Clinical Epigenetics

BRIEF REPORT

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HOXA9 methylation is not associated with survival in Brazilian patients with lung adenocarcinoma

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Abstract

Homeobox A9 promoter methylation (*HOXA9*) has been reported as a biomarker for early lung adenocarcinoma patients' prognosis. We aim to evaluate its prognostic value, regardless of disease stage. Using droplet digital PCR, we measured *HOXA9* methylation in a cohort comprising 161 Brazilian patients. Low *HOXA9* methylation was associated with higher cancer-specific survival but showed no significance after adjustment for clinical covariates. While low *HOXA9* methylation was associated with earlier stages, no survival association was observed in this subset of patients. Overall, *HOXA9* promoter methylation is not an independent prognostic biomarker of cancer-specific survival in Brazilian lung adenocarcinomas patients.

Keywords *HOXA9*, Methylation, Prognostic biomarker, Non-small cell lung cancer, Latin America

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Introduction

Lung cancer is the leading cause of cancer-related deaths worldwide [1]. Despite the development of various systematic treatment strategies, such as targeted therapy and immunotherapy, the prognosis remains poor, with a 5-year survival rate of lower than 20% [2]. This lack of improvement is primarily due to most patients with lung cancer being diagnosed at an advanced stage [2]. A strategy to overcome the low survival rates may be implementing lung cancer screening strategies, and also identifying prognostic biomarkers for patient stratification [2].

Epigenetic biomarkers, such as differentially methylated genes, have emerged with several applications in cancer care [3]. Methylation affects gene transcription thought mechanisms catalyzed by enzymes, which add or remove methyl groups from CpG sites. Hypermethylation usually inhibits gene transcription; while, hypomethylation increases gene transcription. Aberrant methylation of the promoter region of a tumor suppressor genes is a hallmark of cancer [4].

The Homeobox A9 (*HOXA9*) belongs to the Homeobox family that contains 39 genes located on four different chromosomes [5]. *HOXA9* is a transcription factor that plays an essential role in embryonic development. In cancer, the role of *HOXA9* is not entirely elucidated [6]. Hypermethylation of *HOXA9* has been reported as a biomarker to diagnose earlier stages of lung cancer [7]. Studies using liquid biopsy have reported that *HOXA9* promoter methylation could also be used as a diagnostic marker in blood samples [8]. In addition, *HOXA9* promoter methylation has shown a prognostic value for earlier-stage lung adenocarcinoma [9]. Nevertheless, its prognostic value in other tumor stages, including metastatic cases, has never been investigated.

In the present study, we aim to (i) Establish a cutoff in archive formalin-fixed, paraffin-embedded (FFPE) lung adenocarcinoma tissue to discriminate between patients with decreased and increased cancer-specific survival, and (ii) Investigate the prognostic value of *HOXA9* promoter methylation in all stages lung adenocarcinoma.

Methods

Patients

We analyzed 161 patients diagnosed with earlier ($n=65$) and advanced adenocarcinomas ($n=96$), who were treated at Barretos Cancer Hospital (BCH) between 2011 and 2019. We categorized the patients into two groups: 'earlier-stage' combining stages I, II, and IIIA, and 'advanced stage' comprising stages IIIB and IV. Clinical information was extracted from medical records. The study was conducted according to Brazilian national and institutional ethical policies and was previously approved by the Barretos Cancer Hospital IRB (Project #630/2012). Due to its retrospective nature, informed consent was waived.

DNA isolation and bisulfite treatment

Serial 10 µm unstained sections of FFPE blocks were cut, and one hematoxylin and eosin-stained (H&E) section was taken for pathological evaluation and selection of the tumor area for DNA isolation. Briefly, sections were heated at 80 °C, and serial washes with xylene and ethanol (100, 70, and 50%) were performed for paraffin removal. Then, sections were macrodissected using a sterile needle and carefully collected into a microtube. Next, DNA was isolated from FFPE tissues using the QIAmp DNA micro kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. DNA concentration and quality were evaluated by Qubit fluorometric quantification (Thermo Scientific, Wilmington, USA). Bisulfite conversion was made with 100 ng of DNA using the EZ-DNA Methylation-Direct™ Kit MiniPrep (ZymoResearch, Irvine, USA). Bisulfite-converted DNA was eluted in 11 µl ultrapure water and stored at − 80 °C.

