Virchows Archiv (2018) 472:717–725
https://doi.org/10.1007/s00428-017-2291-z

ORIGINAL ARTICLE

RAS testing for colorectal cancer patients is reliable in European laboratories that pass external quality assessment

V. Tack[1] · M. J. L. Ligtenberg[2,3] · A. G. Siebers[2] · P. D. M. Rombout[2] · P. D. Dabir[2,4] · R. D. A. Weren[2] · J. H. J. M. van Krieken[2] · E. M. C. Dequeker[1]

Received: 5 October 2017 / Revised: 11 December 2017 / Accepted: 28 December 2017 / Published online: 15 January 2018

Abstract

Wild-type status of *KRAS* and the *NRAS* gene (exon 2, 3, and 4) in the tumor should be determined before treatment of metastatic colorectal cancer (mCRC) patients with EGFR-targeting agents. There is a large variation in test methods to determine *RAS* status, and more sensitive detection methods were recently introduced. Data from quality assessment programs indicate substantial error rates. This study assessed the completeness and correctness of *RAS* testing in European laboratories that successfully passed external quality assessment (EQA). Participants were requested to send material of their most recent ten patients with mCRC who had been tested for *RAS* status. Isolated DNA, a hematoxylin and eosin stained tissue slide with a marked area for macrodissection and accompanying patient reports were requested. Samples were reevaluated in a reference laboratory by using a next-generation sequencing approach. In total, 31 laboratories sent in the requested material ($n = 309$). Despite regulations for anti-EGFR therapy, one institute did not perform full *RAS* testing. Reanalysis was possible for 274 samples with sufficient DNA available. In the hotspot codons of *KRAS* and *NRAS*, seven discordant results were obtained in total, five of them leading to a different prediction of anti-EGFR therapy efficacy (2%; $n = 274$). Results show that oncologists can rely on the quality of laboratories with good performance in EQA. Oncologists need to be aware that the testing laboratory participates successfully in EQA programs. Some EQA providers list the good performing laboratories on their website.

Keywords Reproducibility · Quality assurance · Molecular pathology · Biological markers · Colorectal neoplasms

Introduction

Today, variants in the *KRAS* and the *NRAS* genes (exon 2, 3, and 4) are considered to be important biomarkers for anti-EGFR therapy decisions for patients with metastatic colorectal cancer (mCRC). Targeted anti-EGFR agents, such as panitumumab and cetuximab, are indicated only for patients with a wild-type *RAS* tumor [1, 2]. Pathology laboratories perform *KRAS* variant analysis since 2008, when it became mandatory to confirm the wild-type status of *KRAS* exon 2 before anti-EGFR treatment is prescribed [3]. The outcome of phase III trials (PRIME and FIRE) in 2013 resulted in addition of the mutational status of *NRAS* and extra exons of *KRAS* as predictive biomarkers for anti-EGFR therapy decisions [4].

Another important biomarker is a somatic activating variant affecting the *BRAF* gene. This is a well-known negative prognostic marker in mCRC, but testing is not mandatory prior to anti-EGFR therapy decisions. Even though many laboratories include *BRAF* in their testing strategy for anti-EGFR

This article is part of the Topical Collection on *Quality in Pathology*

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00428-017-2291-z) contains supplementary material, which is available to authorized users.

✉ E. M. C. Dequeker
els.dequeker@kuleuven.be

[1] Department of Public Health and Primary Care, Biomedical Quality Assurance Research Unit, University of Leuven, Kapucijnenvoer 35 blok d, 3000 Leuven, Belgium

[2] Department of Pathology, Radboud University Medical Center, Nijmegen, The Netherlands

[3] Department of Human Genetics, Radboud University Medical Center, Nijmegen, The Netherlands

[4] Department of Pathology, Randers Regional Hospital, Randers, Denmark

therapy, there is no consensus about the specific effect of activating variants in *BRAF* on this therapy [5, 6].

Shortly after publication of the more stringent rules for panitumumab and cetuximab usage, results of external quality assessments (EQAs) showed concerning results [7–9]. Full *RAS* testing (defined as codons 12, 13, 59, 61, 117, and 146 of *KRAS* and *NRAS*) was only implemented by half of the laboratories participating in the European Society of Pathology (ESP) program, and there were numerous errors in testing the new gene segments. False positive and false negative results can have detrimental consequences for the patient's treatment and must be avoided at all times [10].

There is a large variation in the testing methods used to determine the *RAS* and *BRAF* status and more sensitive detection methods were introduced in routine testing [11]. There is a risk of reporting false negative or false positive results due to different sensitivities of methods, lack of experience in the laboratory, and other, partly unknown, factors. In the Netherlands, where few laboratories fail EQA programs, the result of retesting routine samples was reassuring [12].

In this study, the completeness and correctness of *RAS* testing (*KRAS* and *NRAS* exons 2, 3, and 4) was assessed in a subset of European laboratories, from 17 different countries, that successfully participated in previous EQA schemes.

Materials and methods

All European laboratories that were successful in at least one of their two last ESP Colon EQA scheme participations [13], with a known accreditation status that did not change in the last 3 years, were invited to participate in this study ($n = 126$). From 50 candidature forms, a final selection of 42 laboratories was made depending on the most recent participation in the ESP Colon EQA scheme.

