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ORIGINAL ARTICLE

Changes in the concentration of EGFR-mutated plasma DNA in the first hours of targeted therapy allow the prediction of tumor response in patients with EGFR-driven lung cancer

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Abstract

Purpose This study aimed to analyze changes in the plasma concentration of EGFR-mutated circulating tumor DNA (ctDNA) occurring immediately after the start of therapy with EGFR tyrosine kinase inhibitors (TKIs).

Methods Serial plasma samples were collected from 30 patients with EGFR-driven non-small cell lung cancer before intake of the first tablet and at 0.5, 1, 2, 3, 6, 12, 24, 36 and 48 h after the start of the therapy. The content of EGFR alleles (exon 19 deletions or L858R) in ctDNA was measured by ddPCR.

Results ctDNA was detected at base-line in 25/30 (83%) subjects. Twelve (50%) out of 24 informative patients showed > 25% reduction of the ctDNA content at 48 h time point; all these patients demonstrated disease control after 4 and 8–12 weeks of therapy. The remaining 12 individuals showed either stable content of EGFR-mutated ctDNA ($n = 5$) or the elevation of ctDNA concentration ($n = 7$). 10 of 12 patients with elevated or stable ctDNA level achieved an objective response at 4 weeks, but only 5 of 10 evaluable patients still demonstrated disease control at 8–12 weeks ($p = 0.032$, when compared to the group with ctDNA decrease). The decline of the amount of circulating EGFR mutant copies at 48 h also correlated with longer progression-free survival (14.7 months vs. 8.5 months, $p = 0.013$).

Conclusion Comparison of concentration of EGFR-mutated ctDNA at base-line and at 48 h after the start of therapy is predictive for the duration of TKI efficacy.

Keywords NSCLC · EGFR · Circulating tumor DNA · TKI therapy · Tumor response

Abbreviations

CR	Complete response;
CT	Computed tomography
ctDNA	Circulating tumor DNA
DCR	Disease control rate
ddPCR	Droplet digital PCR
MRI	Magnetic resonance imaging
NSCLC	Non-small cell lung cancer
PD	Progressive disease
PR	Partial response
SD	Stable disease
PFS	Progression free survival
TKI	Tyrosine kinase inhibitors

Introduction

Peripheral blood obtained from oncological patients often contains tumor fragments, such as circulating malignant cells, tissue-specific proteins and cancer-derived nucleic acids. The analysis of circulating tumor DNA (ctDNA) is particularly promising, given that somatic mutations are highly specific for malignant cells and that current methods are capable of detecting even single mutated molecules present within a huge excess of normal tissues [1–3]. There are intensive efforts to utilize so-called "liquid biopsy" for early diagnosis and monitoring of cancer disease [4, 5]. It

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is more or less established, that the concentration of ctDNA is generally proportional to the overall tumor mass [6–9]. Consequently, ctDNA is relatively easily detectable in patients with extensive tumor disease, and its content usually declines after successful treatment [10–13].

The reduction of tumor size, which is achieved with the use of cancer drugs, is attributed to several biological effects. Conventional chemotherapy and targeted agents have a cytostatic action, i.e. they prevent proliferation of cancer cells [14, 15]. In addition, both cytotoxic and targeted drugs may provoke tumor shrinkage by inducing programmed cell death [16–18]. The reduction of overall tumor volume, which is usually achieved after weeks of systemic therapy, is almost always accompanied by the decrease of the concentration of circulating markers, be it tumor-specific proteins or ctDNA [19, 20]. However, the immediate marker response to the cancer therapy is less studied, which is at least in part attributed to the difficulties in collecting multiple serial blood samples within short time intervals.

EGFR tyrosine kinase inhibitors (TKIs; gefitinib, erlotinib, afatinib, osimertinib, etc.) are highly efficient in non-small cell lung carcinomas (NSCLCs), which harbor activating mutation in exon 19 or 21 of the EGFR gene [21–25]. Administration of EGFR TKIs for the treatment of EGFR-mutated NSCLC is almost always accompanied by the objective tumor response or the disease stabilization. EGFR inhibitors are also characterized by the "Lazarus effect", i.e. dramatic symptomatic relief occurring within first hours after the drug administration [26, 27]. It is well established that the reduction of tumor size, which is observed during regular patient check-ups, is paralleled by the decline of the amount of EGFR-mutated ctDNA. However, short-term effects of EGFR TKIs on the level of ctDNA have not been systematically analyzed. We aimed to investigate, how the administration of EGFR TKIs influences the concentration of plasma ctDNA within the first hours after the uptake of the drug, and whether these changes are predictive for the long-term effects of systemic therapy.

Materials and methods

Patients

The study considered consecutive treatment-naïve patients with EGFR-mutated locally advanced or metastatic NSCLC, who were referred to the St.-Petersburg City Cancer Center between August 2018 and March 2020. EGFR mutations testing in tumor tissue was performed as described in [28]. Briefly, EGFR deletions in exon 19 (19del) were analyzed using the primers 5′-CTGTCATAGGGACTCTGGAT-3′ and 5′-CAGCAAAGCAGAAACTCACAT-3′; PCR products were electrophoretically separated in 10% polyacrylamide gel; a 127 bp fragment corresponded to the wild-type sequence, and an additional band of smaller size was observed in the case of deletion. Testing for EGFR L858R mutation in exon 21 was performed by allele-specific real-time PCR with the wild-type-specific primer 5′-CACCCAGCAGTTTGGCCA-3', mutation-specific primer 5′-CACCCAGCAGTTTGGCCC-3′, and common primer 5'-GCATGAACTACTTGGAGGAC-3.

All patients provided informed consent for the participation in the study. The study was approved by the local Ethics Committee (protocol #20 "Evaluation the clinical value of ctDNA testing in patients with EGFR-mutated NSCLC"; approval date November 23, 2017).

