Int J Clin Exp Med 2016;9(5):7729-7742
www.ijcem.com /ISSN:1940-5901/IJCEM0018586

Original Article

The application of RASSF1A promoter methylation as a biomarker in breast cancer: a comprehensive literature review

Hua Geng[1], Longtao Wu[2]

[1]*Tianjin Research Center of Basic Medical Sciences, Tianjin Medical University, Tianjin, P. R. China;* [2]*Department of Surgery, The University of Chicago, Chicago, Illinois, United States of America*

Received October 27, 2015; Accepted March 24, 2016; Epub May 15, 2016; Published May 30, 2016

Abstract: Frequent hypermethylation of RASSF1A promoter has been reported to account for its expression silencing in multiple human malignancies including breast cancer. The methylated RASSF1A was found both in cancer tissues and in paired serum DNA samples of breast cancer patients, but was rare in the serum of non-neoplastic counterparts. In addition, RASSF1A methylation appears to correlate with poor prognostic features and impaired survival of cancer patients, thus indicating RASSF1A promoter methylation could serve as a useful and valuable biomarker for cancers. We made a comprehensive systemic review of publications in the past 15 years. With the meta-analysis and identification of outlier studies that significantly contributed to between-study heterogeneity, we reach the following conclusion: 1) Methylation of RASSF1A promoter could happen in adjacent normal samples. And it should be cautious to use well-validated adjacent normal samples as reference in study the methylation of RASSF1A in breast cancer patients. 2) Methylation of RASSF1A promoter is an early event in breast cancer development. And most importantly, its methylation remains constant across all stages during breast cancer development. 3) Methylation of RASSF1A promoter is positively associate with ER and PR status, but not with HER2 and LN status. That is, the methylation of RASSF1A promoter is lower in triple-negative subtype of breast cancer. 4) RASSF1A methylation in body fluid including serum and nipple fluid is usefully but with limited sensitivity. 5) Peripheral blood leukocytes or white blood cell genomic DNA is not suitable to be used as control for RASSF1A promoter methylation in breast cancer patients. Through the systemic review of the RASSF1A promoter methylation studies, we not only summarize the current discoveries; also, we pinpoint the pitfalls in the application of RASSF1A as biomarker for diagnosis or prognosis purposes.

Keywords: RASSF1A, promoter methylation, epigenetics, breast cancer

Introduction

Breast carcinoma is a multi-factorial disease and becomes the most common malignancy among women worldwide [1]. Carcinogenesis is a multi-step process with the accumulation of numerous genetic and epigenetic changes involving oncogenes and tumor suppressor genes. The molecular differences may lead to distinct clinical outcomes, such as tumor progression, recurrence and response to treatment. Although genetic alterations play a critical role in tumor initiation and progression, the germline mutations of breast cancer susceptibility genes, including BRCA1, BRCA2, and TP53, account for only less than 25% of excess risk [2, 3]. Recent data implicated epigenetic events, including DNA methylation and chromatin changes, may be in part responsible for tumor initiation, progression, metastasis and patient survival as well [4].

Ras association domain family protein 1A (RASSF1A) is located at 3p21.3, a highly allelic loss chromosomal segment in a variety of cancers, and proposed as a putative tumor suppressor gene. RasGTPases are a superfamily of molecular switches that regulate cell growth, cell cycle, differentiation and survival. As a negative effector of Ras, RASSF1A plays a crucial role in Ras-related pro-apoptotic signalling [5]. In the last decade, several studies found that

RASSF1A expression was reduced in both human cancer cell lines and primary tumors, including breast cancers. Subsequently, the frequent hypermethylation of RASSF1A promoter has been reported to account for its expression silencing in multiple human malignancies, rather than genetic events such as somatic mutation or deletion. The methylated RASSF1A was found both in cancer tissues and in paired serum DNA samples of breast cancer patients, but was rare in the serum of non-neoplastic counterparts [6]. In addition, RASSF1A methylation appears to correlate with poor prognostic features and impaired survival of cancer patients, thus indicating RASSF1A promoter methylation could serve as a useful and valuable biomarker for cancers [7-9].

As an ideal biomarker could be easily detectable and obtained from patients by non-invasive techniques, analyses of specific DNA methylation in patient sera might be a useful tool of prognostic prediction and monitoring the outcome of patient treatment. Recent advances in technologies for high-throughput genome-wide DNA methylation analyses have facilitated epigenetic profiling of human malignancies. Among them, RASSF1A methylation appears to be an independent predictor of poor prognosis in several breast cancer study cohorts [10]. However, there is still a great deal of controversy regarding whether RASSF1A methylation is an unfavourable prognostic factor of breast cancer, probably due to case enrolment criteria, cohort size, sample type, methylation analysis methods and different statistical models. Thus, we performed a literature-based meta-analysis and aimed to give a comprehensive evaluation of the prognostic impacts of RASSF1A methylation on survival of breast cancer patients, especially analysing the data obtained from sera and tissue samples separately. We found RASSF1A methylation would be worth of further development as a biomarker for prognostic prediction of breast cancer, but translating this finding into clinical practice still needs large-scale clinical estimation.

Materials and methods

Publication selection

A comprehensive literature search was carried out by two independent reviewers (LW and HG) using the PubMed, Web of Science and Highware databases. The search ended on 1st Jan 2015. The following keywords were used in alone or various combinations: 'breast cancer', 'biomarkers', 'methylation', 'RASSF1', 'RASSF1A'. We also used a manual reference search for relevant studies, including all original articles, reviews, letters, and commentaries with no language restrictions. Abstracts were excluded because of insufficient data for meta-analysis. To be eligible for inclusion, studies should meet the following criteria: (1) studies measured RASSF1A methylation status using semi-quantitative or quantitative methods; (2) studies investigated the relationship between *RASSF1A* methylation and breast cancer risk with sufficient and detailed data for meta-analysis; (3) if the same patient population reported in several publications, only the most recent or most complete one was included to avoid overlapping among cohorts.

Data assessment

Data retrieved from the publications included first author name, year of publication, patient sources, population size, sample type, disease stage, histology, ER/PR status, LN metastatic status, and methylation analysis methods (Supplementary Table 1). The eligible publications selected for meta-analysis were evaluated independently by two reviewers (HG and LW), and consensual agreement about information regarding extracted data was made after comprehensive discussion between the two investigators.

Statistical analysis

Relative risk (RR) with 95% confidence interval were used to evaluate the associations between the RASSF1A methylation and breast cancer risks in both tissue and sera samples, patients ER and PR status, as well as the LN metastatic status. A χ^2 based Q statistic test was performed to assess the between study heterogeneity. If $P<0.10$ of the heterogeneity test, the pooled RRs were analysed using random-effects model, otherwise the fixed-effects model was applied. Heterogeneity between the studies was tested using Q-statistics. It was considered statistically significant if p value less than 0.10 and was also quantified using the I^2 metric ($I^2<25\%$, no heterogeneity; $I^2=25$-50%, moderate heterogeneity; and $I^2>50\%$, strong heterogeneity). If the heterogeneity was

existed, we used a random-effects model in place of a fixed-effects model. Publication bias was examined with funnel plots and Egger's linear regression test. All statistical analyses were performed using Stata10.0 software, College Station TX.

Results

Study selection and characteristics

Ninety-two relevant publications (from Jan 2001 to Dec 2014) were retrieved for initial review using search strategies as described previously. Of these, twenty-nine publications (refs) were excluded due to the following reasons: (1) Detail datacan not be extracted for meta-analysis (13 refs); (2) Patients could not be well defined as categories for analysis (6 refs); (3) Independent experiment design that could not be comparable with other studies (3 refs); (4) Methods discussion only (3 refs); (5) Studies using peripheral blood leukocyte DNA for methylation analysis that could not be comparable with tissue/serum DNA methylation studies (2 refs); (6) Study that not cancer risk related (1 ref); (7) Overlapping patient cohort (1 ref). Ultimately, the remaining 63 studies comprising 5727 patients were eligible for final meta-analysis (Supplementary Table 1). The eligible studies were conducted in multiple countries or regions. DNA methylation status of RASSF1A promoter was assessed in tumor tissues or patient sera by MSP or quantitative MSP methods. ER and PR status could be extracted from 13 and 9 studies, respectively. Patient LN metastatic status was available in 8 studies and extracted for meta-analysis. Funnel plot and Egger's linear regression test were performed in each study groups and results provided no evidence of overt publication bias (data not shown).

Methylation of RASSF1A promoter in adjacent normal samples causes heterogeneity

As the canonical source for determining tumor suppressor gene promoter methylation status, the genomic DNA from solid tissues of breast cancer patients provides the most accurate and reliable reference data. Therefore, we first analyze the methylation of RASSF1A in studies involving the comparison between normal breast tissue and primary tumor samples, totally 28 studies were included in this analysis. Surprisingly, although the meta-analysis clearly shows that RASSF1A is methylated more in primary tumor tissues against normal breast tissues, the overall relative risk (RR=1.184, 95% CI=1.093 to 1.282, P<0.001) with fixed effect model is pretty marginal (Supplementary Figure 1). As the forest plot suggests the possibility of heterogeneity, the I^2 statistic was examined, which is the percentage of between-study heterogeneity that is attributable to variability in the true treatment effect, rather than sampling variation. Here there is substantial between-study heterogeneity with the fixed effect model (P<0.0001). Even, there is strong evidence showing that RASSF1A is highly methylated in breast cancer patients; however, the presence of between-study heterogeneity means that the fixed-effect assumption (that the true treatment effect is the same in each study) is incorrect. Random effect model is used as alternative to avoid this problem, and the relative risk (RR=8.368, 95% CI=3.718 to 18.834, P<0.001) with random effect model is significantly improved (Supplementary Figure 2) and consistent with the observation from most of individual study.

Since significant between-study heterogeneity was detected with both fixed and random effect models, we take a further step to investigatepotential cause of such heterogeneity. Interestingly, in 25 out of the total 28 studies, the methylation of RASSFA1 promoter in health or adjacent normal tissues ranges from 0 to 20%, with an average of 13.26% (95% CI=3.88% to 22.64%). In contrast, the average methylation rate in thesecorresponding primary tissue samples is 69.56% (95% CI=62.7% to 76.42%). However, for the rest three studies, RASF1A promoter methylation pattern is rather different. Park SY et al [11] showed a relative high proportion methylation of RASSF1A (40%) in normal tissue. The methylation of RASSF1A promoter in the health samples of the other two studies is even higher. Yeo W et al [12] reported that 92.5% of normal breast was methylated at RASSF1A promoter, and Jeronimo C et al [13] revealed that weak but positive methylation of RASSF1A was observed in 12 out of 12 (100%) health group. The weight from each study is Yeo W 2005, 49.59%; Jeronimo C 2008 (39.93%) and Park SY 2011 (3.14%).

After removal of these three outlier studies, the overall estimation of methylation in tumor

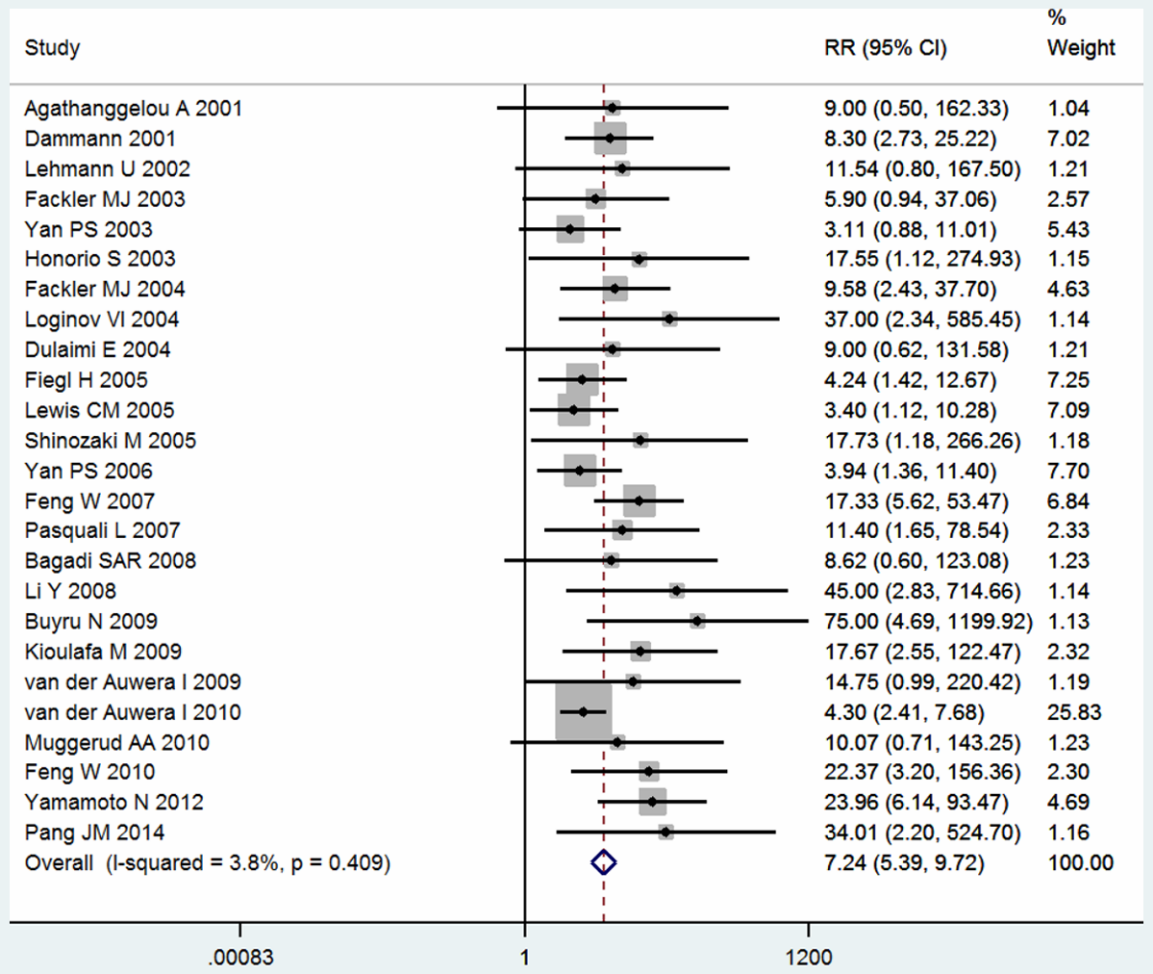


Figure 1. Meta-analysis shows that RASSF1A is methylated more in primary tumor tissues against normal breast tissues, with fixed effect model and removal of three outlier studies.

patients is significantly improved (**Figure 1**). The relative risk estimation from fixed effect model (RR=7.242, 95% CI=5.394 to 9.724, P<0.001) is close to the result with random effect model. Most importantly, heterogeneity between studies was not significant (*P*=0.409).

As high percentage of RASSF1A methylation is observed in multiple independent groups as well as different detection methods.It is highly possible that the methylation of RASSF1A occurred even in adjacent pathological normal tissues in breast cancer patients, which significantly contributes to the between-study heterogeneity observed. These analyses show that RASSF1A promoter is highly methylated in primary breast cancer samples. And it should be cautious to use well-validated “adjacent normal samples”.

