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PCR-based detection of EGFR, ALK, KRAS and BRAF mutations in Russian patients with lung adenocarcinoma: a single-center experience

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In contrast to other countries with predominantly white populations, Russian smoking-related lung cancers (LC) are mainly squamous cell carcinomas and approximately half lung adenocarcinomas (AdCa) are not related to tobacco consumption. Given that smoking significantly influences the probability of presence of actionable mutations in LC, one would expect that Russian lung AdCa patients would differ from other white populations in distribution of EGFR, ALK, KRAS and BRAF mutations. Herein, 2,336 consecutive lung AdCa cases, including 1,203 patients with known smoking status, were subjected to sequential testing for the above mutations. One quarter of lung AdCa patients carried either EGFR or ALK mutation with combined prevalence of 42% in those who had never smoked but only 8% in smokers. There was only a moderate difference in KRAS mutation frequency between ever- and never-smokers in EGFR/ALK-negative cases (31% vs. 23%), and this was mainly attributed to increased prevalence of G12C substitution in the former group. The occurrence of BRAF V600E mutation was 1.7% and 4% in EGFR/ALK/KRAS mutation-negative ever- and never-smokers, respectively. ALK testing of 470 EGFR-mutated tumors revealed only 1 (0.2%) instance of translocation. Similarly, KRAS testing identified 1 (1.25%) mutation in 80 EGFR-mutated AdCa and none in 48 ALK-rearranged AdCa. Therefore, concurrent actionable mutations in lung adenocarcinoma are exceptionally rare and sequential gene testing can be regarded as a reliable option.

Key words: lung adenocarcinoma, EGFR, ALK, KRAS, BRAF

The development of targeted therapies has provided crucial progress in the treatment of patients with lung adenocarcinoma (AdCa). A substantial portion of AdCa is caused by actionable mutations in the EGFR gene which sensitise the tumor to EGFR tyrosine kinase inhibitors [1–3]. ALK translocations are associated with pronounced tumor response to crizotinib, ceritinib and alectinib [4]. KRAS mutated proteins are not pharmaceutically targeted in clinical settings, but a subset of KRAS-associated lung cancers (LC) respond to inhibition of the MEK, a downstream member of the KRAS signaling cascade [5–7]. Importantly, intensive search for more effective therapies for this specific category of cancers is currently underway (https://www.cancer.gov/research/key-initiatives/ras). The inhibitors of mutated BRAF, either alone or in combination with MEK inhibitors, are active against the LC carrying BRAF V600E mutation [8–10]. In addition, some lung AdCa carry activated ROS1, RET, HER2 and MET kinases which can also be efficiently down-regulated by available targeted drugs [11–14].

Multigene testing for LC remains complicated because the majority of patients are diagnosed at an inoperable stage, so the tumor material is in tiny biopsies. In addition, the distribution of actionable mutations strongly depends on patient race, smoking, gender, age and country of residence. For example, lung adenocarcinomas in Russia appear to be significantly distinct from other countries with predominantly white populations. AdCa histology is a prevailing LC type in Europe and America, and the majority of AdCa diagnosed in these regions are related to tobacco smoke. In contrast, smoking-related cancers in Russia are usually squamous cell because of previous popularity of high-tar cigarettes. Accordingly, up to half of Russian lung AdCa is seen in never-smokers, and Russian AdCa patients demonstrate approximately twice higher rate of the EGFR mutations and ALK translocations compared to Western countries [15,

16]. These findings cause expectation that the distribution of other actionable genetic lesions has characteristic features, and this study therefore analyses the pattern of common somatic mutations in various Russian AdCa patient categories.

Patients and methods

This study considered all consecutive patients with lung AdCa referred to the N.N. Petrov Institute of Oncology for molecular genetic analysis from November 2013 to July 2016. Patients provided written informed consent. Nucleic acids were extracted from FFPE tissue using the TRIzol reagent (Invitrogen), and RNA was subjected to reverse transcription by RevertAid Reverse Transcriptase (Thermo Fisher Scientific) in the presence of random hexamers in accordance with the manufacturer's recommendations. The molecular tests were performed in a sequential manner (Figure 1), given that the driver mutations are mutually exclusive in most instances [17, 18]. All PCR primer sequences, reaction conditions and pictures illustrating positive and negative tests results are provided in the Supplementary Methods file.

EGFR mutations (exon 19 deletions and L858R point mutation) were detected as described in our earlier report [19], and the EGFR wild-type tumors were further subjected to analysis of ALK translocations using the two-step procedure: samples were screened for unbalanced expression of the 5'/3'-ends of the ALK transcript [20] and those with evidence of ALK rearrangement were subsequently genotyped for 18 known ALK fusion variants. The EGFR/ALK mutation-negative lung AdCa were further screened for the presence of KRAS mutations in codons 12–13, 61 and 146; high-resolution melting (HRM) analysis was used in the prescreening and allele-specific PCR and/or direct sequencing identified the type of mutation [21]. Similarly, combined allele-specific PCR for V600E substitution and HRM/sequencing for identification of rare exon 15 mutations were used for BRAF gene analysis [21]. Sanger sequencing was conducted with the GenomeLab GeXP Genetic Analysis System (Beckman Coulter) and PyroMark Q24 instrument (Quiagen) was used for pyrosequencing. In addition to sequential gene testing, we performed a separate study to evaluate the probability of concurrent occurrence of EGFR, ALK and KRAS mutations. Statistical analysis was done with R software (https://www.r-project.org/).

Results

Lung AdCa patient characteristics are presented in Table 1. Patterns of EGFR and ALK mutations in Russian patients

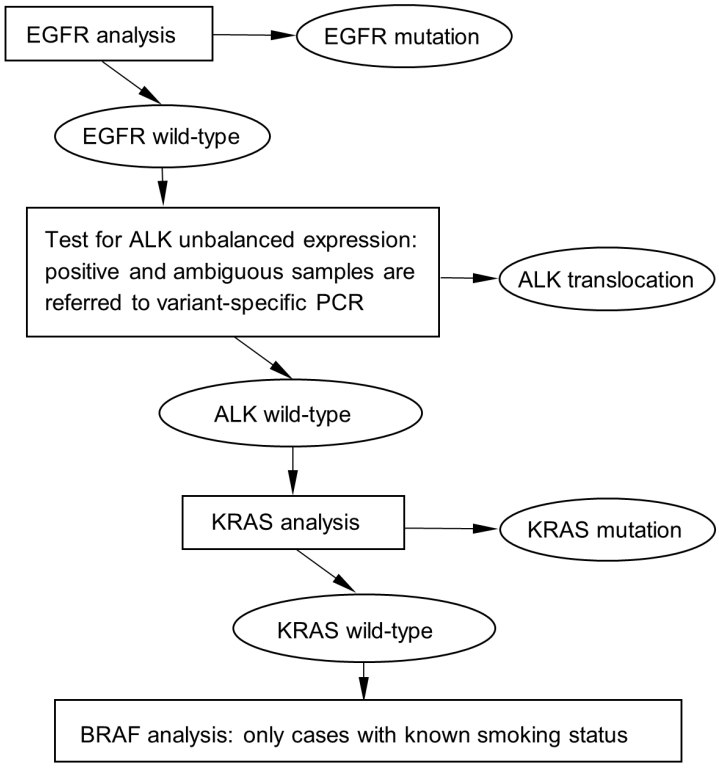

Figure 1. Genotyping workflow. Sequential genetic testing was implemented throughout this study: EGFR-mutations were studied first followed by the analysis of ALK, KRAS and BRAF genes.

with lung AdCa were already described in our previous reports [15, 16]. The EGFR and ALK mutations in this dataset agree with our previous results of being more prevalent in women and non-smokers (Table 2, Supplementary Table S1). In addition, ALK gene fusions were associated with younger patient age (median age was 55 years in ALK-positive group vs 61 years in the ALK-negative group, Mann-Whitney U test p-value = 2.53×10^{-8}) (Supplementary Table S2).

KRAS mutations were detected in 394/1,370 (29%) patients, who lacked actionable lesions in EGFR and ALK kinases. KRAS substitutions were only slightly more common in smokers (31%) than in non-smokers (23%).

The distribution of particular KRAS mutations subtypes strongly depended on smoking history (Table 3). Transversions were characteristic for smokers (29% vs 17.8%, Fisher's exact test p-value = 2.6×10^{-4}), and this was entirely attributed to G12C (GGT>TGT) exchange because the prevalence of non-G12C transversions did not depend on a history of tobacco exposure (Table 4). G12C (GGT>TGT) mutations occurred significantly more often in male than in female non-smokers and these mutations were associated with older

Table 1. Characteristics of patients with lung adenocarcinoma.

	Ever-smokers	Never-smokers	Smoking status unknown	All
Number	613	590	1133	2336
Gender (% females)	9.1%	78.1%	39.4%	41.2%
Age: median (range)	61 (30–86)	63 (26–85)	62 (23–88)	62 (23–88)

Table 2. Main genotyping results and comparison between 'ever- and never'-smokers.

	Ever-smokers	Never-smokers	Smoking status unknown	All	Ever-smokers vs never-smokers, chi-square test
EGFR mutations: L858R or ex19del	45/613 (7.3%)	214/590 (36.3%)	211/1133 (18.6%)	470/2336 (20.1%)	p<**2.2×10^{-16}**
ALK translocations (EGFR-negative cases)	6/568 (1.1%)	32/376 (8.5%)	57/922 (6.2%)	95/1866 (5.1%)	p=**3.1×10^{-8}**
KRAS mutations (EGFR/ALK-negative cases)	151/483 (31.3%)	71/303 (23.4%)	172/584 (29.5%)	394/1370 (28.8%)	p=**0.022**
BRAF V600E mutations[a] (EGFR/ALK/KRAS-negative cases)	5/294 (1.7%)	8/195 (4.1%)	1/54 (age ≥70, 1.9%)	14/543 (2.6%)	p=0.183

[a]In addition, two cases with K601E mutation in the BRAF gene were also identified (0.7%).

Table 3. KRAS mutation frequencies in EGFR/ALK mutation-negative lung adenocarcinomas[a].

