A Gene Expression Signature Associated With Overall Survival in Patients

With Hepatocellular Carcinoma Suggests a New Treatment Strategy

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HCC, hepatocellular carcinoma; HBV, hepatitis B virus OS, HCV, hepatitis c virus; overall survival; MDR, multidrug resistance; PEI, percutaneous ethanol injection; RFA, radiofrequency ablation; TACE, transcatheter arterial chemoembolization; TLDA, TaqMan low density array
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ABSTRACT

Despite improvements in the management of liver cancer, the survival rate for individuals with hepatocellular carcinoma (HCC) remains dismal. The survival benefit of systemic chemotherapy for the treatment of liver cancer is only marginal. Although the reasons for treatment failure are multifactorial, intrinsic resistance to chemotherapy plays a primary role. Here, we analyzed the expression of 377 multidrug resistance-associated genes in two independent cohorts of patients with advanced hepatocellular carcinoma, with the aim of finding ways to improve survival in this poor-prognosis cancer. Taqman-based qPCR revealed a 45-gene signature that predicts overall survival (OS) in patients with HCC. Using the Connectivity Map Tool, we were able to identify drugs that converted the gene expression profiles of HCC cell lines from ones matching patients with poor OS to profiles associated with good OS. We found three compounds that convert the gene expression profiles of three HCC cell lines to gene expression profiles associated with good OS. These compounds increase histone acetylation, which correlates with the synergistic sensitization of those multidrug-resistant tumor cells to conventional chemotherapeutic agents including cisplatin, sorafenib and 5-fluorouracil. Our results indicate that it is possible to modulate gene expression profiles in HCC cell lines to those associated with better outcome. This approach also increases sensitization of HCC cells towards conventional chemotherapeutic agents. This work suggests new treatment strategies for a disease for which few therapeutic options exist.
Introduction

Liver cancer is the third most common cancer in the world, causing approximately 745,000 deaths per year (Ferlay et al., 2015). Hepatocellular carcinoma (HCC) is by far the most prevalent type, accounting for approximately 80 to 85% of primary liver cancer cases (Singal and El-Serag, 2015), whereas cholangiocarcinoma (Ghouri et al., 2015) and fibrolamellar carcinoma (Cornella et al., 2015; Lim et al., 2014) occur only at a frequency of ~14% and ~1%, respectively. The epidemiology of HCC is well known, and in the vast majority of cases, it arises as a consequence of underlying liver disease, usually a viral hepatitis (Singal and El-Serag, 2015). In the case of hepatitis B, integration of the viral DNA into the hepatocyte genome results in loss of chromosomal stability, deregulation of tumor suppressor genes and activation of proto-oncogenes, eventually leading to the development of HCC (Su et al., 2014).

Patients with early stage tumors undergo either surgical resection or liver transplantation if their HCC meets the so-called Milan criteria (Mazzaferro et al., 1996; Waller et al., 2015). When the patients are not suitable for surgery, local ablation including radiofrequency ablation (RFA) and percutaneous ethanol injection (PEI) is standard treatment. TACE, transcatheter arterial chemoembolization, is recommended for patients with intermediate stage HCC (EASL-EORTC, 2012; Villanueva et al., 2013). With the exception of sorafenib, a multi-tyrosine kinase inhibitor for which a survival benefit of three months was demonstrated, no effective systemic therapy exists for patients with advanced HCC (Llovet et al., 2008; Llovet et al., 2015). Although sorafenib is now established as the first line of therapy for advanced HCC, it was shown to be a substrate of ABCB1 and ABCG2, two major ABC transporters involved in multidrug resistance (MDR) and expressed in hepatocytes and hepatomas (Lagas et al., 2010; Tang et al., 2013). Comprehensive molecular profiling contributed to substantial improvement in our knowledge of
the biology of liver cancer and provide a road map to facilitate the development of targeted therapies (Andersen and Thorgerisson, 2012; Bruix et al., 2015; Pinyol et al., 2014; Simon et al., 2015). Beside proof-of-concept trial testing signaling pathway inhibitors or biomarker-based trial enrichment for defining cancer subpopulations, there are still a need for unspecific drugs that target all patients (Llovet and Hernandez-Gea, 2014).

