Original Article

Hypoxia induces universal but differential drug resistance and impairs anticancer mechanisms of 5-fluorouracil in hepatoma cells

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Abstract

Hepatocellular carcinoma (HCC) is one of the most refractory cancers. The mechanisms by which hypoxia further aggravates therapeutic responses of advanced HCC to anticancer drugs remain to be clarified. Here, we report that hypoxia (1% O_2) caused 2.55–489.7-fold resistance to 6 anticancer drugs (sorafenib, 5-fluorouracil [5-FU], gemcitabine, cisplatin, adriamycin and 6-thioguanine) in 3 HCC cell lines (BEL-7402, HepG2 and SMMC-7721). Among the 6 drugs, sorafenib, the sole one approved for HCC therapy, inhibited proliferation with little influence from hypoxia and displayed the smallest variation among the 3 HCC cell lines tested. By contrast, the inhibition of proliferation by 5-FU, which has been extensively tested in clinical trials but has not been approved for HCC therapy, was severely affected by hypoxia and showed a large variation among these cell lines. In 5-FU-treated HCC cells, hypoxia reduced the levels of basal thymidylate synthase (TS) and functional TS, leading to decreased dTMP synthesis and DNA replication. Hypoxia also affected the accumulation of FdUTP and its misincorporation into DNA. Consequently, both single-strand breaks and double-strand breaks in DNA were reduced, although hypoxia also inhibited DNA repair. In 5-FU-treated HCC cells, hypoxia further abated S-phase arrest, alleviated the loss of mitochondrial membrane potential, diminished the activation of caspases, and finally resulted in reduced induction of apoptosis. Thus, hypoxia induces universal but differential drug resistance. The extensive impacts of hypoxia on the anticancer mechanisms of 5-FU contributes to its hypoxia-induced resistance in HCC cells. We propose that hypoxia-induced drug resistance and interference of hypoxia with anticancer mechanisms could be used as candidate biomarkers in selecting and/or developing anticancer drugs for improving HCC therapy.

Keywords: hepatocellular carcinoma; hypoxia; 5-fluorouracil; sorafenib; drug resistance

Introduction

Hepatocellular carcinoma (HCC) has had the highest increase in death rate and the second highest increase in incidence rate among all cancers in recent years in the United States[1]. HCC is also among the cancers that are commonly diagnosed and one of the leading causes of cancer death in China[2]. However, only one anticancer drug, *ie*, sorafenib, has been approved for the treatment of advanced HCC, and it improves survival in patients with HCC by just 2.3–2.8 months[3, 4]. Therefore, systemic therapy for advanced HCC is still a highly unmet medical need. This situation relates to the basic fact that HCC is among the most refractory cancers to anticancer drugs. One likely reason for such drug resistance in HCC could be that HCC cells generally express various drug transporters such as P-glycoprotein (P-gp), which leads to the reduction of cellular drug accumulation[5]. Another possible reason could be that hypoxia occurs extensively in advanced HCC[6-9].

Within solid tumors, the convoluted vasculature and robust proliferation of tumor cells result in an imbalance between the supply and the consumption of oxygen, thereby leading to hypoxia[7]. In HCC, hypoxia might be further aggravated due to differential oxygen supplies between the periportal zone and the perivenous zone of the liver[10]. Hypoxia-inducible factor-1 (HIF-1) is a critical regulator responding to hypoxia. HIF-1 is a transcription factor that regulates the expression of

more than 100 genes, including those involved in drug resistance such as the *MDR1* gene encoding P-gp[7, 8]. Hypoxia can induce drug resistance in tumors by both HIF-1-dependent and HIF-1-independent mechanisms[9]. Many studies have extensively investigated the former mechanism; for example, HIF-1 has been demonstrated to be required for the clinically acquired resistance of HCC to sorafenib[11]. However, relatively few studies have explored the latter, especially in HCC.

To improve the survival of HCC patients with sorafenib treatment, clinical exploration of sorafenib-based combinations was proposed in 2008[12]. Since then, many clinical trials have been reported, which have tested the combination of sorafenib with different anticancer drugs, including fluoropyrimidines [5-fluorouracil (5-FU)[13, 14], capecitabine[15], tegafur[16] and S1][17], gemcitabine[18, 19], oxaliplatin[19] and adriamycin[20]. Relative to sorafenib alone, most of these combinations have shown favorable improvements during the treatment of advanced HCC. In addition, various drug combinations that did not contain sorafenib were tested in clinical trials in patients with advanced HCC, including sorafenib-refractory HCC[21-26]. Because of widespread hypoxia in advanced HCC, knowledge of the characteristics and mechanisms of hypoxia-induced drug resistance in HCC is important for the selection of potential anticancer drugs for clinical combination therapies, whether sorafenib-based or not. Moreover, insights into hypoxia-induced drug resistance in HCC will lay a solid foundation for the development of new therapeutics against HCC.

Irregular blood flow due to convoluted vasculature and varying distances between cancer cells and functional blood vessels[7] in HCC patients and *in vivo* HCC models might prevent the homogeneous exposure of HCC cells to oxygen and/or anticancer drug(s). This situation is not advantageous to exact evaluation of the impact of hypoxia on drug resistance in HCC. In this study, therefore, we used an *in vitro* system of cultured HCC cells, in which the concentrations of both oxygen and the drug(s) to which the cells were exposed were controllable. We found that exposure to hypoxia (1% O_2) caused universal but differential drug resistance of 3 HCC cell lines to 6 anticancer drugs, *ie*, sorafenib, 5-FU, gemcitabine, adriamycin, 6-thioguanine and cisplatin. Further investigations with 5-FU showed that hypoxia profoundly impaired 5-FU-mediated multistep mechanisms of proliferative inhibition and cell killing. Particularly, the hypoxic effects were found to be basically independent of HIF-1(HIF-1α) but were closely correlated with the generation of toxic intermediates of 5-FU, DNA misincorporation, inhibition of thymidylate synthase (TS) and DNA replication, induction of DNA strand breaks, S-phase arrest and induction of apoptosis.

Materials and methods
Cell culture
Both BEL-7402 (2002) and SMMC-7721 (2006) cell lines were kept in the Shanghai Institute of Materia Medica of the Chinese Academy of Sciences (Shanghai, China). The HepG2 (2008) cell line was purchased from the American Type Culture Collection (ATCC). The BEL-7402/5-FU (2011) cell line was obtained

from KeyGen Biotech Co Ltd (Keygen, Nanjing, China). BEL-7402, SMMC-7721 and HepG2 cell lines were authenticated by short tandem repeat (STR) profiling at Shanghai Genesky Biotech Co, Ltd (SMMC-7721 and BEL-7402, July 2013; HepG2, December 2013). The cells were normally cultured according to the suppliers' instructions. Hypoxia treatments were performed by placing the cells in a humidified atmosphere containing 5% CO_2 at 1% oxygen partial pressure (Ruskin Invivo 400 system; Ruskin Technology Ltd, Bridgend, UK).

