Methylation status of NEUROG2 and NID2 improves the diagnosis of stage I NSCLC

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Received November 29, 2011; Accepted January 26, 2012
DOI: 10.3892/ol.2012.587

Abstract. In our previous study, we attempted to develop a tool for the early diagnosis of non-small cell lung cancer (NSCLC) using DNA methylation biomarkers. With the aim of improving the diagnostic potential by optimizing the composition of the target set, in this study, 13 candidate genes (ACTA1, AIDH1A2, CBX8, CDH8, EVX1, MGC16275, NEUROG1, NEUROG2, NID2, OTX2OS1, PGAM2, PHOX2B and TOX) were analyzed by methylation-specific PCR to determine the methylation status of each gene in 5 NSCLC cell lines and in lung tissue samples from 15 healthy volunteers, 103 stage I NSCLC patients and 26 non-cancerous control patients. Results showed that NEUROG2 and NID2 were hypermethylated in stage I NSCLC tissues (31.07 and 46.60%, respectively) and unmethylated in normal lung tissues (0/15) and non-cancerous tissues (0/26). Following recombination, an optimized 5-gene panel (NEUROG2, NID2, RASSF1A, APC and HOXC9) achieved a sensitivity of 91.26% with a specificity of 84.62% in the detection of stage I NSCLC. The optimized 5-gene panel greatly improved the diagnostic power for stage I NSCLC.

Introduction

Although the incidence and mortality rates for lung cancer have declined in the USA in the past decade, with the aging of the population and rapid industrialization, the incidence of this malignancy has been on the increase in China where the mortality rate for lung cancer rose to the highest among all types of cancer (1). In addition to the toxic substances found in tobacco, a number of other chemical substances, including chromium, formaldehyde and nickel, can increase the risk of developing lung cancer (2). Non-small cell lung cancer (NSCLC), which accounts for 85% of all lung cancer cases, consists of three primary histological types: squamous cell carcinoma, adenocarcinoma and large cell carcinoma (3). The prognosis of NSCLC varies greatly depending on the clinical stage at diagnosis and the use of appropriate treatments. The overall 5-year survival rate is 73% for patients with pathological stage IA NSCLC, whereas this rate is only 25% for those patients with stage IIIA NSCLC (4,5). Therefore, if biomarkers are established for the early detection and diagnosis of NSCLC, we may expect a marked decrease in mortality due to NSCLC.

Epigenetics is the study of mitotically and/or meiotically heritable changes in gene expression that are unrelated to changes in the DNA sequence (6). The dysregulation of epigenetic mechanisms is known to be involved in numerous diseases, including cancer (7). Epigenetic aberrations may occur at an earlier stage in tumor development than classical genetic transformation events, including mutations or deletions in tumor suppressor genes and proto-oncogenes. DNA methylation, a widely studied topic in epigenetics, was found to be abnormal in benign neoplasms and early stage tumors and both global DNA hypomethylation and promoter-specific hypermethylation have been observed. These attributes may qualify abnormal DNA methylation as a potential biomarker in the detection of cancer, especially at an early stage in carcinogenesis (8).

In our previous study, we identified a 5-gene panel (RASSF1A, APC, ESR1, HOXC9 and ABCB1) for the DNA methylation-based detection of stage I NSCLC with a sensitivity of 79.8% and a specificity of 73.1% for 124 cases of stage I NSCLC and 26 cases of non-cancerous respiratory disease (9). To further improve the diagnostic potential of this marker panel, in this study we screened more gene targets that
are aberrantly methylated in NSCLC tissues and optimized the diagnostic panel. Since tumor-specific gene methylation may exist in different types of cancer (10,11), we investigated 13 genes that were identified from data from a bladder methylation chip in our laboratory in NSCLC tissues. We found that the addition of 2 genes (NEUROG2 and NID2) to the diagnostic panel was of great benefit to the detection of stage I NSCLC.

Materials and methods

Cell lines and collection of the clinical tissue samples. The clinical tissue samples from 103 stage I NSCLC patients and 26 non-cancerous controls used in this study were the same as those used in our previous study and patient samples were obtained from the Shanghai Chest Hospital (Shanghai, China). Tumor-node-metastasis (TNM) staging/classification of the patient was performed according to the WHO classification (12). Normal lung tissue samples from 15 healthy organ donors were collected from the chest surgery department of the First Affiliated Hospital of Guangxi Medical University (Nanning, China) with the informed consent of the patients and the approval of the ethics committee. Table I shows the patient profiles, including the pathological types, ages and gender.

The lung cancer cell lines A549 [lung adenoma, ATCC (American Type Culture Collection, Manassas, VA, USA) no. CCL-185™], NCI-H1299 (NSCLC, ATCC no. CRL-5803™), LTEP-a-2 (lung adenoma, Cell Bank in Shanghai no. TCHu 33), SPC-A-1 (lung adenoma, Cell Bank in Shanghai no. TCHu 53) and NCI-H460 (large cell lung cancer, ATCC no. HTB-177™) were cultured in L-DMEM medium containing 10% FBS at 37°C in a 95% air, 5% CO2 humidified incubator to the log phase of proliferation prior to the collection of the cells.

