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Implementation of a Multicenter Biobanking Collaboration for Next-Generation Sequencing-Based Biomarker Discovery Based on Fresh Frozen Pretreatment Tumor Tissue Biopsies

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Disclosures of potential conflicts of interest may be found at the end of this article.

Abstract

Background. The discovery of novel biomarkers that predict treatment response in advanced cancer patients requires acquisition of high-quality tumor samples. As cancer evolves over time, tissue is ideally obtained before the start of each treatment. Preferably, samples are freshly frozen to allow analysis by next-generation DNA/RNA sequencing (NGS) but also for making other emerging systematic techniques such as proteomics and metabolomics possible. Here, we describe the first 469 image-guided biopsies collected in a large collaboration in The Netherlands (Center for Personalized Cancer Treatment) and show the utility of these specimens for NGS analysis.

Patients and Methods. Image-guided tumor biopsies were performed in advanced cancer patients. Samples were fresh frozen, vital tumor cellularity was estimated, and DNA was isolated after macrodissection of tumor-rich areas. Safety of the image-guided

biopsy procedures was assessed by reporting of serious adverse events within 14 days after the biopsy procedure.

Results. Biopsy procedures were generally well tolerated. Major complications occurred in 2.1%, most frequently consisting of pain. In 7.3% of the percutaneous lung biopsies, pneumothorax requiring drainage occurred. The majority of samples (81%) contained a vital tumor percentage of at least 30%, from which at least 500 ng DNA could be isolated in 91%. Given our preset criteria, 74% of samples were of sufficient quality for biomarker discovery. The NGS results in this cohort were in line with those in other groups.

Conclusion. Image-guided biopsy procedures for biomarker discovery to enable personalized cancer treatment are safe and feasible and yield a highly valuable biobank. *The Oncologist* 2017;22:33–40

Implications for Practice: This study shows that it is safe to perform image-guided biopsy procedures to obtain fresh frozen tumor samples and that it is feasible to use these biopsies for biomarker discovery purposes in a Dutch multicenter collaboration. From the majority of the samples, sufficient DNA could be yielded to perform next-generation sequencing. These results indicate that the way is paved for consortia to prospectively collect fresh frozen tumor tissue.

INTRODUCTION

In oncology, the prediction of treatment outcome remains an important issue. The number of available treatments steadily increases and re-emphasizes our need for guidance on which treatment to choose for a specific patient. Hypothesis-driven biomarkers have been successful: For example, *BRAF* mutations in melanoma predict response to BRAF inhibitors [1]. However, other effective treatments such as immune checkpoint blockers and novel targeted treatments often lack obvious hypothesis-driven biomarkers. Therefore, unbiased, large-scale approaches such as next-generation DNA/RNA sequencing (NGS), proteomics, and metabolomics may improve the search for more and better predictive biomarkers. To enable the use of these large-scale technologies on clinical samples, it is essential to start the systematic collection of well-annotated tissue samples. Because snap freezing is considered the most optimal preservation method for nucleic acids as well as proteins and metabolites, this should be the preferred way clinical samples intended for current and future biomarker discovery are processed.

One major issue in biomarker discovery remains the heterogeneity of tumors. Genetic heterogeneity has been described extensively, and this heterogeneity spans both temporal and spatial differences [2–5]. Consequently, any biomarker discovery study should try to minimize the time elapsing between sampling and treatment and document the sampling site. Moreover, imaging and pathology studies have shown extensive intralesional heterogeneity with respect to important features such as angiogenesis, oxygen supply, energy consumption, and stromal content [6–8]. This heterogeneity will cause a baseline variability despite any effort to homogenize the sampling time and location. Thus, obtaining a large enough sample size to average out our baseline variation is required for the detection of true differences. The actual sample size needed to detect a meaningful difference remains an elusive matter. However, despite all these potential factors that may cause failure to find novel biomarker profiles, there is an increasing number of successful examples of biomarker detection using NGS, including a study that showed that novel T-cell epitopes predict efficacy of immune therapy, in which the authors were able to detect a meaningful difference in a sample of 11 responders and 14 non-responders [9]. Therefore, collecting materials from patients who undergo specified treatments could yield interesting results even when only limited patient numbers are included, given the proper preservation of such materials.

