COX-2 induces apoptosis-resistance in hepatocellular carcinoma cells via the HIF-1α/PKM2 pathway

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Abstract. The pyruvate kinase M2 isoform (PKM2) is a key component of aerobic glycolysis and has been reported to regulate apoptosis. However, it is unclear whether PKM2 is involved in cyclooxygenase-2 (COX-2) induced apoptosis-resistance in hepatocellular carcinoma (HCC) cells. In the present study, it was observed that COX-2 and PKM2 were significantly elevated in hepatocellular carcinoma tissues compared with adjacent liver tissues (P<0.05). Furthermore, their expression was positively associated with worse clinicopathological characteristics, which indicates poor prognosis in patients with HCC. COX-2 knockdown significantly reduced the expression of PKM2 and hypoxia inducible factor-1α (HIF-1α) at the mRNA and protein levels in addition to inhibiting proliferation (P<0.05), whereas apoptosis was notably increased. Furthermore, HIF-1α and PKM2-knockdown increased cell apoptosis without inhibiting COX-2 expression. PKM2 inhibition did not have a marked effect on COX-2 and HIF-1α expression. In conclusion, the results of the present study suggested that HIF-1α/PKM2 pathway-associated metabolic changes may facilitate COX-2-induced apoptosis resistance in HCC cells.

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Introduction

Hepatocellular carcinoma (HCC) is a common malignant tumor and the second most common cause of cancer-associated mortality globally (1). The overall survival rate is poor for patients with HCC, with a 5-year survival rate of <10% (2). Surgery is the most effective treatment for HCC, however ~80% of patients with HCC are ineligible for surgery due to advanced disease and 20-25% of patients will experience post-operative recurrence within 5 years (2). Chemotherapy serves an important role in the treatment of patients with advanced HCC, however it has low efficacy primarily due to apoptosis resistance (3). However, the mechanism of apoptosis resistance remains unclear.

Cyclooxygenase-2 (COX-2) is a rate-limiting enzyme that serves a role in the conversion of arachidonic acid to prostaglandin H2 (4). COX-2 is typically undetectable in healthy tissues under normal physiological conditions, however, cytokines including tumor growth factor, interleukin (IL)-1 and IL-6, hypoxia, *Helicobacter pylori* infection and carcinogenic substances cells may trigger the rapid and transient expression of COX-2 to modulate inflammation and carcinogenesis (5). COX-2 is associated with a number of tumor progression processes, including the stimulation of tumor cell growth, the inhibition of tumor cell apoptosis, the promotion of tumor angiogenesis and the enhancement of tumor invasion and metastasis (6). A number of studies have demonstrated that COX-2 is overexpressed in HCC cells and serves an important role in apoptosis resistance (7,8). It has been reported that COX-2 overexpression inhibits tumor cell apoptosis; Leng *et al* (9) revealed that transfecting HCC cells with COX-2 promotes cell growth and resistance to butyrate-induced apoptosis. In addition, the COX-2 inhibitor celecoxib suppresses the carcinogen diethyl nitrosamine, which induces HCC growth (10). However, the potential mechanisms underlying COX-2-mediated apoptosis resistance have not yet been fully elucidated.

To maintain a survival advantage in the tumor microenvironment, tumor cells generate energy mainly via aerobic glycolysis even under aerobic conditions-this is called the 'Warburg effect' (11,12). Pyruvate kinase (PK) is a

rate-limiting enzyme in the glycolysis pathway and has four isoenzymes, including L, R, M1 and M2. M1 PK is mainly expressed in the skeletal muscle and brain tissue, which have high energy and oxygen consumption (13,14). A critical mediator of the Warburg effect is the PK M2 isoform (PKM2), a tumor-specific isoform of PK that catalyzes the synthesis of pyruvate and ATP using phosphoenolpyruvate (PEP) and ADP as substrates (15). PKM2 is primarily expressed in embryonic tissues and proliferative cells, and elevated PKM2 expression has been reported in several tumor types, including HCC (16,17). One previous study confirmed that HCC cells express higher level of PKM2 compared with normal adjacent tissues or benign tumor types, while PKM2 knockout has been reported to inhibit the growth of HCC cells (18). Dong *et al* (19) demonstrated that PKM2 was overexpressed in cancer tissues compared with adjacent normal tissues and PKM2 downregulation in HepG2 cells inhibited cell growth via targeting hypoxia inducible factor-1α (HIF-1α) and B-cell lymphoma-extra large expression, suggesting that PKM2 may promote HCC cell proliferation by exerting a protein kinase function. However, whether COX-2 mediated resistance to apoptosis is associated with PKM2 upregulation is unclear.

A number of studies have demonstrated that COX-2 serves an important role in apoptosis resistance (20,21). However, whether the HIF-1α/PKM2 pathway is involved in COX-2-induced apoptosis resistance remains to be elucidated. In the present study, the association between COX-2, PKM2 and HIF-1α was assessed.

Materials and methods

Patients and tissue microarray construction. The present study was approved by the Ethics Committee of the First Affiliated Hospital of Anhui Medical University and conformed to the 1975 Declaration of Helsinki. All patients provided written informed consent. Primary HCC tissues and paired adjacent normal liver tissues were obtained from the archives of the Department of Pathology, the First Affiliated Hospital of Anhui Medical University between March 2010 and June 2016 in 143 patients with HCC (105 male, 38 female; mean age 51, range 35 to 78 years), embedded in paraffin and 10% neutral-buffered formalin was used to fix the tissues at room temperature for 12 h. Histological tumor differentiation was determined using the Edmondson-Steiner scoring system (22) and tumor types were classified using the World Health Organization classification system (23) and the International Anti-cancer Coalition Tumor Node Metastasis classification system (24). Tissue sections were dyed using hematoxylin staining solution (containing 0.2% hematoxylin) and 1-2% eosin at room temperature for 5 min. The sections were reviewed for the identification of the target area by two pathologists independently. Three to five representative 1-mm cores were produced from each sample and then inserted into a new recipient paraffin block in a grid pattern using a manual tissue array (Boyikang Instruments, Beijing, China).

Reagents. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), basement membrane matrix and PBS were obtained from Hyclone (GE Healthcare Life Sciences, Logan, UT, USA). Antibodies against COX-2 (cat. no. ab15191), HIF-1α (cat. no. ab8366) and PKM2 (cat. no. ab150377) were purchased from Abcam (Cambridge, MA, USA). Si-COX-2, Si-PKM2 and Si-HIF-1α were purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China). The Plus Western Blotting Detection System was obtained from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). Reagents for reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis were as follows: The RT kit was purchased from Invitrogen (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and the Thunderbird SYBR RT-PCR Mix was from Toyobo Life Science (Osaka, Japan). Annexin V/propidium iodide (PI) apoptosis detection kit was obtained from BD Biosciences (San Jose, CA, USA). The TUNEL system was purchased from Roche Diagnostics (Basel, Switzerland). Cell Counting Kit-8 (CCK-8) was obtained from BioTek Instruments, Inc. (Winooski, VT, USA).

Cell culture. The HepG2 cell line is originated from hepatoblastoma, however it was frequently used as a HCC cell (23). The HepG2 cell line was purchased from the Shanghai Cell Bank (Chinese Academy of Sciences, Shanghai, China) and cultured in DMEM supplemented with 10% (v/v) heat-inactivated FBS at 37˚C in a humidified atmosphere containing 5% CO_2.