Methylation of RASSF1A promoter is an early event in breast cancer development

To investigate whether there is a progressive RASSF1A promoter methylation at different tumorigenesis stages, that is, higher methylation in advanced stage of breast cancer patients, we analyzed the methylation status of RASSF1A promoter in a collection of studies with data available from different cancer stages.

Firstly, we test whether RASSF1A is methylated more in primary tumor tissues than benign samples. Totally, we collected 7 studies that measured the methylation of RASSF1A in both benign and tumor samples. The average methylation rate in benign samples is 67.64% (95% CI=48.7% to 86.58%). For primary tumor sample the average methylation rate is 78.2% (95%

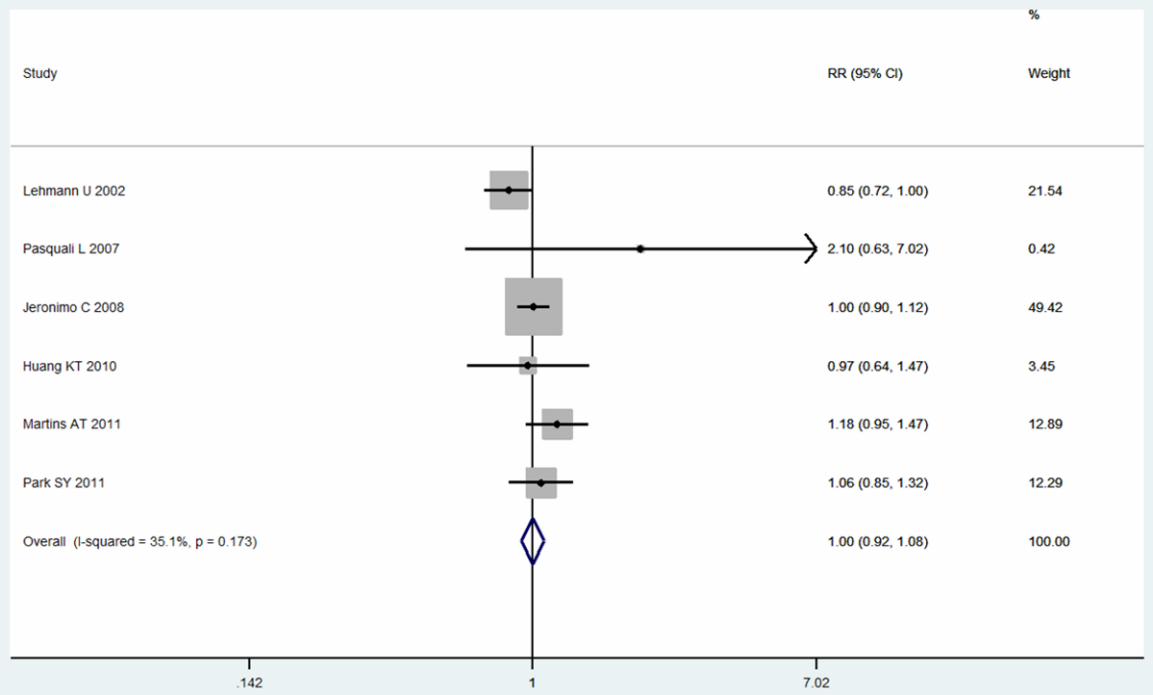


Figure 2. Meta-analysis result with the fixed model shows there is no significant difference between benign and primary tissues in RASSF1A promoter methylation status.

CI=66.42% to 89.98%). Interestingly, the meta-analysis result with the fixed model shows there is no significant difference (RR=1.039, 95% CI=0.963 to 1.120, *P*=0.321) between benign and primary tissues (Supplementary Figure 3). Since the fixed effect model also suggests significant heterogeneity (*P*<0.001), the null hypothesis of fixed model is incorrect, so, alternative random effect model was used. The random effect model also shows there is no difference (RR=1.123 95% CI=0.916 to 1.376, *P*=0.266) between benign and primer tumor tissues (Supplementary Figure 4).

Since significant between-study heterogeneity was also identified in this set of studies, we sought out to explore which study contributed-to such heterogeneity. We then remove study one by one from our analysis, and find that the removal of any study will not affect the heterogeneity status (*P*<0.0001), except Kim JH et al [14]. This study profiled the RASSF1A promoter methylation in borderline and malignant phyllodes tumors, which are rare tumors characterized by myoepitehlial and luminal epithelial component arranged in clefts surrounded by an overgrowing hypercellular mesenchymal component typically organized in leaf-like structure. After removal of this study, the between-study heterogeneity is not significant (*P*=0.173) any more. Then, we applied the fixed effect model to the rest studies, and the relative risk from this model is 0.997 (95% CI=0.922 to 1.078, *P*=0.941). So, RASSF1A promoter is highly methylated in both benign and primary breast cancer tissues (**Figure 2**), and there is no significant difference.

Another important question associated with the using RASSF1A promoter methylation as a potential biomarker for breast cancer diagnosis is whether it is methylated more in patients of advanced infiltration stage tumors. Therefore, we collected 6 studies involving both local and invasive breast cancer samples. The average methylation rate in localized breast cancer tissues is 69.93% (95% CI=52.33% to 87.53%). While for invasive samples, the average methylation level is 75.52% (95% CI=68.51% to 82.53%). We then use fixed model to compare RASSF1A promoter methylation in these two types of tumor tissues. However, meta-analysis result reveals no significant difference (RR=0.981, 95% CI=0.879 to 1.095, *P*=0.736) between localized samples and invasive samples (**Figure 3**). Also, no significant between-

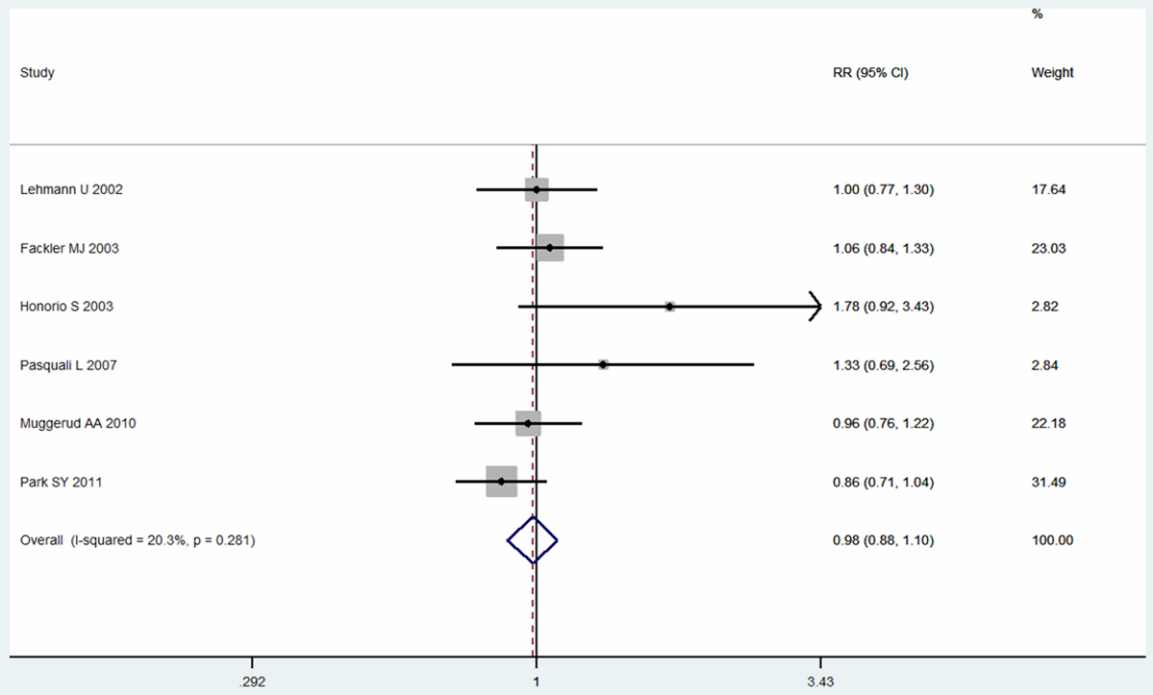


Figure 3. Meta-analysis result with the fixed model shows there is no significant difference between localized samples and invasive samples in RASSF1A promoter methylation status.

study heterogeneity detected (*P*=0.281), which suggesting the consistent results from all studies.

Finally, we asked the question whether the methylation of RASSF1A promoter is different between primary breast cancer tissues and corresponding distant metastatic tissues. Therefore, we collected 4 studies involving both primary and metastatic breast cancer tissues. The average methylation rate in primary tissues is 65.27% (95% CI=55.3% to 75.25%), and in distant metastatic samples, the average methylation rate of RASSF1A promoter is slightly lower (62.94%, 95% CI=57.97% to 67.92%). We then use fixed model to compare these two types of tumor samples. The meta-analysis result shows no significant difference (RR= 1.091, 95% CI=0.888 to 1.340, *P*=0.408) between primary tissues and distant metastatic tissues (Supplementary Figure 5). Also, no significant between-study heterogeneity was detected (*P*=0.491).

Therefore, with a serial of comprehensive comparisons between different tumor types or stages, the meta-analysis suggests that methylation of RASSF1A promoter is an early event as it is highly methylated in benign tissues. However, its methylation remains constant across all stages during breast cancer tumorigenesis.

Methylation of RASSF1A promoter is associated with clinical characteristics

Triple-negative breast cancer refers to these breast cancers that does not express the genes for estrogen receptor (ER), progesterone receptor (PR) or human epidermal growth factor receptor 2 (HER2). The absence of hormone receptors makes it more difficult to treat since most chemotherapies target either one of the three receptors, so triple-negative cancers often require combinatorial therapies [15]. Triple-negative breast cancers have a relapse pattern that is very different from these positive breast cancers. Therefore, it would be interesting to investigate the association between RASSF1A promoter methylation and hormone status. We collected 13 samples with complete record of hormone status or LN status.

We start with the possible association between ER status and RASSF1A promoter methylation, and a collection of 13 studies was analyzed. With the ER positive patients the average meth-

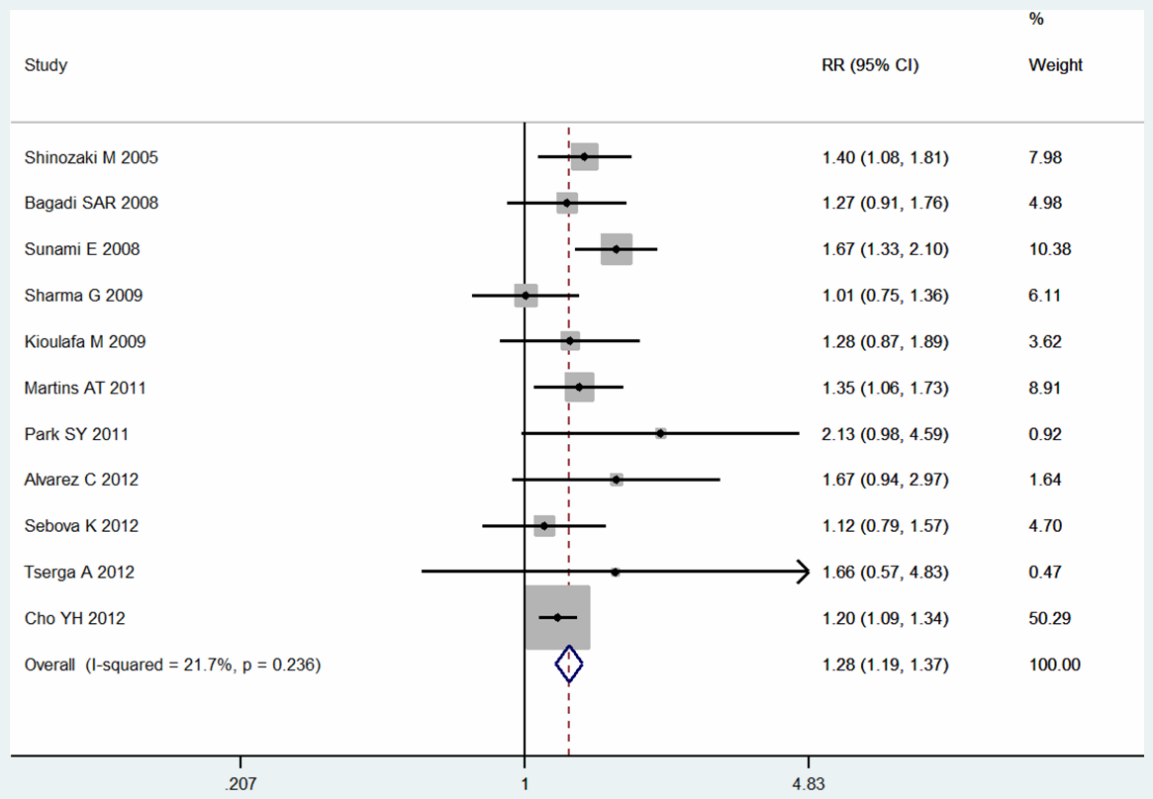


Figure 4. Meta-analysis with fixed effect model shows significant difference between ER positive and negative patients in RASSF1A promoter methylation status.

ylation rate of RASSF1A promoter is 75.96% (95% CI=66.5% to 85.42%). And the ER negative patients the methylation of RASSF1A is on average 60.48% (95% CI=50.95% to 70.01%). We then use fixed effect model to compare these two types of ER+/ER- samples. Meta-analysis result (Supplementary Figure 6) suggests significant difference between ER positive and negative patients (RR=1.185, 95% CI=1.109 to 1.266, *P*<0.001). Since the fixed effect model also revealedthe presence of heterogeneity (*P*=0.001), alternative random effect model was used. The random effect model also shows there is significant difference (RR=1.221, 95% CI=1.060 to 1.406, *P*=0.006) between ER positive and negative patients (Supplementary Figure 7).

Karray-Chouayekh S et al [16] reported the aberrant methylation of RASSF1A is associated with poor survival in Tunisian breast cancer patients. And Rasti M et al [17] discussed the association between RASSF1A promoter and clinicopathological features of breast cancer in Iranian patients. Therefore, the strong heterogeneity that contributed by these two studies may reflect the regional difference of breast cancer patients. After removing them, weapplied fixed effect model to evaluatethe methylation of RASSF1A promoter in ER+ and ER- patients. And meta-analysis result indicates there is indeed significant difference (RR=1.277, 95% CI=1.186 to 1.375, P<0.001) between ER positive and negative patients (**Figure 4**). RASSF1A promoter is hypermethylated more in ER+ patients than ER- patients. Also, no significant between-study heterogeneity detected (*P*=0.236), which suggesting the consistent results from all studies.

