Effects of obatoclax combined with gemcitabine on the biological activity of pancreatic cancer cells under hypoxic conditions

XIN-FANG HOU, SHUAI LI, CHEN WU, KE LI, SHU-NING XU and JU-FENG WANG

Department of Medical Oncology, Affiliated Cancer Hospital of Zhengzhou University, Henan Cancer Hospital, Zhengzhou, Henan 450008, P.R. China

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Abstract. The present study aimed to investigate the effects of obatoclax (OBX) combined with gemcitabine (GEM) treatment on the proliferation, migration, invasion and epithelial-mesenchymal transition (EMT) related proteins of pancreatic cancer cell line BxPC-3 under hypoxic conditions. Protein expression levels of hypoxia-inducible factor 1α (HIF-1α) in BxPC-3 pancreatic cancer cells under normoxic and hypoxic conditions were detected by western blotting. Cells were divided into four groups: Normoxia group, hypoxia group, OBX group and OBX + GEM group. The proliferation activity of BxPC-3 cells was detected by Cell Counting kit-8. The migratory and invasive abilities of BxPC-3 cells were detected by the scratch test and Matrigel assay, respectively. The protein expression levels of vimentin, E-cadherin and p53 in BxPC-3 cells were also detected by western blotting. HIF-1α expression under hypoxic conditions was significantly increased compared with expression under normoxic conditions. Under hypoxic conditions, OBX treatment reduced cell activity, decreased cell migration and invasion, promoted the expression of E-cadherin and p53. In the OBX + GEM group, BxPC-3 cell activity decreased significantly, cell migration and invasion decreased significantly, the expression of vimentin was significantly reduced and the expression of E-cadherin and p53 further increased. In conclusion, the present results demonstrated that under hypoxic conditions, OBX combined with a small dose of GEM may be able to inhibit the growth, migration and invasion of pancreatic cancer cells, possibly via inhibition of EMT process. These results may provide a promising strategy for pancreatic cancer therapy.

Correspondence to: Dr Ju-Feng Wang, Department of Medical Oncology, Affiliated Cancer Hospital of Zhengzhou University, Henan Cancer Hospital, 127 Dongming Road, Jinshui, Zhengzhou, Henan 450008, P.R. China
E-mail: wangjf_0241@163.com

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Introduction

Pancreatic cancer is a malignant tumor that exhibits a high degree of malignancy, a low cure rate and very poor prognosis. In western countries, pancreatic cancer is the fourth leading cause of cancer-associated mortalities (1). Pancreatic cancer incidence ranked the eighth and the mortality ranked the sixth in China in 2013 (2). Although the technologies used for diagnosis and treatment of pancreatic cancer have been greatly improved, the majority of patients are diagnosed at the later stages, owing to a lack of specific clinical manifestations of pancreatic cancer and therefore the five-year survival rate is currently <5% (3-5). Gemcitabine (GEM) is a standard first-line treatment for advanced pancreatic cancer. However, its strong side effects and the drug resistance of tumor cells affect the efficacy of treatment and the quality of life of patients (6).

During the process of malignant tumor development, tumor cells are often in a state in which there is a lack of oxygen and nutrients owing to excessive growth (7). In solid tumors, hypoxia is a common characteristic that may be powerful driving force that promotes tumor progression and is one of the main reasons for failure of treatment (8,9). Hypoxia may induce alterations in the biological characteristics and microenvironment of tumor cells, which may lead to faster tumor growth and stronger invasive abilities of the tumoral cells (10). Pancreatic cancer is hypoxic and hypoxia impacts on the ability of pancreatic cancer cells to invade and metastasize (11). The expression level of HIF-1α in pancreatic cancer is linked to tumor progression, angiogenesis, invasion, and metastasis (12,13).

