RNA-seq transcriptome analysis of breast cancer cell lines under shikonin treatment

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Shikonin is a naphthoquinone isolated from the dried root of *Lithospermum erythrorhizon*, an herb used in Chinese medicine. Although several studies have indicated that shikonin exhibits antitumor activity in breast cancer, the mechanism of action remains unclear. In the present study, we performed transcriptome analysis using RNA-seq and explored the mechanism of action of shikonin in regulating the growth of different types of breast cancer cells. The IC₅₀ of shikonin on MCF-7, SKBR-3 and MDA-MB-231 cells were 10.3 μM, 15.0 μM, 15.0 μM respectively. Our results also demonstrated that shikonin arrests the progression of cell cycle and induces apoptosis in MDA-MB-231 cells. Using RNA-seq transcriptome analysis, we found 38 common genes that significantly express in different types of breast cancer cells under shikonin treatment. In particular, our results indicated that shikonin induces the expression of dual specificity phosphatase (DUSP)-1 and DUSP2 in both RNA and protein levels. In addition, shikonin also inhibits the phosphorylation of JNK and p38, the downstream signaling molecules of DUSP1 and DUSP2. Therefore, our results suggest that shikonin induces the expression of DUSP1 and DUSP2 which consequently switches off JNK and p38 MAPK pathways and causes cell cycle arrest and apoptosis in breast cancer cells.

Breast cancer is one of the most common cancers and the second leading cause of cancer death among women in the United States¹. One in eight women will be diagnosed with breast cancer in her lifetime. Approximately 70% of breast cancer patients are inoperable because of advanced tumor growth or bone metastasis². Therefore, new strategies for the treatment of breast cancer are necessary. Many agents extracted from Traditional Chinese medicine (TCM) have been shown to possess anticancer activities and can be considered as alternative treatments for breast cancer³.

Shikonin, a naphthoquinone isolated from the Chinese herbal plant *Lithospermum erythrorhizon*, has been used to treat a variety of inflammatory and infectious diseases⁴. Several biological and pharmacological actions of shikonin have been reported, including anti-inflammatory⁵, antibacterial⁶, antiviral⁷, and antioxidant⁸ activities. In particular, shikonin has been shown to exert anticancer properties via different mechanisms on various cell types⁹.

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neoplastic cells such as inducing cellular apoptosis through mitochondria-mediated pathway in human prostate cancer cells, leukemia cells and gastric cancer cells, inhibiting migration and metastasis in human prostate cancer cells, breast cancer cells and lung cancer cells, attenuating angiogenesis in murine melanoma and lung carcinoma.

Transcriptome analysis associated with bioinformatics data mining tools provides an opportunity to simultaneously analyze a large number of genes/targets and identify the mechanisms of action after treatments. RNA-seq has many advantages over microarray due to it being free from the probe-specific hybridization of microarrays and has expansive coverage, allowing the unbiased detection of both coding and noncoding novel transcripts as well as low-abundance transcripts.

Several breast cancer cell lines used in biological studies have been classified based on the following measures: histological type, tumour grade, lymph node status and the presence of predictive markers such as estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). Yet, some studies have provided intriguing insights into anti-breast cancer activity of shikonin. Hou et al. indicated that shikonin inhibits cell proliferation and induces apoptosis in MCF-7 cells (Luminal A; ER+, PR−/+, HER2−)20. Zhang et al. demonstrated that shikonin attenuates the proliferation of both MCF-7 cells (Luminal A; ER+, PR−/+, HER2−) and SK-BR-3 cells (HER2; ER+, PR−, HER2+)21. In addition, Li et al. indicated that shikonin exhibits the cytotoxic effect in MDA-MB-231 cells (Claudin-low; ER−, PR−, HER2−)22. Although shikonin has been demonstrated as inhibiting the proliferation in different types of breast cancer cells, the mechanism of action has not been investigated. Herein, the aim of this study is to examine the mechanism of action of shikonin in regulating the growth of different types of breast cancer cells using the RNA-seq approach.

