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ORIGINAL RESEARCH

The clinicopathological significance of *RUNX3* hypermethylation and mRNA expression in human breast cancer, a meta-analysis

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Abstract: Aberrant promoter methylation of *RUNX3* has been reported in several tumors including human breast cancer (BC). However, the association between *RUNX3* hypermethylation and incidence of BC remains elusive. In this study, a detailed literature search was performed in Medline and Google Scholar for related research publications. Analysis of pooled data were executed. Odds ratios with corresponding confidence intervals were determined and summarized, respectively. Finally, 13 studies were identified for the meta-analysis. Analysis of the pooled data showed that *RUNX3* hypermethylation was significantly higher in both ductal carcinoma in situ and invasive ductal carcinoma (IDC) than in normal breast tissues. In addition, *RUNX3* methylation was significantly higher in IDC than in benign tumor. However, *RUNX3* methylation was not significantly higher in IDC than in ductal carcinoma in situ. We also determined that *RUNX3* hypermethylation was significantly higher in ER positive BC than in ER negative BC. In addition, high *RUNX3* mRNA expression was found to be correlated with better overall survival and relapse-free survival for all BC patients. Our results strongly support that *RUNX3* hypermethylation may play an important role in BC incidence. *RUNX3* methylation is a valuable early biomarker for the diagnosis of BC. Further large-scale studies will provide more insight into the role of *RUNX3* hypermethylation in the carcinogenesis and clinical diagnosis of BC patients.

Keywords: breast cancer, estrogen receptor, *RUNX3*, meta-analysis, methylation, odds ratio

Introduction

Breast carcinogenesis is a multi-step process that originates as flat epithelial atypia, progresses to atypical ductal hyperplasia, advances to ductal carcinoma in situ (DCIS), and culminates as invasive ductal carcinoma (IDC).[1,2] Although early diagnostic tools, surgical approaches, and molecular targeted therapy have undergone considerable improvements, the incidence of breast cancer (BC) is still increasing, and the outcome of BC patients remains disappointing due to the high postoperative recurrence rate and metastasis.[3,4] Thus, the identification of the molecular mechanisms of incidence and development of BC is still required and will help to provide better prognostic prediction and individualized treatments of patients with higher chances of BC recurrence.

RUNX family genes consist of *RUNX1*, *RUNX2*, and *RUNX3*. They are essential regulators of cell fate in the regulation of p53-dependent DNA damage response and/or tumorigenesis.[5–7] RUNX3 interacts with CTNNB1/TCFs and prevents the transactivation by inhibiting CTNNB1/TCFs' DNA binding.[8] Ito has observed that RUNX3 downregulates Wnt signaling by directly preventing β-catenin/TCFs in gastric and colon cancers.[9] Therefore, RUNX3 via Wnt signaling pathway contributes to carcinogenesis as a tumor suppressor. The activation of the Wnt/CTNNB1 pathway was observed following

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knockdown of PTEN in human breast cells.[10] Furthermore, the downregulation of the secreted Wnt inhibitor Sfrp1 was reported in most invasive human breast carcinomas.[11] RUNX3 was first reported as a tumor suppressor because of the causal link between the loss of RUNX3 and gastric carcinogenesis.[12] Since then, *RUNX3* has been observed as a suppressor that is inactivated in a wide variety of pre-invasive and invasive epithelial and mesenchymal neoplasms.[13] RUNX3 protein regulates the growth-suppressive effects of TGF-β by associating with SMAD, a downstream protein in the signaling pathway.[14] Taken together, RUNX3 as a suppressor plays a critical role in the development of BC via TGF-β[15] and Wnt signaling pathway.[16] Even if previous reports showed that inactivation of the *RUNX3* gene is mainly caused by its promoter hypermethylation in BC, the positive rates of *RUNX3* hypermethylation in BC were extraordinarily diverse. In addition, it remains elusive whether or not *RUNX3* gene hypermethylation is correlated with the early stage of BC. In this study, we performed a meta-analysis to determine the effects of *RUNX3* hypermethylation on the incidence of BC. In addition, we evaluated *RUNX3* mRNA as a prognostic marker in BC patients.

Methods

Search strategy and methodological assessment

We searched Embase, PubMed, and ISI web of knowledge to select studies from January 1, 1998 to October 2015 using the terms: "breast" and "cancer or tumor or neoplasm or carcinoma", "methylation", and "RUNX3". We also manually searched the reference lists of the retrieved reviews and articles for additional studies.

