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Citation: Cell Death and Disease (2014) 5, e1316; doi:10.1038/cddis.2014.281
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Usp9x- and Noxa-mediated Mcl-1 downregulation contributes to pemetrexed-induced apoptosis in human non-small-cell lung cancer cells

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Pemetrexed, a folate antimetabolite, combined with cisplatin is used as a first-line therapy for malignant pleural mesothelioma (MPM) and locally advanced or metastatic non-small-cell lung cancer (NSCLC). Pemetrexed arrests cell cycle by inhibiting three enzymes in purine and pyrimidine synthesis that are necessary for DNA synthesis. Pemetrexed also promotes apoptosis in target cells, but little is known about its mechanism in cancer cells. We have previously shown that pemetrexed can result in endoplasmic reticulum (ER) stress, and it can lead to downstream apoptosis. In this study, we further elucidate this mechanism. Our data show that pemetrexed increases Noxa expression through activating transcription factor 4 (ATF4) and activating transcription factor 3 (ATF3) upregulation. Furthermore, pemetrexed induces apoptosis by activating the Noxa–Usp9x–Mcl-1 pathway. Inhibition of Noxa by small interfering RNA (siRNA) promotes Usp9x (ubiquitin-specific peptidase 9, X-linked) expression. Moreover, downregulation of the deubiquitinase Usp9x by pemetrexed results in downstream reduction of myeloid cell leukemia 1 (Mcl-1) expression. Mechanistically, Noxa upregulation likely reduces the availability of Usp9x to Mcl-1, thereby promoting its ubiquitination and degradation, leading to the apoptosis of neoplastic cells. Thus, our findings demonstrate that Noxa–Usp9x-Mcl–1 axis may contribute to pemetrexed-induced apoptosis in human lung cancer cells.
Cell Death and Disease (2014) **5,** e1316; doi:10.1038/cddis.2014.281; published online 3 July 2014

Lung cancer is the most prevalent cancer worldwide, and last year 1.6 million people died from this disease.[1] In the United States, more people die of lung cancer than the next three most common cancers (prostate, breast and colon) combined.[2] Non-small-cell lung cancer (NSCLC) accounts for 80% of all lung tumors. Cisplatin-based combination chemotherapy is currently the first-line therapy for NSCLC. In September 2008, the Federal Drug Administration granted approval for combining cisplatin with pemetrexed as a first-line treatment against locally advanced and metastatic NSCLC in the United States.[3]

Pemetrexed plays a critical role in cell cycle arrest and apoptosis.[4–9] It disrupts DNA synthesis by inhibiting thymidylate synthase (TS), dihydrofolate reductase (DHFR) and glycinamide ribonucleotide formyltransferase (GARFT) that are necessary for purine and pyrimidine synthesis.[10,11] Furthermore, pemetrexed induces apoptosis and has been shown to be associated with p53,[4,7,9] upregulation of death receptor 5 (DR5) and degradation of c-FLIP (cellular FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein).[12] The detailed mechanism of pemetrexed-mediated cancer cell apoptosis has not been fully elucidated, and it may offer insight into future directions to treat patients with NSCLC.

Cellular apoptosis typically occurs along two signaling pathways: the mitochondria-mediated intrinsic pathway and the death receptor-induced extrinsic pathway.[13] The mitochondria-mediated pathway is sensitive to various stress signals such as cytotoxic drugs. The Bcl-2 family of proteins plays a major role in this pathway.[14,15] Apoptotic signals in the intrinsic pathway lead to transcriptional changes or/and post-translational modifications that stimulate pro-apoptotic BH3-only proteins.[16] For instance, Noxa and Puma (p53 upregulated modulator of apoptosis) are the key members of pro-apoptotic BH3-only protein family.[16] BH3-only proteins selectively combine with specific anti-apoptotic Bcl-2 members and result in apoptosis. More specifically, Puma associates almost equally well with all the pro-survival proteins, whereas Noxa binds strongly with myeloid cell leukemia 1 (Mcl-1) and weakly with A1, but not with other pro-survival proteins.[16] In response to apoptosis stress, the pro-apoptotic protein Bak will be displaced from the Bak–Mcl-1 complex because of rising levels of Noxa that, in turn, leads to the rapid unraveling and degradation of Mcl-1.[17,18] Mcl-1 plays a pivotal role in protecting cells from apoptosis and is overexpressed in a variety of human cancers. Targeting Mcl-1 for degradation in these cancers using pharmacological approaches represents a potentially effective way to treat cancers.[19] Mcl-1 downregulation was shown to be critical in the initial step of mitochondrial events in UV-induced apoptosis.[20] The destruction of Mcl-1 is required for the

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Abbreviations: NSCLC, non-small-cell lung cancer; ATF4, activating transcription factor 4; ATF3, activating transcription factor 3; PARP, poly (ADP-ribose) polymerase; DR5, death receptor 5; ER, endoplasmic reticulum; c-FLIP, cellular FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein; Mcl-1, myeloid cell leukemia 1; Puma, p53 upregulated modulator of apoptosis; Usp9x, ubiquitin-specific peptidase 9, X-linked; siRNA, small interfering RNA

