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

Fumitaka Taniguchi

Okayama University - Tsushima Campus: Okayama Daigaku

Akihiro Nyuya

Kawasaki Medical School: Kawasaki Ika Daigaku

Toshiaki Toshima

Okayama University: Okayama Daigaku

Yoshiko Mori

Saitama Ika Daigaku Sogo Iryo Center

Hiroyuki Kishimoto

Okayama University: Okayama Daigaku

Yuzo Umeda

Okayama University: Okayama Daigaku

Toshiyoshi Fujiwara

Okayama University: Okayama Daigaku

Takeshi Nagasaka (✉ takeshin@cc.okayama-u.ac.jp)

Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences

<https://orcid.org/0000-0002-4236-350X>

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Abstract

Background: Acquired mutations are detected in circulating cell-free DNA or circulating tumor cells in blood. However, there are still few reports that examine the concordance between liquid biopsy and metastatic lesions with acquired resistance.

Methods: 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 displayed initial early tumor shrinkage and finally progressed to disease (PD). The 7 metastatic lesions showing acquired resistance to chemotherapy were extracted at morbid autopsy. Blood samples were collected before the development of PD during first-line chemotherapy and after acquiring resistance to second- and third-line chemotherapies. Acquired mutations were analyzed using two methodologies: conventional Sanger sequencing and MEBGENTM RASKET KIT based on Luminex® technology.

Results: Metastatic tumor specimens harbored diverse acquired mutations in the KRAS gene in all of the 7 (100%) metastases in the patient resistant to EGFR-targeted treatment. Of the 7 diverse acquired mutations, only one acquired mutation observed in a liver metastasis was clearly detected in the blood collected at third-line chemotherapies. Two other acquired mutations were also detected but did not reach meaningful values.

Conclusions: Liquid biopsy is a successful procedure for capturing acquired mutations that may arise from progressive metastatic lesions with acquired resistance over time.

Introduction

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 plasma[14, 15]. 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 had *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, heterogeneity of *RAS* mutations must be considered. However, there are few reports evaluating the concordance of *RAS* mutational status in all metastatic lesions located in multiple organs and ctDNA.

In this study, we used PCR-rSSO, which can detect an MAF of 1–5% of the *RAS* mutant allele, and is in concordance with the detection of *RAS* mutations with reports of 93.9% using a 5% cut-off of tissue. OncoBEAMing was used and examined the concordance of *RAS* mutations observed between multiple metastatic lesions in multiple organs and ctDNA in plasma throughout anti-EGFR therapy in an mCRC patient[15].

Methods

Tumor and Blood Samples

mCRC patients with primary tumors without activating *RAS* mutations were enrolled in the study. The patients were treated between 2011 and 2015 at the Okayama University Hospital, Japan. This study was approved by the Institutional Review Board (IRB) at Okayama University (IRB number, genome 270 and 271) and Kawasaki Medical School (IRB number, 3196-1 and 3239). The patients provided written informed consent.

Primary tumors were obtained by biopsy and surgical resection before acquiring resistance during anti-EGFR treatment. Metastatic lesions were excised at morbid autopsy and included those of the liver (MS, S2, and S3), lung (Lu), and kidney (Kd). Tissues obtained from autopsy or surgical resection were immediately stored at – 80 °C.

Blood samples were collected before developing PD during first-line chemotherapy (*20) and after acquiring resistance to second- and third-line chemotherapies (*105 and *123, respectively; Fig. 1). 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 the discovery cohort. Primary tumors from 1,070 CRC patients were

analyzed to identify mutations in *KRAS* exon 2 and *BRAF* exon 15. Primer sequences for *KRAS*, *NRAS* exons 2 to 4, and *BRAF* exon 15 and PCR conditions are described in **Supplementary File 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)

Extended *RAS* mutations (both *KRAS* and *NRAS* mutations) of DNA purified from FFPE, fresh frozen tissues, and plasma were evaluated using the MEBGEN™ RASKET KIT based on Luminex® technology (MBL, Nagoya, Japan). The assay was performed according to the manufacturer's protocol. We simultaneously examined 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].

