A panel of promoter methylation markers for invasive and noninvasive early detection of NSCLC using a quantum dots-based FRET approach

Yunfei Ma^{a,b,1}, Yanan Bai^{a,1}, Hailei Mao^c, Qunying Hong^d, Dawei Yang^d, Honglian Zhang^a, Fangming Liu^a, Zhenhua Wu^a, Qinghui Jin^a, Hongbo Zhou^a, Jian Cao^e, Jianlong Zhao^a, Xinhua Zhong^{b*}, Hongju Mao^{a*}

^aState Key Laboratory of Transducer Technology, Shanghai Institute of Microsystem and Information Technology, Chinese Academy of Science, Shanghai, 200050, China.

^bInstitute of Applied Chemistry, East China University of Science and Technology, Shanghai, 200237, PR China

^cDepartments of Anesthesiology and Critical Care Medicine, Zhongshan Hospital, Fudan University, Shanghai 200032, China

^dDepartment of Pulmonary Medicine, Zhongshan Hospital, Fudan University, Shanghai, 200032, China

^eDepartment of Medicine/Cancer Prevention, Stony Brook University, Stony Brook, NY 11794, USA

¹Yunfei Ma and Yanan Bai contribute to this work equally.

***Corresponding Authors:**

Xinhua Zhong, E-mail: zhongxh@ecust.edu.cn

Tel: +86 21 6425 0281; Fax: +86 21 6425 0281

Hongju Mao, E-mail: hjmao@mail.sim.ac.cn

Tel: +86-21-62511070-8707; Fax: +86-21-62511070-8714

ABSTRACT

Non-small-cell lung cancer (NSCLC) leads to a significant proportion of cancer-related deaths, and early detection of NSCLC can significantly increase cancer survival rates. A promising approach has been studied to exploit DNA methylation, which is closely correlated to early cancer diagnosis. Herein, in order to realize the early detection of NSCLC, we utilized the developed quantum dots-based (QDs-based) fluorescence resonance energy transfer (FRET) nanosensor technique to analyze the promoter methylation in early stage NSCLC tissue samples and noninvasive bronchial brushing specimens. Using this method, the methylation levels can be quantitatively determined by measuring the signal amplification during FRET. A panel of three tumor suppressor genes (PCDHGB6, HOXA9 and RASSF1A) was assessed in 50 paired early stage NSCLC and their adjacent nontumorous tissue (NT) samples, and 50 early stage NSCLC bronchial brushing and normal specimens. The combined detection was able to identify not only tissue samples but noninvasive bronchial brushing specimens from control cases with a high degree of sensitivity of 92% (AUC=0.977, $P<0.001$) and 80% (AUC=0.907, $P<0.001$) respectively, indicating the versatility of promoter expression in invasive and noninvasive NSCLC samples. Therefore this approach can be used to sensitively analyze the methylation levels of cancer-related genes, which might be a potential tool for noninvasive early clinical diagnosis of cancers.

Keywords: Promoter methylation, Early detection, NSCLC, Bronchial brushing, Quantum dots, FRET

1. Introduction

Non-small cell lung cancer (NSCLC) includes both adenocarcinoma (ADC) and squamous cell carcinoma (SCC) (Jemal et al., 2010). In men and women, it comprises the most common malignancy and is characterized by a high worldwide incidence couple with low survival rate (Jemal et al., 2010). The diagnosis and treatment of NSCLC is guided primarily by an assessment of the tumor stage. Patients with either Stage I or II are treated by surgical resection, while Stage III patients require a combined strategy featuring surgery and chemotherapy and/or radiation. Despite these aggressive approaches, the overall five-year survival rate for patients diagnosed with NSCLC remains relatively low (Guo et al., 2014). This is due in large part to the difficulty in the early detection of lung cancer (Guo et al., 2014). Thus, finding an effective method of early detection is necessary to prevent tumor progression, which will ultimately leading to a reduction in lung cancer mortality.