Taking the same strategy, we also assess the methylation of RASSF1A promoter in PR+ and PR- patients. With PR positive patients the average methylation rate of RASSF1A promoter is 70.61% (95% CI=59.12% to 82.11%). For the PR negative patients the methylation of RASSF1A is on average 63.83% (95% CI=49.27% to 78.4%). We then use fixed model to compare these two types of tumor samples. Surprisingly unlike the association of RASSF1A

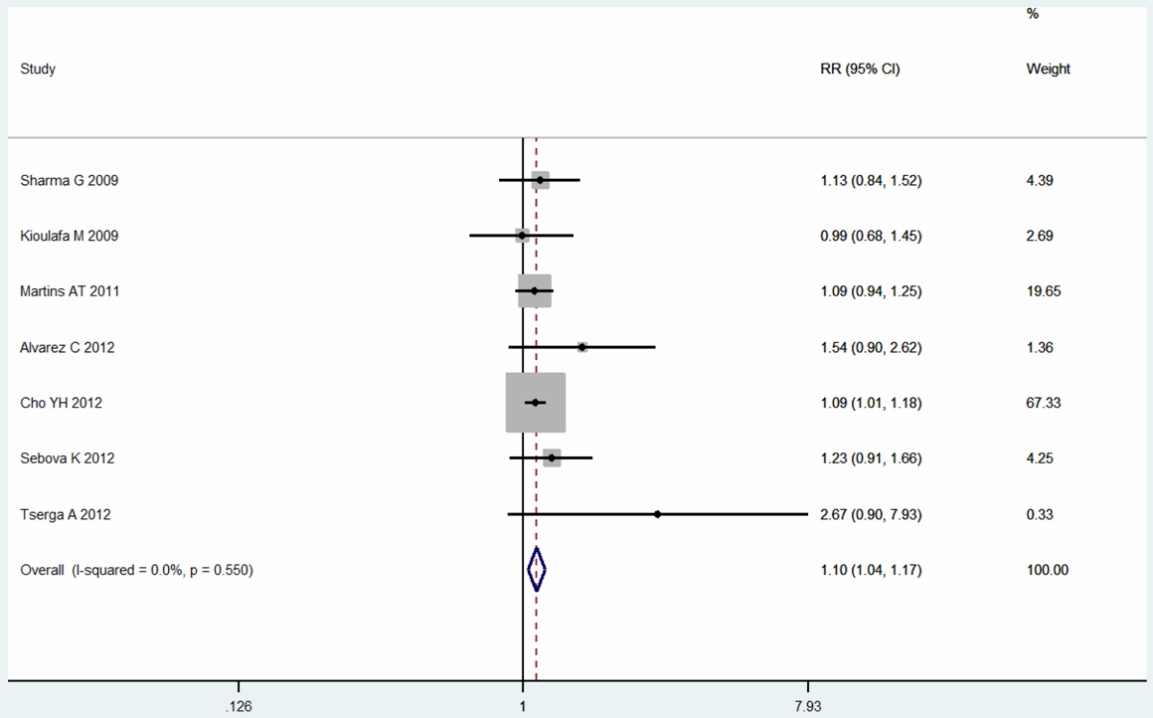


Figure 5. Meta-analysis with fixed effect model shows significant difference between PR positive and negative patients in RASSF1A promoter methylation status.

promoter methylation with ER status, the initial meta-analysis result (Supplementary Figure 8) suggested that there is no significant difference between PR positive and negative patients (RR=1.057, 95% CI=0.998 to 1.119, *P*=0.060). Similar conclusion is also obtained with random effect model (RR=1.050, 95% CI=0.929 to 1.188, *P*=0.434), It looks like noassociation betweenPR status with RASSF1A promoter methylation (Supplementary Figure 9).

Since Karray-Chouayekh S et al and Rasti M et al significantly contributed to the overall heterogeneity in analyzing ER status, which probably reflects the regional or racial difference among breast cancer patients. These two studiesare also removed from the PR status analysis in an effort to reduce between-study heterogeneity. We then apply the fixed model to re-evaluate the association between PR status and RASSF1A promoter methylation. Interestingly, with the re-defined study list, meta-analysis indicates that there is significant difference (RR=1.104, 95% CI=1.037 to 1.175, *P*=0.002) between PR positive and negative patients (**Figure 5**). Also, no significant between-study heterogeneity detected (*P*=0.550), which suggesting the consistent results from all studies.

Finally, we analyzed the association of RASSF1A promoter methylation with HER2 positive or negative patients. The average methylation rate of RASSF1A promoter in PR+ patients is 73.21% (95% CI=54.12% to 92.31%). While for HER2 negative patients the methylation of RASSF1A is on average 69.33% (95% CI= 55.78% to 82.89%). We then use fixed model to compare these two types of tumor samples. Although meta-analysis result (Supplementary Figure 10) suggested significant difference between HER2 positive and negative patients (RR=1.145, 95% CI=1.035 to 1.267, *P*=0.009), and significant heterogeneity (*P*=0.036) was also detected. So, random effect model was used, and analysis with random effect model failed to find any significant difference (RR= 1.101, 95% CI=0.923 to 1.313, *P*=0.284) between HER2 positive and negative patients (Supplementary Figure 11).

The lymph nodes in the underarm (the axillary lymph nodes) are the first place breast cancer is likely to spread. And lymph node status is highly related to prognosis. We collected 8

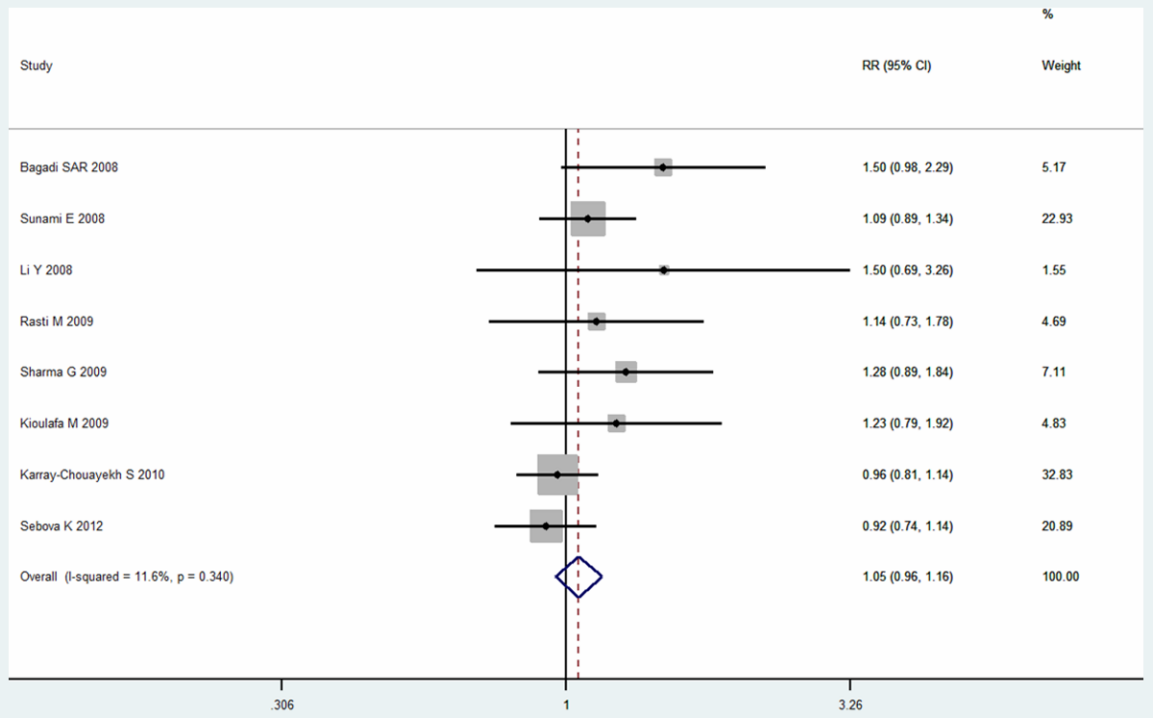


Figure 6. Meta-analysis with fixed effect model shows no significant difference between LN metastatic status positive and negative patients in RASSF1A promoter methylation.

studies involved in the RASSF1A promoter methylation and LN status. With LN positive patients the average methylation rate of RASSF1A promoter is 71.47% (95% CI=63.6% to 79.34%). While the LN negative patients the methylation of RASSF1A is on average 61.61% (95% CI=49.38% to 73.84%). We then use fixed effect model to compare the methylation of RASSF1A in LN+ and LN- patients. And meta-analysis (**Figure 6**) indicates that there isno significant difference between LN positive and negative patients (RR=1.054, 95% CI=0.956 to 1.161, *P*=0.291). Also, no significant between-study heterogeneity detected (*P*=0.340), which suggesting the consistent results from all studies.

Through a serial of comparisons between different tumor types, we made a comprehensive association analysis of RASSF1A promoter with various clinical characteristics. The meta-analysis reveals that methylation of RASSF1A promoter is associate with ER and PR status, but not with HER2 and LN status.

RASSF1A methylation in body fluid is usefully but with limited sensitivity

Changes in the status of DNA methylation, known as epigenetic alterations, are one of the most common molecular alterations in human neoplasia, including breast cancer. The presence of abnormally high DNA concentrations in the serum of breast cancer patients has been recorded long time ago [18-20]. The discovery that cell-free DNA can be shed into the bloodstream has generated great interest. Lots of studies have suggested that tumor-specific alterations in DNA recovered from plasma or serum of patients (or other body fluid), and that these circulating nucleic acids may represent potential biomarkers for molecular diagnosis and prognosis.

To investigate whether circulating DNA from various body fluid including serum, nipple fluid, as well as bone marrow, peripheral blood leukocyte (PBL) and milk. We analyzed a collection of studies involvedmeasuring the methylation of RASSF1A promoter in various the body fluid samples from both health and tumor patients.

Firstly, we tested whether genomic DNA from patient serum is as reliable as these from solid tumor tissues (the canonical reference). Totally, we collected 14 studies that investigated the methylation of RASSF1A in serum from both health and tumor patients. The average methylation rate in health donor serum is 4.59%

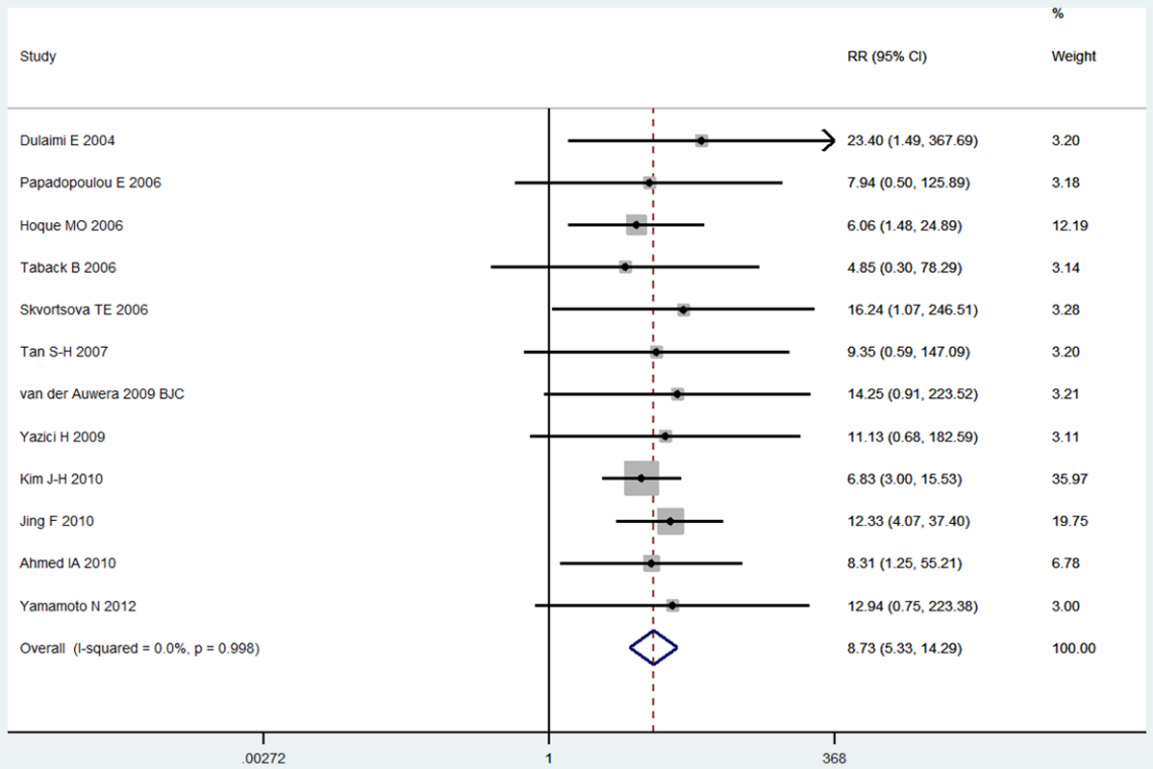


Figure 7. Meta-analysis shows that RASSF1A is methylated more in primary tumor patients' serum against normal or health donors' serum, with fixed effect model.

(95% CI=0.83% to 8.36%). For tumor patient serum the average methylation rate is 38.31% (95% CI=26.84% to 49.79%). Consistent with the result usinggenomic DNA from solid tumor tissues, the meta-analysis result with the fixed model shows there is significant difference (RR=3.509, 95% CI=2.446 to 5.035, *P*<0.001) between health and tumor patients (Supplementary Figure 12). Since the fixed effect model also suggests significant heterogeneity (*P*=0.004), the null hypothesis of fixed model is incorrect. So, alternative random effect model was used. The random effect model also shows there is significant difference (RR=5.704, 95% CI=2.815 to 11.555, *P*<0.001) between health and tumor patients' serum (Supplementary Figure 13).

Since significant between-study heterogeneity was detected with both fixed and random effect models, we take a further step to investigate potential cause of such heterogeneity. And we find that two studies significantly contributed to the overall heterogeneity. Matuschek C et al [21] and Brooks JD [22] had reported unusual high level of RASSF1A promoter methylation in health donors (22.7% and 17.2% respectively). Therefore, these two studies were removed from our list. We then apply fixed model to evaluate the relative risk of RASSF1A promoter methylation in patient's serum. And meta-analysis result indicates there is significant difference (RR=8.728, 95% CI=5.331 to 14.290, *P*<0.001) between health and tumor patients' serum (**Figure 7**). Also, no significant between-study heterogeneity detected (*P*=0.998), which suggesting the consistent results from all studies.

As the genomic DNA from solid tissues of breast cancer patients provides the most accurate and reliable data. We collected 6 reports that studied the RASSF1A promoter methylation with paired tumor tissues and serum samples. The mean methylation rate in tissue samples is 82.65% (95% CI=73.65% to 91.66%), while the methylation in corresponding serum is much lower; the average methylation rate is 55.18% (95% CI=31.83% to 78.52%). Since significant heterogeneity (*P*<0.001) was identified in studies, we performed meta-analysis with random effect model, and the analysis (Supplementary

Figure 14) showed that there is no significant difference (RR=1.603, 95% CI=0.814 to 3.155, *P*=0.172) between tissue samples and serum samples, which proved the feasibility of using patient serum as source of DNA to measure RASSF1A promoter methylation.

