Circulating tumor DNA evaluated by Next-Generation Sequencing is predictive of tumor response and prolonged clinical benefit with nivolumab in advanced non-small cell lung cancer

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ABSTRACT
Nivolumab is an anti-PD1 antibody, given in second-line or later treatment in advanced non-small cell lung cancer (NSCLC). The objective of this study was to describe the predictive value of circulating tumor DNA (ctDNA) on the efficacy of nivolumab in advanced NSCLC. We prospectively included all consecutive patients with advanced NSCLC treated with nivolumab in our Department between June 2015 and October 2016. Plasma samples were obtained before the first injection of nivolumab and at the first tumor evaluation with nivolumab. ctDNA was analyzed by Next-Generation Sequencing (NGS), and the predominant somatic mutation was followed for each patient and correlated with tumor response, clinical benefit (administration of nivolumab for more than 6 months), and progression-free survival (PFS). Of 23 patients, 15 had evaluable NGS results at both times of analysis. ctDNA concentration at the first tumor evaluation and ctDNA change correlated with tumor response, clinical benefit and PFS. ROC curve analyses showed good diagnostic performances for tumor response, clinical benefit and PFS. At the first tumor evaluation (tumor response; positive predictive value (PPV) at 100.0% and negative predictive value (NPV) at 71.0%; clinical benefit: PPV 83.3% and NPV 77.8%) and the ctDNA change (tumor response: PPV 100.0% and NPV 62.5%; clinical benefit: PPV 100.0% and NPV 80.0%). Patients without ctDNA concentration increase ≥9% at 2 months had a long-term benefit of nivolumab. In conclusion, NGS analysis of ctDNA allows the early detection of tumor response and long-term clinical benefit with nivolumab in NSCLC.

Introduction
Lung cancer is the leading cause of cancer-related death in the world.1 Recently, immune checkpoint inhibitors (ICI) have shown efficacy in the treatment of non-small cell lung cancer (NSCLC). Nivolumab is an anti-PD1 antibody, which was tested in a second-line setting in advanced NSCLC. In the pivotal randomized phase III trials, overall response rate (ORR) was 20% and disease control rate (DCR) was 50%.2,3 Of interest, some patients experienced a prolonged (i.e., more than 6 months) clinical benefit with nivolumab. However, no biomarker has been currently identified to predict the efficacy of nivolumab in second-line treatment or more. Programmed death-ligand 1 (PD1) immunohistochemistry (IHC) has been often used. A recent study has shown the benefit of another ICI (pembrolizumab) in first-line treatment in high-PD-L1 expression (≥50%) advanced NSCLC, compared to platinum-based chemotherapy (hazard ratio (HR) for overall survival at 0.60; 95%CI 0.41–0.89).4 PD-L1 IHC was associated with ORR in Checkmate-057, but not in Checkmate-017.2,3 Moreover, patients with negative PD-L1 expression in IHC may however experience tumor response with nivolumab. There is an urgent need to identify new biomarkers associated with nivolumab efficacy.

The monitoring of circulating tumor DNA (ctDNA) may be an interesting tool in this setting. For cytotoxic chemotherapy, several studies have failed to prove a predictive role of ctDNA concentration for response.5,6 However, in case of oncogenic addiction, the monitoring of the mutated allelic fraction can be used to follow the efficacy of targeted therapies and look for the appearance of resistance mutation, especially in EGFR-mutated NSCLC.7-12 Next-generation sequencing (NGS) is also feasible in ctDNA.13 With regard to ICIs, recent reports suggested that the decrease of the ctDNA concentration during treatment was associated with tumor response.14-16 However, most of these studies used digital-droplet Polymerase chain reaction (ddPCR) to monitor only one specific mutation.
In this study, we tested the hypothesis that the change in ctDNA concentration as measured using large-cancer gene panel screening by NGS between baseline and evaluation at 2 months correlated with tumor response and clinical benefit of nivolumab in advanced NSCLC.