Although the reasons for treatment failure are multifactorial, intrinsic resistance to chemotherapy plays a primary role. Here, the expression of multidrug resistance-associated genes were analyzed in two independent cohorts of patients with advanced hepatocellular carcinoma. We hypothesized that the Connectivity Map Tool might reveal compounds that reverse gene expression profile of cancers from patients with poor prognosis to that of cancers from patients who respond well to treatment (Lamb, 2007; Lamb et al., 2006; Zhang and Gant, 2008). The ultimate aim is to find a new strategy to sensitize intrinsically multidrug-resistant cancer.

**Materials and Methods**

**Tumor samples**

Anonymized clinical samples of 38 hepatocellular carcinomas (HCCs) and 13 normal liver tissues were provided by Lee et al. (Lee et al., 2004). The majority of the patients had an HBV background, but a few were HCV and alcoholic. All the samples originated from untreated primary resected tumors. Importantly, even though the majority of patients in this cohort were infected with HBV (which is also the common background for Chinese HCC patients), the cohort also includes HCV and alcoholic-related HCC cases, simulating a “true” clinical situation,
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as patients with HCC are not normally a homogeneous group. Thirty-eight HCC samples were randomly selected to be reanalyzed among samples that were previously classified into two groups based on overall survival (Lee et al., 2004). It was demonstrated by Lee et al. that although patients may originate from different ethnic groups, the cohort could still be considered homogeneous at the molecular level (Lee et al., 2006). A total of seventeen normal liver samples were analyzed. Total RNA for N1-N4 was purchased commercially, N1 from Ambion (catalog number AM7960), N2 from Stratagene (catalog number 540017), N3 from Clontech (catalog number 636531), N4 from Biochain Institute (catalog number R1234149-50). RNA from N5-N17 was provided by the Laboratory of Experimental Carcinogenesis, National Cancer Institute.

**TaqMan low density arrays (TLDAs)**

Expression levels of 377 MDR-associated genes were measured in the above-mentioned samples using a custom-made Taqman Low Density Array (Applied Biosystems, Foster City, CA, USA), as previously reported (Calcagno et al., 2010).

**Normalization and filtering**

The median expression of each sample was subtracted from all gene expression data for that sample. Two of the normal (N1, 2) and seven of the HCC samples (A1, 5, 8, 9, 11 and B16, 18) were analyzed in duplicate. For these 9 samples, the Pearson correlation between the duplicates was greater than 99%. These duplicates were averaged together. One of the genes (18S) was present as multiple probes. The expression data from the multiple probes for that gene were averaged together. Genes that were expressed in 10 or fewer samples were removed from the analysis.

**Comparison of HCC and Normal Liver Samples**
Genes expressed differentially in HCC and normal samples were detected using the t-statistic. The obtained p-values were adjusted for multiple comparisons using the Benjamini-Hochberg method (Benjamini and Hochberg, 1995).

**Comparison of samples from groups A and B**

Unsupervised clustering (Average Linkage algorithm with 1-Pearson correlation as the distance) was done on the normalized, filtered data. Genes differentially expressed in groups A and B were detected using the t-statistic, with the p-values adjusted for multiple comparisons using the Benjamini-Hochberg method.

**Validation of the 45 MDR-linked genes as a prognostic signature for poor overall survival**

The clinical data of this cohort and the analysis of the gene expression profile was published by Andersen et al. (Andersen et al., 2010). Analysis of survival data was performed by Kaplan-Meier using Mantal-Cox (log-rank) statistics (GraphPad.Prism v5).

**Connectivity Map analysis**

The up- and down-regulated genes found in 38 HCC samples that had an adjusted p-value<0.05 in the TLDA data were used as input to the Connectivity Map online web tool (http://www.broadinstitute.org/science/projects/connectivity-map/connectivity-map).

