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High sensitivity of both sequencing and real-time PCR analysis of *KRAS* mutations in colorectal cancer tissue

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Abstract

The *KRAS* mutation status predicts the outcome of treatment with epidermal growth factor receptor targeted agents, and therefore the testing for *KRAS* mutations has become an important diagnostic procedure. To optimize the quality of this test, we compared the results of the two most commonly used *KRAS* mutation tests, cycle sequencing and a real-time PCR-based assay, in DNA extracted from formalin-fixed paraffin-embedded (FFPE) colorectal cancer samples of 511 patients. The results were interpreted in the context of the tumour cell percentage and the assay parameters. In 510 samples *KRAS* mutation status assessment was successful. A *KRAS* mutation was detected in 201 tumours (39.4%). Sequencing and the real-time PCR-based assay generated the same result in 486 samples (95.3%). The sequencing result was considered false positive in one (0.2%) and false negative in nine samples (1.8%). The assay result was considered false positive in six (1.2%) and false negative in seven samples (1.4%). Explanations for discrepant test results were a higher sensitivity of the assay in samples with a low tumour cell percentage, occurrence of mutations that are not covered by the assay and δ Ct values approximating the cut-off value of the assay. In conclusion, both sequencing and the real-time PCR-based assay are reliable tests for *KRAS* mutation analysis in FFPE colorectal cancer samples, with a sensitivity of 95.5% (95% confidence interval [CI] 91.7–97.9%) and 96.5% (95% CI 93.0–98.6%), respectively. The real-time PCR based assay is the method of choice in samples with a tumour cell percentage below 30%.

Keywords: *KRAS* • diagnostic test • EGFR • colorectal cancer • sequencing • real-time PCR-based assay

Introduction

Targeted therapy has become increasingly important in the treatment of several solid tumour types such as colorectal cancer, breast cancer, renal cell carcinoma, head and neck cancer and lung cancer. The search for biologic characteristics determining the response to these agents is of great interest. This requires the development of new diagnostic tools and biomarkers. The selection of patients that are likely to benefit from targeted treatment will ultimately result in a more optimal patient outcome and a reduction of toxicity and costs. Several biomarkers have already been identified, such as overexpression of the gene encoding HER2 (*ERBB2*) as a predictor for the response to trastuzumab (a HER2 monoclonal antibody) in breast cancer [1], and an activating *KIT* mutation in gastrointestinal stromal tumours mediating the response to imatinib [2].

Involvement of the epidermal growth factor receptor (EGFR) signalling pathway has been identified in several cancer types. Binding of a ligand (EGF, transforming growth factor-α, epiregulin, amphiregulin) to the receptor induces dimerization and autophosphorylation and subsequent stimulation of several intracellular signalling pathways such as the RAS/RAF/MAPK pathway and the phosphoinositide-3 kinase (PI3K) pathway. This ultimately results in the stimulation of cell cycle progression, proliferation,

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angiogenesis and the inhibition of apoptosis [3]. Several drugs targeting the EGFR have shown a clinical benefit in cancer patients and have been approved for the use in clinical practice. *KRAS* encodes the KRAS protein which is involved in the MAPK signalling pathway. An oncogenic mutation in *KRAS* results in constitutive activation of the RAS/RAF signalling pathway independent from EGFR activation by binding of the ligand [4]. Patients with advanced colorectal cancer and a tumour harbouring a *KRAS* codon 12 or 13 mutation are resistant to treatment with the EGFR monoclonal antibodies cetuximab [5–9] and panitumumab [10]. Therefore, the European Medicines Agency has restricted the use of these antibodies to patients with wild-type *KRAS* tumours.

KRAS mutations are observed in approximately 38% of colorectal tumours [11]. Seven specific mutations in codon 12 (c.34G>A [p.Gly12Ser], c.34G>T [p.Gly12Cys], c.34G>C [p.Gly12Arg], c.35G>A [p.Gly12Asp], c.35G>T [p.Gly12Val], c.35G>C [p.Gly12Ala]) and 13 (c.38G>A [p.Gly13Asp]) comprise approximately 96% of the observed *KRAS* mutations whereas mutations in codon 61 comprise about 3% of the mutations [12]. Whether other than codons 12 and 13 mutations result in similar resistance to EGFR monoclonal antibodies remains to be assessed.

