Feasibility study of cancer genome alterations identified by next generation sequencing: ABC study

Yoichi Naito1,*, Hideaki Takahashi1, Kohei Shitara1, Wataru Okamoto1, Hideaki Bando1, Takeshi Kuwata1, Yasutoshi Kuboki1, Shingo Matsumoto1, Izumi Miki1, Takeharu Yamanaka2, Atsushi Watanabe3, and Motohiro Kojima1

1National Cancer Center Hospital East, 6-5-1 Kashiwanoha, Kashiwa, Chiba 277-8577, Japan, 2Yokohama City University, Kanagawa, Japan, and 3Nippon Medical School, Tokyo, Japan

*For reprints and all correspondence: Yoichi Naito, National Cancer Center Hospital East, Chiba, Japan, 6-5-1 Kashiwanoha, Kashiwa, Chiba, 277-8577, Japan. E-mail: ynaito@east.ncc.go.jp

Received 26 January 2018; Editorial Decision 26 March 2018; Accepted 5 April 2018

Abstract

Background: To confirm the feasibility and explore the clinical applicability of amplicon sequencing by next generation sequencing (NGS) of biopsy samples from patients with advanced solid tumors, we conducted a prospective study.

Methods: Patients with unresectable, advanced, or recurrent solid tumors were included. Key eligibility criteria were as follows: 20 years or older, any planned systemic therapy, adequate lesion for biopsy, and written informed consent. Samples were fixed in 10% buffered formalin and embedded in paraffin. Cancer-derived DNA was extracted, and amplicon sequencing was performed using Ion AmpliseqTM Cancer Hotspot Panel version 1.0 or version 2.0 by central vendor. We evaluated the success rate of sequencing, and the proportion of the patients with actionable mutations. We organized an expert panel to share the results of targeted sequence, make annotations and reports, and discuss concomitant ethical/legal/social issues.

Results: A total of 232 patients were included, and 208 were successfully analyzed (success rate of 89.7%). The biopsy procedures were safe, with only one case of Grade 3 vasovagal reaction. The proportion of actionable/druggable mutations was 38.9% (81/208), which was not significantly different between the cancer panel version 1.0 and version 2.0 (P = 0.476). Expert panel could discuss the findings and make sufficient reports.

Conclusions: We confirmed the feasibility of NGS-based amplicon sequencing using biopsy samples, making the basis for nationwide genome screening for cancer patients using biopsy samples. Our results suggest that focused panel may be sufficient to detect major mutations.

Key words: neoplasms, genome, genetic variation

Introduction

Recent advances in molecular medicine for patients with advanced solid tumors revealed that cancer genome alteration was crucial in the selection of systemic therapy, especially molecular-targeting agents. For example, patients with non-small cell lung cancer harboring epidermal growth factor receptor (EGFR) mutation could greatly achieve clinical benefit from EGFR tyrosine kinase inhibitors (TKI) (1–3). In addition, in colorectal cancer patients whose tumor...
possesses any RAS mutation, anti-EGFR antibody is not effective (4,5). An increasing number of such druggable or actionable gene alterations, which could affect treatment choice, have been discovered recently across cancer types. Therefore, there is an urgent need to examine gene alterations using small amount of tumor samples obtained from biopsies.

Compared to Sanger sequencing and real-time PCR, next generation sequencing (NGS) is much faster and costs lesser for sequencing DNA and RNA (6–9). However, the feasibility of analyzing pan-cancer gene panel by NGS using small amount of biopsy materials is not fully established in terms of analytical validity, clinical validity, clinical utility, and ethical/legal/social issues (10). To introduce pan-cancer gene panel by NGS into clinical practice, feasibility including safety of biopsy, success rate of sequencing, and annotation should be established prospectively. For annotation, a previous study employed multi-disciplinary tumor board to interpret the sequencing results (11). We conducted an ‘ABC study (Analyses of Biopsy Samples for Cancer Genomics)’ to investigate the feasibility and explore the efficacy of a pan-cancer gene panel by NGS from biopsy samples in patients with advanced solid tumors.

Materials and methods

Objective and study design

The ABC study was a prospective observational study to identify the profile of targeted somatic mutations in solid tumors. It was approved by the institutional review board (2012–20) in accordance with the Japanese Ethical Guidelines for Epidemiological Research.

