

Integrated multi-omics data analysis identifying novel drug sensitivity-associated molecular targets of hepatocellular carcinoma cells

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Abstract. Hepatocellular carcinoma (HCC) is the most common type of liver cancer and the third-leading cause of malignancy-associated mortality worldwide. HCC cells are highly resistant to chemotherapeutic agents. Therefore, there are currently only two US Food and Drug Administration-approved drugs available for the treatment of HCC. The objective of the present study was to analyze the results of previously published high-throughput drug screening, and *in vitro* genomic and transcriptomic data from HCC cell lines, and to integrate the obtained results to define the underlying molecular mechanisms of drug sensitivity and resistance in HCC cells. The results of treatment with 225 different small molecules on 14 different HCC cell lines were retrieved from the Genomics of Drug Sensitivity in Cancer database and analyzed. Cluster analysis using the treatment results determined that HCC cell lines consist of two groups, according to their drug response profiles. Continued analyses of these two groups with Gene Set Enrichment Analysis method revealed 6 treatment-sensitive molecular targets (epidermal growth factor receptor, mechanistic target of rapamycin, deoxyribonucleic acid-dependent protein kinase, the Aurora kinases, Bruton's tyrosine kinase and phosphoinositide 3-kinase; all $P < 0.05$) and partially effective drugs. Genetic and genome-wide gene expression data analyses of the determined targets and their known biological partners revealed 2 somatically mutated and 13 differentially expressed genes, which differed between drug-resistant and drug-sensitive HCC cells. Integration of the obtained data into a short molecular pathway revealed a drug treatment-sensitive signaling axis in HCC cells. In conclusion, the results of the present study provide novel drug sensitivity-associated

molecular targets for the development of novel personalized and targeted molecular therapies against HCC.

Introduction

Hepatocellular carcinoma (HCC) is the most common type of liver cancer globally (1). It is also the sixth most frequently diagnosed type of neoplasm and the third most common cause of cancer associated-mortality globally (2). The late diagnosis of HCC at advanced stages of the disease (3,4), the heterogeneous background of HCC cells (5) and high resistance of HCC cells to conventional chemotherapeutic agents (3,6,7) are considered to be the primary reasons for the high mortality rates observed in patients with HCC.

At present, two drugs (sorafenib and regorafenib) are approved by the US Food and Drug Administration (FDA) for treatment of HCC (8,9). Sorafenib and regorafenib are multi-kinase targeting drugs, exhibiting only a moderate effect on HCC cells (5,8). However, the initial approval of sorafenib by the FDA attracted attention to the development of novel targeted molecular therapies for the effective treatment of HCC (3,5,10). Thus far, the development of other systematic chemotherapeutic treatments for HCC has largely been unsuccessful (11,12). The primary reason for this failure is a lack of comprehensive knowledge on the underlying molecular mechanisms responsible for drug sensitivity and the resistance of HCC cells to chemotherapeutic drugs. Thus, understanding of the underlying molecular mechanisms of the high resistance of advanced stage HCC cells, or the partial sensitivity of early-stage HCC cells to these drugs, requires resolution prior to development of successful novel chemotherapeutic treatments for HCC.

Previous developments in systematic high-throughput drug screening and genomic and transcriptomic profiling studies on cancer cell lines and patient tumor samples have provided a number of publicly available processed and unprocessed datasets, which are accessible through online databases (13-15). Analysis of these datasets may reveal previously unidentified effective small molecules and molecular targets, which may aid the development of novel strategies for cancer treatment. Integration of these separate datasets also enhances understanding of these pathways and mechanisms by revealing novel biological associations via systems biology approaches.

The purpose of the present study was to analyze publicly available drug screening results, genetic and gene expression

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datasets of *in vitro* HCC cell lines and to integrate the obtained data to define molecular players of drug sensitivity and resistance in HCC cells. Systematic drug treatment results, genomic alteration data and transcriptomic differences of 14 different HCC cell lines were analyzed, and the obtained results were integrated into a biological network. These analyses revealed that there were two sub-groups of HCC cells, which each responded differently to drug treatments. The results also provided more comprehensive data regarding drug sensitivity- and resistance-associated molecular targets in HCC cells, enabling the development of effective chemotherapeutic strategies.

Materials and methods

Cell lines and drug treatment results. The Z-score values of 225 different small molecule treatments on 14 HCC cell lines, 7 epithelial-like and 7 mesenchymal-like cell lines (Table I), were downloaded from Genomics of Drug Sensitivity in Cancer (GDSC) database (<http://www.cancerrxgene.org/downloads>; date of access, July 2016) (14). Each normalized Z-score value of a drug indicates the sensitivity (near to -2) or resistance (near to +2) of HCC cell lines to applied drug treatment.

Cluster analyses. The results of drug treatments were used during cluster analyses. Cluster analyses were performed using an unsupervised hierarchical average linkage clustering method with Cluster software (version 3.0) (16). Obtained results were visualized using Java Tree View software (version 1.1) (17).