**Results**

**Patient characteristics**

Between June 2015 and October 2016, 34 patients received nivolumab in second-line or later treatment for an advanced NSCLC. From them, 20 patients had plasma samples for both C1 nivolumab and M2. Of these 20 patients, 15 (75%) had interpretable data in NGS at the two time-points and were used for analyses. There were 9 men (60.0%), 14 current or former smokers (93.0%) with 35 packet-years (median), 11 stage IV NSCLC (73.0%) and 10 non-squamous carcinoma (66.7%). On 15 patients, 14 had evaluable tumor sample for PD-L1 expression assessment (immunohistochemistry, IHC). Seven patients (50.0%) and 2 patients (14.3%) had PD-L1 expression on tumor cells, respectively. The patients received nivolumab mainly in second-line treatment (n = 13, 86.7%) and less often in fourth-line treatment (n = 2, 13.3%). The median number of nivolumab injections was 13 (4–20). The overall response rate was 33.3%. After a median follow-up of 17.3 months, 10 patients (66.7%) exhibited tumor progression, with a ctDNA at baseline for patients with objective response (OR) at the first tumor evaluation and patients without OR, and between patients with clinical benefit (i.e., nivolumab given > 6 months) and patients without clinical benefit. No difference was seen in terms of clinical and pathological features between the groups.

Table 2 displays the PD-L1 status and the mutations found in NGS for each patient.

**ctDNA concentration at baseline**

The ctDNA concentration at baseline (median) was 0.006 ng/μl (0.003–0.013). The ctDNA concentration at baseline was not different between patients with OR and patients without OR: 0.005 ng/μl (0.001–0.007) versus 0.009 ng/μl (0.004–0.029), respectively (p = 0.501) (Table 1). No difference was observed between ctDNA at baseline according to subsequent clinical benefit, with a ctDNA at baseline for patients with clinical benefit at 0.005 ng/μl (0.001–0.008) versus 0.012 ng/μl (0.006–0.046) for patients without clinical benefit of nivolumab (p = 0.148) (Table 1). Baseline ctDNA concentrations were no different according to PD-L1 expression in IHC: median 0.007 ng/μl (0.006–0.012) in PD-L1 ≥1% patients

| Table 1. Patients’ characteristics and comparison between patients with tumor response and without tumor response, and between patients with clinical benefit of nivolumab and patients without clinical benefit of nivolumab. |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                                | all patients    | OR (n = 5)      | no OR (n = 10)  | p-value         | clinical benefit | no clinical benefit |
|                                | (n = 15)        |                 |                 |                 | (n = 8)          | (n = 7)          |
| age (years)                    | 66.0 ± 5.1      | 68.8 ± 1.7      | 64.6 ± 5.7      | 0.143           | 0.264           | 0.264           |
| gender                         |                |                 |                 |                 |                 |                 |
| male                           | 9 (60.0)        | 4 (80.0)        | 5 (50.0)        | 0.013           | 0.046           | 0.046           |
| female                         | 6 (40.0)        | 1 (20.0)        | 5 (50.0)        | 0.145           | 0.019           | 0.019           |
| tobacco-use                    |                |                 |                 | 0.180           | 0.542           | 0.542           |
| ever                            | 7 (46.7)        | 1 (20.0)        | 6 (60.0)        | 3.448           | 0.001           | 0.001           |
| former                         | 7 (46.7)        | 3 (60.0)        | 4 (40.0)        | 0.011           | 0.001           | 0.001           |
| never                           | 1 (6.6)         | 1 (20.0)        | 0 (0.0)         | 0.000           | 0.000           | 0.000           |
| histology                      |                |                 |                 | 0.000           | 0.000           | 0.000           |
| squamous                       | 5 (33.3)        | 1 (20.0)        | 4 (40.0)        | 0.000           | 0.000           | 0.000           |
| non-squamous                   | 10 (66.7%)      | 4 (80.0)        | 6 (60.0)        | 0.000           | 0.000           | 0.000           |
| stage                           |                |                 |                 | 0.680           | 0.876           | 0.876           |
| III                            | 1 (6.6)         | 1 (20.0)        | 3 (30.0)        | 0.000           | 0.000           | 0.000           |
| IV                             | 11 (73.3%)      | 4 (80.0)        | 7 (70.0)        | 0.000           | 0.000           | 0.000           |
| Tumor burden (mm)‡             | 97.5 (59.5–185.8) | 82.0 (56.0–134.0) | 103.0 (700–188.0) | 0.790          | 0.336           | 0.336           |
| mutation status at diagnosis   |                |                 |                 | 0.336           | 0.506           | 0.506           |
| Kras                           | 3 (20.0)        | 1 (20.0)        | 2 (20.0)        | 0.000           | 0.000           | 0.000           |
| BRAF                           | 1 (6.6)         | 1 (20.0)        | 0 (0.0)         | 0.000           | 0.000           | 0.000           |
| WT                             | 11 (73.3%)      | 3 (60.0)        | 8 (80.0)        | 0.000           | 0.000           | 0.000           |
| number of previous line        | 1 (1–1)         | 1 (1–1)         | 1 (1–1)         | 0.000           | 0.000           | 0.000           |
| number of nivolumab injections | 13 (4–20)       | 13 (8–14)       | 10.5 (1.8–28.3) | 0.000           | 0.000           | 0.000           |
| PD-L1 expression in IHC ≥1%**  | 7 (50.0)        | 3 (75.0)        | 4 (40.0)        | 0.027           | 0.012           | 0.012           |
| ctDNA concentration at baseline (ng/μl) | 0.006 (0.003–0.013) | 0.005 (0.001–0.007) | 0.009 (0.004–0.029) | 0.501 | 0.012 | 0.012 |
| ctDNA concentration at 2 months (ng/μl) | 0.002 (0.001–0.011) | 0.001 (0.000–0.001) | 0.007 (0.003–0.078) | 0.032 | 0.008 | 0.008 |
| ctDNA relative augmentation    | yes             | 6 (40.0)        | 0 (0.0)         | 0.025           | 0.125           | 0.125           |
| no                             | 9 (60.0)        | 5 (100.0)       | 4 (40.0)        | 7 (87.5)        | 2 (28.6)        | 2 (28.6)        |