Immunohistochemical (IHC) analysis. The expression of COX-2, HIF-1α and PKM2 in HCC samples from 143 patients was detected using IHC. Following deparaffinization and antigen retrieval, sections were blocked with 5% goat serum (OriGene Technologies, Inc., Beijing, China) at 37˚C for 30 min. All sections were incubated at 4˚C overnight with rabbit antibodies against COX-2 and PKM2 (all 1:100), and mouse monoclonal antibody against HIF-1α (1:100). Sections (4-μm-thick) were then incubated with horseradish peroxidase (HRP)-labeled goat anti-rabbit immunoglobulin G (IgG) (H+L) or goat anti-mouse IgG (H+L) secondary antibodies (1:200, cat. no. BS13278/BS12478; Bioworld Technology Inc., St. Louis Park, MN, USA) at 37˚C for 30 min. The secondary antibodies were diluted by PBS phosphate buffer (0.01 mol/l, pH: 7.4-7.6). Then the sections were stained using a DAB detection kit (cat. no. ZLI-9018; OriGene Technologies, Inc.) at room temperature for 30 sec and hematoxylin staining solution (containing 0.2% hematoxylin) at room temperature for 5 min. Cytoplasm or karyon staining was considered to indicate a positive expression of COX-2, HIF-1α or PKM2. Staining intensity was graded as follows: 0, no staining; 1, weak intensity; 2, moderate intensity; and 3, strong intensity. The degree of positive staining was determined as follows: 0, <24%; 1, 25-49%; 2, 50-74%; and 3, ≥75%). Patients with HCC were classified into two groups according to the total score (staining intensity + positive staining); the negative expression group (total score 0-2) and the positive expression group (total score 3-6). IHC results were quantitatively analyzed using a biological image analysis system that consisted of an Olympus CX31 light microscope (magnification, x200; Olympus Corporation, Tokyo, Japan), Nikon Digital Camera DXM 1200F (Nikon Corporation, Tokyo, Japan), Image-ProPlus6.0 (Media Cybernetics, Inc., Rockville, MD, USA) and JEOA 801D morphological biological image analysis software

version 6.0 (Zhejiang Jieda Technology, Co., Ltd., Zhejiang, China).

siRNA transfection. HepG2 cells were seeded into 6-well plates ($1x10^5$/ml) and cultured until the adherent cells reached 70% confluence. COX-2 (forward, 5'-GGAACGUUGUGAAUAACAUTT-3' and reverse, 5'-AUGUUAUUCACAACGUUCCTT-3'), HIF-1α (forward, 5'-GCCGCUCAAUUUAUGAAUATT-3' and reverse, 5'-UAUUCAUAAAUUGAGCGGCTT-3') and PKM2 (forward, 5'-GGCUGGACUACAAGAACAUTT-3' and reverse, 5'-AUGUUCUUGUAGUCCAGCCTT-3') siRNAs were designed and synthesized by Shanghai GenePharma Co., Ltd. siRNA was transfected into the cells at a concentration of 20 nM using Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.) at 37˚C for 24 h. Cells in the negative control group were treated with Lipofectamine® 2000. Cells were incubated with si-COX-2, si-HIF-1α or si-PKM2 at 37˚C for 8 h and then cultured in DMEM at 37˚C for a further 16 h.

RT-qPCR. Following transfection, COX-2, HIF-1α and PKM2 mRNA expression was assessed using RT-qPCR. Briefly, total RNA was extracted using a TRIzol® kit (Thermo Fisher Scientific Inc.) according to the manufacturer's protocol. The total RNA concentration was assessed by measuring absorbance at 260 and 280 nm using a NanoDrop spectrophotometer (TU-1810; Thermo Fisher Scientific Inc.). RT-qPCR was performed using a two-step reaction using TransStart® All-in-One First-Strand cDNA Synthesis SuperMix and TransStart® Top Green qPCR SuperMix kit (Beijing Transgen Biotech Co., Ltd., Beijing, China). Following normalization to β-actin expression, the relative gene expression was determined using a comparative standard curve. PCR primers specific for human genes were provided by Shanghai GenePharma Co., Ltd. and were as follows: β-actin forward, 5'-CTCTTCCAGCCTTCCTTCCT-3' and reverse, 5'-AGCACTGTGTTGGCGTACAG-3'; COX-2, forward 5'-TAAAAACCCCATAACCCCGCC-3' and reverse 5'-TTGGGCTTTTCTCCTTTGGTT-3'; HIF-1α, forward 5'-CCACCTCTGGACTTGCCTTT-3' and reverse 5'-ACTTATCTTTTTCTTGTCGTTCGC-3'; PKM2, forward 5'-ACTCGGGCTGAAGGCAGTGA-3' and reverse 5'-TGTGGGGTCGCTGGTAATGG-3'. Following denaturation at 95˚C for 3 min, amplification was performed for 40 cycles of 95˚C for 10 sec, 57˚C for 30 sec and 65˚C for 10 sec, with a single fluorescence measurement. All reactions were performed in triplicate. The expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method (25).

Western blotting. To measure the expression of COX-2, HIF-1α and PKM2 proteins, western blotting was performed following transfection with si-COX-2, si-HIF-1α or si-PKM2. Briefly, total protein was extracted from HepG2 cells using radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology, Haimen, China) at 4˚C for 30 min. Protein concentration was determined using a Lowry Protein assay (Thermo Fisher Scientific Inc.). Approximately 20 μg proteins were loaded in each lane and subjected to electrophoresis using 10% SDS-PAGE, and were transferred onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA) at 110 V for 60 min. The membrane was blocked for 2 h at room temperature in blocking solution (5% non-fat milk in Tris-buffered saline with 0.05% Tween-20) and incubated with primary antibodies (1:500) at 4˚C overnight. Following three washes in tris-buffered saline with 0.05% Tween-20, the membrane was incubated with HRP-conjugated mouse anti-rabbit IgG (cat. no. sc-2357) or HRP-conjugated goat anti-mouse IgG (cat. no. sc-2031) secondary antibodies (Santa Cruz Biotechnology Inc., Dallas, TX, USA) at a dilution of 1:10,000 for 60 min at room temperature. The following antibodies were used: COX-2 (Abcam; cat. no. ab15191), HIF-1α (Abcam; cat. no. ab8366), PKM2 (Abcam; cat. no. ab150377) and β-actin (Abcam; cat. no. ab8226). The membranes were incubated in a chromogenic substrate (Thermo Fisher Scientific Inc.) 2 min in room temperature to develop protein bands. Results were quantified by densitometric analysis using ImageJ software (V1.48u; National Institutes of Health, Bethesda, MD, USA).

Flow cytometry. At 24 h following transfection, non-adherent cells were removed by gentle washing, the remaining cells were collected in a 15 ml sterile tube and centrifuged at 400 x g at room temperature for 5 min. Cells were washed twice with cold PBS and resuspended in 400 μl binding buffer at a concentration of $1x10^5$ cells/ml. The cell suspensions were then mixed with 5 μl Annexin V-fluorescein isothiocyanate solution and 10 μl PI, incubated for 15 min at 4˚C in the dark and analyzed using flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA) within 1 h. Flow cytometric analysis was performed using FlowJo7.6.1 software (FlowJo LLC, Ashland, OR, USA). A total of 10,000 cells from each sample were analyzed and the experiment was repeated at least three times.

TUNEL assay. HepG2 cells ($1x10^5$) were seeded into six well plates. Following transfection, cover slips were washed twice with PBS and then fixed in 4% paraformaldehyde solution at room temperature for 25 min. Apoptotic cells were detected using a TUNEL assay (TUNEL System kit; Roche Diagnostics) according to the manufacturer's protocol. The results were quantitatively analyzed using a biological image analysis system, which consisted of an Olympus CX31 light microscope (magnification, x400), Nikon Digital Camera DXM 1200F and ACT-1 version 2.63 software (Nikon Corporation).

Cell proliferation assay. HepG2 cells were seeded into 96-well plates at a density of $2x10^4$ cells/well for 24 h to allow for cell adherence. Transfection with specific siRNA was performed as described above. Subsequently, 10 μl CCK-8 reagent (Shanghai BestBio Beibo Bio, Shanghai, China) was added to each well at 37˚C for 4 h. The optical density was measured using a scanning multi-well spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA) at 490 nm.

