Trial Protocol

A Trial Protocol of Precision Medicine for Patients with RAS Wild Metastatic Colorectal Cancer Using Liquid Biopsy (RAS-liquid Study): A Prospective, Multicenter Observational Study

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Abstract

Background: Anti-epidermal growth factor receptor (EGFR) therapy has been identified to prolong the survival of metastatic colorectal cancer (mCRC) patients without *RAS* mutations. However, its efficacy is not always consistent for these patients. Genomic profiles of primary tumors and metastases are not always concordant; thus, chemotherapeutic agents can alter the tumor molecular profile. This molecular heterogeneity may explain resistance to anti-EGFR therapy. Liquid biopsy using circulating tumor DNA (ctDNA) is a novel, non-invasive diagnostic tool that can accommodate this molecular heterogeneity, providing a comprehensive, real-time view of the molecular landscape. In this study, we evaluated the predictive value of genomic mutations in ctDNA for primary and acquired resistance to anti-EGFR therapy.

Methods/Design: This study is a prospective, multicenter, observational study of mCRC patients with wild-type tissue *RAS* treated with cytotoxic agents and anti-EGFR antibodies as first-line therapy. Genomic mutations, including *RAS*, *BRAF*, *PIK3CA*, and *EGFR* in ctDNA, are assessed via Droplet Digital PCR before starting chemotherapy and every 3 months thereafter until disease progression. The target sample size is estimated to be 100. The primary endpoint is the response rate in patients without *RAS* mutation in their blood sample before starting chemotherapy.

Discussion: This study will clarify the predictive value of baseline *RAS* mutation in ctDNA for responses to anti-EGFR therapy; the frequency of emerging *RAS*, *BRAF*, *PIK3CA*, and *EGFR* mutations in ctDNA; and the association with secondary resistance to anti-EGFR therapy in first-line therapy for wild-type tissue *RAS* mCRC patients.

Keywords

liquid biopsy, metastatic colorectal cancer, cell-free DNA, circulating tumor DNA, anti-EGFR antibody

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Background

Colorectal cancer (CRC), which is responsible for approximately 880,000 deaths worldwide in 2018, has been determined as the leading cause of cancer death [1]. Anti-epidermal growth factor receptor (EGFR) therapy has been found to prolong survival of metastatic CRC (mCRC) patients without *RAS* mutation[2,3]. However, its efficacy is not always consistent for these patients, and spatial heterogeneity (disparate *RAS* mutational profiles between primary and metastatic tumors) is thought to be the reason. Approximately, 10% of metastatic tumors have *RAS* mutations in patients with *RAS* wild-type primary tumors [4]. Additionally, continuous administration of chemotherapeutic and/or targeted agents can alter tumor genetic profiles (temporal heterogeneity), which could also promote resistance [5]. Therefore, reliable detection of *RAS* mutational status is essential just before initialization of anti-EGFR therapy. However, routine, serial tissue biopsies have limited capacity to comprehensively obtain real-time information.

Liquid biopsy, which analyzes circulating nucleic acids, cell-free DNA (cfDNA), and circulating tumor DNA (ctDNA) in body fluids, permits comprehensive, real-time profiling of the tumor genetic landscape [5]. Our observational studies have found that approximately 10% of mCRC patients with *RAS* wild-type primary tumors had *RAS* muta-

tions in cfDNA. In addition to *RAS* mutations, we have reported spatial heterogeneity other than *RAS* mutations, using liquid biopsy [6-8].

Recently, spatial heterogeneity of *RAS* mutations in cfDNA has been reported as a negative predictor of anti-EGFR therapy [9-11]. Also, emerging mutations in *RAS*, *BRAF*, *PIK3CA*, and *EGFR* (temporal heterogeneity) have been identified by liquid biopsy in patients who have acquired resistance to anti-EGFR therapy [12-14]. However, these studies were limited to patients who had undergone second- or third-line chemotherapy. Theoretically, the detected heterogeneity in these clinical settings cannot be unequivocally classified as spatial or temporal heterogeneity, owing to the influence of previous anticancer drugs administered. Our previous pilot study of first-line therapy with a small sample size (n = 30) suggested a consequence that spatial and temporal heterogeneity can cause primary and secondary resistance independently [15]. This multicenter, prospective, observational, and relatively large study, comprising 100 mCRC patients, evaluates the efficacy of serial assessment of *RAS*, *BRAF*, *PIK3CA*, and *EGFR* mutations in cfDNA to predict responses and to explore definitive mechanisms of primary and secondary resistance to anti-EGFR therapy in first-line therapy for *RAS* wild-type primary tumors.

