

Photodynamic Therapy in Combination with Green Tea Polyphenol EGCG Enhances Antitumor Efficacy in Human Papillomavirus 16 (E6/E7) Immortalized Tumor Cells

Mohammad Raish^{1,2}

Syed Zeaul Abrar Husain³

Su Mi Bae¹

Sei Jun Han⁴

Choong Hak Park⁵

Jong Chul Shin⁶

Woong Shick Ahn⁵

¹ Catholic Research Institute of Medical Science,
The Catholic University of Korea.

² Department of Pharmaceutics, Collage of Pharmacy,
King Saud University, Riyadh, Kingdom of Saudi Arabia.

³ Faculty of Pharmacy, Jamia Hamdard New Delhi-110062.

⁴ Department of Obstetrics and Gynecology,
College of Medicine, Chosun University, Gwangju.

⁵ Department of Obstetrics and Gynecology,
College of Medicine, Dankook University, Cheonan.

⁶ Department of Obstetrics and Gynecology,
College of Medicine, The Catholic University of Korea, Seoul, Korea

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ABSTRACT

A constituent of green tea, (-)-epigallocatechin-3-gallate (EGCG), or polyphenol (GTP) has been known to possess anti-cancer properties. Our goal was to investigate the enhanced anti-tumor effects of photodynamic therapy (PDT) plus green tea extract in TC-1 tumor cells implanted into mice. The MTT assay, Western blot analysis and tumor growth inhibition study were evaluated at various time intervals after the PDT

plus EGCG or GTP. Following Radachlorin injection after 3 hr, the tumors were then treated with external light treatment (300 J/cm²), and then, EGCG or GTP was administered daily for 20 days. PDT or EGCG was found to be more effective compared to control groups. Moreover, the PDT combined with EGCG demonstrated a significant suppression of tumor growth in vitro and in vivo. The tumor growth by the PDT combined with EGCG was significantly reduced. The PDT combined with GTP was also suppressed the tumor size compared with the control group. However, the tumor volume after EGCG treatment was significantly

suppressed compared to GTP. In addition, EGCG lowered the uptake of Radachlorin. Thus, an EGCG supplement prior to photodynamic irradiation is not desirable. The PDT combination with EGCG in vitro and in vivo led to a significant decrease in levels of COX-2 at 48 hr post PDT plus EGCG combination treatment. These data suggest that EGCG plus PDT can induce a significant tumor suppression response compared with PDT alone. Also, it can be an effective approach to induce anti-cancer therapy strategy.

INTRODUCTION

Photodynamic therapy (PDT) is a method of treating malignant tumors based on the photodynamic damage of tumor cells resulting from a photochemical reaction.^{1,2} Several chemicals are used as photosensitizers in PDT for a wide range of malignant tumors, as well as nonmalignant diseases. A tumor treated by PDT is reabsorbed and gradually substituted by connective tissue. The location of photodynamic damage in a tumor is provided by the photosensitizer's ability to accumulate in tumor tissues and by the direction and location of precise laser irradiation. PDT has been successfully used in the treatment of a variety of cancers to induce apoptosis in tumor cells.³⁻¹⁰ PDT causes the photochemical generation of cytotoxic reactive oxygen species such as singlet oxygen within the target tissue. PDT clinical trials using a photosensitizer, as well as a variety of second-generation photosensitizer, have shown promise in treating malignancies of the esophagus, bronchus, brain, peritoneal cavity, skin, bladder, head and neck, as well as in treating nononcologic disorders such as age-related macular degeneration.^{11,12} Clinical results of PDT are normally positive. However, use of this technology requires further improvements as tumor recurrences can occur.¹¹⁻¹³ This may be due to the increase of cyclooxygenase (COX)-2, HIF-1 α , or VEGF gene expression. Recently, it has been shown that inhibitors of cyclooxygenase (COX)-2, HIF-1 α , or VEGF can be effective in combination with

PDT therapy, and that Green tea extract may play a major role in suppression of these, potentially harmful, genes¹⁴

