Inhibitory effects of epigallocatechin-3-gallate on cell proliferation and the expression of HIF-1α and P-gp in the human pancreatic carcinoma cell line PANC-1

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Abstract. The aim of this study was to verify the inhibitory effects of epigallocatechin-3-gallate (EGCG) on cell proliferation and the expression of hypoxia-inducible factor 1 (HIF-1α) and multidrug resistance protein 1 (MDR1/P-gp) in the human pancreatic carcinoma cell line PANC-1, thereby, reversing drug resistance of pancreatic carcinoma and improving its sensitivity to cancer chemotherapy. The human pancreatic carcinoma cell line PANC-1 was incubated under hypoxic conditions with different concentrations of epigallocatechin-3-gallate (EGCG) for indicated hours. The effects of EGCG on the mRNA or protein expression of HIF-1α and MDR1 were determined by RT-PCR or western blotting. Cellular proliferation and viability assays were measured using Cell Counting Kit-8. Western blotting revealed that EGCG inhibits the expression of the HIF-1α protein in a dose-dependent manner, while RT-PCR showed that it does not have any effects on HIF-1α mRNA. In addition, EGCG attenuated the mRNA and protein levels of P-gp in a dose-dependent manner, reaching a peak at the highest concentration. Furthermore, EGCG inhibited the proliferation of PANC-1 cells in a concentration- and time-dependent manner. The attenuation of HIF-1α and the consequently reduced P-gp could contribute to the inhibitory effects of EGCG on the proliferation of PANC-1 cells.

Introduction

MDR (multidrug resistance) is one of the most important reasons leading to the failure of chemotherapy for cancers. A common form of the multidrug resistance in human cancer results from expression of the MDR1 gene, which encodes a 170 kDa product called P-glycoprotein (P-gp) functioning as a plasma membrane energy-dependent multidrug efflux pump that reduces the intracellular accumulation of chemotherapeutic agents by transporting them across the plasma membrane (1,2). Hypoxia is a common phenomenon that exists in the microenvironment of a developing tumor due to decreased vascular supply and increased energy demand to maintain proliferation (3). Lots of events occur during the adaptation of a tumor to hypoxia through a group of hypoxia inducible factors (HIFs), of which HIF-1 is the most widely studied (4,5). Hypoxia-inducible factor 1 (HIF-1) is a heterodimeric protein that consists of a highly regulated HIF-1α subunit (120 kDa) and a constitutively expressed HIF-1β subunit (91-94 kDa). HIF-1α is generated at a high level and degraded in an oxygen-dependent manner in normoxia, while accumulates and dimerizes with HIF-1β to form HIF-1, then it translocates to the nucleus to activate the expression of many adaptive genes, which code for proteins that are involved in angiogenesis, glucose metabolism, cell proliferation/survival and invasion/metastasis (6). Many studies have shown a correlation between tumor hypoxia and chemotherapeutic resistance (7-9). Hypoxic cells exhibit increased expression of drug-resistance genes, such as MDR1 and P-glycoprotein (10,11). Recent evidence have demonstrated that MDR1 is one of the target genes of HIF-1 which binds to the hypoxia responsive element (HRE) of the MDR1 gene to activate the expression of P-gp (3,12). Therefore, HIF-1 has become a hot target for the development of anti-cancer drugs (3,13).

Epigallocatechin-3-gallate (EGCG) is an anti-oxidant polyphenol flavonoid isolated from green tea. It has demonstrated chemopreventive and chemotherapeutic benefits in cellular and animal models of cancer (14,15). Some studies have investigated the positive or negative effects of EGCG on HIF-1 in different cell lines (16-19) but similar impact of EGCG has not been demonstrated in human pancreatic carcinoma cell lines. In our study, we chose PANC-1 (20) to investigate the effects of EGCG. We preliminarily demonstrated that EGCG inhibits the proliferation of PANC-1 cells possibly by diminishing HIF-1α synthesis and consequently reducing the production of

P-gp. However, this deserves further investigation to develop a new therapeutic strategy for cancers of multidrug resistance.

