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Sorafenib induces renal cell carcinoma apoptosis *via* upregulating activating transcription factor 4

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Previous studies have shown sorafenib to function as a multitargeted tyrosine kinase inhibitor in different tumors. However, whether sorafenib improves renal cell carcinoma (RCC) through activating transcription factor 4 (ATF4) has never been explored. In the current study, we showed that sorafenib could suppress RCC cell viability in a time- and dose-dependent manner. Furthermore, sorafenib is demonstrated to enhance the mRNA and protein levels of ATF4. Meanwhile, overexpression of ATF4 was demonstrated to induce ACHN cell cycle arrest and cell apoptosis. Moreover, treatment with sorafenib could enhance the expression of CCAAT/enhancer-binding protein-homologous protein (CHOP) and p53 upregulated modulator of apoptosis (PUMA), thereby leading to ACHN cell apoptosis. More importantly, silencing of ATF4 could largely abolish sorafenib-induced upregulation of CHOP and PUMA in ACHN cells. Meanwhile, sorafenib-induced cell apoptosis may be dependent on the activation of ATF4 since knockdown of ATF4 partially reversed sorafenib-induced ACHN cell apoptosis. In summary, the present study demonstrates that sorafenib activates ATF4-CHOP-PUMA pathway in RCC cells, resulting in enhanced ER stress-related cell apoptosis.

1. Introduction

It is reported that renal cell carcinoma (RCC) accounts for approximately 3%–4% of all human malignancies (Siegel et al. 2016). Among RCC, clear cell RCC (CC-RCC) is the most common pathology (Zhao et al. 2013). Currently, the primary therapy for early renal cancer is surgical resection. However, there are still 30% of RCC patients who are not suitable for surgery due to the lack of early diagnosis and distant metastasis (Coppin et al. 2005). Therefore, it is necessary to explore effective therapy methods for patients with advanced RCC.

Because of the rapid and uncontrollable growth, tumors are growing in extracellular environments that lack of nutrients and oxygen, leading to the homeostasis disruption and the activation of unfolded protein response (UPR)(Burton et al. 2016; Zhao et al. 2016). UPR reduces the detrimental effects of accumulated unfolded proteins by enhancing protein degradation and reducing protein synthesis (Kumar et al. 2016; Rajapaksa et al. 2016). Increasing study results suggest that UPR promotes tumor development, angiogenesis, cell growth, and cell migration (Storm et al. 2016; Zhao et al. 2016). Activating transcription factor 4 (ATF4) belongs to the ATF/CREB family, which is widely involved in the regulation of genes that participate in redox homeostasis and UPR (Liu et al. 2015; Luo et al. 2016).

Preclinical and clinical studies have shown sorafenib to function as a multitargeted tyrosine kinase inhibitor that targets several tyrosine kinases, including vascular endothelial growth factor (VEGFR), platelet-derived growth factor receptor-β (PDGFR), and so on (Chouhan et al. 2007; Wilhelm et al. 2008). However, whether sorafenib improves RCC through ATF4 in RCC has never been explored.

In the present study, we found that sorafenib treatment enhanced ATF4 expression in RCC cells. A further study evaluates the specific mechanism in which ATF4 enhances RCC cell apoptosis, which may shed light on the novel treatment method of RCC among advanced patients.

2. Investigations, results and discussion

2.1. Sorafenib suppresses ACHN cell viability in a time- and dose-dependent manner

Firstly, we evaluated the effects of sorafenib on ACHN cell viability. MTT assay indicated that 1, 10, 100 μM sorafenib decreased ACHN cell viability by 16.7%, 34.8%, 53.2%, respectively (Fig. 1A). Next, pre-incubation with 10 μM sorafenib reduced ACHN cell viability by 22.2 %, 34.2 %, 45.6 % at 24 h, 48 h, 72 h, respectively (Fig. 1B).

