**Diagnostic Role of APC Promoter Methylation in Non-Small Cell Lung Cancer (NSCLC): An Integrated-Analysis of Published Articles and Microarray Data**

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**Abstract [269 words]**

Background: Adenomatous polyposis coli (APC) has been reported to be a candidate tumor suppressor in many cancers. However, the diagnostic role of APC promoter methylation in NSCLC remains unclear.

Methods: We systematically integrated published articles and DNA methylation microarray data to discover the diagnosis performance of APC methylation test for NSCLC. 2259 NSCLC and 1039 controls were collected from 17 published studies (1338 cases, 913 controls) and TCGA NSCLC data (921 cases, 126 controls). The association between APC promoter methylation and Lung cancer was quantified using meta-analysis methods. Then independent DNA methylation microarray data, from which five CpG sites located in the promoter region of APC were involved, were used to validate the results of the meta-analysis. Summary ROC curves were fitted to show the performance of sensitivity and specificity and the area under the curve (AUC) of APC methylation test for NSCLC in meta-analysis.

Results: A significant association was observed between APC promoter methylation and NSCLC, with an aggregated odds ratio (OR) of 3.79 (95% CI: 2.22-6.45) in random effect model. Pooled sensitivity and specificity were 0.548 (95% CI:0.42-0.67, P< 0.0001) and 0.776 (95% CI:0.62-0.88, P<0.0001), respectively. The AUC of the APC methylation test in NSCLC was 0.64, meanwhile larger AUC were found in serum group (0.67) than in tissues (0.64). Each of the five CpG site in lung adenocarcinoma (Ad) was much better in prediction (AUC: 0.71-0.73) than that in lung squamous cell carcinoma (Sc) (AUC: 0.45-0.61). In addition, the AUCs of the logistic prediction model based on these 5 CpGs were 0.73 and 0.60 for Ad and Sc, respectively. Integrated analysis showed CpG site location, heterogeneous or autogenous controls and the proportion of adenocarcinoma in samples were most significant heterogeneity sources. However, gender, TNM stage, methylation detection methods, and sample type (tissue or serum) showed no significant associations with APC methylation in NSCLC diagnosis.

Conclusions: The methylation status of APC promoter was strongly associated with NSCLC, especially adenocarcinoma. APC methylation test could be applied in the clinical diagnosis for adenocarcinoma.

Key words: APC, DNA methylation, Diagnosis, Meta-analysis, TCGA, NSCLC, Biomarker, Adenocarcinoma

**Introduction**

Non-small cell Lung cancer(NSCLC), including adenocarcinoma(Ad) and squamous cell carcinoma(Sc), is the leading cause of cancer death in men and women in the United States[[1](#_ENREF_1)]. Over 159,480 Americans die of this disease every year in U.S.A[[1](#_ENREF_1)]. The 5-year relative survival rate varies markedly depending on the stage at diagnosis, from 49% to 16% to 2% for patients with local, regional, and distant stage disease, respectively (SEER Cancer Statistics Review 1975-2002). Early detection is a key bottleneck in increasing lung cancer patient survival[[2](#_ENREF_2)]. DNA hypermethylation has beenrecognized as an important mechanism for tumor suppressor gene inactivation in cancer and could yield powerful biomarkers for early detection of lung cancer and own incomparable advantage than other traditional markers for its stable chemical property, detectabilityin remote patient media, quantitative signal, convenient low cost in detection, etc. [[3](#_ENREF_3)]. Several revolutionary steps has been made to push methylation biomarkers into cancer screening[[4](#_ENREF_4), [5](#_ENREF_5)] which indicated DNA methylation would become a powerful tools for lung cancer diagosis.

APC gene encodes a tumor suppressor protein that acts as an antagonist of the Wnt signaling pathway. It is also involved in other processes including cell migration and adhesion, transcriptional activation, and apoptosis[[6](#_ENREF_6)]. Defects in this gene cause familial adenomatous polyposis (FAP), an autosomal dominant pre-malignant disease that usually progresses to malignancy which suggestsit is a potential predictor for cancer initial or development. Promoter methylation inhibits APC gene expression mediated by changes of chromatin conformation and aberrant binding of CCAAT-box binding transcription factors[[7](#_ENREF_7)].

Following P16INK4A[[8](#_ENREF_8)], the relationship between hypermethylation of APC with NSCLC was extensively estimated and APC methylation test in NSCLC was believed as a effective biomarker for diagnosis[[9](#_ENREF_9), [10](#_ENREF_10)]. However, the results were dramatically different among each researches which may be caused by the difference of gender proportion, age distribution, race source and some other epidemiological characteristics in samples, detection methods, etc. In addition, there was not any quantitative assessment of the relationship between the hypermethylation in the promoter region of APC gene and NSCLC yet.

