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Promoter hypermethylation of RARβ2, DAPK, hMLH1, p14, and p15 is associated with progression of breast cancer

A PRISMA-compliant meta-analysis

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Abstract

Background: Numerous studies have investigated the associations between RARβ2, DAPK, hMLH1, p14, and p15 promoter hypermethylation and clinical progression of patients with breast cancer, however the results remained uncertain due to the small sample size. Therefore, we performed a meta-analysis to explore the role of RARβ2, DAPK, hMLH1, p14, and p15 promoter hypermethylation in the susceptibility and clinical progression of breast cancer.

Methods: Eligible studies were obtained by searching Medicine, Embase, Web of knowledge, and Chinese National Knowledge Infrastructure (CNKI) databases. The odds ratios (OR) and 95% confidence intervals (CI) were calculated to evaluate the associations of RARβ2, DAPK, hMLH1, p14, and p15 promoter hypermethylation with breast cancer pathogenesis. Trial sequential analysis (TSA) was applied to observe the reliability of pooled results of RARβ2 gene, and obtain a conservative required information size (RIS).

Results: In primary screened 445 articles, 39 literatures with 4492 breast cancer patients were finally enrolled in the final meta-analysis. The results indicated that the frequency of RARβ2 promoter hypermethylation in case group was significantly higher than the frequency of control group (OR = 7.21, 95% CI = 1.54–33.80, $P < .05$). The RARβ2 promoter hypermethylation had a significant association with lymph node metastasis of breast cancer (OR = 2.13, 95% CI = 1.04–4.47, $P < .05$). And, the RARβ2 promoter hypermethylation was more common in the breast cancer patients of TNM III–IV stage than those patients of TNM I–II stage (OR = 1.85, 95% CI = 1.33–2.57, $P < .05$). In addition, the promoter hypermethylation of DAPK, hMLH1, and p14 genes were significantly associated with the susceptibility of breast cancer (for DAPK, OR = 4.93, 95% CI = 3.17–7.65; for hMLH1, OR = 1.84, 95% CI = 1.26–1.29; for p14, OR = 22.52, 95% CI = 7.00–72.41; for p15, OR = 2.13, 95% CI = 0.30–15.07).

Conclusions: Our findings revealed that the RARβ2 promoter hypermethylation significantly increased the risk of breast cancer. In the meantime, the meta-analysis demonstrated that there were significant associations of RARβ2 promoter hypermethylation with lymph node metastasis and TNM-stage of breast cancer patients. In addition, DAPK, hMLH1, and p14 genes promoter hypermethylation were significantly associated with the susceptibility of breast cancer.

Abbreviations: CI = confidence intervals, CNKI = Chinese National Knowledge Infrastructure, ER = estrogen receptor, HER2 = human epidermal growth factor receptor 2, HYAL2 = hyaluronoglucosaminidase 2, NOS = Newcastle-Ottawa Scale, OR = odds ratios, PR = progesterone receptor, RA = retinoic acid, RARβ2 = retinoic acid receptor beta, RIS = required information size, TSA = trial sequential analysis.

Keywords: breast cancer, DAPK, hMLH1, meta-analysis, p14, p15, promoter hypermethylation, RARβ2

1. Introduction

Although the improvements of early diagnosis and treatment significantly decrease the mortality rate of breast cancer, breast cancer is still the most common cancer in woman.[1] In addition, early detection of breast cancer provided more options of treatment, which included surgical treatment, radiotherapy, hormonal therapy, and chemotherapy.[2] As is known to all, environmental factors and genetic events played an important role in the development of breast cancer. Recently, the advances of early detection and treatment benefited from the finding of many biomarkers such as: estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2), BRCA1, and BRCA2. PR and ER, typical tumor markers of breast cancer, had a great impact on the therapy of breast cancer. Although the 2 biomarkers had poor prognostic, they were considered as strong predictive factors of response to the treatment of hormonal therapy in breast cancer.[3,4] HER2,

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oncogenic protein, was a transmembrane protein, which was encoded by ERBB2.[5] Under normal situation, low expression level of HER2 gene was presented in normal epithelia of breast. It was reported that 15% to 20% of breast cancer patients had an abnormal amplification and high expression of HER2.[6] HER2 was often considered as a predictive factor for the chemotherapeutic of breast cancer. If some single chemotherapeutics were used or these drugs were combined to treat breast cancer, HER2-targeted drugs had a significant effect on chemotherapy.[7] However, HER2 status had a significant heterogeneity among individuals. Previous reports have found that 5% but no more than 50% nonclustered tumor cells presented HER2 heterogeneity.[8] In addition, BRCA1 and BRCA2, benefitting the early discovery of breast cancer, were the most notable biomarkers of breast cancer patients.[9] Although many other biomarkers were also applied to find breast cancer, a large proportion of breast cancer patients were found in late stage. Thus, in order to research the associations between genetic alterations and breast cancer pathogenesis, numerous biomarkers should be found and studied.

RARβ2 (retinoic acid receptor beta), a member of retinoic acid receptor subfamily and encoded by RARβ2 gene, primarily mediated the retinoic acid (RA) activity. RA was a major bioactive metabolite of vitamin A, which had an important influence in cell growth and differentiation.[10] The abnormity of RA signaling pathways might result in occurrence of disease, such as abnormal embryo development and cancers. Of note, previous studies have suggested that the RARβ2 gene hypermethylation was presented in several cancers such as: skin cancer, head/neck cancer, lung cancer, ovarian cancer, prostate cancer, renal cell carcinoma, pancreatic cancer, and liver cancer.[11] In addition, some clinical studies were also performed to explore the relationships between DAPK, hMLH1, p14, and p15 promoter hypermethylation and breast cancer risk and clinical progression. But the results were unconvincing due to some factors such as sample size and race. So, the sensibility and specificity of one gene might not be so strong to accurately diagnose the occurrence of breast cancer. However, if several genes were applied to diagnose the breast cancer or assess the prognosis of breast cancer together, the accuracy of results should be increased greatly. To find more relevant genes and clarify the relationship between promoter hypermethylation of these genes and susceptibility and clinical progression of breast cancer, we performed this meta-analysis.

2. Materials and methods

2.1. Publication search

On the basis of PRISMA guideline, PubMed, Embase, Web of knowledge, and Chinese National Knowledge Infrastructure (CNKI) databases were retrieved to search eligible articles, investigating the associations of RARβ2, DAPK, hMLH1, p14, and p15 promoter hypermethylation with susceptibility and clinical progression of breast cancer.[12] The following terms were used: "Breast Neoplasms," "breast cancer," "breast tumor," "retinoic acid receptor beta," "RARβ2," "Hypermethylation," "methylation," "DAPK," "hMLH1," "p14," "p15," and "Epigenomics." The references of included articles and related reviews were checked to identify additional articles. All relevant articles were searched up to June 2018.