2.2. Sorafenib enhances ATF4 expression in ACHN cells

ACHN cells were treated with 1, 10, 100 μM sorafenib for 48 h. ATF4 mRNA and protein levels were then examined using RT-PCR and western blot analysis, respectively. As shown in Fig. 2A and 2B, treatment with 1, 10, 100 μM Sorafenib significantly enhanced the mRNA and protein level of ATF4 in ACHN cells. Meanwhile, ACHN cells were incubated with 10 μM sorafenib for 24 h, 48 h, 72h. Here, our data showed that incubation with 10 μM sorafenib increased the mRNA and protein expression of ATF4 in ACHN cells (Fig. 2C and 2D).

2.3. Overexpression of ATF4 exerts anti-proliferative and pro-apoptotic roles in ACHN cells

Furthermore, we overexpressed ATF4 in ACHN cells and evaluated its effect on ACHN cell proliferation. Transfection with p-ATF4 for 48 h significantly enhanced the mRNA and protein level of ATF4 in ACHN cells (Fig. 3A and 3B). More importantly, flowcytometry analysis demonstrated that overexpression of ATF4 significantly enhanced G0-G1 cell arrest (Fig. 3C). Annexin V-PI staining indicated that overexpressed ATF4 increased ACHN cell apoptosis by 24.5% (Fig. 3D). These data indicated the anti-proliferative and pro-apoptotic roles of ATF4 in ACHN cells.

2.4. Knockdown of ATF4 enhances ACHN cell proliferation

In contrast, we silenced ATF4 in ACHN cells. Real time PCR and Western blot analysis showed the mRNA and protein level of ATF4 were reduced by the specific siRNA targeting ATF4 (Fig. 4A and 4B). Moreover, the proportion of cells in G0/G1 phases was lower while the proportion in the S phase was higher, suggesting that knockdown of ATF4 induced ACHN cell proliferation (Fig. 4C). In addition, flow cytometry analysis indicated that ATF4 knockdown decreased apoptotic rate by 1.2% (Fig. 4D).

