Knockdown of EBP1 promotes doxorubicin-induced apoptosis in renal clear cell carcinoma cells through activation of the p38/HIF-1α pathway

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Abstract. Kidney clear cell carcinoma (KIRC) is a prevalent urological cancer. Despite substantial improvements in KIRC care, patients with intermediate and advanced stages of the disease lack access to appropriate medications. Doxorubicin is widely used as a chemotherapy drug for the treatment of multiple types of cancer. However, its use is associated with harmful side effects and drug resistance. ErbB3-binding protein (*EBP1*) is highly expressed in KIRC, and the knockdown of *EBP1* reduces the phosphorylation of p38 mitogen-activated protein kinase (p38MAPK) and the expression of HIF-1α. Therefore, the present study aimed to evaluate the effectiveness of combined doxorubicin administration and *EBP1* knockdown in KIRC cell lines. The KIRC cell lines 786-O and 769-P were used for the experiments, and short hairpin RNA technology was employed to specifically knock down the expression of the *EBP1* gene. After treatment, cells were analyzed by western blotting to detect changes in p38MAPK phosphorylation levels and HIF-1α expression. The results showed that *EBP1* knockdown significantly enhanced the antitumor effect of doxorubicin on KIRC cells through the p38MAPK/HIF-1α pathway. In conclusion, the knockdown of *EBP1* in combination with doxorubicin may be a potential strategy for the treatment of KIRC.

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Introduction

Kidney clear cell carcinoma (KIRC) is a malignant tumor with high morbidity and an increasing mortality rate. Notably, the incidence rate of KIRC has been increasing by 1-2% annually, which is due to factors such as unhealthy lifestyle habits, delays in diagnosis and treatment, and the development of resistance to treatment (1). Treatments frequently used for KIRC include surgical treatment and chemotherapy (2); however, given that most individuals diagnosed with KIRC are in the advanced stages of the disease, chemotherapy is vital in the treatment regimen (3). Doxorubicin is used to treat a variety of malignancies (4); however, its therapeutic applicability is limited because it can cause adverse effects and the development of drug resistance (5). Consequently, it is critical to enhance the ability of doxorubicin to target cancer cells and reduce the side effects associated with its use.

Elevated levels of ErbB3-binding protein 1 (EBP1) have been reported in numerous types of cancer, in which this protein is associated with a poor prognosis, treatment resistance and tumor development (6). Hypoxia-inducible factor-1α (HIF-1α) also plays a pivotal role as a transcription factor in the onset of cancer and serves as a target for the advancement of personalized cancer therapies (7). HIF-1α is crucial in controlling the expression of a number of downstream target genes, which influences the progression of various types of cancer (8). The knockdown of *EBP1* in hepatocellular carcinoma cells has been shown to lead to reduced p38 mitogen-activated protein kinase (p38MAPK) phosphorylation and the downregulation of HIF-1α expression, thereby affecting the proliferation and migration ability of the cells (9). In addition, EBP1 has been demonstrated to promote the occurrence and development of KIRC by regulating the p38/HIF-1α signaling pathway (10).

p38MAPK is essential in various aspects of cell proliferation, differentiation, death and embryogenesis, in addition to tumorigenesis (11). A novel antitumor drug targeting p38MAPK, named ralimetinib, is currently being developed and has entered clinical trials for the treatment of ovarian cancer (12). However, p38MAPK inhibitors are not effective when administered alone, and so are often used in combination with other drugs (13). Doxorubicin penetrates cells and

regulates a variety of target genes to prevent cancer cell growth and proliferation and to trigger apoptosis (14). The effects of doxorubicin include the promotion of reactive oxygen species formation (15), DNA modification and topoisomerase II inhibition (4). Previous research has also shown that the administration of doxorubicin alters the phosphorylation level of p38 in gastric cancer cells (16), with a time-dependent inhibitory effect. Furthermore, the p38MAPK-specific inhibitor SB203580 has been demonstrated to increase the sensitivity of gastric cancer cells to doxorubicin (17). It could be hypothesized that the inhibition of p38MAPK expression may be associated with increased sensitivity of KIRC to doxorubicin chemotherapy. In addition, we further hypothesize that the EBP1 protein may regulate the sensitivity of KIRC cells to doxorubicin via the p38MAPK/HIF-1α pathway.

Therefore, the present study knocked down *EBP1* in two human KIRC cell lies, 786-O and 769-P, with the aim of investigating the effect of *EBP1* on doxorubicin-induced apoptosis and exploring the underlying mechanism of action.

Materials and methods

Bioinformatics. The UALCAN (https://ualcan.path.uab.edu/index.html) and GEPIA (gepia.cancer-pku.cn) databases were used to evaluate the expression of *EBP1* in the tissues of patients with KIRC and its association with tumor grade, stage and overall survival. In GEPIA, the 'match TCGA normal and GTEx data' setting was used.

Cell culture and processing. Preliminary data were obtained demonstrating that EBP1 is expressed at high levels in 786-O and 769-P human KIRC cell lines. Specifically, the mining and analysis of relevant public databases revealed that the expression level of EBP1 in these two cell lines was significantly higher compared with that in most other renal cancer cell lines, a trend consistently validated across multiple bioinformatics platforms, including the Cancer Cell Line Encyclopedia database (https://sites.broadinstitute.org/ccle/), The Cancer Genome Atlas (TCGA; https://www.cancer.gov/tcga; TCGA-KIRC project) and Gene Expression Omnibus (GEO; https://www.ncbi.nlm.nih.gov/geo/). The GSE15641 (18) dataset was downloaded from the GEO.