2.2. Inclusion criteria and exclusion criteria

The eligible studies were selected using the following inclusion criteria: the studies investigating the associations of RARβ2, DAPK, hMLH1, p14, and p15 genes promoter hypermethylation with susceptibility and clinical progression of breast cancer. Case–control studies or cohort studies; the studies which contained full data of RARβ2, DAPK, hMLH1, p14, and p15 hypermethylation frequency; English or Chinese publications. The exclusion criteria were as follows: reviews or meta-analysis; studies which were conducted in cells or animals; articles that contained duplicate data.

2.3. Data collection and quality assessment

According to inclusion criteria, 2 reviewers independently searched eligible studies. The following studies' characteristics were extracted: the name of first author, race, methylation frequency of control and case, detection method of gene methylation, source of control, cancer type, and clinical information. The information of breast tumor differentiation, age, and distant metastasis were not extracted since the information was too few. Furthermore, Newcastle-Ottawa Scale (NOS, http://www.ohri.ca/programs/clinical_epidemiology/oxford.asp) was applied to assess the methodological quality of included studies.[13] Two investigators assessed the included studies and scored them according to the NOS table. If some divergences existed, 2 authors discussed these divergences and reached a consensus score. Methodological qualities of included studies were estimated according to 3 parts such as: sample selection, sample comparability, and sample exposure. The score of quality assessment ranged from 0 to 9 stars.

2.4. Trial sequential analysis

To observe the stable of results and estimate the required information size, TSA was performed on the basis of the RARβ2 promoter hypermethylation frequency of control group and case group. TSA 0.9 software (Copenhagen Trial Unit, Center for Clinical Intervention Research, Denmark, http://www.ctu.dk/tsa/downloads.aspx) was applied to assess statistical significance, using type I errors of 5% and type II errors of 10%.[14] If the Z-curve did not cross any boundary, the result suggested that no significant association existed. In addition, the Z-curve crossed the traditional boundary and the trial sequential monitoring boundary, which indicated the sample size was enough large and a significant association was observed. However, if the Z-curve only crossed the traditional boundary, it showed a lack of firm evidence. When new studies were added or repeating tests were performed, a meta-analysis commonly leaded to type I errors or type II errors. To detect and minimize the risk type I errors and type II errors, TSA was a powerful statistical method.

2.5. Statistical analysis

All statistical analysis were conducted, using Stata 12.0 (StataCorp LP, College Station, TX) software and TSA 0.9 software. The ORs and 95% CIs were used to evaluate the strength of associations between RARβ2, DAPK, hMLH1, p14, and p15 promoter hypermethylation and breast cancer pathogenesis.[15] I^2 statistics and Cochrane Q were calculated to assess the interstudy heterogeneity, while P-value of $<.05$ or I^2 value of $>50\%$ indicated a significant heterogeneity.[16,17] The fixed effects model was utilized when heterogeneity was detected; otherwise, random effects model was used.[18,19] Further, subgroup analysis based on race was conducted to evaluate the strength of associations in different population. In addition,

Begg test and Egger test were performed to observe publication bias of included studies.[20,21] Forest plot and funnel plot were drawn to visually observe differences among included studies.

2.6. Ethnical statement

The consent of ethics committee or institutional review board was not required in the meta-analysis, because the study was a review article.

3. Results

3.1. Literature selection

A total of 39 articles with 4492 breast cancer patients were finally included. Medicine, Embase, Web of knowledge, and Chinese National Knowledge Infrastructure (CNKI) databases were all retrieved to search eligible studies.[22–60] Initially, 445 articles were remained after repeated retrievals were excluded. Then, 312 articles were eliminated after title and abstract were read. Further, 53 literatures were removed because they were not related with the RARβ2, DAPK, hMLH1, p14, and p15 genes hypermethylation. Moreover, 41 articles were excluded since they had no enough data to conduct a meta-analysis. Finally, 39 articles which compared the frequency of RARβ2, DAPK, hMLH1, p14, and p15 genes promoter hypermethylation in control group with those in case group were included.[21–59] In these included studies, 19 studies were involved in RARβ2 gene promoter hypermethylation, 9 studies were about DAPK gene promoter hypermethylation, hMLH1 gene promoter hypermethylation were studied in 5 studies, 6 studies were about p14 gene promoter hypermethylation, and 4 studies were about p15 gene promoter hypermethylation. The included studies were mainly carried out in Asians and Caucasians in the present meta-analysis. Figure 1 illustrates the flow diagram of literature selection (Table 1).

3.2. Quality assessment

The NOS score of included studies was from 6 to 8 stars. The results showed that studies of moderate or high quality were included in this meta-analysis, which greatly improve the power of statistics.

3.3. Results of meta-analysis and trial sequential analysis about RARβ2 promoter hypermethylation

According to results of the meta-analysis, RARβ2 promoter hypermethylation significantly associated with the risk of breast