**Validation of the compounds highlighted by utilizing the connectivity map**

All 20 cell lines were grown to 70-80% confluence. Twenty-four hours prior to RNA extraction using an RNeasy Micro kit (Qiagen, Valencia, CA), all cell lines were grown in DMEM/F12 (Invitrogen, Carlsbad, CA, USA). The RNA was prepared and profiled as previously mentioned in the section entitled “Validation of the 45 MDR-linked Genes as a Prognostic Signature for Poor Overall Survival.” Integration was performed by z-transforming
each data set separately and the hierarchical clustering was performed using Cluster 3.0 and
TreeView1.6.

**Cell lines**

HUH7, PLC and HEP3B, which clustered with samples from patients with poor overall
survival, and HUH1, SNU182, and FOCUS clustering with samples from patients with better
overall survival, were maintained in RPMI-1640 (Life Technologies, Invitrogen), supplemented
with 10% FBS, 100 units of penicillin/streptomycin/mL at 37°C in 5% CO2 humidified air.

**Cytotoxicity Assay to Determine Synergism of Added Drugs**

To assess the synergistic effects of sorafenib, 6TG, 8-Aza, doxorubicin, apigenin, cisplatin,
and 5-FU in combination, Huh7, Hep3B and PLC cells were each treated with a matrix of two
different drugs with serial 1:2 dilutions from 100 µM to 0.001526 µM. Five thousand cells were
seeded per well 16 h before addition of the drug combinations. According to the manufacturer's
protocol, an MTT assay (Trevigen, Gaithersburg, MD) was performed to measure proliferation
of cells at 72 h after drug addition. To evaluate sensitization effects, 20 µM of one drug was
added for 24 h and removed. The second drug was added in a serial 1:2 dilution from 100 µM to
0.3906 µM and proliferation was measured at 72 h using the MTT assay. Three technical
replicates were performed for the sensitization experiments. All additions of cells to 96-well
plates, drug dilutions/additions and the MTT assay were carried out using a Hamilton Star Liquid
Handler (Reno, Nevada). Synergy calculations were done using custom scripts on R (version
2.15.2).

**Western-blot immunoassay**

The following antibodies were used for a Western blot immunoassay: rabbit anti-acetyl
histone H3 (1:1,000; Millipore, Billerica, MA, Cat. #06-599), rabbit anti-GAPDH (1:5,000; Cell
Signaling, Danvers, MA, Cat. #14C10). Horseradish peroxidase-linked secondary antibodies (1:10,000) were from DakoCytomation (Carpinteria, CA). Bands were visualized by chemiluminescence using X-ray film.

Silencing the histone acetyltransferase GCN5

A pGIPZ shRNA construct for stable knockdown of human GCN5 (KAT2A gene) was obtained from Open Biosystems-Thermo Scientific (catalog # RHS4430-99293180). A non-silencing pGIPZ shRNA (catalog # RHS4346) was used as a control. Lentiviral particles were made via Lipofectamine 2000 (Invitrogen)-mediated triple transfection of 293T cells with pGIPZ shRNA plasmids along with the lentiviral envelope plasmid (pMD2.G, Addgene plasmid 12259) and the lentiviral packaging plasmid (psPAX2, Addgene plasmid 12260). Liver cancer cells were transduced with either non-silencing or GCN5-specific shRNA containing lentiviral particles in the presence of 8 μg/mL polybrene and stable cells were selected using 3 μg/mL puromycin for one week and pooled before determining knockdown efficiency. Knockdown efficiency was determined via Western blot analysis using a GCN5-specific antibody (#3305) from Cell Signaling Technology.
Results

