A survey of optimal strategy for signature-based drug repositioning and an application to liver cancer

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20 **Abstract**

21 Pharmacologic perturbation projects, such as Connectivity Map (CMap) and Library
 22 of Integrated Network-based Cellular Signatures (LINCS), have produced many
 23 perturbed expression data, providing enormous opportunities for computational
 24 therapeutic discovery. However, currently there is no consensus on which
 25 methodologies and parameters are the most optimal to conduct such analysis. Aiming
 26 to fill this gap, we developed new benchmarking standards for quantitatively
 27 estimating drug retrieval performance. Investigations of potential factors influencing
 28 drug retrieval were conducted based on these standards. As a result, we determined an
 29 optimal strategy for LINCS data-based therapeutic discovery. With this approach, we
 30 further identified new therapeutics for liver cancer of which the current treatment
 31 modalities remain imperfect. Both computational and experimental results
 32 demonstrated homoharringtonine (HHT) could be a promising anti-liver cancer agent.
 33 In summary, our findings will not only impact the future applications of LINCS data
 34 but also offer new opportunities for therapeutic intervention for liver cancer.

35

1. Introduction

Despite the major advances in drug research and development (R&D), the cost for *de novo* drug development remains high, ranging from \$3 billion to more than \$30 billion. Moreover, it usually takes over 10 years to bring a new drug from bench to bedside, reflecting the complex challenges in this area [1]. Within this context, exploring new indications for existing drugs (drug-centric) or identifying effective drugs for certain disease (disease-centric) represent an appealing concept, namely ‘drug repositioning’ (or ‘drug repurposing’), that can greatly shorten the gap between preclinical drug research and clinical applications [2]. Leveraging big data-driven approaches, drug repositioning can be performed computationally, which has the potential to complement traditional therapeutic discovery means and further improve the cost-effectiveness of drug development [3]. The most notably data resources supporting the *in silico*-based therapeutic discovery campaigns would be the Connectivity Map (CMap) [4] and its recent extension called Library of Integrated Network-Based Cellular Signatures (LINCS) [5]. These two projects have generated large-scale drug-induced gene expression profiles on multiple cancer cell lines under different treatment conditions (CMap Build 2: 3 cell lines, 1,309 compounds; LINCS: 77 cell lines, 19,811 compounds), representing a treasure trove for *in silico* therapeutic exploration [6]. As a 1,000-fold scale-up of the original CMap, LINCS contained dramatic increases in both cell line types and perturbations, making it the focus of the present investigation.

The computational drug discovery approach using LINCS (also CMap) data is based upon the basic concept called ‘signature reversion’ [3]. Specifically, to identify candidate agents for treating a certain disease, molecular signature characterizing the states of this disease needs to be generated first. Then, through comparing compound signatures with disease signatures, inverse compound-disease relationships can be identified. Compounds with the ability to reverse disease-specific gene expression pattern are considered as therapeutic candidates (Fig. 1). Several successful applications of this approach include the discovery of rapamycin as combination

partner of glucocorticoids in treating lymphoid malignancies [7]; topiramate as a potential agent against inflammatory bowel disease [8]; citalopram as a therapeutic option for patients with metastatic colorectal cancer [9]; niclosamide and pyrvinium pamoate as potential therapeutics for liver cancer [10]. Despite its success, there are still many unsolved problems with this approach. Currently, due to the lack of appropriate benchmarking standards, limited studies have focused on investigating the potential factors that influence this computational strategy, let alone quantitatively evaluating whether these factors affect the accuracy of drug prediction. Therefore, across current signature reversion studies, no consensus regarding the implementation details has been reached. Constructing the rational benchmarking standards and developing the best practice approach can facilitate the development of computational pharmacology area and help to identify more effective therapeutic strategies for refractory diseases.

Herein we mainly focused on the disease of liver cancer. As one of the most lethal malignancies worldwide, liver cancer directly accounts for nearly one million deaths each year (8.2% of new cases of cancer-related death) [11]. Hepatocellular carcinoma (HCC) is the major type of liver cancer, representing approximately 90% of all liver cancer cases [12]. Although many standard of care therapies, including Lenvatinib [13], regorafenib [14], cabozantinib [15], ramucirumab [16], pembrolizumab [17], nivolumab [18] and atezolizumab-bevacizumab [19], have been approved for treating HCC in recent years, most of them can yield only marginal survival benefit. Thus, more effective therapeutics treatments for HCC are highly desired. The objectives of the present study were threefold. The first objective was to develop novel benchmarking standards for evaluating drug retrieval performance. The second one was to determine the best practice approach for LINCS data-based computational drug repositioning. For the last objective, through exploiting the findings from the second objective, we aimed to identify novel drug candidates against liver cancer.

94 2. Results

95 2.1. Summary of influencing factors and compound experiments in LINCS

96 Many factors may affect the accuracy of signature-based drug retrieval. We have
 97 categorized these factors into three main aspects: acquisition of compound signature
 98 (reference signature), generation of disease signature (query signature), and selection
 99 of disease-compound matching methods ([Additional file 2: Figure S1](#)). Although all
 100 factors were mentioned and discussed, not all of them were included into the present
 101 analyses, considering that some factors have been covered elsewhere and some were
 102 challenging to explore due to data and method restrictions. In this study, systematic
 103 analyses were carried out to assess the influences of four major factors on signature
 104 matching-based drug discovery, including source of cell line, clinical phenotype of
 105 query signature, query signature size, and signature matching method. Detailed
 106 descriptions and discussions about all included factors were stated in [Additional file 1:](#)
 107 [Supplementary Discussion](#).

108 Since only compound-induced expression data was the focus of this study, we
 109 first excluded experiments of other perturbagens, including gene knockdown (or
 110 knockout) and gene overexpression manipulations. Subsequently, the distribution of
 111 compound profiles was visualized based on their perturbation time, perturbation dose,
 112 and cell line used. Most of the measurements were made in the treatment durations of
 113 6h (43%) or 24h (56.6%), and under the concentrations of 5 μ M (21%) or 10 μ M (63%)
 114 ([Additional file 2: Figure S2a](#)). The count distribution of all cell lines in LINCS was
 115 also presented in [Additional file 2: Figure S2b](#). Although 71 cell lines were included
 116 in LINCS project in total, not all of them were extensively profiled, and only nine cell
 117 lines contained more than 5,000 profiles, which, however, account for 77.8% of all
 118 compound profiles. There were 2,912 compounds shared by these nine cell lines. We
 119 further integrating annotation of the most profiled cell lines with treatment duration
 120 and concentration information and illustrated the specific profile numbers of each cell
 121 line under the conditions of certain time and dose ([Additional file 2: Figure S2c](#)).
 122 Notably, all the following analyses were performed using LINCS data based on a

fixed perturbation condition of 10 μ M for 6 hours. Besides, compound profiles of all top nine cell lines were only utilized when investigating the factor ‘Source of cell line’. In other cases, we focused exclusively on the cell line of HepG2, as in this study, our main point was to uncover novel therapeutics for liver cancer. A systematic summary of included datasets for analyses was presented in [Additional file 3: Table S1](#) and [S2](#).