Received 31.12.13; revised 17.5.14; accepted 27.5.14; Edited by M Agostini

translocation of Bcl-x_L and Bax from the cytosol to the mitochondria, Bak and Bax oligomerization and cytochrome *c* release.[20] The E3 ligases SCF^{FBW7} and Huwe1 have been found to regulate the ubiquitination and degradation of Mcl-1.[21] Recently, deubiquitinase Usp9x (ubiquitin-specific peptidase 9, X-linked) has been shown to stabilize Mcl-1 by removing the lysine 48 (Lys48)-linked polyubiquitin chains.[22] However, a detailed interaction pattern for the apoptosis mediated by Noxa, Mcl-1 and Usp9x has not been elucidated.

In this study, we investigated the underlying mechanism of pemetrexed-induced apoptosis in NSCLC cell lines. We were able to describe an important pathway involving Noxa–Usp9x–Mcl-1 axis in pemetrexed-induced cancer cell death in NSCLC cells. Our data showed that pemetrexed increases Noxa expression through activating transcription factor 4 (ATF4) and activating transcription factor 3 (ATF3) upregulation and results in downregulation of Usp9x (a deubiquitinase) and reduction of Mcl-1 expression. Mechanistically, Noxa upregulation likely reduces the availability of Usp9x to Mcl-1, thereby promoting its ubiquitination and degradation, and leading to the apoptosis of neoplastic cells.

Results

Pemetrexed induces Noxa upregulation that contributes to apoptosis. We have previously shown that pemetrexed induces apoptosis in NSCLC cell lines.[12] To further elucidate this mechanism, H1792 and A549 cell lines were treated with increasing concentrations of pemetrexed for 48 h (Figure 1a). Noxa expression dramatically increased after pemetrexed exposure. In time-course experiments, pemetrexed induced Noxa by 12 h, and remained elevated for 48 h (Figure 1b). We then knocked down Noxa expression by small interfering RNA (siRNA) technique, and we measured the effect on several apoptotic proteins. Clearly, in control siRNA knockdown cells, pemetrexed induces caspase-9, caspase-3 and poly (ADP-ribose) polymerase (PARP) cleavage, indicating both A549 and H1792 showed apoptosis after treatment with 2.5 μmol/l pemetrexed (Figure 1c). Selective targeting of Noxa with siRNA abrogated Noxa expression despite pemetrexed exposure, and it markedly reduced caspase-9, caspase-3 and PARP cleavage products (Figure 1c). The proportion of cells undergoing apoptosis following pemetrexed treatment dropped significantly with Noxa siRNA transfection (Figures 1d and e). In summary, pemetrexed upregulates Noxa that contributes to apoptosis in NSCLC cells.

Pemetrexed downregulates Mcl-1 and leads to apoptosis in NSCLC cells. Previous studies have indicated that BH3-only protein Noxa binds the pro-survival Bcl-2 protein family member Mcl-1 with high affinity.[16] Thus, we examined whether pemetrexed-induced Noxa expression affected Mcl-1. First, H1792 and A549 cell lines were treated with pemetrexed, and Mcl-1 expression was noted to decrease at 2.5 μmol/l pemetrexed (Figure 2a). Mcl-1 levels began to decrease by 24 h and remained low for 48 h (Figure 2b). When we added Mcl-1 siRNA, the cleaved forms of caspase-9, caspase-3 and PARP were markedly elevated (Figure 2c), and the fraction of apoptotic cells was increased (Figures 2d and e). Together, our findings suggest that downregulation of Mcl-1 accelerates pemetrexed-induced apoptosis in NSCLC cells.

Noxa is regulated by ATF3 and ATF4 in pemetrexed-induced apoptosis. We have shown that pemetrexed can activate endoplasmic reticulum (ER) stress that leads to apoptosis.[12] In our model, low concentrations of pemetrexed (2.5 μmol/l) induced ATF4 and ATF3 expression in A549 and H1792 cell lines (Figure 3a). Time-course experiments showed that ATF4 and ATF3 were increased at 12 and 24 h, and increased protein expression was sustained for 48 h (Figure 3b). Experiments were repeated and co-cultured with *ATF4* and *ATF3* siRNA. The expression of cleaved caspase-9, caspase-3 and PARP was reduced despite pemetrexed exposure (Figures 3c and d). In addition, the fraction of apoptotic cells was reduced in A549 and H1792 cells in which *ATF4* or *ATF3* was knocked down using siRNA technique compared with the control siRNA knockdown cells (Figures 4a and b).

Previous reports have shown that some ER stress proteins play a key role in upregulating Noxa, and Noxa induces Mcl-1 degradation.[23] We have shown that pemetrexed results in ER stress in NSCLC cell lines.[12] Thus, in order to determine whether the ER stress response regulated Noxa, NSCLC cells were transfected with siRNAs for *ATF4* and/or *ATF3* and then treated with or without pemetrexed. Western blot analysis indicated that pemetrexed-induced expression of Noxa declined after *ATF3* or *ATF4* knockdown despite pemetrexed treatment (Figures 3c and d). Together, these results demonstrate that pemetrexed induces upregulation of ATF4 and ATF3 that, in turn, activates Noxa and consequently leads to apoptosis.

