

ЛАБОРАТОРНЫЕ И ЭКСПЕРИМЕНТАЛЬНЫЕ ИССЛЕДОВАНИЯ LABORATORY AND EXPERIMENTAL STUDIES

DOI: 10.21294/1814-4861-2018-17-5-52-59

УДК: 616.24-006.6-076:575.113:577.213

Для цитирования: Шаманин В.А., Карпов И.В., Гервас П.А., Чердынцева Н.В., Симолина Е.И., Козлов В.В., Коваленко С.П. Мониторинг мутаций в гене *EGFR* в циркулирующей ДНК плазмы крови больных немелкоклеточным раком легкого. Сибирский онкологический журнал. 2018; 17 (5): 52–59. – doi: 10.21294/1814-4861-2018-17-5-52-59.

For citation: Shamanin V.A., Karpov I.V., Gervas P.A., Cherdyntseva N.V., Simolina E.I., Kozlov V.V., Kovalenko S.P. Monitoring of *EGFR* mutations in the circulating tumor DNA from blood plasma of patients with non-small cell lung cancer. Siberian Journal of Oncology. 2018; 17 (5): 52–59. – doi: 10.21294/1814-4861-2018-17-5-52-59.

МОНИТОРИНГ МУТАЦИЙ В ГЕНЕ *EGFR* В ЦИРКУЛИРУЮЩЕЙ ДНК ПЛАЗМЫ КРОВИ БОЛЬНЫХ НЕМЕЛКОКЛЕТОЧНЫМ РАКОМ ЛЕГКОГО

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Аннотация

Активирующие мутации гена *EGFR* связаны с чувствительностью немелкоклеточного рака легкого (НМКРЛ) к ингибиторам тирозинкиназ (ИТК). Жидкостная биопсия с использованием циркулирующей внеклеточной опухолевой ДНК (цодНК) предлагается в случаях, когда опухолевая ткань недоступна, а также для мониторинга статуса гена *EGFR*. В данном исследовании мы опробовали новый метод на основе количественной ПЦР (кПЦР) для выявления мутаций гена *EGFR* в цодНК плазмы. Чувствительность метода составила 1 % мутантного аллеля *EGFR* L858R, L861Q, S768I и делеций экзона 19 и 5 % мутантного аллеля *EGFR* G719X или T790M. В плазме крови, взятой до операции, мутации были выявлены в 4 из 7 (57 %) случаев с мутацией гена *EGFR* в ткани опухоли. При этом в двух случаях мутации в цодНК полностью соответствовали мутациям, которые были обнаружены в ткани опухоли. В одном случае при наличии мутаций G719X и S768I в ткани опухоли только S768I была выявлена в цодНК. Еще в одном случае мутация T790M была выявлена в плазме дополнительно к мутации L858R, которая была в ткани опухоли. Мутации гена *EGFR* не были обнаружены в ДНК плазмы крови 12 здоровых доноров и 13 больных НМКРЛ, имевших ген *EGFR* дикого типа, что означает 100 % специфичность теста. Жидкостная биопсия выявила мутации *EGFR* в 8 из 16 случаев НМКРЛ, которые несли мутации гена *EGFR* и проходили курс терапии с использованием ИТК. В этой группе в 7 случаях мутации в жидкой биопсии соответствовали тем, которые были в опухолевой ткани, а еще в одном случае мутация T790M была выявлена дополнительно к мутации L858R. В 3 случаях увеличение частоты мутантного аллеля было выявлено за 2–12 мес до клинической прогрессии.

Ключевые слова: жидкостная биопсия, qPCR, *EGFR*, мутация, рак легких.

