A genome-wide CRISPR activation screen reveals Hexokinase 1 as a critical factor in promoting resistance to multi-kinase inhibitors in hepatocellular carcinoma cells

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Abstract
Hepatocellular carcinoma (HCC) is often diagnosed at an advanced stage and is, therefore, treated with systemic drugs, such as tyrosine-kinase inhibitors (TKIs). These drugs, however, offer only modest survival benefits due to the rapid development of drug resistance. To identify genes implicated in TKI resistance, a cluster of regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 activation screen was performed in hepatoma cells treated with regorafenib, a TKI used as second-line therapy for advanced HCC. The screen results show that Hexokinase 1 (HK1), catalyzing the first step in glucose metabolism, is a top candidate for conferring TKI resistance. Compatible with this, HK1 was upregulated in regorafenib-resistant cells. Using several experimental approaches, both in vitro and in vivo, we show that TKI resistance correlates with HK1 expression. Furthermore, an HK inhibitor resensitized resistant cells to TKI treatment. Together, our data indicate that HK1 may function as a
1 | INTRODUCTION

Hepatocellular carcinoma (HCC) is common primary liver cancer and a major cause of cancer-related death. HCC typically develops in patients with liver cirrhosis, an end-stage of chronic liver disease. The major risk factors for chronic liver disease include chronic infection with hepatitis B or C virus and nonalcoholic fatty liver disease, a prevalent disease in the western world. Patients with advanced liver disease may be unaware of their condition or may not be screened for liver cancer and are, therefore, often diagnosed when they already suffer from advanced HCC. Consequently, these patients are not eligible for curative therapies, and their only treatment option is systemic therapy. Tyrosine-kinase inhibitors (TKIs) are an important class of drugs in the arsenal of systemic HCC therapies. Sorafenib was the first TKI approved in 2007. Sorafenib inhibits vascular endothelial growth factor receptors, platelet-derived growth factor receptors, and RAF kinases, among others, leading to inhibition of proliferation, angiogenesis, and invasion (for review, see5). Following a decade in which sorafenib was the sole TKI approved for HCC, regorafenib was approved in 2017 as a second-line TKI for patients progressing on sorafenib. Two additional TKIs, lenvatinib (first line), and cabozantinib (second line) were approved later on. Despite the minor chemical difference between sorafenib and regorafenib, the latter shows a broader inhibitory profile and a more comprehensive pharmacological activity. Both drugs, however, have modest efficiency that translates into a survival benefit of only a few months. In addition, their use is implicated in high costs and serious adverse effects. This low efficacy is attributed to both intrinsic and acquired drug resistance.

The molecular events leading to drug resistance are largely unknown and, thus, there is a dire need to identify the cellular mechanism(s) underlying drug resistance. Therefore, we performed a genome-wide CRISPR/Cas9 activation screen in hepatoma cells (Huh7) to identify genes that confer resistance to regorafenib when overexpressed. CRISPR activation uses single-guide RNAs (sgRNAs) directed to an endogenous promoter to recruit a catalytically inactive Cas9 (dCas9) fused to a transcription activator. Recruitment of the activator to these locations activates the gene controlled by the targeted promoter. This approach could be combined with a genome-wide sgRNA library to activate all known coding isoforms. This technique might identify novel pathways underlying TKI resistance that cannot be detected by loss of function studies. CRISPR activation was previously used to identify host factors conferring chemotherapy resistance and other applications.

Our screen identified several genes conferring regorafenib resistance. Hexokinase 1 (HK1), the enzyme that catalyzes the first step in glucose metabolism, that is, the phosphorylation of glucose to glucose-6-phosphate, was among the top hits of our screen. Four HK isoforms (HK1–4) have been characterized in mammalians. Normal liver tissue expresses glucokinase (HK4), HCC cells, on the other hand, do not express glucokinase but rather HK1 or HK2, or a combination of both. Our experiments indicated that overexpression of HK1 in Huh7 cells increased resistance to regorafenib. Furthermore, HK1 levels correlated with regorafenib resistance in additional HCC cell lines differing in HK1 expression levels. Interestingly, regorafenib-resistant cells overexpressed additional glycolysis-related and other metabolic genes. Taken together our results, indicate that metabolic reprogramming is most likely a crucial aspect of regorafenib resistance.

2 | MATERIALS AND METHODS

2.1 | Cell culture, compounds, plasmids, and transfections

Huh7 cells were propagated in Dulbecco’s Modified Eagle’s Medium (DMEM, Gibco). JHH-6 cells were propagated Williams’ E medium (Gibco, #22551-022). HLF cells were propagated in DMEM supplemented with pyruvate (Gibco, #41966-052). JHH-6 (JCRB1030) and HLF (JCRB0405) cells were obtained from the Japanese Collection of Research Bioresources Cell Bank. The growth media of all the cell lines was supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco), 1% (v/v) penicillin/streptomycin, and 1% (v/v) glutamine (Biological Industries, Bet-Haemek, Israel). The Huh7 cells stably expressing the MS2-P65-HSF1 activator plasmid (Addgene #89308) were grown in the presence of hygromycin (0.8 mg/ml, Invivogen). shRNA lentiviral plasmids targeting HK1 (Mission shRNA TRCN0000197140, TRCN0000199971, TRCN0000037656, TRCN0000291063, TRCN000037658) or nontargeting (NT, Sigma, SHC002) were from Sigma. Lentiviruses produced from these plasmids
were used to infect JHH6 cells, followed by one week of puromycin selection (0.5 µg/ml, Invivogen). All cell lines were maintained in a humidified incubator at 37°C with 5% CO₂. Polyethyleneimine (Polysciences) was used for plasmid DNA transfections of subconfluent cells. Regorafenib was purchased from Abcam. Sorafenib and lonidamine were purchased from the Cayman Chemical Company.

