Concordance of Acquired Mutations between Metastatic Lesions and Liquid Biopsy in Metastatic Colorectal Cancer Treated with Anti-EGFR Therapy

Fumitaka Taniguchi  
Okayama University: Okayama Daigaku

Akihiro Nyuya  
Kawasaki Medical School: Kawasaki Ika Daigaku

Toshiaki Toshima  
Okayama University: Okayama Daigaku

Kazuya Yasui  
Okayama University: Okayama Daigaku

Yoshiko Mori  
Saitama Medical Center: Saitama Ika Daigaku Sogo Iryo Center

Makoto Okawaki  
Kawasaki Medical School: Kawasaki Ika Daigaku

Hiroyuki Kishimoto  
Okayama University: Okayama Daigaku

Yuzo Umeda  
Okayama University: Okayama Daigaku

Toshiyoshi Fujiwara  
Okayama University: Okayama Daigaku

Hiroaki Tanioka  
Kawasaki Medical School: Kawasaki Ika Daigaku

Yoshiyuki Yamaguchi  
Kawasaki Medical School: Kawasaki Ika Daigaku

Ajay Goel  
Beckman Research Institute City of Hope: Beckman Research Institute

Takeshi Nagasaka (✉️ takeshin@med.kawasaki-m.ac.jp)  
Kawasaki Medical University  https://orcid.org/0000-0002-4236-350X

Research

Keywords: Acquired mutations, liquid biopsy, colorectal cancer, RAS, BRAF, PCR-rSSO
Abstract

Background: Acquired mutations are detected in plasma. However, still few reports examine the concordance between liquid biopsy and metastatic lesions with acquired resistance. Herein we evaluated whether a polymerase chain reaction-reverse sequence-specific oligonucleotide (PCR-rSSO) method can examine the concordance between liquid biopsy and metastatic lesions with acquired resistance.

Methods: Firstly, we examined the presence of acquired mutations in 7 chemoresistant metastatic lesions and blood samples obtained from a metastatic colorectal cancer (mCRC) patient without RAS activating mutations treated with anti-EGFR treatment. The patient (patient 1) displayed initial early tumor shrinkage and finally progressed to disease (PD). Blood samples were collected before the development of PD and after acquiring resistance. Next, we evaluated RAS and BRAF mutational status among blood samples, primary tumors, and metastatic lesions obtained from three additional mCRC patients without RAS activating mutations. Acquired mutations were examined using Sanger sequencing and the PCR-rSSO approach.

Results: Of patient 1, metastatic tumor specimens harbored diverse acquired mutations in the KRAS gene in all of the 7 (100%) metastases, and the three acquired mutations were detected in blood specimens collected after acquiring resistance. Next, we analyzed primary tumors, metastatic lesions after chemotherapy, and blood samples from three additional mCRC patients but noted that none of the patients exhibited mutations in liquid biopsy except for one case with BRAF V600E mutation, which was confirmed in both primary tumor and peritoneal dissemination. Of the four cases, acquired mutations of RAS, as well as BRAF V600E mutation, was detected in the blood obtained only after confirmation of acquiring resistance by radiological examinations.

Conclusions: Our results suggest liquid biopsy based on the PCR-rSSO is a successful procedure for capturing acquired mutations with precise information of mutational spectrum that may lend us to reach selective target agents for RAS mutations.

Background

Acquired resistance and primary resistance play major roles in anticancer treatment[1–3]. The epidermal growth factor receptor (EGFR)-targeted antibodies cetuximab and panitumumab are used to treat metastatic colorectal cancer (CRC) negative for mutations in KRAS and NRAS exons 2–4[4–6]. Although patients with metastatic CRC without activating RAS mutations generally show a clinical response to anti-EGFR antibodies, acquired resistance may eventually develop. Several studies have identified acquired genetic alterations; e.g., KRAS, HER2, or MET amplification or KRAS, NRAS, BRAF, or EGFR mutations[1, 3, 7–12].

Recent studies have suggested that genomic alterations in solid tumors can be characterized by analyzing circulating tumor DNA (ctDNA), which is released from cancer cells into the plasma[13]. Currently, OncoBEAM-based liquid biopsy is a standard procedure for the detection of RAS mutations in
Indeed, a previous study used OncoBEAM technology, which can detect mutant allele frequency (MAF) as low as 0.1% of acquired RAS mutant alleles[1, 16]. However, even when using this procedure, approximately 10–18% of patients harbored RAS mutations in tissue that could not be detected in plasma[14, 15]. The reason for this discordance may be attributed to tumor heterogeneity, lower circulating tumor DNA shedding or lower tumor burden. Clinically, mCRC patients at advanced stages possess multiple metastatic lesions in multiple organs. Therefore, in such cases, the heterogeneity of RAS mutations must be considered. However, only a handful of studies have evaluated the concordance of RAS mutational status in all metastatic lesions located in multiple organs and ctDNA.

