

RESEARCH PAPER



Artesunate Sensitizes human hepatocellular carcinoma to sorafenib via exacerbating AFAP1L2-SRC-FUNDC1 axis-dependent mitophagy

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ABSTRACT

Sorafenib is the most widely used first-line drug for the treatment of the advanced hepatocellular carcinoma (HCC). Unfortunately, sorafenib resistance often limits its therapeutic efficacy. To evaluate the efficacy of artesunate against sorafenib-resistant HCC and to investigate its underlying pharmacological mechanisms, a “sorafenib resistance related gene-ART candidate target” interaction network was constructed, and a signaling axis consisting with artesunate candidate target AFAP1L2 and sorafenib target SRC, and the downstream FUNDC1-dependent mitophagy was identified as a major contributor to the sorafenib resistance and a potential way of artesunate to mitigate resistance. Notably, our clinical data demonstrated that AFAP1L2 expression in HCC tissues was markedly higher than that in adjacent non-cancerous liver tissues ($P < 0.05$), and high AFAP1L2 expression was also significantly associated with an unfavorable overall survival of HCC patients ($P < 0.05$). Experimentally, AFAP1L2 was overexpressed in sorafenib resistant cells, leading to the activation of downstream SRC-FUNDC1 signaling axis, further blocking the FUNDC1 recruitment of LC3B to mitochondria and inhibiting the activation of mitophagy, based on both in vitro and in vivo systems. Moreover, artesunate significantly enhanced the inhibitory effects of sorafenib on resistant cells and tumors by inducing excessive mitophagy. Mechanically, artesunate reduced the expression of AFAP1L2 protein, suppressed the phosphorylation levels of SRC and FUNDC1 proteins, promoted the FUNDC1 recruitment of massive LC3B to mitochondria, and further overactivated the mitophagy and subsequent cell apoptosis of sorafenib resistant cells. In conclusion, artesunate may be a promising strategy to mitigate sorafenib resistance in HCC via exacerbating AFAP1L2-SRC-FUNDC1 axis-dependent mitophagy.

Abbreviations: AFAP1L2, actin filament associated protein 1 like 2; ANOVA, analysis of variance; ANXA5, annexin V; ART: artesunate; CETSA, cellular thermal shift assay; CI: combination index; CO-IP: co-immunoprecipitation; CQ: chloroquine; CT, computed tomography; [¹⁸F]-FDG, fluoro-2-D-deoxyglucose F18; FUNDC1: FUN14 domain containing 1; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; HCC, hepatocellular carcinoma; H&E Staining: hematoxylin – eosin staining; HepG2^R, sorafenib resistant HepG2; IF, immunofluorescence; IHC, immunohistochemistry; LAMP1: lysosomal associated membrane protein 1; MAP1LC3B/LC3B: microtubule associated protein 1 light chain 3 beta; miR, microRNA; mRNA: messenger RNA; OE, overexpression; OS, overall survival; PET, positron emission tomography; qRT-PCR: quantitative real-time PCR; sh, short hairpin; shNC: negative control shRNA; shAFAP1L2: short hairpin AFAP1L2; SORA, sorafenib; SPR, surface plasmon resonance; SRC, SRC proto-oncogene, non-receptor tyrosine kinase; SUV, standardized uptake value; TEM, transmission electron microscopy; TOMM20: translocase of outer mitochondrial membrane 20.

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Introduction

Hepatocellular carcinoma (HCC) is the second leading cause of cancer-related death worldwide [1]. Despite the current use of an oral multi-kinase inhibitor, sorafenib, in the care of advanced HCC, multiple clinical trials revealed its limited survival benefits, which is attributed to the primary and acquired drug resistance [2]. Only 30% of patients with HCC can benefit from sorafenib treatment, whereas the resistance in above population is usually acquired within 6 months [3]. In the current clinical settings, the strategies to overcome the sorafenib resistance are mainly the

combined medication with other drugs, including drugs targeting specific molecules, anti-EGFR (epidermal growth factor receptor) antibody (cetuximab), cytotoxicity chemotherapy drugs (epirubicin, cisplatin, 5-FU and capecitabine) and immunotherapy drugs (anti-PDCD1/PD-1 [programmed cell death 1] antibody) [4]. However, severe adverse reactions, such as diarrhea and organ damage, usually restrict the clinical use of these therapeutics. Therefore, it is extremely necessary to develop novel and safe drugs to mitigate and overcome the sorafenib resistance in HCC.

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Accumulating studies have indicated that HCC patients prone to sorafenib resistance often show the increased EGFR expression, JUN/C-JUN (jun proto-oncogene, AP-1 transcription factor subunit) and AKT1/AKT (AKT serine/threonine kinase 1) activation, enhanced epithelial-mesenchymal transition (EMT), increasing number of cancer stem cells, and the advanced hypoxic environment [5]. In recent years, mitophagy, which is responsible for regulating mitochondrial homeostasis, has attracted an increasing attention in tumor drug resistance. The mild activation of mitophagy can help tumor cells to renew drug-damaged mitochondria and promote drug resistance to maintain cell survival. In contrast, the overactivation of mitophagy may lead to autophagic cell death [6]. It has also reported that mitophagy may promote the sorafenib resistance through hypoxia-inducible ATAD3A (ATPase family AAA domain containing 3A) dependent axis [7]. Thus, mitophagy may play a role during the sorafenib resistance in HCC, which may help to discover new treatments that overcome this drug resistance.

Artesunate (ART), a semi-synthetic water-soluble artemisinin derivative, is the first-line drug for malaria treatment in clinical practice [8]. Drug repurposing studies have found that ART may markedly inhibit a variety of malignancies, such as HCC and prostate cancer, by regulating cell autophagy, ferroptosis, cell cycle and DNA damage [8]. ART may mainly stay in the mitochondria of Hela cells, covalently bind to a large number of mitochondrial membrane proteins or enzymes, and induce cellular mitophagy through the PINK1 (PTEN induced kinase 1)-PRKN/Parkin (parkin RBR E3 ubiquitin protein ligase) pathway [9]. In our previous study, we also confirmed the anti-HCC efficacy of ART based on the *in vivo* and *in vitro* experimental systems, and also revealed that the lysosomal hydrolase GBA1/GBA (glucosylceramidase beta 1)-mediated autophagic degradation may be one of its targets against HCC [10]. Of note, ART has gradually attracted more and more attentions in sensitizing several anti-cancer drugs [11,12]. Li et al. [13] and Yao et al. [14] found that ART may markedly enhance the cytotoxicity of sorafenib based on HCC cells *in vitro*. However, whether ART might be a potential strategy to mitigate sorafenib resistance in HCC and the underlying pharmacological mechanisms remain unknown.

To address this problem, we herein constructed the “sorafenib-resistance related gene-ART candidate target” interaction network using clinical transcriptomic data and chemical proteomic data. Following the calculation of network topological features and functional relevance analysis, we hypothesized that the signaling axis consisting with ART candidate target AFAP1L2 (actin filament associated protein 1 like 2) and sorafenib target SRC (SRC proto-oncogene, non-receptor tyrosine kinase), and the downstream FUNDC1 (FUN14 domain containing 1)-dependent mitophagy might contribute to the sorafenib resistance and be a potential way of ART to mitigate resistance. Experimentally, both the *in vivo* (based on sorafenib-resistant HCC orthotopic xenograft mouse model) and *in vitro* (based on sorafenib-resistant HepG2 cells) experiment systems were used to validate the above hypothesis.

Results

ART may have a potential to mitigate sorafenib resistance via targeting AFAP1L2-SRC-FUNDC1 axis-mediated mitophagy

As described in our previous study [10], a total of 58 ART target proteins with a *P* value less than 0.05 and a competition ratio greater than 1.5 were identified by a large-scale chemoproteomic experiment using the ART probe. Detailed information of ART target proteins is provided in Table S1. In addition, there were 53 sorafenib targets against HCC and 141 differentially expressed genes between sorafenib-resistant and sensitive HCC patients obtained from literature mining and GEO dataset analysis, respectively (Table S2 and S3). Following the establishment of the “sorafenib-resistance related gene-ART candidate target” interaction network, and the calculation of topological features, 149 major nodes were screened and functionally enriched into autophagy and angiogenesis (Table S4 and S5, Figure 1A). Among them, AFAP1L2, one of ART candidate targets, and SRC, one of sorafenib targets, were both the major nodes of the above network.

AFAP1L2, an adaptor protein, has been indicated to play a critical role in the regulation of cell proliferation, survival, invasion, metastasis, and apoptosis [13]. It has been revealed as a SRC-binding partner that plays a role in a signaling cascade by enhancing the kinase activity of SRC [14]. Of note, FUNDC1-mediated mitophagy is inhibited by its phosphorylation at the Tyr 18 position in the LIR motif by SRC kinase [15]. Herein, we firstly evaluated the clinical relevance of AFAP1L2 in human HCC based on both Kaplan Meier plotter online database (<http://kmplot.com/analysis/>, 2021) [16] and our clinical cohorts. As shown in Figure 1B, the expression levels of AFAP1L2 mRNA in HCC tissues were significantly higher than that in the adjacent non-cancerous liver tissues ($P < 0.05$). In addition, HCC patients with high AFAP1L2 mRNA expression often had shorter overall survival than those with low expression (HR = 1.94; 95%CI: 1.04 to 3.63; $P = 0.035$, Figure 1C). Thus, we hypothesized that ART might mitigate the sorafenib resistance via targeting AFAP1L2-SRC-FUNDC1 axis-mediated mitophagy.

