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Clinical significance of FoxO1 expression and its regulation in dihydroartemisinin treatment in liver cancer

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Abstract: **Background**: The transcription factor Forkhead Box O 1 (FoxO1) is crucial to numerous cellular and biological functions. Dihydroartemisinin (DHA) is a derivative of artemisinin extracted from Chinese medicinal plants. The regulatory mechanism of FoxO1 in liver cancer and its relationship with DHA treatment remain unclear. **Objectives**: This study aims to investigate the role of FoxO1 in liver cancer and DHA treatment. **Methods**: The expression levels of FoxO1 and its correlation with overall survival were evaluated using the public databases and experiments. The regulation of DHA on FoxO1 was investigated by MTT, Western blotting, immunofluorescence staining, colony formation assays, CRISPR/Cas9 and siRNA-mediated gene knockdown. **Results**: FoxO1 expression was markedly reduced in hepatoma tissues and associated with higher overall survival. FoxO1 expression was diminished in advanced-stage hepatoma tissues. DHA enhanced FoxO1 expression, concomitant with a reduction in p-AKT and its downstream target p-mTORC1. DHA activity was decreased in FoxO1-knockdown cells. Interestingly, knockdown of Sirt2 abolished DHA-induced FoxO1 expression and impaired the anticancer effect of DHA, which may be correlated with FoxO1 ubiquitination regulation. The p38 MAPK signalling pathway is crucial for the tumor-suppressing effects of DHA and the translocation of FoxO1. **Conclusion**: The findings indicated that DHA could impede the development of liver cancer through FoxO1 regulation, suggesting that targeting FoxO1 may represent a promising therapeutic approach for liver cancer treatment.

Keywords: Dihydroartemisinin; FoxO1; Liver Cancer; p38 MAPK; Sirtuin 2

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INTRODUCTION

Liver cancer ranks among the foremost causes of cancer-related mortality, with projections of over one million cases identified worldwide (Lu *et al.*, 2025). Hepatocellular carcinoma (HCC) constitutes the principal type of liver cancer, representing roughly 85% to 90% of all primary liver malignancies (Zanuso *et al.*, 2025). Various established risk factors, such as chronic infection with the hepatitis B virus (HBV) and hepatitis C virus (HCV), alcohol misuse and metabolic syndrome, significantly contribute to the incidence and progression of hepatocellular carcinoma (HCC) (EASL, 2025). Unlike other solid tumours, including breast cancer, colon cancer and lung cancer, the distinct hallmark of HCC is the paucity of clear dependencies on an oncogene, which impedes the development of targeted therapies to some degree (Zhang *et al.*, 2022). Therefore, the exploitation and identification of novel therapeutic targets for HCC are necessary.

The transcription factor FoxO1 is an essential regulator of cellular stress responses and can be induced by extracellular stress, growth factors and multiple therapeutic agents to mediate cell survival, oxidative stress, metabolic disorders, autophagy and apoptosis (Calissi *et al.*, 2021; Santos *et al.*, 2023; Rodriguez-Colman *et al.*, 2024). A variety of signalling pathways, such as the Wnt/β-catenin, TGF-β and NF-kB pathways, are involved in the regulation of FoxO1. In the Wnt pathway, the conversion between FoxO1/β-catenin and the β-catenin/TCF interaction is indispensable for balancing the cell cycle process (Essers *et al.*, 2005). In the TGF-β signalling pathway, activated TGF-β signalling can induce Smad/FoxO complex formation to activate the expression of the growth-suppression gene p21Cip1. Integrated Smad/FoxO signalling is further negatively regulated by the PI3K pathway and the telencephalic development factor FoxG1 in neuroepithelial cells (Seoane *et al.*, 2004). In addition, FoxO1 is closely related to the Hippo–YAP and NF-κB signalling pathways (Xu *et al.*, 2024; Hu *et al.*, 2024). Through interaction with MDM2, FoxO1 also participates in the regulation of the p53/MDM2 pathway (Tomiyasu *et al.*, 2024). The acquisition of sustained proliferation signals and the suppression of growth-inhibiting signals are two major features of tumour cells (Hanahan *et al.*, 2011). An effective interaction occurs between FoxO1 and many signals related to proliferation and growth inhibition, suggesting that FoxO1 is a key participant and regulator of tumour development. In addition, FoxO1 was found to participate in lineage differentiation and immune regulation of Treg cells as well as in M1/M2 typing transformation of tumour-related macrophages (Ren *et al.*, 2023;Wang *et al.*, 2024),

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suggesting that FoxO1 may be a potential target for tumour immunotherapy. In light of the ability of the FoxO1 protein to promote cell proliferation, cell survival and immune system function, FoxO1 is a promising consideration for strategies directed against a wide variety of cancers, including liver cancer (Kim *et al.*, 2018; Jiramongkol *et al.*, 2020). A previous study demonstrated that the reduction of Aurora A kinase via RNA interference (RNAi) in hepatocellular carcinoma elevated FoxO1 in a p53-dependent manner, leading to cell cycle arrest and the inhibition of cell growth (Lee *et al.*, 2013). In clinical HCC tissues, FoxO1 was downregulated and positively correlated with NK3 homeobox 1 (NKX3.1) expression (Jiang *et al*., 2017). After gene overexpression or drug stimulation, the expression of FoxO1 in HCC tissue is restored, and HCC growth is greatly inhibited (Jiang *et al*., 2017). A recent study has shown that oxaloacetate inhibits tumor proliferation and induces apoptosis in cancer cells by regulating FoxO1 (Miao *et al.*, 2025). Analysis of the TCGA database demonstrated that FoxO1 serves as favorable factor for survival rates in patients with HCC (Xie *et al.*, 2025). Although studies have implicated a role for FoxO1 in liver cancer, the mechanistic link of FoxO1 to liver cancer progression has not been established and more importantly, FoxO1 has not been therapeutically targeted in liver cancer.

