Salvianolic Acid B Targets Mortalin and Inhibits the Migration and Invasion of Hepatocellular Carcinoma via RECK/STAT3 Pathway

Mengying Teng
Nanjing Medical University
Chunyan Hu
Nanjing Medical University
Qian Zhou
Nanjing Medical University
Bingmo Yang
Nanjing Medical University
Wei Xiao
Nanjing Medical University
Yuan Li
Nanjing Medical University
Zhong Li (lz-ny@njmu.edu.cn)
Nanjing Medical University https://orcid.org/0000-0001-5979-2776

Primary research

Keywords: hepatocellular carcinoma, mortalin, RECK, Salvianolic acid B, MMPs, invasion and migration.

Posted Date: June 11th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-594706/v1

Version of Record: A version of this preprint was published at Cancer Cell International on December 1st, 2021. See the published version at https://doi.org/10.1186/s12935-021-02367-z.

Abstract

1) Background: Tumor migration and invasion is a complex and diverse process, including epi-thelial-mesenchymal transition (EMT) of tumor cells, and degradation of extracellular matrix by matrix metalloproteases (MMPs). Mortalin is an important oncogene. It has been reported that it plays an important role in tumor migration and invasion through various signaling pathways; however, the underlying mechanism still needs to be further explored.

2) Methods: At present, we studied the role of mortalin in the migration of HCC cell lines HepG2 and HCCLM3 cells.

3) Results: Our results showed that overexpression of Mortalin in HepG2 cells, decreases the protein level of reversion-inducing-cysteine-rich protein with Kazal motifs (RECK), activates the phos-phorylation and acetylation of STAT3, thereby upregulates matrix metalloproteinase 9 (MMP9) and promotes the cell migration and invasion. On the contrary, in HCCLM3 cells, mortalin knockdown increases the expression of RECK, inhibits the STAT3 pathway and the activity of MMP9, and promotes the cell migration and invasion. Furthermore, we found that Salvianolic acid B, a caffeic acid phenethyl ester analogue, specific binds to mortalin, and increases the degradation of mortalin proteasome through ubiquitination, thereby up-regulating RECK, inhibiting STAT3 and finally inhibiting the migration and invasion of HCC.

4) Conclusion: Our work suggests that mortalin is a potential therapeutic target for hepatocellular carcinoma.

1. Background

Hepatocellular carcinoma (HCC) is currently the third leading cause of cancer-related death worldwide (Siegel, Miller, & Jemal, 2018). The main treatment of HCC is surgical resection and liver transplantation (Dhanasekaran, Venkatesh, Torbenson, & Roberts, 2016). However, HCC has a low 5-year survival rate, which is mainly due to a high postoperative recurrence rate and high migration and invasion rates (Llovet et al., 2016). Therefore, the identification of new therapeutic strategies for HCC is urgent.

Mortalin /mtHsp70, a member of the Hsp70 protein family, is highly expressed in many cancers. Studies have shown that overexpression of mortalin increases the malignant degree of breast cancer cells, and promotes their invasion and metastasis through PI3K/AKT or JAK/STAT signaling pathway (Na et al., 2016). More importantly, mortalin is associated with metastasis of hepatocellular carcinoma and is considered as a tumor marker for predicting early recurrence (Yi et al., 2008). At present, the mechanism of mortalin promoting migration and invasion has not been deeply studied. Studies have shown that the classic way for mortalin to inhibit the occurrence and development of cancer is to inhibit the expression and function of p53 (Nagpal et al., 2017). However, studies have found that the probability of p53 mutation is very high in HCC in China (G. Kang, Fang, & Volkmann, 2001). Therefore, there are other mechanisms of mortalin affecting the occurrence and development of HCC remains to be explored.

In the present study, we have shown that mortalin promotes the migration and invasion of HCC cells lines by regulating RECK/STAT3 signaling pathway; mortalin negatively regulates the expression of RECK protein, and the down-regulation of mortalin leads to the up-regulation of RECK protein, which leads to the down-regulation of its downstream STAT3 and MMP2/MMP9 signaling pathway. The inhibition of STAT3 and MMP2/MMP9 signaling pathway reversed EMT and inhibited the migration and invasion of hepatocellular carcinoma cells. We also found that Sal B inhibits the downstream RECK/STAT3 pathway by targeting mortalin, and finally inhibits the migration and invasion of HCC.

2. Materials And Methods

2.1. Reagents and cell culture

Sal B (purity ≥ 98%) was purchased from Spring and Autumn Biological Co., Ltd. (China), dissolved in absolute ethanol to a stock concentration of 50 mM, and stored at -80°C. Human HCC cell lines, HepG2 and HCCLM3, were purchased from the Institute of Basic Medical Sciences of the China Science Academy, Shanghai. HCCLM3 cells were maintained in high glucose Dulbecco's Modified Eagle's Medium (DMEM) and HepG2 cells were maintained in Modified Eagle's Medium (MEM) supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 mg/mL streptomycin. HepG2 and HCCLM3 cells were grown in the presence of 5% CO_2 at 37°C.

2.2. Determination of cell viability

The HCCLM3 cells (2×10^4 cells) were inoculated into 96 well plates for 24 h, and then treated with 0, 100, 200, 300, 400, 500, or 600 μM Sal B for 24 or 48 h. After the treatment, the culture solution containing Sal B was poured out and replaced with 100 μL of cell culture medium containing 10% Cell Counting Kit-8 reagent (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) for 4 h. Absorbance at 450 nm was detected using a multi-well plate reader (Model 680; Bio-Rad; Hercules, CA, USA). Untreated cells were used as controls.

2.3. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated from cells with different treatments using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations. All primers were synthesized by RiboBio Co. (Guangzhou, China), and primer sequences are shown in Supplementary Table S1. Total RNA (2 μg) was transcribed into cDNA using AMV Reverse Transcriptase (Promega, Madison, WI) for detection of mRNAs. The reverse transcription procedure was as follows: 25°C for 10 min, 42°C for 15 min, 85°C for 5 min, followed by chilling on ice. The newly synthesized first-strand cDNA was ready for immediate downstream applications or for long-term storage at -20°C. The qRT-PCR assays were performed with the LightCycler 96 SYBR Green I Master Mix (Roche) for 40 cycles of 95°C for 300 s, 95°C for 10 s, and 60°C for 30 s. It was carried out with SYBR Green master mix (Vazyme Biotech Co., Ltd).

2.4. Western blot analysis

Total protein was extracted using Radio-Immunoprecipitation Assay (RIPA) buffer (Beyotime Co. Ltd). A bicinchoninic acid (BCA) kit (Beyotime Co. Ltd) was used to measure protein concentration. Proteins (20 μg) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by transfer to polyvinylidene fluoride membranes (PVDF; Millipore, Billerica, MA, USA). Antibodies used were RECK, Mortalin, E-cadherin, N-cadherin, p-STAT3^{Tyr705}, Ac-STAT3^{K685} and vimentin (Cell Signaling Technology, 1: 1000), GAPDH and β-actin (Beyotime Co. Ltd, 1: 500) (Supplementary Table S2). Densitometric analysis was performed using Image-Pro-Plus 6.0 software, and GAPDH or β-actin served as the internal controls to correct for differences in protein loading.

2.5. Scratch assay

HepG2 and HCCLM3 cells (5 x 10^5 cells/well) were seeded in 6-well plates and cultured in DMEM (containing 5% FBS) for 24 hours. Then we used a pipette tip to create wound and then washed with PBS for 3 time, followed by replacing to DMEM with 0.0–200.0 Mm Sal B. Cells were viewed and photographed under a phase-contrast microscope (Olympus) in the same spots at 0, 24 or 48h.

