ONCOTARGET (ONCOALVO), a Custom NGS Panel for Therapeutic Decision in Solid Tumors Refractory to Conventional Therapy

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Abstract

Next-generation Sequencing (NGS) of tumor biopsies of both solid tumors, as well as hematological malignancies, using commercially available platforms has broadly entered routine clinical practice in medical oncology. This molecular diagnostic approach is now used mainly to test for predictive biomarkers for patients with no available further standard therapies, as diagnostics rely on genomic testing of molecular alterations to enable effective cancer treatment. However, the high cost of large multigenic panels and especially the sequencing of the entire tumor exome is a concern, especially in a developing country such as Brazil. Here we report the clinical application of a panel of 57 genes that we call ONCOTARGET (ONCOALVO) which is based on the Thermo Fisher Oncomine Focus Assay, an integrated, commercially available NGS assay for the rapid and simultaneous detection of single nucleotide variants, short insertions and deletions, copy number variations, and gene rearrangements in 52 cancer genes with therapeutic relevance, but with the addition of 5 additional genes: the Homologous Recombination Repair (HRR) genes *BCRA1, BRCA2, ATM* and *CHEK2* that determine tumor sensitivity to inhibitors of the PARP enzyme and also KDR, which determines sensitivity to regorafenib. Twenty-five diagnostic samples of formalin-fixed, paraffin-embedded material submitted for molecular testing over a 8-month time period were analyzed so far. All patients had advanced solid tumors already refractory to conventional systemic therapy. Libraries were prepared from isolated nucleic acids and sequenced on the Ion Torrent S5 sequencer. Sequencing datasets were analyzed using the Ion Reporter software. From the samples of the 39 patients tested, we found 30 potential therapy target pathogenic gene aberrations in 22 (56.4%) patients: *KRAS*-7 (23.3%), *BRCA1*-6 (20%), *BRCA2*-5 (16.6%), *PIK3CA*-3 (10%), *MAP2K1*-2 (6.6%), *BRAF-V600E*-2 (6.6%) mutations; and also one of each: *ERBB2* (3.3%), *mTOR* (3.3%), *KIT* (3.3%), *ALK* (3.3%) mutations and one (3,3%) *CCND1* amplification. In 3 (7.69%) patients there were double mutations: *BRCA1* and *BRCA2* in 2 cases and *BRCA2* and *KRAS* and one case. The Oncotarget workflow enabled a turnaround of 18½ days. Taken together, ONCOTARGET was found to be a convenient tool for fast, reliable, broadly applicable and cost-effective targeted NGS of tumor samples in routine diagnostics and therapeutic decisions with a potential to become an important asset for precision oncology in Brazil.

Keywords: Next-generation sequencing; Molecular diagnostics; Somatic copy number variations; Oncomine Focus Assay

Introduction

Next-generation Sequencing (NGS) is an emerging technology for molecular diagnostics. It enables the parallel identification of multiple genomic variants even from small tissue samples [1,2]. Scalable and cost-effective NGS solutions to reliably identify therapeutically relevant genomic driver alterations of tumors are the prerequisite for precision oncology. However, implementing multiplexed and comprehensive NGS assays into the clinical routine is challenging because data analysis and interpretation require specialty infrastructure and expertise [3]. In addition, most established routine tests cannot assess Somatic Copy Number Variations (SCNVs) and/or gene fusions, which presently guide treatment selection for several common cancers [4]. To make precision medicine approaches available for all cancer patients, there is a need for fast, reliable, and cost-effective NGS systems that can detect all classes of currently clinically relevant genomic targets from routine Formalin-fixed, Paraffin-embedded (FFPE) tissues [5-7]. To address these challenges, targeted NGS solutions have been developed to identify recurrently altered oncogenes as well as tumor suppressors, genes with frequent high-level amplifications or deletions, and driving gene fusions in a variety of cancers [8]. However, this emerging approach has so far not been sufficiently evaluated on routine diagnostic FFPE material in terms of feasibility, reliability, cost, and capacity [6,9,10].

