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Vitamin E promotes breast cancer cell proliferation by reducing ROS production and p53 expression

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Abstract. – OBJECTIVE: The role of antioxidant in cancer cell proliferation is still controversial. This study aimed to explore the effects of antioxidant vitamin E on the proliferation of breast cancer cells and the possible underlying mechanisms.

MATERIALS AND METHODS: Orthotopic breast cancer model was established by inoculating MCF7 cells in mice and *in vitro* MCF7 culture system. CM-H2DCFDA fluorescence probe and Western blot analysis were used to detect ROS changes and p53 expression. p53 knockdown in MCF7 cells by siRNA transfection was also used to determine the combination effect of vitamin E and p53 on MCF7 cell proliferation.

RESULTS: Vitamin E supplement in the chow significantly accelerated breast cancer cell growth in vivo. ROS level and p53 expression were decreased in tumor tissues. Water-solvable vitamin E Trolox significantly promoted MCF7 cell proliferation *in vitro*, while reducing intracellular ROS level and p53 expression. p53 knowdown by p53-siRNA transfection inMCF7 cells significantly reduced p53 expression and increased MCF7 cell proliferation.

CONCLUSIONS: Vitamin E accelerated breast cancer growth by reducing ROS production and p53 expression.

Key Words: Vitamin E, Breast cancer, MCF7, ROS, p53.

Introduction

Breast cancer is the most common malignant tumor in women. Under the influence of multiple oncogenic factors, mammary epithelial cells acquire genetic mutations and lose the normal proliferative characteristics, resulting in disorganized structures and loose connection, which further promotes cancer cell metastasis. The number of women diagnosed with breast cancer incidence is on the rise annually, with more occurrences in the younger population. The occurrence of breast cancer is known to be closely related to gene regulations. Recent studies showed that reactive oxygen species (ROS) and p53were the key regulators in breast cancer. ROS signal transduction has been shown to be correlated with tumor metastasis. ROS is the byproducts of cellular metabolism. It is not only a signal transducer of apoptosis and necrosis, but also alters gene expression and promotes cell proliferation and differentiation. The consequences of ROS-regulated gene expression changes can lead to tumorigenesis by either curbing apoptosis or increasing proliferation. Thus, it is believed that ROS promotes cancer by inducing DNA damage or activating oncogenes. However, recent studies also showed that ROS could inhibit tumor growth[1]. Besides, ROS reduction by activation of endogenous anti-oxidation program can also promote tumor growth[2,3].

p53 is one of the earliest identified tumor suppressors by regulating the cell cycle. Mutation of p53 will cause abnormal cellular growth, apoptosis and DNA repairs. p53 activity can be induced by DNA damage, oxidative stress, osmotic pressure, etc. Volkan et al[4] showed that ROS reduction by anti-oxidants or p53 inactivation could reduce tumor growth. Currently, the role of ROS-p53 signaling is still not clearly known in breast cancer. In this study, we used vitamin E to block ROS-p53 signaling in tumor cells to explore the proliferative changes in breast cancer cell, which could provide new insight for breast cancer treatment.

Materials and Methods

Animals

4-6 weeks old female NOD/SCID mice were purchased from Institute of Laboratory Animal of Chinese Academy of Medical Sciences (Beijing,

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China). All animal experiment protocols complied with National Health Guidelines of animal protection and use, which were approved by the IACUC Committee of Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Beijing, China).

Reagents

Vitamin E (DL-α-tocopherol acetate) was purchased from Zhejiang Medicine Company (Shaoxing, Zhejiang, China). Trolox (water-solvable vitamin E) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) (sc-200810, USA). siRNA p53 (sc-29435), Control siRNA (sc-37007), p53 antibody (fl393) and Goat-anti-rabbit antibody (second antibody) were also purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Lipofectamine 2000 was purchased from Invitrogen (Carlsbad, CA, USA). CM-H_2DCFDA (C6827) was from Life Technologies (Carlsbad, CA, USA). Phospho-p53 antibody (9284) was from Cell Signaling Technology (Danvers, MA, USA) and β-tubulin (926-68072) was purchased from Li-Cor (Lincoln, NE, USA). Fetal bovine serum (FBS), Roswell Park Memorial Institute-1640 (RPMI-1640) medium, 0.25% trypsin were purchased from Gibco (Grand Island, NY, USA). MCF7 cells were obtained from Institute of Cell Biology, Chinese Academy of Sciences (Beijing, China). These cells were cultured with 10% FBS in RPMI-1640 with penicillin (100 U/ml) and streptomycin (100 μg/ml) in 5% CO_2 humidified incubator at 37°C and passaged once or twice/week.