Therefore, these cell lines were chosen for analysis. The 786-O and 769-P cell lines were sourced from the Cell Bank of Type Culture Collection of The Chinese Academy of Sciences. They were cultured at 37˚C in an atmosphere consisting of 5% CO_2 and 95% air, using RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) enriched with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), along with 100 μg/ml streptomycin and 100 U/ml penicillin. The 786-O and 769-P cells were seeded into culture dishes, and the medium was supplemented with doxorubicin (3 μM; cat. no. E2516; Selleck Chemicals) and the p38MAPK inhibitor SB203580 (10 μM; cat. no. S1076; Selleck Chemicals). All cells were incubated at 37˚C with 5% CO_2 in a humidified incubator for 48 h to ensure effective drug treatment.

Cell Counting Kit-3 (CCK-8) assay. In order to assess the effect of doxorubicin on the viability of the 786-O and 769-P cell lines, 200 μl complete growth medium was added to the cells in each well of a 96-well plate, and the cells were then incubated for 24 or 48 h with 3 μM doxorubicin. The CCK-8 assay kit from Shanghai Biyuntian Biotechnology Co., Ltd. China was then used to analyze the cells. In summary, each sample was treated with 10 μl CCK-8 solution and incubated at 37˚C for 2 h. The optical density at 450 nm was then assessed using a microtiter plate reader, and cell survival curves were plotted.

Colony formation assay. A colony formation assay was performed to assess the ability of doxorubicin to inhibit 786-O and 769-P cell proliferation. The cells were inoculated into 6-well plates at a density of $3x10^3$ cells/well and the plates were incubated for 1 week. After aspirating the liquid from the 6-well plate, the cells were fixed with 4% paraformaldehyde for 15 min at room temperature and washed with PBS. Subsequently, the cells were stained with Giemsa (Beijing Solarbio Science & Technology Co., Ltd.) at room temperature for 20 min, followed by two washes with PBS. Colonies were defined as containing ≥50 cells and were quantified using ImageJ bundled with Java8 64-bit (National Institutes of Health).

Migration assay. The scratch test was performed to determine the effect of doxorubicin on the migration of 786-O and 769-P cells. The 769-P and 786-O cells were seeded in 6-well plates at a density of $1x10^6$ cells/well and grown to 90% confluence. Subsequently, the cells were cultured overnight in serum-free medium. In brief, a sanitized grid scale was placed over the confluent cells in a 6-well plate, and a 1,000-μl pipette tip was applied from the top to bottom of the grid to create a scratch in the cells. The cells were washed twice with PBS and then cultured in serum-free medium containing doxorubicin for 48 h. Using a Nikon Eclipse Ti-S/L100 inverted phase contrast fluorescence Microscope (Nikon Corporation), images of cell migration into the wound were captured at 0, 24 and 48 h at the same lesion site. Wound measurement was performed using ImageJ, and statistical analysis was conducted using GraphPad Prism 10.0 (Dotmatics). Each experiment was repeated at least three times.

TUNEL assay of apoptosis. Apoptosis was assessed using the DeadEnd™ Fluorometric TUNEL System (Promega Corporation) and fluorescence microscopy. Six-well plates were seeded with $1x10^5$ 786-O or 769-P cells/well, and the cells were cultured at 37˚C in an incubator with 5% CO_2. The cells were treated with doxorubicin for 24 h before analysis using the TUNEL assay kit. First, the cells were fixed with 4% paraformaldehyde at room temperature for 15 min, washed three times with PBS, then incubated with rTdT enzyme incubation buffer at 37˚C for 70 min in the dark. Subsequently, the cells were stained with DAPI solution (1 ml/well) at room temperature for 15 min. The samples were then immediately examined under a fluorescence microscope and images were captured (magnification, x20). Quantification was performed from ~10 fields of view using ImageJ, and statistical analysis was conducted using GraphPad Prism 10.0. Each experiment was repeated at least three times.

Western blotting. Cells were lysed and then washed twice with ice-cold PBS (pH 7.4). Protein lysates were extracted using RIPA buffer (Beijing Solarbio Science & Technology Co., Ltd.),

and nuclear and cytoplasmic extracts were prepared using the Nuclear and Cytoplasmic Extraction Reagents Kit (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The total protein concentration of the lysate was determined using a BCA kit (Thermo Fisher Scientific, Inc.). The protein lysates (30 μg/lane) were then separated by SDS-PAGE on 8-10% gels to ensure effective protein separation and accurate quantification. The proteins were subsequently transferred to a PVDF membrane. The membranes were blocked using 5% skimmed milk (Yuanda Mountain Dairy Co.) in TBS with 10% Tween-20 (TBS-T) for 2 h at room temperature. After blocking, the membranes were incubated with the following primary antibodies in TBS-T overnight at 4˚C: EBP1 (cat. no. sc-393114; Santa Cruz Biotechnology, Inc.), phosphorylated (p-)p38 (cat. no. sc-166182; Santa Cruz Biotechnology, Inc.), p38 (cat. no. sc-81621; Santa Cruz Biotechnology, Inc.), HIF-1α (cat. no. sc-53546; Santa Cruz Biotechnology, Inc.), Bcl-2 (cat. no. sc-7382; Santa Cruz Biotechnology, Inc.), Bax (cat. no. sc-70407; Santa Cruz Biotechnology, Inc.), cleaved-caspase 3 (cat. no. sc-373730; Santa Cruz Biotechnology, Inc.), caspase 3 (cat. no. sc-56053; Santa Cruz Biotechnology, Inc.) and β-actin (cat. no. sc-47778; Santa Cruz Biotechnology, Inc.). The primary antibodies were diluted 1:1,000 in antibody diluent buffer (Beyotime Institute of Biotechnology). Subsequently, the membranes were incubated with HRP-conjugated goat anti-mouse secondary antibodies (1:5,000; cat. no. A0216; Beyotime Institute of Biotechnology) at 4˚C for 2 h. The proteins were visualized using the ECL Western Blotting Substrate system (Beijing Solarbio Science & Technology Co., Ltd.). Protein grayscale values were measured using ImageJ, and statistical analysis was performed using GraphPad Prism 10.0, with each experiment repeated ≥3 times.

