Validation and utilisation of high-coverage next-generation sequencing to deliver the pharmacological audit trail

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Background: Predictive biomarker development is a key challenge for novel cancer therapeutics. We explored the feasibility of next-generation sequencing (NGS) to validate exploratory genomic biomarkers that impact phase I trial selection.

Methods: We prospectively enrolled 158 patients with advanced solid tumours referred for phase I clinical trials at the Royal Marsden Hospital (October 2012 to March 2013). After fresh and/or archived tumour tissue were obtained, 93 patients remained candidates for phase I trials. Results from tumour sequencing on the Illumina MiSeq were cross-validated in 27 out of 93 patients on the Ion Torrent Personal Genome Machine (IT-PGM) blinded to results. MiSeq validation with Sequanom MassARRAY OncoCarta 1.0 (Sequenom Inc., San Diego, CA, USA) was performed in a separate cohort.

Results: We found 97% concordance of mutation calls by MiSeq and IT-PGM at a variant allele frequency ≥13% and ≥500× depth coverage, and 91% concordance between MiSeq and Sequenom. Common ‘actionable’ mutations involved deoxyribonucleic acid (DNA) repair (51%), RAS-RAF-MEK (35%), Wnt (26%), and PI3K-AKT-mTOR (24%) signalling. Out of 53, 29 (55%) patients participating in phase I trials were recommended based on identified actionable mutations.

Conclusions: Targeted high-coverage NGS panels are a highly feasible single-centre technology well-suited to cross-platform validation, enrichment of trials with molecularly defined populations and hypothesis testing early in drug development.

The development of biomarkers in modern anticancer drug development is a critically important challenge. Recent successes in targeted therapy have defined specific gene and/or protein pathways dysregulated and ‘driving’ tumourigenic processes (Romond et al, 2005; Fong et al, 2009; Flaherty et al, 2010; Kwak et al, 2010), but lack of molecular selection has also led to drug attrition (Mateo et al, 2013). Of the increasing number of novel cancer therapeutics developed (Workman et al, 2013), few reach successful late-stage trials (Williams et al, 2012), and biomarker selection may be a necessary tool to improve drug development success (Garraway, 2013). We have previously described the importance of biomarker selection for early-phase trials in the pharmacological audit trail (PhAT), both through utilisation of known predictive biomarkers and through hypothesis testing of putative ‘enrichment’ biomarkers (Yap et al, 2010; Garcia et al, 2011).

Next-generation sequencing (NGS) has brought unprecedented opportunities for biomarker development by vastly expanding the capability and feasibility of molecularly characterising tumours (Ross and Cronin, 2011; Tran et al, 2013). However, despite the potential of whole-genome, whole-exome, or whole-transcriptome sequencing to personalise therapeutic selection, these approaches are not yet practical for routine use in early-phase trials. One recent study showed a median time to NGS results of 91 days (range 43–243) (Weiss et al, 2013), which remains an unreasonable wait time for the patient with treatment-refractory cancer.
Major challenges also hindering the routine application of NGS include bioinformatic interpretation of enormous quantities of data, incomplete functional understanding of multiple pathway interactions or uncommon mutations, and the need to validate sequenced mutations (Braggio et al, 2013).

Nevertheless, phase I trial candidates and physicians highly support biomarker selection (Miller et al, 2013), hoping that rational trial selection will improve the odds of response and relevance to cancer treatment. Challenges to testing of biomarkers in the phase I population include a highly heterogeneous population with a mixture of tumour types, heavy pre-treatment burden, multiple sites of disease likely to be molecularly heterogeneous, and varied life expectancy (Carden et al, 2010). The promise of NGS-guided therapeutic selection will depend on overcoming these challenges in a reasonable timeframe with relevance to therapeutic selection of trials (Arkenau et al, 2008).

