

A comparison of four methods for detecting *KRAS* mutations in formalin-fixed specimens from metastatic colorectal cancer patients

MOTOTSUGU MATSUNAGA¹, TOSHIKADO KANETA², KEISUKE MIWA¹,
WATARU ICHIKAWA², KEN-ICHI FUJITA², FUMIO NAGASHIMA³, JUNJI FURUSE³,
MASAYOSHI KAGE⁴, YOSHITO AKAGI⁵ and YASUTSUNA SASAKI²

¹Multidisciplinary Treatment Cancer Center, Kurume University Hospital, Kurume, Fukuoka 830-0011;

²Division of Medical Oncology, Department of Internal Medicine, Showa University Hospital, Hatanodai, Shinagawa, Tokyo 142-8666; ³Department of Medical Oncology, Kyorin University School of Medicine, Hinkawa, Mitaka, Tokyo 181-8611; Departments of ⁴Diagnostic Pathology and ⁵Surgery, Kurume University Hospital, Kurume, Fukuoka 830-0011, Japan

Received April 2, 2015; Accepted April 29, 2016

DOI: 10.3892/ol.2016.4576

Abstract. There is currently no standard method for the detection of Kirsten rat sarcoma viral oncogene homolog (*KRAS*) mutation status in colorectal tumors. In the present study, we compared the *KRAS* mutation detection ability of four methods: direct sequencing, Scorpion-ARMS assaying, pyrosequencing and multi-analyte profiling (Luminex xMAP). We evaluated 73 cases of metastatic colorectal cancer (mCRC) resistant to irinotecan, oxaliplatin and fluoropyrimidine that were enrolled in an all-case study of cetuximab. The *KRAS* mutation detection capacity of the four analytical methods was compared using DNA samples extracted from tumor tissue, and the detection success rate and concordance of the detection results were evaluated. *KRAS* mutations were detected by direct sequencing, Scorpion-ARMS assays, pyrosequencing and Luminex xMAP at success rates of 93.2%, 97.3%, 95.9% and 94.5%, respectively. The concordance rates of the detection results by Scorpion-ARMS, pyrosequencing and Luminex xMAP with those of direct sequencing were 0.897, 0.923 and 0.900 (κ statistics), respectively. The direct sequencing method could not determine *KRAS* mutation status in five DNA samples. Of these, Scorpion-ARMS, pyrosequencing and Luminex xMAP successfully detected three, two and one *KRAS* mutation statuses, respectively. Three cases demonstrated inconsistent results, whereby Luminex xMAP detected mutated *KRAS* in two samples

while wild-type *KRAS* was detected by the other methods. In the remaining case, direct sequencing detected wild-type *KRAS*, which was identified as mutated *KRAS* by the other methods. In conclusion, we confirmed that Scorpion-ARMS, pyrosequencing and Luminex xMAP were equally reliable in detecting *KRAS* mutation status in mCRC. However, in rare cases, the *KRAS* status was differentially diagnosed using these methods.

Introduction

Cetuximab is a monoclonal antibody that targets the extracellular domain of the epidermal growth factor receptor (EGFR), and is an essential treatment option in patients with metastatic colorectal cancer (mCRC). Numerous researchers have reported that anti-EGFR agents have extremely poor antitumor effects in chemotherapy for mCRC with mutated Kirsten rat sarcoma viral oncogene homolog (*KRAS*) (1-5), providing clear evidence that administration of anti-EGFR agents is recommended only for mCRC with wild-type *KRAS*. However, although a number of methods may be used for *KRAS* mutation testing with varying sensitivity and specificity levels, no standard method has yet been recommended for clinical practice. Therefore, the use of these detection assays is somewhat erratic worldwide.

In Japan, cetuximab was administered for ~18 months following its launch in September 2009 without determination of *KRAS* mutation status, since the above-mentioned analytical methods were not covered by health insurance. The direct sequencing method (6) was covered in April 2010, followed by multi-analyte profiling (Luminex xMAP) technology (7) in March 2011 and Scorpion-ARMS assays (8) in May 2011. Pyrosequencing analysis methods (9) have also been evaluated and are already on the market in other countries. All four methods use the polymerase chain reaction (PCR) method but have different assay techniques. A number of sequencing- and PCR-based methods for detecting *KRAS* mutations

Correspondence to: Dr Keisuke Miwa, Multidisciplinary Treatment Cancer Center, Kurume University Hospital, 67 Asahi-Machi, Kurume, Fukuoka 830-0011, Japan
E-mail: miwakeisuke@gmail.com

Key words: colorectal cancer, *KRAS* mutation, direct sequencing, Scorpion-ARMS, pyrosequencing, Luminex xMAP

are currently in clinical use, although it is not clear which technique offers the best performance in terms of sensitivity, specificity, reproducibility and success rates (10). The aim of this retrospective study was to compare the analytical performances of the four methods (direct sequencing, Scorpion-ARMS assaying, pyrosequencing and Luminex xMAP) using extracted DNA from formalin-fixed paraffin-embedded (FFPE) tissues, and to clarify whether there are cases in which mutant *KRAS* status results differ among the examined methods.