Participating laboratories were requested the following material of the most recent ten patients tested for *RAS* variant status (January–September 2016): a hematoxylin and eosin (H&E) slide marked with the area used for macrodissection and DNA isolation, 50 μl DNA (10 ng/μl) to accommodate automatic processing in the reference laboratory, and the original test reports. The participants received a survey regarding the testing strategy and the techniques used to analyze the samples.

The DNA samples received from the participating laboratories were retested in the Department of Pathology at Radboudumc, Nijmegen, the Netherlands. This laboratory is accredited according to ISO 15189 for their NGS activities and will be further referred to as "the reference laboratory". The anonymized original reports were received and analyzed in the Biomedical Quality Assurance Research Unit of the KU Leuven (Belgium). Any discordant result between the participant's result and the reference laboratory was repeated in the reference laboratory.

The histological slides were reviewed individually for neoplastic cell percentage by two pathologists who used a recently developed algorithm, which was finalized by a consensus to a single value. Quantification of the DNA in the samples was done using the dsDNA broad range kit on the Qubit 3.0 platform (Thermo Fisher Scientific). DNA samples with a measurable amount of DNA ($n = 294$) were analyzed in duplicate using a Cancer Hotspot panel based on single-molecule molecular inversion probes (smMIPs) [14]. Analysis was done with SeqNext software v.4.1.2. (JSI medical systems GmbH). Only codons 4–23, 42–71, 98–119, and 135–150 of the *KRAS* gene (NM_004985.4), codons 1–21, 55–81, and 105–150 of the *NRAS* gene (NM_002524.4), and codons 582–615 of the *BRAF* gene (NM_004333.4) were included in the data analysis. For variant calling a threshold of 11 unique reads and 3 variant reads was applied. Due to the high level of formalin-fixed paraffin-embedded artifacts in some samples, the cutoff for the variant allele frequency was set at ≥ 5%. This data analysis was slightly different from the routine strategy, where a cutoff of 1% is used. The mean amount of unique reads varied between 530 (*KRAS* codon 61) and 1553 (*BRAF* codon 594–601) (for details, see Supplementary Table 2). The data of any discrepant result were reanalyzed in detail, according to the routine procedures. The evaluation was blinded for original test results.

Results from the reference laboratory were compared with results in the original patient reports of the participating laboratories. Information such as sensitivity and neoplastic cell percentage were also available from these reports.

Statistical analysis was done with IBM SPSS statistics version 22. Comparison of means was done with a *t* test.

Results

From the 42 selected laboratories for this study, 31 laboratories sent in their DNA samples along with respective patient reports. One laboratory did not fill in the requested survey, and for one laboratory, only nine samples were received. A total of 309 samples were received, of which 274 gave reliable results after reanalysis by the reference laboratory. The participants belonged to 17 different countries. More detailed information on the countries and characteristics can be found in Table 1. The majority of the participants (61%) had a university (hospital) and research background, 36% was a laboratory in a public or a private hospital, and 3% were private laboratories. Only 61% of the institutes was accredited according to an international standard (ISO 15189) or a national equivalent [15–17].

The neoplastic cell percentage on the HE slides was reassessed by two pathologists for 270 samples of which the H&E slides were available and ranged between 10 and 90% after consensus. The majority of the samples had an estimation between 30 and 70%. The percentage of neoplastic cells was

Table 1 Characteristics of the participating laboratories. U&R University and research background. HRM (high resolution melting). CAP (American national standard) College of American Pathologists. CCKL (Dutch national standard) "Coördinatie Commissie ter bevordering van de Kwaliteitsbeheersing op het gebied van Laboratoriumonderzoek in de Gezondheidszorg"

