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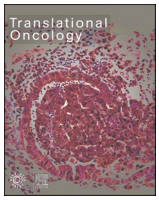

Original Research

Clinical characteristics of EGFR-ctDNA shedders in EGFR-mutant NSCLC patients

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ABSTRACT

Background: Circulating tumor DNA (ctDNA) revolutionized the molecular diagnostics of lung cancer by enabling non-invasive, sensitive identification of actionable mutations. However, ctDNA analysis may be challenging due to tumor shedding variability, leading to false negative results. This study aims to understand the determinants for ctDNA shedding based on clinical characteristics of lung cancer patients, for a better interpretation of false negative results to be considered when ordering ctDNA analysis for clinical practice.

Methods: Blood samples were collected from patients with stage IV EGFR-mutated (mEGFR) NSCLC before treatment and monitored until disease progression. EGFR was assessed on tissue by standard procedures, while EGFR status on ctDNA was tested using dPCR at baseline and at the first reassessment. NGS was used to evaluate patients mutational status at the progression of the disease.

Results: A total of 40 mEGFR tissue samples were collected. Plasma samples were analyzed for mEGFR before starting the first line, 65 % of patients had detectable mEGFR in ctDNA ("shedders"). Higher ECOG PS ($p = 0.04$), bilateral localization of primary tumor ($p = 0.04$), and the presence of intrathoracic/extrathoracic disease ($p = 0.05$), were associated to mEGFR shedding. Shedders had shorter PFS compared to non-shedders ($p = 0.03$). Patients with detectable mEGFR in ctDNA at the first radiological assessment exhibited worse PFS compared to patients with ctDNA clearance ($p = 0.05$).

Conclusion: Our preliminary data demonstrate that specific clinical characteristics predict mEGFR shedding in ctDNA of NSCLC, suggesting a potential clinical applicability for understanding potential false negative results and appropriate reporting in clinical practice.

Introduction

Epidermal Growth Factor Receptor (EGFR) mutations represent one of the most prevalent oncogenic driver mutations in Non-Small Cell Lung Cancer (NSCLC), observed in approximately 15–40 % of lung adenocarcinomas [1–3]. Mutations occurring in exon 19–21, including exon 19 deletion subtypes (ex19del) and p.L858R, have been observed in metastatic NSCLC, particularly in association with younger age, non-smoking history, and female gender [4–6]. Three generations of EGFR Tyrosine Kinase Inhibitors (TKIs) have been developed and introduced into clinical practice for the treatment of EGFR-mutated (mEGFR) lung cancer, which significantly improved the clinical outcomes of these patients [4,7]. Unfortunately, patients will eventually experience disease progression [8]. In this context, liquid biopsy has

Abbreviations: TKIs, Tyrosine Kinase Inhibitors; dPCR, digital Polymerase Chain Reaction; NGS, Next-Generation Sequencing; PFS, Progression-free Survival; ROC, Receiver operating characteristic; AUC, Area under ROC curves; mEGFR, mutated EGFR.

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Table 1
Clinical characteristics of patients.

	Number of patients ($n = 40$)
Histology at diagnosis	
Lung adenocarcinoma, NSCLC	40 (100)
Stage, n (%)	
IV	40 (100)
Age at the diagnosis, median (range)	70 (32–89)
Gender, n (%)	
Male	9 (22.5)
Female	31 (77.5)
Smoking status	
Smokers	6 (15)
Former smokers	14 (35)
Non-smokers	20 (50)
ECOG PS, n (%)	
0	13 (32.5)
1	20 (50)
2	3 (7.5)
3	4 (10)
Primary Tumor Site, n (%)	
Right lobe	21 (52.5)
Left lobe	16 (40)
Bilateral	3 (7.5)
Size of the primary tumor, median cut-off= 33 mm, n (%)	
$\leq$ 33 mm	18 (45)
$>$ 33 mm	22 (55)
Metastatic sites, n (%)	
Pleura	7 (17.5)
Brain	3 (7.5)
Bone	6 (15)
Lymph nodes	6 (15)
Pericardium	1 (2.5)
Multiple anatomical sites	17 (42.5)
Number of metastatic sites, median cut-off= 2, n (%)	
$\leq$ 2	34 (85)
$>$ 2	6 (15)
Disease burden, median cut-off= 15 mm, n (%)	
$\leq$ 15 mm	14 (35)
$>$ 15 mm	23 (57.5)
Unknown	3 (7.5)
First-line EGFR TKIs, n (%)	
First-line therapy	
Osimertinib	28 (70)
Afatinib	8 (20)
Gefitinib	4 (10)

Table 2
Comparison of clinical characteristics of shedders at baseline. Patients were considered as "shedders" if at least one mEGFR was detectable in ctDNA, and non-shedder, if no mEGFR were detected in ctDNA.

