Functional Precision Medicine Identifies Novel Druggable Targets and Therapeutic Options in Head and Neck Cancer

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Abstract

Purpose: Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide, with high mortality and a lack of targeted therapies. To identify and prioritize druggable targets, we performed genome analysis together with genome-scale siRNA and oncology drug profiling using low-passage tumor cells derived from a patient with treatment-resistant HPV-negative HNSCC.

Experimental Design: A tumor cell culture was established and subjected to whole-exome sequencing, RNA sequencing, comparative genome hybridization, and high-throughput phenotyping with a siRNA library covering the druggable genome and an oncology drug library. Secondary screens of candidate target genes were performed on the primary tumor cells and two nontumorigenic keratinocyte cell cultures for validation and to assess cancer specificity. siRNA screens of the kinome on two isogenic pairs of p53-mutated HNSCC cell lines were used to determine generalizability. Clinical utility was addressed by performing drug screens on two additional HNSCC cell cultures derived from patients enrolled in a clinical trial.

Results: Many of the identified copy number aberrations and somatic mutations in the primary tumor were typical of HPV(−) HNSCC, but none pointed to obvious therapeutic choices. In contrast, siRNA profiling identified 391 candidate target genes, 35 of which were preferentially lethal to cancer cells, most of which were not genomically altered. Chemotherapies and targeted agents with strong tumor-specific activities corroborated the siRNA profiling results and included drugs that targeted the mitotic spindle, the proteasome, and G2/M kinases WEEL1 and CHK1. We also show the feasibility of ex vivo drug profiling for patients enrolled in a clinical trial.


Introduction

Genomic profiling of tumors holds great promise to guide cancer therapy. However, a central challenge is the difficulty of matching effective drugs to genomic profiles, which are often complex and unique to each patient. Furthermore, many commonly mutated cancer genes, such as tumor suppressor genes, are difficult to target. Even for genes that might be targetable, it is not always clear which one should be prioritized or which drug would be effective. A second, no less significant challenge is the shortage of druggable targets and associated therapeutic agents. A recent estimate of the number of genes currently targeted by FDA-approved drugs is 109 genes, yet there are approximately 22,000 genes in the human genome, of which approximately 10,000 are considered druggable (1). Even in cases where genome-guided targeted therapy works, development of resistance is common, highlighting the need for new targeted agents or combination therapies.

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide, with about 600,000 new cases each year and a mortality rate around 50% (2). Patients are often
diagnosed with locally advanced disease and subjected to intensive treatment with a combination of surgery, radiation, and chemotherapy. Although these treatments can increase locoregional control, they often induce high-grade toxicities (3). Resistance to chemotherapy and radiation contributes to tumor recurrence and options for those patients are limited.

Genomic analysis of the same tumor facilitates the nomination of driver from passenger genomic aberrations and identifies many candidate targets and potentially effective drugs that could not be predicted a priori. We also show the feasibility of this approach to inform an ongoing clinical trial. Although here we focused on aggressive treatment-resistant head and neck squamous cell carcinoma (HNSCC), this functional precision oncology approach is applicable to other cancer types for which live biopsy specimens can be obtained.

**Translational Relevance**

For most cancer patients, genomic analysis of their tumor is insufficient to point to obvious therapeutic options. To advance precision oncology, there is a need to identify additional targetable vulnerabilities and effective therapies matched to patient-specific features. Here we show for the first time the feasibility of high-throughput functional testing with both siRNA and drug libraries on patient-derived tumor cultures. Combining this large-scale functional data with genomic analysis of the same tumor facilitates the nomination of driver from passenger genomic aberrations and identifies many candidate targets and potentially effective drugs that could not be predicted a priori. We also show the feasibility of this approach to inform an ongoing clinical trial. Although here we focused on aggressive treatment-resistant head and neck squamous cell carcinoma (HNSCC), this functional precision oncology approach is applicable to other cancer types for which live biopsy specimens can be obtained.

**Materials and Methods**

**Cell lines**

This study was approved by the institutional review board (IRB) of the University of Washington (UW, Seattle, WA) and the IRB of the Fred Hutchinson Cancer Research Center (FHCRC, Seattle, WA). Tissue specimens were collected with written consent obtained from a previously untreated 59-year-old male with a floor-of-mouth squamous cell carcinoma (T2N2b, HPV-negative) during surgical treatment. All procedures were conducted in accordance with International Ethical Guidelines for Biomedical Research Involving Human Subjects (CIOMS). Tumor tissue was disinfected in 1% povidone-iodine solution for 5 minutes, miced with a scalpel, and digested with 2–4 mg collagenase and 0.5 mg DNase I (Sigma) in PBS at 37°C for 120 minutes. Cells were obtained by filtration through a 70-μm strainer and centrifugation at 1,000 rpm for 3 minutes. The cells were plated onto collagen IV (Sigma) coated tissue culture flasks and cultured in Keratinocyte-SFM medium supplemented with penicillin-streptomycin, fungizone, glutamine, and nonessential amino acids (Thermo Fisher Scientific). Epithelial tumor cells were enriched by selective culturing and magnetic-activated cell sorting with EpCAM microbeads (Miltenyi Biotec). The established primary tissue culture, which was named FHCRC-SCC-1, was immunohistochemically stained with anti-cytokeratin antibody ab9612 and anti-vimentin antibody EPR8682(2) (Abcam) to confirm epithelial enrichment. Tumorigenicity was confirmed by inoculating cultured cells subcutaneously into the tongue of NOD/SCID IL2 gamma null mice. When tumors reached a palpable mass of >50 mm³, the tumor was harvested for histologic analysis. Isolation and propagation of FHCRC-SCC-7A and FHCRC-SCC-10A HNSCC primary cultures was performed as above. These two cultures were established from two patients enrolled in our phase I clinical trial testing AZD1775 alone and in combination with neoadjuvant chemotherapy in previously untreated, metastatic HNSCC (NCT02508246). The normal oral keratinocyte (NOK) cell line (6) was obtained from Dr. Duvvuri at University of Pittsburgh (Pittsburgh, PA). The cells were maintained in the same medium as FHCRC-SCC-1. Primary human foreskin keratinocytes (HFK-1) were obtained from Dr. Galloway at FHCRC (Seattle, WA; ref. 7). The cells were maintained in Epilife supplemented with human keratinocyte growth supplement (HKGS), glutamine, and nonessential amino acids (Thermo Fisher Scientific).

