

5-Aminolevurinic acid inhibits the proliferation of bladder cancer cells by activating heme synthesis

YASUSHI NAKAI¹, YOSHIHIRO TATSUMI¹, SHUNTA HORI¹, YOSUKE MORIZAWA¹, KOTA IIDA¹, KENTA ONISHI¹, MAKITO MIYAKE¹, YUKI ODA¹, TAKUYA OWARI¹, TOMOMI FUJII², SAYURI ONISHI¹, NOBUMICHI TANAKA¹ and KIYOHIDE FUJIMOTO¹

Departments of ¹Urology and ²Pathology, Nara Medical University, Kashihara, Nara 634-8522, Japan

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Abstract. Iron is an essential nutrient that facilitates cell proliferation and growth, and it can contribute to tumor growth. Although iron chelators have shown great potential in preclinical cancer models, they can cause adverse side-effects. The aim of the present study was to determine whether treatment with 5-aminolevurinic acid (5-ALA) has antitumor effects in bladder cancer, by reduction of mitochondrial iron without using an iron chelator, through activation of heme synthesis. T24 and MGH-U3 cells were treated with 5-ALA. Ferrochelatase uses iron to convert protoporphyrin IX into heme, thus additional groups of T24 and MGH-U3 cells were transfected with synthesized ferrochelatase small interfering RNA (siRNA) either to silence ferrochelatase or to provide a negative siRNA control group, and then cell viability, apoptosis, mitochondrial Fe²⁺, the cell cycle, and ferritin expression were analyzed in all groups and compared. As an *in vivo* assessment, mice with orthotopic bladder cancer induced using N-butyl-N-(4-hydroxybutyl) were treated with 5-ALA. Bladder weight and pathological findings were evaluated, and immunohistochemical analysis was performed for ferritin and proliferating cell nuclear antigen (PCNA). In the cells treated with 5-ALA, proliferation was decreased compared with the controls, and apoptosis was not detected. In addition, the expression of Fe²⁺ in mitochondria was decreased by 5-ALA, expression of ferritin was also reduced by 5-ALA, and the percentage of cells in the S phase of the cell cycle was significantly increased by 5-ALA. In T24 and MGH-U3 cells with silenced ferrochelatase, the inhibition of cell proliferation, decreased expression of Fe²⁺ in mitochondria, reduced expression of ferritin, and increased percentage of cells in the S phase by treatment with 5-ALA were weakened. *In vivo*, no

mouse treated with 5-ALA developed muscle-invasive bladder cancer. The expression of ferritin was weaker in mice treated with 5-ALA and that of PCNA was higher than that in mice treated without 5-ALA. It was concluded that 5-ALA inhibited proliferation of bladder cancer cells by activating heme synthesis.

Introduction

Iron is an essential nutrient that facilitates cell proliferation and growth (1), and it can contribute to tumor growth (1). Campbell *et al* (2) demonstrated that exposure to iron oxide dust tripled the incidence of pulmonary tumors in mice. Richmond *et al* (3) showed that intramuscular injection of iron-dextran induced sarcoma in rats. Hann *et al* (4) reported that the growth rate of tumor xenografts could be influenced by levels of dietary iron. Regarding bladder cancer, there have been few studies concerning the association between bladder cancer and iron. Seligman *et al* (5) reported that bladder cancer cellular proliferation was dependent on iron. Therefore, chelating iron, such as desferrioxamine (DFO), has been evaluated for its antitumor effects (1,6), including in bladder cancer (5). Seligman *et al* (5) demonstrated that DFO inhibited proliferation of bladder cancer cells. Although iron chelators have shown great potential in preclinical cancer models (6), they can cause adverse side-effects, such as infection and gastrointestinal bleeding (7). Therefore, these challenges must be overcome to improve the therapeutical efficacy of iron chelators for tumor treatment (7).

Iron is used in cells for DNA synthesis (cell proliferation), heme synthesis in mitochondria, and iron storage as ferritin (1). Compared with healthy cells, cancer cells have inhibited or defective heme synthesis in mitochondria (8-10). Considering these factors, it appears that iron contributes to tumor growth by its use in DNA synthesis and its storage in cancer cells. Therefore, activation of heme synthesis with consequent reduction of iron in mitochondria may potentially be a new treatment for cancer without the use of an iron chelator.

In bladder cancer, photodynamic diagnosis using 5-aminolevurinic acid (5-ALA) is widely used in clinical practice (11). 5-ALA is distributed ubiquitously in mammalian cells and is a precursor of heme, which is essential in aerobic

Correspondence to: Dr Kiyohide Fujimoto, Department of Urology, Nara Medical University, 840 Shijo-cho, Nara 634-8522, Japan
E-mail: kiyokun@naramed-u.ac.jp

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energy metabolism and the electron-transport system (12). In cancer cells, 5-ALA causes significantly higher accumulation of fluorescent endogenous porphyrins, mainly protoporphyrin IX, than in healthy cells due to certain effects, such as decreased ferrochelatase activity (8,12). The enzyme ferrochelatase converts protoporphyrin IX into heme with iron (13). Treatment with 5-ALA significantly increases protoporphyrin IX in cancer cells and activates heme synthesis (14). Therefore, the aim of the present study was to determine whether treatment with 5-ALA has antitumor effects in bladder cancer by reduction of mitochondrial iron without using chelating iron through activation of heme synthesis.

Materials and methods

Cell culture. Two human bladder cancer cell lines, T24 (ATCC no. HTB-4 derived from an undifferentiated grade 3 carcinoma) and MGH-U3 (a generous gift from Dr. H. LaRue at Laval University Cancer Research Centre, Quebec, Canada; derived from a grade 1 tumor), were maintained in RPMI-1640 growth medium (Nissui Pharmaceutical Co., Ltd.) supplemented with 10% fetal bovine serum (FBS) (ICN Biomedicals, Inc.), 100 U/ml penicillin, and 100 μ g/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc.) in a standard humidified incubator at 37°C in a 5% CO₂ atmosphere for 24 h.

Cell toxicity. T24 and MGH-U3 cells were seeded into 96-well plates at 2x10³ cells/well and incubated overnight. The growth medium was removed, and serum plus medium with or without 5-ALA (0.1 and 1 mM; SBI Pharmaceuticals Co., Ltd.) under dark conditions was applied. Cells at 48 h were assessed using a Cell Counting Kit-8 (Dojindo Laboratories, Inc.) according to the manufacturer's protocol. The absorbance was measured at 490 nm with a reference at 630 nm using an Infinite 200M PRO microplate auto-reader (Tecan Group, Ltd.). Experiments were performed three times with duplicate samples.

Apoptosis analysis. Annexin V assays using Muse® Annexin V & Dead Cell Assay Kit (cat. no. MCH100105; Merck KGaA) following the manufacturer's instructions were performed. Briefly, after treatment with T24 and MGH-U3 cells (1x10⁵ cells) with 1 mM 5-ALA for 9 h, the detached and adherent cells were collected and incubated with Annexin V and 7-amino-actinomycin D, a dead cell marker, for 20 min at room temperature in the dark. The events for live, early, and late apoptotic cells were counted with the Muse™ Cell Analyzer (software version. 1.5.0.0; Merck KGaA).

Transfection of small interfering RNA (siRNA). T24 and MGH-U3 cells in 6-well plates at 1x10⁵ cells/well were transfected with synthesized siRNA ferrochelatase (si-FECH; cat. no. sc-60631; Santa Cruz Biotechnology, Inc.) or siRNA negative control (si-NC; cat. no. 4390843; Invitrogen; Life Technologies; Thermo Fisher Scientific, Inc.) with 50 pmol of siRNA and 5 μ l of Lipofectamine 2000 (Life Technologies; Thermo Fisher Scientific, Inc.) in 6-well plates according to the manufacturer's instructions at 37°C for 48 h. Following transfection, protein was extracted, and the expression of

ferrochelatase was measured in each cell line by western blotting.