Dihydroartemisinin (DHA) is a major active metabolite of the well-known artemisinin, which is traditionally recognized as an effective antimalarial drug (Hanboonkunupakarn *et al.*, 2022). In addition to preventing malaria, DHA is also capable of inhibiting inflammation and protecting against liver injury and fibrosis (You *et al.*, 2022; Chen *et al.*, 2019). Recent reports have shown that DHA has potential anticancer activity in multiple tumours via complex anticancer mechanisms (Bai *et al.*, 2021; Zhang *et al.*, 2021; Ji *et al.*, 2024). Based on the above information, we hypothesized that the antitumour effect of DHA in liver cancer may be related to FoxO1 expression levels. The objective of this work was to examine the role of FoxO1 expression in the antitumor effects of DHA and to determine the mechanisms by which DHA or FoxO1 exert their antitumor effects on liver cancer.

MATERIALS AND METHODS

Antibodies and reagents

Primary antibodies, specifically anti-FoxO1 (C29H4, #2880), anti-p-AKT (Ser473, D9E, #4060), anti-p-mTOR (Ser2448, D9C2, #5536) were obtained from Cell Signalling Technology (CST); anti-Tubulin (AC012) was sourced from ABclonal (Wuhan, China); and anti-PARP1 (66520-1-Ig), anti-Ubiquitin (10201-2-AP) and anti-GAPDH (60004-1-Ig) were acquired from Proteintech Group (Wuhan, China). Secondary antibodies, comprising HRP-conjugated anti-rabbit and anti-mouse antibodies as well as Cy3-conjugated anti-rabbit antibodies, were acquired from Invitrogen. *Reagents and pharmaceuticals*: Lipofectamine 2000 transfection reagents were obtained from Invitrogen; enhanced chemiluminescence (ECL) and protein A/G agarose were sourced from Thermo Fisher Scientific; the p38 MAPK inhibitor SB202190 was acquired from Selleck Chemicals; 3-(4,5)-dimethylthiahiazo (z-y1)-3,5-di-phenytetrazoliumromide (MTT), dihydroartemisinin (DHA) and 4,6-diamidino-2-phenylindole (DAPI) were procured from Sigma–Aldrich; and a cocktail of proteinase and phosphatase inhibitors was purchased from Apexbio. Dulbecco's Modified Eagle Medium (DMEM) and foetal bovine serum (FBS) were acquired from Gibco.

Cell culture and transfection

ATCC provided the HepG2 (HB-8065) cells, which were subsequently confirmed using STR profiling. The cells were cultured at 37°C with 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Foetal Bovine Serum (FBS). 100 U/mL of penicillin/streptomycin was added to the cultured cells. Lipofectamine 2000 was used for transfections in compliance with the manufacturer's instructions.

Generation of stable lines

Lentiviral vectors were used to generate cell lines with FoxO1 knockdown. Using the envelope plasmid VSV-G (Addgene, #8454) and the packaging plasmid psPAX2 (Addgene, #12260), HEK293T cells were transfected with Lenti-CRISPR-V2-sgFoxO1. 48 hours after transfection, the lentiviral supernatant was extracted and used to infect HepG2 cells. Puromycin (Apexbio) at a concentration of 0.8 μg/mL was then used to identify stable cell lines. By using Western blotting, the expression levels of FoxO1 in knockdown cells were evaluated. The following are guide sequences that target the human FoxO1 genes: FoxO1: 5’-GGT TGC CCC ACG CGT TGC GG-3’.

HEK293T cells were first transfected with either the vector or pCDH-CMV-MCS-EF1-puro-FoxO1, along with the envelope plasmid VSV-G and the packaging plasmid psPAX2, to produce FoxO1-overexpressing cell lines. 48 hours after transfection, the lentiviral supernatant was extracted and the HepG2 cells were subsequently infected. Puromycin was eventually used to create stable cell lines, which were then analysed by Western blotting.

MTT assay

As previously described, the MTT test was used to evaluate cell viability (Zhang *et al.*, 2020).

Colony formation

HepG2 and FoxO1 knockdown cells (≤500 cells/well) were cultured in six-well plates and subsequently exposed to 5 μM DHA for two weeks. The cells were subsequently fixed in 4% paraformaldehyde and stained with a 0.5% crystal violet solution for examination.

siRNA synthesis

The siRNA sequences for Sirt2 (AUG UUU CUU GAA AUA GCU GAU) and the nontargeting sequence (UGA ACU UGU GGC CGU UUA CGU) were synthesized by Sangon (Shanghai).

Sirt2 siRNA or scrambled siRNA (50 nM) was transfected using Lipofectamine 2000 and the knockdown efficacy was confirmed using Western blotting.

Western blotting

Relative protein expression levels were assessed using western blotting. Cell lysates were acquired following a 30-minute treatment with RIPA buffer (#P0013, Beyotime) on ice. The lysates were subjected to centrifugation at 12,000 × g at 4 °C for 15 minutes and thereafter collected for protein concentration assessment using a BCA Protein Analysis Kit (#P0010; Beyotime). Approximately 40 µg of total protein was resolved using 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and subsequently transferred to polyvinylidene difluoride (PVDF) membranes (0.22µm, Millipore).

The membranes were blocked with 5% nonfat milk for 1 hour and then incubated with primary antibodies at 4 °C overnight, followed by a 2-hour incubation with an HRP-conjugated secondary antibody (1:5000, ABclonal, China) at room temperature. GAPDH served as a loading control. The band signals were visualised using a ChemiDoc™ imaging equipment (Bio-Rad, USA) and subsequently analysed with Quantity One software. Nuclear and cytoplasmic proteins were isolated from the cells using a Nuclear and Cytoplasmic Protein Extraction Kit (#P0028; Beyotime) in accordance with the manufacturer's guidelines.

Immunofluorescence staining

After being cleaned with PBS, the treated cell slides were incubated for ten minutes in 0.1% Triton X-100. After being incubated for one hour with 5% bovine serum albumin (BSA) to prevent nonspecific binding, the slides were then incubated with a primary antibody that targets FoxO1 (1:200) for a whole night at 4 °C. Following washing, the sections were treated with the nuclear dye DAPI and then incubated for two hours at room temperature with a Cy3-conjugated secondary antibody. In the end, a confocal microscope was used to inspect and take pictures of the slices.