The Oncomine Focus Assay (OFA, Thermo Fisher Scientific, San Francisco, CA) is a targeted, multi-biomarker NGS assay that enables fast simultaneous detection of hundreds of variants across 52 genes relevant to solid tumors [8,11]. These variants are treatable by on-market oncology drugs approved by the U.S. Food and Drug Administration as well as drugs that are part of the National Comprehensive Cancer Network guidelines or are currently listed in clinical trials [8,11]. The assay analyzes clinically relevant gene alterations including single nucleotide variants, short insertions and deletions, SCNVs, and gene fusions from DNA and RNA in a single workflow. It enables the detection of tumor-specific genomic alterations using low-input FFPE samples such as needle biopsies and fine needle aspirates and is compatible with bench top Ion Torrent sequencers. The power of the OFA technology for identification of genetic alterations is underlined by the present application in the nationwide NCI–Molecular Analysis for Therapy Choice Trial [12]. This molecular diagnostic approach is now used mainly to test for predictive biomarkers for patients with no available further standard therapies, as diagnostics rely on genomic testing of molecular alterations to enable effective cancer treatment. However, the

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high cost of large multigenic panels and especially the sequencing of the entire tumor exome is a concern, especially in a developing country such as Brazil. The most commonly used NGS platform in Brazil is the Foundation One (Foundation Medicine). However, its employment is limited by its high cost, besides the inconvenience of being carried out in USA.

On the other hand, DNA Double-strand Breaks (DSBs) are toxic DNA lesions that can be repaired by Non-homologous End-joining (NHEJ) or Homologous Recombination (HR). Mutations in HR genes elicit a predisposition to cancer; yet, they also result in increased sensitivity to certain DNA damaging agents and Poly (ADP-ribose) Polymerase (PARP) inhibitors. To optimally implement PARP inhibitor treatment, it is important that patients with HR-deficient tumors are adequately selected [13]. Here we evaluated the performance and applicability of this novel targeted NGS assay for transfer into daily diagnostic practice.

Materials and Methods

Here we report the clinical application of a panel of 57 genes that we call ONCOTARGET (ONCOALVO) which is based on the Thermo Fisher Oncomine Focus Assay, an integrated, commercially available NGS assay for the rapid and simultaneous detection of single nucleotide variants, short insertions and deletions, copy number variations, and gene rearrangements in 52 cancer genes with therapeutic relevance (Figure 1), but with the addition of 5 additional genes: the Homologous Recombination Repair (HRR) genes *BCRA1, BRCA2, ATM* and *CHEK2* that determine tumor sensitivity to inhibitors of the PARP enzyme and also *KDR*, which determines sensitivity to regorafenib (Figure 2).
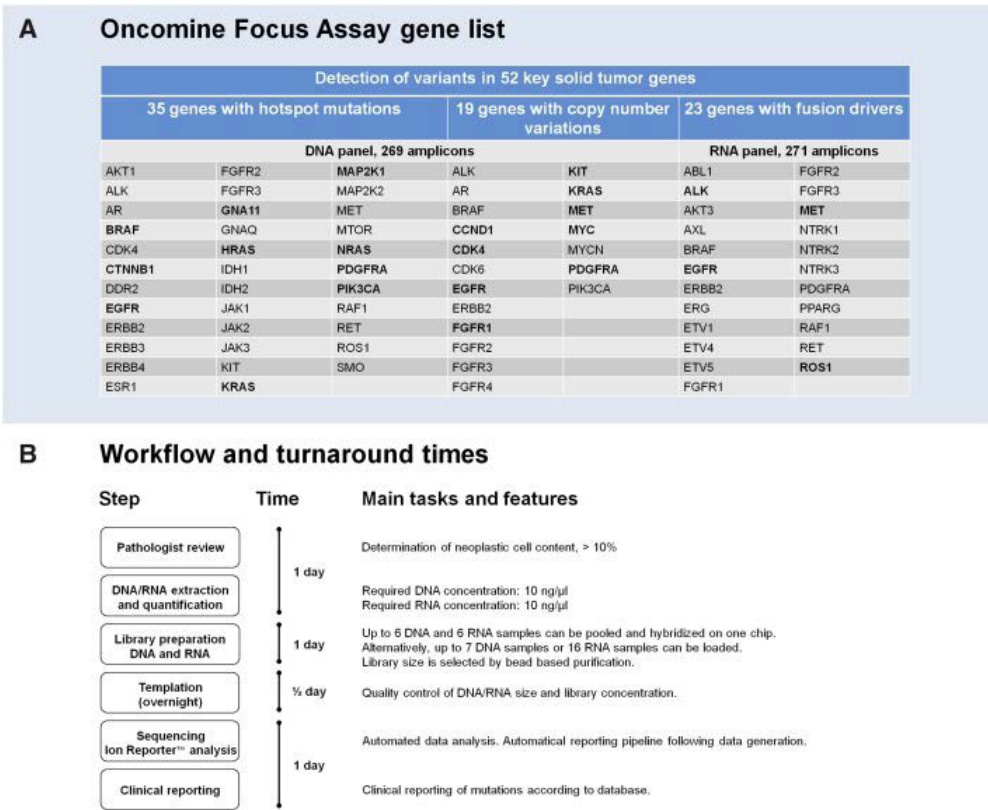

