Mitochondrial uncoupling protein 2 regulates the effects of paclitaxel on Stat3 activation and cellular survival in lung cancer cells

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Growing evidence suggests that Stat3 contributes to chemoresistance. However, the impact of chemotherapy on Stat3 activity is unclear. We found that paclitaxel activated Stat3 in the human lung cancer cell lines PC14PE6AS2 (AS2) and H157, whereas it reduced Stat3 activation in A549 and H460 cells. Pretreatment of AS2 and H157 cells with rotenone, an inhibitor of mitochondrially produced reactive oxygen species (ROS), or carbonyl cyanide p-(trifluoromethoxy)-phenylhydrazone (FCCP), a mitochondrial uncoupler, suppressed the paclitaxel-induced activation of Stat3. Uncoupling protein 2 (UCP-2), localized in the inner membrane of the mitochondria, can reduce ROS production in four conditions of oxidative stress. UCP-2 protein expression in the four cancer cell lines was higher than that in normal lung epithelial cells (NL-20), but its expression was lower in AS2 and H157 cells relative to A549 and H460 cells. Silencing high UCP-2 expression with small interfering RNA (siRNA) in A549 and H460 cells restored paclitaxel-induced Stat3 activation. In addition, paclitaxel-induced Stat3 activation led to the upregulation of survivin and Mel-1, which in turn facilitated cell survival. Moreover, the CL1-5 subline had lower UCP-2 expression relative to the parental CL1-0 cells. Treatment with paclitaxel activated Stat3 in CL1-5 but not in CL1-0 cells, whereas in CL1-5 cells, the overexpression of UCP-2 with complementary DNA (cDNA) blocked Stat3 activation. In lung cancer patients, low UCP-2 expression in cancer cells was a predictor of a poor response to chemotherapy. Therefore, UCP-2 modulates the ROS/Stat3 signaling pathway and response to chemotherapy treatment in lung cancer cells. Targeting UCP-2, ROS and Stat3 pathways may improve anticancer therapies.

Introduction

Lung cancer is a major cause of cancer deaths worldwide. Advances in chemotherapy and targeted therapy have significantly improved tumor control rates (1), but cancer cells may evade treatment-induced death through activation of various pathways. For example, cancer cells were reported to utilize the stimulation provided by chemotherapy to activate the phosphatidylinositol 3’ kinase-Akt, nuclear factor-kappaB (NF-xB) or activator protein 1 (AP-1) signaling pathways (2,3), thereby facilitating cell survival.

Abbreviations: AS2, PC14PE6AS2; DFI, diphenylene iodonium chloride; FCCP, carbonyl cyanide p-(trifluoromethoxy)-phenylhydrazone; MITT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NAC, N-acetylcysteine; NF-xB, nuclear factor-kappaB; ROS, reactive oxygen species; siRNA, small interfering RNA; SOCS3, suppressor of cytokine signaling 3; Stat proteins, signal transducer and activator of transcription proteins; UCP, uncoupling protein.

Signal transducer and activator of transcription (Stat) proteins are latent cytoplasmic transcription factors that regulate cell proliferation, migration and differentiation (4). Stat3 upregulates genes that encode apoptosis inhibitors, cell cycle regulators and angiogenesis inducers (4,5). Stat3 also regulates cell respiration by mediating the mitochondrial electron transport chain function of complexes I and II (6). In cancer cells, downregulation of Stat3 decreases cell viability and increases apoptosis (7), whereas overexpression of Stat3 results in chemoresistance (8). Walker et al. demonstrated that antimicrotubule chemotherapeutic agents inhibit Stat3 signaling (9). However, we still have only a basic understanding of how chemotherapeutic agents affect Stat3 activity.

Anticancer agents, such as paclitaxel and cisplatin, generate reactive oxygen species (ROS) in a concentration-dependent manner (10,11). Treatment with 100 nM paclitaxel induces ROS production, but the maximal accumulation of H2O2 occurred when cells were exposed to 5 μM paclitaxel for 1 h (11). Chemotherapeutic agent-induced ROS production can overwhelm cell antioxidant defenses, leading to cell damage and cell death. In contrast, ROS may act as secondary messengers in intracellular signaling cascades to induce and maintain the oncogenic phenotype of cancer cells (12). For example, ROS accumulation may induce the constitutive activation of transcription factors such as NF-xB, activator protein AP-1, hypoxia-inducible factor 1 (HIF-1), and Stat3, which in turn lead to aberrant extracellular signal-regulated protein kinase (ERK1/2) activation or inhibition of tyrosine phosphatase, thereby promoting cell proliferation (13,14). Furthermore, paclitaxel-induced ROS production has been shown to increase vascular endothelial growth factor expression (15). Mitochondria are a major source of ROS. Uncoupling proteins (UCPs) located in the inner mitochondrial membrane can decrease the mitochondrial membrane potential and catalyze the reentry of protons into the matrix, thereby reducing ROS generation (16). Uncoupling protein 2 (UCP-2) is one of five known members of the UCP family and its expression is ubiquitous in the lung and other types of human tissue (16). Studies have also found that overexpression of UCP-2 contributes to human colon carcinogenesis (17,18) and promotes chemoresistance in cancer cells (19,20). As found in immune cells, the increased expression of UCP-2 negatively regulates mitochondrial ROS generation to decrease the inflammatory response (21). However, it is still unknown whether the level of UCP-2 expression determines the cell response to chemotherapy-induced redox stress in cancer cells.

