STAT3 Silencing Enhances Sorafenib Efficacy in SK-Hep1 Hepatocellular Carcinoma Cells: A Preliminary Study Postprint

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Abstract

Objective: To investigate the role of STAT3 in sorafenib resistance of hepatocellular carcinoma SK-Hep1 cells and to explore the potential of STAT3 gene silencing in enhancing sorafenib efficacy against hepatocellular carcinoma.

Methods: shRNA-based gene silencing technology was employed to knock down STAT3 in SK-Hep1 hepatocellular carcinoma cells. Cell growth and sorafenib sensitivity were assessed using the CCK-8 assay. Western blot analysis was performed to detect expression changes in STAT3, p-STAT3 (Y705), p-STAT3 (S727), and their downstream proteins.

Results: We successfully established the STAT3-knockdown cell line SK-Hep1-shSTAT3. In these cells, STAT3 protein expression was reduced and cell proliferation was significantly inhibited. Sorafenib treatment downregulated STAT3 phosphorylation levels and the expression of downstream proteins Mcl-1 and Cyclin D1. STAT3-knockdown SK-Hep1 cells exhibited enhanced sensitivity to sorafenib.

Conclusion: shRNA-based STAT3 gene silencing significantly inhibits SK-Hep1 cell proliferation and enhances cellular sensitivity to sorafenib, representing a promising novel strategy to improve sorafenib efficacy in hepatocellular carcinoma treatment.

Full Text

A Preliminary Study on Enhancing the Efficacy of Sorafenib by Silencing the STAT3 Gene in SK-Hep1 Hepatocellular Carcinoma Cells

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Abstract

Objective: To investigate the role of STAT3 in sorafenib resistance in hepatocellular carcinoma SK-Hep1 cells and explore whether STAT3 gene silencing can enhance the therapeutic efficacy of sorafenib.

Methods: STAT3 expression was knocked down in SK-Hep1 cells using shRNA-based gene silencing technology. Cell proliferation and sorafenib sensitivity were assessed using the CCK-8 assay. Western blot was employed to detect the expression levels of STAT3, p-STAT3(Y705), p-STAT3(S727), and their downstream target proteins.

Results: The STAT3-knockdown cell line SK-Hep1-shSTAT3 was successfully established, showing reduced STAT3 protein expression and significantly inhibited cell proliferation. Sorafenib treatment decreased STAT3 phosphorylation levels and downregulated the expression of downstream proteins Mcl-1 and CyclinD1. SK-Hep1 cells with STAT3 knockdown exhibited enhanced sensitivity to sorafenib.

Conclusion: shRNA-mediated STAT3 gene silencing significantly suppresses SK-Hep1 cell proliferation and increases cellular sensitivity to sorafenib, suggesting a potential novel strategy for improving sorafenib efficacy against hepatocellular carcinoma.

Keywords: Hepatocellular carcinoma; Sorafenib; STAT3; Gene silencing

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Introduction

Sorafenib (Nexavar) is a targeted therapeutic agent recently approved for clinical treatment of advanced hepatocellular carcinoma (HCC), capable of simultaneously inhibiting multiple kinases including Raf-1, B-Raf, and VEGFR [1]. Although this oral multikinase inhibitor can prolong survival in some patients

with advanced HCC, its therapeutic efficacy varies considerably among different patients [2], and the outcomes remain unsatisfactory for most HCC patients [3]. Therefore, investigating the drug resistance mechanisms in different HCC cell types is of paramount significance for providing a basis for personalized precision therapy.

Signal transducer and activator of transcription 3 (STAT3) is a transcription factor also considered an oncogene. STAT3 is constitutively activated in cancer cells but requires induced activation in normal hepatocytes, suggesting that STAT3 could serve as a therapeutic target for HCC [4-6]. Moreover, STAT3 activation is essential for HCC cell survival, and its knockout induces cell death, making gene knockdown an appropriate approach for studying STAT3 function in HCC cells [7]. Importantly, normal hepatocyte survival does not depend on STAT3, indicating that STAT3-targeted therapy for HCC would be safe [8].

