Communication

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Abstract: Notwithstanding the fact that there is some improvement for an earlier detection of patients with lung cancer, the majority of them still present with a late-stage disease at the time of diagnosis. Next to the most frequently used factors affecting the prognosis of lung cancer patients (stage, performance and age) the recent application of biomarkers obtained by liquid profiling gained more acceptance. In our study we aimed to answer these questions: i) is the quantification of free-circulating methylated PTGER4 and SHOX2 plasma DNA an useful method for the therapy monitoring and is this also possible for patients treated with different therapy regimens?, and ii) is this approach possible when blood drawing tubes are used which allow for a delayed processing of blood samples? Baseline values for *mPTGER4* and *mSHOX2* do not allow for a clear discrimination between different response groups. In contrast the combination of the methylation values for both genes show a clear difference between responders vs non-responders at the time of re-staging. Additionally, blood drawing into tubes stabilizing the sample give researchers more flexibility.

Keywords: liquid profiling; plasma; methylation; *mSHOX2; mPTGER4*

1. Introduction

The last years had seen some progress for the treatment of advanced stage lung cancer patients, specifically, for patients harboring druggable genetic alterations like activating *EGFR* gene mutations, mutations in *BRAF* and *MET* genes, and variants of *ALK* and *ROS1* [1]. This applies to non-small cell (NSCLC) and small cell (SCLC) lung cancer patients who are treated with targeted or immunotherapeutic agents [2]. Notwithstanding there are different resistance mechanisms in place leading to a therapy failure also for these novel treatments. This underscores the need for the search and establishment of reliable tumor markers for therapy monitoring.

Recently liquid profiling has emerged as a promising tool for a longitudinal analysis of response to a given therapy particularly as it can be performed in real-time. After the first description of cell-free nucleic acids detectable in plasma and serum of humans [3], the group of Anker and Stroun laid the foundation for the current interest in this method [4–7]. Currently, there are several

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methods for the analysis of extracellular nucleic acids quantitatively and qualitatively with whole genome sequencing, exon sequencing, genome-wide methylation analysis and others [8,9].

An easier, faster and more straightforward approach is the quantitative measurement of a smaller gene panel of methylated sequences in cell-free DNA with real-time PCR. The usefulness of this approach to demonstrate a relationship between the presence of methylated plasma DNA and therapy response in lung cancer patients has been shown in several papers [10–13].

Together with Wang et al [14], our group was among the first in demonstrating the potential of a longitudinal analysis of methylated cell-free DNA for therapy monitoring. We demonstrated a good correlation between the longitudinal measurement of extracellular plasma mSHOX2 DNA and the response to cytotoxic treatment in late stage lung cancer patients [15]. In this study we used EDTA tubes which were processed within one to two hours after blood drawing. Previous studies had demonstrated that EDTA blood should not be stored for more that six hours as a longer storage leads to cell lysis and a „contamination" of cell-free DNA with genomic DNA. In order to allow for an extended storage of blood tubes (including shipping to a remote laboratory) it is necessary to stabilize the blood sample with special additives. Several companies have developed specially designed blood drawing tubes such as the PAXgene Blood ccfDNA tubes which received market approval in 2016.

In this paper we extended our former study as we included more patients from several hospitals (multicenter), and used PAXgene Blood ccfDNA tubes (Qiagen, Germany) for blood sampling. Additionally we used a modified marker panel for the detection of methylated cell-free circulating DNA in the plasma of late-stage lung cancer patients undergoing a systemic therapy.

2. Materials and Methods

2.1. Patients

In total, 96 patients, among them 78 from the DRK Kliniken Berlin-Mitte and 18 from the other participating clinics, with late stage histologically confirmed non-small cell lung cancer (NSCLC) who received a first-line treatment consisting of chemo +/- radiotherapy, anti-*EGFR* therapy or immunotherapy were enrolled prospectively in the present study. The patients were consecutively referred to the participating clinics for diagnosis and treatment from August 2016 until October 2020. The study was approved by the Ethics Committee of the University Halle/Saale on June 19, 2014 (Reference number 2014-52) and all patients gave informed written consent prior to inclusion in the study. This study was set up as a multicentric trial and the majority of patients were treated at the DRK Kliniken Berlin-Mitte (78 patients). The blood of these 78 patients was drawn in PAXgene Blood ccfDNA tubes (Qiagen, Germany), while EDTA tubes were used for 18 patients treated in the other participating centers. For the evaluation of the therapy response the data from all 96 patients were used while the analysis for the prognostic relevance was limited to the 78 patients enrolled at the DRK Kliniken Berlin-Mitte.

