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Kou, Tadayuki; Kanai, Masashi; Yamamoto, Yoshihiro; Kamada, Mayumi; Nakatsu, Masahiko; Sakuma, Tomohiro; Mochizuki, Hiroaki; ... Kosugi, Shinji; Okuno, Yasushi; Muto, Manabu

Kou, Tadayuki ...[et al]. Clinical sequencing using a next-generation sequencing-based multiplex gene assay in patients with advanced solid tumors. Cancer Science 2017, 108(7): 1440-1446

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Clinical sequencing using a next-generation sequencing-based multiplex gene assay in patients with advanced solid tumors

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Key words actionable mutation, genotype-directed therapy, multiplex gene assay, next-generation sequencing, precision cancer medicine

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Funding information
Japan Agency for Medical Research and Development, AMED.

Received December 14, 2016; Revised April 12, 2017; Accepted April 20, 2017
Cancer Sci 108 (2017) 1440–1446
doi: 10.1111/cas.13265

Advances in next-generation sequencing (NGS) technologies have enabled physicians to test for genomic alterations in multiple cancer-related genes at once in daily clinical practice. In April 2015, we introduced clinical sequencing using an NGS-based multiplex gene assay (OncoPrime) certified by the Clinical Laboratory Improvement Amendment. This assay covers the entire coding regions of 215 genes and the rearrangement of 17 frequently rearranged genes with clinical relevance in human cancers. The principal indications for the assay were cancers of unknown primary site, rare tumors, and any solid tumors that were refractory to standard chemotherapy. A total of 85 patients underwent testing with multiplex gene assay between April 2015 and July 2016. The most common solid tumor types tested were pancreatic (n = 19; 22.4%), followed by biliary tract (n = 14; 16.5%), and tumors of unknown primary site (n = 13; 15.3%). Samples from 80 patients (94.1%) were successfully sequenced. The median turnaround time was 40 days (range, 18–70 days). Potentially actionable mutations were identified in 69 of 80 patients (86.3%) and were most commonly found in TP53 (46.3%), KRAS (23.8%), APC (18.8%), STK11 (7.5%), and ATR (7.5%). Nine patients (13.0%) received a subsequent therapy based on the NGS assay results. Implementation of clinical sequencing using an NGS-based multiplex gene assay was feasible in the clinical setting and identified potentially actionable mutations in more than 80% of patients. Current challenges are to incorporate this genomic information into better therapeutic decision making.

With the paradigm shift to precision cancer medicine, there is a growing recognition that understanding of genomic architecture enables the adoption of better therapeutic strategies and that genotype-directed therapy can improve the clinical outcomes of cancer patients.1 In current clinical practice, hotspot-based, single-gene testing approaches, such as those testing for EGFR mutations in non-small cell lung cancer or RAS mutations in colorectal cancer, have commonly been used.2,3 However, several recent studies have reported that more comprehensive characterization of genomic alterations is necessary for successful identification of patients who may benefit from molecularly targeted therapies, and could provide more clinical benefits for individual patients.4–8

Recent technological innovations in next-generation sequencing (NGS) have facilitated comprehensive genomic profiling of human cancers through whole-genome, whole-exome, and whole-transcriptome sequencing, making it possible to deliver genomically informed personalized cancer therapy to individual patients.9–12 In particular, NGS-based multiplex gene assays can analyze a large number of pre-selected genes with clinical relevance to human cancers at once and are powerful tools for the simultaneous screening of numerous cancer-related genes in the clinical setting.

Clinical sequencing generally refers to sequencing of a genome or exome by NGS technologies for clinical applications.13 To date, this approach has been adopted not only in oncology but also in a variety of medical fields such as genetic analysis of neurologic disorders or genetic phenotyping of infectious diseases. In this study, we have defined clinical sequencing as the characterization of the tumor genomic variants that may confer sensitivity to a specific molecularly targeted therapy using an NGS-based multiplex gene assay.

In April 2015, we introduced clinical sequencing using an NGS-based multiplex gene assay (OncoPrime) into daily clinical practice and no modifications or adaptations are made.
clinical practice.\textsuperscript{(14)} We describe here the feasibility and diagnostic yield of the NGS assay in an initial cohort of patients with advanced solid tumors.