2.2 | CRISPR activation screen

A pooled genome-scale library of lentiviral vectors expressing sgRNAs for CRISPR activation was purchased from Addgene (#1000000078, a gift from Feng Zhang13). The library was amplified according to the depositor’s protocol.20 sgRNA distribution was verified following amplification by next-generation sequencing (NGS). HEK293T cells were used for lentivirus production as previously described.20 For transduction, Huh7 cells stably expressing the MS2-P65-HSF1 activator plasmid (Addgene, #61426) were seeded on 12-well plates at a density of 10⁶ cells per well. The cells were transduced with lentiviral pseudo-particles at a multiplicity of infection of 0.3 by spinoculation at 1000 g for 45 min at 37°C in media containing 3% FBS, 20 mM HEPES, and 4 µg ml⁻¹ polybrene (Sigma). The next day, the cells were replated at low confluency in 15-cm dishes and selected with 7 µg/ml blasticidin for 7 days. At this time point, all untransduced control cells were dead. Following selection, surviving cells were pooled, and 4 x 10⁷ cells were collected and kept frozen at −80°C as an untreated control. The rest of the cells were replated at 40% confluency and treated with 7 µM regorafenib for ten days. The media and drug were replaced every three days during the incubation period. Regorafenib concentration was predetermined using a killing curve (Figure S1). This concentration is clinically relevant as, at a steady-state, regorafenib reaches mean peak plasma levels of about 8.1 µM, following oral administration of 160 mg regorafenib.21 Genomic DNA was prepared from the surviving cells and used to amplify the sgRNAs for NGS as described.20 The resulting reads from the NGS experiments were analyzed using MAGECK v0.5.6.22 The screen was repeated twice. Raw sgRNA sequencing read numbers, MAGECK scores, and a ranked list of genes from the two screen repeats are added in the supplemental data (Supplemental files 1–4).

2.3 | Validation of screen and RNA-seq hits

Screen and RNA-seq hits were further validated by recloning individual sgRNAs of the relevant genes into the library backbone plasmid (lentiSAMv2, Addgene #75112, see Table 1 for sgRNA sequences). NT sgRNA was used as a control.

| TABLE 1 Library sgRNAs used for cluster of regularly interspaced short palindromic repeats activation of individual genes |
|---|---|
| **Top** | **Bottom** |
| PAX6 | CACCGGGAGAGGAGCCGGGACCCAC |
| HK1 | CACCGTGGCCGTCGAGGAGTGGGT |
| ITGB5 | CACCGTCCCCAGGAGCCTCGCC |
| GEMIN7 | CACCGCGGCGGCCAGGTGGCGACCC |
| ZNF296 | CACCGAGGGCAGAGGGCGGAGGC |
| CDKN2C | CACCGTGTGATCCTGAGGCGTGTG |
| CRB2 | CACCGCTAGGGGGAGTGGACTGAG |
| ZBTB42 | CACCGGGCGAGGACCTCCGGGAG |
| ATF3 | CACCGCGAGCGAGTACGCACATC |
| KLF2 | CACCGGAAGGCCCAGCAGGCGCAG |
| ESSRG | CACCGCTGGCTGGCTGCTGCTG |
| ACSLS(1) | CACCGTCAACTGTGGTGAGCAAGC |
| ACSLS(2) | CACCGTGATAGGAGCAGAGGAGGC |
| CESI(1) | CACCGGCACTACTGTGGTACCATATA |
| CESI(2) | CACCGGAGGCTGGGATGAAGAG |
| ANXA1(1) | CACCGAGACTTCTGCTGCTGTTTT |
| ANXA1(2) | CACCGCTACTAAACCATTCAG |
| HKDC1(1) | CACCGGTTGTGGACCTGGAGG |
| HKDC1(2) | CACCGGTTGTGGACCTGGAGG |

Note: For genes with a number in parenthesis following the gene name, two guides were used simultaneously to activate each gene.
Lentiviruses produced from these plasmids were used to infect Huh7 cells stably expressing the MS2-P65-HSF1 activator plasmid. First, the cells were selected with 7 μg/ml blasticidin for seven days to create stable cell lines. Then, the activation of the relevant genes in the obtained cell lines was tested using qPCR with specific primers (Table 2). The verified cell lines were treated withregorafenib at the indicated concentrations for 3 days. Viability was determined using CellTiter-Glo® (Promega) or crystal violet. Crystal violet staining quantification was performed by extracting the dye using 10% acetic acid, followed by measurement of absorbance at 570 nm using a plate reader.