MONITORING OF *EGFR* MUTATIONS IN THE CIRCULATING TUMOR DNA FROM BLOOD PLASMA OF PATIENTS WITH NON-SMALL CELL LUNG CANCER

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Abstract

Activating mutations of *EGFR* are associated with sensitivity of non-small cell lung cancer (NSCLC) to tyrosine kinase inhibitors (TKI). Liquid biopsy using circulating cell-free tumor DNA (cfDNA) is proposed in cases when formalin fixed paraffin embedded (FFPE) tumor tissue is not available and for monitoring of *EGFR* status. In the study we evaluated new qPCR assay for *EGFR* mutations in plasma cfDNA. Sensitivity of the assay was 1 % of the mutant allele for L858R, L861Q, S768I mutations and deletions in exon 19, and 5 % of the mutant allele for G719X or T790M mutations. Before surgery, mutation was detected in plasma of 4 out of 7 patients (57 %) with mutant *EGFR* in FFPE tumor tissue. Mutations found in cfDNA completely matched those found in tumor tissue in 2 cases. In one case with G719X and S768I mutations in FFPE tissue, only S768I was found in cfDNA. In another case, T790M was detected in plasma in addition to L858R that was present in tumor tissue. No *EGFR* mutations were detected in plasma DNA from 12 healthy volunteers and 13 cases of NSCLC with wt *EGFR* suggesting 100 % specificity of the assay. Liquid biopsy detected *EGFR* mutations in cfDNA in 8 of 16 cases of NSCLC with mutant *EGFR* being under therapy with TKI. Among them, 7 cases had mutations in liquid biopsy that matched those in tumor tissue and another case had T790M in addition to L858R. In 3 cases increased mutant allele frequency was detected 212 months before clinical progression.

Key words: Liquid biopsy, qPCR, *EGFR*, mutation, lung cancer.

Non-small cell lung cancer (NSCLC) is the most common type of lung cancer. Activating somatic mutations within the tyrosine kinase (TK) domain in the epidermal growth factor receptor (EGFR) protein are associated with the sensitivity of tumors to EGFR-TK inhibitors [1]. Most of these mutations (~90 %) occur as either in-frame microdeletions that affect codons 746–750 in exon 19 (19del), or a missense mutation (L858R) in exon 21 of *EGFR*. In addition, rare activating mutations (L861Q, G719S/A/C, S768I and others) make up 10–15 % of cases with mutant *EGFR*. Besides, T790M mutation occurs in ~50 % of cases of acquired resistance to the first generation *EGFR* TK inhibitors (TKI). Tumors with T790M mutation are sensitive to therapy with osimertinib, which is the third generation TKI [2]. The European Society for Medical Oncology (ESMO) and American Society of Clinical Oncology (ASCO) recommend an *EGFR* mutation testing for selection of NSCLC patients who could benefit from anti-*EGFR* therapy [3, 4].

DNA from formalin fixed paraffin embedded (FFPE) tumor tissue is usually used to test mutations. In cases when tumor tissue is not available, the liquid biopsy using blood plasma that contains circulating

cell-free tumor DNA (cfDNA) from tumor cells can be tested for clinically actionable mutations [5]. Major challenges of liquid biopsy are low concentration of cfDNA in plasma, and low frequency of mutant allele in cfDNA due to high background of DNA from normal cells. The assays for liquid biopsy are based on different methods including quantitative PCR (qPCR), digital PCR (dPCR), next generation sequencing (NGS) [6].

NGS can test a broad range of mutations with high sensitivity but the assay is expensive, technically demanding and has turnaround time of 12 weeks. Tests based on qPCR and dPCR detect only hot spot mutations but provide fast turnaround time of several days. dPCR has a high sensitivity and can detect mutations with mutant allele frequency (MAF) below 0,1 %. However, the high analytical sensitivity of dPCR often cannot be fully explored due to not sufficient concentration of cfDNA in plasma. The sensitivity of qPCR can be 1 % MAF. Importantly, equipment and reagents for qPCR are commonly available.

The aim of our study was to evaluate liquid biopsy for *EGFR* mutations using new test based on qPCR. We used limited multiplex PCR (mPCR) of regions in 1821 exons of *EGFR* with hot spots for mutations

to increase amount of cfDNA targets so that multiple tests for different mutations can be made in clinical setting without necessity for additional blood draw. After mPCR, amplicons of cfDNA were tested for *EGFR* mutations by qPCR. The developed assay was used for the monitoring of mutations in *EGFR* gene in cfDNA of patients with NSCLC treated with gefitinib or erlotinib.

Material and methods

Ethics statement

The study was approved by the Ethics Committee of the Cancer Research Institute (Tomsk, Russia). Informed consents were received from all patients.

