HSP90α Mediates Sorafenib Resistance in Human Hepatocellular Carcinoma by Necroptosis Inhibition Under Hypoxia

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Research

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Abstract

Background

As one of the most common malignancies worldwide, Hepatocellular carcinoma (HCC) has been treated by Sorafenib, which is the first approved target drug by FDA for advanced HCC. However, drug resistance is one of the obstacles to its application. As a typical characteristic of most solid tumors, hypoxia has become a key cause for resistant to chemotherapy and radiotherapy. It is important to elucidate the underlying mechanisms of Sorafenib resistance under hypoxia. 17-AAG, an inhibitor of HSP90α, and is in clinical test at present in anticancer.

Methods

Routine laboratory experimental methods including cell culture, cell transfection, western blot, immunohistochemistry (IHC), and immunofluorescence (IF) were used; the morphological changes of hepatocellular carcinoma cell was observed by Live Cell Imaging System and Transmission Electron Microscope; coimmunoprecipitation (Co-IP) were used for confirmation of interactions of RIPK3/MLKL/HSP90α and HSP70/LAMP2/HSP90α/MLKL. Patient-derived tumor xenograft (PDX) model and human HCC cells xenograft model have been established, and primary resistant cell line was induced to investigate the potential therapeutic strategies to overcome Sorafenib resistance.

Results

In this study, Sorafenib was found to induce necroptosis in liver cancer. Under hypoxia, the distribution of necroptosis related proteins was changed, which contributed to Sorafenib resistance. HSP90α binds with RIPK1/RIPK3/MLKL complex and promotes chaperone-mediated autophagy (CMA) degradation, which leads necroptosis blocking. Ultimately, the decrease of necroptosis resulted in Sorafenib resistance. 17-AAG inhibited HSP90α and presented obvious reversal effects of Sorafenib resistance in vivo and in vitro. All the results emphasized that the blockage of HSP90α can significantly improve the effect of Sorafenib under hypoxia.

Conclusions

HSP90α plays a critical role in Sorafenib resistance under hypoxia by blocking necroptosis. 17-AAG combining with Sorafenib is a promising therapy for hepatocellular carcinoma.

1. Background

Hepatocellular carcinoma (HCC) is reported to be the fifth most common cancer with the second highest mortality among all cancers in adults [1]. At present, the common treatment strategies for liver cancer are surgical resection, radiation therapies, and chemotherapy [2].
The multikinase inhibitor Sorafenib, originally developed as a Raf kinase inhibitor, targets the MAPK/ERK pathway but also the vascular endothelial growth factor receptors (VEGF-R) and the platelet-derived growth factor receptor (PDGF-R) [3]. Sorafenib contributes to a survival benefit of patients through reducing tumor angiogenesis and increasing cancer cell apoptosis [4-6]. However, its efficacy always has been hampered by the occurrence of drug resistance [7-9], and HCC is much more difficult to cure after relapse. Therefore, the urgent problem is to explore the mechanism of Sorafenib resistance and work out an effective treatment.

At present, it seems that necroptosis can be one of the important mechanisms of Sorafenib in treatment of cancer[10-13]. Necroptosis is non-apoptotic cell death, which depends on the receptor interacting protein kinase 3 (RIPK3). RIPK3 and RIPK1 can activate each other, promotes its conversion to an amyloid-like filamentous structure termed the necrosome, resulting in the recruitment of another necroptosis mediator, mixed lineage kinase domain-like (MLKL) [14, 15]. Phosphorylated MLKL forms oligomers translocate to intracellular membranes and the plasma membrane, which eventually leads to membrane rupture [13-15]. Recent evidence indicates that inhibition of caspase-dependent apoptosis sensitizes many cancer cells to necroptosis. This has led to widespread interest in exploring necroptosis as an alternative strategy for anti-cancer therapy.

