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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

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Conflict of interest
None.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.lungcan.2018.05.021>.

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.

Keywords

Prognostic biomarkers; Post-operative recurrence; Lung adenocarcinoma; HOXA9 promoter methylation; Droplet digital PCR

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 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 μ m) 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 μ l 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'-CCGATACCACCAAAATTATTACATA-3', Probe: 6FAM-5'-TGGTTCGTTTCGGTTCGATTACGGA-3'-NFQ, and C-LESS [26,27] Forward: 5'-TTGTATGTATGTGAGTGTGGGAGAGA-3', Reverse: 5'-TTTCTTCCACCCCTTCTCTTCC-3', Probe: 6FAM-5'-CTCCCCCTCTAACTCTAT-3'-NFQ. Each reaction was prepared in monoplex in a final volume of 20 μ l, consisting of 250 nM forward and reverse primers, 900 nM probe, IX 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 software (QuantaSoft v1.7.4, Bio-Rad). All experiments included a no-DNA template control well, and 10 ng of a bisulfite-converted fully-methylated control DNA. Either 10 or 20 ng of DNA were used to quantify methylation in FFPE samples. To obtain a measure of HOXA9 methylation for each sample, we calculated the percentage of methylated reference (PMR) using the number of copies/20 μ l well, as follows:

$$\text{PMR} = \frac{\text{HOXA9/C-LESS sample}}{\text{HOXA9/C-LESS fully methylated DNA}} \times 100$$

For quality control purposes, we excluded samples generating less than 100 positive droplets for the C-LESS reaction.

2.4. Blood vessel invasion (BVI) analysis

For each patient, one FFPE section (5 μm) was deparaffinized and hydrated with distilled water. Slides were immersed in 1% chloric acid in 70% ethanol, prior to be placed in a Midas® III Automated Microscope Slide Stainer (EMD Millipore). Briefly, slides were stained in the instrument with Resorcin-Fuchsin Solution (Electron Microscopy Sciences, Hatfield, PA) for 60 min, immersed in 100% ethanol for 1 min, washed with distilled water for 15 s, stained with Weigert's Iron Hematoxylin Working Solution (equal mix of Weigert's Iron Hematoxylin A and Weigert's Iron Hematoxylin B, Electron Microscopy Sciences) for 5 min, washed with distilled water for 15 s, and stained with Van Gieson's Solution (Electron Microscopy Sciences) for 10 min. Slides were then washed with distilled water for 1 s, dehydrated quickly through 95% ethanol followed by two changes of 100% ethanol, and two changes of xylene for 3 min each. Slides were then covered with resinous mounting medium and coverslips. Blood vessels were identified by the presence of erythrocytes in the lumen, or an endothelial cell lining, or the presence of elastic tissue around larger vessels. Tumors were classified as vascular-invasion positive when tumor cells were identified in the vessel lumens, and negative otherwise. The evaluation of vascular invasion was performed blindly by two clinicians, and if there was a discrepancy, consensus was made by discussion.

2.5. TP53 sequencing and mutation status analysis

Genomic DNA isolated from lung cancer tissues was submitted for TP53-targeted sequencing using the MiSeq Illumina platform. The assay was targeted at the exons and proximal splice sites. Sequence results were processed and aligned to human genome and underwent quality control requiring coverage >100 reads with the variant and minimum allele frequency > 10%. The variants called included all common polymorphisms. Because only tumor was sequenced, in order to score somatic mutations, those deemed to be germline were filtered out. These included single nucleotide variants (SNVs) present in The Single Nucleotide Polymorphism database (dbSNP) with high reported allele frequency (common polymorphisms). Also, SNVs in untranslated regions and introns were not considered, as their somatic status and functional implications are unclear. The presence of putative somatic exonic and splicing variants was corroborated in The Cancer Genome Atlas (TCGA) and the Catalogue Of Somatic Mutations in Cancer (COSMIC) datasets.