**Results**

**Shikonin inhibits the growth of human breast cancer cells.** To examine whether shikonin affects the growth of human breast cancer cells, different types of human breast cancer cell lines, MCF-7, SK-BR-3 and MDA-MB-231 were treated with shikonin for 24 hr. The cell viability was analyzed by MTT assay. As shown in Fig. 1, shikonin revealed significant cytotoxic effects in different types of human breast cancer cells in a dose-dependent manner. The IC50 of shikonin at 24 hr on MCF-7, SKBR-3 and MDA-MB-231 cells were 10.3 μM, 15.0 μM and 15.0 μM respectively (Fig. 1 A–C). In addition, we also examined whether shikonin affects the growth...
of human mammary epithelial cells, M10 cells. Our results indicated that human mammary epithelial cells were more resistant to shikonin-induced cytotoxicity compared to human breast cancer cells (Fig. 1D).

**Shikonin arrests the progression of cell cycle and induces apoptosis in human breast cancer cells.**

To investigate the mechanisms underlying shikonin-induced inhibition of cell growth, changes in cell cycle progression of human breast cancer cells were determined after shikonin treatment using flow cytometry. As shown in Fig. 2A,B, cells in sub-G1 phase were increased under shikonin treatment in a dose-dependent manner. These results suggest that shikonin inhibited cellular proliferation of human breast cancer cell lines, MCF-7, SKBR-3 and MDA-MB-231 via arrested G1 phase of the cell cycle. Moreover, we also performed Annexin V/PI apoptosis assay. Our results showed that shikonin induced apoptosis in MDA-MB-231 cells (Fig. 2C).

**RNA-seq transcriptome analysis of different human breast cancer cell lines under shikonin treatment.**

To study the gene expression profiling of human breast cancer cells under shikonin treatment, different human breast cancer cell lines, MCF-7, SK-BR-3 and MDA-MB-231 were treated with 10μM shikonin for 6 hr. The gene expression profiling was performed using RNA-seq. As shown in Fig. 3A, numbers of significantly differentially expressed (SDE) genes (>2-fold change) in different human breast cancer cell lines under shikonin treatment were identified. The results of Venn diagrams analysis showed 38 SDE genes (termed as common genes), which were expressed in different types of breast cancer cells under shikonin treatment (Fig. 3A). Thirty-six common genes were consistently upregulated and one common gene was consistently downregulated in different types of breast cancer cells under shikonin treatment (Table 1). Only RN7SL1 was inconsistently expressed in different types of breast cancer cells under shikonin treatment (Table 1).

**Analysis of common genes using both functional enrichment analysis and KEGG enrichment analysis after shikonin treatment by MCF-7, SK-BR-3 and MDA-MB-231 cells.**

We analyzed the common genes by functional enrichment analysis. The results showed that these genes participated mainly in adjustment of cell death, apoptosis, cell cycle and cell growth (Table 2). We further analyzed the common genes by KEGG enrichment analysis. The results showed that these common genes participated mainly in the MAPK signaling pathway, GADD45G, CDKN1A, GADD45G, DUSP1, DUSP2, CDKN1A, SESN2, PGR, HMOX1 (Table 3 and Fig. 3B).

**Validation of RNA-seq data by qRT-PCR.**

To further validate the results of RNA-seq, qRT-PCR was performed on 5 genes (DUSP1, DUSP2, CDKN1A, SESN2, PGR) randomly selected from common genes using the same RNA samples that were used in RNA-seq. A total of 15 RNA-seq samples were validated by qRT-PCR (5 representative genes in three different types of human breast cancer cells. Correlation analysis of expression ratios from the RNA-seq and qRT-PCR data were highly correlated (R = 0.9; P = 5.7 × 10^-6) (Fig. 4).

**Shikonin enhances the expression of DUSP1 and DUSP2 in both RNA and protein levels and decreases the phosphorylation of JNK and p38.**

The results of RNA-seq showed that shikonin induces the expression of DUSP1 and DUSP2 in breast cancer cells (Table 1). We confirmed the results of RNA-seq using qRT-PCR. As shown in Fig. 5A, the expression of DUSP1 and DUSP2 was increased in MCF-7, SK-BR-3 and MDA-MB-231 cells after shikonin treatment. However, there was no effect on the expression of DUSP1 and DUSP2 in M10 cells after shikonin treatment. In addition, we examined the expression of DUSP1 and DUSP2 in MDA-MB-231 cells after shikonin treatment. As shown in Fig. 5B, shikonin induced the expression of DUSP1 and DUSP2 in MDA-MB-231 cells. Furthermore, our results also showed that shikonin decreased the phosphorylation of JNK 1/2 and p38 in MDA-MB-231 cells. On the other hand, we analyzed the expression of DUSP1 and DUSP2 using DriverDB23,24. As shown in Fig. 5D, DUSP1 and DUSP2 were down-regulated in several types of cancers.