This study provided evidence that the level of UCP-2 expression in cancer cells affects paclitaxel-induced ROS production and subsequent Stat3 activation. In addition, we demonstrated that patients with advanced lung cancer and whose cancer cells had low UCP-2 expression experienced inferior treatment responses, with poor prognoses for progression-free survival and overall survival in response to cisplatin-based chemotherapy.

Materials and methods

Antibodies and reagents

Supplementary Materials and methods is available at Carcinogenesis Online.

Small interfering RNA and cDNA transfection

For Stat3 silencing, human Stat3 small interfering RNA (siRNA) were purchased from Ambion (Austin, TX), (sense: 5’-CGAUCUAGAACGAAA- AUGTGT-3’; anti-sense: 5’-CAUUUCCUGUCCAGAAGUCCG-3’). Scrambled control for Stat3 siRNA was purchased from Applied Biosystems (Foster City, CA). Tumor cells were transfected with Stat3 siRNA or scrambled control using Lipofectamine 2000 (Invitrogen) and evaluated after 48 h. For UCP-2 silencing, tumor cells were transfected with siCONTROL non-targeting siRNA or ON-TARGET plus SMART pool human UCP-2 siRNAs (Dharmacon) (22) using Lipofectamine and evaluated after 48 h. For UCP-2 overexpression,
tumor cells were transfected with vector control or UCP-2 cDNA (a gift from György Baffy) (20) using Lipofectamine and evaluated after 48h.

Cell lines
The human lung cancer cell lines A549, H157 and H460 were obtained from the American Type Culture Collection (ATCC; Rockville, MD). We established the human lung adenocarcinoma PC14PS6CAS2 (AS2) cell line, which has increased metastatic susceptibility and constitutively activated endogenous Stat3, in our laboratory (23). Lung adenocarcinoma CL1-0 cells and the more invasive CL1-5 cell subline were a gift from Prof. Yang Pan-Chyr (24). The CL1-5-UCP-2 cell line was also established from CL1-5 cells transfected with the UCP-2 cDNA plasmid, whereas the CL1-5-Veg cell line was the vector control for CL1-5-UCP-2.

Cytotoxicity assay
Cytotoxicity was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (25) (Supplementary Materials and methods is available at Carcinogenesis Online).

Cell viability
Cell viability was assessed using the trypan blue dye (0.2%) exclusion method (26) (Supplementary Materials and methods is available at Carcinogenesis Online).

Immunoblotting
Supplementary Materials and methods is available at Carcinogenesis Online.

Nuclear extraction
Cytoplasmic and nuclear extracts were prepared according to Chang et al. (27).

Luciferase reporter assays
The Stat3 reporter gene (pm67-Luc) was provided by Dr James Darnell Jr (23) and the NF-κB reporter gene (NF-kB-Luc) was provided by Dr Pei-Jane Tsai (27). The pm67-Luc Stat3 reporter gene (1 μg) or NF-kB-Luc report gene (0.5 μg) and the Renilla luciferase-encoding plasmid pRL-TK (0.1 μg/well) were cotransfected into the cancer cells (1 × 10^5) using Lipofectamine. Sixteen (0.5 μl) of the pm67-Luc Stat3 reporter gene (1 × 10^5) or the NF-κB reporter gene (NF-κB-Luc) was also cotransfected from the UCP-2 cDNA plasmid, whereas the CL1-5-Veg cell line was the vector control for CL1-5-UCP-2.

Cytoplasmic and nuclear extracts were prepared according to Chang et al. (27).

Measuring the intracellular ROS generation
We used non-polar 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA; D-399; Molecular Probes) for the determination of intracellular ROS (Supplementary Materials and methods is available at Carcinogenesis Online).

Measuring the mitochondrial membrane potential
The mitochondrial membrane potential was detected using 5,5′,6,6′,tetrachloro-1,1′,3′,3′-tetraethyl-benzimidazolylcarbocyanine (JC-1 assay reagent; Molecular Probes) as a probe (28) (Supplementary Materials and methods is available at Carcinogenesis Online).

Measuring the adenosine triphosphate synthesis rate
The rate of adenosine triphosphate (ATP) synthesis was recorded as the ratio of citrate synthase activity (29) (Supplementary Materials and methods is available at Carcinogenesis Online).

Immunohistochemistry
We adopted a semiquantitative scoring system to evaluate staining intensity (18). Others are mentioned in Supplementary Materials and methods, available at Carcinogenesis Online.

Study subjects
The patient treatment response was evaluated periodically according to the response evaluation criteria in solid tumors (RECIST) criteria (30) using computed tomography. Clinicopathological information was obtained from medical records and pathological reports. Others are mentioned in Supplementary Materials and methods, available at Carcinogenesis Online.