Any large-scale technology that uses unbiased data collection suffers from difficulty in analysis because of the amount of data generated. This problem needs to be addressed at the start of any sample collection project. For NGS-based DNA sequencing the collection of adequate germline samples is essential for the detection of somatic genetic alteration. Also, sampling multiple times from the same patient allows detection of resistance mechanisms [10–12]. Thus any protocol should encourage repetitive sampling.

In The Netherlands, all large oncology centers, including the nine academic centers, are now collaborating in the Center for Personalized Cancer Treatment (CPCT). The CPCT has set up a pipeline for the collection of fresh frozen tumor tissue and for storage in a central biobank. In parallel, all relevant clinical data are recorded in an electronic case record form and can be linked to the results of the tests performed on the tumor material. The primary objective of this biobanking effort is to analyze the individual cancer genome in advanced cancer patients to develop future predictors for response to systemic treatment. Here, we show that it is feasible to set up such a multicenter initiative by presenting the safety of the first 469 image-guided tumor biopsy procedures and by providing the DNA sequencing results of a selected set of 73 biopsy specimens.

PATIENTS AND METHODS

Study Design

To obtain research-related biopsies from advanced cancer patients without curative treatment options, the institutional review board of the participating centers approved a protocol. An important characteristic of the protocol was that it allowed the recruitment of patients with all solid tumor types and multiple treatment protocols. Therefore, it was called the "umbrella" biopsy protocol (NCT01855477). This umbrella protocol was a prospective multicenter trial protocol in which biopsies are obtained to perform next-generation sequencing on fresh frozen biopsy specimens to allow for biomarker detection as well as exploratory biomarker discovery. Patients did not receive systemic treatment as part of this protocol itself. Patients participated in the umbrella protocol and received systemic treatment, either standard of care or experimental treatment, within a different protocol.

Within the umbrella protocol a baseline biopsy procedure was performed, and clinical data were collected, including radiological response data. The protocol allowed for multiple biopsy procedures at different time points to document changes in genetic profiles upon treatment. Study related procedures were (a) screening procedures to ascertain eligibility and safety of the biopsy procedure, (b) biopsy procedures, and (c) a blood draw to determine germline DNA. The umbrella protocol defined radiological tumor assessments within 8 to 12 weeks after the start of the first initiated treatment after baseline biopsy. The study was conducted in accordance with the latest versions of the Declaration of Helsinki and Good Clinical Practice guidelines.

Patient Selection

All patients provided written informed consent before any of the study-related procedures. Patients ages ≥18 years with a locally advanced or metastatic solid tumor without curative treatment options were eligible for inclusion. Patients were eligible only if systemic treatment according to standard of care or with experimental anticancer agents was planned. Eligible patients had an Eastern Cooperative Oncology Group performance status of 0 (asymptomatic) to 2, measurable lesions according to Response Evaluation Criteria in Solid Tumors [13], and adequate renal and hepatic functions. Patients with a history of bleeding disorders or bleeding complications, using anticoagulant medication in which discontinuation of anticoagulants was unadvisable, and patients with a contraindication for lidocaine and, if applicable, midazolam or phentanyl (or their derivatives) were excluded. Biopsy of a locally advanced or metastatic lesion had to be considered safe according to the intervening physician.

Blood Sample Collection and Processing

Tumor-matched blood samples were collected to determine patient's germline variation. This information was used to differentiate between somatic and germline mutations in the tumor and was specifically not used to detect cancer predisposition. Venous blood was collected in K2EDTA tubes. Blood samples were shipped at room temperature to the central core facility of the CPCT for subsequent processing.

