Activation of NF-κB by SOD2 promotes the aggressiveness of lung adenocarcinoma by modulating NFκX2-1-mediated IKKB expression

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Manganese superoxide dismutase (SOD2) has been shown to cause dysfunction of p53 transcriptional activity, whereas, in turn, SOD2 expression is regulated by p53 to modulate lung tumorigenesis. In this study, we found that the level of SOD2 expression in a panel of lung cancer cells was negatively correlated with that of NK2 homeobox 1 (NKX2-1) but was not associated with p53 status. Mechanistic studies indicated that a decrease in NKX2-1 caused by SOD2-activated IKKβ transcription was achieved by derepression of binding of Sp1 to the IKKβ promoter. Immunoprecipitation, glutathione S-transferase pull-down experiments and electrophoretic mobility shift assays demonstrated a direct interaction between NKX2-1 and Sp1, blocking Sp1-mediated IKKβ transcription. SOD2-mediated nuclear factor-kappaB activation, via elevation of IKKβ transcription, promoted anchorage-independent soft-agar growth, invasion and xenograft tumor formation, because of development of the epithelial-to-mesenchymal transition. The expression level of NK2-1 messenger RNA was negatively associated with the extent of SOD immunostaining and the IKKβ messenger RNA expression level in lung tumors. The extent of SOD immunostaining and IKKβ messenger RNA levels may independently predict overall survival and relapse-free survival in lung adenocarcinoma patients. In summary, we found that SOD2 activates nuclear factor-kappaB signaling by increasing IKKβ transcription, which results in progression of lung adenocarcinoma and poorer patient outcomes. We suggest that IKKβ may potentially be targeted to improve outcomes in patients with SOD2-positive tumors.

Introduction

Manganese superoxide dismutase (SOD2), encoded on human chromosome 6q25, is a mitochondrial matrix protein catalyzing the dismutation of superoxide radicals into hydrogen peroxide (1). Therefore, SOD2 may be expected to exhibit tumor-suppressor properties; indeed, overexpression of SOD2 in pancreatic adenocarcinoma, breast cancer and colorectal cancer cells results in marked suppression of cell growth (2–7). In animal models, increases in the SOD2 gene dose significantly decreased aneuploidy in premalignant thymocytes and slowed the onset of tumor formation in Bax-transgenic mice (8). Also, SOD2 promoted invasion and migratory activity of HT-1080 fibrosarcoma and 253J transitional bladder carcinoma cells; invasion was enhanced via upregulation of matrix metalloproteinase (MMP)-1 transcription (9). In breast cancer, the expression levels of SOD2 are higher in aggressive cells than in nonaggressive cells, and MMP-2 upregulation by SOD2 was reported to be potentially responsible for increased cell invasiveness (10–12). Similarly, SOD2 promoted breast tumor metastasis by increasing the level of MMP-2 expression, and MMP-2 may be upregulated by the action of the nuclear factor-kappaB signaling pathway (13,14). SOD2 has been shown to promote prostate cancer progression by driving neuroendocrine differentiation and cell growth (15). Therefore, SOD2 may act as a tumor promoter gene; however, the underlying mechanism of action remains poorly understood.

A significant proportion of lung adenocarcinomas appears to involve activity of NK2 homeobox 1 (NKX2-1) (also termed thyroid transcription factor, TTF-1); this is a novel candidate proto-oncogene encoded on chromosome 14q13.3. However, any oncogenic role for NKX2-1 has not been fully supported in work on lung cancer cell lines (16). In contrast, NKX2-1 plays a tumor-suppressor role in progression of lung adenocarcinoma, as has been demonstrated in (KrasG12D/LSLG12D plus p53flox/flox)-transgenic mice (17). Such contradictory findings complicate the definition of NKX2-1 as an oncogene or tumor-suppressor gene involved in the tumorigenesis of lung adenocarcinomas.

SOD2 has been shown to cause dysregulation of p53 transcription in breast cancer cells (18). Reciprocal regulation by p53 has been demonstrated in terms of SOD2 expression in such cells (18). SOD2 expression is regulated by p53; the SOD2 protein, thus, plays entirely different roles in early and late-stage skin tumorigenesis (19). In addition, SOD2 expression is regulated by the IKKB/NF-kB signaling pathway (20). A previous report found that H2O2 production by SOD2 can reduce expression of the SP-A, SP-B and SP-C surfactants via a decrease in NKX2-1 expression level (21). However, our preliminary data indicate that SOD2 expression is not associated with the p53 status of lung cancer cells. In addition, the SOD2 expression level was negatively correlated with that of NKX2-1. Therefore, we hypothesized that SOD2 could suppress NKX2-1 to modulate progression of lung adenocarcinoma. Our mechanistic studies indicated that the NF-kB signaling pathway was activated via SOD2-mediated NKX2-1 suppression, in turn triggered by upregulation of IKKβ transcription, subsequently causing tumor progression and poor patient outcomes.