Evaluation of Fe²⁺ in mitochondria. In 6-well plates at 1x10⁵ cells/well, T24 and MGH-U3 cells transfected with si-FECH or si-NC were treated with 0, 0.1 and 1 mM 5-ALA in an RPMI-1640 growth medium supplemented with 10% FBS and incubated for 48 h. Fe²⁺ in mitochondria was then evaluated using Mito-FerroGreen (Dojindo Laboratories, Inc.) according to the manufacturer's protocol. Fluorescence of Fe²⁺ in mitochondria was evaluated using a fluorescence microscope (EVOS FL Auto; Life Technologies; Thermo Fisher Scientific, Inc.). The intensity ratio was evaluated by measuring the intensity divided by the number of cells in an x400 field of vision in the three views that exhibited the strongest intensity detected by three observers (YN, TO and YO). ImageJ software (version 1.8.0_172; National Institutes of Health) was used for the quantitative assessments.

Cell cycle analysis. Following transfection of siRNA into T24 and MGH-U3, the cells in 6-well plates at 1x10⁵ cells/well were treated with 0 mM, 0.1 mM, or 1 mM 5-ALA for 48 h. The cell cycle was analyzed using a Muse™ Cell Cycle Kit (MilliporeSigma) following the manufacturer's instructions. The results were analyzed using the Muse™ Cell Analyzer. Experiments were performed three times.

Western blotting. Proteins were extracted using RIPA buffer (cat. no. R0278, Sigma-Aldrich; Merck KGaA), which contained 20 mM of Tris-HCl (pH 7.5), 150 mM of NaCl, 1% of NP-40, 1% of sodium deoxycholate, 2.5 mM of sodium pyrophosphate, 1 mM of Na₃VO₄, 1 mM of phenylmethylsulfonyl fluoride, and 1 μ g/ml of leupeptin in T24 and MGH-U3 cells transfected with siRNA. Protein concentrations were quantified using a Protein Assay BCA kit (Nacalai Tesque, Inc.), and immunoblotting was performed as previously described (15). Total protein of 10 μ g was diluted with sodium dodecyl (SDS) loading buffer containing 2.5% of β -mercaptoethanol, boiled at 95°C for 5 min, and electrophoresed onto 10% SDS-polyacrylamide gels using a Mini-Protean Tetra Cell (Bio-Rad Laboratories, Inc.) at 200 V for 35 min. Gels were subjected to transfer onto polyvinylidene difluoride membranes (Hybond-P; GE Healthcare; Cytiva) using a semidry transfer apparatus (Trans-Blot SD Semi-Dry Transfer Cell; Bio-Rad Laboratories, Inc.) at 15 V for 45 min. Following blocking in Tris-buffered saline (pH 7.6) that contained 5% skim milk for 1 h at room temperature, the membrane was incubated overnight at 4°C with an anti-ferritin heavy chain rabbit monoclonal antibody (product code ab75972; dilution 1:1,000; Abcam), an anti-ferrochelatase mouse monoclonal antibody (cat. no. sc-377377; dilution 1:100; Santa Cruz Biotechnology, Inc.) or an anti-actin mouse monoclonal antibody (cat. no. A2066; dilution 1:3,000; Sigma-Aldrich; Merck KGaA), which was used as an internal loading control, followed by 1 h with horseradish peroxidase-conjugated goat anti-mouse IgG (cat. no. SA00001-1; dilution 1:10,000) or anti-rabbit IgG antibody (cat. no. SA00001-2; dilution 1:10,000; both from ProteinTech Group, Inc.) at room temperature.

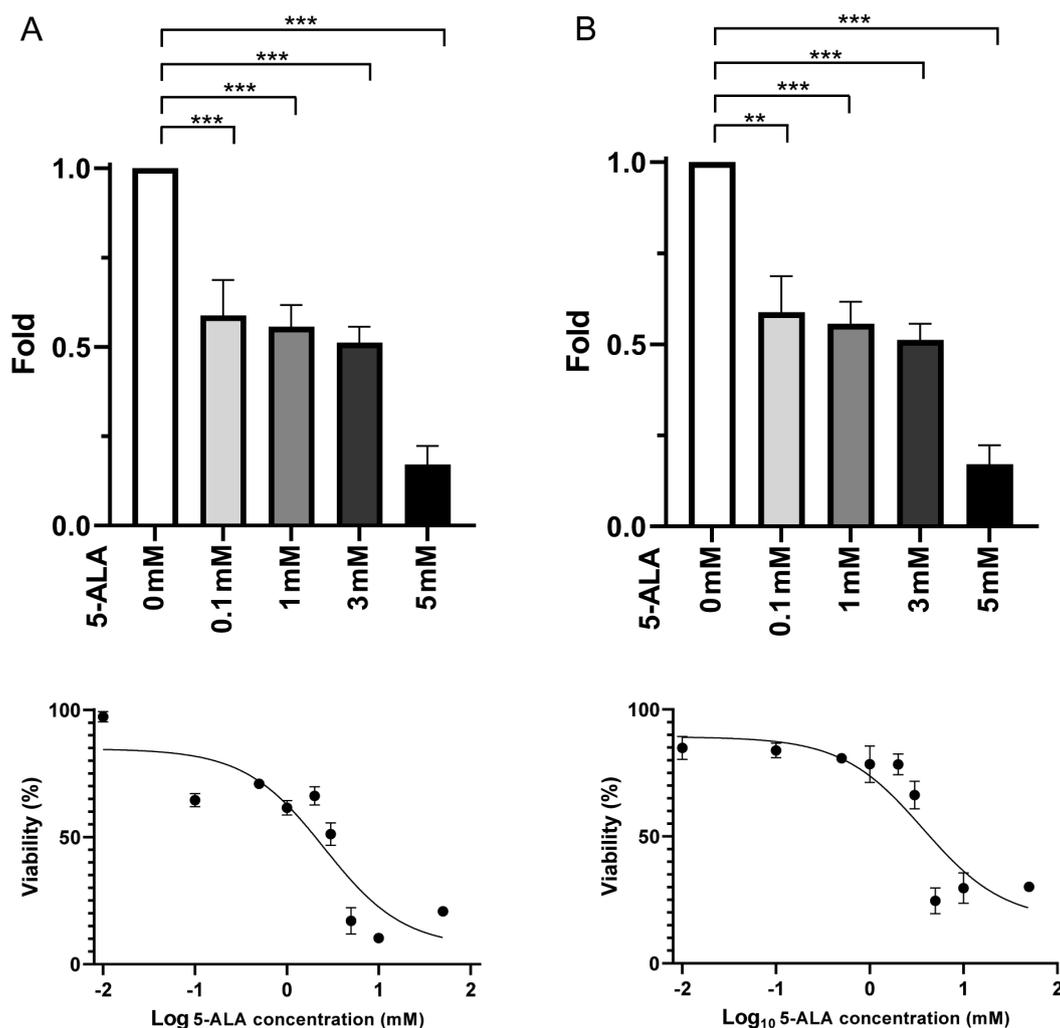


Figure 1. Cell toxicity of (A) T24 and (B) MGH-U3 cells treated with 5-aminolevulinic acid. ** $P < 0.01$ and *** $P < 0.001$. 5-ALA, 5-aminolevulinic acid.

Finally, the bound secondary antibody was detected using the SuperSignal West Pico Chemiluminescent Substrate (Pierce Chemical; Thermo Fisher Scientific, Inc.). Band densities were quantified using ImageJ (version 1.8.0_172). Protein levels were calculated in reference to the protein levels of actin. Experiments were performed three times.

Bladder tumor mouse model. A total of eight C57BL/6J male mice (5 weeks old; 20 g) were obtained from OrientalBioService, Inc. Treatment was started 2 weeks later. The mice were kept in a temperature (24°C)- and humidity (60%)-controlled room, with a 12/12-h light/dark cycle, and food and water were provided *ad libitum*. The eight mice were randomly divided into two groups as follows: Control group, four mice received 0.05% N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN) in drinking water for 20 weeks to develop muscle-invasive bladder cancer; and 5-ALA group, four mice received 0.05% BBN plus 6 mM ALA (16) in drinking water for 20 weeks. Following treatment for 20 weeks, all mice were euthanized by exsanguination under anesthesia with 2-3% isoflurane and their tissues were harvested for the subsequent experiments. The animal study was approved (approval no. 11921, 2017/2/23) by the Ethics Committee on

Animal Research of Nara Medical University (Nara, Japan). All animal experiments were conducted in accordance with the Guidelines for the Welfare of Animals in Experimental Neoplasia (17).