We used the following criteria for identification of studies: 1) *RUNX3* methylation examined in the primary BC tissues, 2) research showed the correlation between *RUNX3* methylation and BC incidence, 3) studies contained enough data to determine odds ratio (OR) and 95% confidence interval (CI). The exclusion criteria were: 1) reviews, letters, case reports, editorials, conference abstracts, and expert opinions, 2) all articles regarding cell lines, in vitro/ex vivo studies, and human xenografts were also excluded.

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

For the methodological evaluation of the studies, we read through each article independently, and assessed and scored them according to the Newcastle–Ottawa Quality Assessment Scale (NOQAS), for cohort and case-control studies.[17] Studies are rated from one to nine stars in the NOQAS, with nine stars indicating a high-quality study. Any discrepancies or disagreements were discussed, and if consensus could not be achieved, a third reviewer resolved the issue. The Institutional Review Board of Beijing Chest Hospital does not require ethics approvals for these case studies.

Prognostic value of *RUNX3* mRNA expression in BC patients

An online database[18] was used to determine the relevance of *RUNX3* mRNA expression to prognosis of BC patients. We used the database that was established using gene expression data and survival information of 3,455 BC patients. *RUNX3* gene was entered into the database (http://kmplot.com/analysis/index.php?p=service&cancer=breast) to get Kaplan–Meier survival plots. The mRNA expression above or below the median separates the cases into high expression and low expression. Hazard ratio and 95% CIs, as well as logrank *P* were calculated.

Statistical analysis

Analysis was performed using the Review Manager 5.2 (Cochrane Collaboration, Oxford, UK) and STATA 12.0 (StataCorp LP, College Station, TX, USA). Heterogeneity among studies was determined with Cochran's Q test[19] and the I^2 statistic.[20,21] 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. When heterogeneity was not an issue (I^2 values <50%), a fixed effect model was used to calculate parameters. The pooled frequency of *RUNX3* hypermethylation and 95% CIs were estimated. *P*-values tailed less than 0.05 were considered statistically significant.

A method reported by Egger et al was used for assessment of publication bias.[22] The analysis of meta-regression and publication bias was evaluated using STATA version 10.0. We also examined reasons for statistical heterogeneity using meta-regression, subgroup analysis, and sensitivity analysis.

Results

Identification of relevant studies

Six hundred and twenty-three articles were identified by the search method as described earlier. Six hundred and ten of those were excluded due to laboratory studies, non-original

articles (review), or studies irrelevant to the current analysis. Finally, there were 13 studies included in final analysis, as shown in Figure 1.

Study characteristics

Thirteen reports published from 2005 to 2015 were selected and eligible for this meta-analysis. A total of 718 BC patients from the People's Republic of China, Japan, Singapore, South Korea, and USA were enrolled. Basic study characteristics are shown in Table 1.

The correlation of *RUNX3* hypermethylation with carcinogenesis

Comparison of *RUNX3* hypermethylation in DCIS, IDC tissue, and normal breast tissue

We first assessed whether *RUNX3* hypermethylation was significantly higher in DCIS than in normal breast tissues. The pooled OR from four studies including 134 DCIS and 103 normal breast tissue is shown in Figure 2A (OR =18.27, 95% CI =7.67–43.54, *z* score=6.56, $P<0.00001$). There was no evidence of heterogeneity across the studies (*P* for heterogeneity =0.13; I^2=47%). *RUNX3* hypermethylation was also significantly higher in IDC than in normal breast tissues. The pooled OR from five studies including 160 IDC and 123 normal breast tissue is shown in Figure 2B (OR =29.2, 95% CI =12.52–68.14, *z* score=7.81, $P<0.00001$). There was also no evidence of heterogeneity across the studies (*P*-value for heterogeneity =0.23; I^2=29%).

Comparison of *RUNX3* hypermethylation in IDC vs benign tumor, IDC vs DCIS

We then determined that *RUNX3* methylation was significantly increased in IDC compared to a benign tumor, OR was 30.90 with 95% CI =4.20–227.48, *z* score=3.37, *P*=0.0008, I^2=66%, *P*=0.03 (Figure 3A). However, *RUNX3* methylation was not significantly increased in IDC compared to DCIS, OR was 1.61 with 95% CI =0.94–2.76, *z* score=1.73, *P*=0.08, I^2=0%, *P*=0.8 (Figure 3B).