Immunoprecipitation and ubiquitination assays

HepG2 cells were transfected with Sirt2-siRNA or control siRNA using Lipofectamine 2000 after DHA treatment. Cells were then lysed in RIPA lysis buffer. Immunoprecipitation was conducted with anti-FoxO1 antibody-conjugated protein A/G agarose beads, as previously outlined. The precipitates were heated in loading buffer and subsequently applied to SDS-PAGE gels for separation and immunoblotting.

Immunohistochemistry

HCC tissue chips were acquired from Shanghai Outdo Biochip Technology Co., Ltd. (HLivH020PG01). The slices were initially deparaffinized, dried, exposed to antigen retrieval and subsequently incubated overnight at 4°C with a primary antibody targeting FoxO1 (1:200). Following incubation, an immunohistochemical staining kit (Fuzhou Maixin Biotechnology Co., Ltd., Fuzhou, China) was employed for 3,3'-diaminobenzidine tetrahydrochloride (DAB) staining, succeeded by haematoxylin counterstaining in accordance with the manufacturer's instructions.

Online database analysis

The relative abundance of the FoxO1 protein in liver cancer was examined using the Cancer Proteogenomic Data Analysis Site (https://cprosite.ccr.cancer.gov/#/). The relationship between FoxO1 expression and overall survival (OS) in hepatocellular carcinoma (HCC) patients was analysed using the online Kaplan-Meier plotter (http://kmplot.com/analysis/).

Statistical analysis

The mean and standard deviation are used to display the data. With GraphPad Prism 8 (version 8.0, GraphPad Software, USA), an unpaired Student's t-test was used to assess significant differences. Statistical significance was defined as a *P* value of less than 0.05. The statistical analysis was carried out utilising the independent variables that make up all of the supplied data.

RESULTS

FoxO1 expression is decreased in HCC and correlated with patient survival

The Cancer Proteogenomic Data Analysis Site (cProSite) of the National Cancer Institute (https://cprosite.ccr.cancer.gov/#/) provides protein expression data for eleven types of solid tumours. Initially, we assessed the comparative abundance of FoxO1 protein in liver cancer utilizing the cProSite database. The relative protein content of FoxO1 was reduced in cancer tissues compared to neighboring normal tissue (Fig. 1A). The online Kaplan-Meier plotter (http://kmplot.com/analysis/) study indicated that FoxO1 expression was positively correlated with overall survival (OS) in patients with HCC (Fig. 1B). FoxO1 expression was additionally confirmed by immunohistochemistry (IHC) using commercial hepatocellular carcinoma (HCC) tissue chips. Our data demonstrated that the expression levels of FoxO1 were dramatically lower in these advanced HCC samples (Fig. 1C). Taken together, our results suggested that FoxO1 may have a tumour suppressor role in liver cancer progression.