Immunohistochemical (IHC) staining. All resected bladders were filled with 150 μ l of 10% neutral-buffered formalin, and all specimens were fixed in 10% neutral-buffered formalin at room temperature for 48 h. Paraffin blocks were cut into 5- μ m thickness and placed on SuperFrost Plus microslides (Thermo Fisher Scientific, Inc.). Sections were deparaffinized and antigen retrieval was carried out in citric acid buffer (pH 6.0) using an autoclave. IHC staining was performed using a Histofine SAB-PO kit (Nichirei Biosciences, Inc.) according to the manufacturer's instructions. Briefly, slides were treated with 1% hydrogen peroxide in methanol at room temperature for 10 min to block endogenous peroxidase activity. The slides were incubated overnight at 4°C with rabbit monoclonal antibodies against ferritin (product code ab75972, dilution 1:50; Abcam); rabbit polyclonal antibodies against cytokeratin 20 (CK20) (cat. no. bs-1588R; dilution 1:100; Bioss Antibodies); rabbit polyclonal antibodies against survivin (cat. no. 10508-1-AP; dilution 1:500; ProteinTech Group, Inc.),

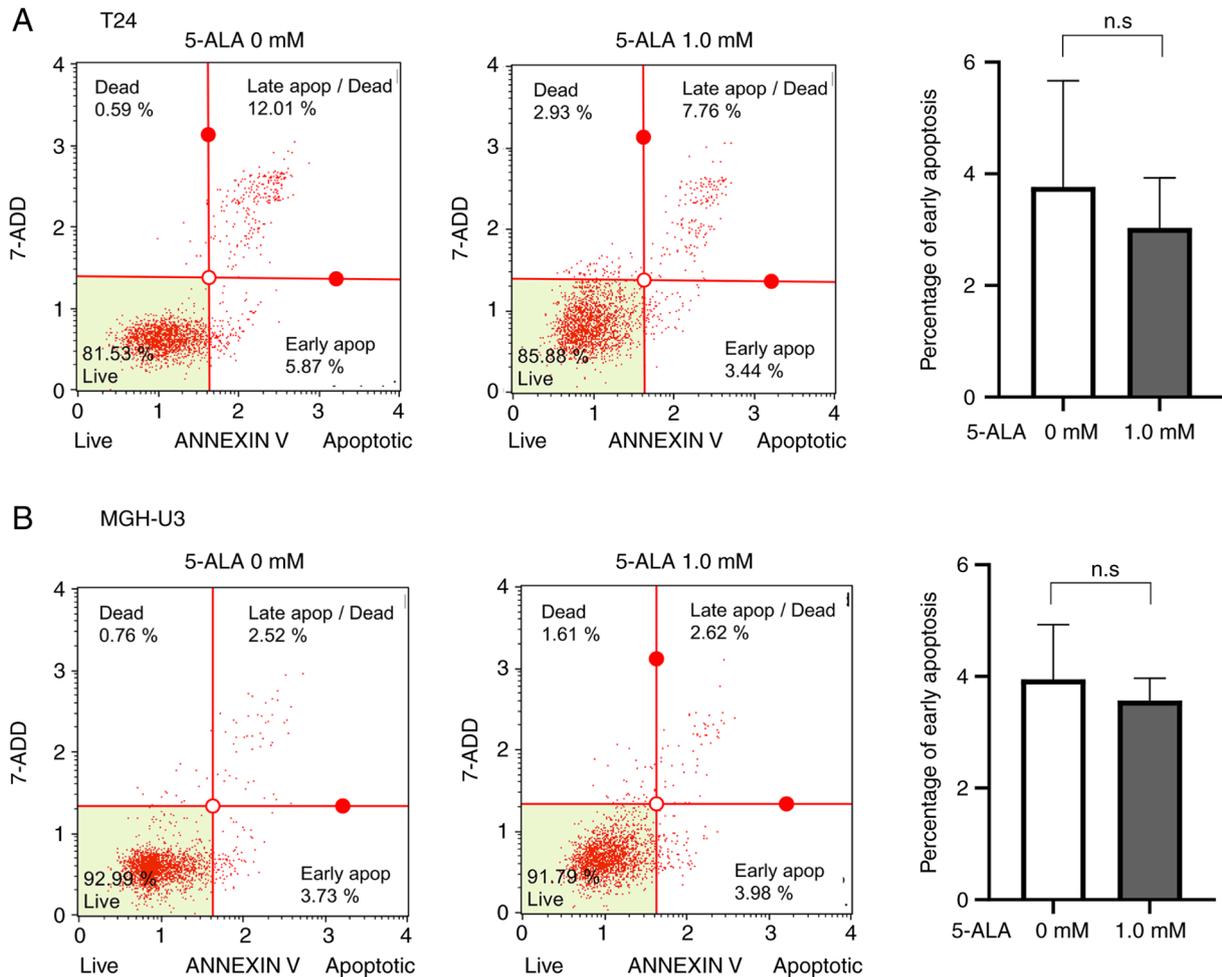


Figure 2. Analysis of apoptosis in (A) T24 and (B) MGH-U3 cells treated with 1 mM 5-aminolevulinic acid. 5-ALA, 5-aminolevulinic acid; apop, apoptosis; n.s., not significant; 7-ADD, 7-amino-actinomycin D.

which is specific to the G2/M phase in cells; or rabbit monoclonal antibodies against proliferating cell nuclear antigen (PCNA) (product code ab92552; dilution 1:100; Abcam), which is specific to the S phase in cells. The slides were then incubated at room temperature for 10 min with 100 μ l of the secondary antibody [anti-mouse and anti-rabbit IgG antibodies, included in the Histofine SAB-PO kit (Nichirei Biosciences, Inc.), at 1 μ l/ml]. The slides were counterstained with Meyer's hematoxylin (Muto Pure Chemicals Co., Ltd.) at room temperature for 1 min, dehydrated, and sealed with a cover slide. Immunoreactive cancer cells were counted based on five independent high-power microscopic fields (HPF; magnification, x400; 0.0625 μ m²), and the number of positive cells was divided by the total number of cancer cells (1-100%) using a microscope (EVOS FL Auto) by two investigators (SH and YM) to quantify the expression level of ferritin, CK20, survivin, and PCNA in cancer cells.

Statistical analysis. PRISM software version 7.00 (GraphPad Software, Inc.) was used for the statistical analysis. The data are presented as the mean \pm standard deviation (SD). The unpaired t-test was used for binary comparisons between two groups. A P-value of <0.05 was considered to indicate a statistically significant difference.

Results

Cell cytotoxicity. In the T24 and MGH-U3 cells, treatment with 5-ALA inhibited cell viability relative to that in the samples not treated with 5-ALA. The IC₅₀ values of T24 and MGH-U3 by 5-ALA were 2.5 and 3.7 mM, respectively (Fig. 1).

Apoptosis analysis. To evaluate the effect of apoptosis induced by photodynamic therapy, apoptosis was evaluated. Early apoptosis was not higher in the T24 (Fig. 2A) or MGH-U3 (Fig. 2B) cells treated with 1 mM 5-ALA than in the samples treated without 5-ALA.

Cell cytotoxicity in cells with gene silencing of ferrochelatase. Gene silencing of ferrochelatase was demonstrated successfully in T24 (Fig. 3A) and MGH-U3 (Fig. 3B) cells. To confirm the effect of silencing of ferrochelatase, a cell cytotoxicity assay was performed. Inhibition of cell viability by 5-ALA was significantly decreased by silencing of ferrochelatase in T24 (Fig. 3C) and MGH-U3 (Fig. 3D) cells treated with 0.1 and 1 mM 5-ALA.