Clinical samples

Blood samples from 12 healthy volunteers (6 males and 6 females) were collected in the medical center “Status” (Novosibirsk). Paired samples of blood before surgery and formalin-fixed paraffin-embedded (FFPE) tumor tissue after surgical resection from 20 patients with non-small cell lung cancer (NSCLC) were collected at the Novosibirsk Regional Clinical Oncology Center (NRCOC; Novosibirsk, Russia). Blood from 16 patients with *EGFR*-mutant NSCLC treated with tyrosine kinase inhibitors (TKI) was collected at the NRCOC and Cancer Research Institute (Tomsk, Russia). Demographics of patients and histotypes of tumors are provided in Table 1.

Table 1

Demographics of patients with NSCLC

Case	Patient ID	Sex	Age	Tumor histology
Cases with mutant <i>EGFR</i> in FFPE tumor tissue DNA, plasma before surgery				
1	BK7	F	53	NSCLC-NOS
2	BK16	F	68	NSCLC-NOS
3	BK35	F	54	AC
4	BK40	F	74	AC
5	BK54	M	60	AC
6	BK59	M	59	AC
7	BK74	F	62	AC
Cases without <i>EGFR</i> mutation in FFPE tumor tissue DNA, plasma before surgery				
8	BK1	M	53	NSCLC-NOS
9	BK3	M	47	AC
10	BK8	F	59	NSCLC-NOS
11	BK9	F	63	AC
12	BK10	M	63	NSCLC-NOS
13	BK17	M	60	AC
14	BK20	M	54	SCC
15	BK23	M	64	SCC
16	BK24	M	43	SCC
17	BK25	M	73	AC
18	BK29	M	64	AC
19	BK53	M	68	SCC
20	BK60	M	56	SCC
Cases with mutant <i>EGFR</i> under TKI therapy				
21	BK57	F	53	AC
22	TMB1	F	60	AC
23	XAH2	M	80	NSCLC-NOS
24	MAT3	F	70	NSCLC-NOS
25	GLF4	F	73	AC
26	SLF5	F	62	AC
27	KVI6	M	na	NSCLC-NOS
28	CAB7	F	64	AC
29	PIM8	F	52	AC
30	DVI9	M	60	AC
31	IEV10	F	69	AC
32	BVI11	M	60	AC
33	FVY12	M	67	AC
34	G013	M	na	AC
35	KMG14	F	77	AC
36	B16	F	na	NSCLC-NOS

Note: NSCLC-NOS – non-small cell lung cancer not otherwise specified, AC – adenocarcinoma, SCC – squamous cell cancer; na – not available.

Blood was collected in 8 ml Vacutainer tubes with EDTA solution and stored at +4°C before preparation of plasma. Plasma was prepared on the same day of the blood draw by centrifugation for 10 min at 2000 g at 4°C and 0.5–1.0 ml aliquots were stored at -70°C.

DNA purification

DNA from FFPE tissue was purified using FFPE DNA kit (Biolink, Russia) and stored at -20°C. cfDNA was purified from 1.0 ml blood plasma using “PME free-circulating DNA Extraction Kit” (Analytik Jena, Germany). cfDNA was dissolved in 50 µl of PCR-grade water and stored at -20°C.

Concentration of cfDNA was estimated by real-time PCR for *EGFR* exon 21 using control PCR reaction mixture from “Real-time-PCR-EGFR-7RP Kit” (Biolink, Russia) and human placenta DNA (2ng/µl) as external standard. The average concentration of cfDNA was 9.3 ± 14 ng/ml (range 0.5–81.0 ng/ml) plasma. In a few samples, the concentration of cfDNA was above 250 ng/ml; the samples had

pink color due to hemolysis and were excluded from further analysis.