Cell Culture

MCF7 cells were maintained in RPMI-1640 medium with 10% FBS in 5% CO_2 humidified incubator at 37°C. The medium was changed every 2-3 day. Cells were passaged when they reached 80-90% confluence. Experiments were performed with cells at log phase growth.

Orthotopic Breast Cancer Model

MCF7 cells were trypsinized with 0.25% trypsin for 1-2 min, washed with phosphate buffered saline (PBS) and resuspended to $5x10^5$ cells/μl with PBS. NOD/SCID mice were anesthetized with intraperitoneal injection of 30 mg/kg 0.3% sodium pentobarbital. Abdominal skin was lifted with forceps and 200 μl cell suspension was injected into the third pairs of fat pads under the mouse mammary groin (Figure 1). Animal weight and tumor size were recorded every three days after implantation.

Grouping

Animals were divided into two groups (n=8 for each group). 0.1 g/kg and 0.5 g/kg of vitamin E (DL-α-tocopherol acetate was supplemented in the food and fed to the experimental group. The control group was fed with food that was without vitamin E.

Tumor Measurement

The tumor was measured with caliper every week. Tumor volume was calculated as (length X $(width)^2$)/2. Mice were sacrificed with CO_2 36 days after implantation, and tumors were taken out and weighed.

ROS Measurement

Mammary tissues were taken out and immediately frozen in liquid nitrogen, embedded in optimal cutting temperature (OCT) and sectioned with cryostats. Sections were incubated with 5 μM CM-H_2DCFDA at 37°C for 90 minutes. Images in the random fields were taken with the same exposure and converted into pixel intensities. Average intensity was analyzed by Image J 1.48b.

4×10^5 cells/ml MCF7 cells were seeded in 6-well plates. After 24-hour treatment with indicated drugs, the cells were trypsinized with 0.25% trypsin for 1-2 min, washed with PBS and resuspended in 1 ml PBS. Fluorescent probe CM-H_2DCFDA was added to the final concentration of 5 μM and cells were incubated at 37°C for 30 min. The cells were then washed with serum-free medium or warmed PBS for three times to remove the free DCFH-DA, and collected for flow cyto-

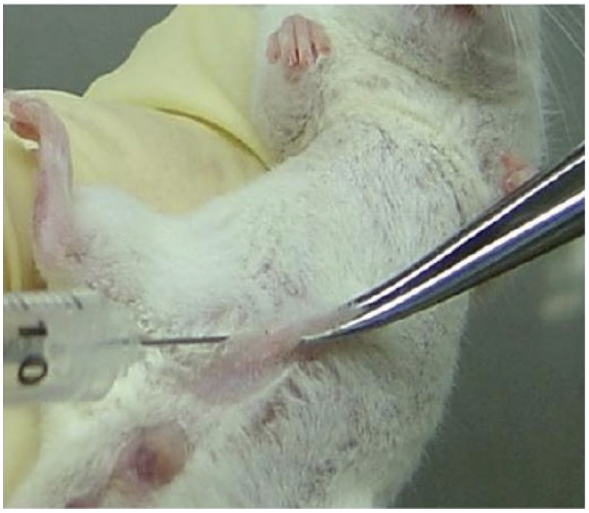

Figure 1. Implantation of breast cancer cells into mammary glands in mice.

meter analysis (BD Bioscience, Franklin Lakes, NJ, USA) of 2',7'dichlorofluorecdein (DCF) intensity. The intracellular fluorescence intensity positively correlated with intracellular ROS.