2.6. Statistical analysis

Agreement between HOXA9 promoter methylation status in paired fresh frozen and FFPE tissues was evaluated by the Cohen's kappa coefficient, implemented in Stata/SE 15.0 (Stata-Corp LP, College Station, TX). Results were classified as concordant if both biospecimens from the same patient had the same methylation status (high or low). The association of HOXA9 promoter methylation with patient smoking, age, race, tumor stage

(IB vs. IA), and tumor size was evaluated by logistic regression, implemented in Stata. The methylation threshold was determined in the test cohort by the minimum P-value approach, which has been used before to systematically find optimal cut-points in continuous gene expression measurements for grouping patients for survival analysis [28]. First, patients in the test cohort were ordered by PMR. Next, patients were dichotomized into High and Low PMR groups at each potential cut-point, and the risk differences of the two groups were estimated by log-rank test. Then, the optimal cut-point that gives the most pronounced P-value was selected. The selected cut-point was subsequently applied to the validation cohort. Patients were also dichotomized on the basis of BVI positivity, to evaluate the association between this biomarker and survival. A score combining HOXA9 promoter methylation and BVI was generated based on the cumulative number of high values for the two biomarkers. Survival time was calculated as the number of days from the date of surgery to the date of lung cancer-specific death, or last known follow-up. Causes of death other than lung cancer were censored. Survival effects were evaluated with Kaplan-Meier survival plots and Log-rank tests using Graphpad Prism v6.0 (Graphpad Software Inc., La Jolla, CA). Hazard ratios and 95% confidence interval were estimated with univariable and multi-variable Cox proportional hazards regression modeling using Stata. Multivariable analyses were adjusted to the following covariates: age (continuous), gender (male/female), race (African American/Caucasian), tumor stage (IA/IB), smoking status (never/former and current) and pack-years (continuous), when appropriate. Regarding smoking status, two groups were defined: never smokers, who reported to have smoked less than 100 cigarettes in their lifetime, and current or former smokers, who had quit smoking at least one year prior to enrollment. The concordance index (C-index) and the nested likelihood ratio test Stata modules were used to evaluate the predictive accuracy of the biomarkers. The C-index is a test for discrimination that quantifies the ability of a model to distinguish events versus non-events. C-index values can range from 0.5 to 1.0, where 1 means perfect accuracy and 0.5 random estimation. A model with C-index ranging from 0.70 to 0.80 is considered to have adequate power of discrimination. The clinical and pathologic model used in the nested likelihood ratio test included age, gender, race, stage, and smoking status. Statistical significance was defined as $P < 0.05$.

Transcriptome profiles of lung adenocarcinomas generated as part of our earlier study [10] were interrogated to evaluate the molecular differences between tumors with high and low HOXA9 promoter methylation using Ingenuity Pathway Analysis (IPA Ingenuity Systems, www.ingenuity.com).

3. Results

3.1. ddPCR assay development and optimization for the analysis of HOXA9 promoter methylation in FFPE samples

The initial series of experiments were aimed at adapting an HOXA9 promoter methylation assay originally developed for methylation-specific PCR [25] to a ddPCR format and assess the critical parameters of its analytical performance in terms of sensitivity, specificity, analytical range, and reproducibility.

Sensitivity and analytical range of the HOXA9 methylation assay were determined by measuring the lower limit of detection. We prepared 10-fold serial dilutions of a fully-methylated (FM) control DNA in water (Fig. 1A, B>) or in a background of unmethylated control DNA (Supplementary Fig. S1), as well as titrations of DNA extracted from FFPE tumors with various methylation levels (Supplementary Fig. S2). The assay could be performed with input DNA amount as low as 5 ng and shows linearity over the titration series. Methylation of HOXA9 down to 0.1% could be detected by ddPCR (Supplementary Fig. S1).