Materials and methods

Reagents and cell line. The human pancreatic carcinoma cell line PANC-1 (20) was purchased from ATCC. Dulbecco's modified Eagle's medium (DMEM) with high glucose was purchased from Gibco, USA. Fetal bovine serum (FBS) was purchased from Sijiqing Inc., Hangzhou, China. EGCG was purchased from Zhejiang Yixin Pharmaceutical Co., Ltd., Zhejiang, China. The polyclonal antibody HIF-1α was purchased from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA. The monoclone antibody P-gp was purchased from Abcam Inc., UK. The monoclonal antibody β-actin was purchased from Santa Cruz Biotechnology. Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Laboratories, Kumamoto, Japan.

Cell culture and EGCG treatment. Human pancreatic carcinoma cell line PANC-1 was cultured in high glucose DMEM supplemented with penicillin (100 U/ml)/streptomycin (100 mg/ml) and 10% fetal bovine serum as previously described (20). Cells were cultured at 37°C in a humidified atmosphere under hypoxic (1% O_2) or normoxic (21% O_2) conditions with an atmospheric balance of 95% N2/5% CO_2. Cells cultured in hypoxic conditions were added with EGCG at the concentrations of 0, 20, 40 and 80 μg/ml, respectively, while with no EGCG under normoxic conditions. Total mRNA and protein were isolated from PANC-1 cells after continuously incubation for 24 h, and then subject the extracts to analysis by semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) or western blotting.

Cellular proliferation and viability assays. Single-cell suspensions were prepared by enzymatic dissociation with trypsin-EDTA. The cells were then allowed to pass through a 40 μm nylon net filter, 100 μl of which is plated in 96-well microplates at a density of 1x10^4 cells/ml. After culturing under normoxic conditons for 12 h EGCG was added at the final concentration of 10, 20l, 40, 60, 80, 100 and 160 μg/ml. Continue inbucating under hypoxic conditions for 12, 24 and 48 h, respectively. Then measured the cell proliferation and viability using the WST-8 assay with Cell Counting Kit-8 (Dojindo Molecular Technologies.)

WST-8 assay. The number of viable cells was estimated using the WST-8 assay, which provides effective and reproducible determination of the proliferative activity of PANC-1 (21). WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] is reduced by the mitochondrial enzyme NAD-dependent succinate dehydrogenase to form a colored formazan product, which is soluble in the culture medium. The amount of formazan dye generated by the activity of the dehydrogenases in cells is known to be directly proportional to the number of living cells. To measure the proliferative activity of PANC-1 in 96-well microplates, 10 μl of the Cell Counting Kit solution was added into each well, followed by incubation of the microplates at hypoxic conditons for 2 h. Absorbance was measured at 450 nm using a microplate reader (Molecular Devices) with a reference wavelength of 650 nm.

Western blot analysis. Western blot anaslysis was used to semi-quantify the level of HIF-1α and P-gp expression in PANC-1 cells of different groups. Cells were recovered from the cultures, washed thrice with PBS, resuspended in cell lysate containing 1 mmol/l Tris-HCl (pH 8.0), NaCl, NaN_3, SDS, 1.06 g/ml NP-40, dd_2H_2O, 1 μl 1.74 mg/ml PMSF and 1 μl 100 mg/l aprotinin, and then left at 4°C for 30 min. After centrifugation at 12,000 rpm at 4°C for 5 min, the precipitations were recovered as total protein. Protein (80 μg) was eletrophoresed under reducing conditions on a 10% and a 5% SDS/polyacrylamide gel in Tris-glycin buffer and transferred to nitrocellulose membrane. The nitrocellulose membrane was then blocked at 37°C for 2 h in blocking buffer (5% skim milk in 0.05, Tween-20-TBST) and probed with rabbit anti-HIF-1α polyclonal antibody (diluted 1:200 in TBST), and mouse anti-P-gp monoclonal antibody (diluted 1:20 in TBST). Following nitrocellulose membrane washing thrice with TBST, horseradish peroxidase-conjugated goat anti-rabbit antiserum and goat anti-mouse antiserum (Beijing Zhongshan Jinqiao Biotechnology Co., Ltd., Beijing, China) was used as a second antibody, diluted 1:2000 in TBST. After extensive washing with TBST, the complex was visualized by Enhanced Chemiluminescent Detection Kit according to the manufacturer's instructions. β-actin was used to semi-quantify the expression of HIF-1α and P-gp.