**Discussion**

The use of Chinese herbal medicine for health promotion and adjuvant therapy is becoming increasingly popular worldwide. Zicao, the dried root of Lithospermum erythrorhizon, is a Chinese herbal medicine widely used for its anti-inflammatory properties in China, Japan, Korea, etc.25. Shikonin is a major component of zicao and has been reported to suppress the growth of several types of cancer through a wide spectrum of anticancer mechanisms26. However, the mechanism of action of shikonin in regulating the growth of breast cancer cells is limited. Various subtypes of breast cancer have distinct prevalence and outcomes. In the present study, we used three different types of breast cancer cell lines, MCF-7 (Luminal A; ER^+, PR^+, HER2^−, good outcome), SK-BR-3 (HER2; ER^−, PR^−, HER2^+, poor outcome), MDA-MB-231 (Claudin-low; ER^−, PR^−, HER2^−, poor outcome)27 and demonstrated that shikonin inhibits the growth of these cancer cells including arresting the progression of the cell cycle and inducing apoptosis. To further explore the mechanism of action of shikonin in regulating the growth of different types of breast cancer cells, we analyzed the gene expression profiling of different types of breast cancer cells using RNA-seq. We found 38 common genes regulated by shikonin in different types of breast cancer cells and further analyzed these common genes using KEGG enrichment analysis. The analytic results indicated that these common genes were significantly involved in the MAPK signaling pathway, P53 signaling pathway, antigen processing and presentation, spliceosome, bladder cancer, endocytosis, HIF1 signaling pathway, cell cycle, pathways in cancer and the PI3K-AKT signaling pathway (Table 3). Several common genes were involved in regulating these pathways such as HSPA1B, HSPA6, GADD45G, DUSP1, DUSP2, CDKN1A, SESN2, PGR, HMOX1 (Table 3 and Fig. 3B).

In particular, the results of RNA-seq pointed out that shikonin induced the expression of both DUSP1 and DUSP2, the upstream regulators of MAPK signaling pathway. Also, the results of qRT-PCR confirmed that
Figure 2. Effect of shikonin on the cell cycle progression and apoptosis in breast cancer cell lines. (A) Different breast cancer cells, MCF-7, SK-BR-3 and MDA-MB231, were incubated with different concentrations of shikonin (0–10 μM) for 24 h. Representative cell cycle distribution of each cell type was analyzed by flow cytometry. (B) Percentage of sub-G1 in different breast cancer cells under shikonin treatment was assessed by Student’s t-test. The statistical significance of the difference between two experimental measurements was represented as follows: *P < 0.05 vs. shikonin 0 μM (DMSO control). (C) MDA-MB231 cells were treated with different concentrations of shikonin (0–10 μM) for 24 h. Cells were collected, stained with Annexin V and PI, and analyzed by flow cytometry. Data are representative of at least three independent experiments with similar results.
shikonin induced the expression of both DUSP1 and DUSP2 in different types of breast cancer cells. The expression ratios from RNA-seq and qRT-PCR data were highly correlated. Moreover, our experimental results also demonstrated that shikonin induced the protein expression of both DUSP1 and DUSP2 in different types of breast cancer cells. In addition, we also found that DUSP1 and DUSP2 were down-regulated in several types of cancers. Therefore, induction of DUSP1 and DUSP2 might be a therapeutic strategy for treating cancer.

