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Sanguisorba officinalis L. enhances the 5-fluorouracil sensitivity and overcomes chemoresistance in 5-fluorouracil-resistant colorectal cancer cells via Ras/MEK/ERK and PI3K/Akt pathways

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ABSTRACT

Sanguisorba officinalis L., a traditional Chinese medicine (TCM) called DiYu (DY) in China, has a strong tradition of utilization as a scorching, blood-cooling, and hemostatic medication, and was used for cancer prevention and treatment due to its potential immune-enhancing and hematological toxicity-reducing effects. Previous studies have reported significant effects of DY on cancers including colorectal cancer (CRC), which is one of the most common malignancies worldwide. The first-line cure 5-fluorouracil (5-FU) plays decisive commerce in the sedative of CRC as a clinically available chemotherapeutic agent. One of the primary causes of cancer treatment failure is the acquisition of chemotherapy drug resistance. In order to successfully combat the emergence of chemoresistance, it is essential to identify herbs or traditional Chinese medicine that have adjuvant therapeutic effects on CRC. Therefore, this study aimed to determine whether DY could improve the sensitivity, conquer the chemoresistance of 5-FU-resistant CRC cells, and investigate its intrinsic mechanism.

Materials and methods: MTT, Hoechst 33258 staining, and flow cytometry assays were used to determine the anticancer activity of DY alone or in combination with 5-FU against 5-FU-resistant CRC cells (RKO-R and HCT15-R) and wound healing assays were conducted to detect cell migration. Transcriptomic techniques were carried out to explore the effect and mechanism of DY

Abbreviations: 5-FU, 5-Fluorouracil; CRC, Colorectal cancer; DEGs, Differentially expressed genes; DMEM, Dulbecco's modified Eagle medium; DMSO, dimethyl sulfoxide; DY, Sanguisorba officinalis L; ERK, Extracellular-signal-regulated kinase; FBS, fetal bovine serum; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; MAPK, mitogen-activated protein kinase; MEK, Mitogen-activated protein kinase; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; Ras, rat sarcoma; PBS, phosphate-buffered saline; PI3K, phosphatidylinositol 3-kinase; PPI, protein-protein interaction; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; TCM, Traditional Chinese medicine.

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on drug-resistant CRC cells. Western Blot and RT q-PCR assays were performed to validate the mechanism by which DY overcomes drug-resistant CRC cells.
Results: These results indicated that DY alone or in combination with 5-FU significantly inhibited the proliferation and the migration of resistant CRC cells, and potentiated the susceptibility of 5-FU to drug-resistant CRC cells. GO and KEGG enrichment analysis showed that the mechanisms of drug resistance in CRC cells and DY against drug-resistant CRC cells highly overlapped, involved in the modulation of biological processes such as cell migration, positive regulation of protein binding and cytoskeleton, and MAPK (Ras-ERK-MEK), PI3K/Akt, and other signaling pathways. Moreover, DY can mediate the expression of p-R-Ras, p-ERK1/2, p-MEK1/2, p-PI3K, p-AKT, HIF-1A and VEGFA proteins. In addition, DY significantly suppressed the expression of *AKT3*, *NEDD9*, *BMI-1*, and *CXCL1* genes in resistant CRC cells.
Conclusion: In conclusion, DY could inhibit the proliferation and migration of 5-FU-resistant cells and strengthen the sensitivity of 5-FU to CRC-resistant cells. Furthermore, DY may prevail over chemoresistance through the Ras/MEK/ERK and PI3K/Akt pathways. These findings imply that DY may be a potential drug for clinical treatment or adjuvant treatment of drug-resistant CRC.

1. Introduction

The dried root of *Sanguisorba officinalis* L. (known as Di Yu in Chinese, DY), a herb that is traditionally used in Chinese medicine as a cooling agent to stop bleeding, clearing heat, detoxification, hemostasis, and detumescence. As a traditional Chinese medicine (TCM), it is recorded in Chinese Pharmacopoeia that is extensively utilized for immunomodulation, therapeutic hemato-toxicity, treatment of hepatitis B, as well as cancer [1]. In modern medicine, it is widely used in the treatment of cancer to prevent the development and metastasis of tumors because of its immune-boosting properties [2]. Previous studies proved that DY improved the hematopoiesis and enhanced immunity [3–5]. Besides, it has been reported that DY extraction exhibits antiproliferative activity in prostate cancer [6], oral cancer [7], breast cancer [8], and hepatocellular carcinoma [9]. Additionally, the inhibitory effect of DY on the growth and metastasis of 5-fluorouracil (5-FU)-sensitive and resistant colorectal cancer (CRC) was determined by our team [10].

CRC is a malignant intestinal cancer with a growing tendency of mortality in young adults, which is a crucial hazard to human health [11]. Modern systemic treatments for CRC have been effective, but the 5-year survival rate of patients remains low due to the development of innate or acquired drug resistance [12]. 5-Fluorouracil is a broadly used first-line chemotherapeutic agent for the therapy of CRC. However, with the occurrence of 5-FU tolerance, its clinical efficacy has been greatly diminished. In clinic, combination therapy with Chinese medicine can effectively tackle the limitations of chemotherapy drugs.

In our previous studies, DY synergistically enhanced 5-FU cytotoxicity in 5-FU sensitive CRC cells (RKO and HCT 116) [13]. However, it remains unclear whether DY can improve the susceptibility of 5-FU-resistant CRC cells or overcome chemoresistance. In addition, it is unknown which key proteins are regulated by DY to play a decisive role in colorectal cancer, especially drug-resistant CRC. Considering all the facts, this study intended to evaluate the role of DY on 5-FU-resistant CRC cells and to explore its potential mechanisms.

2. Materials and methods

2.1. Chemicals and reagents

These materials using in this study are mentioned in prior study, such as 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), DMSO 5-FU, RIPA lysis buffer, crystal violet, 4% paraformaldehyde solution, Dulbecco's modified eagle medium (DMEM), penicillin-streptomycin, Phosphate buffered saline (PBS), Fetal Bovine Serum (FBS), Pierce BCA protein assay kit, and Super Signal™ West Pico Chemiluminescent Substrate kit, TRIzol lysis buffer, and The Prime Script RT reagent Kit and TB Green™ Premix Ex Taq™ II (Tli RNaseH Plus) [10,14]. Hoechst 33258 reagent was from Beyotime Biotechnology (Jiangsu, China). The primary antibodies against Bcl-2, Bax, cleaved-caspase 3, cleaved caspase 9, GAPDH and β-actin were purchased from Cell Signaling Technology (Beverly, MA, USA). P-R-Ras, P-ERK1/2, P-PI3K, VEGFA, P-AKT, HIF-1A, and P-ERK1/2 antibodies were purchased from Affinity Biosciences (Cincinnati, OH, USA).