Country	*KRAS* samples per year	*NRAS* samples per year	*NRAS* testing since	Setting	Standard	Method *KRAS* analysis	Method *NRAS* analysis	Method *BRAF* analysis
Austria	10–99	10–99	1/02/2014	Hospital	No	KRAS mutation detection kit (Amoy Dx)	NRAS mutation detection kit (Amoy Dx)	Cobas 4800 BRAF V600 Mutation Test (Roche)
	100–249	10–99	1/03/2014			Therascreen KRAS Pyro kit (Qiagen) + Therascreen RAS extension kit (Qiagen)	Therascreen NRAS Pyro kit (Qiagen) + Therascreen RAS extension kit (Qiagen)	Cobas 4800 BRAF V600 Mutation Test (Roche)
	10–99	10–99	1/08/2015			TruSight Tumor 15 + MiSeq (Illumina)	TruSight Tumor 15 + MiSeq (Illumina)	TruSight Tumor 15 + MiSeq (Illumina)
Belgium	100–249	100–249	3/11/2014	Hospital	Yes, ISO 15189	Therascreen KRAS Pyro kit (Qiagen) + Therascreen RAS extension kit (Qiagen)	Therascreen NRAS Pyro kit (Qiagen) + Therascreen RAS extension kit (Qiagen)	BRAF pyro kit (Qiagen)
	100–249	100–249	8/02/2015	Hospital		GS Junior (Roche)	GS Junior (Roche)	GS Junior (Roche)
	10–99	10–99	9/03/2015	Hospital		TruSight Tumor 26 + MiSeq (Illumina)	TruSight Tumor 26 + MiSeq (Illumina)	TruSight Tumor 26 + MiSeq (Illumina)
	250–499	250–499	1/08/2013	U&R		TruSight Tumor 26 + MiSeq (Illumina)	TruSight Tumor 26 + MiSeq (Illumina)	TruSight Tumor 26 + MiSeq (Illumina)
	10–99	10–99	2/10/2013	U&R		Idylla KRAS (Biocartis)	Idylla NRAS (Biocartis)	Idylla BRAF (Biocartis)
Czech Republic	250–499	250–499	1/09/2013	U&R	Yes, ISO 15189	Eligene Colorectum NGS panel (Elisaneth Pharmacom) + MiSeq (Illumina)	Eligene Colorectum NGS panel (Elisaneth Pharmacom) + MiSeq (Illumina)	Eligene Colorectum NGS panel (Elisaneth Pharmacom) + MiSeq (Illumina)
	250–499	250–499	2/09/2013			Eligene Colorectum NGS panel (Elisaneth Pharmacom) + MiSeq (Illumina)	Eligene Colorectum NGS panel (Elisaneth Pharmacom) + MiSeq (Illumina)	Eligene Colorectum NGS panel (Elisaneth Pharmacom) + MiSeq (Illumina)
Finland	250–499	250–499	1/10/2013	Hospital	Yes, ISO 15189	Ion Ampliseq Custom panel + Ion Torrent (Life Technologies)	Ion Ampliseq Custom panel + Ion Torrent (Life Technologies)	Ion Ampliseq Custom panel + Ion Torrent (Life Technologies)
France	500–999	250–499	1/10/2013	U&R	Yes, ISO 15189	Taqman assay + Sanger sequencing	Sanger sequencing	Taqman assay
Germany	250–499	100–249	1/03/2013	U&R	No	In house primers + MiSeq (Illumina)	In house primers + MiSeq (Illumina)	Not tested
	100–249	10–99	1/09/2013	Hospital		Therascreen KRAS Pyro kit (Qiagen) + Therascreen RAS extension kit (Qiagen)	Therascreen NRAS Pyro kit (Qiagen) + Therascreen RAS extension kit (Qiagen)	BRAF pyro kit (Qiagen)
Greece	100–249	100–249	1/01/2015	U&R	No	Therascreen KRAS Pyro kit (Qiagen) + Therascreen RAS extension kit (Qiagen)	Therascreen NRAS Pyro kit (Qiagen) + Therascreen RAS extension kit (Qiagen)	BRAF pyro kit (Qiagen)
Hungary	500–999	500–999	1/01/2012	U&R	No	Cobas 4800 KRAS mutation test (Roche) + Sanger sequencing	Sanger sequencing	Cobas 4800 BRAF V600 Mutation Test (Roche)
Italy	100–249	100–249	1/10/2013	U&R	No	Myriapod Colon status (Diatech Pharmacogenetics)	Myriapod Colon status (Diatech Pharmacogenetics)	Not tested
Poland	100–249	100–249	1/10/2013	U&R	No	KRAS mutation analysis kit (Entrogen)	NRAS mutation analysis kit (Entrogen)	Not tested
Portugal	>1000	>1000	1/10/2013	U&R	Yes, CAP	Sanger sequencing	Sanger sequencing	Not tested

Table 1 (continued)

