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Personalized Oncology Through Integrative High-Throughput Sequencing: A Pilot Study

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Abstract

Individual cancers harbor a set of genetic aberrations that can be informative for identifying rational therapies currently available or in clinical trials. We implemented a pilot study to explore the practical challenges of applying high-throughput sequencing in clinical oncology. We enrolled patients with advanced or refractory cancer who were eligible for clinical trials. For each patient, we performed whole-genome sequencing of the tumor, targeted whole-exome sequencing of tumor and normal DNA, and transcriptome sequencing (RNA-Seq) of the tumor to identify potentially informative mutations in a clinically relevant time frame of 3 to 4 weeks. With this approach, we detected several classes of cancer mutations including structural rearrangements, copy number alterations, point mutations, and gene expression alterations. A multidisciplinary Sequencing Tumor Board (STB) deliberated on the clinical interpretation of the sequencing results obtained. We tested our sequencing strategy on human prostate cancer xenografts. Next, we enrolled two patients into the clinical protocol and were able to review the results at our STB within 24 days of biopsy. The first patient had metastatic colorectal cancer in which we identified somatic point mutations in NRAS, TP53, AURKA, FAS, and MYH11, plus amplification and overexpression of cyclin-dependent kinase 8 (CDK8). The second patient had malignant melanoma, in which we identified a somatic point mutation in HRAS and a structural rearrangement affecting CDKN2C. The STB identified the CDK8 amplification and Ras mutation as providing a rationale for clinical trials with CDK inhibitors or MEK (mitogenactivated or extracellular signal–regulated protein kinase kinase) and PI3K (phosphatidylinositol 3-kinase) inhibitors, respectively. Integrative high-throughput sequencing of patients with advanced cancer generates a comprehensive, individual mutational landscape to facilitate biomarker-driven clinical trials in oncology.

INTRODUCTION

The management of patients with cancer is well suited to a personalized approach, as reinforced by recent genomic studies that reveal a disease composed of numerous heterogeneous mutations. Although hallmark mutations such as inactivation of TP53 or activation of BRAF occur frequently, they often appear in concert with a host of uncommon oncogenic events. Further, expanding catalogs of cancer mutations dispel the notion that cancer mutations are tissue-specific (1–7). For example, activating BRAF mutations have been described in more than 50% of cutaneous melanoma and papillary thyroid carcinoma, and the mutant proteins are potential targets for BRAF inhibitors (8, 9). However, BRAF mutations also occur at a lower frequency (5 to 20%) in multiple myeloma, lung cancer, cholangiocarcinoma, and testicular cancer (10, 11). Moreover, a low to moderate fraction of major targetable kinases—including PIK3CA, EGFR (epidermal growth factor receptor), and ERBB2—may be aberrant in several cancers (12, 13). We therefore hypothesize that the clinical management of cancer may be suited to a form of personalized medicine in which the mutational landscape of an individual's cancer informs clinical decision-making, particularly the selection of targeted therapies (14–16).

Translating high-throughput sequencing for biomarker-driven clinical trials for personalized oncology presents unique logistical challenges, including (i) the identification of patients

who could benefit, (ii) the development of an informed consent process that includes a way to deal with incidental findings, (iii) the implementation of efficient and integrative computational pipelines for data analysis, (iv) the selection of the results that should be disclosed to patients, and (v) the completion of the sequencing analysis in a cost-effective and clinically relevant time frame (Table 1). We implemented an exploratory study that we call the Michigan Oncology Sequencing Project (MI-ONCOSEQ) to address these challenges.

RESULTS

Description of the clinical study

Our MI-ONCOSEQ study focused on a patient population who were considering participation in clinical trials and in whom integrative sequencing could have a potential positive impact. We set a clinically relevant time frame of 4 weeks from biopsy to availability of validated results as per the Clinical Laboratory Improvement Amendments (CLIA) (**Fig. 1**). Four weeks is a standard washout period that patients must wait between clinical trials to allow drugs from any previous therapies to dissipate. For our first four cases, the estimated cost for reagents was $5400 per patient (table S1), a practical amount for production of correlative data in clinical trials. Further, the study instituted a Sequencing Tumor Board (STB) that incorporated expertise in clinical oncology, pathology, cancer biology, bioethics, bioinformatics, and clinical genetics (Fig. 1B). The STB is an expanded version of a traditional tumor board that focuses on a single tissue of origin and uses a molecular classification of cancers. For each case, the referring medical oncologist provided a clinical presentation of the patient's history of cancer and previous therapies. Ad hoc faculty who were expert in diseases and pathways discussed at each meeting provided disease- or pathway-specific expertise. Bioinformaticians, genomics experts, cancer biologists, pathologists, and medical oncologists presented findings and reviewed their potential clinical significance. Geneticists and bioethicists provided insight regarding issues such as controversial, incidental, or unexpected findings (**table S2**).

Integrative sequencing strategy

Cancer arises from diverse genetic alterations including nucleic acid substitutions, gene fusions and rearrangements, amplifications and deletions, and other aberrations that perturb gene expression (1). Therefore, a sequencing strategy should comprehensively identify clinically significant alterations while remaining cost-effective. Whole-genome sequencing can identify copy number alterations (CNAs) and structural rearrangements at relatively shallow depth (17), but accurate point mutation identification requires significantly more coverage and remains costly (2). To fill this niche, we used targeted whole-exome sequencing to capture most human protein-coding exons, including clinically informative genes in cancer such as BRAF, EGFR, JAK2, PIK3CA, and ALK (18). Because tumors are often admixtures with normal tissue or contain multiple tumor clones, the high sequencing depth afforded by exome sequencing was advantageous for the detection of variants. Finally, transcriptome sequencing (RNA-Seq) captured the functional or “expressed” genome of a tumor sample and enabled detection of dysregulated genes and the functional products of genomic alterations (19). We included (i) shallow (5× to 15×) paired-end whole-genome sequencing of the tumor, (ii) targeted exome sequencing of the tumor and matched germline samples (blood or buccal smear), and (iii) pairedend transcriptome sequencing of the tumor (**Fig. 1D**).

