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Original Article

Cisplatin sensitivity mediated by NKX2-1 in lung adenocarcinoma is dependent on p53 mutational status via modulating TNFSF10 expression

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Abstract: NKX2-1 was shown to enhance cisplatin sensitivity in KRAS-mutated cells, but it conferred cisplatin resistance in EGFR-mutated lung adenocarcinoma cells. However, NKX2-1 as a dual role in tumor progression depended on p53 mutational status via modulation of the NF-κB pathway. We hypothesized that NKX2-1 may confer cisplatin resistance in p53-mutated (p53-MT) lung adenocarcinoma cells but may enhance cisplatin sensitivity in wild-type (p53-WT) cells. In the present study, six p53-MT and -p53-WT cell lines were treated with various concentrations of cisplatin to calculate the inhibitory concentration of cisplatin for 50% cell viability (IC_{50}). The IC_{50} value was positively correlated with NKX2-1 expression in the p53-MT cells but negatively correlated in the p53-WT cells. TNFSF10 was identified in a microarray analysis as a potential candidate responsible for NKX2-1-mediated apoptosis induced by cisplatin. The retrospective study evaluated 97 surgically resected lung adenocarcinoma patients receiving cisplatin-based chemotherapy to explore the possible association between NKX2-1 expression and tumor response. Patients with higher TNFSF10 mRNA levels, as determined by real-time reverse transcription-polymerase Chain Reaction (RT-PCR), typically showed an favorable response when compared with patients with lower TNFSF10 mRNA levels. Additionally, the association of higher TNFSF10 mRNA levels with favorable response was only revealed in p53-WT patients, not in p53-MT patients. Higher NKX2-1 mRNA levels were associated with an unfavorable response in patients with p53-MT tumors but a favorable response in patients with p53-WT tumors. In summary, modulation of TNFSF10 expression by NKX2-1 may be a potential indicator for predicting the response to cisplatin-based chemotherapy in patients with lung adenocarcinomas.

Keywords: NKX2-1, TNFSF10, cisplatin, p53 mutational status, lung adenocarcinoma

Introduction

NKX2-1, a homeobox-containing transcription factor also known as thyroid transfection factor (TTF-1), plays an oncogenic role in lung adenocarcinomas [1-3]. About 70% of adenocarcinomas express NKX2-1, independent of disease stage, and they retain features of the terminal respiratory unit [4]. These terminal respiratory unit type adenocarcinomas exhibit a distinctively higher prevalence of EGFR mutations; however, their p53 and KRAS mutations are inversely associated with NKX2-1 expression [5, 6]. NKX2-1 transgenic mice exhibit hyperplasia of type II alveolar cells, and the lung adenocarcinoma metastatic potential in $KRAS^{LSL\text{-}G12D/+}$; $p53^{flox/flox}$ mice is suppressed by NKX2-1 [7]. Conversely, haploinsufficiency of NKX2-1 reduces tumor formation in EGFR-mutant transgenic mice [8]. These results suggest that NKX2-1 has either an oncogenic or tumor suppressor role, depending on the mutational status of KRAS, p53, or EGFR.

We previously reported that NKX2-1 serves as a transcription factor to upregulate p53 tran-

scription and consequently to modulate IKKβ transcription and the NF-κB signaling pathway [9]. We also showed that lung adenocarcinoma progression mediated by NKX2-1 was dependent on the p53 mutational status. In other words, NKX2-1 acted as an oncogene in p53-mutated (p53-MT) lung adenocarcinomas, whereas it acted as a tumor suppressor role in p53 wild-type (p53-WT) lung adenocarcinomas. Activation of the NF-κB signaling pathway by mutations of p53 and/or KRAS has been demonstrated to confer cisplatin resistance [10-13]. A recent study indicated that NKX2-1 may modulate cellular sensitivity to cisplatin by modulating the β-catenin pathway through phosphorylation of AKT [14]. We here hypothesized that the modulation of cisplatin sensitivity by NKX2-1 might occur through the modulation of the NF-κB signaling pathway.

