SMP30 Regulates HCC Cell Migration via Affecting the Expression of MMPs and Binding to ROCK1

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SMP30 Regulates HCC Cell Migration via Affecting the Expression of MMPs and Binding to ROCK1

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Abstract

Background: SMP30, as a calmodulin, is differentially expressed in hepatocellular carcinoma (HCC) tissues and adjacent tissues. Previous studies have indicated that the expression of SMP30 is closely related to the prognosis of patients with HCC. However, little is known about the detailed role of SMP30 on HCC. **Methods:** The SMP30 overexpression and silenced cell lines were constructed through lentivirus transfection, the effects of SMP30 on migration and invasion of HCC were observed by Transwell assay. Then subcloned cell line with different migration abilities were undergone four rounds of screening from SMP30 overexpressing cell lines, and the transcriptome and proteome were detected and analyzed. The interactions of proteins with SMP30 were validated by AP-MS and COIP-WB techniques. **Results:** High expression of SMP30 can effectively inhibit the migration and invasion of HCC, but has no significant effect on cell proliferation and cell cycle. The cell lines with higher metastatic abilities have significantly higher calcium ion concentrations than those with lower metastatic abilities and higher expression of MMP9, MMP14 and MMP15 proteins. There is an interaction between SMP30 and tumor metastasis-related protein ROCK1, which reduces the phosphorylation level of cytoskeleton-related MLC protein. The SMP30 regulates the downstream molecule MLC protein phosphorylation and affects the formation of cytoskeletal structure.

Conclusion: On the one hand, SMP30 regulates the expression of MMPs protein by regulating the Ca^{2+} concentration; on the other hand, SMP30 affects the phosphorylation level of MLC protein by interacting with ROCK1 protein. The combined effect of the two aspects inhibits the metastatic ability of HCC.

Keywords

Hepatocellular Carcinoma (HCC), SMP30, MMPs, Ca^{2+} , ROCK1

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors, and the mortality rate is second only to gastric cancer and lung cancer^[1]. The pathogenesis of HCC is closely related to metabolic diseases such as chronic viral hepatitis, alcoholic hepatitis and liver cirrhosis, but the mechanisms of its occurrence and metastasis of HCC is still unclear^[2].

Our research group has successfully screened out HCC related antigen-senescence marker protein 30 (SMP30) which was first identified from Guangxi human hepatocellular carcinoma cDNA expression library using a serological analysis of recombinant cDNA expression library (SEREX) approach^[3]. The SMP30 protein is a novel calcium-binding protein that gradually decreases with age. The results of immunohistochemical analyses have demonstrated that SMP30 is generally showing low expression in tumor tissues and high expression in adjacent tissues. This is closely related to the shorter survival rate of patients. How this differential expression pattern affects the biological function of liver cancer cells is still not very clear^[4]. The main objective of this study is to confirm the effect of SMP30 on HCC through cell behavior experiments and further investigating the mechanisms of SMP30 on HCC.

Methods

1. Construction of cells with overexpressed and silenced SMP30.

The 5×10^5 cells per well were inoculated into 6-well culture plates (Corning, USA). The 5 μ l polybrene helper and 10 μ l lentivirus recombined SMP30 gene or SMP30 shRNA were added when cells reached 70% confluency. Fluorescence intensity was observed under fluorescence inverted microscope. The DMEM (Hyclone, USA) complete culture medium with 2.0 μ g/mL puromycin was used for cell screening. After 3 days of continuous screening, the inoculated cells were replaced with DMEM whole medium when each cell carried green fluorescence under a fluorescence microscope. The SMP30 overexpressed cells (SK-SMP30) and silenced cells (97L-shRNA) were successfully constructed.

2. Cell proliferation assay

The 96-well cell culture plate was used to seed cells (1000 cells/well) and incubated in different time (0, 24, 48, 72, 96 and 128 hrs). The 1 μ l of CCK8 solution (Dojindo, Japan) was added to each well and incubated for 2 hr in incubator. The absorbance values were measured daily by microplate reader at 450 nm and each assay was repeated three times.

