evaluated in the full-text version for inclusion and exclusion criteria and for relevant articles in the reference lists. All searched data were retrieved. Authors' bibliographies and references of selected studies were also searched for additional relevant studies. The most complete study was chosen to avoid duplication if same patient populations were reported in different publications.

Criteria that an eligible study had to meet were as follows: 1) *RUNX3* methylation evaluated in the primary BC tissues, 2) research revealed the relationship between *RUNX3* methylation and BC incidence, and 3) studies provided sufficient information to estimate an odds ratio (OR) and 95% confidence interval (CI). The exclusion criteria included the following: 1) letters, reviews, case reports, conference abstracts, editorials, and expert opinion; and 2) all publications regarding in vitro/ex vivo studies, cell lines, and human xenografts were also excluded.

Data extraction and methodological assessment
We reviewed and extracted data from eligible studies. Disagreements were resolved by discussion and consensus. The following information were recorded for each study: the first author name, year of publication, authors' country, sample source, number of cases, clinicopathological parameters, methylation detection method, methylation rate and/or expression, and follow up. Data for study characteristics were summarized in a table format. Heterogeneity of investigation was evaluated to determine whether the data of the various studies could be analyzed for a meta-analysis.

For the methodological evaluation of the studies, we read through each publication independently and assessed and scored the publications according to REMARK guidelines and ELCWP quality scale.[23,24] We shared the quality scores and compared them. Then, we reached a consensus value for each item.

Patient survival analysis
An online database[25] was used to assess the relevance of *RUNX3* mRNA expression to relapse-free survival. The database was established using gene expression data and survival information of 3,455 BC patients downloaded from Gene Expression Omnibus. Briefly, *RUNX3* gene was entered into the database (http://kmplot.com/analysis/index.php?p=service&cancer=breast) to obtain Kaplan–Meier survival plots where the number-at-risk is indicated below the

main plot. Hazard ratio (and 95% CIs) and log rank *P* were calculated and displayed on the webpage.

Statistical analysis

Analysis was conducted using the STATA 12.0 (StataCorp LP, College Station, TX, USA) and Review Manager 5.2 (Cochrane Collaboration, Oxford, UK). The pooled frequency of *RUNX3* hypermethylation and 95% CIs were estimated. The frequency of *RUNX3* hypermethylation was compared in BC and normal tissues. Heterogeneity among studies was evaluated with Cochran's Q test[26] and the I^2 statistic.[27,28] When heterogeneity was not an issue (I^2 values <50%), a fixed effect 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 *RUNX3* hypermethylation in BC and in normal breast tissues. *P*-values tailed less than 0.05 were considered statistically significant.

Publication bias was assessed by using a method reported by Egger et al.[29] 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 version 10.0.

Results

Identification of relevant studies

Fifty-five publications were identified by the search method as described above. Forty-four of those were excluded due to laboratory studies, non-original articles (review), or studies irrelevant to the current analysis. Eventually, there were eleven studies included in the final meta-analysis, as shown in Figure 1.