		Ever-smokers	Never-smokers	Smoking status unknown	All	Ever-smokers vs never-smokers, Fisher's exact test
1	G12C (**G**GT>**T**GT)	64 (13.3%)	7 (2.3%)	58 (9.9%)	129 (9.4%)	p=**3.0×10^{-8}**
2	G12D (G**G**T>G**A**T)	20 (4.1%)	26 (8.6%)	35 (6.0%)	81 (5.9%)	p=**0.012**
3	G12V (G**G**T>G**T**T)	25 (5.2%)	14 (4.6%)	34 (5.8%)	73 (5.3%)	p=0.866
4	G12A (G**G**T>G**C**T)	8 (1.7%)	11 (3.6%)	11 (1.9%)	30 (2.2%)	p=0.096
5	Q61H (CA**A**>CA**T**/CA**C**)	6 (1.2%)	4 (1.3%)	7 (1.2%)	17 (1.2%)	p=1.000
6	G13C (**G**GC>**T**GC)	3 (0.6%)	1 (0.3%)	9 (1.5%)	13 (0.9%)	p=1.000
7	G12S (**G**GT>**A**GT)	2 (0.4%)	1 (0.3%)	8 (1.4%)	11 (0.8%)	p=1.000
8	G12R (**G**GT>**C**GT)	5 (1.0%)	1 (0.3%)	4 (0.7%)	10 (0.7%)	p=0.414
9	G13D (G**G**C>G**A**C)	5 (1.0%)	2 (0.7%)	1 (0.2%)	8 (0.6%)	p=0.713
10	Q61L (C**A**A>C**T**A)	3 (0.6%)	0	1 (0.2%)	4 (0.3%)	p=0.288
11	Q61R (C**A**A>C**G**A)	1 (0.2%)	2 (0.7%)	1 (0.2%)	4 (0.3%)	p=0.563
12	G12F (**GG**T>**TT**T)	2 (0.4%)	0	1 (0.2%)	3 (0.2%)	p=0.526
13	A146T (**G**CA>**A**CA)	1 (0.2%)	1 (0.3%)	0	2 (0.1%)	p=1.000
14	G13V (G**GC**>G**TT**)	2 (0.4%)	0	0	2 (0.1%)	p=0.526

[a]This table does not include variants, which were detected only in one case each: G12E, G13E, L19F, Y64C, A146P, A146V, A11G+G12C, G12C+G12D.

Table 4. Nucleotide changes in KRAS gene among patients with EGFR and ALK-negative lung adenocarcinomas.

	Ever-smokers	Never-smokers	Smoking status unknown	All	Ever-smokers vs never-smokers, Fisher's exact test
Transversions	142 (29.4%)	54 (17.8%)	167 (28.6%)	363 (26.5%)	p=0.00026
G>T	92 (19.0%)	22 (7.3%)	101 (17.3%)	215 (15.7%)	p=**$3.6 \cdot 10^{-6}$**
G>T, not G12C	28 (5.8%)	15 (5.0%)	43 (7.4%)	86 (6.3%)	p=0.748
G>C	14 (2.9%)	13 (4.3%)	15 (2.6%)	42 (3.1%)	p=0.319
A>C	3 (0.6%)	3 (1.0%)	4 (0.7%)	10 (0.7%)	p=0.681
A>T	6 (1.2%)	1 (0.3%)	4 (0.7%)	11 (0.8%)	p=0.259
Transitions	29 (6.0%)	32 (10.6%)	47 (8.0%)	108 (7.9%)	p=0.028
G>A	28 (5.8%)	30 (9.9%)	44 (7.5%)	102 (7.4%)	p=**0.036**
A>G	1 (0.2%)	2 (0.7%)	1 (0.2%)	4 (0.3%)	p=0.563
C>T	0	0	1 (0.2%)	1 (0.1%)	-
T>C	0	0	1 (0.2%)	1 (0.1%)	-
Complex	7 (1.4%)	0	2 (0.3%)	9 (0.7%)	p=0.048
dinucleotide	6 (1.2%)	0	1 (0.2%)	7 (0.5%)	p=0.087
double	1 (0.2%)	0	1 (0.2%)	2 (0.1%)	p=1.000

Table 5. Overview of large-scale studies on actionable mutations in lung adenocarcinomas[a].

Study	Lee et al. [26]	Hsu et al. [27]	Zheng et al. [28]	Kris et al. [29]	Barlesi et al. [25]	Current study
Country	Korea	Taiwan	China	USA	France	Russia
Number of lung adenocarcinoma cases	5015	1772	1407	1007	13425	2336
% of never-smokers	ND	66.5%	67%	34%	ND	49%
EGFR mutations	46%	55.7%; L858R+ex19del: 54.3%	61.5%	17%; L858R+ex19del: 16.6%	11.2%	L858R+ex19del: 20%
ever-smokers	ND	39.5%	41%	9.5%	ND	7%
never-smokers	ND	63.9%	71.5%	33%	ND	36%
KRAS mutations	9.2%	5%	8.1%	24%	31.5%	22%
ever-smokers	ND	12.3%	16.8%	34.9%	ND	28.5%
never-smokers	ND	1.7%	3.8%	4%	ND	13.5%
ALK rearrangements	7.2%	9.8% (29/295 for EGFR wild-type cases)	5.3%	8%	5.1%	4%
ever-smokers	ND	6.8% for EGFR wild-type cases	4.3%	4.4%	ND	1%
never-smokers	ND	11.9% for EGFR wild-type cases	5.7%	15%	ND	5.4%
BRAF exon 15 (V600E)	ND	0.6%	ND	1%	2.1%	1.5%
ever-smokers	ND	0.7%	ND	1.7%	ND	1.1%
never-smokers	ND	0.7%	ND	1%	ND	1.8%
Comment on the study design	Single-center study; no sufficient details describing the way of testing (sequential vs. parallel)	Multicenter study; all genetic tests were performed in parallel	Single-center study; all genetic tests were performed in parallel	Multicenter study; genetic tests were performed in parallel in most cases; racial distribution is not specified	Multicenter study; no sufficient details describing the way of testing (sequential vs. parallel)	Single-center study; sequential testing

ND: no data; [a]A systematic Pubmed search was conducted using the phrase *"EGFR AND ALK* AND KRAS AND (lung* OR pulmon* OR bronch*) AND (cancer* OR carcinoma* OR tumor* OR tumor* OR adenocarcinoma*) AND English [language]"*. We considered all original reports describing frequencies of EGFR, ALK and KRAS mutations within a single study. By February 15th, 2018 the search retrieved 515 results, which were subjected to manual analysis. We included in the table all studies, which described distribution of EGFR, ALK and KRAS in more than 1000 lung adenocarcinomas.

patient age (Supplementary Tables S1 and S2). Transitions tended to be associated with patient non-smoking status. Interestingly, dinucleotide substitutions were detected in 6 of 483 tumors observed in ever-smokers and in 1 of 584 tumors from patients with unknown smoking history, but not in any of 303 tumors from never-smokers (Fisher's exact test p-value = 0.087).

BRAF V600E mutations constituted a significant proportion of EGFR/ALK/KRAS mutation-negative lung AdCa from non-smokers (8/195, 4%) and were less common in smokers (5/294, 1.7%). The frequency of BRAF V600E substitutions in female non-smokers was as high as 6.1%. Median age of subjects with BRAF V600E mutations was evidently higher than in BRAF wild-type cases (72.5 vs. 63 years, p = 0.008).

We therefore analyzed an additional cohort of 54 elderly lung AdCa patients (70 years and over), however only one additional case of BRAF V600E mutation was identified (1.9%). In addition, two instances of rare K601E mutation in BRAF were found by sequencing of HRM-positive samples: one sample was from a 79 years old non-smoking woman and the other from a 57 years old male smoker. Although the predictive role of K601E mutation has not been systematically studied, available evidence suggests that it is not associated with response to inhibitors of mutated BRAF [22].

We then assessed if EGFR, ALK and KRAS mutations are mutually exclusive. ALK rearrangements were detected in 95/1,866 (5%) EGFR mutation-negative tumors but only 1/470 (0.2%) was found in AdCa with EGFR mutation. Similarly, KRAS mutations were common in EGFR wild-type AdCa (394/1418, 28%), but there was only one instance (1.25%) of concurrent KRAS lesion when we tested 80 carcinomas carrying EGFR mutation. KRAS testing of 48 AdCa with ALK rearrangements did not reveal tumors with KRAS gene lesion. Therefore, sequential gene testing is an acceptable approach for lung cancer molecular diagnosis. The frequency of EGFR, ALK, KRAS and BRAF mutations in the Russian consecutive series of AdCa is estimated to be 20%, 4%, 22% and 1.5%, respectively, being 7%, 1%, 28.5% and 1.1% in smokers and 36%, 5.4%, 13.5% and 1.8% in non-smokers.

Discussion

Although many studies describe the distribution of actionable mutations in lung cancer, most have limitations. For example, insufficient study size prevents consideration of important details such as explicit analysis of lung cancer clinical subsets and/or the distribution of some rare mutation categories. While many clinical investigations present relatively large data sets, they pool distinct histological types (for example, see [23, 24]) or do not specify frequencies of mutations separately for smokers vs. non-smokers or men vs. women [25, 26]. When comparing our results with published studies, we selected reports which recruited more than a thousand lung AdCa and provided data of EGFR, ALK and KRAS mutation distribution. Only 5 prior studies met this criteria [25–29], with three performed in Asians [26–28] and two in countries with predominantly white population [25, 29]. Furthermore, only three of these studies considered AdCa mutation frequency separately in smokers and non-smokers [27–29] (Table 5).

Referral bias can also significantly affect the results of mutation analysis in lung cancer. EGFR and ALK mutations are well known to be highly over-represented in non-smokers and women, therefore these categories of patients are likely to be preferred when considering molecular tests in clinical decision-making. Furthermore, the probability of finding EGFR mutations is significantly increased for Asians, thus providing a further source of referral bias in multi-racial countries. For example, the study of Kris et al. [29], which encouraged the tailoring of patients to approved or investigational targeted drugs, included large number of patients with AdCa and considered smoking history when calculating mutation frequencies (Table 5). Interestingly, the proportion of never-smokers, EGFR mutations and ALK translocations in the study of Kris et al. [29] is somewhat higher than in the majority of Western studies [25, 30, 31]; authors did not comment on the race of the recruited patients [29]. Therefore, it is possible that the inclusion of Asian descent patients could contribute to the elevated frequency of EGFR mutations.

The series presented in this study consisted of patients who were referred for genetic analysis by their primary physicians. In order to evaluate referral bias, we compared our current data set (years 2013–2016) with our previous study, where all surgically resected lung AdCa cases (years 2000 – 2005) were consecutively collected from the pathological archive [32]. Here, it is essential to emphasize that all relevant data from the current clinical collection was virtually identical to that in our previous investigation involving consecutive lung AdCa (EGFR mutation frequency 470/2336 (20.1%) vs. 38/192 (19.8%); proportion of 'never-smokers': 590/1203 (49%) vs. 94/192 (49%); proportion of women: 962/2336 (41.2%) vs. 76/192 (39.6%)). This is an important indicator, demonstrating that all patients with lung AdCa are currently referred for genetic testing irrespective of smoking history and gender. This lack of referral bias reflects current situation with lung cancer treatment in Russia, where first-generation EGFR tyrosine kinase inhibitors are fully accessible to all patients with TKI sensitizing mutations and health professionals are encouraged to perform EGFR testing for all patients with non-squamous lung cancer.

