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# Effects of ALA-PDT on HPV16-immortalized cervical epithelial cell

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Current treatments for high-grade cervical intraepithelial neoplasia (CIN) and persistent infection with high-risk human papillomavirus (HR-HPV) type are mainly surgical interventions. However, such treatments are associated with adverse side effects and pose risks for future pregnancies. In order to reduce the requirement for excisional procedures, an effective and noninvasive therapy is needed for women at reproductive age. ALA-PDT has proved to be effective in the treatment of HPV-associated disease in several clinical investigations. In this study, the anti-proliferative effect of ALA-PDT was investigated in HPV16-immortalized cervical epithelial H8 cells. CCK-8 assay was used to measure cytotoxicity in H8 cells. The $IC_{50}$ of ALA-PDT on H8 cells was about 120.75 ± 1.18 μM. We have now evaluated the mechanism by which ALA-PDT induces cell death. Annexin V-FITC/PI staining showed a significant dose-dependent induction of apoptosis by ALA-PDT in H8 cells, associated with accumulation of the tumor suppressor protein p53 and the cyclin-dependent kinase inhibitor p21. Furthermore, ALA-PDT down-regulates expression of HPV E6/E7 oncogene as well as up-regulate tumor suppressor RbAp48 protein. Together, our data provides a basis for understanding and developing ALA-PDT as a cure for HPV infection-associated diseases and prevention of cervical cancer.

*Key words: cervical intraepithelial neoplasia, human papillomavirus, photodynamic therapy, p53, p21, HPV16 E6, HPV16 E7, RbAp48*

Cervical cancer is the third most common cause of cancer morbidity and mortality for women globally. According to the World Health Organization (WHO) International Agency for Research on Cancer (GLOBOCAN 2012), about 528,000 women worldwide were diagnosed with cervical carcinoma and 266,000 were dead each year. Moreover, cervical cancer cases show increasing prevalence in young patients [1]. Fortunately, it is now clear that cervical intraepithelial neoplasia (CIN) is the precancerous lesion of invasive cervical cancer and persistent infection with high-risk human papillomavirus (HR-HPV) type is necessary but not sufficient for the development of cervical cancer and its precursors. The process of CIN cancerous transformation *in situ* usually takes 3-5 years, which providing enough time for the adequate diagnostics and treatment of cervical lesions at early stage. However, most HPV infection is sexually transmitted and transient, with a high spontaneous regression rate of 91% in 2 years. Only a small fraction of HR-HPV infections are persistent which may lead to development of CIN with increasing risk of progression to cancer [2]. The current therapies for high-grade CIN or persistent HR-HPV infection lesions focus on removing the abnormal HPV-associated neoplasia and transformation-susceptible cells including a loop electrosurgical excision procedure (LEEP), cold knife cone biopsy, and focal destruction by electrofulgaration or cryotherapy rather than specific target HPV infection. The major disadvantages of all of these ablative and excisional treatments are recurrence and subsequent cervical insufficiency and cervical scar stricture due to substantial destruction of cervical stroma. The combination of a high prevalence of high-grade CIN and persistent HR-HPV infection among young women and extensive use of excisional procedures leads to significant perinatal morbidity. The development of an effective and safe therapy for high-grade CIN or persistent HR-HPV infection is urgently needed in women at reproductive age.

Photodynamic therapy (PDT) involves the activation of a photosensitizer selectively accumulated in target cells by

a specific wavelength of light that will generate cytotoxic reactive oxygen species, which leads to specific cell damage and tissue necrosis [3]. There is evidence that PDT can also be active against bacteria, viruses and fungi [4]. 5-Aminolevulinic acid (5-ALA), a derivative from the heme pathway, is often used as photosensitizer in PDT. They have shown great promise in the treatment of HPV-associated cancers as well as precancerous lesions in several clinical investigations [5-8]. In addition, PDT, as an ideal adjuvant therapy, can be applied before or after chemotherapy or surgery and can be repeated as needed without cumulative toxicity [9]. However, the specific mechanism(s) of PDT on HPV-infected host cells has not been fully understood and its efficacy is remarkably varied. Complete understanding of the molecular mechanism(s) of PDT-mediated cell destruction provides novel insights for improving its therapeutic efficacy.