Results

Clinical Outcome

One patient had multiple unresectable liver-limited metastases at their first medical examination and was treated with first-line systemic chemotherapy with anti-EGFR therapy (Fig. 1). The primary tumor had no mutations in *KRAS*, *NRAS* or *BRAF* exon 15 (Fig. 2). Overall survival after first-line treatment initiation was 34 months. The patient was administered bevacizumab with cytotoxic agents as the second-line regimen and was rechallenged with anti-EGFR antibody as the third-line chemotherapy. Despite initial tumor shrinkage in the metastases, the patient ultimately developed PD.

Identification of Acquired *RAS* Mutations by Conventional Sanger Sequencing

In the patient, no activating *RAS* mutation was observed in the primary tumor biopsy specimen obtained prior to treatment, in 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. 2). In addition, *KRAS* sequences in metastatic specimens obtained during autopsy revealed diverse acquired mutations at different metastatic sites, indicating resistance to the systemic chemotherapy used, 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), in a hepatic lymph node (HN), and in the left lung (Lu), respectively. No samples from primary and metastatic lesions harbored mutations in *NRAS* or *BRAF*.

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. 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. 3 and **Supplementary File 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 *KRAS*G12C allele captured from the Kd by PCR-rSSO analysis was 114, *i.e.*, lower than the conventional cut-off value (300), which might be influenced by the immediate synonymous mutation of A11Ag clearly demonstrated by Sanger sequencing, as previously reported (Fig. 2) [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. The 7 diverse acquired mutations found in the resistant tumors were not detected before or after PD during the initial course of panitumumab treatment (*20 and *105 in Fig. 4 and **Supplementary File 3**), but only one of the 7 diverse acquired mutations was detected at a significant level following the confirmation of PD during third-line panitumumab rechallenge (*123). The *KRAS*G12R frequency, which was confirmed in the resistant tumor in S3, was strikingly elevated in circulating cell-free DNA in a plasma sample collected 123 weeks after treatment initiation. Additionally, *KRAS*G13D in the HN and *KRAS* Q61Hc in the Lu and S2 were also detected in the plasma sample *123, but the indices were not reached at the cut-off value. All the acquired mutations detected in the plasma sample *123 were not detected in plasma at 20 weeks (*20, approximately as early as 10 months before PD during first-line therapy) or at 105 weeks (*105, when PD was confirmed radiologically during first-line treatment).

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 prior to radiographic documentation of PD as well as individuals who already have possessed 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 a patient who was treated with first- and third-line chemotherapy, including panitumumab, until PD.

In a previous study, an acquired mutation was detected in plasma as early as 10 months before radiological PD[1]; therefore, we attempted to confirm the detection of acquired *RAS* variants in plasma that were identified in metastatic tumor lesions. However, none of the 7 acquired mutations found in the resistant tumors were detected either before or after PD during the initial panitumumab treatment course (*20 and *105), but three of the 7 acquired mutations were detectable after PD was confirmed during third-line panitumumab rechallenge (*123). Indeed, the two of three acquired *RAS* mutations confirmed in the PCR-rSSO were under the cut-off values estimated by tumor tissue analysis. Our inability to detect ctDNA in the plasma samples might be explained partially by a difference 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[1, 16]. However, OncoBEAMing missed the detection of *RAS* mutations in plasma, and approximately 10–18% of patients had *RAS* mutations in tumor tissue[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[15, 17, 18]. In line with this, our results also showed that the degree of *RAS* mutational concordance varied according to the metastatic site; e.g., *KRAS* G12R clearly 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 [3, 9]. First, resistant cells harboring these acquired mutations may be present at very low numbers upon treatment initiation (Fig. 5a). Second, in response to continued molecular-targeted therapy-based chemotherapy, cells may have acquired a *de novo* activating mutation (Fig. 5b). In the first model, metastatic CRC response to EGFR-targeted therapies is accompanied by a selection of preexisting resistant clones metastasizing to the initial metastatic lesion; therefore, if the acquired *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 *RAS* mutations. However, almost all resistant metastatic lesions harbored one acquired *RAS* mutation, as demonstrated by conventional Sanger sequencing and the highly sensitive PCR-SSO procedure, except for RL, which displayed multiple *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 *KRAS* mutants could explain why the RL visibly continued shrinking with calcification during the sequential chemotherapy treatment, as visualized by computed tomography.zA