Hypoxia is one of the characteristics of most solid tumors, which plays an important role in the occurrence and development of cancers. Adaptation of tumor cells to hypoxia has important biological effects on drug resistance. Previous studies have reported that sustained Sorafenib treatment may promote hypoxia within tumors, which has been associated with Sorafenib resistance to HCC patients as well as subcutaneous mice model on HCC [17]. Hypoxia usually results in resistance of various tumors to therapy through inducing activation of the HIF signaling pathway and survival of tumor cells [18, 19]. Molecular chaperones are a heterogeneous class of proteins unified by their primary function of assisting the cellular proteome to achieve and maintain a conformationally mature and functional state [20, 21]. HSP90α is a chaperone protein that interacts with client proteins that it is closely related to cell apoptosis, metastasis, invasion and chemotherapy resistance, it protects cells from damage and stimuli, promotes tumor cells growth, make tumor cells tolerate chemotherapy, heat treatment and other traumatic stimuli, and finally leads to treatment failure. In HCC, HSP90α expression positively correlated with HIF-1. Down-regulation of HIF-1α or HIF-1β completely blocks HSP90α secretion, indicating HIF-1 as a critical upstream regulator of HSP90α secretion [22]. In recent years, HSP90α was also proved to play an important role in drug resistance under hypoxia [23-26]. Demethoxygeldanamycin (17-AAG) is a derivative of geldanamycin that is currently undergoing clinical development as a novel anticancer agent for the treatment of human cancers [27, 28]. 17-AAG induces tumor apoptosis and inhibit tumor proliferation in leukemia cells and prostate cancer has already been studied. Combination of 17-AAG and oxaliplatin or capecitabine in colorectal cancer cell lines has been studied. 17-AAG in combination with paclitaxel on anaplastic thyroid carcinoma cells has also been reported[29-31]. In this study, we confirmed that necroptosis was one of the important reasons of Sorafenib attacking HCC. Besides, we elucidated HSP90α binds with RIPK1/RIPK3/MLKL complex to promotes chaperone-mediated autophagy (CMA) degradation, which would be the main causes of Sorafenib resistance. 17-AAG, as a specific inhibitor of
HSP90α, could overcome Sorafenib resistance on HCC. Combining 17-AAG with Sorafenib might be a potential therapeutic strategy to enhance Sorafenib efficacy for the treatment of HCC.

2. Materials And Methods

2.1 Reagents

Sorafenib Bay 43-9006, Sigma-Aldrich), 17-AAG (Tanespimycin, MCE, HSP90 inhibitor), Z-VAD-FMK (Selleck, Caspase Inhibitor), Necrostatin-1 (Selleck, RIPK1 inhibitor), Necrosulfonamide (Selleck, MLKL inhibitor).

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenytetrazoliumbromide) was obtained from Fluka Chemical Corp (Ronkonkoma, NY, USA) and was dissolved in 0.01 M phosphate-buffered saline (PBS).

Antibodies against RIPK1, RIPK3, MLKL, were purchased from Abclonal Technology (Wuhan, China). Antibodies against HIF-1α, HSP90α, p-MLKL, and GAPDH were products of Bioworld Technology (USA). Antibodies against AFP and LAMP2 were products of Cell Signaling Technology (Beverly, MA, USA). Antibodies against Lamin A, β-Tubulin and β-Actin were products of Santa Cruz Biotechnology (USA). Normal mouse and rabbit IgG-HRB secondary antibodies were purchased from Santa Cruz Biotechnology.

2.2 Cell Culture

In this study, different human HCC cell lines (Huh7, HepG2, SMMC-7721, and BEL-7402) were purchased from Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). The human HCC cells were cultured in Dulbecco's Modified Eagle Medium (Gibco, Carlsbad, CA, USA) with 10% (v/v) heat-inactivated fetal bovine serum (Gibco, Paisley, Scotland), 100U/ml streptomycin and 100 U/ml penicillin at 37°C with 5% CO₂. Primary tumor cells were isolated from tumor tissues, cultured with the special medium for primary human liver cancer culture medium (iCell Bioscience Inc, Shanghai) with 10% fetal bovine serum.

Cells were maintained at 37 °C in a humidified incubator containing 20% O₂, 5% CO₂ and 75% N₂ in normoxia. The hypoxic condition was achieved at 37 °C with a gas mixture containing 1% O₂, 94% N₂ and 5% CO₂ in a humidified atmosphere.

2.3 Clinical Samples

Fresh primary liver cancer tissue and adjacent nontumor liver tissue samples were obtained from HCC patients undergoing hepatectomy, hepatic tumor ablation or percutaneous transhepatic biopsy and similar liver surgery at Jiangsu Cancer Hospital and NanJing Drum Tower Hospital.

2.4 PDX (Patient-Derived tumor Xenograft) Model and Sorafenib- Resistance Model in vivo

PDX model is constructed by transplantation of tumor tissue from patients into severe NSG immunodeficiency mice, 5-6-week-old NSG mice were purchased from Vital River Laboratory Animal
Technology (Beijing, China). Liver cancer samples are inserted into the mice's armpit, then fed with normal feed and drinking water. As shown in Supplement Data 1A, when the tumor size reached 400-500mm$^3$, tumor tissues are stripped and inoculated into another immunodeficiency mice, which is the first generation. For the inducing resistant group, when the tumor size reached 100mm$^3$, the mice were given an oral dosage of Sorafenib 80 mg/kg, once a day. At the fourth generation, the Sorafenib resistance was detected obviously. Data in Supplement Data 1B showed the successive processes of Sorafenib inducing resistance. The sensitive primary cells were extracted from the tumors of non-treated group. The resistant primary cells were extracted from the tumors of the Sorafenib induced resistant group. The drug resistance was tested by MTT assay (Supplement Data 1C), flowcytometry (Supplement Data 1D), and trypan blue staining (Supplement Data 1E). The morphology of primary resistant cells was also observed by Transmission Electron Microscope. Sorafenib could not induce cell death including necroptosis in primary resistant cells (Supplement Data 1F).

