Original Article

The *mSHOX2* is capable of assessing the therapeutic effect and predicting the prognosis of stage IV lung cancer

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Background: Instant monitoring of the therapeutic effect of systematic therapy in late-stage lung cancer is crucial for response assessment and strategy adjustment. Previous study found that specific plasma methylation markers may be applied to therapeutic effect assessment. In order to investigate the performance of plasma *mSHOX2* in assessing the therapeutic effect and predicting the prognosis of stage IV lung cancer, we performed the study focusing on patients underwent chemotherapy or tyrosine kinase inhibitor (TKI)-based targeted therapy.

Methods: Blood samples from 163 subjects, including 30 stage I, 29 stage II, 26 stage III and 68 stage IV lung cancer patients, were recruited in this study. Quantitative relationship between primary tumor size and the plasma *mSHOX2* level was established. Blood samples before therapy and two cycles after therapy were obtained from 68 stage IV patients, and the *mSHOX2* level was quantified as ΔΔCt.

Results: Sharp decrease of plasma *mSHOX2* level was seen in patients with partial response (PR) while not in those with stable disease (SD). The plasma *mSHOX2* level change reflected the degree of response and correlated with the maximal diameter of primary tumors in linear relationship. The *mSHOX2* levels before and two cycles after therapy were predictors of the overall survival, while the *mSHOX2* level change or the tumor size change were not predictors of the overall survival. Furthermore, univariable and multivariable Cox regression revealed that *mSHOX2* level before therapy was the only independent predictor of the overall survival with a hazard ratio of 1.414.

Conclusions: *mSHOX2* is effective for therapeutic effect assessment and prognosis prediction of stage IV lung cancer patients underwent systematic therapy.

Keywords: SHOX2; *mSHOX2*; lung cancer; methylation; circulating tumor DNA (ctDNA); therapy; prognosis

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Introduction

Lung cancer ranks the highest in cancer morbidity and mortality in the world. Low-dose computed tomography (LDCT) has been recommended as the screening method for lung cancer (1). However, in scenarios that LDCT is not accessible, the *in vitro* diagnostic (IVD) methods may provide options for lung cancer early detection. The CE-approved Epi proLung is a recently developed assay for lung cancer early detection (2-14). Many other IVD methods for lung cancer screening or early detection, including those using the next-generation sequencing (NGS) technology and blood-based circulating tumor DNA (ctDNA) analysis, are currently under development and exhibit promising application perspectives (15-20). However, there is no effective IVD assay so far for therapeutic effect assessment or prognosis prediction in lung cancer. Clinically used protein markers, such as cyfra21-1, neuron-specific enolase (NSE), squamous cell carcinoma (SCC) and progastrin-releasing peptide (proGRP), are not appropriate for these applications, as their detection sensitivity is not satisfactory, and patients with negative results before therapy cannot be assessed after therapy. Furthermore, they are more sensitive to late stage lung cancer than early stage lung cancer, and are used more frequently as a marker for recurrence monitoring than therapeutic effect monitoring. The computed tomography (CT) is another non-invasive method for therapeutic effect assessment. However, CT cannot be used routinely as a monitoring examination, as the radiation method cannot be repeated frequently as an instant test. Therefore, it is lack of effective way for frequent therapeutic effect monitoring and prognosis prediction during and following lung cancer therapy.

The *mSHOX2* assay is the first blood-based test recently developed as a lung cancer screening test. It has been proved as a sensitive and specific assay for blood-based lung cancer early detection (4,10,11). The assay detects abnormally methylated *SHOX2* gene from ctDNA. Studies have found that the detection sensitivity of the m*SHOX2* test was positively correlated with the severity of lung cancer (2,4), suggesting that the plasma *mSHOX2* level could be an indicator for disease progression or relief. However, the observation on blood *mSHOX2* level change following therapy is very limited (21). Since it was found that the level of blood methylation markers can be used as indicators for therapeutic effect assessment and prognosis prediction, we would like to investigate the potentials of blood *mSHOX2* in these applications.

In the present study, we tested whether blood *mSHOX2* is capable of assessing the therapeutic effect and predicting the long-term prognosis of stage IV lung cancer patients undergoing first-line standard chemotherapy, combined radio- and chemotherapy or tyrosine kinase inhibitor (TKI)-based targeted therapy. By collecting the blood samples from lung cancer patients before therapy and two cycles after therapy, we investigated the relationship between the blood *mSHOX2* level change and the degree of patient response. The *mSHOX2* level change exhibited linear correlation with tumor size change, facilitating its use in assessment and monitoring. The blood *mSHOX2* levels before and after two cycles of therapy were also predictors for patient long-term survival. Our study provided strong evidence for the use of *mSHOX2* in the therapeutic effect assessment and prognosis prediction of stage IV lung cancer patients.

Methods

Ethics

The permission for clinical study was granted by the ethics committees of all participating hospitals before the start of sample collection. Informed consent was obtained from all subjects, and the information on the usage of plasma and test results were provided to all subjects.

