

Degraded DNA may induce discordance of *KRAS* status between primary colorectal cancer and corresponding liver metastases

Yuka Kaneko · Hidekazu Kuramochi ·
Go Nakajima · Yuji Inoue · Masakazu Yamamoto

Received: 27 May 2012 / Accepted: 2 December 2012 / Published online: 9 January 2013
© Japan Society of Clinical Oncology 2012

Abstract

Background *KRAS* mutation is widely accepted as a strong, negative predictive marker for anti-epidermal growth factor receptor antibodies, including cetuximab and panitumumab. Previous reports demonstrated approximately 100 % concordance of *KRAS* status between primary colorectal cancer and liver metastases; however, mismatched *KRAS* status still occurs.

Methods *KRAS* status was evaluated in 105 pairs of formalin-fixed primary colorectal cancer and corresponding liver metastases specimens by direct sequencing. DNA quality of patients displaying mismatched *KRAS* status between primary tumors and metastases was assessed using a Bioanalyzer.

Results *KRAS* status was successfully analyzed in 90/105 patients (85.7 %). The concordance rate between primary tumors and metastases was 88.2 % in synchronous metastases ($n = 76$) and 100 % in metachronous metastases ($n = 14$). Discordance in *KRAS* status was observed in nine patients. Independent method validation revealed only five samples showed the same *KRAS* status between the two methods. DNA quality assessment by a Bioanalyzer revealed that the median length of DNA samples in the peak concentration of the mismatched group was significantly shorter than those in the control group (153.5 vs 276.5 bp, $P = 0.0059$). In addition, the median value of

the percentage of degraded DNA (0–200 bp) in each sample in the mismatched group was significantly higher than the control group (35.5 vs 22 %, $P = 0.020$). These data suggest that the discordant results for these nine patients (18 samples) were due to low quality DNA, which may obscure polymerase chain reaction analysis, affecting sequencing reliability.

Conclusion Quality control and assurance of *KRAS* genotyping is critical, and standardization of the methodology is warranted.

Keywords *KRAS* · Mutation · Liver metastases · Bioanalyzer

Introduction

Cetuximab and panitumumab, anti-epidermal growth factor receptor (EGFR) monoclonal antibodies, are widely used for the treatment of colorectal cancer. Previous studies have established that therapy with these treatments is only effective in patients bearing wild-type *KRAS* [1, 2]. *KRAS*, a small, guanosine phosphate-binding protein, is a key component of the RAS/RAF/MAPK pathway, and is activated by phosphorylation of EGFR. Point mutations in codons 12 and 13 of the *KRAS* oncogene are detected in approximately 30–40 % of all patients with colorectal cancer [3].

Previous reports comparing the mutation status of *KRAS* between primary colorectal cancer and distant metastases reported that the percentage of concordance between primary tumors and metastases ranged between 68 and 100 % [4]. One reason for the discordance in *KRAS* status between primary tumors and metastases may be due to limitations in measurement due to poor quality DNA. Because formalin-fixed paraffin embedded (FFPE) samples are typically used

Y. Kaneko · Y. Inoue · M. Yamamoto
Department of Gastroenterological Surgery, Tokyo Women's
Medical University, 8-1 Kawadacho, Shinjuku-ku, Tokyo, Japan

H. Kuramochi (✉) · G. Nakajima
Department of Chemotherapy and Palliative Care,
Tokyo Women's Medical University, 8-1
Kawadacho, Shinjuku-ku, Tokyo 162-8666, Japan
e-mail: Kuramochi@chemo.twmu.ac.jp

in *KRAS* genotyping, poor preservation of DNA may result in fragmentation, preventing accurate genotyping of *KRAS*. Direct sequencing is the most frequently used method for *KRAS* genotyping; however, this process is technically complicated, and may easily lead to errors in measurement.

In this study we examined the *KRAS* status in 105 patients with colorectal cancer in both primary tumors and liver metastases by direct sequencing. Additional analysis was performed in patients displaying discordant *KRAS* status between primary tumors and metastases. To investigate the causes underlying this observation, the DNA quality of these tumors was assessed using a Bioanalyzer.