2.5 Animal Studies

5-6-week-old female BALB/c nude mice were purchased from SLAC Laboratory (Shanghai, China). The transplanted tumors were induced by subcutaneous injection into the flanks of the mice with $3.0 \times 10^6/0.1\text{ml}$ HepG2, Huh7 cells or primary tumor cells. A couple of days later, the tumor volume was measured by micrometer calipers, according to the size of the tumor, the mice were average divided into groups. And mice were treated with 60 mg/kg Sorafenib every 2 days orally. Nec (1.65mg/kg) and 17-AAG·25mg/kg·was administered intra-peritonelly twice a week. 3 weeks later, the mice were killed, and the tumor xenografts were removed and measured. Tumor volume (TV) was calculated using the following formula: $TV (\text{mm}^3) = D/2 \times d^2$, where D is the longest diameter while d is the shortest diameters.

All experiment animals were raised in air-conditioned rooms under controlled lighting (12 h light/day) and provided with food and water at discretion. Animal care and surgery protocols are approved by the Animal Care Committee of China Pharmaceutical University. All animals are treated and used in a scientifically valid and ethical manner.

2.6 Western Blot Analysis

Cells were washed with cold PBS and lysed in RIPA Lysis buffer (ThermoFisher, USA) containing protease/phosphatase inhibitors. After lysates concentration was determined by BCA protein assay (Pierce, Rockford, IL, USA), an equal amount of denatured proteins was subjected to SDS-PAGE gel electrophoresis and then transferred onto a nitrocellulose membrane (BioTrace NT, PallCor, U.S.A.), which was blocked by 5% non-fat milk in PBS, following by incubation at 4 °C with specific primary antibodies overnight. Then, membranes were incubated with HRP goat anti-rabbit immunoglobulin G (IgG; H + L) or anti-mouse IgG (H + L) secondary antibody (Biosharp) for 1 h and finally visualized with chemiluminescence (ThermoFisher). Immunoreactive protein was detected with Tanon-5200 Multi Automatic chemiluminescence/fluorescence image analysis system (Tanon-5200, Tanon).
2.7 Immunohistochemistry (IHC)

Paraffin embedding sections were heated at 60°C for half an hour and dewaxed. For IHC assay, tissue sections were incubated with 0.3% Triton-X 100 for 20 minutes after antigen retrieval. Then, the solution containing goat serum was used to block nonspecific binding sites. Next, primary antibodies were added and incubated at 4°C overnight. Before interacting with DAB solution, tissues were incubated with biotin-labeled secondary antibody at room temperature for 30 minutes. Finally, tissues were stained with hematoxylin and covered by neutral gum, all the process was performed with standard techniques.

2.8 Immunofluorescence Assay

The cells were seeded onto cover glasses in a 6-well plate and fixed by 4% PFA for 15 min, followed by permeabilization using 0.3% Triton-X 100 for 15 min. Then, the cover glasses were blocked by 3% bovine serum albumin for 1 hr and incubated with primary antibody at 4°C overnight. Then cells were incubated with Alexa Fluor-conjugated secondary antibodies (1:200, Invitrogen, CA, USA, M30101, L42001) for 1 hr and stained by DAPI for 20 min. The confocal microscope was employed to photograph the protein expressions or location in the cells. Various organelle fluorescent probes such as ER-Tracker (KeyGen Biotech, China), Mito-Red (KeyGen Biotech, China), were incubated before paraformaldehyde fixed and next steps were same as the above mentioned. After 30 min incubation at 37°C in the dark, coverslips were fixed in 4% paraformaldehyde for 30 min. The images were captured with the Olympus FV1000 confocal microscope.

2.9 Cell Transfection

HIF1-α siRNA was purchased from Santa Cruz Biotechnology. SiRNA transfections were performed according to the manufacturer's instructions using Lipofectamine 2000 reagent (Invitrogen). After that, the transfected system was removed, and it as not until 24 h cultured in normal media were the cells used for further experiment.

To establish HSP90α-knockdown cells, 293T cells were transfected with lentivirus plasmid together with psPAX2 and pMD2G packing plasmids by X-treme GENE 9 (Roche) for 12 hr. Fresh medium was added to 293T cells to replace the previous medium. Then, the supernatants of 293T cells were collected and mixed with fresh medium to infect tumor primary cells along with 8 µg/ml polybrene. Next day, supernatants were replaced with fresh medium containing 2 µg/ml puromycin. Maintain 2 µg/ml concentration for more than one week and the puromycin-resistant cells were isolated and used for further experiments.

