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Concordance in *KRAS* and *BRAF* mutations in endoscopic biopsy samples and resection specimens of colorectal adenocarcinoma

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Abstract ***Background:*** *KRAS* testing is mandatory if anti-*EGFR* therapy is considered in patients with metastatic colorectal cancer (CRC). In addition, *BRAF* mutations seem to be an important negative prognostic factor. The aim of this study is to establish the concordance of *KRAS* and *BRAF* mutational status in paired biopsy and resection specimens of primary CRC using several analytic methods.

Methods: DNA was extracted from paraffin blocks of 126 CRC patients. *KRAS* codon 12/13 and *BRAF* V600E mutational status was assessed using high resolution melting (HRM), direct sequencing (DS) of the HRM polymerase chain reaction (PCR) product. In addition, the Therascreen Amplification Refractory Mutation System (ARMS)-Scorpion *KRAS* assay and *BRAF* pyrosequencing were employed; both assays claim to require less tumour cells in comparison with DS.

Results: *KRAS* and *BRAF* were found to be mutually exclusive. Mutation frequencies were 33.9% for *KRAS*, and for *BRAF* 19.0%, respectively. Concordance of *KRAS* mutational status between biopsy and resection specimens was 97.4% (ARMS), 98.4% (DS) and 99.2% (HRM), respectively. For *BRAF* concordance was 98.4% (Pyro, DS) and 99.2% (HRM).

Conclusions: *KRAS* and *BRAF* mutational status of endoscopic biopsies and resection specimens of CRC showed a >95% concordance. Endoscopic biopsies can be confidently used for molecular analysis.

1. Introduction

The demand for *KRAS* mutational analysis as a predictive marker has increased rapidly. Prior to treatment with epidermal growth factor receptor (EGFR) inhibitors in colorectal cancer (CRC), *KRAS* testing has become mandatory in the European Union[1,2] and is

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recommended in the United States.[3] In addition, *BRAF* mutations are emerging as a very strong negative prognostic factor in CRC.[4]

KRAS is a member of the RAS proteins which are small GTPases that act as molecular switches. *KRAS* binds to Guanosine triphosphate (GTP) in the active state and has an intrinsic enzymatic activity which cleaves the terminal phosphate of the nucleotide converting it to Guanosine diphosphate (GDP).[5,6] Upon conversion of GTP to GDP, *KRAS* is turned off.[5,7] The *KRAS* protein has an important role in Ras/MAPK signalling in which GTP-bound *KRAS* regulates a variety of cellular processes, including proliferation, differentiation and apoptosis.[6,8] In CRC, codon 12 and 13 of the *KRAS* gene are mutated in 35% of cases,[9] transforming the intrinsic GTPase activity of the protein in the constitutively active conformation.[10]

BRAF, a member of the Raf kinase family of serine/threonine-specific protein kinases, is a cytosolic protein kinase and is activated by membrane-bound RAS. Mutated *BRAF* activates a signalling pathway, which causes cell proliferation and inhibits apoptosis.[10] The most common mutation is a single glutamic acid for valine substitution at codon 600 causing the V600E point mutation,[7] the *BRAF* mutation frequency is 11%.[9] Earlier, Roth and colleagues[11] described the mutual exclusivity of *BRAF* and *KRAS* mutations.

The activating mutations in *KRAS* and *BRAF* induce constitutive Ras/MAPK signalling, which cannot be suppressed by EGFR inhibition. Due to prevent undesirable side-effects of the EGFR antibodies and to suppress excessive treatment costs *KRAS* mutation analysis prior to anti-EGFR therapy is indicated.[12] In addition, *BRAF* mutational analysis yields prognostic information.[11]

Currently the most commonly used method for *KRAS* and *BRAF* mutation analysis is direct sequencing (DS). Nevertheless, this method requires high tumour cell percentages and good quality material. Resection specimens normally show large amounts of tumour cells. Biopsies generally consist of small tumour foci that might not meet the requirements for direct sequencing. An alternative is high-resolution-melting (HRM) analysis. High resolution melting (HRM) is advocated to be a rapid and cheap 'pre-screen' method, and can distinguish wild type from mutated genes based on subtle differences in temperature-depended denaturation ('melting') of double strand DNA fragments. This method requires considerably lower tumour cell percentages compared to DS but is unable to define the exact nature of a mutation when non-wild type melting curves are found. Other methods allowing low tumour cell percentages are pyrosequencing and a specific Amplification Refractory Mutation System (ARMS)-Scorpion PCR assay.