2.6. Migration and invasion assays

Transwell assays were performed using a growth factor reduced Matrigel-coated filter and a non-Matrigel coated filter (8 mm aperture, BD, Franklin Lakes, NJ, USA) in a 24-well plate. The treated cells were separately trypsinized and inoculated into the upper chamber (5 × 10^4 cells/well) of Transwells in serum-free MEM and DMEM, and the lower chamber culture of the Transwell contained 15% FBS. Cells were cultured at 37°C for 48 h and 24 h, respectively. Five fields of view were randomly selected using an inverted microscope to take a photograph, and then the cells on the filter were dissolved in 33% acetic acid. Absorbance was measured at 570 nm.

2.7. Gelatin zymography

After removing the treatment drug and the complete medium, HepG2 and HCCLM3 cells were cultured in a medium containing 1% FBS in MEM and DMEN, and the culture supernatant was collected. Protein concentration was measured after centrifugation. Proteins previously mixed with non-deformed non-reducing SDS were isolated under non-reducing conditions by SDS-PAGE (10%) gel containing 1 mg/mL gelatin (Sigma-Aldrich). The separation gel was washed three times in 2.5% Triton X-100/50 mM Tris-HCl (pH 7.6) for 30 min. Then, the gel was incubated in 0.15 M NaCl/10 mM $CaCl_2$/ 50 mM Tris-HCl(pH 7.6/0.05% NaN3 for 2 h at 37°C in a CO_2 -free incubator. The gel was stained with 0.005% Coomassie Blue R250 for 3 h, and decolorized with 10% acetic acid and 10% isopropanol. MMP2 and MMP9 were detected as an effective band on the slab gel.

2.8. Cell transfection

The siRNAs were purchased from Santa Cruz Biotechnology (Supplemrntary Table S3). HCCLM3 cells were seeded in a dish at a density of 5 × 10^5 per well for 24 h, then transiently transfected with Lipofectamine 2000 (Invitrogen) and siRNA for 8 h according to the manufacturer's instructions. After

transfection, the cells were incubated for an additional 24 h in fresh medium with 10% FBS after removal of the medium and then used in other experiments.

The mortalin -Flag plasmid overexpressing both mortalin and Flag was created by inserting the coding sequences of mortalin into the plRES2-3FLAG-EGFP plasmid (Genechem, Shanghai, China). HepG2 cells were seeded in 6-well plates at a density of 1×10^5 per well for 24 h, then transiently transfected with vector control (12 ng/mL) or mortalin (12 ng/mL) using Lipofectamine 2000 (Invitrogen) for 12 h according to the manufacturer's instructions. The medium was then replaced with fresh medium (10% FBS without penicillin-streptomycin), and the treated cells were used for other experiments.

2.9. Docking studies

SYBYL-X software was utilized for molecular-docking of Caffeic acid and its derivatives with mortalin. The crystal structure of mortalin [PDB ID: 4KBO] was obtained from PDB (http://www.rcsb.org/pdb/home/home.do). All ligands and water molecules are first removed, adding polar hydrogen atoms and AMBER7FF99 charge. According to the reported binding region of mortalin and p53 in literature, the binding sites of CAPE and mortalin are Try196, Phe250, Asp251, Thr267, Asn268, Gly269, Asp270 and Pe272. The residues in the receptors are designated to produce protoplasts using this residue pattern.

2.10. Immunoprecipitation combined with High-performance liquid chromatography mass spectrometry

The cells of Logarithmic HCCLM3 were subcultured and treated with proteasome inhibitor MG132 for 2 hours when the cell convergence reached 60%-70%. Cells was cultured for 24 hours after replacement of 200μM Sal B. Then the cell protein was collected for immunoprecipitation. 100μL sterilized double distilled water was added to the precipitate of the Control group and the treatment group, and then the mixture was boiled in the protein dry thermostat at 100°C for 10 min to precipitate the denaturation, and the tested substance was separated from the protein and dissolved in water for UPLC / Ms Analysis. Sal B standard as positive control, sterile double distilled water as control, PBS and Cell Lysate as samples was to be tested.

The UPLC / MS Analysis used Thermo Fisher q active four-stage rod-electrostatic field orbital trap high resolution mass spectrometry system and Dionex UltiMate 3000 High-performance liquid chromatography system. Thermo Scientific Xcaliber and Thermo Scientific Pathfinder are respectively used for data collection and processing.

2.11. Statistical analysis

Data sets were compared using GraphPad-6.0 (GraphPad Software, Inc, La Jolla, CA, USA). Values are presented as the mean ± SD. The difference between two groups was analyzed using a two-tailed Student's *t*-test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Mortalin promotes the migration and invasion of hepatocellular carcinoma cells

It has been reported that mortalin enhances the process of EMT in breast cancer cells (Na et al., 2016). To further determine the effect of mortalin in HCC, we knocked down mortalin with siRNA or overexpressed mortalin with the viral plasmid Flag-mortalin. As shown in Fig. 1A, after mortalin was overexpressed in HepG2, EMT increased, showing that E-cadherin was down-regulated, while N-cadherin and Vimentin were up-regulated, while knock down mortalin in HCCLM3 reversed the process of EMT. In addition, after mortalin was overexpressed in HepG2, the activities of MMPs were increased, while knock down mortalin in HCCLM3 decreased the activities of MMPs. And the transwell assay confirmed that mortalin overexpression increased cell migration ability in HepG2 (Fig. 1B), while mortalin knockdown in HCCLM3 decreased cell migration ability (Fig. 1C). These results suggest that mortalin promotes the EMT process, the activities of MMPs and cell migration of HCC.

3.2. Mortalin promotes the migration of hepatocellular carcinoma cells via RECK/STAT3 signaling pathway

RECK, a tumor suppressor gene, is critical for the regulation of the migratory and invasive capacities of tumor cells (Oh et al., 2001). In order to explore whether there is regulatory effect of mortalin on RECK/STAT3 signal pathway, we firstly transfected the Flag-mortalin into HepG2 cells. As shown in Fig. 2A, mortalin overexperssion significantly decreased the RECK protein level. At the same time, we found that the expression of RECK protein significantly increased after transfection of mortalin siRNA in HCCLM3.

Studies showed that RECK is an important molecule regulating STAT3 (Walsh et al., 2015). STAT3 activation plays an important role in metastasis, and STAT3 increases the expression of MMP2 and MMP9, which act as key mediators of the metastatic process of cancer cells. Na.et al. obtained that JAK-STAT signaling was involved in mortalin-induced migration and invasion of breast cancer through the analysis of DNA microarray (Na et al., 2016). To explore the relationship between STAT3 and mortalin, we used mortalin siRNA and high expression plasmid respectively to knock down or high expression mortalin. We found that the expression levels of p-STAT3 and Ac-STAT3 increased significantly after mortalin overexpression in HepG2, while the expression levels of p-STAT3 and Ac-STAT3 decreased after mortalin knockdown in HCCLM3 (Fig. 2A). In order to further explore the role of STAT3 in cell migration regulated by mortalin, we first used siRNA to knockdown STAT3 in HepG2 cells, and then conducted transwell experiments. We found that the cell migration ability decreased significantly after knocking down STAT3 (Fig. S1). Then we observed the changes of EMT and cell migration after adding p-STAT3 or Ac-STAT3 inhibitors STATTIC and C646 to HepG2 cells with high mortalin expression. We found that E-cadherin was down-regulated, N-cadherin and Vimentin were up-regulated after high mortalin expression,

and EMT process was reversed after adding inhibitors, and the up-regulation of migration induced by mortalin was also inhibited (Fig. 2B-D). These results confirmed that mortanlin, through the RECK/STAT3 signaling pathway, promotes the migration of hepatocellular carcinoma cells.