2.2. Cell-type specific gene expression changes induced by compound treatments

Some previous studies utilized compound profiles from cell lines irrelevant to the disease they studied for performing signature-based drug repositioning. To investigate whether this was a reasonable practice, we conducted following analyses based on the LINCS data of the nine most profiled cell lines. First, we visualized the compound profiles in a cosine distance-based two-dimensional t-distributed stochastic neighbor embedding (t-SNE) plot that represented the overall compound perturbation space wherein each dot was equivalent to a unique perturbation and each cell line was color-coded ([Fig. 2a](#)). As shown in the figure, most dots with the same color clustered together, indicating that most of compound-induced gene expression changes tended to be cell-type specific. Intriguingly, dots with different colors in the white region seemed to mix together, suggesting that some certain compounds might induce similar gene expression changes across cell lines. To figure out which compounds were likely to cause cell-type specific gene expression changes, and which tended to induce universal changes independent of cell lines, we calculated the pairwise cosine similarities (L1) among the profiles from the same compounds measured in different cell lines ([Fig. 2b](#)). The cosine similarity measures range from -1 to 1, where higher values indicate increased similarity. The similarity scores (compound-level, L2) of the 2,912 unique compounds were determined by calculating the median pairwise cosine similarity values (L1) across the most profiled nine cell lines mentioned before ([Additional file 3: Table S3](#)). As a result, a high degree of cell-specificity was observed for most compounds, with a median L2 similarity score of 0.078 ([Fig. 2c](#)).

152 Furthermore, we retrieved the mechanism of action (MOA) information and mapped
153 them to the compounds to determine the MOA-level similarity scores (L3). L3
154 similarity scores were calculated based on the median values of L2 similarity scores
155 of compounds within the same MOA. Results suggested that inhibitors targeting core
156 cellular processes (e.g., cell cycle, RNA transcription, protein synthesis) tended to
157 induce similar changes across all cell lines, generally in agreement with previous
158 findings (Fig. 2d and Additional file 3: Table S3) [5, 20]. We then marked the dots
159 representing the compounds of top five MOAs in the t-SNE plot. As might be
160 expected, most of marked dots fell in clusters within the nonspecific region (Fig. 2e).

161 Apart from investigating similarity of perturbed expression profiles at compound
162 level, we sought to further investigate the cell line pair/cell line-level similarity. Nine
163 cell lines contributed a total of 36 unique cell line pairs. The cell line pair-level
164 perturbed expression similarities (L4) were determined through calculating the
165 median value of similarity scores of all compound pairs between two cell lines, and
166 the corresponding basal expression similarities were computed using Spearman
167 ranked correlation on expression data from CCLE project (Fig. 2f). Based on these 36
168 cell line pairs, we found that there was a significant, albeit not very remarkable,
169 association between the perturbed expression similarities (cell line pair-level, L4) and
170 basal expression similarities ($\rho = 0.344$; $P = 0.040$), suggesting that cell lines with
171 similar molecular features were more likely to have consistent gene expression
172 changes upon perturbation (Fig. 2g). Similarities within the nine cell lines were also
173 explored (cell line-level, L5). Among the nine cell lines we tested, PC3 cell line
174 showed the highest L5 similarity score (median value = 0.122) (Fig. 2h). Notably, the
175 cosine similarity of 0.122 still denoted a weak relationship, which further supported
176 the conclusion that compound-induced gene expression changes were highly cell line
177 specific.

178 Among the nine most profiled cell lines, HepG2 was the only one derived from
179 liver. To investigate whether HepG2 was an appropriate cell line model for
180 computational therapeutics discovery for liver cancer or other liver-associated

diseases, we calculated the expression correlation between HepG2 and other cell lines (921 CCLE cell lines) or tissues (17,382 normal tissues from GTEx and 9,701 tumor tissues from TCGA PanCancer). Compared to other tissue-derived cancer cell lines or normal/tumor tissues, HepG2 exhibited a significantly higher expression correlation with liver cancer cell lines (median correlation coefficient = 0.729), normal liver tissues (median correlation coefficient = 0.616), and liver cancer tissues (median correlation coefficient = 0.631) ([Additional file 2: Figure S3](#)). Coupled with above finding of the significant relationship between perturbed expression similarities and basal expression similarities, we supposed that the use of LINCS-derived HepG2 data was preferable to be limited within liver diseases.

2.3. Developing new benchmarking standards

Owing to the lack of benchmarking standards, accurate assessment of retrieval performance of signature matching methods remains challenging. Inspired by previous findings [10, 21], we proposed two novel benchmarking standards, namely area under curve (AUC)-based standard and Kolmogorov-Smirnov (KS) statistic-based standard. They were built upon different notions and thus independent of each other, which helped to avoid potential bias introduced by single standard. The corresponding benchmarking datasets were developed mainly based on preclinical/clinical data of liver cancer. Detailed processes regarding data collection and metrics calculation have been described in Methods and visualized in [Additional file 2: Figure S4a](#).

The development of AUC-based standard was based on the finding that there existed correlation between the reversal potency and treatment efficacy, which has been reported by several previous studies [10b, 21a]. In order to further validate whether this correlation remained significant in other conditions, we retrieved drug response data from CTRP dataset in which area under the dose-response curve (AUDRC) values were used as measurements of drug sensitivity, and utilized two different HCC signatures as query signatures to obtain KS-based similarity scores [10].

210 A total of 109 compounds shared by two datasets were selected to conduct correlation
211 analyses. As a result, statistically significant correlation could still be observed
212 between similarity scores and AUDRC values in these scenarios, further proving the
213 reliability of this standard ([Additional file 2: Figure S4b](#)). A benchmark dataset was
214 then generated, composing of 117 unique compounds with both LINCS and drug
215 efficacy (IC₅₀) data available, which was taken as a basis for the application of
216 AUC-based standard ([Additional file 2: Table S4](#)). The resultant AUC from this
217 standard were termed as drug retrieval-associated AUC (DR-AUC). Higher DR-AUC
218 value indicated better performance.

219 As for KS statistic-based standard, we assumed that agents under evaluation in
220 clinical trial for HCC treatment, namely HCC agents, might possess an increased
221 reversal capacity [10a]. In other words, HCC agents were more likely to cause
222 negative enrichment in KS test. To verify this hypothesis, we compiled a set of 27
223 potential HCC agents which were both included in LINCS and under clinical trials for
224 liver cancer treatment. Besides, similarity scores of all compounds tested in HepG2
225 were also calculated, which were then used as ranked list for KS test. The results of
226 KS test demonstrated the above assumption that the HCC agent set was negatively
227 enriched significantly ([Additional file 2: Figure S4c](#)). Accordingly, KS statistic-based
228 standard could be implemented based on this 27-agent set and the resultant
229 enrichment scores (ES) here were termed as drug retrieval-associated ES (DR-ES)
230 ([Additional file 3: Table S5](#)). Of note, in contrast to DR-AUC, lower DR-ES values
231 denoted better performance.

232

233 **2.4.Benchmarking signature matching methods for drug retrieval**

234 These two independent benchmarking standards enabled us to quantitatively assess
235 the retrieval performance of different signatures matching methods and thus aided in
236 the identification of optimal methodology for computational drug repositioning. In
237 total, six available methods including eXtreme Sum (XSum) [21b], eXtreme Cosine
238 (XCos) [21b, 22], eXtreme Pearson (XCor) [23], eXtreme Spearman (XSpe) [23],

239 Kolmogorov-Smirnov (KS) test [4], and the Reverse Gene Expression Score (RGES)
 240 [10b] were included for performance comparison. To minimize technical bias
 241 introduced by different query signatures, four HCC signatures with different sizes
 242 generated from distinct datasets were utilized for benchmarking (Additional file 3:
 243 Table S6). Of these, Sig_{gastro} [10a] and Sig_{NC} [10b] were directly obtained from
 244 previously publications, while Sig_{LIRI} and Sig_{GSE54236} were generated using RNAseq
 245 data from LIRI cohort and microarray data from GSE54236, respectively. A brief
 246 summary of above essential components involved in the evaluation process was
 247 illustrated in Fig. 3a.

