TP53 upregulates α-smooth muscle actin expression in tamoxifen-resistant breast cancer cells

SANGMIN KIM[1], DAEUN YOU[2], YISUN JEONG[2], JONGHAN YU[1,3], SEOK WON KIM[1,3], SEOK JIN NAM[1,3] and JEONG EON LEE[1-3]

[1]Breast Cancer Center, Samsung Medical Center; [2]Department of Health Sciences and Technology, Samsung Advanced Institute for Health Sciences and Technology (SAIHST), Sungkyunkwan University; [3]Department of Surgery, Samsung Medical Center, Gangnam-gu, Seoul 06351, Republic of Korea

Received June 14, 2018; Accepted November 22, 2018

DOI: 10.3892/or.2018.6910

Abstract. In a previous study, we reported that α-smooth muscle actin (α-SMA), one of the mesenchymal marker proteins, is highly expressed in tamoxifen-resistant breast cancer (TamR) cells. However, the exact mechanism of α-SMA expression in TamR cells is not fully understood. Here, we investigated the effect of TP53 on α-SMA expression in breast cancer cells. The levels of α-SMA mRNA and protein expression were analyzed by real-time PCR and western blotting, respectively. In estrogen receptor-positive [ER(+)] breast cancer patients, aberrant α-SMA expression was found to be associated with a poor prognosis. The level of α-SMA expression was significantly increased in established TamR cells compared to tamoxifen-sensitive (TamS) cells. To verify the regulatory mechanism of α-SMA expression, we analyzed diverse kinase activities between TamS and TamR cells. The activity of TP53 was markedly increased in the TamR cells. When TamS cells were treated with TP53 activator, Nutlin3 (Nut3), α-SMA expression was increased in the TamS cells. In addition, α-SMA expression was significantly increased by TP53 overexpression in breast cancer cells. On the contrary, the basal level of α-SMA expression was decreased by the TP53 inhibitor, pifithrin-α (PFT-α). Taken together, we demonstrated that α-SMA expression is regulated by TP53 activity in TamR cells.

Introduction

α-Smooth muscle actin (α-SMA) is encoded by the ACTA2 gene and contributes to tumor cell migration and invasion through the suppression of E-cadherin (1-3). The abnormal induction of α-SMA expression was found to be associated with shorter disease-free survival in lung, colorectal and pancreatic cancers (4-6). Recently, we reported that breast cancer patients with high α-SMA and HER2 levels had a poorer prognosis than patients with low α-SMA and HER2 levels (2). The level of α-SMA expression is significantly increased during epithelial-mesenchymal transition (EMT) and is regulated by various stimuli such as IL-1β, IL-6 and TGF-β1 (7,8).

The *TP53* gene encoding p53 is the single most frequently inactivated gene in human cancers, with somatic missense mutations being present in approximately 50% of all invasive tumors (9). These mutation frequencies of TP53 are lower in breast cancers than in other solid tumors (10). The level of TP53 protein expression is stabilized and activated in response to a wide variety of cellular stresses such as DNA damage, ultraviolet irradiation, hypoxia and nucleotide depletion (11-13). TP53 works as a transcriptional activator and regulates cell cycle arrest, senescence, apoptosis, DNA replication and repair through the expression of its downstream target genes such as p21, Bax, NOXA and p53R2 (14,15).

In the present study, the clinical significance of α-SMA expression was investigated in estrogen receptor-positive [(ER(+)] breast cancer patients and the effect of TP53 on α-SMA expression was evaluated in tamoxifen-resistant breast cancer cells. In this study, it was found that α-SMA expression is associated with the survival of ER(+) breast cancer patients. In addition, we observed that wild-type TP53 upregulates α-SMA expression in tamoxifen-resistant breast cancer cells.

Materials and methods

Reagents. Dulbecco's modified Eagle's medium (DMEM) was purchased from Thermo Fisher Scientific, Inc. (Hemel Hempstead, UK). Fetal bovine serum (FBS) was purchased from HyClone/GE Healthcare Life Sciences (Logan, UT, USA). Phenol red-free DMEM, penicillin (100 U/ml), and 100 mg/ml streptomycin were purchased from Life Technologies (Rockville, MD, USA). 4-Hydroxytamoxifen (4-OHT) was purchased from Sigma-Aldrich;Merck KGaA (Darmstadt, Germany). Proteome Profiler Human Phospho-Kinase Antibody Array Kits were purchased from R&D Systems (Minneapolis, MN, USA). Nutlin3 (TP53 activator, Nut3)

Correspondence to: Dr Jeong Eon Lee, Breast Cancer Center, Samsung Medical Center, 50 Irwon-dong, Gangnam-gu, Seoul 06351, Republic of Korea
E-mail: paojlus@hanmail.net

Key words: α-SMA, TP53, tamoxifen resistance, estrogen receptor, breast cancer

and pifithrin-α (TP53 inhibitor, PFT-α) were purchased from Selleck Chemicals (Houston, TX, USA). Anti-p53 (1:1,000 dilution; cat. no. sc-126) and mouse monoclonal anti-β-actin (1:1,000 dilution; cat. no. sc-47778) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-α-SMA (1:20,000 dilution; cat. no. ab124964) antibody was purchased from Abcam (Cambridge, UK). ECL Western Blotting Detection Reagents (West-Q Chemiluminescent Substrate Plus kit) were obtained from GenDepot (Barker, TX, USA).

Cell culture. Tamoxifen-sensitive and -resistant MCF-7 (TP53 wild-type), MDA-MB-231 (TP53-mutant R280K), and Hs578T (TP53-mutant V157F) human breast cancer cells were grown in a humidified atmosphere of 95% air and 5% CO_2 at 37˚C in DMEM supplemented with 10% FBS, 2 mM glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin. Each cell line was maintained in culture medium without FBS for 24 h before the experiments.

