Published in final edited form as:

Cancer Cytopathol. 2015 May ; 123(5): 289–297. doi:10.1002/cncy.21522.

Comprehensive Mutation Profiling by Next-Generation Sequencing of Effusion Fluids From Patients With High-Grade Serous Ovarian Carcinoma

Ronak H. Shah, MS¹, Sasinya N. Scott, MPH¹, A. Rose Brannon, PhD¹, Douglas A. Levine, MD², Oscar Lin, MD, PhD¹, and Michael F. Berger, PhD^{1,3}

¹Department of Pathology, Memorial Sloan Kettering Cancer Center, New York, New York

²Department of Surgery, Memorial Sloan Kettering Cancer Center, New York, New York

³Human Oncology and Pathogenesis Program, Memorial Sloan Kettering Cancer Center, New York, New York

Abstract

BACKGROUND—Mutation analysis for personalized treatment has become increasingly important in the management of different types of cancer. The advent of new DNA extraction protocols and sequencing platforms with reduced DNA input requirements might allow the use of cytology specimens for high-throughput mutation analysis. In this study, the authors evaluated the use of effusion fluid for next-generation sequencing-based, multigene mutation profiling.

METHODS—Four specimens from each of 5 patients who had at least stage III, high-grade serous ovarian carcinoma were selected: effusion fluid; frozen tumor; formalin-fixed, paraffin embedded tumor; and matched normal blood. Frozen tumors from each patient were previously characterized by The Cancer Genomic Atlas (TCGA). DNA was extracted from all specimens and was sequenced using a custom hybridization capture-based assay. Genomic alterations were compared among all specimens from each patient as well as with mutations reported in TCGA for the same tumors.

RESULTS—In total, 17 distinct somatic mutations were identified in the cohort. Ten of 17 mutations were reported in TCGA and were called in all 3 malignant specimens procured from the patients. Of the remaining 7 mutations, 2 were called in all 3 specimens, and the other 5 were sample-specific. Two mutations were detected only in the cytology specimens. Copy number profiles were concordant among the tumors analyzed.

CONCLUSIONS—Cytology specimens represent suitable material for high-throughput sequencing, because all mutations described by TCGA were independently identified in the

Corresponding authors: Oscar Lin, MD, PhD, Department of Pathology, Memorial Sloan Kettering Cancer Center, 1275 York Avenue, New York, NY 10065; Fax: (212)-639-6318; lino@mskcc.org; Michael F. Berger, PhD, Department of Pathology, Oncology and Pathogenesis Program, Memorial Sloan Kettering Cancer Center, 1275 York Avenue, New York, NY 10065; Fax: (646)-888-2595; bergerm1@mskcc.org.

Additional Supporting Information may be found in the online version of this article.

CONFLICT OF INTEREST DISCLOSURES

The authors made no disclosures.

effusion fluid. Differences in mutations detected in samples procured from the same patient may reflect tumor heterogeneity.

Keywords

next-generation sequencing; cytology; ovarian; carcinoma

INTRODUCTION

Molecular profiling of oncogenic aberrations involving genes and pathways critical to tumor progression increasingly influences the diagnosis and treatment of cancer. The comprehensive identification of DNA mutations and chromosomal alterations allows clinicians to determine the origin of the cancer, its potential for metastasis, its specific drug responsiveness, and the probability of its recurrence.¹ By subjecting patients to molecular profiling as part of their care, clinicians may select personalized therapies according to the specific mutations detected in their tumors.

The advent of next-generation sequencing (NGS) has enabled broader scale genomic profiling because of its lower cost per base pair and higher throughput.² NGS has proven to be a cost-effective method for identifying clinically actionable genetic alterations across multiple genes in solid and hematopoietic tumors as well as alterations associated with inherited cancer-predisposition syndromes.^{3–5} In addition, studies have demonstrated that NGS is capable of detecting the full range of mutation types, including substitutions, insertions and deletions (indels), copy number alterations, and translocations.^{6–9} Coupled with targeted DNA enrichment methods, NGS provides comprehensive, unbiased, and inexpensive mutation analysis of multiple target genes with a single test.^{10–13}

Traditionally, NGS has been used to characterize tissue, partly because, typically, more DNA can be obtained. However, cytologic samples can serve as a suitable alternative to surgical specimens for detecting genetic or epigenetic abnormalities.^{14–16} Cytology specimens are usually safer and more cost effective to obtain than other biopsy modalities. Nevertheless, the suitability of cytologic specimens for large-scale, capture-based NGS applications has been questioned.