In this article, firstly, we conducted ameta-analysis of the sensitivity and specificity of APC methylation on NSCLC diagnosis. The factors which led heterogeneity to the sensitivity and specificity were discovered with meta-regression. We also found that the cancer genome atlas project (TCGA) had provided hundreds of whole genome DNA methylation microarray data of NSCLC samples with comprehensive clinical and demographic information, which could be integrated with the data in published articles to evaluate the diagnosis ability of APC methylation test in NSCLC. Therefore, integrated analysis of all these existing data were conducted to make an unbiased conclusion on the relationship between APC methylation and NSCLC.

**MATERIALS AND METHODS**

**Search strategy, selection of studies and data extraction**

This pooled study involved searching a range of computerized databases, including Pubmed, Cochrane Library, OVID Medline and TMC ProSearch for articles published in English or Chinese to March 2013. The study used a subject and text word strategy with (APC OR BTPS2 OR DP2 OR DP2.5 OR DP3 OR PPP1R461) AND ((Lung OR NSCLC) AND (cancer OR neoplasm)) as the primary search terms. Wildcard character of star, dollar or some other truncations were applied according to the rule of the databases to make effective collection of the articles.

Two independent reviewers (Guo, Tan) screened the titles and abstracts derived from the literature search to identify relevant studies. The following types of studies were excluded: animal experiments, case reports, reviews or meta-analyses and studies of non-case-control study or with insufficient data or be inaccessible after the contact with the authors. The remained articles were further examined to see if they met the inclusion criteria: 1) the patients had to be diagnosed with NSCLC(Ad and Sc), 2) the studies had to have APC gene promoter methylation data from tissue, blood or serum, 3) case-control study, which included tissue-tissue, blood-blood or serum-serum in case and controls respectively. The reference sections of all retrieved articles were searched to identify further relevant articles. Potentially relevant papers were obtained and the full text articles were screened for inclusion by two independent reviewers (Guo, Tan). Disagreements were resolved by discussion with KX,JJW, JHW. Included studies were summarized in data extraction forms. Authors were contacted when relevant data were missing. The name of the first author, year of publication, sample size, age (mean or median), gender proportion (male/female, M2F), the proportion of TNM stage I samples (proportion of early stage of NSCLC samples), publication aim (for diagnosis or not), analyzing multiple genes or not (one or more genes detected simultaneously in studies design), control type (autogenous or heterogenous counterpart) and methylation status of the APC promoter in human NSCLC and normal or control tissues were extracted.

**Meta analysis and Summary receiver operating characteristics analysis**

Data were analyzed and visualized mainly using R Software (R version 2.15.3) including meta, metefor and mada packages. The strength of association was expressed as pooled odds ratio (OR) with corresponding 95% confidence intervals (95% CI). Data were extracted from the original studies and recalculated if necessary. The data were pooled using the DerSimonian and Laird random effects model (I2 > 50%, P ≤ 0.1) or fixed effects model (I2 < 50%) according to heterogeneity statistic I2 [[11](#_ENREF_11)]. A two-sided P ≤ 0.05 was considered significant without special annotation. Heterogeneity was tested using the I2 statistic with values over 50% and Chi-squared test with P ≤ 0.1 indicating strong heterogeneity between the studies[[12](#_ENREF_12)]. Tau-squared (τ2) was used to determine how much heterogeneity was explained by subgroup differences. Meta-regression analyses were employed to analyze the sources of the heterogeneity when the heterogeneity was significant[[13](#_ENREF_13)]. Subgroup analyses of the ORs of APC promoter methylation in cancer tissue versus normal tissue were performed according to control types (autogenous and heterogeneous), gender proportion, proportion of TNM stage I samples, age, single or multiple target detection, serum or tissue, methylation detection method and study design. Sensitivity analyses were performed to assess the contributions of single studies to the final results with the abandon of one article each time. Publication bias was analyzed by funnel plot with mixed-effects version of the Egger test. If bias was suspected, the conventional meta-trim method was used to re-estimate the effect size.

Compared with traditional SNP association study, methylation-associated research might be involved with different methylation-definition thresholds. In these cases, traditional weighted averages (pooled sensitivity and specificity) would not reflect the overall accuracy of the test, as the extremes of threshold criteria could skew the distribution, that is so called threshold effect[[14](#_ENREF_14)]. Thus, summary receiver operating characteristics (SROC) analysis can be applied to meta-analysis of diagnostic tests[[14](#_ENREF_14), [15](#_ENREF_15)]. It was plotted to show the performance of the diagnosis ability of APC methylation to NSCLC. Each study produces values for sensitivity, specificity and therefore true positive rate (TPR) and false positive rate (FPR). The SROC curve is placed over the (TPR, FPR) points to form a smooth curve. Linear regression model were selected to fit the SROC curve where sensitivity and (1-specificity) are transformed into complex logarithmic variables. The exact area under the curve (AUC) for the SROC function was used to assess the accuracy of the test [[14](#_ENREF_14)].