Establishment of tamoxifen-resistant MCF-7 breast cancer cells. Tamoxifen-sensitive (TamS) and -resistant (TamR) breast cancer cell lines were kindly provided by Professor Keon Wook Kang (Seoul National University, Seoul, Korea). The TamR cells were established using methodology as previously reported (16,17). Briefly, MCF-7 cells were washed with PBS, and the culture medium was replaced with phenol red-free DMEM containing 10% charcoal-stripped steroid-depleted FBS and 0.1 μM 4-OHT. The cells were continuously exposed to this treatment regimen for two weeks, and the 4-OHT concentration was increased gradually up to 3 μM over a 9-month period. Initially, cell growth rates were depressed. However, after exposure to the medium for 9 months, the rate of cell growth increased gradually, indicating the establishment of tamoxifen-resistant cells.

Analysis of a public database. Expression data were downloaded from a public database [Kaplan-Meier plotter database (http://kmplot.com/breast)] (18). The clinical value of ACTA2 mRNA expression was analyzed by Kaplan-Meier survival plots in 209 ER(+) breast cancer patients with tamoxifen treatment (GSE2034) (19). The hazard ratios with 95% confidence intervals and log-rank P-values were calculated.

Human Phospho-Kinase Antibody Array. TamS and TamR cells were seeded at $1x10^6$ cells/plate in two separate 100-mm dishes. Protein lysates from TamS and TamR cells were prepared using the supplied buffers and 300 μg of protein was hybridized to each array from the Proteome Profiler Human Phospho-Kinase Antibody Array (R&D Systems, Minneapolis, MN, USA). Further steps were performed based on the manufacturer's protocol.

Soft agar colony formation assay. TamR breast cancer cells were seeded at a density of $5x10^4$ cells/well in 6-well plates in growth medium containing 0.7% agar (1.5 ml/well). The cells were seeded on top of a layer of growth medium containing 1.4% agar (2 ml/well). Next, growth medium (500 μl) with 10% FBS was added on top of the agar. In addition, 3 μM 4-OHT was added on top of the agar for some of the plates. The cells were plated and cultured in a 37˚C incubator for two weeks. After two weeks, the viable colonies were stained with 0.01% crystal violet and observed using an Olympus CK40 inverted microscope at x10 magnification (Olympus Corp., Tokyo, Japan).

Flow cytometric analysis (FACS). As in a previous study, we performed an apoptosis assay using the Annexin V-Fluorescein Isothiocyanate (FITC) Apoptosis Kit-I (BD Pharmingen; BD Biosciences, San Diego, CA, USA) (3). Briefly, cells ($1x10^6$ cells/ml) were collected and washed twice with PBS, and then resuspended in 500 μl of staining solution containing 5 μl FITC-conjugated Annexin V and propidium iodide (PI). After incubation for 15 min at room temperature (RT) in the dark, the cells were immediately analyzed on a flow cytometer. Apoptotic cells were double-stained with Annexin V and PI and then they were analyzed using the FACS Vantage system (Becton-Dickinson, San Diego, CA, USA). The percentage of cells undergoing apoptosis was determined.

Western blotting. Cell lysates were prepared to detect α-SMA, TP53, and β-actin expression. Equal amounts of protein (50 μg) were boiled for 5 min in Laemmli sample buffer, and then electrophoresed on 10% sodium dodecyl-sulfate polyacrylamide (SDS-PAGE) gels. Separated proteins were transferred to polyvinylidene fluoride (PVDF) membranes, and the membranes were blocked with 10% skim milk in Tris-buffered saline (TBS) containing 0.01% Tween-20 (TBS/T) for 15 min. Blots were washed three times in TBS/T and then incubated overnight with antibodies against α-SMA, TP53 and β-actin in TBS/T buffer at 4˚C. The blots were then washed three times in TBS/T and subsequently incubated with secondary HRP-conjugated antibodies (1:2,000 dilution; cat. nos. sc-2004 and sc-2005; Santa Cruz Biotechnology) in TBS/T buffer. After 1 h of incubation at room temperature (RT), the blots were washed three times in TBS/T and positive immunoreactive proteins were detected using the West-Q Chemiluminescent Substrate Plus kit.

Real-time PCR. Total RNA was extracted from the cells using Invitrogen™ TRIzol reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Isolated RNA samples were then used for RT-PCR. Samples of total RNA (1 μg) were reverse transcribed into cDNA in 20 μl reaction volumes using a first-strand cDNA synthesis kit for RT-PCR according to the manufacturer's instructions (MBI Fermentas, Hanover, MD, USA). Gene expression levels were quantified by real-time PCR using a SensiMix SYBR kit (Bioline Ltd., London, UK) and 100 ng of cDNA per reaction. The primer sequences used for this analysis were as follows: human TP53 (forward, 5'-GGCCCACTTCACCGTACTAA-3' and reverse, 5'-AAGCGAGACCCAGTCTCAAA-3'); human α-SMA (forward, 5'-AGACATCAGGGGGTGATGGT-3' and reverse, 5'-CATGGCTGGGACATTGAAAG-3'), and GAPDH was used as an internal control (forward, 5'-ATT GTT GCC ATC AAT GAC CC-3' and reverse, 5'-AGT AGA GGC AGG GAT GAT GT-3'). An annealing temperature of 60˚C was used for all the primers. PCR was performed in a standard 384-well plate format with an ABI 7900HT Real-Time PCR Detection System (Thermo Fisher Scientific, Inc.). For data analysis,

the raw threshold cycle (C_T) value was first normalized to the housekeeping gene for each sample to obtain a ΔC_T. The normalized ΔC_T was then calibrated to the control cell samples and to obtain the $\Delta\Delta C_q$ values (20).

Adenovirus induction. Adenovirus expressing *Lac Z* and human TP53 cDNA (Ad-TP53) was a gift from Dr Hyunil Ha (Korean Institute of Oriental Medicine, Daejeon, Korea). Recombinant adenovirus expressing human TP53 was reproduced in 293A cells. The expression of this construct was confirmed by western blot analysis. Each construct was transfected into Hs578T and BT549 cells for 24 h and incubated for 24 h in fresh culture media. Ad-TP53-overexpressing MDA-MB-231 and Hs578T cells were further incubated for 24 h in serum-free culture media. The cell lysates and culture medium were then harvested for analysis of α-SMA and TP53 expression.