On potential solution to early detection is the use of molecular markers. Among the wide range of possibilities, the use of epigenetic DNA methylation patterns holds great promise. DNA methylation is an important epigenetic mark that involves the transfer of a methyl group to the fifth carbon position of the cytosine, resulting in 5-methylcytosine (Bird, 2002). DNA methylation regulates gene expression, particularly those containing CpG-rich areas (termed CpG islands) that can be found within the promoter regions (Jones, 2012). Intensive work over the past years has shown that aberrant DNA methylation in these regions is one of the most well-defined epigenetic changes in human cancers (Lister et al., 2009). Not only could this feature be utilized to distinguish cancer cells from normal tissue, but also exploited for further use in early cancer diagnosis.

Given the current knowledge on the importance of DNA methylation, numerous detection methods have been developed to better assay any changes. For instance, restriction enzyme-based methods allow for simple and sensitive detection of DNA methylation through PCR amplification (Mikeska et al., 2012; Melnikov et al., 2005). However, this approach is not easily to achieve quantitative results (Mikeska et al., 2012; Melnikov et al., 2005). Bisulfite-based methods provide qualitative and/or quantitative DNA methylation analyses and also allow for single-nucleotide resolution. Methylation specific-PCR (MSP) is a common bisulfite-based detection method with PCR-assistance. It is a sensitive methodology,

but only gives a binary report (e.g. yes/no) for methylation status (Herman et al., 2008). Some quantitative approaches have also been established, including quantitative MS-PCR (qMSP) (Lehmann et al. 2002), methylation-sensitive high-resolution melting (MS-HRM) (Wojdacz and Dobrovic, 2007), MethyLight (Eads et al., 2000), methylation-specific fluorescent amplicon generation (MS-FLAG) (Bonanno et al., 2007), methylation-sensitive single-nucleotide primer extension (Ms-SNuPE) (Gonzalvo and Jones, 1997), combined bisulfite restriction analysis (COBRA) (Xiong and Laird, 1997), bisulfite genomic sequencing (Shiraishi and Hayatsu, 2004), and pyrosequencing (Colella et al., 2003; Tost and Gut, 2007). All of these methods undergo chemical treatment with sodium bisulfite and are able to achieve quantitative detection of DNA methylation. However, the aforementioned approaches are not without problems, which can include one or more of the followings: design of dye-labeled DNA probes, the use of radioactive materials, high cost for detection, limited read length of DNA, and the need for complicated procedures and/or specialized instruments. These wide-ranging disadvantages severely limit their widespread application.

Recently, our group has established a novel strategy for the optical detection of DNA methylation based on fluorescence resonance energy transfer (FRET) of quantum dots (QDs) (Ma et al., 2015). This method relies on a methylation-sensitive restriction enzyme for the differential digestion of methylated and unmethylated DNAs. Digested DNA is subjected to PCR amplification for the incorporation of Alexa Fluor-647 (A647) fluorophores. DNA methylation levels can be quantitatively determined by measuring the signal amplification from QDs to A647 during FRET. In our previous study, the method was established and few random lung ADC samples were tested to prove the feasibility of this technique. Herein, further early clinical testing was performed to verify the reliability of the established approach. Moreover, in order to observe the ability for early cancer detection in two types of NSCLC samples by the developed QDs-based FRET method, we utilized the method to analyze the gene promoter methylation in both early stage tumor tissues and noninvasive bronchial brushing specimens (including Stage I and Stage II, ADC and SCC). Considering the possible risks of bleeding or other contraindications during biopsy, the examination in noninvasive endobronchial brushings offers a more meaningful test for the detection of lung cancer (Huang et al. 2012). It should be noted that most reports on the examination of DNA

methylation in NSCLC require invasive biopsy tissue samples, few studies have been reported on noninvasive bronchial brushing specimens. The methylation levels of three potential tumor suppressor genes in lung cancer (PCDHGB6, HOXA9 and RASSF1A) were examined in these specially selected early stage samples. By using subsequent associated analysis of the methylation levels, the detection sensitivity and specificity of tumor tissue and bronchial brushing specimens were obtained and compared respectively.

2. Material and methods:

2.1 Synthesis of amino-CdSe/CdS/ZnS QDs

The amino-QDs were started with initial oil-soluble CdSe/CdS/ZnS QDs and further modified with cysteamine hydrochloride by a thiol exchange method. The detailed procedure has been described in published report (Ma et al., 2015).

