Pemetrexed exerts anticancer effects by inducing G_0/G_1-phase cell cycle arrest and activating the NOXA/Mcl-1 axis in human esophageal squamous cell carcinoma cells

XINYING LI¹, HONGXIA SONG², FENG KONG³, YANXIA GUO³, YUAN CHEN³, LU ZHANG³, DONGFANG GAO³, XIAOFEI ZHAO¹,³ and HAN ZHANG¹

¹Department of Ophthalmology, Second Hospital of Shandong University, Jinan, Shandong 250033; ²Department of Ozone Treatment, Jinan Infectious Disease Hospital, Jinan, Shandong 250021; ³Department of Central Research Laboratory, Second Hospital of Shandong University, Jinan, Shandong 250033, P.R. China

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Abstract. Esophageal squamous cell carcinoma (ESCC) is a dominant histological subtype of esophageal cancer with notably high incidence and mortality rates. Pemetrexed is a clinical antifolate therapeutic agent with anticancer properties. The present study aimed to understand whether pemetrexed is able to exert anticancer effects on ESCC cells, and to determine the underlying molecular mechanism. ESCC cells were treated with pemetrexed and cell survival was assessed with MTT assays. The cell cycle and apoptosis were evaluated using flow cytometry analysis, and proteins were detected using western blotting. It was demonstrated that pemetrexed inhibited cell survival and induced G_0/G_1 cell cycle arrest and apoptosis in human ESCC cells. Furthermore, the results demonstrated that the phorbol-12-myristate-13-acetate-induced protein 1/induced myeloid leukemia cell differentiation protein Mcl-1 axis is involved in intrinsic apoptosis induced by pemetrexed. The protein expression of endoplasmic reticulum stress markers inositol-requiring enzyme 1α, binding immunoglobulin protein and CCAAT-enhancer-binding protein homologous protein were upregulated following treatment with pemetrexed. These results suggest that pemetrexed may induce an endoplasmic reticulum stress response while activating intrinsic apoptosis. The present study provided important mechanistic insights into potential cancer treatments involving pemetrexed and enhanced the understanding of human ESCC.

Introduction

Esophageal cancer, which consists of two principal subtypes, adenocarcinoma and esophageal squamous cell carcinoma (ESCC), is the sixth leading cause of cancer-associated mortality worldwide (1). Compared with adenocarcinoma, ESCC is more common in East Asia, and ~50% of ESCC cases occur in China (2-4). Due to the rapid progression and high malignancy, the prognosis of patients with ESCC remains poor (5).

Chemotherapy is the most common treatment for cancer (1). Unfortunately, the majority of chemotherapeutic drugs have a limited effect on ESCC due to side effects and drug resistance (6). Thus, there is an urgent need to develop safe, efficacious agents with limited harmful effects for the treatment of ESCC.

Pemetrexed is a novel multi-targeted antifolate that targets a number of crucial enzymes involved in folate metabolism (7). Recent studies have reported that pemetrexed is also able to act as an antitumor drug, and its cytotoxicity has been demonstrated in advanced non-small cell lung cancer and malignant pleural mesothelioma (8,9). However, the effects of pemetrexed on human esophageal cancer and the possible mechanisms of such effects remain to be elucidated. Determining whether pemetrexed exhibits anticancer effects on ESCC cells and understanding the underlying molecular mechanism are important for developing better chemotherapeutics for ESCC.

Cell cycle arrest and apoptosis induced by cytotoxic agents are critical in cancer treatment (10,11). The intrinsic mitochondrial pathway is an important signaling cascade associated with apoptosis. Members of the apoptosis regulator Bcl-2 (Bcl-2) family serve crucial roles in the regulation of apoptotic processes in various cancer cells (12). Phorbol-12-myristate-13-acetate-induced protein 1 (NOXA), a crucial pro-apoptotic protein in the Bcl-2 family, has been reported to be involved in chemotherapeutic agent-induced apoptosis (13). NOXA is able to interact with the anti-apoptotic Bcl-2 protein induced myeloid leukemia cell differentiation protein Mcl-1 (Mcl-1), interfering with the polymerization of apoptosis regulator BAX and Bcl-2.
homologous antagonist/killer to trigger the mitochondrial apoptosis pathway (14). The combination of NOXA and Mcl-1 may also facilitate the proteasomal degradation of Mcl-1 and thereby strengthen intrinsic apoptosis (15).

