Prognostic role of methylated GSTP1, p16, ESR1 and PITX2 in patients with breast cancer
A systematic meta-analysis under the guideline of PRISMA

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Abstract
Background: BRCA1 and RASSF1A promoter methylation has been reported to be correlated with a worse survival in patients with breast cancer. However, the prognostic values of GSTP1, p16, ESR1, and PITX2 promoter methylation in breast cancer remain to be determined. Here, we performed this study to evaluate the prognostic significance of GSTP1, p16, ESR1, and PITX2 promoter methylation in breast cancer.

Methods: A range of online databases was systematically searched to identify available studies based on the Preferred Reporting Items for Systematic reviews and Meta-Analyses (PRISMA) guideline. The pooled hazard ratios (HRs) with their 95% confidence intervals (95% CIs) were applied to estimate the prognostic effect of GSTP1, p16, ESR1, and PITX2 promoter methylation in breast cancer for multivariate regression analysis.

Results: 13 eligible articles involving 3915 patients with breast cancer were analyzed in this meta-analysis. In a large patient population, GSTP1 showed a trend toward a worse prognosis in overall survival (OS) (HR=1.64, 95% CI=0.93–2.87, P=.085). PITX2 promoter methylation was significantly correlated with a worse prognosis in OS (HR=1.57, 95% CI=1.15–2.14, P=.004), but no association between p16 promoter methylation and OS (HR=0.92, 95% CI=0.31–2.71, P=.884). PITX2 promoter methylation was significantly correlated with an unfavorable prognosis of patients with breast cancer in metastasis-free survival (MFS) (HR=1.73, 95% CI=1.33–2.26, P<.001). The result from 3 studies with 227 cases showed that ESR1 promoter methylation was linked to a worse prognosis in OS (HR=1.55, 95% CI=1.06–2.28, P=.025).

Conclusions: Our findings suggest ESR1 and PITX2 promoter methylation may be correlated with a worse survival of patients with breast cancer (ESR1: OS, PITX2: OS and MFS). The clinical utility of aberrantly methylated ESR1 and PITX2 could be a promising factor for the prognosis of breast cancer.

Abbreviations: 95% CI = 95% confidence interval, BRCA1 = breast cancer susceptibility gene 1, DFS = disease-free survival, ESR1 = estrogen receptor-α, GSTP1 = glutathione S-transferase P 1, HER2 = human epidermal growth factor receptor-2, HR = hazard ratio, MFS = metastasis-free survival, OS = overall survival, p16 = cyclin-dependent kinase inhibitor 2A, PITX2 = paired-like homeodomain transcription factor 2, RARbeta2 = retinoic acid receptor beta 2, RASSF1A = Ras association domain family 1 isoform, RFS = relapse-free survival, TSG = tumor suppressor gene.

Keywords: breast cancer, metastasis-free survival, multivariate analysis, overall survival, promoter methylation

1. Introduction
Breast cancer is the most common malignant tumor and the leading cause of cancer-related deaths among women with human cancers.[1] Based on global cancer estimates, approximately 1,676,600 new cases were diagnosed with breast carcinoma, leading to an estimate of approximately 521,900 deaths around the world in 2012.[2] Despite improvements in the early detection and treatment of breast cancer, patients with distant stage breast cancer remain to have an unfavorable 5-year survival rate of 26%.[3] In routine clinical practice, several clinicopathological features are applied as strong prognostic factors in the assessment of patients with breast cancer, such as lymph node metastasis, histological grade, tumor size, so on.[4] However, the basic molecular mechanism of this disease has not been fully understood. Thus, more noninvasive factors should be investigated to better predict prognosis.