2.2 Sample preparation

Paired lung ADC tissues and NT samples and paired SCC and NT samples were obtained from 50 patients who underwent primary surgical resection of lung ADC and SCC in 2014 at Shanghai Zhongshan Hospital. 50 bronchial brushing specimens including 40 ADC and SCC and 10 normal specimens were also obtained from 50 patients at Zhongshan Hospital. Patients' demographic and clinical characteristics are summarized in Table 1. All patients gave informed consent prior to sample collection. The pathological stage grade of each sample was appraised by an experienced pathologist. Staging was determined according to the TNM (Tumor Node Metastasis) Classification of Malignant Tumors. All aspects of this study were approved by the Institutional Review Board of Shanghai Zhongshan Hospital, China.

[Insert Table 1]

Genomic DNA of tissues samples and bronchial brushing specimens were extracted from frozen samples using an AllPrep DNA/RNA Mini Kit (50) (QIAGEN) according to the manufacturer's instructions. The DNA concentration for each sample was quantified using a NanoDrop ND-1000. Then the genomic DNA was divided into two separate samples. The first sample was treated with the methylation-sensitive restriction enzyme, HpaII (New England Biolabs). Briefly, 100 ng of extracted DNA, 10 units of HpaII, 1×NEBuffer and deionized water were mixed in a tube. The second sample was subjected to non-HpaII

treatment, which contained an equal amount of extracted DNA and 1×NEBuffer, but not HpaII. Deionized water was added to the tube to until it was at an equal volume to the first HpaII-treated sample. Both reaction systems were incubated at 37°C overnight, followed by heat inactivation of HpaII at 80°C for 20 min. The treated DNA samples were stored at 4°C until subsequent PCR amplification.

2.3 Gene selection and primer design

In this study, three potential suppressor genes in NSCLC—PCDHGB6, HOXA9 and RASSF1A—were selected to determine their methylation levels in the extracted DNA samples. All genes should have covered at least two 5'-CCGG-3' sites. For some specific genes that did not reach this criterion, other enzymes such as HhaI (5'-GCGC-3' recognition site) or BstUI (5'-CGCG-3' recognition site) were used instead of HpaII. All primer sequences are listed in Table S1.

2.4 Methylation level detection

The detection of promoter methylation levels was performed using the QDs-based FRET method. Both of the DNA samples (HpaII-treated and untreated) were amplified using one-round PCR (Takara Gradient PCR equipment). The PCR reaction mixture contained the following: 10 ng DNA sample, 1×Taq buffer, 0.5 mmol/L MgCl₂, forward and reverse primers (0.25 μM each), 1×dNTPs/A647-dNTPs mix (16 μmol/L dATP, 16 μmol/L dGTP, 14 μmol/L dCTP, 14 μmol/L dTTP, 2 μmol/L A647-dCTP, and 2 μmol/L A647-dUTP) and 1 unit Hot Start Taq polymerase (Takara). The final reaction volume was 20 μL. As the negative control, water was used in place of DNA sample and was amplified in an identical manner to experimental samples. The thermocycling program was as follow: 95°C for 10 min, followed by 40 cycles of 95°C for 10 s, 55°C for 10 s, and 72°C for 30 s, and a final elongation step at 72°C for 10 min. To degrade the excess dNTPs and A647-dNTPs, 1.5 units of SAP (Takara) and 1.8 μL of 10×SAP buffer were mixed with 15 μL PCR products. The mixture was incubated at 37°C for 60 min, after which the enzyme was heat inactivated (65°C for 20 min). After SAP digest, 1 μL of prepared amino-QDs (5 μmol/L) was diluted in 180 μL HEPES buffer (25 mmol/L, pH 7.0, Sigma) and then mixed with digested DNA samples in a 96-well plate. The mixture was incubated at 37°C for 30 min, fluorescence signals were finally measured using a microwell plate reader (Horiba fluoromax-4 spectrofluorimeter) with an

excitation wavelength of 500 nm. Methylation levels could be calculated through the fluorescence amplification signal from QDs to A647. To do this, parameter E was used, in which FRET ratios represent the ratio of emission intensities of A647 to QDs ($I_{670\text{ nm}}/I_{600\text{ nm}}$). Mathematically, parameter E was defined as follows:

$$E = \frac{\text{FRET ratio}_{\text{HpaII}} - \text{FRET ratio}_{\text{blank}}}{\text{FRET ratio}_{\text{non-HpaII}} - \text{FRET ratio}_{\text{blank}}}$$