While the result using patient serum could largely recapitulate the data with solid tumor tissue, the association studies of RASSF1A promoter methylation with various clinical features is not conclusive. The association of RASSF1A methylation with ER status (Supplementary Figure 15) was not significant (RR=1.292, 95% CI=0.712 to 2.344, *P*=0.400). The association of RASSF1A methylation with ER PR status (Supplementary Figure 16) is also not significant (RR=1.065, 95% CI=0.692 to 1.638, *P*=0.776).

Beside serum, several studies have attempted to use nipple fluid [23-25] as the source of genomic DNA to interrogate RASSF1A promoter methylation. Interestingly, with random effect model (RR=1.412, 95% CI=0.465 to 4.286, *P*=0.542), no significant difference between nipple fluid and solid tumor tissue samples was detected (Supplementary Figure 17), which proved the potential feasibility of using patient nipple fluid as source of DNA to measure RASSF1A promoter methylation. However, due to small sample size, the sensitivity of using nipple fluid still need more data to support the conclusion.

Through a serial of comparisons of the RASSF1A methylation level with different origin of DNA, including serum and nipple fluid, we proved the feasibility of using circulating DNA from various body fluids to measure RASSF1A promoter methylation.

Discussion and conclusion

Accurately screening women with increased risk of developing breast cancer will significantly facilitate early detection and prevention of this fetal disease. And there have been more and more accumulating evidences showing that epigenetic defects in breast cancer may be potentially used as prognostic biomarker with cancer progression. Lots of efforts have been made in searching for potential epigenetic biomarkers with high sensitivity and specificity. Especially, we are interested in these candidate genes, which could be used as a prognostic indicator for breast cancer.

Ideally, the promoter methylation correlated with the progression of breast cancer will be of greater clinical application value. The epigenetic methylation of RASSF1A promoter has been one example of these earliest applicationsthat measured the DNA methylation level and correlated it with both disease free survival and overall survival in female breast cancer [10]. In the past 15 years, the exploration of using RASSF1A promoter methylation in various clinical applications has provide us enough independent study to re-evaluate this important asset. In this study, we make a comprehensive assessment to all past publications. And several important conclusions were reached, and potential pitfalls wereidentified.

Although the dense promoter methylation of RASS1F1A gene in breast cancer has been firmly established, we still unexpectedly detected significant heterogeneity the initial meta-analysis. Three outlier studies have significantly raised the question about the feasibility of using RASSF1A as good diagnosis biomarker [7-10]. As high percentage of RASSF1A methylation is observed in multiple independent groups as well as different detection methods. It is highly possible that the methylation of RASSF1A occurred even in adjacent pathological normal tissues in breast cancer patients, which significantly contributes to the between-study heterogeneity observed. Yan et al has reported that a field of methylation changes extending as far as 4 cm from primary tumors [26]. While the methylation of RASSF1A promoter proposed the potential mechanism for local recurrence, it also brings the question that we need to use “adjacent normal tissue” more cautiously. Thus, carefully defining the normal level of RASSF1A promoter methylation will make a major impact about how we could draw the conclusion.

The candidate gene or locus would be ideal if its promoter methylation also shows progressive methylation with the development of breast cancer from local to invasive and metastatic stages. Such epigenetic biomarker will be of great clinical value to be used as predictive factor. To investigate whether RASSF1A promoter methylation meets these criteria, we make a serial of meta-analysis by pairwise

comparison the methylation in health tissue, benign tissue, primary local tissue, primary invasive tissue as well as distant metastatic samples. The meta-analysis results revealed that there is no higher methylation in primary samples than benign tissue. Also, in terms of the RASSF1A promoter methylation, there is no difference across primary, invasive as well as metastatic stages. Therefore, it is well established that RASSF1A is an early epigenetic change in female breast cancer. Also, its promoter methylation does not evolve with the progression of breast cancer from benign to local, to invasive as well as metastatic cancer. So, methylation of RASSF1A is not an ideally prognostic biomarker, as it fails to distinguished advanced stages of breast cancer.

There have been several individual reports about the association of RASSF1A promoter methylation with various clinical risk factors *i.e.,* age, tumor grade, stage, and ER/PR/HER2 status with methylation status of RASSF1A promoter. However, the conclusion is quite controversial [27-29]. While several publications showing the methylation correlated with ER+/PR+, negative report also exists [30]. Therefore, we make further meta-analysis of published data about the association of ER, PR, HER2 as well as LN status with RASSF1A promoter methylation. Interestingly, our analysis clearly shows that the methylation is higher in ER positive patients (75.96%) than the ER negative patients (60.48%). Also it is higher in PR positive patients (70.61%) than the PR negative patients (63.83%). And no association of RASSF1A promoter methylation with HER2 was found. This is quite opposite to some reports that RASSF1A is highly methylated in triple negative breast cancer, which was one of the most aggressive and refractory form of breast cancer. Again, our data shows that the promoter methylation is not associated with aggressive stage/group of breast cancer.

DNA methylation analysis has been one of the rapidly developing field, however, a reproducible epigenetic blood-based assay for diagnosis and follow-up of breast cancer has yet to be successfully. Although serum is readily accessible for molecular diagnosis in all individuals from a peripheral blood sample, the percentage of samples tested that shows a methylation response for any specific gene is highly variable. One of the possible reasons is at least in part the result of the different sensitivities and the difference of the various methods used to measure methylation.

With our meta-analysis using solid tissue as reference, it was shown that there is no significant difference between tissue samples and serum samples as useful biomarker for early diagnosis, which proved the feasibility of using patient serum as source of DNA to measure RASSF1A promoter methylation. However, it was noteworthy that 1) the average methylation level in serum (55.18%) is much lower than paired tissue samples (82.65%). 2) There is significant difference with the fixed model (RR=1.367, 95% CI=1.205 to 1.552, $P<0.001$), though the difference is not significant with random effect model. 3) While the result using patient serum could largely recapitulate the data with solid tumor tissue, the association studies of RASSF1A promoter methylation with various clinical features is not conclusive. The same problems are also observed with nipple fluid samples. The problems raise the concern about the sensitivity of detecting epigenetic biomarker in body fluid.

Other sources of body fluid have been reported such as bone marrow [31, 32], peripheral blood leukocyte [33, 34], even milk [35, 36]. However, these samples either show quite low sensitivity or not suitable for breast cancer. Especially, since peripheral blood leukocytes do not share any same origin with breast cancer cells, and genome DNA from these cells has shown no any methylation in either health donor or tumor patients. Therefore, unusual methylation conclusion could be drawn based on the methylation from peripheral blood leukocytes or white blood cells [33, 34].

In summary, our comprehensive systemic reviewof related publication has reached the following conclusions: 1) Methylation of RASSF1A promoter could happen in adjacent normal samples. And it should be cautious to use well-validated adjacent normal samples as reference in study the methylation of RASSF1A in breast cancer patients. 2) Methylation of RASSF1A promoter is an early event in breast cancer development. And most importantly, its methylation remains constant across all stages during breast cancer development. 3) Methylation of RASSF1A promoter is positively asso-

ciate with ER and PR status, but not with HER2 and LN status. That is, the methylation of RASSF1A promoter is lower in triple-negative subtype of breast cancer. 4) RASSF1A methylation in body fluid including serum and nipple fluid is usefully but with limited sensitivity, and their applications, as non-invasive biomarker should be used with cautions. 5) Peripheral blood leukocytes or white blood cell genomic DNA is not suitable to be used as control for RASSF1A promoter methylation in breast cancer patients.

Acknowledgements

This work was kindly supported by grants (81202100) from the National Natural Science Foundation of China, grants (12JCYBJC15600) from Tianjin Research Program of Application Foundation and Advanced Technology.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Hua Geng, Tianjin Research Center of Basic Medical Sciences, Tianjin Medical University, 22 Qi Xiang Tai Road, Tianjin 300070, China. Tel: +86-22-23542068; Fax: +86-22-23542775; E-mail: huahuageng@gmail.com

References

[1] Siegel RL, Miller KD and Jemal A. Cancer statistics, 2015. CA Cancer J Clin 2015; 65: 5-29.

[2] Carraro DM, Koike Folgueira MA, Garcia Lisboa BC, Ribeiro Olivieri EH, Vitorino Krepischi AC, de Carvalho AF, de Carvalho Mota LD, Puga RD, do Socorro Maciel M, Michelli RA, de Lyra EC, Grosso SH, Soares FA, Achatz MI, Brentani H, Moreira-Filho CA and Brentani MM. Comprehensive analysis of BRCA1, BRCA2 and TP53 germline mutation and tumor characterization: a portrait of early-onset breast cancer in Brazil. PLoS One 2013; 8: e57581.

[3] Maxwell KN and Nathanson KL. Common breast cancer risk variants in the post-COGS era: a comprehensive review. Breast Cancer Res 2013; 15: 212.

[4] Pouliot MC, Labrie Y, Diorio C and Durocher F. The Role of Methylation in Breast Cancer Susceptibility and Treatment. Anticancer Res 2015; 35: 4569-4574.

[5] Pefani DE and O'Neill E. Safeguarding genome stability: RASSF1A tumor suppressor regulates BRCA2 at stalled forks. Cell Cycle 2015; 14: 1624-1630.

[6] Hesson LB, Cooper WN and Latif F. The role of RASSF1A methylation in cancer. Dis Markers 2007; 23: 73-87.

[7] Gao T, Wang S, He B, Pan Y, Song G, Gu L, Chen L, Nie Z, Xu Y and Li R. The association of RAS association domain family Protein1A (RASSF1A) methylation states and bladder cancer risk: a systematic review and meta-analysis. PLoS One 2012; 7: e48300.

[8] Pan J, Chen J, Zhang B, Chen X, Huang B, Zhuang J, Mo C and Qiu S. Association between RASSF1A promoter methylation and prostate cancer: a systematic review and meta-analysis. PLoS One 2013; 8: e75283.

[9] Shi H, Li Y, Wang X, Lu C, Yang L, Gu C, Xiong J, Huang Y, Wang S and Lu M. Association between RASSF1A promoter methylation and ovarian cancer: a meta-analysis. PLoS One 2013; 8: e76787.

[10] Jiang Y, Cui L, Chen WD, Shen SH and Ding LD. The prognostic role of RASSF1A promoter methylation in breast cancer: a meta-analysis of published data. PLoS One 2012; 7: e36780.

[11] Park SY, Kwon HJ, Lee HE, Ryu HS, Kim SW, Kim JH, Kim IA, Jung N, Cho NY and Kang GH. Promoter CpG island hypermethylation during breast cancer progression. Virchows Arch 2011; 458: 73-84.

[12] Yeo W, Wong WL, Wong N, Law BK, Tse GM and Zhong S. High frequency of promoter hypermethylation of RASSF1A in tumorous and non-tumourous tissue of breast cancer. Pathology 2005; 37: 125-130.

[13] Jeronimo C, Monteiro P, Henrique R, Dinis-Ribeiro M, Costa I, Costa VL, Filipe L, Carvalho AL, Hoque MO, Pais I, Leal C, Teixeira MR and Sidransky D. Quantitative hypermethylation of a small panel of genes augments the diagnostic accuracy in fine-needle aspirate washings of breast lesions. Breast Cancer Res Treat 2008; 109: 27-34.

[14] Kim JH, Choi YD, Lee JS, Lee JH, Nam JH, Choi C, Park MH and Yoon JH. Borderline and malignant phyllodes tumors display similar promoter methylation profiles. Virchows Arch 2009; 455: 469-475.

[15] van Rooijen JM, Stutvoet T, Schroder CP and de Vries EG. Immunotherapeutic options on the horizon in breast cancer treatment. Pharmacol Ther 2015; 156: 90-101.

[16] Karray-Chouayekh S, Trifa F, Khabir A, Boujelbane N, Sellami-Boudawara T, Daoud J, Frikha M, Jlidi R, Gargouri A and Mokdad-Gargouri R. Aberrant methylation of RASSF1A is associated with poor survival in Tunisian breast cancer patients. J Cancer Res Clin Oncol 2010; 136: 203-210.

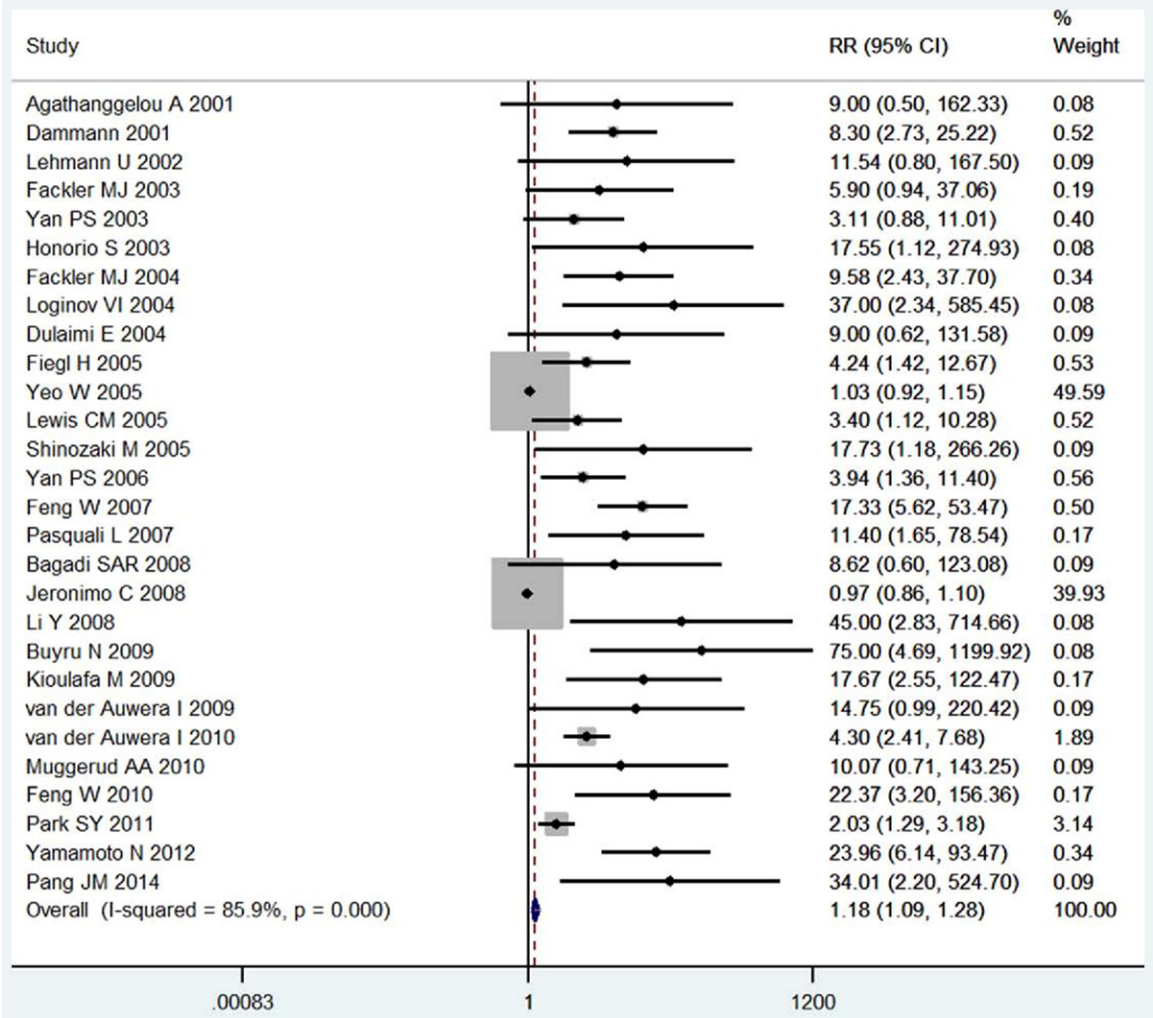
[17] Rasti M, Tavasoli P, Monabati A and Entezam M. Association between HIC1 and RASSF1A