More importantly, although the OncoBEAM-based liquid biopsy has such a higher sensitivity for RAS mutant alleles and can provide which exon is mutated, this could not provide a precise RAS mutational spectrum; e.g., G12C or G12A. Concern to RAS mutations, KRAS mutations are often associated with resistance to targeted therapies and poor outcomes in patients with cancer, no selective KRAS inhibitor has been approved despite more than three decades of scientific effort [17–23]. However, recent advances have led to the development of a small molecule, sotorasib, which specifically and irreversibly inhibits KRASG12C through a unique interaction with a pocket of the switch II region and exhibited encouraging anticancer activity in patients with heavily pretreated advanced solid tumors harboring KRAS G12C mutations [24–27].

Thus, in the clinical setting, easier identification of RAS mutational spectrum will continue to rise in numbers as their role in multiple cancers is further recognized. In contrast to OncoBEAM technology, a polymerase chain reaction-reverse sequence-specific oligonucleotide (PCR-rSSO) method for the detection of RAS mutations is now of use in the clinical setting to detect RAS mutations in tumor tissues. The PCR-rSSO approach can detect a minor allele frequency (MAF) of 1–5% of the RAS mutant alleles, and could identify all RAS mutational spectrum all at once. In this study, the PCR-rSSO method was examined for its concordance for the identification of RAS mutations present in multiple metastatic lesions in multiple organs and ctDNA in plasma throughout anti-EGFR therapy in mCRC patients [15].

**Methods**

**Tumor Samples**

Four mCRC patients with primary tumors without activating RAS mutations were analyzed in this study. The patients were treated between 2011 and 2017 at the Okayama University Hospital, Japan. Each patient was enrolled as a research subject in the clinical trials (UMIN [University Hospital Medical Information Network Center] ID, 8377, 9698, or 11954), which were approved by the ethics committee of Okayama University and Kawasaki Medical School the Institutional Review Board [IRB] number, 3172, 3193, 3194, 3196-1, and 3239). The patients provided written informed consent and all studies were conducted in accordance with the Declaration of Helsinki.
In patient 1, primary tumors were obtained through biopsy before initiation of any treatment (Tb) and by surgical resection before acquiring resistance during anti-EGFR treatment (Ts). Metastatic lesions were excised at morbid autopsy and included those of the liver (MS, S2, and S3), hepatic lymph node (HN), lung (Lu), and kidney (Kd) (Fig. 1A).

The primary tumors from patient 2 (Fig. 2A) and patient 3 (Fig. 2D) were obtained by surgical resection before chemotherapy. A metastatic liver lesion from patient 2 was obtained following subsequent liver resection and that from patient 3 at the initial liver resection.

The primary tumor of patient 4 was obtained by biopsy before initiation of any treatment. A metastatic lesion of peritoneal dissemination was obtained at surgical resection after anti-EGFR treatment (Fig. 2G).

Tissues obtained from autopsy or surgical resection were immediately stored at −80°C.

**Blood samples**

Blood samples from patient 1 were collected before developing progressive disease (PD) during first-line chemotherapy (point 1) and after the acquisition of resistance to second- and third-line chemotherapies (point 2 and point, Fig. 1A). Of patient 2, the blood sample at time point 1 was collected after surgical resection of liver metastases at the right lobe. Blood samples from timepoints 2 and 3 were collected after confirmation of recurrence in the liver and after second-time resection of liver metastasis (Fig. 2A). Of patient 3, the blood sample of point 1 was collected before initiation of treatment. The blood sample of point 2 was collected after developing PD during bevacizumab (an anti-vascular endothelial growth factor antibody)-based chemotherapy. The blood sample of time point 3 was collected after cetuximab treatment with tumor shrinkage (Fig. 2D). Of patient 4, the blood sample of point 1 was collected before developing PD. The blood sample of point 2 was collected after surgical resection. The blood sample of point 3 was collected after acquiring resistance to cetuximab treatment (Fig. 2G). Plasma was separated immediately and stored at −80°C.

**Extraction of Genomic DNA**

Genomic DNA was extracted from fresh-frozen samples using the QIAamp DNA Mini Kit (Qiagen NV, Hilden, Netherlands). Tumor DNA of several metastatic lesions was microscopically extracted from formalin-fixed paraffin-embedded (FFPE) specimens. These specimens included metastases located in the liver (RL) and hepatic lymph node (HN). DNA derived from FFPE specimens was extracted using the QIAamp DNA FFPE Tissue Kit (Qiagen NV, Hilden, Netherlands). Circulating cell-free DNA was extracted from 200 µl of plasma using the QIAamp Blood Kit (Qiagen) according to the manufacturer’s instructions.

**Conventional Sequencing**

Sanger sequencing was also performed to confirm mutations in *KRAS*, *NRAS* exons 2 to 4, and *BRAF* exon 15 (including codon 600) in all samples. Primer sequences for *KRAS*, *NRAS* exons 2 to 4, and *BRAF* exon 15 and PCR conditions are described in Supplementary Table 1. PCR products were purified using
the QIAquick PCR Purification Kit (Qiagen) and were directly sequenced using the ABI PRISM® 3100-Avant (Applied Biosystems) and a SeqStudio Genetic Analyzer (Thermo Fisher Scientific).