Drugs, antibodies, and reagents
5-FU and 6-thioguanine were from Sigma-Aldrich (St Louis, USA). Sorafenib was from Selleck (Houston, TX, USA). Adriamycin and gemcitabine were from Dalian Meilun Biology Technology Co, Ltd (Dalian, China). Cisplatin was from Mayne Pharma Pty Ltd (Salisbury, Australia). The antibody against HIF-1α was from BD Biosciences. The antibody against PAR was from Trevigen. Antibodies against γH2AX, P-gp, ChK1, ChK2, ATM, p-Ser1981-ATM, PARP, MCL-1, BCL-2, dUTPase, UMP-CMPK and β-Actin were all from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against BCRP, MRP and TS were from Abcam (Cambridge, UK). Antibodies against p-Ser317-Chk1, p-Ser345-Chk1, p-T68-Chk2, Caspase-3, Caspase-7, Caspase-9, BCL-XL, BAK and BIM were from Cell Signaling Technology (Danvers, MA, USA).

Proliferation assays
Proliferative inhibition was measured by sulforhodamine B (SRB, Sigma, St Louis, MO, USA) assays. Cells were seeded in 96-well plates, cultured overnight and treated with a concentration gradient of the tested drugs under normoxic or hypoxic conditions for 72 h. IC_{50} was calculated by the logit method. The resistance factor (RF) for each drug was calculated as the ratio of the IC_{50} value in hypoxia to that in normoxia.

Bromodeoxyuridine (BrdU) incorporation assays
Cells treated with 5-FU for 36 h were incubated with serum-free medium containing 10 μmol/L BrdU for 45 min, then collected and washed with PBS and fixed with pre-cooled 75% ethanol at 4°C. The fixed samples were denatured with 2 mol/L HCl, resuspended in 0.1 mol/L sodium tetraborate, then incubated with anti-BrdU antibody (BD Biosciences, Franklin Lakes, NJ, USA) and stained with fluorescence-conjugated secondary antibody (Invitrogen, Carlsbad, CA, USA) and PI. The samples were analyzed using a FACSCalibur platform (BD Biosciences, Franklin Lakes, NJ, USA).

Western blotting
Western blotting was performed as previously described[27, 28]. Cells were seeded in 6-well plates and cultured overnight. After treatment with 5-FU at the indicated conditions, the cells were lysed with SDS loading buffer and prepared for Western blotting.

Cell immunofluorescence
Cells were seeded on glass coverslips in 12-well plates, cul-

tured overnight, and treated with 5-FU for the indicated time. Then, the cells were fixed for 20 min with pre-cooled methanol at -20 °C, blocked with 3% bovine serum albumin for 15 min, incubated with the primary antibody for 1 h, and stained with fluorescence-conjugated secondary antibody for 1 h. Finally, after being counterstained with 4′,6-diamidino-2-phenylindole (DAPI), the cells were imaged under an Olympus confocal microscope (Olympus, Tokyo, Japan).

Comet assays
BEL-7402 cells were seeded in 6-well plates and treated with 5-FU as indicated. Alkaline or neutral comet assays were performed using a comet assay kit (Trevigen, Gaithersburg, MD, USA) according to the manufacturer's instructions.

Transfection of siRNA against HIF-1α
Transfection of siRNA against HIF-1α was performed as previously described[29]. The sequence of siRNA for *hif-1a* was 5′-CACCAUGAUAUGUUUACUAUT-3′ (siHIF-1α), and 5′-UUCUCCGAACGUGUCACGUTT-3′ was used as the negative control (siCtrl). These siRNA sequences were synthesized by Shanghai GenePharma Co, Ltd (Shanghai, China).

Cell cycle assays
The cells treated with 5-FU for 36 h were collected and washed with PBS and fixed with pre-cooled 75% ethanol at 4 °C. Then, the cells were washed with PBS and stained with propidium iodide (PI) in the dark for 15 min. For each sample, at least $1×10^4$ cells were analyzed using a FACSCalibur platform (BD Biosciences, Franklin Lakes, NJ, USA).

Detection of mitochondrial membrane potential (MMP)
The cells treated with 5-FU for 36 h were collected and washed with PBS. Then, the cells were stained using a JC-1 kit (Keygen, Nanjing, China). MMP was analyzed with a FACSCalibur platform (BD Biosciences, Franklin Lakes, NJ, USA).

Annexin V-FITC apoptosis detection
The cells treated with 5-FU for 48 h were collected and washed with PBS. Then, the cells were stained using an Annexin V-PI apoptosis detection kit (Keygen, Nanjing, China). Fluorescence of the cells was determined immediately using a FACS-Calibur platform (BD Biosciences, Franklin Lakes, NJ, USA).

Statistical analysis
All data, if applicable, were expressed as the mean±SD. Comparison between two groups was performed with the Student's *t*-test. $P<0.05$ was considered statistically significant. All the data expressed as the mean±SD were from three independent experiments unless otherwise specified.

Results
Hypoxia leads to universal but differential resistance of HCC cells to anticancer drugs
We evaluated the effects of hypoxia (1% O_2) on the proliferative inhibition of 3 HCC cell lines by 6 anticancer drugs including sorafenib (the only drug approved for HCC), 5-FU, gemcitabine, cisplatin, adriamycin (these 4 have been tested in clinical trials for HCC) and 6-thioguanine. The 3 tested cell lines (BEL-7402, HepG2 and SMMC-7721) have different expression profiles of important anticancer drug transporters, including P-gp, multidrug resistance protein (MRP) and breast cancer resistance protein (BCRP)[5]. Moreover, the expression of P-gp in HepG2 cells is sensitive to hypoxia and HIF-1[30]. Our data showed that all the 3 HCC cell lines displayed hypoxia-induced drug resistance to the tested anticancer drugs with an average RF of 44.75 (range: 2.55–489.70) (Table 1). However, the range of the extent of hypoxia-induced resistance to different anticancer drugs was variable (Table 1). According to the average RF for each tested drug in the 3 tested cell lines, the degree of the hypoxia-induced drug resistance increased from sorafenib (3.59), cisplatin (3.99), 6-thioguanine (7.77), adriamycin (11.69) and 5-FU (56.6) to gemcitabine (184.88). In

Table 1. Hypoxia leads to resistance of HCC cells to different anticancer drugs.

Agents	BEL-7402			HepG2			SMMC-7721		
	IC_{50}[a] (µmol/L)		RF[b]	IC_{50} (µmol/L)		RF	IC_{50} (µmol/L)		RF
	20% O_2	1% O_2		20% O_2	1% O_2		20% O_2	1% O_2	
5-Fluorouracil	4.92±0.45	660.98±83.84	134.29	0.46±0.12	12.06±0.03	26.52	26.93±4.48	242.13±34.05	8.99
Gemcitabine	0.14±0.07	67.42±15.77	489.70	0.37±0.04	7.36±2.95	19.89	0.33±0.05	14.71±5.90	45.04
6-Thioguanine	4.70±0.21	11.99±1.14	2.55	3.26±0.62	54.02±9.94	16.59	4.59±0.11	19.13±0.91	4.17
Adriamycin	0.21±0.12	1.26±0.08	6.05	0.04±0.01	1.06±0.02	24.35	0.42±0.14	1.98±0.04	4.68
Cisplatin	6.62±0.30	18.21±1.18	2.75	4.53±0.45	26.96±0.99	5.95	3.36±0.19	10.97±0.90	3.27
Sorafenib	5.52±0.49	14.58±2.01	2.64	2.44±0.36	12.53±2.89	5.13	4.21±0.77	12.62±0.87	3.00

[a]The drug concentration required for 50% growth inhibition (IC_{50}) of tumor cells was determined from three separate experiments and expressed as mean±SD. Each drug concentration was tested in triplicate for 72 h.
[b]The resistance factor (RF) was calculated as the ratio of the IC_{50} value of the cells treated at hypoxia (1% O_2) to that of the cells treated at normoxia (20% O_2).

