RESEARCH ARTICLE

Metastatic Site Location Influences the Diagnostic Accuracy of ctDNA EGFR-Mutation Testing in NSCLC Patients: a Pooled Analysis

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Abstract: Background: Recent studies evaluated the diagnostic accuracy of circulating tumor DNA (ctDNA) in the detection of epidermal growth factor receptor (EGFR) mutations from the plasma of NSCLC patients, overall showing a high concordance as compared to standard tissue genotyping. However, it is less clear if the location of the metastatic site may influence the ability to identify EGFR mutations in plasma.

Objective: This pooled analysis aims to evaluate the association between the metastatic site location and the sensitivity of ctDNA analysis in detecting EGFR mutations in NSCLC patients.

Methods: Data from all published studies, evaluating the sensitivity of plasma-based EGFR-mutation testing, stratified by metastatic site location (extracranial (M1b) vs intracranial (M1a)) were collected by searching in PubMed, Cochrane Library, American Society of Clinical Oncology, and World Conference of Lung Cancer, meeting proceedings. Pooled Odds ratio (OR) and 95% confidence intervals (95% CI) were calculated for the ctDNA analysis sensitivity, according to metastatic site location.

Results: A total of ten studies, with 1425 patients, were eligible. Pooled analysis showed that the sensitivity of ctDNA-based EGFR-mutation testing is significantly higher in patients with M1b vs M1a disease (OR: 5.09; 95% CI: 2.83–8.84). A significant association was observed for both EGFR-activating (OR: 4.36; 95% CI: 2.53–7.88) and resistant T790M mutations (OR: 11.89; 95% CI: 1.45–97.22), regardless of the use of digital-PCR (OR: 5.83; 95% CI: 3.56–9.69) or non-digital PCR technologies (OR: 2.06; 95% CI: 2.24–5.91).

Conclusions: These data suggest that the location of metastatic sites significantly influences the diagnostic accuracy of ctDNA analysis in detecting EGFR mutations in NSCLC patients.

Keywords: EGFR, ctDNA, liquid biopsy, NSCLC, metastasis, intracranial, extracranial.

1. INTRODUCTION

Targeting the epidermal growth factor receptor (EGFR) by tyrosine-kinase inhibitors (TKIs) has represented a milestone in the treatment of lung cancer. Eight phase III randomized studies have clearly demonstrated that EGFR-TKIs significantly improved response rate (RR), progression-free survival (PFS) and quality of life (QoL) compared to first-line platinum-based chemotherapy in patients with advanced NSCLC harboring EGFR activating mutations [1–8]. Recently a pooled analysis of both Lux-Lung3 (LL3) and Lux-Lung6 (LL6) trials showed also an overall survival (OS) benefit in favour of the second generation EGFR-TKI Afatinib in the subgroup of patients with EGFR exon19 deletion [9]. Overall, the results of all such studies convincingly and consistently demonstrated that, for about 40% of Asian and 12% of Caucasian “EGFR-positive” NSCLC patients, the optimal upfront treatment is an EGFR-TKI, as gefitinib, erlotinib, or afatinib. Testing for EGFR mutations in tumor samples DNA is recommended at the time of diagnosis by all the international guidelines for all patients with advanced NSCLC and non-squamous histology in order to decide the proper therapeutic strategy [10]. Even if tissue biopsy remains the current gold-standard, however, it is limited by several features, such as the difficult access to different tumor sites, the invasiveness of procedures, the tumor heterogeneity, and the low patients’ compliance. Thus, in the last decade, an alternative approach, known as a liquid biopsy, has been proposed to overcome the aforementioned issues. An increasing number of studies and meta-analysis evaluated the diagnostic accuracy of circulating tumor (ct)DNA in the