We sought to pilot the use of targeted, high-coverage NGS technologies at the Royal Marsden Hospital Drug Development Unit (RMH-DDU) and The Institute of Cancer Research (ICR) by testing NGS panels with targeted regions of interest (ROIs) directly relevant to our portfolio of phase I trials. The key issue of validation of genomic mutations was explored through cross-platform comparison of results obtained from two NGS technologies as well as a mass spectrometry-based method. Secondary issues of platform comparison of results obtained from two NGS technologies were explored, including translating NGS results into feasibility of real-time application of this approach in the phase I clinic were explored, including translating NGS results into potentially relevant clinical trials.

## MATERIALS AND METHODS

### Patient consent and enrolment.
Institutional research ethics board approval was obtained. Patients referred for experimental therapy October 2012 to March 2013 at the RMH-DDU were considered for study enrolment. Eligibility criteria were patients with metastatic or locally advanced cancer, age \( \geq \) 18 years, referral for early-phase clinical trials, fitness for clinical trials (Eastern Cooperative Group Performance Status (ECOG-PS) 0–2), no major comorbidity requiring active management, no grade 3–4 laboratory abnormalities, and signed informed consent for genetic analysis of tumours. Patient demographic and disease data were collected and entered into an anonymised database with linking accession numbers.

### Tissue collection and processing.
Tissue analyses were conducted at The ICR Cancer Biomarkers Laboratory according to Human Tissue Act requirements. Archived tumour material (tissue blocks or slides) from primary and/or metastatic samples were retrieved from across 58 referral centres in the UK. If deemed technically safe, fresh tumour tissue biopsies were performed with patient consent by an experienced interventional radiologist. Sample identification including site, collection time, and referring institution were recorded. Fresh tissue samples were paraffin embedded and processed further in the same manner as archived tissue. Tissue samples were reviewed by the histopathology team after haematoxylin and eosin (H&E) staining. The region of tumour was then marked by a pathologist for coring. All samples were labelled with anonymised tissue identification.

### DNA extraction and quality control.
The DNA was manually extracted using the QIAamp DNA FFPE Tissue kit (Qiagen, Venlo, Limburg, Netherlands) following the manufacturer’s protocol. Eluted DNA was measured using nanodrop and Quant-IT high-sensitivity Picogreen double-stranded DNA (dsDNA) Assay Kit (Invitrogen, ThermoFisher Scientific Corp., Waltham, MA, USA), according to the manufacturer’s recommendations.

DNA-QC was performed using the Illumina FFPE QC kit (WG-321-1001; see Supplementary Methods).

### Illumina MiSeq sequencing.
The Illumina MiSeq TrueSeq AmpliSeq Cancer Panel (TSACP) is a highly multiplexed NGS assay covering 212 ROIs in 48 cancer-related genes (Supplementary Table 1), in which 2631 mutations are represented at least twice in the Catalogue of Somatic Mutations in Cancer (COSMIC). Targeted sequencing was performed following the manufacturer’s protocol on samples that passed QC parameters (see Supplementary Methods). Bioinformatic analyses were performed utilising the MiSeq Reporter Software MCS 2.2.0, RTA 1.17 28.0 and Nextgene (from Biogene, Kimbolton, Cambs, UK).

### Ion Torrent Personal Genome Machine (PGM) sequencing.
The Ion AmpliSeq Cancer Hotspot Panel v2 contains a single pool of primers used to perform multiplexed PCR for preparation of amplicon libraries covering 207 ROIs in 50 cancer-related genes (Supplementary Tables 1 and 2, Figure 1A and B), in which 2175 mutations are represented at least twice in COSMIC. Twenty-seven randomly selected samples from the prospective cohort were selected to compare MiSeq and PGM sequencing. Targeted sequencing was performed following the manufacturer’s protocol (see Supplementary Methods). Bioinformatic analyses were performed using the PGM Torrent Suite software (version 3.4.2) including a Torrent Variant Caller for single-nucleotide polymorphisms and indel variants across a reference BED file, and Nextgene (from Biogene) for comparison with the MiSeq data.