Genes Differentially Expressed in HCC compared to Normal Liver Samples

We conducted a study on 17 normal liver samples and 38 HCC samples (generously provided by Thorgeirsson and colleagues (Lee et al., 2004; Lee et al., 2006) to compare the expression profile of 377 MDR-linked genes in normal and HCC samples. These genes, selected from the literature published over the last 30 years, were reported to have a role in multidrug resistance, mostly based on in vitro studies (Calcagno et al., 2010; Gillet et al., 2011). Our analysis revealed 103 genes that are differentially expressed in HCC compared to normal liver samples. Eighty-two genes have a false discovery rate (FDR) < 0.05 and p-value < 0.01, and 21 additional genes fulfill the less stringent criteria of FDR < 0.05 and p-value < 0.05 (Table 1). More precisely, 32 genes were found to be down-regulated in HCC compared to normal samples, while 71 were found to be up-regulated (Table 1). Notably, 8 ABC transporters were found to be over-expressed in HCC. Many of these drug efflux transporters are important mediators of MDR (Gillet et al., 2007). Within this group of 8 genes, the involvement of ABCC1, ABCC4, ABCC5 and ABCC10 in MDR has been well characterized (Gillet et al., 2007). Our analysis also highlighted a cluster of genes involved in cell cycle regulation, including CDKN2A, CDK2 and 4, CCD42 and CCNE1. Moreover, the cell cycle checkpoints CHEK 1 and 2, and RAD1 were found to be over-expressed, as well as a large cluster of DNA repair genes including TOP2A, over-expressed 67-fold.

Four main groups of down-regulated genes were uncovered, including several ABC transporters, CYP450s, metallothioneins and solute carriers. None of the down-regulated ABC transporters were found to be involved in MDR except ABCC11, whereas several down-regulated SLCs have been previously identified as drug transporters. They include...
SLC21A8/SLCO1B3, SLC22A1/OCT1, SLC28A1/CNT1 and the copper transporter
SLC31A1/CTR1, which also transports cisplatin, oxaliplatin and carboplatin (Huang, 2007; Kuo et al., 2007).

Identification of an MDR-linked gene signature in a previously established group of poor
overall survival patients

We characterized the differential MDR-linked gene expression of the 38 HCC samples, which
were previously classified into two groups based on overall survival (Lee et al., 2004). Group A
consisted of 20 samples taken from Chinese patients with a poor overall survival, while Group B
contained eleven samples taken from Chinese patients and 7 samples from Belgian patients. All
of the Group B patients demonstrated better overall survival.

Unsupervised clustering of all the genes expressed in more than 10 of the 38 samples yields
distinct clusters for the HCC group A subtype (poor overall survival), the HCC group B subtype
(good overall survival), and the normal samples, with few exceptions (Fig. 1A). Supervised class
comparisons highlighted 45 genes that are differentially expressed in Groups A and B
(FDR<0.05), of which 29 were found to be up-regulated in patients with poor overall survival
compared to patients with good overall survival, while 16 were found to be down-regulated
(Table 2). Perhaps the most striking finding is the up-regulation of 12 genes related to DNA
repair, including cell cycle checkpoints CHEK1 and ATM, the regulators BRCA1 and 2, the
double-strand break repair genes MRE11A, TOP2, RAD51, XRCC1, 2 and 5, and the single
strand DNA repair gene TOP1. Three gene families were found to be down-regulated. They
include five ABC transporter genes (ABCA6, B4, B11, C9 and G8), 4 CYP450s (CYP2A6, 2C8,
2C9 and 2C19) and three SLCs (SLC10A1, 22A1 and 28A1), which could be attributed to the
concomitant down-regulation of the nuclear receptor genes NR1I2 and NR1I3 (di Masi et al., 2009).

Validation of the 45 MDR-linked genes as a prognostic signature for poor overall survival

We assessed the predictive power of the 45-gene signature identified as differentially expressed in A vs. B subtypes on an independent cohort of 53 HCCs obtained from Caucasian and Chinese patients (Andersen et al., 2010). The gene expression profiling was performed using Illumina bead chips (Andersen et al., 2010). Fig. 1B shows that the 45-gene signature effectively predicts overall survival of patients with HCC (p<0.02), validating the clinical relevance of the gene signature.