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

Then, we determined whether or not *RUNX3* hypermethylation rate in BC was associated with ER status in BC patients. The pooled OR from four studies including 207 ER positive BC and 146 ER negative BC is shown in Figure 4 (OR =8.16, 95% CI =4.53–14.71, *z* score=6.99, $P<0.00001$, I^2=43%, *P*=0.15),

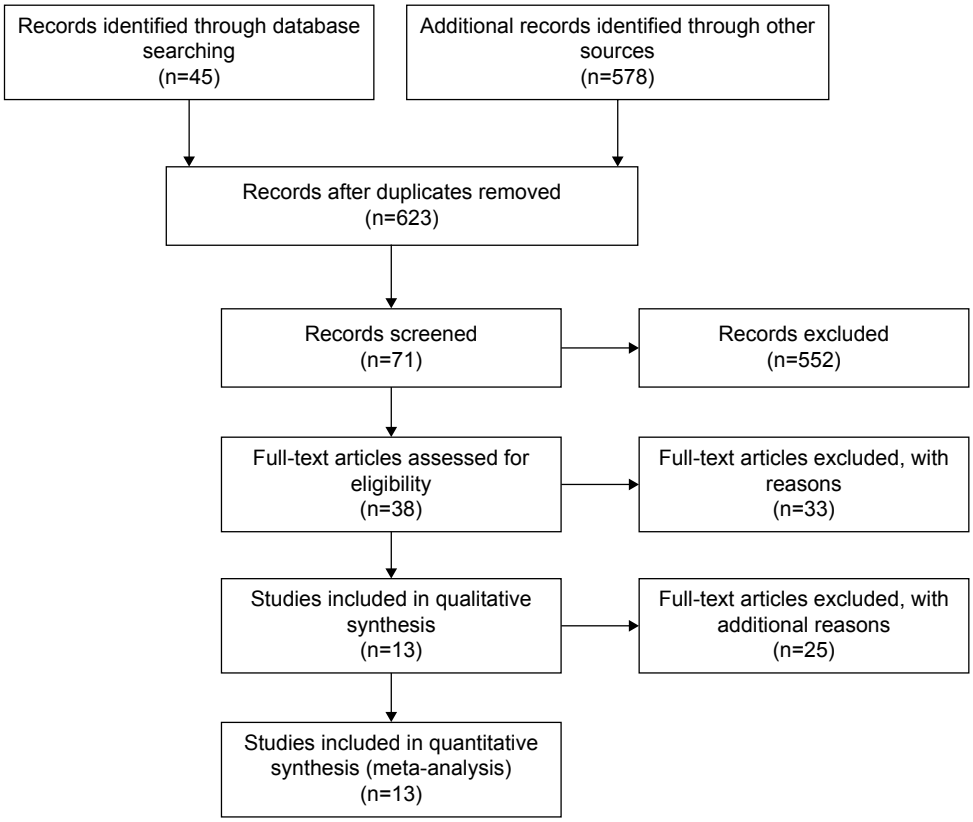

Figure 1 Schematic flow diagram for selection of included studies.

Table 1 Main characteristics of included studies

Study	Country	Patients (n)	Methods	Primary aims
Yan et al[54]	People's Republic of China	113	Methylation specific PCR (MSP)	To investigate the relationship between hypermethylation of *RUNX3* gene promoter and ER in BC patients.
Wang et al[46]	People's Republic of China	40	MSP	To investigate the methylation status of *RUNX3* promoter and RUNX3 expression in breast lesion tissues.
Li et al[47]	People's Republic of China	48	MSP	To investigate the methylation status of the *RUNX3* gene and protein expression in BC patients.
Park et al[48]	South Korea	129	Methylight	To investigate the methylation status of 15 tumor suppressors in BC patients.
Qiao et al[49]	People's Republic of China	60	MSP	To investigate the methylation status of the *RUNX3* gene in early diagnosis of BC patients.
Park et al[50]	South Korea	35	Methylight	To assess the role of seven tumor suppressors in BC patients.
Subramaniam et al[51]	Singapore	40	MSP, IHC	To investigate the role of *RUNX3* gene and protein in the progression of BC patients.
Du et al[52]	People's Republic of China	40	MSP, IHC	To investigate the role of *RUNX3* gene and protein in the prediction of BC.
Tian and Chen[53]	People's Republic of China	56	MSP	To determine the correlation of *RUNX3* methylation in BC and its pathologic features.
Subramaniam et al[31]	Singapore	61	MSP, IHC	To investigate the role of *RUNX3* gene and protein in the progression of BC patients.
Jiang et al[41]	People's Republic of China	15	MSP, IHC	To determine the correlation of *RUNX3* methylation and protein in BC and its pathologic features.
Lau et al[39]	USA	44	MSP, RT-PCR	To investigate the role of *RUNX3* gene and protein in the progression of BC patients.
Suzuki et al[13]	Japan	37	MSP	To investigate inactivation of TGFβ-related genes *DRM/Gremlin, RUNX3, and HPP1* in human cancers.