248 Considering that the performance of eXtreme methods (including XSum, XCos,
 249 XCor, and XSpe) may be affected by the number of top genes (topN), we thus
 250 calculated the DR-AUC or DR-ES values of each eXtreme methods iteratively, using
 251 topN ranging from 50 to 489. In the condition of using Sig_{LIRI} as query signature, both
 252 benchmarking standards demonstrated that XSum outperformed other five methods
 253 across almost all candidate topNs (Fig. 3b). Concordantly, when using other three
 254 query signatures, XSum also achieved better performance compared with other
 255 methods, except in the case of using Sig_{Gastro} as query signature and AUC-based
 256 standard for benchmarking, where RGES showed a similar performance with XSum
 257 (Additional file 2: Figure S5a-c). Generally, XSum exhibited a consistently
 258 satisfactory performance, independently of the query signature and benchmarking
 259 standard, and thus could be used as the optimal methodology for drug retrieval
 260 (Additional file 3: Table S7 and S8). In addition, our analyses also demonstrated that
 261 the recently developed RGES (a modification of the KS method) appeared superior to
 262 the KS method in most cases and might serve as an alternative approach for KS-based
 263 connectivity mapping [10b].

264 Since we have proved that XSum could be used as the optimal matching method,
 265 we next sought to find the most appropriate topN value for applying XSum method to
 266 achieve the best retrieval performance. Directly selecting the exact topN value where
 267 corresponding DR-AUC/DR-ES reached their maximum/minimum might cause bias

and could be prone to over fitting. Given the continuous trait of candidate topNs, we chose to divide them into several smaller groups, typically 50 topN values in each group. The DR-AUC/DR-ES of a certain group was defined as the mean DR-AUC/DR-ES values within this group, which could decrease potential influences brought about by the outliers. With this normalization approach, relatively consistent results across all conditions could be obtained. The optimal topN group which yielded best performance were either ‘top150-200’ or ‘top200-250’ groups (Additional file 3: Table S9). Besides, we also observed a biphasic pattern of fitting curves, with the inflection points appearing where topNs were around 200 (Fig. 3c and Additional file 2: Figure S5d-f). Based on these results, we supposed that topN of 200 could serve as a rough guide.

2.5.Determining the optimal query signature size

Above analyses determined XSum as the optimal method and tuned the parameter of topN to 200. Next, we intended to further discern the optimal gene number in query signatures. Considering that Sig_{gastro} (n=44) and Sig_{NC} (n=73) had fixed and relatively small signature sizes, we only utilized signatures generated from LIRI and GSE54236 cohorts for subsequent investigation. We adopted two complementary approaches: (i) iterative fold change-based and (ii) random sampling-based approaches, to obtain query signatures with different sizes (Fig. 3d). The iterative fold change-based approach could create a number of signatures with discontinuous sizes through setting iterative threshold values of fold changes. The exact sizes of optimal signatures identified by this approach varied substantially (including 55, 79, 140, and 167). Despite this, similar trends of biphasic pattern with inflection points at around 100 under different conditions could still be observed (Fig. 3e and Additional file 2: Figure S6a). The approach based on random sampling was adopted as a complement. The results showed that as the signature sizes increased, the DR-AUC/DR-ES values also increased/decreased and eventually converged when the signature size was more than 100 (Fig. 3f and Additional file 2: Figure S6b). Based on these observations, we

considered that the signature size of 100 could be selected as a good compromise. This conclusion remained valid in the conditions when other topN values were applied, such as 100 and 400 (results not shown).

300

301 **2.6. Investigating the optimal clinical phenotype underlying query signature**

Many previous studies chose to compare normal versus diseased states to define disease signatures. However, signatures derived from other clinical phenotypes, such as prognosis and metastasis, could also be used to query LINCS. Aiming to figure out whether this factor could also affect the performance of drug retrieval, we designed a forward and a backward strategy respectively ([Additional file 2: Figure S7a](#)). The application of forward strategy was based on two types of signatures, general signatures (representing discordant expression pattern between normal and tumor tissues) and prognostic signatures (associated with survival outcomes). Through adopting above random sampling-based approach, we compared the above two signature phenotypes across varied signature sizes. Unfortunately, the results under different datasets and benchmarking standards were highly inconsistent. This strategy, consequently, failed to provide a definitive conclusion ([Additional file 2: Figure S8 a and b](#)).

Opposed to the forward strategy, backward strategy started from creating a collection of 10,000 random signatures, followed by identification of the optimal signature for clinical implication evaluation. Specifically, the optimal random signature was determined according to both benchmarking standards, and exploring the clinical values of this signature might, to some extent, reveal some necessary features possessed by a ‘good’ query signature ([Additional file 2: Figure S7b](#)). A comprehensive clinical evaluation on the optimal signature was carried out based on five RNAseq and five microarray clinical cohorts from three perspectives. First, the ability of this signature to distinguish tumors from non-tumors was investigated. Briefly, we extracted the first principal components (PC1) of this signature to represent its overall expression pattern. AUC was used here as a reflection of the

classification capability. The results showed that more than 0.90 of AUC can be obtained in seven out of eight cohorts (87.5%), indicating that the ability to discern the difference between diseased and normal states might be an indispensable property for achieving good retrieval performance (Additional file 2: Figure S7c). Next, we intended to find out whether the optimal signature should be a prognostic indicator. Cox regression analyses were conducted to investigate the association between the signature expression (PC1) and clinical outcome. As a result, significant prognostic implications (Cox $P < 0.05$) of the optimal signature could be observed in six out of eight cohorts (75%), suggesting that prognostic significance could also be a necessary characteristic (Additional file 2: Figure S7d). At last, the association between signature expression and other clinical features was explored. Considering that CHCC and LIHC cohorts held the most abundant clinical information, corresponding analyses were thus conducted on these two cohorts. The results showed that there was a significant correlation between the optimal signature and multiple clinical features, including BCLC stage ($P = 0.011$), tumor thrombus ($P = 0.001$), AFP level ($P = 0.022$), TNM stage ($P < 0.001$), and histologic grade ($P < 0.001$) (Additional file 2: Figure S7e). According to the above results, we concluded that quality query signature should possess the ability to comprehensively recapitulate the clinical features of corresponding disease, rather than only reflect the disease characteristic from single perspective.

346

2.7. Linking above findings to the discovery of new anti-liver cancer therapeutics

The knowledge acquired from above analyses was then applied to establish a signature characterizing liver cancer initiation and development, so as to identify computationally prioritized compounds with potential therapeutic as well as chemopreventive effects against liver cancer. The generation of this evolution-associated signature was based on the notion that the initiation and progression of liver cancer was a stepwise process with gradually acquired advantageous biological capabilities (Fig. 4a). Therefore, conceptually, antagonizing

genes that were most related to these stages could be a potential therapeutic strategy. Through implementing Random Forests algorithm on GSE89377 cohort, preliminary screening was performed to include stage-associated genes, where genes with greater predictive power were selected for further analysis. This screening yielded a total of 6017 stage-associated genes (23.9%), of which 309 were landmark genes (Fig. 4b). Next, we conducted weighted gene co-expression network analysis (WGCNA) to obtain co-expressed modules with diverse expression patterns (Additional file 2: Figure S9a). Seven gene modules were discerned by WGCNA analysis (Additional file 2: Figure S9 b and c), and two of them, which we termed the ‘ascending’ module (n = 1,738) and the ‘descending’ module (n = 350) for their greatest relevance to stages and patterns of linear evolution from normal to cancer, were retained for further analyses (Fig. 4 c and d). Biological processes associated with genes in these two modules were investigated. We found that the ‘ascending’ module was closely associated with proliferation (Fig. 4c), while the ‘descending’ module was enriched in several different types of processes (Fig. 4d). There were 159 genes in common between these two modules and landmarks. Based on the aforementioned recommendation about the optimal query signature size, we sought to further reduce the size of 159 to 100 genes. This procedure was carried out using HCC occurrence-related clinical and molecular data in GSE15654 cohort. In brief, ten thousand random signatures, each containing 100 genes, were generated based on the 159-gene panel. The one which had the most significant association with HCC occurrence was considered as the optimal query signature (Additional file 2: Figure S10 a and b). This analysis yielded a signature comprised 82 ascending genes and 18 descending genes, with cox *P* value of 0.009, which was then termed as Sig_{evo} (Additional file 3: Table S10). The linear evolution pattern of Sig_{evo} remained present in training (Additional file 2: Figure S10c) as well as an internal validation cohort (Additional file 2: Figure S10d).