Multiplex PCR of 18-21 exons of *EGFR*

Multiplex PCR (mPCR) was done in 50µl in reaction mixture with 1x Buffer for Taq-DNA-polymerase; 0.2 mM each of dATP, dGTP, dTTP, dCTP; 1.5 mM MgCl₂, 0.5 µM each forward and reverse primers for 18-21 exons of *EGFR* (Table 2), 2.0 U Taq DNA-polymerase (Biolink), and 20 µl cfDNA from blood plasma. mPCR was performed in iCycler iQ5 (Bio-Rad) using the following protocol: 1 cycle – 95°C 3 min; 8–12 cycles – 95°C 15 sec, 58°C 30 sec, 72°C 20 sec; 1 cycle 72°C 3 min. After mPCR, reaction mixture was diluted 1:25 in PCR-grade water and 5µl was used in qPCR.

Detection of *EGFR* mutations in cfDNA by real-time PCR

Amplicons after multiplex PCR of *EGFR* sequences in cfDNA were tested for *EGFR* L858R, L861Q, G719A/C/S, S768I, T790M mutations and deletions

Table 2

Primers for multiplex PCR of sequences in 1821 exons of *EGFR*

Exon	Forward primer	Reverse primer	Amplicon size, b.p.
18	tccaaccaagctctctga	ctgtgccagggacctacc	109
19	tggatcccagaaggtgagaaag	cccacacagcaaagcagaa	118
20	cctccctccaggaagccta	gccgaaggcctatgagctg	116
21	ccgcagcatgtcaagatcac	aatgctggctgacctaagc	115

Table 3

Mutations of *EGFR* in FFPE tumor DNA and plasma DNA from NSCLC patients

Case ID	FFPE Tumor DNA ^(a)	Plasma cfDNA ^(a, b)
Cases with <i>EGFR</i> mutation in FFPE tumor tissue		
BK7	G719X; S768I	S768I
BK16	19del	19del
BK35	19del	19del;
BK40	L861Q	wt
BK54	L858R	wt
BK59	L858R	L858R;
BK74	19del	wt
Cases with <i>EGFR</i> mutation, n (%)	7/7 (100.0)	4/7 (57.1)
Cases without <i>EGFR</i> mutation in FFPE tumor tissue		
BK1	wt	wt
BK3	wt	wt
BK8	wt	wt
BK9	wt	wt
BK10	wt	wt
BK17	wt	wt
BK20	wt	wt
BK23	wt	wt
BK24	wt	wt
BK25	wt	wt
BK29	wt	wt
BK53	wt	wt
BK60	wt	wt
Cases with <i>EGFR</i> mutation, n (%)	0/13 (0,0)	0/13 (0,0)

Note: ^(a) – allele-specific real-time PCR for mutations *EGFR* L858R, L861Q, G719X, T790M, S768I and wild-type blocking PCR for mutations *EGFR* 19del; ^(b) – blood draw was done during a week before surgery.

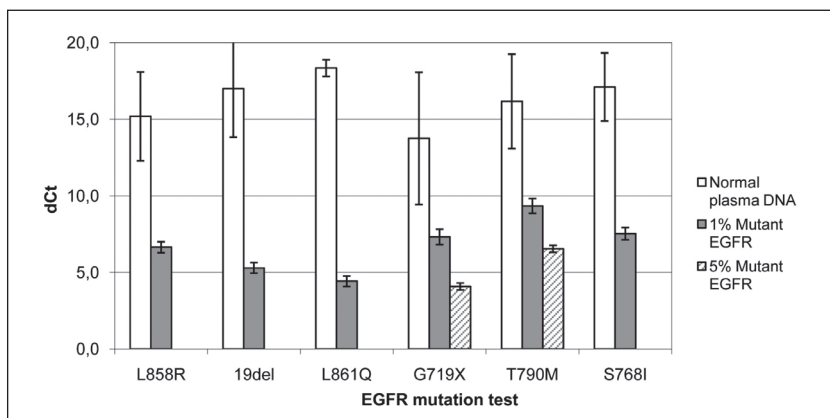


Fig. 1. Sensitivity and specificity of real-time PCR for *EGFR* mutations. Mutations *EGFR* L858R, L861Q, G719X, T790M, S768I and deletions in *EGFR* exon 19 (19del) were detected by “Real-time-PCR-EGFR-7RP Kit” (Biolink, Russia). DNA of positive controls (PC) with 1–5 % mutant allele *EGFR* L858R, E746_A750delELREA, L861Q, G719S, T790M, S768I and 12 samples of cfDNA from blood plasma of 12 healthy volunteers were tested using six replicates of PC and two replicates of amplicons of each cfDNA. dCt mean and dCt standard variation are shown

in exon 19 using “Real-time-PCR-EGFR-7RP Kit” (Biolink, Russia). In the kit, allele-specific qPCR (as-PCR) is used to detect L858R, L861Q, G719A/C/S, S768I, T790M mutations, and wild-type blocking PCR [8] was used to detect deletions in exon 19.