TP53 siRNA transfection. TP53 siRNA was purchased from Bioneer Corporation (Daejeon, Korea). A synthetic siRNA against two different TP53 mRNAs was used to inhibit endogenous TP53 gene expression. The duplex sequences of TGFBRI and TGFBRII siRNA used for this experiment are as follows: Human #1 TP53 siRNA [sense, CACUACAACUA CAUGUGUA (dTdT) and antisense, UACAUAUGUAGUUG UAGUG (dTdT)]; and human #2 TP53 siRNA [sense, GA GGUUGGCUCUGACUGUA (dTdT) and antisense, UACAG UCAGAGCCAACCUC (dTdT)]. It was found that the optimal siRNA knockdown conditions involved transfecting TamR cells. Effectene (Qiagen, Inc., Valencia, CA, USA) was used for transfections with siRNA following protocols provided by the manufacturer. After the 72 h transfection, the levels of TP53 and α-SMA mRNA expression were analyzed by real-time PCR.

Statistical analysis. Statistical significance was determined using Student's t-test. The results are presented as the mean ± SEM. All the quoted P-values are two-tailed and the differences were considered statistically significant when the P-value was <0.05. Statistical analyses were performed using the Microsoft Excel 2016.

Results

α-SMA expression is associated with a poor prognosis in estrogen receptor-positive breast cancer patients. In a previous study, we reported that the levels of mesenchymal marker proteins such as fibronectin and α-SMA were significantly increased in TamR cells (3). In the present study, we investigated the clinical significance and the regulatory mechanism of α-SMA in breast cancer models. We found that the levels of α-SMA mRNA expression were associated with a poor prognosis in 209 ER(+) breast cancer patients using the GSE2034 dataset (Fig. 1A). ER(+) breast cancer patients with a high expression of α-SMA exhibited poorer relapse-free survival compared to the patients with low expression (P=0.0058, Fig. 1A).

Next, we analyzed the effect of tamoxifen on TamS and TamR cells. As shown in Fig. 1B and C, the anchorage-independent growth and apoptotic cell death of TamS were completely inhibited by 4-OHT treatment. However, cell growth and the death of TamR cells were not affected by 4-OHT. Furthermore, we examined the level of α-SMA expression in the TamS and TamR cells. α-SMA expression was significantly increased in the TamR cells (Fig. 1D). Under the same condition, the level of α-SMA mRNA expression was increased by 71.8-fold compared with the TamS cells (Fig. 1E). Therefore, we demonstrated that α-SMA expression was significantly increased in TamR cells and was associated with survival in ER(+) breast cancer patients.

Analysis of kinase activities between TamS and TamR cells. To characterize α-SMA expression-related protein kinases, we analyzed the phosphorylated levels of various protein kinases in TamS and TamR cells using the Proteome Profiler Human Phospho-Kinase Array. Protein lysates were loaded to the Proteome Profiler Human Phospho-Kinase Array Kit membranes. A schematic model of the membrane is indicated in Fig. 2A. In the present study, we found that the phosphorylation levels of Akt and TP53 were significantly increased in TamR cells when compared with TamS cells (Fig. 2B, red square). To confirm the results obtained by the Phospho-Kinase array, we examined the levels of pS392-p53 and pS46-p53 by western blot analysis. As expected, our results showed that the levels of pS392-p53 and pS46-p53 were markedly increased in the TamR cells (Fig. 2C). In addition, we observed that the level of TP53 protein (Fig. 2C) and mRNA (Fig. 2D) expression were slightly increased in the TamR cells. However, the phosphorylation level of p70 S6 kinase (T421/S424) was observably decreased in the TamR cells (Fig. 2B, green square). Based on these results, it was theorized that TP53 activity may be involved with α-SMA expression in breast cancer cells.

TP53 activator, nutlin3, upregulates α-SMA expression in breast cancer cells with wild-type TP53. To verify the role of TP53 on α-SMA expression, activator of TP53, Nut3, was administered to block the interaction of MDM2 and TP53 in TamS cells with wild-type TP53 for 24 h. After 24 h, we harvested whole cell lysates for the detection of α-SMA and TP53 protein and mRNA expression. As shown in Fig. 3A, the level of α-SMA expression was observably increased by Nut3 treatment. Under the same conditions, we analyzed the levels of α-SMA and TP53 mRNA expression. As expected, the level of α-SMA mRNA expression increased by 63.5±14.3-fold with Nut3 relative to the control (Fig. 3B). However, TP53 mRNA levels were not affected by Nut3 treatment, although the basal level of TP53 expression was slightly increased (Fig. 3A and B).

Next, we examined the effect of Nut3 on α-SMA expression in TP53-mutant breast cancer cells. The TP53 status of breast cancer cell lines was determined from the database http://p53.free.fr/Database/Cancer_cell_lines/Breast_cancer.html, which revealed the TP53-mutant breast cancer cells (MDA-MB-231 and Hs578T). As shown in Fig. 3C, the levels of α-SMA and TP53 protein expression were not observably altered by Nut3 treatment in MDA-MB-231 and Hs579T cells. In addition, the levels of α-SMA and TP53 mRNA expression were also markedly changed by Nut3 (Fig. 3D). Therefore, we demonstrated that the TP53 status plays an important role on α-SMA expression in breast cancer cells.