As previously discussed, a ‘good’ query signature should reflect the clinical features of corresponding disease comprehensively. To evaluate whether Sig_{evo} was suitable for querying chemopreventive and therapeutic agents for liver cancer, we

384 systematically surveyed the association between this signature and the clinical
 385 phenotypes of precancerous/cancerous liver lesions using clinical and experimental
 386 data from both human and animal datasets. First, based on clinical cohorts of HCC,
 387 we demonstrated that Sig_{evo} had a remarkable capability for distinguishing between
 388 tumors from non-tumors, with a median AUC of 0.972 in all eight cohorts (Fig. 4e).
 389 Besides, this signature also held great prognostic power in HCC, as suggested by the
 390 results of Cox analyses (Fig. 4f). Next, in view of the crucial role of fibrosis in driving
 391 hepatocarcinogenesis, further investigation was performed to validate its relevance to
 392 fibrosis-related phenotype. The result suggested that Sig_{evo} could also effectively
 393 differentiate between mild (S0/S1) and severe (S3/S4) fibrosis, thus demonstrating its
 394 relationship with fibrosis progression (Fig. 4g). Additionally, we collected four
 395 experimental datasets, including two carbon tetrachloride (CCl₄)-treated mouse
 396 datasets and two diethylnitrosamine (DEN)-treated rat datasets, to assess the
 397 enrichment extent of Sig_{evo} in mouse and rat fibrosis models. It could be observed that
 398 ascending genes in Sig_{evo} were significantly enriched in both CCl₄-treated
 399 (GSE27640) and DEN-treated (GSE19057) liver tissues (Fig. 4 h and i). However,
 400 descending genes did not exhibit any significant enrichment pattern in all included
 401 datasets, possibly due to the limited gene number (Fig. 4 h and i). Notably, the
 402 expression profiles in GSE63726 were derived from non-parenchymal cell fractions
 403 which had abundant hepatic stellate cells (HSCs), and thus the significant enrichment
 404 could provide the evidence that this signature might reflect the molecular feature of
 405 HSC activation (Fig. 4i). In summary, the Sig_{evo} fully complied with the criteria of
 406 ‘good’ query signature and was thus employed for querying LINCS.

407 Subsequently, using the optimal method (XSum) and a compromising parameter
 408 (topN = 200), we matched Sig_{evo} with HepG2-derived compound signatures in LINCS
 409 and obtained the similarity scores of each compound (lower scores implied higher
 410 reversal potency and greater potential for application). After excluding preclinical
 411 agents or agents withdrawn from the market according to the drug annotation
 412 achieved from the Drug Repurposing Hub, 793 agents remained, which were then

413 considered as repositioning candidates ([Additional file 3: Table S11](#)) [24].
 414 Interestingly, some agents which were previously proved to have chemopreventive
 415 effects, including erlotinib [25], caffeine [26], and fasudil [27], dominated relatively
 416 high rankings on the list ([Fig. 5a](#)). Besides, anti-HCC agents were also found to be
 417 enriched significantly in compounds with reversal potency ([Fig. 5b](#)). These findings
 418 converged together to support the reliability of the prediction results.

419

420 **2.8.Characterization of homoharringtonine as anti-liver cancer agent**

421 According to the computational results, homoharringtonine (HHT) ([Fig. 5c](#)), a protein
 422 synthesis inhibitor targeting RPL3, had the highest reversal potency among 793
 423 repositioning candidates [28]. To ensure the reliability of L1000 platform-derived
 424 HHT signature, we treated HepG2 cells with the same condition used in LINCS and
 425 utilizing RNA sequencing to measure HHT-induced expression changes. Comparison
 426 between L1000-based and RNA sequencing-based HHT signatures was made and a
 427 good agreement between them could be observed, suggesting that our prediction using
 428 L1000-based HHT signature was comparable and reliable ([Additional file 2: Figure](#)
 429 [S11](#)). Further validation was then conducted to investigate the potency of HHT against
 430 liver cancer.

431 As the drug target of HHT, RPL3 were first characterized and delineated for its
 432 clinical and biological implications. The comparison of mRNA expression between
 433 normal and tumor tissues suggested that RPL3 exhibited a higher expression in tumor
 434 tissues ([Additional file 2: Figure S12a](#)). The increase of protein expression of RPL3
 435 could also be observed in tumor tissues, as shown by immunohistochemical images
 436 from the Human Protein Atlas ([Additional file 2: Figure S12b](#)) [29]. Higher
 437 expression of RPL3 also indicated a worse prognosis ([Additional file 2: Figure S12c](#)).
 438 In addition to the clinical implications, RPL3 also had essential biological
 439 implications in liver cancer. Leveraging the clustered regularly interspaced short
 440 palindromic repeats (CRISPR)-based screening data from Project Achilles, we found
 441 that RPL3 was essential for maintaining the survival and growth of all liver cancer

cell lines ([Additional file 2: Fig. S12 d and e](#)) [30]. Above results demonstrated the rationality of RPL3 inhibition for treating liver cancer.

Subsequently, we analyzed the drug response data from PRISM which measured the HHT activities in a large panel of cell lines [31]. It could be observed that, compared with molecular-targeted agents and non-oncology agents, HHT had a lower distribution of IC₅₀ values across 482 PRISM cell lines ([Fig. 5d](#)). Of note, in 15 liver cancer cell lines, HHT has exhibit a powerful anti-cancer capacity with a median IC₅₀ value of 0.408 μ M, which was numerically lower than three FDA approved HCC agents (lenvatinib: 0.617 μ M; regorafenib: 2.009 μ M; sorafenib: 3.348 μ M) ([Fig. 5e](#)). The anti-liver cancer activity of HHT was also corroborated by the long-term cell proliferation assay ([Fig. 5f](#)) and IncuCyte cell proliferation assay ([Additional file 2: Figure S13](#)). Given that the co-administration of HHT with other approved agents was more likely to have clinical utility, we also interrogated whether HHT had the ability to augment the tumor-killing effect of sorafenib. Three different statistical models were adopted for synergy estimation. The results suggested that HHT could indeed synergize with sorafenib in many conditions, albeit not very remarkable in general ([Fig. 5g-h](#) and [Additional file 2: Figure S14](#)). Collectively, our results demonstrated the therapeutic potential of HHT, either administrated as monotherapy or as combination with sorafenib.

2.9. Investigation of the anti-fibrotic effects of homoharringtonine

Liver fibrosis occurs when the liver tissue is repeatedly and continuously injured, which is a crucial risk factor for hepatocarcinogenesis [32]. The activation of hepatic stellate cells (HSCs) is one of the key steps in fibrosis development [33]. Since we have proved that Sig_{ev0} was associated with liver fibrosis using clinical and animal-derived data, it could be postulated that HHT, a compound with the ability to reverse Sig_{ev0}, might also be able to inhibit the activation of HSCs and thereby alleviate liver fibrosis. To test this hypothesis, the anti-fibrotic effects of homoharringtonine on TGF- β 1-activated human HSC line LX-2 were investigated.