3. Cell migration and invasion assays

Cells were collected and the concentration was adjusted to 1×10^5 cells/ml with serum-free medium. The 200 μ l of the cell suspension was added to the upper chamber of Transwell while 700 μ l of DMEM medium containing 10% FBS (Gibco, USA) was added to the lower chamber. In invasion assay, the matrix gel was diluted with serum-free DMEM (1:8) and added to the chamber before cell seeding. After culture

at 37°C for 24 hours, the liquid in the chamber was discarded, washed three times with PBS and then fixed in formaldehyde for 15min. Cells on the lower layer chamber surface of the membrane were stained with 0.1% crystal violet for 10 minutes, then washed twice with dddH₂O and air dried. Five random fields were observed under a 200-magnification microscope for cell counting, and the average number of cells in each field was calculated for cell migration and invasion ability evaluation.

4. Flow cytometry analysis of cell cycle

Cells were collected and washed twice with precooling PBS buffer and fixed overnight in 70% ice-cold ethanol at 4°C. The cells were centrifuged and resuspended in 400µl of cold PBS containing 20µl of RNaseA solution (Vazyme Biotech, China). The mixture was placed in a water bath at 37°C for half of an hour and then 400µl of PI staining solution (Vazyme Biotech, China) was added. After incubation for a further half of an hour at 4°C in the dark, the mixture was subjected to flow cytometry analysis.

5. Screening of subcloned cell line with different migration abilities in SMP30 overexpressed cells

Applying Transwell method, 1ml of SMP30 over expressed SK-Hep-1 cell suspension was added to the upper chamber (Corning, USA). The 2.5ml of DMEM complete medium was added to the lower chamber. Transwell plate was cultured at 37°C in 5% CO₂ humidified atmosphere for 24hrs. The cells stayed in the upper and lower chambers were extendedly cultured separately. The two groups of cells were subjected to the above-mentioned repeated Transwell operations to finally select a subcloned cell line (SK-Down) with strong migration ability and a subcloned cell line with weak migration ability (SK-Up).

6. Homogeneous and heterogeneous adhesion tests of different migration ability of subcloned cell line.

For homogeneous adhesion ability test, the SK-Up and SK-Down cells were adjusted to 100/ml in DMEM medium, then added to a 96-well cell culture plate with 100 μ l per well. Cells were cultured at 37°C until complete fusion. Culture medium was discarded and the cells were washed twice with D-Hanks preheated at 37°C. The 100ul of SK-UP and SK-Down cell suspensions with a concentration of 1×10^5 /ml were added to wells full of the same cells and cultured in a 5%CO₂ incubator for 60, 90 and 120min respectively. For heterogeneous adhesion ability test, Fibronectins (FN) with 50 μ g/ml was added to 96-well cell culture plate with 100 μ l per well, kept at 4°C overnight and 1%BSA was used as contrast base; the liquid in the wells was removed using pipette and then washed twice using D-Hanks preheated to 37°C; 100 μ l of 1%BSA was added to each well and then sealed and kept for 1 hour. The 100ul of SK-UP and SK-Down cell suspensions with a concentration of 1×10^5 /ml were added to wells and cultured for 60, 90 and 120min respectively. Both homogenous and heterogeneous adhesion tests, unbound cells were aspirated; 100uL DMEM medium and 10 μ l CCK-8 solution were added to each well, and the OD value of 450nm was determined after incubation for half of an hour.

7. Transcriptome analyses of subcloned cell line with different migration capacities.

Total RNAs from SK-Up and SK-Down cells were extracted to convert cDNA by reverse transcription method and the cDNA library was constructed to prepare for the next sequencing analysis. The 10 μ l library

cDNA was mixed with 10 μ l NaOH for 5 minutes at room temperature and 980 μ l of pre-cooled hybridization buffer was added to the mixture. The molecules in the library were combined with primers immobilized on the Flowcell for bridge PCR amplification to generate clusters. The generated Flowcell was transferred to the sequencing platform and the corresponding recipe was selected for sequencing.

8. Proteomics analyses of subcloned cell line with different migration capacities.

The total protein of SK-Up and SK-Down cells was extracted and quantified and 100 μ g of each protein was placed in a 1.5ml EP tube. Samples were processed and labeled according to the PierceTM T[®] Mass Labeling Kit (Pierce, USA) and the mixed labeled samples were fractionated using C18 chromatographic column. The sample after fully dissolved was loaded onto the mass spectrometer for identification.

