IJC
International Journal of Cancer

Feasibility of real time next generation sequencing of cancer genes linked to drug response: Results from a clinical trial

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The successes of targeted drugs with companion predictive biomarkers and the technological advances in gene sequencing have generated enthusiasm for evaluating personalized cancer medicine strategies using genomic profiling. We assessed the feasibility of incorporating real-time analysis of somatic mutations within exons of 19 genes into patient management. Blood, tumor biopsy and archived tumor samples were collected from 50 patients recruited from four cancer centers. Samples were analyzed using three technologies: targeted exon sequencing using Pacific Biosciences PacBio RS, multiplex somatic mutation genotyping using Sequenom MassARRAY and Sanger sequencing. An expert panel reviewed results prior to reporting to clinicians. A clinical laboratory verified actionable mutations. Fifty patients were recruited. Nineteen actionable mutations were identified in 16 (32%) patients. Across technologies, results were in agreement in 100% of biopsy specimens and 95% of archival specimens. Profiling results from paired archival/biopsy specimens were concordant in 30/34 (88%) patients. We demonstrated that the use of next generation sequencing for real-time genomic profiling in advanced cancer patients is feasible. Additionally, actionable mutations identified in this study were relatively stable between archival and biopsy samples, implying that cancer mutations that are good predictors of drug response may remain constant across clinical stages.

International efforts to quantify and catalogue mutations, gene expression and epigenetic data for multiple forms of cancer, coupled with the successes of targeted agents in patients with molecularly defined tumors and improvements in genomic technology, have increased enthusiasm to adopt genomic profiling into clinical cancer practice.[1] As the numbers of clinically significant genetic variants have increased, clinical testing has evolved, moving from single mutations to multiplex hotspot evaluations in multiple cancer genes. Several pilot studies have demonstrated the feasibility and potential benefits of real-time multiplex hotspot evaluations in various cancer types.[2–8] However, as improvements in genomic technology overcome previous concerns of cost, complexity, time and tissue requirements, an increasing interest in adopting next generation sequencing (NGS) for genomic profiling in clinical cancer practice has developed.[6,9,10] Roychowdhury *et al.* recently reported the use of integrative sequencing in the clinic and demonstrated its potential to facilitate biomarker driven clinical trials.[11] However, it remains unclear whether the use of high-throughput, real-time NGS for genomic profiling is capable of generating results in a timeframe that allows for changes to patient management. Furthermore, the additional value of NGS over the multiplex hotspot genotyping approach is unclear, and

Key words: molecular profiling, cancer genomes, next generation sequencing, personalized medicine
Additional Supporting Information may be found in the online version of this article
*B.T., A.M.K.B., L.L.S., J.D.M., S.K.-R. and J.E.D contributed equally to this work.
Grant sponsor: The PMH Foundation, The Birmingham Cancer Research Fund, Cancer Care Ontario, The Ontario Institute for Cancer Research through Ontario Ministry of Economic Development and Innovation
DOI: 10.1002/ijc.27817
History: Received 2 Aug 2012; Accepted 3 Aug 2012; Online 5 Sep 2012
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What's new?

Advances in next generation sequencing (NGS) technologies and cancer genetics have led to considerable enthusiasm for genomic profiling as a tool for the clinical management of cancer. This multi-center clinical trial, which examined different sequencing technologies, demonstrated the feasibility of incorporating NGS technology clinically. Both fresh biopsy and archived samples were found to be suitable for these analyses. If proven cost-effective and clinically beneficial, NGS technologies could result in more effective treatment regimens and improved outcomes for patients.

amidst the growing concerns over intrapatient and intratumoral heterogeneity,[12] the clinical relevance of identified mutations is clouded. These issues must be addressed before the clinical use of NGS advances further.

Princess Margaret Hospital–University Health Network and the Ontario Institute for Cancer Research developed a genomic pathway strategy to address these concerns through systematically evaluating the use of NGS for personalized cancer medicine.[12–16] Herein, we report results from a multicenter clinical trial arising from the genomic pathway strategy, that examines the feasibility of biopsying patients with advanced solid cancers for molecular profiling using targeted exon sequencing (Pacific Biosciences PacBio RS) together with somatic mutation genotyping (Sequenom MassARRAY).