Noxa regulates Usp9x and Mcl-1 level in pemetrexed-induced apoptosis. In order to identify the role of Noxa in pemetrexed-induced apoptosis, we blocked Noxa expression in NSCLC cell lines treated with or without pemetrexed. As shown in Figure 5a, Mcl-1 expression was negatively correlated with the expression of Noxa. Both basal Mcl-1 and induced Mcl-1 levels were increased compared with control siRNA-transfected cells. Furthermore, Usp9x expression increased in *Noxa* knockdown cells, suggesting Noxa may regulate Mcl-1 expression by controlling the expression of Usp9x.

To evaluate the role of Usp9x in pemetrexed-induced apoptosis, we teased out Usp9x expression in A549 and H1792 cells following pemetrexed treatment. Usp9x levels decreased in both H1792 and A549 cells with pemetrexed (2.5 μmol/l) (Figure 5b). Usp9x levels were downregulated by 24 h and remained low at 48 h (Figure 5c). Usp9x siRNA transfection downregulated Mcl-1 levels and increased the activated levels of apoptosis-related proteins (caspase-9, caspase-3 and PARP) (Figure 6a). Following pemetrexed treatment, the proportion of apoptotic cells increased in *Usp9x* knockdown cells compared with control knockdown cells (Figures 6b and c). In conclusion, after pemetrexed treatment in NSCLC cells, Noxa downregulated Usp9x, resulting in a decrease in Mcl-1 and eventually leading to apoptosis. Together, these results indicate that pemetrexed activates the Noxa–Usp9x–Mcl-1 axis.

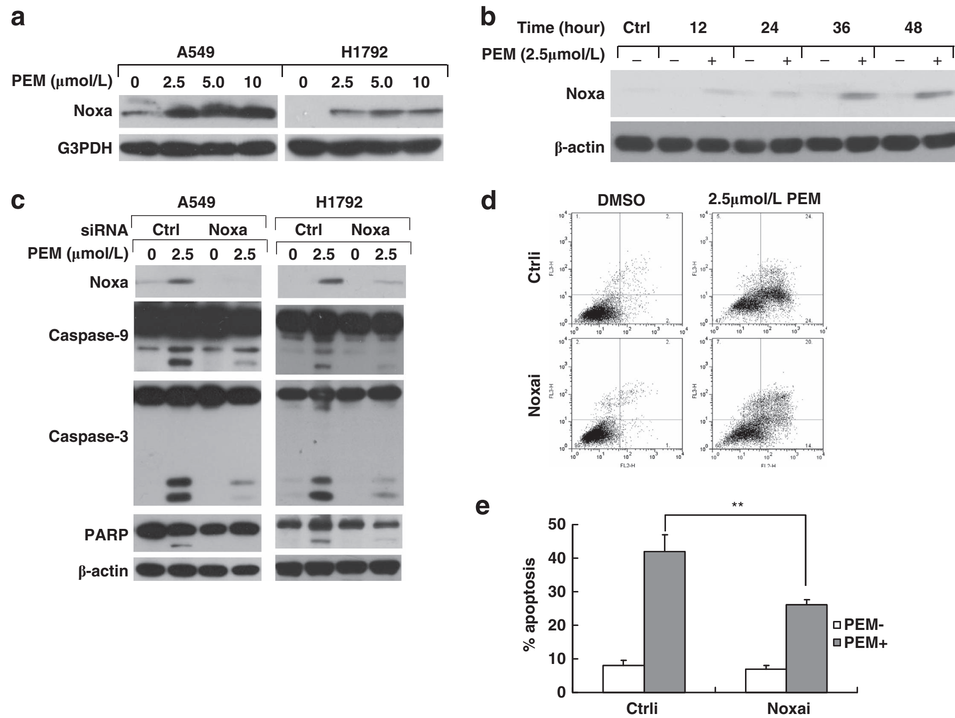

Figure 1 Pemetrexed induces Noxa expression and leads to apoptosis. A549 and H1792 cell lines were treated with pemetrexed at the indicated concentrations for 48 h (**a**). A549 cell lines were treated with 2.5 μmol/l pemetrexed for various time periods (**b**). A549 and H1792 cell lines were cultured in six-well plates. On day 2, cells were transfected with control (Ctrl) or Noxa siRNA. On day 3, a single well was divided between two wells. On day 4, 2.5 μmol/l pemetrexed was given to A549 and H1792 cell lines for 48 h (**c**). Cells were harvested and lysates of whole-cell protein were used for western blot analysis (**a**–**c**). A549 cell line was transfected with control and Noxa siRNA using the same protocol. Apoptosis was measured by Annexin V-PE/7-AAD staining and analyzed by flow cytometry (**d**). For the flow cytometry analysis, the % positive cells in the upper right (Annexin V + /7-AAD + : late apoptotic cells) and lower right quadrants (Annexin V + /7-AAD − : early apoptotic cells) were summed to give the total number of apoptotic cells. The experiment was performed in triplicate and repeated three times. The data are presented as mean ± S.D. of three replicates in one experiment (**$P<0.01$, Student's *t*-test) (**e**). Representative pictures from three independent experiments are shown