Materials and methods

Patients. The eligibility criteria of patients enrolled in this study were as follows: Cases aged 20 years or over and less than 80 years who had been enrolled in an all-case study of cetuximab conducted between September 2008 and January 2010 following the Good Post-marketing Study Practice (GPSP) of the Japanese Pharmaceutical Affairs Act; diagnosis of mCRC with histological findings of primary colorectal adenocarcinoma; Eastern Cooperative Oncology Group performance status (ECOG PS) of grade 0-2; clinically unresponsive or intolerant to irinotecan, oxaliplatin and fluoropyrimidine; treated with cetuximab alone or cetuximab plus irinotecan; appropriate and usable FFPE sections available, consisting of ten undyed 10- μ m-thick sections and two 4- μ m-thick sections for hematoxylin and eosin (HE) staining. Cetuximab was administered to all subjects once a week according to the package insert. The initial dosage was 400 mg/m² and other dosages were 250 mg/m².

Four institutions in Japan participated in this study: Saitama Medical University International Medical Center (Hidaka, Saitama, Japan), the National Defense Medical College Hospital (Tokorozawa, Saitama, Japan), Kyorin University Hospital (Mitaka, Tokyo, Japan) and Showa University Hospital (Shinagawa, Tokyo, Japan). The protocol was reviewed and approved by the independent ethics committee or the institutional review board of each participating institution, and the study was conducted according to the Declaration of Helsinki alongside local ethical and legal requirements. The study was conducted between 1 July 2010 and 30 September 2011. Specific study termination criteria were not determined in advance, but a simple guideline was implemented to immediately halt the study should an ethically serious problem occur during the course of the study, such as in the event of a subject's personal information being compromised.

Pathological assessment and DNA extraction. All FFPE tissue blocks from the primary CRC site were prepared at each institution. First, 10 undyed 10- μ m-thick serial sections were prepared from each FFPE tissue block, and two 4- μ m-thick sections for HE staining were removed from either side of each prepared 10- μ m-thick section. Then, microscopic examination was conducted at the Department of Diagnostic Pathology, Saitama Medical University International Medical Center, Japan. Pathologists marked areas where tumor tissue accounted for more than 50% of the prepared slides, and confirmed the results by observing tumor areas on two HE-stained sections sandwiching the marked slide between them. Following this, DNA extraction was performed after manual microdissection

from five of the ten 10- μ m-thick serial sections and without manual microdissection from the latter five, according to the manufacturer's instructions for DNA extraction using the QIAamp DNA FFPE tissue kit (Qiagen, Venlo, Netherlands). DNA concentrations were measured using a NanoDrop ND 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

Mutation testing methods. DNA extracted from serial sections by manual microdissection was used for direct sequencing. Based on the manufacturer's instructions, DNA extracted from serial sections by manual microdissection was used for Luminex xMAP, and DNA extracted without manual microdissection was used for Scorpion-ARMS assays and pyrosequencing. The four detection assays were conducted at the same institution under the same conditions. Direct sequencing for exon 2 of the *KRAS* gene was carried out using PCR and 2X bidirectional direct sequencing following previously described protocols (11,12). Tumor DNA for exon 3 was amplified using the following primers: forward, 5'-CACTGTAATAATCCA GACTGTG-3' and reverse, 5'-CCCACCTATAATGGTGAA TATC-3'. Sequencing reactions were performed in direct and reverse directions, and electropherograms were reviewed manually to detect any genetic alterations. All variants were confirmed by resequencing of independent PCR products. In the study, analyses were carried out using home-brew primers and the following *in vitro* research use only reagents: Expand High Fidelity PCR system (Roche Diagnostics, Basel, Switzerland), BigDye terminator Cycle Sequencing Ready Reaction (Life Technologies, Carlsbad, CA, USA) and BigDye XTerminator purification kit (Life Technologies). The other tests were performed according to each measurement manual. In this study, Scorpion-ARMS assays, pyrosequencing and Luminex xMAP were carried out using a TheraScreen kit (Qiagen), a *KRAS* Pyro kit (Qiagen) and a MEBGEN *KRAS*TM mutation detection kit (Medical and Biological Laboratories, Nagoya, Aichi, Japan) as *in vitro* diagnostic tests, respectively.

