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

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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 assay, 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
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 Lumines 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-µm-thick sections and two 4-µm-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-µm-thick serial sections were prepared from each FFPE tissue block, and two 4-µm-thick sections for HE staining were removed from either side of each prepared 10-µm-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-µm-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 Lumines xMAP, and DNA extracted without manual microdissection was used for Scorpion-ARMS 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’-CAGCTGTAATAATCCAGACTGGT-3’ and reverse, 5’-CCCCACCTTATAATGTGAAATATC-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 XTermi-nator purification kit (Life Technologies). The other tests were performed according to each measurement manual. In this study, Scorpion-ARMS assays, pyrosequencing and Lumines xMAP were carried out using a TheraScreen kit (Qiagen), a KRAS Pyro kit (Qiagen) and a MEBGEN KRAS™ 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 1. 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
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...
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

| Parameter | Direct sequencing | Scorpion-ARMS | Pyrosequencing | Luminex xMAP |
|-----------|-------------------|---------------|---------------|--------------|
| Detected cases (%) | 68 (93.2%) | 71 (97.3%) | 70 (95.9%) | 69 (94.5%) |
| Wild-type *KRAS* (%) | 40 (54.8%) | 42 (57.5%) | 41 (56.2%) | 38 (52.1%) |
| Mutated *KRAS* (%) | 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.

| 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 28 0 0 28 0 0 | NE 3 0 2 2 0 3 1 0 4 |
| Scorpion-ARMS | | | | |
| W 41 0 1 37 2 3 | M 0 29 0 29 0 0 | NE 0 0 2 1 0 1 | | |
| Pyrosequencing | | | | |
| W 37 2 2 | M 0 29 0 | NE 1 0 2 | | |

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

| Case | Direct sequencing | Scorpion-ARMS | Pyrosequencing | Luminex xMAP | DNA (ng/μl) without MD | DNA (ng/μl) with MD | ORR (days) | 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.
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 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 χ2=0.974 and χ2=0.845, respectively. Since we classified the χ values according to the Landis and Koch methods (13), as previously mentioned, the comparison of χ 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/µl, respectively. All analytical methods accurately detected DNA samples prepared at a concentration of 100 ng/µl or more. It is considered that while detectability depends on DNA concentrations of 100 ng/µl or more, it is reliant on the quality of DNA when the concentration is less than 100 ng/µl. 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/µl 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 G12D 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 WAVE-based 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.

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