Pemetrexed regulates Mcl-1 ubiquitination via reducing the availability of Usp9x to Mcl-1. To determine whether pemetrexed affects Mcl-1 levels through ubiquitination and degradation, we transfected A549 cells with a plasmid pcDNA3.1-Myc-Mcl-1 containing *Myc–Mcl-1* fusion gene and a pcDNA3.1 as control. Myc–Mcl-1 protein was immunoprecipitated using anti-Myc antibody, and its ubiquitination levels were examined with an anti-HA antibody by western blot assay. The data showed that Mcl-1 ubiquitination levels were increased after pemetrexed treatment compared with controls. At the same time, we found more Noxa was precipitated with Mcl-1 following pemetrexed treatment (Figure 7a).

Based on these data, we hypothesized that the binding of Noxa to Mcl-1 actually decreases the availability of Usp9x to Mcl-1; as a result, this leads to augmentation of its ubiquitination level. To test the hypothesis, we overexpressed the *Myc–Mcl-1* fusion gene in A549 cells. After immunoprecipitation using anti-Myc antibody, western blot analysis showed that the binding levels of Noxa were increased whereas Usp9x levels were decreased (Figure 7b). Together, this suggests that Noxa promotes Mcl-1 ubiquitination by reducing the availability of Usp9x to Mcl-1.

Puma regulates pemetrexed-induced apoptosis in human lung cancer cells. As Puma is another critical BH3-only protein that is usually modulated at transcriptional level and similar to Noxa,[16] we wonder whether Puma is also involved in the apoptosis induced by pemetrexed in human lung cancer cells. Thus, we examined the Puma expression level in H1792 and A549 cells that were treated with increasing concentrations of pemetrexed for 48 h (Supplementary Figure S1a). Puma expression level was dramatically increased after pemetrexed exposure in these two cell lines. In time-course experiments, pemetrexed induced Puma by 24 h, and it was maintained to 48 h we measured (Supplementary Figure S1b).

To assess the influence of Puma on pemetrexed-induced apoptosis, A549 cells were transfected with control (Ctrl), *Puma*, *Noxa* or combination of *Puma* and *Noxa* siRNA respectively for 48 h and then were treated with 2.5 μmol/l pemetrexed for 48 h. Selective knockdown of *Noxa* and *Puma* alone both abrogated their expression despite pemetrexed exposure and reduced apoptotic proteins such as caspase-9, caspase-3 and PARP cleavage, respectively (Supplementary Figure S2a). Moreover, it seemed that the apoptotic protein cleavage in combination knockdown cells was somewhat more reduced compared with the one in *Puma* or *Noxa* alone knockdown cells (Supplementary Figure S2a). However, *Noxa* knockdown recovers the level of Mcl-1 after pemetrexed exposure, whereas *Puma* knockdown does not. In addition, the fraction of apoptotic cells was reduced in the *Puma* or *Noxa* or combination knockdown cells compared with the control siRNA