Material and Methods

Our primary objective was to demonstrate the feasibility and optimize processes and procedures for collection and genomic analyses of core biopsies from advanced cancer patients in a multicenter trial setting. Five target criteria were preselected to assist in assessing feasibility: (*i*) Recruit ≥50% of patients approached (patient acceptability); (*ii*) attain sufficient DNA from biopsy specimens in ≥90% of patients (sample quality); (*iii*) complete molecular analysis (defined as successfully generating a molecular profile from a clinical laboratory) in ≥90% of patients (sample analysis); (*iv*) generate a report in ≤21 calendar days (including weekends) from consent in ≥90% of patients (timeliness); and (*v*) Identify mutations in ≥30% of patients (utility). These criteria did not apply to the analysis of archival specimens. Patients were recruited from four centers throughout Ontario, Canada. A sample size of 50–80 patients was selected to demonstrate feasibility based on assumed recruitment of 10–20 patients per center. Figure 1 outlines the study design. The study was approved by institutional research ethics boards.

Patient eligibility

Patient eligibility criteria included, histological or cytological diagnosis of advanced solid cancer, potential candidates for phase I/II clinical trials, at least one biopsiable lesion, laboratory parameters safe for tumor biopsy and written voluntary informed consent.

Sample collection and processing

A fresh tumor biopsy, blood sample and archived tumor specimens were sought from consenting patients. Three to six

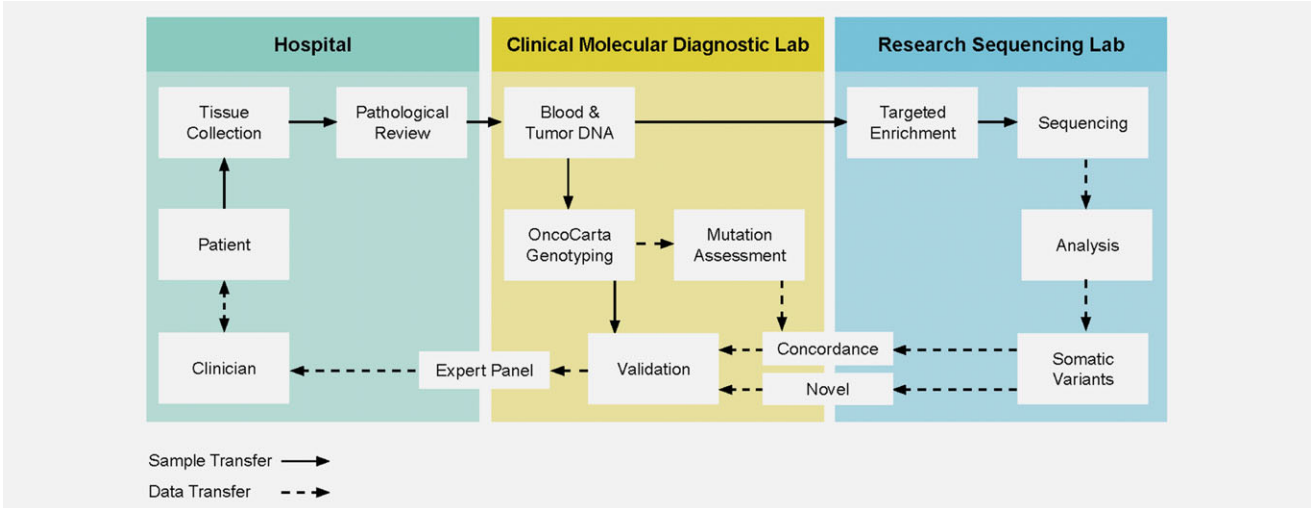

Figure 1. Study design. Patients are consented and biopsied in their treating hospital and samples are sent to the CAP/CLIA diagnostic laboratory where DNA is extracted. In the clinical molecular diagnostic laboratory an aliquot is sent to the sequencing laboratory and the rest remains in the clinical laboratory. Following sequencing, results are compared to genotyping results to assess concordance. Sequencing data that are generated in a research laboratory is never reported directly to the patient. All variants uncovered, including those found on both platforms and those only seen in the sequencing data are validated in the clinical laboratory prior to reporting. Once a sample leaves the clinical laboratory, it never returns. Only data are shared from the research laboratory to the clinical laboratory. Validated somatic variants are reviewed by an expert panel where a report is generated and sent to the treating physician.

core biopsies were collected from each patient with an 18 gauge or larger needle. Fresh tumor samples were initially divided for snap freezing and formalin fixation then paraffin embedding (FFPE). Later specimens were only processed by FFPE, as FFPE provided easier handling and greater quantity of DNA without loss of quality or difference in results. A pathologist reviewed H&E slides from all tissue samples to quantify tumor material prior to macrodissection to enrich for tumor DNA. DNA was extracted in a College of American Pathologists (CAP) and Certified Laboratory Improvements Amendments (CLIA) certified laboratory as described in Supporting Information Methods 2. The DNA sample was split: an aliquot was sent to the research laboratory for sequencing and a second aliquot remained in the clinical laboratory. Figure 1 describes the molecular profiling pipeline.