The Green tea polyphenols have been shown to have a protective effect with prostate cancer in pre-clinical animal models. It has also been reported to be effective in several other cancer types as well.¹⁵⁻¹⁸ Green tea is composed of several catechins, including (-)-Epigallocatechin-3-gallate (EGCG), epicatechin (EC), epicatechin-3-gallate (ECG), and epigallocatechin (EGC). Among them, (-)-Epigallocatechin-3-gallate (EGCG), the major catechin found in green tea, has been recognized as a potential therapeutic agent.¹⁹⁻²⁹ Tea polyphenols have been shown to inhibit carcinogenesis in many animal models, and the significance of catechins, the main constituents of green tea, which has been shown to be important for cancer prevention.¹⁹⁻²⁴

Interest in the study of EGCG as an anticancer agent has increased in recent years due to their effects in vitro and in vivo on tumor cell signaling pathways regulating growth and apoptosis, including the suppression of cyclooxygenase (COX)-2, HIF-1 α , and VEGF gene expression.³⁰⁻³⁶

In this study, we evaluated the effectiveness of the PDT plus EGCG or GTP on tumor regression in vitro and in vivo, which showed that the PDT combined with EGCG demonstrated a significant suppression of tumor growth. It was found that the PDT, using Radachlorine, exerts its antitumor activity through EGCG supplement after the PDT, rather than by EGCG supplement prior to the PDT.

MATERIALS AND METHODS

Cell Cultures

The cell lines were obtained from the cell line bank at Johns Hopkins University (a kind gift from Dr. Wu, Johns Hopkins University, MD, USA). The cells were routinely propagated in monolayer cultures in RPMI-1640 medium, supplemented with 5% heat-inactivated fetal bovine serum, 0.22 % sodium bicarbonate and penicillin/

streptomycin. The cells were cultured in a 5% CO₂ incubator at 37 °C.

PDT

The PDT was carried out using a diode laser generator apparatus (Won-PDT D662, Won Technology, Daejon, Korea) equipped with a halogen lamp, a band-pass filter, and a fiber optics bundle. The wavelength was set at 662± 2 nm. Duration of the light irradiation, under PDT treatment, was calculated taking into account the effective dose of light energy in J/cm². Radachlorin (RADA-PHARMA Co, Ltd., Moscow, Russia) was used as a photosensitizer.

Cell Growth Inhibition Assay by EGCG and GTP

For the inhibition assay of cell growth, EGCG (a kind gift from Dr. Yukihiko Hara of Mutsui Norin Co, Fujied, Japan) and green tea polyphenol (GTP, 85% polyphenol, a kind gift from Dr. Sejun Han of Chosun University, Korea) was diluted in Dulbecco's Phosphate-Buffered Saline (DPBS) and stored at -20°C before use. For viable cell counting, 3 × 10³ cells per well (96 well plate) were treated with EGCG ranging from 25, 50, 100 µM and GTP ranging from 5, 10, 20 µg/ml for 24 hr. Cell growth inhibition was determined by MTT assay. For the MTT assay, 20 µl of MTT was added to each cell-culture well and cultured for 4 hr. Next, 100 µl of dimethylsulfoxide was added to the culture, shaken for 10 sec, and the absorbance measured with an ELISA-reader at 570 nm. Measurements were performed 24 and 48 hr after laser irradiation. Each group consisted of three wells. The means of their values were used as the measured values.

Cell Growth Inhibition Assay by PDT Combination with EGCG, GTP

There were 3 × 10³ cells per well (96well plate) treated with 50 µM EGCG, 10 µg/ml GTP and 2.5 µg/ml, 5 µg/ml Radachlorin (RADA-PHARMA Co, Ltd), and then the cells were exposed to PDT 12 hrs later. Laser irradiation in this experiment was performed at 6.25 J/cm². From the analysis of three replicates, the typical measurement deviations were observed to be less than

±3.0% for each assay.

