Abstract. Background: Direct sequencing (DS) has often been used for detection of v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS) mutations. High-resolution melting analysis (HRMA) is another method to detect mutations by using a light scanner, and is more rapid, lower in cost, and more sensitive than DS. We confirmed correspondence between DS and HRMA for KRAS mutation detection in colorectal cancer (CRC). Patients and Methods: From 102 patients with CRC, intended to receive cetuximab treatment at the National Cancer Center Hospital between September 2008 and July 2009, formalin-fixed paraffin-embedded tissues were retrospectively analyzed for KRAS status using HRMA and DS. Cetuximab efficacy was evaluated. Results: Success rates of analysis by DS and HRMA were 100 out of the 102 patients (98.0%) and 99 out of the 102 patients (97.1%), respectively. The cases which failed by one method were analyzed by the other. KRAS mutant-type was detected by DS in 47 out of 100 samples (47.0%), and by HRMA in 45 out of 99 samples (45.5%). The concordance between the two methods was 94.8%. Forty-six out of the 53 patients with wild-type KRAS, as detected by DS received cetuximab and the response and disease control rates were 19.6% and 63.0%, respectively. With a median follow-up of 8.8 months, the median progression-free survival was 5.6 months and overall survival was 11.1 months. The efficacy of two discordant cases which received cetuximab showed that the best responses were stable disease and progressive disease in one each, progression-free survival was 2.9 and 1.1 months and overall survival was 5.3 and 1.2 months, respectively. Conclusion: HRMA is a useful optional method for detection of KRAS mutations in CRC in light of accuracy, cost performance and rapidity.

Colorectal cancer (CRC) is the third most common cancer in morbidity and the fourth most common in mortality in the world (1). In Japan, CRC has been rapidly increasing in recent years and is now the second most common malignancy. In 2008, it was estimated that there were 101,656 new cases of CRC in Japan and 43,349 deaths because of this disease (2). Although metastatic CRC has been considered as a non-curable disease, the duration of survival has been prolonged due to new antibody drugs. Cetuximab is used for metastatic CRC that expresses epidermal growth factor receptor (EGFR), but has not shown any treatment benefit in patients with v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS) mutations (3, 4). KRAS mutation occurs in about 40% of patients with CRC. Therefore, the Guidelines from the American Society of Clinical Oncology (ASCO) and the National Comprehensive Cancer Network (NCCN) endorse KRAS mutation testing for all individuals with advanced CRC (5, 6). However, there is no specific methodology recommended for KRAS analysis in those guidelines.

Direct sequencing (DS) has often been used for the analysis of KRAS mutations. However, DS has two drawbacks in clinical practice. Firstly, although the sequencing methods require a high ratio of tumor-to-normal tissue DNA for optimal results, the diagnostic specimens obtained from colonoscopy may contain only a small amount of tumor cells and may be contaminated with other cells. Secondly, KRAS mutation analysis based on DS requires special instruments and is also time-consuming and expensive. Therefore, more simple and highly sensitive non-sequencing methods to detect KRAS mutations are required.

High-resolution melting analysis (HRMA) is another method of mutation determination by using Light Scanner and is faster, simpler, and less expensive than DS (7).
Although DS determines the sequence of bases in amplified DNA without subcloning, HRMA measures differences in melting point temperatures between matched and mismatched double-stranded DNA. In a previous study, HRMA was already shown to have a high degree of accuracy in detecting EGFR mutations in patients with non-small cell lung cancer (NSCLC) receiving gefitinib, and the method is routinely used (8-10).

In this study, we investigated the consistency between DS and HRMA in detecting KRAS mutations in CRC using DNA extracted from formalin-fixed paraffin-embedded (FFPE) tissues.

Patients and Methods

For 102 patients with CRC who were scheduled to receive cetuximab at the National Cancer Center Hospital between September 2008 and July 2009, FFPE tissues were retrospectively analyzed.