Test sequencing

To test our integrative sequencing strategy, we evaluated tumor xenografts from two living patients (patients 1 and 2) with metastatic prostate cancer that had been grown in mice (table

S4). After sequencing and bioinformatics analysis, we convened a mock STB meeting to interpret the results. Table 2 summarizes key findings for each patient and highlights clinically relevant pathways and matching therapies available.

Patient 1 is a 67-year-old man with castrate-resistant metastatic prostate cancer who had predominantly nodal disease. Before xenograft establishment, the patient's previous therapies included leuprolide plus bicalutamide, diethylstilbestrol, a NY-ESO-1 vaccine trial, and a 5-azacytidine plus valproic acid trial. Human cells in the xenograft accounted for greater than 90% of total cells (fig. S6). Integrative sequencing uncovered 146 point mutations, 54 CNAs, 52 structural rearrangements, and 8 gene fusions (**tables S5, S6, and S12 to S14**). Genomic events were chosen for presentation at the mock STB on the basis of predetermined criteria (table S3) for a potential role in cancer (**Fig. 1A**). These included amplification of the androgen receptor (AR), homozygous deletion of the PTEN (phosphatase and tensin homolog) tumor suppressor, and an inactivating point mutation of the TP53 tumor suppressor (**fig. S1, B to E**). RNA-Seq provided orthogonal support for some of the DNA-based findings by detecting low PTEN expression in this patient relative to an existing prostate RNA-Seq cohort (**fig. S2B**). In addition, the patient's tumor harbored the canonical prostate cancer– specific rearrangement of TMPRSS2 (transmembrane protease, serine 2) and ERG (ETS transcription factor) (20) (**fig. S1, F and G**), and a novel gene fusion between copine IV (CPNE4, a calcium-dependent membrane-binding protein) and NEK11 (NIMA-related kinase 11) (**fig. S1H**). The fusion product preserved the full NEK11 open reading frame and resulted in marked up-regulation of NEK11 expression (**fig. S1I**). These findings were only evaluated in the xenograft specimen.

The mock STB evaluated the sequencing results in preparation for the clinical study. A predetermined list of potentially informative genes was used to filter the results for discussion in STB (**table S3**). The STB noted that recent preclinical studies support a rationale for treatment with poly(adenosine diphosphate–ribose) polymerase (PARP) inhibitors in ERG-rearranged prostate cancer (21). Further, amplification of AR implies intact androgen signaling in the tumor, and loss of PTENsupports a possible role of the PI3K (phosphatidylinositol 3-kinase) pathway (12). Recent data suggest that combined blockade of these two pathways may provide additional benefit over single-agent therapy (22). The STB determined that the gene fusion involving NEK11 has unknown clinical significance and requires further biological validation, but could potentially lead to a therapeutic approach.

Patient 2 is a 60-year-old man with metastatic prostate cancer not yet treated with hormonal therapies. Our major findings for his cancer included homozygous loss of PTEN and the TMPRSS2-ERG gene fusion (**Table 2**). Details, including mock STB deliberations, are presented in the Supplementary Results (**fig. S3 and tables S5, S7, S12, S13, and S15**).

Clinical sequencing study

We enrolled two patients in the MIONCOSEQ pilot study (**Fig. 1A, table S4**). Patient 3 is a 46-year-old man diagnosed with colorectal cancer (CRC) in March 2009, who presented with metastatic disease in the liver, bladder perforation, and innumerable polyps upon flexible sigmoidoscopy. His tumor's KRAS genotype was wild type at time of diagnosis. After failing or progressing on standard therapies (**Table 2**), he opted to participate in a phase 1 trial with an Aurora kinase B inhibitor, TAK-901 (NCT00935844). After two cycles of treatment, there was stable disease in the liver with slight progression in the lungs. After two more cycles, there was clear evidence of progression, and he was taken off the study. He enrolled in the MI-ONCOSEQ protocol and had four core liver biopsies taken through interventional radiology, each confirmed by a pathologist to consist of 60 to 70% tumor

(**Fig. 2, A and B**). We completed integrative sequencing and analysis for presentation at the STB within 24 days of biopsy.

The tumor analyses identified 160 nonsynonymous somatic point mutations, 49 CNAs, 20 rearrangements, and 2 gene fusions (**fig. S4 and tables S5, S8, S10, S12, S13, and S16**). Variants were grouped for established clinical significance or literature-supported relevance in cancer for presentation at the STB. These included a canonical activating mutation in NRAS (Q61L), homozygous inactivation of TP53 (via point mutation and copy number loss), dual copy number gain and point mutation in Aurora kinase A (AURKA), point mutations in smooth muscle myosin heavy chain (MYH11) and FAS death receptor, amplification of CDK8 (cyclin-dependent kinase 8), and copy number gains of EGFR (**Fig. 2C**). A global landscape of copy number alternations was generated from whole-genome and exome sequencing (**Fig. 2D**). The integrative sequencing approach afforded opportunities for cross-validation of results through orthogonal analyses. In this patient, integrative copy number analysis (**Fig. 2, D and E**) revealed a large region of chromosome 13 containing CDK8 that was prominently amplified, on the basis of whole-genome and exome data. CDK8 was also overexpressed in the RNA-Seq outlier analysis (**Fig. 2F**). Although originally nominated by exome sequencing, the NRAS-activating mutation was also identified by whole-genome and transcriptome data (**Fig. 2G**). Finally, RNA-Seq revealed an intrachromosomal gene fusion between acetylserotonin O-methyltransferase–like antisense RNA 1 (ASMTL-AS1) and protein phosphatase regulatory subunit 2 (PPP2R3B) on chromosome X that abrogated the open reading frame of PPP2R3B (**Fig. 2H**). No somatic mutations were observed in the prevalent CRC oncogenes KRAS, BRAF, or PIK3CA. No significant germline aberrations were observed for the polyposis-related genes APC or MUTYH (23).

