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Review Article

Value of concentration and gene methylation analyses of circulating cell-free DNA as diagnostic method for breast cancer: a systematic review

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Abstract: *Background and aims:* Many studies focus attention on circulating cell-free DNA (cfDNA) concentration and methylation as means for early detection of breast cancer. However, the results of existing studies are different and it remains difficult to determine the diagnostic role of circulating cfDNA in breast cancer. Therefore, this study performed a meta-analysis to integrate the findings of these published studies and establish the diagnostic value of circulating cfDNA in diagnosis of breast cancer. *Methods:* A total of 19 studies were included and divided into three subgroups. Sensitivity, specificity, and other important measures of cfDNA accuracy for breast cancer diagnosis were pooled using random-effects models. Summary receiver operating characteristic curve analysis techniques were used to summarize overall accuracy. *Results:* Summary estimates for cfDNA concentration and gene methylation in diagnosis of breast cancer were as follows: in the subgroup of quantitative analysis, sensitivity and specificity were 0.889 (95% CI = 0.858-0.915) and 0.806 (95% CI = 0.767-0.841); PLR was 5.058 (95% CI = 2.723-9.392); NLR was 0.150 (95% CI = 0.090-0.251) and DOR value was 42.156 (95% CI = 19.364-91.774). In the subgroup of single-gene indicators, sensitivity and specificity were 0.406 (95% CI = 0.390-0.421) and 0.848 (95% CI = 0.836-0.860); PLR was 4.569 (95% CI = 3.329-6.270); NLR was 0.643 (95% CI = 0.588-0.704) and DOR value was 8.608 (95% CI = 5.679-13.046). In the subgroup of multiple-gene panel, sensitivity and specificity were 0.778 (95% CI = 0.760-0.796) and 0.835 (95% CI = 0.819-0.850); PLR was 6.158 (95% CI = 4.432-8.556); NLR was 0.194 (95% CI = 0.139-0.272) and DOR value was 39.353 (95% CI = 21.139-73.260). *Conclusion:* Circulating cfDNA concentration and gene methylation analysis might be applied in breast cancer detection since the test has a relatively high level of diagnostic accuracy. If only the single-gene methylation detection assay is used for breast cancer diagnosis, extra caution should be applied. Evaluation of circulating cfDNA concentration and multiple-gene methylation analyses might improve breast cancer early diagnosis.

Keywords: Breast neoplasms, circulating cell-free DNA, concentration, methylation, meta-analysis

Introduction

Breast cancer is the most common cause of cancer deaths in women, worldwide. As with other types of cancer, early detection is crucial for successful treatment. Hence, non-invasive testing has been a goal for many researchers. In the past decade, many researchers [1-5] have focused their attention on circulating cell-free DNA (cfDNA) concentration and methylation as means for early detection of a variety of cancers, including breast cancer.

Circulating cell-free DNA (cfDNA) is released from living eukaryotic cells or is derived from apoptotic and necrotic cells. Many studies have shown that abnormal cfDNA concentration and methylation are associated with many cancers [6]. Therefore, cfDNA can be regarded as a promising diagnostic tool for many cancers. Circulating cfDNA, its concentration, and tumor-specific methylations have been reported early events in breast cancer [7].

However, results of cfDNA analysis of existing studies are different so it has remained difficult to determine the diagnostic role of circulating cfDNA concentration and methylation patterns in breast cancer. Furthermore, it has not yet been systematically evaluated.

Table 1. Summary of included studies

Author	Year	Region	Breast cancer	Benign lesions	Healthy women	Sample	Assay methods	Assay indicators	Cutoff	Tumor stage	QUADAS score
Kloten et al. [13]	2013	Germany	138	39	135	Serum	MSP	Methylation: (ITIH5, DKK3 and RASSF1A)	0.085%	I-IV	10
Liu et al. [14]	2015	China	36	30	30	Serum	MS-HRM	Methylation (FHIT)	> 50%	Unknown	8
Agostini et al. [15]	2012	Italy	39	0	49	Plasma	RQ-PCR	Quantitative analysis	2.0 or > 9.3 ng/ml$^{\Psi}$	I-III	8
Hashad et al. [16]	2012	Egypt	42	30	27	Plasma	RQ-PCR	Quantitative analysis	1,866 GE/ml	I-IV	8
Wu et al. [17]	2015	USA	47	0	42	Plasma	RQ-PCR	Quantitative analysis	91.40$^{\zeta}$	Unknown	9
Shan et al. [18]	2016	China	268	236	245	Serum	MethyLight	Methylation (six-genes)$^{\delta}$	NA	Unknown	10
Liu et al. [19]	2009	China	80	40	97	Plasma	RQ-PCR	Quantitative analysis	≤ 12.90 μg/L	I-III	5
Fu et al. [20]	2014	China	86	36	0	Plasma	MSP	Methylation (Sox17)	NA	Unknown	5
Jin et al. [21]	2007	China	61	33	27	Plasma	RQ-PCR	Quantitative analysis	19 μg/L	Unknown	7
Huang et al. [22]	2007	China	61	33	25	Plasma	RQ-PCR	Quantitative analysis	NA	Unknown	6
Li et al. [23]	2010	China	78	20	30	Serum	RQ-PCR	Quantitative analysis	96.0 μg/L	Unknown	6
Li et al. [24]	2016	China	86	0	67	Plasma	MiSeqsequencing	Methylation (six-gene)$^{\theta}$	NA	(I~II)	8
Fackle et al. [25]	2014	USA	57	0	55	Serum	MSP	Methylation (ten-gene)$^{\lambda}$	NA	IV	8
Chimonidou et al. [26]	2013	Greece	114	0	60	Plasma	MSP	Methylation (SOX17)	NA	Unknown	10
Chimonidou et al. [27]	2013	Greece	73	0	37	Plasma	MSP	Methylation (CST6)	NA	I-III	8
Radpour et al. [28]	2011	Switzerland	36	0	30	Plasma	EpiTYPER assay	Methylation (ten-gene)$^{\&}$	Cut-off value$^{\Phi}$	I-III	6
Brooks et al. [29]	2010	USA	50	50	50	Serum	MSP	Methylation (RASSF1A, GSTP1, APC, RARβ2)	NA	Unknown	9
Kim et al. [30]	2010	Korea	119	0	125	Serum	MSP	Methylation (CST6)	NA	Unknown	10
Jing et al. [31]	2010	China	50	40	10	Serum	MSP	Methylation (eight-gene)*	NA	Unknown	10