Study design, patients, and therapy

The study was designed and implemented in four Chinese hospitals using the *mSHOX2* test in Epi proLung assay (Epigenomics AG, Berlin, Germany). Clinical status was determined before blood draw for *mSHOX2* assay, and blood samples were obtained from all subjects who met the selection criteria. All technicians were blinded to the clinical information of subjects. A total of 163 subjects were recruited in this study, including 30 stage I, 29 stage II, 26 stage III and 68 stage IV lung cancer patients (*Table 1*). The staging was based on the National Comprehensive Cancer Network (NCCN) guidelines on cancer stage (22). Determination of stage was based on pathological examinations of biopsies from needle aspiration or surgery. First-line standard chemotherapy, combined radio- and chemotherapy, or TKI-based targeted therapy was performed for stage IV patients. The primary tumors were the target tumors in this study, and the diameter of the primary tumors were assessed based on RECIST 1.1

Table 1 Number of enrolled subjects and demographic characteristics by diagnosis group in this study

Diagnosis group	Total	Gender		Age, years			
		Male	Female	<50	50–59	60–69	≥70
Overall	163	104	59	26	53	59	25
Stage							
I	30	21	9	2	11	12	5
II	29	17	12	4	9	10	6
III	26	25	11	9	14	8	5
IV	68	41	27	11	19	29	9
SCLC	24	16	8	5	7	8	4
NSCLC	44	25	19	6	12	21	5
EGFR M+	16	8	8	2	5	7	2
EGFR M–	28	17	11	4	7	14	3

SCLC, small cell lung cancer; NSCLC, non-small cell lung cancer; *EGFR M+*, with EGFR-sensitive mutations; *EGFR M–*, without EGFR-sensitive mutations.

criteria, and correlated with the level of mSHOX2.The assessment of non-target lesions, lymph node metastasis, distal metastasis or new lesions was not included in this study.

Sample size estimation

The equation for known positive detection rate was used for sample size estimation: $N=Z^2*[p*(1-p)]/E^2$. Z is a statistical parameter (Z =1.96 for 95% CI) and E represented the error (10% was chosen in this study), and p represented the putative positive detection rate. The p value represented the known sensitivity for Epi proLung assay on lung cancer, and was obtained from a previous pilot study. If the known sensitivity for stage IV lung cancer equals to 0.90, an estimated 35 lung cancer cases were required. The study goal was to recruit 58 patients, anticipating a 40% loss of follow-up rate (*Table 1*). Stage IV lung cancer involved 24 small cell lung cancer (SCLC) and 44 non-small cell lung cancer (NSCLC) patients, and NSCLC involved 16 patients with *EGFR*-sensitive mutations (*EGFR* M+) underwent TKI-based first-line therapy and 28 patients without *EGFR*-sensitive mutations (*EGFR* M–) underwent standard chemotherapy or combined chemo- and radiotherapy.

Sample collection and storage

Blood samples were collected before the start of therapy and two cycles after the therapy. One cycle of chemotherapy lasted for 21 days, while patients for TKI therapy took medicine every day. The blood collection point after therapy was therefore set at day 42 for all patients, just before the start of the third cycle of chemotherapy. A 10 mL peripheral blood sample was collected with a 10 mL K_2EDTA anticoagulant tube (BD biosciences, Franklin Lakes, NJ, USA). Sample storage and transportation followed the instructions for use of the Epi proLung assay. The sample information was recorded in sample collection forms. Plasma samples from all participating hospitals were prepared in individual hospitals and stored under –20 ℃ before they were delivered to Beijing BioChain Medical Laboratory, and all assays were performed in the same laboratory within three weeks from the sample collection date. The sample quality was examined when the samples arrived at the medical laboratory. Samples with plasma volume less than 3.5 mL, or with apparent hemolysis, high bilirubin, chylemia, or visible particles or pellets were not tested, and repeated blood draw was requested.

DNA extraction and qualitative PCR analysis of SHOX2

DNA extraction and bisulfite conversion were performed manually following the manufacturer's instructions of Epi proLung assay. The bisDNA (bisulfite-converted DNA before methylation analysis) was assayed on an ABI7500 Fast Dx Real Time PCR device (Life Technologies) with Epi proLung kits. The PCR assay was modified by Beijing

BioChain Medical Laboratory to examine the level of *mSHOX2* alone, instead of both *mSHOX2* and *mPTGER4*. The reaction condition and PCR parameters remained the same as the Epi proLung kits. PCR was performed in triplicate with 15 µL template DNA per well and run for 45 cycles. The validity of each sample batch was determined on the basis of methylated *SHOX2* and *ACTB* threshold count (Ct) values for the positive and negative controls. *ACTB* was used as an internal reference to assess the integrity of each sample.

Data analysis and interpretation

Test data of the Epi proLung assay were analyzed by calculating the ΔΔCt values using the Ct values from samples, *ACTB* internal controls and the positive controls. Statistical analysis was performed and figures were plotted with GraphPad Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA 92037, USA). For each sample, a relative methylation value was determined using the ΔΔCt method adapted for DNA methylation analyses as previously described (6). In brief, ΔΔCt values were calculated as below:

$\Delta\Delta Ct_{Sample} = \Delta Ct_{Sample} - \Delta Ct_{Calibrator}$, where $\Delta Ct_{Sample} = Ct_{ACTB\ of\ sample} - Ct_{SEPT9\ of\ sample}$ and $\Delta Ct_{Calibrator} = Ct_{ACTB\ of\ calibrator} - Ct_{SEPT9\ of\ calibrator}$

In order to identify the distribution pattern of *mSHOX2* ΔΔCt values, histogram analysis, probability-probability (P-P) plot and the Kolmogorove-Smirnov (KS) test were performed (*Figure S1*). The *mSHOX2* ΔΔCt values conformed to the normal distribution in all stages of lung cancer, stage IV lung cancer before therapy and stage IV lung cancer after therapy. The P values from the KS test for the above three groups were 0.473, 0.485 and 0.675, respectively (P>0.05 indicates no significant difference to normality). Therefore, student *t*-test was used in this study for significance analysis when comparing two groups.

