Role of p53 in antioxidant defense of HPV-positive cervical carcinoma cells following H_2O_2 exposure

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Role of p53 in antioxidant defense of HPV-positive cervical carcinoma cells following H_2O_2 exposure

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ABSTRACT

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In HPV-positive cervical carcinoma cell lines, p53 protein is functionally antagonized by the viral E6 oncoprotein; however, normal p53 function and p53 associated pathways can be demonstrated in several experimental model systems, suggesting that p53 is not completely inactivated in cervical cancer cells. Here, we investigated the possible role of p53 protein in antioxidant defense of HPV-positive cervical cancer cell lines. We found that SiHa cells containing integrated HPV 16 had higher expression of p53 and exhibited the greatest resistant to H_2O_2-induced oxidative damage, as compared with HeLa, CaSki and ME180 cell lines. Downregulation of p53 using RNA interference (RNAi) resulted in the inhibition of p53-regulated antioxidant enzymes and elevated intracellular ROS in SiHa cells. In contrast, the ROS level was not

affected in HeLa, CaSki and ME180 cell lines after inhibition of p53 protein. Under mild or sever H_2O_2-induced stress, p53-deficient SiHa cells exhibited much higher ROS levels than control SiHa cells. Furthermore, we analyzed cell viability and apoptosis after H_2O_2 treatment and found that p53 deficiency sensitized SiHa cells to H_2O_2 damage. Inhibition of p53 resulted in excessive oxidation of DNA; control SiHa cells exhibited a more rapid removal of 8-oxo-7,8-dihydro-2'-deoxyguanosine from DNA than p53-deficient SiHa cells exposed to the same level of H_2O_2 challenge. Taken together, this present study provides evidence that endogenous p53 in SiHa cells has an antioxidant function and involves in the reinforcement of the antioxidant defense.

Key words : p53, SiHa, H_2O_2, ROS, oxidative stress, RNA interference (RNAi)

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I. INTRODUCTION

Reactive oxygen species (ROS) such as superoxide, hydroxyl and peroxyl radicals, and hydrogen peroxide (H_2O_2) are more reactive than molecular oxygen[1] and therefore can indiscriminately oxidize biological molecules, providing a constant threat to cells in an oxygen-rich environment[2]. In living cells, endogenous ROS are generated as byproducts of cellular metabolism and through leakage of electrons from the mitochondrial electron transport chain[3]; they are also derived from exogenous sources such as UV radiation, γ-irradiation, and chemicals. Present in all aerobic cells, ROS exist in a physiological balance with biochemical antioxidants. Oxidative stress occurs when this critical balance is disrupted, increasing intracellular ROS levels. In order to maintain intracellular redox homeostasis and protect against oxidative damage, cells have developed a sophisticated antioxidant defense system by

enlisting functional antioxidant buffers through redox-coupled enzymatic networks that regenerate oxidized substrates[4,5]. In this system, superoxide dismutase (SOD) converts the superoxide anion radical (O_2^-) to H_2O_2 which is subsequently eliminated by catalase (CAT), glutathione peroxidase 1 (Gpx1) and peroxiredoxin (Prx). Gpx1, a selenoprotein, is considered the primary enzyme responsible for the removal of H_2O_2, and may have a role in the regulation of cellular redox status[6,7]. Likewise, Prx is a major reductant of endogenously produced peroxides in eukaryotes and catalyzes the conversion of H_2O_2 into H_2O by using reducing equivalents provided by thioredoxin (Trx)[8-10]. During the peroxiredoxin catalytic cycle, peroxidatic cysteine is oxidized to a sulfenic acid form which typically reacts with a proximal thiol to form a disulfide bond[11,12]. this intermolecular disulfide bond is subsequently reduced by Trx; however, because the formation of the resolving disulfide bond is slow, high concentrations of ROS cause further oxidation of the peroxidatic cysteine to sulfinic acid, yielding an inactive form of Prx, that cannot be reduced by typical cellular reductants such as glutathione or thioredoxin[13-15]. Sestrins, a family of cysteine sulfinyl reductases, are essential for reactivating Prx by reducing of the cysteine sulfinic acid to thiol, thus reestablishing the antioxidant firewall[16].

The tumor suppressor protein p53 is an important sensor of cellular stress and is involved in regulating cellular responses to DNA damage. Depending on the cell type and the context of stimuli, p53 can either induce cell growth arrest to allow repair or alternatively, triggers apoptosis to prevent DNA damage in abnormal or stress-exposed cells from becoming fixed as a mutation[17]. Additional evidence exists suggesting that p53 works in a more positive way, participating in the maintenance of intracellular redox

homeostasis and protection of the genome from oxidative damage. First, there are several p53-regulated genes such as Gpx1, SOD_2, and aldehyde dehydrogenase 4 family, member A1 (ALDH4A1) that, encode products that act as antioxidants[18-20]. Second, reactivation of overoxidized Prx is mediated by two p53-regulated sestrins, namely, PA26 and Hi95 (encoded by SESN1 and SESN2, respectively). Third, in the absence of stress or after mild stress, a relatively low level of p53 is sufficient enough to upregulate several antioxidant genes that decrease ROS levels and protect cells from DNA damage[21].

Decreasing intracellular levels of ROS has long been a goal for cancer prevention. Owing to the effects of many cancer therapeutics, cancerous cells can be subjected to oxidative stress[22], increasing the understanding of how ROS homeostasis is achieved in cancer cells which may be critical to developing more effective cancer therapies. Cervical cancer is the second most common cancer among women in the world in both incidence and mortality, and high-risk human papillomavirus (HPV), mainly serotypes 16 and 18, is present in more than 90% of such tumors[23, 24]. Most HPV-positive cervical carcinoma cells possess the wild-type p53 gene, but it is often rendered non-functional by the E6 oncoprotein which complexes with cellular proteins E6-AP and p53 to facilitate p53 degradation via an ubiquitin-dependent proteolytic system[25]. When studied in cervical carcinoma cells expressing E6 in the context of its natural promoter, endogenous p53 protein could exert transcriptional function following DNA damage, despite coexpression of the viral E6 protein, suggesting that the amount of expressed HPV E6 is proportionate to the inhibition of p53 activity. Basal p53 activity, as measured with luciferase reporter assays in HPV-positive cervical cancer

cells, showed that these cells have residual p53 activity and that p53 is not completely inactivated in cervical cancer cells[26-28].

To provide new insights into the possible role of endogenous p53 in the antioxidant defense of HPV-positive cervical cancer cells, we used a plasmid-mediated short hairpin RNA (shRNA) to knock down p53 expression in SiHa, HeLa, CaSki and ME180 cells (all of which integrated with high risk HPV sequences) and investigated whether p53 could modulate intracellular ROS levels under both nonstressed and stressed conditions. We found that endogenous p53 in SiHa cells was involved in the modulation of ROS levels, and may participate in maintaining ROS homeostasis. These findings may help to facilitate future clinical studies of HPV-positive cervical cancer therapy.