Adenocarcinoma is a predominant type of lung cancer in the Western world, and its high incidence is largely attributed to the consumption of low-tar cigarettes. In contrast to Western countries, where most lung AdCa patients are smokers, approximately half of Russian lung AdCa are observed in non-smokers. This study is therefore one of the largest series of smoking-unrelated lung AdCa collected in European patients.

Distribution of KRAS mutations in lung AdCa has also been analyzed in a number of studies. It is frequently stated that the occurrence of KRAS mutations is significantly higher in smokers compared to non-smokers [33, 34]. However, this conclusion is certainly true only for non-selected patients with lung AdCa. If one considers the most common actionable LC mutations , which affect EGFR and ALK genes, these are characterized by low occurrence in smokers but account for almost half of AdCa in white non-smokers. Accordingly, when we exclude patients who are candidates for tyrosine kinase inhibitor therapy, smoking is no longer a factor considerably affecting the likelihood of finding the KRAS mutation.

Furthermore, while some KRAS mutations are indeed more prevalent in smokers, other types of substitutions in this gene are clearly associated with no tobacco consumption. The lung tumors in smokers usually carry a large overall number of transversions, while transitions are characteristic for smoking-independent cancers [35]. Although several reports demonstrate that this trend applies to KRAS mutations [36–38], we found that only KRAS G12C transversion is more commonly observed in smokers vs. non-smokers, while frequencies of other KRAS transversions are independent of smoking status. If drugs targeting KRAS-mutated lung cancers eventually enter clinical practice, it will be essential to consider all these data in order to ensure that both smokers and non-smokers have equal chance of being tested for KRAS status.

Use of combined inhibition of mutated BRAF kinase and MEK kinase was shown to be a viable approach in treating lung cancer with BRAF V600E substitution. Unlike with EGFR, ALK and KRAS, associations with clinical parameters for BRAF mutations are less defined and this is mainly due to the limited number of published studies. Two meta-analyses indicated higher prevalence of BRAF V600E mutation in females and never-smokers [39, 40], thus supporting our findings. The overall frequency of BRAF V600E mutation in Russian lung AdCa patients is estimated to be approximately 1.5% which is at the lower limit of variations reported in patients of European origin (1.6–2.8%) [41–43], but it is higher than the frequency observed in Asian patients [27, 44]. This difference is likely to be explained by the larger proportion of EGFR-mutant lung AdCa cases in East Asia compared to Europe [45] with intermediate frequency characteristic of the Russian series [16, 32].

Several studies [46–48] demonstrated frequent co-occurrence of driver mutations in lung AdCa. Notably, one of these studies utilized extremely sensitive methods to detect concomitant mutations present in rare sub-populations of tumor cells [48]. The clinical significance of such findings is yet to be determined. Our data highlights that concurrent presence of EGFR, ALK and KRAS mutations is very rare, at least when moderately sensitive assays, such as high-resolution melting analysis (HRM), are used in mutation screening; and this finding is in agreement with most published data.

While next-generation sequencing and simultaneous testing of all potentially relevant genes is likely to replace current diagnostic methods in lung cancer molecular analysis in the near future, now personalized prescription of molecular tests remains standard clinical practice in most cancer clinics worldwide. This study confirms the reliability of sequential lung cancer testing for actionable mutations, and describes the previously unrecognized peculiarities in their distribution.

Supplementary information is available in the online version of the paper.

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Supplementary Methods

Supplementary Methods Table 1. PCR tests design: primers, probes and conditions.

Test	**PCR composition and conditions**	**Primers: forward; reverse; TaqMan probe / pyrosequencing primer (where applicable)**		**PCR fragment length**
EGFR gene[a]				
PCR followed by gel-electrophoresis	10 mkl volume reaction: 1-x GeneAmp PCR buffer I (Applied Biosystems), 0.5U Taq M hot-start DNA polymerase (AlkorBio), 2.5 mM $MgCl_2$, 250 mkM dNTP, 200 nM primers. PCR program: 95°C 10 min, followed by 45 cycles (95°C 20 sec, 58°C 30 sec, 72°C 30 sec). Device: MyCycler (Bio-Rad).	CTGTCATAGGGACTCTGGAT; CAGCAAAGCAGAAACTCACAT		127 bp (WT allele)
Real-time AS-PCR for L858R mutation	20 mkl volume reaction: 1-x GeneAmp PCR buffer I (Applied Biosystems), 1U Taq M hot-start DNA polymerase (AlkorBio), 2.5 mM MgCl2, 250 mkM dNTP, 1-x SYBR Green I, 100 nM primers. PCR program: 95°C 10 min, followed by 45 cycles (95°C 15 sec, 60°C 30 sec, 72°C 30 sec), then melting from 65°C to 95°C. Device: CFX96 (Bio-Rad).	WT-specific reaction	GCATGAACTACTTGGAGGAC; TCCGCACCCAGCAGTTTGGCTA	120 bp
		L858R-specific reaction	GCATGAACTACTTGGAGGAC; TCCGCACCCAGCAGTTTGGCTC	120 bp
ALK gene[b]				
Test for unbalanced 3'/5'-end ALK expression (qPCR)	20 mkl volume reaction: 1-x GeneAmp PCR buffer I (Applied Biosystems), 1U Taq M hot-start DNA polymerase (AlkorBio), 2.5 mM $MgCl_2$, 250 mkM of dNTP, 175 nM primers and TaqMan probe. PCR program: 95°C, 10 min – 50 cycles (95°C 15 sec, 60°C 1 min) Device: CFX96 (Bio-Rad)	5'-end amplification (exons 9-11)	CCTCTCCTCGATGTGTCTGA; CTTGTCCTCTCCGCTAATGGT; FAM-CATCGTGGCTTTTGACAATATCTC-BHQ1	135 bp
		3'-end amplification (exons 22-23)	TGTGCTCTGAACAGGACGAACT; TGAGCTCCAGCAGGATGAACC; FAM-ATGGAAGCCCTGATCATCAGCAAAT-BHQ1	132 bp

(continued on the next page)

Test	PCR composition and conditions	Primers: forward; reverse; TaqMan probe / pyrosequencing primer (where applicable)		PCR fragment length
Detection of specific ALK fusions (qPCR)	20 mkl volume reaction: 1-x GeneAmp PCR buffer I (Applied Biosystems), 1U Taq M hot-start DNA polymerase (AlkorBio), 2.5 mM $MgCl_2$, 250 mkM dNTP. 175 nM primers and TaqMan probe. PCR program: 95°C, 10 min – 50 cycles (95°C 15 sec, 60°C 1 min) Device: CFX96 (Bio-Rad)	EML4ex13; ALKex20 (V.1)	TGGAGCAAAACTACTGTAGAG; GTCGAGGTGCGGAGCTTG; FAM-CTTGCTCAGCTTGTACTCAGGGC-BHQ1	134 bp
		EML4ex20; ALKex20 (V.2)	CTAACTCGGGAGACTATGAAAT; GTCGAGGTGCGGAGCTTG; FAM-CTTGCTCAGCTTGTACTCAGGGC-BHQ1	118 bp
		EML4ex6; ALKex20 (V.3a/b)	CATAAAGATGTCATCATCAACCA; GTCGAGGTGCGGAGCTTG; FAM-CTTGCTCAGCTTGTACTCAGGGC-BHQ1	V3a – 113 bp, V3b – 146 bp
		EML4ex15; ALKex20 (V.8)	AGTATGGCACAATCAGAGCTG; TAGTTGGGGTTGTAGTCGGT; FAM-ATTTTTAGTAGGCAAGCTCCGCAC-BHQ1	100 bp
		EML4ex18; ALKex20 (V.9)	ACACAGACGGGAATGAACAG; GTCGAGGTGCGGAGCTTG; FAM-CTTGCTCAGCTTGTACTCAGGGC-BHQ1	133 bp
		EMLex2; ALKex20 (V.5a)	GCAATCTCTGAAGATCATGTG; GTCGAGGTGCGGAGCTTG; FAM-CTTGCTCAGCTTGTACTCAGGGC-BHQ1	140 bp
		EMLex2; ins117ALKex20 (V.5b)	GCAATCTCTGAAGATCATGTG; TACACAGGCCACTTCCTACA; FAM-CAGTCTCAAGTAAAGGTTCAGAGC-BHQ1	115 bp
		EMLex14; ins11del49ALKex20 (V.4), EMLex14; del12ALKex20 (V.7)	TGGAGGAGGGAAAGACAGA; GTCGAGGTGCGGAGCTTG; FAM-CTTGCTCAGCTTGTACTCAGGGC-BHQ1	V4 – 117 bp, V7 – 142 bp
		EMLex13; ins69 ALKex20 (V.6)	TGGAGCAAAACTACTGTAGAG; TGGCCCTTGAAGCACTACAC; FAM-GGAAAGGACCTAAAGGAAGTGGC-BHQ1	76 bp
		KIF5Bex24; ALKex20	CGCATAAAGGAAGCAGTCAG; GTCGAGGTGCGGAGCTTG; FAM-CTTGCTCAGCTTGTACTCAGGGC-BHQ1	149 bp
		KIF5Bex17; ALKex20	CGATGCCCTCAGTGAAGAAC; GTCGAGGTGCGGAGCTTG; FAM-CTTGCTCAGCTTGTACTCAGGGC-BHQ1	129 bp
		KIF5Bex15; ALKex20	AGCAGCTGAGATGATGGCA; GTCGAGGTGCGGAGCTTG; FAM-CTTGCTCAGCTTGTACTCAGGGC-BHQ1	164 bp
		TFGex3; ALKex20	AGTAGGATACTGAAACTGACAT; GTCGAGGTGCGGAGCTTG; FAM-CTTGCTCAGCTTGTACTCAGGGC-BHQ1	116 bp
		KLC1ex9; ALKex20	TCTCACTCGTGCACATGAAAG; GTCGAGGTGCGGAGCTTG; FAM-CTTGCTCAGCTTGTACTCAGGGC-BHQ1	129 bp
		DCTN_ex26; ALKex20	CTGGTCTCTGGCATTGCTG; GTCGAGGTGCGGAGCTTG; FAM-CTTGCTCAGCTTGTACTCAGGGC-BHQ1	104 bp
		SQSTM1ex5; ALKex20	TGAAGAACGTTGGGGAGAGT; GTCGAGGTGCGGAGCTTG; FAM-CTTGCTCAGCTTGTACTCAGGGC-BHQ1	127 bp

(continued on the next page)