Thirty patients were recruited to the investigation; their characteristics are described in Table 1 [see also Tables 1S and 2S in the electronic supplementary material (ESM)] for the description of individual patient data and response to TKI treatment). Serial plasma samples were collected before intake of the first tablet (at base-line) and at 0.5, 1, 2, 3, 6, 12, 24, 36 and 48 h after the "starting point" (Fig. 1). In

Table 1 Clinical characteristics of NSCLC patients included in the study

	n	%
Gender		
F	26	87
M	4	13
Mean age (min–max)	68.4 (52–81)	
EGFR mutation		
ex19del	20	67
L858R	10	33
Lung metastases	16	53
Liver metastases	3	10
Pleural involvement	6	20
Adrenal metastases	2	7
Bone metastases	10	33
Lymph node metastases	6	20
Brain metastases at base-line	7	25
Mean number of metastatic sites (min–max)	1.7 (1–4)	
ECOG		
0	1	3
1	25	83
2	2	7
3	2	7
4	0	0
Mean sum of lesions *V* (mm^3)	60841 (23–490590)	
Mean *V* of max lesion (mm^3)	54833 (14–490509)	
Drug		
Gefitinib	20	67
Erlotinib	5	17
Afatinib	3	10
Osimertinib	2	7

addition, all patients were invited to donate blood after 14 and 28 days of the treatment. EGFR TKIs were given at regular daily doses (gefitinib: 250 mg; erlotinib: 150 mg; afatinib: 40 mg; osimertinib: 80 mg). Early response evaluations were performed with spiral computed tomography (CT) on the week 4, and the routine check-ups occurred within weeks 8–12 after the beginning of the therapy. Chest scans were performed before and after the administration of the contrast agent (100 mL of non-ionic iodinated contrast with a 100 mL saline chaser at 4.5–5 ml/s). All nodules with the size of more than 10 mm were measured. The images were based on venous-phase scans. Image reconstructions were performed on a CT workstation (Vitrea). Tumor burden was evaluated using the slice-by-slice pathology volume measurement with the slice thickness of 1.0 mm and assistance of the RadiAnt DICOM Viewer V.4.5.9.18463 software. Tumor responses RECIST v.1.1 criteria and progression-free survival (PFS) were evaluated according to standard guidelines by the study investigators [29].

ctDNA analysis

Blood samples (10 mL) were collected in cf-DNA/cf-RNA Preservative Tubes (Norgen) and the plasma was separated from the rest of the specimen by the two-step centrifugation protocol (400 g for 10 min at room temperature followed by 14,400 g for 10 min at 4 °C). Cell-free DNA was extracted with the QIAamp Circulating Nucleic Acid kit from 3–5 ml of plasma according to the manufacturer's instructions and dissolved in 50 µl of water.

The fractions of *EGFR* mutant alleles (exon 19 deletions or L858R substitutions) were measured by droplet digital PCR (ddPCR) using the QX100 Bio-Rad System [30]. ddPCR reactions were performed in triplicate and contained 2X ddPCR Supermix for Probes (no UTP, Bio-Rad), mutation-specific oligonucleotides (see Table 3S in the ESM) and 2–3 µl of the template DNA in a total reaction volume of 22–23 µl. Data analyses were performed with the QuantaSoft Software version 1.7.4 as recommended by the manufacturer. All ddPCR reactions, which yielded 10 or

