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Comparative Response of HCC Cells to TKIs: Modified in vitro Testing and Descriptive Expression Analysis

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Introduction: Although the treatment paradigm for hepatocellular carcinoma (HCC) has recently shifted in favour of checkpoint inhibitor (CPI)-based treatment options, the tyrosine kinase inhibitors (TKI) currently approved for the treatment of HCC are expected to remain the cornerstone of HCC treatment alone or in combination with CPIs. Despite considerable research efforts, no biomarker capable of predicting the response to specific TKIs has been validated. Thus, personalized approaches to HCC may aid in determining optimal treatment lines for 2nd and 3rd lines. To identify new biomarkers, we examined differential sensitivity and investigated potential transcriptomic predictors.

Methods: To this aim, the sensitivity of nine HCC cell lines to sorafenib, lenvatinib, regorafenib, and cabozantinib was evaluated by a prolonged treatment scheme to determine their respective growth rate inhibition concentrations (GR_{50}). Subgroups discriminated by GR_{50} values underwent differential expression and gene set enrichment analysis (GSEA).

Results: The nine cell lines showed broadly different sensitivities to different TKIs. GR_{50} values of sorafenib and regorafenib clustered closer in all cell lines, whereas treatments with lenvatinib and cabozantinib showed diversified GR_{50} values. GSEA showed the activation of specific pathways in sensitive vs non-sensitive cell lines. A signature consisting of 14 biomarkers (GAGE12H, GJB6, PTCHD3, PRH1-PRR4, C6orf222, HBB, C17orf99, GOLGA6A, CRYAA, CCL23, RP11-347C12.3, RP11-514O12.4, FAM180B, and TMPRSS4) discriminates the cell lines' response into three distinct treatment profiles: 1) equally sensible to sorafenib, regorafenib and cabozantinib, 2) sensible to lenvatinib, and 3) more sensible to regorafenib than sorafenib.

Conclusion: We observed diverse responses to either of the four TKIs. Subgroup analysis of TKI effectiveness showed distinct transcriptomic profiles and signaling pathways associated with responsiveness. This prompts more extensive studies to explore and validate pharmacogenomic and transcriptomic strategies for a personalized treatment approach, particularly after the failure of CPI treatment.

Keywords: biomarkers, sorafenib, lenvatinib, regorafenib, cabozantinib

Introduction

Hepatocellular carcinoma is the sixth most common and third most deadly malignant disease.[1] The tyrosine kinase inhibitors sorafenib,[2] lenvatinib,[3] regorafenib,[4] and cabozantinib[5] are approved for the treatment of advanced HCC for first or second-line treatment. But with the advent of immune oncology (I/O)-based therapies in the first line, the previously established empirical order of TKI treatment has changed. However, even before CPIs, there was an unmet need for adequate predictors of response for either of the four TKIs. Although TKIs impinge on several specific

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intracellular signal transduction mechanisms, their clinical use in monotherapy or in combination with CPIs is based on empirical clinical evidence.[6,7] Previous attempts to establish clinically validated biomarkers of response for these agents have not been implemented in clinical routine so far, leaving an unmet need for biomarkers capable of guiding the choice of TKI.[8–11]

The TKIs' mechanism of action consists of the inhibition of various receptor tyrosine kinases by disrupting their autophosphorylation, as well as inhibiting Raf serine/threonine kinases, eventually leading to a disruption of the PI3K/AKT/mTOR- and MAPK/ERK-pathways, which are believed to play a significant role in the genesis of hepatocellular carcinoma.[12–14] The four TKIs' differences in mechanisms of action and toxicity can be explained through a varying potency towards one target point, as well as slightly different points of target.[15–18] However, despite the well-characterized mechanism of action, there are little to no clinical, phenotypical, or genetic features to guide us to the right therapy.

The lack of predictive biomarkers in the clinical routine may be attributed to the lack of histological specimen acquisition in the initial pharmaceutical studies. Another explanation may be that candidate biomarkers were generated from an analysis made in artificial in vitro systems, and the lack of microenvironment hinders transfer to the in vivo situation. For example, the treatment duration (in vitro 48–72 h) is not remotely close to the clinical situation (weeks to months). In addition, it has recently been shown that pharmacogenomic examinations are strongly confounded by the cell lines' individual proliferative speed. To increase biological relevance, Hafner et al developed the GR_{50} metric, a new approach to assess cellular drug efficacy.[19,20] Another rationale for using the GR50 metric instead of ED50 or AUC, is that it also corrects for cell-line specific differences in read-out measurements by adjusting for read-outs of initial cell populations.[21]

In this study, we used the GR_{50} metric and a prolonged treatment scheme to examine the response of nine hepatoma cell lines to the four viable tyrosine kinase inhibitors to compare responsiveness to either drug and narrow down potential markers that could be further examined in subsequent studies as predictors of response for one of the four TKIs.

Materials and Methods
Cell Lines, Cell Culture and Chemicals
Nine human hepatoma cell lines with different clinical and histological features were used for this research project (Table 1).