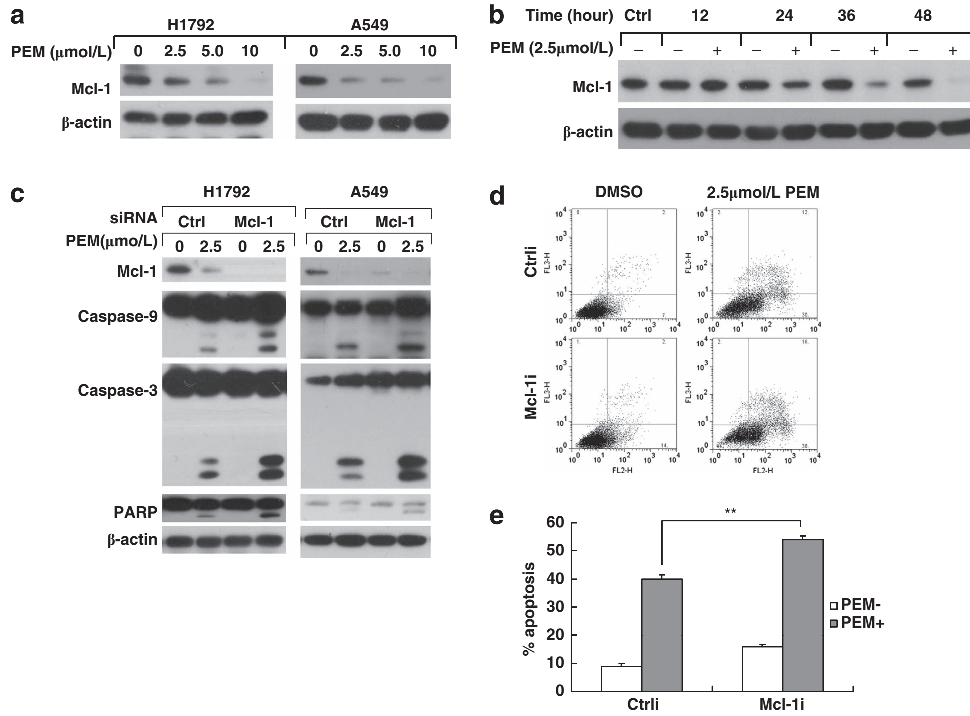

Figure 2 Downregulation of Mcl-1 accelerates pemetrexed-induced apoptosis. H1792 and A549 cells were treated with pemetrexed for 48 h (**a**). A549 cells were treated with 2.5 μmol/l pemetrexed for the indicated times (**b**). Both H1792 and A549 cells were transfected with control (Ctrl) or Mcl-1 siRNA. At 2 days after transfection, cells were treated with 2.5 μmol/l pemetrexed for 48 h (**c** and **d**). Whole-cell protein lysates were harvested for western blot analysis (**b–d**). A549 cells were transfected with control and Mcl-1 siRNA and treated with pemetrexed using the same method. The cells were stained by Annexin V-PE/7-AAD staining and analyzed by flow cytometry (**d** and **e**). The experiment was performed in triplicate and repeated three times. The data are presented as mean ± S.D. of three replicates in one experiment (**$P<0.01$) (**e**). Representative pictures from three independent experiments are shown

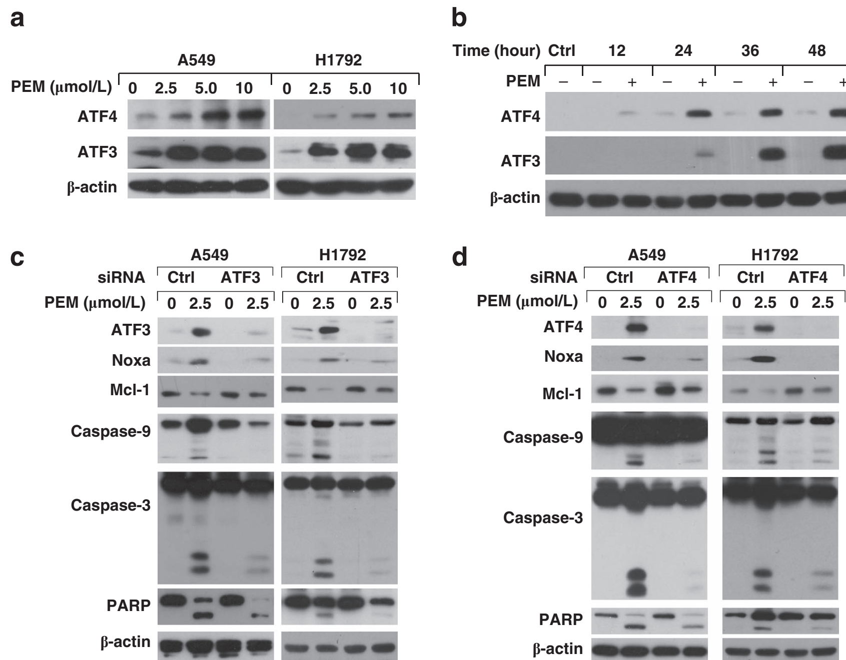

Figure 3 ATF3 and ATF4 are upregulated by pemetrexed, and knockdown of their expression by siRNA protects NSCLC cells from pemetrexed-induced apoptosis. A549 and H1792 cells were treated with the indicated pemetrexed doses for 48 h (**a**). A549 cells were treated with 2.5 μmol/l pemetrexed for the indicated times (**b**). A549 and H1792 cells were transfected with control (Ctrl) or ATF3/ATF4 siRNA for 48 h and then treated with 2.5 μmol/l pemetrexed for 48 h (**c** and **d**). Cells were harvested and lysates of whole-cell protein were used for western blot analysis (**a–c**). Representative pictures from three independent experiments are shown

knockdown cells (Supplementary Figure S2b). Surprisingly, *Puma* and *Noxa* combination knockdown did not reduce apoptosis further. Taken together, our data suggest that pemetrexed induces multiple apoptotic signaling pathways, and blocking intrinsic apoptotic proteins such as Puma or/and Noxa cannot thoroughly inhibit the apoptosis.

Discussion

Pemetrexed is a first-line agent against NSCLC patients, yet little is known about its underlying apoptotic mechanism. In this study, we demonstrate that pemetrexed upregulates the expression of ATF4 and ATF3 that leads to Noxa expression. The activation of Noxa makes Mcl-1 degrade by decreasing deubiquitinase Usp9x after pemetrexed exposure. This work shows the Noxa–Usp9x–Mcl-1 pathway contributes to pemetrexed-induced apoptosis.