OR: Overall response. WT: EGFR, Kras, Braf and ALK wild-type. Variables are expressed as mean (±SD) or median (IQR). *Tumor burden was evaluable for 14 patients at baseline and for 12 patients at the first tumor evaluation. **PD-L1 IHC was available for 14 patients.
Predominant mutation was chosen according to the concentration of each mutated allele, based on the allelic frequency and circulating DNA concentrations. PD-L1 (Supplementary Fig. 1).


correlated with baseline tumor burden (patients (ctDNA concentration at the

m 50% patients versus 0.005 ng/ml (0.0005–0.01) in PD-L1 ≥50% patients versus 0.007 ng/ml (0.005–0.018) in PD-L1 <50% patients (p = 0.927). Baseline ctDNA concentrations were not correlated with baseline tumor burden (p = 0.371) (Supplementary Fig. 1).

**ctDNA concentration at the first evaluation**

At the first tumor evaluation, the ctDNA concentration was statistically different between patients with OR versus patients without OR: 0.001 ng/ml (0.0006–0.001) versus 0.007 ng/ml (0.003–0.078), respectively (p = 0.032) (Table 1). ROC curve analysis determined a positivity threshold at 0.006 ng/ml, allowing the detection of an absence of clinical benefit in case of ctDNA > 0.006 ng/ml with a sensitivity of 71.4%, a specificity of 87.5%, a positive predictive value at 83.3% and a negative predictive value at 77.8% (AUC = 0.839) (Fig. 1B).

Median PFS for patients with ctDNA > 0.006 ng/ml at first tumor evaluation was 1.8 months, versus not-reached for patients with ctDNA < 0.006 ng/ml (p = 0.003) (Fig. 2A). Median OS for patients with ctDNA > 0.006 ng/ml at first tumor evaluation was 2.2 months, versus not-reached for patients with ctDNA < 0.006 ng/ml (p = 0.044) (Fig. 2B).

cDNA concentrations at the first evaluation were no different according to PD-L1 expression in IHC: median 0.003 ng/ml (0.000–0.012) in PD-L1 ≥1% patients versus 0.006 ng/ml (0.001–0.085) in PD-L1 <1% patients (p = 0.695); median 0.007 ng/ml (0.004–0.131) in PD-L1 ≥50% patients versus 0.005 ng/ml (0.000–0.031) in PD-L1 <50% patients (p = 0.926). ctDNA concentrations at the first tumor evaluation were not correlated with tumor burden at the first tumor evaluation (p = 0.543) (Supplementary Fig. 2).