Biopsy Procedure

Percutaneous biopsy procedures were performed under ultrasound or computed tomographic guidance after local anesthesia (and in incidental cases under conscious sedation). Whether a guiding needle was used mainly depended on tumor localization and on the preference of the individual physician. We aimed to retrieve two to four core biopsy specimens, preferably with at least an 18-gauge biopsy needle. If appropriate, a gastroenterologist performed an endoscopic (ultrasound) guided procedure using a 19-gauge endoscopic ultrasound histology needle under sedation with midazolam and opioids (phentanyl) for pain relief. When we suspected possible complications with patients, we used ultrasound or computed tomography (CT) to check for major complications (e.g., pneumothorax or initial bleeding complications).

Biopsy Sample Processing

Biopsy specimens were labeled and snap-frozen directly after the biopsy procedure. Subsequently, the specimens were stored at $-80\,°C$ until they were shipped on dry ice to the central core facility of the CPCT.

Histological Assessment

From each biopsy, 4-μm frozen sections were cut and stained for hematoxylin and eosin. A dedicated pathologist (S.M.W. or P.J.D.) performed histological assessment to confirm the presence of tumor tissue as well as the percentage of tumor cells based on the quantity of nuclei and tumor cell vitality. Tissue morphology was comparable to frozen sections and allowed for reliable confirmation of the presence of cancer. Obvious tumor-rich islands within the sections were marked to obtain an optimal tumor cellularity and quality and to facilitate macrodissection, during which regions of interest were scraped off with a scalpel and collected in phosphate-buffered saline solution. Only when the percentage of vital tumor cells was at least 30%, we proceeded to DNA isolation after macrodissection of indicated areas.

DNA Isolation

DNA was isolated from 500 μL of whole blood and from approximately five macrodissected 20-μm sections using the NorDiag Arrow machine (Isogen Life Science, De Meern, The Netherlands, http://www.isogen-lifescience.com) for isolation and purification of the DNA. DNA extraction was performed in batches (1 to 12 samples per run) using 230 μL of lysis buffer and 20 μL of proteinase K and comprised two washing steps with a final elution volume of 100 μL, according to the manufacturer's protocol. DNA quantity was measured with the Qubit 2.0 fluorometer (Thermo Fisher Scientific Life Sciences, Waltham, MA, http://www.thermofisher.com). Depending on DNA quantity, the protocol was repeated on additional tissue

sections to aim for a DNA quantity of at least 500 ng of DNA. DNA was stored at $-20\,°C$ until sequencing was performed.

Safety Evaluation

Observation after the biopsy procedure was performed according to local protocols. No observation was required for patients undergoing superficial tumor biopsies (e.g., biopsy of a subcutaneous lesion or low-risk biopsy of a superficial lymph node). After a percutaneous lung biopsy, a chest x-ray was routinely performed after 1 to 4 hours, depending on local protocols, which in some cases required overnight hospitalization. After all other biopsy procedures, patients were clinically observed for 1 to 4 hours.

Biopsy procedures of individual patients were included in the safety evaluation if specimens for research purposes had been retrieved. All major complications, defined as any adverse events grade 3 or higher related to the biopsy procedure, and all serious adverse events (SAE) occurring within 14 days after tumor biopsy, were registered prospectively. Adverse events were graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events (version 4) [14]. An SAE was defined in the protocol as any complication that resulted in death, was life threatening, required prolonged hospitalization, resulted in persistent or significant disability or incapacity, or was a congenital anomaly or birth defect. Clinical observation or hospitalization to facilitate biopsy procedures was not considered a criterion for seriousness. Special attention was paid to the occurrence of bleeding complications and to pneumothorax after transthoracic biopsy. Pneumothorax and hematothorax are treated invasively at grade 2 and were therefore registered at that grade or higher.

Feasibility

Within the umbrella protocol, retrieval of research specimens for biomarker analyses could be combined with a biopsy procedure for diagnostic assessment. All the image-guided biopsy procedures during which research specimens were retrieved were evaluable for quantification of vital tumor cellularity. Sequencing was performed if DNA yield was at least 500 ng. Performing extended sequencing on paired blood samples (germline DNA) allowed for filtering for true somatic mutations in tumor samples.

DNA Sequencing

Two different platforms have been used for DNA sequencing, that is, a targeted panel analysis using SOLiD sequencing and exome sequencing analysis using Illumina sequencing.

