Sorafenib-Induced Exosome Secretion Promotes Chemotherapy Resistance in Hepatoma Cell Line

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1. Abstract

Hepatocellular Carcinoma (HCC) is a most common cause of cancer-related deaths globally. Traditional HCC treatments, including surgery, radiation therapy, and chemotherapy, could provide potential options; however, Sorafenib has been used for advanced HCC treatment. Unfortunately, most patients with advanced-stage HCC do not benefit from Sorafenib for its easy progression to resistance. Although numerous studies have addressed that mechanism of Sorafenib resistance, many questions remain. Exosomes are membrane vesicles secreted from diverse organisms, especially tumors, and they communicate with their surroundings. Studies have found that cancer cell-derived exosomes are important factors leading to HCC drug resistance. Our study demonstrated that Sorafenib could promote HCC release of exosomes by enhancing Rab27a activity. Thereby, the secreted exosome promoted the behavior of recipient hepatoma cells and activated the AKT signaling pathway, resulting in decreased sensitivity for chemotherapy. In addition, blocking Rab27a through knockdown of Rab27a expression or treatment with a Rab27a inhibitor (Nexinhib20) significantly increased sensitivity of hepatoma cells to Sorafenib. Our study provides novel insight into the mechanism of Sorafenib-induced chemoresistance in exosomes derived from Hepatoma cells.

2. Introduction

Currently, liver cancer is the fourth major cause of cancer-related deaths around the world. Hepatocellular carcinoma (HCC) accounts for 75-85% of primary liver [1]. Traditional HCC treatment, including surgery, radiofrequency ablation(RFA)and chemotherapy, could provide potential clinical cures; however, patients still experience many limitations. As such, alternative therapeutic strategies must be further developed. Sorafenib is a multi-target inhibitor, taken orally, that inhibits kinases, including Ras/Raf kinase to exert anti-proliferative effects and vascular endothelial growth factor receptor (VEGFR) for anti-angiogenic effects [2]. Sorafenib is first approved systemic treatment choice in patients with advanced HCC. Although the treatment has shown a significant benefit in advanced HCC, only approximately 30% patient benefits from this therapy due to a high resistance rate [3, 4]. Therefore, a better understanding of the mechanism about acquired resistance is urgent to overcome tumor progression and improve the efficiency of Sorafenib.

Exosomes are bilayer membrane vesicles range between 50–150 nm. The exosome is released from intracellular multivesicular bodies (MVBs) which fused with plasma membrane into the extracellular space by almost all cells, especially cancer cells [5]. Exosomes play an essential role in cell-to-cell communication in multicellular organisms, even outside the tumor microenvironment. Exosome cargo can carry a great number of components, including proteins, nucleic acids, and lipids [6]. It has been reported that exosome release is regulated by ESCRT and ESCRT-independent pathways, such as the small GTPase protein family. Rab27a is a well-studied small GTPase family member, which play an essential role in exosome secretion [7].

Studies have found that exosomes derived from tumor cells are risk factors leading to HCC drug resistance [8, 9]. The potential positively association between exosomes and drug resistance has provided important insights into a promising new strategy for HCC chemoresistance. Our study demonstrated that Sorafenib can promote HCC release of exosomes by increasing Rab27a activity. The HCC-derived exosomes were induced by Sorafenib, thereby reducing the sensitivity of recipient HCC cells to Sorafenib.

3. Materials and Methods

3.1. Cell Culture, Reagents, and Plasmids

The human hepatoma cell line Huh7 was preserved in our laboratory as described previously [10]. Huh7 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco), 10% Fetal Bovine Serum (FBS; Gibco Laboratories) and 1% penicillin/streptomycin at 37°C under 5% CO_2 incubator. Sorafenib was obtained from Santa Cruz Biotechnology (CAS 284461-73-0). Nexinhib20 was purchased from Tocris (#6089). Plasmids pGEX-4T-1 vector (GST) and GST-JFC1 were cloned by TSINGKE Bio Tec. Rab27a and Control siRNA pairs were obtained from Santa Cruz Biotechnology (sc-41834).

