The Effect of Compound Kushen Injection on Cancer Cells: Integrated Identification of Candidate Molecular Mechanisms.

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Abstract

Background: Because Traditional Chinese Medicine (TCM) preparations are often combinations of multiple herbs containing hundreds of compounds, they have been difficult to study. Compound Kushen Injection (CKI) is a complex mixture cancer treatment used in Chinese hospitals for over twenty years.

Purpose: To demonstrate that a systematic analysis of molecular changes resulting from complex mixtures of bioactives from TCM can identify a core set of differentially expressed (DE) genes and a reproducible set of candidate pathways.

Study Design: We used a cancer cell culture model to measure the effect of CKI on cell cycle phases, apoptosis and correlate those phenotypes with CKI induced changes in gene expression.

Methods: We treated cancer cells with CKI in order to generate and analyse high-throughput transcriptome data from two cancer cell lines. We integrated these differential gene expression results with previously reported results.

Results: CKI induced cell-cycle arrest and apoptosis and altered the expression of 363 core candidate genes associated with cell cycle, apoptosis, DNA replication/repair and various cancer

pathways. Of these, 7 are clinically relevant to cancer diagnosis or therapy and 14 are cell cycle regulators, and most of these 21 candidates are downregulated by CKI. Comparison of our core candidate genes to a database of plant medicinal compounds and their effects on gene expression identified one-to-one, one-to-many and many-to-many regulatory relationships between compounds in CKI and DE genes.

Conclusions: By identifying promising candidate pathways and genes associated with CKI based on our transcriptome-based analysis, we have shown this approach is useful for the systematic analysis of molecular changes resulting from complex mixtures of bioactives.

Keywords: Compound Kushen Injection, cancer cell, transcriptome, multiple targets, cell cycle, apoptosis

Abbreviations:

DE, differentially expressed; TCM, traditional Chinese medicine; CKI, compound Kushen injection; GO, Gene Ontology; DO, Disease Ontology; KEGG, Kyoto Encyclopedia of Gene and Genomes; PI, propidium iodide.

1   Introduction

The treatments of choice for cancer are often radiotherapy and/or chemotherapy, and while these can be effective, they can cause quite serious side-effects, including death. These side-effects have driven the search for adjuvant therapies to both mitigate side-effects and/or potentiate the effectiveness of existing therapies. Traditional Chinese Medicine (TCM) is one of the options for adjuvant therapies, particularly in China, but increasingly so in the West. While clinical trial data on the effectiveness of TCM is currently limited, it remains an attractive option because of its long history and because its potential effectiveness is believed to result from the cumulative effects of multiple compounds on multiple targets [13]. Because TCM often has not been subjected to rigorous evidence-based assessment and because it is based on an alternative theoretical system compared to Western medicine, adoption of its plant derived therapeutics has been slow.

In this report, we continue to characterize the molecular effects of Compound Kushen Injection

13 (CKI) on cancer cells. CKI has been approved by the State Food and Drug Administration

14 (SFDA) of China for clinical use since 1995 [31] (State medical license no. Z14021231). CKI is an

15 herbal extract from two TCM plants, Kushen (*Sophora flavescens*) and Baituling (*Smilax Glabra*)

16 and contains more than 200 different chemical compounds. These compounds include alkaloids

17 and flavonoids such as matrine, oxymatrine and kurarinol that have been reported to have

18 anti-cancer activities [31, 47, 35, 48]. Some of these activities have been shown to influence the

19 expression of TP53, BAX, BCL2 and other key genes known to be important in cancer cell growth

20 and survival [45, 38, 25, 17].

21 We have previously characterized the effect of CKI on the transcriptome of MCF-7 breast

22 carcinoma cells and in this report, we extend our previous results to two additional human cancer

23 cell lines (MDA-MB-231, breast carcinoma and Hep G2, hepatocellular carcinoma). Both cell lines

24 have also been shown to undergo apoptosis in response to the ingredients of CKI [35, 48, 38, 44].

25 Hep G2 is one of the most sensitive cancer cell lines with respect to exposure to CKI [39] and CKI

26 is often used in conjunction with Western chemotherapy drugs for the treatment of liver cancer

27 patients in China. While the specific mechanism of action of CKI is unknown, several recent

28 studies have reported that CKI or its primary compounds affect the regulation/expression of

29 oncogene products including β-catenin, TP53, STAT3 and AKT [31, 35, 22, 18, 41].

30 However, these and other reports did not evaluate the entire range of molecular changes from

31 treatment with a multi-component mixture such as CKI [10, 8]. Whilst several research databases

32 and tools for TCM research have been developed [32, 34, 5], they are limited by the fact that most

33 of the studies that contribute to the corpus of these databases are from different experimental

34 systems, use single compounds or measure effects based on one or a handful of genes/gene

35 products.

36 In contrast to previous studies, our strategy was to carry out comprehensive transcriptome

37 profiling and network reconstruction from cancer cells treated with CKI. Instead of focusing on

38 specific genes or pathways in order to design experiments, we have linked phenotypic assessment

39 and RNA-seq analysis to CKI treatment. This allows us to present an unbiased, comprehensive

40 analysis of CKI specific responses of biological networks associated with cancer. Our results

41 indicate that different cancer cell lines that undergo apoptosis in response to CKI treatment can

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exhibit different CKI induced gene expression profiles that nonetheless implicate similar core genes and pathways in multiple cell lines.

The current study presents the effects of CKI on gene expression in cancer cells with an aim to identify candidate pathways and regulatory networks that may be perturbed by CKI *in vivo*. To this end we primarily use concentrations of CKI higher than used *in vivo* in order to be able to detect effects in the short time frames available to tissue culture experiments. We also combine our current analysis with previously published data to focus on a shared, much smaller set of candidate genes and pathways.

Material and Methods

Cell culture and reagents

CKI (total alkaloids concentration of 25 mg/ml) in 5 ml ampoules was provided by Zhendong Pharmaceutical Co. Ltd. (Beijing, China). Chemotherapeutic agent, Fluorouracil (5-FU) was purchased from Sigma-Aldrich (MO, USA). A human breast adenocarcinoma cell line, MDA-MB-231 and a hepatocellular carcinoma cell line Hep G2 were purchased from American Type Culture Collection (ATCC, VA, USA). The cells were cultured in Dulbecco's Modified Eagle Medium (Thermo Fisher Scientific, MA, USA) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific). Both cell lines were cultured at 37°C with 5% CO_2.

For all *in vitro* assays, 4×10^5 cells were seeded in 6-well trays and cultured overnight before being treated with either CKI (at 1 mg/ml and 2 mg/ml of total alkaloids) or 5-FU (150 μg/ml for Hep G2 and 20 μg/ml for MDA-MB-231). As a negative control, cells were treated with medium only and labelled as "untreated". After 24 and 48 hours of treatment, cells were harvested and subjected to the downstream experiments.

Cell cycle and apoptosis assay

The assay was performed as previously described [28]. For each cell line, three operators replicated the assay twice in order to ensure reproducibility of the observations. The results were obtained by flow cytometry using either FACScanto or LSRII (BD Biosciences, NJ, US).

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RNA isolation and sequencing

The treated cells were harvested, and the cell pellets were snap frozen with liquid nitrogen and stored at -80°C. Total RNA was isolated with PureLink^{TM} RNA Mini Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. After quantified using a NanoDrop Spectrophotometer ND-1000 (Thermo Fisher Scientific), the quality of the total RNA was verified on a Bioanalyzer by Cancer Genome Facility (SA, Australia) ensuring all samples had RINs>7.0.

For both cell lines, the sequencing was performed in Ramaciotti Centre for Genomics (NSW, Australia). The sample preparation for each cell line was TruSeq Stranded mRNA-seq with dual indexed, on the NextSeq500 v2 platform. The parameter was 75bp paired-end High Output. The fastq files were generated and trimmed through Basespace with application FASTQ Generation *v1.0.0.*

Bioinformatics analysis of RNA sequencing

The clean Hep G2 reads were aligned to reference genome (hg38) using STAR v2.5.1 with following parameters: –outFilterMultimapNmax 20 –outFilterMismatchNmax 10 –outSAMtype BAM SortedByCoordinate –outSAMstrandField intronMotif [6]. The clean MDA-MB-231 reads were aligned to reference genome (hg19) using TopHat2 v2.1.1 with following parameters: –read-gap-length 2 –read-edit-dist 2 [15]. Differential expression analysis for reference genes was performed with edgeR and differentially expressed (DE) genes were selected with a False Discovery Rate<0.05 [29].