For SOLiD, single nucleotide variants and insertions or deletions (INDELs) were detected by targeted sequencing of a designed "cancer mini-genome" consisting of 1,977 cancer genes (supplemental online Table 1). Barcoded fragment libraries were generated from 2 μg of isolated DNA from tumor and control samples, as described previously [15]. Pools of libraries were enriched for 1,977 cancer-related genes (cancer mini-genome [16] using SureSelect technology [Agilent, Santa Clara, CA, http://www.agilent.com/home]). Enriched libraries were sequenced on a SOLiD 5500xl instrument, according to the manufacturer's protocol. Reads were mapped on the human genome (GRCh37) by using Burrows-Wheeler Aligner (BWA) [17] with the following parameters: -c $-$l 25 $-$k 2 $-$n 10. Variant calling was done using a custom pipeline identifying

Table 1. Baseline characteristics

| Characteristic	Biopsied patients (N = 450)	Sequenced biopsies (N = 73)
Age, mean (SD)	59 (11)	58 (11)
Sex		
Male	239 (53%)	37 (51%)
Female	211 (47%)	36 (49%)
Primary tumor		
GI: CRC	99 (22%)	16 (22%)
Lung cancer	61 (14%)	3 (4%)
Breast cancer	49 (11%)	5 (7%)
Melanoma	44 (10%)	16 (22%)
Hepatobiliary cancer	37 (8%)	6 (8%)
GI: other	32 (7%)	7 (10%)
Gynecological cancer	31 (7%)	5 (7%)
GU cancer	31 (7%)	2 (3%)
Other	27 (6%)	6 (8%)
Sarcoma	26 (6%)	4 (5%)
Head/neck cancer	13 (3%)	3 (4%)

Abbreviations: CRC, colorectal cancer; GI, gastrointestinal tract; GU, genitourinary tract.

variants with at least $10\times$ coverage, an allele frequency of 15%, and multiple (>2) occurrences in the seed (the first 25 base pairs [bp] most accurately mapped part of the read), as well as support from independent reads (>3). All variant positions identified were subsequently genotyped in the raw datasets of all samples using SAMtools mpileup (SourceForge.net, http://samtools.sourceforge.net/mpileup.shtml) to ensure the presence or absence of possible low-frequency variants. To identify somatic mutations, we excluded all variants identified in both tumor and blood from further analysis.

The Illumina data were processed with an in-house developed pipeline (version 1.2.1) (https://github.com/CuppenResearch/IAP), including GATKv3.2.2 [18], according to the best-practices guidelines [19]. Briefly, we mapped the pairs with BWA-MEM v0.7.5a [17], marked duplicates, merged lanes, and realigned INDELs. Base recalibration did not improve our exome results, so this step was skipped. Next, GATK Haplotypecaller was used to call single nucleotide polymorphisms (SNPs) and INDELs. Variants are flagged as PASS only if they do not meet the following criteria: $QD < 2.0$, $MQ < 40.0$, $FS > 60.0$, $HaplotypeScore > 13.0$, $MQRankSum < -12.5$, $ReadPosRankSum < -8.0$, snpclusters ≥ 3 in 35 bp. For INDELs: $QD < 2.0$, $FS > 200.0$, $ReadPosRankSum < -20.0$. Effect predictions and annotation were added using snpEFF [20] and dbNSFP [21]. Somatic mutation is determined by providing the reference and tumor sequencing data to the following algorithms: Strelka v1.0.14 [22], Varscan v2.3.7 [23], and Freebayes v0.9.20 [24]. High-confident variants are determined by the tool-filtering steps and merged to a single .vcf file.