Reverse-transcription PCR. HIF-1α and MDR1 mRNA expression in PANC-1 cells were measured by semi-quantitative RT-PCR. Total mRNA was isolated from PANC-1 cells by TRIzol Reagent according to the manufacturer's instructions, resuspended in nuclease-free water, and the absorbance was read at A260 and A280. Briefly each RNA sample (2 μl, 1 μg/μl) was subjected to cDNA synthesis in 20 μl of reaction mixture containing 4 μl 5X RT reaction buffer, 1 μl dNTPs (10 mmol/l), 1 μl Oligo(dT), 1 μl M-MLV, 10 μl DEPC water. The reverse transcription was processed at 70°C for 5 min, 42°C for 1 h and 95°C for 10 min. PCR amplification of cDNA (2 μl) was performed in 50 μl of reaction mixture containing 5 μl 10X PCR buffer, 3 μl $MgCl_2$ (25 mmol/l), 1.25 μl Taq DNA polymerase (1 U/μl), 1 μl dNTPs (10 mmol/l), 2 μl Primer I and II and 33.75 μl ddH_2O. The reaction involved cDNA denaturation at 94°C for 3 min and then 30 cycles of denaturation at 94°C for 45 sec, annealing 58°C for 45 sec, and elongation at 72°C for 45 sec, followed by extension at 72°C for 10 min. Mixtures containing 5 μl of the resulting PCR fragments and 1 μl 6X loading buffer were electrophoresed on a 1.5% agarose gel in 1X TAE buffer at 110 V for 30 min at room temperature. The β-actin was used to evaluate the semi-quantification of HIF-1α and MDR1 mRNA expression. The nucleotide sequences of the primers were: 5'-GATGTAATGCTCCCCTCAC-3' (sense) and 5'-GCTGGAATACTGTAACTGTGC-3' (antisense) for HIF-1α; 5'-CAGGAGATAGGCTGGTTTGATGGT-3' (sense) and 5'-TTAGCTTCCAACCACGTGTAAATC-3' (antisense) for MDR1; 5'-GTTGCGTTACACCCTTTCTTG-3' (sense) and 5'-GACTGCTGTCACCTTCACCGT-3' (antisense) for β-actin. All the primers were sythesized by Shanghai Sangon
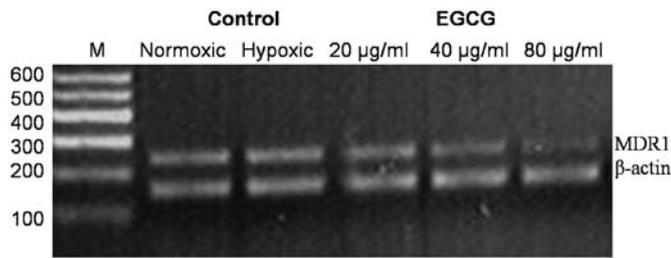
Figure 1. Effects of EGCG on the mRNA expression of MDR1. MDR1 mRNA expression of five groups were detected by semi-quantitative RT-PCR after 24 h normoxic or hypoxic incubation. Lane M, DNA marker; lane 1, normoxic controls; lane 2, hypoxic controls; and lanes 3-5, cells incubated with EGCG at the concentrations of 20, 40 and 80 µg/ml, respectively.
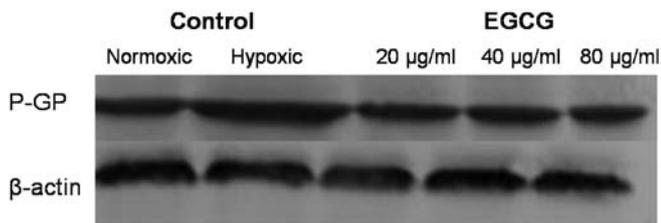