Methods/Design

This prospective, multicenter, observational study is organized by the Department of Gastrointestinal and Hepato-Biliary-Pancreatic Surgery, Nippon Medical School, Tokyo, Japan. Patients are being recruited at 15 centers in Japan. Written informed consent will be obtained from each patient prior to any procedures.

Study schedule

This project was registered with the University Hospital Medical Information Network (UMIN 000031177) on July 1, 2018. Its estimated completion date is December 31, 2023 for patient recruitment, follow-up, and data analysis.

Patient selection criteria

Inclusion criteria

1. Histologically proven colorectal adenocarcinoma
2. *RAS* wild-type in the primary tumor
3. With distant metastatic tumors, one or more of which are unresectable
4. Chemotherapy naïve for distant metastasis
5. Age between 20 and 80 years
6. Eastern Cooperative Oncology Group Performance Status of 0 or 1
7. With measurable lesions, based on the Response Evaluation Criteria in Solid Tumors, version 1.1
8. Able to take food by mouth
9. With a life expectancy of at least 3 months
10. Exhibiting sufficient organ function for up to weeks prior to enrollment in the study with the following parameters considered:
    - Leukocyte count ≥3,500/mm^3
    - Absolute neutrophil count ≥1,500/mm^3
    - Platelet count ≥75,000/mm^3
    - Hemoglobin level ≥8.0 g/dL
    - Aspartate aminotransferase level ≤2.5 × upper limit of normal
    - Alanine aminotransferase level ≤2.5 × upper limit of normal
    - Total bilirubin level ≤1.5 mg/dL
    - Serum creatinine level ≤1.5 mg/dL
    - No active infectious disease
    - No recognizable diarrhea or non-hematological adverse events (except for alopecia, dysgeusia, or pigmentation)
11. Providing signed, written informed consent prior to enrollment in the study

Exclusion criteria

1. Contraindications for anti-EGFR antibody or history of treatment with anti-EGFR antibody
2. History of severe drug allergy
3. Females who are pregnant or planning a pregnancy and males intending to impregnate their partners
4. With serious complications (interstitial pneumonia, pulmonary fibrosis, renal failure, liver failure, uncontrollable diabetes mellitus, or uncontrollable hypertension)
5. With pleural effusion and ascites, requiring treatment
6. With active duplicated cancers
7. With serious abnormalities in an electrocardiogram, clinically problematic heart failure, or ischemic heart diseases
8. Clinical or radiological evidence of brain metastases
9. Synchronous or metachronous multiple malignancy within the last 5-year disease-free interval
10. Any other criteria for which the investigator deems patients unsuitable for this present study

Treatment regimens

The combined regimens of cytotoxic drugs (i.e., doublet regimen) and anti-EGFR antibodies listed below are to be administered as first-line therapy for included patients.

1) mFOLFOX* and cetuximab or panitumumab
   * (400 mg/m^2 bolus of 5-fluorouracil [FU]; 2400 mg/m^2 continuous infusion of 5-FU; 200 mg/m^2 of levofolinate; 85 mg/m^2 of oxaliplatin) every 2 weeks
2) FOLFIRI* and cetuximab or panitumumab
   * (400 mg/m^2 bolus of 5-FU; 2400 mg/m^2 continuous infusion of 5-FU; 200 mg/m^2 of levofolinate; 150 mg/m^2 of irinotecan) every 2 weeks
3) CapOX* and cetuximab or panitumumab
   * (130 mg/m^2 bolus of oxaliplatin on day 1; 2000 mg/m^2/day of oral capecitabine on days 1-14) every 3 weeks
4) SOX* and cetuximab or panitumumab
   * (130 mg/m^2 bolus of oxaliplatin on day 1; 80-120 mg/day, body surface area (BSA) of less than 1.25 m^2, 80 mg/day; 100 mg/day for BSA=1.25-1.5 m2, and 120 mg/day for BSA ≥1.5 m^2, of oral S-1 on days 1-14) every 3 weeks
5) IRIS* and cetuximab or panitumumab
   * (125 mg/m^2 bolus of irinotecan on day 1, 14; 80-120 mg/day of oral S-1 on days 1-14) every 4 weeks

Cetuximab is administered weekly (initial dose of 400 mg/m^2 on day 1 and followed by weekly doses of 250 mg/m^2) or biweekly (500 mg/m^2), and panitumumab is administered biweekly (6 mg/kg).