Sequenom MassARRAY somatic mutation genotyping

In the clinical laboratory, the Sequenom MassARRAY and OncoCarta Panel v1.0 were used as per the manufacturer's protocol. The panel consists of 24 multiplex assays that detect 238 mutations in 19 oncogenes. Supporting Information Methods 3 describes the methods used for somatic mutation genotyping by Sequenom MassARRAY.

PacBio RS-targeted exon sequencing

The PacBio RS instrument was selected as the NGS platform for this study based on its rapidity of analysis and following validation using a set of 30 blood and FFPE samples (27 with known cancer mutations). In the initial testing, 26/27 mutations were called correctly and led to improvements in assay design, sequencing protocol and analysis pipeline utilized for the clinical trial. When possible, 300–400 ng of DNA was used to PCR amplify 63 amplicons corresponding to the 60 exons containing all 238 mutations on the OncoCarta Panel v1.0. The average size of amplicons is 340 bp (range: 230–442 bp) in length (PCR primers are described in Supporting Information Table 2). There were 23 reactions (five 5-plex, seven 4-plex and 11 single-plex). PCR products were pooled using 6 μl for each multiplex and 1.5 μl for each single-plex (total 82.5 μl per sample). Purified pools were quantified using Qubit Fluorometric Quantitation (Invitrogen). Library preparation used 500 ng of each pool. Further details regarding targeted exome sequencing performed by PacBio RS is described in Supporting Information Methods 4.

Review, reporting and follow-up

All identified mutations were verified in a CAP/CLIA laboratory by Sanger Sequencing or an alternate validated technology. An expert panel of clinicians, genome scientists, bioinformaticians and pathologists deliberated weekly to determine which mutations were reportable and actionable (defined as having potential to alter patient management based upon known prognostic, predictive or diagnostic implications). Reports of results from biopsy specimens were sent to clinicians and updated when archival specimen results became available.

Baseline demographic data were collected. Biopsy related adverse events were graded using Common Terminology Criteria for Adverse Events (CTCAE) version 4.0. The impact of profiling on treatment recommendations and patient response to matched treatment was recorded initially and every 3 months for 2 years.

Bioinformatics tools

Three interconnected components to assist with logistics of sample handling, validation of the pipeline and reporting mechanisms were developed. First, a web-enabled variant mutation knowledge base to manage data reporting on each mutation was designed. This combines expert annotated literature with statistical data on mutation frequencies within cancer types from COSMIC data.[17] Prior to sending to clinicians, reports were reviewed and updated following expert panel deliberations. The second component was a collaborative web-based spreadsheet for patient and sample tracking through the study workflow, sending reminders when required, and making the elapsed time and status immediately visible. The third component managed the genotyping and sequencing results. It was used to record and integrate observed mutations from the different genomics technologies, and associate these genomics results with the samples, the patients, and the knowledge base. These components were integrated into a single uniform interface that provides an effective bridge between clinical and genomic information, creating personalized reports for treating physicians

Results

From March to December 2011, 51 of 56 (91%) advanced cancer patients approached consented to the study (Figs. 1 and 2*a* details patient and specimen flow). One patient was ineligible as no lesion was judged to be biopsiable. Table 1 details the characteristics of enrolled patients. There were more female patients reflecting the proportions of breast (16%) and ovarian cancer (16%) recruited. ECOG performance status was 0–1 in 92% of patients and median number of prior treatments was 3.

Forty-nine patients had successful biopsies. Three patients underwent repeat biopsies. Biopsies were predominantly performed under radiological guidance (72%). Most common biopsy sites were soft tissue (30%) and liver (28%). Eight biopsy-related adverse events occurred: six were minor, while two were serious (Table 1). Pathological review identified insufficient tumor in five biopsy specimens and one archival specimen. Median tumor cellularity was 60% for both biopsy (range: 0–90%) and archival specimens (range: 5–100%). Median amount of DNA extracted was greater in archival specimens (9.5 μg, range: 0.6–103.0 μg) compared to biopsy specimens (3.9 μg, range: 0.09–88.2 μg). Biopsy specimens providing sufficient DNA for analysis were obtained from 43 (86%) patients. While 80% of the first 15 biopsies provided sufficient