Materials and methods

Patients and samples

Paired samples of primary colorectal adenocarcinoma and corresponding liver metastases were obtained from 105 patients following surgical resection between 1995 and 2008 at the Department of Gastroenterology, Tokyo Women's Medical University, Tokyo, Japan. All patients were Japanese and gave their written informed consent according to institutional regulations. Of these, sequencing data were successfully obtained from 90 patients (57 males and 33 females; median age 62.9 years (range 38–91)). Synchronous liver metastases, defined as metastases diagnosed within a year from the primary tumor resection, were observed in 76 patients. Metachronous liver metastases, defined as metastases appearing >1 year after the primary tumor resection, were observed in 14 patients. The characteristics of the 90 patients are shown in Table 1.

This study was approved by the institutional ethics committee and was performed in accordance with the Declaration of Helsinki.

Microdissection

FFPE tumor specimens were cut into serial sections with a thickness of 10 μ m. Manual microdissection was performed using a scalpel if the histology was homogeneous and the tissue contained >90 % cancer cells. For all other samples, laser capture microdissection (P.A.L.M. Micro-laser Technologies AG, Munich, Germany) was performed to ensure that only tumor cells were dissected.

KRAS mutation screening by direct sequencing

DNA was extracted from FFPE specimens using the QIAamp DNA FFPE Tissue Kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. DNA concentration and quality was assessed using the ND-1000

Table 1 Demographic and clinical parameters of patients with metastatic colorectal cancer

Characteristics	Synchronous	Metachronous
Age		
Mean (range)	62.9 (38–91)	62.4 (40–75)
Gender		
Male	48 (63.2 %)	9 (64.3 %)
Female	28 (36.8 %)	5 (35.7 %)
Anatomical site		
Right colon	22 (28.9 %)	5 (35.7 %)
Transverse colon	4 (5.3 %)	1 (7.1 %)
Left colon	43 (56.6 %)	8 (57.2 %)
Rectum	7 (9.2 %)	0
Histology ^a		
Well	57 (75.0 %)	9 (64.3 %)
Moderately	16 (21.0 %)	4 (28.6 %)
Poorly	2 (2.6 %)	1 (7.1 %)
Mucinous	1 (1.4 %)	0
Dukes grade		
A	2 (2.6 %)	0
B	16 (21.1 %)	7 (50.0 %)
C	58 (76.3 %)	7 (50.0 %)
Number of metastases		
Single	30 (39.4 %)	8 (57.1 %)
Multiple	46 (60.5 %)	6 (42.9 %)

^a Well, well-differentiated tubular adenocarcinoma; moderately, moderately differentiated tubular adenocarcinoma; poorly, poorly differentiated adenocarcinoma; mucinous, mucinous adenocarcinoma

Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Polymerase chain reaction (PCR) was performed in a final volume of 50 μ l containing 500 ng of DNA, 0.6 μ l (6 pmol) each of forward and reverse primers, and 25 μ l of Quick Tag HS DyeMix (Toyobo, Osaka, Japan). Primers spanned codons 12 and 13 of the *KRAS* gene, as previously described [5]. Primer sequences were as follows: forward, 5'-GAATGGTCCTGCACCAGTAA-3', and reverse, 5'-GTGTGACATGTTCTAATATAGTCA-3'. The length of the amplified product was expected to be 217 bp. PCR was performed using the following cycling conditions: 94 °C for 3 min, 40 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 45 s, and 72 °C for 10 min.

PCR products were purified using the MinElute PCR Purification Kit (Qiagen) and used as a template for cycle sequencing using the Dye Terminator Cycle Sequencing (DTCS) Quick Start Kit (Beckman Coulter, Tokyo, Japan) according to the manufacturer's instructions. Nested PCR primer sequences were: forward, 5'-GTCCTGCACCAGT AATATGC-3', and reverse, 5'-ATGTTCTAATATAGTCA CATTTC-3'. A total of 4.4 μ l stop solution (3 μ l 3 M-NaOAc₂, 0.4 μ l 0.5 M Na₂EDTA, 1 μ l 20 mg/ml

glycogen) and 60 μ l 99.5 % (v/v) ethanol was added to sequencing reactions and samples were centrifuged at 14,000 rpm for 15 min at 4 °C. The supernatant was decanted and DNA was washed twice with 200 μ l 70 % (v/v) ethanol, followed by centrifugation at 14,000 rpm for 2 min at 4 °C. DNA pellets were vacuum dried for 5 min and DNA was dissolved in 40 μ l sample loading solution provided in the DTCS Quick Start Kit. Sequencing reactions were run on a CEQ-8800 Genetic Analyzer (Beckman Coulter). All sequencing reactions were performed in both forward and reverse directions. Direct sequencing was performed in duplicate for each sample.

