



# Trunk or branch? Identifying and targeting intratumoral heterogeneity in hepatocellular carcinoma using genomics and patient derived primary cancer cells

Kelsey E. Wuensch<sup>1,2</sup>, Aik Choon Tan<sup>1,2,3</sup>

<sup>1</sup>Translational Bioinformatics and Cancer Systems Biology Laboratory, Division of Medical Oncology, Department of Medicine, <sup>2</sup>Cancer Biology Graduate Program, School of Medicine, <sup>3</sup>University of Colorado Cancer Center, University of Colorado Anschutz Medical Campus, Aurora, CO, USA

Correspondence to: Aik Choon Tan, PhD. Division of Medical Oncology, Department of Medicine, University of Colorado Anschutz Medical Campus, Aurora, CO 80045, USA. Email: aikchoon.tan@ucdenver.edu.

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Precision oncology aims to deliver personalized treatment to individual patients based on genomic profiling of tumors (1). Molecular targeted therapies have been developed based on the identification of “oncogene drivers” from large-scale genomic studies; leading to several successful genotype-directed clinical applications of targeted therapies (2,3). However, complete and durable responses to these therapies are rare, and the residual tumors eventually acquired new mutations or rewired signaling pathways to by-pass blockade of these targeted therapies (4-6). Moreover, intratumoral heterogeneity (ITH) and tumor evolution play key roles in resistance mechanisms in targeted therapies (7). Therefore, current research efforts have been focusing on identifying these molecular changes in resistant tumors in search for more effective rational combination treatment to overcome resistance mechanisms.

In hepatocellular carcinoma (HCC), sorafenib is the only FDA approved targeted therapy, has had little success in this disease (8). There has been a large effort to identify targetable molecular drivers in HCC, however translating these finding into the clinic remains a challenge. HCC has been documented to have morphologic and genetic ITH, which is thought to be an obstacle preventing targeted therapies from being successful, and hypothesized to contribute to resistance to targeted therapies (9). To study this, Gao *et al.* recently characterized the landscape of spatial and temporal ITH and its impacts in HCC progression and response, or lack thereof, to targeted therapies (10). A total of 55 fresh multi-regional tissue samples collected from 10 cases of HBV-related HCC

treatment naïve patients, ranging from 4–9 samples per case. These samples were subjected to establish an *in vitro* cell culture system of patient derived primary cancer cells (PDPCs) and subsequently profiled by next generation sequencing to determine molecular drivers and for drug discovery (10). By sequencing multiple specifically distinct regional samples across the tumor, the mutational landscape of these samples could be phylogenetically mapped to understand the branched evolution of tumor growth. This method of combining multi-regional samples for genetic analysis and subsequent *in vitro* drug response is a novel method for functionally testing the effects of ITH on drug responses in HCC.

The PDPC model has been used in lung cancer to determine therapeutic targets to overcome acquired resistance induced by targeted therapies (11). Specifically, this cell culture method was used to model acquired resistance to EGFR and ALK inhibition directly from primary patient tumors. This short primary culture is amenable for high-throughput drug screening, allowing for rapid identification of rational drug combinations to overcome resistance (11). This method is attractive as it provides the ability to functionally assess genetic alterations and acquisition of resistance that occurred in a clinical setting. This study highlights the importance of using patient derived cell lines as a clinically relevant model to use for next generation sequencing in conjunction with pharmacological screens to determine rational combination therapies to overcome resistance. For example, in the instance of resistant ALK-positive lung cancer, drug screening identified that

inhibition of SRC restored ALK inhibitor sensitivity, which would not have been identified predicted by genetic analysis due to no mutations in SRC or its regulators, rather SRC upregulation at the protein level (11). Additionally, this study supports how functional assessment of PDPCs can enhance genetic profiling, as in the instance where fibroblast growth factor receptor (FGFR) inhibitors were effective in a sample with a previously uncharacterized FGFR3 mutation (11). Without the functional data to compliment the genetics, the biological effect of this uncharacterized mutation would remain unclear.