Materials and methods

Abbreviations: cDNAs, complementary DNAs; ChiP, chromatin immunoprecipitation; CI, confidence interval; GST, glutathione S-transferase; HR, hazard ratio; MMP, matrix metalloproteinase; NKX2-1, NK2 homeobox 1; mRNA, messenger RNAs; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; PBS, phosphate-buffered saline; RT–PCR, reverse transcriptase–polymerase chain reaction; SOD2, superoxide dismutase,
Plasmid construction

Full-length human SOD2 and NKKX2-1 complementary DNAs (cDNAs) were amplified from A549 and TL-4 total messenger RNAs (mRNAs) via reverse transcriptase–PCR (RT–PCR) using primers based on published mRNA sequences (GenBank: BC012423.1). The SOD2 and NKKX2-1 cDNAs were cloned into pcDNA3.1 Zeo+ (Invitrogen, Carlsbad, CA). The shSOD2, shNKKX2-1, and shCtrl plasmids were obtained from the National RNAi Core Facility of the Institute of Molecular Biology/Genomics Research Center, Academia Sinica, Taiwan. The IKKβ–Luc plasmid was constructed by inserting a Kpnl/XhoI fragment into the Kpnl/XhoI-treated pGL3 vector (Promega, Madison, WI). IKKβ promoter–driven luciferase reporter vectors (the promoters contained point mutations in Sp1 sites) were generated using the Quick Change site-directed mutagenesis system (Stratagene, Invitrogen). Site-directed mutagenesis was used to generate mutations in the Sp1-binding sites of the IKKβ promoter (Supplementary Figure 1, available at Carcinogenesis Online).

Different levels of expression plasmids were transiently transfected into lung cancer cells (1 × 10⁶) using the Transfast reagent (Fermentas). After 48 h, cells were harvested, and whole-cell extracts were assayed in subsequent experiments. The NF-kB–luciferase construct was kindly provided by Dr T.-C. Tsou (Division of Environmental Health and Occupational Medicine, National Health Research Institutes, Zhunan, Miaoli 350, Taiwan).

Measurement of SOD2 activity

At least 10 cells were washed using phosphate-buffered saline (PBS) and lysed in ice-cold 0.1 M Tris/HCl (pH 7.4) containing 0.5% Triton, 5 mM β-mercaptoethanol and 0.1 mg/ml phenylmethylsulfonyl fluoride. Lysates were clarified by centrifugation at 14 000g at 4°C for 5 min and cell debris was discarded. SOD activity was detected using a commercial ‘SOD Assay Kit-WST’ according to the manufacturer’s protocol (Dojindo Molecular Technologies, Japan). SOD2 activity was measured in the presence of a CuZnSOD inhibitor (3 mM NaCN) and normalized to total protein content. The highly water-soluble tetrazolium salt WST-1 (2-[4-(iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2-tetrazolium, monosodium salt], which produces a water-soluble formazan dye upon reduction by the superoxide anion, was used to measure SOD2 activity. Absorbance values at 450 nm were measured using a microplate reader; the experiments were performed in triplicate.

Electrophoretic mobility shift assay

Three micrograms of an anti-Sp1 antibody, 100 pmol of surfactant protein B promoter DNA and (in test samples) 3 μg glutathione S-transferase (GST)-NKKX2-1 (amino acids 101–230) were added to nuclear extracts (10 μg of protein) and the mixtures were incubated at 25°C for 20 min. Reactions were further incubated at 25°C for 20 min after addition of 10 pmol of a biotin-labeled Sp1 oligonucleotide from the binding site of the IKKβ promoter; the oligonucleotide was as follows: 5′-ACGCCACCCCCGCCGC-3′. The reaction mixture was electrophoresed at 4°C in a 6% polyacrylamide gel using Tris–borate-ethylenediaminetetraacetic acid as the running buffer. The detection solution was purchased from Pierce (Washington) and used according to the instructions of the manufacturer.

Real-time RT–PCR and western blotting

These assays were performed as described previously (22).

Soft-agar colony formation assay and Boyden chamber invasion assay

These assays were performed as described previously (23).

Luciferase reporter assay

The luciferase assay was performed using a luciferase reporter assay system (Promega) 48 h after transfection. Luciferase activity was normalized to that of β-galactosidase and (luciferase activity/β-galactosidase activity) ratios are reported.