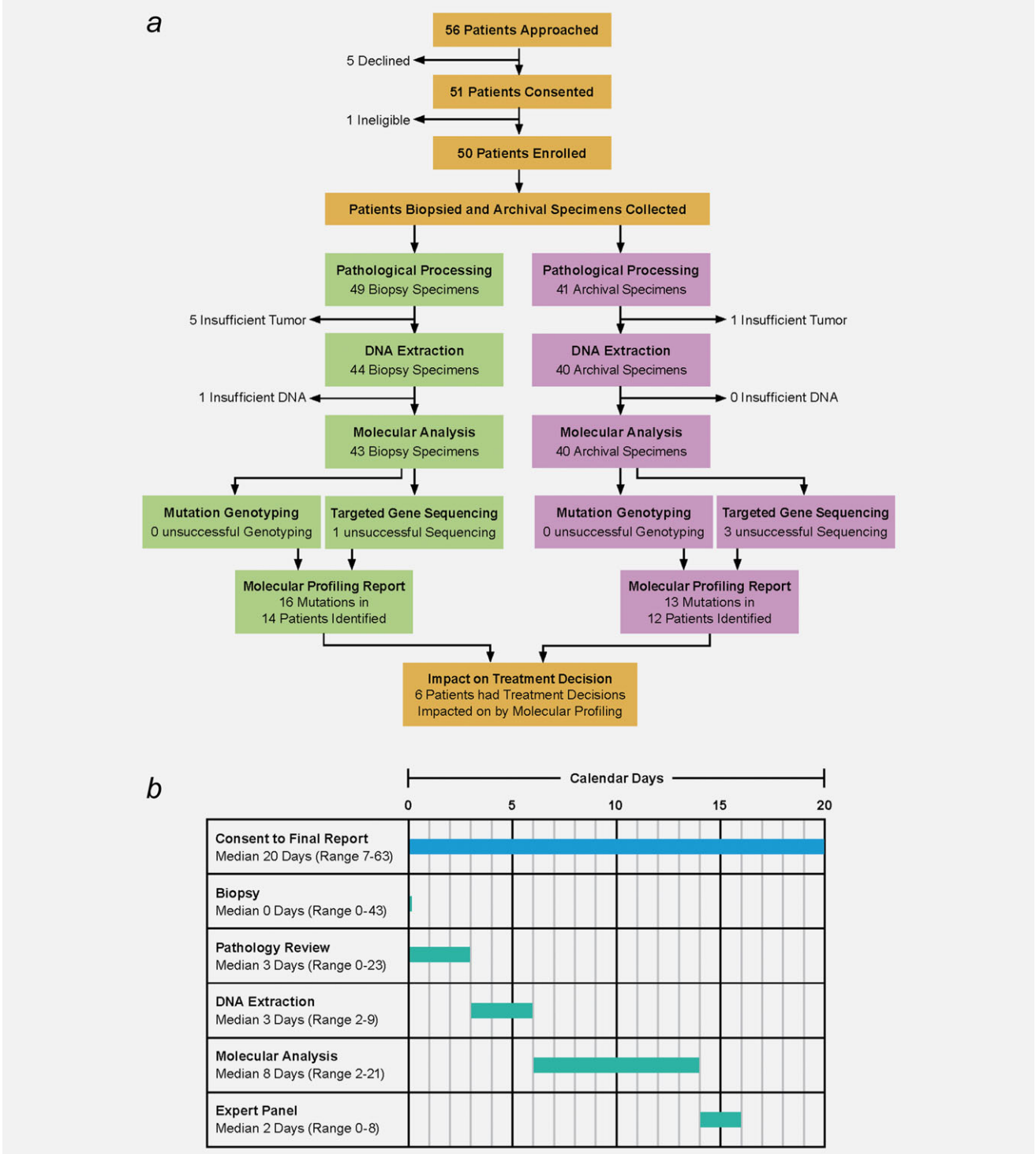

Figure 2. (*a*) Clinical trial CONSORT diagram. Among the 50 enrolled patients, 49 patients had successful biopsies. Initial biopsy was unsuccessful in four patients; three underwent repeat biopsies. Five samples had insufficient tumor and one sample had insufficient DNA for analysis. (*b*) Timelines for clinical trial patient, specimen, analysis and reporting.

DNA, this increased to 89% in the subsequent 35 biopsies. Archival specimens were analyzed from 40 patients: 31 were from primary tumor and 9 from metastases. Retrieval of archival specimens is pending in four patients, while six patients had insufficient archival specimens.