The primer set and probe used were designed to only hybridize with bisulfite-converted fully-methylated HOXA9 promoter. Although we optimized the assay conditions to minimize 'rain', in terms of annealing temperature, primers and probe concentration, droplets with intermediate fluorescence were still visible (Fig. 1B). Various factors may be the source of rain droplets, including non-specific amplification, sequence alteration in the target template due to DNA fragmentation after bisulfite treatment, or variances in the recognition site. In that regard, we noticed that creating a primer-template mismatch at CpG sites reduced the PCR amplification efficiency (data not shown). Nevertheless, no positive droplets were detected when unmethylated control DNA or non-bisulfite treated FM control DNA were used as input (Fig. 1C, D), confirming the specificity of the assay for methylated DNA.

In our earlier study, we evaluated HOXA9 methylation on a set of fresh-frozen tumor and adjacent non-tumor tissues by pyrosequencing [10]. To determine whether ddPCR methylation results were comparable to those obtained by pyrosequencing, we quantified HOXA9 promoter methylation from eleven fresh-frozen paired tissue samples (Fig. 1E). As previously observed, fresh-frozen tumors showed a higher level of methylation compared to matched non-tumors (paired *t*-test, $P < 0.0001$). The difference in methylation was maintained when comparing twenty pairs of FFPE tumor and adjacent non-tumor tissues (paired *t*-test, $P < 0.0001$; Fig. 1F), indicating that our ddPCR assay retains the ability to discriminate between various levels of HOXA9 promoter methylation in FFPE samples, although overall the methylation measured from FFPE samples was lower than from fresh-frozen tissues. Taken together, these results suggest that the ddPCR assay is robust to assess HOXA9 promoter methylation in FFPE tissue samples.

3.2. High HOXA9 promoter methylation is associated with poor prognosis

In our previous study, we reported that high HOXA9 promoter methylation identified stage I lung adenocarcinoma patients at high risk of poor outcome [10]. In order to adapt this biomarker for clinical use, we applied ddPCR to quantify HOXA9 promoter methylation in FFPE samples from that same cohort. Patient characteristics are summarized in Table 1. We selected 107 patients as part of a test cohort (69 of them overlapping with our previous study), to establish the optimal threshold value of methylation for survival association analyses, as the threshold described in our previous study to dichotomize patients on the basis of the mean percent methylation was no longer applicable. HOXA9 promoter methylation was calculated as PMR using FM control DNA as a reference (see methods section). We implemented a minimum P-value approach to empirically identify the optimal

PMR cut-point (cut-off value) in the test cohort. For each potential cut-point of PMR, patients in the test cohort ($n = 107$) were dichotomized into High and Low PMR groups and survival difference between them was calculated by log-rank test (Supplementary Fig. S3). The resulting dose-response curve had an elbow point at PMR 27–28% with P -value = $7.E-5$. Thus, a cutoff value PMR of 30% was selected. Using this threshold, the concordance rate between ddPCR analysis of FFPE samples and pyrosequencing analysis of matched fresh frozen specimens was 72% (50/69) (kappa statistic 0.2887, $P = 0.0054$) (Supplementary Table S1). High HOXA9 promoter methylation (PMR > 30%) was associated with worse cancer-specific outcome in the Kaplan-Meier (Log-rank test, $P = 0.0004$; Fig. 2A) and multivariable Cox regression analyses, after adjustment for age, gender, race, tumor stage, smoking history and pack years (Hazard Ratio [HR], 3.52; $P = 0.003$; Table 2 and Supplementary Table S2).

The test's cut-off value and clinical performance were further verified in a validation set of 70 independent cases from the same cohort (Table 1). HOXA9 promoter methylation was measured using the same method as for the test set and converted to PMR. High HOXA9 promoter methylation was observed in 27.1% (19/70) patients and was associated with shorter cancer-specific survival in the Kaplan-Meier analysis compared to those with low PMR (log-rank test, $P = 0.002$; Fig. 2B). After controlling for age, gender, race, tumor stage, smoking history and pack years the results remained statistically significant (HR, 4.28; $P = 0.006$; Table 2 and Supplementary Table S2).