KRAS mutation validation

To confirm the results obtained by direct sequencing as described above, we also assessed the mutational status of *KRAS* using a *KRAS* Mutation (codon 12, 13) Detection kit (Exciton ver.) (Dnaform, Tokyo, Japan). This kit is based on smart amplification process version 2 (SmartAmp2) technology [6–9]. It was used in accordance with the manufacturer's instructions. This method was performed once for each sample, but when the first analysis showed 'no amplification', a second analysis was performed. If both the first and second analysis showed 'no amplification', the sample was determined as 'not detected'. A third analysis was performed to investigate reproducibility if the second analysis showed successful amplification.

DNA quality analysis using Bioanalyzer

DNA quality was assessed using the 2100 Bioanalyzer (Agilent Technologies, Tokyo, Japan) and Agilent High Sensitivity DNA kit, according to manufacturer's instructions.

Statistical analysis

Comparisons of the median length of DNA fragments and the median percentage of degraded DNA between the mismatched group and the control group were assessed using the Mann–Whitney's *U* test. A *P* value of <0.05 was considered statistically significant. All values were two-sided.

Results

KRAS status was successfully measured in both primary tumors and liver metastases of 90/105 (85.7 %) patients examined in this study. The lack of available data for the remaining 15 patients was due to either low DNA yield (<500 ng), misalignment of the sense and antisense strands or low sequencing reproducibility.

Concordance of *KRAS* mutation status in primary colorectal adenocarcinoma and corresponding liver metastases

Of the 90 patients analyzed, 76 displayed synchronous metastases and 14 displayed metachronous metastases. In patients with synchronous metastases, *KRAS* mutation was observed in the primary tumors of 34 patients (44.7 %). Analysis of concordance between *KRAS* status in primary tumors and metastases was 88.2 %: 42 patients (55.3 %) had wild-type *KRAS* in both primary and liver, 25 patients (32.9 %) had mutations in both, three patients (3.9 %) had wild-type *KRAS* in primary and mutant *KRAS* in liver, and six patients (7.9 %) had mutant *KRAS* in primary and wild-type *KRAS* in liver. In patients with metachronous metastases, *KRAS* mutation was observed in the primary tumors of eight patients (57.1 %). The percentage of concordance was 100 % in these patients: six patients were wild-type in both primary colorectal adenocarcinoma and liver metastases, and eight patients were mutant in both primary tumors and liver (Table 2).

Validation of *KRAS* status in patients with discordant *KRAS* status in primary tumors and metastases

Nine patients displayed discordance in *KRAS* status between primary tumors and liver metastases. To confirm the *KRAS* status of these patients using an independent technique, their DNA was re-evaluated using the *KRAS* Mutation Detection Kit (Dnaform). In primary tumors, samples from six of nine patients were successfully analyzed and all patients displayed wild-type *KRAS*. In metastatic samples, data were obtained for five out of nine samples, with three patients displaying wild-type and two patients displaying mutant *KRAS* status (Table 3). Thus, using this independent method, we show that at least three patients previously shown by sequencing to have discordant *KRAS* status in primary and metastatic tumors had

Table 2 *KRAS* status in primary tumor and liver metastases in synchronous and metachronous metastases

	Primary <i>KRAS</i> wild-type	Primary <i>KRAS</i> mutant
Synchronous metastases (<i>n</i> = 76) ^a		
Liver metastases		
<i>KRAS</i> wild-type	42	6
<i>KRAS</i> mutant	3	25
Metachronous metastases (<i>n</i> = 14) ^b		
Liver metastases		
<i>KRAS</i> wild-type	6	0
<i>KRAS</i> mutant	0	8