MSP, methylation-specific polymerase chain reaction, RQ-PCR, Real-time quantitative PCR, MethyLight, Fluorogenic PCR, MiSeq sequencing, desktop sequencer that can produce 2 x 300 paired-end reads in a single run. Φ, Cut-off points were calculated according to 90% specificity. Ψ, 2.0 ng/ml (ALU247); > 9.3394 ng/ml (ALU115). ζ, 91.40 (T/R copy ratio). δ, Methylation (HOXD13, SFN, RASSF1a, P16, PCDHGB7, and hMLH1). θ, Methylation (EGFR, GREM1, PDGFRB, PPM1E, SOX17 and WRN). λ, Methylation (AKR1B1, ARHGEF7, COL6A2, GPX7, HOXB4, RASGRF2, RASSF1A, TM6SF1, TMEFF2, HIST1H3). &, Methylation (APC, BIN1, BMP6, BRCA1, CST6, ESR-b, GSTP1, P16, P21 and TIMP3). *, Methylation (RASSF1A, CDH1, RARβ2, BRCA1, p16, ER, APC and DAPK).

Table 3. Results for measurement of quantitative analysis, single-gene, and multiple-gene panel indicators analysis in the diagnosis of breast cancer

Group	AUC	Sen (%) (95% CI)	Spe (%) (95% CI)	PLR (95% CI)	NLR (95% CI)	DOR (%) (95% CI)
Quantitative analysis	0.9267	0.889 (0.858-0.915)	0.806 (0.767-0.841)	5.058 (2.723-9.392)	0.150 (0.090-0.251)	42.156 (19.364-91.774)
Single-gene indicators	0.7775	0.406 (0.390-0.421)	0.848 (0.836-0.860)	4.569 (3.329-6.270)	0.643 (0.588-0.704)	8.608 (5.679-13.046)
Multiple-gene panel indicators	0.9279	0.778 (0.760-0.796)	0.835 (0.819-0.850)	6.158 (4.432-8.556)	0.194 (0.139-0.272)	39.353 (21.139-73.260)

Sen, Sensitivity; Spe, Specificity; AUC, Area under the curve; BC, breast cancer; VS, compared with; non-cancer control = begnin and heathy control.

Table 2. Methodological quality of 19 studies

	QUADAS item													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Yes (n)	13	19	19	19	0	19	14	14	0	19	19	16	14	19
No (n)	6	0	0	0	19	0	0	5	19	0	0	0	4	0
Unknown (n)	0	0	0	0	0	0	5	0	0	0	0	3	1	0
Yes (%)	68	100	100	100	0	100	74	74	0	100	100	84	73	100

n, number of studies.

Therefore, the present meta-analysis was performed to integrate the findings of these published studies and analyze the diagnostic value of circulating cfDNA concentration and gene methylation patterns for early detection of breast cancer.

Materials and methods

Literature search strategy

EMBASE (1970-2016), MEDLINE (1966-2016), the Cochrane Library, (issue 2, 2016), VIP Chinese Journals Database (1968-2016), China Biological Medicine Database (CBM-disc, 1979-2016), and CNKI Database (1994-2016) were searched. Recently completed and ongoing trials were searched in Trials registers of Cochrane Breast Cancer Group and WHO International Clinical Trials Registry at www.who.int/ictrp/en/. All searches were up to date as of August 2016. Terms included "breast neoplasms (MeSH)", "breast cancer", "cell-free DNA", "plasma DNA", "concentration", "methylation", "diagnostic marker", and "accuracy". To form a highly sensitive search strategy, related keywords and their synonyms were included. Reference lists were scanned for additional publications. No restrictions were applied regarding study design or publication status. Review of articles published only in Chinese and English was allowed for full-text review and final analysis.

The following exclusion criteria were applied: (1) reviews, conference abstracts, letters, and grey literature; (2) articles in which circulating cfDNA was not related to cancer diagnosis; (3) articles including markers evaluation not used for breast cancer diagnosis; (4) articles containing insufficient data for calculating specificity and sensitivity values; (5) articles considering markers rarely studied.

Among studies collected, only those following the inclusion criteria were used for analyses: studies designed with at least 10 patients and written in English and Chinese. The index diagnosis method was circulating cfDNA concentration and gene methylation pattern. Studies were independently retrieved by two reviewers (Yinghua Yu and Xiao Zhu). When necessary, discrepancies were solved by team discussion.

Data extraction

Two reviewers (Yinghua Yu, Xiao Zhu) performed data extraction, independently. Data included author name, patient characteristics, geographic location, assay indicators, experimental methods, docimastic samples, cutoff values, and breast cancer stage (**Table 1**). TP (true-positive), TN (true-negative), FP (false-positive), FN (false-negative), and the results are displayed in Supplementary Tables 1, 2 and 3.

In the present meta-analysis, subgroups were evaluated as follows: studies about abnormal concentrations of total circulating cfDNA were quantitatively analyzed; studies about single-gene methylation patterns were analyzed by single-gene subgroup; and studies about multiple-gene panel methylation patterns were analyzed by multiple-gene panel subgroup.

Quality assessment

Methodological quality assessment used QUADAS tool (quality assessment for studies of diagnostic accuracy) [8]. For criteria, expert opinions, empirical evidence, and formal consensus were used to assess the quality of primary studies. If data was not reported in primary studies, a formal request to the authors was arranged. If the authors did not respond to the letters, "unknown" items were treated as "no".