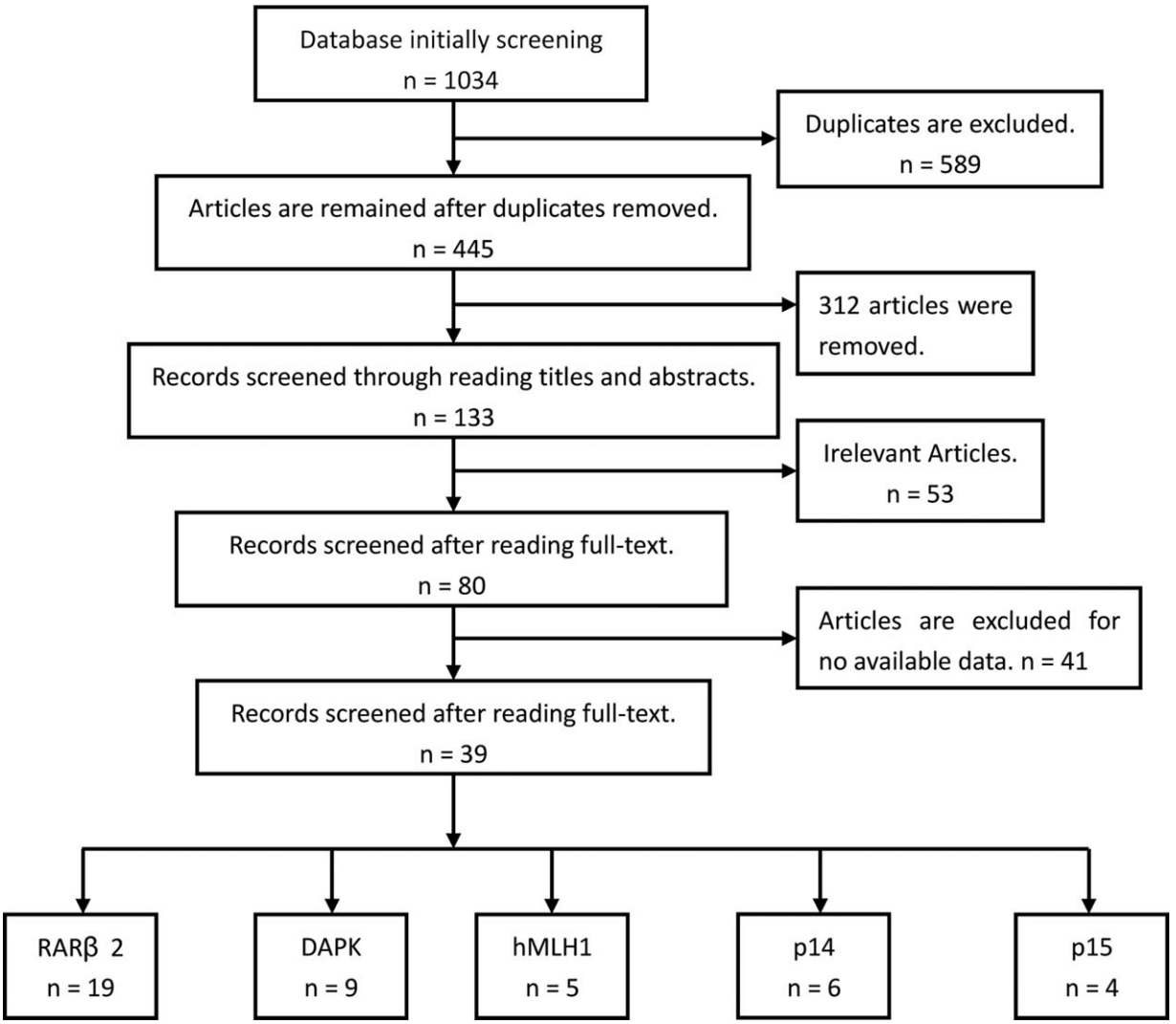

Figure 1. Flow chart of included studies searching.

Table 1

Characteristics of included studies evaluating the associations of RARβ2, DAPK, hMLH1, p14, and p15 promoter hypermethylation with breast cancer susceptibility.

						Control			Breast cancer						
Study	Year	Country	Race	Histology	Gene	U	M	Total	U	M	Total	Methods	Case	Control	NOS
Li[22]	2014	China	Asian	BC	RAR-β2	40	0	40	142	50	192	MSP	BCT	ABT	8
Xie[23]	2011	China	Asian	BC	RAR-β2	10	0	10	31	31	62	MSP	BCT	NBT	8
Xu[24]	2010	China	Asian	BC	RAR-β2	20	0	20	33	27	60	MSP	BCT	NBT	8
Bagadi[25]	2008	India	Asian	BC	RAR-β2	5	0	5	46	8	54	MSP	BCT	NBT	6
Khodyrev[26]	2008	Russia	Caucasian	BC	RAR-β2	49	2	51	30	26	56	MSRA	BCT	NBT	6
Shukla[27]	2006	India	Asian	PBC	RAR-β2	20	0	20	18	2	20	MSP	PBCT	NBT	7
Lewis[28]	2005	USA	Caucasian	BC	RAR-β2	17	10	27	50	5	55	MSP	BCT	NBT	8
Fackler[29]	2003	USA	Caucasian	BC	RAR-β2	8	0	8	61	42	103	MSP	BCT	NBT	8
Widschwendter[30]	2001	Austria	Mixed	PBC	RAR-β2	16	0	16	10	6	16	MSP	PBCT	NBT	6
Bovenzi[31]	1999	Canada	Caucasian	BC	RAR-β2	12	1	13	9	4	13	MSP	BCT	NBT	8
Xie[32]	2011	China	Asian	BC	RAR-β2	15	0	15	34	28	62	MSP	BCB	BD	7
Rykova[33]	2008	Russia	Caucasian	BC	RAR-β2	25	0	25	14	10	24	MSP	BCB	HB	7
Hoque[34]	2006	Senegal	Africa	BC	RAR-β2	38	0	38	43	4	47	qMSP	BCB	HB	7
Shukla[27]	2006	India	Asian	PBC	RAR-β2	20	0	20	20	0	20	MSP	BCB	HB	7
Skvortsova[35]	2006	Russia	Caucasian	BC	RAR-β2	10	0	10	30	5	35	MSP	BCB	HB	6
Pirouzpanah[36]	2015	Iran	Caucasian	PBC	RAR-β2	—	—	—	60	41	101	MSP	PBCT	—	—
Tao[37]	2011	USA	Caucasian	PBC	RAR-β2	—	—	—	514	201	715	QMSP	PBCT	—	—
Yang[38]	2001	Japan	Asian	BC	RAR-β2	—	—	—	29	21	50	MSP	BCT	—	—
Marzese[39]	2012	Argentina	Caucasian	IDCs	RAR-β2	—	—	—	49	21	70	MS-MLPA	BCT	—	—
Cho[40]	2012	USA	Caucasian	BC	RAR-β2	—	—	—	441	209	750	MethyLight	BCT	—	—
Zhu[41]	2017	China	Asian	BC	DAPK	10	5	15	7	8	15	MSP	BCT	ABT	8
Jung[42]	2013	Korea	Asian	BC	DAPK	60	0	60	58	2	60	MSMLDP	BCT	NT	8
Feng[43]	2010	China	Asian	BC	DAPK	50	0	50	41	9	50	MSP	BCB	HB	7
Ahmed[44]	2010	Egypt	Mixed	BC	DAPK	12	0	12	3	23	26	MSP	BCB	HB	6
Auwera[45]	2010	Belgium	Caucasian	BC	DAPK	30	26	56	12	44	56	qMSP	BCT	NBT	8
Auwera[46]	2009	Belgium	Caucasian	BC	DAPK	9	0	9	69	31	100	qMSP	BCT	NBT	6
Jeronimo[47]	2008	Portugal	Caucasian	BC	DAPK	2	7	9	11	41	52	qMSP	BCT	NBT	6
Jee[48]	2007	Korea	Asian	BC	DAPK	53	14	67	31	36	67	MSP	BCT	NBT	8
Krassenstein[49]	2004	USA	Caucasian	BC	DAPK	22	0	22	21	1	22	MSP	BCT	NBT	8
Shan[50]	2016	China	Asian	BC	hMLH1	210	35	245	193	75	268	MethyLight	BCB	HD	6
Klajic[51]	2013	Norway	Caucasian	BC	hMLH1	6	0	6	188	27	215	Pyrosequencing	BCT	NBT	6
Dejeux[52]	2010	France	Caucasian	BC	hMLH1	6	0	6	140	23	163	Pyrosequencing	BCB	NBT	6
Feng[53]	2007	USA	Caucasian	BC	hMLH1	27	6	33	33	0	33	Pyrosequencing	BCT	NBT	8
Jee[48]	2007	Korea	Asian	BC	hMLH1	60	7	67	57	10	67	MSP	BCT	NBT	8
Askari[54]	2013	India	Asian	BC	p14	149	1	150	134	16	150	MSP	BCB	HD	6
Sharma[55]	2007	India	Asian	BC	p14	4	0	4	19	17	36	MSP	BCB	HD	6
Krassenstein[49]	2004	USA	Caucasian	BC	p14	22	0	22	17	5	22	MSP	BCT	NBT	8
Dominguez[56]	2003	Spain	Mixed	BC	p14	100	0	100	76	24	100	MSP	BCT	NBT	8
Silva[57]	2003	Spain	Mixed	BC	p14	30	0	30	76	24	100	PSMA	BCT	NBT	8
Zemlyakova[58]	2003	Russia	Caucasian	BC	p14	5	0	5	105	0	105	MSP	BCT	NBT	6
Jung[42]	2013	Korea	Asian	BC	p15	43	17	60	45	15	60	MSMLDP	BCT	NBT	8
Buyru[59]	2009	Turkey	Caucasian	BC	p15	77	0	77	66	11	77	MSMLDP	BCT	NBT	8
Zemlyakova[58]	2003	Russia	Caucasian	BC	p15	5	0	5	103	2	105	MSMLDP	BCT	NBT	6
Bisogna[60]	2001	USA	Caucasian	BC	p15	17	0	17	33	5	38	MSP	BCT	NBT	6