The STB convened to deliberate on findings from patient 3. Most of the findings were deemed biologically interesting but not clinically significant. For example, the tumor had a point mutation in MYH11, which is rearranged in acute myeloid leukemia (24) and has been implicated in intestinal cancer (25). Furthermore, the board noted the mutation and amplification of AURKA as a possible mechanism for this patient's tumor progression while on an Aurora kinase inhibitor, although direct evidence was not available to support this hypothesis (assessment of pretreatment tumor specimen or in vitro assays). Patient 3's tumor also had a point mutation in the intracellular domain of the FAS death receptor. Although it is known that FAS intracellular mutations can protect against apoptosis, the functional effect of this mutation (activating, inactivating, or passenger) is unknown. Finally, the gene fusion involving PPP2R3B (a subunit of the protein that regulates the tumor suppressor protein phosphatase PP2A) generated interest from the STB because a closely related regulatory subunit, PPP2R1B, has been implicated in colon and lung tumors (26). Also, the NRAS and CDK8 aberrations were highlighted by the STB as potentially informative genes that could in the future be matched to clinical trials with MEK (mitogen-activated or extracellular signal–regulated protein kinase kinase), PI3K, or CDK inhibitors. Current clinical testing often disregards NRAS because of its low frequency (2%) in CRC, but activating mutations in NRAS are biologically similar to KRAS (35 to 40% of CRC), which predict resistance to antibody therapies against EGFR (27). For example, trials may accrue CRC patients with KRAS or BRAF mutations for Raf inhibitors, but fail to include patients with NRAS mutations (NCT01086267). In addition, the STB noted that amplification of CDK8 has been implicated in 15 to 20% of CRC as a positive regulator of catenin signaling (28) and is a viable target for CDK inhibitors in clinical trials.

Patient 4 is a 48-year-old woman diagnosed with metastatic melanoma who underwent wide local excision for ulcerated spitzoid-type melanoma on her right heel. One of two sentinel lymph nodes was positive, leading to a right inguinal femoral lymph node dissection. She

elected observation but subsequently developed diffuse skin recurrences on her right leg and was enrolled in the MI-ONCOSEQ study. She had four skin punch biopsies, and three of these had tumor content greater than 75 to 80% (**Fig. 3, A and B**). We completed integrative sequencing and analysis for presentation to the STB within 24 days of biopsy.

Tumor analyses identified 36 nonsynonymous point mutations, 269 CNAs, 24 rearrangements, and 4 gene fusions (**tables S5, S9, S11, S12, S13, and S17**). Of these, the following were nominated for presentation to the STB (**Fig. 3C**): an activating mutation of HRAS (Q61L), a point mutation in the ETS transcription factor family member ELK1 (R74C), and a complex rearrangement abolishing the open reading frame of cyclin-dependent kinase inhibitor 2C (CDKN2C or p18INK4C) (**Fig. 3, E to G**). Mutations were not observed in the prevalent melanoma oncogenes BRAF, CKIT, or NRAS (11) (**Fig. 3C**).Copy number analysis from tumor exome and wholegenome sequencing data did not reveal major amplification for genes of interest (**Fig. 3D**). No germline aberrations were observed corresponding to theHumanGene Mutation Database (29).

The STB deliberated on patient 4's findings. Inactivating deletions in CDKN2C, an inhibitor of CDK4, have been reported in glioblastoma multiforme and have prognostic significance in up to 30% of multiple myeloma (30). ELK1 was of interest because ETS transcription factors are downstream targets of a relevant signaling pathway in melanoma [Ras-MAPK (mitogen-activated protein kinase)], and a recent study demonstrated amplification of another ETS transcription factor oncogene (ETV1) in melanoma (31). Although biologically intriguing, the clinical relevance of ELK1 is not known. Finally, the STB nominated HRAS as a potential target for clinical trials. The HRAS activating mutation was surprising, because HRAS mutations have not been described in malignant melanoma, whereas NRAS mutations are common (15%) (32). Constitutive Ras signaling leads to downstream activation of MAPK/MEK and PI3K/mTOR (mammalian target of rapamycin) cascades and provides the biological rationale for ongoing clinical trialswith inhibitors ofMEK, PI3K, and mTOR for patients with Ras-activated cancers (32–35). It was also noted that this patient's tumor harbored wild-type BRAF and mutantHRAS. Findings that inhibitors of mutant BRAF can paradoxically activate MAPK signaling suggest that this genotype combination could predict outcomes for BRAF or MEK inhibitors in a clinical trial (36). This patient could potentially qualify for an upcoming trial of combined treatment with PI3K and MEK inhibitors for specified solid tumor malignancies with KRAS, NRAS, and BRAF mutations (NCT01363232).

DISCUSSION

In the MI-ONCOSEQ study, we aimed to translate high-throughput sequencing into a viable analysis tool for biomarker or mutation-driven clinical trials and, in doing so, addressed important logistical challenges (**Table 1**). First, the study enrolled patients eligible for early clinical trials and completed sequencing efficiently to potentially allow stratification to trial on the basis of the sequencing results. Second, the study addressed ethical implications of genome sequencing through an informed consent process with concurrent input from bioethicists. Third, the study established an STB to deliberate on the clinical value of sequencing results, including those that are unexpected.