Drug sets and Gene Set Enrichment Analysis (GSEA) experiments. Data used in the cluster analyses were re-processed for GSEA studies. Data from 18 small molecule treatments that were missing values for $\geq 25\%$ of the samples (4 cell lines) were discarded to achieve true statistical results. The remaining 207 small molecule treatment datasets were utilized for GSEA studies. All small molecules used in the cluster analyses were grouped according to their known molecular targets to generate drug sets and run GSEA. A total of 33 drug sets, which include data concerning ≥ 3 small molecules targeting the same biological molecule were generated and utilized during GSEA experiments (Table II). Drug treatment responses of Group A and Group B cells, which were divided by cluster analysis, were compared using generated drug sets and GSEA desktop software (version 2.2.3) with the Diff_of_Classes metric ranking method (18). P-values and false discovery rate (FDR) values for each drug set were generated using the GSEA software.

Determination of small molecule treatment sensitivity-associated somatic mutations. Lists of genes that are associated with the determined molecular targets [epidermal growth factor receptor (EGFR), mechanistic target of rapamycin (mTOR), DNA-dependent protein kinase (DNA-PK), aurora kinases (AURK), Bruton's tyrosine kinase (BTK) and phosphoinositide 3-kinase (PI3K); Table III] in different cellular pathways were downloaded from the Molecular Signatures Database (MSigDB; version 6.1; <http://software.broadinstitute.org/gsea/msigdb>; date of access, December 2017) (19). A total of 553 unique genes were determined. Somatic mutations

Table I. HCC cell lines analyzed in the present study.

| Cell line number | Cell line name | HCC sub-type | (Refs.) |
|------------------|----------------|--------------|------------|
| 1 | HEP3B | E/W | (72-74) |
| 2 | HUH-7 | E/W | (72-75) |
| 3 | HUH-1 | E/W | (75) |
| 4 | HLE | E/U | (72,75,76) |
| 5 | JHH-4 | E/W | (74,75) |
| 6 | JHH-6 | E/W | (75) |
| 7 | JHH-7 | E/W | (75) |
| 8 | JHH-2 | M/P | (75) |
| 9 | SNU-475 | M/P | (73,74) |
| 10 | SNU-182 | M/P | (73,74) |
| 11 | SNU-398 | M/P | (73,74) |
| 12 | SNU-387 | M/P | (73,74) |
| 13 | SNU-423 | M/P | (73,74) |
| 14 | SNU-449 | M/P | (73,74) |

High-throughput drug screening z-score results of 14 HCC cell lines were retrieved from GDSC database. Sub-types of the analyzed HCC cell lines are listed based on the literature, and the Japanese Collection of Research Bioresources Cell Bank and American Type Culture Collection databanks. E, epithelial-like; W, well differentiated; U, undifferentiated; M, mesenchymal-like; P, poorly differentiated; HCC, hepatocellular carcinoma.

to these genes in 14 HCC cell lines were screened using the Catalogue of Somatic Mutations in Cancer (COSMIC) database (versions 77 and 78; <http://cancer.sanger.ac.uk/cosmic>; date of access, September 2016) (15). Somatic mutation data for selected genes that were mutated exclusively in $>50\%$ of a group are presented in Table IV.

Gene expression values of HCC cell lines. Whole transcriptome datasets from 14 HCC cell lines, which were generated by the Cancer Cell Line Encyclopedia (20) using Affymetrix Human Genome U133 Plus 2.0 gene chip arrays, were downloaded from Gene Expression Omnibus database (GSE36133 data series; <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE36133>) (13). Raw data were normalized using BRB Array Tools software (version 4.5.1) using a Robust Multi-Array Average quantile normalization method (21). Gene expression values of the aforementioned 553 genes, which were analyzed using the COSMIC database (15), were determined. Genes that exhibited ≥ 1.5 -fold and statistically significant ($P < 0.05$) differential expression between Group A and Group B HCC cells were determined using the class comparison tool of the BRB Array Tools software, with default parameters (Table V).

Integrated pathway analysis. Pathway visualization analyses were performed using PathVisio Software (version 3.2.3) (22). Known molecular interactions of the differentially expressed and mutant genes were retrieved from Wikipathways (23), Consensus Path (24) and Kyoto Encyclopedia of genes and genomes databases (date of access, December 2016) (25); and integrated using PathVisio software. Visualization of the

Table II. List of drug sets.

