MnSOD Promotes Tumor Invasion via Upregulation of FoxM1–MMP2 Axis and Related with Poor Survival and Relapse in Lung Adenocarcinomas

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Abstract

Manganese superoxide dismutase (MnSOD) is an antioxidant enzyme responsible for the elimination of superoxide radical. The role of MnSOD in tumor progression in different human cancers is still controversial. In the present study, MnSOD expression in lung cancer cells was explored by knockdown or overexpression using transfection of a short hairpin RNA (shRNA) or an expression vector, respectively, to determine whether MnSOD expression mediates lung cancer cell migration, invasion, and oncogenic potential by increasing FoxM1 and MMP2 expression. Western blotting showed that FoxM1 and MMP2 expression was dependent on MnSOD expression, suggesting that FoxM1 could be upregulated by MnSOD. Three FoxM1 promoters were constructed to verify this activation of FoxM1 by MnSOD and to determine the transcription factors responsible. Luciferase reporter and chromatin immunoprecipitation assays indicated that MnSOD overexpression in lung cancer cells promoted binding of E2F1 and Sp1 to their putative FoxM1 promoter-binding sites and activated FoxM1 reporter activity. MnSOD also enhanced the potential for cell migration, invasion, and anchorage-independent colony growth on soft-agar plates, again via upregulation of FoxM1 and MMP2 expression. In patients with lung cancer, evaluation of MnSOD expression in lung tumors by immunohistochemistry indicated a positive correlation between FoxM1 and MMP2 mRNA expressions. Kaplan–Meier and Cox regression analysis revealed a poorer overall survival (OS) and relapse-free survival (RFS) in patients with MnSOD-positive tumors than with MnSOD-negative tumors. We conclude that MnSOD may promote tumor aggressiveness via upregulation of the FoxM1–MMP2 axis, and that MnSOD expression can independently predict survival and relapse in patients with resected lung adenocarcinoma. Mol Cancer Res; 11(3); 261–71. ©2012 AACR.

Introduction

The manganese superoxide dismutase (MnSOD) is an important antioxidant enzyme that eliminates superoxide radical (O₂⁻) by converting it into H₂O₂ (1). MnSOD may also function as a tumor suppressor; for example, it may reduce growth of tumors associated with melanoma (2), pancreatic adenocarcinoma (3, 4), colorectal cancer (5), ovarian cancer (6), breast cancer (7), and multiple myeloma (8). Conversely, MnSOD may promote tumor progression in cancers, such as fibrosarcoma (9), gastric cancer (10, 11), glioblastoma (12), T-cell lymphoma (13), breast cancer (14, 15), cervical cancer (16), prostate cancer (17), and lung cancer (18). Therefore, the precise role of MnSOD in tumorigenesis remains elusive.

MnSOD-dependent H₂O₂ production is involved in the regulation of expression of matrix metalloproteinases (MMP; refs. 19, 20), which are major contributors to the stromal degradation associated with tumor invasion. Zhang and colleagues showed activation of MMP2 by MnSOD in human breast cancer MCF-7 cells via the regulation of intracellular reactive oxygen species (ROS; ref. 21). A study on immunodeficient mice showed that MnSOD promoted the metastatic potential of fibrosarcoma cells via increased levels of MMP1 mRNA (22). However, no connection has yet been established between MnSOD upregulation of MMP1 or MMP2 and the
increased metastatic potential of MCF-7 and fibrosarcoma cells.

FoxM1, a member of the forkhead box transcription factor family, is only expressed in proliferating cells and is involved in cell-cycle progression (23). Several studies have implicated FoxM1 in tumorigenesis, as it is overexpressed in various human cancers including lung cancer (24–28). Wang and colleagues showed that proliferation of lung tumor cells was diminished in Mx-Cre FoxM1−/− mouse mutants and that these mice had a significant reduction in the number and size of lung adenomas (28). In contrast, tumors from FoxM1-transgenic mice treated with 3-methylcholanthrene/butylated hydroxytoluene displayed a significant increase in number, size, and DNA replication compared with wild-type mouse tumors (28). Conversely, lung tumor number and size were remarkably reduced when FoxM1 was deleted from mouse respiratory epithelial cells (epFoxM1−/−; ref. 29).

