SUFU suppresses ferroptosis sensitivity in breast cancer cells via Hippo/YAP pathway

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Highlights
SUFU regulates the sensitivity of breast cancer cells to ferroptosis
SUFU associates with LATS1 to downregulate the YAP-ACSL4 axis
Vincristine targets the SUFU-YAP-ACSL4 axis
SUFU suppresses ferroptosis sensitivity in breast cancer cells via Hippo/YAP pathway

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SUMMARY
Ferroptosis is a new kind of regulated cell death that is characterized by highly iron-dependent lipid peroxidation. Cancer cells differ in their sensitivity to ferroptosis. Here we showed that the Suppressor of fused homolog (SUFU), a critical component in Hedgehog signaling, regulates ferroptosis sensitivity of breast cancer cells. Ectopic SUFU expression suppressed, whereas depletion of SUFU enhanced the sensitivity of breast cancer cells to RSL3-triggered ferroptosis through deregulation of ACSL4. Moreover, SUFU depletion promoted the activation of Yes-associated protein (YAP), thereby increasing the expression of ACSL4. Mechanistically, SUFU is associated with LATS1. Deletion of a region comprising residues 174–385 in SUFU disrupted SUFU binding to LATS1, thus abrogating SUFU-mediated downregulation of the YAP-ACSL4 axis and sensitivity to ferroptosis. Noteworthy, we showed that vincristine downregulated SUFU, thus increasing breast cancer cell sensitivity to RSL3 in vitro and in vivo. Together, our findings uncover SUFU as a novel regulator in ferroptosis sensitivity.

INTRODUCTION
Ferroptosis is a non-apoptotic form of regulated cell death characterized by iron dependency and lipid peroxidation (Dixon et al., 2012). To date, only a few key molecular players which involved in ferroptosis such as the phospholipid hydroperoxide glutathione peroxidase (GPX4), cystine/glutamate transporter (SLC7A11), and the acyl-CoA synthetase long-chain family member 4 (ACSL4) have been identified (Dixon et al., 2012; Doll et al., 2017; Poursaitidis et al., 2017). Ferroptosis sensitivity varies markedly among cancer cells from distinct tissue lineages (Hangauer et al., 2017; Yang and Stockwell, 2008; Yi et al., 2020; Zhang et al., 2019). Hence, the understanding of the molecular mechanisms underlying ferroptosis sensitivity will be of significance. The transcriptional co-activators Yes-associated protein (YAP) and its paralog, transcriptional co-activator with the PDZ binding motif (TAZ; encoded by WWTR1), two effectors of the Hippo pathway, has been shown recently to be associated with sensitivity to ferroptosis (Gao et al., 2021; Lin et al., 2021; Wu et al., 2019; Yang and Chi, 2020; Yang et al., 2019, 2021; Zhang et al., 2021). For instance, YAP promotes ferroptosis by upregulating several ferroptosis modulators, including ACSL4 and transferrin receptor 1 (TFRC) in breast and colorectal cancer cells (Wu et al., 2019). In addition, the TAZ-EMP1-NOX4 axis upregulates ferroptosis sensitivity in renal cell carcinoma (Yang et al., 2019). In epithelial ovarian cancer cells, TAZ modulates ferroptotic cell death through the ANGPTL4-NOX2 axis (Yang et al., 2020). These studies highlight targeting the Hippo/YAP signaling might provide therapeutic potential for ferroptosis-inducing cancer therapy. However, the mechanisms underlying the regulation of YAP/TAZ signaling in ferroptosis remain unclear.

The Suppressor of fused homolog (SUFU) is known as a critical component in Hedgehog (Hh) signaling and has been implicated in cancer development (Alimirah et al., 2016; Kasai et al., 2008; Lee et al., 2007; Raducu et al., 2016). In the current study, we show that SUFU regulates ferroptosis sensitivity in breast cancer cells through the Hippo/YAP pathway.

RESULTS
SUFU suppresses the sensitivity of breast cancer cells to RSL3-induced ferroptosis
To date, whether Hh signaling or components regulate ferroptosis remains unknown. We systematically correlated sensitivity data (determined by the area under the curve (AUC)) of the ferroptosis inducers...
Figure 1. SUFU regulates the sensitivity of breast cancer cells to RSL3-triggered ferroptosis

(A) Pharmacoc-transcriptomic correlation analysis identified key components of the SHH pathway associated with sensitivity to ferroptosis inducers across solid cancer cell lines (n = 658). Blue dots indicate the significantly negatively correlated genes while red dots indicate the positively correlated ones. Here, a negative correlation indicates the association of a larger AUC (area under the curve) with lower gene expression and vice versa. (B and C) Viability (%) of breast cancer cells MCF-7 and T47D stably depleted of SUFU (B), or HCC1954 and MDA-MB-231 stably overexpressing SUFU (C), following RSL3 treatment at indicated concentrations for 24 h. (D and E) Cell death determined by Sytox Green staining of cells in D and E, respectively, cells were treated with RSL3 at indicated concentrations for 24 h; scale bar, 200 μm. (F and G) RSL3-induced ROS levels (F) and GSH/GSSG ratios (G) were measured in MCF-7 and T47D cells in which SUFU was knocked down by siRNA (upper panels), or HCC1954 and MDA-MB-231 cells overexpressing SUFU (lower panels). ROS levels were assessed by flow cytometry with C11-BODIPY. GSH/GSSG ratios were measured by a GSH/GSSG-Glo assay. Data are presented as mean ± SD (*p < 0.05, **p < 0.01, ***p < 0.001, one-way ANOVA with Tukey’s multiple comparisons test or Unpaired t test). Data are presented as mean ± SD (*p < 0.05, **p < 0.01, ***p < 0.001). (one-way ANOVA with Tukey’s multiple comparisons test or Unpaired t test).

The production of the lipid-based reactive oxygen species (lipid ROS), is a hallmark of ferroptosis. Lipid oxidation can be measured by staining with C11-BODIPY, a dye that detects lipid ROS in cell membranes. This experiment demonstrated that RSL3-induced accumulation of lipid ROS was elevated in both MCF-7 and T47D cells upon transient knockdown of SUFU with siRNA targeting SUFU, compared to non-targeting siRNA controls (Figure 1F, upper panels). On the contrary, ectopic expression of SUFU in HCC1954 and MDA-MB-231 cells led to a significant decrease in ROS levels induced by RSL3 (Figure 1F, lower panels).