2.10 Cytoplasmic and Nuclear Protein Extraction

The cells were normally collected, and the cells were added with buffer A and mixed evenly. After that, the cells were cracked on the ice for 15 minutes, and the cells were mixed with intermittent shocks. At 4°C, 13000rpm, 5min, the transferred supernatant was stored as the cytoplasmic part, washed three times with buffer A, and after washing the residual, the transferred supernatant was lysed with buffer B for
10min, at 4 °C, 13000rpm, 10min, the transferred supernatant was the nuclear component, protein concentration was measured by BCA, and after protein denaturation then stored with -20°C or subsequent WB experiments.

2.11 Co-Immunoprecipitation (Co-IP)

The nuclear extracts were incubated with 1 μg control anti-IgG and

20 μl of Protein A/G PLUS-Agarose (Santa Cruz) at 4°C for 30 min. After eliminating beads by centrifugation at 2,500 rpm, 10 μl primary antibody was incubated with nuclear extracts at 4°C for 1 hr and 20 μl beads were added to rotate at 4°C overnight. Next day, samples were centrifuged at 2,500 rpm for 5 min at 4°C, and then the supernatants were carefully discarded. PBS was used to wash pellet three times, and the samples were mixed with 20 μl 2× loading buffer and boiled for 8 min. Finally, samples were analyzed by SDS-PAGE.

2.12 Statistical Analyses

The data shown in the study were expressed as means ± standard errors (SEM) from at least 3 independent experiments, each in triplicate samples for individual treatment or dosage. Statistical analyses were performed using ANOVA coupled with a post hoc test.

3. Results

3.1 Sorafenib Induced HCC Necroptosis.

Soft agar assay (Fig. 1A) and Flow cytometry (Fig. 1B) were used to detect the inhibitory effect of Sorafenib on HCC cell lines. Results suggested that after being treated with 10 μM Sorafenib for 24 hours, HepG2 and Huh7 cells growth was decreased to 9.5% and 16% compared to control group respectively. The dead cells were increased to 35.1% and 18.1% respectively. Fig. 1C-D showed the morphological changes of HepG2 cells by Live Cell Imaging System and Transmission Electron Microscope. We can see cell experienced normal mitotic proliferation in control group. While in Sorafenib treated group, cells suffered a blocked proliferation, cellular content aggregates, and eventually swelled until dead (Fig. 1C). More accurate views of various changes in cell death patterns were investigated by Transmission Electron Microscope. As shown in Fig. 1D, healthy state of liver cancer cells was presented in the control group. Picture No.1 showed the complete organelles like mitochondria and it has complete cell membrane structures. However, Sorafenib induced cells death including necroptosis, apoptosis, and autophagic death, etc. In Picture No.2, cell membrane and organelle destroyed were shown in Sorafenib treated group. Cell swelled, cell content was released, the morphology of the cell main part presented necrosis like. We also observed apoptotic body in Picture No.3 and autophagosomes in Picture No.4. In Fig. 1E-F, the expression of necroptosis marker proteins such as RIPK1, RIPK3, and MLKL were detected by western blot and immunofluorescence. Results showed that RIPK1, RIPK3, and MLKL were increased in treated cells, which suggested Sorafenib activated necroptosis pathway strongly. Also, necroptosis
Marker proteins were detected in tumor tissues of PDX model. WB (Fig. 1G) and IHC (Fig. 1H) results showed that Sorafenib induced necroptosis in vivo as well. Besides, HepG2 cells xenograft model was used to confirm the results (Supplement data 2A-B). All the results indicated that Sorafenib induced HCC necroptosis in vitro and in vivo.

Apoptotic cell death involves the engagement on pathways that result in the activation of caspase proteases that ultimately cause the morphological features of cell death. In contrast, necroptosis was recognized as a caspase-independent cell death that can be triggered by tumor necrosis factor (TNF) in the presence of a pan-caspase inhibitor such as zVAD-fluoromethylketone (VAD). To detect the necroptosis inducing effect of Sorafenib, Nec, a specific inhibitor of RIPK1 kinase was used. As shown in Fig. 1I, Sorafenib induced obvious cell death even when VAD exists, and the effect was reversed by Nec, which suggested that the necroptosis inducing effect of Sorafenib on HCC cells was apoptosis independent. The morphological changes of HepG2 cells under Sorafenib were observed by Live Cell Imaging System (Fig. 1J). Transmission Electron Microscope showed the similar results (Supplement Data 2C). In HepG2 xenograft model, Sorafenib inhibited tumor growth significantly, while Nec weakened the effect (Supplement Data 2D-E). All the results showed that Sorafenib induced hepatocellular carcinoma death partly via the necroptosis pathway in vivo and in vitro.