Two pairs of HNSCC cell lines were used: UM-SCC-14A and UM-SCC-14C, obtained from Dr. Carey at University of Michigan (Ann Arbor, MI), and PCI-15A and PCI-15B, obtained from Dr. Ferris at University of Pittsburgh (Pittsburgh, PA). Each pair was derived from the same patient’s primary tumor and subsequent recurrence in the case of UM-SCC-14C or the metastatic lymph node in the case of PCI-15B (8). The HNSCC cell lines were maintained in DMEM supplemented with 10% FBS, glutamine, and nonessential amino acids, and penicillin/streptomycin (Thermo Fisher Scientific).

**DNA and RNA extraction**

DNA and RNA from the primary tumor and HNSCC cell lines were extracted using the AllPrep DNA/RNA Mini kit (QIAGEN). DNA was also extracted from the patient’s tumor-free salivary gland formalin-fixed block using QIAamp DNA FFPE Tissue kit (QIAGEN). Extracted DNA and RNA were assessed with ND-1000 Spectrophotometer (NanoDrop Technologies) and Agilent 2200 TapeStation (Agilent Technologies). Quantity of double stranded DNA was determined by Quant-it PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific). Quantity of RNA was also determined by Trinean DropSense96 spectrophotometer (Caliper Life Sciences) before sequencing.
Exome sequencing

Next-generation exome sequencing was performed on FHCRSCC-1 at the UW Northwest Genomics Center using the NimbleGen SeqCap probes on an Illumina platform. Paired tumor-free DNA was used as reference to remove germline SNPs. Exome sequencing results were analyzed using VarScan (v2.3.6; ref. 9) and MuTect (v1.1.4; ref. 10) with default parameter settings. SNP and indel calls were generated in reference to human genome build hg19 (Supplementary Table S1; Fig. 1E track 2). Targeted sequencing of FHCRSCC-7A and FHCRSCC-10A was done using UW Oncoplex version 5 (http://tests.labmed.washington.edu/UW-OncoPlex).

Comparative genome hybridization

Comparative genome hybridization (CGH) was performed at the FHCR Genomics Core using Agilent’s SurePrint G3 Human High-Resolution Discovery Microarray 1 × 1M (Design ID 023642, Agilent Technologies). Male human genomic DNA G147A (Promega) was used as normal genome reference for making copy number aberration calls. The array was scanned at 2 μm resolution using Agilent DNA Microarray Scanner. Hybridization signal was extracted from raw images and normalized using Feature Extraction software (Agilent Technologies, v9.5). Copy number aberrations were detected using the ADM-2 algorithm (Threshold = 6, Fuzzy Zero = on) in Agilent’s Genomic Workbench Software (Agilent Technologies, v7.0). Genomic regions with signal ≥3/2 were deemed amplified and those with signal ≤1/2 were deemed deleted (Supplementary Table S2; Fig. 1E track 4).

RNA sequencing

Next-generation RNA sequencing was performed at the FHCR Genomics Core. Briefly, RNA-seq libraries were prepared from total RNA using the TruSeq RNA Sample Prep Kit (Illumina, Inc.) and a Sciclone NGSX Workstation (PerkinElmer). Library size distributions were validated using an Agilent 2200 TapeStation (Agilent Technologies). Additional library QC, blending of pooled indexed libraries, and cluster optimization were performed using Invitrogen Qubit 2.0 Fluorometer (Life Technologies). RNA-seq libraries were pooled (5-plex) and clustered onto a flow cell lane. Sequencing was performed using HiSeq 2500 (Illumina) in a “rapid run” mode employing a paired-end, 100 base read length (PE100) sequencing strategy. Image analysis and base calling were performed using Real Time Analysis software (Illumina, v1.18), followed by “demultiplexing” of indexed reads and generation of FASTQ files, using bcl2fastq Conversion Software (Illumina, v1.8.4).

Confirmation of tumor genetic profile

Copy number aberrations and mutations of selected cancer-related genes were confirmed using next-generation sequencing-based technologies by Resolution Bioscience Inc. Mutation in TP53 was further confirmed by capillary electrophoresis-based sequencing method using Applied Biosystems (ABI) 3730xl DNA Analyzers (Thermo Fisher Scientific) with primer sets as previously described (8).

Integrative analysis of patient tumor and TCGA HNSCC profiles

For genome mutations, TCGA HNSCC mutation data (277 patients × 14,693 genes) were obtained from Synapse (syn1571579) as part of the Pan-Cancer 12 TCGA data (11) and used to calculate gene mutation frequency. For transcriptome analysis, to facilitate incorporation of patient tumor FHCRSCC-1 RNA-seq data with that from TCGA for downstream analysis, the “TCGA mRNA-seq Pipeline for UNC data” was implemented locally to generate “Level 3, RNASeq Version 2” processed data from our samples. In brief, reads of poor quality were discarded prior to alignment to hg19 using MapSplice (v2.1.8; ref. 12), followed by quantitation with RSEM (v1.1.13; ref. 13). Genes with undetected transcripts were excluded and the remaining signals were normalized using the weighted trimmed mean of M-values approach from the edgeR package (14). A small constant was added to all normalized counts and the mean value of the normal samples was calculated to obtain base 2 log-fold change. Genes with log fold-change >1 were deemed highly expressed and those with log fold change < −1 were deemed lowly expressed. We used a CIRCOS plot with 4 concentric tracks to visualize our integrative findings (Fig. 1E). Track 1 presents the mutation frequency of genes in the TCGA HNSCC patient cohort (11). Tracks 2–4 visualize the genomic profile of FHCRSCC-1. Track 2 presents the mutation results as detected by WES, including: non/missense, readthrough, splice site, flanking, UTR, RNA and intergenic mutations, as well as in-frame and frame-shift indels. Track 3 shows the base 2 log-normalized RNA-seq transcript counts. Track 4 visualizes the normalized base 2 log ratio of tumor to normal CNV signal as detected by CGH. The amplified and deleted regions are highlighted with black border. On the periphery of the CIRCOS plot, we highlight genes of interest. We first compiled a list “cancer genes” by aggregating three sets of genes that may be actionable for precision oncology (i.e., Foundation One, MSK-IMPACT, and UW Oncoplex). We used this list to filter the highly expressed genes (shown in red), the highly expressed and amplified genes (shown in bold red), and the lowly expressed genes, which were also harboring deletions (shown in green). Mutated genes in FHCRSCC-1 that were also mutated in TCGA