Despite these efforts, we anticipate the need for improvements and modifications to the process and procedures used here. Because the pilot study was implemented in a research setting, we did not offer testing as a routine or billable service. Any results that affect clinical decision-making must be validated using a CLIA-certified test. As a next step, we anticipate that the molecular genetics and pathology communities will move high-throughput sequencing toward CLIA certification, which will ultimately reduce costs and

improve turnaround time of results. Additionally, declining sequencing costs will make our approach even more practical. The per-patient price tag decreased from $5400 six months ago to $3600 at present. This cost is comparable to routine clinical tests such as OncotypeDx and is financially practical for every patient who is considering clinical trials.

MI-ONCOSEQ has used a combination of DNA and RNA sequencing to reveal a broad view (11) of an individual's genetic aberrations. Moving forward, we anticipate that incorporation of global epigenetic and small RNA analyses, as well as evolving bioinformatics algorithms, will provide complementary information and enable cross-validation (37). Alternative strategies that assess a limited panel of genes or genetic aberrations (38–40) optimize sensitivity to detect aberrations in clinically informative genes, but are of limited use for basic science research. This trade-off between breadth and sensitivity will be an important consideration in heterogeneous samples with multiple subclones as well as biopsies with low tumor purity. For patients 3 and 4, samples were of acceptable purity and clinically informative variants were captured at substantial depth (fig. S7), but this does not rule out missed mutations. Therefore, it will be important to develop approaches to assess samples with low tumor purity. Aside from increasing sequencing depth to compensate for low tumor content, one could enrich for tumor-relevant DNA through microdissection, cell-based enrichment, or ploidy-based sorting.

Although others have demonstrated the potential benefits of highthroughput assays for individual patients with cancer (41), the next logical step is facilitating clinical trials in oncology with biomarker informed therapies. Clinical investigators are increasingly recognizing the importance of patient selection by mutation assessment when using targeted therapeutic agents (42, 43). The proven effect of this approach in the recent BRAF and ALK phase 1 trials demonstrates the need for molecular stratification (44, 45). Here, integrative sequencing identified informative oncogenes that would have been missed by standard single-gene clinical assays or approaches with a limited panel of genes. Both patients 3 and 4 had potentially informative aberrations, but these patients did not fit into available trials. Patient 3's CDK8 amplification and NRAS activating mutation provided a good rationale for use of investigational agents such as CDK inhibitors and combined MEK/PI3K inhibition (46, 47). A phase 1 trial is pending for doxorubicin plus seliciclib (a CDK inhibitor with activity for CDK8) in patients with breast cancer. However, because of the study's limited eligibility for breast cancer, the patient was not eligible (NCT01333423). Similarly, we identified a phase 1 study for a MEK inhibitor in patients with CRC who have BRAF or KRAS, but not NRAS, activating mutations (NCT00959127). This lack of suitable trials for our two patients may be an early warning that we need to restructure the eligibility criteria for trials of molecularly targeted therapies. We envision an array of available mutation and pathway-based trials for targeted therapies, with eligibility based on molecular assessment.

In addition to identifying aberrations in informative genes, integrative sequencing permits discovery research, such as the NEK11 gene fusion (patient 1) and the AURKA alterations (patient 3). Although difficult to interpret at present, these events could plausibly represent rare or “private” drivers or resistance mechanisms. In this context, the sequencing results can serve as a source of correlative data for trials with molecularly targeted therapies. If patients are treated with matching targeted therapies and develop secondary resistance, repeat tumor biopsy and assessment could reveal mechanisms of resistance, for example, the emergence of a resistant subclone. These data can inform the rational combination of targeted therapies to maximize efficacy and response (47) and minimize resistance. This suggests a future need for the systematic inclusion of tumor biopsies for patients on trials.

Although state-of-the-art technology in genomic sequencing has markedly accelerated biomedical research, translation to the clinical setting has numerous barriers that limit

potential benefits. Therefore, we must strive to develop evidence-based, ethically sound guidelines for implementing genomic sequencing in clinical medicine. This multidisciplinary endeavor provides an early road map for translating high-throughput sequencing into biomarker-driven clinical trials in oncology.

MATERIALS AND METHODS

Study design

Research was performed under Institutional Review Board (IRB)–approved studies. Experiments were performed on tumor xenografts from patients 1 and 2 with metastatic prostate cancer at M. D. Anderson Cancer Center. Patients 3 (metastatic CRC) and 4 (metastatic melanoma) were enrolled and consented through our University of Michigan IRB-approved protocol for integrative tumor sequencing (**Supplementary Methods**).

Briefly, medically fit patients 18 years or older with advanced or refractory cancer were eligible for the study (**Fig. 1**). Informed consent detailed the risks of integrative sequencing and included up-front genetic counseling (**Supplementary Methods**). In collaboration with experts in bioethics and genetic testing (S.Y.K. and J.S.R.), we developed a flexible-default consent process that facilitates both patient autonomy and flexibility. A biopsy was arranged for a safely accessible tumor site. A board-certified pathologist (L.P.K.) evaluated histologic sections for minimum tumor content of 60%. Nucleic acid preparation and high-throughput sequencing were performed with standard protocols. Aberrations were identified by a set of bioinformatics pipelines (**Supplementary Methods**).