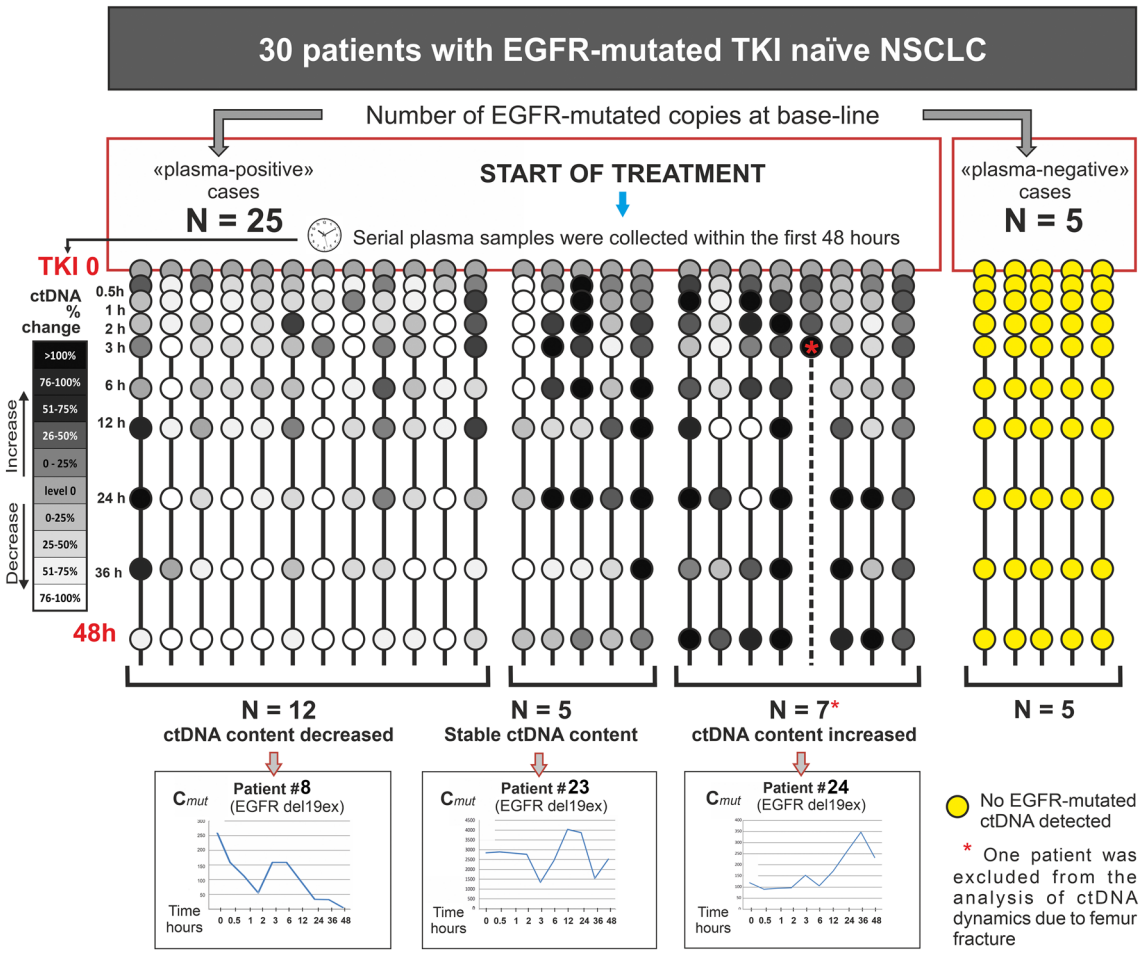

Fig. 1 Work-flow of the study

more droplets with the target DNA molecule, were considered informative.

The absolute number of tumor-derived "mutated" DNA copies in 1 mL of plasma (C_{mut}) was calculated according to the formula:

$$N\ mut\ \text{copies}/1\text{mL plasma} = \frac{\text{Concentration}\left(\frac{\text{copies}}{\mu L} cf\text{DNA}\right) \times V_{\text{template}} \times V_{\text{dilution}}}{V_{\text{plasma}}}$$

where: Concentration—number of «mutated» droplets per 1 µL of ddPCR reaction. V_{template}—volume of *ct*DNA aliquot taken into ddPCR, µL. V_{dilution}—total volume of diluted ctDNA sample collected from the plasma, µL. V_{plasma}—volume of processed plasma, mL.

Statistics

Quantitative data were present as a median values/range or means ± 95% confidence interval ($1.960\sigma\bar{x}$). Non-parametric Wilcoxon Signed Rank Test and Mann–Whitney *U* Test were utilized to compare the medians. *p* value of < 0.05 was considered statistically significant. All calculations were performed using IBM SPSS v.23 software package.

Results

Clinical responses to EGFR TKI therapy

All 30 included patients attended CT examination after 4 weeks of TKI treatment. 25 subjects demonstrated partial tumor response, 3 had stable disease and 2 progressed during the treatment (See Table 2 in the ESM). Twenty-nine patients continued TKI therapy (28 cases with the disease control (objective tumor response or stable disease) and 1 case beyond progression) after the first check-up. Twenty-five patients managed to attend the second CT examination, which was performed within 8–12 weeks after the beginning of the treatment; among 5 missing subjects, 3 patients underwent cytoreductive surgery, 1 subject refused examination due to COVID-19 epidemic precautions, and 1 patient died on the 6th week of treatment. The death of the patient occurred after sudden and rapid symptomatic deterioration; the cause of the death was unknown as the family of this subject refused an autopsy.

ctDNA analysis at base-line

Thirty patients were subjected to the ctDNA analysis at base-line. EGFR-mutated DNA was detected in 25/30 (83%) subjects (Table 2, Fig. 1). As expected, the sum volume of the tumor lesions was evidently higher in patients with detectable mutated ctDNA level as compared with "plasma-negative" patients, but the difference did not reach the statistical significance (29,463 mm^3 *vs.* 9963 mm^3, $p = 0.552$, Mann–Whitney *U* test). The probability of detecting ctDNA at base-line did not correlate with the patient age or gender, number of metastatic sites or EGFR mutation type (Table 2). The first CT evaluation of tumor response at 4th week after the beginning of anti-EGFR therapy documented a trend towards more pronounced tumor volume decrease in the "ctDNA-positive" group as compared with "ctDNA-negative" patients (−61% vs. −18.5%, $p = 0.208$, Mann–Whitney *U* test). This tendency was not maintained after 8–12 weeks of treatment (Table 2). Patients with detectable EGFR-mutated DNA at base-line had shorter PFS than "ctDNA-negative" cases, however this difference was also below the level of statistical significance [11.4 months vs. 21.0 months, $p = 0.238$, Breslow (generalized Wilcoxon) test for comparison of Kaplan–Meier curves].

Changes of ctDNA concentration during the first hours of TKI exposure

None of 5 patients, who were negative for plasma EGFR-mutated DNA at base-line, showed the presence of ctDNA (at least 5 mutation-specific signals per reaction) in the serial samples, which were taken in the first hours after the beginning of the treatment. The remaining 25 subjects demonstrated some changes in the amount ctDNA (Table 3, Fig. 2, see also Fig. 1S in the ESM). One of these subjects, patient #Pt22, experienced in the 1st day of treatment the femur fracture at the site of the metastatic lesion; the trauma was accompanied by the increase of the concentration of EGFR mutation signals in the plasma; this patient was considered not informative for further analysis.

The analysis of changes in ctDNA concentrations occurring within the first 48 h of treatment revealed a few patterns (Table 3, Fig. 2, see also Fig. 1S in the ESM). Some patients demonstrated more or less consistent decline of the ctDNA content during first two days of therapy (#Pt7, #Pt8, #Pt10). A minority of cases showed a trend towards continuous increase in the number of circulating EGFR mutant copies (#Pt21, #Pt24). There were instances of relatively steady level of ctDNA over the first 48 h (#Pt23). The majority of patients showed less consistent variations in the ctDNA contents, with a number of spikes and drops (#Pt3, #Pt4, #Pt25, #Pt30, etc.).