Surgically-resected or biopsy specimens containing sufficient tumor tissues were selected. Each block was sliced into 30-μm slices, or tumor tissues on the slides were scraped off with a needle. DNA was extracted from the specimens with a QIAGEN Micro kit (Venlo, Netherlands). Primers were designed to span codon 12 and 13 of the KRAS gene, with a product size of 92 bp on the basis of a previously published study (11). The sequences of the primers for HRMA were TTATAAGGCCTGAAAATGACTGAA (forward: primer F) and TGAATTAGCTGTATCGTCAAGGCACT (reverse: primer R). A polymerase chain reaction (PCR) for HRMA was performed in a 9 μl volume containing 4 μl 2.5 × Light Scanner Master Mix, 1 μl primer F, 1 μl primer R, and 3 μl double distilled water. The 9 μl of PCR product was transferred to a 96-well plate and mixed with a drop of light mineral oil. The plate was centrifuged (1,300 × g, 1 min). The PCR was run according to the following conditions: one cycle of 94˚C for 2 min; 45 cycles of 94˚C for 30 sec, 70˚C for 30 sec; one cycle of 94˚C for 30 sec, 25˚C for 30 sec. HRMA was performed on a Light Scanner (Idaho Technology: Salt Lake City, Utah, USA) and the acquired data were analyzed using the provided software (Idaho Technology: Salt Lake City, Utah, USA). The graph normalized to the software showed the degree of the reduction in fluorescence over a temperature range of 70-98˚C. A difference plot was generated using serial dilutions of DNA from a mutated cell line compared to wild-type DNA to assess HRMA sensitivity. The melting profiles of each sample were compared with those of the reference samples. Samples were considered to be of the MT if the shape of their difference melting plot differed significantly from that of the WT sample. The shapes of the HRMA curves varied according to the proportion of mutated DNA in the samples (Figure 1). A higher percentage of cells with mutated DNA in the samples caused an upward shift. The presence of only 1% of cells with mutated DNA was regarded as negative due to a high possibility of detecting a false positive.

DS, which had been outsourced, was carried out on samples from 102 patients with metastatic CRC. For these patients, HRMA was also performed using formalin-fixed specimens, and the results were compared blindly with the results of DS. In regard to the patients who received cetuximab, we evaluated its efficacy. Patients were received either cetuximab monotherapy or cetuximab + irinotecan. Cetuximab monotherapy was selected when the physician judged a patient as intolerable of irinotecan. Computed tomography (CT) scans of measurable lesions were assessed before treatment as the baseline, and they were repeated every 8 cycles until progression of disease. RECIST criteria (ver. 1.0) were used to assess tumor response. This study was approved by the Ethics Committee of the National Cancer Center Hospital #031.

Results

A total of 102 specimens were tested by each of the two mutation detection methods. The duration of formalin fixation was from one to seven days. The number of surgically-resected
specimens: biopsy specimens was 90:12 cases. The results of each of the methods are shown in Table I. Successful analyses were performed in 100 and 99 out of the 102 patients for DS (98%) and HRMA (97.1%), respectively. However, we were unable to analyze five cases because we were unable to amplify the DNA by one of the methods; the failed cases were successfully-analyzed by the other method. MT KRAS was detected by DS in 47 out of the 100 samples (47.0%), and by HRMA in 45 out of the 99 samples (45.5%). Forty-seven MT samples detected by DS were classified as a six different point mutation group (Table II). Both of the methods were able to analyze 97 out of the 102 samples. The results of the comparison between HRMA and DS for detection of KRAS mutations are shown in Table III. There was a concordance between the two methods for 92 out of the 97 samples and the concordance rate for results between the two methods was 94.8% (95% confidence interval (CI)=88.4-98.3).

Forty-six out of the 53 patients with WT KRAS detected by DS received cetuximab and the patient characteristics are shown in Table III. The response and disease control rates were 19.6% (95% CI=9.4-33.9) and 63.0% (95% CI=47.6-76.8), respectively (Table V). With a median follow-up of 8.8 months, the median progression-free survival was 5.6 (95% CI=3.9-7.3) months and the median survival time was 11.1 (95% CI=9.1-13.2) months (Figure 2).

Regarding the discordant cases, WT KRAS detected by DS and MT detected by HRMA, comprised two cases (2.1%), both of which received cetuximab. Best responses of the two cases were stable disease and progressive disease in one each, progression-free survival was 2.9 and 1.1 months, and overall survival was 5.3 and 1.2 months, respectively. MT detected by DS and WT detected by HRMA comprised three cases (3.1%), none of which received cetuximab because we treated on the basis of DS results.

**Discussion**

We compared two methods for the determination of KRAS mutations in FFPE tissue samples. We conclude that HRMA is also a highly accurate method for detecting KRAS mutations in patients with CRC. The rates of KRAS mutations detected by DS and HRMA in this study were almost the same as those in data published by others (12). HRMA and DS showed a high degree of concordance.