The objective of this study was to use a hybridization capture-based NGS assay to analyze cytology specimens and compare the results with those obtained from patient-matched specimens, such as formalin-fixed, paraffin-embedded (FFPE) tissue and frozen tissue. Our results clearly demonstrate that it is possible to detect genomic alterations in cytology specimens with high accuracy and sensitivity and that such material is promising for the identification of biomarkers for targeted cancer therapy.

MATERIALS AND METHODS

Patients and Samples

Five patients from Memorial Sloan Kettering Cancer Center who were diagnosed with high-grade serous carcinoma (HGSC) of the ovary and who presented with effusion fluid (EF) were selected for this study. All 5 patients had their ovarian carcinoma previously analyzed

by The Cancer Genome Atlas (TCGA).¹⁷ DNA was obtained from samples of EF, frozen tumor (FZ), FFPE tumor, and blood as a genetically matched control (Fig. 1, Table 1). EF samples were initially fixed in Cytolyt (Hologic, Marlborough, Mass). Cytospin slides were prepared from the residual material after the cytologic diagnosis was rendered. The slides were subsequently frozen at -80°C until the DNA was extracted. FZ and FFPE specimens were obtained from the surgically resected tumor. Morphologic confirmation of the presence of tumor cells was performed in all specimens. All cytospins were cellular and contained at least 1000 neoplastic cells each. The neoplastic cells represented at least 50% of the cell population in the cytospin. Fifty microliters of Qiagen ATL buffer (Qiagen Inc., Valencia, Calif) were dispensed onto each slide, and cells were scraped into a 1.5-mL tube using a sterile, steel surgical blade. This was repeated twice for each slide, and the blade was rinsed with 30 μL of the buffer, resulting in a final volume of 180 μL . Tumor DNA was extracted from cytospins (1 slide per patient) using the Qiagen QIAmp DNA FFPE Tissue Kit according to the manufacturer's protocol (released April 2010; Qiagen Inc.). Extracted DNA samples were quantified using Life Technologies dsQubit DNA Assay (Life Technologies Corporation, Grand Island, NY).

Targeted NGS by IMPACT

DNA samples from tumors and matched normal tissues were subjected to NGS analysis using our custom IMPACT assay.¹⁸ IMPACT is a targeted exome-capture assay with deep sequencing coverage involving 281 oncogenes, tumor suppressor genes, and components of pathways deemed actionable by targeted therapies (Supporting Table 1; see online supporting information). Target-specific probes for hybrid selection were designed to capture all protein-coding exons and selected introns of these genes. We prepared barcoded sequence libraries (New England BioLabs, Ipswich, Mass; Kapa Biosystems, Inc., Wilmington, Mass) and performed exon capture on barcoded pools by hybridization (Nimblegen SeqCap; Roche Diagnostics USA, Indianapolis, Ind) using an input of 50 to 250 ng of DNA as described previously.¹⁹ The input amount for the cytologic samples was 50 ng DNA; whereas, for virtually all other samples, it was 250 ng (Table 1). Captured pools were sequenced on an Illumina HiSeq 2000 (2×75 -base pair reads; Illumina Inc., San Diego, Calif). To identify mutations and indels, we have developed a unified analytic framework to discover and genotype variation among multiple samples simultaneously with high sensitivity and specificity.

Our framework incorporates many elements that have become standard practice for NGS data analysis, such as: 1) adaptor trimming using Cutadapt²⁰; 2) mapping to the reference human genome (hg19) using the Burrows-Wheeler Alignment tool^{21,22} and duplicate masking using Picard Tools (available at: <http://broadinstitute.github.io/picard/>; accessed January 9, 2015); 3) local realignment around indels and base quality score recalibration using the Genome Analysis Toolkit (GATK) according to GATK best practices^{23,24}; 4) identification of single-nucleotide variants using MuTect,²⁵ small indels using SomaticIndelDetector,^{23,24} and copy number alterations using CNVkit²⁶; 5) annotation of mutation using ANNOVAR²⁷; 6) filtering calls using in-house scripts; and 7) manual review of candidate mutations and indels using the Integrative Genomics Viewer.^{28,29} Genomic alterations are called from tumor-normal pairs to distinguish somatic mutations from

inherited variants. Local realignment is performed jointly for all samples from the same patient to maximize the sensitivity and specificity for detecting somatic indels. To distinguish true low-frequency somatic mutations from systematic sequencing artifacts, we genotype each candidate sequence variant in a collection of unmatched normal samples from multiple sequence runs and remove recurrent false-positive calls.