ABT = adjacent breast tissue, ABT = adjacent breast tissue, BC = breast cancer, BCB = breast cancer peripheral blood, BCT = breast cancer tissue, BD = benign blood, HB = healthy blood, M = methylation, MS-MLPA = methylation-specific multiplex ligation-dependent probe amplification, MSMLDP = methylation-specific multiplex ligation-dependent probe, MSP = Methylmion Specific PCR, qMSP = quantitative Methylation Specific PCR, NT = normal breast tissue, PBC = primary breast cancer, PBCT = primary breast cancer tissue, PSMA = PCR–SSCP mutational analysis, RARβ2 = retinoic acid receptor beta, U = unmethylation.

cancer (OR = 7.21, 95% CI = 1.54–33.80, $P < .05$). TSA showed that the sample size has exceeded the required information size (RIS = 528), while the Z-curve has crossed conventional boundary and trial sequential monitoring boundary. Therefore, the result was stable and no further studies were conducted. In addition, we found that the frequency of RARβ2 promoter hypermethylation in tissue sample was lower than the frequency of RARβ2 promoter hypermethylation in blood sample. The subgroup analysis based on race was conducted due to significant heterogeneity. The results showed that heterogeneity among studies in Asians disappeared, and the result still presented a significant association in Caucasians (OR = 12.51, 95% CI = 3.39–46.15, $P < .05$). From the analysis of clinical progression of breast cancer, significant associations of RARβ2 promoter hypermethylation with lymph node metastasis and TNM-stage of breast cancer were detected in Caucasians (for node metastasis, OR = 2.13, 95% CI = 1.04–4.47, $P < .05$; for TNM-stage, OR = 1.85, 95% CI = 1.33–2.57, $P < .05$). At the same time, no heterogeneity among studies was observed on breast cancer pathogenesis. In addition, no associations of RARβ2 promoter hypermethylation ER status, PR status, and menopause of breast cancer were observed (Table 2, Fig 2, 3, 4).

3.4. Results of DAPK, hMLH1, p14, and p15 promoter hypermethylation in susceptibility of breast cancer

In order to observe the strength of associations between DAPK, hMLH1, p14, and p15 promoter hypermethylation and breast

Table 2

Meta-analysis results of associations between RARβ2, DAPK, hMLH1, p14, and p15 promoter hypermethylation and clinical features of breast cancer.

Group	OR	95% CI	Publication bias		Begg test (*P*)	Egger test (*P*)
			I^2	*P*		
RARβ						
Risk (overall)	7.21	1.54–33.80	77.10%	.00	.42	.18
Risk in Caucasians	3.57	0.22–58.76	89.40%	.00	.50	.51
Risk in Asians	12.51	3.39–46.15	0.00%	.59	.14	.09
TNM (I–II vs III–IV) (overall)	1.85	1.33–2.57	43.10%	.09	.81	.17
TNM (I–II vs III–IV) in Caucasians	1.91	1.17–3.10	0.00%	.46	.31	—
TNM (I–II vs III–IV) in Asians	1.81	1.16–2.82	57.50%	.04	.19	.28
Lymph node metastasis (N0 vs N1–N2)	2.15	1.04–4.47	63.50%	.02	.35	.70
Menopausal (premenopausal vs postmenopausal)	1.04	0.82–1.32	0.00%	.94	.60	.13
ER status (ER (–) vs ER (+)) (overall)	1.05	0.83–1.33	27.40%	.22	.45	.72
ER status (ER (–) vs ER (+)) in Caucasians	1.14	0.87–1.49	60.50%	.11	.32	—
ER status (ER (–) vs ER (+)) in Asians	0.79	0.49–1.30	6.70%	.37	.14	.27
PR status (PR (–) vs PR (+)) (overall)	0.87	0.69–1.09	23.30%	.27	.62	.98
PR status (PR (–) vs PR (+)) in Caucasians	0.89	0.69–1.09	23.00%	.27	.32	—
PR status (PR (–) vs PR (+)) in Asians	0.78	0.45–1.35	0.00%	.66	.6	.92
DAPK						
Risk (overall)	4.93	3.17–7.65	26.30%	.21	.68	.09
Risk in Caucasians	3.60	1.83–7.08	0.00%	.48	.50	.21
Risk in Asians	4.71	2.51–8.84	0.00%	.55	1.00	.19
hMLH1						
Risk (overall)	1.84	1.26–2.69	37.20%	.17	.33	.052
Risk in Caucasians	0.47	0.15–1.44	46.90%	.15	.60	.23
Risk in Asians	2.18	1.45–3.28	0.00%	.44	.32	—
p14						
Risk (overall)	22.52	7.00–72.41	0.00%	.89	.14	.27
Risk in Caucasians	14.14	0.73–273.39	—	—	—	—
Risk in Asians	14.47	2.72–77.08	0.00%	.67	.32	—
p15						
Risk (overall)	2.13	0.30–15.07	65.40%	.03	1.00	.58
Caucasians	3.67	0.23–58.40	61.20%	.08	.12	.00
Asians	0.84	0.37–1.90	—	—	—	—

ER = estrogen receptor, OR = odds ratios, PR = progesterone receptor, RARβ2 = retinoic acid receptor beta, TNM = T: scope of the tumor, N: lymph node metastasis, M: distant metastasis.