Recently, many researchers have reported on simple and highly sensitive non-sequencing methods for detecting KRAS mutations using small tumor samples, and the results of several mutation analyses were correlated with the clinical outcome of EGFR antibody treatment (13-15). HRMA can detect mutations in samples containing ~1% to 10% cells.
with mutated DNA, as opposed to DS which requires the presence of at least 10% to 30% in the samples. Additionally, HRMA provided a higher sensitivity than did DS in identifying the mutations in clinical samples (16). HRMA is an easy, quick (PCR for ~2 hours and HRMA for 10 minutes), and inexpensive (at a running cost per sample of approximately $4) method that might be useful in clinical practice with a great advantage over DS, which requires the extraction of high-quality DNA from an adequate amount of pure tumor cells, takes a long time, and is expensive. The previous study we performed showed that HRMA is a highly accurate method for detecting EGFR mutations in patients with NSCLC. The results of HRMA on surgically resected specimens as well as DNA sequencing revealed 100% sensitivity and specificity. On the other hand, the sensitivity and specificity of HRMA from the small diagnostic specimens were 83.3% and 100%, respectively (8). In practice we treated patients with NSCLC with EGFR tyrosine kinase inhibitors according to the HRMA results. In the other previous study, Ma et al. also compared DS and HRMA in KRAS analysis in patients with CRC. The concordance rate between the two methods was 95%, which is similar to the results of our study. However, Ma et al. did not test their samples blindly (17). In another study, Wilbur et al. compared DS, HRMA, and ARMS/Scorpion for KRAS analysis. Discrepancies between HRMA and the other two methods were due to the higher frequency of mutation in the HRMA groups. The Amplified Refractory Mutation System (ARMS)/Scorpion method also showed greater sensitivity than did DS, but HRMA is less expensive and faster than ARMS/Scorpion (16).

We developed the KRAS analysis with HRMA using primers for codon 12 and 13 provided by Idaho Technology. However, at first we were unable to amplify DNA from FFPE tissues. Hence we adopted a primer that amplifies DNA for codons 12 and 13 from FFPE tissues, as used in a previously published study (11). We were then able to amplify the DNA with a temperature-gradient PCR.

The current study has some limitations. Firstly, with HRMA, the success rate of analysis for KRAS mutation was not 100%, thus a confirmation by DS was needed when HRMA failed to amplify DNA or formed an abnormal curve pattern that could not be analyzed. On the other hand, cases that were not amplified by DS were successfully analyzed by HRMA in this study. Because more than one week of formalin fixation may fragment DNA, the period of formalin fixation should be as short as possible in order to increase the success rate for amplifying DNA. Secondly, there were discrepant results between HRMA and DS. There were two cases of HRMA positives that were not detected by DS. This might have been caused by a low level of mutated DNA that could only be detected by HRMA. In addition, there were three cases of DS positives that were not detected by HRMA. As two out of the three cases showed slightly higher shifts upward than did the WT samples, they might have been false-negatives. Additionally, in HRMA it may be difficult to identify exactly...
which codon is mutated. De Roock et al. showed that the use of cetuximab was associated with longer overall and progression-free survival among patients with chemotherapy-refractory CRC with codon 13 mutated tumors than with other KRAS-mutated tumors (18). Further investigations, such as scrutiny of HRMA curve patterns should be performed to clarify the methods best suited to discriminate between codon 12 and codon 13 in KRAS mutations.

Concerning efficacy, we found a response rate of 19.6% and a disease control rate of 63.0%. Moreover, the median progression-free survival was 5.6 months, the median overall survival was 11.1 months. Almost all (93.5%) patients received cetuximab as third line or later of therapy, which was compatible with prior published data (19). The efficacy of two discordant cases which received cetuximab showed that the best responses were stable disease and progressive disease (1:1) with progression-free survival of 2.9 and 1.1 months and overall survival of 5.3 and 1.2 months, respectively. These results were inferior to that for the entire data.

In the future, it may be possible to utilize HRMA for detection of other mutation, such as V-raf murine sarcoma viral oncogene homolog-B1 (BRAF) mutation and DNA methylation in CRC. Additionally, we will be able to scan not only for somatic but also germline mutations. Personalized medicine is developing more and more in terms of pharmacogenomics by scanning for mutations.

In conclusion, HRMA is an optional method for detection of KRAS mutations in CRC in light of its accuracy, cost performance and rapidity. As analysis failed or showed discordance between HRMA and DS in some cases, the two methods should complement each other in scanning for KRAS mutations in FFPE tumor tissues.

**Conflicts of Interest**

The Authors declare no conflict of interests.

**Acknowledgements**

We would like to thank Ms. Hideko Morita, Ms. Karin Yokozawa, and Ms. Shoko Nakamura for their help in collecting and organizing the clinical data for this study.

**References**