To compare copy number profiles for TCGA, FZ, FFPE, and EF samples, we used the published segmented copy number values from TCGA and used CNVkit to compute segmented copy number values for each IMPACT data set. All plots and statistical analyses were performed using the freely available R statistics package (R version 2.15.1; R Project for Statistical Computing, Vienna, Austria).

TCGA Data Set

TCGA Binary Alignments/MAP (BAM) format files were obtained using CGHub (available at: <https://cghub.ucsc.edu/>; accessed January 9, 2015), which hosts all of the processed files associated with TCGA data sets. The TCGA mutation files (MAFs) were obtained from the Broad Institute's tumor portal (available at: <http://cancergenome.broadinstitute.org>). The TCGA copy number segmentation data for CN Array were obtained from the TCGA data portal (available at: <https://tcga-data.nci.nih.gov/tcga/dataAccessMatrix.htm>; accessed January 9, 2015).

RESULTS

Performance of Cytology-Derived DNA

For each of 5 patients with metastatic ovarian HGSC, we used our IMPACT assay to sequence genomic DNA from 4 samples: EF, FZ, FFPE, and matched normal blood. All samples were sequenced to >250× unique sequence coverage across 281 target genes, except for the FFPE sample from patient 4, which was covered at only 20× in part because of the older age of the specimen (7 years) and the low input amount of DNA. That sample was retained for comparative purposes only. The mean unique sequence coverage for the 5 EF samples was 879×. It is noteworthy that this high depth of coverage was achieved for all 5 EF samples despite using lower input amounts of DNA (range, 37–50 ng) compared with the solid tumor and blood samples (range, 109–250 ng) (Fig. 2). The greater coverage for the EF samples was achieved by deliberately adding more sequence library into the pooled exon-capture reaction. By comparison, the average sequence coverage produced by whole-exome sequencing for the genes in these samples in TCGA was 166×.

Detection of Known Mutations

First, we examined whether the somatic mutations reported by TCGA were detected by IMPACT analysis of the corresponding samples. Ten of 10 (100%) mutations reported by TCGA in the 281 target genes were called in all 3 tumor-derived samples (EF, FZ, and FFPE) from the same patient (Table 2). (Note that the FFPE sample from patient 4 was an exception, because the sequence coverage was not sufficient for mutation calling; however, all 4 mutations in this patient were detected at 24% allele frequency or higher.) All 5 patients harbored mutations in the tumor protein 53 (*TP53*) gene. It is noteworthy that, in 2 patients

(patients 1 and 3), the allele frequencies of the *TP53* mutations were highest in the cytologic EF samples (82% and 64%, respectively), suggesting that these samples harbored a higher fraction of tumor DNA compared with the corresponding solid tumors (Table 2, Fig. 3a).

Detection of Novel Mutations

Next, we searched for additional mutations that were not called by TCGA but were present in at least 1 of the 3 samples sequenced by IMPACT. We detected 7 such mutations (Table 3). Two mutations (B-cell chronic lymphocytic leukemia/lymphoma 6 [*BCL6*] leucine to serine change at position 677 [L677S] in patient 1 and tumor necrosis factor, α -induced protein 3 [*TNFAIP3*] proline to serine change at position 481 [P481S] in patient 5) were called in all 3 samples that were sequenced. Upon further examination of the TCGA whole-exome data, both mutations were detectable, but the sequence coverage and mutation allele frequencies were not sufficient for them to have been called (Fig. 3b). A third mutation that was detected in the FZ and FFPE tumors from patient 2 (AT-rich interactive domain A1 [*ARID1A*] threonine frameshift mutation at position 292 [T282fs]) also was missed by TCGA, because the sequence coverage at that particular site was very poor (1 $\times$) relative to the average coverage across the whole exome. Together, these results illustrate the increased sensitivity of IMPACT for detecting mutations because of the high depth and uniformity of coverage across target genes.

