**Quantitative assessment of the diagnostic role of CDH13 promoter methylation in non-small cell lung cancer**

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**Abstract:**

Background: Aberrant methylation of CpG islands acquired in tumor cells in promoter regions plays an important role in carcinogenesis. Accumulated evidence demonstrates *CDH13* gene promoter hypermethylation is involved in non-small cell lung carcinoma (NSCLC), indicating it may be a potential biomarker for NSCLC diagnosis. The aim of this study is to evaluate the degree of *CDH13* gene promoter methylation between cancer samples and normal controls by summarizing published studies and public datasets.

Methods: By searching PubMed, Cochrane Library, OVID Medline and Web of Science databases, the open published studies about *CDH13* gene promoter methylation and NSCLC were identified using a systematic search strategy. The pooled odds of *CDH13* promoter methylation in lung cancer tissues versus normal controls were calculated by meta-analysis method. Simultaneously, four independent DNA methylation datasets of NSCLC from TCGA and GEO database were downloaded and analyzed to validate the results from meta-analysis.

Results: Fifteen studies, including 2109 samples were included in this meta-analysis. The pooled odds ratio of *CDH13* promoter methylation in cancer tissue was 6.06 (95% CI: 4.45 to 8.26, z =11.38, P<0.00001) compared to controls under fixed-effect model. In the validation stage, 63 paired samples from TCGA were analyzed and 5 out of the 6 CpG sites near the *CDH13* promoter were significantly hypermethylated in lung adenocarcinoma tissues but none of the 6 CpG sites was hypermethylated in the lung squamous cell carcinoma tissues. Similarly, other three datasets were subsequently obtained from GEO database consisting of 568 tumors and 256 normal tissues also consisted with the result from TCGA dataset.

Conclusion: The pooled data provides strong evidence that the methylation status of the *CDH13* promoter is strongly associated with NSCLC, especially for adenocarcinoma. And the significant difference of methylation level between lung adenocarcinoma and normal tissues is further validated with four independent datasets from TCGA and GEO database. While the association between *CDH13* methylation and lung squamous cell carcinoma is inconsistent between the meta-analysis and microarray results, which needs more comprehensive and advanced methods to draw a robust conclusion. In conclusion, the *CDH13* methylation test could be a promising diagnostic biomarker which could be applied in the clinical diagnosis of lung adenocarcinoma with remote non-invasive media detection.

## Key words

CDH13, DNA methylation, Non-small cell lung cancer, NSCLC, Diagnosis

## Introduction

Lung cancer is a complicated disease involving genetic and epigenetic variation, and is the leading cause of cancer death all over the world[[1](#_ENREF_1)]. Lung cancer is often silent in its early stages and difficult to diagnose in the early stages when treatment would be much more effective. Non-small cell lung cancer (NSCLC) comprises the majority of lung cancer and has an increasing incidence and mortality in the last two decades in China and in the world. The overall five-year survival rates for late stage III and IV of NSCLC patients were just 5%-14% and 1% respectively, however, the rate could come up to 63% for the early stage Ia if treated with surgery properly [[2](#_ENREF_2), [3](#_ENREF_3)]

DNA methylation is one of the key epigenetic modifications in eukaryote, which regulates genes and microRNAs expression [[4](#_ENREF_4)], gene alternative splicing[[5](#_ENREF_5)], playing important role in the developing of cancer. Moreover, with the advantages of stable chemical property, detection ability in remote patient media, quantitative signal, relatively low cost in detection, DNA methylation has been regarded as a promising non-invasive biomarker to detect lung cancer in the early stage[[6](#_ENREF_6)].

The *CDH13* gene, an atypical member of the cadherin superfamily, was isolated recently and has been mapped to 16q24 [[5](#_ENREF_5)], which was devoid of a transmembrane domain and anchored to the exterior surface of the plasma membrane via a glycosylphosphatidylinositol anchor [[7](#_ENREF_7)]. Evidence showed that promoter methylation, which inhibits *CDH13* gene expression, is mediated by DNA methyltransferases Dnmt3A [[8](#_ENREF_8)]. Researchers have reported that hypermethylation and loss of function of *CDH13* was detected in in breast [[9](#_ENREF_9)] and lung cancers [[10-12](#_ENREF_10)], in pituitary adenomas [[13](#_ENREF_13)], diffuse large B cell lymphoma [[14](#_ENREF_14)], and nasopharyngeal carcinoma [[15](#_ENREF_15)]. Furthermore, *CDH13* gene has been suggested as an early detecting marker for lung cancers [[16](#_ENREF_16)].