**Patients and Methods**

**Patient population.** Between April 2015 and July 2016, 85 patients with histopathologically confirmed solid tumors underwent an NGS-based multiplex gene assays (OncoPrime) at Kyoto University Hospital. The principal indications for the assay were cancers of unknown primary site, rare tumors, and any solid tumors refractory to standard chemotherapy. This study was approved by the Ethics Committee of the Kyoto University Graduate School of Medicine (G692) and all patients provided written informed consent for the use of genomic and clinical data for research purposes.

**NGS-based multiplex gene assay (OncoPrime).** OncoPrime is an NGS-based multiplex gene assay designed for clinical tumor genomic analyses. This NGS assay can sequence the entire coding region of 215 genes and concurrently examine the rearrangement of 17 frequently rearranged genes with clinical or preclinical relevance in human solid tumors (Table S1).

After the NGS assay was ordered by the treating physician, 5–10 slices of 10 μm sections of archival formalin-fixed paraffin-embedded (FFPE) tumor tissue (tumor content ≥20%) or DNA extracted from fresh frozen tumor tissue at our institution were shipped to a Clinical Laboratory Improvement Amendment (CLIA)-certified laboratory of EA Genomics (Morrisville, NC, USA). DNA extraction was performed by EA Genomics. Solution hybridization targeted 3861 exons of 215 cancer-related genes and 59 introns of 17 genes commonly rearranged in human cancers. Sequencing was performed on an Illumina HiSeq 2500 machines (San Diego, CA, USA). Variant calling was done using variant calling software (VARSPOWLY) in a CLIA-certified laboratory of EA Genomics.

The turnaround time (TAT) was defined as the period between the date of ordering OncoPrime and that of receiving NGS assay results by the treating physician.

**Definition of actionability.** Actionability implies that a protein product of the mutated gene can impact clinical decision making for patient treatment. The NGS-based multiplex gene assay provides an enormous amount of information about genomic alterations within tumors; however, sometimes it is challenging to determine whether identified genomic alterations are actionable or not.\textsuperscript{(15)} In addition, the evidence behind actionability ranges from sufficient clinical data to only preclinical evidence, and several actionability classification schemes have been proposed.\textsuperscript{(12,16–18)}

In this study, we defined a genomic alteration as actionable if the identified alterations met any of several criteria:

1. It can be directly targeted by a United States Food and Drug Administration (FDA)-approved drug (such as a BRAF inhibitor targeting a *BRAF* mutation, an EGFR inhibitor targeting an *EGFR* mutation, or an HER2 kinase inhibitor targeting an *HER2* mutation).
2. It is a signaling pathway component that can be targeted by an FDA-approved drug (such as a mammalian target of rapamycin inhibitor for *PIK3CA* mutation, a smoothened homolog inhibitor for the *PTCH1* mutation, or a mitogen-activated protein kinase kinase inhibitor for *RAS* mutation).
3. It predicts treatment response to an FDA-approved drug (such as a poly [ADP-ribose] polymerase inhibitor for *BRCA1*/*2* mutation).
4. It can be targeted directly or indirectly by an investigational agent that is available in early clinical trials. For instance, this category includes such genes as *CTNNB1* or *TP53*.
5. It is a biomarker for which only preclinical data is available. For instance, this category includes such genes as *IGF2R* or *SMAD4*.

**Variant filtering and reporting.** The procedure for variant prioritization and filtering was as follows (Fig. S1). First, all silent mutations in non-reference alleles were removed, keeping mutations that were missense, nonsense, or involved splicing junctions. Second, all non-reference alleles that appeared in >1% of the population were removed, as these were likely germline events. Third, all non-reference alleles with allele frequencies <4% and >95% were removed, as these were below the specified limit of detection of the assay and likely germline events, respectively. Finally, the importance of variants was prioritized based on membership in the following databases: Online Mendelian Inheritance in Man (https://www.omim.org/),\textsuperscript{(19)} ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/),\textsuperscript{(20)} Clinical Trial.gov (https://clinicaltrials.gov/), Drug Bank (https://www.drugbank.ca/), Catalogue of Somatic Mutations in Cancer (http://cancer.sanger.ac.uk/cosmic),\textsuperscript{(21)} and the Cancer Genome Atlas (https://cancergenome.nih.gov/). NGS data were annotated by N-of-One, Inc. (Concord, MA, USA). Genomic alterations that were potentially targetable with an FDA-approved drug or an investigational agent tested in early clinical trials were reported as potentially actionable mutations. Including variants of unknown significance, a maximum of 14 variants was listed in a final report and returned to the treating physician.

