Tumor Suppressor Protein p53-dependent Cell Death Induced by 5-Aminolevulinic Acid (ALA)-based Photodynamic Sensitization of Cancer cells in Vitro

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Summary

Recently, photodynamic therapy using 5-aminolevulinic acid (ALA-PDT) is widely used in cancer therapy, owing to the tumor-specific accumulation of photosensitizing protoporphyrin IX (PpIX) after administration of ALA. In the present study, by focusing on ALA-based photodynamic challenge (ALA-PDC) with 640 nm of LED, we examined the potential involvement of p53-mediated apoptosis in 3 human gastric cancer cell lines. We found the cytotoxic effects of ALA-PDC are dependent on both the ALA concentration and the irradiation dose of LED. Typical morphological changes of apoptotic and/or necrotic cell death caused by ALA-PDC were observed by propidium iodide and Hoechst 33258 staining. At the higher concentrations of ALA, necrotic cells were increased whereas apoptotic cells were decreased. Among three cell lines tested, we found that MKN45 cells which possess wild-type $p53$ gene are highly sensitive to ALA-PDC. However, MKN45 cells after treatment with $p53$ specific inhibitor pifithrin-$\alpha$ showed significantly decreased sensitivity to ALA-PDC. Immunoblotting analysis confirmed that Ser15 in p53 was phosphorylated following ALA-PDC in MKN45 cells, suggesting $p53$ protein stabilization and transcriptional up-regulation of $p53$ target genes. Conversely the photosensitivities of KKLS and MKN28 cells which harbor mutant $p53$ gene were little affected by pifithrin-$\alpha$. Thus, we propose that the gene status of $p53$ is potential to predict the patient’s response to ALA–PDT and the patient who has wild-type $p53$ gene would be sensitive for ALA–PDT.

Keywords:
Photodynamic therapy, p53, 5-aminolevulinic acid, apoptosis, necrosis, pifithrin-$\alpha$

Introduction

Photodynamic therapy (PDT) is an advantageous anti-tumor treatment that utilizes the generation of reactive oxygen species in tumor tissue [1-3]. 5-Aminolevulinic acid (ALA) is recently used for PDT, owing to the tumor-specific accumulation of photosensitizing
protoporphyrin IX (PpIX) after administration of ALA [3; 4]. As ALA can be applicable orally or topically, ALA administration has become a widely acceptable and popular procedure, particularly for the treatment of skin cancer [4]. Accumulation of ALA in cancer cells rapidly increases the intracellular porphyrin levels preferentially in neoplastic regions. ALA-induced intracellular accumulation of protoporphyrin IX (PpIX) is also used for fluorescence diagnosis of premalignant and malignant diseases in dermatology, urology, neurosurgery, otorhinolaryngology, gynecology, and gastroenterology [4; 5].

p53 plays the central role of tumor suppressor protein to restrict tumor development by serving as a sensor of cellular stress, such as DNA damage and oxidative stress [6; 7]. p53 is also involved in triggering apoptosis. p53 protein is composed of a core DNA-binding domain and several regulatory domains such as trans-activation domain, proline-rich domain, tetramerization domain, and carboxy terminal regulatory domain. Over 50% of human cancers reportedly possess p53 mutation. Most of p53 mutations are missense mutations found in DNA-binding domain known as hot spot, showing the ablation of apoptosis. In this regard, it is of importance that loss of function of wild-type p53 decreases the efficacy of anti-cancer treatments such as radiation therapy and chemotherapy [8; 9]. Hitherto, the signal transduction involved in PDT-mediated cell death using several types of photosensitizers has been reportedly investigated so as to make the consideration to the efficacy of PDT [10-15]. Fisher et al reported that p53 expression does not directly modulate tumor cell sensitivity to Photofrin-PDT in either apoptosis-responsive (LS513) or nonresponsive (MCF-7) cells. Lee et al also suggested that the gene status of p53 may not be important in hypericin-PDT-mediated cell killing or induction of apoptosis [11; 15].

In this work, we investigated the involvement of p53 on ALA-based photodynamic challenge (ALA-PDC) with 640 nm of LED irradiation. We examined the potential involvement of p53-mediated apoptosis in ALA-PDT using a p53 specific inhibitor pifithrin-α [16] showing pifithrin-α inhibited p53-mediated apoptosis. The present study provides evidence that the status of p53 gene in cancer cells is practical and useful biomarkers for predicting the photo-sensitivity to ALA-PDT.