2.4 | qPCR

Total RNA was extracted from each cell line using TRI-Reagent (Sigma). The extracted RNA (1 μg) was reverse transcribed using the iScript cDNA Synthesis Kit (Bio-Rad). qPCR was performed using a SYBR Green Master mix (Fast Syber® ABI). Specific primers were used to quantify the expression levels of each activated gene (Table 2). The housekeeping gene hypoxanthine phosphoribosyltransferase 1 (HPRT) was used as a control. The results are presented as a fold change from NT sgRNA expressing cells calculated using ΔΔCt.

2.5 | Western blot analysis

Total protein was harvested from cells using radioimmunoprecipitation assay buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton x-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 1 mM EDTA) containing a protease inhibitor cocktail (Sigma). Extracts were clarified by centrifugation at 12 000×g for 15 min at 4°C, followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of equal amounts of total protein. Proteins were transferred to a nitrocellulose membrane and blocked with 5% low-fat milk or 5% bovine serum albumin. Membranes were incubated with primary antibodies overnight at 4°C, washed with phosphate-buffered saline (PBS) containing 0.1% Tween-20 (PBST), and incubated with the appropriate horseradish peroxidase (HRP)—conjugated secondary antibody for 1 h. After washing with PBST, the HRP signal was detected using enhanced chemiluminescence.

2.6 | Antibodies

The following primary antibodies were used: rabbit anti-hexokinase I (C35C4, CellSignaling Technology, 1:1000), rabbit anti-hexokinase II (Cell Signaling Technology, C64G5, 1:1000), rabbit anti-hexokinase II (Proteintech, 22029-1-AP, 1:1000), rabbit anti-PARP (Cell Signaling Technology, 9542T, 1:1000), and mouse anti-tubulin (Sigma, T-9026, 1:10000). Secondary antibodies were goat anti-mouse or goat anti-rabbit HRP secondary antibodies (Jackson ImmunoResearch, 1:5000).

2.7 | Lactate production assay

For lactate production assay, 20 000 cells were plated in a 96-well plate. Lactate levels in the HK1 overexpressing
cells and media were determined after 20 h. Lactate levels in the regorafenib treated cells were determined after 72 h. Lactate levels were determined using the Lactate-Glo™ Kit (Promega J5021) according to the manufacturer’s instructions. The results were normalized to cell viability levels, as determined using presto blue (Thermo Fisher).

2.8 | RNA sequencing

mRNA sequencing was performed at the Weizmann Institute of Science Crown Genomics institute of the Nancy and Stephen Grand Israel National Center for Personalized Medicine. Total RNA was prepared from three biological replicates of regorafenib resistant or sensitive Huh7 cells using Tri-reagent (Sigma) and Direct-zol™ RNA Miniprep Kit (Zymo Research). RNA quality was confirmed using the Tapestation (Agilent). Poly(A) + mRNA library was prepared using the mRNA-Seq kit (Illumina). Sequencing was performed using NextSeq high-output SR75. Data normalization and differential expression analyses were performed using the DESeq2 package.23 The resulting data is provided (Supplemental file 5). Deregulated genes were subjected to DAVID for pathway analyses.24

2.9 | Patient-derived xenografts

Specimens for patient-derived xenografts (PDX) model preparation were obtained by tumor resection from a patient diagnosed with HCC at the Rabin Medical Center (RMC). The tissue collection was performed per institutional review board-approved protocols with written informed consent from the patient. All mice were treated following RMC guidelines for the care and use of experimental animals with approval from the RMC Institutional Animal Care and Use Committee (IACUC).

Tumor samples were placed in cold DMEM supplemented with 10% FBS and 1% penicillin/streptomycin and maintained on ice until processing. Within 0.5–2 h, tumor fragments were cut into 2–3-mm pieces using sterile surgical instruments. Several pieces were used for implantation, and one was formalin-fixed for histological examination. Typically, several implantations were carried out (subcutaneously on the flanks, intraperitoneal implantation). The recipients were 6–7-week-old immunodeficient male NSG mice (NSG, NOD.Cg-25 Prkdc<sup>scid</sup>Il2rgtm1Wjl/SzJ, Jackson Laboratories). Before implantation, the tumor fragments were coated with Cultrex Basement membrane matrix, type 3 (Trevigen). Mice were kept under pathogen-free conditions and received sterilized food and water ad libitum.

Fresh tumors excised from mice were dissociated by GentleMACS and implanted by subcutaneous injection into the neck of 7–10-week-old NRG mice. When tumors reached a size of 60–200 mm<sup>3</sup>, mice were assigned to the various treatment groups (6–7 mice each) based on the tumor size, growth rate, and mouse body weight, creating groups with similar average parameters. Control mice were treated with the vehicle, whereas a different group was orally treated with sorafenib (20 mg/kg) 5 days per week. Tumor volume and body weight were measured twice a week throughout the treatment period. The treatment continued up to a maximal tumor size of 1500 mm<sup>3</sup> or when clinical signs met those defined by the IACUC. Then, the mice were euthanized according to the IACUC-approved protocol. The tumors were harvested and examined histologically to confirm their human origin and morphological similarity to the corresponding engrafted tumor. Additional slides were stained with HK1 antibody (1:800) or HK2 antibody (1:400) using the Bond III immunostaining instrument (Leica). Slides were scanned using the Aperio-Slide Scanner (VERSA) at a 20× magnification.