Results

The plasma level of mSHOX2 was quantitatively correlated with tumor size in lung cancer

In order to discover the correlation between tumor size and the plasma *mSHOX2* level, we first performed a validation study by looking at the relationship between lung cancer tumor size and the *mSHOX2* ΔΔCt values. The relationship was shown in *Figure 1* by studying the diameter and the *mSHOX2* level in stage I to stage IV lung cancer. It appeared that the *mSHOX2* level (*Figure 1B*) increased with the elevation of tumor stage and tumor size (*Figure 1A*). The relationship between the mean diameter or the median diameter for each stage and the ΔΔCt values was shown in *Figure 1C,D*, respectively. Good linear relationship was found between the tumor size and quantified *mSHOX2* level (ΔΔCt values), with r^2 at 0.92 and 0.99 for mean and median diameter, respectively. These results proved the linear correlation between lung cancer tumor size and the plasma *mSHOX2* level.

The therapeutic effect of stage IV lung cancer can be quantitatively assessed by the plasma mSHOX2

The plasma *mSHOX2* levels were then measured before and after the therapy of stage IV lung cancer patients to study whether the assay can be used to assess the therapeutic effect of these patients. Stage IV patients underwent first-line chemotherapy, combined radio- and chemotherapy or TKI-based targeted therapy (for subjects with *EGFR*-sensitive mutations) in this study. Blood draws were performed within one week before therapy and after two cycles of therapy (before the start of the third cycle), and therapeutic effect assessment was performed after two cycles of therapy (before the start of the third cycle) based on RECIST 1.1. Primary tumors were target tumors in the therapy and their maximal diameter was measured for the assessment.

It can be observed from *Figure 1A* that in 68 stage IV patients recruited in the study, most patients exhibited *mSHOX2* level decrease following two cycles of therapy, while some exhibited slightly increased or steady *mSHOX2* level after therapy (*Figure 2A*). The mean ΔΔCt values decreased from –1.25 (95% CI: –2.39 to –0.10) to –3.78 (95% CI: –4.83 to –2.73) (*Figure 2B*), exhibiting an overall significant decrease of *mSHOX2* level following two cycles of therapy (student *t*-test, P=0.0014). The *mSHOX2* level change for each individual is clearly shown in *Figure 2C*, in which the patients were ranked from highest to lowest based on pre-therapeutic plasma *mSHOX2* level. Although most patients showed decreased *mSHOX2* level, the extent of decrease exhibited large variation among individuals.

The details of the correlation between *mSHOX2* level change and the therapeutic effect were further investigated by dividing the patients into two groups based on

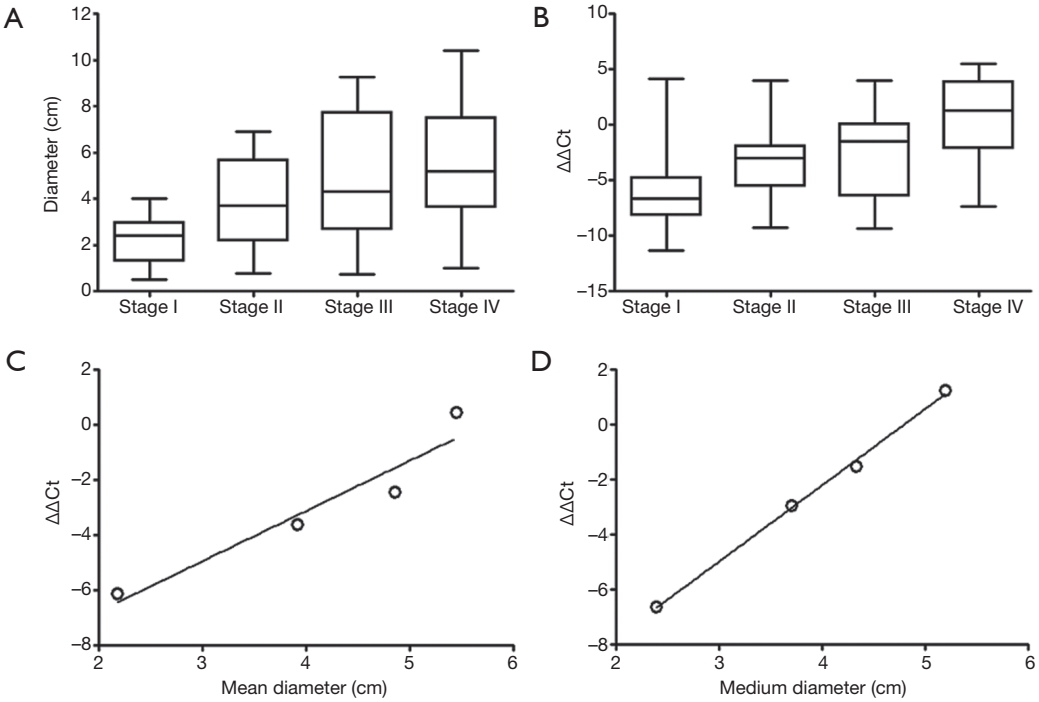

Figure 1 The relationship between tumor diameter and plasma *mSHOX2* methylation level (ΔΔCt). Box and Whisker plots show the diameter (A) and the ΔΔCt values (B) from stage I to stage IV lung cancer. The linear relationship between the mean values of diameter and the ΔΔCt was shown in (C) and the linear relationship between the medians of diameter and the ΔΔCt was shown in (D).

therapeutic response. In the partial response (PR) group (*Figure 3A*,*B*), it was very clear that most patients exhibited sharply decreased or steady *mSHOX2* level after two cycles of therapy (*Figure 3A*), and the mean *mSHOX2* level decreased from –0.68 (95% CI: –2.44 to 1.08) to –4.99 (95% CI: –6.34 to –3.64) (*Figure 2B*) (student *t*-test, P=0.0002). In contrast, the mean *mSHOX2* level changed from –1.89 (95% CI: –3.38 to –0.40) to –2.42 (95% CI: –3.98 to –0.85) (*Figure 2B*) in the stable disease (SD) group, exhibiting essentially no change following therapy (student *t*-test, P=0.62). These observations clearly suggest that *mSHOX2* level change was a sensitive indicator for therapeutic response, which was consistent with the assessment by computed tomography based on RECIST 1.1.