Statistical analysis
Data were analyzed using SPSS 13.0 software (SPSS, IL). Fisher’s exact test and Chi-square test were used to evaluate correlations between clinicopathological variables and UCP-2 expression. Survival analysis was based on the Kaplan–Meier method, whereby statistical significance was assessed using the log-rank test. Univariate and multivariate Cox regression models were used to determine the clinical predictors of survival. In cell line studies, differences between two independent groups were determined using the Mann–Whitney U-test. Significance was set at P < 0.05.

Results
Paclitaxel induced Jak2/Stat3 activation in AS2 and H157 cells, but not in A549 and H460 cells
AS2, A549, H157 and H460 are human lung carcinoma cell lines with constitutive Stat3 activation (23). In the AS2 and H157 cell lines, paclitaxel treatment gradually increased Stat3 tyrosine phosphorylation, peaking at 3h. In contrast, treatment of A549 and H460 cells with paclitaxel led to a time-dependent reduction in phospho-Stat3 levels (Figure 1A). Serine phosphorylation (Ser-727) of the C-terminal transcriptional activation domain enhanced the transcriptional activity of Stat3 (31). In our studies, paclitaxel induced Stat3 serine phosphorylation equally in AS2, A549, H157 and H460 cells (Figure 1A).

Paclitaxel induced tyrosine phosphorylation of Stat3 in AS2 and H157 cells at a clinically achievable dosage (0.5 μM; Figure 1B). However, the degree of phospho-Stat3 expression was weaker and the Stat3 activation peak occurred about 5h later in cells treated with 0.5 μM paclitaxel than in cells treated with 5 μM paclitaxel.

To study the role of Stat3 activation in the cell susceptibility to paclitaxel, we exposed AS2, A549, H157 and H460 cells to scrambled siRNA or Stat3 siRNA, treating the cells with or without paclitaxel. The viabilities of the cells were measured at 48h after paclitaxel treatment. Silencing Stat3 expression with siRNA enhanced the cytotoxicity of paclitaxel against these four cancer cell lines (Figure 1C).

Alexandre et al. showed that 5 μM paclitaxel provided maximal accumulation of H$_2$O$_2$ in cancer cells and that paclitaxel treatment induced the generation of ROS in a dose-dependent manner (11). We found that ROS generation by 5 μM paclitaxel was greater than that produced by 0.5 μM paclitaxel in the AS2, H157, A549 and H460 cell lines (data not shown). Exposure to 5 μM paclitaxel for 8h did not cause significant cell death in the studied cell lines (data not shown). Therefore, we used 5 μM paclitaxel in our experiments to ensure clear signaling changes (11,32).

Jak2 activation often precedes Stat3 phosphorylation in cell signaling. We found that paclitaxel activated Jak2 in AS2 and H157 cells within 5min, with an activation peak at 30min (Figure 1D). In contrast, there was no detectable Jak2 activation in A549 and H460 cells in response to paclitaxel treatment.

Paclitaxel treatment affected tyrosine and serine phosphorylation of Stat3 quite differently (Figure 1A and 1B), so we determined how paclitaxel influenced the activity of the Stat3 gene using a luciferase reporter assay. We detected elevated luciferase activities of the Stat3 reporter in AS2, A549, H157 and H460 cells at baseline (data not shown). After 3h treatment with paclitaxel, luciferase activities were induced in AS2 and H157 cells but suppressed in A549 and H460 cells (Figure 1E). These data were compatible with changes in the Tyr-phosphorylation of Stat3 after paclitaxel treatment in the four cancer cell lines.

Incubation with AG490, a Jak2 kinase inhibitor, resulted in a dose-dependent suppression of paclitaxel-induced Stat3 activation in AS2 and H157 cells (Figure 1F). The data described here indicate that paclitaxel activates Jak2, which in turn induces Stat3 phosphorylation in AS2 and H157 cells.

Paclitaxel induced significant mitochondrial ROS production to activate Stat3 in AS2 and H157 cells
Oxidative stress can activate Stat3 in cancer cells (13). We determined ROS levels during paclitaxel treatment of AS2, A549, H157 and H460 cells using a fluorophotometric method with DCFH-DA fluorescent dye. Five minutes after exposing cells to paclitaxel (5 μM), there was a 1.5-fold increase in intracellular ROS levels in AS2 and H157 cells, and this increase was sustained for at least 4h (Supplementary Figure 1A is available at Carcinogenesis Online). There was only a modest increase in the intracellular ROS levels in A549 and H460 cells. There was a significant difference in ROS levels of these two groups of cells (P < 0.05) (Supplementary Figure 1A is available at Carcinogenesis Online).

Rotonein is an electron transport inhibitor of the mitochondrial respiratory chain complex I. Pretreating AS2 cells with rotonein for 1h significantly reduced the degree of paclitaxel-induced ROS
production (Supplementary Figure 1B is available at Carcinogenesis Online) and Stat3 activation (*P < 0.001; Figure 2A). We observed similar results for H157 cells (Supplementary Figure 1B is available at Carcinogenesis Online; Figure 2A).