In this article, we conducted a meta-analysis of the sensitivity and specificity of *CDH13* methylation on NSCLC diagnosis. The studies included were homogeneous according to the low I−squared value. We also found that The Cancer Genome Atlas project (TCGA) and Gene Expression Omnibus (GEO) had collected hundreds of whole genome DNA methylation microarray datasets of NSCLC samples with comprehensive clinical and demographic information, providing additional resources that may be without publication bias. Several studies have showed the improved robustness of combining data from papers and databases[[17](#_ENREF_17)]. In our work, we innovatively integrated these microarray data with the data from published articles to evaluate the diagnostic ability of the *CDH13* methylation test in NSCLC.

## Results

### **Study characteristics**

The electronic search strategy identified 365 potentially relevant articles (PubMed, 73; Web of science, 177; OVID Embase, 115; Cochrane Library, 0), 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, 13 studies with data on the relationship between *CDH13* gene promoter methylation and NSCLC were pooled for analysis (Table 1) [[12](#_ENREF_12), [16](#_ENREF_16), [18-28](#_ENREF_18)]. All these articles were written in English. In total, 1379 lung cancer tissues/serum and 730 normal counterpart tissues/serum were collected. The age of the subjects in the 14 studies ranged from 26 to 87 years, with mean or median ranging from 59 to 70 years. As for the study aim, 5 articles were especially aiming at diagnosis, while the others were for prognosis, survival research, and so on. Among 14 studies, the proportions of stage I samples differed from 9.52 to 68.57%, and the percentage of male individuals in the NSCLC samples has a range of 52 to 80%. For the experimental methods to explore *CDH13* promoter methylation status, 9 of 14 inclusions used methylation-specific polymerase chain reaction (MSP), while others used quantitative MSP (qMSP, such as Methylight, Prosequencing, and so on). Four kinds of methylation detection primers or probes were found to be utilized for most of the 14 studies (Table S1).

### **Meta-analysis**

The ORs for *CDH13* methylation in cancer samples (tissue or plasma) compared with that in normal controls were 6.74 (95% CI: 4.46 to 10.20, z =9.07, P<0.00001) in random effects model pooled using Inverse Variance method, and 6.06 (95% CI: 4.45 to 8.26, z =11.38, P<0.00001) in fixed effects model using Inverse Variance method, demonstrating a statistically significant increasing in likelihood of methylation in lung cancer tissues comparing to controls. A homogeneity analysis revealed that the variation among them was not significant (I2=35%, Chi2=21.51) (Figure 1).

Subgroup analyses were conducted for different subtypes, which included sample types (tissue or serum), age, counterpart categories, proportion of early stage, aim of the study (for diagnosis or non-diagnosis), proportion of adenocarcinoma (Ad%) and other possible confounding factors (Table 2). Significant differences were found only between the ORs of the diagnosis (3.48, 95% CI: 2.18 to 5.58) and non-diagnosis (9.26, 95% CI: 6.13 to 13.97) subgroup (P = 0.0004) (Figure 2A). Both tissue and serum groups showed significant association between *CDH13* methylation and NSCLC (OR = 6.15 and 5.60, respectively) (Figure 2B) which suggested that *CDH13* methylation can be taken as a potential biomarker for NSCLC diagnosis using either tissue or serum samples. No significant difference was found between subgroups of MSP and qMSP (OR = 5.98 and 6.34, respectively; P = 0.62), which suggested that the two methods were equivalent in methylation detection (Figure 2C). In addition, there were no significant differences between the subgroups of ratio of male to female, counterpart categories, proportion of adenocarcinoma, the primer set as well as other factors (Table 2).

Because of the significant differences between the diagnosis and non-diagnosis subgroup, we conducted further research in the non-diagnosis subgroup. However, when we focused on the studies not aiming at diagnosis, the OR was found significantly reduced in subgroups of the proportion of adenocarcinoma <70% (9.55, 95% CI: 6.22 to 14.67) than >70% subgroup (21.03, 95% CI: 5.79 to 7) (p=0.26) (Figure 2D).