Results and discussion

Sensitivity and specificity of qPCR test for *EGFR* mutations

Analytical sensitivity and specificity of the assay was evaluated using human genomic DNA spiked with recombinant plasmid DNA as positive controls (PC) to have 1–5 % mutant allele for tested mutations (*EGFR* L858R, E746_A750delELREA, L861Q, G719S, T790M, S768I). As negative controls, we used 12 samples of cfDNA from the blood plasma of healthy volunteers without *EGFR* mutations.

To have sufficient DNA-copies for mutation test, sequences of 1821 exons of *EGFR* in cfDNA were amplified by multiplex PCR. Two replicates of amplicons of each cfDNA and 6 replicates of PC were tested for *EGFR* mutations using ~3000 copies of *EGFR* DNA in reaction. For each DNA, dCt was calculated as $dCt = Ct_{AS} - Ct_C$, where Ct_{AS} is Ct of the DNA in asPCR for the tested mutation, and Ct_C is Ct of the DNA in control PCR for constant *EGFR* sequence.

dCt values were much larger for wt cfDNA in comparison to PC (Fig. 1) indicating that the method differentiated DNA with 1 % of mutant *EGFR* alleles: L858R, E746_A750delELREA, L861Q, S768I or 5 % of mutant *EGFR* alleles: G719S or T790M from wt cfDNA with probability $p > 0.95$.

Mutations of *EGFR* in paired samples of the DNA from FFPE tumor tissue and blood plasma from patients with NSCLC. Clinical sensitivity of liquid biopsy for *EGFR* mutations was evaluated using FFPE tumor samples from patients with NSCLC and matched blood plasma of the patients that was collected before surgery (Table 3).

Among 7 cases that carried *EGFR* mutations in the DNA from FFPE tumor tissue, 3 cases had the same mutations in plasma cfDNA. Interestingly, in a case BK7 with G719X and S768I mutations in FFPE tissue, only S768I was found in plasma presumably due to tumor heterogeneity. In the other three cases (BK40, BK54, BK74) mutations present in FFPE tumor tissue were not detected in plasma. Importantly, *EGFR* mutations were not found in cfDNA of 13 cases without *EGFR* mutation in FFPE tumor tissue. These data showed that in comparison to FFPE tissue test, the liquid biopsy for *EGFR* driver mutations had sensitivity of 4/7 (57 %) and specificity of 13/13 (100 %).

Monitoring of *EGFR* mutations in plasma cfDNA from patients with NSCLC treated with TKI

Changes in MAF of the *EGFR* in blood plasma of patients treated with TKI can be a molecular marker of sensitivity or development of resistance of tumor to TKI treatment. The accumulation of T790M results in the development of resistance to the first generation TKI that can be treated with the third generation TKI (osimertinib). We started monitoring of *EGFR* mutations in plasma cfDNA from 16 patients with NSCLC treated with gefitinib or erlotinib. This is ongoing study and interim results are shown in Table 4.

Among 16 cases with NSCLC, 12 carried mutation 19 del and 4 cases carried L858R in FFPE tumor tissue. In 8 cases, *EGFR* mutations were not detected in plasma cfDNA. Lack of or low concentration of mutant *EGFR* in the cfDNA of the cases could be due to: 1) tumors being under TKI suppression or 2) metastasis to the brain with the cfDNA level in plasma below the detection limit of our test.