II. MATERIALS AND METHODS

1. Cell lines and cell culture

Four cervical carcinoma cell lines, namely, SiHa, HeLa, CaSki, and ME180 were used in this study. SiHa and CaSki contain integrated HPV 16; HeLa cells carry integrated HPV 18; and ME180 cells contain HPV 68 sequences. SiHa and HeLa cells were grown in DMEM medium (GIBCO-BRL, Grand Island, NY, USA), CaSki and ME180 cells were maintained in RPMI 1640 medium (GIBCO-BRL, Grand Island, NY, USA), supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin. Cultures were maintained at 37°C in a 5% CO_2 atmosphere, at 100% humidity.

2. Western blot analysis

Cell lysates were made using the PRO-PREP protein extraction solution (Intron Biotech, Korea). Lysates were clarified by centrifugation at 15,000 × g at 4°C for 20 min, and the protein concentration of the supernatant was determined with a Bradford assay (BioRad). For sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) protein lysates were mixed with Laemmli's sample buffer and boiled for 20 min. Total protein was separated on a 12% gel and transferred to nitrocellulose (Amersham, Germany). After blocking in TBST (20 mM Tris/137 mM NaCl/0.1% Tween 20, pH 7.6) with 5% skim milk, membranes were incubated with the primary antibodies diluted in TBST with 3% skim milk for 2 h at room temperature. Next, membranes were washed three times with TBST, and incubated for an additional 2 hours with horseradish peroxidase-linked secondary antibody (1:2,000) diluted in TBST with 3% skim milk. Labeled protein bands were

visualized with the enhanced chemiluminescence reagent (ECL) (Pierce, Rockford). Mouse monoclonal DO1 antibody against p53 (1:2000) and goat polyclonal antibody against GAPDH (1:2000) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse monoclonal antibody against GPX1 (2μg/ml) was obtained from MBL (Japan). Rabbit polyclonal antibody against SESN2 (2μg/ml) was purchased from Quark Biotech (Korea). Horseradish peroxidase-labeled secondary antibodies were purchased from Zymed. Immunoblotting for GAPDH was performed to verify equivalent protein loading. Densitometry analysis of Western blots was performed using TINA 2.0 Software (Raytest Isotopenmessgerate Gmbh, Straubenhardt, Germany).

3. RNA isolation and RT-PCR analysis

Cells were collected and total RNA was extracted using an UltraspecTM-II RNA isolation kit (Biotecx, Houston, Texas, USA). To prepare cDNA, five hundred nanograms of total RNA was mixed with the SuperScript RT-PCR System (Invitrogen). Five uL of the RT reaction was then used for PCR using the HotStarTaq DNA polymerase (Qiagen). The PCR reactions were carried out under the following temperature profile: 1) denaturation at 95°C for 15 min; 2) 35 cycles of 95°C for 40 s, 56°C for 1 min, 72°C for 1 min; 3) a final extension for 10 min at 72°C. To detect the corresponding gene expression, we used the following primers: TP53, 5'-TCCACTACAACTACATGTGTAAC-3' and 5'-GTGAAATATTCTCCATCCAGTG-3'; SESN1 (T2), 5'-CGACCAGGACGAGGAACTT-3' and 5'-CCAATGTAGTGACGATAATGTAGG-3' ACTB, 5'-AAGAGAGGCATCCTCACCCT-3' and

5'-TACATGGCTGGGGTGTTGAA-3'.

4. RNA interference

In order to generate a short hairpin RNAi specific for p53, we used the pSuppressorNeo p53 plasmid (IMG-803, Imgenex, San Diego, CA). A negative control plasmid (IMG-800) with a scrambled sequence was supplied from Imegenex. The following sequences, representing 19 bp of the mRNAs, were present in the hairpin transcripts: TP53, 5′-GACTCCAGTGGTAATCTAC-3′; negative control, 5′-AGTCACGTTAATGGTCGTT-3′. Cells were cultured overnight and transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the protocol of the manufacturer. Briefly, both the DNA and Lipofectamine 2000 were diluted with serum-free medium. The diluted DNA was added to the diluted Lipofectamine 2000, mixed gently, and incubated at room temperature for 30 min. Lastly, the DNA-Lipofectamine 2000 mixture was carefully added to cells at 95% conflueny in fresh medium without antibiotics and incubated at 37°C.

5. Cell proliferation and viability analysis

In order to measure the number of viable cells, a Trypan blue exclusion assay was used. Cells were seeded in a 24-well plate and incubated for 24h. At the indicated time points, cells were collected and stained with 0.2% Trypan blue. Cell numbers were determined by direct counting of cells under the microscope, using a standard haemocytometer. Cell viability was measured using a WelCountTM Cell Viability Assay Kit (WelGENE Inc, Seoul, Korea). Briefly, we defrosted the XTT reagent and PMS at 37°C, prepared the reaction mixture (40 µl PMS and 2 ml XTT reagent per plate), added 20 µl of the reaction mixture to each well (each containing 100 µl media), and incubated

at 37°C for 4 h. After observing a change in color of the solution, we measured these samples at a wavelength of 490nm using a Molecular Devices VERSAmax microplate reader (Molecular devices, Sunnyvale, CA, USA). All experiments were performed in triplicate.

6. Measurement of ROS accumulation

Intracellular production of ROS was measured using a cell-permeable fluorescent dye, 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H2DCFDA, Molecular Probes). When CM-H_2DCFDA is oxidized by ROS in cells, its fluorescent signal increases. For the assay, cells were plated in six-well plates and loaded with CM-H_2DCFDA in the dark for 30 min at 37°C. Next, cells were washed twice with PBS, trypsinized. And fluorescence was measured using flow cytometry (excitation at 488 nm, emission at 515–545 nm). Data analysis was performed with CELLQuest software and the mean fluorescence intensity was used to quantify the responses. A minimum of 10,000 cells were acquired for each sample.

7. Cell Cycle Distribution Observation

Flow cytometry analysis of PI-stained cells was performed to demonstrate the progression of the cell cycle. Briefly, cells were harvested, washed, and fixed in 70% ethanol overnight at 4°C. Prior to flow cytometry, cells were washed and stained with 1 ml of PI (50 μg/ml) containing 0.1 mg/ml RNase A. DNA content was determined with a FACScan flow cytometer (Becton Dickinson) and the proportion of cells in a particular phase of cell cycle was determined with CellQuest software.

8. Apoptosis assay by Annexin V/PI staining

An Annexin V-FITC Apoptosis Kit I (BD, San Jose, CA, USA) was used to

detect apoptosis. Annexin V is a $Ca2^{+}$-dependent phospholipid-binding protein that has a high affinity for phosphatidylserine (PS). In apoptotic cells, PS is translocated from the inner leaflet of the plasma membrane to the outer leaflet. Propidium iodide (PI), a standard flow cytometric viability probe, was used to distinguish between viable and nonviable cells. Cells that were stained were detected by a flow cytometer. Briefly, 1×10^{6} cells were harvested, washed with ice-cold PBS, and resuspended in 500 µl of Annexin binding buffer. A 100 µl aliquot (2×10^{5} cells) was taken, 5 µl each of Annexin V-FITC and PI were added, and cells were incubated for 15 min at room temperature in the dark. 400 µl of binding buffer (10 mM HEPES/NaOH pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) was added and samples were acquired on a FACScan flow cytometer (FACSCalibur, Becton Dickinson) and analyzed using CELLQuest software with in 1 hour. Cells that were Annexin V^{-}/PI^{+} were counted as necrotic, those that showed up as Annexin V^{+}/PI^{+} were counted as late apoptotic or secondarily necrotic, and Annexin V^{+}/PI^{-} cells were recognized as apoptotic. All procedures stated above were performed according to the manufacturer's instructions.