Oxidized form glutathione (GSSG) is one of the end products of lipid peroxidation during ferroptosis (Stockwell et al., 2020). GSH/GSSG-Glo assay revealed that SUFU overexpression significantly reversed the decreased GSH/GSSG ratio induced by RSL3 in HCC1954 and MDA-MB-231 cells (Figure 1G, lower panels), whereas depletion of SUFU in MCF-7 and T47D cells decreased further the GSH/GSSG ratio by RSL3 (Figure 1G, upper panels), comparing with their respective controls. Knockdown or overexpression of SUFU in these cells was confirmed by immunoblot assay (Figures S1I and S1J). Together, these data support that SUFU regulates the susceptibility of breast cancer cells to RSL3-triggered ferroptosis.

SUFU mediates resistance to RSL3-induced ferroptosis via downregulation of ACSL4

To explore the mechanistic connection between SUFU and ferroptosis, we performed RNA-seq analysis of control and SUFU-overexpressing SK-BR-3 cells (GEO accession number: GSE18880X) and found 53 differentially expressed genes (oeSUFU-DE-Gs) (Table S1). Differentially expressed genes upon overexpression of SUFU compared to control in SK-BR-3 cells. Related to Figure 2). Among those oeSUFU-DE-Gs, three
SUFU compared to control in HCC1954 cells. And Table S3. Gene set enrichment analysis (GSEA) of these SUFU-depleted cells robustly restored the levels of AXL and ACSL4 to the control level (Figure 3C), HCC1954, MCF-7, and MDA-MB-231 cells (Figure 3B). Importantly, re-expression of V5-tagged SUFU in upregulation of AXL and ACSL4, two YAP-regulated genes (Wu et al., 2019), in SUFU-depleted as a YAP-dependent target gene, was highlighted in the display (Figure 3A). IB analysis showed a robust YAP gene set upon knockdown of SUFU in HCC1954 cells. Related to Figure 3, p < 0.05, tion in HCC1954 and MCF-7 cells exhibited a significantly increased expression from the luciferase reporter led to a marked decrease in the protein levels of AXL and ACSL4 (Figure S3A), suggesting that SUFU eleva-

ACS4 predicts sensitivity to RSL3-triggered ferroptosis in a panel of basal-like breast cancer cell lines and sensitizes breast cancer cells to ferroptosis (Doll et al., 2017). The ACS4 expression in a panel of breast cancer cell lines was examined by IB (Figure S2B). We stably ablated ACS4 expression in MDA-MB-231 and HCC1954 cells using lentiviruses expressing shRNA targeting ACS4 (Figure S2C) and treated these cells with RSL3 for 24h. As expected, ACS4 depletion markedly mitigated RSL3-induced cell death in MDA-MB-231 and HCC1954 cells (Figure S2D), confirming an essential role of ACS4 in RSL3-induced ferroptosis. Moreover, upon RSL3 exposure, the increase in ferroptosis caused by SUFU ablation in MCF-7 and T47D cells were significantly mitigated by siRNA-mediated knockdown of ACS4 (Figure 2D), whereas the decrease in ferroptosis caused by SUFU overexpression in HCC1954 and MDA-MB-231 cells was rescued by ectopic ACS4 expression (Figure 2E). Consistently, ACS4 knockdown significantly abrogated SUFU depletion-induced decrease in the GSH/GSSG ratio upon RSL3 treatment (Figure 2F), whereas forced expression of a Flag-tagged ACS4 construct significantly mitigated SUFU overexpression-induced increase in the GSH/GSSG ratio in the presence of RSL3 (Figure 2G). Ectopic expression or siRNA-mediated knockdown of ACS4 in these breast cancer cells were examined by IB respectively (Figure S2E).

SUFU depletion upregulates YAP activity and target gene expression

To gain insights into how SUFU regulates ACS4 expression in breast cancer cells, we analyzed differential gene expression between SUFU-depleted and shControl HCC1954 cells (GEO accession number: GSE188806). Gene set enrichment analysis (GSEA) using a curated set of YAP-dependent signatures showed that depletion of SUFU in HCC1954 cells results in a highly significant enrichment of active YAP gene expression signatures (Figure 3A and Table S2). Differentially expressed genes upon knockdown of SUFU compared to control in HCC1954 cells. And Table S3. Gene set enrichment analysis (GSEA) of YAP gene set upon knockdown of SUFU in HCC1954 cells. Related to Figure 3, p < 0.05, q < 0.25). AXL as a YAP-dependent target gene, was highlighted in the display (Figure 3A). IB analysis showed a robust upregulation of AXL and ACS4, two YAP-regulated genes (Wu et al., 2019), in SUFU-depleted HCC1954, MCF-7, and MDA-MB-231 cells (Figure 3B). Importantly, re-expression of V5-tagged SUFU in these SUFU-depleted cells robustly restored the levels of AXL and ACS4 to the control level (Figure 3C), indicating that the increase in the levels of AXL and ACS4 upon SUFU knockdown is a consequence of SUFU deficiency. Consistently, overexpression of SUFU in HCC1954, MDA-MB-231, and SK-BR-3 cells led to a marked decrease in the protein levels of AXL and ACS4 (Figure S3A), suggesting that SUFU elevation is sufficient to downregulate YAP activity in breast cancer cells. To further confirm this notion, breast cancer cells with SUFU manipulation were transfected with a reporter encoding a YAP/TAZ-responsive luciferase gene (8xGTIIC-luciferase reporter), in which eight TEAD-YAP-binding sites were cloned into a promoter which drives the expression of firefly luciferase (Dupont et al., 2011). We found that SUFU depletion in HCC1954 and MCF-7 cells exhibited a significantly increased expression from the luciferase reporter.
Figure 3. SUFU downregulates YAP activity

(A) Gene set enrichment analysis (GSEA) of YAP target gene set upon knockdown of SUFU in HCC1954 cells. Gene symbols of the YAP common MCF10 and 3T3_Zhao gene set and detailed information on GSEA results were provided in Table S2: AXL, as a YAP-dependent target gene, was highlighted in the display.