3.2. Cell Proliferation Assay and Colony Formation

Cells were seeded onto 96-well dishes at a density of $5–8 \times 10^3$ cells/well. On the following day, with different treatments, each well was added with CCK8 reagent (# K1018, APExBio) and incubated for 2 h at 37°C. The cell proliferation status was determined by measuring the absorbance at 450 nm using a Microplate reader (Bio-Tek, USA). To perform colony formation, 500-600 cells were plated to each 6-well dish. After 14-day incubation with different treatment, the colonies were fixed with paraformaldehyde, stained with crystal violet, and imaged using a Leica Microscope (Germany).

3.3. Exosome Purification and Characterization

Exosome secreted by hepatoma cells were isolated, as previously reported [11]. Briefly, conditioned medium was collected and transferred to 50ml tubes to remove the cell debris by centrifugation at $2000 \times$ rpm for 20 min. Then the supernatants were harvested and centrifuged at $10,000 \times$ g for 40 min to discard the macrovesicles. Exosomes were collected by ultracentrifugation at $120,000 \times$ g for 2 h and washed in PBS under the same ultracentrifugation conditions. Pelleted exosome was re-suspended in PBS and characterized by Transmission electronic microscope (TEM) (Hitachi, Tokyo, Japan), Nanoparticle tracking analysis (NTA) by Zeta View and immunoblot analysis.

3.4. Immunoblotting and Antibody

For immunoblot analysis, cells or exosome pellets were lysed by cell lysis buffer with a protease inhibitor (Roche). The protein measurement was using the Bradford protein assay. Equal amounts of extracts were separated by 12% SDS–PAGE gel, blotted onto the PVDF membrane, blocked with 5% bovine serum albumin (BSA) for 1-2 h at room temperature, and incubated overnight with primary antibody. Anti-CD63 (ab134045) and anti-CD9 (ab92726) antibodies were purchased from Abcam. Anti-Tsg101 (sc-136111), anti-Rab27 (sc-136995), and anti-Gapdh (sc-47724) antibodies were purchased from Santa Cruz Biotechnology. Anti-Akt (#4685), anti-P38(#9212), and anti- ribosomal protein S6 kinase (#9202) antibodies were obtained from Cell Signal Technology.

3.5. GST Pull-Down Assay

GST and GST-JFC1 fusion protein were purified as described previously [12]. For the GST pull-down assay, the purified protein was verified by Ponceau staining. Appropriate amounts of purified GST-tagged proteins were incubated with different cell lysates at 4⁰C overnight. The resins were washed three times with cold wash buffer. Resin-bound complexes were eluted and boiled for immunoblot analysis.

3.6. PKH67 Staining

Cells were pre-seeded in 6-well plates with glass slides at a density of 30–40% confluence. To fluorescently label exosomes, suspended exosomes were incubated with a green fluorescent dye (PHK67, Sigma) following the manufacturer's instructions. On the next day, the cells were treated with labeled exosomes for an additional 6 h. After washing with PBS three times, plates were fixed with 4% PFA for 10 min. Finally, cells were stained with DAPI (C3362, Apex BIO), and images were visualized by fluorescence Microscope (DMI6000B, Germany).

3.7. Wound Healing and Migration Assay

To assess the migration ability of hepatoma cell line. Cells were seeded onto 6-well plates to reach 70–80% confluence. The scratch was conducted as previous described [13]. Following, the plate was washed with PBS to remove debris and incubation for 48 h. Images were taken every 24 h, and the gap distance was measured. Further, 100 μL of cell suspension solution (at the density of $1 \times 10^6/$) was plated without serum on upper chamber of a Transwell insert. Subsequently, 500 μL of the cell culture medium was added with serum into the bottom chamber of the plate. After incubation for 24 h at 37°C, the cells were washed twice with 1× PBS. The Transwell insert was removed from the plate and the remaining cells on top of the membrane were gently removed. Finally, the cells were fixed with 4% PFA for 10 min and stained with crystal violet.

3.8. Statistics Analysis

All data are expressed as the mean ± Standard Deviation (SD). The difference between groups was determined by Student's T-test using GraphPad prism version8 (GraphPad Software, USA). All experiments were repeated for three times.

