Plasma cell-free DNA methylation combined with tumor mutation detection in prognostic prediction of patients with non-small cell lung cancer (NSCLC)

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Abstract

Background: Lung cancer is one of the most common cancers with high degree of malignancy, is a devastating disease with a poor prognosis worldwide. Prognostic prediction for patients with non-small-cell lung cancer (NSCLC) is still challenge.

Material and methods: The cohort consisted of 64 consecutive patients with NSCLC identified from June 1, 2014, to June 30, 2018. Liquid biopsy samples were collected. Genomic mutation DNA was calculated by including all substitutions and indels over the entire somatic, coding, sequencing length. Statistical evaluations were carried out using SPSS software.

Results: Quantity of total ctDNA was successfully determined in all 64 patients from whom baseline circulating DNA was available. ctDNA concentration ranged from 4000 to 3,562,000 genome equivalents per milliliter. Treatments induced a significant decrease in cancer specific markers in most patients with response to treatments, while the methylated DNA demonstrated favorable prediction efficiency regardless of the response status. Patients with ctDNA mutation and methylated DNA decreasing have favorable overall survival (P < .05), combination of genetic and methylated DNA decreasing had high reliability in predicting overall survival of patients with NSCLC.

Conclusions: We have detected both tumor mutations and methylated DNA in plasma of patients with NSCLC. Combined genetic and methylated DNA decreasing after treatment was an independent risk factor for prognosis of patients with NSCLC. Meanwhile, it had favorable predict value and had potential to be defined as a novel biomarker for patients with NSCLC.

Abbreviations: ctDNA = circulating tumor DNA, NSCLC = non-small-cell lung cancer.

Keywords: circulating tumor DNA, methylated DNA, non-small-cell lung cancer

1. Introduction

Lung cancer is one of the most common cancers with high degree of malignancy, is a devastating disease with a poor prognosis worldwide.\cite{1,2} Approximately 85% of lung cancers are classified as non-small-cell lung cancer (NSCLC), which consists of 2 major histology subtypes: squamous and non-squamous carcinoma. A large proportion of patients are diagnosed at advanced-stage in this world, which would be the leading cause of cancer-related mortality, in where curative treatment, such as resection is not feasible.\cite{3,4} Even in patients with early-stage and resectable disease receiving definitive chemoradiation, up to 90% of patients eventually relapse.\cite{5} NSCLC remains a challenge to cure, thus investigations and attempts at identifying novel molecular target therapy to improve patients’ outcomes still continue.

Tumor mutational has emerged as a potential measure of genomic instability and neoantigens to predict response to treatments including resection, chemotherapy, radiotherapy and immunotherapy. Neoantigens arise as a result of tumor-specific mutations that are absent from the normal human genome.\cite{6,7} Recent research and commercialization efforts in the field of detecting circulating tumor DNA (ctDNA) in bodily fluids, that is, “liquid biopsies”, particularly blood plasma, have demonstrated the clinical utility of detecting ctDNA as an aid for clinical management of cancer patients. The detection of ctDNA can be used for obtaining diagnostic, prognostic and theragnostic information concerning cancer.\cite{6-11} Both tumor mutations detection including tumor tissues testation and liquid biopsy
were used for comprehensive genomic DNA testation.\[^{12,13}\] Aberrant DNA methylation is a characteristic of most types of solid cancer with common hypermethylation events occurring more frequently than most mutations.\[^{14,15}\] As a driving force in tumorigenesis, methylation occurs at early stage during cancer formation. Moreover, DNA methylation pattern in plasma ctDNA is similar with that derived from cancer tissue. These observations suggest that ctDNA methylation could serve as a useful biomarker for cancer detection.\[^{16-18}\]

While there are many challenges associated with direct neoantigen prediction, tumor mutation DNA and ctDNA methylation may serve as an indirect, probabilistic measure of immunogenicity. Here we propose the evaluation of the circulating tumor DNA and DNA methylation markers in ctDNA, not as an early detection method, but rather as a non-invasive treatment-monitoring assay for prognostic prediction for patients with NSCLC. While using innovative approaches and being very informative, studies which aimed at identifying cancer-specific methylated markers usually relied on platforms with low genomic coverage, or small sample datasets, or exclusively assessed patient tissue which might have been partially infiltrated with stroma.\[^{19-21}\] We therefore employed genome-wide assessment of DNA methylation in collection of 64 patients with NSCLC. Longitudinal assessment of this panel in ctDNA was performed to monitor disease burden in patients with NSCLC over diverse treatment courses and we further compared the prognostic value of both tumor mutational DNA and methylated DNA after follow-up.