2.5 Statistical analysis

A conventional statistical package (SPSS version 17.0) was used to determine the receiver operating characteristic (ROC) curve and the area under the curve (AUC). The ROC and AUC were operated to obtain the highest sensitivity and specificity. Statistical differences between tumor and control samples were evaluated by Student's t-test. All p-value were two-sided and $p < 0.05$ was considered statistically significant.

3. Results and discussion

3.1 Principle and feature of QDs-based FRET method for methylation detection

To date, genome-wide methylation studies in NSCLC have concentrated on specific areas of the promoter regions to detect early stage lung cancer. This early detection is crucial, since it allows for a dramatic increase in survival rates. In this study, we used a QDs-based FRET technique for the detection of promoter methylation levels in early stage NSCLC in tumor tissues and bronchial brushing specimens. The principle for methylation detection using QDs-based FRET method is illustrated in Fig. 1. Genomic DNA was first divided into two separate samples. The first sample was treated with the methylation-sensitive restriction enzyme HpaII, which cleaves at unmethylated 5'-CCGG-3'sites, while leaving methylated DNA intact. The second sample was not subjected to HpaII cleavage. Both samples were then amplified using conventional PCR, itself accompanied by the incorporation of A647-dNTPs. Our negative blank control consisted of using water instead of genomic DNA in the reaction mixture. In the first sample, methylated DNA in the sample could be amplified since it was insensitive to HpaII cleavage, thus retaining an intact DNA strand. Contrastingly, unmethylated DNA was cleaved without PCR amplification. In the second part, both

methylated and unmethylated DNAs were intact for amplification and incorporation of A647-dNTPs. PCR amplification will not occur in the negative control due to the absence of any DNA template. After SAP degradation of excess dNTPs and A647-dNTPs, the resulting PCR products were mixed with amino-QDs. The positively charged amino-QDs are able to form a complex with negatively charged A647-labeled DNA. This brings the A647 fluorophores in close proximity to the QDs due to their electrostatic interactions. This feature meets the requirement for FRET, therefore the FRET signal from the QDs to A647-labelled DNA could be generated. For the HpaII-treated products, the FRET signal was determined by the A647-labeled DNA generated by methylated DNA templates. The more methylated DNA in a given sample, the stronger the resulting FRET signal was. In the case of HpaII-untreated products, both methylated and unmethylated DNAs were amplified, leading to highly efficient FRET signals. There was no FRET signal for the negative control, since the lack of a DNA template led to random dispersion of A647 fluorophores. Based on the principle discussed above, parameter E was defined to measure methylation levels with a subtracted background, where the $\text{FRET ratio}_{\text{HpaII}}$ refers to the ratio of emission intensities of A647 to QDs ($I_{670 \text{ nm}}/I_{600 \text{ nm}}$) for the HpaII-treated sample and $\text{FRET ratio}_{\text{non-HpaII}}$ represents the FRET ratio for the HpaII-untreated sample. The $\text{FRET ratio}_{\text{blank}}$ refers to the FRET ratio for the negative non-template control.

[Insert Fig.1]

Previous work by the Wang research group used cationic conjugated polymers (CCP) as the energy donor to quantitatively detect gene methylation and achieve high sensitivity for colon cancer detection (Feng et al., 2008; Feng et al., 2010; Yang et al., 2012; Zhang et al., 2014). When compared with organic fluorophores, QDs have several advantages, including a narrow emission spectrum, broad absorption spectrum, negligible photobleaching, and high photochemical stability (Wang et al., 2010; Clapp et al., 2006; Resch-Genger et al., 2008; Hötzer et al., 2012; Su et al., 2014). They are also within the size-dependent fluorescence emission spectra. This is beneficial to the accommodation of spectral overlap between donors and acceptors since particle size can be adjusted during synthesis (Goldman et al., 2004). Therefore QDs were selected for our bioanalyses. The QDs used in this study were positively charged, water-soluble amino-CdSe/CdS/ZnS QDs to facilitate the connection with