Statistical analysis

STATA version 10.0 (STATA Corporation, TX, USA) and Meta-DiSc (Barcelona, Spain) were used to analyze data. Accuracy criteria such as specificity, sensitivity, negative likelihood ratio (NLR), positive likelihood ratio (PLR), and diagnostic odds ratio (DOR) were evaluated for each

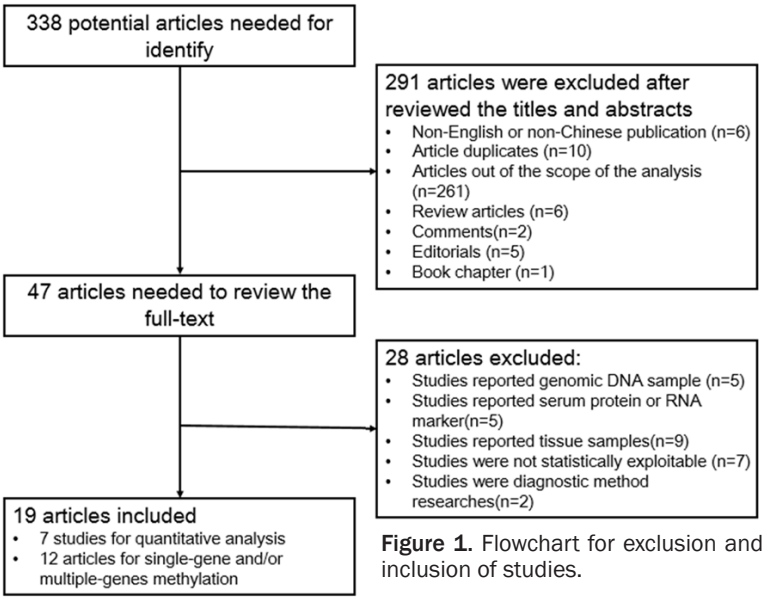

Figure 1. Flowchart for exclusion and inclusion of studies.

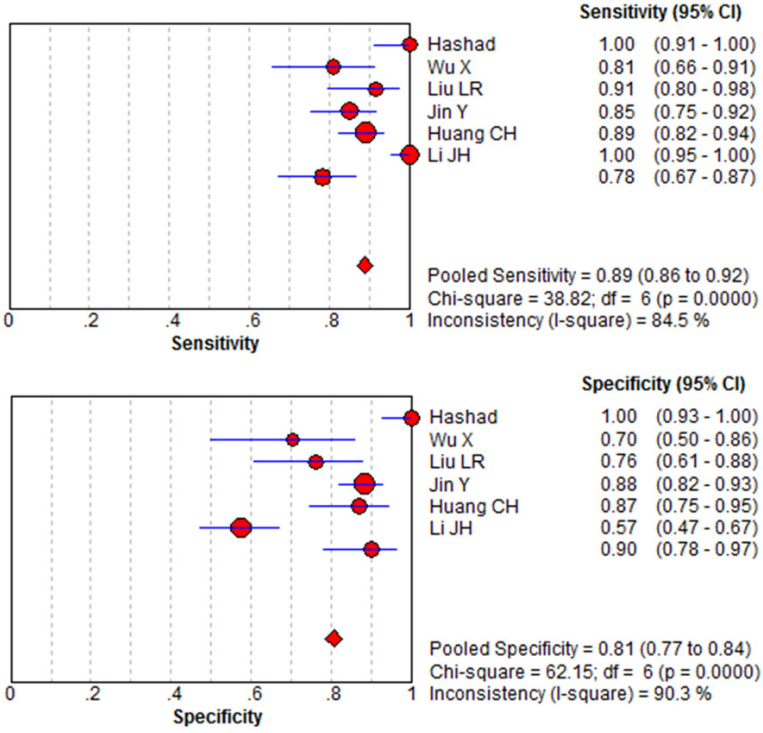

Figure 2. Forest plot of estimates of sensitivity and specificity for cfDNA in the subgroup of quantitative analysis. The point estimates of sensitivity and specificity from each study are shown as solid circles. Error bars are 95% confidence intervals. Author names indicate the studies.

study: PLR was defined as: PLR = sensitivity/(1-specificity). NLR was defined as: NLR = (1-sensitivity)/specificity. SROC curve was plotted by sensitivity and specificity for the single test threshold. Random-effects model was used to analyze accuracy criteria such as average specificity, sensitivity, and other measures across studies.

Heterogeneity refers to the degree of variability of results across studies. Cochran's Q test ($p < 0.05$ or $I^2 > 50\%$) [9] was used to detect statistical heterogeneity. Effects of covariance in univariate meta-regression analysis (i.e., geographic location, experimental methods, assay indicators, docimastic sample, cutoff values, breast cancer stage, methodological quality, and QUADAS scores) were analyzed. To investigate change in diagnostic precision, relative DOR (RDOR) was calculated per unit increase in the covariate [10, 11]. Since publication bias is important regarding the validity of diagnostic meta-analyses, funnel plots were used to test potential presence of bias [12].

Results

After review, a total of 19 studies [13-31], involving cfDNA for breast cancer, were eligible. Exclusion and inclusion criteria flowchart is shown in **Figure 1**.

Study characteristics and studies quality

The 19 studies selected for this study included 1,521 patients with breast cancer, 587 benign breast lesions, and 1,141 healthy women (**Table 1** and Supplementary Tables 1, 2 and 3). There were ten studies in Asia and the remaining studies were in the United States and Europe. All studies were prospective. Clinical characteristics are presented in **Table 1**. Among these 19 studies, 7 trials [15-17, 19, 21-23] evaluated abnormal concentrations of circulat-

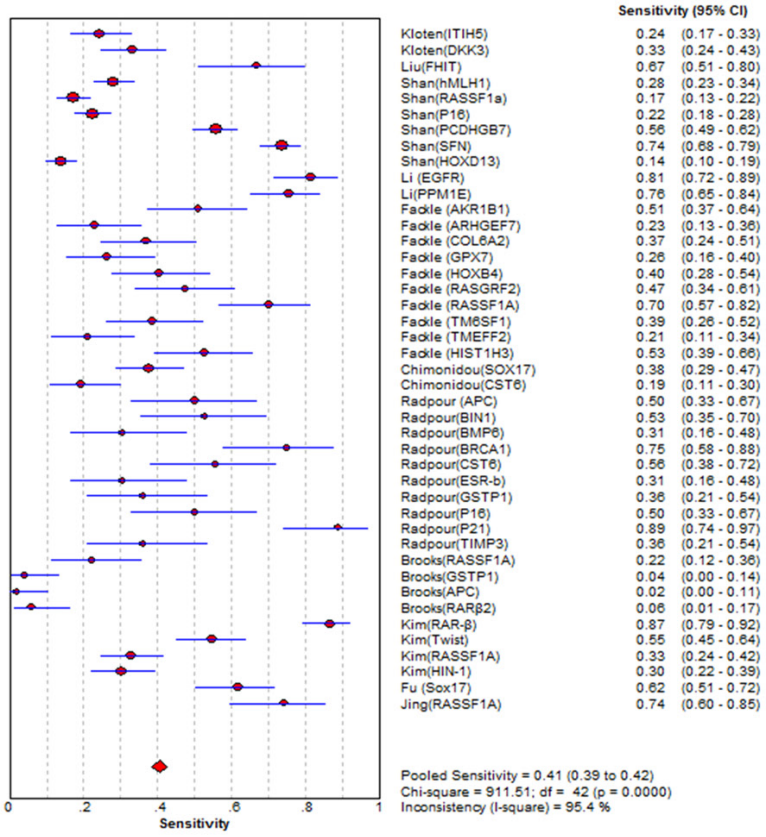

Figure 3. Forest plot of estimates of sensitivity and specificity for cfDNA in the subgroup of single-gene indicators analysis. The point estimates of sensitivity and specificity from each study are shown as solid circles. Error bars are 95% confidence intervals. Author and gene names indicate the studies.