| Drug set name/molecular targets | Size | Small molecules of drug sets |
|---------------------------------|------|---|
| PI3K | 10 | AS605240, AZD6482_1, AZD6482_2, BEZ235, CAL-101, GDC0941, GSK2126458, PI-103, PIK-93, ZSTK474 |
| HDAC | 9 | AR-42, Belinostat, CAY10603, CUDC-101, JQ12, LAQ824, Tubastatin_A, VNLG/124, Vorinostat |
| EGFR | 7 | Afatinib_1, Afatinib_2, Cetuximab, CUDC-101, EKB-569, Gefitinib, OSI-930 |
| KIT | 7 | AMG-706, Axitinib, Masitinib, Midostaurin, OSI-930, Pazopanib, XL-184 |
| CDK9 | 6 | AT-7519, JNK-9L, KIN001-270, NG-25, THZ-2-49, TL-1-85 |
| MEK1-2-5 | 6 | BIX02189, PD-0325901, RDEA119_1, Selumetinib_1, Selumetinib_2, Trametinib |
| VEGFR | 6 | AMG-706, Axitinib, OSI-930, Pazopanib, Tivozanib, XL-184 |
| JAK1-2-3 | 5 | CEP-701, KIN001-055, QL-X-138, Ruxolitinib, TG101348 |
| PARP1-2 | 5 | AG-014699, Olaparib_1, Olaparib_2, Talazoparib, Veliparib |
| PDGFR | 5 | AMG-706, Axitinib, MP470, OSI-930, Pazopanib |
| AKT | 4 | AKT_inhibitor_VIII, GSK690693, KIN001-102, MK-2206 |
| BRAF | 4 | Dabrafenib, PLX4720_1, PLX4720_2, SB590885 |
| BRD2-3-4 | 4 | I-BET-762, JQ1_1, JQ1_2, PFI-1 |
| CDK1-4-6-7-pan | 4 | PD-0332991, PHA-793887, RO-3306, THZ-2-102-1 |
| FLT1-3-4 | 4 | AC220, CEP-701, WZ3105, XL-184 |
| HSP70-90 | 4 | 17-AAG, AUY922, Elesclomol, SNX-2112 |
| IGF1R | 4 | BMS-536924, BMS-754807, GSK1904529A, Lisitinib |
| IKK | 4 | BMS345541, BX-795, KIN001-260, TPCA-1 |
| Microtubules | 4 | Docetaxel, Epothilone_B, Vinblastine, Vinorelbine |
| ALK | 3 | CH5424802, SB505124, SB52334 |
| AURK | 3 | BX-795, Genentech_Cpd_10, GSK1070916 |
| BCL2-XL-W | 3 | Navitoclax, Obatoclax_Mesylyate, TW-37 |
| BTK | 3 | LFM-A13, QL-X-138, QL-XII-47 |
| DNA-PK | 3 | NU-7441, PI-103, QL-X-138 |
| ERBB2 | 3 | Afatinib_1, Afatinib_2, CP724714 |
| JNK | 3 | AS601245, JNK-9L, JNK_Inhibitor_VIII |
| MDM2 | 3 | JNJ-26854165, NSC-207895, Nutlin-3a (-) |
| mTOR | 3 | GSK2126458, QL-X-138, Temsirolimus |
| mTORC1-2 | 3 | AZD8055, BEZ235, OSI-027 |
| PDK1 | 3 | BX-912, KIN001-244, OSU-03012 |
| RET | 3 | AMG-706, CEP-701, XL-184 |
| ROCK1-2 | 3 | GSK269962A, GSK429286A, Y-39983 |
| TOP1-2 | 3 | Camptothecin, Etoposide, SN-38 |

A total of 33 drug sets, which include ≥ 3 different small molecule treatments that have the same biological target. Size refers to the total number of small molecule treatment results in the drug set.

differentially expressed genes was performed based on the microarray gene expression results.

Results

HCC cell lines consist of two groups according to their drug response profiles. To identify drug treatment response characteristics of HCC cell lines and determine effective drugs and molecular targets for the treatment of HCC, small molecule treatment Z-score values of 14 HCC cell lines were downloaded

from the GDSC database (14). Results of 225 treatments on 14 HCC cell lines were analyzed using the aforementioned clustering method to determine the global treatment response profiles of HCC cells. Unsupervised hierarchical clustering analysis revealed two main groups of HCC cell lines according to their sensitivity to drug treatments (Fig. 1). The first group of HCC cells, Group A cells, was comprised of 7 epithelial-like HCC cell lines; whereas the second group, Group B cells, was comprised of 7 mesenchymal-like HCC cells (Table I). This result indicates that although all cells analyzed were

Table III. List of significantly enriched drug sets.

| Rank | Name | Size | ES | NOM P-value | FDR q-value |
|------|--------|------|-------|-------------|-------------|
| 1 | EGFR | 7 | 0.869 | 0.007 | 0.151 |
| 2 | mTOR | 3 | 0.957 | 0.013 | 0.118 |
| 3 | DNA-PK | 3 | 0.957 | 0.016 | 0.136 |
| 4 | AURK | 3 | 0.952 | 0.016 | 0.164 |
| 5 | BTK | 3 | 0.902 | 0.039 | 0.173 |
| 6 | PI3K | 10 | 0.829 | 0.046 | 0.218 |

Among all 33 drug sets, 6 (18%) were statistically significantly ($P < 0.05$ and $FDR < 0.25$) enriched in Group A HCC cells. Drug sets were ranked according to their P-value scores. ES, enrichment score; NOM, nominal; FDR, false discovery rate; EGFR, epidermal growth factor receptor; mTOR, mechanistic target of rapamycin; DNA-PK, deoxyribonucleic acid-dependent protein kinase; AURK, aurora kinases; BTK, bruton tyrosine kinase; PI3K, phosphoinositide 3-kinase; HCC, hepatocellular carcinoma.

HCC cells, epithelial-like and mesenchymal-like HCC cells responded differently to the same drug treatments.

