

# Low-Level Laser Therapy Promoted Aggressive Proliferation and Angiogenesis Through Decreasing of Transforming Growth Factor- $\beta$ 1 and Increasing of Akt/Hypoxia Inducible Factor-1 $\alpha$ in Anaplastic Thyroid Cancer

Yun-Hee Rhee, PhD,<sup>1</sup> Jeong-Hwan Moon, MD, PhD,<sup>1,2</sup> Sun-Hyang Choi, BS,<sup>3</sup> and Jin-Chul Ahn, PhD<sup>4,5</sup>

## Abstract

**Objective:** We assessed the cause of increased tumor after low-level laser therapy (LLLT) by histological analysis. **Background data:** LLLT is a nonthermal phototherapy used in several medical applications, including wound healing, reduction of pain, and amelioration of oral mucositis. We discovered by accident that LLLT increased tumor size while testing a photodynamic therapy (PDT) model for the treatment of thyroid cancer. Although therapeutic effects of LLLT on cancer or dysplastic cells have been studied, LLLT has been recently reported to stimulate the aggressiveness of the tumor. **Methods:** The anaplastic thyroid cancer cell line FRO was injected into thyroid glands of nude mice orthotopically and then laser irradiation was performed with 0, 15, and 30 J/cm<sup>2</sup> (100 mW/cm<sup>2</sup>) on the thyroid after 10 days. The tumor volume was measured for 4 weeks and the thyroid tissues underwent histological analysis. We observed that proliferation of FRO cells and macrophage infiltration was increased with energy delivery to the thyroid glands. We also assessed overproliferated FRO cells using an immunohistochemical staining with hypoxia inducible factor 1 $\alpha$  (HIF-1 $\alpha$ ), p-Akt, vascular endothelial growth factor (VEGF), and transforming growth factor  $\beta$ 1 (TGF- $\beta$ 1). **Results:** HIF-1 $\alpha$  and p-Akt were elevated after LLLT, which suggested that the phosphorylation of Akt by LLLT led to the activation of HIF-1 $\alpha$ . Moreover, TGF- $\beta$ 1 expression was decreased after LLLT, which led to loss of cell cycle regulation. **Conclusions:** In conclusion, LLLT led to a decrease in TGF- $\beta$ 1 and increase of p-Akt/HIF-1 $\alpha$  which resulted to overproliferation and angiogenesis of anaplastic thyroid carcinoma (ATC). Therefore, we suggest that LLLT can influence cancer aggressiveness associated with TGF- $\beta$ 1 and Akt/HIF-1 $\alpha$  cascades in some poorly differentiated head and neck cancers.

## Introduction

LOW-LEVEL LASER THERAPY (LLLT) has been reported to modulate the process of tissue repair by stimulation of cellular reactions such as migration, proliferation, and cell differentiation. Most of studies of LLLT have been reported to have suppressive effect on cancer cells;<sup>1–4</sup> however, LLLT also has been reported to modify cell behavior and proliferation in dysplastic lesions.<sup>5–7</sup> Moreover, head and neck cancer patients receiving LLLT for oral mucositis may have residual tumor cells that will be stimulated by LLLT.<sup>8</sup> Thyroid cancer is the most frequent endocrine cancer, and poorly differentiated anaplastic thyroid carcinoma (ATC) cell lines respond by increased telomerase activity if they harbor a p53 mutation.<sup>9,10</sup> Therefore, transforming growth factor  $\beta$ 1 (TGF- $\beta$ 1) exerts

opposing effects on telomerase activity in ATC cell lines, possibly reflecting deregulation of TGF- $\beta$ 1 signaling in more malignant genotypes.<sup>11,12</sup> Interestingly, we found that ATC cells had aggressive proliferation responses after laser irradiation when performing photodynamic therapy of thyroid tumors. We hypothesize that LLLT led to elevated cell proliferation caused by increased stability of hypoxia inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ), and a reduction of TGF- $\beta$ 1 in ATC cells. In this study, we evaluate the effects of laser irradiation on ATC and discuss how LLLT leads to hyperplasia of thyroid cancer using an *in vivo* study. We performed orthotopic injection of FRO cells into the murine thyroid gland, and analyzed alterations of key thyroid cancer proteins by immunohistochemical (IHC) staining to detect HIF-1 $\alpha$ , p-Akt, vascular endothelial growth factor (VEGF), and TGF- $\beta$ 1.