	Shedders ($n = 26$)	Non-shedders ($n = 14$)	p-value
Age at the diagnosis, median (range)	70 (43–89)	70 (32–86)	0.42
Gender, n (%)			
Male	4 (15.4)	5 (35.7)	0.28
Female	22 (84.6)	9 (64.3)	
Smoking history			
Non-smokers	13 (50)	7 (50)	0.74
Former smokers/smokers	13 (50)	7 (50)	
ECOG PS, n (%)			
0	5 (19.2)	8 (57.1)	0.04
1	14 (53.8)	6 (42.9)	
2	3 (11.6)	-	
3	4 (15.4)	-	
Primary tumor site, n (%)			
Right lobe	10 (38.4)	11 (78.6)	0.04
Left lobe	13 (50)	3 (21.4)	
Bilateral	3 (11.6)	-	
Size of the primary tumor, median cut-off = 33 mm, n (%)	10 (38.4)	8 (57.1)	0.42
$\leq$ 33 mm	16 (61.6)	6 (42.9)	
$>$ 33 mm			
Metastatic sites, n (%)	3 (11.6)		
Pleura	3 (11.6)	4 (28.6)	0.24
Brain	5 (19.2)	-	
Bone	3 (11.6)	1 (7.2)	
Lymph nodes	-	3 (21.4)	
Pericardium	12 (46)	1 (7.2)	
Multiple		5 (35.7)	
Number of metastatic sites, median cut-off = 2, n (%)	21 (80.8)	13 (92.8)	0.57
$\leq$ 2	5 (19.2)	1 (7.2)	
$>$ 2			
Intrathoracic and extrathoracic disease, n (%)	9 (34.6) 17 (65.4)	10 (71.4)	0.05
Intrathoracic		4 (28.6)	
Intrathoracic + extrathoracic			
Disease burden, median cut-off = 15 mm, n (%)	6 (23.1)	8 (57.1)	0.06
$\leq$ 15 mm	18 (69.3)	5 (35.7)	
$>$ 15 mm	2 (7.6)	1 (7.2)	
Unknown			

emerged as a revolutionary tool in the landscape of cancer diagnostics and monitoring, providing real-time information on both spatial and temporal tumor heterogeneity, tracking genetic alterations in a non-invasive, sensitive manner and with fast turn-around-time [9,10]. Moreover, liquid biopsy overcomes tissue sampling limitations, including invasiveness and feasibility, shifting its use in earlier settings for tumor molecular assessment [10,11]. In particular, circulating tumor DNA (ctDNA), described as a fraction of cell-free DNA (cfDNA) circulating in plasma and released by tumor cells, gained importance in NSCLC as a tool for molecular assessment at diagnosis and at progression of the disease [12,13]. Elevated levels of ctDNA have been found in cancer patients, with an increase in ctDNA shedding correlated with advanced stages, number of metastatic sites, and high tumor burden, providing valuable insights into tumor's heterogeneity, and adding a new layer of prognostic information [14,15]. Moreover, patients who lose the mEGFR at the first reassessment, known as "ctDNA clearance", have been associated with better survival outcomes compared to those who retain the mutation [16]. Nevertheless, ctDNA presents limitations and challenges especially due to its low abundance, depending on biological and anatomic characteristics of the tumor, which is reflected by the high variability in shedding [15,17]. In fact, very low ctDNA shedding, can lead to false negative results [17–19]. Despite the introduction of highly sensitive techniques for ctDNA analysis, including Next-Generation Sequencing (NGS) and digital Polymerase Chain Reaction (dPCR), the interpretation of ctDNA results remains an important challenge both in research and in the clinical setting [20,21].

In this regard, in order to select patients for appropriate therapy and to monitor them during treatment, a better understanding, in case of a negative result, if this is a true negative or a non-shedder tumor is a strong unmet need.

Therefore, the present study aims to explore the association between the clinical characteristics of mEGFR NSCLC patients and their shedding status by means of ctDNA analysis, with the objective of providing insights into the likelihood of an individual being a shedder or not, avoiding the trouble of false negatives.

Patients and methods

Patients and data collection

Blood samples obtained from patients affected by stage IV NSCLC with previously confirmed EGFR mutation in tissue were collected before first line therapy (at baseline) and during scheduled follow-ups as a part of clinical practice, until disease progression disease (PD). All patients received EGFR-TKIs including gefitinib, afatinib or osimertinib as first-line treatment as per approved labels.