(B and C) Immunoblot (IB) analysis of AXL and ACSL4 abundance upon SUFU depletion in HCC1954, MCF-7, and MDA-MB-231 cells (B). A rescue experiment was performed by ectopic expression of a VS-tagged SUFU construct in SUFU-depleted cells (C). GAPDH as a loading control. Numbers indicate the normalized ratio of ACSL4 or AXL over GAPDH signals obtained by densitometric analysis.

(D) Expression of TEAD luciferase reporter in SUFU-depleted MCF-7 and HCC1954 cells and their respective control cells. Cells were transfected with the 8xGTIIC-luciferase reporter and pRL-TK Renilla luciferase. Renilla luciferase was used as an internal control. Data were normalized to vector control.

(E) Confocal analysis of YAP nuclear localization in SUFU-depleted HCC1954 (left panels) and SUFU-depleted MCF-7 (right panels) cells; scale bar, 50µm. (F and G) IB analysis of P-YAP127 and total YAP levels in SUFU-overexpressed HCC1954 and MDA-MB-231 cells (F), in SUFU-depleted HCC1954, MCF-7, and MDA-MB-231 cells (G). Densitometric analysis of the normalized ratio of P-YAP127 over total YAP signals was shown. Data are presented as mean ± SD (*p < 0.05, **p < 0.01, ***p < 0.001, one-way ANOVA with Tukey’s multiple comparisons test).

(Figure 3D), whereas overexpression of SUFU in HCC1954, MDA-MB-231, and SK-BR-3 cells led to an opposite effect (Figure S3B), suggesting that SUFU indeed regulates YAP activity.

The increase in YAP activity upon SUFU depletion in breast cancer cells indicates YAP activation. To confirm this, we examined the nuclear localization of active YAP. Confocal analysis showed that depletion of SUFU led to significant increases in nuclear YAP in HCC1954 and MCF-7 cells (Figure 3E), whereas overexpression of SUFU resulted in significant decreases in nuclear YAP in these cells (Figure S3C), compared with their respective controls. Consistently, subcellular fractionation analysis revealed a remarkable decrease in nuclear YAP in SUFU-overexpressing HCC1954 and MCF-7 cells (Figure S3D). The phosphorylation status of YAP at Ser127, which is the LATS target site that mediates YAP cytoplasmic sequestration, is an indicator of YAP activation (Zhao et al., 2010). We observed an increase in P-YAP/YAP ratio in SUFU-expressing HCC1954 and MDA-MB-231 cells (Figure 3F). On the contrary, SUFU depletion led to a decrease in P-YAP/YAP ratio in HCC1954, MCF-7, and MDA-MB-231 cells (Figure 3G). Overall, these data reveal that SUFU plays a role in regulating YAP activation in at least breast cancer cells.

SUFU interacts with LATS1 to regulate YAP activity

We next dissect how SUFU regulates YAP activity in breast cancer cells. In the canonical Hippo pathway, YAP S127 phosphorylation is regulated by the MST/LATS kinase cascade. We found that SUFU overexpression led to an increase in P-LATS1/LATS1 ratio in HCC1954 and MDA-MB-231 cells (Figure 4A), whereas depletion of SUFU resulted in a decrease in P-LATS1/LATS1 ratio (Figure 4B). Surprisingly, SUFU overexpression induced a strong decrease in LATS1 abundance in HCC1954 and MDA-MB-231 cells, whereas SUFU ablation increased LATS1 abundance in these cells (Figures 4A and 4B). In addition, phosphorylation of MST1/2 remained unchanged upon SUFU overexpression or depletion in these cells (data not shown). Therefore, these data suggest that SUFU regulates LATS1 activity and/or abundance. To examine whether SUFU regulates YAP signaling via LATS1, we ectopically expressed SUFU in MDA-MB-231 and T47D cells with stable knockdown of LATS1. As expected, LATS1 knockdown led to the upregulation of YAP target genes AXL and ACSL4 (Figure 4C), comparing lane 1 with 2, three in left and right panels, respectively), whereas ectopic SUFU expression downregulated the levels of AXL and ACSL4 in control cells (Figure 4C, comparing lane 1 with 4 in left and right panels respectively). Notably, ectopic SUFU expression failed to impair the upregulation of AXL and ACSL4 in LATS1 knockdown cells (Figure 4C, comparing lane 4 with 5, six in left and right panels respectively), indicating that SUFU regulates YAP activity in a LATS1-dependent fashion.

We next examined how SUFU regulates the Hippo/YAP signaling by first testing for physical interaction between SUFU and key components of Hippo signaling including Merlin, Kibra, MST1/2, SAV1, LATS1/2, MOB1 as well as the Hippo effector YAP. Immunoprecipitation of VS-tagged-SUFU revealed that Myc-tagged LATS1, but not other tested Hippo components, robustly interacted with SUFU at the exogenous level in transfected HEK293T cells (Figures 4D and S4A). A faint interaction between merlin and SUFU at the exogenous level was also observed. In addition, we observed cytoplasmic co-localization between Flag-tagged SUFU and Myc-tagged LATS1 in MDA-MB-231 (Figure 4E) and MCF-7 cells (Figure S4B). Moreover, the physiologic association between endogenous SUFU and endogenous LATS1 was detected in HCC1806 cells (Figure 4F). To map the determinants of the interactions between SUFU and LATS1, we constructed a series of SUFU deletion mutants and assessed the interaction by immunoprecipitation and IB. A schematic depicting the regions in SUFU is shown in Figure 4G. As shown in Figure 4H, deletion of a region comprising residues 174–385 (hereafter as d174-385), but not other regions in SUFU, dramatically diminished its...
interactions with LAT51. We next examined the ability of SUFU deletion mutants in regulating YAP activity. The SUFU d174-385 deletion mutant which disrupts SUFU binding to LAT51 failed to inhibit the TEAD luciferase reporter activity as wild-type SUFU and other deletion mutants did in MCF-7 (Figure 4I), and HCC1954 (Figure S4C) cells. In addition, SUFU depletion-mediated increases in YAP nuclear localization as well as in the levels of AXL and ACSL4 in HCC1954 cells could not be rescued by the SUFU d174-385 deletion mutant (Figures S4D and S4E), while wild SUFU and other deletion mutants could do so.