the 3 tested cell lines, the extent of hypoxia-induced resistance to sorafenib and cisplatin was the least variable, with the RF ranges from 2.64 to 5.13 for sorafenib and from 2.75 to 5.95 for cisplatin. By contrast, the extent of hypoxia-induced resistance to gemcitabine was the most variable with an RF range from 19.89 to 489.7 (Table 1). Of note, under hypoxic conditions,
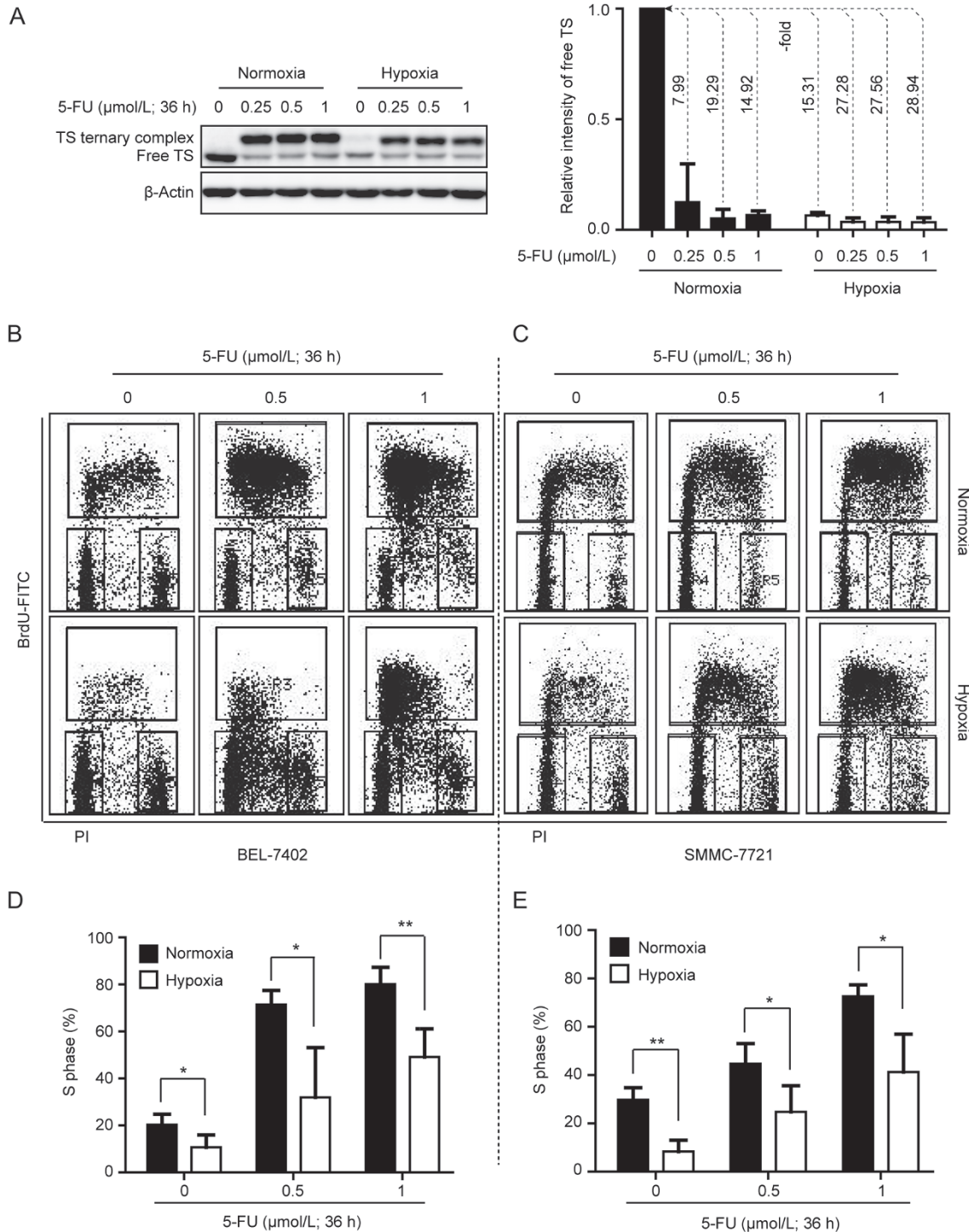

Figure 1. Hypoxia impairs DNA synthesis. (A) BEL-7402 cells were treated with the indicated concentrations of 5-FU for 36 h. The cells were collected for Western blotting of TS. The relative intensity of free TS in each group was quantified with the Image-Pro Plus software, and the intensity of free TS in the untreated cells under normoxia was set as 1. The data from three independent experiments are shown as the mean±SD. (B) BEL-7402 cells and (C) SMMC-7721 cells were treated with 5-FU for 36 h. The cells were analyzed for BrdU incorporation by flow cytometry. (D and E) The data from three independent experiments are shown as the mean±SD. *P<0.05; **P<0.01.

HepG2 cells were more resistant to sorafenib, cisplatin, 6-thioguanine and adriamycin than BEL-7402 and SMMC-7721 cells; by contrast, BEL-7402 cells were more resistant to 5-FU and gemcitabine than the other two cell lines (Table 1). The results indicate that hypoxia leads to universal but differential resistance in the 3 HCC cell lines to the tested anticancer drugs.
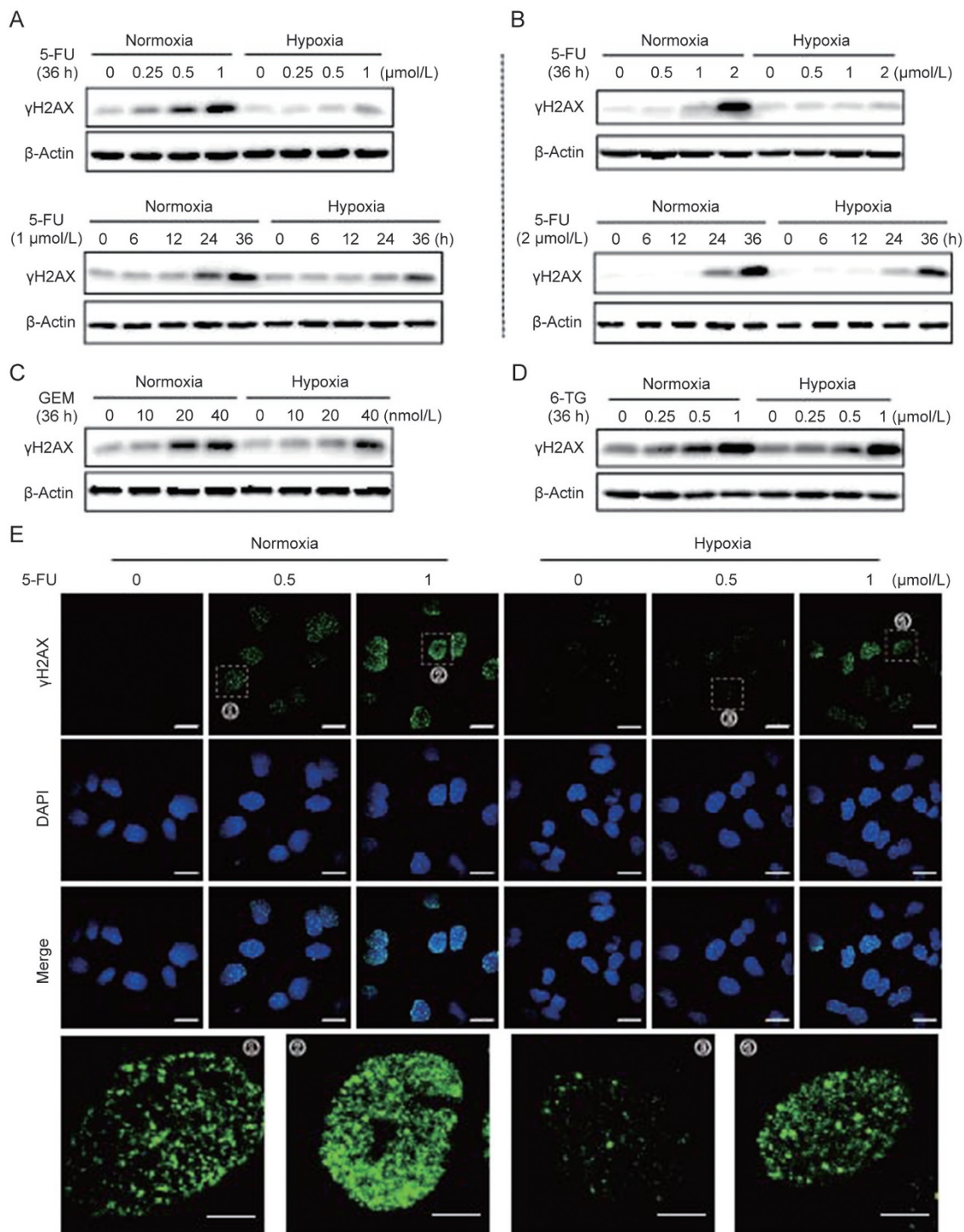