2.2. Preparation of the water extract of Sanguisorba officinalis L.

DY (Jiangsu, China, No.201902) was authenticated by Prof. Depo Yang (Sun Yat-sen University, China), mentioned in prior study [14] and stored in the School of Pharmaceutical Sciences (Shenzhen), Sun Yat-sen University, China. The preparation of the aqueous extracts of DY and the UPLC-MS/MS analysis results (in the Supplementary Material file) were described in the previous study [14]. The DY dried powder is obtained by vacuum drying of the filtered extraction concentrate liquid and stored at −20 °C for subsequent use. The HPLC analysis of DY was carried out based on a previous study [13], and the results were provided in the supplementary material (Fig. S1).

2.3. Cell culture

The 5-FU-sensitive RKO and HCT15 cells (RKOP and HCT15P) and 5-FU-resistant RKO and HCT15 cells (RKOR and HCT15R) were culture in DMEM medium with 10% FBS, which is described in the previous study [10]. Additionally, 5-FU was added into the culture medium for resistant cells to maintain drug resistance.

2.4. Cell viability assay

Cells were cultured in 96-well plates and following treatment with various doses of DY extraction (0, 31.3, 62.5, 125, 250, 500 μg/mL) or 5-FU (0, 62.5, 125, 250, 500, 1000 μM), or a combination of both (50 or 100 μg/mL of DY with various concentrations of 5-FU) for 24 h or 48 h. For details of cell viability assay, please refer to the previous study [10].

2.5. Cell colony assay

Cells were plated in 6-well plates (1000 cells/well) for 24 h, and treated with DY (100 μg/mL), 5-FU (100 μM), and both (100 μg/mL DY+100 μM 5-FU) for 9 days. The next experimental steps are consistent with the previous study [10].

2.6. Hoechst 33258 staining assay

Cells were seeded into 12-well plates (2×10^5 cells/well). After 48 h exposure to DY (100 μg/mL), 5-FU (100 μM), and both (100 μg/mL DY + 100 μM 5-FU), the cells were washed twice with PBS and fixed in 4% paraformaldehyde for 20 min before staining with Hoechst 33258 solution. Cells were photographed by an inverted fluorescence microscope (Olympus, Tokyo, Japan).

2.7. Wound healing assay

Cells were plated in 6-well plates. Then cells were scratched and photographed for 0 h before DY, 5-FU single, or both treatment for 12 h or 24 h (the doses were as above). For more details, please refer to the previous study [10].

2.8. Cell apoptosis assay

Cells were treated with DY (100 μg/mL), 5-FU (100 μM), or both agents together (100 μg/mL DY + 100 μM 5-FU) for 48 h. The Annexin V- FITC apoptosis kit was employed to determine the number of apoptotic cells according to manufacturer protocols on a flow cytometry system (Temecula, CA, USA).

2.9. Transcriptomic analysis

Cells were treated with DY (100 μg/mL) for 24 h. The RNA extraction, RNA sequencing, Gene ontology (GO), and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were all as described previously [14].

2.10. Construction of pathway-gene target network

Cytoscape 3.7.1 software was used to construct the network among the pathway-gene target. The nodes represent gene targets or signal pathways, and the edges represent the interaction between nodes.

2.11. Construction of protein-protein interaction (PPI) network

The PPI network of DEGs was conducted by STRING software (https://string-db.org/). Then the nodes and score information was

Table 1
The genes primers sequences of RT-PCR.

	Forward	Reverse
AKT3	5′-AGATGCAGCCACCAT GAAGACATTC-3′	5′-ACCAGTCTACTGC TCGGCCATAG-3′
NEDD9	5′-GCTGGATGGATG ACTACGATTA-3′	5′-GCTGCATCTTGTT CTGTTTCAT-3′
BMI-1	5′-CAAGACCAGACCA CTACTGAAT-3′	5′-TATCTTCATCTGC AACCTCTCC-3′
CXCL1	5′-AAGAACATCCAA AGTGTGAACG-3′	5′-CACTGTTCAGCATC TTTTCGAT-3′
GADPH	5′-AGGTCGGAGT CAACGGATTTG-3′	5′-TGTAAACCATGT AGTTGAGGTCA-3′