AFAP1L2-SRC-FUNDC1 axis activation promotes the proliferation and inhibits the apoptosis of sorafenib-resistant HCC cells

Fig. S1A showed the IC₅₀ values of sorafenib to four types of HCC cell lines, including Huh-7 (8.5 μ M), Hep3B (13.2 μ M), MHCC-97 H (15.4 μ M) and HepG2 (5.5 μ M), implying that HepG2 might be the most sensitive cell line to sorafenib. Following the establishment of the sorafenib-resistant HepG2 cell line (HepG2^R), we confirmed that the IC₅₀ value of HepG2^R was 15 μ M (Fig. S1B), which was approximately three-fold higher than HepG2 cells. In addition, the expression levels of multidrug resistance proteins ABCB1/MDR1 (ATP binding cassette subfamily B member 1) and ABCC1/MRP1 (ATP binding cassette subfamily C member 1) were detected, and revealed that their expression levels in

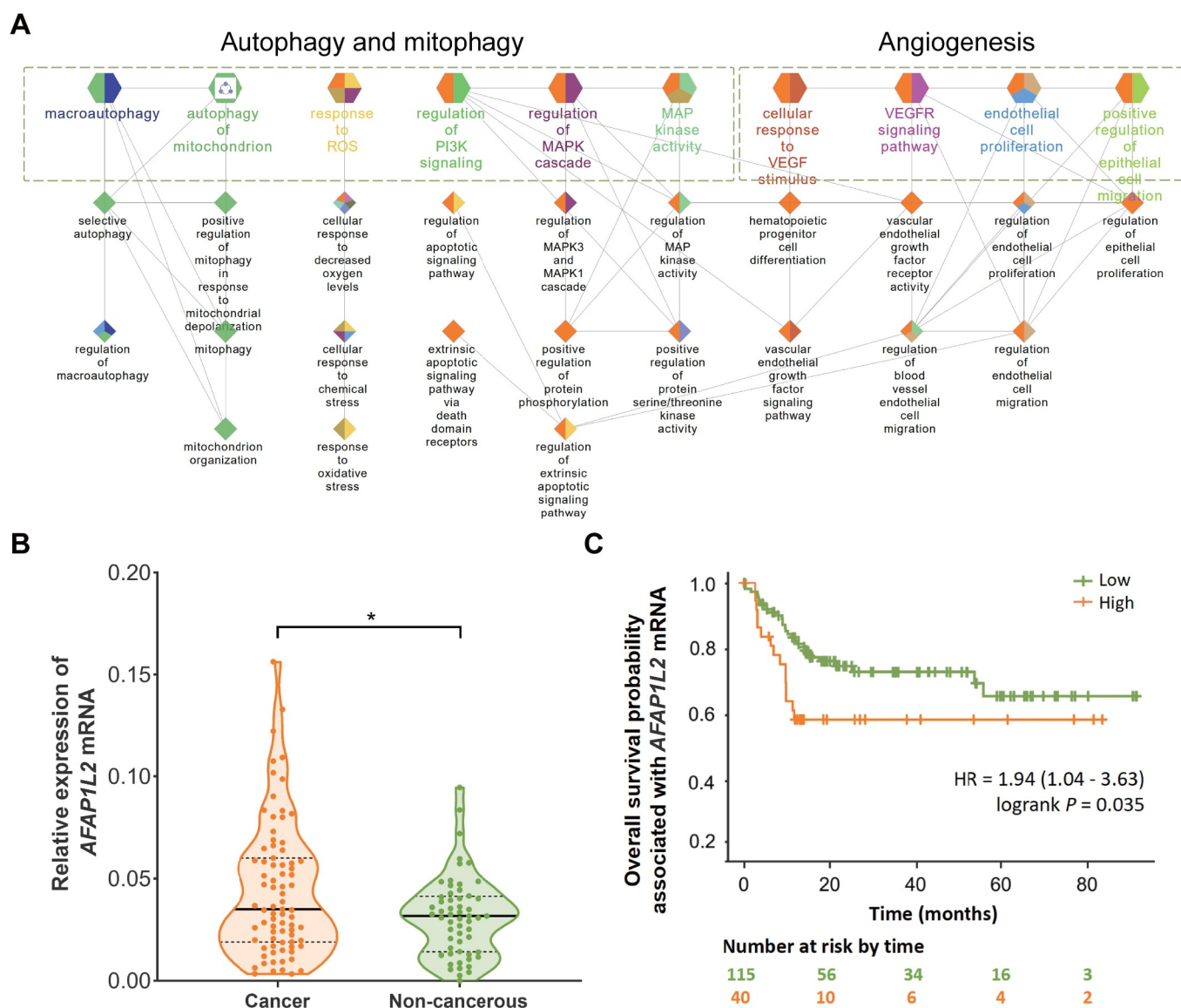


Figure 1. ART has the potential to alleviate sorafenib resistance, and AFAP1L2-SRC-FUNDC1 signaling and mitophagy may play an important role in sorafenib resistance. High AFAP1L2 mRNA expression confers poor prognosis on HCC patients. (A) the gene ontology (GO) analysis tool in cytoscape software was used to enrich the biological processes involved in target genes. (B) expression levels of AFAP1L2 mRNA in HCC and the adjacent non-cancerous liver tissues ($p < 0.05$). (C) HCC patients with high AFAP1L2 mRNA expression often had shorter overall survival than those with low AFAP1L2 mRNA expression (HR = 1.94; 95%CI: 1.04 to 3.63; $P = 0.035$).

HepG2^R were both significantly higher than that in HepG2 (both $P < 0.05$, Fig. S1C and D). The above data indicated the successful establishment of the HepG2^R cells.

The different characteristics between HepG2 and HepG2^R cells were compared based on the results of proliferation assay and flow cytometry. As shown in Figure 2A and Fig. S1E, 3.5 μ M sorafenib clearly suppressed the cell proliferation and prevented the colony formation in HepG2 cells compared to HepG2^R cells (all $P < 0.05$). Meanwhile, we also observed that the cell cycle of HepG2 cells were more arrested at the G₀/G₁ phase than resistant cells ($P < 0.05$, Fig. S1F). Moreover, flow cytometry demonstrated that the apoptosis rate in HepG2^R cells after exposing to sorafenib was much lower than that in parental cells ($P < 0.05$, Figure 2B).

To verify the above *in vitro* findings, the sorafenib-resistant animals bearing orthotopic HepG2 xenograft tumors were

established as shown in Figure 2D. According to the results of living imaging assay, we found that the liver fluorescence intensity of the HCC group (HCC) and sorafenib-resistant group (RES-SORA) continued to increase, while the sensitive group (SEN-SORA) decreased or even disappear, indicating that the sorafenib-resistant animal model was successfully established (Figure 2E,F). It was demonstrated that 30 mg/kg/2 days sorafenib had the highest success rate in inducing drug-resistant animals (about 70%), and this dose was used in subsequent experiments. The similar trends on tumor size and weight were found in both HCC and RES-SORA groups (Figure 2G,H). Similar pathological changes were also observed in both HCC and RES-SORA groups, including necrotic area in the tumor focus, vacuole-like changes for a few normal cells and high infiltration of inflammatory cells, while the tumor focus and the inflammatory cell

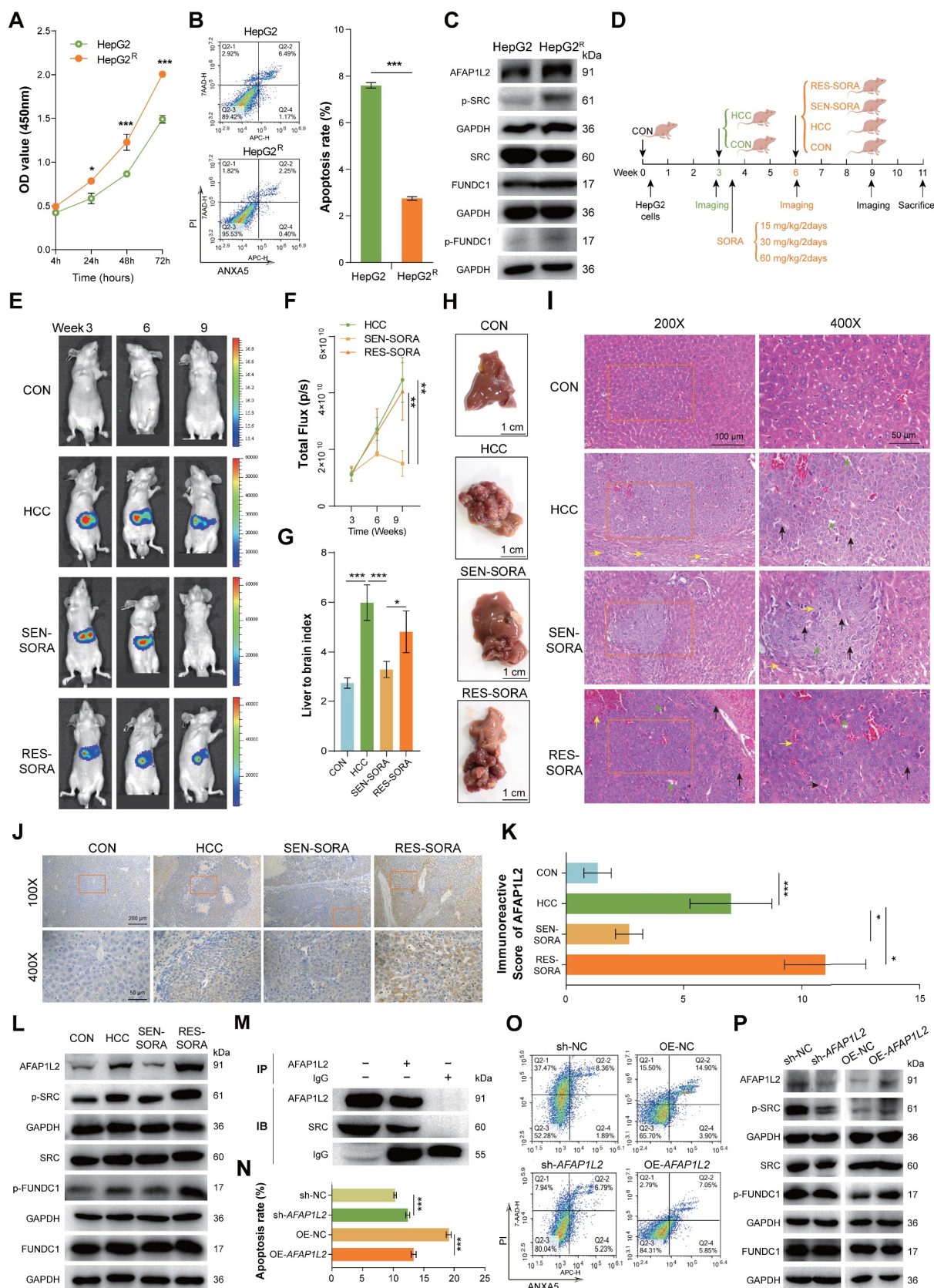


Figure 2. AFAP1L2-SRC-FUNDC1 axis activation promotes the proliferation and inhibits the apoptosis of sorafenib-resistant HCC cells. (A) and (B) proliferation ability and apoptosis of HepG2 and HepG2^R cells to 3.5 μ M sorafenib. (C) western blot analysis of the expression of AFAP1L2-SRC-FUNDC1 signaling. (D) schematic representation of the sorafenib-resistant model timeline. (E) and (F) Representative bioluminescent images and bioluminescence analysis results of mice. (G) and (H) the weight ratio of liver to brain and representative images of the orthotopic HCC tumors. (I) Representative H&E staining images of liver tissues of different treatment groups. Black, green, and yellow arrows indicated necrotic areas, vacuole-like changes, and inflammatory infiltrates, respectively. Scale bar: 50 or 100 μ m. (J) and (K) the protein level of AFAP1L2 in HCC tissues was detected by immunohistochemical staining assay. Scale bar: 50 or 200 μ m. (L) western blot analysis of proteins expression in HCC tissues. (M) co-IP assay was used to determine the interaction between AFAP1L2 and SRC. (N) and (O) the effects of AFAP1L2 gain or loss of function on apoptosis of HepG2 were measured by flow cytometric analysis of ANXA5/annexin V/PI staining. (P) The western blot analysis of the expression of AFAP1L2 signaling in HepG2-sh-AFAP1L2 and HepG2-OE-AFAP1L2 cells. The data is represented as mean $\pm$ SD. * p < 0.05, ** p < 0.01, *** p < 0.001.

