The prognostic impact of circulating homeobox A9 methylated DNA in advanced non-small cell lung cancer

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Background: The homeobox A9 gene encodes a transcription factor, and aberrantly methylated homeobox A9 in the circulation has been suggested as a prognostic marker in early stage non-small cell lung cancer (NSCLC). The aim of the present study was to investigate the prognostic impact of methylated homeobox A9 in plasma from patients with advanced NSCLC.

Methods: Blood samples were prospectively collected from patients with NSCLC stage III and IV receiving standard first line chemotherapy. Sampling took place before treatment initiation and subsequently before each treatment cycle. Plasma was stored at –80 ℃ until analysis. DNA was extracted, and following bisulfite conversion methylated homeobox A9 was analyzed by methylation specific droplet digital polymerase chain reaction. Detection of methylated homeobox A9 was assessed as a binary variable. The primary endpoint was overall survival (OS).

Results: A total of 231 patients were included. At baseline methylated homeobox A9 was detected in 78.5% of the patients with a clear correlation to survival. The median OS for patients with and without detectable methylated homeobox A9 was 7.4 and 11.1 months, respectively [hazard ratio (HR) 1.79, 95% confidence interval (CI): 1.35–2.38, P<0.001]. The difference increased after the first cycle of treatment. At this time point the median OS was 6.2 and 15.6 months for patients with and without detectable methylated homeobox A9, respectively (HR 2.07, 95% CI: 1.58–2.73, P<0.001). The independent prognostic impact of detectable methylated homeobox A9 after one treatment cycle assessed by multiple Cox regression including known prognostic factors resulted in a HR of 3.79 (2.19–6.54, P<0.001) compared to undetectable methylated homeobox A9.

Conclusions: Measurable methylated homeobox A9 after the first treatment cycle may serve as a valuable prognostic marker in patients with advanced NSCLC. Routine clinical application with treatment reconsideration calls for further studies, preferably in prospective clinical trials.

Keywords: Circulating tumor DNA; methylation; HOXA9; biomarkers; non-small cell lung cancer (NSCLC)

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856

Wen et al. Prognostic impact of circulating methylated HOXA9

Introduction

Lung cancer is estimated to have the highest cancer incidence and mortality worldwide (1). Non-small cell lung cancer (NSCLC) accounts for more than 80% of all cases. Almost half the Danish patients have metastases at the time of diagnosis, and data from England and the USA show that more than 70% are diagnosed at stage III or IV (2-4). Age-adjusted 5-year survival rates for lung cancer stage III and IV are 9% and 3%, respectively, in the Danish population (2) with similar rates in both England and the USA (4,5). Overall survival (OS) has generally improved over the past years, but there is no improvement for stages III and IV in the Danish population (2) despite introduction of new treatment options.

Patients with incurable NSCLC are eligible for systemic palliative treatment if they have an Eastern Cooperative Oncology Group performance status (PS) of 0–2 (6). If there are no actionable oncogenic driver mutations and the expression level of Programmed death ligand 1 (PD-L1) is <50%, the choice is usually a double-agent chemotherapy regimen (6). A meta-analysis of 16 randomized, controlled trials showed an increase in 1-year OS from 20% to 29% when comparing chemotherapy with best supportive care (7). This translates to an increase in median OS from 4.5 to 6 months.

One of the most well-established prognostic factors in NSCLC is the tumor-node-metastasis (TNM) classification (8). However, the group of patients with advanced or locally advanced disease is large, and prognostic biomarkers are needed for further risk stratification. Liquid biopsy, a term established in recent years, has been suggested as a method for obtaining tumor material through body fluids such as blood or sputum (9-11). One way to analyze a liquid biopsy is detection of circulating tumor DNA (ctDNA) (12). Aberrant methylation patterns can be detected in most malignant tumor cells, and methylated ctDNA has been suggested for both diagnostic and prognostic purposes in patients with lung cancer (13-17). A wide range of genes have been investigated with different results, but very few assays have been clinically validated (12).

The homeobox A9 gene (HOXA9) is located on chromosome 7. It encodes a DNA-binding transcription factor involved in regulating gene expression, but its specific function is unknown. HOXA9 has been suggested as a target for microRNA-196b, with HOXA9 suppression resulting in increased cell invasiveness *in vitro* (18). Another study showed that HOXA9 was involved in cell migration but not proliferation *in vitro* (19). Methylated HOXA9 (metHOXA9) in blood and other body fluids has been suggested as a diagnostic biomarker for NSCLC (13-15,20). A few studies have investigated metHOXA9 as a prognostic marker in early stage NSCLC with promising results (13,16,21). However, little is known about the methylation pattern of HOXA9 in advanced NSCLC and the prognostic information it may hold.

The aim of the present study was to investigate the prognostic impact of metHOXA9 in plasma from patients with advanced NSCLC cancer receiving standard first line treatment. We present the following article in accordance with the REMARK reporting checklist (22) (available at http://dx.doi.org/10.21037/tlcr-20-826).

Methods

Study population

The present study is a retrospective analysis of prospectively collected plasma samples from a cohort of lung cancer patients enrolled from June 2011 to September 2015 at the Department of Oncology, Vejle Hospital, Denmark. Inclusion criteria comprised age >18 years, histologically confirmed diagnosis of NSCLC, disease stage III or IV, PS ≤2, no previous chemotherapy, and eligibility for standard first line treatment. The exclusion criteria were other malignant disease within five years prior to study enrolment except non-melanoma skin cancer, experimental treatment within 30 days prior to study enrolment, and planned radiotherapy against target lesions.

The study was conducted in accordance with the Declaration of Helsinki and the Harmonized Tripartite Guideline for Good Clinical Practice from the International Conference on Harmonization. The study was reviewed and approved by the Regional Committee on Health Research Ethics for Southern Denmark (S-20110005) and performed in accordance with Danish data protection legislation. All enrolled patients gave written, informed consent.