Nuclear/cytoplasmic fractions of lung cancer cells

Nuclear/cytoplasmic lysates were prepared using a nonionic detergent method. Briefly, nuclear extracts were prepared in extraction buffer (10 mM KCl, 10 mM N-2-hydroxethylpiperazine-N′-2-ethanesulfonic acid, 1.5 mM MgCl₂, 0.5 mM dithiothreitol and protease inhibitors; pH 7.9). Extracts were centrifuged at 14 000g for 1 min and the supernatants (the cytoplasmic extracts) were aspirated into fresh tubes. Pellets were suspended in extraction buffer [420 mM NaCl, 20 mM N-2-hydroxethylpiperazine-N′-2-ethanesulfonic acid, 1.5 mM MgCl₂, 0.2 mM ethylenediaminetetraacetate acid, 25% (v/v) glycerol, 0.5 mM dithiothreitol, and 0.5 μM phenomenol, and pH 7.9] and recentrifuged at 14 000g for 5 min. The supernatants were the nuclear extracts. Both types of extracts were stored at −70°C for later evaluation.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) analysis was conducted according to a report published previously (Supplementary Table 1, available at Carcinogenesis Online) (22).

Xenograft tumors in nude mice

For in vivo tumor growth assays, female immunodeficient nude mice (BALB/c nu/nu mice), 5 weeks of age and weighing 18–22 g, were purchased from the National Laboratory Animal Center, Taiwan. Mice were housed under pathogen-free conditions with a 12 h light/12 h dark cycle and fed an autoclaved diet of standard rodent chow with ad libitum access (LabDiet, 5001). To establish A549 tumor xenografts, mice were injected subcutaneously with 3 × 10⁶ A549 (shGFP) or A549 (shSOD2) stable cell lines (100 μl amounts) plus 100 μl Matrigel (BD Biosciences, catalog no. 354234).

Cell proliferation assay

One thousand cells, seeded into wells of 96-well plates, were stained at the indicated time points with 100 μl amounts of sterile 3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) dye (0.5 mg/ml Sigma) for 4 h at 37°C, followed by removal of culture medium and addition of 150 μl amounts of dimethyl sulfoxide (Sigma). Absorbance was measured at 570 nm; 655 nm served as the reference wavelength. All experiments were performed in triplicate. Cells were counted with the aid of the MTT assay at each time point. The two-tailed Student’s t-test was used to explore the significance of simple comparisons between two values, when appropriate.

In vivo tumor metastasis assay

Female immunodeficient nude mice (BALB/c nu/nu mice), 5 weeks of age and weighing 18–22 g, were injected with A540/shGFP and A549/shSOD2 cells (n = 6 per group) via the tail vein (10⁶ cells in 0.1 ml amounts of PBS). After 62 days, mice were killed by administration of an overdose of anesthetic and lungs were fixed in 10% (v/v) formalin prior to hematoxylin and eosin staining. The numbers of lung tumor nodules in each mouse were counted.

Statistical analysis

Chi-square analysis was conducted using SPSS software (Version 13.0; SPSS, Chicago, IL). The significance of survival differences was calculated using the log-rank test. Survival curves were evaluated by the Kaplan–Meier method and variables affecting survival were analyzed using Cox proportional hazards regression model. A P value less than 0.05 was considered to be statistically significant.

Results

SOD2 expression was not related to p53 status but was negatively associated with NKKX2-1 and positively correlated with IKKβ expression

As mentioned above, SOD2 expression is regulated by p53 status and activity of the IKKβ/NF-kB signaling pathway, and SOD2–induced H₂O₂ production may suppress NKKX2-1-mediated SP-A, SP-B and SP-C expression. We therefore examined relationships between SOD2 expression and IKKβ, NKKX2-1 and p53 status in a panel of lung cancer cells. Western blotting showed that SOD2 expression was not affected by p53 status in a panel of eight lung cell types but was positively correlated with IKKβ expression (Figure 1A). As expected, SOD2 expression was negatively correlated with NKKX2-1 expression (Figure 1A). The level of SOD2 enzyme activity was linearly correlated with the SOD2 protein expression level in these eight lung cancer cell lines (R = 0.714, P = 0.047, Figure 1B: Upper panel) and the level of SOD2 enzyme activity in the cells was negatively correlated with NKKX2-1 mRNA expression (R = −0.74, P = 0.013; Figure 1B: Lower panel). Two cell lines that expressed wild-type p53 and two that did not, expressing high or low levels of SOD2, were selected to explore whether SOD2 could modulate NKKX2-1 expression in lung cancer cells of different p53 status. A549 and H1355 cells (expressing SOD2 at high levels) were transfected with two types of short hairpin RNAs targeting SOD2. TL-4 and CL-1-0 cells (expressing SOD2 at low levels) were transfected with a SOD2 cDNA plasmid. Western blotting and real-time RT–PCR analysis showed that the level of SOD2 protein expression was negatively correlated with that of NKKX2-1 protein and NKKX2-1 mRNA expression in these four cell types (Figure 1C). The results showed that SOD2 expression might decrease expression.
SOD2 promotes tumor malignancy via activation of NF-κB

Reduction of NKX2-1 expression by SOD2 promoted soft-agar growth and the invasive capacity of tumor cells via NF-κB activation, in turn increasing IKKβ expression.