ing cfDNA in serum or plasma. Twelve trials [16-17, 21, 23, 27-34] assessed the validity of using single-gene methylation alterations, with six trials [16, 21, 27-28, 33-34] using multiple-gene panel methylation alterations. DNA was isolated from different samples in these studies. Eleven studies [18-20, 22-25, 27, 29-31] isolated cfDNA from plasma, while the others from serum. Eight studies [16, 23, 28-30, 32-34] used MSP (real-time methylation-specific PCR) as assay method and the remaining studies used RT-qPCR (Real-time quantitative PCR), MethyLight, EpiTYPER assay, or other methods. Cutoff values and tumor stages were reported in 9 studies [16-20, 22, 24, 26, 31] and 8 studies [16, 18-19, 22, 27-28, 30-31], respectively.

Assessment of quality of the included studies was made using QUADAS. Among 14 QUADAS items, assessing variability of the studies was according to item 1 (spectrum composition) and item 2 (selection criteria); assessing the quality of reporting was using items 8 (index test execution), 9 (reference standard execution), and 13 (uninterpretable test results). The rest of the items were adopted to analyze the bias of studies. If a study fulfilled the criterion, the item was marked as “yes”; if the criterion was not ful-

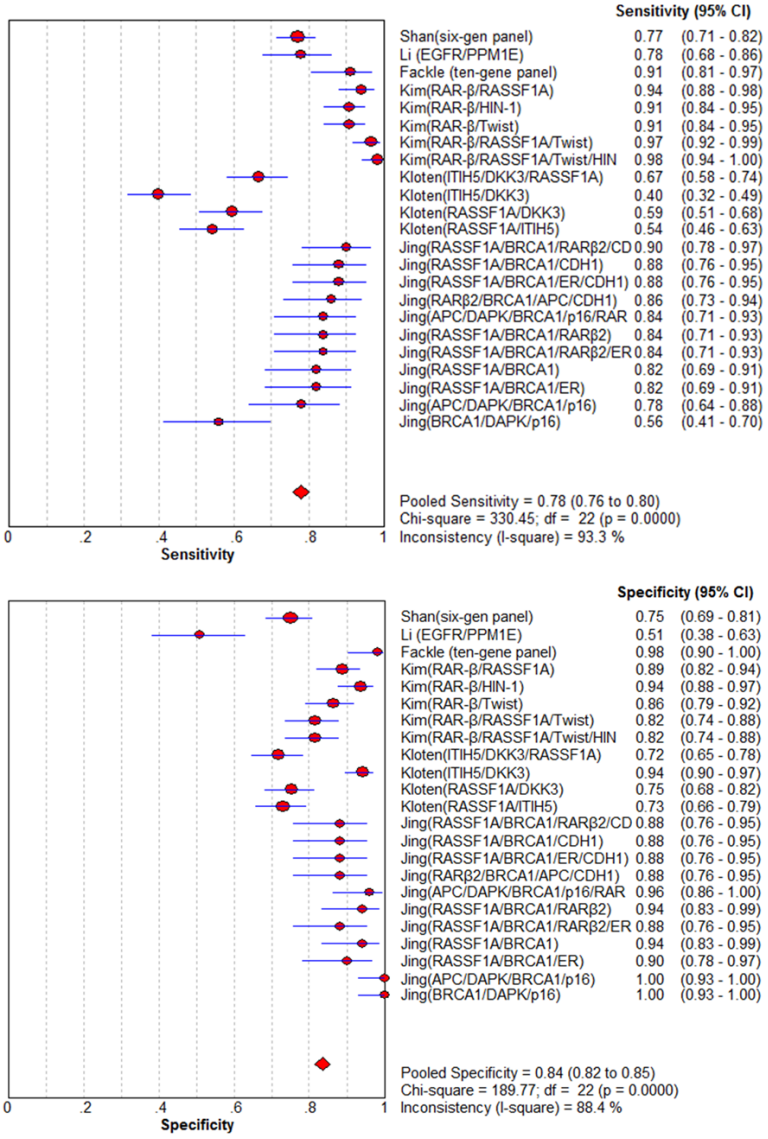

Figure 4. Forest plot of estimates of sensitivity and specificity for cfDNA in the subgroup of multiple-gene panel indicators analysis. The point estimates of sensitivity and specificity from each study are shown as solid circles. Error bars are 95% confidence intervals. Author and gene names indicate the studies.

filled, the item was marked as "no"; if a study was not clearly reported, the item was marked as "unknown". Of the included studies, 68% and 100% fulfilled the criterion of items 1 and 2, respectively while 74%, 73% and 0% fulfilled the criterion of items 8, 9 and 13, respectively. The rest of the items were reached at high levels, except for item 5 (partial verification) (**Table 2**).

Diagnostic performance

Diagnostic parameters such as pooled sensitivity, specificity, PLR, NLR, and DOR were used to analyze the accuracy of cfDNA in three subgroups (quantitative, single-gene, and multiple-gene panel subgroups).

As presented in **Figure 2**, in the subgroup quantitative analysis, pooled sensitivity and specificity were 0.889 (95% CI: 0.858-0.915) and 0.806 (95% CI = 0.767-0.841), respectively. PLR was 5.058 (95% CI = 2.723-9.392), NLR was 0.150 (95% CI = 0.090-0.251), and DOR was 42.156 (95% CI = 19.364-91.774). I^2 values of sensitivity, specificity, PLR, NLR, and DOR were 84.5%, 90.3%, 87.9%, 68.5% and 60.4%, respectively.