promoter hypermethylation with MTHFD1 G1958A polymorphism and clinicopathological features of breast cancer in Iranian patients. Iran Biomed J 2009; 13: 199-206.
[18] Johnson PJ and Lo YM. Plasma nucleic acids in the diagnosis and management of malignant disease. Clin Chem 2002; 48: 1186-1193.
[19] Van De Voorde L, Speeckaert R, Van Gestel D, Bracke M, De Neve W, Delanghe J and Speeckaert M. DNA methylation-based biomarkers in serum of patients with breast cancer. Mutat Res 2012; 751: 304-325.
[20] Wittenberger T, Sleigh S, Reisel D, Zikan M, Wahl B, Alunni-Fabbroni M, Jones A, Evans I, Koch J, Paprotka T, Lempiainen H, Rujan T, Rack B, Cibula D and Widschwendter M. DNA methylation markers for early detection of women's cancer: promise and challenges. Epigenomics 2014; 6: 311-327.
[21] Matuschek C, Bolke E, Lammering G, Gerber PA, Peiper M, Budach W, Taskin H, Prisack HB, Schieren G, Orth K and Bojar H. Methylated APC and GSTP1 genes in serum DNA correlate with the presence of circulating blood tumor cells and are associated with a more aggressive and advanced breast cancer disease. Eur J Med Res 2010; 15: 277-286.
[22] Brooks JD, Cairns P, Shore RE, Klein CB, Wirgin I, Afanasyeva Y and Zeleniuch-Jacquotte A. DNA methylation in pre-diagnostic serum samples of breast cancer cases: results of a nested case-control study. Cancer Epidemiol 2010; 34: 717-723.
[23] Krassenstein R, Sauter E, Dulaimi E, Battagli C, Ehya H, Klein-Szanto A and Cairns P. Detection of breast cancer in nipple aspirate fluid by CpG island hypermethylation. Clin Cancer Res 2004; 10: 28-32.
[24] Euhus DM, Bu D, Ashfaq R, Xie XJ, Bian A, Leitch AM and Lewis CM. Atypia and DNA methylation in nipple duct lavage in relation to predicted breast cancer risk. Cancer Epidemiol Biomarkers Prev 2007; 16: 1812-1821.
[25] Twelves D, Nerurkar A, Osin P, Dexter T, Ward A, Gui GP and Isacke CM. DNA promoter hypermethylation profiles in breast duct fluid. Breast Cancer Res Treat 2013; 139: 341-350.
[26] Yan PS, Venkataramu C, Ibrahim A, Liu JC, Shen RZ, Diaz NM, Centeno B, Weber F, Leu YW, Shapiro CL, Eng C, Yeatman TJ and Huang TH. Mapping geographic zones of cancer risk with epigenetic biomarkers in normal breast tissue. Clin Cancer Res 2006; 12: 6626-6636.
[27] Alvarez C, Tapia T, Cornejo V, Fernandez W, Munoz A, Camus M, Alvarez M, Devoto L and Carvallo P. Silencing of tumor suppressor genes RASSF1A, SLIT2, and WIF1 by promoter hypermethylation in hereditary breast cancer. Mol Carcinog 2013; 52: 475-487.
[28] Cho YH, Shen J, Gammon MD, Zhang YJ, Wang Q, Gonzalez K, Xu X, Bradshaw PT, Teitelbaum SL, Garbowski G, Hibshoosh H, Neugut AI, Chen J and Santella RM. Prognostic significance of gene-specific promoter hypermethylation in breast cancer patients. Breast Cancer Res Treat 2012; 131: 197-205.
[29] Tserga A, Michalopoulos NV, Levidou G, Korkolopoulou P, Zografos G, Patsouris E and Saetta AA. Association of aberrant DNA methylation with clinicopathological features in breast cancer. Oncol Rep 2012; 27: 1630-1638.
[30] Mehrotra J, Ganpat MM, Kanaan Y, Fackler MJ, McVeigh M, Lahti-Domenici J, Polyak K, Argani P, Naab T, Garrett E, Parmigiani G, Broome C and Sukumar S. Estrogen receptor/progesterone receptor-negative breast cancers of young African-American women have a higher frequency of methylation of multiple genes than those of Caucasian women. Clin Cancer Res 2004; 10: 2052-2057.
[31] Muller HM, Widschwendter A, Fiegl H, Ivarsson L, Goebel G, Perkmann E, Marth C and Widschwendter M. DNA methylation in serum of breast cancer patients: an independent prognostic marker. Cancer Res 2003; 63: 7641-7645.
[32] Gobel G, Auer D, Gaugg I, Schneitter A, Lesche R, Muller-Holzner E, Marth C and Daxenbichler G. Prognostic significance of methylated RASSF1A and PITX2 genes in blood- and bone marrow plasma of breast cancer patients. Breast Cancer Res Treat 2011; 130: 109-117.
[33] Cho YH, Yazici H, Wu HC, Terry MB, Gonzalez K, Qu M, Dalay N and Santella RM. Aberrant promoter hypermethylation and genomic hypomethylation in tumor, adjacent normal tissues and blood from breast cancer patients. Anticancer Res 2010; 30: 2489-2496.
[34] Wojdacz TK, Thestrup BB, Overgaard J and Hansen LL. Methylation of cancer related genes in tumor and peripheral blood DNA from the same breast cancer patient as two independent events. Diagn Pathol 2011; 6: 116.
[35] Wong CM, Anderton DL, Smith-Schneider S, Wing MA, Greven MC and Arcaro KF. Quantitative analysis of promoter methylation in exfoliated epithelial cells isolated from breast milk of healthy women. Epigenetics 2010; 5: 645-655.
[36] Browne EP, Punska EC, Lenington S, Otis CN, Anderton DL and Arcaro KF. Increased promoter methylation in exfoliated breast epithelial cells in women with a previous breast biopsy. Epigenetics 2011; 6: 1425-1435.

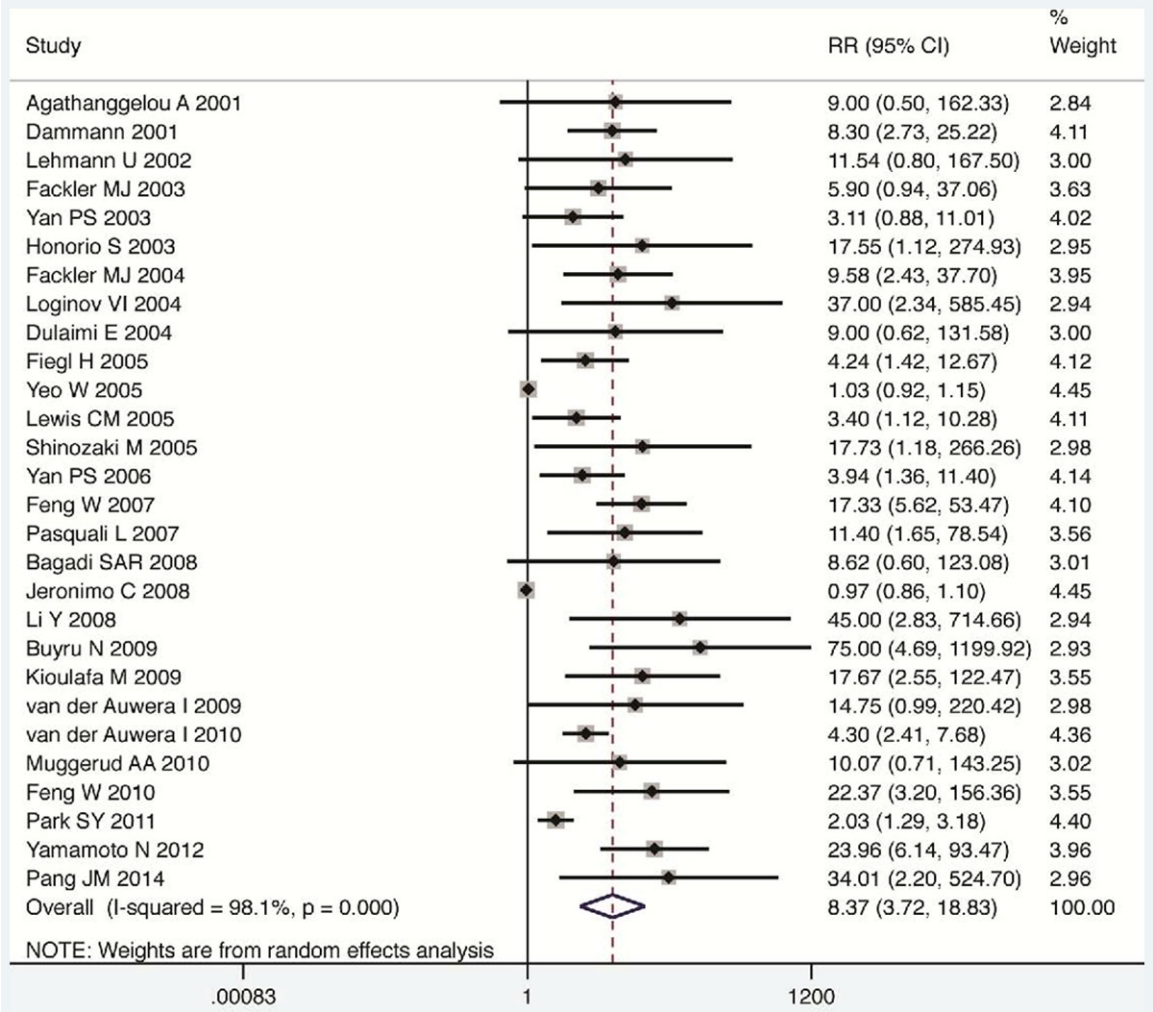
Supplementary Table 1. Summary of publications eligible for meta-analysis

ID/Author	Year	Pubmed ID	Patient sources	Population size	Serum Stud	y ER/PR status	LN metastat-ic status	Methylation analysis methods
Agathanggelou A	2001	11313894	Glenfield Hospital NHS Trust, UK	44				Direct sequencing or restriction enzyme digestion
Dammann R	2001	11306494	Beckman Research Institute, USA	45				Direct sequencing
Lehmann U	2002	11839581	Medizinische Hochschule Hannover, Germany	57				Quantitative MSP (qMSP)
Fackler MJ	2003	14601057	Johns Hopkins Medical Institutions, USA	103				Methylation specific PCR (MSP)
Honorio S	2003	12527916	University of Birmingham, Italy	20				MSP
Müller HM	2003	14633683	University of Innsbruck, Innsbruck, Austria	132		Yes	Yes	MethyLight
Yan PS	2003	14559801	Ellis Fischel Cancer Center, USA	37				COBRA
Dulaimi E	2004	15448006	Fox Chase Cancer Center Tumor Bank Facility	34	Yes			MSP
Fackler MJ	2004	15231653	Johns Hopkins Hospital	19				QM-MSP
Krassenstein R	2004	14734448	Fox Chase Cancer Center Tumor Bank Facility	22				MSP
Loginov VI	2004	15456137	Blokhin Cancer Research Center, Russia	59				MSP
Mehrotra J	2004	15131050	Johns Hopkins Hospital	55				MSP
Fiegl H	2005	15734995	University of Innsbruck, Innsbruck, Austria	148				MethyLight PCR
Lewis CM	2005	15671542	University of Texas Southwestern	17				MSP
Shinozaki M	2005	15788661	John Wayne Cancer Institute	151		Yes		MSP
Yeo W	2005	16028839	Prince of Wales Hospital, Hong Kong	40				MSP
Hoque MO	2006	16908936	University of Dakar Tumor Institute	93	Yes			QM-MSP
Papadopoulou E	2006	17108217	Molecular Biology Research Center, Greece	50	Yes			MSP
Shukla S	2006	17998817	All India Institute of Medical Sciences	20				MSP
Skvortsova TE	2006	16641902	Novosibirsk Regional Oncologic Dispensary, Ru	35	Yes			MSP
Taback B	2006	17108214	John Wayne Cancer Institute	33	Yes			qMSP
Yan PS	2006	17121881	The Ohio State University	23				MSO microarray and QM-MSP
Euhus DM	2007	17855699	University of Texas Southwestern Medical Cent	34				QM-MSP
Feng W	2007	17764565	MD Anderson Cancer Center	80				Pyrosequencing
Pasquali L	2007	17706863	University of Pittsburgh Med-ical Cente	21				Pyrosequencing
Tan SH	2007	17914577	National University Hospital, Singapore	19	Yes			MSP
Bagadi SA	2008	18538349	All India Institute of Medical Sciences	54		Yes	Yes	MSP
Jeronimo C	2008	17549626	Portuguese Oncology Institute, Porto, Portugal	97				qMSP
Li Y	2008	18425370	First Affiliated Hospital of Nanjing Medical Uni	36			Yes	MSP
Sunami E	2008	18485221	John Wayne Cancer Institute	65		Yes	Yes	MSP
Wu JM	2008	18381931	Johns Hopkins Hospital	10				qMSP
Buyru N	2009	19194828	Istanbul University Cerrahpasa Medical Faculty	77				Methylation-specific multiplex ligationdependent probe amplification (MS-MLPA)
Kim JH	2009	19924440	Chonnam National University Medical, Korea	84				MSP
Kioulafa M	2009	19374895	Hellenic Oncology Research Group Greece	93		Yes	Yes	MSP
Rasti M	2009	19946345	Shiraz University Hospital, Iran	81		Yes	Yes	MSP
Sharma G	2009	19940364	All India Institute of Medical Sciences	101		Yes	Yes	MSP
Van der Auwera I	2009	19367284	General Hospital Sint-Augustinus, Belgium	80	Yes			qMSP