Figure 2. Hypoxia decreases 5-FU-induced DNA damage. (A) BEL-7402 cells and (B) HepG2 cells were treated with 5-FU under normoxic or hypoxic conditions. The cells were collected for Western blotting. (C and D) BEL-7402 cells were treated with gemcitabine (GEM) for 24 h (C) or with 6-thioguanine (6-TG) for 36 h (D). Then, the cells were collected for Western blotting analysis of γH2AX. (E) BEL-7402 cells were treated with 5-FU for 36 h. γH2AX foci were imaged by immunofluorescence-based laser confocal microscopy. Scale bar: 20 μm. The magnified images of γH2AX foci in the dashed rectangles are shown in the lower panels. The images shown in this figure are representative of those from three independent experiments.

Fluoropyrimidine drugs are the most commonly tested anticancer drugs for combination therapies in clinical trials of HCC[13-17, 21-24]. 5-FU is a classical fluoropyrimidine drug from which almost all the other drugs in this class have been derived. Table 1 shows that hypoxia induced the 2nd highest degree of drug resistance to 5-FU. Therefore, we chose 5-FU as the primary drug to further explore the mechanisms by which hypoxia induces drug resistance in HCC.

Exposure to hypoxia decreases the protein level of thymidylate synthase (TS) and attenuates the 5-FU-induced suppression of TS

In cells, 5-FU can be converted to fluorodeoxyuridine monophosphate (FdUMP), a primary active metabolite. FdUMP binds to and forms a stable ternary complex with TS, causing a reduction of free (functional) TS to which the endogenous substrate deoxyuridine monophosphate (dUMP) binds, thereby inhibiting the synthesis of deoxythymidine monophosphate (dTMP) and suppressing DNA replication. This is the main mechanism of proliferative inhibition by 5-FU[31]. The 36-h exposure of HCC BEL-7402 cells to hypoxia (1% O_2) resulted in a 15.31-fold reduction in cellular TS protein levels (Figure 1A). No TS ternary complexes could be detected in the control groups. Treatment with 5-FU led to a sharp increase in TS ternary complexes and a 14.92–28.94-fold reduction in free TS under both normoxic and hypoxic conditions. When the protein level of free TS in the 5-FU-treated cells was compared with that of the untreated cells under normoxic and hypoxic conditions, the ratio was found to be much higher in

normoxia, indicating that the inhibition of TS under normoxia was more effective (Figure 1A). BrdU, a thymidine analog, is a marker of DNA synthesis[32]. Exposure to hypoxia reduced the incorporation of BrdU into DNA in both HCC BEL-7402 (Figure 1B) and SMMC-7721 (Figure 1C) cells and thus decreased the number of cells in S phase (Figure 1D and 1E) because cellular DNA synthesis occurs in this phase. 5-FU caused an increase in the number of BrdU-labeled cells in a concentration-dependent fashion, which was significantly inhibited by hypoxia in both cell lines (Figure 1B -1E).

Hypoxia decreases DNA strand breaks caused by treatment with 5-FU

FdUMP can be further converted to fluorodeoxyuridine triphosphate (FdUTP), which can be misincorporated into DNA. Misincorporation of FdUTP into DNA together with DNA-repair inhibition arising from dTMP reduction leads to DNA-strand-broken damage. This is another mechanism of anticancer activity of 5-FU[31]. In both HCC BEL-7402 and HepG2 cells (Figure 2A-2E; Supplementary Figure S1), hypoxia (1% O_2) itself did not seem to change the levels of γH2AX, an established marker for DNA double-strand breaks (DSBs)[33]. Under normoxia, 5-FU increased the levels of γH2AX in a concentration- and/or a time-dependent manner in both BEL-7402 (Figure 2A and 2E) and HepG2 (Figure 2B) cells, which was clearly inhibited when these cells were exposed to hypoxia. The data reveal that hypoxia reduces DSBs induced by treatment of HCC cells with 5-FU. The results were further
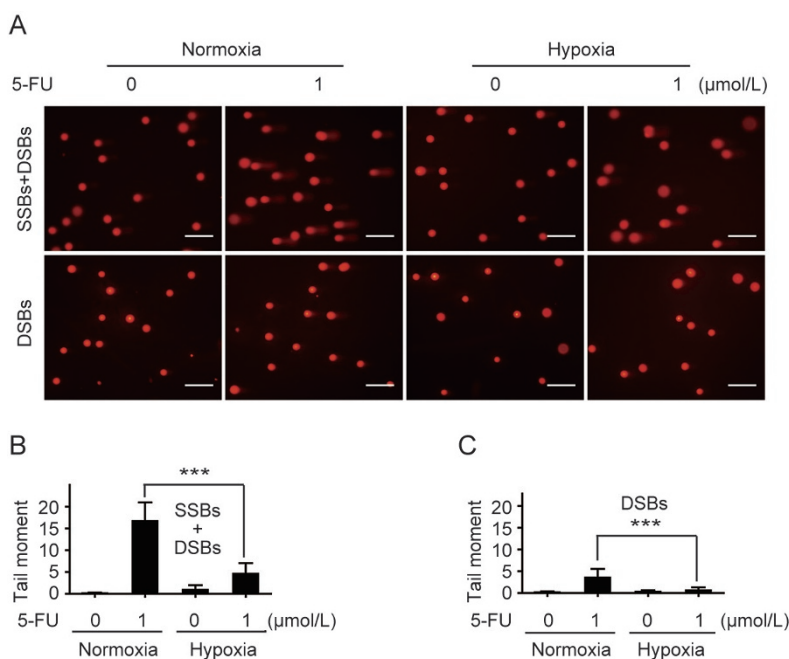

Figure 3. Hypoxia reduces 5-FU-induced SSBs and DSBs. BEL-7402 cells were treated with 5-FU for 36 h. (A) The cells were collected for comet assays to detect SSBs+DSBs (at alkaline conditions) and DSBs (at neutral conditions). The images shown in this figure are representative of those from three independent experiments. (B and C) The tail moment was calculated from 50 cells in each sample with the CASP software. The data are shown as the mean±SD. ***P<0.001.

validated in BEL-7402 cells treated with 2 other antimetabolite drugs, gemcitabine (Figure 2C) and 6-thioguanine (Figure 2D).