Figure 1: Oncomine Focus Assay Gene List (A), Workflow and Turnaround Times (B).

Figure 2: Oncotarget Panel.

Genomic profiling of samples by targeted NGS

All samples in this study were analyzed using the commercially available OFA platform. The genes targeted in this panel are carefully selected biomarkers derived from expertly curated cancer genomics data [8]. The assay analyzes a maximum of six parallel samples per run for DNA and RNA. It can be used on FFPE samples (10 ng DNA and 10 ng DNase-treated RNA per reaction) and is compatible with bench top Ion Torrent sequencers (Ion Personal Genome Machine, Ion Proton System, Ion S5 System, and Thermo Fisher Scientific).

The percentage of tumor cells relative to other cells (e.g., stromal, inflammatory, and preexisting epithelial cells) was estimated on one Hematoxylin and Eosin (H&E) stained tumor section by a board-certified molecular pathologist (KDM), and an area with a minimum tumor cell content of >20% was designated for the analysis. FFPE tissue sections (4 μm) on positively coated slides were deparaffinized by a standard procedure (2×15 min in xylol, 5 min in absolute EtOH). The defined tumor area was transferred into an Eppendorf LoBind PCR tube (Eppendorf, Hamburg, Germany). For the direct DNA extraction, the dissected tissue was mixed with digestion buffer (50 mM Tris-HCl pH 8.5, 1 mM EDTA pH 8.0, 5% Tween 20, 6 mg/mL of proteinase K; Qiagen, Hilden, Germany) followed by thermomixer incubation (1 h at 56°C and 5 min at 95°C). This crude extract was directly processed or frozen at −20°C for long-term storage. RNA was extracted from FFPE samples using the Recover All Total Nucleic Acid Isolation kit by the "RNA Only" protocol as described by the manufacturer (Ambion, Thermo Fisher Scientific). Nucleic acid concentrations were measured by the Qubit dsDNA HS and the Qubit RNA HS Assay kits, respectively, with the Qubit Fluorometer, and RNA was reverse transcribed to cDNA using the SuperScript VILO cDNA synthesis kit (all from Thermo Fisher Scientific). Libraries were prepared using the Ion PGM Select Library kit and were equalized to 100 pm concentration using the Ion PGM Select Library Equalizer kit. Templation and enrichment were performed using the Ion OneTouch Select Template Kit (all from Thermo Fisher Scientific) on Ion OneTouch 2 and Ion OneTouch ES instruments. The library was sequenced using the Ion PGM sequencer (Thermo Fisher Scientific). The run was considered successful and the sequencing quality adequate when the following quality metrics were met: 1) mapped reads ≥ 300,000; 2) average base coverage depth ≥1000; 3) amplicons having at least 500 reads: ≥ 90%; 4) no strand bias: ≥ 90%; 5) amplicons read end-to-end: ≥ 85%. Five thousand mapped reads were set as the limit for a single RNA library. Primary analysis was performed by Torrent Server™ (v 5.0) and further by Ion Reporter™ Server hosting informatic tools (Ion Reporter™ Software v5.0) for variant analysis, filtering, and annotations. Automatic workflow (Oncomine Focus v2.0,