Droplet digital PCR (ddPCR) analysis

HOXA9 promoter methylation was analyzed by ddPCR as described by Lissa and colleagues [9]. Briefly, the ddPCR reaction was performed with 2X ddPCR Supermix for Probes (Bio-Rad, Hercules, CA, USA), 250 nM of each primer, 900 nM of the probe, and 1 µL bisulfite-converted DNA in a final volume of 22,1 µL followed by droplet generation using an automated droplet generator (Bio-Rad Laboratories, Hercules, CA, USA). Cycling conditions included preheating at 95 °C for 10 min followed by 40 cycles of denaturation at 94 °C for 30 s, annealing and extension at 56 °C for 60 s, a final heating at 98 °C for 10 min for DNA polymerase deactivation and 4 °C for cooling. After amplification, the PCR plate was transferred to a QX100 droplet reader (Bio-Rad Laboratories, Hercules, CA, USA), and fluorescence amplitude data were obtained by QuantaSoft software (Bio-Rad Laboratories, Hercules, CA, USA). A valid result was considered when more than 10,000 droplets were generated in each reaction. All experiments included a bisulfite-converted methylated and non-methylated control DNA, and a No Template Control (NTC). Primers and probe sequences were as follows: for *HOXA9* Forward: 5′-GTG GTTATTATCGTGTTTAGCGT-3′, Reverse: 5′-CCG ATACCACCAAATTATTACATA-3′, Probe: 6FAM-5′-TGGTTCGTTCGGTTCGATTTACGGA-3′-NFQ, and *C-LESS* Forward: 5′-TTGTATGTATGTGAGTGTGGG AGAGA-3′, Reverse: 5′-TTTCTTCCACCCCTTCTC TTCC-3′, Probe: 6FAM-5′-CTCCCCCTCTAACTCTAT -3′-NFQ.

To obtain a measure of *HOXA9* methylation for each sample, we calculated the percentage of methylation reference (PMR) using the number of copies/20 µl well, as follows:

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

Statistical analysis

The methylation threshold was determined using the minimum p value as previously reported [9], in which 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 logrank test (cancer-specific survival). Then, the optimal cut point that gives the most pronounced p value was selected. This optimal PMR was used to evaluate its ability to stratify patients into low and high cancer-specific survival groups in both earlier (IA-IIIA) and advanced stages (IIIB-IV).

Univariate survival analysis was performed using Kaplan–Meier survival curves, Logrank tests, and Cox regression. Survival time was calculated as the number of months from the date of diagnosis to the date of lung cancer-specific death. Causes of death other than lung cancer were censored. Multivariable analysis was performed using the Cox regression method using variables that presented a p-value ≤ 0.05 in the univariate analysis. In the final model, those that presented a p-value ≤ 0.05 remained. Hazard ratios (HR) and 95% confidence interval were estimated using univariable and multi-variable Cox proportional hazards regression modeling.

Statistical analysis was performed using the IBM SPSS Statistics software version 26.0 (Chicago, IL, USA) and RStudio. Statistical significance was defined as p-value ≤ 0.05.

Systematic literature search

We performed a systematic literature search on PubMed and Scopus to select papers published until January 2024 analyzing *HOXA9* promoter methylation in non-small cell lung cancer (NSCLC) clinical samples (Supplementary Table 1). Using the syntax: (("Lung Neoplasms"[Mesh]) AND (hoxa9)) AND ("DNA Methylation"[Mesh]), we identified 24 studies on PubMed and 26 on Scopus, with 21 overlapping. Fourteen were included; while, ten were excluded: one for not evaluating HOXA9 methylation, five for focusing on new detection methodologies, one using cell lines, one a meta-analysis, one a review, and one for not analyzing *HOXA9* methylation in NSCLC.