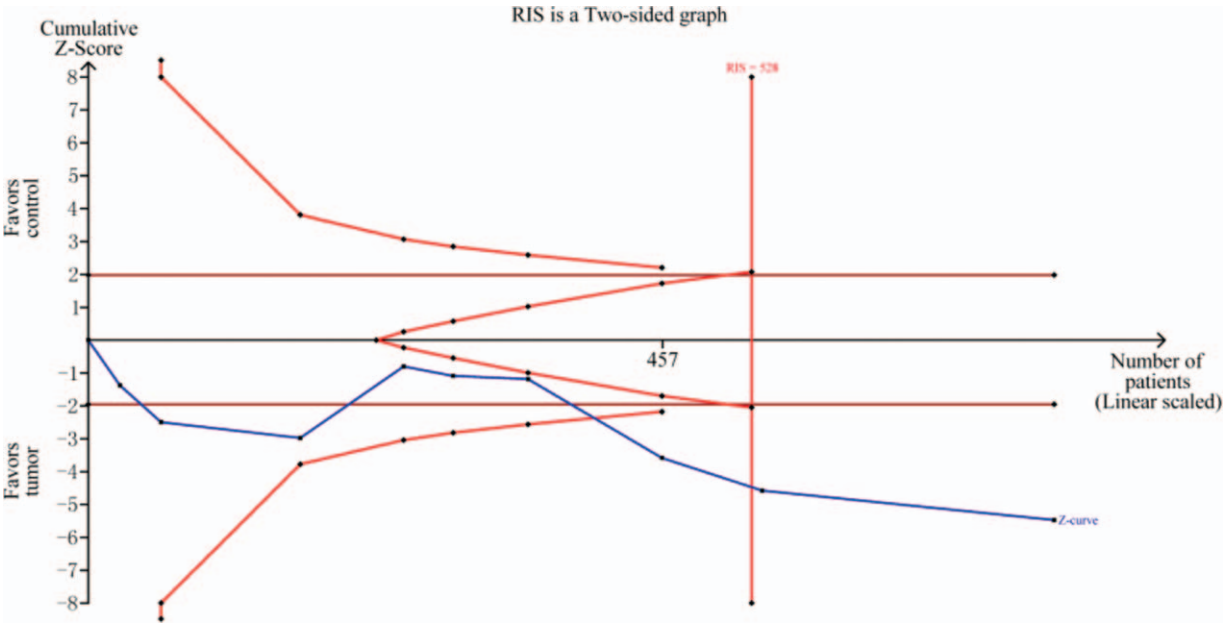

Figure 2. TSA on pooled results for effects of RARβ2 promoter hypermethylation on risk of breast cancer. RIS = required information size.

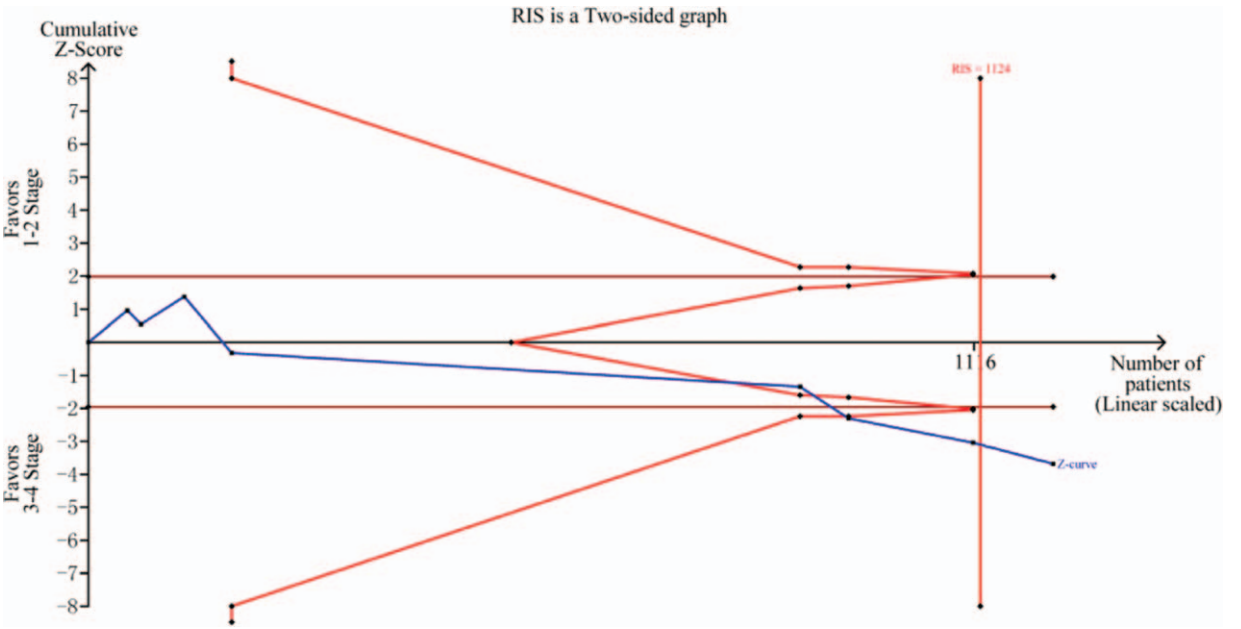

Figure 3. TSA on pooled results for effects of RARβ2 promoter hypermethylation on TNM-stage of breast cancer. RIS = required information size.