9. Screening of interaction proteins of SMP30 by AP-MS technology.

The biotin-labeled Flag-SMP30 overexpressing cell SK-Hep-1-Flag-SMP30 (Flag-SMP30) and negative control cells (Control) were successfully constructed by using lentivirus as a vector. Streptavidin magnetic beads were added to the cellular protein, 4 $^{\circ}$ C overnight on a mixer, and the supernatant was discarded on a magnetic separator. Magnetic beads were washed with cell lysate for 3 times then washed with PBS again and eluted with biotin for 10min.

The eluate was precipitated with pre-cooled acetone and the precipitate was re-dissolved in the cell lysate. After that, an appropriate amount of Loading buffer was added, bathed in 100 $^{\circ}$ C water for 5min and SDS-PAGE electrophoresis was used for protein separation. After the

SDS-PAGE colloid was stained with coomassie bright blue, the heavy chain and light chain of the antibody were excised and all the remaining bands were placed in EP tube for liquid phase tandem mass spectrometry identification.

10. Reverse validation of the interacting proteins by CO-IP and WB experiments.

Plasmids (PCMV-Myc) with Myc-ROCK1 gene were constructed and transferred into SK-Hep-1-Flag-SMP30 cells and SK-Hep-1-Flag-SMP30-Myc-ROCK1 cells (SMP30-ROCK1) was successfully constructed. We added anti-Myc tagged protein antibody to the total protein extracted from SMP30-ROCK1 cells and placed it on a mixer at 4°C overnight. The protein A/G magnetic beads washed with PBS were added to the mixture and placed on a mixer at 4°C for 2hrs. Magnetic beads were washed 3 times with cell lysate and eluted with glycine. Loading Buffer was added to the eluent and magnetic beads respectively and boiled, centrifuged at 12000rpm for 5min, and the supernatant was subjected to Western Blot detection.

11. Detection of intracellular Ca^{2+} concentration

We use a commercially available QuantiChrom™ Calcium Assay Kit (BioAssay Systems, USA) and follow the relevant operation for quantitative calcium ion detection. The 5μl diluted standard substance and 5μl extracted cytoplasm from the cells of SK-Up and SK-Down were added to the 96-well plate respectively and 200μl working reagent was added to each well. Tap the orifice plate to mix and incubate at room temperature for 3 minutes. The absorbance values were read at 570-650nm.

12. Immunofluorescence assay.

SK-SMP30 and Control cells were seeded into cell culture plates, respectively. When the confluence of cells reached 50%, they were washed twice with pre-warmed PBS. Cells were fixed in 4% paraformaldehyde for 10 min at room temperature and washed twice with PBS. Subsequently, cells were permeabilized with 0.5% Triton-X-100 for 5 min. After washing with PBS twice, TRITC-labeled phalloidin (1: 1000; Yeasen) was added to the culture plate, and incubated at room temperature for 30 minutes in the dark. The cells were also washed with PBS, and 200ul of Hoechst 33342 solution (1: 100; Sigma) was added to counterstain the cells for 1 min. Finally, the cells were washed once with PBS, and the fluorescence was observed under a laser confocal microscope.

13. Statistical Analysis

The SPSS 17.0 software was adopted for statistical analysis in this study. The data obtained were expressed as mean $\pm$ standard deviation (SD) and T test was used for comparison between groups. The P-value of less than 0.05 was considered having statistical difference.

Results

1. The effects of SMP30 gene overexpression on the cellular behavior of SK-Hep-1.

In order to investigate the effects of SMP30 on SK-Hep-1 cell, we have successfully constructed SMP30 overexpressed cell lines (SMP30) and negative Control (Control) (Figure 1A.1B). Proliferation experiments of CCK8 have shown that SMP30 cannot effectively inhibit the proliferation of SK-Hep-1 cell (Figure 1C). Transwell experiments have found that the migration and invasion ability in SMP30 overexpressed group is

significantly lower than that of control group, indicating that SMP30 can effectively inhibit the migration and invasion ability of HCC (Figure 1D.1E). Cell cycle detections by flow cytometry have shown no significant difference in the proportion of G1, S and G2 cells between the two strains (Figure 1F).