**Molecular tumor board.** Our institutional molecular tumor board comprises medical and surgical oncologists, pathologists, bioinformaticians, and medical geneticists. The molecular tumor board meetings are held for an hour every one or 2 weeks, and in general, 15–20 board members and clinicians attend the meeting. The molecular tumor board discusses genetically informed treatment options and other issues such as the possibility of germline variants in two to three patients and supports the treating physician for appropriate use of sequencing data. T.K, M.K, Y.Y, M.K, M.N, E.N, H.M, S.M, K.T, S.M, H.H, H.S, S.K, Y.O and M.M were the board members.

**Results**

**Patient characteristics.** The patient characteristics are summarized in Table 1. The median age was 58 years (range, 8–82 years). Most patients (80.0%) had solid tumors refractory to standard chemotherapy, and the remainder had cancers of unknown primary site (15.3%) or rare tumors (4.8%). The most common solid tumor types tested were pancreatic (15.3%) or rare (4.8%). The most common solid tumor types tested were pancreatic tumors (15.3%) or rare tumors (4.8%). The most common solid tumor types tested were pancreatic (15.3%) or rare tumors (4.8%). The most common solid tumor types tested were pancreatic tumors (15.3%) or rare tumors (4.8%).
of 80 patients, the median of DNA yield and concentration of
(94.1%) and the final report could be completed. In this group
As a result, the NGS assay was successful for 80 patients
(tive DNA extracted from fresh frozen tumor tissue was used.
first assay, the second NGS assay was successful when alterna-
patient from whom fresh frozen tumor tissue was used in the
(high duplicate read rate (>80%) and/or low insert size
extracted from fresh frozen tumor tissue. In 12 of 85 patients
(14.1%), the initial NGS assay failed because of insufficient
tissue quantity (<120 bp) (n = 5). Among these 12 patients, for six patients
from whom archival FFPE tumor tissue was used and one patient from whom fresh frozen tumor tissue was used in the
first assay, the second NGS assay was successful when alterna-
tive DNA extracted from fresh frozen tumor tissue was used.
As a result, the NGS assay was successful for 80 patients
(94.1%) and the final report could be completed. In this group
of 80 patients, the median of DNA yield and concentration of
library DNA were 1853 ng (range, 125–31850) and 5.40 nM
(range, 0.20–36.93), respectively (Fig. S2). The mean depth
for sequencing reactions were analyzed in 58 patients, and its
median figure was 4397 (range, 2123–6674) (Fig. S3). The
median TAT was 40 days (range, 18–70) (Fig. S4).
Identification of potentially actionable mutations. Of the 80
patients with NGS success, at least one potentially actionable
mutation was identified in 69 patients (86.3%). The median
number of actionable mutations per patient was 2 (range, 0–6).
Potentially actionable mutations were identified throughout dif-
different tumor types and were most commonly found in TP53
(46.3%), KRAS (23.8%), APC (18.8%), STK11 (7.5%), and
ATR (7.5%) genes (Fig. 2, Table S2).
Therapeutic implications of actionable mutations. To explore
the impact of the NGS assay results on subsequent treatment
decision making, we classified the patients based on the avail-
ability of drugs suggested by genomic testing (Fig. 3). Forty-
two patients (60.9%) had genomic alterations that were poten-
tially targetable with an approved or off-label drug in Japan.
Nineteen patients (27.5%) had genomic alterations that were
classified as not targetable with an approved or off-label drug in Japan, but
were targetable with an FDA-approved drug. Eight patients
(11.6%) had genomic alterations that were potentially tar-
targetable with an investigational agent only available in early
clinical trials.
After the NGS assay, nine patients (13.0% of those with
actionable mutations) received subsequent therapy based on
the NGS assay results (Table 2). At the start of treatment
based on the NGS assay results, we discussed the therapeutic
indications and possible treatment options at our molecular
tumor board meeting. The doses and regimen schedules were
adjusted at the discretion of the treating physicians according
to the general condition of each patient. Six patients received
genotyped-directed therapy with molecularly targeted agents
generated by the NGS assay results. Notably, among these
patients, a patient with cancer of unknown primary site was
found to harbor an EGFR mutation, which had not been identi-
cified by conventional hotspot-based gene assays, most likely
because of low allele frequency. This patient was treated with an
EGFR tyrosine kinase inhibitor (erlotinib) and consequently
experienced a remarkable tumor response with improvement in
symptoms. Three patients with BRCA1 or BRCA2 mutations
did not receive genotype-directed therapy with molecularly tar-
targeted agents, such as olaparib, because they were not approved
in Japan at that time. Instead, they received platinum-based