The DE genes in common for both Hep G2 and MDA-MB-231 cell lines at 24 hours and 48 hours after CKI treatment were selected as "shared" genes. These shared genes were utilized to describe the major anti-cancer functions and principal mechanisms of CKI.

Gene Ontology (GO), and Kyoto Encyclopedia of Gene and Genomes (KEGG) over-representation analyses of both cell lines were carried out using the online database system ConsensusPathDB [14] with the following settings: "Biological process" at third level (for GO); q values (<0.01) were corrected for multiple testing with the system default settings. Disease ontology (DO) over-representation analyses of both cell lines were performed by using the Bioconductor R package clusterProfiler v3.5.1 [40]. For the function analyses of shared/core genes,

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96 the method was as similar as our previous study [28] using ClueGO app 2.2.5 in Cytoscape v3.6.0.

97 We enriched our GO terms in the biological process category level 3 and KEGG pathways,

98 showing only terms/pathways with p values less than 0.01. Specific Over-represented

99 terms/pathways and gene expression status mapping in KEGG pathways were visualised with the

100 R package "Pathview" [21].

101 *Gene expression-based investigation of bioactive components in CKI*

102 To integrate with previous data from MCF-7 cells [28], all the shared DE genes regulated by

103 CKI identified in all three cell lines using edgeR were mapped to the BATMAN-TCM database

104 [19]. The pharmacophore modelling method [16] was used to generate the interaction network

105 between the key genes and TCM components using R package igraph [4].

106 *Reverse transcription quantitative polymerase chain reaction (RT-qPCR)*

107 RT-qPCR was performed as previously described [28]. The list of target genes selected for this

108 study and the sequences of all primers are shown in Additional file 1: Table S1.

Results

110 *Effect of CKI on the cell cycle and apoptosis*

111 In our previous study, CKI significantly perturbed/suppressed cancer cell target genes/networks.

112 In the current study we present results that confirm and generalize our previous work. We

113 observed in the MCF-7 study, low concentrations of CKI in our short-term cell assay showed

114 no/little phenotypic effect within 48 hours, and very high doses resulted in excessive cell death at

115 48 hours precluding the isolation of sufficient RNA for transcriptome analysis [28]. Therefore, in

116 our current study with the two additional cell lines, to ensure consistency, we also selected 1

117 mg/ml and 2 mg/ml total alkaloid concentrations of CKI for our assays because they generated

118 reproducible and significant phenotypic effects in our cell culture assay.

119 We used flow cytometric analysis of propidium iodide stained cells to assess both CKI induced

120 alterations to the cell cycle and apoptosis. In Hep G2 cells, CKI treatment resulted in an overall
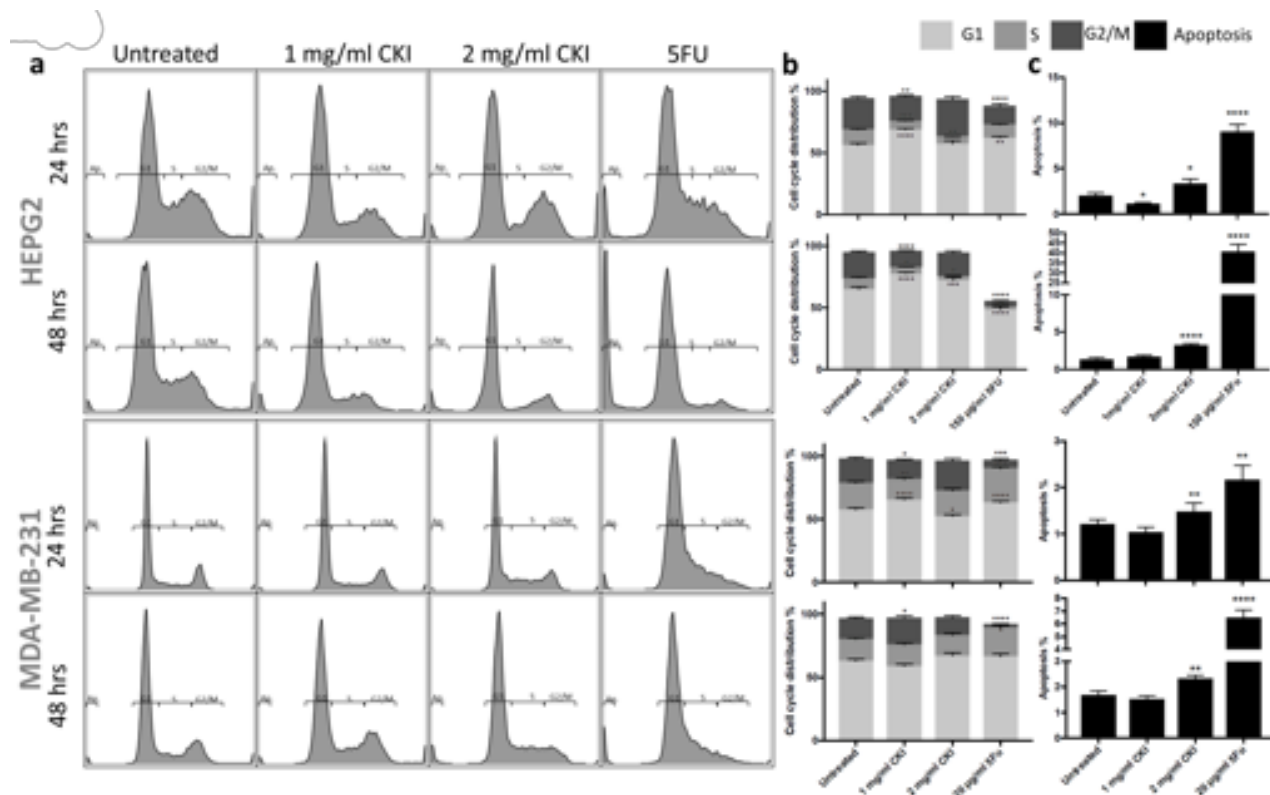
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Figure 1. Effects of different treatment on cell cycle and apoptosis of Hep G2 and MDA-MB-231 cells. A) The apoptosis and cell cycle distribution of each cell line after 24- and 48-hour treatments with CKI or 5-Fu assessed PI staining. B) Percentages of cells in different phases of cell cycle resulting from treatment. c Percentage of apoptotic cells after treatment. Results shown are mean ±SEM (n=6). Statistically significant differences from untreated control were identified using two-way ANOVA (*p<0.05, **p<0.01, ****p<0.0001).

121 increase in the proportion of cells in G1 phase and decrease in S phase (Fig. 1a and b). Similarly,

122 in MDA-MB-231 cells, although a consistent increase in G1 phase was not observed, CKI caused a

123 decrease in S phase particularly at the 24-hour time point (Fig. 1a and b) indicating possible

124 incidence of cell cycle arrest at G1 phase. Furthermore, at 2 mg/ml of total alkaloids, CKI

125 consistently induced significantly higher level of apoptosis in both cell lines at both time points

126 compared to untreated controls (Fig. 1c). These data together suggest that CKI has effects on the

127 cell cycle by interfering with the transition between G1 to S phase as well as by acting on the

128 apoptosis pathway and promoting cell death.

129 *CKI perturbation of gene expression*

130   In order to elucidate the molecular mechanisms of action of CKI on these cancer cells,

131 transcriptome analysis of CKI treated cells was performed. As mentioned above, RNA samples

132 from two cell lines were sequenced with 2×75 bp paired-end reads. We had previously sequenced

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133 transcriptomes from CKI treated MCF-7 cells [28] and have included those results for comparison

134 below. The samples from each cell line contained 7 groups at 3 time points (Fig. 2a), in triplicate

135 for every group. In the multidimensional scaling (MDS) analysis, each cell line clustered

136 independently and generally, within the cell line clusters, untreated cells clustered apart from

137 treated cells (Additional file 2: Fig. S1).