Abbreviations: BC, breast cancer; IHC, immunohistochemistry; RT-PCR, reverse transcription polymerase chain reaction; ER, estrogen receptor.

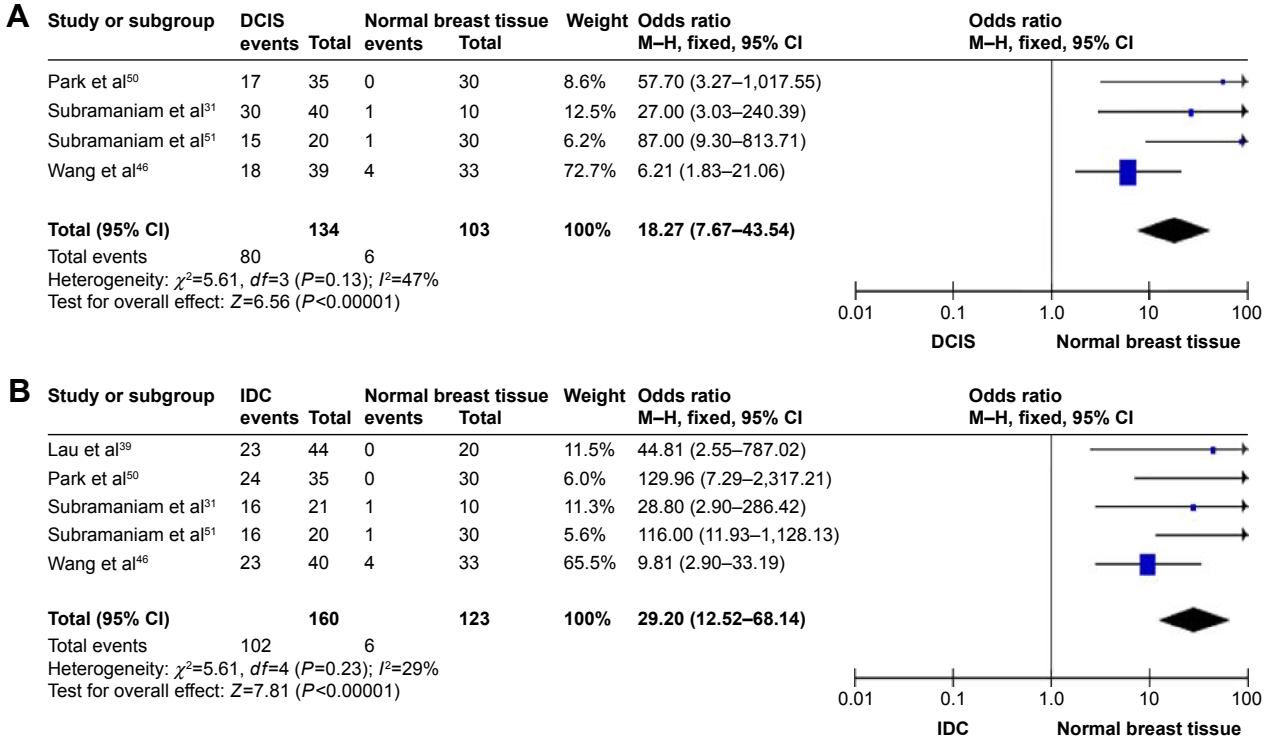

Figure 2 Comparison of *RUNX3* hypermethylation in DCIS, IDC tissue and normal breast tissue.
Notes: (**A**) Forest plot for *RUNX3* methylation in DCIS and normal breast tissue. (**B**) Forest plot for *RUNX3* methylation in IDC and normal breast tissue.
Abbreviations: DCIS, ductal carcinoma in situ; IDC, invasive ductal carcinoma; CI, confidence interval; M–H, Mantel–Haenszel; *df*, degrees of freedom.