Lentiviral transduction. The 786-O and 769-P cells were transduced with two different short hairpin RNA (shRNA) lentiviral vectors targeting EBP1 (sh-EBP1*1 and sh-EBP1*2) and two different shRNA lentiviral vector negative controls (sh-NC*1 and sh-NC*2; Shanghai GeneChem Co., Ltd.). The sh-EBP1 target sequence was 5'-ACCAGCATTTCGGTAAATA-3', and the sh-EBP1 sequences were as follows: sh-EBP1*1 (PA2G4-RNAi(23071-1)-a), 5'-CCGGCCACCAGCATTTCGGTAAATACTCGAGTATTTACCGAAATGCTGGTGGTTTTTG-3' and sh-EBP1*2 (PA2G4-RNAi(23071-1)-b), 5'-AATTCAAAAACCACCAGCATTTCGGTAAATACTCGAGTATTTACCGAAATGCTGGTGG-3'. The control gene insertion sequence was 5'-TTCTCCGAACGTGTCACGT-3', and the sequences of the shRNA controls were as follows: sh-NC*1, 5'-CCGGTTCTCCGAACGTGTCACGTCTCGAGACGTGACACGTTCGGAGAATTTTTG-3' and sh-NC*2, 5'-AATTCAAAAATTCTCCGAACGTGTCACGTCTCGAGACGTGACACGTTCGGAGAA-3'. An *EBP1* overexpression lentiviral vector (pHS-AVC-LY124) and control vector (pHS-AVC-ZQ328) were purchased from SyngenTech Co., Ltd. Lentiviral infection was carried out immediately after virus preparation. The virus titers were as follows: shNC1, $8x10^8$ TU/ml; shNC2, $8x10^8$ TU/ml; sh1 (sh*EBP1*1), $7x10^8$ TU/ml; sh2 (sh*EBP1*2), $7x10^8$ TU/ml. The multiplicity of infection (MOI) values in 786-O cells for shNC1, shNC2, sh1 and sh2 were 5, 10, 10 and 15, respectively. In 769-P cells, the MOI values for shNC1, shNC2, sh1 and sh2 were 10, 15, 15 and 20, respectively. The lentiviruses were added to the cells and incubated for 24 h at 37˚C for gene transduction. Prior to adding the virus to the cells, it was mixed with 2 μg/ml polybrene to enhance infection efficiency. Selection was performed with 1 g/ml puromycin to obtain successfully transduced cells. Transduction efficiency was assessed by western blotting immediately.

Statistical analysis. Images were analyzed using ImageJ software. Differences between two groups were compared by unpaired Student's t-test while differences among multiple groups were compared using one-way or two-way ANOVA with Tukey's post hoc analysis. Statistical evaluation of the results was performed using GraphPad Prism 10.0. Experimental results are presented as the mean ± SD. Each experiment was replicated ≥3 times. $P<0.05$ was considered to indicate a statistically significant difference.

Results

EBP1 is highly expressed in KIRC. The UALCAN and GEPIA databases were used to evaluate the expression of *EBP1* in KIRC. The results showed a marked upregulation in *EBP1* expression in KIRC tissues compared with normal tissues (Fig. 1A). The association between KIRC status and *EBP1* expression was then assessed. The results revealed that *EBP1* expression was highly associated with tumor grade (Fig. 1B). Moreover, the analysis of pathological staging results demonstrated that *EBP1* expression was associated with the stage of KIRC (Fig. 1C). In addition, survival curves (Fig. 1D) showed that the survival rate of patients with high *EBP1* expression was significantly lower compared with that of patients with low *EBP1* expression, indicating a robust association between *EBP1* expression and patient survival.

To clarify the regulatory role of *EBP1* in KIRC, *EBP1* in 786-O and 769-P cells was stably knocked down, and the transduction effect was verified using western blotting. The results revealed that sh-EBP1*1 had a stronger knockdown effect than sh-EBP*2. Therefore, sh-EBP1*1 and sh-NC*1 were selected for use in subsequent experiments (Fig. 1E).

Knockdown of EBP1 decreases viability and proliferation and increases apoptosis in KIRC cells treated with doxorubicin. CCK-8 assay results showed that 786-O and 769-P cells in the sh-EBP1 group grew more slowly than those in the sh-NC group, with a significant difference detected between the groups on days 4-6; following doxorubicin treatment, the viability of cells with *EBP1* knockdown was significantly lower than that of the cells transduced with sh-NC (Fig. 2A). The scratch assay demonstrated that 786-O and 769-P cells in the sh-EBP1 group had a significantly lower capacity to repair scratches after receiving doxorubicin treatment for 24 and 48 h in comparison with those in the sh-NC group (Fig. 2B), indicating that the inhibitory effect of doxorubicin on the migratory ability of KIRC cells was stronger following the knockdown of *EBP1*. The results of the colony formation assay showed that the number of colonies formed by doxorubicin-treated 786-O and 769-P cells was lower in the sh-EBP1 group than in the sh-NC group (Fig. 2C). This result illustrates that