Statistical Analysis

All baseline patient characteristics, image-guided biopsy procedure characteristics, and other described analyses were performed using descriptive statistics (Microsoft Excel 2010;
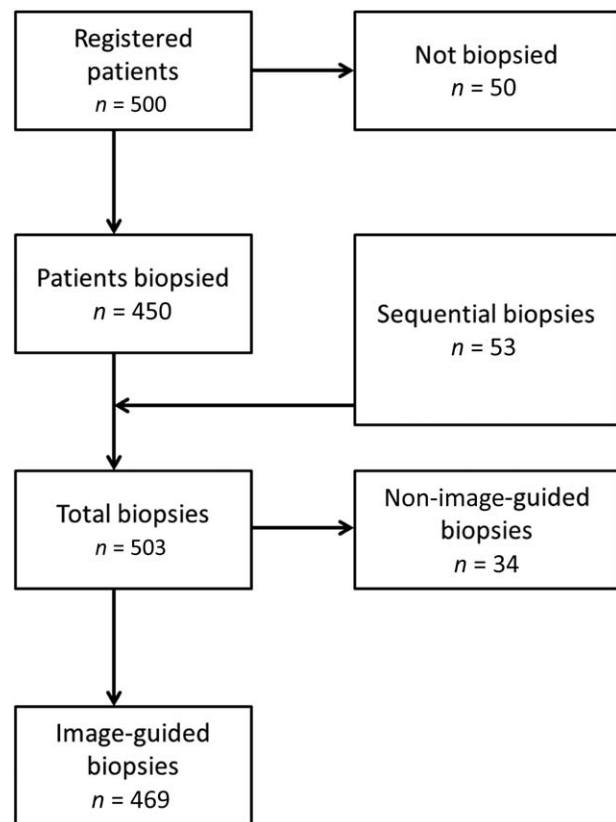
Figure 1. Study flowchart.

Microsoft, Redmond, WA, https://www.microsoft.com/en-us). Tumor cellularity and DNA yield were recorded as continuous variables but were grouped (on the basis of our preset criteria) to allow for descriptive analysis.

RESULTS

Baseline Characteristics

From August 17, 2011, until December 31, 2013, a total of 500 patients signed informed consent and were included in the study. In 50 patients the biopsy procedure was not performed, because the procedure was not deemed safe or because of clinical progression before the planned biopsy. In Table 1, baseline characteristics are depicted for the 450 biopsied patients, of which the majority had been diagnosed with breast cancer, lung cancer, colorectal cancer, or melanoma.

Biopsy Procedures

In order to be evaluable for this study, patients had to be biopsied at least once before the start of designated treatment. We attempted to obtain other biopsies during or directly after treatment. Multiple biopsies for study purposes were performed in 44 patients, that is, two biopsies in 37 patients, three biopsies in 5 patients, and four biopsies in 2 patients. Of the 503 biopsy procedures in this study, 469 were performed under image guidance of ultrasonography, CT scan, or endoscopy (Fig. 1, Table 2). Most image-guided biopsies were performed on the liver ($n = 185$; 39%). Other abdominal organs ($n = 94$; 20%) and intrathoracic organs ($n = 56$; 12%) were also biopsied frequently. Superficial lesions such as cutaneous, subcutaneous,

Table 2. Biopsy characteristics

Characteristic	Image-guided biopsies (N = 469)
Biopsy timing	
Baseline	419 (89%)
On/post-treatment	50 (11%)
Biopsy localization	
Abdominal: liver	185 (39%)
Abdominal: other	94 (20%)
Superficial[a]	120 (26%)
Thoracic[b]	56 (12%)
Bone	14 (3%)
Imaging modality	
CT scan	101 (22%)
Ultrasonography	360 (77%)
Endoscopy[c]	8 (2%)

[a]Superficial lesions include all subcutaneous tumors, superficial lymph nodes, and breast tumors.
[b]Thoracic lesions include pulmonary tumors, pleural tumors, intra-thoracic lymph nodes, and thymic tumors.
[c]Endoscopy was performed as gastroscopy ($n = 5$), colonoscopy ($n = 1$), bronchoscopy ($n = 1$), or endoscopic ultrasonography ($n = 1$).
Abbreviation: CT, computed tomography.

and soft tissue lesions were biopsied in 120 procedures (26%) and osseous lesions in 14 (3%).

Treatment Details
Of all biopsied patients, 324 (72%) were subsequently treated with systemic therapy. The majority of these patients were treated with targeted agents (Table 3).