<sup>1</sup>Beckman Laser Institute Korea, Dankook University, Cheonan, Chungnam, Republic of Korea.

<sup>2</sup>Department of Otolaryngology-Head and Neck Surgery, College of Medicine, Dankook University, Cheonan, Republic of Korea.

<sup>3</sup>Department of Medical Laser, Graduate School, Dankook University, Cheonan, Republic of Korea.

<sup>4</sup>Medical Laser Research Center, Dankook University, Cheonan, Republic of Korea.

<sup>5</sup>Department of Biomedical Science, College of Medicine, Dankook University, Cheonan, Republic of Korea.

## Materials and Methods

### Chemicals

RPMI1640 and phosphate-buffered saline (PBS) were purchased from WelGENE (Daejeon, Korea). Trypsin, fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Hyclone (South Logan, UT). TGF- $\beta$ 1, HIF- $\alpha$ , and phospho-Akt were purchased from cell signaling (Beverly, MA). Hematoxylin and eosin (H&E) were purchased from IHC world (Woodstock, MD). For IHC staining, ABC kit and 3,3'-diaminobenzidine (DAB) substrate were purchased from Vector (Burlingame, CA).

### Cell culture

The human anaplastic thyroid cell line FRO was donated from the Department of Endocrinology, Chungnam National University. Cells were maintained in RPMI 1640 containing 10% FBS with 1% penicillin streptomycin.

### In vivo study

Six-week-old female athymic nude mice were purchased from Orient Bio (Suwon, Korea) ( $n=30$ ) and all animal procedures used in the present study were performed according to the institutional review board (IRB), which was approved by the Ethics Committee of Dankook University. To evaluate the effect of nonthermal laser irradiation (LLT) on tumor volume, FRO cells were injected into the thyroid gland with  $1 \times 10^6$  cells per mouse orthotopically and the mice were divided into three groups ( $n=10$  each). Tumor sizes were measured with calipers for 1 month. After 10 days, tumor growth was confirmed and irradiated with 0, 15, and 30 J/cm<sup>2</sup> (100 mW/cm<sup>2</sup>). The tumor sizes were measured with a caliper for 1 month. The tumor volume was calculated according to the formula  $V=0.25a^2b$ , where  $a$  is the smallest superficial diameter and  $b$  is the largest superficial diameter. After 1 month, mice were euthanized and thyroid tissues were fixed with 10% neutral buffered formalin.

### Laser operation

The light source was a 650 nm diode laser (WON Technology Co., Ltd, Deajeon, Korea). The elliptical fiber shape of the diode laser output had a diameter of 1.7 mm. The laser device information is described in Table 1. The irradiance at the surface of the cell monolayer was measured by a power meter (FieldMAXII, Coherent Inc. Santa Clara, CA). We attached supplementary data of the laser diode (See supplementary Fig. S1 at [www.liebertpub.com/pho](http://www.liebertpub.com/pho)) and the irradiation parameter evaluation is provided in Table 2. The irradiance or power density was measured as 100 mW/cm<sup>2</sup> and the operating mode was continuous wave. The light

TABLE 1. DEVICE INFORMATION

Manufacturer	Wontech Co. Ltd., Daejeon, Republic of Korea
Year produced	2012
Number of emitters	1
Emitter type	Laser diode
Spatial distribution of emitter	none
Beam delivery system	Hand-held probe

TABLE 2. IRRADIATION PARAMETERS

Parameter (unit)	Information source
Center wavelength (nm)	650
Spectral bandwidth (nm)	650 $\pm$ 10
Operating mode	Continuous wave (CW)
Aperture diameter (mm)	1.7
Irradiance at aperture (mW/cm <sup>2</sup> )	100
Beam shape	Circular
Beam profile	Gaussian

dose measure for energy density or fluence was 15 or 30 J/cm<sup>2</sup>, with the duration of each treatment set at 150 and 300 sec. This irradiation treatment was delivered once 10 days after FRO cell orthotopic injection into the murine thyroid gland. Total radiant energy was 0.3 and 0.6 J. The LLLT conditions are described in Table 3.