Figure 1. Showing the effects of SMP30 overexpression on SK-Hep-1 cell behavior. A). qPCR tested relative expression level of SMP30mRNA after overexpression of the gene. B). WB tested

expression level of SMP30 protein after overexpression of SMP30 gene. C).CCK8 proliferation assay showing the effects of SMP30 on the proliferation of SK-Hep-1 cell. D). Transwell migration experiments confirming that SMP30 can effectively inhibit the migration ability of SK-Hep-1 cells. E). Transwell invasion assay showing that SMP30 has a certain inhibitory effect on the invasion ability of SK-Hep-1 cells. F). Flow cytometry results showing the effects of SMP30 on the cell cycle.

2. The effects of SMP30 gene silenced on the cellular behavior of 97L

We have also successfully constructed SMP30 silencing cell lines (97L-shRNA) and negative controls (Figure 2A.2B) in order to further investigate the effects of SMP30 on HCC cells. The CCK8 proliferation assay shows no significant abnormalities in cell proliferation after SMP30 is silenced (Figure 2C). Transwell assay shows that the migration and invasion ability of 97L-shRNA cells are significantly higher than those of the control group (Figure 2D.2E). We have employed flow cytometry to analyze the distributions of cell cycles. As shown in Figure 2F, there is no significant difference in the proportion of G1, S and G2 cells between the two groups.

Figure 2. Showing the effects of SMP30 gene silenced on 97L cell behavior. A). qPCR tested relative expression level of SMP30 mRNA after the gene silenced. B). WB tested expression of SMP30 protein after silenced of SMP30 gene. C). CCK8 experiment showing that the proliferation of 97L cell is not affected after SMP30 gene silenced. D). Transwell migration experiments showing that SMP30 can effectively inhibit the migration ability of 97L cells. E). Transwell invasion assay shows that SMP30 has a certain inhibitory effect on the invasive ability of 97L cells. F). Flow cytometry detects the effect of SMP30 on the cell cycle.

3. Screening subcloned cell line with different migration abilities

Two groups of subcloned cell lines with strong migration ability (SK-Down) and weak metastatic ability (SK-Up) have been successfully screened by four rounds screening of Transwell experiments. The expression levels of SMP30 protein in subcloned cell line are significantly different as shown in Figure 3A. Migration experiments confirmed that the migration ability of SK-Down cells was significantly higher than that of SK-Up cell lines (Figure 3B). Adhesion test results showed that the homophilic adhesion ability of SK-UP cell line was significantly stronger than that of SK-Down, but the heterophilic adhesion ability was weaker than that of SK-Down cell line (Figure 3C, 3D). Scanning electron microscopy has revealed that the pseudopodia of the SK-Up group are

significantly less than those of the SK-Down group(Figure 3E).

Figure 3. Screening and capacity verification of subcloned cell line with different migration abilities. A). Expression of SMP30 in two subcloned cell lines with different metastatic capacities. B). Transwell assay confirmed the migration ability of the two subcloned cell lines. C). Homophilic adhesion experiments of two groups of cell lines. D). Heterophilic adhesion experiments of two groups of cell lines. E). Pseudopods of two groups of cells observed under a scanning electron microscope.

4. Analyses of differentially expressed gene and protein in subcloned cell line with different migration abilities by transcriptome and proteome.

The genes with more than twice differential expressions in transcriptome have been analyzed. There are 302 differentially expressed genes in SK-Down and SK-Up groups, including 154 up-regulated genes and 148 down-regulated genes. Those differentially expressed genes are mainly involved in the cellular component of extracellular region and extracellular space, as shown in Figure 4A. Analyses of the differential proteins using the MADIL-TOF-TOF-MS is based on 1.5 times of the difference. Among them, 49 up-regulated proteins and 2 down-regulated proteins are closely related to Ca^{2+} signaling pathway and extracellular region components (Figure 4B).

Figure 4. Showing transcriptome and proteome detection of subcloned cell lines with different migration capacities. A). Analysis of mRNA expression of subcloned cell line with different migration abilities using transcriptome sequencing. B). The MADIL-TOF-TOF-MS has been used to analyze the protein expression of subcloned cell line with different migration abilities.