Patients

Inclusion criteria for the present study were as follows: (i) Patients with unresectable, advanced, or recurrent solid tumors including malignant lymphoma; (ii) 20 years or older; (iii) Systemic therapy (chemotherapy, endocrine therapy, or molecular-targeted therapy) is conducted or planned; (iv) Adequate lesion for biopsy; (v) Written informed consent; (vi) Expected adequate amount of DNA for sequencing (at least 10 ng of double-stranded DNA). If the biopsy was not assumed to be safe, the patient would be excluded.

Sample preparation

Biopsy of primary or metastatic site was planned in all participants. Acquired tissues were fixed in 10% buffered formalin within 48 hours and embedded in paraffin. Pathologists evaluated the proportion of tumor cells in the specimen. In general, samples including 10% or more tumor cells were considered eligible for further analyses. Thereafter, DNA was extracted from whole formalin-fixed paraffin embedded (FFPE) blocks. In case the whole FFPE block was unavailable, thin-sectioned samples (5 μm for five sections) were used. If the biopsy sample was not appropriate for further analysis, re-biopsy was recommended. In case re-biopsy was not feasible, an archival sample from a previous biopsy or surgery was used.

DNA extraction and amplicon sequencing

DNA extraction and subsequent procedure including amplicon sequencing by NGS were performed by SRL (Tokyo, Japan), certified by CAP IS015189. Ion Ampliseq™ Cancer Hotspot Panel was used to examine 739 mutations in 47 genes (version 1.0) or 2855 mutations in 50 genes (version 2.0) (Tables S1 and S2) (12,13). About 10 ng of double-stranded genomic DNA was applied for both versions of Ion Ampliseq™ Cancer Hotspot Panel. Sequencing data were analyzed with Coverage Analysis and Variant Caller plugins available within the Ion Torrent Suite software TS 4.0.2 and contextually with Ion Reporter. The variant caller is designed to detect single nucleotide variants (SNVs) with 2% or more variant frequency or indels with 8%.

Expert panel for annotation and report

We organized an expert panel, which was a multi-disciplinary intra-mural cancer board consisting of specialists in medical oncology, pathology, molecular medicine, genetics, and bioinformatics. The meetings of the expert panel were held twice a month to discuss the results, give annotation and make reports.

Feasibility and efficacy

To explore the feasibility, we assessed the success rate (defined as the ratio of the number of successfully sequenced samples to the number of all enrolled patients), the safety of biopsy, and the time from sending tissues to the clinical laboratory for sequencing to receiving results from the laboratory. To evaluate the efficacy of the cancer panel by NGS, we employed the proportion of the patients with actionable mutations, defined as deleterious mutations in KRAS, NRAS, HRAS, BRAF, EGFR, ERBB2, KIT, PIK3CA, AKTI, PTEN, and FGFR as a consensus of the expert panel.

We compared patient characteristics and proportions of mutations investigated by Ion Ampliseq™ Cancer Hotspot Panel version 1.0 and version 2.0 by univariate and multivariate analyses.

Results

Patient characteristics

Between July 2012 and September 2014, samples from 208 of 232 patients (success rate of 89.7%) were successfully sequenced (Fig. 1). Patient characteristics of sequenced cases are listed in Table 1. The median age was 64.0 years (range 20–87). Eighty patients (38.5%) were female. One hundred and eighty seven (89.9%) were Eastern Cooperative Oncology Group (ECOG) performance status 0/1. 159 (76.4%) were chemotherapy-naïve. The most common cancer was gastric cancer (65 patients, 31.3%), followed by colorectal cancer (41 patients, 19.7%), breast cancer (20 patients, 9.6%) and lung cancer (15 patients, 7.2%).

Adequate samples were obtained from biopsies

Two hundred and twenty-nine patients underwent biopsies, and adequate samples were obtained from 214 (93.4%) patients. Endoscopic biopsies were performed in 135 patients (58.2%), and CT- or ultrasound-guided percutaneous biopsied in 60 patients (25.9%). Our procedures were generally safe, with only one case of Grade 3 adverse event related to the biopsy procedure (vasovagal reaction). In 7 of 18 cases where the tissue was not considered adequate for subsequent analyses (mainly because samples did not contain any tumor cells), the archival tissue was used; therefore, a total of 221 samples were forwarded to DNA extraction and subsequent amplicon sequencing. The mean proportion of tumor cells in the specimen was 56.6% (Table S3). Among the 208 successfully sequenced samples, whole FFPE blocks were used in 171 cases, and thin-sectioned samples were used in 37 cases (Table 2), and between them the proportions of tumor cells were not significantly different.
The mean amount of double-stranded DNA in 208 patients was 4651.9 ng. The mean amount of DNA was significantly larger ($P < 0.001$) in samples from FFPE blocks (5369.9 ng) than that from thin-sections (1333.8 ng) (Table 2). In terms of biopsy site, the amount of DNA tended to be smaller in samples from liver biopsy and core-needle biopsy from Figure 1. CONSORT of patients and samples. A total of 232 patients were enrolled, and in 208 patients (89.7%) sequencing was successfully performed.