To date, no systematic study has been performed to ascertain the validity of *KRAS* and *BRAF* mutational analysis of endoscopic biopsy material in comparison to resection specimens.

The aim of our study was to establish the concordance of *KRAS* and *BRAF* mutational status between paired endoscopic biopsy and resection specimens in an unselected group of CRC patients. Three molecular assessment methods were used, e.g. DS and HRM for both genes, and in addition DxS ARMS-Scorpion Therascreen for *KRAS* and Qiagen Therascreen Pyro kit for *BRAF*.

2. Patients and methods

2.1. Patient selection

Colorectal adenocarcinoma cases were retrieved from the Department of Pathology, Isala klinieken, Zwolle, The Netherlands from the 2002 to 2005 period. Next, cases were selected: (i) colorectal endoscopic biopsies with an unequivocal report stating 'colorectal adenocarcinoma', and (ii) subsequent colorectal resection without any prior treatment stating unequivocally 'colorectal adenocarcinoma'. Archival formalin fixed paraffin-embedded (FFPE) tumour blocks were retrieved of both biopsy and resection specimens; 5 μm slides were cut and stained with haematoxylin & eosin (H&E); cases lacking adequate tumour tissue (defined as less than approx. 10% tumour percentage) were excluded. In total, 126 cases remained for the present study. Patient gender, age, location of CRC and stage were provided; right sided CRC was defined as: coecum, colon ascendens and colon transversum and left sided CRC as colon descendens and sigmoid. *KRAS* and *BRAF* mutational status was correlated with gender, age, CRC location and stage; statistical analysis was performed using Chi-square test for categorical variables or Fisher's in the case of dichotomous variables with small groups, all 2-tailed using alpha 0.05 as significance level. Analysis was performed using PASW version 18 (SPSS Inc., Chicago, IL, USA).

The Ethical Committee of the Isala klinieken declared that the study was not subjected to their approval being exempted from Wet Medisch-Wetenschappelijk Onderzoek (Law Medical Research) as a retrospective anonymous study.

2.2. DNA extraction

After the initial H&E stained slides, 4 (resection specimens) or 12 (biopsy specimens) additional slides were cut and mounted on slides for DNA extraction, followed by a final H&E stained section in order to check tumour availability. Guided by the H&E stained slides macrodissection was performed on the unstained slides discarding areas without tumour tissue. Genomic DNA was extracted with the QIAmp DNA FFPE Tissue kit (Qiagen, Venlo, NL) using the Qiacube automated method; concentration and purity of DNA was checked

by spectrophotometry (Nanodrop, Thermo Scientific, Landsmeer, NL). If DNA concentration exceeded 50 ng/μl then the DNA concentration was normalised to a concentration of approx. 50 ng/μl. No minimum concentration was defined. Multiplex polymerase chain reaction (PCR) (with 100, 200, 300, and 400 bp as reference) was performed in order to analyse the DNA quality of the assays that failed repeatedly in the HRM-PCR of *KRAS* and/or *BRAF*.

