Combination treatment of Cetuximab and photodynamic therapy in SNU-1041 squamous cancer cell line

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Abstract. Cetuximab (Erbitux) has been highlighted for its anti-proliferative effects in solid tumors and it is currently used as an adjuvant modality with other anti-cancer treatments. Photodynamic therapy (PDT) is used widely in many specialties of medicine. This study evaluated the efficacy of a combination treatment of two modalities (Cetuximab, PDT) both *in vivo* and *in vitro*. The SNU-1041 cell line was used for both the *in vitro* and *in vivo* studies. The *in vivo* and *in vitro* experiments were each classified into four groups, control group, Cetuximab applied group, PDT applied group and combined modality group. A migration study was performed to determine the anti-migration effect of Cetuximab, and a MTT assay was performed to compare the anti-proliferative effect of the modalities *in vitro*. For the *in vivo* study, the cells were implanted into 5-week-old nude mice. The measured volume of the tumor for each group was compared as a function of time. In the migration study, the control group showed a longer migration length than the Cetuximab applied group. In the MTT assay, the combination modality group showed less survival than the uni-modality groups. The measured tumor size after treatment showed that the combination treatment was more effective than the single modalities. PDT and Cetuximab are treatment modalities that target different molecular pathways. A combination of these two treatment modalities was found to more effective than an individual treatment. However, further studies will be needed to determine the optimal dose of the photosensitizer and Cetuximab.

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Introduction

The Epidermal Growth Factor (EGF) combines the EGF receptor (R) that exists in the cell membrane and delivers a signal inside the cell that promotes cell division. EGFRs are found on the cell membranes attached to a phosphoglyco-protein with a molecular weight of 170 kDa. The intracellular area exhibits tyrosine kinase activity in the part where the extracellular area of the EGF is. If EGF and EGFR are combined, cell division and proliferation occur to phosphorylate a large number of target proteins. In addition, it has been reported that EGFR is associated with an increase in tumorigenesis because EGF promotes cell division, and works through EGFR. EGFR has been observed in various normal human body organizations, such as the skin, muscle, pancreas, breast, prostate, but not in hematopoietic cells (1).

EGFR is related to malignant changes in human brain cancer and poorly differentiated or invasive bladder cancer, and has been observed in colon and rectal cancer (2-5). In addition, the overexpression of EGFR suggests an increase in cell proliferation. Therefore, EGFR has attracted a great deal of attention as a tumor marker indicating a poor prognosis.

Cetuximab (Erbitux, C225) is a monoclonal antibody of EGFR and has been studied extensively in a large number of preclinical or clinical experiments (6,7). A preclinical study showed that Cetuximab can inhibit the proliferation of colon, head and neck, and pancreatic cancer (8-10). In addition a comparison of a combined radiation-Cetuximab group with a radiation only group of head and neck cancer patients in a three-stage clinical experiment revealed an extended survival period in the combined radiotherapy group (11). The synergistic effect of DNA damaging factors, such as Cetuximab, anti-cancer drugs and radiotherapy has attracted considerable attention. In particular, a combination therapy of Cetuximab and irinotecan to colon cancer patients showing no response to irinotecan recently received approval from the FDA (12).

Photodynamic therapy (PDT) was recently introduced as an anticancer treatment with excellent treatment effects, and is used widely in a similar manner to radiotherapy or chemotherapy. Photodynamic therapy destroys cancer after being

photosensitized to light of a specific wavelength by the administration of a photosensitizer (13-15). The development of a photosensitizer with the optimal treatment effect and minimal side effects is important. The intracellular distribution of a photosensitizer is affected by physical chemical properties, such as the molecular weight of the photosensitizer, and quantity of electric charge during photodynamic therapy (16). A lipophilic photosensitizer migrates after combining with serum lipoprotein, is absorbed through intracellular endocytosis after combining with the LDL receptor of a cell and accumulates mainly in the endoplasmic mitochondria, endoplasmic reticulum, nucleus, and lysosomes (17). In addition, the response of a cell appearing after photodynamic therapy differs according to the type of photosensitizer, method used and type of a tumor cell (18). Photofrin is a recently derived hematophorphyrin derivative that has gradually improved its coverage as photosensitizer with a variety of effects reported (19). This study compared the synergic effect of Cetuximab, which inhibits the growth of tumor cells, when used in conjunction with photodynamic therapy, which is being used increasingly as a new anticancer treatment both *in vitro* and *in vivo*.