### Histology

Fixed tissues were dehydrated by processing with xylene and gradient alcohol. Samples were embedded in paraffin blocks and sliced into 5  $\mu$ m thick sections using a microtome (Leica, Buffalo Grove, IL). The sections were stained in Harris hematoxylin solution for 2 min, then differentiated in 1% acid alcohol after deparaffinizing. The sections were rinsed with tap water and counterstained in eosin Y solution. The sections were observed by microscope and photographed after mounting.

### IHC staining

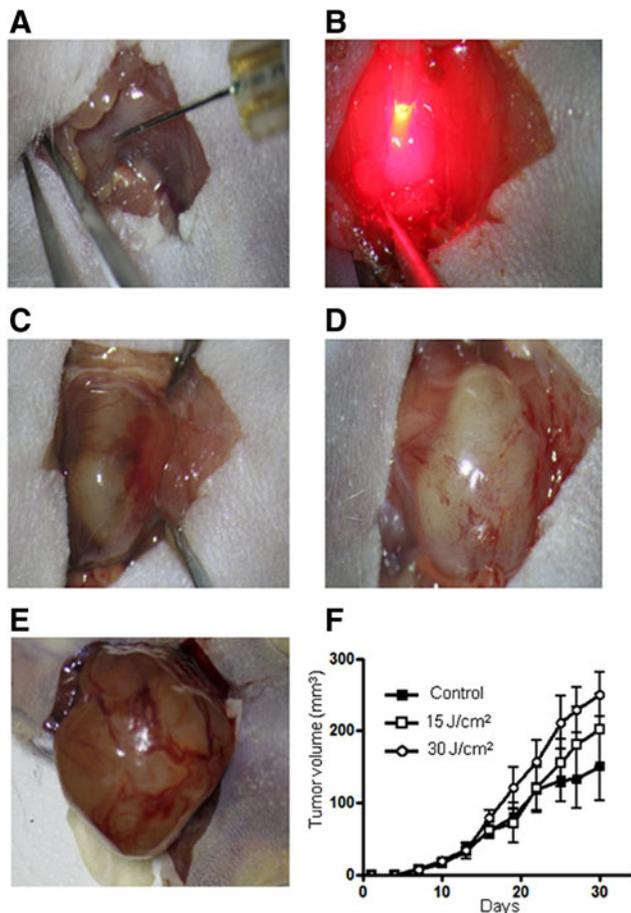
The sections were blocked with 1% normal horse serum and then incubated with specific antibodies to HIF-1 $\alpha$  (1:100), VEGF (1:100), phospho-Akt (1:100), and TGF- $\beta$ 1 (1:50) for overnight at 4°C in a humidified chamber. All sections were then incubated in a 1:100 dilution of biotinylated anti-rabbit immunoglobulin (Ig)G and detected with DAB substrate. The sections were all counterstained with hematoxylin except TGF- $\beta$ . The TGF- $\beta$  samples were detected with nickel-added DAB substrate and counterstained with H&E.

### Statistical analysis

The results are expressed as mean $\pm$ SD. Unpaired Student's  $t$  tests were used for comparisons between two means.

TABLE 3. TREATMENT PARAMETERS

Parameter (unit)	Value
Beam spot size at target (cm <sup>2</sup> )	0.02
Irradiance at target (mW/cm <sup>2</sup> )	100
Exposure duration (sec)	150, 300
Radiant exposure (J/cm <sup>2</sup> )	15, 30
Radiant energy (J)	0.3, 0.6
Number of points irradiated	1
Area irradiated (cm <sup>2</sup> )	0.02
Application technique	Epithelial contact
Number and frequency of treatment sessions	1 treatment delivered
Total radiant energy (J)	0.3, 0.6



**FIG. 1.** (A) FRO anaplastic thyroid cancer cells were injected into the thyroid gland orthotopically. (B) The light source was a diode laser with output of 650 nm, and irradiation was assessed at different time intervals (0, 15, 30 J/cm<sup>2</sup>, 25 mW) on murine thyroid glands at 10 days after injection of anaplastic thyroid carcinoma (ATC) cells. (C) FRO cells only. (D) FRO +15 J/cm<sup>2</sup>. (E) FRO +30 J/cm<sup>2</sup>. (F) The tumor sizes were measured every 2–3 days with calipers for 1 month. The results are expressed as mean ± SD.