Epithelial mesenchymal transition (EMT) serves a key role in tumor invasion and metastasis. EMT can make tumor cells lose their epithelial cell-like polarity characteristics, and obtain interstitial cell characteristics and accordingly increase the metastatic and invasive potential of tumor cells. E-cadherin is an important intercellular adhesion molecule, mainly expressed on the surface of cell membranes and serves an important role in maintaining intercellular adhesion. As the decrease in the intercellular adhesion can increase the migration ability of cells and promote tumor cells to invade surrounding tissues, a decrease in E-cadherin is considered to serve an important role in tumor metastasis (14). As a major member of the intermediate filament protein family, vimentin is expressed in almost all normal interstitial cells, and serves

an important role in maintaining cell integrity and resisting external emergency injury (15). A decrease in the expression of E-cadherin and an abnormal increase in the expression of vimentin are considered as major molecular markers for the initiation of the EMT program (16). In previous years, certain scholars have proposed that, p53 is also involved in the regulation of the EMT process, loss of p53 function can occur in tumor cells with a mutation in the p53 gene, which facilitates the occurrence of EMT (17).

Through pre-clinical trials, it has been confirmed that obatoclax (OBX) may be used to inhibit tumor cells and the growth of transplanted tumors (12). Therefore, a better therapeutic effect for treatment may be obtained with therapeutic strategies against cellular characteristics under hypoxic conditions. In the present study, pancreatic cancer BxPC-3 was used as a study subject, to investigate the effect of OBX combined with gemcitabine on the proliferation, migration and invasion of BxPC-3 cells under hypoxia, and the effects on EMT-related molecular markers including E-cadherin, vimentin and p53, in order to provide a theoretical basis for improving the curative effect of GEM in the treatment of pancreatic cancer.

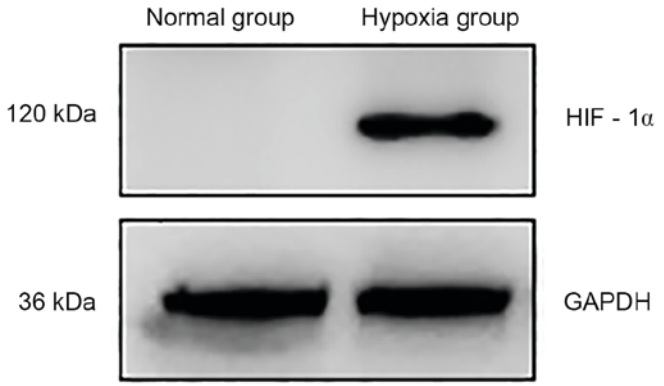

Figure 1. Expression of HIF-1α protein. Western blot analysis was used to detect the HIF-1α protein expression levels in BxPC-3 pancreatic cancer cells under normoxic and hypoxic conditions, respectively. GAPDH served as an internal control. HIF, hypoxia inducible factor.

Materials and methods

Main reagents. RPMI-1640 culture medium was purchased from HyClone (GE Healthcare Life Sciences, Logan, UT, USA). Fetal bovine serum (FBS) was purchased from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). GEM and OBX were obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Penicillin, streptomycin and Cell Counting kit-8 (CCK-8) were obtained from Beyotime Institute of Biotechnology (Haimen, China). The hypoxia-inducible factor (HIF)-1α antibody was obtained from BD Biosciences (Franklin Lakes, NJ, USA; cat. no. 610958), GAPDH antibody was obtained from Sigma-Aldrich (Merck KGaA; cat. no. G9545), and antibodies against vimentin (cat. no. sc-6260), E-cadherin (cat. no. sc-71009) and p53 (cat. no. sc-126) were purchased from Santa Cruz Biotechnology, Inc., (Dallas, TX, USA).

Cell culture. The human pancreatic cancer cell line BxPC-3 was purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI-1640 medium supplemented with 10% FBS, 100 U/l penicillin and 100 μg/ml streptomycin. Cells were cultured in an incubator with 5% CO_2 at 37˚C, and passaged every three days. Cells in the logarithmic growth phase were used in subsequent experiments.