Materials and methods

A chemical reagent, photosensitizer and a laser. The photofrin was used as the photosensitizer in the *in vitro* and *in vivo* experiments, and a 632 nm diode laser (Biotec AG, Jena, Germany) was used as the light source. Cetuximab (ImClone Systems Inc., New York, NY, USA) was used undiluted (2 mg/ml).

Cell culture. The SNU-1041 squamous cancer cell line of the head and neck, was cultured in a culture flask (Nunc, Denmark) containing 500 ml of RPMI (Gibco BRL, Grand Island, NY, USA) mixed with 50 ml of fetal bovine serum (Gibco BRL), and 5.5 ml of antibiotic antimycotic (Gibco BRL) in a 5% CO_2 incubator.

Anti-growth effect of Cetuximab for SNU-1041 cell. A MTT assay was used to evaluate the cytotoxic effect of Cetuximab on SNU-1041 cells. SNU-1041 cells in the exponential period were grown to 10^5 cells/ml and diluted with the culture medium. The cells were then grown in a 96-well plate at 100 μl per well in a 5% CO_2 thermo-hygrostat. The cells were divided into 5 groups; a control group and four density groups (3.9, 31.3, 250, 500 μg/ml). The cells were cultured for approximately 48 h. Subsequently, 50 μl of pH 7.3 DPBS MTT[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] (2 mg/ml, Sigma, St Louis, MO) was added to each well and cultured in a thermo-hygrostat for 4 h. After culturing, the culture medium was removed from each well and 150 μl of DMSO was added. The wells were shaken for 5 min and the formazan was melted using a microplate mixer (Amersham Pharmacia Biotech, Sweden). The optical density of formazan was measured at 540 nm using a microplate reader (Bio-Rad Laboratories Inc., Hercules, CA, USA). The survival rate was calculated using the following formula, and statistical analysis was carried out using a statistical package.
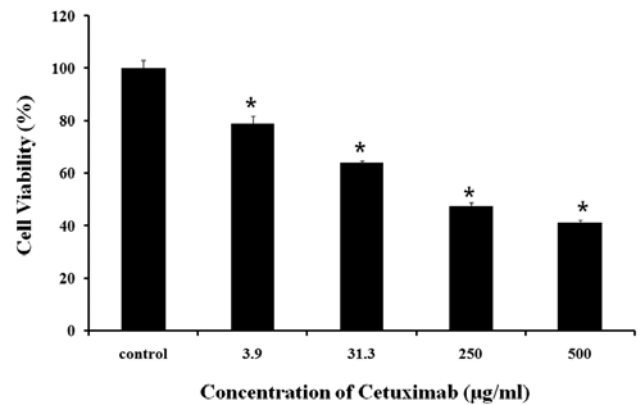

Figure 1. The antiproliferative effect of Cetuximab in SNU-1041 human head and neck squamous cancer cells. The viability of SNU-1041 cells treated with Cetuximab decreased in a dose-dependent manner.

$$\text{Cell viability (\%)} = \frac{\text{Mean optical density in the test well}}{\text{Mean optical density in the control well}} \times 100$$

Cell migration assay. The SNU-1041 cells were cultured until the monolayer was 70-80% confluent in a culture flask (Nunc). The culture medium was then removed, and a regular distance between cells of approximately 1.2 mm was made in flask using the yellow tip of a pipette. After washing the cells that failed in the DPBS solutions (0, 2, 20 and 200 μg/ml) Cetuximab at different concentration was then added. The distance was measured at 0, 12, 24, 36, 48 and 72 h from photographs, using a digital camera (Samsung, Korea) and the degree of cell migration was compared.

The synergistic effect of Cetuximab and photodynamic therapy. SNU-1041 cells in the exponential period were grown to 10^5 cells/ml. After diluting the cells with the culture medium, 100 μl per well of the cells were added to four 96-well plates and cultured in a 5% CO_2 thermo-hygrostat. After adding Cetuximab to the four plates at the set concentration (50, 125, 250 and 500 μg/ml), photofrin, which was diluted from 1.0 to 0.001 mg/ml, was added. After 6 h, the upper 10 cm area of the plates was exposed to 632 nm diode laser light (3.2 J/cm^2). After exposure, the culture medium was changed, and the same concentration of Cetuximab was added. After 48 h, the groups treated individually with either photodynamic therapy or Cetuximab were compared with the groups treated with both photodynamic therapy and Cetuximab.