DNA and fusions/DNA/fusions, Single Sample) with preconfigured parameter settings (Oncomine Variants 5% CI SCNV ploidy ≥ gain of 2 over normal) was utilized.

The performance of the OFA panel was ascertained using a highly multiplexed test panel, the AcroMetrix Oncology Hotspot Control (Thermo Fisher Scientific) targeting various mutations in 25 of the genes represented in the OFA panel (*AKT1, ALK, BRAF, CTNNB1, EGFR, ERBB2, ERBB4, FGFR2, FGFR3, GNA11, GNAQ, HRAS, IDH1, IDH2, JAK2, JAK3, KIT, KRAS, MAP2K1, MET, NRAS, PDGFRA, PIK3CA, RET, SMO*), plus the 5 additional genes: *BRCA1, BRCA2, ATM, CHEK2* and *KDR*, with a frequency near the limit of detection (5%-35%). The performance of the OFA RNA part was ascertained using the Lung Panel (*ALK-RET-ROS1*) FFPE RNA Reference Standard (Horizon Discovery, Cambridge, UK).

Results

These results are still preliminary: so far, 39 diagnostic samples of formalin-fixed, paraffin-embedded material submitted for molecular testing over a 8-month time period were analyzed so far. All patients had advanced solid tumors already refractory to conventional systemic therapy. Libraries were prepared from isolated nucleic acids and sequenced on the Ion Torrent S5 sequencer. Sequencing datasets were analyzed using the Ion Reporter software. From the samples of the 39 patients tested, we found 30 potential therapy target pathogenic gene aberrations in 22 (56.4%) patients: *KRAS*-7 (23.3%), *BRCA1*-6 (20%), *BRCA2*-5 (16.6%), *PIK3CA*-3 (10%), *MAP2K1*-2 (6.6%), *BRAF-V600E*-2 (6.6%) mutations; and also one of each: *ERBB2* (3.3%), *mTOR* (3.3%), *KIT* (3.3%), *ALK* (3.3%) mutations and one (3,3%) *CCND1* amplification. In 3 (10%) patients there were double mutations: *BRCA1* and *BRCA2* in 2 cases and *BRCA2* and *KRAS* and one case (Table 1). The Oncotarget workflow enabled a turnaround of 18½ days. All 4 patients with *BRCA* 1 and 2 mutations received treatment with Olaparibe because they had refractory advanced ovarian adenocarcinoma, thus demonstrating the utility of the addition of HRR genes in our panel.

Discussion and Conclusions

Molecular profiling of cancer has become essential to predict therapeutic response to targeted therapies. NGS has enabled genome wide personalized oncology efforts for actionable variant identification and prioritization. Here, we conducted a comprehensive analysis and validation of a novel amplicon-based NGS solution for identification of relevant somatic alterations in solid tumors. Multi-biomarker NGS assays from numerous commercial and academic providers are rapidly reaching clinical application. Lower cost and wider availability of NGS

Molecular Aberration (n=30)	Number (%)	Primary Tumor
KRAS	7 (23.3%)	Colorectal (3), Ovarian (1), Pancreatic (2), Unknown (1)
BRCA1	6 (20%)	Ovarian (4), Breast (1), Prostate(1)
BRCA2	5 (16.6%)	Ovarian (3). Breast (1), Prostate (1)
PIK3CA	3 (10%)	Ovarian (1), Prostate (1) Pancreatic (1)
MAP2K1	2 (6.6%)	Ovarian (2)
ERBB2 amplification	1 (3.3%)	Pancreatic (1)
BRAF V600E mutation	2 (6.6%)	NSCLC (1), Ovarian(1)
KIT mutation	1 (3.3%)	GIST (1)
ALK mutation	1 (3.3%)	Sarcoma (1)
CCND1 amplification	1 (3.3%)	Colorecta (1)
BRCA1 and 2 mutations	3 (10%)	Breast (3)
BRCA1 and *KRAS* mutations	1 (3.3%)	Ovarian (1)