Both ATF3 and ATF4 are pro-apoptotic proteins that are induced during chronic ER stress.[24,25] For example, with pemetrexed, the expression of ATF3 and ATF4 increase by 36 h and remain high level at 48 h. Inhibition of ATF3 or ATF4 expression reduces the Noxa level and confers human lung cancer cell resistant to pemetrexed, suggesting that ATF3 and ATF4 play critical roles in apoptosis induced by pemetrexed. Consistent with this finding, ATF4 and ATF3 can form a complex and regulate Noxa by binding to the *Noxa* promoter.[23] Other studies have shown ER stress specifically activates BH3-only protein Noxa by p53 at transcriptional level in mouse embryo fibroblasts (MEFs).[26] As pemetrexed induces Noxa expression in H1792 cells, which are known to have mutant p53, this indicates that the activation of Noxa is p53 independent in NSCLC. To our knowledge, this work first describes pemetrexed results in Noxa upregulation through activation of ATF3 and ATF4 in NSCLC.

Mcl-1 is the main pro-survival binding protein of Noxa, and its expression is essential for NSCLC cell survival.[27] Mcl-1 has emerged as a potential and promising target for cancer therapy.[19] Our data demonstrated that pemetrexed-mediated apoptosis is enhanced by Mcl-1 knockdown, and inhibiting Noxa expression increases basal Mcl-1 levels and pemetrexed-induced Mcl-1 levels. These results are consistent with the finding that a discrete C-terminal sequence in the Noxa BH3 domain is requisite for Mcl-1 degradation.[28]

Moreover, in these data we found that Usp9x acts as a mediator between Noxa and Mcl-1. Blocking Noxa increased Usp9x levels in H1792 and A549 cells. It has been reported that Usp9x deubiquitinates Mcl-1 by removing the conjugated ubiquition.[22] Moreover, Noxa overexpression decreases the interaction of Mcl-1/Usp9x, resulting in the increase of Mcl-1 polyubiquitinated forms.[29] We found that Usp9x was down-regulated in lung cancer cells following pemetrexed because of an unclear mechanism. Eventually, Noxa promoted Mcl-1 ubiquitination by reducing the availability of Usp9x to Mcl-1 after pemetrexed exposure, according to our immunoprecipitation assay. The fine mechanism underlying the relationship of Noxa/Mcl-1/Usp9x requires future exploration.

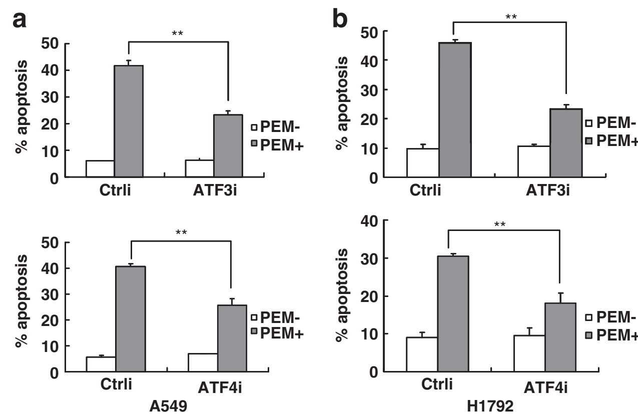

Figure 4 Knockdown of ATF3 or ATF4 expression by siRNA protects NSCLC cells from pemetrexed-induced apoptosis. A549 (**a**) and H1792 (**b**) cells were transfected with control (Ctrl) or ATF3/ATF4 siRNA for 48 h and then treated with 2.5 μmol/l pemetrexed for 48 h. Cells were harvested and subjected to Annexin V-PE/7-AAD staining and flow cytometry analysis. The % positive cells in the upper right (Annexin V+/7-AAD+: late apoptotic cells) and lower right quadrants (Annexin V+/7-AAD−: early apoptotic cells) were summed to give the total number of apoptotic cells. The experiment was performed in triplicate and repeated three times. The data are presented as mean ± S.D. of three replicates in one experiment (**$P<0.01$)

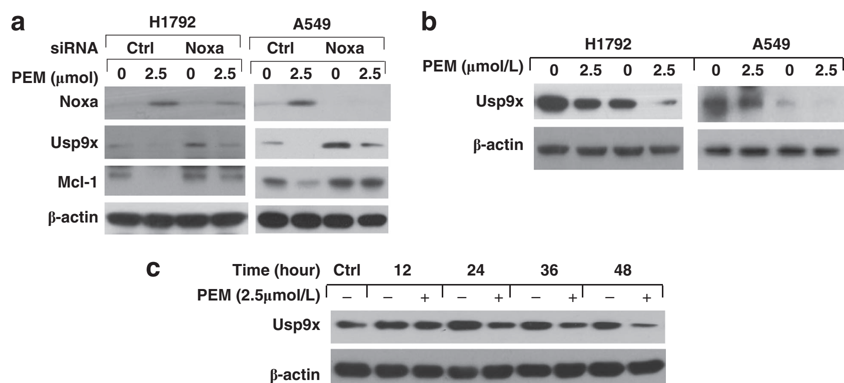

Figure 5 Noxa regulates Usp9x and Mcl-1 level after pemetrexed treatment. A549 and H1792 cells were cultured in six-well plates and transfected with control (Ctrl) or Noxa siRNA. Then, 24 h later, single wells were equally divided into two wells. On the second day, cells were treated with 2.5 μmol/l pemetrexed for 48 h (**a**). The indicated concentrations of pemetrexed were given to H1792 and A549 cells for 48 h (**b**). A549 cells were treated with 2.5 μmol/l pemetrexed for the indicated time (**c**). Whole-cell protein of lysates were harvested and used for western blot analysis. Representative pictures from three independent experiments are shown