These results suggest a clear association between FoxM1 and lung tumorigenesis. However, FoxM1 also promotes the MMP2 expression that is associated with enhanced invasion by glioma cells (30). On the basis of the available evidence, we hypothesized that MnSOD might upregulate FoxM1 transcription, thereby promoting tumor aggressiveness via increased MMP2 expression. In the present study, we enrolled a panel of lung cancer cells to examine: (i) whether MnSOD expression was positively correlated with expression of MMP2 and FoxM1; (ii) the effects of MnSOD-knockdown and MnSOD-overexpression [by transfection of a short hairpin RNA (shRNA) and an expression vector, respectively] on expression of MMP2 and FoxM1; (iii) the potential upregulation of FoxM1 by MnSOD (by construction of promoter fragments of FoxM1 to study the binding activity of E2F1 and S1 to the putative binding site of the FoxM1 promoter region); and (iv) whether FoxM1-mediated MMP2 expression in MnSOD-overexpressed lung cancer cells could be responsible for the observed increase in potential for cell invasion and anchorage-independent colony growth on soft-agar plates. In addition, the relationships between MnSOD and both FoxM1 and MMP2 in human lung tumors were examined by immunohistochemistry and real-time reverse transcriptase–PCR (Q-PCR). The influence of MnSOD expression on overall survival (OS) and relapse-free survival (RFS) in patients with resected lung adenocarcinoma was statistically analyzed by Kaplan-Meier and Cox regression models.

Materials and Methods

Patients

Lung tumor specimens were collected from patients with lung cancer at the Department of Thoracic Surgery, Taichung Veteran’s General Hospital (TVGH, Taichung, Taiwan, ROC) between 1994 and 2002. Written informed consent for a biology study was obtained from all patients before sample collection. Tumor stages were determined according to the World Health Organization (WHO) classification. Tissues were stored at −80°C, immediately after resection. In total, 48 females, 53 males, 76 nonsmokers, 25 smokers, 51 patients with stage I and II cancers, and 50 patients with stage III cancers were enrolled in this study (Supplementary Table S1).

Cell culture and reagents

The TL5 and TL6 lung cancer cell lines were established from pleural effusions of Taiwanese patients with lung cancer; both cell types were previously identified as adenocarcinoma cells (31). The CH27, A549, and H1299 cancer cell lines were maintained in Dulbecco’s modified Eagles’ medium (DMEM). The TL4, TL5, TL6, CL3, H23, H157, H358, H441, H460, H1355, and CL1-0 lung cancer cell lines were maintained in RPMI-1640 medium containing 10% FBS and supplemented with penicillin (100 U/mL) and streptomycin (100 mg/mL). Cells were grown in a 37°C humidified incubator in a 5% CO2 atmosphere.

Quantitative real-time RT-PCR

The detail procedures were conducted as described previously (32).

Plasmid construction

A full-length human MnSOD cDNA was amplified from A549 mRNA by the RT-PCR using primers based on published mRNA sequences (GenBank: BC012423.1) as follows: sense oligonucleotide 5′-GAATTCTATGGTTAGCCGGGCA-3′ and antisense oligonucleotide 5′-GGATCCTTACCTTTTGGCAGCCATGATC-3′. The PCR product was cloned into the KpnI and HindIII sites of the pGL3-Basic vector from A549. The resulting construct was confirmed by DNA sequencing. The 5′-flanking deletion constructs of the FoxM1 promoter, (−330/+26) FoxM1 and (−178/+26) FoxM1, were similarly generated using the (−600/+26) FoxM1 construct as a template (Supplementary Table S2).

Soft-agar colony formation assay and Boyden chamber invasion assay

These assays were conducted as described previously (33).

Luciferase reporter assay

Luciferase assays were conducted using the luciferase reporter assay system (Promega) 48 hours after transfection. Normalized luciferase activity was reported as luciferase activity/β-galactosidase activity.

Site-directed mutagenesis and transient transfection

Detailed procedures have been described previously (34). Briefly, the expression plasmid was a derivative of pCDNA3 (Invitrogen) constructed by ligation of a 1,192 bp EcoRI and Xhol fragment derived from pCM3SN by the PCR. Primer A (5′-GAATTCTATGGTTAGCCGGGCA-3′) and primer B (5′-AGCTTAGATCGTGTTGAATCGGCCCTCTG-3′) contained the entire wild-type p53 (WT-p53) coding sequence in frame, and these were used to insert the fragment into the EcoRI/Xhol sites of pCDNA3.1. The nucleotide sequences and amino acid sequence.

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substitutions of the mutated primers used for the construction of mutant recombinant p53 were 5'-GCATCGTAT-CCGAGTTGGAG-3' for L194R and 5'-GGACCGGAG-TCCCATCCTC-3' for R249S. Constructs were verified by direct sequencing. The negative control plasmid containing no p53 was pCDNA3.1. The H1299 cells were seeded at 1 × 10^6 cells in 6-cm dish and incubated at 37°C for 18 hours. All transfections were carried out in triplicate in 6-cm dishes using Transfast transfection reagent (Promega), according to the manufacturer's instructions. The WT-p53, L194R, R249S, and control expression plasmids were transiently transfected at different concentrations into H1299 cells. The Transfast reagent/plasmid mixture was then added directly to the cells, followed by incubation at 37°C and 5% CO2. After 48 hours, cells were harvested and whole-cell extracts were assayed in the following experiments.