Based on the data of Figure 1A, we hypothesized that reduction in NKX2-1 expression caused by SOD2 might promote tumor malignancy via NF-κB activation. Western blotting showed that the NKX2-1 expression level reduced in SOD2-overexpressing TL-4 cells and elevated in SOD2-knockdown A549 cells (Figure 2A). IKKβ expression increased in SOD2-overexpressing TL-4 cells and decreased in SOD2-knockdown A549 cells in a dose-dependent manner (Figure 2A). Concomitantly, phosphorylation of the IκBα protein increased in SOD2-overexpressing TL-4 cells and decreased in SOD2-knockdown A549 cells. As a result, an increase in nuclear p65 level in SOD2-overexpressing TL-4 cells and a decrease in such expression in SOD2-knockdown A549 cells were observed (Figure 2A: middle panel). However, such a change in p65 expression level was not observed in SOD2-overexpressing TL-4 cells or SOD2-knockdown A549 cells (Figure 2A: upper panel). The use of a luciferase reporter assay further confirmed that SOD2 overexpression or knockdown in lung cancer cells modulated NF-κB reporter activity (Figure 2A). Interestingly, IKKβ levels, phosphorylation of IκBα, nuclear p65 levels, the DNA-binding activity of NF-κB and NF-κB reporter activity of SOD2-overexpressing TL-4 cells were all reduced by transfection of an NKX2-1-expressing plasmid (5 μg) (Figure 2A). The opposite was seen when SOD2-knockdown A549 cells were transfected with

Fig. 1. NKX2-1 expression is deregulated by SOD2. (A) The expression levels of SOD2, NKX2-1 and IKKβ in a panel of lung cancer cells with or without p53 mutations were evaluated by western blotting. (B) SOD2 enzyme activity and SOD2 protein levels in these cells were measured as described in Materials and methods. Data are presented with the Kendall tau-b correlation coefficient (R = 0.714, P = 0.047); SOD2 enzyme and NKX2-1 mRNA levels in these cells were measured as described in Materials and methods. Data are presented with the Kendall tau-b correlation coefficient (R = –0.74, P = 0.013). Cells were collected, stained or lysed, as indicated. (C) SOD2 expression was depleted upon transfection of A549 and H1355 cells with either of two types of SOD2 short hairpin RNA and SOD2 was overexpressed upon transfection of TL-4 and CL1-0 cells with either of two levels of a plasmid carrying SOD2 cDNA. The levels of the SOD2 and NKX2-1 proteins were evaluated by western blotting; β-actin was used as a loading control. NKX2-1 mRNA levels were determined by real-time RT–PCR. VC, vector control; NC, non-specific short hairpin RNA control (shGFP vector). Means ± standard deviations were calculated using the data from three independent experiments.

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The effects of NKX2-1 on SOD2-mediated IKKβ expression, and activation of the NF-kB signaling pathway, also observed in p53-mutated CL1-0 and H1355 cells (Supplementary Figure 2A, available at Carcinogenesis Online). These results clearly indicate that the reduction in NKX2-1 level caused by SOD2 promotes NF-kB activation by increasing IKKβ transcription.

We next examined whether the reduction in NKX2-1 expression caused by SOD2 could promote anchorage-independent soft-agar colony growth and enhance the invasive capacity of tumor cells, as a result of increased IKKβ expression. As expected, the reporter activities of the IKKβ (–1105/+1)-Luc and IKKβ (–480/+1)-Luc promoters were significantly higher than in transfected TL-4 and CL1-0 cells and the luciferase reporter assay was performed 48 h later. The two putative Sp1-binding sites on IKKβ (–480/+1)-Luc were changed by site-directed mutagenesis and the mutated promoters were transfected into A549 and TL-4 cells to evaluate reporter activity.

NKX2-1 negatively regulated IKKβ transcription via suppression of Sp1 binding to the IKKβ promoter

We next asked whether the observed reduction in NKX2-1 level caused by SOD2, and promotion of tumor malignancy, might occur via upregulation of IKKβ transcription. We constructed two fragments [IKKβ (–1105/+1)-Luc and IKKβ (–480/+1)-Luc] using the promoter region of IKKβ. Software analysis identified two putative AP1 and two putative Sp1 binding sites in the promoter region.