Table 1. Patient demographics and clinical characteristics

| Characteristics                        | Number of patients (%) |
|----------------------------------------|------------------------|
| Sex, no. (%)                           |                        |
| Female                                 | 47 (55.3)              |
| Male                                   | 38 (44.7)              |
| Age, years                             |                        |
| Median                                 | 58                     |
| Range                                  | 8-82                   |
| Indication, no. (%)                    |                        |
| Cancers of unknown primary site        | 13 (15.3)              |
| Rare tumors                            |                        |
| Liposarcoma                            | 1 (1.2)                |
| Malignant schwannoma                   | 1 (1.2)                |
| Calcifying fibrous tumor               | 1 (1.2)                |
| Thymic                                 | 1 (1.2)                |
| Solid tumors refractory to standard chemotherapy |              |
| Pancreatic                             | 19 (22.4)              |
| Biliary tract                          | 14 (16.5)              |
| Colorectal                             | 10 (11.8)              |
| Gastric                                | 6 (7.1)                |
| Lung                                   | 4 (4.7)                |
| Esophageal                             | 3 (3.5)                |
| Liver                                  | 3 (3.5)                |
| Breast                                 | 2 (2.4)                |
| Ovarian                                | 2 (2.4)                |
| Brain                                  | 1 (1.2)                |
| Melanoma                               | 1 (1.2)                |
| Neuroendocrine tumor                   | 1 (1.2)                |
| Peritoneum                             | 1 (1.2)                |
| Uterine body                           | 1 (1.2)                |

Fig. 1. Feasibility of the next-generation sequencing (NGS)-based multiplex gene assay. NGSsuccess means that NGS was successfully completed, and the treating physician could receive the NGS assay results from the laboratory. Also, NGS failure means that NGS was not successfully completed, and the treating physician could not receive the NGS assay results.
therapies because these have been reported to elicit better responses in these patients.\(^22\)\(^–\)\(^24\)

Incidental findings. It is possible that the clinical NGS assay incidently revealed germline variants. With regard to these incidental findings, the American College of Medical Genetics and Genomics (ACMG) has published their recommendations on the management of incidental germline findings from somatic mutation profiling in the clinical setting.\(^25\) Because our gene panel contained 20 of 56 genes for which the ACMG recommends return of pathogenic germline variants, we carefully reviewed whether the mutations found in these 20 genes were derived from germline variants based on the patients’ personal and family histories of cancer and reported allele frequencies. Pathogenicity was determined based on ClinVar and gene-specific databases, such as Insight (https://www.insight-group.org/), LOVD (http://www.lovd.nl/3.0/home), or the database of the University of Utah Department of Pathology and ARUP Laboratories (http://arup.utah.edu/database/BRCA/). Consequently, among 80 patients with NGS success, five patients (6.3%) had a suspected pathogenic germline variant in at least one of the 20 genes: \(BRCA1\) \(n = 1\), \(BRCA2\) \(n = 1\), \(TP53\) \(n = 2\), and \(BRCA1\) and \(TP53\) \(n = 1\) (Table 3).

Patients 1 and 2 did not desire to undergo germline testing because they considered that the result did not influence their subsequent cancer treatment. By Sager sequencing using genomic DNA extracted from peripheral lymphocytes, we confirmed that \(TP53\) p.Gly245Asp in patient 3 and \(TP53\) p.Arg175His in patient 5 were somatic variants and \(BRCA1\) p.Gln934* in patient 5 was a germline variant. Patient 4 had already been diagnosed with hereditary breast and ovarian cancer (HBOC) elsewhere.