The details of the clinical data of the patients are shown in Table 1 (Table 1). The re-staging was done after the first two therapy cycles of chemo +/- radiotherapy, or 6 - 8 weeks after start of therapy, respectively. The response evaluation was carried out according the RECIST v1.1 criteria.

2.2. Plasma preparation

Blood was taken from patients at the time of diagnosis (pre-treatment) and thereafter in intervals of 7 to 10 days until the time of re-staging. The samples which were obtained at the DRK Kliniken Berlin-Mitte were collected in PAXgene Blood ccfDNA tubes and shipped by regular mail or transported via a courier (at room temperature in any case) to the laboratory in Halle/Saale. The range of delay in processing the blood was 0 to 8 days (median 5 days). The blood was spun once at 1500 rpm for 10 min, the supernatant was carefully transferred into a new tube and re-centrifuged at 3500 rpm for 10 min. The cell-free plasma was aliquotted and stored at -80°C before processing. The blood samples drawn at the other three participating study centers were processed as described above and the plasma was stored at -80°C and shipped on dry ice to the laboratory in Halle/Saale.

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2.3. DNA isolation, purification and bisulfite conversion

We used the Epi Bis-kits (Epigenomics, Berlin, Germany) according to the protocol of the manufacturer with an initial input of 3.5 mL plasma for the isolation, purification and bisulfite treatment of the DNA. For the detection and quantification of the gene products, a PCR kit developed by Epigenomics for the simultaneous quantification of *mSHOX2, mPTGER4 and ß-actin* as a reference gene [Weiss et al J Thorac Oncol 2017] was used. All samples were measured in triplicate. The quantification of *mSHOX2, mPTGER4* and *ß-actin* (as the reference gene for the calculation) was performed according to Kneip et al [17]. The quantification of the cell-free methylated sequences was performed after all prospectively collected plasma samples were complete, i.e. making this analysis an observational study.

2.4. Statistical analysis

Distribution of PTGER4 and SHOX2 methylation values is demonstrated as boxplots for all response groups at time of first radiological staging being partial remission (PR), stable disease (SD) and progressive disease (PD). Prediction of therapy response was evaluated in two settings. For detection of progression, PD was compared with SD+PR while for detection of response, PR was compared with SD+PD. Power of discrimination was calculated as area under the curve (AUC) of receiver operating characteristic (ROC) curves and sensitivity at 90% specificity. Significance of statistical differences was calculated by Wilcoxon-test. For combination of markers, decision tree models were established using appropriate cutoffs and marker order and probabilities for therapy response was shown at every node. Survival was evaluated by Kaplan-Meier-curves and log-rank test. All comparisons were performed two-sided and statistical significance was set at $p<0.05$. Data analysis was performed using R (version 4.2.0; https://www.R-project.org, free software foundation, Inc., USA).

3. Results

All patients received serial sample collection during the treatment with an average of 6 samples per patients. Treatment was performed for a minimum of 6 months; the median follow-up of the patients was 22 months with a range from 6 to 58 months. We evaluated the biomarker values before start of therapy, at re-staging exams and the relative changes were considered and correlated with radiological response to treatment and survival of the patients.

Of those 96 patients with advanced NSCLC, 83 received regular chemo +/- radiotherapy, 9 a combination of chemo- and immunotherapy and 4 patients an immuntherapy only. Thirty four patients responded well to the treatment at staging exams and showed partial remission (PR), 15 had stable disease (SD), and 33 progressive disease (PD). The therapy response of 14 patients is unknown. The median overall survival (OS) was 8.5 months with a range from 1 to 37 months (Table 1).

Table 1. Clinical data for all enrolled patients.