### Sequenom OncoCarta v1.0 sequencing.
The OncoCarta v1.0 panel comprises 24 multiplex assays that detect 238 mutations in 19 oncogenes (Supplementary Table 1). Twenty-five archived tumour samples from patients referred between 2009 and 2012 who were previously enrolled into a molecular characterisation study were analysed by the Sequenom OncoCarta v1.0 panel (Sequenom Inc.) and MiSeq TSACP (Illumina, San Diego, CA, USA). The samples were processed as described in the methods above for DNA extraction, quantification, QC and sequencing on the MiSeq TSACP. Sequenom OncoCarta v1.0 sequencing was performed using the PGM Torrent Suite software (WG-321-1001; see Supplementary Methods).

### Materials and Methods

**Abbreviations:** NGS, next-generation sequencing; IT-PGM, Ion torrent personal genome machine.

![Patient and sample flow diagram. Abbreviations: IT-PGM = Ion Torrent Personal Genome Machine; NGS = next-generation sequencing.](https://www.bjcancer.com/FIG1.jpg)
Mutation report for multidisciplinary tumour board. DNA mutations reported by each sequencing platform were entered into an anonymised database that was linked to patient and sample data. To minimise false-positive reporting, somatic mutations were only reported if a minimum of 500× coverage depth had been achieved with a variant allele frequency of at least 5%. Mutations were also coded with results of functional knowledge from the COSMIC database and prior literature review. Mutation data from multiple tumour samples from a single patient were linked. Patient mutation reports were generated containing identifying data, tumour primary, histopathology summary, type of sample(s) (FFPE, fresh, or other), sequencing platform(s) used, mutation(s) detected (gene, frequency, and prior description in literature), and potential functional significance of mutations. ‘Actionability’ of mutations was defined by a literature search of each gene covered by the MiSeq and PGM panels, with potential therapeutics having entered late-stage preclinical or clinical stage (Supplementary Table 3).

Phase I clinical trial allocation. Patient cases were discussed at multidisciplinary rounds including oncologists, radiologists, histopathology staff, nurses, and biomedical scientists, integrating information from the patient mutation report. Only mutations previously reported in COSMIC were considered actionable, and relevance of phase I clinical trials to the identified mutations was discussed. Specific clinical trial information was presented to patients provided that they fit eligibility criteria for participation, and while it was disclosed if relevant mutation data influenced the decision for trial allocation, the experimental nature of this approach was emphasised. Patients ineligible for mutation-relevant clinical trials were offered phase I trials without required selection biomarkers if available, or transferred back to the care of the referring health care team.

RESULTS

Characteristics of patients analysed by NGS. Overall, 207 patients were seen in consultation at the RMH-DDU for phase I trials between October 2012 and March 2013, of which 158 patients (76%) initially met study inclusion (Figure 1). Tumour tissue was collected in 118 patients (75%); 103 archived samples alone, 9 with both fresh tissue (including ascites or pleural fluid) and archival tissue collected, and 7 with a fresh tissue biopsy without archived sample. Median age of tumour samples was 3 years. Upon obtaining tumour tissue, 22 patients were excluded as they were no longer eligible or interested in a phase I clinical trials, and another 3 patients had tissue samples without sufficient tumour for analysis.

In total, 93 patients remained eligible for phase I trials and were processed by NGS (Table 1). The most common tumour primary types were ovarian/peritoneal (n = 22, 24%), colorectal (n = 19, 20%), breast (n = 7, 8%) and bladder (n = 6, 7%); 92% of patients had metastatic disease, most commonly to the lungs (45%) and lymph nodes (41%). Two patients had previously treated stable blockage or referral centre (n = 58) (Figure 2), although the best QC scores were obtained from fresh tissue sampled in 2013 at site 32 (RMH-DDU). Three hundred and ninety-six mutations were detected from 69 of the 85 samples (81%) that passed QC, while 16 (19%) patients had no mutations detected despite excellent tumour QC parameters (Supplementary Table 4). In total, 232 of these 396 (59%) mutations had been previously described as oncogenic in the COSMIC database; 69 out of 396 (17%) mutations were unreported but impacting an amino acid previously described to be mutated in cancer; 95 out of 396 (24%) were mutations not previously described. Most frequently mutated genes were TP53 (11%), PIK3CA (12%), APC (11%), KRAS (9%), ATM (6%), and FBXW7 (5%) (Figure 3), although a substantial proportion of the mutations seen in ATM and FBXW7 had not been previously described as oncogenic. Mutations in BRAF (3%), MET (3%), KIT (3%), EGFR (3%), ERBB2 (2%), and AKT1 (1%) were also detected.