Study characteristics

Eleven studies published from 2005 to 2013 were eligible for meta-analysis. A total of 565 BC patients from the People's Republic of China, South Korea, Japan, Singapore, and USA were enrolled. Their basic characteristics were summarized in Table 1.
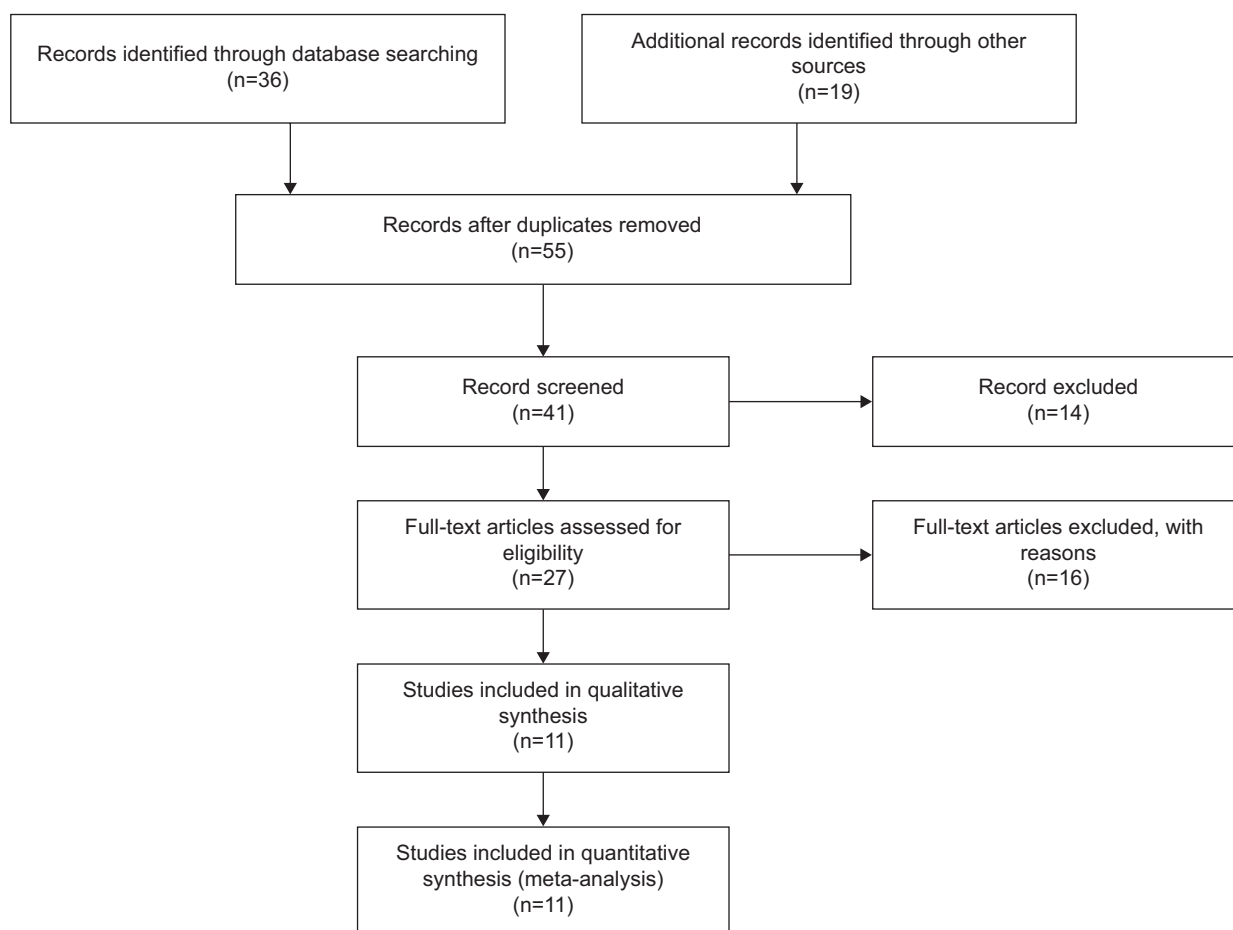

Figure 1 Flowchart of study selection.

Table 1 Basic characteristics of the included studies

Study	Country	Patients	Methods	Primary aim	Methylation site	*RUNX3* expression
Li et al[48]	People's Republic of China	48	Methylation specific PCR (MSP)	Determine the methylation status of the *RUNX3* gene and protein expression in BC patients	Promoter, CpG islands	+
Park et al[49]	South Korea	129	MethyLight	Determine the methylation status of 15 tumor suppressors in BC patients	Promoter, CpG islands	−
Qiao et al[50]	People's Republic of China	60	MSP	Determine the methylation status of the *RUNX3* gene in early diagnosis of BC patients	Promoter, CpG islands	−
Park et al[51]	South Korea	35	MethyLight	To elucidate the role of seven tumor suppressors in BC patients	Promoter, CpG islands	−
Subramaniam et al[21]	Singapore	40	MSP, IHC	Determine the role of *RUNX3* gene and protein in the progression of BC patients	Promoter, CpG islands	+
Du et al[52]	People's Republic of China	40	MSP, IHC	Determine the role of *RUNX3* gene and protein in the prediction of BC	Promoter, CpG islands	+
Tian et al[53]	People's Republic of China	56	MSP	To examine the correlation of *RUNX3* methylation in BC and its pathologic features	Promoter, CpG islands	−
Subramaniam et al[37]	Singapore	61	MSP, IHC	Determine the role of *RUNX3* gene and protein in the progression of BC patients	Promoter, CpG islands	+
Jiang et al[54]	People's Republic of China	15	MSP, IHC	To examine the correlation of *RUNX3* methylation and protein in BC and its pathologic features	Promoter, CpG islands	+
Lau et al[42]	United States	44	MSP, RT-PCR	Determine the role of *RUNX3* gene and protein in the progression of BC patients	Promoter, CpG islands	+
Suzuki et al[55]	Japan	37	MSP	Determine inactivation of TGFβ-related genes *DRM/Gremlin*, *RUNX3*, and *HPP1* in human cancers	Promoter, CpG islands	−