We first treated LX-2 cells with vehicle or HHT (1 μ M and 5 μ M) for 6 hours. Then, RNA sequencing was conducted to quantify the mRNA expression level of vehicle-treated or HHT-treated LX-2 cells (Additional file 2: Table S12). HHT-induced expression changes were obtained based on the resultant data. Nine well-acknowledged fibrotic genes from previous publications were collected for subsequent analysis, the high expression level of which represented the activation status of HSCs. Apparently, the mRNA expression of almost all the fibrosis-associated genes were downregulated after HHT treatments (Fig. 6a and Additional file 2: Figure S15). The downregulated tendency of two most critical genes which encoded collagen I and α -SMA was further corroborated by the quantitative real-time PCR (Fig. 6b). Additionally, the protein-level expression of collagen I and α -SMA was also detected using western blot and immunofluorescence. The results showed that HHT could inhibit the protein expression of collagen I and α -SMA as well (Fig. 6 c and d). Overall, the ability of suppressing HSC activation markers of HHT indicated it had potential anti-fibrotic activity and thus may hold promise for preventing hepatocarcinogenesis.

487

488 **3. Discussion**

In recent years, the explosive growth of pharmacogenomic data enables the development of the new frontier of research named computational drug discovery and repositioning, leading to many remarkable findings of novel therapeutics [34]. Owing to the success of CMap and LINCS projects which profiled numerous compound-induced expression data [4-5], ‘signature reversion’-based computational drug discovery approach has been extensively adopted [7-10]. However, lack of suitable benchmarking standards for evaluating drug repositioning performance limits further improvement of this approach. Although some studies proposed that the benchmarks assessing drug-drug similarity, such as the Anatomical Therapeutic Chemical system (ATC fourth level codes), could be taken as alternative standards to indirectly determine the optimal methodologies and parameters of computational

repositioning where gold standards for benchmarking were more complicated [22-23], considering the great difference between these two situations, developing specifically tailored benchmarking standards for assessing disease-drug similarity would be more desirable [21b]. In this study, we proposed two novel benchmarking standards, AUC-based standard and KS statistic-based standard. According to the aforementioned observations, despite being mutually independent, there still existed good agreement of the evaluation results between these two standards, demonstrating the rationality and robustness of the novel standards.

These two standards enable the establishment of a standardized procedure for performing more effective signature-based computational repositioning. We first suggested that using reference signatures from one of the most relevant cell lines with the disease of interest instead of from a non-touchstone cell line or aggregation-based consensus results was a preferable option to exploit LINCS data, in view of a high degree of cell-type specific effects of perturbations. Next, XSum was identified as an optimal method for matching compound and disease signatures. Notably, a prior study which made a comparison of drug retrieval performance between XSum, XCos, and KS methods using a totally different benchmarking standard from ours also come to the same conclusion [21b]. This consistency not only further demonstrated the reliability of the two newly developed benchmarking standards, but also could support our identification of using XSum as the optimal method. Furthermore, we also uncovered the parameter choice (topN = 200) of XSum, which lacked guidance previously.

Within the computational repositioning framework, most of current investigations and methodological developments were focused on reference signatures and signature matching methods. Nevertheless, relatively limited efforts have been made to standardize the procedures for generating quality query signatures [35]. In this study, two potential factors, signature phenotypes and the signature size that might be associated with the retrieval performance of query signatures, were systematically analyzed. First, we intended to figure out how many genes in the query signatures

could best characterize a disease state. Through adopting two independent approaches, an appropriate query signature size of 100 was determined. However, some prior studies considered a reduced number of 50 as the optimal length of query signatures [10b, 36]. It is reasonable to speculate that the utility of different signature matching methods (XSum in this study and KS-based methods in other studies) and also the different benchmarking standards may be responsible for the discrepancy. Next, we sought to determine the influences brought by the clinical phenotypes of query signatures, so as to identify the necessary properties a ‘good’ signature needed to possess. Interestingly, the results suggested that a quality query signature should hold the ability to draw a comprehensive picture of clinical features of corresponding disease. This finding seemed to be reasonable since disease was highly likely to be under-represented when creating molecular signature from only one perspective of this disease.

Based on these findings, we summarized the best practice approach for LINCX-based computational drug repositioning. The application of this approach to liver cancer was then carried out. An evolution-associated query signature related to the development and progression of liver cancer was constructed for drug retrieval. Following the best practice approach, HHT was identified as the candidate agent for its highest reversal potency. Since the query signature (Sig_{evo}) possessed the liver cancer initiation and development-associated properties simultaneously, we considered that HHT might have both chemotherapeutic and chemopreventive effects for liver cancer accordingly. For validating the chemotherapeutic effect of HHT, drug sensitivity data from PRISM was analyzed and cell proliferation assays were conducted. The remarkable tumor-killing activity of HHT suggested it could be novel therapeutics for liver cancer. For validating the chemopreventive effect of HHT, an indirect approach focused on investigating the anti-fibrotic effect of HHT was adopted. Our results showed that the expression level of fibrosis-associated genes was dramatically decreased when challenging HSCs with HHT treatments. Inhibition of liver fibrogenesis might prevent the progression of cirrhosis and thereby suppress

HCC tumorigenesis [25]. Therefore, we supposed that HHT had the potential to be taken as chemopreventive agents for liver cancer as well. Notably, in view of the grim prognosis and imperfect treatment modalities of liver cancer, prevention of HCC development in patients at high risk of primary malignancy or following curative treatment rather than treating patients at advanced stages with limited health benefit is theoretically the most desirable approach to improve patient prognosis [27, 37]. As HHT has been approved by FDA for the treatment of chronic myelogenous leukemia (CML) and widely used in clinical practice without serious adverse effects, it may provide a new chemopreventive strategy that merits further validation in clinical trials [38].

In this study, we have performed the most comprehensive surveys so far about the influencing factors involved in ‘signature reversion’-based drug repositioning. Two novel benchmarking standards are proposed, which provide insight into the evaluation of methodologies developed for signature reversion purpose. Moreover, all the findings in this study are verified independently by at least two different approaches, which can eliminate potential bias and guarantee the reliability of the results. Nevertheless, we also recognize several important limitations. First, with our design, our conclusions are conditional and hold only under the conditions of using compound profiles of HepG2 in LINCS as reference signatures. Further investigations using other LINCS data are required to extend current conclusions to other conditions. Second, the parameters recommended by us, including topN of 200 and query signature size of 100, are more or less based on our subjective judgments and should be taken as a rough guide. Although there are sufficient non-quantitative estimates supporting the use of these two parameters, more efforts are still needed to quantitatively and accurately determine the optimal parameters for this computational approach. Third, in this study we only focused on analyzing the data from the project that utilized transcriptomic platforms to measure cell responses during perturbation experiments, and other omics data which are actively being generated by different LINCS centers might also be a good choice for computational drug discovery and

587 repositioning [39]. Recently, large-scale resources (CPPA) of perturbed protein
588 responses have been generated [40]. Considering that proteins are the components of
589 the basic functional units in biological pathways, investigating the optimal
590 repositioning strategy based on proteomic resources should also have important
591 implication.

592

593 **4. Conclusions**

594 In summary, our findings fill a knowledge gap in the area of LINCS-based
595 computational repositioning. Through exploiting these findings, we also determined a
596 promising anti-liver cancer agent HHT, of which the chemotherapeutic and
597 chemopreventive effects have been validated experimentally.