DUSP1 and DUSP2 are the members of the threonine-tyrosine dual-specificity phosphatase family which play an important role in regulating the dephosphorylation of threonine and tyrosine residues on MAPKs27. MAPKs are signaling components that link extracellular signals to regulate a wide range of cellular processes

Figure 3. Intersectional analysis of SDE genes from breast cancer cells after shikonin treatment identified by RNA-seq and analysis of common genes using KEGG enrichment analysis. (A) Numbers of SDE genes from MCF-7, SK-BR-3, and MDA-MB-231 cells after shikonin treatment for 6 h were 408, 714, and 323, respectively. (B) 38 common genes were further used for KEGG enrichment analysis. The blue circles indicate the common genes from different types of breast cancer cells after shikonin treatment. The yellow circles show the biological functions and signaling pathway regulated by shikonin in breast cancer cells.
in cancer cells including growth, differentiation, migration and apoptosis\(^28\). Our experimental results indicated that shikonin reduced the phosphorylation of JNK \(1/2\) and P38 in MDA-MB-231 cells. Previous studies pointed out that JNK and P38 MAPK pathways regulated the progression of cell cycle, modulated the cell survival and differentiation, and controlled the balance of apoptosis and autophagy in response to chemotherapeutic agents in cancer cells\(^29,30\). Therefore, we suggest that shikonin induces the expression of DUSP1 and DUSP2 which consequently switches off JNK and p38 MAPK pathways and causes cell cycle arrest and apoptosis in breast cancer cells.

In summary, our results showed that shikonin inhibits cell growth and induces apoptosis in different types of breast cancer cells. We further examined the transcriptome regulation of shikonin in different types of breast cancer cells using the RNA-seq. We firstly reported that shikonin affects the expression of common genes among different types of breast cancer cells and is involved in regulating several anticancer mechanisms of action. Particularly, our results indicated that shikonin induces the expression DUSP1 and DUSP2 and reduces the activity of their downstream signaling molecules, JNK and p38. These results suggest that shikonin induces apoptosis through enhancing the expression of DUSP1 and DUSP2 (Fig. 5E).

### Materials and Methods

#### Chemicals and reagents.

Cell culture medium, Dulbecco’s modified Eagle’s medium (DMEM), DMEM/F12, alpha-Minimum essential medium, trypsin, penicillin–streptomycin, and Dulbecco’s Phosphate Buffered Saline (DPBS) were purchased from Corning Cellgro (Manassas, VA, USA). Fetal bovine serum (FBS) was

| Genes     | Entrez ID | MCF-7 | SK-BR-3 | MDA-MB-231 |
|-----------|-----------|-------|---------|-------------|
| RMRP      | 6023      | Infinity | Infinity | Infinity   |
| HSPA6     | 3310      | 8.48  | Infinity | 8.41        |
| HMOX1     | 3162      | 6.18  | 5.60    | 2.31        |
| PGF       | 5228      | 5.32  | 3.83    | 1.01        |
| HSPA1A    | 3303      | 3.92  | 6.11    | 4.69        |
| HSPA1B    | 3304      | 3.75  | 6.12    | 4.04        |
| ATF3      | 467       | 3.69  | 2.03    | 4.08        |
| DNAJB1    | 3337      | 3.37  | 4.70    | 3.51        |
| OSGIN1    | 29948     | 3.19  | 2.79    | 1.36        |
| TNSF9     | 8744      | 3.03  | 2.28    | 3.91        |
| PPP1R15A  | 23645     | 2.84  | 2.89    | 2.06        |
| ARC       | 23237     | 2.70  | 2.84    | Infinity    |
| MIR222, MIR22HG | 407004 | 2.64 | 4.00 | 2.09 |
| SNAI1     | 6615      | 2.64  | 2.06    | 4.52        |
| RN7SK     | 125050    | 2.54  | 1.01    | Infinity    |
| UBC       | 7316      | 2.53  | 2.40    | 1.02        |
| IER5      | 51278     | 2.42  | 1.21    | 1.99        |
| MAFF      | 23764     | 2.19  | 2.28    | 1.33        |
| ZFAND2A   | 90637     | 2.12  | 4.07    | 2.99        |
| SESN2     | 83667     | 2.00  | 1.28    | 1.22        |
| CDKN1A    | 1026      | 1.91  | 3.56    | 2.19        |
| HSPH1     | 10808     | 1.91  | 2.73    | 1.25        |
| BAG3      | 9531      | 1.89  | 3.68    | 1.33        |
| IDI2-AS1  | 55853     | 1.70  | 1.54    | Infinity    |
| SIK1      | 150094    | 1.61  | 1.00    | 1.30        |
| DUSP2     | 1844      | 1.60  | 3.01    | 5.32        |
| DUSP1     | 1843      | 1.55  | 2.88    | 1.20        |
| GADD45G   | 10912     | 1.53  | 2.42    | 3.17        |
| PIM1      | 5292      | 1.43  | 2.00    | 1.09        |
| MAP1LC3B  | 81631     | 1.39  | 1.75    | 1.17        |
| SLC25A25  | 314788    | 1.34  | 1.51    | 1.26        |
| OSER1     | 51526     | 1.34  | 1.80    | 1.61        |
| TSPYL2    | 64061     | 1.32  | 1.41    | 1.41        |
| RND3      | 390       | 1.30  | 2.34    | 1.06        |
| MAP1LC3B2 | 643246    | 1.24  | 1.31    | 1.34        |
| CSRNP1    | 64651     | 1.06  | 2.25    | 1.79        |
| RN7SL1    | 6029      | −1.22 | 1.15    | 1.32        |
| ETAA1     | 54465     | −1.45 | −1.15   | −1.68       |