2.3. KRAS analysis

2.3.1. High resolution melting (HRM)

Prior to this study, dilution experiments were performed with FFPE tissue of patients with known *KRAS* mutational status (CAIRO2 study material provided by Dr. J. Tol, University of Nijmegen, NL). A minimum tumour cell percentage of 12.5% was established without macrodissection. Presumably, the macrodissection performed in the present study achieves even higher sensitivity. Primers for *KRAS* analysis – previously defined by Krypuy et al.[5] – span *KRAS* exon 2, codons 12 and 13, with an amplicon length of 189 bp: Forward: 5′-TCATTATTTTTATTATAAGGCCTGCTGAA-3′; Reverse: 5′-CAAAGACTGGTCCTGCACCAGTA-3′. HRM was performed in duplicate in a final volume of 25 μl using 12.5 μl HRM Mastermix (Roche Diagnostics, Almere, The Netherlands), 1 μl genomic DNA, 200 nM of each primer, 3.5 mM HRM MgCl2 (Roche Diagnostics), 1.2 U HRM Uracil-DNA glycosylase (UNG) (Roche Diagnostics) and ddH_2O.

PCR cycling and HRM analysis was performed on a LightCycler 480 II (Roche Diagnostics) using the following conditions: PCR: first cycle UNG incubation for 10 min at 40 °C; second cycle for 10 min at 95 °C; 45 subsequent cycles for 10 s at 95 °C, 10 s at 60 °C and 30 s at 72 °C. Melt from 70 to 95 °C at 0.2 °C per second, the HRM curve analysis was performed using the Gene Scanning Software (Roche Diagnostics). For sample analysis, melting curves were normalised, temperature-shifted and, finally, a difference plot was generated. *KRAS* mutations were identified by comparison of each patient's plot with that of a mutant and a wild type control. Samples were considered mutated when a significant difference of fluorescence level for both duplicates fell outside the range of variation of the wild-type control. When duplicate samples failed to show an identical signal, new duplicate analysis was performed. When the difference plots failed to show an unequivocal signal of either 'normal/wild type' or 'abnormal/mutation', the HRM analysis was deemed as a 'failed analysis/non-analysable'.

2.3.2. Direct sequencing

After HRM analysis in duplicate, the PCR products were purified using the QIAquick PCR purification kit (Qiagen) and used as template for direct sequencing using the Big Dye Terminator v3.1 kit (Applied Biosystems, Nieuwerkerk a/d Ijssel, The Netherlands). The reaction mix consisted of 2 μl Sequencing RR-100, 3 μl 5× Sequencing buffer, 4 μl ddH2O), 1 μl purified PCR product and 0.2 nM primer (*KRAS* forward primer 5′-TCATTATTTTTATTATAAGGCCTGCTGAA-3′ or *BRAF* reverse primer 5′-TGATTAAATTTTTGGCCCTGA-3′) in a final volume of 20 μl. The PCR reaction prior to sequencing was run on a Veriti 96-well Thermal Cycler (Applied Biosystems) according the following conditions: one cycle of 96 °C for 1 min; 25 cycles of 96 °C for 10 s, 60 °C for 125 s; 4 °C 'forever'. The sequence reactions were purified using the DyeEx 2.0 Spin Kit (Qiagen), run on a 3130 Genetic Analyzer (Applied Biosystems); sequences were analysed using the Sequencer 3.0 software (Applied Biosystems). Read outs of the duplicates were compared and repeated if discordant read outs were generated. Read outs were analysed without prior knowledge of the HRM results.

2.3.3. DxS ARMS-Scorpion K-RAS Therascreen

The FDA-approved K-RAS Therascreen Conformité Européenne – In vitro diagnostic use (CE-IVD) Kit (DxS, Manchester, UK) is an allele-specific PCR-based technology with specific primers for the seven most common *KRAS* codon 12 and 13 mutations. The assay screens for the following mutations: 12ALA, 12ASP, 12ARG, 12CYS, 12SER, 12VAL, and 13ASP. Analysis was performed on the LightCycler 480 II (Roche Diagnostics) according to instructions in the TheraScreen *K-RAS* Mutation Kit manual. Eight assays are supplied for each sample (seven assays each separate for each of the seven mutations, one control assay). In total 8×5 μl genomic DNA was used for each sample. The quality thresholds were applied according the manual, e.g. samples with a cycle threshold (Ct) of the HEX control $\geqslant 35$ were rejected, and samples with a 5′-fluorescein dye mutation signal of Ct $\geqslant 38$ were scored as negative for mutation/wild-type. Prior to this study, we established a confident read out up to 1% dilution with patient material derived from the CAIRO2 study.