infiltration of the SEN-SORA group was markedly less than that of the HCC or RES-SORA group (Figure 2I). Mechanically, AFAP1L2 protein, and its downstream effectors SRC and FUNDC1, were all highly activated in HepG2^R cells (Figure 2C and Fig. S6A), which was consistent with the *in vivo* data shown in Figure 2J–L and Fig. S7A. The results of CO-IP assay demonstrated that SRC was effectively pulled down by AFAP1L2, indicating that SRC may interact directly with AFAP1L2 in HepG2^R cells (Figure 2M).

To specifically address the potential role of AFAP1L2 in the sorafenib resistance of HCC, we established AFAP1L2-overexpressing and AFAP1L2-knockdown HepG2 cells, respectively (Fig. S1G and H). CCK8 assay demonstrated that the proliferation of HepG2 was markedly suppressed after AFAP1L2 knockdown (Fig. S1I, left panel). Apoptosis assays showed the increased apoptosis rate of HepG2 cells following the suppression of AFAP1L2 compared to the control groups (Figure 2N,O). To determine the relevance of AFAP1L2 in sorafenib resistance, the drug sensitivity to sorafenib of AFAP1L2-depleted HepG2 cells was compared with that of parental control cells. As shown in Fig. S1J, the IC₅₀ curve of the AFAP1L2-suppressed HepG2 cells to sorafenib shifted toward the left of control cells, implying the expected increase in sorafenib sensitivity. The knockdown-based functional assays demonstrated that AFAP1L2 might not only modulate growth and progression but also influence sorafenib sensitivity of HepG2. In contrast, the enforced expression of AFAP1L2 in HepG2 cells led to a significant promotion of cell proliferation (Fig. S1I right panel), and a marked reduction in the percentage of total apoptotic cells (Figure 2N,O). Mechanically, the knockdown of AFAP1L2 significantly reduced the activation of SRC and FUNDC1 proteins (Figure 2P, left panel, Fig. S6E), while the phosphorylation levels of SRC and FUNDC1 proteins were markedly enhanced in HepG2 cells with AFAP1L2 overexpression (Figure 2P, right panel, Fig. S6F).

AFAP1L2 overexpression inhibits mitophagy in sorafenib-resistant cells and tumors

As shown in Figure 3A,B, our flow cytometry results indicated that ROS level was decreased and mitochondrial membrane potential was increased in HepG2^R cells compared to HepG2 cells, suggesting a better mitochondrial status in resistant cells. To verify whether AFAP1L2 might be involved into mitophagy during sorafenib resistance, we demonstrated an increased expression level of the mitochondrial membrane protein TOMM20 (translocase of outer mitochondrial membrane 20), but a reduced ratio of LC3B-II:LC3B-I in both HepG2^R and resistant tumors (Fig. S2A and S6A, Fig. S2B and S7A). In addition, our observation through the confocal colocalization analysis found that TOMM20 was predominantly colocalized with a substantial amount of LC3B and lysosome degradation marker LAMP1 (lysosomal associated membrane protein 1) in HepG2, whereas the trend was reversed in HepG2^R cells (Figure 3C,D). Moreover, the western blot analysis demonstrated the reduced expression of TOMM20 protein, and the autophagy marker cytosolic LC3B-I became conjugated phosphatidylethanolamine to form LC3B-II in HepG2 cells transfected with sh-AFAP1L2

(Fig. S2C and S6E). Consistently, the enforced expression of AFAP1L2 in HepG2 cells led to the accumulated TOMM20 and weakened LC3B lipidation (Fig. S2D and S6F). Furthermore, the fluorescence intensity of TOMM20 and LC3B colocalization in HepG2 cells with the knockdown of AFAP1L2 was markedly stronger than the control cells while it dropped drastically in HepG2 cells with the overexpression of AFAP1L2 (Figure 3E,F). These results indicated that AFAP1L2 May play an important role in mitophagy, which may tightly connect with sorafenib resistance.

ART significantly enhances the anti-HCC effects of sorafenib in the drug resistant cells and animal models

The different doses of sorafenib and ART were combined at a constant ratio, and the combination index (CI) plot (Fig. S3A) showed that the CI calculated for sorafenib (3.5 μ M) and ART (25 μ M) was 0.850 (CI < 1), indicating a synergistic effect. We treated the HepG2^R cells with increasing dosages of sorafenib in the presence or absence of ART for 24 h, and found that ART enhanced the inhibitory effects of sorafenib on the growth of the cells in a dose-dependent manner (Fig. S3B). The cell viability of HepG2^R cells with the treatment of sorafenib and ART combination was also lower than that with sorafenib or ART treatment alone (Fig. S3C). Similarly, the apoptosis assays also revealed that the treatment of sorafenib and ART combination effectively induced the apoptosis of HepG2^R cells (Fig. S3D and 3E).

In addition, the sorafenib-resistant mouse model was used to determine whether ART could alleviate the sorafenib resistance. As shown in Fig. S3F, Figure 4A,B the fluorescence intensity of the tumor tissues in the liver of the drug-sensitive HCC group (SEN-SORA) was significantly decreased or even disappeared compared with that of the HCC model group (HCC) after 8 weeks of administration. On the contrary, the tumors in the drug-resistant HCC group (RES-SORA) continued to grow. The combined treatment of sorafenib and ART (SORA, 30 mg/kg/2 days; L/H-ART, 30 or 60 mg/kg/2 days, respectively) in the RES-SORA+L/H-ART groups effectively slowed down the growth of drug-resistant tumors of the sorafenib-resistant mice ($P < 0.001$). To support the evidence of the living imaging assay, ¹⁸F-FDG MicroPET/CT imaging was further performed to verify the efficacy of ART in alleviating drug-resistant tumors. As shown in Figure 4C,D, high-dose ART (RES-SORA+H-ART) significantly reduced the glucose metabolic levels of drug-resistant tumors comparing with the drug-sensitive controls. Accordingly, the treatment of ART also markedly extended the overall survival of the drug-resistant mice (Figure 4E). Moreover, both the number of tumors in the liver tissues and the liver:brain ratio in the RES-SORA+L/H-ART groups were lower than those in the RES-SORA group, in line with the data of tumor necrosis areas, vacuolated changes and inflammation extent (Fig. S3G-I).

To further determine whether ART treatment could enhance the sensitive response of HCC to sorafenib, we demonstrated that the tumor fluorescence intensity of HCC-SORA+L/H-ART group (with the combined treatment of 30 mg/kg/2 days sorafenib and 30/60 mg/kg/2 days ART) was significantly reduced or even disappeared after 8 weeks of