Numerous studies suggest that epigenetic alterations are found to be an early and common event in cancer.[5–8] DNA methylation, a reversible epigenetic change, plays a crucial role in the carcinogenesis, progression, and prognosis of various
human malignant tumors. Tumor suppressor genes (TSGs) have been indicated to be frequently methylated in the promoter regions in breast cancer. Gene with aberrant promoter methylation is identified to be closely correlated with breast cancer development and progression. Located on human chromosome 11q13, the glutathione S-transferase P 1 (GSTP1) gene, a tumor suppressor gene, involves in the prevention of development of malignant tumors upon exposure to various carcinogens or electrophilic compounds. The human cyclin-dependent kinase inhibitor 2A (p16) gene is a key cyclin-dependent kinase inhibitor that plays an important role in the regulation of cell cycle. Estrogen receptor-α (ESR1) mediates the biological action of estrogen and dysregulation of its expression is found to be strongly implicated in breast cancer development and progression. The paired-like homeodomain transcription factor 2 (PITX2), a bicoid-related homeobox transcription factor, has been suggested to be associated with the regulation of pituitary-specific gene and normal embryonic development. Promoter methylation of the GSTP1, p16, ESR1, and PITX2 genes has been frequently reported in breast cancer.

Previous studies have revealed that promoter methylation of BRCA1 and RASSF1A is linked to a poor prognosis of breast cancer patients in OS and DFS. There were some inconsistent and conflicting results on multivariate regression analysis of GSTP1, p16, ESR1, or PITX2 promoter methylation for the prognosis of breast cancer. For example, GSTP1 promoter methylation was not correlated with the prognosis of breast cancer in DFS. A significant correlation was found between GSTP1 promoter methylation and DFS in breast cancer. p16 promoter methylation was associated with the prognosis of breast cancer in OS, whereas p16 promoter methylation was not correlated with the prognosis of breast cancer in OS. PITX2 promoter methylation was correlated with a poor OS of breast cancer in tissue or blood samples. ESR1 promoter methylation showed a trend toward a poor prognosis of breast cancer in OS.

No significant correlation was reported between ESR1 promoter methylation and OS in breast cancer. Therefore, we performed this meta-analysis to summarize the prognostic significance of GSTP1, p16, ESR1, or PITX2 promoter methylation in breast cancer for multivariate regression analysis.

2. Materials and methods

2.1. Publication search

A systematic search strategy of the relevant publications was conducted in the PubMed, Embase, EBSCO, and Cochrane Library databases up to January 19th, 2017. The following combinations of key words and search terms were applied: (breast OR mammary) AND (cancer OR tumor OR carcinoma OR neoplasm) AND (methylation OR epigenetic silencing OR epigenetic inactivation) AND (prognos’ OR survival OR outcome). Additionally, we also carefully scanned the references of the included studies to get other additional eligible papers.

2.2. Selection criteria

The eligible papers were included in this meta-analysis if they satisfied the following selection criteria: (1) all patients were limited to breast cancer using the diagnostic criteria; (2) studies provided sufficient information regarding the clinical outcome of GSTP1, p16, ESR1, or PITX2 promoter methylation in overall survival (OS), disease-free survival (DFS), relapse-free survival (RFS), or metastasis-free survival (MFS) for multivariate regression analysis; (3) hazard ratio (HR) with its 95% confidence interval (95% CI) was reported from the original paper. If the original data were not recorded, we calculated the presented data in the survival plots by the methods described by Tierney et al. Four articles published in English were included in this meta-analysis. Methylated genes were excluded as follows: (1) a meta-analysis published involving the survival analysis of gene methylation in breast cancer; (2) methylated genes with fewer than 3 studies in survival analysis.

2.3. Ethical review

The present study was not primary research involving human samples, but rather a secondary analysis of human subject data published in the public domain.

2.4. Data extraction

The following information was collected from the included studies: first author’s surname, publication year, country, ethnic population, age, tumor stage, testing method of methylation, the frequency of promoter methylation, the number of patients, OS, DFS, RFS, and MFS for multivariate regression analysis.