Percentage of concordance: ^a 88.2 %, ^b 100 %

Table 3 Discrepancy of *KRAS* genotype comparing direct sequencing and the SmartAmp2 kit

	Primary tumor		Liver metastases	
	DS	Kit	DS	Kit
#1	Mutant	Wild-type	Wild-type	Wild-type
#2	Mutant	Wild-type	Wild-type	ND
#3	Mutant	Wild-type	Wild-type	Wild-type
#4	Mutant	ND	Wild-type	ND
#5	Wild-type	Wild-type	Mutant	ND
#6	Wild-type	ND	Mutant	Mutant
#7	Wild-type	Wild-type	Mutant	Wild-type
#8	Mutant	ND	Wild-type	ND
#9	Mutant	Wild-type	Wild-type	Mutant

DS direct sequencing, *kit* KRAS Mutation Detection Kit (SmartAmp2 kit), ND not detected

concordant status using the *KRAS* mutation detection kit. Interestingly, however, we found that in primary tumors only two patients showed equivalent *KRAS* status comparing direct sequencing and the SmartAmp2 kit, whereas four patients showed different status. Similarly, analysis of liver metastases showed that *KRAS* status was concordant in three patients using these two methods, whereas two patients showed different statuses.

Background data of nine patients with mismatched *KRAS* status between primary tumors and metastases

To investigate whether specific factors may account for the discordance observed in the *KRAS* status of these nine patients, we examined background data for these patients compared with all patients. Age, gender, location of tumor, pathological type, and number of metastases were not differentially distributed between these nine mismatched *KRAS* status patients and all 90 patients (data not shown). The median year for collection of samples from these nine patients was 2004 (range 1998–2008), which was similar to collection times for the entire patient cohort (median year 2003; range 1995–2008). Similarly, the median concentration of DNA for these nine patients (including both primary and metastases; total 18 samples) was 230.96 ng/ μ l, which again was comparable to the median concentration of the entire 90 patients (233.93 ng/ μ l). Last, we compared the median UV 260/280 and 260/230 absorbance ratios in these patients, which are a measure of protein and organic contaminants, respectively, that may affect downstream applications. In the mismatch patients these values were 1.98 and 0.86, respectively, which were similar to the entire patient cohort (1.99 and 0.91, respectively).

DNA quality analysis using Bioanalyzer

Since no differences were observed when background data and DNA quality as assessed by NanoDrop were compared between the mismatched samples and all samples, we hypothesized that this discrepancy may be due to DNA degradation. To test this, we analyzed DNA quality using a Bioanalyzer. The Agilent 2100 Bioanalyzer and High Sensitivity DNA Kit are capable of analyzing the size and quantity of fragmented DNA and the quality control of DNA sequencing using only 100 pg/ μ l of DNA. Electrophoresis results are visualized as migration-time plots or computer-generated virtual gels. Figure 1 displays typical electropherograms of degraded and well-preserved DNA samples extracted from FFPE specimens. Table 4 indicates the size of fragmented DNA in the peak concentration. It also shows the analysis of DNA from seven patients (control group) randomly selected from the matched samples used in this study.

The median length of DNA samples in the peak concentration in the mismatched group was significantly shorter than those in the control group (153.5 vs 276.5 bp, $P = 0.0059$). In the mismatched group, five out of 18 samples contained DNA that was significantly degraded (>50 % degraded fragments in the range of 0–200 bp). In contrast, zero out of 14 samples was degraded in the control group. The median value of the percentage of degraded DNA in each sample was 35.5 % in the mismatched group, which was significantly higher than the control group (22 %, $P = 0.020$). Because the PCR amplicon size generated using primers for the direct sequencing method was 217 bp, it is likely that the low concordance of results observed in the mismatched group was due to the low quality of the starting DNA template, resulting in insufficient PCR amplification and unreliable sequencing data.