5. The association analyses of differentially expressed gene and protein in subcloned cell lines with different migration capacities.

Correlation analyses of differentially expressed gene and protein identified by transcriptome and proteome have shown that MMP9, MMP14 and MMP15 are differentially expressed as confirmed by data analyses. We have further used RT-PCR and Western Blot procedures to confirm that the expression levels of those three differential genes in high metastatic cells are significantly higher than that in low metastatic cells (Figure 5A and B). The MMP9, MMP14 and MMP15 belong to the matrix metalloproteinase family (MMPs), which can degrade the extracellular matrix and are closely related to the metastasis of tumor cells. Intracellular Ca²⁺ detections have revealed that the concentration of Ca²⁺ with high migration capacity was significantly increased (Figure 5 C).

Figure 5. Showing transcriptome and proteome association analyses of subcloned cell lines with different migration capacities. A). RT-PCR detection of relative mRNA expression levels of differential genes in SK-Down and SK-Up cell lines. B) Comparison of translation levels of tumor metastasis related genes in different migrating cells. C). Comparison of Ca²⁺ concentrations in cells with different metastatic capacities.

6. Screening and reverse validation of SMP30 interacting proteins

Bioinformatics analyses of the interaction protein obtained by affinity purification-mass spectrometry (AP-MS), ROCK1, a key gene related to tumor cell metastasis, has been screened out in the regulation of actin cytoskeleton signaling pathway, see Figure 6. We have used COIP-WB experiments to further confirm the interaction between SMP30 and ROCK1 (Figure 7A.B.C). Myosin light chain (MLC) is a downstream regulatory target gene of ROCK1, which is associated with the regulation of cell movement. WB results have shown that the phosphorylation levels of MLC have decreased after the expression of SMP30 increased, see Figure 7D. Meanwhile, we have also discussed the possible role of SMP30 in the formation of stress fibers. The SMP30 overexpression has significantly inhibited the formation of stress fibers, while cells with low expression of SMP30 have exhibited stronger stress fibers as illustrated in Figure 8.

Figure 6. Showing GO analyses and pathway enrichment analyses of differentially expressed genes obtained from AP-MS.

Figure 7. Showing verification of the interaction between SMP30 and ROCK1 protein. A). Anti-SMP30 antibody and anti-flag antibody were used to detect the expression of flag-SMP30 protein in SK-Hep-1-Flag-SMP30 cells. B). After Flag-SMP30 was transfected into SK-Hep-1, the protein was extracted for affinity purification. The expression of Flag-SMP30 before and after affinity purification detected by anti-Flag antibody. C). Myc-ROCK1 was transferred to SK-Hep-1-Flag-SMP30 cells. Anti-Myc antibody was used to detect the expression of Myc-ROCK1 in co-immunoprecipitation. Anti-Flag antibody was used to detect the expression of Flag-SMP30 protein in co-immunoprecipitation. D). Phosphorylation level of MLC was decreased after the overexpression of SMP30 gene.

Figure 8. Immunofluorescence was carried out to detect the stress fiber rearrangement. F-actin were stained by FITC-Phalloidine (red) and the nuclei were stained by Hoechst 33342 (blue).

Discussion

SMP30 is a new type of calcium-binding protein, which is involved in the regulation of various intracellular metabolic processes and is closely related to the occurrence and development of HCC^[5]. In the early stages, our research group have confirmed through immunohistochemistry that the expression levels of SMP30 in HCC tissues are significantly lower than that in the corresponding paracancerous tissues. The expression level of SMP30 is closely related to tumor size, grade and prognosis of patients^[6]. Previous studies have also confirmed that the proliferation and migration ability of HepG2 cells increased after SMP30 is silenced by shRNA^[7].

In this study, we have demonstrated the ability of SMP30 to inhibit the migration and invasion of HCC cells from the following aspects. First, Transwell assays have showed that SMP30 can effectively reduce the migration and invasion ability of SK-Hep-1 cells. Second, the results of

subcloned cell line adhesion experiments confirmed that SMP30 can increase the homogeneous adhesion capacity of tumor cells and reduce its heterogeneous adhesion capacity. Third, Immunofluorescence assay have shown that the tension fibers of SK-Hep-1 cells became thinner and the pseudopodia decreased after the introduction of SMP30 gene. At the same time, we observed by scanning electron microscopy that the pseudopods in subcloned cells with high expression of SMP30 were significantly less than that cells with low expression of SMP30.