138   With the mapping rate were around 90% (Additional file 3: Table S2), a p-value based ranked

139 list of DE genes (compared to untreated from each time point) was generated for both cell lines

140 (Additional file 4: Table S3, sheet 1-4). This list was used to select the shared DE genes. This

141 analysis generated thousands of DE genes (Additional file 4: Table S3, sheet 5) across Hep G2 and

142 MDA-MB-231 cell lines.

143   Because for each cell line the respective treatment groups clustered together on the MDS plot,

144 there were large numbers of shared genes between them. As a result, we identified a set of 6852

145 shared DE genes by identifying common DE genes from Hep G2 and MDA-MB-231 cell lines, at 24

146 hours and 48 hours (Fig. 2b). These shared genes might predict a common molecular signature for

147 CKI's activity. However, there were still a large number of DE genes that were not shared by both

148 cell lines, as seen in the heatmap in Fig. 2c. The expression of the shared gene set in both Hep G2

149 and MDA-MB-231 is highly consistent. Interestingly, this consistency is with respect to treatment

150 time, rather than with respect to cell line.

151 *RT-qPCR validation and dose response of gene expression to CKI*

152   Based on our previous results [28], and analysis below, we selected the 4 top ranked DE genes

153 expressed in G1-S phase of the cell cycle (TP53 and CCND1 for expression level validation and

154 E2F2 and PCNA for low dose response), as well as the proliferation and differentiation relevant ras

155 subfamily encoding gene (RAP1GAP1) for low dose response. We also selected a prominently

156 expressed gene (CYP1A1) for validation because of its sensitivity to CKI treatment. CYP1A1,

157 TP53 and CCND1 expression changes were validated with RT-qPCR with all three genes showing

158 similar patterns of expression in the transcriptome data and RT-qPCR (Fig. 3a).

159   Because low dose treatment with CKI did not cause significant gross phenotypic effects in either

160 cell line, we decided to use gene expression as a more sensitive measure of phenotype to look at
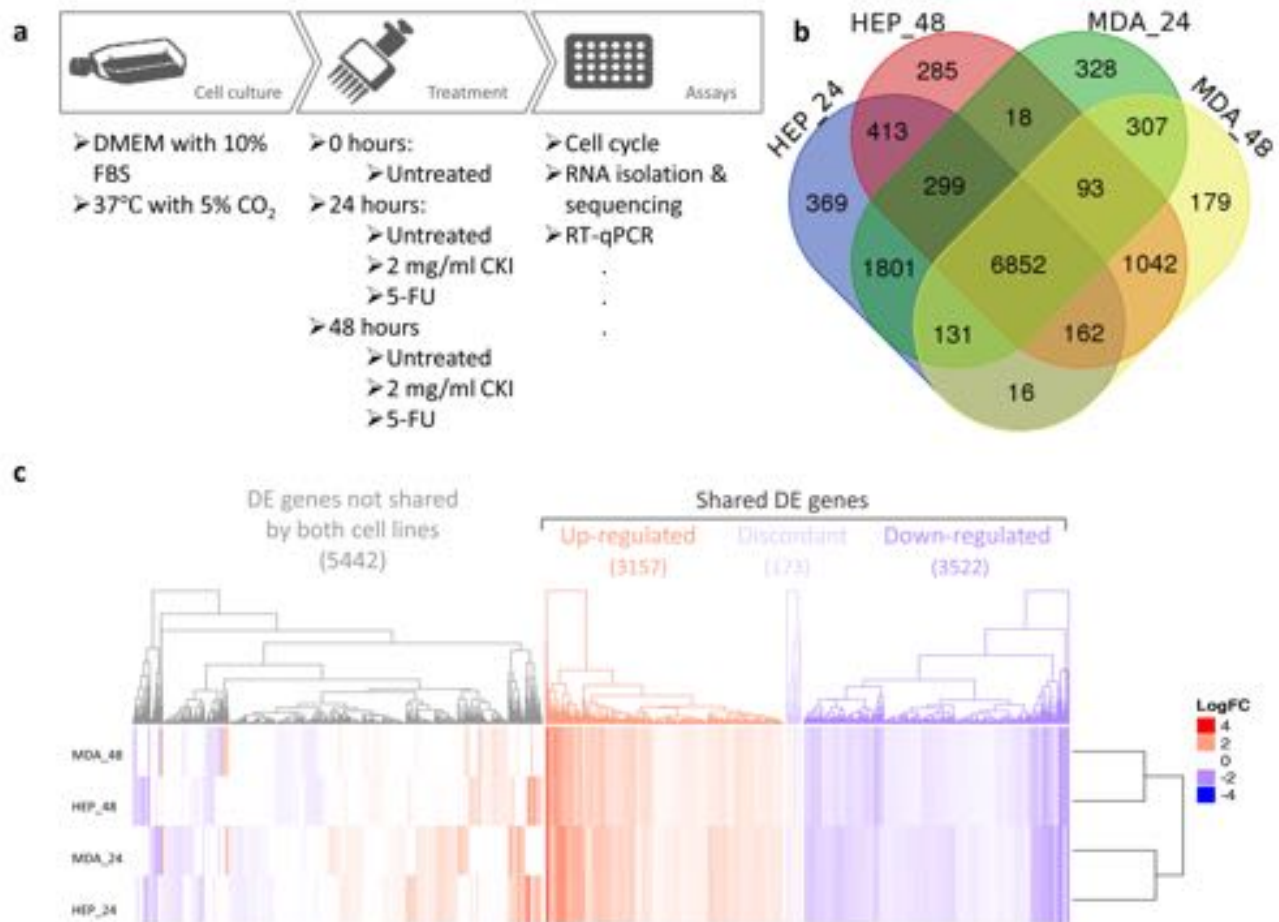
Figure 2. DE genes shared in both cell lines at both time points. A) Work flow diagram showing experimental design and sample collection. B) Venn diagram showing the number of shared DE genes between Hep G2 and MDA-MB-231. C) Heatmap presenting the overall gene expression pattern in both cell lines treated with CKI. Heatmap is split into four parts based on gene content and expression pattern: 5442 differentially regulated genes with expression not shared between the two cell lines, 3157 upregulated genes shared between both cell lines, 3522 down-regulated genes shared between both cell lines, and 173 discordantly regulated genes with differential expression shared between both cell lines.

161 the effect of lower doses of CKI. We used 0.125 mg/ml, 0.25 mg/ml, 0.5 mg/ml and 1 mg/ml

162 concentrations to look for dose dependency of gene expression. Our results showed an obvious

163 dose-dependent expression trend (Fig. 3b) in both cell lines. Because the 0.125 mg/ml

164 concentration of CKI is equivalent to what cancer patients are treated with, our results are

165 potentially clinically relevant.

166 *Function enrichment analysis*

167 To identify candidate mechanisms of action of CKI, we carried out functional enrichment

168 analysis. We used ConsensusPathDB [14] and Clusterprofiler [16] along with GO and KEGG

169 pathways for over-representation analysis, along with disease ontology (DO) [30] enrichment.

170 GO over-representation test was determined based on Biological Process level 3 and q value

171 <0.01. The results for both cell lines at both time points were summarized and visualized based

172 on semantic analysis of terms in Fig. 4a. From this result, it was obvious that there were a large

173 proportion of enriched GO terms relating to cell cycle, such as "cell cycle checkpoint",

174 "negative/positive regulation of cell cycle process" and so on prominently featured for all data sets

175 (Additional file 5: Fig. S2, Additional file 6: Table S4, sheet 1-4).

176 We then used KEGG pathways to determine the specific pathways altered by CKI in cancer.

177 The most regulated over-representative KEGG pathways are summarized according to KEGG

178 Orthology (KO) (Fig. 4b). Cell cycle related pathways such as "cell cycle", "DNA replication",

179 and "apoptosis" were also consistently seen in the KEGG enrichment results (Additional file 6:

180 Table S4, sheet5-8) at both 24 and 48 hours. Moreover, in addition to the cell cycle relevant

181 pathways, some cancer related pathways were also observed, such as "prostate cancer" and

182 "chronic myeloid leukaemia", and a large number of DE genes (283) from the two cell lines were

183 relevant in "pathways in cancer".