STB activity

We carried out a mock STB by evaluating prostate cancer xenograft results to prepare for the clinical study. The STB expands upon traditional tumor boards that focus on a single tissue of origin and uses a molecular classification of cancers based on somatic mutations. For each case, the referring medical oncologist provided a clinical presentation of the patient's history of cancer and previous therapies. Disease-specific expertise is incorporated through ad hoc faculty for diseases and pathways discussed at each meeting. Bioinformaticians, genomics experts, cancer biologists, pathologists, and medical oncologists present findings and review their potential clinical significance. Geneticists and bioethicists have been incorporated to provide insight regarding issues such as controversial, incidental, or even unexpected findings.

The STB classifies results into categories including “Direct impact on care of current cancer,” “Conditions other than cancer of interest,” or “Significance unknown.” For example, genomically significant amplifications may not be considered clinically significant if there are no existing data for that region (significance unknown). Disclosure of results depends on category assignments and the patient's consent preference. Select mutations could be CLIA-validated and disclosed to patients through oncologists, board-certified clinical geneticists, and counselors as appropriate. Currently, if a finding has potential to affect clinical decision-making, the findings are referred for specific CLIA-certified laboratory validation. After accumulating further data and experience, we anticipate that the high-throughput sequencing and analyses will move into CLIA-certified labs. Thus, the STB provides a mechanism to review and interpret the results.

Approach for stratification of aberrant genes

We have curated a gene list to prioritize specific genes for review by the STB (table S3). The list includes genes from the Sanger Institute's Cancer Gene Census (May 2011), which is a catalog of genes (currently 427) for which mutations have been causally implicated in

cancer. This is complemented by the Catalog of Somatic Mutations in Cancer (COSMIC) (4), which curates mutation data and associated information extracted from primary literature. This database provides information about published sequence variants and also estimation of mutational frequencies. We have supplemented this list with additional informative genes on the basis of best clinical practices and genes targeted in clinical oncology trials (48). There are more than 40 locally available early clinical trials through University of Michigan and Wayne State University's Cancer Centers. Last, because nearly half of targeted therapies are aimed at protein kinases, we have also included a comprehensive list of the human kinome (49). The list is updated monthly, and analyses are run iteratively so that new clinically relevant findings can return to STB for discussion if needed.

Sample preparation, sequencing, and validation

Nucleic acids were prepared from tumor and germline tissues with standard commercially available kits (Supplementary Methods). RNA integrity was confirmed by an Agilent Bioanalyzer. For solid tumors, a board-certified pathologist (L.P.K.) evaluated histologic sections for tumor content. We generated whole-genome and transcriptome libraries for tumors according to Illumina protocols. Exome capture was performed for tumor and germline DNA with SureSelect Human Exon Target Enrichment kit (version 2, Agilent) (patients 1 and 2) or NimbleGen Sequence Capture kits (Roche) (patients 3 and 4). Each library was sequenced on one lane of an Illumina HiSeq 2000. Somatic point mutations and indels nominated as clinically informative by the STB were amplified and sequenced for validation (**Supplementary Methods**). For the exploratory study, next-generation sequencing was not completed in a CLIA-certified lab, and therefore, any findings that could be used for clinical decision-making would require separate CLIA-certified validation. Our CLIA lab (J.W.I., director) currently has capacity for 95 amplicons per day or 1900 per month. We are accumulating data to facilitate the CLIA certification of next-generation sequencing for a defined set of genetic aberrations.

Bioinformatics analyses overview

We identified somatic mutations, CNAs, structural variations, gene fusions, and highly overexpressed genes through a set of bioinformatics pipelines (**fig. S5 and Supplementary Methods**). Briefly, for whole genome data analysis, we used the BreakDancer method to call structural variants, the DNAcopy circular binary segmentation algorithm to call CNAs, ChimeraScan (50) to discover gene fusion, and Cancer Outlier Profile Analysis (COPA) approach (20) to nominate overexpressed genes. Finally, point mutations were called using the Genome Analysis Toolkit (GATK) and in-house algorithms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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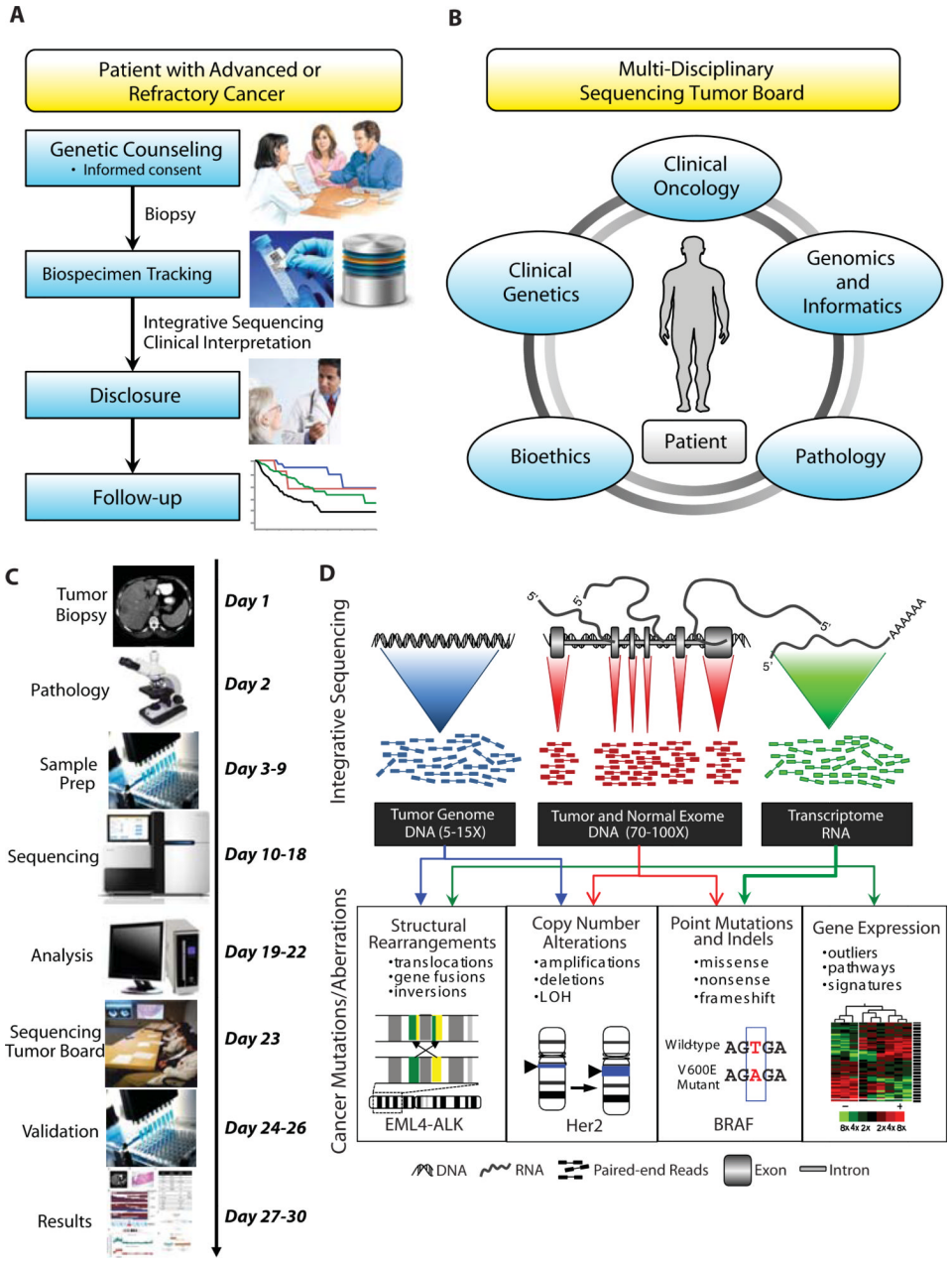