9. Detection of 8-oxo-dG residues in DNA

After transfection with the pSuppressorNeo p53 or negative control plasmids for 48 hrs, cells were treated with H_2O_2 at concentrations of 0.1 mM and 0.5 mM for 1 hr. The levels of 8-oxo-dG residues in cellular DNA before and following treatment with H_2O_2 were determined using the OxyDNA assay kit (EMD Biosciences, San Diego, CA). This assay is based upon the direct binding of a fluorescent probe to 8-oxoguanine in the DNA of fixed cells[29, 30]. Cells were harvested, washed with PBS, and fixed with 2% paraformaldehyde on ice for 15 minutes. The samples then were washed with PBS and fixed in

70% ethanol at -20°C overnight. The following day, the cells were washed once with PBS, followed by the wash solution provided by the manufacturer. Next, the cells were incubated with blocking solution, and then treated with the 8-oxo-dG specific FITC-labeled probe and analyzed by flow cytometry according to the manufacturer's protocol. The intensity of the FITC fluorescent signal was proportional to the level of 8-oxoG residues in DNA.

10. Analysis of 8-oxo-dG residue removal from DNA

A pulse-chase experiment was used to assay the removal of 8-oxo-dG residues from DNA in whole cells. First, cells were transfected with pSuppressorNeo p53 or negative control plasmids for 48 hrs. Following the transfection, cells were treated with 0.5 mM H_2O_2 for 1 hr. Afterwards, cells were rinsed with fresh medium (without H_2O_2) incubated. Cells were harvested before or at various times after incubation in fresh medium. Relative abundance of 8-oxo-dG in cellular DNA was measured as described previously. The decrease of 8-oxoG signal as a function of the chase time was taken as 8-oxoG removal from DNA in whole cells.

11. Statistical analysis

Student's *t*-test was used for statistical analysis. Statistical significance was accepted if the null hypothesis was rejected with a P value < 0.05.

III. RESULTS

1. Analysis of p53 and p53-regulated antioxidant enzymes in several cervical carcinoma cell lines

Levels of p53 protein were examined in several HPV-positive cell lines by immunoblot analysis with the mouse monoclonal antibody DO1 to human p53. SiHa cells, which contain only one copy of HPV 16 per cell, exhibited higher amounts of p53 than Caski cells containing 600 copies of HPV 16, HeLa cells with 25 copies of HPV 18, and ME180 cells with HPV 68 (Fig.1a). Further, we investigated the expression of p53-regulated antioxidant enzymes GPX1, SESN1 and SESN2, and found very high levels of GPX1 protein, SESN2 protein, and SESN1 mRNA in SiHa cells (Fig. 1a, b).

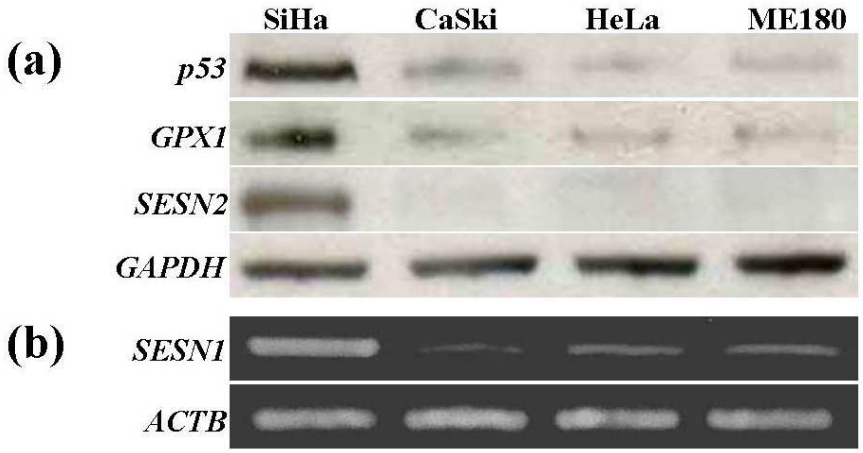


Figure 1. Expressions of p53 and p53-regulated antioxidant enzymes in human cervical carcinoma cell lines. (a) Western blot analysis for p53, GPX1, and SESN2 (Hi95). GAPDH was used as a loading control. (b) RT-PCR for SESN1 and ACTB (β-actin). The SiHa cell line showed the highest expression of p53 and p53-regulated antioxidant enzymes: GPX1, SESN1 and SESN2.

2. Sensitivity of cervical carcinoma cells to H_2O_2 challenge

Having shown the status of p53 and its regulated antioxidant enzymes in various cell lines, we next analyzed the sensitivity of HPV positive cervical carcinoma cells to H_2O_2-induced oxidative damage. As shown in Fig. 2, we observed differences in the response to the H_2O_2 challenge in the four cell lines. The SiHa cell line was the most resistant to H_2O_2, while the other three cell lines displayed similarly reduced survival. The sublethal doses of H_2O_2 to SiHa, HeLa, CaSki and ME180 were 0.8 mM, 0.2 mM, 0.2mM, and 0.1mM, respectively.

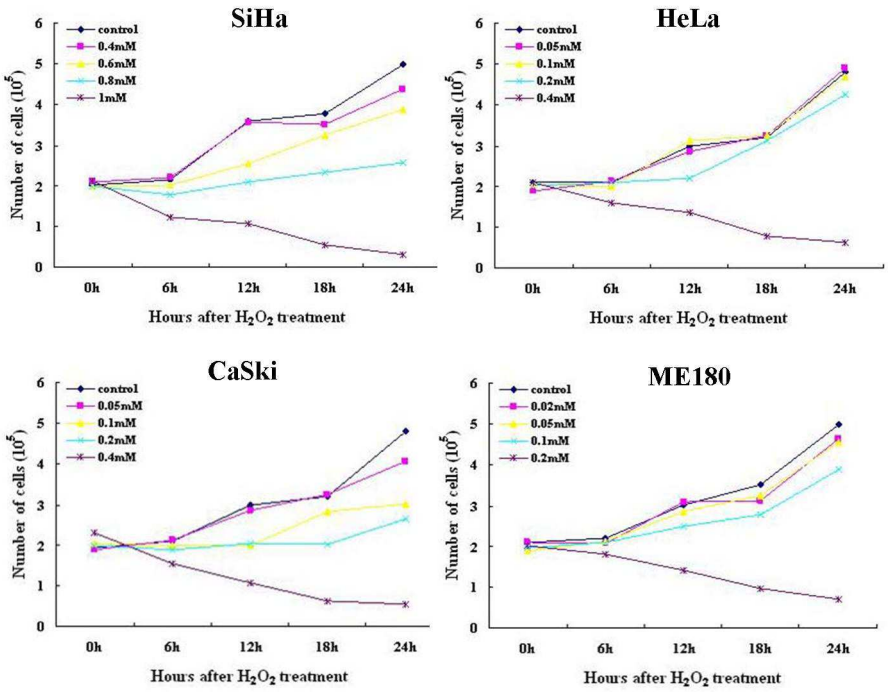


Figure 2. Cell viability of SiHa, HeLa, CaSki and ME180 after H_2O_2 treatment as detected by Trypan blue assay. The data represented the means of pooled results from five independent experiments.