Western Blot Analysis

Western blot is often used in research to separate and identify proteins by staining cells or biological tissue samples which treated by specific antibodies, and analyzing the location of the color and the color depth, so that the information of the specific protein expression in the cell or tissue is obtained. In this case, mammary tumor tissue and MCF7 cells were lysed with cold radioimmunoprecipitation assay (RIPA) buffer. Protein concentration was quantitated by Bradford assay. 30 μg protein was loaded onto 12% SDS-PAGE gel for electrophoresis and then transferred onto nitrocellulose (NC) membrane. The membrane was blocked with 5% skimmed milk and incubated with p53, phosphor-p53 and β-tubulin antibodies at 4°C overnight. After three washes with tris buffered saline-tween (TBS-T), the membrane was incubated with secondary antibodies for 1h at room temperature. Band intensity was analyzed with Quantity One software.

Trolox Treatment

Trolox (±)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) is a water-soluble analog of vitamin E. We used $1x10^4$ cells/ml MCF7 cells were seeded onto 12-well plate. 100 μM Trolox6 was added to medium 6 hours after. Cells were trypsinized and analyzed by Vi-cell viability machine on day 0, Day 3, day 7 and day12 after treatments.

siRNA Transfection

Small-interfering RNA (siRNAs), which operates within the RNA interference pathway, can be also introduced into cells by transfection to knockdown the expression of a protein of interest. At the beginning, any gene can be knocked down by a synthetic siRNA with a complementary sequence; therefore, siRNA has become a major application in biological research with potential applications in gene therapy. In this study, 1×10^6 cells/ml MCF7 cells were cultured in RPMI-1640 medium with 10% FBS in 5 6-well plates. The cells were transfected 6 hours after seeding. The cells were divided into five groups: 1) Lipofectamine 2000 (empty vector control); 2) Control siRNA (negative control); 3) siRNA p53; 4) Control siRNA +Trolox; 5) siRNA p53+ Trolox. The procedures were performed according to manufacturer's instructions of lipofectamine 2000. After Lipofectamine 2000 transfection of siRNA p53, cells were cultured for 72h, washed with medium twice and cultured for another 6h. The cells were then collected by centrifugation at 1200 rpm for 10 min, groups 1 to 3 were collected for Western blot analysis. Groups 4 and 5 were collected on day 0, Day 3, day 7 and day 1, and analyzed by Vi-cell viability machine after trypsinization and PBS resuspension.

Statistical Analysis

Statistical analysis was performed with GraphPad Prism 5 software. All data are shown as $\bar{x}\pm s$. Multiple groups were compared using ANOVA t-test (analysis of variance) with Dunnett's test and the two groups were compared using independent samples *t*-test. $p<0.05$ was considered as statistically significant.

Results

Vitamin E Increased Tumor Growth in Orthotopic Breast Cancer Model in a Dose-Dependent Manner

To explore if vitamin E had any effects on the tumor growth in orthotopic breast cancer model, 0.1 g/kg and 0.5 g/kg vitamin E were supplemented into the food and fed to the mice. The unsupplemented food was used as the control one week after inoculation. Tumors were measured every 2-3 days since day 9. The mice were euthanized with CO_2 on day 36 and the tumors were weighed. Our results showed that the tumors were significantly larger ($p<0.05$) with 0.1 g/kg and 0.5 g/kg vitamin E supplemented compared with those from the control group. Also, the dissected tumors in the treated group were significantly heavier than those from the control group ($p<0.05$, Figures 2 and 3).

Vitamin E Reduced ROS Activity in Breast Cancer Tissue

To further uncover the mechanism underlying the increased tumor growth by vitamin E, tumor tissues from vitamin E treatment group were immediately frozen and sectioned by Cryostats. After incubation with CM-H_2 DCF-DA, images from the sections were taken under a fluorescent microscope, and the fluorescence of DCF was analyzed by Image J. Our results showed that vitamin E could significantly decrease ROS level in the breast cancer tissue ($p<0.05$, Figures 4 and 5).