**Western blotting**
Cells were washed twice on ice with PBS before adding protein lysis buffer (1× protease inhibitor cocktail; Roche; 1.5 mmol/L EDTA, 1 mmol/L Diethiothreitol (DTT), 10% glycerol, 25 mmol/L HEPES, pH 7.6). The protein concentration was determined by the Bradford assay (BioRad) using bovine serum albumin (BSA) as a standard. Total protein (20 μg) was resolved by 10% SDS-PAGE for subsequent Western blot analysis using antibodies against the following proteins [diluted in Tween-Tris Buffered Saline (TTBS): 0.02% Tween-20 in 100 mmol/L Tris-CL, pH 7.5 as indicated]: β-actin (1:100,000), MnSOD (1:1,000), FoxM1 (1:1,000), p21WAF-1 (1:1,000), retinoblastoma (Rb: 1:1,000), MMP1 (1:1,000), MMP2 (1:1,000), MMP7 (1:1,000), Sp1 (1:1,000), and p53 (1:1,000). The gel was transferred to a Hybond-C Extra membrane and immunoblotted with primary antibody, as indicated in the figure legends. Anti-mouse or rabbit immunoglobulin G (IgG) conjugated to horseradish peroxidase was used as the secondary antibody for detection using an ECL Western blotting detection system.

**Chromatin immunoprecipitation assays**
Chromatin immunoprecipitation (ChIP) analysis was conducted according to a published procedure, with the following modifications (35). Immunoprecipitated DNA was ethanol precipitated and resuspended in 20 μl double-distilled water (ddH2O). Total input samples were resuspended in 100 μL ddH2O and diluted 1:100 before PCR analysis. PCR amplification of immunoprecipitated DNA was carried out with diluted aliquots using the oligonucleotides (5'-GGAGCAGAGGAGCTTGAG-3') and (5'-AAAAGTGCCGGTGGGGC-3') as primers, which encompass the 153-bp promoter of FoxM1 (~350/198). The PCR products were separated on 2% agarose gels and analyzed by ethidium bromide staining. All ChIP assays were conducted at least twice and produced similar results. Cells were seeded at 1 × 10^6 cells in 6-cm dishes and incubated at 37°C for 18 hours. Cells were equally cotransfected with GFP and the MnSOD plasmids for 24 hours.

**Flow-cytometry analysis**
For flow-cytometry analysis, cells (5 × 10^5) were seeded onto 6-cm dishes for 24 hours. The cultured cells were then harvested and centrifuged at 200 × g for 5 minutes. The cell pellets were washed twice with 1× PBS and fixed with 70% ethanol at 4°C for 30 minutes. After centrifugation at 200 × g for 5 minutes, the cell pellets were washed with 1× PBS to remove any residual ethanol. Finally, the cells were resuspended in 1 mL of solution containing 0.5 mg/mL RNase A, 1% Triton X-100, and 40 μg/mL propidium iodide, and incubated at 37°C for 30 minutes. The cells were filtered through a 40-μm nylon mesh before flow-cytometry analysis of cell-cycle distribution using a FACS-Calibur flow cytometer (Becton Dickinson).

**Statistical analysis**
Chi-square analysis was conducted using SPSS software (Version 13.0; SPSS Inc.) for statistical analysis. Statistical differences for survival data were analyzed using the log-rank test. Survival curves were plotted using the Kaplan–Meier method and variables related to survival were analyzed using Cox's proportional hazards regression model using SPSS software. A value of P less than 0.05 was considered to be statistically significant.

**Results**
MnSOD is positively correlated with FoxM1 and MMP2 expression
The potential association of MnSOD with MMP2 expression was evaluated by Western blot analyses of MnSOD and MMP2 protein expressions in 14 lung cancer cell lines. The expression of MnSOD was markedly higher in the A549, H157, H1355, TXA, TXL, TX6, and CL3 cells than in the CH27, H460, H441, H1299, CL1-0, H23, and H358 cells. Interestingly, MMP2 expression was also higher in cells that expressed high levels of MnSOD than in cells expressing low levels of MnSOD (Fig. 1A). A positive correlation was therefore indicated between MnSOD and the expression of both FoxM1 and MMP2 in different lung cancer cell lines, which suggested that the capability of lung cancer cells for invasion might be promoted via upregulation of FoxM1 and MMP2 by MnSOD.

**FoxM1 and MMP2 expressions in lung cancer cells are dependent on MnSOD expression**
FoxM1 is a known transcriptional factor that upregulates MMP2 expression (30). We hypothesized that MnSOD may increase FoxM1 expression and thereby lead to upregulation of MMP2 expression. We used 4 lung cancer cell lines (A549, H1355, CL3, and TL4) that showed high MnSOD expression levels, and we knocked down the MnSOD expression using a shRNA (shMnSOD) (Fig. 1B). Another 4 lung cancer cell lines (H23, H358, H460, and CL1-0) with low MnSOD expression were
transfected with a MnSOD expression vector to increase their expression of MnSOD (Fig. 1C). All of the cells, except for H460 (a large lung cancer cell line), were lung adenocarcinoma cells. Western blotting showed that MMP2 and FoxM1 expression levels were decreased in MnSOD-knockdown cells and were increased in MnSOD-overexpression lung cancer cells in a dose-dependent manner. In addition, p21 and Rb expression were negatively regulated by FoxM1 expression, which was consistent with previous reports (36).