Abbreviations: BC, breast cancer; IHC, immunohistochemistry; MSP, methylation-specific PCR; PCR, polymerase chain reaction; RT-PCR, reverse transcription PCR.

The correlation of *RUNX3* hypermethylation with carcinogenesis
Comparison of *RUNX3* hypermethylation in BC tissue and normal breast tissue

First, we determined that *RUNX3* hypermethylation was significantly higher in BC than in normal breast tissues. There was no evidence of heterogeneity across the studies (*P* for heterogeneity =0.42; I^2 =2%). The pooled OR from nine studies including 339 BC and 248 normal breast tissue is shown in Figure 2 (OR =24.12, 95% CI =13.50–43.11, *Z*=10.75, *P*<0.00001), indicating that *RUNX3* inactivation through hypermethylation plays an important role in the pathogenesis of BC.

Correlation of *RUNX3* hypermethylation with estrogen receptor status in BC

Then, we determined whether or not *RUNX3* hypermethylation rate in BC was correlated to estrogen receptor (ER) status in

BC patients. The pooled OR from three studies including 139 ER-positive BC and 101 ER-negative BC is shown in Figure 3 (OR =5.67, 95% CI =2.69–11.95, *Z*=4.57, *P*<0.00001), indicating that *RUNX3* hypermethylation was significantly higher in ER-positive BC than in ER-negative BC.

Sensitivity analyses and publication bias

A sensitivity analysis, in which one study was removed at a time, was conducted to assess the result stability. The pooled OR was not significantly changed, indicating the stability of our analyses. The funnel plots were largely symmetric (Figure 4A and B), suggesting there were no publication biases in the meta-analysis.