Thus, the aim of this study was to evaluate the anti-proliferative activity of ALA-PDT against HPV16-immortalized cervical epithelial H8 cells and investigate the underlying molecular mechanisms of action. Here, we demonstrate repression of HPV E6 and E7 oncogenes and up-regulation of p53, p21 and RbAp48 levels induced by ALA-PDT, leading to cell apoptosis in HPV-infected cells. We provide the evidence that ALA-PDT could play as successful noninvasive therapeutic modality for the regression and elimination of HPV infections associated with lesions.

## Materials and methods

**Cell culture and photodynamic therapy.** The HPV16 immortalized human cervical epithelial H8 cell line was originated from the Institute of Virology of Chinese Academy of Medical Sciences and was a generous gift from Professor Weiming Zhao from the Department of Medical Microbiology of Shandong University. This cell line is anchorage dependent with otherwise normal structure and function except for the characteristics of transformation by HPV16 E6 and E7, which cannot form tumors within 6 months after injection into nude mice. The H8 cells were cultured in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 100 μg/mL of streptomycin, 100 U/ml of penicillin and heat-inactivated FBS (Invitrogen, Carlsbad, CA, USA) under standard culture conditions. And 80-90% confluent cells were used in all the experiments.

5-Aminolevulinic acid hydrochloride obtained from Aldrich-Sigma Chemical (St. Louis, MO, USA) was dissolved in DMEM. Various concentrations of ALA in DMEM were added to the cells, which were incubated 6 h in the dark under standard culture conditions. After the administration of ALA, all the samples were performed through three washing steps with phosphate buffered saline (PBS), and fresh culture medium (DMEM supplemented with 10% fetal bovine serum) was then added to each well, followed by Light-Emitting Diode laser irradiation (Yage, wuhan, Chinese) at a wavelength of 630nm. The laser energy was 10 J/cm$^2$. And then the cells were maintained in the dark at 37˚C, 5% $CO_2$.

**Cell proliferation assay.** The effect of ALA-PDT on cell viability was determined by the Cell Counting Kit-8 (CCK-8) assay and cell counting. Briefly, cells were seeded $2.0\times10^3$ per well in a 96-well culture plate (corning costar, USA) for Cell viability assay and $1.0\times10^4$ per well in a 24-well culture plate for Cell counting assay, and then treated with the various concentrations (0-200 μM) of ALA for 6 h followed by light irradiation at a wavelength of 630nm. 24 h after PDT, the medium was changed and the cell viability was evaluated by the CCK-8 (Dojindo, Japan) following the manufacturer's protocol. Experiments were performed in triplicate to ensure the reproducibility of the results.

**Apoptosis rate and cell cycle analysis by flow cytometry.** For cell apoptosis analysis, cells were plated in 35-mm dishes ($4.0\times10^5$ per dish) followed by various concentrations of ALA-PDT treatment and incubated in the dark for 12 h. Then, the medium was removed, the cells were washed twice with PBS and collected in tubes ($1\times10^6$) followed by staining using cell apoptosis kit (BestBio, Shanghai, China) according to the manufacturer's protocols. For cell cycle analysis, cells were harvested at 12 h after PDT-treatment and then fixed and stained using cell cycle kit (BD, Biosciences, USA) according to the manufacturer's protocols. Cells were then analyzed by flow cytometry (BD, Biosciences, USA).

**Western blot assay.** After PDT treatment, cells were harvested at 24 h and then washed with cold PBS. Total protein was extracted from cells by the use of NP-40 lysis buffer (Beyotime, China) following the manufacturer's recommendation as described. For Western blot assay, 25 μg protein was electrophoresed on 12% polyacrylamide-SDS gels and transferred onto a 0.22 μm polyvinylidene fluoride membrane (Immobilon, Millipore, MA, USA). After blocking with TBST (Tris-buffered saline, 0.1% Tween 20) containing 5% non-fat milk for 1 h at room temperature, the blot was washed with TBST (10 mM Tris, 100 mM NaCl, 0.1% Tween-20) for 3×10 min, followed by incubating with primary antibody overnight at 4˚C. And primary antibodies: RbAp48 monoclonal mouse antibody (1:1000, Abcam, UK), $p^{21}$ monoclonal mouse antibody (1:1000, Abcam, UK), GAPDH (1:2000, Abcam, UK). The blot was washed for 3×10 min and then incubated with the horseradish peroxidase-conjugated secondary antibody for 1 hour at room temperature. The blot was washed for 3×10 min and immune complexes were visualized by chemiluminescence using an ECL kit (Amersham Biosciences). Equal loading in whole cell lysates was confirmed by GAPDH.