In the subgroup of single-gene indicators, the forest plot of sensitivity and specificity of cfDNA is shown in **Figure 3**. Pooled sensitivity and specificity were 0.406 (95% CI = 0.390-0.421) and 0.848 (95% CI = 0.836-0.860), respectively. PLR was 4.569 (95% CI = 3.329-6.270), NLR was 0.643 (95% CI = 0.588-0.704), and DOR was 8.608 (95% CI = 5.679-13.046). As shown in **Figure 3**, I^2 values of sensitivity and specificity indicated significant heterogeneity between studies.

Compared with the subgroup of single-gene indicators, the diagnostic value of cfDNA in the multiple-gene panel subgroup improved dramatically. The forest plot of sensitivity and specificity of cfDNA is shown in **Figure 4**. Pooled sensitivity and specificity were 0.778 (95% CI = 0.760-0.796) and 0.835 (95% CI = 0.819-0.850), respectively. PLR was 6.158 (95% CI = 4.432-8.556), NLR was 0.194 (95% CI = 0.139-0.272), and DOR was 39.353 (95% CI = 21.139-73.260). As shown in **Figure 4**, I^2 values of sensitivity and specificity indicated significant heterogeneity between studies. All data above are summarized in **Table 3**.

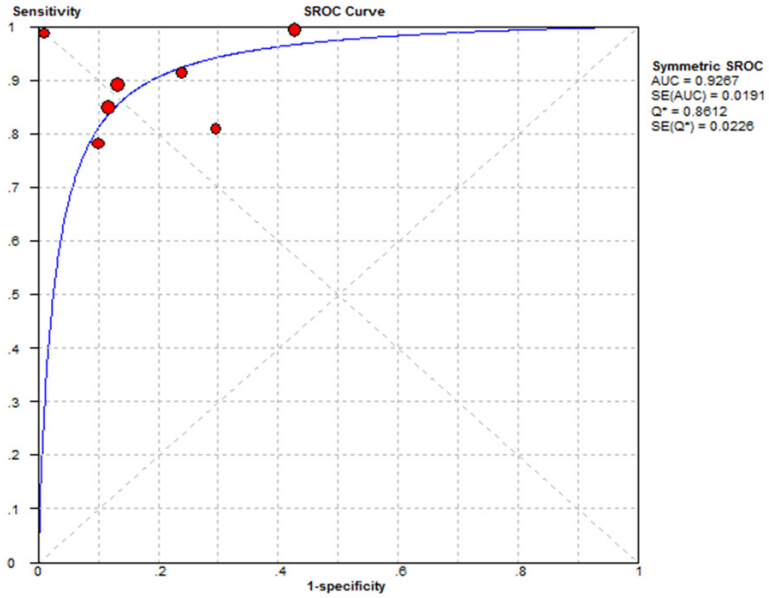

Figure 5. SROC curves for cfDNA in the subgroup of quantitative analysis. Each study is represented by each solid circle in the meta-analysis. The size of the solid circle indicates the size of each study. SROC curves summarize the overall diagnostic accuracy.

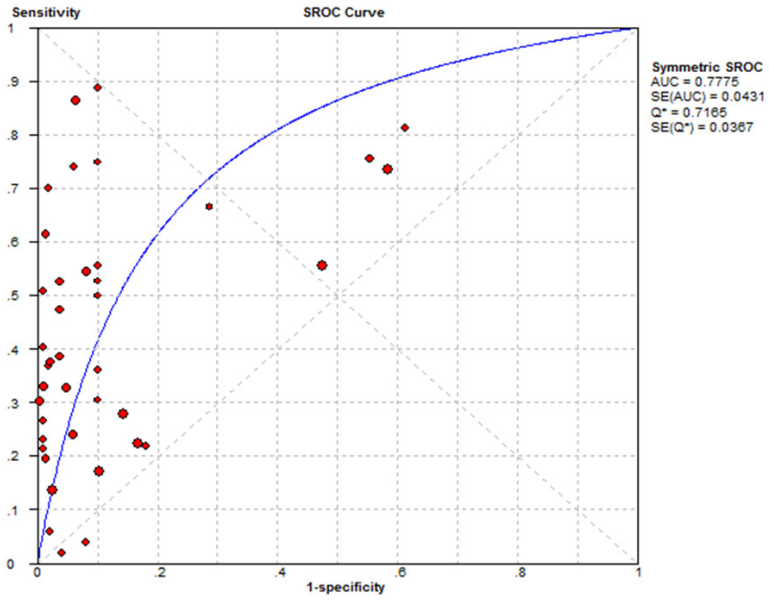

Figure 6. SROC curves for cfDNA in the subgroup of single-gene indicators analysis. Each study is represented by each solid circle in the meta-analysis. The size of the solid circle indicates the size of each study. SROC curves summarize the overall diagnostic accuracy.

SROC curve, presenting a global summary of test performance, showed a trade-off between sensitivity and specificity. SROC curve graphs for cfDNA diagnostic accuracy in the three subgroups are shown in **Figures 5**-**7**. The present results showed that SROC curves of quantitative analysis and multiple-gene panel indicators subgroups were placed near the desirable upper left corner. Their maximum joint sensitivity and specificity (i.e., the Q-value) were 0.861 and 0.862, respectively; areas under the curve (AUC) were 0.927 and 0.928, respectively, indicating a high level of overall accuracy. On the contrary, Q-value of the single-gene indicators subgroups was 0.717 and AUC was 0.778, indicating a relatively lower level of overall accuracy compared to the quantitative analysis and multiple-gene panel subgroups.

Multiple regression analysis and publication bias

We used meta-regression to assess different aspects in the subgroups to find the source of heterogeneity. Study characteristics included "study location (Region: Asia or not)", "docimastic sample (Sample: Plasma or Serum)", "experimental methods (MSP or not)", "cutoff value (mentioned or not)", "tumor stage (mentioned or not)", and QUADAS scores. QUADAS was used to analyze quality scoring [32], in which a score of 1 was given when a criterion was fulfilled, 0 if a criterion was unclear, and -1 if the criterion was not achieved (**Table 1**). Studies were considered to be of relatively high quality if their score was more than 8 (**Table 1**).

In meta-regression analysis, scores were used to assess the effects of study quality on RDOR of cfDNA. As presented in **Table 4**, differences of the aspects mentioned above did not reach statistical significance and they did not affect diagnostic accuracy.