3 | RESULTS

3.1 | CRISPR activation screen identifies genes conferring regorafenib resistance

The lentiviruses expressing the CRISPR activation library were used to infect Huh7 cells stably expressing the activation domains.13 Following selection, the cells were treated with 7 µM regorafenib for ten days (Figure 1A). Genomic DNA was prepared from the cells used to amplify the sgRNAs for NGS. Figure 1B,C show the detailed ranking of the top screen hits. To validate the screen results, the top-ranking individual sgRNAs were recloned and used to stably overexpress the respective genes in Huh7 cells. All the genes were significantly activated at varying levels except for ATF3, which was significantly downregulated for an unknown reason (Figure 2A). The obtained cell lines were treated with regorafenib for 72 h, and viability was determined (Figure 2B). For 5/10 tested genes, viability levels were significantly elevated above the NT control. Significant activation was observed for all genes showing a protective effect.

3.2 | HK1 and ITGB5 overexpression promote regorafenib resistance

To confirm the role of the identified genes in regorafenib resistance, regorafenib resistant Huh7 cells were prepared...
using repeated rounds of treatment with 10 μM regorafenib until resistance was achieved. Expression levels of the confirmed screen hits in the resistant cells were determined by qPCR (Figure 3A). HK1 and integrin subunit β5 (ITGB5) were the only significantly elevated genes from the screen hits. A western blot analysis further confirmed HK1 induction at the protein level in the resistant cell line compared with a DMSO-treated control. At the same time, no difference was observed in HK2 levels between regorafenib-resistant and control cells (Figure 3B).

Because HK1 was a top screen hit conferring resistance, and one of the only screen hits significantly elevated in the resistant cells, we further focused on HK1.

First, we confirmed its activation in the CRISPR-activated cell line at the protein level compared with NT control cells (Figure 4A). Our results showed that while HK1 protein is not detected in the control cells, it is highly expressed in the activated cells. In contrast, HK2 was expressed in both CRISPR-activated and control cells. Next, the effect of HK1 activation on cells’ resistance to increasing doses of regorafenib and sorafenib was determined (Figure 4B,C). As shown, HK1 activation conferred resistance to both regorafenib and sorafenib, compared with an NT control, indicating that HK1-mediated TKI resistance may have a class effect.

The hallmark of cancer cells is the production of large amounts of lactate in the presence of oxygen, through aerobic glycolysis.\(^\text{25}\) To test if HK1 overexpression or regorafenib treatment induced a further shift toward this aberrant glycolysis, we tested lactate production levels in these cells (Figure 4D,E). Lactate levels were significantly increased in both the HK1 activated cells and their growth media compared with NT control cells (Figure 4E). Similar results were obtained with Huh7 cells treated with regorafenib for 72 h (Figure 4D).

ITGB5 was the only screen hit that was also elevated in the Huh7 regorafenib-resistant cells (Figure 3A). Since ITGB5 was previously found to facilitate glycolysis,\(^\text{15}\) we asked whether HK1 and ITGB5 synergize in conferring resistance. A stable Huh7 cells line in which both HK1 and ITGB5 were activated was prepared to test this possibility. To confirm the expression levels of both genes in these cells, we tested their mRNA levels compared with an NT control (Figure S2A). Both genes were significantly activated in this cell line, and the effect of their activation on regorafenib resistance was determined (Figure S2B). The HK1+ITGB5 expressing cell line showed increased resistance levels compared with an NT control. This resistance, however, was comparable with activation of HK1 alone, indicating that there is no additive or synergistic activities between these two genes.

**Figure 1** A genome-wide cluster of regularly interspaced short palindromic repeats screen identifies genes conferring resistance to regorafenib. (A) A scheme summarizing the screen conditions. (B) A comparison of the top hit ranks in the two screen repeats. Values represent gene ranks assigned by MAGeCK. (C) Top candidates (colored dots) using the MAGeCK p-value analysis versus log2 fold change from the first screen repeat.
genes were identified using the DESeq2 package. Out of 328 genes with a known function answering these criteria, the largest group of 35 genes were classified by DAVID as metabolic genes (Figure 5A). Several gene families previously known to be involved in regorafenib metabolism were significantly overexpressed. These include several members of the cytochrome P450 family and the UDP glucuronosyltransferase family, confirming our approach. HK1 was overexpressed by 4.75-fold, confirming our previous results. ITGB5, however, was not detected under the filtering conditions used. Interestingly, an additional hexokinase (HKDC1) involved in liver metabolism was overexpressed in regorafenib-resistant cells at levels similar to HK1. The Aldolase B, a different glycolytic enzyme, was overexpressed by 4.9-fold. Furthermore, several other metabolic gene families were overexpressed, including aldehyde dehydrogenases, aldo-keto reductase acyl-CoA synthetase, and acyl-CoA synthetases. To confirm the RNA-seq results, we focused on four of the elevated genes. Carboxylesterase 1 (CES1), a major liver enzyme that functions in liver drug metabolism, was upregulated in the resistant cells by 86-fold and, thus, might be involved in regorafenib metabolism. Annexin A3 was previously shown to confer sorafenib resistance to HCC cells. ANXA1, a family member, was significantly upregulated in the resistant cells by almost sevenfold compared with controls. Thus, we tested if other members of the family could confer resistance. Long-chain-fatty-acid-CoA ligase 5 (ACSL5), a metabolic gene, is an enzyme that plays a crucial role in lipid biosynthesis and fatty acid degradation. CRISPR activation system was used to validate the RNA-seq results by generating a cell line expressing individual sgRNAs for each of the genes mentioned above. Increased expression at the mRNA level of all activated genes was confirmed (Figure 5B and Table 2). The obtained cell lines were treated with regorafenib for 72 h, and viability was determined (Figure 5C). As expected, all of the tested genes conferred increased resistance to regorafenib compared with an NT control indicating that additional metabolic genes are involved in mediating regorafenib resistance.