In the present study, we have explored the nature of the gene alterations that might contribute to NKX2-1-mediated cisplatin sensitivity in lung adenocarcinoma cells via the NF-κB signaling pathway. Microarray analysis data revealed that tumor necrosis factor superfamily factor 10 (TNFSF10), which is regulated by the NF-κB signaling pathway [15], may play a crucial role in modulation of NKX2-1-associated cisplatin sensitivity in p53-MT and p53-WT lung adenocarcinoma cells. In addition, a retrospective analysis of 97 lung adenocarcinoma patients who had received cisplatin-based chemotherapy was conducted to confirm the potential association of NKX2-1 and TNFSF10 mRNA expression levels with the chemotherapeutic response observed in this study population.

Materials and methods

Study subjects

Lung tumors were enrolled from 157 lung adenocarcinoma patients who underwent surgical therapy at the Division of Thoracic Surgery, Taichung Veterans General Hospital, Taiwan, between 1993 and 2004. In total, 97 of the 157 patients received cisplatin-based chemotherapy. This study was approved by the Institutional Review Board, Taipei Medical University Hospital (TMUH No: 201301051). The tumor type and stage of each collected specimen were histologically determined according to the WHO classification system. Cancer relapse data and tumor response to cisplatin-based chemotherapy were obtained by chart review and further confirmed by two clinical physicians.

Tumor response

The tumor response was classified according to our previous report [16]. Tumor responses were categorized as follows: complete response was a complete disappearance of all the tumors; a partial response was a decrease in size or number of the tumor lesions by > or = 50%; progressive disease was at least a 25% increase in the size or number of the tumor lesions; and stable disease was neither a sufficient shrinkage to qualify as a partial response nor a sufficient increase to qualify as progressive disease. Therefore, a favorable response (complete response and partial response) was a ≥ 50% decrease in tumor size.

Cell lines

The A549, H23, H358, H441, H1355, and H1975 lung adenocarcinoma cells were obtained from the American Type Culture Collection (ATCC) and cultured as described previously [16]. The CL-3 and CL1-5 cells were kindly provided by Dr. P. C. Yang (Department of Internal Medicine, National Taiwan University Hospital, Taipei, Taiwan). The TL-4, TL-5, TL-6, TL-10, and TL-13 lung adenocarcinoma cells were established from pleural effusions from Taiwanese lung cancer patients and cultured as described previously [17, 18].

The 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cytotoxicity assay

The cell lines were maintained at 37°C in a humidified incubator containing 95% air and 5% CO_2. The cells were cultured in 96-well flat-bottomed microtiter plates containing RPMI-1640 (Roswell Park Memorial Institute-1640) and Dulbecco's modified Eagle's mediums (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 U/ml streptomycin. The cells were cultured in the exponential growth phase before cisplatin treatment. After 48 h of incubation, the in vitro cell viability induced by cisplatin was determined by MTT assay (at 570 nm) and the cell viability was expressed as a percentage of cells without cisplatin treatment (% of control).

Western blotting

The detailed procedures were described previously [16]. Cells were washed twice on ice with PBS before adding protein lysis buffer (1 × protease inhibitor cocktail [Roche, Basel, Switzerland], 1.5 mM EDTA, 1 mM DTT, 10% glycerol, 25 mM HEPES, pH 7.6). Total protein (20 μg) was resolved by 10% SDS-PAGE for subsequent western blot analysis using antibodies diluted in TTBS. The antibodies for detection of NKX2-1, TNFSF10, caspase 8, and β-actin were purchased from Santa Cruz CA (USA). The gel was transferred to a Hybond-C Extra membrane (GE Healthcare, Little Chalfont, UK) and immunoblotted with primary antibody, as indicated in the figure legends. Anti-mouse or rabbit IgG conjugated to horseradish peroxidase was used as the secondary antibody for detection using an ECL western blot detection system.

Microarray analysis

Total RNA from the H441 and H441 shNKX2 cells was isolated using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. The quantity and quality of extracted RNA was assessed using a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). First-strand cDNA was synthesized from the total RNA using SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA). The labeled cDNAs of the H441 and H441 shNKX2 were then used in the microarray analysis (Phalanx Biotech, Taiwan).