Given its important role for the selection of patients for anti-EGFR treatment, the number of patients in whom the assessment of the *KRAS* mutation status is indicated is increasing. Until recently sequencing was the most common method for *KRAS* mutation analysis. Recently, a real-time PCR-based assay targeting only the seven most prevalent *KRAS* mutations in codons 12 and 13 has become commercially available (DxS, Manchester, UK). Clinical studies already use a real-time PCR-based assay [6, 10, 13] but the assay has not been directly compared with sequencing. Although several different techniques to detect *KRAS* mutations are available [14], international guidelines for the performance and interpretation of *KRAS* mutation analyses are still to be developed [15]. In this study we compare the performance of a cycle sequencing approach with dye terminators of the region surrounding *KRAS* codons 12 and 13 and the commercially available real-time PCR-based assay using formalin-fixed paraffin-embedded (FFPE) colorectal cancer tissue from a randomized clinical trial, and give recommendations for interpretation of the *KRAS* test results obtained with both approaches.

Materials and methods

Selection of tumour material

Suitable tumour samples from 511 primary tumours were collected from 755 patients with previously untreated advanced colorectal cancer who participated in a multicentre phase 3 study (CAIRO2, CKTO 2005–02) of the Dutch Colorectal Cancer Group, and who were treated with capecitabine, oxaliplatin and bevacizumab, with or without cetuximab [16, 17]. The written informed consent required for all patients before study entry also included translational research on tumour tissue. The samples were collected from more than 50 different laboratories of pathology. The histology of all tumour samples was centrally reviewed by a pathologist (I.D.N., J.H.J.M.v.K.). Of 244 patients no suitable tumour tissue was available, because no resection of the primary tumour was performed ($n = 164$), the amount of available tissue was too low for DNA isolation ($n = 10$), the tissue was received too late to be included in this analysis ($n = 17$), the patient was excluded from all analyses due to ineligibility to the study protocol ($n = 19$), or no material was made available for this study ($n = 34$).

DNA extraction

Genomic DNA was extracted from four to eight manually microdissected 50-μm sections of FFPE tissue. Four micrometre haematoxylin-stained tissue sections, obtained before and after the withdrawal of the 50-μm sections, were used to estimate the tumour cell percentage in the microdissected tumour specimen. DNA was extracted as previously described [18]. After the ProtK incubation, RNA-free genomic DNA was obtained by adding 10 μl RNase A (100 mg/ml, Qiagen, West Sussex, UK) for 1 hr at 37°C. Further DNA isolation was performed according to the manufacturer's protocol (DNeasy blood and tissue kit, Qiagen), except for the wash step with 500 μl buffer AW2 which was repeated once. DNA was eluted in 50 μl AE buffer (provided by the Qiagen kit). DNA concentration was determined at 260 nm using the Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Inc., Wilmington, DE, USA).

Multiplex control PCR

DNA quality was assessed by performing a multiplex PCR using four primer sets, resulting in fragments of 100, 200, 300 and 400 base pairs (bp) [19]. A 50 μl reaction mixture contained 50 ng DNA; 0.2 μM of each primer (Invitrogen, Paisley, UK); dATP, dCTP, dGTP, dTTP (GE Healthcare, Piscataway, NJ, USA) at 500 mM each; 50 mM KCl; 10 mM Tris-HCl (pH 8.3); 2 mM $MgCl_2$ (Applied Biosystems, Foster City, CA, USA) and 1 U AmpliTaq Gold (Applied Biosystems). The PCR conditions were as follows: 94°C for 10 min.; 92°C for 30 sec., 60°C for 40 sec., 72°C for 40 sec. (35 cycles) and 72°C for 30 min.

The PCR products were analysed on a 2% agarose gel containing 0.3 μg/ml ethidium bromide (Sigma, St Louis, MO, USA). DNA quality classification was based on the largest of the four possible fragments detected.

KRAS mutation analysis by dye terminator cycle sequencing

A 50 μl reaction mixture, containing the oligonucleotide primers KRAS-F and KRAS-R (Invitrogen) at 0.2 μM each; dATP, dCTP, dGTP, and dTTP (GE Healthcare) at 500 μM each; 50 mM KCl; 10 mM Tris-HCl (pH 8.3); 2.5 mM $MgCl_2$; 1 U of AmpliTaq Gold polymerase (Applied Biosystems); and 50 ng of template DNA, was used to generate a 198 bp PCR product of the *KRAS* gene (GenBank accession number NM_004985). The PCR conditions were as follows: 94°C for 10 min.; 92°C for 1 min., 60°C for 45 sec., 72°C for 45 sec. (35 cycles); and 72°C for 10 min. PCR products were purified with the

MinElute 96 UF PCR Purification Kit (Qiagen) and subsequently used for sequencing with fluorescently labelled terminators (BigDye® Terminators (v 1.1); Applied Biosystems) with both the M13-F and M13-R sequencing primers. The following primers were used for *KRAS* amplification:

5′-*TGTAAAACGACGGCCAGT*GGTACTGGTGGAGTATTTGATAGTG-3′ (forward) and 5′-*CAGGAAACAGCTATGACC*TGGATCATATTCGTCCACAAAA-3′ (reverse). The nucleotides presented in italic correspond to M13 consensus sequences and were used for the cycle sequencing with M13 consensus primers.