598

599 **5. Experimental Section**

600 **5.1.Data collection**

601 LINC data were downloaded from Gene Expression Omnibus (GEO) database
 602 (Phase I: GSE92742, Phase II: GSE70138). Molecular data of clinical and animal
 603 datasets were obtained from The Cancer Genome Atlas (TCGA), the Genotype-Tissue
 604 Expression (GTEx), GEO and the ArrayExpress databases. Dependency and
 605 pharmacogenomic data of cell lines were achieved from the Dependency Map
 606 (DepMap) portal. For detailed description, please see the [Additional file 1:](#)
 607 [Supplementary Methods.](#)

609 **5.2.AUC-based benchmarking standard**

610 Since establishing benchmarking standards and datasets across all LINC cell lines
 611 seems to be an enormous undertaking, we mainly focused exclusively on one cell line
 612 (HepG2) and one disease (liver cancer) in this study. Two benchmarking standards,
 613 namely area under curve (AUC)-based standard and Kolmogorov-Smirnov (KS)
 614 statistic-based standard, were generated for evaluating the retrieval performance of
 615 disease-compound similarity metrics across different conditions. For establishing
 616 AUC-based standard, we collected the drug response data from multiple data sources.
 617 Compound IC₅₀s tested in HepG2 cell line was achieved from ChEMBL (version 27)
 618 [41] and Liver Cancer Model Repository (LIMORE) [42] datasets. Compounds
 619 among LINC, ChEMBL, and LIMORE were mapped using compound name
 620 followed by manual inspection. Each experiment provided in ChEMBL was also
 621 manually checked to ensure the compliance with our requirement. Due to the
 622 redundancy of IC₅₀s, the median IC₅₀s of certain compound among duplicates was
 623 used for representing the activity of this compound. We categorized the compounds
 624 into effective (IC₅₀ < 10μM) and ineffective group (IC₅₀ ≥ 10μM) according to
 625 previous study [10b]. The ability to distinguish between effective and ineffective
 626 compounds was taken as a measurement of retrieval performance of different
 627 similarity metrics (namely AUC value) [10b]. Notably, some have argued that partial

AUC (limiting false positive rate at 0.1/0.01) might be a more relevant statistic for actual application of drug repositioning [21b, 22]. However, due to the limited size of our benchmarking dataset, adopting partial AUC with even less information could result in loss of statistical precision, and thus the use of the full AUC is preferred in this situation. The statistical significance of AUCs were calculated through performing permutation test. Briefly, we randomly permuted the class labels of the feature vectors and created 10,000 permutations to form a distribution of ‘random’ AUCs. Then, the *P* value was determined according to the fraction of ‘random’ AUCs greater than or equal to the observed AUC [21b]. For distinguishing, AUC used for evaluating drug retrieval performance was renamed as drug retrieval-associated AUC (DR-AUC), the higher values of which indicate better performance.

5.3.KS statistic-based benchmarking standard

To avoid confusion, it should be noted that KS-based method was also used for calculating disease-compound similarity scores, and the specific details were described below. For generating the benchmarking dataset required for the KS statistic-based standard, we systematically surveyed clinical trials involved in HCC treatment and compiled a set of potential HCC agents (clinicaltrials.gov). Preliminary retrieval yielded 1,999 results, and after removing trials failing to fulfil our requirements, we obtained 254 potential therapeutic agents for HCC. The detailed retrieval process was presented in Figure 3A. To minimize potential selection bias, this process was performed independently by two investigators (C.Y. and X.H.). Perturbagen-induced expression profiles of 27 agents among these tested in HepG2 cell line are available in LINCS dataset. Based on the assumption that HCC-associated agents are more likely to reverse HCC signature than random agent combinations, the enrichment capabilities of different similarity metrics could be used to assess their repositioning performance. The calculation of enrichment score (ES) of HCC agents was generally identical with that in gene set enrichment analysis [43]. Considering that there was no need accounting for the size of the agents set, we did

not calculate the normalized enrichment score (NES) that might introduce additional randomization. To obtain a nominal P value, we created 10,000 permutations and recomputed the ES for each permutation to form a null distribution. The significance P of the observed ES was then determined relative to the null distribution. Notably, ES used in retrieval performance evaluation was renamed as drug retrieval-associated ES (DR-ES), the lower values of which represent better performance.

5.4. Signature matching methods

The retrieval performance of six different matching methods, including eXtreme Sum (XSum) [21b], eXtreme Cosine (XCos) [21b, 22], eXtreme Pearson (XCor) [23], eXtreme Spearman (XSpe) [23], Kolmogorov-Smirnov (KS) test [4], and the Reverse Gene Expression Score (RGES) [10b], was systematically benchmarked. Based on the consideration that small variations in expression changes may be noise without biological significance, the eXtreme methods only utilized top up- and down-regulated genes in compound signatures for similarity score calculation (all remaining genes were assigned the values of zero). By comparison, KS and RGES methods use complete compound profiles as reference signatures. The detailed features and scoring schemes of these methods are described as follows.

The XSum method handles the up- and down-regulated genes separately. In brief, the sums of the change values in reference/compound signatures relative to up-regulated query/disease genes (sum_{up}) and down-regulated query/disease genes (sum_{down}) are first calculated. Then, XSum is defined as following: $\text{XSum} = \text{sum}_{\text{up}} - \text{sum}_{\text{down}}$. Other three eXtreme methods, including XCos, XCor, and XSpe, take disease signatures as a whole to query compound signatures, and they calculated the correlation between the numeric vectors of disease and compound signatures using cosine similarity, Pearson correlation, and spearman correlation, respectively. Notably, cosine similarity is nearly identical with Pearson correlation except without centering vectors around the mean values. The KS method was adopted by the first CMap study and has been the most widely used method for connecting disease signatures to

compound signatures [4]. Similar to XSum, KS method also needs to sperate disease signatures into two gene sets, up-regulated gene set and down-regulated gene set, and ignores the magnitude of differential expression. Briefly, using complete compound profiles as reference, maximum deviation (MD)-based enrichment scores of up-regulated gene set (es_{up}) and down-regulated gene set (es_{down}) are first computed. If es_{up} and es_{down} have the same algebraic sign then $KSscore = 0$, otherwise, $KSscore = es_{up} - es_{down}$. The RGES method is a recently proposed modification of the original KS method, which was demonstrated to perform better in drug response prediction than KS method [10b]. In contrast to original KS method, RGES focuses on the reversal relation between the disease and agents, and RGES is defined as $es_{up} - es_{down}$ regardless of the sign of es_{up} and es_{down} . In addition to the above six methods, there also exist many other methods, such as WSS/sscMap [44], TES [45], ProbCmap [36], NFFinder [46], EMUDRA [23], and so on, for calculating the similarity between disease and compound signatures. However, some of them are not accessible currently and some are developed based on the data of initial CMap dataset (1,309 compounds), and accordingly, we did not include these methods into our analyses.

702

703 **5.5.Generation of query signatures for performance evaluation**

For evaluating retrieval performance of similarity metrics at different conditions, we prepared four HCC-associated gene signatures to query LINCS. Two of them, Sig_{gastro} and Sig_{NC} , are achieved from previously published studies [10]. Given that the development of these two signatures were mainly based on LIHC cohort, as a complement, the other two were generated from another RNAseq cohort (LIRI) and a microarray cohort (GSE54236), respectively. The differentially expressed genes in LIRI cohort were computed using *edgeR* package (version 3.26.5) on raw count data [47]. For microarray data, we used *limma* package (version 3.40.2) to conduct differential expression analysis on normalized data [48]. The statistically significant differential genes in above analyses were defined as adjusted $P < 0.01$ and absolute $\log_2$ fold change (FC) > 1 . As a result, we obtained a 70-gene signature (Sig_{LIRI}) with

715 48 up- and 22 down-regulated genes from LIRI and a 28-gene signature (Sig_{GSE54236})
 716 with 22 up- and 6 down-regulated genes from GSE54236 respectively, which could
 717 represent discordant expression pattern of HCC. Considering that the gene numbers in
 718 signatures created through differential expression analysis were much less than that in
 719 prognostic signatures (see section below), to make comparison of retrieval
 720 performance between these two signature phenotypes without being subject to the
 721 signature size, we relaxed the significance threshold of differential genes to $P < 0.01$
 722 and $\log_2FC > 0.5$, and built two increased signatures which included 125 genes (LIRI)
 723 and 116 genes (GSE54236) respectively. These two increased signatures were also
 724 used to explore the potential influences of signature size.