The results were analysed with the LightCycler® Adapt Software v1.1 (Roche Diagnostics).

2.4. BRAF analysis

2.4.1. High resolution melting

BRAF HRM was performed in duplicate according to equal conditions and volumes as the *KRAS* HRM. Primers were designed by us using Primer3 software (Applied Biosystems) and span exon 15, codon 600 with an amplicon length of 154 bp: Forward primer: 5′-CATGAAGACCTCACAGTAAAAA-3′; Reversed primer: 5′-TGATTAAATTTTTGGCCCTGA-3′.

2.4.2. Direct sequencing

DS was performed as described above for *KRAS*. Note that the primer pair used in *KRAS*-HRM and

BRAF-HRM sequencing was identical to the sequencing primers using the forward and reverse primers, respectively. Sequencing was performed in duplicate. Read out of output was performed blinded for HRM results.

2.4.3. Qiagen Therascreen Pyrosequencing

The CE-IVD Therascreen *BRAF* Pyro Kit (Qiagen) was performed on a PyroMark Q24 System according to the manufacturer's Handbook. Primers for codon 600 were used to detect the exon 15, codon V600E mutation. Briefly, 5 μl of genomic DNA were used in a total volume of 25 μl using 12.5 μl of 2× PyroMark PCR Master Mix, 2.5 μl 10× CoralLoad Concentrate, 1 μl PCR Primer *BRAF* codon 600 and 4 μl of water supplied in the Kit. PCR was initiated by 15 min of 95 °C, followed by 42 cycles denaturation at 95 °C for 20 s, annealing 53 °C for 30 s and extension 72 °C for 20 s, followed by final extension at 72 °C for 5 min. Ten microlitre of the PCR product was subjected to the pyrosequencing reaction. The quality thresholds for the mutational analysis were a required peak height of 30 relative light units (RLU) for 'passed' quality and 10 RLU for 'check' quality. Samples with an initial 'check' status, or with an indicated mutation signal of 2–5%, were subjected to a second round of analysis performed in triplicates. In addition, samples that failed the initial PyroMark *BRAF* analysis were subjected to a second round of analysis.

3. Results

3.1. KRAS mutations

Direct sequencing (DS) was successfully performed in all 126 resection specimens resulting in 41 (32.5%) *KRAS* mutated cases: 7 (5.8%) 12VAL, 16 (13.3%) 12ASP, 4 (3.3%) 12CYS, 3 (2.5%) 12SER, 9 (7.5%) 13ASP and 1 (0.8%) 13CYS. DS failed in one biopsy specimen that was *KRAS* wild type in the resection specimen; DS in biopsy specimens resulted in 39 (31.2%) *KRAS* mutations (see Table 1). One resection sample with DS clearly showing a 12ASP appeared to be *KRAS* wild type in the biopsy sample; another case, a 12CYS mutation in the resection sample did not match with *KRAS* wild type in the biopsy specimen. Thus, in 125 analysable cases there was a 123/125 (98.4%) concordance between resection and biopsy specimens.

HRM was successful in 121 resection specimens of which 40 cases (33.1%) showed an abnormal melting curve deemed to be mutated. One of the biopsy specimens could not be analysed by HRM; 41 cases (32.8%) of the 125 analysable specimens showed a *KRAS* melting curve abnormality. Paired sample analysis of both resection and biopsy specimens was available in 120 cases showing 40/120 (33.3%) *KRAS* melting curve abnormalities. Concordance was 120/120 (100.0%) between resection and biopsy specimens.