Test	PCR composition and conditions	Primers: forward; reverse; TaqMan probe / pyrosequencing primer (where applicable)		PCR fragment length
KRAS gene[c]				
HRM analysis and pyrosequencing for KRAS codons 12 -13	20 mkl volume reaction: 1-x GeneAmp PCR buffer I (Applied Biosystems), 1U Taq M hot-start DNA polymerase (AlkorBio), 3.5 mM $MgCl_2$, 250 mkM dNTP, 1-x EvaGreen (Biotium), 200 nM primers. PCR program: 95°C 10 min, followed by 50 cycles (95°C 15 sec, 60°C 30 sec, 72°C 30 sec), then melting from 65°C to 95°C. Device: LightCycler 96 instrument (Roche Life Science).	AATGACTGAATATAAACTTGTGG; biotin-CAAGATTTACCTCTATTGTTGG; TGTGGTAGTTGGAGC		122 bp
Real-time AS-PCR for frequent KRAS mutations in codons 12-13	20 mkl volume reaction: 1-x GeneAmp PCR buffer I (Applied Biosystems), 1U Taq M hot-start DNA polymerase (AlkorBio), 2.0 mM MgCl2, 250 mkM dNTP, 1-x SYBR Green I, 150 nM primers. PCR program: 95°C 10 min, followed by 50 cycles (95°C 15 sec, 62°C 30 sec, 72°C 30 sec), then melting from 65°C to 95°C. Device: CFX96 (Bio-Rad).	WT-specific reaction	CTTGTGGTAGTTGGAGCTGG; TGTATCAAAGAATGGTCCTGC	149 bp
		G12D-specific reaction	CTTGTGGTAGTTGGAGCTGA; TGTATCAAAGAATGGTCCTGC	149 bp
		G12V-specific reaction	CTTGTGGTAGTTGGAGCTGT; TGTATCAAAGAATGGTCCTGC	149 bp
		G13D-specific reaction	GGTAGTTGGAGCTGGTGA; TGTATCAAAGAATGGTCCTGC	144 bp
		G12C-specific reaction	ACTTGTGGTAGTTGGAGCTT; TGTATCAAAGAATGGTCCTGC	150 bp
		G12A-specific reaction	CTTGTGGTAGTTGGAGCTGC; TGTATCAAAGAATGGTCCTGC	149 bp
		G12S-specific reaction	CTTGTGGTAGTTGGAGCTA; TGTATCAAAGAATGGTCCTGC	149 bp
		G12R-specific reaction	CTTGTGGTAGTTGGAGCTC; TGTATCAAAGAATGGTCCTGC	149 bp
		G13C-specific reaction	TTGTGGTAGTTGGAGCTGGTT; TGTATCAAAGAATGGTCCTGC	148 bp
		G13R-specific reaction	GTGGTAGTTGGAGCTGGTGC; TGTATCAAAGAATGGTCCTGC	146 bp
HRM analysis and pyrosequencing for KRAS codons 59-61[d]	20 mkl volume reaction: 1-x GeneAmp PCR buffer I (Applied Biosystems), 1U Taq M hot-start DNA polymerase (AlkorBio), 2.5 mM $MgCl_2$, 250 mkM dNTP, 1-x EvaGreen (Biotium), 200 nM forward primer, 70 nM reverse primer. PCR program: 95°C 10 min, followed by 50 cycles (95°C 15 sec, 60°C 30 sec, 72°C 30 sec), then melting from 65°C to 95°C. Device: LightCycler 96 instrument (Roche Life Science).	biotin-TGTAAAACGACGGCCAGTACCTGTCTCTTGGATATTCTC; AGCGGATAACAATTTCACACAGGTACTGGTCCCTCATTGCAC; GTCCCTCATTGCACTGT		106 bp

(continued on the next page)

Test	**PCR composition and conditions**	**Primers: forward; reverse; TaqMan probe / pyrosequencing primer (where applicable)**		**PCR fragment length**
Real-time AS-PCR for frequent mutations in codons 59-61	20 mkl volume reaction: 1-x GeneAmp PCR buffer I (Applied Biosystems), 1U Taq M hot-start DNA polymerase (AlkorBio), 2.0 mM MgCl2 in total, 250 mkM dNTP, 1-x SYBR Green I dye, 130 nM primers. PCR program: 95°C 10 min, followed by 50 cycles (95°C 15 sec, 62°C 30 sec, 72°C 30 sec), then melting from 65°C to 95°C. Device: CFX96 (Bio-Rad).	WT-specific reaction	GACTGTGTTTCTCCCTTCTCA; CTGTACTCCTCTTGACCTGC	105 bp
		A59G-specific reaction	GACTGTGTTTCTCCCTTCTCA; CACTGTACTCCTCTTGACCTC	107 bp
		A59T-specific reaction	GACTGTGTTTCTCCCTTCTCA; CTGTACTCCTCTTGACCTGT	105 bp
		Q61H (CAA>CAC)-specific reaction	GACTGTGTTTCTCCCTTCTCA; TCATTGCACTGTACTCCTCG	113 bp
		Q61H (CAA>CAT)-specific reaction	GACTGTGTTTCTCCCTTCTCA; CTCATTGCACTGTACTCCTCA	114 bp
		Q61L-specific reaction	GACTGTGTTTCTCCCTTCTCA; TCATTGCACTGTACTCCTCTA	113 bp
		Q61R-specific reaction	GACTGTGTTTCTCCCTTCTCA; TCATTGCACTGTACTCCTCTC	113 bp
		Q61K-specific reaction	GACTGTGTTTCTCCCTTCTCA; TCATTGCACTGTACTCCTCTTT	113 bp
		Q61E-specific reaction	GACTGTGTTTCTCCCTTCTCA; TCATTGCACTGTACTCCTCTTC	113 bp
HRM analysis and pyrosequencing for KRAS codon 146	20 mkl volume reaction: 1-x GeneAmp PCR buffer I (Applied Biosystems), 1U Taq M hot-start DNA polymerase (AlkorBio), 3.5 mM $MgCl_2$, 250 mkM dNTP, 1-x EvaGreen (Biotium), 200 nM primers. PCR program: 95°C 10 min, followed by 50 cycles (95°C 15 sec, 60°C 30 sec, 72°C 30 sec), then melting from 65°C to 95°C. Device: LightCycler 96 instrument (Roche Life Science).	biotin-GTAGACACAAAACAGGCTCAG; TGTATTTATTTCAGTGTTACTTAC; TGTTACTTACCTGTCTTG		102 bp
Real-time AS-PCR for frequently occurring mutations in codon 146	20 mkl volume reaction: 1-x GeneAmp PCR buffer I (Applied Biosystems), 1U Taq M hot-start DNA polymerase (AlkorBio), 2.5 mM $MgCl_2$, 250 mkM dNTP, 1-x SYBR Green I, 175 nM primers. PCR program: 95°C 10 min, followed by 50 cycles (95°C 15 sec, 62°C 30 sec, 72°C 30 sec), then melting from 65°C to 95°C. Device: CFX96 (Bio-Rad).	WT-specific reaction	AGATGTACCTATGGTCCTAGTA; ACTTACCTGTCTTGTCTTTGC	136 bp
		A146T-specific reaction	AGATGTACCTATGGTCCTAGTA; ACTTACCTGTCTTGTCTTTGT	136 bp
		A146P-specific reaction	AGATGTACCTATGGTCCTAGTA; ACTTACCTGTCTTGTCTTTGG	136 bp
		A146V-specific reaction	AGATGTACCTATGGTCCTAGTA; GTTACTTACCTGTCTTGTCTTTA	139 bp

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Test	PCR composition and conditions	Primers: forward; reverse; TaqMan probe / pyrosequencing primer (where applicable)		PCR fragment length
BRAF gene[c]				
HRM analysis and pyrosequencing for BRAF exon 15	20 mkl volume reaction: 1-x GeneAmp PCR buffer I (Applied Biosystems), 1U Taq M hot-start DNA polymerase (AlkorBio), 3.5 mM $MgCl_2$, 250 mkM dNTP, 1-x EvaGreen (Biotium), 200 nM primers. PCR program: 95°C 10 min, followed by 50 cycles (95°C 15 sec, 60°C 30 sec, 72°C 30 sec), then melting from 65°C to 95°C. Device: LightCycler 96 instrument (Roche Life Science).	CCTTTACTTACTACACCTCAG; biotin-CACAAAATGGATGCAGACAACT; GACCTCACAGTAAAAATAG		136 bp
Real-time AS-PCR for V600E mutation	20 mkl volume reaction: 1-x GeneAmp PCR buffer I (Applied Biosystems), 1U Taq M hot-start DNA polymerase (AlkorBio), 2.5 mM $MgCl_2$, 250 mkM dNTP, 1-x SYBR Green I, 130 nM primers. PCR program: 95°C 10 min, followed by 50 cycles (95°C 15 sec, 62°C 30 sec, 72°C 30 sec) and melting from 65°C to 95°C. Device: CFX96 (Bio-Rad).	WT-specific reaction	GGTGATTTTGGTCTAGCTACAGT; ATAGCCTCAATTCTTACCATCC	101 bp
		V600E-specific reaction	GGTGATTTTGGTCTAGCTACAGA; ATAGCCTCAATTCTTACCATCC	101 bp

Abbreviations: PCR – polymerase chain reaction; AS-PCR – allele-specific PCR; qPCR – quantitative real-time PCR; HRM – high resolution melting; dNTP – deoxynucleotide; WT – wild-type.

[a]Primer sequences and reaction conditions were taken from [Mitiushkina et al., Cancer Cytopathol 2013; 121(7): 370-376]. Allele-specific primers for EGFR L858R mutation detection were modified to increase the reaction specificity.

[b]This method was described in detail in [Iyevleva et al., Cancer Lett 2015; 362(1): 116-121].

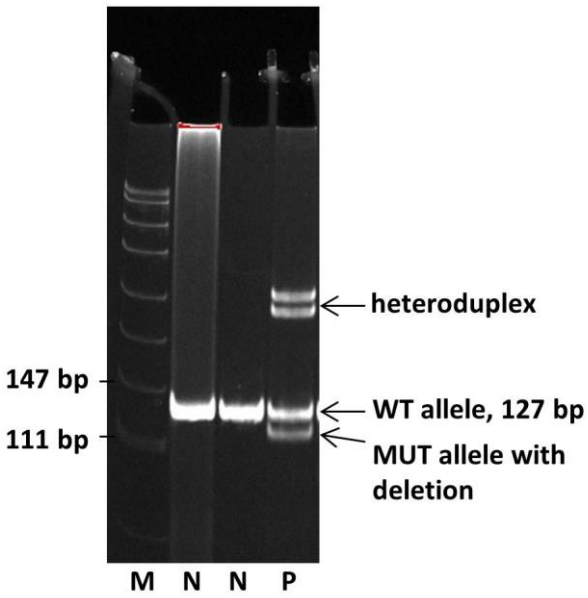
[c]Analysis of mutations in KRAS codons 12-13, 61, 146 and BRAF exon 15 was previously described in [Yanus et al., Med Oncol 2013; 30(3): 686].