We then merged the test and validation sets for added power in the stratified analysis. No significant associations with patient smoking, age, race and tumor stage (IB vs. IA) were observed by logistic regression. HOXA9 promoter methylation was positively associated with tumor size (Odds Ratio [OR], 1.47; $P = 0.02$). Kaplan-Meier analysis and Log-rank test showed that stage I patients with PMR values above 30% (47/177, 26.6%) had worse 5-year prognosis ($P < 0.0001$; Fig. 3A). The association remained significant after stratification for stage IA ($P = 0.0002$; Fig. 3B) and stage IB ($P = 0.031$; Fig. 3C). In multivariable Cox regression analysis, high HOXA9 promoter methylation negatively impacted survival, after adjustment for age, gender, race, tumor stage, smoking history, pack-years and cohort (HR, 3.37; $P = 1.5.E-04$; Table 2 and Supplementary Table S2). Thus, HOXA9 promoter methylation is an independent biomarker of prognosis for patients with Stage I, as well as Stage IA or Stage IB, lung adenocarcinoma. Because the majority of Stage I patients did not receive therapy, we focused on therapy naïve patients, and similarly found HOXA9 methylation to be associated with shorter cancer-specific survival (HR, 3.45; $P = 4.E-04$; Table 2). In addition, the association remained significant when the analysis was adjusted for therapy (Supplementary Table S3). We also found HOXA9 promoter methylation to be predictive of poor outcome in the subset of patients who received therapy. However, the number of patients confirmed to have received therapy is relatively small (Table 1) and the type of therapy received is heterogeneous (chemotherapy, radiation or both). It is worth noting that when HOXA9 promoter methylation was added to clinical and pathological characteristics (*i.e.*, age, gender, race, stage, and smoking history), the risk model was significantly improved (nested likelihood ratio test, $P = 0.0004$), whereas addition of the same characteristics to the molecular marker did not lead to higher predictive accuracy (nested likelihood ratio test, $P = 0.64$). Furthermore, including HOXA9 methylation

increased the C-index from 0.60 (95% CI 0.51–0.69) for a model with clinical and pathological variables only, to 0.68 (0.60–0.76). Thus, adding HOXA9 methylation status improves the power of the model to predict patient outcome.

3.3. Highly methylated tumors harbor molecular characteristics of aggressive tumors

The *TP53* gene is frequently mutated in lung cancer and *TP53* mutation is a predictor of poor clinical outcome in stage I NSCLC patients [29]. We observed a higher frequency of *TP53* mutations in tumors with high HOXA9 promoter methylation, both when considering all stage I ($P = 0.0002$; Supplementary Fig. S4A) or stage IA only ($P < 0.0001$; Supplementary Fig. S4B). To further evaluate the molecular differences between tumors with high and low HOXA9 promoter methylation, we interrogated the transcriptomic profiles generated in our earlier study [10]. After applying a 25%FDR and absolute 1.2-fold expression thresholds, there were 620 genes and microRNAs differentially expressed between those two groups (Supplementary Table S4). We found that, although *HOXA9* was not differentially expressed, several *HOX* genes from other clusters were upregulated, including its paralogue, *HOXC9* and others within this cluster (i.e., *HOXC6*, *HOXC4*, *HOXC8*). This finding is consistent with that of Ooki et al. (16). Several microRNA species from intergenic regions of *HOX* clusters were also up-regulated (i.e., miR-196a, miR-10a). In addition, miR-9 was upregulated in tumors with high methylation. This microRNA has been described as an OncomiR [30] and is associated with metastasis and poor prognosis of NSCLC [31–34]. Furthermore, Ingenuity Pathway Analysis identified lysine acetyltransferase 6A (KAT6A), known to regulate *HOX* family gene expression including *HOXA9*, as the top activated upstream regulator in high methylation tumors (Supplementary Information IPA analysis). Canonical Pathways affected include: *Complement System*, *Cell Cycle*, *DNA damage response*, and *Natural Killer Cell Signaling*. However, significance values associated with these pathways were only moderate (10^{-5} to 10^{-3}). Altogether, these results suggest that tumors identified with high HOXA9 promoter methylation have specific molecular features and characteristics of highly aggressive tumors.