Western Blotting

The cells grown in 6-well culture dishes (5 × 10⁵) were treated with several concentrations of EGCG. After 24 hr, the cells were lysed and then the cells were exposed to PDT 12 hr later. The protein concentration of the clear supernatant collected by centrifugation was evaluated using the BioRad Protein-Assay kit (Bio-Rad, Hercules, CA) and was adjusted for a final concentration of 2 mg/ml. After addition of 2-mercaptoethanol (2%), samples were boiled for 5 min and used for the experiments.

The 40 µg of each protein sample underwent electrophoresis for 2.5 hr with SDS-PAGE at 10 mA, and the Western blotting was performed with a Hybond-ECL membrane (Amersham, Uppsala, Sweden) at 100 V. The blotted membrane was blocked with 5% skim milk and reacted with a primary antibody (COX-2, VEGF, HIF-1α, MMP 9, actin; Santa Cruz Biotechnology Inc, Santa Cruz, CA). After washing with Tris-buffered saline containing 0.1% Tween 20, the membrane was then incubated with the horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Protein bands were visualized using an ECL Kit (Amersham, Arlington Heights, IL).

Photosensitizer Uptake

Radachlorin was injected intravenously via the tail vein for different periods of time, ranging from 0.5 to 48 hr when the tumor size was 7-9 mm. Cell debris was removed by centrifugation (3,000 rpm). Fluorescence (recorded at 642 nm following excitation at 595 nm) was measured and concentrations were deduced from a calibration curve. Experiments were carried out at least in triplicate.

Volume of the Tumors by PDT, EGCG, GTP or Combination

The volume of the tumors was measured after laser irradiation for each protocol. This experiment consisted of seven different experimental groups: the EGCG only group (E), GTP only group (G), PDT only group

(P), P+E group, P+G group, E+P group and G+P group. Each were subjected to combination therapies, in which EGCG or GTP was administered for 7 days 3 hours before PDT. The P+E group or P+G group also received combination therapy. EGCG or GTP was administered for 7 days immediately after PDT. The control group did not receive any photosensitizer, EGCG, GTP, or PDT. There were seven mice in each group.

STATISTICAL ANALYSIS

Statistical analysis included ANOVA and the Student's t-test. The values for the different groups were compared. P values of less than 0.05 were considered significant.

RESULTS

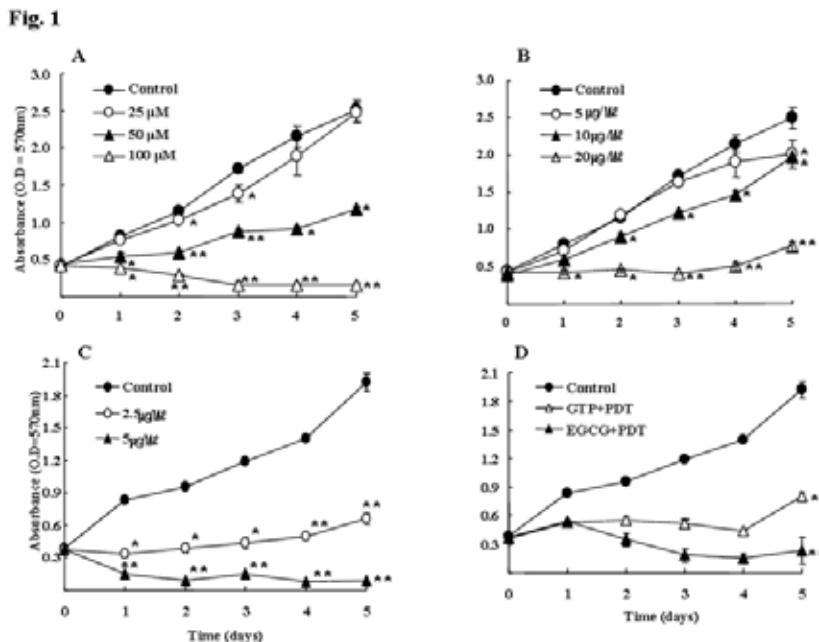
Cell Growth Inhibition by EGCG, GTP, and PDT Alone or PDT Combination with EGCG and GTP

First, we compared the effects of the two

treatments, EGCG and GTP, on growth inhibition in the TC-1 cell line. The cell growth inhibition effect was evaluated using MTT assays with increasing amounts of EGCG, and showed a significant increase in the cell growth inhibition over time (Fig. 1A). However, GTP did not affect suppression compared to the EGCG group (Fig. 1B).