### 3.4 | Regorafenib resistance correlates with HK1 levels in hepatoma cells

HCC tumors are molecularly heterogeneous and display distinct expression patterns of metabolic genes. Liver cell lines were previously shown to mimic the metabolic gene expression patterns of the corresponding human tumors. With regards to hexokinase expression, liver cell lines were found to divide into two main categories: HK1+HK2+ (Huh7) and HK1+HK2− (HLF, JHH6). To test if HK1 expression levels correlate with regorafenib resistance, we tested viability levels in response to

| **FIGURE 2** Functional validation of the screen results. (A) Expression levels of the activated genes as determined by qPCR using gene-specific primers. HPRT1 was used as a housekeeping control. Shown are mean values ± SD. *p < .05; **p < .01; multiple, two-tailed, unpaired t tests. (B) Huh7 cell lines expressing the indicated sgRNAs were treated with 7 μM regorafenib for 72 h. Viability was tested using a commercial kit. Results are mean ± SD from three independent experiments student’s t test. *p < .05; **p < .01 |

### 3.3 | Essential metabolic genes are differentially overexpressed in regorafenib-resistant cells

We next asked if the induction of HK1 is part of a more extensive metabolic change occurring in the Huh7 regorafenib-resistant cells and if we can identify additional genes conferring similar resistance. To this end, we performed RNA-seq analysis of the regorafenib-resistant cells compared with DMSO-treated controls. Differentially expressed genes were identified using the DESeq2 package. The analysis revealed 943 upregulated genes (log2-fold change>1) and 501 downregulated genes (log2-fold change<−1) in the regorafenib resistant. The DAVID functional annotation tool was used to further classify the overexpressed genes (log2-fold change>2) in the regorafenib-resistant cells. Out of 328 genes with a known function answering these criteria, the largest group of 35 genes were classified by DAVID as metabolic genes (Figure 5A). Several gene families previously known to be involved in regorafenib metabolism were significantly overexpressed. These include several members of the cytochrome P450 family and the UDP glucuronosyltransferase family, confirming our approach. HK1 was overexpressed by 4.75-fold, confirming our previous results. ITGB5, however, was not detected under the filtering conditions used. Interestingly, an additional hexokinase (HKDC1) involved in liver metabolism was overexpressed in regorafenib-resistant cells at levels similar to HK1. The Aldolase B, a different glycolytic enzyme, was overexpressed by 4.9-fold. Furthermore, several other metabolic gene families were overexpressed, including aldehyde dehydrogenases, aldo-keto reductase acyl-CoA synthetase, and acyl-CoA synthetases. To confirm the RNA-seq results, we focused on four of the elevated genes. Carboxylesterase 1 (CES1), a major liver enzyme that functions in liver drug metabolism, was upregulated in the resistant cells by 86-fold and, thus, might be involved in regorafenib metabolism. Annexin A3 was previously shown to confer sorafenib resistance to HCC cells. ANXA1, a family member, was significantly upregulated in the resistant cells by almost sevenfold compared with controls. Thus, we tested if other members of the family could confer resistance. Long-chain-fatty-acid-CoA ligase 5 (ACSL5), a metabolic gene, is an enzyme that plays a crucial role in lipid biosynthesis and fatty acid degradation. CRISPR activation system was used to validate the RNA-seq results by generating a cell line expressing individual sgRNAs for each of the genes mentioned above. Increased expression at the mRNA level of all activated genes was confirmed (Figure 5B and Table 2). The obtained cell lines were treated with regorafenib for 72 h, and viability was determined (Figure 5C). As expected, all of the tested genes conferred increased resistance to regorafenib compared with an NT control indicating that additional metabolic genes are involved in mediating regorafenib resistance.
HK1 and ITGB5 are elevated in regorafenib-resistant cells. (A) Expression levels of the confirmed genes were tested by qPCR. HPRT1 was used as a housekeeping control. Results are mean ± SD from three independent experiments performed in triplicates. Student’s t test. **p < .01; ***p < .001. (B) Protein expression levels of HK1 and HK2 in regorafenib-resistant cells were determined by western blot using HK1- and HK2-specific antibodies. Tubulin was used as a housekeeping control.
regorafenib treatment in HCC cell lines that endogenously express different HK1 levels. To this end, we used cell lines from the two groups mentioned above and compared Huh7 cells with HLF and JHH6 cells. First, we tested HK1 expression at the mRNA and the protein levels (Figure 6A,B). As previously reported, Huh7 cells do not express HK1, whereas HLF and JHH6 cells express HK1. HK2 levels were similar between Huh7 and JHH6, whereas HLF cells expressed somewhat higher levels. Viability levels in response to regorafenib treatment showed a clear correlation between HK1 expression levels and the degree of resistance (Figure 6C,D). Huh7 cells were the most sensitive to regorafenib treatment, HLF showed an intermediate phenotype, whereas JHH6 cells were the most resistant.