Protein extraction and Western blotting. The SNU-1041 cells were divided into six groups. Group 1 was the control group. Group 2 was treated with 3.9 μg/ml of Cetuximab. Group 3 was treated with photodynamic therapy at a photosensitizer concentration of 0.8 μg/ml. Group 4 was treated with photodynamic therapy at a photosensitizer concentration of 3.1 μg/ml. Group 5 was treated with photodynamic therapy and 3.9 μg/ml Cetuximab at a photo-sensitizer concentration 0.8 μg/ml. Group 6 was treated with photodynamic therapy and 3.9 μg/ml Cetuximab at a photo-sensitizer concentration

of 3.1 μg/ml. After culturing for 24 h, the protein was extracted and treated with a RIPA buffer (Sigma). The protein was analyzed using the Bradford assay (Bio-Rad). A protein of equivalence was placed in the sample buffer solution, boiled for 5 min, and loaded onto 10% poly-acrylamide gel. SDS-PAGE electrophoresis was performed at 100 V. Subsequently, the protein in the polyacrylamide gel was shifted to a PVDF membrane (Bio-Rad) at 100 V for 90 min. The PVDF membrane was placed in a PBST buffer solution (0.1% Tween-20 in PBS) that included 5% non-fat dry milk and shaken for 1 h at a normal temperature. The first antibody EGFR (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) was added at 4˚C for 24 h. After cleaning the PVDF membrane 3 times with a PBST buffer solution for 15 min each, the HRP-conjugated second antibody, which suited the 1st antibody, was added at a normal temperature for 1 h. The sample was washed again 3 times with a PBST buffer solution for 15 min each. The samples were then reacted in an ECL kit (Amersham, UK), and analyzed using an image analyzer (Kodak, Japan).

Laboratory animal and breeding condition. Five-week old nude mice (Charles River Laboratories, Wilmington, MA, USA) were maintained in a thermo-hygrostat (Jeung Do B&P Co., Korea) at 22-23˚C and a relative humidity of 40-60%. The mice were housed four mice/cage in a clean rack. The hall and sterile chamber was cleaned with Hibitan solutions every 7-10 days, and the straw was exchanged with sterile straw every 3-4 days. The researchers' hands were cleaned with 70% ethanol, and clean gloves were used.

Xenotransplantation of the SNU-1041 cancer cell line. After the cell concentration was diluted to 10^8 cells/ml, the cells were injected into back of the nude mice aged 5 weeks using a 30 gauge (G), 1 ml insulin syringe. The cancer volume was measured 3 times per week.

Cetuximab and results after photodynamic therapy. The mice were divided into four experimental groups after the cancer volume reached 100-300 mm³. The 1st group (n=10) was the control group. The 2nd group (n=10) was treated with 2 mg (equal to 50 kg man) Cetuximab every 2 days for 3 weeks. The 3rd group (n=10) was treated with photodynamic therapy using photofrin (1 mg/kg) diluted in DPBS fluid injected into the abdominal cavity through a 30 gauge (G) 1 ml insulin syringe. After 24 h, the cancer area was then exposed to 632 nm diode laser light at four directions for 5 min. In Group 4, photodynamic therapy and Cetuximab were performed simultaneously. The other areas of the mice were blocked to prevent other complications. Photodynamic therapy was performed on the 1st day. A 2nd photodynamic therapy was performed on the 14th day for the mice with tumors that did not respond to the 1st photodynamic therapy. The anti-cancer effect was judged by measuring the volume of the tumor twice per week. The volume was calculated using the following formula:

$$V = (4/3 \times A \times B \times C) \times 1/2$$

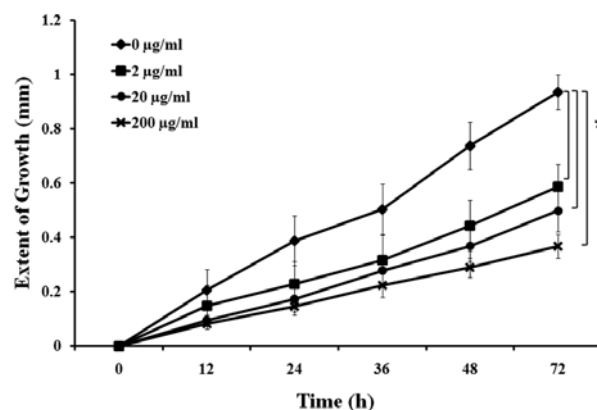(V, volume; A, major axis; B, minor axis; C, height)

Figure 2. The effect of Cetuximab on the migration of SNU-1041 human head and neck squamous cancer cells. Migration was inhibited in SNU-1041 cells treated with Cetuximab in a dose-dependent manner.