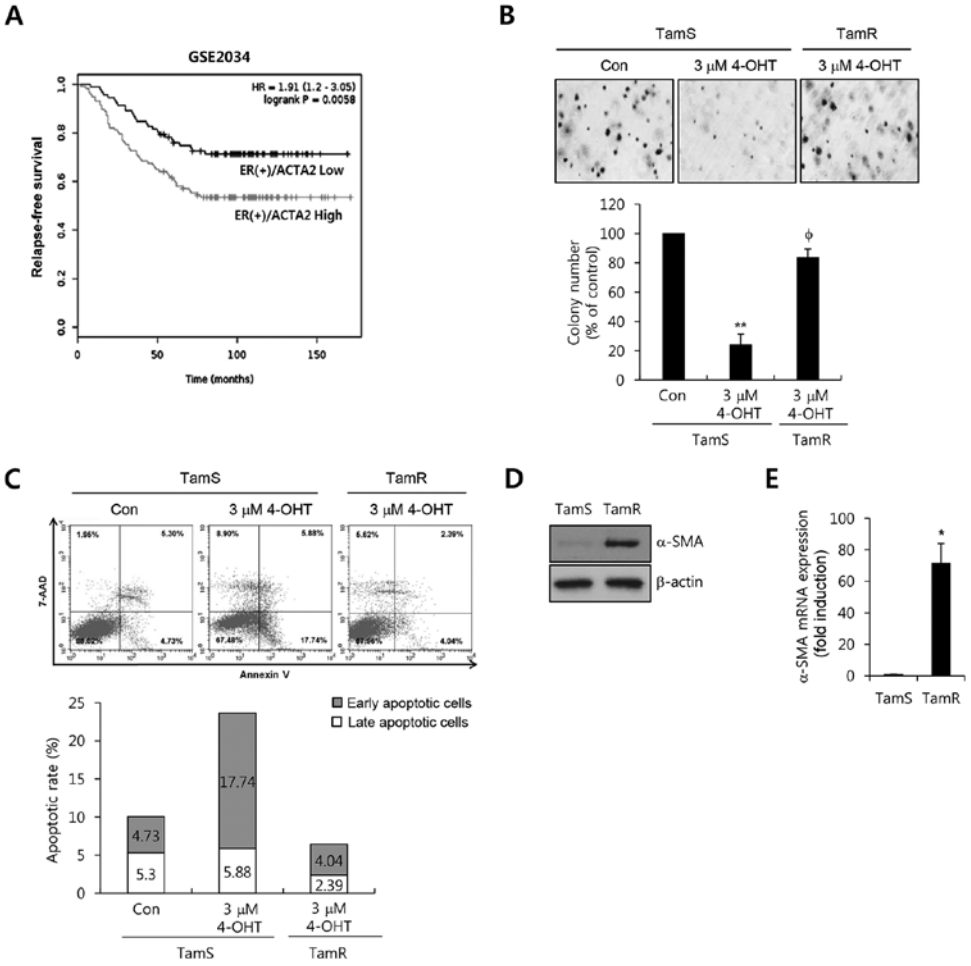

Figure 1. α-SMA expression is associated with a poor prognosis in estrogen receptor-positive [ER(+)] breast cancer patients. (A) The clinical value of α-SMA mRNA expression was analyzed in ER(+) breast cancer patients using a public database [Kaplan-Meier plotter database (http://kmplot.com/breast)]. (B) Established TamS and TamR cells were seeded in a 6-well soft agar plate with or without 3 μM 4-OHT for 2 weeks for establishment of the colony formation. (C) Established TamS and TamR cells were seeded with or without 3 μM 4-OHT for 24 h. Apoptotic cells were analyzed by flow cytometry. (D and E) Levels of α-SMA protein and mRNA expression were analyzed by western blot analysis (D) and real-time PCR (E), respectively. The results are representative of three independent experiments. Values shown are the mean ± SEM. *P<0.05, **P<0.01 vs. the control; $^{\phi}$P<0.05 vs. 4-OHT treated TamS. α-SMA, α smooth muscle actin; TamS, tamoxifen-sensitive cells; TamR, tamoxifen-resistant cells; Con, control.

A

	11	12	13	14	15	16	17	18
A			TP53 (S392)	TP53 (S392)			Reference Spot	Reference Spot
B	Akt1/2/3 (T308)	Akt1/2/3 (T308)	TP53 (S46)	TP53 (S46)				
C	p70 S6 kinase (T389)	p70 S6 kinase (T389)	TP53 (S15)	TP53 (S15)	c-Jun (S63)	c-Jun (S63)		
D	p70 S6 kinase (T421/S424)	p70 S6 kinase (T421/S424)	RSK1/2/3 (S380/S383/S377)	RSK1/2/3 (S380/S383/S377)	eNOS (S1177)	eNOS (S1177)		
E	STAT3 (Y705)	STAT3 (Y705)	p27 (T198)	p27 (T198)	PLC-γ1 (Y783)	PLC-γ1 (Y783)		
F	STAT3 (Y727)	STAT3 (Y727)	WNK1 (T60)	WNK1 (T60)	PYK2 (Y402)	PYK2 (Y402)		
G	HSP60	HSP60					Neg.	Neg.

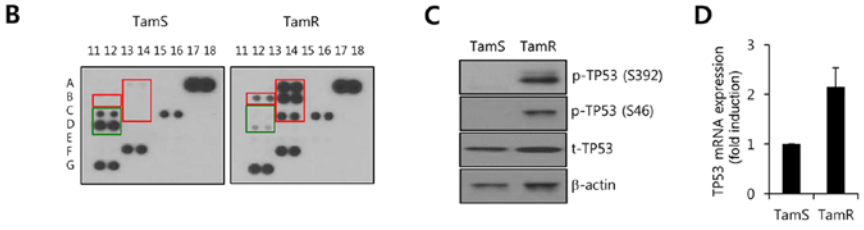

Figure 2. Analysis of kinase activities between TamS and TamR cells. (A) Schematic model of the kinase array membrane. (B) Protein lysates of TamS and TamR cells were subjected to a human phospho-kinase array. (C) Levels of p-TP53 (S392 and S46), t-TP53 and β-actin protein expression were analyzed by western blot analysis. (D) Levels of TP53 mRNA expression were analyzed by real-time PCR. The results are representative of three independent experiments. TamS, tamoxifen-sensitive cells; TamR, tamoxifen-resistant cells.