184 Because the KEGG enrichment revealed many pathways relating to diseases, most of which

185 were cancers, we decided to explore the enrichment of DE genes with respect to DO terms (Fig.

186 4c). In the DO list (Additional file 6: Table S4, sheet 9-12), all top ranked terms listed are cancers.

187 Interestingly, most cancer types listed are from the lower abdomen, for example "ovarian cancer",

188 "urinary bladder cancer "and "prostate cancer" etc. occurring in genitourinary organs (Additional
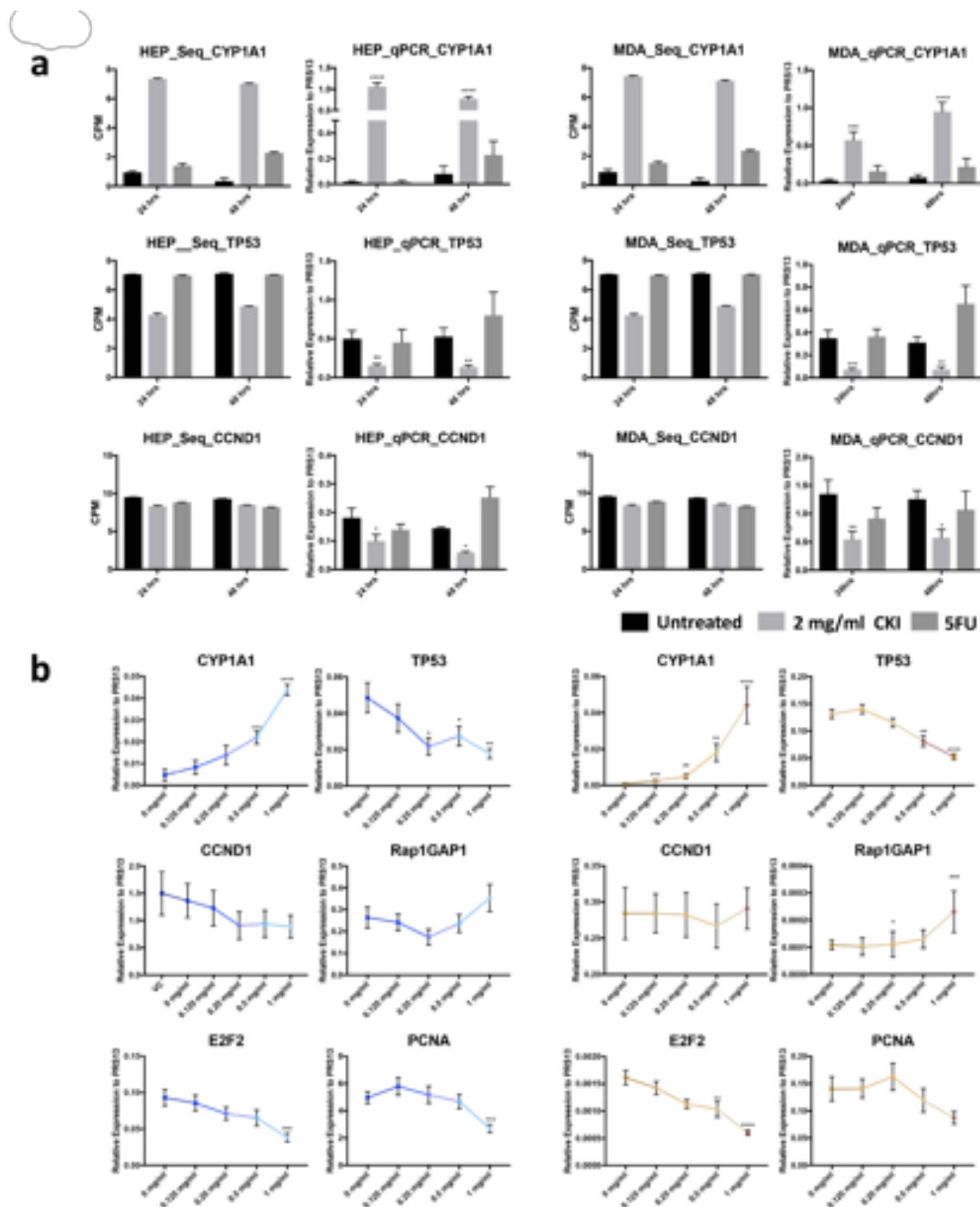
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Figure 3. Validation of gene expression and effects of low dose CKI using RT-qPCR. A) Comparison of DE genes between RNA-seq results (left) and RT-qPCR validation (right) for each cell line at 2 time-points. Three DE genes (CYP1A1, TP53 and CCND1) were chosen for validation. Gene expression was generally consistent between transcriptome data and qPCR data. B) Dose response of CKI using a subset of genes with conserved expression in Hep G2 (left), and MDA-MB-231 (right) from 0 mg/ml to 1 mg/ml of total alkaloids. Six genes (CYP1A1, TP53, CCND1, Rap2GAP1, E2F2 and PCNA) were selected based on their relevance to important pathways perturbed by CKI. RT-qPCR results are presented as expression relative to RPS13. Data are represented as mean ±SEM (n>3). A t-test was used to compare CKI doses with "untreated" (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

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189 file 6: Table S4, sheet 9-12). For both KEGG pathway and DO enrichment, the effects of CKI on
190 both cell lines were similar.

191   In addition to cell line specific functional enrichment of DE genes, we also analyzed the
192 over-represented GO terms for shared DE genes (Fig. 5a). The most significant clusters were
193 highly relevant to metabolic process, such as "cellular macromolecule metabolic process", as well as
194 the corresponding positive/negative regulatory biological process (Additional file 6: Table S4, sheet
195 13). Moreover, various signaling pathways, though not forming a large cluster, were also significant,
196 for example, "regulation of signal transduction" and "intracellular receptor signaling pathway".
197 Finally, some "cell cycle" related terms constituted relatively large sub-clusters, including "cell
198 division" and "mitotic cell cycle process". The enriched GO analysis was consistent with the cell
199 line specific enriched results, and with our previous analysis of MCF-7 cells [28]. It is worth noting
200 that for "cell cycle" related terms, most of the participating genes were down-regulated by CKI.

201   Similar results were observed from KEGG analysis (Fig. 5b, and Additional file 6: Table S4,
202 sheet 14) of shared genes. Various pathways related to cancer, formed a large cluster. Pathways
203 such as "DNA replication", "Ribosome" and "cell cycle" were mostly down-regulated, while
204 up-regulated pathways included "inositol phosphate metabolism" and "protein processing in
205 endoplasmic reticulum".

206   We also carried out over-representation analysis of DO terms (Fig. 5c) for all shared DE genes.
207 The analysis results were consistent with the single cell line DO term analysis with mostly cancer
208 related terms; in particular genitourinary or breast cancer terms. While this was also partially
209 similar to the KEGG results for shared DE genes, there were some differences in the KEGG
210 results for disease pathways compared to the DO results, such as "bacterial invasion of epithelial
211 cells", "Fanconi anemia pathway" and "AGE-RAGE pathway in diabetic complications".