Figure 1. Integrative genomics for personalized medicine. A, Schematic overview of an integrative high-throughput strategy combining genomic profiling with genome-scale siRNA and drug profiling of a patient with advanced HNSCC. B, Enrichment of cytokeratin-positive tumor cells (red) from vimentin-positive stromal cells (green). C, Tumorigenicity of FHCRSCC-1: mouse xenograft confirms tumor morphology (left), prominent nuclear p53 staining (middle), and mitotic figures (right). D, Cisplatin treatment did not induce p21 in FHCRSCC-1 cells but did induce p21 in p53 wild-type UMSCC-74A cells; E, Integration of patient tumor genomics and TCGA HNSCC mutation profiles. Track 1: gene mutation frequency in TCGA HNSCC patient cohorts. Track 2: selective mutations in FHCRSCC-1 as detected by WES, including nonsense and readthrough (red), missense (blue), splice site (green), flanking, UTR, RNA and intergenic (gray) mutations, in-frame (orange), and frame-shift (green) indels. Track 3: FHCRSCC-1 log-scale transcript level as detected by RNA-seq, with highly expressed genes highlighted with black border. Track 4: FHCRSCC-1 genome copy number aberrations as detected by CGH: log-ratio of tumor to normal CNV signal is shown, where green and red tracks indicate partial deletion and amplification regions, respectively, and the line in between showing no change. A total of 87 deleted (ratio ≤1/2) and 56 amplified (ratio ≥3/2) regions are highlighted with black borders. On the periphery, highlighted genes of interest were filtered by potentially actionable gene lists aggregated from Foundation One, MSK-IMPACT, and UW Oncoplex. These included highly expressed genes (red), highly expressed genes in amplified regions (bold red), lowly expressed genes in deleted regions (green), mutated genes that were also mutated in TCGA HNSCC (>15%) (black), and mutated genes that were also lowly expressed or in an amplified or deleted region (bold black).
at 1% or higher frequency are shown in black. A subset of genes that were also amplified on one hand, or lowly expressed and deleted on the other, are highlighted in bold.

To identify frequently overexpressed genes in the TCGA HNSCC patient cohort, we used the tumor (syn1571420) and normal (syn1571422) RNA-seq transcript profiles and after filtering nondetected transcripts calculated the average transcript levels for each gene. Genes with average tumor expression at least one SD above the average normal (syn1571428) RNA-seq transcript profile were deemed overexpressed. Similarly, we identify frequently amplified genes in TCGA using tumor (syn1571428) and normal (syn1571430) genome-wide SNPs array (CNV) profiles. Genes with average tumor CNV at least one SD above the average normal were deemed amplified.

RNA interference druggable genome screen
High-throughput robotic siRNA-mediated gene knockdown on FHRCSC-SCC-1 cells was performed at the UW Quellos Screening Core as described previously (8). Briefly, optimal transfection conditions were established by comparing cell viability after knockdown of KIF11 with Hs_KIF11_6 siRNA (QIAGEN Inc, catalog no. SI02653693), which causes cell arrest in mitosis, to nontargeting control siRNA (NTC) and reagent-only mock controls. siRNA-mediated druggable genome profiling was performed using the MISSION siRNA Human Druggable Genome library (Sigma, n = 6,659; Supplementary Fig. S2) and an additional panel of DNA damage and repair genes (n = 318). In total, 6,977 genes were screened (Supplementary Table S7; Supplementary Fig. S3). Each gene was targeted by a pool of three siRNAs at a final concentration of 25 nmol/L in a one-gene-per-well, 384-well plate format with 0.1% Lipofectamine RNAiMAX transfection reagent (Thermo Fisher Scientific). All siRNA-mediated gene knockdown tests were performed in triplicate with Hs_KIF11_6 and NTC siRNA as positive and negative controls, respectively. Cell viability was quantified 96 hours after gene knockdown with CellTiter-Glo Luminescent Cell Viability Assay (Promega). Raw luminescence signal was mock-normalized per plate to calculate the percent viabilities for each gene and a homoscedastic t-test p-value was computed. Replicate signals were summarized into a mean value, Z-transformed and used to rank genes. Genes that were both in the bottom 10% and had a P < 0.05 (FDR < 0.23) were deemed strong hits. Genes were also analyzed on a per plate basis. A robust Z-score (MAD = 1.4826) was computed using the plate signal as reference (15). Genes with at least one replicate with a robust Z-score value < 2 and P < 0.05 were deemed weak hits. A total of 391 candidate target genes (CTG) were prioritized.

We performed functional annotation and pathway enrichment analysis of the identified genes using the gProfilerR package version 0.5.3 with FDR as the multiple testing correction method (16), which integrates data from the Gene Ontology (17), Corum (18), KEGG (19), and REACTOME (20) databases, among others.

RNA interference kinome screening of HNSCC cell lines
High-throughput RNA interference kinome screens were performed using MISSION siRNA Human Gene Family Set Kinase Panel (713 kinases) on four HNSCC cell lines as described above for the druggable genome screen. A total of 71, 71, 66, and 77 CTGs were prioritized, respectively, in UM-SCC-14A, PCI-15A, PCI-15B, and UM-SCC-14C cells (Supplementary Table S4).