Response evaluation

All patients were treated and followed according to national guidelines. Treatment effect was evaluated by computed tomography (CT) scans of the chest and abdomen, and images were assessed according to the RECIST 1.1 criteria (23) by experienced radiologists.

If no measurable target lesions were present, response status was based on non-target lesions and appearance of new malignant lesions. Treatment effect was evaluated after every two cycles or earlier as needed. Patients were censored in the event of radiation therapy against target lesions; the censor date was the date of the last evaluable CT scan. Radiologists were blinded to the biomarker status of the patients.

Specimen sampling and storage

Blood was drawn before initiation of first line medical oncology treatment (baseline), before each subsequent treatment cycle, and at confirmed disease progression. Two 9 ml peripheral blood samples were collected into EDTA-containing tubes and centrifuged at 2,000 g for 10 minutes. Plasma was stored at –80 ℃ until analysis. The samples were analyzed in the period from 2017 to 2019 resulting in storage time ranging from three to eight years.

Analysis of metHOXA9

Plasma was thawed and upon reaching room temperature centrifuged at 10,000 g for 10 minutes. Cell free DNA was extracted from 4 mL plasma as previously reported (24) using the DSP Circulating DNA kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The purified DNA was eluted in 60 µL and diluted with water to a total volume of 400 µL. The HOXA9 methylation specific analysis was previously published by our group (25). Briefly, DNA extraction was assessed by spike-in of an internal control [CPP1, a gene from soybean (26)]. The β2 microglobulin gene was used as a surrogate for the total amount of cell free DNA. Both CPP1 and 2 microglobulin were analyzed in triplet by quantitative PCR on 3 µL of the diluted, purified DNA. The purified DNA was concentrated to a final volume of 20 µL. All of the purified DNA was bisulfite converted using the EZ DNA Methylation-Lightning Kit (Zymo Research, Irvine, California, USA) as recommended by the manufacturer. The converted DNA was analyzed using an in-house, HOXA9 methylation specific, droplet digital polymerase chain reaction (ddPCR) assay (24,25) with albumin as reference gene (27). The primer and probe sequences as well as the PCR settings are available in Appendix 1 online. Water served as negative control, a pool of lymphocyte DNA from healthy donors (20 µL corresponding to approximately 20 ng) as non-cancer control, and Universal Methylated DNA Standard (Zymo Research) as positive control. See Appendix 1 for a detailed description of the positive control. The controls were bisulfite converted and analyzed by ddPCR in parallel with the patient samples. Droplets were generated on a QX200 Automated Droplet Generator (Bio-Rad, Hercules, California, USA). Results were read on a QX100 Droplet Digital Reader (Bio-Rad). Data were exported from QuantaSoft™ (Bio-Rad) as a percentage of metHOXA9 {calculated as [metHOXA9 copies/albumin copies] ×100} including a 95% confidence interval (CI) derived from a Poisson distribution (28). If the CI included 0, metHOXA9 was considered undetectable in the sample. Analysis of metHOXA9 was performed blinded to the clinical endpoints.

Analysis of lactate dehydrogenase (LDH)

LDH was measured as part of the standard blood work before treatment initiation. Peripheral blood was drawn into a 4 mL tube containing lithium-heparin. Plasma was kept at room temperature until analysis. LDH was analyzed on a Roche/Hitachi Cobas 8000 c702 using a UV assay measuring photometrical increase in the absorbance. The results were reported as units/liter (U/L).

Endpoints

The primary endpoint was OS defined as the number of months from blood sampling to death of any cause. The secondary endpoint was progression free survival (PFS) defined as the number of months from blood sampling to disease progression as defined by the protocol (see 2.2. Response evaluation) or death of any cause. Patients who did not experience an event were censored at the time of data workup (1 July 2019).

Statistical analyses

Categorical variables are presented as fractions (percentages) and continuous variables as medians (interquartile range, IQR). Comparisons between groups were made with Wilcoxon rank-sum test for numeric variables and Fisher's Exact test or Pearson's Chi-Square test for categorical variables. MetHOXA9 was assessed both as a continuous (percentage) and binary (detectable or undetectable) variable. Changes in the levels of metHOXA9 were compared with Wilcoxon signed-rank test. The correlation between metHOXA9 and LDH was analyzed
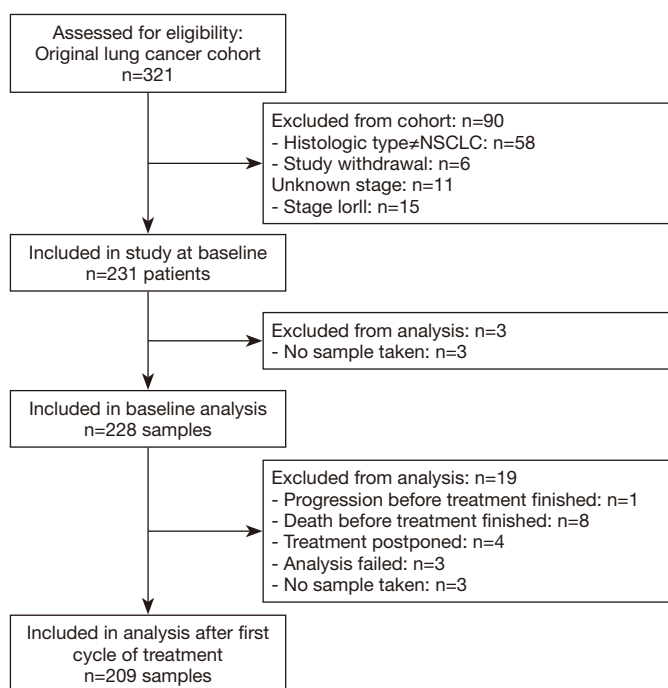
858

Wen et al. Prognostic impact of circulating methylated HOXA9

Figure 1 Flowchart illustrating the selection of patients from the original lung cancer cohort and reasons for exclusion from analysis after one cycle of treatment.

with Spearman Rank Correlation. Associations between metHOXA9 and endpoints were investigated using Kaplan-Meier curves and Log rank tests, with hazard ratio (HR) calculated by simple Cox regression. Independent prognostic impact was tested in multiple Cox regression analysis. The Cox regression model was constructed by the backward selection method including potentially prognostic factors such as sex, age, histologic cancer type, etc. Please refer to Appendix 1 online for a more detailed description of the development of the multiple Cox regression model. The model was assessed for violation of the proportional hazards assumption. All tests were two-sided with P values <0.05 considered significant. All analyses except Cox regression were performed using NCSS (NCSS, Utah, USA). Cox regression modelling was performed using STATA 16IC (StataCorp LLC, Texas, USA).