In assessing potential publication bias, funnel graphs were drawn for the quantitative analy-

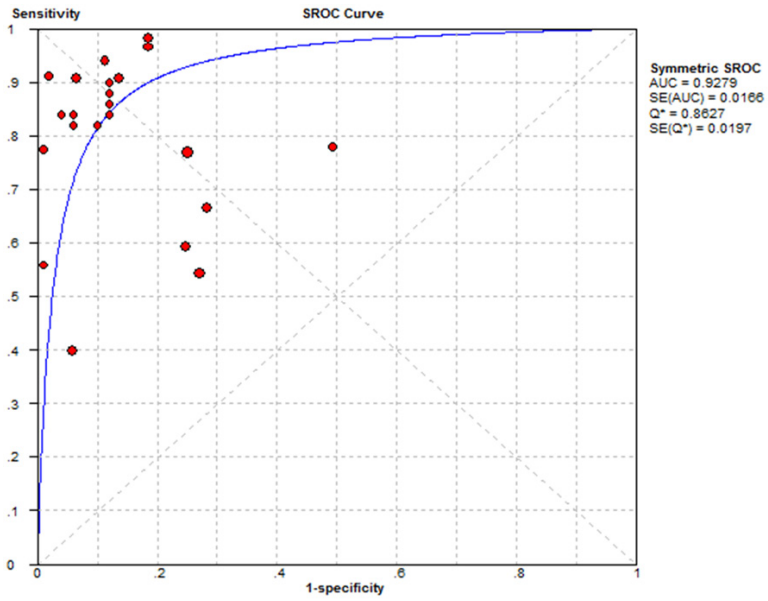

Figure 7. SROC curves for cfDNA in the subgroup of multiple-gene panel indicators analysis. Each study is represented by each solid circle in the meta-analysis. The size of the solid circle indicates the size of each study. SROC curves summarize the overall diagnostic accuracy.

sis, single-gene, and multiple-gene panel indicators subgroups. In **Figure 8**, some asymmetry is shown in funnel plots for publication bias. For these three subgroups, potential publication bias still existed.

Discussion

Circulating cfDNA, especially regarding its concentration and gene methylation pattern, has attracted new enthusiasm in different types of cancer [33, 34], including breast cancer. Until now, a meta-analysis to systematically evaluate the diagnostic potential of circulating cfDNA concentration and methylation in breast cancer has not existed.

The current systematic review and meta-analysis analyzed pooled diagnostic sensitivities and specificities of breast cancer in the quantitative subgroup, multiple-gene panel subgroups, and single-gene subgroups. In comparison, the two former subgroups showed better sensitivity values. On the contrary, diagnostic sensitivity in single-gene subgroups showed low sensitivity levels, no more than 50%. Pooled specificity values were acceptable in all those three subgroups, all above 80%.

In this meta-analysis, overall diagnostic performance was analyzed using SROC curves and corresponding AUC. Evaluation criteria were as follows: AUC ranging from 0.9 to 1.0 was defined as high accuracy; AUC ranging from 0.7 to 0.9 was defined as moderate accuracy; AUC ranging from 0.5 to 0.7 was defined as low accuracy [35]. AUC values for quantitative and multiple-gene panel analyses showed an accuracy of more than 0.9 in the present meta-analysis. Results showed that overall diagnostic performance with quantitative and multiple-gene panel analyses presented a relatively high diagnostic level of overall accuracy and were superior to single-gene analyses (AUC = 0.778).

DOR is an important indicator of test accuracy. It is the ratio of the odds of positive test results with disease relative to that without disease [36]. The DOR of a test ranges from 0 to infinity. The higher values of DOR, the better discriminatory test performs. A value of 1.0 indicates that a test is not able to distinguish patients with the disorder from those without it. In this meta-analysis, mean DOR for quantitative and multiple-gene panel subgroups analyses were 42.2 and 39.4, respectively, indicating a high part of moderate levels of overall accuracy. In contrast, the mean DOR for single-gene analyses was 8.6, considered unsatisfactory.

Regarding clinical practice, likelihood ratios are considered to be more meaningful than SROC curves and DOR [37, 38]. Hence, PLR and NLR were included as measures of diagnostic accuracy. To evaluate if a positive or negative result changed the probability of a disease state, likelihood ratios are used to combine both sensitivity and specificity of a test. Likelihood ratios > 10 or < 0.1 indicate high accuracy [38]. As shown in the subgroup of quantitative analysis, PLR value of 5.058 suggested that patients with breast carcinoma have an approximately 5-fold higher chance of seeing positive results compared with patients without breast carcinoma. The NLR value of 0.150 indicated that if the quantitative analysis result was negative,

Table 4. Meta-regression of the effects of different study aspects on diagnosis value of cfDNA in three subgroups

Subgroup	Covariates	Study no.	Coefficient	RDOR	95% CI	*P* value
Quantitative analysis	QUADAS ≥ 8	3	-0.953	0.39	0.08-1.78	0.1586
	Plasma	6	0.187	1.21	0.02-79.69	0.9075
	Cutoff value	6	-3.302	0.04	0.00-194.33	0.3451
	Stage I-III	2	1.557	4.75	0.17-134.61	0.2657
	Asia	4	0.953	2.59	0.56-11.97	0.1586
Single-gene	QUADAS ≥ 8	8	-0.963	0.38	0.01-17.74	0.5717
	Plasma	4	0.624	1.87	0.09-37.90	0.6392
	MSP	6	1.764	5.84	0.26-128.79	0.2195
	Cutoff value	3	-0.771	0.46	0.02-10.55	0.5781
	Stage I-III	1	-0.14	0.87	0.01-108.04	0.9473
	Asia	5	0.761	2.14	0.06-71.75	0.6242
Multiple-gene panel	QUADAS > 8	4	-2.508	0.08	0.00-7.29	0.1963
	Plasma	1	4.834	125.71	0.25-64423.84	0.0979
	MSP	4	-0.275	0.76	0.00-228.99	0.9002
	Cutoff value	2	-1.715	0.18	0.00-72.85	0.4722
	Asia	5	-0.132	0.88	0.00-765.29	0.9593

MSP, real-time methylation-specific PCR.

the probability of breast carcinoma was approximately 15%. In multiple-gene panel analyses, PLR was 6.158 and NLR was 0.194, similar to results of quantitative analysis. Compared with the former two subgroups, PLR and NLR of single gene analyses were relatively poor (PLR was 4.569 and NLR was 0.643). The NLR value of 0.643 indicated that an approximate 64% error rate would be present when the true negative was determined in the negative test. Although pooled specificity was higher in this group, poor robustness was indicated by unsatisfactory negative likelihood ratios. In other words, when single-gene methylation is used independently for detection of breast cancer, a negative cfDNA assay result should be interpreted with caution.