Real-time reverse transcription (RT)-polymerase chain reaction (PCR)

The detailed procedures were described previously [9]. The expression of NKX2-1 and TNFSF10 mRNA levels were determined by real-time RT-PCR analysis using 18S rRNA as an internal control. The relative amounts of the target gene, standardized against the amount of 18S rRNA, were expressed as ΔCt = Ct (target) - Ct (18S rRNA). The ratio of gene mRNA copies to 18S rRNA copies was then calculated as $2^{-\Delta Ct} \times K$ (K = 104, a constant).

Statistical analysis

The χ^2 test was performed using SPSS software (version 13.0; SPSS, Inc., Chicago, IL, USA). Statistical analysis was performed using Chi-square test and $P < 0.05$ compared to the control was considered statistically significant.

Results

Association of NKX2-1 expression levels with cisplatin sensitivity of p53-MT and p53-WT lung adenocarcinoma cells

A panel of p53-MT and p53-WT lung adenocarcinoma cell lines was collected to explore the possible association between NKX2-1 expression and cisplatin sensitivity. The MTT assay was performed to evaluate the inhibitory concentration of cisplatin for 50% cell viability (IC_{50}) in each cell line. Western blotting indicated various NKX2-1 expression levels in the different lung adenocarcinoma cell lines, regardless of their p53 mutational status (**Figure 1A**). The expression levels of NKX2-1 and the IC_{50} values are presented at the bottom of **Figure 1A**. The IC_{50} values of all 12 cell lines showed no association with NKX2-1 expression levels (**Figure 1B** left panel). When the cell lines divided into two subgroups according to their p53 mutational status, the IC_{50} value of the p53-MT cells was positively correlated with NKX2-1 expression levels ($R^2 = 0.6117$, $P = 0.019$), whereas the IC_{50} values were negatively correlated with NKX2-1 expression levels in the p53-WT cells ($R^2 = 0.701$, $P = 0.010$; **Figure 1B**, middle and right panels). We further selected two p53-MT cells, a higher NKX2-1-expressing CL1-0 and a lower NKX2-1-expressing H1299 cells [19], to confirm that a higher IC_{50} value was observed in CL1-0 cells than in H1299 cells (24.6 μM vs. 10.5 μM). These results suggest that higher NKX2-1 expression may confer cisplatin resistance in p53-MT cells, whereas higher NKX2-1 expression may enhance cisplatin sensitivity in p53-WT cells, when compared with cells with lower NKX2-1 expression.

NKX2-1-silenced p53-MT H441 cells show decreased BUB1B and caveolin 1 and increased GADD45G and TNFSF10 expression

Microarray analysis was used to identify potential gene alterations that could contribute to NKX2-1-mediated cell apoptosis. P53-MT H441 cells with higher expression of NKX2-1 were selected for silencing NKX2-1 expression with a small hairpin RNA (shRNA). The NKX2-1-knockdown H441 cells showed an approximately 3-fold decrease in BUB1B and caveolin 1 (CAV1) expression and an approximately 3-fold