Statistical analysis. At least three independent experiments were performed for all assays. Statistical analysis was performed using SPSS version 17.0 software (SPSS Inc., Chicago, IL, USA). The results are expressed as the mean ± standard error of the mean. Differences between two or multiple groups were compared using a independent t-test or a one-way analysis of variance and a post-hoc test (Bonferroni's). Associations

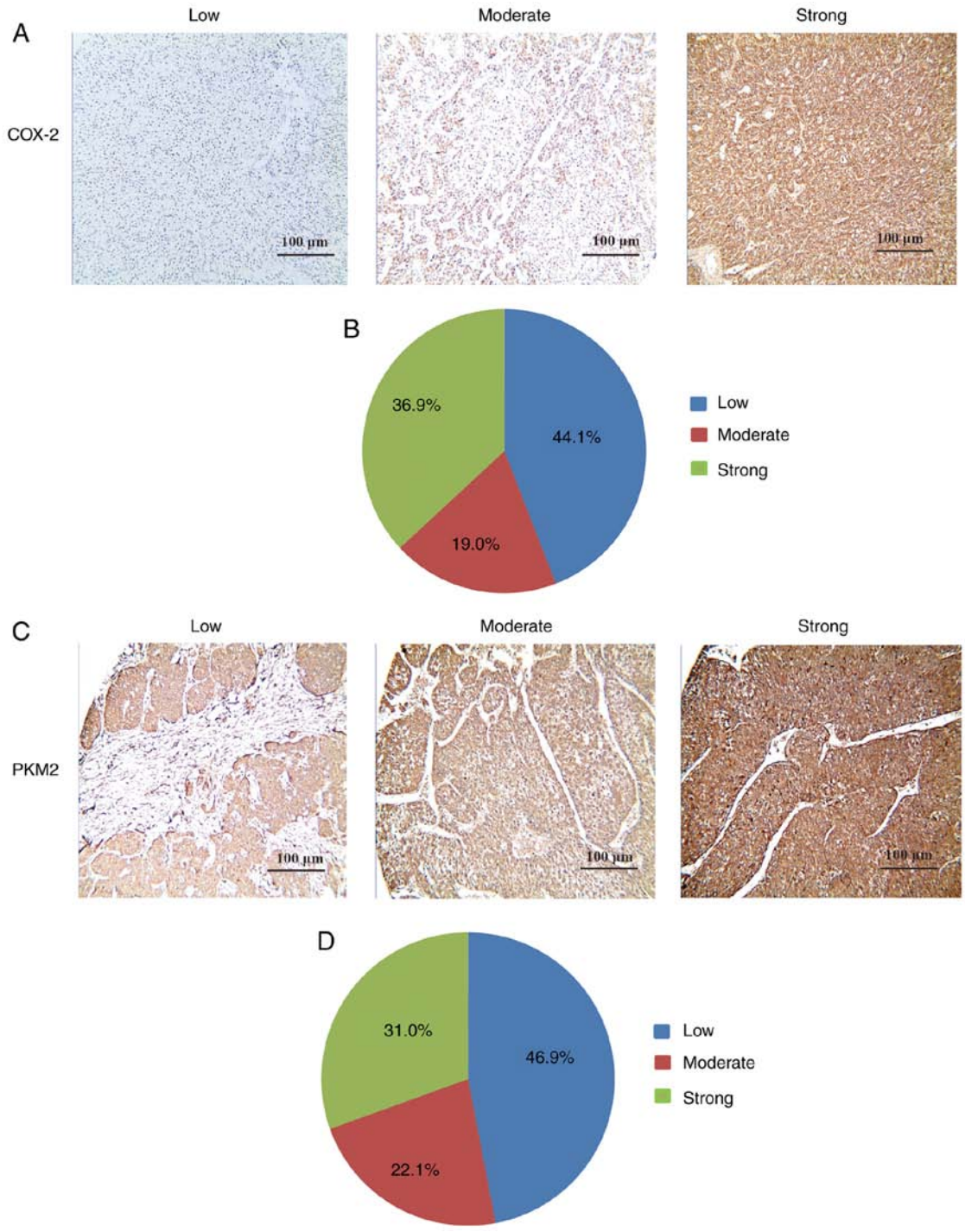

Figure 1. COX-2 and PKM2 are overexpressed in HCC tissues and are positively associated with worse clinicopathological characteristics. (A) Immunohistochemical analysis of COX-2 expression in paraffin-embedded HCC tissue samples (scale bar =100 μm). (B) Percentage of COX-2^{strong}, COX-2^{moderate} and COX-2^{low} HCC tissues. (C) Immunohistochemical analysis of PKM2 expression in paraffin-embedded HCC tissue samples (scale bar=100 μm). (D) Percentage of PKM2^{strong}, PKM2^{moderate} and PKM2^{low} HCC tissues. HCC, hepatocellular carcinoma; COX-2, cyclooxygenase-2; PKM2, pyruvate kinase M2 isoform.

between protein expression and clinicopathological parameters were assessed using Spearman's correlation analysis. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Expression of PKM2 protein is positively associated with COX-2 expression and worse clinicopathological characteristics in patients with HCC. To investigate the function of COX-2 in patients with HCC, formalin-fixed paraffin-embedded HCC specimens were analyzed using IHC staining. It was demonstrated that COX-2 was primarily expressed in the cytoplasm of tumor cells in a diffused pattern (Fig. 1A). Furthermore, 55.9% (80/143) of tumor tissues had a high COX-2 expression (moderate or strong) compared with adjacent liver tissues (Fig. 1B). The association between clinicopathological characteristics and COX-2 expression is presented in Table I. The histological grade

Table I. Association between COX-2 expression level in hepatocellular cancer and clinicopathological factors.

Characteristics	Samples (n)	COX-2		Positive rate (%)	χ^2	P-value
		Low	High			
Sex					2.708	0.100
Male	105	34	71	67.6		
Female	38	18	20	52.6		
Age (years)					0.001	0.973
<60	101	46	55	54.5		
≥60	42	19	23	54.8		
Tumor size (cm)					2.292	0.130
<5	52	13	39	75.0		
≥5	91	34	57	62.6		
Hepatitis					0.008	0.931
Positive	122	36	86	70.5		
Negative	21	6	15	71.4		
Liver cirrhosis					0.133	0.706
Positive	123	42	81	65.9		
Negative	20	6	14	70.0		
Tumor number					0.037	0.848
Single	24	6	18	75.0		
Multiple	119	32	87	73.1		
Portal vein invasion					0.590	0.443
Present	60	18	42	70.0		
Absent	83	30	53	63.9		
AFP					0.277	0.599
High	137	36	101	73.7		
Normal	6	1	5	83.3		
Clinical stage					2.197	0.533
I	14	6	8	57.1		
II	100	25	75	75.0		
III	25	8	17	68.0		
IV	4	1	3	75.0		
Histological grade					18.663	<0.001
Well-differentiated	54	34	20	37.0		
Moderately-differentiated	57	19	38	66.7		
Poorly-differentiated	32	6	26	81.2		

COX-2, cyclooxygenase-2; AFP, α-fetoprotein.

of HCC cells was significantly positively associated with COX-2 expression; 81.2% of poorly-differentiated tumor tissues had a high expression of COX-2, while only 37 and 66.7% of the well- and moderately-differentiated tumor cells had a high COX-2 expression (P<0.001). However, no association was observed between the expression of COX-2 and other clinicopathological characteristics, including tumor size and clinical stage (P>0.05).