**Polymerase Chain Reaction-Reverse Sequence-Specific Oligonucleotide (PCR-rSSO)**

Extensive RAS mutations (both KRAS and NRAS mutations) of DNA purified from FFPE, fresh frozen tissues, and plasma were evaluated using the MEBGEN™ RASKET or RASKET-B KIT based on the Luminex® technology (MBL, Nagoya, Japan). The assay was performed according to the manufacturer's protocol. The MEBGEN™ RASKET KIT can simultaneously examine 12 types of RAS exon 2 (G12S, G12C, G12R, G12D, G12V, G12A, G13S, G13C, G13R, G13D, G13V, and G13A), eight types of RAS exon 3 (A59T, A59G, Q61K, Q61E, Q61L, Q61P, Q61R, and Q61H) and four types of RAS exon 4 (K117N, A146T, A146P, and A146V) mutations[16]. The MEBGEN™ RASKET-B KIT was examined 12 types of RAS exon 2, eight types of RAS exon 3, four types of RAS exon 4, and BRAF V600E mutation.

**Results**

**Identification of Acquired RAS Mutations by Conventional Sanger Sequencing**

In patient 1, no activating RAS mutation was observed in the primary tumor biopsy specimen obtained before treatment, in the primary tumor tissue obtained after 1 year of panitumumab administration, or in a metastatic tumor in the right liver lobe (RL) that responded continuously to panitumumab with first-line systemic chemotherapy and shrank in response to third-line panitumumab rechallenge (Fig. 1A). Besides, KRAS sequences in metastatic specimens obtained during autopsy revealed diverse acquired mutations at different metastatic sites, indicating resistance to the systemic chemotherapy, including anti-EGFR antibody treatment. Acquired activating KRAS mutations resulting in G61Hc, G12R, and G12V were detected by Sanger sequencing in liver segments II (S2), III (S3), and middle segment (MS), respectively. The KRAS G12C, G13D, and G61Hc mutations were also detected in metastases in the left kidney (Kd), a hepatic lymph node (HN), and the left lung (Lu), respectively. No samples from primary and metastatic lesions harbored mutations in NRAS or BRAF (Fig. 1B).

**Identification of Acquired RAS Mutations by a PCR-rSSO Method**

By Sanger sequencing, acquired mutations were detected in 6 (87%) of the 7 metastatic lesions in patient 1. Because of the lower sensitivity for the detection of mutant alleles by Sanger sequencing (~ 20%), we could not exclude the possibility of associated metastatic lesions caused by resistant cells already existing in the primary lesion in extremely low numbers.
We further analyzed all samples using the PCR-rSSO method, RASKET, which has high sensitivity in terms of detecting extended RAS mutant alleles at lower frequencies, 1–5%; a sample was considered mutation-positive when the index was estimated over the cut-off value, the sensitivity of which for the mutant allele was 1–5%, for each mutant allele[16].

Using this procedure, KRAS or NRAS mutations with indices higher than the cut-off values were not found in DNA purified from pretreated primary tumor cells (Fig. 1C and Supplementary Table 2). Additionally, PCR-rSSO analysis revealed the same mutation spectrum obtained by Sanger sequencing in the patient except for the metastatic lesion in the Kd and the RL. The index of the KRASG12C allele captured from the Kd by PCR-rSSO analysis was 114, i.e., lower than the conventional cut-off value (index value: 300), which might be influenced by the immediate synonymous mutation of A11Ag demonstrated by Sanger sequencing, as previously reported (Fig. 1A) [16]. Interestingly, the metastatic tumor in the RL, which responded consistently to panitumumab and showed no mutation by Sanger sequencing, revealed multiple KRAS mutations, Q61Ht, G12A, and G12R, with indices higher than the cut-off values. Although these mutant alleles were not frequent enough to be detected by Sanger sequencing, heterogeneity in RAS mutant cancer cells may exist in the RL tumor mass.

In summary, by Sanger sequencing and PCR-rSSO, the 7 metastatic lesions were found to harbored diverse acquired mutations in the KRAS gene: Q61Ht, G12A, and G12R in RL; G12V in MS; Q61Hc in S2; G12R in S3; G12C in Kd; G13D in HN; Q61Hc in Lu.

Detection of Acquired Mutations in Circulating Cell-Free DNA

We investigated whether acquired RAS mutations could be detected in the plasma samples using PCR-rSSO (Fig. 1D). The seven diverse acquired mutations found in the resistant tumors were not detected before or after PD during the initial course of panitumumab treatment (point 1 and point 2 in Fig. 1A and Supplementary Table 3), but two of the seven diverse acquired mutations were detected at a significant level following the confirmation of PD during third-line panitumumab rechallenge (point 3). The KRAS G12R and Q61Hc frequency, which were confirmed in the resistant tumor in S3 and S2, respectively, were strikingly elevated in circulating cell-free DNA in a plasma sample collected at point 3. All the acquired mutations detected in the plasma of point 3 were not detected in the plasma of points 1 and 2.