Flow-cytometry analysis of cell distribution showed that the MnSOD-knockdown H1355 cells had a greater number of cells in the G0–G1 phase and fewer cells in the S-phase as compared with H1355 (NC) cells transfected with a nonspecific shRNA (Fig. 1D). Conversely, the distribution of MnSOD-overexpressing CL1-0 cells showed a fewer cells in the G0–G1–phase cells and a much greater number of cells in the S-phase cells as compared with vector control (VC) cells (Fig 1D). These results supported a change in p21 expression due to MnSOD-knockdown and MnSOD-overexpression in both cell lines.

We also used p53-mutated H1355 and CL1-0 cell lines to investigate whether MnSOD could decrease Rb expression by increasing the release of E2F1 from the Rb–E2F1 complex and subsequent upregulation of FoxM1 expression. As shown in Fig. 1E, MnSOD expression was decreased by MnSOD knockdown in p53-mutated H1355 cells and increased by MnSOD overexpression in p53-mutated CL1-0 cells. The expression of p21 and Rb was elevated in

Figure 1. FoxM1 and MMP2 expression in lung cancer cells are dependent on MnSOD expression (A) MnSOD, FoxM1, and MMP2 expressions were evaluated by Western blotting in a panel of lung cancer cells. B, MnSOD in A549, H1355, CL3, and TL4 cells, which had high MnSOD expression, was knocked down by transfection of MnSOD shRNA and changes in p21, Rb, FoxM1, and MMP2 expressions were evaluated by Western blotting. C, MnSOD in H23, H358, H460, and CL1-0 cells, which had low MnSOD expression, was ectopically expressed by transfection of a MnSOD cDNA plasmid and the changes in p21, Rb, FoxM1, and MMP2 expressions were analyzed by Western blotting. β-Actin was used as a loading control in all experiments. D, the change in cell distribution of different cell-cycle phases in MnSOD-knockdown H1355 and MnSOD-overexpression CL1-0 cells compared with their control cells. The cell-cycle phase was determined by fluorescence-activated cell sorting (FACS). The mean ± SD for G0–G1, S, and G2–M phase cells was calculated by data obtained from 3 independent experiments. E, Western blotting was used to evaluate MnSOD, Rb, and E2F1 expressions in H1355 and CL1-0 cells after transfection with MnSOD expression vector and shMnSOD. These lysates were immunoprecipitated with anti-Rb-conjugated beads. The immunoprecipitates were analyzed by SDS-PAGE, followed by immunoblotting with anti-E2F1 antibody. Each experiment was carried out in triplicate. VC: pCDNA3.1 vector control; NC: nonspecific shRNA vector control. The mean ± SD was calculated from data of 3 independent experiments.
MnSOD-knockdown H1355 cells but reduced in MnSOD-overexpressing CL1-0 cells. A consistent interaction was seen between Rb and E2F1, which increased markedly in MnSOD-knockdown H1355 cells and decreased in MnSOD-overexpressing CL1-0 cells in a dose-dependent manner. The expression of E2F1 was not changed in MnSOD-knockdown and MnSOD-overexpressing cells. These results suggest that MnSOD may promote the release of E2F1 from the Rb–E2F1 complex by suppressing p21 expression, thereby promoting FoxM1 expression. Therefore, we expected that MnSOD would upregulate FoxM1, thereby increasing MMP2 expression in lung cancer cells.

**FoxM1 transcription is upregulated by MnSOD via increased binding activity of E2F1 and Sp1 onto the FoxM1 promoter**

Previous studies indicated the existence of putative E2F1-binding sites on the FoxM1 promoter, and showed that FoxM1 transcription was decreased by E2F1-knockdown (36, 37). However, no evidence was presented to confirm E2F1 as a transcription factor of FoxM1. In the present study, 3 promoter regions of the FoxM1 gene (−600/+26, −330/+26, and −178/+26) were constructed, and 3 E2F1 and 1 Sp1 putative binding sites were detected on the −600/+26 promoter region using the software available at http://molinsinstlouis.missouri.edu/Research/TFSEARCH. html (Fig. 2A, top). An effect of p53 on binding of E2F1 and Sp1 to the FoxM1 promoter was verified by transfecting the 3 promoter sequences into p53-mutated or null H1355, H23, and H1299 cells, and WT-p53 A549 and H460 cells. The luciferase reporter assay showed that the reporter activity of the −330/+26 promoter in p53-mutated or null cells accounted for approximately 80% of the reporter activity of the −600/+26 promoter. However, in WT-p53 cells, the reporter activity of the −330/+26 promoter was decreased to approximately 50% of the reporter activity of the −600/+26 promoter. These findings indicated that the binding activity of E2F1 and Sp1 to the −330/+26 promoters could be deregulated by WT-p53.