Antimycin A is another mitochondrial electron transport inhibitor, whereas diphenylene iodonium chloride inhibits both mitochondrial-dependent and -independent ROS generation (33). Pretreatment of AS2 cells with either antimycin A or diphenylene...
Mitochondrial uncoupling decreases the proton gradient across the inner mitochondrial membrane, impairs ATP synthesis and reduces ROS production. FCCP (1, (trifluoromethoxy)-phenylhydrazone (FCCP) is a mitochondrial uncoupler. We treated cells with FCCP to determine whether mitochondrial uncoupling was related to paclitaxel-induced mitochondrial ROS production. At 5 μM concentration, FCCP had little effect on cell viability (data not shown), but it significantly reduced the rate of ATP synthesis in AS2, H157, A549 and H460 cells (P < 0.01; Figure 2B). However, there was a significantly greater reduction in baseline ATP synthesis in AS2 and H157 cells compared with that in A549 and H460 cells (Figure 2B). These findings suggest that baseline mitochondrial uncoupling was low in AS2 and H157 cells but very strong in A549 and H460 cells. The degree of paclitaxel-induced ROS production was significantly reduced in FCCP-treated AS2 and H157 cells compared with that in FCCP-treated A549 and H460 cells (P < 0.001; Figure 2C). One-hour pretreatment with FCCP also suppressed paclitaxel-induced Stat3 activation in AS2 and H157 cells (Figure 2D).

High expression of UCP-2 in A549 and H460 cells prevented paclitaxel-induced ROS production and subsequent Stat3 activation. UCP-2 is a major negative regulator of mitochondrial ROS, and UCP-2 is expressed abundantly by lung mitochondria (16,21). We found that UCP-2 protein levels were greater in all the four lung cancer cell lines (AS2, H157, A549 and H460) compared with normal lung epithelial cells (NL-20), whereas UCP-2 protein levels were higher in A549 and H460 cells compared with those in AS2 and H157 cells (Figure 3A). The ATP synthesis rates increased in all four cancer cell lines after silencing UCP-2 protein with UCP-2 siRNA, although the magnitude of the increase was greater in A549 and H460 cells (P < 0.01) than in AS2 and H157 cells (P < 0.05) (Figure 3A and 3B). In addition, after UCP-2 knockdown, A549 and H460 cells exhibited more significant increases in ROS production than AS2 and H157 cells (Figure 3C). Stat3 activation was restored after the increase in paclitaxel-induced ROS production in A549 and H460 cells that had been exposed to UCP-2 siRNA (Figure 3D). However, treatment with rotenone suppressed Stat3 activation in the A549 and H460 cell lines (Figure 3D). Therefore, high UCP-2 expression in cancer cells under oxidative stress may enhance mitochondrial uncoupling and reduce ROS production.

Fig. 2. Paclitaxel induced significant mitochondrial reactive oxygen species (ROS) production in AS2 and H157 cells, resulting in Stat3 activation. Rotenone, a mitochondrial ROS inhibitor, and FCCP, a mitochondrial uncoupler, blocked Stat3 activation. (A) Changes in tyrosine-activated Stat3 protein and total Stat3 protein were evaluated in AS2 and H157 cells that had been exposed to 5 μM paclitaxel for 3 h after 1 h pretreatment with various doses of rotenone (1 and 5 μM). β-actin was used as the loading control. (B) The rates of cell ATP synthesis in AS2, H157, A549 and H460 cells were measured at baseline and after 5 μM FCCP treatment for 3 h (**P < 0.01). (C) In AS2, H157, A549 and H460 cells, fluorescent 2',7'-dichlorofluorescein was measured as cell ROS at baseline levels after 5 μM FCCP treatment for 1 h; 5 μM paclitaxel treatment for 3 h and 1 h pretreatment with FCCP, followed by paclitaxel treatment for 3 h. The experiment was repeated three times (***P < 0.001). (D) Changes in tyrosine-activated Stat3 protein expression and total Stat3 protein were evaluated in AS2 and H157 cells that had been exposed to 5 μM paclitaxel for 3 h after 1 h pretreatment with various doses of FCCP (1 and 5 μM).
Paclitaxel induced survivin and Mcl-1 expression in cancer cells with low UCP-2 expression

To determine whether the paclitaxel-induced Stat3 activation in cancers with low UCP-2 expression led to changes in downstream gene expression, we selected three genes for further study, i.e. survivin, MCL-1 and suppressor of cytokine signaling 3 (SOCS3). Survivin, a Stat3 transcriptional target, is associated with cell resistance to paclitaxel, which is independent of cell cycle arrest (5,35). Upregulation of Mcl-1, another direct target of Stat3, also leads to chemoresistance to paclitaxel (36). SOCS3 is induced by Stat3 activation and acts as a negative regulator of the Jak/Stat pathway (37). We found that paclitaxel upregulated survivin, Mcl-1 and SOCS3 expression in AS2 and H157 cells but not in A549 and H460 cells (Figure 4A). Pretreatment with AG490, rotenone or FCCP prevented paclitaxel-induced expression of survivin in cells in a dose-dependent manner (Figure 4B). Therefore, the ROS/Jak2/Stat3 pathway appeared to govern paclitaxel-induced cell survival in the AS2 and H157 cell lines.