Four mutations were specific to only 1 of the 3 samples sequenced by IMPACT. Notably, mutations in fibroblast growth factor receptor 3 (*FGFR3*) (valine to leucine change at position 745 [V754L]) and v-myb avian myeloblastosis viral oncogene homolog (*MYB*) (glutamic acid to glycine change at position 194 [E194G]) were called only in the EF sample for patient 1 (Fig. 3c). The absence of these mutations from the FZ and FFPE samples despite sequence coverage exceeding 260 $\times$ at the corresponding genomic sites suggests the existence of tumor heterogeneity and the ability of cytologic specimens to occasionally reveal genomic alterations that are not detected in individual primary lesions from patients with metastatic cancer.

Detection of Copy Number Alterations

Finally, we tested the feasibility of detecting somatic copy number alterations from cytologic specimens. We used CNVkit³⁰ to derive segmented copy number profiles for EF, FZ, and FFPE samples from patient 1. Genes were assigned the copy number value of the segment in which they resided. To assess the reliability of copy number profiles derived from targeted sequencing by IMPACT, we compared the gene-level copy number values from the FZ tumor with the microarray-derived copy number values reported by TCGA. We observed a very high correlation (R^2) between IMPACT and TCGA ($R^2 = 0.907$) (Fig. 4, left). The copy number profiles for the FZ and FFPE samples also were very similar ($R^2 = 0.912$) (Fig. 4, middle). Although the EF sample exhibited a copy number profile that was less concordant ($R^2 = 0.514$) (Fig. 4, right), the same copy number gains and losses were observed at both the chromosome arm level and the focal level (Fig. 5). The correlation of copy number profiles among all specimens from patient 1 is displayed in Supporting Figure 1 (see online supporting information). In summary, our current analysis suggests that, although it is not as precise as sequence analysis of solid tissue specimens, sequence analysis of cytologic

specimens has the potential to reveal structural genomic alterations in addition to sequence mutations.

DISCUSSION

Ovarian HGSCs usually present as advanced-stage disease and are highly aggressive. Despite the initial high response rate to platinum-based chemotherapy in HGSCs, most patients develop recurrent disease after a median progression-free interval of 18 months. Clinically useful genomic predictors of the risk for platinum-resistant relapse are lacking. Understanding the molecular background of ovarian tumorigenesis and chemoresistance may help identify effective means for preventing chemoresistance and improving treatment for and outcomes of ovarian cancer. The molecular characterization of tumors provides a basis for personalizing cancer treatment, resulting in increased clinical benefit, reduced toxicity, and overcoming therapeutic resistance.³¹ Examples of genotype-directed therapies for specific tumor subtypes, such as trastuzumab, pertuzumab, or lapatinib for human epidermal growth factor 2 (*HER2*)-amplified breast and gastric cancers³² or vemurafenib for advanced v-raf murine sarcoma viral oncogene homolog (BRAF) valine at position 600 (V600)-mutant melanoma,³³ have highlighted the potential of personalized medicine.

Our results demonstrate that cytology specimens may represent a suitable alternative for molecular studies, because the tumor concentration might be higher in cytology specimens than in tissue specimens. Cytology specimens are devoid of stromal cells, which can decrease the concentration of tumor cells. In our study, 2 EF samples had higher allele frequencies of *TP53* mutations, suggesting that these samples harbored a higher fraction of tumor DNA compared with the corresponding solid tumors.

Our data also highlighted the increased detection sensitivity of IMPACT because of the deep and uniform sequence coverage afforded by the assay. IMPACT revealed 7 additional mutations that were not reported by TCGA, in part because of differences in sequence coverage. These mutations exhibited supporting evidence in TCGA whole-exome data but not sufficient evidence for the mutations to be called on their own. It should be noted that the inconsistent coverage observed in TCGA data are caused in part by the relative novelty of whole-exome sequencing at the time the analysis was performed.