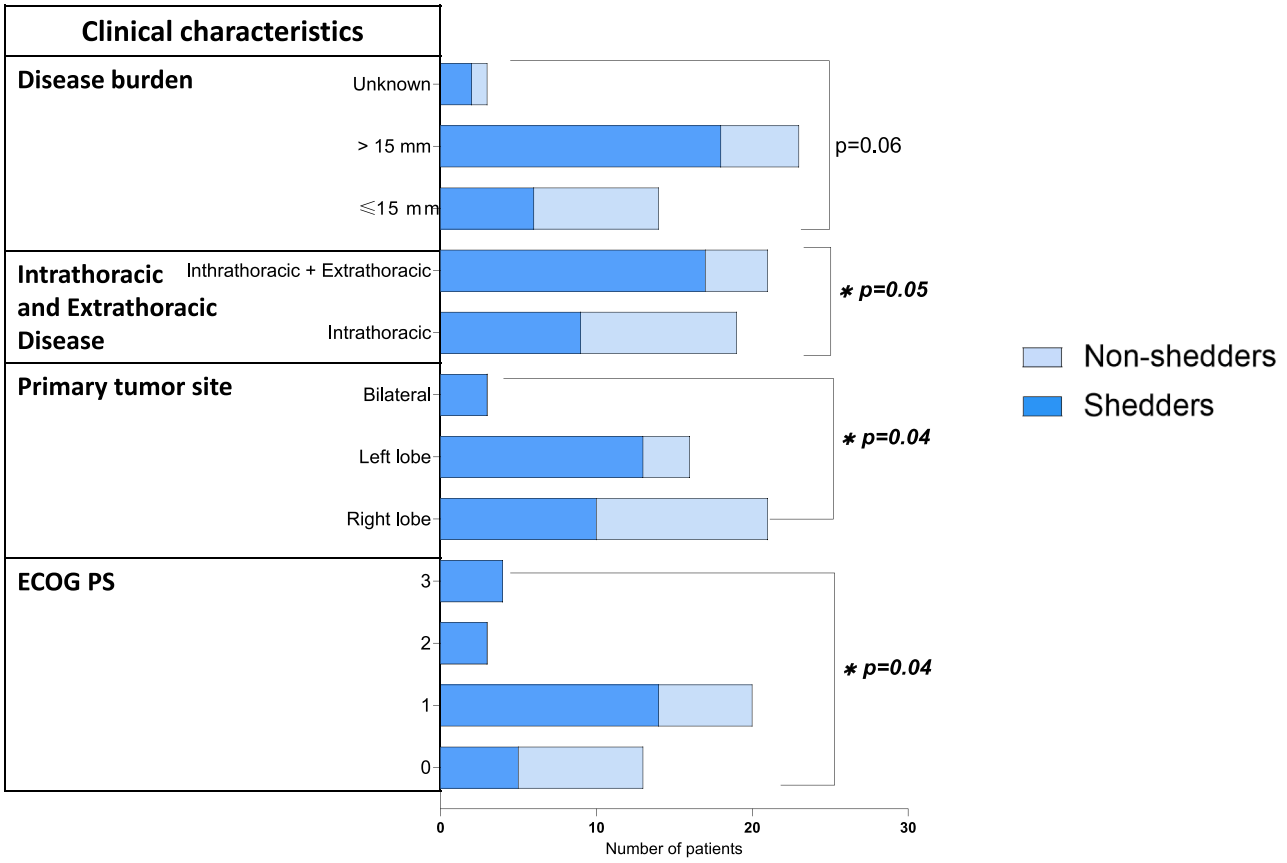

Fig. 1. Associations between shedding status and clinical characteristics of patients.

Patients were classified as "shedders", if at least one activating mEGFR was detectable in ctDNA at baseline, and as "non-shedders", if no EGFR activating mutations were detected in ctDNA at baseline. Patients were also classified into ctDNA positive patients at baseline that turned into negative at first re-evaluation (clearance), and patients which retained the mEGFR (non-clearance).

Circulating-free DNA extraction and analysis

Twelve ml of blood were collected in EDTA tubes and centrifuged for 10 min at 1900 × *g* within 2 h to isolate plasma, and consequently stored at −80 °C until molecular analysis. Plasma samples were centrifuged again for 15 min at 1900x*g* to remove cellular debris. cfDNA was isolated from 3 ml of plasma using the QIAamp Circulating Nucleic Acid Kit (Qiagen, Valencia, CA). The EGFR mutational analyses at baseline and at the first reassessment were performed on the QIAcuity One Digital PCR System (dPCR) (Qiagen, Hilden, Germany). At the progression of the disease, comprehensive mutational profiling of patients, beyond EGFR mutations, was performed in all cases with Next-Generation Sequencing (NGS) by using the AVENIO ctDNA Expanded kit (Roche Diagnostics, Basel, Switzerland) on the Illumina NextSeq 550 platform (San Diego, CA).

The concentration of mutant alleles was reported as Allelic Fraction (AF%). All the procedures for the molecular analysis were conducted following the specific manufacturer's instructions.

Statistical analysis

Absolute and relative frequency distributions, as well as median and interquartile ranges, were used to describe the qualitative and quantitative characteristics of patients, respectively. Primary tumor volume was evaluated according to RECIST criteria version 1.1 [22,23]. Chi-square test was used to assess potential associations between shedding status and clinical characteristics. Correlations were calculated using Spearman's coefficient of rank correlation. Progression-Free Survival (PFS) was defined as the duration of time between initiation of treatments and the progression of the disease. The Kaplan-Meier method was employed to estimate the PFS of patients, and differences between the curves were calculated using the Log-rank test. A cut-off of 0.38 was found using the median of the AF% of EGFR. Univariate and multivariate Cox hazard regression model were performed to evaluate the effect of shedding status and independent risk factors (i.e., clinical characteristics of the cohort) for PFS. Logistic regression model using Cox-Snell's (R^2) was computed to estimate predictive diagnostic performance of shedding status in ctDNA, combined with the other clinical features analyzed. Receiver operating characteristic (ROC) curve analysis was computed to estimate diagnostic performance in terms of sensitivity and specificity of clinical characteristics with shedding status. Comparison of ROC curves was also performed as described by DeLong et al. [24] to test the statistical significance of the difference between the areas under ROC curves (AUC). Differences were considered significant at $p < 0.05$. Statistical analyses were performed using MedCalc Statistical Software version 14.8.1 (MedCalc Software bvba, Ostend, Belgium; http://www.medcalc.org; 2014) and GraphPad Prism 9.0.0 (GraphPad Software, San Diego, California USA, www.graphpad.com).

Results

EGFR-cfDNA analysis at baseline

A total of 40 blood samples from patients affected by grade IV mEGFR NSCLC on tissue and candidate to treatment with EGFR-TKIs as first-line were collected and analyzed before the administration of therapy, by means of dPCR. The median time between tissue collection

