Down-regulations of both HSP27 and TGF-β expression overcome the resistance of hepatocellular carcinoma cells to Sorafenib

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Down-regulations of both HSP27 and TGF-β expression overcome the resistance of hepatocellular carcinoma cells to Sorafenib

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2011년 처음 실험실에 들어왔을 때가 엊그제 같은데 벌써 5년이란 시간이 지나 드디어 졸업을 앞두고 감사의 글을 쓰게 되였습니다. 많은 분들의 도움으로 유학생 생활을 즐겁고 충실하게 보낼 수 있었습니다. 이 글을 통해 조금이나마 그분들께 감사한 마음을 전하고자 합니다.

먼저 부족한 저를 학생으로 받아주시고 많은 새로운 지식을 배울 수 있는 기회를 주시고 항상 격려해주신 김주항 교수님께 감사 드립니다. 직접 실험에 대한 방향을 제시해주시고 저의 부족한 부분에 대해서 따끔한 충고와 함께 늘 칭찬도 잊지 않으시고 생활 방면에서도 항상 관심을 주신 송재진 교수님께도 감사 드립니다. 그리고 항상 저의 연구 방향

과 내용에 대하여 조언을 해주시고 진행 과정에 많은 도움을 주시고 유학 생활에서도 많은 도움을 주시고 저의 지도 교수님을 맡아 주신 최혜진 교수님께 감사 드립니다. 또한, 제 논문을 심사하시면서 저의 연구 내용에 대한 조언과 격려를 아끼지 않으셨던 이재면 교수님과 허만욱 교수님께 진심으로 감사를 드립니다.

한국에서 유학 생활을 시작했을 때 많은 걱정이 앞서기도 했지만 좋은 선후배와 동료를 만나 실험실 생활을 즐겁게 해 나갈 수 있었습니다. 언제나 귀찮은 내색 없이 저에게 많은 실험 기법과 지식을 가르쳐준 소영이, 지금은 다른 실험실에 계시지만 항성 많이 걱정해 주시고 챙겨 주신 보경 누나, 실험에 대한 조언과 도움을 많이 주고 항상 즐거운 분위기

를 만들면서 시간을 함께 하였던 지나씨와 수진씨, 예의 바르고 성실하고 열심히 실험실 생활을 하였던 은경씨, 승하씨, 수연씨, 같은 고향에서 와서 서로 힘이 되고 많은 격려를 해준 철수, 시간이 짧지만 친동생처럼 관심해주시고 많은 도움을 주시고 조언을 해주신 경주 누나, 졸업에 대한 압박감으로 실험이 가장 힘들 때 도움을 준 연수, 최근에 연구실에 왔지만 열심히 배우려하고, 착하고 성실한 지현이 모두에게 진심으로 감사의 마음을 전합니다. 그리고 항상 옆 방 연구실에서 저에게 많은 것을 알려주시고 많은 도움을 주시고 따뜻하게 대해주신 박규현 선생님, 김태수 선생님께도 감사를 드립니다.

제가 어떠한 선택을 하고 결정을 하더라도 언제나 저를 응원해 주시고 격려해주신 부모님, 언제나 희

로애락을 함께하고, 저를 사랑해 주는 아내, 영원히 사랑하고 감사합니다.

제가 중국에서 한국으로 유학 와서 공부를 할 수 있고 성과를 이룰 수 있는 길을 마련해주신 심웅호 과장님과 장송남 선배님께도 감사 드립니다.

한국 유학생활을 보내면서 많은 일들을 배울 수 있었고 좋은 추억을 쌓을 수 있었습니다. 여기서 보낸 시간은 앞으로 중국에서 제가 발전하는데 있어서 커다란 밑거름이 되고 추진력이 될 것입니다. 배운 지식을 토대로 나가서 더 많은 것을 배우고 훌륭한 의사가 되여 더 좋은 치료방법으로 많은 환자들의 고통을 치료 해드릴 수 있도록 노력하겠습니다.

2017년 1월 강 동 욱 올림

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<ABSTRACT>

Down-regulations of both HSP27 and TGF-β expresion overcome the resistance of hepatocellular carcinoma cells to Sorafenib

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(Directed by Professor Hye Jin Choi)

Hepatocellular carcinoma (HCC) is one of the most common malignances worldwide. Although there have been developments in surgical strategies and percutaneous techniques, such as ethanol injection and radiofrequency ablation and transcatheter arterial chemoembolization (TACE), the overall outcome for HCC patients remains poor, as HCC is commonly detected at a late stage, when therapeutic options are limited. These patients show extremely poor prognoses, where the overall 5-year survival rate is poor. More than 630,000 patients die from HCC annually, representing the third highest number of cancer-related deaths. Therefore, it is necessary to improve anticancer therapies that effectively and specifically target liver

tumor cells.

Sorafenib, a multikinase inhibitor, is the standard therapy for patients with advanced-stage HCC. Although the results of sorafenib for patients with advanced HCC are encouraging, the benefits of sorafenib are limited by the low response rates of HCC cells, owing to a resistance to the treatment. Therefore, it is essential to develop more effective therapeutic strategies to overcome resistance and improve the efficacy of sorafenib in treating HCC patients.

In this study, we tried to unravel the mechanism underlying the resistance of HCC cells to sorafenib via the development of a novel anti-cancer gene therapy by using two short hairpin RNAs (shRNAs) against heat shock protein 27 (HSP27) and transforming growth factor-β (TGF-β) delivered by an adenovirus.

We found that p38MAPkinase activity was inhibited by low concentrations of sorafenib, which could potentially lead to sorafenib resistance in HCC cell lines. Subsequently, we used constitutive form of MKK3/6(MKK3/6E) to confirm that massive cell death was induced by the activation of p38, which demonstrated the ability to activate p38 without any stimulation. In addition, sorafenib resistance was reduced by the activation of p38. Then we used shRNAs against

TGF-β and HSP27 to increase the activity of p38 and overcome sorafenib resistance in HCC cell lines.

TGF-β expression was decreased by treatment with high concentration of sorafenib, thereby inducing effective cell death. Subsequently, we confirmed that TGF-β shRNA effectively recovered the phosphorylation of p38 inhibited by sorafenib, and increased the sensitivity of HCC cells to sorafenib, thereby inducing cell death and overcoming the resistance of HCC cells to sorafenib. We also observed a HSP27 shRNA-induced reduction in the expression of HSP27, a protein involved in general resistance, which also enhanced the cell death response of HCC cells to sorafenib.

There are many features of adenoviruses make them well suited for gene delivery, as recombinant adenoviruses can be grown to high titers, and have a relatively high capacity for transgene insertion, usually without the incorporation of viral DNA into the host cell genome.

The co-expression of both the shTGF-β and shHSP27 delivered by adenovirus, combined with low concerntration of sorafenib, effectively recovered p38 activity and the phosphorylation of HSP27 (p-HSP27), and resulted in a reduction in the levels of HSP27 protein,

all of which synergistically lead to HCC cell death.

Our study provides a new therapeutic strategy for HCC that not only overcomes the resistance of HCC to sorafenib, but also utilizes cutting edge of gene therapies.

Keywords: HCC, sorafenib, resistance, TGF-β, HSP27, p38, adenovirus

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I. INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignancies across the globe, especially in Asia and southern Africa[1]. Via a diagnosis followed by systemic examination, HCC can be classified into early, mid, and late (advanced) stages. Surgical resection, liver transplantation[2], or local ablation, have been generally used to treat early- and mid-stage HCC, where the 5-year survival rate could be as high as to 60~70%. However, owing to the lack of effective treatment options and underlying liver disease, patients who are diagnosed at an advanced stage or with progression experience a much more dismal prognosis after locoregional therapy[3]. Until sorafenib was used as a first-line therapy, there was no systemic therapy to improve survival in patients with late-stage HCC.[4,5,6]

Sorafenib, which has anti-proliferative and anti-angiogenic effects, is an oral multikinase inhibitor. It can inhibit several cellular signaling pathways, including those of Raf/mitogen-activated protein kinase (MAPK), extracellular signaling-regulated kinase (ERK), as well as the receptors of vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF). As the standard care for patients with advanced HCC, sorafenib has been proven to have the capacity to inhibit HCC cell proliferation, angiogenesis, and so on.[7,8,9] However, the promising treatment of HCC with sorafenib has limited benefits to survival and very low rates of tumor response, where some patients with HCC even exhibit no initial response to sorafenib[3,10], indicating the existence of both primary and acquired resistances of HCC cells to sorafenib[11]. The primary resistance of HCC to sorafenib has been shown to be attributed to genetic heterogeneity.[9] While sorafenib inhibits several cellular signaling pathways as a multikinase inhibitor, it simultaneously or sequentially activates the addiction switches and compensatory pathways.[9] As for the acquired resistance of HCC cells to sorafenib, although several mechanisms have been proposed, the exact mechanisms underlying sorafenib resistance in HCC cells remain unclear.[12,13]

The multifunctional cytokine transforming growth factor-β (TGF-β) orchestrates an intricate signaling network to modulate tumorigenesis and progression by exerting a dynamic effect on cancer cells.[14] Early in the carcinogenesis process, TGF-β1 suppresses tumors and arrests cell growth,

whereas in later and more advanced tumor stages, TGF-β1 potentiates epithelial-to-mesenchymal transition (EMT), angiogenesis, tumor progression, invasion, and metastasis. Previous studies have reported that TGF-β1 was overexpressed in HCC cells.[15] In clinical studies, the levels of TGF-β1 in blood have been shown to be higher in patients with HCC than in patients with chronic hepatitis or cirrhosis. HCC cells with a higher IC50 in response to sorafenib tended to have higher TGF-β1 mRNA expression.[15] A previous study reported that TGF-β2 was overexpressed in some HCC cell lines and patient cohorts and found a correlation between high TGF-β2 expression and lower survival rates ($p < 0.01$) by analyzing patients with defined TGF-β2 regulation in GSE1898/4024.[16] Thus, the TGF-β signaling pathway should be explored as a therapeutic target for patients with advanced HCC.

MAPKs are essential components of intracellular signal transduction, in which p38MAPK plays an essential role in the regulation of gene expression and in controlling cellular responses to the environment, cell growth, and apoptosis. These features have previously made p38MAPK a molecular target for drug development in the treatment of many human diseases, and most notably in the treatment of a variety tumors.[17] The involvement of the p38MAPK cascade in the apoptotic pathway has been demonstrated in two hepatoma cell lines, HepG2 and HuH7, wherein the activation of p38MAPK inhibited cell growth and induced apoptosis.[18] The apoptosis of

hepatoma cell lines is the result of increased caspase 3 activity via the activation of p38MAPK. It is reported that Bid cleavage and cytochrome c release was modulated by p38MAPK activation to induce apoptosis in human hepatoma cell lines.[18] In human HCC patients, the activities of p38MAPK and MKK6 were significantly lower in comparison to adjacent uninvolved liver tissue,[18] whereas the activity of ERK1/2 was significantly increased in malignant lesions.[19] Treatment with sorafenib has been shown to inhibit p38α kinase activity in vitro by targeting the DFG-out conformational state of p38α.[20,21,22,23] The reduction in p38 activity as a result of sorafenib treatment could be one of the causes of resistance, and should be a potential target to induce more effective cell death in HCC patients.

Heat shock protein 27 (HSP27), an important chaperone molecule, belongs to the heat shock protein family, which is responsible for modulating more than 200 client proteins.[24] The most common functions of HSP27 are chaperone activity, thermotolerance, inhibition of apoptosis, regulation of cell development, and cell differentiation. It has also been reported that HSP27 functions as a pro-metastatic protein in different types of cancer and is closely linked to the aggressiveness of tumor behavior, metastasis, and poor prognosis.[25,26] HSP27 is overexpressed in many different types of human cancers, including prostate, ovarian, gastric, breast, and liver cancers.[24] HSP27 accumulation has been shown to reduce the chemosensitivity induced by vincristine and adriamycin in gastric cancer cells.[14] In contrast, a

combination of traditional chemotherapeutic agents (cisplatin and gemcitabine) and an HSP27 inhibitor (quercetin) exerts a surprisingly greater chemotherapeutic effect in lung stem-like cells.[15] In addition, a recent study from our laboratory showed that a reduction in HSP27 expression, delivered by an adenoviral vector containing shHSP27, can sensitize gemcitabine-induced cell death in pancreatic cancer cells.[26]

In this study, recombinant adenoviral vectors were used as a gene delivery tool for the transfer of shRNAs against TGF-β and HSP27 to cancer cells. The initial attachment of adenovirus virion particles to the cell surface occurs by the binding of the fiber knob to the coxsackievirus B and adenovirus receptor (CAR).[27] Therefore, effective therapeutic gene delivery can be induced by using the adenoviral vector construct without any further artificial operation. Most notably, the main advantage of an adenoviral vector is that it can be used in gene therapy without toxicity to normal (non-tumor) cells.[28,29]

In this research, we designed adenovirus-delivered HSP27 and TGF-β shRNAs to knock down HSP27 and TGF-β expression, respectively. The subsequent reductions in HSP27 and TGF-β expression result in an increase p38 activity, not only to activate the cellular death signal, but also to synchronously reduce the ability of HSP27 to protect cells, where an increase in HSP27 phosphorylation resulted in cell death cooperatively. When both HSP27 shRNA and TGF-β shRNA were combined with sorafenib, HCC

cells were no longer resistant to sorafenib, and the efficiency of sorafenib against advanced HCC was significantly increased.

II. MATERIALS AND METHODS

1. Cell culture

Hep-3B, Huh-7, and SK-Hep-1 cells were cultured in DMEM with 10% fetal bovine serum (FBS). SNU-182, SNU-398, and SNU-449 cells were cultured in RPMI with 10% FBS. Cells were maintained in a 37℃ humidified atmosphere containing 5% CO_2.