Table 1 Clinical and Histological Features of Nine Hepatoma Cell Lines

	Ethnicity	Sex	Age	HBV	AFP	Histological Type	Source	Ref
Hep3B	African	M	8	(+)	(+)	HCC	ATCC	[40]
HepG2	Caucasian	M	15	(-)	(+)	Hepatoblastoma	ATCC, JCRB	[40]
HLE	Asian	M	68	(-)	(-)	HCC	JCRB	[41]
HLF	Asian	M	68	(-)	(-)	HCC	JCRB	[41]
HuH1	Asian	M	53	(+)	(+)	HCC	JCRB	[42]
HuH7	Asian	M	57	(-)	(+)	HCC	RIKEN, JCRB	[43]
PLC-PRF5	African	M	24	(+)	(+)	HCC	ATCC, JCRB	[44]
Snu398	Asian	M	42	(+)	(-)	HCC	ATCC	[45]
Snu475	Asian	M	43	(+)	(-)	HCC	ATCC	[45]

Notes: Modified and recreated from Hirschfield et al,[46] Qiu et al,[8] and Caruso et al.[9]
Abbreviations: AFP, alpha-fetoprotein; ATCC, American Type Culture Collection; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; JCRB, Japanese Collection of Research Bioresources; M, male; RIKEN, short for Rikagaku Kenkyujo – a Japanese research institute.

HuH1, HuH7, HepG2, PLC-PRF5, HLE, and HLF were cultivated in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, USA), Hep3B in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 (DMEM/ F12, Sigma-Aldrich, USA), supplemented with 200mM L-glutamine (Sigma-Aldrich, USA), and Snu398 and Snu475 in RPMI-1640 medium (Sigma-Aldrich, USA). The disparity of media for the various cell lines is due to our intent to continue using the same medium compound for one cell that has been used in the original publications and in our laboratory in the past, in order to protect the cells from sudden unfamiliarity. All the media were supplemented with 10% Filtrated Bovine Serum (FBS, PAN-Biotech GmbH, Germany) and 1% Penicillin–Streptomycin (P/S, Sigma-Aldrich, USA). Cells were constantly maintained at 37°C and 5% CO_2. Genetic fingerprinting was performed on all cell lines by the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures to confirm authenticity. Furthermore, all cell lines have been tested negative for mycoplasms by polymerase chain reaction. Sorafenib tosylate, lenvatinib (E7080), regorafenib (BAY 73–4506), and cabozantinib (XY184 free base) were acquired from AdooQ Bioscience, USA, dissolved in 100% dimethyl sulfoxide (DMSO, Carl Roth GmbH + Co. KG, Germany) at a stock concentration of 100μM and stored at −80°C until used.

Cell Viability Assay

After seeding 800–3000 cells from each cell line in 96-well-plates and leaving the cells to attach for approximately 24 hours, the cells were then treated with seven different concentrations of each drug, ranging from 0.03μM to 20μM in a 1:2–3 serial dilution. The individual concentration ranges for each drug were determined in preliminary experiments that have been conducted beforehand. A DMSO medium solution was used as a control group. After six days, the cell count was estimated using SYBR green assay (Lonza, USA), and fluorescence was measured using the Cytofluor Series 4000 (Applied Biosystems, Germany), as previously described.[22] All experiments were performed in triplicates with at least three individual biological replicates per drug.

To minimize the effect of proliferation, we decided on using the growth rate method established by Hafner et al. To calculate the normalized GR(c)-value at concentration c, the following equation is being used:

$$GR(c) = 2^{\frac{log_2(x(c)/x_0)}{log_2(x_{ctrl}/x_0)}} - 1$$

with x(c) being the cells treated with one drug at concentration c at day 7, x_{ctrl} being the DMSO treated control wells from the same plate at day 7, and x_0 being the untreated cells from the time of treatment at day 1. Typically, the GR value lies between −1 and +1, whereas a positive value (0 to +1) correlates to partial growth inhibition, GR equals 0 stands for complete cytostasis, ie, no increase or decrease in cell number, and negative values (0 to −1) stand for cytotoxicity, that is cell death. Afterwards, the GR values were fitted to a sigmoid curve, and the GR_{50} and GR_{max} values were calculated. The GR_{max} value, which is a metric for drug efficacy, represents the GR value for one drug at the highest concentration of this drug. The GR_{50} value is a metric for drug potency and represents the concentration at which GR(c) = 0.5.[19]

Processing of Gene Expression Data

To determine the driving genes affecting the response to our medication groups (sorafenib, lenvatinib, regorafenib and cabozantinib) and of an additional "regorafenib over sorafenib" (RoS) subset, differential expression analysis was performed using the "DESeq2" package in R Version 4.1.1 and RStudio Desktop 2021.09 release,[23] evaluating our treatment responses in a binary "sensitive vs resistant" notation against the established cell lines RNA-seq gene expression profiles obtained from the Liver Cancer Model Repository (LIMORE, https://www.picb.ac.cn/limore/), a cell models compilatory project with 81 human liver cancer cell models.[8] The resulting information was filtered to contain only genes with ±2-fold changes (FC), and a cut-off value of 0.001 was set as statistically significant using the Bonferroni-Holm adjusted p-value.

Gene Clustering and Pathway Enrichment Analysis

To visualize the DESeq2 data, a heatmap focusing on the gene groups of interest was created using the Heatmapper webtool (http://www.heatmapper.ca).[23,24] Clustering was performed using the Complete Linkage method, and the Manhattan distance method was selected due to the high dimensionality of the data. Furthermore, KEGG pathways enrichment analysis was performed on the resulting differential expression data using a cut-off value of 0.05 in the ShinyGO webtool version 0.64,[25] and further pathway analysis and filtering of our data with the same cut-off value was performed using the commercial tool QIAGEN's Ingenuity Pathways Analysis (IPA).[26] A selection of the most statistically significant over- and under-expressed genes was performed by filtering the genes with a fold change ±2 and an adjusted p-value under 0.001.