Detection of Acquired Mutations in Circulating Cell-Free DNA in other mCRC patients

We next evaluated other three mCRC patients with no activating RAS mutations. Patient 2 was initially resected for the primary tumor and then treated with FOLFOX plus cetuximab. After 1.5 years of chemotherapy administration, metastatic tumors in the right liver lobe that responded continuously were resected (Fig. 2A). Following liver resection, patient 2 was carefully followed without chemotherapy. However, unfortunately, the patient experienced a recurrence of single metastasis at the middle segment
of the liver. FOLFOX plus cetuximab was re-introduced and made led to effective shrinkage of the metastatic lesion. Thus, second-time liver resection was performed. Both the primary tumors obtained before initiation of treatment and the metastatic liver lesion after the second-time liver resection showed no activating RAS mutations by the PCR-rSSO method (Fig. 2B). The importance is that patient 2 never experienced acquired resistance during the treatment course. Therefore, liquid biopsies among the 3 points also showed no activating RAS mutations (Fig. 2C).

Patient 3 was initially resected for their primary tumor and then treated with FOLFOX plus bevacizumab. Ten months after treatment, this first-line systemic chemotherapy failed; hence, FOLFIRI plus cetuximab was selected as the second-line treatment. The metastatic tumors in the right liver lobe responded rapidly to cetuximab. Thus, liver resection was performed. Both the primary tumor obtained before initiation of treatment and a metastatic liver lesion after response to cetuximab showed no activating RAS mutations by the PCR-rSSO method (Fig. 2E). Similar to patient 2, patient 3 also never experienced acquired resistance to cetuximab. Therefore, it is reasonable that liquid biopsies among the 3 points showed no activating RAS mutations (Fig. 2F).

Patient 4 was initially diagnosed that the primary tumor possessed BRAF V600E mutation and treated with FOLFIRI plus cetuximab as the first-line chemotherapy (Fig. 2G and H). The primary tumor and peritoneal dissemination responded rapidly to cetuximab. After tumor shrinkage, the primary tumor and peritoneal dissemination were surgically resected. After surgery, FOLFIRI plus cetuximab was continuously administrated. Approximately, 3 months after surgery, residual metastatic lesions grew, indicating resistance to the systemic chemotherapy. Liquid biopsies at time points 1 and 2 were collected at the time when tumors responded rapidly to cetuximab. On the other hand, the liquid biopsy at point 3 was collected after confirmation of acquired resistance to cetuximab. No BRAF V600E mutation nor RAS mutations were confirmed in plasma DNA (points 1 and 2) while the tumors responded to cetuximab. In contrast, BRAF V600E mutation was detected in the plasma DNA collected after cetuximab refractory (point 3, Fig. 2I).

Discussion

PD is generally determined by radiological evaluation. Liquid biopsy has been suggested for the early identification of individuals at risk of developing drug resistance before radiographic documentation of PD as well as individuals who already possess certain mutations in tumor burden[1, 13–15]. Here, PCR-rSSO analysis was conducted on serial plasma samples and all metastatic lesions in multiple organs from patients who were treated with chemotherapies, including panitumumab or cetuximab.

In patient 1, none of the 7 acquired mutations found in the resistant tumors were detected either before or after PD during the initial panitumumab treatment course (point 1 and 2), but three of the 7 acquired mutations were detectable after PD was confirmed during third-line panitumumab rechallenge (point 3). Indeed, the two of three acquired RAS mutations confirmed in the PCR-rSSO were under the cut-off values estimated by tumor tissue analysis.
In support of the results obtained from patient 1, among liquid biopsies of patient 4, only the blood of point 3, which was collected after confirmation of radiological PD, demonstrated \textit{BRAF} V600E mutation that the primary tumor and peritoneal dissemination possessed. In contrast, as all blood specimens of the patient 2 and 3 were collected at the time that chemotherapies were effectively causing tumor regression, these specimens did not show any activating mutations which cause acquired resistance.

In a previous study, an acquired mutation was detected in plasma as early as 10 months before radiological PD\cite{1}; we also attempted to confirm whether acquired \textit{RAS} variants in plasma could detect before radiological PD. Unfortunately, by the conventional cut-off value of the PCR-rSSO, we could not detect acquired mutations before radiological PD. Our inability to detect ctDNA in the plasma samples might be explained partially by the differences in analytical technologies. The PCR-rSSO method used in the present study could detect MAFs of 1–5%, whereas the previous study with OncoBEAMing technology considered 0.1% MAFs as mutation positive\cite{1,16}.

Although the OncoBEAM-based liquid biopsy has such a high sensitivity for \textit{RAS} mutant alleles and can identify which exon is mutated, this could not provide a precise \textit{RAS} mutational spectrum, e.g., G12C or G12A. In contrast, although the PCR-rSSO strategy had less sensitivity for MAF and the index values are semi-quantitative, the advantage of the PCR-rSSO is to be able to confirm all \textit{RAS} mutation spectrum at a time.