2. Construction and recombinant adenoviral vectors

A. Construction of adenoviral vectors

For the expression of siRNAs targeting human TGF-β1, TGF-β2, HSP27, each shRNA (short hairpin RNA) construct was cloned into a pSP72ΔE3-U6/H1 vector using BamHI/HindIII digestion. These vectors, designated pSP72ΔE3-U6-shhTGF-β1, pSP72ΔE3-U6-shhTGF-β2, and pSP72ΔE3-H1-shhHSP27 (E3 shuttle vector), were linearized by XmnI digestion, and co-transformed into *E. coli* BJ5183 together with the SpeI-digested adenoviral vector (dl324-IX) for homologous recombination.

For the expression of siRNA targeting human TGF-β1, TGF-β2, and HSP27 together, H1-shhHSP27 was cloned into HindIII/KpnI-digested pSP72ΔE3-U6-shhTGF-β1 or pSP72ΔE3-U6-shhTGF-β2. pSP72ΔE3-U6-shhTGFβ1-H1-shHSP27 and pSP72ΔE3-U6-shhTGF-β2-H1-shhHSP27 (E3 shuttle vector) were linearized by XmnI digestion, and co-transformed

into *E. coli* BJ5183 together with the SpeI-digested adenoviral vector (dl324-IX) for homologous recombination.

For the expression of HSP27(3A) and HSP27(3D) mutation types, the vectors designated as pCA14-HSP27(3A) and pCA14-HSP27(3D) were linearized by XmnI digestion, and co-transformed into *E. coli* BJ5183 together with the Bsp1191-digested adenoviral vector (dl324-BstBI) for homologous recombination.

B. Recombinant adenoviral vectors

NC: Ad-IX-ΔE1--ΔE3, control virus

shT1: Ad-IX-ΔE1-ΔE3-U6-shhTGFβ1, virus expressing shRNA of human TGFβ1 (shTGFβ1)

shT2: Ad-IX-ΔE1-ΔE3-U6-shhTGFβ2, virus expressing shRNA of human TGFβ2 (shTGFβ2)

shHSP27: Ad-IX-ΔE1-ΔE3-H1-shhHSP27, virus expressing shRNA of humanHSP27 (shHSP27)

ShT1-shHSP27: Ad-IX-ΔE1-ΔE3-U6-shhTGFβ1-H1-shHSP27, virus expressing shRNA of TGFβ1 and HSP27

ShT2-shHSP27: Ad-IX-ΔE1-ΔE3-U6-shhTGFβ2-H1-shHSP27, virus expressing shRNA of TGFβ2 and HSP27

3. MTS viability assays

The CellTiter 96® Aqueous Assay kit (Promega, Madison, WI, USA)

is composed of solutions of a novel tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS) and an electron coupling reagent (phenazine ethosulfate; PES). MTS is bioreduced by cells into a formazan product that is soluble in tissue culture media. Subsequently, HCC cell lines were treated with varying doses of sorafenib (0, 2.5, 5, 10, 15, 20, and 25 μM) to in a 96-well plate for 24 h. The absorbance of formazan at 490 nm can be measured directly from 96-well plates without additional processing. The conversion of MTS into aqueous, soluble formazan is accomplished by dehydrogenase enzymes found in metabolically active cells. The quantity of formazan product measured at 490 nm is directly proportional to the number of living cells in culture.

4. Western blot analyses

Cells were lysed with 1X Laemmli sample buffer (62.5 mM Tris, pH 6.8, 2% sodium dodecyl sulfate, 10% glycerol, 0.002% bromophenol blue) and protein concentration was determined using the BCA Protein Assay Kit (Thermo Scientific, Fremont, CA, USA). Then, protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS PAGE), and gels were electro-transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). Immunodetection was performed using anti-phoshoAkt (pAkt), anti-phoshoSrc (pSrc), anti-

phoshoP65 (pP65), anti-phoshoERK (pERK), anti-phoshoP38 (pP38), anti-phoshoHSP27(pHSP27), anti-P38, anti-HSP27, anti-MKK3, anti-MKK6 and anti-GAPDH parimary antibodies, with the chemiluminescent and fluorescent image analysis system (Syngene, Cambridge, UK).

5. Real-time quantitative polymerase chain reactions

Cells were lysed with Trizol reagent (Life Technologies, Carlsbad, CA, USA) and the total RNA was isolated via chloroform extraction. RNA concentration was determined with a Nanodrop 2000 (Thermo Scientific). The real-time polymerase chain reaction (PCR) reaction was performed using a Power SYBR Green RNA-to-CT 1-Step Kit (Life Technologies). The reaction mixture contained the reverse transcriptase enzyme mix, reverse transcription PCR mix, forward primer, reverse primer, RNA template, and nuclease-free water. Human TGF-β1 cDNA was amplified using the forward primer 5'-CAAGGGCTACCATGCCAACT-3' and the reverse primer 5'-AGGGCCAGGACCTTGCTG-3'. Human TGF-β2 cDNA was amplified using the forward primer 5'-GCTGCCTACGTCCACTTTACAT-3' and the reverse primer 5'-ATATAAGCTCAGGACCCTGCTG-3'. Human β-actin was amplified using the forward primer 5'-ACTCTTCCAGCCTTCCTT-3' and the reverse primer 5'-ATCTCCTTCTGCATCCTGTC-3'.

6. Enzyme-linked immunosorbent assays (ELISA)

Cells were plated in the wells of six-well plates at a density of 1×10^5 cells/well. Supernatants were collected after 48 hr. Levels of TGF-β1 and TGF-β2 expression were determined by an ELISA according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA).

7. Clonogenic assays

Cells were plated into six-well plates at a density of 1×10^5 cells/well. Subsequently, HCC cell lines were treated with sorafenib (24 hr) at a variety of concentrations (0, 2.5, 5, 10, 15, or 20 μM) or treated with 2.5 μM sorafenib (12 hr) followed by a pre-treatment with adenoviral vectors (NC, shT1, shT2, shHSP27, shT1-shHSP27, shT2-shHSP27, HSP27-3A, or HSP27-3D) for 24 hr. Cells were trypsinized and plated into six-well plates at densities of 5×10^3 or 1×10^4 cells/well. Cells were subsequently monitored daily by microscopy. When cells exhibited colonies, surviving cells were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet.

8. Animal studies

Tumors were implanted subcutaneously in the abdomen of BALB/c nude mice via the injection of SNU-449 human liver cancer cells (1×10^7) in 100 μL of Hank's balanced salt solution (HBSS; Gibco BRL, Carlsbad, CA, USA). In the experiment, when tumors reached a size range of 70–100 mm^3, animals were randomized into 8 groups of 8 animals per group (PBS,

sorafenib, NC + sorafenib, shT1 + sorafenib, shT2 + sorafenib, shHSP27 + sorafenib, shT1-shHSP27 + sorafenib, and shT2-shHSP27 + sorafenib). Animals in adenoviral groups or the control group (PBS) were administered adenoviruses intratumorally (virus; 1×10^9 PFU [plaque-forming units] per tumor in 50μL of PBS) on days 1, 3, and 5. Sorafenib (30 mg/kg) was administered once daily by gavage for 10 days. The regression of tumor growth was assessed by taking measurements of the length (L) and width (W) of each tumor. Tumor volume was calculated using the following formula: volume = $0.52 * L * W^2$.

9. Immunohistochemistry (IHC)

Imunohistochemistry studies were performed on paraffin-embedded tumor tissues using anti-TGF-β1 (Abcam, MA, USA), anti-TGF-β2 (Abcam, MA, USA), anti-HSP27 (Santa Cruz Biotechnology), and anti-Ad5 (Novus Biologicals) antibodies to determine the expression of these proteins in the tumor tissue. The tumor tissue slides were deparaffinized by incubation in xylene for 10 min and rehydrated serially in alcohol (100%, 90%, and 70%). Endogenous peroxidase was blocked by incubation with 3% H_2O_2 for 15 min at room temperature and antigen retrieval was achieved by incubating the slides in citrate buffer for 10 min in a steamer. For permeabilization, the slides were incubated in PBS containing 0.5% Triton X-100 for 30 min and then washed three times with PBS. To reduce nonspecific

background staining due to endogenous peroxidases, the slides were incubated with a hydrogen peroxide block (Thermo Scientific) for 10 min. After washing, an ultra V block (Thermo Scientific) was applied to the slides for 5 min at room temperature to further block nonspecific background staining. The slides were incubated with an anti-TGF-β1 antibody (1:400 dilution), an anti- TGF-β2 antibody (1:1000), anti-HSP27 antibody (1:50) and an anti-Ad5 antibody (1:800 dilution) for 12 h at 4°C and further with a horseradish peroxidase polymer (Thermo Scientific) for 15 min at room temperature. To detect protein expression, the tissue sections were stained with diaminobenzidine tetrahydrochloride and minimally counterstained with hematoxylin (for visualization of antigen-antibody complexes). Sections were mounted under a coverslip using an mounting solution (shandon synthetic mountant (thermo scientific) + xylene = 1:1).

10. Statistical analyses

The data are expressed as mean ± standard error (SE). Statistical comparisons(Students *t* -test) were made using Graph Pad (Systat Software, Inc., Chicago, IL, USA). P-values less than 0.05 were considered statistically significant (*, $P < 0.05$; **, $P < 0.01$;***, $P < 0.001$)

III. RESULTS

1. Sorafenib treatment induced changes in several signaling pathways in HCC cell lines

Sorafenib is a standard chemotherapy treatment for advanced HCC patients, and can target many different signaling pathways. To evaluate the impact of sorafenib in vitro, we first identified the IC50 of sorafenib in different HCC cell lines. As the MTS assay results showed that the IC50 of sorafenib in HCC cell lines ranged from 7.5 μM to 15 μM (Fig. 1A). Subsequently, we scanned various key signaling pathway molecules, including p38, HSP27, p65, Src, and ERK via western blot analyses, in HCC cell lines after treatment with sorafenib IC50 concentrations. We observed that ERK activity was reduced by sorafenib treatment. The activity of phospho-p38 (p-p38) was also decreased, which may inhibit the death-related signaling pathway (Fig. 1B). We then performed a clonogenic assay to confirm the cell viability of HCC cells after dose-dependent treatments with sorafenib. We observed that many HCC cells survived treatment, even in the presence of high doses of sorafenib, signifying the low sensitivity of HCC cells to sorafenib and their resistance to this form of treatment. (Fig. 1C). We also confirmed that p-AKT and p-P65 activity was increased by incubating cell lines with 2.5 μM of sorafenib for extended periods of time, and

that p-p38 levels were also reduced (Fig. 1D).

The increased activities of p-AKT and p-P65 signify that cell lines were in fact resistant to sorafenib. Likewise, the inhibition of p38 activity was observed under conditions of sorafenib resistance, suggesting that it may play a role in inducing resistance to sorafenib in HCC cell lines..

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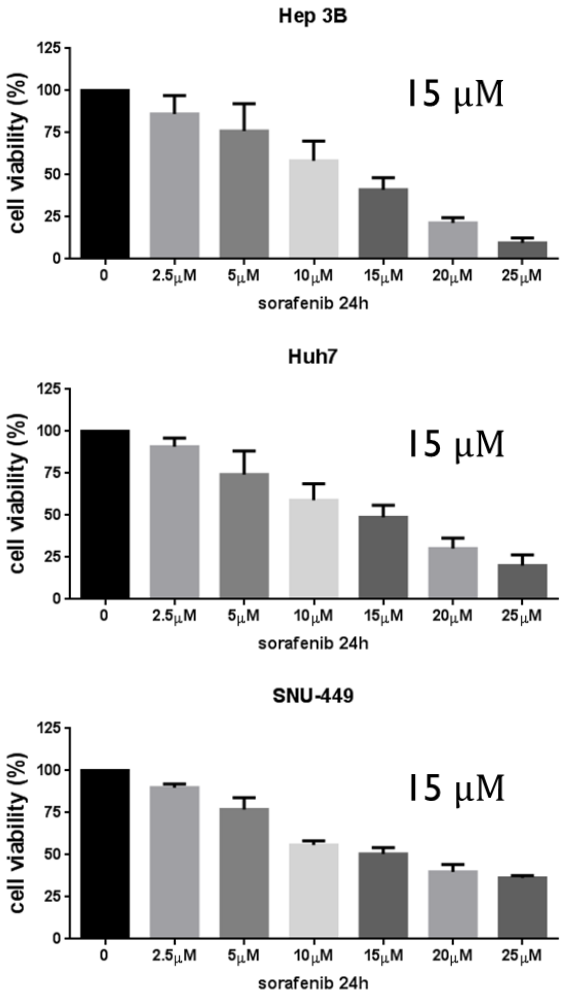

