Lung Cancer 69 (2010) 172–179

Contents lists available at ScienceDirect

Lung Cancer

journal homepage: www.elsevier.com/locate/lungcan

DNA hypermethylation of tumors from non-small cell lung cancer (NSCLC) patients is associated with gender and histologic type☆

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ARTICLE INFO

Article history:
Received 20 May 2009
Received in revised form 25 August 2009
Accepted 3 November 2009

Keywords:
Hypermethylation
Lung cancer
Gender
Histology

ABSTRACT

Background: We previously identified a number of genes which were methylated significantly more frequently in the tumor compared to the non-cancerous lung tissues from non-small cell lung cancer (NSCLC) patients. Detection of methylation profiles of genes in NSCLC could provide insight into differential pathways to malignancy and lead to strategies for better treatment of individuals with NSCLC.
Methods: We determined the DNA methylation status of 27 genes using quantitative MethyLight assays in lung tumor samples from 117 clinically well-characterized NSCLC patients.
Results: Hypermethylation was detected in one of more of the genes in 106 (91%) of 117 cases and was detected at high levels (percentage methylation reference (PMR) $\geq$ 4%) in 79% of NSCLC cases. Methylation of APC, CCND2, KCNH5 and, RUNX was significantly more frequent in adenocarcinomas compared to squamous cell carcinomas (SCC), while methylation of CDKN2A was more common in SCC. Hypermethylation of KCNH5, KCNH8, and RARB was more frequent in females compared to males. Hypermethylation of APC and CCND2 was inversely associated with proliferation score assessed by Ki-67 level.
Conclusions: Our findings of differential gene hypermethylation frequencies in tumor tissues from patients with adenocarcinoma or squamous cell cancers and in females compared to males suggests that further investigation is warranted in order to more fully understand the potential disparate pathways and/or risk factors for NSCLC associated with histologic type and gender.

1. Introduction

Primary lung cancer is a major cause of death worldwide [1] and remains the leading cause of cancer death in the United States, with an estimated 215,020 new patients diagnosed and 161,840 deaths expected in 2008 [2]. Non-small cell lung cancer (NSCLC) accounts for 80% of these new cases and includes the following histologic types: adenocarcinoma, squamous cell carcinoma, large cell carcinoma and mixed histologies. Approximately 25–33% of NSCLC patients present with stage I or II disease, which permits surgical resection with curative intent. However, despite a complete and presumably curative resection, approximately 40–50% of patients with resected NSCLC die of recurrent disease [3].

In recent years, much attention has focused on whether women and men differ with respect to the epidemiologic and clinicopathologic features of NSCLC. Recent trends in the United States show that while incidence in men has declined during the past decades, lung cancer has continued to increase in women [4]. Further, much of the current evidence suggests that women have an increased susceptibility to lung cancer, are younger at age of diagnosis, are more likely to have adenocarcinoma compared to squamous cell carcinomas, and have better response to therapy and improved survival [5–9]. Identifying the molecular profiles of these patients could provide insight into differential pathways to malignancy in females compared to males and could lead to strategies for better treatment of individuals with NSCLC.

It has recently become clear that epigenetic alterations play an important role in cancer development and result in changes in gene function that occur without changes in nucleotide sequence [10]. One of the most well studied epigenetic changes is DNA methylation, which adds a methyl group to cytosines preceding guanidines (also called CpG dinucleotides). Methylation of CpG islands in the promoter region of the gene leads to gene silencing

☆ Informed consent was obtained according to procedures approved by the Human Subjects Committee of the University of Washington.

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doi:10.1016/j.lungcan.2009.11.002

and inactivation, and it has been proposed that DNA methylation of promoter regions of tumor suppressor genes plays an important role in tumor development [11,12]. A number of genes appear to be aberrantly methylated in tumor as opposed to non-tumor tissues from NSCLC patients [13–19]. Multiple studies have attempted to assess the clinical significance of one gene, or a small set of genes, in differentiating the clinicopathological features of these tumors [16,18,20–25]. However, most of these studies utilized qualitative methylation-specific PCR (MSP) in order to detect DNA methylation, a method that can sometimes lead to false positive results and does not distinguish between low and high level methylation [26]. Furthermore, results in these studies have been inconsistent due to varying methylation detection protocols, PCR primers, and study populations, and none have comprehensively studied more than 10 genes.

In the present study, we chose to quantify methylation of genes which were potentially important in lung cancer, many of which have been assessed by others but some which have not been previously evaluated (Appendix 1). The selected genes affect apoptosis (DAPK1, RUNX3, TMS1, PTEN, and SOCS3), cell adhesion, invasion and/or metastasis (OPCML, CDH13, BVES, APC, CDH1, IGSF4, KCNH5, and PCSK6), cell cycle control (CCND2, RASSF1, APC, FHIT, CDKN2A, CDKN2B, P14, and PTEN), cell proliferation and/or differentiation (RARB, IGFBP3, KCNH5, KCNH8, SOCS3 and PTGS2), and DNA-repair and/or detoxification of DNA adducts (FHIT, MLH1, MGMT, FANCF, and GSTP1). In order to assess more broadly the clinical significance of gene hypermethylation in NSCLC, we determined the DNA methylation status of these 27 genes using quantitative MethyLight assays in lung tumor samples from 117 clinically well-characterized NSCLC patients.