3. Downregulation of p53 elevated intracellular ROS in SiHa cells

As SiHa cells had a higher expression of p53 and were much more resistant to H_2O_2 challenge than other HPV positive cell lines, we attempted to analyse the impact of p53 RNAi on intracellular ROS. We used the human U6 promotor to drive the expression of shRNA targeting p53 in a plasmid-based system (pSuppressorNeo p53 plasmid). A negative control plasmid, which contained a scrambled sequence, was also included in experiments. Each construct produced a shRNA composed of two 19-nucleotide repeats in an inverted orientation as shown in Fig. 3 a.

When the pSuppressorNeo p53 plasmid and negative control plasmid were transfected into SiHa cells, the p53 protein level was markedly decreased after 24-72 hr, as compared to normal expression of p53 in the control SiHa transfection (Fig.3 b). In contrast to p53, GAPDH was not affected with either construct, indicating the reduction of p53 by applying U6-driven shRNA constructs was specific. We further tested how p53 deficiency affects the levels of p53-regulated antioxidant enzymes. Inhibition of p53 in SiHa cells 24-72 hr after transfection resulted in a notable decrease in the p53-inducible transcript T2[12] of SESN1 and a virtual disappearance of GPX1 and SESN2 (Fig. 3 b).

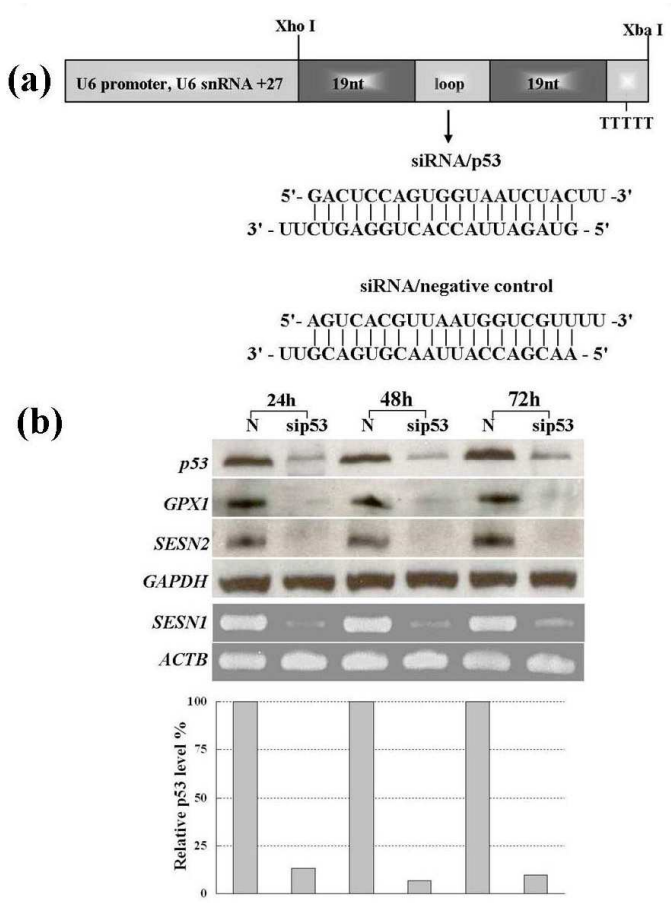


Figure 3. Reduction of p53 levels in SiHa cells transfected with p53 siRNA or nonspecific siRNA. (a) Schematic illustration of the U6 promoter-driven shRNA constructs containing the sequence targeting p53 and the scrambled sequence. (b) Upper panel: Expression levels of p53, GPX1 and SESN2 in control or in si-p53-expressing cells, as detected by western blot analysis. GAPDH was used as a loading control. Middle panel: Expression of SESN1 (T2) and ACTB as detected by RT-PCR. Lower panel: Quantification of p53 in western blots above normalized to GAPDH expression.

To show the effects of p53 on ROS levels in nonstressed cells, SiHa cells were labeled with the cell-permeable fluorescent dye CM-H_2DCFDA, the fluorescence of which increases following oxidation by H_2O_2 and hydroxyl radical in the cells. Fluorescent signals were analyzed by flow cytometry. CM-H_2DCF staining indicated that there was an approximately twofold increase in ROS levels 24 hr after inhibition of p53, and the high levels of ROS remained for 72 hr (Fig 4 a and b), in agreement with the above observations that the expressions of GPX1, SESN1 and SESN2 were markedly decreased after p53 inhibition by siRNA. Next, we examined whether N-acetyl cysteine (NAC, a potent antioxidant) inhibits ROS accumulation in SiHa cells. As shown in Fig. 4 c, the increase in ROS induced by p53 specific siRNA 48 hr after transfection was almost completely reversed by incubation with 5 mM NAC. When tested in cell culture, there was no notable change in the growth rate (Fig. 5 a) or cell cycle distribution (Fig.5 b) of SiHa cells with inhibited p53, and addition of NAC (5 mM) did not affect cell proliferation. We also found the expression of p53-specific siRNA did not increase ROS levels in HeLa, CaSki, and ME180 cell lines, similar to negative control plasmid (Fig. 6).