**Figure 1.** ROC curves for PFS (A) and OS (B) according to ctDNA concentration at the first tumor evaluation.

| Patient | PD-L1 (%) | Predominant mutation | Other mutations |
|---------|-----------|----------------------|----------------|
| 1       | 0         | TP53 p.Pro190Arg c.569C>G | — |
| 2       | N.A.      | NOTCH1 c.5019-35C>T    | — |
| 3       | 0         | NOTCH1 p.His1591Gln c.4773C>G | — |
| 4       | 0         | FBXW7 p.Val6445Val c.1935G>A | TS3 p.Val173Met c.517G>A |
| 5       | 30        | TP53 p.Lys101* c.301A>T | TS3 (NM_000546.5) p.Gly245Val c.734G>T |
| 6       | 0         | KRAS p.Gly12Val c.35G>T | FGFR2 (NM_022970.3) / c.939–43C>T |
| 7       | 30        | KRAS p.Gly12Cys c.34G>T | — |
| 8       | 0         | TP53 p.Arg337Leu c.1010G>T | TP3 (NM_000546.5) p.Arg175His c.524G>A |
| 9       | 0         | SMP4 p.Gly467Arg c.1399G>A | PIK3CA (NM_006218.3) p.Glu545Gly c.1634A>G |
| 10      | 80        | KDR p.Gln472His c.1416A>T | — |
| 11      | 50        | DDR2 c.1505-14G=A | — |
| 12      | 20        | BRAF p.Asn616Val c.1846C>T | — |
| 13      | 5         | PTEN p.Thr233 c.697C>T | CTNNB1 (NM_000190.4) p.Ser37Phe c.110C>T |
| 14      | 0         | TP53 p.Val754Met c.2262A>T | CTNNB1 (NM_000190.4) p.Ser37Phe c.110C>T |
| 15      | 20        | PTEN p.Arg233* c.697C>T | SMAD4 (NM_005359.5) p.Tyr338* c.1193G>A |

Predominant mutation was chosen according to the concentration of each mutated allele, based on the allelic frequency and circulating DNA concentrations. PD-L1 expression was evaluated by immunohistochemistry on tumor cells. N.A.: non available.

Table 2. PD-L1 expression in immunohistochemistry and mutations found in NGS for the 15 patients.

N.B. The concentration of ctDNA was not statistically different between patients with OR versus patients without OR.
Evolution of the ctDNA concentration

The change in the ctDNA concentration was strongly associated with tumor response. All the patients with tumor response (n = 5/5) had a decrease in ctDNA concentration, whereas 60% (n = 6/10) of patients without tumor response had an increase in the ctDNA concentration (p = 0.025) (Table 1). In patients with tumor response, the median of the relative change in ctDNA was −87.8% (−91.4% − −45.9%), whereas in patients without tumor response at first tumor evaluation, it was +34.1% (−39.8% + +188.5%) (p = 0.032). The corresponding ROC curve analysis defined a threshold of 30% of ctDNA decrease at first tumor evaluation, allowing the detection of an absence of OR if ctDNA relative evolution >−30%, with a sensitivity at 70%, a specificity at 100%, a positive predictive value at 100% and a negative predictive value at 62.5% (AUC = 0.860) (Fig. 3A).

For the prediction of clinical benefit with nivolumab, 87.5% (n = 7/8) patients with clinical benefit had a decrease of their ctDNA concentration at the first tumor evaluation, whereas 71.4% (n = 5/7) of patients without clinical benefit with nivolumab had an increase of their ctDNA concentration (p = 0.020) (Table 1). In patients with clinical benefit, the median of the relative change in ctDNA concentration was −45.8% (−63.3% − −28.3%), whereas in patients without tumor response at first tumor evaluation, it was +166.6% (−14.0% + +229.2%) (p = 0.118). The corresponding ROC curve analysis defined a threshold of 9% of ctDNA increase at first tumor evaluation, allowing the detection of an absence of clinical benefit with nivolumab if ctDNA concentration relative evolution >9%, with a sensitivity at 71.4%, a specificity at 100%, a positive predictive value at 100% and a negative predictive value at 80% (AUC = 0.750) (Fig. 3B).