4. Results

4.1. Exosome Isolation and Characterization

Exosome were isolated from condition medium by differential centrifugation. The morphology and size of isolated exosome from Huh7 cell culture medium was detected by transmission electron mi-

croscopy. (Figure 1A) presents the round-shaped membrane vesicle, with a size of approximately 100 nm. To further determine the exosome characteristics, Nano-tracking analysis was used to detect the distribution and size of extracellular vesicles. As shown in (Figure 1B), the distribution of exosomes was around 50–150 nm. Western blotting was used to verify the presence of exosome markers. Compared to the medium control, exosomes were positive for CD63, CD9, and TSG101 markers (Figure 1C).
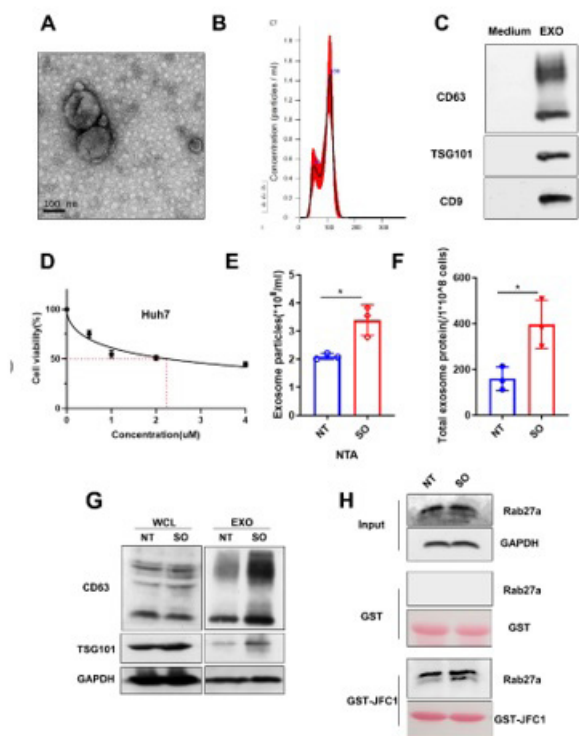
Figure 1: Sorafenib increases exosome secretion by activating Rab27a. A: Isolated exosome was determined by transmission electron microscopy, presenting as a round-shaped membrane vesicle, with a size of around 100 nm. B: The distribution and size of exosome was verified by Nano-tracking analysis. C: Exosomes were lysed for immunoblotting analysis. Exosome showing positive for anti-CD63, anti-TSG101, and anti-CD9.D: Huh7 cells were treated with Sorafenib at different concentrations for 48 h. IC50 was determined as 2. 24uM.E: Exosome particles quantified by NTA method in Sorafenib and Control groups. F: Exosome total proteins were quantified. G: Exosome pellets were analyzed by immunoblotting with exosome markers. H: The purified GST-JFC1 co-precipitated with Rab27a from Huh7 cell lysates with or without Sorafenib treatment. The purified GST and GST-JFC1 proteins are stained with Ponceau S. NT: non-treatment. SO: Sorafenib. *: P<0.05, **: P<0.01

4.2. Sorafenib Increases Exosome Secretion by Activating Rab27a

Under stress conditions, cancer cell-derived exosomes contribute much to the tumor environment. It has been shown that exosomes secreted by chemotherapy-induced breast cancer were positively associated with tumor progression [14]. Sorafenib is the first line systemic chemotherapy of HCC. However, the response of Sorafenib is

limited due to its easy progression to tolerance in patients. Exosomes have been reported that can carry and transfer various contents to promote acquired resistance in cancer cells [15]. In our study, we addressed the influence of exosome secretion by Sorafenib. First, we explored the effectivity of hepatoma cell line (Huh7) with Sorafenib treatment for 48 h. Further, different concentrations (0, 0.5, 1.0, 2.0, 4.0 μM) of Sorafenib were used to determine the IC_{50}. As shown in (Figure 1D), the IC_{50} was determined as 2.24 μM. Therefore, we chose 2.0 μM for subsequent experiments. Exosomes were isolated from the condition medium of Sorafenib- treated or Control Huh7 cells. From the NTA result (Figures 1E and 1F), as well as total protein measurement, Sorafenib treatment significantly increased exosome secretion. The band of exosome marker (CD63, TSG101) was more intense in the Sorafenib group than the control group (Figure 1G). Rab27a, a member of the small GTPases family, is a well reported protein that can regulate exosome release [16]. However, we did not observe changes in Rab27a protein level upon Sorafenib treatment. JFC-1, a Rab27a-binding protein, is a downstream effector of Rab27a [17] Therefore, we constructed the truncated JFC1 plasmid containing the Rab-binding domain, which can directly represent Rab27a activity. As shown in GST Pull-down assay (Figure 1H), Rab27a activity increased after Sorafenib treatment, indicating that Sorafenib can induce exosome release by activating Rab27a.