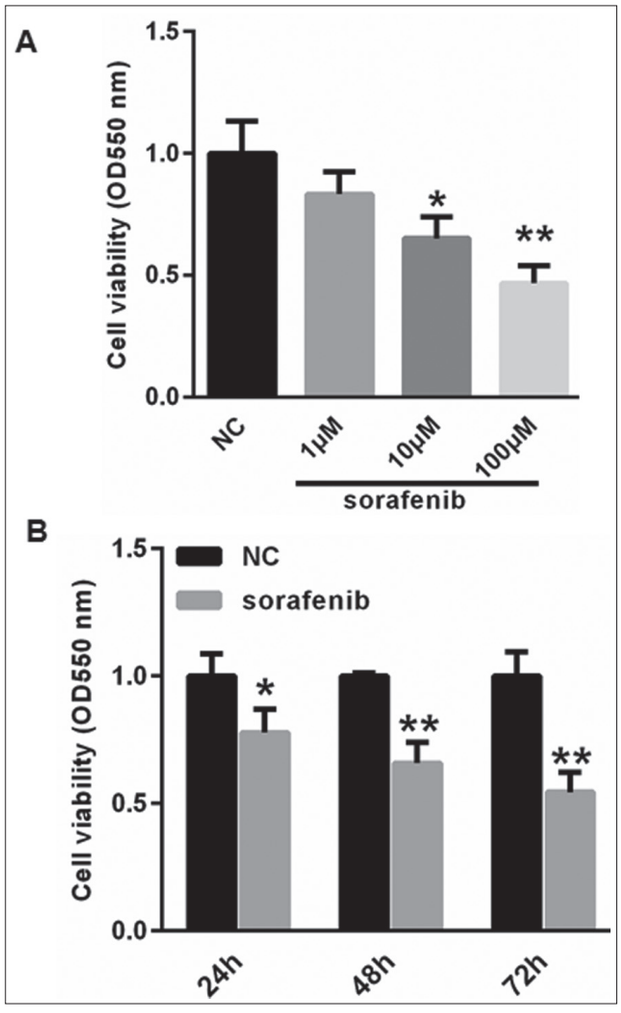

Fig. 1: Sorafenib suppresses ACHN cell viability in a time- and dose-dependent manner. (A) MTT assay indicated that 1, 10, 100 μM sorafenib decreased ACHN cell viability by 16.7 %, 26.8 %, 43.2 %. (B) Pre-incubation with 10 μM sorafenib for reduced ACHN cell viability by 22.2 %, 34.2 %, 45.6 % at 24 h, 48 h, 72 h. $^{*}p<0.05$, $^{**}p<0.01$ vs. con.

2.5. Silencing of ATF4 could reverse sorafenib induced apoptosis effects

To determine whether sorafenib suppressed ACHN cell proliferation mainly by upregulating ATF4 expression, we silenced ATF4 with or without the presence of 10 μM sorafenib for 48 h. Western blot analysis showed that transfection with si-ATF4 significantly suppressed the expression of ATF4 even in ACHN cells treated with 10 μM Sorafenib (Fig. 5A). Meanwhile, we also tested the expression of CCAAT/enhancer-binding protein-homologous protein (CHOP) and p53 upregulated modulator of apoptosis (PUMA) after treatment with 10 μM sorafenib for 48 h. Our data indicated that sorafenib increased the protein levels of CHOP and PUMA in ACHN cells (Fig. 5A). However, knockdown of ATF4 could significantly decrease the expression of CHOP and PUMA even in cells pretreated with 10 μM sorafenib (Fig. 5A). More importantly, sorafenib-induced ACHN cell apoptosis could be significantly abolished by transfection with si-ATF4 in ACHN cells (Fig. 5B). These data indicated that sorafenib-induced apoptosis is mainly achieved through upregulating ATF4.

As a common malignant neoplasm in the urinary system, RCC ranks the sixth leading cause of cancer mortality in Western countries (Siegel et al. 2016). Approximately 63,920 new cases of kidney cancer were diagnosed in 2016 in the USA (Siegel et al. 2016). Even with advancement of clinical management of RCC in recent years, 20–40% of RCC patients still suffer from cancer progression because of high resistance of chemotherapy and radiotherapy (Han et al. 2010). Thus, it is of great importance to explore the underlying mechanism that can effectively suppress RCC cell growth and metastasis.

Sorafenib is an Raf1/Mek/Erk kinase inhibitor and is approved for the therapy of RCC (Halbach et al. 2016; Karovic et al. 2016). A recent study has shown that incubation with sorafenib induces ER stress and results in cell death in human leukemia cells (Rahmani et al. 2007). ATF4 plays a key role in ER stress-induced cell death and cancer progression (Wang et al. 2015; Wu et al. 2015). Thus, ATF4 may serve as an attractive target for anticancer therapeutics. However, whether sorafenib leads to ER stress induced cell apoptosis via ATF4 in RCC cells has never been explored. In the current study, we showed that sorafenib could suppress RCC cell viability in a time- and dose-dependent manner. Given the importance of ATF4 in cancer development, we investigated the role of sorafenib on the expression of ATF4 in RCC cells. Here, sorafenib is demonstrated to enhance the mRNA and protein level of ATF4 in ACHN cells. Meanwhile, overexpression of ATF4 was demonstrated to induce ACHN cell cycle arrest and cell apoptosis.