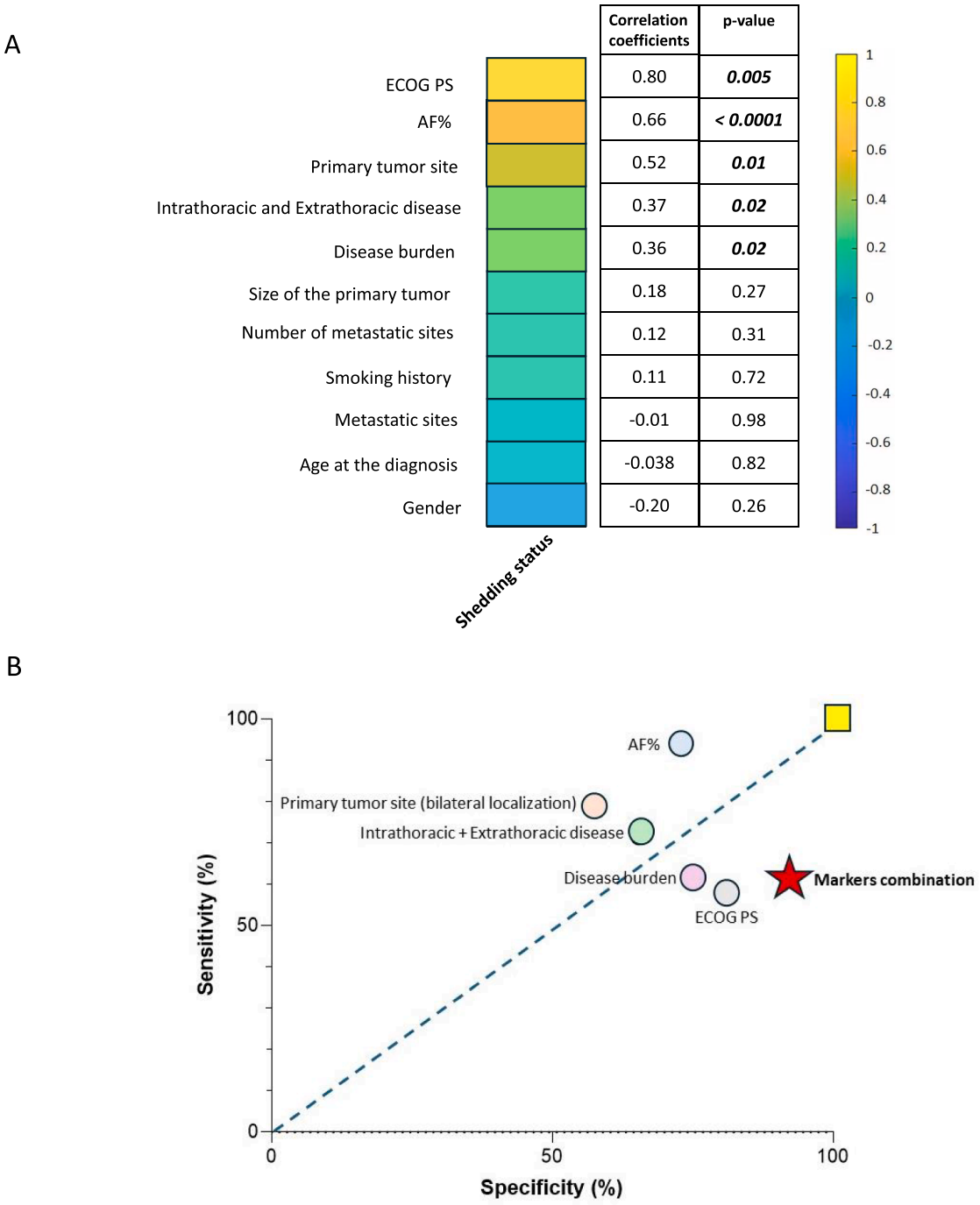

Fig. 2. Application of logistic regression estimating the predictive capability of clinical characteristics for shedding status in EGFR-mutated lung cancer patients at baseline (A), demonstration of specificity and sensitivity of shedding status combined with statistically significant clinical characteristics identified from the regression model (B) and median PFS of patients based on the clearance status. Univariate Cox regression analysis of the impact of shedding status and patients' clinical characteristics on the PFS (C).

for diagnosis and liquid biopsy samples was 34 days (CI 5–56); no treatment was started between tissue and liquid biopsy assessment. Clinical characteristics of patients were summarized in Table 1.

At baseline, 26 out of 40 samples (65 %) were shedders for an EGFR mutation in ctDNA, and 14 (35 %) resulted as non-shedders (Table 2). A statistically significant association was found between shedding status and ECOG Performance Status (PS), localization of the primary tumor site, and the presence of intrathoracic and/or extrathoracic disease (Fig. 1). In particular, worse ECOG PS levels (2 and 3) ($p = 0.04$), bilateral localization of the primary tumor ($p = 0.04$), and the presence of both intrathoracic and extrathoracic disease ($p = 0.05$), were associated with ctDNA shedding of mEGFR.

A trend of association ($p = 0.06$) was also identified between shedder patients and larger tumor burden (> 15 mm, median cut-off). Furthermore, larger disease burden as well as the presence of both intrathoracic and extrathoracic disease were correlated with higher AF% of mEGFR ($p = 0.04$ and $p = 0.03$, respectively) (Fig. 2A). Logistic regression demonstrated a direct correlation between shedding status and ECOG PS, the primary tumor site involving a bilateral localization, the presence of both intrathoracic and extrathoracic disease, higher tumor