Figure 2. Effects of EGCG on protein expression of P-gp. P-gp protein expression of five groups were detected by western blot analysis after 24-h normoxic or hypoxic incubation. Lane 1, normoxic controls; lane 2, hypoxic controls; and lanes 3-5, cells incubated with EGCG at the concentrations of 20, 40, 80 µg/ml, respectively.

Figure 3. Effects of EGCG on mRNA and protein expression level of the HIF-1α. (A) Five groups of HIF-1α and mRNA expression level were detected by semi-quantitative RT-PCR after incubated in normoxic or hypoxic conditions for 24 h. Lane M, DNA marker; lane 1, normoxic control groups; lane 2, hypoxic control groups; lanes 3-5, experimental groups (the concentration of EGCG at lanes 3-5 were 20, 40 and 80 µg/ml). (B) Five groups of HIF-1α and protein expression were detected by western blotting after incubated in normoxic or hypoxic conditions for 24 h. Lane 1, normoxic control groups; lane 2, hypoxic control groups, lanes 3-5, experimental groups (the concentration of EGCG at lanes 3-5 were 20, 40 and 80 µg/ml).

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Statistical analysis. Statistical analysis was performed using one-way ANOVA or either paired or unpaired Student's t-test with $P<0.05$ for independent samples considered to be statistically significant. The data were evaluated statistically with SPSS 12.0 software.

Results

Effects of EGCG on the expression of MDR1 mRNA. RT-PCR was used to detect the expression of MDR1 in PANC-1 cells after treated with EGCG for 24 h. MDR1 mRNA was weakly expressed in cells exposed to to normoxic conditions, but the synthesis of the mRNAs was significantly increased in hypoxic controls (Fig. 1). To examine the effect of EGCG on the hypoxic induction of the mRNAs, the cells were treated with EGCG with increasing concentration of 20, 40 and 80 µg/ml for 24 h under hypoxia. We found that EGCG reduced the mRNA levels of MDR1 in a dose-dependent manner under hypoxic conditions (Fig. 1). Following the increasing concentrations of EGCG, the expression level of MDR1 mRNA was gradually inhibited. The expression of MDR1 reached the lowest level when the EGCG concentration was at the highest dose.

Effects of EGCG on expression of P-gp protein. P-gp protein was significantly more highly expressed in cells incubated in hypoxic conditions than in the normoxic controls (Fig. 2). It revealed that hypoxia condition can induce the expression of
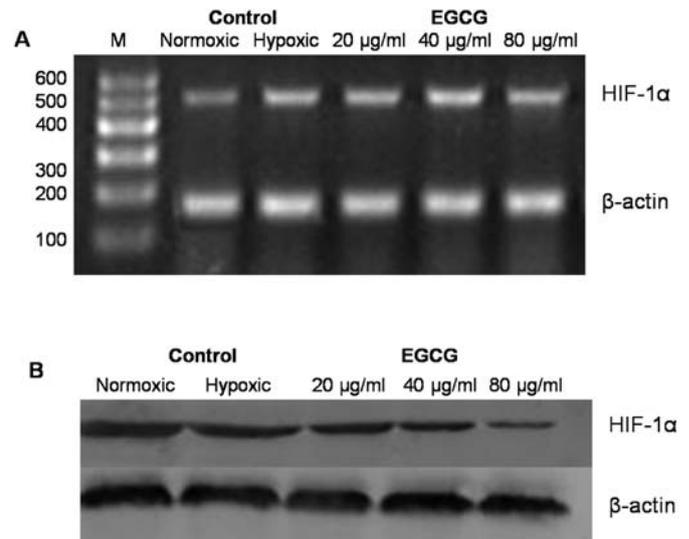
P-gp protein. EGCG reduced the expression of P-gp protein. Following the increasing doses of EGCG, the level of P-gp protein expression was gradually inhibited. P-gp protein showed a dose-dependent reduction in PANC-1 cells incubated in hypoxia in the presence of EGCG, strongly expressed in hypoxic controls, but cells incubated in normoxic conditions only slightly.