Impact of *RUNX3* mRNA expression on prognosis of BC

The clinical relevance of *RUNX3* was further corroborated in a patient survival analysis using an online database containing
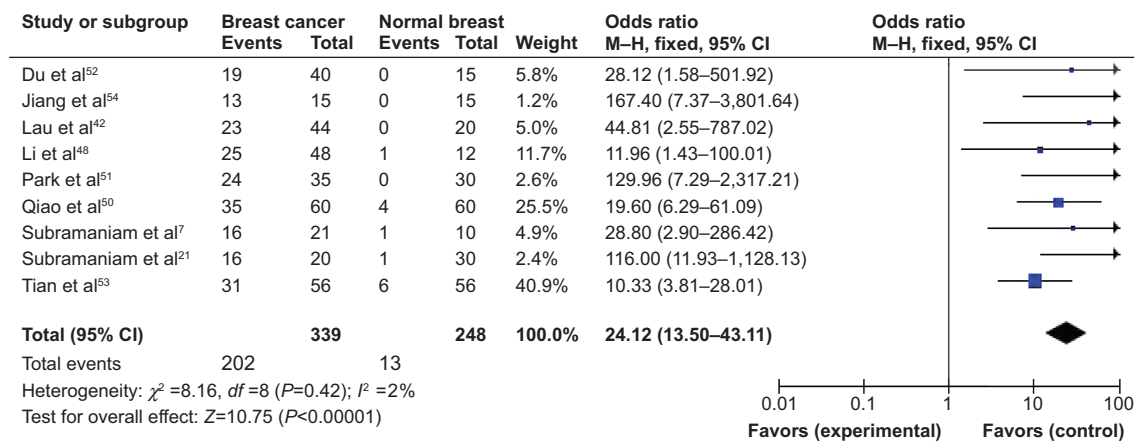
Study or subgroup	Breast cancer Events	Total	Normal breast Events	Total	Weight	Odds ratio M–H, fixed, 95% CI	Odds ratio M–H, fixed, 95% CI
Du et al[52]	19	40	0	15	5.8%	28.12 (1.58–501.92)	
Jiang et al[54]	13	15	0	15	1.2%	167.40 (7.37–3,801.64)	
Lau et al[42]	23	44	0	20	5.0%	44.81 (2.55–787.02)	
Li et al[48]	25	48	1	12	11.7%	11.96 (1.43–100.01)	
Park et al[51]	24	35	0	30	2.6%	129.96 (7.29–2,317.21)	
Qiao et al[50]	35	60	4	60	25.5%	19.60 (6.29–61.09)	
Subramaniam et al[7]	16	21	1	10	4.9%	28.80 (2.90–286.42)	
Subramaniam et al[21]	16	20	1	30	2.4%	116.00 (11.93–1,128.13)	
Tian et al[53]	31	56	6	56	40.9%	10.33 (3.81–28.01)	
Total (95% CI)		**339**		**248**	**100.0%**	**24.12 (13.50–43.11)**	
Total events	202		13				
Heterogeneity: χ^2 =8.16, *df* =8 (*P*=0.42); I^2 =2%							
Test for overall effect: *Z*=10.75 (*P*<0.00001)							

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

Figure 2 The pooled OR from nine studies including 339 BC and 248 normal breast tissues.
Notes: OR =24.12, 95% CI =13.50–43.11, Z=10.75, P<0.00001.
Abbreviations: BC, breast cancer; CI, confidence interval; *df*, degrees of freedom; M–H, Mantel–Haenszel; OR, odds ratio.

the expression of 22,277 genes and 20-year survival information of 3,455 BC patients.[25] *RUNX3* mRNA high expression was found to be correlated to better overall survival (OS) for all BC patients followed for 20 years (Figure 5A; hazard ratio [HR] 0.79, *P*=8.8×10^{−5}). In addition, *RUNX3* mRNA high expression was not found to be correlated to better OS in ER-positive patients (Figure 5B; HR 0.87, *P*=0.13) but only in ER-negative patients (Figure 5C; HR 0.72, *P*=0.01).

Discussion

Inactivation of RUNX3 by promoter hypermethylation plays an important role during normal development and tumorigenesis in several types of tumors including BC.[11,30–40] To date, there have been some studies describing the precise expression and methylation status of *RUNX3* in BC; however, the roles of *RUNX3* hypermethylation in BC and its correlation with carcinogenesis have not been thoroughly investigated. Analysis of the pooled data showed that BC had a higher *RUNX3* hypermethylation than normal breast tissues. *RUNX3* hypermethylation was also significantly higher in

ER-positive BC than in ER-negative BC. The results from the current study indicated that the hypermethylation rate of *RUNX3* gene promoter in BC was strongly correlated to BC incidence. Since changes in *RUNX3* promoter hypermethylation are reversible, drug treatment through demethylation may be useful to delay carcinogenesis and progression. Trichostatin A and/or 5-Aza-CdR (5-aza-2′-deoxycytidine) are capable of inhibiting BC cell proliferation and inducing apoptosis by eliminating the methylation status of *RUNX3* promoter and restoring its expression.[41,42] Reintroduction of RUNX3 into UNX3-deficient BC cells suppresses cancer cell proliferation and their tumorigenic potential.[22,42,43] Therefore, restoring RUNX3 activation by specific small molecules or inhibitors to block *RUNX3* hypermethylation might constitute a novel therapeutic strategy for the treatment of BC. This approach brings a new direction and hope for cancer treatment through epigenetic modulation and/or gene-targeted therapy.