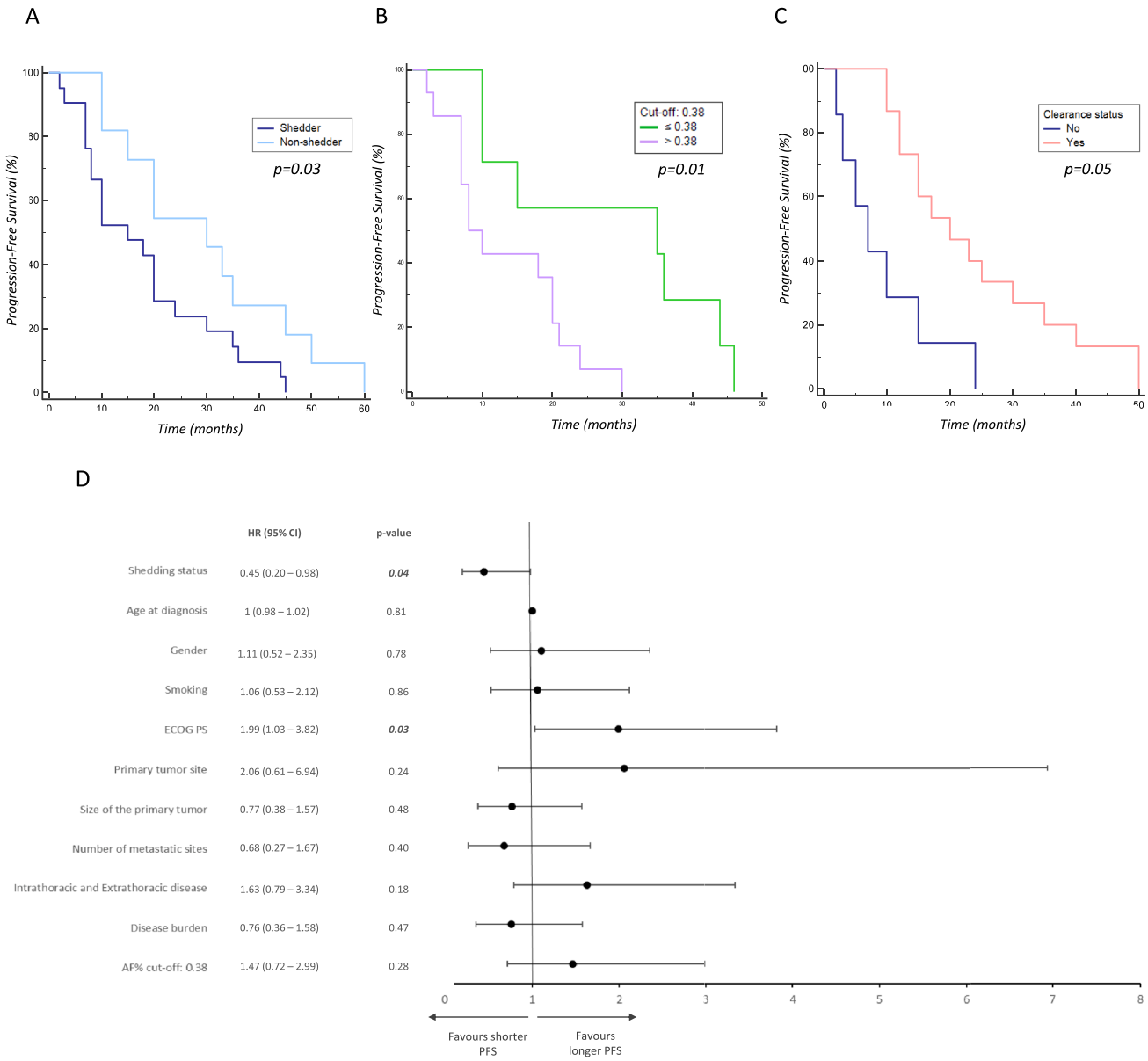

Fig. 3. Median PFS of shedders compared to non-shedders (A), and shedders with AF% ≥0.38 (median cut-off), compared to shedders with AF% <0.38 (B).

burden, and higher ctDNA AF% (Fig. 2A). No other clinical characteristics were correlated with the shedding status (i.e. age at diagnosis, gender, smoking history, size of the primary tumor, localization, and number of metastatic sites). The combination of the identified predictive biomarkers, incorporating all the features together, resulted in a more specific and sensitive predictive signature for ctDNA shedding (specificity 91.7 %, sensitivity 61.6 %, and AUC 0.89) (Fig. 2B).

Shedder patients revealed a statistically significant shorter median PFS compared to non-shedders patients (15 vs. 30 months, respectively; $p = 0.03$) (Fig. 3A). Furthermore, among shedders, patients with higher mEGFR AF% in ctDNA (> 0.38, median cut-off) were associated with worse PFS compared to patients with lower AF% (≤ 0.38, median cut-off) (8 vs. 35 months, respectively; $p = 0.01$) (Fig. 3B). The PFS univariate model revealed a statistically significant association between shedding status (HR=0.45; 95 % CI=0.20–0.98; $p = 0.04$) and ECOG PS (HR=1.99; 95 % CI=1.03–3.82; $p = 0.03$) (Fig. 3D). In multivariate analysis, statistically significant associations were not confirmed ($p = 0.57$). Overall, this study shows that 65 % of patients are mEGFR shedders in ctDNA at baseline, and that higher ECOG PS, bilateral localization of primary tumor, and the presence of intrathoracic/extrathoracic disease are associated to the occurrence of mEGFR shedding.

EGFR-ctDNA analysis at the first reassessment and at the progression of the disease

The ctDNA of 35 patients was analyzed at the first assessment (Supplementary Table 1). mEGFR clearance in plasma occurred in 16 patients (45.7 %), whereas in 7 patients (20 %) the mEGFR persisted. Chi-square test revealed no statistically significant associations between clearance status and related clinical characteristics (Supplementary Table 2). Nevertheless, patients who experienced mEGFR clearance showed longer PFS compared to patients with persistently detectable mutation in ctDNA at the first reassessment (17 vs. 7 months, respectively; $p = 0.05$) (Fig. 3C).

Thirty-three patients were monitored until the progression of the disease. The comprehensive mutational profile of these patients was analyzed by NGS (Table 3). Among EGFR-shedder patients at baseline, at least one EGFR mutation was detected in the ctDNA of 12 patients, in 4 patients the mEGFR co-occurred with other mutations (EGFR ex19del,

Table 3
Mutations detected in the ctDNA of patients ($n = 33$) at the time of the progression of the disease, and their respective AF%, analyzed through digital PCR or NGS.