725

726 **5.6.Construction of size-diversified query signatures**

727 We adopted two independent approaches to explore whether the differences of
 728 query/disease signature size could affect subsequent drug retrieval. The first approach
 729 was based on iterating the threshold of fold change values, ranging from 0.1/-0.1 to
 730 the maximum/minimum with a increment/decrement of 0.05, which could obtain a
 731 number of query signatures with a diversity of signature size (duplicates were
 732 removed). As for the second approach, above two increased signatures, 125-gene
 733 signature from LIRI and 116-gene signature from GSE54236, were taken as the basis
 734 for generating smaller-size testing signatures. Briefly, we randomly extracted testing
 735 signatures from complete signatures, with the size ranging from the minimum of five
 736 to the maximum of 124 or 115. To avoid bias, this process was repeated 1,000 times to
 737 generate 1,000 testing signatures for each signature size.

738

739 **5.7.Construction of query signatures representing different clinical phenotypes**

740 To investigate whether the clinical phenotype of signature was potential factor
 741 affecting the retrieval performance, we developed two strategies, a forward strategy
 742 starting from generation of signatures with distinguishing clinical phenotypes to the
 743 evaluation of retrieval performance and a backward strategy starting from obtaining

signature with the best performance to the comprehensive investigations of its clinical implication. For the first strategy, to compare with general HCC signature representing discordant expression pattern, two prognostic signatures based on LIRI and GSE54236 cohorts were constructed. We integrated survival data with expression data and performed Cox proportional hazards regression to assess association between overall survival and gene expression. The statistically significant prognostic genes were defined as $P < 0.005$. A 133-gene prognostic signature with 117 poor- and 16 good-outcome genes was generated based on LIRI, while analysis on GSE54236 resulted in a 107-gene prognostic signature with 79 poor- and 28 good-outcome genes. Comparisons of drug retrieval performance between these two types of signatures were carried out subsequently. For the second strategy, taking 978 landmark genes as a basis, simple random sampling without replacement (SRSWOR) was performed to extract genes from landmarks for forming candidate signatures. The size of randomized signatures was set at 100 and the process of random sampling was repeated 10,000 times to obtain a collection of 10,000 randomized signatures. The DR-AUC and DR-ES values were then calculated for each signature, and the optimal one was defined as the signature with the minimum of DR-AUC multiplying DR-ES.

761

762 **5.8.Generation of evolution-associated query signature**

To find compounds with potential in the prevention and treatment of liver cancer, we developed a hepatocarcinogenesis and progression-associated signature. GSE89377 cohort was utilized to build this signature while GSE6764 cohort was taken for external validation. To identify genes associated with developmental stages, we constructed Random Forests (RF) model taking stages as dependent variable. Variable importance was assessed with the mean decrease accuracy (MDA) measures for individual factors in RF model. Variables with positive MDA values are of high importance in predicting stages. In other words, these variables are more likely to be related with liver cancer development and progression (negative MDA values can be regarded as equivalent to zero importance with no predictive power). The RF analysis

773 was independently repeated 1,000 times with 1,000 trees growing each time, and
774 genes with positive MDA values incorporated in more than 200 iterations were kept
775 for subsequent analyses.

776 We next performed weighted gene co-expression network analysis (WGCNA) to
777 assign resultant genes into modules according to expression similarity, and
778 simultaneously, to recognize the trajectories of gene expression during liver cancer
779 development [49]. First, an appropriate soft threshold was estimated by using the
780 *pickSoftThreshold* function in *WGCNA* package. Then, we constructed WGCNA
781 network and detected gene expression modules using *blockwiseModules* function with
782 a minimum module gene number of 50, soft thresholded power of 12, and a
783 dendrogram cut height of 0.3. Genes without assignment to specific modules were
784 assigned the color of grey. Module eigengenes (MEs) representing the first principal
785 components (PC1) of each module were returned, and the module-trait relationship
786 (MTR) analysis was conducted by calculating the correlation between MEs and
787 developmental stages. The expression trend of each module across seven stages of
788 HCC development was visualized through using mean PC1 values of samples in each
789 stage to generate trend curves. According to the correlation coefficient of MTR
790 analysis and the visualized expression trend of each module, two modules exhibiting
791 the highest positive/negative correlation with developmental stages as well as
792 showing gradually increasing or decreasing expression trends were selected for their
793 potential functional roles during HCC initiation and development. Subsequently, to
794 explore the biological processes associated with genes in these two modules, we
795 conducted hypergeometric test based on the hallmark gene sets (h.all.v7.0.symbols)
796 downloaded from the Molecular Signatures Database (MSigDB) using *enricher*
797 function in *clusterProfiler* package [50]. The *P* values from the hypergeometric tests
798 were adjusted for multiple comparison testing and an adjusted *P* value less than 0.05
799 was considered significantly enriched.

800 Genes in these two modules were mapped to the 978 landmark genes, resulting in
801 a 159-gene panel (134 genes in ascending module and 25 genes in descending

module). According to the findings described in *Results* section, we decided to further narrow down this panel to create a query signature with the size of 100 genes. The molecular and clinical data in GSE15654 were utilized to determine the association between the expression patterns of signatures and the occurrence of HCC. Briefly, we first performed SRSWOR to extract a subset of 100 genes from the 159-gene panel, repeated 10,000 times. The processes of random sampling were conducted separately for ascending and descending genes, to ensure a minimum descending gene number of two in the randomized signatures. As a result, 10,000 randomized signatures with 100 genes per signature were generated. Next, PC1 values of all randomized signatures were extracted based on expression data from GSE15654 to represent the overall expression patterns of these signatures, and the follow-up data using HCC occurrence as endpoint was then integrated with above expression pattern data for subsequent Cox proportional hazards regression (COXPH). The signature which had the minimum *P* value across the 10,000 COXPH analyses was considered as the optimal signature and retained for querying LINCS. The expression trend of this signature was further validated GSE6764 cohort.

5.9. Experimental methods

All experimental methods, including cell proliferation assays, quantitative real-time PCR, western blotting analysis, immunofluorescence, RNA sequencing, etc., were described in the [Additional file 1: Supplementary Methods](#).

5.10. Statistical analysis

All the computational analyses and graphical visualization were performed in R software, version 3.6.0 (<https://cran.r-project.org/>). Unless stated otherwise, correlation between two continuous variables was measured by Spearman's rank-order correlation, and pairwise comparisons were conducted using Kruskal-Wallis and Wilcoxon sum rank tests. ROC curves and AUC values were visualized and calculated using the pROC package[51]. The hazard ratio (HR) was

831 estimated using Cox regression model in *survival* R package. Cumulative hazard
832 curve was carried out using *jskm* package and the log-rank test was used to determine
833 the statistical significance of differences. A two-tailed $P < 0.05$ was considered
834 significant unless indicated otherwise.

835

836 **Additional files**

837 Additional file 1: Supplementary methods and Supplementary discussion. (DOCX 113
838 kb)

839 Additional file 2: Supplementary figures 1-15. (PDF 4333 kb)

840 Additional file 3: Supplementary tables 1-12. (XLS 2279 kb)

841

842 **Availability of data and materials**

843 Sequencing data necessary for the analysis are available within Supplementary Data.

844 The rest of the data supporting the findings of this study are available from the

845 corresponding author upon reasonable request. The codes are available at

846 https://github.com/YangJAT/Best_practices_for_LINCS.

847

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851

852 **Conflict of interest**

853 The authors declare no conflict of interest.

854

855 **Authors' Contributions**

856 C.Y. and M.C. designed the study and performed the computational analyses. S.W.

857 and X.H. and R.Q. performed the *in vitro* validation experiments. C.Y., J.W. and Z.L.