Effects of EGCG on mRNA and protein expression level of the HIF-1α. The expression of HIF-1α was weakly expressed in cells incubated in normoxic conditions on the mRNA and protein level, but strongly expressed in hypoxic controls (Fig. 3). We also observed that EGCG reduced the protein levels of HIF-1α in a dose-dependent manner under hypoxic conditions, whereas there was no effect on the HIF-1α mRNA level. Following the increasing concentrations of EGCG, the expression level of HIF-1α protein was gradually inhibited, but the level of HIF-1α mRNA was nearly unchanged in the presence of different concentration of EGCG in hypoxic PANC-1 cells.

Effects of EGCG on proliferation of the PANC-1 cells. Each concentration of the EGCG inhibited the proliferation of PANC-1 cells and the inhibition effect was concentration-dependent (Fig. 4). After incubation in hypoxic conditions for 48 h, the inhibition ratio of the PANC-1 cells were (7.7±3.11%), (10.67±2.22%), (56.74±1.97%), (79.06±1.89%), (85.47±2.94%), (91.03±2.32%), respectively, in cells treated with EGCG at 10, 20, 40, 60, 80, 100 and 160 µg/ml. Following the increasing doses of the EGCG (10-160 µg/ml),
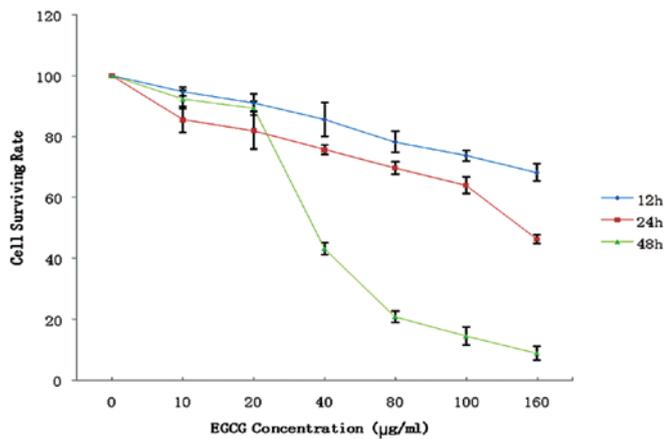
Figure 4. Effects of EGCG on the cell viability of PANC-1. Cells were incubated in hypoxic conditions for 12, 24 and 48 h, respectively. The inhibitory rates in different groups were indirectly reflected by viable cell absorbance rates at 450 nm using a microplate reader (Molecular Devices) with a reference wavelength of 650 nm. EGCG was added 5 min prior to hypoxic incubation.

the proliferation rates of PANC-1 cells was gradually inhibited. By increasing the time period (12 h/24 h/48 h) the inhibition rates of PANC-1 cells were gradually increased.

Discussion

Human pancreatic cancer is an aggressive and devastating disease with poor prognosis, which is frequently diagnosed at advanced stage and generally resistant to chemotherapeutic agents (22,23). There are many reasons for the poor effects of chemotherapy in pancreatic cancer, including the local pharmacokinetics, blood-pancreatic barrier, as well as the natural property of pancreatic cancer to resist chemotherapeutic drugs, which is commonly thought as the main reason. Currently, the most active agent appears to be the DNA chain terminator gemcitabine (2',2'-diflurodeoxycytidine). However, even with this drug, the objective response rate in human pancreatic cancer is less than 20% in clinic (24). This broad spectrum of drug resistance is inherent rather than acquired during the course of initially successful chemotherapy in pancreatic cancer (25).