Results

Patient characteristics

A total of 231 patients met the inclusion criteria out of 321 patients from the original cohort (*Figure 1*). At baseline 228 samples were available, and after the first cycle of

treatment and at disease progression 209 and 134 samples, respectively, were included for analysis. Of the 231 patients included, 194 had disease progression (84%) and 226 had died (97.8%). Only one patient neither experienced progression nor death during the follow-up period. Median follow-up time was not calculated since the vast majority of patients experienced an event.

Patient characteristics are shown in *Table 1*. There was no significant difference between the methylation groups regarding sex, histology, stage, smoking status, and treatment. The group with no detectable metHOXA9 at baseline was characterized by significantly older patients and lower LDH compared to the group with detectable metHOXA9.

Dynamics of metHOXA9 in advanced NSCLC

The median level of metHOXA9 at baseline was 1.65% (IQR: 0.30–5.85%) with 21.5% of the patients having undetectable metHOXA9. After the first treatment cycle the median metHOXA9 was 0.53% (IQR: 0–2.80%) and the percentage of patients with undetectable metHOXA9 had significantly increased to 34.4% (P<0.001). At progression, the median level of metHOXA9 was 1.49% (IQR: 0.20–

Table 1 Patient characteristics of the whole cohort and as divided into subgroups by metHOXA9 status at baseline

Patient characteristics	Total, n=231	Baseline metHOXA9 =0, n=49	Baseline metHOXA9 >0, n=179	P value
Sex, male	126/231 (55%)	25/49 (51%)	99 (55%)	0.629
Age, years	67 [61–74]	71 [64–75]	66 [60–73]	0.017*
Histologic type				0.064
Adenocarcinoma	180 (78%)	43 /88%)	134 (75%)	
Squamous cell carcinoma	33 (14%)	2 (4%)	31 (17%)	
Other[†]	18 (8%)	4 (8%)	14 (8%)	
Stage				1.000
III	32 (14%)	7 (14%)	25 (14%)	
IV	199 (86%)	42 (86%)	154 (86%)	
Treatment				1.000
Platinum + vinorelbine	202 (87%)	43 (88%)	157 (88%)	
Other[‡]	29 (13%)	6 (12%)	22 (12%)	
Smoking status				0.241
Never	16 (7%)	6 (12%)	10 (6%)	
Previous	136 (59%)	26 (53%)	109 (61%)	
Active	74 (32%)	16 (33%)	56 (31%)	
Unknown[§]	5 (2%)	1 (2%)	4 (2%)	
LDH baseline, units/L	202 [171–254]	175.5 [160–224]	205 [176–275]	<0.001*
LDH baseline, ≥250 U/L	60/215 (28%)	4/46 (2%)	56/169 (26%)	<0.001*

Results are presented as a median (IQR) for numeric variables and a fraction (percentage) for categorical variables. Differences between groups were tested by Wilcoxon rank-sum test for numeric variables and by Fisher's exact test or Pearson's Chi square test for categorical variables as appropriate. *, statistically significant difference between groups; [†], category covers poorly differentiated non-small cell carcinoma and tumors with mixed histology treated as NSCLC; [‡], category covers vinorelbine monotherapy and tyrosine kinase inhibitors; [§], treated as missing values in analyses. LDH, lactate dehydrogenase; NSCLC, non-small cell lung cancer.

7.06%) with 17.9% of the patients having undetectable metHOXA9. The level of metHOXA9 increased in the three samples before disease progression. The median values were 0% (0–0.88%), 0.19% (0–0.90%), and 0.44% (0–1.88%), respectively. This increase, however, was not statistically significant (sample 3 compared to sample 5, P=0.054, *Figure 2*).

LDH and survival

There was a weak correlation between LDH and metHOXA9 (r=0.31, 95% CI: 0.18–0.43, P<0.001). There was no significant association between PFS and elevated LDH (LDH ≥250 U/L, P=0.150, n=218). Patients with normal range LDH (<250 U/L) had a median OS of

8.4 months while patients with elevated LDH had a median OS of 6.2 months, HR 1.40, (95% CI: 1.01–1.93, P=0.027, n=218). Kaplan-Meier plots for LDH status and other patient characteristics can be found in Appendix 1 online.