Predicted compounds for target prioritization
To further prioritize targets for intermediate-scale validation, we used predictive modeling to associate CTGs without targeted compounds with alternative compounds. To extract drug response biomarkers, we trained a Gene-wise Prior Bayesian Group Factor Analysis model (21) with 200 hidden components, using copy number, expression, mutation, and drug response data for a total of 138 compounds and 16,291 genes from the Genomics of Drugs Sensitivity in Cancer data portal (22). The top-ranked (0.1%) predicted biomarkers were selected for each compound, intersected with the 391 CTGs and further filtered yielding a set of 49 targets (Supplementary Table S5).

Confirmatory retesting of 178 prioritized gene candidates
siRNAs were independently designed and manufactured by GE Dharmacon (Supplementary Table S6). Each prioritized gene target was targeted with a pool of 4 siRNAs. Gene knockdown was manually performed in triplicate as described above in a one-gene-per-well, 96-well plate format and cell viability was quantified 72 hours after siRNA-mediated gene knockdown. A homoscedastic t test P value was computed for each gene against NTC siRNA. Targeted genes with less viability than NTC siRNA (P < 0.05) were deemed confirmed (Supplementary Table S10).

Drug screens and confirmatory retesting
High-throughput robotic drug screening on FHRCSC-SCC-1 and NOK cells was performed at the UW Quellos Screening Core. All drugs were tested in duplicate using a series of 10-point dilutions ranging from 0.5 nmol/L to 10 μmol/L in a 384-well plate format. Cell viability was quantified 72 hours after drug treatment using CellTiter-Glo Luminescent Cell Viability Assay. Prioritized small molecules were manually retested in triplicate in a 96-well plate format following the same procedure. Raw luminescence signals were normalized to vehicle control for each drug and analyzed with XFit (IDBS). A four-parameter dose–response curve was fit for each drug. The area under the curve (AUC), IC50, EC50, Y-minimum, Y-maximum, and R-squared were calculated for each compound using default parameters. Drug screening of FHRCSC-7A and FHRCSC-10A cells was performed using a drug library assembled by SEngine Precision Medicine that includes FDA-approved targeted agents and chemotherapies as well as agents in clinical development for the majority of solid tumors (see Supplementary Table S9). Cells were tested in 2D under optimal growth condition for HNSCC and data evaluated as described in Pauli and colleagues (23).

Results
Patient background and primary tumor culture for ex vivo functional genomic profiling
The overall strategy is shown in Fig. 1A. To determine whether comprehensive functional profiling could inform therapeutic strategies in a clinical setting, we chose an aggressive, treatment-
resistant tumor as a case study. The patient presented with a previously untreated, HPV-negative, floor-of-mouth T2N2b squamous cell carcinoma. This patient received surgery followed by concurrent radiation and cisplatin treatment. The tumor recurred, however, and the patient died within a year of diagnosis.

A primary culture was established from a biopsy taken at the initial surgery and designated as "FHCRC-SCC-1." Following a purification step, the culture was composed mainly of epithelial cells with no significant stromal cell contamination (Fig. 1B). Upon intraluminal injection into immunodeficient NOD SCID gamma (NSG) mice, these cells formed tumors with typical HNSCC pathologic characteristics including abundant abnormal mitotic figures (Fig. 1C). Immunostaining showed high levels of nuclear p53 and sequencing identified a TP53 mutation P278S (Fig. 1B-C). The original tumor was intrinsically cisplatin resistant and gain-of-function p53 mutations are associated with chemoresistance (24, 25), so we examined the effect of cisplatin on p53 activity. Cisplatin treatment of FHCRC-SCC-1 cells did not increase the already high levels of p53 nor did it lead to induction of the p53 target gene p21, while it did so in p53 wild-type HNSCC cells (Fig. 1D).

Molecular profiling of FHCRC-SCC-1

We characterized the mutation spectra, copy number aberrations, and gene expression patterns of FHCRC-SCC-1 with whole-exome sequencing (WES), comparative genome hybridization (CGH), and RNA sequencing, respectively. The CIRCOS plot in Fig. 1E serves as a bird’s eye view of the mutational landscape of this tumor with respect to these three parameters. In total, 581 somatic point mutations and 102 indels were identified in 568 genes (Supplementary Table S1). Of these, 210 genes harbor somatic point mutations (n = 192) and/or indels (n = 21) that could potentially alter the gene function (Supplementary Table S1). CGH analysis detected copy number changes, several of which are also commonly observed in HNSCC, such as amplification in 3p12.1 - p11.2, 11p13 - p12, 11q12.3 - q13.4, and 20q11.21 - q12, and deletion in 9p21.3 and 18q11.2 - q23. A total of 1,299 and 831 genes were located, respectively, in amplified and deleted regions (Supplementary Table S2). Compared with the normalized gene expression levels in 44 HNSCC normal controls (see Materials and Methods), 773 genes in FHCRC-SCC-1 were highly expressed and 12,063 genes were lowly expressed, as identified by RNA-seq (Supplementary Table S3).

Identification of potential cancer-associated genetic events in FHCRC-SCC-1

Multi-gene panel tests are increasingly used to identify aberrations in common cancer genes that may be actionable for precision oncology (i.e., Foundation One, MSK-IMPACT, and UW OncoPlex). The outside ring of the CIRCOS plot in Fig. 1E highlights 104 genomic alterations or expression changes from FHCRC-SCC-1 for genes tested by these panels. These include tumor suppressor genes (TP53 and CDKN2A/B); DNA repair genes (RAD21, 51 and 54L; FANCD2 and FANCF); mitotic regulation/complex (AU1RA, AU1RB, CHEK2, and DNAHS); FAT2, and receptor tyrosine kinases and oncogenic drivers (EPHA3, E1F3E, and DDR2), among others.