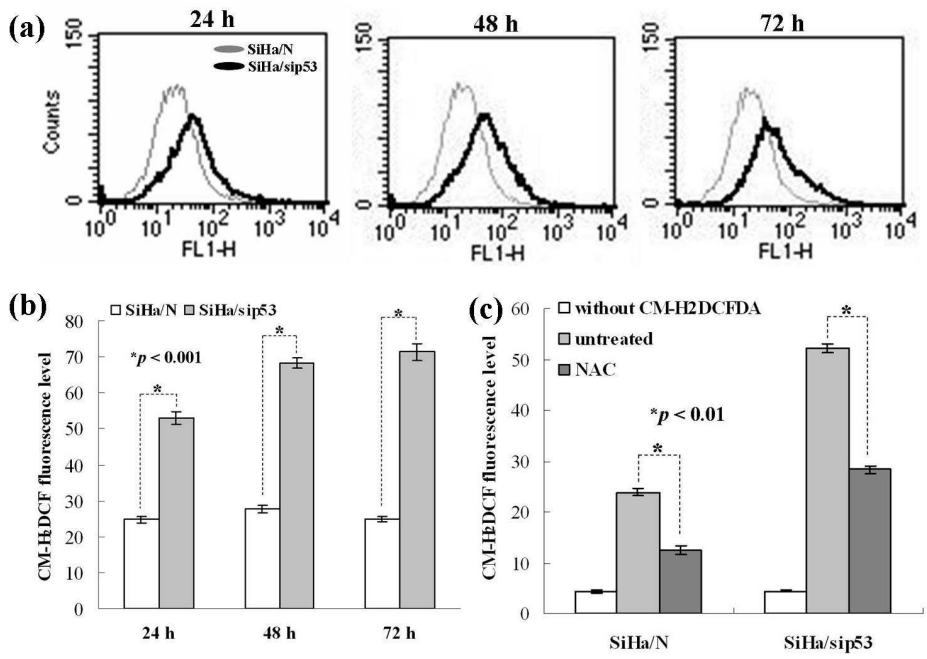


Figure 4. Effect of deficiency in p53 on intracellular ROS level in SiHa cell line. (*a*) Intracellular ROS levels in SiHa cells measured by CM-H_2DCFDA staining and FACS-analysis at different time intervals after transfection with p53 siRNA or nonspecific siRNA. (*b*) ROS levels are expressed as the mean ± SD intensity of cell fluorescence. *$p < 0.001$ compared to the corresponding negative control by Student's t test. (*c*) FACS-analysis of CM-H_2DCFCA-stained cells treated with NAC (5mM, 6 hours) 48 h after transfection with p53 siRNA or nonspecific siRNA. *$p < 0.01$ compared to the corresponding untreated group by Student's *t*-test.

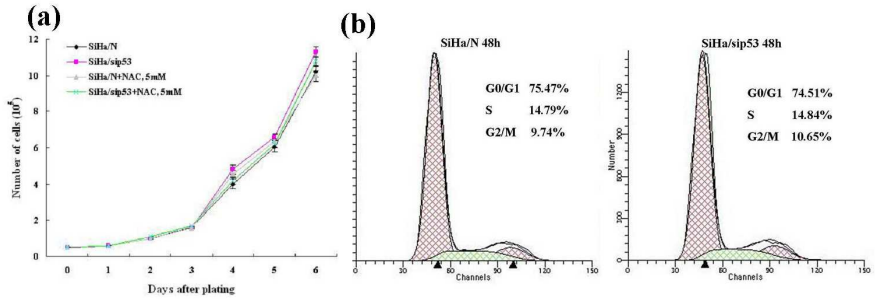


Figure 5. Characterization of SiHa cells with inhibited expression of p53. (*a*) Growth curves of SiHa/N and SiHa/sip53 cells untreated or treated with 5mM NAC. *$p > 0.98$ by Student's *t*-test. (*b*) Cell cycle distribution of SiHa/N and SiHa/sip53 was analyzed 48 h after transfection.

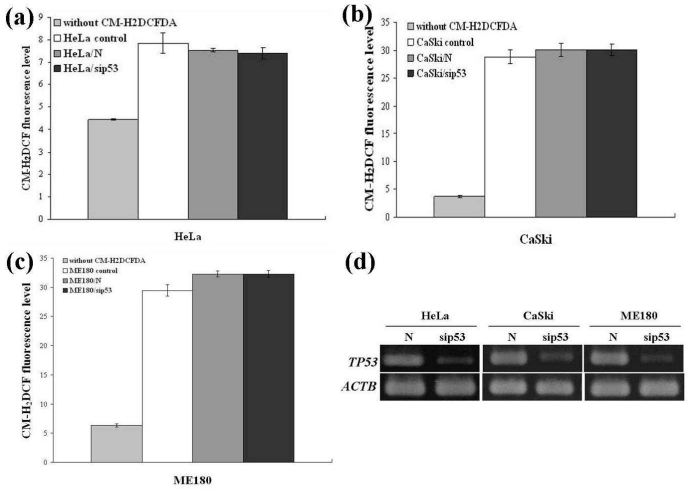


Figure 6. ROS levels in HeLa (a), CaSki (b), and ME180 (c) cells 48 hr after inhibition of p53 with siRNA (sip53). For a negative control we used nonspecific siRNA. *$p > 0.95$ compared to the corresponding cells with negative control vector (Student's *t*-test). (d) Expression levels of p53 mRNA in both control and si-p53-expressing cells 48 h after transfection with corresponding plasmid, as detected by RT-PCR.

4. p53 deficiency promoted intracellular ROS level in SiHa cells under oxidative stress

The results presented above suggest that p53 in the SiHa cell line may provide a protective function by participating in antioxidant defense. To test the role of p53 in antioxidant defense according to the severity of oxidative stress, we compared ROS levels in control and p53-deficienct SiHa cells stimulated with different concentrations of H_2O_2. 48 hours after transfection with either the negative control plasmid (SiHa/N) or p53 shRNA plasmid (SiHa/sip53), cells were mock treated or treated with different concentrations of H_2O_2 for 12 hr, and intracellular levels of ROS were analyzed. As compared to SiHa/N cells with negative siRNA, both untreated and H_2O_2-treated SiHa/sip53 cells (in which p53 was inhibited by siRNA) exhibited markedly increased ROS levels. Intriguingly, we also observed that even at a lethal dose of H_2O_2 (1 mM), ROS level in SiHa/sip53 cells was much higher than that in SiHa/N cells (Fig. 7). Taken together, the data strongly suggest that endogenous p53 in HPV-positive SiHa cells functions as an antioxidant and may play an important role following oxidative stress.

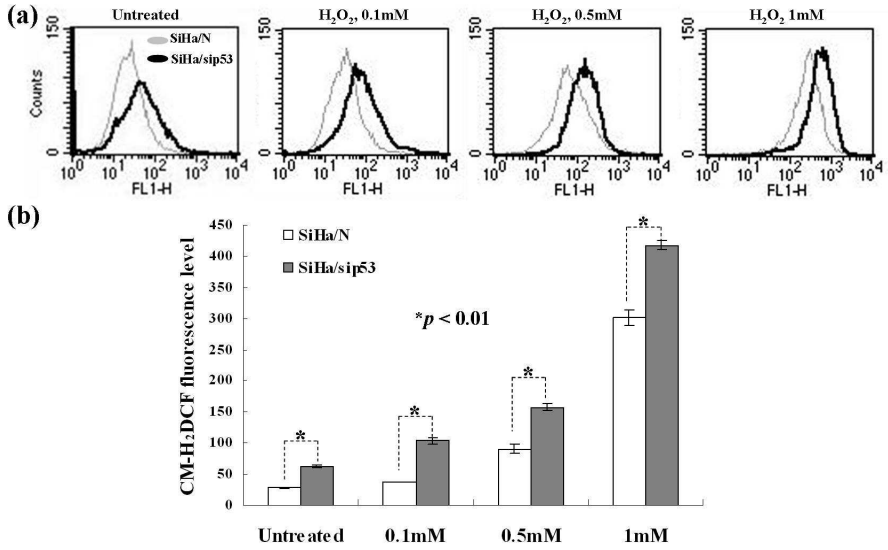


Figure 7. Effect of p53 deficiency on intracellular ROS level in SiHa cells undergoing oxidative stress. (*a*) ROS levels in untreated and H_2O_2-treated (12 h) SiHa and SiHa cells with p53 inhibited with siRNA, as detected by FACS after CM-H_2DCFCA-staining. (B) ROS levels were expressed as the mean ± SD intensity of cell fluorescence. *$p < 0.001$ compared to the corresponding negative control by Student's *t*-test.