Effective molecular targets of drug-sensitive Group A epithelial-like HCC cells were identified. To determine the list of molecular targets, which are associated with the treatment response characteristics of Group A and Group B cells, GSEA studies were performed. Since GSEA compares two sample groups and determines statistically significantly enriched sets in each group, 33 drug sets that included ≥ 3 different small molecules with the same biological target were generated using the data downloaded from GDSC (Table II). GSEA results revealed that 28/33 drug sets (85%) were enriched (effective) on Group A cells, and that 6 of them (18%) were significantly enriched ($P < 0.05$; Table III); whereas the remaining 5 drug sets (15%) were enriched in Group B cells; however, none of them were statistically significant. The significantly drug-sensitive molecular targets in Group A HCC cells were; EGFR, mTOR, DNA-PK, AURK, BTK and PI3K (Table III). Therefore, GSEA results identified molecular targets and drugs associated with drug sensitivity in epithelial-like Group A HCC cells.

Somatic mutations associated with treatment response profiles of Group A and Group B HCC cells were determined. To identify genetic factors that were possibly associated with drug sensitivity in Group A cells and drug resistance of Group B cells, genetic variation data (somatic mutations, fusions, breakpoints) of all genes biologically function with the determined 6 molecular targets were analyzed. First, a list of all biologically functioning genes with 6 determined molecular targets was retrieved from MSigDB. A total of 553 unique genes, which directly or indirectly interact with the determined molecular target in at least one signaling pathway, were identified. Genetic variation data of all 553 genes in 14 HCC cell lines were assessed using the COSMIC database. The two genes that were exclusively mutated in $>50\%$ of a group were considered to be potentially associated with the drug response profile of HCC cell lines and groups (Table IV). The inositol

1,4,5-trisphosphate receptor type 2 (ITPR2) gene exhibited 5 distinct mutations in 4 Group A HCC cell lines (HUH-7, JHH-6, JHH-7 and HEP3B); whereas phosphoinositide-3-kinase regulatory subunit 4 (PIK3R4) gene exhibited 4 distinct mutations in 4 Group B HCC cell lines (JHH-2, SNU-182, SNU-387, SNU-423; Table IV). As a result, 553 genes that function with the 6 determined molecular targets were identified; and 2 genes with somatic mutations possibly associated with small molecule treatment responses of Group A and Group B HCC cells were identified.

Molecular targets and highly differentially expressed genes were determined. To identify differentially expressed genes that had potential functions in sensitivity and resistance responses of Group A and Group B HCC cells, respectively, when the determined 6 molecules were targeted, whole genome transcriptomics data of the same 14 HCC cell lines were downloaded and analyzed. Gene expression profiles of the aforementioned 553 genes were determined for Group A and Group B HCC cells, and 13 genes that exhibited ≥ 1.5 -fold and statistically significant ($P < 0.05$) differential expression between the two groups were selected (Table V). Among the 13 selected genes, 6 genes were upregulated in Group A HCC cells, and 7 genes were upregulated in Group B HCC cells (Table V). In conclusion, gene expression profiles of untreated Group A and Group B HCC cells were determined and 13 differentially expressed genes associated with previously defined molecular targets were identified.

Integrated multi-omics results of Group A HCC cells revealed a drug-sensitive molecular network of HCC. To identify a simple molecular interaction network of drug-sensitive molecular targets for Group A HCC cells, the determined pharmacogenomics and transcriptomic results were integrated into one molecular pathway. Enriched drug targets and drugs (Table III), mutant genes (Table IV) and differentially expressed genes (Table V) results for drug-sensitive Group A HCC cell lines were integrated into one pathway and visualized based on their known interactions in pathway databases (23-25) (Fig. 2). The integration of multi-omics data of Group A HCC cells revealed the molecular network of drug-sensitive HCC cells (Fig. 2).

Discussion

The FDA approval of the multi-kinase inhibitor sorafenib for the treatment of HCC led to an acceleration in the search for effective molecular targets and molecularly targeted chemotherapeutic drugs against HCC. Thus far, only one additional drug, regorafenib, has been approved for HCC treatment by the FDA. The complex and heterogeneous characteristics of HCC cells are considered to be among the main obstacles to identification of effective drugs for use against HCC (26). The present study determined that there were two main subgroups of HCC cells in terms of drug treatment response profiles (Fig. 1). The first group (Group A) consisted of epithelial-like HCC cells, and the second group (Group B) consisted of mesenchymal-like HCC cells (Fig. 1; Table I). Mesenchymal-like HCC cells emerge following epithelial-to-mesenchymal transition, and their presence corresponds to an advanced stage of HCC (27).

Table IV. Somatic mutations, possibly associated with drug response profiles of hepatocellular carcinoma cell lines and Groups.

| Gene | Mutant cell lines, n | Cell line | Group | AA mutation | Transcript ID | CDS mutation | Zygoty | Val. | Mut. type |
|--------|----------------------|-----------|-------|-----------------|-----------------|------------------|--------|------|-----------|
| ITPR2 | 4 | HUH-7 | A | p.L1859L | ENST00000381340 | c.5577A>G | Het. | U. | S.-C. |
| | | JHH-6 | A | p.T969I | ENST00000381340 | c.2906C>T | Het. | V. | S.-M. |
| | | JHH-7 | A | p.E1614_M1621>V | ENST00000381340 | c.4841_4861del21 | Het. | V. | Comp. |
| | | HEP3B | A | p.T728N | ENST00000381340 | c.2183C>A | Het. | U. | S.-M. |
| | | HEP3B | A | p.V1508I | ENST00000381340 | c.4522G>A | Het. | U. | S.-M. |
| PIK3R4 | 4 | JHH-2 | B | p.D473V | ENST00000356763 | c.1418A>T | Het. | V. | S.-M. |
| | | SNU-182 | B | p.R1033S | ENST00000356763 | c.3099A>T | Het. | V. | S.-M. |
| | | SNU-387 | B | p.R495R | ENST00000356763 | c.1483A>C | Het. | U. | S.-C. |
| | | SNU-423 | B | p.V345F | ENST00000356763 | c.1033G>T | Het. | V. | S.-M. |