212   Specific to the therapeutic potential of CKI for cancer treatment, we applied our data set
213 mapping to KEGG cancer pathways: pathways in cancer - homo sapiens (Additional file 7: Fig.
214 S3). The R package Pathview [21] was used to integrate log fold change values of all the gene
215 expression patterns into these target pathways. Within the 21 pathways in cancer, the "cell cycle"
216 still featured prominently (Fig. 6a). The expression of almost every gene in the cell cycle pathway
217 was affected by CKI, with most of them suppressed. We did not observe this kind of overall
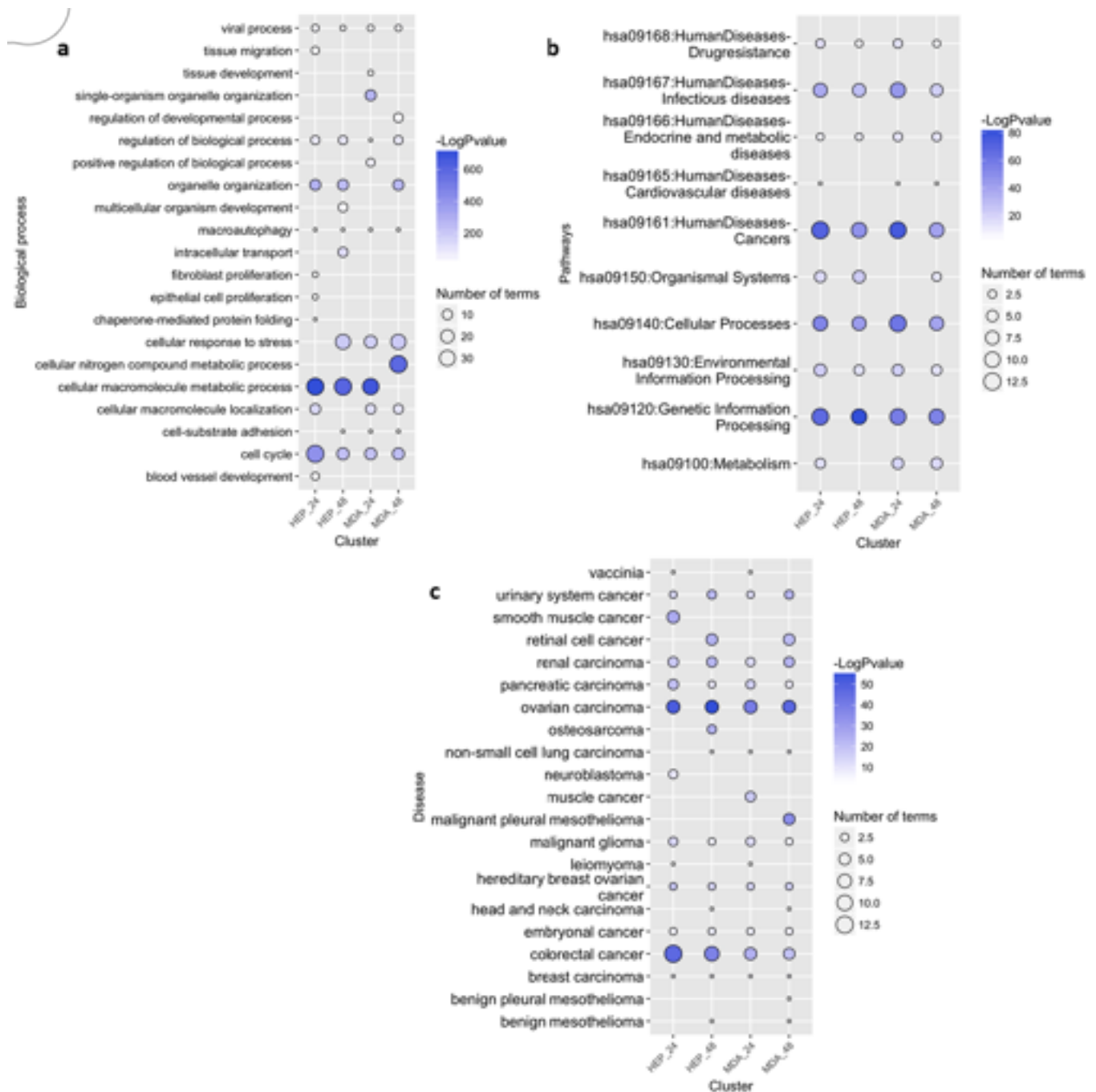
Figure 4. Functional annotation of DE genes for each cell line as a result of CKI treatment. Summary of over-represented A) GO terms for Biological Process, B) KEGG pathways and C) DO terms for DE genes as a result of CKI treatment in each cell line at two time points. For GO semantic and enrichment analysis, Lin's algorithm was applied to cluster and summarize similar functions based on GO terms found in every treatment. Similarly, by back-tracing the upstream categories in the KEGG Ontology, we were able to obtain a more generalized summary of KEGG pathways for each treatment. The size of each bubble represents the number of GO terms/pathways, and the colour shows the statistical significance of the relevant function or pathways. The DO summary for each treatment was determined by back-tracing to parent terms.
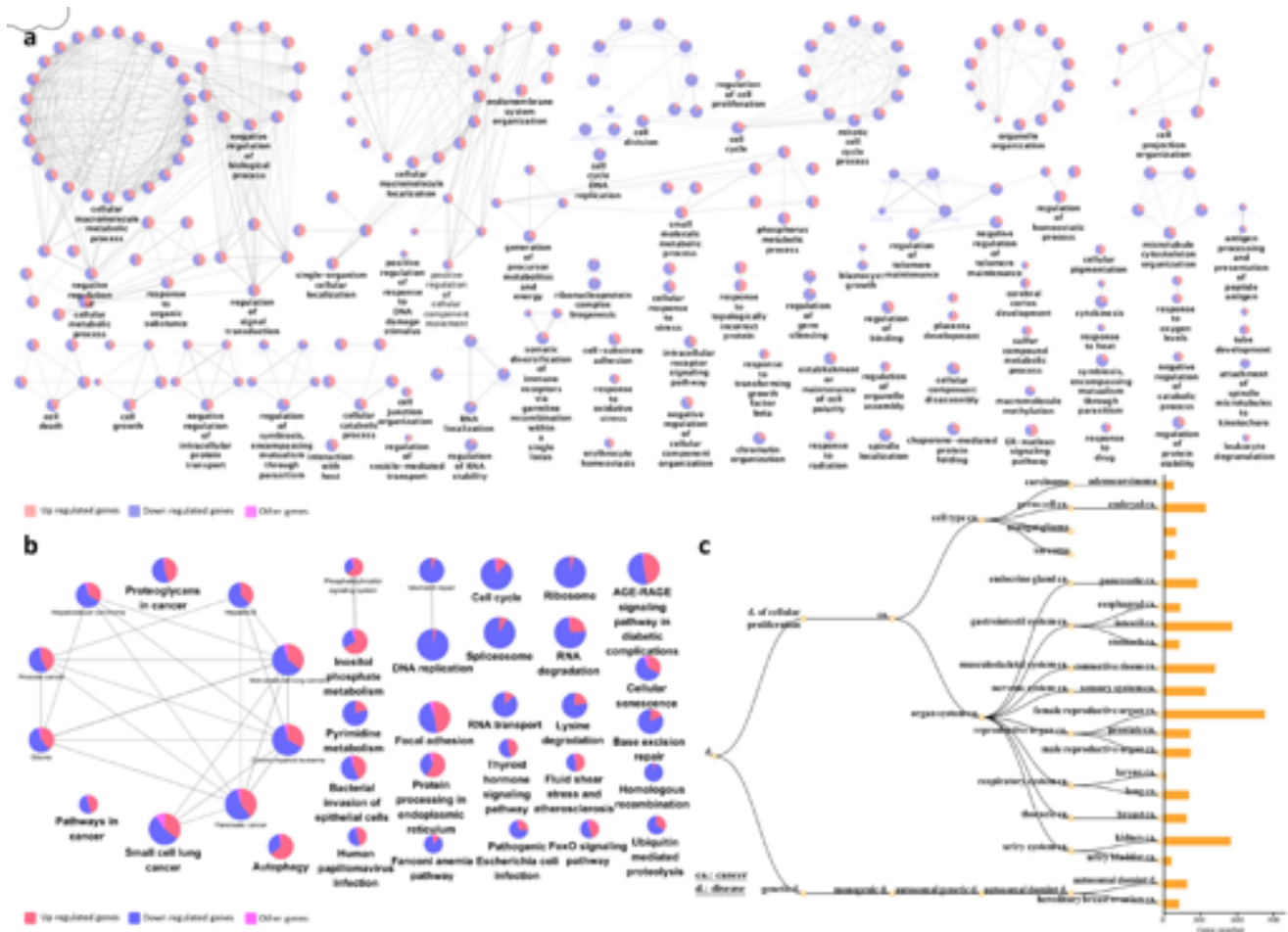
Figure 5. Functional annotation of DE genes with shared expression in both cell lines as a result of CKI treatment. Over-representation analysis was performed to determine A) GO terms for Biological Process, B) KEGG pathways, and C) DO terms for DE genes shared in both cell lines. In nodes for both GO terms and KEGG pathways, node size is proportional to the statistical significance of over-representation. For DO terms, all the enriched terms are statistically significant ($p < 1 \times 10^{-5}$) in each category, and the bar length represents the number of expressed genes that map to the term.