Previous studies have demonstrated that the activation of the intrinsic mitochondrial apoptosis pathway is associated with persistent endoplasmic reticulum (ER) stress (16). Once inositol-requiring enzyme 1α (IRE1α) and other ER sensors are released from binding immunoglobulin protein (Bip), downstream effectors, including CCAAT-enhancer-binding protein homologous protein (CHOP) may trigger pro-apoptotic signals by targeting numerous apoptotic genes (17,18). However, whether the intrinsic mitochondrial apoptosis pathway and ER stress are active following treatment with pemetrexed in human ESCC cells remains unknown.

The present study investigated whether pemetrexed exerted anticancer effects on ESCC cells. The role of the cell cycle and the NOXA/Mcl-1 axis in the regulation of this effect was also studied. The present study revealed the therapeutic potential of pemetrexed for ESCC and enriched the understanding of this cancer type.

Materials and methods

Antibodies and reagents. Pemetrexed (cat no. CDS024404), was purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany), and was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich; Merck KGaA) at a concentration of 10 mmol/l (19). The stock solutions were stored at -20°C and diluted to the desired concentrations with growth medium prior to use. All the antibodies were previously described (13). Antibodies targeting caspase (casp8) (cat no. 9746; 1:1,000 dilution), casp9 (cat no. 9502; 1:1,000 dilution), poly(ADP-ribose) polymerase (PARP; cat no. 9542; 1:1,000 dilution), IRE1α (cat no. 3294; 1:2,000 dilution) and Bip (cat no. 3183; 1:1,000 dilution) were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). Antibodies targeting casp3 (cat no. NB100-56708; 1:1,000 dilution) and NOXA (cat no. OP180; 1:500 dilution) were purchased from Imgenex (Novus Biologicals, LLC, Littleton, CO, USA) and Calbiochem; Merck KGaA, respectively. Anti-actin antibody (cat no. A5441; 1:20,000 dilution) was obtained from Sigma-Aldrich; Merck KGaA. Antibodies against CHOP (cat no. sc-7351; 1:100 dilution) and Mcl-1 (cat no. sc-12756; 1:1,000 dilution) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA).

Cell lines and cell culture. The human ESCC cell lines Eca-109 and EC9706 were obtained from the American Type Culture Collection (Manassas, VA, USA) and were grown in monolayer cultures at 37°C in a humidified atmosphere consisting of 5% CO2 and 95% air. The cells were cultured with RPMI-1640 medium containing 5% fetal bovine serum (both Gibco; Thermo Fisher Scientific Inc., Waltham, MA, USA).

Cell treatment and MTT assay. A total of 7x10⁴ cells were seeded in 96-well microtiter plates and treated with 0, 0.625, 1.25, 2.5, 5 and 10 µM pemetrexed on the 2nd day. Following culturing with chemotherapeutics for 24, 36 or 48 h, cells were subjected to the MTT assay. Each sample was incubated with 20 µl (5 mg/ml) MTT (Sigma-Aldrich; Merck KGaA) at 37°C for 4 h. Following incubation, the solution was discarded and 100 µl DMSO was added. Cell viability was determined by measuring the absorbance at 495 nm using an ELISA Multiskan reader (Thermo Fisher Scientific, Inc.).

Cell cycle analysis. The cell cycle was evaluated through DNA flow cytometry analysis. Overall, 2x10⁶ cells were seeded in six-well plates and treated with different concentrations of pemetrexed (0, 2.5, 5 and 10 µM) for 24 h. Following treatment, cells were harvested and washed twice with ice-cold PBS and fixed in 70% ethanol at -20°C overnight. Prior to analysis, cells were washed with ice-cold PBS and incubated at 4°C with 5 µl propidium iodide (PI; 100 µg/ml) and 5 µl RNase (50 µg/ml; both Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) for 30 min in the dark. The samples were analyzed using a FACScan flow cytometer and the BD FACSuite™ flow cytometry software (version 1.0.5; both BD Biosciences, San Jose, CA, USA). Data analysis was performed using FlowJo software (version 7.2.2; Tree Star, Inc. San Carlos, CA, USA) (13).