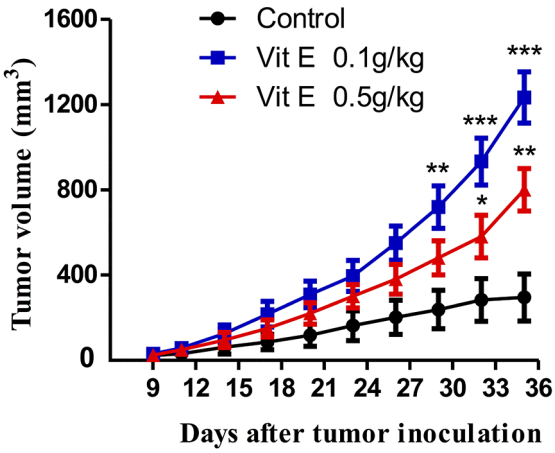

Figure 2. Growth curve of orthotopic breast tumor after inoculation ($^{*}p<0.01<^{**}$ $p<0.01$, $^{***}p<0.001$, compared with the control group).

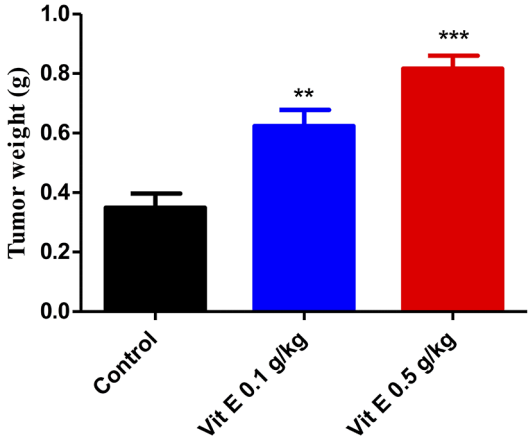

Figure 3. Tumor weights at the end of the experiment ($^{**}p<0.01$, $^{***}p<0.001$, compared with the control group).

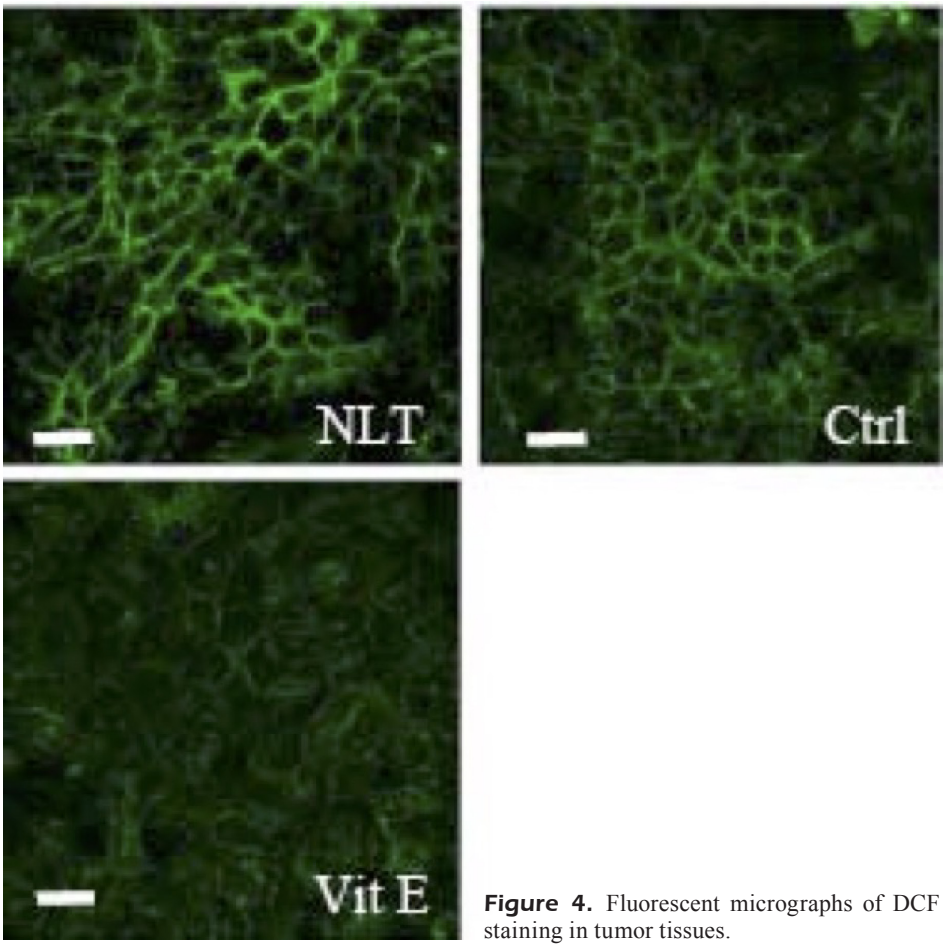

Figure 4. Fluorescent micrographs of DCF staining in tumor tissues.