Results

Most patients were male (57.8%), diagnosed with advanced stages (59.6%; IIIB-IV), current/quitter smokers (74.5%), and did not harbor *EGFR* mutations (76.4%). Comparing the characteristics of patients with earlier and advanced stages, the age distribution is similar, with most patients being ≤ 64 years. However, female representation is lower in the advanced group. The proportion of patients with *HOXA9* methylation ≤ 83 is higher in earlier stages (55.4%) than in advanced stages (42.7%). Smoking history shows the most current/quitter smokers in both groups. As expected, PS-ECOG scores indicate a higher performance status impact in advanced stages. *EGFR* status shows a higher percentage of wild-type in both stage groups (Supplementary Table 2).

We identified an optimal PMR cutoff value for *HOXA9* promoter methylation in our cohort by determining the minimum p value from the logrank test, using several *HOXA9* promoter methylation thresholds to distinguish between low and high cancer-specific survival groups (Fig. 1A). Based on this analysis, we identified a PMR 83 with p-value = 0.027 (Fig. 1A–B) for downstream analysis.

Previous studies have suggested the potential of *HOXA9* methylation as a prognostic biomarker in earlier-stage lung cancer (Supplementary Table 1); therefore, its potential was evaluated in this Brazilian series. *HOXA9* methylation levels were lower in earlier stages (Supplementary Fig. 1A; Fisher's exact test $p = 0.005$; Supplementary Table 3), although they showed no significant difference according to cancer-specific survival (Supplementary Fig. 1B). Consistently, no association was observed between dichotomous *HOXA9* promoter methylation (PMR < 83) and high cancer-specific survival in Kaplan–Meier analysis ($p = 0.130$; Fig. 1C) and univariable Cox regression analysis (Hazard Ratio [HR], 1.89; p-value = 0.141; Table 1). Similarly, multi-variable Cox regression analysis showed no association between *HOXA9* methylation and disease outcome (Hazard Ratio [HR], 1.35; p-value = 0.600; Table 1). We further performed the analysis in only the advanced stage subset. Similar to the earlier-stage group, we observed no significant correlation between low *HOXA9* promoter methylation (PMR < 83) and high cancer-specific survival (Fig. 1D; Table 1).

When we combined all stages patients, the low *HOXA9* promoter methylation (PMR < 83) was associated with high cancer-specific survival in the Kaplan–Meier (Logrank test, p-value = 0.027; Fig. 1B) and univariable Cox regression analysis (Hazard Ratio [HR], 1.52; p-value = 0.029; Table 1). After adjustment for age, sex, tumor stage, smoking history, PS-ECOG, and *EGFR* mutation status, the multivariable Cox regression analysis did not demonstrate the *HOXA9* promoter methylation as an independent prognostic biomarker