DNA isolation and methylation-specific PCR (MSP). Genomic DNA was isolated from the cell lines and the frozen clinical tissues, using conventional proteinase K/organic methods, as described in a previous study (13-15).

The primer pairs for MSP analysis used in this study were designed using software available online (http://www. urogene.org/methprimer/index1.html; Table II). The bisulfite conversion and PCR analyses were performed as previously
described (13,15) and the representative PCR products were TA-cloned and then sequenced for validation.

**Statistical analysis.** Statistical calculations were performed with the use of SPSS 13.0 software. The incidence of hypermethylation in NSCLC tissues versus the non-cancerous tissues was calculated by a 2x2 Fisher’s exact test. The associations among the pathological variables and the methylation status of the genes were assessed by means of univariate logistic-regression analysis. Multivariate regression models were then constructed to adjust for gender (female vs. male), smoking status (never smoked vs. smoker) and tumor histology.

The area under the receiver operating characteristics (ROC) curve (AUC) is a measure of the ability of a continuous marker to accurately classify tumor and non-tumor tissue. In this study, cut-off values were obtained to increase the sensitivity [% TP/(TP + FN), (TP, true positive; FN, false negative)] and the specificity [% TN/(TN + FP), (TN, true negative; FP, false positive)]. The sensitivity and the specificity of each gene set were calculated and plotted.

**Results**

**MSP profiling of 13 genes in 5 NSCLC cell lines and 15 normal lung tissues.** The 13 genes listed in Table II were analyzed by MSP to determine their methylation status in the CpG sites within their promoter or the first exon region. These genes were methylated in various NSCLC lines, ranging from 2 lines to all NSCLC cell lines (Table III).

In the 15 normal lung tissues, the methylation status of each gene ranged from 0 to 100%. NEUROG2 and NID2 were unmethylated in all 15 normal lung tissues; by contrast, another 5 genes (CBX8, MGC16275, NEUROG1, PGAM2 and PHOX2B) were methylated in all the normal lung tissues, while the remaining 6 genes [AIDH1A2 (20.00%), ACTA1 (33.33%), TOX (40.00%), EVX1 (46.70%), OTX2OS1 (60.00%) and CDH8 (73.30%)] were methylated in some of the normal lung tissues (Table III).

Consequently, we hypothesized that NEUROG2 and NID2 have the potential to discriminate lung cancer from healthy controls as these genes were methylated in several lung cancer cell lines but not in any of the normal lung tissues.

**MSP profiling of NEUROG2 and NID2 in stage I NSCLC tissues and non-cancerous lesion controls.** To further assess the potential of NEUROG2 and NID2 as biomarkers for the early detection of NSCLC, we MSP-profiled the two genes in the tissue samples from 103 stage I NSCLC patients and 26 patients suffering from non-cancerous lung disease; the cohorts were the same as those mentioned in our previous study (9). In this study, the methylation of NEUROG2 was detected in 32 of the 103 (31.07%) cancer tissues but none (0%) of the 26 non-cancerous control tissues, indicating a significant association between NEUROG2 methylation and cancer. NID2 was methylated in 48 of the 103 (46.60%) cancer tissues and unmethylated in all 26 non-cancerous control tissues; thus NID2 was also a cancer-specific gene (Table IV). Therefore, these two genes were demonstrated to be ideal biomarkers for the diagnosis of NSCLC.
Univariate analysis was carried out to assess the correlation between the methylation frequencies of NEUROG2 and NID2 with each of the clinical characteristics; the result was graphed as a forest plot. Notably, the methylation status of NEUROG2 differed greatly between ADC (adenocarcinoma) and SSC (squamous cell carcinoma) with an odds ratio of 9.66 (95% CI 100.00).
Multivariate regression models were then established to consolidate the correlation between the methylation of NEUROG2 and ADC which was adjusted for other clinical characteristics, such as gender and smoking history. If NEUROG2 was methylated, the adjusted odds ratio for ADC was 4.28 (95% CI 1.19-15.30; P=0.0255; Table V). No statistically significant correlation was observed between the methylation of either NEUROG2 or NID2 and either gender or smoking status.

By analyzing the ROCs, we found that the addition of NID2 to the previous panel of 5 genes (RASSF1A, APC, ESR1, HOXC9 and ABCB1) detected 11 additional stage I NSCLC patients, who were false negatives for the previous panel, thus improving the sensitivity from 79.61 to 90.29% with no decline in specificity. By adding another gene (NEUROG2), another 2 patients were diagnosed, raising the sensitivity to 92.23%, and no additional false-positive cases were added. This combination of 7 genes (RASSF1A, APC, ESR1, HOXC9, ABCB1, NEUROG2 and NID2) provided 92.23% sensitivity and 73.08% specificity (Table IV).