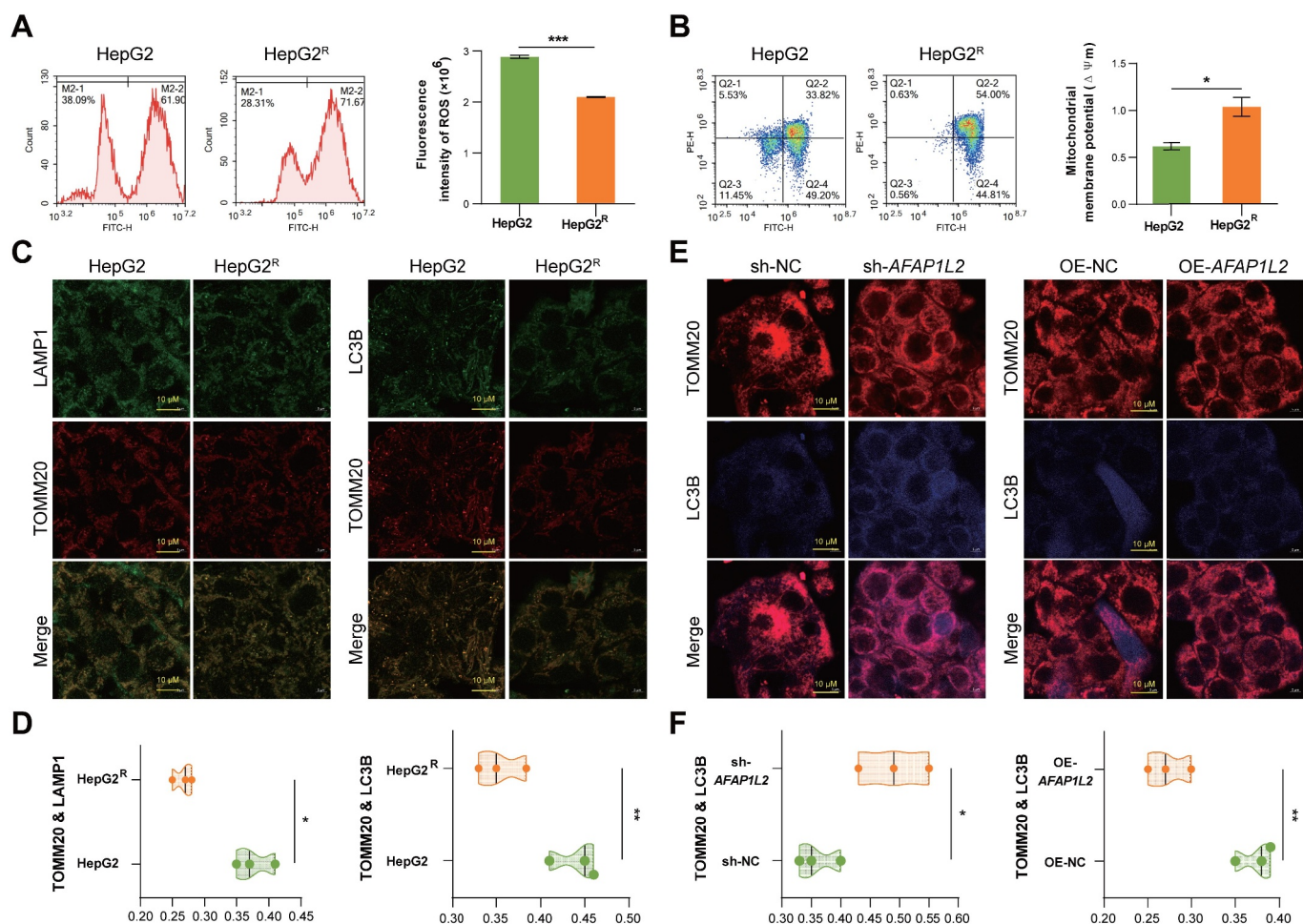


Figure 3. AFAP1L2 overexpression inhibits mitophagy in sorafenib-resistant cells. (A) and (B) flow cytometry was used to determine the level of ROS accumulation and mitochondrial membrane potential of HepG2 and HepG2^R. (C) confocal microscopy was performed to detect spatial colocalization of mitochondrial protein TOMM20 (red) with LAMP1 (green, left panel) and LC3B (green, right panel) in HepG2 and HepG2^R cells. (D) the quantitative results of relative fluorescence intensity of colocalized regions. (E) confocal microscopy was performed to detect spatial colocalization of mitochondrial protein TOMM20 (red) with LC3B (blue) in HepG2-sh-AFAP1L2 and HepG2-OE-AFAP1L2 cells (scale bars: 10 μm). (F) the quantitative results of relative fluorescence intensity of colocalized regions. The data is represented as mean ± SD. **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

administration compared with the HCC-SORA group (30 mg/kg/2 days sorafenib), indicating that the drug combination exerted a significantly stronger inhibitory effect on HCC than sorafenib treatment alone (Fig. S3J, Figure 4F,G). Consistently, the survival curve shown in Figure 4H displayed a longer overall survival of the drug combination group than that of the sorafenib group. The number and size of tumors in the liver tissues, and liver brain ratio values of the drug combination group were all obviously smaller than that of the sorafenib treatment alone group (Fig. S3K and L). Histopathologically, the observations of the necrosis areas, vacuolar changes, and inflammatory infiltration were also dramatically reduced in the drug combination group (Fig. S3M).

ART improves the response of drug resistant cells and tumor tissues to sorafenib via exacerbating AFAP1L2-SRC-FUNDC1 axis-dependent mitophagy

The above “sorafenib-resistance related gene-ART candidate target” interaction network analysis hypothesized that ART

might mitigate sorafenib resistance by targeting AFAP1L2-SRC-FUNDC1 Axis-dependent mitophagy. To verify this hypothesis, we observed the changes of mitophagy in HCC cells following the treatment of ART. The observation of confocal microscopy showed that both the ART treatment alone or the combined treatment of ART and sorafenib significantly increased the fluorescence colocalization level of TOMM20 with LC3B and LAMP1 in HepG2^R cells (*P* < 0.001, Figure 5A, Fig. S4A), in line with the findings of western blot analysis as shown in Fig. S4B, S6B, C and D (*P* < 0.05). In addition, TEM observed that both the low and high dose of ART treatment increased the number of autophagosomes and autolysosomes in sorafenib-resistant tumor tissues (Figure 5B). Meanwhile, ART dose-dependently increased LC3B-II:I ratio and decreased the expression of TOMM20 protein (Fig. S4C and S7B), which displayed the same trends in the sorafenib sensitive tumor tissues (Figure 5C, Fig. S4D and S7C), suggesting that ART may markedly enhance the level of mitophagy in both sorafenib-resistant and sorafenib-sensitive tumors. Moreover, the mitophagy inhibitor chloroquine was employed to verify the regulatory effects of ART on

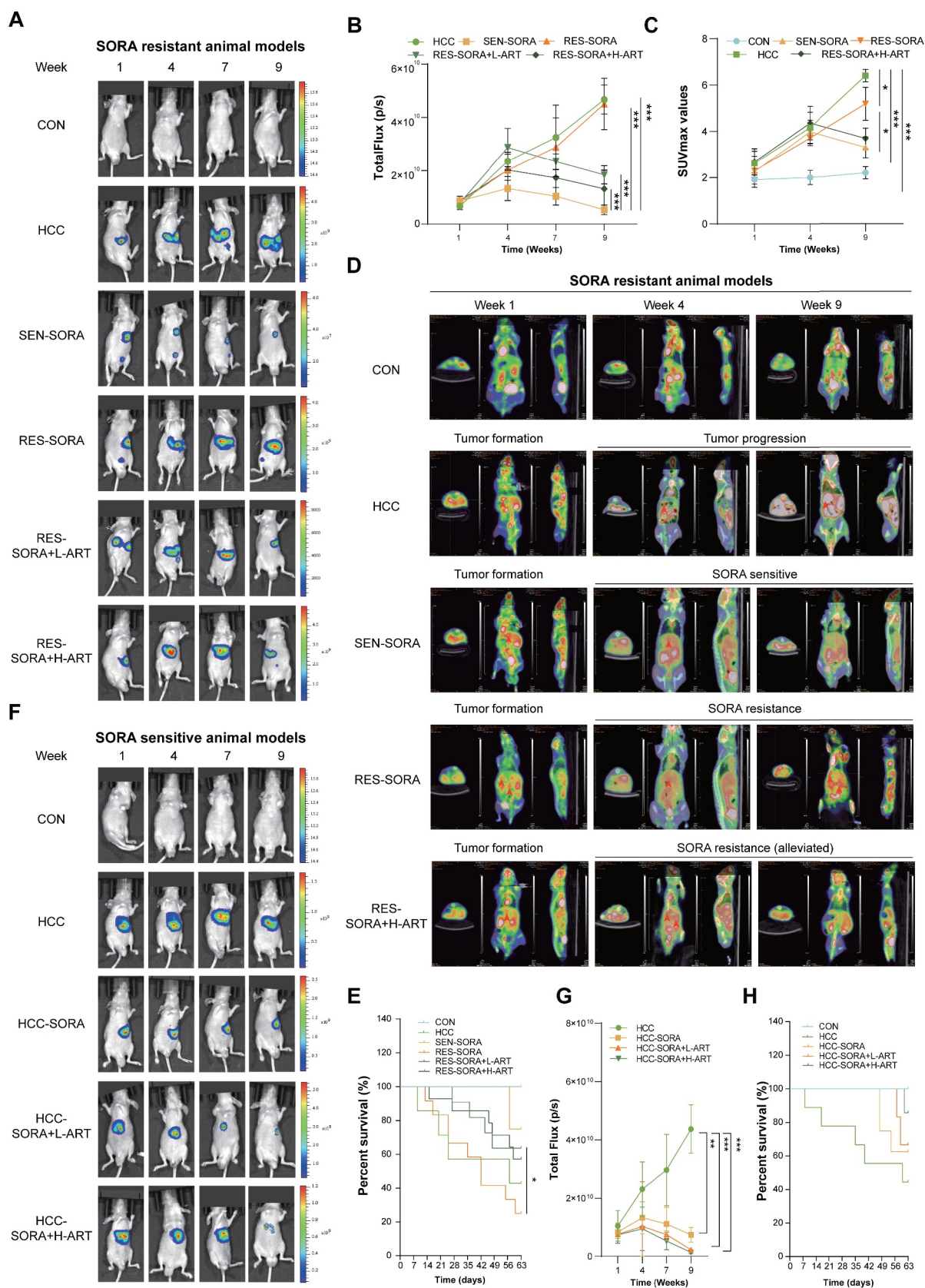


Figure 4. Evaluation of the anti-HCC efficacies of the combined treatment of sorafenib and ART in both *in vivo* and *in vitro* experiments. (A), (B), (F) and (G) Representative bioluminescence images (A, F) and bioluminescence semi-quantitative results (B, G) of sorafenib combined with ART inhibiting the sorafenib-resistant tumors (A, B) or sensitive tumors (F, G). (C) and (D) Representative images of tumor vascularization and metabolism acquired by 18F-FDG MicroPET/CT are shown in (D). The tumors are indicated with red arrows. The quantitative data are also presented at (C). (E) and (H) Kaplan – Meier survival curves were used to analyze the survival time of the mice in each group in the experiment of sorafenib combined with ART inhibiting resistant (E) or sensitive (H) tumor progression. The data is represented as mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