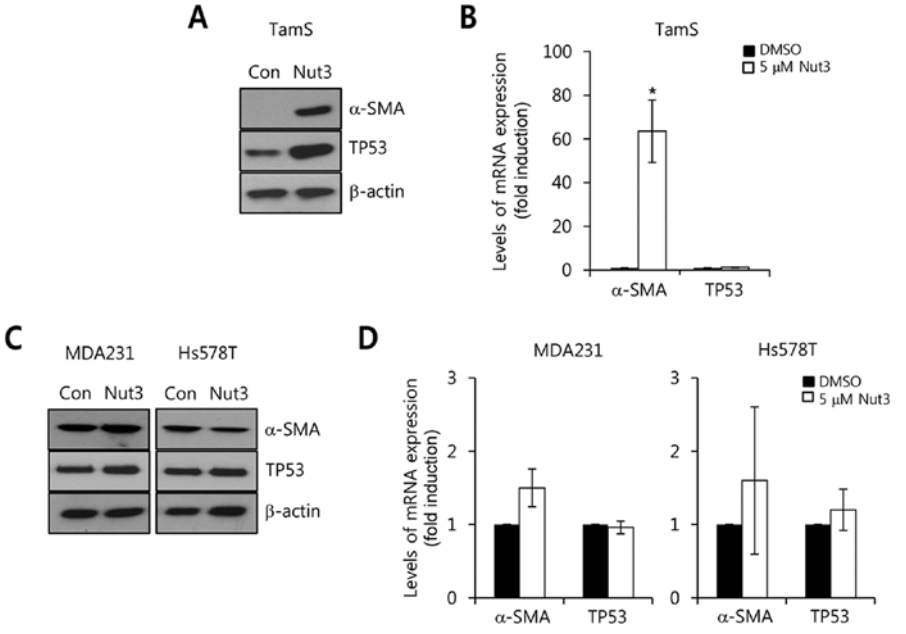

Figure 3. TP53 activator, Nutlin3 (Nut3), upregulates α-SMA expression in breast cancer cells with wild-type TP53. (A and C) TamS, MDA-MB-231 (MDA231) and Hs578T cells were treated with 5 μM Nut3 for 24 h. Levels of α-SMA, TP53 and β-actin protein expression were analyzed by western blot analysis. (B and D) Levels of α-SMA and TP53 mRNA expression were analyzed by real-time PCR. The results are representative of three independent experiments. Values shown are the mean ± SEM. *P<0.05 vs. control. α-SMA, α smooth muscle actin; Con, control; Nut3, nutlin3; TamS, tamoxifen-sensitive cells.

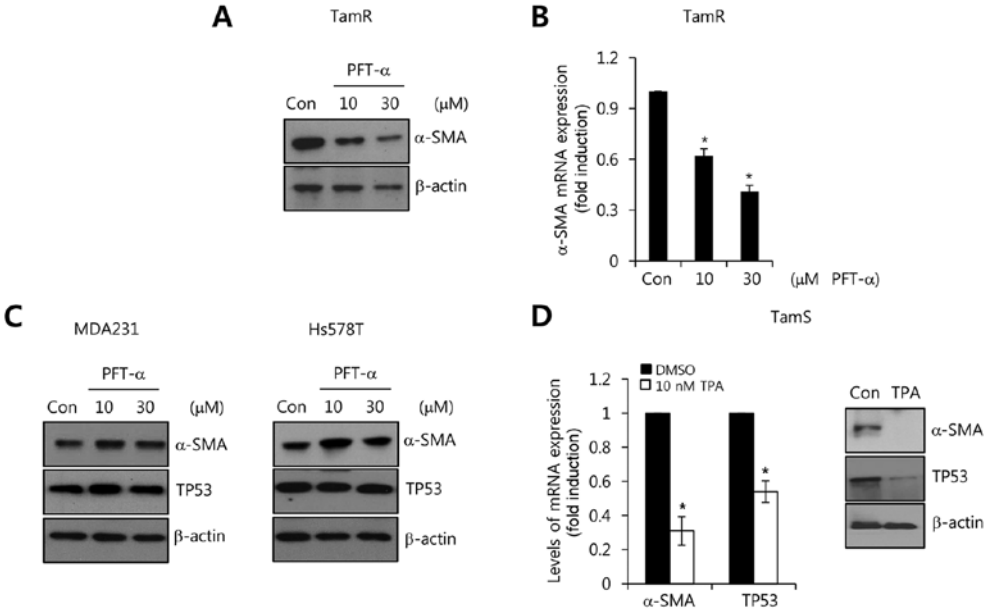

Figure 4. Suppression of TP53 downregulates α-SMA expression in TamR cells. (A-C) TamR, MDA-MB-231 (MDA231) and Hs578T cells were treated with the indicated doses of PFT-α for 72 h. (D) TamS cells were treated with 10 nM TPA for 24 h. Levels of α-SMA and TP53 protein and mRNA expression were analyzed by western blot analysis and real-time PCR, respectively. The results are representative of three independent experiments. Values shown are the mean ± SEM. *P<0.05 vs. control. α-SMA, α smooth muscle actin; TamS, tamoxifen-sensitive cells; TamR, tamoxifen-resistant cells; Con, control; PFT-α, pifithrin-α (TP53 inhibitor).

Suppression of TP53 downregulates α-SMA expression in tamoxifen-resistant cells. Conversely, we investigated the effect of a TP53 inhibitor, pifithrin-α (PFT-α), on α-SMA expression in TamR cells. We treated the TamR cells with the indicated concentrations (10 and 30 μM) of PFT-α for 72 h. As shown in Fig. 4A, the basal levels of α-SMA protein expression were dose-dependently decreased by PFT-α treatment. In addition, the levels of α-SMA mRNA expression were also decreased by PFT-α (Fig. 4B). The reduction of α-SMA mRNA expression by PFT-α was decreased to 0.62 and 0.41-fold relative to the control (Fig. 4B). However, the MDA-MB-231 and Hs578T cells with mutant TP53 were not altered by PFT-α treatment (Fig. 4C). In a previous study, TPA stimulated the ubiquitination and degradation of TP53 through the downregulation of PKC-d and the suppression of TP53 transcriptional activity (21,22). Therefore, we treated the