IHC was used to investigate the association between PKM2 and COX-2 in HCC cells. PKM2 was mainly expressed in the cytoplasm of tumor tissues (Fig. 1C). Furthermore, PKM2 expression was upregulated in 53.1% (76/143) of tumor tissues compared with adjacent liver tissues (Fig. 1D). The association between clinicopathological characteristics and PKM2 expression is presented in Table II. The histological grade of HCC cells was significantly positively associated with PKM2 expression; 75.0% of poorly-differentiated tumor cells had a high expression of PKM2, while only 29.6 and 63.1% of well- and moderately-differentiated tumor cells had a high expression of PKM2 proteins (P<0.001). To assess whether PKM2 expression is associated with COX-2 activation, Spearman's correlation analysis was performed. As illustrated in Table III, 92.5% of COX-2 positive tumor cells had a significantly high PKM2 expression (P<0.001), while only 3.2% of COX-2 negative tumor cells highly expressed

Table II. Association between PKM2 expression level in hepatocellular cancer and clinicopathological factors.

Characteristics	Samples (n)	PKM2		Positive rate (%)	χ^2	P-value
		Low	High			
Sex					3.122	0.077
Male	105	28	77	73.3		
Female	38	16	22	57.9		
Age (years)					0.098	0.754
<60	101	51	50	49.5		
≥60	42	20	22	52.3		
Tumor size (cm)					0.949	0.330
<5	52	11	41	78.8		
≥5	91	26	65	71.4		
Hepatitis					0.096	0.756
Positive	122	33	89	72.9		
Negative	21	5	16	76.1		
Liver cirrhosis					0.284	0.594
Positive	123	38	85	69.1		
Negative	20	5	15	75.0		
Tumor number					0.163	0.686
Single	24	7	17	70.8		
Multiple	119	30	89	74.8		
Portal vein invasion					0.817	0.366
Present	60	16	44	73.3		
Absent	83	28	55	66.3		
AFP					0.371	0.542
High	137	31	106	77.4		
Normal	6	2	4	66.7		
Clinical stage					4.235	0.237
I	14	6	8	57.1		
II	100	22	78	78.0		
III	25	6	19	76.0		
IV	4	0	4	100.0		
Histological grade					20.425	<0.001
Well-differentiated	54	38	16	29.6		
Moderately-differentiated	57	21	36	63.1		
Poorly-differentiated	32	8	24	75.0		

PKM2, pyruvate kinase M2 isoform; AFP, α-fetoprotein.

PKM2. Altogether, these results suggest that COX-2 activation is associated with a high PKM2 expression and worse clinicopathological characteristics in HCC patients.

Reduced COX-2 expression promotes apoptosis and downregulates proliferation in HepG2 cells. To investigate whether COX-2 influences apoptosis in HCC cells, HepG2 cells were transfected with COX-2 siRNA for 24 h. As presented in Fig. 2A, apoptosis was significantly promoted in COX-2 siRNA-transfected HepG2 cells compared with untransfected cells (P<0.05). The results of a TUNEL assay revealed that COX-2 knockdown significantly

Table III. Correlation between COX-2 and PKM2.

PKM2	COX-2		r	P-value
	-	+		
Negative	61	6	0.889	<0.001
Positive	2	74		

PKM2, pyruvate kinase M2 isoform; COX-2, cyclooxygenase-2.

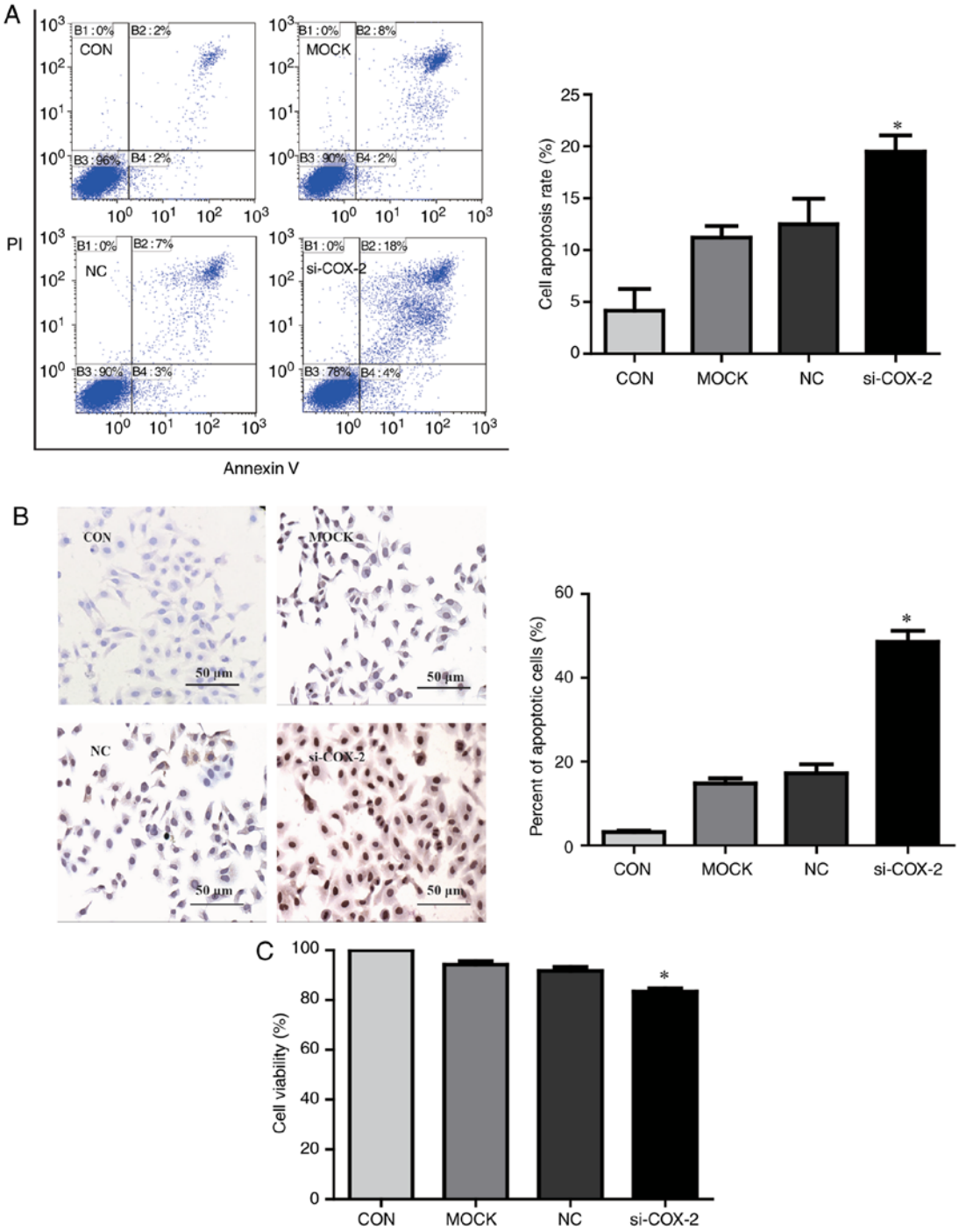

Figure 2. COX-2 knockdown increases apoptosis and inhibits viability in HepG2 cells. HepG2 cells were transfected with COX-2 siRNA for 24 h. (A) Apoptosis of HepG2 cells was assessed using an Annexin V/propidium iodide double-staining assay. (B) Apoptotic HepG2 cells subsequent to transfection with si-COX-2 were examined using TUNEL staining (scale bar=50 μm) and semi-quantitative analyzed. (C) Cell viability was determined using a Cell Counting Kit-8 assay. Data are presented as the mean ± standard error of the mean for at least three independent experiments. *P<0.05 vs. CON. COX-2, cyclooxygenase-2; siRNA, small interfering RNA; CON, untreated hepatocellular carcinoma cells; MOCK, hepatocellular carcinoma cells treated with Lipofectamine® 2000 alone; NC, hepatocellular carcinoma cells treated with negative control siRNA.

enhanced the apoptosis in HepG2 cells compared with paired controls (P<0.05; Fig. 2B). Furthermore, a CCK-8 assay revealed that COX-2 knockdown significantly reduced cell viability compared with untransfected HepG2 cells (P<0.05; Fig. 2C).