Overall, 49 (98%) patients had successful molecular analysis of either archival or biopsy specimens. Analysis was successful for the 43 (86%) biopsy specimens and 40 (80%) archival specimens with sufficient DNA by Sequenom MassARRAY; 40 biopsy (80%) and 37 archival samples (74%) by

Table 1. Patient characteristics and biopsy details

Characteristic	Number of patients	%
Sex		
Male	21	42
Female	29	58
Age (years)		
Median	57	
Range	44–74	
Hospital		
PMH (Toronto)	29	58
LHSC (London)	9	18
JCC (Hamilton)	6	12
OCC (Ottawa)	6	12
Time since metastatic diagnosis (months)		
Median	17.2	
Range	1.2–81.8	
No. of prior treatment regimens		
Median	3	
Range	0–8	
Primary tumor type		
Colorectal	9	18
Breast	8	16
Ovarian	8	16
NSCLC	5	10
Prostate	3	6
Anal	2	4
Mesothelioma	2	4
Head and neck	2	4
Other	1 each	2 each
Pancreas, thyroid, esophageal, cervix, mucoepidermoid lung, bladder, unknown primary, GIST, skin (basal cell carcinoma), parotid, adenocystic parotid		
Biopsy method		
Radiological	36	72
Clinician at bedside	10	20
Surgical	4	8
Site of biopsy		
Soft tissue	15	30
Liver	14	28
Abdominal mass	10	20
Superficial lymph node	5	10
Lung	4	8
Other	1 each	2 each
Oral cavity, pleura		
Biopsy complications		
Minor adverse events	6	12
Grade 1 pain	3	4
Grade 1 bruising	1	
Grade 1 bleeding	1	
Grade 2 wound infection	1	
Serious adverse events (SAE)	2	
Grade 2 pneumothorax	1	
Grade 3 cellulitis	1	
Archival specimens		
Sufficient for analysis	40	80
Primary site	31	8
Metastatic site	9	12
Pending	4	
Not available or insufficient	6	

Abbreviations: PMH: Princess Margaret Hospital; LHSC: London Health Sciences Centre; JCC: Juravinski Cancer Centre; OCC: Ottawa Hospital Cancer Centre; NSCLC: non-small cell lung cancer; GIST: gastrointestinal stromal tumor.

PacBio RS (Table 2). Profiling was successful in paired specimens from 34 patients, biopsy only in 9, archival only in 6 and neither in 1. The average depth of sequence coverage with PacBio RS was 600× (range: 114× to 972×), 100% of target bases were covered with 98.8% covered at >10×. High-throughput targeted gene sequencing was unsuccessful in 1 (2%) biopsy specimen due to insufficient DNA and 3 (8%) archival specimens due to DNA degradation. Impact of DNA degradation on sequencing alone may relate to the larger amplicons required for PacBio RS compared to MassARRAY. Somatic mutation genotyping by Sequenom MassARRAY was successful in all specimens with sufficient DNA.

In total, 19 mutations, including five non-OncoCarta mutations, were identified in 16 (32%) patients (Table 3, Supporting Information Table 1). There were 16 mutations (four non-OncoCarta mutations) identified in biopsy specimens, from 14 patients. The ratio of mutated to germline alleles ranged from 13 to 94%, likely reflecting normal cell contamination and clonal heterogeneity in tumor specimens. Across technologies, OncoCarta mutation calls agreed for 100% of biopsy and 95% of archival specimens. Results from paired archival/biopsy specimens obtained a median 33 months apart were concordant in 30/34 (88%) patients. Three somatic mutations, PDGFRA R718W, AKT1 E341K and EGFR Q787L, had unknown function based on review of public databases and published literature, although *in silico* studies suggest that an EGFR Q787R substitution may be activating.[18] *In vitro* functional studies of these mutations are ongoing.

Median time from consent to final report was 20 calendar days (range: 7–63) (Fig. 2*b*). Reports were generated within 21 calendar days for 31 (62%) patients. The 21-day target was met in 27% of the initial 15 patients and 74% for the

Table 2. Results from molecular analysis

	Biopsy specimen	Archival specimen
Molecular analysis		
Successful somatic mutation genotyping (MassARRAY)	43 (86%)	40 (80%)
Successful targeted gene sequencing (PacBio RS)	42[1] (84%)	37[1] (74%)
Patients		
No. of patients with at least 1 mutation	14[2] (28%)	12[2] (22%)
Mutations		
No. of reported mutations	16 (32%)	13 (24%)
No. of actionable mutations	13[3] (26%)	10[4] (18%)
No. of OncoCarta mutations	12	8
Genes involved		
KRAS	5	3
EGFR	4	1
PIK3CA	3	3
RET	0	1
No. of non-OncoCarta mutations	4	5
Genes involved		
PDGFRA	1	1
AKT1	1	1
EGFR	1	1
KRAS	0	1
KIT	1	1
Agreement		
Agreement between platforms	42/42 (100%)	35/37 (95%)[5]
Biopsy versus archival specimens		
Available for comparison	34	
No difference	30/34 (88%)	
Difference	4/34 (12%)	