**SUFU modulates susceptibility of breast cancer cells to ferroptosis through YAP signaling**

We next investigate whether SUFU regulates breast cancer cell growth and susceptibility to RSL3 through YAP signaling. To test this, we knocked down YAP with short interfering RNA (siRNA) in SUFU-depleted cells or treated the cells with verteporfin, which inhibits TEAD-YAP association by disrupting YAP/TEAD-mediated transcription (Liu-Chittenden et al., 2012). As shown in Figure 5A, knockdown of YAP with siRNA markedly attenuated the increase in the levels of AXL and ACSL4 triggered by SUFU depletion in MDA-MB-231 and T47D cells. Similar effects were detected in SUFU-depleted HCC1954 and MDA-MB-231 cells treated with verteporfin (Figure 5B). Surprisingly, verteporfin robustly decreased SUFU abundance in control cells. YAP is phosphorylated on multiple serine residues by LAT51/2, and the conversion of all these serine residues into alanine creates constitutively active YAP (YAP5SA) mutant that are no longer inhibited by LAT51/2 (Zhao et al., 2007). The YAP S94A mutant without the TEAD binding site, selectively abolishes YAP’s ability to activate TEADS (Zhao et al., 2008). We also employed another complementary method, i.e. ectopic expression of YAP-SSA or YAP-S94A in SUFU-overexpressing HCC1954 and MDA-MB-231 cells. Ectopic expression of YAP-SSA, but not YAP-S94A, substantially impaired the decrease in the levels of AXL and ACSL4 by SUFU overexpression (Figure 5C). Noteworthy, the increase in the sensitivity to RSL3 (i.e. more cell death) and the decrease in GSH/GSSG ratios caused by SUFU depletion in MCF-7 and T47D cells were reversed by YAP knockdown (Figures 5D and 5E). Moreover, we observed an increase in YAP nuclear localization upon VCR treatment in HCC1954 and MDA-MB-231 cells (Figure 6C). Noteworthy, siRNA-mediated knockdown of YAP abrogated VCR-induced nuclear localization of YAP (Figure 5F and 5G). Knockdown or overexpression of YAP in the tested cell lines was examined by immunoblot assay (Figure 5H). Together, these data highlight that YAP abundance and activity are essential for SUFU-mediated effects on breast cancer cell growth and sensitivity to RSL3.

**Vincristine downregulates SUFU and increases breast cancer cell sensitivity to RSL3**

Our above findings suggest that targeting SUFU might enhance breast cancer cell sensitivity to RSL3. To obtain a pharmacologic modulation of SUFU expression, we tested several clinically relevant drugs including 5-fluorouracil, cisplatin, doxorubicin hydrochloride, metformin, paclitaxel, and vincristine. HCC1954, MCF-7, and MDA-MB-231 cells were treated with these compounds at different doses for 24h. Treatment with these compounds alone did not induce substantial cell death (data not shown). We found that among these tested drugs, vincristine (VCR) robustly decreased SUFU levels in all three cell lines (Figure 6A). Furthermore, VCR treatment led to a dose-dependent decrease in SUFU levels in HCC1954, MCF-7, and MDA-MB-231 cells with a concomitant decrease in P-YAP127 levels and an increase in AXL and ACSL4 levels (Figure 6B). In addition, VCR treatment also decreased the level of ectopic expressed SUFU in HCC1954 and MCF-7 cells with a concomitant increase in the levels of AXL and ACSL4 (Figure S5A). Moreover, we observed an increase in YAP nuclear localization upon VCR treatment in HCC1954 and MDA-MB-231 cells (Figure 6C). Noteworthy, siRNA-mediated knockdown of YAP abrogated VCR-induced nuclear localization of YAP (Figure 5F and 5G). Knockdown or overexpression of YAP in the tested cell lines was examined by immunoblot assay (Figure 5H). Together, these data highlight that YAP abundance and activity are essential for SUFU-mediated effects on breast cancer cell growth and sensitivity to RSL3.
increases in the levels of AXL and ACSL4 in HCC1954 and MDA-MB-231 cells (Figure 6D), while VCR-triggered downregulation of SUFU was not affected by YAP knockdown. Altogether, these data suggest that VCR might upregulate the YAP-ACSL4 axis at least partly via targeting SUFU.

Next, we asked whether VCR would increase breast cancer cell sensitivity to RSL3. We observed that combined treatment with VCR and RSL3 resulted in synergistic response in MCF-7 and T47D cells which are insensitive to RSL3 (Figure 6E), while no significant cell death was observed when treated with VCR or RSL3 alone. Notably, the combination of VCR and RSL3 significantly enhanced YAP nuclear localization in MCF-7 cells compared with RSL3 alone treatment (Figure S5B). Finally, mice bearing MCF-7-derived tumors were used to evaluate the in vivo response to RSL3 and VCR. RSL3 at high concentrations significantly suppressed tumor growth compared to either vehicle or RSL3 at low concentrations. However, the combination of VCR and RSL3 resulted in significant tumor regression compared with any single modality (RSL3 at low concentrations) (Figures 6F and S6A). IHC analysis revealed a decrease in both Ki67 and SUFU staining and an increase in ACSL4 staining in tumor sections from mice treated with the combination of VCR and RSL3, compared with the drug alone group (Figure 6G).

DISCUSSION

Here, we show that SUFU, a key component of Hh signaling, suppresses the sensitivity of breast cancer cells to RSL3-induced ferroptosis. Mechanistically, SUFU interacts with LATS1 thus downregulating the YAP-ACSL4 axis. We further demonstrate that targeting SUFU may represent a strategy to sensitize breast cancer cells to ferroptosis induction.