Safety
Adverse events occurred after 10 image-guided biopsy procedures (2.1%; Table 4). Four patients experienced grade 3 pain, one patient had grade 3 hypertension, and one patient experienced grade 3 vasovagal reaction. Of the 41 patients who underwent percutaneous CT-guided lung biopsy, three patients (7.3%) suffered from pneumothorax, for which drainage was indicated (grade 3 in two patients and grade 2 in one patient). Grade 2 pleural hemorrhage was observed once after a CT-guided liver biopsy of a metastatic lesion that was situated directly subdiaphragmatic. In this case, drainage was required, but treatment was not delayed.

Tumor Cells and DNA Yield
From 20 patients who underwent image-guided biopsies, no samples were sent in for analysis, because all material was used for standard-of-care treatment. In 363 of the remaining 449 image-guided biopsy-retrieved specimens (81%), we found a tumor cell percentage of 30% or more. Of the 86 tumor specimens with an insufficient percentage of tumor cells, 40 did not contain tumor cells at all.

A sufficient amount of DNA (i.e., 500 ng or more) was obtained from 331 of the 363 biopsy specimens containing > 30% tumor cells. From 14 of these specimens, DNA had to be isolated a second time to retrieve the required amount of DNA. These 331 specimens (74% of the 449 image-guided biopsy-retrieved specimens received at the central core

Table 3. Treatment details of all biopsied patients

Treatment detail	Subjects (N = 450)
Treatment	
Classical chemotherapy	83 (18%)
Phase I drug(s)	76 (17%)
Everolimus	51 (11%)
VEGF TKI (sunitinib/sorafenib/pazopanib)	37 (8%)
Monoclonal antibody	26 (6%)
Vemurafenib	23 (5%)
Antihormonal therapy	16 (4%)
Other TKI	12 (3%)
No treatment started	126 (28%)
Treatment duration (months)	
Observations	301
Median (range)	1.91 (0.00–20.24)
Median (IQR)	1.91 (0.92–3.88)
Lost to follow-up	23
Response at first evaluation	
Complete response	1 (0%)
Partial response	39 (13%)
Stable disease	129 (43%)
Progressive disease	110 (37%)
Not evaluable	2 (1%)
Not done	12 (4%)
Lost to follow-up	8 (3%)

Abbreviations: IQR, interquartile range; TKI, tyrosine kinase inhibitor; VEGF, vascular endothelial growth factor.

facility) met our preset criteria to perform DNA sequencing. For all three centers individually, the proportion of samples that met the criteria was 70% or higher and did not differ significantly between the centers ($p = .77$; chi-square test).

DNA Sequencing
At data cut-off for this analysis, the sequencing results from 73 biopsied specimens were available. DNA data could be retrieved from all specimens. On SOLiD ($n = 54$) we sequenced samples for the 1,977-gene panel until a minimum mean coverage of $150 \times$ was reached (mean of $185\times$). For exome analysis on Illumina ($n = 19$) we sequenced reference samples at least $\sim75 \times$ (mean of $95\times$) and tumor $\sim150 \times$ (mean of $185\times$). The most frequently mutated genes were *TP53, APC*, and *BRAF* (Table 5).

DISCUSSION
With these results we have shown that it is feasible to set up large, multicenter logistics to biobank image-guided retrieved tumor biopsies. In several other retrospective studies, it has been shown that research-related biopsies are safe and feasible [25–28]. Description of large biopsy series have generally reported on comparable frequencies of major complications [29–31]. The incidence of pneumothorax requiring drainage after percutaneous lung biopsies (3 of 41 biopsied patients) was similar to that described by El-Osta et al. [32] (2 of 42). Importantly, the additional value of our series is that we have

Table 4. Adverse events

Adverse event	Grade	Related to biopsy	Biopsied organ	Duration of hospitalization
Pain	3	Definite	Abdomen (US guided)	NA
Pain	3	Definite	Liver (US guided)	Hours[a]
Pain	3	Definite	Paravertebral mass (US guided)	NA
Pain	3	Possible	Liver (US guided)	Days
Vasovagal reaction	3	Definite	Liver (US guided)	NA
Hypertension	3	Possible	Abdomen (US guided)	Hours[a]
Pneumothorax	3	Definite	Lung (CT guided)	Days
Pneumothorax	3	Definite	Lung (CT guided)	Days
Pneumothorax	2	Definite	Lung (CT guided)	Days
Pleural hemorrhage	2	Definite	Liver (CT guided)	Days

[a]Two patients were admitted to the hospital for several hours after the biopsy procedure and were discharged on the same day.
Abbreviations: CT, computed tomography; NA, not applicable; US, ultrasonography.