The therapeutic response and the corresponding *mSHOX2* level change are shown in *Figure 4A*,*B*, respectively. Thirty-six patients out of 68 achieved PR while the rest 32 achieved SD following two cycle of therapy, regardless of the personalized strategies (*Figure 4A*). The *mSHOX2* level (ΔΔCt) change was shown in descending order in *Figure 4B*, in which patients with PR (solid bars) generally exhibited bigger ΔΔCt change than those with SD (blank bars). The relationship between the response and the ΔΔCt change was plotted in *Figure 6* and linear relationship can be obtained (r^2=0.57). These results indicate that *mSHOX2* level change was consistent with the response and was a good indicator for non-invasive therapeutic effect assessment.

The plasma mSHOX2 level predicted the long-term survival of stage IV lung cancer patients

Generally speaking, most stage IV lung cancer patients who receive first-line therapy will move to multiline therapy due to the development of resistance to first-line therapy. The multiline therapy include chemotherapy, combined chemo- and radiotherapy, TKI-based targeted therapy and immunotherapy. Here we would like to investigate the potential of *mSHOX2* as a biomarker for early prediction of patient long-term prognosis before and at early-stage of the first-line therapy, no matter what type of multiline therapy was implemented.

We therefore followed the patients for up to 871 days and collected the overall survival data from 31 patients

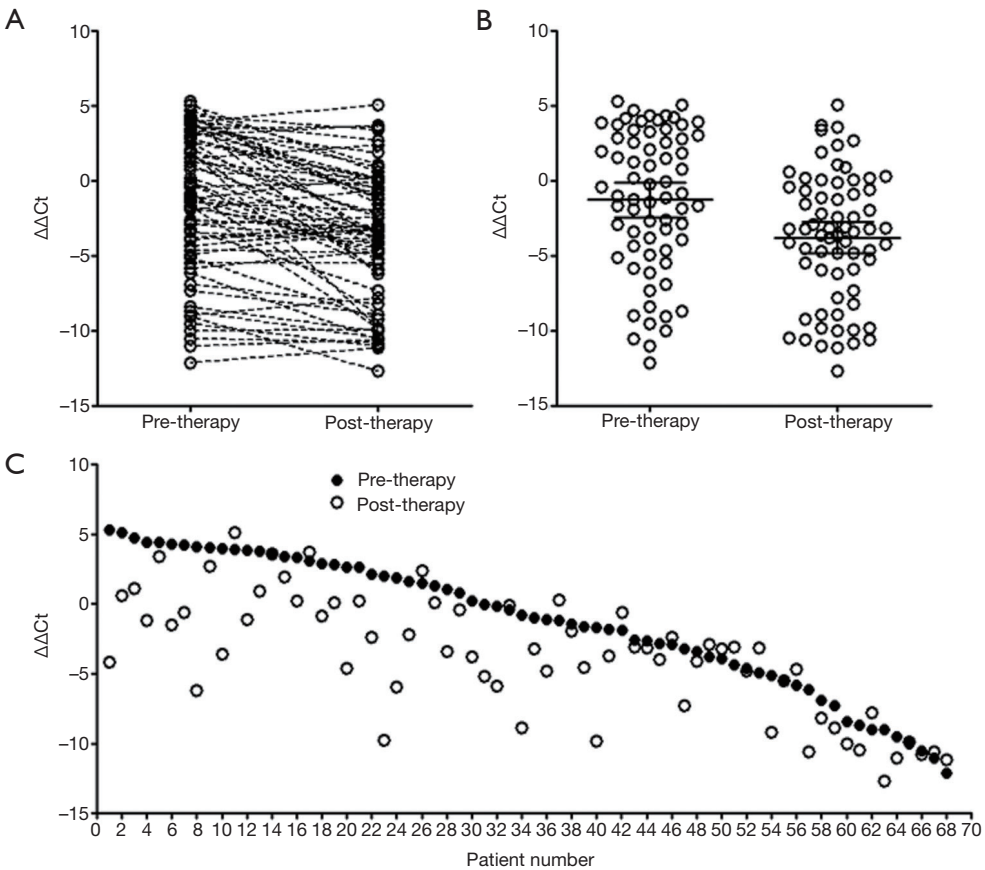

Figure 2 The *mSHOX2* levels before and two cycles after therapy for stage IV lung cancer patients. The before-after plot is shown in (A), and data from the same patient is connected by dashed line to show the *mSHOX2* level change. The scatter plot is shown in (B), and bars represent mean values with 95% CI. The paired *mSHOX2* levels before and two cycles after therapy were shown in (C) in descending order of the pre-therapeutic *mSHOX2* level. Solid dots and blank dots represent *mSHOX2* level before and two cycles after therapy, respectively.