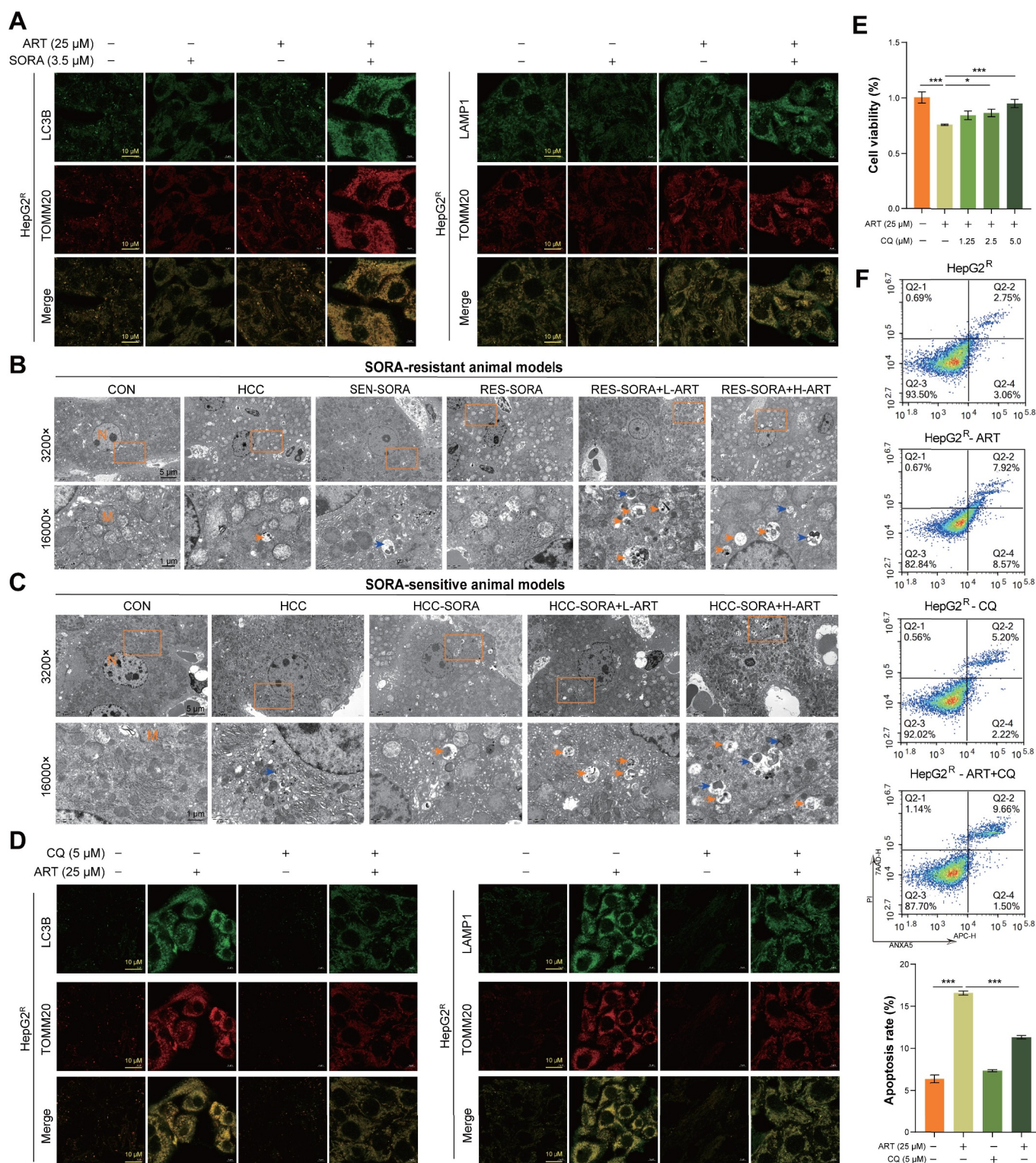


Figure 5. ART improves the response of drug resistant cells and tumor tissues to sorafenib via exacerbating mitophagy. (A) the combined effects of sorafenib and ART on mitophagy were measured via the confocal microscopy, which was performed to detect spatial colocalization of mitochondrial protein TOMM20 (red) with LC3B (green, left panel) and LAMP1 (green, right panel) in HepG2^R cells under the treatment of sorafenib and ART (scale bars: 10 μ m). (B) and (C) Representative TEM images depicted ultrastructure in cells from sorafenib resistant and sensitive tumor tissues, respectively. Blue arrows and orange arrows indicated autophagosome and autolysosome, respectively (scale bars: 1 or 5 μ m). (D) the effect of the combination of sorafenib and chloroquine on mitophagy was observed by confocal microscopy, and the spatial colocalization of mitochondrial protein TOMM20 (red) with LC3B (green, left) and LAMP1 (green, right) in HepG2^R cells was detected (scale bars: 10 μ m). (E) and (F) the combined effects of sorafenib and chloroquine on cell viability and apoptosis were measured via the CCK8 and flow cytometry, respectively. The data is represented as mean $\pm$ SD. * p < 0.05, ** p < 0.01, *** p < 0.001.

mitophagy. As shown in Figure 5D and Fig. S4E, chloroquine significantly inhibited ART-induced mitophagy in HepG2^R cells ($P < 0.05$), and the blockade of mitophagy by chloroquine markedly reversed the efficacy of ART treatment on proliferation and apoptosis of HepG2^R cells (Figure 5E,F).

Mechanically, the western blot analysis demonstrated that ART dose-dependently inhibited the expression of AFAP1L2 protein, which subsequently induced the dephosphorylation of SRC and FUNDC1 proteins in HepG2^R cells (all $P < 0.05$, Figure 6A, Fig. S6B, C and D), which was in line with the findings of immunohistochemistry and western blot analysis based on the drug-resistant (Figure 6B, Fig. S5A, B and S7B) and drug-sensitive (Figure 6C, Fig. S5C, D and S7C) tumor tissues (all $P < 0.05$). Notably, the enforced expression of AFAP1L2 in HepG2 cells obviously attenuated the inhibitory effects of ART in cell proliferation (Figure 6D) and its apoptosis-inducing effect (Figure 6E), as well as the downstream proteins (SRC and FUNDC1, Figure 6F and Fig. S6G).

Furthermore, the confocal imaging observed that the overexpression of AFAP1L2 markedly inhibited mitophagy in HepG2 cells, including attenuated fluorescence colocalization of TOMM20 and LC3B (Figure 6G), decreased expression of LC3B-II:I and upregulation of TOMM20, compared to empty vector transfected cells, similar to the western blot data (Figure 6H and Fig. S6G), indicating that the ART-enhanced mitophagy level was restored by the overexpression of AFAP1L2.

ART directly binds to AFAP1L2 protein

To verify whether ART could regulate AFAP1L2 directly in HepG2^R cells, the biotin-ART was synthesized and used to pull down the target proteins of ART. As shown in Figure 7A, biotin-ART bound to AFAP1L2, which competed with unlabeled ART. As a result of CETSA, the level of AFAP1L2 in ART treatment group was higher than that in the untreated group under 10% protease and phosphatase inhibitors after the exposure to 25 μ M ART for 4 h. AFAP1L2 degraded at 49°C and almost disappeared at 61°C in untreated HepG2^R cells, while it started to be degraded at 61°C in ART-treated HepG2^R cells (Figure 7B). To quantify the interaction between ART and AFAP1L2 protein, SPR by Biacore T200 system was employed. The result of the real time analysis showed that there was a specific interaction between ART and AFAP1L2 protein [$K_d = 28 \mu$ M, coefficient of determination (r^2) = 0.99] (Figure 7C). Schematic representation of the major molecular mechanism was shown in Figure 7D.

Discussion

Sorafenib resistance is one of the great clinical challenges in the effective treatment of advanced HCC. Until now, the molecular mechanisms underlying sorafenib resistance and its interventions have not been fully elucidated. In the current study, we revealed that the sorafenib-resistant HCC cells and tissues were often accompanied by the elevated expression of AFAP1L2, leading to the activation of the downstream SRC/FUNDC1 signaling axis, thereby blocking the recruitment of LC3B by FUNDC1 and the subsequent activation of mitophagy. Interestingly, ART dramatically

enhanced the inhibitory effects of sorafenib on the proliferation of drug-resistant cells and the growth of drug-resistant tumors, and effectively mitigated sorafenib resistance, which was dependent on the over-induction of mitophagy by ART. Mechanistically, we demonstrated that ART reduced the expression of AFAP1L2 protein and inhibited the phosphorylation of SRC and FUNDC1 proteins, subsequently promoted the recruitment of LC3B and induced the mitophagy, which may contribute to the apoptosis of drug-resistant HCC cells and tumors. To the best of our knowledge, this is the first study that identified AFAP1L2-SRC-FUNDC1 axis as one of the critical factors leading to sorafenib resistance of HCC through regulating mitophagy, and also clarified a novel mechanism of ART against sorafenib-resistant HCC.

The establishment of the sorafenib-resistant animal model is one of the necessary tools for investigating the mechanisms of sorafenib resistance and the solutions to sorafenib resistance. In the previous studies, drug-resistant HCC animal models were subcutaneously inoculated with transplanted drug-resistant tumor tissues or drug-resistant tumor cells in nude mice, but the method could not well simulate the formation process of clinical tumor drug resistance [17–19]. To better simulate the clinical status of sorafenib-resistant HCC, we herein successfully established a drug resistant orthotopic xenograft HCC animal model. The liver of nude mice was inoculated orthotopically with luciferase-labeled HepG2 cells, and then the animals were treated with chemotherapeutic drug sorafenib, and the tumor growth was monitored continuously by bioluminescence imaging and MicroPET/CT. Those animals whose tumors continued to grow after the treatment of sorafenib were regarded as the drug-resistant animals; on the contrary, others were regarded as drug-sensitive animals. In order to determine the optimal dose to induce sorafenib resistance in HCC, three dosages including 15 mg/kg/2 days, 30 mg/kg/2 days, and 60 mg/kg/2 days were set simultaneously. As a result, the dosage of 30 mg/kg/2 days sorafenib displayed the highest success rate in inducing sorafenib resistance in animals. The liver tumors of our sorafenib-resistant mouse model may be formed orthotopically, and the development and progression of its chemotherapeutic drug resistance may be similar to the clinical status, although the achievement ratio of this model was 70% (Table S6 and S7).