Country	*KRAS* samples per year	*NRAS* samples per year	*NRAS* testing since	Setting	Standard	Method *KRAS* analysis	Method *NRAS* analysis	Method *BRAF* analysis
Slovakia	500–999	250–499	17/12/2013	Private lab	No	Cobas 4800 KRAS mutation test (Roche) + Lightmix Kit KRAS 117, 146 (Tib Molbiol)	LightMix Kit NRAS 12-13. 59-61. 117. 146 (TIB Molbiol)	Cobas 4800 BRAF V600 Mutation Test (Roche)
Slovenia	250–499	100–249	1/09/2013	U&R	No	KRAS mutation analysis kit (Entrogen)	NRAS mutation analysis kit (Entrogen)	BRAF mutation analysis kit (Entrogen)
Spain	250–499	100–249	6/04/2015	U&R	Yes, ISO 15189	Therascreen KRAS Pyro kit (Qiagen) + Therascreen RAS extension kit (Qiagen) + Cobas 4800 KRAS mutation test (Roche)	Therascreen RAS extension kit (Qiagen)	Not tested
Sweden	100–249	100–249	6/01/2014	U&R	Yes, ISO 15189	K-Ras/B-Raf mutation analysis kit (Entrogen)	NRAS Mutation Detection Kit (EntroGen)	Pyrosequencing
Switzerland	100–249	100–249	16/11/2012	U&R	No	Ion Ampliseq Custom panel + Ion Torrent (Life Technologies)	Ion Ampliseq Custom panel + Ion Torrent (Life Technologies)	Ion Ampliseq Custom panel + Ion Torrent (Life Technologies)
The Netherlands	100–249	100–249	1/01/2009	U&R	Yes, ISO 15189	Ion Ampliseq Custom panel + Ion Torrent (Life Technologies)	Ion Ampliseq Custom panel + Ion Torrent (Life Technologies)	Ion Ampliseq Custom panel + Ion Torrent (Life Technologies)
	250–499	250–499	1/09/2013	U&R		Ion Ampliseq Custom panel + Ion Torrent (Life Technologies)	Ion Ampliseq Custom panel + Ion Torrent (Life Technologies)	Ion Ampliseq Custom panel + Ion Torrent (Life Technologies)
	100–249	100–249	1/07/2013	U&R	Yes, CCKL	Pyrosequencing + Sanger sequencing	Pyrosequencing + Sanger sequencing	Pyrosequencing
	500–999	100–249	1/01/2012	U&R		Amliseq Cancer Hotspot Panel (Life Technologies) + Ion Proton (Life Technologies)	Amliseq Cancer Hotspot Panel (Life Technologies) + Ion Proton (Life Technologies)	Amliseq Cancer Hotspot Panel (Life Technologies) + Ion Proton (Life Technologies)
	10–99	10–99	1/03/2013	Hospital		Sanger sequencing + Snapshot	Sanger sequencing + Snapshot	Snapshot
	10–99	10–99	1/01/2014	Hospital		HRM + Sanger sequencing	HRM + Sanger sequencing	HRM + Sanger sequencing
	10–99	10–99	10/12/2013	Hospital		HRM + Sanger sequencing	HRM + Sanger sequencing	HRM + Sanger sequencing

assessed in total by three different pathologists, two from the reference laboratory and one from the participating laboratory. Paired statistical analysis of these assessments, excluding cases where a range is given instead of an exact percentage of neoplastic cells, showed no significant differences between the original estimate and the consensus estimate in the reference laboratory ($p = 0.817$, $n = 195$). Analyses on the neoplastic cell content were performed using the pathology estimations of the reference laboratory.

Even though confirmation of the wild-type status of *NRAS* and *KRAS* (codons 12, 13, 59, 61, 117, 146) is mandatory before anti-EGFR therapy, one institute did not perform full *RAS* testing. They did not include codon 59 of the *KRAS* gene. The majority of the laboratories (84%, $n = 31$) performed *BRAF* variant analysis for one or more of the samples. However, 13 of these 26 participants did not perform *BRAF* testing on all the samples. For one laboratory, none of the *BRAF*-tested samples could be reanalyzed by the reference laboratory and these were therefore not taken into account for further analysis.

The use of NGS-based methods and non-NGS commercial kits is similar for variant analysis of the *KRAS* gene, 38% and 39%, respectively. For *NRAS* and *BRAF* variant analysis, NGS (42% and 55% respectively) is more popular than commercial non-NGS kits (34 and 19%, respectively). Laboratory-developed tests (non-NGS) were used by a quarter of the participants for *KRAS*, *NRAS*, and *BRAF* variant analysis (20%, 23%, and 26%, respectively). One laboratory (3%) used a combination of a commercial non-NGS kit with Sanger sequencing for *KRAS* variant analysis.

The concordance between the genotype reported by the participating and reference laboratories is presented in Table 2. Taken into account the tested regions and the samples for which the reanalysis by the reference laboratory gave reliable results, 24 laboratories (77%) were able to correctly type all samples. Of these, four laboratories sent in samples that contained a *BRAF* variant that was identified by the reference laboratory, but was not included in the tested region of that particular laboratory.

There were seven discordant genotypes at clinically relevant positions: four false negatives, one false positive, one insertion-deletion instead of a single nucleotide substitution, and one sample with a second *KRAS* mutation. Not more than one genotyping error was made by each laboratory. Table 3 gives a more detailed information on these variants, which all involved a *KRAS* variant. One of the variants that was missed was due to the limit of detection of the laboratory's method, which was 10% while the missed variant was present at a frequency of 6%. The percentage of tumor cells in this sample was 70%. All seven laboratories with genotyping errors had a university (hospital) and research background and four of them were accredited. The average neoplastic cell content of these seven mistyped samples was 47%, with five of the seven samples above 40%. The percentage of neoplastic cells was known for 233 of the 267 concordant samples. The average neoplastic cell content of the samples that were correctly typed was 52%. The two groups, samples with discordant results and those without do not significantly differ from each other regarding neoplastic cell content ($p = 0.535$). In total six *BRAF* p.(V600E) variants and two variants in codon 594 of *BRAF* were missed by the participants as they were not included in their test regimen. Moreover, seven variants at positions without known clinical relevance were identified (Table 2).

In Table 4, an overview is shown for the frequency of the variants in each relevant codon of *KRAS*, *NRAS*, and *BRAF* as determined in the reference laboratory ($n = 274$). Details on all specific variants can be found in Supplemental Table 1.

Discussion

The aim of this study was to assess quality of routine *RAS* testing in Europe, by verifying its reproducibility. In addition, implementation of complete *KRAS* and *NRAS* (exons 2, 3, and 4) testing in diagnostic setting was evaluated.