Apoptosis analysis. Apoptosis was evaluated according to a previously described protocol (13). The Annexin V-fluorescein isothiocyanate (FITC)/PI apoptosis detection kit was purchased from Nanjing Biobox Biotech (Nanjing, China). Following treatment with different concentrations of pemetrexed (0, 2.5, 5 and 10 µM) for 36 h, 2x10⁶ cells were harvested, washed with pre-chilled PBS and resuspended in 500 µl binding buffer. A total of 5 µl each of Annexin V-FITC and PI were added to each sample, and the samples were incubated for 10 min in the dark at room temperature. Analysis was performed by flow cytometry with the aforementioned flow cytometer and software.

Western blot analysis. Whole-cell protein lysates were prepared and analyzed by western blotting according to a previously described protocol (13). Cells were treated with different concentrations of pemetrexed (0, 2.5, 5 and 10 µM) for 36 h, harvested and rinsed with pre-chilled PBS. Cell extracts were lysed and centrifuged at 12,000 x g for 15 min at 4°C, and the protein concentration was determined via the bicinchoninic acid assay. Whole-cell protein lysates (40 µg) were separated by SDS-PAGE on a 12% gel and transferred to Hybrid-enhanced chemiluminescence (ECL) membranes via electroblotting. Following blocking with 5% skimmed milk at room temperature for 1 h, the proteins were first probed with the appropriate primary antibodies at 4°C overnight followed by secondary goat anti-rabbit/mouse monoclonal antibodies (cat nos. SA00001-1 and SA00001-2; 1:8,000 dilution; ProteinTech Group, Inc., Chicago, IL, USA). Antibody binding was detected using an ECL system (EMD Millipore, Billerica, MA, USA), according to the manufacturer’s protocol. The expression levels of the proteins were quantified using ImageJ software (version 1.6.0_24; National Institute of Health, Bethesda, MD, USA).

Statistical analysis. SPSS statistical software (version 20.0; IBM Corp., Armonk, NY, USA) was used for all statistical analyses. The data obtained represent the mean ± standard error of the mean (SEM) based on three independent experiments.
deviation of at least three independent assays performed in duplicate or triplicate. A one-way analysis of variance followed by a Least Significant Difference post hoc test was performed for testing for all data. P<0.05 was considered to indicate a statistically significant difference.

Results

**Pemetrexed inhibits the survival of human ESCC cells.** The cytotoxicity of pemetrexed in ESCC cells was analyzed by treating Eca-109 and EC9706 cells with various concentrations of pemetrexed for various times and performing an MTT assay. The results demonstrated that pemetrexed may induce survival inhibition in human ESCC cells. When the exposure time was prolonged, the inhibitory effect was enhanced in Eca-109 and EC9706 cells (Fig. 1). These results suggested that pemetrexed may effectively suppress the survival of human ESCC cells.

**Pemetrexed induces G₀/G₁ cell cycle arrest in ESCC cells.** To determine the molecular mechanism by which pemetrexed induced survival inhibition in human ESCC cells, the ability of the agent to induce cell cycle arrest was analyzed by DNA flow cytometry analysis. The results demonstrated that exposure to pemetrexed may trigger G₀/G₁ cell cycle arrest in human ESCC cells.
Pemetrexed induces apoptosis in human ESCC cells. The present study also sought to determine whether apoptosis was involved in the inhibition of survival induced by pemetrexed. Therefore, an apoptosis assay was performed via Annexin V/PI staining. The resulting data revealed that pemetrexed induced apoptosis in a concentration-dependent manner in Eca-109 and EC9706 cells (Fig. 3). When treated with pemetrexed (0-10 μM), the frequency of apoptosis was increased from 4.317% to 32.43% in Eca-109 cells and 9.795% to 43.48% in EC9706 cells. Furthermore, the expression levels of apoptotic proteins were detected via western blot analysis. The data demonstrated that pemetrexed induced cleavage and activation of the apoptotic proteins casp8,
casp9, casp3 and PARP in a concentration-dependent manner in human ESCC cells (Figs. 4 and 5). These results indicated that exposure to pemetrexed may trigger apoptosis. In conclusion, pemetrexed triggers G\(_0\)/G\(_1\)-phase cell cycle arrest and apoptosis in human ESCC cells.