Fe²⁺ in mitochondria. The expression of mitochondrial Fe²⁺ in T24 (Fig. 4) and MGH-U3 (Fig. 5) cells treated with the

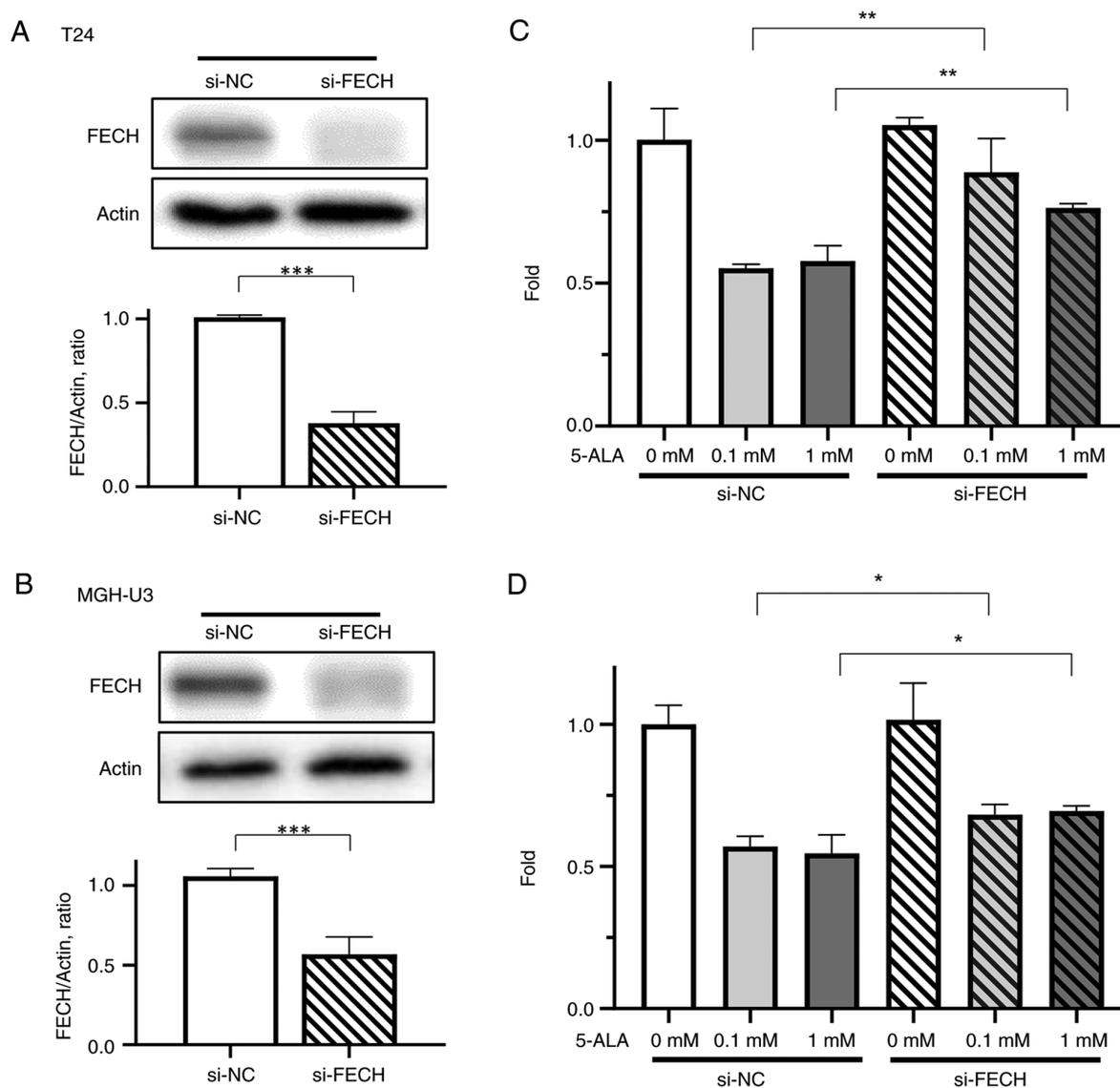


Figure 3. The expression of FECH in (A) T24 and (B) MGH-U3 cells treated to silence ferrochelatase. Cell toxicity of (C) T24 and (D) MGH-U3 cells with silenced FECH treated with 5-aminolevulinic acid. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. FECH, ferrochelatase; NC, negative control; si-, siRNA; 5-ALA, 5-aminolevulinic acid.

si-NC decreased when treated with 0.1 mM in T24 and MGH-U3 cells, and 1 mM 5-ALA in T24 and MGH-U3 cells. The effect of 5-ALA, which decreased the expression of Fe^{2+} in mitochondria in T24 (Fig. 4) and MGH-U3 (Fig. 5) cells, was significantly suppressed by gene silencing of ferrochelatase.

Ferritin. The expression of ferritin in T24 (Fig. 6A and B) and MGH-U3 (Fig. 6C and D) cells treated with si-NC was decreased by treatments with 0.1 mM in MGH-U3 cells, and 1 mM 5-ALA in T24 and MGH-U3 cells. The effect of 5-ALA in T24 (Fig. 6A and B) and MGH-U3 (Fig. 6C and D) cells, which decreased the expression of ferritin, was significantly suppressed by gene silencing of ferrochelatase.

Cell cycle. The percentage of the S phase in T24 and MGH-U3 cells treated with si-NC was increased by treatments with 0.1 mM in MGH-U3 cells and 1 mM 5-ALA in

T24 and MGH-U3 cells (Figs. 7 and 8). The effect of 5-ALA in T24 and MGH-U3 cells, which increased the percentage of the S phase, was significantly suppressed by gene silencing of ferrochelatase after treatment with 1 mM 5-ALA (Figs. 7 and 8).

Effect of oral intake of 5-ALA in mice treated with BBN.

The body weight (mean, 28.3 ± 1.5 g) of the mice treated with ALA at 20 weeks of treatment was not significantly different from that of the mice in the control group (median, 30 ± 1.4 g) (Fig. 9A). Liver fibrosis, necrosis, or filtration of lymphocytes or plasma cells was not detected in the liver of mice treated with 5-ALA (Fig. 9B). The bladder weights were lower in mice treated with 5-ALA (mean, 0.089 ± 0.009 g) than in mice not treated with 5-ALA (median, 0.14 ± 0.058 g) although the difference was not significant. Bladder cancer was not found in two out of the four mice treated with 5-ALA, and muscle-invasive cancer was not found in the other two mice (Table I). Between the mouse with T1 high grade bladder