### **Summary receiver operating characteristic curve for diagnostic capacity of CDH13 methylation**

Pooled sensitivity and specificity were 0.410 (95%CI: 0.344 - 0.479) and 0.897 (95%CI: 0.839 - 0.936) for all the studies based on the presupposition of the fixed effects model. The sensitivity of the tissue group was higher than that of the serum group, 0.430 (0.348 to 0.516) versus 0.355 (0.247 to 0.482), while the specificity of the tissue group was higher than that of the serum group, 0.936 (0.792 to 0.982) versus 0.89 (0.814 to 0.938), which suggested biopsy in tissue still have advantage over non-tissue samples.

Although sensitivity and specificity were two of the most important features of a diagnostic test, in some occasions, pooling sensitivity or specificity could be misleading as mentioned in the Methods section. Therefore, we constructed the summery receiver operating characteristic (SROC) curve to depict the stability and accuracy of the methylation test’s diagnostic ability. The area under the curve (AUC) of the SROC was 0.674, suggesting a fair ability for NSCLC diagnosis (Figure 2E).

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

A funnel plot of methylation status of lung cancer tissues versus normal tissues showed significant publication bias (Egger test, z = 3.1605, P = 0.007519) and 1 study exceeded the 95% confidence limits (Additional file 2: Figure S1).

In order to eliminate the effect of publication bias, trim and fill analysis was performed with the random effects model. The adjusted pooled OR were 5.04 (95% CI: 3.20 to 7.96) in the random effects model and 4.93 (95% CI: 3.69 to 6.60) in the fixed effects model. Both results demonstrate a significantly positive association between *CDH13* methylation and NSCLC (Additional file 2: Figure S2).

In sensitivity analysis to determine the effect of omitting a single study on the overall effect, the overall ORs were between 6.01 (95% CI: 4.15 to 8.70) and 7.40 (95% CI: 4.89 to 11.18) in the fixed effects method, which suggested that combined OR was consistent and reliable (Additional file 2: Figure S3).

### **Validation by independent TCGA and GEO lung cancer dataset**

Data from The Cancer Genome Atlas (TCGA) and Gene Expression Omnibus (GEO) was used to verify the findings from the meta-analysis. In TCGA dataset, lung adenocarcinoma (LUAD) and lung squamous cell carcinoma (LUSC) methylation datasets were obtained for further analysis. There were 6 CpG sites sharing the same CGI (CpG Island) with the primers in the papers collected for meta-analysis, and the overall methylation status of the six CpG sites could be used to represent the methylation status of *CDH13* gene. Surprisingly, the result from LUAD dataset and LUSC dataset differed and differential methylation profiles were shown between these two subtypes (Figure 3). In LUAD methylation dataset, 5 out of the 6 CpG sites showed significantly differential methylation level between cancer tissues and paired adjacent normal tissues according to the criteria (See Method). While in LUSC dataset, though 3 of the 6 CpG sites had p-values less than 0.05 after multiple correction, the absolute mean difference was less than 0.1 for all and thus couldn’t be considered as significantly methylated as well (Table 3).

In order to draw a more robust conclusion, GEO dataset GSE39279 as well as GSE52401 were then downloaded and combined from Gene Expression Omnibus. There were 322 lung adenocarcinomas and 122 lung squamous cell carcinomas and 244 normal tissues in the combined dataset, which is of sufficient sample size to be an independent validation database. We performed the same analysis as before and obtained the result consistent with the TCGA dataset. Due to the large sample size, p-values of all the CpG sites in each dataset were less than 0.05 after multiple corrections. However, In LUAD dataset, 5 out of the 6 CpG sites showed absolute mean difference above 0.1 and thus could be regarded as significantly methylated while none of the CpG sites passed the criteria in LUSC dataset (Table S3). Moreover, another independent GEO dataset GSE56044 with 83 lung adenocarcinoma and 23 lung squamous cell carcinoma tissues and 12 adjacent normal tissues was also downloaded for further validation. Unsurprisingly, the result was almost the same with the previous two datasets. According to our criteria, 6 out of 7 CpG sites were significantly hypermethylated in LUAD dataset while none of the 7 CpG sites was differentially methylated in LUSC dataset (Table S4).