### 2. Patients and methods

#### 2.1. Study design and participants

The cohort consisted of 64 consecutive patients with NSCLC identified from June 1, 2014, to June 30, 2018. Liquid biopsy samples were collected prospectively and analyzed retrospectively in double blind fashion for patient outcome. The study was approved by the Regional Ethical Review Board Henan Cancer Hospital, Affiliated Cancer Hospital of Zhengzhou University. Patients were treated according to the Declaration of Helsinki’s ethical principles for medical research involving human subjects. All patients provided an informed written consent prior to study entry. Patients were required to meet the following inclusion criteria: Participants were age 18 to < 80 years; Eastern Cooperative Oncology Group performance status (ECOG-PS)\[^{22}\] was evaluated; histologically or cytologically confirmed NSCLC. No prior chemotherapy, radiotherapy and immunotherapy before radical resection were allowed. Patients were excluded if they had a concurrent malignancy other than NSCLC, a serious, uncontrollable medical condition, or a psychiatric disorder that would limit ability to comply with study requirements.

#### 2.2. Pretreatment Evaluation and treatment scheme

Medical history and physical findings were documented in each patient. Each patient also had an ECG, computed tomography of the abdomen and pelvis (and thorax, if needed), serum chemistry and CBC, and urine analysis. Most patients received operations and after the surgery, all patients received adjuvant chemotherapy, including TP (Paclitaxel+Cisplatin), GP (Gemcitabine+Cisplatin), DP (Docetaxel+Cisplatin), PC (Pemetrexed+Cisplatin), and SP (S1+Cisplatin).

#### 2.3. Sample collection and ctDNA extraction

All liquid samples of patient with NSCLC were collected from Henan Cancer Hospital, Affiliated Cancer Hospital of Zhengzhou University. All blood samples from patients (~5 mL) were collected in tubes containing EDTA as anticoagulant, centrifuged for 15 minute at 1500 × g. The purified plasma was then stored at −80°C. Plasma was collected before any treatment. If the patients have received surgery, plasma was collected at the day after operation and patients who do not have surgery have their plasma collection after the first cycle of chemotherapy or immunotherapy. The ctDNA was extracted from plasma using QIAamp Circulating Nucleic Acid kit (Qiagen, 55114) according to manufacturer’s protocol. The quality of plasma ctDNA was evaluated by Bioanalyzer 2100 (Agilent Technologies).

#### 2.4. Genomic mutation DNA and methylated DNA enrichment detection

Customized targeted multiple gene panel consisting of 90 genes was used for next-generation sequencing (NGS). The results of NGS were obtained after the following steps: separation of plasma from the samples, isolation of ctDNA from the plasma, target region enrichment in an appropriate quality and quantity, library preparation, clonal amplification and NGS steps. Afterwards, bioinformatic analyses were performed to determine the quality and variant analysis according to the clinical information of the patients to interpret the variants. Genomic mutation DNA was calculated by including all substitutions and indels over the entire somatic, coding, sequencing length.\[^{7,23}\] Synonymous mutations were included given their potential to promote genomic instability. Noncoding alterations were excluded. Separate pools of male and female human genomic DNA were created by combining equal amounts of DNA. Samples of pooled DNA in a 300 μL volume of Tris-EDTA buffer (TE) were sonicated for 10 minute in a Diagenode Bioruptor (Liege, Belgium) set on high. Sonication was performed for 20 cycles consisting of a 30 second pulse followed by a 30 second rest. 1.8 to 3 μg of sonicated DNA (500bp average size) was enriched for methylated fragments using the MethylMiner kit (Invitrogen, Carlsbad, CA, Catalogue # ME10025) according to the manufacturer’s instructions and both bound and unbound fractions kept. The fractionated DNA was then subjected to bisulphite treatment using the EZ DNA Methylation-Gold Kit (Zymo Research, Irvine, CA Cat # D5006) according to the manufacturer’s instructions.