R rottenone and FCCP treatment enhanced paclitaxel cytotoxicity to cancer cells with low UCP-2 expression

To study the effects of paclitaxel-induced ROS, Jak2, Stat3 and survivin signaling on cell responses to paclitaxel, we pretreated AS2, A549, H157 and H460 cells with rotenone or FCCP for 1 h and exposed them to paclitaxel for 48 h. Cell viability was evaluated using the MTT assay (25). We found that in cells pretreated with rotenone or FCCP, paclitaxel was significantly more cytotoxic to AS2 and H157 cells than A549 and H460 cells ($P < 0.01$; Figure 4C). The loss of the mitochondrial membrane potential correlated well with drug-induced cell death (38), so we assayed the mitochondrial membrane potential after treating cells with FCCP for 1 h, paclitaxel for 24 h or FCCP pretreatment for 1 h, followed by exposure to paclitaxel for 24 h. Treatment with FCCP or paclitaxel alone caused a mild or moderate reduction in the mitochondrial membrane potential in all four lung cancer cells. When FCCP was given before exposure to paclitaxel, we observed a significantly greater reduction in the mitochondrial membrane potential of AS2 and H157 cells compared with the reduction observed in A549 and H460 cells ($P < 0.01$; Figure 4D).

UCP-2 modulated paclitaxel-induced Stat3 activation in highly invasive lung adenocarcinoma CL1-5 cells

Parental CL1-0 and its subclone CL1-5 are lung adenocarcinoma cell lines with different invasive activities. CL1-5 cells are more aggressive than CL1-0 cells (24). At baseline, the CL1-5 cells expressed lower UCP-2 (Figure 5A). Paclitaxel treatment (0.5 and 5 μM) induced increased ROS generation and luciferase activity of the Stat3 reporter in CL1-5 cells but not in CL1-0 cells (Figure 5A–B).
Silencing UCP-2 in CL1-0 cells with UCP-2 siRNA restored paclitaxel-induced ROS production (Supplementary Figure 4A is available at Carcinogenesis Online) and Stat3 activation (Figure 5C), whereas pretreatment with rotenone prevented reactivation of Stat3 (Figure 5C).

In CL1-5 cells, both FCCP and rotenone pretreatment suppressed paclitaxel-induced ROS production (Supplementary Figure 4B is available at Carcinogenesis Online), whereas pretreatment with rotenone prevented reactivation of Stat3 (Figure 5C). We overexpressed UCP-2 in CL1-5 cells by cDNA transfection and found that upregulation of UCP-2 attenuated paclitaxel-induced ROS production (Supplementary Figure 4B is available at Carcinogenesis Online) and suppressed both Stat3 activation and survivin expression (Figure 5D). Pretreatment with rotenone alone greatly enhanced paclitaxel cytotoxicity toward CL1-5 cells (Supplementary Figure 4C is available at Carcinogenesis Online).