Multiple proteins are suggested to be involved in the ER stress-induced cell apoptosis and the transcription factor ATF4 is one of the key regulators (Milani et al. 2009; Nagelkerke et al. 2013). In the process of ERS-related cell apoptosis, the transcription of CHOP is induced, which eventually leads to cell apoptosis (Martinez-Reyes et al. 2012; Fan et al. 2014). Furthermore, CHOP increases the expression of PUMA, a member of the Bcl2 protein family, which plays a crucial role in the apoptotic process in different cancer cells (Um et al. 2011). PUMA is a pro-apoptotic protein, that acts as an important regulator in cell death in response to ER stress (Chatwichien et al. 2016; Liu et al. 2016). Here, we found that treatment with sorafenib could enhance the expression of CHOP and PUMA, thereby leading to ACHN cell apoptosis. More importantly, silencing of ATF4 could largely abolish sorafenib-induced upregulation of CHOP and PUMA in ACHN cells. Meanwhile, sorafenib-induced cell apoptosis may be dependent on the activation of ATF4 since knockdown of ATF4 partially reversed sorafenib-induced ACHN cell apoptosis.

To the best of our knowledge, the present study is the first to demonstrate that sorafenib activates ATF4-CHOP-PUMA pathway in RCC cells, resulting in enhanced ER stress-related cell apoptosis. We hypothesize that ATF4 protects RCC cells from malignant transformation and that ATF4 is therefore a potential therapeutic target for RCC patients. However, further studies are required to determine the underlying mechanism.

3. Experimental

3.1. Cell culture

RCC cells (ACHN and Caki-1) were purchased from American Type Culture Collection (ATCC, VA, USA). The cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 (Forma Series II 3110 Water-Jacketed CO_2 Incubator, Thermo Fisher Scientific Inc., USA) in Dulbecco's modified Eagle's medium (31600-034, HyClone Company, Logan, UT, USA) containing 10% heat-inactivated fetal bovine serum (FBS) (16000-044, Gibco Company, Grand Island, NY, USA) and 100 U/mL penicillin-streptomycin (15140122, GibcoCompany, Grand Island, NY, USA).

3.2. Analysis of apoptosis

To analyze cell apoptosis, an Annexin V apoptosis kit (BD Pharmingen) was applied. Firstly, cells were washed with ice-cold PBS and then re-suspended in binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, and 2.5 mM $CaCl_2$) at a concentration of 1×10^6 cells/ml. Then, the cells were incubated with 5 μl each of annexin V and PI. The samples were then examined using fluorescence-activated cell sorting (FACS), and the results were analyzed using CellQuest software (Becton Dickinson, San Jose, CA).

3.3. Transfection

The siRNA targeting ATF4 or a non-specific siRNA (NC) were purchased from Genepharma (Shanghai). Transfection of si-ATF4 or NC was performed with HiperFect transfection reagent (QIAGEN) according to the instructions. In brief, 6 × 105 cells were equally seeded in the 6-well plates with 2 ml DMEM culture medium containing serum and antibiotics. At the same time, si-ATF4 or NC were mixed with HiperFect transfection reagent (QIAGEN) and incubated at room temperature for 10 min. Then, the complex was respectively transfected into cells for 48 h.