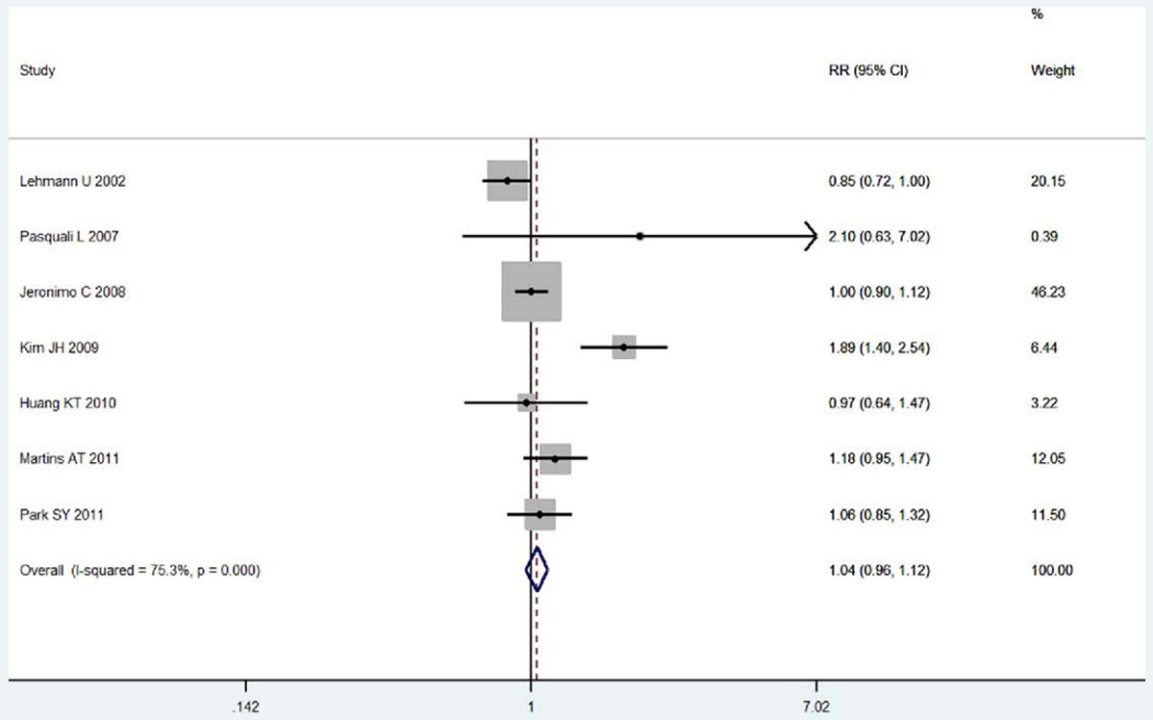
Van der Auwera I	2009	19829046	General Hospital Sint-Augustinus, Belgium	100				qMSP
Yazici H	2009	19755643	New York site of the Breast Cancer Family Reg	100	Yes			MSP
Ahmed IA	2010	20471512	Tubingen University Hospital Germany	26	Yes			MSP
Brooks JD	2010	20627767	New York University Women's Health Study, N	100	Yes			qMSP
Cho YH	2010	20682973	University of Istanbul in Turkey	40				qMSP
Feng W	2010	20642860	Fondazione IRCCS Istituto Nazionale dei Tumo	38				Pyrosequencing
Huang KT	2010	20563638	Peter MacCallum Cancer Centre, Australia	112				Methylation-sensitive high resolution melting (MS-HRM)
Jing F	2010	20490964	Laboratory Medicine Center of Taihe Hospital, S	90	Yes			MSP
Karray-Chouayekh S	2010	19657672	CHU Habib Bourguiba of Sfax in Tunisia	78		Yes	Yes	MSP
Kim JH	2010	20466412	Chonnam National University Hwasun Hospital	119	Yes			QM-MSP
Matuschek C	2010	20696638	University of Dusseldorf, Germany	85	Yes			qMSP
Muggerud AA	2010	20056007	Uppsala University Hospital, Sweden	89				Pyrosequencing
Van der Auwera I	2010	20226036	General Hospital Sint-Augustinus, Wilrijk, Belg	56				qMSP
Göbel G	2011	21221769	Medical University Hospital in Innsbruck, Austr	428				qMSP
Martins AT	2011	20842524	Portuguese Oncology Institute, Porto, Portugal	211		Yes		qMSP
Park SY	2011	21120523	Seoul National University Bundang Hospital. Se	125		Yes		MethyLight PCR
Alvarez C	2013	22315090	Pontificia Universi-dad Cato' lica de Chile	47		Yes		MSP
Avraham A	2012	22407753	Assaf Harofeh Medical Center, Zerifin, Israel	52				Methylation-sensitive high resolution melting (MS-HRM)
Cho YH	2012	21837480	Long island, NY, USA	765		Yes		qMSP
Sebova K	2012	22297548	Slovakia and the St. Elizabeth Cancer Institute,	92		Yes	Yes	QM-MSP
Tserga A	2012	22159596	Hippocration Hospital of Athens, Greece	49		Yes		Pyrosequencing
Yamamoto N	2012	21594664	Osaka University Hospital, Osaka, Japan	253	Yes			One-step MSP (OS-MSP) assay
Kajabova V	2013	23730409	University Hospital in Bratislava, Slovakia	151				Quantitative multiplex MSP
Klajic J	2013	24093668	Haukeland University Hospital in Norway	238				Pyrosequencing
Twelves D	2013	23674191	Royal Marsden Hospital	42				MSP
Pang JM	2014	25331261	Peter MacCallum Cancer Centre and Royal Prin	72				Methylation-sensitive high resolution melting (MS-HRM)
			Total	5727				



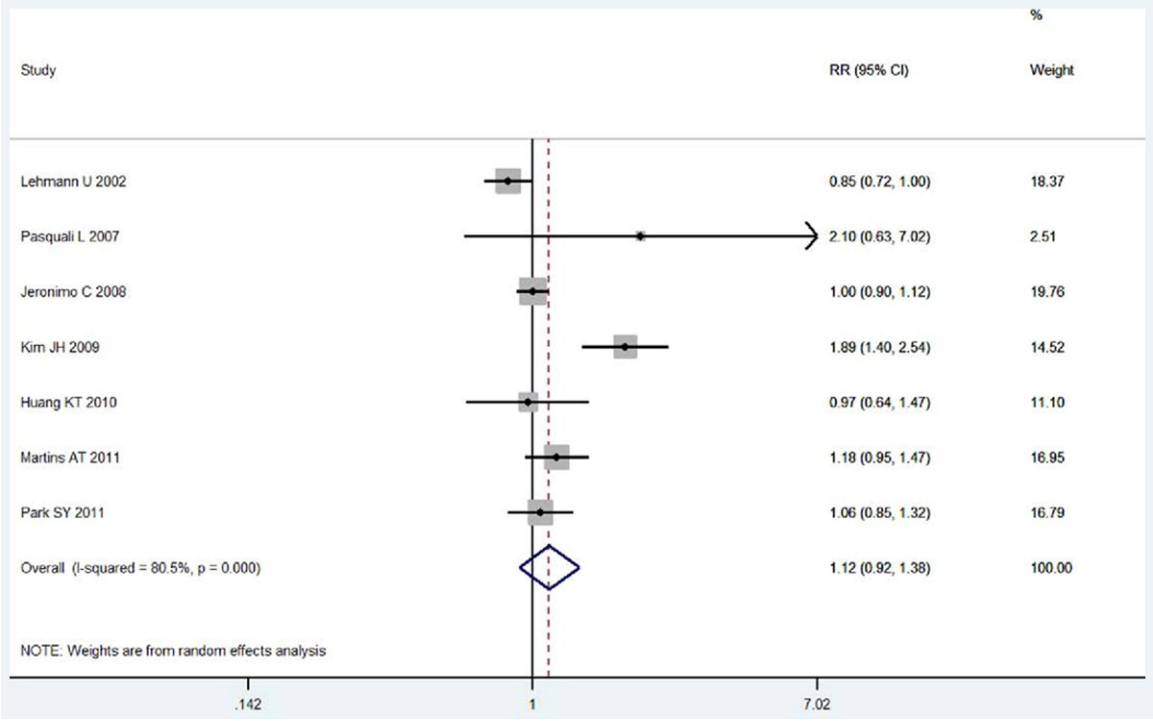
Supplementary Figure 1. Metaanalysis shows that RASSF1A is methylated more in primary tumor tissues against normal breast tissues, with fixed effect model.



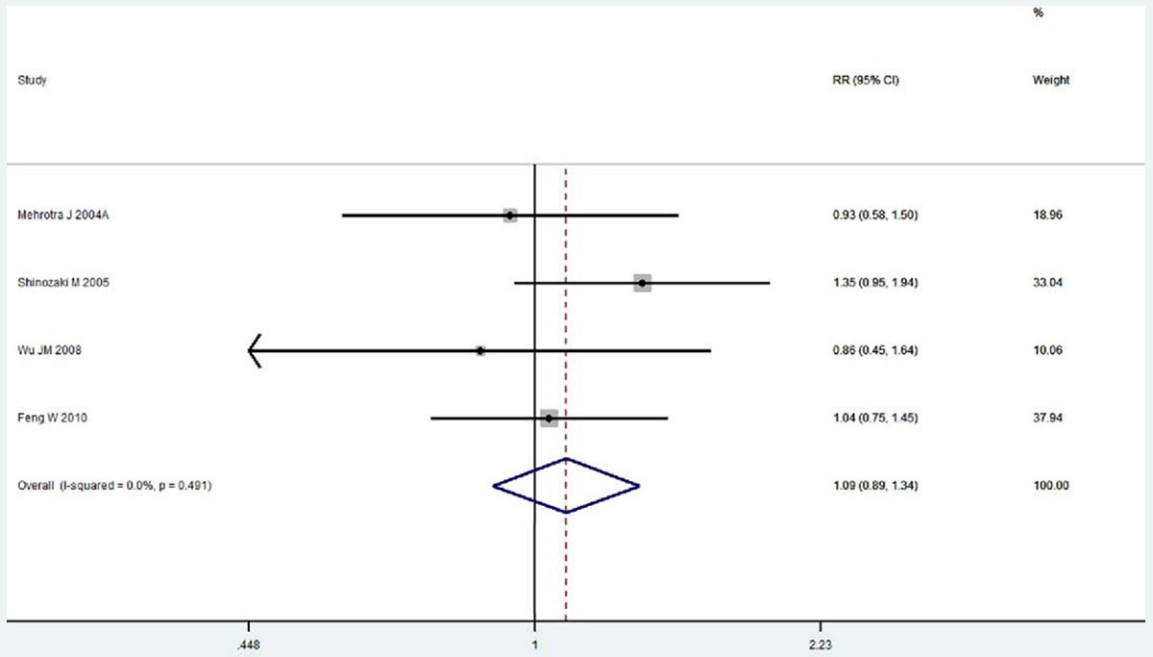
Supplementary Figure 2. Metaanalysis shows that RASSF1A is methylated more in primary tumor tissues against normal breast tissues, with random effect model.



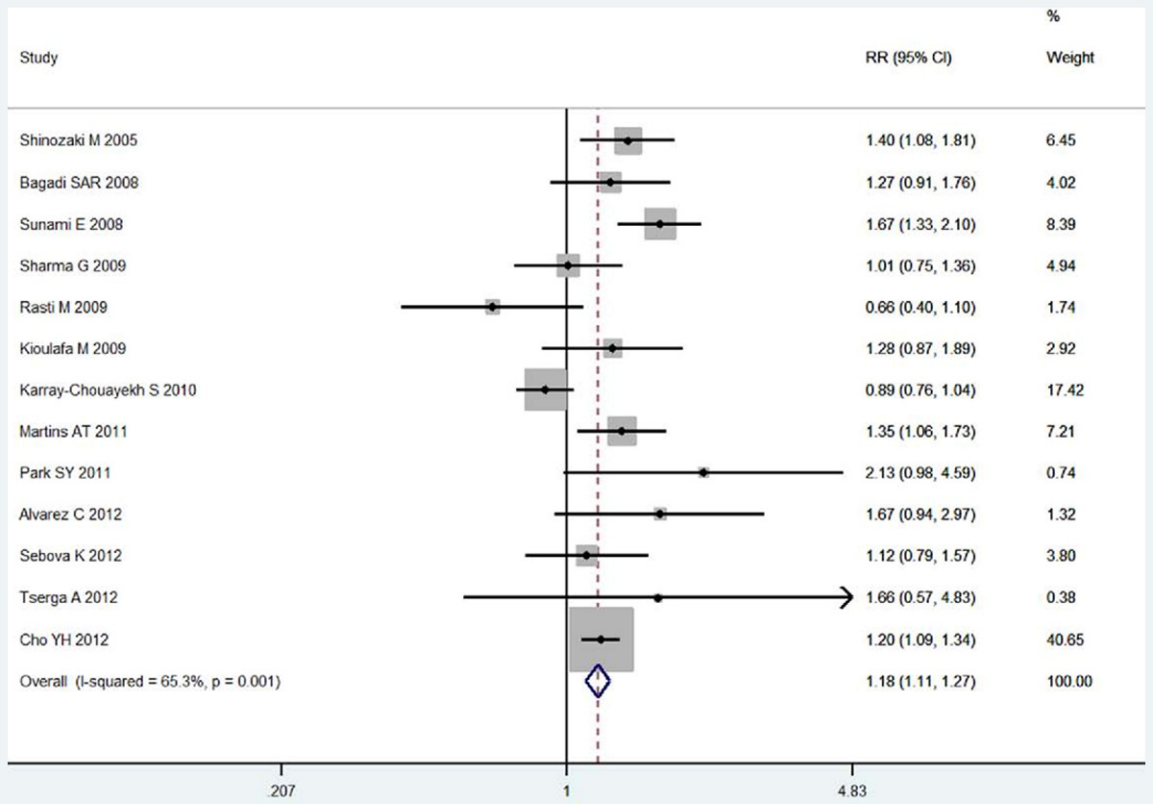
Supplementary Figure 3. Metaanalysis result with the fixed model shows there is no significant difference between benign and primary tissues in RASSF1A promoter methylation status.