Statistical analyses. The significance of the concordance of mutation detection by the different methods for the two categories (wild type and mutated type) was assessed by κ statistics. We classified the κ values according to Landis and Koch (13): <0.00, poor; 0.00-0.20, slight; 0.21-0.40, fair; 0.41-0.60, moderate; 0.61-0.80, substantial; and 0.81-1.00, almost perfect.

Results

In this study, we recruited and analyzed 73 mCRC patients. All subjects had been enrolled in an all-case study of cetuximab, the results of which enabled us to calculate their progression-free survival (PFS) and overall survival (OS). Among these, 69 patients completed the study and could be followed up until mortality, while four cases dropped out. Of these 73 cases, 42 cases received cetuximab alone and 31 cases received cetuximab plus irinotecan. Patient characteristics are detailed in Table I. The objective response rate of cetuximab for all subjects was 15%. The median PFS and OS were 77 and 228 days, respectively. The median PFS of wild-type *KRAS* cases detected by direct sequencing was 112 days and that of

Table I. Characteristics of eligible patients.

Characteristic	No. of patients (n=73)
Age (range)	65 (39-80)
Gender	
Male	58 (79%)
Female	15 (21%)
ECOG performance status	
0	41 (56%)
1	29 (40%)
2	3 (4%)
No. of previous chemotherapy regimens	
2	40 (55%)
3	21 (29%)
≥4	12 (16%)
Objective response rate	
CR	0
PR	11 (15%)
SD	24 (33%)
PD	35 (48%)
NE	3 (4%)
Median progression-free survival (range), days	77 (8-682)
Median overall survival (range), days	228 (25-1058)

ECOG, Eastern Cooperative Oncology Group; CR, complete response; PR, partial response; SD, stable disease; PD, progression disease; NE, not evaluated.

mutated *KRAS* cases was 53 days [log-rank, $P=0.001$; hazard ratio (HR), 0.416; 95% confidence interval (CI), 0.244-0.718]. The median OS of wild-type *KRAS* cases detected by direct sequencing was 318 days and that of mutated *KRAS* cases was 196 days (log-rank, $P=0.0149$; HR, 0.523; 95% CI, 0.307-0.897).

The median concentrations of extracted DNA after and without manual microdissection were 119.5 ng/μl (range, 2.8-358.9 ng/μl) and 130.1 ng/μl (range, 2.1-500.4 ng/μl), respectively. The success rates of detection by direct sequencing, Scorpion-ARMS, pyrosequencing and Luminex xMAP were 93.2, 97.3, 95.9 and 94.5%, respectively. With respect to *KRAS* mutation, direct sequencing, Scorpion-ARMS, pyrosequencing and Luminex xMAP detected mutated *KRAS* in 28 (38.4%), 29 (39.7%), 29 (39.7%) and 31 (42.5%) subjects, respectively (Table II). All mutation sites in cases detected as mutated *KRAS* by the four methods were in complete accordance with each method.

Pairwise concordances between each method for *KRAS* status are shown in Table III. The concordance rates of direct sequencing with Scorpion-ARMS, pyrosequencing and Luminex xMAP were 0.897, 0.923 and 0.900 as the κ values, respectively. The κ value of Scorpion-ARMS with pyrosequencing and Luminex xMAP, and that of pyrosequencing with Luminex xMAP were sufficient to demonstrate good concordances.

The direct sequencing method could not detect *KRAS* mutations in five cases (Table IV). There was one case (case 3) in which *KRAS* mutation status was determined by all four methods. Notably, the remaining four cases were diagnosed as wild-type *KRAS* by all three methods. Scorpion-ARMS failed to detect two cases, pyrosequencing three and Luminex xMAP four. The cases that could not be detected by Scorpion-ARMS, pyrosequencing and Luminex xMAP were all included in the five cases that were undetectable by direct sequencing. Among those, Scorpion-ARMS, pyrosequencing and Luminex xMAP successfully detected three, two and one cases, respectively. All of these cases had wild-type *KRAS*. One case (case 2) was detected only by Scorpion-ARMS and had a PFS and OS of 383 days and 740 days, respectively, while another case (case 4) was detected only by Luminex xMAP, with a PFS and OS of 61 and 147 days, respectively.