Statistical analysis. The results are reported as the mean ± SD. A t-test and paired t-test ANOVA test were performed for statistical analysis. A $p < 0.05$ was considered significant.

Results

Anti-growth effect of Cetuximab on the SNU-1041 cells. The cell survival rate (Photoluminescence measurements) of the control group was converted to an average numerical value of 100%. The survival rate of the cells treated with 3.9, 31.3, 250 and 500 μg/ml Cetuximab was 78.8, 64.1, 47.7 and 41%, respectively. Overall, the cell survival rate decreased with increasing Cetuximab concentration. In addition, the rate of SNU-1041 cell proliferation decreased with increasing Cetuximab concentration ($p < 0.05$, Fig. 1).

Cell migration assay. The migration distance of a cell was measured at the lowest magnification using a digital camera. The image was then enlarged 4 times. The distance between the cells was measured. The whole distance minus the measured distance was defined as the migration distance. The migration distance was measured at 12, 24, 36, 48 and 72 h. The migration distance of the control group was 0.2, 0.39, 0.5, 0.74 and 0.94 mm, respectively. The migration distance of the group treated with 2 μg/ml Cetuximab (Erbitux) was 0.15, 0.23, 0.32, 0.44 and 0.59 mm, respectively. After 12 h, there was no significant difference between the two groups. However, after 24 h, there was a significant decrease in migration distance compared with the control group. In the group treated with 20 μg/ml Cetuximab, the migration distance decreased to 0.09, 0.17, 0.28, 0.37 and 0.50 mm. The migration distance of the group treated with 200 μg/ml Cetuximab after 12 h was 0.08, 0.15, 0.22, 0.29 and 0.37 mm, and was significantly lower than the control group ($p < 0.05$, Fig. 2 and 3).

The photodynamic therapy in SNU-1041 cell line and the synergistic cytotoxic effect of combined therapy with Cetuximab. The cell survival rate of the group given the combined photodynamic therapy with 3.9 μg/ml Cetuximab was measured. The cell survival rate was very low when a high concentration of photofrin was used. However, when
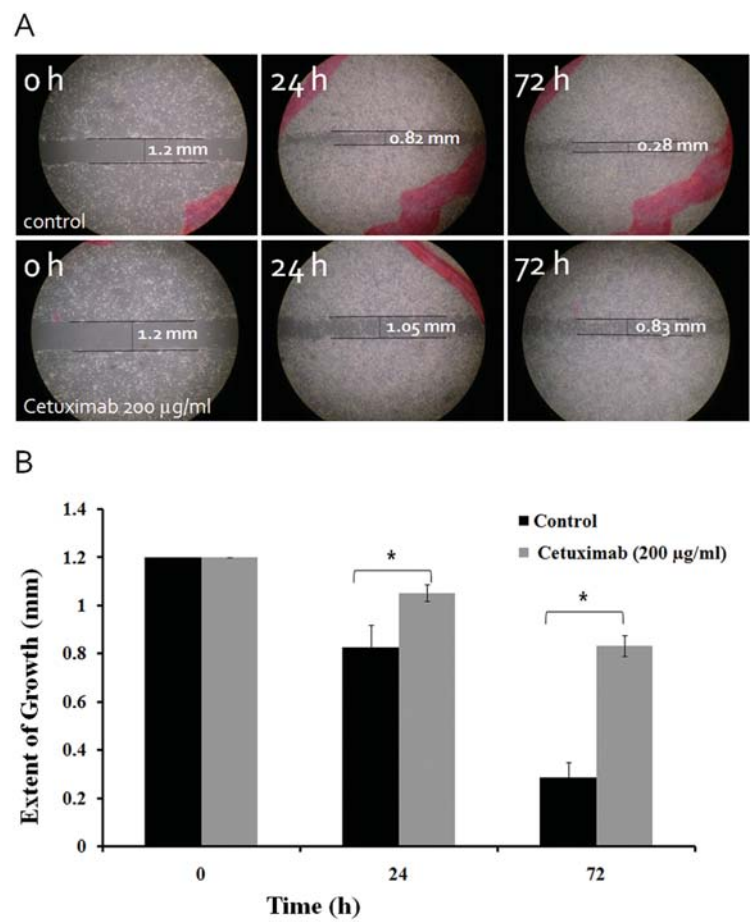
Figure 3. The effect of Cetuximab on the migration of SNU-1041 human head and neck squamous cancer cells. Migration was significantly suppressed in the SNU-1041 cells treated with 200 μg/ml of Cetuximab compared with control morphologically.
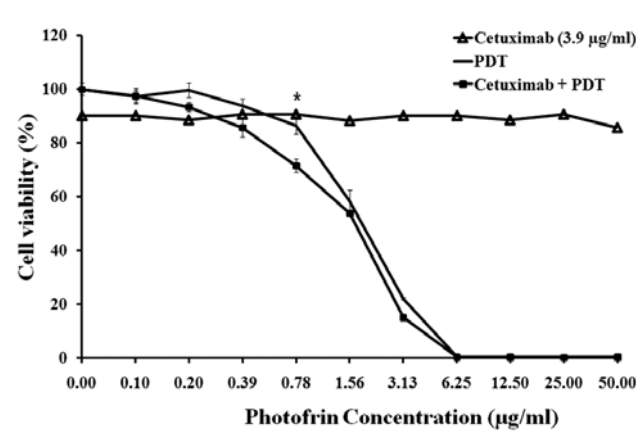