3.3. Mortalin is a specific target of Salvianolic acid B

We initially predicted the binding of caffeic acid and its derivatives to mortalin protein by molecular docking software SYBYL-X. We used three combinations to predict the interaction of caffeic acid and its derivatives with mortalin. The results showed that the binding score of Sal B was the highest among the three binding modes, indicating that the binding ability of Sal B and mortalin was the strongest. Then we chose Sal B for further software analysis. As shown in Fig. 3A, Sal B contains four benzene rings with hydroxyl groups attached to them. Two hydroxyl groups form two hydrogen bonds with Tyr196 and Phe197. One hydroxyl group forms one hydrogen bond with Asp270 and the other two hydroxyl groups form two hydrogen bonds with Ile252 and Thr267 respectively, thus forming a stable bond (shown in the white arrow). And sal B is located in a hole in mortalin from the steric hindrance, which is also located between the residue 253–282 in the binding region of mortalin and p53. Mortalin's classical inhibitor MKT-077 also binds mortalin in this region (Wadhwa et al., 2000). In order to further confirm the interaction between Sal B and mortalin in cells, we designed an immunoprecipitation-high performance liquid chromatography-mass spectrometry technique based on classical experiments to confirm the specific binding of Sal B with mortalin. As shown in Fig. 3B, Sal B had no effect on HCC cell viability at concentrations of 0.0, 100.0, 200.0, and 300.0 μM for 24 and 48 h. The cell samples were used Sal B of 100.0 μM for 24h. At the same time, we used Sal B of 10 μM as positive control and sterilized double steamed water as negative control. Other confounding factors such as PBS, cell lysate and the last PBS cleaning solution were also tested. The results showed that the retention time of Sal B was 4.88 min, the accurate mass number m/z was 717.1459 in the negative ion mode and the area is 9.7×10^4. Sal B was detected in cell samples, while Sal B was not detected in other samples (Fig. 3C).

It is suggested that Sal B binds mortalin specifically when other confounding factors are excluded from the immunoprecipitation process. However, it is not clear whether Sal B can affect mortalin expression. Current studies have shown that ubiquitin-like protein UBXN2A promotes hydroxyl-terminal-dependent mortalin ubiquitination of HSP70 interacting protein (CHIP), and UBXN2A can increase mortalin proteasome degradation (Sane et al., 2018). Therefore, we speculated whether Sal B degraded mortalin protein through ubiquitination. After immunoprecipitation of HCCLM3 cells treated with Sal B and mortalin antibody, we found that the ubiquitination level of mortalin in cells treated with Sal B was significantly higher than that in control group (Fig. 3D). And Sal B could significantly reduce mortalin protein expression, but this regulation was significantly inhibited in cells pretreated with MG132, a proteasome inhibitor (Fig. 3E). These results indicated that Sal B can specifically bind to mortalin, and increased the degradation of mortalin proteasome through ubiquitination, thus inhibiting the expression of mortalin protein.

3.4. Salvianolic acid B inhibits the migration and invasion of HCC via RECK/STAT3 pathway

Considering the important link between EMT/RECK-STAT3 signaling pathway/MMPs and tumor cell migration/invasion, HCCLM3 cells were treated with Sal B at 0.0, 50.0, 100.0, and 200.0 μM for 48 h, and the expression of EMT-related proteins, including E-cadherin, N-cadherin, and vimentin was analyzed. Sal B up-regulated the expression of E-cadherin and down-regulated the expression of N-cadherin and vimentin, suggesting that Sal B could reverse the EMT process. At the same time, the protein levels of RECK increased and the protein levels of mortalin decreased in response to treatment of HCCLM3 with Sal B at different concentrations up to 200.0 μM for 48 h, and Sal B efficiently decreased the expression of p-STAT3^{Y705} and Ac-STAT3^{K685} in HCCLM3 (Fig. 4A). Furthermore, Sal B downregulated the expressions of MMP2 and MMP9. Gelatin zymography analysis demonstrated that Sal B inhibited the activities of MMP2 and MMP9 (Fig. 4B). In the scratch assay, 50.0, 100.0, and 200.0 μM Sal B inhibited the scratch healing abilities of HCC cells in a dose-dependent manner (Fig. S2). And as shown in Fig. 4C, Sal B decreased the migratory and invasive potential of HCCLM3 cells in a dose-dependent manner. These data indicated that Sal B inhibits the EMT, RECK/STAT3 signaling pathway and MMPs activities of HCCLM3, and attenuate the migratory and invasive abilities of HCCLM3.

3.5. Salvianolic acid B inhibits the migration and invasion of HCC by regulating mortalin.

As we have shown in the previous, mortalin promoted migration and invasion of hepatocellular carcinoma cells via RECK/STAT3 signaling, and that Sal B inhibited EMT, RECK/STAT3 pathway, MMP9/MMP2, and migration and invasion of hepatocellular carcinoma cells. And we found that Sal B specific binds to mortalin and degrade it, we hypothesized that Sal B inhibits the migration and invasion of hepatocellular carcinoma cells by mortanlin.

As shown in Fig. 5A, siRNA-mediated silencing of RECK was performed to determine the effect of RECK on EMT markers and STAT3/MMP signaling in HCC cells. Cells were transiently transfected with control or RECK siRNA for 8 h, then exposed to 0 or 100 μM Sal B for 48 h. As shown in Fig. 5A, knockdown of RECK significantly reduced the Sal B-induced upregulation of E-cadherin and downregulation of N-cadherin and vimentin. In addition, we determined the role of RECK in the STAT3/MMP axis. Sal B downregulated p-STAT3^{Y705} and Ac-STAT3^{K685}, and knockdown of RECK abolished the Sal B-induced downregulation of p-STAT3^{Y705} and Ac-STAT3^{K685}. Gelatin zymography analysis demonstrated that knockdown of RECK significantly increased the Sal B-induced repression of MMP2 and MMP9. Collectively, these results suggested that Sal B modulated EMT markers and STAT3/MMP signaling in HCC cells by upregulating RECK. Based on the results described above, we hypothesized that RECK was involved in the Sal B-induced inhibition of migration and invasion in HCC cells. To verify this hypothesis, we treated RECK knockdown in HCCLM3 cells with Sal B to determine their migratory/invasive abilities. As shown in Fig. 5B, knockdown of RECK blocked the Sal B-induced inhibition of the migratory and invasive abilities of HCC cells. These results suggested that RECK is involved in the anti-metastatic effects of Sal B in HCC cells. Immunocoprecipitation assay showed that mortalin binds to RECK, which was weakened by Sal B (Fig. 5C).

To further confirm the effect of mortalin on Sal B inhibiting migration and invasion of HCC, we overexpressed mortalin on the basis of Sal B treatment. As shown in Fig. 5D, high expression mortalin combined with Sal B significantly reduced the up-regulation of E-cadherin induced by Sal B, and reversed the down-regulation of N-cadherin and Vimentin induced by Sal B. At the same time, Sal B downregulated p-STAT3^{Y705} and Ac-STAT3^{K685}, and high expression mortalin abolished the Sal B-induced downregulation of p-STAT3^{Y705} and Ac-STAT3^{K685}. In addition, gelatin zymogram also confirmed that high expression mortalin combined with Sal B significantly reversed the decrease of MMP2/MMP9 regulated by Sal B (Fig. 5D). Transwell experiment also confirmed that high expression of mortalin combined with Sal B could significantly reverse the migration ability of hepatocellular carcinoma cells inhibited by Sal B (Fig. 5E). These results suggest that mortalin is involved in the process of migration and invasion of HCC inhibited by Sal B.

4. Conclusion

In summary, our results showed that mortalin plays an important role in the migration and invasion of HCC cells, and RECK/STAT3 signal pathway is regulated by mortalin. Furthermore, we found that Salvianolic acid B, a caffeic acid phenethyl ester analogue, specific binds to mortalin, and increases the degradation of mortalin proteasome through ubiquitination, therepy up-regulating RECK, inhibiting STAT3 and finally inhibiting the migration and invasion of HCC. Our work suggests that mortalin is a potential therapeutic target for hepatocellular carcinoma.