It has been suggested that cancer heterogeneity may restrict the full potential of precision medicine given that assessment of biomarkers and therapeutic targets based on the analysis of single biopsies may differ according to the area of the sampled tumor.³⁴ This multiregional separation of molecular aberrations can lead to sampling bias,^{35,36} potentially influencing the interpretation of the molecular characterization of tumors and the selection of treatment. To date, most studies have relied on the identification of key molecular biomarkers in 1 representative area. Therefore, the usual practice of analyzing 1 representative area of primary tumors is unlikely to reveal the complete profile of genomic alterations in a given tumor. In the current study, 4 mutations were specific to only 1 of the 3 samples sequenced by IMPACT, suggesting the existence of tumor heterogeneity. This also suggests that cytologic specimens are able to reveal genomic alterations not detected in other

sites. Therefore, sampling of multiple sites would provide a more comprehensive genetic representation of the tumor affecting a particular patient.

With the advent of targeted therapeutics in personalized medicine, NGS embodies a powerful tool for molecular diagnostics and the selection of appropriate therapies and clinical trials for patients with advanced disease. This study evaluated the application of NGS directly to cytology samples. We demonstrated that, with very little DNA (<50 ng), cytology samples can yield extremely high coverage sequence data. Consequently, all mutations that were called by TCGA in the corresponding frozen samples from the same patients were also called in the cytology specimens. In addition, this study identified mutations that were previously missed by TCGA because of low coverage and/or low mutation allele frequencies in those specific regions. In summary, this study demonstrates the feasibility of using NGS-based methods to perform gene mutation analysis in routine cytologic specimens. We believe that NGS-based gene mutation analysis is a valuable addition to the workup of cytology specimens obtained from patients with various tumors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank the Memorial Sloan Kettering Cancer Center Genomics Core Laboratory for their assistance in sequencing and Cyriac Kandoth and Nikolaus Schultz for their help in obtaining The Cancer Genome Atlas data sets and their insights into The Cancer Genome Atlas results.

FUNDING SUPPORT

This work was supported by the Laura Mercier Ovarian Cancer Fund, the Entertainment Industry Foundation Revlon Run/Walk for Women, the Geoffrey Beene Cancer Research Center, and the Farmer Family Foundation.

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Figure 1.

These images of representative samples from a patient with ovarian serous carcinoma include (A) a frozen section stained with hematoxylin and eosin (H&E); (B) a formalin-fixed, paraffin-embedded section of the same tumor (H&E stain); and (C) pleural fluid from the same patient obtained during recurrence (ThinPrep slide; Papanicolaou stain).

Figure 2.

Mean unique target coverage is illustrated for all samples (P1–P5) that were sequenced with the IMPACT assay. All cytology samples (effusion fluid [EF]) samples were sequenced to >800× unique sequence coverage (light blue). FFPE indicates formalin-fixed, paraffin-embedded; FZ, frozen section.

Figure 3.

Evidence supporting selected mutations in patient 1 (P1) is illustrated. The first and second rows display whole-exome data sets of The Cancer Genome Atlas (TCGA) from the Broad Institute (BROAD) and Washington University (WASHU), respectively, using frozen tissue; and the third, fourth, and fifth rows display frozen tumor (FZ); formalin-fixed, paraffin-embedded (FFPE); and effusion fluid (EF) samples, respectively, that were analyzed with the IMPACT assay. DP indicates the total depth of coverage; VF, variant frequency. (a) Tumor protein 53 (*TP53*) mutation H193Y (histidine to tyrosine at position 193) called in all samples is shown. (b) B-cell chronic lymphocytic leukemia/lymphoma 6 (*BCL6*) mutation L677S (leucine to serine change at position 677) called in FZ, FFPE, and EF samples by IMPACT (and not called by TCGA because of lower coverage and mutant allele frequency) is shown. (c) The v-myb avian myeloblastosis viral oncogene homolog (*MYB*) mutation E194G (glutamic acid to glycine change at position 194) was present only in the EF sample.

Figure 4.

The correlation of copy number profiles derived in samples from patient 1 (P1) is illustrated. The mean segmented values from each specimen for each of the 281 target genes are plotted for (*Left*) IMPACT-frozen tumor (FZ) specimens versus The Cancer Genome Atlas (TCGA)-FZ specimens (correlation coefficient [R^2] = 0.91); (*Middle*) IMPACT-FZ samples versus IMPACT-formalin-fixed, paraffin-embedded (FFPE) specimens (R^2 = 0.910); and (*Right*) IMPACT-FZ specimens versus IMPACT-effusion fluid (EF) specimens (R^2 = 0.52).