We further attempted to correlate, which of the ctDNA measurements provides the best correlation with the tumor response and PFS. We have conditionally chosen 25% difference between the numbers of EGFR-mutated signals as

Table 2 Clinical characteristics of NSCLC patients, tumor response to TKI treatment and changes in ctDNA content

	Plasma-positive patients (at base-line)	Plasma-negative patients (at base-line)	*p* value positive *vs.* negative	Changes in ctDNA after the start of TKI treatment (patients with detectable ctDNA at base-line)				
	n = 25	*n* = 5		Decrease (*n* = 12)	Increase/stable (*n* = 12)[a]	*p* value "Decrease" *vs.* "Increase/stable"	Increase (*n* = 7)	Stable (*n* = 5)
ctDNA, C*mut*[b]								
Base-line Median [min–max]	161 [16 to 4351]	0 [0]	–	299 [7 to 2071]	117 [6 to 4093]	NS (Mann–Whitney *U* test)	114 [27 to 4093]	127 [6 to 2841]
ctDNA, Percentage change (%)[c] C*mut* (0 h) to C*mut* (48 h) Median [min–max]	− 45 [− 100 to 254.5]	–	–	-85 [− 100.0 to − 48.7]	37 [− 21.4 to 276.3]	–	95 [35.6–276.3]	-11 [− 21.4 to 16.7]
Gender								
Male	3 (12%)	1 (20%)	NS (Fisher exact test)	2	1	NS (Fisher exact test)	1	0
Female	22 (88%)	4 (80%)		10	11		6	5
Age, y.o Median [min–max]	70 [52 to 82]	67 [59 to 81]	NS (Mann–Whitney *U* test)	70 [63 to 79]	69.5 [52 to 82]	NS (Mann–Whitney *U* test)	70 [52 to 82]	66 [61–70]
Distant metastases (M)								
M1	20 (80%)	4 (75%)	NS (Fisher exact test)	11	8	0.312 (Fisher exact test)	4	4
M0	5 (20%)	1 (25%)		1	4		3	1
Number of metastatic zones Median [min–max]	2 [0 to 4]	1 [0 to 4]	NS (Fisher exact test)	1 [1 to 3]	2 [0 to 4]	NS (Fisher exact test)	2 [0 to 4]	2.5 [0 to 3]
EGFR mutation								
ex19del	16 (64%)	4 (80%)	NS (Fisher exact test)	7	8	NS (Fisher exact test)	5	3
L858R	9 (36%)	1 (20%)		5	4		2	2
Drug								
Gefitinib	16 (64%)	4 (80%)	N (Chi-Square test)	8	7	0.254 (Chi-Square test)	6	1
Erlotinib	4 (16%)	1 (20%)		3	1		0	1
Afatinib	3 (12%)	0		0	3		1	2
Osimertinib	2 (8%)	0		1	1		0	1
Base-line: Sum of lesion, *V* (mm^3) Median [min–max]	29,463 [23–490590]	9963 [37–175455]	0.552 (Mann–Whitney *U* test)	34,527.5 [23–159943]	23,575 [37–490590]	NS (Mann–Whitney *U* test)	24,360 [166–111270]	4875 [37–490590]
4th week: 1st RECIST response								
Total, *n*	25 (100%)	5 (100%)		12 (100%)	12 (100%)		7 (100%)	5 (100%)
CR	0	0	0.045 (Fisher exact test)	0	0	NS (Fisher exact test)	0	0
PR	22 (88%)	3 (63%)		11 (92%)	10 (83%)		7 (100%)	3 (60%)
SD	1 (4%)	2 (25%)		1 (8%)	0		0	0
PD	2 (8%)	0		0	2 (17%)		0	2 (40%)
DCR (CR + PR + SD), *n* (%)	23 (92%)	5 (100%)	NS (Fisher exact test)	12 (100%)	10 (83%)	NS (Fisher exact test)	7 (100%)	3 (60%)

Table 2 (continued)

	Plasma-positive patients (at base-line) $n=25$	Plasma-negative patients (at base-line) $n=5$	p value positive *vs.* negative	Changes in ctDNA after the start of TKI treatment (patients with detectable ctDNA at base-line)				
				Decrease ($n=12$)	Increase/stable ($n=12$)[a]	p value "Decrease" *vs.* "Increase/stable"	Increase ($n=7$)	Stable ($n=5$)
4th week:						NS		
Tumor percentage change (%)[c]	−61.1	−18.5	0.214 (Mann–Whitney U test)	−62.1	−65.7	(Mann–Whitney U test)	−61.1	−81.4
Median [min–max]	[−95.0 to 27.3]	[−97.5 to 0.89]		[−86.9 to 8.4]	[−95.0 to–0.02]		[−95.0 to -43.9]	[−91.4 to 27.30]
8-12th week: 2nd RECIST response								
Not-available[d]	4	1		2	2		0	2
Total, n	21 (100%)	4 (100%)		10 (100%)	10 (100%)		7 (100%)	3 (100%)
CR	0	0	0.173 (Fisher exact test)	0	0	0.017 (Fisher exact test)	0	0
PR	14 (67%)	2 (50%)		8 (80%)	5 (50%)		3 (43%)	2 (67%)
SD	2 (10%)	2 (50%)		2 (20%)	0		0	0
PD	5 (24%)	0		0	5 (50%)		4 (57%)	1 (33%)
DCR (CR + PR + SD), n (%)	16 (76%)	4 (100%)	NS (Fisher exact test)	10 (100%)	5 (50%)	0.032 (Fisher exact test)	3 (43%)	2 (67%)
8–12th week:								
Tumor percentage change (%)[c]	0.0	−2.64	NS (Mann–Whitney U test)	0	−6.7	NS (Mann–Whitney U test)	41.0	−6.7
Median [min–max]	[−99.4 to 2661.1]	[−42.8 to 19.0]		[−70.6 to 108.8]	[−99.4 to 2622.1]		[−75.4 to 2660.1]	[−99.4 to 153.2]
Continuation of decrease of tumor volume (from 4 to 8th weeks of treatment)								
Yes	8 (38%)	2 (50%)	NS (Fisher exact test)	4	4	Ns (Fisher exact test)	3	1
No	13 (62%)	2 (50%)		6	6		4	2
PFS, months, [95% CI] Kaplan–Meier method	11.37 [11.24–11.70]	21.03 [na]	0.238 (Breslow test)	14.7 [10.33–15.81]	8.5 [6.27–8.07]	0.013 (Breslow test)	9.2 [5.57–12.87]	6.1 [4.07–8.19]
Progression[e]								
Yes	18 (72%)	2 (40%)	0.300 (Fisher exact test)	8 (67%)	9 (75%)	Ns (Fisher exact test)	6 (86%)	3 (60%)
No	7 (28%)	3 (60%)		4 (33%)	3 (25%)		1 (14%)	2 (40%)