**Experimental**

**Biochemicals**

5-Aminolevulinic acid (ALA) hydrochloride was purchased from COSMO OIL CO., LTD. (Tokyo, Japan). Pifithrin-α was purchased from ALEXIS (Lausen, Switzerland). MTT reagent, propidium iodide (PI), and Hoechst 33258 were purchased from Sigma–Aldrich (St. Louis, MO). RPMI-1640 medium and protease inhibitor cocktail were obtained from Nacalai Tesque (Kyoto, Japan). Penicillin–streptomycin and FBS were purchased from Invitrogen (Carlsbad, CA). All other chemicals used were of analytical grade.

**Cells and cell culture**
The human gastric cancer cell lines KKLS [17] were kindly provided by Dr. Mai of Cancer Research Institute of Kanazawa University (Kanazawa, Japan). MKN45 and MKN28 cells were kindly provided by Dr. Suzuki (Fukushima Medical College, Fukushima, Japan). Cells were maintained at 37°C under 5% CO₂ gas in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin.

**Detection of non-synonymous p53 mutation in human gastric cancer cells**

To detect non-synonymous mutation of p53 in MKN45, MKN28, KKLS cells, the coding region of p53 cDNA (NM_000546) were amplified by RT-PCR using the sense primer p53-37seq: 5’-TTG CCG TCC CAA GCA ATG GAT TTG ATG CTG TC-3’ and the antisense primer p53-2seq: 5’-GCG GAG ATT CTC TTC CTC TGT GCG CCG GTC TCT CCC A-3’. The sequence of most common amino acid residues altered region in cancer known as hotspots in p53 was determined by direct sequence method using the sense primer p53-37seq: 5’-TTG CCG TCC CAA GCA ATG GAT TTG ATG CTG TC-3’, p53-seq5: 5’-GAT GAA GCT CCC AGA ATG CCA GAG GCT GCT-3’, p53-1seq: 5’-ACC TGC CCT GTG CAG CTG TGG GTT GAT TCC ACA-3’, and p53-seq4: 5’-TTG CGT GT G GAG TAT TTG GAT GAC AGA AAC ACT TTT-3’, then the anti-sense primer p53-44seq: 5’-GCA AGT CAC AGA CTT GGC TGT CCC AGA ATG CAA G-3’, p53-seq6: 5’-TCT CCC AGG ACA GGC ACA AAC ATG CA-3’, and p53-2seq: 5’-GCG GAG ATT CTC TTC TCT GTC TGT GCG CCG GTC TCT CCC A-3’. We confirmed the p53 status of those cell lines; MKN45 cells possess wild-type p53 whereas KKLS cells (Gly245Asp) and MKN28 cells (Ile251Leu) possess non-synonymous p53 mutation.

**Incubation of human gastric cancer cells with ALA and exposure to light-emitting diode (LED) irradiation**

Cells were seeded in a 96-well plate (1 × 10⁴ cells/well) and cultured at 37°C under 5% CO₂ gas for 24 h. ALA was added to the culture medium at increased concentrations, and incubation continued in the dark for 4 h. Thereafter, cells were exposed to LED irradiation for designated time (630 nm, 3.6 mW/cm²) by placing the cell culture plate below an LED irradiation unit provided by SBI ALA PROMO (Tokyo, Japan). Cells were further incubated in the dark for 24 h, and cell viability measured by the MTT assay as described previously [18]. The absorbance of formazan (a metabolite of MTT) in the resulting solution was measured photometrically at a test wavelength of 570 nm and a reference wavelength of 655 nm in a Microplate reader Model 550 (Bio-Rad Laboratories, Hercules, CA). IC₅₀ values were calculated from dose–response curves (i.e., cell survival vs. ALA concentration) obtained in duplicate experiments.

In this context, in order to investigate classification of cell death - *i.e.* apoptosis and/or necrosis, MKN45 cells incubated for 24 h following photodynamic treatment were stained with 5 μM of PI and Hoechst 33258 in culture medium for 1 h. Fluorescence of PI or Hoechst 33258 was detected with a fluorescence microscope (IX70/FLUOVIEW; Olympus, Tokyo, Japan).
**Western blot analysis**

For immunoblot analysis, cell lysates were prepared as described previously [18]. Samples were first treated with the SDS-PAGE sample buffer solution. Thereafter, sample proteins were separated electrophoretically by using a 12% polyacrylamide gel and electroblotted onto an Immobilon-P PVDF membrane (Millipore Corp., MA). The membrane was incubated in a blocking solution containing 5% (w/v) skim milk in TTBS [20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.05% (v/v) Tween 20] at room temperature for an hour.