To further test the effect of HK1 depletion on sensitivity to regorafenib, we knocked down HK1 expression in JHH6 cells, which express high levels of endogenous HK1. First, we tested the efficiency of various shRNAs in knocking down HK1 expression. We identified two shRNAs that efficiently downregulated HK1 expression (#6 and 8). These shRNAs were further used in our experiments (Figure 7A). Of note, these shRNAs were HK1-specific and did not affect HK2 protein levels (Figure 7B). JHH6 cells expressing HK1 shRNAs or control NT shRNAs were treated with increasing doses of regorafenib, and viability was determined 72 h following treatment. Viability was significantly reduced in the HK1 knock-down cell lines compared with control cells (Figure 7C). These results indicate that HK1 levels may have a significant role in mediating regorafenib resistance.

3.5 Modulation of HK1 levels does not affect regorafenib-induced apoptosis

HK1 was previously reported as a potent pro-survival factor that counters tumor necrosis factor α (TNF α)-induced apoptosis.34 Thus, HK1 might confer regorafenib resistance by inhibiting apoptosis. To test this possibility, we tested the effect of HK1 overexpression or knockdown on apoptosis in regorafenib-treated cells compared with controls. As a surrogate marker for apoptosis, we used poly (ADP-ribose) polymerase 1 (PARP1) cleavage. Staurosporine, a well-known inducer of apoptosis, served as a positive control.35

As indicated by cleaved PARP1 levels, the degree of apoptosis was similar in HK1 activated cells and NT controls (Figure 8A), suggesting that overexpression of HK1 did not confer resistance to regorafenib by inhibiting apoptosis. Furthermore, HK1 knockdown did not increase
apoptosis in regorafenib-treated cells, as indicated by the similar amounts of cleaved PARP1 in cell lines expressing HK1 shRNA compared to cells expressing NT shRNA controls (Figure 8B).

3.6 Lonidamine resensitizes regorafenib-resistant cells

Lonidamine is a glycolysis inhibitor that inhibits the activity of mitochondrially bound hexokinases.36 Our results indicate that Huh7 regorafenib-resistant cells overexpress HK1, HKDC1, and aldolase B, an additional glycolytic enzyme. Thus, we tested the ability of lonidamine to resensitize regorafenib-resistant cells. The effect of regorafenib and lonidamine were first tested on control Huh7 cells that are not resistant to regorafenib (Figure 9A). Regorafenib treatment resulted in substantial cell death in the control cells, reducing viability by 11-folds. Lonidamine was less efficient, with the lower dose showing a minor nonsignificant effect and the higher dose reducing viability by 3.9-folds. In the resistant cells, regorafenib treatment reduced viability by 2.1-fold. Lonidamine treatment alone showed similar levels

| Gene Symbol | log2FC | padj |
|-------------|--------|------|
| A4GALT      | 2.77   | 9.13E-48 |
| ACSL5       | 2.66   | 5.94E-59 |
| ADH4        | 5.73   | 1.99E-51 |
| AKR1B10     | 5.55   | 0.00E+00 |
| AKR1C3      | 3.41   | 9.83E-115 |
| ALDH1A2     | 3.07   | 9.59E-26 |
| ALDH3B1     | 2.00   | 2.61E-47 |
| ALDOB       | 2.29   | 1.52E-12 |
| B3GNT5      | 6.81   | 4.96E-158 |
| CD38        | 4.85   | 0.00E+00 |
| CDA         | 4.27   | 9.75E-111 |
| CES1        | 6.43   | 1.68E-229 |
| CMPK2       | 3.31   | 1.42E-37 |
| CPS1        | 3.26   | 2.41E-17 |
| CYP19A1     | 2.29   | 1.47E-17 |
| CYP8B1      | 4.27   | 4.45E-22 |
| ENPP3       | 2.80   | 2.39E-20 |
| ENPP7       | 2.15   | 1.45E-24 |
| GDA         | 2.41   | 6.86E-89 |
| GPAT3       | 2.60   | 8.73E-83 |
| HK1         | 2.26   | 2.13E-13 |
| HKDC1       | 2.16   | 7.27E-34 |
| HPD         | 3.23   | 4.53E-41 |
| HSD3B7      | 3.02   | 4.89E-24 |
| MAT1A       | 2.29   | 2.30E-59 |
| ME3         | 2.70   | 1.07E-13 |
| NNMT        | 3.11   | 1.20E-32 |
| PTGES       | 4.16   | 8.96E-29 |
| RDH16       | 3.70   | 9.85E-57 |
| TDO2        | 3.78   | 1.95E-30 |
| TUSC3       | 6.35   | 4.37E-09 |
| UGT1A7      | 4.21   | 5.11E-15 |
| UGT2A3      | 2.07   | 3.20E-21 |
| UGT2B7      | 2.89   | 2.31E-96 |
| ANXA1       | 4.86   | 1.78E-99 |