The comparisons among the three groups were performed by one way ANOVA, Kruskal–Wallis test (Graph Pad, Prism<sup>®</sup>, La Jolla, CA).  $p < 0.05$  and  $p < 0.0001$  were considered statistically significant.

## Results

As shown in Fig. 1, tumor volumes of the LLLT groups were increased compared with controls, and were proportional to LLLT irradiation energy delivery.

When evaluated by histological analysis with H&E staining, ATC cell proliferation was increased after LLLT exposure, and macrophage infiltration also was increased at higher dosages. Further, hemolysis and necrotic regions were detected by microscopy at the margins of macrophage infiltrated areas after 30 J/cm<sup>2</sup> LLLT (Fig. 2, red arrow).

To assess whether HIF-1 $\alpha$  was increased after LLLT as a result of hypoxia, we performed the IHC staining with HIF-1 $\alpha$  and phospho-Akt. As shown by microscopy in Figs. 3 and 4, HIF-1 $\alpha$  and phospho-Akt were elevated after

LLLT in a dose-dependent manner. The phosphorylation of Akt also influences expression of HIF-1 $\alpha$ , which induces the proangiogenic factor VEGF. Expression of VEGF was also increased after LLLT (Fig. 5). VEGF expression was more intense and concentrated in smaller regions at 15 J/cm<sup>2</sup> LLLT, but VEGF expression was more often found at 30 J/cm<sup>2</sup>.

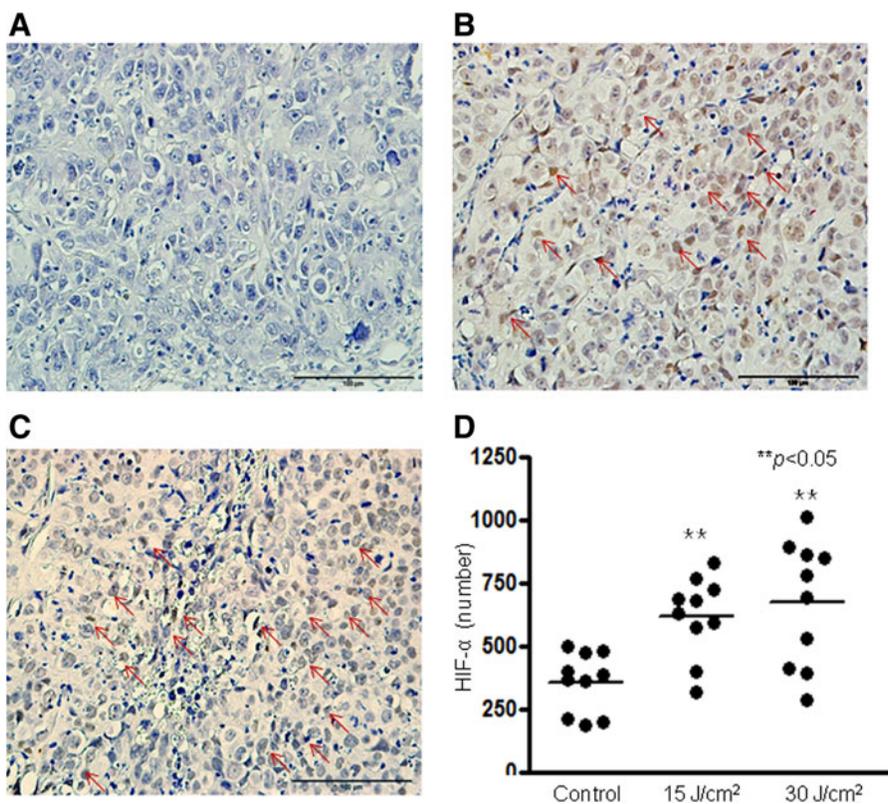
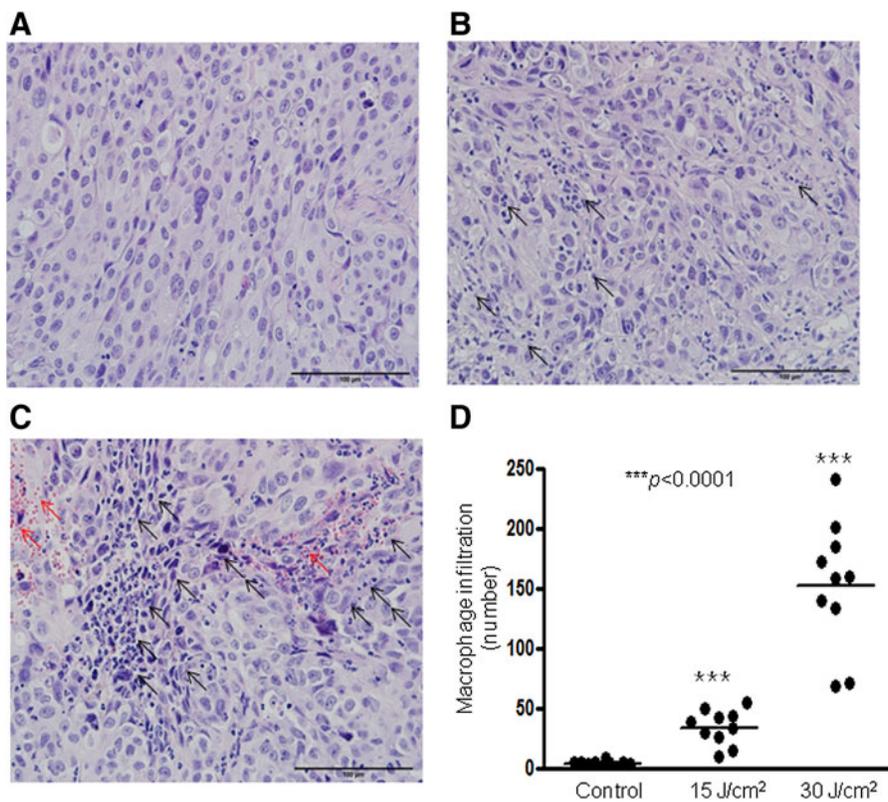
Meanwhile, TGF- $\beta$  was decreased after LLLT and was light dose dependent (Fig. 6). TGF- $\beta$ 1 was expressed on some cells in the control group (gray spots), whereas TGF- $\beta$ 1 expression was decreased after LLLT at an energy density of 30 J/cm<sup>2</sup>. Our results suggest that decrease of TGF- $\beta$ 1 led to overproliferation of ATC cells after low-level laser treatment.