**TCGA data extraction and analysis**

DNA methylation information for NSCLC which included two sets of samples (535 Ad and 50 Control, and 385 Sc and 67 control) were collected from TCGA Project including methylation 27K and 450K dataset ([http://cancergenome.nih.gov/). The](http://cancergenome.nih.gov/).%20The) estimate of methylation for each CG probe was calculated with the traditional function: beta. M and U represent the mean signal intensities for about 30 replicate methylated (M) and unmethylated (U) probes on the array. The methylation signalsof the 25978 shared CpG sites by 27K and 450K datasets were extractedand the methylation status of each probe was defined according to the beta value. The CpG site will be considered methylated when the beta value is greater than the empirical threshold of 0.3 for tissue data[[16](#_ENREF_16" \o "Sproul, 2011 #14)]. Six CpG sites located in the promoter region of APC gene (cg01240931, cg15020645, cg16970232, cg20311501, cg21634602 and cg24332422) were taken as the object of study (S. Table s1). Adjustment for multiple testing of differential methylation was conducted with the method of Benjamini and Hochberg at the 5% FDR level.

**RESULTS**

**Study characteristics**

The electronic search strategy identified 506 potentially relevant articles(Pubmed, 315; Scopus, 112; Cochrane Library, 3; OVID Medline, 53; TMC ProSearch, 23), which were further screened for inclusion on the basis of their titles, abstracts, full texts, or a combination of these terms. The electronic search was supplemented from reference lists of relevant articles including reviews. Finally, 17 studies included data on the relationship between APC gene promoter methylation and NSCLC were pooled for analysis (**Table 1**) [[9](#_ENREF_9), [17-32](#_ENREF_17)]. All the included articles were written in English. Totally, 1338 lung cancer tissues /serum and 913 normal counterpart tissues/serum were collected. Theage of the subjects in the 17 studies ranged from25 to 86 years while mean or median age was 53-67. Among the 17 retrieved studies (13 articles were especially for diagnosis, while the others were for prognosis, survival research, etc), 7 observations used methylation-specific polymerase chain reaction (MSP) while others used quantitative MSP (qMSP, suh as Methylight, Prosequencing, etc) to explore APC promoter methylation status. The proportions of the samples in stage I were countedand the ranges were 32.1-100% and 70-100% respectively. The pencentage of male individuals in the NSCLC samples ranged from 53% to 81%. Two kinds of methylation detection primers or probes were found to be utilized for most of the 17 studies. The information of the two sets of primers (set I: chr5:112073421-112073518, 7 studies; and set II: chr5:112101379-112101452, 7 studies) was listed in **S Table s1**. In addition, no CpG sites from the methylation microarrays was found located in the above primers, while cg20311501 is covered by the replication region of Set II primers.

**Meta-analysis, subgroup analysis and meta-regression**

The ORs for APC methylation in cancer tissues compared with that in normal controls were 4.67 (95% CI: 2.66-8.22, z=5.35, P < 0.0001), and 2.74 (95%CI:1.99-3.23, z = 8.10, P < 0.0001) in random effect model pooled and fixed effect model, respectively,indicating an increased likelihood of methylation in Lung cancer tissues **(Figure 1)**.

Subgroup analysis were conducted for different subtypes, which included sample type ( tissue or serum), counterparts category (autogenous and heterogeneous), proportion of stage I, aim of the study (for diagnosis or non-diagnosis), ratio of adenocarcinoma to squamous(Ad2Sc), primer categories (set I and II) and other possible interference factors **(Table 2)**. Significant difference were found between the OR of younger (5.03, 95%CI: 2.53-10.0) and older group (0.91, 95% CI: 0.57-1.41) subgroup (P <0.0001). High proportion of adenocarcinoma group had a significantly bigger OR than that of low subgroup (P=0.0077), which suggested that APC methylation might have subtype specificity in NSCLC. Significant difference was found between primer set I and II (P=0.0137), which supported primers were one of the most important heterogeneity sources in the APC methylation test. When we see the effects of sample type and control type on the OR of APC methylation, it can be found that both tissue and serum groups had showed significant association between APC methylation and NSCLC (OR=3.72, 11.54, respectively) which suggested APC methylation can be taken as a potential biomarker for NSCLC diagnosis using either tissue or serum samples. In addition, significant difference were found between the ORs of heterogeneous (ORh=8.33, 95% CI: 3.77-18.39) and autogenous (ORa=2.25, 95% CI: 1.06-4.77) subgroups (P=0.0187). One possible reason might be the impure composition of the adjacent normal specimens which might have been slightly contaminated by cancer cells or it have been transformed to precancerous status, while normal serum samples came from healthy individuals in general. The subgroup of high Ad2Sc group had a larger OR than that of low Ad2Sc **(Table 2)**, indicating that methylation of APC might occurred or functioned at the early stage of the tumorigenesis, which had been founded in endometrial cancer [[33](#_ENREF_33)]. Differences in the OR of diagnosis (OR=6.79) or non-diagnosis group(OR=2.59) was very large, which might be caused by unbalanced distribution of proportion of early stage samples (P = 0.0218, wilcox test) **(Table 2)**. No significant difference was found between the subgroups of MSP and qMSP (P=0.77), which suggested both of the methods were equivalent in methylation detection **(Table 2)** and the result was consistent with Wu’s conclusion [[34](#_ENREF_34)].