The innermost ring of the CIRCOS plot shows mutational frequencies from the TCGA HNSCC dataset for comparison (11). Among the altered genes in FHCRC-SCC-1 genome, 18 were also altered at frequency of 3% or higher in HNSCC, providing preliminary evidence that these genes could be generally involved in HNSCC pathogenesis (Table 1). FHCRC-SCC-1 harbors a TP53 mutation, consistent with the high prevalence of TP53 mutation in HPV-negative HNSCC (4). Amplification of 11q13 in FHCRC-SCC-1 is also frequently observed in solid tumors including one quarter of HNSCC cases. Several genes in this amplicon, such as the FGF3, FGF4, FGF19, and CCND1, are associated with tumorigenesis in several cancers including HNSCC (26, 27). Other alterations in FHCRC-SCC-1 that are also frequently observed in HNSCC include deletion of the tumor suppressors CDKN2A and CDKN2B, amplification of oncogenes PIK3CA and MYC, and mutation in the receptor tyrosine kinase (RTK) ephrin receptor EPHA3. Another mutated RTK was DDR2 encoding the discoidin domain receptor 2, which is involved in a wide range of carcinogenic processes such as epithelial-to-mesenchymal transition. Mutations in this gene were recently shown in advanced and recurrent HNSCC tumors and conferred sensitivity to the kinase inhibitor dasatinib (28, 29). However, the DDR2 mutation in FHCRC-SCC-1 was in the 3’UTR making it less likely to be functional.

The frequent alteration of these eighteen genes in HNSCC is suggestive of a functional role in FHCRC-SCC-1 pathogenesis, but
beyond well-studied genes such as TP53, CDKN2A, or MYC, this evidence is not definitive and insufficient to guide treatment. In summary, the genomic landscape of FHCRC-SCC-1 is typical for HPV-negative HNSCC in that it has a high mutation load, has substantial copy number variation, and carries aberrations in known cancer genes. This patient's genomic profile is also typical in that despite carrying many somatic mutations, these do not point to obvious therapeutic choices. Given deletion of CDKN2A and amplification of CCND1, one could hypothesize an overactive CDK4/6 pathway and sensitivity to CDK4 inhibitors such as palbociclib. Alternatively, mutations in FGFR genes might confer sensitivity to FGFR1/FGFR2/RTK inhibitors and mutations in other RTKs such as EPHA3 and DDR2 might predict sensitivity to dasatinib. In the absence of functional information, there is no straightforward way to prioritize these targets or potential therapeutic options.

**Functional testing to prioritize driver events and identify new targets**

We used two complementary approaches to identify novel targets and potentially effective therapeutic strategies: functional genomic profiling using genome-scale arrayed siRNA in a one gene per well approach, and drug profiling using an oncology focused drug library.

We performed siRNA functional profiling of FHCRC-SCC-1 cells using siRNAs targeting 6,977 genes, which includes a 6,659-gene druggable genome library and a 318-gene DNA damage and repair library (Supplementary Fig. S1-3). Compared to the reagent controls, the knockdown of 1,023 genes affected cell viability with 391 causing growth inhibition and 632 growth stimulation (Fig. 2A; Supplementary Table S4). Here, we focused only on genes whose knock-down caused growth inhibition, which we will refer to as 'candidate target genes' (CTGs). Functional annotation and pathway enrichment analysis of the 391 CTGs revealed enrichment of genes involved in cell cycle regulation, DNA damage response, microtubule spindle complex, proteolysis, cell signaling, morphogenesis, immune system, and locomotion (Fig. 2B).

We next sought to identify actionable targets among somatically mutated genes by testing whether siRNA-mediated knockdown impacted viability of FHCRC-SCC-1 derived cell cultures. Because functional testing and genomic characterization were both performed on the same patient derived cells, we could address functionality in the same cells in which these mutations arose. Of the 210 genes with potential function-altering mutations, 60 were included in the siRNA screen. siRNA-mediated knockdown of nine of these genes significantly reduced cell viability (Fig. 2C, left panel), while siRNAs to the remaining 51 were neutral or even growth enhancing. Interestingly, four of the nine genes that were both mutated and CTGs, TP53, DNAH5, EPHA3, and FAT2, are also mutated in the TCGA HNSCC dataset at a frequency of 3% or higher (Table 1). siRNA to EPHA2, related to EPHA3, also reduced viability of FHCRC-SCC-1 cells and EPHA2 is mutated in HNSCC at ~4% frequency and FAT1, related to FAT2, is mutated at ~23% frequency (4). This combination of mutational and functional evidence suggests that, in addition to TP53, mutations in DNAH5, EPHA3/EPHA2, and FAT2/FAT1 could have a driver role in FHCRC-SCC-1 and possibly other head and neck cancers with these mutations.

Similarly, intersection of the genes included in the siRNA library with genes amplified or highly expressed in FHCRC-SCC-1 revealed that knockdown of only approximately 5.3% (20/379) of copy number amplified genes and approximately 7.6% (18/238) of highly expressed genes led to a reduction in viability (Fig. 2C, middle and right). As in the case of mutated genes, the majority of amplified or highly expressed genes tested were phenotypically neutral in this assay.

In summary, only a small fraction of mutated, amplified, or highly expressed genes showed evidence of essentiality, suggesting most events are phenotypically neutral. In contrast, functional genomic testing with siRNA profiling identified novel vulnerabilities that would not be revealed by descriptive genotyping approaches.

**Cancer specificity of gene targets**

From the set of 391 CTGs derived from our siRNA profiling, we prioritized 174 genes for further testing based on the following criteria: (i) the top 1% of profiled genes according to negative viability Z-score (n = 69); (ii) genes with available or predicted (see Materials and Methods) small-molecule inhibitors (n = 49); (iii) genes that harbor nonsilent point mutations and that were also mutated or deleted at rate of 1% or higher in TCGA HNSCC cases (n = 14); (iv) genes that are frequently overexpressed (n = 7) or amplified (n = 53) in HNSCC; (v) genes that were also identified as CTGs from siRNA kinase screens of HNSCC cell lines (described below; n = 19); and (vi) genes based on biological rationale (n = 14; Supplementary Table S5). We retested these prioritized targets using an independent set of siRNAs with at least three of four siRNAs targeting a different region of the gene as compared with the siRNAs used for the primary screen. siRNA-mediated knockdown of 121 of 174 of the retested genes (70%) significantly reduced viability of FHCRC-SCC-1 cells (P<0.05, Fig. 3A), and the remaining 53 trended in the same direction indicating a high level of reproducibility of results from the primary screen. To determine whether the candidate targets showed preferential activity against tumor cells, the same set of 174 siRNAs was tested against two nonmutorigenic keratinocyte cell lines, NOK and HFK1 (6, 7). siRNAs to 35 of the tested genes resulted in greater lethality in tumor cells compared with both normal cells and an additional 23 to one or the other normal cells (P<0.05, Fig. 3A; Supplementary Table S10). This set of "tumor-specific" targets included proteasome genes, G2/M regulators (AURKA, CHEK1, WEE1), mitotic spindle genes, TP53, the Notch ligand JAG1, the Wnt pathway gene WISP2, the antiapoptotic gene MCL1, and others (Fig. 3A–B). These confirmed tumor-specific gene targets were not necessarily the most lethal hits from the primary siRNA screen, demonstrating the value of using multiple parameters besides the top Z-score to prioritize targets. In summary, we tested 6,977 genes and identified 391 candidate target, from which we selected 174 genes for confirmation. Retesting confirmed 121 genes as potential targets against FHCRC-SCC-1 and 35 genes were identified as tumor-specific gene targets (Fig. 3C). TP53 was both mutated (P278S) and identified as a CTG in FHCRC-SCC-1 (Fig. 3A), consistent with a gain-of-function and prosurvival activity for this mutation as previously shown in lung cancer (24).