3.2 Hypoxia Contributed to HCC Resistance to Sorafenib.

Hypoxia can promote proliferation, invasion, metastasis, apoptosis, drug resistance and other malignant biological behaviors of tumor cells. The IC50 values of Sorafenib under normoxia and hypoxia were tested (Fig. 2A). In HepG2 cells, the IC50 was 12.8 μM under normoxia and 194.2 μM under hypoxia. In Huh7 cells, the values were 11.1 μM and 34.7 μM. Clone formation experiment also confirmed that hypoxia can induce Sorafenib resistant under hypoxia (Fig. 2B). And results of trypan blue staining also showed that cell death induced by Sorafenib was significantly reduced under hypoxia condition (Supplement Data 2F). WB results proved that necroptosis pathway induced by Sorafenib was attenuated under hypoxia (Fig. 2C). The results of Co-IP also revealed that the capacity of RIPK1 binding with RIPK3/MLKL was upregulated in normoxia and decreased in hypoxia microenvironment after Sorafenib treatment, which indicated hypoxia reduced the activation necroptosis pathway of Sorafenib (Fig. 2D).

HIF1α (hypoxia inducible factor-1) is a key regulator of many signals in tumor occurrence, development, and chemotherapy resistance. In order to figure out the effect of HIF1α on Sorafenib resistance, HIF1-α siRNA was used. Soft Agar Cloning experiment showed that HIF1α knockdown can enhance the inhibitory effect of Sorafenib (Fig. 2E). Results in Fig. 2F showed that RIPK3 and MLKL in HepG2 cells were upregulated by Sorafenib with HIF1α being silenced under hypoxia. In PDX model (Fig. 2G), HIF1α was much more expressed in internal hypoxia tumor tissue than in external normoxia tumor. And Sorafenib could not activate the necroptosis pathway in tumor internal area, which means hypoxia blocked the necroptosis induced by Sorafenib in vivo.

3.3 Hypoxia Impeded the Distribution of RIPK1/RIPK3/MLKL Complex in Cytoplasm.
Necroptosis key protein MLKL usually acts on lipid and cell membrane structures, causing membrane damage and eventual leading cell death [32,33]. In order to figure out the mechanisms of Sorafenib resistance under hypoxia, the changes of MLKL expression and the complex of RIPK1/RIPK3/MLKL in cytoplasm were studied. In Fig. 3A, under normoxia, RIPK1, RIPK3, and MLKL were increased by Sorafenib in cytoplasm. However, under hypoxia, necroptosis related proteins were down-regulated in cytoplasm. The RIPK1/RIPK3/MLKL complex was also detected by Co-IP (Fig. 3B). Results showed that in cytoplasm, Sorafenib increased RIPK1/RIPK3/MLKL complex under normoxia and had no obvious effect under hypoxia. Therefore, Sorafenib could not increase necrosome in cytoplasm, which might be an important reason for Sorafenib resistance under hypoxia. In this study, Sorafenib-induced necroptosis destroy the membrane structures in hepatocellular carcinoma cells. Sequently we detected the distribution of MLKL in several kinds of organelles intracellular which has abundant membranous structure, such as cell membrane, mitochondria, endoplasmic reticulum, lysosomes. As shown in Fig. 3C-F, MLKL increased after Sorafenib treatment in normoxia, and located in various organelles. However, the location was decreased in organelles and increased in nucleus under hypoxia. Similar results were observed in primary tumor cells (Supplement Data 3A-D). All the results indicated that MLKL was strongly located in kinds of organelles which has a membranous structure in the process of Sorafenib induced necroptosis, then the damaged membranes finally lead cell death. Hypoxia reduced MLKL expression in organelles, which might be one of the main causes of why necroptosis induced by Sorafenib was attenuated under hypoxia.

3.4 HSP90α Promotes Chaperone-Mediated Autophagy (CMA) Degradation by directly binding to MLKL in hypoxia.

To investigate whether hypoxia could affect the necroptosis protein degradation under Sorafenib treatment, protein synthesis inhibitor CHX was performed. As shown in Fig. 4A, MLKL did not change when CHX was used in normoxia. While under hypoxia, MLKL was time-dependently decreased by Sorafenib. It seemed MLKL was more susceptible to degrade under hypoxia than under nomoxia when cells co-cultured with Sorafenib. Generally, there are three main ways of protein degradation: proteasome pathway, caspase pathway, and autophagy-lysosome pathway. To verify which pathway contributes to the MLKL degradation under hypoxia, proteasome inhibitor MG132, caspase inhibitor z-VAD-fmk, and autophagy inhibitor chloroquine were used. As shown in Fig. 4A and Supplement Data 3E, rather than MG132 or z-VAD-fmk, the chloroquine abrogated the degradation of MLKL, indicating that autophagy-lysosome pathway was the main degradation of MLKL in hypoxia microenvironment.