There were three cases for which the *KRAS* mutation status was inconsistently detected by the different methods (Table V). In two of these three cases, only Luminex xMAP detected mutated *KRAS* (G12D for case 1 and G12S for case 2), whereas the other three methods detected wild-type *KRAS*. These two cases appeared to be clinically responsive to cetuximab therapy in terms of disease control and survival. The remaining case (case 3) with poor prognosis was diagnosed as mutated *KRAS* (G12C) by the other three methods, although direct sequencing revealed a wild-type *KRAS* status.

Discussion

Retrospective analyses of pivotal clinical trials for the anti-EGFR monoclonal antibodies cetuximab and panitumumab have revealed that patients with CRC-containing activating mutations in the downstream *KRAS* gene do not benefit from these therapies (14,15). The association between defined mutations and response to therapy provides a clear opportunity to increase response rates and reduce the likelihood of treating patients who are unlikely to respond to certain drugs, which is costly and unnecessarily exposes them to potential adverse effects. Therefore, mutant *KRAS* has been demonstrated to be a strong negative predictive biomarker to indicate whether a CRC patient is likely to respond to anti-EGFR treatment, and administration of cetuximab is recommended only for patients with a wild-type *KRAS* tumor. In addition, a previous study demonstrated that cetuximab is ineffective for tumors harboring any *RAS* mutations except in exon 2 of *KRAS* (16).

A number of sequencing- and PCR-based methods to detect *KRAS* mutations are currently in clinical use. At present, there are numerous ways of testing for *KRAS* mutations, and there have been comparative studies and analyses of the sensitivity of these assays in the clinical setting (16-19). However, it is not clear which technique offers the best performance in terms of sensitivity, specificity, reproducibility and success rates. We confirmed the high performance of more sensitive methods including Scorpion-ARMS, Luminex xMAP and pyrosequencing in analyzing *KRAS* mutation status in DNA extracted from FFPE tissues compared with the detection sensitivity of 20% by direct sequencing. Additionally, to our knowledge, this is the first study to report rare cases in which

Table II. *KRAS* mutation statuses detected by each analytical method (n=73).

Parameter	Direct sequencing	Scorpion-ARMS	Pyrosequencing	Luminex xMAP
Detected cases (%)	68 (93.2%)	71 (97.3%)	70 (95.9%)	69 (94.5%)
Wild-type <i>KRAS</i> (%)	40 (54.8%)	42 (57.5%)	41 (56.2%)	38 (52.1%)
Mutated <i>KRAS</i> (%)	28 (38.4%)	29 (39.7%)	29 (39.7%)	31 (42.5%)
Undetectable cases (%)	5 (6.8%)	2 (2.7%)	3 (4.1%)	4 (5.5%)

KRAS, Kirsten rat sarcoma viral oncogene homolog.

Table III. Pairwise concordance between methods of *KRAS* mutation detection.

Method	Scorpion-ARMS			Pyrosequencing			Luminex xMAP		
Direct sequencing	W	M	NE	W	M	NE	W	M	NE
W	39	1	0	39	1	0	37	3	0
M	0	28	0	0	28	0	0	28	0
NE	3	0	2	2	0	3	1	0	4
	$\kappa=0.89672$			$\kappa=0.92347$			$\kappa=0.90004$		
Scorpion-ARMS									
W				41	0	1	37	2	3
M				0	29	0	0	29	0
NE				0	0	2	1	0	1
				$\kappa=0.97355$			$\kappa=0.84502$		
Pyrosequencing									
W							37	2	2
M							0	29	0
NE							1	0	2
							$\kappa=0.87238$		

KRAS, Kirsten rat sarcoma viral oncogene homolog; W, wild-type *KRAS*; M, mutated *KRAS*; NE, not evaluated.

Table IV. Detection capability of each analytical method for undetectable cases.

Case	Direct sequencing	Scorpion-ARMS	Pyrosequencing	Luminex xMAP	DNA (ng/ μ l) without MD	DNA (ng/ μ l) with MD	ORR	PFS (days)	OS (days)
1	NE	Wild	Wild	NE	95.6	100.5	SD	83	157
2	NE	Wild	NE	NE	39.0	57.7	SD	383	740
3	NE	NE	NE	NE	26.6	56.2	PR	116	317
4	NE	NE	NE	Wild	2.1	4.3	SD	61	147
5	NE	Wild	Wild	NE	93.5	126.5	PD	17	95

NE, not evaluated; Wild, wild-type *KRAS*; ORR, objective response rate; PFS, progression-free survival; OS, overall survival; PR, partial response; SD, stable disease; PD, progression disease; MD, manual microdissection.

the status of *KRAS* was differentially diagnosed by the more sensitive methods.