Figure 5.

Segmented copy number profiles are illustrated for different samples from patient 1 (P1). Copy number profiles for separate samples are displayed in rows, with gains indicated in red and losses indicated in blue. Color shading corresponds to the amplitude of the copy number alteration. Genes that had amplifications and deletions consistently detected in all tumor samples are highlighted. TCGA indicates The Cancer Genome Atlas; EF, effusion fluid; FZ, frozen tumor; FFPE, formalin-fixed, paraffine-mbedded; *EPHB3*, EPH receptor B3; *MAP3K1*, mitogen activated protein kinase kinase 1, E3 ubiquitin protein ligase; *MYC*, *v-myc* myelocytomatosis viral oncogene homolog; *TET1*, tet oncogene 1; *GRIN2A*, glutamate receptor, ionotropic, N-methyl D-aspartate 2A.

Table 1

Description of the Samples Analyzed

Sample	TCGA ID	Tissue Site	Sample Class	Sample Type	Tumor Type	DNA Input Amount, ng	Year
P1	TCGA-13-0894	Peritoneal cavity	Metastasis	Cytospin	Peritoneal effusion	49.8	2008
		Blood	Normal	Blood	NA	250.0	
		Ovary	Primary	Frozen	Resection	250.0	
P2	TCGA-13-0795	Ovary	Primary	FFPE	Resection	250.0	
		Peritoneal cavity	Metastasis	Cytospin	Peritoneal effusion	50.0	2008
		Blood	Normal	Blood	NA	250.0	
P3	TCGA-13-0924	Ovary	Primary	Frozen	Resection	250.0	
		Ovary	Primary	FFPE	Resection	250.0	
		Pleural cavity	Metastasis	Cytospin	Pleural effusion	50.2	2008
P4	TCGA-13-1404	Blood	Normal	Blood	NA	250.0	
		Ovary	Primary	Frozen	Resection	250.0	
		Ovary	Primary	FFPE	Resection	250.0	
P5	TCGA-13-1412	Pleural cavity	Metastasis	Cytospin	Pleural effusion	50.1	2006
		Blood	Normal	Blood	NA	250.0	
		Ovary	Primary	Frozen	resection	250.0	
P5	TCGA-13-1412	Ovary	Primary	FFPE	resection	109.5	
		Pleural cavity	Metastasis	Cytospin	Pleural effusion	37.2	2008
		Blood	Normal	Blood	NA	250.0	
P5	TCGA-13-1412	Ovary	Primary	Frozen	Resection	250.0	
		Ovary	Primary	FFPE	Resection	250.0	

Abbreviations: FFPE, formalin-fixed, paraffin-embedded; ID, identifier; NA, not applicable; TCGA, The Cancer Genome Atlas.

Table 2

Mutations Reported by The Cancer Genome Atlas^a

Sample	TCGA ID	Gene	Protein Change	TCGA		Frozen		FFPE		Cytology (EF)	
				Coverage	Frequency	Coverage	Frequency	Coverage	Frequency	Coverage	Frequency
P1	TCGA-13-0894	<i>TP53</i>	H193Y	139×	0.35	383×	0.50	493×	0.45	1272×	0.82
P2	TCGA-13-0795	<i>TP53</i>	C135Y	113×	0.49	666×	0.47	474×	0.63	1718×	0.09
P3	TCGA-13-0924	<i>DNMT3A</i>	F414S	184×	0.23	796×	0.31	636×	0.40	1641×	0.28
P3	TCGA-13-0924	<i>NCOA2</i>	L1206R	327×	0.24	414×	0.24	586×	0.15	1187×	0.26
P3	TCGA-13-0924	<i>TP53</i>	F270L	153×	0.49	392×	0.39	426×	0.50	935×	0.64
P4	TCGA-13-1404	<i>SETD2</i>	A1699V	286×	0.46	442×	0.38	15×	0.27	312×	0.13
P4	TCGA-13-1404	<i>PTCHI</i>	V988E	24×	0.33	470×	0.36	14×	0.29	1467×	0.12
P4	TCGA-13-1404	<i>TP53</i>	C176Y	50×	0.70	457×	0.63	19×	0.58	1823×	0.17
P4	TCGA-13-1404	<i>JAK3</i>	Q600*	10×	0.40	721×	0.38	21×	0.24	1909×	0.15
P5	TCGA-13-1412	<i>TP53</i>	M237I	288×	0.70	268×	0.59	529×	0.66	1595×	0.07