### **Gene Expression data with TCGA RNA-Seq dataset**

DNA methylation is one of the key regulators for gene expression. It is found that DNA methylation change especially in promoter region would affect gene expression level. As a result, we downloaded the level 3 RNA-Seq dataset from TCGA. Per million mapped reads (RPKM) was used as the measurement for gene expression. After calculating the fold change and p-value with multiple correction, significantly differential expression was shown in LUAD (p-value: 4.53e-12, fold change: 0.437) but not shown in LUSC (p-value: 0.16, fold change: 1.120) samples when compared with normal tissues, which was also concordant with the result from the microarray data analysis (Figure 3).

## Discussion

The *CDH13* gene has been reported hypermethylated in many types of cancers, such as breast [10] and lung cancers [11-13], in pituitary adenomas [14], diffuse large B cell lymphoma [15], and nasopharyngeal carcinoma [16]. However, the diagnostic role of the methylation status of the *CDH13* gene in lung cancer lacks quantitative assessment. We therefore performed an integrated analysis to quantify the ability for the *CDH13* promoter methylation test in NSCLC diagnosis, and a significant association was identified between *CDH13* methylation and NSCLC (OR = 6.06, 95% CI: 4.45 to 8.26, P < 0.00001). Five imputed studies were filled when trim and fill tests were performed to eliminate the influence of publication bias on the fixed effects model, and the overall OR (4.93, 95% CI: 3.69 to 6.60) was still significant, although it was slightly smaller than that in the crude meta-analysis, indicating the existence of a strong association between *CDH13* promoter methylation and lung cancer.

In order to validate the result from the meta-analysis, we downloaded four independent datasets from TCGA and GEO database. And unexpected, the methylation profile in the two subsets of lung cancer differed dramatically. All of the datasets from TCGA and GEO showed significant hypermethylation in the promoter CpG sites of lung adenocarcinoma tissues when compared with normal tissues. However, none of the CpG sites was significantly differential methylated between lung squamous cell carcinoma tissues and normal tissues. Moreover, the expression data from TCGA level 3 RNA-Seq data were also concordant with this conclusion. The expression level of *CDH13* was significantly lower in lung adenocarcinoma tissues but not in lung squamous cell carcinoma tissues when compared with normal tissues. This result was partially confirmed when we focused on the studies not aiming at diagnosis, the OR was found to be largely reduced in subgroups of the proportion of adenocarcinoma <70% (9.55, 95% CI: 6.22 to 14.67) than >70% subgroup (21.03, 95% CI: 5.79 to 7).

To summarize, according to the previous results drawn from meta-analysis and microarray data analysis, *CDH13* may be a powerful potential biomarker for the diagnosis of lung adenocarcinoma and may be played a role in the carcinogenesis of lung adenocarcinoma while the association of *CDH13* methylation with lung squamous cell carcinoma needs more data to draw a robust conclusion.

**Conclusion**  
In conclusion, this integrated analysis of the pooled data provides strong evidence that the methylation status of the *CDH13* promoter is strongly associated with NSCLC, especially for adenocarcinoma. And the significant difference of methylation level between lung adenocarcinoma and normal tissues is further validated with four independent datasets from TCGA and GEO database. However, different results were drawn from meta-analysis and microarray dataset for the association of *CDH13* and lung squamous cell carcinoma. It was found that none of the CpG sites in the *CDH13* promoter was significantly differential methylated from the microarray dataset. However, significant association was found between *CDH13* methylation and lung squamous cell carcinoma through meta-analysis though the ORs were largely reduced in subgroups of the proportion of adenocarcinoma <70% than >70% subgroup. As for the relatively inconsistent result, firstly, qMSP is the semi-quantitative method especially being used in low dose expression. Secondly, the sparseness of CpG sites in HumanMethylation 450K array may be a key factor. For the microarray was based on the designed probes to detect the methylation of selected CpG sites on the genome and couldn’t cover the whole CpG island region of *CDH13* and therefore might be misleading. As a result, more comprehensive and advanced methods like WGBS (whole genome bisulfite sequencing) or RRBS (restricted region bisulfite sequencing) are needed to draw a more robust conclusion. In conclusion, the *CDH13* methylation test could be a promising diagnostic biomarker which could be applied in the clinical diagnosis of lung adenocarcinoma with remote non-invasive media detection and more data are needed to explore and confirm the correlation between *CDH13* methylation and lung squamous cell carcinoma.