PKM2 knockdown increases apoptosis in HCC cells and down-regulates proliferation. To investigate whether PKM2 affects apoptosis in HCC cells, HepG2 cells were transfected with PKM2 siRNA. Annexin V/PI double-staining and TUNEL assays were performed to assess apoptosis. As presented in Fig. 3A, PKM2 knockdown resulted in a significant increase in apoptosis compared with untransfected HepG2 cells (P<0.05). The results of a TUNEL assay further confirmed that PKM2 knockdown in HepG2 cells significantly increased apoptosis compared with untransfected control cells (P<0.05; Fig. 3B). Furthermore, a CCK-8 assay revealed that PKM2 knockdown

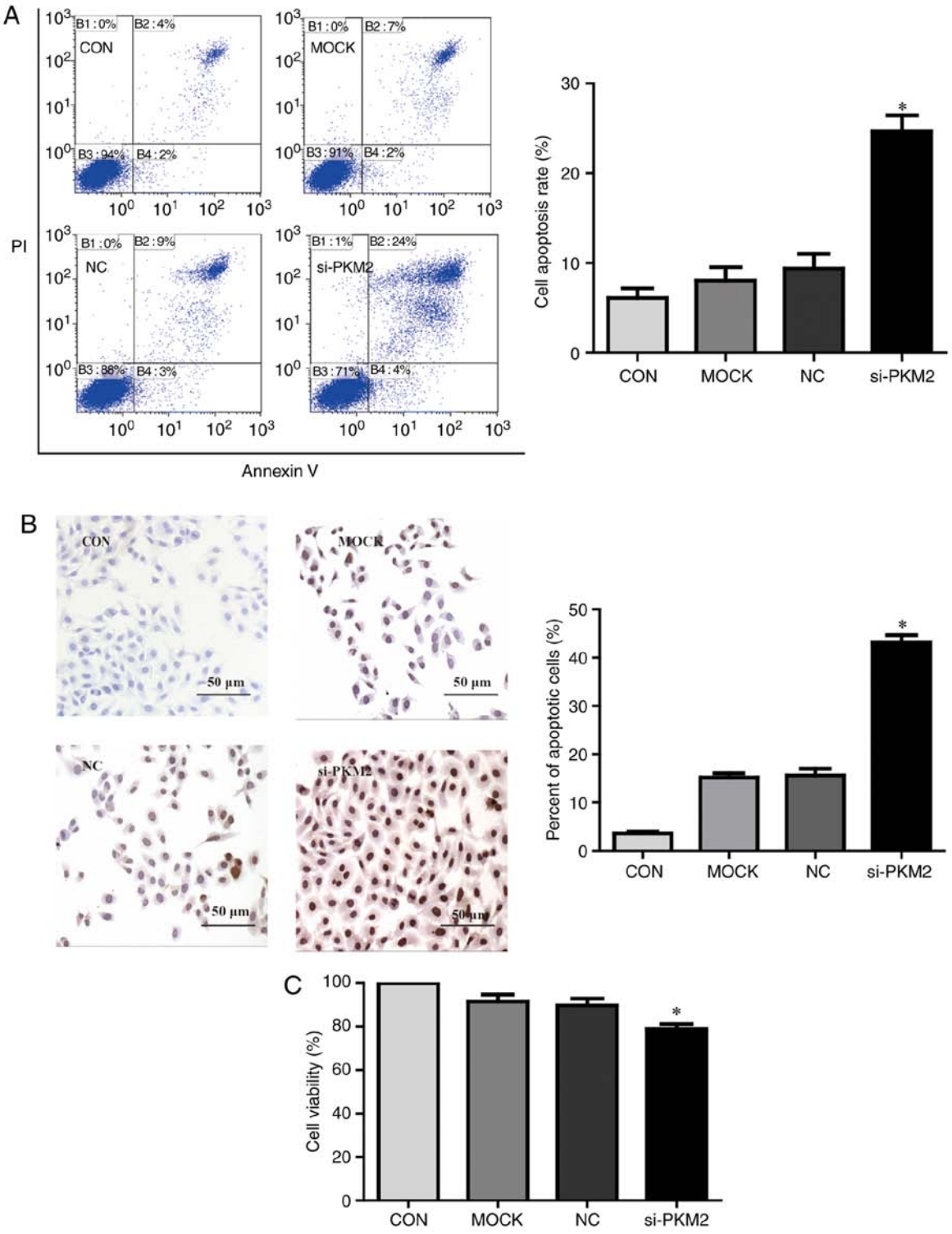

Figure 3. PKM2 knockdown increases apoptosis and inhibits viability in HepG2 cells. HepG2 cells were transfected with PKM2 siRNA for 24 h. (A) Apoptosis of HepG2 cells was assessed using an Annexin V/propidium iodide double-staining assay. (B) Apoptotic HepG2 cells following transfection with si-PKM2 were examined using TUNEL staining (scale bar=50 μm) and semi-quantitative analyzed. (C) Cell viability was determined using a Cell Counting Kit-8 assay. Data are presented as the mean ± standard error of the mean for at least three independent experiments. *P<0.05 vs. CON. PKM2, pyruvate kinase M2 isoform; si/siRNA, small interfering RNA; CON, untreated hepatocellular carcinoma cells; MOCK, hepatocellular carcinoma cells treated with Lipofectamine® 2000 alone; NC, hepatocellular carcinoma cells treated with negative control siRNA.

significantly reduced cell viability compared with untransfected HepG2 cells (P<0.05; Fig. 3C).

COX-2 regulates PKM2 expression in HCC cells. To investigate the association between COX-2 and PKM2 in HCC cells *in vitro*, HepG2 cells were transfected with COX-2 siRNA, RT-qPCR was performed and the results revealed that the expression levels of COX-2 (P<0.05; Fig. 4A) and PKM2 (P<0.05; Fig. 4B) mRNA were significantly reduced compared with untransfected cells. Western blotting also