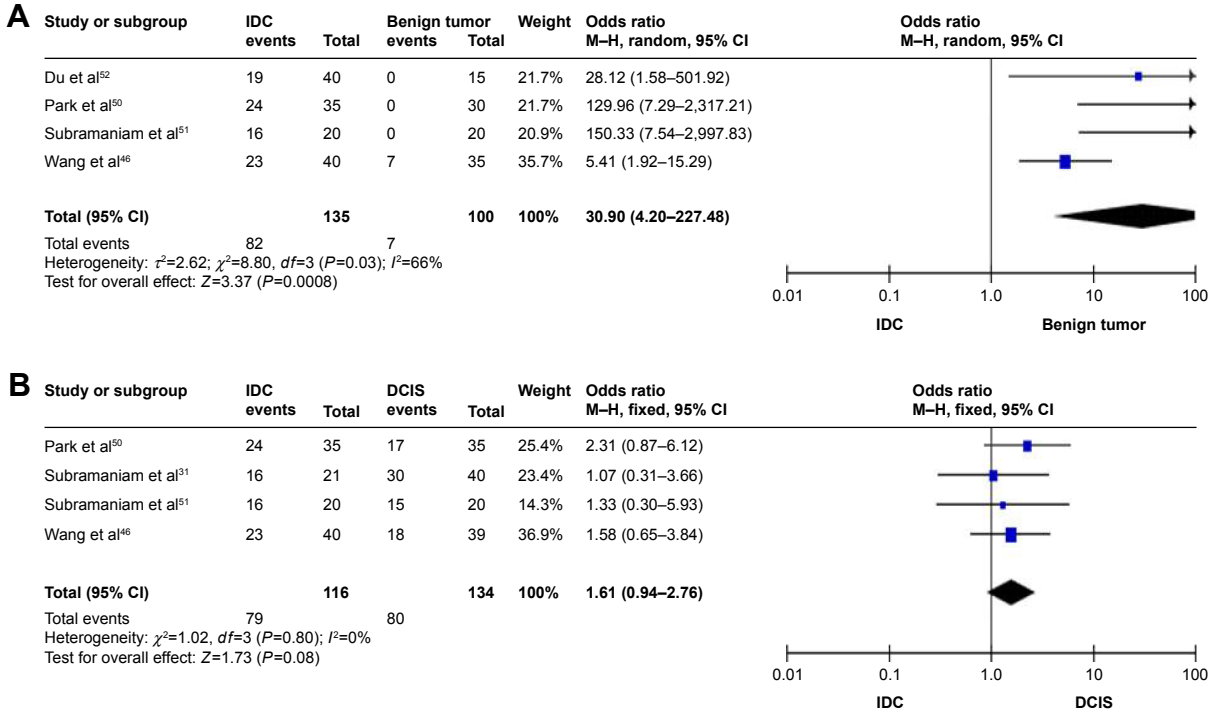

Figure 3 Comparison of *RUNX3* hypermethylation in IDC vs benign tumor and IDC vs DCIS.
Notes: (**A**) Forest plot for *RUNX3* methylation in IDC and benign tumor. (**B**) Forest plot for *RUNX3* methylation in IDC and DCIS.
Abbreviations: DCIS, ductal carcinoma in situ; IDC, invasive ductal carcinoma; CI, confidence interval; M–H, Mantel–Haenszel; *df*, degrees of freedom.

indicating that *RUNX3* hypermethylation was significantly higher in ER positive BC than in ER negative BC.

Sensitivity analyses and publication bias

A sensitivity analysis 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 5A–E) suggesting there were no publication biases in the meta-analysis. We used the NOQAS for assessment of the quality of each study. Of 13 studies, three scored 8 points, six scored 7 points, and three scored 6 points. Therefore, the selected studies were of a relatively high quality (Table 2).

Prognostic values of high *RUNX3* mRNA expression in BC patients

We finally assessed the clinical relevance of *RUNX3* in a patient survival analysis using an online database containing