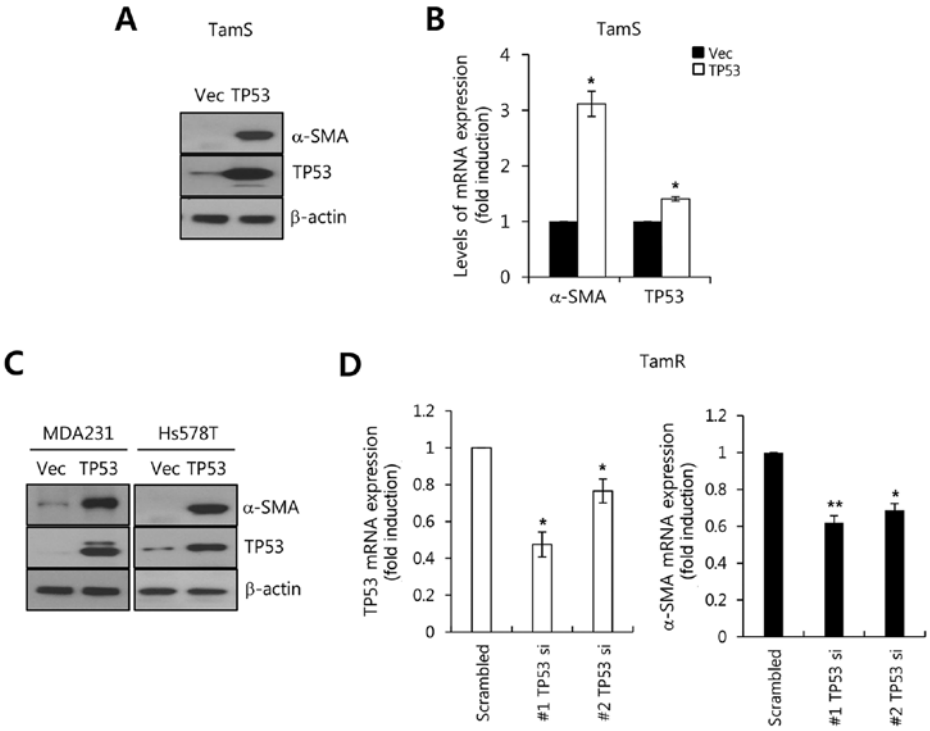

Figure 5. Wild-type TP53 upregulates α-SMA expression in breast cancer cells. (A and C) After Ad-*Lac Z* and Ad-TP53 infection for 48 h using whole cell lysates, levels of α-SMA, TP53 and β-actin protein expression were measured by western blot analysis. (B) Levels of α-SMA and TP53 mRNA expression were analyzed by real-time PCR. (D) TamR cells were transfected with two different TP53 siRNA, respectively, for 72 h. The levels of TP53 and α-SMA mRNA expression were analyzed by real-time PCR. The results are representative of three independent experiments. Values shown are the mean ± SEM. *P<0.05, **P<0.01 vs. control (vector transfected or scrambled cells). α-SMA, α smooth muscle actin; Con, control; TamR, tamoxifen-resistant cells.

TamS cells with 10 nM TPA for 24 h with wild-type TP53. As expected, the level of α-SMA mRNA expression was decreased to 0.31-fold relative to the control (Fig. 4D). In addition, the level of TP53 mRNA expression decreased 0.54-fold relative to the control (Fig. 4D). Based on these results, we demonstrated that the level of wild-type TP53 plays an important role in the α-SMA expression in breast cancer cells.

Wild-type TP53 upregulates α-SMA expression in breast cancer cells. We investigated whether TP53 upregulates α-SMA expression in breast cancer cells. In the present study, breast cancer cells were transfected with adenoviral *LacZ* and *TP53* genes for 48 h and further incubated for 24 h in fresh serum-free media. As expected, our results showed that the level of α-SMA protein expression was significantly increased by TP53 overexpression (Fig. 5A). Under the same conditions, the level of α-SMA mRNA expression was increased by TP53 overexpression (Fig. 5B). The level of α-SMA mRNA expression increased 3.11±0.23-fold relative to the Vec alone (Fig. 5B). As shown in Fig. 3C and D, triple-negative breast cancer (TNBC) cells with mutant TP53 did not exhibit changes in the levels of α-SMA expression by Nut3 treatment. However, these cells also exhibited significantly increased levels of α-SMA expression following wild-type TP53 overexpression (Fig. 5C). In contrast, we examined the effects of TP53 knockdown by two different TP53 siRNAs on α-SMA expression in TamR cells. As expected, our results showed that the downregulation of TP53 by TP53 siRNA decreased the levels of α-SMA mRNA expression in TamR cells (Fig. 5D). Therefore, we demonstrated that wild-type TP53 expression upregulates the level of α-SMA expression in breast cancer cells.

Discussion

Breast cancer is the most common cancer in women worldwide accounting for approximately 25% of all cancer cases and 15% of all cancer-related deaths (23). Although therapeutic strategies for breast cancer patients involve systemic therapy including chemotherapy, hormonal therapy and targeted therapy, their therapeutic efficacy is still limited by either intrinsic or acquired resistances (24,25). To date, the level of α-SMA expression has been known as a prognostic marker for a variety of cancers, including oral tongue squamous cell carcinoma and lung adenocarcinomas (4,26). Aberrant α-SMA expression is significantly associated with worse overall survival and metastasis-free survival rates in lung adenocarcinomas (4). Consistent with these reports, we also observed that α-SMA expression is associated with survival in breast cancer. ER-α(+) breast cancer patients with high expression of α-SMA exhibited decreased relapse-free survival compared to α-SMA-low expressing patients. In addition, we found that the levels of α-SMA mRNA and protein expression were increased in TamR cells when compared to TamS cells. Therefore, we focused on the underlying mechanism of α-SMA expression which is highly expressed in tamoxifen-resistant breast cancer cells.