All subjects in the study were enrolled in an all-case study of cetuximab following the GPSP of the Japanese Pharmaceutical Affairs Act, and the effects of cetuximab administration and prognoses of these patients were already

described in specified studies, which enabled us to expect a small selection bias. Mutant *KRAS* is observed in ~35-45% of CRC (1,5,14,15,20-22), and codon 12 and 13 are two hotspots that account for ~95% of all mutation types (5,23,24); our results were within this range. Moreover, the results of *KRAS* analysis by direct sequencing demonstrated

Table V. Details of inconsistent results.

Case	Direct sequencing	Scorpion-ARMS	Pyro-sequencing	Luminex-xMAP	Mutation site	DNA (ng/ μ l) without MD	DNA (ng/ μ l) with MD	ORR	PFS (days)	OS (days)
1	Wild	Wild	Wild	Mutant	G12D	78.3	68.7	PR	287	344
2	Wild	Wild	Wild	Mutant	G12S	217.5	215.5	SD	108	208
3	Wild	Mutant ^a	Mutant ^a	Mutant ^a	G12C	138.7	182.5	PD	42	90

^aAll cases had *KRAS* G12C mutation. Wild, wild-type *KRAS*; mutant, mutated *KRAS*; ORR, objective response rate; PFS, progression-free survival; OS, overall survival; PR, partial response; SD, stable disease; PD, progression disease; MD, manual microdissection.

significant prolongation of PFS and OS in wild-type *KRAS* cases compared with mutated *KRAS* cases, consistent with the published data (4,25).

In this study, we evaluated the differences between four PCR-based analytical methods using the same DNA samples. The success rates in *KRAS* status detection ranged from 93.2% to 97.3% by the four methods (Table II) without statistical significance due to the small sample size. However, among the five cases in which the *KRAS* mutation was not detected by direct sequencing, the mutation status in four of these cases was detectable by the other more sensitive methods (Table IV). This might be simply explained by the differences in sensitivities to detect *KRAS* mutation status between direct sequencing and the other three methods. It has already been reported that direct sequencing has poor sensitivity for low levels of mutation (26). Thus, the direct sequencing method should not be applied to detect *KRAS* mutation status in clinical practice. The detection sensitivity by direct sequencing, Scorpion-ARMS, Pyrosequencing and Luminex xMAP is ~20%, 1%, 5-10% and 5-10%, respectively. Three cases were diagnosed as wild-type *KRAS* by Scorpion-ARMS among four cases in which the *KRAS* status could not be determined by Luminex xMAP. We were able to diagnose *KRAS* status by Scorpion-ARMS in one case among three in which *KRAS* status was not determined by pyrosequencing. These results may reflect the higher sensitivity in detecting *KRAS* mutation status in Scorpion-ARMS compared with pyrosequencing and Luminex xMAP.

Scorpion-ARMS is a real-time PCR-based assay that combines the amplified refractory mutation system (ARMS) with Scorpion probes (seven probes for seven different mutations in *KRAS*), eliminating the requirement for post-PCR confirmation by direct sequencing. Until recently, this was considered to be the most sensitive method, with a sensitivity of 1% compared with the other three methods (27). In this study, the concordance rates of Scorpion-ARMS with pyrosequencing and Luminex xMAP were $\kappa=0.974$ and $\kappa=0.845$, respectively. Since we classified the κ values according to the Landis and Koch methods (13), as previously mentioned, the comparison of κ values has no statistical significance if the values were over 0.80 in our analysis. Pairwise analysis results were almost perfect among the three sensitive methods, inferring that these methods are equally useful and reliable.