AA, amino acid; CDS, coding sequence; Val., validation status; Mut, mutation; Het, heterozygous; U, unvalidated; V, validated; S.- M, substitution-missense; S.- C, substitution-coding silent; Comp, complex-deletion inframe; ITPR2, inositol 1,4,5-trisphosphate receptor type 2; PIK3R4, phosphoinositide-3-kinase regulatory subunit 4.

Table V. List of 13 differentially expressed genes between Group A and Group B HCC cells.

| Rank | ProbeSet | Gene symbol | Group A | Group B | Fold change | P-value | FDR |
|------|--------------|-------------|---------|---------|-------------|---------|------|
| 1 | 226213_at | ERBB3 | 9.39 | 5.54 | 3.85 | 0.002 | 0.12 |
| 2 | 228912_at | VIL1 | 7.33 | 4.15 | 3.19 | 0.015 | 0.22 |
| 3 | 228716_at | THRB | 8.79 | 6.77 | 2.03 | 0.001 | 0.07 |
| 4 | 232530_at | PLD1 | 7.90 | 6.07 | 1.83 | 0.001 | 0.11 |
| 5 | 238441_at | PRKAA2 | 7.84 | 6.13 | 1.72 | 0.001 | 0.07 |
| 6 | 202609_at | EPS8 | 11.37 | 9.83 | 1.54 | 0.014 | 0.22 |
| 7 | 38037_at | HBEGF | 6.00 | 7.60 | -1.61 | 0.005 | 0.14 |
| 8 | 219383_at | PRR5L | 5.04 | 6.69 | -1.65 | 0.009 | 0.18 |
| 9 | 202742_s_at | PRKACB | 7.67 | 9.38 | -1.71 | 0.001 | 0.10 |
| 10 | 203085_s_at | TGFB1 | 7.97 | 9.75 | -1.78 | 0.014 | 0.22 |
| 11 | 212912_at | RPS6KA2 | 3.68 | 6.08 | -2.40 | 0.006 | 0.14 |
| 12 | 1556499_s_at | COL1A1 | 7.10 | 11.85 | -4.76 | 0.006 | 0.14 |
| 13 | 201842_s_at | EFEMP1 | 6.14 | 10.94 | -4.81 | 0.005 | 0.14 |

A total of 13/553 genes were ≥ 1.5 -fold differentially expressed between two groups. Among 13 significantly ($P < 0.05$ and $FDR < 0.25$) differentially expressed genes, six of them were upregulated in Group A cells, while seven genes were upregulated in Group B cells. Genes were ranked according to their fold-change values. FDR, false discovery rate; HCC, hepatocellular carcinoma.

In contrast, the epithelial-like HCC cells retain the original hepatocyte epithelial morphology. These two groups of HCC cells exhibited distinct sensitivities to identical drug treatments. There was no drug that was effective on all HCC cell types among the 225 small molecules analyzed.

A comparison of drug treatment responses of the two groups via GSEA revealed the molecular targets sensitive to drug treatment in the two groups of HCC cells (Table III). GSEA results demonstrated that Group A cells, which are comprised of early-stage HCC cells, are more sensitive than Group B cells, which are comprised of advanced-stage HCC cells, to small molecule treatments that target six molecules (Table III). Thus, targeting these molecules with the analyzed drugs cannot yield successful results in advanced-stage

HCC cells, but it may be a useful strategy for the treatment of early-stage HCC cells. Since the majority of the identified treatment-sensitive molecular targets in the present study have been studied previously as potential treatment targets for HCC and other types of cancer (10,11,28), it is known that they are ineffective targets in HCC cells, although drugs targeting these molecules are effective in other types of cancer, (5,26). In addition, the results of the present study indicated that early-stage HCC cells are more sensitive to drug treatments than advanced-stage HCC cells. Thus, the identification of altered molecular mechanisms and novel molecules responsible for the observed differences between the two groups of HCC cells is required to improve treatment outcomes. The results of GSEA in the present study provide valuable information to further