Even if we use the highest sensitivity method to detect mutant alleles of resistance in liquid biopsy, we missed the mutant alleles from blood from 10–18% of patients who had \textit{RAS} mutations in tumor tissue \cite{14,15}, owing to tumor heterogeneity, lower ctDNA shedding or lower tumor burden. In particular, the location of metastatic tumors may be important for the detection of mutant alleles. Discrepancies of mutations between tumor tissue and ctDNA in patients with lung-only metastases were observed, but a higher agreement in liver metastases was found\cite{15,28,29}. In line with this, our results of the patient 1 also showed that the degree of \textit{RAS} mutational concordance varied according to the metastatic site, e.g., \textit{KRAS} G12R detected in ctDNA was confirmed in resistant tumors in S3, looking like more aggressively progressed by radiographic findings.

At least two possibilities account for the development of acquired mutations \cite{3,9}. First, resistant cells harboring these acquired mutations may be present at incredibly low numbers upon treatment initiation. Second, in response to continued molecularly-targeted therapy, cells may have acquired a \textit{de novo} activating mutation. In the first model, metastatic CRC response to EGFR-targeted therapies is accompanied by a selection of preexisting resistant clones metastasized to the initial metastatic lesion; therefore, if the acquired \textit{RAS} mutations observed in our case were present at treatment initiation but at a frequency sufficiently low to be undetectable by the two different procedures, at least some resistant metastatic lesions that grew after treatment should harbor not one but multiple \textit{RAS} mutations. However, almost all resistant metastatic lesions harbored one acquired \textit{RAS} mutation, as demonstrated by conventional Sanger sequencing and the sensitive PCR-SSO procedure, except for RL in patient 1, which displayed multiple \textit{KRAS} mutants by PCR-rSSO with a lower population and was not detected by Sanger
sequencing, suggesting the existence of many types of cancer cells. The lower population of multiple \textit{KRAS} mutants could explain why the RL visibly continued shrinking with calcification during the sequential chemotherapy treatment, as visualized by computed tomography.

Since only a small part of the tumor lesions could be examined, we cannot exclude sampling bias regarding the existence of mutant alleles precluding assessment of genetic heterogeneity within or among lesions. However, concerning \textit{RAS} mutations in CRC, \textit{RAS} mutations are believed to occur in the early phase of tumorigenesis, such as in the development of a small adenoma into a larger adenoma\cite{30}. Thus, an \textit{RAS} mutation spreads homogeneously within the tumor mass, resulting in more than 95% concordance in \textit{RAS} mutation status across different sites of a tumor mass\cite{31}. Therefore, \textit{RAS} mutation-based drug resistance may be attributable to new mutations arising rather than the selection and clonal amplification of an exceedingly small number of preexisting \textit{RAS} mutant cells. This scenario was also supported by previously reported in vitro studies\cite{1,32}. Shaffer SM et al. presented that in a \textit{BRAF}-mutant melanoma cell line, the population of resistant cells may arise upon the selection of multiple clones that were already present before \textit{BRAF} inhibitor treatment\cite{33}. Of note, these resistant cells arise from profound transcriptional variability at the single-cell level, which involves infrequent, semi-coordinated transcription of several resistance markers at high levels in a small percentage of cells. The addition of the drug then induced epigenetic reprogramming in these cells, converting the transient transcriptional state to a stably resistant state.

In this study, although we analyzed only four cases and were unable to identify acquired mutations via liquid biopsy before radiographic documentation of PD, we detected acquired drug resistance-inducing mutations in liquid biopsy collected after radiographical PD by the PCR-rSSO. Interestingly, the patient 1 and 3 experienced radiographic PD during bevacizumab contained chemotherapy. The blood obtained after confirmation of bevacizumab resistance did not show any \textit{RAS} acquired mutations. This is very reasonable and thus strongly emphasizing the clinical utility of liquid biopsy to the detection of \textit{RAS} mutations for decisions regarding anti-EGFR antibody administration to individuals at risk of drug resistance.

\textbf{Conclusions}

Our results indicate that anti-EGFR treatment induces the development of resistant cancer cells with acquired resistance heterogenetic mutations. At the time that a certainly acquired mutation was detected in plasma by the PCR-rSSO method, it might strongly suggest that tumor cells with such acquired mutations exist in progressive metastatic lesions.

\textbf{Declarations}

\textit{Ethics approval and consent to participate}
Each patient was enrolled as a research subject in the clinical trials (UMIN [University Hospital Medical Information Network Center] ID, 8377, 9698, or 11954), which were approved by the ethics committee of Okayama University and Kawasaki Medical School the Institutional Review Board [IRB] number, 3172, 3193, 3194, 3196-1, and 3239). The patients provided written informed consent and all studies were conducted in accordance with the Declaration of Helsinki.

**Availability of data and materials**

The datasets used during the current study are available from the corresponding author on reasonable request. All data generated by the PCR-rSSO during this study are included in this published article and its supplementary information files.

**Competing interests**

The authors declare that they have no competing interests.