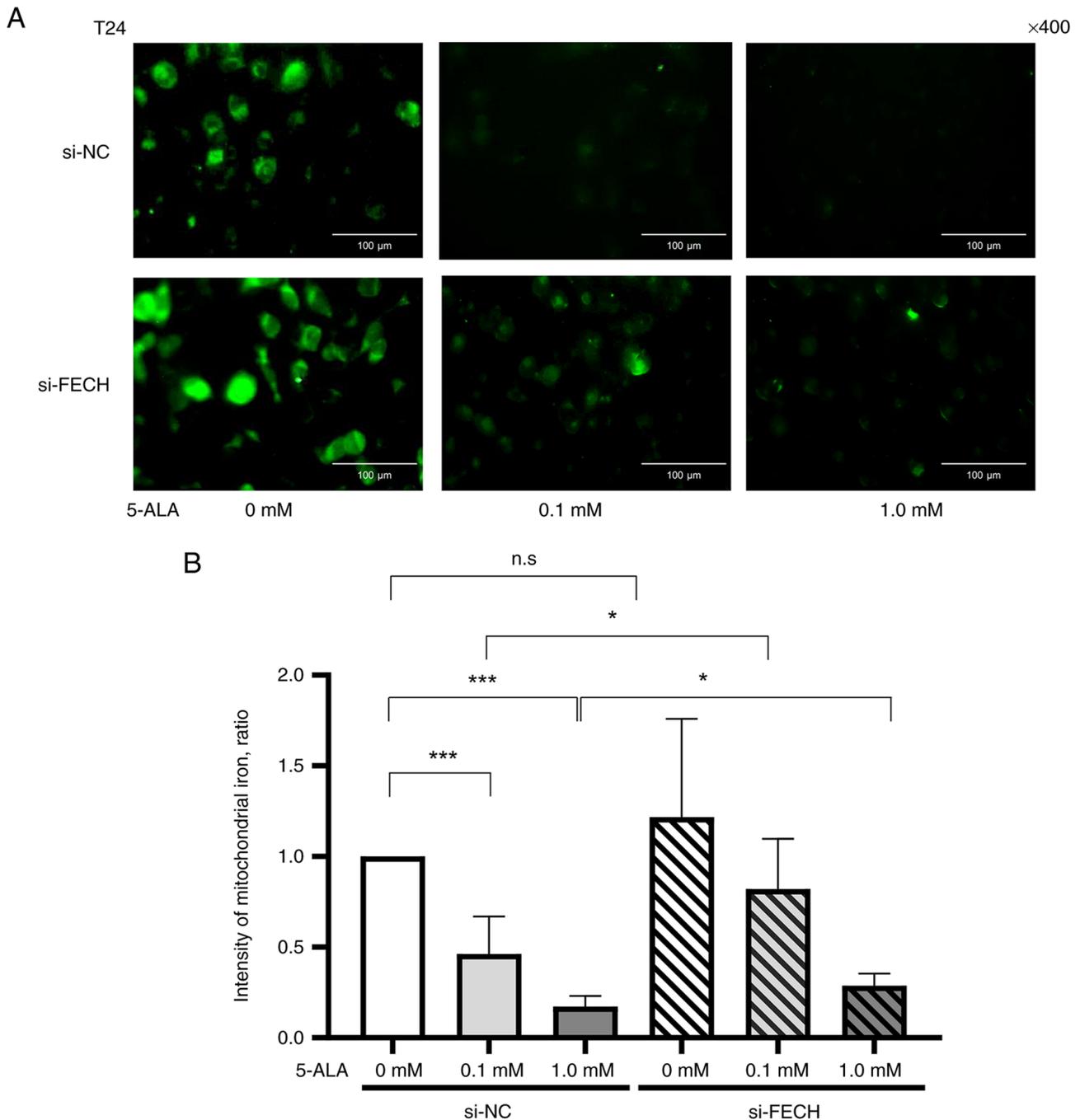


Figure 4. (A) Images and (B) intensity of mitochondrial Fe^{2+} in T24 cells with silenced ferrochelatase treated with 1 mM 5-aminolevulinic acid. * $P < 0.05$ and *** $P < 0.001$. si-, siRNA; NC, negative control; FECH, ferrochelatase; 5-ALA, 5-aminolevulinic acid; n.s., not significant.

cancer treated with ALA and without ALA, the expression of ferritin was significantly lower in the mice treated with 5-ALA (positive cells: mean, $18.5 \pm 6.6\%$) than in the mice in the control group (positive cells: mean, $38.3 \pm 6.9\%$), the expression of PCNA (marker of the S phase in the cell cycle) was significantly higher in the mice treated with 5-ALA (positive cells: median, $29.5 \pm 6.4\%$) than in the control group (positive cells: median, $15.3 \pm 6.2\%$), the expression of survivin in the mice treated with 5-ALA (positive cells: median, $35.8 \pm 4.8\%$) was not significantly different from that in the mice in the control group (positive cells: median, $35.3 \pm 6.7\%$), and the expression of CK20 was significantly

lower in the mice treated with 5-ALA (positive cells: mean $30.5 \pm 5.9\%$) than that in the mice in the control group (positive cells: mean $56.8 \pm 4.9\%$) (Fig. 10).

Discussion

The results of the present study demonstrated that 5-ALA inhibited the viability of bladder cancer cells, decreased the level of Fe^{2+} in mitochondria, and increased the expression of ferritin and the percentage of cells in the S phase of the cell cycle. Furthermore, by silencing ferrochelatase, which converts Fe^{2+} and protoporphyrin IX to heme (13), the effects

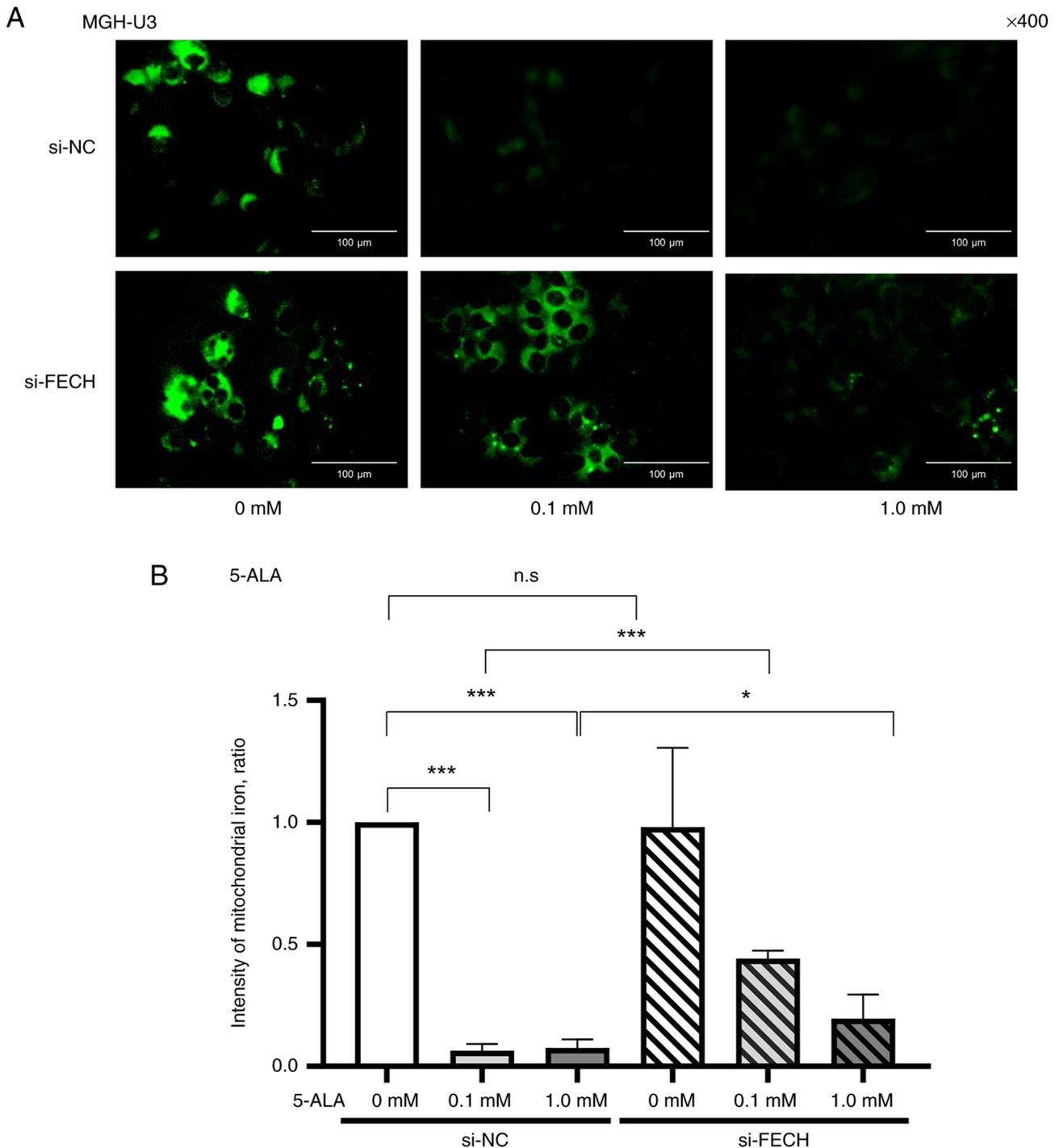


Figure 5. (A) Images and (B) intensity of mitochondrial Fe^{2+} in MGH-U3 cells with silenced ferrochelatase treated with 1 mM 5-aminolevulinic acid. * $P < 0.05$ and *** $P < 0.001$. si-, siRNA; NC, negative control; FECH, ferrochelatase; 5-ALA, 5-aminolevulinic acid; n.s, not significant.

of inhibition of viability by 5-ALA and the decreased level of Fe^{2+} in mitochondria by 5-ALA were reduced, and the increased expression of ferritin and percentage of cells in the S phase of the cell cycle were also decreased. These results indicated that 5-ALA inhibited the viability of bladder cancer cells by reducing iron in mitochondria through activation of heme synthesis. Previous studies (18,19) have shown the biological significance of heme metabolites, the mechanism of protoporphyrin IX accumulation in tumor cells, and the therapeutic potential of ALA-induced photodynamic therapy

alone and combined with hyperthermia and immunotherapy in cancer treatment. The present study, to the best of our knowledge, is the first to demonstrate the possibility of 5-ALA as an antitumor agent targeting iron in bladder cancer cells without the use of an iron chelator, and decreasing mitochondrial iron through the activation of heme synthesis and inhibiting bladder cancer cell viability.