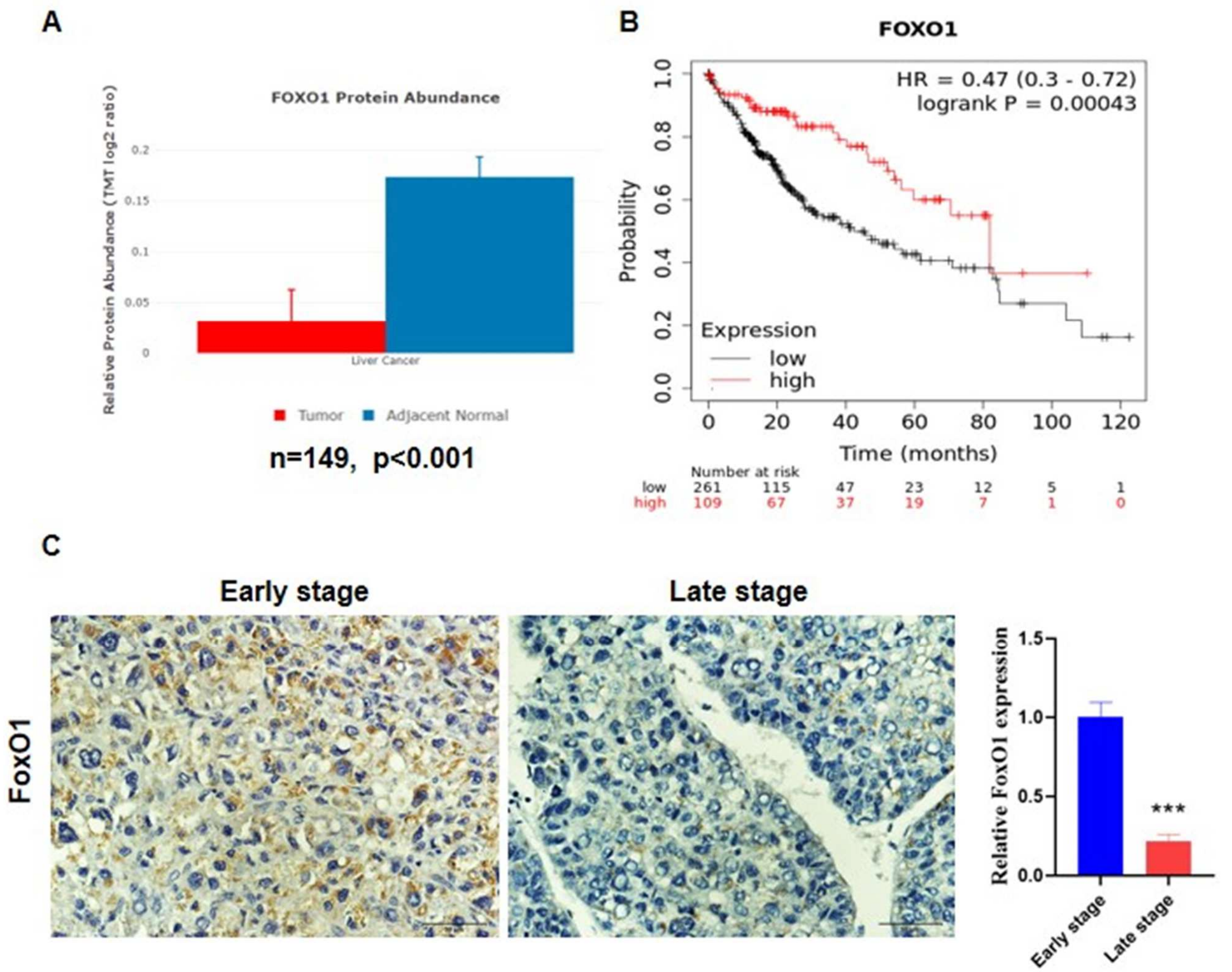

Fig. 1: FoxO1 is downregulated and positively associated with patient OS in HCC.
(A) The relative FoxO1 protein abundance in liver cancer tissues was analysed via the Cancer Proteogenomic Data Analysis Site (cProSite) database. The protein levels of FoxO1 were calculated and compared between tumours and adjacent liver tissues (n=149); p < 0.001. (B) The correlation of FoxO1 with overall survival (OS) in HCC patients was determined by an online Kaplan–Meier plotter analysis tool. The cut-off level of FoxO1 was set at the median level. The FoxO1 expression levels were divided into high and low-expression groups. OS was compared between the two groups. HR (hazard ratio) =0.47, P = 0.00043. (C) The protein levels of FoxO1 in early- or late-stage HCC tissues were analysed by immunohistochemistry. Patients were diagnosed and pathologically staged according to the AJCC TNM staging criteria.

DHA induced FoxO1 expression in a dose- and time-dependent manner

DHA has been widely investigated for its anticancer effects on multiple malignancies (Slezakova *et al.*, 2017). However, its effects and mechanisms in liver cancer remain largely unknown. In HepG2 cells, we found that a high dose of DHA induced PARP cleavage, which is an indicator of apoptosis (Fig. 2A). DHA potently inhibited the expression of p-AKT and p-mTOR (Liu *et al.*, 2019; Zhu *et al.*, 2019). In addition, treatment with DHA may increase the expression of the transcription factor FoxO1 (Zhu *et al.*, 2019). In accordance with prior findings, DHA administration significantly elevated FoxO1 protein levels in a dose- and time-dependent manner, while concurrently reducing p-AKT and downstream p-mTOR expression (Fig. 2A-D). These findings indicate that FoxO1 may play a role in the anticancer effects of DHA.

FoxO1 mediated the antitumour effects of DHA in liver cancer cells

To confirm the change in FoxO1 protein expression induced by DHA treatment, DHA-treated and control HepG2 cells were subjected to immunofluorescence (IF) staining. IF analysis demonstrated that both the nuclear and cytoplasmic expression of the FoxO1 protein increased after DHA treatment (Fig. 3A). The FoxO1 levels in the nuclear and cytoplasmic fractions of cells treated with DHA were analyzed by subcellular separation.