In the other 8 cases, *EGFR* mutations were detected in cfDNA. The same *EGFR* mutations that were present in FFPE tumor DNA were detected in cfDNA in 7 cases. In another case (patient TMB1) mutation T790M was detected in plasma cfDNA in addition to *EGFR* driver mutation L858R. Four

Table 4

EGFR mutations in cfDNA of NSCLC patients under treatment with TKI

Patient ID	Treatment	EGFR mutation	
		FFPE DNA	cfDNA
BK57	S,TKI	L858R	L858R
TMB1	TKI	L858R	L858R, T790M
XAH2	ChT, TKI	19del	wt
MAT3	S, TKI	19del	wt
GLF4	TKI	19del	wt
SLF5	S, ChT, TKI	19del	wt
KVI6	TKI	19del	wt
CAB7	TKI	19del	wt
PIM8	TKI, RT	19del	19del
DVI9	TKI, G	19del	19del
IEV10	S, TKI	19del	19del
BVI11	S, TKI	19del	wt
FVY12	S, RT, ChT, TKI	19del	wt
G013	TKI	L858R	L858R
KMG14	S, ChT, TKI	19del	19del
B16	TKI	L858R	L858R

Note: ChT – polychemotherapy, G – gamma knife radiosurgery, RT – radiation therapy, S – surgery, TKI – therapy with gefitinib or erlotinib; BM – brain metastasis.