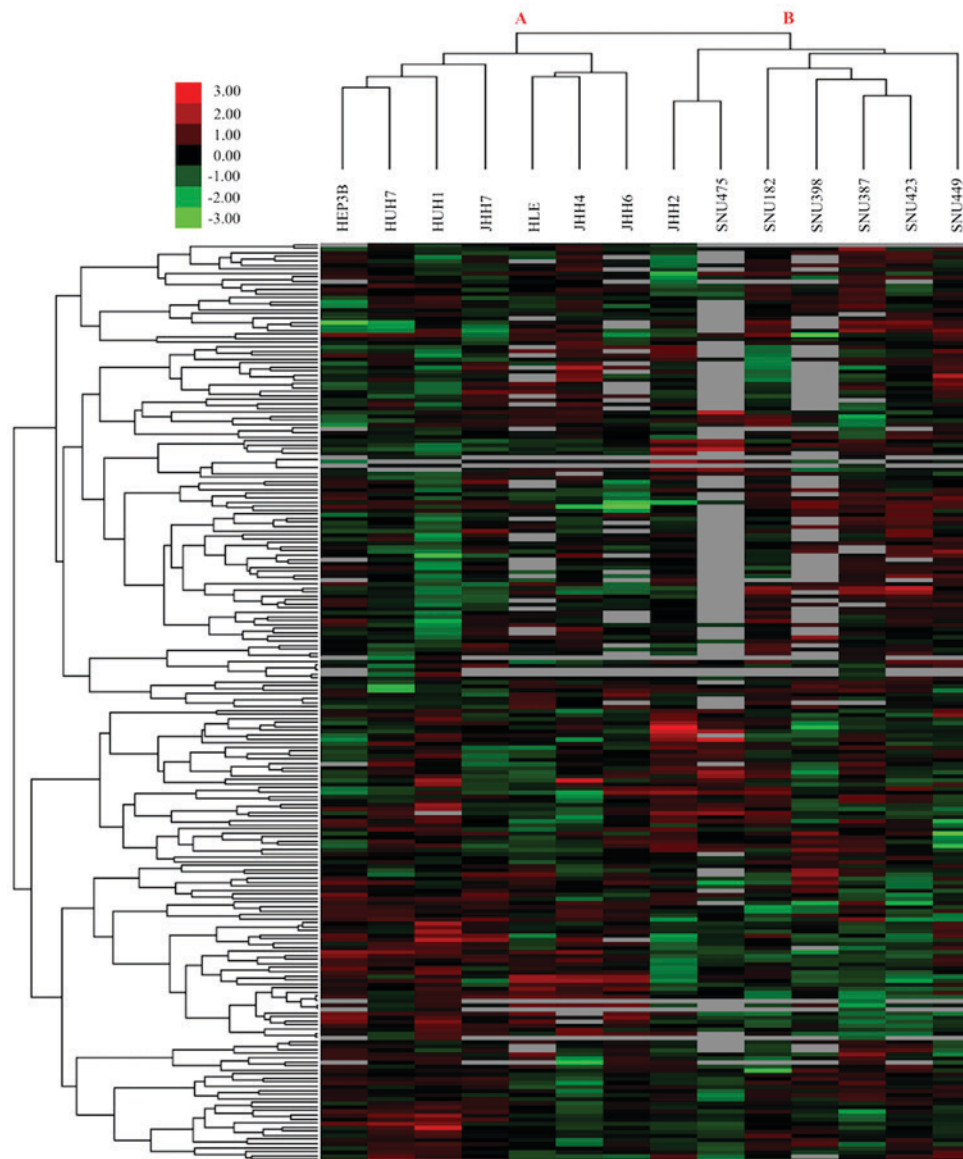


Figure 1. Results of cluster analysis for 225 drug treatments in 14 HCC cell lines. A heatmap was generated using drug treatment Z-score values of HCC cell lines. Unsupervised clustering of small molecules and samples resulted in two main sample clusters, Group A and Group B. Each group comprises 7 cell lines. Red, resistant; green, sensitive; grey, missing value; HCC, hepatocellular carcinoma.

analyze and understand the underlying molecular mechanisms of drug sensitivity and resistance in HCC cells. Therefore, molecules that cooperate with the defined molecular targets and genes that are somatically mutated (Table IV) and differentially expressed were determined (Table V).

The PI3K/RAC serine/threonine-protein kinase (AKT)/mTOR signaling pathway serves a function in cell growth, proliferation, angiogenesis, metabolism and mechanisms of anti-apoptosis in hepatocytes (29-31). The data in the present study revealed that mTOR and PI3K are effective molecular targets for the treatment of epithelial-like (early) HCC cells, but not for mesenchymal-like (advanced) HCC cells. PI3K may be activated following the activation of certain receptors, including the insulin receptor and EGFR, which is another target molecule identified in the present study (32,33). Active PI3K catalyzes the generation of phosphatidylinositol (3,4,5)-trisphosphate (PIP3) from phosphatidylinositol (4,5)-bisphosphate (PIP2) and

causes the activation of AKT (34-36). Activated AKT phosphorylates and activates several molecules, including mTOR (29,35). Active mTOR increases cell proliferation, survival and angiogenesis as a component of mTOR complex 1 (mTORC1) and mTORC2 (36,37). This signaling pathway is negatively regulated by phosphatase and tensin homolog (35,38). The PI3K/AKT/mTOR pathway is activated in 15-41% of HCCs, and inhibitors of this signaling pathway exhibited anti-neoplastic activities in experimental HCC models (11). The results of the present study identified that the EGFR/PI3K/AKT/mTOR signaling pathway serves a central function in the regulation of drug sensitivity and resistance in HCC cells (Fig. 2; Table III).