5. Inhibition of p53 sensitized SiHa cells to H_2O_2-induced oxidative damage

Since the reduction of p53 induced a marked increase in the level of ROS under both unstressed condition and oxidative challenge, we investigated whether p53 deficiency could sensitize SiHa cells to oxidative damage. In order to address this question, we transfected SiHa cells with a negative control plasmid (SiHa/N) or p53 shRNA plasmid (SiHa/sip53). After 48 hr post-transfection, cells were treated with varying concentrations of H_2O_2 for either 12 hr or 24 hr. As expected, the proliferation in SiHa/sip53 cells by 0.5 mM H_2O_2 was reduced to 57.5 and 56.5% at the time point 12 and 24 hr treatment, respectively, compared to control SiHa/N cells. Likewise, treatment of control SiHa/N cells with 0.5 mM H_2O_2 had almost no effect on cell viability. When tested at a lethal dose of H_2O_2 (1 mM) for 12 and 24 hr, we found that SiHa/sip53 cells were more susceptible to H_2O_2-induced damage. For example, treatment with 1 mM H_2O_2 for 12 hr induced death in 68% of SiHa/sip53 cells, whereas the rate was much lower (27%) in SiHa/N cells (Fig. 8 a).

Though the XTT assay is a convenient method to measure cell death, it doe s not discriminate between apoptosis and necrosis. To determine whether apop tosis was involved in H_2O_2-induced cell death, SiHa/sip53 cells were further e xamined by annexin V and propidium iodide staining. Under defined salt and calcium concentrations, annexin-V is predisposed towards binding externalize d phosphatidyl serine (PS) that is present on the cell surface during the early st ages of apoptosis. Addition of PI helps to distinguish between early apoptotic cells and late apoptotic or necrotic cells because PI cannot enter the cells in th e early stages of apoptosis when the membrane integrity is intact. Cells were t

reated with H_2O_2 for 12 hr, and apoptosis studies indicated that there was a str ong induction in early stage apoptosis (annexin V positive) as well as in late a poptosis (annexin V and PI double staining positive) in SiHa/sip53 cells, as de picted in Fig. 8 b. Collectively, the data suggest that the reduction of p53 sensi tized SiHa cells to oxidative damage.

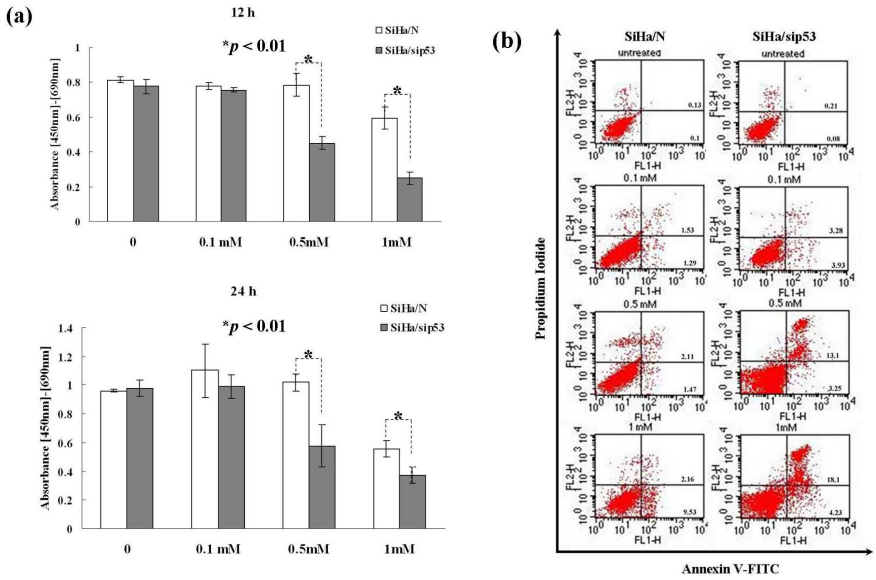


Figure 8. Inhibition of p53 sensitized SiHa cells to H_2O_2-induced oxidative damage. (*a*) 48 hours after transfection with negative control plasmid (SiHa/N) or p53 shRNA plasmid (SiHa/sip53), cells were treated with various concentrations of H_2O_2 for either12 hr or 24 hr, after which time cell viability was measured with an XTT assay. Results shown are expressed as mean ± SD of triplicate microcultures. *$p < 0.01$ compared to the corresponding negative control by Student's *t* test. (*b*) Apoptosis and death levels of SiHa/N and SiHa/sip53 cells 12 hr after treatment with 0.1, 0.5 and 1 mM of H_2O_2 as detected by FACS after Annexin V/PI staining. Apoptotic (Annexin V^+/PI^-), dead (Annexin V^+/PI^+) and alive (Annexin V^-/PI^-) populations were readily identified. The rates of apoptotis and death are shown in upper right and lower right panels respectively. *$p < 0.01$ compared to the corresponding untreated cells (Student's *t*-test).

6. p53 deficiency increased DNA oxidation in SiHa cells

To determine whether p53 is required for the protection of DNA from oxidative damage, we monitored the rate of formation of 8-oxoguanine (8-oxo-dG), the major product of DNA oxidation, using an assay kit as described in the Materials and Methods section. SiHa cells with siRNA inhibited p53 displayed a twofold increase in levels of 8-oxo-dG as compared to control cells (Fig. 9 a). We also examined whether p53 is associated with oxidative DNA damage induced by challenge with H_2O_2, and evaluated the possible role of p53 in the removal of such DNA damage in SiHa cells. Treatment of cells with 0.1 and 0.5 mM H_2O_2 for 1 hr resulted in an increase in 8-oxo-dG levels, as indicated by the increase in fluorescence of the 8-oxo-dG signal. However, there was a significant difference in the levels of 8-oxo-dG in SiHa/N and SiHa/sip53 cells. SiHa/sip53 cells consistently exhibited higher 8-oxo-dG content than SiHa/N cells (Fig. 9 b). Therefore, these data suggest that in the in SiHa cell line, p53 may participate in protecting DNA from oxidative damage.