Chaperone-mediated autophagy (CMA) is a lysosomal pathway of proteolysis that is responsible for the degradation of 30% of cytosolic proteins[32,33]. HSP70 HSP90 is the most essential component for protein transport across the lysosomal membrane in process of CMA[34, 35]. They can not only locate the substrate protein but also recognition of substrate protein. HSP90α can also formed a polymer structure on the membrane of lysosome cavity side to maintain the stability of transport complex. LAMP2/HSP70/HSP90α complex is the marker and finally identified as the lysosomal membrane receptor of CMA pathway to combine and transport substrate proteins. In Fig. 4B-D, we observed
lysosomes with lyso-tracker and detected the expression of HSP70, LAMP2, and HSP90α by immunofluorescence. Notably, comparing to normoxia, HSP70, LAMP2, and HSP90α were strikingly increased under hypoxia and located in lysosomes. Similarly, WB results indicated that HSP70, LAMP2, and HSP90α were high expressed under hypoxia (Fig. 4E). Furthermore, the capacity of HSP90α binding with RIPK3 and MLKL was much more in hypoxia than that in normoxia (Fig. 4F). The HSP70/HSP90α/LAMP2/MLKL complex was also detected by Co-IP. We tested the capacity of MLKL binding with HSP70/HSP90α/LAMP2 was increased obviously under hypoxia condition in HepG2 cells (Fig. 4G). The similar results were found in primary tumor cells (Fig. 4H). Besides, the capacity of LAMP2 binding with HSP70/HSP90α/MLKL was increased in hypoxia. LAMP2 and HSP90α were also highly expressed in tumor internal hypoxia area compare to normoxia tumor area in vivo, however the expression of MLKL was decreased in hypoxia (Supplement Data 3F). Collectively, MLKL as a customer, was recognized by HSP70/HSP90α/LAMP2 transporter and transported into lysosome then degraded in the end, all these results suggested that MLKL could be degraded under hypoxia through chaperone-mediated autophagy (CMA) degradation pathway.

To test the role of HSP90α in necroptosis pathway, siRNA was used. As shown in Fig. 4I, loss of HIF1α inhibited HSP90α, while loss of HSP90α did not change HIF1α expression. Besides, both inhibition of HIF1α and HSP90α can promote MLKL recoverd under hypoxia. In Fig. 4J, necroptosis related proteins was down-regulated obviously and HSP90α was highly expressed in primary resistant cells, which suggested necroptosis was dull in Sorafenib-resistant tumor cells. Taken together, these results indicated that HSP90α played a direct and important role in Sorafenib resistance by blocking necroptosis.

3.5 17-AAG Combining with Sorafenib Enhanced Necroptosis Pathway in vitro.

In order to study whether the inhibitory of HSP90α can improve the necroptosis induced by Sorafenib, 17-AAG, an inhibitor of HSP90α, was used. As shown in Fig. 5A, when combining with 17-AAG, the PI-positive cells were increased to 30.5% and 30.45% respectively in HepG2 and Huh7. While in Sorafenib group, only about 4.65% and 9.03% of cells death were increased in hypoxia. MTT results in Fig.5B also showed that hepatoma cell lines were insensitive to Sorafenib in hypoxia, the survival of HepG2 and Huh7 was 32.3% and 9.5% respectively when 17-AAG combining with Sorafenib. In general, the inhibitory effect of Sorafenib was increased significantly under hypoxia. In Fig. 5C, 17-AAG decreased HSP90α and safeguard necroptosis induced by Sorafenib. Immunofluorescence presented the similar phenomenon in HCC cell lines (Fig. 5D). All the results revealed that 17-AAG reversed the Sorafenib resistance in hypoxia and enhanced the inhibitory effect of Sorafenib on HCC.

3.6 HSP90α Could be an Important Target in Sorafenib Resistance in vivo.

To further verify the pivotal role of HSP90α in Sorafenib Resistance in vivo, HSP90α knockdown primary cell line was established. In the Sorafenib resistant xenograft model, we tested the effect of 17-AAG combined with Sorafenib and HSP90α knockdown combined with Sorafenib, the results indicated that Sorafenib reduced the tumor growth most significantly when HSP90α was inhibited (Fig. 6A-C). Compared to the control group, the tumor weight has also been significantly reduced to 57% and 48% in
the 17-AAG combined Sorafenib group and Sorafenib combined HSP90α knockdown group respectively. While the Sorafenib alone has poor efficacy in resistant tumor. The expression of HIF1α/HSP90α and RIPK3/MLKL were also detected by immunofluorescence and IHC tissue sections (Fig. 6D-E). Necroptosis related proteins were low expressed in resistant tumors and HIF1α/HSP90α was high expressed. However, in ShHSP90α group, necroptosis induced by Sorafenib was surprisingly reactivated. All these results indicated that HSP90α can be a useful target of Sorafenib resistance therapy via recovering the necroptosis pathway in liver cancer.