Literature also supports that sorafenib' s therapeutic mechanism in gliomas [9], liver fibrosis [10], and non-small cell lung cancer [11] involves inhibiting STAT3 activation and downregulating STAT3-associated signaling pathways. However, whether the STAT3 gene is a critical factor in sorafenib resistance of HCC cells remains unknown.

Therefore, this study investigated whether STAT3 knockdown enhances the sensitivity of SK-Hep1 cells to sorafenib, explored the role of STAT3 in sorafenib drug resistance in HCC cells, and provided a theoretical basis for targeting STAT3 to improve sorafenib efficacy against HCC.

Materials and Methods

1.1 Materials Human embryonic kidney cells HEK293T, human hepatocellular carcinoma cell line SK-Hep1, and lentiviral packaging plasmids (pREV, pVSVG, and pMDL) were kindly provided by the State Key Laboratory of Cellular Stress Biology, Xiamen University. STAT3-shRNA plasmids were purchased from Shanghai GeneChem Co., Ltd. Sequencing services were provided by Xiamen Minbo Biotechnology Co., Ltd. Sorafenib was obtained from Santa Cruz Biotechnology (USA). The CCK-8 cell viability assay kit was purchased from Beijing TransGen Biotech Co., Ltd. Fetal bovine serum was from Biological Industries (Israel). DMEM medium and other cell culture reagents, as well as TurboFect transfection reagent, were from Thermo Fisher Scientific (USA). Dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich (USA). All other reagents were from Shanghai Sangon Biotech Co., Ltd. Antibodies against STAT3, Mcl-1, and CyclinD1 were from Wuhan Sanying Biotechnology; p-STAT3(Tyr705) antibody was from Cell Signaling Technology (USA); p-STAT3(Ser727) antibody was from Shanghai Sangon Biotech; GAPDH antibody was from Beijing Zhongshan Golden Bridge Biotechnology; and HRP-conjugated secondary antibodies were from Fuzhou Kenuo Biotechnology.

1.2.1 Cell Culture and Treatment HEK293T cells, human hepatocellular carcinoma SK-Hep1 cells, and SK-Hep1-shSTAT3 cells were cultured in DMEM high-glucose medium supplemented with 10% fetal bovine serum. Cells were maintained at 37°C in a 5% CO incubator and passaged using 0.25% trypsin.

1.2.2 Construction of SK-Hep1-shSTAT3 Cell Line The purchased STAT3 knockdown plasmid pLv-shSTAT3 and lentiviral packaging plasmids (pREV, pVSVG, and pMDL) were mixed at a 3:1:1:1 ratio in 200 l serum-free DMEM to prepare a DNA mixture, with empty vector used as a negative control. The mixture was transfected into HEK293T cells using TurboFect transfection reagent for virus packaging. Viral supernatants were collected 48 hours later. SK-Hep1 cells were infected with a 1:1 mixture of viral supernatant and complete medium containing 10 g/ml polybrene. After 12-24 hours, cell morphology was observed and the medium was replaced with complete medium. Infection efficiency was assessed 48 hours later by observing green fluorescence under a fluorescence microscope. SK-Hep1 cells infected with STAT3 shRNA-expressing or control empty vector viruses were designated as SK-Hep1-shSTAT3 and control cells, respectively.

1.2.3 Western Blot Analysis of Protein Expression After cell counting using Countstar, cells were collected, lysed, and total protein was extracted. Proteins were separated by SDS-PAGE gel electrophoresis, transferred to PVDF membranes, blocked, and incubated with primary antibodies (1:1000) overnight at 4°C. Membranes were then incubated with secondary antibodies (1:10,000) for 1 hour at room temperature and visualized using ECL reagent.