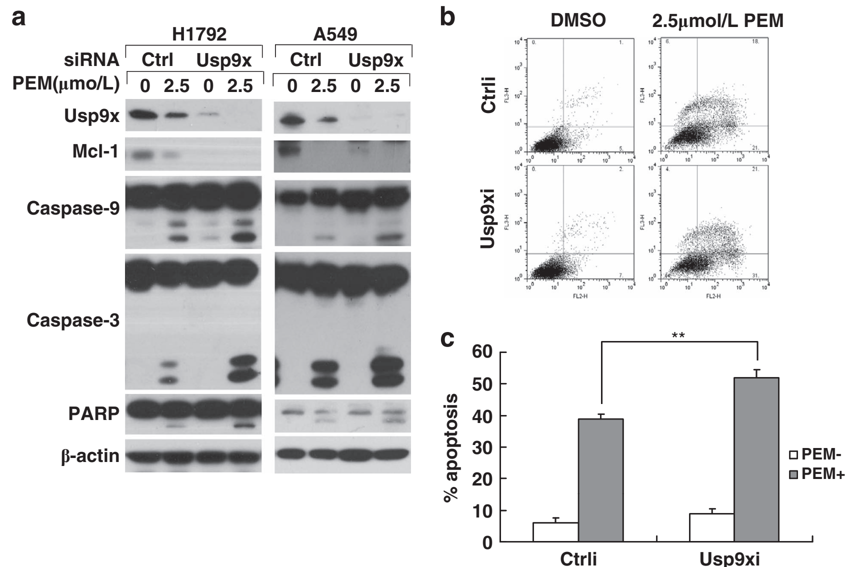

Figure 6 Knockdown of Usp9x expression attenuates Mcl-1 expression and increases pemetrexed-induced apoptosis in NSCLC cells. H1792 and A549 cells were transfected with control (Ctrl) or Usp9x siRNA for 2 days and then were treated with 2.5 μmol/l pemetrexed for 48 h. Cells were subjected to preparation of the whole-cell protein lysates for western blot analysis (**a**). A549 cells were prepared to flow cytometry analysis (**b**). Apoptosis was measured by Annexin V-PE/7-AAD staining. In the flow cytometry analysis, the percent positive cells in the upper right and lower right quadrants were summed to yield the total number of apoptotic cells. The experiment was performed in triplicate and repeated three times. The data are presented as mean ± S.D. of three replicates in one experiment (**$P<0.01$) (**c**)

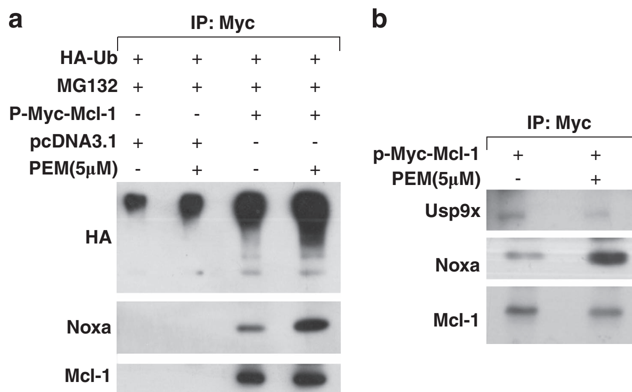

Figure 7 Pemetrexed treatment reduces the availability of Usp9x to Mcl-1 and enhances Mcl-1 ubiquitination in lung cancer cells. A549 cells were transfected with plasmids pcDNA3.1-Myc-Mcl-1 or pcDNA3.1 and Ub-HA that could express HA-tagged ubiquitin. After 12 h, the cells were treated with pemetrexed (5 μmol/l) for 8 h, and MG132 (10 μmol/l) was cotreated for another 4 h. For immunoprecipitation, cells were lysed and incubated with Myc antibody for 1 h and then incubated with protein A/G agarose (1 : 1 mix) at 4°C overnight. The beads were washed three times and then boiled in the loading buffer. The Mcl-1 ubiquitination level were analyzed by western blot assay using anti-HA antibody (**a**). Plasmid pcDNA3.1-Myc-Mcl-1 was transfected into A549 cells. After 12 h, the cells were treated with DMSO or pemetrexed (5 μmmol/l) for 12 h. For immunoprecipitation, cells were lysed and incubated with Myc antibody for 1 h and then incubated with protein A/G agarose (1 : 1 mix) at 4°C overnight. The beads were washed three times and then boiled in the loading buffer. Western blot was performed to examine the levels of proteins binding to Mcl-1 such as Usp9x and Noxa (**b**). Representative pictures from two independent experiments are shown