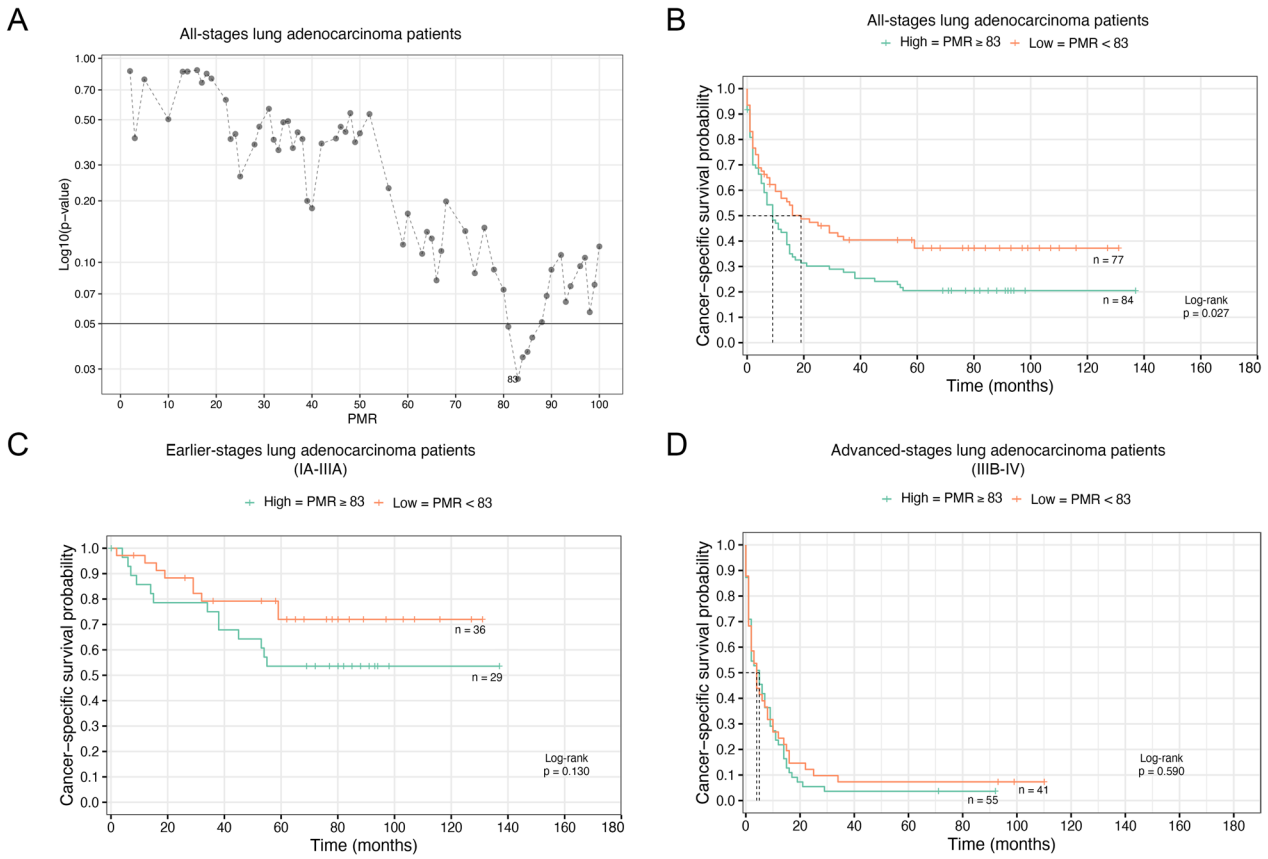

Fig. 1 Prognostic significance of HOXA9 promoter methylation in patients with adenocarcinoma. A: Patients are dichotomized for each potential PMR cutoff, and the survival difference between high and low PMR groups was calculated by logrank. The X-axis represents each PMR cutoff, and the Y-axis represents raw p values on a log scale. The cut point to minimize the p value was determined (83%). B: Kaplan–Meier survival analysis of adenocarcinoma patients was conducted using a cutoff of 83% to classify patients into low- and high-methylation groups, based on whether their HOXA9 promoter methylation levels were below or above the cutoff, respectively. C and D represent the identical analysis described in B in earlier-stage (IA-IIIA) and advanced stage (IIIB-IV), respectively

of cancer-specific survival (Hazard Ratio [HR], 1.18; p-value = 0.400; Table 1).

In line with this, the ROC curve demonstrated low specificity and sensibility of *HOXA9* promoter methylation in distinguishing between low and high cancer-specific survival (Supplementary Fig. 1C).

The presence of *EGFR* mutation was an independent variable for predicting high cancer-specific survival in our multivariable Cox regression model (Hazard Ratio [HR], 0.32; p-value < 0.001; Table 1). Considering that half of the *EGFR*-mutated patients had received TKi therapies in any line of treatment, we investigated whether *HOXA9* promoter methylation could stratify *EGFR* wild-type patients into those with high and low cancer-specific survival. Our multivariable Cox regression analysis did not indicate the *HOXA9* promoter methylation as an independent biomarker for predicting higher cancer-specific survival in this *EGFR* wild-type subgroup of patients (Hazard Ratio [HR], 0.99; p-value > 0.900; Supplementary Table 4).