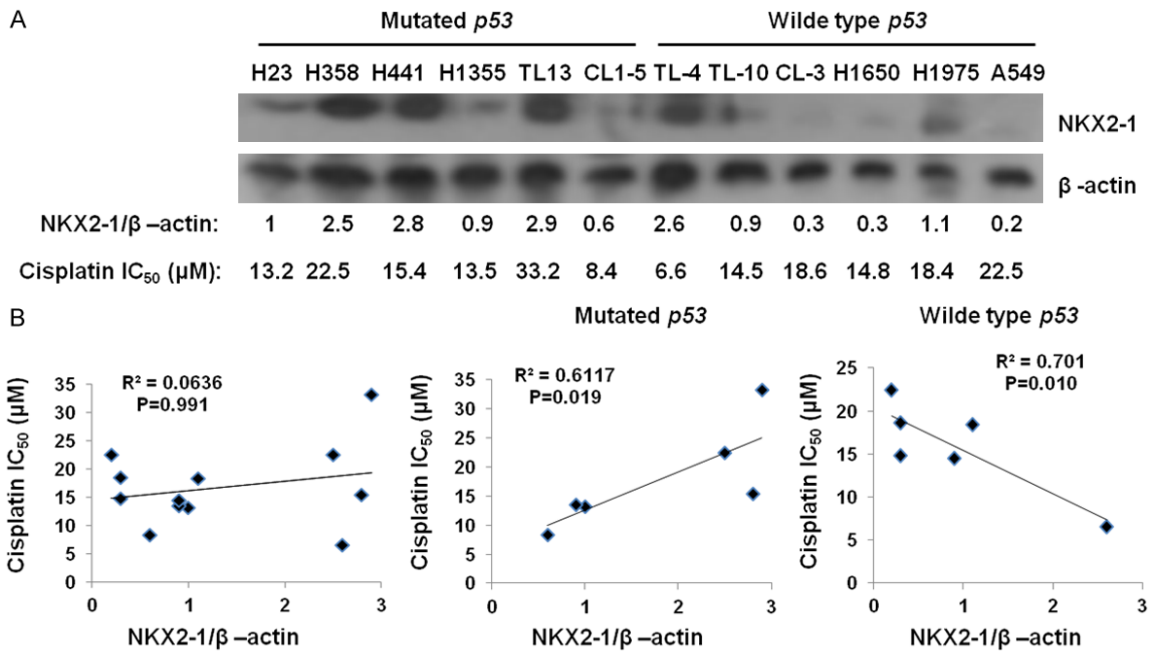

Figure 1. The IC_{50} values for p53-MT and p53-WT lung adenocarcinoma cells, evaluated by the MTT assay, were calculated from dose-response curves. Western blotting was used to detect the expression of NKX2-1 in a panel of p53-MT and p53-WT cell lines. β-catenin was used as a protein loading control.

increase in GADD45G and TNFSF10 expression when compared with H441 cells transfected with non-specific shRNA (H441 NC) (**Figure 2** left panel). To confirm the results of microarray analysis, other two cell lines, p53-WT A549 and p53-MT H358, were selected for overexpression and knockdown, respectively, using two doses (2 and 5 μg) of the NKX2-1 expression vector and its shRNA, respectively. As expected, a dose-dependent decrease in CAV1 and BUB1B expression and a dose-dependent increase in GADD45G and TNFSF10 expression were observed in NKX2-1-knockdown H441 cells, in NKX2-1-knockdown H358 cells, and in NKX2-1-overexpressing A549 cells (**Figure 2** right panel). These results suggest that these four genes (CAV1, BUB1B, GADD45G, and TNFSF10) might be significant contributors to NKX2-1-modulated cisplatin-induced apoptosis in p53-MT and p53-WT lung adenocarcinoma cells.

TNFSF10 may be responsible for NKX2-1-modulated cisplatin-induced apoptosis

TNFSF10 is a p53 target gene that mediates p53-dependent cell death [20]. We therefore selected to further examine whether TNFSF10 could be responsible for NKX2-1-mediated apoptosis induced by cisplatin. The H441, H358, and A549 cell lines were transfected with shNKX2-1 or NKX2-1 expression vectors and/or transfected with TNFSF10 shRNA and then treated with or without cisplatin (0 and 5 μg). The MTT assay was performed to calculate the IC_{50} vales. Western blotting showed the expected changes in NKX2-1 and TNFSF10 expression in response to NKX2-1 knockdown and/or TNFSF10 knockdown in p53-MT H441 and H358 cells. Similarly, both NKX2-1 and TNFSF10 expressions were increased and decreased, respectively, in NKX2-1-overexpressing and/or TNFSF10-knockdown p53-WT A549 cells (**Figure 3** upper panel). The caspase 8 precursor was expressed in NKX2-1-knockdown and/or TNFSF10-knockdown H441 as well as in H358 cells and in NKX2-1-overexpressing and/or TNFSF10-knockdown A549 cells in the presence or absence of cisplatin. Interestingly, the expression of cleaved caspase 8 was significantly elevated in NKX2-1-knockdown H441 as well as in H358 cells and in NKX2-1-overexpressing A549 cells. The expression of cleaved caspase 8 was also increased by NKX2-1 knockdown in H441 as well as in H358 cells and in NKX2-1-overexpressing A549 cells, but the increase of cleaved caspase 8 expression was rescued by TNFSF10