**Kinome siRNA profiling of TP53-mutant HNSCC cell lines**

To further prioritize and nominate highly druggable gene targets, we performed kinome-focused siRNA profiling on four TP53-mutant HNSCC cell lines (8). One pair of cells was derived from a primary tumor and a subsequent recurrence (1UM-SCC-14A/C) and another pair from a primary tumor and metastatic lymph node (PCI-15A/B). Comparison of the top target kinases...
from the four cell lines revealed 19 kinases that were also essential for FHCRC-SCC-1, including AURKA, WEE1, CHK1, EPHA3, and EPHA2 (Fig. 4). The shared kinases were involved in the regulation of microtubule-based processes and cytoskeleton organization, mitotic centrosome separation, and G2–M transition of mitotic cell cycle, confirming that pathway dependencies noted in the patient tumor apply to other HNSCC cell lines. Two kinases implicated in response to radiation, TLK2 and CHK1 (30, 31), were essential in FHCRC-SCC-1, the recurrent UM-SCC-14C and metastatic lymph node–derived PCI-15B cells. Of the 19 shared candidate target kinases, 15 were retested, of which 9 successfully validated as targets in FHCRC-SCC-1 (Fig. 3A).

**Drug response profiling**

To complement the siRNA profiling results and to enhance the potential for clinical translation, we performed drug profiling of FHCRC-SCC-1 cells using 332 small molecules, including FDA-approved drugs, drugs in clinical development, and experimental agents targeting cancer pathways (Supplementary Table S8). The majority of the drug library was assembled by The National Cancer Institute’s Cancer Target Discovery and Development Network (CTD2, manuscript in preparation). On the basis of potency, preferential toxicity to FHCRC-SCC-1 relative to NOK cells, and potential for clinical application, we prioritized 41 candidate tumor-specific drugs, including 25 chemotherapies and...
15 targeted agents (Fig. 5). Drugs that showed strong tumor-specific activities included chemotherapies that target the mitotic spindle (paclitaxel, docetaxel, Ixabepilone, vincristine, vinblastine) and targeted agents that inhibit G2–M kinases WEE1 and CHK1 (AZD1775 and AZD7762). FHCRC-SCC-1 tumor cells were 15 times more sensitive to the WEE1 inhibitor AZD1775.
IC50 = 0.13 μmol/L vs. 1.9 μmol/L (Fig. 5B) relative to NOK cells. Additional drugs that showed preferential activity toward FHCRC-SCC-1 included the proteasome inhibitor carfilzomib, the AKT inhibitor (−) Degrulin, topoisomerase inhibitors etoposide and topotecan, the NFκB activation inhibitor IV, a JNK inhibitor IX, a PDE4 inhibitor ML-030, and a TRPC4/5 inhibitor ML204.

We also tested dasatinib, a pan-kinase inhibitor that inhibits EPHA receptor kinase activity as EPHA2 and EPHA3 were both identified as candidate targets of FHCRC-SCC-1 with some tumor specificity (Fig. 3A). FHCRC-SCC-1 tumor cells were 7 times more sensitive to dasatinib compared with NOK cells (IC50 = 0.06 μmol/L vs. 0.44 μmol/L, Fig. 5B).

Interestingly, despite amplification of FGF3, FGF4, FGF19, and FGFR1, FHCRC-SCC-1 cells were not responsive to FGFR1 inhibitors AZD4547, dovitinib, erdafitinib, lucitanib, PD173074, Ki8751, thalidomide, FGF RTK inhibitor 341608 (CAS #192705-79-6), and the FGF/PDGF/VEGF RTK inhibitor 341610 (CAS #144335-35-5; Supplementary Table S8).

Similarly, despite FHCRC-SCC-1 harboring a CDKN2A deletion which includes p16INK4A, cells' response to CDK4 inhibitor palbociclib, did not show preferential activity toward FHCRC-SCC-1 relative to NOK cells (Supplementary Table S8). Additional CDK4 inhibitors, such as abemaciclib and ribociclib, did not significantly affect the viability of FHCRC-SCC-1 cells at any of the doses tested (up to 10 μmol/L) after 3 or 6 days of drug treatment. Consistent with the lack of effect of these targeted agents, siRNAs to either FGFR1 or CDK4 did not reduce viability of FHCRC-SCC-1 cells (Supplementary Table S4). This lack of response to palbociclib is also observed in metastatic breast cancers harboring CDKN2A genetic alterations (32). The drug library contained five mTOR inhibitors, rapamycin, PI-103, PP121, GSK1059615, and BEZ235. FHCRC-SCC1 tumor cells were resistant to all these compounds relative to NOK cells despite increased PIK3CA copy number (Supplementary Table S8).