| Variables                        | All sequenced patients (N = 208) (%) | Ion Ampliseq ver 1.0 (N = 95) (%) | Ion Ampliseq ver 2.0 (N = 113) (%) | P-value |
|----------------------------------|-------------------------------------|-----------------------------------|-----------------------------------|---------|
| Median age (range)               | 64 (20–87)                          | 64 (29–81)                        | 65 (20–87)                        | 0.814   |
| Female                           | 80 (38.5)                           | 36 (37.9)                         | 44 (38.9)                         | 0.878   |
| PS                               |                                     |                                   |                                   | 0.285   |
| 0                                | 128 (61.5)                          | 56 (58.9)                         | 72 (63.7)                         |         |
| 1                                | 59 (28.4)                           | 29 (30.5)                         | 30 (26.5)                         |         |
| 2                                | 17 (8.2)                            | 7 (7.4)                           | 10 (8.8)                          |         |
| ¾                                | 4 (1.9)                             | 3 (3.2)                           | 1 (0.9)                           |         |
| Stage                            |                                     |                                   |                                   | 0.150   |
| 0–3                              | 63 (30.3)                           | 27 (28.4)                         | 36 (31.9)                         |         |
| 4                                | 145 (69.7)                          | 68 (71.6)                         | 77 (68.1)                         |         |
| Disease                          |                                     |                                   |                                   | 0.336   |
| Gastric                          | 65 (31.3)                           | 26 (27.4)                         | 39 (34.5)                         |         |
| Colorectal                       | 41 (19.7)                           | 19 (20.0)                         | 22 (19.5)                         |         |
| Breast                           | 20 (9.6)                            | 9 (9.5)                           | 11 (9.7)                          |         |
| Lung                             | 15 (7.2)                            | 12 (12.6)                         | 3 (2.7)                           |         |
| Liver                            | 12 (5.8)                            | 8 (8.4)                           | 4 (3.5)                           |         |
| Pancreas                         | 7 (3.4)                             | 2 (2.1)                           | 5 (4.4)                           |         |
| Unknown primary                  | 7 (3.4)                             | 3 (3.2)                           | 4 (3.5)                           |         |
| Esophagus                        | 6 (2.9)                             | 2 (2.1)                           | 4 (3.5)                           |         |
| Lymphoma                        | 6 (2.9)                             | 3 (3.2)                           | 3 (2.7)                           |         |
| GIST                             | 5 (2.4)                             | 2 (2.1)                           | 3 (2.7)                           |         |
| Soft tissue sarcoma              | 4 (1.9)                             | 2 (2.1)                           | 2 (1.8)                           |         |
| Other                            | 20 (9.6)                            | 7 (7.4)                           | 13 (11.5)                         |         |
| Line of previous chemotherapy    |                                     |                                   |                                   | 0.797   |
| 0                                | 159 (76.4)                          | 73 (76.8)                         | 86 (76.1)                         |         |
| 1                                | 29 (13.9)                           | 12 (12.6)                         | 17 (15.0)                         |         |
| 2                                | 14 (6.7)                            | 6 (6.3)                           | 8 (7.1)                           |         |
| ¾                                | 6 (2.9)                             | 4 (4.3)                           | 2 (1.8)                           |         |
| Distant metastasis              |                                     |                                   |                                   |         |
| Liver                            | 92 (44.2)                           | 45 (47.4)                         | 47 (41.6)                         | 0.403   |
| Brain                            | 7 (3.4)                             | 3 (3.2)                           | 4 (3.5)                           | 0.879   |
| Lung                             | 40 (19.2)                           | 25 (26.3)                         | 15 (13.3)                         | 0.017   |
| Bone                             | 25 (12.0)                           | 16 (16.8)                         | 9 (8.0)                           | 0.050   |

(58.1% vs. 50.0%, $P = 0.129$). The mean amount of double-stranded DNA in 208 patients was 4651.9 ng. The mean amount of DNA was significantly larger ($P < 0.001$) in samples from FFPE blocks (5369.9 ng) than that from thin-sections (1333.8 ng) (Table 2). In terms of biopsy site, the amount of DNA tended to be smaller in samples from liver biopsy and core-needle biopsy from...
breast (P < 0.001); however, only three cases were under 100 ng, so further analyses were successfully performed. In 24 cases, DNA was not retrieved by the laboratory-determined procedure, so amplicon sequencing was successfully performed in a total of 208 cases. The main reason of failure in DNA extraction was inadequate tumor cells in specimens.