Abbreviations: A1699V, alanine to valine change at position 1699; C135Y, cysteine to tyrosine change at position 135; C176I, cysteine to threonine change at position 176; *DNMT3A*, DNA (cytosine-5-)-methyltransferase 3α; EF, effusion fluid; F270L, phenylalanine to leucine change at position 270; F414S, phenylalanine to serine change at position 414; FFPE, formalin-fixed, paraffin-embedded; H193Y, histidine to tyrosine change at position 193; ID, identifier; *JAK3*, Janus kinase 3; L1206R, leucine to arginine change at position 1206; M237I, methionine to isoleucine change at position 237; *NCOA2*, nuclear receptor coactivator 2; *PTCHI*, patched homolog 1; Q600*, nonsense glutamine substitution at position 600; *SETD2*, SET domain containing 2; TCGA, The Cancer Genome Atlas; *TP53*, tumor protein 53; 988E, valine to glutamic acid change at position 988.

^aTen of 10 (100%) mutations identified in TCGA were detected in the frozen, FFPE, and cytology samples that were sequenced with the custom hybridization capture-based IMPACT assay. Total sequence coverage at the site of each mutation and the mutant allele frequency are listed. TCGA data represent the collective aggregate of data from the Broad Institute and Washington University.

Table 3

Mutations Unique to the Custom Hybridization Capture-Based IMPACT Assay^a

Patient	TCGA ID	Gene	Protein	TCGA		Frozen		FFPE		Cytology(EF)	
				Coverage	Frequency	Coverage	Frequency	Coverage	Frequency	Coverage	Frequency
P1	TCGA-13-0894	<i>BCL6</i>	L677S	145×	0.08	432×	0.19	456×	0.12	2435×	0.52
P1	TCGA-13-0894	<i>FGFR3</i>	V754L	5×	0.0	359×	0.0	324×	0.0	1236×	0.09
P1	TCGA-13-0894	<i>MYB</i>	E194G	521×	0.0	260×	0.0	286×	0.0	96×	0.26
P2	TCGA-13-0795	<i>ARID1A</i>	T292fs	1×	0.0	59×	0.10	173×	0.20	168×	0.01
P3	TCGA-13-0924	<i>GLI3</i>	R989Q	7×	0.0	60×	0.0	73×	0.16	182×	0.005
P3	TCGA-13-0924	<i>NFI</i>	V153fs	148×	0.0	134×	0.007	169×	0.10	96×	0.0
P5	TCGA-13-1412	<i>TNFAIP3</i>	P481S	57×	0.14	278×	0.33	575×	0.28	1235×	0.03

Abbreviations: *ARID1A*, AT-rich interactive domain A1; *BCL6*, B-cell chronic lymphocytic leukemia/lymphoma 6; E194G, glutamic acid to glycine change at position 194; EF, effusion fluid; FFPE, formalin-fixed paraffin-embedded; *FGFR3*, fibroblast growth factor receptor 3; *GLI3*, GLI family zinc finger 3; ID, identifier; L677S, leucine to serine change at position 677; *MYB*, v-myb avian myeloblastosis viral oncogene homolog; *NFI*, neurofibromin 1; P481S, proline to serine change at position 481; R989Q, arginine to glutamine change at position 989; T292fs, threonine frameshift mutation at position 292; TCGA, The Cancer Genome Atlas; *TNFAIP3*, tumor necrosis factor, α -induced protein 3; V153fs, valine frameshift mutation at position 153; V754L, valine to leucine change at position 745.

^aListed are 7 mutations not reported by TCGA that were detected in 1 or more samples analyzed using the IMPACT assay. The depth of coverage and mutant allele frequency are listed. Shaded cells represent mutations that were present in the indicated sample.