Cell groupings. The concentrations of OBX (18) and GEM (19) were determined according to these studies. The cells were first cultured at 37˚C under normal oxygen conditions until they covered 50% of the bottom, then, divided into four groups under different conditions of treatment. Cells were separated into the following groups: i) Normoxia group, the cells continued to cultured at 37˚C under normal oxygen condition; ii) hypoxia group, the cells were cultured under hypoxic conditions (induction conditions of 37˚C, 1% O_2, 5% CO_2, and 94% N_2); iii) OBX group, the cells were added with 1.25 μM of OBX, then cultured at 37˚C under hypoxia condition; and the fourth group; and iv) OBX + GEM group, the cells were added with 1.25 μM of OBX and 0.3 μM of GEM, then cultured at 37˚C under hypoxia conditions.

Proliferation in the four groups detected by CCK-8. The cells in the four groups were inoculated into 96-well plates at the dose of 1,000/well, continued to culture for 24 and 48 h at 37˚C, then the cells were harvested, respectively. CCK-8 solution (100 μl) was added into each well and incubated at 37˚C for 2 h; optical density was detected by a microplate reader at 450 nm.

Scratch test for cell migration. Cells at a density of $5x10^5$/well in 2 ml were inoculated in a 6-well plate and incubated for 12 h at 37˚C. A straight line was scratched onto the bottom of Petri dish using a syringe needle. Cells were incubated in serum-free medium for 48 h at 37˚C, and cell migration was observed under a microscope and images were captured.

Detection of cell invasive ability by Matrigel assay. The cells in each group were incubated in serum-free culture medium at 37˚C for 24 h. The surface of the upper chamber of the Transwell plate was coated with Matrigel diluted with serum-free culture medium (8:1), and the chamber was incubated at 37˚C for 24 h. The cells in each group ($5x10^4$ cells/100 μl) were inoculated with serum-free culture medium in the upper chamber of the Transwell chamber for 24 h. The cells in the upper chamber were wiped off with a cotton swab, whereas cells on the lower membrane were washed twice with PBS and fixed with 4% paraformaldehyde at 4˚C for 30 min. The membrane was stained with 0.1% crystal violet for 20 min, and washed with PBS twice. Subsequently, cells were observed under a microscope and images were captured. The number of cells that had migrated was quantified by counting them in five distinct randomly chosen fields using a light microscope. The number of invading cells was calculated.

Western blot assay. The western blotting method was used to detect the HIF-1α protein expression in the hypoxia group and normoxia group, and the vimentin, E-cadherin and p53 protein expression levels in each group. Following incubation, the culture medium was discarded, Cells were lysed

