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

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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.[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 (VarPROWL) 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.[15] In addition, the evidence behind actionability ranges from sufficient clinical data to only preclinical evidence, and several actionability classification schemes have been proposed.[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/),[19] ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/),[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),[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 (*n* = 19; 22.4%), followed by biliary tract (*n* = 14; 16.5%).

Feasibility of the NGS-based multiplex gene assay. Archival FFPE tumor tissue was available for the NGS assay in 62 patients (Fig. 1). Because the remaining patients did not have appropriate archival FFPE tumor tissue, they underwent endoscopic biopsy (*n* = 7), fine needle biopsy (*n* = 12), or excisional biopsy (*n* = 4) of their primary or metastatic lesions. DNA extracted from the archival FFPE tumor tissue or fresh frozen tumor tissue was used for the NGS assay. The success rate of the NGS assay was 82.3% (51 of 62 patients) when using DNA extracted from archival FFPE tumor tissue, whereas it was 95.7% (22 of 23 patients) when using DNA

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)

extracted from fresh frozen tumor tissue. In 12 of 85 patients (14.1%), the initial NGS assay failed because of insufficient tissue quantity ($n = 3$), failed library preparation ($n = 4$), or high duplicate read rate (>80%) and/or low insert size (<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 alternative 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 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 availability of drugs suggested by genomic testing (Fig. 3). Forty-two patients (60.9%) had genomic alterations that were potentially targetable with an approved or off-label drug in Japan. Nineteen patients (27.5%) had genomic alterations that were 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 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 as suggested 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 identified 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 targeted agents, such as olaparib, because they were not approved in Japan at that time. Instead, they received platinum-based
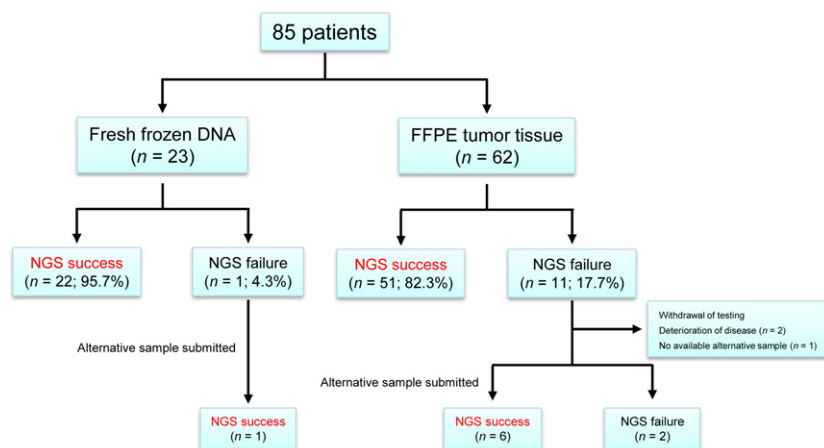