3.7 Clinical Analysis of HIF1α/HSP90α as a Therapeutic Target.

Results of Fig. 7A showed that the overall survival of HSP90α, HIF-1α, RIPK1 and RIPK3 in Kaplan-Meier Plotter. Database showed the higher HIF1α/HSP90α expression, the worse of patient prognosis. And HIF1α/HSP90α showed positive correlation in GEPIA (Gene Expression Profiling Interactive Analysis) database (Fig. 7B). The expression of HSP90α and HIF1α was stronger in cancer tissues than liver tissues, and RIPK3 was opposite. RIPK1 may get closed to cancer development (Fig. 7C). We also analyzed the samples of patients with liver cancer by IHC and WB in Fig. 7D-F, the similar results were found. All these results suggested that HSP90α would be a promising and useful target in hepatocellular carcinoma therapy.

4. Discussion

At present, effective treatment of liver cancer is still very limit, the recurrence rate of liver cancer is still high. Sorafenib remains the only FDA-approved systemic drug for patients with advanced HCC. Long-term exposure to Sorafenib often results in drug resistance. The understanding of the mechanism of Sorafenib resistance will enable us to use it more effectively in clinic.

Here, we demonstrated necroptosis would be one of the main targets of Sorafenib on HCC. Necroptosis related proteins RIPK1, RIPK3 and MLKL were highly expressed in hepatocellular carcinoma cells and tumor tissues with Sorafenib treatment, which means Sorafenib activated the necroptosis pathway in vitro and in vivo. Hypoxia is a hallmark of solid tumors due to the rapid growth of cancer cells and the abnormal angiogenesis. Recent studies have confirmed that the adaptation of tumors to hypoxic microenvironment is not only to maintain the survival or growth of tumors but also play an important role in drug resistance. In the present study, we demonstrated that hypoxia rendered resistance to Sorafenib in human HCC cells by attenuating necroptosis. We observed that abundant HSP90α binds with necrosome directly, and MLKL was found degraded by autophagy lysosomal degradation pathway in hypoxia. Further study revealed that the expressions of HSP70/HSP90α/LAMP2/MLKL complex was increased under hypoxia and located in lysosomes. all our results presented that MLKL was degraded , which resulted in the decreasing of RIPK1/RIPK3/MLKL complex. Then necroptosis was interrupted and drug resistance appeared. Clinical data and patient samples also suggest that HSP90α expression in hepatocellular carcinoma was associated with the prognosis and Sorafenib resistance. The blockage of HSP90α can significantly overcome Sorafenib resistance under hypoxia in vitro and in vivo.
17-AAG, a derivative of geldanamycin, is currently undergoing clinical development as a novel anticancer agent for the treatment of human cancers. It has been reported that 17-AAG could be an effective anticancer drug whether used alone or in combination with other drugs[36, 37]. Here we found that 17-AAG decreased HSP90α, then safeguard the activated necroptosis fluently. 17-AAG combining with Sorafenib showed great inhibitory effect in HCC in vivo and in vitro.

**Conclusions**

Sorafenib induced necroptosis in liver cancer and HSP90α plays a critical role in Sorafenib resistance under hypoxia by blocking necroptosis. 17-AAG combining with Sorafenib is a promising therapy for hepatocellular carcinoma.

**Abbreviations**

HCC: Hepatocellular carcinoma

17-AAG: Demethoxygeldanamycin

IF: immunofluorescence

WB: Western Blot

IHC: immunohistochemistry

i.g.: intragastrically

Co-IP: Coimmunoprecipitation

CMA: chaperone-mediated autophagy

DAB: 3, 3’-diaminobenzidine

DAPI: 2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride

PI: Propidium Iodide

PBS: Phosphate-Buffered Saline

MTT: 3- (4, 5-Dimethylthiazol-2-yl) -2, 5-diphenyltetrazolium bromide

DMSO: dimethyl sulfoxide

NEC: necrostatin-1

VAD: Z-VAD-FMK
RIPK1: Receptor-interacting protein kinase 1
MLKL: mixed lineage kinase domain-like
RIPK3: receptor-interacting protein kinase-3
HSP90α: Heat shock proteins 90α
HIF1α: hypoxia inducible factor-1α
AFP: Alpha fetoprotein
LAMP2: Lysosomal-associated membrane protein 2
VEGF-R: vascular endothelial growth factor receptors
PDGF-R: platelet-derived growth factor receptor
PDX: Patient-Derived tumor Xenograft
GEPIA: Gene Expression Profiling Interactive Analysis

Declarations

Ethics approval and consent to participate: We have ethics approval to participate in related animal experiment. Animal care and surgery protocols are approved by the Animal Care Committee of China Pharmaceutical University.

Consent for publication: Not applicable.