**Funding:** This work was supported by KAKENHI (20590572, 25860409, 26462016, and 15H03034).

**Authors' contributions**

FT performed genetic analyses and drafted the manuscript. AN, TT, KY, and MO extracted DNA and analyzed the genetic mutations. YM, HK, YU, TF, and HT treated patients, provided the patient samples, and summarized all clinicopathological data. YY, and AG assisted with the interpretation of all data and drafted the manuscript. TN designed the project, performed analyses, assisted with the interpretation of all data, secured the funding and drafted the manuscript. All authors read and approved the final manuscript.

**Acknowledgments**

The authors would like to thank Dr. Futoshi Uno, Dr. Ayako Watanabe, Mr. Toru Nakai, Mrs. Tae Yamanishi, and Mrs. Kikue Tokuda for their technical assistance.

**Abbreviations**

CRC: colorectal cancer

ctDNA: circulating tumor DNA

EGFR: epidermal growth factor receptor

FFPE: formalin-fixed paraffin-embedded

FOLFIRI: fluorourasil, leucovorin and irinotecan

FOLFOX: fluorourasil, leucovorin, and oxaliplatin
IRB: Institutional Review Board

MAF: mutant allele frequency

mCRC: metastatic colorectal cancer

PCR: polymerase chain reaction

PCR-rSSO: polymerase chain reaction-reverse sequence-specific oligonucleotide

PD: progressive disease

UMIN: University Hospital Medical Information Network Center

References

1. Misale S, Yaeger R, Hobor S, Scala E, Janakiraman M, Liska D, Valtorta E, Schiavo R, Buscarino M, Siravegna G, et al. Emergence of KRAS mutations and acquired resistance to anti-EGFR therapy in colorectal cancer. Nature. 2012;486(7404):532–6.

2. Kobayashi S, Boggon TJ, Dayaram T, Janne PA, Kocher O, Meyerson M, Johnson BE, Eck MJ, Tenen DG, Halmos B. EGFR mutation and resistance of non-small-cell lung cancer to gefitinib. N Engl J Med. 2005;352(8):786–92.

3. Misale S, Di Nicolantonio F, Sartore-Bianchi A, Siena S, Bardelli A. Resistance to anti-EGFR therapy in colorectal cancer: from heterogeneity to convergent evolution. Cancer discovery. 2014;4(11):1269–80.

4. Douillard JY, Oliner KS, Siena S, Tabemero 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. 2013;369(11):1023–34.

5. Heinemann V, von Weikersthal LF, Decker T, Kiani A, Vehling-Kaiser U, Al-Batran SE, Heintges T, Lerchenmuller C, Kahl C, Seipelt G, et al. FOLFIRI plus cetuximab versus FOLFIRI plus bevacizumab as first-line treatment for patients with metastatic colorectal cancer (FIRE-3): a randomised, open-label, phase 3 trial. The Lancet Oncology. 2014;15(10):1065–75.

6. Van Cutsem E, Lenz HJ, Kohne CH, Heinemann V, Tejpar S, Melezinek I, Beier F, Stroh C, Rougier P, van Krieken JH, et al. Fluorouracil, leucovorin, and irinotecan plus cetuximab treatment and RAS mutations in colorectal cancer. Journal of clinical oncology: official journal of the American Society of Clinical Oncology. 2015;33(7):692–700.

7. Yonesaka K, Zejnullahu K, Okamoto I, Satoh T, Cappuzzo F, Souglakos J, Ercan D, Rogers A, Roncalli M, Takeda M, et al. Activation of ERBB2 signaling causes resistance to the EGFR-directed therapeutic antibody cetuximab. Science translational medicine. 2011;3(99):99ra86.

8. Montagut C, Dalmases A, Bellosillo B, Crespo M, Pairet S, Iglesias M, Salido M, Gallen M, Marsters S, Tsai SP, et al. Identification of a mutation in the extracellular domain of the Epidermal Growth Factor
Receptor conferring cetuximab resistance in colorectal cancer. Nature medicine. 2012;18(2):221–3.

9. Diaz LA Jr, Williams RT, Wu J, Kinde I, Hecht JR, Berlin J, Allen B, Bozic I, Reiter JG, Nowak MA, et al. The molecular evolution of acquired resistance to targeted EGFR blockade in colorectal cancers. Nature. 2012;486(7404):537–40.

10. Bardelli A, Corso S, Bertotti A, Hobor S, Valtorta E, Siravegna G, Sartore-Bianchi A, Scala E, Cassingena A, Zecchin D, et al. Amplification of the MET receptor drives resistance to anti-EGFR therapies in colorectal cancer. Cancer discovery. 2013;3(6):658–73.

11. Bertotti A, Papp E, Jones S, Adleff V, Anagnostou V, Lupo B, Sausen M, Phallen J, Hruban CA, Tokheim C, et al: The genomic landscape of response to EGFR blockade in colorectal cancer. Nature 2015.

12. Siravegna G, Mussolin B, Buscarino M, Corti G, Cassingena A, Crisafulli G, Ponzetti A, Cremolini C, Amatu A, Lauricella C, et al. Clonal evolution and resistance to EGFR blockade in the blood of colorectal cancer patients. Nature medicine. 2015;21(7):795–801.