Chelating iron in cancer cells has been reported to contribute to inhibition of DNA synthesis (1,20), S phase arrest (21,22), G1/S arrest (23), and cell proliferation. Additionally, in the

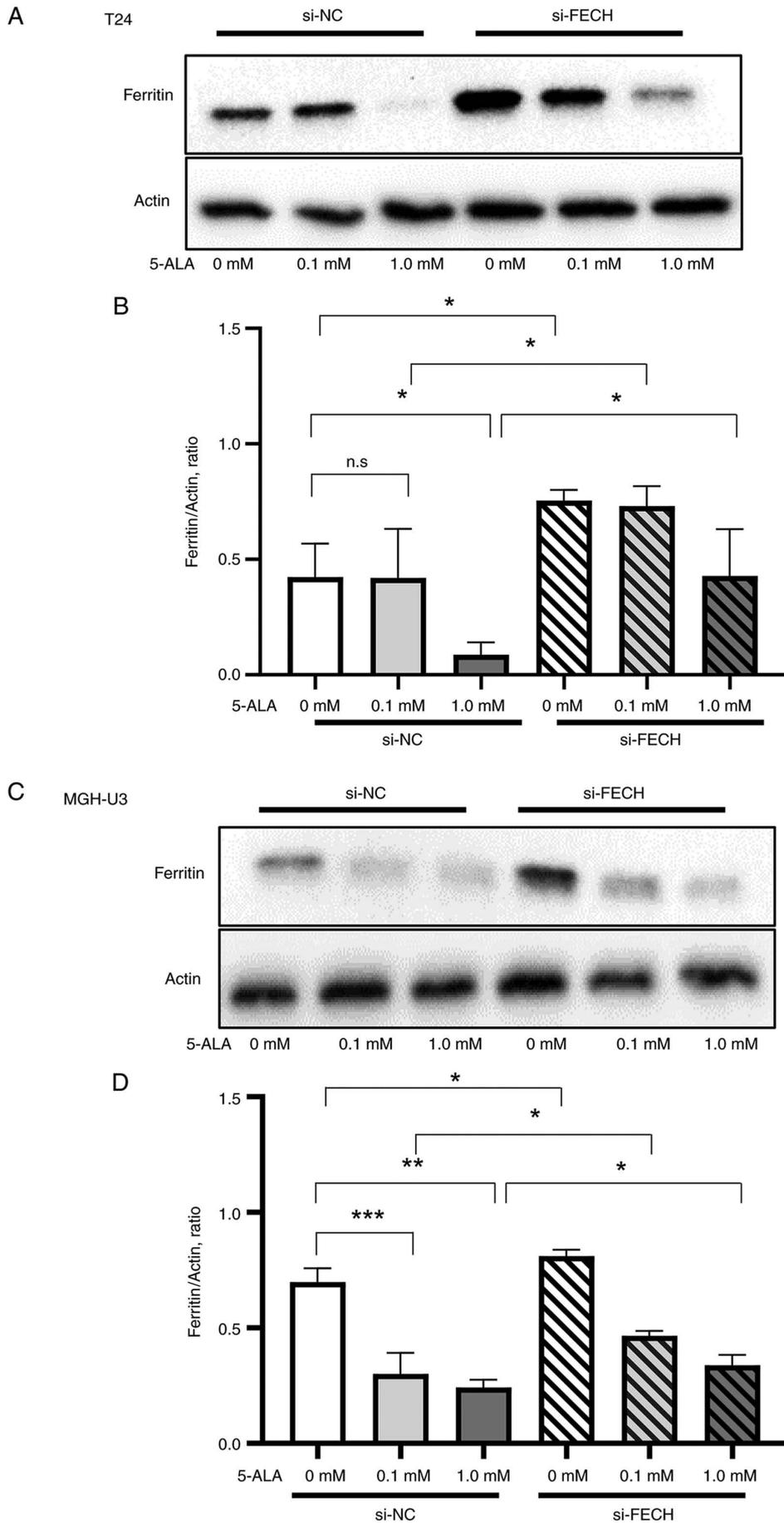


Figure 6. Images and expression of ferritin in (A and B) T24 and (C and D) MGH-U3 cells with silenced ferrochelatase treated with 5-aminolevulinic acid. *P<0.05, **P<0.01 and ***P<0.001. si-, siRNA; NC, negative control; FECH, ferrochelatase; 5-ALA, 5-aminolevulinic acid; n.s, not significant.

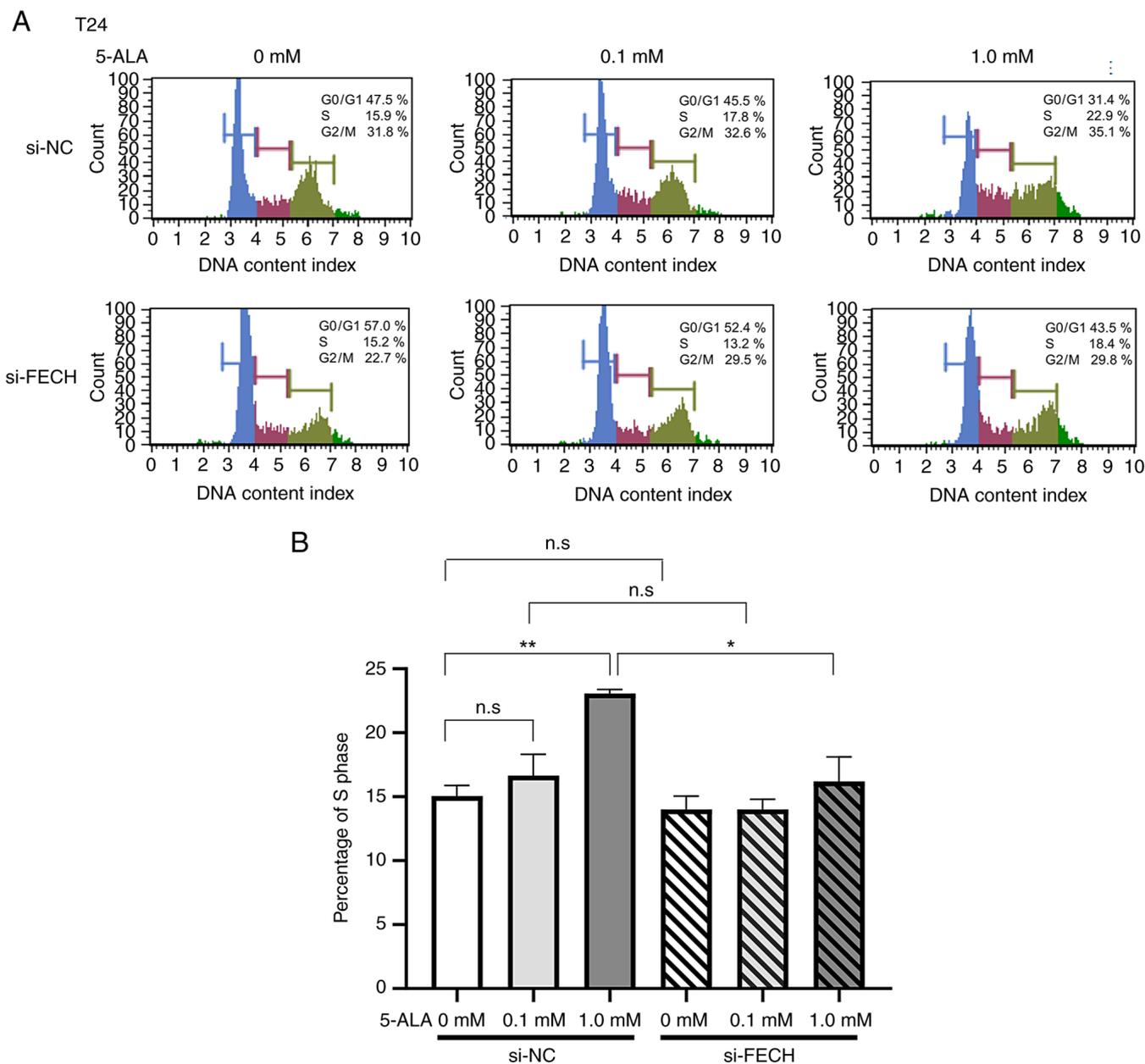


Figure 7. (A) Images of the cell cycle and (B) percentage of the S phase in T24 cells with silenced ferrochelatase treated with 5-aminolevulinic acid. * $P < 0.05$ and ** $P < 0.01$. si-, siRNA; NC, negative control; FECH, ferrochelatase; 5-ALA, 5-aminolevulinic acid; n.s, not significant.

present study, S phase arrest was observed upon treatment with 5-ALA, and by silencing ferrochelatase, S phase arrest induced by 5-ALA was weakened. These results indicated that 5-ALA activated heme synthesis in bladder cancer cells, which converted mitochondrial Fe^{2+} to heme resulting in inhibition of DNA synthesis and S phase arrest from inhibition of cell viability. The data support the theory that 5-ALA inhibits the viability of bladder cancer cells by reducing mitochondrial iron through activation of heme synthesis without the use of an iron chelator.