To further clarify whether the 5-FU-induced DSBs are derived from DNA single-strand breaks (SSBs), we conducted comet assays[34] at neutral and alkaline conditions. The comet assay detects only DSBs at neutral condition but both DSBs and SSBs at alkaline condition[35]. In normoxia, 5-FU increased the degree of both types of DNA damage in the BEL-7402 cells, but it increased DNA strand breaks at the alkaline condition (DSBs+SSBs) [Figure 3A (upper panel) and 3B] to a much greater extent than that at the neutral condition (DSBs only) [Figure 3A (lower panel) and 3C]. In the cells exposed to 5-FU, hypoxia significantly reduced DNA strand breaks; however, the remaining DNA strand breaks were still more abundant at the alkaline condition (DSBs+SSBs) than at the neutral condi-

tion (DSBs only). The data mechanistically indicate that the DSBs originate mainly from SSBs caused by 5-FU and that hypoxia suppresses the generation of 5-FU-induced SSBs, leading to the reduction of DSBs.

5-FU does not impair hypoxia-induced changes in the levels of HIF-1α and drug transporters

HIF-1α is a subunit of HIF-1[30]. As expected, hypoxia enhanced the levels of HIF-1α in all 3 tested HCC cell lines (Figure 4A). However, treatment with 5-FU did not disturb the process of these hypoxia-induced HIF-1α changes (Figure 4A). Consistent with the prior results, hypoxia increased the level of HIF-1α and reduced the generation of 5-FU-driven DSBs in the BEL-7402 cells transfected with scramble siRNA (siCtrl). By contrast, knockdown of HIF-1α with specific
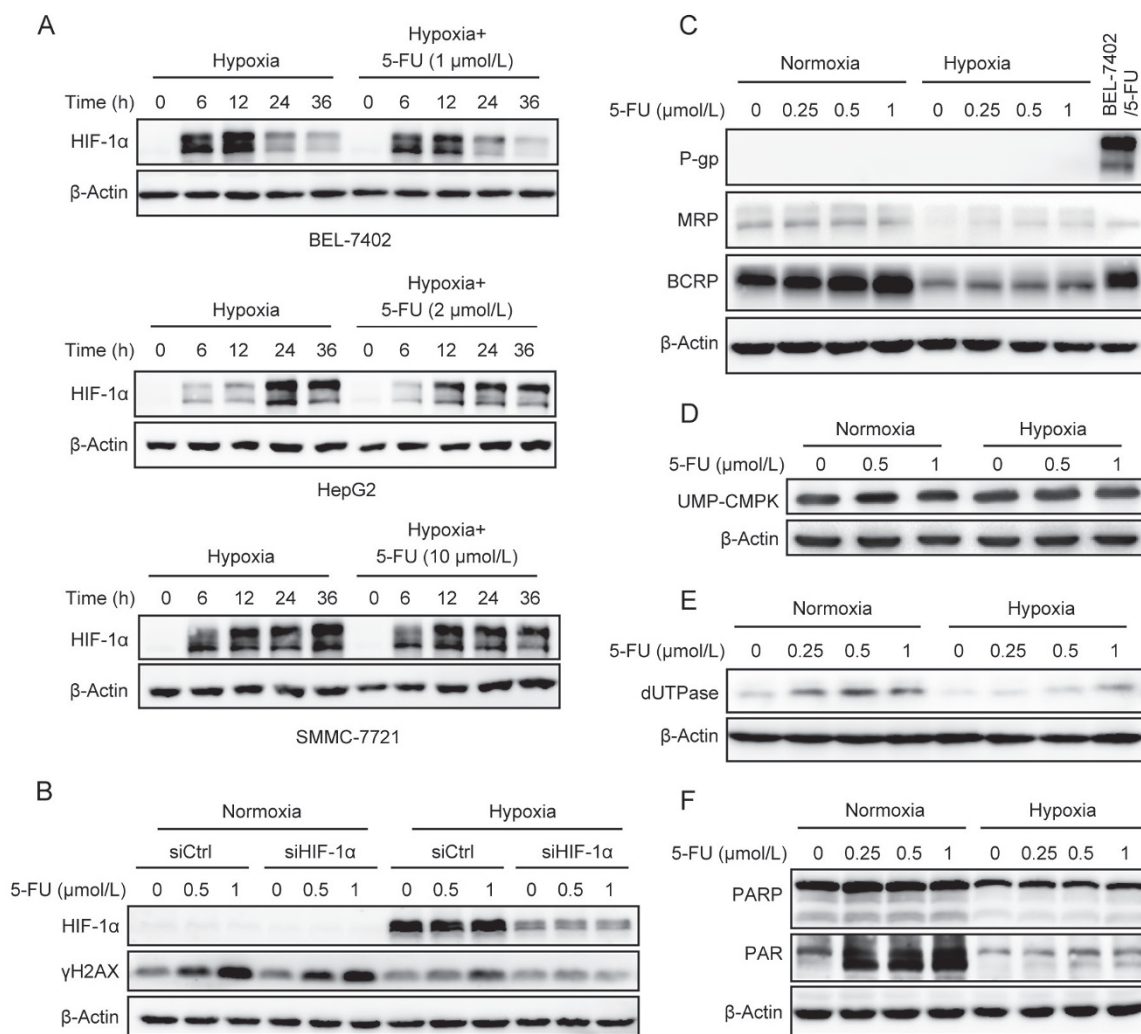

Figure 4. Effects of hypoxia on the levels of HIF-1α, drug transporters, dUTPase, UMP-CMPK and PARP. (A) BEL-7402, HepG2 and SMMC-7721 cells were treated with 5-FU under hypoxic conditions. The cells were collected for Western blotting. (B) BEL-7402 cells were treated with scrambled siRNA (siCtrl) or specific siRNA targeting human *HIF-1α* gene (siHIF-1α) for 48 h. The cells were treated with 5-FU for 36 h under normoxic or hypoxic conditions and then collected for Western blotting. (C-F) BEL-7402 cells were treated with 5-FU for 36 h under normoxic or hypoxic conditions. Then, the cells were collected for Western blotting. BEL-7402/5-FU cells were normally cultured for 36 h and collected for Western blotting as the control in (C). The images shown in this figure are representative of those from three independent experiments.

siRNA against the *HIF-1a* gene did not increase the accumulation of γH2AX induced by 5-FU under hypoxic conditions (Figure 4B), suggesting that 5-FU-driven DSBs might be independent of HIF-1α in the tested conditions.