218 pathway suppression in any of the other pathways. We have displayed the summaries for the
219 remaining 20 pathways in the heatmap in Fig. 6b. Although all the pathways were all perturbed
220 by CKI, they include both over and under expressed genes in roughly equal proportions.

221 Collectively, these results suggest a direct anti-cancer effect of CKI, and implicate specific
222 candidate mechanisms of action based on the perturbed molecular networks. The most obvious
223 example is the cell cycle, where G1-S phase is significantly altered, resulting in the induction of
224 apoptosis. The downstream process triggered by CKI is the suppression of gene expression of cell
225 cycle regulators, including TP53 and CCND1. The other perturbed cancer pathways provide
226 additional candidate mechanisms of action for CKI. In the following section we integrate these
227 results with previous results reported in the literature to refine the core set of genes and pathways
228 perturbed by CKI.

Discussion

230 Although Hep G2 (liver cancer – mesodermal tissue origin) and MDA-MB-231 (mammary
231 epithelial adenocarcinoma – ectodermal tissue origin) are different cancer types, they shared a
232 large number of CKI DE genes with similar expression profiles, presumably these shared genes
233 include CKI response genes that are essential to the apoptotic response triggered by CKI.
234 However, the number of shared CKI DE genes is too high to allow straight forward identification
235 of genes critical to the CKI response. We therefore decided to combine these data with previously
236 reported CKI DE genes from MCF-7 cells [28] in order to reduce the number of core CKI response
237 genes. The intersection of MCF-7 CKI DE genes with the shared CKI DE genes yielded 363 core
238 CKI DE genes (Additional file 8: Fig. S4).

239 Among the 363 core CKI DE genes, cytochrome P450 family 1 subfamily A member 1
240 (CYP1A1) gene is the most over-expressed. This gene is consistently up-regulated by CKI in all
241 three cell lines, and showed significant dose response. In liver cancer cells, over-expression of
242 CYP1A1 induced by plant natural products has been associated with Aryl-hydrocarbon Receptor
243 transformation [2, 49]. Furthermore, as a steroid-metabolizing enzyme, CYP1A1 is part of cancer
244 metabolic processes relevant to steroid hormone responsive tumors, such as breast cancer, ovarian
245 cancer and prostate cancer [24, 26, 23, 27]. Therefore, CYP1A1 may be of particular interest for

Figure 6. Comparison of shared genes expression in specific pathways across two cell lines. A) Cell cycle pathways, where each coloured box is separated into 4 parts, from left to right representing 24 hour CKI treated Hep G2, 48 hour CKI treated Hep G2, 24 hour CKI treated MDA-MB-231 as well as 48 hour CKI treated MDA-MB-231. B) Heatmap of pathways in cancer. The top two heatmaps summarise the effects of CKI on Hep G2 cells for two time-points, and the bottom two heatmaps show the effects of CKI on MDA-MB-231 cells. In addition to the cell cycle pathway, there were 21 associated pathways in cancer that were perturbed by CKI. The effects of CKI on both cell lines were similar, with changes in TARGET database genes indicated by arrows. Compared to other pathways in cancer, the effects of CKI on the cell cycle pathway showed overall down-regulation.

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246 understanding the mechanism of action of CKI on cancer cells.

247 Comparison of the 363 core genes to the 135 Tumor Alterations Relevant for Genomics-driven
248 Therapy (TARGET) genes (version 3) from The Broad Institute (https://www.broadinstitute.org/
249 cancer/cga/target) identified 7 DE genes that were shared across the three cell lines and two time
250 points (Fig. 7a). Of these seven genes, six (TP53, CCND1, MYD88 (Myeloid differentiation
251 primary response gene 88) , EWSR1, TMPRSS2 and IDH1 (isocitrate dehydrogenase 1) were
252 similarly regulated (either always over-expressed or under-expressed), while CCND3 was
253 over-expressed in all three cell lines at both time points except at 48 hours in MCF-7 cells, where
254 it was under-expressed.

255 The TP53 gene encodes a tumor suppressor protein, that can induce apoptosis [11]. However,
256 in all cell lines TP53 was down-regulated, and all cell lines showed increased apoptosis. This
257 suggests that CKI induced apoptosis was not TP53-dependent. Support for this comes from the
258 fact that transcripts for PCNA (proliferating cell nuclear antigen), and a group of transcription
259 factors: MCM (mini-chromosome maintenance) complex and the E2F family are down-regulated.
260 The E2F transcription factors regulate the cell cycle and TP53-dependent and -independent
261 apoptosis [37, 12, 42, 33]. In addition, other core genes present in the TARGET database have
262 also been shown to induce apoptosis. For example, inhibition of MYD88 induces apoptosis in both
263 triple negative breast cancer and bladder cancer [3, 43]. The increased expression of IDH1 may be
264 important, as IDH1 is frequently mutated in cancers [16] and when mutated, it causes loss of
265 α-ketoglutarate production and may be important for the Warburg effect. TMPRSS2
266 (transmembrane protease, serine 2) has also been shown to regulate apoptosis in cancer [1].
267 Therefore, CKI may induce apoptosis through a variety of means.

268 In the GO (Fig. 7b) and KEGG (Fig. 7c) over-representation analysis of the 363 core genes
269 yielded enrichment for cell cycle and cancer pathways. In the GO enriched genes, cell cycle and
270 related pathways accounted for the majority of functional sub-clusters. In the KEGG enriched
271 pathways, cell cycle and cancer pathways predominated in a single cluster. Most of the core genes
272 in GO and KEGG clusters were down-regulated by CKI. In addition to the cell cycle, CKI
273 treatment also caused enrichment for terms or pathways related to cancer progression, such as
274 "focal adhesion" and "blood vessel development". (Additional file 6: Table S4, sheet 5-8). These

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275 developmental processes contribute to tumorigenesis and metastasis [20, 36]. It is tempting to

276 speculate that CKI may alter these functions *in vivo*, possibly altering angiogenesis which is

277 critical for tumors [9]. In addition, there were metabolic pathways and terms that were also

278 identified as perturbed by CKI. Effects on many targets/pathways is one of the expected features

279 of TCM drugs which likely hit multiple targets [7].

280 We have examined the effect of a complex mixture of plant natural products (CKI) on different

281 cancer cell lines and have identified specific, consistent effects on gene expression resulting from

282 this mixture. However, the complexity of CKI makes it difficult to determine the mechanism of

283 action of individual components, and often testing of individual components has resulted in either

284 no effect or contradictory results in the research literature. In spite of this complexity, it is possible

285 to map our results on to a pre-existing corpus of work that links individual natural compounds to

286 changes in gene expression. We have used BATMAN [19], an online TCM database of curated

287 links between compounds and gene expression. Based on this resource, we have identified 14

288 components of CKI that have been linked to the regulation of 52 of our core genes (Fig. 7d). We

289 can see from the network diagram in Fig. 7d that one to one, one to many and many to many

290 relationships exist between CKI components and genes which is consistent with previous studies

291 [38, 18, 46]. As more information becomes available for individual components, we will be able to

292 construct a more comprehensive model of CKI mechanism based on network analysis.

Conclusion

294 Our systematic analysis of gene expression changes in cancer cells caused by a complex herbal

295 extract used in TCM has proven to be effective at identifying candidate molecular pathways. CKI

296 has consistent and specific effects on gene expression across multiple cancer cell lines and it also

297 consistently induces apoptosis *in vitro*. These effects show that CKI can suppress the expression of

298 cell cycle regulatory genes and other well characterized cancer related genes and pathways.