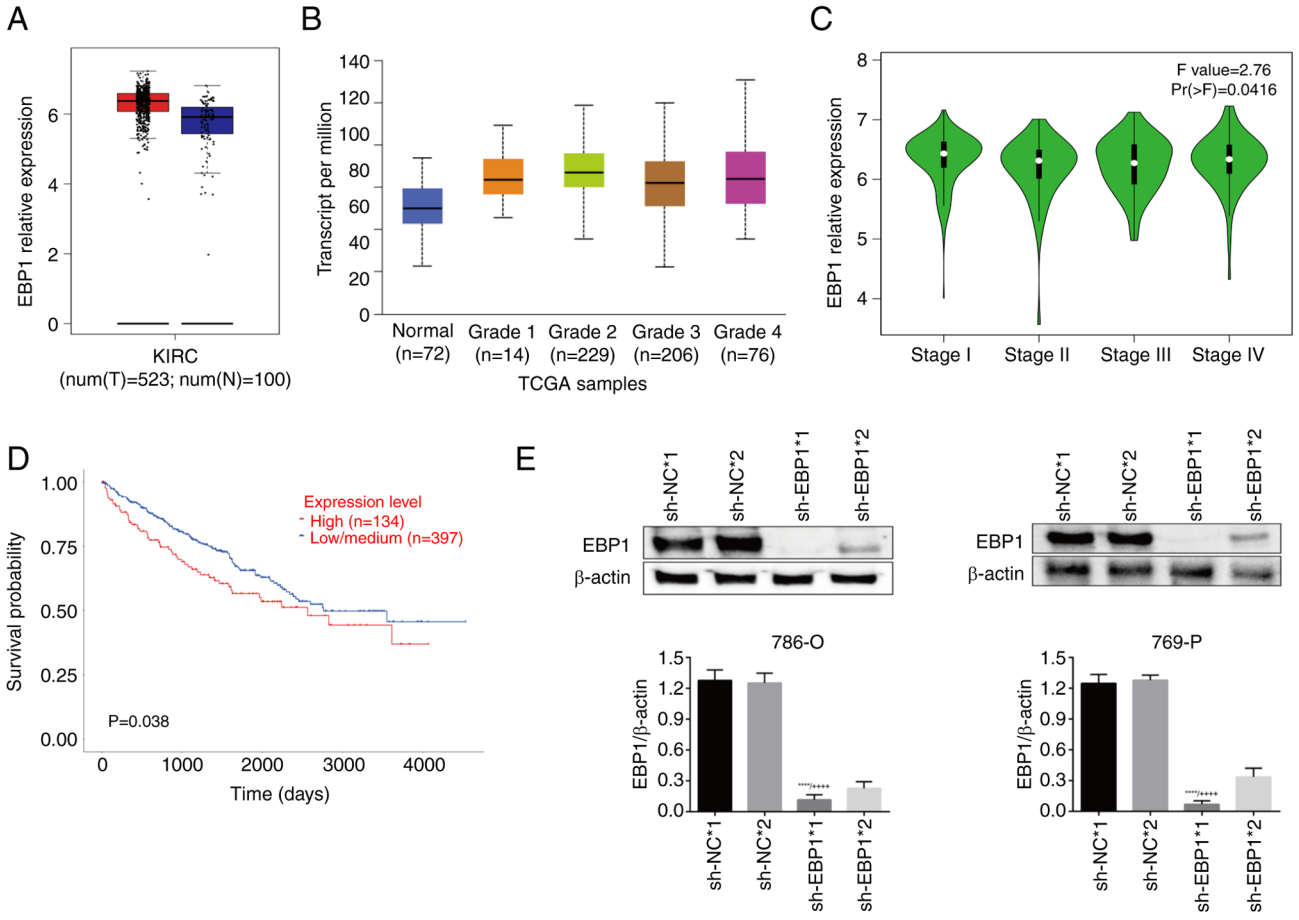

Figure 1. *EBP1* is highly expressed in KIRC. (A) Analysis of *EBP1* expression in KIRC and normal tissues using the GEPIA database. (B) Expression of *EBP1* according to the pathological grade of KIRC assessed using UALCAN. (C) Expression of *EBP1* according to the stage of KIRC assessed using GEPIA. (D) Association of *EBP1* expression with the survival of patients with KIRC assessed using UALCAN. (E) Western blotting detection of lentiviral transduction efficiency in KIRC cell lines. Data are presented as the mean ± SD, n=3. ****P<0.0001 vs. sh-NC*1 and $^{++++}$P<0.0001 vs. sh-NC*2. EBP1, ErbB3-binding protein; KIRC, kidney clear cell carcinoma; T, tumor; N, normal; TCGA, The Cancer Genome Atlas; Pr(>F), P-value for the F-statistic; sh-NC, short hairpin negative control; sh-EBP1, short hairpin EBP1.

the inhibition of KIRC cell proliferation by doxorubicin was enhanced following the knockdown of *EBP1*. TUNEL staining showed that few 786-O and 769-P cells underwent apoptosis in the sh-NC group treated with doxorubicin, and the proportion of apoptotic cells was significantly higher in the sh-EBP1 group (Fig. 2D). These results indicate that *EBP1* knockdown promotes doxorubicin-induced cell death in KIRC cells, suggesting that the anticancer effects of doxorubicin may be potentiated by *EBP1* knockdown.