**FIGURE 5** Differentially overexpressed genes in regorafenib-resistant cells. (A) The table shows the metabolic and other genes overexpressed in the regorafenib resistant cells (log2 fold-change >2) and their adjusted p-values. Genes that were further confirmed are labeled in red. (B) Expression levels of the activated genes used for further validation as determined by qPCR using gene-specific primers, HPRT1 was used as a housekeeping control. Results are mean ± SD from three independent experiments performed in triplicates. Student’s t test *p < .05; **p < .01; ***p < .001. (C) Huh7 cell lines expressing the indicated sgRNAs were treated with 7 µM regorafenib for 72 h. Viability was tested using Cell-titer Glo. Results are mean ± SD from three independent experiments performed in triplicates. Student’s t test. ***p < .001, ****p < .0001.
of cell death (2.6-folds). At the same time, a combination of both drugs significantly reduced cell viability at all lonidamine concentrations used, up to 12-fold in the highest lonidamine concentration (Figure 9B). These results show that lonidamine, an HK inhibitor, resensitizes the resistant cells to regorafenib.
and its magnitude of induction may be associated with tumor growth rate.

4 | DISCUSSION

In this study, a CRISPR activation screen was used to identify genes that confer resistance to regorafenib upon overexpression in Huh7 hepatoma cells. HK1, catalyzing the first step in glycolysis, that is, converting glucose to glucose-6-phosphate, was a top hit in our screen. Interestingly, HK1 was one of the genes from the screen hits that was also upregulated in regorafenib-resistant Huh7 cells. Both, HK1-activated cells and regorafenib-treated cells showed increased lactate production, compatible with an increased rate of glycolysis in cancer cells. Using RNA-seq analysis, we found that a large group of metabolic genes was significantly upregulated in regorafenib-resistant Huh7 cells. Of note, HKDC1, another hexokinase, and aldolase B, an additional glycolytic enzyme, were among these significantly upregulated genes. Using our HK1 CRISPR activated cell line, shRNA mediated downregulation, and different liver cancer cell lines with varying HK1 expression levels, we show that regorafenib resistance correlated with HK1 expression levels. Furthermore, lonidamine, a hexokinase inhibitor, resensitized the resistant cells to regorafenib. A correlation between HK1 expression and resistance to TKI treatment was also suggested by in vivo experiments in PDX mice. Thus, our results indicate a crucial role for HK1 in regorafenib resistance.

Mechanistically, our results suggest that HK1 does not confer resistance by inhibiting apoptosis, one of its known functions. Therefore, further studies should explore the potential mechanism(s) for HK1-mediated resistance to TKI in hepatoma cells.

Under aerobic conditions, in healthy cells, glycolysis is inhibited, and glucose is catabolized by oxidative phosphorylation. In cancer cells, however, glucose is catabolized to lactate even in the presence of oxygen, a phenomenon known as the “Warburg effect” or aerobic glycolysis.

The fact that cancer cells use glycolysis as their primary energy source provided a therapeutically selective target, which led to increasing efforts to target glycolysis as an anticancer treatment for many cancer types.

Most liver cancer cells express both HK1 and HK2, with smaller subsets expressing only one of these isoforms. Because HK1 is ubiquitously expressed in many nontumor tissues, inhibition of HK2 activity was the focus of most therapeutic efforts for liver cancer. Interestingly, in a previous study, deletion of HK2 in HK1+HK2+ tumors demonstrated that tumors expressing only HK1 still
**Figure 9** Lonidamine resensitizes regorafenib-resistant cells. (A) Huh7 DMSO-treated control cells were treated with 10 µM regorafenib or increasing concentrations of lonidamine for 72 h. Viability was determined using Cell-titer Glo. (B) Regorafenib-resistant cells were treated with 10 µM regorafenib and increasing concentrations of lonidamine. Viability was determined as described above. Results are mean ± SD from two independent experiments performed in triplicates. Two-way ANOVA Dunnett’s multiple comparison test. n.s., not significant, **p = .002, ***p = .0003, ****p < .0001

**Figure 10** Sorafenib treatment in hepatocellular carcinoma patient-derived xenograft (PDX) mice. (A) Representative HK1 and HK2 staining results from 5 PDX mice treated with sorafenib (20 mg/kg) 5 days per week. (B) The tumor growth rate during sorafenib treatment.
progress, similar to HK1^{+}/HK2^{+} tumors, despite reduced glucose consumption. This observation suggests a pivotal role for HK1 in promoting tumorigenesis. Huh7 cells express only HK2 and not HK1, whereas our results show that HK1 activation in these cells promoted resistance.