QUADAS is regarded as a quality evaluation criterion for diagnostic meta-analysis. It has been adopted for this present study. According to QUADAS, although many studies did not reach item 5 (partial verification) and 9 (reference standard execution), the overall quality of included studies was higher than median level. By analyzing study details, different standard methods (pathology or other method) were found to be adopted by the included studies. These aspects did not achieve item 5. Most studies did not match item 13 because they did not mention uninterpretable test results. These biases would affect analysis of the accuracy of cfDNA assays.

Exploring sources of heterogeneity was a major goal of this meta-analysis [39]. As shown in the results, various study characteristics did not reach statistical significance in meta-regression results. Therefore, other sources of heterogeneity might have contributed to the significant heterogeneity observed in this study.

However, this meta-analysis has some limitations. First, only including Chinese and English language studies might have resulted in publication bias. Second, in evaluation of all sources of heterogeneity, some covariates such as tumor size and metastasis were not included. The results of this meta-analysis increases confidence that circulating cfDNA concentration and gene methylation might be useful for detection of breast cancer. However, unsatisfactory sensitivity and NLR of single-gene methylation analyses indicated that single-gene methylation analysis in breast cancer cfDNA was not robust enough, as with the results of Liao et al. [40]. If using quantitative or multiple-gene panel analyses, the accuracy of circulating cfDNA could be improved. It is believed that, with the development of new technologies, cfDNA as a tool for detection of breast cancer would be more practical.

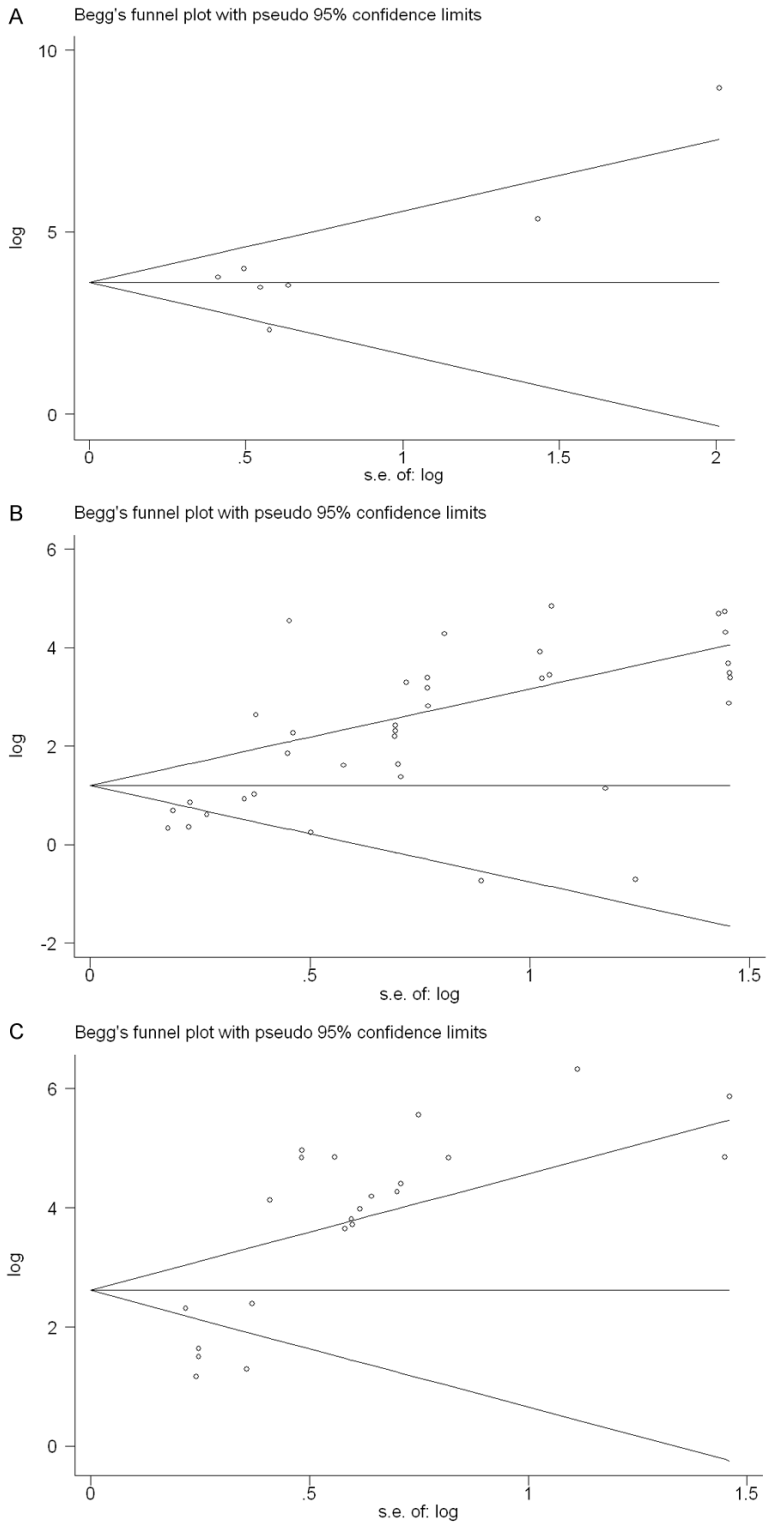

Figure 8. Funnel graphs for assessment of potential publication bias in the quantitative analysis single-gene and multiple-gene panel indicators subgroups. A. Funnel plots for the subgroup of quantitative analysis. B. Funnel plots for the subgroup of single-gene analysis. C. Funnel plots for the subgroup of multiple-gene panel analysis. The funnel graph plots the log of the DOR against the SE of the log of the DOR (an indicator of sample size). The line in the center indicates the summary diagnostic odds ratio.