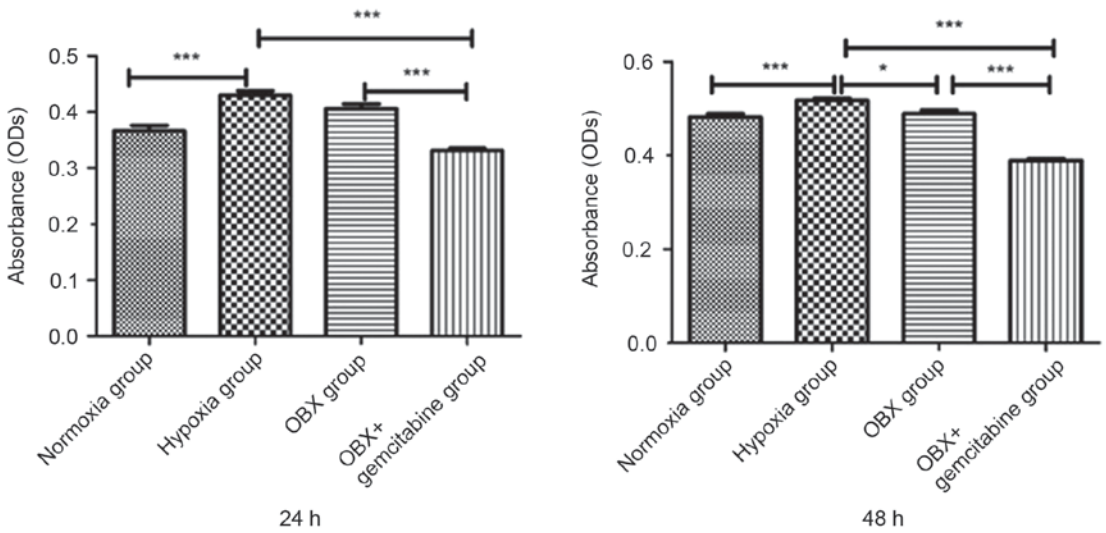

Figure 2. Effect of *OBX* and OBX+GEM combined treatments on the proliferation of BxPC-3 cells detected by a Cell Counting Kit-8 assay. A total of four group cells were cultured for 24 h or 48 h, respectively. Data was expressed as the mean ± standard deviation from triplicate experiments. Data was expressed as the mean ± standard deviation from triplicate experiments. *P<0.05, ***P<0.001. OBX, obatoclax; GEM, gemcitabine; OD, optical density.

on ice for 30 min in 500 μl RIPA lysis solution [150 mM NaCl, 1% Nonidet P-40, 1% deoxycholate, 0.1% SDS, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, (pH 8.0)] and the total protein was extracted supplemented with protease inhibitors (1 mM phenylmethylsulfonyl fluoride (PMSF), 0.2 mM sodium orthovanadate and 1 μg/ml aprotinin). Lysates were centrifuged at 12,000 x g for 20 min at 4˚C. The protein concentration was determined using the Bradford protein assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with bovine serum albumin (BSA; Wako Pure Chemical Industries, Ltd., Osaka, Japan) as the standard. Equal amounts of proteins (30 μg) were separated on 12% acrylamide gels (Bio-Rad Laboratories, Inc.) and transferred onto polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA) and the membranes were blocked with 5% skimmed milk at room temperature for 1 h, followed by incubation with antibodies against HIF-1α (1:500; cat. no. 610958; BD Biosciences, Franklin Lakes, NJ, USA), vimentin (1:500; cat. no. sc-6260), E-cadherin (1:500; cat. no. sc-71009), p53 (1:500; cat. no. sc-126; all Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and GAPDH (1:10,000; cat. no. G9545; Sigma-Aldrich, Merck KGaA) at 4˚C overnight. Membranes were subsequently incubated with horseradish peroxidase-conjugated goat anti-rabbit (1:5,000; cat. no. sc-2004) or goat anti-mouse (1:5,000; cat. no. sc-2005) secondary antibodies (all Santa Cruz Biotechnology, Inc.) at room temperature for 1.5 h. Odyssey film scanning was performed by the Odyssey Fc Imaging System (LI-COR Biosciences; Lincoln, NE, USA) and Image J Software, version 1.48 (National Institutes of Health, Bethesda, MD, USA) was used for densitometric analysis.

Statistical analysis. SPSS 13.0 software system (SPSS, Inc., Chicago, IL, USA) was used for statistical evaluation. Data are presented as the mean ± standard deviation of at least three independent experiments. Statistical significance was evaluated by analysis of variance followed by Tukey's post-hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Expression of HIF-1α under hypoxic conditions. Western blotting results revealed that HIF-1α protein expression in BxPC-3 cells grown under normoxic conditions was almost undetectable, whereas a notable increase in expression was observed in cells grown under hypoxic conditions (Fig. 1).