## 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 Web of Science for articles published in English by December 2014. The study used a subject and text word strategy with (CDH13 OR CDHH OR P105 OR H-cadherin OR Cdht OR T-cadherin OR Tcad OR CH211-122A20.1 OR BOS\_16969 OR cdhh) AND (lung or non-small) as the primary search terms. Wildcard character of star, dollar or some other truncations were applied according to the rules of the databases to allow effective article collection.

Two independent reviewers (Geng, Guo) screened the titles and abstracts derived from the literature search to identify relevant studies. The following types of studies were excluded: animal and cell experiments, case reports, reviews or meta-analyses and studies of non-case-control studies or studies with insufficient data or those proving inaccessible after making contact with the authors. The remaining 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 contain *CDH13* gene promoter methylation data from tissue, blood or serum, 3) the studies had to be case-control studies 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 (Geng, Guo). Disagreements were resolved by discussion with LXT, SDC, and LJ. 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), the percentage of adenocarcinoma (Ad%), publication aim (for diagnosis or not), analyzing multiple genes or not (one or more genes detected simultaneously in studies design), control type (autogenous or heterogeneous counterpart) and methylation status of the *CDH13* promoter in human NSCLC and normal or control tissues were extracted.

### **Meta-analysis and SROC 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. 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. Tau-squared (τ2) was used to determine how much heterogeneity was explained by subgroup differences. 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. A two-sided P ≤ 0.05 was considered significant without special annotation. Sensitivity analyses were performed to assess the contributions of single studies to the final results with the abandonment 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 studies, 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, because the extremes of threshold criteria could skew the distribution, known as the threshold effect. Thus, SROC analysis was applied to meta-analysis of diagnostic tests. The SROC curve shows the performance of the diagnostic ability of *CDH13* methylation to NSCLC. Each study produces values for sensitivity, specificity and therefore true positive rate (TPR) and false positive rate (FPR), and the plots were placed over the TPR and FPR points to form a smooth curve. A linear regression model was selected to fit the SROC curve where sensitivity and (1-specificity) are transformed into complex logarithmic variables. The exact AUC for the SROC function was used to assess the accuracy of the test.

### **TCGA data extraction and analysis**

TCGA DNA methylation datasets which included 23 lung adenocarcinoma and 40 lung squamous cell carcinoma tissues as well as 63 paired adjacent tissues, were collected from TCGA project [http://cancergenome.nih.gov/]. And GEO datasets including GSE39279 and GSE52401 and GSE56044 were downloaded from Gene Expression Omnibus [http://www.ncbi.nlm.nih.gov/geo/], including a sum of 568 NSCLC tissues and 256 adjacent or normal lung tissues. Illumina HumanMethylation450K Beadchip was used to detect the methylation level for all of the above datasets. The estimation of methylation for each CG probe was calculated between methylated (M) and unmethylated (U) alleles. Specifically:

beta =

Both M and U represent mean signal intensities for about 30 replicates on the array. Beta value of the CpG sites are used as the measurement of methylation. CpG site would be immediately omitted if there was one missing samples or more in the dataset. CpG sites of CDH13 gene in TCGA dataset and GEO datasets were not completely the same due to the quality control procedure previously mentioned. 6 or 7 CpG sites located in the same CpG island with the primers mentioned in the meta-analysis were the signatures for the methylation status of *CDH13* (Table S1). Wilcoxon rank sum test along with logistic regression were conducted and generated a p-value for each comparison. Multiple comparison of the differential methylation was conducted with Benjamini and Hochberg at 5% FDR as the threshold. The statistical analysis was performed using R version3.1.0.

### **RNA-Seq data extraction and analysis**

Level 3 RNA-Seq dataset was obtained from TCGA database, which includes 114 lung adenocarcinoma and 104 lung squamous cell carcinoma as well as 218 normal tissues. Per million mapped reads (RPKM) was regarded as the measurement for gene expression. We assessed the significance of the differential gene expression by comparing the tumor tissues with paired adjacent normal tissues using Wilcoxon rank sum test. For identification of differentially expression genes, p-value0.05 and fold change 2.0 or was set as the criteria. All the data analysis was conducted with open-source R software (version 3.1.0)

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