Ferroptosis is increasingly recognized as a promising avenue to kill cancers resistant to standard treatments. However, regulators that predict the sensitivity and/or resistance of ferroptosis and the underlying mechanisms are not yet completely investigated. Recent studies reported that the Hippo/YAP pathway regulates ferroptosis sensitivity in several types of cancer such as mesothelioma (Wu et al., 2019), ovarian cancer (Yang et al., 2020), and renal cell carcinoma (Yang et al., 2019). Thus, modifiers of the Hippo/YAP pathway might be related to ferroptosis sensitivity. Supporting this notion, we revealed that SUFU regulates breast cancer cell sensitivity to RSL3-triggered ferroptosis via perturbing the Hippo/YAP signaling, as demonstrated by our gain- and loss-of-function analyses. Given that SUFU acts primarily as a negative regulator of the Hh signaling pathway via interacting with Gli proteins (Kim et al., 2019), our findings uncover a new mechanism of action for SUFU in cancer.

Our findings suggest that elevated SUFU in breast cancer might serve as a predictor for responsiveness to ferroptosis induction for at least RSL3. We recently reported that SASH1 suppressed breast cancer cell invasiveness by targeting the Hippo/YAP pathway (Jiang et al., 2020). It will be interesting to investigate whether SASH1 and other reported modulators of the Hippo/YAP signaling would contribute to the ferroptosis sensitivity in cancer. In search of potential avenues to overcome SUFU-mediated resistance to RSL3, we screened out vincristine, which could target SUFU and its regulated YAP-ACSL4 axis among several clinically relevant chemotherapeutic agents. Currently, vincristine is mostly used in combination with other anti-cancer drugs in active clinical trials (Skubnik et al., 2021), therefore our finding may have potential implications for ferroptosis-inducing cancer therapies.

To be taken together, our study demonstrates for the first time a crucial role for SUFU in regulating ferroptosis sensitivity in breast cancer cells. We suggest that vincristine warrants further clinical development in combination with RSL3 for the treatment of breast cancer that is insensitive to RSL3-triggered ferroptosis.
A

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B

Vincristine μM (24h)

HCC1954

| Treatment | 0.01 | 0.1 | 0.2 |
|-----------|------|-----|-----|
| DMSO      |      |     |     |
| Mitomycin | 50μM |     |     |
| Vinorelbine| 10μM |     |     |
| Cisplatin | 25μM |     |     |
| SUFU      |      |     |     |
| GAPDH     |      |     |     |

MDA-MB-231

| Treatment | 0.01 | 0.1 | 0.2 |
|-----------|------|-----|-----|
| DMSO      |      |     |     |
| Mitomycin | 50μM |     |     |
| Vinorelbine| 10μM |     |     |
| Cisplatin | 25μM |     |     |
| SUFU      |      |     |     |
| GAPDH     |      |     |     |

MCF-7

| Treatment | 0.01 | 0.1 | 0.2 |
|-----------|------|-----|-----|
| DMSO      |      |     |     |
| Mitomycin | 50μM |     |     |
| Vinorelbine| 10μM |     |     |
| Cisplatin | 25μM |     |     |
| SUFU      |      |     |     |
| GAPDH     |      |     |     |

C

**Article**

D

Vincristine (0.1μM)

HCC1954

| Treatment | 0.01 | 0.1 | 0.2 |
|-----------|------|-----|-----|
| DMSO      |      |     |     |
| Mitomycin | 50μM |     |     |
| Vinorelbine| 10μM |     |     |
| Cisplatin | 25μM |     |     |
| SUFU      |      |     |     |
| GAPDH     |      |     |     |

MDA-MB-231

| Treatment | 0.01 | 0.1 | 0.2 |
|-----------|------|-----|-----|
| DMSO      |      |     |     |
| Mitomycin | 50μM |     |     |
| Vinorelbine| 10μM |     |     |
| Cisplatin | 25μM |     |     |
| SUFU      |      |     |     |
| GAPDH     |      |     |     |

E

Tumor growth in MCF-7 and T47D cells

F

Cell viability in MCF-7 and T47D cells

G

Histological analysis of tumor sections

| Tumor Type | Treatment |
|------------|-----------|
| MCF-7      | H&E       |
| T47D       | H&E       |
| Ki67       |           |
| SUFU       |           |
| ACSL4      |           |
| RSL3 (100mg/kg) | VCR (30μg/kg) |
| RSL3 (40mg/kg) | VCR (30μg/kg) |
| RSL3 (40mg/kg) | +VCR (10μg/kg) |
Limitations of the study
Although our study suggests that SUFU regulates the Hippo pathway in breast cancer cells via interacting with LATS1, how the interaction between SUFU and LATS1 is regulated in breast cancers remains unknown. In addition, more work is needed to dissect how this regulation impacts on SUFU-mediated ferroptosis sensitivity in breast cancers.

STAR★METHODS
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SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.104618.

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AUTHOR CONTRIBUTIONS
D.J., W.C., Y.W., and S.M. participated in the design of the study. K.F., S.D., D.S., Z.X., K.J., H.X., L.H., X.Z., J.W., Y.J., C.X., and Z.X. collected the data. D.L. performed bioinformatics analysis. K.F., D.S., and K.J. built...
the animal model. D.L., K.F., D.J., W.C., and S.M. participated in the data analysis. Y.W. and S.M. helped to draft the manuscript. All authors read and approved the final manuscript.

DECLARATION OF INTERESTS
The authors declare no competing financial interests.