Discussion

In this study, we performed a detailed investigation in patients displaying discordant *KRAS* status between the primary colorectal cancer and the corresponding liver metastases. Our initial prediction was that these results may be caused by low DNA yield or contamination of the DNA sample with other agents that may interfere with the PCR reaction. However, spectrophotometric analysis of these DNA specimens using a NanoDrop revealed that the yield and presence of protein or organic contaminants was similar between the mismatched samples and all patients. Instead, we demonstrated that there was significant DNA degradation in the primary and/or metastases specimens in the mismatched group. This DNA degradation was difficult

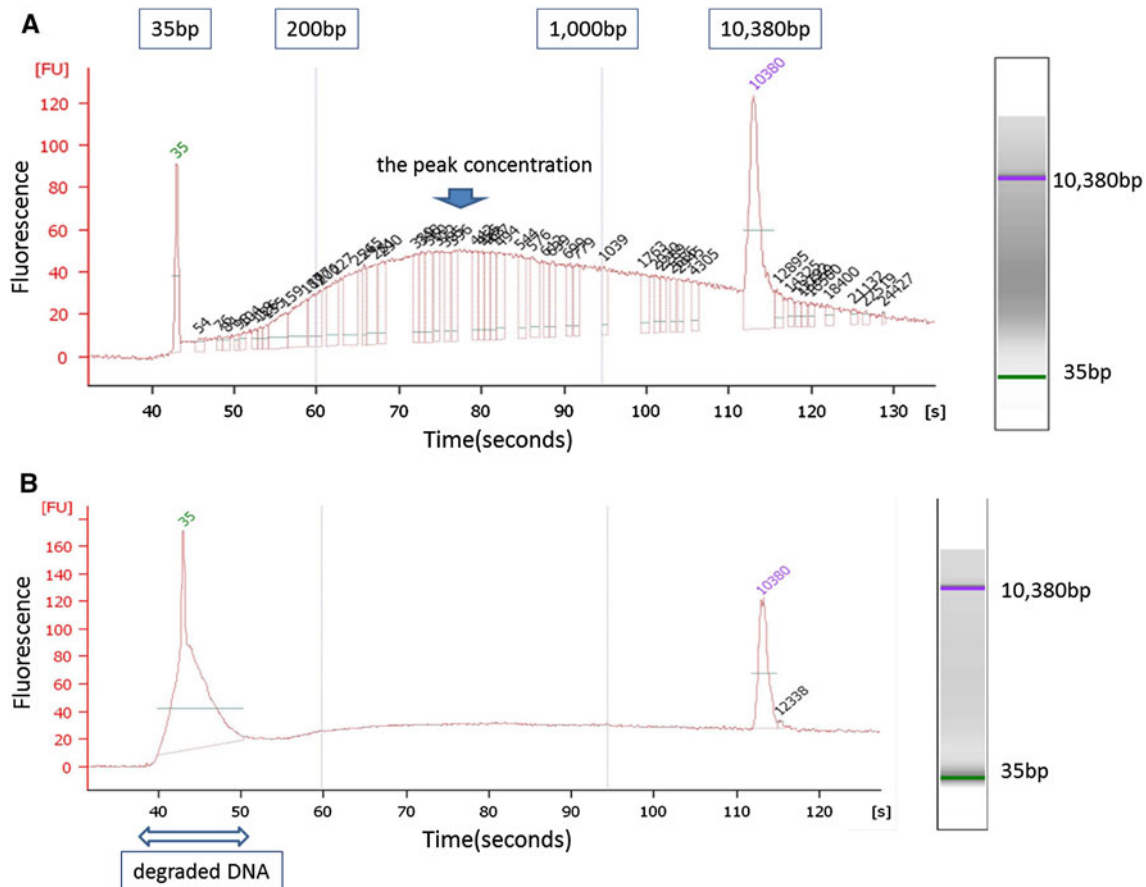


Fig. 1 Electropherograms (left panel) and gel-image (right panel) displaying DNA quality assessment by the Agilent Bioanalyzer. The horizontal axis represents the migration time of DNA fragments in seconds, and the vertical axis represents fluorescence. The left peak represents a DNA ladder at 35 bp and the right peak a DNA ladder at

10,380 bp. **a** Examples of well-preserved DNA: the size of DNA samples in the peak concentration was 396 bp. The majority of the DNA fragments were >200 bp. **b** Examples of poorly preserved DNA: the majority of DNA fragments were <200 bp

to detect by NanoDrop, whereas the Bioanalyzer was very useful for this level of quality control.