[1]Targeted gene sequencing not successful in one biopsy specimen due to insufficient DNA and three archival specimens due to DNA degradation. [2]Two patients had two mutations from molecular analysis of biopsy specimens and one patient had two mutations from molecular analysis of archival specimens. [3]Of the four non-OncoCarta mutations identified by sequencing in the biopsy specimens, three were novel and not classified actionable, while one was previously reported with known function and classified actionable. [4]Of the five non-OncoCarta mutations identified by sequencing in the archival specimens, three were novel and not classified actionable, while two were previously reported with known function and classified actionable. [5]In one archival specimen, PacBio RS could not detect a PIK3CA mutation that was detected by MassARRAY and confirmed by a CAP/CLIA laboratory. In another archival specimen, PacBio RS detected a FGFR3 mutation that was not detected by MassARRAY and not confirmed by the CAP/CLIA laboratory.

remaining 35 patients. Most common reasons for exceeding the 21-day target were delays in molecular analyses ($n = 7$), expert panel reporting ($n = 4$) and repeating of biopsies ($n = 3$). Median time from consent to receipt of archival specimens was 22 days (range: 2–143).

Data regarding clinical impact are limited, as median follow-up is 3 months (Table 3). Table 4 details the six patients who were prescribed treatments matched to their reported mutation and their best responses. Of the remaining 10 patients with reported mutations, six had mutations known through prior routine testing, three were not eligible for matched treatments and one continues on standard treatment.

Discussion

We demonstrated feasibility by successfully generating timely results in the majority of advanced cancer patients in a multicenter setting using processes that are amenable to larger scale studies. Study outcomes for successful biopsy sample analysis (86%) and clinical utility (28%) of patients with actionable mutations did not quite meet their targets due to sample quality; when samples with insufficient DNA were excluded from the analysis, both criteria were met. Greater attention to biopsy site selection resulted in an improvement from 80% of biopsy specimens providing sufficient DNA in the first 15 patients, to 89% in the final 35 patients. The 3-week interval from patient consent to return of results improved following the introduction of a customized web-based tracker to minimize delays, increasing the proportion of patients meeting the target from 27 to 74%. Timeliness can be improved further by reducing the interval between consent and date of biopsy and eliminating the requirement for confirmation of all mutations by Sanger sequencing or an alternate validated technology. The latter should be possible as NGS and multiplex genotyping technologies are taken up and validated within CAP/CLIA clinical laboratories. Including results from both archived and tumor biopsies, 32% of patients had actionable mutations. While this proportion is highly dependent on the tumor types selected, we believe our cohort is a good representation of advanced cancers that may benefit from a personalized cancer medicine approach.

Sequencing results were concordant with genotyping technologies for recurrent mutations and provided additional value by identifying five novel variants not present on the OncoCarta v1.0 mutation panel. Furthermore, DNA extracted from FFPE was sequenced successfully, thus satisfying the dual requirements for the pathological confirmation of diagnosis and DNA analyses with a single biopsy sample. In patients whose tumors contained novel mutations solely detected by sequencing, two were prescribed matched treatment, leading to one partial response (unconfirmed) and one minor response classified as stable disease. These results suggest an advantage for NGS over genotyping in the clinical setting, although ultimately, further study is required to determine whether identifying novel mutations translates into patient benefit.

A key issue we sought to address was the level of concordance for actionable mutations detected by targeted sequencing between archived diagnostic samples versus new