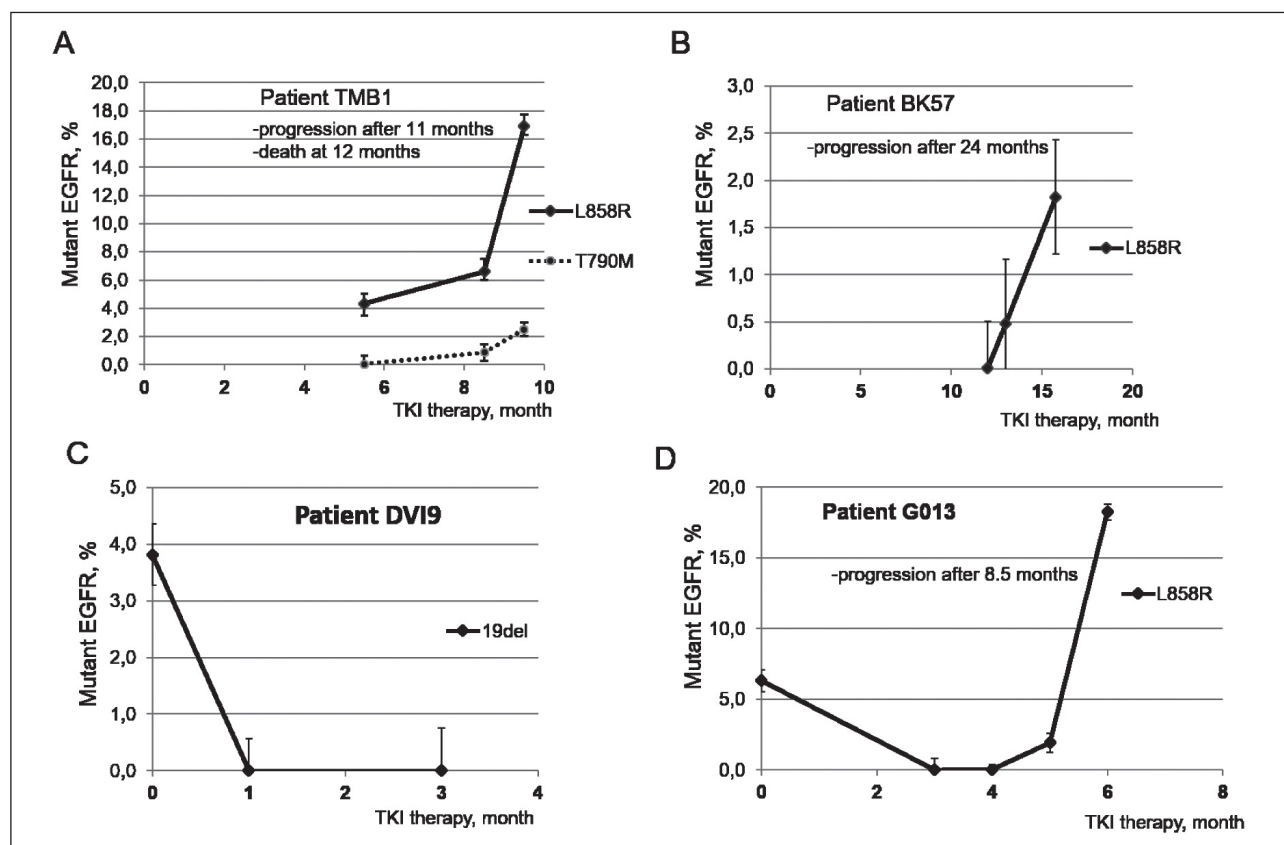


Fig. 2. Mutant allele frequency of the EGFR in plasma cfDNA of patients under TKI therapy. EGFR mutations were detected by qPCR. A. Patient TMB1. B. Patient BK57. C. Patient DVI9. D. Patient G013

representative cases with changes in MAF of *EGFR* in cfDNA during TKI therapy are shown in Fig. 2. In case TMB1 (Fig. 2A), L858R and T790M mutations were detected 5.5 and 8.5 months after starting TKI therapy, respectively thus suggesting the development of T790M-associated resistance in the tumor. Disease progression was manifested 11 months from the start of TKI therapy, resulting in death at 12 months. Of note, higher MAF of L858R in cfDNA in comparison to T790M suggests heterogeneity of TKI-resistant tumor with subpopulation(s) of resistant tumor cells with and without the T790M mutation. In case BK57, the increased MAF of L858R mutation in cfDNA was detected 12 months after therapy with erlotinib (Fig. 2B), while clinical progression with metastasis to bones developed 24 months after therapy. In case DVI9, tumor with 19del responded to treatment with gefitinib and the level of 19del became undetectable after the first month of TKI therapy. Later, the patient had metastasis to the brain without accumulation of mutant *EGFR* in plasma cfDNA (data not shown). In case G013, tumor with the L858R mutation responded to treatment with gefitinib. Mutation in plasma cfDNA dropped to undetectable level 3 and 4 months after starting TKI therapy. However, the L858R mutation in cfDNA was detected 5 months after starting TKI therapy, and the level of the mutation continued to increase 6 months from the start of TKI therapy.

Clinical disease progression was manifested 8.5 months from starting TKI therapy.

In this study we evaluated liquid biopsy test for *EGFR* mutations in plasma DNA of patients with NSCLC. Our qPCR test detected *EGFR* mutations in plasma cfDNA collected before surgery in 57 % of the cases that carried mutation in FFPE tumor tissue. Other study that used validated allele-specific qPCR kit (Therascreen *EGFR* 29; Qiagen) reported 60 % detection rate of mutations in plasma [7]. In our study, no *EGFR* mutations were detected in plasma DNA from 12 healthy volunteers and 13 cases of NSCLC with wt *EGFR* suggesting 100 % specificity of the assay. High specificity of a test for *EGFR* mutations is critical since false positives can result in use of TKI that will be without benefit or harmful for a patient. The data suggested that liquid biopsy test could be used for selection of patients for therapy with TKI, however, in about half of the cases *EGFR* mutation may not be detected in plasma when it was found in tumor tissue after surgery. Liquid biopsy using plasma cfDNA of patients treated with TKI therapy showed that the test could detect *EGFR* mutations during the first month after starting therapy. Importantly, increased mutant *EGFR* in cfDNA was detected several months before clinical progression. This suggests that liquid biopsy for *EGFR* mutations in patients receiving TKI treatment may have reasonable prognostic value.

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Поступила/Received 25.06.18
Принята в печать/Accepted 17.09.18

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Финансирование

Исследование было поддержано грантом № 15-14-10004 Российского научного фонда.

Конфликт интересов

С.П. Коваленко и В.А. Шаманин представляют интересы ООО «Биолинк». Другие авторы заявляют об отсутствии потенциального конфликта интересов.

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Funding

The study was supported by grant № 15-14-10004 from the Russian Science Foundation.

Conflict of interest

S.P. Kovalenko and V.A. Shamanin owe shares of the Biolink Ltd. Other authors declare lack of the potential conflicts of interests.