ChIP assays indicated that the binding of E2F1 and Sp1 onto the −330/+26 promoter was not detectable in WT-p53 A549 and H460 cells; however, the binding onto −330/+26 was always detected in p53-mutated or p53-null cells (Fig. 2B). The impact of wild-type or mutant p53 on FoxM1 transcription was further examined by transfecting H1299 cells with wild-type and mutant L194R and R249S p53. As expected, the reporter activity of the −330/+26 promoter was markedly decreased by transfection with WT-p53; however, the reporter activity of the −330/+26 promoter was not changed by transfection with either mutant p53 when compared with their control cells (Fig. 2C). The ChIP assay further revealed that the binding activity of E2F1 and Sp1 onto the −330/+26 promoter was significantly decreased by WT-p53, but not by either mutant p53 (Fig. 2C). The reporter activity of FoxM1 (−330/+26) was decreased by transfection with WT-p53 but not by transfection with mutant p53 (L194R, R249S) in H1299 cells (Fig. 2D). To further verify whether FoxM1 could be regulated by MnSOD, FoxM1 reporter activity (−330/+26) was decreased in MnSOD-knockdown H1355 cells and increased in H460 cells overexpressing MnSOD. In addition, the binding activities of E2F1 and Sp1 on the FoxM1 promoter evaluated by ChIP analysis were significantly decreased in MnSOD-knockdown H1355 cells and increased in H460 cells overexpressing MnSOD (Fig. 2E). We used 2 WT-p53 A549 and H460 cell lines to explore whether MnSOD expression would increase FoxM1 transcription via increased E2F1 and Sp1 binding to FoxM1 promoter. As shown in Fig. 2F, MnSOD expression was higher in A549 cells than in H460 cells. The p53 and Rb expression levels were significantly lower in A549 cells than in H460 cells. However, FoxM1 expression was higher in A549 cells than in H460 cells. ChIP analysis indicated that the capability of E2F1 and Sp1 binding to the FoxM1 promoter was greater in A549 cells than in H460 cells. These results suggest that MnSOD could modulate the expression of p53 and Rb, thereby promoting the release E2F1 from the Rb–E2F1 complex and consequently increasing Sp1 and E2F1 binding to the FoxM1 promoter. We explored this possibility further by transfecting H460 cells with an MnSOD expression vector and further cotransfection with shSp1 or shE2F1. Western blotting showed that p53 and Rb expressions were markedly decreased by MnSOD overexpression in H460 cells compared with vector control cells. As expected, the expression of Rb, Sp1, and E2F1 were decreased by p53−, Sp1−, and E2F1-knockdown, respectively, in H460 cells. The binding activity of E2F1 onto the FoxM1 promoter was evident in p53-knockdown H460 cells and MnSOD-overexpressing H460 cells transfected with shSp1 and nonspecific shRNA but was not observed in MnSOD-overexpressing H460 cells transfected with shE2F1. Similarly, Sp1 binding to the FoxM1 promoter was also shown in p53-knockdown and MnSOD-overexpressing H460 cells transfected with shE2F1 and nonspecific shRNA but was not observed in MnSOD-overexpressing H460 cells transfected with shSp1 (Fig. 2G). Consistent results were also observed in p53-overexpressing and MnSOD-knockdown A549 cells following further cotransfection with the 2 types of shp53 (Fig. 2H). These results clearly indicate that the MnSOD may reduce p53 and Rb expression, which would derepress Sp1 and release E2F1 from the Rb–E2F1 complex, allowing it to bind to the FoxM1 promoter and consequently to upregulate FoxM1 transcription.

**Cell migration, invasion, and anchorage-independent soft-agar colony growth are dependent on MnSOD-induced FoxM1 and MMP2 expressions**