HOXA9-methylation and survival

The status of metHOXA9 was highly associated with survival. The median PFS in patients with and without baseline detectable metHOXA9 was 4.2 and 6.0 months, respectively, HR 1.57 (95% CI: 1.18–2.09, p=0.0041, n=228, *Figure 3A*). After one cycle of treatment the median PFS was 2.7 and 5.3 months in patients with and without metHOXA9, respectively, HR 1.74 (95% CI: 1.33–2.29, P=0.0001, n=209, *Figure 3B*). This difference also applied
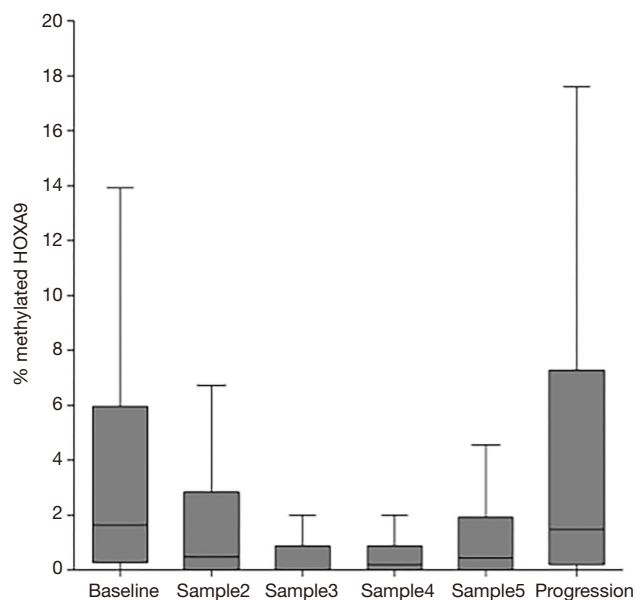
Figure 2 Box plot illustrating the dynamics of methylated HOXA9 from baseline, after the first cycle of treatment (sample 2), at the three samples before progression (samples 3–5), and after disease progression as confirmed by CT scan.
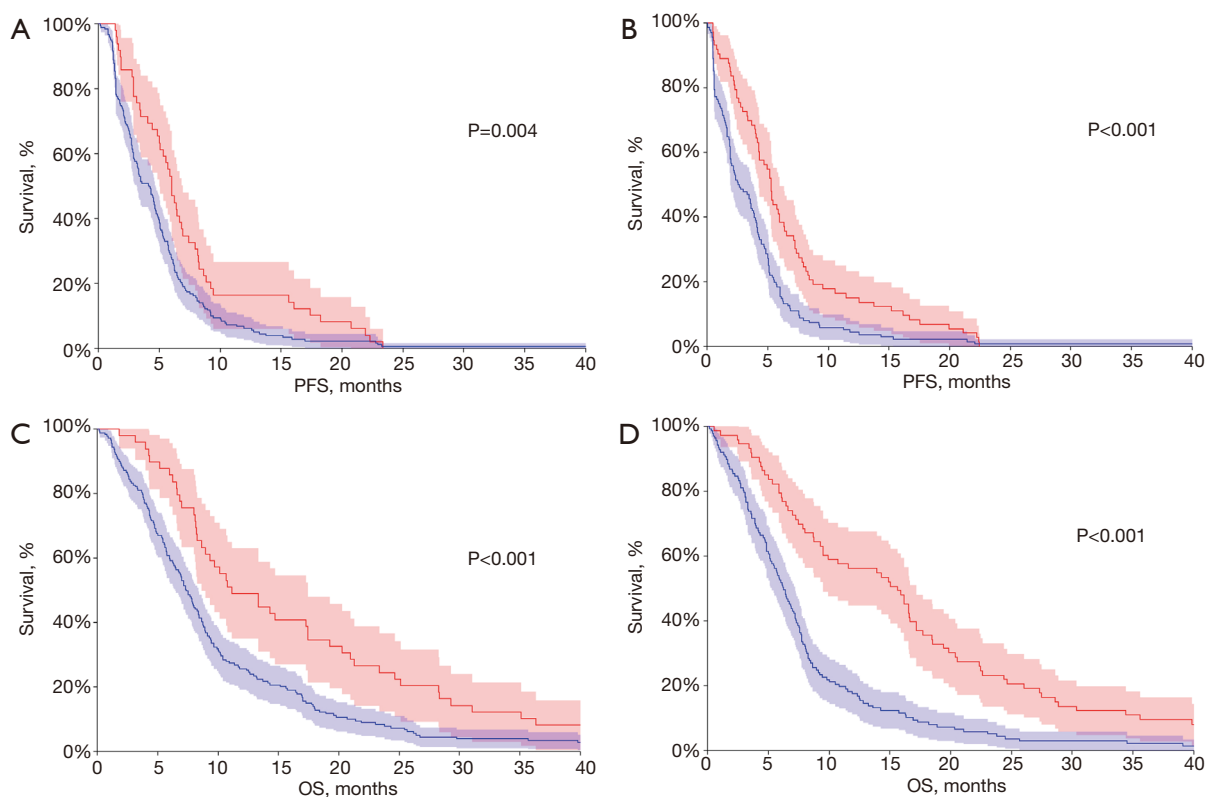

Figure 3 Prognostic impact of metHOXA9 status. Kaplan-Meier plots illustrating PFS and OS probability as a function of metHOXA9 status at baseline (A,C) and after the first treatment cycle (B,D). Red line: metHOXA9 =0. Blue line: metHOXA9 >0. Colored areas represent 95% CIs. P values for Log rank tests are shown. PFS, progression free survival; OS, overall survival.

Table 2 Multiple Cox regression analysis performed on 196 patients and 193 events with complete data for all covariates

Covariate	HR	95% CI (lower)	95% CI (upper)	P value
metHOXA9 status, 1 cycle of treatment				
Undetectable (reference)	1			
Detectable	3.788	2.192	6.544	<0.001*
Sex				
Female (reference)	1			
Male	1.299	0.970	1.740	0.079
Treatment				
Platinum and vinorelbine (reference)	1			
Other than platinum and vinorelbine	0.637	0.405	1.002	0.051
LDH baseline (numeric)	1.002	1.001	1.003	0.004*
Time-varying coefficient, metHOXA9	0.998	0.997	0.999	0.002*

Table presents hazard ratios (HR), 95% CIs, and P values. *, statistically significant covariates. CI, confidence interval.

to OS with a median of 7.4 and 11.1 months, respectively, for the patients with and without detectable metHOXA9, HR 1.79 (95% CI: 1.35–2.38, P=0.0003, n=228, *Figure 3C*). The prognostic impact became clearer after the first cycle of treatment with an OS of 6.2 and 15.6 months for patients with and without detectable metHOXA9, respectively, HR 2.07 (95% CI: 1.58–2.73, P<0.0001, n=209, *Figure 3D*).