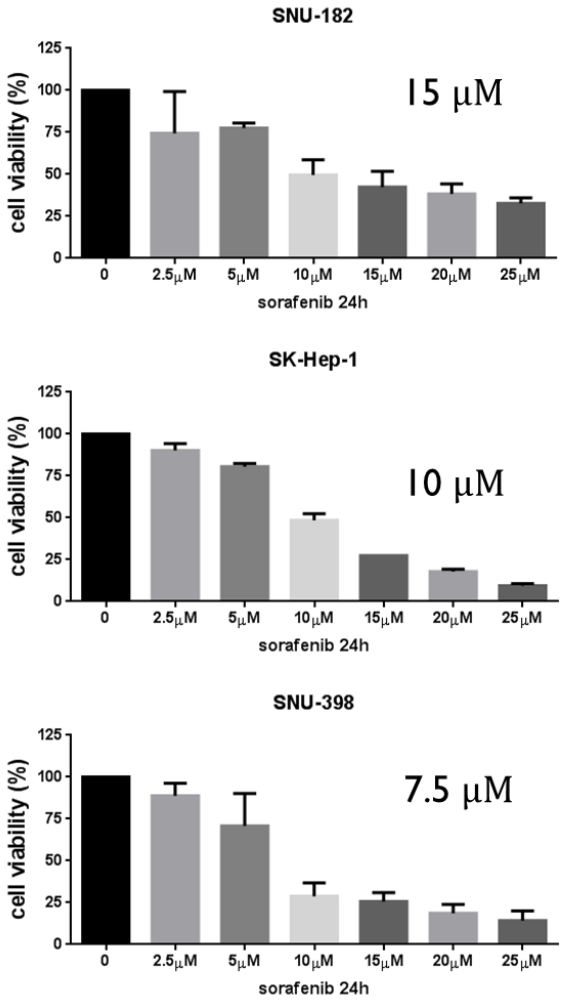

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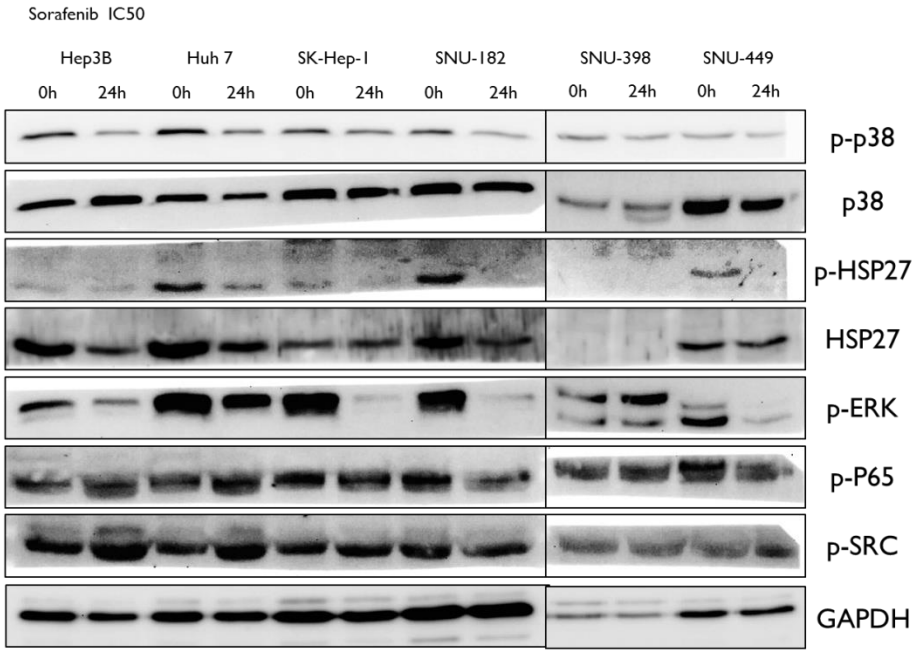

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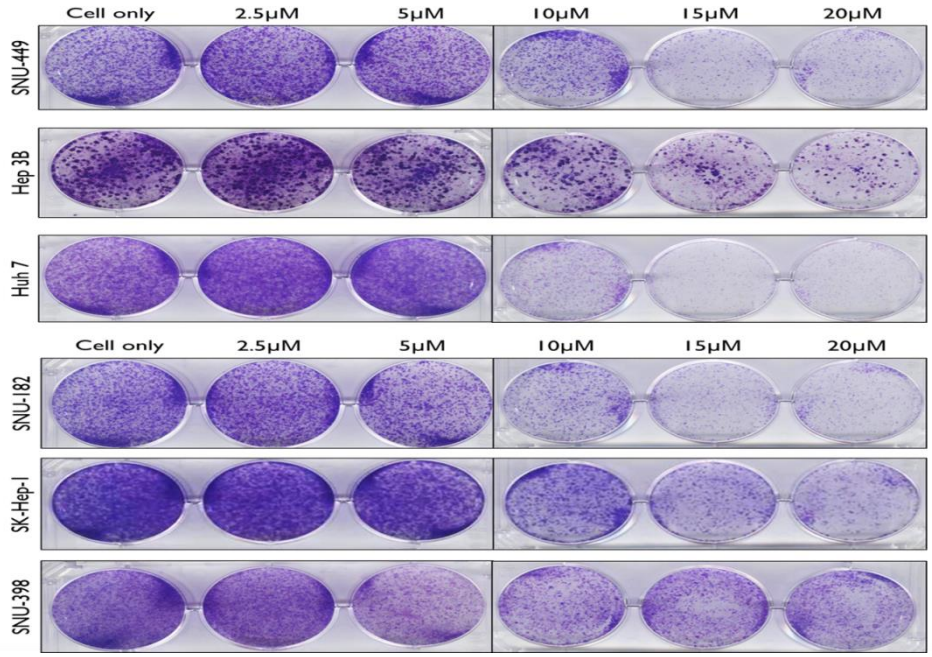

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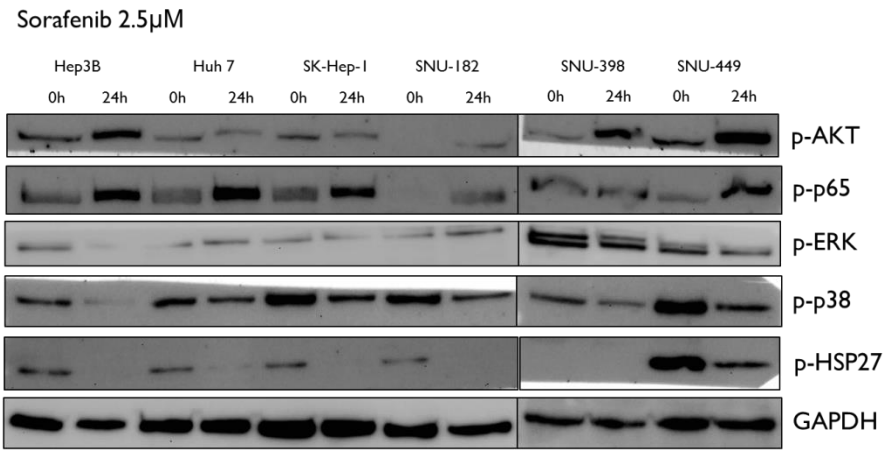

Figure 1. Effect of sorafenib in different HCC cell lines. (A) Hep 3B, Huh7, SK-Hep-1, SNU-182, SNU-398, and SNU-449 cells were treated with sorafenib in a dose-dependent manner. After 24 hr, cell viability was tested via a MTS viability assay. Error bars represent the standard error from three independent experiments. (B) HCC cell lines were treated using sorafenib at IC50 concentrations respectively. After 24 hr, the expression of p-p38, p38, p-HSP27, HSP27, p-ERK, p-Src, p-p65, and GAPDH in HCC cell lines were detected via western blot analysis. (C) HCC cell lines were treated by sorafenib in a dose-dependent manner for 24 hr. and incubated for an additional 14 days for clonogenic assays. (D) HCC cell lines were treated with a low dose of sorafenib (2.5 μM) for 24 hr and incubated for an additional 14 days for cell colony formation. Then, changes in levels of p-AKT, p-p65, p-ERK, p-p38, and p-HSP27 expression in HCC cell lines

were detected by western blot analysis.

2. p38-mediated cell death pathway was inhibited by treatment with sorafenib

In this study, we used the constitutive form of MKK3/6(MKK3/6E), which has the ability to induce p38 phosphorylation without any stimulation, to increase the activity of p38. Our results show that HCC cell lines transfected with the MKK3/6E plasmid exhibited an obvious increase in p38 activity (Fig. 2A, 2D), while the cell viability assay showed that the activation of p38 induced massive cell death in HCC cell lines (Fig. 2B). In addition, when treated with both the MKK3/6E plasmid and sorafenib, cell viability in HCC cell lines was significantly reduced in comparison to cell lines treated only with sorafenib (Fig. 2B). Clonogenic assays also confirmed that cell colonies were reduced by MKK3/6E transfection in comparison to control plasmid, and that fewer cell colonies formed in treatment groups co-treated with MKK3/6E and sorafenib than in cells treated with sorafenib alone (Fig. 2C). In addition, p-AKT and p-p65 activities were reduced in response to co-treatment with MKK3/6E and sorafenib, suggesting that p38 activity, which was inhibited by sorafenib, effectively reduced the cytotoxicity of sorafenib and increased the survival potential of HCC cell lines (Fig. 2D). Thus, we can surmise that the inhibition of p38 activity as a result of treatment with sorafenib was the mechanism of sorafenib resistance in HCC

cell lines. Therefore, an increase in the activity of p-p38 as a result of treatment with sorafenib overrode sorafenib resistance in HCC cell lines.

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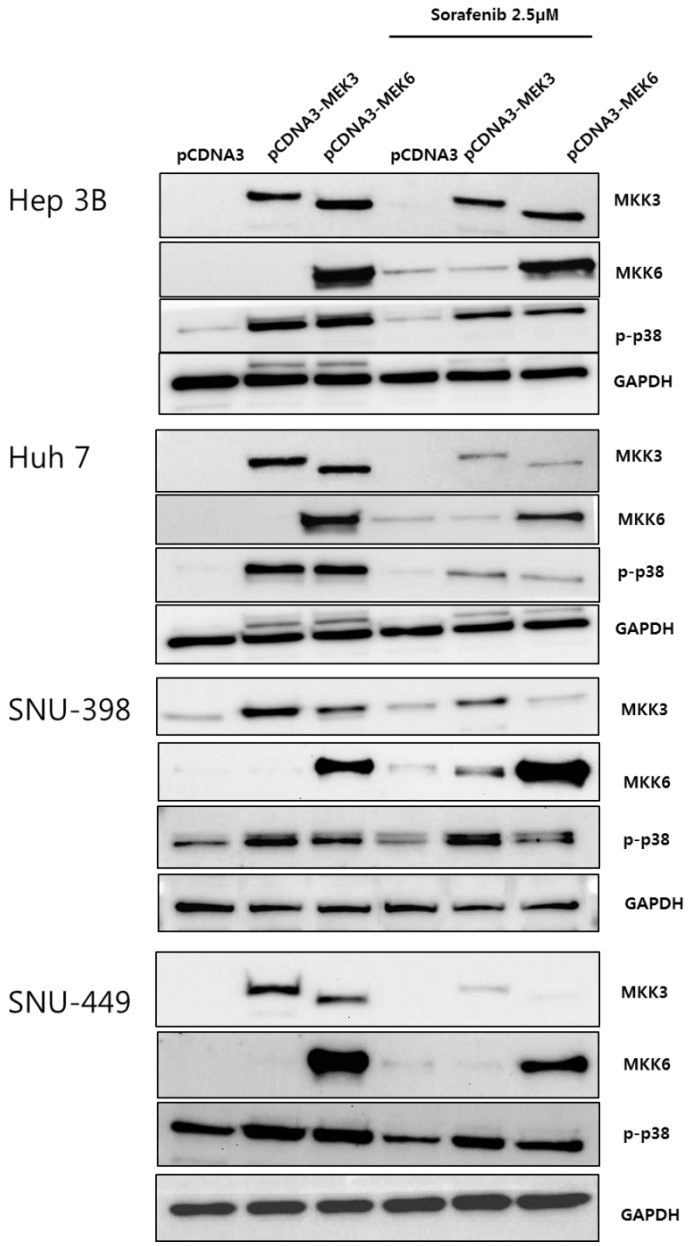

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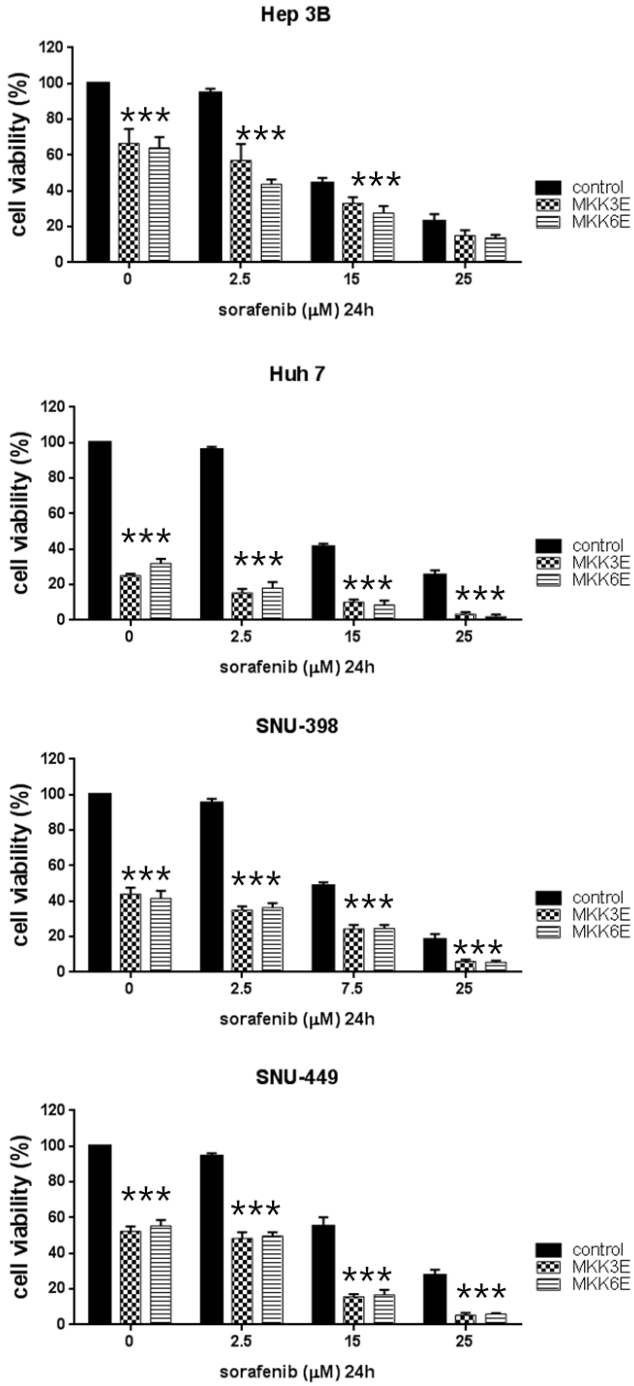