DxS ARMS-Scorpion K-RAS Therascreen (DxS) was successful in all 126 resection and biopsy specimens. Since the 13CYS mutation is not detectable in the DxS Therascreen kit this assay failed to report this mutation in the single case present in our study. In the resection specimens 40 (31.7%) *KRAS* mutations were found. In the biopsy specimens, 38 (30.2%) *KRAS* mutations were found. The two discrepant cases had abnormal curves indicating a possible mutation but were below the 1% detection limit required for this assay. As a consequence, concordance between biopsy and resection specimens was 124/126 (98.4%) using DxS.

3.2. BRAF mutations

Direct sequencing succeeded in 125 resection specimens showing 23 (18.4%) *BRAF* mutations. All 126 biopsy specimens were analysable with 21 (16.7%) *BRAF* mutations. Concordance between 125 paired resection and biopsy specimens was 98.4% (123/125) (Table 2). High resolution melting, successful in all specimens, detected 23 (18.3%) *BRAF* V600E abnormalities in resection and 22 (17.5%) in biopsy specimens yielding a concordance in paired resection and biopsy samples of 99.2% (125/126). Qiagen's Therascreen *BRAF* Pyro kit showed 23 (18.4%) mutations in 126 resection specimens, and 22 (17.5%) mutations in 125 biopsy specimens; concordance between resection and biopsy samples was 99.2% (124/125). In all three assays, there was one case showing discrepancy of *BRAF* analysis between the resection specimen (with a V600E mutation) and the biopsy (wild type). It might be suggested that this case could be an example of tumour heterogeneity but further research is needed to confirm this speculation.

3.3. Correlation of KRAS and BRAF mutations and clinical data

KRAS mutational status showed no correlation with clinical parameters (Table 3). However, *BRAF* mutations were more frequent in females, especially in the

Table 1
Results of *KRAS* analysis with the three assays. Concordance between resection and biopsy specimens.

KRAS analysis	HRM	DS	DxS
Biopsy mutated	41	39	38
Resection mutated	40	41	40
Biopsy wild type	84	86	88
Resection wild type	81	85	86
Concordance (%)	100%	98.4%	98.4%
Biopsies successfully analysed	125	125	126
Resection successfully analysed	121	126	126
Paired resection/biopsy analyses available	120	125	126
Biopsy mutated	32.8%	31.2%	30.2%
Resection mutated	33.1%	32.5%	31.7%
Biopsy wild type	67.2%	68.8%	69.8%
Resection wild type	66.9%	67.5%	68.3%

HRM, high resolution melting; DS, direct sequencing; DxS, ARMS-Scorpion Therascreen.

Table 2
Results of *BRAF* analysis with three assays.

	HRM	DS	Pyro
Biopsy mutated	22	21	22
Resection mutated	23	23	23
Biopsy wild type	104	105	104
Resection wild type	103	102	102
Concordance (%)	99.2%	98.4%	99.2%
Biopsies successfully analysed	126	126	126
Resection successfully analysed	126	125	125
Paired analyses available	126	125	125
Biopsy mutated	17.5%	16.7%	17.5%
Resection mutated	18.3%	18.4%	18.4%
Biopsy wild type	82.5%	83.3%	82.5%
Resection wild type	81.7%	81.6%	81.6%

HRM, high resolution melting; DS, direct sequencing; Pyro, Qiagen Therascreen Pyro.

ages 70+. Also, a striking correlation was found with location. *BRAF* mutations were almost exclusively seen in the right sided colon. Thus, *BRAF* mutational status in the study group was relatively high (18%) but does show the known correlations with clinical parameters.

4. Discussion

In the present study, *KRAS* and *BRAF* mutational analysis was performed in paired samples of biopsy and resection specimens of 126 CRC patients. The aim of the study was to observe the concordance of both *KRAS* exon 2, codon 12/13 and *BRAF* exon 15, p.V600E mutational status between resection and biopsy specimens of colorectal cancers, using three molecular assays including one commercially available assay for both *KRAS* and *BRAF*.