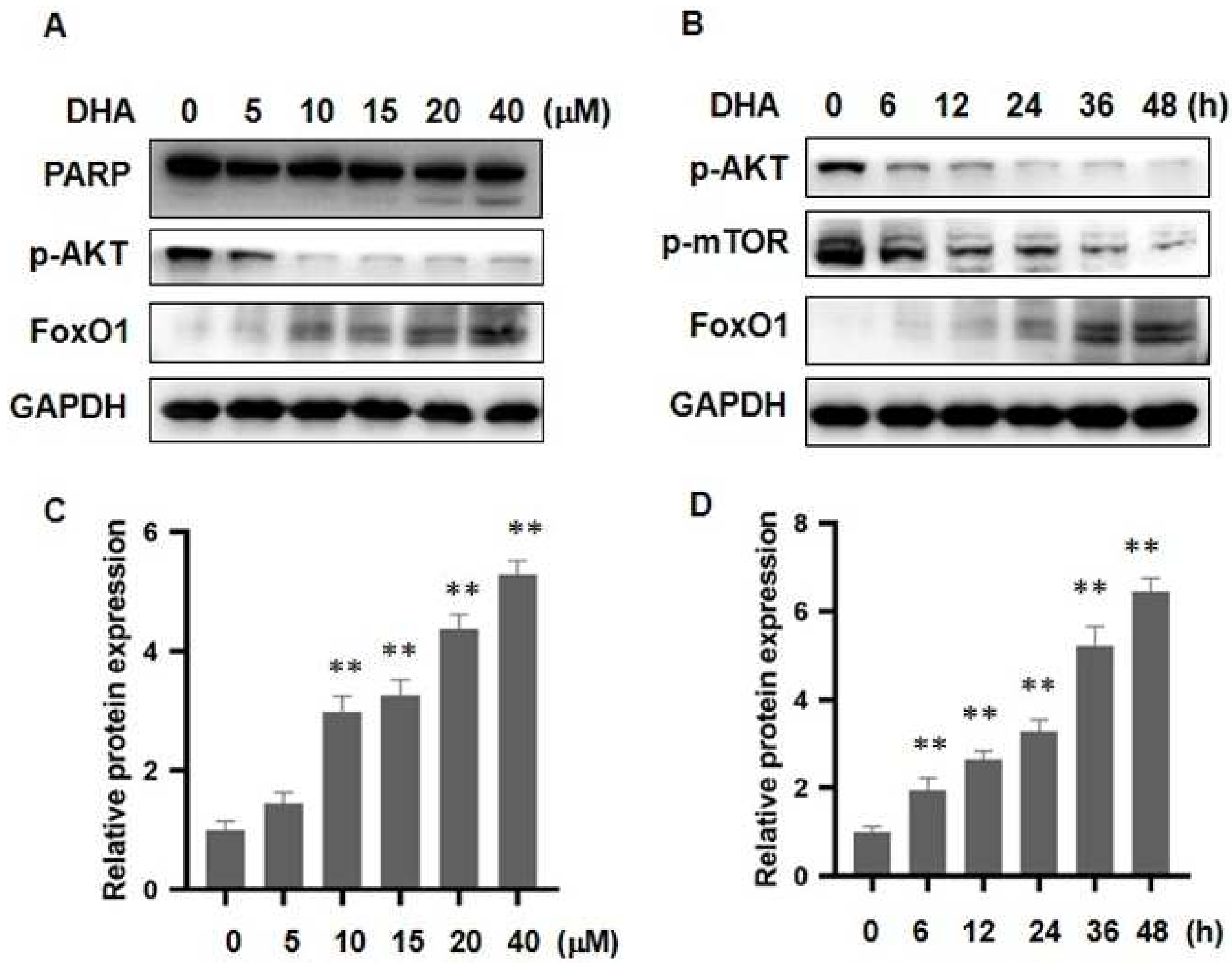

Fig. 2: DHA induced FoxO1 expression in a dose- and time-dependent manner.
(A) Western blot analysis of FoxO1, PARP, and p-AKT protein expression in HepG2 cells after treatment with different doses of DHA for 12 h. GAPDH was used as a loading control. (B) HepG2 cells were treated with vehicle or 10 μM DHA for different durations. The protein expression levels of FoxO1, p-AKT and p-mTOR in the cells were detected by western blotting. (C-D) Quantification of FoxO1 levels after DHA treatment using Quantity One software. The data are presented as the mean ± S.D.; $^{**}P < 0.01$, the DHA-treated group vs. the vehicle group.

Consistent with the immunofluorescence results, treatment of cells with DHA increased the amount of nuclear and cytoplasmic FoxO1 (Fig. 3B).

We further explored whether FoxO1 was required for the anticancer activity of DHA. FoxO1-knockdown and parental HepG2 cells were treated with various doses of DHA, and cell viability was subsequently examined. Our results showed that FoxO1 interference largely blocked the anticancer activity of DHA (Fig. 3C). The colony formation assay also indicated that silencing FoxO1 strongly impaired the ability of DHA to suppress the proliferation of HepG2 cells (Fig. 3D). The aforementioned data indicate that FoxO1 expression is pivotal in facilitating the anticancer effects of DHA.

Sirt2 is required for the antitumour effects of DHA on liver cancer cells

The family of mammalian Sirtuin proteins comprises seven members widely involved in cell stress and apoptosis regulation (Zhang *et al.*, 2023). Cytoplasmic Sirt2 is an important regulator of FoxO1 acetylation activity (Guo *et al.*, 2022). To investigate the underlying mechanism by which DHA affects FoxO1 expression, we knocked down Sirt2 using specific siRNA technology. DHA-induced FoxO1 accumulation in the nucleus and cytoplasm was strongly suppressed in Sirt2-knockdown cells (Fig. 4A), indicating the critical role of Sirt2 in DHA-induced FoxO1 expression. Sirt2 knockdown also reduced the sensitivity of HepG2 cells to DHA (Fig. 4B). Furthermore, the knockdown of Sirt2 induced the ubiquitination of FoxO1 in the context of DHA treatment (Fig. 4C).