Further analyses were performed using meta-regression method with Knapp-Hartung modification to determine the sources of heterogeneity, which significantly existed among all studies (I2= 79.2%, Q = 52.78, P < 0.0001) (**Figure 1**). It showed that the trend in ORs was inversely correlated with age, suggesting that the age accounted for some of the heterogeneity (beta = -0.3, P = 2.0×10-5). It was consistent with the subgroup analysis in which the OR of older group (OR = 2.24) was smaller than younger group (OR=4.65). However, other factors such as sample type, proportion of males, proportion of stage I and detection methods could not explain the heterogeneity (**Table 3**).

**Summary Receiver Operating Characteristic Curve for diagnosis capacity of APC methylation**

Pooled sensitivity and specificity were 0.548 (95% CI: 0.42-0.67, P<0.0001) and 0.78 (95% CI: 0.62-0.88, P<0.0001) for the whole studies based on the presupposition of fixed effect model. The sensitivity of tissue subgroup was higher than that of serum subgroup, 0.61 (0.45-0.75) versus 0.396 (0.26-0.56), while the specificity of serum subgroup was higher than that of tissue subgroup, 0.92 (0.86-0.96) versus 0.68 (0.49-0.83), which suggested the advantage of this biomarker for its high ability in diagnosis using remote non-invasive media.

However, although sensitivity and specificity were two of most important features of a diagnosis test, in some occasions, pooling sensitivity or specificity could be a misleading event as mentioned in the section of “Meta analysis and Summary receiver operating characteristics analysis“. Therefore, the ability in diagnosis of the methylation test was assessed with SROC curve to depict its stability and accuracy. The AUC of the SROC was 0.64, suggesting the potential ability for NSCLC diagnosis (**figure 2**). Meanwhile, the AUC of the SROC for serum and tissue group were 0.67 and 0.64 respectively which indicated the different performance for APC methylation test in serum and tissue samples.

**Bias analysis and robust estimation of pooled OR**

A funnel plot of methylation status of Lung cancer tissue versus normal tissue showed significant publication bias (Egger test, z = 4.3, P<0 .0001) and eight studies exceeded the 95% confidence limits **(S Figure 3)**. In order to eliminate the effect of publication bias, trim and fill analysis was performed using the random effects model. The adjusted pooled OR were 2.50 (95% CI: 1.43-4.38, P = 0.0013) in random effect model and 2.19 (95% CI: 1.74-2.77, P < 0.0001) in fixed effect model, respectively, indicating a significantly positive association between APC methylation and NSCLC. In sensitivity analysis to determine the effect of omitting a single study on the overall effect, it showed the overall ORs were between 4.3 (95% CI: 2.46-7.52) and 5.27 (95% CI: 2.92-9.53) in the random effect method, which suggested that combined OR was consistent and reliable**(S Figure S1)**.

A cumulative meta-analysis by the time of the published literature were also conducted, and we found the OR was tending to be stable (**figure 3**), which was suggested the result of the meta-analysis might more credible when add more incoming researches.

Using the similar methodology, the influence on meta-regression was analyzed by omitting one study each time to explore heterogeneity sources. The sample type of tissue or serum would be one of the heterogeneity sources when Begum et al (2011, USA) were removed from the meta studies (P< 0.026), likewise, the proportion of stage I and aim of the study would become heterogeneity source when Lin et al (2009, China), Zhang et al (2011,China) or Yanagawa et al (2003, Japan) was removed (P-value were 0.0046, 0.029 and 0.039 respectively), which suggested these factors should be considered in the future case-control association study.

**Validation by independent TCGA Lung cancer Dataset**

In order to make independent validation of the above results, we collected the data of the methylation status of 6 CpG sites (**S Table 1**) from lung cancer samples of TCGA Project. There is no significant difference in age and gender between the cases and controls (**S Table 1**). The methylation percentage of cg15020645, cg16970232, cg20311501, cg21634602 and cg24332422 were dramatically different between the two groups, especially in adenocarcinoma. The methylation of all these 6 CpG sites were significantly different between Ad and its counterparts based on t-test after FDR adjustment (P<10-17), whereas, only two CpG sites (cg16970232, cg20311501) were significantly different between Sc and its counterparts (P = 1.6×10-6 and 3.9×10-3), and the significant level in Sc was dramatically lower than that in Ad (**Table 4**). In addition, logistic regression analysis also supported the above results: the ORs in Ad were from 23.3 to 1.2×103, while those were from 0.15 to 7.54 in Sc **(Table 5)**.