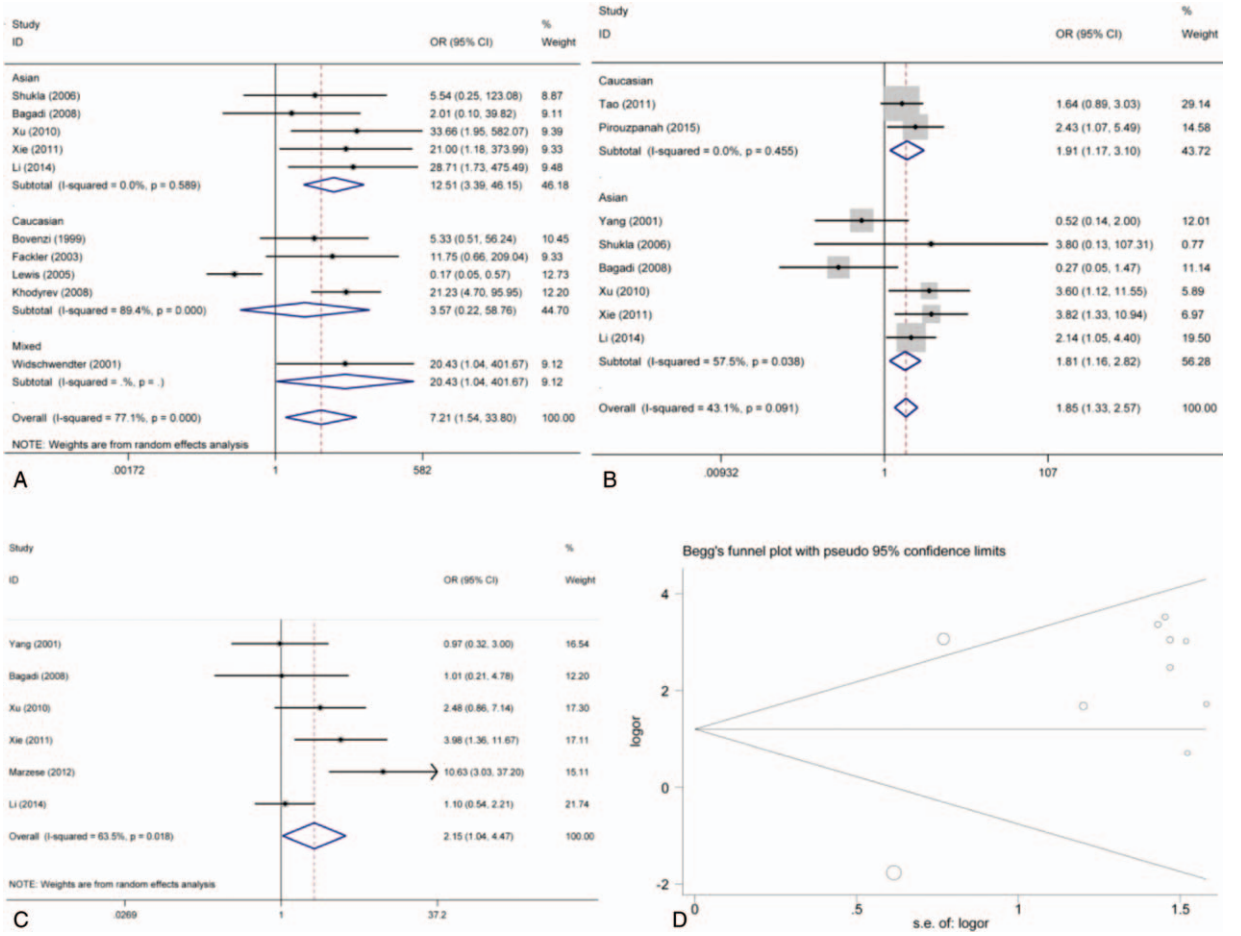

Figure 4. Forest plot and funnel plot for associations of RARβ2 promoter hypermethylation with risk, TNM-stage, and lymph node metastasis of breast cancer. (A) Forest plot for risk; (B) forest plot for TNM-stage; (C) forest plot for lymph node metastasis; (D) funnel plot for risk. CI = confidence intervals, Log OR = log odds ratio, OR = odds ratio, s.e. of logOR = standard error of log odds ratio.

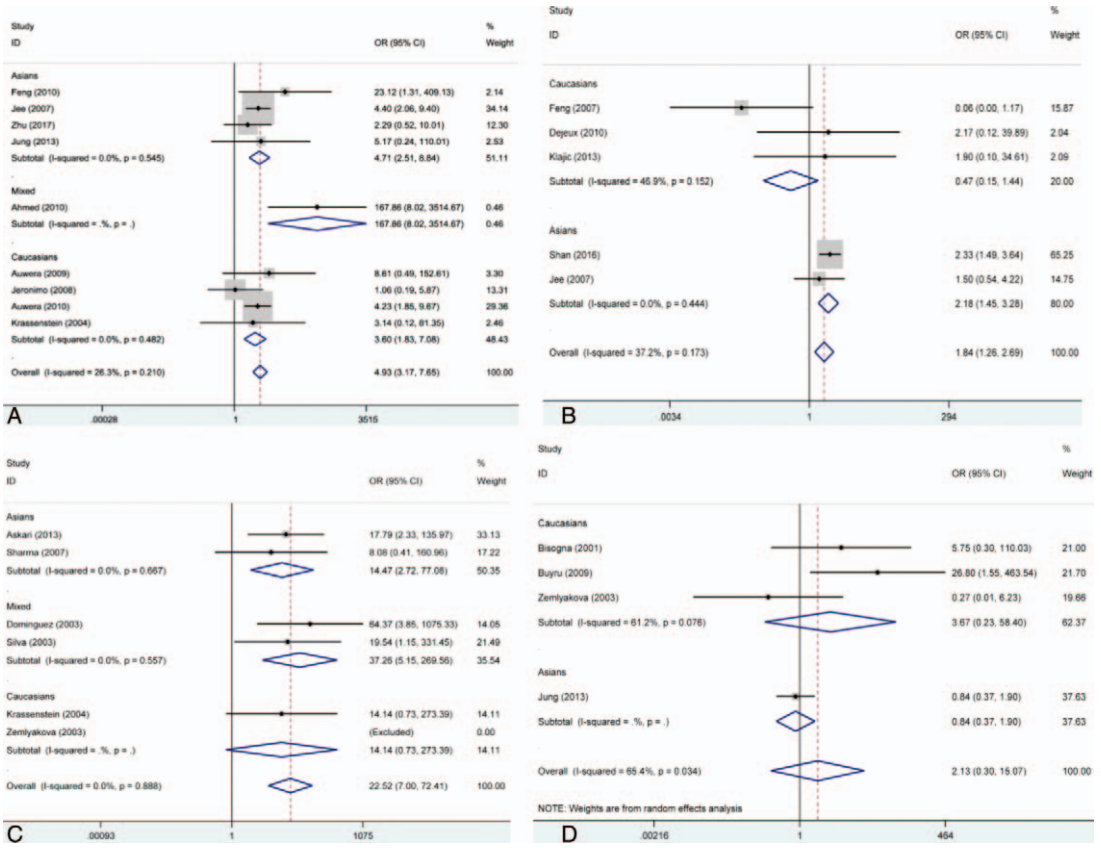

Figure 5. Forest plot for associations of DAPK, hMLH1, p14, and p15 promoter hypermethylation with risk of breast cancer. (A) Forest plot for DAPK; (B) forest plot for hMLH1; (C) forest plot for p14; (D) forest plot for p15. CI = confidence intervals, OR = odds ratio.

cancer risk, we drawed the forest plots to acquire the overall OR and 95% CI. The results showed that there were significant associations of DAPK, hMLH1, and p14 gene promoter hypermethylation with breast cancer risk, but not p15 gene promoter hypermethylation (DAPK, OR = 4.93, 95% CI = 3.17–7.65; for hMLH1, OR = 1.84, 95% CI = 1.26–1.29; for p14, OR = 22.52, 95% CI = 7.00–72.41; for p15, OR = 2.13, 95% CI = 0.30–15.07). Moreover, no significant heterogeneity was found in the analysis of DAPK, hMLH1, and p14 gene promoter hypermethylation. However, significant heterogeneity was found in the analysis about p15 gene promoter hypermethylation. In the sensitivity analysis, we found the study of Jung et al contributed a lot to the heterogeneity. But there was still no significant association between p15 gene promoter hypermethylation and breast cancer risk after the study of Jung was eliminated (OR = 3.67, 95% CI = 0.23–58.40) (Table 2, Fig. 5).