Doxorubicin acts by inhibiting p38MAPK phosphorylation and HIF-1α expression. The 786-O and 769-P cells were cultured in media containing doxorubicin (3 μM) and the p38MAPK inhibitor SB203580 (10 μM) at 37˚C and 5% CO_2 for 48 h. When doxorubicin was administered to cells in the sh-NC group, the p-p38MAPK/p38MAPK ratio and HIF-1α expression levels significantly decreased (Fig. 3). In the sh-EBP1 group, significant reductions in the p-p38MAPK/p38MAPK ratio and HIF-1α expression levels were also observed after the addition of doxorubicin compared with those in the sh-NC group. In the simultaneous presence of doxorubicin and SB203580, the suppression of the p-p38MAPK/p38MAPK ratio and HIF-1α expression in the sh-NC group was greater compared with that achieved with doxorubicin alone; however, a stronger suppression was observed in the sh-EBP1 group. These results suggest that doxorubicin acts by inhibiting p38MAPK phosphorylation and HIF-1α expression.

Combination of doxorubicin and EBP1 knockdown increases KIRC cell apoptosis and reduces cell viability. The expression of apoptosis-associated proteins Bax and Bcl-2, and the cleavage of caspase-3 in doxorubicin-treated KIRC cells were examined using western blotting. When 786-O and 769-P cells were treated with doxorubicin, the proportion of caspase-3 that was cleaved and the expression of pro-apoptotic factor Bax increased significantly. Conversely, there was a notable downregulation in the expression of Bcl-2, an inhibitory apoptotic component. Furthermore, the Bax/Bcl-2 ratio increased significantly when SB203580 was combined with doxorubicin (Fig. 4A and B). These results suggest that the knockdown of *EBP1* promotes doxorubicin-induced apoptosis in KIRC cells by altering the levels of apoptosis-associated proteins via the p38MAPK pathway.

The viability of KIRC cells in the sh-NC and sh-EBP1 groups following treatment with doxorubicin and SB203580 was assessed using the CCK-8 assay. When the sh-EBP1 group