Patients	Gene	Mutation	AF%
1	CDK6	p.A326G	0.16
2	EGFR	p.L858R	0.45
3	TP53	p. I251M	0.27
	MET	p.M1131F	0.27
4		Absence	0
5	EGFR	ex19del	0.27
6		Absence	0
7	EGFR	p.T790M	0.8
9	EGFR	p.R958H	0.16
	TP53	p.N235D	0.15
11	EGFR	ex19del	0.22
12	EGFR	p.L858R	2.57
	EGFR	p.L747P	2.71
	EGFR	p.T790M	1.61
	EGFR	p.C797S	1.53
13	EGFR	ex19del	0.4
16	EGFR	ex19del	3.37
17	EGFR	ex19del	0.08
	EGFR	p.C797S	0.08
18	EGFR	ex19del	19.2
	TP53	p.D100V	8.65
20	EGFR	p.L858R	0.48
21	EGFR	p.T790M	0.06
23	EGFR	p.L858R	0.5
	EGFR	p.T790M	20.2
24	EGFR	ex19del	17.2
25	BRAF	p.L485F	0.14
26	EGFR	p.L858R	4.6
	EGFR	p.C797S	0.15
28	EGFR	ex19del	2.2
	EGFR	p.T790M	0.94
29		Absence	0
30	EGFR	p.L858R	3.9
	EGFR	p.T790M	0.3
	EGFR	p.C797S	2.1
31	EGFR	ex19del	33.8
	EGFR	p.C797S	19.93
	EGFR	p.D384V	0.07
	TP53	p.V113L	14.46
	MET	CNV	14.2
	EGFR	CNV	14.4
32	EGFR	ex19del	0.28
33	EGFR	ex19del	2.5
	EGFR	p.C797S	0.74
34		Absence	0
35	EGFR	ex19del	20
	EGFR	p.C797S	1.3
36		Absence	0
37		Absence	0
38	EGFR	ex19del	2.13
	TP53	p.H193Y	1.26
	KRAS	p.Q61H	0.11
39	EGFR	ex19del	11.5
	EGFR	p.C797S	5.29
	TP53	p.D149Y	2.31
40	EGFR	p.L858R	42.3
	MET	CNV	18.22
	EGFR	CNV	18.4

TP53 p.H193Y and KRAS p.Q61H; EGFR ex19del and TP53 p.D149Y; p. L858R, MET CNV and EGFR CNV), mutations other than EGFR were found in 2 patients (TP53 p. I251M and MET p.M1131F; BRAF p.L485F), and 5 patients became ctDNA negative. Additionally, among non-shedder patients at baseline, at least one mEGFR at disease progression was detected in the ctDNA of 6 patients, in 3 patients the mEGFR co-occurred with other mutations (EGFR ex19del and TP53 p.D100V; EGFR ex19del, EGFR p.C797S, EGFR p.D384 V, TP53 p.V113 L, MET CNV and EGFR CNV; EGFR p.R958H and TP53 p.N235H), mutations other than EGFR were found in 1 patient (CDK6 p. A326G), and 1 patient remained ctDNA negative.

Overall, clinically and functionally relevant alterations were detected, including EGFR mutations ex19del (31.1 %), p. C797S (17.8 %), p. T790M (13.3 %), p.L858R (15.6 %), p.L747P (2.2 %), followed by TP53 p.V113L (2.2 %), p.H193Y (2.2 %), p.D149Y (2.2 %), BRAF p.L485F (2.2 %), KRAS p.Q61H (2.2 %), MET CNV (4.5 %) and EGFR CNV (4.5 %) (Fig. 4A). In addition, 5 patients were considered as ctDNA-positive for mutations of uncertain significance, including EGFR p.D384V (1.7 %), EGFR p.R958H (1.7 %), CDK6 p.A326G (1.7 %), MET p.M1131F (1.7 %), TP53 p.D100V (1.7 %), TP53 p.N235D (1.7 %), and TP53 p.I251M (1.7 %) (Fig. 4B).

Discussion and conclusions

Liquid biopsy entered the clinical practice of solid tumors, including NSCLC, being used at disease diagnosis and at the PD to identify predictive biomarkers of response and resistance to treatment helping clinicians in the choice of therapies, particularly their sequence following first line. Therefore, a proper understanding and interpretation of the liquid biopsy results is of extreme importance, especially to report false negative results considering the biological and clinical factors that affect ctDNA shedding. In this context, our study was aimed at identifying the clinical features of patients correlated with shedding status, in order to predict whether a tumor may be a ctDNA shedder or a non-shedder, and avoid the trouble of false negative results [25]. Clinical features of NSCLC patients associated with ctDNA release have been correlated in previously published data including tumor burden, mitotic or metabolic activity, histological type, and tumor grade [17]. The identification of clinical factors associated to a non-shedder profile, would be extremely useful to guide clinicians in understanding the liquid biopsy result and choosing the most appropriate therapy.

In our study, 40 patients were enrolled, plasma samples were collected at baseline and ctDNA was analyzed. Twenty-six out of 40 patients (65 %) were found to be shedders for mEGFR, harbouring specific clinical characteristics and having a negative correlation with PFS, including higher ECOG PS, bilateral localization of primary tumor, the presence of intrathoracic/extrathoracic disease and a worse PFS.