Outcomes

1) *Primary endpoint*
   - Response rate in patients without *RAS*, *BRAF*, *PIK3CA*, and *EGFR* mutations in cfDNA (before starting chemotherapy)
2) *Secondary endpoint*
   - Early tumor shrinkage rate in patients without *RAS*,

- *BRAF*, *PIK3CA*, *and EGFR* mutations in cfDNA (before starting chemotherapy)
- Progression-free survival (PFS) in patients without *RAS*, *BRAF*, *PIK3CA*, *and EGFR* mutations in cfDNA (before starting chemotherapy)
- Overall survival (OS) in patients without *RAS*, *BRAF*, *PIK3CA*, *and EGFR* mutations in cfDNA (before starting chemotherapy)
- Frequency of *RAS*, *BRAF*, *PIK3CA*, and *EGFR* mutation in cfDNA (before starting chemotherapy) of patients without RAS and BRAF mutations in their tissue sample
- Frequency of *RAS*, *BRAF*, *PIK3CA*, and *EGFR* mutation in cfDNA (after acquired resistance)

3）*Safety evaluation*
- Adverse event with the worst grade

Study outline and variable measures

1）*Before enrollment*
- Patient characteristics: gender, performance status, medical history, comorbidity, allergy, and blood pressure
- Tumor characteristics: primary/recurrent, location of primary tumor, histological findings, clinical stage, location of metastatic tumor, number of metastatic organs, and *RAS* mutation status of primary tumor

2）*Within 14 days before enrollment*
- Blood examination: white blood cell, neutrophils, platelet count and hemoglobin, AST, ALT, total bilirubin, LDH, creatinine clearance (CCr), and electrolyte levels
- Tumor marker: CEA and CA19-9

3）*Within 21 days before enrollment*
- Radiological findings: computed tomography and magnetic resonance imaging

4）*Before treatment initiation, but after enrollment*
- Liquid biopsy: *RAS*, *BRAF*, *PIK3CA*, and *EGFR* mutations in cfDNA

5）*During treatment until termination of first-line treatment*
- Blood examination: every treatment cycle
- Tumor markers: every month
- Radiological findings: every 3 months
- Liquid biopsy: every 3 months and at treatment termination
- Adverse events:

cfDNA extraction and assay for RAS, BRAF, PIK3CA, and EGFR mutations

Blood samples are collected, and cfDNA is extracted from 1 mL of plasma into 30 μL of elution buffer using a Maxwell® RSC cfDNA Plasma Kit (Promega, Madison, WI, USA). Genetic mutations (i.e., ctDNA) of *RAS*, *BRAF*, *PIK3CA*, and *EGFR* were detected in cfDNA using QX200 Droplet Digital PCR (ddPCR, Bio-Rad Laboratories, Hercules,

California, USA). The threshold was defined as a mutation allele frequency (MAF) >0.1%.

Data collection

Researchers at each hospital maintain individual patient data, including images, laboratory data, and other records. All data are collected by the Nippon Medical School Data Center. The data center oversees data sharing within the trial. Clinical data entry, central monitoring, and data management are performed. All data aggregation and statistical analysis are performed at Nippon Medical School Data Center. Only clinical data managers of Nippon Medical School Data Center can access reported case data. Interim analysis and auditing are not planned for the study.

Sample size calculation

We assumed a threshold response rate of 78% of included patients without assessment of liquid biopsy, based on a previous study evaluating responses to anti-EGFR therapy in mCRC patients with *RAS* wild-type primary tumors (PEAK trial) [16]. With an expected response rate of 90% using liquid biopsy, simulation results require a sample size of 78 with α = 0.05 (two-sided) for 90% power, based on the one-arm binominal test using the SWOG statistical tool. Our previous pilot study showed that the response rate of patients without *RAS* mutations in tissue samples or liquid biopsy was 92% (23/25) [15]. If the estimated dropout is 8%, a target sample size of 85 is thus necessary. Considering the reported detection rate of *RAS* mutations in cfDNA as 10% of mCRC patients with *RAS* wild-type primary tumor, at least 100 patients should be recruited so as to include 85 patients without *RAS* mutations in cfDNA.

Statistical considerations

All patients receiving anti-EGFR antibody are subjected to analysis. After enrollment, ineligible patients are excluded from this study. Response rates with 95% confidence intervals are calculated for all eligible patients. The Kaplan-Meier method is used to calculate PFS and OS, whereas univariate analyses are performed using the log-rank test. Correlations are analyzed using Spearman's rank correlation coefficient.