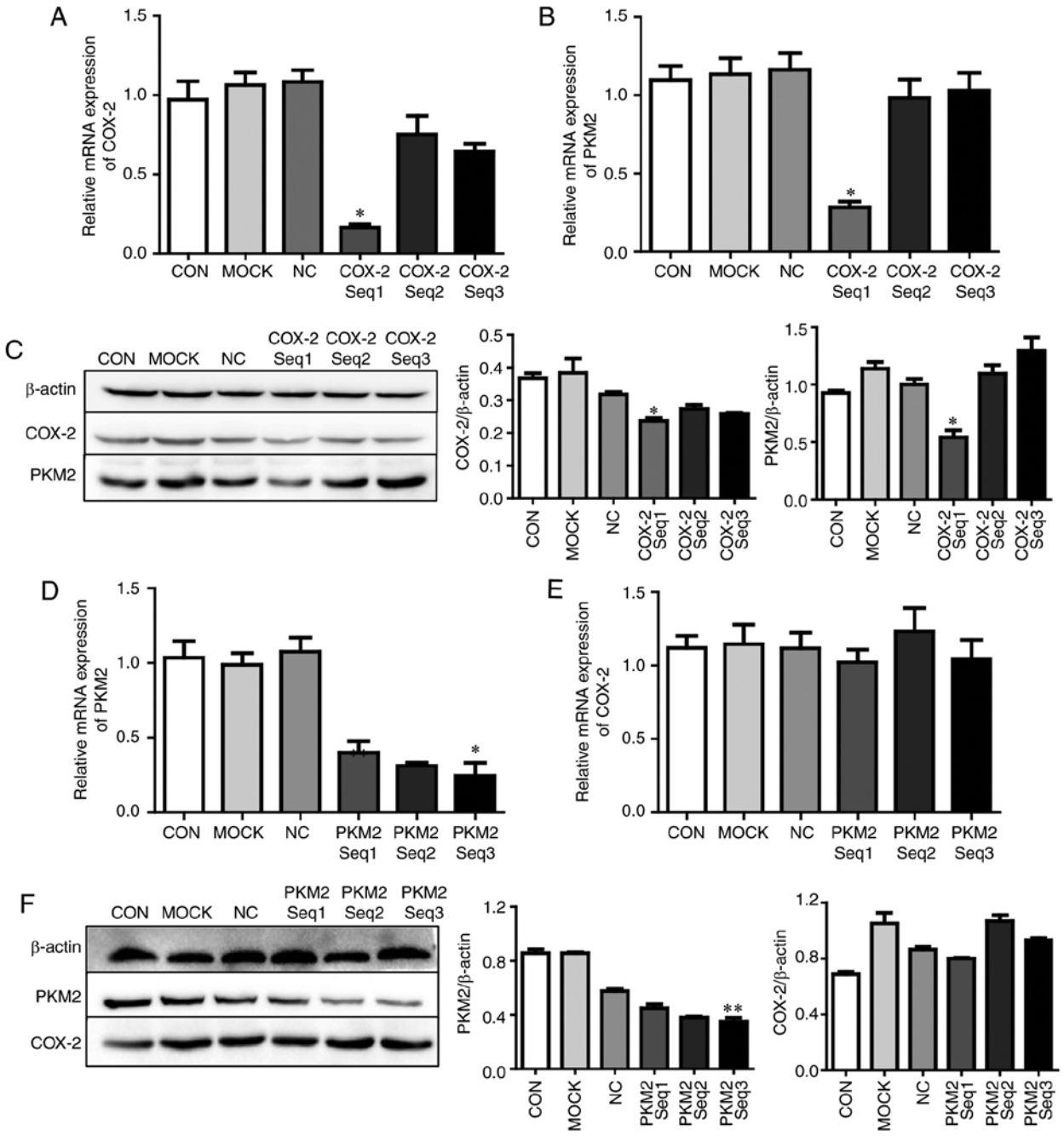

Figure 4. COX-2 regulates PKM2 expression in HepG2 cells *in vitro*. Firstly, HepG2 cells were transfected with COX-2 siRNA for 24 h. (A) COX-2 and (B) PKM2 mRNA was measured using RT-qPCR. (C) COX-2 and PKM2 protein expression was measured by western blotting. Secondly, HepG2 cells were transfected with PKM2 siRNA for 24 h, and (D) PKM2 and (E) COX-2 mRNA was also measured using RT-qPCR, and (F) COX-2 and PKM2 protein expression was measured using western blotting. *P<0.05 and **P<0.01 vs. CON. COX-2, cyclooxygenase-2; PKM2, pyruvate kinase M2 isoform; siRNA, small interfering RNA; CON, untreated hepatocellular carcinoma cells; MOCK, hepatocellular carcinoma cells treated with Lipofectamine® 2000 alone; NC, hepatocellular carcinoma cells treated with negative control siRNA; reverse transcription-quantitative polymerase chain reaction.

confirmed that COX-2 knockdown significantly reduced PKM2 expression at the protein level (P<0.05; Fig. 4C). Subsequently, RT-qPCR revealed that PKM2 mRNA was significantly downregulated in HepG2 cells following PKM2 siRNA transfection (P<0.001; Fig. 4D); however, COX-2 mRNA expression levels were unaffected (P>0.05; Fig. 4E). Western blotting also revealed that PKM2 siRNA was able to effectively downregulate PKM2 expression levels (P<0.05) but had no impact on COX-2 expression levels (P>0.05; Fig. 4F). These results suggest that COX-2 is able to modulate PKM2 expression in HCC cells.

HIF-1α is associated with the COX-2/PKM2-mediated regulation of cell apoptosis. To investigate whether HIF-1α is associated with modulation of the COX-2/PKM2 pathway, HIF-1α expression in HCC tissues was measured using IHC. HIF-1α was mainly expressed in the cytoplasm of tumor cells and was upregulated in 46.2% (66/143) of HCC specimens compared with adjacent liver tissues (Fig. 5A). The associations between HIF-1α, COX-2 and PKM2 were assessed and the results revealed that 54.3% of COX-2-positive tissues and 60.5% of PKM2-positive tissues expressed HIF-1α, whereas only 35.5% and 29.9% of COX-2 and PKM2-negative HCC