Table 3. Mutation analysis of study samples and clinical outcomes

		Identified mutation(s)						Clinical impact		
Patient sample no.	Primary tumor	Gene and amino acid change	In biopsy	In archival	In both	Biopsy specimen site	Archival specimen site	Impacted management	Matched treatment	Best response (RECIST)
3	Colorectal	PIK3CA E542K[2]	Yes	Yes	Yes	Liver met	Colorectal primary	Yes	Phase I trial: PI3K inhibitor and MEK inhibitor; Cetuximab and Irinotecan[3]	PD
										PD
5	Pancreas	KRAS G12D[2]	Yes	NA	NA	Liver met	–	No	–	–
6	Colorectal	KRAS G12A[2]	Yes	No	No	Liver met	Colorectal primary	No	–	–
8	Breast	PIK3CA H1047R[2]	Yes	Yes	Yes	Breast primary	Breast primary	Yes	Phase I trial: PI3K inhibitor and MEK inhibitor	PD
10	NSCLC	EGFR E746-A750del[2]	Yes	NA	NA	Lung met	–	No	–	–
11	Cervix	PIK3CA H1047L[2]	Yes	No	No	RPLN met	Cervical primary	No	–	–
		PI3KCA E542K[2]	No	Yes	No					
15	Colorectal	KRAS G12A[2]	Yes	Yes	Yes	Liver met	Colorectal primary	No	–	–
		PDGFRA R718W[1]	Yes	Yes	Yes					
16	Ovary	KRAS G12D[2]	Yes	Yes	Yes	Subcut met	Subcut met	Yes	Phase I trial: PI3K inhibitor and MEK inhibitor	cPR
18	Breast	AKT1 E341K[1]	Yes	Yes	Yes	Breast primary	Breast primary	Yes	Everolimus, paclitaxel and trastuzumab	SD
20	Thyroid (medullary)	RET M918T[2]	No	Yes	No	Liver met	LN met	Yes	Sorafenib	uPR
25	Colorectal	KRAS G12V[2]	Yes	Yes	Yes	Subcut met	Colorectal primary	No	–	–
31	CUP (Squamous cell)	EGFR Q787L[1]	Yes	Yes	Yes	LN met	LN met	Yes	Erlotinib	uPR
35	NSCLC	EGFR L858R[2]	Yes	Yes	Yes	Lung met	Lung primary	No	–	–
38	NSCLC	EGFR L747_E749del[2]	Yes	No	No	Liver met	Lung primary	No	–	–
		EGFR T790M[2]	Yes	No	No					
44	GIST	KIT N822K[1],[2]	Yes	Yes	Yes	Liver met	Small bowel primary	No	–	–
45	Colorectal	KRAS Q22K[1],[2]	NA	Yes	NA	–	Colorectal primary	No	–	–

On study KRAS testing of biopsy sample of liver metastasis and one archived sample identified KRAS wildtype; one additional archived sample identified KRAS mutation at 4%.
[1]Non-OncoCarta mutations.
[2]Mutations deemed actionable by expert panel.
[3]Patient 3 had KRAS mutant colon cancer based on prior clinical testing.
Abbreviations: Spec: specimen; Freq: frequency; RECIST: response evaluation criteria in solid tumors; Met.: metastasis; PI3K: phosphoinositide 3-kinase; PD: progressive disease; NA: not applicable; NSCLC: non-small cell lung cancer; RPLN: retroperitoneal lymph node; Subcut: subcutaneous; cPR: confirmed partial response; SD: stable disease; LN: lymph node; uPR: unconfirmed partial response; CUP: carcinoma of unknown primary; GIST: gastrointestinal stromal tumor.

Table 4. Features of patients with mutations matched to therapies

Patient sample no.	Sex	Age	ECOG	Primary	Time with metastatic disease (months)	Number of previous treatment lines	Prior systemic therapy	Mutation results	Months between biopsy and archive	Matched treatment	Best response	Follow up (months)
3[1]	F	69	0	Colorectal	33	4	5-Fluorouracil, leucovorin, oxaliplatin, irinotecan, bevacizumab	PIK3CA E542K	33	PI3K + MEK inhibitors; cetuximab + irinotecan	PD, PD	6
8	F	46	1	Breast	9	3	Doxorubicin, cyclophosphamide, paclitaxel, trastuzumab, lapatinib, capecitabine, navelbine	PIK3CA H1047R	31	PI3K + MEK inhibitors	PD	3
16	F	53	0	Ovarian	21	2	Paclitaxel, carboplatin, gamma secretase inhibitor	KRAS G12D	21	PI3K + MEK inhibitors	PRc	3
18	F	48	0	Breast	12	4	5-Fluorouracil, epirubicin cyclophosphamide, docetaxel, trastuzumb, tamoxifen, lapatinib, letrozole, capecitabine	AKT1 E341K	15	mTOR inhibitor + paclitaxel + trastuzumab	SD	3
20	M	57	1	Medullary thyroid	42	2	MET-VEGFR2 inhibitor, Notch inhibitor, cedarinib, radiation	RET M918T	38	Sorafenib	Pru	3
31	M	67	0	CUP (squamous cell carcinoma)	12	1	Docetaxel, cisplatin, 5-fluorouracil	EGFR Q787L	12	Erlotinib	PR	3