Figure 1. Exploratory integrative sequencing of tumors for personalized oncology

A, The MI-ONCOSEQ study recruits cancer patients and provides up-front genetic counseling. Patients are tracked through a biospecimen and clinical database. **B**, A multi-disciplinary Sequencing Tumor Board was instituted including expertise in clinical oncology, genomics, bioinformatics, pathology, bioethics, and genetics. **C**, Clinically relevant timeframe from tumor biopsy to available results. **D**, Integration of whole genome sequencing (blue), whole exome capture sequencing for 1-2% of the genome (red), and transcriptome or messenger RNA sequencing (green). Each sequencing strategy can be integrated (bottom) for analysis of tumor aberrations including structural rearrangements, copy number alteration, point mutations, and gene expression.

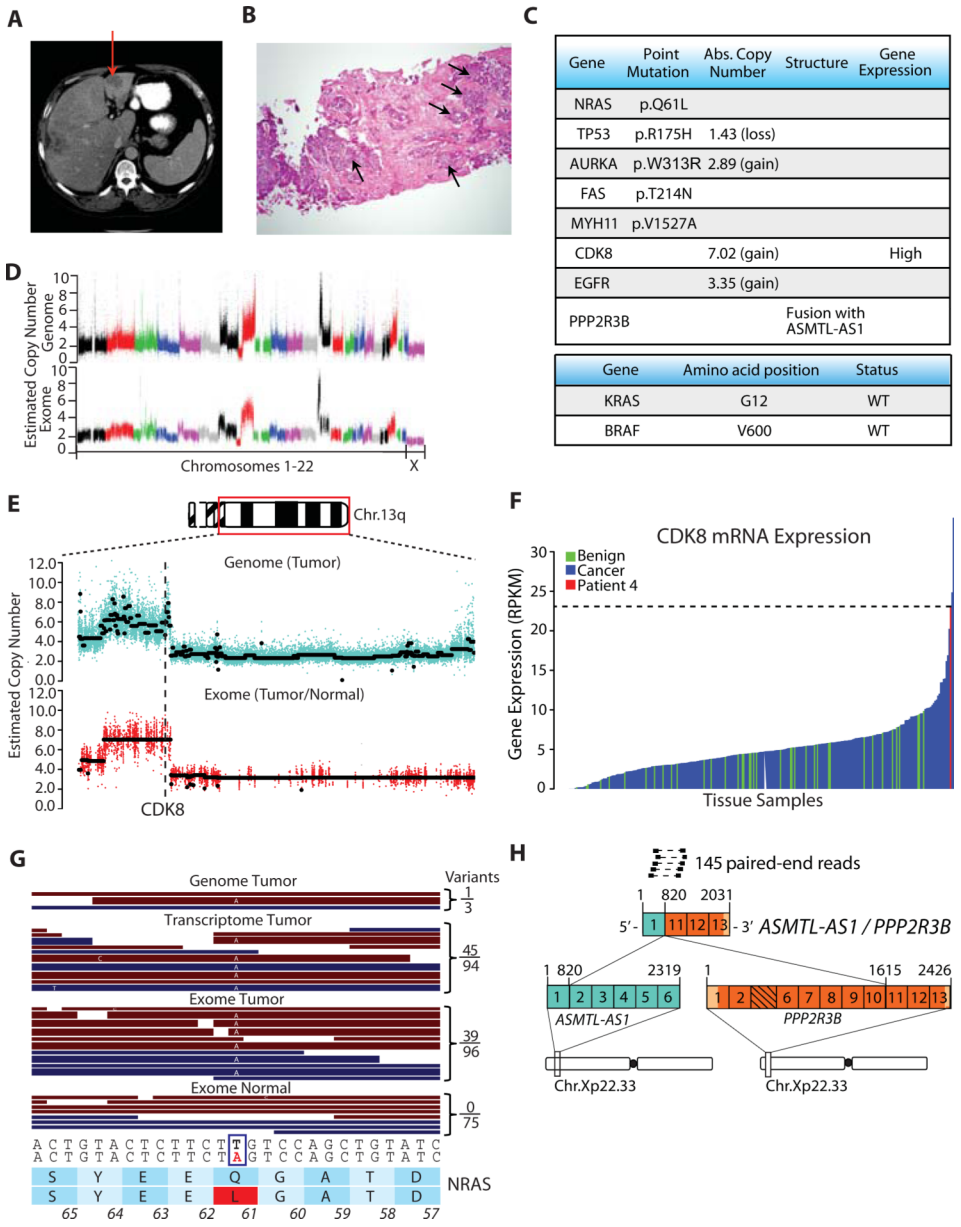

Figure 2. Integrative sequencing of a patient with metastatic colorectal cancer enrolled on the MI-ONCOSEQ protocol