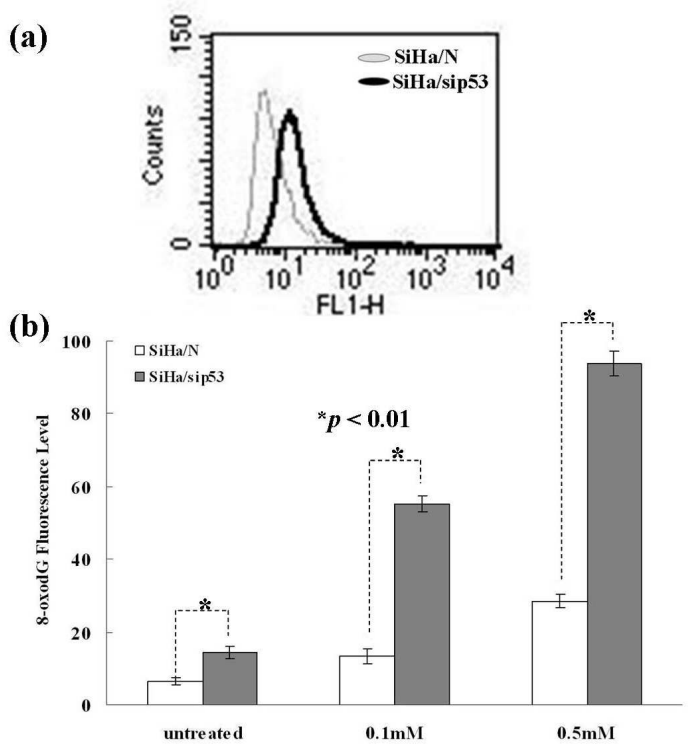
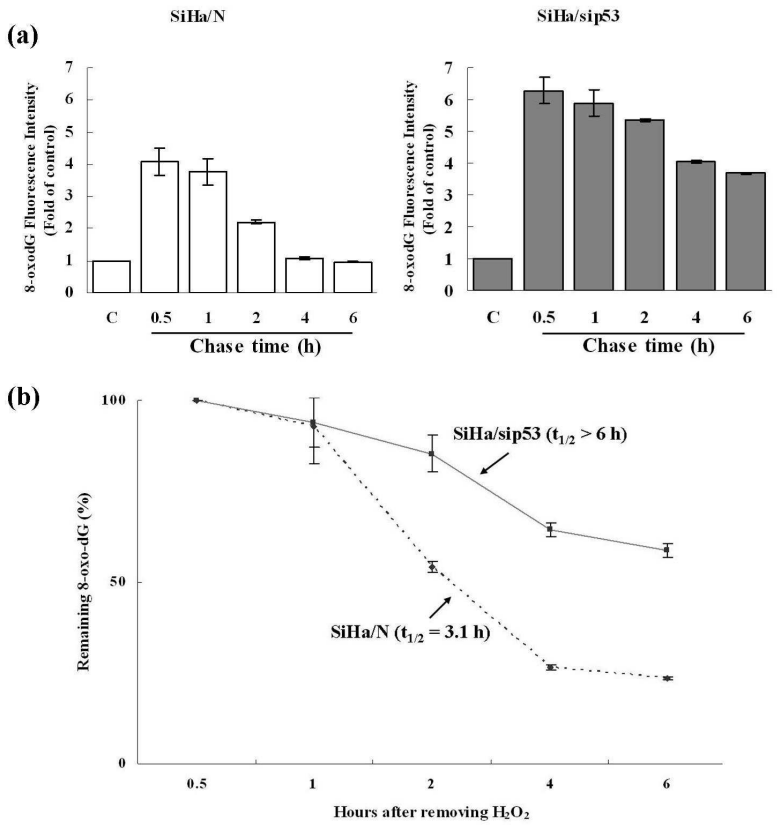


Figure 9. Reduction of p53 expression increased DNA oxidation in SiHa cells. (*a*) 8-oxo-dG level in SiHa cells expressing negative control plasmid (SiHa/N) or p53 shRNA plasmid (SiHa/sip53) 48 hr after transfection. (*b*) 48 hr after transfection with negative control plasmid or p53 shRNA plasmid, cells were treated with the indicated concentrations of H_2O_2 for 1 hr. Quantification of 8-oxo-dG staining was performed after FACScan analysis using CellQuest software. 8-oxo-dG levels are expressed as the mean ± SD intensity of cell fluorescence. *$p < 0.01$ compared to the corresponding cells with negative control vector by Student's *t* test.

7. p53 enhanced the removal of 8-oxo-dG residues from DNA in whole SiHa cells

Lastly, we performed pulse-chase experiments to test whether p53 enhances the removal of 8-oxoG residues from DNA in the SiHa cell line. 48 hr after transfection with negative control plasmid or p53 shRNA plasmid, cells were treated with 0.5 mM H_2O_2 for 1 hr to induce 8-oxo-dG accumulation. After treatment, the medium was changed and the cells were incubated without H_2O_2 for various times. To evaluate the rate of 8-oxo-dG elimination, the level of 8-oxo-dG remaining was detected at each time point. SiHa/sip53 cells were slower than SiHa/N cells to remove 8-oxo-dG from the DNA, with the time for the level of 8-oxo-dG to decrease by 50% ($t_{1/2}$) was 3.1 hours and more than 6 hours, respectively. (Fig. 10).



Figur 10. p53 facilitated the removal of 8-oxo-dG residues from DNA in whole SiHa cells. (*a*) 48 hr after transfection with negative control plasmid (SiHa/N) or p53 shRNA plasmid (SiHa/sip53), cells were treated with 0.5 mM H_2O_2 for 1 hr. The cells were washed with fresh medium and then incubated in fresh medium for the indicated times. 8-oxo-dG levels were measured at each time point as indicated and expressed as fold of the control sample. (*b*) The percentage of remaining 8-oxo-dG residues in cellular DNA was plotted as a function of chase time. The $t_{1/2}$ value reflects the estimated time for levels of 8-oxo-dG to decrease to 50% of their original levels.

IV. DISCUSSION

Persistent infection by high-risk types of HPV has been associated with the development of human cervical cancer. Based on epidemiological and experimental evidence, it is widely accepted that carcinogenesis of HPV-infected cells is a process involving integration of the viral genome in cancer cells, resulting in the loss of expression of the viral E2 gene and the persistence of E6 and E7 oncoproteins expression[31]. The oncogenic activity of high-risk HPVs is explained in part by the ability of the viral E6 oncoprotein to target p53 for degradation and thus to inhibit p53-mediated transcription[32]. However, studies investigating the functional status of p53 in a series of HPV-positive cancer cells expressing E6 gene from chromosomally integrated viral sequences under its natural promoter have produced differing results, indicating that despite co-expression of the viral E6 oncogene, there is residual p53 activity, indicating that endogenous p53 is not completely inactivated in HPV-positive cancer cells[26-28, 33, 34]. To date, there are no suitable anti-E6 antibodies available for reliable quantitation, and thus the presence of viral E6 protein has not been unambiguously demonstrated in HPV-positive cancers. Therefore, it is currently widely assumed that E6 protein levels are very low in HPV-positive carcinoma cell lines and thus may be limited in their capacity to interfere with p53 function. In the present study, we found that endogenous p53 in SiHa cells is involved in the modulation of intracellular ROS levels, participates in maintaining ROS homeostasis in response to oxidative stress, and protects DNA against oxidative damage.

Direct comparison of p53 protein levels between HPV-positive cervical

carcinoma cell lines, as examined by immunoblot analysis, indicated that SiHa cells had the highest steady-state level of p53. This result is in full agreement with previous findings Scheffner et al.[35] who showed that p53 level in SiHa cells is 3-5 times higher than that in other HPV-positive cervical cancer cells. A Recent study from Sablina et al.[21] provided evidence that even the very low levels of p53 present in normal tissues in the absence of stress were sufficient enough to drive the expression of several antioxidant genes. Therefore, we next addressed the hypothesis that high levels of p53 in SiHa cells may contribute to some antioxidant enzymes. Indeed, among the series of HPV-positive cancer cell lines investigated in this study, SiHa cells exhibited the highest expression of GPX1, SESN1 and SESN2, implying that SiHa cells may have a powerful antioxidant defense system and are thus tolerant of severe oxidative insults. By employing H_2O_2 to induce oxidative stress, we found that HeLa, CaSki and ME180 cells were severely impaired in their antioxidant capacity, whereas, SiHa cells, as expected, exhibited a higher resistance to H_2O_2 damage. Given the evidence that p53 is a major regulator of the H_2O_2 response in human cells[36], the above findings raised the question whether the presence of high steady-state level of p53 in SiHa cells has an antioxidant function.