negatively charged DNA. As FRET is strongly distance dependent, the electrostatic interaction is the basis to connect FRET pairs to generate FRET signals in this study, and is notably more labour-saving and cost-saving than conventional covalent linking (Jiang et al., 2009). The QD emission wavelength was selected at 600 nm, which partially overlaps with the absorption spectrum of the A647 fluorophore, thus meeting the requirement for FRET system. When compared with conventional methods for methylation detection, the superior features of our QDs-based approach are as follows: its quantitative capacity, high sensitivity, simple procedure, and low cost. In addition, only a small amount DNA sample (~10 ng) is required. The experimentation was simplified by avoiding bisulfite converting, primer labeling, complicated post-treatment or sophisticated instruments to achieve detection. This method is different from most other fluorescence-based techniques in that it introduces A647-labeled nucleotide triphosphates to replace the dye-labeled DNA probes used in the MethyLight (Eads et al., 2000) and MS-FLAG (Bonanno et al., 2007) techniques. This switch not only simplifies the experimental design, but also reduces experimental cost. Furthermore, the quantitative analysis of methylation levels is accomplished by the commonly-used fluorescence spectrometer, which replaces the expensive, specialized quantitative real-time PCR (Wojdacz and Dobrovic, 2007) or sequencing machines (Shiraishi and Hayatsu, 2004; Colella et al., 2003; Tost and Gut, 2007) previously used in other methods. Moreover, high-throughput detection can also be achieved by using a microwell plate reader.

[Insert Fig.2]

3.2 Quantitative spectral analysis of different methylation levels between cancer sample and control sample

The promoter methylation of 50 paired early stage NSCLC and NT tissue samples, and 50 early stage NSCLC and normal bronchial brushing specimens were tested, both of which included ADC and SCC samples. Based on the aforementioned method, we performed a quantitative comparison of methylation levels between cancer and control samples. The HpaII-treated, non-HpaII-treated, and blank PCR products of cancer and control samples were respectively mixed with amino-QDs solution to conduct fluorescence analysis. When QDs were excited with a wavelength of 500 nm, they efficiently transferred energy to the fluorophores via FRET because of the electrostatic attraction, producing an amplified A647

fluorescence signal that could be used to detect the presence of DNA products. The exemplar spectra for different methylation levels generated from one pair of NT and cancer samples are shown in Fig. 2. Generally, the promoter-associated CpG islands in NT samples have lower levels of methylation, resulting in poor amplification by the treatment with HpaII. Thus, a low FRET ratio ($I_{670\text{ nm}}/I_{600\text{ nm}}=0.127$) was observed in HpaII-treated NT sample, i.e. $\text{FRET ratio}_{\text{HpaII}}=0.127$, which was attributed to the low amount of amplified dye-labeled DNA. When compared with the high FRET signal of HpaII-untreated NT sample ($\text{FRET ratio}_{\text{non-HpaII}}=0.432$) and the background signal ($\text{FRET ratio}_{\text{blank}}=0.085$) of the negative control, a low methylation level ($\sim 12\%$) was observed using parameter E (Fig.2A). In contrast, promoter CpG islands are usually highly methylated in cancer cells, leading to amplification of numerous methylated DNAs in HpaII-treated samples. Thus a higher FRET ratio was observed in HpaII-treated cancer sample ($\text{FRET ratio}_{\text{HpaII}}=0.295$) than that in NT sample. After the comparison with the FRET ratio of HpaII-untreated cancer sample ($\text{FRET ratio}_{\text{non-HpaII}}=0.450$) and the background signal ($\text{FRET ratio}_{\text{blank}}=0.085$), we finally found a robust optical amplification of the A647 fluorophore with a corresponding high methylation level ($E=\sim 58\%$) in tested cancer sample (Fig.2B). The corresponding schematic illustrations of different levels of FRET signal in NT and cancer samples are shown in the right of Fig. 2A, B, respectively. Furthermore, in order to confirm the levels of methylation detected by QDs-based FRET method, two known control samples with high and low methylation in PCDHGB6 gene detected by pyrosequencing method were mixed to define varying levels of methylation. Pyrosequencing is considered as the gold standard for methylation detection and the experimental process was described previously (Ma et al., 2015). With the known methylation levels, a standard curve was obtained versus the E value and is shown in Fig. 3. The standard error bars were calculated from three separate experiments. It can be seen that the E value showed a linear relationship with the methylation level with the correlation coefficient of $R^2=0.996$, indicating that the general trends of the E values obtained by QD-based FRET and the pyrosequencing results are consistent. With the standard curve, the E values detected by our method can realize the calibration and precision. Moreover, the figure shows that E values detected by the QDs-based FRET method are slightly higher than the results of the mixed pyrosequencing, which may be due to the incomplete digestion of DNA