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detection of EGFR activating mutations in plasma of patients with advanced NSCLC overall showing a sensitivity of 0.62 and a specificity of 0.98 as compared with the standard tissue genotyping, thus suggesting a high concordance rate between these two testing approaches [11-14]. These evidence have led to the analytical validation and the clinical approval of EGFR mutation testing by using ctDNA isolated from plasma or serum for about 25-30% of NSCLC patients whose tissue is not available at the time of diagnosis or tissue analysis results are not available. Following the clinical approval of osimertinib, re-biopsy at progression became mandatory, in order to re-analyze the tumor molecular profile and identify T790M mutation. Osaheid et al. first demonstrated an adequate accuracy of plasma genotyping by digital PCR (dPCR) in 180 patients with advanced NSCLC, including 60 patients with acquired resistance to EGFR-TKI [15]. The predictive value of plasma ctDNA genotyping for T790M was prospectively confirmed in Phase III AURA3 trial, showing a longer PFS and higher ORR for osimertinib as compared with platinum-pemetrexed chemotherapy in patients who progressed to first-generation TKI and were T790M positive on plasma [16]. However, because of 30% potential false negative rate associated with this method, the ctDNA analysis is currently recommended as the first step of tumor genotyping, but it must be always followed by tissue biopsy for those patients who are T790M-negative on plasma [10]. Increasing evidence have recently suggested that the ability to identify EGFR activating and resistant mutations by ctDNA analysis is significantly higher in patients who have extra-thoracic metastasis as compared with patients with intra-thoracic disease [15, 17-24], but the low number of patients has limited the scientific reliability of these data. Thus it has not been clearly demonstrated if the metastatic site location may significantly influence the sensitivity of plasma ctDNA analysis in detecting EGFR mutations. Integrating data from multiple studies, in order provide evidence with greater statistical value may lead to practical implications for the management of NSCLC patients. This pooled analysis combined and analyzed simultaneously all the studies which evaluated the sensitivity of ctDNA in the detection of EGFR mutations comparing patients with extra-thoracic (Mib) versus intrathoracic (Mia) disease, with the main aim to demonstrate a significant association between the metastatic site location and the diagnostic accuracy of ctDNA analysis in NSCLC patients.

2. MATERIALS AND METHODS

2.1. Search for Clinical Trials

We searched for all published studies reporting the sensitivity of plasma-based EGFR-mutation testing by ctDNA, stratified according to the metastatic site location (extra-thoracic (Mib) vs intra-thoracic (Mia) disease). We searched for clinical trials using Medline (PubMed), Embase-databases and Cochrane-Library up to May 2017, with no language restrictions. We used the following search terms: “EGFR” or “Epidermal growth factor receptor” and “T790M”, and “circulating tumor DNA” or “ctDNA”, and “non-small cell lung cancer”, or “NSCLC” or “lung cancer.” The search was limited to human studies in the English language. Relevant abstracts from the American Society of Clinical Oncology (ASCO), European Society of Medical Oncology (ESMO), and World Conference on Lung Cancer (WCLC) were included. We also explored the ClinicalTrials.gov website (www.clinicaltrials.gov) to search for unpublished data and ongoing studies.

2.2. Selection Criteria

According to the aforementioned search, clinical trials were taken into account if they met the following inclusion criteria: 1) patients with histologically-proven diagnosis of advanced NSCLC; 2) studies performing EGFR mutation testing in matched tumor tissue and plasma samples; 3) studies evaluating the sensitivity of EGFR mutation testing by ctDNA analysis; 4) studies reporting the sensitivity of ctDNA mutation testing according to the metastatic site location (extra-thoracic (Mib) vs intra-thoracic (Mia) disease).

2.3. Data Extraction

Data extraction and assessment were performed by two different authors (F.P. and S.R.) and disagreements were solved by a discussion with another author (A.R.). Following data were collected from eligible studies: first author name, journal and year of publication, study design, study treatment, baseline characteristics of patients (i.e. age, sex, stage, metastatic site location), true positive (TP) and false negative (FN) rates stratified according to the metastatic site location (Mib vs Mia disease). The meta-analysis was designed according to the PRISMA - guidelines for reporting of systematic reviews [25].

2.4. Statistical Analysis

Patients were stratified according to the metastatic site location into 2 groups: extra-thoracic (Mib) versus intrathoracic (Mia) metastatic disease. The outcome measure was the sensitivity of ctDNA analysis, defined as the proportion of EGFR-positive patients by tumor tissue testing correctly identified by the ctDNA analysis. We extracted the number of events over total patients included in each arm using odds ratio (OR) as a measure of association between ctDNA sensitivity and the metastatic site location (extra-thoracic (Mib) versus intrathoracic (Mia) disease). Thus, an OR greater than 1 indicates that extra-thoracic (Mib) disease is associated with a higher sensitivity of EGFR-mutation testing by ctDNA analysis in patients with advanced NSCLC. We calculated a pooled OR performing a meta-analysis of ORs emerging from the included studies. Heterogeneity between studies was explored using Chi-square test with a predefined significance threshold of 0.1. We used the random-effect or fixed-effect, based on statistical significance of Q-test, according to Mantel-Haenszel method. We performed a publication bias analysis using both Begg's funnel plots and Egger's test, with P<0.05 suggesting a statistically significant publication bias. When publication bias was found, the DerSimonian and Laird method was used to adjust it. The methodological quality of included trials was assessed by QUADAS-2. We used Cochrane RevMan ver. 5.3 statistical software to perform the meta-analysis and Comprehensive Meta-Analysis ver. 2.0 to assess the risk of publication bias.
3. RESULTS

3.1. Literature Search

The search of literature updated in May 2017, identified a total of 93 records. Among these, nine studies met our inclusion criteria and were included in our pooled-analysis. In the study by Karlovich et al. [16] the EGFR mutation analysis by ctDNA was performed both for EGFR-activating and T790M resistant mutations, thus the data were reported as two different studies. Overall ten eligible studies (1425 patients) were included in our pooled analysis (Fig. 1).