13. Bettegowda C, Sausen M, Leary RJ, Kinde I, Wang Y, Agrawal N, Bartlett BR, Wang H, Luber B, Alani RM, et al. Detection of circulating tumor DNA in early- and late-stage human malignancies. Science translational medicine. 2014;6(224):224ra224.

14. Garcia-Foncillas J, Tabernero J, Elez E, Aranda E, Benavides M, Camps C, Jantus-Lewintre E, Lopez R, Muinelo-Romay L, Montagut C, et al. Prospective multicenter real-world RAS mutation comparison between OncoBEAM-based liquid biopsy and tissue analysis in metastatic colorectal cancer. Br J Cancer. 2018;119(12):1464–70.

15. Bando H, Kagawa Y, Kato T, Akagi K, Denda T, Nishina T, Komatsu Y, Oki E, Kudo T, Kumamoto H, et al. A multicentre, prospective study of plasma circulating tumour DNA test for detecting RAS mutation in patients with metastatic colorectal cancer. Br J Cancer. 2019;120(10):982–6.

16. Yoshino T, Muro K, Yamaguchi K, Nishina T, Denda T, Kudo T, Okamoto W, Taniguchi H, Akagi K, Kajiwara T, et al: Clinical Validation of a Multiplex Kit for RAS Mutations in Colorectal Cancer: Results of the RASKET (RAS KEy Testing) Prospective, Multicenter Study. EBioMedicine 2015, 2(4):317–323.

17. Nadal E, Chen G, Prensner JR, Shiratsuchi H, Sam C, Zhao L, Kalemkerian GP, Brenner D, Lin J, Reddy RM, et al. KRAS-G12C mutation is associated with poor outcome in surgically resected lung adenocarcinoma. J Thorac Oncol. 2014;9(10):1513–22.

18. Massarelli E, Varella-Garcia M, Tang X, Xavier AC, Ozburn NC, Liu DD, Bekele BN, Herbst RS. Wistuba, Il: KRAS mutation is an important predictor of resistance to therapy with epidermal growth factor receptor tyrosine kinase inhibitors in non-small-cell lung cancer. Clinical cancer research: an official journal of the American Association for Cancer Research. 2007;13(10):2890–6.

19. Fiala O, Buchler T, Mohelnikova-Duchonova B, Melichar B, Matejka VM, Holubec L, Kulhankova J, Bortlicek Z, Bartouskova M, Liska V, et al. G12V and G12A KRAS mutations are associated with poor outcome in patients with metastatic colorectal cancer treated with bevacizumab. Tumour Biol. 2016;37(5):6823–30.
20. Lievre A, Bachet JB, Le Corre D, Boige V, Landi B, Emile JF, Cote JF, Tomasic G, Penna C, Dureux M, et al. KRAS mutation status is predictive of response to cetuximab therapy in colorectal cancer. Cancer Res. 2006;66(8):3992–5.

21. Cox AD, Fesik SW, Kimmelman AC, Luo J, Der CJ. Drugging the undruggable RAS: Mission possible? Nat Rev Drug Discov. 2014;13(11):828–51.

22. Ostrem JM, Shokat KM. Direct small-molecule inhibitors of KRAS: from structural insights to mechanism-based design. Nat Rev Drug Discov. 2016;15(11):771–85.

23. Suzawa K, Offin M, Lu D, Kurzatkowski C, Vojnic M, Smith RS, Sabari JK, Tai H, Mattar M, Khodos I, et al. Activation of KRAS Mediates Resistance to Targeted Therapy in MET Exon 14-mutant Non-small Cell Lung Cancer. Clinical cancer research: an official journal of the American Association for Cancer Research. 2019;25(4):1248–60.

24. Hong DS, Fakih MG, Strickler JH, Desai J, Durm GA, Shapiro GI, Falchook GS, Price TJ, Sacher A, Denlinger CS, et al. KRAS(G12C) Inhibition with Sotorasib in Advanced Solid Tumors. N Engl J Med. 2020;383(13):1207–17.

25. Ostrem JM, Peters U, Sos ML, Wells JA, Shokat KM. K-Ras(G12C) inhibitors allosterically control GTP affinity and effector interactions. Nature. 2013;503(7477):548–51.

26. Lito P, Solomon M, Li LS, Hansen R, Rosen N. Allele-specific inhibitors inactivate mutant KRAS G12C by a trapping mechanism. Science. 2016;351(6273):604–8.

27. Patricelli MP, Janes MR, Li LS, Hansen R, Peters U, Kessler LV, Chen Y, Kucharski JM, Feng J, Ely T, et al. Selective Inhibition of Oncogenic KRAS Output with Small Molecules Targeting the Inactive State. Cancer discovery. 2016;6(3):316–29.

28. Kim MJ, Lee HS, Kim JH, Kim YJ, Kwon JH, Lee JO, Bang SM, Park KU, Kim DW, Kang SB, et al. Different metastatic pattern according to the KRAS mutational status and site-specific discordance of KRAS status in patients with colorectal cancer. BMC Cancer. 2012;12:347.