[d]Primers used for this reaction contain additional 5'-sequences to increase PCR fragment length; that was done to improve results of direct Sanger sequencing.

Examples of mutation tests

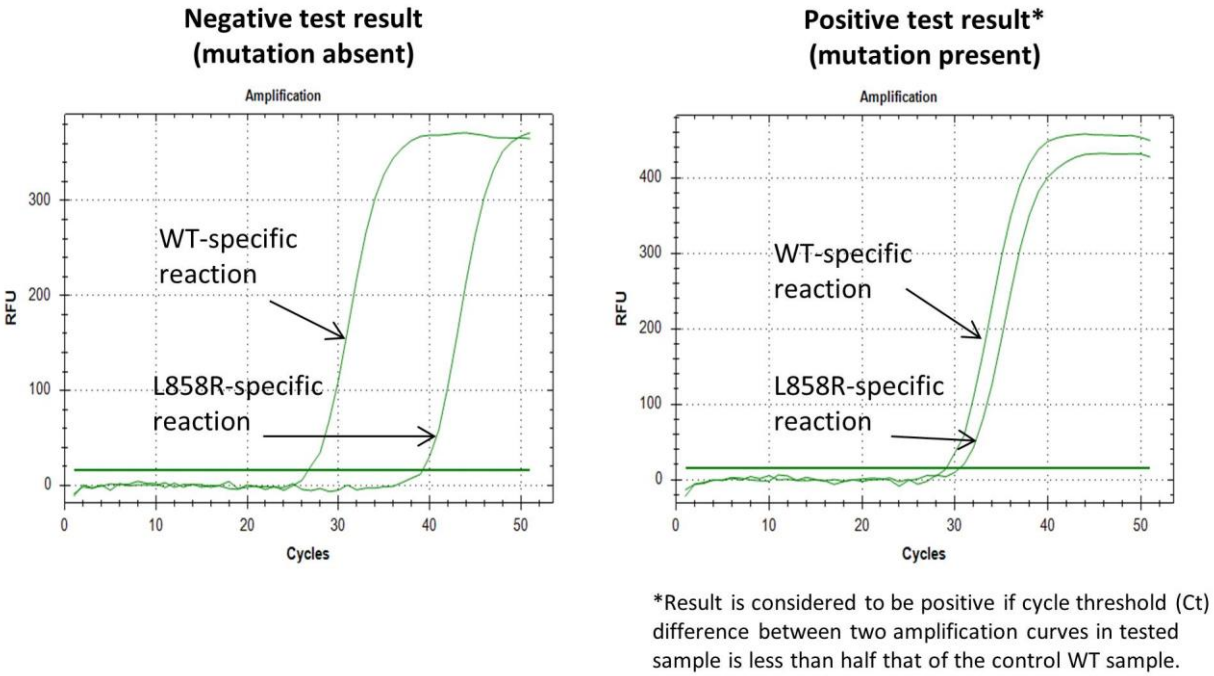
EGFR gene

a) Test for deletions and insertions in exon 19 (PCR, followed by polyacrylamide gel electrophoresis)



Abbreviations: P – positive result; N – negative result; M – molecular weight marker; WT – wild-type; MUT - mutant

b) Test for L858R point mutation (allele-specific PCR)

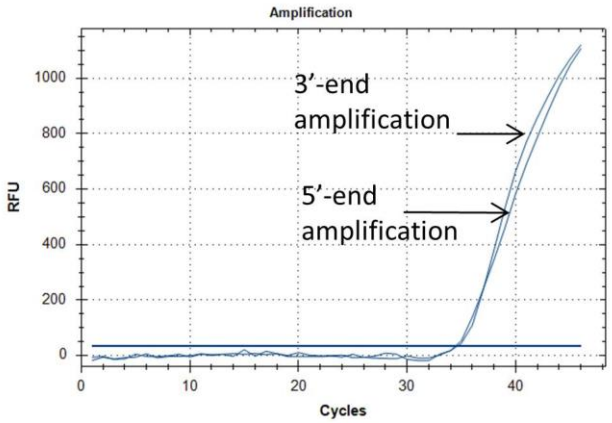


Abbreviations: WT – wild-type

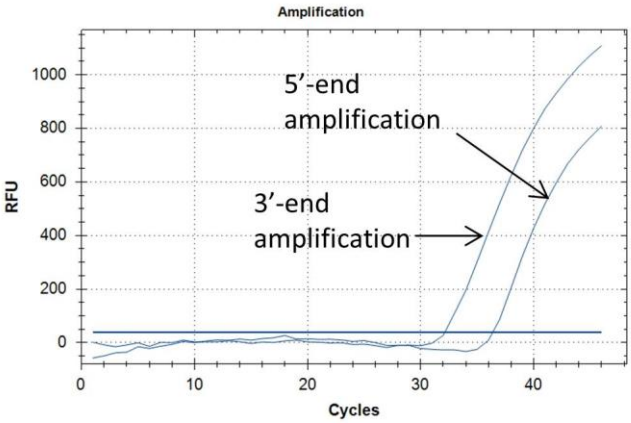
ALK gene

a) Test for unbalanced 3’/5’ ALK expression*

Negative test result
(balanced 3’/5’-ends expression
or no ALK expression)



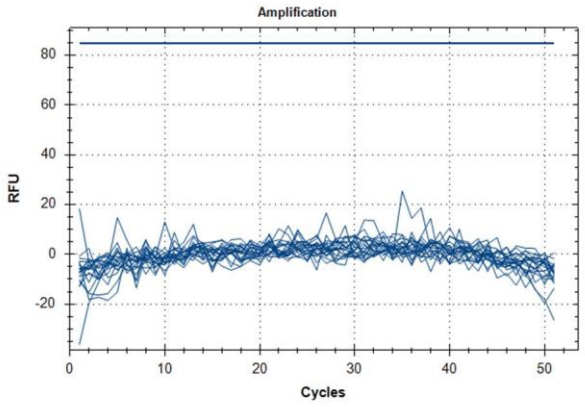
Positive test result
(unbalanced 3’/5’-ends expression)



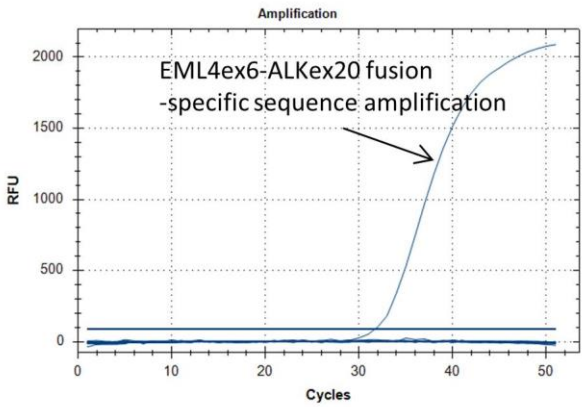
*This test is based on a method originally suggested by Wang R. et al [Clin Cancer Res. 2012; 18(17):4725-4732] and is described in detail in Iyevleva A.G. et al. [Cancer Lett 2015; 362(1): 116-121].

b) PCR tests for specific ALK gene rearrangements (applied to cases with unbalanced 3’/5’-ends ALK expression)

Negative test result
(sample tested negative for all
known ALK fusions)

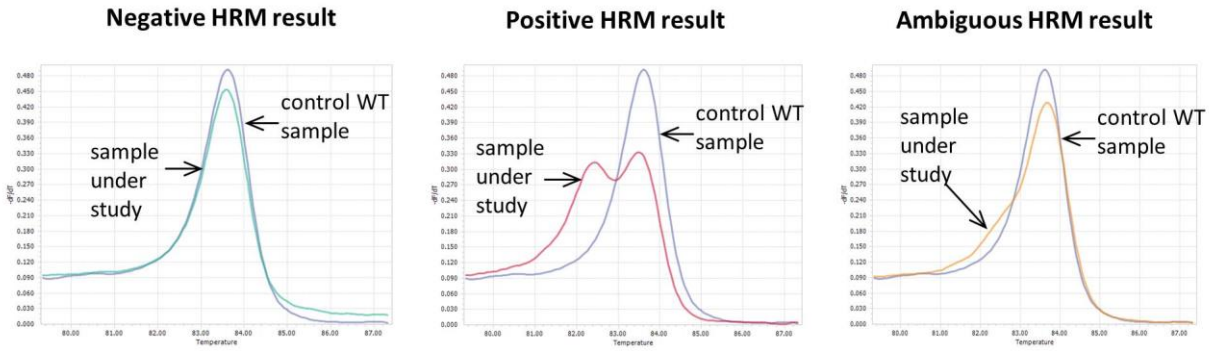


Positive test result



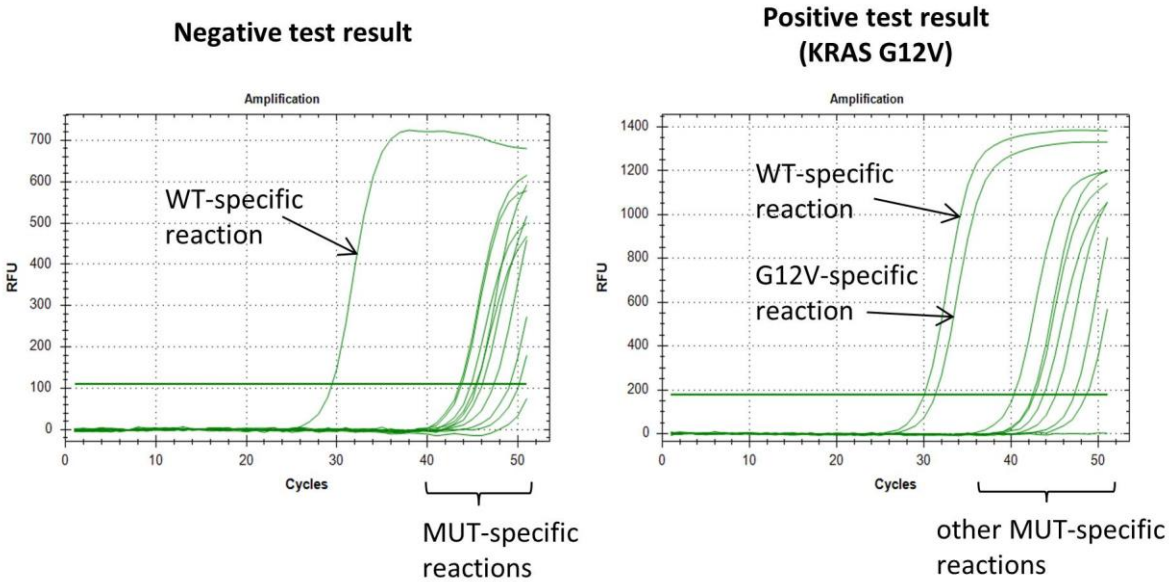
KRAS gene

a) HRM analysis for exon 2 (KRAS codons 12-13)



Abbreviations: HRM – high resolution melting curve analysis; WT – wild-type

b) Allele-specific PCR (AS-PCR) test for nine frequently occurring mutations in codons 12 and 13 (applied to cases with positive or ambiguous HRM test results).