Several factors may underlie the observed DNA degradation. First, DNA fragmentation may occur between the time of sample collection and time of analysis. All samples are paraffin-embedded, which is not ideal for DNA preservation and DNA fragmentation increases over time. In this study, patient samples in the mismatched group were not only collected at an older age, but were also distributed widely from 1998 to 2008. Second, the type of fixative used for DNA preservation can also affect DNA integrity. In most cases, formalin fixative is used; however, its cross-linking impairs DNA and RNA quality. Higher formalin concentration causes more damage to DNA quality. In addition, previous reports have shown that neutral-buffered formalin is a superior preservative for DNA compared with non-buffered formalin [10, 11]. In our institute, 10 % buffered formalin was used before 2000. Since 2001, however, 15 % non-buffered formalin was commonly used

in the fixation of surgical specimens, which is not optimal for DNA preservation. In our study, we observed no difference in the success rate of *KRAS* screening before and after 2000 (data not shown). As previously mentioned, the mismatched samples were collected between 1998 and 2008, suggesting that the type of fixative used was not a significant factor affecting DNA integrity and subsequent *KRAS* genotyping in this study. Third, the sample fixation time is crucial. Basically, the shorter the duration of formalin fixation, the better DNA is preserved [10–13]. In our institute, fixation time was usually within 3 days. But there were no data for the length of formalin fixation for each of these nine samples, nor the time from surgical resection to formalin fixation. Thus, this factor might be one of the reasons for DNA degradation. In a previous study, DNA fragments ranging between 268 and 1327 bp were PCR-amplified from FFPE samples, demonstrating these samples performed well only for shorter length amplicons [13]. Thus, to increase the success rate of the *KRAS* screening

Table 4 Bioanalyzer data of mismatched group and control group

Sample ID		Fragment length in peak concentration (bp)	Proportion of DNA (%)		
			0–200 bp	201–400 bp	>400 bp
Mismatched group					
#1	Primary	42	60	20	20
	Metastases	43	45	20	35
#2	Primary	170	59	21	20
	Metastases	173	66	22	12
#3	Primary	No peak	22	38	40
	Metastases	311	12	33	55
#4	Primary	53	73	20	6
	Metastases	173	49	41	10
#5	Primary	40	40	34	26
	Metastases	137	31	39	30
#6	Primary	No peak	ND	ND	ND
	Metastases	No peak	16	20	64
#7	Primary	No peak	29	37	33
	Metastases	No peak	20	30	49
#8	Primary	No peak	51	29	19
	Metastases	No peak	30	36	34
#9	Primary	No peak	ND	ND	ND
	Metastases	5034	0	0	100
Median		153.5	35.5	29.5	31.5
Control group					
#1	Primary	270	31	43	25
	Metastases	305	18	42	40
#2	Primary	220	22	44	34
	Metastases	254	13	36	51
#3	Primary	272	28	36	36
	Metastases	180	28	38	33
#4	Primary	497	24	26	50
	Metastases	281	31	32	37
#5	Primary	200	22	44	33
	Metastases	250	31	15	54
#6	Primary	589	0	4	96
	Metastases	960	0	0	100
#7	Primary	1523	35	0	65
	Metastases	1684	14	1	85
Median		276.5	22	34	38.5

test, it may be useful to design primers generating a smaller amplicon compared with our study, which amplified a 217 bp product.

In this study, an independent *KRAS* genotyping method, SmartAmp2, was used to validate the data obtained from direct sequencing. The basic principle of this method is that DNA amplification equals detection. Araki et al. [8] validated *KRAS* status with four different methods (direct sequencing, SmartAmp2, enzyme-enriched sequencing, and PNA-enriched sequencing) and reported that

SmartAmp2 was the most sensitive and accurate method that can detect as little as 1 % of the mutant DNA with no non-specific amplification, which often reduces the sensitivity of sequencing by elevating the background noise. In our data, 18 samples from nine patients (primary and metastases) were analyzed with both direct sequencing and SmartAmp2 kit; seven out of 18 samples showed no amplification, and of 11 samples that were successfully amplified, six showed discordant *KRAS* status. From the Bioanalyzer data, these 18 samples had degraded low-quality DNA, which led to

unsuccessful PCR amplification and/or poor sequencing with high background noise when analyzed by direct sequencing. We obtained reproducible data from the SmartAmp2 kit, which seemed to be more reliable than direct sequencing for these degraded samples. In a clinical setting, however, sensitive methods are not always ideal, because they are sometimes so sensitive that they may exclude the patients who are potentially sensitive to anti-EGFR antibody agents.