Median PFS for patients with ctDNA increase >9% at first tumor evaluation was 0.7 months, versus 12.0 months for patients with ctDNA decrease (p < 0.001) (Fig. 4A). Median OS for patients with ctDNA increase >9% at first tumor evaluation was 2.1 months, versus not-reached for patients with ctDNA decrease (p < 0.001) (Fig. 4B). The majority of patients without increase of ctDNA >9% at the first tumor evaluation had a prolonged use and benefit of nivolumab, as shown in Fig. 5.

Concerning the other mutations found in NGS (Table 2), their concentrations had the same evolution as the predominant mutation selected for follow-up, except for one patient with a minor mutated clone (SMAD4 mutation), which concentration increased whereas others (PTEN mutation and CTNNB1 mutation) decreased between baseline and the first evaluation.

Discussion

In this study, we showed that the ctDNA concentration at the time of first tumor evaluation and the relative change in this concentration was associated with tumor response, PFS and
Threshold at baseline and the PFS according to the relative change in ctDNA concentration between whereas PDL1 staining is heterogeneous in the tumor.17 Moreover, PDL1 IHC raises several issues: different antibodies used in different trials depending on the ICI, different types of analyses (tumor cells with or without immune cells), different positivity thresholds, and tests often performed on small biopsies, resulting in the possibility of performing longitudinal studies of ctDNA samples with multiple mutations.13

Beyond the association to tumor response, we clearly showed that the evaluation of ctDNA allowed an early detection of the patients who will have a clinical benefit of nivolumab, as assessed by a treatment given more than 6 months. The evaluation of the clinical benefit seems more adapted for ICI treatment evaluation rather than tumor response, as several patients can experience significant clinical improvement with prolonged stable disease, or a tumor control after an initial augmentation of tumor volumes on CT-scan.2,4

In our population, we found an ORR at 33.3%, which is higher than ORR reported in the Checkmate trials (around 20.0%).2,3 Our rate of PD-L1 positive tumors (≥1% and ≥50%) was however comparable to those reported in the literature.2-4 Because of the small number of patients (n = 15), this high ORR could be due to hazard of patients inclusion.

The monitoring of ctDNA in ICI treatment opens a wide range of possible applications. The possibility to detect as soon as the first tumor evaluation, those patients who will not have tumor response or clinical benefit with nivolumab could offer the possibility of alternative treatment strategies for these patients, such as ICI combinations. In addition, ctDNA monitoring could also be used for early detection of secondary progression, and maybe reveal the appearance of resistance mutation, as described in melanoma with JAK1/JAK2 and beta-2 microglobulin mutations.20 Further studies are needed in NSCLC to confirm these hypotheses.
In conclusion, ctDNA is a useful biomarker during nivolu-
amb treatment in advanced NSCLC. NGS allow the detection
and the follow-up of a wide variety of somatic mutations. A
high ctDNA concentration at 2 months (i.e., first tumor evalua-
tion) and an increase in concentration compared with baseline
are associated with poor response and no long-term clinical
benefit. Further studies with larger population and longer fol-
low-up are needed to confirm our results.

Patients and methods

Patients and plasmas

We analyzed all consecutive patients in our hospital’s depart-
ment of Thoracic Oncology who received nivolumab in sec-
ond-line or later treatment for a stage IIIb-IV NSCLC, between
June 2015 and October 2016. Demographical, pathological and
treatment-related data were collected from a prospective data-
base. Patients received nivolumab at 3 mg/kg intravenously
every 2 weeks until progression or intolerable toxicity. Tumor
evaluation was performed at baseline with brain, thoracic and
abdominal CT-scan at 2 months (after 4 injections of nivolu-
mab) and then every 2 months under nivolumab. Tumor
response was assessed according to irRECIST.21 Patients who
did not have restaging due to clinical disease progression before
2 months, as assessed by their clinician, were classified as pro-
gressive disease, with plasma taken at the moment of the cli-
nical progression and included in the analysis. Clinical benefit
was defined as patients treated with nivolumab for more than
6 months. Tumor burden was calculated by the sum of the larg-
est diameter of all evaluable tumor lesions (more than 1 cm), at
baseline and at the first tumor evaluation.