More and more attentions have been attracted to elucidating the mechanisms of sorafenib resistance. For example, several new insights including a higher EGFR, autophagy, hypoxic environment, JUN and AKT1 activation in HCC cells, as well as increased EMT, cancer stem cells, and exosomes have been recently revealed to be associated with the occurrence of sorafenib resistance [19,20]. In the current study, we focused on the mitophagy since the decreased mitophagy levels in the HepG2^R cells were observed compared to HepG2 cells, which were also supported by the evidence of the reduced autophagosomes and autolysosomes during sorafenib resistance in sorafenib-resistant HCC cells. In addition, we employed an integrated research strategy with chemical proteomics and clinical transcriptomic data, and identified AFAP1L2-SRC-FUNDC1 axis-dependent mitophagy to be associated with the occurrence of sorafenib resistance to HCC. It has been reported that AFAP1L2

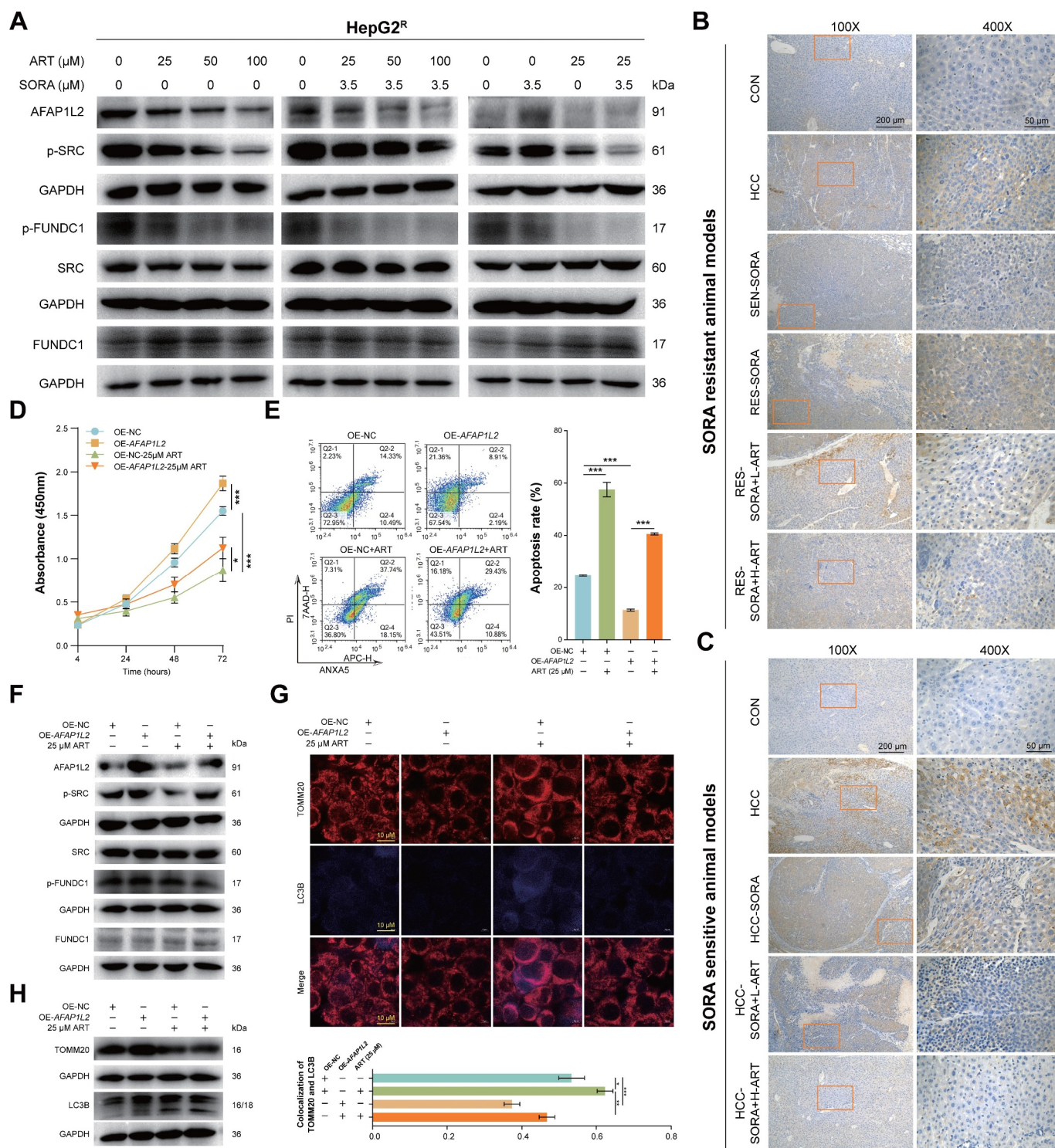


Figure 6. ART improves the response of drug resistant cells and tumor tissues to sorafenib through inhibiting AFAP1L2-SRC-FUNDC1 axis. (A) western blot analysis of the expression of AFAP1L2-SRC-FUNDC1 signaling in HepG2^R treated with sorafenib and different doses of ART. (B) and (C) the protein level of AFAP1L2 in resistant tumor (B) and sensitive tumor (C) was detected by immunohistochemical staining assay. (D) and (E) CCK8 (D) and flow cytometry (E) were used to detect the effects of ART treatment on cell proliferation and apoptosis after the overexpression of AFAP1L2 in HepG2. (F) After the overexpression of AFAP1L2 in HepG2 cells, the inhibition of ART on the expression of each member of the AFAP1L2 signaling was detected by WB. (G) the induced effects of ART on mitophagy were measured via the confocal microscopy, which was performed to detect spatial colocalization of mitochondrial protein TOMM20 (red) with LC3B (blue) in HepG2-OE-AFAP1L2 cells (up panel, scale bars: 10 μm). The relative fluorescence intensity of colocalization of TOMM20 with LC3B was also shown (down panel). (H) western blot analysis of mitophagy marker proteins expression in HepG2-OE-AFAP1L2 treated with ART. A total of 6 to 10 mice were included in each group. The data is represented as mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.01$.

overexpression was involved into development of multiple cancer types, such as esophageal cancer, gastric cancer, HCC and prostate cancer [14]. As a binding partner of AFAP1L2, SRC has been indicated to be able to enhance FUNDC1 phosphorylation

and subsequently weaken the strength of mitophagy [21], which may be consistent with our mechanical findings.

Addressing sorafenib resistance requires the continued development of safer drug combinations because existing

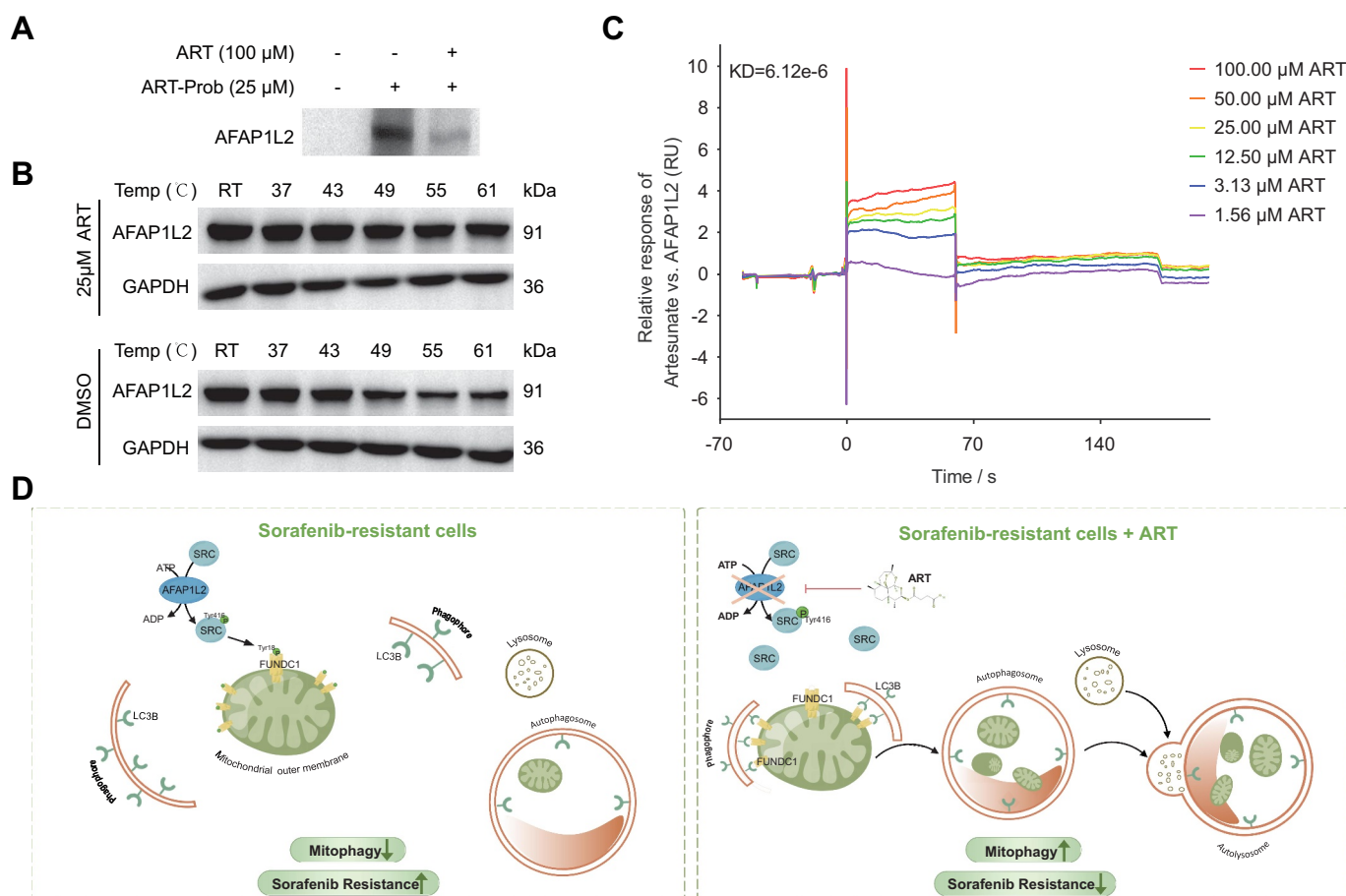


Figure 7. ART directly binds to AFAP1L2 protein. (A) the effect of ART-probe (25 μ M) in the presence or absence of ART (4 $\times$, 100 μ M) on AFAP1L2 expression in HepG2^R cell lysates was examined by affinity-isolation assays. (B) HepG2^R cells incubated with or without ART (25 μ M) for 4 h were subjected to CETSA assay. AFAP1L2 was normalized with GAPDH. (C) interaction between ART and AFAP1L2 was determined via SPR analysis. (D) the schematic representation of the major molecular mechanism.

combination therapies to overcome or alleviate sorafenib resistance are often limited by severe adverse side effects. Although the administration of oral ART is currently applied in cancer patients in several clinical studies, such as advanced HCC (ClinicalTrials.gov identifier: NCT00764036, NCT02304289) [22,23], its benefit on sorafenib-resistant HCC patients is still uncertain. Recent studies have revealed that ART markedly enhanced the cytotoxicity of sorafenib in HCC cells [24,25], and was used only against the sorafenib-sensitive HCC cells [24,26]. The current study demonstrated for the first time that the sensitivity of the HepG2^R cells to the sorafenib was recovered by the cotreatment with ART, suggesting that ART may effectively enhance the anti-HCC effects of sorafenib. It's a bit of a shame that the inhibitory effect of ART on sorafenib-resistant tumors was still weaker than that of sorafenib on sorafenib-sensitive tumors. Mechanically, mitophagy may contribute to cell survival by degrading damaged mitochondria, whereas excessive mitophagy may degrade normal mitochondria and contribute to cell apoptosis [27]. Consistently, our results show that ART-induced excessive mitophagy potentiates cell apoptosis in sorafenib-resistant HCC. Furthermore, Wu et al. [7] revealed that highly activated mitophagy induced HCC resistance to sorafenib,

whereas we observed the opposite results – mitophagy was attenuated in drug-resistant cells. It has been reported that ART treatment activates the PINK1-PRKN/Parkin-dependent pathway, ultimately leading to mitophagy in Hela cells [9]. Importantly, our data provide another novel pathway underlying ART-induced mitophagy-the AFAP1L2-SRC-FUNDC1 axis, in which a high AFAP1L2 expression phenotype may not only account for the pharmacological effect of ART, but also function as an upstream regulator for mitophagy in sorafenib-resistant cells.