29. Thierry AR, El Messaoudi S, Mollevi C, Raoul JL, Guimbaud R, Pezet D, Artru P, Assenat E, Borg C, Mathonnet M, et al. Clinical utility of circulating DNA analysis for rapid detection of actionable mutations to select metastatic colorectal patients for anti-EGFR treatment. Ann Oncol. 2017;28(9):2149–59.

30. Jones S, Chen WD, Parmigiani G, Diehl F, Beeremwinkel N, Antal T, Traulsen A, Nowak MA, Siegel C, Velculescu VE, et al: **Comparative lesion sequencing provides insights into tumor evolution.** *Proceedings of the National Academy of Sciences of the United States of America* 2008, 105(11):4283–4288.

31. Krol LC, t Hart NA, Methorst N, Knol AJ, Prinsen C, Boers JE. Concordance in KRAS and BRAF mutations in endoscopic biopsy samples and resection specimens of colorectal adenocarcinoma. European journal of cancer. 2012;48(7):1108–15.

32. Misale S, Arena S, Lamba S, Siravegna G, Laloo A, Hobor S, Russo M, Buscarino M, Lazzari L, Sartore-Bianchi A, et al. Blockade of EGFR and MEK intercepts heterogeneous mechanisms of acquired
resistance to anti-EGFR therapies in colorectal cancer. Science translational medicine. 2014;6(224):224ra226.

33. Shaffer SM, Dunagin MC, Torborg SR, Torre EA, Emert B, Krepler C, Beqiri M, Sproesser K, Brafford PA, Xiao M, et al. Rare cell variability and drug-induced reprogramming as a mode of cancer drug resistance. Nature. 2017;546(7658):431–5.

Figures

Figure 1

Summary of a rectosigmoid cancer patient (patient 1) with unresectable multiple liver metastases A) Timeline of the treatment course of patient 1 The line graph abscissa indicates time, and the ordinate indicates carcinoembryonic CEA levels, scaled logarithmically. The computed tomography scan of the liver metastases (above) and chemotherapy regimen (below) are described for each time point. Blood was collected before the administration of chemotherapy at point 1, 2, and 3 after the initiation of first-line chemotherapy. B) Status of RAS and BRAF mutational metastases by Sanger sequencing The status of KRAS, NRAS, and BRAF. Sanger sequencing results are described for all samples, including primary lesions, with focal distant metastasis. Numbers in red and gray boxes indicate the codon numbers. Codons in red boxes indicate hot spots. Red arrows indicate observed missense mutations. C) Status of
RAS mutational metastases by PCR-rSSO Panels show index values of 12 types of RAS exon 2 (G12S, G12C, G12R, G12D, G12V, G12A, G13S, G13C, G13R, G13D, G13V, and G13A), eight types of RAS exon 3 (A59T, A59G, Q61K, Q61E, Q61L, Q61P, Q61R, and Q61H) and four types of RAS exon 4 (K117N, A146T, A146P, and A146V) mutations by PCR-rSSO indices of metastatic specimens in the patient. D) Detection of circulating RAS mutant DNA The detection of circulating RAS mutant alleles in plasma by PCR-rSSO. The KRAS G12R mutant allele index value increased to 2,600 in plasma obtained at point 3. The index values of the KRAS G13D and Q61Hc mutant alleles also increased to 159 and 471, respectively, in plasma obtained at point 3.

Figure 2

Summary of three mCRC patients A-C) Timeline of the treatment course of patient 2, 3, and 4 The line graph abscissa indicates time, and the ordinate indicates carcinoembryonic CEA levels, scaled logarithmically. The magnetic resonance imaging (patient 2, A), computed tomography (patient 3, B), and positron emission tomography (patient 4, C) scan of the liver metastases or peritoneal dissemination are described for each time point. Blood was collected at point 1, 2, and 3. D-F) RAS and BRAF mutational status in primary tumor and metastases by PCR-rSSO Panels show index values of 12 types of RAS exon 2 (G12S, G12C, G12R, G12D, G12V, G12A, G13S, G13C, G13R, G13D, G13V, and G13A), eight types of RAS exon 3 (A59T, A59G, Q61K, Q61E, Q61L, Q61P, Q61R, and Q61H) and four types of RAS exon 4 (K117N, A146T, A146P, and A146V) mutations by PCR-rSSO indices of the primary tumor and metastatic specimens in the patients. G-I) Detection of Circulating RAS and BRAF mutant DNA The detection of circulating RAS and BRAF mutant alleles in plasma by PCR-rSSO. Patient 2 (G) and 3 (H) showed no mutation in liquid biopsy. In patient 4 (I), the BRAF V600E mutant allele index value increased to 1,242 in plasma obtained at point 3.
Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- TaniguchiFetal.SupplementaryTable1.xlsx
- TaniguchiFetal.SupplementaryTable2.xlsx
- TaniguchiFetal.SupplementaryTable3.xlsx