The median concentrations of extracted DNA with and without manual microdissection were 119.5 and 130.1 ng/ μ l,

respectively. All analytical methods accurately detected DNA samples prepared at a concentration of 100 ng/ μ l or more. It is considered that while detectability depends on DNA concentrations of 100 ng/ μ l or more, it is reliant on the quality of DNA when the concentration is less than 100 ng/ μ l. Research has demonstrated that DNA quality is influenced by the concentration of formic acid used to fix tissues and the fixation time (28,29). In this study, among the five cases in which *KRAS* status could not be detected by direct sequencing, there was one case (case 3) that could not be determined by all three sensitive methods (Table IV). This was due to the low concentration of extracted DNA. It was therefore notable that one case with 2.1 ng/ μ l DNA obtained without manual microdissection was diagnosed as wild-type *KRAS* only by Luminex xMAP and not by the other two sensitive methods. We do not have any explanation for this observed result. It may be that the fixation time was longer in the undetectable cases, or that the DNA sample contained excess fragmentation. However, we were unable to investigate these aspects due to the retrospective nature of this study.

There were three cases in which the status of *KRAS* was differentially diagnosed by the examined methods (Table V). One case (case 3) was judged to be *KRAS*-mutant (G12C) by the three sensitive methods, although the *KRAS* status was diagnosed as wild type by direct sequencing; this discordance is likely due to the levels of sensitivity. Two cases were judged to have *KRAS* mutations (G12D for case 1 and G12S for case 2) by Luminex xMAP, although Scorpion-ARMS and pyrosequencing diagnosed these cases to be wild type. These two cases clinically responded to cetuximab-alone therapy. Although patients with G13D mutations are reported to benefit more from cetuximab than patients with tumors harboring *KRAS* codon 12 mutations (30), these cases had mutations of G12D or G12S. If clinicians took account of the results of Luminex xMAP and did not use cetuximab, positive outcomes were not achieved. We assumed the *KRAS* status of these two cases to be wild type. These conflicting results might be explained by non-specific reactions of the primer probe used in Luminex xMAP.

Certain limitations exist in our study. One is the retrospective nature of the study, including the small number of patients treated by cetuximab alone or the combination therapy with irinotecan. Second, more sensitive and specific methods than those used in our study, including the BEAMing method (31) and WAVEbased Surveyor Scan kits (32), are available to detect *KRAS* mutation status. However, it was

technically difficult to apply these in this study. Third, our data were limited to *KRAS* mutations in exon 2, while we are now at the point where there is technology available to detect all *RAS* mutations beyond *KRAS* mutations. At the same time, considering those false results on exon 2 mutations, it is necessary to bear in mind that similar false-positive or false-negative test results may also be obtained for other mutation sites.

In conclusion, all three sensitive methods (Scorpion-ARMS, pyrosequencing and Luminex xMAP) were equally useful and reliable in detecting *KRAS* mutation status, with high success and concordance rates between each method. However, there were rare incidences in which the *KRAS* status was differentially diagnosed by the three methods, even though the same DNA samples were used. Further large prospective studies are necessary to clarify the clinical factors responsible for the discordant *KRAS* results between the different methods.

Acknowledgements

The authors thank Professor Michio Shimizu for his assistance at the Department of Diagnostic Pathology, Saitama Medical University International Medical Center, and also thank Ms. Kaori Kawara for her assistance at the Department of Medical Oncology, Saitama Medical University International Medical Center.