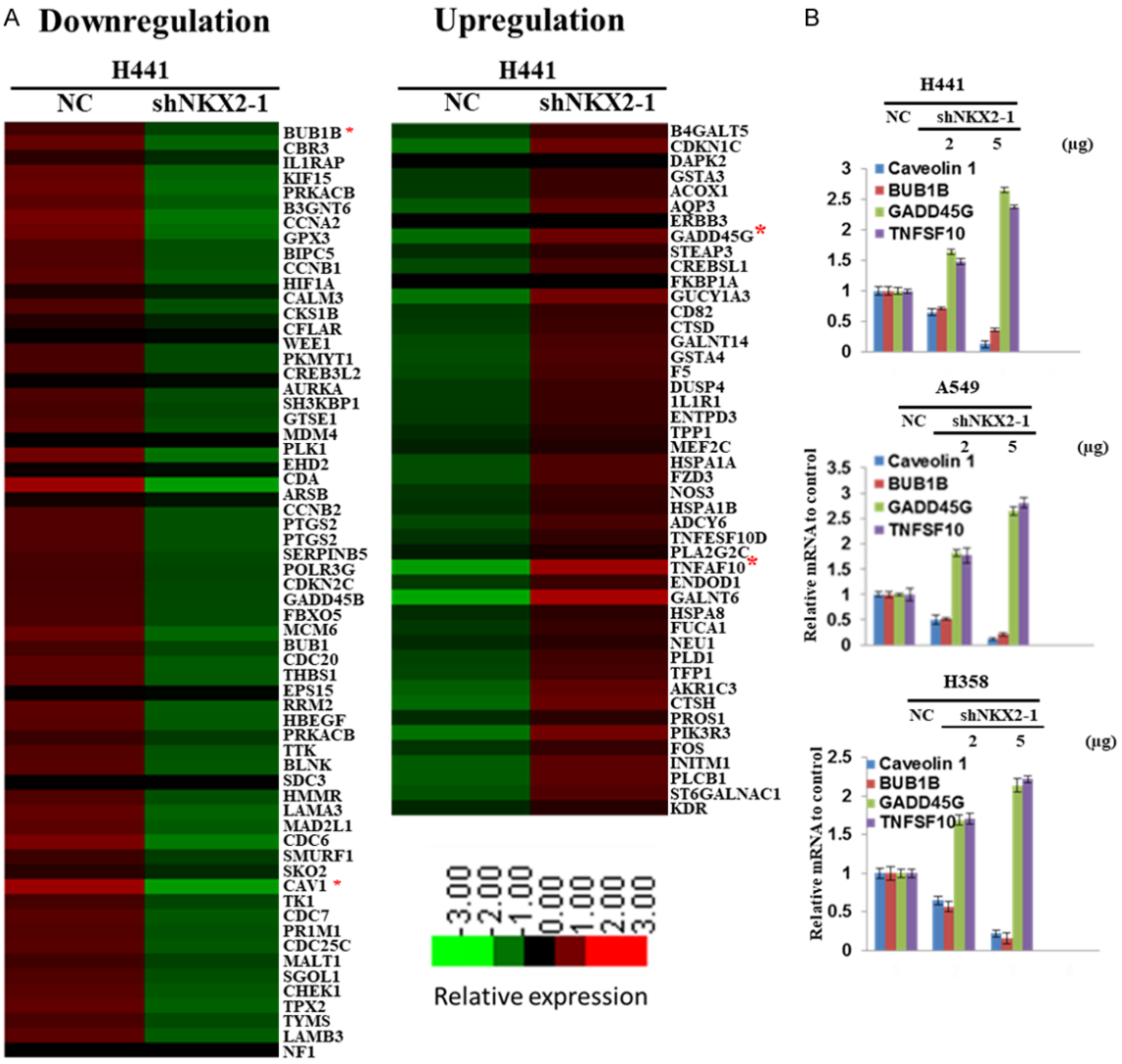

Figure 2. Microarray analysis was performed to compare the downregulation and upregulation of genes in NKX2-1-knockdown H441 cells versus their control cells (H441 NC) (downregulation: BUB1B and CAV1; upregulation: GADD45G and TNFSF10) (A). Two other cell lines, A549 and H358, were selected to verify whether these four genes detected in the microarray analysis (BUB1B, CAV1, GADD45G, and TNFSF10) could have similar effects in NKX2-1-knockdown H358 and NKX2-1-overexpressing A549 cells when compared to NKX2-1-knockdown H441 cells (B).