Overall, a very good concordance was observed between the participating laboratories and the reference laboratory (97%). For five patients (2%), the reference laboratory generated a genotype that leads to a different anti-EGFR therapy advice. In two patients (1%), the anti-EGFR therapy advice remained the same. In case the four patients with a false negative result actually received anti-EGFR therapy, this could have had adverse effects instead of improvement of progression-free survival [4]. The participants with discordant results all used different methods (Table 3). Laboratories using NGS technologies made no mistakes which lead to false negative or false positive results for therapy decisions. This observation may be due to the higher familiarity with genotyping technologies.

A limitation of our study is that not all laboratories had sufficient residual DNA that could be evaluated in the automatic workflow of the reference laboratory. This may have caused a selection bias towards samples with large tumor areas and laboratories that isolated a surplus of DNA in their routine workflow. Actually, six participants indicated that the requested DNA amount was higher than what was extracted in their routine practice. This did not seem to cause much problems, as only for eight samples of these participants the repeat NGS analysis gave low coverage. On the other hand, the amount of DNA supplied by some of the participating laboratories was so low that the concentration could not be measured by Qubit analysis.

The frequency of the variants in the mCRC samples used in this study (Table 4 and Supplemental Table 1) is in accordance with previously reported results [4, 12, 18–20]. *KRAS* exon 2 variants were most commonly identified (41%) and no *NRAS* exon 4 variants occurred. *BRAF* variants were present in 10% of the samples, of which *BRAF* c.1799T>A p.(Val600Glu)

Table 2 Overview of the results after reanalysis by the reference laboratory. Major genotyping errors include false negatives, false positives, or identification of a different *RAS* variant. *NCR* no clinical relevance, *NA* not applicable

Participant	Total number of samples reanalyzed by the reference laboratory	*KRAS* and *NRAS* genotype			*BRAF* genotype			
		Number of samples with concordant annotation	Number of discordant samples	Number of samples with variants without clinical relevance (NCR)	Number of samples tested for *BRAF* by the participant	Number of samples with concordant annotation	Number of discordant samples	Number of samples with variants not tested by participant
1	10	10 (100%)	0 (0%)	0	6	6 (100%)	0 (0%)	0
2	10	10 (100%)	0 (0%)	0	1	1 (100%)	0 (0%)	0
3	10	10 (100%)	0 (0%)	1, NCR, *KRAS* c.64C>A p.(Q22K)	1	1(100%)	0 (0%)	1, c.1799T>A p.(V600E)
4	3	3 (100%)[a]	0 (0%)	0	0	NA	NA	0
5	10	10 (100%)	0 (0%)	0	10	10 (100%)	0 (0%)	0
6	10	10 (100%)	0 (0%)	1, NCR, *KRAS* c.313G>T p.(D105Y)	9	9 (100%)	0 (0%)	1, c.1799T>A p.(V600E)
7	10	10 (100%)[a]	0 (0%)	0	10	10 (100%)[a]	0 (0%)	0
8	9	8 (89%)	1 (11%)	0	1	1 (100%)	0 (0%)	2, c.1799T>A p.(V600E)
9	10	10 (100%)	0 (0%)	0	10	10 (100%)	0 (0%)	0
10	9	8 (89%)	1 (11%)	0	9	9 (100%)	0 (0%)	0
11	5	5 (100%)	0 (0%)	0	0	NA	NA	1, c.1781A>G p.(D594G)
12	9	9 (100%)	0 (0%)	0	8	8 (100%)	0 (0%)	0
13	10	10 (100%)	0 (0%)	0	10	10 (100%)	0 (0%)	0
14	10	9 (90%)	1 (10%)	0	2	2 (100%)	0 (0%)	1, c.1780G>A p.(D594N)
15	9	8 (89%)	1 (11%)	0	9	9 (100%)	0 (0%)	0
16	10	10 (100%)[a]	0 (0%)	1, NCR, *NRAS* c.394G>A p.(E132K)	2	2 (100%)[a]	0 (0%)	0
17	8	8 (100%)	0 (0%)	0	0	NA	NA	1, c.1799T>A p.(V600E)
18	8	8 (100%)	0 (0%)	0	8	8 (100%)	0 (0%)	0
19	10	10 (100%)[a]	0 (0%)	0	10	10 (100%)[a]	0 (0%)	0
20	10	9 (90%)	1 (10%)	1, NCR, *KRAS* c.24A>G p.=	3	3 (100%)	0 (0%)	0
21	10	10 (100%)[a]	0 (0%)	0	10	10 (100%)[a]	0 (0%)	0
22	10	10 (100%)	0 (0%)	0	10	10 (100%)	0 (0%)	0
23	3	3 (100%)	0 (0%)	1, NCR, *NRAS* c.360G>A p.=	3	3 (100%)	0 (0%)	0
24	10	10 (100%)	0 (0%)	0	10	10 (100%)	0 (0%)	0
25	7	6 (86%)	1 (14%)	0	0	NA	NA	0
26	10	9 (90%)	1 (10%)[a]	0	0	NA	NA	1, c.1799T>A p.(V600E)
27	9	9 (100%)	0 (0%)	0	9	9 (100%)	0 (0%)	0
28	9	9 (100%)	0 (0%)	1, NCR, *KRAS* c.198A>G p.=	3	3 (100%)	0 (0%)	0
29	6	6 (100%)	0 (0%)	0	0	NA	NA	0
30	10	10 (100%)	0 (0%)	1, NCR, *KRAS* c.64C>A p.(Q22K)	8	8 (100%)	0 (0%)	0
31	10	10 (100%)[a]	0 (0%)	0	1	1 (100%)[a]	0 (0%)	0
Total	274	267 (97%)	7 (2.5%)	7	163	163 (100%)	0 (0%)	8