**Fig. 4.** Activation of ROS, Jak2 and Stat3 resulted in cell resistance to paclitaxel in AS2 and H157 cells. (A) Expression of survivin, Mcl-1 and SOCS3 was assessed during 5 μM paclitaxel treatment of AS2, AS49, H157 and H460 cells. (B) Changes in survivin expression was evaluated after 5 μM paclitaxel treatment for 3h, with or without pretreatment with various doses of inhibitors, i.e. 24h pretreatment with 10, 20 and 40 μM AG490, 1h pretreatment with rotenone (1 and 5 μM) and 1h pretreatment with FCCP (1 and 5 μM). (C) Cell viability was measured using an MTT assay were AS2, H157, AS49 and H460 cells were treated with paclitaxel alone for 48h and after 1h pretreatment with 5 μM rotenone or FCCP, followed by paclitaxel for another 48h (**P < 0.01). (D) AS2, H157, AS49 and H460 cells were treated as follows: (i) FCCP for 1h, (ii) paclitaxel for 24h, (iii) FCCP pretreatment for 1h, followed by paclitaxel and (iv) no treatment. JC-1 was used as a probe to measure red fluorescence (emission: 585 nm) and green fluorescence (emission: 530 nm) with a fluorescence reader (excitation: 488 nm). The mitochondrial membrane potential was calculated as the ratio of red to green fluorescence (**P < 0.01).
Fig. 5. UCP-2 mediated paclitaxel-induced Stat3 activation in highly invasive lung adenocarcinoma CL1-5 cells. (A) Baseline UCP-2 protein was assessed in lung adenocarcinoma CL1-0 cells and the more invasive subline CL1-5. The band density ratio (UCP-2 to β-actin) represents data from three experiments as the mean ± SD. Changes in tyrosine-activated and serine-phosphorylated Stat3 protein and total Stat3 protein were evaluated in CL1-0 and CL1-5 cells that had been exposed to 0.5 and 5 μM paclitaxel for 3 h. ROS levels were also measured during paclitaxel treatment by detecting 2,7'-dichlorofluorescein fluorescence. The experiment was performed three times (**P < 0.01). (B) CL1-0 and CL1-5 cells were cotransfected with the pm67-Luc Stat3 reporter gene and the Renilla luciferase-encoding plasmid pRL-TK. Cells were treated with 5 μM paclitaxel for 3 h. Normalized luciferase activity was measured (**P < 0.01). (C) Changes in the expression of UCP-2, total Stat3 and tyrosine-activated and serine-phosphorylated Stat3 protein were analyzed in CL1-0 cells at baseline and after the following treatments: (i) 5 μM paclitaxel treatment for 3 h, (ii) UCP-2 siRNA transfection, (iii) UCP-2 siRNA transfection followed by 5 μM paclitaxel treatment for 3 h and (iv) UCP-2 siRNA transfection followed by 1 h pretreatment with 5 μM rotenone and 5 μM paclitaxel treatment for 3 h. (D) The changes in the expression of UCP-2, survivin, total Stat3 and tyrosine-activated and serine-phosphorylated Stat3 protein in CL1-5 cells were analyzed at baseline and after the following: (i) 5 μM paclitaxel for 3 h, with or without 1 h pretreatment with inhibitors (5 μM rotenone or FCCP), (ii) UCP-2 cDNA transfection and (iii) UCP-2 cDNA transfection followed by 5 μM paclitaxel for 3 h. (E) Cell viability was evaluated using the MTT and the trypan blue assays when UCP-2 expression in CL1-0 and CL1-5 cells was enhanced by transfection with 0.2 μg UCP-2 cDNA or vector control cDNA for 48 h, followed by different doses (0.001, 0.01, 0.1, 1 and 5 μM) of paclitaxel treatment for another 48 h (*P < 0.05).
rendered cells sensitive to paclitaxel treatment (Figure 5E). We concluded that lung cancer cells with lower UCP-2 expression utilize chemotherapymduced ROS to activate survival signals.

Overexpression of UCP-2 may suppress NF-κB activation and UCP-2 regulated the effect of paclitaxel on Stat3/survivin signaling, although possibly not through NF-κB activity

In addition to Stat3 activation, NF-κB activation could also be induced by paclitaxel treatment (3). The direct physical interaction of Stat3 and p65 NF-κB is well known and could be involved in the modulation of their trans-acting properties (39). Therefore, we tested the role of UCP-2 in NF-κB regulation. We found that at baseline, there was more activated NF-κB in CL1-5 cells (cancer cells with low UCP-2 expression) than in CL1-0 cells. Paclitaxel treatment also activated NF-κB in CL1-5 cells (Supplementary Figure 5A is available at Carcinogenesis Online). When overexpressing UCP-2 in CL1-5 cells by cDNA transfection (CL1-5-UCP-2 cells), we found that the baseline NF-κB activity declined, compared with vector control cells (CL1-5-Vec cells; Supplementary Figure 5B is available at Carcinogenesis Online).

Next, we examined the role of UCP-2 in concert with Stat3 induced by paclitaxel treatment. In CL1-5-Vec cells, paclitaxel enhanced tyrosine-phosphorylated Stat3, SOCS3 and survivin expression. When CL1-5-Vec cells had been pretreated with the NF-κB inhibitor BAY 11–7082, the paclitaxel-induced upregulation of SOCS3 was abolished, whereas the upregulation of tyrosine-activated Stat3 and survivin was unchanged (Supplementary Figure 5C is available at Carcinogenesis Online). Because SOCS3 expression requires the interaction between Stat3 and NF-κB (39), Stat3 plays a major role in regulating paclitaxel-induced survivin expression. When overexpressing UCP-2 in CL1-5 cells by cDNA transfection (CL1-5-UCP-2 cells), the upregulation of tyrosine-activated Stat3, SOCS3 and survivin by paclitaxel treatment was suppressed (Supplementary Figure 5C is available at Carcinogenesis Online). We also performed NF-κB luciferase activity assays to confirm that NF-κB was activated by paclitaxel and that the elevation of NF-κB activity was reduced by the overexpression of UCP-2 and BAY 11–7082 (Supplementary Figure 5C is available at Carcinogenesis Online). Collectively, these results show that the level of UCP-2 in a cell after paclitaxel treatment may affect the activities of NF-κB and Stat3, whereas the interaction between NF-κB and Stat3 differentially regulates specific gene expression.