References

- Bokemeyer C, Bondarenko I, Makhson A, Hartmann JT, Aparicio J, de Braud F, Donea S, Ludwig H, Schuch G, Stroh C, *et al*: Fluorouracil, leucovorin, and oxaliplatin with and without cetuximab in the first-line treatment of metastatic colorectal cancer. *J Clin Oncol* 27: 663-671, 2009.
- Ciardiello F, Tejpar S and Papamichael D: Implications of *KRAS* mutation status for the treatment of metastatic colorectal cancer. *Target Oncol* 4: 311-322, 2009.
- De Roock W, Piessevaux H, De Schutter J, Janssens M, De Hertogh G, Personeni N, Biesmans B, Van Laethem JL, Peeters M, Humblet Y, *et al*: *KRAS* wild-type state predicts survival and is associated to early radiological response in metastatic colorectal cancer treated with cetuximab. *Ann Oncol* 19: 508-515, 2008.
- Harbison CT, Horak CE, Ledezine JM, Mukhopadhyay P, Malone DP, O'Callaghan C, Jonker DJ, Karapetis CS, Khambata-Ford S, Gustafson N, *et al*: Validation of companion diagnostic for detection of mutations in codons 12 and 13 of the *KRAS* gene in patients with metastatic colorectal cancer: analysis of the NCIC CTG CO.17 trial. *Arch Pathol Lab Med* 137: 820-827, 2013.
- Van Cutsem E, Köhne CH, Hitt E, Zaluski J, Chang Chien CR, Makhson A, D'Haens G, Pinter T, Lim R, Bodoky G, *et al*: Cetuximab and chemotherapy as initial treatment for metastatic colorectal cancer. *N Engl J Med* 360: 1408-1417, 2009.
- Jonker DJ, O'Callaghan CJ, Karapetis CS, Zalcberg JR, Tu D, Au HJ, Berry SR, Krahn M, Price T, Simes RJ, *et al*: Cetuximab for the treatment of colorectal cancer. *N Engl J Med* 357: 2040-2048, 2007.
- Khan IH, Krishnan VV, Ziman M, Janatpour K, Wun T, Luciw PA and Tuscano J: A comparison of multiplex suspension array large-panel kits for profiling cytokines and chemokines in rheumatoid arthritis patients. *Cytometry B Clin Cytom* 76: 159-168, 2009.
- Kimura H, Fujiwara Y, Sone T, Kunitoh H, Tamura T, Kasahara K and Nishio K: High sensitivity detection of epidermal growth factor receptor mutations in the pleural effusion of non-small cell lung cancer patients. *Cancer Sci* 97: 642-648, 2006.
- Ahle JD, Barr S, Chin AM and Battersby TR: Sequence determination of nucleic acids containing 5-methylisocytosine and isoguanine: identification and insight into polymerase replication of the non-natural nucleobases. *Nucleic Acids Res* 33: 3176-3184, 2005.
- Whitehall V, Tran K, Umapathy A, Grieff F, Hewitt C, Evans TJ, Ismail T, Li WQ, Collins P, Ravetto P, *et al*: A multicenter blinded study to evaluate *KRAS* mutation testing methodologies in the clinical setting. *J Mol Diagn* 11: 543-552, 2009.
- Conde E, Angulo B, Tang M, Morente M, Torres-Lanzas J, Lopez-Encuentra A, Lopez-Rios F and Sanchez-Cespedes M: Molecular context of the EGFR mutations: evidence for the activation of mTOR/S6K signaling. *Clin Cancer Res* 12: 710-717, 2006.
- Fernandez P, Carretero J, Medina PP, Jimenez AI, Rodriguez-Perales S, Paz MF, Cigudosa JC, Esteller M, Lombardia L, Morente M, *et al*: Distinctive gene expression of human lung adenocarcinomas carrying *LKB1* mutations. *Oncogene* 23: 5084-5091, 2004.
- Landis JR and Koch GG: The measurement of observer agreement for categorical data. *Biometrics* 33: 159-174, 1977.
- Karapetis CS, Khambata-Ford S, Jonker DJ, O'Callaghan CJ, Tu D, Tebbutt NC, Simes RJ, Chalchal H, Shapiro JD, Robitaille S, *et al*: K-ras mutations and benefit from cetuximab in advanced colorectal cancer. *N Engl J Med* 359: 1757-1765, 2008.
- Amado RG, Wolf M, Peeters M, Van Cutsem E, Siena S, Freeman DJ, Juan T, Sikorski R, Suggs S, Radinsky R, *et al*: Wild-type *KRAS* is required for panitumumab efficacy in patients with metastatic colorectal cancer. *J Clin Oncol* 26: 1626-1634, 2008.
- Sorich MJ, Wiese MD, Rowland A, Kichenadasse G, McKinnon RA and Karapetis CS: Extended RAS mutations and anti-EGFR monoclonal antibody survival benefit in metastatic colorectal cancer: a meta-analysis of randomized, controlled trials. *Ann Oncol* 26: 13-21, 2015.
- Angulo B, García-García E, Martínez R, Suárez-Gauthier A, Conde E, Hidalgo M and López-Ríos F: A commercial real-time PCR kit provides greater sensitivity than direct sequencing to detect *KRAS* mutations: a morphology-based approach in colorectal carcinoma. *J Mol Diagn* 12: 292-299, 2010.
- Franklin WA, Haney J, Sugita M, Bemis L, Jimeno A and Messersmith WA: *KRAS* mutation: comparison of testing methods and tissue sampling techniques in colon cancer. *J Mol Diagn* 12: 43-50, 2010.
- Tol J, Dijkstra JR, Vink-Börger ME, Nagtegaal ID, Punt CJ, Van Krieken JH and Ligtenberg MJ: High sensitivity of both sequencing and real-time PCR analysis of *KRAS* mutations in colorectal cancer tissue. *J Cell Mol Med* 14: 2122-2131, 2010.
- Douillard JY, Siena S, Cassidy J, Tabernero J, Burkes R, Barugel M, Humblet Y, Bodoky G, Cunningham D, Jasssem J, *et al*: Randomized, phase III trial of panitumumab with infusional fluorouracil, leucovorin, and oxaliplatin (FOLFOX4) versus FOLFOX4 alone as first-line treatment in patients with previously untreated metastatic colorectal cancer: the PRIME study. *J Clin Oncol* 28: 4697-4705, 2010.
- Peeters M, Price TJ, Cervantes A, Sobrero AF, Ducreux M, Hotko Y, André T, Chan E, Lordick F, Punt CJ, *et al*: Randomized phase III study of panitumumab with fluorouracil, leucovorin, and irinotecan (FOLFIRI) compared with FOLFIRI alone as second-line treatment in patients with metastatic colorectal cancer. *J Clin Oncol* 28: 4706-4713, 2010.
- Van Cutsem E, Köhne CH, Láng I, Folprecht G, Nowacki MP, Cascinu S, Shchepotin I, Maurel J, Cunningham D, Tejpar S, *et al*: Cetuximab plus irinotecan, fluorouracil, and leucovorin as first-line treatment for metastatic colorectal cancer: updated analysis of overall survival according to tumor *KRAS* and *BRAF* mutation status. *J Clin Oncol* 29: 2011-2019, 2011.
- Asaka S, Arai Y, Nishimura Y, Yamaguchi K, Ishikubo T, Yatsuoka T, Tanaka Y and Akagi K: Microsatellite instability-low colorectal cancer acquires a *KRAS* mutation during the progression from Dukes' A to Dukes' B. *Carcinogenesis* 30: 494-499, 2009.
- Russo A, Rizzo S, Bronte G, Silvestris N, Colucci G, Gebbia N, Bazan V and Fulfaro F: The long and winding road to useful predictive factors for anti-EGFR therapy in metastatic colorectal carcinoma: the *KRAS/BRAF* pathway. *Oncology* 77 (Suppl 1): S57-S68, 2009.
- Li S, Schmitz KR, Jeffrey PD, Wiltzius JJ, Kussie P and Ferguson KM: Structural basis for inhibition of the epidermal growth factor receptor by cetuximab. *Cancer Cell* 7: 301-311, 2005.
- Gonzalez de Castro D, Angulo B, Gomez B, Mair D, Martinez R, Suarez-Gauthier A, Shieh F, Velez M, Brophy VH, Lawrence HJ and Lopez-Rios F: A comparison of three methods for detecting *KRAS* mutations in formalin-fixed colorectal cancer specimens. *Br J Cancer* 107: 345-351, 2012.