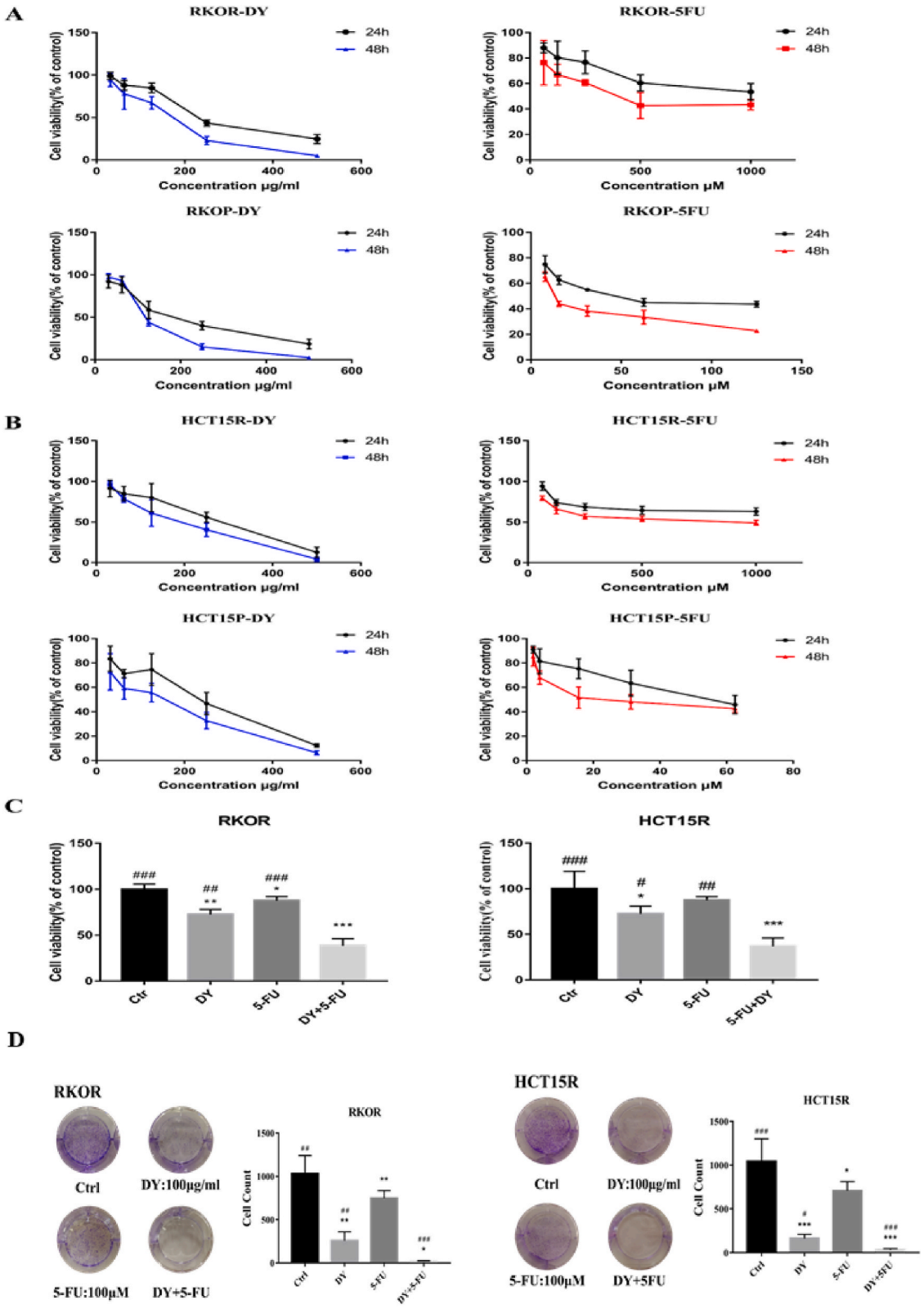

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Fig. 1. *Sanguisorba officinalis* L. enhanced the chemosensitivity of 5-FU resistant CRC cells to 5-FU. (A&B) Cells were treated with DY or 5-FU at different dose alone for 24 h or 48 h and then cell viability was detected by MTT assay. (C) RKOR and HCT15R cells were treated with 5-FU (100 μM) or DY (100 μg/mL) alone or in combination with each other for 24 h, cell viability was assessed by MTT. (D) Cell colony assay was performed to observe the effects of 5-FU (100 μM) or DY (100 μg/mL) alone or in combination with each other for 9 days. Results are presented as mean ± SD ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared with Ctrl group. And $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, compared with DY + 5-FU group.

combined into the Cytoscape3.7.1 software for visual analysis.

2.12. Western blotting assay

Cells were treated with DY (100 μg/mL), 5-FU (100 μM), or both agents together (100 μg/mL DY + 100 μM 5-FU) for 24 h. Next, cells were collected and lysed by RIPA buffer. The western blotting assay was performed as previously described [14].

2.13. Real time quantitative PCR analysis

Cells were extracted by TRIzol reagent to obtain total RNA, then total RNA were reversed by using Prime Script RT reagent kit to collect cDNA. Next, cDNA was used for the template and the real time quantitative assay was performed as previously described [10]. These sequences of primers are listed in Table 1.

2.14. Statistical analysis

The data were shown as mean ± SD (n = 3). The statistical results were analyzed using a *t*-test by GraphPad Prism 8.0 software and $P < 0.05$ was considered a significant difference [14]. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared with Ctrl group; $\#P < 0.05$, $\#\#P < 0.01$, $\#\#\#P < 0.001$, compared with DY+5-FU group.

3. Results

3.1. Sanguisorba officinalis L. enhanced the chemosensitivity of 5-FU resistant CRC cells to 5-FU

To assess the cell viability of DY and 5-FU, cells were treated with different doses of DY or 5-FU for 24 h or 48 h (Fig. 1A and B). The effect of DY on cell viability was concentration- and time-dependently decreased in all cell lines. The IC_{50} values of DY at 48 h in RKOR and HCT15R were 141.0 μg/mL and 157.1 μg/mL, respectively, and at 24 h 270.5 μg/mL and 279.3 μg/mL (Table 2). But treatment with 5-FU had a very limited effect on RKOR and HCT15R cells, especially for 24 h. And the IC_{50} values of 5-FU on resistant cells were over 20 times larger than those of sensitive cells, which indicated that the resistant cell model was successfully established. So DY (100 μg/mL) and 5-FU (100 μM) were selected for further combinative studies according to the results of the MTT assay. Furthermore, exposure to 100 μg/mL DY or 100 μM 5-FU alone resulted in a slight decrease in cell viability in RKOR and HCT15R cells compared to untreated cells (control group), whereas co-treatment with DY and 5-FU led to a significant decrease in cell viability compared to the control, DY and 5-FU groups (Fig. 1C). From these data, it is demonstrated that DY increased the sensitivity of 5-FU-resistant cells to 5-FU.

To further confirm the growth-inhibitory effect of DY and 5-FU, the colony formation assay was employed to assay long-term effects on cell viability (Fig. 1D). It was shown that DY have an evident inhibitory effect on the cell colony. Besides, 5-FU resistance can be reversed by treatment with DY via causing the predominantly reduction of number of the cell colony formation.

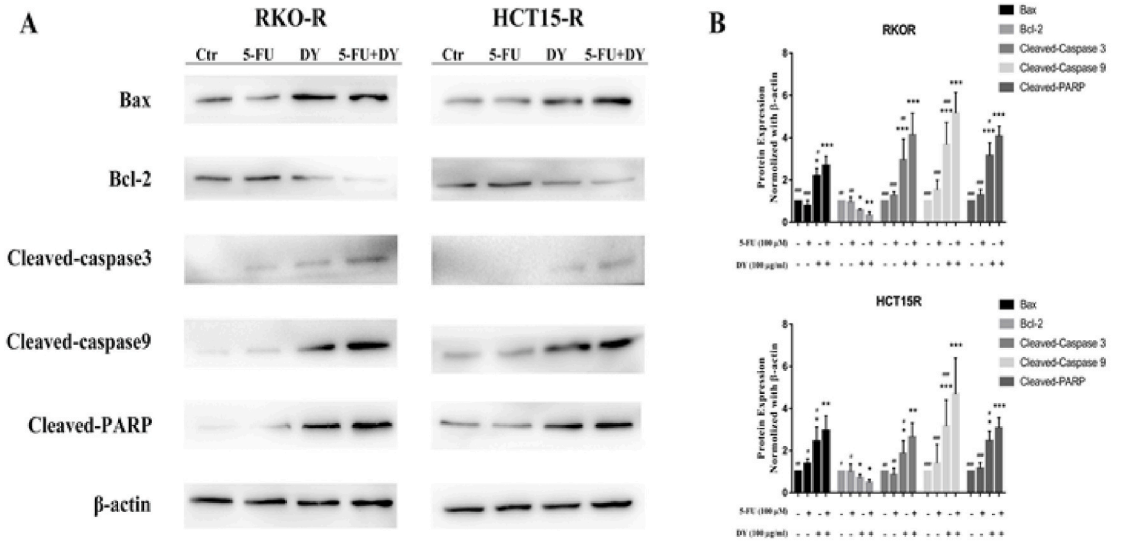
3.2. Sanguisorba officinalis L. augmented the effect of 5-FU in inducing resistant CRC cells apoptosis