Low UCP-2 expression in tumor cells predicted an inferior response and lower survival in advanced lung cancer patients who received cisplatin-based chemotherapy

Cisplatin-based chemotherapy is a standard treatment for lung cancer (1). We found that cisplatin induced Stat3 activation in lung cancer cells with low UCP-2 expression (Supplementary Figure 3 is available at Carcinogenesis Online). To study the clinical significance of UCP-2 expression in lung cancer, we obtained tumor samples from 43 patients with inoperable, advanced lung cancer who had received cisplatin-based chemotherapy as the first-line treatment. UCP-2 was expressed at higher levels in cancer cells than in the adjacent normal lung tissue (Figure 6A). We stratified patients into high UCP-2-expression (grades 2 and 3) and low UCP-2-expression (grades 0 and 1) groups, as described previously (19). UCP-2 expression was not associated with histology, sex, age or smoking (Supplementary Table 1 is available at Carcinogenesis Online). The high UCP-2-expression group comprised 27 patients (62.8%) and the low UCP-2-expression group comprised 16 patients (37.2%). Among the patients in the high UCP-2-expression group, six (37.5%) responded poorly to chemotherapy. In the low UCP-2-expression group, 10 (62.5%) patients suffered disease progression during treatment. Patients with low UCP-2 expression had an inferior response to cisplatin-based chemotherapy (P = 0.011) and had poorer progression-free survival (P = 0.008; Figure 6B). Log-rank testing revealed that the overall survival was significantly shorter among patients with low UCP-2 expression compared with the survival of those with high UCP-2 expression (P = 0.016; Figure 6C).

Discussion

Cancer cells can acquire a degree of mitochondrial uncoupling through the expression of uncoupling proteins. Our findings indicated that UCP-2 expression determines the susceptibility of cancer cells to chemotherapy, at least in part. We stratified human lung cancer cells into a low UCP-2-expression group (AS2 and H157 cells) and a high UCP-2-expression group (A549 and H460 cells). Treatment of AS2 and H157 cells with paclitaxel enhanced the generation of mitochondrial ROS and resulted in Stat3 activation and the expression of survivin/Mcl-1. However, treatment of A549 and H460 cells with paclitaxel did not generate adequate ROS nor did it induce Stat3 activation or expression of survivin/Mcl-1. Human lung adenocarcinoma CL-5 cells, a subclone of CL-5 cells, had greater invasive ability and expressed less UCP-2 protein compared with the parental cell expression level. Thus, paclitaxel induced significant ROS production, which led to Stat3 activation and survivin expression in CL1-5 cells but not in CL1-0 cells. Furthermore, low UCP-2 expression in the lung cancer cells of patients with advanced lung cancer predicted an inferior response to cisplatin-based chemotherapy and a poor survival prognosis. Thus, UCP-2 regulates both paclitaxel-induced activation of Stat3 and the cell sensitivity to paclitaxel in lung cancer cells.

The lung cancer cell lines AS2, H157, A549 and H460 used in our study expressed higher levels of UCP-2 protein than normal bronchial cells (NL-20). NL-20 cells were more sensitive to paclitaxel treatment than the four lung cancer cell lines (Supplementary Figure 6A is available at Carcinogenesis Online). We found that UCP-2 expression was higher in human lung tumor tissues than adjacent non-diseased cells. Our findings support the hypothesis that cancer cells undergo a degree of mitochondrial uncoupling during carcinogenesis, which minimizes oxidative stress through the expression of UCPs including UCP-2 (16–19).

Several defense systems have evolved to combat ROS-induced oxidative stress (40). These include a number of non-enzymatic molecules (e.g. glutathione; vitamins A, C and E; and flavonoids), a number of enzymatic ROS scavengers (e.g. superoxide dismutases, catalase and glutathione peroxide) and activation of the p53 pathway (41). Interestingly, cancer cells with high UCP-2 expression, such as A549 and H460, harbor wild-type p53 (42). High concentrations of UCP-2 in these cells may protect the cells from oxidative stresses and subsequent cell damage by reducing ROS production. A previous report that UCP-2 inhibits p53-induced apoptosis via post-translational modification of p53 (20) highlights other mechanisms whereby UCP-2 protects cells from death. We found that knockdown of UCP-2 expression by siRNA restored Stat3 activation and the expression of survivin in A549 and H460 cells, although the restored Stat3 activation did not prevent cell death in this study. Downregulation of UCP-2 in these two cell lines resulted in significantly increased cell death in A549 and H460 cells following paclitaxel treatment (Supplementary Figure 6B is available at Carcinogenesis Online). Our data indicate that UCP-2 plays a more important role in cell survival than Stat3 in A549 and H460 cells.

Cells with a p53 mutation can tolerate greater ROS stresses than cells with wild-type p53 and they are more biologically aggressive (43). In the current study, H157 and AS2 lung cancer cells with low UCP-2 expression were found to harbor mutant p53. The H157 cells had a nonsense mutation in codon 298 (44), whereas the AS2 cell had mutations in codons 72 and 248 of the p53 gene (unpublished data). Paclitaxel induction increased ROS production in both cell lines, but the cells were not killed by oxidative stress. ROS activated Stat3 and induced the expression of survivin. This finding is intriguing, although the association between UCP-2 expression and the p53 gene status requires further confirmatory work.