		Number
Sex	female	62
	male	34
Age	Range	48 - 81
	median	63
Smoker	Yes	41
	No	4
	Ex-smoker	30
	Unkown	21

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Histology	NSCLC (unclassified)	25
	Adenocarcinoma	54
	Squamous cell carcinoma	17
Treatment	Chemo +/- Radiotherapy	83
	Chemo + Immunotherapy	9
	Immunotherapy	4
Treatment response	partial remission	42
	stable disease	23
	progressive disease	31

3.1. Distribution of mPTGER4 and mSHOX2 methylation values in response groups

Before start of therapy (V1), patients who showed a partial remission had slightly lower methylated *PTGER4* levels than SD and PD patients, however this difference was not statistically significant. However, at time of re-staging exam (VS), *mPTGER4* levels of PR patients were significantly lower than of SD and even more than of PD patients – and also the ratio of *mPTGER4* VS/V1 was significantly different between the response groups (Figure 1A–C). Similar tendencies were observed for *SHOX2* methylation with no significant differences between response groups for the pre-therapeutic assessment and lower values for PR and SD than PD patients. In contrast and comparable to the *mPTGER4* values, the differences seen for the ratio of *mSHOX2* at VS and VS/V1 values reached a low significance level (Figure 1D–F).

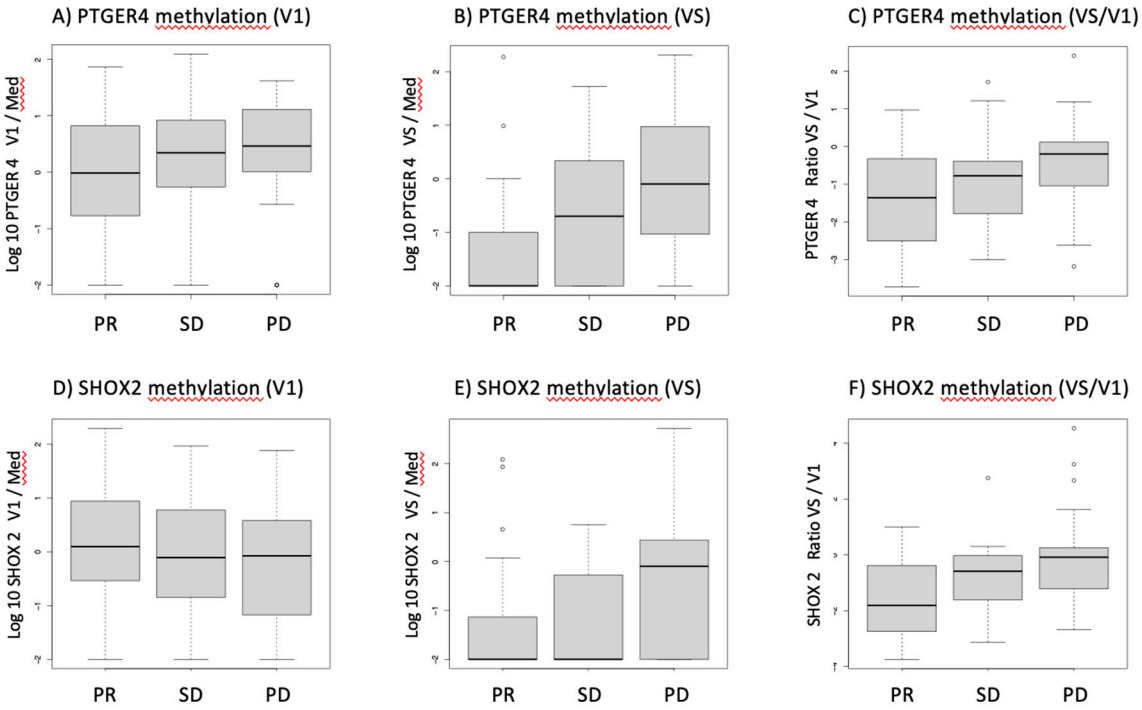

Figure 1. Boxplots for the distribution of methylated PTGER4 (A-C) and SHOX2 (D-F) before start of therapy (V1), at time of first radiological staging (VS) and the ratio between both time points (VS/V1) for patients with partial remission (PR), stable disease (SD) and progressive disease (PD) in staging exams.