NS not significant (p value > > 0.05), *PFS* progression-free survival; RECIST abbreviations: *CR* complete response, *PR* partial response, *SD* stable disease, *PD* progressive disease, *DCR* disease control rate (frequency of objective response (CR + PR) + SD)

[a]Patient #Pt22 was excluded from the analysis of ctDNA dynamics because of trauma (see explanation in the text)

[b]C*mut*—number of mutated copies per 1 mL of plasma

[c]Percentage change = (New Value − Initial Value)/(Initial Value) × 100%

[d]5 patients missed the second tumor response evaluation at the 8–12th weeks of treatment: patient #Pt3 died on the 6th week, 3 patients underwent cytoreductive surgery between the 4th and 8th weeks, and 1 subject refused examination because of epidemiologic situation

[e]Disease status on the 20 July, 2020

a threshold. This cut-off was evaluated by the analysis of intrapatient differences in ctDNA concentration in 8 paired blood samples obtained at 0.5 h before the treatment start and at the time of TKI administration (see Table 4S in the ESM); none of the these pairs showed difference exceeding 25%. The samples were classified for 3 groups according to change of the ctDNA content between the base-line and a given time point; accordingly, there were groups with the increased, decreased and stable concentration of tumor DNA in plasma. The measurements made at 0.5, 1, 2, 3, 6, 12, 24 and 36 h did not produce statistically significant correlations with the disease outcome (p-values (Breslow test) for

Table 3 Serial measurement of ctDNA content in plasma of NSCLC patients in first hours after EGFR-TKI treatment initiation (the individual graphics are presented on Fig. 1S *in the ESM,* for the detailed clinicopathological features of patients see Table 2S *in the ESM*)

Pts code	EGFR mut	Circulating tumor cell-free DNA level (C*mut*[a])												Changes in ctDNA content at 48 h vs. base-line[b]	Tumor response at 4 wks	Tumor response at 8–12 wks	PFS, months
		0	30'	1 h	2 h	3 h	6 h	12 h	24 h	36 h	48 h	14 days	28 days		RECIST	RECIST	
Pt1	*ex19del*	0	0	0	0	0	4	0	0	0	0	nd	nd	No ctDNA at base-line	PR	PR	8.47
Pt2	*ex19del*	328	456	267	257	388	326	587	715	659	153	0	0	Decrease	PR	PR	11.47
Pt3	*L858R*	127	142	142	209	167	713	1273	636	360	129	8	nd	No changes	PD	na	2.30
Pt4	*L858R*	29	50	72	38	30	39	56	90	35	84	40	67	Increase	PR	PD	2.67
Pt5	*ex19del*	114	67	52	80	53	65	0	193	100	157	0	0	Increase	PR	PD	3.23
Pt6	*ex19del*	13	6	4	3	0	3	6	0	13	0	0	0	Decrease	PR	PR	21.80
Pt7	*L858R*	48	58	24	44	32	45	48	30	22	0	nd	nd	Decrease	PR	PR	13.77
Pt8	*ex19del*	270	159	112	55	159	159	96	33	32	5	nd	nd	Decrease	PR	PR	11.37
Pt9	*L858R*	14	0	0	0	0	0	11	11	0	11	nd	nd	No changes	PD	PD	7.00
Pt10	*ex19del*	1180	929	677	736	880	982	445	309	167	291	0	0	Decrease	PR	PR	13.07
Pt11	*ex19del*	479	392	305	785	437	395	498	266	435	199	0	0	Decrease	PR	na (pCR, MPR 4)[c]	13.37
Pt12	*ex19del*	0	0	0	0	0	0	0	0	0	0	0	0	No ctDNA at base-line	SD	SD	3.23
Pt13	*L858R*	42	61	135	81	46	66	0	0	53	82	3	0	Increase	PR	PD	11.43
Pt14	*ex19del*	1278	1340	593	2222	3168	2042	728	6798	400	1004	nd	nd	No changes	PR	PR	7.17
Pt15	*L858R*	7	0	4	0	8	0	0	0	0	0	0	nd	Decrease	PR	SD	8.77
Pt16	*ex19del*	0	0	0	0	0	0	0	0	0	0	0	0	No ctDNA at base-line	PR	na	21.03
Pt17	*ex19del*	49	23	51	10	0	15	31	28	29	0	nd	nd	Decrease	PR	na (pPR, MPR 2)[c]	11.53
Pt18	*ex19del*	6	19	26	18	9	23	3	21	2	7	0	0	No changes	PR	na	3.70
Pt19	*L858R*	1584	1619	1100	924	1734	2033	1698	1910	859	730	nd	nd	Decrease	PR	PR	13.30
Pt20	*L858R*	0	0	0	0	0	0	0	0	0	0	nd	nd	No ctDNA at base-line	PR	PR	15.77
Pt21	*ex19del*	4093	4093	6367	8318	5751	6352	15,404	13,408	29,340	15,404	nd	nd	Increase	PR	PR	9.97
Pt22	*ex19del*	4351	6077	5023	5559	9949	18,883	12,576	11,099	12,115	18,403	nd	nd	Not informative[d]	PR	PR	13.97
Pt23	*ex19del*	2841	2891	2828	2766	1340	2451	4035	3872	1545	2523	nd	nd	No changes	PR	PR	6.97
Pt24	*ex19del*	119	90	94	96	153	105	171	261	347	232	21	21	Increase	PR	PR	13.90
Pt25	*ex19del*	27	24	26	13	19	23	15	67	21	78	0	0	Increase	PR	PD	6.53
Pt26	*ex19del*	161	65	80	132	138	129	96	104	9	60	nd	nd	Decrease	SD	SD	3.9
Pt27	*L858R*	845	787	48	32	15	239	394	150	46	47	nd	nd	Decrease	PR	PR	16.76
Pt28	*ex19del*	0	0	0	0	0	0	0	0	0	0	nd	nd	No ctDNA at base-line	SD	SD	9.40