Graphics

Graphics and figures were generated using GraphPad Prism 8 (GraphPad Software Inc., USA), Heatmapper (University of Alberta, Canada), or QIAGEN's Ingenuity Pathway Analysis (QIAGEN Inc., USA).

Statistical Analysis

Data were analyzed using IBM SPSS statistics software version 27 (SPSS Inc., IBM, USA) or GraphPad Prism 8 (GraphPad Software Inc., USA). Results are shown as mean ± standard deviation for at least three individual experiments. One-way analysis of variance (ANOVA) or the more robust Welch-ANOVA, in case equal variances using Levene's test could not be shown, followed by a Tukey- or Games Howell post hoc analysis, was conducted. If $p < 0.05$ between two cell lines, these two cell lines showed a statistically significant difference in response to the drug that has been examined.

Additionally, the correlation coefficients, which show the strength of correlation between our GR_{50} values, our GR_{max} values, Qiu et al's IC_{50} values,[8] and Caruso et al's GI_{50} values,[9] for every drug, as well as overall, were calculated. According to the linearity of the correlation between two factors, Pearson's correlation or the more robust Spearman's Rho correlation were calculated.

Results

Differential Responsiveness to Clinically Viable TKIs Sorafenib, Lenvatinib, Regorafenib, and Cabozantinib

The effect of four tyrosine kinase inhibitors, sorafenib, lenvatinib, regorafenib, and cabozantinib, on cell viability was assessed on nine hepatoma cell lines, exhibiting different clinical and molecular features (Table 1). We used a six-day treatment protocol to mimic the clinical application of TKIs. Each TKI induces a decrease in cell viability in a dose-dependent manner in all cell lines (Figure 1A–D). In addition, we observed a diversified response to the four TKIs (Figure 1E–H).

Upon sorafenib treatment (Figure 1A), HLE, Snu398, Hep3B, and HepG2 are more sensitive to GR_{50} concentrations below 2µM and HLF, HuH1, HuH7, Snu475, and PLC-PRF5 are less sensitive to GR_{50} concentrations above 2µM. The difference in sensitivity between the most (1.3µM) and the least sensitive cell line (2.9µM) only differs by a factor of 2.2. Almost all cell lines display a slight cytotoxic effect at the highest concentration of sorafenib (Table 2).

Lenvatinib displays a heterogeneous pattern of response (Figure 1B), dividing all cell lines but Sun475 into two groups. The first group, containing HuH7, Snu398, HLE, and HLF, shows GR_{50} values between 0.7µM and 1.0µM. The second group, containing HepG2, Hep3B, PLC-PRF5, and HuH1, displays GR_{50} values between 6.7µM and >20µM. Hence, the GR_{50} value of the most sensitive cell lines (HuH7 and HLF) differs from the most resistant cell line (PLC-PRF5) by more than 28.5 times.

Regorafenib's range of efficacy is similar to sorafenib's (Figure 1A and C). However, some cell lines, such as Hep3B, HepG2, HLF, HuH7, and Snu475, show a difference in potency when treated with sorafenib and
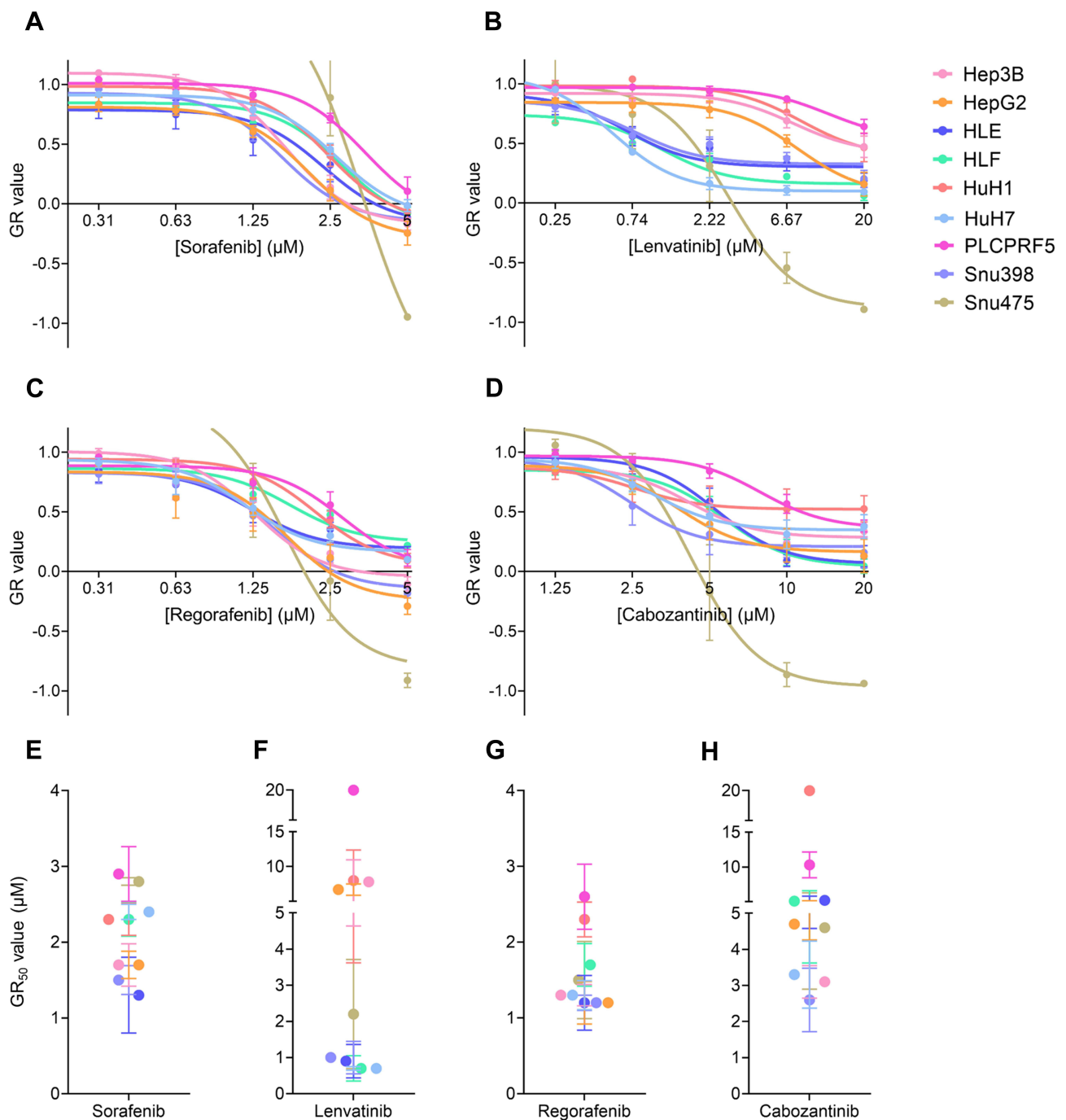
Figure 1 The four tyrosine kinase inhibitors, sorafenib, lenvatinib, regorafenib, and cabozantinib, cause a reduction in cell viability in all nine cell lines. (**A**) Effect of sorafenib on all cell lines with a concentration range of 0.31μM to 5μM in a 1:2 serial dilution. (**B**) Effect of lenvatinib on all cell lines with a concentration range of 0.25μM to 20μM in a 1:3 serial dilution. (**C**) Effect of regorafenib on all cell lines with a concentration range of 0.31μM to 5μM in a 1:2 serial dilution. (**D**) Effect of cabozantinib on all cell lines with a concentration range of 1.25μM to 20μM in a 1:2 serial dilution. Potency (GR$_{50}$ values) for (**E**) sorafenib, (**F**) lenvatinib, (**G**) regorafenib, and (**H**) cabozantinib. Results are presented as the mean and standard deviation for at least three experiments. $P < 0.05$ for all drugs by analysis of variance (ANOVA). Due to the increased proliferation of Snu475 under sorafenib and regorafenib at the lower concentrations and to better fit the representation in this graph, we do not show Snu475 at said lower concentrations. The complete dose–response curves of Snu475 can be seen in Supplementary Figure 1.