knockdown in these three cell lines. The MTT assay indicated that the IC_{50} value was markedly decreased by NKX2-1 knockdown in H441 and H358 cells, as well as in NKX2-1-overexpressing A549 cells, when compared to their control cells (H441: 15.6 vs. 5.2 μM; A549: 20.4 vs. 6.0 μM; H358: 21.6 vs. 8.4 μM), but the decrease in the IC_{50} value by NKX2-1 knockdown or overexpression in these three cell lines was nearly completely rescued by TNFSF10 silencing (H441: 12.8 vs. 15.6 μM; A549: 18.0 vs. 20.4 μM; H358: 19.2 vs. 21.6 μM). These results strongly suggest that NKX2-1 modulation of TNFSF10 expression may be responsible for cisplatin-induced apoptosis in p53-MT and p53-WT lung adenocarcinoma cells.

Association of NKX2-1 and TNFSF10 mRNA expression levels with the tumor response in patients with lung adenocarcinoma who received cisplatin-based chemotherapy

A total of 97 lung adenocarcinoma patients who had received cisplatin-based chemotherapy were enrolled to verify the possible association of NKX2-1 and TNFSF10 expressions with the therapeutic response to cisplatin-bas-

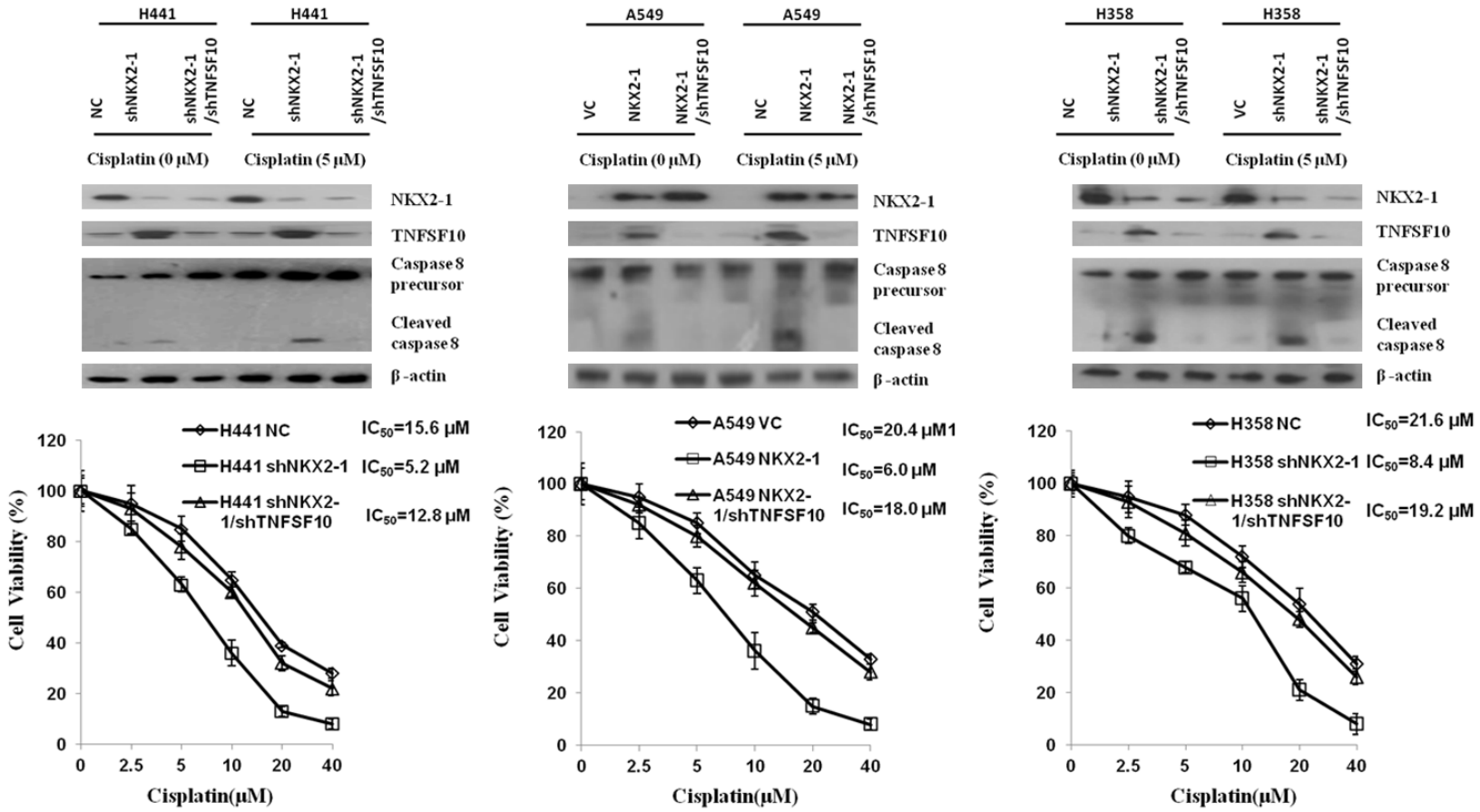

Figure 3. TNFSF10 may be responsible for NKX2-1-modulated apoptosis induced by cisplatin in H441, A549, and H358 cells. The expression of NKX2-1 in H441 and H358 cells was silenced by transfection with shNKX2-1 and/or shTNFSF10 in the presence or absence of cisplatin treatment (5 μM). The A549 cells were transfected with NKX2-1 expression vector, and/or