EGFR shedding was correlated with worse clinical status (ECOG PS level 2 and 3; $p = 0.04$). Accordingly, several studies have previously identified an association between the presence of mutations and/or a higher number of mutations in ctDNA, a worse ECOG PS level, and shorter outcomes in mEGFR lung adenocarcinoma patients [26–28]. Also, the presence of both extra- and intrathoracic disease has demonstrated to be a clinical predictor for EGFR-ctDNA shedding ($p = 0.05$), according to recent literature [29–31]. A study conducted by Jee and colleagues demonstrated that 74 % of NSCLC patients with extrapulmonary disease had detectable ctDNA, whereas only 40 % with intrapulmonary disease had detectable ctDNA ($p < 0.001$) [30]. Furthermore, Passiglia and colleagues emphasized the reliability, high sensitivity (approximately 80 %), and accuracy of ctDNA tests in mEGFR NSCLC patients with extrathoracic disease compared to those with intrathoracic disease only, irrespective of different technologies [29].

Larger volume and diameter of the primitive tumor associated with higher shedding of mEGFR NSCLC compared to non-shedding tumors have also been demonstrated [17,32]. In accordance, our study revealed that the 3 shedder patients presenting a bilateral localization of the tumor ($p = 0.04$), also presented a larger volume of the primary disease (> 33 mm). Additionally, the majority of shedders exhibited a larger primary tumor size (> 33 mm, $n = 16$ vs. ≤ 33 mm, $n = 10$) compared to non-shedders (> 33 mm, $n = 6$ vs. ≤ 33 mm, $n = 8$). However, this difference did not reach statistical significance, probably due to the limited number of patients in this study. Moreover, a trend of association between higher disease burden and shedding status was also found in our cohort ($p = 0.06$), and higher AF% was correlated with larger tumor burden ($p = 0.04$). Indeed, while a high AF% indicates a significant tumor burden, it has been observed that a high tumor burden is not necessarily predictive of shedding in ctDNA, since ctDNA is also affected

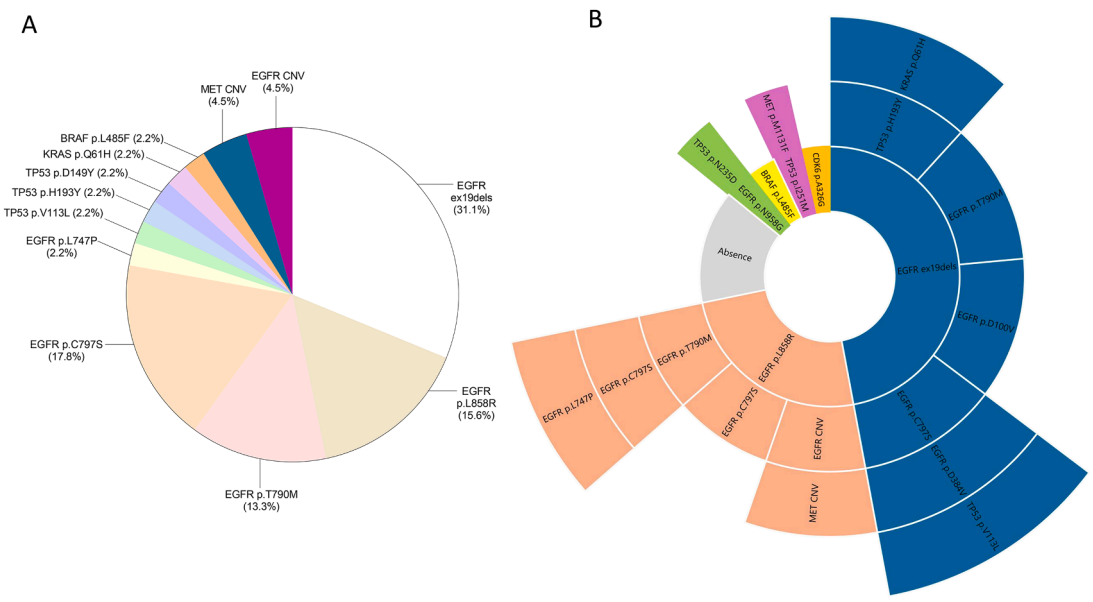

Fig. 4. Clinically and functionally relevant mechanisms of resistance identified at the progression of the disease (A), and the overall mutations (%) detectable in ctDNA at the clinical progression of the disease (B).

by the disease localization (i.e. visceral vs bone) [33,34]. Accordingly, several studies found an association between mEGFR AF% in the ctDNA of advanced NSCLC patients and larger tumor burden [34,35]. No other association between EGFR shedding status at baseline and clinical characteristics including age, gender, and the type and number of metastatic sites were found in recent literature [16,36,37]. It is crucial to emphasize that the localization of metastatic sites plays a significant role in influencing the diagnostic accuracy of ctDNA analysis for detecting mEGFR in NSCLC patients, even in presence of high tumor burden [29]. Indeed, in our cohort, the majority of shedding patients presenting with both extrathoracic and intrathoracic disease exhibited widespread involvement, with visceral metastases (such as the liver, spleen, and adrenal glands), which are sites associated with increased shedding [29,37,38]. On the contrary, non-shedder patients with exclusively intrathoracic disease exhibited metastases to lymph nodes and bone, known as sites of lower ctDNA shedding [29,30]. Concerning survival, in line with our previous study conducted in an independent cohort of patients (treated with osimertinib as second line), which found a longer median PFS of 19.5 months for non-shedder patients compared to 3.7 months for EGFR-shedder patients [36], the present data also show that non shedders have a PFS of 30 vs 15 months. A statistically significant difference in the median PFS of shedders and non-shedders (5 vs. 17 months; $p = 0.05$) was also found in a study conducted by Verzè and colleagues [16]. Additionally, in our cohort, shedder patients with lower AF% of EGFR mutation (AF% $\leq$ 0.38, cut-off) showed better PFS compared with shedders with higher amount of mEGFR ($p = 0.01$). In accordance, patients who exhibited higher amount of mEGFR in ctDNA (AF% $\geq$ 6.1, cut-off generated by ROC curve analysis), showed better PFS (17.8 vs. 4.3 months, respectively; $p = 0.02$) and OS (23.6 vs. 7.7 months, respectively; $p = 0.016$) compared to patients with lower amount of EGFR mutations [36]. A study stratifying patients into complete shedders, defined as those shedding both activating and p.T790M mutations, and shedders for the activating mutation only, revealed a statistically significant difference in terms of PFS for shedders for the activating mutation only at baseline (5.5 vs. 1.6 months, respectively; $p = 0.01$) [16]. However, shedding status and ECOG PS were also identified as predictive factors of PFS in the univariate model, but not in the multivariate, as reported in recent studies [39,40]. Moreover, a difference towards better outcomes in patients who underwent clearance of the EGFR plasmatic mutations ($p = 0.05$) was observed, as confirmed previously [16,36,37]. However, no clinical characteristic was correlated with the clearance status, probably due to the small sample size of the patients considered for this subgroup analysis. For the sake of comprehensiveness, we also interrogated TCGA but meaningful correlations were found.