Table 5. Overview of mutated genes categorized by histological origin shown as n (%)

Gene	Total ($N = 73$)	Breast cancer ($n = 5$)	GI cancer, CRC ($n = 16$)	GI cancer, non-CRC ($n = 7$)[a]	Gynecological cancer ($n = 5$)[b]	Head/ neck cancer ($n = 3$)[c]	Hepatobiliary cancer ($n = 6$)[d]	Lung cancer ($n = 3$)[e]	Melanoma ($n = 16$)	Other ($n = 12$)[f]
TP53	34 (47)	2 (40)	11 (69)	5 (71)	2 (40)	1 (33)	4 (67)	1 (33)	3 (19)	5 (42)
APC	15 (21)	1 (20)	9 (56)	0 (0)	0 (0)	1 (33)	0 (0)	0 (0)	3 (19)	1 (8)
BRAF	13 (18)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	13 (81)	0 (0)
CCNB3	12 (16)	1 (20)	2 (13)	0 (0)	2 (40)	0 (0)	1 (17)	0 (0)	4 (25)	2 (17)
LRP2	12 (16)	1 (20)	4 (25)	0 (0)	2 (40)	1 (33)	1 (17)	0 (0)	2 (13)	1 (8)
ATRX	11 (15)	1 (20)	1 (6)	0 (0)	1 (20)	0 (0)	1 (17)	0 (0)	5 (31)	2 (17)
CTBP2	11 (15)	0 (0)	0 (0)	2 (29)	0 (0)	0 (0)	2 (33)	0 (0)	5 (31)	2 (17)
FAT3	11 (15)	1 (20)	2 (13)	2 (29)	1 (20)	0 (0)	1 (17)	0 (0)	3 (19)	1 (8)
KRAS	11 (15)	0 (0)	6 (38)	1 (14)	0 (0)	0 (0)	1 (17)	1 (33)	0 (0)	2 (17)
OBSCN	11 (15)	1 (20)	0 (0)	0 (0)	2 (40)	0 (0)	2 (33)	0 (0)	4 (25)	2 (17)
PIK3CA	10 (14)	3 (60)	2 (13)	1 (14)	2 (40)	0 (0)	0 (0)	0 (0)	1 (6)	1 (8)

[a]Esophageal cancer ($n = 3$), stomach cancer ($n = 2$), and small intestine cancer ($n = 2$).
[b]Cervical cancer ($n = 2$), endometrial cancer ($n = 2$), and ovarian cancer ($n = 1$).
[c]Laryngeal cancer ($n = 1$), oral cavity cancer ($n = 1$), and pharyngeal cancer ($n = 1$).
[d]Liver cancer ($n = 3$) and pancreatic cancer ($n = 3$).
[e]Non-small cell lung cancer ($n = 2$) and other lung cancer ($n = 1$).
[f]Thyroid cancer ($n = 1$), soft tissue sarcoma ($n = 4$), kidney cancer ($n = 2$), and cancer of unknown primary ($n = 5$).
Abbreviations: CRC, colorectal cancer; GI, gastrointestinal.

also shown that it is feasible to extract sufficient DNA from the majority of the biopsy specimens to perform analyses such as NGS. Moreover, because all samples are processed at a central location within the CPCT, uniformity of the analyses is ensured. We could retrieve sufficient DNA for NGS from 74% of the image-guided retrieved biopsy specimens. Although this may be too low for a regular diagnostic test, we feel this hit rate justifies systematic tissue collection in this manner, because similar proportions have been reported in other series [26] and because this hit rate is therefore likely to represent the true potential of image-guided tumor biopsies. For the specimens that did not meet the criteria for DNA sequencing, we found that 86 of the 118 specimens contained less than 30% tumor cells. Retrospectively, we cannot discern whether this low tumor cell percentage is due to issues with the biopsy procedure or due to intratumoral aspects, such as heterogeneity. As sequencing techniques advance, specimens with lower tumor cell percentage can probably be sequenced in the future, but

especially in these specimens it will remain challenging to determine the clinical relevance of infrequent aberrations.