Table 1. Differentially expressed common genes after shikonin treatment by MCF-7, SK-BR-3, and MDA-MB-231 cells. (log2 ratio).
| Term                                      | Category | Number of genes | %    | P-value | Genes                                     |
|-------------------------------------------|----------|-----------------|------|---------|-------------------------------------------|
| Response to unfolded protein              | B.P.     | 5               | 1.57 | 1.2 × 10⁻⁵ | HSPA1A, HSPA1B, HSPA1A, DNAJB1, HSPA1B, PPP1R15A |
| Negative regulation of apoptosis          | B.P.     | 6               | 1.89 | 6.7 × 10⁻⁵ | CDKN1A, HMOX1, BAG3, PIM1, UBC, HSPA1A   |
| Negative regulation of programmed cell death | B.P.     | 6               | 1.89 | 7.2 × 10⁻⁴ | CDKN1A, HMOX1, BAG3, PIM1, UBC, HSPA1A, HSPA1B |
| Negative regulation of cell death         | B.P.     | 6               | 1.89 | 7.2 × 10⁻⁴ | CDKN1A, HMOX1, BAG3, PIM1, UBC, HSPA1A, HSPA1B |
| Positive regulation of anti-apoptosis     | B.P.     | 3               | 0.94 | 1.6 × 10⁻³ | CDKN1A, DUSP1, HMOX1                     |
| Intracellular part                        | C.C.     | 25              | 7.86 | 3.2 × 10⁻³ | MAF, ARC, PIM1, HSPA1A, HSPA1B, SESN2, HSPH1, TSPYL2, SLC25A25, MAP1LC3B, HMOX1, BAG3, ETTA1, SIK1, MAF, ARC, PIM1, MAP1LC3B, SNA1, RND3, CDKN1A, DUSP2, ATF3, DUSP1, UBC, DNAJB1, SIK1, PPP1R15A |
| Regulation of cell cycle                  | B.P.     | 5               | 1.57 | 4.3 × 10⁻³ | CDKN1A, TSPYL2, GADD45G, PIM1, SIK1      |
| Regulation of programmed cell death       | B.P.     | 7               | 2.20 | 5.2 × 10⁻³ | CDKN1A, DUSP1, HMOX1, BAG3, PIM1, UBC, HSPA1A, HSPA1B |
| Regulation of cell death                  | B.P.     | 7               | 2.20 | 5.3 × 10⁻³ | CDKN1A, DUSP1, HMOX1, BAG3, PIM1, UBC, HSPA1A, HSPA1B |
| Regulation of transferase activity        | B.P.     | 5               | 1.57 | 6.5 × 10⁻³ | CDKN1A, TSPYL2, DUSP2, GADD45G, PIM1     |
| Apoptosis                                 | B.P.     | 6               | 1.89 | 6.8 × 10⁻³ | BAG3, CSRNP1, GADD45G, UBC, TNFSF9, PPP1R15A |
| Intracellular membrane-bounded organelle  | C.C.     | 21              | 6.60 | 7.3 × 10⁻³ | MAF, ARC, PIM1, HSPA1A, HSPA1B, SESN2, HSPH1, TSPYL2, SLC25A25, MAP1LC3B, HMOX1, BAG3, ETTA1, SIK1, MAF, ARC, PIM1, MAP1LC3B, SNA1, RND3, CDKN1A, DUSP2, ATF3, DUSP1, UBC, DNAJB1, SIK1, PPP1R15A |
| Nucleus                                   | C.C.     | 16              | 5.03 | 8.2 × 10⁻³ | MAF, ARC, PIM1, HSPA1A, HSPA1B, SESN2, HSPH1, TSPYL2, SLC25A25, MAP1LC3B, HMOX1, BAG3, ETTA1, SIK1, MAF, ARC, PIM1, MAP1LC3B, SNA1, RND3, CDKN1A, DUSP2, ATF3, DUSP1, UBC, DNAJB1, SIK1, PPP1R15A |
| Intracellular organelle                   | C.C.     | 22              | 6.92 | 1.2 × 10⁻² | MAF, ARC, PIM1, HSPA1A, HSPA1B, SESN2, HSPH1, TSPYL2, SLC25A25, MAP1LC3B, HMOX1, BAG3, ETTA1, SIK1, MAF, ARC, PIM1, MAP1LC3B, SNA1, RND3, CDKN1A, DUSP2, ATF3, DUSP1, UBC, DNAJB1, SIK1, PPP1R15A |
| Negative regulation of cell growth        | B.P.     | 3               | 0.94 | 1.5 × 10⁻² | CDKN1A, TSPYL2, OSGIN1                  |
| Negative regulation of cellular process   | B.P.     | 9               | 2.83 | 1.5 × 10⁻² | CDKN1A, TSPYL2, HMOX1, BAG3, PIM1, UBC, OSGIN1, HSPA1A, HSPA1B, SIK1 |
| Regulation of phosphorus metabolic process| B.P.     | 5               | 1.57 | 1.6 × 10⁻² | CDKN1A, TSPYL2, DUSP2, GADD45G, PIM1    |
| Negative regulation of cell size          | B.P.     | 3               | 0.94 | 1.7 × 10⁻² | CDKN1A, TSPYL2, OSGIN1                  |
| Negative regulation of catalytic activity | B.P.     | 4               | 1.26 | 1.8 × 10⁻² | CDKN1A, DUSP2, GADD45G, UBC            |
| Cell cycle arrest                         | B.P.     | 3               | 0.94 | 1.8 × 10⁻² | CDKN1A, SESN2, PPP1R15A                |
| Cytoplasm                                 | C.C.     | 19              | 5.97 | 2.1 × 10⁻² | ARC, PIM1, HSPA1A, HSPA1B, MAP1LC3B2, HSPA1B, SESN2, RND3, HSPH1, CDKN1A, TSPYL2, SLC25A25, MAP1LC3B, HMOX1, ZFAND2A, BAG3, ETTA1, SIK1, MAF, ARC, PIM1, MAP1LC3B2, SNA1, RND3, CDKN1A, DUSP2, ATF3, DUSP1, UBC, DNAJB1, SIK1, PPP1R15A |
| Negative regulation of growth             | B.P.     | 3               | 0.94 | 2.1 × 10⁻² | CDKN1A, TSPYL2, OSGIN1                  |
| Cellular protein metabolic process        | B.P.     | 10              | 3.14 | 3.9 × 10⁻² | DUSP2, DUSP1, MAP1LC3B, BAG3, GADD45G, PIM1, UBC, MAP1LC3B2, DNAJB1, SIK1 |

