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HOXA9 methylation and blood vessel invasion in FFPE tissues for prognostic stratification of stage I lung adenocarcinoma patients

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ABSTRACT

Objectives: Surgery with curative intent is the standard treatment for stage I lung adenocarcinoma. However, disease recurrence occurs in a third of patients. Prognostic biomarkers are needed to improve postoperative management. Here, we evaluate the utility of Homeobox A9 (HOXA9) promoter methylation, alone or in combination with Blood Vessel Invasion (BVI) assessment, for prognostic stratification of stage I lung adenocarcinoma patients.

Materials and methods: We developed a Droplet Digital PCR (ddPCR) assay to measure HOXA9 promoter methylation in formalin-fixed paraffin-embedded (FFPE) biospecimens generated during routine pathology. The prognostic value of HOXA9 promoter methylation and BVI, alone and in combination, was evaluated by Kaplan-Meier survival and Cox regression analyses in a cohort of 177 stage I lung adenocarcinoma patients from the NCI-MD study.

Results: The ddPCR assay showed linearity, sensitivity and specificity for measuring HOXA9 promoter methylation down to 0.1% methylated DNA input. The HOXA9 promoter was methylated *de novo* in FFPE tumors ($P < 0.0001$). High methylation was independently associated with worse cancer-specific survival (Hazard Ratio [HR], 3.37; $P = 0.0002$) and identified high-risk stage IA and IB patients. Addition of this molecular marker improved a risk model comprised of clinical and pathologic parameters (age, gender, race, stage, and smoking history; nested likelihood ratio test; $P = 0.0004$) and increased the C-index from 0.60 (95% CI 0.51–0.69) to 0.68 (0.60–0.76). High methylation tumors displayed high frequency of TP53 mutations and other molecular characteristics associated with aggressiveness. BVI was independently associated with poor outcome (HR, 2.62; $P = 0.054$). A score that combined BVI with HOXA9 promoter methylation further stratified high-risk patients (trend $P = 0.0001$ comparing 0, 1 or 2 positive markers).

Conclusions: ddPCR can be used to quantify HOXA9 promoter methylation in FFPE samples. Alone or combined with BVI in a prognostic classifier, HOXA9 promoter methylation could potentially inform the clinical management of patients with early-stage lung adenocarcinoma.

1. Introduction

Lung cancer is the leading cause of cancer death worldwide [1]. Non-small cell lung cancer (NSCLC) represents more than 80% of all lung cancers. Although progress has been made in the management of lung cancer, early detection and timely treatment remain the most effective strategy to reduce mortality. Recently, it was demonstrated that low-dose computed tomography (LDCT) scan for lung cancer screening can reduce mortality among high-risk individuals [2]. Candidates eligible for LDCT screening are heavy smokers (at least a 30 pack-year smoking history, current smokers or with no more than 15 years since quitting) aged 55–74 years [3]. LDCT is highly sensitive and about 60% of cases diagnosed are stage I. Treatment by surgical resection remains the only curative option for these patients. However, after complete resection patients with disease at the same stage experience different outcomes. In fact, about a third of patients develop recurrence and die

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of their disease within 5 years [4]. This implies that the tumor-node-metastasis (TNM) staging system, that is currently used for prognosis, is not sufficient to predict the behavior of some early-stage tumors [5]. Adjuvant chemotherapy has been extensively explored to prevent recurrence in patients with early-stage NSCLC. No beneficial effect on the prognosis of stage IA patients was demonstrated, and its benefit in stage IB is controversial [6,7]. Accordingly, adjuvant chemotherapy is generally not recommended for patients with stage I in the US and optional for stage IB in Europe. Prognostic indicators to better risk-stratify early-stage NSCLC patients is a field of active investigation. The combination of TNM staging with prognostic biomarkers would allow stratification of patients into those at high risk of recurrence who could potentially benefit from adjuvant chemotherapy, and those at low risk who should be spared the toxicity of treatment.

DNA methylation-based biomarkers have emerged as promising candidates for early cancer detection, prognostication and real-time follow-up of tumor dynamics [8]. Altered DNA methylation pattern is frequent in lung cancer, and plays an important role in cancer initiation and progression [9]. DNA methylation occurs on cytosine within a CpG site. CpG sites are found throughout the genome but are enriched in regions referred to as CpG islands, located at transcription start sites. Global hypomethylation of the genome and hypermethylation of the promoter regions of several genes, including tumor suppressor genes, are hallmarks of cancer. Gene promoter methylation is generally associated with decreased gene expression. A number of gene promoters have been described as being hypermethylated in early-stage lung cancer, including Homeobox A9 (HOXA9) promoter [10–16]. *HOXA9* belongs to the *HOX* family of genes, which encodes transcription factors that are critical for embryogenesis and are associated with carcinogenesis in multiple different cancer types [17]. In a recent study, we found that high HOXA9 promoter methylation was associated with worse cancer-specific survival of patients with stage I lung adenocarcinoma [10]. The prognostic significance of HOXA9 promoter methylation for early-stage NSCLC has also been reported in two other studies [15,16]. Collectively, HOXA9 appears to be a reliable prognostic biomarker for stage I NSCLC. Neovascularization of the primary tumor is a critical step for tumor cell dissemination and metastasis. Blood Vessel Invasion (BVI) has been recognized as an important prognostic factor in many cancers, including NSCLC. Stage I NSCLC patients harboring BVI are at higher risk for poor outcomes [18–21].