The sequencing products were analysed on an ABI 3730 DNA Analyzer (Applied Biosystems) and the data analysis was performed with Sequencing Analysis Software Sequencing Analysis Software v5.3.1 with KB™ Basecaller. Sequence results were scored by visual inspection of the chromatograms.

KRAS mutation analysis using the real-time PCR-based assay

In this study we used a commercially available real-time PCR-based assay for *KRAS* mutation detection (DxS) which combines the ARMS® and Scorpions® technique to identify the seven following *KRAS* mutations, located in codon 12 and 13: c.34G>A (p.Gly12Ser), c.34G>T (p.Gly12Cys), c.34G>C (p.Gly12Arg), c.35G>A (p.Gly12Asp), c.35G>T(p.Gly12Val), c.35G>C (p.Gly12Ala) and c.38G>A (p.Gly13Asp). In a real-time or quantitative PCR the amount of product is monitored during the reaction. The number of amplification cycles required to obtain a certain amount of PCR product is registered as the threshold cycle (Ct) [20]. A mutation is present when the δ Ct (which is the Ct of the mutation specific PCR minus the Ct of a PCR of an endogenous control gene) is below a mutation specific threshold. The δ CT values that were used as a cut-off level for the presence of a *KRAS* mutation were based on the detection of at least 1% tumour cells and were provided by the manufacturer of the kit. This δ Ct cut-off values were as follows for the different mutations: c.34G>A cut-off 9, c.34G>T cut-off 7, c.34G>C cut-off 8, c.35G>A cut-off 8, c.35G>T cut-off 6.5, c.35G>C cut-off 6.5 and c.38G>A cut-off 9. The assay was validated for analytical and diagnostic use and performed according to manufacturer's instructions on a 7500 Fast Real-Time PCR System (Applied Biosystems). The exact location of the primers used in this kit is not available. The amount of input DNA was based on both the concentration of isolated DNA and the quality of DNA, as determined by the multiplex control PCR. Duplicate analyses were only performed in case of a discrepancy between the result of the sequencing and the assay.

Statistical analysis

The tumour cell percentages in previously irradiated tumours compared to not irradiated tumours was compared using a Wilcoxon rank sum test. The correlations between poor DNA quality and previous radiotherapy, between a low tumour cell percentage and previous radiotherapy, and between a low tumour cell percentage and *KRAS* mutation frequency were calculated using a Fisher's exact test. All tests were performed two-sided with a significance level of 0.05. The statistical tests were performed with SPSS version 16.0 (SPSS, Inc,, Chicago, IL, USA).

Results

Sample description

FFPE colorectal cancer tissue was obtained from 511 patients. The baseline characteristics of these patients were comparable with the total study population (data not shown). The median DNA fragment length was 300 bp (range 100–400 bp). Forty samples (7.8%) contained poor quality DNA (less than 200 bp on the multiplex control PCR). Poor DNA quality was not associated with previous radiotherapy on the primary tumour (10% poor quality DNA in 110 irradiated tumours *versus* 7.2% in 390 not irradiated tumours, $P = 0.32$). The frequency of the different tumour cell percentages as assessed on a haematoxylin-stained tissue section is shown in Fig. 1A. The median tumour cell percentage was 60% (range 3–90%). A low tumour cell percentage was associated with previous radiotherapy on the primary tumour (Fig. 1B). In 109 previously irradiated tumours the median tumour cell percentage was 40%, compared to 60% in 375 not irradiated tumours ($P < 0.001$). Ten samples (9.2%) of patients who received previous radiotherapy contained 10% or less tumour cells *versus* four samples (1.1%) of patients without previous radiotherapy ($P < 0.001$). The radiotherapy status of 10 samples was unknown.