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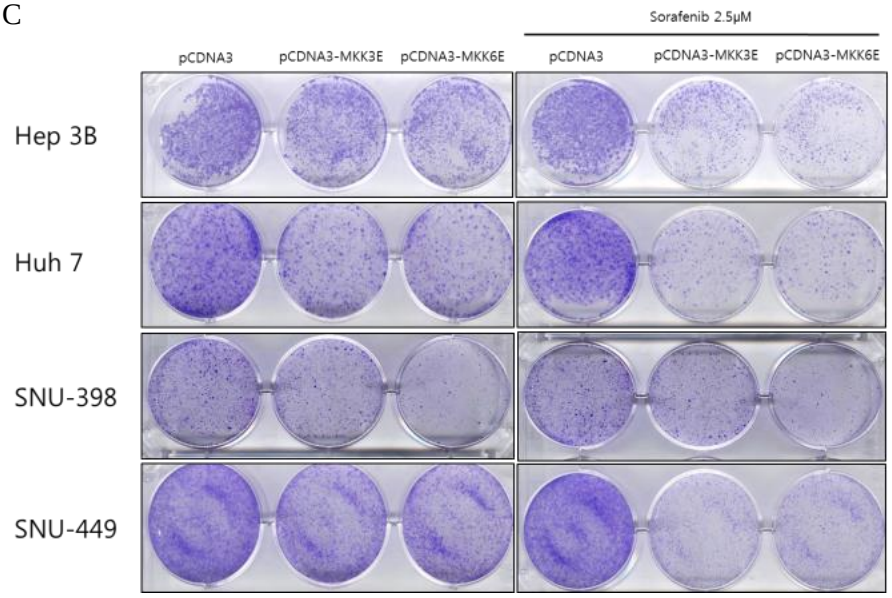

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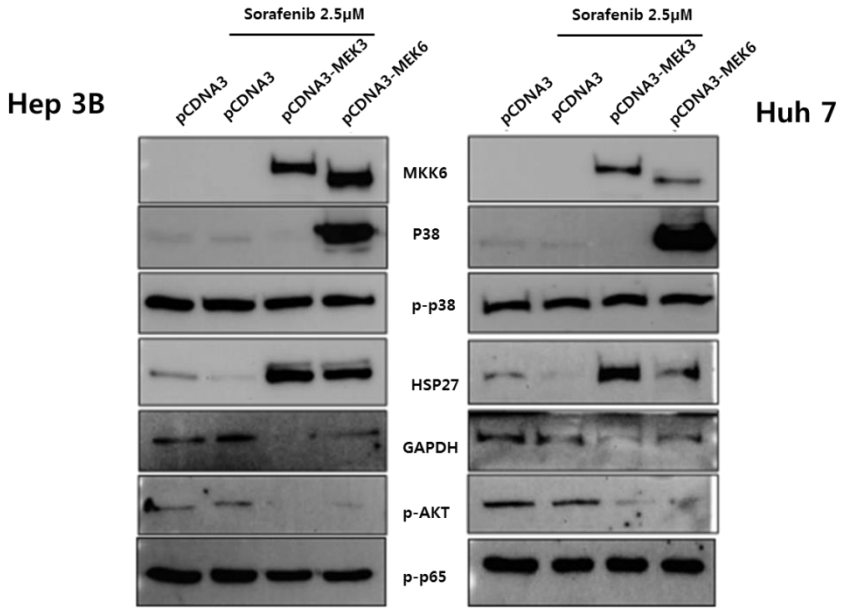

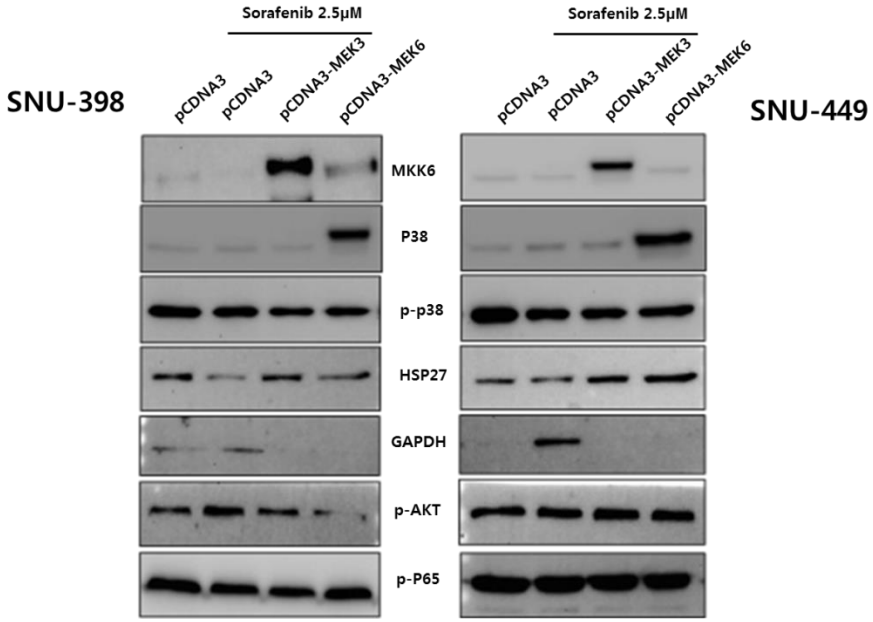

Figure 2. MKK3/6E induced p-p38 activation and massive cell death in HCC cell lines. (A) Hep 3B, Huh7, SNU-398, and SNU-449 cells were transfected with the pCDNA3-MKK3/6E plasmid (1 μg) for 24 hr, and treated with sorafenib (2.5 μM) for 24 hr. Protein expression of MKK3, MKK6, p-p38, and GAPDH were estimated via western blot analysis. (B) Hep 3B, Huh7, SNU-398, and SNU-449 cells were transfected with the pCDNA3-MKK3/6E plasmid (1μg) for 24 hr, and treated with sorafenib in a dose-dependent manner for 24 hr. Cell viability was tested using a MTS viability assay. Error bars represent the standard error from three independent experiments. (C) Hep 3B, Huh7, SNU-398, and SNU-449 cells were transfected with the pCDNA3-MKK3/6E plasmid (1μg) for 24 hr, then

treated with sorafenib in a dose-dependent manner for 24 hr, and incubated for an additional 14 days for clonogenic assays.

3. TGF-β expression was reduced by sorafenib treatment in HCC cell lines

In order to clarify the changes in TGF-β expression as a result of sorafenib treatment, we analyzed changes in the mRNA levels of TGF-β by real-time PCR. Our results show that while treatment with sorafenib at low concentrations(2.5 μM) did not significantly alter TGF-β expression($p > 0.05$), TGF-β expression is significantly ($p < 0.05$) reduced as a result of treatment with sorafenib at high concentrations (20 μM) (Fig. 3A). In addition, TGF-β expression was increased under conditions of sorafenib resistance (Fig. 3B), suggesting that decreases in TGF-β expression could reduce the resistance of HCC cell lines to sorafenib and more effectively induce cell death. Interestingly, when we attempted to knock down TGF-β expression by shRNA, we found that p-p38 activity was increased (Fig. 3C).

4. Sorafenib combined with adenovirus-expressed shRNA against TGF-β is more effective at inducing cell death in HCC cell lines

Next, we investigated whether the cell death was increased by sorafenib in low concentrations (2.5 μM) when combined with shRNA against TGF-β. Our results show that p-p38 activity was not reduced in response to

this combined treatment, and that cell viability was lower than in the NC virus-infected group (Fig. 3D and E).

This suggests that shTGF-β may increase the cytotoxicity of sorafenib. However, the emergence of the clones originating from HCC cells was not dramatically reduced, indicating that sorafenib resistance was not fully overcome by TGF-β downregulation alone.

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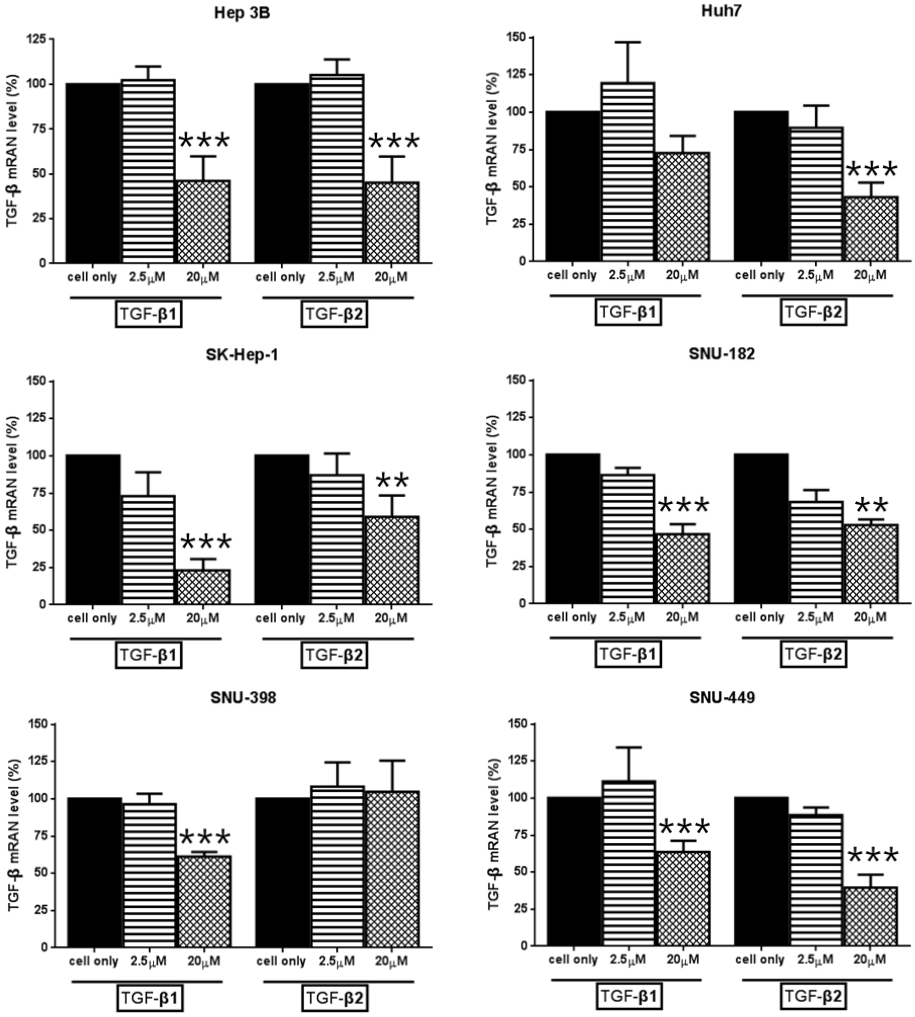

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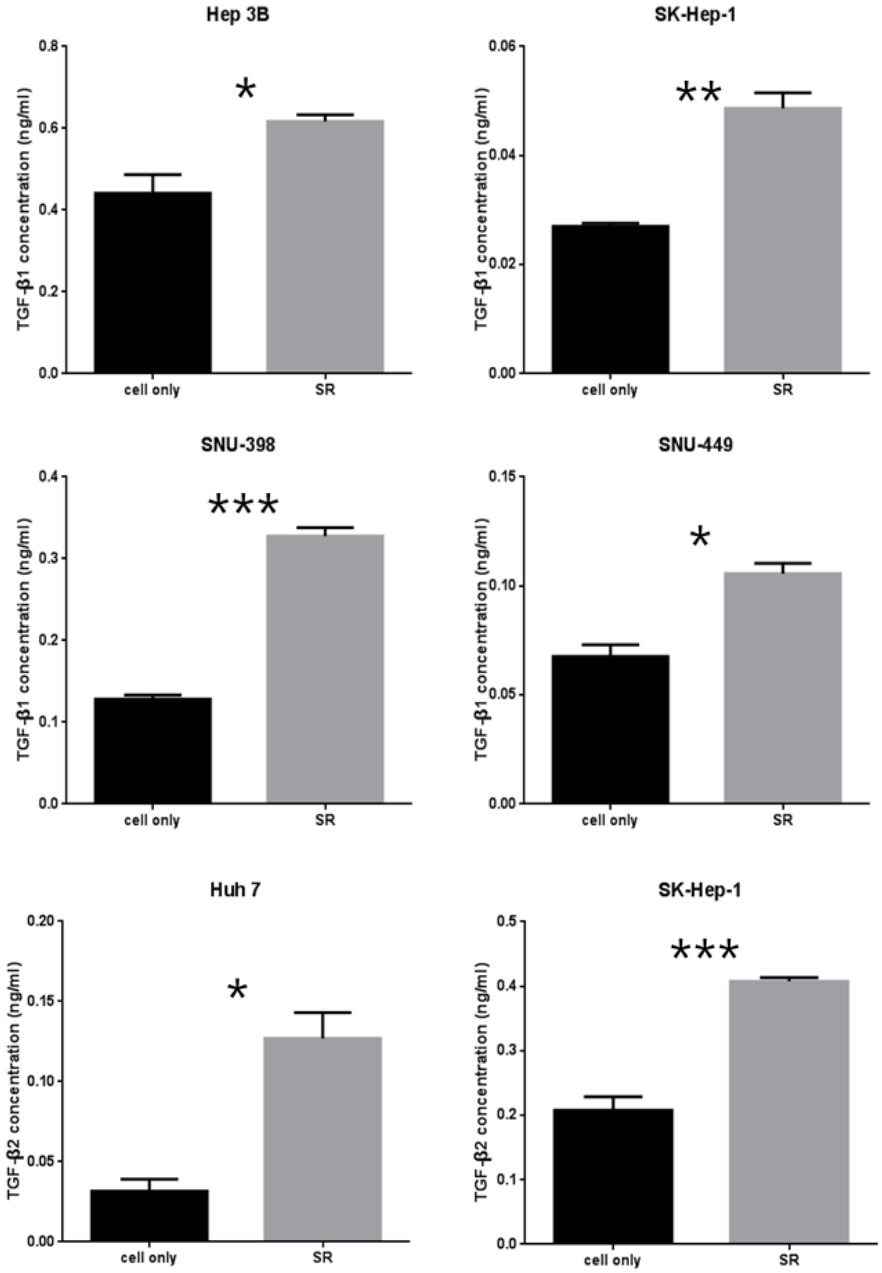