The level of TP53 expression is also regulated by a wide variety of cellular stress, such as UV, hypoxia and phorbol ester (11,22,27). Our results showed that the basal level of

TP53 expression decreased while α-SMA mRNA and protein expression were significantly increased through TPA treatment. In previous studies, the basal level of α-SMA expression was upregulated through focal adhesion kinase (FAK) as well as the JAK2/STAT1-dependent pathway in breast cancer cells and fibroblasts (2,28,29). In the present study, we analyzed kinase activities to identify the regulatory kinases on α-SMA expression in TamS and TamR cells. Our results showed that the activities of TP53 and Akt were significantly increased in TamR cells, but did not affect the activity of p70 S6 kinase. Therefore, we investigated the role of TP53 in the regulation of α-SMA expression in breast cancer cells.

Nutlin3 (Nut3), a *cis*-imidazoline analog, interferes with the binding between MDM2 and p53 as an antagonist of MDM2, an E3 ubiquitin ligase of protein of the TP53 family (30). Nut3 induces prominent $p21^{WAF1}$ expression by upregulating the phosphorylation of Ser 46 on p53 (31). Although we did not assess the phosphorylation of TP53 by Nut3, our results showed that the basal level of TP53 expression was slightly increased through Nut3 treatment in breast cancer cells with wild-type TP53, but not in breast cancer cells with mutant TP53. In addition, α-SMA expression was significantly increased following Nut3 treatment in breast cancer cells with wild-type TP53. In contrast, the basal level of α-SMA mRNA and protein expression dose-dependently decreased with PFT-α in TamR cells that blocked the transcriptional activity of TP53. Therefore, we demonstrated that the activity and expression of wild-type TP53 plays an important role in the regulation of α-SMA expression in breast cancer cells.

The tumor-suppressor protein TP53 is a transcription factor that regulates anti-carcinogenesis programs associated with apoptosis, cell cycle arrest, and DNA repair in genotoxic and not-genotoxic cellular injuries (32,33). In recent studies, we reported that the level of fibronectin expression is associated with the status and expression of TP53 in breast cancer cells (34,35). Wu *et al* reported that Slug is transcriptionally induced by TP53 and then escapes from apoptosis by repressing the TP53-mediated transcription of Puma (36). We found for the first time that the levels of α-SMA expression are increased by wild-type TP53 overexpression in all breast cancer cells with wild-type or mutant TP53. Therefore, we demonstrated that wild-type TP53 is involved with α-SMA expression in breast cancer cells.

In conclusion, aberrant α-SMA expression is associated with the survival of ER(+) breast cancer patients. Nut3 significantly increased the levels of α-SMA mRNA and protein expression in TamS cells with wild-type TP53, but not in MDA-MB-231 and Hs578T cells with mutant TP53. In addition, the overexpression of wild-type TP53 augmented the level of α-SMA expression in all breast cancer cells with wild-type or mutant TP53. In contrast, we observed that PFT-α dose-dependently suppressed α-SMA expression in TamR cells. Based on these results, we demonstrated that wild-type TP53 expression augments the level of α-SMA expression in breast cancer cells. We also will investigate whether TP53 directly regulates α-SMA expression in breast cancer cells.

Acknowledgements

Not applicable.

Funding

The present study was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (2016R1D1A1B01010508) and by a National Research Foundation of Korea (NRF) grant funded by the Korean Gove rnment (MSIP) (2016R1A5A2945889).

Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

SK and JEL designed the study, interpreted the data and wrote the manuscript. DY and YJ were responsible for the laboratory experiments such as real-time PCR and western blotting; JY, SWK and SJN analyzed the data and developed the prognostic signature. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors state that they have no competing interests.

References

1. Lambrechts A, Van Troys M and Ampe C: The actin cytoskeleton in normal and pathological cell motility. Int J Biochem Cell Biol 36: 1890-1909, 2004.
2. Jeon M, You D, Bae SY, Kim SW, Nam SJ, Kim HH, Kim S and Lee JE: Dimerization of EGFR and HER2 induces breast cancer cell motility through STAT1-dependent ACTA2 induction. Oncotarget 8: 50570-50581, 2016.
3. Kim S, Lee J, Oh SJ, Nam SJ and Lee JE: Differential effect of EGFR inhibitors on tamoxifen-resistant breast cancer cells. Oncol Rep 34: 1613-1619, 2015.
4. Lee HW, Park YM, Lee SJ, Cho HJ, Kim DH, Lee JI, Kang MS, Seol HJ, Shim YM, Nam DH, *et al*: Alpha-smooth muscle actin (ACTA2) is required for metastatic potential of human lung adenocarcinoma. Clin Cancer Res 19: 5879-5889, 2013.
5. Sinn M, Denkert C, Striefler JK, Pelzer U, Stieler JM, Bahra M, Lohneis P, Dorken B, Oettle H, Riess H and Sinn BV: α-Smooth muscle actin expression and desmoplastic stromal reaction in pancreatic cancer: results from the CONKO-001 study. Br J Cancer 111: 1917-1923, 2014.
6. Tsujino T, Seshimo I, Yamamoto H, Ngan CY, Ezumi K, Takemasa I, Ikeda M, Sekimoto M, Matsuura N and Monden M: Stromal myofibroblasts predict disease recurrence for colorectal cancer. Clin Cancer Res 13: 2082-2090, 2007.
7. Aoki H, Ohnishi H, Hama K, Shinozaki S, Kita H, Osawa H, Yamamoto H, Sato K, Tamada K and Sugano K: Cyclooxygenase-2 is required for activated pancreatic stellate cells to respond to proinflammatory cytokines. Am J Physiol Cell Physiol 292: C259-C268, 2007.