However, to facilitate the screening for cancer in the general population, a panel of biomarkers should have a high specificity to minimize false positives; otherwise, numerous unnecessary follow-up procedures, which may be expensive and invasive, may be performed (16). A 3-gene panel (NEUROG2, NID2 and HOXC9) may provide 100% specificity; however, the 66.02% sensitivity was barely satisfactory. To optimize the panel, we constructed another panel of 5 genes (NEUROG2, NID2, RASSF1A, APC and HOXC9), achieving a sensitivity of 91.26% and a specificity of 84.62% (Table IV).

ROC curves were constructed for each of the two panels of 5 DNA methylation biomarkers from our previous study (RASSF1A, APC, ESR1, HOXC9 and ABCB1) and the present study (NEUROG2, NID2, RASSF1A, APC and HOXC9). The ROC curve for the previous panel used to classify stage I NSCLC and non-cancerous lung disease yielded an AUC of 0.763 (P<0.0001; 95% CI, 0.655-0.872); by contrast, the present panel yielded an AUC of 0.869 (P<0.0001; 95% CI, 0.782-0.957; Fig. 2).

The increase in the AUC demonstrates the superiority of the optimized panel of biomarkers.

Discussion

In our previous study, 5 genes (RASSF1A, APC, ESR1, HOXC9 and ABCB1) were combined as a panel for the diagnosis of stage I NSCLC by the method of MSP, which was able to achieve 79.8% sensitivity and 73.1% specificity, based on 124 cases of stage I NSCLC and 26 cases of non-cancerous respiratory disease (9).

Aiming to increase the sensitivity and specificity of DNA methylation as a biomarker for the diagnosis of NSCLC, we analyzed additional gene targets in this study in order to optimize the gene panel.

Recently, MBD-MethylCap/sequencing of bladder cancer cell lines and normal bladder mucosa was carried out in our
laboratory, through which we identified numerous aberrantly methylated genes in bladder cancer cells (data unpublished). Aberrant methylation of CpG sites in the promoter region was found in more than one type of cancer and we selected 13 genes (ACTA1, ADH1A2, CBX8, CDH8, EVX1, MGC16275, NEUROG1, NEUROG2, NID2, OTX2OS1, PGAM2, PHOX2B and TOX) from among the top 100 bladder cancer-specific genes to conduct further studies concerning the early detection and diagnosis of NSCLC. In this study, the tissue samples from the 103 stage I NSCLC patients and those from the 26 non-cancerous lung disease patients were from the same cohorts as used in our previous study, but the tissue DNA samples from some of the patients were no longer available.

The methylation of NEUROG2 and NID2 was associated with NSCLC. NEUROG2 and NID2 were found to be potential methylation biomarkers in stage I NSCLC diagnosis, being hypermethylated (NEUROG2, 31.07%; NID2, 46.60%) in cancer tissue samples but completely unmethylated in the non-cancerous controls.

Neurogenin-2, encoded by the NEUROG2 gene (4q25), is affiliated with the neurogenin subfamily of basic helix-loop-helix (bHLH) transcription factors, which are essential in neurogenesis from migratory neural crest cells (17). However, no evidence thus far has shown that NEUROG2 is correlated with the process of carcinogenesis.

The gene NID2 (1q43) encodes nidogen 1, which belongs to the nidogen family of basement membrane proteins and may play a role in maintaining the structure of the basement membrane (18). A number of studies have already reported that NID2 is correlated with cancer. The results of one study revealed that NID2 was methylated in 29% of colon cancer tissues and 95% of gastric cancer tissues, whereas it was completely unmethylated in the corresponding normal mucosa (19). Another study found that the concentration of nidogen-2 in the serum of patients suffering from ovarian cancer was elevated relative to that of normal controls and patients with benign conditions (20). At present, no report of an association between the methylation of NID2 and lung cancer is available.

This study was the first to demonstrate an association of these two genes with stage I NSCLC. Moreover, NEUROG2 showed an ADC-specific methylation.

An optimized 5-gene panel (NEUROG2, NID2, RASSF1A, APC and HOXC9) was developed for the early detection of NSCLC. Adding NEUROG2 and NID2 to the previous 5-gene panel markedly increased the sensitivity for stage I NSCLC (from 79.61 to 92.23%), but the 73.08% specificity of the 7-gene panel (RASSF1A, APC, ESR1, ABCB1 and HOXC9, but not p16INK4A, DAPK1, PTEN and MT1G genes) were frequently methylated in the stage I non-small cell lung cancer in China. J Cancer Res Clin Oncol 135: 1675-1684, 2009.


Acknowledgements

This study was supported in part by grants from the National Science Foundation (grant 30872963, J. Yu), the State Key Laboratory of Oncogenes and Related Genes (grant no. 91-11-01, J. Yu), the Shanghai Science Foundation (grant 09ZR1429900, Y. Zhao) and the Shanghai Cancer Institute Master & Doctoral Foundation (SB09-07, Y. Zhao).

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