The effects of MnSOD expression on FoxM1 and MMP2 expressions and the subsequent potential for cell invasion and anchorage-independent soft-agar colony growth were examined using Boyden chambers and soft-agar assays of MnSOD-knockdown and MnSOD-overexpressing cells and comparison with their control cells. As expected, MnSOD expression levels were decreased in the MnSOD-knockdown H1355 and A549 cells and increased in the MnSOD overexpression CL1-0 and H358 cells (Fig. 3). A dose-dependent
Figure 2. FoxM1 transcription is upregulated by MnSOD via an increased binding of E2F1 and Sp1 onto FoxM1 promoter. A, FoxM1 promoter-driven luciferase reporters: FoxM1 (−600/+26)-Luc, FoxM1 (−330/+26)-Luc, FoxM1 (−178/+26)-Luc and involvement of E2F1 and Sp1 of putative binding site. After transient transfection with the FoxM1 promoter for 24 hours, the luciferase activity of the H1299, A549, H1355, H23, and H460 cells was determined using an AutoLumat LB953 luminometer (Berthold) and normalized against the cotransfected β-gal activity. B, binding of E2F1 and Sp1 onto the FoxM1 promoter (−350/+198) was evaluated by ChIP in H1299, A549, H1355, H23, and H460 cells. The immunoprecipitated DNA was amplified by PCR for 25 cycles. C, Western blotting and ChIP assays were used to evaluate p53 protein levels and binding activity of E2F1 and Sp1 to the FoxM1 promoter in MnSOD-knockdown H1355 and MnSOD-overexpressing H460 cells. The immunoprecipitated DNA was amplified by PCR for 25 cycles. F, MnSOD, p53, Rb, and FoxM1 expressions were evaluated by Western blotting and the Sp1 and E2F1 binding to the FoxM1 promoter in A549 and H460 cells were evaluated by ChIP. The immunoprecipitated DNA was amplified by PCR for 30 cycles. G, H460 cells were cotransfected with shp53 (5 μg), MnSOD (5 μg), shSp1 (5 μg), and shE2F1 (5 μg) plasmids, and the whole-cell lysates used for evaluating the expression of MnSOD, p53, p21, Rb, Sp1, E2F1, and FoxM1 by Western blotting. β-Actin was used as a protein loading control. The binding activity of Sp1 and E2F1 onto the FoxM1 promoter was evaluated by ChIP. The immunoprecipitated DNA was amplified by PCR for 30 cycles. H, A549 cells were cotransfected with p53 (5 μg), shMnSOD (5 μg), and 2 kinds of shp53 (5 μg). The cell lysates of A549 cells with different transfections were used to determine the expressions of MnSOD, p53, p21, Rb, and FoxM1 by Western blotting. β-Actin was used as a protein loading control. The binding activity of Sp1 and E2F1 to the FoxM1 promoter was evaluated by ChIP analysis. The input control was 30% of the cell extract without any treatment. Chromatin was isolated and immunoprecipitated with 2 antibody specific for Sp1 and E2F1.

decrease in migration, invasion, and soft-agar colony growth was found in the MnSOD-knockdown H1355 and A549 cells than in the nonspecific shRNA control (NC) cells. A dose-dependent increase in migration, invasion, and soft-agar colony growth was found for MnSOD-overexpressing CL1-0 and H358 cells when compared with vector control cells. We verified whether upregulation of the MMP2 and FoxM1 by MnSOD was responsible for the increased capability for migration, invasion, and soft-agar colony growth potential by knockdown of FoxM1 and MMP2 by their respective shRNAs in MnSOD-overexpressing CL1-0 and H358 cells. Soft-agar and Boyden chamber assays indicated that the anchorage-independent soft-agar colony growth, cell migration, and invasion of both MnSOD-overexpressing cell
lines were restored to the same levels as observed in their respective control cells. These results clearly indicate that the FoxM1–MMP2 axis is upregulated by MnSOD and may be responsible for the migration, invasion, and oncogenic potential of lung cancer cells.

MnSOD was positively correlated with FoxM1 and MMP2 mRNA expression levels in lung adenocarcinomas

We next examined whether the upregulation of FoxM1 and MMP2 by MnSOD observed in the cell model...
would also be evident in lung tumors from patients with lung cancer. Here, we enrolled 101 tumors from patients with lung adenocarcinoma to evaluate MnSOD expression by immunohistochemistry of paraffin sections. A cutoff point, established as 10% or less than 10% positive immunoreactive tumor cells in the paraffin sections, was used to divide tumors into negative or positive MnSOD immunostaining groups. Correlation between MnSOD protein expression and MnSOD, FoxM1, and MMP2 mRNA expression was verified by real-time RT-PCR in these lung tumors. The median value of MnSOD, FoxM1, and MMP2 mRNA expression was used as a cutoff point to categorize tumors into high or low mRNA expression groups. As shown in Table 1, MnSOD protein expression was positively correlated with mRNA expression \( (P = 0.005) \). FoxM1 and MMP2 mRNA levels in MnSOD-positive tumors were significantly higher than in MnSOD-negative tumors \( (P = 0.048 \text{ for FoxM1; } P = 0.017 \text{ for MMP2}) \). These observations from lung tumors as agreed with the findings from the lung cancer cell lines, suggesting that the involvement of the FoxM1–MMP2 axis and upregulation by MnSOD might be part of an in vivo mechanism for metastasis of lung tumors.