Availability of data and materials: All data generated or analysed during this study are included in this article. All data are fully available without restriction on reasonable request.

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Authors' contributions: YL, Y Yang and LZ conceived the study. YL and Y Yang performed the experiments and collected the data. YL analyzed the data and drafted the manuscript. YL,Y Yang and LZ participated in the design of the study and performed the statistical analysis. YL, Y Yang, DP, YD, HZ, Y Ye, JL and LZ discussed the results. YL, Y Yang and LZ participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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**Figures**
Figure 1

Sorafenib Induced HCC Necroptosis. (A) The inhibitory effect of Sorafenib to Hepatocellular carcinoma cell lines by Soft Agar Assay. (B) Detection of PI-positive cells in Huh7 and HepG2 cells with Sorafenib treatment by Flow Cytometry. (C) To observed the morphological changes of HepG2 cells by Live Cell Imaging System. (D) The different types of death in HepG2 cells induced by Sorafinib observed by Transmission Electron Microscope. (E-F) The expressions of necroptosis pathway in hepatocellular...
carcinoma cell lines after Sorafenib treated detected by Western blot and Immunofluorescence. (G-H) The expression of RIPK1, RIPK3 and MLKL were detected in PDX-xenograft respectively by Western blot and IHC. (I) Detection of PI positive cells by flow cytometer to observe the dead cell when Nec and VAD exists. (J) Live cell Imaging System observed morphological changes in HepG2 cells when Nec and VAD exist.

**Figure 2**

Hypoxia Induced Hepatocellular Carcinoma Resistant to Sorafenib. (A) Inhibition of Sorafenib under normoxia and hypoxia assessed by MTT assay. (B) Inhibition of Sorafenib under normoxia and hypoxia assessed by Clone formation experiment. (C) The expression of necroptosis proteins in normoxia and hypoxia detected by Western Blot. (D) The binding capacity of the necroptosis complex under normoxia and hypoxia. (E-F) HIF1α effect on Sorafenib observed by Soft Agar Cloning experiment. (F) HIF1α effect on Sorafenib in necroptosis pathway detected WB. (G) Detected HIF1α and necroptosis protein in PDX-xenograft tumor tissues by WB.
Figure 3

Hypoxia Impeded the Distribution of RIPK1/RIPK3/MLKL Complex in Cytoplasm. (A) Distribution of necroptosis proteins in cytoplasmic and nuclear observed by WB. (B) Distribution of RIPK1/RIPK3/MLKL complex in cytoplasm by Co-IP. (C-F) Expression of MLKL on different organelles which has rich membrane structure such as cell membrane, mitochondria, endoplasmic reticulum, lysosome observed by Immunofluorescence.
Figure 4

HSP90α Promotes Chaperone-Mediated Autophagy (CMA) Degradation by directly binding to MLKL in hypoxia. (A) The degradation stability of MLKL detected by Western blot. (B-D) The expressions of LAMP2, HSP90α and HSP70 located in lysosomal observed by immunofluorescence. (E) The expression of LAMP2, HSP90α and HSP70 in hypoxia. (F) HSP90α binding with necroptosis protein. (G-H) The expression of LAMP2/HSP90α/HSP70/MLKL complex detected by Western blot in HepG2 and primary
tumor cells. (I) Silenced HIF-1α and HSP90α affected necroptosis in hypoxia. (J) Necroptosis pathway in primary resistant cells detected by Western blot.

Figure 5

17-AAG Combined with Sorafenib Treatment Enhanced Necroptosis Pathway in vitro. (A) Detection of death cells under hypoxia by Flow Cytometry. (B) The survival of liver cancer cells under hypoxia assessed by MTT assay. (C-D) The detection of HSP90α and MLKL by Western blot and Immunofluorescence.
HSP90α can be a Useful Target in Sorafenib Resistance Therapy in vivo. (A) Tumor volume was recorded in primary Sorafenib resistant xenograft model. (B) Tumor weight was recorded in primary Sorafenib resistant xenograft model. (C) Bioluminescence images of primary Sorafenib resistant xenograft model. (D-E) The expression of HIF1α, HSP90α and necroptosis pathway in tumor detected by IF and IHC.
Figure 7

Clinical Analysis of HIF1α/HSP90α as a Therapeutic Target. (A-B) The overall survival and correlation of several proteins in GEPIA (Gene Expression Profiling Interactive Analysis) database. (C) Expressions of HSP90α, HIF-1α, RIPK1 and RIPK3 in Kaplan-Meier Plotter database. (D) Protein expressions in tumor, para-carcinoma, and normal tissues of patient samples detected by IHC. (E) Analysis the expressions of
HSP90α, HIF-1α, RIPK1 and RIPK3 in patient sample. (F) Protein expressions in tumor, para-carcinoma, and normal tissues of patient samples detected by WB.

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