The DNA sequencing data for the first 73 biopsy specimens are largely concordant with the results from the Cancer Genome Atlas (TCGA) [33]. Alterations in *TP53, APC, KRAS*, and *PIK3CA* were among the most frequently found genomic aberrations across all tumor types. The higher incidence of *PTEN* and *VHL* in the TCGA set and of *BRAF* in our set is likely to be caused by the difference in tumor types between the two sets: The TCGA set contains glioblastoma multiforme samples and many samples, relatively, from gynecological and kidney cancers, whereas our set contains a large number of melanoma samples. By sequencing germline DNA as a reference for the intratumoral findings, we were bound to detect hereditary mutations, as had been foretold almost a decade ago [34]. The way these findings have been handled in our consortium has been published separately [35].

By establishing a multi-institutional pipeline for large-scale collection of fresh frozen tumor material, we have shown that it

is possible for consortia to prospectively collect high-quality fresh frozen tumor tissue. In our collaboration, we have set up a unique framework in which tumor biopsies are obtained prior to standard-of-care systemic treatment and in which these biopsies are stored in a way that enables us to perform not only NGS, but also many other analyses on RNA, protein, epigenetic processes, or even metabolite concentrations if sufficient tissue remains. Because the biopsy specimens are obtained just before the start of the treatment, we are able to capture the most accurate status of genetic and metabolic processes within a tumor. The process of obtaining fresh frozen samples is seemingly simple but requires significant investment when introduced into the clinical setting. The effort we describe is meaningful if intended to serve as a discovery tool. Although many groups have shown that NGS and other molecular techniques such as RNA sequencing are possible from formalin-fixed, paraffin-embedded (FFPE) tissue samples [36], there are still discordances between RNA sequencing results from FFPE and fresh frozen tissue [37], and our experience is that NGS results from fresh frozen tissue are more consistent. However, the logistical process needed to implement our protocol in itself represents added value for discovery purposes and large-scale biobanking.

Patient accrual is one of the major issues in gathering biopsies in the context of a clinical study in which there is no direct benefit for an individual patient. Both the willingness of patients and the reluctance of the treating physician to ask their patients for research biopsies play a role here. This is a common phenomenon in the process of acquiring research biopsies and has recently been described elsewhere [38]. Consequently, many of the early-phase clinical trials that include mandatory biopsies fail to report on biomarker analysis [39, 40]. Despite the scarcity of adequately collected tumor material, many tumor biopsies are still collected in small initiatives or by industry studies, looking predominantly at only *RAS, RAF*, or the *ERBB* family [41]. An alternative would be to identify predictive markers in preclinical model systems, but here the major discrepancies between pharmacologic drug responses for identical cell lines in the two largest pharmacogenomics cell line studies suggest that preclinical studies often lack predictive power [42]. Thus, current and future clinical research should be aimed at collecting tumor tissue and at correlating molecular data to clinical outcome to identify true predictive biomarkers.

In this study we have shown that it is feasible to perform next-generation sequencing on fresh frozen biopsies for biomarker discovery in a multi-institutional setting. Additionally, we have confirmed that acquiring fresh frozen tumor biopsies under image guidance is safe in advanced cancer patients.

AUTHOR CONTRIBUTIONS

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DISCLOSURES

Stefan M. Willems: Pfizer, Roche (C/A, RF); **Ron H.J. Mathijssen:** Astellas (C/A, RF), Bayer, Prostakan, Roche, Sanofi (RF); **Martijn P. Lolkema:** Sanofi, Astellas, Johnson and Johnson (RF). The other authors indicated no financial relationships.

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