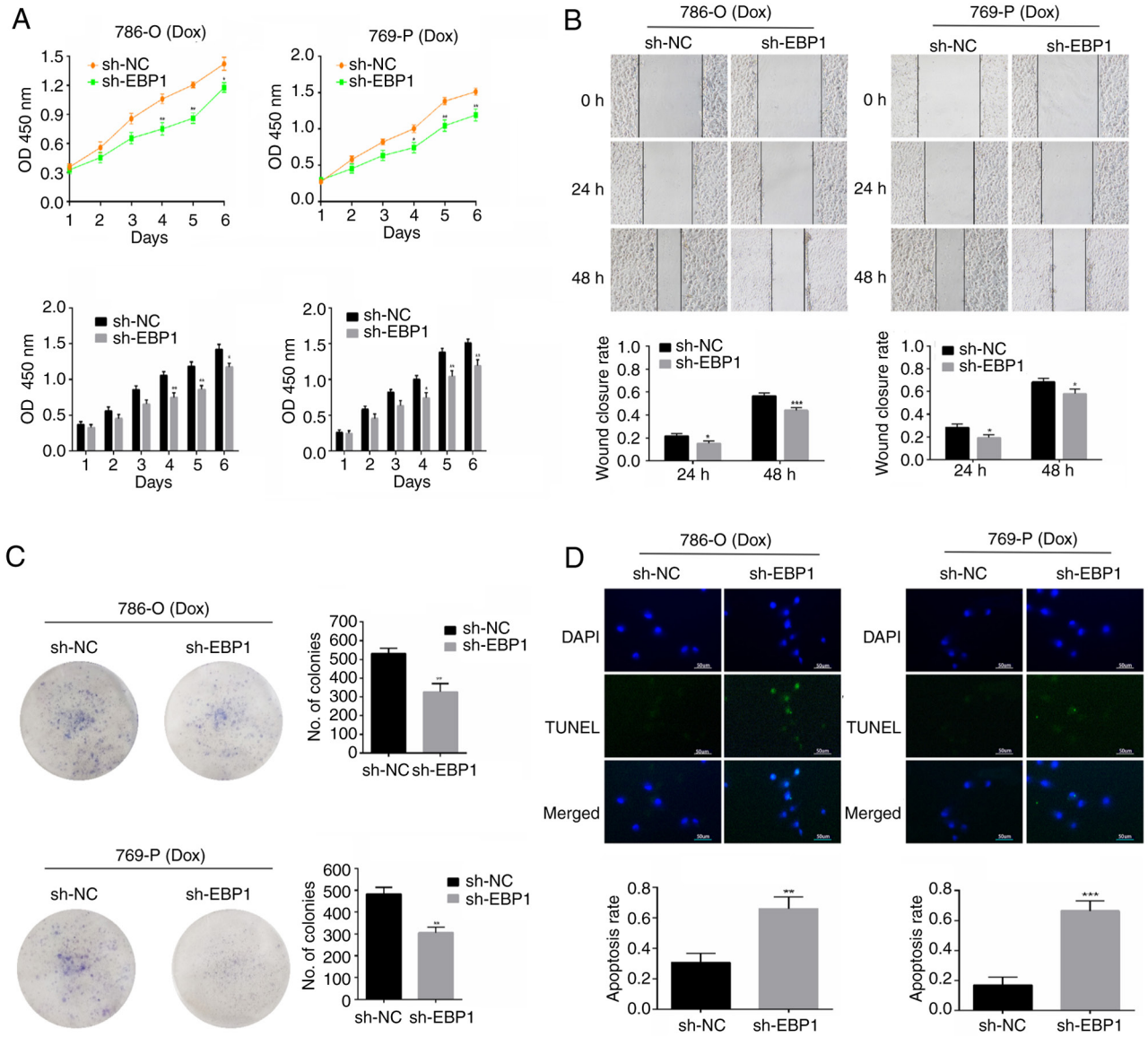

Figure 2. Knockdown of *EBP1* decreases the viability and proliferation of doxorubicin-treated KIRC cells. (A) Cell Counting Kit-8 assay, (B) scratch assay (magnification, x10), (C) colony formation assay and (D) TUNEL assay (scale bar, 50 μm) showing the effect of *EBP1* knockdown on the proliferation, migration and apoptosis of KIRC cells treated with doxorubicin. Data are presented as the mean ± SD, n=3. *P<0.05, **P<0.01 and ***P<0.001 vs. sh-NC. EBP1, ErbB3-binding protein; KIRC, kidney clear cell carcinoma; Dox, doxorubicin; OD450 nm, optical density at 450 nm; sh-NC, short hairpin negative control; sh-EBP1, short hairpin EBP1.

was compared with the sh-NC group in each cell line, it was observed that doxorubicin treatment reduced cell viability and that this effect was even more pronounced following the addition of SB203580 (Fig. 4C and D). These findings indicate that doxorubicin and *EBP1* knockdown cooperate via the p38MAPK pathway to increase the inhibitory effect of doxorubicin on KIRC cell survival.

Discussion

KIRC is a frequently occurring type of kidney cancer (1). The treatment of advanced kidney cancer with radiation and chemotherapy is complicated by tumor resistance to these treatments (19). Doxorubicin is a broad-spectrum anticancer medication that has been reported to inhibit the growth of a variety of cancer cells (4), and shown to have significant efficacy in the treatment of kidney cancer (20). However, the toxic side effects of doxorubicin and the development of drug resistance have limited its clinical application (5). Therefore, the present study attempted to identify a means of increasing the susceptibility of cancer cells to doxorubicin and lowering the dosage of doxorubicin to lessen its harmful side effects. In preliminary experiments, the cytotoxicity of doxorubicin to KIRC cell lines was evaluated, and its half-maximal inhibitory concentration was calculated to be 3 μM (data not shown). At this concentration, doxorubicin effectively induced cell death but had a minimal impact on normal cells, showing suggesting that doxorubicin at this concentration has good selectivity and efficacy. Therefore, this doxorubicin concentration was selected for use in the experiments (21). The anticancer effects of doxorubicin include the induction of apoptosis via the inhibition of RNA and DNA synthesis (22). Doxorubicin