The novelty of Gao *et al.* current study is the combination of multi-regional biopsies with the PDPC model to determine molecular drivers of HCC, which highlights the branched evolution and ITH (10). Genetic analysis, utilizing whole exome sequencing and copy number variation analysis, was performed on a total of 55 tumor samples, derived from 10 distinct tumors. The genetic variations found in the different areas of the tumor can be represented as a branched tree pattern to visualize and represent tumor evolution in HCC (12). Mutations at the trunk are present in all regions of the tumor, mutations found in branches are heterogeneous and present in at least two regions, whereas mutations in a private branch are unique to one region in an individual tumor. Gao *et al.* found that all ten HCC tumors displayed ITH with branch and private branch mutations comprising, on average ~40% of total mutations (10).

To study the ITH, Gao *et al.* focused on 26 genes previously characterized as drivers in HCC, and mapped them to the phylogenetic trees (10). Not surprisingly, all ten cases showed a long trunk with multiple (private) branches. About half of the mutations were indicated to be early events as they mapped to the trunk. Specifically, *TP53*, the top mutated gene in HCC, was mapped to all trunks. *TERT* promoter mutation, an early genetic event in HCC, was mapped to five trunks. Interestingly, half of the drivers mapped to the branches, indicating that these mutations are only found in the subclonal populations, further highlighting a need to target these populations. Copy number alteration analysis did not identify significant large-scale differences in chromosomal alterations, however examination of specific smaller segments identified amplification in the potential driver genes *FGF19* and *DDR1* in branches of two distinct tumors.

To test the drug responses of ITH in HCC, the multi-regional PDPC model was utilized for high-throughput drug screening (10). None of the PDPCs showed sensitivity to the HCC standard of care drugs, the tyrosine kinase inhibitor sorafenib and the chemotherapy oxaliplatin,

indicating that all samples have intrinsic resistance to standard therapy. Resistance to sorafenib was supported by the genomic profiles, where all PDPCs lacked the suggested sorafenib sensitivity biomarkers such as *ARAF* mutations and amplifications in *VEGFA* or *FGF3/FGF4* (10). Analysis of trunk mutations, which are considered to be the early genetic events, revealed very limited druggable targets, which is consistent with previous HCC sequencing data (9). This can be explained by the fact that HCC differs from many other cancers with clear targetable oncogenic drivers that occur early in tumor progression such as *EGFR* mutations in lung cancer.

Gao *et al.* data also supports the inter-patient heterogeneity, showing that targetable alterations, such as amplification of *FGF19* and *DDR2*, were only identified in the subclonal branch and putative branch groups in separate cases (10). This finding reinforces the need to deploy personalized rational therapy for individual patients based on their genetic profiles. For example, in one patient sample, *FGF19* was found to be amplified in 7 of the 9 subclones, however only subclones expressing relatively high mRNA levels of *FGF19* (3/7 samples) were sensitive to the FGFR inhibitor, LY2874455, as compared to subclones with low or medium mRNA *FGF19* expression. To validate these findings, Gao *et al.* screened 105 independent liver cancer cell lines for *FGF19* mRNA levels and correlated them with the sensitivity of LY2874455. They found that cell lines with high *FGF19* mRNA levels corresponded to LY2874455 sensitivity whereas low expressing cells were resistant to this FGFR inhibitor, validating the findings in the PDPC model. These findings corroborate with previous studies, showing that *FGF19* amplification and expression may be indicative of sensitivity to FGFR inhibition (13). The remaining PDPCs resistant to FGFR inhibitions and lacking a targetable genetic alteration were subjected to high throughput drug compound screening to identify potential therapies. Gao *et al.* found that these PDPCs resistant to FGFR inhibitions were sensitive to the BRD4 inhibitor, JQ1 (10). In order to investigate the effects of the combination therapy, cells expressing high and low levels of *FGF19* were mixed in culture to recapitulate ITH *in vitro*. They showed that combination therapy of FGFR inhibition and JQ1 was most effective at reducing cell growth, and equally reducing both subpopulations of *FGF19* low and high expressing cells, as compared to monotherapy. They demonstrated that the combination of FGFR and JQ1 inhibitors is a rational combination for eliminating ITH in HCC (10).