Figure 4. The antiproliferative effect of Cetuximab and PDT in SNU-1041 human head and neck squamous cancer cells. The cell viability after combination treatment with Cetuximab (50 μg/ml) and PDT was decreased in the SNU-1041 cells.
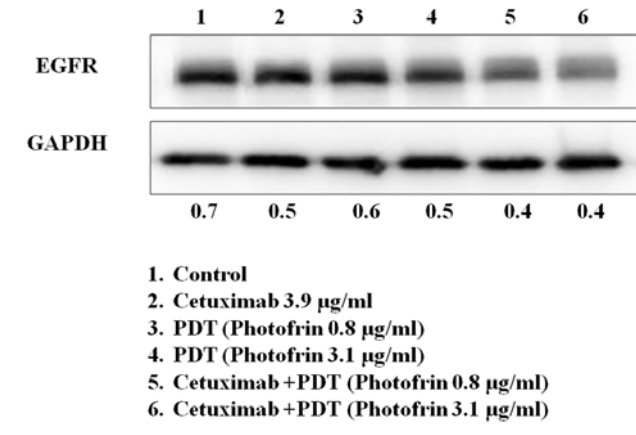

Figure 5. The expression of EGFR in SNU-1041 human head and neck squamous cancer cells treated with the combination therapy. The EGFR of SNU-1041 cells was inhibited by the combination treatment of Cetuximab and PDT.