Patient 3 is a 46-year-old man with metastatic colorectal cancer and the first patient enrolled. **A**, CT scan abdomen demonstrates liver metastases and biopsy site. **B**, Representative histology from liver biopsy demonstrates poorly differentiated adenocarcinoma and estimated tumor content 60-70%. **C**, Summary of genetic aberrations identified includes an activating point mutation of NRAS, an inactivating point mutation of TP53, and amplification of CDK8. Wildtype genes included KRAS and BRAF. **D**, Integrated copy number analysis based on exome and whole genome data. **E**, Amplification in region of chromosome 13q including CDK8 is displayed as estimated copy number based on integrative analysis of whole genome (green) and exome (orange) data. **F**, CDK8 is highly expressed based on RNA-seq compared with benign or other cancer samples. **G**, Schema shows integrative analysis used to identify activating NRAS mutation with number of

variant reads on right. **H**, Schema of probable inactivating rearrangement involving PPP2R3B based on integrative analysis of RNA-seq and whole genome data.

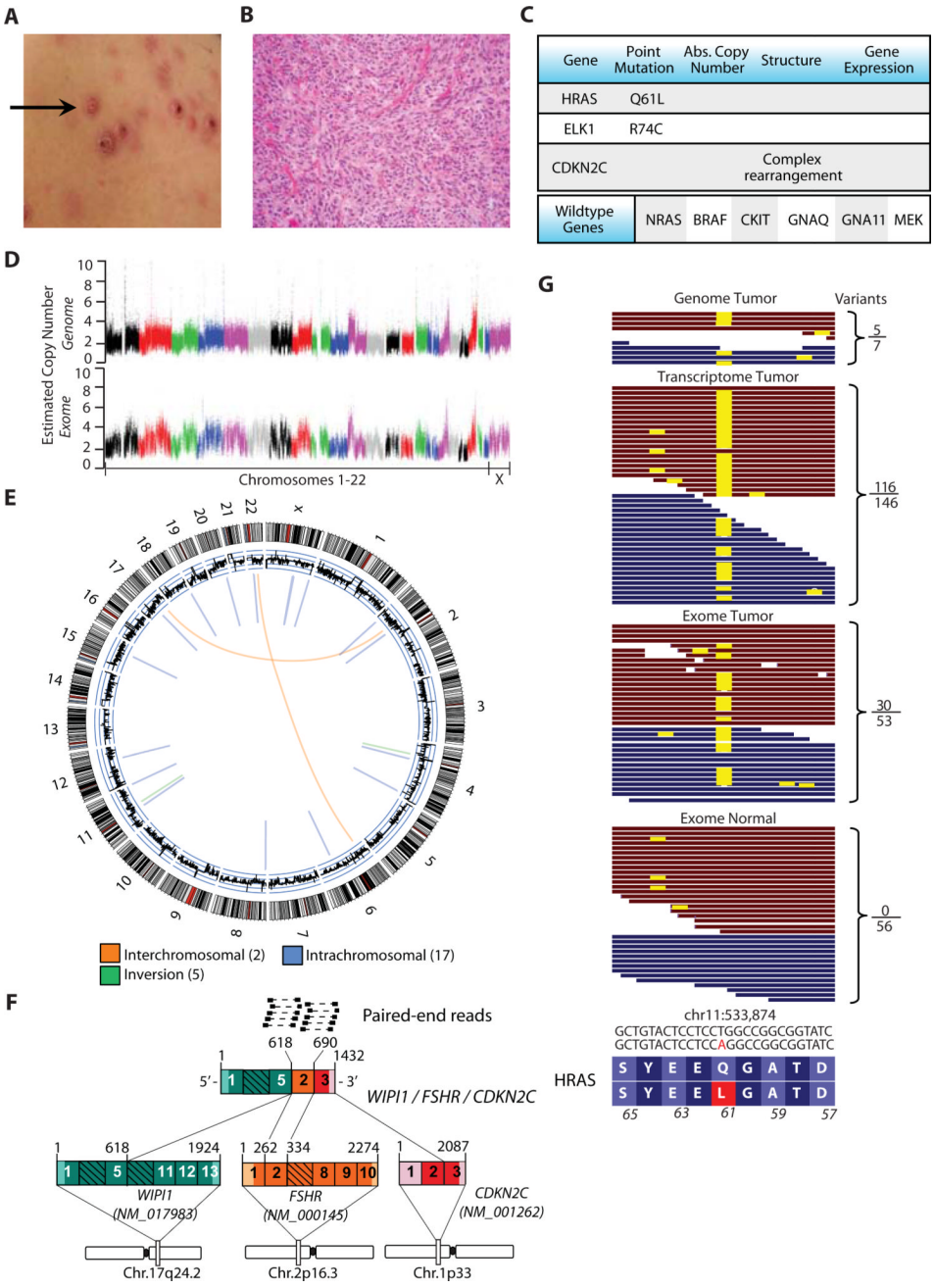

Figure 3. Aberrations reported in Patient 4 (Melanoma)