*FGF19* amplification has been previously identified in a small subset (5%) of HBV-associated HCC, and was correlated with cirrhosis, however, the functional impact of *FGF19* in

patient tumors was not analyzed (14). Previously, another study screened and identified 124 amplified genes from patient HCC samples, and performed a forward genetic screen to identify potential oncogenic drivers. To determine the tumor promoting capabilities of these genes, each gene was individually overexpressed in a relevant mouse hepatoblast model, which established FGF19 as a tumor promoter, showing that genetic or pharmacologic inhibition of FGF19 impedes cancer growth (13). While this identifies FGF19 as a potential biomarker for FGFR inhibition, the biological role of FGF19 amplification in HCC patient samples remained to be elucidated. Gao *et al.* not only established a role for FGF19 amplification, additionally this study identified that FGF19 amplifications may be events occurring later in tumor evolution and are heterogeneous, which may provide insight as to why FGF19 amplifications in HCC are seen at a low frequency in other studies (10).

Recently, multiple studies have demonstrated that kinase dependency could occur in the absence of the kinase mutation. For example, FGF19 amplification in HCC as identified in Gao *et al.* activates the FGFR signaling pathway that can mediate downstream signaling to oncogenic pathways such as the MAPK pathway to induce cellular proliferation or PI3K-AKT pathway to control cell survival (15). Specifically, FGFR 1–4 have been identified to function as RTKs through binding of the FGF ligand on the extracellular domain, resulting in auto-phosphorylation of FGFR, and subsequent downstream signaling. FGF19 has been identified as being responsible for FGFR4 activation, resulting in increased hepatocyte proliferation and induction of HCC in an *in vivo* mouse model (16). Other studies identified FGFR1 mRNA expression as a biomarker for predicting FGFR inhibitor sensitivity in lung, head and neck cancers (17,18). Moreover, these signaling pathways act in concert as networks with multiple cross-talks and feedbacks when perturbed by kinase inhibitors. Therefore, it is no longer sufficient to study individual kinases; instead they should be investigated as complex networks working in a concerted fashion in cancer (6).

To decipher these signaling networks, functional genomic screens represent a powerful approach to identify oncogenic drivers and rational targeted therapies in cancer cells. For example, using functional genomic screens, Singleton *et al.* identified synthetic lethal interactions between MTOR and FGFR1 pathway in lung, head and neck cancer cells (19); Manchado *et al.* identified FGFR1 as a compensatory mechanism to overcome MEK inhibition in KRAS mutant lung cancer (20); and Spreafico *et al.* identified WNT as a compensatory mechanism to overcome MEK inhibition in

KRAS mutant colorectal cancer (21) and co-targeting the WNT and MEK pathways is now being tested in clinical trial (ClinicalTrials.gov ID: NCT02188264). Another approach to decipher the complex signaling networks is to integrate computational approach with high-throughput screening data. For example, Ryall *et al.* developed the Kinase Addiction Ranker (KAR) method to identify kinase dependency in cancer cells by integrating high-throughput drug screening and kinase inhibition data (22,23); Szwajda *et al.* developed the Kinase Inhibition Sensitivity Score (KISS) to map kinase signaling addiction in cancer cells for predicting drug combinations (24). We anticipate that going forward, using these methods in the PDPC model may provide insights to potential mechanisms of resistance to targeted therapies that are clinically relevant.

In summary, to eliminate oncogenic signaling in cancer cells, it is necessary to identify drug combinations that target different nodes in cancer signaling networks according to individual mutational profiles including ITH. Gao *et al.* and others have highlighted that PDPCs are a model system to study heterogeneity and tumor evolution, genetic drivers and rational combination therapies. Comprehensive genomics profiling and analysis coupled with high-throughput drug screening on PDPCs represent an attractive approach to study ITH, identify rational drug combinations and the development of predictive biomarkers. All these approaches are needed in the research quest for precision medicine in oncology.

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