The two putative Sp1-binding sites on IKKβ (–480/+1)-Luc were changed by site-directed mutagenesis and the mutated promoters were transfected into A549 and TL-4 cells to evaluate reporter activity. The binding activity of Sp1 to the IKKβ promoter was evaluated by ChIP in SOD2-knockdown A549 cells and SOD2-overexpressing TL-4 cells. Chromatin was isolated and immunoprecipitated using an anti-Sp1 antibody.
SOD2 promotes tumor malignancy via activation of NF-xB

SOD2 promotes tumor malignancy via activation of NF-κB (Figure 4B). An IKKβ (–480/+1)-Luc promoter was decreased in SOD2-knockdown A549 cells and increased in TL-4 cells overexpressing SOD2 (Figure 2E). Binding of Sp1 to the IKKβ (–480/+1)-Luc promoter decreased in SOD2-knockdown A549 cells and in TL-4 cells overexpressing SOD2 (Figure 2E). Lower panel). These results clearly indicate that the increase in IKKβ levels caused by SOD2 expression is mediated via derepression of the DNA-binding activity of Sp1 by SOD2.

NKX2-1 directly interacts with Sp1 to block Sp1-mediated IKKβ transcription

We next explored whether NKX2-1 could interact with Sp1 to modulate IKKβ transcription by decreasing the binding activity of Sp1 to the IKKβ promoter. As shown in Figure 3A, IKKβ expression decreased in NKX2-1-overexpressing A549 cells but increased in NKX2-1-knockdown TL-4 cells. We therefore hypothesized that SOD2 could promote lung tumor progression via NF-κB activation triggered by a reduction in NKX2-1 expression. The extent of interaction between NKX2-1 and Sp1, evaluated using a co-immunoprecipitation approach, elevated significantly in NKX2-1-overexpressing A549 cells but reduced in NKX2-1-knockdown TL-4 cells. ChIP analysis showed that Sp1-binding activity increased markedly in NKX2-1-knockdown TL-4 cells and decreased in NKX2-1-overexpressing A549 cells in a dose-dependent manner (Figure 3A: lower panel). To further explore whether interaction of NKX2-1 with Sp1 influenced IKKβ transcription, various levels of Sp1, NKX2-1 and a combination of both were used to treat A549 cells transfected with the IKKβ (–480/+1)-Luc promoter. Expression of Sp1 and NKX2-1 and both combinations in A549 cells was confirmed by western blotting (Figure 3B: upper panel). The reporter activity of the IKKβ (–480/+1)-Luc promoter was elevated by Sp1 transfection and reduced by NKX2-1 transfection; however, reporter activity was restored by transfection with Sp1 combined with NKX2-1. The IKKβ mRNA levels in A549 cells following the various treatments were consistent with IKKβ (–480/+1)-Luc reporter activity levels (Figure 3B: lower panel). We identified the domain of the NKX2-1 protein that interacted with Sp1 using GST pull-down assays. Three NKX2-1 domains (activation domain, amino acids 1–100; homeodomain: amino acids 101–230; and inhibitor domain-activation domain, amino acids 231–371; Figure 3C) were constructed and linked to GST. The molecular weights of these three domains of NKX2-1 were evaluated by western blotting, followed by Coomassie Blue staining (Figure 3C). The GST pull-down assay indicated that the NKX2-1 domain that interacted with Sp1 was located in the HD domain of the NKX2-1 protein. Sp1 in nuclear extracts of A549 cells served as a control (Figure 3C).

We next investigated whether GST-NKX2-1 (amino acids 101–230) could interact with Sp1 and inhibit DNA binding to the Sp1-binding site of the IKKβ promoter (a 15 bp oligonucleotide was used as probe). EMSA analysis showed that the Sp1 in nuclear extracts of A549 cells bound to the oligonucleotide; however, this binding of Sp1 was almost abolished upon addition of GST-NKX2-1 or anti-Sp1 antibody. In addition, the inhibition of Sp1 oligonucleotide-binding activity (thus to GST-NKX2-1; amino acids 101–230) was completely restored by addition of the SP-B promoter (–110 to –95 oligonucleotides) but such restoration was markedly reduced by anti-Sp1 antibody (Figure 3D). This was attributable to the fact that SP-B transcription is regulated by NKX2-1 (24). Therefore, addition of the SP-B promoter can decrease the interaction of NKX2-1 with Sp1, in turn increasing Sp1 binding to the IKKβ promoter. Thus, Sp1 binding to the oligonucleotide GST-NKX2-1 can be completely restored by addition of the SP-B promoter. These results clearly indicate that an interaction between NKX2-1 and Sp1 was responsible for suppression of Sp1-mediated IKKβ transcription.