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3.2. Differentiation of patients according to their therapy response

The power of discrimination between patients with good and poor response to therapy was objectified by areas under the curves (AUC) of receiver operating characteristic (ROC) curves.

For the comparison of patients with progression (PD) versus no progression (PR+SD), AUCs for *mPTGER4* were AUC=0.60 (V1), AUC=0.72 (VS), and AUC=0.66 (VS/V1) and for *mSHOX2* AUC=0.57 (V1), AUC=0.67 (VS), and AUC=0.71 (VS/V1). Sensitivities for the detection of progression at a 90% specificity vs. non-progression were for *mPTGER4* 0.60 at V1, 0.74 at VS and 0.65 for VS/V1 (Figure 2 A-C) as well as for *mSHOX2* 0,59 at V1, 0.64 at VS and 0.69 for VS/V1, respectively (Figure 2 D-F). For the comparison of patients with remission (PR) at a 90% specificity versus no remission (SD+PD), AUCs for *mPTGER4* were AUC=0.60 (V1), AUC=0.74 (VS), and AUC=0.65 (VS/V1) (Figure 3 A-C) and for *mSHOX2* AUC=0.59 (V1), AUC=0.64 (VS), and AUC=0.69 (VS/V1) (Figure 3 D-F).

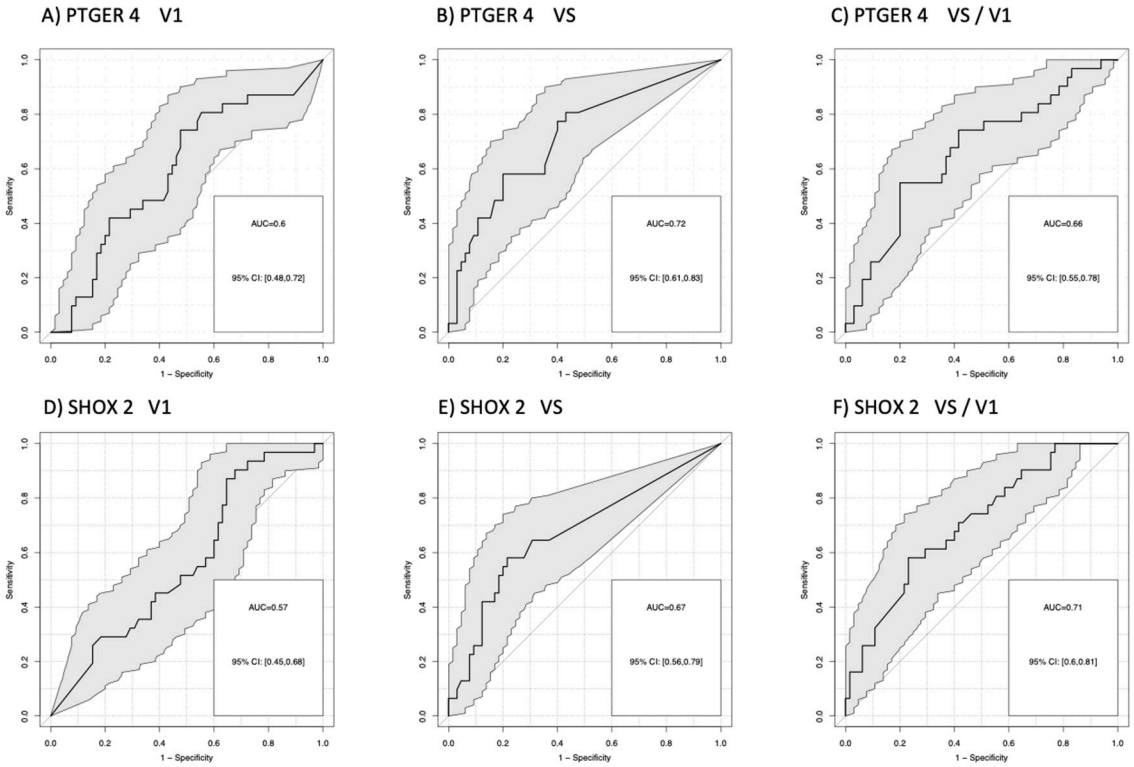

Figure 2. Receiver-operating characteristic (ROC) curves for the discrimination of patients with progressive disease (PD) from patients with no progression (stable disease + partial remission) for methylated PTGER4 (A-C) and SHOX2 (D-F) before start of therapy (V1), at time of first radiological staging (VS) and the ratio between both time points (VS/V1).