858 wrote the manuscript. Q.W. provided guidance on manuscript preparation. H.W., H.H.

859 and C.W. supervised the study.

860

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1066 **Figure Legends**

1067 **Fig. 1 Overview of LINCS data-driven therapeutic discovery.** The working
1068 principle of ‘signature reversion’-based computational approach. A disease signature
1069 representing discordant expression pattern needs first to be identified (G1, G2, and G3
1070 stand for up-regulated genes while G4, G5, and G6 stand for down-regulated genes in
1071 disease state). With this signature, pharmacologic perturbation datasets can be queried
1072 to find compounds with the ability to reverse disease expression pattern (suppress
1073 expression of G1, G2, and G3 and induce expression of G4, G5, and G6). After
1074 determining the candidate compounds, experimental and clinical validation are
1075 required to translate computational findings to clinical applications.

1076
1077 **Fig. 2** Highly cell-type specific compound-induced expression changes. **a**
1078 Two-dimensional t-SNE projection based on cosine distance between compound
1079 signatures. Each dot represents a unique perturbation-induced expression profile, and
1080 each color represents one type of cell line. **b** Schematic diagram displaying the
1081 calculation process of compound-level (L2) and MOA-level (L3) similarity scores. **c**
1082 Distribution of compound-level (L2) cosine similarity scores, which ranging from -1
1083 (completely opposite pattern) to 1 (perfectly identical pattern). Three examples are
1084 presented (left to right: etodolac, geldanamycin, and doxorubicin). **d** Illustration of
1085 MOA-level (L3) similarities. Only MOAs with more than five compounds included
1086 are shown in figure. **e** A t-SNE projection showing the distribution of compounds
1087 (indicated by purple dots) in top ranked five MOAs (including HDAC inhibitors, IKK
1088 inhibitors, mTOR inhibitors, CDK inhibitors, and topoisomerase inhibitors). **f**
1089 Schematic diagram displaying the calculation process of cell line pair-level (L4)
1090 similarity scores. **(g)** Correlation between basal expression similarities and perturbed
1091 expression similarities (L4) of 36 cell line pairs (nine cell lines in total). Statistical
1092 significance and correlation coefficient were determined by ranked-based Spearman
1093 correlation. **h** Schematic view of the calculation of cell line-level (L5) similarity
1094 scores (upper) and the presentation of L5 similarity scores of nine cell lines in the

1095 boxplot (lower).

1096 **Fig. 3** Benchmarking different methodologies and parameters to determine the
 1097 optimal computational strategy. **a** Diagram summarizing the workflow and the
 1098 important components involved in the evaluation process of drug retrieval
 1099 performance of six different signature matching methods. **b** Retrieval performance of
 1100 six matching methods evaluated by AUC-based benchmarking standard (left) and KS
 1101 statistic-based benchmarking standard (right). **c** Visualization of AUC-based (left) and
 1102 KS statistic-based (right) performance measurements of XSum method on
 1103 standardized data for discerning the optimal operating parameter. **d** Diagram
 1104 summarizing the workflow and the important components associated with the
 1105 investigation process of the optimal query signature size. **e** Relationship between
 1106 query signature size determined by iterative fold change-based approach and retrieval
 1107 performance evaluated by AUC-based standard (left) and KS statistic-based standard
 1108 (right). **f** Relationship between query signature size determined by random
 1109 sampling-based approach and retrieval performance evaluated by AUC-based
 1110 standard (left) and KS statistic-based standard (right). LOESS polynomial regression
 1111 analysis was performed for curve fitting. Query signature for above analyses was
 1112 generated based on LIRI cohort, and evaluation results using other query signatures
 1113 were presented in Supplementary Figure 5.

1114

1115 **Fig. 4** Developing a novel signature, Sig_{evo}, for drug repositioning. **a** Schematic of the
 1116 stepwise process of liver cancer initiation and progression. **b** Preliminary screening of
 1117 developmental stage-associated genes by Random Forests algorithm. **c** The expression
 1118 pattern of the ‘ascending’ module discerned by WGCNA analysis (left) and the
 1119 enriched biological processes determined by hypergeometric test (right). **d** The
 1120 expression pattern of the ‘descending’ module (left) and the enriched biological
 1121 processes (right). **e** The performance evaluation of the Sig_{evo} for discerning the
 1122 difference between tumor and normal tissues based on RNA sequencing cohorts (left)

1123 and microarray cohorts (right). **f** The association between the Sig_{evo} and the clinical
1124 phenotype of prognosis. Color toward gray indicates no statistical significance. **g** The
1125 association between the Sig_{evo} and fibrosis-related phenotype suggested by ROC
1126 curve. **h** The association between Sig_{evo} and CCl4-induced expression changes in liver
1127 tissues of mice. The enrichment scores and statistical significance were determined by
1128 gene set enrichment analysis. **i** The association between Sig_{evo} and DEN-induced
1129 expression changes in liver tissues of rats.

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1131 **Fig. 5** Identifying HHT as repositioning candidate with *in vitro* tumor killing activity.

1132 **a** Results of best practice approach-based computational drug repositioning using
1133 Sig_{evo} as query signature. Top ranked 10 compounds with highest reversal potency
1134 were illustrated in the right panel. **b** Enrichment of HCC agents in compounds with
1135 reversal potency (XSum score < 0). The statistical significance was determined based
1136 on the null distribution formed by 10,000 permutations. **c** 2D (left) and 3D (right)
1137 chemical structure of homoharringtonine (HHT). Data was derived from DrugBank. **d**
1138 Comparison of distribution of compound activity between HHT and three different
1139 drug categories, including chemotherapy (n = 45 compounds), targeted cancer agents
1140 (n = 419 compounds), and non-oncology (n = 362 compounds). The IC₅₀ value of each
1141 drug category in each cell line (n = 482) was determined through calculating the
1142 median IC₅₀ value across all the compounds in this category. **e** The drug sensitivity
1143 data of HHT (achieved from PRISM dataset) across different HCC cell lines. The
1144 drug sensitivities of two HCC agents in the first-line (sorafenib and lenvatinib) and
1145 one HCC agent in the second-line (regorafenib) were also illustrated for comparison.
1146 Areas with different colors denote the interquartile range of median IC₅₀ values of
1147 compounds within different drug categories. **f** Long-term cell proliferation assay for
1148 testing the anti-tumor activity of HHT across 11 liver cancer cell lines. Of these, five
1149 cell lines have not been profiled by PRISM for the sensitivity to HHT. **g** Therapeutic
1150 potency of the co-administration of HHT and sorafenib across 11 cell lines. **h** Two
1151 representative surface plots showing the overall synergistic (left) and antagonistic

1152 (right) effects of the combination treatments.

1153

1154 **Fig. 6** Assessing the anti-fibrotic effects of HHT. **a** Differential expression of nine
 1155 fibrosis-associated genes between HHT-treated and HHT-untreated LX-2 cells. **b**
 1156 Quantitative real-time PCR-based mRNA expression level of collagen I (upper) and
 1157 α -SMA (lower) of LX-2 cells treated with gradient concentrations of HHT for 6 hours.
 1158 **c** Western blot-based protein expression level of collagen I and α -SMA of LX-2 cells
 1159 treated with gradient concentrations of HHT for 24 hours. **d** Representative images of
 1160 immunofluorescence stainings of LX-2 cells with antibody against collagen I (upper)
 1161 and α -SMA (lower).

Computational drug discovery and repurposing

Collect disease samples for obtaining molecular profiles (e.g., liver cancer)

Establish disease gene expression signature

Identify compounds that antagonize the disease signature

Validation for computational results

Approved for marketing

FDA Review

Clinical trials (Phase I/II/III)

Preclinical evaluation

In vitro

In vivo