In summary, a subset of chemotherapies and targeted agents that were active against FHCRC-SCC-1 cells impinge on the same cellular processes and genes that were identified from the siRNA functional profiling providing two independent lines of evidence that FHCRC-SCC-1 cells are vulnerable to disruption of G2–M regulation, the mitotic spindle, and proteasome function (Supplementary Fig. S4). In contrast, and in agreement with the siRNA
Figure 5.
Drug profiling of FHCRC-SCC-1 and NOK cells. A, The heatmap shows clustering of the 320 drugs tested on the basis of their AUC values for the two cell lines. The scatterplot shows a further comparison of drug response between FHCRC-SCC-1 (x-axis) and NOK cells (y-axis). Drugs showing tumor-specific inhibitory effect, as defined by at least twofold AUC difference, are labeled and highlighted in blue. The size of the dot is proportional to the absolute differential of AUC values between the two cell cultures. B, Twenty selected tumor-specific agents were retested. Shown are dose–response curves (compound concentration [log(mol/L)] vs. percent viability) of FHCRC-SCC-1 cells (blue) and NOK cells (red).
results, an additional set of targeted agents that were predicted to be active based on FHCRC-SCC-1 genomics did not show preferential activity.

High-throughput drug profiling and genomic analysis of tumor cells derived from patients on a clinical trial

To determine whether ex vivo functional testing can identify patient-specific vulnerabilities in a clinically relevant setting, we generated two additional primary HNSCC cell cultures, FHCRC-SCC-7A and FHCRC-SCC-10A, from patients enrolled in a phase I clinical trial. This trial tests the WE1 inhibitor AZD1775 alone followed by a combination with cisplatin/docetaxel given to previously untreated advanced HNSCC patients (NCT02508246). During the 4-week trial period, the tumor from the patient corresponding to cell culture FHCRC-SCC-7A had a partial response (Fig. 6A, left). In contrast, the tumor from the patient corresponding to cell culture FHCRC-SCC-10A progressed rapidly through treatment (Fig. 6A, right). Cell cultures from these two patients were subjected to the SEngine Precision Medicine CLIA approved PARIS test, which determines sensitivity to 120 oncology-focused drugs (23). In parallel, tumor biopsies corresponding to these primary cultures were interrogated with the CLIA approved UWOncoPlex test that provides next-generation sequencing data for 262 cancer relevant genes (tests.labmed.washington.edu/UW-OncoPlex). The overall drug sensitivities for these two patients are shown in Fig. 6B and Supplementary Table S9. Responses are compared with a database of additionally tested cells to evaluate relative sensitivities across patients. Cells FHCRC-SCC-7A derived from the patient with a positive clinical response, showed relative sensitivity to AZD1775 (normalized AIC = 0.42, IC50 = 9.1 x 10^-7 M, Z-score = -0.44), while FHCRC-SCC-10A cells, from the patient who did not respond to treatment, showed relative resistance (normalized AUC = 0.51, IC50 = 1.2 x 10^-6 M, Z-score = -0.07; Fig. 6C). We have found that the Z-score based on AUC is the most relevant metric to determine the relative sensitivity to a given drug (23). FHCRC-SCC-7A cells were also sensitive to the PIK3CA inhibitor apitolisib. Of note, this patient’s tumor carried a mutation in PIK3CA (E545K) and a recent phase I study of apitolisib reported a partial response in two patients whose tumors carried the same PIK3CA mutation (33). Finally, as seen in our FHCRC-SCC-1 primary culture, despite FHCRC-SCC-7A having a mutation in CDKN2A (R58X), this was not associated with sensitivity to any of the CDK2/4/6 inhibitors tested. Therefore, broadening these studies to additional cases will help define biomarkers of response to these targeted agents.

In summary, we show the feasibility of combining genomics with ex-vivo drug testing using cultures established from patients enrolled in a clinical trial. Depending on the size and quality of the biopsy, the establishment of primary cultures, drug screening, and analysis can occur in under four weeks, which is a similar time frame for genomic analysis. For the two patients tested, the ex vivo responses to the trial drug AZD1775 were consistent with patient responses.

Discussion

Here we demonstrate the feasibility and potential for clinical translation of high-throughput functional profiling of patient-derived tumor cells using both siRNA- and oncology-focused drug libraries together with in-depth genomic profiling. To illustrate this approach on the subtype of head and neck cancers with the greatest unmet need, we studied a patient with HPV-negative p53-mutant oral cavity HNSCC who died of disease in less than one year despite the most aggressive treatments with surgery and cisplatin chemoradiation.

Comprehensive genomic analysis of this patient’s tumor revealed a heterogeneous mutational profile that is typical for HPV-negative HNSCC, with mutations or deletions in tumor suppressor genes such as TP53 and CDKN2A, amplification of oncoproteins such as MYC and CCND1 and over 200 additional somatic variants, and many copy number aberrations overall pointing to defects in cell-cycle regulation, cell adhesion/polarity, and growth factor signaling (Supplementary Fig. S4). Despite this comprehensive genomic characterization, identifying a drug or drug target that might be effective for this patient remained elusive.

Functional analysis of mutated or amplified genes found in cancers has historically relied on the use of unrelated cell lines or animal models. Because we used the same tumor-derived cells for both genomic and functional profiling, we were able to study the functional consequences of somatically altered genes in the appropriate genetic, epigenetic, and cellular context. This comprehensive and patient-focused approach led to several general conclusions. First, there was a high rate of confirmation of the candidate targets identified from the druggable genome siRNA screen (>70%). Second, it enabled the nomination of several mutated driver genes, such as EPHA3, a highly druggable tyrosine kinase receptor for preclinical exploration. Third, the majority of genes that were mutated or amplified in this patient had minimal impact on viability when targeted by siRNA, suggesting most of these alternations are bystander events. Fourth, siRNA profiling that was unbiased, yet focused on the druggable genome, identified a much greater number of potential targets than did genomic profiling. Many of these novel targets lack clinically available inhibitors highlighting opportunities for drug development. Finally, drug profiling in combination with siRNA testing was useful to confirm gene targets and pathway dependencies and also to prioritize therapeutic strategies as described below.