| Table 1. Characteristics of patients analysed using NGS platforms |
|---------------------------------------------------------------|
| **Demographic** | **Patients (%)** |
| Median age (range, years) | 57 (19–79) |
| **Primary site** |  |
| Ovarian/primary peritoneal | 22 (23) |
| Colorectal | 19 (20) |
| Breast | 7 (8) |
| Bladder | 6 (6) |
| Pleural | 5 (5) |
| Sarcoma | 5 (5) |
| Cervical | 4 (4) |
| Head and neck | 4 (4) |
| Lung | 4 (4) |
| Oesophageal, renal, gallbladder, vaginal, melanoma, pancreatic, penile and prostate | 17 (18) |
| **Female** | 58 (62) |
| **ECOG-PS at consultation** |  |
| 0–1 | 91 (99%) |
| 2 | 2 (2%) |
| **Locally advanced disease** | 7 (8%) |
| **Metastatic disease** | 86 (92%) |
| **Number of metastatic sites** |  |
| 0 | 7 (8%) |
| 1 | 26 (28%) |
| 2 | 37 (40%) |
| 3+ | 23 (24%) |
| **Sites of metastases** |  |
| Lung | 42 (45%) |
| Lymph node | 38 (41%) |
| Liver | 30 (32%) |
| Bone | 9 (10%) |
| Brain | 2 (2%) |
| **RMH Prognostic Index** |  |
| 0–1 | 74 (80%) |
| 2–3 | 19 (20%) |

Abbreviations: ECOG-PS = Eastern Cooperative Group Performance Status; NGS = next-generation sequencing; RMH = Royal Marsden Hospital. All values are expressed as n (%) unless otherwise specified.
Mutations were detected at a frequency similar to the expected mutation rates in the ovarian and colorectal cancer cohorts (Supplementary Table 5), considering the limited sample size. Histological subtypes influenced mutation rates, for example, in the ovarian cancer cohort where high rates of KRAS and PIK3CA mutations were detected: in the low-grade serous carcinoma group (seven patients, 38%), 3 out of 7 (43%) had a KRAS mutation and 4 out of 7 (57%) had PIK3CA mutation, and in both a clear cell and a mucinous ovarian carcinoma, concurrent KRAS and PIK3CA mutations were detected.

Blinded analysis of mutations from patients with multiple samples shows the ability of MiSeq TSACP to detect mutations reproducibly between primary tumour, synchronous metastases, and metachronous metastases (Table 2; patients 1–5 and 7).

Interestingly the detected variant allele frequencies were strikingly similar between multiple tumour samples from the same patient. Only in one patient mutations were not reproducibly detected between samples (patient 6), in which all mutations were found at low variant allele frequencies (5–10%).

Cross-validation of MiSeq results by IT-PGM ACP. Concordance of IT-PGM results with MiSeq results was very high for overlapping ROIs, with 21 out of 27 patient samples (78%) demonstrating 100% concordance of mutation results, and only 2 out of 27 (7%) patient samples having fully discordant results (Table 3). Overall, 84% of mutations detected by MiSeq TSACP were also detected by PGM AmpliSeq at the pre-defined coverage depth (500 ×) and frequency parameters (5%). Notably, 14 out of 15
(93%) of discordant mutation calls had allele frequencies of 12% or less, whereas 38 out of 39 (97%) of concordant mutation calls had allele frequencies of 13% or greater. Variant allele frequencies of gene mutations detected on both NGS platforms were strikingly similar.

Cross-validation of MiSeq results with Sequenom OncoCarta v1.0 mutations detected on both NGS platforms were strikingly similar. Concordance of Sequenom OncoCarta v1.0 mutations with v1.0.