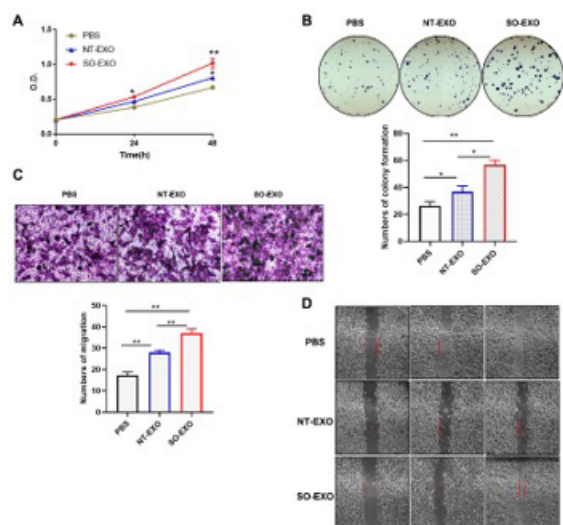### 4.3. Sorafenib-Induced Exosomes Promote Growth and Migration of Huh7 Cells

Previous study has shown that the cargo of cancer-secreted exosomes may mediate dynamic communication between cancer cells in response to environmental changes, thereby contributing to cancer progression [18]. We addressed the hypothesis that the behaviors of neighboring tumor cells would be altered by cancer cell-derived exosomes under Sorafenib treatment. First, we explored whether exosome secretion induced by Sorafenib treatment could stimulate tumor cell growth. As shown in (Figure 2A), Sorafenib-induced exosomes stimulated proliferation of Huh7 cells, as compared to the control group. As expected, the colony formation assay showed a consistent result (Figure 2B). Subsequently, we tested the potential function of exosomes on recipient Huh7 cell migration. As shown in (Figures 2C and 2D), co-culture with Sorafenib-induced exosomes enhanced recipient Huh7 migration, while exosomes derived from cancer cells showed slight differences compared to the control group. The data suggested that Sorafenib-induced exosomes can promote growth and migration of recipient Huh7 cells.

4.4. Exosome Can be Uptaken by Neighboring Tumor Cells and Activate ATK Signaling Pathway

Exosomes possess a phospholipid bilayer membrane. To evaluate whether secreted exosomes can be taken up by recipient cells, exosomes from Huh7 cells were collected and incubated with PKH67. PKH67-labeled exosomes were added to the culture medium of Huh7 cells for 6 h, staining was detected by microscope (Figure 3A),

suggesting that exosomes were taken up by recipient cells. One of the explanations for the mechanism of Sorafenib resistance in HCC is the AKT activation, thereby resulting in its downstream factors activation, including ribosomal protein S6 kinase and MAPK p38 signal. In our study, we determined whether exosome induced by

Sorafenib could activate AKT signaling pathway in recipient cells. As shown in (Figure 3B), Akt and its downstream S6 kinase were enhanced by Sorafenib treatment as compared to the control group. Therefore, the result indicates that exosomes induced by Sorafenib resulted in Sorafenib resistant.