In conclusion, our data provides the evidence that ART may be a potential strategy to mitigate sorafenib resistance in HCC via exacerbating AFAP1L2-SRC-FUNDC1 Axis-dependent mitophagy.

Materials and methods

Ethics statement

This study was approved by the Research Ethics Committee of the Institute of Chinese Materia Medica and the Fifth Medical Centre of Chinese PLA General Hospital (license no: 2016003D). Animal experiments were carried out according

to the guidelines for the care and use of laboratory animals of the Animal Ethical and Welfare Committee, Guangzhou Forevergen Medical Laboratory Animal Center, Guangdong, China (license no: IACUC-AEWC-F2203003 and IACUC-AEWC-F2111003).

Data collection & network analysis

ART target profiling was identified using the ART probe by large-scale chemoproteomic experiments [9]. Then, the mRNA expression profile in the liver tissue samples obtained from the sorafenib sensitive and resistant HCC patients were collected from the NCBI Gene Expression Omnibus under accession number GSE143235 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE143235>). Differentially expressed genes between the two groups referring to the criteria of P value < 0.05 and $|\log_2$ folds change (FC)| > 1 were screened to be the sorafenib resistance related genes according to t -test analysis. After that, the “sorafenib-resistance related gene-ART candidate target” interaction network was constructed based on the gene-gene links obtained from the public database STRING (Search Tool for Known and Predicted Protein-Protein Interactions, version 10.0, <http://string-db.org/>). The interaction network was visualized by our ETCM v2.0 network visualization tool [28] and Cytoscape 3.8.0 software [29]. The nodes' degree, betweenness and closeness were calculated to evaluate the topological importance of the nodes in the networks as described in our previous studies [30,31], and the biological functions of the network targets were enriched using the ClueGO in Cytoscape.

Reagents

Sorafenib (284461-73-0) was purchased from Sigma-Aldrich, chloroquine (14774S) was purchased from Cell Signaling Technology and artesunate (88495-63-0) was purchased from Sigma-Aldrich.

Cell culture

Human HCC cell lines HepG2 (SCSP-510), Hep3B (SCSP-5045), MHCC-97H (SCSP-573), and HUH7 (SCSP-5011) were purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences, and maintained in high-glucose Dulbecco's Modified Eagle Medium (DMEM; HyClone, SH30285.02) supplemented with 10% fetal bovine serum (Gibco, 10099141C), 100 U/mL of penicillin (Gibco, 15070063), and 100 g/mL of streptomycin (Gibco, 15070063). All cells were grown at 37°C in a humidified incubator with 5% CO₂.

Establishment of sorafenib-resistant HCC cells

The resistant HCC cell lines with acquired sorafenib resistance, HepG2^R, were established based on HepG2 cells as described previously [19]. Briefly, the cells were cultured in a step-wise increase in sorafenib concentration (1–8 μM), by 10% every two weeks until the maximum

tolerated dose (8 μM) had been reached. HepG2^R cells were cultured in the presence of 8 μM sora, which was withdrawal for two days before using.

Establishment of overexpression or knockdown cell lines

The shAFAP1L2 and AFAP1L2 cDNA lentiviral plasmids were purchased from Shanghai Genechem Company Ltd. The respective primers sequence for AFAP1L2 knock-down and overexpression are shown in Table S8. Lentivirus was produced using a three-plasmid packaging system. Briefly, the indicated plasmids were transfected into 293T cells using PEI reagent (Sigma-Aldrich, 9002-98-6) following manufacturer's protocols. Lentivirus was collected 48 h after transfection. HepG2 cells were infected with lentiviral supernatants in the presence of 5 μg/mL Polybrene Infection/Transfection Reagent (Sigma-Aldrich, TR-1003), and were selected under 0.8 μg/mL puromycin dihydrochloride from *Streptomyces alboniger* (Sigma-Aldrich, 58-58-2) for 2 weeks. Stably transfected clones were validated by immunoblotting and qPCR analysis. Other transfection experiments were conducted by using Lipofectamine 3000 (Thermo Fisher Scientific, L3000015) following the manufacturer's instructions.

CCK8

Cell viabilities were assessed by Cell Counting Kit-8 (Beyotime Biotechnology, C0038) assay. Briefly, cells were seeded in 96-well plates overnight and subjected to different treatments. CCK8 reagents was then added to each well for 2 h at 37°C, and absorbance was examined with a microplate reader (Bio-Tek Instruments, USA).

Colony formation

Three thousand cells were seeds in 24-wells plate per well. After being cultured for 14 days, we washed the plate with PBS (Applygen Technology, C1053) for two times and fixed cells with 4% paraformaldehyde (Servicebio, G1101) for 15 min, incubated cells with Crystal Violet Staining Solution (Beyotime Biotechnology, C0121) for 15 min and washed the staining solution with distilled water.

Cell cycle

Cells were fixed overnight at −20°C in 75% ethanol. The washed, precipitated cells were then resuspended in PBS containing 0.1% RNaseA (ThermoFisher Scientific, R1253) and 100 μL of 10 μg/mL propidium iodide (PI; Sigma, 25535-16-4) for 30 min at room temperature. They were subsequently analyzed by NovocyteFlow Cytometer (ACEA Bioscience, USA).

Apoptosis

The Annexin V-APC/7-AAD Apoptosis Detection Kit (MULTI SCIENCES, AP105) was used as recommended by manufacturer's instruction. And the cell suspension was subsequently analyzed by Novocyte Flow Cytometer (ACEA Bioscience, USA).

ROS and mitochondria membranepotential analysis

Intracellular ROS was determined using DCFH-DA (BeyotimeBiotechnology, S0033S) stain and mitochondrial membrane potential was measured by staining with JC-1 (Beyotime Biotechnology, C2003S). After treatment with ART, cells were washed with 0.01 M PBS and incubated with 10 μ M DCFH-DA or JC-1 (200X) at 37°C for 30 min, and then cells were measured with Novocyte Flow Cytometer (ACEA Bioscience, USA).

Analysis of synergistic activity

To analyze the combinatorial effect of ART and sorafenib on HepG2^R cells, the combination index (CI) of ART and sorafenib were evaluated as described previously with our own tools based on the Chou-Talalay theory [32]. CI < 1, CI = 1, CI > 1 represents synergistic, additive, and antagonistic effects, respectively.

qRT-PCR

Total RNA was isolated from cell lines using TRIzol RNA isolation reagent (Invitrogen, 15596026) and reverse transcribed using a RevertAid FirstStrand cDNA Synthesis Kit (Thermo Fisher Scientific, K1622) according to the manufacturer's instructions. RT-qPCR was performed with an UltraSYBR One Step RT-qPCR Kit (CWBIO, CW2624) using 7900HT Fast Real-Time PCR System (Thermo Fisher Scientific, 4329001). The respective primers used are shown in Table S8 and all primers were synthesized by Sangon Biotech (Shanghai, CHN). Relative gene expression analysis was performed using the eq. $2^{-\Delta\Delta CT}$, with ACTB used as an internal control.

Western blot

Cells or tissue specimens were rinsed in precooled PBS and lysed in RIPA lysis buffer (Beyotime Biotechnology,

P0013B) containing protease inhibitor phenylmethanesulfonyl fluoride (PMSF; Beyotime Biotechnology, ST506). Total lysates were quantified by a Bradford Protein Assay Kit (Beyotime Biotechnology, P0006). Equal amounts of the protein samples were subjected to western blot analysis. After incubating with appropriate primary and secondary antibodies, the immunoreactions were visualized with SuperSignal[™] West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, 34095). The images were obtained using a Tanon 5200 (Shanghai, CHN). Antibodies are listed in Table 1.

Immunofluorescence (IF) staining

HCC cells were seeded on glass coverslips in 6-well plates, incubated overnight and then fixed in 4% paraformaldehyde (Solarbio, P1110) for 15 min, permeabilized with 1% Triton X-100 (Solarbio, 9002-93-1) for 5 min, blocked in 1% bovine serum albumin (BSA; Sigma, A9418) for 60 min, and incubated with primary antibodies overnight at 4°C, followed by secondary antibodies for 60 min at RT. Photographs were captured with a laser confocal microscopy (Leica Microsystems AG, GEN). Antibodies applied in this experiment are listed in Table 1.

Co-immunoprecipitation (CO-IP)

After ART treatment, proteins were extracted using cold RIPA followed by centrifuging at 14,000× g 4°C for 20 min. 5 μ g of primary antibody against AFAP1L2 and 2 μ L of 2.5 μ g/ μ L protein A/G plus agarose (Thermo Fisher Scientific, 88803) were added into the supernatant at 4°C overnight. After centrifugation at 8× g for 1 min, the pellet was washed three times with pre-cooled IP buffer, and the beads were boiled in 4× loading buffer. Then the supernatants were collected and subject to western blot analysis for detecting the expression levels of AFAP1L2 and SRC proteins.

Table 1. Summary of antibodies used in western blot, immunochemistry, immunofluorescence, co-immunoprecipitation, affinity isolation, and CETSA.