2.5. Statistical analysis

The current meta-analysis was conducted with Stata 12.0 software (Stata Corporation, College Station, TX). The pooled HRs and their 95% CIs were calculated to evaluate the strength of association between methylated genes and the prognosis of patients with breast cancer in OS, DFS, RFS, and MFS using multivariate regression analysis. Heterogeneity of the eligible studies was estimated based on Cochran’s Q test. The random-effects model was used when there was obvious evidence of heterogeneity (P ≤ .1); otherwise, the fix-effects model was determined in this meta-analysis (P > .1).

3. Results

3.1. Characteristics of the eligible studies

Figure 1 shows the detailed procedure of the relevant literature. According to the above selection criteria, final 13 eligible articles...
using multivariate regression analysis\cite{30,33–38,43–48} were identified in the present meta-analysis, including 3915 patients with breast cancer. Of the included studies, 6 studies with 1509 breast cancer patients analyzed the prognostic role of GSTP1 promoter methylation in OS, DFS, and RFS.\cite{33,34,43–45,47} Four studies with 1480 breast cancer patients analyzed the prognostic value of PITX2 promoter methylation in OS and MFS.\cite{30,36,37,46} Three studies involving 1107 patients with breast cancer evaluated the correlation between p16 promoter methylation and the prognosis in OS and DFS.\cite{33,35,43} Three studies involving 227 patients with breast cancer assessed the association between ESR1 promoter methylation and the prognosis in OS, DFS, and MFS.\cite{33,38,48} The baseline characteristics of the eligible studies are listed in Table 1.

### 3.2. GSTP1 promoter methylation and the prognosis of patients with breast cancer

As depicted in Fig. 2, the result from 4 studies with 1235 breast cancer patients demonstrated that GSTP1 promoter methylation had a trend toward a poor prognosis in OS (HR=1.64, 95% CI=1.22–6.04).
CI=0.93–2.87, \( P = .085 \)). Significant correlation was found between GSTP1 promoter methylation and DFS, and RFS (HR = 2.09, 95% CI = 1.19–3.67, \( P = .011 \); HR = 2.71, 95% CI = 1.22–6.03, \( P = .015 \); respectively), including 2 studies with 201 breast cancer patients and 1 study with 174 patients with breast cancer, respectively. GSTP1 promoter methylation was notably correlated with a poor prognosis in breast cancer in OS.

3.3. PITX2 promoter methylation and the prognosis of patients with breast cancer

The data involving PITX2 promoter methylation included 3 studies with 1052 breast cancer patients in MFS and 2 studies with 840 breast cancer patients in OS (Fig. 3). The results showed that PITX2 promoter methylation was significantly associated with the prognosis in MFS and OS (HR = 1.73, 95% CI = 1.33–2.26, \( P < .001 \); HR = 1.57, 95% CI = 1.15–2.14, \( P = .004 \), respectively). Thus, PITX2 promoter methylation was significantly correlated with a poor prognosis of breast cancer patients in MFS and OS.

3.4. p16 promoter methylation and the prognosis of patients with breast cancer

No significant relationship was observed between p16 promoter methylation and the prognosis in OS and DFS (HR = 0.92, 95% CI = 0.31–2.71, \( P = .884 \); HR = 1.49, 95% CI = 0.73–3.05, \( P = .274 \); respectively) (Fig. 4), including 3 studies with 1107 breast cancer patients in OS and 1 study with 101 breast cancer patients. The analysis revealed that p16 promoter methylation was not linked to the prognosis of breast cancer patients in OS.

3.5. ESR1 promoter methylation and the prognosis of patients with breast cancer

The results showed that ESR1 promoter methylation was significantly correlated with the prognosis in OS and MFS (HR = 1.55, 95% CI = 1.06–2.28, \( P = .025 \); HR = 2.76, 95% CI = 1.02–7.45, \( P = .046 \), respectively) (Fig. 5), including 3 studies with 227 cases in OS and 1 study with 69 cases in MFS. The result from 2 studies involving 158 breast cancer cases revealed that ESR1 promoter methylation had a trend toward an unfavorable prognosis in DFS (HR = 1.41, 95% CI = 0.94–2.10, \( P = .096 \)) (Fig. 5). Promoter methylation of the ESR1 gene may be significantly linked to a poor prognosis of patients with breast cancer in OS.