Independent prognostic impact of metHOXA9

The independent prognostic impact of detectable metHOXA9 after the first cycle of treatment was assessed by multiple Cox regression. There was an independent prognostic impact of LDH but only borderline significant impact of sex and treatment (*Table 2*). Since the status of metHOXA9 violated the proportional hazards assumption, a time-varying coefficient (metHOXA9 status interacting with time) was introduced into the Cox model. The HR of detectable metHOXA9 is 3.79 (2.19–6.54, P<0.001) at time =0. The HR declines by 0.002 at each unit of time. This means that the HR would be 3.06 at time =365 days or 2.33 at time =730 days. The Cox modelling process is described in further detail in Appendix 1 online.

Discussion

This study, to the best of our knowledge, is the first to explore the prognostic impact of circulating metHOXA9 in advanced NSCLC. We show that detection of metHOXA9

at baseline is a negative prognostic factor in patients with NSCLC stage III and IV, and the impact is enhanced after the first treatment cycle.

Previous studies have shown metHOXA9 to be a promising prognostic marker. Ooki *et al.* (13) reported a tendency towards worse OS in patients with detectable metHOXA9. However, the cohort was rather small (n=53) and metHOXA9 was detected by quantitative methylation specific PCR (Q-MSP), which is a well-established method but not as precise or accurate as ddPCR (29). Lissa *et al.* (30) found that metHOXA9 was independently associated with worse cancer specific survival in patients with stage I adenocarcinoma of the lung. Sandoval *et al.* (16) reported a significantly shorter relapse free survival for operated stage I lung cancer patients with detectable metHOXA9 in both their discovery and validation cohort. All of these studies used tumor tissue for metHOXA9 analysis in relation to prognosis, and the patients had localized disease. We have now shown that metHOXA9 is a prognostic biomarker in advanced NSCLC as well, and that it can be detected in plasma. This finding is supported by Peng *et al.* (31), who detected methylated ctDNA in the form of short stature homeobox 2 (SHOX2). They reported a significantly better OS in stage IV NSCLC patients with low pre-treatment and post-treatment SHOX2 levels compared to patients with higher levels.

The diagnostic sensitivity of metHOXA9 tends to be higher in tissue samples than ctDNA from blood or pleural effusion (13), but this might be countered by choosing a

more sensitive detection method or increasing the volume of samples. We used ddPCR for ctDNA detection, which is considered a reproducible method (32), and included both internal and external controls for validation. The method is cost-efficient and relatively simple, making implementation into routine clinical practice feasible. metHOXA9 has generally shown good performance and sensitivity in previous studies (13-15,20,33). Nevertheless, the assay does not have perfect sensitivity, and the patient may have a lung cancer which does not release metHOXA9. Sensitivity seems to increase with a panel of genes, though a trade-in on specificity has been observed (13). By building a gene panel analyzed with ddPCR the sensitivity may be improved and more accurate results generated.

Interestingly, we found the prognostic impact of metHOXA9 status to interact with time, meaning that it violated the proportional hazards assumption. This can be interpreted as a declining prognostic impact of metHOXA9 status; i.e., detectable metHOXA9 after the first treatment cycle has a certain impact on prognosis, but the impact gradually decreases over time. Accounting for a time-varying coefficient can lead to more solid estimates in the Cox regression model, if it makes clinical and logical sense (34,35). An even more precise prognosis estimate may be derived from real-time monitoring with serial blood sampling. This has shown clinical potential with a relevant lead time compared to standard follow-up with CT scans in the progression of colon cancer (36) and recurrence of bladder cancer (37).

We observed an independent prognostic impact of LDH in the multiple Cox regression confirming previous findings. An elevated LDH level was associated with shorter OS in a meta-analysis of 76 studies of patients with solid tumors and the same applied to their subgroup analysis of seven studies on lung cancer patients (38). Similar results were found in a recent meta-analysis of lung cancer studies (39). A study in colorectal cancer found a correlation between methylation of two genes and LDH to be a surrogate marker for cell death (40). However, this was not the case for a third gene investigated, suggesting that ctDNA can occur by other routes than cell death.

After the first cycle of treatment the fraction of patients with undetectable metHOXA9 was higher than at baseline, i.e., the treatment changed the biomarker status for some patients. The difference in OS between patients with and without detectable metHOXA9 was more than doubled after the first treatment cycle. Similar results were recently reported by our group in a study of circulating metHOXA9

in ovarian cancer, in which the most pronounced effect was observed after the third treatment cycle with an OS of 9.4 months versus 19.4 months in patients with detectable and undetectable metHOXA9, respectively (25). This implies that a change in biomarker status reflects a change of prognosis. The difference in metHOXA9 was observed after only three weeks in the present study while the first response evaluation by CT scan was performed after six to eight weeks depending on treatment regimen. The large difference in OS according to metHOXA9 after the first treatment cycle raises the question of whether treatment should be reconsidered for patients with detectable metHOXA9. There is a possibility of this marker being predictive as well as prognostic. The patients with detectable metHOXA9 after the first treatment cycle potentially do not benefit from the administered chemotherapy regimen and may be better served by moving on to second line treatment, e.g., immunotherapy. This remains to be elucidated in a prospective trial ideally randomizing patients to standardized follow-up with CT evaluation of treatment response or follow-up by metHOXA9 measurement for treatment reconsideration.

The primary limitation of the present study is its retrospective nature increasing the possibility of selection bias. However, the plasma samples were prospectively collected with the purpose of future biomarker analysis, and the translational study was pre-planned. The clinical endpoints were unknown at the time of collection and laboratory staff were blinded to clinical outcome status of the individual patient. The samples were stored for up to eight years, which is rather a long time. They were never thawed and refrozen, and we did not experience any problems with DNA yield. However, it was previously reported that the loss of cell free DNA in frozen plasma samples was about 30% per year of storage (41). Mean and median cell free DNA yield in the present study can be found in Appendix 1 online. We believe that the storage time did not affect our samples to a degree that would significantly change our results, although shorter storage time would have been preferable. While the cohort is of intermediate size, the samples were collected at a single institution with no validation cohort. These results should therefore be interpreted with caution until further validated. The cohort is Danish and thus quite uniformly Caucasian, meaning that the results should be applied to other countries with caution, as genes and methylation patterns may vary between ethnic groups. And finally, we only investigated methylation of one gene as elaborated above.