Effects of OBX and OBX + GEM treatments on cell proliferation under hypoxic conditions. Results of CCK-8 detection identified that, compared with normal oxygen group, the proliferation ability of BxPC-3 cells in the hypoxia group was significantly increased (P<0.001); at 24 h, the cell proliferation ability of OBX group was not altered compared with the hypoxia group, but at 48 h, cell proliferation decreased; in the OBX + GEM group, cell proliferation markedly decreased from 24 h compared with not only hypoxia group but also OBX group (Fig. 2).

Effects of OBX and OBX + GEM treatments on cell migration ability under hypoxic conditions. Scratch test results revealed that the healing rate of BxPC-3 cells in the hypoxia group was higher compared with the normoxic group, and the difference was statistically significant (P<0.05). Compared with the hypoxia group, the migratory ability of cells in the OBX group and OBX + GEM group decreased (P<0.001); and the migratory ability of cells in the OBX + GEM group decreased more significantly (P<0.05), which indicated that co-treatment with OBX and GEM may reduce markedly the ability of BxPC-3 cells to migrate under hypoxic conditions (Fig. 3).

Effects of OBX and OBX + GEM treatments on the invasiveness of cells under hypoxic conditions. Results from the Matrigel invasion assay revealed that the number of invading BxPC-3 cells significantly increased in the hypoxia group (P<0.001; Fig. 4). Compared with cells in the hypoxia group, the number of invading cells in the OBX group and

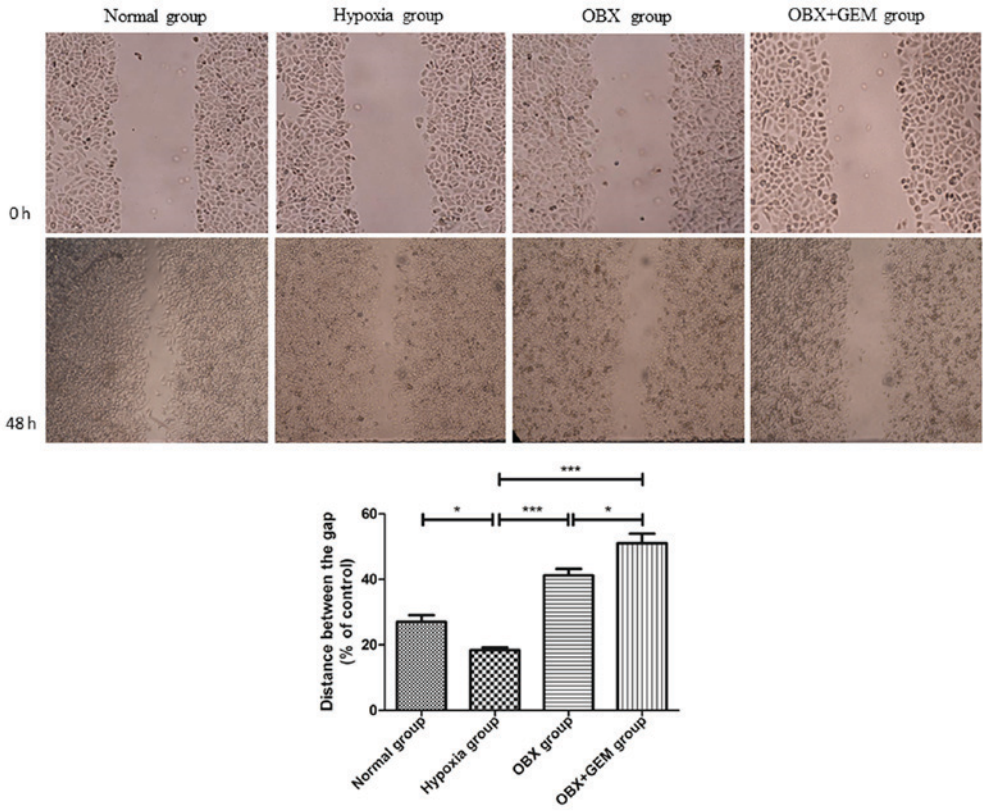

Figure 3. Effects of *OBX* and OBX+GEM combined treatments on migration ability of BxPC-3 cells detected by scratch experiment. The confluent monolayer was wounded with a sterile pipette tip, and the cells were allowed to migrate for 48 h. Data was expressed as the mean ± standard deviation from triplicate experiments. *P<0.05, ***P<0.001. OBX, obatoclax; GEM, gemcitabine.