299 Validation of a subset of DE genes at lower doses of CKI has shown a dose-response relationship

300 that suggests that CKI may have similar effects *in vivo* at clinically relevant concentrations. Our

301 results provide a molecular basis for further investigation of the mechanism of action of CKI.

19
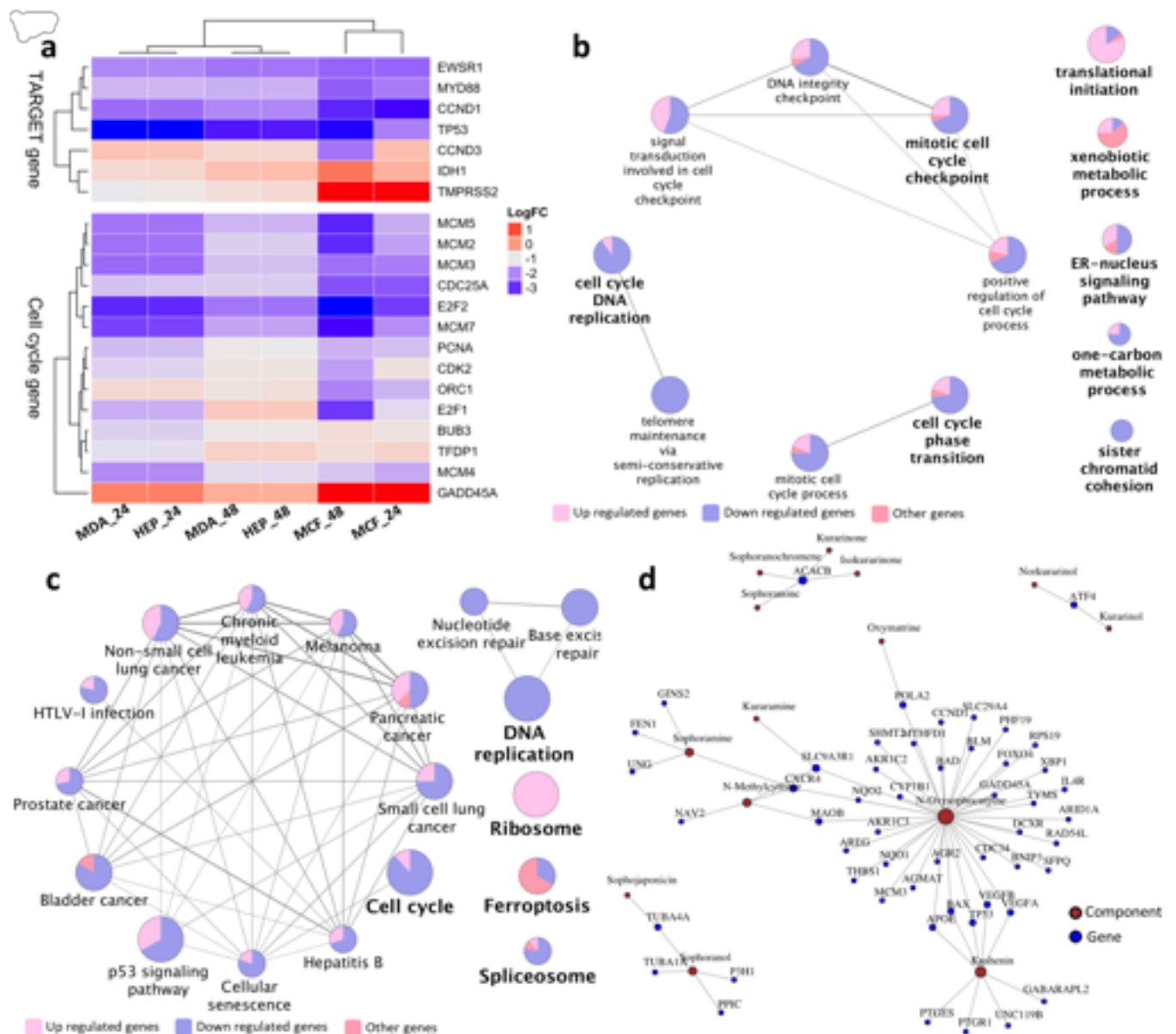
Figure 7. Analysis of CKI regulated core genes from this report combined with previous available data. A) Fold changes of TARGET and cell cycle regulatory gene expression in MDA-MB-231, Hep G2 and MCF-7 [28] cell lines 24 and 48 hours after CKI treatment. Only seven TARGET genes are affected by CKI in all three cell lines. Most of the 14 cell cycle regulatory genes differentially expressed in all three cell lines are down-regulated. B) GO term enrichment analysis of 363 core genes from MDA-MB-231, Hep G2 and MCF-7 cell lines. C) KEGG pathway enrichment of 363 core genes from MDA-MB-231, Hep G2 and MCF-7 cell lines. D) Some individual compounds present in CKI linked to genes they regulate that are also found in this report and our previous study [28]. Node size is proportional to the number of related components/genes.

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Conflict of Interest

We wish to draw the attention of the Editor to the following facts which may be considered as potential conflicts of interest and to significant financial contributions to this work. While a generous donation was used to set up the Zhendong Centre by Shanxi Zhendong Pharmaceutical Co Ltd, they did not determine the research direction for this work or influence the analysis of the data. JC: no competing interests, ZQ: no competing interests, YHL: no competing interests, HS: no competing interests, TNA: no competing interests, WW: is an employee of Zhendong Pharma seconded to Zhendong Centre to learn bioinformatics methods, RDK: no competing interests, DLA: Director of the Zhendong Centre which was set up with a generous donation from the Zhendong Pharmaceutical Co Ltd. Zhendong Pharmaceutical has had no control over these experiments, their design or analysis and have not exercised any editorial control over the manuscript.

Author contributions statement

JC experimental design, carried out experiments, analyzed data, wrote paper, ZQ experimental design, assisted with experiments, assisted with data analysis, wrote paper, YHL experimental design, assisted with experiments, assisted with data analysis, wrote paper, HS assisted with experiments, TNA assisted with experiments, WW assisted with experimental design, assisted with experiments, RDK experimental design, and DLA supervised the research, acquired funding for the experiments, experimental design, wrote paper.

Data Availability

All RNA-seq data raw and processed data were deposited at the Gene Expression Omnibus (GEO) data repository (XXXXXXX).