Discussion

The present study showed that *HOXA9* promoter methylation was not an independent prognostic biomarker in Brazilian lung adenocarcinoma patients from a single Institution.

It has been described that *HOXA9* promoter methylation is a prognostic biomarker in early-stage NSCLC patients (Supplementary Table 1). High *HOXA9* promoter methylation was associated with stage I lung adenocarcinoma patients at risk for cancer-specific death and shorter relapse-free survival [7, 9]. Due to the limited sample size for each stage, we could not assess the prognostic value of *HOXA9* promoter methylation in stage I adenocarcinoma patients. Then, we classified the patients as 'earlier-stage' combining stages I, II and IIIA. This approach has been used in several studies, since the 'earlier-stage' group comprises only patients with localized disease. We did not find an association between *HOXA9* promoter methylation and earlier-stage adenocarcinoma.

Table 1 Univariate and multivariate Cox regression analysis across the 3 groups using the *HOXA9* promoter methylation cutoff delivered by minimum *p*-value approach

All stages adenocarcinoma patients (*n* = 161)					**Earlier-stages (IA-IIIA) adenocarcinoma patients (*n* = 65)**					**Advanced stages (IIIB-IV) adenocarcinoma patients (*n* = 96)**				
Variables	**Univariable**		**Multivariable**		**Variables**	**Univariable**		**Multivariable**		**Variables**	**Univariable**		**Multivariable**	
	HR(95%CI)	***p*-value**	**HR(95%CI)**	***p*-value**		**HR(95%CI)**	***p*-value**	**HR(95%CI)**	***p*-value**		**HR(95%CI)**	***p*-value**	**HR(95%CI)**	***p*-value**
Age					Age					Age				
≤ 64	Ref	–	Ref	–	≤ 64	Ref	–	Ref	–	≤ 64	Ref	–	Ref	–
> 64	0.87(0.60–1.26)	0.456	1.34(0.88–2.04)	0.200	> 64	1.00(0.43–2.31)	0.998	1.09(0.38–3.11)	0.900	> 64	1.42(0.93–2.16)	0.104	1.24(0.75–2.03)	0.400
Sex					Sex					Sex				
Female	Ref	–	Ref	–	Female	Ref	–	Ref	–	Female	Ref	–	Ref	–
Male	**1.62(1.10–2.37)**	**0.014**	1.09(0.73–1.64)	0.700	Male	1.53(0.66–3.54)	0.321	0.71(0.24–2.13)	0.500	Male	1.27(0.82–1.96)	0.282	1.06(0.66–1.70)	0.800
HOXA9-Meth					HOXA9-Meth					HOXA9-Meth				
≤ 83	Ref	–	Ref	–	≤ 83	Ref	–	Ref	–	≤ 83	Ref	–	Ref	–
> 83	**1.52(1.05–2.21)**	**0.029**	1.18(0.79–1.77)	0.400	> 83	1.89(0.81–4.43)	0.141	1.35(0.46–3.96)	0.600	> 83	1.12(0.74–1.71)	0.590	0.96(0.60–1.51)	0.800
Smoking history					Smoking history					Smoking history				
Never smoker	Ref	–	Ref	–	Never smoker	Ref	–	Ref	–	Never smoker	Ref	–	Ref	–
Current/quitter smoker	**2.12(1.32–3.42)**	**0.002**	1.63(0.96–2.77)	0.070	Current/quitter smoker	**3.63(1.07–12.30)**	**0.038**	**3.67(1.02–13.20)**	**0.047**	Current/quitter smoker	**2.16(1.27–3.67)**	**0.005**	1.39(0.76–2.53)	0.300
TNM Stage					TNM Stage					TNM Stage				
IA-IIIA	Ref	–	Ref	–	IA	Ref	–	Ref	–	IIIB	Ref	–	Ref	–
					IB	0.74(0.12–4.44)	0.744	0.99(0.14–7.02)	> 0.900					
IIIB-IV	**8.86(5.41–14.5)**	**< 0.001**	**10.90(5.89–20.30)**	**< 0.001**	IIA	2.22(0.23–21.30)	0.491	6.16(0.54–70.02)	0.140	IV	1.62(0.66–3.99)	0.296	1.99(0.74–5.35)	0.200
					IIB	2.69(0.60–12.00)	0.196	**3.47(0.72–16.80)**	**0.120**					
					IIIA	**5.04(1.42–17.90)**	**0.012**	**10.60(2.34–47.80)**	**0.002**					
EGFR status					EGFR status					EGFR status				
WT	Ref	–	Ref	–	WT	Ref	–	Ref	–	WT	Ref	–	Ref	–
Mutated	0.67(0.43–1.06)	0.085	**0.32(0.18–0.54)**	**< 0.001**	Mutated	0.32(0.08–1.39)	0.129	0.27(0.04–1.66)	0.200	Mutated	**0.39(0.23–0.64)**	**< 0.001**	**0.28(0.15–0.51)**	**< 0.001**