Epigenetic alterations, particularly aberrant DNA methylation, one of the best-characterized epigenetic modifications,
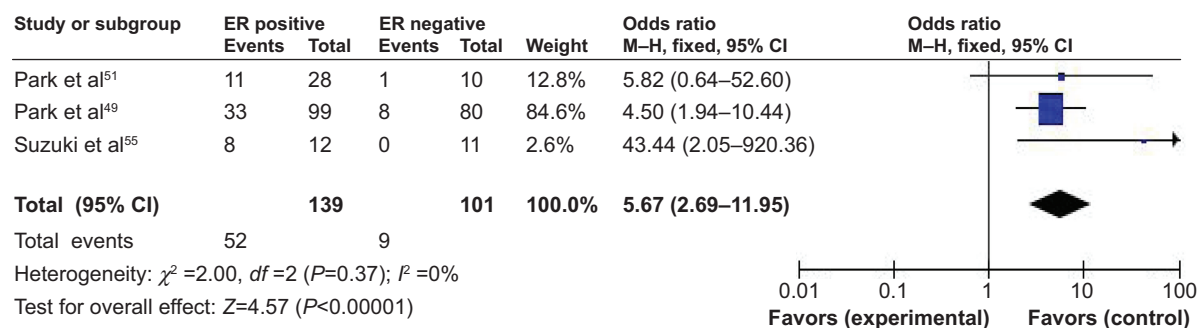
Study or subgroup	ER positive Events	Total	ER negative Events	Total	Weight	Odds ratio M–H, fixed, 95% CI	Odds ratio M–H, fixed, 95% CI
Park et al[51]	11	28	1	10	12.8%	5.82 (0.64–52.60)	
Park et al[49]	33	99	8	80	84.6%	4.50 (1.94–10.44)	
Suzuki et al[55]	8	12	0	11	2.6%	43.44 (2.05–920.36)	
Total (95% CI)		**139**		**101**	**100.0%**	**5.67 (2.69–11.95)**	
Total events	52		9				
Heterogeneity: χ^2 =2.00, *df* =2 (*P*=0.37); I^2 =0%							
Test for overall effect: *Z*=4.57 (*P*<0.00001)							

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

Figure 3 The pooled OR from three studies including 139 ER-positive BC and 101 ER-negative BC.
Notes: OR =5.67, 95% CI =2.69–11.95, Z=4.57, P<0.00001.
Abbreviations: BC, breast cancer; CI, confidence interval; *df*, degrees of freedom; ER, estrogen receptor; M–H, Mantel–Haenszel; OR, odds ratio.
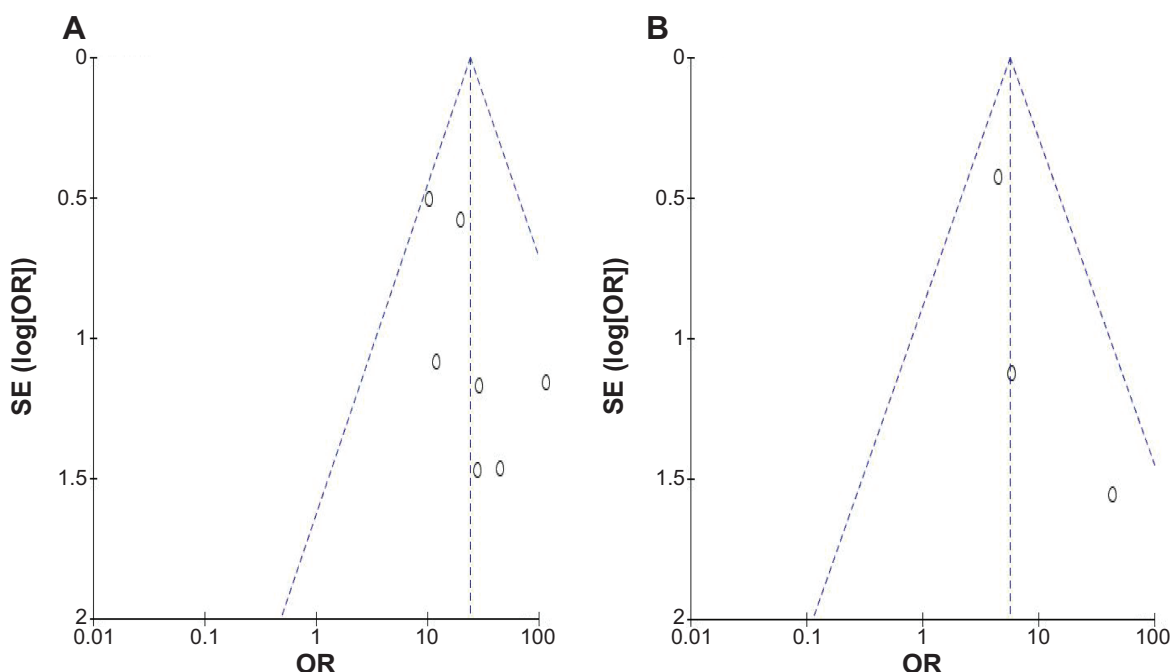
Figure 4 The funnel plots were largely symmetrical, suggesting there were no publication biases in the meta-analysis.
Notes: The funnel plot from nine studies comparing *RUNX3* hypermethylation between BC and normal breast tissue (**A**). The funnel plot from three studies comparing *RUNX3* hypermethylation between ER-positive BC and ER-negative BC (**B**).
Abbreviations: BC, breast cancer; ER, estrogen receptor; OR, odds ratio; SE, standard error.