Table 1: Molecular Aberrations found in 22 (56.4%) patients tested (30 molecular aberrations).

now raise the debate over the merit of routine tumor genomic analysis. Although some genetic lesions are targeted by a new generation of cancer therapies and certain treatment regimens are coupled to single gene assays, we still do not know if the vast majority of information on other genomic alterations is worth the added cost and if assignment of patients to off-label treatment with a targeted agent might carry potentially serious side effects. We tested the OFA NGS solution which focuses on a carefully selected panel of genomic alterations with therapeutic relevance in comparison to other amplicon-based assays on the market [8]. The addition of 5 additional genes in our panel (the homologous recombination repair (HRR) genes *BCRA1, BRCA2, ATM* and *CHEK2* that determine tumor sensitivity to inhibitors of the PARP enzyme) was successful: 4 patients with BRCA 1 and 2 mutations received treatment with Olaparibe because they had refractory advanced ovarian adenocarcinoma. Besides, the two patients with *BRAF V600E* mutation received anti-*BRAF* therapy. The patient with *KIT* mutation was treated with sunitinib and the patient with sarcoma and *ALK* mutation was treated with crizotinib.

The OFA thereby promises to avoid time consuming and costly analyses of molecular changes without known predictive value. As defined molecular alterations are currently used to drive treatment selection, the OFA appears to be a highly suitable tool for molecular routine diagnostics [14-17]. Before NGS technologies can be applied in the daily routine, systematic studies are needed to ensure consistent and reliable assay performance. Comparison with current conventional tests with respect to turnaround time, sensitivity, specificity, mutation detection limits, costs, and feasibility is required. Molecular profiling of cancer has become essential to predict therapeutic response to targeted therapies. NGS has enabled genome wide personalized oncology efforts for actionable variant identification and prioritization. Here, we conducted a comprehensive analysis and validation of a novel amplicon-based NGS solution for identification of relevant somatic alterations in solid tumors. First, we characterized the workflow, turnaround times, feasibility, and reliability in the analysis of routine clinical tissue samples. OFA performance was assessed using independent methods to confirm the detected alterations. Second, we tested the retrospective analysis of archival FFPE tissue samples in an independent cohort of well-characterized malignant melanoma cases. In both settings, the OFA was found to be a convenient tool for fast, reliable, easy-to-use, broadly applicable, and cost-effective targeted NGS analysis. Multi-biomarker NGS assays from numerous commercial and academic providers are rapidly reaching clinical application. Lower cost and wider availability of NGS now raise the debate over the merit of routine tumor genomic analysis. Although some genetic lesions are targeted by a new generation of cancer therapies and certain treatment regimens are coupled to single gene assays, we still do not know if the vast majority of information on other genomic alterations is worth the added cost and if assignment of patients to off-label treatment with a targeted agent might carry potentially serious side effects. As a preliminary analysis we should conclude:

- The addition of 5 additional genes in our panel (the homologous recombination repair (HRR) genes *BCRA1, BRCA2, ATM* and *CHEK2* that determine tumor sensitivity to inhibitors of the PARP enzyme) was successful: 3 patients with *BRCA* 1 and 2 mutations received treatment with Olaparibe because they had refractory advanced ovarian adenocarcinoma.

- Taken together, ONCOTARGET was found to be a convenient tool for fast, reliable, broadly applicable and cost-effective targeted NGS of tumor samples in routine diagnostics and therapeutic decisions with a potential to become an important asset for precision oncology in Brazil. The final cost is about 1,400 USD.

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