As a multitargeted antifolate drug, pemetrexed induces cell cycle arrest by p21 upregulation and multiple apoptotic signaling pathways in human cancer cells including extrinsic death receptor DR5 pathway and intrinsic Bcl-2/Bax pathway as well as the caspase-independent pathway and so on.[7,12,30] In order to assess how much the Noxa/Mcl-1 pathway contributes to the pemetrexed-induced apoptosis, we explored the influence of pemetrexed on the important intrinsic apoptotic protein Puma. Although it seemed that the apoptotic protein cleavage in the *Noxa/Puma* combination knockdown cells was somewhat more reduced compared with single *Noxa* or *Puma* alone knockdown cells, *Noxa/Puma* combination knockdown did not reduce the percentage of apoptotic cells thoroughly. This finding further suggests that pemetrexed induces multiple apoptotic signaling pathways in cancer cells including the extrinsic apoptotic pathway, and knockdown of intrinsic *Noxa* and/or *Puma* cannot sufficiently inhibit apoptosis by pemetrexed in human cancer cells. This may explain why pemetrexed has a potent anticancer effect on cells resistant to conventional therapeutic agents.[7] However, whether blocking extrinsic and intrinsic apoptotic pathways simultaneously can inhibit the pemetrexed-induced apoptosis will require further investigation.

Materials and Methods

Reagents. Pemetrexed was purchased from Toronto Research Chemicals, Inc. (Toronto, ON, Canada). Caspase-3 antibody (clone: 31A1067) was obtained from Imgenex (San Diego, CA, USA). Caspase-9 (cat. no. 9502s), PARP (cat. no. 9542L) and Usp9x (cat. no. 5751s) antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Mcl-1 (cat. no. sc-12756), ATF4 (sc-200) and ATF3 (sc-81189) antibodies were obtained from Santa Cruz (Santa Cruz, CA, USA). Puma (clone no.114C307) and β-actin (clone no. AC-15) antibodies were purchased from Sigma (St. Louis, MO, USA). Noxa (clone no.114c307) antibody was purchased from EMD Millipore (Billerica, MA, USA).

Cell lines. All human lung cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). The A549 and H1792 cell lines were recently authenticated in Microread Gene Technology (Beijing, China) by STR analysis. These cell lines were cultured in RPMI-1640 medium containing 5% newborn calf serum at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

Western blot analysis. Preparation of whole-cell protein lysates and the western blot were performed as previously described.[31]

Gene silencing with siRNA. All siRNA duplexes were synthesized by GenePharma (Shanghai, China). Control (Ctrl), ATF3 and ATF4 siRNA target sequences have been previously described.[32] The target sequences of Noxa, Mcl-1, Usp9x and Puma are 5′-GGAAGUCGAGUGUGCUACU-3′, 5′-GAAATTCT TTCACTTCATT-3′, 5′-CAATCAAGTTCAATGATTA-3′ and 5′-GGAGGGUCCUGU ACAAUCU-3′, respectively. HighPerfect Transfection Reagent (Qiagen, Hilden, Germany) was used to transfect siRNAs per the manufacturer's protocol. Gene silencing effects were evaluated by western blot analysis.

Construction of the plasmid. Myc-tagged *Mcl-1* gene was amplified from A549 cells cDNA with PCR technique using the primers described as below: 5′-CGGATCCGCCGCCACCATGGAACAAAAACTCATCTCAGAAGAGGATCTGA TGTTTGGCCTCAAAAGAAACG-3′ (sense); 5′-CGGGCCCCTATCTTATTAGATA TGCCAAAC-3′ (antisense). The fragments then were cloned into the pcDNA3.1 (+) vector by using *Bam*HI and *Apa*I restriction sites. This resultant construct was named pcDNA3.1-Myc-Mcl-1.

Apoptosis assays. Apoptosis was evaluated by an Annexin V-PE/7-AAD apoptosis detection kit (BD Biosciences, San Jose, CA, USA). For the flow cytometry analysis, the percent positive cells in the upper right (Annexin V+/7-AAD+: late apoptotic cells) and lower right quadrants (Annexin V+/7-AAD−: early apoptotic cells) were summed to give the total number of apoptotic cells. Caspase activation was detected by western blot.

Immunoprecipitation. A549 cells were transfected with plasmids pcDNA3. 1-Myc-Mcl-1 or pcDNA3.1 and Ub-HA that could express HA-tagged ubiquitin. After 12 h, the cells were treated with pemetrexed (5 μmol/l) for 8 h, and MG132 (10 μmol/l) was cotreated for another 4 h. Cells were lysed and incubated with Myc antibody for 1 h and then incubated with protein A/G agarose (1 : 1 mix; Roche, Basel, Switzerland) at 4°C overnight. The beads were washed three times and then boiled in the loading buffer. The Mcl-1 ubiquitination level were analyzed by western blot assay using anti-HA antibody. The proteins Noxa, Usp9x and Mcl-1 were also detected by western blot.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements. This work was supported by grants from the National Natural Science Foundation of China (31371402, 81000947), National Key Basic Research Program of China (2013CB910900 project 3), the Shandong Natural Science Foundation (JQ201007) and the Independent Innovation Foundation of Shandong University (IIFSDU2012TS010).

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