Similar findings were also reported by Milica and colleagues, who showed no significant survival differences associated with *HOXA9* promoter methylation in primary NSCLC [10].

HOXA9 promoter methylation has been associated with other molecular features, such as *TP53* mutations [9]. In our study, the *TP53* status was available only for a small subset of patients, hampering any statistical analysis. Nevertheless, we associated it with the *EGFR* status, and observed that *HOXA9* methylation was not useful for prognostic stratification of *EGFR* wild-type cases. However, *EGFR* mutation was an independent variable for predicting favorable outcomes regardless of *HOXA9* methylation.

A challenge in establishing a methylation biomarker is the absence of a standard method to calculate a cutoff value for methylation, as observed in our meta-analysis. Most studies only evaluate the ´presence´ of methylation or stratify the methylation levels in quartile. Thus, the prognostic value of *HOXA9* promoter methylation in all stages lung adenocarcinoma remains to be investigated in different clinical settings using a minimum p value approach for establishing an optimal cutoff.

Our study has some limitations, including the analysis of a single, retrospective, limited-size patient cohort. Also, combining different disease stages may lead to issues in survival analysis. Therefore, evaluating *HOXA9* promoter methylation as a prognostic biomarker in a real-world clinical setting of NSCLC should be investigated in other populations with larger sample size.

Finally, although we applied the same methylation cutoff for different groups of adenocarcinomas—all stages, earlier, and advanced stages—the methylation cutoff did not stratify patients according to cancer-specific survival, adjusting for clinically relevant covariates. This suggests that while *HOXA9* promoter methylation might be a valuable biomarker, especially in lung cancer screening programs, its effectiveness may be limited in real-world clinical setting when most of the patients are still diagnosed at advanced stages.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13148-025-01831-1.

Additional file 1.

Additional file 2.

Additional file 3.

Additional file 4.

Additional file 5.

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Author contributions
Conceptualization contributed by D.L., A.C.C., R.M,R. and L.F.L. Patient recruitment and clinical data collection contributed by F.A.S.S.,R.O.C., A.L.V.S., E.D.A.S., F.A.F.S., J.D.M., P.M. Investigation contributed by A.L.S.A.V., F.A.S.S., R.O.C.,A.L.V.S and M.B.R. Formal analysis contributed by A.L.S.A.V., F.A.S.S. and W.Y.H. Data interpretation contributed by A.L.S.A.V., F.A.S.S., W.Y.H, L.F.L. and R.M.R. Supervision contributed by R.M,R. and L.F.L. Writing—original draft contributed by A.L.S.A.V. and L.F.L. Editing and reviewing the paper contributed by all authors.

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Availability of data and materials
Data will be available upon request from the corresponding authors.

Declarations

Ethics approval and consent to participate
The study was conducted according to Brazilian national and institutional ethical policies. The present study was previously approved by the Barretos Cancer Hospital IRB (Project #630/2012) and informed consent was waived.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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