in the OBX + GEM group decreased significantly (P<0.001); Futhermore, compared with the OBX group, the number of invasive cells in the OBX + GEM group decreased significantly (P<0.001), which indicated that co-treatment with OBX and GEM may reduce markedly the invasive ability of BxPC-3 cells under hypoxic conditions (Fig. 4).

Effects of OBX and OBX + GEM treatments on EMT related proteins expression under hypoxic conditions. Compared with BxPC-3 cells in the normoxia group, the protein expression level of vimentin in cells in hypoxia group was significantly increased (Fig. 5). However, the expression of E-cadherin was significantly decreased, and no significant changes were found in the protein expression of p53. Compared to the hypoxia group, the expression of E-cadherin and p53 was upregulated in the OBX group, and the expression of vimentin did not change. Compared with the hypoxia group and OBX group, the expression of E-cadherin and p53 was upregulated in the OBX + GEM group, and the expression of vimentin was significantly downregulated (P<0.01). These data indicated that when BxPC-3 cells were treated with OBX combined with GEM in hypoxic conditions, the EMT related proteins altered markedly.

Discussion

Pancreatic cancer is a fatal disease with poor prognosis; in China, as in other countries worldwide, the health burden of pancreatic cancer continues to grow (13). Furthermore, the annual mortality rate is almost equal to its incidence rate, and the annual incidence and mortality rates in China have exceeded those of the United States (13). GEM is a standard first-line treatment for advanced pancreatic cancer; however, whether treated with GEM monotherapy or combination therapy, the survival periods of patients is <9.8 months (20,21).

The characteristics of pancreatic cancer include reduced blood supply and the continuous exposure of cells to hypoxic conditions (11). A previous study determined the oxygen state of tumor cells in patients with pancreatic cancer during surgery, and demonstrated that all seven of the collected tumor tissues were in an hypoxic state, whereas the adjacent normal pancreatic tissues were in a normoxic state (22). The lack of oxygen is usually due to excessive growth in tumor volume. The inner tumor cells, particularly the tumor center, do not obtain enough blood supply, which results in insufficient oxygen supply to meet the growth needs of the tumor (22). In mouse models of pancreatic ductal adenocarcinoma (PDAC), measurement of hypoxia following systemic administration of pimonidazole (a chemical that becomes reduced in low oxygen environments and binds to thiol-containing molecules inside cells, forming adducts that can be detected by antibodies) revealed the presence of frequent intratumoral hypoxic areas (23). A similar result was observed in orthotopic implants of human PDAC samples into mice supporting the idea that intratumoral hypoxia is an important component of the PDAC microenvironment (24). Hypoxia may induce alterations in the biological characteristics of tumor cells, as well as changes in the tumor microenvironment,