Vitamin E significantly Decreased p53 Expression

We used Western blot analysis to further determine the effects of vitamin E on p53 protein expression. In the vitamin E treatment group, p53 expression was significantly lower than that in the control group ($p<0.05$, Figure 6 and Table I).

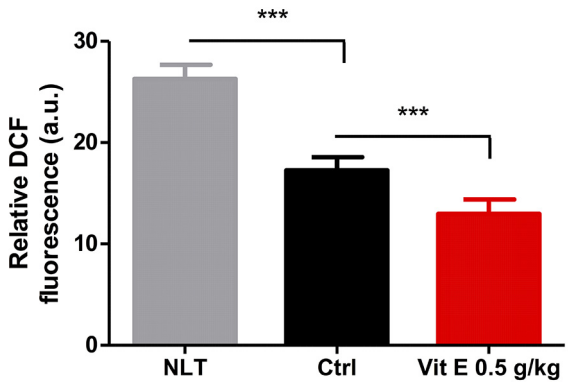

Figure 5. The relative DCF fluorescence intensity in tumor tissues (NLT: normal tissueCtrl:tumor tissue from chow-fed animals.Vit E: tumor tissue from vitamin E-fed animals $^{***}p<0.001$,compared with NLT group, control group).

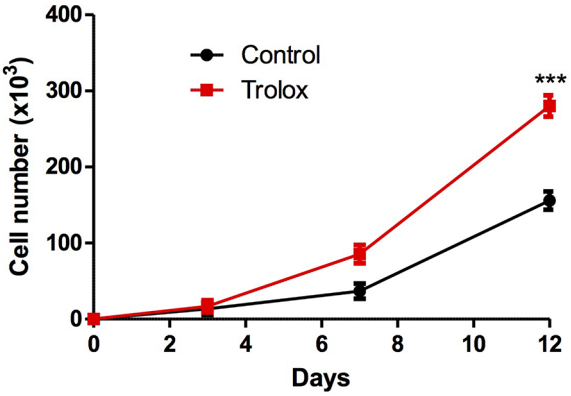

Figure 7. Proliferation curve of MCF7 cells under 100 μMTrolox treatment ($^{***}p<0.001$, compared with the Control group (withoutTrolox).

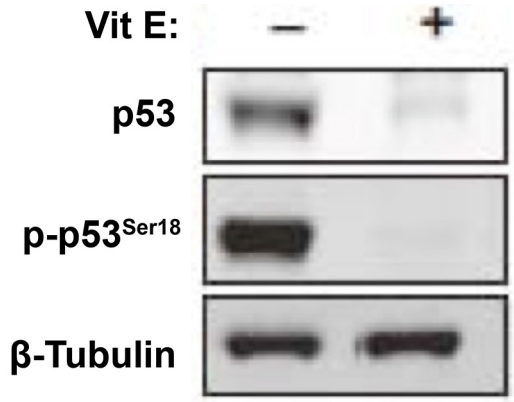

Figure 6. Representative Western blot of p53 expression.

Table I. Band intensity ratio of Western blot ($\bar{x}$ ± s, n = 8).

Group	p53/β-Tubulin	p-p53/β-Tubulin
NA	0.354±0.034	0.663±0.026
Vit E	0.067±0.021**	0.002±0.012***

$^{**}p<0.01$, $^{***}p<0.001$, compared with the control group (NA: without Vit E)

Water-Solvable Trolox increased MCF7 cell Growth in vitro

To determine the proliferation changes of breast cancer cell after vitamin E treatment, water-solvable vitamin E (Trolox) was added in the MCF7 culture medium. Cell viability was analyzed by Vi-cell analyzer on day 0, day 3, day 7 and day 12, after centrifugation and resuspension by PBS. As shown in Figure 7, Trolox significantly increasedMCF7 proliferation after 12-day treatment, compared with the control group ($p<0.05$).