The type of biospecimens and the choice of assay platform are key issues that may hinder clinical development of cancer biomarkers. Here, we developed and optimized an assay that utilizes Droplet Digital PCR (ddPCR) technology to assess HOXA9 promoter methylation in formalin-fixed paraffin-embedded (FFPE) samples. In recent years, ddPCR has become increasingly used clinically due to its ability to reliably detect and quantitate rare alleles, as well as its technical simplicity, rapidity and cost-effectiveness [22]. In addition, FFPE tissues generated after surgery for cancer diagnosis and staging constitute a valuable resource that is generally available for the assessment of biomarkers. In the present study, we evaluate the prognostic value of HOXA9 promoter methylation and BVI using FFPE tissues generated during routine pathological assessment of resected stage I lung adenocarcinoma patients. Using a partly independent set of patients from our previous study [10], we validate that HOXA9 promoter is hypermethylated in tumors, and that high HOXA9 promoter methylation is associated with poor prognosis. Highly methylated tumors harbored molecular characteristics of aggressive tumors, notably the high frequency of *TP53* mutations and the upregulation of the OncomiR miR-9. We assessed BVI in a subset of FFPE tumor samples in this cohort for the first time and show its prognostic significance. Finally, combining HOXA9 promoter methylation with BVI improves the risk stratification of patients with early-stage lung adenocarcinoma.

2. Materials and methods

2.1. Study population

Formalin-fixed paraffin-embedded (FFPE) tissue samples resected from patients with stage I lung adenocarcinoma were selected from the National Cancer Institute-Maryland (NCI-MD) lung cancer case-control study. We evaluated HOXA9 promoter methylation in two independent sets, consisting of 107 patients for the test cohort and 70 patients for the validation cohort. In the test cohort, 69 cases were overlapping with our previous study that used frozen tissue samples [10]. BVI was evaluated in a nested cohort of 113 patients. Patients were recruited from hospitals in the Metropolitan Baltimore area between 1985 and 2012. Tumors and adjacent noncancerous tissues were obtained at time of surgical resection. Detailed background and medical information including age, gender, race, smoking history, tumor size, and staging were collected. Survival time was determined as described elsewhere [10,23]. This study was approved by the Institutional Review Board of the National Institutes of Health, and informed consents were obtained for each participant.

2.2. DNA extraction and bisulfite treatment

For each patient, one to five sequential FFPE tissue sections (ranging from 5 to 50 µm) were deparaffinized and genomic DNA was purified using Quick-DNA™ FFPE MiniPrep (ZymoResearch, Irvine, CA), according to the manufacturer's protocol. Tissue sections subsequent to those used for analysis of methylation and stained with hematoxylin and eosin were reviewed by a pathologist to confirm the presence of tumor cells. In over 80% of the cases, a 50% tumor content or higher was observed. Tumor content of at least 50% is commonly considered appropriate in published studies for analysis of DNA methylation on FFPE biospecimens [24]. DNA concentration and integrity were evaluated using the 2200 TapeStation System (Agilent Technologies, Inc., Santa Clara, CA). Between 200–500 ng of DNA were used for bisulfite conversion using the EZ-DNA Methylation-Direct™ Kit MiniPrep (ZymoResearch), following the manufacturer's recommendations. Bisulfite-converted DNA was eluted in 10 µl of M-elution buffer and stored at −80 °C.

2.3. Droplet digital PCR (ddPCR) analysis

Methylated copies of HOXA9 promoter and total DNA (C-LESS reaction) were quantified using the QX200™ AutoDG Droplet Digital™ PCR system (Bio-Rad, Hercules, CA). Primers and probe sequences were as follows: for HOXA9 [25] Forward: 5′-GTGGTTATTATCGTGTTTAG CGT-3′, Reverse: 5′-CCGATACCACCAAATTATTACATA-3′, Probe: 6FAM-5′-TGGTTCGTTCGGTTCGATTTACGGA-3′-NFQ, and C-LESS [26,27] Forward: 5′-TTGTATGTATGTGAGTGTGGGAGAGA-3′, Reverse: 5′-TTTCTTCCACCCCTTCTCTTCC-3′, Probe: 6FAM-5′-CTCCCCCTCTAA CTCTAT-3′-NFQ. Each reaction was prepared in monoplex in a final volume of 20 µl, consisting of 250 nM forward and reverse primers, 900 nM probe, 1X ddPCR ™ SuperMix for Probes (Bio-Rad) and bisulfite converted DNA. Samples were loaded into the DG32™ Automated Droplet Generator Cartridges (Bio-Rad). Automated Droplet Generation Oil for Probes (Bio-Rad) was then added, and droplets were generated using the Automated Droplet Generator (Bio-Rad). Droplets were transferred to a 96-well PCR plate and placed into a C1000 Touch™ Thermal Cycle with 96-Deep Well Reaction Module (Bio-Rad). PCR cycling conditions were as follows: 10 min at 95 °C for DNA polymerase activation, followed by 40 cycles of 30 s at 94 °C for denaturation and 1 min at 56 °C for annealing and extension, and ending at 98 °C for 10 min for DNA polymerase deactivation and 4 °C for cooling. PCR plates were then loaded into the QX200™ Droplet Reader (Bio-Rad) and droplet signal was read as being either positive or negative for HOXA9 or C-LESS amplification. Data were analyzed using the QuantaSoft