Identification of compounds that sensitize chemo-resistant HCC

The next step in this study was to pinpoint drugs that might efficiently alter the poor prognosis gene signature in HCC. For this, we used the Connectivity Map tool published by Lamb and colleagues (Lamb et al., 2006), designed to reveal connections among drugs, genes, and pathological states. The Connectivity Map algorithm (Lamb et al., 2006) compares the direction of gene expression change from one disease state to another with the change due to a drug treatment. Drugs that cause an expression change similar to the change between poor-prognosis and better-prognosis tumors may be able to change the outcome in those with HCC, possibly by causing the resistant cells to become more drug-sensitive, or by changing the physiology of the tumor.

We were interested in drugs that cause a change in gene expression that matches the gene expression change from group A to group B. From the up- and down-regulated genes obtained by TLDA that had an adjusted p-value <0.05, we found 4 drugs with high positive concordance, low p-value (p<0.001) and a low specificity score (specificity score<0.05). These drugs were 8-
azaguanine, 6-thioguanosine, apigenin, and 0175029-0000, a pyrimidine derivative (2-[4-(2-diethylaminoethyloxy)anilino]-8-phenyl-pyrido[2,3-d]pyrimidin-7-one) (Supplementary Table S1).

To confirm our findings regarding these drugs, we performed an integrative clustering using the 45-gene signature in 20 HCC cell lines and the 53 HCC clinical samples of our validation set (Fig. 2A). Two HCC cell lines randomly selected, HUH7 and PLC, which clustered with samples from patients with poor overall survival, were treated for 72 hours with a sub-cytotoxic dose of each of the drugs individually except for compound 0175029-0000, which is unavailable. The data indicate that the treatment caused a change in the gene expression profile of the cell lines from that of poor overall survival to that of better overall survival (Fig. 2B).

6-TG, 8-AZG and apigenin mediate increased acetylation of histone protein

We next hypothesized that the mechanism underlying the ability of these three compounds to change gene expression patterns might be associated with increased acetylation of histone protein. This was confirmed using an antibody directed against acetylated histone H3 in three HCC cell lines (HUH7, PLC, and HEP3B) treated for 6 hours with 10 μM of either one of these drugs (Fig. 3A). Optimization of the treatment (24 hours with 20 μM) dramatically increased the effect observed, with a 3- to 6-fold increase in acetylated histone H3, and as much as a 13.5-fold increase when treated with depsipeptide as a positive control (Fig. 3B).

To explore further the hypothesis that changes in gene expression patterns were due to increased histone H3 acetylation, we knocked down the expression of the major histone H3 acetyltransferase GCN5 (Fig. 4, C-F). As hypothesized, drug treatment-induced histone acetylation decreased in the HUH7 and PLC cell lines when GCN5 was knocked down and to some extent in the HEP3B cell line, when treated with apigenin.
Drug combinations show synergistic cytotoxicity

For each pair of drugs that were tested, we used combinations of varying concentrations to determine whether the combination of these drugs resulted in increased cytotoxicity and whether this effect was additive or synergistic. The combination of three drug pairs had a significant synergistic effect on all three HCC cell lines: 6-TG/Apigenin, doxorubicin/apigenin and sorafenib/Apigenin, and to some extent, 6-TG/5-FU (Fig. 4), indicating that the changes we observed in gene expression patterns were also associated with increases in treatment efficacy over and above the toxicity of the drugs themselves.
Discussion