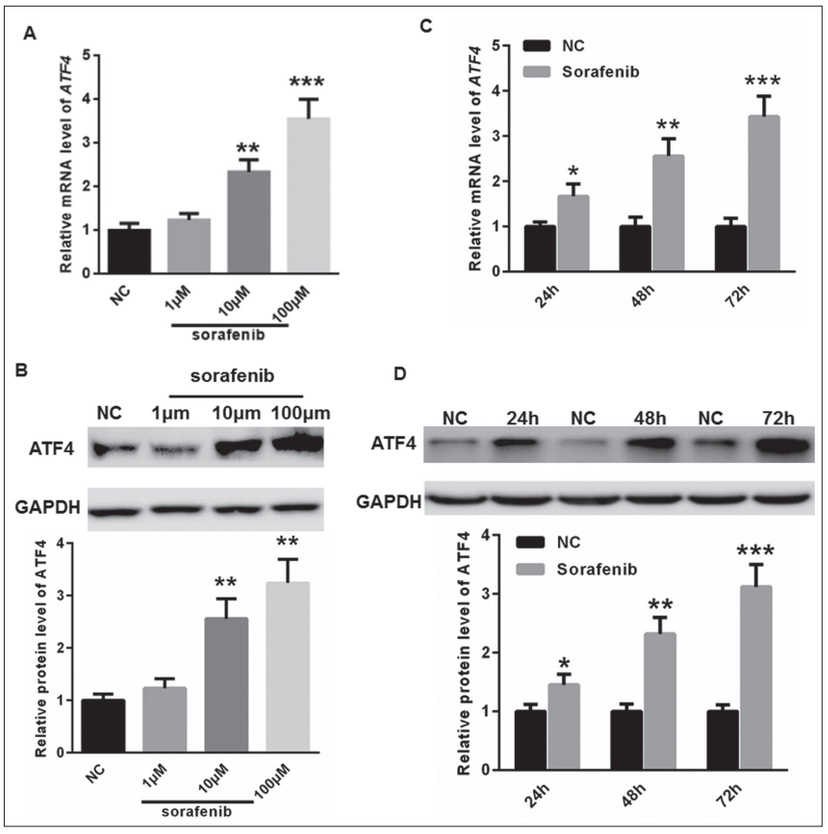

Fig. 2: Sorafenib enhances ATF4 expression in ACHN cells. RT-PCR (A, C) and western blot analysis (B, D) were applied to determine the mRNA and protein level of ATF4 after treatment with sorafenib at different concentration and time. $^{*}p<0.05$, $^{**}p<0.01$ vs.con.