Expression of p53 was knocked down by transfecting U6 promoter–driven shRNA constructs into SiHa cells. We were able to demonstrate that p53 was reduced 24-72 hr post-transfection, compared to that in the control. Likewise, the levels of GAPDH protein and ACTB (encoded β-actin) mRNA remained unchanged, indicating that the reduction of p53 was specific. Interestingly, we also noted that the expression of GPX1, SESN1 and SESN2 were also inhibited following p53 RNAi in SiHa cells. These observations further

supported the idea that p53 is transcriptionally competent despite co-expression of the viral E6 protein and could induce antioxidant gene expressions in SiHa cells in the absence of stress. Elevated levels of ROS were also found in p53-deficient SiHa cells, although this could be reversed by NAC supplementation. These data strengthened the idea that the antioxidant function of p53 is mediated through a set of antioxidant gene products, and the depletion/downregulation of p53 damped down the antioxidant defense in SiHa cells.

Intriguingly, while p53-deficieny promoted ROS levels in SiHa cells, we did not observe a similar correlation between p53 protein and ROS levels in the other three HPV-positive cervical cancer cells HeLa, CaSki and ME180. Different scenarios could be envisioned to explain the differences in the modulation of ROS by p53 in the HPV-positive cancer cells investigated in the present study. The transcriptional activity of p53 to activate antioxidant genes is necessarily correlated with p53 protein abundance, and a steady-state level of p53 may be required for its transcriptional activity in HPV-positive cells under physiological conditions. In addition, there may also be cell specific differences in the regulation of cellular antioxidant abilities between these cell lines.

In concordance with the notion that the p53 protein is functional in SiHa cells, we observed a much higher level of ROS in p53-deficient SiHa cells following H_2O_2 exposure than in SiHa cells containing wild type p53. Indeed, it has been shown previously that p53 induces ROS accumulation and a number of genes induced by p53 are associated with the metabolism of ROS[37, 38]. In the present study, the p53-deficient cells showed higher ROS level than control cells did, even at lethal concentrations of H_2O_2 (1 mM). These

findings were in contrast with a previous study[21] in which p53 showed pro-oxidant activity and thus promoted ROS levels in HPV-negative cell types under severe oxidative damage. One possible explanation may be that the antioxidant and pro-oxidant function of p53 was temporally separated[39, 40] and the pro-oxidant function of p53 is severely impaired in SiHa cells.

Furthermore, cells containing reduced p53 were more susceptible to H_2O_2-induced cell death. As shown in this study, a more rapid and greater magnitude of cell death was observed in SiHa cells with p53 deficiency after H_2O_2-challenge, indicating that the antioxidant defense of the SiHa cell line was achieved , at least in part, by endogenous p53, and therefore depletion of p53 followed by inhibition of the expression of some antioxidant enzymes disintegrated the antioxidant firewall and sensitized cells to oxidative stress.

The p53 tumor suppressor, a “guardian of the genome”, restricts abnormal or stress-exposed cells before DNA damage becomes fixed as a mutation[17]. A key target of ROS in cells is the DNA, and under physiological conditions, the endogenous ROS that are byproducts of normal respiration modify approximately 20,000 bases of DNA per day in a single cell[41-43]. Thus, endogenous ROS are a major source of DNA damage and a substantial factor contributing to chromosomal instability, accumulation of mutations[44, 45], and deletions that may lead to cancer[46, 47]. A frequent oxidative modification of DNA is the hydroxylation of guanine at C-8, leading to the formation of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxo-dG)[48, 49]. In the present study, we showed that p53 protein in SiHa cell line has a protective role against DNA oxidation. SiHa cells deficient in p53 displayed significantly increased 8-oxo-dG levels under both non-stressed and oxidative stressed conditions, a finding which was consistent with the observation that 8-oxo-dG residues

were removed more rapidly from cellular DNA in whole SiHa cells with wild-type p53. Together, these results suggested that the endogenous p53 in SiHa cell line plays a role of in protecting DNA and facilitating cellular responses to ROS-induced DNA damage.

V. CONCLUSION

In conclusion, we have provided evidence that endogenous p53 in SiHa cells had an antioxidant function and was involved in the reinforcement of the antioxidant defense. Depletion of p53 by RNAi in SiHa cell line resulted in disintegration of the antioxidant firewall and increased oxidative damage. As ROS are also involved in cancer chemoradiotherapy, these findings may help to facilitate clinical studies of HPV-positive cervical cancer therapy.

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Abstract (in Korean)

H_2O_2 처치후 인유두종 바이러스 감염 자궁암세포주의 p53의 항산화방어기전

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정파효

HPV양성 자궁암세포주에서 p53단백은 기능적으로 E6 단백과 길항작용을 하는 것으로 알려져 있으나 실제 정상적인 p53단백과 관련 경로들은 다양한 실험모델을 통해 규명한 바에 의하면 p53은 자궁암세포주에서 완전히 비활성화되는 것은 아닌 것으로 밝혀졌다. 이 점을 착안하여 본 연구는 인유두종 바이러스가 감염된 자궁암세포주의 p53단백의 항산화기능을 확인하는데 목적을 두었다. 특히 인유두종 바이러스 16형이 감염된 SiHa세포주가 p53단백이 다른 자궁암세포주에 비해 현저히 많이 발현되고 H_2O_2의 고농도에도 비교적 저항하여 생존하는 특징을 보였다. 또한 SiHa

세포주의 p53을 RNA간섭으로 저하시켜 p53관련 항산화 인자들의 발현저하를 확인하였고 ROS가 세포내 축적되는 것을 확인하였다. 반면에 다른 자궁암세포주인 HeLa, CaSki, ME180세포주는 동일한 RNA간섭 방법으로도 ROS가 변하지 않았다. 경미하거나 고농도의 H_2O_2로 인한 자극에 p53 저하를 유도한 SiHa세포주가 mock SiHa세포주에 비해 더 높은 ROS치수를 보였다. H_2O_2투여 후 세포의 활성도와 세포자연사를 측정한 결과 p53저하 SIHa세포주가 H_2O_2에 더 민감하게 반응함을 확인하였다. P53의 저하는 SiHa세포주에서 과도한 양의 DNA 산화를 유도했고, mock SiHa 세포주는 p53-저하 SiHa세포주에 비해 같은 농도의 H_2O_2자극에도 DNA로부터 8-oxo-7,8-dihydro-2'-deoxyguanosine을 급속히 처리하는 결과를 보였다. 본 연구의 모든 결과를 종합하면, SiHa세포주의 내재적인 p53이 항산화기능이 았을 뿐 아니라 항산화방어기전을 더욱 공고히하는 역할을 하는 것으로 생각된다.

핵심되는 말: p53, SiHa, H_2O_2, ROS, 산화자극, RNA간섭

PUBLICATION LIST

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