2. Methods

2.1. Study population

The subjects in the present study are a subset of patients included in a larger prospective study of fluorodeoxyglucose (FDG) PET imaging in NSCLC conducted under University of Washington Human Subjects Division approval [27]. Briefly, 208 patients were enrolled into the imaging trial and were followed using the standard NSCLC care algorithm previously described [28]. Results of this imaging trial have been recently reported [27]. In 117 of the 208 subjects, a paraffin-embedded tumor block of the primary tumor was available for methylation analysis, the results of which are presented here.

2.2. Pathology

All biopsy and resection specimens were reviewed by the pathology department of the University of Washington Medical Center or the Veterans Affairs Puget Sound Health Care System to verify non-small cell histology of the lung cancer samples and to determine the histologic subtype. NSCLC histology was classified as adenocarcinoma, squamous, large cell, bronchoalveolar adenocarcinoma (BAC), and NSCLC with NOS/other/mixed histology. Histologic BAC was defined using the WHO definition: a variant of adenocarcinoma characterized by surface growth over alveolar septa and without an invasive component. The tumor proliferation rate was assessed by the Ki-67 score as described previously [28]. For each subject, the tissue size and stage had been thoroughly assessed as described previously [29].

2.3. DNA isolation from paraffin blocks

Six 20 μm sections were cut from each block and deparaffined by xylene extraction. The resulting tissue pellets were digested with proteinase K at 48 °C overnight. The genomic DNA was isolated by phenol/chloroform extraction and ethanol precipitation. Finally the DNA was purified using QIAamp DNA mini-column according to the manufacturer's protocol (Qiagen, Valencia, CA).

2.4. Sodium bisulfite conversion

Unmethylated human sperm DNA (U-DNA) and in-vitro fully methylated DNA (M-DNA) were converted with clinical samples as described before [30]. Briefly, about 1 μg DNA was modified by 5 M sodium bisulfite, desulfonated with NaOH, then purified and resuspended in 80 μl elution buffer (10 mM Tris-HCl, pH 8.0, Qiagen).

2.5. DNA methylation (MethyLight) analysis

For each gene, the primers and probe were designed specifically for bisulfite-converted fully methylated DNA (Appendix 2). Amplification of bisulfite-converted β-actin (ACTB) was used to normalize for input DNA. Samples that were negative for ACTB were excluded in the methylation analysis. A plasmid containing bisulfite-converted ACTB gene of known concentration was diluted and used as the standard curve for quantification. The assay for a given set of samples was considered valid only if the converted U-DNA was not amplified, while the converted M-DNA was amplified. The percentage methylated reference (PMR) for each locus was calculated by dividing the GENE: reference ratio of a sample by the GENE: reference ratio of M-DNA and multiplying by 100 [31]. Twenty-seven genes, plus the control gene ACTB, were analyzed in this study.

2.6. Statistical analysis

MethyLight data were dichotomized semi-quantitatively, initially classifying a specific gene positive for hypermethylation using both a PMR > 0% cutoff as well as a PMR ≥ 4% cutoff, as we have used in our previous study of lung cancer methylation [19]. However, for assessing associations of hypermethylation with behavioral and clinical data, we used a cutoff value of PMR > 4% which has been validated in the literature as a standard cutoff and is associated with loss of protein expression [31–34]. When multiple cancerous tissue samples were available, we randomly chose one of the cancerous blocks to be used in this analysis. Factors associated with patient and tumor characteristics were assessed univariately with chi-square tests for trend and logistic regression. In the final analysis, multivariable adjustments were made to adjust for the potentially confounding effects of histologic type, gender, and tumor size. Exact logistic regression was utilized to compute risk estimates and confidence intervals when the prevalence of gene methylation was low. In the multivariable analyses, *p*-values and 95% confidence intervals were adjusted to take into account multiple comparisons by setting the false discovery rate (FDR) equal to 0.05, using PROC MULTTEST [35] and subsequently recalculating confidence intervals [36]. A two-sided 0.05 test level determined statistical significance for all analyses. All analyses were conducted using SAS version 9.1 (SAS Institute Inc., Cary, NC).

3. Results

3.1. Demographics

In the present study, we chose genes which were potentially important in lung cancer and determined the DNA methylation status of these 27 genes using quantitative MethyLight assays in lung tumor samples from 117 well-described patients. The mean age of the 117 NSCLC cases at the time of enrollment in the imaging trial was 64.8 years (Table 1). Somewhat less than half were