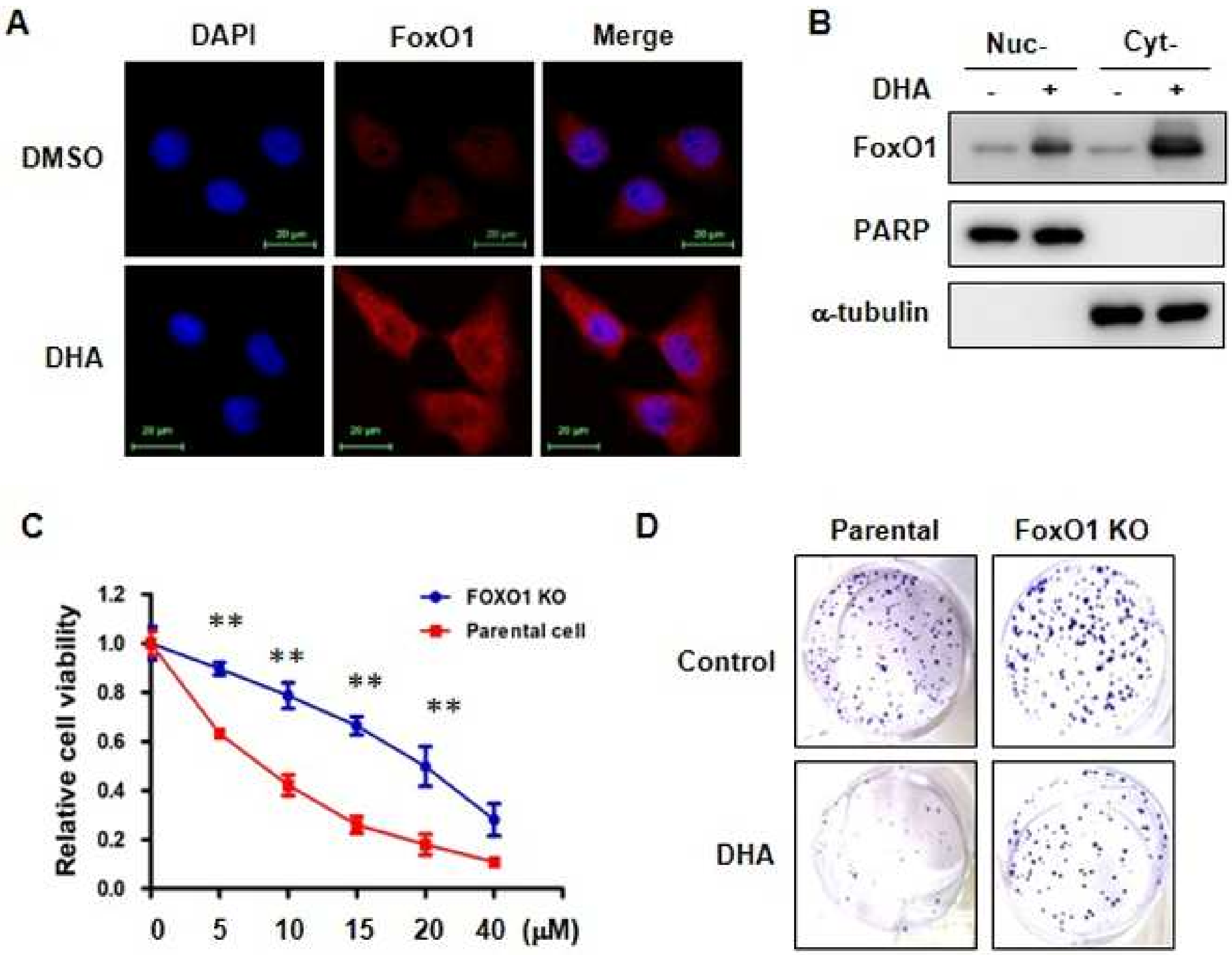

Fig. 3: FoxO1 mediated the antitumour effects of DHA in liver cancer cells.
(A) HepG2 cells were treated with 10 μM DHA for 24 h. After fixation, the cells were immunostained with an anti-FoxO1 antibody and observed under a laser scanning confocal microscope. DAPI counterstaining was performed. (B) HepG2 cells were treated with 10 μM DHA for 24 h. The FoxO1 expression levels in the cytoplasm and nucleus were examined in the subcellular fraction. Cyt-: cytoplasm; Nuc-: nucleus. PARP was used as a nuclear marker, and tubulin was used as a cytosolic marker. (C) FoxO1-knockdown cells and their parental cells were treated with vehicle or increasing concentrations of DHA for 48 h. Cell proliferation was detected with MTT. The data are presented as the mean ± S.D.; $^{**}P < 0.01$, the FoxO1 knockdown group vs. the parental group. (D) FoxO1-knockdown cells and their parental cells were treated with 5 μM DHA and grown in 6-well plates for two weeks. The cells were fixed with paraformaldehyde and stained with crystal violet.

Collectively, these findings indicate that Sirt2 is essential for the expression of FoxO1 and the antitumor effects of DHA.

p38 MAPK participates in the anticancer effect of DHA and translocation of FoxO1

Alongside its impact on AKT/mTOR signalling, we also investigated the effect of DHA on additional signalling pathways. DHA administration stimulated p38 MAPK signalling in a dose-dependent manner (Fig. 5A). We subsequently examined the influence of p38 MAPK on DHA activity. Our data demonstrated that DHA-induced PARP cleavage was abolished when p38 MAPK was inhibited by SB202190 (Fig. 5B). Interestingly, immunofluorescence staining showed that inhibition of p38 MAPK strongly prevented FoxO1 protein localization in the cytoplasm (Fig. 5C). Thus, our results demonstrated that the p38 MAPK signalling pathway was responsible for DHA activity and FoxO1 translocation.

DISCUSSION

A growing body of research has illustrated the essential role of FoxO1 in metabolic functions (Peng *et al.*, 2020). Therefore, the design of FoxO1 modulators for metabolic diseases has been widely attempted and is urgently needed (Zhang *et al.*, 2021). Hepatocarcinogenesis is closely associated with abnormal metabolic status and signalling pathways and there are very limited treatment options.