3.4. Combining HOXA9 promoter methylation and BVI risk-stratifies stage I lung adenocarcinoma patients

BVI has been shown to be a predictor of survival for NSCLC [18–21]. Staining for BVI was performed in FFPE tissues (Supplementary Fig. S5) from a subset of stage I patients also evaluated by HOXA9 methylation and with similar characteristics (Table 1). BVI was present in 15% (17/113) patients. Five-year survival was shorter in patients with BVI, in the Kaplan-Meier analysis (log-rank $P = 0.001$; Fig. 4A) and by multi-variable Cox regression analysis (HR, 2.62; $P = 0.054$; Table 2 and Supplementary Table S5). Since the combination of statistically and mechanistically independent biomarkers may provide a more robust prognostic signature, we evaluated the performance of HOXA9 promoter methylation together with BVI. Patients were dichotomized on the basis of the cumulative number of high values for both biomarkers. An increasing combined score was associated with worse clinical outcome in the Kaplan-Meier analysis (log-rank for trend $P = 0.0001$; Fig. 4B) and multivariable Cox regression analyses (trend $P = 9.7 \times 10^{-5}$; Table 2), after adjusting for covariates. Of note, compared to HOXA9 promoter methylation alone, a score that

combined BVI and HOXA9 promoter methylation showed higher predictive accuracy in an adjusted model (C-index, 0.68 vs 0.72).

4. Discussion

LDCT screening results in an increased detection of early-stage lung cancer, and therefore holds promise for improvements in the prognosis of the disease. Surgery alone is the standard of care for stage I NSCLC, as the benefit of adjuvant chemo- or radio-therapy to reduce the risk of recurrence in these patients remains controversial [6,7]. Relapse eventually occurs in a significant proportion of patients within 5 years [4]. Consequently, prognostic biomarkers are urgently needed to predict recurrence following surgery and improve clinical management of high-risk patients. In a previous study, we reported a prognostic classifier comprising mRNA, microRNA and DNA methylation biomarkers [10]. Here, we move towards the clinical application of HOXA9 promoter methylation, by (i) evaluating its prognostic significance in an extended set of stage I NSCLC patients, (ii) developing a ddPCR assay suitable for clinical application and (iii) using FFPE tissue samples generated by routine pathology. Moreover, we provide further evidence of the prognostic role of BVI and propose a combined classifier to better risk-stratify stage I patients using FFPE tissue.

HOXA9 promoter methylation was evaluated in a total of 177 patients, including a subset of patients for which we had previously evaluated methylation using a pyrosequencing-based assay in fresh-frozen tissue samples [10]. Compared to our past study, we observed lower values for HOXA9 promoter methylation, due to the use of different technologies and biospecimens. In fact, the level of HOXA9 promoter methylation measured with ddPCR was lower than pyrosequencing, despite covering the same CpG sites (an additional CpG was analyzed by ddPCR). Similar results have been reported before [35], and can be in part explained by the different readouts that are used to quantify methylation in the two methods. Contrary to pyrosequencing, that measures the percentage of methylation at each of several CpG sites within a given locus, ddPCR reports on the complete methylation of the target sequence [36]. We observed that primer-template mismatches, due for instance to partial methylation of the promoter, led to reduced PCR efficiency. Moreover, we noticed decreased values of HOXA9 promoter methylation in FFPE tissues. This is most likely due to the lower quality of FFPE DNA, as formalin fixation induces several artifacts, such as DNA fragmentation and base modification. Therefore, the reference gene (C-LESS reaction) accompanied each ddPCR reaction to control for total input and quality of amplifiable DNA. Of note, even though FFPE and fresh frozen represent different sections of a tumor that were each interrogated by a different technique, HOXA9 promoter methylation status was concordant in 72% of paired samples after adjusting the methylation cut-off value. This suggests that robustness of the biomarker across different tissue sources and technologies.