Involvement of the NOXA/Mcl-1 axis in pemetrexed-induced apoptosis. The NOXA/Mcl-1 axis has been reported to be involved in chemotherapeutically-induced apoptosis in numerous types of tumor cells (12,13). To characterize the molecular mechanism of pemetrexed-induced apoptosis in human ESCC cells, the expression levels of proteins following treatment with pemetrexed were analyzed. Western blot analysis revealed that the expression of NOXA was upregulated in a concentration-dependent manner following treatment with pemetrexed. By contrast, the expression levels of Mcl-1 decreased (Fig. 6A and B). These results indicated that the NOXA/Mcl-1 axis may be involved in pemetrexed-induced apoptosis in human ESCC cells. The expression level of Bcl-2, which is another member of the Bcl-2 family, was also detected and the western blotting results demonstrated that Bcl-2 was downregulated following treatment with pemetrexed (Fig. 6C and D). In summary, pemetrexed may induce mitochondrial apoptosis in human ESCC cells.

Pemetrexed triggers ER stress in human ESCC cells. The ER response has been reported to be activated by chemotherapeutics while inducing apoptosis in cancer cells (12,20). To determine whether pemetrexed triggers ER stress in human ESCC cells, a number of relevant proteins in the ER stress pathway were examined. The results illustrated that the marker proteins IRE1\(\alpha\), Bip and CHOP were upregulated in a concentration-dependent manner following treatment with pemetrexed (Fig. 7). These results indicated that pemetrexed may trigger ER stress in human ESCC cells.

Discussion

Esophageal cancer is the third leading cause of cancer-associated mortality in China (21). The incidence and mortality rates of esophageal cancer in China have increased in recent years (21,22). ESCC is the predominant histological subtype of esophageal cancer. Due to its high malignancy and the inadequate efficacy of conventional therapy, ESCC frequently has an unfavorable prognosis (23,24). Thus, the development of effective novel agents and treatments is of the utmost importance.

Pemetrexed is a clinically available multi-target antifolate cytotoxic agent (19). It is able to inhibit the synthesis of purine and pyrimidine by blocking dihydrofolate reductase, thymidylate synthase and glycinamide ribonucleotide formyltransferase (25). Recently, pemetrexed has been reported to exhibit antitumor effects in various solid tumors (19,25). The present study aimed to determine whether pemetrexed was able to exert anticancer effects in ESCC and to elucidate the underlying molecular mechanism.

In the present study, the cytotoxicity of pemetrexed against ESCC cells was analyzed, and it was reported that pemetrexed displayed a time-dependent inhibitory effect on the survival of ESCC cells. Subsequently, it was demonstrated that pemetrexed induced G\(_0\)/G\(_1\)-phase cell cycle arrest and apoptosis in a concentration-dependent manner in human ESCC cells. These results indicated that pemetrexed may exert anticancer effects by inducing G\(_0\)/G\(_1\)-phase cell cycle arrest and apoptosis in human ESCC cells.

Involvement of the NOXA/Mcl-1 axis in pemetrexed-induced apoptosis. The NOXA/Mcl-1 axis has been reported to be involved in chemotherapeutically-induced apoptosis in numerous types of tumor cells (12,13). To characterize the molecular mechanism of pemetrexed-induced apoptosis in human ESCC cells, the expression levels of proteins following treatment with pemetrexed were analyzed. Western blot analysis revealed that the expression of NOXA was upregulated in a concentration-dependent manner following treatment with pemetrexed. By contrast, the expression levels of Mcl-1 decreased (Fig. 6A and B). These results indicated that the NOXA/Mcl-1 axis may be involved in pemetrexed-induced apoptosis in human ESCC cells. The expression level of Bcl-2, which is another member of the Bcl-2 family, was also detected and the western blotting results demonstrated that Bcl-2 was downregulated following treatment with pemetrexed (Fig. 6C and D). In summary, pemetrexed may induce mitochondrial apoptosis in human ESCC cells.

Figure 5. Pemetrexed activates casp9 and PARP in human esophageal squamous cell carcinoma cells. (A) Eca-109 and (B) EC9706 cells were treated with 0, 2.5, 5 or 10 µM pemetrexed and incubated for 36 h. Following treatment, the protein levels of the cells were measured via western blot analysis. All data are presented as the mean ± standard deviation. *P<0.05, **P<0.01, ***P<0.001. Casp, caspase; PARP, poly(ADP-ribose) polymerase; NS, not significant.
Figure 6. Involvement of the NOXA/MCL-1 axis in pemetrexed-induced apoptosis. (A and C) Eca-109 and (B and D) EC9706 cells were treated with 0, 2.5, 5 or 10 µM pemetrexed and incubated for 36 h. Following treatment, NOXA and Mcl-1 expression was quantified via western blot analysis. All data are presented as the mean ± standard deviation. *P<0.05, **P<0.01, ***P<0.001. PEM, pemetrexed; NOXA, phorbol-12-myristate-13-acetate-induced protein 1; Mcl-1, induced myeloid leukemia cell differentiation protein Mcl-1; Bcl-2, apoptosis regulator Bcl-2; NS, not significant.