Table 3 (continued)

Pts code	EGFR mut	Circulating tumor cell-free DNA level (*Cmut*[a])										Changes in ctDNA content at 48 h vs. base-line[b]	Tumor response at 4 wks	Tumor response at 8–12 wks	PFS, months
		0	30'	1 h	2 h	3 h	6 h	12 h	24 h	36 h	48 h	14 days	28 days				
Pt29	*ex19del*	3623	4819	4715	4094	4612	4129	4025	4911	4727	4911	3105	nd	Increase	PR	PR	16.77
Pt30	*L858R*	2071	2074	3168	3014	3212	1141	3590	1672	781	1063	nd	nd	Decrease	PR	PR	5.97

nd no data, *na* patients were lost from the second CT examination: #Pt3 died on the 6th week, #Pt11, #Pt16, #Pt17 underwent the cytoreductive surgery between the 4th and the 8–12th weeks of the treatment, #Pt18 refused examination due to COVID-19 epidemic precautions

[a]*Cmut*—number of mutated copies per 1 mL of plasma (evaluated by ddPCR)

[b]Changes in ctDNA concentration at 48 h *vs.* base-line: "decrease"—*Cmut* % change ≤ -25%; "increase"—*Cmut* % change ≥ 25%; "no changes"—*Cmut* % change ~ 0 ± 25%; "no ctDNA at base-line"—"plasma-negative" cases at base-line

[c]Surgical cytoreduction of the primary tumor/MPR—Major Pathological Response

[d]Patient #Pt22 was excluded from the analysis of ctDNA dynamics because of trauma (see explanation in the text)

comparison of Kaplan–Meier curves in different subgroups: 0.714, 0.841, 0.206, 0.798, 0.255, 0.276, 0.161 and 0.737, respectively; p-values (Fisher exact test) for tumor response rates: 0.697, 0.697, 0.283, 0.657, 0.444, 0.978, 0.408 and 0.319, respectively). However, there were clinical correlations with the change of ctDNA level registered at 48 h after the start of the treatment (see Fig. 2S in the ESM).

Changes of ctDNA concentration at 48 h are predictive for TKI clinical efficacy

Twelve (50%) out of 24 informative patients showed > 25% reduction of the plasma ctDNA concentration (median decrease: − 85%; range: from − 100% to − 49%) at 48 h after the start of treatment. All these patients demonstrated disease control after 4 and 8–12 weeks of therapy (at 4 weeks: 11 PR and 1 SD; at 8–12 weeks: 8 PR and 2 SD; two patients underwent surgery and were not evaluable by RECIST) (Tables 2, 3). One of two patients, who underwent surgery between 1st and 2nd assessments, demonstrated complete pathologic tumor response.

The remaining 12 individuals showed either stable content of circulating EGFR-mutated DNA ($n = 5$) or the elevation of ctDNA concentration ($n = 7$) at 48 h after the start of the therapy (Table 2, 3). The median increase of the ctDNA level in the latter group was 95% (range: from 36 to 276%). 10 of 12 patients with elevated or stable ctDNA level achieved an objective response at 4 weeks, but only 5 of 10 evaluable patients still demonstrated disease control at 8–12 weeks of the treatment (Fisher exact test: $p = 0.032$, when compared to the group with ctDNA decrease). Progressive disease (PD) at weeks 8–12 was documented in 5/10 (50%) patients, who showed increased or stable ctDNA content at 48 h after the treatment; one additional patient died before the second assessment (Tables 2, 3).

The decline of concentration of EGFR-mutated DNA in plasma, which was observed at 48 h after the start of the TKI treatment, predicted for longer PFS as compared with patients with increasing or stable level of ctDNA (14.7 months vs. 8.5 months, $p = 0.013$, Kaplan–Meier method; Table 2; Fig. 3).

Discussion

EGFR TKIs are characterized by a relatively rapid absorption, with peak plasma concentrations achieved within a few hours after the uptake of the tablet [31, 32]. The therapeutic doses of the EGFR TKIs are significantly higher than the minimal drug concentrations, which exert some antitumor effect [33–35]. Consequently, it is explainable, that some patients experience evident symptomatic relief within the first hours after the beginning of the treatment [26, 27, 36].