Results from two orthogonal functional profiling schemes converged on the same cellular processes and gene targets as vulnerabilities for FHCRC-SCC-1 (Supplementary Fig. S4). Nearly a third (20/69) of the top 1% of genes, based on viability Z-score, were components of the ubiquitin proteasome system highlighting both the sensitivity of FHCRC-SCC-1 cells to disruption of protein recycling machinery (34) and the ability of our functional screening platform to identify not just isolated genes but entire pathways. Similarly, nearly one third (11/35) of essential tumor-specific genes were involved in G2/M regulation or mitotic spindle function including kinases and spindle components. siRNAs to WE1 and CHK1 as well as specific inhibitors to WE1 and CHK1 and five chemotherapies that disrupt the mitotic spindle also showed preferential sensitivity toward FHCRC-SCC-1 over nontumorigenic keratinocytes. In vivo tumor growth of FHCRC-SCC-1 showed frequent abnormal mitotic figures (Fig. 1C), including lagging chromosomes, tripolar mitotic figures, and anaphase bridges consistent with aberrant regulation of mitosis.

TP53 mutations result in loss of the G1 checkpoint leading to an increased reliance on the G2 checkpoint to maintain genome stability. Thus, WE1 and CHK1 inhibitors have been developed with the hopes that they would be selectively lethal against p53-mutant cancer cells, particularly in combination with genotoxic

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Druggable Target Discovery in Head and Neck Cancer

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Figure 6.
High-throughput ex vivo drug profiling of patients enrolled in a clinical trial. A, Selected genomic features and images from two patients enrolled in clinical trial NCT02508246. Images were taken before and after neoadjuvant therapy (4 weeks apart) with AZD1775 alone on the first week, and in combination with cisplatin/docetaxel for 3 additional weeks. Left, PET scans of patient who corresponds to culture FHCRC-SCC-7A showed a RECIST response of \(-16\%\) and histologic response of \(-95\%\). Right, CT images of patient who corresponds to cultures FHCRC-SCC-10A showed a RECIST progression of 21%. Yellow arrows depict tumor in the lateral tongue for FHCRC-SCC-7A and buccal space and lymph node for FHCRC-SCC-10A. Compared with the partial loss of PET signal in the former, there is significant progression of disease in the latter. B, Primary tumor cultures from these patients were subjected to SEngine PARIS drug test. Shown are normalized AUC and Z-score for 120 agents tested. C, Drug response curves to AZD1775 in FHCRC-SCC-7A and 10-A (red curves) compared with the average response of other tumor lines (black curves).
therapy (35–37). We and others have shown that inhibiting WEE1 in p53-mutant cells leads to mitotic cell death and treatment with AZD1775 either alone or in combination with cisplatin blocked growth of p53-mutant HNSCC xenografts in mice (8, 37, 38). On
the basis of these results, we opened a phase I clinical trial with
AZD1775 in combination with neoadjuvant chemotherapy in
previously untreated, metastatic HNSCC (NCT02508246). The
results of this trial are encouraging and support further investiga-
tion of WEE1 as a target in the metastatic setting of HNSCC
(39). The relative sensitivity to AZD1775 of tumor cells cultured
from two of these patients was concordant with the patient
response or nonresponse in vivo. Both tumors had TP53
mutations, while the nonresponder had an HRAS mutation, potentially
linked to the lack of response to targeted agents (40). Antitumor
activity of AZD1775 as a single agent has been shown in a recent
phase I trial with con
sistance. Gain-of-function mutations in
neoplastic testing of individual tumor samples to de-
terminations, while the nonresponder had an
malignant target (45, 46). In addition, gain-of-function mutations
in p53 can protect cells from deregulated MYC and chemother-
apy-induced apoptosis (44). FHRCSCC-1 cells had amplifi-
cation of MYC and were very sensitive to depletion of the
antiapoptotic gene MCL1, indicating these cells are primed to
undergo apoptosis, which is blocked by MCL1. This finding is
consistent with the identification of MCL-1 as a MYC synthetic
lethal target (45, 46). In addition, gain-of-function mutations in
p53 lead to abrogation of the G2–M checkpoint after DNA
damage, centrosome amplification, and aberrant mitosis
(44, 47), which could explain the strong dependence of
FHRCSCC-1 cells on disruption of spindle function and
G2–M regulation. In summary, targeting both the G2–M
vulnerability of p53-mutant cells, as well as inhibiting anti-
apoptotic proteins such as MCL-1, may provide a valuable
drug combination approach for head and neck cancer with
dual alterations of MYC and TP53.

Integrating genomic, siRNA, and drug profiling implicate EPH
receptor signaling as another potentially targetable pathway.
EPHA3 was mutated in FHRCSCC-1 and both EPHA2 and
EPHA3 are mutated in approximately 3% of HNSCC cases. Both
EPHA2 and EPHA3 were identified as vulnerabilities by siRNA
profiling in both FHRCSCC-1 and HNSCC cell lines. Dasatinib,
which inhibits EPH receptor kinase activity (48), also showed
preferential activity to FHRCSCC-1 consistent with reliance of

FHRCSCC-1 cells on EPH kinase activity. EPHA3 is over-
expressed in a number of cancer types and both EPHA2 and EPHA3
are candidate anticancer targets (49–51). However, context may
be important as EPHA2 is reported to have tumor suppressor
activity in murine squamous cell carcinoma (52). Functional
profiling also helped clarify that DDR2 mutation was not likely
driver mutation as knockdown did not decrease viability.

In conclusion, we demonstrate the utility of genome-scale
functional testing as an adjunct to genomic analysis in the
execution of precision oncology for treatment-refractory HNSCC.
Functional profiling prioritizes candidate actionable targets but
also reveals novel targets that are not genomically altered. Func-
tional profiling using high-throughput drug screening with a
clinically relevant collection of drugs within the context of a
clinical trial offers the opportunity to address mechanisms of
resistance and biomarkers of response in real time. Finally, the
dependency of p53 mutant HNSCC cells on WEE1 and CHK1
reinforces the therapeutic potential of targeting G2–M checkpoint
regulators as a therapeutic option for these aggressive cancers.

Disclosure of Potential Conflicts of Interest
F.X. Schaab, V.K. Gadi, and C.J. Kemp have ownership interests (including patents) in Engine Precision Medicine. E. Méndez reported receiving commer-
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