**MnSOD expression may independently predict survival and relapse in lung adenocarcinomas**

We further evaluated the potential for an association between MnSOD expression and the outcome of patients with lung adenocarcinomas. We enrolled 101 patients for OS analysis, and of these, 89 were available for RFS analysis, with a median number of follow-up months of 15.1. In the RFS analysis group, the condition relapsed in 30 patients (4, local recurrence; 22, distant metastasis; 4, local recurrence and distant metastasis) and 24 patients died from the disease. Detailed information for the 30 patients with local tumor recurrence and/or metastasis is listed in Supplementary Table S3. No patients received adjuvant treatment before surgical therapy. Kaplan–Meier survival analysis showed shorter OS and RFS periods in patients with MnSOD-positive or low MMP2 mRNA tumors than in patients with MnSOD-negative or high MMP2 mRNA tumors (Fig. 4; MnSOD: \( P = 0.046 \) for OS, \( P = 0.005 \) for RFS; MMP2 mRNA: \( P = 0.004 \) for OS, \( P = 0.019 \) for RFS). In a Cox model for OS, the median survival in patients with MnSOD-negative tumors was 23 months, which was longer than for the OS for patients with MnSOD-positive tumors (15 months). The 5-year survival rate was 40% for patients with MnSOD-negative and 23% for patients with MnSOD-positive tumors (Table 2). The HR for patients with MnSOD-positive tumors versus MnSOD-negative tumors was 1.77 \( (95\% \text{ confidence interval (CI), 1.06–2.96; } P = 0.030; \text{ Table 2}) \). In a Cox model for RFS, the median survival of patients with MnSOD-negative tumors was 17 months, which was longer than RFS for patients with MnSOD-positive tumors (12 months). The 5-year survival rate was 30% for patients with MnSOD-negative and 11% for patients with MnSOD-positive tumors. The HR of patients with MnSOD-positive tumors versus those with MnSOD-negative tumors was 1.94 \( (95\% \text{ CI, 1.17–3.21; } P = 0.010; \text{ Table 2}) \).

We also examined the influence of MMP2 mRNA on OS and RFS of these patients. In a Cox model for OS, poorer median survival and 5-year survival rate was observed in patients with high MMP2 mRNA than with low MMP2 mRNA (16 vs. 22 months for median survival; 21% vs. 48% for 5-year survival; HR, 2.17; 95% CI, 1.28–3.69; \( P = 0.004; \text{ Table 2}) \). In a Cox model for RFS, the median survival and 5-year survival rate were marginally worse in patients with high MMP2 mRNA than with low MMP2 mRNA (14 vs. 18 months for median survival; 10% vs. 35% for 5-year survival; HR, 1.68; 95% CI, 1.00–2.80; \( P = 0.049; \text{ Table 2}) \). These results clearly indicate that MnSOD immunostaining and MMP2 mRNA may independently predict both survival and relapse in patients with lung adenocarcinoma.

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**Table 1. Relationship of MnSOD IHC with MnSOD, FoxM1, and MMP2 mRNA expression in patients with lung adenocarcinoma**

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<tr>
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<td>16</td>
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<tr>
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<td>MnSOD mRNA</td>
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<td>High</td>
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NOTE: MnSOD IHC: MnSOD immunostaining in paraffin section of lung tumors was determined by immunohistochemical analysis. \( P \) value was obtained from \( \chi^2 \) test.
Discussion

In the present study, we provide evidence, from both in vitro cell studies and in vivo human lung tumors, to indicate that metastasis of lung adenocarcinoma is promoted by MnSOD and that the mechanism involves upregulation of the FoxM1–MMP2 axis. Two putative E2F1-binding sites have previously been identified in the FoxM1 promoter, but direct activation of FoxM1 by E2F1 has not yet been shown (36). We showed that release of E2F1 from the Rb–E2F1 complex due to suppression of p53 and p21 expressions by MnSOD resulted in increased E2F1 binding to the FoxM1 promoter in MnSOD-overexpressing H460 cells (Fig. 2G, top). The capability of Sp1 binding to FoxM1 promoter was also increased by suppression of p53 expression by MnSOD (Fig. 2G, bottom). This was due to a derepression of Sp1 DNA-binding activity by reduction of p53 expression by MnSOD (38, 39). Conversely, marked elevation of p53 and Rb expression, but reduced FoxM1 expression, was observed in MnSOD-knockdown A549 cells. However, FoxM1 expression was restored in A549

Table 2. Cox regression analysis for the influence of MnSOD immunostaining and MMP2 mRNA on OS and RFS in patients with lung adenocarcinoma

<table>
<thead>
<tr>
<th>Variable</th>
<th>OS</th>
<th>RFS</th>
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<tr>
<td>MnSOD</td>
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<tr>
<td>Negative</td>
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<tr>
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<tr>
<td>High</td>
<td>51</td>
<td>46</td>
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</table>

*HR: adjusted for the parameters of age, gender, smoking status, and tumor stage.

Figure 4. Kaplan–Meier analysis of the influence of MnSOD expression and MMP2 mRNA expression on OS and RFS in patients with lung adenocarcinomas.
cells with a double knockdown of MnSOD and p53 expression (Fig. 2H, top). This restoration of FoxM1 expression was consistent with a decrease in Rb expression and an increase in E2F1 and Sp1 binding to the FoxM1 promoter in A549 cells with double knockdown of MnSOD and p53 (Fig. 2H, bottom). These results clearly indicate that FoxM1 suppression by p53 is mediated through a reduced binding capability of E2F1 and Sp1 to the FoxM1 promoter.