3.2. Studies’ Characteristics

All these studies prospectively collected matched blood and tumor tissue from patients with a histologically-confirmed diagnosis of advanced NSCLC. Real-time polymerase chain reaction (RT-PCR) was the most commonly used method to detect EGFR mutations in the plasma of NSCLC patients, while the studies of Oudard [15], Therasse [21], Karlovich [17] and Kasthani [23] used digital PCR technologies. RT-PCR was the most commonly used to detect EGFR mutations in FFPE (formalin-fixed paraffin-embedded) tumor tissue. The sample sizes of the analyzed population ranged from 38 to 397, while the percentage of patients with intrathoracic (M1a) disease ranged from 23% to 61% across the different studies. Baseline characteristics of selected studies are described in Table 1.

3.3. Diagnostic Accuracy of ctDNA

All included studies compared the sensitivity of EGFR mutation analysis by ctDNA in patients with extra-thoracic (M1b) versus intrathoracic (M1a) disease. Pooled analysis showed that the sensitivity of ctDNA was significantly higher in patients with extra-thoracic (M1b) as compared to intrathoracic (M1a) disease (OR: 5.69; 95% CI: 2.93 – 8.84) (Fig. 2). The pooled OR for sensitivity was calculated using random-effect model, because of a significant heterogeneity between treatment effects (Q-test: P < 0.0001). A significant association between the “M-status” and the diagnostic accuracy of ctDNA analysis was observed for both EGFR-activating (OR: 4.30, 95% CI: 2.35–7.88) (Fig. 3) and
Table 1. Characteristics of the trials included in the pooled analysis.

<table>
<thead>
<tr>
<th>Study/Reference</th>
<th>EGFR Mutation (ctDNA)</th>
<th>Sensitivity (M1b) n(%)</th>
<th>Sensitivity (M1a) n(%)</th>
<th>Odds Ratio (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oudard et al. 2014 [5]</td>
<td>Del19/L858R</td>
<td>13/16 (81%)</td>
<td>3/18 (17%)</td>
<td>2.11 (0.95-4.66)</td>
</tr>
<tr>
<td>Norman et al. 2014 [6]</td>
<td>Del19/L858R</td>
<td>14/26 (54%)</td>
<td>13/65 (20%)</td>
<td>3.87 (1.73-8.59)</td>
</tr>
<tr>
<td>Yu et al. 2016 [7]</td>
<td>Del19/L858R</td>
<td>15/20 (75%)</td>
<td>6/35 (17%)</td>
<td>2.21 (1.35-3.56)</td>
</tr>
<tr>
<td>Kress et al. 2017 [9]</td>
<td>Del19/L858R</td>
<td>11/15 (73%)</td>
<td>11/15 (73%)</td>
<td>2.40 (0.76-7.53)</td>
</tr>
<tr>
<td>Kress et al. 2018 [10]</td>
<td>Del19/L858R</td>
<td>23/30 (77%)</td>
<td>7/11 (64%)</td>
<td>3.71 (1.03-13.48)</td>
</tr>
<tr>
<td>Kress et al. 2018 [11]</td>
<td>Del19/L858R</td>
<td>35/38 (92%)</td>
<td>13/16 (81%)</td>
<td>2.31 (1.09-5.04)</td>
</tr>
<tr>
<td>Kress et al. 2018 [12]</td>
<td>Del19/L858R</td>
<td>35/38 (92%)</td>
<td>13/16 (81%)</td>
<td>2.31 (1.09-5.04)</td>
</tr>
<tr>
<td>Total (95% CI)</td>
<td></td>
<td></td>
<td></td>
<td>2.52 (1.63-3.88)</td>
</tr>
</tbody>
</table>

The number of patients reported corresponds to the number of patients evaluable.

![Fig. 2](image2.png)

Fig. (2). Forest plot showing odds ratio for overall sensitivity of plasma ctDNA EGFR-mutation testing by metastatic sites location (M1b vs M1a).