**Discussion**
We reviewed our experience with the first consecutive 85 patients who underwent an NGS-based multiplex gene assay in a CLIA-certified laboratory at our institution. The success rate

| Gene | Patients |
|------|----------|
| TP53 | 46.3%    |
| KRAS | 23.8%    |
| APC  | 14.8%    |
| ATR  | 7.5%     |
| STK11| 7.5%     |
| BRCAl| 6.3%     |
| BRCa2| 6.3%     |
| PIK3CA| 6.3%  |
| RB1  | 6.3%     |
| SMAD4| 6.3%     |
| TSHR | 5.6%     |
| AXIN1| 3.8%     |
| CROCN1A| 3.8%  |
| EP300| 3.8%     |
| EPHB2| 3.8%     |
| PTCH1| 3.8%     |
| PTEN | 3.8%     |
| ATM  | 2.5%     |
| BRAF | 2.5%     |
| CDH1 | 2.5%     |
| CEBPA| 2.5%     |
| CTNNB1| 2.5%  |
| CYP2D6| 2.5%  |
| FGFR1| 2.5%     |
| NDRG1| 2.5%     |
| FTO  | 2.5%     |
| SMO  | 2.5%     |
| ATR  | 1.3%     |
| AR   | 1.3%     |
| EGF   | 1.3%     |
| FGFR2| 1.3%     |
| FGFR3| 1.3%     |
| FLJ   | 1.3%     |
| NRAS | 1.3%     |
| IDH1 | 1.3%     |
| IDH2 | 1.3%     |
| IDH3 | 1.3%     |
| JAK2 | 1.3%     |
| MLH1 | 1.3%     |
| NFI  | 1.3%     |
| NF1  | 1.3%     |
| NFE2L2| 1.3%   |
| NFE2L3| 1.3%   |
| RET  | 1.3%     |
| SF3B1| 1.3%     |
| SMARCA4| 1.3%  |
| SRC  | 1.3%     |
| TSC1 | 1.3%     |
Several studies have reported that genomic information facilitates the adoption of better therapeutic strategies and that genotype-directed therapy can improve the clinical outcomes of cancer patients. In fact, we observed notable treatment response in a few patients. This regard, rigorous studies are needed to investigate whether genotype-directed therapy proposed by comprehensive genomic profiling can result in better clinical outcome in cancer patients. Several prospective studies are now underway for verifying the clinical utility of comprehensive genomic profiling.

The number of patients who could receive a therapy according to NGS assay results was relatively limited (13.0% of those with actionable mutations). In general, to receive genotype-directed therapy based on NGS assay results, it is necessary to use an off-label drug or an investigational agent that is available only in early clinical trials. However, in Japan, there are several barriers, such as a high costs and statutory regulations, to accessing these drugs in the clinic. Moreover, available clinical trials are open in limited institutions and for selected patients, thus limiting patient enrollment. In accordance with our experience, previous studies have also pointed out these problems as obstacles to promoting precision medicine in the field of cancer treatment.

To overcome these problems, amendments of social rules and regulations would be needed, to push the application of NGS assay from the research setting into daily clinical practice.

**Table 2. Patients who received subsequent therapy based on next-generation sequencing (NGS) assay results**

| Tumor type | Gene | Mutation | Drugs | Treatment response | References |
|------------|------|----------|-------|-------------------|------------|
| Lung       | PIK3CA | Splice site | AZD5363 | Discontinuation due to adverse effects | Li et al. (35) |
| Gastric    | PIK3CA | p.Glu542Lys | Everolimus | Progressive disease | Janku et al. (36), Loi et al. (37), Deming et al. (38) |
| Unknown primary site | EGFR | p.Leu858Arg | Erlotinib | Remarkable response | Rosell et al. (39), Mok et al. (40), Tsao et al. (41) |
| Pancreatic | PTEN | p.Asp92Glu, p.Cys130Phe | Everolimus | Termination of treatment before evaluation of response due to poor general condition | Wu et al. (42) |
| Biliary tract | ERBB2 | p.Ser130Phe, p.Gly660Asp | Afatinib | Stable disease | Sequist et al. (43), Suzawa et al. (44) |
| Biliary tract | ERBB2 | p.Gly776Arg | Afatinib | Discontinuation due to adverse effects | Sequist et al. (43), Suzawa et al. (44) |
| Unknown primary site | BRCA2 | p.Ser76* | Gemcitabine plus cisplatin | Termination of treatment before evaluation of response due to poor general condition | Lowery et al. (22), Maxwell et al. (23), Golan et al. (24) |
| Pancreatic | BRCA2 | p.Gln3026* | S-1 plus oxaliplatin | Partial response | Lowery et al. (22), Maxwell et al. (23), Golan et al. (24) |
| Liver | BRCA1 | p.Leu52Phe | S-FU plus cisplatin | Termination of treatment before evaluation of response due to poor general condition | Lowery et al. (22), Maxwell et al. (23), Golan et al. (24) |