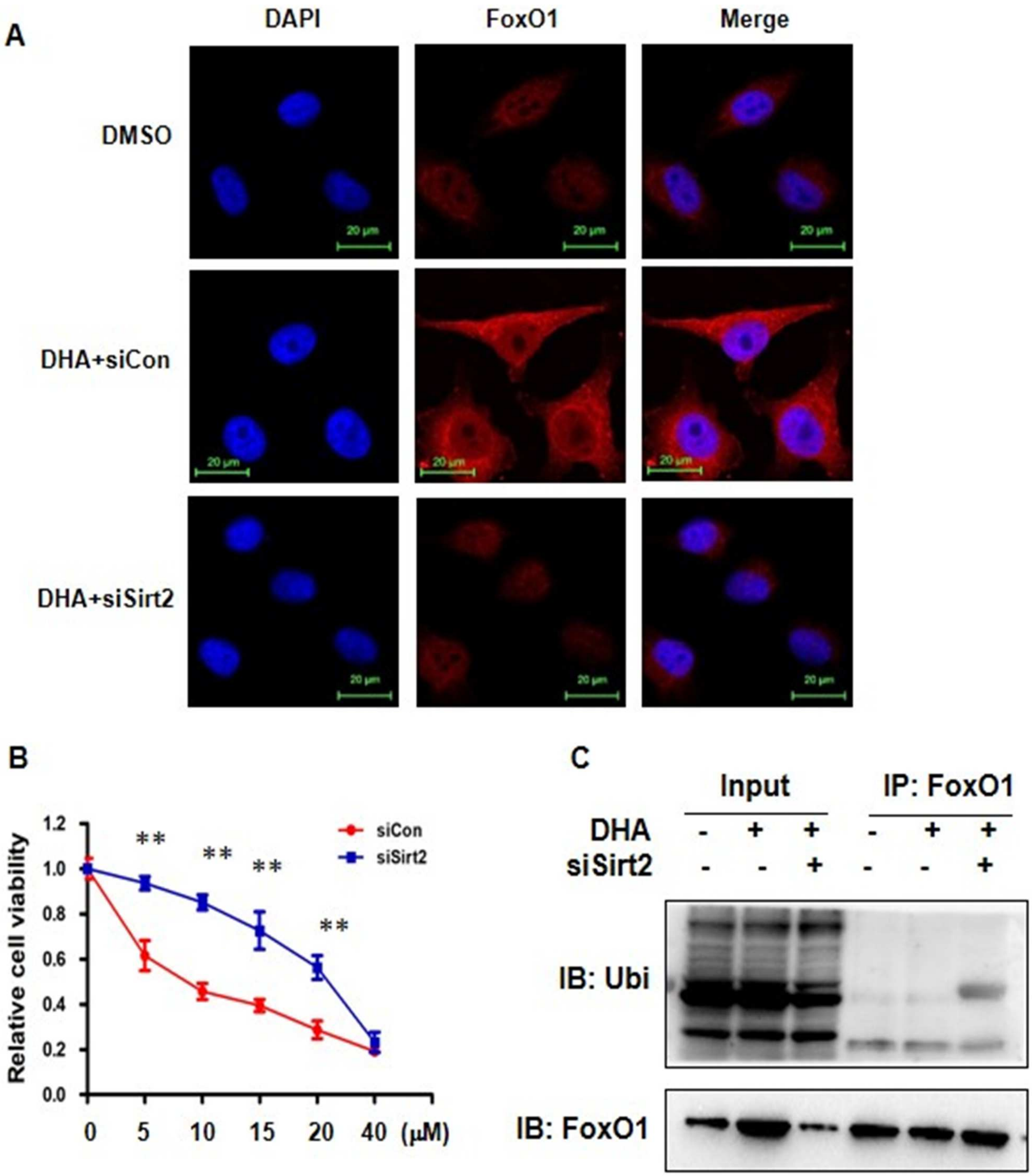

Fig. 4: Sirt2 is required for the antitumour effects of DHA in liver cancer cells.
(A) HepG2 cells were transfected with Sirt2 siRNA or scramble siRNA and then treated with 10 μM DHA for 24 h. After fixation, the cells were immunostained with an anti-FoxO1 antibody and observed under a laser scanning confocal microscope. DAPI counterstaining was performed. (B) HepG2 cells were transfected with Sirt2 siRNA or scramble siRNA and then treated with increasing concentrations of DHA for 48 h. Cell proliferation was detected with MTT. The data are presented as the mean ± S.D.; $^{**}P < 0.01$, the Sirt2 siRNA group vs. the control siRNA group. (C) HepG2 cells were transfected with Sirt2 siRNA or scramble siRNA and then treated with 10 μM DHA for 24 h. The cell lysates were immunoprecipitated with an anti-FoxO1 antibody. The IP products were detected for FoxO1 ubiquitination with an anti-ubiquitin antibody. Whole-cell lysates were loaded as the input group.

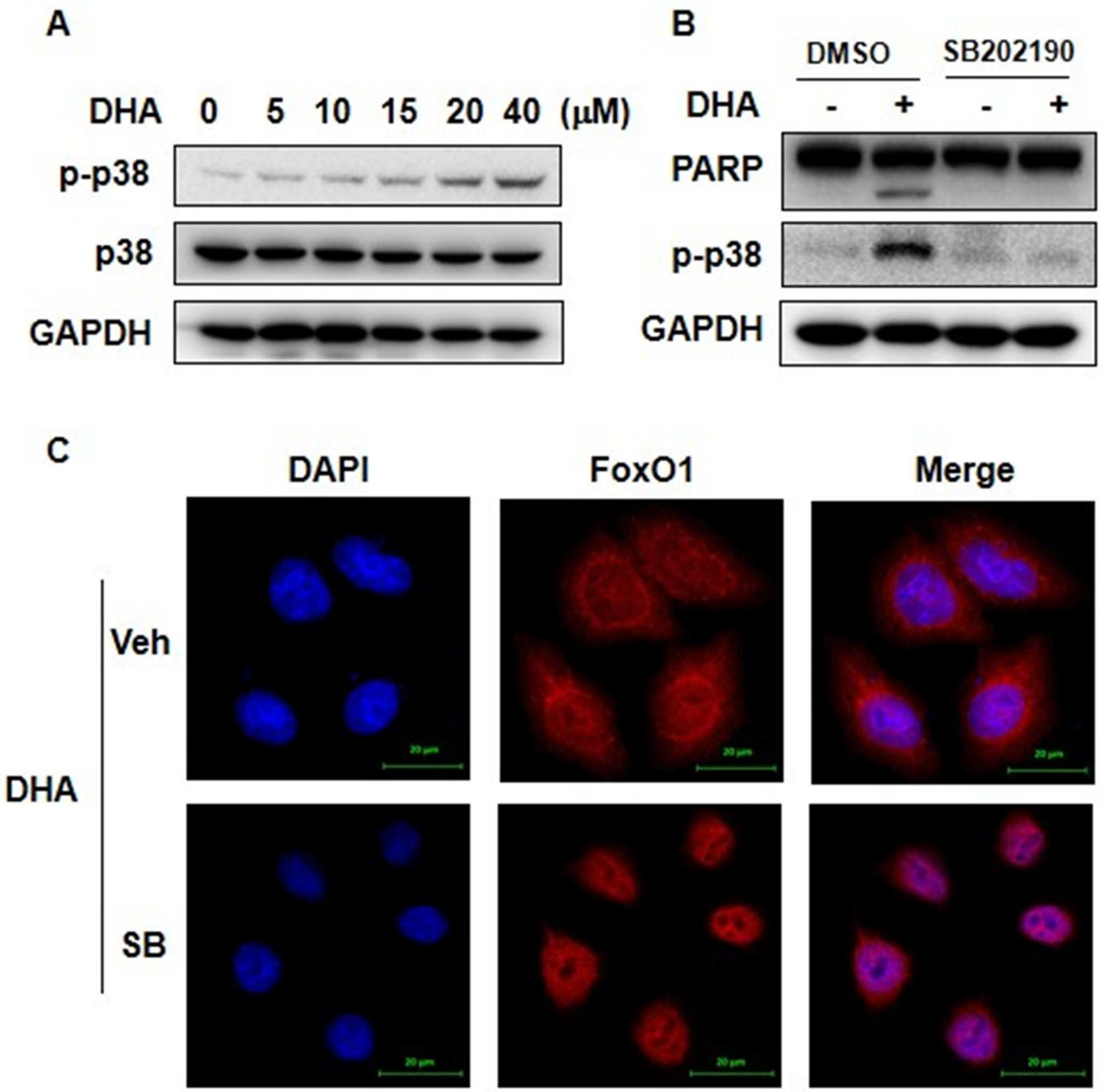

Fig. 5: p38 MAPK participates in the anticancer effect of DHA and the translocation of FoxO1.
(A) Western blotting detection of p-p38 and p38 proteins in HepG2 cells after treatment with different doses of DHA. (B) HepG2 cells were pretreated with 10 μM SB202190 for 3 h and then treated with vehicle or 10 μM DHA for 24 h. The protein expression levels of PARP and p-p38 in the cells were detected by western blotting. (C) HepG2 cells were pretreated with 10 μM SB202190 (SB) for 3 h and then treated with 10 μM DHA for 24 h. After fixation, the cells were immunostained with an anti-FoxO1 antibody and observed under a laser scanning confocal microscope. DAPI counterstaining was performed.