**Quantitative real-time PCR assay.** Total RNA was extracted from the cells with the Trizol reagent (Invitrogen, Carlsbad, CA, USA), and then 5 μg total RNA was purified and reversely transcribed to cDNA with the real-time PCR Kit AMV (TaKaRa Bio, Tokyo) following the manufacturer's protocols. Real-time PCR was performed on the LightCycler 2.0 Instrument (Roche, Penzberg, Germany). The quantified mRNA levels of β-actin were used as an endogenous control and normalization. Primers used for real time RT-PCR were

as follows: RbAp48, 5′-ATGCCCCAGAACCCTTGTATC- 3′ and 5′-GCCCATAGCCTTCCTTCTGAT- 3′; HPV-16 E6, 5′-AATGTTTCAGGACCCACAGG- 3′ and 5′-TCACGTCGCAGTAACTGTTG-5′; HPV-16 E7, 5′-AGTGTGACTCTACGCTTCGG-5′ and 5′-TGTGCCCATTAACAGGTCTT-3′; β-actin, 5′-TGGCATTGCCGACAGGATGCAGAA-3′ and 5′-CTCGTCATACTCCTGCTTGCTG AT-5′.

**Statistical analysis.** The data is presented as mean ± SD. Two-way ANOVA or Student's t-test by using GraphPad Prism v6.0 (GraphPad, San Diego, CA, USA) were used for Statistical comparisons. A *P* value less than 0.05 was considered as statistically significant.

## Results

**The anti-proliferative effect of PDT in H8 cells.** To examine the cytotoxicity of ALA-PDT under a certain light dose of 10 J/cm$^2$, H8 cells were incubated with various concentrations of ALA for 6 h followed by photoactivation. Since there was no effect of light upon cell destruction, we used cells that had been treated with light irradiation (0 ALA, i.e., cells that had not been treated with ALA) as our control group. Cell viability was measured by Cell Counting Kit-8 and cell counting assay 24h after PDT. Both CCK-8 assay (Figure. 1A) and cell counting (Figure 1B) revealed that significant cytotoxicity was observed in H8 cells subjected to ALA-PDT treatment. It increased in a dose dependent manner, and the $IC_{50}$ values were about 120.75 ± 1.18 μM. In order to determine the dark cytotoxicity of ALA on H8 cells, cells were incubated for 30 h with various concentrations (0-600 μM) of ALA in dark and evaluated by Cell Counting Kit-8 assay. No significant dark cytotoxicity was observed at the dose range of 0-600 μM.

**Induction of cell cycle arrest by ALA-PDT on H8 cells.** To precisely assess the effects of ALA-PDT on cell cycle distribution, we performed flow cytometric analysis using propidium iodide stain at various concentrations of ALA-PDT (0, 50, 100, 150 μM). The analyses indicated a dose-dependent accumulation in G1 phase population and reduction in S and G2/M phase population (Figure 2).

**Induction of apoptosis by ALA-PDT in H8 cells.** The antitumor effect of ALA-PDT is predominantly performed *via* apoptosis. Therefore, we analyzed whether the cytotoxic effect of ALA-PDT on H8 cells was mediated by apoptosis. To confirm the rate of apoptosis, flow cytometric analysis using annexin V-FITC/PI staining was performed. A dose-dependent induction of apoptosis was observed after PDT treatment (Figure 3A, 3B). Subsequently, Western-blot analysis was performed to determine whether the apoptosis induced by ALA-PDT was associated with protein 53 (p53) and cyclin-dependent kinase (CDK) inhibitors p21 in H8 cells. Notably, the accumulation of protein level of p53 and p21 was observed after PDT treatment in a dose-dependent manner (Figure 3C). Thus, our data demonstrated the involvement of p53-dependent pathway of apoptosis induced by ALA-PDT in HPV-immortalized cervical epithelial cells.