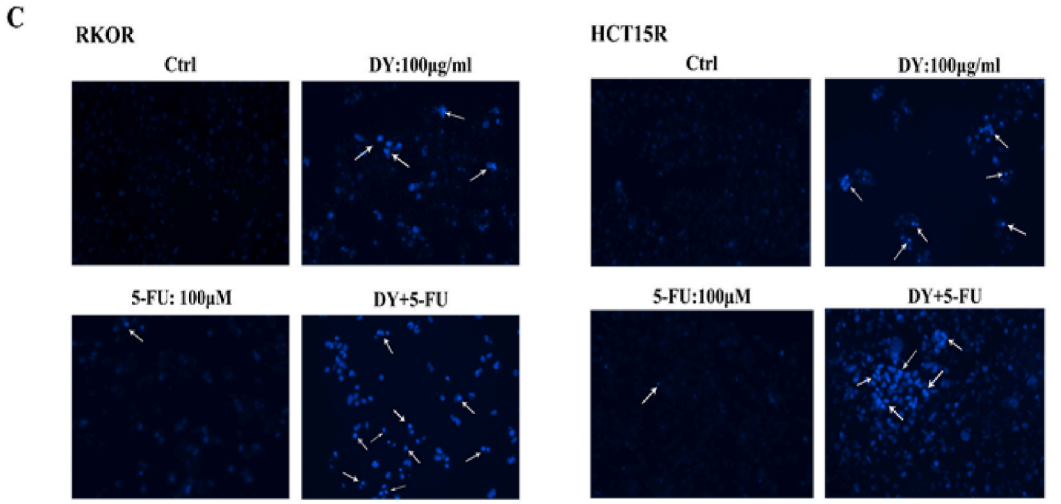
First of all, apoptosis pathway-related proteins were determined by western blot assays (Fig. 2A and B). Compared to the 5-FU alone group, the expressions of Bax, cleaved-caspase 3, cleaved-caspase 9 and cleaved PARP proteins were markedly promoted in the combination group, while the Bcl-2 protein was decreased, indicating DY might induce and enhance the apoptosis effect. The effect of

Table 2
The IC_{50} values of DY, 5-FU alone or in combination.

Cell	Drug	IC_{50} - 24 h	IC_{50} - 48 h
RKOR	5-FU (μM)	–	429.3 ± 30.7
	DY (μg/mL)	270.5 ± 12.6	141.0 ± 16.8
RKOP	5-FU (μM)	41.25 ± 2.9	16.0 ± 1.9
	DY (μg/mL)	187.2 ± 5.2	124.2 ± 12.5
HCT15P	5-FU (μM)	54.8 ± 5.7	26.6 ± 3.9
	DY (μg/mL)	234.2 ± 21.9	112.7 ± 17.6
HCT15R	5-FU (μM)	–	607.2 ± 27.7
	DY (μg/mL)	279.3 ± 19.1	157.1 ± 22.4

-: means the IC_{50} value was over 1000 μM.





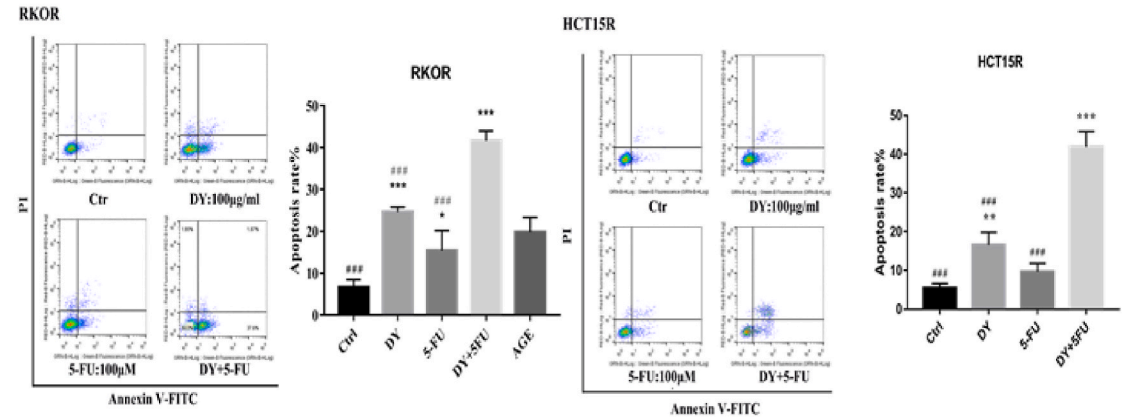


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Fig. 2. Effect of DY on the apoptosis of 5-FU resistant cells. RKOR and HCT15R cells were exposed to 100 μg/mL DY, 100 μM 5-FU, or in combination. (A&B) Effects of 48 h treatment with drugs on the expression of Bax, Bcl-2, cleaved-caspase 3, cleaved-caspase 9, cleaved-PARP proteins in resistant cells. (C) Cells were treated with Hoechst 33258 after 48 h, then observed under a fluorescence microscope (200×). The arrows refer to condensed and bright chromatins; (D) Cells were treated for 48 h, then cell apoptosis was analyzed by flow cytometry. The cell apoptosis rate was shown on the right. Results are expressed as mean ± SD ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared with Ctr group. And $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, compared with DY + 5-FU group.

DY on cell apoptosis was further evaluated by Hoechst 33258 staining (Fig. 2C) and flow cytometry analyses (Fig. 2D). After 48 h treatment, 5-FU alone only induced limited apoptosis (condensed and bright chromatins) in both resistant cells, while DY alone slightly induced apoptosis. As shown in combination of DY and 5-FU group, the condensed and bright chromatin were displayed obviously. Additionally, the cell apoptosis were significantly induced in combination of DY and 5-FU group. From these results, it is proved that DY enhanced 5-FU sensitivity through dramatically promoting the cell apoptosis rate. Collectively, it was identified that DY extremely augmented the potency of 5-FU on the apoptosis of resistant CRC cells.

3.3. *Sanguisorba officinalis L. potentiated the effect of 5-FU in inhibiting resistant CRC cells migration*

Moreover, the migratory ability of RKOR and HCT15R cells was investigated after 48 h drug treatment (Fig. 3A and B). It was displayed that co-treated 5-FU with DY visibly widened the wound width in RKOR and HCT15R cell lines. It was validated that DY can act synergistically with 5-FU thereby leading to the suppression of the migration in drug-resistant cells.

3.4. *Functional analysis of DEGs between 5-FU resistant HCT15 cells (HCT15R) and sensitive HCT15 cells (HCT15P)*

To uncover the mechanism of insusceptibility of 5-FU, whole transcriptome sequencing was exploited and profiles of genes were analyzed. The exhibited data revealed that 804 genes were significantly differentially expressed in HCT15P and HCT15R cells (Fig. 4A). The screened differential genes by RNA-sequence technical analysis were used for pathway prediction and displayed in a manner of GO and KEGG pathway enrichment. The GO results showed that they were enriched in response to multicellular organism development, semaphoring-plexin signaling pathway and cell migration in biological process (BP), protein binding, chemorepellent