Table 2. Functional enrichment analysis of common genes by GO-terms. B.P., biological process; C.C., cellular component.

purchased from Gibco (Invitrogen, Carlsbad, CA, USA). Purified shikonin (>98%), dimethyl sulfoxide (DMSO), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), hydrochloric acid (HCl), isopropanol, RIPA buffer, protease inhibitor cocktail and Tris-buffered saline/Tween 20 (TBST) were purchased from Sigma (St. Louis, MO, USA). Antibodies against dual specificity phosphatase (DUSP)-1, DUSP-2, β-actin and horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Antibodies against mouse mouse phospho-JNK 1/2, JNK 1/2, phospho-p38 mitogen-activated protein kinase (MAPK), and p38 MAPK, and phospho-ERK 1/2, ERK 1/2 were purchased from Cell Signaling (Farmingdale, NY, USA). Pierce BCA Protein Assay Kit and ECL chemiluminescence substrate were purchased from Thermo Scientific (Rockford, IL, USA). TRIZol reagent, SuperScript® VILO™ cDNA Synthesis kit, SYBR® GreenER™ qPCR SuperMixes were purchased from Life Technologies (Carlsbad, CA, USA). RNA 6000 Nano LabChip kit was obtained from Agilent Technologies (Palo Alto, CA, USA).