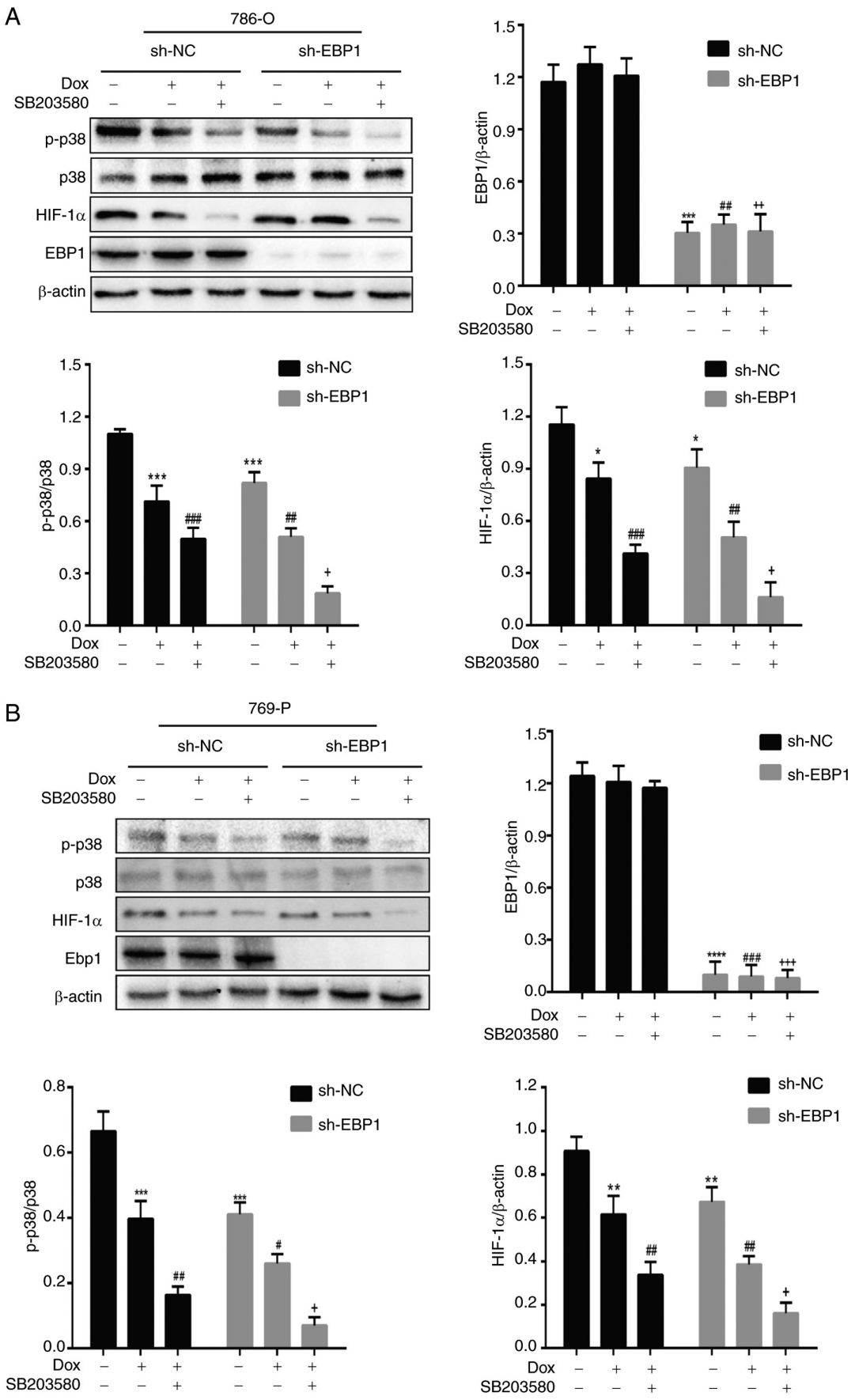

Figure 3. Doxorubicin acts by inhibiting p38 phosphorylation and HIF-1α expression in kidney clear cell carcinoma cells. Effect of doxorubicin and SB203580 on p-p38 and HIF-1α protein levels in (A) 786-O cells and (B) 769-P cells. Representative western blots and quantitative analyses are shown, normalized to β-actin. Data are presented as the mean ± SD, n=3. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001 vs. sh-NC; $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. sh-NC + Dox; $^{+}$P<0.05, $^{++}$P<0.01 and $^{+++}$P<0.001 vs. sh-NC + Dox + SB203580. p38, p38 mitogen-activated protein kinase; HIF-1α, hypoxia-inducible factor-1α; EBP1, ErbB3-binding protein; Dox, doxorubicin; sh-NC, short hairpin negative control; sh-EBP1, short hairpin EBP1; p-, phosphorylated.