However, pairwise methylation correlation analysis showed that methylation status was high correlated among these CpG sites except cg01240931. Meanwhile, cg01240931 were hypermethylated in both the cancer and normal specimens. Therefore, this CpG site were excluded in the following analysis. The AUCs of the 5 CpG methylation test were calculated to assess their prediction ability. As shown in Table 5, each of the CpG site in Ad was much better in prediction (AUC: 0.71-0.73) than that in Sc (AUC: 0.45-0.61) **(Table 5).** In addition, the AUCs of the logistic prediction model based on all the 5 CpGs were 0.73 and 0.60 for Ad and Sc, respectively. It revealed from all the above evidences that APC methylation test would have better performance in adenocarcinoma than that in Sc, and therefore, the different proportion of Ad and Sc in the samples might bring certain bias for the association between APC methylation and NSCLC. Generally, 25% to 30% of lung cancers were Sc while 40% were Ad. Thus, we resampled the Ad and Sc from TCGA data to simulate the effect of different proportion of Ad versus Sc (Ad2Sc) at 2:1, 4:3, 3:4 and 1:2 on the odds ratio of APC methylation for NSCLC. The ORs were dramatically varied within group and between groups of the 5 CpGs by 10000 times of resampling simulations **(S Table s2**). As expected, cg16970232 and cg20311501, the significant sites in both Ad and Sq, were consistently significant risk factors for NSCLC, while the other three CpGs would loss association with NSCLC in certain vignettes (**S Table s2**). Moreover, ORs from logistic regression based on heterogeneous samples were significantly greater than those on the autogenous samples in the condition of Ad2Sc of 4:3, which was concordant with the above subgroup meta-analysis (**S Table s3**). Logistic based interaction analysis among age, gender with NSCLC showed there were no significant interaction in NSCLC risk between APC methylation and these covariates (**S Table s4**).

**DISCUSSION**

APC gene has been supposed as an important tumor suppressor gene in colorectal cancer[[35](#_ENREF_35)], and the aberrant of APC methylation had been reported in numeric of cancers, such as bladder[[36](#_ENREF_36)], prostate [[37](#_ENREF_37)], breast and lung cancer[[29](#_ENREF_29)]. However, the diagnostic role of the methylation status of APC gene in Lung cancer is lack of quantitative estimation. We therefore performed an integrated analysis to quantify the ability for APC promoter methylation test in NSCLC diagnosis, and a significant association was identified between APC methylation and NSCLC (OR = 4.67,P < 0.0001). Seven virtual studies were filled when trim and fill tests were performed to eliminate the influence of the publication bias in the random effects model, and the overall OR (2.49, 95%CI:1.18-5.26) was still significant, although it was slightly smaller than that in the crude meta-analysis (4.67,95%CI:2.66-8.22), indicating the existence of a strong association between APC promoter methylation and Lung cancer. The pooled sensitivity, specificity and AUC of APC methylation test in the present meta-analysis were 0.548, 0.78 and 0.64, respectively, which revealed that APC methylation status is a good biomarker in NSCLC diagnosis.

Integrated analysis showed that the age at the diagnosis, control type (autogenous or heterogeneous), the ratio of the adenocarcinoma to squamous cell carcinoma (Ad2Sc), and primer set (CpG site) were the most important heterogeneity sources, while sample type (tissue or serum), proportion of males, proportion of stage I, and detection methods could not explain the heterogeneity.

Age was one of the most important heterogeneity sources from meta-regression analysis (beta = -0.3, P = 2.0×10-5), meanwhile, the OR in the younger subgroup (OR = 4.65) was greater than that in older subgroup(OR = 2.24). However, we didn’t obtain the same conclusion in TCGA NSCLC dataset. Furthermore, neither Ad nor Sc data support that age would affect the odds ratio of the APC methylation to the risk of NSCLC in logistic regression model (P > 0.05), and thus much more evidence should be collected to make an eventual decision.

As to the contribution of Ad2Sc, both subgroup analysis and TCGA analysis showed significantly greater OR in high Ad2Sc than that in low Ad2Sc group, which suggested APC methylation test have better diagnosis performance for adenocarcinoma.

Since the late 1980s, various studies showed that the same genetic/epigenetic alterations, such as DNA methylation, in the primitive tumours were also found in the circulating DNA of the patients affected with tumours [[38-40](#_ENREF_38)]. Interestingly, in the present study, the odds ratio of the serum subgroup was greater than that of the tissue group and the AUC of APC methylation test for serum was greater than that for tissue in both meta- and microarray analysis, which indicated APC methylation test would be a promising serum biomarker for NSCLC diagnosis.

Meta-analysis has been widely applied in SNP-disease risk association study because SNPs had specific genome location, meanwhile, it is also booming in the realm of DNA methylation gradually. The primers for methylation detection have been considered when extracting information from studies, however, it was difficult to be analyzed in the following subgroup or meta-regression analysis sometimes since the high diversity of the primers used in each individual article. For example, at least 3 different primer sets were observed in the 17 studies we selected for meta-analysis (S table 5). Moreover, in order to expatiate on the divergence of different CpG sites, we collected the methylation signals of 5 CpGs from methylation 27K and 450K microarray dataset from TCGA project(Ad and Sc). It was found that the methylation status of the five different CpG sites were dramatically different, which the methylation ratio in cancers were from 14.8% to 48.4% (Table 4). Subgroup analysis further showed significantly different ORs in different primer set. It reminded that future DNA methylation detection in case-control studies should be designed more accurately and comprehensively to some certain CpG site or blocks and the location information should be clearly noted when published to facilitate the re-analysis of the published data.