5. Discussion

HCC is a highly metastatic tumor that is associated with high recurrence rates and low survival rates (Siegel et al., 2018). The molecular mechanisms underlying the suppression of invasion and migration in HCC have been extensively studied to discover new therapeutic targets and predictive markers. The EMT is an important step in tumor migration and invasion, and it is characterized by the loss of the epithelial properties of cells such as adhesion and the expression of the epithelial marker E-cadherin, and the acquisition of mesenchymal properties, such as increased cell motility and the upregulation of the mesenchymal markers N-cadherin and vimentin (Lamouille, Xu, & Derynck, 2014).

The extracellular matrix (ECM) is an important tissue barrier for tumor metastasis. The migration and invasion of malignant tumors are often accompanied by changes in the expression of the ECM and its cell surface receptors (Lu, Takai, Weaver, & Werb, 2011). The degradation of the ECM by MMPs is one of the key aspects of tumor cell metastasis. Many malignant tumors are associated with increased secretion and activity of MMPs (Shay, Lynch, & Fingleton, 2015).

Mortalin, a member of the Hsp70 family, plays an important carcinogenic role in cancer cells through a variety of mechanisms. In addition, mortalin is considered as a target for many kinds of cancer therapy (Ando et al., 2014; Jin et al., 2016; Starenki, Hong, Lloyd, & Park, 2015; Wadhwa et al., 2006; Yang, Li, Jiang, Zuo, & Liu, 2013). It is important to find specific mortalin inhibitors for the treatment of tumors.

Current studies have shown that mortalin has good surface properties and can make small molecules dock with high affinity and specificity. Wadhwa et al. (Wadhwa et al., 2016) used molecular docking software Autodock to analyze that caffeic acid phenylethyl ester (CAPE) could dock with mortalin and destroy the interaction of mortalin-p53 complex, resulting in nuclear ectopia of mortalin and activation of p53 anti-cancer function, and inhibited the growth of cancer cells through p53-GAD45a-p21 pathway. Over-inhibition of matrix metalloproteinase inhibits cell invasion and metastasis. At the same time, CAPE can increase the sensitivity of anticancer drugs by targeting mortalin in many ways (Yun et al., 2017). CAPE and Sal B in this study belong to caffeic acid derivatives, so we speculate whether Sal B also has the same targeting effect, targeting mortalin to play its anti-cancer role. We also found that another specific inhibitor of mortalin, MKT-077, binds to mortalin in this region, and MKT-077 inhibits its interaction with p53 without affecting mortalin expression, thus activating the anti-cancer function of p53 (Grover et al., 2012; Wadhwa et al., 2000). As shown in Fig. 6, we found that Sal B can significantly combine with mortalin protein. Therefore, we consider that Sal B can be used as a new inhibitor of mortalin protein. So how does Sal B affect mortalin expression? It has been reported that ubiquitin-like protein UBXN2A promotes the carboxyl end of mortalin's HSP70 interacting protein (CHIP) dependent ubiquitination. Subsequently, it was found that UNXN2A increased mortalin proteasome degradation. Subcellular regionalization experiments showed that induction of UNXN2A reduced mortalin and its partner HSP60 levels. Upregulation of UNXN2A by small molecules of veratridine (VTD) can decrease mortalin level in cancer cells. Consistent with the results in vitro, UNXN2A$^{+/-}$ mice showed increased mortalin selectivity in colon tissues. Recombinant UNXN2A can enhance the degradation of mortalin proteasome in mouse colon tissue (Sane et al., 2018). Our results also proved that Sal B degrades mortalin protein through ubiquitination.

More and more studies have been done on mortalin and migration and invasion of tumors recently. It has been proved that mortalin can promote migration and invasion of tumors, including breast cancer, liver cancer, cholangiocarcinoma, etc (Q. Kang et al., 2017; Na et al., 2016; Yi et al., 2008). However, the mechanism of its effect on the migration and invasion of tumors is still unclear. Our current experiments confirm that mortalin affects the migration and invasion of HCC through EMT and matrix metalloproteinase pathways. RECK and STAT3 are also involved as upstream signaling molecules. RECK is down-regulated in many tumors, and the mechanism of this down-regulation is multifactorial and tumor-specific. One of the common targets of inhibition is the Sp1 site in the promoter sequence of RECK. Early studies have speculated that the oncogene Ras promotes the phosphorylation or other modification of Sp1/Sp3 factor by activating extracellular signal-regulated kinase (ERK) pathway, increases the affinity of Sp1 site on RECK promoter, and reduces the expression of RECK (Oh et al., 2001; Sasahara, Takahashi, & Noda, 1999). Other studies hypothesized that the interaction between histone deacetylase and Sp1 might help to inhibit RECK transcription (Liu, Chang, Chiang, & Hung, 2003). However, our study showed that Sal B could up-regulate the expression of RECK and down-regulate the expression of mortalin, and mortalin could affect the protein level of RECK. This indicated that mortalin could affect RECK not through the transcriptional regulation of Sp1, but through other ways.

Overactivation of STAT3 is essential for metastasis. RECK regulates STAT3 activation, cytokine signaling and induction of vascular endothelial growth factor and uPA by forming complex with cell surface receptors (Walsh et al., 2015). MMP9 is secreted paracrine in cancer cells with high STAT3 signal transduction, and STAT3 can regulate MMP9 (Orgaz et al., 2014). In addition, STAT3 blocked MMP2 expression and MMP9 promoter activity by using chromatin immunoprecipitation of anti-STAT3 antibody (Zhang et al., 2015). We suggest that Sal B induces the up-regulation of RECK, which leads to the down-regulation of p-STAT3^{Y705} and Ac-STAT3^{K685}, and inhibits the ability of MMPs to destroy ECM, thus inhibiting the migration and invasion of HCC (Fig. 6). Yuki et al. showed that RECK was up-regulated after EMT in non-malignant epithelial cells, but in cancer-derived cell lines, the down-regulation of E-cadherin and up-regulation of RECK were not related. Our current studies suggest that Sal B regulates EMT markers and STAT3/MMPs signal transduction in HCC by up-regulating RECK. All changes in EMT-related molecular markers knocked down by RECK were based on Sal B treatment. Whether RECK can affect EMT in HCC cells without Sal B remains to be further confirmed.

Na et al. (Na et al., 2016)showed that JAK-STAT signaling was involved in mortalin-induced migration and invasion of breast cancer through DNA microarray analysis. However, the relationship between mortalin and STAT signaling pathway is still lack of experimental evidence. It is the first time that Sal B can significantly reduce the levels of p-STAT3 and Ac-STAT3, mortalin can regulate the levels of p-STAT3 and Ac-STAT3, and high expression of mortalin can reverse the down-regulation of p-STAT3 and Ac-STAT3 by Sal B. However, further mechanism research is still needed.

6. List Of Abbreviations

Sal B	Salvianolic acid B
RECK	The reversion-inducing cysteine-rich protein with kazal motifs
MMPs	Max metalloproteinases
STAT3	Signal transducer and activator of transcription 3
HCC	Hepatocellular carcinoma
EMT	Epithelial-mesenchymal transition
ECM	Extracellular matrix
CAPE	Caffeic acid phenethyl ester

Declarations

7.1 Ethics approval and consent to participate:

This study does not include animal studies.

7.2 Consent for publication:

All of the authors agree for publication.

7.3 Availability of data and material:

Availability of data	Template for data availability statement
Data available on request from the authors	The data that support the findings of this study are available from the corresponding author upon reasonable request.

7.4 Competing interests:

The authors declare that there is no conflict of interest regarding the publication of this paper.

7.5 Funding:

This work was supported by National Natural Science Foundation of China (81573183, 81673205) .

7.6 Authors' contributions:

Zhong Li, Yuan Li and Mengying Teng contributed to the conception of the study;

Mengying Teng, Chunyan Hu and Qian Zhou performed the experiment;

Mengying Teng, Bingmo Yang and Wei Xiao contributed significantly to analysis and manuscript preparation;

Mengying Teng and Chunyan Hu performed the data analyses and wrote the manuscript;

Qian Zhou, Bingmo Yang and Wei xiao helped perform the analysis with constructive discussions.