samples by restriction enzyme HpaII or the potential dimers and by-products generated in PCR amplification.

[Insert Fig.3]

3.3 Statistical analysis of promoter methylation levels in tumor tissue and bronchial brushing specimens

Based on the superior property of our QDs-based FRET method, we sought to determine the relationship of histological analysis to tumor aggressiveness in early stage lung cancer tissues and bronchial brushing specimens. In this study, the DNA methylation status of a selected panel of three tumor suppressor genes (PCDHGB6, HOXA9, and RASSF1A) was assessed in lung cancer samples. These three genes were selected due to their potential relationship to lung cancer. PCDHGB6 is a member of the protocadherin gamma gene cluster, and has an immunoglobulin-like organization (Sandoval et al., 2013). Therefore, a novel mechanism may be involved in its regulation and expression. To our knowledge, few studies have been reported on this gene methylation expression in lung cancer. Only Sandoval et al. reported a signature based on the number of hypermethylated events by a single-methylation pyrosequencing assay to distinguish patients with high- and low-risk stage I NSCLC (Sandoval et al., 2013). HOXA9 gene is part of the homeobox A cluster on chromosome 7 and encodes for a DNA-binding transcription factor that may regulate gene expression, morphogenesis, and differentiation (Hwang et al., 2011). Some methods have been reported to evaluate the prevalence of HOXA9 promoter methylation in samples with lung cancer. For instance, Hwang et al. utilized pyrosequencing methylation analysis to demonstrate that the HOXA9 gene was hypermethylated in 32 of 40 tumors (80%) (Hwang et al., 2011). Olaussen et al. indicated that 64% (14 of 22) of the cytologically negative sputum samples from NSCLC patients had abnormal methylation in HOXA9 by using MSP method (Olaussen et al. 2005). Finally, RASSF1A encodes for a protein similar to the RAS effector proteins and has been found to be correlated with the hypermethylation of its CpG-island promoter region. Either loss or altered expression of this gene has been associated with the pathogenesis of a variety of cancers, which suggests a tumor suppressor function for RASSF1A (Agathangelou et al., 2005). Of these functions, RASSF1A is frequently investigated and many studies have been conducted to evaluate its epigenetic regulation in lung cancer. For example, Damman et

al. used COBRA method to conclude that hypermethylation of RASSF1A was detected in 10 out of 14 (71%) cancer cell lines and in 7 out of 16 (44%) primary tumors, but not found in any normal lung samples (Dammann et al. 2005). Lee et al. found that the RASSF1A promoter was aberrantly methylated in 44.7% (92/206) of NSCLC tissue samples by pyrosequencing, while in 37.4% (77/206) of the same NSCLC tissue samples when tested by MSP method (Lee et al. 2012). The list of three selected genes reported to be methylated in NSCLC by using conventional methods is shown in Table S2. It should be noted that most of the tests of gene promoter methylation were performed in tumor tissue samples. Few were in noninvasive bronchial brushing specimens. Biopsies have a potential risk of bleeding and may not be an option in some clinical cases. Thus bronchial brushing offers a more suitable way to be used by clinicians to make diagnoses. In this study, both tumor tissues and bronchial brushing specimens were tested.