Antibody	Concentration				Cat. No.	Company
	for WB	for IHC	for IF	for Co-IP		
AFAP1L2	/	1:200	/	/	Ab106433	Abcam
	1:1000	/	1:200	/	17183-1-AP	Proteintech
	/	/	/	1:50	12684	Cell Signaling Technology
p-SRC	1:1000	/	/	/	AF3161	Affinity bioscience
	/	/	/	1:50	4211	Cell Signaling Technology
SRC	1:1000	/	/	1:50	2109	Cell Signaling Technology
p-FUNDC1	1:1000	/	/	/	TP30110	Huabio
FUNDC1	1:1000	/	/	/	ab224722	Abcam
LC3B	1:1000	/	/	/	3868	Cell Signaling Technology
	/	/	1:200	/	Ab51520	Abcam
GAPDH	1:5000	/	/	/	60004-1-Ig	Proteintech
TOMM20	1:1000	/	1:200	/	Ab186735	Abcam
LAMP1	/	/	1:200	/	55273-1-AP	Proteintech
ABCC1	1:1000	/	/	/	ab180960	Abcam
ABCBI	1:1000	/	/	/	ab170904	Abcam
Normal rat IgG	/	/	/	1:50	39005	Cell Signaling Technology
HRP-Conjugated AffiniPure Goat Anti-rabbit IgG (H+L)	1:10000	/	/	/	BA1054	Boster
HRP-Conjugated AffiniPure Goat Anti-mouse IgG (H+L)	1:10000	/	/	/	BA1050	Boster
AMCA – conjugated AffiniPure Goat Anti-Rabbit IgG(H+L)	/	/	1:500	/	SA00010-2	Proteintech
Goat Anti-Rabbit IgG H&L (Alexa Fluor [®] 488)	/	/	1:500	/	5257	Cell Signaling Technology
Goat Anti-Mouse IgG H&L (Alexa Fluor [®] 647)	/	/	1:500	/	ab150077	Abcam

Affinity isolation of ART-bound proteins

HepG2^R cell lysates were incubated with DMSO (Solarbio, D8371), 25 μ M biotin-ART [33] and 25 μ M biotin-ART plus 100 μ M ART overnight at 4°C, respectively. The lysates were pulled down with streptavidin-conjugated beads (Softlink soft release Avidin Resin; PROMEGA, V7820) at 4°C for another 4 h. After an extensive wash with PBS, the beads were boiled in 5×loading buffer. Then, the supernatants were collected and subjected to western blot for detecting the expression levels of AFAP1L2 protein [9].

Cellular thermal shift assay (CETSA)

HepG2^R cells were incubated with or without ART (25 μ M) for 4 h, and then the cells were collected and subjected to CETSA assay. Briefly, incubated cells were equally divided into 6 parts, each part was heated for 3 min under different temperature (room temperature, 37, 43, 49, 55, and 61°C), which was followed by repeatedly frozen and thawed three times in liquid nitrogen (1 min each time with 1 min interval) [34]. The procedure was repeated three times with an interval of 1 min between each pass. After that, cell lysates were extracted by centrifugation at 20,000×g, 20 min. Levels of AFAP1L2 were assessed by western blot.

Surface plasmon resonance (SPR)

SPR assay was performed using the Biacore T200 (Cytiva, USA) as follows. Recombinant human AFAP1L2 protein (HUABIO, TP20553) was immobilized on a Biacore CM5 sensor chip via the primary amine groups. The compounds were superfused at a rate of 30 μ L·min⁻¹ for 60 s to allow for association, followed by 150 s for dissociation over immobilized protein in PBS, 5% DMSO running buffer (1.05× PBS, 0.5% P20 surfactant [Cytiva, BR100054], 5% DMSO, pH 7.4). ART was tested for binding at 1.56 to 50 μ M. Normalization of the data involved transformation of the y-axis such that the theoretical maximum amount of binding for a 1:1 interaction with the protein surface corresponded to a sensor response of 100 relative units (RU) [35].

Establishment of sorafenib-resistant HCC orthotopic xenograft mouse model

BALB/c mice were housed five mice per cage in a specific pathogen-free room with a 12 h light/dark schedule at 25°C ± 1°C and were fed an autoclaved chow diet and water ad libitum. *In vivo* experiments were conducted using 4 weeks-old BALB/c nude mice. HepG2 derived orthotopic xenograft HCC model was established as described in our previous study [10,35]. Briefly, mice were anesthetized by tribromoethanol. Abdominal median incision was used. After liver exposure, a total of 5 million HepG2/Luc (Immocell, IML-064) cells were implanted into one lobe of liver, and medical OB glue (MEYER-HAAKE GmbH, MHG-0482) was used to bind the wound. The incision was closed using a suture of 5-0 silk (Jinhuan Medical, CR537). To monitor the growth of tumors, D-luciferin (MedChemExpress, HY-12591A) was injected through the tail vein (15 mg/mL, 200 μ L, i.v.), and the

bioluminescence from the tumor was monitored with IVIS Spectrum imaging system (PerkinElmer, USA) after three weeks of surgery. Then those mice carrying the tumor were taken as HCC group, and started to receive different doses of sorafenib (15/30/60 mg/kg/2 days) treatment at week 4. After three weeks of administration, using the living imaging assay to monitor the therapeutic effects of sorafenib. Compared with the first imaging, the animals with continuous tumor shrinkage were classified as sorafenib sensitive group, and the animals the tumor of which continuing to grow were considered as sorafenib-resistant group. The optimal dosage to induce sorafenib resistance was determined and used in the subsequent formal experiments. After 9 weeks, the third living imaging assay was performed to verify the accuracy of group division. Animals were killed under anesthesia at week 11 after operation.

ART treatment for sorafenib resistant and sensitive mice

The experimental procedure is shown in the body of the article. Bioluminescence imaging were taken at 1, 4, 7, 9 weeks after operation. For experiments to demonstrate that ART alleviates sorafenib resistance, the methods of building the sorafenib resistant model were the same as described above, except for the time node and dosage of sorafenib (30 mg/kg/2 days). After getting the sorafenib sensitive group and resistant group, then the sorafenib sensitive group was still administered with sorafenib by intragastric administration, the resistant group were randomized into three groups according to tumor fluorescence intensity and body weights, one group continued to receive sorafenib, and the other two groups received 30 or 60 mg/kg/2 days ART on the basis of sorafenib.

For experiments to demonstrate that ART enhances the sensitivity of HCC to sorafenib, the methods of building the sorafenib sensitive mice were the same as the orthotopic HCC model that described above. Then the HCC group was divided into four groups, including one group given water, one group given sorafenib, and two other groups given 30 or 60 mg/kg/2 days ART in combination with sorafenib.

MicroPET/CT imaging

MicroPET/CT imaging of mice was performed using an Inveon MicroPET/CT (Siemens, GER). Briefly, tumor-bearing mice were injected with 11.1 MBq (300 mCi) of ¹⁸F-FDG via the tail vein. Scanning began 45 min after injection. Mice were subjected to a 10 min MicroCT scan and then to a 10-min MicroPET scan. Images of mice were reconstructed using a three-dimensional ordered subset expectation maximization (OSEM3D)/maximum algorithm. The Inveon Research Workplace was used to calculate the percentage injected dose per gram (%ID/g) and the SUVs as previously described [36]. The SUV_{max} was then calculated.

H & E

To observe the pathological changes of liver tissues in different groups, the extracted livers were fixed in 4% paraformaldehyde. Following routine procedures, 3- μ m-thick sections were obtained from the resulting paraffin blocks. After

deparaffinization and rehydration, sections were stained with Hematoxylin and Eosin Staining Kit (Beyotime Biotechnology, C0105S). All sections were examined under a ML31 Mshot light microscope (Guangzhou, CHN).

Transmission electron microscopy (TEM)

TEM was used to observe the autophagosomes in HCC cells and orthotopic HCC tissues, which were fixed in 2.5% glutaraldehyde (Merck, 340855):1% paraformaldehyde mixture for 1 h at 4°C in 100 mM phosphate buffer, pH 7.2, post-fixed in 1% osmium tetroxide (Merck, 201030) for 2 h at 4°C, dehydrated in graded acetone (Merck, 179124) and embedded in Araldite (Fluka, 10951), sectioned, doubly stained with uranyl acetate (HEAD, SPI-02624) and lead citrate (HEAD, 17810), and analyzed using JEM1230 transmission electron microscope (JEOL, JPN) [7].

Immunohistochemical (ihc) staining

Briefly, after deparaffinized with xylene (Chemical Book, CB0130912), rehydrated in graded ethanol (Merck, 1.11727), immersed in 0.3% hydrogen peroxide (Merck, 1.08600), and heat-mediated antigen retrieval in citric acid (BOSTER, AR0024) at pH 6.0, tissue sections were incubated with the antibody for AFAP1L2 at 4°C overnight, labeled by HRP (rabbit) second antibody at room temperature for 60 min. Finally, sections were developed in DAB (BOSTER, AR1000) solution under microscopic observation and counterstained with hematoxylin (BOSTER, AR0005). Immunohistochemical scoring criteria are detailed in Supplementary material. A proportion score, which represents the estimated proportion of positively stained tumor cells, was assigned as follows: < 10%, 0; 10 to 25%, 1; 26 to 50%, 2; 51 to 75%, 3; and > 75%, 4. An intensity score, which represents the average intensity of the positive tumor cells, was assigned as follows: 0 (no staining), 1 (intensity lower than positive control), 2 (intensity equal to positive control), 3 (intensity higher than positive control). The proportion and intensity scores were then multiplied to obtain a total score, which ranged from 0 to 12. A total score of 0, 1 to 4, 6 to 8, and 9 to 12 was defined as being negative (-), weak positive (+), moderate positive (++), and strong positive (+++), respectively. The final scores were designated as low or high expression as follows: low expression (negative and weak positive), high expression (moderate positive, strong positive).

Statistical analyses

Statistical analyses were performed using GraphPad Prism 8.0 Software (San Diego, USA). Data are expressed as the mean $\pm$ S.D. and analyzed by one-way ANOVA with Bonferroni's or Dunnett's post hoc test for comparison of multiple columns. Differences were considered statistically significant when the *p* value was less than 0.05.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

The original contributions proposed in the study are stored in articles and Supplementary Materials, and further inquiries can be made directly to the corresponding author.

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