## Discussion

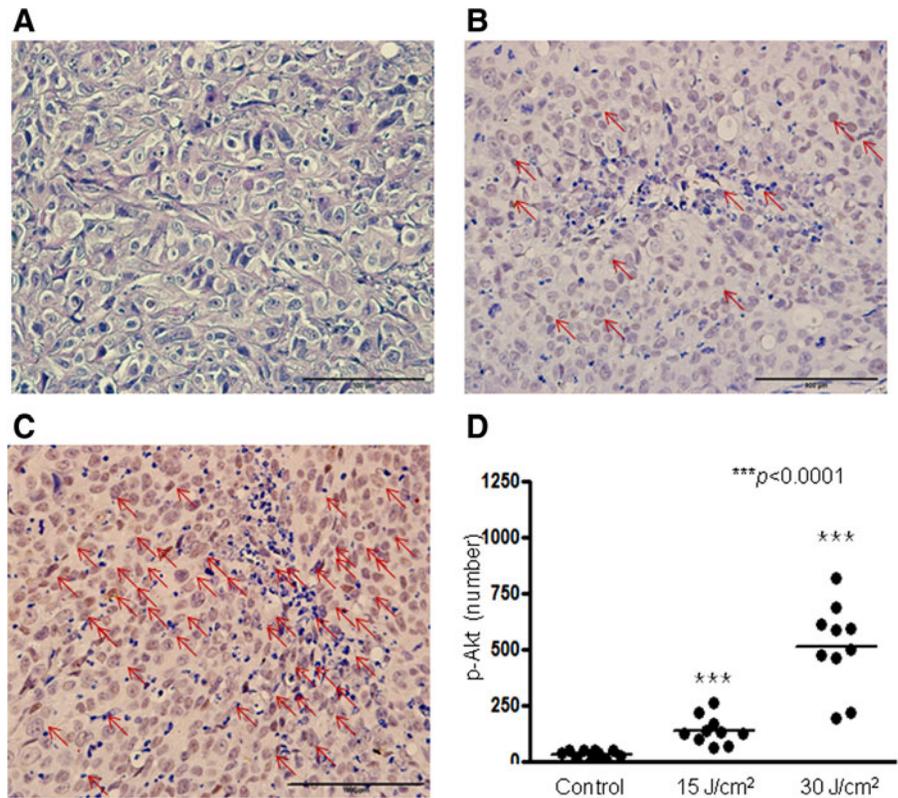
At first, we planned to develop an orthotopic photodynamic therapy *in vivo* model of thyroid cancer using an FRO cell line; the poor differentiation type of thyroid cancer. We injected FRO cells into the murine thyroid gland orthotopically and irradiated with 650 nm light from a diode laser at a low energy density of 15 and 30 J/cm<sup>2</sup> as laser control groups. By accident, we observed that the tumor volume was increased more rapidly in the LLLT groups than in the untreated control group (Fig. 1). This tumor overproliferation appeared proportional to increases in energy dose ( $p = 0.057$ ). Moreover, we could not analyze the data at a higher dose (60 J/cm<sup>2</sup>) because tumor growth was increased so rapidly that it progressed to tissue necrosis. We were surprised by this phenomenon because this observation was in great contrast with numerous data published. We searched the experimental study of effects of laser therapy on malignant tumors and discovered some studies supporting our observation. de C Monteiro et al. reported that earlier laser phototherapy (660 nm) induced oral chemical carcinogenesis in the hamster cheek pouch model through histological study,<sup>5</sup> and Sperandio and colleagues suggested that low-level therapy can produce increased aggressiveness of dysplastic and oral cancer cell lines by modulations of the Akt/molecular target of rapamycin (mTOR) signaling pathway.