Using a TaqMan-based qRT-PCR array, we studied the expression profile of 377 MDR-linked genes and found a signature of 103 genes differentially expressed in normal liver cells and HCC. The MDR genotype consists of the up-regulation of several members of the ABCC family (known as MRPs), of genes involved in cell proliferation through regulation of the G1/S cell cycle transition, and of DNA repair genes. We also identified down-regulation of several solute carriers involved in platinum drug uptake, potentially resulting in a dramatic decrease in the cellular entry of this drug (Huang, 2007; Kuo et al., 2007). The intrinsic expression of several additional ABC transporters known to efflux standard chemotherapeutics including ABCB1, ABCB4, ABCB11 and ABCG2, leaves limited treatment modalities to clinicians when coupled with the MDR-linked gene signature of HCC. Many of these ABC transporters have been shown to transport doxorubicin (Szakacs et al., 2006). It should be noted that this poor prognosis MDR gene signature probably reflects a biological state of the HCC rather than being the sole cause of the poor prognosis, since the patients who were the source of the analyzed HCC samples were not treated with chemotherapeutic agents. However, the presence of these drug-resistance mechanisms in poor prognosis HCC makes it difficult to design chemotherapy that might be effective against these cancers. On the other hand, this 103-gene signature not only confirms the expression of known markers of HCC such as TOP2A (Wong et al., 2009), which is a target for topoisomerase inhibitors, but also highlights new markers including the solute carriers SLC2A5/GLUT5, SLC16A3/MCT, SLC7A11 and the melphalan transporter SLC7A5/LAT1 (del Amo et al., 2008). It is possible that these uptake transporters might facilitate cellular entry of certain yet unidentified drug species, therefore facilitating therapy.
The HCC samples analyzed were comprised of two groups defined by overall survival rate. Since previous studies indicated the superiority of TLDAs over high density microarrays (confirmed in this work), we used this technique to investigate the differences in gene expression profiles for patients with good and poor overall survival (Gillet and Gottesman, 2011). Interestingly, our analysis revealed a novel 45-gene signature that was shown to predict overall survival. In addition to well-established markers, we demonstrated 13-fold and 5-fold overexpression of SLC29A2 and SLC16A3/MCT, respectively, in the poor overall survival group. SLC29A2, a nucleoside uptake transporter, mediates the transport of gemcitabine, cladribine, and zidovudine (AZT) (Baldwin et al., 2004; Huang and Sadee, 2006). Huang and colleagues correlated SLC gene expression profiles in the NCI-60 cancer cell line panel with the potencies of 119 standard anticancer drugs and identified new transporter substrates (Huang et al., 2004). More recently, a similar approach was carried out by Okabe et al. to investigate the SLC22 and SLCO gene families using qRT-PCR, rather than oligonucleotide array (Okabe et al., 2008). That work revealed the role of SLC22A4 in cellular sensitivity to doxorubicin and mitoxantrone. Studies of this type may help to reveal additional compounds that could be used to treat SLC over-expressing cancer cells, because they are substrates for up-regulated uptake transporters.

The ultimate goal of highlighting MDR-linked gene signatures is to be able to sensitize drug-resistant cancers. One strategy is the development of targeted therapy using newly identified biomarkers associated with drug resistance, as indicated above. Another approach highlighted in this work is to utilize a resource published earlier by the Golub group, the Connectivity Map, which allows the identification of compounds that might sensitize MDR cancers with a specific gene signature (Lamb, 2007; Lamb et al., 2006; Zhang and Gant, 2008). We found three
compounds, a flavone (Apigenin) and two nucleoside analogs (8-AZG and 6-TG), that convert the gene expression profiles of three HCC cell lines classified with poor overall survival patients into gene expression profiles associated with good overall survival. Application of these compounds, which we have demonstrated in this work are histone deacetylase inhibitors in the cell lines studied, resulted in the synergistic sensitization of those MDR tumor cells to conventional chemotherapeutic agents including cisplatin, sorafenib and 5-fluorouracil. Of note, we made this observation using cell lines with three different TP53 mutational profiles, TP53 null (HEP3B), mutated (HUH7), and WT (PLC). It was shown by Thorgeirsson and colleagues that HCC patients with p53 mutations had a shorter overall survival time compared with patients with WT p53 (Woo et al., 2011). Here we show that the efficiency of the proposed strategy is not dependent on TP53 status.