Consistency between sequencing and real-time PCR-based assay

In all 511 samples the *KRAS* mutation status was assessed both by using sequencing and the real-time PCR-based assay. In 486 samples (95.3%) the two test methods generated the same outcome. In the discrepant samples both tests were repeated and, providing the availability of sufficient tissue, new DNA was isolated in which again *KRAS* mutation analysis was performed.

Description of discrepant test results (Table 1)

In seven samples a mutation was detected by sequencing but not by the assay (no. 1–7). In four of these samples in which a mutation was only detected by sequencing, the duplicate analyses generated the same discrepant results (no. 1–4). In three cases the mutations remained undetected by the real-time PCR-based assay because these alterations were not targeted by the assay (c.35_36delinsAG (p.Gly12Glu); c.38_39delinsAG (p.Gly13Glu); c.[38G>A; 40G>A] (p.[Gly13Asp; Val14Ile]; no. 1–3). In the fourth case a new DNA isolation did no longer reveal a mutation by sequencing, thus the initial sequencing was considered false positive due to a sample tracking problem (no. 4). In the other three samples the second analysis with the kit confirmed the sequencing results, and thus the kit was considered false negative (no. 5–7).

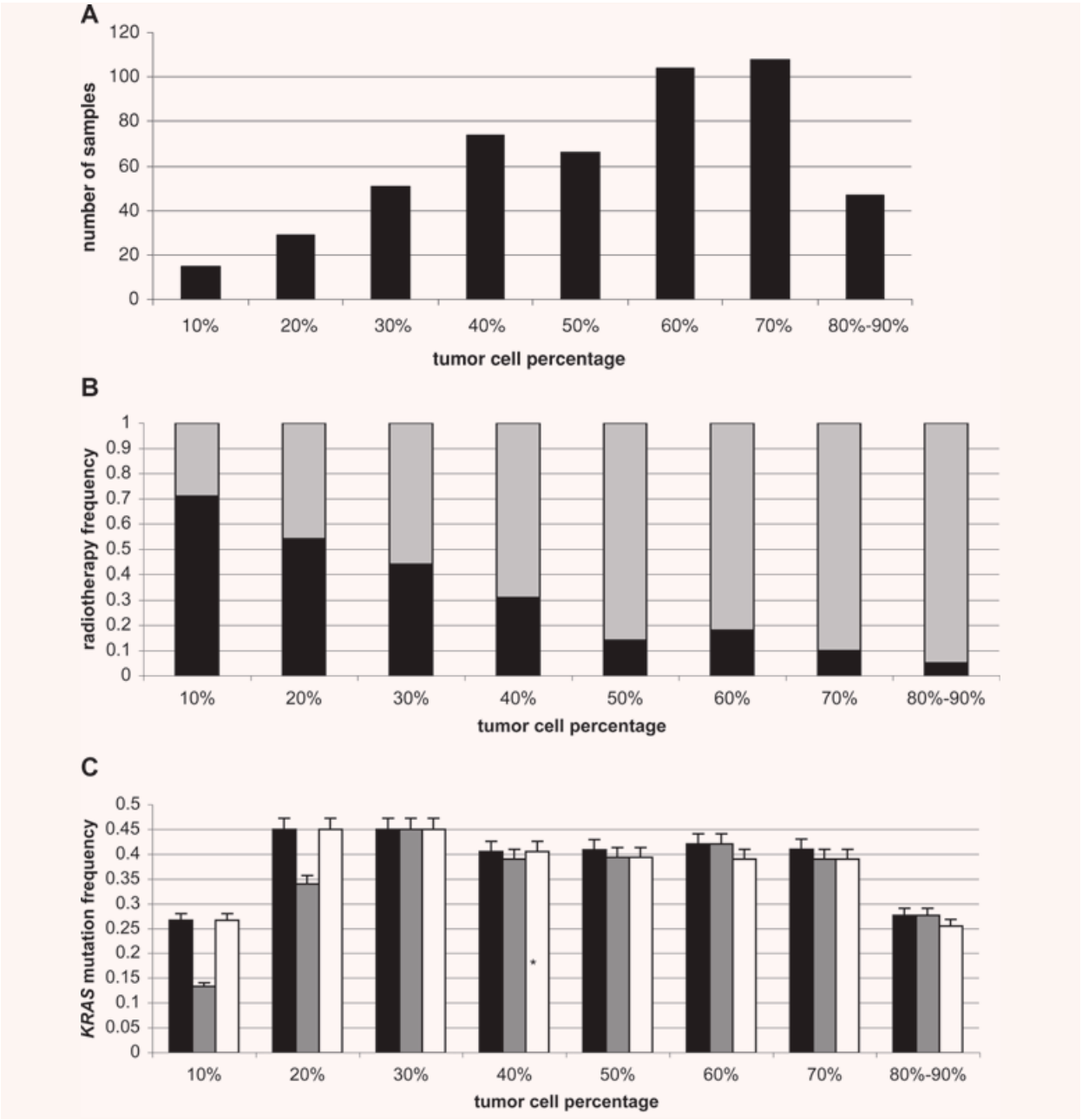

Fig. 1 (**A**) The number of samples per tumour cell percentage. (**B**) The frequency of irradiated (black bars) *versus* not irradiated tumours (grey bars) according to tumour cell percentage. (**C**) The *KRAS* mutation frequency (with 5% confidence level) according to the final test result (black bars), according to the sequencing results (grey bars) or the results with the real-time PCR-based assay (white bars) according to tumour cell percentage.

In one sample the mutation c.37G>T (p.Gly13Cys), which was found in duplicate by sequencing was detected as c.35G>T (p.Gly12Val) in the first and as wild-type in the second real-time PCR experiment (no. 8). This rare mutation is not targeted by the kit.

In 16 samples a mutation was detected by the assay and not by sequencing. In eight of these samples the inconsistent results were persistent in the second analyses. In one sample the mutation probably remained below the detection limit of our sequencing method due to heterogeneity of the primary tumour, which