The prognostic significance of HOXA9 promoter methylation in early-stage NSCLC has been reported in several studies, although the mechanisms explaining its effect on survival remain unclear. Sandoval et al., found that hypermethylation of a panel of 5 genes, including HOXA9, was associated with recurrence and shorter relapse-free survival (RFS) [15]. We also observed an association between high HOXA9 promoter methylation, and worse cancer-specific survival and RFS [10]. Finally, Ooki et al. recently reported poor prognosis

associated with HOXA9 promoter methylation together with three other genes [16]. Here we show consistent results, as high HOXA9 promoter methylation identified a subset of stage I lung adenocarcinoma patients at risk for cancer-specific death. Furthermore, we observed that adding HOXA9 methylation status improved a risk model based on clinical and pathological characteristics. Analysis of *TP53* mutation status and gene expression profiles revealed that high methylation tumors harbor molecular features associated with more aggressive tumors and worse outcome. Thus, high HOXA9 methylation could be a useful surrogate marker for highly invasive tumors. To foster translation of this biomarker to the clinic, we used a platform that is suitable for implementation in clinical practice. ddPCR is becoming increasingly used for analysis of cancer biomarkers, including methylation and mutations, as it is more sensitive than pyrosequencing and quantitative methylation-specific PCR and, compared to next-generation sequencing, is less labor intensive, less prone to errors and more cost-efficient [8,22].

The analysis and integration of different types of biomarkers is a core principle of precision oncology [37,38]. FFPE is the standard preservation method, and as such, FFPE samples are very useful in the discovery and clinical development of biomarkers. However, the use of FFPE tissues for molecular analysis can be limited when DNA is degraded or contains too many lesions. Therefore, evaluating an immunohistochemical biomarker like BVI [18–21], in parallel with an epigenetic biomarker, is a valuable strategy for prognostic prediction. Here, we report further evidence that the presence of BVI is associated with worse cancer-specific survival, and that its combination with HOXA9 promoter methylation improves the predictive accuracy for stage I lung adenocarcinoma patients.

Our study has several limitations, including analysis of a single, retrospective patient cohort, comprised of patients with lung adenocarcinoma. No standard method exists to calculate a cut-off value for methylation, as most studies only evaluate the 'presence' of methylation. Thus, validation in FFPE samples from independent patient cohorts will be necessary to establish reproducibility in different clinical settings and robustness of the methylation cut-off value, set here at 30% PMR. The cut-off value determined in the present study could differ slightly depending on the quality of the FFPE DNA or the methylated control DNA used to calculate the PMR.

In conclusion, a combined classifier that includes HOXA9 promoter methylation and BVI, can robustly risk-stratify early-stage NSCLC patients. The biomarker assay we developed could easily be translated into clinical settings. If validated in a larger independent study, our findings could help inform patient management in prospective clinical trials that evaluate the benefit of adjuvant therapy in high risk early-stage NSCLC patients to prevent recurrence.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Sensitivity, specificity and reproducibility of the HOXA9 promoter methylation ddPCR assay. The HOXA9 assay is highly sensitive, linear and reproducible. (A) Serial 1:10 dilution of bisulfite-converted fully methylated control DNA ($n = 3$ independent replicates). (B) 1-D ddPCR analysis plot showing methylated HOXA9 promoter in serial dilutions (blue dots, positive amplification droplets; grey dots, negative amplification droplets). The HOXA9 reaction is specific for methylated DNA, and does not amplify (C) unmethylated control DNA and (D) non-converted DNA. The C-LESS assay was used to determine the relative amount of input DNA. HOXA9 ddPCR assay detects a higher HOXA9 promoter methylation level in tumors from (E) fresh frozen and (F) FFPE samples, compared to adjacent non-tumor tissues. Mean + Standard Deviation; paired t -test.

Fig. 2.
Kaplan-Meier survival analyses of HOXA9 promoter methylation in stage I lung adenocarcinoma patients. In the test set (A) and the validation set (B), high methylation of HOXA9 promoter was associated with worse clinical outcome. Low methylation, PMR values < 30%; high methylation, PMR values > 30%.