regorafenib, with all of them being more sensitive to the second-line treatment option regorafenib (Figure 1A and C, Table 2).

Lastly, cabozantinib shows a little more variety of its effect on different cell lines (Figure 1D). The most sensitive (Snu398, GR$_{50, \text{C-Snu398}}$ = 2.6μM) and the least sensitive cell line (HuH1, GR$_{50 \text{ C-HuH1}}$ >20μM) differ by a factor greater than 7.6, respectively.

Table 2 GR Values for Potency and Efficacy

Cell-Line	Sorafenib			Lenvatinib			Regorafenib			Cabozantinib			RoS
	GR_{50}	GR_{max}	R	GR_{50}	GR_{max}	R	GR_{50}	GR_{max}	R	GR_{50}	GR_{max}	R	R
Hep3B	1.7	−0.17	1	7.8	0.47	0	1.3	−0.11	1	3.1	0.34	1	1
HepG2	1.7	−0.25	1	6.7	0.16	0	1.2	−0.29	1	4.7	0.13	1	1
HLE	1.3	−0.12	1	0.9	0.19	1	1.2	0.12	1	5.2	0.13	0	0
HLF	2.3	−0.08	0	0.7	0.06	1	1.7	0.22	0	5.1	0.05	0	1
HuH1	2.3	−0.06	0	8.0	0.20	0	2.3	0.09	0	20	0.53	0	0
HuH7	2.4	−0.01	0	0.7	0.09	1	1.3	0.10	1	3.3	0.38	1	1
PLC-PRF5	2.9	0.11	0	20	0.64	0	2.6	0.12	0	10.3	0.38	0	0
Snu398	1.5	−0.16	1	1.0	0.47	1	1.2	−0.18	1	2.6	0.16	1	0
Snu475	2.8	−0.95	0	2.2	−0.95	1	1.5	−0.92	0	4.6	−0.98	1	1

Notes: This table shows the potency (GR_{50} in μM), efficacy (GR_{max}), and response (R) for each cell line and drug, as well as the response for one regorafenib over sorafenib (RoS) group. The response of the four TKIs is subgrouped into less sensitive (0) and more sensitive (1). The cut-off points for response chosen for each drug were determined by looking at the spread of GR_{50} values of the different cell lines, factoring in the steady-state plasma concentration of each drug, as well as trying to achieve a 4:5 ratio for the two subgroups: 2μM for sorafenib, 1.5μM for regorafenib, 5μM for lenvatinib and cabozantinib. For the RoS-group, the cell lines were divided into being equally responsive to regorafenib and sorafenib (0) and showing a statistically significant difference in response to those two drugs, with all of them being more sensitive to regorafenib (1). Results are presented as the mean of at least three experiments. $P<0.05$ for all drugs by analysis of variance (ANOVA). GRmax values can only be compared if the highest concentrations are equal. Therefore, they can be used for interpretation when comparing the efficacy of different cell lines to one drug, but not when comparing how one cell line reacts to different drugs.