This study provides in vitro-based evidence that, if validated in vivo, suggests that the management of cancers intrinsically resistant to standard chemotherapeutic treatments would improve by changing transcriptional profiles, including genes whose expression is known to be associated with drug resistance. We can also envision applying such a strategy to any cancer highly resistant to standard chemotherapy with the goal of reversing its gene expression profile to that of cancers from patients who respond well to treatment.
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Authorship Contributions

Participated in research design: Gillet, Gottesman, Ambudkar, Bagni, Madigan, Thorgeirsson

Conducted experiments: Gillet, Andersen, Madigan, Bagni, Powell, Burgan, Wu, Calcagno

Performed data analysis: Gillet, Varma, Andersen, Wu

Wrote or contributed to the writing of the manuscript: Gillet, Gottesman, Ambudkar, Andersen
References


Footnotes

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Figure Legends

**Figure 1.** Identification of MDR-linked gene signature. A, Unsupervised hierarchical clustering of gene expression data from clinical samples of HCC stratified into two main groups, with few exceptions (7 good overall survival samples clustered with poor overall survival samples. i.e. B3, 4, 5, 7, 11, 12 and 19). Red and green cells reflect high and low expression levels, respectively. B, Kaplan-Meier Survival Curve. The panel shows overall survival of patients with HCC using the 45 MDR-linked genes (P<0.02).

**Figure 2.** Unsupervised hierarchical clustering based on expression data of 45 MDR-linked genes from 53 clinical samples of HCC and 20 HCC cell lines. A, Dendrogram presenting the clustering of the 20 HCC lines into two groups according to patient survival. B, Dendrogram showing the reversal of the 45-gene expression profile of two HCC cell lines (HUH7 and PLC) clustered with samples from patients with poor overall survival to a gene expression profile found in patients with good prognosis, following a 72-hr drug treatment (HUH7 6TG, HUH7 8-AZA, HUH7 API and PLC 6TG, PLC 8-AZA, PLC API). HUH7 cells were treated with 6.25 µM 6-TG, 1.56 µM 8-AZ and 15 µM Apigenin. PLC cells were treated with 12.5 µM 6-TG, 50 µM 8-AZ and 25 µM Apigenin. Each drug was tested individually.

**Figure 3.** Western blot analysis of histone H3 acetylation. A, Analysis using initial drug-treatment regimen, in three HCC cell lines. The untreated cell lines were compared with their treated counterparts (10 µM, 6-hrs). DP: depsipeptide, and TA: trichostatin were used as positive controls (5 and 10 nM). B, Analysis using optimized drug-treatment regimen, in the same three HCC cell lines. Optimization of the treatment conditions increased the effect observed (20 µM, 24-hrs). The untreated cell lines were compared with their treated counterparts. DP: depsipeptide,
was used as positive control (5 nM). Histone acetyltransferase, GCN5, is involved in chemosensitizing drug-induced Histone-H3 acetylation. C, Protein lysates from the HCC cell lines HUH7, PLC and HEP3B, stably expressing either control or GCN5-specific shRNA, were resolved by SDS-PAGE and blotted for GCN5, β-actin and acetylated Histone-H3 antibodies. Percent knockdown of GCN5 and fold changes in acetylated Histone-H3 levels between control and GCN5 knockdown cells, for all three cell lines, are indicated. Control and GCN5-specific shRNA expressing HUH7 (D), PLC (E), and HEP3B (F) cell lines were treated with either vehicle control or 20 μM of either 6-Thioguanine, apigenin or 8-Azaguanine for 24 hours. Protein lysates were resolved by SDS-PAGE and blotted for GCN5, β-actin and acetylated Histone-H3 antibodies. Fold changes in acetylated Histone-H3 levels of drug-treated cells, compared to vehicle control-treated, for both control and GCN5 knockdown cells are indicated.

Figure 4. Assessment of the synergistic effect of drug combinations. Presented are the 2-D plots of synergy for one drug pair that showed significant synergy for all three cell lines, HUH7, PLC, and HEP3B. The 2D plots show the difference between the measured values and additive null surface (Loewe and Muischne, 1926). Blue and black boxes are regions of synergy, whereas pink shows antagonism. Green and yellow areas are close to the null surface (neither synergy nor antagonism).
Table 1. Differentially expressed genes in HCC samples compared with normal liver samples

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Figure 1

A

B

Good survival (median=29.3)
N=31 (20 deaths)

Poor survival (median=9.7)
N=22 (22 deaths)
Figure 4

HUH7

PLC

HEP3B

Apigenin

Doxorubicin

6TG

5-FU

Sorafenib