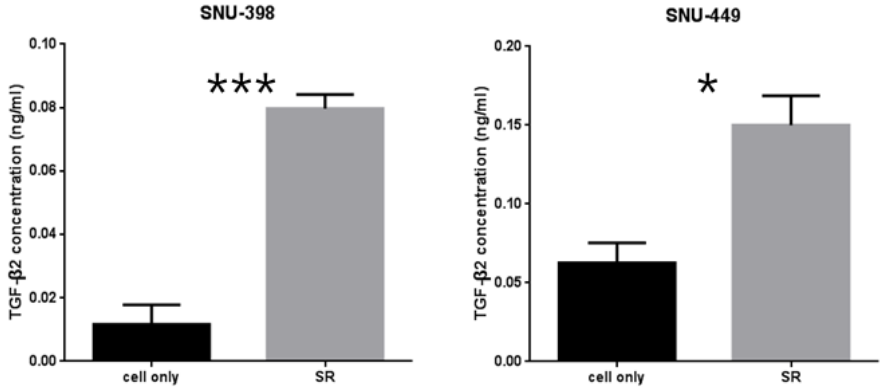

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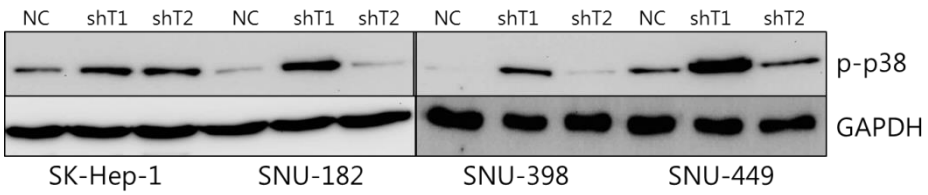

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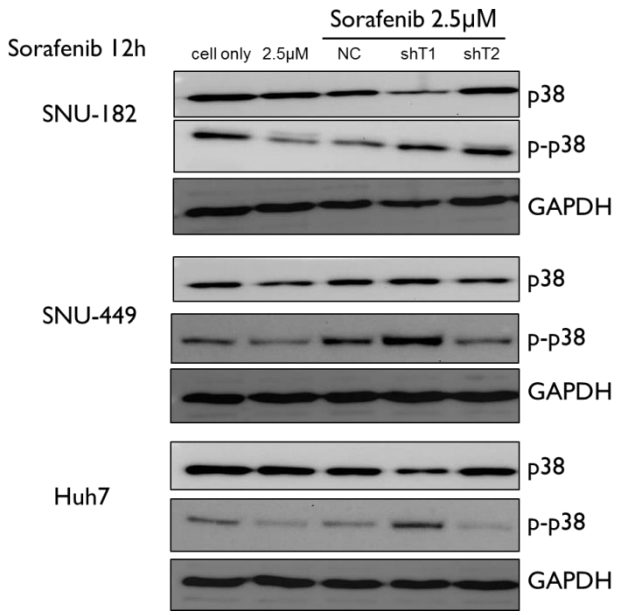

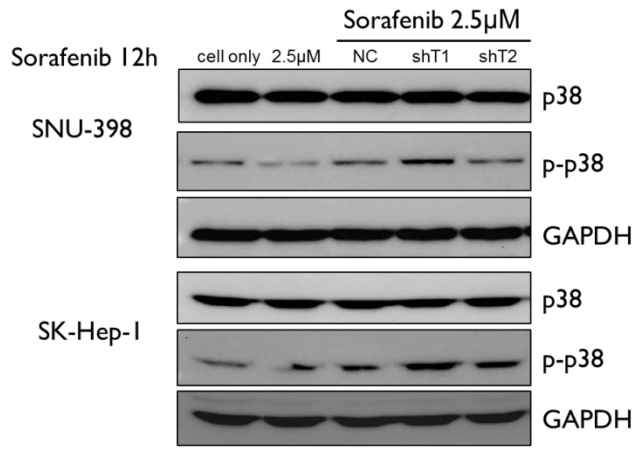

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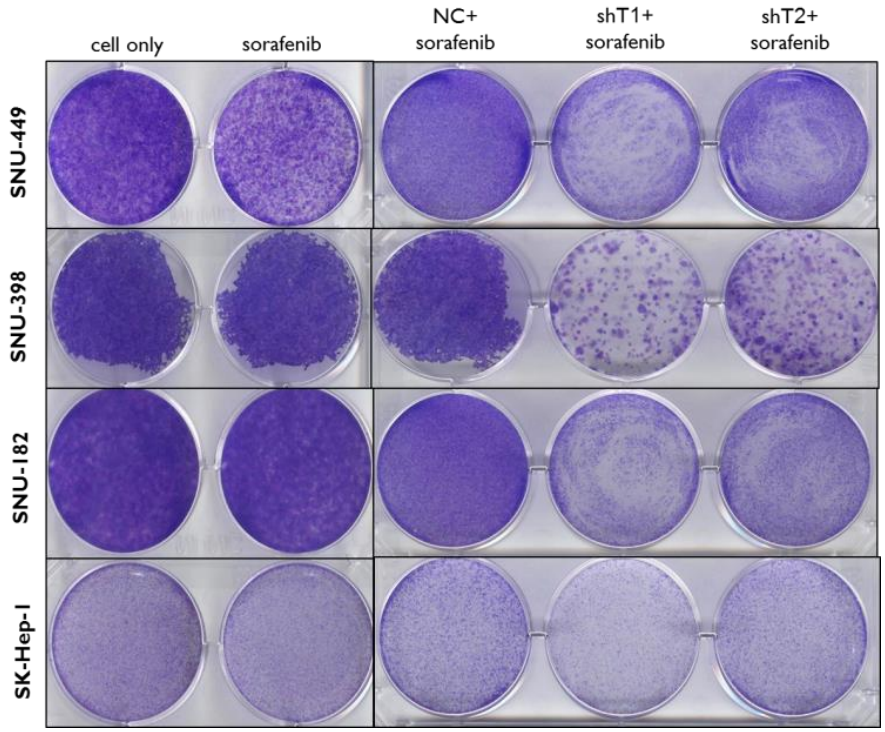

Figure 3. Changes in TGF-β expression in response to sorafenib treatment in HCC cell lines. (A) Hep 3B, Huh7, SK-Hep-1, SNU-182, SNU-398, and SNU-449 cells were treated with sorafenib (2.5 μM, IC50) for 24 hr, and TGF-β mRNA was estimated by RT-PCR. Error bars represent the standard error from three independent experiments. (B) Hep 3B, Huh7, SK-Hep-1, SNU-398, and SNU-449 cells were treated with sorafenib (2.5 μM) for 24 hr, and were incubated for additional 14 days for colony formation. Then TGF-β1/2 expression was detected via ELISA. Error bars represent the standard error from three independent experiments. (C) SK-Hep-1, SNU-182, SNU-398, and SNU-449 cells were infected by defective adenoviruses (NC, shT1, and shT2) at 50 MOI. After 2 days, protein expression of p-p38 was detected via western blot analysis. (D) Huh7, SK-Hep-1, SNU-182, SNU-398, and SNU-449 cells were infected by defective adenoviruses (NC, shT1, and shT2) at 50 MOI. After 36 hr, cells were treated with sorafenib at a low concentration (2.5 μM) for 12 hr. Changes in the protein expression of p38 and p-p38 were detected by western blot analysis. (E) SK-Hep-1, SNU-182, SNU-398, and SNU-449 cells were infected by defective adenoviruses (NC, shT1, and shT2) at 50 MOI. After 36 hr, cells were treated with sorafenib at a low concentration (2.5 μM) for 12 hr, and were incubated for additional 14 days for clonogenic assays.

5. HSP27 expression in HCC cell lines reduces sorafenib sensitivity

We screened HSP27 expression in HCC cell lines and analyzed its relationship with the IC50 of sorafenib (Fig. 4A). As expected, we observed that cell lines with elevated levels of HSP27 expression usually had low sensitivities to sorafenib, indicating that there is a negative correlation between HSP27 expression and the IC50 of sorafenib. In addition, the expression of HSP27 was increased under conditions of sorafenib resistance (Fig. 4B).

As our previous study reported, the proportion of p-HSP27 and HSP27 is a key biological switch, which modulates cell survival and death in pancreatic cancer cell lines. Subsequently, we used adenovirus-expressing HSP27-3A (HSP27 nonphosphorylation) or HSP27-3D (HSP27 phosphorylation) genes, to investigate whether p-HSP27 exerted an effect on the fate of HCC cells in response to sorafenib treatment. We observed that cell clones originating from the SNU-449 cell line were reduced in the treatment group infected with the HSP27-3D virus in comparison to the treatment group infected with the HSP27-3A virus (Fig. 4C, 4D).

Then we also demonstrated that knock down of HSP27 expression via adenovirus-expressing shRNA against HSP27 could increased the sensitivity of HCC cells to soarfenib treatment.

This suggests that an increase in the activity of p-HSP27 and/or knock down of HSP27 expression could reduce sorafenib resistance and induce effective cell death in HCC cell lines.

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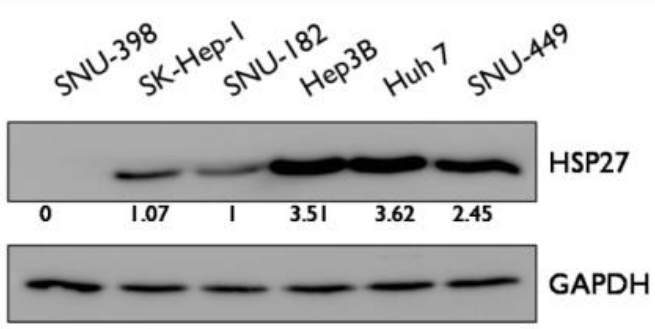

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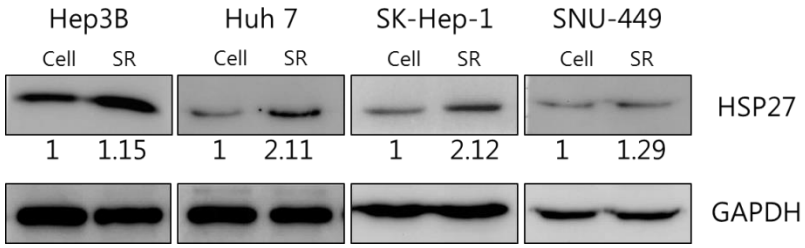

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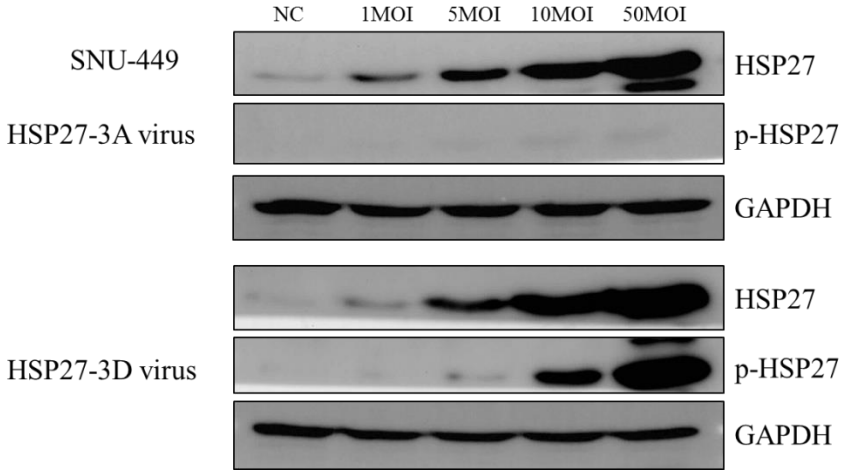

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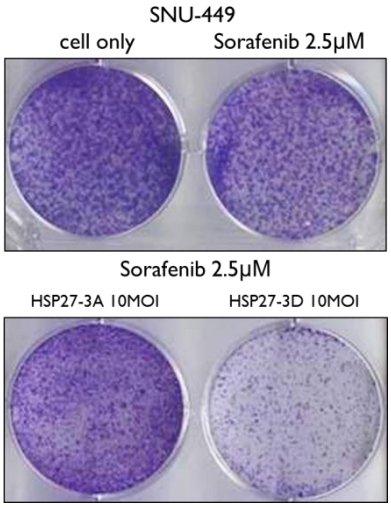