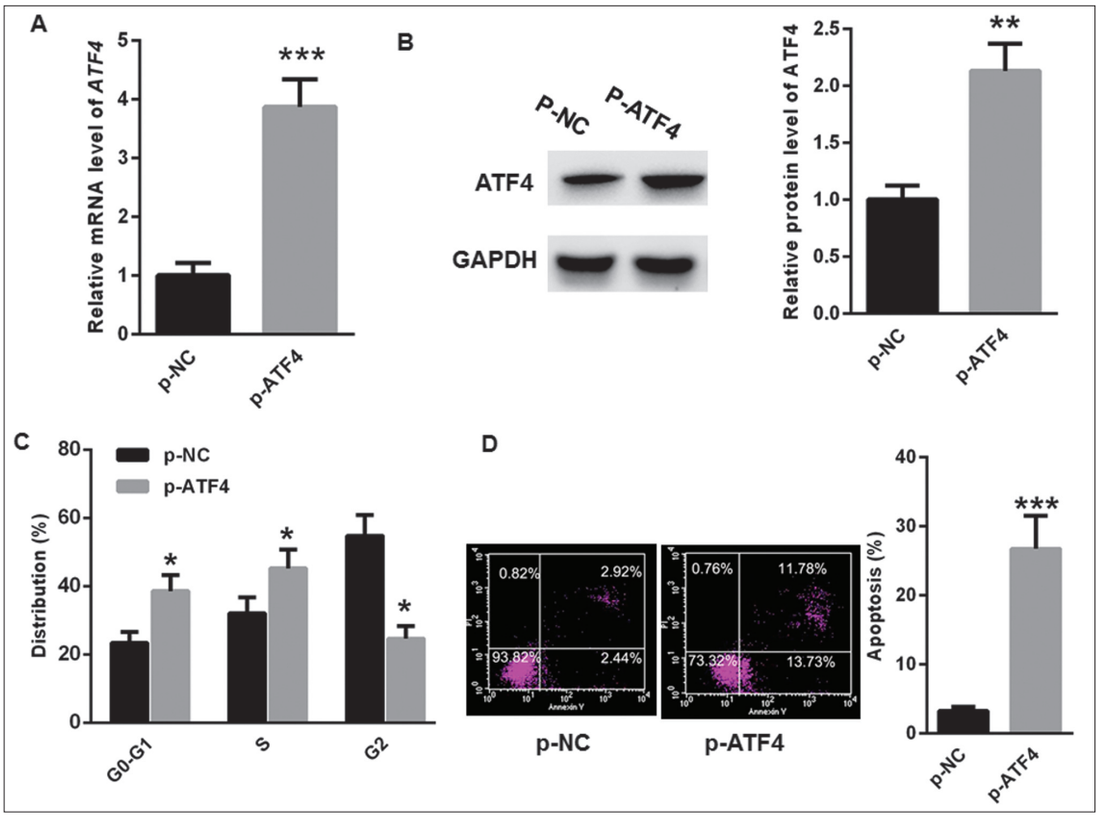

Fig. 3: Overexpression of ATF4 exerts anti-proliferative and pro-apoptotic roles in ACHN cells. Transfection with p-ATF4 for 48 h significantly enhanced the mRNA (A) and protein level (B) of ATF4 in ACHN cells. (C) Flow cytometry analysis demonstrated that overexpression of ATF4 significantly enhanced G0-G1 cell arrest. (D) Annexin V-PI staining indicated that overexpressed ATF4 increased ACHN cell apoptosis compared with control. $^{*}p<0.05$, $^{**}p<0.01$ vs.con.

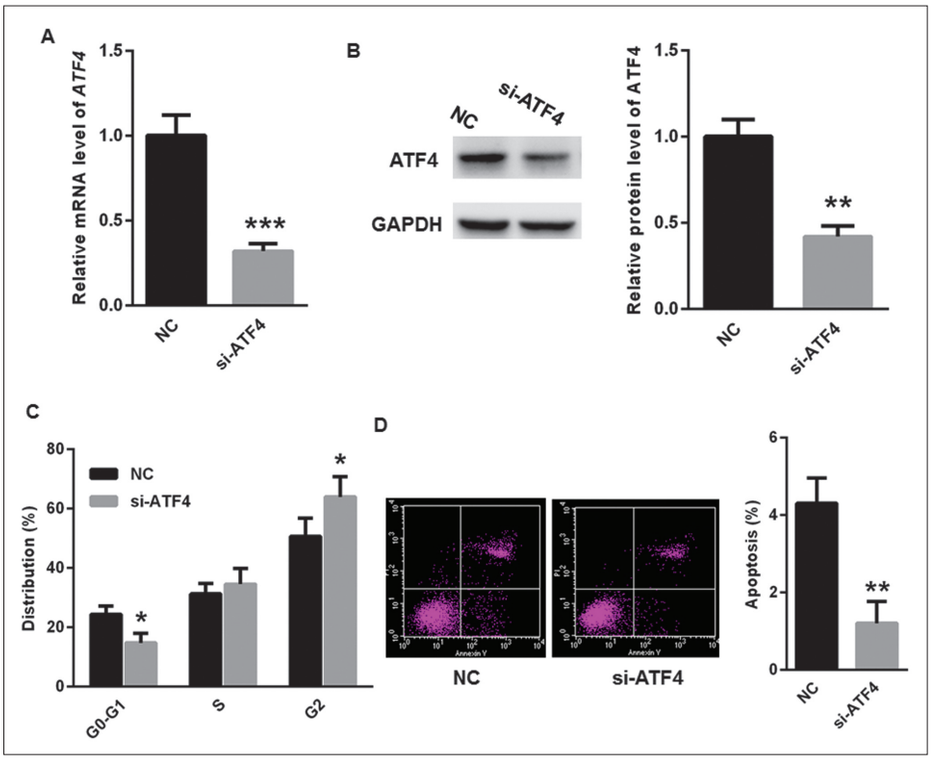

Fig. 4: Knockdown of ATF4 enhances ACHN cell proliferation. Real time PCR (A) and Western blot analysis (B) showed the mRNA and protein level of ATF4 were reduced by the specific siRNA targeting ATF4. (C) The proportion of cells in G0/G1 phase cells was lower after knockdown of ATF4 in ACHN cells. (D) Flow cytometry analysis indicated that ATF4 knockdown decreased apoptotic rate by 1.2 %. *p<0.05, **p<0.01 vs.con.