7.7 Acknowledgments:

This work was supported by National Natural Science Foundation of China (81573183, 81673205) and the Center for Global Health, School of Public Health, Nanjing Medical University. The project funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD).

References

1. Ando K, Oki E, Zhao Y, Ikawa-Yoshida A, Kitao H, Saeki H,.. . Maehara Y. Mortalin is a prognostic factor of gastric cancer with normal p53 function. Gastric Cancer. 2014;17(2):255–62. doi:10.1007/s10120-013-0279-1.

2. Dhanasekaran R, Venkatesh SK, Torbenson MS, Roberts LR. Clinical implications of basic research in hepatocellular carcinoma. J Hepatol. 2016;64(3):736–45. doi:10.1016/j.jhep.2015.09.008.
3. Grover A, Priyandoko D, Gao R, Shandilya A, Widodo N, Bisaria VS,.. . Sundar D. Withanone binds to mortalin and abrogates mortalin-p53 complex: computational and experimental evidence. Int J Biochem Cell Biol. 2012;44(3):496–504. doi:10.1016/j.biocel.2011.11.021.
4. Jin H, Ji M, Chen L, Liu Q, Che S, Xu M, Lin Z. The clinicopathological significance of Mortalin overexpression in invasive ductal carcinoma of breast. J Exp Clin Cancer Res. 2016;35:42. doi:10.1186/s13046-016-0316-0.
5. Kang G, Fang Z, Volkmann M. (2001). [Study on p53 gene mutation in hepatocellular carcinoma patients in China]. *Zhonghua Shi Yan He Lin Chuang Bing Du Xue Za Zhi, 15*(2), 163–165. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/11436649.
6. Kang Q, Cai JB, Dong RZ, Liu LX, Zhang C, Zhang PF,.. . Shi GM. Mortalin promotes cell proliferation and epithelial mesenchymal transition of intrahepatic cholangiocarcinoma cells in vitro. J Clin Pathol. 2017;70(8):677–83. doi:10.1136/jclinpath-2016-204251.
7. Lamouille S, Xu J, Derynck R. Molecular mechanisms of epithelial-mesenchymal transition. Nat Rev Mol Cell Biol. 2014;15(3):178–96. doi:10.1038/nrm3758.
8. Liu LT, Chang HC, Chiang LC, Hung WC. (2003). Histone deacetylase inhibitor up-regulates RECK to inhibit MMP-2 activation and cancer cell invasion. *Cancer Res, 63*(12), 3069–3072. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12810630.
9. Llovet JM, Zucman-Rossi J, Pikarsky E, Sangro B, Schwartz M, Sherman M, Gores G. Hepatocellular carcinoma. Nat Rev Dis Primers. 2016;2:16018. doi:10.1038/nrdp.2016.18.
10. Lu P, Takai K, Weaver VM, Werb Z. (2011). Extracellular matrix degradation and remodeling in development and disease. Cold Spring Harb Perspect Biol, 3(12). doi:10.1101/cshperspect.a005058.
11. Na Y, Kaul SC, Ryu J, Lee JS, Ahn HM, Kaul Z,.. . Wadhwa R. Stress chaperone mortalin contributes to epithelial-mesenchymal transition and cancer metastasis. Cancer Res. 2016;76(9):2754–65. doi:10.1158/0008-5472.CAN-15-2704.
12. Nagpal N, Goyal S, Dhanjal JK, Ye L, Kaul SC, Wadhwa R,.. . Grover A. Molecular dynamics-based identification of novel natural mortalin-p53 abrogators as anticancer agents. J Recept Signal Transduct Res. 2017;37(1):8–16. doi:10.3109/10799893.2016.1141952.
13. Oh J, Takahashi R, Kondo S, Mizoguchi A, Adachi E, Sasahara RM,.. . Noda M. (2001). The membrane-anchored MMP inhibitor RECK is a key regulator of extracellular matrix integrity and angiogenesis. *Cell, 107*(6), 789–800. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11747814.
14. Orgaz JL, Pandya P, Dalmeida R, Karagiannis P, Sanchez-Laorden B, Viros A,.. . Sanz-Moreno V. Diverse matrix metalloproteinase functions regulate cancer amoeboid migration. Nat Commun. 2014;5:4255. doi:10.1038/ncomms5255.
15. Sane S, Hafner A, Srinivasan R, Masood D, Slunecka JL, Noldner CJ,.. . Rezvani K. UBXN2A enhances CHIP-mediated proteasomal degradation of oncoprotein mortalin-2 in cancer cells. Mol Oncol.

2018;12(10):1753–77. doi:10.1002/1878-0261.12372.

16. Sasahara RM, Takahashi C, Noda M. Involvement of the Sp1 site in ras-mediated downregulation of the RECK metastasis suppressor gene. Biochem Biophys Res Commun. 1999;264(3):668–75. doi:10.1006/bbrc.1999.1552.
17. Shay G, Lynch CC, Fingleton B. Moving targets: Emerging roles for MMPs in cancer progression and metastasis. Matrix Biol. 2015;44–46:200–6. doi:10.1016/j.matbio.2015.01.019.
18. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2018. CA Cancer J Clin. 2018;68(1):7–30. doi:10.3322/caac.21442.
19. Starenki D, Hong SK, Lloyd RV, Park JI. Mortalin (GRP75/HSPA9) upregulation promotes survival and proliferation of medullary thyroid carcinoma cells. Oncogene. 2015;34(35):4624–34. doi:10.1038/onc.2014.392.
20. Wadhwa R, Nigam N, Bhargava P, Dhanjal JK, Goyal S, Grover A,.. . Kaul SC. Molecular Characterization and Enhancement of Anticancer Activity of Caffeic Acid Phenethyl Ester by gamma Cyclodextrin. J Cancer. 2016;7(13):1755–71. doi:10.7150/jca.15170.
21. Wadhwa R, Sugihara T, Yoshida A, Nomura H, Reddel RR, Simpson R,.. . Kaul SC. (2000). Selective toxicity of MKT-077 to cancer cells is mediated by its binding to the hsp70 family protein mot-2 and reactivation of p53 function. *Cancer Res, 60*(24), 6818–6821. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11156371.
22. Wadhwa R, Takano S, Kaur K, Deocaris CC, Pereira-Smith OM, Reddel RR, Kaul SC. Upregulation of mortalin/mthsp70/Grp75 contributes to human carcinogenesis. Int J Cancer. 2006;118(12):2973–80. doi:10.1002/ijc.21773.
23. Walsh LA, Roy DM, Reyngold M, Giri D, Snyder A, Turcan S,.. . Chan TA. RECK controls breast cancer metastasis by modulating a convergent, STAT3-dependent neoangiogenic switch. Oncogene. 2015;34(17):2189–203. doi:10.1038/onc.2014.175.
24. Yang L, Li H, Jiang Y, Zuo J, Liu W. Inhibition of mortalin expression reverses cisplatin resistance and attenuates growth of ovarian cancer cells. Cancer Lett. 2013;336(1):213–21. doi:10.1016/j.canlet.2013.05.004.
25. Yi X, Luk JM, Lee NP, Peng J, Leng X, Guan XY,.. . Fan ST. Association of mortalin (HSPA9) with liver cancer metastasis and prediction for early tumor recurrence. Mol Cell Proteomics. 2008;7(2):315–25. doi:10.1074/mcp.M700116-MCP200.
26. Yun CO, Bhargava P, Na Y, Lee JS, Ryu J, Kaul SC, Wadhwa R. Relevance of mortalin to cancer cell stemness and cancer therapy. Sci Rep. 2017;7:42016. doi:10.1038/srep42016.
27. Zhang N, Duan WD, Leng JJ, Zhou L, Wang X, Xu YZ,.. . Dong JH. STAT3 regulates the migration and invasion of a stemlike subpopulation through microRNA21 and multiple targets in hepatocellular carcinoma. Oncol Rep. 2015;33(3):1493–8. doi:10.3892/or.2015.3710.