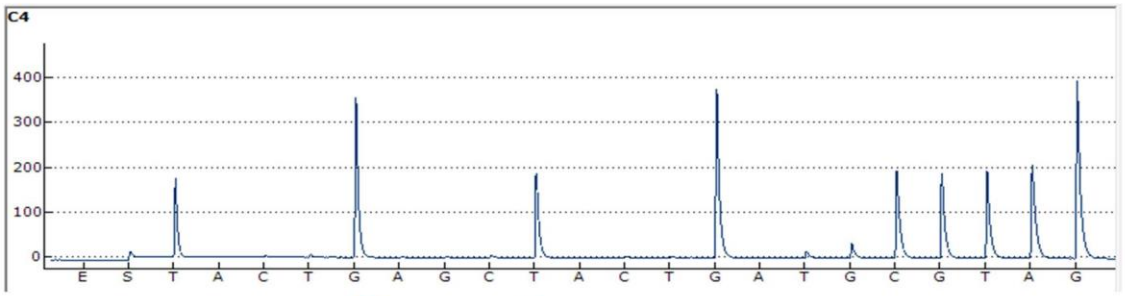


Abbreviations: WT – wild-type; MUT - mutant

c) Pyrosequencing or Sanger sequencing of the DNA fragment containing KRAS codons 12-13 (applied to cases with positive or ambiguous HRM test results) if:

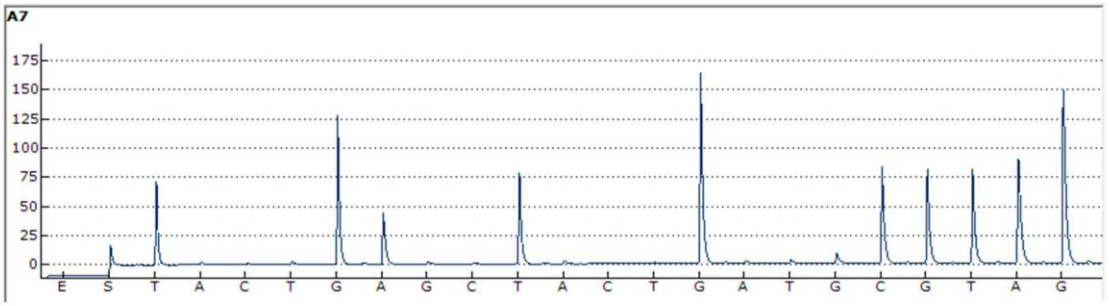
- DNA quality or quantity was insufficient for AS-PCR
- AS-PCR results were negative for all tested mutations
- AS-PCR result was inconclusive.

Negative pyrosequencing result



WT allele: T GG T GG C G T A GG

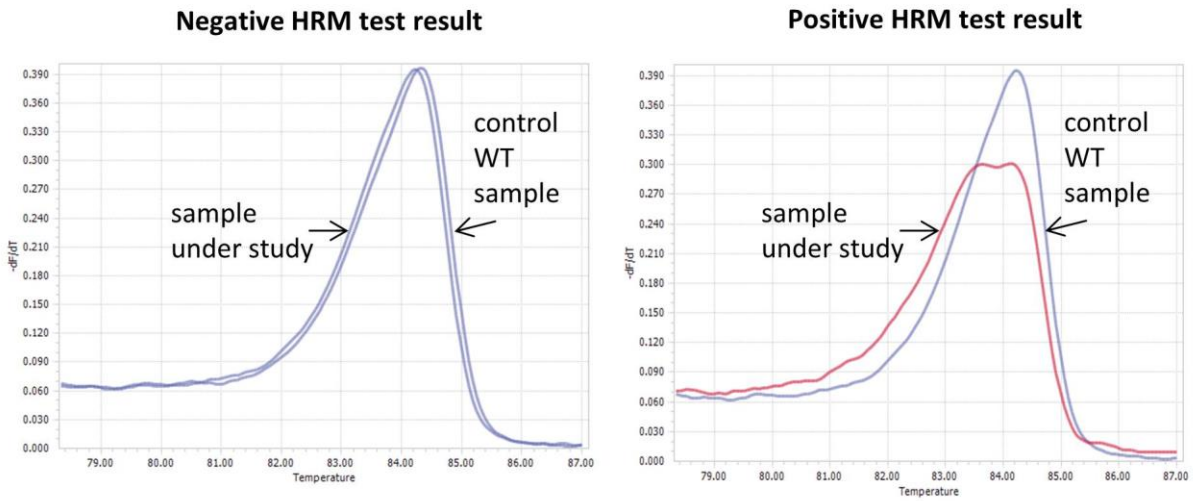
Positive pyrosequencing result
(codon 12 GGT > GAT, KRAS G12D mutation)



WT allele: T GG T GG C G T A GG
MUT allele: T G A T GG C G T A GG

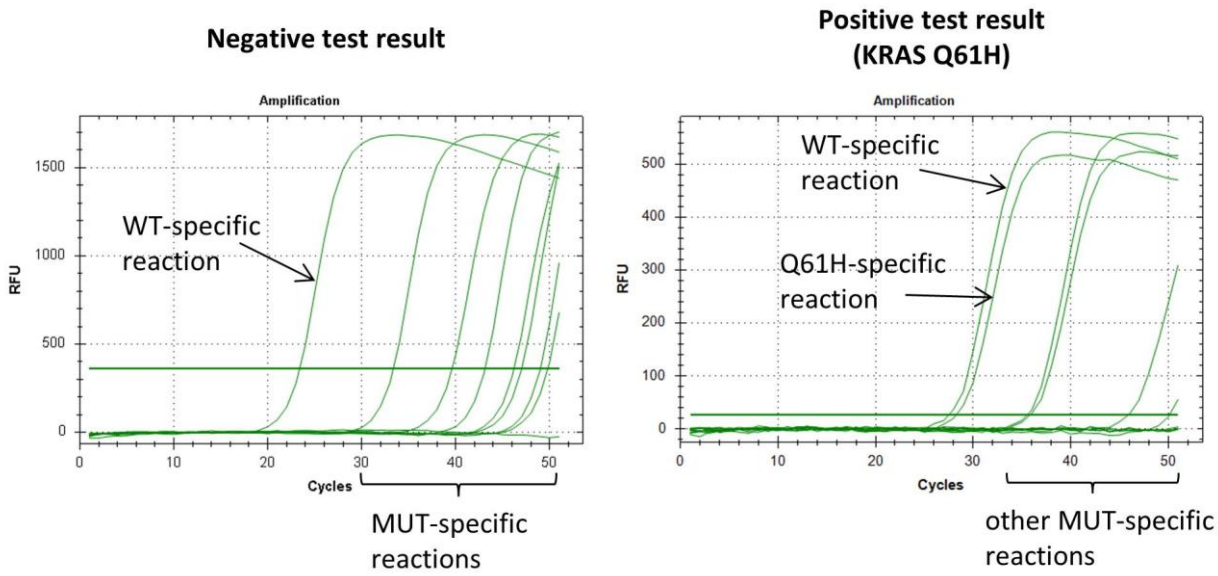
Abbreviations: WT – wild-type; MUT - mutant

d) HRM analysis for KRAS exon 3 (codons 59-61)



Abbreviations: HRM – high resolution melting curve analysis; WT – wild-type

e) Allele-specific PCR (AS-PCR) test for eight frequently occurring mutations in KRAS codons 59-61 (applied to cases with positive or ambiguous HRM test results).



Abbreviations: WT – wild-type; MUT - mutant

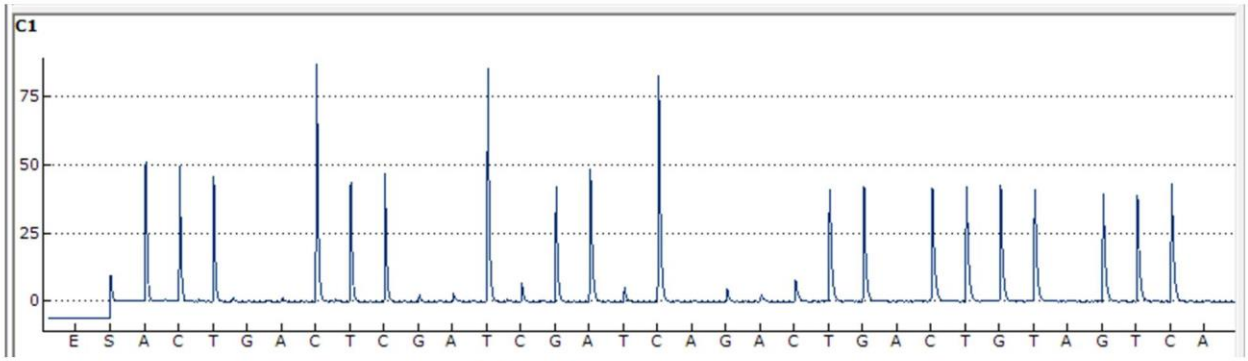
f) Pyrosequencing or Sanger sequencing of the DNA fragment containing KRAS codons 59-61 (applied to cases with positive or ambiguous HRM test results) if:

- DNA quality or quantity was insufficient for AS-PCR

- AS-PCR results were negative for all tested mutations

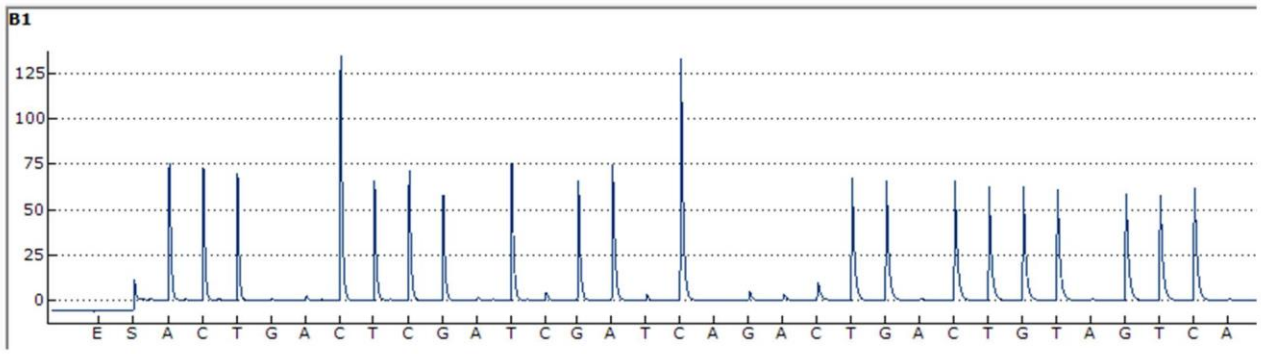
- AS-PCR result was inconclusive.