<sup>8</sup> Therefore, we decided to evaluate differences in cell proliferation between untreated tumor controls and LLLT illuminated tumors by histology. As shown in Figure 2, tumor cell proliferation was increased after irradiation and prominent macrophage infiltration was observed by microscopy following H&E staining. The cell morphology was irregular and dense after LLLT compared with the control group. There was a hemolysis region identified in the 30 J/cm<sup>2</sup> irradiated group (red arrow in Fig. 2C), which demonstrated that the aggressive cell proliferation induced an acute inflammation environment.

Whether overproliferation of FRO cells after irradiation is the result of the survival transcription or growth factors, we assessed the IHC staining with HIF-1 $\alpha$ , p-Akt, VEGF, and TGF- $\beta$ 1. Because aggressive proliferation of tumors occurs under hypoxic conditions of tissue for angiogenesis, we observed the expression of HIF-1 $\alpha$  of thyroid tissue. As shown in Fig. 3, the expression of HIF-1 $\alpha$  was dose dependently increased with the energy dose.

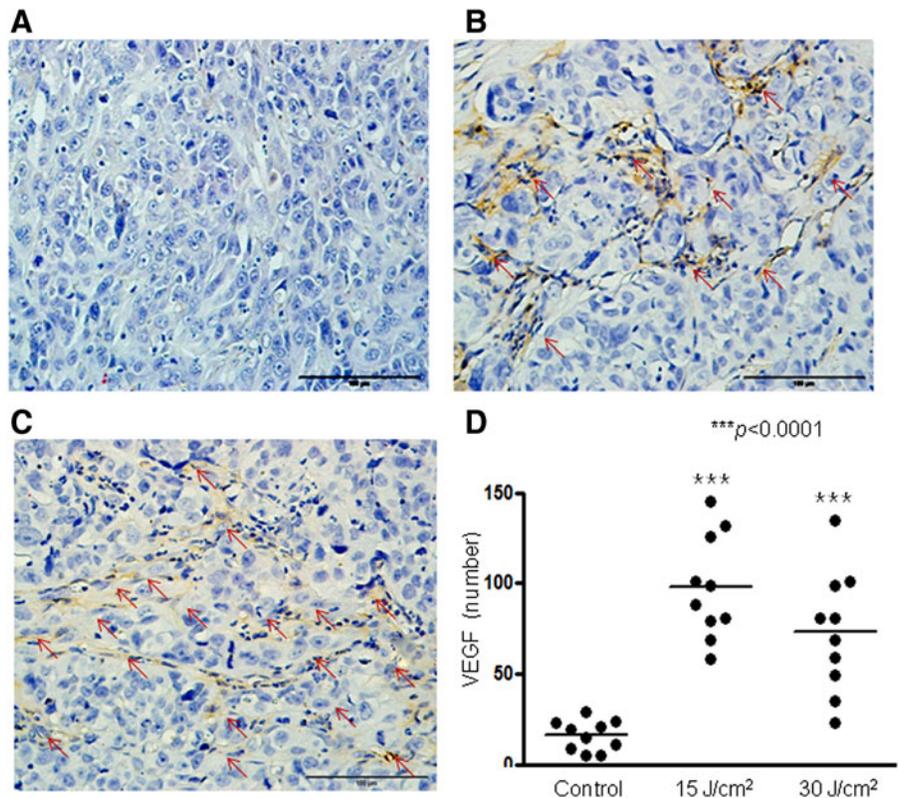
Hypoxia is a manifestation of cellular stress in all mammalian cells, and also is a hallmark of aggressive growth by