Figures

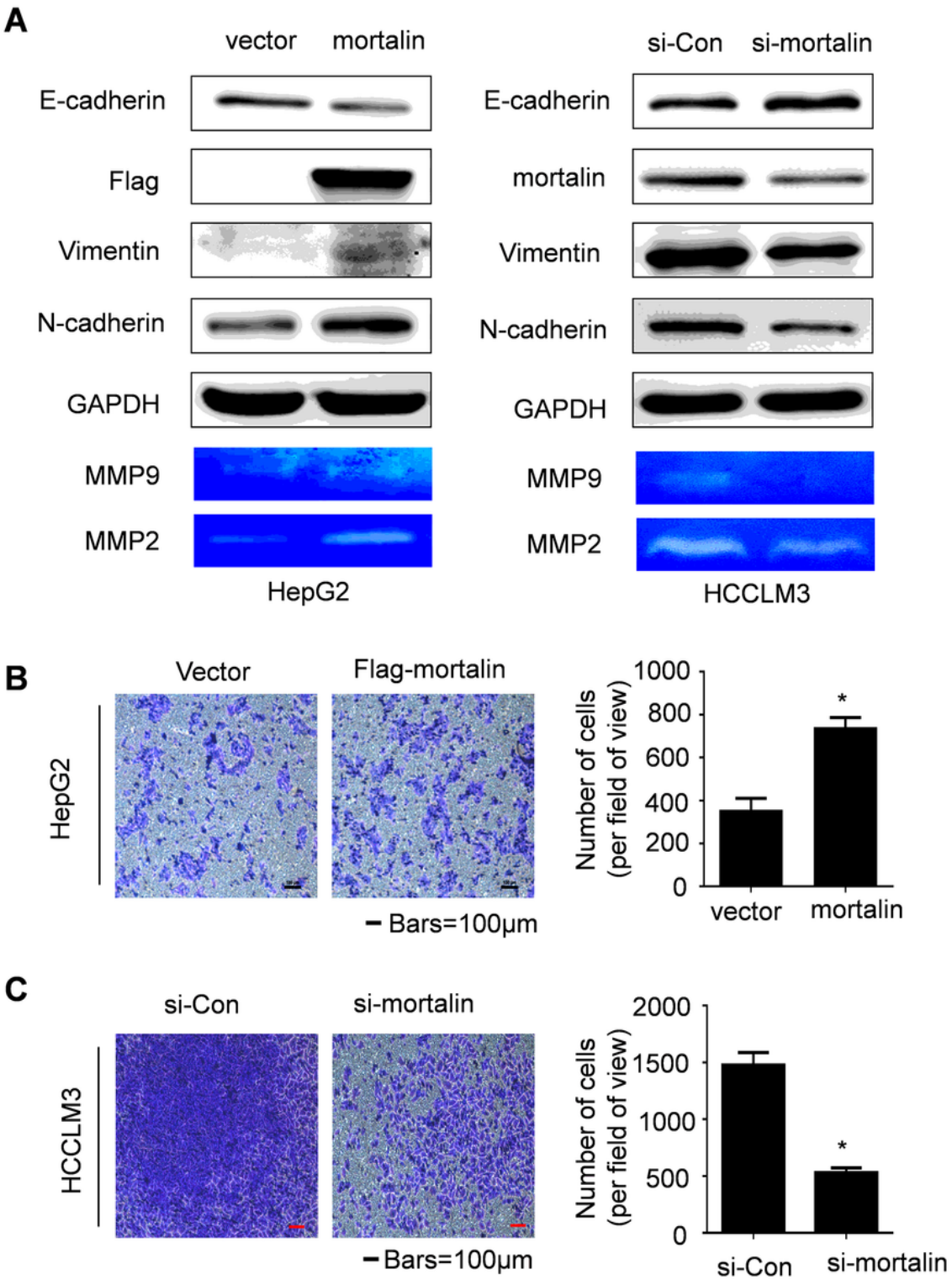

Figure 1

Mortalin can promote the migration of hepatocellular carcinoma cells. (A) Western blot analyses of the expressions of E-cadherin, Vimentin, N-cadherin, mortalin, Flag and GAPDH in HCC with different mortalin levels. MMP2 and MMP9 activities were investigated by gelatin zymography assays. (B) HepG2 cells were subjected to the migration assays after overexpressed of mortalin, and migrated cells were counted with Stat Monitor in photoshop (mean ± SD, n=3). *P < 0.05, statistically significant difference vs.

untreated cells. Bars = 100 μm. (C) HCCLM3 cells were subjected to the migration assays after si-mortalin, and migrated cells were counted with Stat Monitor in photoshop (mean ± SD, n=3). *P < 0.05, statistically significant difference vs. untreated cells. Bars = 100 μm.