3.5. Publication bias and sensitivity analysis

According to the results of Begg test and Egger test, no obvious publication bias among studies was found in the analysis (Table 2, Fig. 6). Sensitivity analysis revealed that the pooled ORs did not have a significant change by eliminating each study in the analysis of RARβ2, DAPK, hMLH1, and p14 genes promoter hypermethylation. Therefore, it showed a robust result of the meta-analysis.

4. Discussion

Although digital mammography and magnetic resonance imaging have screened numerous breast cancer patients, these physical methods lacked specificity and sensitivity.[61] As alternative biomarkers, DNA methylation was extensively studied in many tumor suppressor genes, especially in gene promoter region. DNA methylation usually was detected in the early stage of breast cancer and had a significant association with clinical characteristics of breast cancer, which was helpful for early detection and treatment of breast cancer.[62] Many biomarkers, benefitting the classification of clinical features in breast cancer, were identified through genome-wide approach, using tissue sample and blood sample.[63] For example, hyaluronoglucosaminidase 2 (HYAL2) had a lower methylation frequency in breast cancer group than control group. And the hypomethylation of HYAL2 was different with the methylation frequency of HYAL2, which the HYAL2 methylation was significant higher than control group. Therefore, these results demonstrated that abnormity of HYAL2 methylation might not originate from the tumor cycle DNA.[64] In this meta-analysis, the data of RARβ2 promoter hypermethylation in blood sample was extracted and analyzed, which indicated the frequency of RARβ2 promoter hypermethylation was higher than those in control group (OR = 15.04, 95% CI = 3.59–63.00, $P < .05$).[32–35] Given the condition of HYAL2, the hypermethylation of RARβ2 promoter hypermethylation in blood sample might not originate from tumor cells or tumor cycle DNA. Thus, the association of RARβ2 promoter hypermethylation with breast cancer pathogenesis was evaluated on the basis of the results of tissue sample.

According to results of tissue sample, the RARβ2 promoter hypermethylation had a significant association with the susceptibility of breast cancer, in which the breast cancer group had a higher frequency of RARβ2 promoter hypermethylation than

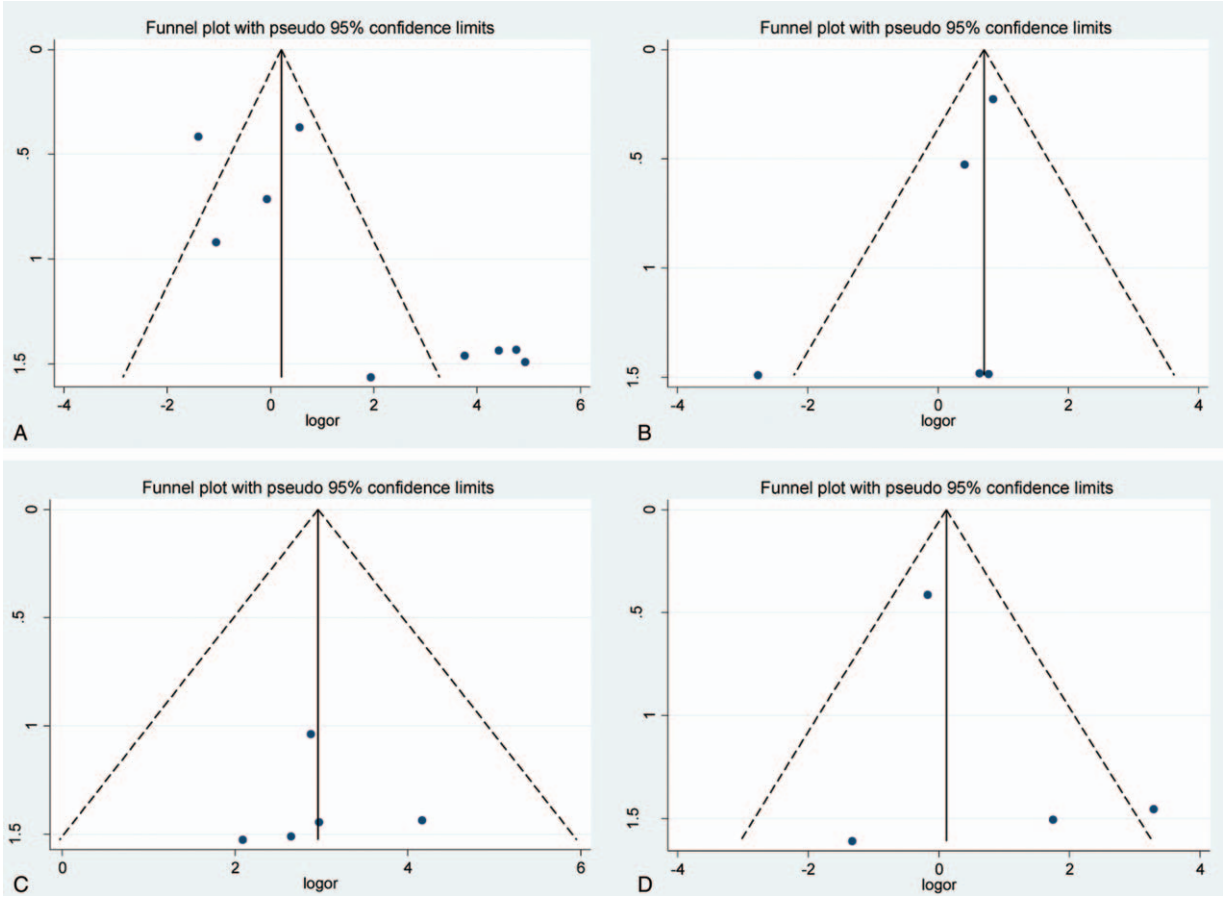

Figure 6. Funnel plot for associations of DAPK, hMLH1, p14, and p15 promoter hypermethylation with risk of breast cancer. (A) DAPK; (B) hMLH1; (C) p14; (D) p15. Log OR = log odds ratio, s.e.of logOR = standard error of log odds ratio.