27. Jimeno A, Messersmith WA, Hirsch FR, Franklin WA and Eckhardt SG: KRAS mutations and sensitivity to epidermal growth factor receptor inhibitors in colorectal cancer: practical application of patient selection. *J Clin Oncol* 27: 1130-1136, 2009.
28. Srinivasan M, Sedmak D and Jewell S: Effect of fixatives and tissue processing on the content and integrity of nucleic acids. *Am J Pathol* 161: 1961-1971, 2002.
29. Yokota T, Shibata N, Ura T, Takahari D, Shitara K, Muro K and Yatabe Y: Cycleave polymerase chain reaction method is practically applicable for V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS)/V-raf murine sarcoma viral oncogene homolog B1 (BRAF) genotyping in colorectal cancer. *Transl Res* 156: 98-105, 2010.
30. De Roock W, Jonker DJ, Di Nicolantonio F, Sartore-Bianchi A, Tu D, Siena S, Lamba S, Arena S, Frattini M, Piesseaux H, *et al*: Association of KRAS p.G13D mutation with outcome in patients with chemotherapy-refractory metastatic colorectal cancer treated with cetuximab. *JAMA* 304: 1812-1820, 2010.
31. Bokemeyer C, Kohne C-H, Ciardiello F, *et al*: Treatment outcome according to tumor RAS mutation status in OPUS study patients with metastatic colorectal cancer (mCRC) randomized to FOLFOX4 with/without cetuximab. *ASCO Meeting Abstracts* 32: 3505, 2014.
32. Douillard JY, Oliner KS, Siena S, Tabernero J, Burkes R, Barugel M, Humblet Y, Bodoky G, Cunningham D, Jassem J, *et al*: Panitumumab-FOLFOX4 treatment and RAS mutations in colorectal cancer. *N Engl J Med* 369: 1023-1034, 2013.