Fig. 3. Kaplan-Meier cancer-specific survival for stage I lung adenocarcinoma patients according to HOXA9 promoter methylation. In the merged analysis, high HOXA9 promoter methylation was associated with worse prognosis in stage I (A), and within the subgroups stage IA (B) and stage IB (C). Low methylation, PMR values < 30%; high methylation, PMR values > 30%.

Fig. 4.

Kaplan-Meier survival analyses of blood vessel invasion (BVI) and the combined prognostic classifier, in patients with stage I lung adenocarcinoma. The presence of BVI (A) and an increasing combined score (B) were associated with greater risk for poor outcome. Low risk, BVI negative and PMR values < 30%; intermediate risk, BVI negative and PMR values > 30%, or BVI positive and PMR values < 30%; high risk, BVI positive and PMR values > 30%.

Table 1

Demographic and clinical characteristics of the National Cancer Institute-Maryland cohort.

	HOXA9 Test set (N = 107)	HOXA9 Validation set (N = 70)	BVI set (N = 113)
Age (years)			
Mean (SD)	65 (10.5)	61 (9.7)	64.6 (10.9)
Range	38–88	39–84	32–88
Gender (%)			
Male	49 (45.8)	38 (54.3)	56 (49.6)
Female	58 (54.2)	32 (45.7)	57 (50.4)
Race (%)			
Caucasian	70 (65.4)	54 (77.1)	72 (63.7)
African-American	37 (34.6)	16 (22.9)	41 (36.3)
TNM stage (%)			
IA	65 (60.7)	43 (61.4)	71 (62.8)
IB	39 (36.4)	23 (32.9)	40 (35.4)
Unspecified	3 (2.8)	4 (5.7)	2 (1.8)
Tumor size (mm)			
Mean (SD)	2.6 (1.1)	2.6 (1.1)	2.4 (1.1)
Range	0.8–5	0.5–4.6	0.8–5
Smoking history (%)			
Never	6 (5.6)	5 (7.1)	6 (5.3)
Former/current	86 (80.4)	63 (90)	91 (80.5)
Unknown	15 (14)	2 (2.9)	16 (14.2)
Pack-years (%)			
< 30 pack-years	17 (15.9)	10 (14.3)	19 (16.8)
> 30 pack-years	67 (62.6)	56 (80)	70 (62)
Unknown	23 (21.5)	4 (5.7)	24 (21.2)
Adjuvant Therapy (%)			
No	78 (80.4)	42 (80.8)	80 (78.4)
Yes	19 (19.6)	10 (19.2)	22 (21.6)
Unknown	10	18	11

Table 2

Univariable and multivariable Cox proportional-hazard regression of HOXA9 promoter methylation, blood vessel invasion (BVI) and the combined prognostic classifier. Included in the Cox model: age, gender, race, tumor stage, smoking history (never, former/current) and pack-years. HR, hazard ratio; CI, confidence interval.

Variables	Univariable	<i>P</i>	Multivariable	<i>P</i>
	HR (95%CI)		HR (95%CI)	
HOXA9 methylation (high)				
Test set (n = 107)	3.49 (1.68–7.27)	0.001	3.52 (1.53–8.10)	0.003
Validation set (n = 70)	3.09 (1.40–6.84)	0.005	4.28 (1.53–11.97)	0.006
Merge set (n = 177)	3.31 (1.93–5.66)	1.3.E–05	3.37 (1.8–6.31)	1.5.E–04
Therapy naive (n = 120)	3.45 (1.74–6.84)	4.E–04	4.18 (1.8–9.67)	0.001
BVI (positive)	2.74 (1.21–6.19)	0.016	2.62 (0.98–6.97)	0.054
Combined classifier				
Low	Reference		Reference	
Intermediate	2.24 (0.95–5.28)	0.065	2.68 (0.98–7.32)	0.055
High	21.17 (6.55–68.37)	< 0.0001	23.59 (5.52–100)	< 0.0001
	Trend <i>p</i> = 2.9.E–05		Trend <i>p</i> = 9.7.E–05	