HCC is a highly invasive tumor with intrahepatic proliferation and extrahepatic metastasis. Therefore, effective inhibition of tumor metastasis is very important for the prognosis of HCC^[8-9]. SMP30 can effectively inhibit the migration and invasion of liver cancer cells, but its mechanism is not yet clear. Based on literature reports and our research, we speculate that SMP30 can play a role in inhibiting the migration and invasion of liver cancer cells through the following two aspects. On one hand, we have screened out subcloned cell lines with different migration abilities and performed transcriptome and proteome sequencing analysis. Our studies have confirmed that the low level of SMP30 expression in SK-Down subcloned cell lines with strong metastasis ability led to the increase of intracellular Ca^{2+} concentration. Meanwhile, high concentration of Ca^{2+} induces cells to produce MMP9, MMP14 and MMP15, and participate in the regulation of extracellular matrix signaling pathway^[10-12]. Therefore, SMP30, as a calmodulin, can activate the cellular calcium pump. When the expression level of SMP30 decreases, the activity of the cellular calcium pump decreases and the intracellular Ca^{2+} concentration increases^[13-14]. High concentrations of intracellular Ca^{2+} promote the synthesis and release of MMPs family proteins and the MMPs protein family can degrade and damage collagen, fibrin, laminin and other extracellular matrix components, and play an important role in

cell invasion and metastasis^[15-17]. On the other hand, we have screened ROCK1 protein as SMP30 interacting protein by affinity purification-mass spectrometry (AP-MS) and confirmed the existence of interaction between SMP30 and ROCK1 through Co-Immunoprecipitation-Western Blot (COIP-WB). The ROCK1, similar to Rho (Ras homolog), is a small molecule protein with GTPase activity and a helical structure, which can phosphorylate myosin light chain (MLC) to cause contraction of cell myofilament^[18-19]. At the same time, ROCK1 can also phosphorylate MLCP. Phosphorylated MLCP cannot dephosphorylate MLC, and the contractile force of cell muscle filaments will be further increased^[20-21]. Therefore, we speculate that ROCK1 binding to SMP30, resulting in weakened intracellular tension fiber synthesis, decreased MLC phosphorylation level and reduced cell migration ability.

We believe that SMP30 can effectively reduce the migration ability of liver cancer cells. The interaction between SMP30 and ROCK1 regulates the synthesis of stress fibers and phosphorylation of MLC. At the same time, SMP30 regulates the expression of MMP and affects the dynamic equilibrium of the Extracellular Matrix (ECM) network. Therefore, SMP30 inhibits the migration ability of liver cancer cells through multiple effects on extracellular matrix regulation, cytoskeleton formation, and MLC phosphorylation levels.

Abbreviations

SMP30: Senescence marker protein 30; HCC: Hepatocellular carcinoma; SEREX: Serological analysis of recombinant cDNA expression library; FN: Fibronectins; ROCK1: Rho-associated protein kinase 1; MMPs: Matrix metalloproteinase family; AP-MS: Affinity purification-mass

spectrometry; MLC: Myosin light chain;

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Authors' contributions

SZ was responsible for the concept, curation, funding acquisition, writing the manuscript. SZ and YZ assisted with project administration, generated and analyzed experimental data. ZO, XL, ZL, assisted with analysis of experimental data. All authors read and approved the final manuscript.

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Availability of data and materials

All data analyzed and generated during the current study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

The Ethics approval and consent to participate of the current study was approved and consented by the ethics committee of Guangxi Medical University.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interest.