BEL-7402 cells do not express detectable levels of P-gp but express both MRP and BCRP (Figure 4C)[5]. The overexpression of P-gp in BEL-7402/5-FU cells (Figure 4C) mechanistically contributes to the acquired resistance of BEL-7402 cells to 5-FU[36]. Although hypoxia induced the apparent resistance of BEL-7402 cells to 5-FU (Table 1), it did not increase the expres-
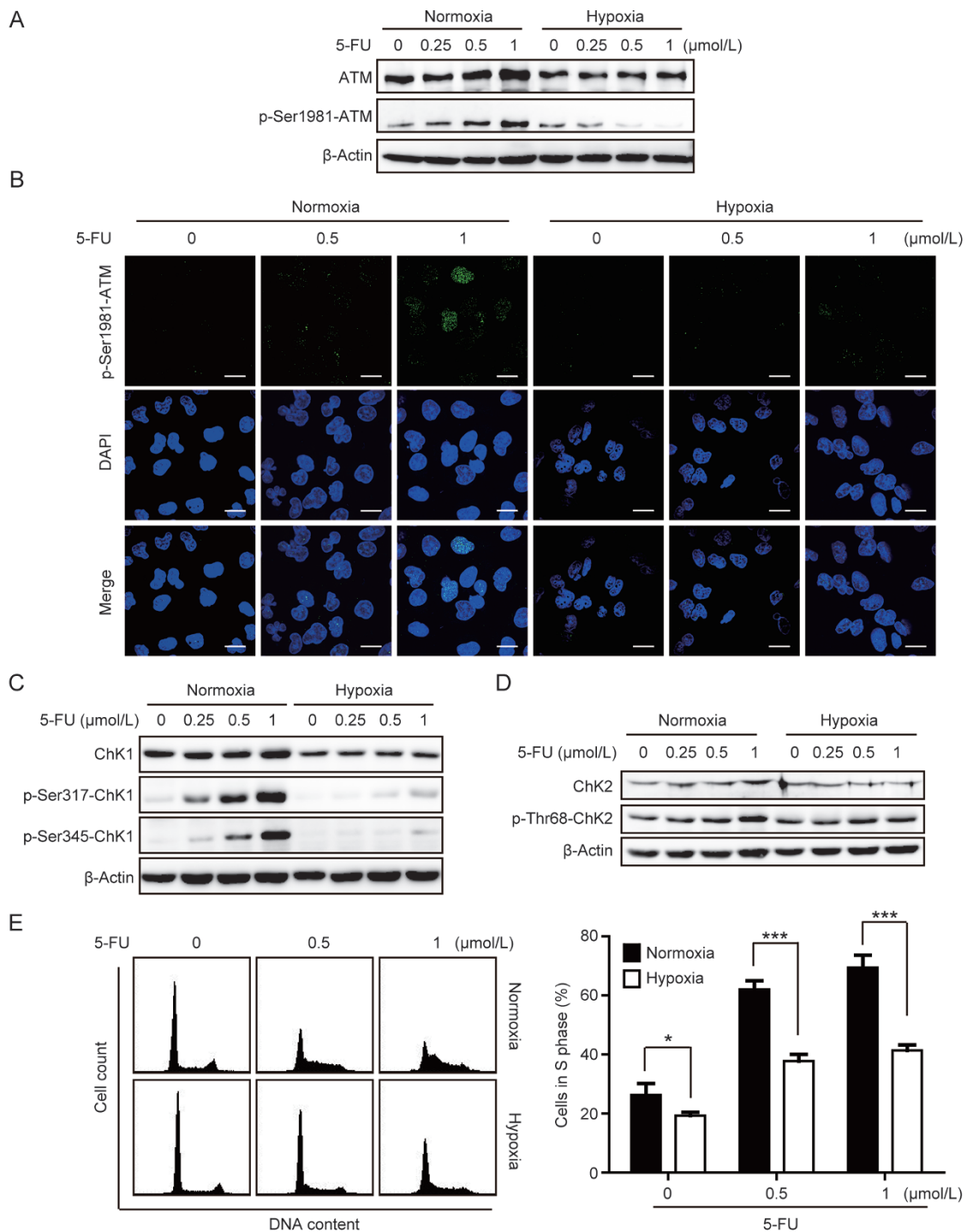

Figure 5. Impact of hypoxia on the cell cycle. BEL-7402 cells were treated with 5-FU for 36 h. (A) The cells were collected for Western blotting analyses of ATM and p-Ser1981-ATM levels. (B) p-Ser1981-ATM was imaged by immunofluorescence-based laser confocal microscopy. Scale bar: 20 μm. (C and D) The cells were collected for Western blotting analyses of the indicated proteins. (E) Cell cycle distribution was analyzed by flow cytometry. Data from three independent experiments are shown as the mean±SD. *$P<0.05$; ***$P<0.001$.

sion of P-gp; moreover, it reduced the levels of MRP and BCRP in the cells exposed to concentration gradients of 5-FU (Figure 4C). The data indicate that these drug transporters may not have been associated with the hypoxia-induced resistance to 5-FU.

Effects of 5-FU and hypoxia on the levels of UMP/CMP kinase (UMP/CMPK), the pyrophosphatase dUTP nucleotidohydrolase (dUTPase), poly(ADP-ribose) polymerase-1 (PARP-1) and poly(ADP-ribose) (PAR)

UMP/CMPK, using ATP as a phosphate donor to physiologically catalyze the phosphorylation of UMP, CMP and dCMP, has been shown to phosphorylate diphosphate and triphosphate metabolites of pyrimidine anticancer drugs. The level of UMP/CMPK protein is correlated with cellular sensitivity to 5-FU[37]. FdUTP can be converted to FdUMP by dUTPase, which prevents the misincorporation of FdUTP into DNA[38]. PARP-1 is a critical repair factor in the nucleotide excision repair (NER) pathway[39] that is responsible for repairing DNA-misincorporated FdUTP[31]. However, the treatment with 5-FU did not alter the levels of UMP/CMPK protein in BEL-7402 cells either at normoxia or hypoxia (Figure 4D). In contrast, 5-FU led to concentration-dependent increases in the levels of dUTPase protein and PAR, the product generated from PARP-1 catalysis, though the level of PARP-1 protein remained unchanged in normoxia. Hypoxia decreased the levels of dUTPase and PARP-1 proteins and the generation of PAR and weakened the 5-FU-induced increase in the levels of dUTPase protein and PAR (Figure 4E and 4F). These data indicate that hypoxia affects the conversion between FdUMP and FdUTP and inhibits the repair of 5-FU-induced SSBs via the NER pathway.

Hypoxia suppresses the activation of checkpoint proteins and S-phase arrest induced by 5-FU

Ataxia telangiectasia mutated (ATM) kinase can be activated by both DSBs and DNA replication stress in a self-phosphorylating manner. Activated ATM further activates cell cycle checkpoints by phosphorylating checkpoint kinase (ChK)1/ChK2, leading to S-phase arrest[40]. In normoxia, as expected, 5-FU resulted in enhanced phosphorylation levels of ATM at Ser1981, ChK1 at both Ser317 and Ser345 and ChK2 at Thr68 and typical S-phase arrest in a concentration-dependent fashion in BEL-7402 cells (Figure 5). Hypoxia reduced the total protein levels of ATM and ChK1, and dramatically inhibited 5-FU-triggered phosphorylation of these 2 proteins, thereby abating the S-phase arrest (Figure 5A-5C and 5E). Moreover, only hypoxia significantly reduced the number of cells in S phase (Figure 5E). However, it did not alter the level of total ChK2 protein and 5-FU-driven phosphorylation of ChK2 at Thr68 (Figure 5D). The effect of hypoxia on S-phase arrest (Figure 5E) was consistent with its impact on the incorporation of BrdU (Figure 1D and 1E).