An increase in IKKβ levels caused by SOD2 expression is responsible for epithelial-to-mesenchymal transition, invasiveness and xenograft tumor formation

The possibility that SOD2-mediated IKKβ expression might be responsible for the epithelial-to-mesenchymal transition and cell invasion was explored by transflecting TL-4 cells with an SOD2 expression vector (5 μg), followed by co-transfection with two different levels of shIKKβ. Western blotting showed that the levels of vimentin and snail both fell upon shIKKβ transfection but that E-cadherin levels increased in SOD2-overexpressing TL-4 cells (Supplementary Figure 4A: upper panel, available at Carcinogenesis Online). The Matrigel invasion capacity of SOD2-overexpressing TL-4 cells was decreased by shIKKβ in a dose-dependent manner (Supplementary Figure 4A: lower panel, available at Carcinogenesis Online). Compared with cells transfected with the empty vector, SOD2-expressing cells exhibited a spindle-like morphology (Supplementary Figure 4B: first line, available at Carcinogenesis Online). Confocal microscopy indicated that the level of E-cadherin expression in the cytoplasm of TL-4 cells overexpressing SOD2 was increased upon shIKKβ transfection (Supplementary Figure 4B: second line, available at Carcinogenesis Online).

We established stable SOD2-knockdown A549 clones to allow xenograft tumor formation in nude mice. The expression levels of SOD2 and IKKβ in these clones were very low, but both molecules were expressed in A549 cells upon shGFP transfection (A549shGFP). The expression levels of mRNAs encoding SOD2 and IKKβ, evaluated by real-time RT–PCR, were consistent with the levels of protein expression (Figure 4A). The use of the MTT assay revealed that proliferation of the stable SOD2-knockdown A549 clone was significantly lower than that of A549 (shGFP) cells (Figure 4B). The tumor volume in nude mice injected with a stable A549 transfectant with shGFP linearly increased over 8 weeks, but tumor growth was slower than was noted when the stable A549 transfectant expressed shSOD2 (Figure 4B). The tumor burdens in mice injected with the stable transfectants A549 (shSOD2) and A549 (shGFP) are shown in Figure 4C. The SOD2 and IKKβ expression levels in protein extracts of eight xenograft tumors were determined by western blotting and real-time PCR (Figure 4D). In addition, an in vivo tumor metastasis assay was performed in nude mice injected with stable A549 (shSOD2) and A549 (shGFP) clones, via the tail vein, over 62 days. The number of lung tumor nodules in nude mice injected with the stable A549 (shGFP) clone was significantly higher than that in animals injected with the stable A549 (shSOD2) clone (16 ± 4 versus 6 ± 3, P < 0.05; Figure 4E). No lung tumor nodule was found in nude mice injected with PBS. Collectively, the results clearly show that an increase in IKKβ level caused by SOD2 expression triggered the epithelial-to-mesenchymal transition, invasiveness and xenograft tumor formation.

The NKX2-1 mRNA expression level is negatively correlated with the extent of SOD2 immunostaining and the IKKβ mRNA expression level in tumors from lung adenocarcinoma patients

We further explored whether our cell model findings were relevant in the study of tumors from 104 patients with lung adenocarcinomas. NKX2-1 and IKKβ mRNA expression levels were determined by real-time RT–PCR and the extent of SOD2 immunostaining was evaluated immunohistochemically (Supplementary Figure 5, available at Carcinogenesis Online). As shown in Table 1, NKX2-1 mRNA expression levels were higher in SOD2-negative tumors than in SOD2-positive tumors (P = 0.015), but the IKKβ mRNA expression levels were lower in SOD2-negative tumors than in SOD2-positive tumors (P = 0.001). Tumors with low NKX2-1 mRNA levels more commonly showed higher IKKβ mRNA expression levels than

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P.-M. Chen et al. did tumors with high NKX2-1 mRNA levels ($P = 0.006$). Therefore, the extent of SOD2 immunostaining was negatively correlated with NKX2-1 mRNA levels, but positively related to IKKβ mRNA expression levels, in lung adenocarcinoma patients. SOD2 immunostaining levels are associated with OS and RFS in lung adenocarcinoma patients

We investigated whether the SOD2 immunostaining level could estimate OS and RFS in lung adenocarcinoma patients. Kaplan–Meier and Cox regression models were used to evaluate the prognostic value of this marker. Kaplan–Meier analysis indicated that patients exhibiting higher SOD2 expression levels had shorter OS and RFS periods than patients with lower SOD2 expression levels ($P = 0.040$ for OS; $P = 0.008$ for RFS; Supplementary Figure 6A, available at Carcinogenesis Online). Cox regression analysis further showed that a shorter median survival time, a lower 5-year survival percentage and a higher hazard ratio (HR) were exhibited by patients with higher levels of SOD2 immunostaining and IKKβ mRNA expression than was the case when these levels were lower (SOD2: HR = 1.72 for OS, HR = 1.92 for RFS; IKKβ: HR = 2.69 for OS and HR = 2.48 for RFS; Table II). As expected, the prognostic significance of stage III versus stage I and II was also noted in our study population (HR = 2.36, $P = 0.001$ for OS; HR = 2.97, $P < 0.001$ for RFS; Table II). To explore whether combined analysis of SOD2/IKKβ expression levels would be more predictive of patient prognosis, the effects of these levels on OS and RFS were further analyzed by Cox regression. The results showed that patients with tumors that were positive for SOD2 and that expressed high levels of IKKβ mRNA exhibited the worst OS and RFS among the four subgroups analyzed (OS: HR = 4.20, 95% confidence interval (CI) 2.02–8.73, $P < 0.001$; RFS; Supplementary Figure 6B, available at Carcinogenesis Online).