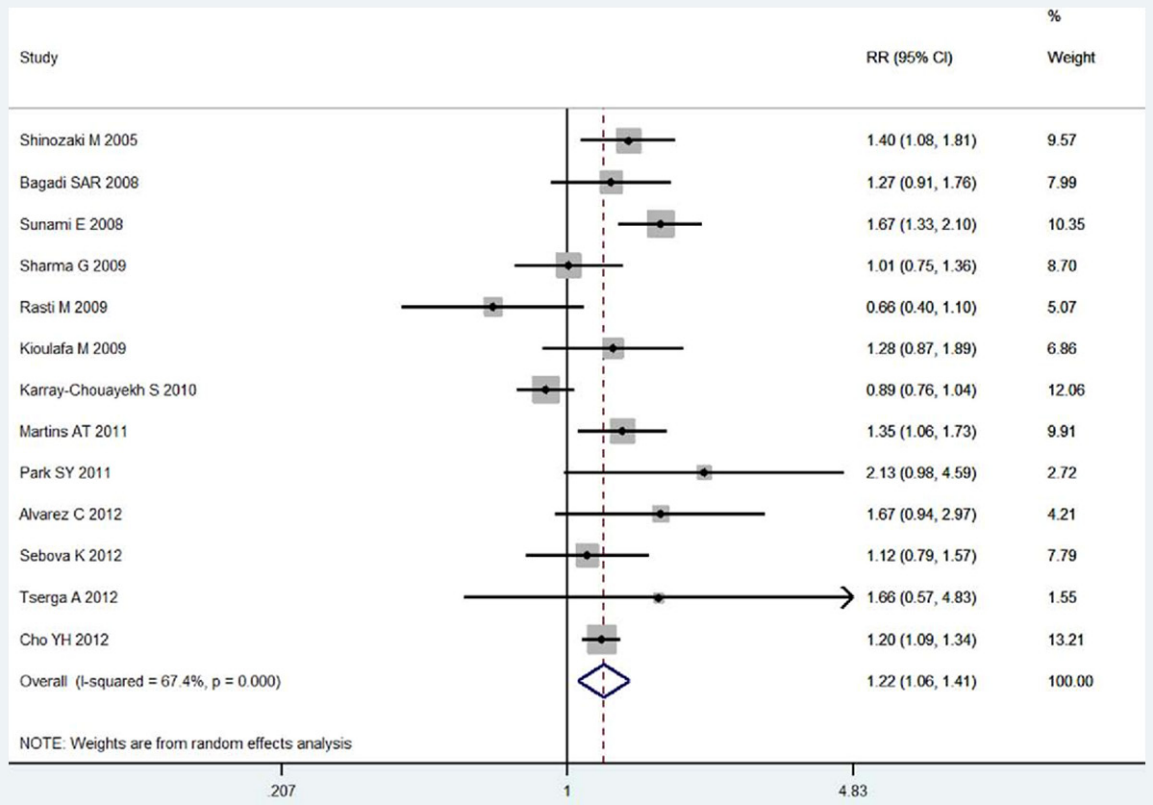
Supplementary Figure 4. Metaanalysis result with the random effect model shows there is no significant difference between benign and primary tissues in RASSF1A promoter methylation status.



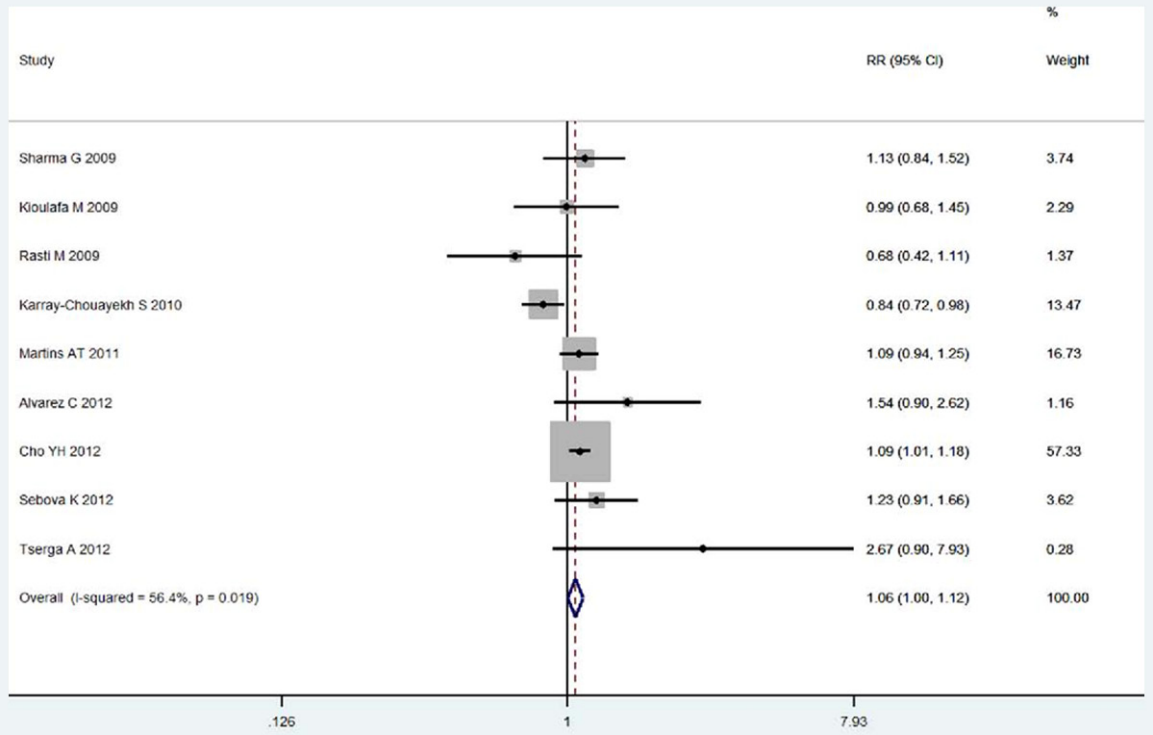
Supplementary Figure 5. Metaanalysis result with the fixed model shows there is no significant difference between primary tissues and distant metastatic tissues in RASSF1A promoter methylation status.



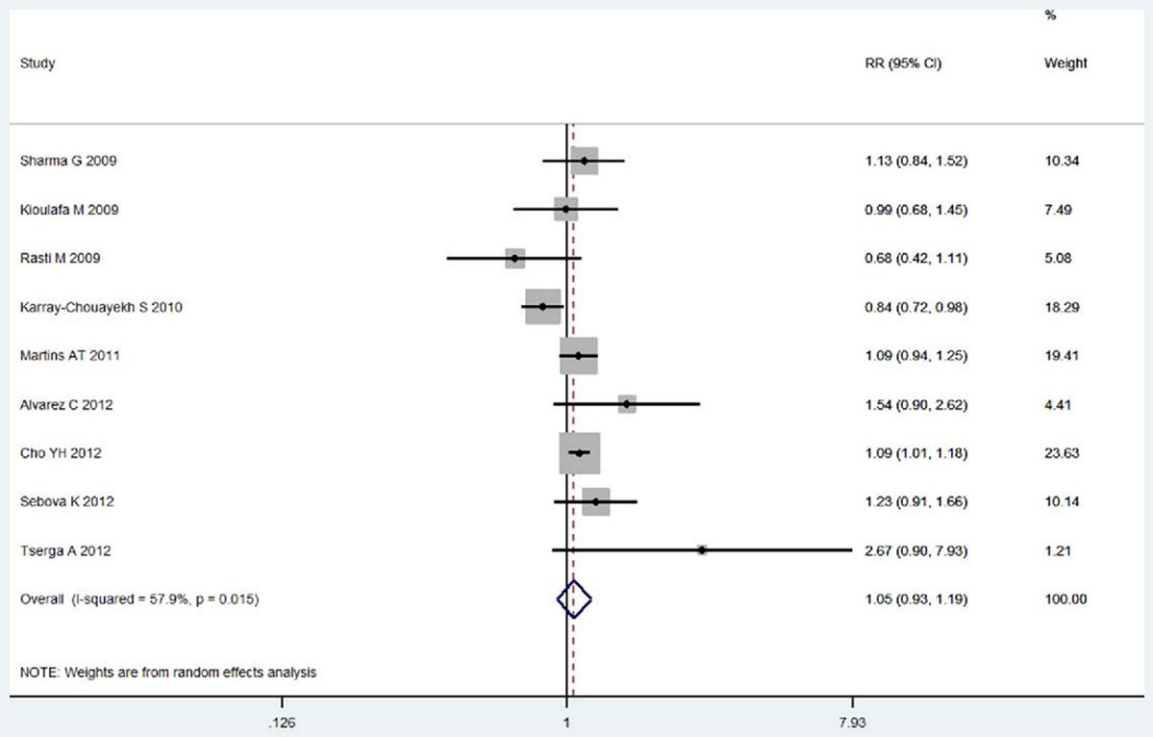
Supplementary Figure 6. Metaanalysis with fixed effect model shows significant difference between ER positive and negative patients in RASSF1A promoter methylation status.



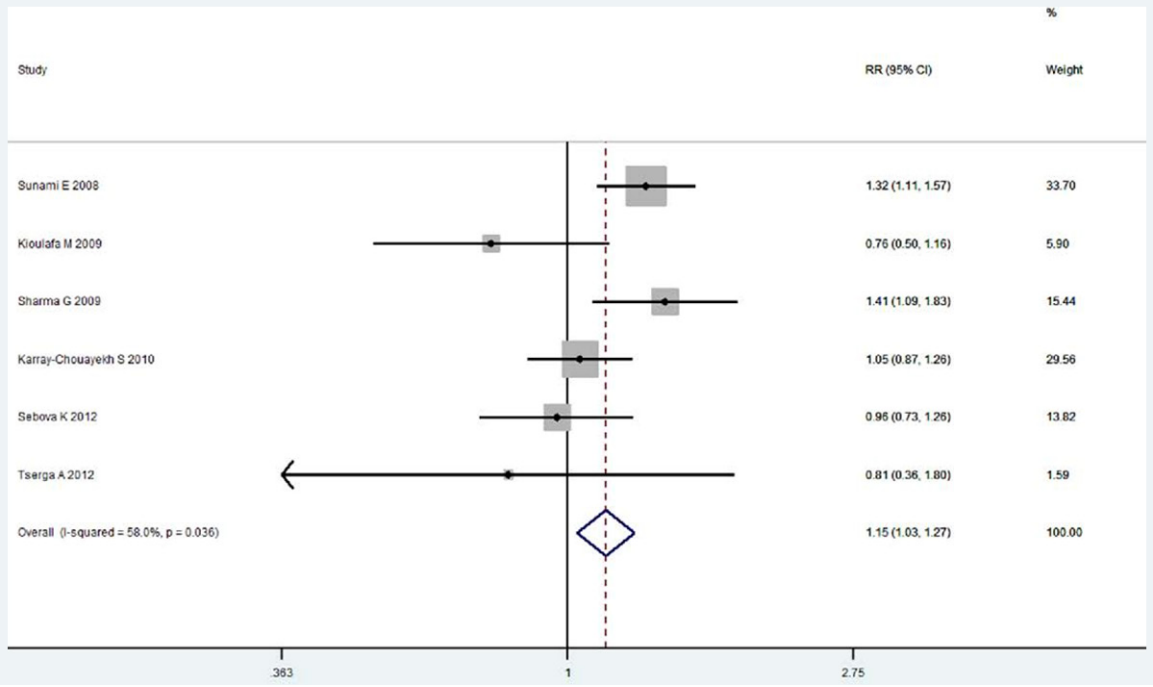
Supplementary Figure 7. Metaanalysis with random effect model shows significant difference between ER positive and negative patients in RASSF1A promoter methylation status.



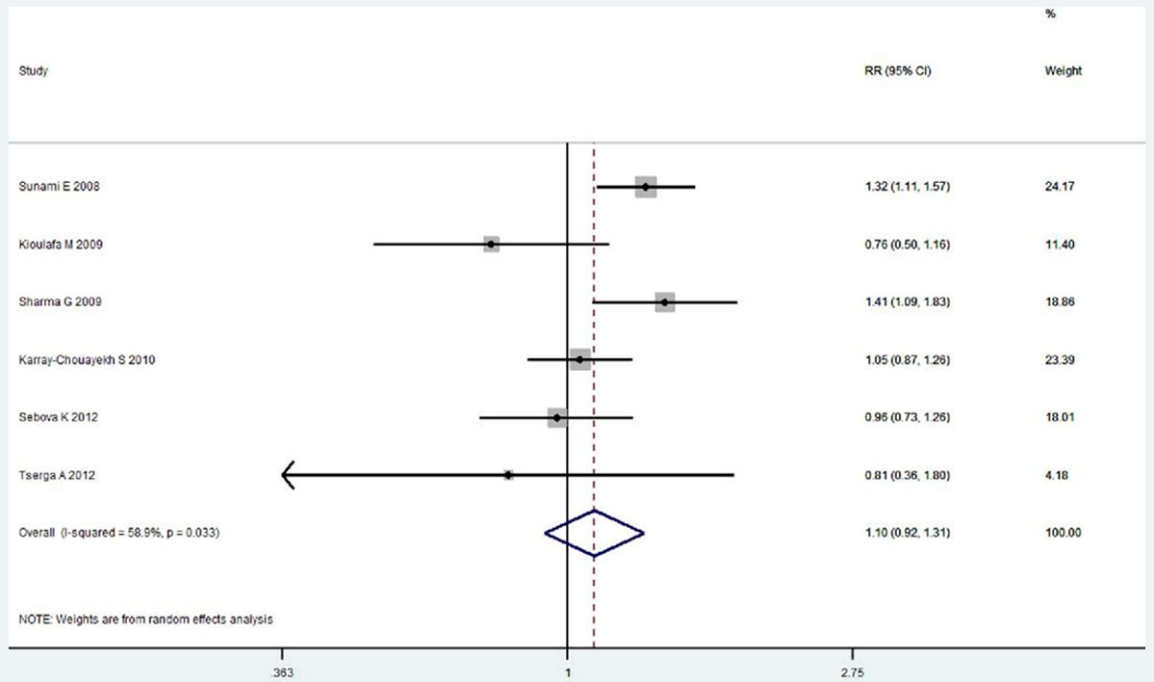
Supplementary Figure 8. Metaanalysis with fixed effect model shows significant difference between PR positive and negative patients in RASSF1A promoter methylation status.



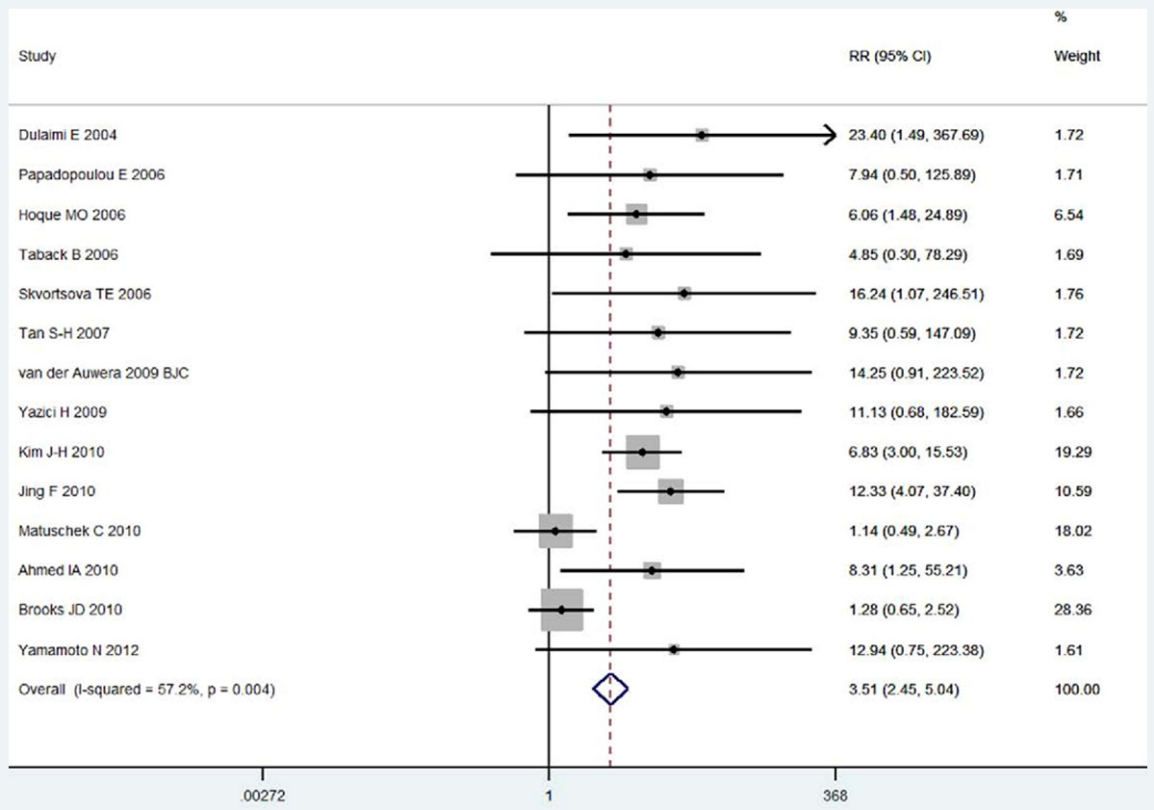
Supplementary Figure 9. Metaanalysis with random effect model shows significant difference between PR positive and negative patients in RASSF1A promoter methylation status.