The concordance of *KRAS* and *BRAF* mutational status in biopsy and resections specimens – regardless of the molecular assay performed – was very high. Two *KRAS* assays had a concordance of 98.4% (direct sequencing and the DxS ARMS-Scorpion Therascreen Kit) with 125 out of 126 paired samples that were successfully analysed, while high resolution melting analysis had a 100% concordance in 120 analysable cases. Using *BRAF* molecular assays, concordance between biopsy and resection was 99.2% in high resolution melting (all 126 cases analysable) and Qiagen's Therascreen *BRAF* Pyrosequencing kit (125 cases analysable), while direct sequencing had a concordance of 98.4% of 125 analysable cases.

The inter-assay concordance was very high in both *KRAS* and *BRAF* analysis. In *KRAS*, concordance was at least 97.4%, with slightly lower concordance in the biopsy specimens, and a somewhat lower number of analysable cases in the resection specimens. In *BRAF*, concordance was at least 99.2% in which one case was excluded.

In our study population, *KRAS* was found mutated in 33% and *BRAF* in 18%. In the COSMIC database (accessed 13th June 2011) the reported mutational frequency for large intestinal adenocarcinoma is 36% for *KRAS* and 12% for *BRAF*. Correlation of *BRAF* mutations with clinical parameters shows the known association[13] of *BRAF* mutations with female gender, higher age and right sided tumour location. A probable explanation is that our population shows a skewed distribution in demographical data.

Direct sequencing is the method to detect mutations in molecular pathology and dates back to the 1970s. It is thus the 'golden standard' to which newer methods are compared. As a consequence, a large number of studies describe the results of direct sequencing in *KRAS* mutational analysis.[5,6,14–25] In general, direct sequencing is a method with good performance with relatively low discordance to more sensitive assays, especially when tumour cell percentage exceeds 50%. In 'difficult' cases with lower tumour cell percentages, direct sequencing is reported to have a lower sensitivity[14,20] in detecting *KRAS* mutations in comparison with other assays. In clinical studies, direct sequencing was carried out retrospectively in the CO.17 trial[12] showing a predictive value of *KRAS* mutational status in which third line cetuximab monotherapy was compared to best supportive care in pretreated mCRC patients. Retrospective *KRAS* mutational analysis of 394 patients showed a significant improvement of both Progression Free Survival and overall survival (OS) in the cetuximab arm. There are a number of reasons why direct sequencing was not used as a test in other clinical studies, and is compared in others with newer assays. Sequencing requires expensive lab equipment, is contamination liable, requires relatively high tumour percentages, and is laborious. Nevertheless, in our study 39 mutations were found in the biopsy samples compared to 41 in resection specimens (98.4% concordance in 125 paired samples) with superior performance in resections after microdissection. In addition, direct sequencing is flexible for detecting new mutations.

HRM has been described in over 15 studies in CRC[5,6,14–17,20–26] in which two or more molecular methods were compared in 5[15] up to 263[16] cases. In general, high concordance between HRM and other molecular assays is reported. HRM was also used as a powerful predictive molecular test for the efficaciousness of anti-EGFR therapy in clinical studies: both the CRYSTAL study[9,27] and the OPUS trial[28,29] employed the commercially available assay of TIB Molbiol retrospectively. HRM in our study was – in concordance with the literature – an extremely effective tool for screening for *KRAS* mutations having a 100% negative predictive value. When HRM is deemed 'positive for mutation', a second test has to be employed in order to define the exact nature of the mutation. If direct sequencing is used as a second test, the HRM PCR product can be used after a DNA purification step. HRM is effective in

Table 3
Correlation of *KRAS* and *BRAF* mutational status with gender, age, colorectal cancer (CRC) location and stage.