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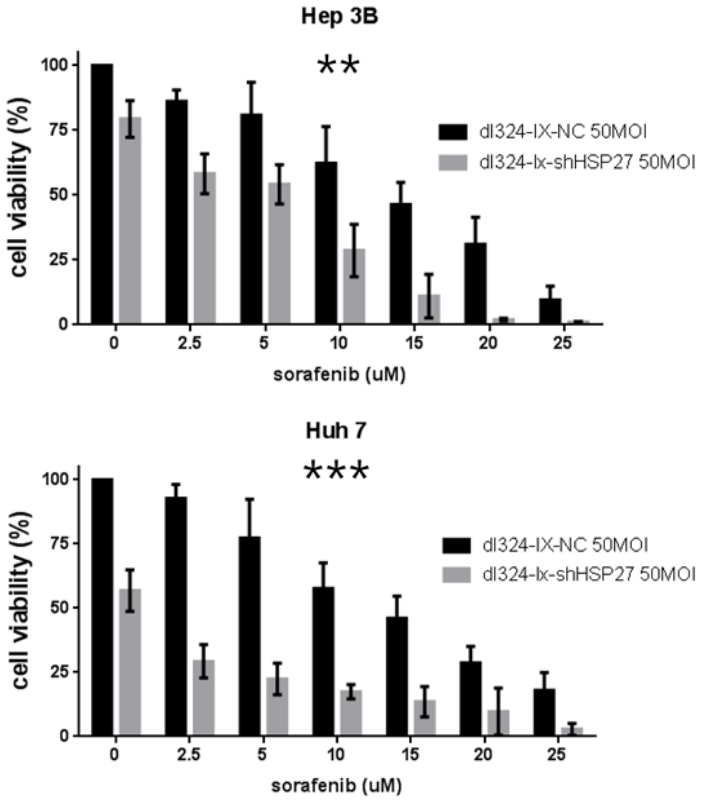

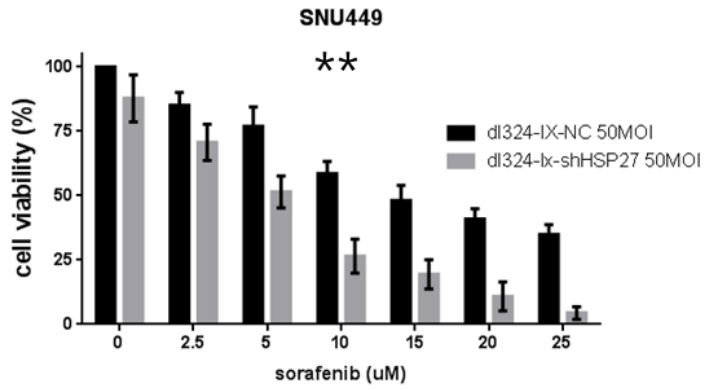

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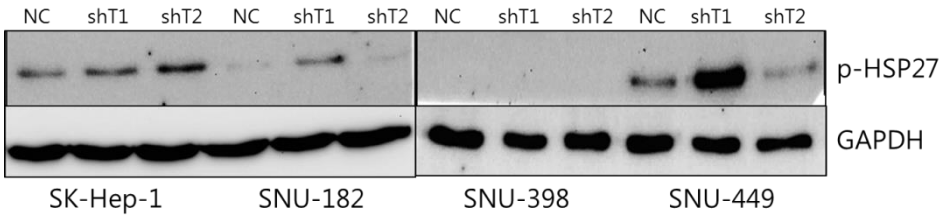

Figure 4. Relationship of HSP27 expression and sorafenib treatment. (A) Endogenous levels of HSP27 expression in HCC cell lines were detected via western blot analysis. (B) Hep 3B, Huh7, SK-Hep-1, and SNU-449 cells were treated with sorafenib (2.5 μM) for 24 hr, and were incubated for additional 14 days for colony formation. Then HSP27 expression was detected via western blot analysis. (C) SNU-449 cells were infected with HSP27-3A and HSP27-3D viruses at various MOIs. After 48 hr, the expression of HSP27 and p-HSP27 were detected by western blot analysis. (D) SNU-449 cells were infected by defective

adenoviruses (NC, HSP27-3A, and HSP27-3D) at 10MOI. After 36 hr, cells were treated with sorafenib at a low concentration (2.5 μM) for 12 hr, and incubated for an additional 14 days for clonogenic assays. (E) Hep 3B, Huh7, and SNU-449 cells were infected with NC and shHSP27 viruses at various MOIs. After 24 hr, cells were treated with sorafenib in a dose-dependent manner. After 24 hr, cell viability was tested via a MTS viability assay. Error bars represent the standard error from three independent experiments. (F) SK-Hep-1, SNU-182, SNU-398, and SNU-449 cells were infected by defective adenoviruses (NC, shT1, and shT2) at 50 MOI. After 2 days, p-HSP27 expression was detected by western blot analysis.

6. Levels of p-p38 and p-HSP27 were markedly increased in response to treatment with sorafenib combined with an adenovirus expressing both shTGF-β and shHSP27

We used shTGF-β combined with sorafenib to increase p-p38 activity in order to more effectively induce cell death in HCC cells. As a result, we demonstrated that the increased phosphorylation of HSP27 combined with a low concerntration (2.5 μM) of sorafenib did effectively induce increased cell death in comparison to treatment with sorafenib alone. However, HSP27-3D could not be used clinically owing to the increased expression of

HSP27. Under conditions of stress, HSP27 phosphorylation can be induced by the phosphorylation of p38. Interestingly, we found that the down-regulation of TGF-β expression by shTGF-β resulted in an increase in the activity of p-HSP27 (Fig. 4F). Next, we used the shRNA against HSP27 to knock down HSP27 expression. Thus, we designed adenovirus that co-expressed shTGF-β and shHSP27 and combined this with a low concerntration (2.5 μM) treatment of sorafenib. Our results show that p-p38 activity was increased while that of HSP27 was reduced, thereby leading to an increase in the activity of p-p38 and p-HSP27 (Fig. 5A). We observed that many of the clones originating from HCC cells lines emerged from the NC virus group, while there were fewer clones in the shTGF-β or shHSP27 treatment groups. Furthermore, we observed a complete disappearance of colony formation in the co-expressed shTGF-β and shHSP27 virus group (Fig. 5B).

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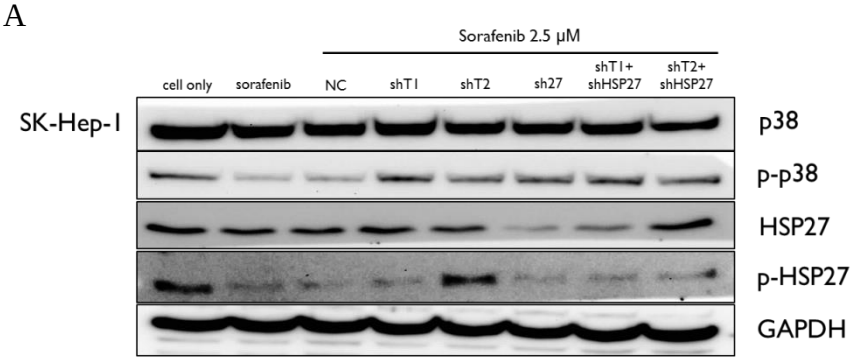

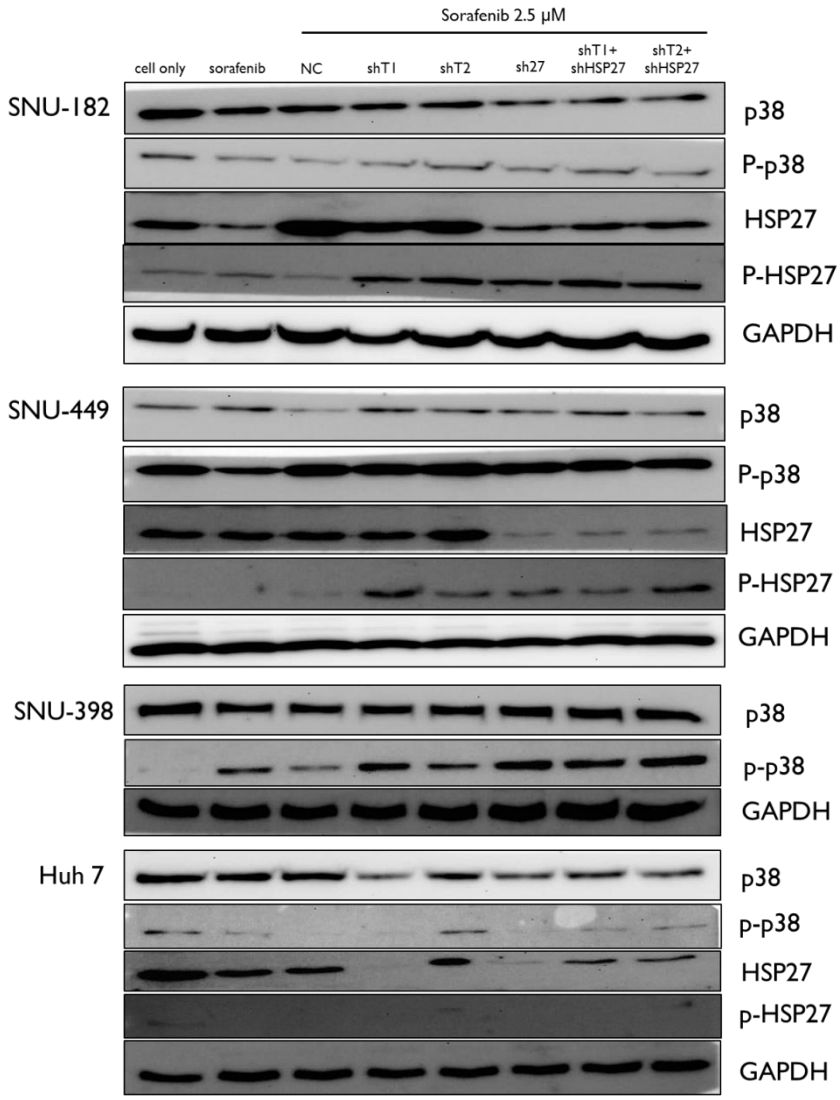

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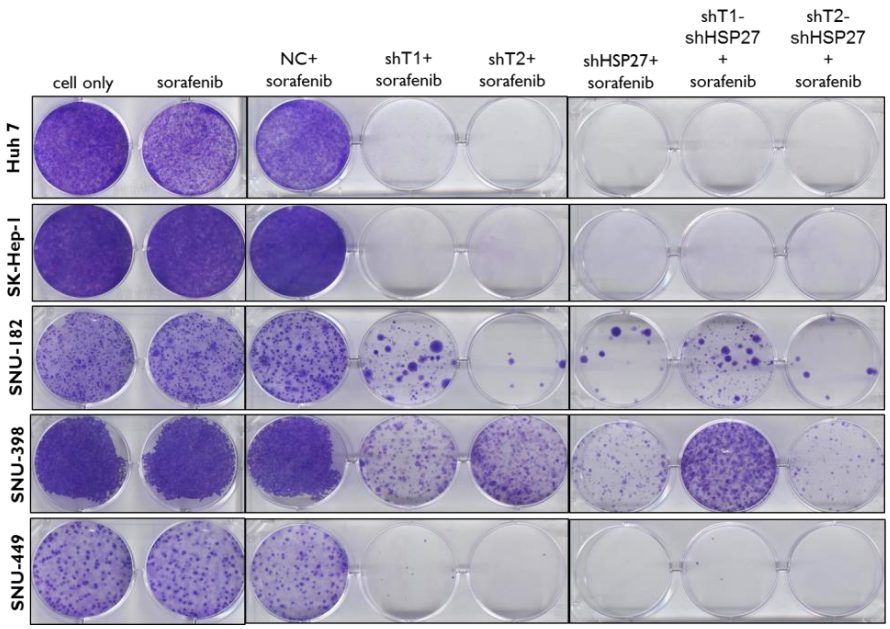

Figure 5. Combined treatment of adenovirus and a low concentration of sorafenib induces massive cell death in HCC cell lines. (A) Huh7, SK-Hep-1, SNU-182, SNU-398, and SNU-449 cells were infected by defective adenoviruses (NC, shT1, shT2, shHSP27, shT1-shHSP27, and shT2-shHSP27) at 50 MOI. After 36 hr, cells were treated with a low concentration of sorafenib (2.5 μM) for 12 hr, and changes in the expression of p38, p-p38, HSP27, and p-HSP27 were detected via western blot analysis. (B) SK-Hep-1, SNU-182, SNU-398, and SNU-449 cells were infected by defective adenoviruses (NC, shT1, shT2, shHSP27, shT1-shHSP27, and shT2-shHSP27) at

50 MOI. After 36 hr, cells were treated with a low concentration of sorafenib (2.5 μM) for 12 hr and incubated for an additional 14 days for clonogenic assays.

7. Anti-tumor effect of sorafenib combined with an adenovirus co-expressing shTGF-β and shHSP27 in xenograft animal models

After a series of in vitro experiments, we confirmed that an adenovirus co-expressing shTGF-β and shHSP27 increased the activity of p-p38 and decreased the expression of HSP27, thereby overcoming the resistance of HCC cell lines to sorafenib. Subsequently, we designed an in vivo experiment in xenograft animal models to confirm the anti-tumor effect of this combination therapy, and whether this therapy is able to override the resistance of HCC tumor cells to sorafenib.