#### 2.5. Data collection

The following data were collected from medical records: patient characteristics, PS, presence of tumor, number and sites of metastases, mutational status of EGFR, KRAS, TP53, PI3KCA and BRAF, c-Met, amplification of HER2, and MSI status, baseline carcinoma antigen-199 (CA-199) carcinoembryonic antigen (CEA). Tumor response was evaluated according to Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1. CT scan was performed every 8 weeks from treatment start. Overall survival was defined as the time from initiation treatment to death from any reason.
2.6. Statistical methods

Continuous variables were expressed as mean ± SD (standard deviation) and compared using a 2-tailed unpaired Student t test; categorical variables were compared using x^2 or Fisher analysis. The predictive performance of genetic DNA, methylated DNA were measured using the area under ROC curve.[24] Life-table estimates of survival time were calculated according to the Kaplan and Meier methodology.[25,26] The Greenwood formula was used for the standard deviation. A Cox proportional hazards regression approach[26] was chosen for the evaluation of DFS and OS as the primary end-point. Potential prognostic variables were analyzed both univariately with 1 factor taken at a time, and then in a multivariate model combining all factors. Results are reported as hazard ratios (HR) and their 95% confidence intervals (CI). A HR > 1 indicated an elevated risk with respect to the reference category. A confidence interval which did not include the value 1 indicated statistical significance at the 5% level. All statistical evaluations were carried out using SPSS software (Statistical Package for the Social Science, version 15.0, SPSS Inc, Chicago, IL). A value of \( P < .05 \) was considered to be statistically significant in all the analyses.

3. Results

3.1. Baseline characteristics

Patient characteristics are summarized in Table 1. A total of 64 patients were enrolled, and the median age was 57.3 years old. A majority of patients exhibited a good PS (57% with ECOG PS = 0). The number of patients received previous chemotherapy before tumor resection was 10. 45 patients had prior resection of the primary tumor and among these patients, 10 patients had further targeted therapy using gefitinib and erlotinib, 25 patients had chemotherapy and radiotherapy, 9 patients received immunotherapy. Among patients not received operation, 6 patients received targeted therapy, 8 patients received chemotherapy and radiotherapy and 5 patients received immunotherapy.

| Variable | All NSCLC patients (n = 64) |
|----------|-----------------------------|
| Age      | 57.3 ± 10.2                 |
| Gender   |                             |
| Female   | 27                          |
| Male     | 37                          |
| ECOG-PS  |                             |
| 0/1      | 57                          |
| ≥ 2      | 7                           |
| Histologic subtype |                   |
| Squamous | 38                          |
| Nonsquamous | 26                     |
| Smoking status |                        |
| Minimal/never | 16                     |
| Current/former | 48                     |
| Patients receiving chemotherapy before surgery | |
| Yes      | 10                          |
| No       | 54                          |
| Treatment options |                   |
| Surgery  | 45                          |
| Targeted therapy | 6                      |
| Chemotherapy and radiotherapy | 8                   |
| Immunotherapy | 5                      |
| Circulating DNA |                         |
| Mutational DNA | 60                   |
| Methylated DNA | 61                 |
| Presence of EGFR/KRAS mutations |             |
| EGFR mutant/wild type | 16/48           |
| KRAS mutant/wild type | 25/39           |
| No. prior lines of treatment |          |
| 0–1      | 8                           |
| ≥ 2      | 2                           |
| TNM staging |                         |
| I–II     | 50                          |
| III–IV   | 14                          |

Data are presented as n (%) or median (range).