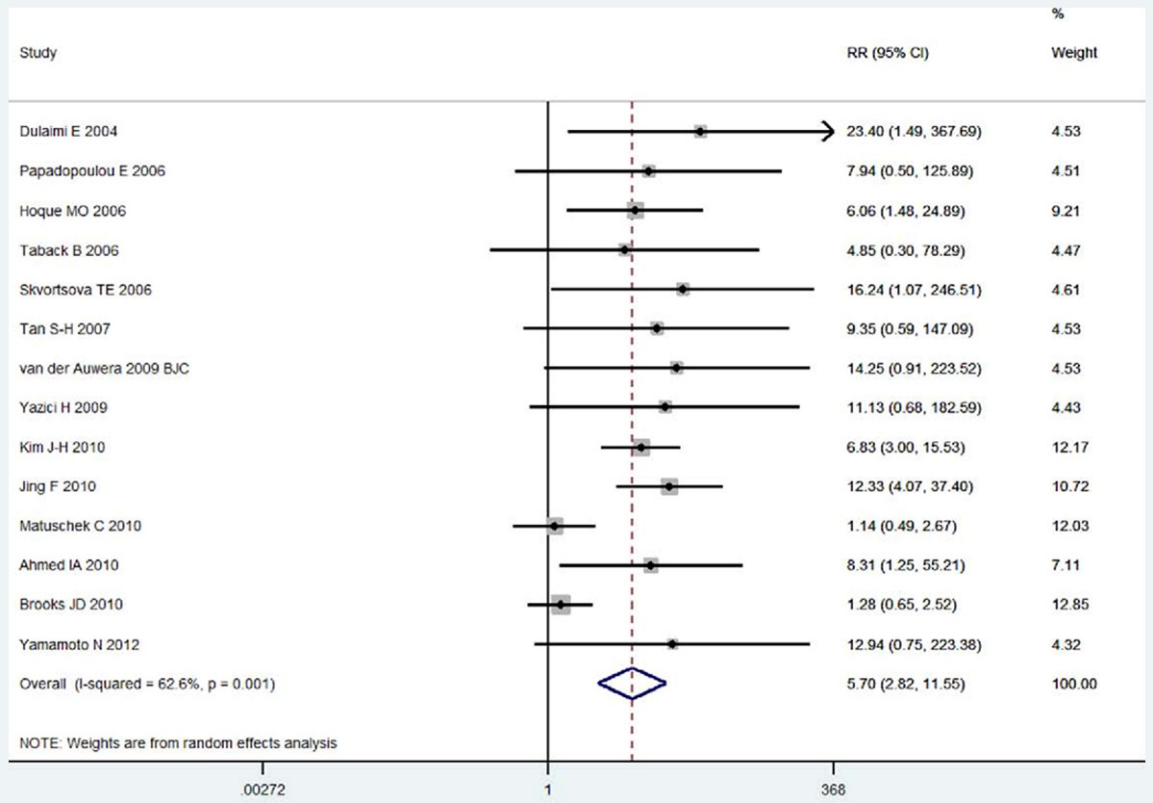
Supplementary Figure 10. Metaanalysis with fixed effect model shows no significant difference between HER2 positive and negative patients in RASSF1A promoter methylation status.



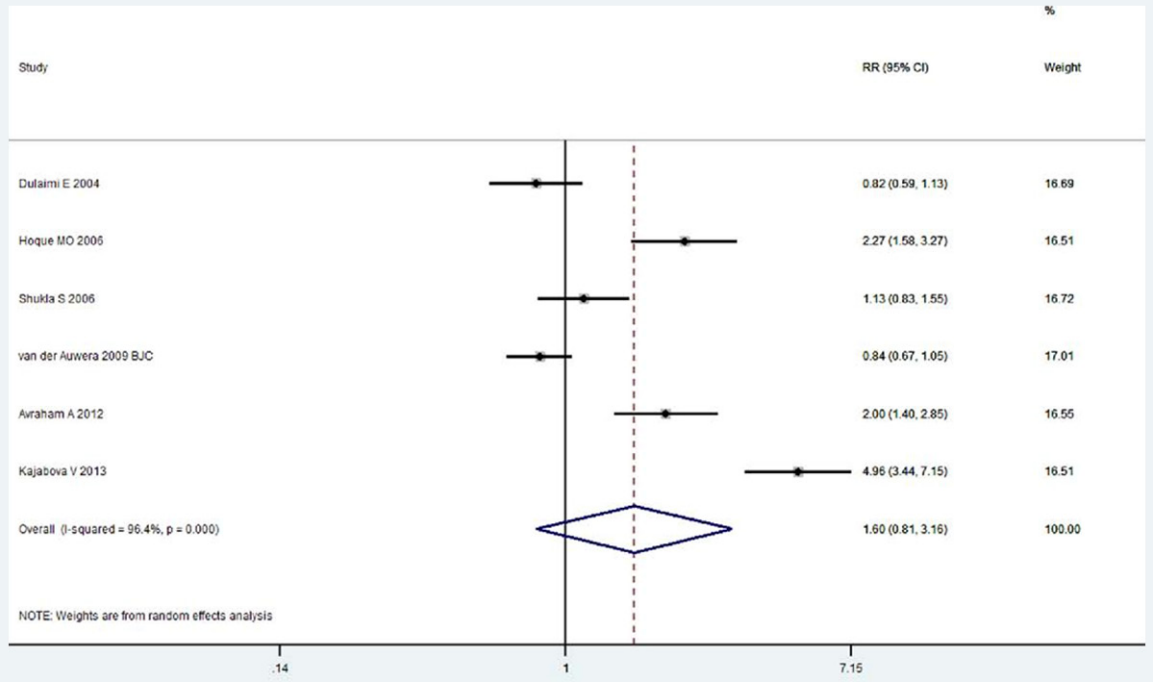
Supplementary Figure 11. Metaanalysis with random effect model shows no significant difference between HER2 positive and negative patients in RASSF1A promoter methylation status.



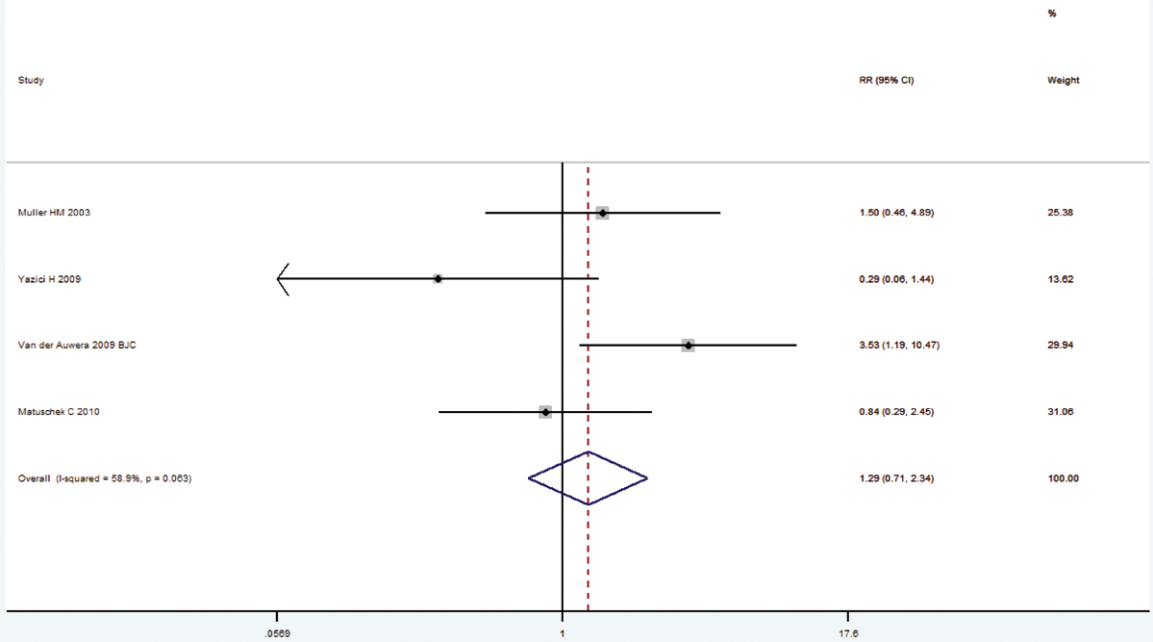
Supplementary Figure 12. Metaanalysis shows that RASSF1A is methylated more in primary tumor patients' serum against normal serum, with fixed effect model.



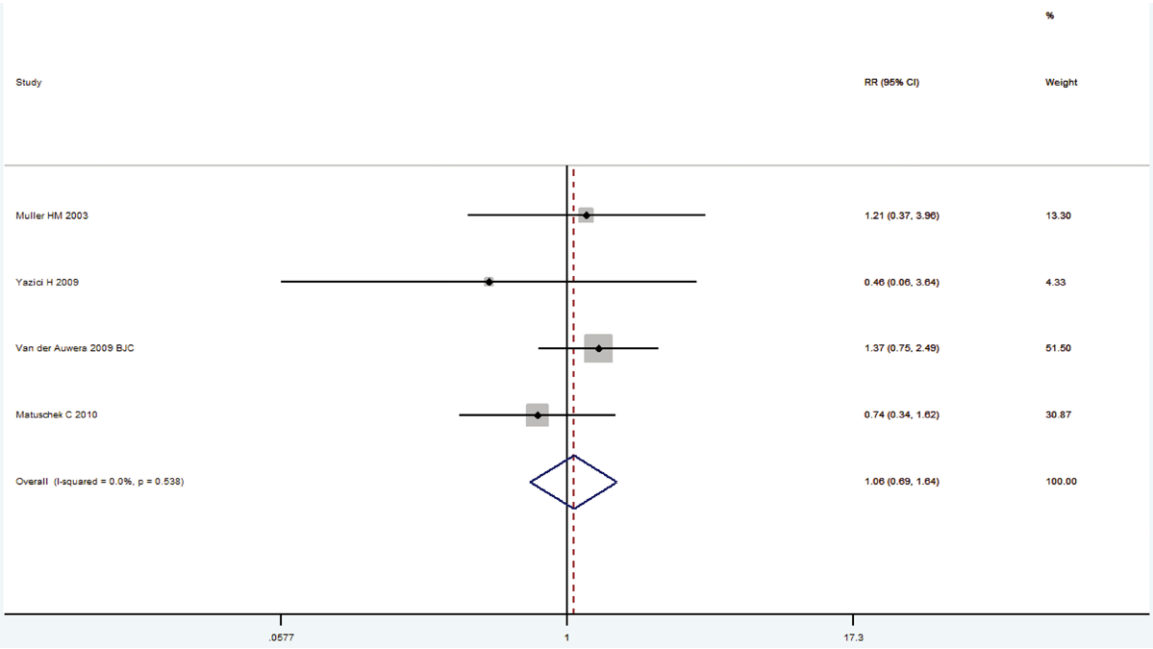
Supplementary Figure 13. Metaanalysis shows that RASSF1A is methylated more in primary tumor patients' serum against normal serum, with random effect model.



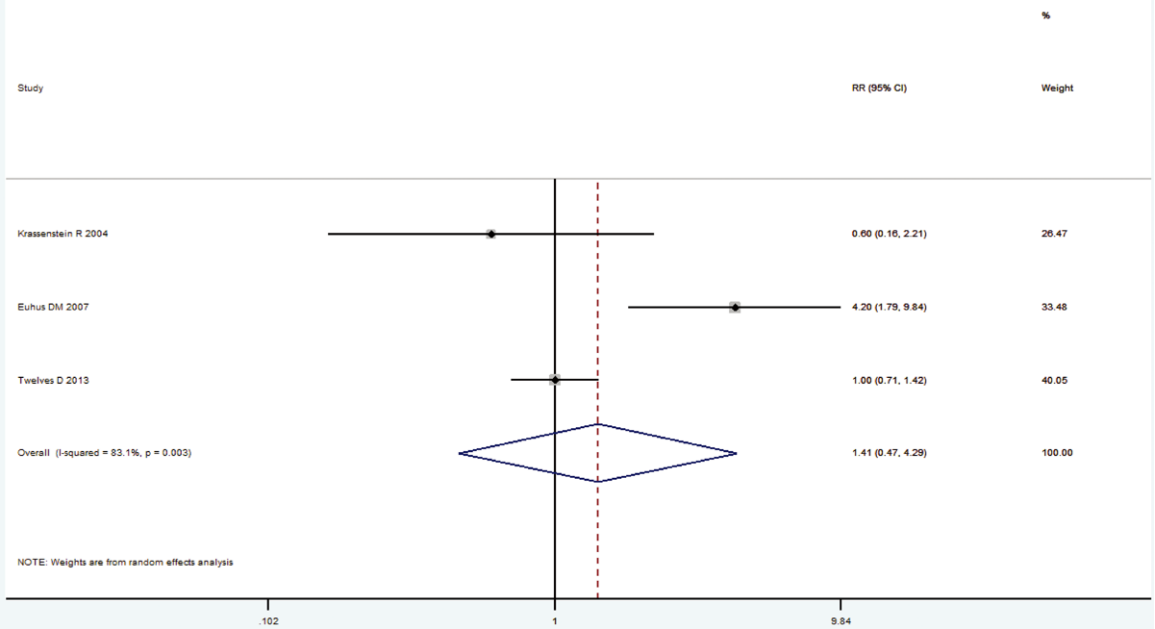
Supplementary Figure 14. Metaanalysis with random effect model shows no significant difference between paired tumor tissues and serum samples in RASSF1A promoter methylation status.



Supplementary Figure 15. Metaanalysis with random effect model shows no significant difference between ER positive and negative patients in RASSF1A promoter methylation status, with genomic DNA from patients' serum.



Supplementary Figure 16. Metaanalysis with random effect model shows no significant difference between PR positive and negative patients in RASSF1A promoter methylation status, with genomic DNA from patients' serum.



Supplementary Figure 17. Metaanalysis with random effect model shows no significant difference between tumor tissues and nipple fluid samples in RASSF1A promoter methylation status.