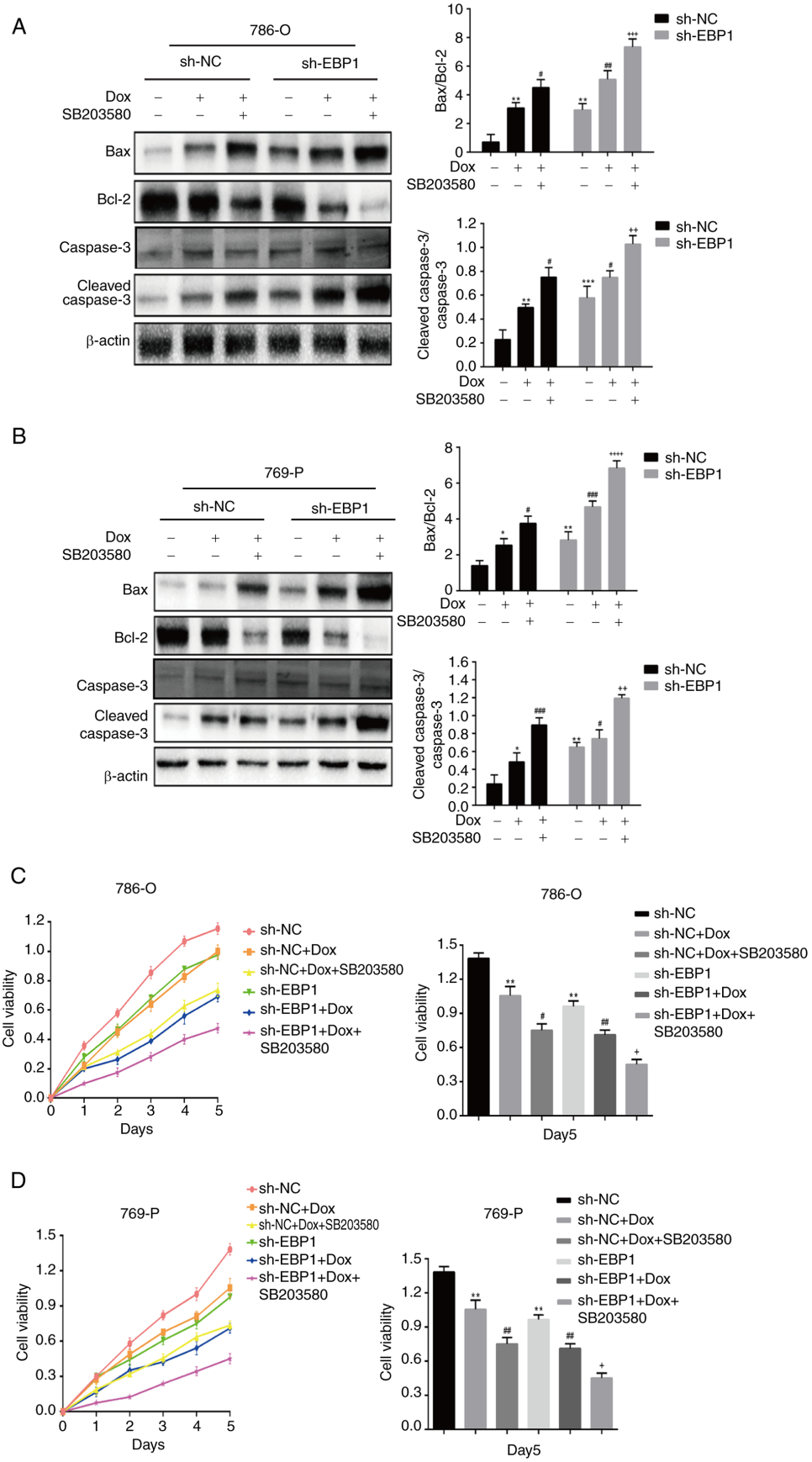

Figure 4. Concurrent doxorubicin and *EBP1* knockdown together promote apoptosis and reduce the viability of kidney clear cell carcinoma cells. Effect of doxorubicin on protein levels associated with apoptosis in (A) 786-O cells and (B) 769-P cells. Representative western blots and quantitative analyses are shown, normalized to β-actin. Effect of doxorubicin on (C) 786-O and (D) 769-P cell viability. Changes in viability over a 5-day period, measured daily, and a quantitative assessment on day 5 are shown. Data are presented as the mean ± SD, n=3. *P<0.05, **P<0.01 and ***P<0.001 vs. sh-NC; $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. sh-NC + Dox; $^{+}$P<0.05, $^{++}$P<0.01, $^{+++}$P<0.001 and $^{++++}$P<0.0001 vs. sh-NC + Dox + SB203580. EBP1, ErbB3-binding protein; Dox, doxorubicin; sh-NC, short hairpin negative control; sh-EBP1, short hairpin EBP1.

has also been demonstrated to inhibit cancer progression via the modulation of several signaling pathways, including the p53 pathway (23), PI3K/Akt/mTOR pathway (24), NF-κB pathway (25) and HIF-1α pathway (26). The inhibition of these pathways is essential to efficiently suppress tumor development and accelerate the process of apoptosis.

The therapeutic strategy of combining oncogene knockdown with drug administration has been a topic of in-depth research in the field of cancer treatment. By specifically combining the targeting and reducing the expression of oncogenes with the use of chemotherapy, targeted therapy and other drug interventions, this strategy can effectively inhibit tumor cell proliferation and metastasis. In addition, it may help to overcome the issue of drug resistance that arises when single treatment methods are used (27,28). Numerous cancers are regulated by *EBP1*, which affects cell proliferation and differentiation (29). Furthermore, *EBP1* has the potential to operate both as an oncostatic and an oncogenic regulator (30). Studies have shown that EBP1 protein intensifies the carcinogenic properties of cancer cells, including those in hepatocellular carcinoma (9) and melanoma (31). In addition, the inhibition of *EBP1* expression has been shown to suppress tumor formation in colon cancer (32). Although database analysis reveals that KIRC has significantly upregulated levels of *EBP1* expression, the combined effects of EBP1 and doxorubicin in KIRC and the underlying mechanism are unclear. EBP1 has been indicated to contribute to KIRC onset and progression via the regulation of cell cycle-related genes or apoptosis-related signaling pathways (10). Therefore, we hypothesized that *EBP1* knockdown may enhance the sensitivity of KIRC to doxorubicin chemotherapy. In the current study, the combination of *EBP1* knockdown and doxorubicin treatment was observed to have a significant inhibitory effect on KIRC cells, suggesting that *EBP1* promotes the progression of KIRC, which is consistent with previous research. This suggests that combination therapy may be critical for the effective management of KIRC.

EBP1 has previously been demonstrated to promote the occurrence and development of KIRC by regulating the p38MAPK/HIF-1α signaling pathway (10). Cellular inflammatory responses are mainly regulated by mechanisms involving apoptosis, differentiation and the p38MAPK intracellular signaling cascade (33). In addition, p38MAPK and HIF-1α are strongly associated with the medication resistance, invasiveness and angiogenesis of tumors (34). p38MAPK has been indicated to control HIF-1α activity, which in turn may control tumor development and metastasis (35). Notably, research has shown that p38MAPK inhibitors increase the susceptibility of cancer cells to doxorubicin (36). Also, Ye *et al* (37) have shown that the activation of p38MAPK is associated with doxorubicin-induced apoptosis. Therefore, we hypothesized that doxorubicin might act synergistically with the knockdown of *EBP1* through its effects on the p38MAPK pathway to increase the sensitivity of KIRC cells to doxorubicin. To demonstrate the contribution of the p38MAPK/HIF-1α pathway to the effect of doxorubicin on KIRC cells, a p38MAPK inhibitor was administered to 786-O and 769-P cells. Western blotting results indicated that the p-p38MAPK/p38MAPK ratio and HIF-1α expression level were reduced when both doxorubicin and the p38MAPK inhibitor were applied to the cells, compared with their levels following treatment with doxorubicin alone. Furthermore, EBP1 protein expression was not affected by the addition of doxorubicin alone or with the p38MAPK inhibitor, indicating that EBP1 acts upstream of p38MAPK and HIF-1α. The effect of *EBP1* knockdown on the p38MAPK pathway was indicated to increase the sensitivity of KIRC cells to doxorubicin. These findings provide novel insights for the treatment of KIRC.

Although the present study reveals the potential role of EBP1 in KIRC cell lines, it has certain limitations, in particular, the lack of *in vivo* validation and clinical data. While cell line experiments can provide valuable molecular mechanisms and preliminary data, they cannot fully replicate the complex tumor microenvironment *in vivo* or the interactions between the tumor and the host. Therefore, it is necessary to conduct further validation in mouse or other animal models in future studies to confirm the biological function and clinical relevance of EBP1 in KIRC.

In conclusion, the present study indicates that doxorubicin acts by inhibiting p38MAPK phosphorylation and HIF-1α expression. *EBP1* knockdown and doxorubicin act together via the p38MAPK pathway, with *EBP1* knockdown further enhancing the inhibitory effect of doxorubicin on KIRC cell viability.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

LM, JH and SC performed the experiments. SC and JH made significant contributions to the conception or design of the work. YY and XL prepared the figures and contributed to the analysis of experimental results. LL and ST wrote the main manuscript and made substantial contributions to the bioinformatics analysis. The manuscript was critically reviewed for important intellectual content. JH and SC confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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