In the present study, luciferase reporter and ChIP assays revealed that FoxM1 transcription is directly upregulated by E2F1 and Sp1, and that the binding activity of both transcription factors on the putative binding sites of FoxM1 promoter are attenuated by p53 status. This result was consistent with previous reports that indicated a requirement for p53 in the downregulation of FoxM1 (26, 35). The FoxM1 mRNA expression levels substantially decreased when E2F1 was knocked down in MCF7 cells; this indicated a possible involvement of E2F1 in FoxM1 transcription (35).

A previous study showed that reduced levels of p21 and degradation of Rb might play a role in E2F1-mediated FoxM1 transcription (36). Consistent with previous results, the expression of p21 and Rb was also remarkably decreased in MnSOD-knockdown lung cancer cells. We provided further evidence to show that the release of E2F1 from the Rb–E2F1 complex, which occurs due to decreased expression of p53 and Rb in response to MnSOD, might be involved in the upregulation of FoxM1 expression (Fig. 2G and H). This observation is supported by the fact that the number of cells in the G1–S transition was elevated in MnSOD-overexpressing CL1-0 cells and was reduced in MnSOD-knockdown H1355 cells (Fig. 1D). Therefore, modulation of p21 expression in MnSOD-overexpressing cells, due to decreased p53 and Rb expression, may cause alterations in the cell cycle at the G1–S transition. The lack of a change in E2F1 expression in MnSOD-knockdown H1355 cells seems to support this speculation (Supplementary Fig. S1).

FoxM1 seems to be a transcription factor for MnSOD in human fibroblast cells. In the present study, MnSOD expression was unchanged by FoxM1-knockdown in A549 and H1355 cells (Supplementary Fig. S2A), whereas MnSOD was able to activate FoxM1 transcription via increased binding of E2F1 and Sp1 onto the FoxM1 promoter. A negative feedback loop involving FoxM1 may regulate ROS in proliferating human fibroblast cells. Induction of FoxM1 requires ROS, whereas elevated FoxM1, in turn, reduces ROS levels by promotion of MnSOD expression. Therefore, promotion of FoxM1 expression by MnSOD may play a crucial role in the control of oxidative stress responses during oncogenesis (27). On the basis of the findings of the present and previous studies (27), we suggest that upregulation of FoxM1 by MnSOD is required to overcome ROS-induced oxidative stress that occurs during lung tumor progression and metastasis.

An early report on MCF-7 breast cancer cells indicated that MnSOD induced MMP2 expression by regulation of intracellular ROS (21). Another report showed that MMP2 transcription was directly upregulated by FoxM1 and that the invasion of glioma cells was enhanced by FoxM1-induced MMP2 expression (30). In the present study, MMP2 expression was markedly decreased in FoxM1-knockdown cells (Fig. 3D), but no change was seen for MnSOD expression (Supplementary Fig. S3A). Moreover, FoxM1 and MMP2 expressions induced by MnSOD in lung cancer cells were responsible for an increase in cell invasion capability and enhanced potential for anchorage-independent colony growth (Fig. 3D). However, the enhancement of invasive and migratory activity of HT-1080 fibrosarcoma and 253J bladder cancer cells by MnSOD overexpression was mediated through MMP1. Our data indicated that MMP1 and MMP2 expression levels were concomitantly decreased in MnSOD-knockdown A549 cells, but no change was seen for MMP7 and MMP9 expressions (Supplementary Fig. S2B), which suggests that MMP1 might partially contribute to MnSOD-induced cell invasiveness and oncogenic activity of lung cancer cells. A recent meta-analysis report indicated that MMP2 expression was associated with poorer prognosis in patients with lung adenocarcinoma (40). Similar findings reveal that MMP2 acts as a critical accelerator to the progression of lung adenocarcinoma (Table 2).

In summary, we provide mechanistic evidence to support the possibility that lung adenocarcinoma metastasis is enhanced by MnSOD expression and is mediated predominantly through the FoxM1–MMP2 axis. Our findings from cell models were further supported by evidence that MnSOD-positive tumors were associated with shorter survival and relapse periods in patients with lung adenocarcinoma, as MnSOD expression in lung tumors from patients with lung cancer was positively correlated with MMP2 expression. Thus, both of these molecules may be useful as predictors of the clinical outcome of patients with lung adenocarcinoma.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

Conception and design: Y.-H. Wu, Y.-W. Cheng, H. Lee
Development of methodology: M.-C. Li, H. Lee, S.-H. Shih, G.-T. Shue
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y.-H. Wu, C.-Y. Chen, H. Lee, T.-C. Wu, S.-H. Shih
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): P.-M. Chen, Y.-H. Wu, C.-Y. Chen, H. Lee
Writing, review, and/or revision of the manuscript (i.e., reporting or organizing data, constructing databases): M.-C. Li, H. Lee
Study supervision: H. Lee

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References


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