	Total	*KRAS* mutated			*BRAF* mutated		
	N=	*N*=	%	*p*-value	N=	%	*p*-value
All cases	126	40	86				
Male	75	26	35	0.393	11	15	0.206
Female	51	14	27		12	24	
<70 yr	50	34	68	0.960	5	10	**0.052**
70+ yr	76	52	68		18	24	
Right sided	53	35	66	0.685	20	38	**0.000**
Left sided	63	43	68		3	5	
Rectum	10	8	80		0	0	
Stage II	69	50	72	0.285	12	17	0.618
Stage III	51	31	61		9	18	
Stage IV	6	5	83		2	33	
Male							
<70 yr	31	10	32	0.713	3	10	0.346
70+ yr	44	16	36		8	18	
Right sided	27	11	41	0.514	9	33	**0.003**
Left sided	42	14	33		2	5	
Rectum	6	1	17		0	0	
Stage II	42	12	29	0.238	5	12	0.244
Stage III	28	13	46		4	14	
Stage IV	5	1	20		2	40	
Female							
<70 yr	19	6	32	0.611	2	11	0.171
70+ yr	32	8	25		10	31	
Right sided	26	7	27	0.986	11	42	**0.005**
Left sided	21	6	29		1	5	
Rectum	4	1	25		0	0	
Stage II	27	7	26	0.774	7	26	0.805
Stage III	23	7	30		5	22	
Stage IV	1	0	0		0	0	

Bold face: p-value < 0.05.

samples with relatively low tumour cell percentage (down to about 10%). In addition, other codons of the *KRAS* gene such as codon 61 can be added. Furthermore, PCR efficiency can be improved by adding a wild type probe.

A commercially available CE-IVD approved test was developed by DxS Genotyping in Manchester, UK. It is a real time PCR assay for the detection of all six possible *KRAS* exon 2, codon 12 point mutations; in addition, one of the codon 13 mutations (13ASP) is included. A large number of studies comparing DxS Therascreen with other assays have been published. Carotenuto et al.[14] provides a detailed description of the assay as compared to direct sequencing. Good concordance with HRM, direct sequencing and pyrosequencing is described in a number of reports.[17,18,20,22,30] Several large clinical studies used DxS Therascreen for determining *KRAS* mutational status[31–33] retrospectively, establishing *KRAS* as a strong predictive marker for anti-EGFR therapy in metastatic CRC. Arguments to use DxS Therascreen as a molecular assay are the relative robustness of the test, early CE-IVD approval and that no confirmatory test is required. Disadvantages are the large amount of DNA needed, the relatively high cost of the test and the fact that the kit contains only a finite number of 7 mutations excluding 13CYS (encountered once in our study population) and codon 61. Furthermore, in our experience the AdaptSoftware designed by Roche to 'automatically' generate final results does encounter very frequent read out problems requiring a 'manual' re-analysis.

BRAF mutational status – retrospectively performed in the CRYSTAL study[9] using high resolution melting analysis – was shown not to be of predictive value of response to anti-EGFR therapy but indicated a poor prognosis in *KRAS* wild type patients. A more modest negative prognostic value was found in the MRC FOCUS trial using pyrosequencing as the molecular assay.[34] In two studies in which *BRAF* mutational status was analysed using direct sequencing as the molecular assay,[35,36] *BRAF* mutated patients were unresponsive to anti-EGFR therapy. At present, *BRAF* mutational

testing is neither mandatory nor recommended though it might be used for prognostic purposes. In our study, a single case showed tumour heterogeneity for *BRAF* mutation, with a discrepant result between the biopsy specimen (*BRAF* wild type) and the resection specimen (*BRAF* mutated) in all three molecular assays.

5. Conclusion

Endoscopic biopsies of colorectal carcinoma can be used to establish the mutational status of both *KRAS* and *BRAF* genes. High resolution melting is a fast and cheap method with an excellent negative predictive value for *KRAS* and *BRAF* mutations, thus limiting confirmatory tests of mutations to samples with abnormal melting curves. Direct sequencing, DxS *KRAS* Therascreen and Qiagen Pyromark *BRAF* prove to be efficacious as confirmatory assays.

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Conflict of interest statement

None declared.

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