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# STAR Methods

## Key Resources Table

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| SUFU                | Cell Signaling Technology | C81H7; AB_2302728 |
| LATS1               | Cell Signaling Technology | 3477S; AB_2133513 |
| P-LATS1             | Cell Signaling Technology | 9157S; AB_2133515 |
| P-YAP127            | Cell Signaling Technology | 4911S; AB_2218913 |
| V5-tag              | Cell Signaling Technology | 13202S; AB_2687461 |
| Myc-tag             | Cell Signaling Technology | 2278S; AB_490778 |
| xCT/SLC7A11         | Cell Signaling Technology | 12691; AB_2687474 |
| Anti-V5             | Invitrogen Thermo Fisher Scientific | 46-0705; AB_10804584 |
| YAP1                | NOVUS | NB110-58358; AB_922796 |
| GAPDH               | Proteintech | 10494-1-AP; AB_2263076 |
| AXL                 | Proteintech | 13196-1-AP; AB_10642006 |
| Flag-tag            | Proteintech | 20543-1-AP; AB_11232216 |
| HA-tag              | Sigma | H6908; AB_260070 |
| LAMIN A+C           | Abcam | ab133256; AB_2813767 |
| ACSL4               | Abcam | ab155282; AB_2714020 |
| GPX4                | Abcam | ab125066; AB_10973901 |
| Ki67                | Abcam | ab16667; AB_302459 |
| SUFU                | Santa Cruz Biotechnology | sc-137014; AB_2197315 |
| IgG                 | Santa Cruz Biotechnology | sc-69786; AB_1124809 |
| **Chemicals, peptides, and recombinant proteins** |        |            |
| Verteporfin         | Selleck | S1786; CAS: 129497-78-5 |
| Z-VAD-FMK           | Selleck | S7023; CAS: 187389-52-2 |
| Necrostatin-1       | Selleck | S8037; CAS: 4311-88-0 |
| Ferrostatin-1       | Sigma | SML0583; CAS: 347174-05-4 |
| liproxstatin-1      | Sigma | SML1414; CAS: 950455-15-9 |
| 15,3R-RL3           | Sigma | SML2234; CAS: 1219810-16-8 |
| Doxorubicin Hydrochloride | Sigma | D1515; CAS: 25316-40-9 |
| 3-Methyladenine     | CALBIOCHEM | 189490; CAS: 5142-23-4 |
| Metformin           | TargetMol | T0740; CAS: 1115-70-4 |
| Vincristine         | J&K Scientific | 594515; CAS: 2068-78-2 |
| 5-Fluouracil        | Aladdin | 51-21-8; CAS: 51-21-8 |
| Paditaxel           | Solarbio | SP8020; CAS: 33069-62-4 |
| Cisplatin           | Solarbio | SC5170; CAS: 15663-27-1 |
| **Critical commercial assays** |        |            |
| CCK8                | MedChemExpress | Cat#HY-K0301 |
| Sytox Green staining | Thermo | Cat#S34860 |
| Dual-Luciferase Reporter Assay System | Promega | Cat# E1910 |
| BODIPY S81/591 C11 (Lipid Peroxidation Sensor) | Thermo | Cat# D3861 |
| GSH/GSSG-Glo™ assay | Promega | Cat# V6911 |

(Continued on next page)
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Raw and analyzed data (mRNA-seq) | This paper | GEO:GSE188806 |

**Experimental models: Cell lines**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| HCC1954 | ATCC | CRL-2338 |
| MDA-MB-231 | ATCC | HTB-26 |
| MCF-7 | ATCC | HTB-22 |
| SK-BR-3 | ATCC | HTB-30 |
| T47D | ATCC | HTB-133 |
| 293T | ATCC | CRL-11268 |
| MCF-10A | ATCC | CRL-10317 |
| MDA-MB-468 | ATCC | HTB-132 |
| HCC1806 | ATCC | CRL-2335 |
| HCC1937 | ATCC | CRL-2336 |

**Experimental models: Organisms/strains**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| BALB/c Nude mice | Beijing Vital River Laboratory Animal Technology Co., Ltd | N/A |

**Oligonucleotides**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| V5-SUFU-WT Forward: 5’-AGAA GCTTATGCGAGCTGGGC-3’ | This paper | N/A |
| V5-SUFU-WT Reverse: 5’-CGGGAT CCGTGTAGCGGACTGTCGAAC-3’ | This paper | N/A |
| V5-SUFU-d(1-173) Forward: 5’-AGAAGC TTATGCTAGCATCTGCGACTGACA-3’ | This paper | N/A |
| V5-SUFU-d(1-173) Reverse: 5’-CGGGATCC GTTAGCGAGCTGCGAACC-3’ | This paper | N/A |
| V5-SUFU-d (174–385) Forward: 5’-CAGGAG CCTGATCCCTTCTCCTCCTG-3’ | This paper | N/A |
| V5-SUFU-d (174–385) Reverse: 5’-AAAGATG AGTCAGAAGGAGGGCGACTCGGCT-3’ | This paper | N/A |
| V5-SUFU-d (386–484) Forward: 5’-AGAAAG CTATTGGCGAGCTGCGC-3’ | This paper | N/A |
| V5-SUFU-d (386–484) Reverse: 5’-CGGGATCT CCTAGGCGAGAGAATGAGGGC-3’ | This paper | N/A |
| V5-SUFU-d (1-173)+ (386–484) Forward: 5’-AGAAGCTTATGAGCTGGAGCTGCTGACA-3’ | This paper | N/A |
| V5-SUFU-d (1-173)+ (386–484) Reverse: 5’-CGGGATCTGATGGCGAGCTGCGACA-3’ | This paper | N/A |
| siRNA targeting sequence: siSUFU-1: 5’-CCUCAUUUCCUCCUCUATT-3’ | Ribobio(Guangzhou, China) | N/A |
| siRNA targeting sequence: siYAP-1: 5’-CCUCUCCUCCUCUATT-3’ | Ribobio(Guangzhou, China) | N/A |
| siRNA targeting sequence: siACSL4-1: 5’-CCUCUCCUCCUCUATT-3’ | Ribobio(Guangzhou, China) | N/A |

(Continued on next page)
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Songshu Meng (ssmeng@dmu.edu.cn).

Materials availability
This study did not generate new unique reagents and all materials in this study are commercially available.

Data and code availability
- Data: All data reported in this paper will be shared by the lead contact upon request.
- Code: This paper does not report original code.
- Additional information: Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon reasonable request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell lines and plasmids
Human breast cancer cell lines HCC1954, HCC1806, HCC1937, MCF-7, MDA-MB-231, MDA-MB-468, SK-BR-3, and T47D, human mammary epithelial cell line MCF-10A and human embryonic kidney (293T) cell line were purchased from the American Type Culture Collection. Cells were cultured at 37°C in 5% CO₂ with DMEM or RPMI-1640 (Gibco) medium supplemented with 10% fetal bovine serum, 100 unit/mL penicillin and 100 mg/mL streptomycin. MCF-10A cells were in the DMEM/F12 media (Invitrogen) supplemented with horse serum (5% final, Invitrogen) insulin (10 μg/mL, Sigma), EGF (20 ng/mL, Peprotech), hydrocortisone (0.5 μg/mL, Sigma), 1% penicillin–streptomycin (Invitrogen), and cholera toxin (100 ng/mL, Sigma).
The HA-tagged SUFU cDNA construct was kindly provided by Prof. Vincenzo D’Angiolella (Raducu et al., 2016). V5 or Flag-tagged SUFU wild type and deletion mutants were constructed by standard molecular cloning procedures.