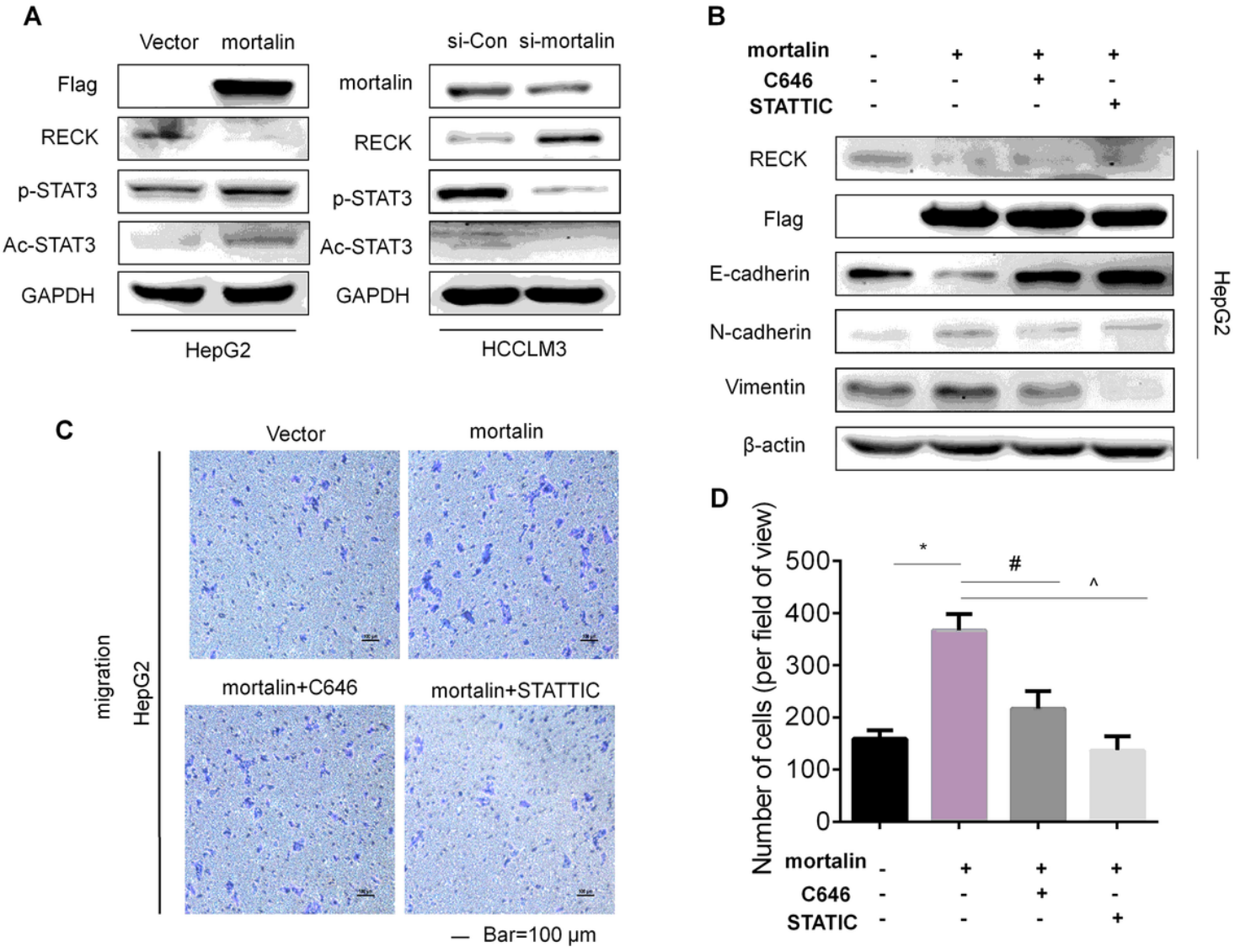

Figure 2

Mortanlin regulates the RECK/STAT3 signaling pathway to promote the migration of hepatocellular carcinoma cells. (A) Western blot analyses of the expressions of mortalin, RECK, Flag-mortalin, p-STAT3, Ac-STAT3 and GAPDH in HCC with different mortalin levels. (B) Western blot assays of RECK, Flag, E-cadherin, N-cadherin, Vimentin and β-actin were performed. (C) HepG2 cells were subjected to migration assays, and (D) migrated cells were counted with Stat Monitor in photoshop (mean ± SD, n=3). *P < 0.05, statistically significant difference vs. vector cells. #P < 0.05 and ^P < 0.05, statistically significant difference vs. vector cells treated by Flag-mortalin.

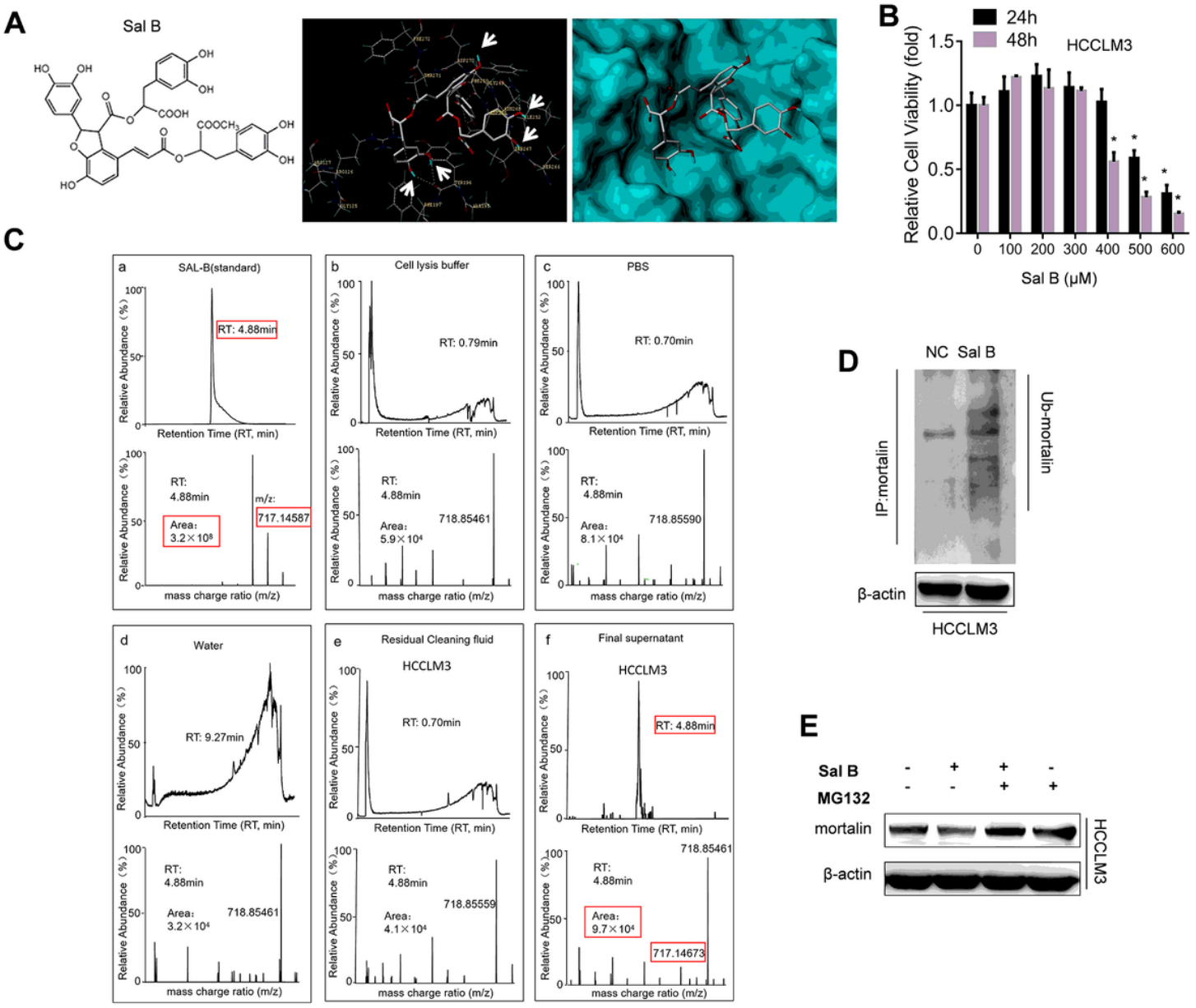

Figure 3

Mortanlin is degraded by salvianolic acid B, a caffeic acid phenethyl ester analogue, which is found to combine with mortanlin. (A) Prediction of combination of sal B and mortalin by SYBYL-X software. (B) HCCLM3 cells were treated with 0- 600 μM Sal B for 24h or 48 h. Cell viability of HCCLM3 cells was measured, *P < 0.05, statistically significant difference vs. 0 μM Sal B group. (C) Sal B of 10μM was used as positive control and sterilized double steamed water as negative control. The retention time of sal B was 4.88 min, the accurate mass number m/z was 717.1459 and the area was 9.7×104. Red marker represents the detection of Sal B samples. (D) After treatment with Sal B, cells were harvested for ubiquitination analysis of mortalin by immunoprecipitation. (E) Western blots of mortalin expression after pretreatment of MG132. After pretreatment with 20 μM proteasome inhibitor MG132 for 2 h, HCCLM3 cells were treated with Sal B for 48 h, and then cells were harvested for western blot analysis.

Figure 4

Salvianolic acid B can inhibit the migration and invasion of hepatocellular carcinoma cells. (A) Western blots assays of E-cadherin, Vimentin, N-cadherin, mortalin, RECK, p-STAT3, Ac-STAT3 and β-Actin in HCCLM3 were performed. (B) Western blot assays of MMP2, MMP9, and GAPDH were performed. MMP2 and MMP9 activities in HCCLM3 were investigated by gelatin zymography assays. The mRNA levels of MMP2 and MMP9 were investigated by quantitative real-time polymerase chain reaction (qRT-PCR) analysis (mean ± SD, n=3). *P < 0.05, statistically significant difference vs. untreated cells. (C) HCCLM3 cells were subjected to the migration and invasion assays, and migrated and invaded cells were counted with Stat Monitor in photoshop (mean ± SD, n=3). *P < 0.05, #P < 0.05, statistically significant difference vs. untreated cells. Bars = 100 μm.