[1]Patient 3 had KRAS mutant colon cancer based on prior clinical testing. On study KRAS testing of biopsy sample of liver metastasis and one archived sample identified KRAS wildtype; one additional archived sample identified KRAS mutation at 4%.

biopsy samples obtained at advanced stages. While molecular differences between primary and metastatic tumors have been studied in several tumor types, these studies have either reported large-scale analyses (not restricted to actionable mutations) in small numbers of patients[12,19–21] or compared early versus late lesions for a few genes and/or specific mutations.[22–25] This study reports the largest time-series comparing multigene profiling between biopsies obtained at different stages of disease (median 33 months apart). The 88% concordance of results are reassuring from a clinical perspective, as using archival specimens when new biopsies are unfeasible may be justified (although we note that there are disadvantages such as longer time to obtain archived samples from distant hospitals and DNA degradation over time). The high concordance observed between archival and current biopsy samples does not contradict the evidence that there is extensive clonal evolution as tumors progress and disseminate. We assessed 60 exons in 19 genes, thus it is likely that there is genetic heterogeneity that we did not evaluate. However, the discrepancy between reports of high genetic differences in the global mutation load in different tumor samples over time, and the relative stability of actionable mutation profiles reported here, may reflect that clinically successful therapies are more likely to target strong driver mutations that remain operative through clonal evolution of cancer cells from early to late stages of disease. While profiles generated from archived diagnostic samples may be suitable for clinical trial and treatment decisions at disease relapse or progression, analysis of a new tumor sample may be preferred in certain situations. We and others[7,26] detected drug resistant mutations in biopsy samples from patients with disease progression following exposure to targeted therapies (KRAS mutation in colon cancer patient treated with panitumumab; EGFR T790M mutation in lung cancer patient treated with erlotinib, Table 4). These mutations were not present in the archival tumor samples.

The potential for clinical benefit associated with identifying somatic mutations through NGS depend upon several key criteria: (*i*) genetic variants must be identified that can be acted upon; (*ii*) the clinical implications of the mutation must be understood; (*iii*) there must be access to treatments that are matched to the identified mutation; and (*iv*) patients should be willing and fit to receive these agents. Currently, most matched treatments are likely to be available through off-label use of targeted drugs or through enrollment on early phase clinical trials evaluating investigational drugs. In our study, six patients met these criteria and had treatment recommendations based upon the identified mutation. Best responses observed included three partial responses (one confirmed and two unconfirmed) and one stable disease. These results suggest that our study's 21-day target for profiling is appropriate, as results are available within a timeframe that allows treatment/trial decisions prior to patient deterioration; furthermore, 21 days is aligned with other profiling studies.[4,8,11,27] Finally, these results are consistent with the

hypothesis that matching drugs to mutation may improve clinical outcomes, as reported by other investigators.[28]

We, like others,[11] utilized an expert panel to deliberate on the functional and clinical significance of identified mutations and developed standardized summary mutation reports for clinicians. The most challenging deliberations involved novel mutations with unknown significance, occurring in the kinase domain of known driver oncogenes. In our study, these results were reported to clinicians as unknown function and clinical significance. Knowledge sharing among researchers, ideally through a publically available database where function and clinical data regarding novel mutations can be uploaded, would facilitate deliberations.

We have shown that high-throughput NGS, when accompanied by verification of results in a clinical laboratory, can be incorporated into a clinical care workflow using readily acquired biopsy specimens fixed in formalin and that this approach adds value to the information generated by traditional genotyping methods. Limitations of the current study include its focus on technologies that only detect simpler somatic mutations affecting protein structure in specific exons of actionable genes, which could have led to our missing potentially actionable mutations in other exons or genes or other mutation forms (methylation, expression, copy number alterations, fusion proteins or other rearrangements). In addition, the goal of our study was to demonstrate feasibility of NGS in a clinical setting, thus, we have relatively small numbers of patients and short follow-up that limit drawing conclusions on the clinical value of the approach. Having established the feasibility of incorporating genomic sequencing into clinical workflow, we will be expanding the numbers of genes and types of genomic alternations to reflect targets of experimental therapeutics in development as the genomic pathway strategy moves to its next phase of demonstrating patient benefit from NGS use in the clinic. With the feasibility of rapid NGS profiling demonstrated, evaluation of its clinical benefit and cost-effectiveness is now required through proof-of-concept studies aimed at testing the hypothesis that treatment recommendations based upon profiling will result in superior patient outcomes.

Acknowledgements

We thank the numerous clinicians, scientists, technicians and data managers who contributed to the PMH-OICR led feasibility study.

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