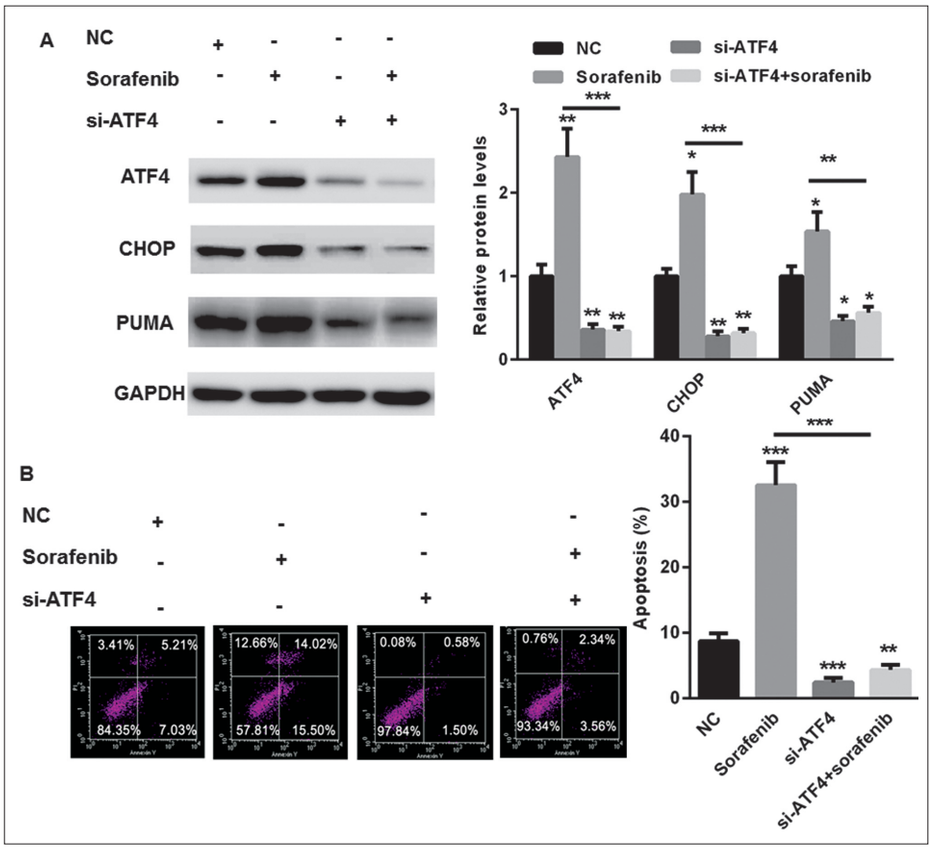

Fig. 5: Silencing of ATF4 could reverse sorafenib induced apoptosis effects. (A) Knockdown of ATF4 could significantly decreased the expression of CHOP and PUMA even in cells pretreated with 10 μM sorafenib. (B) Sorafenib-induced ACHN cell apoptosis could be significantly abolished by transfection with si-ATF4 in ACHN cells. *p<0.05, **p<0.01 vs.con.