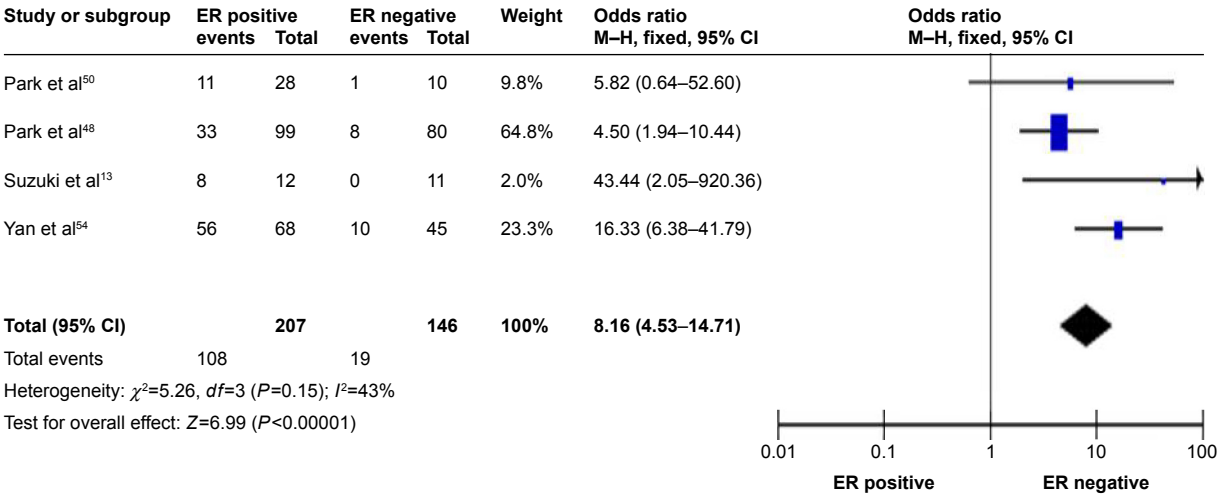

Figure 4 Forest plot for *RUNX3* methylation in ER positive and negative of BC.
Abbreviations: BC, breast cancer; CI, confidence interval; M–H, Mantel–Haenszel; *df*, degrees of freedom; ER, estrogen receptor.

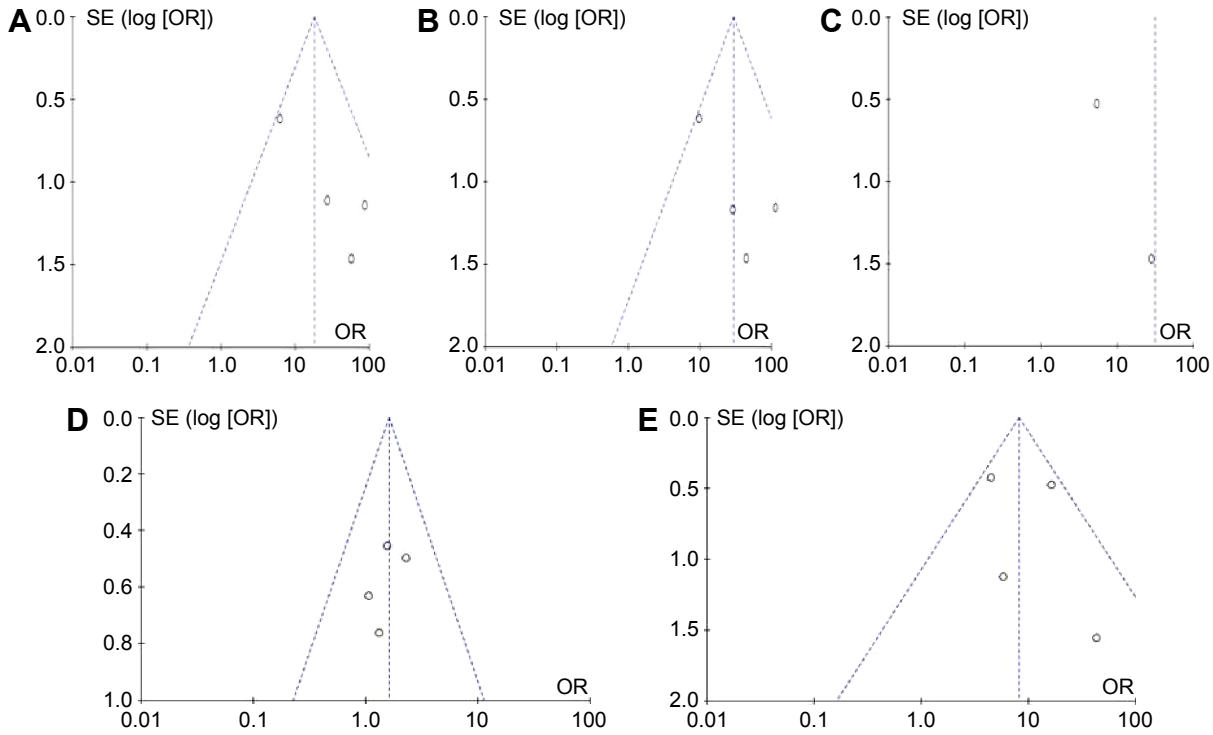

Figure 5 Funnel plot for publication bias.
Notes: (**A**) *RUNX3* methylation in DCIS and normal breast tissue; (**B**) *RUNX3* methylation in IDC and normal breast tissue; (**C**) *RUNX3* methylation in IDC and benign tumor; (**D**) *RUNX3* methylation in IDC and DCIS; (**E**) *RUNX3* methylation in ER (+) and ER (–) breast cancer.
Abbreviations: DCIS, ductal carcinoma in situ; IDC, invasive ductal carcinoma; SE, standard error; OR, odds ratio; ER, estrogen receptor.