Fig. 3. NKX2-1 protein interacts with Sp1 protein. (A) Immunoprecipitation of Sp1 with NKX2-1. (B) Western blotting and real-time RT–PCR were used to explore expression levels in A549 cells after transfection with the indicated plasmids (expressing wild-type Sp1 and wild-type NKX2-1). (C) The N- and C-terminal activation domains (ADs), the homeodomain (HD) and the inhibitor domain (ID) of NKX2-1 protein are shown. Bacterial expression vectors featuring GST fused to NKX2-1 fragments were constructed by inserting the appropriate PCR fragments between the EcoRI–XhoI sites of vector pGEX-4T1. A GST pull-down assay showed that interaction of a subset of GST-NKX2-1 fusion constructs with Sp1 occurs in the nuclear extracts of A549 cells. Upper panel: Coomassie Blue–stained gel of proteins eluted from glutathione–sepharose using a GST Fusion Protein Purification Kit. Immunoblot of eluates of glutathione-sepharose beads to which GST-NKX2-1 constructs had bound Sp1 by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and probed using a polyclonal anti-Sp1 antibody and antibodies recognizing various cell proteins. (D) Binding of Sp1 to the putative Sp1-binding site of the IKKβ promoter was analyzed using an EMSA. Binding of NKX2-1 to Sp1 was eliminated by addition of anti-Sp1 antibody. The SP-B promoter (−110 to −95) was used as a positive control (the promoter contains an NKX2-1 binding site).
SOD2 promotes tumor malignancy via activation of NF-κB

In addition, patients with tumors that were negative for SOD2 but that expressed high levels of high IKKβ mRNA had shorter OS and RFS periods than did patients with tumors negative for SOD2 and with low levels of IKKβ mRNA (OS: HR = 2.46, 95% CI 1.22–4.98, P = 0.012; RFS: HR = 2.27, 95% CI 1.20–4.31, P = 0.012). However, the HR values of patients with tumors positive for SOD2 and with high levels of IKKβ mRNA, in terms of OS and RFS, were higher than those in patients with tumors negative for SOD2 but with high levels of IKKβ mRNA. Therefore, the tumor progression promoted by SOD2-mediated IKKβ/NF-κB activation noted in our cell model seems to be in line with the human data. SOD2 and IKKβ combine to exert effects on OS and RFS that were more marked than those exerted by SOD2 alone.

Discussion

In this study, SOD2 expression was not dependent on p53 status (Figure 1A). However, both NF-κB and Sp1 were shown to affect SOD2 transcription. As expected, the DNA-binding activity of both transcription factors (NF-κB and Sp1) on the SOD2 promoter was suppressed by wild-type p53 but not by mutated p53 (19,25,26). However, the SOD2 expression level in p53-mutated cells was no higher than in p53 wild-type cells (Figure 1A). This seemed to suggest that binding of NF-κB and Sp1 to the SOD2 promoter was directly enhanced by NKX2-1, thus not by p53. As shown in Figure 2, Sp1 binding to the IKKβ promoter was derepressed by SOD2-mediated NKX2-1 reduction, and this in turn activated the NF-κB/IKKβ pathway. We thus

Table I. Correlation among SOD2 immunostaining, IKKβ mRNA and NKX2-1 mRNA levels in tumors from lung adenocarcinoma patients

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<td></td>
<td>38</td>
<td>25 (66)</td>
<td>13 (34)</td>
<td></td>
<td>11 (29)</td>
</tr>
<tr>
<td>NKX2-1 mRNA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>52</td>
<td>19 (37)</td>
<td>33 (63)</td>
<td>0.006</td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>52</td>
<td>33 (63)</td>
<td>19 (37)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Median value of NKX2-1 and IKKβ mRNA expression in lung tumors was used as a cutoff point to divide tumors into ‘low’ and ‘high’ categories.