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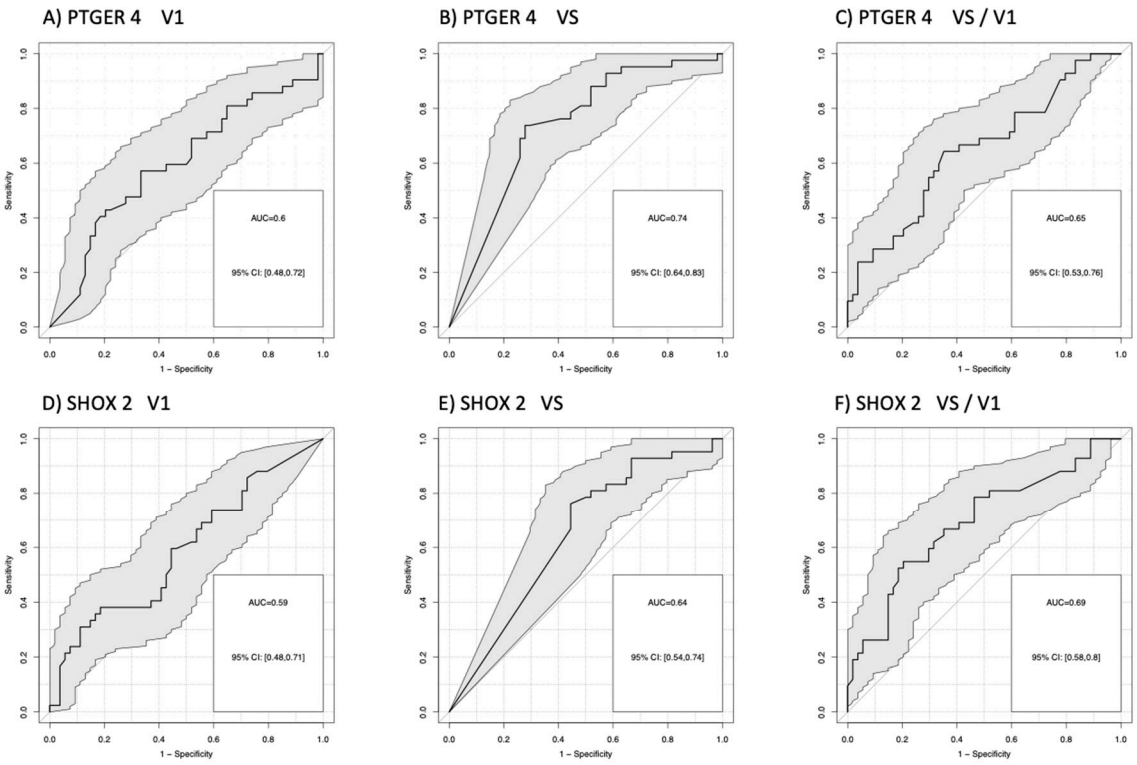

Figure 3. Receiver-operating characteristic (ROC) curves for the discrimination of patients with remission (PR) from patients with no remission (stable + progressive disease) for methylated PTGER4 (A-C) and SHOX2 (D-F) before start of therapy (V1), at time of first radiological staging (VS) and the ratio between both time points (VS/V1).

3.3. Combination of markers in decision trees

In order to maximize the information for a therapy prediction, markers were combined in a decision tree model by use of appropriate cutoffs and marker order. In using this model we demonstrated that patients with low m*SHOX2* ratio (VS/V1) and low *mPTGER4* levels at the time of staging (VS) had a high chance of achieving PR at staging exams (Figure 4, Node 3). In contrast, patients with high *mSHOX2* ratio (VS/V1) and high *mPTGER4* ratio (VS/V1) had a high probability of demonstrating a progressive disease (Figure 4, Node 7). Patients with either low *mSHOX2* ratio (VS/V1) and high *mPTGER4* (VS) levels or alternatively high *mSHOX2* ratio (VS/V1) and low *mPTGER4* ratio (VS/V1) ranged intermediate (Figure 4, Nodes 4 and 6).