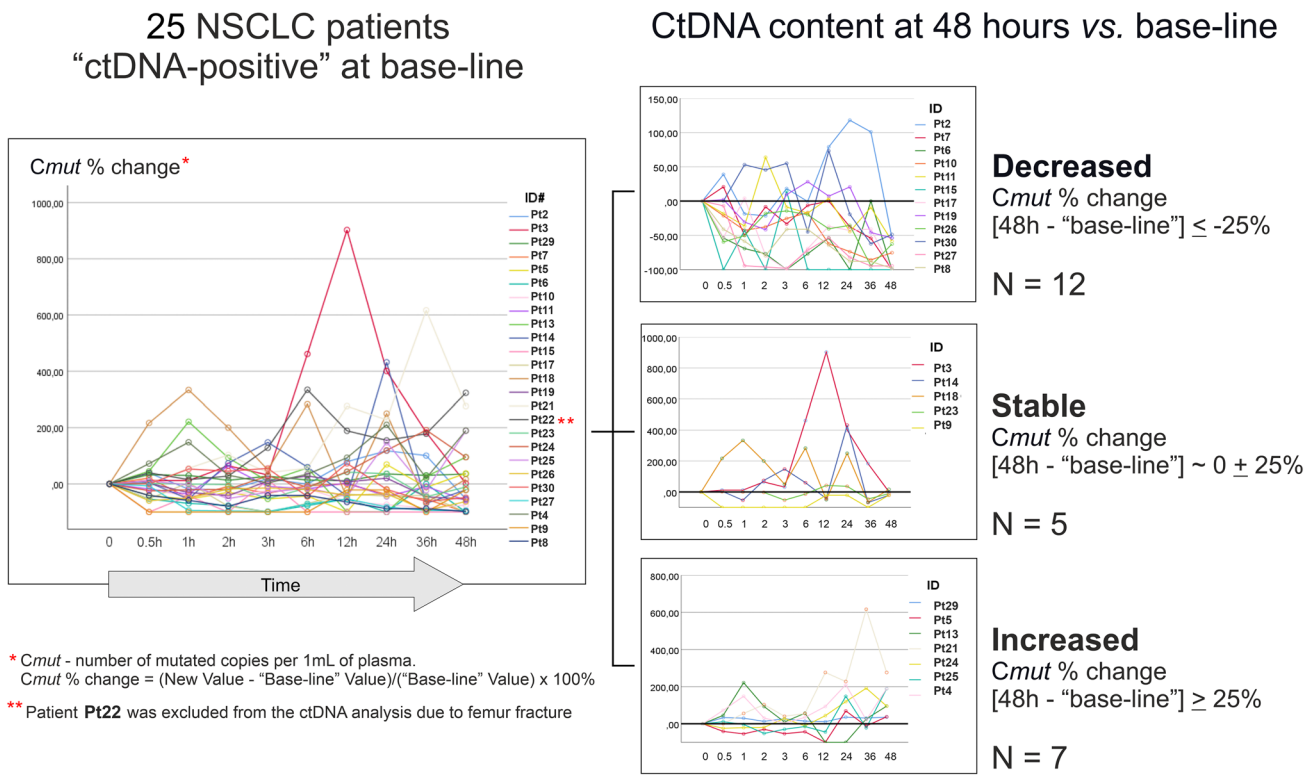

Fig. 2 Spider plots of changes in ctDNA concentrations occurring in the plasma obtained from the NSCLC patients within the first 48 h of anti-EGFR treatment

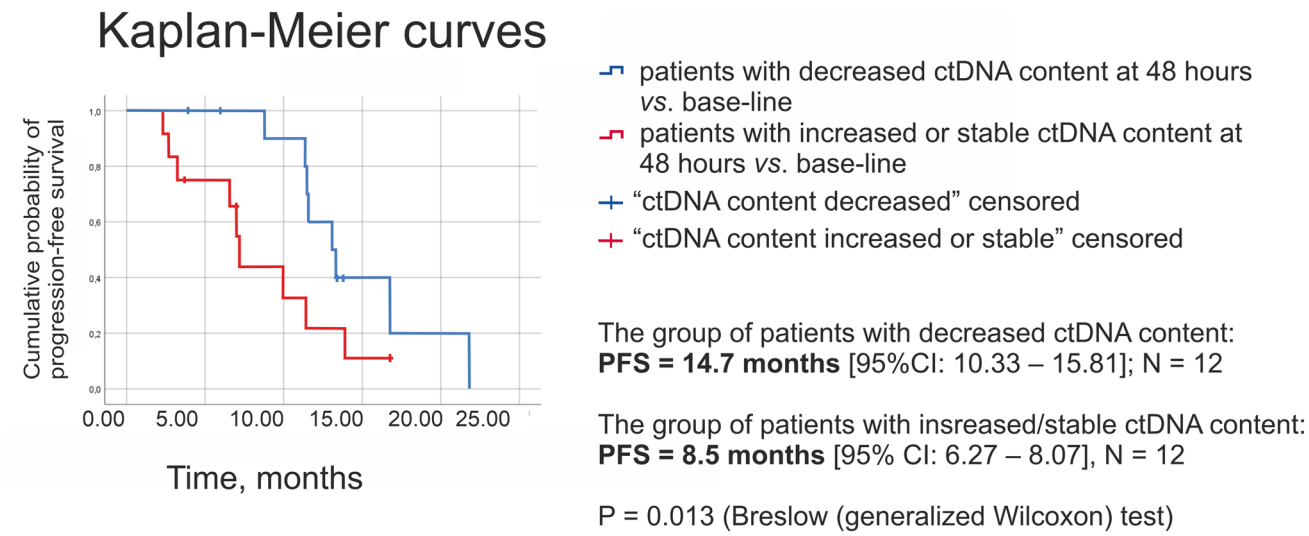

Fig. 3 Probability of survival in NSCLC patients with different patterns of early ctDNA dynamics during the first 48 h of anti-EGFR treatment

Studies of EGFR inhibition in cell lines revealed, that the administration of anti-EGFR drugs results in immediate biological consequences. Exposure to TKI causes the decrease of EGFR autophosphorylation, followed by down-regulation of ERK, AKT, STAT3 and other signaling proteins; all these events are observed within 10–30 min after the addition of TKI to the cell culture medium [37–39]. Activation of the apoptotic signaling cascade can also be observed within first 10 min of TKI exposure [40, 41]. The reduction of the tumor mass upon EGFR TKI therapy is likely to be attributed both

to the cessation of cell proliferation and to the induction of programmed cell death [17, 42, 43]. Some data indicate that immune-related mechanisms may also contribute to the tumor shrinkage [44, 45]. Use of MRI in animal experiments revealed evidence for tumor regression occurring already within 1–7 days after TKI administration [46–48]. Consequently, the mere fact of the existence of rapid plasma ctDNA response to TKI treatment is in agreement with preclinical observations.