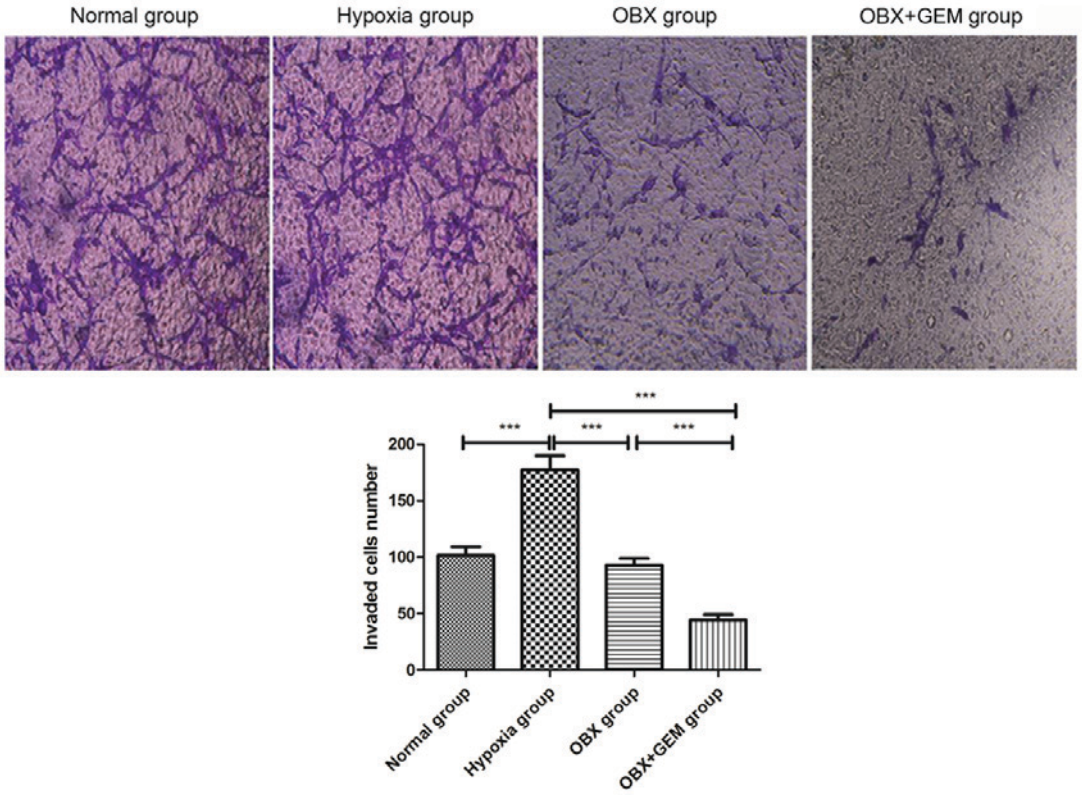

Figure 4. Effects of *OBX* and OBX+GEM combined treatments on invasive ability of BxPC-3 cells detected by Matrigel assay. The images exhibit the bottom side of the filter inserts with the stained cells that had migrated through the filter pores following 24 h. The invasion ability was determined by counting the stained cells on the bottom surface. A total of five random fields were captured at a magnification of x20 (n=5). Data was expressed as the mean ± standard deviation from quintuplicate determinations.. ***P<0.001. OBX, obatoclax; GEM, gemcitabine.