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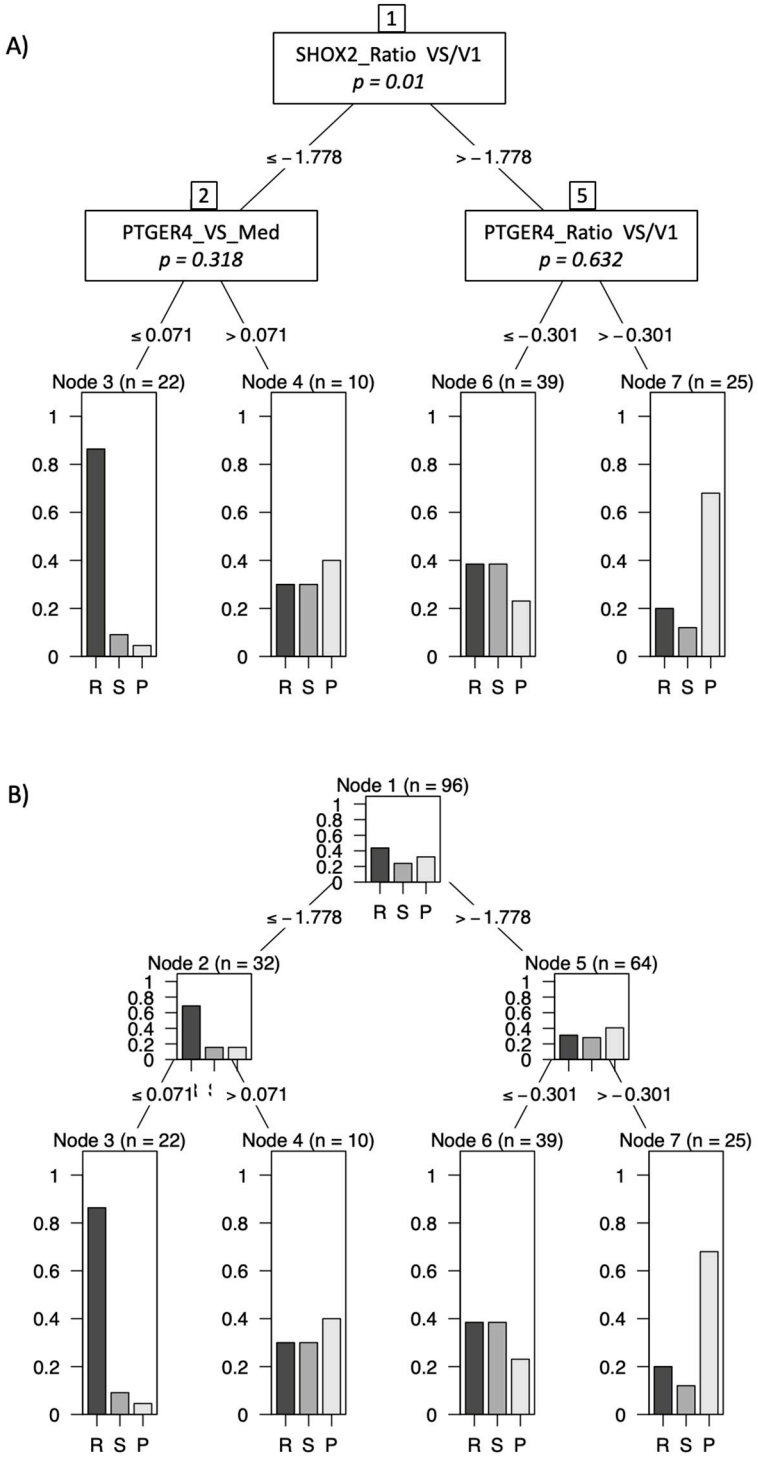

Figure 4. Decision tree model using combination of markers mSHOX2 ratio (VS/V1), mPTGER4 at staging (VS) and mPTGER4 ratio (VS/V1) for best prediction of therapy response. Patients with low mSHOX2 VS/V1 and low mPTGER4 VS had a high chance of achieving partial remission (R) whereas patients with high mSHOX2 VS/V1 and high mPTGER4 VS/V1 had a high probability for progressive disease (P). A) shows the decision rules and B) the distribution of responses at every decision node.