Snu475 appeared to be the overall least responsive cell line. Snu475 proliferates approximately 12 times slower than the next slowest proliferating cell line, assuming exponential growth. Snu475 is the only cell line, that shows a complete cytotoxic response to all four drugs, with GR_{max} values almost reaching −1 ($GR_{max,S-Snu475}$ = −0.95, $GR_{max,L-Snu475}$ = −0.95, $GR_{max,R-Snu475}$ = −0.92 and $GR_{max,C-Snu475}$ = −0.98, respectively) (Table 2).

Differential Expression Analysis of the Four Treatment Groups and a Fifth Category

The bioinformatics evaluation following the responsiveness analysis allowed the addition of a fifth category ("regorafenib over sorafenib") to determine the genes involved in the differential potency response of a specific subset of cell lines to the second-line treatment regorafenib in comparison to the first-line treatment sorafenib. Expression data is filtered for significance as previously described in the methods. Our differential gene expression data are translated into an expression heatmap (Figure 2). On the far left of Figure 2, we can see two distinct clusters that show a highly over- and under-expression (FC > ±20) consistent in all our cell lines (Figure 2, blue rectangle), showing two groups of identifiable gene expression signatures concerning the distinct drug response groups.

Gene Sets Enrichment Analysis

Out of the 19.761 human genes evaluated on the RNA-seq data, a total of 966 fulfilled our differentially expressed genes (DEG) filtering criteria (*FC >|2|, pAdj > 0.001*) in at least one of the treatment groups. Specifically, there were 217 DEG for sorafenib, 296 for lenvatinib, 201 for regorafenib, 240 for cabozantinib, and 210 for the RoS group (ie those related to the differential response between sorafenib and regorafenib).

These gene groups were analyzed for pathway enrichment using ShinyGO (to evaluate KEGG) and QIAGEN's IPA. The KEGG pathway enrichment analysis (Table 3) showed three pathways – two of them associated with drug metabolism in the sorafenib responding group, one – related to neurotransmitters – in the lenvatinib group, seven pathways – most of them related to cardiac function – in the regorafenib group, three receptor-related pathways in the
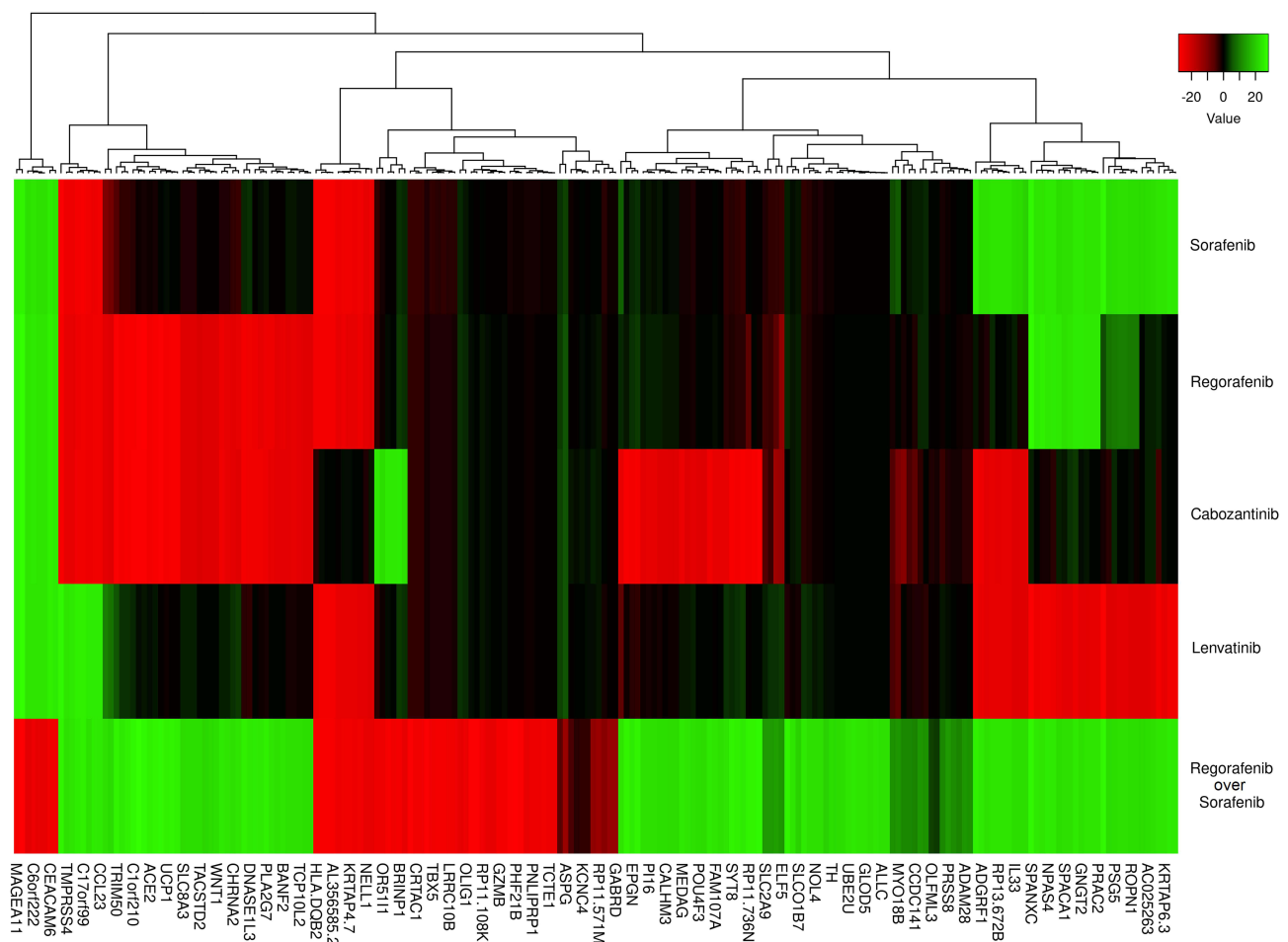
Figure 2 Gene differential expression values compared to treatment groups. This heatmap shows the differential expression values for each drug. The scale is measured in expression fold changes (FC) in relation to the standardized expression profiles of the original cell lines after being analyzed with the DESeq2 algorithm. The dark colors represent FC values between 0 and ± 10, and the lighter colors present FC values over ± 10. At the left, the blue rectangle includes two clusters tinted in light green and red in all the treatment groups, suggesting two identifiable gene expression signatures: the left one, with overexpression in four groups (sorafenib [S], regorafenib [R], cabozantinib [C] and lenvatinib [L]) and underexpression in those that responded differentially better to regorafenib than sorafenib (RoS), and the right one, with genes highly underexpressed in the first three groups ((S), (R), and (C)) and overexpressed in the last two (L and RoS).