Clarifying the pivotal role and mechanism of FoxO1 in HCC will contribute to the development of new therapeutic strategies. The expression levels and therapeutic relevance of FoxO1 in liver cancer are frequently overlooked. This investigation consistently revealed that a reduction in FoxO1 is significantly correlated with diminished overall survival (OS) (Fig. 1). We found that an artemisinin derivative, dihydroartemisinin (DHA), exhibited an antitumour effect on liver cancer. DHA mechanistically inhibited the development and proliferation of liver cancer cells by elevating FoxO1 protein levels (Figs. 2-3), indicating that targeting FoxO1 could be a viable therapeutic strategy for liver cancer treatment. Given that AKT activation confers resistance to specific chemotherapeutics, such sorafenib (Tan *et al.*, 2022), targeting FoxO1 may prove advantageous for additional anticancer agents.

FoxO1 is regarded as a crucial regulatory element of the insulin signalling cascade. During fasting, FoxO1 is activated within the nucleus of hepatocytes, leading to the upregulation of genes associated with gluconeogenesis and glycogenolysis (Ge *et al.*, 2021). Upon insulin signalling activation, phosphorylated AKT modifies FoxO1, prompting its translocation to the cytoplasm for destruction (Pan *et al.*, 2017). It is therefore meaningful to elucidate the role of FoxO1 in HCC, where the PI3K-AKT signalling pathway, the master regulatory switch for FoxO1, is usually activated. Theoretically, FoxO1 is believed to be an ideal cancer-specific target for HCC therapy. However, FoxO1 is subject to numerous posttranslational modifications, including phosphorylation, acetylation and ubiquitination, which further alter its protein activity upon exposure to extracellular stimuli and may also potentially prevent its AKT dependency. Sirtuins are NAD^+-dependent deacetylase enzymes. Among the sirtuin members, Sirt2 is reported to play crucial roles in cellular proliferation, migration and apoptosis related to the promotion of tumour initiation, progression and metastasis in HCC (Chen *et al.*, 2019). As FoxO1 has been identified as a substrate of Sirt2, knockdown of Sirt2 may promote FoxO1 acetylation and transcriptional activity (Guo *et al.*, 2022). Sirt2 knockdown suppressed FoxO1 expression and facilitated the ubiquitination of FoxO1 (Fig. 4). Therefore, our findings suggested that Sirt2 may influence FoxO1 protein stability, which needs to be explored in future works.

In our study, a significant increase in FoxO1 protein was observed in DHA-treated liver cancer cells, which occurred not only in the nucleus but also in the cytoplasm (Fig. 3). From our perspective, the downregulation of p-AKT may lead to a nuclear increase in FoxO1 expression, while the cytoplasmic increase in FoxO1 expression may be irrelevant to the AKT pathway. Recently, several studies have demonstrated that the MAPK p38 signalling pathway is closely associated with the induction of cancer cell apoptosis (Chuang *et al.*, 2022). MAPK-p38 phosphorylates and regulates FoxO1, but the role of MAPK-p38 in FoxO1-mediated biological regulation is not fully understood (Wu *et al.*, 2024). The simultaneous inhibition of PARP breakage and FoxO1 translocation by pharmacological blockage of p38 kinase (Fig. 5) suggests that p38-mediated cytoplasmic localization of FoxO1 is pivotal to the antitumor efficacy of DHA. Consistent with our findings, a prior investigation indicated that cytosolic FoxO1 was crucial for the initiation of autophagy and tumour suppressor function in reaction to oxidative stress or serum deprivation (Zhao *et al.*, 2010). Given that the predominant localization of FoxO1 was in the cytoplasm of early HCC tissue (Fig. 1), we assert that cytosolic FoxO1 primarily facilitates its tumour suppressor function in HCC. Based on the above observations, we suggested that FoxO1 can shuttle between the cytoplasm and nucleus, exerting various nongenomic effects beyond its transcriptional activity. Consequently, it is essential to elucidate the diverse roles of FoxO1 in various physiological and pathological contexts.

Dihydroartemisinin (DHA) is an active metabolite of artemisinin, extensively utilized in the treatment of malaria. DHA exhibits anticancer properties against numerous tumour types through multiple molecular pathways, including the inhibition of proliferation, induction of apoptosis and promotion of autophagy and endoplasmic reticulum (ER) stress (Dai *et al.*, 2021). The anticancer mechanism of DHA in HCC may encompass various regulatory processes. Considering the anti-inflammatory properties of DHA and the typical association of HCC with inflammation (Hammerich *et al.*, 2023), we conclude that DHA may serve as a promising multifunctional lead compound that mitigates hepatitis and inhibits HCC progression.

Our data indicate that FoxO1 may facilitate the antitumor impact of DHA on liver cancer. Furthermore, we uncovered a previously unrecognized control of the FoxO1 protein mediated by Sirt2 and involving p38, which may be critical for DHA-induced FoxO1 expression and the inhibition of liver cancer. The binding connection between FoxO1 and DHA, as well as the precise amino acid residues in the FoxO1 protein that are subject to ubiquitination and phosphorylation, remains ambiguous and need additional elucidation. Future discoveries of novel FoxO1 modulators for drug design may represent a promising therapeutic avenue for the treatment of liver cancer. These findings establish a scientific basis for targeting FoxO1 as a viable pharmacological target for liver cancer, presenting a potential new therapeutic strategy for practical use.

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Authors' contributions
Conception, funding acquisition and data curation: SZ, SL. Collection, investigation and analysis of data: YY, SL and YZ. Writing of draft, editing and revision of the manuscript: YY, MZ and SZ. All authors have read and agreed to the final version of the manuscript.

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Data availability statement
The data presented in this study are available upon request from the corresponding author.

Ethical approval
The tissue microarrays (HLivH020PG01) used in this study were approved by the Ethics Committee of Shanghai Outdo Biotech Company (Approval number: YB M-05-02).

Conflict of interest
All the authors declare that there are no conflicts of interest.

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