Table 1 Description of 24 discrepant test results

No.	Tumour cell%	Sequence 1	Assay 1	Sequence 2	Assay 2	Sequence new DNA	Final interpretation	Test	Explanation
1	80	c.35_36delinsAG	wt	c.35_36delinsAG	wt	c.35_36delinsAG	**c.35_36delinsAG**	FNA	not targeted by assay
2	80	c.38_39delinsAG	wt	c.38_39delinsAG	wt		**c.38_39delinsAG**	FNA	not targeted by assay
3	90	[c.38G>A; 40G>A]	wt	[c.38G>A; 40G>A]	wt	[c.38G>A; 40G>A]	**[c.38G>A; 40G>A]**	FNA	not targeted by assay
4	80	c.35G>A	wt	c.35G>A	wt	wt	**wt**	FPS	
5	60	c.34G>T	wt	c.34G>T	c.34G>T	c.34G>T*	**c.34G>T**	FNA	
6	50	c.35G>A	wt	c.35G>A	c.35G>A	c.35G>A*	**c.35G>A**	FNA	
7	70	c.38G>A	wt	c.38G>A	c.38G>A	c.38G>A	**c.38G>A**	FNA	
8	80	c.37G>T	c.35G>T	c.37G>T	wt		**c.37G>T**	FNA	not targeted by assay
9	70	wt	c.35G>T	wt	c.35G>T	wt/ c.35G>T[#]	**c.35G>T**	FNS	heterogeneity
10	20	wt	c.34G>C	wt	c.34G>C		**c.34G>C**	FNS	low tumour cell%
11	20	wt	c.35G>A	wt	c.35G>A	c.35G>A**	**c.35G>A**	FNS	low tumour cell%
12	10	wt	c.35G>A	wt	c.35G>A	c.35G>A*	**c.35G>A**	FNS	low tumour cell%
13	20	wt	c.35G>T	wt	c.35G>T		**c.35G>T**	FNS	low tumour cell%
14	10	wt	c.35G>T	wt	wt	c.35G>T**	**c.35G>T**	FNS	low tumour cell%
15	80	wt	c.35G>A	wt	c.35G>A	wt	**wt**	FPA	high δ Ct
16	50	wt	c.35G>A	wt	wt		**wt**	FPA	high δ Ct
17	80	wt	c.38G>A	wt	c.38G>A	wt	**wt**	FPA	high δ Ct
18	30	wt	c.38G>A	wt	wt	wt	**wt**	FPA	high δ Ct
19	50	wt	c.34G>T	wt	wt	wt[%]	**wt**	FPA	.
20	50	wt	c.38G>A	wt	wt	wt[%]	**wt**	FPA	
21	80	wt	c.34G>T	c.34G>T	c.34G>T		**c.34G>T**	FNS	
22	50	wt	c.35G>A	c.35G>A	c.35G>A	c.35G>A	**c.35G>A**	FNS	
23	50	wt	c.35G>T	c.35G>T	c.35G>T	c.35G>T	**c.35G>T**	FNS	
24	10	wt	c.38G>A	wt	c.38G>A		**Inconclusive**		low tumour cell% and high δ Ct

*For the second DNA isolation tissue was derived from a different cube of the same tumour.
**For the second DNA isolation tissue derived from metastatic tissue was used with a higher tumour cell percentage.
[#]DNA isolated from two different regions of the tumour showed wild-type with sequencing and with the assay wild-type in one sample and a c.35G>T mutation in another sample indicating intratumoral heterogeneity.
[%]In the newly isolated DNA the real-time PCR based assay also showed wild-type KRAS.
wt = wild-type.
FNA = false negative result assay.
FPS = false positive result sequencing.
FNS = false negative result sequencing.
FPA = false positive result assay.
Bold figures indicate difference in significance *vs* other columns.