cabozantinib group, and no enriched pathways in the RoS group. Noticeably, "Neuroactive ligand-receptor interaction" was shared among lenvatinib, regorafenib, cabozantinib, and "Cell adhesion molecules" among regorafenib and cabozantinib.

Furthermore, among the top five canonical pathways detected using the IPA analysis (Table 3), two were enriched in all the treatment groups: "CREB Signalling in Neurons" and "Breast Cancer Regulation by Stathmin1", and three were partially shared: "Hepatic Fibrosis/Hepatic Stellate Cell Activation" between lenvatinib and sorafenib, "Synaptic Long-Term Depression" between sorafenib and regorafenib, and "Calcium Signaling" between cabozantinib and RoS.

Resulting Gene Expression Signature

After filtering the DEG consistently significant in the five treatment groups, 14 genes (GAGE12H, GJB6, PTCHD3, PRH1-PRR4, C6orf222, HBB, C17orf99, GOLGA6A, CRYAA, CCL23, RP11-347C12.3, RP11-514O12.4, FAM180B, and TMPRSS4) fulfilled our criteria. Moreover, they all presented a highly significant level of over or under expression (FC > |20|). When organized as in the heatmap clusters, three different expression profiles were formed: one comprehending those that respond to sorafenib, regorafenib, and cabozantinib; one comprehending those that respond to lenvatinib; and one with those that respond better to regorafenib compared to sorafenib (Table 4).

Table 3 KEGG and IPA enriched pathways by treatment group.

KEGG = **Fold enrichment** / [DEGs / Total genes in pathway]
IPA = **Impact (%)** / [DEGs / Total genes in pathway]
— = Enriched pathways' and medications intersections
⊙ = Cardiovascular-related pathways
⊙ = Liver-specific pathways
⊙ = Neurologic signaling pathways
⊙ = Others cellular pathways

A) KEGG Pathways Enrichment

Sorafenib	Lenvatinib	Regorafenib	Cabozantinib	RoS	Pathway
FE: 3.5 [14/350]	FE: 3.8 [10/350]	FE: 5 [16/350]			Neuroactive ligand-receptor interaction
	FE: 5.3 [6/149]	FE: 4.8 [5/149]			Cell adhesion molecules
FE: 8.7 [5/74]					Metabolism of xenobiotics by cytochrome P450
FE: 9.3 [5/69]					Drug metabolism
FE: 6.5 [5/99]					Hematopoietic cell lineage
		FE: 8.6 [5/77]			Arrhythmogenic right ventricular cardiomyopathy
		FE: 5.3 [6/150]			Adrenergic signaling in cardiomyocytes
		FE: 8.3 [6/96]			Dilated cardiomyopathy
		FE: 8.8 [6/90]			Hypertrophic cardiomyopathy
		FE: 3.8 [5/87]			Cardiac muscle contraction
			FE: 4.3 [10/294]		Cytokine-cytokine receptor interaction

B) IPA Canonical Pathways

Sorafenib	Lenvatinib	Regorafenib	Cabozantinib	RoS	Pathway
12.8% [77/602]	16.1% [97/602]	13.6% [82/602]	15.0% [90/602]	13.3% [80/602]	CREB Signaling in Neurons
11.3% [67/592]	14.0% [83/592]	12.0% [71/592]	12.8% [76/592]	13.3% [68/592]	Breast Cancer Regulation by Stathmin1
11.9% [23/194]	20.6% [40/194]				Hepatic Fibrosis / Hepatic Stellate Cell Activation
			15.3% [33/216]	14.4% [31/216]	Calcium Signaling
12.4% [24/194]		14.4% [28/194]			Synaptic Long-Term Depression
11.6% [22/189]					Granulocyte Adhesion and Diapedesis
	21.5% [32/149]				Cellular Effects of Sildenafil
	22.3% [29/130]				Atherosclerosis Signaling
		15.2% [21/138]			Gαi Signaling
		15.3% [20/131]			GABA Receptor Signaling
			20.3% [24/118]		Airway Pathology in COPD
			14.8% [28/189]		Hepatic Cholestasis
				25.0% [14/56]	Chondroitin Sulfate Biosynthesis
				13.5% [34/252]	Mediated Inhibition of RXR Function

Notes: This table shows the enriched pathways for every treatment group as detected by **A**) KEGG pathways enrichment and **B**) QIAGEN'S IPA analysis. To establish each pathway's relevance, Fold Enrichment (FE; the percentage of DEGs belonging to a pathway divided by the corresponding percentage in the background) is used on KEGG, and Impact (the number of DEGs divided by the total number of pathway genes) on IPA pathways. The intersections of pathways between treatments are shown with a line, and the main type of enriched pathways is expressed by colors: red denotes cardiovascular-related pathways, gray the neurological, brown the liver-specific, and black other pathways.