Figure2: Sorafenib-induced exosome promotes growth and migration of Huh7 cells. **A:** Cell viability was evaluated by CCK8 assays. HCC cell-derived exosomes promote cell growth. B: HCC cell-derived exosomes promote cell proliferation by colony formation assay. Cell plates were treated with medium. Exosomes derived from Control- and Sorafenib-treated Huh7 cells. C and D: HCC cell-derived exosomes promote recipient cells migration. Cell plates were treated with medium, Exosomes derived from Control- and Sorafenib-treated cells. NT-EXO: exosome derived from non-treatment Huh7 cells. SO-EXO: exosome derived from Sorafenib-treated Huh7 cells *: P<0.05, **: P<0.01.
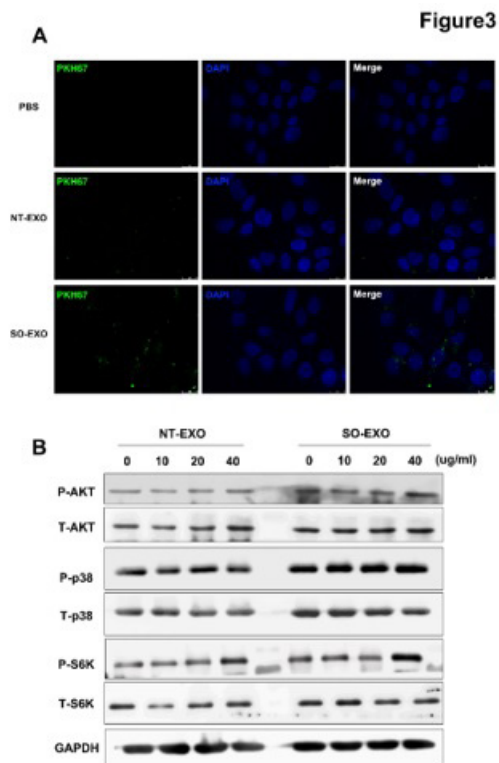

Figure3: Exosome derived from cancer cells can be uptaken by recipient cells and activates AKT signaling pathway. A. Exosomes were labeled with PKH67 and added to the culture medium of recipient Huh7 cells and incubated (40ug/ml) for 6 h. Staining was detected by microscope. B. Exosomes were harvested from Huh7 cells with or without Sorafenib treatment. The recipient cells were treated with different exosome concentration for 24 h. Cell lysates were collected for immunoblotting analysis.

4.5. Knock-Down or Inhibition of Rab27a Could Increase Sensitivity to Sorafenib

Rab27a is a known key factor that can regulate exosome release. We, therefore, determined whether inhibiting Rab27a in Huh7 cells reduced exosome secretion. As shown in (Figures 4A and 4B), knock-down of Rab27a expression resulted in a significant decrease of exosome secretion by NTA and immunoblotting method. In addition, Nexinhib20, a small molecule inhibitor, is designed to suppress the interaction between Rab27a and its effector JFC1. It has been reported that Nexinhib20 plays a major role in Rab27a and its effector-mediated neutrophil exocytosis [19]. In our study, we tested whether Nexinhib20 would inhibit exosome secretion. As expected, Nexinhib20 treatment suppressed exosome release (Figures 4C and

4D). As mentioned earlier, we determined that Sorafenib could induce exosome secretion by enhancing Rab27a activity, thereby stimulating neighboring tumor cell growth. Subsequently, we explored whether inhibition of Rab27a could increase Sorafenib sensitivity in Huh7 cells. As shown in (Figures 4E and 4F), the treatment of either Sorafenib or Rab27a knock-down slightly inhibited proliferation, while treatment with Sorafenib and siRab27a greatly suppressed the growth rate of Huh7 cells. Moreover, Nexinhib20 did not induce cell damage, even at higher concentration (10 μM), for 4 h [19]. We also found that Nexinhib20 treatment itself only slightly inhibited growth of Huh7 cells, while combined Sorafenib and Nexinhib20 greatly increased the inhibitory effect. Taken together, our result indicates that Rab27a inhibition could increase Sorafenib sensitivity in hepatoma cells.
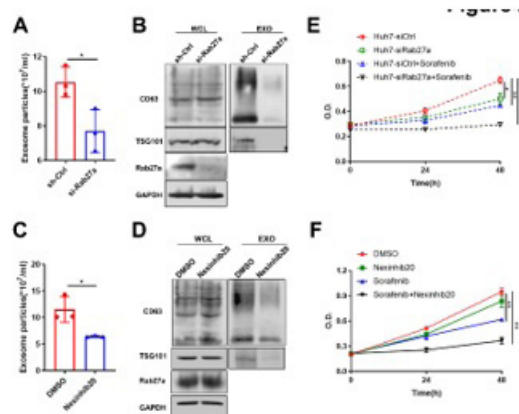
Figure4: Knock-down or inhibition of Rab27a increases Sorafenib sensitivity. A: Exosome particles quantified by NTA method in Rab27a siRNA and control groups. B: Collected exosome were subjected to immunoblotting from Rab27a siRNA cells or control cells culture medium. C: Exosome particles quantified by NTA method in Nexinhib20 and DMSO groups. D: Harvested exosomes from culture medium were subjected to immunoblotting after DMSO or Nexinhib20 treatment for 48 h. E: Cell viability was evaluated by CCK8 assays. Huh7 cells were treated with Sorafenib in Rab27a siRNA group or control group. F: Cell viability was evaluated by CCK8 assays. Huh7 cells were treated with Sorafenib combined with DMSO or Nexinhib20. *: P<0.05, **: P<0.01.