MDR (multidrug resistance) is one of the most important reasons leading to the failure of chemotherapy for cancers. In many mechanisms of MDR, the most important and thoroughly studied are human multi-drug resistant gene 1 (MDR1) and the MDR-associated protein P-gp. P-gp, encoded by the MDR1 gene, and molecular weight of 170 kDa membrane glycoprotein, is one of ATP-binding cassette (ABC) transporter super-family members, with a specific drug binding and ATP binding sites. Its activity is associated with ATP, which can take advantage of the energy released from ATP hydrolysis to transfer a large number of drugs to the extracellular membrane, so that to reduce the intracellular drug concentration, resulting in drug resistance.

Many chemotherapy drugs not only kill tumor cells, but also cause a great deal of damage to normal cells, with strong side effects. Side effects of chemotherapy drugs and drug-resistance of tumor cells both are difficult to resolve

in cancer therapy. Research and development of new types of anti-cancer drugs that can specifically attack tumor cells, reverse drug-resistance of tumor cells is expected to be a good way to solve the problem. In this regard, the dietary botanicals have attracted considerable attention because of their intriguing biological activities at non-toxic levels. Therefore, natural materials for screening and separation to the active ingredients to cure tumors, and looking for agents to induce tumor cell apoptosis are the current hot topic in oncology. Tea as a natural plant and its antitumor effects of tumor prevention and treatment have been widely studied. It is rich in polyphenolic compounds, including flavanols, flavonoids and polyphenols, the majority of which are flavanols, commonly known as catechins. The main catechins in green tea are: epigallocatechin gallate (epirigallocatechin-3-gallate, EGCG), epigallocatechin (epicatechin-3-gallate, EGC), and epicatechin (epicatechin, EC). EGCG content is the highest, accounting for 80% of catechins. Numerous studies have shown that green tea extract and EGCG possess obvious antiproliferative (26,27), antiangiogenic (28-31), antimetastatic (27,32,33), proapoptotic (34,35), and cell cycle perturbation (36) activities *in vitro* and *in vivo* tumor models. These data together with several epidemiologic studies have suggested that green tea extracts and EGCG harbor strong anticancer and cancer preventive effects in numerous human cancers, including skin cancer, lung cancer, oral cavity, stomach, small intestine, colon, liver, pancreas cancer, colorectal cancer, prostate cancer (37-41). Ullmann *et al* (42) studied different doses (50-1,600 mg) of EGCG in healthy volunteers, to check for safety, tolerance and pharmacokinetics. The results showed that, even at EGCG dose of 1,600 mg, the volunteers tolerated it well.

Hypoxia is a common phenomenon that exists in the microenvironment of a developing tumor due to decreased vascular supply and increased energy demand to maintain proliferation. The expression of hypoxia-responsive genes is regulated primarily by the transcription factor hypoxia-inducible factor 1 (HIF-1). Hypoxia-inducible factor 1 (HIF-1) is a heterodimeric transcription factor containing an inducibly expressed HIF-1α subunit and a constititutively expressed HIF-1β subunit. HIF-1α appears to play a general role in the transcriptional regulation of all cells in response to hypoxia, whereas HIF-1β plays a more limited or specialized role in hypoxia conditions. HIF-1 activity in tumors depends on the availability of the HIF-1α subunit, the levels of which increase under hypoxic conditions and through the activation of oncogenes and/or inactivation of tumor suppressor genes, which code for proteins that are involved in angiogenesis, glucose metabolism, cell proliferation/survival and invasion/metastasis. Overexpression of the HIF-1α subunit, resulting from intratumoral hypoxia and genetic alterations, has been demonstrated in common human cancers and is correlated with tumor drug-resistance and patient mortality, which led to the current interest in HIF-1α as a cancer drug target.