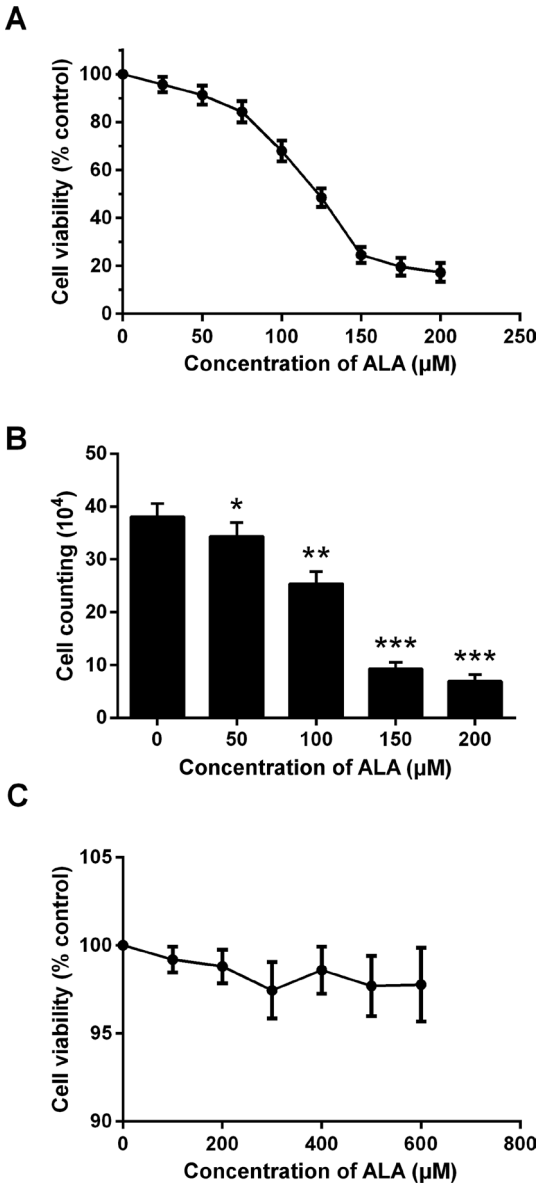


**Figure 1. The effect of ALA-PDT on proliferation**
**Cell cytotoxicity of various concentrations of ALA-PDT in H8 cells was measured by CCK-8 assay (A) and cell counting (B). Cell cytotoxicity of ALA without photoactivation was evaluated using CCK-8 assay (C). Data is presented as mean ± SD of three independent experiments. * P < 0.05; ** P < 0.01; *** P < 0.001 versus the control group (0 μM ALA).**

**ALA-PDT down-regulates the expression of HPV oncogenes– E6 and E7 in H8 cells.** In order to determine a possible mechanism of action of ALA-PDT on HPV16 immortal cervical epithelial H8 cells, the HPV oncogene E6 *and* E7 were analyzed by Real-time PCR. As shown in Figure 4, ALA-PDT down-regulated the mRNA levels of HPV E6 and E7 in a dose-dependent manner in H8 cells. Nearly 4-fold and