Patient 4 is a 48-year-old woman with metastatic melanoma. **A**, Multiple skin metastases and sites of biopsy. **B**, Representative histology from skin biopsy demonstrates dermal proliferation of ovoid to spindle cells with frequent prominent nucleoli. **C**, Summary of mutations reveals an activating HRAS mutation and an ETS transcription factor (ELK1) mutation. Wildtype genes included BRAF, CKIT, MEK, and NRAS. **D**, Copy number landscape across chromosomes derived from whole genome and exome sequencing. **E**, Circos plot derived from whole genome sequencing depicts structural variations including deletions (green), interchromosomal (orange) and intrachromosomal (blue) rearrangements. **F**, RNA-seq data support a possible rearrangement involving CDKN2C, WIPI1, and FSHR,

and is predicted to inactivate CDKN2C. **G**, Integrative analysis identifies the activating HRAS mutation.

Table 1

Challenges for Translating High-Throughput Sequencing into Clinical Oncology

Challenges	Approach
Which patients could benefit?	■ Focus evaluation on patients with advanced refractory cancer considering clinical trials ■ Focus evaluation on patients with rare poorly defined disease with no standard therapy
How will informed consent for integrative sequencing be obtained? How will incidental findings be dealt with?	■ Consent through a flexible default consent form, developed in conjunction with bioethicists and genetic counselors, which includes upfront genetic counseling, and discussion of risks for incidental findings, and preservation of patient autonomy to accept or decline incidental findings.
What type of sequencing should be performed?	■ Comprehensive and cost-effective assessment of tumor structural rearrangements, copy number alterations, point mutations, insertions, deletions, and gene expression.
How will computational analysis be completed for each patient?	■ Multiple bioinformatics pipelines rapidly assess data and provide orthogonal support for calling mutations. ■ Focus analyses on genes that could have known clinical significance including genes utilized in best clinical practices, identified as tumor suppressors or oncogenes through the Sanger Cancer Census, and all potentially druggable kinases.
How will sequencing be completed within a clinically relevant timeframe?	■ Complete integrative sequencing within 4 weeks to match the typical time frame that patients must wait between oncology clinical trials.
How will results be interpreted?	■ Assemble multi-disciplinary team for a Sequencing Tumor Board with expertise in clinical oncology, clinical genetics, genomics, bioinformatics, clinical pathology, social and behavioral sciences, and bioethics.

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Table 2

Summary of integrative sequencing results for each patient

Key actionable or informative mutations nominated for the four individual tumors described in this study. Patients 1 and 2 were carried out as pilot samples while patients 3 and 4 were enrolled on the MI-ONCOSEQ clinical protocol.

No	Diagnosis	Age	Prior Therapies	Sequence Results	Potential Pathways for Therapeutic Intervention	Examples of Approved or Investigational Agents
1	Metastatic Castrate Resistant Prostate Cancer	67	Leuprolide + bicalutamide Diethylstilbestrol NY-ESO vaccine study Azacytidine + valproic acid study	PTEN deletion	PI3K inhibitors	BEZ235, GDC-0941, XL147
				AR amplification	Androgen Signaling	Abiraterone, MDV3100
				TMPRSS2-ERG rearrangement	PARP inhibitors	Olaparib, BSI-201, ABT-888
				CPNE4-NEK11 rearrangement	(NIMA kinases?)	??
				TP53 mutation		
2	Metastatic Prostate Cancer	61	Hormone Naïve (Newly diagnosed)	PTEN deletion	PI3K inhibitors	BEZ235, GDC-0941, XL147
				TMPRSS2-ERG rearrangement	PARP inhibitors (UMich trial)	Olaparib, BSI-201, ABT-888
				PLK1 outlier expression	Polo kinase inhibitors	BI2536, GSK461364A, ON-01910
				TP53 mutation		
3	Metastatic Colorectal Cancer	46	FOLFOX + Cetuximab Irinotecan + Cetuximab Phase 1: TAK-901	NRAS mutation	BRAF and MEK inhibitors PI3K inhibitors	PLX4032, GSK2118436, AZD6244 BEZ235, GDC-0941, XL147
				CDK8 amplification	CDK inhibitors	Flavopiridol, PD0332991
4	Metastatic Melanoma	48	Multiple Surgical Resections	HRAS mutation	BRAF and MEK inhibitors PIK3 inhibitors	PLX4032, GSK2118436, AZD6244 BEZ235, GDC-0941, XL147
				CDKN2C rearrangement	CDK inhibitors	Flavopiridol, PD0332991

Abbreviations: PTEN, Phosphatase and tensin homolog; PI3K, Phosphatidylinositol 3-kinase; AR, androgen receptor; TMPRSS2-ERG fusion involving TMPRSS2, transmembrane protease, serine 2 and ERG, ETS transcription factor; CPNE4-NEK11 fusion, involving Copine-4 and NIM A kinase family member; PARP, Poly(ADP-ribose) polymerase; PLK1, polo-like kinase 1; CDK8, cyclin-dependent kinase 8; BRAF, v-raf murine sarcoma viral oncogene homolog B1; MEK, Mitogen-activated protein (MAP) kinase kinase; CDKN2C, Cyclin-dependent kinase inhibitor C.