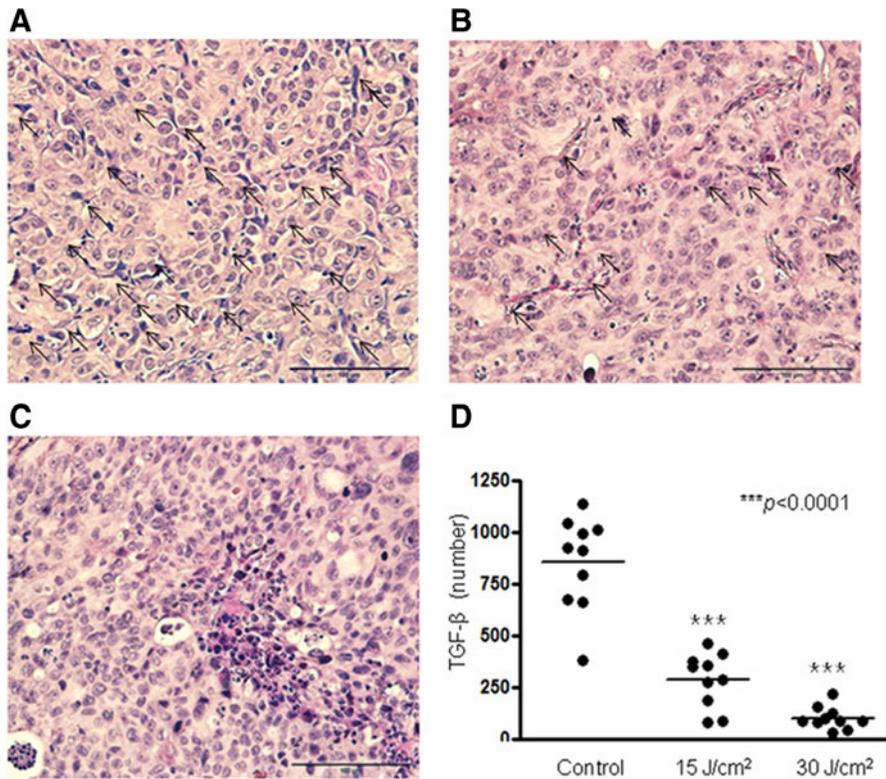


**FIG. 4.** The sections underwent immunohistochemical staining with antibody to p-Akt and detected by biotinylated antiglobulin with 3,3'-diaminobenzidine (DAB) substrate. (A) FRO only. (B) FRO +15 J/cm<sup>2</sup>. (C) FRO +30 J/cm<sup>2</sup>. (D) Measurement of positive p-Akt cell numbers. The results are expressed as mean ± SD. Arrows, positive p-Akt.



**FIG. 5.** The sections underwent immunohistochemical staining with antibody to vascular endothelial growth factor (VEGF) and detected by biotinylated antiglobulin with 3,3'-diaminobenzidine (DAB) substrate. (A) FRO only. (B) FRO +15 J/cm<sup>2</sup>. (C) FRO +30 J/cm<sup>2</sup>. (D) Measurement of positive VEGF cell numbers. The results are expressed as mean ± SD. Arrows, positive VEGF.