contribute to tumor initiation and progression.[44,45] RUNX3 inhibits the oncogenic Wnt signaling pathway via the formation of a complex with the TCF4-β-catenin complex and hampering it from binding to target genes such as c-myc and cyclin D1.[18,30] RUNX3 interacts with SMAD3/SMAD4 to activate TGF-β-dependent proliferation inhibition and apoptosis by the activation of p21 and Bim. In RUNX3 (−/−) p53 (−/−) murine gastric epithelial (GIF-14) cells, concurrent activation of an EGFR/Ras gene expression signature was observed during TGF-β-induced epithelial-mesenchymal transition in GIF-14 cells.[19] RUNX3 also destabilizes HIF-1α protein by promoting the proline hydroxylation of HIF-1α through binding to HIF-1α/PHD2, thus RUNX3 could be an inhibitor of HIF-1α and of hypoxia-mediated angiogenesis.[46] RUNX3 acts as a novel co-activator for p53 through regulating its DNA damage-induced phosphorylation at Ser-15 and mediates tumor suppression.[47] In addition, RUNX3 could also inhibit ERα-dependent transactivation by reducing the stability of ERα in BC.[22] Therefore, RUNX3 can be considered as a tumor suppressor, and its inactivation could contribute to tumor initiation and progression. Based on this meta-analysis, we may conclude that *RUNX3* hypermethylation in BC tends to indicate higher incidence of BC. We further determined the clinical relevance of *RUNX3* mRNA expression in a patient survival analysis using an online database containing the expression of 22,277 genes and 20-year survival information

of 3,455 BC patients.[25] *RUNX3* mRNA high expression was found to be correlated to better OS for all BC patients (HR 0.79, $P=8.8\times10^{-5}$). Interestingly, *RUNX3* mRNA high expression was not found to be correlated to better OS in ER-positive patients (HR 0.87, $P=0.13$) but only in ER-negative patients (HR 0.72, $P=0.01$). *RUNX3* mRNA low expression might be due to the *RUNX3* hypermethylation in BC patients. Since ER-negative BC patients lack the prognostic marker and drug target, RUNX3 will be more important for future study in ER-negative BC.