Hypoxia contributes to drug resistance in chemotherapy, including many complex mechanisms, mainly in the following areas: i) abnormal vascular structure and function in solid tumor due to hypoxia leads to tumor necrosis, bleeding, formation of thrombosis, and thus it is difficult for a drug to reach inside the tumor to achieve antitumor effect because of the slow blood flow. ii) hypoxia can inhibit tumor

cell proliferation. *In vitro* hypoxia treatment can specially inhibit S-phase cells to start DNA replication, showing that the cell proliferation rate is reduced with increasing distance from blood vessels, and most of the major role of antineoplastic agents are aimed at the rapid phase of cell splitting, leading to decreased sensitivity to anticancer drugs. iii) hypoxic tissue changes in pH value (pH value of hypoxic tissue is about 7.05, the normal tissue is about 7.3) (12), can affect the distribution of cells inside and outside the drugs. iv) hypoxia induces an increase in the expression of multidrug resistance gene (43). v) hypoxia is the initiator leading to tumor metastasis or malignant transformation. Hypoxia induces genetic instability of tumor cells, loss of apoptosis potential inducing a variety of tumor cells involved in the malignant transformation of the gene and protein expression, the occurrence of malignant transformation of tumor cells, tumor metastasis or even malignant transformation reducing the sensitivity of anticancer drugs. Comerford *et al* (44) have confirmed that epithelial tumor cells under the conditions of hypoxia can directly increase the expression of MDR1/P-gp from the level of transcription and translation, in a time-dependent manner. Further study has shown that there is a HIF-1 binding domain and auxiliary sequence, which is similar to hypoxia-responsive element (HRE) in the MDR1 gene promoter. MDR1 may be a hypoxia-responsive gene. It has also been demonstrated that HIF-1 and P-gp expression have an obvious correlation in human prostate cancer (45). Comerford *et al* (44) also found that hypoxia through the activation of MAPK signal transduction pathway of c-Jun-NH2-terminal kinase (JNK), result in high expression of HIF-1α, which induced increased expression of MDR1/P-gp. Therefore, some signaling pathway may exist in the course of hypoxic regulation of MDR1/P-gp expression.

Our study shows that under the conditions of hypoxia, the expression of MDR1/P-gp in the pancreatic PANC-1 cells was significantly higher than in normoxic conditions. This suggests that hypoxia can induce PANC-1 cell MDR1/P-gp expression, which may be through the HIF-1α pathway. EGCG can inhibit HIF-1α expression and regulation of its downstream gene expression, including MDR1. In the mRNA level, EGCG had no significant impact of the expression of HIF-1α; but in the protein level, EGCG significantly inhibited HIF-1α protein expression, and this inhibition also showed a dose-dependent manner, suggesting that EGCG inhibited HIF-1α protein expression through a post-transcriptional mechanism (by affecting HIF-1α protein synthesis and/or degradation). Different concentrations of EGCG can inhibit the MDR1 mRNA and P-gp protein expression, and these inhibitory effects are increased with higher doses of drugs. At the largest dose EGCG, MDR1 mRNA and P-gp protein expression was at the minimum level.

In conclusion, in this study we report, to our knowledge for the first time, that EGCG inhibited HIF-1α protein and MDR1/P-gp expression in PANC-1 cells may be via promoting HIF-1α protein degradation and/or interfering with protein translational path, thus providing a novel mechanism for the reversing effect of EGCG in drug-resistance. Thomas and Kim reported that treatment of prostate cancer cells with EGCG (20-40 *μ*g/ml, 43.6-87.3 *μ*mol/l) led to an increased HIF-1α protein level and

HIF-1-mediated gene transcription even under normoxic conditions (46). These findings together with our present data suggest that the effects of EGCG on HIF-1α expression and its downstream transcriptional activity are probably the specific tumor targets in pancreatic cancer. EGCG is expected to be a new pancreatic cancer drug to reverse natural drug resistance.

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