**In vivo xenograft model**

All animal studies were carried out at Dalian Medical University Laboratory Animal Center, and conducted in accordance with the national guidelines for the care and use of laboratory animals with approval from the experimental animal ethics committee: Dalian Medical University.

For subcutaneous tumor formation, MDA-MB-231 cells (1 \( \times \) 10^7) were injected subcutaneously into flanks of nude mice (female, 5–6 weeks-old, n = 5, Beijing Vital River Laboratory Animal Technology Co., Ltd). Tumor diameters were measured every three days and the tumor volume was calculated using the equation: V = 0.52 \( L \times W^2 \). Tumor-bearing mice were euthanized by CO2 inhalation at the tumor burden endpoint.

For RSL3 and vincristine combination experiments, MCF-7 cells (1 \( \times \) 10^7) were implanted subcutaneously into the flank of 5-to 6-week-old nude mice. Mice with established tumors with approximately 50–80 mm³ average volumes were randomized into treatment groups (n = 5), including vehicle (DMSO), RSL3 (100 mg/kg in 20mL DMSO plus 80mL corn oil), RSL3 (40 mg/kg), vincristine (30 \( \mu \)g/kg), or the combination of RSL3 (40 mg/kg) with vincristine (30 \( \mu \)g/kg). Compounds or vehicle were administered through intra-tumor injection biweekly.

**METHOD DETAILS**

**Antibodies and reagents**

The following antibodies were purchased from Cell Signaling Technology (America): SUFU (C81H7), LATS1 (3477S), P-LATS1 (9157S), P-YAP127 (4911S), V5-tag (13202S), xCT/SLC7A11 (12691). Anti-V5 (P/N46-0705) was obtained from Invitrogen Thermo Fisher Scientific (America). YAP1 (NB110-58358) was purchased from NOVUS (America). GAPDH (10494-1-AP), AXL (13196-1-AP) and Flag-tag (20543-1-AP) were bought from Proteintech (America). LAMIN A + C (ab133256), ACSL4 (ab155282), GPX4 (ab125066) and Ki67 (ab16667) were bought from Abcam (UK). SUFU (sc-137014) and IgG (sc-69786) were purchased from Santa Cruz Biotechnology (America). Verapamilin (S1786), Z-VAD-FMK (S7023) and Necrostatin-1 (S8037) were purchased from Selleck Chemicals (America). Ferrostatin-1 (SML0583), liproxstatin-1 (SML1414), 1S,3R-RSL3 (SML2234) and Doxorubicin Hydrochloride (D1515) were obtained from Sigma (America). 3-Methyladenine (189490) was bought from CALBIOCHEM (Germany). Metformin (T0740) was purchased from TargetMol (America). Vincristine (594515) was bought from J&K Scientific (China). 5-Fluorouracil (51-21-8) was obtained from Aladdin (China). Paclitaxel (SP8020) and Cisplatin (SC5170) were purchased from Solarbio (China). Drugs were dissolved and stored at –20°C or –80°C according to the manufacturer’s instructions.

**Bioinformatics analysis**

For pharmaco-transcriptomic correlation analysis, gene expression data of human solid cancer cell lines were obtained from the Cancer Cell Line Encyclopedia (CCLE) project. The CTRP2.0 contains the sensitivity data for ferroptosis inducers (erastin, sorafenib, RSL3, ML162, ML210) and provides area under the curve (AUC), determined by fitted concentration-response curves (2-fold dilution, over a 16-point concentration range), as a measure of drug sensitivity. AUC values were rescaled into a range of [0, 1] (AUC/30). Pearson correlation was calculated between SHH signaling gene expression and ferroptosis inducers AUC.

RNA-Seq was performed by the Novogene Corporation (Beijing, China). The sequencing libraries were constructed using NEBNext® UltraTM RNA Library Prep Kit for Illumina® (NEB, USA) according to the manufacturer’s instructions. The heatmap of differentially expressed genes (DEGs) was generated using the R ComplexHeatmap package. The association between SUFU and YAP was analyzed with Gene Set Enrichment Analysis (GSEA) using YAP-dependent gene signatures collected from YAP common MCF10 and 3T3_Zhao (Zhao et al., 2008). Metrics for ranking the key mRNAs were calculated based on log2 fold change in SUFU knockdown vs control. YAP dependent gene signatures were significantly enriched at p-value < 0.05 and q-value < 0.25.
RNA-sequencing (RNA-seq) for transcriptional profiles of SKBR3 control and SUFU-overexpressing cells (GSE188806) and HCC1954 control and cells with SUFU silencing (GSE188806) have been deposited in the NCBI Genome Expression Omnibus under SuperSeries GSE 188806. Differentially expressed genes upon overexpression of SUFU compared to control in SK-BR-3 cells are listed in Table S1. Differentially expressed genes upon knockdown of SUFU compared to control in HCC1954 cells are listed in Table S2. GSEA of YAP gene set upon knockdown of SUFU in HCC1954 cells is listed in Table S3.

**RNA interference**

siRNA oligonucleotides were purchased from Ribobio (Guangzhou, China) and sequences are as follows:

- siSUFU-1: 5′-CCUCAUCCUCUCUGCCUATT-3′
- siSUFU-2: 5′-GGAGAGGACUCGAGAUCAATT-3′
- siYAP-1: 5′-GACAUCUUCUGGUCAGAGA-3′
- siYAP-2: 5′-GAGATGGAATGAACATAGA-3′
- siACSL4-1: 5′-GAGCGATTGAAATTCCAA-3′
- siACSL4-2: 5′-ACAGCATGCAATCAGTAGA-3′

siRNAs or negative control (siControl) were transfected with Lipofectamine 3000 (Invitrogen) according to the manufacturer’s instructions.