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References

- [1] Ferlay J, Colombet M, Soerjomataram I, Mathers C, Parkin DM, Piñeros M, Znaor A, Bray F. Estimating the global cancer incidence and mortality in 2018: GLOBOCAN sources and methods. *Int J Cancer*. 2019. 144(8): 1941-1953.
- [2] Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. *CA Cancer J Clin*. 2015. 65(2): 87-108.
- [3] Stenner-Liewen F, Luo G, Sahin U, Tureci O, Koslovski M, Kautz I, Liewen H, Pfreundschuh M. Definition of tumor-associated antigens in hepatocellular carcinoma. *Cancer Epidemiol Biomarkers Prev*. 2000. 9(3): 285-90.
- [4] Zhou SF, Mo FR, Bin YH, Hou GQ, Xie XX, Luo GR. Serum immunoreactivity of SMP30 and its tissues expression in hepatocellular carcinoma. *Clin Biochem*. 2011. 44(4): 331-6.
- [5] Ishigami A, Fujita T, Inoue H, Handa S, Kubo S, Kondo Y, Maruyama N. Senescence marker protein-30 (SMP30) induces formation of microvilli and bile canaliculi in Hep G2 cells. *Cell Tissue Res*. 2005. 320(2): 243-9.
- [6] Mo Z, Zheng S, Lv Z, Zhuang Y, Lan X, Wang F, Lu X, Zhao Y, Zhou S. Senescence marker protein 30 (SMP30) serves as a potential prognostic indicator in hepatocellular carcinoma. *Sci Rep*. 2016. 6: 39376.
- [7] Zhang SC, Liang MK, Huang GL, Jiang K, Zhou SF, Zhao S. Inhibition of SMP30 gene expression influences the biological characteristics of human Hep G2 cells. *Asian Pac J Cancer Prev*. 2014. 15(3): 1193-6.
- [8] Chen D, Li Z, Song Q, Qian L, Xie B, Zhu J. Clinicopathological features and differential diagnosis of hepatocellular carcinoma in extrahepatic metastases. *Medicine (Baltimore)*. 2018. 97(50): e13356.
- [9] Han TS, Ban HS, Hur K, Cho HS. The Epigenetic Regulation of HCC Metastasis. *Int J Mol Sci*. 2018. 19(12).
- [10] Sun SJ, Wang N, Sun ZW, Chen J, Cui HW. MiR-5692a promotes the invasion and metastasis of hepatocellular carcinoma via MMP9. *Eur Rev Med Pharmacol Sci*. 2018. 22(15): 4869-4878.
- [11] Li T, Xie J, Shen C, et al. miR-150-5p inhibits hepatoma cell migration and invasion by targeting MMP14. *PLoS One*. 2014. 9(12): e115577.
- [12] Inagaki Y, Shiraki K, Sugimoto K, Yada T, Tameda M, Ogura S, Yamamoto N, Takei Y, Ito M. Epigenetic regulation of proliferation and invasion in hepatocellular carcinoma cells by CBP/p300 histone acetyltransferase activity. *Int J Oncol*. 2016. 48(2): 533-40.
- [13] Lai P, Yip NC, Michelangeli F. Regucalcin (RGN/SMP30) alters agonist- and thapsigargin-induced cytosolic $[Ca^{2+}]$ transients in cells by increasing SERCA $Ca(2+)$ ATPase levels. *FEBS Lett*. 2011. 585(14): 2291-4.
- [14] Fujita T, Inoue H, Kitamura T, Sato N, Shimozawa T, Maruyama N. Senescence marker protein-30 (SMP30) rescues cell death by enhancing plasma membrane $Ca(2+)$ -pumping activity in Hep G2 cells. *Biochem Biophys Res Commun*. 1998. 250(2): 374-80.
- [15] Guo YS, Tang J, Chen B, Huang W, Li Y, Cui HY, Zhang X, Wang SJ, Chen ZN, Jiang JL. β ig-h3 regulates store-operated Ca^{2+} entry and promotes the invasion of human hepatocellular carcinoma cells. *Cell Biol*