Multiplex amplicon sequencing successfully detected genomic alterations

Turnaround time, defined as the median time from the submission of tissue to the clinical laboratory for sequencing to receiving results, was 9 (7–24) business days. The results are shown in Fig. 2. TP53 mutations (41.8%) were the most frequently observed protein-altered mutations, followed by KRAS (16.3%), STK11 (12.5%), PIK3CA (12.0%), KIT (12.0%), MLH1 (7.2%), APC (5.8%), CTNNB1 (5.8%), MET (5.8%), and BRAF (4.8%) mutations. The first 95 cases were analyzed by Ion AmpliseqTM Cancer Hotspot Panel version 1.0 (739 mutations in 47 genes) (Group 1), and the rest 113 were analyzed by version 2.0 (2855 mutations in 50 genes) (Group 2). The mean number of mutations registered at COSMIC database was 2.16 per patient (1.63 for Group 1 and 2.61 for Group 2, P < 0.01). The mean number of the nonsynonymous, nonsense mutations or indels was 1.54 per patient (Figures S1 and S2, 1.36 for Group 1 and 1.69 for Group 2, P = 0.059). Numerically, the proportion of common mutations, which could be detected in more than 5% of patients such as TP53, KRAS, STK11, KIT, and PIK3CA, were not different between Groups 1 and 2; however, minor mutations (detected in 5% or less patients) were detected more in Group 2. The proportion of actionable/druggable mutations (Table S4) was 38.9% (81/208), with 35.8% (34/95) for Group 1 and 41.6% (47/113) for Group 2, with no statistically significant difference (Fisher’s exact test P = 0.476). After patient characteristics were adjusted by multivariate analysis, the proportion of actionable/druggable mutations was also not significantly different between the two groups (Odds ratio 1.308, P = 0.458). Figure 3 shows the results of each case. In our study, 54 (26.0%) out of 208 patients participated in at least one investigational new drug trials, and 35 (16.8%) participated in phase I trials.

Expert panel

A total of 51 meetings of the expert panel were held. The expert panel discussed patient characteristics, biopsy procedures, and pathological findings (including proportions of tumor cells, amount of obtained DNA, and results of sequencing), made annotations and reports, and discussed related ethical/legal/social issues. The report included biopsy site, proportions of tumor cells, amount of double-stranded DNA.

Table 2. Amount of double-stranded DNA

| Variables                | Number of patients | Total amount of double-strand DNA (mean) (ng) | P-value |
|--------------------------|-------------------|---------------------------------------------|---------|
| FFPE Block               | 171               | 5369.9                                      | <0.001  |
| Thin section             | 37                | 1333.8                                      |         |
| (10 μ × 5 slices)        |                   |                                             |         |
| Biopsy site              |                   |                                             | <0.001  |
| Lymph nodes              | 25                | 6747.4                                      |         |
| Stomach (endoscopic)     | 66                | 5210.2                                      |         |
| Colon (endoscopic)       | 39                | 6577.9                                      |         |
| Liver (US-guided)        | 41                | 1499.1                                      |         |
| Breast (core-needle biopsy) | 6              | 1905.8                                      |         |
| Other                    | 31                | 4051.8                                      |         |

Figure 2. Frequency of nonsynonymous/nonsense mutations and indels in the entire cohort. TP53 mutations (41.8%) were the most frequently observed protein-altered mutations, followed by KRAS (16.3%), STK11 (12.5%), PIK3CA (12.0%), KIT (12.0%), MLH1 (7.2%), APC (5.8%), CTNNB1 (5.8%), MET (5.8%), and BRAF (4.8%) mutations.
DNA, COSMIC (Catalogue of Somatic Mutations in Cancer)-registered mutations detected in individual cases (14) and variant frequencies, top five common tumor types harboring the detected mutations, annotations of mutations, information of matched drugs (including investigational new drug trials), and comments by the expert panel.