The numerical data obtained in our study correspond well to the results observed in similarly designed investigations. In particular, we were able to detect EGFR mutations in plasma in 25/30 (83%) patients at base-line, which is close to the observations made in other studies [49–53]. Our data also validate previous findings, which suggest that the absence of detectable EGFR-mutated copies in the plasma at base-line correlates with improved PFS [13, 51, 54]. Similarly to the reports of Riediger et al. [55] and Phallen et al. [56], we observed temporary increase of the level of ctDNA during the first hours of treatment in some although not all cases. It is unclear whether these changes are attributed to the massive tumor cell shedding in response to the drug, or caused by other reasons.

Several studies monitored ctDNA concentration in the beginning of the treatment by EGFR inhibitors. Lee et al. [51] analyzed EGFR-mutated ctDNA at 8 weeks after the TKI administration, and observed the decline of its concentration in all 40 patients analyzed. Subjects with complete clearance of ctDNA at 8 weeks had significantly longer PFS as compared to patients with residual amounts of EGFR-mutated copies in the bloodstream. Molina-Vila et al. [54] examined 74 patients at 6 weeks after the start of therapy, and observed the presence of EGFR-mutated DNA only in 3 (4%) subjects. Monitoring of ctDNA at earlier time points is significantly more complicated. Riediger et al. [55] obtained serial blood samples at 1-day intervals during the first week of therapy from a patient receiving afatinib. They observed an increase of ctDNA concentration at 26 h, and subsequent continuous decline of the number of EGFR-mutated copies starting from 48 h time point. Husain et al. [57] monitored the amount of ex19del, L858R and T790M mutation in patients, who acquired resistance to erlotinib or afatinib, and started to receive osimertinib. Serial urinary samples were obtained at 1-day intervals during the first week of therapy. Some of these patients showed temporary spikes of urine tumor DNA concentration within days 1–7, however the subsequent observation revealed a consistent decline of the amount of EGFR-mutated DNA by the end of the second week. Our study has a novelty as compared to the mentioned above investigations, as it included a relatively large number of patients and involved a serial blood-takes performed within first hours after TKI administration. The justification of this effort was based on published observations describing a very rapid treatment response in a subset of patients [26, 27, 36] as well as on the data obtained in preclinical experiments [37–39]. Our results suggest that good responders to TKIs can be identified already at 48 h after the start of the EGFR-targeted therapy. Some NSCLCs treated by first- or second-generation EGFR TKIs demonstrate emergence of EGFR T790M mutations before clinical disease progression [58, 59]. However, treatment-naïve tumors usually do not contain EGFR T790M mutation as base-line [60] therefore our study considered only monitoring of ex19del- and L858R-mutated ctDNA.

This study has some limitations. Blood-take at 48 h after the beginning of the treatment was the latest time point in the early ctDNA response analysis. This was due to convenience reasons, as the patients started to receive TKI while been in a hospital, and the 2 days was a period between the first tablet and the hospital discharge. While 48 h was the only informative point for clinical prediction within the range 0.5–48 h, one could expect that the analysis of ctDNA at somewhat longer time intervals could have even better predictive value. The design of our study initially considered blood-takes at 2 and 4 weeks after the beginning of TKI treatment, however the compliance of patients was incomplete and the obtained data did not provide additional information (Table 3). It is also desirable to validate the obtained findings in larger studies involving serial blood-takes from NSCLCs patients undergoing EGFR TKI therapy.

Early monitoring of ctDNA after the start of treatment allows to evaluate whether the tumor will indeed consistently respond to TKI. It appears that although the majority of EGFR-mutated tumors demonstrate some initial disease control upon TKI administration, they can broadly be divided into two categories. In approximately a half of tumors the majority of cells constituting neoplastic lumps are vulnerable to TKI exposure, and these tumors demonstrate prolonged tumor response. Another half of EGFR-mutated NSCLCs is characterized by some intratumoral heterogeneity caused by compromised access to the drug for some tumor cells or by various in-built signaling mechanisms for TKI resistance. In these NSCLCs only a fraction of cells composing the tumor mass respond to TKI, while the remaining malignant clones facilitate rapid disease progression after initial short-term disease control.

Conclusions

The present study demonstrates that the clinical response to TKI can be predicted by the analysis of changes in the plasma ctDNA concentration at 48 h after the start of EGFR-targeted therapy. It is not obvious whether the results of this plasma test could call to some action. While the prediction for good response clearly supports the continuation of the

treatment, it is unclear what options can be offered to potential poor responders. There are studies demonstrating promising results of the combined use of EGFR TKI inhibitors and antiangiogenic or cytotoxic drugs [61, 62]. Perhaps, lack of rapid response to a single-agent EGFR TKI may justify the addition of another antitumor compound to the front-line therapy. Current clinical trials often involve liquid biopsy; therefore testing of this concept is compatible with the available medical resources.

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Author contributions EK, FM and EI designed the study. The processing of biological materials was done by: KZ, AM. Data collection was performed by AZ, SB, ES, EA, TS, and NV. Molecular genetic analysis was carried out by: TL, TS, MK, and SC. Statistical analysis was performed by: EK, GY and EI. The first draft of the manuscript was written by FM, EK and EI. All authors read and approved the final manuscript.

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Availability of data and material The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

Code availability Not applicable.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethics approval The study design was approved by the local Ethical Committee of N.N. Petrov Institute of Oncology. All procedures performed in study were in accordance with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Consent to participate Informed consent was obtained from all individual participants included in the study.

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