As an exploratory aim, we investigated the resistance mechanisms to EGFR-TKIs. Of the overall population, 33 patients experienced the progression of the disease, and we found that almost all of them (81.8 %) were positive for at least one mutation on ctDNA, whereas only 6 patients were negative. Indeed, most of the shedders at baseline retained at least one mutation in ctDNA at disease progression ($n = 18$). Furthermore, 9 non-shedder patients at baseline acquired at least one additional mutation at the progression. Among the mechanisms of resistance there was a mutation in EGFR or involving different pathways [41]. The concomitant presence of ex19del and p.C797S was found in 14 patients, as reported in a recent study [42]. At lower frequency, other clinically relevant variants such as BRAF p.L485F, KRAS p.Q61H, MET CNV, and EGFR CNV were detected [41]. Other mutations of uncertain significance have also been found. Among these, EGFR p.R958H and p.D384V mutations have been linked to germline EGFR-related lung cancer [43]. However, no documented occurrence of these variants have been reported to date. Low levels of the CDK6 p.A326G [44,45] have been associated with a better prognosis in lung cancer, whereas MET p.M1131T [46,47] is known to increase kinase activity and leads to phenotypic transformation in papillary renal cell carcinoma. Furthermore, the TP53 mutations p.D100V, p.N235D, and p.I251M have been interpreted as disease-causing mutations in glioma, breast cancer, and childhood adrenocortical carcinoma [48–52].

Despite the present study identified correlations between clinical characteristics and shedding status in accordance with previously published literature, it presents some limitations. In addition to the small sample size analyzed, one limitation of this study is that the shedding was assessed only in patients with mEGFR tumor. This limitation arises from the evaluation of the EGFR driver mutation in the ctDNA by dPCR. As a consequence, this does not rule out the potential presence of other mutations released in the ctDNA of these patients. Therefore, some of the patients we called “non-shedders” may have been “shedders” for mutations other than EGFR. Indeed, NGS assessment at baseline would have offered a more comprehensive understanding of the mutational profile of these patients. However, for a matter of cost-savings, it is not

always feasible to implement NGS technology for all the patients in clinical practice, while a dPCR approach may help when a few biomarkers – such as EGFR – are needed to be tested with a short turnaround time, at a lower cost.

Nevertheless, analyzing this subset of shedder patients for the EGFR driver mutation in ctDNA may provide clinically relevant information on the therapy to be administered, for which the EGFR mutation represents the target.

The introduction of the tumor fraction (TF) parameter in the reports, which refers to the proportion or amount of tumor-derived genetic material present in a ctDNA sample, addresses the challenge of false negative results [53,54]. In fact, a higher TF identifies samples with sufficient ctDNA amount and suggests a comprehensive tumor profiling, increasing the percentage of concordance with tissue testing. This approach can potentially reduce the need for confirmatory tissue testing in patients with negative liquid biopsy results, ensuring confidence in promptly initiating therapy [53,54]. In conclusion, these data offer valuable insights for clinical applicability, reinforcing the significant role of liquid biopsy in testing EGFR mutational status, suggesting clinicians and laboratory personnel to carefully consider the ECOG PS, bilateral localization of primary tumor, and the presence of intrathoracic/extrathoracic disease, for the ctDNA test interpretation in the case of a negative result.

CRediT authorship contribution statement

Martina Ruglioni: Writing – original draft, Formal analysis, Data curation. **Iacopo Petrini:** Writing – original draft, Visualization, Data curation. **Stefania Crucitta:** Visualization. **Andrea Sbrana:** Visualization, Resources. **Giovanna Irene Luculli:** Visualization. **Leila Sadeghi Gol:** Visualization. **Carola Forte:** Visualization. **Antonio Chella:** Writing – review & editing, Visualization. **Christian Rolfo:** Writing – review & editing, Visualization, Validation, Supervision. **Romano Danesi:** Writing – review & editing, Validation, Supervision, Project administration, Funding acquisition, Conceptualization. **Marzia Del Re:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Investigation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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Informed consent statement

This study was conducted in accordance with the Declaration of Helsinki and approved by the local Ethics Committee of Area Vasta Nord-Ovest, Tuscany Region, Italy (reference code 2014–363).

Data availability and statement

The data presented in this study are available in this article.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.tranon.2024.102228.

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