Next we investigated the effects of the PDT on growth inhibition in the TC-1 cell line. A significant decrease in cell survival was detected with PDT compared to the control group (Fig. 1C). In addition, to compare the combination effects of the two treatments, 50 μ M EGCG and 10 μ g/ml GTP were used with 2.5 μ g/ml of Radachlorin (Fig. 1D). The cell growth was significantly suppressed in the EGCG plus PDT group. The GTP plus PDT group showed good cell growth inhibition compared to the control group.

Figure 1. Cell growth inhibition by PDT, EGCG, GTP alone or PDT combination with EGCG and GTP



TC-1 cells (3×10^3) were treated with (A) EGCG, (B) GTP, (C) Radachlorin/PDT, and (D) PDT combination with EGCG or GTP for five days. The control group represents cells only. Samples were assayed in triplicate. Vertical bars indicate SD ($n = 3$). Statistically significant inhibition of cell growth was measured by the t-test. *, $P < 0.05$, **, $P < 0.01$ compared with the control.

In-

fluence of Time on the PDT Effect After Injection

Influence of PDT time after Radachlorin injection on anti-cancer effect was investigated. We evaluated tumor suppression at 3, 6, 9, 12, and 15 days after PDT treatment. Figure 2 shows the time course of the changes in the tumor sizes. A significant difference was confirmed on day 15, and a tendency to grow larger was observed. In the control group, the tumor sizes increased almost linearly with time, until the end of the observation period. The results showed that tumor suppression was most effective at 3 hr post Radachlorin injection.

Radachlorin Uptake

Here we tested whether intravenous Radachlorin injection plus EGCG supplement affected Radachlorin accumulation in serum or tumor *in vivo*. As Radachlorin was injected intravenously, it is natural that Radachlorin was highly accumulated in serum in the first time periods (Fig. 3A). We confirmed that Radachlorin was accumulated in sera, and then excreted in each Radachlorin and/or EGCG group after 48 hrs. For tumors, the highest accumulation was at 3 hr post-intravenous injection (Fig. 3B). In addition, there was a significant difference with the levels of Radachlorin in either treatment. This was consistent with the anti-tumor effect of PDT, as shown in Fig. 2.

Under the same conditions, Radachlorin accumulation was lower in the combination group (EGCG plus Radachlorin) compared to Radachlorin alone in tumors. The results showed that EGCG supplement prior to Radachlorin injection could inhibit Radachlorin uptake *in vivo*. The time lapse post Radachlorin injection in tumors is also very important for controlling the effect of PDT and/or EGCG combination.

Tumor Suppression by EGCG or GTP Supplement Prior to PDT

Next, we investigated the effects of EGCG and GTP supplement prior to PDT *in vivo*. As shown in Fig. 4, the tumor volume after the EGCG and GTP supplements were relatively suppressed, but not significantly

compared to the PDT alone. The results showed that EGCG lowered the uptake of Radachlorin. This suggested that an EGCG supplement prior to photodynamic irradiation is not desirable. Therefore, EGCG supplementation prior to photodynamic irradiation may play a role as an interference factor against uptake of Radachlorin.

Tumor Suppression by PDT, EGCG, and GTP Alone or PDT Combination with EGCG, and GTP *in vivo*

To further enhance the anti-cancer effect with PDT, we investigated the effects of the combination of PDT and EGCG or GTP *in vivo*. Tumor volume after the PDT was suppressed compared with controls, as shown in Fig. 2.