In conclusion, the present study suggests that detectable circulating metHOXA9 is a negative prognostic factor in patients with advanced NSCLC and could be used for risk stratification after one cycle of treatment. Routine clinical application requires further investigation in prospective clinical trials.

Footnote

Reporting Checklist: The authors have completed the REMARK reporting checklist. Available at http://dx.doi.org/10.21037/tlcr-20-826

Data Sharing Statement: Available at http://dx.doi.org/10.21037/tlcr-20-826

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/tlcr-20-826). SW, TH, RA, CN, OH, and AJ report non-financial support from NKMax America, outside the submitted work. The authors have no other conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013) and the Harmonized Tripartite Guideline for Good Clinical Practice from the International Conference on Harmonization. The study was reviewed and approved by the Regional Committee on Health Research Ethics for Southern Denmark (S-20110005) and performed in accordance with Danish data protection legislation. All enrolled patients gave written, informed consent.

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Appendix 1

1. Investigators

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2. Methods

Methylated HOXA9 was analyzed with an in-house ddPCR assay. (Primers and probe from LGC Biosearch technologies, Aarhus, Denmark).

Primer sequence
Forward   GAGTATTTCGATTTTAGTTCGTGT
Reverse   CGCGTACACTAAATTCCAC

Probe sequence
Probe   FAM-TTAGTTTAAGGCGACGGTGTT-BHQ-1

Positive control:
Universal Methylated Human DNA Standard, 2 µl (DNA concentration 250 ng/µl, Zymo Research, Irvine, California, USA), healthy donor lymphocyte DNA 199 µl (DNA concentration approximately 20 ng/µl) and water 800 µl.
For each reaction, 20 µl of this mixture was added and bisulfite converted in parallel with the patient samples.

PCR conditions for the methylation specific droplet digital PCR assay

Steps	Temperature	Time
Step 1	95ºC	10 minutes
Step 2: 44 cycles	95ºC 56ºC	15 seconds 1 minute
Step 3	98ºC	10 minutes

Machine: Veriti Thermal Cycler (Applied Biosystems, Thermo Fisher Scientific, Foster City, California, USA).
Ramp rate: 1.5ºC/second.

Cell free DNA yield:
We have measured total cell-free DNA by qPCR in the form of the β2 microglobulin gene in the present patient cohort. We found a median of 2825 copies/ml (mean 4920 copies/ml, range 279-106656 copies/ml) across the 228 baseline samples.