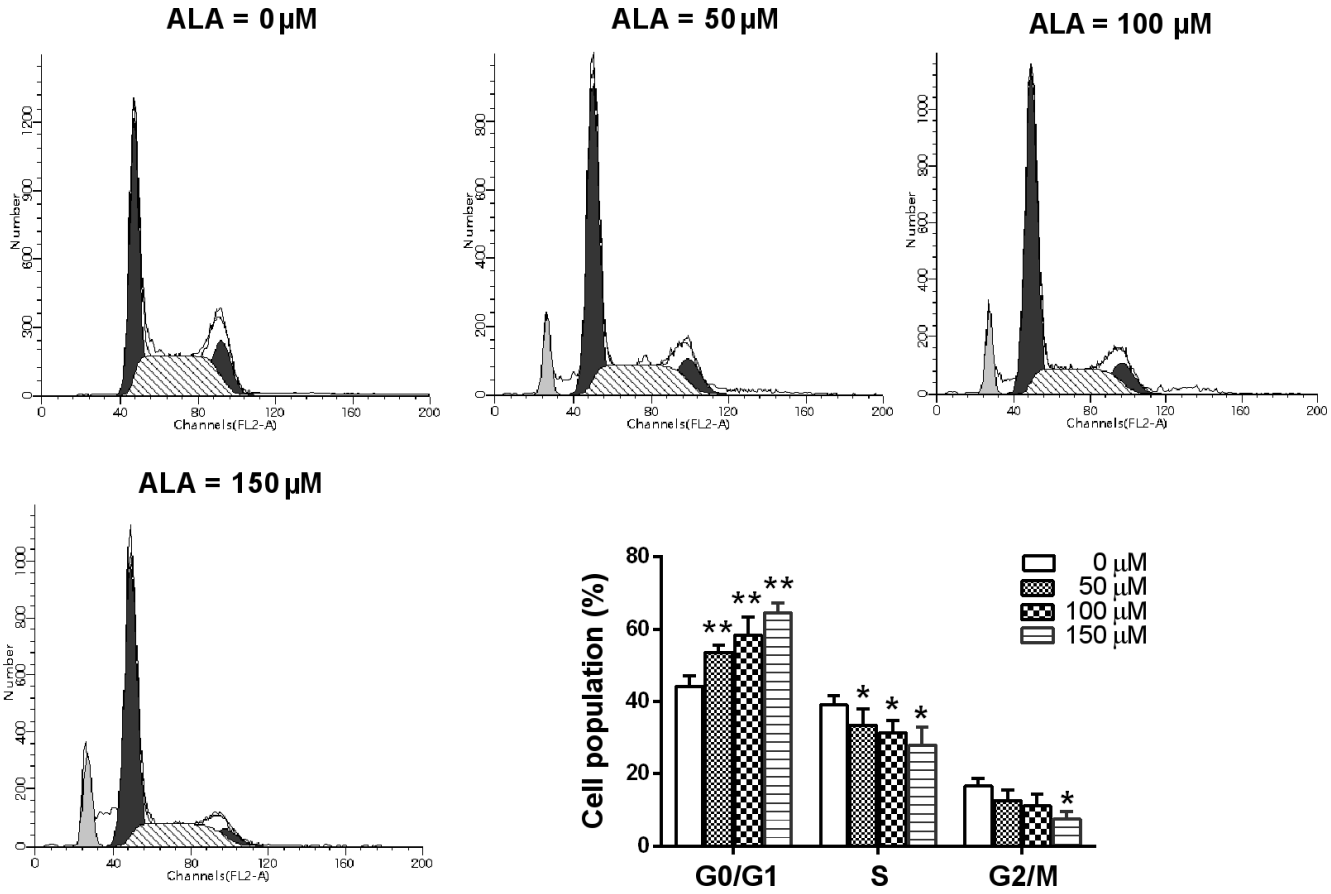


**Figure 2. The effect of ALA-PDT on cell cycle cell cycle distribution**
**After ALA-PDT treatment, the cells were stained fixed and stained using cell cycle kit and then analysed by flow cytometry. A remarkable accumulation of PDT-treated H8 cells in the G1/G0 phase of the cell cycle was observed, and adversely in the S and G2/M phases, compared to those in the control group ($p<0.05$). Furthermore, G1/G0 phase arrest was in concentration dependent manner. * $P < 0.05$; ** $P < 0.01$ versus the control group (0 μM ALA).**

2.2-fold reduction in the mRNA expression levels of HPV16 E6 and E7 respectively were observed with 150 μM ALA-PDT after 24 h.

**ALA-PDT induced RbAp48 expression in H8 cells.** Earlier studies have shown that RbAp48 controls the transforming activity of HPV16 in cervical cancer and may be employed as a therapeutic target for treating HPV-associated lesions. We determined the expression pattern of RbAp48 during ALA-PDT on H8 cells. The mRNA levels of RbAp48 (Figure 5A) were significantly up-regulated in H8 cells subjected to ALA-PDT treatment. The induced expression of RbAp48 during ALA-PDT in H8 cells were also verified by Western blot (Figure 5B).

## Discussion

Adequate treatment of persistent HR-HPV infectious lesions and high-grade CIN is the most effective measure for preventing the incidence of cervical cancer. The traditional surgical excision and physical ablation have shown effective in treating localized lesions, but they are invasive and inefficient with high recurrence rate. High frequency of recurrences and complications after conservative therapy dictates the necessity of searching new treatment methods for persistent high-risk HPV infections and CIN.