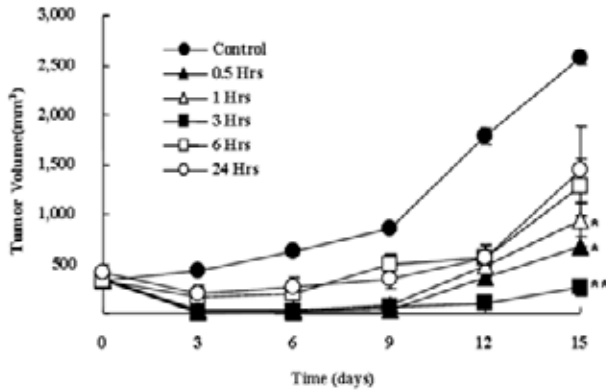
Next, we compared the effects of the two treatments, EGCG, and GTP on tumor volume *in vivo* (Fig. 5A). Tumor volume after EGCG treatment was significantly suppressed compared to GTP. Enhanced tumor suppression was observed in the combination group (Fig. 5B). Tumor volume with EGCG combination treatment was significantly suppressed compared with the control. In addition, we attempted to determine which molecules are responsible for the enhanced anticancer effects changed after the PDT combination with EGCG *in vitro* and *in vivo*. We have shown that the PDT combination with EGCG *in vitro* and *in vivo* led to a significant decrease in levels of COX-2 at 48 hr post PDT plus EGCG combination treatment (Fig. 6). However, we did not observe similar trends for the PDT combination with GTP in Western blotting experiments. Also, we evaluated the roles of the molecules that were responsible for angiogenesis function such as VEGF, HIF-1 α , and MMP9. The changes in the levels of proteins were not shown after the PDT combination with EGCG.

DISCUSSION

The main finding of this study was that the combination therapy approach using PDT and one of the green tea constituents, (-)-Epigallocatechin-3-gallate (EGCG), was effective for reducing tumor growth.

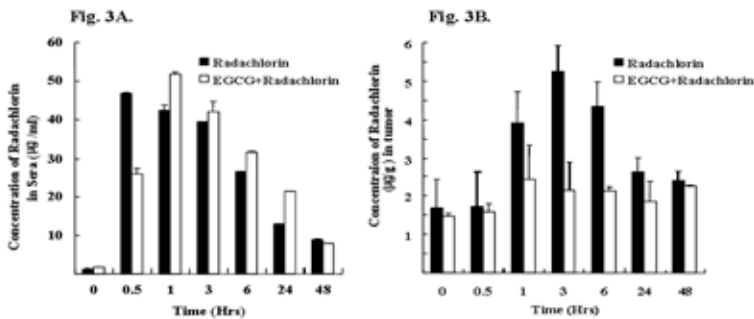
Figure 2. Antitumor effects by PDT intervals after Radachlorin injection in mice

Fig. 2



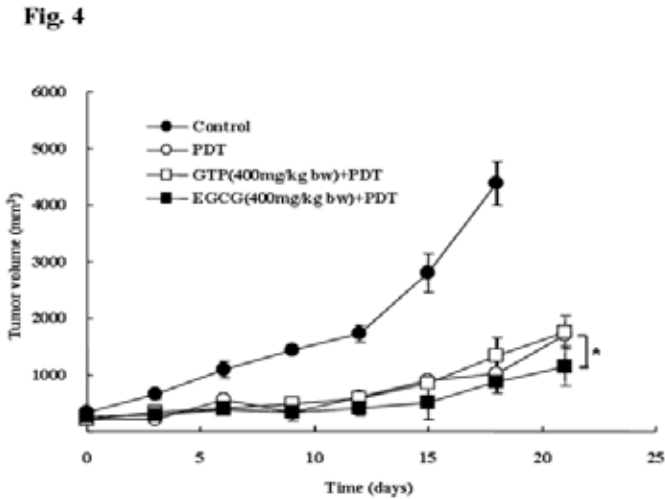
The tumor-bearing mice were light-irradiated (300 J/cm²) at 0.5, 1, 3, 6, and 24 hr after Radachlorin injection (10 mg/kg, iv). Tumor volumes were measured at 3, 6, 9, 12, 15 days after Radachlorin/PDT treatment. *, $P < 0.05$, **, $P < 0.01$ compared with the control.