Figure 7. Pemetrexed triggers endoplasmic reticulum stress in human esophageal squamous cell carcinoma cells. (A) Eca-109 and (B) EC9706 cells were treated with 0, 2.5, 5 or 10 µM pemetrexed and incubated for 36 h. Following treatment, the protein expression of IRE1α, Bip and CHOP was quantified via western blot analysis. All data are presented as the mean ± standard deviation. *P<0.05, **P<0.01, ***P<0.001. PEM, pemetrexed; IRE1α, inositol-requiring enzyme 1α; CHOP, CCAAT-enhancer-binding protein homologous protein; Bip, binding immunoglobulin protein; NS, not significant.
human ESCC cells. It has also been demonstrated that pemetrexed combined with cisplatin displays an effective synergistic effect in a wide variety of solid tumors (12,26,27). However, how this combination may exert a beneficial synergistic effect in human ESCC cells, and the underlying molecular mechanism, requires further research.

The results of the present study revealed that necrosis was also increased following treatment with pemetrexed. Cell death occurs via necrosis in addition to apoptosis. Research has reported that cell apoptosis and necrosis may be led by ER stress and mitochondrial membrane permeability (28,29). In the present study, persistent ER stress and increasing mitochondrial membrane permeability may be involved in the induction of necrosis. The molecular mechanism and effect of necrosis in pemetrexed-induced survival inhibition requires further investigation.

To understand the underlying molecular mechanism by which pemetrexed induces apoptosis in human ESCC cells, the expression levels of apoptosis-associated proteins were analyzed. NOXA and Mcl-1 are key proteins in intrinsic mitochondrial apoptosis. It was reported that the expression levels of the pro-apoptotic protein NOXA was upregulated in a concentration-dependent manner following treatment with pemetrexed, while the expression levels of the anti-apoptotic protein Mcl-1 decreased. The results indicated that pemetrexed may induce intrinsic mitochondrial apoptosis via the NOXA/Mcl-1 axis in human ESCC cells. The expression levels of Bcl-2 were also measured and the western blotting results demonstrated that Bcl-2 was downregulated following treatment with pemetrexed. In summary, pemetrexed may induce mitochondrial apoptosis in human ESCC cells. In addition to intrinsic mitochondrial apoptosis, extrinsic death receptor apoptosis is another key apoptotic signaling pathway. However, further research is required to determine whether pemetrexed induces extrinsic death receptor apoptosis in human ESCC cells.

A number of chemotherapeutics activate an ER response while inducing apoptosis in cancer cells (12,20). To determine whether pemetrexed triggers ER stress in human ESCC cells, a number of relevant proteins in the ER stress pathway were investigated. The results demonstrated that the marker proteins IRE1α, Bip and CHOP were upregulated in a concentration-dependent manner following treatment with pemetrexed, which indicates that pemetrexed triggers ER stress in human ESCC cells. Cyclic AMP-dependent transcription factor ATF-3 (ATF3) is also a key protein marker of ER stress (30). ATF3 has been reported to act as a suppressor of CHOP in a number of cancer types, whereas it may promote the expression of CHOP in others (31,32). Previous studies have reported that the expression of ATF3 is downregulated in certain ESCCs, and this decreased expression is negatively correlated with a poor prognosis in vivo, and with cell growth and invasion in vitro (33,34). However, further research is required to determine whether ATF3 is involved in pemetrexed-induced ER stress in human ESCC cells.

In conclusion, the results of the present study demonstrated that pemetrexed was able to inhibit cell survival and induce G0/G1-phase cell cycle arrest and apoptosis in human ESCC cells. The NOXA/Mcl-1 axis may be involved in intrinsic apoptosis induced by pemetrexed. Furthermore, pemetrexed induced an ER stress response while activating apoptosis. The present study provided important mechanistic insights into pemetrexed as a potential cancer treatment and enhanced the current understanding of human ESCC.