In conclusion, this integrated analysis of the pooled data provided strong evidence that the APC promoter hypermethylation is significantly associated with NSCLC, especially with adenocarcinoma, and it would be a promising diagnosis biomarker for lung adenocarcinoma with remote non-invasive media detection.

**Authors’ contributions**

All authors have made substantial contributions to this article: ShichengGuo and JiuCun Wang, Li Jin, Jibin Xu contributed to the conception, design and final approval of the submitted version. ShichengGuo, Lixing Tan, KuanXu, JunJie Wu, Jinhui Wu contributed to the meta-analysis and interpretation of data, ShichengGuo and WeilinPu contributed to the TCGA NSCLC data analysis, All authors read and approved the final manuscript.

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Table 1, Characteristics of eligible studies considered in the report

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Author (Published Year) | Sample Type | Agea (years) | Stages I % | Gender (M/F) | Patients  (M+/M-) | Control (M+/M-) | Methods | Aim | Multiple Targe | Ad2Sc | Control  Design | ref. |
| **Zhanget al (2011,China)b** | tissue | 59 | 32.05 | 29/39 | 44/34 | 10/68 | MSP | Diagnose | Yes | 0.83 | hom | [[32](#_ENREF_32)] |
| Wang et al (2008, China) | tissue | NA | NA | 17/28 | 19/9 | 1/11 | 3-D PCR | Diagnose | Yes | 2.14 | heter | [[30](#_ENREF_30)] |
| Jin et al (2009, Japan) | tissue | 66.7 | NA | 17/24 | 27/45 | 22/41 | MethyLight | Non-diagnose | Yes | 1.87 | heter | [[20](#_ENREF_20)] |
| Feng et al (2008, USA) | tissue | 64.3 | 42.86 | 26/49 | 26/23 | 21/28 | MethyLight | Diagnose | Yes | 1.43 | hom | [[19](#_ENREF_19)] |
| Brabender et al (2001, USA) | tissue | 63.3 | 49.45 | 69/91 | 86/5 | 80/11 | qMSP | Non-diagnose | Single | 0.77 | hom | [[18](#_ENREF_18)] |
| Virmani et al (2001, USA) | tissue | NA | NA | NA | 22/26 | 0/18 | MSP | Diagnose | Yes | NA | heter | [[29](#_ENREF_29)] |
| Yanagawa et al (2003, Japan) | tissue | 67.3 | 66.67 | 18/25 | 28/47 | 36/39 | MSP | Diagnose | Yes | 1.48 | hom | [[31](#_ENREF_31)] |
| Topaloglu et al (2004, USA) | tissue | NA | 54.84 | NA | 17/14 | 5/17 | qMSP | Diagnose | Yes | 3.00 | heter | [[27](#_ENREF_27)] |
| Kim et al (2007, Korea) | tissue | 63 | 56.57 | 64/79 | 48/41 | 33/66 | MSP | Non-diagnose | Yes | 0.62 | hom | [[21](#_ENREF_21)] |
| Vallbohmer et al (2006, USA) | tissue | 63 | 49.45 | 69/91 | 86/5 | 80/3 | PCR | Non-diagnose | Yes | 0.77 | hom | [[28](#_ENREF_28)] |
| Lin et al (2009, China) | tissue | 61.1 | 100.00 | 20/31 | 49/75 | 2/24 | MSP | Diagnose | Yes | 1.84 | heter | [[22](#_ENREF_22)] |
| Shivapurkar et al (2007, USA) | tissue | NA | NA | NA | 35/5 | 23/17 | qMSP | Diagnose | Yes | 1.22 | heter | [[25](#_ENREF_25)] |
| Suzuki et al (2006, Japan) | tissue | 64 | 34.00 | 33/49 | 53/97 | 3/57 | MSP | Non-diagnose | Yes | NA | heter | [[26](#_ENREF_26)] |
| **Zhang et al (2011, China) b** | serum | NA | NA | NA | 54/56 | 5/45 | MSP | Diagnose | Yes | NA | heter | [[32](#_ENREF_32)] |
| Pan et al (2009,China) | serum | 53 | NA | 17/26 | 40/38 | 0/31 | qMSP | Diagnose | Single | NA | heter | [[23](#_ENREF_23)] |
| Begum et al (2011, USA) | serum | 65 | NA | 10/19 | 12/64 | 3/27 | qMSP | Diagnose | Yes | NA | heter | [[17](#_ENREF_17)] |
| Rykova et al (2004, Russia) | serum | NA | NA | NA | 3/6 | 0/16 | MSP | Diagnose | Yes | NA | heter | [[24](#_ENREF_24)] |
| Usadel et al (2002, USA) | serum | 64.2 | NA | NA | 42/47 | 0/50 | qMSP | Diagnose | Single | NA | heter | [[9](#_ENREF_9)] |

Agea, mean or median age from articles; Zhang et al (2011, China) b with two records since there are tissue and serum data simultaneously in this article.Sampe type represent the match method for the samples: tissue means tissue-tissue pairs, serum means serum-serum pairs.Methods were classified into two category: qualitativemethod denotes“MSP” while quantitativemethod denotes “qMSP”.