Hypoxia inhibits apoptosis induced by 5-FU

B cell lymphoma 2 (BCL-2) family proteins include anti-apop-

totic (such as BCL-2, MCL-1 and BCL-XL) and pro-apoptotic (such as BAK and BIM) members. The balance between these two groups of proteins determines the permeabilization of the outer mitochondrial membrane and thus MMP, thereby regulating apoptosis[41, 42]. In normoxia, treatment with 5-FU decreased the cellular levels of the anti-apoptotic proteins MCL-1 and BCL-2 in a concentration-dependent manner, slightly reduced the level of both the anti-apoptotic protein BCL-XL and the pro-apoptotic protein BIM but had almost no effect on the pro-apoptotic protein BAK in BEL-7402 cells (Figure 6A). These effects subsequently caused the loss of MMP (Figure 6B) and induced the activation of caspase-3, caspase-7 and caspase-9, leading to the cleavage of PARP (Figure 6C) and eventually induced apoptosis (Figure 6D). The loss of MMP, activation of caspases, cleavage of PARP and induction of apoptosis in the cells exposed to 5-FU in normoxia were significantly inhibited by hypoxia (Figure 6B-6D). However, the effects of hypoxia on the BCL-2 family proteins were relatively complicated. Hypoxia counteracted the 5-FU-induced reduction in the level of the anti-apoptotic protein MCL-1 but did not affect the 5-FU-driven decrease in the level of another anti-apoptotic protein, BCL-2. On the other hand, hypoxia itself reduced the baseline levels of BCL-XL, BAK and BIM proteins, and 5-FU induced an increase in their levels under hypoxia (Figure 6A).

Discussion

Advanced HCC is resistant to the majority of current anticancer drugs, and one of the possible reasons for this is extensive hypoxia within the tumor[6-10]. We report that persistent hypoxia (1% O_2 for 72 h) led to universal but differential resistance of 3 HCC cell lines derived from different patients[43] to 6 common anticancer drugs with different mechanisms of action. Among these tested drugs, sorafenib inhibited cellular proliferation with little influence by hypoxia and displayed the smallest variation among these cell lines. By contrast, the inhibition of proliferation by 5-FU and gemcitabine was severely affected by hypoxia with the largest variation among the cell lines. Moreover, hypoxia-induced resistance to 5-FU in colon, oral and pancreatic cancer cells[44-46] has also been found, which further supports our observation that the anticancer activity of 5-FU is easily affected by hypoxia. This might be one of the reasons why sorafenib has been approved for HCC systemic therapy while 5-FU has not, although the latter has been tested in clinical trials since the 1960s[13, 14, 21, 22, 47]. The data also suggest that hypoxia and the sensitivity of drugs' anticancer activity to hypoxia should be taken into consideration when developing new therapeutics for advanced HCC.

Using 5-FU as an example, we conducted mechanistic investigations of hypoxia-induced drug resistance of HCC cells. The results revealed that hypoxia exerted a profound impact on the following processes by which 5-FU elicits its anticancer activity (Figure 7): (a) Reducing TS and DNA replication. Hypoxia caused an apparent reduction in the levels of TS, the main anticancer target of 5-FU, in HCC BEL-7402 cells possibly by inhibiting the expression of the *TS* gene,

consistent with what has been reported in other tumor cells *in vivo*[48]. Moreover, compared with normoxia, hypoxia clearly reduced TS inhibition efficiency. Consequently, the synthesis of dTMP and dTTP may have been decreased, which led to the inhibition of DNA replication, evidenced by the reduction in BrdU incorporation in both BEL-7402 and SMMC-7721 cells treated with 5-FU under hypoxia. Notably, in these cells,

hypoxia itself significantly diminished BrdU labeling, consistent with what has been shown previously in C3H mammary carcinomas[48]. (b) Decreasing SSBs and DSBs caused by 5-FU. Our data clearly revealed that hypoxia significantly abated 5-FU-driven SSBs and DSBs. However, we observed that on one hand, hypoxia decreased dUTPase, which would cause reduced conversion of FdUTP to FdUMP and thus increase
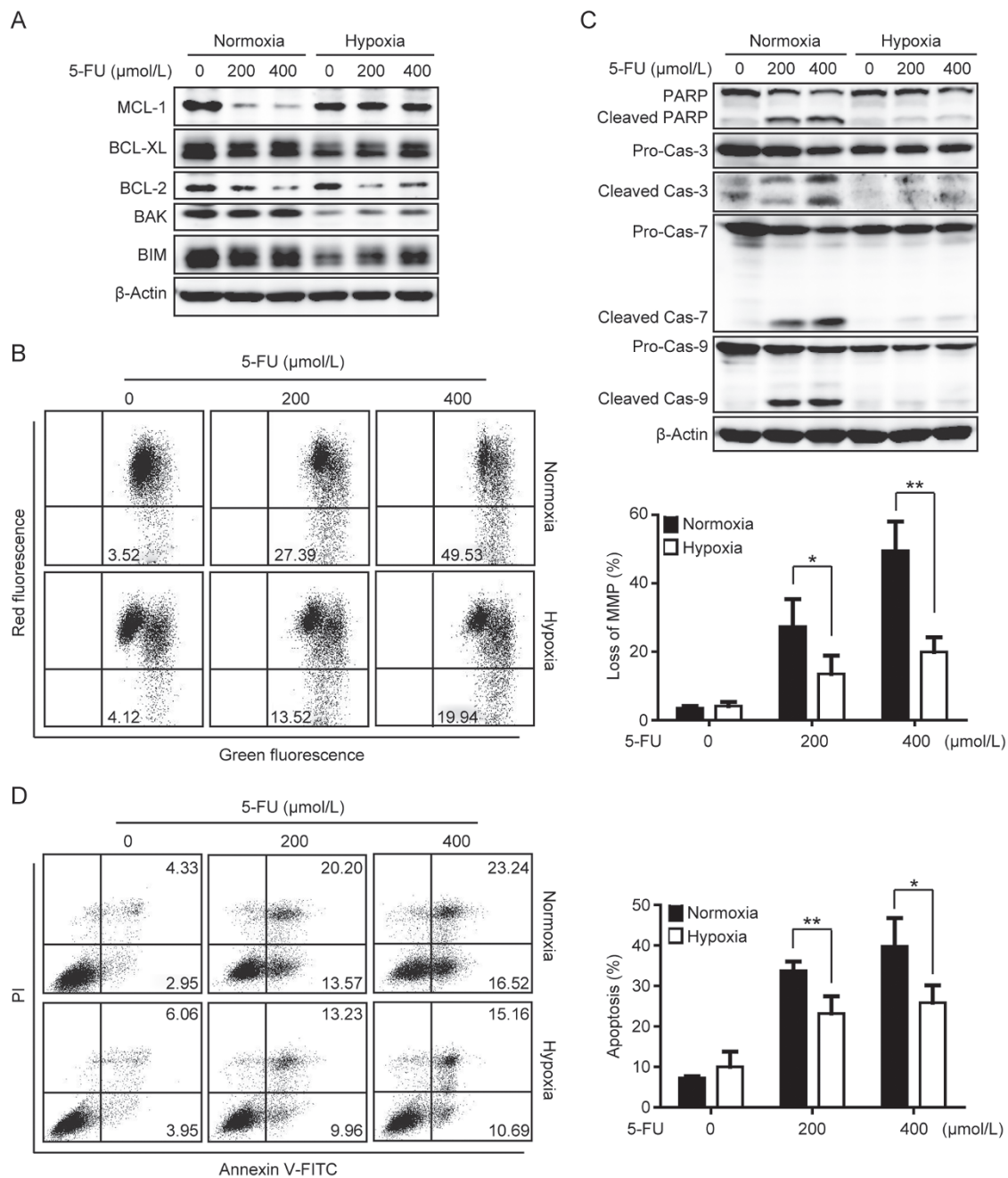