5. Discussion

In the past decades, a great number of kinase inhibitors such as Sorafenib have been adopted for HCC treatment [20]. However, most patients with advanced-stage HCC do not benefit from these therapies due to its limited response. As such, the deficiency motivates us to explore other potential strategies in HCC.

Exosomes can be secreted into the extracellular space and communicate with their surroundings. As a transport carrier, exosomes carrying a variety of biological molecules including proteins, mRNAs, and DNA, participate in the regulation of the tumor microenvironment [21, 22]. Exosomes derived from tumor cells carry specific cytokines, which have been shown to increase multiple myeloma cell proliferation and induce drug resistance to chemotherapy [23]. Studies have found that HCC cell-derived exosomes transfer exosome cargos may also contribute to the regulation of the tumor microenvironment [24].

Sorafenib seems to be an effective target drug in prolonging the

survival duration in HCC patients. Yet, it still causes resistance in many patients. Numerous studies have addressed that mechanism of Sorafenib resistance [25]. One explanation is primary resistance caused by tumor genetic heterogeneity. The other is acquired resistance, relevant to several signals pathways: PI3K-AKT activation, Autophagy, and tumor immune environment. Here, our result revealed that Sorafenib-induced exosomes can promote growth and migration of recipient Huh7 cells. A meaningful studies have revealed that docetaxel-resistant breast cancer cells may transmit resistance capacity to sensitive cells by secreted exosomes which containing specific contents [26]. Researchers also demonstrated that exosomal lncRNA, could act as mediator, involved in modulation of hepatoma cellular chemotherapeutic responses to Sorafenib [27]. We also show a mechanism through which Sorafenib-treated hepatoma cells, by secreting exosomes, communicate with nearby cancer cells to induce Sorafenib resistance. Our studies confirmed that HCC cell-derived exosomes induced by Sorafenib can reverse sensitivity to Sorafenib and activate the AKT signaling pathway in vitro. A study has previously report-

ed that treatment of recipient cells with HCC cell-derived exosomes result in key protein Akt activation [8], which is consistent with our result. Therefore, it is suggested that activated AKT by exosomes derived from Sorafenib-treated cancer cells may be important mechanisms underlying HCC resistance to Sorafenib.

A previous study has demonstrated that Rab27a is strongly relevant with HCC clinical pathology, providing evidence that Rab27a may function during tumor progression [28]. However, many unanswered questions remain, including how Rab27a participates in the progression of HCC. Rab27a is a member of Ras-like small GTPases family, which usually has two status: a GTP-bound active state and a GDP-bound inactive state [29]. Rab27a effectors specifically bind its GTP-bound active form to promote function in secretory pathways. Numerous studies revealed that Rab27a can promote various steps in membrane trafficking, including vesicle budding from the plasma membranes, vesicle transport, etc [30-32]. JFC1 was found to bind specifically to Rab27a as a Rab27a effector. Immunoprecipitation experiments using the Rab-binding domain of JFC1 co-localized with Rab27a exhibited Rab27a activity. Our result shows that Sorafenib treatment resulted in more JFC1-Rab27a binding, suggesting Sorafenib-mediated activation of Rab27a. In addition, blocking Rab27a by Rab27a knockdown or inhibitor Nexinhib20 treatment decreased exosome release. Both treatments in recipient cells reduced exosome secretion, thereby increasing sensitivity to Sorafenib. Our result revealed that Sorafenib resistance mediated by Sorafenib-induced HCC cell-derived exosomes is highly associated with Rab27a function. However, the role of exosomes and the carrier content in mediating Sorafenib resistance in HCC should be explored further.

The study offers novel insight into the mechanism by which exosomes derived from cancer cell treatment with Sorafenib influences the chemoresistance of Hepatoma cells. Further, this study highlights the therapeutic potential of combined Sorafenib and Nexinhib20 in HCC treatment.

6. Funding

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