Table 2. Comparison of mutation calls from patients with multiple samples processed on MiSeq TSACP

| Tumour primary | Sample 1/mutations/freq | Sample 2/mutations/freq | Sample 3/mutations/freq |
|----------------|-------------------------|-------------------------|-------------------------|
| Patient 1      | Archived primary tumour (October 2011) | Archived metastasis biopsy (November 2012) | Fresh metastasis biopsy (April 2013) |
| Ovarian clear cell carcinoma | PIK3CA E545K/45% KIT M541L/40% KRAS G12D/30% | PIK3CA E545K/34% KIT M541L/56% KRAS G12D/26% | PIK3CA E545K/23% KIT M541L/53% KRAS G12D/20% |
| Patient 2      | Archived metastasis biopsy (February 2012) | Fresh metastasis biopsy (March 2013) | Fresh metastasis biopsy (April 2013) |
| Ovarian low-grade adenocarcinoma | PIK3CA E542K/12% | PIK3CA E542K/36% MET7 T1010I/80% | PIK3CA E545K/23% KIT M541L/53% KRAS G12D/20% |
| Patient 3      | Archived primary tumour (November 2010) | Fresh metastasis biopsy (March 2013) | Fresh metastasis biopsy (April 2013) |
| Colon adenocarcinoma | PIK3CA E542K/24% FBXW7 582L/23% APC T1488fs*17/21% | PIK3CA E542K/84% FBXW7 582L/69% TP53 R282W/94% | PIK3CA E545K/23% KIT M541L/53% KRAS G12D/20% |
| Patient 4      | Archived primary tumour (September 2008) | Pleural fluid (May 2013) | Pleural fluid (May 2013) |
| Breast invasive ductal carcinoma, ER negative, Her2 negative | PIK3CA G106_R108del/32% TP53 P142fs*28/25% | PIK3CA G106_R108del/7% TP53 V143fs*35 | PIK3CA G106_R108del/7% TP53 V143fs*35 |
| Patient 5      | Archived metastasis biopsy (March 2012) | Archived primary biopsy (September 2012) | Pleural fluid (May 2013) |
| Breast invasive ductal carcinoma, ER positive, Her2 negative | CDH1 R108fs*7/69% KRAS G125/42% NRAS G60R/9% PTEN E256K/9% ERBB2 R784C/7% SMAD4 Q334Y/6% | CDH1 R108fs*7/57% | PIK3CA N1044S/36% |
| Patient 6      | Archived primary tumour (September 2012) | Archived primary biopsy (September 2012) | Pleural fluid (May 2013) |
| Cervical mucinous adenocarcinoma | APC P1455S/5% BRAF V450*5% FBXW7 V265I/7% | ATM R2459C/8% FBXW7 W486Y/5% | PIK3CA N1044S/36% |
| Patient 7      | Archived primary tumour (March 2009) | Archived metastasis biopsy (March 2009) | Fresh metastasis biopsy (January 2013) |
| Ovarian carcinoma, high-grade serous papillary | ATM D1963N/43% TP53 Y220S/33% | ATM D1963N/47% TP53 Y220S/27% | ATM D1963N/38% TP53 Y220S/53% |

Abbreviations: ER negative = oestrogen receptor negative; TSACP = TruSeq Amplicon Cancer Panel. Bolded gene mutations indicate concordant results between samples.
both rare mutations in the p85 domain and the other in the catalytic domain, each reported only twice previously. This patient was treated with a dual mTOR-Pi3K oral inhibitor and achieved a RECIST partial response but discontinued therapy after 5 months due to drug-related toxicity.