shTNFSF10, followed by treatment with or without cisplatin (5 μM). Western blotting was performed to evaluate the expression of NKX2-1, TNFSF10, caspase-8 precursor, and cleaved caspase-8 following transfection with shNKX2-1, NKX2-1 expression vector, and/or shTNFSF10. β-catenin was used as a protein loading control (upper panel). The cell lines transfected with shNKX2-1, NKX2-1 expression vector, and/or shTNFSF10 were treated with various concentrations of cisplatin. Cell viability curves were used to calculate the IC_{50} values (lower panel).

Table 1. The association of NKX2-1 and TNFSF10 mRNA expression levels with tumor response in patients with lung adenocarcinoma who received cisplatin-based chemotherapy

Variables	Patients No.	Tumor response		*P* value
		Favorable	Unfavorable	
p53 status				
p53-WT	78	28 (36)	50 (64)	0.217
p53-MT	19	4 (21)	15 (79)	
TNFSF10				
Low	48	10 (21)	38 (79)	0.012
High	49	22 (45)	27 (55)	
NKX2-1				
Low	48	12 (25)	36 (75)	0.131
High	49	20 (41)	29 (59)	
p53-WT				
TNFSF10				
Low	36	12 (33)	24 (67)	
High	42	32 (76)	10 (24)	< 0.0001
NKX2-1				
Low	42	8 (17)	34 (83)	0.001
High	36	20 (56)	16 (44)	
p53-MT				
TNFSF10				
Low	12	2 (17)	10 (73)	
High	7	3 (43)	4 (54)	0.103
NKX2-1				
Low	6	4 (66)	2 (34)	0.004
High	13	0 (0)	13 (100)	

The median value of NKX2-1 and TNFSF10 mRNA expression levels were used to as an cutoff point to divide patients' tumors into "low" and "high" subgroups. *P* values were obtained from the statistical analysis by Chi-square test.