#SOD2 immunostaining score: [proportion score (0 = 0/100, 1 = 1/100–1/10, 2 = 1/10–1/3, 3 = 1/3–2/3, 4 = 2/3–1 and 5 = 100/100) + intensity score (0 = negative, 1 = weak, 2 = intermediate and 3 = strong)]. The score of over 4 was defined as ‘positive’ immunostaining, whereas a finding of less than 4 was considered ‘negative’ immunostaining.
expected that Sp1 binding to the SOD2 promoter would be increased by an SOD2-mediated reduction in NKX2-1 levels. Further, binding of NF-xB and Sp1 to the SOD2 promoter was markedly increased by an SOD2-mediated reduction in NKX2-1 levels; this, in turn, upregulated SOD2 transcription. The feedback loop NKX2-1-Sp1-NF-xB imparts a more aggressive phenotype to SOD2-overexpressing lung adenocarcinoma cells. Therefore, SOD2 expression levels may be more affected by NKX2-1 than by p53. We thus suggest that SOD2 expression was not dependent on p53 status in lung adenocarcinoma cells.

SOD2 has been shown to be upregulated by Sp1, and the DNA-binding activity of Sp1 was suppressed by wild-type p53 but not by mutated p53 (19,25,26). This suggested that NKX2-1 not might be required for interaction with Sp1 (to decrease SOD2 expression) in wild-type p53 cells but rather only in cells expressing mutated p53. Therefore, SOD2 expression levels were higher in p53-mutated tumor cells than in p53 wild-type cells. In addition, the same group reported that transgenic mice expressing a luciferase reporter gene exhibited suppression of SOD2 expression at a very early stage of exposure to chemical carcinogens of skin and an increase at the late stage of carcinogenesis (19). Therefore, SOD2 may act at either early or advanced stages of cancer depending on tumor p53 status. In this study, the SOD2 expression level was independent of p53 status; however, a clear negative relationship was found between SOD2 and NKX2-1 levels when a panel of lung cancer cells was investigated (Figure 1A). In addition, the level of SOD2 expression in SOD2-overexpressing TL-4 cells was decreased by NKX2-1 overexpression. Conversely, the level of SOD2 expression in SOD2-knockdown A549 cells was increased upon NKX2-1-knockdown (Figure 2A; upper panel). These changes in SOD2 expression levels upon NKX2-1-overexpression and knockdown were confirmed by EMSA analysis, which showed that Sp1 in A549 nuclear extracts bound to the Sp1-binding site of the SOD2 promoter oligonucleotides to gel shift an Sp1-DNA complex. However, this shift was abolished by addition of GST-NKX2-1 (Supplementary Figure 3, available at Carcinogenesis Online). These results indicate that NKX2-1 may play a more important role than does p53 in modulating SOD2 transcription in lung cancer cells.

NF-xB signaling is required for development of lung adenocarcinomas in mice with a KRAS mutation (G12D) that lack p53. In addition, p53 restoration led to inhibition of activation of NF-xB signaling in lung tumors in vivo and significantly reduced tumor development (27). Earlier, SOD2 activation by all-trans retinoic acid in neuroblastoma cells was mediated via NF-xB signaling (20). Activation of NF-xB-SOD2 cross-signaling induced by a low dose of γ-radiation (an oxidative stressor) has also been reported in an animal model (28). This study revealed a reciprocal relationship between signaling by SOD2 and NF-xB and demonstrated that SOD2-activated NF-xB signaling was mediated via IKKβ transcription because of derepression of Sp1-binding activity by NKX2-1.

Reduction in NKX2-1 levels caused by SOD2 derepresses the DNA-binding activity of Sp1 and permits upregulation of IKKβ transcription and activation of the NF-xB signaling pathway. Conceivably, a positive feedback loop of NF-xB activation is persistently upregulated by expression of SOD2 and causes lung adenocarcinoma cells to exhibit a more aggressive phenotype. Consequently, patients with such cells, especially stage III patients, have poor outcomes (Supplementary Figure 6A, available at Carcinogenesis Online). Therefore, we expect that NKX2-1 may play a more important role than does p53 in activation of NF-xB by SOD2, especially in late-stage lung adenocarcinomas.

In summary, the mechanistic results of this study show that SOD2 can upregulate IKKβ transcription to constitutively activate the NF-xB signaling pathway via alteration of Sp1 DNA-binding activity by NKX2-1. This promotes lung tumor aggressiveness and causes patients to have poor outcomes. Therefore, we suggest that IKKβ may serve as a potential therapeutic target to improve outcomes in patients with SOD2-positive tumors.

**Supplementary material**

Supplementary Table 1 and Figures 1–6 can be found at http://carcin.oxfordjournals.org/
Funding

National Health Research Institute (NHR96-TD-G-111-006, NHR97-TD-G-111-006), National Science Council (NSC-96-2628-B-040-002-MY3, DOH100-TD-C-111-005) of Taiwan, ROC.

Conflict of Interest Statement: None declared.

References


Received November 2, 2012; revised June 3, 2013; accepted June 16, 2013

SOD2 promotes tumor malignancy via activation of NF-xB