Negative pyrosequencing result



WT allele: A C T CC T C **TT** **G** A CC T G C T G T G T C
(r e v e r s e c o m p l e m e n t s e q u e n c e)

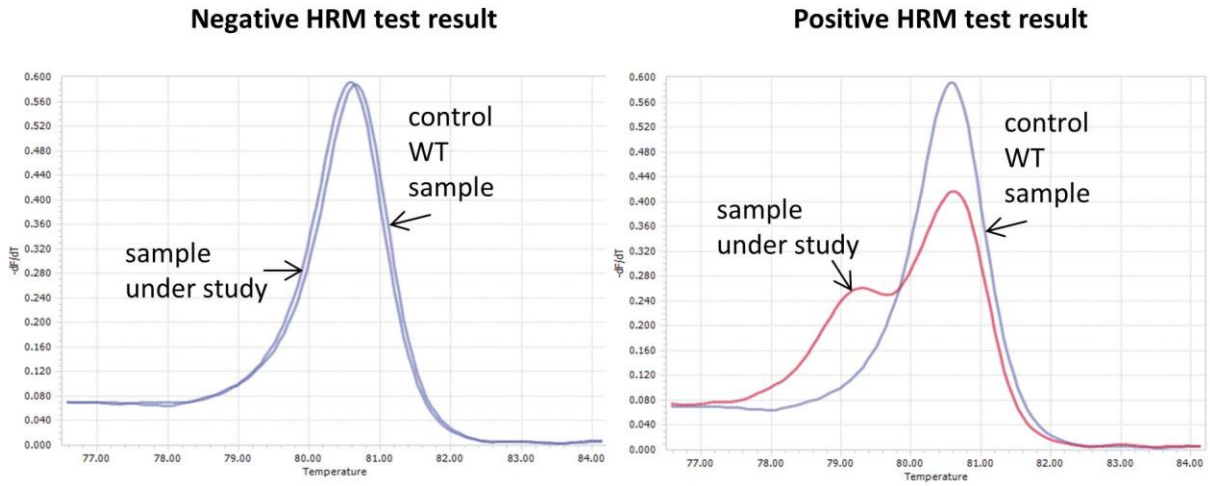
Positive pyrosequencing result
(codon 61 CAA > CAC [reverse complement TTG > GTG],
KRAS Q61H mutation)



WT allele: A C T CC T C **TT** **G** A CC T G C T G T G T C
MUT allele:A C T CC T C **G** **T** **G** A CC T G C T G T G T C

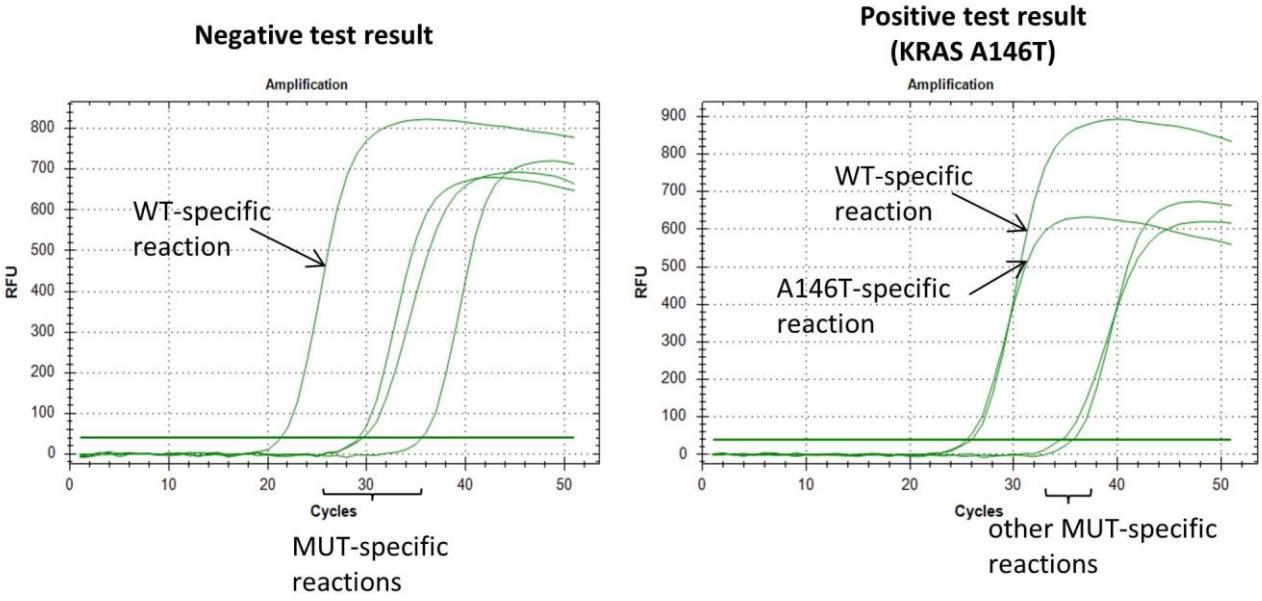
Abbreviations: WT – wild-type; MUT - mutant

g) HRM analysis for KRAS exon 4 (codon 146)



Abbreviations: HRM – high resolution melting curve analysis; WT – wild-type

h) Allele-specific PCR (AS-PCR) test for three frequently occurring mutations in KRAS codon 146 (applied to cases with positive or ambiguous HRM test results)

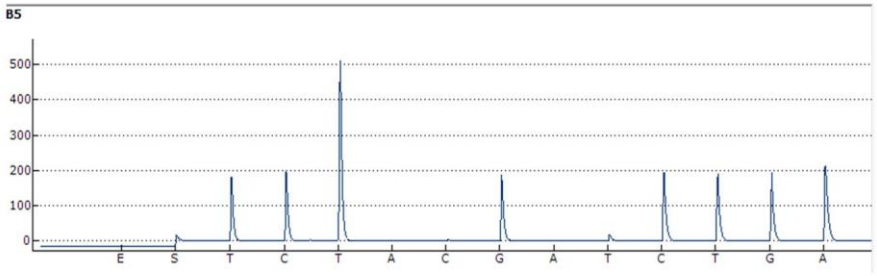


Abbreviations: WT – wild-type; MUT - mutant

i) Pyrosequencing or Sanger sequencing of the DNA fragment containing KRAS codon 146 (applied in cases with positive or ambiguous HRM test results) if:

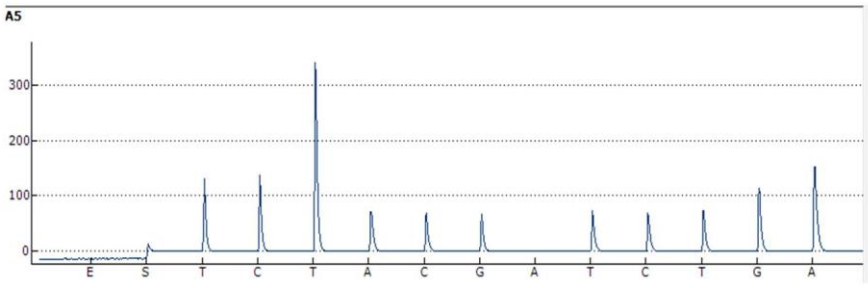
- DNA quality or quantity was insufficient for AS-PCR
- AS-PCR results were negative for all tested mutations
- AS-PCR result was inconclusive.

Negative pyrosequencing result



WT allele: T C TTT G C T G A
(reverse complement sequence)

Positive pyrosequencing result (codon 146 GCA > GTA [reverse complement TGC > TAC], KRAS A146V mutation)