Cell culture. Human breast cancer cell lines, MCF-7, SK-BR-3, and MDA-MB-231 cells were obtained from American Type Culture Collection (Manassas, VA, USA). Human mammary epithelial cell line, M10, was purchased from Bioresource Collection and Research Center (Hsinchu, Taiwan). MCF-7 and MDA-MB-231 cells were maintained in DMEM supplemented with 10% heat-inactivated FBS and 100 μg/mL of penicillin-streptomycin, SK-BR-3 cells were maintained in DMEM/F12 supplemented with 10% heat-inactivated FBS and 100 μg/mL of penicillin-streptomycin, and M10 cells were maintained in alpha-Minimum essential medium supplemented with 10% heat-inactivated FBS and 100 μg/mL of penicillin-streptomycin. All cell lines were cultured in a humidified atmosphere with 5% CO₂ at 37 °C. Shikonin was dissolved in DMSO. All treatments were adjusted to equal concentrations of DMSO between 0.1–0.2%.
Cell viability assay. Cell viability was determined using the MTT colorimetric assay. Cells were seeded in 96-well plates in culture medium at density of $1 \times 10^4$ cells/well overnight and then treated with various concentrations of shikonin between 0~50 $\mu$M for 24 hr. Subsequently, MTT in DPBS (0.1 mg) was added to each well and incubated for 4 hours at 37 °C. The MTT formazan crystals were dissolved with the addition of acid–isopropanol (1 portion of 4 N HCl: 100 portion of isopropanol). After 20 min, the optical density (OD) was measured with a microplate reader (BIO-RAD, Hercules, CA, USA) at 570 nm.

Cell cycle analysis. MCF-7, SK-BR-3, and MDA-MB-231 cells were treated with different doses of shikonin (0, 5, and 10 $\mu$M) for 16 hr. Cells were trypsinized, washed twice by cold PBS, and fixed in 70% cold ethanol overnight at −20 °C. After fixation, cells were washed twice by cold PBS and stained with PI/Triton X-100 staining solution (0.1% Triton X-100, 2 mg/mL PI, and 0.2 mg/mL DNase-free RNase) for 30 minutes. Samples were analyzed using a flow cytometry FC500 (Beckman Coulter, Krefeld, Germany).

Apoptosis assay. MDA-MB-231 cells were treated with different doses of shikonin (0, 5, and 10 $\mu$M) for 24 hr. Cells were trypsinized, washed twice by cold PBS, and stained with Alexa Fluor® 488 Annexin V and propidium iodide (PI) according to manufacturer’s protocol (Thermo Fisher Scientific, Rockford, IL, USA). Apoptotic cells were determined using FC500 flow cytometer (Beckman Coulter, Fullerton, CA, USA). Ten thousand events were collected per sample. Data were analyzed by CXP analysis software (Beckman-Coulter, Fullerton, CA, USA).

RNA preparation and RNA-seq. Total RNA was extracted with TRIzol reagent following the recommendations of the manufacturer. The quality of total RNA was evaluated using the Agilent 2100 bioanalyzer (Agilent, Palo Alto, CA, USA) with the RNA 6000 Nano LabChip kit. RNA-seq libraries were prepared Illumina® TruSeq RNA Library Prep Kit v2 and were sequenced using Illumina® HiSeq 2500 to obtain 150-bp paired-end reads. The sequencing depth for each sample was >20 million reads. The reads were aligned with TopHat 2.0.13 to GRCh37 with default parameters, and then were assembled by Cufflink 2.2.1, using Ensembl v75 annotations. Transcript abundance was measured in fragments per kb of exon per million fragments mapped (FPKM). The RNA-seq data is available at GEO (GSE100687).