[a] One of the samples in this category was accepted for analysis by the reference laboratory but had borderline minimal criteria for variant calling

Table 3 Overview of the major genotyping errors made by the participating laboratories. The reference laboratory analyzed the samples with a Cancer Hotspot panel based on single-molecule molecular inversion probes (smMIPs). *TC* tumor cell content, *VAF* variant allele frequency, as determined by the reference laboratory in Nijmegen, *LOD* limit of detection of the participant's method, *NA* not applicable

Participant ID	TC participant (%)	Variant identified by the participant	LOD (%)	Used mutation detection method by participant	TC reference lab (%)	Variant identified by the reference laboratory	VAF (%)
8	Missing	*KRAS* c.35G>A p.(G12D)	Missing	Idylla KRAS mutation test (Biocartis)	20%	Wild-type	NA
10	Missing	*KRAS* c.181C>A p.(Q61K)	10%	Eligene Colorectum NGS panel (Elisaneth Pharmacom)	60%	*KRAS* c.180_181delinsAA p.(Q61K)	39%
14	60%	Wild-type	10%	TaqMan Allelic Discrimination Assay Sanger sequencing	60%	*KRAS* c.38_39delinsAA p.(G13D)	26%
15	Missing	Wild-type	1%	Therascreen KRAS Pyro kit (Qiagen) Therascreen RAS extension kit (Qiagen)	40%	*KRAS* c.38G>A p.(G13D)	31%
20	Missing	*KRAS* c.34G>T p.(G12C)	10%	Pyrosequencing and Sanger sequencing	70%	*KRAS* c.34G>T p.(G12C)	38%
						KRAS c.35G>C p.(G12A)	6%
25	80%	Wild-type	10%	KRAS mutation analysis kit (Entrogen) NRAS mutation analysis kit (Entrogen)	20%	*KRAS* c.436G>A p.(A146T)	32%
26	> 20%	Wild-type	10%	Sanger sequencing	60%	*KRAS* c.35G>A p.(G12D)	12%

Table 4 Overview of the variants in the samples, as determined by the NGS panel of the reference laboratory in Nijmegen. Samples without a reliable result ($n = 35$) were not taken into account

Variant in sample	Number of samples (%) n = 274	Agreement (%)
KRAS	134 (49%)	127/134 (95%)
Codon 12	90 (33%)[a]	87/90 (97%)
Codon 13	23 (8%)	21/23 (91%)
Codon 59	1 (0%)	1/1 (100%)
Codon 61	8 (3%)	7/8 (88%)
Codon 146	12 (4%)[b]	11/12 (92%)
NRAS	15 (5%)	15/15 (100%)
Codon 12	4 (1%)[b]	4/4 (100%)
Codon 13	2 (1%)	2/2 (100%)
Codon 61	9 (3%)	9/9 (100%)
BRAF	28 (10%)	28/28 (100%)
Codon 600	24 (9%)	24/24 (100%)
Codon 594	4 (1%)[b]	4/4 (100%)
Wild-type	99 (36%)	99/99 (100%)

[a] Two codon 12 variants in one sample

[b] Two samples contained both a *RAS* variant and a *BRAF* variant

was most frequent. One exception to the literature is the parallel occurrence of both *BRAF* and *RAS* variants (one sample with *KRAS* c.436G>A p.(Ala146Thr) and *BRAF* c.1780G>A p.(Asp594Asn), and one sample with *NRAS* c.35G>A p.(Gly12Asp), and *BRAF* c.1780G>A p.(Asp594Gly)). In both samples, this concerned a variant of *BRAF* codon 594 that causes inactivation rather than activation of *BRAF*. In contrast to *BRAF* codon 600 variants, which are considered mutually exclusive with *RAS* variants, *BRAF* codon 594 variants co-occur relatively frequently with *RAS* variants [21].

This study shows that successful participation in EQA, reflects complete and correct reporting of results for *RAS* testing in routine analysis. Despite the label given by EMA to EGFR-targeted agents for patients with mCRC, one laboratory did not test all relevant codons (12, 13, 59, 61, 117, 146) and did not mention this limitation in the reports. This is an improvement compared to studies performed right after the labels changed [7, 12, 20].

Because of the potential negative consequences following a false negative result, laboratories should be careful when reporting a wild-type sample in case of incomplete *RAS* testing. Also, laboratories using less sensitive methods, such as Sanger sequencing, without mentioning the sensitivity of the method in the report are at risk of reporting false negative results. The tested regions and limitations of the test should be clearly mentioned in the methods section of the patient report and further testing should be recommended for the complete *RAS* gene [22]. In addition, the tumor cell content is an important element

on the report to allow an unambiguous interpretation of the result in relation to the used testing method [23].