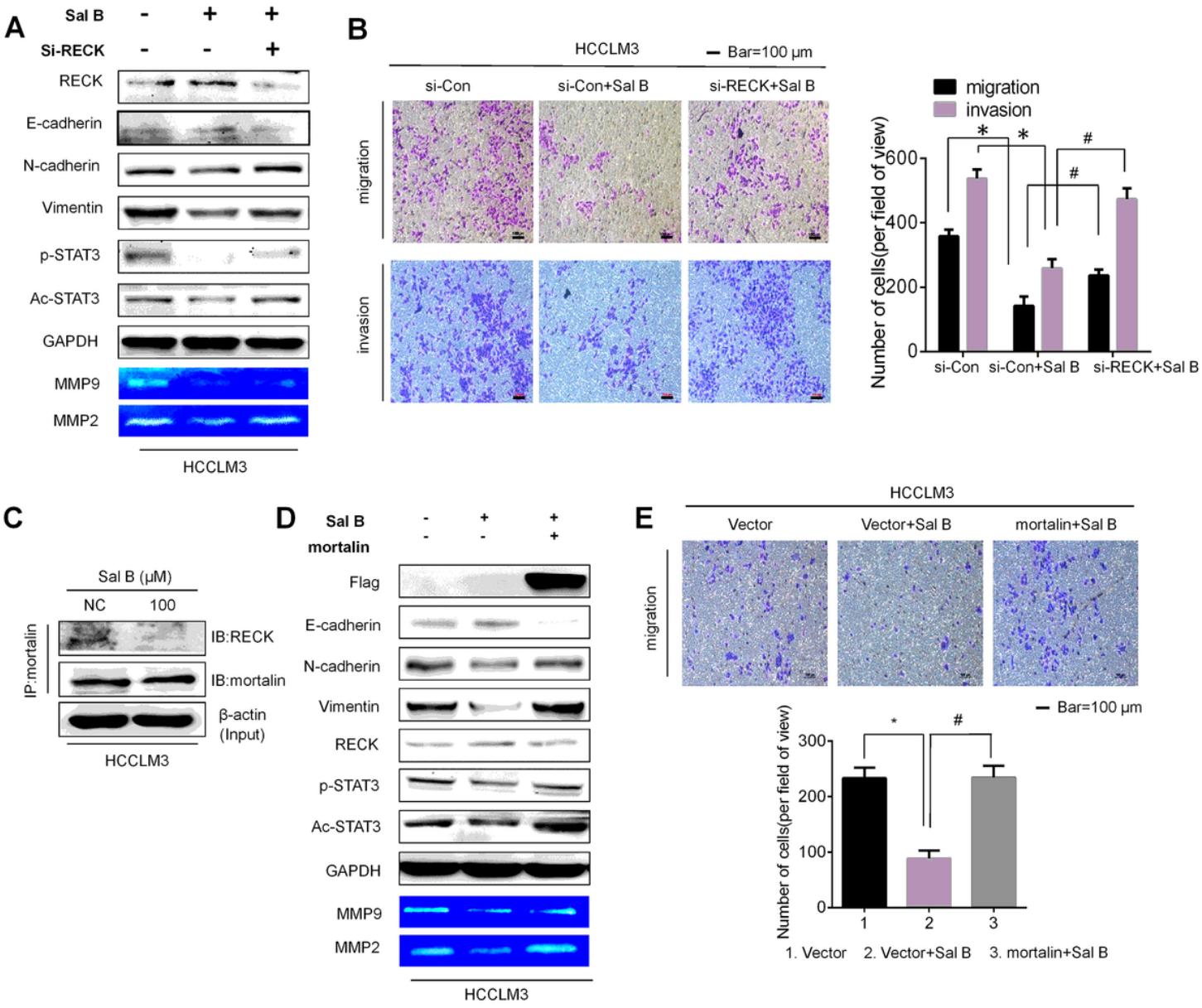

Figure 5

Salvianolic acid B inhibits the migration and invasion of hepatocellular carcinoma cells by regulating mortanlin. (A) Western blot assays of E-cadherin, N-cadherin, Vimentin, p-STAT3, Ac-STAT3 and GAPDH were performed. MMP2 and MMP9 activities in HCCLM3 were investigated by gelatin zymography assays. (B) HCCLM3 cells were subjected to migration and invasion assays, and migrated and invaded cells were counted with Stat Monitor in photoshop (mean ± SD, n=3). *P < 0.05, statistically significant difference vs. si-NC cells. #P < 0.05, statistically significant difference vs. si-NC cells treated with 100 μM Sal B. (C) After HCCLM3 cells were treated with NC or 100.0 μM Sal B for 48 h, the binding of mortalin to RECK was detected by immunoprecipitation. (D) HCCLM3 cells were transiently transfected with vector or Flag-mortalin for 8 h, and then the cells treated with 0 or 100 μM Sal B were incubated for an additional 24 h in fresh medium with 10% FBS. Western blot assays of E-cadherin, N-cadherin, Vimentin, Flag, RECK, p-STAT3, Ac-STAT3 and GAPDH were performed. MMP2 and MMP9 activities were investigated by gelatin

zymography assays. (E) HCCLM3 cells were subjected to the migration assays, and migrated cells were counted with Stat Monitor in photoshop (mean ± SD, n=3). *P < 0.05, statistically significant difference vs. untreated cells. Bars = 100 μm. #P < 0.05, statistically significant difference vs. si-NC cells treated with 100 μM Sal B.

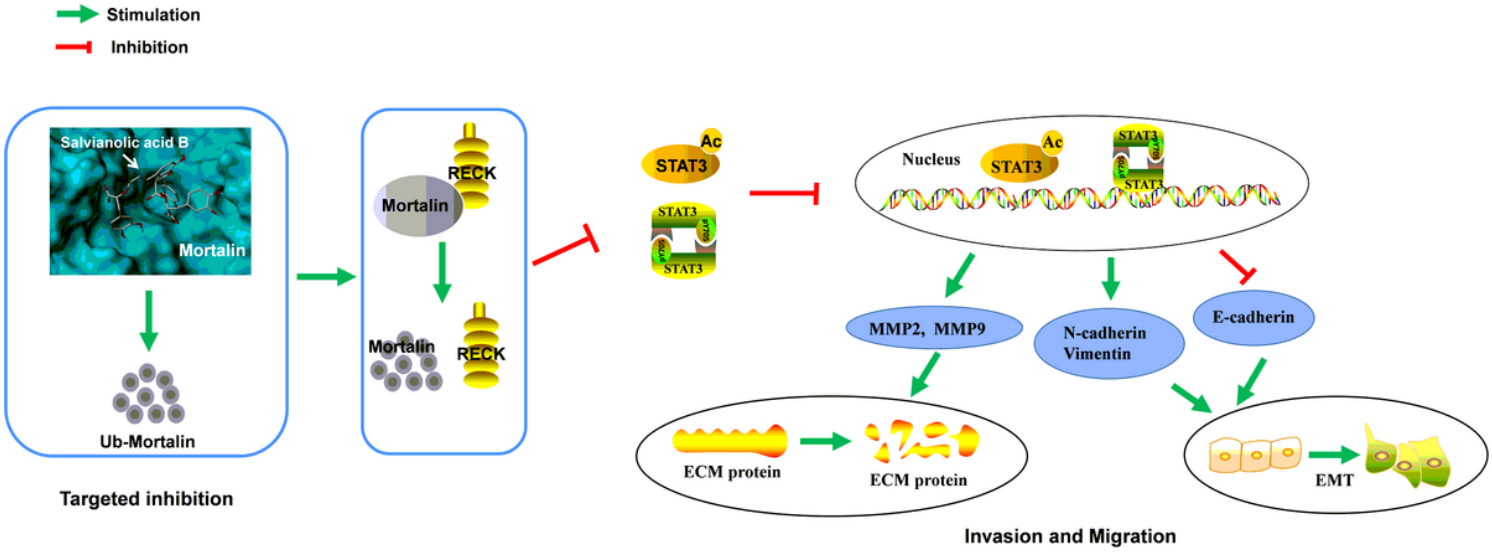

Figure 6

The signaling pathway was discoverd in hepatocellular carcinoma cells after Sal B treatment. Sal B can specifically bind to mortalin, and increased the degradation of mortalin proteasome through ubiquitination. The down-regulation of mortalin can lead to the up-regulation of RECK protein. Then its downstream p-STAT3 and Ac-STAT3 were inhibited to enter the nucleus, resulting in decrease of the invasive and migratory abilities of hepatocellular carcinoma cells.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- supplementaryinformation.docx
- originaldatamRNA.rar
- originaldata2.rar
- Orignaldata3.rar
- Orignaldata4.rar
- Orignaldata5.rar