Ethical considerations

The study protocol was approved by the Ethics Committee of Nippon Medical School (Tokyo, Japan) on October 19, 2017 (Registration number: 229023), and an amendment was approved on October 20, 2020. Written informed consent is obtained from each patient before his/her participation in the study. Each researcher is committed to implementing obligations of the law in accordance with provisions of the Helsinki Declaration.

Dissemination policy

Results of this present study will be submitted for publication in peer-reviewed journals, and important findings will be presented at domestic and international conferences. Authorship is assigned in accordance with guidance from the International Committee of Medical Journal Editors.

Discussion

Spatial and temporal heterogeneities have been determined to affect the outcome of molecular targeted therapy; however, conventional molecular diagnosis based on tissue biopsy is limited in terms of detecting these two heterogeneities. With the use of liquid biopsy, the two heterogeneities can now be diagnosed, thus contributing to the more accurate prediction for molecular targeted therapy. Liquid biopsy includes ctDNA, circulating tumor cells, and circulating non-coding RNAs, with ctDNA being the most widely used because of its high sensitivity [5,17,18]. In this present study, we try to clarify the utility of liquid biopsy in predicting the efficacy of anti-EGFR therapy and mechanism of primary and secondary resistance in terms of spatial and temporal heterogeneity.

Only our previous small study showed that *RAS* spatial heterogeneity can cause primary resistance to anti-EGFR therapy, whereas *RAS*, *BRAF*, and *EGFR* temporal heterogeneity can cause secondary resistance to anti-EGFR therapy in first-line chemotherapy [15]. Some authors showed *RAS* heterogeneity can cause primary resistance to anti-EGFR therapy for mCRC patients undergoing second- or third-line treatment [9-11]. However, the detected heterogeneity in these clinical settings cannot be unequivocally classified as spatial or temporal heterogeneity theoretically, owing to the influence of previous anticancer drugs administered. Also, if it was spatial heterogeneity, we cannot clarify the reason for the occurrence.

Thus, our study design, limited to first-line therapy, would eliminate the influence of previous treatments and temporal heterogeneity on response prediction. We will evaluate the influence of temporal heterogeneity to acquired resistance using samples obtained after acquiring resistance.

Another limitation of our previous study is that *NRAS* and *PIK3CA* were not included. Anti-EGFR therapy reportedly induces both emerging *RAS* and non-*RAS* mutations in EGFR signaling [12-14]. Toledo et al. [19] included 25 mCRC patients who received FOLFIRI and cetuximab as first-line therapeutics and showed that heterogeneity of *NRAS* and *PIK3CA* in addition to that of *KRAS* and *BRAF* cause primary and secondary resistance. Kim et al. [20] reported that cfDNA mutations in non-*RAS* genes should be considered in exploring mechanisms of acquired resistance despite low frequencies of emerging *RAS* mutations. Furthermore, recent studies suggest multiple molecular mechanisms for secondary resistance in individual patients [12,13,21]. Therefore, assessing cfDNA mutations in genes other than *RAS* (*BRAF*, *PIK3CA*, and *EGFR*) in our study with a relatively large sample size should illuminate this genetic complexity and its association with secondary resistance.

This study had some limitations. First, this was a single-arm, not randomized trial, which involves a potential selection bias. Second, the emerging temporal heterogeneity cannot be identified from either cytotoxic or anti-EGFR agent, or both. This study will help clarify the predictive value of baseline *RAS, BRAF*, *PIK3CA*, and *EGFR* mutations in cfDNA for responses to anti-EGFR therapy. It will also characterize the frequency of temporal heterogeneity of these genes and their association with secondary resistance to anti-EGFR therapy.

Conflicts of Interest
There are no conflicts of interest.

Author Contributions
All the listed 15 authors substantially contributed to create the study protocol.

Approval by Institutional Review Board (IRB)
Approval code issued by the IRB: 229023
Name of institution that granted the approval: Nippon Medical School, Tokyo, Japan

Disclaimer
Takeshi Yamada is one of the Associate Editors of Journal of the Anus, Rectum and Colon on the journal's Editorial Board. He was not involved in the editorial evaluation or decision to accept this article for publication at all.

Trial registration information
Registry name: Precision medicine for patients with *RAS* wild metastatic colorectal cancer using liquid biopsy
Trial ID: UMIN000031177
URL: https://upload.umin.ac.jp/cgi-open-bin/ctr/ctr_view.cgi?recptno=R000035597

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