ECOG PS = Eastern Cooperative Oncology Group performance status, EGFR = epidermal growth factor receptor, NSCLC = non-small-cell lung cancer.

Figure 1. A, genomic mutational detection in all 64 patients with non-small-cell lung cancer and the ctDNA concentration ranged from 4000 to 3,562,000 genome equivalents per milliliter; B: Quantity of methylated DNA could be determined in all 64 samples and ranged from 3500 to 2,394,000/mL.
3.2. Circulating DNA markers at baseline

Quantity of total ctDNA was successfully determined in all 64 patients from whom baseline circulating DNA was available. ctDNA concentration ranged from 4000 to 3,562,000 genome equivalents per milliliter. Quantity of methylated DNA could be determined in all 64 samples and ranged from 3300 to 2,394,000/mL. Assessment of mutational DNA and methylated DNA were successful in all samples and positivity were observed in 93.7% (n = 60/64) and 95.3% (n = 61/64) (Fig. 1). In the three negative samples (4.7%), the assay was unable to detect any circulating mutational DNA (Fig. 1A) and methylation signal above the limit of detection (Fig. 1B). This suggests a lack of sensitivity of the assay, possibly due to very limited DNA release from these specific tumors or technical issues related to the DNA extraction process. Another explanation could be the specific site of metastases in those patients.

3.3. Circulating DNA markers during treatment and their dynamics

All 64 blood samples were available both prior to treatment and after treatment including operation and postoperative adjuvant therapy. Consistent to what we previously observed with other studies, treatments induced a significant decrease in cancer specific markers (genetic or methylated DNA; Figs. 2A, B) in most patients with response to treatments, while the methylated DNA demonstrated favorable prediction efficiency regardless of the response status. The individuals dynamical changing during treatments of genetic or methylated DNA were shown in Figure 2C, D.

3.4. Survival analysis for clinical-pathological characteristics and circulating DNA Markers

In the univariate analysis, factors significantly associated with shorter OS were genetic and methylated DNA decreasing after
treatments (Fig. 3). There was no statistically significant association between OS and age, gender, smoking status, histological subtypes, previous chemotherapy line, baseline CEA and CA-199 and adjuvant therapy were not significantly associated with OS ($P = \text{.258}$, $P = \text{.135}$, $P = \text{.227}$, $P = \text{.347}$, $P = \text{.189}$, $P = \text{.756}$, $P = \text{.567}$, and $P = \text{.843}$, respectively). In the multivariate analysis, combined genetic and methylated DNA decreasing and TNM staging remained significant (Table 2).

### 3.5. Prognostic performance of genetic and methylated DNA decreasing in patients with NSCLC

The performance of the genetic DNA, methylated DNA decreasing and the combination of these 2 subtypes decreasing in differentiating the clinical outcomes of patients with NSCLC was evaluated (Fig. 4). The analysis demonstrated that the combination of genetic and methylated DNA decreasing had high reliability in predicting overall survival of patients with NSCLC (area under ROC curve $0.874$; $95\%$ CI: $0.521–0.935$; sensitivity $85.4\%$, specificity $73.6\%$).

### 4. Discussion

One of the surprising aspects of cancer biology that emerged from the Cancer Genome Atlas sequencing projects was the wide diversity of mutations that give rise to cancer.[27,28] Even within a single tumor type the mutation profiles vary from patient to patient, and it’s not unusual for even the most commonly altered genes to be mutated in less than half of the cases. The variability of cancer mutation profiles contrasts with the Stability of CpG island methylation changes. The 14-3-3 sigma Promoter has been found to be methylated in 96% of breast carcinomas, and unmethylated in the breast epithelium of individuals without cancer. While not a good candidate biomarker for a breast cancer blood test,[29,30] since 14-3-3 sigma is also heavily methylated in leukocytes, this level of methylation underlies the homogeneity of DNA methylation changes as compared with mutations. Along the same lines, the HOXA9 promoter and EN1 promoter were found to be methylated in 95% and 80% of HGSOC ovarian cancers, respectively. Given the greater consistency of DNA methylation changes in cancer compared to mutations, methylation is a promising target for biomarker development.[18,31,32]