Our results further show that regorafenib-resistant Huh7 cells show increased expression of two other glycolytic enzymes besides HK1, namely, HKDC1 and aldolase B. Of note, the expression of other intermediate glycolytic enzymes was not tested in this study. These results are generally in line with other studies linking sorafenib resistance to enhanced glycolysis.40–42

Interestingly, Gao et al.41 recently reported yellowing of the cell media in sorafenib-resistant Huh7 cells, indicating increased acidification caused by increased glycolysis and lactate excretion. Furthermore, this study showed an induction of HK2, but not HK1, in these cells at the RNA level. In our hands, however, despite the use of the same cell line and methodology, an induction in HK1, rather than HK2, was observed and our results showed a correlation between HK1 expression and resistance to regorafenib in different cell lines with various expression levels of HK1 strongly support our initial observation. Possible explanations for the difference between our study and Gao et al. might be the inherent differences in Huh7 cells used, originating from different laboratories and linages. However, the most likely explanation is that the drug-induced selection has led to a clonal expansion of different clones with different characteristics in the respective studies. Despite this fact, although different genes were elevated in resistant cells, the outcome of increased glycolysis as a potential driver of resistance remains similar, and its inhibition remains a valid strategy to resensitize resistant cells.

Liver tumors and their derived cell lines display high intra and intertumoral heterogeneity.31,32,44 Huh7, the hepatoma cell line used for our screen, belongs to a group of well-differentiated liver cancer cell lines. In agreement with our results, these well-differentiated cell lines responded better to regorafenib than poorly differentiated cell lines such as HLF and JHH6.32 In addition, these two different groups of cell lines seem to be metabolically distinct.31 Interestingly, depletion of HK1 using shRNA in JHH6 cells increased their sensitivity to regorafenib, further linking HK1 expression to TKI resistance.

Our results point to a correlation between HK1 expression levels and TKI resistance; however, the clinical relevance of these results to TKI resistance in patients and the underlying mechanisms should be further studied. Although the PDX experiments point to a correlation between HK1 and TKI resistance, these experiments are limited by the fact that they were performed on multiple mice transplanted with a tumor from a single origin. Furthermore, it is still unclear whether HK1 expression itself is sufficient to confer resistance or its elevated expression levels contribute to this phenotype. Of note, the majority of the liver tumor samples in the Cancer Genome Atlas were shown to express discernable levels of HK1 (Figure S3).

Our RNA-seq analysis highlighted at least three previously unknown drug targets that might further sensitize liver tumor cells to regorafenib. These genes were all significantly upregulated in the regorafenib-resistant cells, and their activation resulted in resistance to regorafenib treatment in naive cells. The first one is CES1, a major enzyme in liver drug metabolism that might also be involved in regorafenib metabolism.29 The second one is ANXA1, a family member of ANXA3, previously shown to confer sorafenib resistance in HCC cells by activating autophagy and inhibiting apoptosis.30 The third gene is HKDC1, a hexokinase resembling HK1 involved in liver metabolism.28 Future studies are needed to access the role of these genes in regorafenib resistance and to test the feasibility of their inhibition as a therapeutic target.

The fact that these genes were not detected in our CRISPR activation screen most likely stems from the differences between both systems. The CRISPR activation sgRNA library is produced using generalized bioinformatic rules regarding the distance from the transcription start site to provide maximal activation.13 However, differences between genes and the cell lines used for each screen might lead to varying activation levels of various genes. Thus, the two methods used are complementary, as genes not detected in the CRISPR activation screen could be detected by RNA-seq.

In summary, in this study, we identified HK1 as a critical gene promoting regorafenib resistance in hepatoma cells. Our results support a model in which metabolic changes underlie regorafenib resistance and identify additional potential resistance genes. We further demonstrated that lonidamine, a known HK inhibitor, can resensitize regorafenib-resistant cells to regorafenib, indicating that pharmacological inhibition of glycolysis might increase TKI efficiency. However, such inhibitors are yet to be approved. Moreover, HK1 is ubiquitously expressed and thus might not be a good target for clinical use in TKI-resistant patients. Therefore, inhibition of some of the other identified genes, once further validated, might prove useful. Alternatively, the expression levels of HK1 or one of the other targets might serve as valuable biomarkers predicting the outcome of TKI treatment, thereby preventing unnecessary morbidities and costs implicated in the treatment of inadequate candidates.

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DISCLOSURES
The authors have stated explicitly that there are no conflicts of interest in connection with this article.

AUTHOR CONTRIBUTIONS
Ella H. Sklan and Amir Shlomai designed research; Summer Sofer, Shiry Partouche, and Shir Armoza Eilat performed research; Kevin Lamkiewicz and Manja Marz analyzed data; Salomon M. Stemmer and Neta Moskovits contributed samples and assisted with their analysis; Ella H. Sklan and Amir Shlomai wrote the paper.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available in the methods and/or supplementary material of this article.

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