Figure 3. Radachlorin uptake in mice



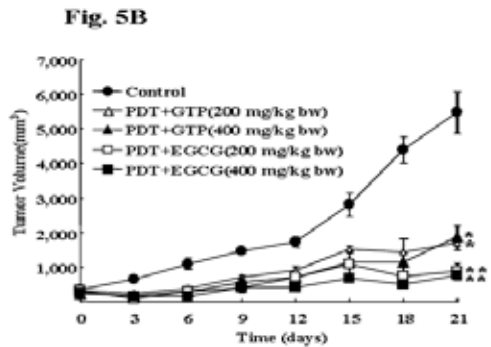
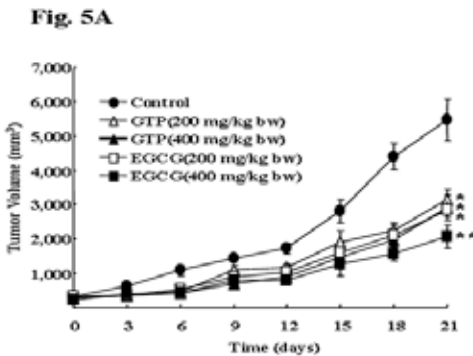
Radachlorin uptake was expressed as µg/g of wet tissue in the tumor and expressed as µg/ml of sera, as a function of the time after intravenous injection at a dose of 10 mg/kg b.w. to the C57BL/6 mice with TC-1 tumors. The bars represent the mean SD of three animals.

Figure 4. Tumor suppression by EGCG or GTP supplement prior to PDT



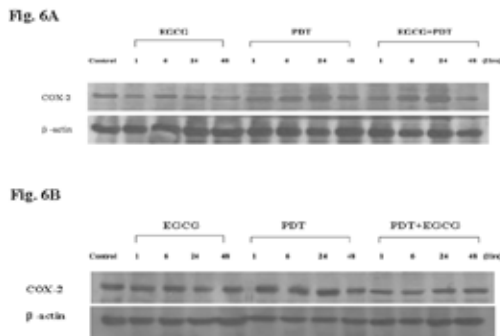
Tumor-bearing mice received EGCG or GTP a week prior to PDT treatment. Seven days after EGCG or GTP administration, the animals were treated with Radachlorin/PDT. EGCG and GTP were administered for 20 days. See Materials and Methods for details. Each group included seven animals. Error bars indicate the SD. *, $P < 0.05$ compared with PDT.

Figure 5. Tumor suppression by PDT, EGCG, and GTP alone or PDT combination with EGCG, and GTP in vivo



(A) Mice were injected subcutaneously with 5×10^5 cells per mouse. When tumor size reached 7 - 9 mm, they were treated with EGCG or GTP. Each group included seven animals. Statistically significant inhibition of tumor growth was evaluated by the t-test. *, $P < 0.05$, **, $P < 0.01$, compared with the control.
 (B) Tumor-bearing mice were treated with Radachlorin/PDT. After the PDT, EGCG or GTP was administered for 20 days. Each group included seven animals. Error bars indicate the SD. **, $P < 0.01$, compared with the control.

Figure 6. Effects of EGCG on PDT-induced COX-2 expression *in vitro* and *in vivo*



Western blot analysis of COX-2 after incubated with EGCG treatment (A) *in vitro* and (B) *in vivo*.

Photodynamic therapy (PDT) is a method of treating malignant tumors that produces photodynamic damage of tumor cells by photochemical reactions.³⁷ Several chemicals are used as photosensitizers of PDT for a wide range of malignant tumors as well as nonmalignant diseases.³⁸

We used Radachlorin, a promising sensitizer for photodynamic therapy. Radachlorin is a second generation photosensitizer with promising physico-chemical properties and high photodynamic efficiency.