![Fig. 3](image3.png)

Fig. (3). Forest plot showing odds ratio for overall sensitivity of plasma ctDNA EGFR-activating (Del19, L858R) mutations testing by metastatic sites location (M1b vs M1a).
resistant 1790M mutation (OR: 1.189, 95% CI: 1.45-97.22) (Fig. 4), and it was confirmed regardless of the use of digital-PCR (OR: 1.189, 95% CI: 1.45-97.22) (Fig. 5) or non-digital PCR technologies (OR: 0.50, 95% CI: 2.24-4.91) (Fig. 6).

3.4. Risk of Bias Assessment

Publication bias has been found either by Hegg's and Fager's test (P<0.01) (Fig. 7). However, the 'trim and fill' analysis did not show significantly different results, confirming that the sensitivity cDNA analysis is associated to the metastatic site location (adjusted OR: 3.129, 95% CI: 1.73-5.88). The methodological quality of each trial was assessed by QUADAS-2, showing a good average quality of all included trials (Fig. 8).

4. DISCUSSION

This meta-analysis included ten studies which evaluated the diagnostic accuracy of EGFR mutation testing by cDNA in patients with advanced NSCLC comparing patients with extra-thoracic vs intra-thoracic disease. The results of this study have shown a significant association between the metastatic site location and the sensitivity of the cDNA analysis. Indeed the ability to identify EGFR mutations in the plasma of NSCLC patients is significantly higher in patients with extra-thoracic disease (M1b) as compared to patients with intrathoracic (M1a) disease. Since the clinical application of cDNA in patients with lung cancer is rapidly evolving, it is important to establish both the accuracy and feasibility of this tool in the practical management of advanced disease. Published studies and meta-analysis revealed an adequate diagnostic accuracy of cDNA in the detection of EGFR activating mutations, showing a sensitivity of 0.64 and specificity of 0.96, with an overall mutation status concordance of about 90% with the standard tissue genotyping [11, 12]. The promising diagnostic performance of cDNA observed in these controlled studies has been confirmed also in a real-world diagnostic setting. The multicenter ASSESS study has recently showed a similar concordance rate of 89%.
(sensitivity 45%, specificity 97%) between plasma and tissue-based EGFR mutation analysis in 1162 patients with advanced NSCLC [18], suggesting the clinical utility and reliability of ctDNA for the detection of EGFR activating mutations in clinical practice. These data have led to the recommendation of EGFR mutation analysis by ctDNA in patients with newly diagnosed, advanced NSCLC when tumor tissue is not available [10] and this was also stated in the approval of Gefitinib. However, some efforts should be needed to raise the sensitivity of this tool, in order to reduce the number of false negative patients who would be wrongly excluded from receiving EGFR-TKI treatment. In this scenario, our work provides additional evidence with potential practical implications for the management of NSCLC patients. Indeed the results of this pooled analysis clearly demonstrated that the ability to detect EGFR activating mutations in the plasma of patients with advanced NSCLC is significantly influenced by the location of tumor metastasis.
Particularly, the sensitivity of ctDNA is significantly lower in patients with intrathoracic (50%) as compared to extra-
thoracic disease (79%), suggesting the “M” status as a clin-
ical predictor of ctDNA accuracy, used to identify the best
candidates for plasma EGFR-testing. As consequence, onc-
ologists should carefully evaluate any negative result ob-
tained in patients with M1a disease. Indeed, the very low
sensitivity of ctDNA analysis in M1a stage could lead to
50% of potentially false-negative patients who would be
wrongly excluded from receiving the best first-line treatment
with an EGFR-TKI. Even if they represent only 30% of the
overall metastatic population, every effort should be made by
clinicians to detect EGFR-mutations in tumor tissue in this
subgroup of patients. Conversely, the ctDNA analysis may
be considered as a routine alternative to EGFR-TKI in
M1a patients with the extra-thoracic disease, since it showed a sensitivity of about
80% and it could be peacefully recommended when tumor tissue is not available. It would be interesting to compare also the sensitivity of ctDNA in M1b (≥1 extrathoracic me-
tastasis) versus M1c (>1 extrathoracic metastasis) disease, as
defined by the last version of the IASLC-TNM staging pro-
ject [26]. Unfortunately, the majority of the studies included in this pooled analysis did not perform this kind of
evaluation, since they were conducted before the publication of the last version of IASLC-TNM staging. Furthermore, none of the included trials evaluated how the number and the specific site of extra-thoracic tumor metastasis could influence the sensitivity of the ctDNA analysis. The detection of EGFR-T790M mutation by ctDNA is currently recom-
ended as a routine test for all patients with advanced
NSCLC who failed first-line EGFR-TKI and are a can-
didate to receive osimertinib as second-line treatment [10]. To date, three published studies [17, 21, 24] revealed a significantly lower sensitivity, ranging from 20%-50%, of plasma ctDNA analysis in the subgroup of patients with M1a intrathoracic disease as compared to 70%-90% in patients with M1b extra-thoracic metastasis. The results of our work confirmed a significantly higher sensitivity nearly to 80% in patients with M1b as compared to 50% in patients with M1a disease, suggesting that T790M mutation testing by ctDNA could result negative in half of the patients without extra-
thoracic metastasis, thus requiring further tissue biopsy and second-line treatment delay. For this reason, tissue biopsy
should be recommended upfront in all EGFR-positive NSCLC patients with M1a disease who progressed after first-
generation TKIs in order to increase the chance to detect
T790M mutation and avoid any subsequent treatment delay. Interestingly, the subgroup analysis performed to evaluate the influence of detection technology on ctDNA analysis sensitivity, revealed that the use of digital-PCR increased ctDNA sensitivity (5%) as compared to neo-digital PCR (73%) only in patients with the M1b disease while no differences have been observed in M1a patients. As previously reported in different studies, these data confirm that the chances to detect EGFR mutations in the blood of NSCLC patients with
intrathoracic metastasis cannot be increased by the use of
more accurate technologies and that the significant association
between M-status and ctDNA accuracy is the result of a
greater release of DNA into the blood of patients with extra-
thoracic metastases. Interestingly the combination of exosa-
mal RNA and ctDNA significantly increased the sensitivity
for EGFR mutation detection in plasma of NSCLC patients
with intrathoracic disease [27], suggesting that RNA-based
liquid biopsy platform as an innovative and promising ap-
proach for this subgroup of patients. This literature-based
analysis highlights the potential role of the metastatic site
location as a clinical predictor of ctDNA ability to identify
EGFR mutations in the plasma of patients with advanced
NSCLC. Nevertheless there are also some limitations: the
cohorts of patients enrolled in the analyzed studies are hetero-
geneous; indeed different selection criteria as well as the
different practices for EGFR mutation testing in plasma
samples have been used among included trials. Genotyping
cfDNA was performed by RT-PCR, including QIAGEN
therasure, Roche Cobas and others in the real word AN-
NESS study, while the adoption of the BEAMING digital
PCR allowed to increase the sensitivity of cfDNA analysis in
the OXOND trial. Furthermore the local practices for plasma
sample testing used in real world setting could have influ-
cenced the inferior cfDNA performance observed in the AS-
NESS study as compared to the expert laboratories included in
the academic controlled clinical trials. Even if the previously
described heterogeneity doesn’t reduce both the clinical
and statistical value of results, further prospective clinical
studies are needed to investigate if and how the “M status”
should be considered by oncologists to identify patients more
suitable for plasma EGFR testing in clinical practice.