Recently, the *KRAS* mutation screening test has become essential in clinical practice for the treatment of unresectable colorectal cancer, since it is a strong negative biomarker for anti-EGFR antibody therapies [1, 2, 14]. Several *KRAS* genotyping methods are commonly employed; however, a standardized method has not yet been fixed. The most frequently used method is direct sequencing, which was also used in this study. A number of commercial kits are currently available for *KRAS* genotyping [6, 7, 15]. A study by Dequeker et al. [16] which aimed to identify variables that need to be assessed in a *KRAS* genotyping quality control scheme, involved sending 14 different colorectal cancer FFPE samples to independent laboratories in 13 different European countries for *KRAS* mutation screening. This study illustrated that only 10 of 13 experienced laboratories correctly identified the *KRAS* mutational status in all 14 cases, and *KRAS* genotyping methods and DNA extraction methods varied considerably between the laboratories. These results indicate a lack of consistency, even in laboratories that routinely perform *KRAS* genotyping, suggesting that quality control and quality analysis are essential, particularly as this result may affect patient prognosis and treatment strategy. Although direct sequencing is the most widely used method for *KRAS* genotyping, the result is affected by several factors including DNA quality and quantity, PCR conditions, primer design, and the condition of the sequencing instrument. While the use of kits is expensive, they may minimize the risk of variation between laboratories.

Previous literature has reported that the concordance rate of *KRAS* status in primary tumors and metastases ranges between 68 and 100 % [4, 17]. Knijn et al. [4] reported that the concordance rate of 892 patients from 18 previously published studies was 96.4 %. In our study, the success rate of *KRAS* diagnosis was 85.7 % (90/105), and the overall concordance rate between primary and metastases was 90 %, which seemed to be lower than previous reports. The reason might be due to DNA degradation; the first 15 had highly degraded DNA that could not be amplified by the first PCR, and even among the 90 patients with successful amplification, some samples had degraded DNA that could not present reliable, reproducible data. In a separate analysis, examination of patients with synchronous and metachronous metastases revealed concordance rates of 81 and 100 %, which was not statistically significant. In previous studies,

the discrepancy in *KRAS* status between primary tumors and metastases has been attributed to intratumoral heterogeneity [18–21]; however, it was also acknowledged this may be due to the incorrect genotyping caused by DNA degradation. Therefore, careful interpretation is required in these studies.

In conclusion, since *KRAS* genotyping has become an essential test for clinical practice, it is crucial to be aware that these results may be influenced by several factors, such as DNA quality and methodology. Thorough quality control and analysis and standardization of methods for DNA preservation, extraction and genotyping are critical for high quality, reliable results, or the restriction of laboratories for *KRAS* testing may be taken into consideration.

Acknowledgments We thank Dr. Hitoshi Kanno for kindly providing the data obtained using the *KRAS* mutation detection kit.