available for follow-up. *Figure 5* shows the overall survival data based on pre-therapy *mSHOX2* level (*Figure 5A*), two cycles post-therapy *mSHOX2* level (*Figure 5B*), *mSHOX2* level change (ΔΔCt change) (*Figure 5C*) or the primary tumor size change (*Figure 5D*). When using the median as the subgrouping threshold, patients with low pre-therapeutic *mSHOX2* level (i.e., ΔΔCt <–1.62) exhibited significantly better survival rate than those with high pre-therapeutic *mSHOX2* level (i.e., ΔΔCt >–1.62) (*Figure 5A*, P=0.04). The median survival for patients with high pre-therapeutic *mSHOX2* level was 598 days, while the median survival for patients with low pre-therapeutic *mSHOX2* level has not been reached. Similarly, patients with low post-therapeutic *mSHOX2* level (i.e., ΔΔCt <–4.0) exhibited significantly better survival rate than those with high post-therapeutic *mSHOX2* level (i.e., ΔΔCt >–4.0) (*Figure 5B*, P=0.008). The median survival for patients with high post-therapeutic *mSHOX2* level was 598 days, while the median survival for patients with low post-therapeutic *mSHOX2* level has not been reached. In contrast, neither the ΔΔCt change (*Figure 5C*, P=0.66) nor the tumor size change (*Figure 5D*, P=0.40) showed significant survival difference, when the median was used as the subgrouping threshold. Univariable and multivariable Cox regression analysis [Forward: likelihood ratio (LR)] revealed that *mSHOX2* level before therapy was the only independent predictor of the overall survival (P=0.032), with a hazard ratio of 1.414. These observations suggest that the pre-therapeutic plasma *mSHOX2* level was a predicting factor for patient long-term survival, while the *mSHOX2* level change or tumor size change before and after therapy were not predicting factors.

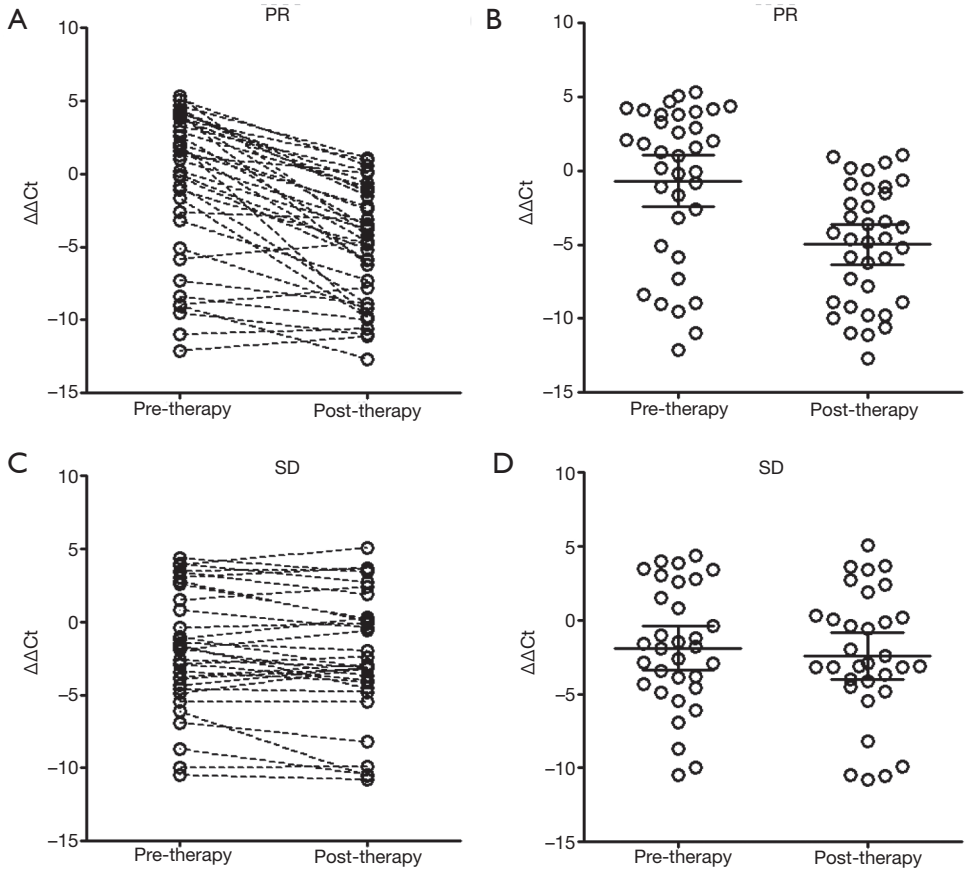

Figure 3 The *mSHOX2* levels before and two cycles after therapy for stage IV lung cancer patients with partial response (PR) or stable disease (SD). The before-after plot is shown in (A) and (C) for patients with PR and SD, respectively, and data from the same patient is connected by dashed line to show the *mSHOX2* level change. The scatter plot is shown in (B) and (D) for patients with PR and SD, respectively, and bars represent mean values with 95% CI.

Discussion

The dynamic alterations of the blood mSHOX2 level following first-line therapy of stage IV lung cancer

The relationship between the level of plasma methylation markers (including *mSHOX2*, *mSEPT9* and *mPTGER4*) and the progression of has been reported in previous studies of lung cancer and colorectal cancer. Generally, the detection rate and the plasma methylation level increase with the elevation of cancer stage, and good correlation was found between tumor size and plasma methylation level (2,4,11,23-25). It has been reported that methylation level of *mSHOX2*, *mSEPT9*, *mPTGER4* and *mPITX2* before or after therapy could be used as predicting markers for patient survival in colorectal cancer, lung cancer, neck squamous cell cancer, and gliomas (6,21,26-30). However, these markers were previous developed as diagnostic markers, and most of the studies focused on relatively early-stage cancers, instead of advanced or late-stage cancers. With the rapid development of targeted therapy and immunotherapy, stage IV lung cancer can be treated, the primary and metastatic cancers can be controlled, and therefore some patients can survive a long time. Our study focused on the monitoring and assessment of these patients and tried to identify a strategy to evaluate the therapeutic effect and predict the long-term outcome of various therapies.