the expression of 22,277 genes and 20-year survival information of 3,455 BC patients.[18] High *RUNX3* mRNA expression was found to be correlated with better overall survival (OS) for all BC patients followed for 20 years (Figure 6A, hazard ratio 0.78, *P*=0.037). In addition, high *RUNX3* mRNA expression was also found to be correlated with better relapse-free survival (RFS) for all BC patients followed for 20 years (Figure 6B, hazard ratio 0.8, *P*=0.00013).

Discussion

The RUNX3 transcription factor is a downstream effector of TGF-β signaling pathway. TGF-β is activated after binding to their respective cognate receptors, phosphorylate transducers named Smads. Smads 2 and Smads 3 are called R-Smads, which associate with a common Smad 4 (co-Smad) and enter the nucleus. R-Smad/co-Smad complex binds to transcription factors and regulates the transcription of target genes. RUNX3 interacts with R-Smads,

Table 2 Quality assessment according to the Newcastle–Ottawa scale of the included studies

Author	Selection	Comparability	Exposure	Total score
Yan et al[54]	2	2	3	7
Wang et al[46]	2	2	3	7
Li et al[47]	2	2	3	7
Park et al[48]	3	2	3	8
Qiao et al[49]	2	1	3	6
Park et al[50]	2	2	3	7
Subramaniam et al[51]	3	2	3	8
Du et al[52]	2	1	3	6
Tian and Chen[53]	2	1	3	6
Subramaniam et al[31]	3	2	3	8
Jiang et al[41]	2	1	3	6
Lau et al[39]	2	2	3	7
Suzuki et al[13]	2	2	3	7