A tumor treated by PDT is reabsorbed and is gradually replaced by connective tissue.³⁸ The location of photodynamic damage of a tumor is determined by a photosensitizer's ability to accumulate in tumor tissues and by the direction and localization of precise laser irradiation. PDT has been used in the treatment of a variety of cancers to induce apoptosis in tumor cells.³⁸

PDT results in the photochemical generation of a cytotoxic reactive oxygen species such as singlet oxygen within the target tissue. PDT clinical trials using a photosensitizer, as well as a variety of second-generation photosensitizer, has shown promise in treating malignancies of the esophagus, bronchus, brain, peritoneal cavity, skin, bladder, head and neck, as well as in treating nononcologic disorders such as age-related macular degeneration.

Dougherty et al (1998) reported that photodynamic therapy (PDT) is a promising cancer treatment modality based on its selective killing of malignant cells by singlet oxygen, 1O_2 , and other reactive products generated by photoactivated photosensitizer (PS) molecules that accumulate in tumor tissue.³⁷

Clinical application of PDT is generally positive. However, further development is needed to improve outcomes as tumor recurrences occur, and tumor size and depth affect the efficiency of PDT irradiation. This may be due to the increase in survival of molecules expressing cyclooxygenase (COX)-2, HIF-1 α and the VEGF gene.

Recently, it has been shown that inhibitors of cyclooxygenase (COX)-2, HIF-1 α , VEGF can be effective in combination with PDT therapy, and that Green tea extract may play a major role in suppression of these genes.

The Green tea polyphenols have been shown to have a protective effect in prostate cancer in a variety of pre-clinical animal models and has been reported to be effective in several other cancer types as well.³⁹⁻⁴¹ Green tea is composed of several catechins, including (-)-Epigallocatechin-3-gallate (EGCG), epicatechin (EC), epicatechin-3-gallate (ECG), and epigallocatechin (EGC).

Among them, (-)-Epigallocatechin-3-gallate (EGCG), the major catechin found in green tea, has been recognized as a potential therapeutic agent. Tea polyphenols have been shown to inhibit carcinogenesis in many animal models, and the significance of catechins, the main constituents of green tea, has been increasingly recognized to play a role in cancer prevention. The study of EGCG as anticancer agents has increased in recent years, due to their effects *in vitro* and *in vivo* on tumor cell signaling pathways regulating growth and apoptosis, including the suppression of cyclooxygenase (COX)-2, HIF-1 α and VEGF gene expression.^{41,42}

In this study, we compared the effectiveness of green tea polyphenols (GTP)

and its constituent Epigallocatechin Gallate (EGCG) in tumor regression using TC-1 tumor cells implanted into mice. EGCG suppressed cancer efficiently and showed enhanced activity in combination with PDT therapy. By contrast, similar effects were not observed in GTP supplemented combination PDT therapy. We confirmed that the tumor growth was significantly delayed in groups treated with Radachlorin/PDT and EGCG compared to either the Radachlorin/PDT or EGCG alone groups.

These findings demonstrated a high anti-cancer activity of photodynamic therapy with green tea constituent, Epigallocatechin Gallate (EGCG), on TC-1 tumor cell implanted mice. In this study, EGCG lowered the uptake of Radachlorin, a promising sensitizer for photodynamic therapy. This suggested that an EGCG supplement prior to photodynamic irradiation is not desirable, and that the supplement after photodynamic irradiation enhances the effect. Therefore, EGCG supplementation prior to photodynamic irradiation may play a role as an interference factor against uptake of Radachlorin.

In conclusion, the present experiments showed that the combination of photodynamic therapy with the green tea constituent, Epigallocatechin Gallate (EGCG), was very useful in cancer therapy by regulation of tumor cell signaling pathways, growth and induction of apoptosis together with the suppression of cyclooxygenase (COX)-2, HIF-1 α and VEGF gene expression. Therefore it is strongly suggested that high anti-cancer activity of photodynamic therapy with green tea constituent, Epigallocatechin Gallate (EGCG) may be a useful treatment for effective cancer therapy; additional studies are needed for further consideration of clinical applications.

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