It was obvious in our study and previous studies that *mSHOX2* can be used for monitoring the progression, relief or response to therapy. This assessment should not be dependent on a single test, but a series of continuous tests at key assessment time points. Dynamic alterations of *mSHOX2* levels are indicative for treatment response. Plasma *mSHOX2* can therefore be used as non-invasive monitoring method for instant therapeutic effect

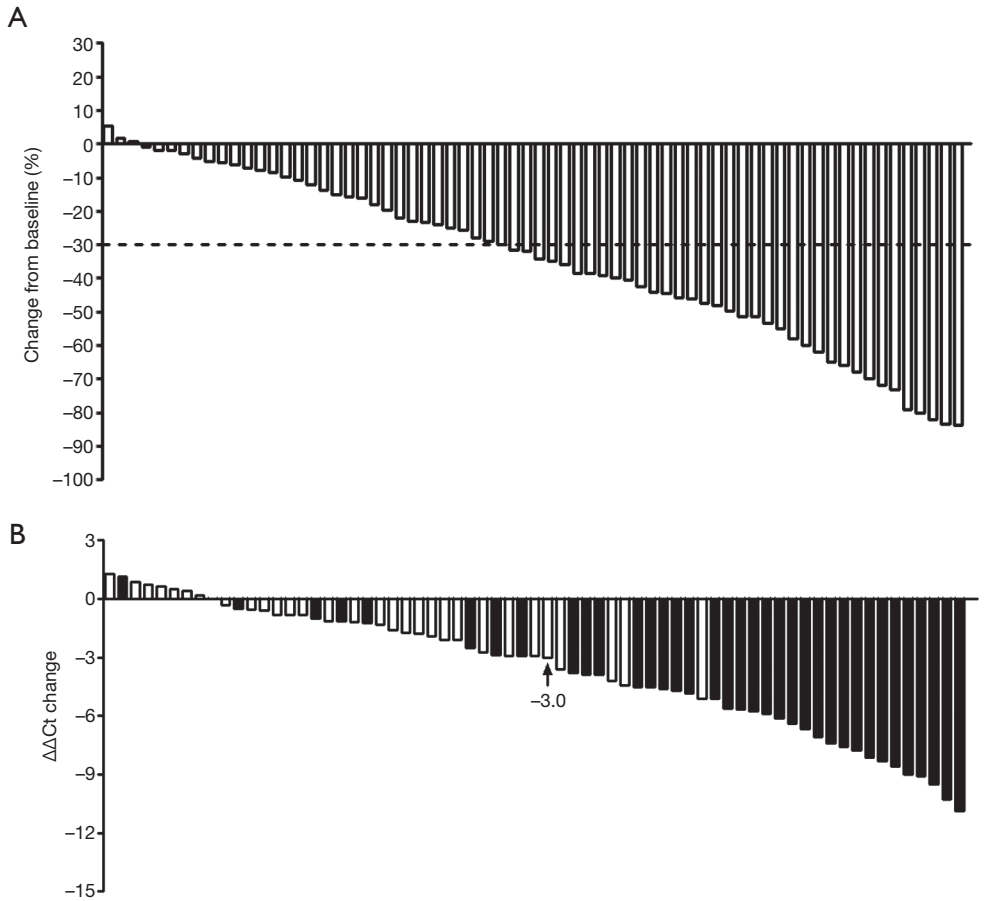

Figure 4 The therapeutic response and the corresponding *mSHOX2* level change (ΔΔCt change) for stage IV lung cancer patients. (A) The therapeutic response of all involved stage IV lung cancer patients in ascending order of the response. 30% threshold was used for distinguishing patients with PR from those with SD. (B) The ΔΔCt change of the corresponding patients in descending order. Positive numbers indicate ΔΔCt increase and negative numbers indicate ΔΔCt decrease. Solid bars represent the ΔΔCt change values for patients with PR and blank bars represent ΔΔCt change values for patients with SD. −3.0 (arrow) was the median value of the dataset, which was used in *Figure 5* for patient dichotomization.

assessment, and can be more safely and frequently used than CT. Although methylation markers have been shown to be sensitive for assessment, they are still not applicable in therapeutic strategy selection, in which mutation detection using NGS technology are currently widely used. Combination of mutation detection and methylation detection may be a good choice for both strategy selection and response monitoring, as methylation detection can also be achieved by NGS.

We found that a large majority of patients exhibited parallel response between tumor shrinkage and *mSHOX2* level decrease (*Figures 2-4*), which achieved the linear relationship between them (*Figure 6*). However, the correlation between plasma *mSHOX2* decrease and maximal diameter reduction for each individual is still hard to predict. This may be due to the way of measurement of tumor size and the *mSHOX2* level. The measurement of maximal diameter at the same CT section may not fully reflect the tumor size change, while the *mSHOX2* quantification for low level of *mSHOX2* may not be very accurate. Ideally, the volume of tumor can be assessed by 3D reconstruction, and the *mSHOX2* level can be quantified by digital PCR.

The application of mSHOX2 assay in predicting the long-term prognosis of metastatic lung cancer