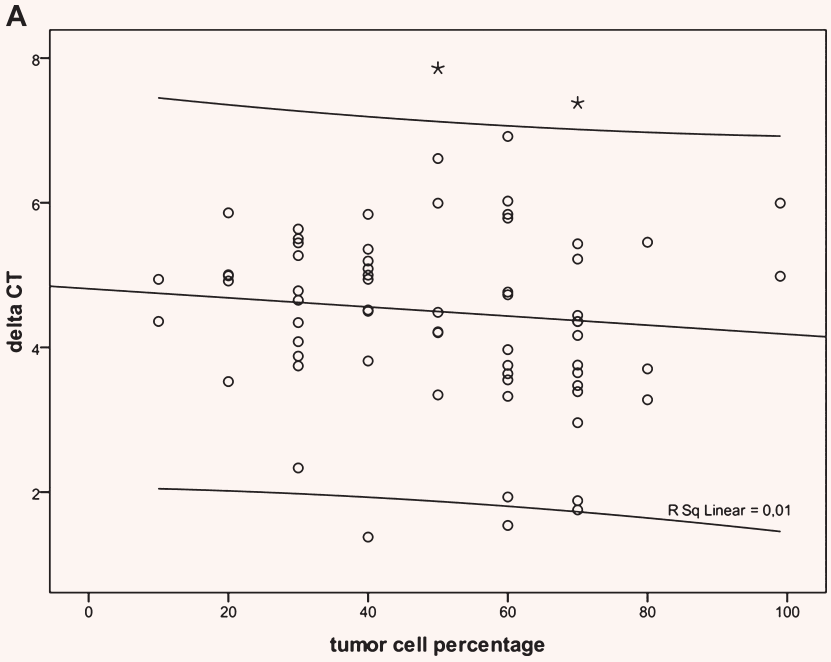

Fig. 2 δ Ct in relation to tumour cell percentage and true positive (○), false positive (*) and inclusive (■) assay results with a fit line plus 95% CI for the individual values. (**A**) Results for *KRAS* c.35G>A (p.Gly12Asp). For this alteration the δ Ct cut-off value as provided by the manufacturer of the kit was 8. (**B**) Results for *KRAS* c.38G>A (p.Gly13Asp). For this alteration the δ Ct cut-off value as provided by the manufacturer of the kit was 9.

could be demonstrated by DNA isolation of two different regions from the tumour (no. 9). In five other persistent cases (no. 10–13, 24) and in one case in which the assay was not consistently positive (no. 14), the tumour cell percentage was 20% or lower. Again the mutation probably remained below the detection limit of our sequencing method, which could be confirmed by analyses of a second isolate with a higher tumour cell percentage in three cases (no. 11, 12, 14). In two cases in which the assay was consistently positive while sequencing was negative and in two other cases in which only the first assay result was positive the δ Ct approximated

the cut-off value as provided by the manufacturer of the assay (c.35G>A (p.Gly12Asp) δ Ct of 7.38 and 7.05 for sample no. 15 and 7.86 for sample no. 16, cut-off of 8 and c.38G>A (p.Gly13Asp) δ Ct of 8.14 and 8.01 for sample no. 17 and 8.59 for sample no. 18, cut-off of 9). The correlation between the δ Ct and tumour cell percentage for these two mutations is given in Fig. 2. These four samples (marked as ⋆ in Fig. 2) are outliers and are considered as false positive results of the assay. No correlation between the δ Ct and tumour cell percentage was observed ($r = 0.01$ and $r = 0.038$ for c.35G>A and c.38G>A, respectively). Also in one of the above mentioned discrepant samples with a low tumour cell percentage a c.38G>A (p.Gly13Asp) mutation was observed with a high δ Ct of 8.02 (no. 24, marked as ■ in Fig. 2B). As we cannot judge whether the sequence analysis is false negative or the assay is false positive, this sample is considered inconclusive. In two other cases the positive result with the assay could not be repeated and thus the kit was considered false positive (no. 19, 20). In three cases the mutation as detected in duplicate by the assay could be confirmed in the second sequence and thus the initial sequence was considered false negative (no. 21–23).