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Douillard JY, Siena S, Cassidy J et al (2010) 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(31):4697–4705
2. Lievre A, Bachet JB, Boige V et al (2008) *KRAS* mutations as an independent prognostic factor in patients with advanced colorectal cancer treated with cetuximab. *J Clin Oncol* 26(3):374–379
3. Monzon FA, Ogino S, Hammond ME et al (2009) The role of *KRAS* mutation testing in the management of patients with metastatic colorectal cancer. *Arch Pathol Lab Med* 133(10):1600–1606
4. Knijn N, Mekenkamp LJ, Klomp M et al (2011) *KRAS* mutation analysis: a comparison between primary tumours and matched liver metastases in 305 colorectal cancer patients. *Br J Cancer* 104(6):1020–1026
5. Pao W, Wang TY, Riely GJ et al (2005) *KRAS* mutations and primary resistance of lung adenocarcinomas to gefitinib or erlotinib. *PLoS Med* 2(1):e17
6. Hoshi K, Takakura H, Mitani Y et al (2007) Rapid detection of epidermal growth factor receptor mutations in lung cancer by the Smart-Amplification Process. *Clin Cancer Res* 13(17):4974–4983
7. Mitani Y, Lezhava A, Kawai Y et al (2007) Rapid SNP diagnostics using asymmetric isothermal amplification and a new mismatch-suppression technology. *Nat Methods* 4(3):257–262
8. Araki T, Shimizu K, Nakamura K et al (2010) Usefulness of peptide nucleic acid (PNA)-clamp smart amplification process version 2 (SmartAmp2) for clinical diagnosis of *KRAS* codon 12 mutations in lung adenocarcinoma: comparison of PNA-clamp SmartAmp2 and PCR-related methods. *J Mol Diagn* 12(1):118–124
9. Tatsumi K, Mitani Y, Watanabe J et al (2008) Rapid screening assay for *KRAS* mutations by the modified smart amplification process. *J Mol Diagn* 10(6):520–526
10. Taguchi M, Inoue H, Motani-Saitoh H et al (2012) DNA identification of formalin-fixed organs is affected by fixation time and type of fixatives: using the AmpF ℓ STR(R) Identifier(R) PCR Amplification Kit. *Med Sci Law* 52(1):12–16

11. Ferrer I, Armstrong J, Capellari S et al (2007) Effects of formalin fixation, paraffin embedding, and time of storage on DNA preservation in brain tissue: a BrainNet Europe study. *Brain Pathol* 17(3):297–303
12. Inoue T, Nabeshima K, Kataoka H et al (1996) Feasibility of archival non-buffered formalin-fixed and paraffin-embedded tissues for PCR amplification: an analysis of resected gastric carcinoma. *Pathol Int* 46(12):997–1004
13. Turashvili G, Yang W, McKinney S et al (2012) Nucleic acid quantity and quality from paraffin blocks: defining optimal fixation, processing and DNA/RNA extraction techniques. *Exp Mol Pathol* 92(1):33–43
14. Van Cutsem E, Kohne CH, Hitre E et al (2009) Cetuximab and chemotherapy as initial treatment for metastatic colorectal cancer. *N Engl J Med* 360(14):1408–1417
15. Bando H, Tsuchihara K, Yoshino T et al (2011) Biased discordance of KRAS mutation detection in archived colorectal cancer specimens between the ARMS-Scorpion method and direct sequencing. *Jpn J Clin Oncol* 41(2):239–244
16. Dequeker E, Ligtenberg MJ, Vander Borgh S et al (2011) Mutation analysis of KRAS prior to targeted therapy in colorectal cancer: development and evaluation of quality by a European external quality assessment scheme. *Virchows Arch* 459(2):155–160
17. Santini D, Loupakis F, Vincenzi B et al (2008) High concordance of KRAS status between primary colorectal tumors and related metastatic sites: implications for clinical practice. *Oncologist* 13(12):1270–1275
18. Albanese I, Scibetta AG, Migliavacca M et al (2004) Heterogeneity within and between primary colorectal carcinomas and matched metastases as revealed by analysis of Ki-ras and p53 mutations. *Biochem Biophys Res Commun* 325(3):784–791
19. Fukunari H, Iwama T, Sugihara K et al (2003) Intratumoral heterogeneity of genetic changes in primary colorectal carcinomas with metastasis. *Surg Today* 33(6):408–413
20. Baldus SE, Schaefer KL, Engers R et al (2010) Prevalence and heterogeneity of KRAS, BRAF, and PIK3CA mutations in primary colorectal adenocarcinomas and their corresponding metastases. *Clin Cancer Res* 16(3):790–799
21. Watanabe T, Kobunai T, Yamamoto Y et al (2011) Heterogeneity of KRAS status may explain the subset of discordant KRAS status between primary and metastatic colorectal cancer. *Dis Colon Rectum* 54(9):1170–1178