Table 4 Gene Expression Signature Candidate

Candidates for Gene Expression Signature (FC >|2|, pAdj > 0.001)	Sorafenib (217 DEG)	Regorafenib (201 DEG)	Cabozantinib (240 DEG)	Lenvatinib (296 DEG)	RoS (210 DEG)
Signature Group		A		B	C
GAGE12H	+	+	+	+	−
GJB6	+	+	+	+	−
PTCHD3	+	+	+	+	−
PRH1-PRR4	+	+	+	+	-
C6orf222	+	+	+	+	-
HBB	+	+	+	+	-
C17orf99	-	-	-	+	+
GOLGA6A	-	-	-	+	+
CRYAA	-	-	-	+	+
CCL23	-	-	-	+	+
RP11-347C12.3	-	-	-	+	+
RP11-514O12.4	-	-	-	+	+
FAM180B	-	-	-	+	+
TMPRSS4	-	-	-	+	+

Notes: This table shows the DEG that fulfills the filtering criteria. Group A (sorafenib, regorafenib, and cabozantinib group) present an overexpression with an FC > 20 in GAGE12H, GJB6, PTCHD3, PRH1-PRR4, C6orf222, and HBB, and an underexpression with an FC < −20 in C17orf99, GOLGA6A, CRYAA, CCL23, RP11-347C12.3, RP11-514O12.4, FAM180B, and TMPRSS4. Group B (lenvatinib) presents an overexpression in all the named genes, and Group C (regorafenib over sorafenib) completely inverts the profile seen in group A.

Discussion

The current gold standard for treating HCC in an advanced stadium are I/O-based therapies, such as atezolizumab plus bevacizumab or durvalumab plus tremelimumab, which are presently investigated in the ongoing randomized Phase III HIMALAYA study and show promising efficacy in the preliminary analysis.[27,28] However, if the tumor is non-responsive to the I/O-treatment or contraindications prohibiting I/O-based therapies are present, inclusion in second-line studies or the usage of TKIs may be considered as the next choice in the treatment algorithm for advanced HCCs.[6] If a patient must be treated with a TKI, there is no established biomarker to guide treatment. Therefore, the use and the application sequence of these agents in different lines of treatment are solely determined by empirical evidence. To narrow potential gene sets and explore the feasibility of biomarker-guided approaches, we investigated the differential responsiveness of nine hepatoma cell lines to the four currently approved TKIs, followed by a differential expression and gene set enrichment analysis.

Cell line-specific heterogeneity of proliferative rates influences the comparison of in vitro efficacy measurements. It is assumed that only 40% of statistically significant associations between IC_{50} values and genomic alterations in large-scale screening experiments are not confounded.[20] The recently developed GR_{50} metric adjusts for proliferation and integrates cytostatic and cytotoxic effects.[19]

In the last years, extensive experiments have been conducted examining the response of various hepatoma cell lines to the four tyrosine kinase inhibitors among an array of other substances.[8,9] Given that we used the GR_{50} metric, a prolonged treatment protocol, and a different assay, a comparison of these two papers to our experiments can only be made to a certain extent.

In our experiments, we used the GR method with the GR_{50} value as a marker for potency, as previously described. Qiu et al used the half-maximal inhibitory concentration (IC_{50}) as a value for potency, which shows the drug

concentration having caused 50% inhibition on cell viability and Caruso et al decided to portray the potency of their substances in the form of the GI_{50} value, which is the renamed version of the IC_{50} value correcting for the cell count at time zero.[29] In their work, some inhibitory concentrations of regorafenib, lenvatinib, and cabozantinib were outside the metrics range (Supplementary Table 1). While we can see an apparent decrease in cell viability in our experiments, both Qiu et al and Caruso et al show that while the four TKIs lead to a decrease in cell viability on all cell lines, their effect on hepatoma cell lines in vitro is among the smallest out of all the agents that were evaluated in their experiments. In addition, comparing the efficacy measurements in all three datasets, that is, Caruso et al's, Qiu et al's and ours, for the four TKIs, a difference in responses can be seen, with our experiments showing the most potent effect in cell decrease for all four TKIs (Supplementary Tables 1 and 2). Duration of treatment also affects the validity and clinical relevance of in vitro studies.[30] In a clinical setting, the four TKIs are usually administered over several weeks to months. Therefore, we decided on using a drug assay with a six-day treatment duration instead of a shorter drug assay to mimic this clinically proven treatment schedule better. Both Qiu et al and Caruso et al decided on a shorter duration of drug exposure (48–72 hours) compared to our experiment (144 h). In contrast, we observed significantly decreased GR_{50} values compared with the other two studies (Supplementary Table 1) and attribute it to the prolonged treatment. Since cytostasis is the primary mode of action of the four TKIs, a transcriptomic lag or cellular accumulation of the cytostatic factors may explain the observed differences with the prolonged treatment scheme. Additionally, we can also assume that the different metrics for efficacy, the varying execution of the drug assays, and the mode of action of the TKIs play a role in the different results of these three studies.[8,9]