Figure 6. Hypoxia inhibits 5-FU-induced apoptosis. BEL-7402 cells were treated with 5-FU for 48 h under normoxic or hypoxic conditions. (A) The cells were collected for Western blotting analyses of MCL-1 and BCL-2 family proteins. (B) Loss of mitochondrial membrane potential (MMP) was analyzed by flow cytometry. The data from three independent experiments are shown as the mean±SD. (C) The cells were collected for Western blotting analyses of caspases and PARP levels. (D) Apoptosis was analyzed by flow cytometry. The data from three independent experiments are shown as the mean±SD. *P<0.05; **P<0.01.
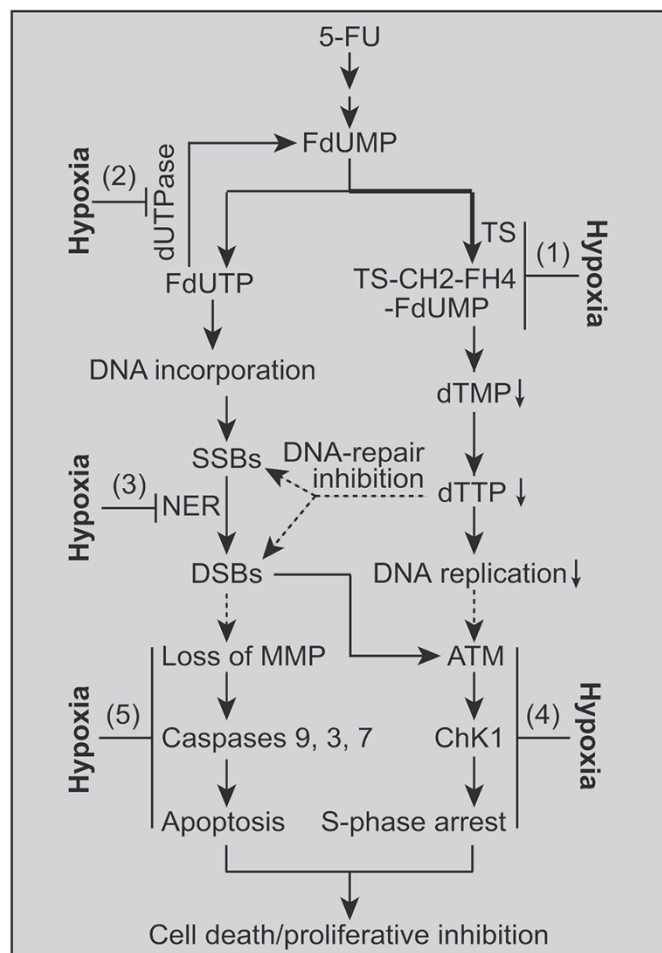
Figure 7. Schematic representation of the possible impacts of hypoxia on the primary anticancer mechanisms of 5-FU.

the level of FdUTP and its subsequent misincorporation into DNA, and on the other hand, hypoxia suppressed the activation of PARP-1 and aggravated the 5-FU-induced depletion of dTTP, which would inhibit DNA repair[31]. Both of these conditions are likely to increase the levels of SSBs and DSBs in the 5-FU-treated cells under hypoxia (1% O_2). However, contradictory results suggest relatively complicated mechanisms by which hypoxia (1% O_2) affected 5-FU-driven SSBs and DSBs. One possible reason could be that hypoxia results in more changes than observed here. For example, hypoxia might disturb the energetic metabolism and thus reduce the cellular levels of ATP in these HCC cells, although exposure to 1% O_2 has been found to preserve ATP levels in other cell lines[49]. Reduction in ATP would inhibit the ATP-dependent catalytic process of kinases, including those that promote the conversion of (d)NMP to (d)NDP and to (d)NTP. Thus, hypoxia might lead to the reduction of FdUTP and its misincorporation into DNA of HCC cells. (c) Suppressing checkpoint signaling and S-phase arrest. Hypoxia lowered the levels of ChK1 and abated the number of cells in S phase on the one hand and suppressed the checkpoint activation and S-phase arrest induced by the treatment with 5-FU on the other hand. These

effects of hypoxia could alleviate the proliferative inhibition of 5-FU. (d) Abating apoptotic cell death. In addition to diminishing DSBs in response to 5-FU, hypoxia reduced the protein levels of the pro-apoptotic factors BIM and BAK and alleviated the 5-FU-triggered reduction of the anti-apoptotic factors MCL-1 and BCL-XL. All these led to reduced activation of the apoptotic cascade signaling and thus decreased the apoptosis induced by 5-FU.

Therefore, hypoxia induces the drug resistance of HCC cells to 5-FU by widely interfering with its anticancer mechanisms. Similar effects might also contribute to hypoxia-induced resistance of HCC cells to other antimetabolites such as gemcitabine and 6-thioguanine because hypoxia also mitigated DNA damage induced by these antimetabolites. Notably, at the tested conditions, hypoxia-induced resistance of the HCC cells to 5-FU seems to be independent of HIF-1α and drug transporters, both of which are factors that are known to be involved in drug resistance. Together with the varying degrees of hypoxia-induced resistance of the HCC cells to 6 drugs, our results further suggest that the sensitivity of the mechanisms of action of the given drugs to hypoxia might be a predominant factor in determining the degree of hypoxia-induced resistance to these drugs. In particular, sorafenib is a multi-targeted kinase inhibitor that inhibits several tyrosine kinases [such as platelet-derived growth factor receptor-β (PDGFR-β), fibroblast growth factor receptor 1 (FGFR1), stem cell factor receptor (c-KIT), FMS-related tyrosine kinase 3 receptor (FLT3) and RET receptor] and serine/threonine kinases (such as wild-type BRAF, mutant BRAF[V600E] and CRAF)[3]. This property of multi-targeting different growth signaling pathways might help to reduce the influence of hypoxia on the anticancer activity of sorafenib. By contrast, 5-FU inhibits the sole molecular target, *ie*, TS, which leads to hypoxia-susceptible effects as mentioned above. Therefore, its anticancer activity could be decreased by hypoxia more easily. Certainly, this conclusion needs to be supported by more evidence; however, if it is eventually confirmed, it could become a critical factor for consideration in selecting and/or developing anticancer drugs for HCC therapy.

Altogether, our results demonstrate universal but differential hypoxia-induced resistance of HCC cells to 6 common anticancer drugs used in the clinic. We also reveal the extensive impact of hypoxia on the primary anticancer mechanisms of 5-FU, which contributes to resistance to 5-FU in the hypoxic HCC cells. These results suggest that hypoxia-induced drug resistance and interference of hypoxia with anticancer mechanisms could be exploited in selecting and/or developing anticancer drugs for improving HCC therapy.

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Author contribution

Jing-qiu LI, Xiang-liang YANG and Ze-hong MIAO conceived and designed this study; Jing-qiu LI and Xian WU conducted the experiments; Jing-qiu LI and Lu GAN analyzed and interpreted the data; Jing-qiu LI, Xiang-liang YANG and Ze-hong MIAO wrote and revised the manuscript.

Supplementary information

Supplementary information is available on the website of Acta Pharmacologica Sinica.

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