Photodynamic therapy (PDT), as an organ-saving therapeutic approach, has shown promise in treatments of high-grade CIN and early cervical cancer [10]. A vast majority of pre-clinical and clinical studies have established that PDT is of particular value to the women who are interested in future child-bearing [11, 12]. In the present study, we used HPV-positive precancerous cells-the HPV16 immortalized human cervical epithelial H8 cells. It is particularly useful for studying of HPV infection associated cervical epithelial lesions in which it has the structure and function of normal human cervical epithelial cells except for the transformation by HPV16 E6 and E7 and they fail to result in carcinogenesis [13, 14]. The main objective of the present study was to evaluate the anti-proliferative activity of

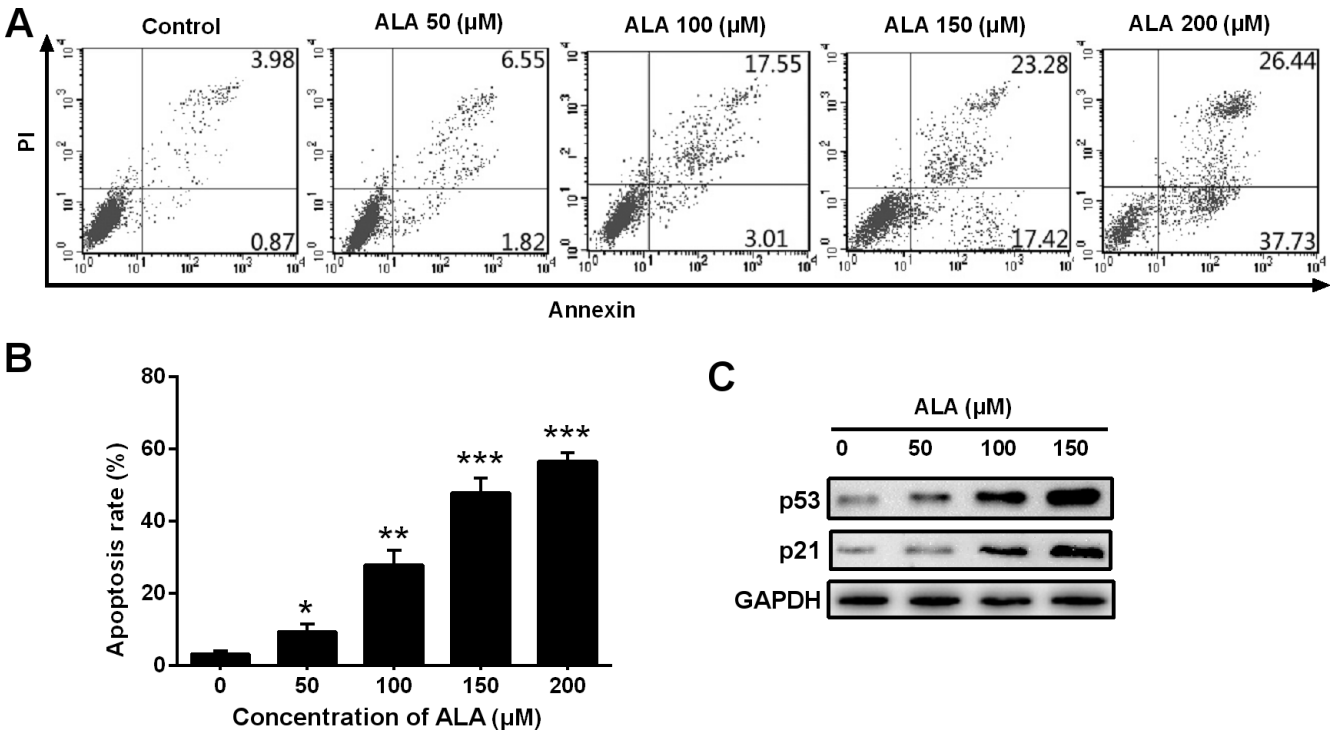


**Figure 3. The effect of ALA-PDT on apoptosis and related gene changes**
**(A) The levels of apoptotic analysis in H8 cells were assayed by flow cytometric analysis of the Annexin-FITC/PI double staining method. The figure is a representative of three independent trials. (B) ALA-PDT induced H8 cells apoptosis, and data is presented as mean ± SD from 3 independent experiments. * P < 0.05; ** P < 0.01; *** P < 0.001 versus control group (0 μM ALA). (C) ALA-PDT induced changes in protein levels of p53 and p21 in H8 cells by Western blot analysis. The protein contents were collected at 24 h after ALA-PDT. The figure is a representative of three independent trials.**