ed chemotherapy. NKX2-1 and TNFSF10 mRNA levels in tumors of these patients were evaluated by real-time RT-PCR. The median value of both gene mRNA expressions was used to divide patients into a "low" or a "high" subgroup. The tumor response to cisplatin-based chemotherapy in this study population was not associated with p53 mutational status (P = 0.217, **Table 1**). Interestingly, a higher prevalence of unfavorable responses was observed in the low TNFSF10 mRNA subgroup than in the high TNFSF10 mRNA subgroup (79% vs. 55%, P = 0.012; **Table 1**). Additionally, the association of lower TNFSF10 mRNA levels with unfavorable response was only revealed in p53-WT patients (P < 0.0001), not in p53-MT patients (P = 0.103). The expression of NKX2-1 mRNA was not associated with tumor response in the overall study population, but when the study population was divided into p53-MT and p53-WT patients, the NKX2-1 mRNA expression was correlated with tumor response. Among the p53-WT patients, a higher frequency of unfavorable responses was seen in low the NKX2-1 subgroup than in the high NKX2-1 subgroup (83% vs. 44%, P = 0.001; **Table 1**). Conversely, among the p53-MT patients, a higher prevalence of unfavorable responses was shown in the high NKX2-1 subgroup than in the low NKX2-1 subgroup (100% vs. 34%, P = 0.004; **Table 1**). These results from patients strongly supported the findings of the cell model, whereby NKX2-1 promoted cisplatin-induced apoptosis in p53-WT lung adenocarcinoma but reduced cisplatin-induced apoptosis in p53-MT lung adenocarcinoma, and these responses occurred through modulation of TNFSF10 expression.

Discussion

The results of this study provide evidence that NKX2-1 plays a dual role in cisplatin-induced apoptosis in p53-MT and p53-WT lung adenocarcinoma cells. Specifically, NKX2-1 expression may confer cisplatin resistance by decreasing TNFSF10 expression in p53-MT cells; however, it may enhance cisplatin sensitivity by increasing TNFSF10 expression in p53-WT lung adenocarcinoma cells (**Figure 3**). This association of NKX2-1 and TNFSF10 mRNA levels with the chemotherapeutic response was observed in lung adenocarcinoma patients who had received cisplatin-based chemotherapy (**Table 1**). These observations indicate that TNFSF10 may be responsible for cisplatin sensitivity mediated by NKX2-1 in p53-MT and p53-WT lung adenocarcinoma cells and in patients with lung adenocarcinoma.

TNFSF10, which is a member of the TNF family, has been viewed as a promising antitumor agent due to its ability to kill many tumor cells while sparing most normal cells [15, 21]. TNFSF10 induces cancer cell death by binding to its receptors to activate the caspase 8-dependent apoptosis pathway [22]. The other three genes (BUB1B, CAV1, and GADD45G) have re-

ported relationships to apoptosis but operate via different mechanisms [23-26]. For example, BUB1B was identified as a downstream gene of the NF-κB signaling pathway and was involved in the growth and survival of lymphoma cells [23, 24]. Similarly, CAV1 was shown to promote resistance to chemotherapy in various cancers, including ovarian cancer, but it acted by activation of the Notch-1/Akt/NF-κB signaling pathway [25]. By contrast, GADD45G acts as one of the master switches in life and death decision in cancer cells, so that NF-κB-mediated cell survival and apoptosis are absolutely dependent on GADD45G [26]. These three genes identified from our microarray analysis might reflect an NKX2-1-modulated NF-κB signaling pathway. This possibility was consistent with our previous findings of an NKX2-1-modulated NF-κB signaling pathway in lung adenocarcinoma cells that was dependent on p53 mutational status [9]. The role of these three genes (BUB1B, CAV1, and GADD45G) on cisplatin sensitivity mediated by NKX2-1 should be further investigated. Nevertheless, the results of the present study clearly indicated that modulation of TNFSF10 by NKX2-1 may be responsible for cisplatin sensitivity in p53-WT and p53-MT lung adenocarcinoma cells.

In summary, we have demonstrated that increases and decreases in TNFSF10 expression by NKX2-1 may be responsible for cisplatin-mediated apoptosis in p53-MT and p53-WT lung adenocarcinoma cells. Therefore, the cisplatin resistance conferred by NKX2-1 in p53-MT lung adenocarcinoma cells and the enhanced cisplatin sensitivity conferred by NKX2-1 in p53-WT lung adenocarcinoma cells may arise by modulation of TNFSF10 expression. The response of NKX2-1 and TNFSF10 mRNA expression levels to chemotherapy in lung adenocarcinoma patients further confirmed the findings obtained using the cell model. We conclude that NKX2-1 may be a potential biomarker for predicting the chemotherapeutic response in p53-MT and p53-WT patients with lung adenocarcinomas.

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Disclosure of conflict of interest

None.

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