5-FU, 5-fluorouracil.

**Table 3. Patients with suspected pathogenic germline variants**

| Patient | Age | Sex | Tumor type | Family history | Gene | Mutation | Germline testing† |
|---------|-----|-----|------------|----------------|------|----------|------------------|
| 1       | 44  | M   | Pancreatic | –              | BRCA2 | p.Gln3026*| –                |
| 2       | 78  | F   | Colorectal | –              | TP53  | p.Arg273His| –                |
| 3       | 57  | M   | Colorectal | –              | TP53  | p.Gly245Asp| Somatic          |
| 4       | 39  | F   | Breast (HBOC) | +             | BRCA1 | p.Leu63* | Germline         |
| 5       | 82  | M   | Gastric    | –              | BRCA1 | p.Gln934* | Germline         |

HBOC, hereditary breast and ovarian cancer. †Patients 1 and 2 did not desire to undergo germline testing.
With regard to incidental germline variants, we observed five patients (6.3%) who had a suspected germline variant. Sanger sequencing using genomic DNA showed that one patient (patient 3) had a somatic variant of TP53 and one (patient 5) had somatic and germline variants of TP53 and BRCA1, respectively. One patient with HBOC (patient 4) had a confirmed germline variant of BRCA1. As for the remaining two patients (patients 1 and 2), we provided detailed information about the results to the patients and their families. Although they understood the significance of germline testing, they did not desire further testing because they considered the results would not influence any subsequent cancer treatment. Meric-Bernstam et al. recently reported that 4.3% patients who underwent clinical NGS assays for advanced cancers probably had pathogenic germline variants in one of 19 genes for which the ACMG recommends return of pathogenic germline variants, with BRCA1, BRCA2, and TP53 being the most common. Although there were a few limitations to the present study such as the limited sample size and bias in the types of tumor tested, we estimate that the frequency of incidental germline variants in the Japanese population may be comparable to or lower than in the series of Meric-Bernstam et al.

The analysis of the cancer genome using NGS provides extensive information about the genetic alterations within tumors. In this regard, molecular tumor boards play a pivotal role in the appropriate understanding and clinical use of NGS assay results. In our institution, we regularly hold an institutional multi-disciplinary molecular tumor board meetings comprising medical oncologists, surgical oncologists, pathologists, bioinformaticians, and medical geneticists, and discuss possible treatment options based on NGS assay results and other issues such as incidental findings.

In conclusion, the implementation of clinical sequencing using an NGS-based multiplex gene assay is feasible in the clinical setting. Although the clinical utility of comprehensive genomic profiling has not yet been completely evaluated, we believe that implementation of clinical sequencing using an NGS-based multiplex gene assay would facilitate the rapid molecular classification of tumors and has a great potential for promoting precision cancer medicine.

Acknowledgments

We gratefully thank M. Imano, E. Sasaki, K. Ashida, and M. Funakoshi for their excellent technical assistance and secretarial help. This study was supported by the Practical Research for Innovative Cancer Control and Program for Integrated Database of Clinical and Genomic Information from Japan Agency for Medical Research and Development, AMED.

Disclosure Statement

The authors have declared no conflicts of interest.

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Clinical sequencing in solid tumor patients

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1. A flow of variant filtering process in OncoPrime™.

Fig. S2. DNA yield and concentration of library DNA of each patient.

Fig. S3. Mean depth for sequencing reactions of each patient.

Fig. S4. Histogram of the turnaround time between the date of ordering OncoPrime™ and that of receiving NGS assay results by the treating physician.

Table S1. Genes sequenced in OncoPrime™.

Table S2. Potentially actionable mutations identified in OncoPrime™.

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Supporting Information