Discussion

In this study, we clearly demonstrated the feasibility of investigating mutations by NGS in clinical practice by showing the safety of biopsy, high success rate (89.7%) and acceptable turnaround time (median 9 days from the submission of samples to obtaining results). Previous reports demonstrated that among 1528 samples submitted for testing, 343 (22.5%) failed to produce results for pre-analytic or analytic reasons (15). Therefore high success rate of our study might be due in part to the adequate samples obtained by afresh biopsies.

Although less amount of DNA was obtained by liver biopsy or core-needle biopsy from breast than endoscopic procedure, it was sufficient to conduct mutational analyses. We used samples from whole FFPE blocks in most cases; however, the amount obtained by samples from thin-section materials was 1333.8 ng in average, which was sufficient for not only targeted resequencing but also whole exon analysis.

Despite of accumulating evidence of analytical and clinical validity (16–18), to elucidate the clinical applicability of multiplex ampli-con sequencing by NGS is somewhat troublesome. One problem is that the appropriate endpoint for clinical utility is yet to be determined. One approach is to evaluate the proportion of patients who could participate in the trial of investigational new drug. However, this endpoint is strongly interfered by accessibility to the trial for new drugs. We alternately employed the proportion of patients with actionable mutations, which enables us to evaluate directly the efficacy of cancer panel. However, obtaining the information of predictive biomarkers of non-responsiveness to targeting therapy, such as RAS mutations, is also important to avoid ineffective treatment, so this endpoint is not valid yet. Although a recent report suggested improved survival in patients receiving a matched targeted agent (19), there is an urgent need to establish the clear and valid endpoint.

We used two versions of cancer panel, Ion Ampliseq™ Cancer Hotspot Panel version 1.0 and version 2.0 in a chorological way. Between the two panels, the proportion of common or druggable mutations was not significantly different, although nonsynonymous mutations were numerically more detected in version 2.0. This means that to detect common druggable mutations, the ‘core’ panel, which covers appropriate common mutations, is sufficient and may be cost-effective. However, to detect minor mutations, NGS-based large panel such as Ion Ampliseq™ Cancer Hotspot Panel version 2.0 may be useful and could be applied to examine novel drugs that potentially target minor mutations.

Our study has several limitations. First, our panel could not detect fusions and amplifications. Currently, we employ another panel which can examine amplification and fusion as actionable genomic alterations (20). Second, the sample size was small. One of the next steps is a nationwide cancer genome screening project (SCRUM-Japan) to detect minor mutations and facilitate enrollment of patients in clinical studies for investigational new drugs (UMIN000010234, UMIN000016343, UMIN000016344) (21).

We organized an expert panel to discuss the procedure and results, make annotations and reports, and discuss concomitant ethical/legal/social issues. We suggest the utility to organize multidisciplinary boards such as our expert panel for discussing the results, as many physicians reported lack of confidence in the interpretation of genomic alterations (22). Moreover, to expand such genomic testing to multi-institutional study, education for individual physician is critical because a multi-institutional expert panel is not
pragmatic as the study scales up. In addition, the reporting system should be established. Consultation for genetic specialists and social maturity for ethical/legal issues including legislation are also essential.

In conclusion, we established the feasibility and suggested the clinical applicability of NGS-based amplicon sequencing using FFPE samples obtained from biopsies. Biopsies were generally safe and median turnaround time of 9 business days was acceptable for introduction to clinical practice. Further investigation is required to improve the potential clinical utility of NGS that covers mutations as well as amplification and fusion drivers.

Supplementary data
Supplementary data are available at Japanese Journal of Clinical Oncology online.

Acknowledgements
We would like to thank the patients and families who participated in this study. We also thank and acknowledge all of the researchers and clinicians.

Funding
This study is supported in part by Grant-in-Aid for National Cancer Center Research and Development Fund 24-A-1, 25-A-6, Grant-in-Aid for Cancer Research from the Ministry of Health, Labour and Welfare Research grant 205-7, Grand-in-Aid for Japan Agency for Medical Research and Development 16kk020512h0001.

Conflict of interest statement
None declared.