### Table 3. KEGG enrichment analysis of common genes after shikonin treatment by MCF-7, SK-BR-3 and MDA-MB-231 cells.

| Pathway                                | P value ($-\log_{10}$) | Genes                                           |
|----------------------------------------|------------------------|-------------------------------------------------|
| MAPK signaling pathway                 | 5.96                   | HSPA1B,HSPA1A,HSPA6,GADD45G,DUSP1,DUSP2         |
| P53 signaling pathway                  | 3.98                   | CDKN1A,GADD45G,SESN2                            |
| Antigen processing and presentation    | 3.67                   | HSPA1B,HSPA1A,HSPA6                            |
| Spliceosome                            | 3.22                   | HSPA1B,HSPA1A,HSPA6                            |
| Bladder cancer                         | 2.85                   | CDKN1A,PGF                                     |
| Endocytosis                            | 2.78                   | HSPA1B,HSPA1A,HSPA6                            |
| HIF1 signaling pathway                 | 2.69                   | CDKN1A,HMox1                                    |
| Cell cycle                             | 1.93                   | CDKN1A,GADD45G                                 |
| Pathways in cancer                     | 1.19                   | CDKN1A,PGF                                     |
| PI3K-AKT signaling pathway             | 1.15                   | CDKN1A,PGF                                     |

Figure 4. Correlation of gene expression ratios between RNA-seq and qRT-PCR. A total of 15 RNA-seq samples were validated by qRT-PCR (5 representative genes, DUSP1, DUSP2, CDKN1A, SESN2, and PGF, in three different types of breast human breast cancer cells. Data from both RNA-seq and qRT-PCR were normalized by setting the expression level of untreated control.
Quantitative real-time PCR (qRT-PCR).

C DNA was synthesized from 1 to 2 μg RNA using SuperScript® VILO™ cDNA Synthesis kit according to the manufacturer’s instructions (Life Technologies, Carlsbad, CA, USA). The PCR reaction was performed by iCycler thermal cycler (Bio-Rad, Hercules, CA, USA) using SYBR® GreenER™ qPCR SuperMixes with PCR primers (CDKN1A: F-TGTCCGTCAGAACCCATGC, R-AAA GTCGAAGTTCCATCGCTC; DUSP1: F-AGTACCCCACTCTACGATCAGG, R-GAAGCGTGATACGC ACTGC; DUSP2: F-GGGCTCCTGTCTACGACCA, R-GCAGGTCTGACGAGTGACTG; PGF: F-GAAC GGGCTCAGAGGTG, R-ACAGTGCAGATTCTCATCGCC; SESN2: F-AAGGACTACCTGCGGTTCG, R-CGCC CAGAGGACATCAGTG; 18S rRNA: F-GGAATTGACGGAAGGGCACCACC, R-GTGCAGCCCCGGACATCAGTG). The relative level of target genes from each sample was determined by normalizing to 18S rRNA. All experiments were repeated at least twice to duplicate results.
Functional enrichment analysis. For the differentially expressed genes associated with treatment responses, we performed functional enrichment analysis, as described in our previous studies23,24 to interpret their biological functions. In brief, we used the topGO and GeneAnswers packages of Bioconductor to calculate the topology of the GO graph, as well as to visualize the many-to-many relationships between GO terms and genes. In the “Pathway” analysis, we used collections from KEGG25, PID26, Biocarta (http://www.biocarta.com/), REACTOME27, and MSigDB28 to annotate driver genes.

Statistical analysis. Data were obtained from at least three independent experiments and expressed as the mean ± standard deviation for each group. Statistical analyses, including Student’s t-test, one-way analysis of variance and regression analysis were performed using GraphPad Prism 4.0 software (GraphPad, Inc., La Jolla, CA, USA). P < 0.05 was considered to indicate a statistically significant difference.

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Author Contributions
K.L., M.H., W.C., S.F. and C.L. conceived and designed the experiments; S.W., M.H., H.T., C.S., Y.H., P.L.C.C., Y.W. and C.L. performed the experiments; W.C., P.L. and C.L. analyzed the data; K.L., W.C. and C.L. wrote the paper; K.L., W.C. and C.L. reviewed and edited the manuscript.

Additional Information
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