8. Lim MJ, Ahn J, Yi JY, Kim MH, Son AR, Lee SL, Lim DS, Kim SS, Kang MA, Han Y, *et al*: Induction of galectin-1 by TGF-β1 accelerates fibrosis through enhancing nuclear retention of Smad2. Exp Cell Res 326: 125-135, 2014.
9. Petitjean A, Mathe E, Kato S, Ishioka C, Tavtigian SV, Hainaut P and Olivier M: Impact of mutant p53 functional properties on *TP53* mutation patterns and tumor phenotype: Lessons from recent developments in the IARC TP53 database. Hum Mutat 28: 622-629, 2007.
10. Davidoff AM, Humphrey PA, Iglehart JD and Marks JR: Genetic basis for p53 overexpression in human breast cancer. Proc Natl Acad Sci USA 88: 5006-5010, 1991.
11. Sugrue MM, Shin DY, Lee SW and Aaronson SA: Wild-type p53 triggers a rapid senescence program in human tumor cells lacking functional p53. Proc Natl Acad Sci USA 94: 9648-9653, 1997.
12. Royds JA and Iacopetta B: p53 and disease: When the guardian angel fails. Cell Death Differ 13: 1017-1026, 2006.
13. Sur S, Pagliarini R, Bunz F, Rago C, Diaz LA Jr, Kinzler KW, Vogelstein B and Papadopoulos N: A panel of isogenic human cancer cells suggests a therapeutic approach for cancers with inactivated p53. Proc Natl Acad Sci USA 106: 3964-3969, 2009.
14. Woods DB and Vousden KH: Regulation of p53 function. Exp Cell Res 264: 56-66, 2001.
15. Gasco M, Shami S and Crook T: The p53 pathway in breast cancer. Breast Cancer Res 4: 70-76, 2002.
16. Choi HK, Yang JW, Roh SH, Han CY and Kang KW: Induction of multidrug resistance associated protein 2 in tamoxifen-resistant breast cancer cells. Endocr Relat Cancer 14: 293-303, 2007.
17. You D, Jung SP, Jeong Y, Bae SY, Lee JE and Kim S: Fibronectin expression is upregulated by PI-3K/Akt activation in tamoxifen-resistant breast cancer cells. BMB Rep 50: 615-620, 2017.
18. Gyorffy B, Lanczky A, Eklund AC, Denkert C, Budczies J, Li Q and Szallasi Z: An online survival analysis tool to rapidly assess the effect of 22,277 genes on breast cancer prognosis using microarray data of 1,809 patients. Breast Cancer Res Treat 123: 725-731, 2010.
19. Wang Y, Klijn JG, Zhang Y, Sieuwerts AM, Look MP, Yang F, Talantov D, Timmermans M, Meijer-van Gelder ME, *et al*: Gene-expression profiles to predict distant metastasis of lymph-node-negative primary breast cancer. Lancet 365: 671-679, 2005.
20. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. Methods 25: 402-408, 2001.
21. Abbas T, White D, Hui L, Yoshida K, Foster DA and Bargonetti J: Inhibition of human p53 basal transcription by down-regulation of protein kinase Cdelta. J Biol Chem 279: 9970-9977, 2004.
22. Kim S, Han J, Kim NY, Lee SK, Cho DH, Choi MY, Kim JS, Kim JH, Choe JH, Nam SJ, *et al*: Effect of berberine on p53 expression by TPA in breast cancer cells. Oncol Rep 27: 210-215, 2012.
23. DeSantis C, Ma J, Bryan L and Jemal A: Breast cancer statistics, 2013. CA Cancer J Clin 64: 52-62, 2014.
24. Musgrove EA and Sutherland RL: Biological determinants of endocrine resistance in breast cancer. Nat Rev Cancer 9: 631-643, 2009.
25. Pohlmann PR, Mayer IA and Mernaugh R: Resistance to trastuzumab in breast cancer. Clin Cancer Res 15: 7479-7491, 2009.
26. Ding L, Zhang Z, Shang D, Cheng J, Yuan H, Wu Y, Song X and Jiang H: α-Smooth muscle actin-positive myofibroblasts, in association with epithelial-mesenchymal transition and lymphogenesis, is a critical prognostic parameter in patients with oral tongue squamous cell carcinoma. J Oral Pathol Med 43: 335-343, 2014.
27. Han J, Kim S, Yang JH, Nam SJ and Lee JE: TPA-induced p21 expression augments G2/M arrest through a p53-independent mechanism in human breast cancer cells. Oncol Rep 27: 517-522, 2012.
28. Chen R, Zhang Z, Xue Z, Wang L, Fu M, Lu Y, Bai L, Zhang D and Fan Z: Focal adhesion kinase (FAK) siRNA inhibits human hypertrophic scar by suppressing integrin alpha, TGF-β and α-SMA. Cell Biol Int 38: 803-808, 2014.
29. Li S, Butler P, Wang Y, Hu Y, Han DC, Usami S, Guan JL and Chien S: The role of the dynamics of focal adhesion kinase in the mechanotaxis of endothelial cells. Proc Natl Acad Sci U S A 99: 3546-3551, 2002.
30. Wade M, Li YC and Wahl GM: MDM2, MDMX and p53 in oncogenesis and cancer therapy. Nat Rev Cancer 13: 83-96, 2013.
31. Enge M, Bao W, Hedstrom E, Jackson SP, Moumen A and Selivanova G: MDM2-dependent downregulation of p21 and hnRNP K provides a switch between apoptosis and growth arrest induced by pharmacologically activated p53. Cancer Cell 15: 171-183, 2009.
32. Levine AJ: p53, the cellular gatekeeper for growth and division. Cell 88: 323-331, 1997.
33. Menendez D, Inga A and Resnick MA: The expanding universe of p53 targets. Nat Rev Cancer 9: 724-737, 2009.
34. You D, Jung SP, Jeong Y, Bae SY and Kim S: Wild-type p53 controls the level of fibronectin expression in breast cancer cells. Oncol Rep 38: 2551-2557, 2017.
35. Jeong Y, You D, Kang HG, Yu J, Kim SW, Nam SJ, Lee JE and Kim S: Berberine suppresses fibronectin expression through inhibition of c-jun phosphorylation in breast cancer cells. J Breast Cancer 21: 21-27, 2018.
36. Wu WS, Heinrichs S, Xu D, Garrison SP, Zambetti GP, Adams JM and Look AT: Slug antagonizes p53-mediated apoptosis of hematopoietic progenitors by repressing *puma*. Cell 123: 641-653, 2005.