ALA-PDT and the associated mechanism of action in H8 cells. We found that ALA-PDT markedly inhibited cell proliferation in HPV16 immortal cervical epithelial H8 cells.

Earlier reports have demonstrated that apoptosis mediated by PDT is crucial for its therapeutic efficacy [15]. As shown in figure 3A, we found that ALA-PDT result in a dose-dependent apoptosis of HPV16 immortal cervical epithelial H8 cells. Induction of apoptosis is crucial to the anti-tumor activity of p53 [16], p53-dependent apoptotic pathways induced by ALA-PDT was further investigated. It is generally believed that p53

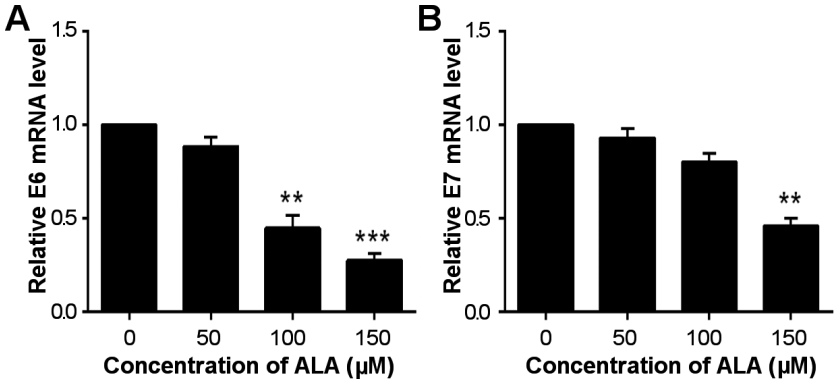


**Figure 4. The effect of ALA-PDT on HPV16 E6 and E7**
**Relative mRNA levels of HPV16 E6 (A) and E7 (B) in H8 cells were subjected to concentrations of ALA assayed by quantitative real-time PCR. Expression of HPV16 E6 and E7 in each sample was normalized to that of β-actin. Data is presented as mean ± SD of three independent experiments. ** P < 0.01; *** P <0.001 versus the control group (0 μM ALA).**