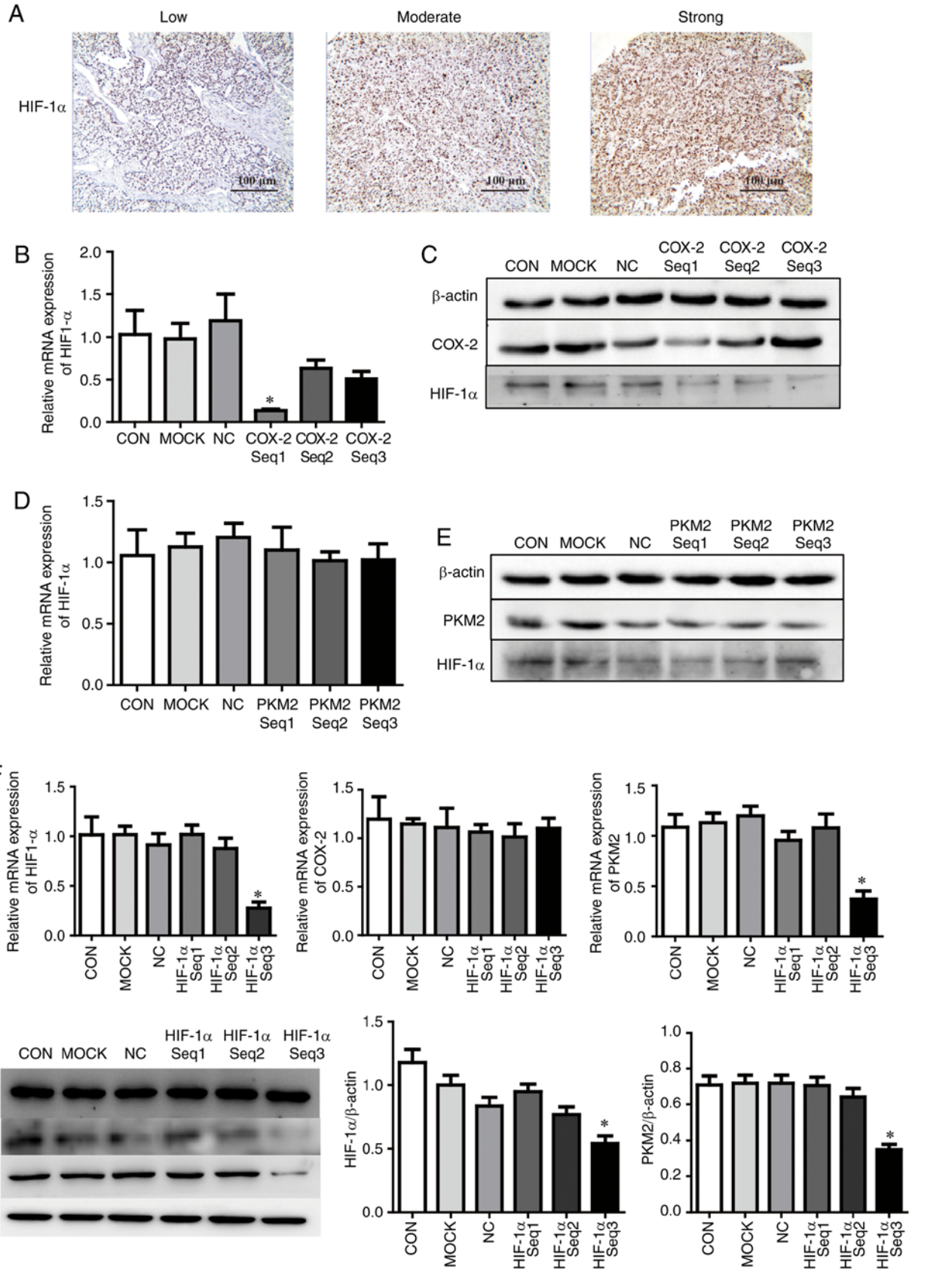

Figure 5. HIF-1α expression is associated with COX-2/PKM2-mediated regulation of cell apoptosis. (A) Immunohistochemical analysis of HIF-1α expression in paraffin-embedded hepatocellular carcinoma tissue samples (scale bar=100 μm). HepG2 cells were transfected with COX-2 siRNA for 24 h, and the expression of HIF-1α (B) mRNA and (C) protein was measured using RT-qPCR and western blotting, respectively. HepG2 cells were transfected with PKM2 siRNA for 24 h and the expression of HIF-1α (D) mRNA and (E) protein was measured using RT-qPCR and western blotting, respectively. HepG2 cells were transfected with HIF-1α siRNA for 24 h and the expression of HIF-1α (F) mRNA and (G) protein was measured using RT-qPCR and western blotting, respectively. *P<0.05 vs. CON. HIF-1α, hypoxia-inducible factor 1-α; COX-2, cyclooxygenase-2; PKM2, pyruvate kinase M2 isoform; siRNA, small interfering RNA; CON, untreated hepatocellular carcinoma cells; MOCK, hepatocellular carcinoma cells treated with Lipofectamine® 2000 alone; NC, hepatocellular carcinoma cells treated with negative control siRNA; reverse transcription-quantitative polymerase chain reaction.

Table IV. Correlation between HIF-1α and COX-2/PKM2.

	COX-2				PKM2			
HIF-1α	-	+	r	P-value	-	+	r	P-value
Negative	40	37	0.187	0.025	47	30	0.307	<0.001
Positive	22	44			20	46		

HIF-1α, hypoxia-inducible factor 1-α; PKM2, pyruvate kinase M2 isoform; COX-2, cyclooxygenase-2.

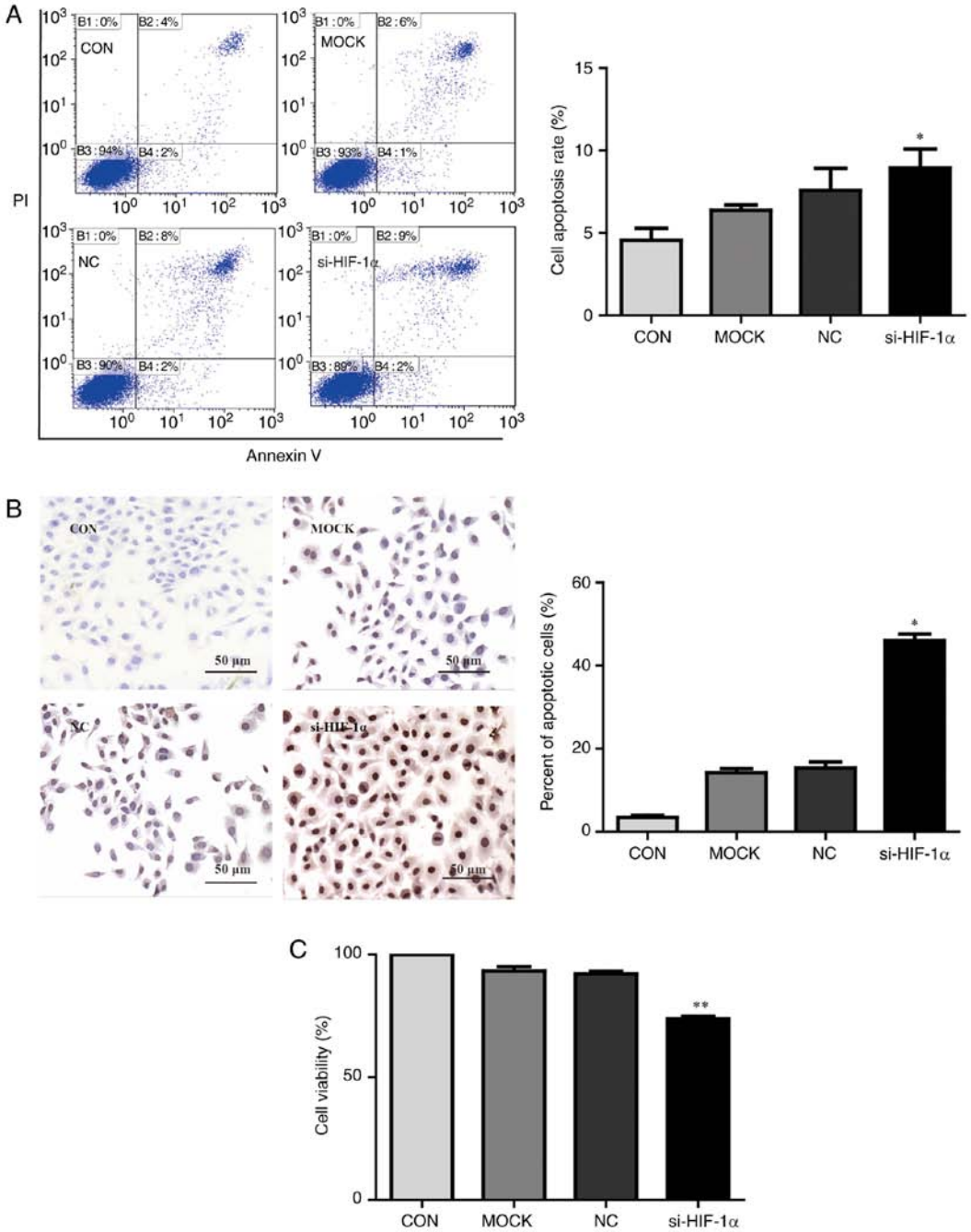

Figure 6. HIF-1α is associated with the COX-2/PKM2-mediated regulation of cell apoptosis. Apoptotic HepG2 cells were assessed using (A) Annexin V/propidium iodide double-staining and (B) TUNEL assays following transfection with HIF-1α siRNA (scale bar=50 μm). (C) Cell viability was determined using a Cell Counting Kit-8 assay. Data are presented as the mean ± standard error of the mean for at least three independent experiments. *P<0.05 and **P<0.01 vs. CON. HIF-1α, hypoxia-inducible factor 1-α; COX-2, cyclooxygenase-2; PKM2, pyruvate kinase M2 isoform; si-/siRNA, small interfering RNA; CON, untreated hepatocellular carcinoma cells; MOCK, hepatocellular carcinoma cells treated with Lipofectamine® 2000 alone; NC, hepatocellular carcinoma cells treated with negative control siRNA.

tissues, respectively, expressed HIF-1α (P<0.001; Table IV). Furthermore, HIF-1α protein expression was positively correlated with COX-2 (r=0.187, P=0.025) and PKM2 (r=0.307, P<0.001) expression, suggesting that HIF-1α may be involved in the modulation of the COX-2/PKM2 pathway.

HepG2 cells were transfected with COX-2 siRNA and RT-qPCR analysis (P<0.05; Fig. 5B) and western blotting (Fig. 5C) demonstrated that HIF-1α was significantly reduced compared with untransfected cells. HepG2 cells were next transfected with PKM2 siRNA and RT-qPCR (P>0.05; Fig. 5D) and western blotting (Fig. 5E) revealed that PKM2 knockdown had no effect on HIF-1α expression. Finally, HepG2 cells were transfected with HIF-1α siRNA and RT-qPCR (Fig. 5F) and western blotting (P<0.05; Fig. 5G) revealed that HIF-1α knockdown did not influence COX-2 expression compared with untransfected cells, whereas PKM2 expression was significantly reduced.