Plasma samples were taken at diagnosis, just before the first
injection of nivolumab (C1), and at the first tumor evaluation
(at 2 months, M2).

cDNA analyses

Two 10 ml-EDTA tubes of peripheral blood were taken, and
plasma was isolated within one hour after and immediately
conserved at −80°C.

DNA Extraction from Tumor and Plasma Samples

DNAs were extracted on a Maxwell® 16 FFPE Plus LEV DNA
Purification Kit (Promega, France) for FFPE samples and the
Maxwell® RSC ccfDNA Plasma Kit (Promega, France) for cir-
culating cell-free DNAs. Quantification was done by Qubit
Fluorometric Quantitation using the Qubit dsDNA HS Assay
Kit and the Qubit dsDNA BR Assay Kit (Life Technologies–Thermo Fisher Scientific, Saint Aubin, France) for circulating
dNA and tumor DNA respectively. DNAs were stored at
−20°C before use.

Sequencing

Colon and Lung Cancer Panel V2 libraries were prepared using
the Ion Ampliseq library preparation kit v2 from 30 ng of
tumor DNA or 6 ul of cell free DNAs. Libraries were normal-
ized (Ion Library Equalizer™ Kit), pooled, processed on a Ion
Chef™ System for template preparation and chip loading (Ion
PI™ Hi-Q™ Chef Kit, Ion PI™ Chip Kit v3, Thermo Fisher
Scientific), and sequenced on a Ion Proton™ System.

Analysis

The FASTQs sequencing data were aligned to the human gene
ome (hg19) and processed by the IonTorrent Suite V5.0.4.0.
VCF files were generated using the built-in “Somatic – low
stringency” parameters to automatically call variants with allele
frequencies (AF) > 2%. Annotation pipeline was developed
internally on a galaxy platform that uses SAFIR 2.4 report tool.
In parallel, samples were analyzed by the BPER method after
BAM recalibration, a specific algorithm developed to detect
AF < 2%.22 It is publicly available at https://cran.r-project.org/
package=PlasmaMutationDetector. Results provided by BPER
method and annotated VCFs were registered blindly from clin-
ical data.

In case of multiple mutations, we chose for follow-up the
predominant mutation found in NGS, according to the concen-
tration of each mutated allele, based on the allelic ratio and cir-
culating DNA concentrations.

IHC technique

IHC was performed using an automated method (Leica) and
the E13LN anti-PD-L1 antibody (Cell signalling Technology)
diluted to the 1/80th on 4 μm-slides from the treatment-naive
diagnostic samples. The assay was performed using human
amygdala as positive control, and IgG as isotype negative con-
control. The IHC was considered as being positive if at least one
tumour cell out of 100 analysed tumour cells was positively
stained.

Ethical considerations

All patients signed a consent form prior to plasma collection.
The plasma collection and analyses were authorized by the
Institutional Review Board CPP IDF n°8 (ID CRB 2014-
A00187-40). The retrieval of data from our prospectively col-
lected database conformed to the rules of the Commission
Nationale de l’Informatique et des Libertés.

Statistical analyses

Clinical and pathological data were compared between patients
with tumor response (overall response, OR) at the first evalua-
tion versus no tumor response, and with clinical benefit of
nivolumab versus no clinical benefit of nivolumab. Associations
with qualitative variables were assessed using the χ2, whereas
comparisons of continuous variables were assessed using the
Student t test (variables with normal distribution) or Mann–
Whitney test (variables without normal distribution). Analyses
of correlation between tumor burden and ctDNA concentra-
tions were made using the Spearman test. ROC curves were
used to determine positivity thresholds to detect OR and clin-
ical benefit, with expression of related sensitivity, specificity,
positive predictive value, negative predictive value, and area
under the curve (AUC) value. PFS and OS were estimated using
the Kaplan–Meier method (p-value calculated using log-rank
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