BTK is a non-receptor intracellular kinase that is mainly expressed in B-lymphocytes. BTK functions via B-cell receptor (BCR) signaling and the PI3K/AKT pathway. Antigen-bound BCRs bind to Lck/Yes novel kinase and spleen tyrosine kinase, and phosphorylate and activate PI3K, which converts PIP2 to

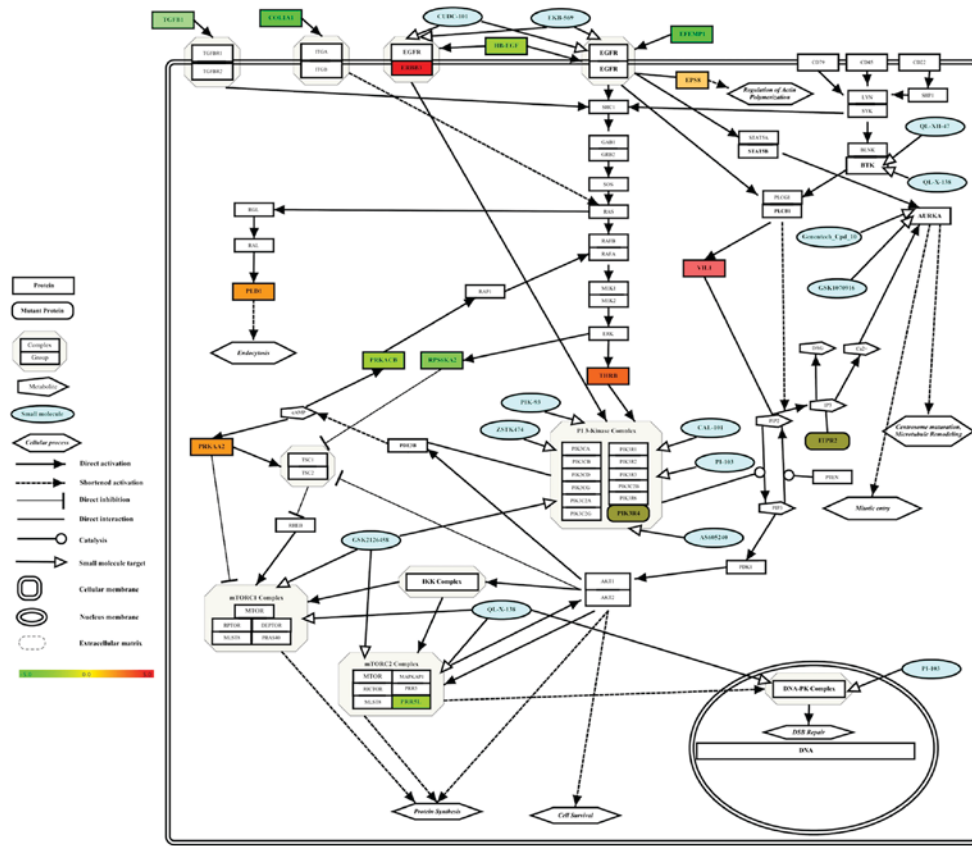


Figure 2. Integrated molecular pathway of small molecule sensitivity in Group A HCC cell lines. Effective drugs (in blue color), enriched drug targets (Table III), target-associated somatically mutant genes (Table IV) and differentially expressed genes (Table V) in HCC cell lines were integrated into one pathway and visualized. Gene expression levels are depicted in a color gradient between -5 (downregulated, color) and +5 (upregulated, red) by the software, as indicated by the color bar. Gene Symbols were retrieved from Human Genome Organisation Gene Nomenclature Committee (71) using the software. PIP3, phosphatidylinositol (3,4,5)-trisphosphate; PIP2, phosphatidylinositol (4,5)-bisphosphate; IP3, inositol trisphosphate; DAG, diacylglycerol; cAMP, cyclic adenosine monophosphate; ER, endoplasmic reticulum; HCC, hepatocellular carcinoma.

PIP3, to which BTK and AKT proteins bind. Numerous BTK inhibitors have exhibited promising therapeutic activities in hematological malignancies; however, further studies are required to identify the roles of BTK in HCC (39).

Aurora kinases (AURKA, AURKB and AURKC) are serine/threonine kinases that control cell division. These kinases serve a pivotal function during the mitotic phase of the cell cycle and are targeted by small molecule inhibitors. AURKC is expressed in the testes, whereas AURKA and AURKB may serve functions in different sub-cellular compartments. However, all three Aurora proteins perform crucial functions during chromosomal arrangement and the control processes of mitotic spindle apparatus formation (40). In addition, all three proteins are overexpressed in numerous types of cancer (41). In cancer cells, including HCC, Aurora kinases inhibit apoptosis and promote cellular proliferation and metastasis (42). AURKA is directly associated with the EGFR/PI3K/Akt/mTOR pathway, since active EGFR signaling is able to upregulate expression of AURKA through the FR/PI3K/Akt/mTOR signaling axis (43).

DNA-PK is a multi-protein complex that is primarily comprised of Ku70 [encoded by X-ray repair cross complementing 6 (XRCC6)], Ku80 (encoded by XRCC5) and the catalytic subunit DNA-PK catalytic subunit (DNA-PKcs), which is encoded by protein kinase DNA-activated catalytic

polypeptide (44). DNA-PKcs may be activated following DNA damage (44-46); it serves a pivotal function in the non-homologous end-joining mechanism of DNA double-strand break repair (47,48). It has been identified that DNA-PK serves a function in the initiation and progression of cancer, and the therapeutic resistance of multiple types of cancer, including HCC (49-52). DNA-PK directly interacts with AKT and increases its activity (53,54). Therefore, DNA-PK may function independently as a member of the PI3K-AKT-mTOR axis. In conclusion, all 6 drug sensitivity-associated targets identified via GSEA study are biologically functional together in the EGFR-PI3K-mTOR-DNAPK signaling axis, and targeting this axis renders early-stage Group A HCC cells sensitive to drug treatments, but not advanced-stage Group B HCC cells.