1.2.4 CCK-8 Assay for Cell Viability Logarithmic-phase SK-Hep1 cells were seeded into 96-well plates at 10,000 cells per well with three replicate wells per condition and cultured under standard conditions. The following day, after cells had attached, experimental groups were treated with sorafenib at a final concentration of 5 mol/L, while control groups received an equivalent volume of DMSO for various durations. Alternatively, cells were treated with different concentrations of sorafenib for 48 hours. After treatment, the medium was removed and replaced with 100 l of fresh medium containing 10% (v/v) CCK-8 reagent, followed by incubation for 1 hour. Absorbance values (A) were measured at 450/630 nm dual wavelengths using a microplate reader. Results were plotted with time on the x-axis and relative absorbance values on the y-axis (for growth curves, relative absorbance = A value at each time point / A value at 0 h; for dose-response curves, relative absorbance = A value of drug-treated group / A value of vehicle group).

1.2.5 Statistical Analysis Statistical analysis was performed using SPSS 22 software. Data are presented as mean ± SD. Comparisons between experimental and control groups were made using independent samples t-test, with $p < 0.05$ considered statistically significant.

Results

2.1 Construction of STAT3-Knockdown SK-Hep1 Cell Line To elucidate the role of STAT3 in sorafenib resistance in SK-Hep1 cells, we knocked down STAT3 expression using RNA interference technology. We designed four STAT3 shRNA sequences and employed viral packaging and infection techniques to construct control and STAT3 shRNA-expressing SK-Hep1 cell lines. Western blot analysis of STAT3 total protein and phosphorylation levels in control and shRNA groups revealed that shRNA groups showed significantly decreased STAT3 protein expression and phosphorylation levels. Two shRNA sequences demonstrated particularly effective knockdown, with target sequences 5' -GCTGACCAACAATCCCAAGAA-3' and 5' -GCAAAGAATCACATGCCACTT-3' (Figure 1 [Figure 1: see original paper]; results for the other two shRNAs are not shown). These results demonstrate that STAT3 shRNA effectively inhibits STAT3 expression and that silencing the STAT3 gene can reduce STAT3 phosphorylation levels.

2.2 Effects of Sorafenib on STAT3 in SK-Hep1 Cells Previous studies have reported that sorafenib inhibits STAT3 phosphorylation in various cancer cells [23]. We confirmed this phenomenon in the hepatocellular carcinoma cell line SK-Hep1. After stimulating SK-Hep1 cells with different concentrations of sorafenib for 24 hours, we observed that STAT3 phosphorylation levels decreased with increasing sorafenib concentration, while total STAT3 protein remained unchanged (Figure 2 [Figure 2: see original paper]). This suggests that STAT3 plays an important role in sorafenib efficacy.

2.3 Effect of STAT3 Knockdown on Sorafenib Sensitivity in SK-Hep1 Cells Clinical studies have shown that increasing sorafenib dosage leads to adverse reactions [12], and our preliminary experiments revealed that SK-Hep1 cells are resistant to sorafenib [13]. Therefore, we investigated whether STAT3 knockdown could enhance sorafenib efficacy in SK-Hep1 cells. As shown in Figure 3A [Figure 3: see original paper], the proliferation of STAT3-knockdown SK-Hep1 cells (shSTAT3+DMSO) was significantly lower than that of control cells (Vector+DMSO), with the difference increasing over culture time. Furthermore, treatment with low-concentration sorafenib (5 mol/L) combined with STAT3 knockdown (shSTAT3+Sorafenib) resulted in significantly slower growth compared to either knockdown alone (shSTAT3+DMSO) or sorafenib treatment alone (Vector+Sorafenib) ($p < 0.05$).

After treating SK-Hep1 cells with various sorafenib concentrations for 48 hours, cell viability was assessed using the CCK-8 assay. STAT3-knockdown cells showed significantly reduced viability compared to control cells (Figure 3B [Figure 3: see original paper], $p < 0.01$). Notably, at a sorafenib concentration of 2.5 mol/L, STAT3-knockdown cells exhibited a two-fold reduction in viability

compared to non-knockdown cells. These results indicate that downregulating STAT3 decreases SK-Hep1 cell viability and increases sensitivity to sorafenib.