The impact of HIF-1α on apoptosis in HepG2 cells was assessed using an Annexin V/PI double-staining assay and TUNEL assay. As presented in Fig. 6A, HIF-1α knockdown significantly increased apoptosis compared with untransfected cells (P<0.05). TUNEL assays further confirmed that HIF-1α knockdown in HepG2 cells significantly increased apoptosis compared with untransfected control cells (P<0.05; Fig. 6B). Furthermore, HepG2 cells transfected with HIF-1α siRNA significantly reduced cell viability compared with untransfected cells (P<0.01; Fig. 6C). Altogether, these results suggest that COX-2 is able to regulate the expression of PKM2 in HCC cells via modulating HIF-1α.

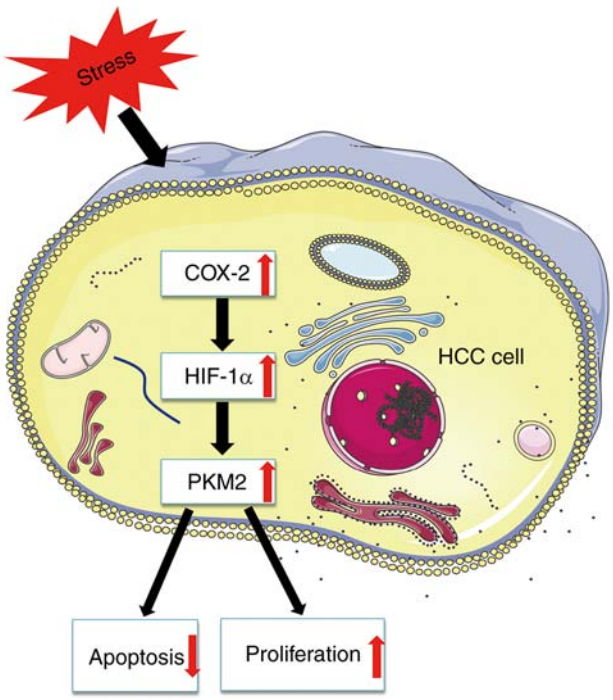

Figure 7. Proposed mechanism of COX-2 mediated apoptosis-resistance in HCC cells. Various stress conditions may upregulate COX-2 expression in HCC cells which subsequently activates PKM2 via the HIF-1α pathway, and the upregulated PKM2 expression further induces apoptosis-resistance and stimulates tumor cell growth to facilitate tumor cell survival and progression. COX-2, cyclooxygenase-2; HCC, hepatocellular carcinoma; PKM2, pyruvate kinase M2 isoform; HIF-1α, hypoxia-inducible factor 1-α.

Discussion

Standard metabolic processes are adapted in cancer cells compared with normal cells in order to maintain their rapid proliferation and progression, and PKM2 serves an important role in cancer cell metabolism (18,19). It has previously been reported that COX-2 is overexpressed in a number of tumor types, including HCC (26). However, whether COX-2 overexpression affects tumor cells metabolism via regulating PKM2 is currently unclear. In the present study, it was demonstrated that COX-2 and PKM2 were significantly upregulated in HCC tissues compared with normal liver tissues (P<0.05), and that their expression was positively associated with worse clinicopathological characteristics. Furthermore, it was demonstrated that COX-2 knockdown significantly reduced the expression of PKM2 and HIF-1α in HepG2 cells, whereas apoptosis was increased. The results of the present study suggest a novel method of metabolism regulation via the COX-2-mediated activation of the HIF-1α/PKM2 pathway in HCC (Fig. 7).

COX-2 is an inducible enzyme that serves an important function in multiple pathophysiological processes, including inflammation, atherosclerosis, tissue injury, angiogenesis and tumorigenesis (14,27). COX-2 is chronically overexpressed in numerous premalignant, malignant and metastatic human cancer types (20,21). One previous study reported that COX-2 regulates multiple cellular processes, including survival, proliferation and apoptosis resistance in cancer (28). In the present study, COX-2 overexpression was observed in liver tumor tissues and was positively associated with poorly differentiated histological grades. COX-2 knockdown significantly reduced cell proliferation and increased HepG2 cell apoptosis (P<0.05). Furthermore, it was revealed that COX-2 expression was positively associated with PKM2 expression, while PKM2 was downregulated and apoptosis was increased in HepG2 cells transfected with COX-2 siRNA. These results suggest that PKM2 may serve a function in COX-2 induced apoptosis resistance in HCC.

The primary function of the PK enzyme is to regulate the final rate-limiting step of glycolysis (29), which catalyzes the transfer of a phosphate group from PEP to ADP, yielding one molecule of pyruvate and one of ATP (30,31). Due to its overexpression in tumor tissues, PKM2 has been widely studied and has been reported to serve an essential role in tumor progression (32,33). A number of studies have indicated that PKM2 regulates apoptosis and proliferation (34,35); however, the precise molecular mechanisms responsible remain elusive (36). In the present study, PKM2 expression was detected in paraffin-embedded HCC specimens using IHC staining. It was revealed that PKM2 is upregulated in HCC tissues compared with normal hepatic tissues and is associated with poor differentiation. PKM2 function was also investigated using *in vitro* knockdown assays. The results demonstrated that PKM2 knockdown resulted in impaired cell viability and augmented tumor cell apoptosis *in vitro*. These data suggest that targeting PKM2 proteins may have potential as a therapeutic strategy for the treatment of HCC.

PKM2 expression varies among different types of cancer, suggesting that the function of PKM2 in tumorigenesis depends on the signaling context (37). Therefore,

a mechanistic insight into the expression of PKM2 and its molecular pathways in HCC may provide important data for developing novel HCC therapies (38,39). HIF-1 is a transcriptional factor that comprises two subunits: HIF-1α and HIF-1β (40). HIF-1α mediates systemic hypoxia by activating the transcription of multiple genes including erythropoietin, glycolytic enzymes and vascular endothelial growth factor (41,42). HIF-1α is known to regulate aerobic glycolysis in a number of cancer types and promotes tumor growth by regulating PKM2 expression (21,43). To determine whether PKM2 expression is modulated by HIF-1α in HCC, HIF-1α was silenced in HepG2 cells via siRNA transfection. The results revealed that si-HIF-1α effectively blocked the expression of PKM2 in HepG2 cells, inhibiting cell growth and promoting apoptosis. Similarly, a significant reduction in HIF-1α expression was observed following COX-2 knockdown ($P<0.05$). These data clearly demonstrate that the HIF-1α/PKM2 pathway may contribute to COX-2-induced apoptosis resistance in HCC.

In conclusion, the present study demonstrates that COX-2 knockdown inhibits HepG2 cell viability and induces apoptosis *in vitro*, and this may be mediated by the HIF-1α/PKM2 signaling pathway. These results suggest that the COX-2/HIF-1α/PKM2 pathway may facilitate apoptosis resistance in HCC cells, suggesting that COX-2-induced apoptosis resistance in HCC may result from metabolic changes associated with the HIF-1α/PKM2 pathway.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

GS and JL designed the experiments and wrote the paper. QW and DL performed the biological experiments with assistance from LF, YuhL and YuL. HY and QW contributed to the immunohistochemical analysis. GS and HW draft the manuscript. GS, HW and JL analyzed data and edited the paper. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of the First Affiliated Hospital of Anhui Medical University and conformed to the 1975 Declaration of Helsinki. All patients provided written informed consent.

Patient consent for publication

All patients consented to the publication of their data.

Competing interests

The authors declare that they have no competing interests.

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