The expression of ferritin was weaker in the mice treated with 5-ALA than in those not treated with 5-ALA. Additionally, the expression of PCNA, which is specific to the S phase in cells, and not survivin, which is specific to the G2/M phase in cells, was higher in the mice treated with 5-ALA. Ferritin is a form of stored iron (1,6), and a decrease in ferritin results in a

shortage of iron in bladder cancer cells. Therefore, the results of the *in vivo* study were consistent with those of the *in vitro* study. Furthermore, bladder cancer was not found in two mice treated with 5-ALA. This phenomenon is not explained fully by inhibition of cell viability caused by 5-ALA. Ferritin protein has been reported to have a key role in nuclear factor- κB (24), which is a widely expressed transcription factor involved in cancer and related to cancer development and progression (25). Therefore, the effect of decreased ferritin by 5-ALA may have another antitumor effect that decreases the iron level in cancer cells, which particularly could have affected the *in vivo* results in the present study, because half of the mice treated with 5-ALA did not develop bladder cancer.

We had assumed that the mechanisms of the antitumor effect of 5-ALA on bladder cancer were due to photodynamic therapy (26) or apoptosis by oxidative phosphorylation (12).

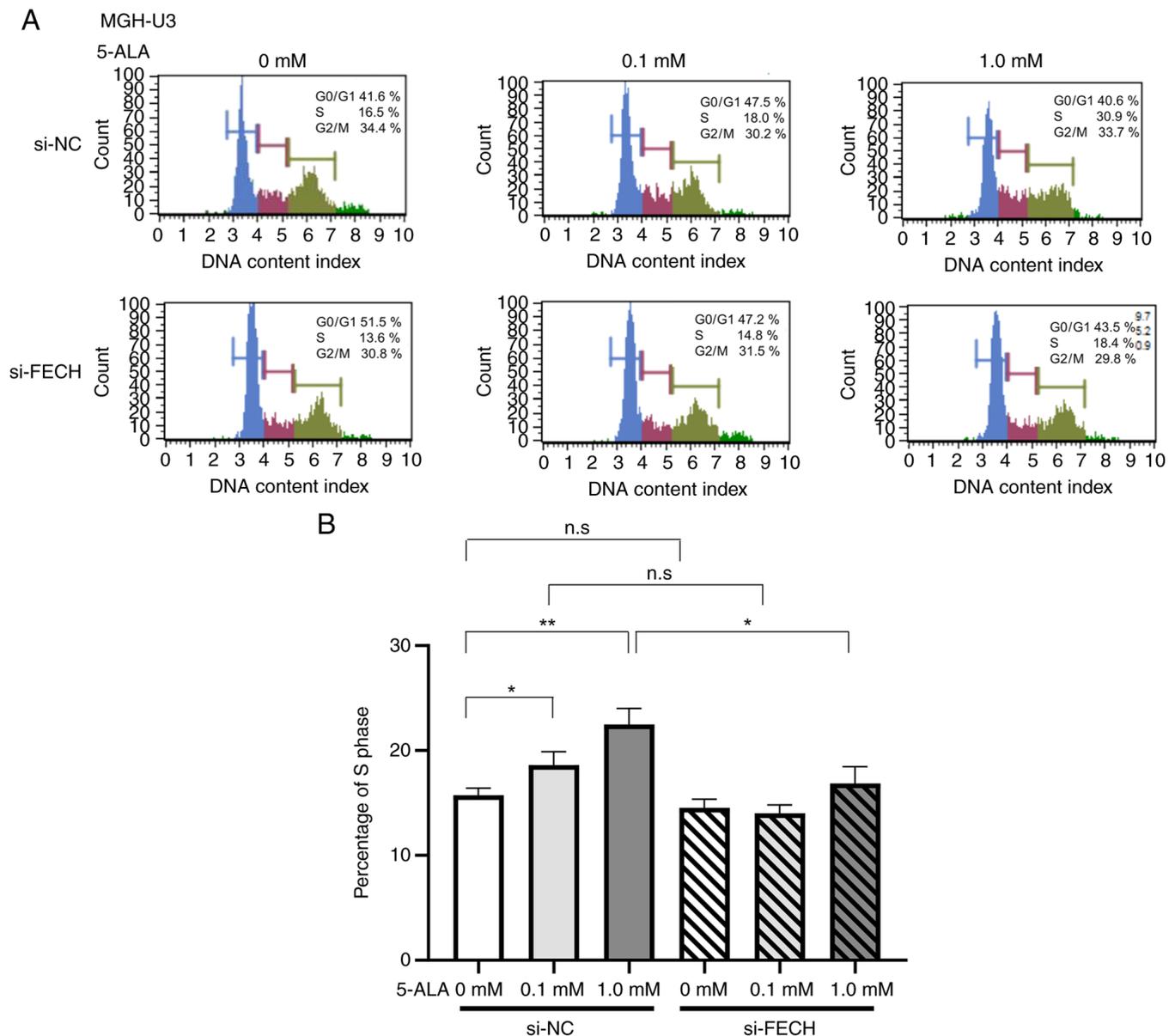


Figure 8. (A) Images of the cell cycle and (B) percentage of the S phase in MGH-U3 cells with silenced ferrochelatase treated with 5-aminolevulinic acid. * $P < 0.05$ and ** $P < 0.01$. si-, siRNA; NC, negative control; FECH, ferrochelatase; 5-ALA, 5-aminolevulinic acid; n.s., not significant.

However, when bladder cancer cells were treated with 5-ALA, apoptosis was not observed. The percentage of the late apoptotic cells was higher in T24 cells treated with 5-ALA than that in T24 cells without 5-ALA, but was not caused by apoptosis due to the same percentages of early apoptosis in the samples treated with or without 5-ALA even 6 h after ALA treatment (data not shown). Furthermore, the effect of photodynamic therapy was not observed in bladder cancer cells with suppressed ferrochelatase expression, although the effect of photodynamic therapy reportedly is enhanced by suppression of ferrochelatase (15). Therefore, the main mechanism underlying the antitumor effect for bladder cancer by 5-ALA is considered to mainly involve decreasing of mitochondrial iron through activation of heme synthesis and inhibiting bladder cancer cell viability.

The viability of T24 cells was higher than that of MGH-U3 cells, and the expressions of mitochondrial Fe^{2+} and ferritin in

T24 cells appeared to be higher than those in MGH-U3 cells. The expression of ferrochelatase was lower in T24 cells than that in MGH-U3 cells, being consistent with the finding of a previous study (8). This result indicated that heme synthesis is more defective in T24 cells than in MGH-U3 cells. Furthermore, the antitumor effect of 5-ALA appeared to be weaker in T24 cells than that in MGH-U3 cells. The results of the present study indicated that 5-ALA can be more effective in cells that express more ferrochelatase.

The present study has some limitations that should be considered. First, only two types of bladder cancer cell lines were evaluated. The effect of 5-ALA, which inhibited the viability of bladder cancer cells by activating heme synthesis, may be in various types of cancers, especially in cancers where chelating iron has been reported to exert an antitumor effect. Therefore, the present mechanism should be evaluated in different cancer cell lines in future studies. Second, the