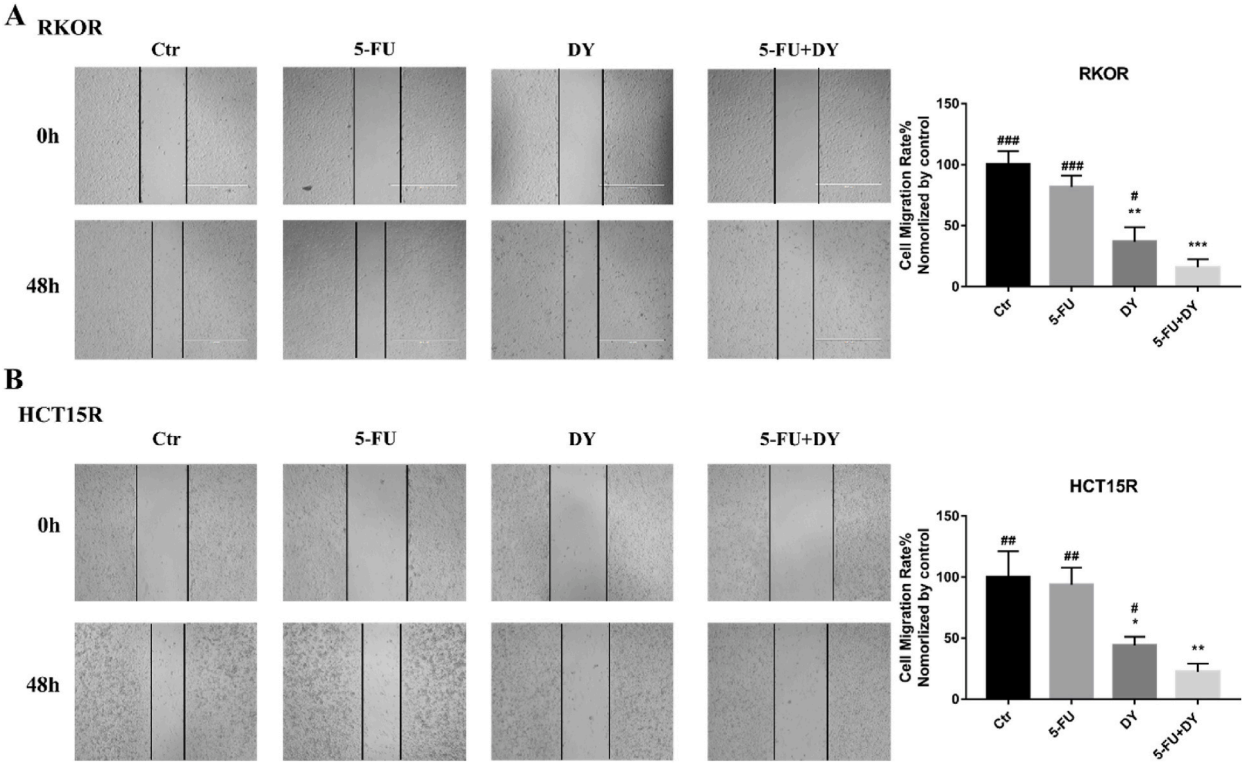

Fig. 3. Effect of DY on the migration of 5-FU resistant cells. RKOR and HCT15R cells were exposed to 100 μg/mL DY, 100 μM 5-FU, or in combination. (A&B), Wound healing assays were applied to detect the effect of DY on cell migration after 48 h treatment. The cell migration rate was on the right. Results are expressed as mean ± SD ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared with Ctr group. And $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, compared with DY + 5-FU group.

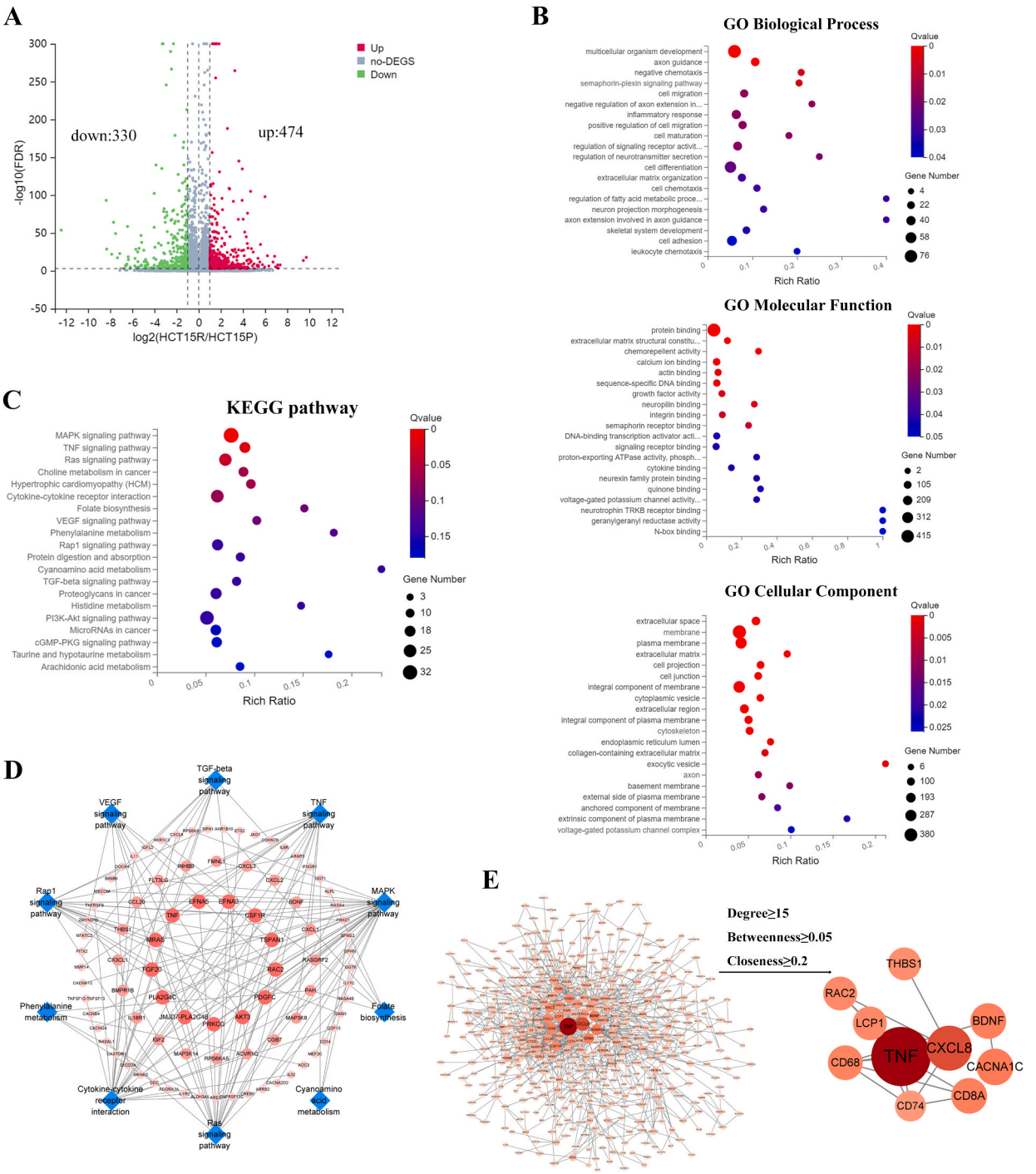

Fig. 4. The GO, KEGG, and network analyses of DEGs between HCT15P and HCT15R cell lines. (A) Volcano plots of DEGs. Red dots indicated the up-regulated genes, and green dots represented the down-regulated genes. The selected thresholds were, fold change >2 and FDR ≤0.001. (B) Top 20 GO enrichment analysis of DEGs. (C) Top 20 enriched KEGG pathways for the DEGs. The color scales indicate the different thresholds of adjusted Q values, and the sizes of the dots represent the gene count of each term. (D) Target-pathway network. The blue diamond represents the pathways and the red ellipse represents the targets. (E) The process of PPI network of significantly differently expressed genes screening. Circles represent targets. The redder the circle is, the larger the degree value of the target. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

activity and et al. (Fig. 4B). What's more, MAPK, TNF, Ras, VEGF, and Rap1 pathways were considerable pathways based on KEGG pathway analysis (Fig. 4C).