2.4 Effects of Sorafenib on Expression of STAT3 Downstream Genes Mcl-1 and CyclinD1 Mcl-1 and CyclinD1 are known downstream targets of STAT3 [14-17]. We treated STAT3-knockdown and control SK-Hep1 cells with 10 mol/L sorafenib and found that sorafenib-induced decreases in STAT3 phosphorylation positively correlated with downregulation of Mcl-1 and CyclinD1. Combined STAT3 knockdown and sorafenib treatment significantly reduced the expression of p-STAT3(Y705), p-STAT3(S727), Mcl-1, and CyclinD1 in a time-dependent manner (Figure 4 [Figure 4: see original paper]). These results suggest that sorafenib may affect cell apoptosis and proliferation by regulating the expression of the cell cycle protein CyclinD1 and the anti-apoptotic protein Mcl-1 through STAT3.

Discussion

Sorafenib is currently the only FDA-approved targeted drug for advanced HCC. However, due to the complex pathogenesis and heterogeneity of liver cancer [18], its therapeutic efficacy is suboptimal. Therefore, identifying effective targets and biomarkers to enhance sorafenib efficacy is critical for advanced HCC treatment. Using gene silencing technology, we reduced STAT3 expression in sorafenib-resistant SK-Hep1 cells and demonstrated that STAT3 silencing enhances sensitivity to sorafenib. Our findings also suggest that high STAT3 expression or activity may predict sorafenib resistance in tumor cells. Thus, STAT3 expression levels or phosphorylation status could help guide clinical identification of target populations for targeted therapy, enabling optimal individualized and precision treatment.

Previous reports indicate that sorafenib inhibits STAT3 activity to regulate downstream proliferation- and apoptosis-related genes (e.g., Mcl-1, Bcl-2, Cyclin D and E, Survivin), thereby suppressing tumor cell proliferation and promoting apoptosis [19]. Our study confirms that in STAT3-knockdown SK-Hep1 cells, the downstream anti-apoptotic protein Mcl-1 and the proliferation-promoting cyclin CyclinD1 are both reduced.

Clinical trials have shown that sorafenib-related adverse reactions increase with dosage, forcing dose reduction or treatment interruption and severely limiting clinical efficacy [20]. For patients insensitive to sorafenib, simply increasing the drug dose is clearly inappropriate. Our results demonstrate that sorafenib treatment of STAT3-knockdown cells is more effective than either STAT3 knockdown or sorafenib treatment alone. This suggests that for HCC cells insensitive to sorafenib, combination therapy targeting STAT3 expression or activity—without increasing sorafenib dosage—may have synergistic effects to promote cell death. This provides theoretical guidance for future individualized and precision ther-

apy to improve efficacy. While in vivo RNA interference requires viral vectors that can trigger host immune responses and even cause tumorigenesis, our results suggest that clinically, STAT3 inhibitors or inhibitors of the upstream JAK kinase family could be used synergistically with sorafenib to enhance sorafenib-induced cell death and improve therapeutic outcomes.

To further elucidate the mechanism of STAT3 in sorafenib-induced HCC cell death, future studies should examine downstream effectors of STAT3, particularly inflammatory factors, and investigate the type of cell death involved—whether apoptosis, necrosis, pyroptosis, or ferroptosis. Additionally, establishing a mouse HCC model to evaluate the therapeutic effect of combined STAT3 inhibition and sorafenib treatment is warranted. Studies have reported that aberrant activation of the HGF pathway can confer sorafenib resistance [21], and that sorafenib can inhibit HGF-induced EMT and cell migration [22]. Therefore, future research clarifying STAT3' s role in other pathways such as HGF and its effects on EMT and migration in HCC cells will help elucidate the underlying mechanisms. While sorafenib-mediated STAT3 downregulation is considered a novel mechanism for HCC therapy [23], our study also demonstrates that STAT3 inhibition enhances sorafenib-induced cell death. These seemingly contradictory observations suggest that STAT3 acts as a "bumper" in sorafenib treatment of HCC: sorafenib reduces STAT3 activity, but once cells lose this "bumper," they become more sensitive to sorafenib.

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23. [Reference incomplete in original]

Note: Figure translations are in progress. See original paper for figures.