**DISCUSSION**

In our study, 158 patients were consented to study, 118 (75%) had tissue retrieved/collected, 93 (59%) were eligible for phase I trials and had NGS done, and 85 (54%) had tumour DNA that passed quality control for NGS. Of these eligible and sequenced 85 patients, 15 (18%) had no mutations detected, 7 (10%) had no ‘actionable’ mutation, and 63 (74%) had ‘actionable’ mutations detected, of which 29 out of 63 (46%) participated in a matched clinical trial. Therefore, the overall rate of success in matching patients to clinical trial within our trial portfolio was 29 out of 158 (18%) considering all of the feasibility hurdles. This single-centre pilot study demonstrates that targeted, high-coverage NGS can deliver biomarker-enriched patient populations for early-phase trials, allowing for early hypothesis testing as part of PhAT. Rapid cross-validation of detected mutations was feasible by different NGS technologies with very high concordance using paraffin tissue. Overall, 74% of successfully sequenced patients had ‘actionable’ mutations, of which 46% of patients with actionable mutations were ‘matched’ to trial.

A major advantage to targeted sequencing on the MiSeq or IT-PGM platforms is that ROIs can be customised to match the specific clinical trial portfolio of the drug development unit, delivering biomarker-enriched patient populations for early-phase trials, allowing for early hypothesis testing as part of PhAT. Rapid cross-validation of detected mutations was feasible by different NGS technologies with very high concordance using paraffin tissue. Overall, 74% of successfully sequenced patients had ‘actionable’ mutations, of which 46% of patients with actionable mutations were ‘matched’ to trial.

A major advantage to targeted sequencing on the MiSeq or IT-PGM platforms is that ROIs can be customised to match the specific clinical trial portfolio of the drug development unit,
allowing a focussed evaluation of potential biomarkers of interest. We also found that the advantages included scalability according to patient volumes, moderate material costs (~$250 per patient sample run for MiSeq TSACP, excluding tissue processing and labour; see Supplementary Table 2), and a fast turnaround time with a median tissue receipt to reporting of results of 5 days. Previous targeted sequencing in the phase I setting have been advocated as a means of personalising therapy in view of the current costs of whole-genome sequencing approaches may be better utilised to define the biological underpinnings of tumours that have an exceptional response to treatment (Roychowdhury et al., 2011), these are currently too resource and time intensive for the majority of treatment-refractory patients considering phase I trials. Whole-genome sequencing analyses frequently require months of bioinformatics analyses before the finalised reports are available. Moreover, these approaches may be better utilised to define the biological underpinnings of tumours that have an exceptional response to therapy in view of the current costs of whole-genome sequencing (Kwak et al., 2013). The reliance of NGS on software for data analyses to interpret the large amount of information generated makes it critically important to validate detected mutations (Ross and Cronin, 2011). Cross-validation of mutations by different NGS sequencing panels was possible due to significant overlap in ROIs. Prior technical studies have suggested variant frequency cut-offs of 10% and minimum of 250 × depth coverage (Singh et al., 2013); we chose 5% variant frequency and a minimum of 500 × coverage depth for reporting, which is conservative given the manufacturer’s labelling of 2% sensitivity. On cross-platform analysis we found discrepancies occurred almost exclusively at low variant allele frequencies (<13%), suggesting that in real-life clinical samples, validation with highly sensitive, multiplexed and unbiased methods such as NGS are feasible and appropriate (Hadd et al., 2013).

In order to conduct this study, we received tumour samples from hospitals across the UK. Therefore a significant limitation to the quality of the DNA analysed included the prior handling of tumour tissue. The quality of the DNA extracted from an archival sample is highly dependent on how the sample has been handled before, during and after fixation and embedding. Great care should be taken during the fixation process to prevent overfixation (not > 24 h) that would result in more extensive crosslinking and make extraction of nucleic acids of good quality more difficult. During the embedding process it is important that the specimen is fully dehydrated to prevent degradation and stored appropriately. Storage at 4 °C compared to room temperature has been shown to better preserve nucleic acids.

Our study shows that there is significant variability in the quality control parameters based on the site of archived tumour retrieval. Nevertheless, the majority of archived tumour samples still achieved good quality control and could be successfully archived. Archived FFPE tissue was the main tissue source because of availability and patient preference. The formalin fixation process does not appear to alter NGS mutation calls if DNA quality is adequate (Spencer et al., 2013), and in this study identical mutations were detected among multiple patient samples (primary and metastatic).