Following identification of the central signaling axis, known genomic variants of the same 14 HCC cells associated with the drug response profiles of Group A and Group B cells were analyzed. Following analysis of genomic variation using the COSMIC database, somatic mutations to 2 genes were determined as possibly biologically associated, since ITPR2 and PIK3R4 genes are exclusively mutated in Group A and Group B cells, respectively (Table IV).

The ITPR2 gene, which encodes a receptor of IP3, is mutated in 4 Group A cell lines (HUH-7, JHH-6, JHH-7 and HEP3B; P=0.039; Table IV). Upregulated ITPR2

expression is a biomarker of poor prognosis in patients with acute myeloid leukemia (55). ITPR2 was also identified as a susceptibility gene for Kashin-Beck disease (56). ITPR2 may cause an increase of cytoplasmic calcium following binding to IP3 (57); it also serves functions in oncogene-induced senescence and replicative senescence by regulating calcium levels, and the loss of ITPR2 causes escape from cellular senescence (57). Since cellular senescence is a mechanism of hepatocellular carcinogenesis (58), the effect of the identified ITPR2 mutation on HCC cells should be further investigated.

PIK3R4, which encodes the serine/threonine-protein kinase VPS15, exhibited 4 distinct mutations in 4 Group B HCC cell lines (JHH-2, SNU-182, SNU-387, SNU-423; $P=0.039$; Table IV). PIK3R4 functions with protein kinase AMP-activated catalytic subunit $\alpha 2$ (PRKAA2; one of the genes upregulated in Group A HCC cells) in the autophagy pathway (25), indicating that it may serve a role in the drug sensitivity of Group A HCC cells. PIK3R4 is also a member of the PI3K complex, and serves a function in autophagy as a member of the class III PI3K complex (59). The over-expression of the PIK3R4 gene in patients with chronic lymphocytic leukemia (CML) has been associated with the prognosis of CML (60). This gene has also been identified to be mutated in certain thymic epithelial tumors and metastatic melanoma samples (61,62). Copy number aberrations of PIK3R4 have been associated with decreased survival rates of patients with ovarian cancer (63). The statistically significant presence of mutated PIK3R4 in the treatment-resistant Group B cells indicates the possible function served by PIK3R4 mutations in the drug response profiles of HCC cells.

Transcriptomics analyses on HCC cells revealed that six genes are significantly upregulated and seven genes are downregulated in Group A cells (Table V). Roles of the transcriptionally deregulated genes in drug sensitivity of Group A cells require further study. Supporting this hypothesis, the activation of PRKAA2 protein sensitizes HCC cells to a number of drug treatments (64-67). Thus, the upregulation of PRKAA2 in drug-sensitive HCC cells may serve a central function in the observed response to treatment. For the other genes differentially upregulated in Group A HCC cells, there is a limited amount of available data concerning the effects of their transcriptional upregulation in HCC. Upregulated expression of villin 1 was identified as a predictive factor for the recurrence of high serum α -fetoprotein-associated HCC following hepatectomy (68). Erb-B2 receptor tyrosine kinase 3 (ERBB3) mRNA was upregulated in 52% of HCC tumors (69), and secreted ERBB3 isoforms were identified as serum markers for early hepatoma in patients with chronic hepatitis and cirrhosis (70). Therefore, the roles of these genes in drug sensitivity and resistance mechanisms of HCC cells should be studied further.

Since all the treatment-sensitive molecular targets identified possess biological functions, and the mutated and differentially expressed genes are also associated with these molecules, the results of multi-omics data analysis were integrated into a simple molecular interaction network to analyze the treatment sensitivity-associated molecular mechanisms of HCC cells better (Fig. 2). This shortened, drug treatment sensitivity-associated molecular network of HCC cells can be used to generate novel hypotheses for further experimentation

to reveal the underlying molecular mechanisms of drug sensitivity and resistance in HCC cells. For example, since the integrated molecular network identifies the molecular pathway of Group A HCC cells (Fig. 2), which are sensitive to treatments with drugs depicted in green, it may be of interest to examine whether activation of the EGFR-PI3K-mTOR-DNAPK axis via external or constitutively active internal signals in Group B HCC cells renders them sensitive to the same drugs. This approach may provide valuable information, enabling the development of novel combined drug treatment approaches against advanced-stage HCC cells.

In conclusion, the results of high-throughput drug treatment experiments on HCC cells analyzed in the present study indicate that molecular targeted, personalized chemotherapeutic approaches should be developed for the treatment of HCC, since distinct HCC cell types respond differently to the same drug treatments. Novel molecular targets and their biological associations identified in the present study should be further investigated to develop targeted molecular drug therapies against HCC.

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Availability of data and materials

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Authors' contributions

GY analyzed the data and wrote the manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Conflict of interest

The author declares that there are no competing interests.

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