Our results showed that treatment with adenovirus-expressed shTGF-β1, shTGF-β2, or shHSP27 in combination with sorafenib displayed increased anti-tumor abilities in comparison to sorafenib alone (Fig. 6A and C). This suggests that while these treatments did effectively reduce the resistance of HCC tumor cells to sorafenib, these treatments did not result in a complete reduction in resistance. Moreover, the combination therapy of adenovirus co-expressed shTGF-β1/2 and shHSP27 with sorafenib exhibited the most effective anti-tumor ability (Fig. 6A and C). Despite these results, we observed no differences in tumor regression in response to shTGF-β1,

shTGF-β2, and shHSP27, or to shTGF-β1-shHSP27 and shTGF-β2-shHSP27 (Fig. 6A and C). The survival rate of the animals in our study indicated that the combination therapy of adenovirus co-expressed shTGF-β1/2 and shHSP27 with sorafenib was the most effective, where no mice died until 19 days post-treatment (Fig. 6B). Treatment groups in which singularly-expressed adenovirus shRNAs combined with sorafenib groups displayed 60–80% survival rates, while the survival rate of the group treated with sorafenib alone group was 60%, and the survival rate of the control (PBS) group was 40% (Fig. 6B).

The result of immunohistochemical analysis showed that TGF-β1, TGF-β2, and HSP27 expression was reduced in treatment with adenovirus-expressed shTGF-β1, shTGF-β2, shHSP27, shTGF-β1-shHSP27 and shTGF-β2-shHSP27 compared with PBS, sorafenib, and NC virus treated tumor tissues (Fig. 6D).

As shown in ex vivo experiments, we confirmed that the combination therapy of the adenovirus co-expressing shTGF-β and shHSP27 with sorafenib noticeably increased the anti-tumor effect, effectively overriding sorafenib resistance in HCC animal models.

In the future, it will be necessary to confirm these results in immune competent animal models. These results bring new hope to HCC patients, and especially those who have exhibited resistance to sorafenib.

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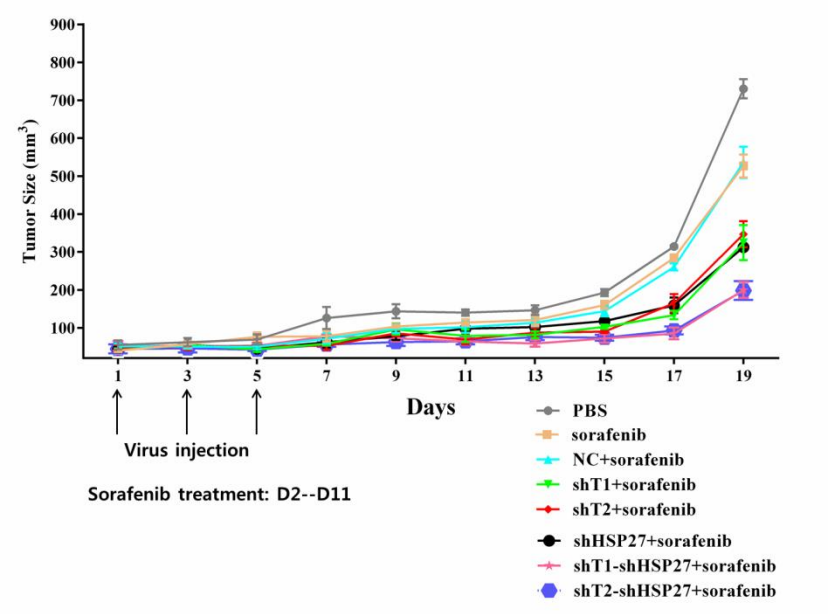

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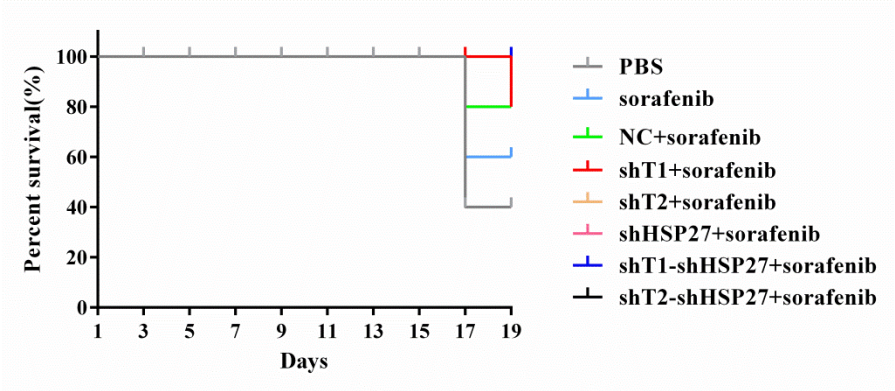

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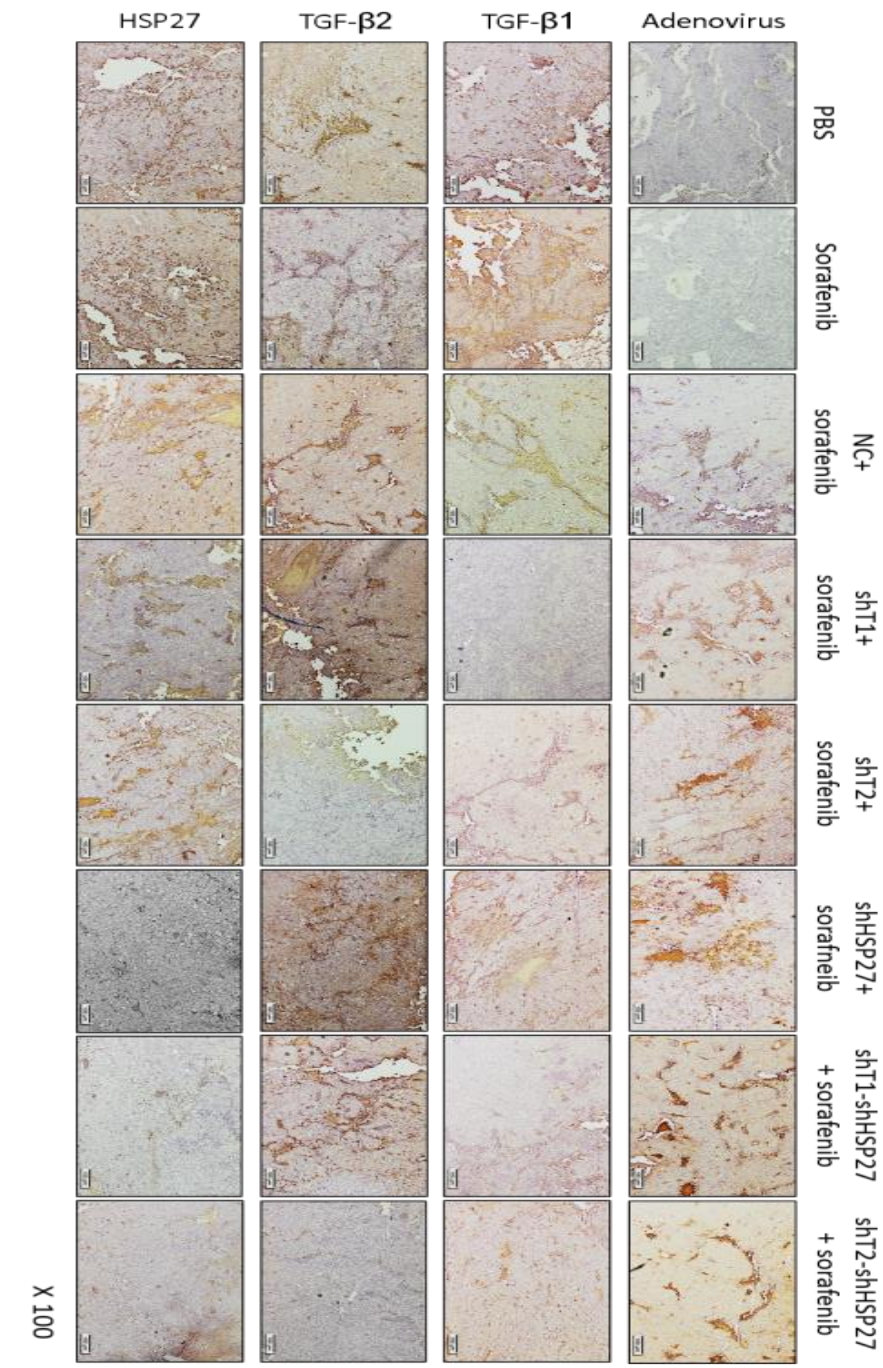

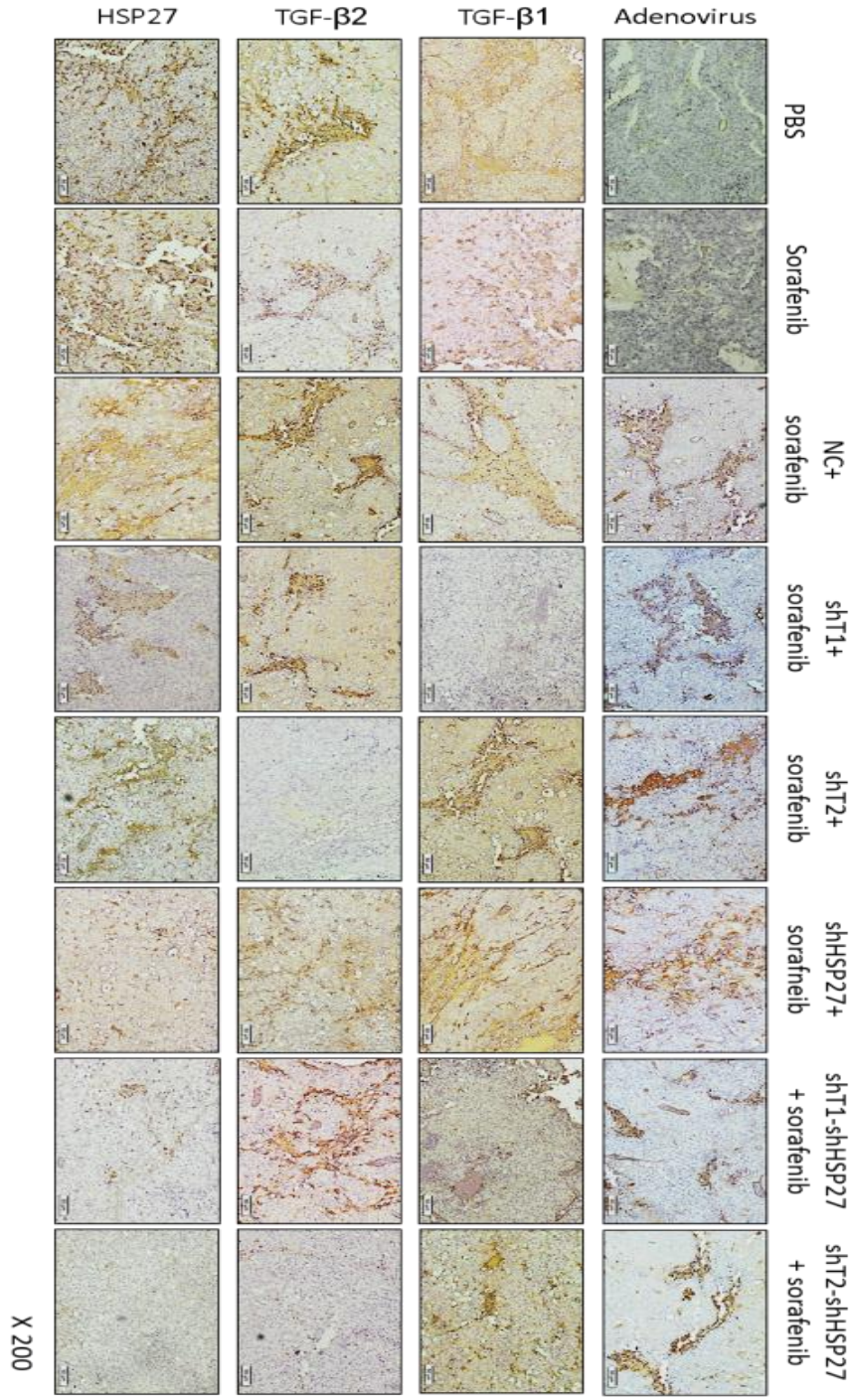

Figure 6. Antitumor effects of the combined treatment of sorafenib and an adenovirus co-expressing shTGF-β and shHSP27 in BALB/c nude mice. (A) SNU-449 tumors were grown in male BALB/c nude mice. Tumors were established by the subcutaneous injection of 1 × 10^7 cells, and were allowed to grow to an average size of 60–100 mm^3. PBS and adenoviruses were intratumorally injected every other day for a total of 3 injections. Sorafenib (30mg/kg) was administered via gavage once daily from days 1 to 10. Tumor growth was measured using calipers every 2 days for more than 19 days. (B) Survival rates were calculated every 2 days for more than 19 days. (C) Images of tumor nodes were captured using a digital camera at 15 day. (D) Tumors were collected at day 11 for histological analysis. Paraffin-embedded sections of tumor tissue were stained with anti-adenovirus type 5 (top row, original magnification: ×100 and ×200), anti-TGF-β1 (second row, original magnification: ×100 and ×200), anti- TGF-β2 (third row, original magnification: ×100 and ×200), and anti-HSP27 (forth row, original magnification: ×100 and ×200) antibodies.