On-study fresh tumour biopsies are ideal specimens for molecular characterisation, but the challenges are well documented in the literature; these include informed patient consent, appropriate selection of biopsy sites, logistical coordination of radiological guided biopsies, monitoring of patients post tumour biopsies, adequate staffing for real-time processing and analysis of tumour specimens, and adequate analytical validation of biomarker assays. The safety of biopsies are generally excellent, and at our site we performed superficial, lymph node or liver biopsies on-site but referred thoracic biopsies to a hospital with thoracic surgical support.

### Table 4. Patient tumours with mutations in actionable pathways as detected by Illumina MiSeq (n = 85)

| Tumour type; any mutation detected (%) | COSMIC-described mutations (%) | DNA repair | RAS-RAF-MEK | PI3K/AKT-mTOR | Wnt signalling | KIT | Angiogenesis | MET | FGFR | ERBB signalling | Actionable mutations (%) |
|---------------------------------------|-------------------------------|-----------|-------------|--------------|--------------|----|--------------|-----|------|----------------|-----------------------|
| Ovarian/pelvic 20 out of 21 (95)      | 19 (90)                       | 52%       | 38%         | 29%          | 5%           | 10%| —            | 5%  | —    | 5%             | 18 (86)                |
| Colorectal 19 out of 19 (100)        | 19 (100)                      | 79%       | 68%         | 32%          | 74%          | —  | 16%          | 5%  | 11%  | 16%            | 19 (100)               |
| Breast 7 out of 7 (100)              | 7 (100)                       | 71%       | 14%         | 29%          | 14%          | —  | 14%          | 14%  | 14%  |                | 7 (100)                |
| Bladder 4 out of 5 (80)              | 3 (60)                        | 40%       | 20%         | 20%          | 20%          | 20%| —            | —   | —    |                | 3 (60)                 |
| Pleural 2 out of 4 (50)              | 1 (25)                        | —         | 25%         | —            | —            | —  | —            | —   | —    |                | 1 (25)                 |
| Sarcoma 2 out of 5 (40)              | 1 (20)                        | 20%       | —           | 20%          | —            | —  | —            | —   | —    |                | 1 (20)                 |
| Cervical 2 out of 4 (50)             | 2 (50)                        | 25%       | 50%         | 25%          | 25%          | —  | —            | —   | —    |                | 2 (50)                 |
| Head and neck 3 out of 3 (100)       | 3 (100)                       | 33%       | 33%         | 33%          | —            | —  | —            | —   | —    |                | 2 (66)                 |
| Lung 3 out of 3 (100)                | 3 (100)                       | 67%       | 67%         | 33%          | —            | —  | —            | —   | —    |                | 3 (100)                |
| Other primary 8 out of 14 (57)       | 7 (50)                        | 36%       | 14%         | 7%           | 21%          | —  | 14%          | 7%   | 7%   |                | 7 (50)                 |
| **Total** 70 out of 85 (82)          | 65 (76)                       | 63 patients (74%) with ‘actionable’; COSMIC-described mutation |

Abbreviations: COSMIC – Catalogue of Somatic Mutations in Cancer; QC – quality control. Mutations described in COSMIC and potentially targetable by a drug in early-phase trials were considered ‘actionable’. Values above represent % of samples analysed which harboured a mutation known to impact the pathways described.
**Table 5. Phase I clinical trial participation and selected relevant mutations**