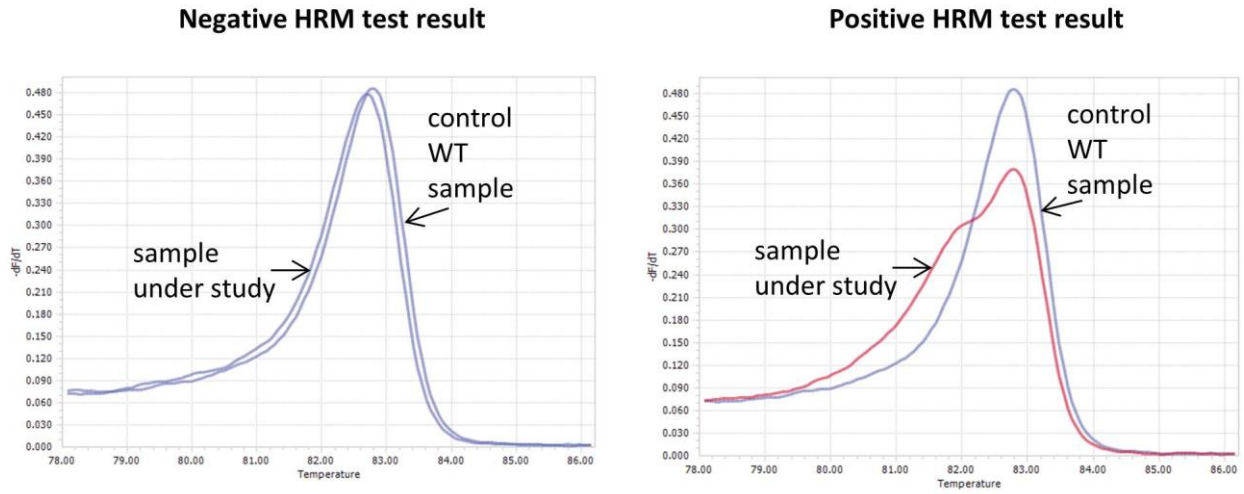


WT allele: T C TTT G C T G A
MUT allele: T C TTT A C T G A

Abbreviations: WT – wild-type; MUT - mutant

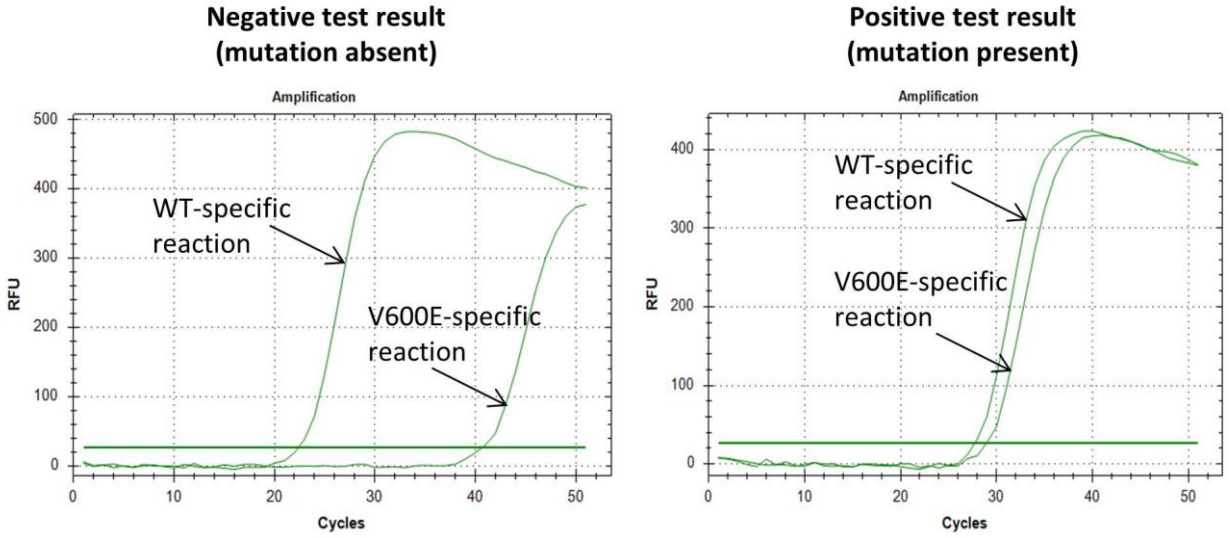
BRAF gene

a) HRM analysis for BRAF exon 15 fragment (codon 600)



Abbreviations: HRM – high resolution melting curve analysis; WT – wild-type

b) Allele-specific PCR (AS-PCR) test for BRAFV600E mutation (applied to cases with positive or ambiguous HRM test results).

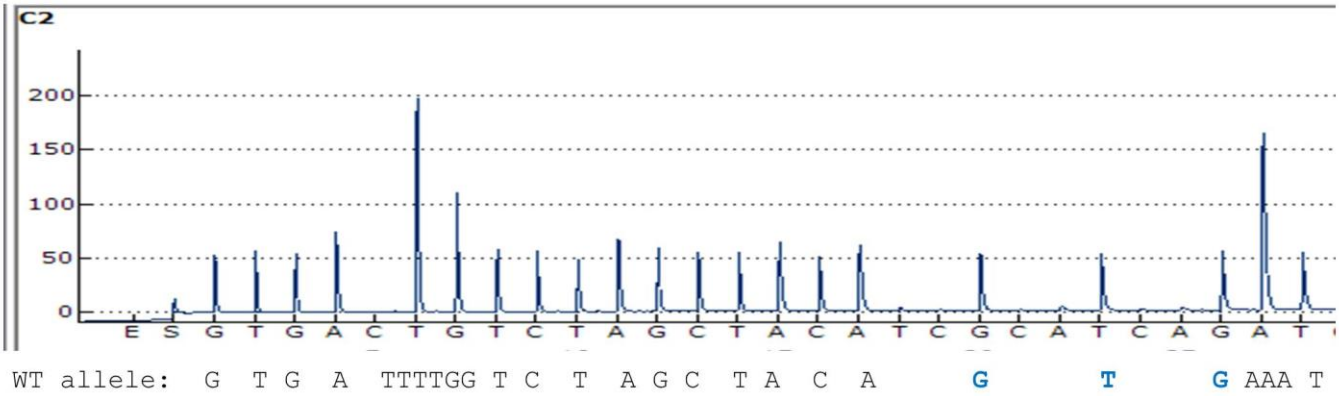


Abbreviations: WT – wild-type

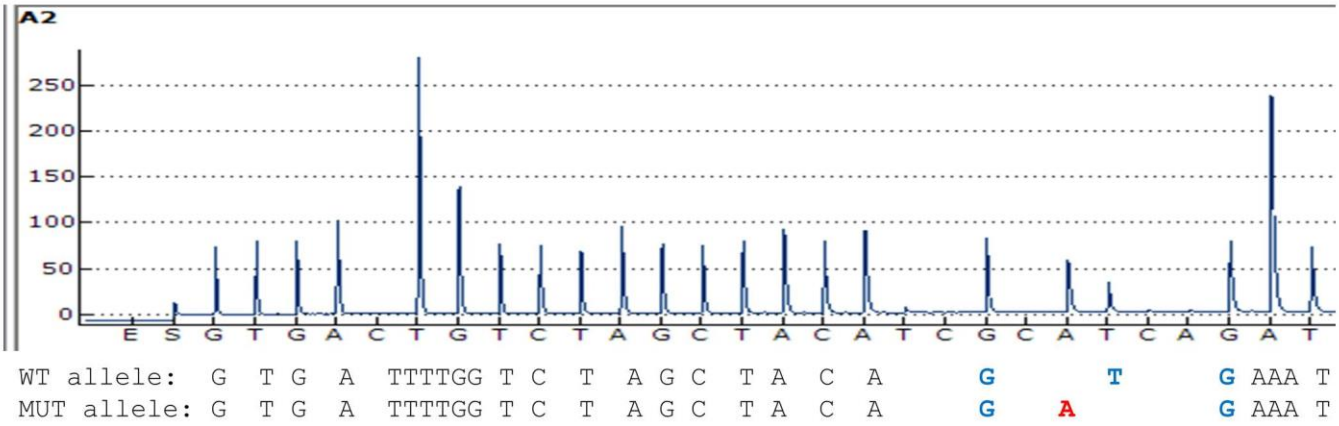
c) Pyrosequencing or Sanger sequencing of the DNA fragment containing BRAF codon 600 (applied to cases with positive or ambiguous HRM test results) if:

- DNA quality or quantity was insufficient for AS-PCR
- AS-PCR results were negative for all tested mutations
- AS-PCR result was inconclusive.

Negative pyrosequencing result



Positive pyrosequencing result
(codon 600 GTG > GAG, BRAF V600E mutation)



Abbreviations: WT – wild-type; MUT - mutant

PCR-based detection of EGFR, ALK, KRAS and BRAF mutations in Russian patients with lung adenocarcinoma: a single-center experience

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Supplemental Material

Suppl. Table 1. Gender difference in the distribution of EGFR, ALK, KRAS and BRAF genotypes.

	Ever-smokers			Never-smokers			Smoking status unknown			All		
	M	F	p-value[a]	M	F	p-value	M	F	p-value	M	F	p-value
EGFR mutations: L858R or ex19del	36/557 (6.5%)	9/56 (16.1%)	**0.033**	18/129 (14.0%)	196/461 (42.5%)	**4.8×10^{-6}**	44/684 (6.4%)	167/445 (37.5%)	**$<2.2\times10^{-16}$**	98/1370 (7.2%)	372/962 (38.7%)	**$<2.2\times10^{-16}$**
ALK translocations (EGFR-negative cases)	6/521 (1.2%)	0/47	1.000	6/111 (5.4%)	26/265 (9.8%)	0.227	23/640 (3.6%)	34/278 (12.2%)	**1.3×10^{-5}**	35/1272 (2.8%)	60/590 (10.2%)	**1.1×10^{-10}**
KRAS mutations (EGFR/ ALK-negative cases)	139/439 (31.7%)	12/44 (27.3%)	0.744	26/94 (27.7%)	45/209 (21.5%)	0.398	135/411 (32.8%)	35/171 (20.5%)	**0.025**	300/944 (31.8%)	92/424 (21.7%)	0.00013
KRAS G12C mutations (EGFR/ALK-negative cases)	56/439 (12.8%)	8/44 (18.2%)	0.367	6/94 (6.4%)	1/209 (0.5%)	**0.005**	49/411 (11.9%)	8/171 (4.7%)	**0.013**	111/944 (11.8%)	17/424 (4.0%)	**1.8×10^{-6}**
KRAS G12D mutations (EGFR/ALK-negative cases)	18/439 (4.1%)	2/44 (4.5%)	0.703	8/94 (8.5%)	18/209 (8.6%)	1.000	21/411 (5.1%)	12/171 (7.0%)	0.434	47/944 (5.0%)	32/424 (7.5%)	0.078
BRAF V600E mutations (EGFR/ALK/KRAS-negative cases)	4/266 (1.5%)	1/28 (3.6%)	0.402	0/63	8/132 (6.1%)	0.060	1/27 (3.7%)	0/27	1.000	5/356 (1.4%)	9/187 (4.8%)	**0.023**

M – males; F – females; [a]Fisher's exact test p-value

Suppl. Table 2. Median age of patients with particular genetic abnormalities in their tumors vs. patients with no such abnormalities (Mann-Whitney U-test p is provided).

	Ever-smokers	Never-smokers	Smoking status unknown	All
EGFR mutations: L858R or ex19del	60 vs. 61 (p=0.378)	65 vs. 62 (p=**0.0002**)	64 vs. 61 (p=**6.5×10^{-6}**)	64 vs. 61 (p=**2.1×10^{-11}**)
ALK translocations (EGFR-negative cases)	58.5 vs. 61 (p=0.697)	56 vs. 63 (p=**0.005**)	54 vs. 61 (p=**$4.2\cdot10^{-7}$**)	55 vs. 61 (p=**2.5×10^{-8}**)
KRAS mutations (EGFR/ALK-negative cases)	59 vs. 61 (p=0.058)	63 vs. 63 (p=0.764)	60.5 vs. 62 (p=0.146)	61 vs. 62 (p=**0.013**)
KRAS G12C mutations (EGFR/ALK-negative cases)	59 vs. 61 (p=0.137)	69 vs. 63 (p=**0.035**)	60 vs. 62 (p=0.063)	60 vs. 62 (p=**0.041**)
KRAS G12D mutations (EGFR/ALK-negative cases)	57 vs. 61 (p=**0.019**)	60.5 vs. 63 (p=0.065)	62 vs. 61 (p=0.527)	60 vs. 61 (p=0.088
KRAS G12V mutations (EGFR/ALK-negative cases)	59 vs. 61 (p=0.334)	63.5 vs. 63 (p=0.814)	63.5 vs. 61 (p=0.676)	62 vs. 61 (p=0.669)
KRAS G12A mutations (EGFR/ALK-negative cases)	64.5 vs. 60 (p=0.238)	65 vs. 63 (p=0.417)	63 vs. 61 (p=0.535)	65 vs. 61 (p=0.119)
BRAF V600E mutations (EGFR/ALK/KRAS-negative cases)	73 vs. 61 (p=0.190)	70.5 vs. 63 (p=**0.011**)	–	72.5 vs. 63 (p=**0.009**)