IV. DISCUSSION

Sorafenib is the first-line systemic drug for advanced hepatocellular carcinoma. For patients in which HCC is diagnosed at advanced stage, sorafenib is the only choice of systemic therapy, as other potentially curative treatments, such as resection and liver transplantation, are mainly only applicable for patients diagnosed at early stage.[30] Recently, the unstable efficacy of sorafenib as a treatment for HCC has raised the concerns of more and more researchers, and 'sorafenib resistance' has become well-known terminology, and is regularly used to describe the impaired efficacy of sorafenib, especially in patients with advanced HCC. However, the exact mechanisms underlying sorafenib resistance in HCC remain unclear, and no effective therapy for late-stage HCC has emerged in the wake of the failure of sorafenib as a therapy.[9]

The P38MAPK pathway is a multifunctional system that cannot only improve cell viability to protect cells, but can also induce apoptosis. The activity of p38 in hepatocellular carcinoma tissues has been shown to be lower in comparison to p38 activity in normal liver tissues, while the increased the activity of p38 is known to induce cell death.[18]

The main purpose of sorafenib is to inhibit the ERK signaling pathway, where the activities of p-AKT and p-p65 displayed signs of increase after long-term treatment with sorafenib, thereby leading to drug resistance.

5,30,31,32

In the present study, we have demonstrated that a low concentration of sorafenib (2.5 μM) can inhibit the activity of p-p38. However, no significant cell death appeared in the low-dose sorafenib treatment. In fact, after the low-dose treatment, the activities of p-AKT and p-P65 was increased. Thus, we can infer that a decrease in p-p38 is the underlying mechanism responsible for sorafenib resistance in HCC cell lines.

We used MKK3/6E to show that when the activity of p-p38 is increased, cell death is induced and sorafenib resistance is overcome. As MKK3/6 is upstream of the p38 signal in the signally pathway, its activity can induce the phosphorylation of p38. It has been shown that the structure of MKK3/6E is such that MKK3/6E is able to activate p38 without an external stimulus.[33] Our results show that MKK3/6E can significantly improve the activity of p-p38 and induce cell death in a significantly large number of cells ($p < 0.05$). When treatment with MKK3/6E was combined with sorafenib, p-p38 activity was significantly increased in comparison to the activity observed in cells treated with sorafenib alone, but was lower in comparison to the p-p38 activity of cells treated with MKK3/6E alone. MTS and clonogenic assays confirmed that the combined application was better than treatment with sorafenib alone. It can be seen that in response to the low-dose sorafenib treatment, p-p38 activity was reduced, thereby resulting in

the efficacy of the sorafenib treatment and indicating the mechanism by which HCC cells exhibit resistance to sorafenib.

The role of sorafenib, a type-II kinase inhibitor, in the inhibition of p38 activity is well known.[20,21,22,23] As the role of the inhibition of p38 activity in the resistance of HCC cells to sorafenib has become more clear, a therapy that utilizes sorafenib while increasing the activity of p38 could not only greatly improve the rate of cell death, it could also reduce the incidence of drug resistance in HCC tumor cells.

After elucidating the importance of p-p38 activity, we employed two types of shRNA, shTGF-b/shHSP27, to stimulate an increase in p-p38 activity, thereby overcoming drug resistance.

It is well known that the elevated expression of TGF-β in patients with advanced cancer can promote tumor growth, metastasis, angiogenesis, and inhibit the anti-tumor immune response.[14,15] Early clinical studies demonstrated the elevated expression of TGF-β1 in patients with sorafenib drug resistance, and showed that TGF-β1 expression and prognosis and survival time were negatively correlated.[15] Moreover, recent studies have shown that TGF-β2 expression was higher in HCC tissues than in normal liver tissues, and is negatively correlated with survival rate.[16]

In this study, we found that cell viability and the expression of TGF-β was increased, where HCC cells exhibited drug resistance after treatment with a low concentration of sorafenib. We have also demonstrated the in-

creased activities of p-AKT and p-p65 under conditions of sorafenib resistance, where TGF-β expression was clearly and positively correlated with sorafenib drug resistance. However, TGF-β expression was significantly decreased and cell viability was noticeably inhibited in response to treatment with a high concentration (20 μM) of sorafenib. These results indicate that the reduction in cell viability in response to the use of a high concentration (20 μM) of sorafenib was associated with decreased TGF-β expression. As a result of this, we made TGF-β our target, and used shRNA to knock down TGF-β expression in order to improve the rate of cell death and reduce drug resistance.

It is interesting to note that in HCC cell lines infected by the adenoviral expression of TGF-β shRNA, the activity of p-p38 was increased significantly. The application of shTGF-β and sorafenib together increased the activity of p-p38 more than treatment with sorafenib alone. In addition, a clonogenic assay showed that the number of cell colonies was more effectively decreased when sorafenib was administered as a single treatment. This result is consistent with the previously proposed theory that an increase in the activity of p-p38 would result in decreased cell viability and reduced drug resistance. Therefore, in this study, the reduced expression of TGF-β by shRNA not only increased the activity of p-p38, it reduced cell viability, and reduced drug resistance. However, although the clonogenic assay showed the combined application effectively improved cytotoxicity,

the combined treatment did not completely eliminate the formation of cell colonies. As a result of this, we considered adding a new target in order to further improve cytotoxicity, and to more effectively overcome sorafenib resistance.

HSP27 is a chaperone protein that protects cells against external stimuli, including anti-tumor drugs.[14] Therefore, HSP27 has become a hot topic in cancer research, where studies involving anti-tumor drug resistance often refer to HSP27 as an drug resistance inducer.[15]

Thus, in this study, we also analyzed the expression of HSP27 in HCC cell lines. Our results show that the expression of HSP27 in HCC cells is negatively correlated with the IC50 of sorafenib, and thus the higher expression of HSP27, the lower the drug sensitivity of the cells. Thus, the expression of both HSP27 and TGF-β increased under conditions of resistance to sorafenib in HCC cell lines. These results indicate a correlation between HSP27 and sorafenib resistance.

Our previous study of gemcitabine drug resistance in pancreatic cancer cells showed that, the increased activity of p-HSP27 and the decreased expression of HSP27 effectively reduced drug resistance and increased cytotoxicity.[26]

In the present study, the use of HSP27-3A/3D virus confirmed that the increased the activity of p-HSP27 reduced the formation of cell colonies and improved the drug resistance of sorafenib in HCC cell lines. However,

as HSP27-3D also increases the expression of HSP27, it cannot be used for the clinical treatment of patients. Fortunately, as p-HSP27 is a downstream signal of p-p38, the increased the activity of p-p38 induced the activity of p-HSP27. In the results section, we mentioned that a decrease in TGF-β expression induces p-p38 activity. We further confirmed, using an adenovirus expressing shTGF-β, that the downregulation of TGF-β expression can also increase the activity of p-HSP27.

Therefore, we confirmed that the combined use of shTGF-β and shHSP27 resulted in the increased activity of p-p38 and p-HSP27, reduced the expression of HSP27, and effectively overcame sorafenib drug resistance, and thus completely prevented the formation of cell colonies. However, as the results of a clonogenic assay showed, the sensitivity of cells to shTGF-β1/2 is different in the six HCC cell lines. This could be the result of the amount of inherent TGF-β1/2 expression in the various HCC cell lines.

The adenoviruses used in the present study as shRNA delivery tools displayed high titers, good infection rates, and no carcinogenicity. Although adenoviral anti-tumor drugs have been previously used in clinical patients, the effects have been limited. Therefore, the improving the antitumor effects of adenovirus-based therapies moved to the forefront of cancer therapy research, where a large number of studies focused on the ideal genes for adenoviral delivery and to induce the most desirable anti-tumor effects. In

this study, we expressed two kinds of shRNA simultaneously using an adenovirus, thereby maximizing the functions of the treatment.

An in vitro experiment proved that sorafenib drug resistance could be overcome by the adenoviral co-expression of shTGF-β and shHSP27. In order to further clarify the anti-tumor effect, as well as the ability of this combination treatment to overcome drug resistance, experiments were performed on live animals. An animal model was established using subcutaneous abdominal injections of an HCC cell line (SNU-449) into BALB/c nude mice. Our results showed that the combination of shTGF-β1/2 plus shHSP27 and sorafenib had the most substantial anti-tumor effect, and effectively overcame the resistance of HCC cells to sorafenib. However, there were no significant differences between shTGF-β1 plus shHSP27 and shTGF-β2 plus shHSP27, while the single virus group also displayed no notable differences. These results may be the result of differences in the levels of expression of target proteins in tumors, or the residence time of virus. In this study, we used defective-type adenoviruses, which cannot replicate in tumor cells, in order to clarify the effect of shRNAs in overcoming drug resistance. In the future, the use of oncolytic adenoviruses should be explored, as well as the use of immune-competent mouse models, which would be more similar to clinical conditions.

In summary, although a series of in vitro experiments demonstrated that the inhibition of p-p38 activity is the underlying mechanism of soraf-

enib drug resistance. Similarly, while in vivo experiments using an animal model showed that the co-expression of shTGF-b1/2 plus shHSP27, can effectively reduce drug resistance and improve the antitumor effect.

Therefore, in the future, it will be necessary to analyze the correlations between the related factors (TGF-β, HSP27) responsible for drug resistance, and to use oncolytic adenovirus to improve antitumor immunity.

V. CONCLUSIONS

The mechanisms underlying the resistance of HCC cells to sorafenib remain complex. In this study we demonstrate that the inhibition of p38 activity by sorafenib treatment is one of the mechanisms responsible for sorafenib resistance.

The increased activity of p38 by MKK3/6E effectively induced massive cell death and reduced the resistance of HCC cell lines to sorafenib. TGF-β shRNA was shown to increase the activities of both p38 and p-HSP27, and increased the cell death induced by treatment with sorafenib. Likewise, a reduction in the expression of HSP27 increases the sensitivity of HCC cell lines to sorafenib.

In this study, we demonstrate that sorafenib chemotherapy combined with adenovirus gene therapy, which co-expressed shRNA against TGF-β and HSP27, effectively overcomes the sorafenib resistance of HCC cells and improves the anti-tumor effect of sorafenib.

This combinational therapy will be a new hope for HCC patients who have sorafenib resistance, can be a better therapeutic strategy to control the malignant liver cancer.

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<ABSTRACT(IN KOREAN)>

HSP27 과 TGF-β 발현 감소를 통한 간암에서의 sorafenib 저항성 극복

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강 동 욱

간암(HCC)은 세계적으로 발병률이 높은 악성종양 중 하나이다. 현재 외과 수술과 고주파 열 치료 및 간동맥 화학 색전술 등이 간암 치료에 이용되고 있다. 하지만 HCC환자가 보통 말기로 발견되어 치료 방법의 선택이 제한되어 있고 예후도 좋지 않아서 5 년 생존율이 낮다. 해마다 630,000명 이상의 환자가 간암으로 사망하여, 암으로 인한 사망의 3위를 차지하고 있다. 따라서, 간암에 대해 효율적인 항암 요법을 개발하는 것이 필요하다.

Sorafenib은 다중 인산화효소 억제제로 개발되어 말기 간암 치료제로 이용되고 있다. 말기 간암 환자에서 sorafenib의 치료 효과가 고무적이지만 많은 환자에서 치료 효과가 없거나 곧 저항성을 나타낸다. 따라서 말기 간암 환자에서 sorafenib의 효능을 개선하고 저항성을 극복할 수 있는 새로운 치료 전략을 개발하는

것이 필요하다.

본 연구에서는 HCC에서 sorafenib 약제 저항성을 극복하기 위하여 adenovirus 전달체에 heat shock protein 27 (HSP27)과 transforming growth factor-β (TGF-β) mRNA를 타겟팅하는 short hairpin RNA를 발현하는 유전자조각을 삽입하여 새로운 항종양 유전자재조합 adenovirus 유전자치료제를 개발하였다.

p38 MAP kinase 활성은 sorafenib을 저농도로 처리시에 억제되는데 이는 저항성을 유도하는 원인으로 판단된다. 본인은 MKK3 / 6 (MKK3 / 6E)의 constitutive active form을 사용하여 p38의 활성화에 의해 세포사멸이 유도됨을 확인하여 sorafenib 내성이 p38의 활성화에 의해 감소 될 수 있다는 것을 발견하게 되었다. 또한 HSP27에 대한 shRNA와 TGF-β에 대한 shRNA를 사용하여 p38의 활성을 증가시켜 HCC 세포주에서 sorafenib에 대한 저항성을 극복할 수 있음을 보였다.

TGF-β발현은 sorafenib을 저농도로 처리하고 저항성이 나타날때 증가된다. 따라서 본 연구에서 TGF-β shRNA는 sorafenib에 의하여 억제되었던 p38 MAP kinase활성을 회복 시킨다는 것을 밝혔고 HCC에서 sorafenib에 대한 저항성을 극복하게 하고

sorafenib에 의하여 유도되는 세포사멸을 증가시킬 수 있었다. 또한 HSP27 shRNA을 이용하여 광범위하게 약제 저항성에 관여하는 HSP27 발현을 감소시켜서 sorafenib에 의한 세포 사멸을 증가시킬 수 있었다.

유전자치료 전달체로서의 아데노바이러스는 치료 유전자를 효율적으로 전달하며, 높은 역가로 바이러스를 생산이 가능하고 숙주 세포에 바이러스 DNA가 삽입되지 않아 비교적 안전한 유전자 전달체이다.

TGF-β shRNA와 HSP27 shRNA를 함께 사용함으로써 sorafenib에 의해 억제되었던 p38 MAP kinase활성이 회복되고, HSP27의 인산화 증가 및 HSP27 감소가 유발되였다. 이러한 변화로 인행 sorafenib에의한 HCC 세포 사멸이 극대화되였다.

본인은 간암의 새로운 치료로 유전자 치료법을 이용한 sorafenib 저항성 극복 방법을 제시하였다.

핵심되는 단어:간암, sorafenib, 저항성, TGF-β, HSP27, p38, 아데노바이러스

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