Since only a small part of the tumor lesions could be examined, we cannot exclude sampling bias with regard to the existence of mutant alleles precluding assessment of genetic heterogeneity within or among lesions. However, with respect to *RAS* mutations in CRC, *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[19]. Thus, an *RAS* mutation spreads homogenously within the tumor mass, resulting in more than 95% concordance in *RAS* mutation status across different sites of a tumor mass[20]. Therefore, *RAS* mutation-based drug resistance may be attributable to new mutations arising rather than the selection and clonal amplification of a very small number of preexisting *RAS* mutant cells. This scenario was also supported by previously reported in vitro studies[1, 21]. Shaffer SM et al. presented that in a *BRAF*-mutant melanoma cell line, the population of resistant cells may arise upon the selection of multiple clones that were already present before *BRAF* inhibitor treatment[22]. Of note, these resistant cells arise from profound transcriptional variability at the single-cell level, which involves infrequent, semicoordinated transcription of a number of resistance markers at high levels in a very 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 one case and were unable to identify acquired mutations via liquid biopsy before radiographic documentation of PD, we clearly detected acquired drug resistance-inducing mutations in liquid biopsy collected during a later phase of the treatment course, thus strongly emphasizing the clinical utility of liquid biopsy for decisions regarding anti-EGFR antibody administration to individuals at risk of drug resistance.

Conclusions

Our results indicate that anti-EGFR treatment induces the development of resistant cancer cells with acquired resistance heterogenous mutations. At the time that a certain acquired mutation was detected by “liquid biopsy”, it might strongly suggest that tumor cells with such acquired mutations definitely exist in progressive metastatic lesions.

Declarations

Authors' contributions

FT performed genetic analyses and drafted the manuscript. AN, TT, and KY extracted DNA and analyzed the genetic mutations. YM, HK, YU, and TF treated patients, provided the patient samples, and summarized all clinicopathological data. 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.

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Figures

Figure 1

Timeline of a rectosigmoid cancer patient with unresectable multiple liver metastases. 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 20 (*20), 105 (*105), and 123 (*123) weeks after the initiation of first-line chemotherapy. White arrows denote metastatic lesions in the hepatic lymph node, the left lung, and the left kidney.

Figure 2

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.

Figure 3

Status of RAS Mutational Metastases by PCR-rSSO. Panels show the radar charts, indicating PCR-rSSO indices of metastatic specimens in the patient. The right-sided light blue semicircle shows KRAS mutations, and the left-sided light orange semicircle shows NRAS mutations.

Figure 4

Detection of Circulating KRAS Mutant DNA The detection of circulating KRAS mutant alleles in plasma by PCR-rSSO. The KRAS G12R mutant allele index value increased to 2,600 in plasma obtained at 123 weeks (*123) after the initiation of the treatment. The index values of the KRAS G13D and Q61Hc mutant alleles also increased to 159 and 471, respectively, in plasma obtained at 123 weeks (*123).

Figure 5

Possible mechanisms of acquired resistance to anti-EGFR therapies in metastatic CRC. (a) A possible mechanism of acquired resistance caused by the already existing heterogeneity and resistant cancer cells carrying distinct molecular alterations, such as KRAS (red), coexist in the initial metastasis and/or primary tumor sites. (b) Another possible mechanism of acquired resistance caused by a cell, which acquires a de novo activating acquired mutation in response to continued EGFR-targeted therapy-based

chemotherapy. Cancer cells that acquire a de novo activating acquired RAS mutation appear after EGFR-targeted therapy-based chemotherapy (red).

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