3.4. Relevance of PTGER4 and SHOX2 for prognosis

Information regarding overall survival (OS) was available from 78 out of the 96 NSCLC patients. For prediction of OS only *SHOX2* methylation at the time of staging (VS) showed borderline significant prognostic information. All other markers and time points did not. Patients with SHOX2

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methylation levels below the median had a tendency to longer survival with a median OS of 11 months as compared with patients with SHOX2 methylation levels above the median who had a median OS of 8 months. This difference showed a trend but was not statistically significant with a value of p=0.058 (Figure 5).

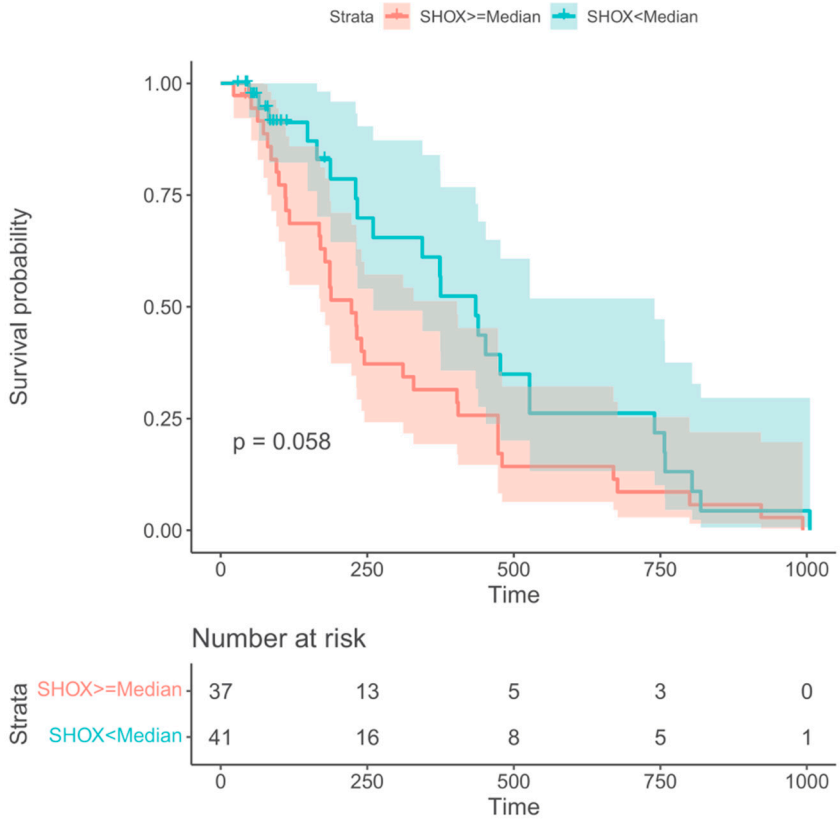

Figure 5. Kaplan-Meier survival curves for the mSHOX2 at time of staging (VS) showing borderline longer survival for patients with low levels (green) than those with high levels (red).

4. Discussion

According to the latest US cancer statistics 46% of the newly diagnosed lung cancer patients demonstrate a late stage disease [18]. Furthermore, a recent survey showed that almost 49% of 210 000 newly diagnosed lung cancer patients died within 2 months after receiving their diagnosis [19]. Thus it is of utmost importance to differentiate between patients with a high risk for early death and patients with a better prognosis.

Since our group and other researchers demonstrated that the detection and quantification of methylated ctDNA is a useful approach for the therapy monitoring in lung cancer patients [14–16], more papers on this subject were published [20,21]. Additionally, the incorporation of liquid profiling as an additional clinical tool for the diagnosis, treatment stratification, detection of resistance mechanisms and as a prognostic indicator in lung cancer patients has been shown [22–25].