3.4. RNA extraction and real-time PCR

RNA was isolated in an RNA TRIZOL (Invitrogen) according to the manufacturer's instructions. Then, the RNA was transcribed into complementarty DNA (cDNA) with TaqMan RNA Reverse Transcription Kit (Applied Biosystems). To quantify the expression of mRNAs, a quantitative real-time PCR assay was carried out with SYBR Green Supermix (Bio-Rad) in a BIO-RAD iCycleriQ real-time PCR detection system as previously described (Guo et al. 2014).The primers used in the current study were listed as follows:
ATF4-F, 5'-CCCCTTCACCTTCTTACAACC-3';
ATF4-R, 5'-GGGCTCATACAGATGCCACTA -3';
PUMA-F, 5'-TGTCCTGGATGAGGATGTGA-3';
PUMA-R, 5'-GCAGTTAGCAGGGGACTGAG-3';
CHOP-F, 5'-GCAGGGTGTGAGAGAAGAGG-3';
CHOP-R, 5'-TGTGCAAAGGCAAACTCTTG-3';
β-actin-F, 5'-CTCCATCCTGGCCTCGCTGT-3';
β-actin-R, 5'-GCTGTCACCTTCACCGTTCC -3'.

3.5. Cell proliferation

MTT assay was carried out to evaluate cell proliferation. Briefly, cells were cultured at 10^3 cells/well in a 96-well plate for 24 h. Then, 100 ìL of DMEM and 20 ìL of MTT (5 mg/mL) (Sigma, USA) were added to each well. After the cells were incubated at 37 °C for 4 h, the medium was discarded and 200 ìL of 0.04 M hydrochloric acid in isopropanol was added to each well. The amount of MTT formazan product in each well was determined by measuring absorbance using a microplate reader at a test wavelength of 490 nm. Each assay was performed in triplicate.

3.6. Cell cycle analysis

Cells were transfected using p-NC or p-ATF4, which were synthetized by Genchem (Shanghai, China), for 48 h and collected using trypsin. Then, the cells were washed, resuspended in PBS and fixed with cold ethanol. Furthermore, the fixed cells were incubated in phosphate–citric acid buffer for 5 min and re-suspended in PI/RNase solution (PBS, RNase, and 50 mg/ml PI) for 15 min at 37 °C in the dark. After that the cells were analyzed using flow cytometry using a BD FACSCalibur (BD Biosciences, San Jose, CA) and FlowJo software (Tree Star, Inc., Ashland, OR).

3.7. Western blot

Proteins samples were isolated from RIPA buffer (1% TritonX-100, 15 mmol/L NaCl, 5 mmol/L EDTA, and 10 mmol/L Tris-HCl (pH 7.0) (Solarbio, China) with a supplementation of a protease and phosphatase inhibitor cocktail (Sigma). Next, the samples were separated by 12% SDS-PAGE, and then electrophoretically transferred onto a PVDF membrane. The membranes were further blocked with 8% milk in PBST (pH 7.5) for 2 h at room temperature and were incubated with the primary antibodies, anti-ATF4, anti-CHOP, anti-PUMA and anti-GAPDH (Cell signaling). Immunodetection was performed using enhanced chemiluminscence detection system (Millipore) according to the manufacturer's instructions. GAPDH was used as the internal control.

3.8. Assay for intracellular ROS

CM-H2DCFDA (Invitrogen) was used as the probe to detect reactive oxygen species (ROS). ACHN and Caki-1cells were infected with p-NC or p-ATF4 for 48 h. The cells were grown to 90% confluence, washed with warm PBS and then harvested using trypsinization (250 µl of trypsin per well). The cells were then washed four times and incubated with 10 µM CM-H2DCFDA for 45 min at 37°C in a relatively high humidity (95%) atmosphere containing a controlled level of CO2 (5%) in the dark. The intensity of fluorescence (490 nm excitation and 527 nm emission) was recorded using a Tecan Genios Microplate Reader (Infinite M200). The obtained values (fluorescence/mg protein) are shown relative to the number of p-CGN infected cells (set to 100%).

3.9. Statistical analysis

The data are represented as the mean ± standard error of the mean (SEM). The two-tailed unpaired student's t-tests were used for comparisons of two groups. The ANOVA multiple comparison test (SPSS 13.0) followed by Turkey post hoc test were used for comparisons of two more groups. $P < 0.05$ was considered to be statistically significant.

Conflicts of interest: None declared.

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