Overall evidence suggests that circulating cfDNA concentration and gene methylation might be used in breast cancer detection since the assay has a relatively high level of diagnostic accuracy. However, the results of single-gene methylation analysis lacked robustness. Thus, extra caution should be applied if only the single-gene methylation detection assay is used for breast cancer diagnosis. Evaluation of circulating cfDNA with quantitative and multiple-gene methylation analyses might improve breast cancer early diagnosis.

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Disclosure of conflict of interest

None.

Abbreviations

cf DNA, circulating cell-free DNA; MSP, methylation-spe-

cific polymerase chain reaction; CI, Confidence interval; DOR, Diagnostic odds ratio; AUC, Area under the curve; IFN, Interferon; PLR, Positive likelihood ratio; NLR, Negative likelihood ratio; QUADAS, Quality assessment for studies of diagnostic accuracy; SROC, Summary receiver operating characteristic; ROC, Receiver operating characteristic; RDOR, Relative diagnostic odds ratio; RQ-PCR, Real-time quantitative PCR.

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Supplementary Table 1. Studies reporting on the application of cfDNA analysis in the subgroup of quantitative analysis

Author	Year	Test result				No. of P/C
		TP	FP	FN	TN	
Agostini	2012	39	0	0	49	39/49
Hashad	2012	34	8	8	19	42/27
Wu X	2015	43	10	4	32	47/42
Liu LR	2009	68	16	12	121	80/137
Jin Y	2007	115	7	14	46	129/53
Huang CH	2007	79	43	0	58	79/101
Li JH	2010	61	5	17	45	78/50

No. of P/C, number of patients and control; TP, true positive; FP, false positive; FN, false negative; TN, true negative.

Supplementary Table 2. Studies reporting on the application of cfDNA analysis in the subgroup of single-gene indicators analysis

Author	Year	Gene	Test result				No. of P/C
			TP	FP	FN	TN	
Vera Kloten	2013	ITIH5	27	6	85	96	112/102
		DKK3	37	1	75	101	112/102
Limei Liu	2015	FHIT	30	6	15	15	36/30
Ming Shan	2016	hMLH1	75	35	193	210	268/245
		RASSF1a	46	25	222	220	268/245
		P16	60	41	208	204	268/245
		PCDHGB7	149	116	119	129	268/245
		SFN	197	143	71	102	268/245
		HOXD13	37	6	231	239	268/245
Zibo Li	2016	EGFR	70	41	16	26	86/67
Zibo Li		PPM1E	65	37	21	30	86/67
Mary Jo Fackle	2014	AKR1B1	29	0	28	55	57/55
		ARHGEF7	13	0	44	55	57/55
		COL6A2	21	1	36	54	57/55
		GPX7	15	0	42	55	57/55
		HOXB4	23	0	34	55	57/55
		RASGRF2	27	2	30	53	57/55
		RASSF1A	40	1	17	54	57/55
		TM6SF1	22	2	35	53	57/55
		TMEFF2	12	0	45	55	57/55
		HIST1H3	30	2	27	53	57/55
Maria Chimonidou	2013	SOX17	43	1	71	48	114/49
Maria Chimonidou	2012	CST6	14	0	59	37	73/37
Ramin Radpour	2011	APC	18	3	18	27	36/30
		BIN1	19	3	17	27	36/30
		BMP6	11	3	25	27	36/30
		BRCA1	27	3	9	27	36/30
		CST6	20	3	16	27	36/30
		ESR-b (ER beta)	11	3	25	27	36/30
		GSTP1	13	3	23	27	36/30

		P16 (CDKN2A)	18	3	18	27	36/30
		P21 (CDKN1A)	32	3	4	27	36/30
		TIMP3	13	3	23	27	36/30
Jennifer D. Brooks	2010	RASSF1A	11	9	39	41	50/50
		GSTP1	2	4	48	46	50/51
		APC	1	2	49	48	50/52
		RARβ2	3	1	47	49	50/53
Jo-Heon Kim	2010	RAR-β	103	8	16	117	119/125
		Twist	65	10	54	115	119/125
		RASSF1A	39	6	80	119	119/125
		HIN-1	36	0	83	125	119/125
Feng Jing	2010	RASSF1A	37	3	13	47	50/50
De-yuan Fu	2014	Sox17	53	0	33	36	86/36

No. of P/C, number of patients and control; TP, true positive; FP, false positive; FN, false negative; TN, true negative.

Supplementary Table 3. Studies reporting on the application of cfDNA analysis in the subgroup of multiple-gene panel indicators analysis

Author	Year	Multiple-gene panel	Test result				No. of P/C
			TP	FP	FN	TN	
Vera Kloten	2013	ITIH5/DKK3/RASSF1A	92	49	46	125	138/174
		ITIH5/DKK3	55	10	83	164	138/174
		RASSF1A/DKK3	82	43	56	131	138/174
		RASSF1A/ITIH5	75	47	63	127	138/174
Feng Jing	2010	RASSF1A/BRCA1/RARβ2/CDH1	45	6	5	44	50/50
		RASSF1A/BRCA1/CDH1	44	6	6	44	50/50
		RASSF1A/BRCA1/ER/CDH1	44	6	6	44	50/50
		RARβ2/BRCA1/APC/CDH1	43	6	7	44	50/50
		APC/DAPK/BRCA1/p16/RARβ2	42	2	8	48	50/50
		RASSF1A/BRCA1/RARβ2	42	3	8	47	50/50
		RASSF1A/BRCA1/RARβ2/ER	42	6	8	44	50/50
		RASSF1A/BRCA1	41	3	9	47	50/50
		RASSF1A/BRCA1/ER	41	5	9	45	50/50
		APC/DAPK/BRCA1/p16	39	0	11	50	50/50
		BRCA1/DAPK/p16	28	0	22	50	50/50
Ming Shan	2016	6-gene panel	201	52	60	156	253/216
Zibo Li	2016	EGFR + PPM1E	67	33	19	34	86/67
Mary Jo Fackle	2014	10-gene panel	52	1	5	54	24/28
Jo-Heon Kim	2010	RAR-β/RASSF1A	112	14	7	111	119/125
		RAR-β/HIN-1	108	8	11	117	119/125
		RAR-β/Twist	108	17	11	108	119/125
		3-gene panel	115	23	4	102	119/125
		4-gene panel	117	23	2	102	119/125

No. of P/C, number of patients and control; TP, true positive; FP, false positive; FN, false negative; TN, true negative.