In our study, paclitaxel treatment induced a 1.5-fold increase in ROS levels in AS2, H157 and CL1-5 cells. The increased ROS levels were sufficient to activate a biological signaling cascade (45). In
breast cancer cells, paclitaxel induces a toxic bystander effect by generating extracellular H₂O₂ through a membrane-associated Nox (46). In AS2 cells, the addition of naloxone, a Nox inhibitor, did not block the paclitaxel-induced generation of ROS or the activation of Stat3. We did not monitor extracellular H₂O₂ or test the effects of other Nox inhibitors in our study; hence, we cannot exclude the possibility that ROS induced by paclitaxel may have been generated by the Nox system, in part.

ROS was reported to facilitate autophagy, which may lead to cell resistance to paclitaxel (47,48); therefore, we tested the expression of autophagy marker-LC3II in the four cancer cell lines after paclitaxel treatment. We found that paclitaxel gradually induced LC3II expression in AS2 and H157 but not in A549 and H460 cells (Supplementary Figure 6C is available at Carcinogenesis Online). Therefore, ROS-induced autophagy may contribute partially to the paclitaxel resistance of AS2 and H157 cells.

Phosphorylation of serine in Stat3 was reported to direct the molecule into mitochondria (6). In our study, treatment of lung cancer cell lines with paclitaxel and cisplatin differentially modulated tyrosine phosphorylation and Stat3-dependent gene activity. Therefore, the effect of chemotherapeutic agents on Stat3 appears to be primarily through tyrosine phosphorylation. In breast cancer cells, Liu et al. found that the abrogation of tyrosine phosphorylation in Stat3 by B7-H3 silencing increased the cancer cell sensitivity to paclitaxel (36) and our findings are consistent with this report.

In order to demonstrate that the UCP-2-regulated paclitaxel-induced activation of Stat3 is a general phenomenon, we studied two more cancer cell lines with the same genetic background. The CL1-5 cell line is a subclone of the CL1-0 cell line, but it is more aggressive than its parental cell line (24). CL1-5 cells had lower UCP-2 expression compared with CL1-0 cells, indicating that UCP-2 expression can be modulated when cells gain a new biological function. A previous report that the loss of transforming growth factor (TGF)-β signaling in breast cancer cells promotes UCP-2 expression (49) supports our hypothesis. Paclitaxel induced increases in ROS production and Stat3 activation in CL1-5 cells but not in CL1-0 cells.

**Fig. 6.** UCP-2 expression was a survival predictor in inoperable lung cancer patients who received cisplatin-based chemotherapy. (A) Representative photomicrographs showing lung cancer and normal lung epithelial cells stained for UCP-2. Cancer cells with UCP-2 expression are marked with arrowheads. Sections were counterstained with hematoxylin. Magnification: ×400 (upper panels) and ×100 (lower panels). (B and C) Plots represent progression-free survival and overall survival in 43 inoperable lung cancer patients who received cisplatin-based chemotherapy as the first-line treatment.
After silencing UCP-2 in CL1-0 cells with UCP-2 siRNA, paclitaxel treatment induced the activation of Stat3, whereas overexpression of UCP-2 by cDNA transfection in CL1-0 cells effectively attenuated paclitaxel-induced Stat3 activation and survivin expression. CL1-5 cells engineered to overexpress UCP-2 were more sensitive to paclitaxel treatment than the control cells. Thus, our findings with CL1-0 and CL1-5 cells were consistent with those for A52, H157, A549 and H460 cells.

In our in vitro study, lung cancer cells (A52, H157 and CL1-5) with lower UCP-2 protein expression were generally more chemoresistant. In the clinical study, we found that low UCP-2 expression in the cancer cells of patients with advanced lung cancer predicted inferior responses to cisplatin-based chemotherapy and poor progression-free and overall survival prospects. Therefore, the in vitro and in vivo data were mutually consistent.

Cells (A52, H157 and CL1-5) with low UCP-2 protein expression showed greater chemoresistance, but pretreatment with FCCP or rotenone blocked paclitaxel-induced ROS generation, Stat3 activation and survivin expression, thereby enhancing paclitaxel cytotoxicity. We propose that compounds with the ability to reduce mitochondrial ROS production also have the potential to overcome drug resistance in cancer cells with low UCP-2 expression. In cancer cells (A549, H460 and CL1-0) with high UCP-2 expression, we showed that the knockdown of UCP-2 enhanced paclitaxel-induced cell death. The report that mesenchymal stromal cells support survival of A549 cells in condition of oxidative stress through the stanniocalcin-1-mediated upregulation of UCP-2 (50) is compatible with our findings. Further studies are warranted to select effective therapeutic modalities that are appropriate to cell UCP-2 expression.

In conclusion, we demonstrated that the level of UCP-2 expression in cancer cells determine the cell response to oxidative stress induced by anticancer drugs. Furthermore, chemotherapeutic agents can modulate Stat3 activation and survivin expression in cancer cells with low UCP-2 expression through mitochondrial ROS generation. Finally, UCP-2 is predictive of the response and survival of patients with advanced lung cancer who had received cisplatin-based chemotherapy. A prospectively clinical study with a large cohort is required to verify the role of UCP-2 during lung cancer progression.

Supplementary material
Supplementary materials and methods, Figures S1–S6 and Supplementary Table I can be found at http://carcin.oxfordjournals.org/.

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