Fig. 1. Feasibility of the next-generation sequencing (NGS)-based multiplex gene assay. NGS success means that NGS was successfully completed, and the treating physician could receive the NGS assay results from the laboratory. Also, NGS failure means that NGS was not successfully completed, and the treating physician could not receive the NGS assay results.
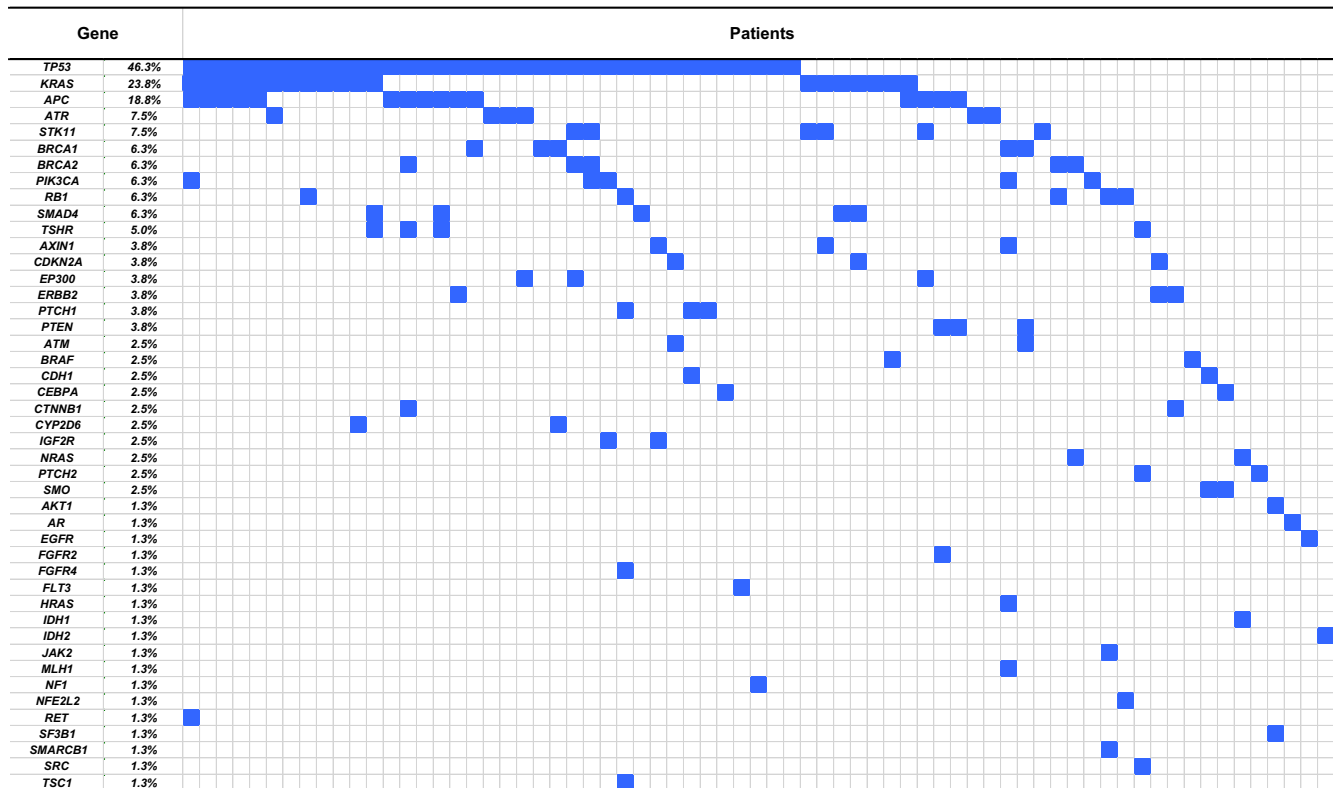
Gene		Patients
TP53	46.3%	
KRAS	23.8%	
APC	18.8%	
ATR	7.5%	
STK11	7.5%	
BRCA1	6.3%	
BRCA2	6.3%	
PIK3CA	6.3%	
RB1	6.3%	
SMAD4	6.3%	
TSHR	5.0%	
AXIN1	3.8%	
CDKN2A	3.8%	
EP300	3.8%	
ERBB2	3.8%	
PTCH1	3.8%	
PTEN	3.8%	
ATM	2.5%	
BRAF	2.5%	
CDH1	2.5%	
CEBPA	2.5%	
CTNNB1	2.5%	
CYP2D6	2.5%	
IGF2R	2.5%	
NRAS	2.5%	
PTCH2	2.5%	
SMO	2.5%	
AKT1	1.3%	
AR	1.3%	
EGFR	1.3%	
FGFR2	1.3%	
FGFR4	1.3%	
FLT3	1.3%	
HRAS	1.3%	
IDH1	1.3%	
IDH2	1.3%	
JAK2	1.3%	
MLH1	1.3%	
NF1	1.3%	
NFE2L2	1.3%	
RET	1.3%	
SF3B1	1.3%	
SMARCB1	1.3%	
SRC	1.3%	
TSC1	1.3%	

Fig. 2. Heat map describing potentially actionable mutations identified in each patient. Each column represents one patient, and each row represents one gene. Potentially actionable mutations are shown in blue.