**Lentiviral constructs and stable cell lines**

The lentiviral vectors encoding short hairpin RNAs (GIPZ shRNAs) targeting SUFU, LATS1, and ACSL4, respectively, and scrambled shRNA were purchased from Dharmacon (America). Flag-tagged SUFU was subcloned into the pCDH-puro lentiviral vector by standard molecular cloning procedures. Stable cell lines knocking down or overexpressing SUFU were established. LATS1-depleted MDA-MB-231 cells were established in our lab (Jiang et al., 2020).

**Cell viability assay, crystal violet staining and SYTOX green cell death assay**

Cells were seeded (2000 cells/well) in 96-well plates for the indicated time, and cell viability was measured by Cell Counting Kit-8 (CCK8, MCE/Y-K0301) or Crystal violet staining. For IC50 assays, a CCK-8 assay was used to measure drug sensitivity at 450 nm using a microplate reader (Thermo Fisher Scientific).

Cytotoxicity was determined by Sytox Green staining (Thermo) according to the manufacturer’s protocol. To analyse cell death, breast cancer cells were seeded at 2 × 10^5 cells/well into 12-well plates. After 24h contact, cells were divided into control group and model group. In the control group, cells were incubated for 24h in complete medium; in the RSL3 group, cells were incubated in the same medium with the presence of RSL3. Following incubation, cells were rinsed twice with PBS. Then adhered cells were incubated with SYTOX Green staining solution (40 nM) in PBS in the dark for 30 minutes at 37°C. Cells were rinsed twice with PBS and imaged by inverted fluorescence microscope.

**Luciferase reporter assay**

For the luciferase assays, cells at 50% confluency were transfected in 24-well plates with 0.1μg of a YAP/TAZ-responsive luciferase gene (8xGTIIC-luciferase reporter) or empty vector control (pGL3), with 0.1μg of pCDNA3.1 empty vector or pCDNA3.1-V5-SUFU and 0.02μg pRL-TKRenilla (Promega) as a transfection control. The cells were lysed in 50μL passive lysis buffer for 3 days after transfection. The soluble fraction was subsequently assayed for luciferase activity with a Dual-Luciferase Reporter Assay System (Promega, USA).

**Lipid ROS and GSH/GSSG-Glo assays**

Lipid ROS levels in breast cancer cells were assessed by BODIPY 581/591 C11 (lipid peroxidation sensor, Thermo scientific, USA) according to the manufacturer's instructions. To analyse lipid peroxidation, breast cancer cells were seeded at 5 × 10^5 cells/well into 6-well plates. After 24h contact, cells were divided into
two groups, the control group and the model group. In the control group, cells were incubated for 24 h in complete medium; in the RSL3 group, cells were incubated in the same medium with the presence of RSL3. Following incubation, the cells were rinsed twice with PBS. Adhered cells were incubated with C11-BODIPY (5 μM) staining solution in PBS in the dark for 30 minutes at 37°C. Cells were then harvested with 0.05% trypsin solution, suspended in fresh medium, and immediately analyzed with flow cytometer. Each sample test was repeated in three independent wells.

Detection and quantification of the GSH/GSSG ratios were carried out with GSH/GSSG-Glo™ assay (Promega, Southampton, UK) according to the manufacturer’s guidelines.

**Immunohistochemistry**

Paraffin-embedded tissue sections were dewaxed using a decreasing xylene/alcohol series. Briefly, the processed sections were blocked with 3% BSA and incubated with anti-SUFU antibody (1:50) and anti-ACSL4 antibody (1:100). The DAB Detection Kit was used to develop the staining signals according to the protocols provided for the streptavidin-peroxidase system (Sangon Biotech, China). Haematoxylin was used for counterstaining.

**Immunofluorescence**

Seed and allow cells to adhere to glass slides in the cell culture medium for 24 h and fixed with 4% paraformaldehyde (PFA) for 20 minutes. To visualize cytoplasmic and nuclear components, 4% PFA fixed cells were permeabilized with 0.5% Triton X-100 for 20 minutes. Then the cells were blocked with 5% BSA for 60 minutes. Primary antibodies were diluted in blocking buffer and cells were incubated overnight at 4°C. Cells were washed with PBS and fluorescently labeled secondary antibodies were diluted in blocking buffer followed by incubation for 30 minutes at room temperature. Finally, the cells were sealed with anti-fluorescence quenching sealing tablets.

**Immunoprecipitation and immunoblotting**

For immunoprecipitation studies, cells were harvested 24 hours after being transfected with plasmids in 1% Triton X-100 lysis buffer, which was supplemented with a mixture of protease and phosphatase inhibitors. Lysates were centrifuged and supernatants collected. Supernatants were precipitated by 30 μL of protein G dynabeads (Roche, 11243233001) for one hour followed by 4°C incubation with rotation for 2.5 hours in the presence of 5 μg/ml antibodies or control IgG. Immunoprecipitated complexes were washed five times with cold lysis buffer before boiling in 1X Loading Buffer. Immunoprecipitates and 2% lysate loading controls were then run on an SDS-PAGE gel and subsequent immunoblotting was conducted.

For immunoblotting (IB), in vitro cultured human breast cancer cells were harvested using RIPA lysis buffer (10 mM Tris-HCl, pH 8.0, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, pH 7.4) supplemented with a cocktail of protease and phosphatase inhibitors. The protein concentrations were assessed using a BCA Protein Assay reagent kit, and after boiling, protein samples were separated via sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Then, proteins were transferred onto a nitrocellulose membrane using electrophoresis (Bio-Rad Laboratories). All membranes were blocked with 5% nonfat dry milk in 1X Tris-buffered saline with Tween 20 for 1 hour and then incubated at 4°C overnight with the primary antibodies: After washing with 1X Tris-buffered saline with Tween 20, membranes were then incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulin G for 1 hour at room temperature and exposed using an enhanced ECL Western Blotting Substrate. GAPDH was used as loading control (Jiang et al., 2016, 2020; Liu et al., 2018), and the detailed information about antibodies were provided in key resources table.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

All data analyses in this experiment were performed by GraphPad Prism 7 software. Differences between variables of two groups were examined by Student’s t test, and one-way ANOVA was used to evaluate the differences among variables of multiple groups. The results were shown as mean ± standard error. Significant difference: *p < 0.05, **p < 0.01, ***p < 0.001.