Trolox Reduced ROS and p53 Expression in MCF7 cells

To further confirm that vitamin E could promote tumor cell growth through the ROS-p53 signaling pathway, we treated MCF7 cells with Trolox. After incubation with 100 μM Trolox for 24h and CM-H_2 DCFDA staining for 30 min, the cells were analyzed with flow cytometer for DCF fluorescence. As shown in Figure 8, intracellular DCF fluorescence in MCF7 cells was significantly lowered upon Trolox treatment, compared with the control group ($p<0.05$). Besides, Trolox significantly lowered p53 and phospho-p53 expression ($p<0.05$, Figure 9 and Table II).

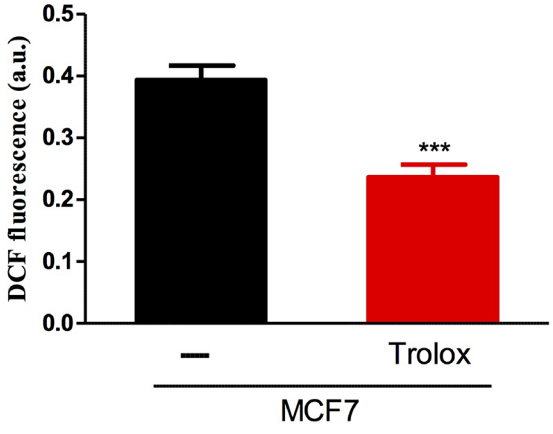

Figure 8. Fluorescence intensity of DCF in MCF7 cells.

Table II. Band intensity ratio of Western blot ($\bar{x}$ ± s, n = 8)

Group	p53/β-Tubulin	p-p53/β-Tubulin
NA	0.454±0.027	0.812±0.035
Trolox	0.084±0.028**	0.004±0.010***

**p<0.01, ***p<0.001, compared with the control group (NA: without Trolox).

siRNA Knockdown of p53 Increased MCF7 cell Proliferation

p53 can be activated by ROS or DNA damage to regulate cell proliferation. To further prove that vitamin E could regulate p53 expression and tumor cell proliferation by reducing ROS, we used p53-siRNA to knockdown p53 expression in MCF7 cells. As shown in Figure 10, transfection of p53-siRNA by Lipofectamine 2000 successfully decreased p53 expression in MCF7 cells. Cell proliferation in siRNA, Ctrl siRNA+ Trolox and siRNA+ Trolox groups was significantly higher than that in the Ctrl siRNA group (p<0.05, Table II).

Discussion

Breast cancer is one of the malignancies with the highest mortality. Recently, the mortality of breast cancer patients and the number of newly diagnosed cases in the younger population are both on the rise. Therefore, breast cancer is receiving more attention. It has been shown that breast cancer is closely related to ROS level. ROS is the by-product of metabolism, including oxygen ions, peroxide and oxygen-free radicals, which plays an important role in maintaining the homeostasis of the body. Environmental factors, such as UV or heat exposure, can activate and increase ROS level, which is termed as oxidative stress. Under such circumstances, cells produce enzymes, such as peroxide dismutase, to reduce the damages caused by ROS. Some small molecules, such as vitamin E, play critical roles as cellular antioxidants. It has been reported that ROS signaling is correlated with tumor cell metastasis[5,6]. Due to the metabolic pathway changes in cancer cells or high oxidative stress caused by gene mutation, a large amount of ROS is produced[7-9]. Increased ROS in cancer cells may up-regulate the expression of redox-sensitive molecules, and promote the cell proliferation, differentiation, metastasis, cell tolerance to anticancer substance and gene mutations to induce oncogenesis[7,10-12]. Therefore, consumption of antioxidant-rich food or food supplement of antioxidants can reduce ROS, which may inhibit tumor growth. However, a recent report[1] showed that ROS could inhibit tumor growth. Volkan et al[4] observed that antioxidant feeding reduced ROS production and promoted tumor growth in lung cancer model. In the present work our results confirmed that vitamin E not only reduced ROS production in tumor tissue and MCF7 cells, but also promoted tumor growth. Schafer et al[13] also proved that ROS reduction facilitated tumor cell survival in the extracellular matrix. It has also been shown that intracellular ROS production was regulated by transcription factor Nrf2. Oncogenes stimulated Nrf2-mediated antioxidant expression to reduce ROS generation and promote tumor cell growth[14,15]. Therefore, antioxidants can clear cellular ROS and can be used to treat cancers. These observations imply the dual roles of ROS in tumorigenesis.