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 incidentally revealed germline variants. With regard to these incidental findings, the American College of Medical Genetics and Genomics (ACMG) has published their recommendations

8 (11.6%)
19 (27.5%)
42 (60.9%)

■ Patients with genomic alterations that are potentially targetable with an approved or off-label drug in Japan

■ Patients with genomic alterations that are not targetable with an approved or off-label drug in Japan, but are potentially targetable with an FDA-approved drug

■ Patients with genomic alterations that are potentially targetable with an investigational agent that can be used in early clinical trials

Fig. 3. Distribution of patients according to the availability of drugs suggested by next-generation sequencing (NGS) assay results.

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.
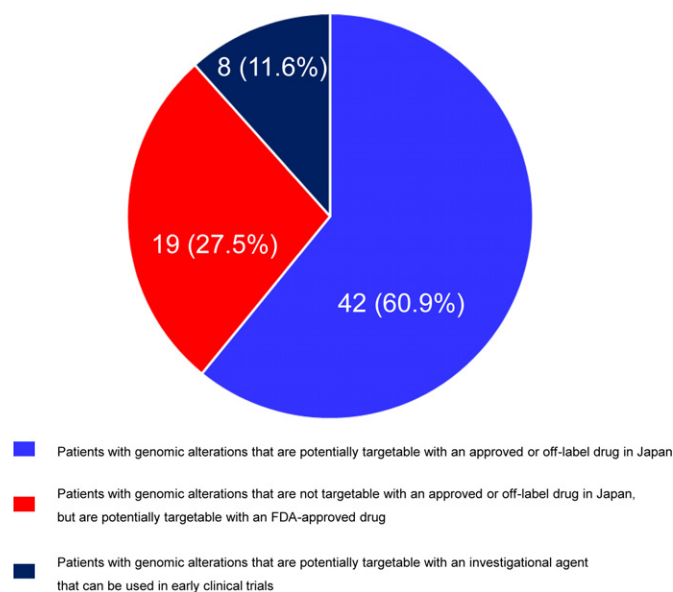
Discussion

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

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

	Tumor type	Gene	Mutation	Drugs	Treatment response	References
Genotype-directed therapy	Lung	PIK3CA	Splice site 814-1G>A	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.Ser310Phe 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]
Genotype-relevant therapy	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	5-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
					TP53	p.Arg175His	Somatic

HBOC, hereditary breast and ovarian cancer. †Patients 1 and 2 did not desire to undergo germline testing.

of the NGS assay using clinical samples such as archival FFPE or fresh frozen tumor tissue was 94.1%, supporting the feasibility of clinical sequencing in the daily clinical practice.

In this study, most (86.3%) of the patients were found to harbor at least one potentially actionable mutation. However, the median TAT was 40 days (range, 18–70), which was not satisfactory for a clinical setting because it caused delay in treatment initiation. One of the main reasons for the long TAT was long transport time because the NGS assay had to be performed in a CLIA-certified laboratory in the United States. A shorter TAT is an essential to promptly initiate cancer treatment, especially for patients with advanced tumors refractory to standard chemotherapy. Indeed, some patients unfortunately missed the chance to receive a treatment based on the NGS assay results because of the deterioration of their disease. Therefore, shortening the TAT is very much required.

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.[26] In fact, we observed notable treatment response in a few patients. In 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.[27,28]

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.[29–31] 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.

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.[32] 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 genomic alterations within tumors. In this regard, molecular tumor boards play a pivotal role in the appropriate understanding and clinical use of NGS assay results.[33,34] 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.

Disclosure Statement

The authors have declared no conflicts of interest.

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Supporting Information

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^{TM}.

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^{TM} and that of receiving NGS assay results by the treating physician.

Table S1. Genes sequenced in OncoPrime^{TM}.

Table S2. Potentially actionable mutations identified in OncoPrime^{TM}.