Fig. 4A and B present the methylation levels of PCDHGB6, HOXA9, and RASSF1A genes in 50 paired early stage NSCLC and NT tissue samples and 50 early stage NSCLC bronchial brushing and normal specimens (40 tumor and 10 normal specimens), respectively. As expected, the average methylation levels of PCDHGB6, HOXA9, and RASSF1A in both tumor tissue samples and bronchial brushing specimens were markedly higher than those in controls. These results suggest that the selected genes were not only closely related to NSCLC gene expression, but had the possibility for use in tumor discrimination between cancer and control samples. We conducted a receiver operating characteristic (ROC) curve analysis to evaluate the diagnostic capability of combining our results from all three tested genes in tumor tissue and bronchial brushing specimens. When differentiating tumor tissues from NT samples, the analysis of single PCDHGB6, HOXA9, and RASSF1A genes showed the area under receiver operating characteristic curve (AUC) values of 0.856, 0.934, and 0.852, respectively ($P < 0.001$ for all comparisons, Table 2). When optimum thresholds were selected, the sensitivity of a single gene was 68% (34/50) for PCDHGB6, 80% (40/50) for HOXA9, and 64% (32/50) for RASSF1A, respectively. The specificity was 96% (48/50), 100% (50/50) and 100% (50/50) for the three genes, respectively. In contrast, the AUC values were 0.810, 0.854, and 0.814 for single PCDHGB6, HOXA9, and RASSF1A genes in identifying bronchial brushing specimens from normal specimens ($P < 0.001$ for all comparisons). The

corresponding single gene sensitivities were 57.5% (23/40) for PCDHGB6, 70% (28/40) for HOXA9, and 60% (24/40) for RASSF1A, with specificities of 90% (9/10), 100% (10/10), and 90% (9/10), respectively.

[Insert Fig.4]

Based on a logistic regression model, we built a combination detection mode for all three biomarkers. With regards to tumor tissue and NT samples, combining all three genes produced an AUC value of 0.977 ($P < 0.001$), which was higher than that of each individual gene. The ROC curve for the three-biomarker combination is shown in Fig. 4C. The ROC curve analysis yielded a sensitivity of 92% when all three genes were used, which was higher than the sensitivity of using any of the three genes alone. In the case of bronchial brushing specimens from normal specimens, the AUC value was 0.907 ($P < 0.001$, Fig. 4D) after combining three genes, with corresponding sensitivity of 80%. Both were higher than the AUC and the sensitivity of each individual gene. Our findings demonstrated that combining detection for three selected genes of promoters could achieve highly effective detection in both tumor tissues and noninvasive bronchial brushing specimens.

[Insert Table 2]

We then investigated if methylation markers are related to tumor stage. The sensitivity for methylation detection at Stages I and II is shown in Table 2. Our results in tissue samples revealed that single PCDHGB6, HOXA9, and RASSF1A gene had sensitivities of 60%, 72%, and 60%, respectively, at Stage I. After combining these three genes, the corresponding sensitivity was 83%. At Stage II, the single sensitivities of these three genes were 72%, 88%, and 80% respectively, and all relatively higher than those at Stage I. The combining sensitivity was 100%, also higher than that at Stage I. The same phenomenon was also observed in our bronchial brushing specimens. The combining detective sensitivity was 94.1% at Stage II, higher than the 69.6% at Stage I. The research on tumor stage indicated that the detection ability in early Stage I was less intensive than that in early Stage II. However, these results still showed that high detection efficiency could be obtained both at early Stage I and early Stage II by QDs-based FRET method. Furthermore, we also investigated the effect of different tumor types on the methylation status. The results showed that both ADC and SCC samples had similar high sensitivities in tumor tissues and bronchial brushing specimens after

the combination of the three genes (shown in Table 2), indicating the versatility of promoter expression in NSCLC types. Additionally, we had a test on the effects of age and gender on methylation status, with results indicating that DNA methylation of the selected genes is not obviously related to age or gender. Taken together, these results confirm that the QDs-based FRET method can be successfully used for early detection of NSCLC. Compared with the gene sensitivities reported in other studies shown in Table S2, ours show relatively high detection rates, accompanied by low cost and high convenience.