Table 2, Subgroup analysis for the main potential interference factors with random effect model

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  | Study | OR | 95%CI | Q | I2 | P-value |
| All | 12 | 3.28 | 1.74-6.17 | 52.78 | 79.2% |  |
| **Age≤ 64.5** | **9** | **5.03** | **2.53-10.0** | **27.96** | **71.4%** |  |
| **Age>64.5** | **3** | **0.91** | **0.57-1.41** | **2.21** | **9.4%** | <0.0001 |
| Stage I>49.45% | 5 | 4.11 | 1.90-8.91 | 12.76 | 68.60% |  |
| Stage I≤ 49.45% | 4 | 2.81 | 0.87-9.09 | 19.42 | 84.60% | 0.5944 |
| M2F≤ 69.1% | 6 | 5.98 | 2.04-17.53 | 16.66 | 70% |  |
| M2F> 69.1% | 6 | 2.13 | 0.99-4.55 | 29.05 | 82.80% | 0.1246 |
| MSP | 8 | 5.16 | 2.01-13.26 | 44.61 | 84.30% |  |
| qMSP | 10 | 4.32 | 2.08-8.94 | 29.28 | 69.30% | 0.7685 |
| Diagnose | 13 | 6.79 | 2.99-15.44 | 59.54 | 79.80% |  |
| Non-diagnose | 5 | 2.59 | 1.33-5.05 | 11.56 | 65.40% | 0.0745 |
| Multiple targets | 15 | 4.08 | 2.28-7.34 | 62.99 | 77.80% |  |
| Single target | 3 | 18.72 | 1.23-283 | 9.03 | 77.80% | 0.2836 |
| **heterogeneous** | **12** | **8.33** | **3.77-18.39** | **35.71** | **69.2%** |  |
| **autogenous** | **6** | **2.25** | **1.06-4.77** | **27.19** | **81.6%** | **0.0187** |
| Serum | 5 | 11.54 | 2.87-46.40 | 10.4 | 61.50% |  |
| Tissue | 13 | 3.72 | 2.03-6.78 | 55.18 | 78.30% | 0.14 |
| **Ad2Sc < 2** | **9** | **2.46** | **1.35-4.48** | **35.79** | **77.0%** |  |
| **Ad2Sc >= 2** | **2** | **17.1** | **4.68-62.7** | **0.11** | **0%** | **0.0077** |
| **Primer Set I** | **5** | **5.41** | **2.43-12.04** | **13.71** | **70.8%** |  |
| **Primer Set II** | **4** | **1.82** | **1.05-3.13** | **4.57** | **34.3%** | **0.0137$** |

Study represent the number of the study in each subgroup; P-value shows the significance of the difference between groups; Mean value of age, stage I proportion, M2F are taken as the separate threshold; $All serum groups and the studies whose sample size lower than 50 were remove when conducted primer type subgroup analysis to decrease the bias.

Table 3, Meta-regression analysis for the main potential interference factors with random-effects model

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | Coefficient(95%CI) | P-value | tau2 | QE | QE.P-value |
| Sample type | -1.03(-2.4,0.34) | 0.14 | 0.9 | 65.59 | 5.84E-08 |
| Age | -0.3(-0.44,-0.16) | **2.00E-05** | 0.18 | 17.57 | 0.062603 |
| Proportion of Stage I | -0.01(-0.05,0.03) | 0.608 | 0.79 | 33.5 | 2.14E-05 |
| Ratio of Male vs Female | -0.69(-8.1,6.71) | 0.855 | 0.98 | 50.51 | 2.15E-07 |
| Detection Methods | -0.09(-1.28,1.1) | 0.88 | 1.11 | 73.89 | 2.06E-09 |
| Study Aim | -0.82(-2.05,0.41) | 0.19 | 1.07 | 71.1 | 6.40E-09 |
| Multiple-Targets | 1.05(-0.71,2.81) | 0.243 | 1.01 | 72.02 | 4.41E-09 |
| Hetero/autogeous control | -1.25(-2.35,-0.15) | **0.026** | 0.89 | 62.9 | 1.68E-07 |
| Ad2Sc | 0.44 (-0.56 , 1.44 ) | 0.387 | 0.89 | 45.78 | 6.62E-07 |