lower concentrations of photofrin were used, the cell survival rate decreased in a concentration-dependent manner. In particular, the cell survival rate was 98, 93, 85 and 71% when 0.1, 0.2, 0.39 and 0.78 μg/ml photofrin was used in conjunction with 3.9 μg/ml Cetuximab. Combined photodynamic therapy with Cetuximab decreased the survival rate at low concentrations of photofrin. In comparison, cell survival rate of the group treated with photodynamic therapy only was 97, 99, 94 and 86%, respectively. The cell survival rate was significantly lower when the cells were treated with photodynamic therapy combined with Cetuximab and 0.78 μg/ml photofrin than those treated with photodynamic therapy or Cetuximab
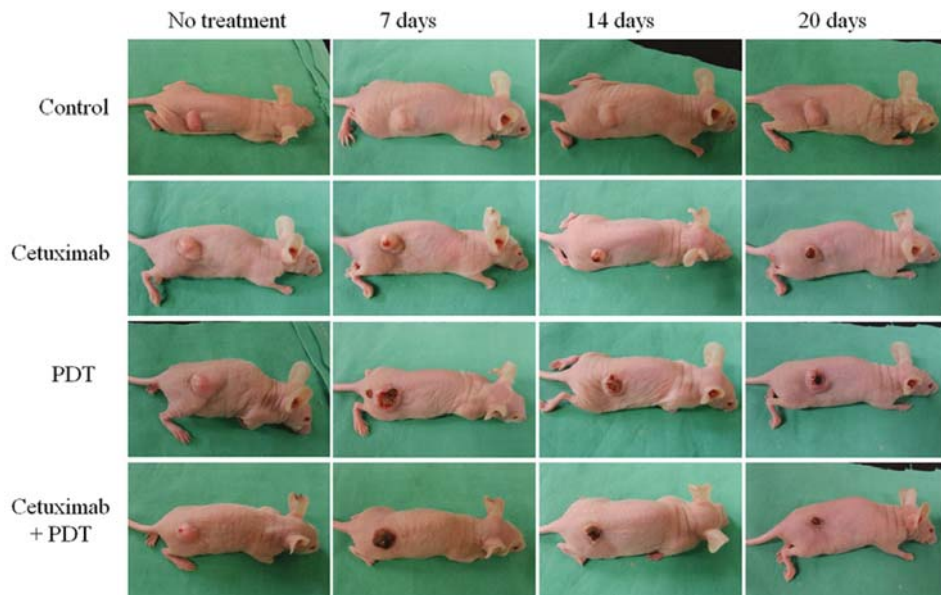
Figure 6. The effect of the combination therapy with Cetuximab and PDT on the tumor volume in a xenograft model.
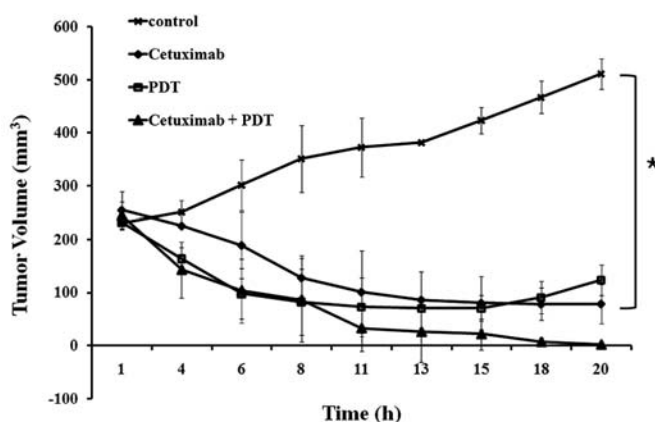

Figure 7. The effect of the combination therapy with Cetuximab and PDT on the tumor volume in a xenograft model. The combination treatment of Cetuximab with PDT showed significant tumor growth inhibition in the xenograft model (p<0.05).

(p<0.05, Fig. 4). This demonstrates the effect of the combination treatment of photodynamic therapy with photofrin and Cetuximab as an EGFR antagonist on the SNU-1041 cell line *in vitro*.

Negative effect of photodynamic therapy on the SNU-1041 cell line and combination treatment with Cetuximab and photodynamic therapy to EGFR. A large amount of EGFR was found in the SNU-1041 cell line treated with Cetuximab only, photofrin 0.8 and 3.1 µg/ml photodynamic therapy, and the control group. However, the group treated with combination treatment showed a low level of EGFR depending on the photofrin concentration used (Fig. 5).

Effect of the combination Cetuximab and photodynamic therapy on the transplanted cell line (in vivo study). Analysis of the combined effect of Cetuximab and photodynamic

therapy was performed using an experimental animal (nude mouse) model *in vivo*. The cancer volume after injecting cancer cells in the control group increased from 252.5 to 300.8 mm^3, 423 mm^3 and 511 mm^3 after 6, 14 and 20 days, respectively. The cancer volume after injecting the cancer cells in the Cetuximab group decreased from 231 mm^3 at start of treatment to 188.6, 79.8 and 78.1 mm^3 after 6, 15 and 20 days, respectively. The cancer volume was significantly lower than the control group. The cancer volume after injecting the cancer cells in the photodynamic therapy group decreased from 181.1 mm^3 at the start of treatment to 97.5 mm^3, 70.5 and 123.2 mm^3 after 6, 15 and the last day, respectively. The cancer volume after injecting the cancer cells to the combination treatment with Cetuximab and photodynamic therapy group decreased from 143.4 mm^3 at the start of treatment to 103.2 mm^3, 20.9 and 1.2 mm^3 after 6, 15 and 20 days, respectively. Overall, the combined treatment had a significantly greater effect than the individual treatments (p<0.05, Fig. 6 and 7).