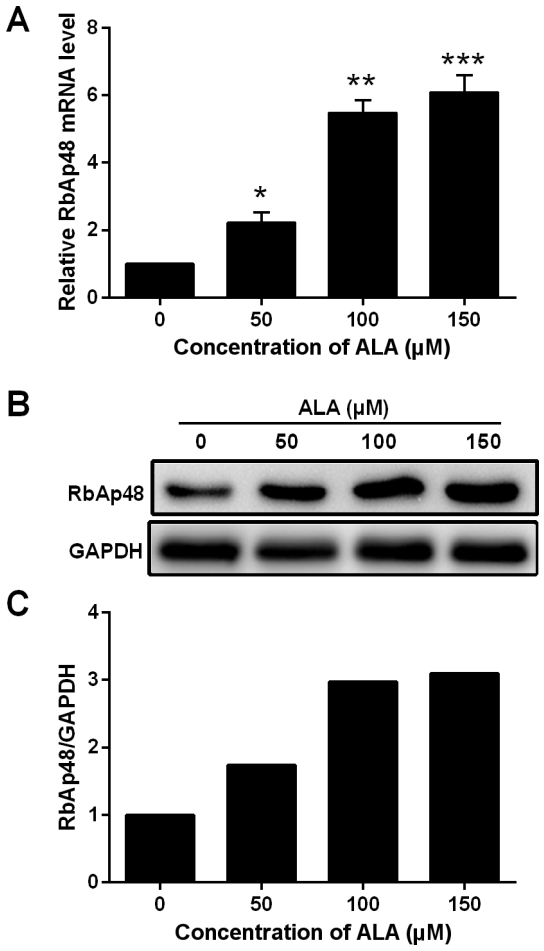


**Figure 5. The effect of ALA-PDT on RbAp48 in H8 cells**
**(A) Relative mRNA levels of RbAp48 in H8 cells were assayed by quantitative real-time PCR. Data is presented as mean ± SD of three independent experiments. (B) The protein levels RbAp48 in H8 cells were assayed by Western blot analysis 24 h after ALA-PDT, and GAPDH was used as a loading control. Data shows a typical experiment, which was repeated three times with similar results. (C) The ratio of RbAp48 /GAPDH for each was analyzed by densitometry scanning of Western blots and shown as bar graphs. Data is presented as mean ± SD from 3 independent experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ versus the control group (0 μM ALA).**

and p21 are crucial regulators of cell proliferation and genome stability, which are associated with several kinds of human cancers and have great significance in cancer therapy [17-19]. Studies have demonstrated that the accumulation of potent tumor suppressor p53 was associated with apoptosis induced by ALA-PDT in the HepG2 cells. The p21, as a downstream target gene of p53, plays a central role in cell cycle control and induction of p21 may result in cell cycle arrest and senescence. Furthermore, p21 is a cellular target of HPV-16 E7 and acts as a tumor suppressor in cervical carcinogenesis [20, 21]. Previous studies showed that accumulation of p53 and p21 was associated with apoptosis induced by cidofovir in HPV-positive cells [22]. In the present study, the expression of p21 was elevated proportionately in relation to p53 increase after ALA-PDT treatment. It is conceivable that accumulation of p53 and p21, at least in part, relates to the apoptosis induced by ALA-PDT.

To elucidate the molecular events underlying ALA-PDT induced cell destruction of HPV16 immortal cervical epithelial H8 cells, we examined the effect of PDT on HPV E6 and E7. It is well known that functions of the viral protein E6 and E7 are crucial for HPV-induced cervical carcinogenesis and for the support of the viral life cycle, in which they are able to combine with tumor suppressor p53 and retinoblastoma (Rb) respectively and act cooperatively to facilitate cell immortalization, genomic instability and malignant conversion [23]. Blocking the expression of E6 and E7 has been shown to have the potential to reprogram the proliferation status of HPV-transformed cells and switch to apoptosis [24]. Our present investigation shows that PDT significantly affects the mRNA levels of E6 and E7 in H8 cells (Figure 4).

A primary target of HPV E7 is the Rb family of proteins that control the activity of E2F transcription factors [25]. Retinoblastoma-associated protein 48 (RbAp48), a highly abundant component of HDAC, is required for the negatively-regulated transcription of E2F activity [26]. Studies have verified that over-expression of RbAp48 in Caski and HeLa cells significantly inhibits cell proliferation *in Vitro* and tumor formation *in Vivo* [13]. RbAp48 is considered to be a tumor suppressor in HPV-induced cervical carcinogenesis. Our earlier study demonstrated that ALA-PDT result in accmulation of RbAp48 through induction of p53, Rb and caspase-3 and inhibition of E6 and E7. Presently we have observed that ALA-PDT increased the expression of RbAp48 expression in a dose-dependent manner. Since RbAp48 has the potential to improve the sensitivity of radiation therapy, up-regulation of RbAp48 induced by ALA-PDT may provide a basis for its clinical prospect of as an ideal radio-adjuvant therapy against cervical cancer [27].

It is concluded that ALA-PDT has proved to induce apoptosis in a dose-dependent manner in the immortalized HPV16 human cervical epithelial H8 cells. The regression of papillomatous lesions observed with ALA-PDT in patients may be due, at least in part, to the induction of apoptosis. Not only can PDT induce direct damage to the host cells of HPV, but also down-regulate the expression of E6 & E7 viral oncogenes and up-regulate RbAp48 which in turn is helpful to eradicate the virus itself and therefore reduce recurrence. Our findings provide a strong basis for the further exploration of ALA-PDT as a therapeutic modality, either alone or adjuvant to standard medical treatment modality, and for treating high grade CIN or persistent HR-HPV infections as well as preventing cervical cancer carcinogenesis.

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