- Int. 2011. 35(8): 811-7.
- [16] Jiang JL, Yao XY, Zhou J, Huang Y, Chen ZN. [Effect of Ca²⁺ mobilization on release and activation of matrix metalloproteinases in hepatocellular carcinoma cells]. *Zhonghua Zhong Liu Za Zhi*. 2004. 26(9): 525-7.
 - [17] Conlon GA, Murray GI. Recent advances in understanding the roles of matrix metalloproteinases in tumour invasion and metastasis. *J Pathol*. 2018 .
 - [18] Liu J, Wada Y, Katsura M, Tozawa H, Erwin N, Kapron CM, Bao G, Liu J. Rho-Associated Coiled-Coil Kinase (ROCK) in Molecular Regulation of Angiogenesis. *Theranostics*. 2018. 8(21): 6053-6069.
 - [19] Lin L, Li M, Lin L, Xu X, Jiang G, Wu L. FPPS mediates TGF- β 1-induced non-small cell lung cancer cell invasion and the EMT process via the RhoA/Rock1 pathway. *Biochem Biophys Res Commun*. 2018. 496(2): 536-541.
 - [20] Hu J, Niu M, Li X, Lu D, Cui J, Xu W, Li G, Zhan J, Zhang H. FERM domain-containing protein FRMD5 regulates cell motility via binding to integrin β 5 subunit and ROCK1. *FEBS Lett*. 2014. 588(23): 4348-56.
 - [21] Vega FM, Ridley AJ. Rho GTPases in cancer cell biology. *FEBS Lett*. 2008. 582(14): 2093-101.

Figures

Figure 1

Showing the effects tested relative expression level of 9 control group, indicating that HCC).detections by flow cytometry haves of SMP30 overexpression on SK-Hep-1 cell behavior elative SMP30mRNA after overexpression of the gene. B ontral SMP30 can (Figure shown no behavior. A). qRCR B). WB tested expression level of SMP30 protein after overexpression of SMP30 gene. C).CCK8 proliferation assay showing the effects of SMP30 on the proliferation of SK-Hep-1 cell. D) Transwell migration experiments confirming that SMP30 can effectively inhibit the migration ability of SK-Hep-1 cells. E). Transwell invasion assay showing that SMP30 has a certain inhibitory effect on the invasion ability of SK-HEP-1 cells. F). Flow cytometryresults showing the effects of SMP30 on the cell cycle.

Figure 2

Showing the effects of SMP30 gene silenced on 97L cell behavior. A). qPCR tested relative expression level of SMP30 mRNA after the gene silenced. B). WB tested expression of SMP30 protein after silenced of SMP30 gene. C). CCK8 experiment showing that the proliferation of 97L cell is not affected after SMP30 gene silenced. D). Transwell migration experiments showing that SMP30 can effectively inhibit the migration ability of 97L cells. E). Transwell invasion assay shows that SMP30 has a certain inhibitory effect on the invasive ability of 97L cells. F). Flow cytometry detects the effect of SMP30 on the cell cycle.

Figure 3

Screening and capacity verification of subcloned cell line with different migration abilities. A). Expression of SMP30 in two subcloned cell lines with different metastatic capacities. B). Transwell assay confirmed the migration ability of the two subcloned cell lines. C). Homophilic adhesion experiments of two groups of cell lines. D). Heterophilic adhesion experiments of two groups of cell lines. E). Pseudopods of two groups of cells observed under a scanning electron microscope.

Figure 4

Showing transcriptome and proteome detection of subcloned cell lines with different migration capacities. A). Analysis of mRNA expression of subcloned cell line with different migration abilities using

transcriptome sequencing. B). The MADIL-TOF-TOF-MS has been used to analyze the protein expression of subcloned cell line with different migration abilities.

Figure 5

Showing transcriptome and proteome association analyses of subcloned cell lines with different migration capacities. A). RT-PCR detection of relative mRNA expression levels of differential genes in SK-Down and SK-Up cell lines. B) Comparison of translation levels of tumor metastasis related genes in different migrating cells. C). Comparison of Ca²⁺ concentrations in cells with different metastatic capacities.

Figure 6

Showing GO analyses and pathway enrichment of differentially expressed genes obtained from AP-MS.

Figure 7

Showing verification of the interaction between SMP30 and ROCK1 protein. A Anti-SMP30 antibody and anti protein in SK-Hep-1-Flag-SMP30 cells protein was extracted for affinity purification. The expression of Flag affinity purification detected by anti SK-Hep-1-Flag-SMP30 cells. Anti Myc-ROCK1 in co-immunoprecipitation. Anti Flag-SMP30 protein in co-immunoprecipitation. after the overexpression of SMP30 gene.

Figure 8

Immunofluorescence was carried out to detect the stress fiber rearrangement. F-actin were stained by FITC-Phalloidine (red) and the nuclei were stained by Hoechst 33342 (blue).