Reliability of both tests in samples with low tumour cell percentages

We observed that *KRAS* mutations can be missed by sequencing in samples with a tumour cell percentage below 30%. The distribution of *KRAS* mutation frequency according to tumour cell percentage for the assay, sequencing and the most likely final test result is shown in Fig. 1C. In 4 of the 15 samples (26.7%) containing 10% or less tumour cells a mutation was detected with the assay, which is not significantly different from the mutation frequency based on the final test result in samples containing more than 10% tumour cells (40.5%; $P = 0.42$). However, in only two of these samples (13.3%) a mutation was detected by sequencing, which is significantly less than in samples with more than 10% tumour cells ($P = 0.035$). In samples with 20% tumour cells still 23% of the *KRAS* mutations were missed by sequencing. However, the *KRAS* mutation frequency in samples containing 20% or less tumour cells was not statistically significant different from that of samples with more than 20% tumour cells (40.2% *KRAS* mutations) neither by sequencing (27.3% *KRAS* mutations, $P = 0.11$), nor by the assay (38.6%, $P = 0.87$). In samples with 30% tumour cells all 23 mutations are detected by sequencing.

Test characteristics

Overall, the mutation status of one sample was inconclusive, sequencing was considered false negative in nine and false positive in one sample and the kit was considered false negative in seven and false positive in six samples. A *KRAS* mutation was detected in 201 out of 510 samples (39.4%). The prevalence of the different *KRAS* mutations is shown in Table 2. The sensitivity of sequencing and of the real-time PCR-based assay was 95.5% (95% confidence interval [CI] 91.7–97.9%) and 96.5% (95% CI 93.0–98.6%), respectively. Further characteristics of both tests are shown in Table 3. Considering the lower sensitivity of sequencing analysis in samples with less than 30% tumour cells, sensitivity and specificity of both tests were also calculated in the subgroup of 398 samples with more than 30% tumour cells, resulting in a sensitivity of sequencing and the assay of 97.5% (95% CI 93.6–99.3%) and 95.6% (91.1–98.2%), respectively.

Table 2 The distribution of *KRAS* mutations in 510 samples

	N	%
c.34G>A (p.Gly12Ser)	8	1.6
c.34G>T (p.Gly12Cys)	20	3.9
c.34G>C (p.Gly12Arg)	5	1.0
c.35G>A (p.Gly12Asp)	67	13.2
c.35G>T (p.Gly12Val)	51	10.0
c.35G>C (p.Gly12Ala)	14	2.8
c.35_36delinsAG (p.Gly12Glu)*	1	0.2
c.37G>T (p.Gly13Cys)*	1	0.2
c.38G>A (p.Gly13Asp)**	33	6.5
c.38_39delinsAG (p.Gly13Glu)*	1	0.2
Total	**201**	**39.4**

*Nucleotide changes not covered by the real-time PCR-based assay.
**One of these samples had an additional alteration c.[38G>A; 40G>A] (p.[Gly13Asp; Val14Ile]).

Table 3 Test characteristics of cycle sequencing and real-time PCR-based assay for *KRAS* mutation assessment ($n = 510$)

	Cycle sequencing	Real-time PCR-based assay
Sensitivity	95.5%	96.5%
(95% CI)	(91.7–97.9%)	(93.0–98.6%)
Specificity	99.7%	98.1%
(95% CI)	(98.2–100%)	(95.8–99.3%)
Positive predictive value	99.5%	97.0%
(95% CI)	(97.1–100%)	(93.6–98.9%)
Negative predictive value	97.2%	97.7%
(95% CI)	(94.7–98.7%)	(95.4–99.1%)

Discussion

In this series of 511 primary colorectal cancer samples we were able to compare the reliability of *KRAS* mutation analysis on FFPE tissue by cycle sequencing and a commercial FDA approved real-time PCR-based assay. Since a concordant result between the tests was obtained in over 95% of samples, both cycle sequencing and the real-time PCR-based assay are reliable methods for *KRAS* mutation assessment in colorectal cancer. Without doing duplicate experiments the *KRAS* mutation status was incorrectly determined by cycle sequencing in 2.0% and by the commercial assay in 2.6% of the samples. These results are well below the threshold commonly used in biomarker testing [21–23].