P-values represent the significant of the coefficient; tau2 is the estimate of residual; QE.P-value represent the significant of the test for residual Heterogeneity;

**Table 4**, Differential APC methylation status (Beta) between adenocarcinoma, squamous cell carcinoma and its counterparts based on t-test

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | Adenocarcinoma | | | | Squamous Cell Carcinoma | | | |
| CpG Site | MCaM  (N=535) | MCoM  (N=56) | P-value | FDR | MCaM  (N=386) | MCoM  (N=70) | P-value | FDR |
| cg01240931 | 0.54 (96.4%) | 0.47 (100%) | 1.9×10-10 | **1.9×10-10** | 0.4 (75.39%) | 0.44 (100%) | 0.000178 | **0.000535** |
| cg15020645 | 0.26(40.7%) | 0.13(0%) | 3.5×10-32 | **1.0×10-31** | 0.13(14.77%) | 0.11(0%) | 0.087466 | 0.131199 |
| cg16970232 | 0.3(45.2%) | 0.11(0%) | 5.0×10-38 | **3.0×10-37** | 0.15(18.91%) | 0.09(0%) | 2.7×10-7 | **1.6×10-6** |
| cg20311501 | 0.33(48.4%) | 0.16(5.3%) | 1.4×10-22 | **2.1×10-22** | 0.18(19.95%) | 0.14(0%) | 0.001955 | **0.003909** |
| cg21634602 | 0.33(47.4%) | 0.16(7.1%) | 3.6×10-17 | **4.3×10-17** | 0.16(20.47%) | 0.14(7.14%) | 0.222306 | 0.266767 |
| cg24332422 | 0.26(40.5%) | 0.16(0%) | 1.0×10-26 | **2.0×10-26** | 0.16(17.36%) | 0.15(0%) | 0.338755 | 0.338755 |

MCaM, MCoM represent the mean of case methylation(Beta) and mean of control methylation(Beta). FDR,false discovery rate.Significant P-values after FDR are bolded. Methylation level are calculated with traditional formula: Beta=(M/M+U).

**Table 5,** significant association between APC methylation with adenocarcinoma and squamous cell carcinoma, respectively, based on logistic regression adjusted with age, gender.

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | Adenocarcinoma | | | | Squamous Cell Carcinoma | | | |
| CpG Site | OR | P-value | 95%CI | AUC | OR | P-value | 95%CI | AUC |
| cg01240931 | 1224.1 | **4.50×10-6** | 64.72-29732 | 0.75 | 0.148 | 0.074 | 0.018-1.20 | 0.58 |
| cg15020645 | 190.57 | **7.7×10-6** | 22.65-2321 | 0.72 | 3.16 | 0.406 | 0.28-68.72 | 0.61 |
| cg16970232 | 108.85 | **5.1×10-6** | 17.64-1043 | 0.73 | 7.54 | **0.0347** | 1.39-64.07 | 0.45 |
| cg20311501 | 61.56 | **4.96×10-6** | 11.94-420 | 0.73 | 2.48 | 0.257 | 0.57-13.74 | 0.49 |
| cg21634602 | 23.34 | **3.6×10-5** | 5.75-116. | 0.71 | 1.27 | 0.726 | 0.35-5.42 | 0.53 |
| cg24332422 | 223.63 | **2.81×10-5** | 21.11-3463 | 0.71 | 1.60 | 0.656 | 0.23-14.30 | 0.52 |

Figure 1. Combined estimates for the association between APC methylation and NSCLC.

Author, year, country of the studies and methylated and un-methylated numbers in case and control were labeled in the left column of the figure.Combined odds ratio(OR), 95% confidence region, weight of the combination for fixed and random model were labeled in the right column.The ORs of random effect model pooled and fixed effect model for APC methylation in cancer tissues compared with normal tissues were 4.67 (95%CI: 2.66-8.22, z=5.3534, P < 0.0001), 2.74 (95%CI:1.99-3.23, z =8.1038, P < 0.0001). The DerSimonian and Laird random effects modelor fixed effects model was selected to conduct combinationwhenheterogeneity statistic I2>50%, P ≤ 0.05 or I2<50%.

Figure 2.SROC of APC methylation test in NSCLC and PCa

The SROC curve is placed over the points to form a smooth curve. Linear regression model were selected to fit the SROC curve where sensitivity and (1-specificity) are transformed into complex logarithmic variables.The result showed that both sensitivity and specificity in diagnosis of prostate cancer(sen=0.75,spe=0.85) were higher than that in NSCLC (sen=0.55,spe=0.78). AUC for NSCLC was 0.671 while that for PCa was 0.82.

Figure 3. Funnel plot to diagnosis of the publication bias

Supplementary Figure 1 Sensitivity analyses of the overall effect by omitting a single study

Supplementary Figure 2 Combined estimates for the association between APC methylation and NSCLC after trimfill treatment

Supplementary Figure 3 Cumulative meta-analysis of studies ordered chronologically by publication year with random effect model