To investigate the relationship between genes and pathways, a gene-pathway network was built (Fig. 4D). The *EFNA5, EFNA3, CSF1R, TSPAN1, RAC2, PDGFC, AKT3, PRKCG, JMJD7-PLA2G4B, PLA2G4C, FGF20, MRAS,* and *TNF* genes were high-degree targets. And the PPI network contained 452 nodes and 978 edges (Fig. 4E). According to three network parameters of "degree ≥ 15", "betweenness ≥0.05" and "closeness ≥ 0.2", the second PPI network containing 10 nodes and 17 edges was then created. And the top 3

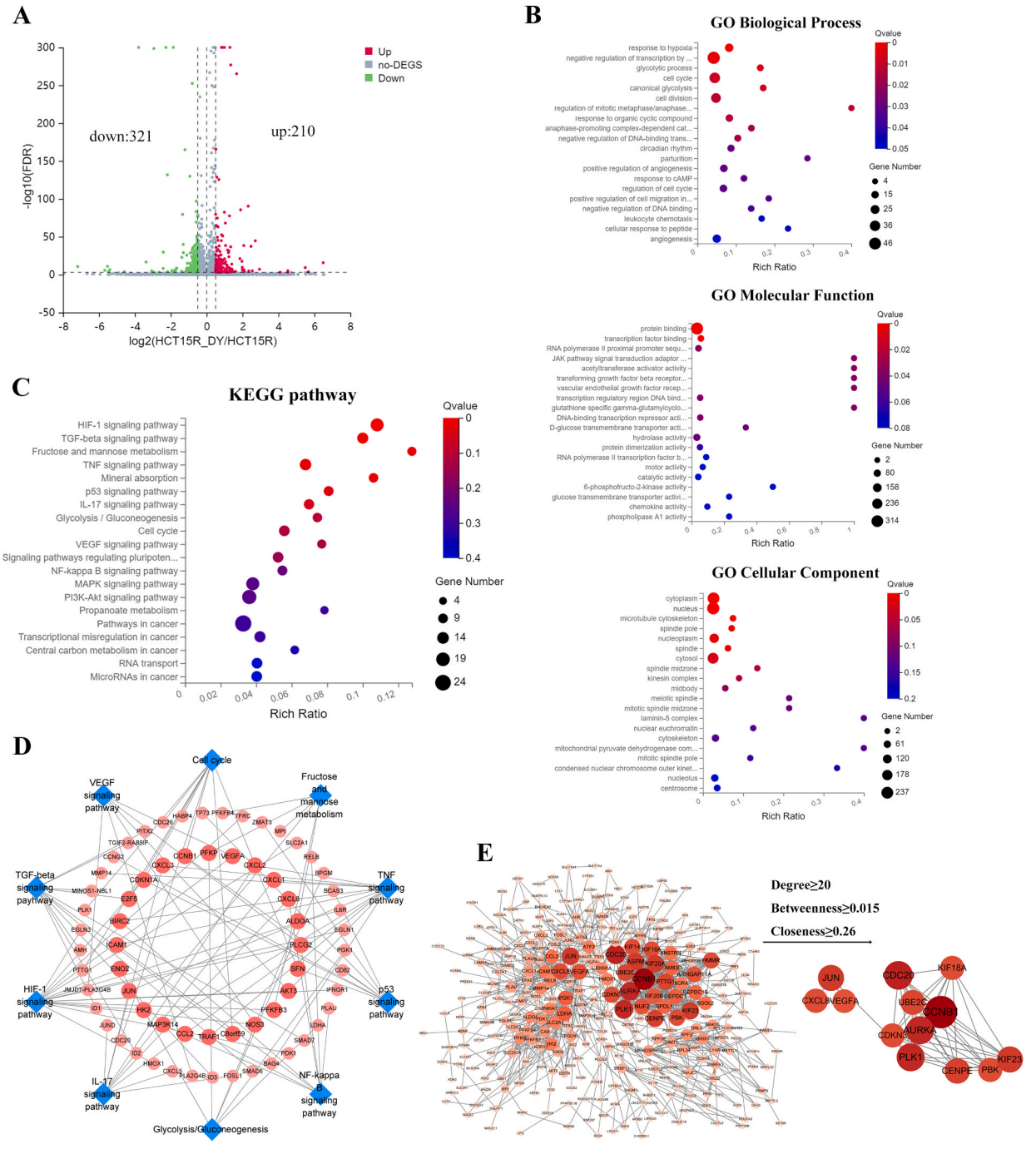

Fig. 5. The GO, KEGG, and network analyses of DEGs between HCT15R and DY-treated HCT15R cells. (A) Volcano plots of DEGs. Red dots indicated the up-regulated genes, and green dots represented the down-regulated genes. The selected thresholds were fold change >2 and FDR ≤0.001. (B) GO enrichment analysis of DEGs. (C) KEGG pathways for the DEGs. (D) Gene-pathway network. (E) The screening process of PPI network of core DEGs. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

core genes were *TNF, CXCL8* and *CACNA1C*, respectively. Thoroughly, drug resistance of HCT15-R cell to 5-FU may be related to the above core genes and pathways, which provides a crucial clue for subsequent studies on drug resistance.

3.5. Sanguisorba officinalis L. suppressed 5-FU resistant cell proliferation, angiogenesis, metastasis, and survival via regulating multiple pathways

Subsequently, transcriptomic sequencing was performed on HCT15-R cell after DY treatment for 24 h. The results disclosed that 531 genes were significantly differentially expressed between HCT15R and DY-treated HCT15R cells, including 210 up-regulated genes and 321 down-regulated genes (Fig. 5A). From Fig. 5B, it was illustrated that response to hypoxia, cell cycle, positive regulation of cell migration and etc. were modulated by DY. Meanwhile, KEGG pathway revealed that HIF-1, TGF-beta, TNF, p53, and IL-17 pathways were notably altered by DY (Fig. 5C). In addition, a gene-pathway network was formed by 75 nodes (10 pathways and 65 genes) and 93 edges (Fig. 5D). The top ten core genes were *PLCG2, HK2, CDKN1A, AKT3, CXCL8, CCNB1, SFN, MAP3K14, TRAF1, and CXCL2*. And PPI network consisted of 313 nodes and 1020 edges (Fig. 5E). Similarly, the second PPI network including 13 nodes and 49 edges was then constructed through filtering conditions of "degree $\geq$ 20", "betweenness $\geq$ 0.015" and "closeness $\geq$ 0.26". Finally, *CCNB1, CDC20, AURKA,* and *PLK1* were selected as the top 4 core differential target genes.