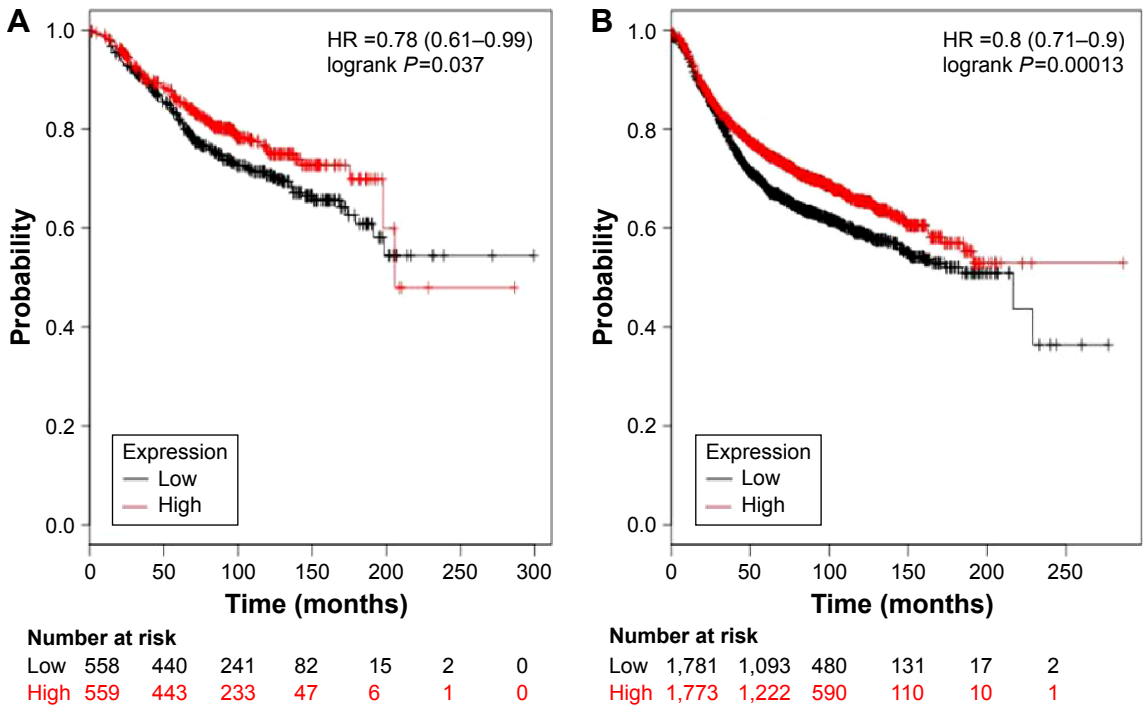

Figure 6 The clinical relevance of *RUNX3* was determined in a patient survival analysis using an online database containing the expression of 22,277 genes and 20-year survival information of 3,455 breast cancer (BC) patients.
Notes: (**A**) High *RUNX3* mRNA expression was found to be correlated with better overall survival for all BC patients followed for 20 years, hazard ratio (HR) 0.78, *P*=0.037. (**B**) High *RUNX3* mRNA expression was also found to be correlated with better relapse-free survival for all BC patients followed for 20 years, HR 0.8, *P*=0.00013.

co-Smads, and p300, a transcriptional co-activator, and fulfills its tumor suppressor activity via TGF-β signaling pathway.[14] *RUNX3* hypermethylation plays an important role during normal development and tumorigenesis in several types of tumors including BC.[23–34] 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 *RUNX3* hypermethylation was significantly higher in both DCIS and IDC compared to normal breast tissues. In addition, *RUNX3* methylation was significantly increased in IDC compared to a benign tumor. However, *RUNX3* methylation was not significantly increased in IDC compared to DCIS. We also determined that *RUNX3* hypermethylation was 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 is an early event during BC carcinogenesis. Thus, *RUNX3* methylation is a valuable early detection biomarker for the diagnosis of BC.

Epigenetic alterations, particularly aberrant DNA methylation, one of the best-characterized epigenetic modifications, contribute to tumor initiation and progression.[35,36] RUNX3 has been reported to downregulate Wnt signaling by directly inhibiting CTNNB1/TCF4 in colon cancer and gastric cancer.[9] Wnt signaling pathway is not only critical for the normal development of the mammary gland, but also for regulating cell proliferation and survival. RUNX3 inhibits the oncogenic Wnt signaling pathway via the formation of a complex with the TCF4-CTNNB1 complex and hampering it from binding to target genes such as *c-myc* and *CCND1*.[24,37]

RUNX3 also acts as a novel co-activator for p53 through regulating its DNA damage-induced phosphorylation at Ser-15 and mediates tumor suppression.[38] Numerous studies have supported that *RUNX3* is a suppressor and is inactivated in BC by protein mislocalization,[31,39] reduced copy number,[40] hemizygous deletion, and gene hypermethylation.[41,42] Based on this meta-analysis, we may conclude that *RUNX3* hypermethylation in BC tends to indicate higher incidence of BC, its inactivation could contribute to tumor initiation and progression.

ER signaling plays an important role in the development of normal mammary gland through the regulation of genes involved in cell cycle and apoptosis.[43] Abnormal ER signaling contributes to initiation and progression of BC.[44] A recent report has shown that RUNX3 inhibits ER signaling through

suppressing the transcription activity of ERα and reducing ERα-dependent cancer cell proliferation.[45] In this meta-analysis, we determined that the pooled OR from four studies including 207 ER positive BC and 146 ER negative BC, OR =8.16, 95% CI =4.53–14.71, *z* score=6.99, $P<0.00001$, I^2=43%, P=0.15, indicated that *RUNX3* hypermethylation was significantly higher in ER positive BC than in ER negative BC. Therefore, *RUNX3* methylation could contribute to the development of BC by modulating ER signaling pathway.

RUNX3 mRNA expression might be due to the *RUNX3* hypermethylation status in BC patients. Thus, we further assessed the prognostic value of high *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.[18] High *RUNX3* mRNA expression was found to be correlated with better OS for all BC patients followed for 20 years, hazard ratio 0.78, P=0.037. In addition, high *RUNX3* mRNA expression was also found to be correlated with better RFS for all BC patients followed for 20 years, hazard ratio 0.8, P=0.00013.

Conclusion

This meta-analysis showed that *RUNX3* methylation is significantly increased in DCIS and IDC. The frequency of *RUNX3* methylation is associated with ER status in patients with BC. In addition, high *RUNX3* mRNA expression was found to be correlated with better OS and RFS for all BC patients. Our results strongly support that *RUNX3* hypermethylation may play an important role in BC incidence. *RUNX3* methylation is a valuable early biomarker for the diagnosis of BC. Further large-scale studies will provide more insight into the role of *RUNX3* in the carcinogenesis and clinical diagnosis 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 paper. The authors report no conflicts of interest in this work.

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