It was interesting in this study that the *mSHOX2* level

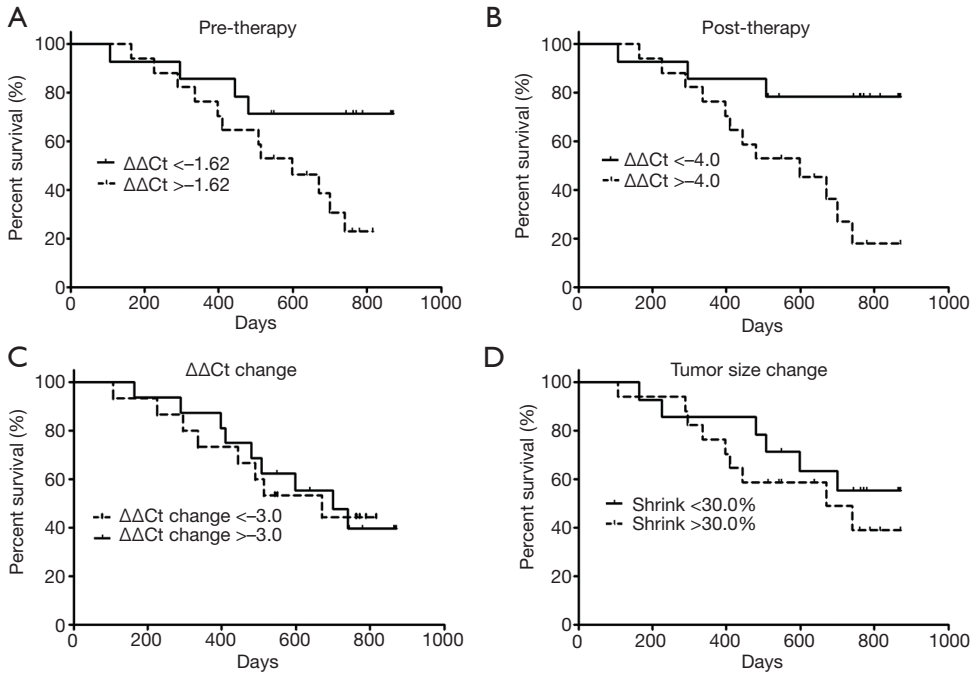

Figure 5 Kaplan-Meier survival analysis of risk factors in stage IV lung cancer patients involved in this study. (A) the overall survival analysis based on pre-therapeutic *mSHOX2* level with stratifying threshold at the ΔΔCt median of –1.62; (B) the overall survival analysis based on two cycles post-therapeutic *mSHOX2* level with stratifying threshold at the ΔΔCt median of –4.0; (C) the overall survival analysis based on *mSHOX2* level change (ΔΔCt change) with stratifying threshold at the median of –2.72; (D) the overall survival analysis based on targeted tumor maximal diameter change with stratifying threshold at the median of 31.6%.

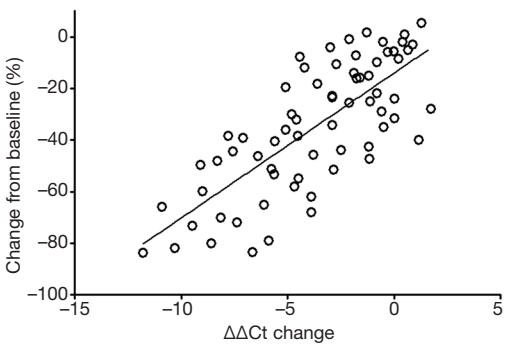

Figure 6 Linear relationship between the therapeutic response and *mSHOX2* level change (ΔΔCt change) for all stage IV patients involved in this study. r^2=0.57 for the linear regression.

before and two cycles after therapy were predictive for patient survival, while the *mSHOX2* level change or tumor diameter change were not predictive. This suggests that the initial *mSHOX2* level before therapy actually predicted long-term survival, and patients with relatively higher pre-therapeutic *mSHOX2* level appeared to live shorter, no matter what types of therapy they receive in the following treatment. The earliest predicting time can be the time that lung cancer is diagnosed before any therapy. This suggests that *mSHOX2* predicts the therapeutic response instead of merely reflecting the clinical course. The *mSHOX2* level after two cycles of therapy was also predictive for survival, and this may be relevant to both pre-therapeutic *mSHOX2* level and the degree of *mSHOX2* level change following therapy. However, the fact that *mSHOX2* level change and tumor diameter change were not predictive suggests that the initial response to first-line therapy did not predict the long-term survival. Patients with initial good response to first-line therapy may not have good prognosis in future.

It was found that the *mSHOX2* level before therapy had huge variation (*Figure 2A*). Since the pre-therapeutic *mSHOX2* level appeared to be predictive for long-term survival, it would be interesting to know the reason for this variation and the decisive factors for *mSHOX2* level. Generally speaking, the plasma level of methylation markers may be relevant to tumor size, tumor growth pattern, and tumor pathological type. We previous found in colorectal

cancer that patients with moderate to low differentiation exhibited significantly higher plasma *mSEPT9* level than those with high differentiation. Patients with infiltrative CRC exhibited significantly higher plasma *mSEPT9* level than those with protrude or ulcerative CRC. Patients with N2 lymph node metastasis exhibited significantly higher plasma *mSEPT9* level than those with N1 lymph node metastasis or no lymph node metastasis. Patients with distal metastasis exhibited significantly higher plasma mSEPT9 level than those with no metastasis (31). Although no such studies have been performed with lung cancer, it can be speculated that similar factors in lung cancer may affect the plasma *mSHOX2* level, which, in turn, affect the long-term survival.

Opportunities and challenges of methylation-based assay in future lung cancer therapeutic effect assessment and prognosis prediction

The plasma single gene-based methylation assay has already exhibited promising clinical perspectives. *mSHOX2* and a couple of other methylation markers have been shown to be effective for therapeutic effect assessment and prognosis prediction. However, it is still difficult to predict the individual risk or prognosis based on methylation markers. The individual prediction may be composed of many factors other than methylation biomarkers, and a quantified scoring system might be used to provide a definite interpretation. Another approach is to build a panel-based methylation model to assess the therapeutic effect and to predict the prognosis. This may overcome the deficiency of a single marker and make the assessment and prediction more accurate. However, this needs validation and optimization of a NGS-based methylation panel, and algorithm, modeling or even artificial intelligence may be required for prediction.