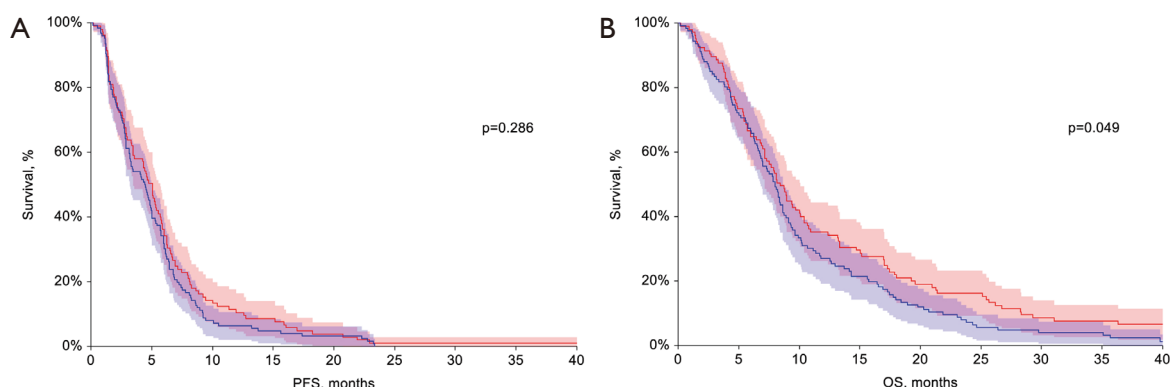### 3. Univariate analyses

Figure S1 Prognostic impact of sex. Kaplan-Meier plots illustrating PFS and OS probability as a function of sex (A, B). Red line: Female. Blue line: Male. Colored areas represent 95% CIs. P-values for Log rank tests are shown.
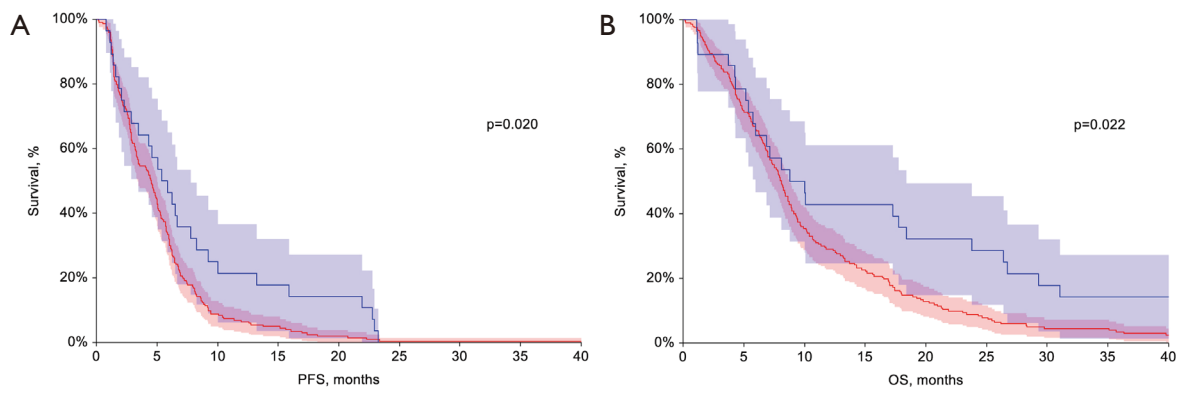
Figure S2 Prognostic impact of treatment. Kaplan-Meier plots illustrating PFS and OS probability as a function of treatment category (A, B). Red line: Platinum and vinorelbine. Blue line: Other treatments. The category 'Other' covers vinorelbine monotherapy and tyrosine kinase inhibitors. Colored areas represent 95% CIs. P-values for Log rank tests are shown.
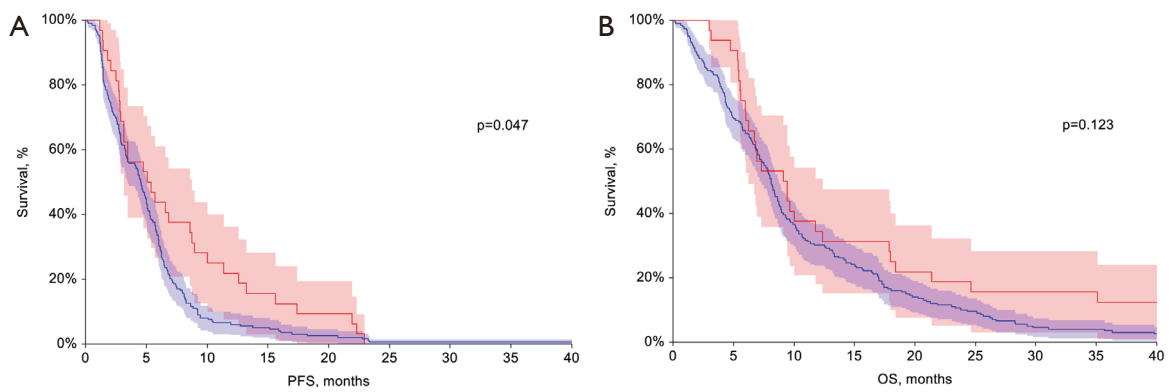

Figure S3 Prognostic impact of stage. Kaplan-Meier plots illustrating PFS and OS probability as a function of stage (A, B). Red line: Stage 3. Blue line: Stage 4. Colored areas represent 95% CIs. P-values for Log rank tests are shown.
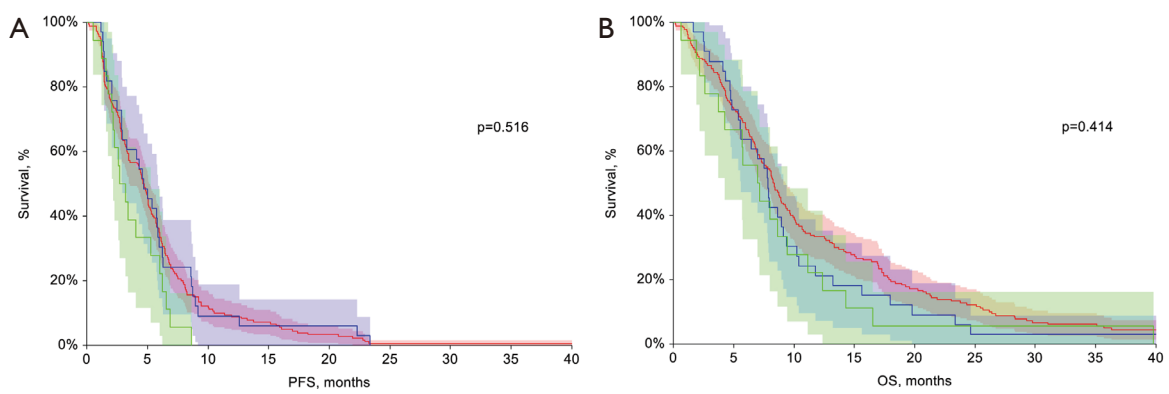

Figure S4 Prognostic impact of histologic type. Kaplan-Meier plots illustrating PFS and OS probability as a function of stage (A, B). Red line: Adenocarcinoma. Blue line: Squamous cell carcinoma. Green line: Other. The category 'Other' covers poorly differentiated non-small cell carcinoma and tumors with mixed histology treated as NSCLC. Colored areas represent 95% CIs. P-values for Log rank tests are shown.
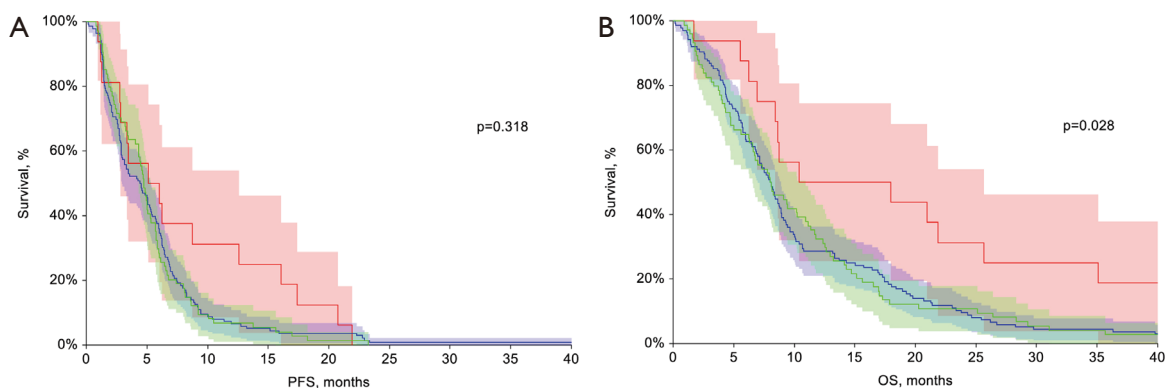