A diversified response can be observed for all four TKIs. In particular, lenvatinib and cabozantinib, but also sorafenib and regorafenib, showed a pronounced spread of GR_{50} values. Thus, the diverse pharmacological response reflects the divergent biology of these cell lines and gives justification to pursue biomarker-guided approaches such as transcriptomic-driven approaches or individualized testing strategies. In this regard, Broutier et al recently showed the feasibility of ex vivo culture of individual tumoroid HCC and cholangiocarcinoma cell lines and the possibility of ex vivo drug testing.[31]

Interestingly, the response in most cell lines to sorafenib and regorafenib correlates strongly, which is not surprising considering their chemical structure only differs by one additional fluorine atom,[16] they display almost identical target points,[32] and regorafenib is only approved for therapy in patients with HCC in the second line after they have previously been treated with sorafenib.[33]

Moreover, the differential responsiveness of each drug allowed us to compare their related canonical pathways and the different gene expression profiles. Among the shared sets, KEGG enrichment analysis identified the "neuroactive ligand-receptor interaction pathways" in the lenvatinib, regorafenib, and cabozantinib responders, and "Cell adhesion molecules" in regorafenib and cabozantinib responders. This further validates the enrichment of these pathways in sorafenib-resistant cell lines as reported by Gan et al, including focal adhesion, neuroactive ligand/receptor, and cytokine/cytokine receptor.[34] The rest of the pathways were enriched only in treatment-specific responses, being the sorafenib responders related to drug metabolism and regorafenib responders to cardiac-related pathways. Notably, no specific KEGG enriched set was identified in the regorafenib over sorafenib group and none of our TKIs' key target pathways in KEGG (eg "Pathways in cancer", "VEGF signaling pathway", "MAPK signaling pathway", among others) was enriched.

On the other hand, Qiagen's IPA analysis showed two pathways enriched in all five groups: "CREB Signalling in Neurons" and "Breast Cancer Regulation by Stathmin 1". As recently reviewed by Zhang et al, the cAMP-PKA-CREB pathway has a versatile role in multiple targets, and it can promote or inhibit HCC in a context-dependent matter, suggesting that CREB's homeostasis is vital for HCC progression.[35] Stathmin 1′s importance for hepatoma seems to be on the rise, since it has recently been proposed as a tumorigenic and progression regulator due to its interaction with YAP1,[36] and, when overexpressed, as a novel treatment target for HCC.[37] Furthermore, sorafenib and regorafenib shared the "Synaptic Long-Term Depression" pathway, which is not surprising given that it encompasses the activation of glutamate and other neurotransmitter receptors,[38] as seen in the KEGG neuroactive ligand/receptor enriched set.

Additionally, we found the "Hepatic Fibrosis/Hepatic Stellate Cell Activation" in both the sorafenib and lenvatinib responders and "Calcium Signalling" in the cabozantinib and "regorafenib over sorafenib" groups.

As discussed in a recent review by Qian et al, the creation of an HCC gene expression signature is challenging because it usually arises from the cirrhotic liver, and the comparison of tumoral samples against non-tumorous tissue is complicated as the control tissue itself may have significant changes in gene expression.[39] Nonetheless, the determination of a common DEG signature capable of predicting multiple cell lines' treatment response into three distinct TKI treatment profiles has been achieved: A) those equally sensible to sorafenib, regorafenib, and cabozantinib, B) those more sensible to lenvatinib, and C) those more sensible to regorafenib than sorafenib. This signature comprised the 14 genes that were statistically significant in each treatment, and additionally, they had a Fold-change of ±20, turning them into easily discernible molecules. Consequently, despite not being able to discern the best responders to cabozantinib, it may be used to decide whether the group sorafenib/cabozantinib, lenvatinib, or regorafenib should be used for a patient's best possible outcome according to their tumor DEG. However, due to the limited number of cell lines investigated, these data must be validated in more extensive cell-line or tumoroid-line collections before being tested in a clinical approach. Another limitation of our study, is that we have conducted our experiments on cell lines that were generated from untreated HCCs and did not undertake any pre-treatments. As the currently preferred first-line therapy for advanced HCCs are CPI therapies, TKIs are mainly used upon progression to the preferred first line. Upon progression to CPI therapies, it is likely that the tumor biology in untreated tumors differs significantly from post-CPI tumors, for example, due to the process of acquiring resistance. Therefore, inference of our results to the post-CPI situation is limited. We are planning on using in vitro systems such as the tumoroid cell culture method in follow-up studies, to better reflect the whole spectrum of tumor biology and the clinical context of the HCC tumor entity.[31]

Upon evaluation of multiple hepatoma cell lines in vitro and in silico, a common gene expression signature was characterized that, if validated in vitro and in vivo, could accurately predict a patient's HCC response to the different available TKIs, and it could help us define the best treatment for the individual patient according to transcriptomic analysis in our near future.

Conclusion

We observed heterogeneous responsiveness to the four currently approved TKIs. This generates a rationale for evaluating genomic and transcriptomic biomarkers and individualized approaches, such as patient-derived tumoroid culture and testing. In addition, interobserver heterogeneity highlights the need for additional investigations evaluating the applicability of modified in vitro strategies (ie, prolonged testing) and new efficacy metrics. The transcriptomic-analysis-generated genes candidating as biomarkers showed distinct signaling pathways associated with sensitivity to either TKI and found a candidate gene signature capable of discriminating response to the TKIs.

Disclosure

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