**FIG. 6.** The sections underwent immunohistochemical staining with antibody to transforming growth factor (TGF)- $\beta$ 1 and detected by biotinylated antiglobulin with nickel-added 3,3'-diaminobenzidine (DAB) substrate. (A) FRO only. (B) FRO +15 J/cm<sup>2</sup>. (C) FRO +30 J/cm<sup>2</sup>. (D) Measurement of positive TGF- $\beta$ 1 cell numbers. The results are expressed as mean  $\pm$  SD. Arrows, positive TGF- $\beta$ 1.

solid tumor cells, wherein they acquire the capacity to survive and proliferate at conditions of low oxygen tension.<sup>13</sup> In responding to hypoxia, each of these heterodimeric protein transcription factors translocate to the nucleus and activate transcription of genes involved in cellular and physiologic adaptation to a low oxygen supply. As shown in Fig. 4, the phosphorylation of Akt was elevated after LLLT and these tumorigenic pathways may stimulate different downstream signaling elements, including phosphatidylinositol 3'-kinase (PI3K)/Akt/mTOR pathways that in turn upregulate the expression and stability of the HIF-1 $\alpha$  subunit.<sup>14,15</sup> A direct and important role of hypoxia in survival and progression of solid tumors is to stimulate tumor angiogenesis induced through HIF-1 $\alpha$  expression of proangiogenic factors, such as VEGF and Akt.<sup>16,17</sup> The expression of VEGF also was increased after LLLT (Fig. 5). Nevertheless, the VEGF-positive spot numbers were lower and the stained area observed in the 30 J/cm<sup>2</sup> -irradiated group was larger than that in the 15 J/cm<sup>2</sup> group. Hypoxia causes secretion of VEGF, an angiogenic/permeability factor, as well as inducing formation of new vessels. VEGF is the key response to hypoxia that induces angiogenesis and cancer formation; it is strongly correlated with the disrupted circulation and rapid growth of cancer.<sup>15,16</sup>

TGF- $\beta$ 1 regulates cell proliferation, growth, differentiation, and cell movement.<sup>12,17,18</sup> TGF- $\beta$ 1 causes synthesis of p15 and p21 proteins, which block the cyclin: cyclin-dependent kinase (CDK) complex.<sup>19</sup> TGF- $\beta$  also suppresses expression of c-myc, a gene that is involved in G1 cell cycle progression.<sup>17</sup> Therefore, we decided to observe the expression of TGF- $\beta$ 1 after LLLT irradiation in thyroid cancer cells. The expression of TGF- $\beta$ 1 was dose dependently de-

creased after LLLT (gray spots around the nucleus) (Fig. 6). Interestingly, the poorly differentiated ATC cell lines responded with increased telomerase activity and harbored a p53 mutation, which allowed TGF- $\beta$ 1 to exert opposing effects on telomerase activity in ATC.<sup>11</sup> This may reflect the important role of TGF- $\beta$ 1 in cell cycle regulating in malignant carcinomas. Consequently, the inhibition of TGF- $\beta$ 1 by irradiation-modulated macrophages and vessel maturation was seen respectively in Figs. 2 and 5.

These results suggest that overproliferation of ATC was caused by inhibition of TGF- $\beta$ 1 and stabilization of HIF- $\alpha$ . Also, it was observed that TGF- $\beta$ 1 has a biphasic function in tumorigenesis: a growth-inhibitory effect in the early stage. Therefore, additional studies are needed to better understanding the role of TGF- $\beta$ 1 on ATC after LLLT.

## Conclusions

In conclusion, LLLT promoted decrease of TGF- $\beta$ 1 and increase of Akt/HIF-1 $\alpha$ , which caused aggressive proliferation and angiogenesis in ATC. This provides unexpected new evidence that low-level laser treatment might be an unsafe procedure in head and neck cancer patients.

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#### Author Disclosure Statement

No competing financial interests exist.

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Address correspondence to:

*Jin-Chul Ahn*  
*Medical Laser Research Center*  
*Dankook University*  
*Cheonan*  
*Republic of Korea 31116*  
*E-mail: jcahn@dankook.ac.kr*