Different elements were taken into account to provide a good estimation of the reproducibility of *RAS* testing. The selected group of participants had different experience levels with *RAS* testing and different settings. Laboratories following a specific standard and laboratories without accreditation were also included. The invited participants were good performers in EQA, which could lead to an overestimation of the general reproducibility of *RAS* testing in Europe. Taken this into account, it could be concluded that a good EQA performance is indicative of reliable performance in routine practice. Some EQA providers publish a list of laboratories that passed the EQA program [24, 25].

Conclusion

The results show that patients and oncologists can rely on the results of routine *RAS* testing in pathology laboratories that successfully participated in EQA in Europe. Nevertheless, laboratories must acknowledge samples with limited quality in the diagnostic report. Providing conclusive results of such samples can result in a wrong therapy decision.

Acknowledgments We thank the participating laboratories for providing the sample information and participating to this study.

We are grateful to the technicians and molecular biologists of the Department of Pathology, Nijmegen, for technical support and support in interpretation of the data. We thank the Radboud Genomics Technology Center for performing the sequencing runs.

Funding information This work was supported by Amgen.

Compliance with ethical standards

Conflict of interest JHJM van Krieken has received research grants and speaker's fees from AMGEN, Merck Serrono.

Research involving human participants and/or animals Not applicable.

Informed consent Not applicable

References

1. European Medicine Agency (EMA) Vectibix: EPAR summary for the Publica 2013. http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Summary_for_the_public/human/000741/WC500047704.pdf. Accessed 1 Aug 2017
2. European Medicine Agency (EMA) Erbitux: EPAR summary for the Publica 2013. http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Summary_for_the_public/human/000558/WC500029111.pdf. Accessed 1 Aug 2017
3. Amado RG, Wolf M, Peeters M, Van Cutsem E, Siena S, Freeman DJ, Juan T, Sikorski R, Suggs S, Radinsky R, Patterson SD, Chang DD (2008) Wild-type KRAS is required for panitumumab efficacy in patients with metastatic colorectal cancer. J Clin Oncol 26(10): 1626–1634. https://doi.org/10.1200/JCO.2007.14.7116
4. Douillard JY, Siena S, Cassidy J, Tabernero J, Burkes R, Barugel M, Humblet Y, Bodoky G, Cunningham D, Jassem J, Rivera F, Kocákova I, Ruff P, Błasińska-Morawiec M, Smakal M, Canon JL, Rother M, Oliner KS, Tian Y, Xu F, Sidhu R (2014) Final results from PRIME: randomized phase III study of panitumumab with FOLFOX4 for first-line treatment of metastatic colorectal cancer. Ann Oncol 25(7):1346–1355. https://doi.org/10.1093/annonc/mdu141
5. Stintzing S, Stremitzer S, Sebio A, Lenz HJ (2015) Predictive and prognostic markers in the treatment of metastatic colorectal cancer (mCRC): personalized medicine at work. Hematol Oncol Clin North Am 29(1):43–60. https://doi.org/10.1016/j.hoc.2014.09.009
6. Ciardiello F, Normanno N, Maiello E, Martinelli E, Troiani T, Pisconti S, Giuliani F, Barone C, Cartenì G, Rachiglio AM, Montesarchio V, Tonini G, Rizzi D, Cinieri S, Bordonaro R, Febbraro A, De Vita F, Orditura M, Fenizia F, Lambiase M, Rinaldi A, Tatangelo F, Botti G, Colucci GL (2014) Clinical activity of FOLFIRI plus cetuximab according to extended gene mutation status by next-generation sequencing: findings from the CAPRI-GOIM trial. Ann Oncol 25(9):1756–1761. https://doi.org/10.1093/annonc/mdu230
7. Tack V, Ligtenberg MJ, Tembuyser L, Normanno N, Vander Borght S, Han van Krieken J, Dequeker EM (2015) External quality assessment unravels interlaboratory differences in quality of RAS testing for anti-EGFR therapy in colorectal cancer. Oncologist 20(3):257–262. https://doi.org/10.1634/theoncologist.2014-0382
8. Normanno N, Pinto C, Castiglione F, Fenizia F, Barberis M, Marchetti A, Fontanini G, De Rosa G, Taddei GL (2015) The Italian external quality assessment for RAS testing in colorectal carcinoma identifies methods-related inter-laboratory differences. J Transl Med 13(1):287. https://doi.org/10.1186/s12967-015-0655-1
9. Richman SD, Fairley J, Butler R, Deans ZC (2017) How close are we to standardised extended RAS gene mutation testing? The UK NEQAS evaluation. J Clin Pathol 70(1):58–62. https://doi.org/10.1136/jclinpath-2016-203822
10. Heinemann V, Douillard JY, Ducreux M, Peeters M (2013) Targeted therapy in metastatic colorectal cancer—an example of personalised medicine in action. Cancer Treat Rev 39(6):592–601. https://doi.org/10.1016/j.ctrv.2012.12.011
11. Ihle MA, Fassunke J, Konig K, Grünewald I, Schlaak M, Kreuzberg N, Tietze L, Schildhaus HU, Büttner R, Merkelbach-Bruse S (2014) Comparison of high resolution melting analysis, pyrosequencing, next generation sequencing and immunohistochemistry to conventional Sanger sequencing for the detection of p.V600E and non-p.V600E BRAF mutations. BMC Cancer 14(1):13. https://doi.org/10.1186/1471-2407-14-13
12. Boleij A, Tops BB, Rombout PD, Dequeker EM, Ligtenberg MJ, van Krieken JH, Dutch RAS EQA Initiative (2015) RAS testing in metastatic colorectal cancer: excellent reproducibility amongst 17 Dutch pathology centers. Oncotarget 6(17):15681–15689. https://doi.org/10.18632/oncotarget.3804
13. Colon External Quality Assessment scheme of the European Society of Pathology. kras.eqascheme.org. Accessed 5 Oct 2017
14. Eijkelenboom A, Kamping EJ, Kastner-van Raaij AW, Hendriks-Cornelissen SJ, Neveling K, Kuiper RP, Hoischen A, Nelen MR, Ligtenberg MJ, Tops BB (2016) Reliable next-generation sequencing of formalin-fixed, paraffin-embedded tissue using single molecule tags. J Mol Diagn 18(6):851–863. https://doi.org/10.1016/j.jmoldx.2016.06.010
15. Coördinatie Commissie ter bevordering van de Kwaliteitsbeheersing op het gebied van Laboratoriumonderzoek in de Gezondheidszorg. CCKL Praktijkrichtlijn 2005 www.cckl.nl. Accessed 21 Sep 2017