Figure S5 Prognostic impact of smoking status. Kaplan-Meier plots illustrating PFS and OS probability as a function of smoking status (A, B). Red line: Never smokers. Blue line: Previous smokers. Green line: Active smokers. Colored areas represent 95% CIs. P-values for Log rank tests are shown.
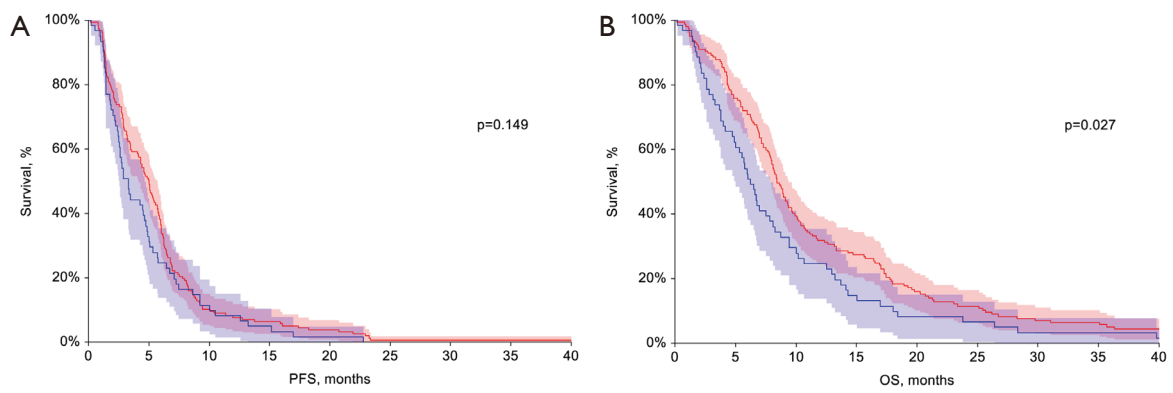
Figure S6 Prognostic impact of LDH status. Kaplan-Meier plots illustrating PFS and OS probability as a function of LDH status at baseline (A, B). Red line: LDH < 250 U/l. Blue line: LDH ≥ 250 U/l. Colored areas represent 95% CIs. P-values for Log rank tests are shown.
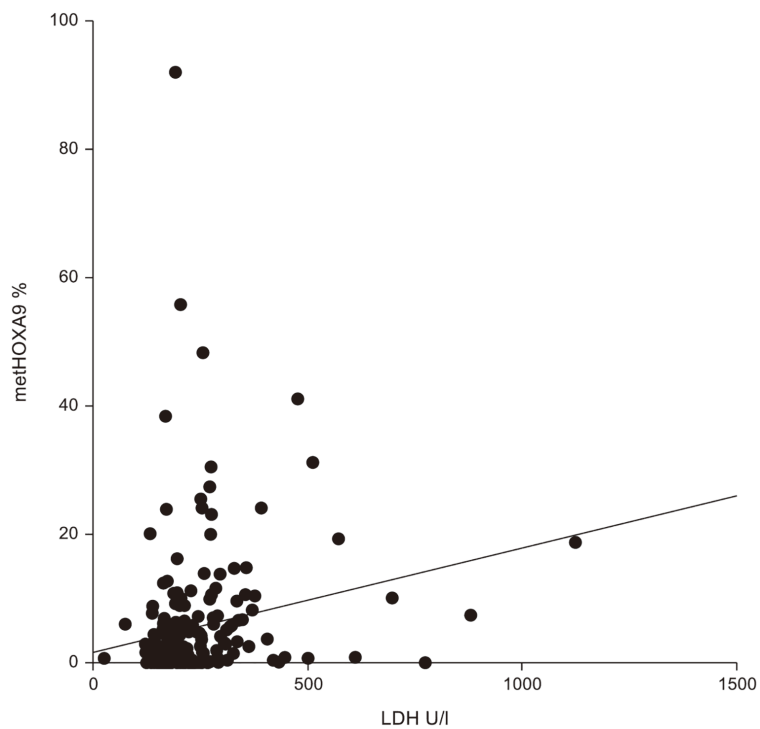

Figure S7 LDH in relation to metHOXA9. Scatter plot of LDH in U/l against metHOXA9 in percent showing a weak correlation depicted by a straight line, r=0.31 (A).

4. Multivariate analyses

Table S1 Multiple Cox regression analysis, all covariates

Covariate	HR	95% CI lower	95% CI upper	P-value
metHOXA9 status, 1 cycle of treatment				
Undetectable (reference)	1			
Detectable	3.619	2.085	6.280	<0.001*
Sex				
Female (reference)	1			
Male	1.237	0.912	1.677	0.172
Smoking status				
Never (reference)	1			
Previous	1.584	0.808	3.103	0.180
Active	1.722	0.859	3.450	0.125
Histology				
Adenocarcinoma (reference)	1			
Squamous cell carcinoma	1.166	0.730	1.862	0.520
Other	1.052	0.582	1.904	0.866
Treatment				
Platinum and vinorelbine (reference)	1			
Other	0.637	0.400	1.014	0.057
Stage				
Stage 3	1			
Stage 4	0.984	0.622	1.556	0.945
Age (numeric)	1.001	0.983	1.020	0.886
LDH baseline (numeric)	1.002	1.001	1.003	0.006*
Time-varying coefficient, metHOXA9	0.998	0.997	0.999	0.003*

Multiple Cox regression analysis performed on n=192 patients and 189 events with complete data for all covariates. Table presents hazard ratios (HR), 95% CIs, and p-values. *Statistically significant covariates.

The Cox regression model as reported in the manuscript (Table 2) was developed by the backward selection method. The full model (Table S1) was narrowed down by taking out one covariate with a high p-value and comparing the larger model with the smaller (nested) model by the likelihood-ratio test. If there was no significant difference between the two models, i.e. the missing covariate did not contribute significantly to the model, the smaller model was kept for further model development. There was no interaction between LDH and metHOXA9 neither as continuous nor binary variables.

The time varying coefficient (metHOXA9 status interacting with time) was included from the beginning. We tested the full model as described above (Table S1) but without the time varying coefficient with the proportional hazards test, and metHOXA9 status violated the proportional hazards assumption. The solution was to include a time varying coefficient in the model.

Missing data were treated as such and not included in the model or in other statistical analyses.