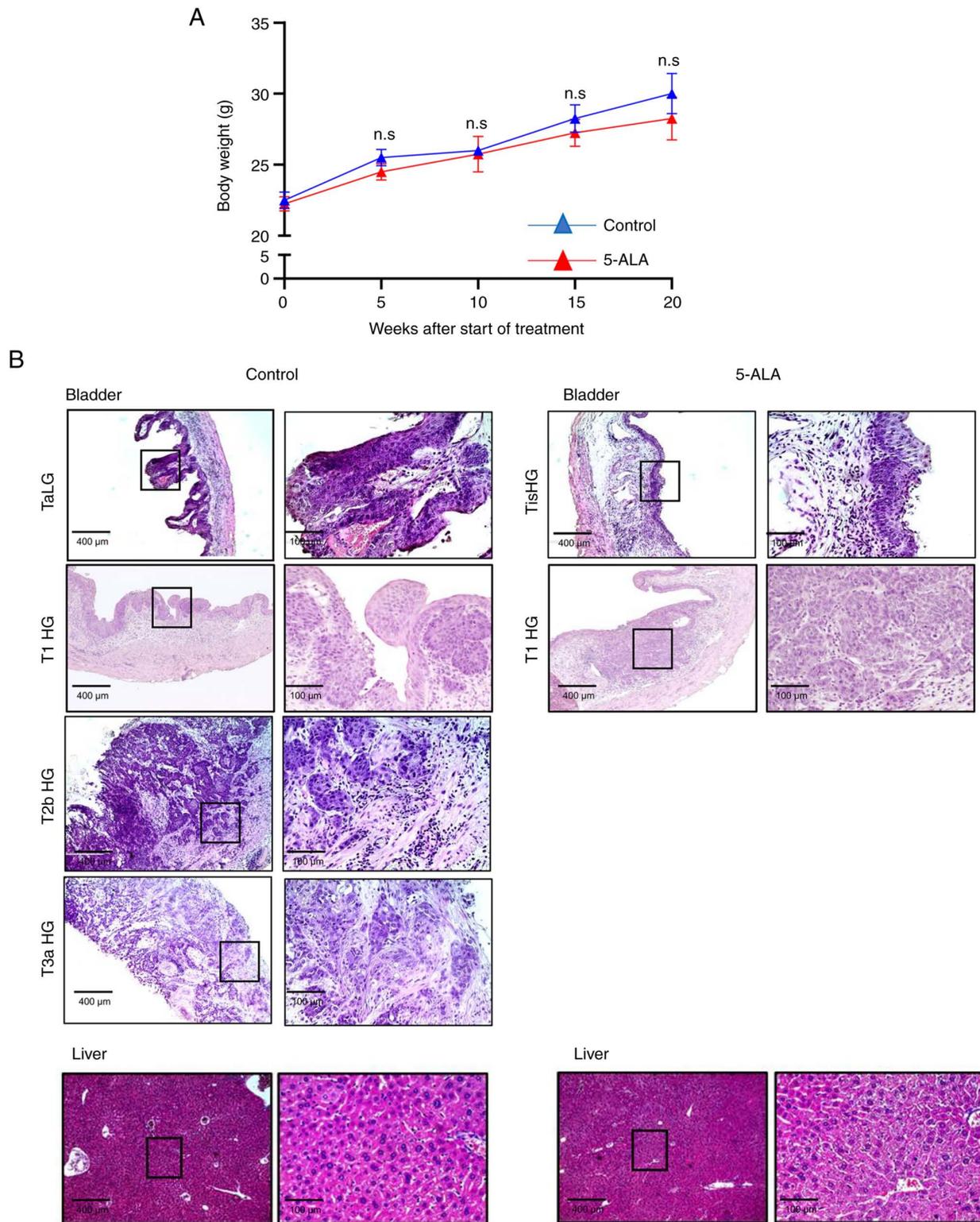


Figure 9. (A) Chronological change of weight in the control (blue) and 5-ALA (red) group. (B) Representative images of a hematoxylin and eosin-stained bladders of mice with bladder cancer, and a liver of a mouse with pT1 high grade (left, control group; and right, 5-ALA group). 5-ALA, 5-aminolevulinic acid; n.s, not significant; LG, low grade; HG, high grade.

number of mice used in the *in vivo* study was small. However, animal use guidelines recommend that the number of animals used in experiments be as limited as possible. Because the results showed significant differences between groups, an additional *in vivo* study was not performed.

In conclusion, the present study demonstrated that 5-ALA decreased iron levels in bladder cancer and inhibited bladder cancer cell viability. These results indicated that 5-ALA may potentially be used as a new anticancer agent for bladder cancer without the use of an iron chelator.

Table I. Pathological findings in the bladders of mice.

Treatment	T stage	Grade	LVI	INF	Body weight (g)	Bladder weight (g)
Control	a	Low grade	0	-	29	0.084
	1	High grade	0	c	30	0.088
	2b	High grade	1	c	32	0.168
	3a	High grade	1	c	29	0.201
5-ALA	No malignancy	-	-	-	30	0.082
	No malignancy	-	-	-	27	0.081
	CIS	High grade	0	-	27	0.099
	1	High grade	0	b	29	0.093

LVI, lymphovascular invasion; INF, pattern of tumor infiltration; 5-ALA, 5-aminolevulinic acid; CIS, carcinoma *in situ*.

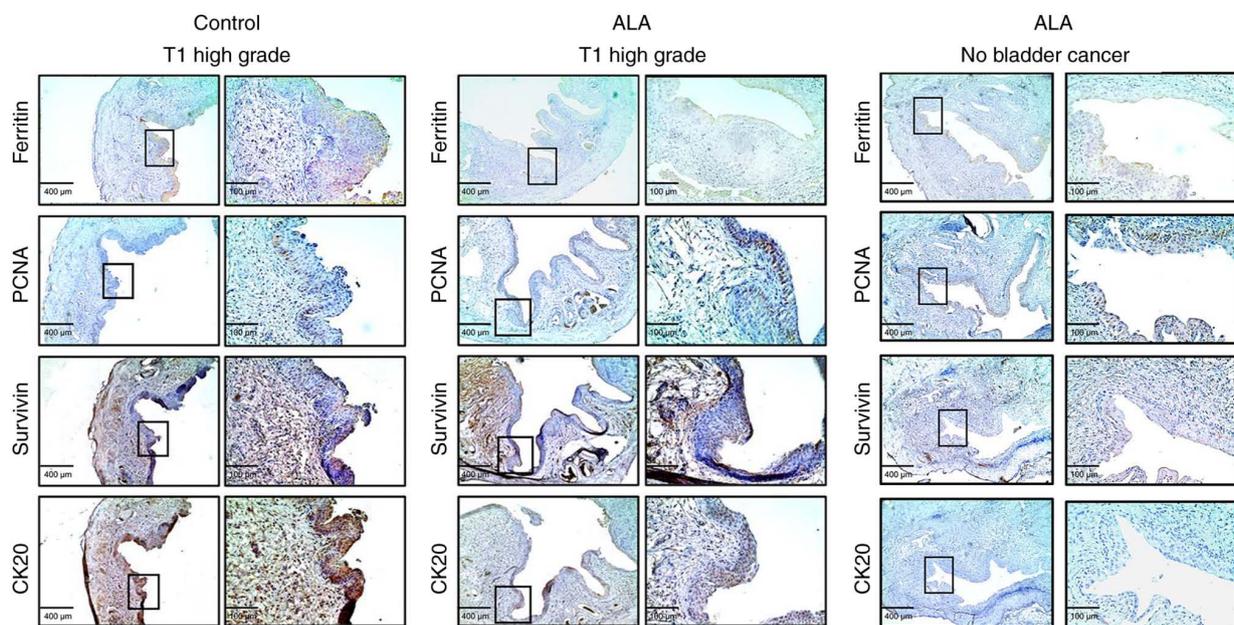


Figure 10. Expression of ferritin, proliferating cell nuclear antigen, survivin, and cytokeratin 20 in bladder tumors in the control group (images on the left) and 5-ALA group (images in the middle) and in a bladder without bladder cancer in the 5-ALA group (images on the right). PCNA, proliferating cell nuclear antigen; CK20, cytokeratin 20; 5-ALA, 5-aminolevulinic acid; Tis, tumor *in situ*.

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Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authors' contributions

YN and KF conceived and designed the study. YN, TO and SO performed the cell cytotoxicity assay and analyzed and interpreted the data. YT and TF performed the apoptosis analysis and analyzed and interpreted the data. YN, TO and YO performed the Fe^{2+} in mitochondria assay and analyzed and interpreted the data. SO performed the western blotting and transfection of small interfering RNA. TF and SO performed cell cycle assay and YN, TF and SO analyzed and interpreted the data. YN, SH, YM, KI, KO and MM performed the *in vivo* study and analyzed and interpreted the data. YN, NT and KF confirm the authenticity of all the raw data. YN wrote the original draft of the manuscript. KF and NT contributed to the writing, review and editing of the manuscript. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The animal study was approved by the Committee on Animal Research of Nara Medical University (approval no. 11921, 2017/2/23). All animal experiments were conducted in accordance with the Guidelines for Welfare of Animals in Experimental Neoplasia. This study did not include patient participation or analysis of patient data.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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