It would also be interesting if methylation markers can be used in patient selection for specific therapy, similar to the *EGFR*-sensitive mutations for selecting patients for TKI therapy or tumor mutation burden (TMB) assay for immunotherapy patient selection. This may be applied to some new therapies or drugs relevant to patient methylation status, or some current therapies in which patients can be selected by methylation. Methylation may be the next revolutionary marker for cancer therapy if it can be used in patient selection. Furthermore, recurrence could be predicted by circulating methylation markers in future, as there is evidence showing that the mutation detection by NGS from ctNA can predict the recurrence of lung cancer before signs from imaging tests can be identified. However, evidence is not available for *mSHOX2* and more investigation is needed.

Conclusions

Quantification of the plasma *mSHOX2* is capable of monitoring and assessing the therapeutic effect of stage IV lung cancer patients undergoing chemotherapy, combined chemo- and radiotherapy, or targeted therapy. Both pre-therapeutic and post-therapeutic plasma *mSHOX2* quantification are effective for patient long-term prognosis prediction.

Future perspective

Individualized risk assessment, therapeutic effect assessment and prognosis prediction by methylation markers would be one of the future directions for blood-based methylation assays. This may require combination of multiple markers with clinical characteristics to achieve model prediction. Methylation panel assay by NGS may be an option for multiple marker detection, and can be applied to all cancers other than lung cancer. Selection of therapeutic strategies based on methylation status might be another future direction with huge potential.

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Footnote

Conflicts of Interest: L Song was previously an employee of BioChain (Beijing) Science and Technology, Inc. BioChain is a collaborator of Epigenomics AG, a Germany-based company that launched the first commercial *mSHOX2* assay. The other authors have no conflicts of interest to declare.

Ethical Statement: The permission for clinical study was

granted by the ethics committees of all participating hospitals before the start of sample collection. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all subjects, and the information on the usage of plasma and test results were provided to all subjects.

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Supplementary

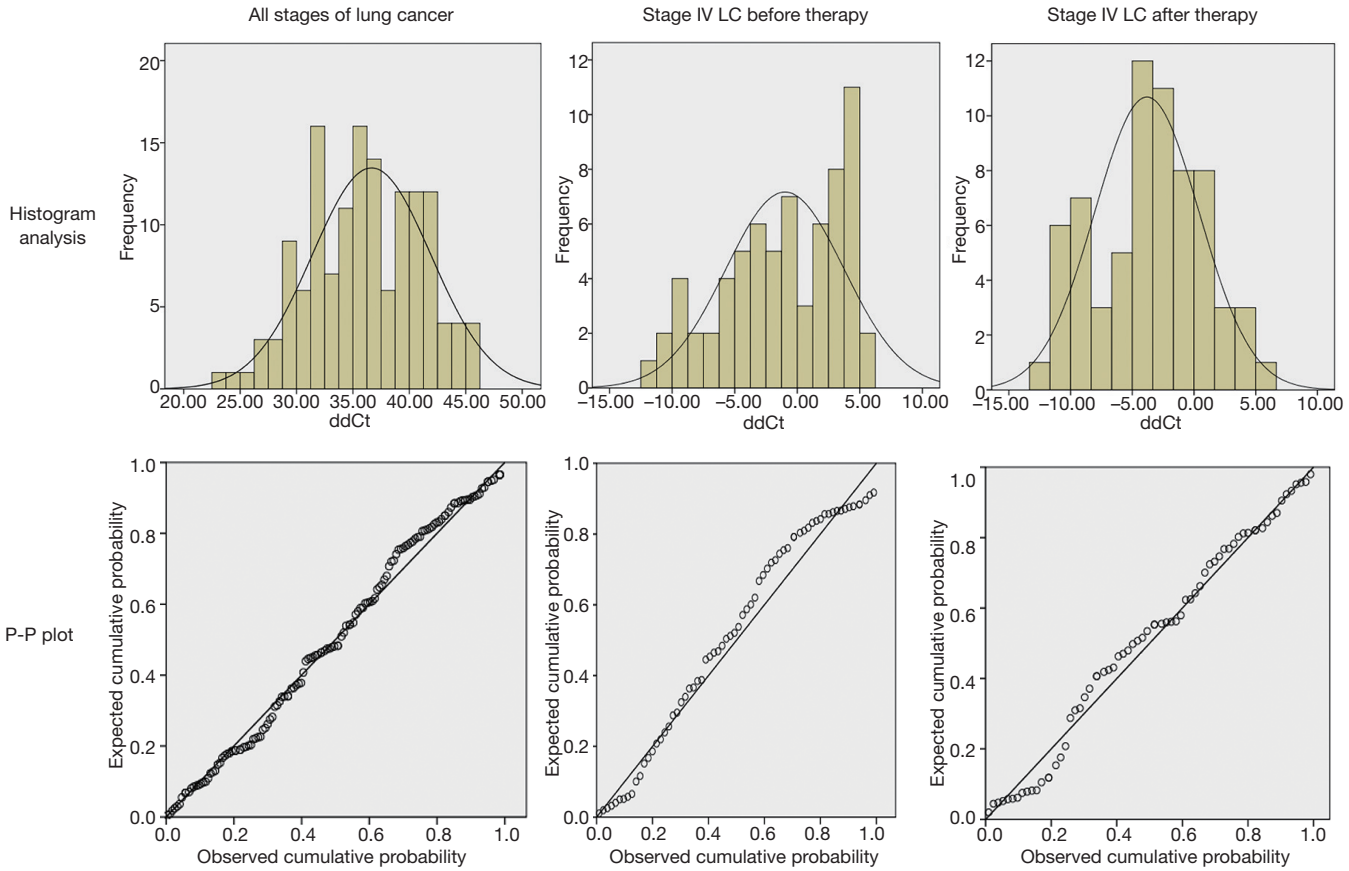

Figure S1 Test of normality using the histogram analysis and the P-P plot methods for three datasets used in this study. LC, lung cancer; P-P, probability-probability.