4. Discussion

The silencing of tumor suppressor genes (TSGs) via promoter methylation may prompt the carcinogenesis and progression of breast cancer. Dedeurwaerder and Fuks et al.\(^{50}\) reported that high expression of some T-cell marker genes was correlated with a better clinical outcome in breast cancer. The Chi-square (and Fisher’s exact) test had a notably \( (P < .05) \) higher percentage of promoter methylation of the ESR1 gene in breast cancer patients with triple negative and HER2 phenotypes with poorer prognosis by Martinez-Galan et al.\(^{51}\) Some methylated genes within the promoter (i.e., RASSF1A, RARbeta2, BRCA1, and GSTP1) have been identified to be associated with poor prognosis of breast cancer patients.\(^{31–33,44}\) suggesting that aberrantly methylated genes may become potential prognostic factors. Therefore, aberrant promoter methylation of a gene may provide more independent prognostic information as a prognostic indicator in the treatment and management of breast cancer.

In the present study, 4 genes consisted of the GSTP1, p16, ESR1, and PITX2 genes. However, the prognostic values of GSTP1, p16, ESR1, and PITX2 promoter methylation in breast cancer remain to be elucidated. Thus, we first determined whether these 4 cancer-related genes with promoter methylation were correlated with the prognosis of patients with breast cancer in multivariate regression analysis.
The results in a large patient population showed that promoter methylation of the GSTP1 gene just showed a trend toward a poor prognosis in OS (HR = 1.64, 95% CI = 0.93–2.87, P = .085). PITX2 promoter methylation was significantly correlated with an unfavorable prognosis of breast cancer patients in OS, but no significant correlation was found between p16 promoter methylation and OS. In addition, PITX2 promoter methylation was also found to be significantly associated with a poor prognosis of patients with breast cancer in MFS. Based on small sample sizes, we found that GSTP1 and ESR1 promoter methylation in 2 studies was correlated with an unfavorable prognosis of patients in DFS, but no association was observed between p16 promoter methylation and DFS in 1 study. A significant correlation was observed between ESR1 promoter methylation and a worse prognosis of patients with breast cancer in 3 studies with a small patient population in OS. Only 1 study...
involving 174 breast cancer patients reported that GSTP1 promoter methylation was linked to a worse prognosis in RFS.\cite{14} ESR1 promoter methylation was reported to be associated with a worse prognosis in 1 study with 69 breast cancer patients in MFS.\cite{15} For the results with small sample sizes, more studies with large sample sizes should be necessary to further validate the prognostic values in OS, DFS, RFS, and MFS.

Some limitations should be addressed in this meta-analysis. First, only publications written in English were identified in our study, which can lead to a bias in literature selection. Articles with positive results were more easily accepted than articles with negative results. Second, the main population included Caucasians in the current study, and other ethnic populations, such as Africans and Asians, were insufficient. In the future, additional studies are still needed to confirm the prognostic role of GSTP1, p16, ESR1, or PITX2 promoter methylation in the African and Asian populations with breast cancer. Third, studies of the blood with large sample sizes should be done to confirm whether GSTP1, p16, ESR1, or PITX2 promoter methylation could be a noninvasive prognostic factor based on blood samples.

In conclusion, our findings show that ESR1 and PITX2 may be notably associated with a worse prognosis of patients with breast cancer in OS, but no significant relationship was found between ESR1 and GSTP1 promoter methylation and OS. Moreover, PITX2 promoter methylation was found to be significantly correlated with an unfavorable prognosis of patients with breast cancer in MFS. ESR1 or PITX2 promoter methylation could serve as a potential drug target in the treatment of breast cancer.

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