References
1. Lynch TJ, Bell DW, Sordella R, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N Engl J Med 2004;350:2129–39.
2. Paez JG, Janne PA, Lee JC, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science 2004;304:1497–500.
3. Mok TS, WuYL, Thongprasert S, et al. Gefitinib or carboplatin-paclitaxel in pulmonary adenocarcinoma. N Engl J Med 2009;361:947–57.
4. Van Cutsem E, Köhne CH, Ling I, et al. Cetuximab plus irinotecan, fluorouracil, and leucovorin as first-line treatment for metastatic colorectal cancer: updated analysis of overall survival according to tumor KRAS and BRAF mutation status. J Clin Oncol 2011;29:2011–9.
5. Douillard JY, Siena S, Cassidy J, et al. 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 2010;28:4697–705.
6. Morozova O, Marra MA. Applications of next-generation sequencing technologies in functional genomics. Genomics 2008;92:235–64.
7. McCourt CM, McArt DG, Mills K, et al. Validation of next generation sequencing technologies in comparison to current diagnostic gold standards for BRAF, EGFR and KRAS mutational analysis. PLoS One 2013;8:e69604.
8. Harismendy O, Ng PC, Strausberg RL, et al. Evaluation of next generation sequencing platforms for population targeted sequencing studies. Genome Biol 2009;10:R32.
9. Tuononen K, Mäki-Nevala S, Sarhadi VK, et al. Comparison of targeted next-generation sequencing (NGS) and real-time PCR in the detection of EGFR, KRAS, and BRAF mutations on formalin-fixed, paraffin-embedded tumor material of non-small cell lung carcinoma-superiority of NGS. Genes Chromosomes Cancer 2013;52:503–11.
10. Kou T, Kanai M, Matsumoto S, et al. The possibility of clinical sequencing in the management of cancer. Jpn J Clin Oncol 2016;46:399–406.
11. Roychowdhury S, Iyer MK, Robinson DR, et al. Personalized oncology through integrative high-throughput sequencing: a pilot study. Sci Transl Med 2011;3:111ra121.
12. Singh RR, Patel KP, Routbort MJ, et al. Clinical validation of a next-generation sequencing screen for mutational hotspots in 46 cancer-related genes. J Mol Diagn 2013;15:607–22.
13. thermofisher.com [Internet]. Ion AmpliSeq™ Cancer Hotspot Panel v2. https://www.lifetechnologies.com/order/catalog/product/4475346 (20 January 2018, date last accessed).
14. Open database: COSMIC (Catalogue of Somatic Mutations in Cancer) [Internet]. http://cancer.sanger.ac.uk/cosmic (20 January 2018, date last accessed).
15. Al-Kateb H, Nguyen TT, Steger-May K, Pfiefer JD. Identification of major factors associated with failed clinical molecular oncology testing performed by next generation sequencing (NGS). Mol Oncol 2015;9:1737–43.
16. Trujillano D, Weiss MF, Schneider J, et al. Next-generation sequencing of the BRCA1 and BRCA2 genes for the genetic diagnostics of hereditary breast and/or ovarian cancer. J Mol Diagn 2015;17:162–70.
17. Bourgon R, Lu S, Yan Y, et al. High-throughput detection of clinically relevant mutations in archived tumor samples by multiplexed PCR and next-generation sequencing. Clin Cancer Res 2014;20:2080–91.
18. Zhang L, Chen L, Sah S, et al. Profiling cancer gene mutations in clinical formalin-fixed, paraffin-embedded colorectal tumor specimens using targeted next-generation sequencing. Oncologist 2014;19:336–43.
19. Kris MG, Johnson BE, Berry LD, et al. Using multiplexed assays of oncogene drivers in lung cancers to select targeted drugs. JAMA 2014;311:1999–2006.
20. Hovelson DH, McDaniel AS, Cani AK, et al. Development and validation of a scalable next-generation sequencing system for assessing relevant somatic variants in solid tumors. Neoplasia 2015;17:385–99.
21. Open database: UMIN (University hospital Medical Information Network (UMIN) Center). http://www.umin.ac.jp/ (20 January 2018, date last accessed).
22. Gray SW, Hicks-Courant K, Cronin A, et al. Physicians’ attitudes about multiplex tumor genomic testing. J Clin Oncol 2014;32:1317–23.

Appendix
Genichiro Ishii, Satoshi Fujii, Seiji Niho, Masafumi Ikeda, NozomuFuse, Izumi Ohno, National Cancer Center Hospital East, 6-5-1 Kashiwanoha, Kashiwa, Chiba 277–8577, Japan.

Takashi Kohno, National Cancer Center Research Institute, Tokyo, Japan.

Akihiro Sato, Atsushi Ohtsu, Atsushi Ochiai, Takayuki Yoshino, Katuya Tsuchihara, National Cancer Center Hospital East, 6-5-1 Kashiwanoha, Kashiwa, Chiba 277–8577, Japan.