| Tumour primary/histology | Actionable gene/mutation | Trial drug mechanism |
|--------------------------|--------------------------|---------------------|
| Ovarian clear cell carcinoma | KIT MS41L, KRAS G12D, PIK3CA E545K | PI3K p110α inhibitor |
| Ovarian clear cell carcinoma | KRAS G12D, PIK3CA E545K | MEK/PI3K combination |
| Ovarian high-grade adenocarcinoma | TP53 A138V, TP53 E271K, TP53 W91* | PARP inhibitor |
| Ovarian high-grade adenocarcinoma | TP53 S96fs*53 | PARP inhibitor |
| Ovarian high-grade adenocarcinoma | TP53 Y220S | PARP inhibitor + chemotherapy |
| Ovarian low-grade adenocarcinoma | PIK3CA E542K | PI3K p110δ inhibitor |
| Ovarian mucinous adenocarcinoma | KRAS G12D, PIK3CA E542K, TP53 R248W | MEK/IGF1R combination |
| Colon adenocarcinoma | KRAS G12V | AGC-kinase inhibitor |
| Colon adenocarcinoma | KRAS G12V, PIK3CA E545K | Dual mTORC inhibitor |
| Colon adenocarcinoma | KRAS G12V | Dual mTORC inhibitor |
| Colon adenocarcinoma | KRAS G12C, PIK3CA Q546K | MEK/IGF1R combination |
| Colon adenocarcinoma | KRAS G12D, PIK3CA S553N | MEK/PI3K combination |
| Colon adenocarcinoma | KRAS G12V | MEK/PI3K combination |
| Colon adenocarcinoma | BRAF V600E | Pan-AKTI |
| Colon adenocarcinoma | BRAF G466A, PIK3CA N1000D, PIK3CA Y1021F | PI3K p110α inhibitor |
| Rectal adenocarcinoma | KRAS G12D | AGC-kinase inhibitor |
| Rectal adenocarcinoma | KRAS G13D, PIK3CA I391M | IGF 1/2 ligand antibody |
| Breast ER+/Her2-negative adenocarcinoma | ERB2 R784C, KRAS G12S, NRAS G60R, PIK3CA N1044S, PTEN E256K | PI3K p110α inhibitor |
| Breast TNBC adenocarcinoma | PIK3CA G106R, R108del | Androgen receptor inhibitor |
| Breast TNBC adenocarcinoma | TP53 H193Y | PARP inhibitor |
| Breast TNBC adenocarcinoma | TP53 D281H, TP53 R175H | PI3K p110β inhibitor |
| Cervix mucinous adenocarcinoma | BRAF W450* | Dual mTORC inhibitor |
| Cervix squamous cell carcinoma | BRAF G450* | PI3K p110α inhibitor |
| Endometrial adenocarcinoma | EGFR G721D | Pan-AKTI |
| Head and neck squamous cell carcinoma | PIK3CA E545K | PI3K/mTOR inhibitor |
| Liposarcoma | p53 wild-type | HDM2 inhibitor |
| Melanoma | BRAF V600E | BRAF inhibitor |
| Penile squamous cell carcinoma | PIK3CA E545K | AGC-kinase inhibitor |

Abbreviations: ER = oestrogen receptor; TNBC = triple-negative breast cancer.

The use of liquid biopsy is showing promise through detection of circulating tumour DNA (ctDNA), but not all types of cancer release equal amounts (Bettegowda et al, 2014). Tumour heterogeneity and evolution are important factors to consider in the treatment-refractory patient (Collisson et al, 2012; Gerlinger et al, 2012; Swanton, 2012), and less invasive tissue sources such as ctDNA and malignant fluid (as explored in this study), may be necessary tools in patients unable or unwilling to undergo a biopsy (Agulnik et al, 2006; Perkins et al, 2012; Murtaza et al, 2013).

**CONCLUSION**

Overall, we show that targeted, multiplexed NGS panels assessing customised ROIs can quickly and cost-effectively allow drug development units to enrich their trials with patients harbouring mutations of interest as suggested by the PhAT. Challenges remain in the clinical implementation of NGS technologies, but in this study a high proportion of patients had actionable mutation results (63 out of 85; 74%) and nearly half of the patients with actionable mutations were directed to relevant trial options (29 out of 63, 46%). Future directions will include further customisation of ROIs to match our trial portfolio, a greater emphasis on prospective biopsy sampling including ctDNA, and application of biomarker data from exceptional responders to inform trial allocation.

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**CONFLICT OF INTEREST**

The authors declare no conflict of interest.
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