Role of the funding source

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28

a

Cell culture → Treatment → Assays
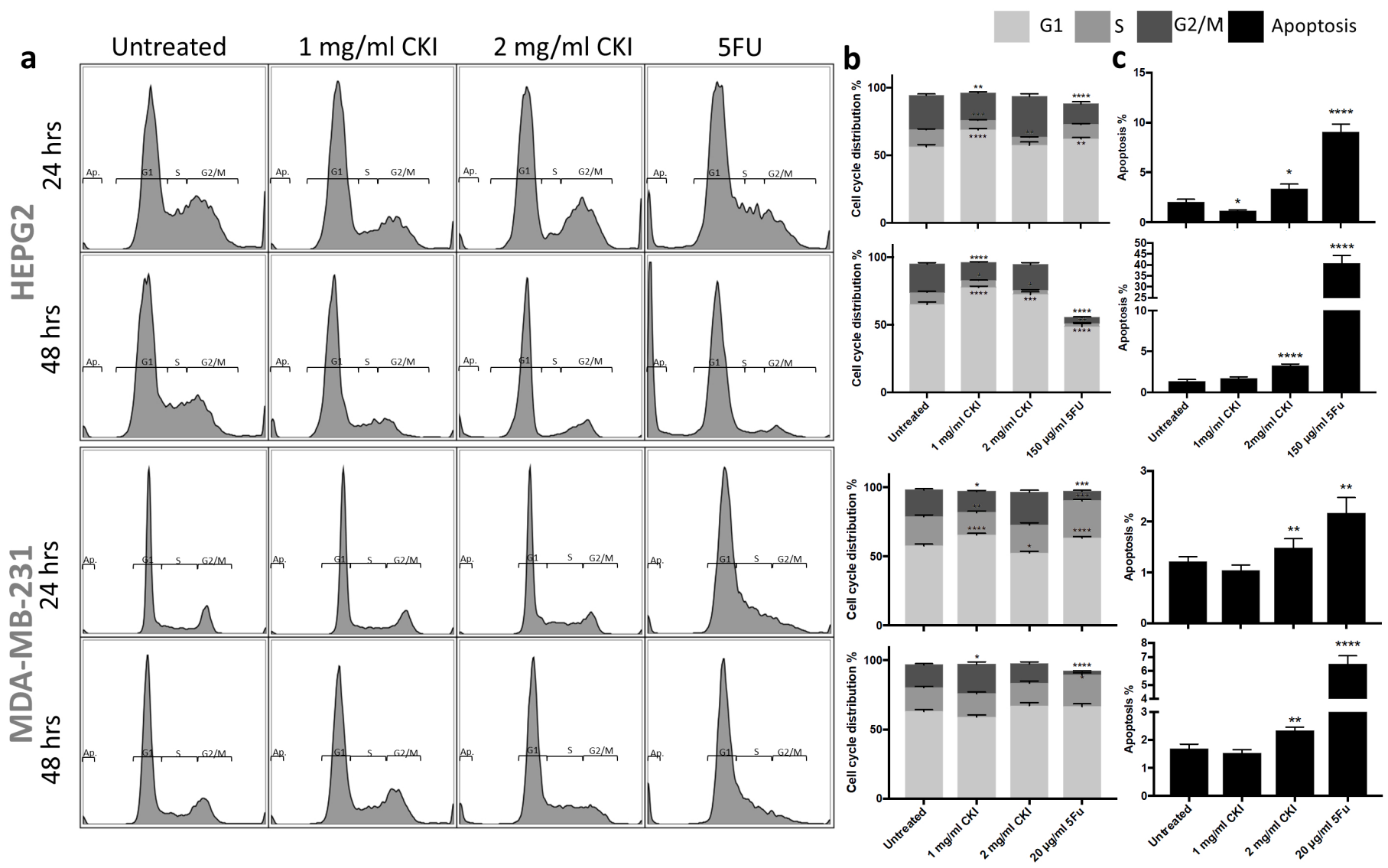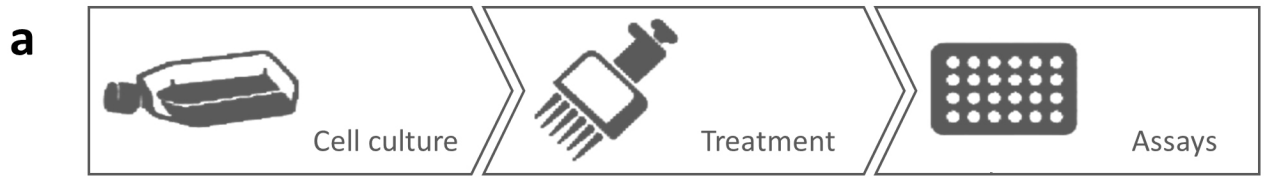
➢DMEM with 10% FBS
➢37℃ with 5% CO_2

➢0 hours:
  ➢Untreated
➢24 hours:
  ➢Untreated
  ➢2 mg/ml CKI
  ➢5-FU
➢48 hours
  ➢Untreated
  ➢2 mg/ml CKI
  ➢5-FU

➢Cell cycle
➢RNA isolation & sequencing
➢RT-qPCR
  .
  .
  .

b

HEP_48   MDA_24
HEP_24   MDA_48

285
328
413
18
307
369
299
93
179
1801
6852
1042
131
162
16

c

DE genes not shared by both cell lines (5442)

Shared DE genes

Up-regulated (3157)　　Discordant (173)　　Down-regulated (3522)

MDA_48
HEP_48
MDA_24
HEP_24

LogFC
4
2
0
-2
-4

a

Up regulated genes   Down regulated genes   Other genes

b

Up regulated genes   Down regulated genes   Other genes

c

ca.: cancer
d.: disease

a

CELL CYCLE

LogFC
-1 0 1

Growth factor Growth factor withdrawal

DNA damage checkpoint

TGFβ

ARF

p300

DNA-PK ATM/ATR

Smc1 | Smc3
Stag1,2
Rad21   Cohesin

Mps1

p107
E2F4,5
DP-1,2

Smad2,3
Smad4

Mdm2

Esp1   Separin

GSK3β

Rb

MAPK signaling pathway

c-Myc

Miz1

SCF
Skp2

p53

Apoptosis

Mad1

PTTG   Securin

Mad2
BubR1
Bub3

p16 p15 p18 p19
Ink4a Ink4b Ink4c Ink4d

p27,57 p21
Kip1,2 Cip1

GADD45

14-3-3σ

Chk1,2

Bub1

APC/C
Cdc20

+u

PCNA

14-3-3

Cdc25A

Cd25B,C

Ubiquitin mediated proteolysis

R-point (START)

CycD
CDK4,6

CycE
CDK2

CycA
CDK2

CycH
CDK7

CycA
CDK1

CycB
CDK1

Plk1

SCF
Skp2

p107,130

Rb

Abl
HDAC

Cdc6

Cdc45

Rb

Wee

Myt1

APC/C
Cdh1

E2F4,5
DP-1,2

E2F1,2,3
DP-1,2

ORC

MCM

Cdc14

Cdc7
Dbf4

Bub2   MEN

DNA → S-phase proteins, CycE

DNA → DNA biosynthesis

ORC (Origin Recognition Complex)
Orc1 | Orc2
Orc3 | Orc4
Orc5 | Orc6

MCM (Mini-Chromosome Maintenance) complex
Mcm2 | Mcm3
Mcm4 | Mcm5
Mcm6 | Mcm7

Data on KEGG graph
Rendered by Pathview

G1   S   G2   M

b

LogFC
-1 0 1

Pathways in cancer

HEP_24

HEP_48

MDA_24

MDA_48

a

TARGET gene

EWSR1
MYD88
CCND1
TP53
CCND3
IDH1
TMPRSS2

Cell cycle gene

MCM5
MCM2
MCM3
CDC25A
E2F2
MCM7
PCNA
CDK2
ORC1
E2F1
BUB3
TFDP1
MCM4
GADD45A

LogFC
1
0
-1
-2
-3

MDA_24 HEP_24 MDA_48 HEP_48 MCF_48 MCF_24

b

DNA integrity checkpoint

signal transduction involved in cell cycle checkpoint

mitotic cell cycle checkpoint

cell cycle DNA replication

positive regulation of cell cycle process

telomere maintenance via semi-conservative replication

mitotic cell cycle process

cell cycle phase transition

translational initiation

xenobiotic metabolic process

ER-nucleus signaling pathway

one-carbon metabolic process

sister chromatid cohesion

Up regulated genes   Down regulated genes   Other genes

c

Chronic myeloid leukemia

Non-small cell lung cancer

Melanoma

HTLV-I infection

Pancreatic cancer

Prostate cancer

Small cell lung cancer

Bladder cancer

Hepatitis B

p53 signaling pathway

Cellular senescence

Nucleotide excision repair

Base excision repair

DNA replication

Ribosome

Cell cycle

Ferroptosis

Spliceosome

Up regulated genes   Down regulated genes   Other genes

d

Kurarinone
Sophoranochromene
Isokurarinone
ACACB
Sophoranone
Norkurarinol
ATF4
Kurarinol

Oxymatrine

GINS2
FEN1
Sophoramine
UNG
Kuraramine
N-Methylcytisine
CXCR4
NAV2
MAOB

POLA2
SLC29A4
CCND1
PHF19
SHMT2 MTHFD1
BLM
RPS19
SLC9A3R1
AKR1C2
BAD
FOXO4
XBP1
NQO2
CYP1B1
GADD45A
IL4R
N-Oxysophocarpine
TYMS
AKR1C3
ARID1A
DCXR
RAD54L
AREG
NQO1
AGR2
CDC34
BNIP3
SFPQ
THBS1
AGMAT
MCM3
APOE
BAX TP53
VEGFB
VEGFA
Kushenin
GABARAPL2
Sophojaponicin
TUBA4A
TUBA1A Sophoranol
P3H1
PPIC
PTGES
PTGR1 UNC119B

Component   Gene