3.6. Sanguisorba officinalis L. down-regulated the Ras/MEK/ERK and PI3K/Akt pathways in 5-FU resistant CRC cells

Based on the results of whole genome sequencing data analysis, it was indicated that Ras/MEK/ERK and PI3K/Akt axis were considered as a dominant role in the regulation of tumor proliferation, progression, and chemoresistance in DY-treated HCT15R cells. Thus, these pathway-related proteins expressions were analyzed after DY treatment by western blot assays (Fig. 6). The experimental results verified that DY dramatically trigger the reduction of p-R-Ras, p-MEK1/2, p-ERK1/2, p-PI3K, p-AKT, VEGFA, and HIF1A proteins.

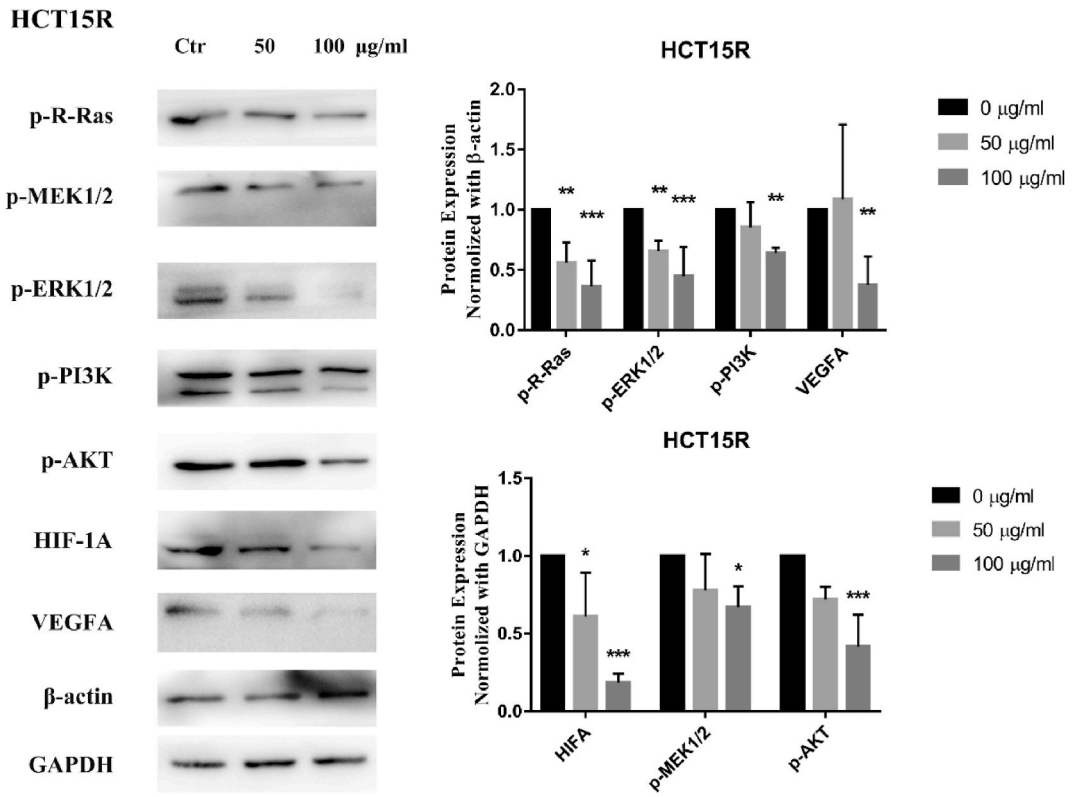

Fig. 6. Effect of DY on the Ras/MEK/ERK and PI3K/Akt pathways in the HCT15R cells. Cells were exposed to 0, 50 and 100 μg/mL DY for 24 h treatment. The histograms of protein expression are on the right. Results are expressed as mean ± SD ($n = 3$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, compared with 0 μg/mL group.

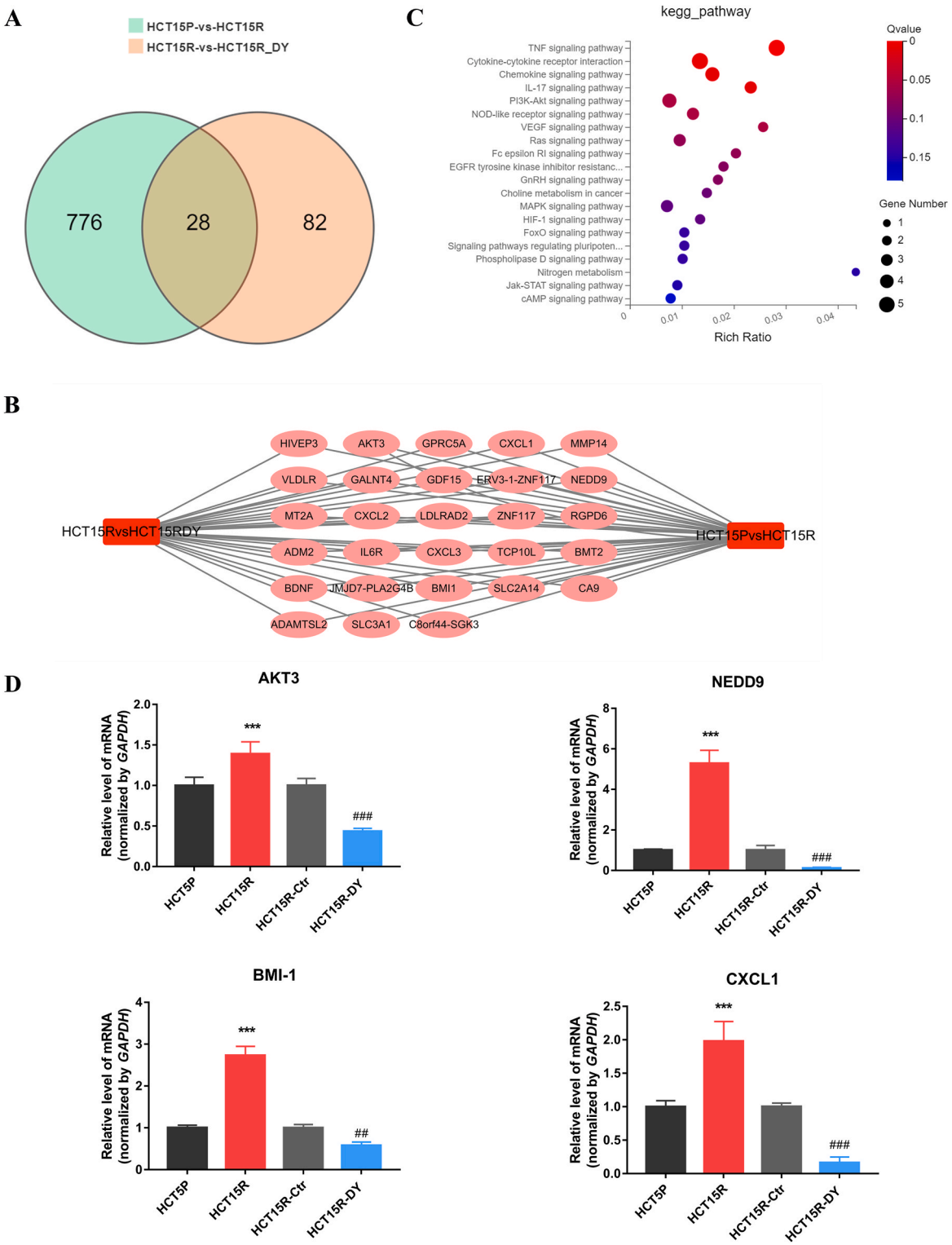

Fig. 7. The KEGG network analyses and validation of DEGs between HCT15P_HCT15R cells group and HCT15R_DY-treated HCT15R cells group. (A) The Venn of DEGs. (B) The network of DEGs. (C) The KEGG analysis of DEGs. (D) The expression of *AKT3*, *NEDD9*, *BMI-1*, and *CXCL1* genes in HCT15P, HCT15R cells or cells after DY 24 h treatment. Results are expressed as mean ± SD (n = 3). ***$P < 0.001$, compared with HCT15P group. ##$P < 0.05$, ###$P < 0.001$, compared with HCT15R group.