We used a polyclonal antibody specific to the phosphorylated p53 at Ser15 (Cell signaling technology, Boston, MA; 1:1000 dilution), or monoclonal antibody against rabbit muscle GAPDH (American Research Products, Belmont, MA; 1:1000 dilution) as the first antibody, depending on the specific purpose of the immunoblot analysis. For the second antibody, we used anti-mouse IgG horseradish peroxidase (HRP)-conjugated antibody (Cell Signaling Technology, Beverly, MA) and anti-rabbit IgG HRP conjugates (Santa Cruz Biotechnology) at 1:3000 dilution.

HRP-dependent luminescence was developed with Western Lightning Chemiluminescent Reagent Plus (PerkinElmer Life and Analytical Sciences, Waltham, MA) and detected with a Lumino Imaging Analyzer LAS-4000mini (GE Healthcare UK, Amersham Place, England).

**Results and Discussion**

**Photosensitivities of human gastric cancer cells are dependent on ALA and irradiation dose.**

In the present study, in order to evaluate the cellular photosensitivity, we first treated cells in the presence of ALA for 4 h in the dark. Cells were then exposed to LED light (630 nm, 3.6 mW/cm²) for 0, 0.5, 1, 2.5, 5, 10 min, and the cell viability was analyzed using MTT assay. Figure 1 demonstrates the LED irradiation and ALA dose-responses of MKN45, KKLS

![Figure 1](image-url)

**Figure 1.** The dose responses of human gastric cancer cells to photodynamic sensitization following incubation with ALA. The cellular sensitivity by ALA-PDC is dependent on the ALA- and irradiation-dose. (A) MKN45 cells (wild-type p53) (B) KKLS cells (mutant p53; Gly245Asp) (C) MKN28 cells (mutant p53; Ile251Leu).
, and MKN28 cells to ALA-PDC. Each cell lines exhibited photosensitivity depending on ALA concentration and irradiation energy of LED light. The cell viability of the control cells was not affected by irradiation with the LED light in the absence of ALA (data not shown). The IC_{50} values for 10 min (2160 mJ/cm^2) of MKN45, KKLS, MKN28 cells were calculated to be 100, 700, and 1000 μM for ALA, respectively. Compared with MKN45 posseeing wild-type p53 gene, KKLS and MKN28 harboring mutant p53 gene showed lower sensitivity to ALA-PDC. These results suggest that gene status of p53 would determine the efficacy of ALA-PDT.

**ALA-PDC induced apoptosis in MKN45 cells associated with chromatin condensation by photosensitization**

Multiple signaling cascades are concomitantly activated in cancer cells exposed to the photodynamic stress. Depending on the subcellular localization of cytotoxic ROS, those signals are transduced into adaptive or cell death responses [19]. To address the question of whether apoptosis occurs in the case of ALA-PDC, we investigated the morphological change of MKN45 cells caused by photosensitization following incubation with 100 μM ALA for 4 h. Using PI and Hoechst33258 staining, typical morphological change of apoptotic and/or necrotic cell death caused by ALA-PDC were observed. Fluorescence staining with Hoechst 33258 (blue) revealed the ALA-PDC induces chromatin condensation leading to apoptosis (Fig. 2). On the other hand, PI staining (red) detected the late apoptotic or necrotic cells. This result demonstrates that apoptosis and necrosis are concomitantly occurred in the case of ALA-PDC. In higher concentration of ALA, necrotic cells were increased and apoptotic cells were decreased. In KKLS and MKN28 cells, apoptotic cells were not observed at the IC_{50} condition of ALA-PDC (data not shown). These results suggest that when the lower amounts of ALA are incubated with cancer cells with wild-type p53, the efficacy of ALA-PDT is highly attributed to apoptosis-mediated cell death.

**Figure 2.** Fluorescence staining of apoptotic and necrotic MKN45 cells using PI and Hoechst 33258. Arrows indicate typical apoptotic cells associated with chromatin condensation and/or cellular fragmentation. PI derived red fluorescence is observed in necrotic cells.