In order to confirm and to extend the results of our original analysis [15] and to demonstrate the robustness of the method we performed the present study. Apart from increasing the number of patients, we included patients who received different treatment regimens, i.e. chemotherapy with/without radiotherapy, chemotherapy plus immunotherapy and immunotherapy exclusively. In addition we changed the pre-analytical process and applied a modified marker panel.

It is known that blood drawn into EDTA tubes should not be stored for more than 4-6 hours before being processed [26]. A delay of more than 6 hours can lead to irregular results due to lysis of blood cells and "contamination" of cell-free DNA with genomic DNA. Several studies had demonstrated that a storage of blood drawn into PAXgene Blood ccfDNA Tubes for up to 7 days does not change the quantity and quality of plasma DNA [27,28]. Additionally, we applied a modified

marker panel which included the detection of cell-free methylated *SHOX2* and *PTGER4* plasma DNA [27]. All of these factors plus a different patient population might explain the different results obtained in this study as compared to our previous paper [15].

It is interesting to note that the baseline values for *mPTGER4* and *mSHOX2* do not allow for a clear discrimination between different response groups (Figure 1). In contrast, the methylation values for both genes show a clear difference between responders vs non-responders at the time of re-staging (Figure 1). This observation still holds true when the ratios of the methylation values (VS/V1) for both genes are plotted (Figure 1 box E and F). This data corroborates our initial observation [15] that the methylation values at the time of diagnosis do not allow a differentiation between the two groups while this is possible at the time of re-staging (i.e. 8 to 12 weeks after therapy start). At the time this study was commenced, Epigenomics AG (Berlin, Germany) had introduced a modified kit for the DNA methylation analysis. This new kit included *mSHOX2* as well as a second marker, *mPTGER4* [27]. Currently, no comparative data exists for the two kits in a head-to-head approach. However, promising results have been published that advocate for the inclusion of a second marker. Indeed, the grouping of patients with low m*SHOX2* ratio (VS/V1) plus low *mPTGER4* levels at the time of re-staging (VS) in decision trees allowed the discrimination of patients responding to the therapy from non-responding patients (Figure 3). When patients with a high m*SHOX2* ratio (VS/V1) and high *mPTGER4* levels at the time of staging (VS) were combined we were able to differentiate patients not responding to the therapy but with less statistical power. When the methylation levels of both genes at the time of re-staging were used for Kaplan-Meier curves only *mSHOX2* demonstrated a trend for statistical significance but not *mPTGER4* (Figure 4). *SHOX2* belongs to the homeobox family and is coding for a transcriptional regulator which is involved in pattern formation in both invertebrate and vertebrate species. In contrast *PTGER4* is a protein coding gene and belongs to the G-protein coupled receptor family. As for both genes no role in the development of lung cancer has been described so far, we assume that only the former has a functional relationship in lung cancer patients.

In conclusion, our findings demonstrate that quantifying of extracellular free-circulating methylated DNA in plasma could be a valuable tool to monitor the response of lung cancer patients undergoing various treatment regimens. The use of specialized blood drawing tubes to stabilize samples at room temperature allows the collection and transport to a remote laboratory without cooling. This approach gives researchers the possibility for enrolling a large number of patients in future studies to confirm the validity of our current findings.

Author Contributions: E.A, D.R., S.E., G.T., J.K., C.S. and C.G., contributed to data acquisition. M.F., J.K., F.K., S.H. and B.S. contributed to the data analysis and writing of the paper. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and has been approved by the Institutional Review Board (IRB) at the University Hospital of Halle/Saale. Informed consent (written) was obtained from all donors (Reference number 2014-52).

Informed Consent Statement: Informed written consent was obtained from all subjects involved in the study.

Data Availability Statement: Not applicable.

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Conflicts of Interest: Michael Fleischhacker and Bernd Schmidt are coinventors of the patent: "Methods for assessing the treatment response of cancer patients and for treating cancer patients by analysing CpG methylation" (Application Number: 62076674, patent pending). There are no further patents, products in development or marketed products to declare. This does not alter the authors' adherence to all the publication policies on sharing data and materials.

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