3.7. Functional analysis and validation of DEGs between HCT15R _ HCT15P cell group and DY-treated HCT15R_DY-treated HCT15R cell group

To further examine and evaluate the transcriptomic sequencing results, the Venn diagram of the common DEGs from the HCT15R _ HCT15P cells group and the DY-treated HCT15R_DY-treated HCT15R cells group was obtained. Overall, 28 genes have been screened as targets of DY against 5-FU drug resistance (Fig. 7A and B). Thereafter, these DEGs were used to carry out KEGG analysis. As shown in Fig. 7C, TNF, Chemokine, IL-17, PI3K/Akt, and NOD-like receptor signaling pathways were the top 5 pathways.

To validate the role of these targets, we used RT-QPCR to determine some drug-resistance genes expression. The results showed that the expression of AKT3, NEDD9, BMI-1, and CXCL1 genes in resistant CRC cells (HCT15R) was higher than those in sensitive cells (HCT15P), and DY induced the reduction of these genes' expressions in resistant CRC (Fig. 7D).

4. Discussion

Although 5-FU is the preferred chemotherapeutic agent in clinical practice, as an easily resistant chemotherapeutic agent, it is no longer suitable for metastatic patients with CRC who have acquired strong tolerance to 5-FU-based therapy, which significantly lowers patient survival rates. Nevertheless, it is a big challenge for reversing drug resistance via a single mechanism due to the complex responses of cancer cells to chemotherapeutic drugs. Studies showed that alterations of numerous pathways contributed to the development of cellular resistance to 5-FU, such as cell cycle, apoptosis, autophagy, and oxidative stress. Absolutely, other major factors in the emergence of drug resistance are the presence of transporters, epigenetic alterations induced by 5-FU [15].

TCM leads to effective clinical treatment as potential or adjuvant drugs against resistant CRC due to the multi-component and multi-target advantages. It has been reported that resistance CRC was repressed by the ethanol extract of *Hedyotis diffusa* through the suppression of P-glycoprotein and ABC subfamily G member 2 [16]. Besides, the ethanol extract of *Scutellaria barbata* could suppress 5-FU resistant HCT-8 cell proliferation and promote apoptosis through PI3K/Akt pathway inhibition [17].

As reported in our previous study, DY was found to have extreme anti-CRC activity [13]. In this present study, DY was discovered to inhibit 5-FU-resistant CRC cells and enhance the sensitivity of resistant CRC cells to 5-FU. Similarly, Goyal et al. disclosed that ellagic acid, which is enriched in DY, mitigated the toxicity of the chemotherapy drug cisplatin in CRC [18]. Likewise, DY resulted in the inferior migration ability of drug-resistant CRC cells, indicating that DY might simultaneously overcome the challenges of metastasis and drug resistance. Additionally, the mechanism of DY against drug-resistant CRC cells was explored by transcriptomics sequencing. It demonstrated that MAPK (MEK/ERK), HIF-1 and VEGF are closely associated with drug resistance. Raf/MEK/ERK/PI3K cascade signals play a crucial role in the modulation of cell cycle, apoptosis, and drug resistance in various cancers [19]. The dysregulated core phosphatase proteins, such as p-R-Ras, p-MEK, p-ERK, p-PI3K, p-Akt, are the causes of resistance to molecular inhibitors, and chemotherapy drugs [20]. The activated Raf facilitates resistance to the doxorubicin and paclitaxel [21]. Besides, another study reported that activation of Akt effector reinforces the drug resistance in advanced prostate cancer cells [22].

It was shown that VEGF expression was up-regulated and induced under hypoxic conditions through the upstream transcription factor HIF-1 [23]. According to the above drug resistance mechanism, DY might exert its effects in the treatment of drug-resistant CRC via inhibiting multiple signaling pathways (Raf/MEK/ERK and PI3K/Akt). Besides, 28 of the drug resistance-associated genes also play a pivotal role in DY-inhibited resistant CRC cells. For example, AKT3 is closely related to AKT1 and AKT2, which is critical players in the PI3K/Akt pathway participating in processes that regulate metabolism, cell survival, and proliferation [24]. Furthermore, high expression of AKT3 in CRC cell lines was found to be linked to enriched expression of EMT marker genes, leading to more resistance to chemo- and targeted-drugs [25]. A study also uncovered that NEDD9 was involved in the migration, invasion, and metastasis of cancer cells [26]. Dysregulation of NEDD9 can mediate the EMT process, suggesting that high expression of NEDD9 may involve in the progression and metastasis of CRC [27]. BMI-1 expression has been reported to be upregulated in liver metastases in a CRC mouse model and knockdown BMI-1 in CRC cells reduced CRC liver metastasis [28]. These studies have indicated that these genes may serve as targets for the regulation of drug resistance and metastasis, and DY may exert its effects by modulating these targets. However, some protein target validation experiments need to be further investigated to explore the core proteins that play a role, thus compensating for the limitations of this study.

5. Conclusion

In conclusion, DY alone or in combination with 5-FU significantly repressed the proliferation of 5-FU-resistant CRC cells and improved the susceptibility of resistant CRC cells to 5-FU. Meanwhile, DY inhibited the migration rate of drug-resistant CRC cells. Additionally, the Ras/MEK/ERK, PI3K/Akt and HIF-1 pathways are crucial pathways in the mechanism of drug resistance in CRC and DY against drug-resistant CRC cells. Western Blot results showed that DY down-regulated the expression levels of p-Ras, p-ERK1/2, p-MEK1/2, p-PI3K, p-Akt, HIF-1A, and VEGFA proteins, and suppress the *AKT3*, *NEDD9*, *BMI-1* and *CXCL1* gene expressions, which potentially interfere with the mentioned signaling pathways and genes, thereby leading to suppression of survival in 5-FU resistant CRC cells.

Author contribution statement

Zhong Feng; Yuanjing Zou; Yuemei Yuan, Hao Li: Contributed reagents, materials, analysis tools or data; Wrote the paper.
Weijia Zhang: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the

paper.

Ling Ou; Chang Peng: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Shuyi Sang: Analyzed and interpreted the data; Wrote the paper.

Guimin Zhang; Meicun Yao: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Data availability statement

Data will be made available on request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

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Appendix A. Supplementary data

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