**Effect of p53 inhibition to the cellular sensitivity to ALA-PDC**

To investigate the potential involvement of p53 on apoptosis after ALA-PDC, we estimated the
effect of p53 inhibition during ALA-PDC on the cell viability. Three cell lines were used in this experiment: MKN45, KKLS, and MKN28. Cells were incubated with 50 μM PFT-α at increased concentration of ALA for 4 h. Cells were then replaced with fresh medium, and exposed to LED light for 10 min. Cell viability 24 h after ALA-PDC was measured by MTT assay. As the results, PFT-α significantly conferred cellular resistance on MKN45 cells (Fig. 3). This result strongly suggests that wild type p53 is critically involved in ALA-PDC induced apoptosis. In contrast, the effects of PFT-α on both KKLS and MKN28 were minimal compared with MKN45. These results suggest that the variant type of p53 could determine the photo-sensitivity to ALA-PDC. It is of importance that mutant p53 might be no efficacy of ALA-PDT.

![Graph](image)

**Figure 3.** Effect of pifithrin-α on cell viability of human gastric cancer cells after ALA-PDC. Cells were incubated with ALA in presence or absence of 50 μM PFT-α. The cellular sensitivity by ALA-PDC is measured by MTT assay as described in Experimentals section.

*Ser15 phosphorylation of p53 following ALA-PDC*

Human p53 possesses a series of serine / threonine phosphorylation sites. It is known the majority of these sites are phosphorylated following cellular stress. Ser15 phosphorylation of p53 is known to reduce p53 affinity for its primary negative regulator HD2M and promotes the recruitment of transcriptional coactivators. In this study, we assessed the phosphorylation status and the stabilization of p53 after ALA-PDC from immunoblotting analysis with specific antibody to p53 and Ser15-phosphorylated p53 (Fig. 4). ALA-PDC to MKN45 cells induced Ser15 phosphorylation in p53. The only LED irradiation hardly affected p53 status. Furthermore, the Ser15 phosphorylation and the deprivation of p53 were completely suppressed with a p53-specific inhibitor pifithrin-α (PFT-α). These results suggest that apoptosis following ALA-PDC in MKN45 is mediated by wild type p53. Previous studies have shown that PFT-α inhibits p53 accumulation in various cell systems subjected to ultraviolet radiation, cisplatin, resveratrol, or doxorubicin treatment [16; 20-23]. To our knowledge, this is the first report showing PFT-α could inhibit ALA-PDC-induced p53 status in MKN45 cells. Reportedly it is suggest that PFT-α interferes with multiple steps of apoptosis such as JNK and ERK [23]. Further study is ongoing in our laboratory.
for the specific mechanism that pretreatment of cells with PFT-α followed by ALA-PDC attenuated p53-dependent apoptosis.

**Conclusion**

We demonstrated the wild type p53 is of importance to predict the efficacy of ALA-PDT because MKN45 cells, wild type p53, showed higher cellular sensitivity by ALA-PDC than KKLS and MKN28 cells expressing mutant p53 (Fig. 1). Many clinical studies have revealed associations between p53 mutation and poor clinical outcome in a variety of cancer. Over 50% of human cancers reportedly possess p53 mutation, showing the ablation of apoptosis. Our results also suggested that mutant p53 decreased the efficacy of ALA-PDT as well. However, ALA-PDT has a potential to induce necrosis when higher amount of PpIX is accumulated in cancer cells (Fig.1 and 2). In this regard, with the effort that cancer cells accumulate PpIX, ALA-PDT would be successful in the case of p53 mutant cancer. In any of these cases, the status of p53 could be a significant factor in determining the therapeutic strategy.

The efficacy of ALA-PDT is also considered to depend on the intracellular PpIX concentration that can produce cytotoxic singlet oxygen and other ROS via photoactivation reactions. In mutant p53 cancer, cellular PpIX accumulation is required to induce necrosis. It is speculated that enhancement of PpIX accumulation would be achieved by an inhibitor of PpIX efflux transporters. The potential to induce both apoptosis and necrosis indicate the clinically advantageous merit of ALA-PDT. In conclusion, we hypothesize that the variant type of p53 is potential to predict the patient's response to ALA-PDT.

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