**CONCLUSION**

In conclusion, the results of this pooled-analysis have
clearly demonstrated that the location of metastatic sites sig-
nificantly influences the diagnostic accuracy of cfDNA
analysis, suggesting that the ability to identify both EGFR
activating and resistant T790M mutations in plasma of
NSCLC patients is significantly higher in presence of extra-
thoracic disease, regardless the use of more accurate dia-
gnostic technologies, like digital-PCR. These observations
should be carefully considered to optimize the clinical man-
agement of EGFR-mutated NSCLC patients.

**LIST OF ABBREVIATIONS**

ASCO = American Society of Clinical Oncology
95% CI  = 95% confidence intervals
CMA = Comprehensive Meta-Analysis software
cfDNA = Circulating tumor
EGFR = Epidermal growth factor receptor
ELCC = European Lung Cancer Conference
ESMO = European Society of Medical Oncology
FFPE = Formalin-fixed paraffin embedded
FN = False negative
L3 = LuxLuL3
L6 = LuxLuL6
M1b = Extra-thoracic disease
M1a = Intrathoracic disease
NSCLC = Non-small cell lung cancer
OR = Odds ratio
ORR = Objective response rate

PFS = Progression free survival

PRISMA = Preferred Reporting Items for Systematic Reviews and Meta-Analyses

QoL = Quality of life

RevMan 5.3.5 = Review Manager 5.3.5

RR = Response rate

RT-PCR = Real-time polymerase chain reaction

TKIs = Tyrosine-kinase inhibitors

TP = True positive

WCLC = World Conference on Lung Cancer

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are base of this research.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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REFERENCES


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