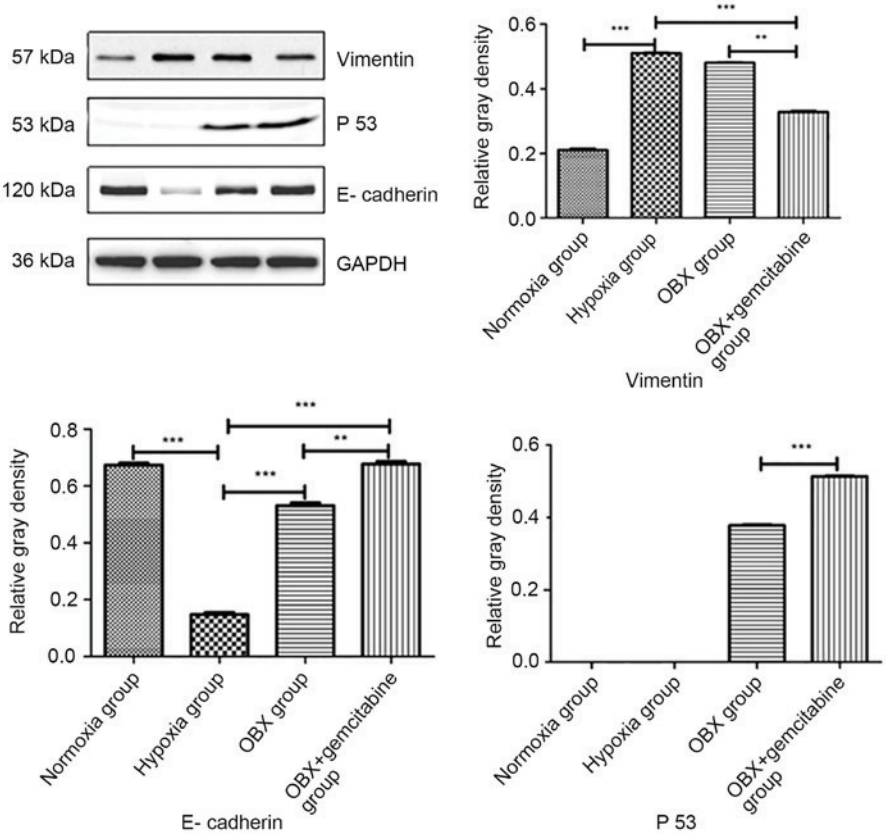

Figure 5. Effects of *OBX* and OBX+GEM combined treatments on EMT related proteins in BxPC-3 cells detected by western blot assay. Protein expression levels of vimentin, E-cadherin and p53 protein in each group of BxPC-3 cells were detected. Data was expressed as mean ± standard deviation from triplicate determinations. **P<0.01, ***P<0.001. OBX, obatoclax; GEM, gemcitabine; E, epithelial.

the occurrence of drug resistance in tumor cells. These alterations may lead to faster growth of tumor cells and the enhanced invasiveness of tumor cells (25-27). Therefore, a better therapeutic effect may be obtained using therapeutic strategies against cellular characteristics under hypoxic conditions. A potential reason for the failure of the classical therapeutic approach may be explained by pancreatic cancer's high metastatic potential (28). The occurrence of EMT can alter the composition of the extracellular matrix, facilitating tumor invasion and metastasis. EMT is one of the important mechanisms of cell invasion, migration and secondary metastasis. The molecular indicators for EMT are the decrease of epithelial markers, including E-cadherin, and the increase in the levels of mesenchymal markers, such as N-cadherin and vimentin (29). Presently, certain scholars consider p53 to also be involved in the occurrence of EMT (30).

Hypoxia may induce changes in the biological characteristics of tumor cells, as well as changes in the tumor microenvironment, and the occurrence of drug resistance in tumor cells. These changes may lead to faster growth of tumor cells and the enhanced invasiveness of tumor cells (15-17). Therefore, a better therapeutic effect may be obtained using therapeutic strategies against cellular characteristics under hypoxic conditions.

It was previously reported in preclinical experiments that OBX is a BH3 peptide analogue that may be used as to inhibit the growth of tumor cells and transplantation of tumors (9). Other studies have demonstrated that the BH3 analogue ABT-737 was able to exert its effect on tumor cells more efficiently under hypoxic conditions compared with cells treated in normoxic conditions (31,32). OBX was reported to reduce the expression level of HIF-1α under hypoxic conditions, thereby enhancing the sensitivity of colon cancer cells to 5-fluorouracil treatment (33). However, whether OBX combined with GEM was able to change the invasive phenotype of BxPC-3 pancreatic cancer cells under hypoxic conditions and improve the efficiency of GEM treatment remained unreported.

Results from the present study revealed that under hypoxic conditions, OBX combined with a small dose of GEM was able to promote the expression of E-cadherin and p53, reduce the expression of vimentin, decrease the migratory and invasive ability, thereby improving the efficiency of GEM.

In conclusion, the present experimental results revealed that under hypoxic conditions, OBX combined with a small dose of GEM may be able to inhibit the growth, migration and invasion of pancreatic cancer cells, possibly via inhibition of EMT process. However, identification of the specific mechanisms of action requires further study.

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