Several explanations for the false test results are relevant for general clinical practice. First, laboratories offering *KRAS* mutation analysis in colorectal cancer should be aware of the relevance of the tumour cell percentage of material used for DNA isolation. In samples containing less than 30% tumour cells *KRAS* mutations can be missed by cycle sequencing. To avoid these low tumour cell percentages it is essential to carefully select and microdissect the tissue sample used for DNA isolation. As a low tumour cell percentage is often due to preoperative radiotherapy, the reliability of the mutation detection will in most cases be higher on biopsies that are taken before the start of radiotherapy. In the rare cases in which a tumour cell percentage of 30% cannot be reached, the commercial kit would be the method of choice. We recommend to include the tumour cell percentage in all reports and to mention the possibility of a false negative result in samples with a tumour cell percentage below 30%.

Second, the standard δ Ct cut-off values of the real-time PCR-based assay, which are designed to detect 1% mutation in a background of wild-type DNA might be too high. This might lead to false positive results. In four samples that were positive in the initial analysis with the kit, but negative by cycle sequencing, the δ Ct of that particular mutation was higher than in all other samples tested positive. The most likely explanation for these four discrepant test results is that the assay is false positive, although tumour heterogeneity with only a small percentage of tumour cells harbouring a *KRAS* mutation cannot be formally excluded. In our series there was no relation between the δ Ct and the tumour cell percentage (Fig. 2), while we had expected to find lower δ Ct values with increasing tumour cell percentages. Although this might partly reflect variations in tumour heterogeneity or aneuploidy, also some samples with a very pronounced mutant allele in the cycle sequencing analysis give relatively high δ Ct values, showing that at least part of this variation is due to intrinsic test characteristics. We recommend to register and report both the δ Ct value of the individual analyses and the cut-off value and to assess the mutation status by cycle sequencing in cases with a δ Ct value approximating the cut-off value.

Third, by the commercial kit only the most abundant *KRAS* mutations are detectable. In our series four out of 510 samples (<1%) were false negative with the assay because the sequence alterations were not covered by the kit. *KRAS* mutations located outside codons 12 and 13 will not be targeted by the assay as well. Cycle sequencing would allow for detecting mutations other than those targeted by the kit and circumvents the problem of possible false positive results due to a high δ Ct. On the other hand, the real-time PCR based kit is the method of choice in samples containing less than 30% tumour cells.

Assessment of *KRAS* mutation status in FFPE tumour material is technically more challenging than in fresh frozen tissue due to DNA fragmentation. However, fresh frozen tissue is usually not available in routine clinical practice. With the primer set we used for amplification a product with a length less than 200 bp including the M13 sequences was obtained. Both amplification of this product and of the control amplicon present in the kit were successful in 100% of the samples. DNA fragment length thus was not a limiting determinant in *KRAS* mutation assessment. We show that FFPE colorectal cancer tissue derived from many different routine pathology laboratories is suitable for *KRAS* mutation detection.

Our data show that analysis by both direct sequencing and the commercial assay provide more optimal results compared to a single test. However, the relatively small increase in accuracy does not appear to justify the associated increase in costs. Most discrepancies would be overcome by performing either of the tests in duplicate, by using the assay in samples with a tumour cell percentage below 30% and by sequence analysis of samples with a higher tumour cell percentage in which the assay results in a high δ Ct. To date, the published data on *KRAS* mutation assessment using a real-time PCR-based assay have either been presented as the result of single testing [10] or with confirmation of all mutations by sequencing [6]. To learn more about the limitations and interlaboratory reproducibility of these analyses and considering the expected increase in the use of *KRAS* testing, a quality assurance program as proposed by the European Society of Pathology is warranted [15]. A high quality of *KRAS* testing is important since false results have major clinical implications. Not only is the benefit of anti-EGFR therapy restricted to patients with *KRAS* wild-type tumours, patients with *KRAS* mutated tumours may even be harmed by this treatment [16, 24, 25]. Lastly, data accumulate that *KRAS* mutation status is not the only determinant for the efficacy of anti-EGFR treatment. Other molecular determinants that are involved in the response to anti-EGFR antibodies, such as *BRAF* mutations [26], *PIK3CA* mutations [27] and *EGFR* gene copy number changes [28], may become relevant selection parameters for this type of treatment.

In conclusion, we show that both cycle sequencing and a commercial real-time PCR-based assay are reliable tests for *KRAS* mutation analysis in FFPE colorectal cancer samples provided an adequate percentage of tumour cells is present in the tissue tested.

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