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 searching strategy was restricted to articles published in English and Chinese. Articles with potentially high-quality data that were published in other languages were not included because of anticipated difficulties in obtaining accurate medical translation. The correlation of *RUNX3* hypermethylation with ER status in BC was based on the pooled OR from only three studies including 139 ER-positive BC and 101 ER-negative BC; the conclusion will need further validation in future study. In addition, most selected publications are from Asia; none are from Europe. Hence, cautions should be
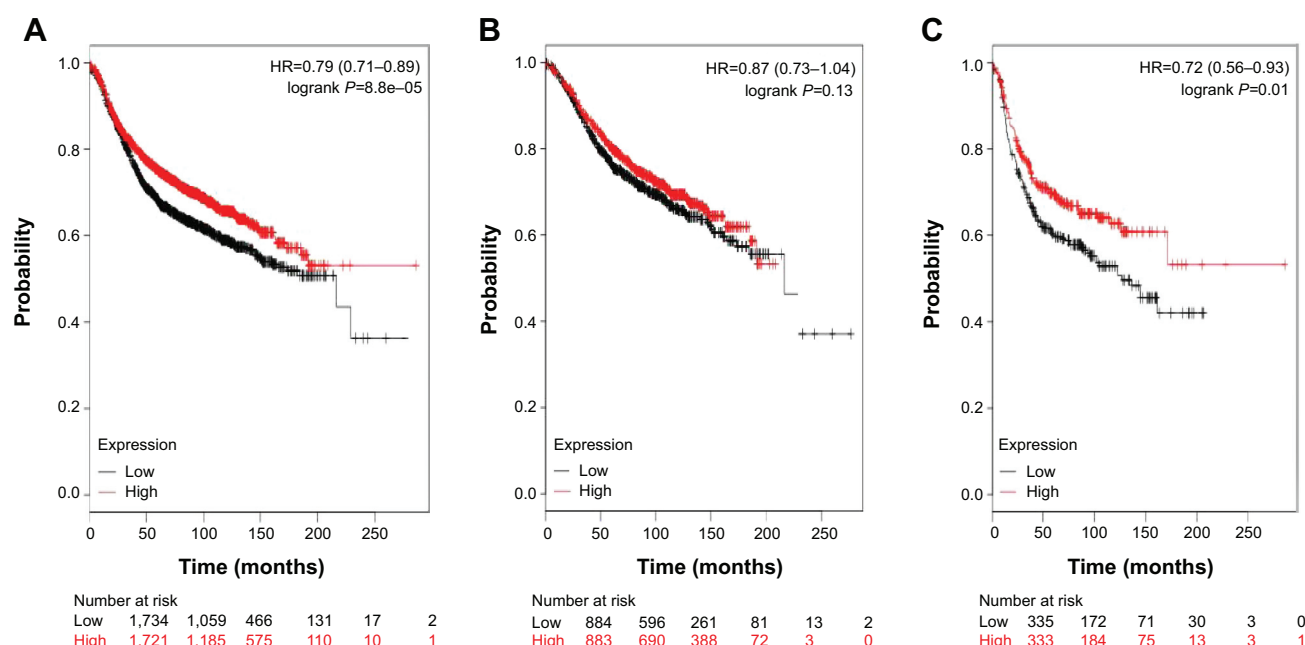
Figure 5 The clinical relevance of *RUNX3* was determined in a patient survival analysis using an online database containing the expression of 22,277 genes and 20-year survival information of 3,455 BC patients.
Notes: *RUNX3* mRNA high expression was found to be correlated to better OS for all BC patients followed for 20 years (HR 0.79, $P=8.8\times10^{-5}$) (**A**). *RUNX3* mRNA high expression was not found to be correlated to better OS in ER-positive patients (HR 0.87, $P=0.13$) (**B**), but only in ER-negative patients (HR 0.72, $P=0.01$) (**C**).
Abbreviations: BC, breast cancer; ER, estrogen receptor; HR, hazard ratio; mRNA, messenger RNA; OS, overall survival.

taken when our findings are interpreted among the general populations.

In conclusion, our meta-analysis showed *RUNX3* may play an important role in BC incidence, and *RUNX3* hypermethylation is strongly correlated with ER status. In addition, *RUNX3* mRNA high expression was found to be correlated to better OS for all BC patients, especially in ER-negative BC patients. Further large-scale studies, especially multicenter and well-matched cohort research, will provide more insight into the role of *RUNX3* in the carcinogenesis and clinical implementation of BC patients.

Disclosure

The authors have no financial involvement with any organization or entity with a financial interest in the subject matter or materials discussed in the manuscript.

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