normal tissue. These results were only indicated in Asians. In these included studies, 5 studies obtained negative results,[25,27–29,31] while other studies found significant associations.[22–24,26,30,32–35] Trial sequential analysis was also performed and the result suggested that the sample size has reached the required information size (RIS = 528) and the significant result was found. The trial sequential analysis was carried out according to the incidence of breast cancer group and control group. On the basis of the cumulative data, the average incidence rate of control group and breast cancer group were 6.19% and 31.85%. Furthermore, no significant associations of RARβ2 promoter hypermethylation with ER status, PR status, and menopause of breast cancer were observed by the present meta-analysis ($P > .05$). Of these studies for TNM-stage of breast cancer, Pirouzpanah et al,[38] Xu et al[24], Xie et al,[32] and Li et al[38] found that the frequency of RARβ2 promoter hypermethylation in III–IV stage was higher than those patients in I–II stage, but contrary results was indicated in the study of Tao et al,[37] Yang et al,[38] Shukla et al,[27] and Bagadi et al.[25] One thing to note was that no significant heterogeneity was found among included studies for the meta-analysis of TNM-stage. The required information size of studies regarding TNM-stage of breast cancer was 1124 based on TSA, but Z-curve has crossed the futility boundary, which showed an inapparent association. According to the data of lymph node metastasis, this epigenetic change of RARβ2 was significantly associated with the lymph node metastasis of breast cancer. In addition, no significant association between RARβ2 promoter hypermethylation and menopausal was presented in Caucasians and Asians. However, considering the limited number and sample sizes of studies both in Caucasians and Asians, more studies were still needed to demonstrate these findings of the meta-analysis. In addition, we did not find that RARβ2 promoter hypermethylation had significant associations with the status of ER and PR in Caucasians and Asians. As is well known, the expression of ER had an important role in predicting the response to endocrine therapy and recurrence of breast cancer.[61] Based on these results, RARβ2 promoter hypermethylation might not be associated with the ER status and PR status. RARβ2 promoter methylation might be a single biomarker to predict the risk and pathogenesis of breast cancer. However, further studies with large sample size and more clinical information were needed to be conducted to clarify these findings. Furthermore, a previous meta-analysis which assessed the association between RARβ2 promoter hypermethylation and breast cancer risk was conducted by Fang et al, and the results were consistent with the meta-analysis.[65] However, no clinical information was included in the previous meta-analysis. Therefore, this meta-analysis demonstrated RARβ2 promoter hypermethylation was significantly associated with the clinical progression of breast cancer.

On the other hand, significant heterogeneity was only found in the analysis of breast cancer risk and RARβ2 promoter hypermethylation in Caucasians. The result of subgroup analysis based on race indicated that race was not the mainly source of

heterogeneity because significant heterogeneity still exist. Perhaps, other clinical information or tumor heterogeneity contributed a lot to the significant heterogeneity. Furthermore, no significant heterogeneity was found among studies about lymph node metastasis and TNM-stage of breast cancer. According to the results of sensitivity analysis, overall results were stable.

In many studies, many genes were used to detect breast cancer, including RASSF1A, APC, RARβ2, GSTP1, BRCA1, HOXA5, HIC-1, E-cadherin, p16, CyclinD2, HIN1, and TWIST genes promoter hypermethylation.[25,28,29,34–38] So the doctor diagnosed the breast cancer and estimated the clinical progression of breast cancer on the basis of the results of the status of methylation in these genes. In addition aberrant hypomethylation of genes such as: RASSF1A, APC, p16, p14, p15 were often detected in cancers. Thus the status of genes aberrant methylation might have a strong influence in the production of tumor cells. In the literature searching, we found that no meta-analysis was conducted to summarize the published data and discuss the relationship of DAPK, hMLH1, p14, and p15 hypermethylation and breast cancer. In the present meta-analysis, we have observed that DAPK, hMLH1, and p14 genes promoter hypermethylation were significantly associated with the susceptibility of breast cancer, and no obvious heterogeneity was found. However, the p15 gene promoter hypermethylation did not have a significant relationship with breast cancer risk. In the pooled analysis about p15 gene promoter hypermethylation, 4 studies with 280 breast tumor samples and 159 control samples were included in the meta-analysis. This might be partly explain the significant heterogeneity among studies ($I^2 = 65.4\%$, $P = .034$).

Several potential limitations should be noted in the present meta-analysis: the small sample size might be the mainly restrictions; no enough and detailed clinical information was included in this analysis, which might affect the evaluation of associations between RARβ2, DAPK, hMLH1, p14, and p15 gene promoter hypermethylation and susceptibility and clinical progression of breast cancer; heterogeneity was mainly found in Caucasians, therefore more studies in Caucasians should be further performed in the analysis of RARβ2; the studied population of included studies was mostly from Asians and Caucasians, thus caution should be taken in studied populations, and more studies in other races should be performed to clarify these results. The detailed molecular mechanisms of the abnormal hypomethylation of these genes should be studied. In addition, the expression of DNMT1 (DNA methyltransferase1), DNMT3a, DNMT3b, and TET double oxygenase in cancer or other disease, regulating genes methylation levels, were often lower than the control group.[66,67] However, it has been reported that some molecules might accurately regulate the methylation of certain target gene such as ncRNA in plants.[65] Published studies often altered the protein expression levels of DNMT1 (DNA methyltransferase1), DNMT3a, DNMT3b, and TET double oxygenase, and then observed relevant genes methylation, which might cause unpredictable influences.[66–68] In arabidopsis, the study of Zhou et al suggested that CLASSY family could control the locus-specific methylation of de novo DNA. And authors of the study speculated that similar regulating system of DNA methylation might exist in a broad range of organisms. Therefore, this might be a new direction to explore the DNA methylation.[69]

5. Conclusion

In conclusion, this meta-analysis demonstrated that RARβ2, DAPK, hMLH1, and p14 promoter hypermethylation was significantly associated with breast cancer risk. At the same time, significant associations of RARβ2 promoter hypermethylation with lymph node metastasis and TNM-stage of breast cancer were found. Considering the heterogeneity among studies, further studies with larger sample size, more clinical information, and environmental factors should be performed to validate these findings.

Author contributions

Data curation: Ming Qi.
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Funding acquisition: Xiang Xiong.
Investigation: Ming Qi.
Methodology: Ming Qi, Xiang Xiong.
Software: Ming Qi, Xiang Xiong.
Writing – original draft: Ming Qi.
Writing – review & editing: Xiang Xiong.

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