16. College of American Pathologists 2011. www.cap.org. Accessed 21 Sep 2017
17. International Organization for Standardization. ISO 15189:2012 Medical laboratories - Particular requirements for quality and competence. Geneva: ISO 2012
18. Vaughn CP, Zobell SD, Furtado LV, Baker CL, Samowitz WS (2011) Frequency of KRAS, BRAF, and NRAS mutations in colorectal cancer. Genes Chromosomes Cancer 50(5):307–312. https://doi.org/10.1002/gcc.20854
19. Molinari F, Felicioni L, Buscarino M, De Dosso S, Buttitta F, Malatesta S, Movilia A, Luoni M, Boldorini R, Alabiso O, Girlando S, Soini B, Spitale A, Di Nicolantonio F, Saletti P, Crippa S, Mazzucchelli L, Marchetti A, Bardelli A, Frattini M (2011) Increased detection sensitivity for KRAS mutations enhances the prediction of anti-EGFR monoclonal antibody resistance in metastatic colorectal cancer. Clin Cancer Res 17(14):4901–4914. https://doi.org/10.1158/1078-0432.CCR-10-3137
20. Boleij A, Tack V, Taylor A, Kafatos G, Jenkins-Anderson S, Tembuyser L, Dequeker E, van Krieken JH (2016) RAS testing practices and RAS mutation prevalence among patients with metastatic colorectal cancer: results from a Europe-wide survey of pathology centres. BMC Cancer 16(1):825. https://doi.org/10.1186/s12885-016-2810-3
21. Heidorn SJ, Milagre C, Whittaker S, Nourry A, Niculescu-Duvas I, Dhomen N, Hussain J, Reis-Filho JS, Springer CJ, Pritchard C, Marais R (2010) Kinase-dead BRAF and oncogenic RAS cooperate to drive tumor progression through CRAF. Cell 140(2):209–221. https://doi.org/10.1016/j.cell.2009.12.040
22. van Krieken JH, Normanno N, Blackhall F, Boone E, Botti G, Carneiro F, Celik I, Ciardiello F, Cree IA, Deans ZC, Edsjö A, Groenen PJ, Kamarainen O, Kreipe HH, Ligtenberg MJ, Marchetti A, Murray S, Opdam FJ, Patterson SD, Patton S, Pinto C, Rouleau E, Schuuring E, Sterck S, Taron M, Tejpar S, Timens W, Thunnissen E, van de Ven PM, Siebers AG, Dequeker E (2013) Guideline on the requirements of external quality assessment programs in molecular pathology. Virchows Arch 462(1):27–37. https://doi.org/10.1007/s00428-012-1354-4
23. Dijkstra JR, Heideman DAM, Meijer GA, Boers JE, 't Hart NA, Diebold J, Hirschmann A, Hoefler G, Winter G, Miltenberger-Miltenyi G, Pereira SV, Richman SD, Quirke P, Rouleau EL, Guinebretiere JM, Tejpar S, Biesmans B, van Krieken JHJM (2013) KRAS mutation analysis on low percentage of colon cancer cells: the importance of quality assurance. Virchows Arch 462(1):39–46. https://doi.org/10.1007/s00428-012-1356-2
24. European Society of Pathology. https://www.esp-pathology.org/. Accessed 21 Sep 2017
25. Deutsche Gesellschaft für Pathologie. https://www.pathologie-dgp.de/. Accessed 1 Aug 2017