As a tumor suppressor, a p53 inactivation is a critical event in tumorigenesis. p53 and ROS mutually regulate each other to induce the downstream signaling cascade[16]. p53 is the most critical factor to regulate the cell cycle. The increase in intracellular ROS can affect the regulatory fun-

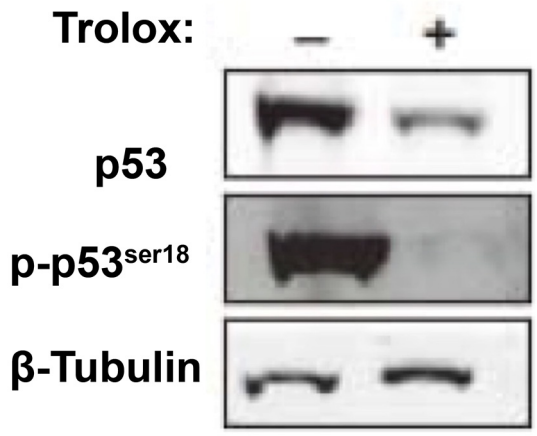

Figure 9. p53 expression in cultured MCF7 cells with Trolox treatment.

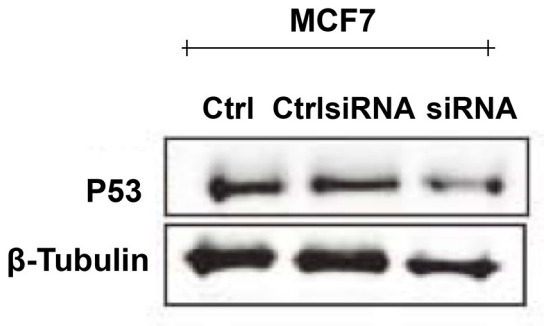

Figure 10. Representative western blot of p53 after siRNA knockdown (Ctrl: Lipofectamine 2000 control Ctrl siRNA: negative control).

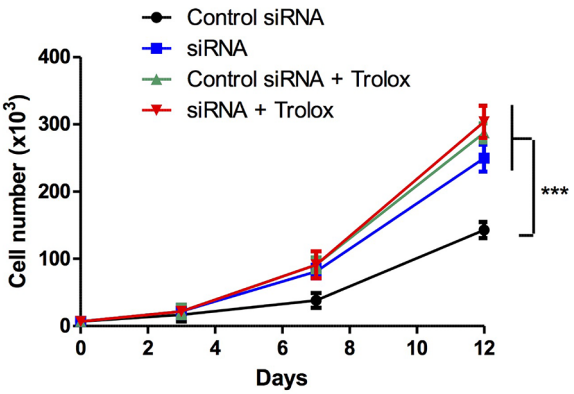

Figure 11. Proliferation curve of MCF7 cell after p53 knowdown by siRNA (***p<0.001, compared with Ctrl siRNA group).

ction of p53 on cell survival. It has been shown[17] that H_2O_2could induce p53 phosphorylation. A study[18] in lung cancer also showed that ROS could increase p53 expression.

Our researches further verified that p53 mediated vitamin E-induced tumor growth. Our findings showed that vitamin E promoted tumor growth by reducing ROS production and p53 expression in breast cancer cells and tissues. Further, vitamin E also promoted wild-type breast cancer cell proliferation, and p53 knockdown by siRNA counteracted the effects of vitamin E. Lee et al[19] showed that antioxidants N-acetylcysteine and GSH significantly inhibited the apoptosis of human liver cancer cell-mediated by p53. Our results were consistent with a previous study in lung cancer showing antioxidants could promote lung cancer growth by reducing ROS and p53 expression[4]. Besides, NF-κB and Fas play an important role in ROS-mediated p53 expression[18,20]. Therefore, we speculated that ROS might have a positive feedback regulation of p53 through unknown mechanisms.

Conclusions

In summary, as an antioxidant, vitamin E can regulate tumor growth through ROS-p53 signaling, which provides new insights for developing anti-cancer drugs based on antioxidants.

Conflicts of interest

The authors declare no conflicts of interest.

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