In this study, we found that treatments induced a significant decrease in cancer specific markers (genetic or methylated DNA) in most patients with response to treatments, while the methylated DNA demonstrated favorable prediction efficiency regardless of the response status. These results were consistent with other studies reported the changing of ctDNA and methylated DNA. Moreover, in the survival analysis we found that the patients with tumor mutational DNA and methylated DNA decreasing had favorable survival after diverse treatments. The performance of the genetic DNA, methylated DNA decreasing and the combination of these 2 subtypes decreasing in differentiating the clinical outcomes of patients with NSCLC was evaluated (Fig. 4). The analysis demonstrated that the combination of genetic and methylated DNA decreasing had high reliability in predicting overall survival of patients with NSCLC.

The collection and processing of clinical samples for the discovery and validation of plasma biomarkers needs to be carefully considered. Blood in EDTA tubes can only be stored for a limited amount of time before leukocytes begin to lyse and contribute their DNA to the plasma DNA fraction. At the very least, same day processing of blood samples is required so that the circDNA does not become contaminated with genomic leukocyte DNA. In clinical settings where patient samples are collected one at a time, sample collection and processing can become a major

### Table 2

| Variables                                | Univariate HR (95% CI) | Univariate P value | Multivariate HR (95% CI) | Multivariate P value |
|------------------------------------------|------------------------|--------------------|--------------------------|----------------------|
| EGOG-PS: 2                               | 1.487 (1.258–2.698)    | .012               | 0.873 (0.761–1.132)      | .833                 |
| TNM staging IV                           | 1.463 (1.282–4.462)    | .638               | 1.847 (1.239–3.674)      | .013                 |
| combined genetic and methylated DNA decreasing | 1.729 (1.448–3.103)    | .027               | 2.104 (1.582–4.372)      | .001                 |
| EGFR mutations                           | 1.224 (1.090–2.156)    | .023               | 0.918 (0.721–1.149)      | .832                 |
| Kras mutations                           | 1.383 (1.127–2.311)    | .031               | 0.823 (0.647–1.650)      | .412                 |
Meanwhile, it had favorable predictive value and had potential to be an independent risk factor for prognosis of patients with NSCLC. Genetic and methylated DNA decreasing after treatment was an indicator of methylated DNA in plasma of patients with NSCLC. Combined mutational DNA and methylated DNA decreased have significant difference in predicting prognosis of patients with non-small-cell lung cancer.

**Figure 4.** Predictive performance of mutational DNA decreased (36 patients), methylated DNA decreased (38 patients), and combined mutational DNA and methylated DNA decreased have significant difference in predicting prognosis of patients with non-small-cell lung cancer.

Furthermore, determining the specificity of a biomarker, that is, its ability to correctly identify negative samples, requires large numbers of cancer-free controls, ideally age-matched and collected within the same setting and in the same way as the cases. If control samples are collected at different centers than the cancer samples, standardized protocols are required so that variations in sample handling between centers do not introduce artifacts. Problems can also arise from discovery and validation strategies that compare samples from patients with cancer to samples from healthy individuals, insofar as in addition to cancer specific biomarkers, the patient samples are also likely to show changes due simply to the presence of inflammation. The resulting biomarker may not be able to differentiate between cancer and other less serious conditions with an inflammation component. These considerations are not specific to methylated DNA biomarkers and apply to biomarker discovery and validation generally.

There are limitations of this study:

1. this prediction model was based on the data of Chinese patients. Whether these 2 biomarkers are applicable to patients from other countries remains to be determined;
2. the sample size is small in this study and further randomized controlled trial with large cohort should be performed to validated the results in this study.

In conclusion, we have detected both tumor mutations and methylated DNA in plasma of patients with NSCLC. Combined genetic and methylated DNA decreasing after treatment was an independent risk factor for prognosis of patients with NSCLC. Meanwhile, it had favorable predictive value and had potential to be defined as a novel biomarker for patients with NSCLC.

**Author contributions**

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