4. Conclusions

In summary, we analyzed the DNA methylation levels in early stage (Stage I and Stage II) NSCLC tissue samples and noninvasive bronchial brushing specimens by a QDs-based FRET method. Through the calculation by parameter E, the promoter methylation levels of PCDHGB6, HOXA9, and RASSF1A genes in these samples were obtained and compared. The statistical analysis showed that the sensitivity of a single gene was 64–80% for differentiating tumor tissues from NT samples, and 57.5–70% for bronchial brushing and normal specimens. While after the combining detection with all three genes, tumor tissues and NT samples, and bronchial brushing and normal specimens can be discriminated with high sensitivity of 92% and 80%, respectively. The ability for early cancer detection in bronchial brushing specimens was slightly insensitive than that in tumor tissues, however still showed a high sensitivity when using the combined promoter methylation detection. The examination in noninvasive endobronchial brushings can avoid the possible risks of bleeding or other contraindications during biopsy, being meaningful for the clinical diagnosis. These results demonstrate that the developed method can be successfully used for noninvasive early cancer detection, and possesses advantages of convenience, low cost and high sensitivity, being expected to be an alternative for early cancer diagnosis in the clinical applications.

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Appendix A. Supplementary material: Primer sequences used for promoter region amplification of lung cancer-related genes. The list of some reports on three selected genes to be methylated in NSCLC by using conventional methods in studies. Tissue type is either TU, tumor tissue, or CL, cell line. N/A means Not Applicable.

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Patient characteristics	Tissue (n=50)	Bronchial brushing (n=50)
Age		
Mean±SD	61.8±10.2	62.1±12.8
Sex		
Male	37	30
Female	13	20
TNM stage		
I	25	23
II	25	17
Type		
ADC	25	24
SCC	25	16

Table 1. Clinical pathological characteristics of the 50 paired early stage NSCLC tissues and their adjacent nontumorous tissue (NT) samples and 50 early stage NSCLC bronchial brushing and normal specimens (40 tumor bronchial brushing specimens and 10 normal specimens).

		ALL subjects				Stage		Tumor type	
		Sensitivity	Specificity	P	AUC	I Sen	II Sen	ADC Sen	SCC Sen
		(Sen, %)	(Spe, %)			(%,)	(%)	(%)	(%)
Tissue (n=50)	PCDHGB6	68	96	8.86×10^{-10}	0.856	60	72	72	60
	HOXA9	80	100	7.44×10^{-14}	0.934	72	88	80	80
	RASSF1A	64	100	1.36×10^{-9}	0.852	60	80	64	76
	Combination	92	100	2.08×10^{-16}	0.977	83	100	92	92
Bronchial brushing (n=50)	PCDHGB6	57.5	90	3.17×10^{-4}	0.810	52.2	64.7	62.5	50
	HOXA9	70	100	2.01×10^{-4}	0.854	60.9	82.3	66.7	75
	RASSF1A	60	90	2.39×10^{-4}	0.814	50	70.6	58.3	62.5
	Combination	80	100	1.71×10^{-5}	0.907	69.6	94.1	79.2	81.3

Table 2. Combined and single biomarker sensitivity and specificity for methylation detection in (top panel) 50 paired tumor tissue and NT samples and (bottom panel) 50 bronchial brushing and normal specimens

Fig. 1. Schematic illustration of the detection of DNA methylation based on QDs-FRET.

Fig. 2. Fluorescence spectra of the methylation levels of one pair of NT (A) and cancer (B) samples, in which QDs were excited at 500 nm. The rights are the corresponding schematic illustrations.

Fig. 3. The standard curve between the known methylation content by mixing known highly methylated and lowly methylated DNA samples measured by pyrosequencing and the E value detected by QDs-FRET method.

Fig. 4. Methylation levels of PCDHGB6, HOXA9 and RASSF1A in 50 paired NSCLC tissue samples (red dots) and corresponding NT samples (black dots) (A), and 50 NSCLC bronchial brushing specimens (40 tumor samples, red dots) and normal specimens (10 normal samples, black dots) (B) as measured by the QDs-based FRET technique. ROC curves analysis for using the combination of the three genes for the discrimination of NSCLC tissue from NT samples (C), and bronchial brushing specimens from normal samples (D).