Discussion

PDT has been studied extensively as an anticancer treatment from 1970. In this treatment, a photosensitizer that is absorbed well by cancer cells is injected into the cancer and activated by a specific wavelength. A photosensitizer is essential for carrying out photodynamic therapy because it is activated by light of a specific wavelength. A laser is used mainly in photodynamic therapy because it has sufficient energy to activate the photosensitizer compared with other light sources, and can emit light of a single wavelength. Hence, it can produce the maximum activity of the photosensitizer.

Currently, a diode laser with a continuous wavelength (CW) is used universally for photodynamic therapy. The diode laser used in this study had a wavelength of 632 nm, which is the maximum absorption spectrum of photosensitizer

sufficient to produce a maximum treatment effect. It is possible that there will be a difference in cytotoxic effect according to the Laser radiation method. The pulse method is more effective in killing tumor cells without damaging normal cells (20). Laser in pulsed mode generally has deeper penetration than a CW Laser and has a lower temperature. Therefore, it is believed to be more suitable for photodynamic therapy.

The start of action electric generation within the cell by photodynamic therapy is understood to be a phenomenon of photophysics. A photosensitizer in the ground state (S0) is activated by a specific wavelength, and excited to the singlet (S1) or triplet state (T1). At this time, the energy that is released when with the floor state reacts with oxygen produces an oxygen free radical (type II reaction), which causes cell destruction. On the other hand, photosensitizer is involved in the electron transport system (type I reaction), which causes cell destruction.

The type I response occurs mainly in polar culture media, and the type II response occurs mainly in high O_2 lipophilic environments. Therefore, the type II response is used mainly in photodynamic therapy. However, Ochsner reported that type II responses can change to type I responses in situations where local hypoxia is caused (24,25).

In addition, while ATP storage runs dry in a cell as a result of direct cell membrane damage (26), the progress process of necrosis occurring after photodynamic therapy impedes cell reproduction or the rehabilitation process, reduces the level of amino acid movement into cells (27), or decrease of the pH in a cell (28). The destruction of a tumor cell and the resulting necrosis due to damage to the lysosome membrane causes the dissociation of a large amount of proteinase (29). According to this phenomenon, damage to the cell membrane causes failure of the arachidonic acid metabolism as well as blood vessel injury. Necrosis is accelerated by stimulation of the complement system as well as the excretion of histamine and other inflammatory materials (30). Direct cell damage induces an imbalance of the proteins transmitting ions through the cell membrane (31). The level of endonuclease is increased when the level of calcium within the cell increases, which causes calcium dependent necrosis, i.e. DNA decomposition (32). This is not observed in all cases after photodynamic therapy, and this type of necrosis is dependent on the type of photosensitizer and target cell line (33).

The advantage of photodynamic therapy is the virtual lack of pain. It is performed simultaneously with chemotherapy, radiotherapy and surgery. In addition, there is no limit to the number of times photosensitizer injection or laser therapy can be performed, so a continuous procedure is possible. Photodynamic therapy is suitable for superficial cancers because the absorption wavelength of most photosensitizers (including photofrin) used in these cases is 630 nm, and the permeability of a laser is 0.5-1 cm.

A total of 171 examples have been reported since Keller *et al* (34) presented 3 examples of complete remission of oral cavity cancers. Of these, photodynamic therapy was used to treat 145 examples (85%) of early stage head and neck cancer. Freche *et al* (36) reported the complete remission of early stage laryngeal cancer in 25 patients (78%) with T1 or less out of 32 examples. Biel *et al* (37)

reported the complete remission of 25 squamous cell cancer examples with T1. However, 17 examples relapsed after radiotherapy. Schweitzer (38) reported 8 cases of complete remission of squamous cell cancer out of 10 examples with T1 and T2. Six out of the 10 cases relapsed. Two cases with T1 that relapsed after LMS with a laser showed complete remission. Of the remaining 4 cases, 1 case with T1 relapsed after radiotherapy and 3 cases with T2 relapsed after radiotherapy. After treatment, 3 of these 4 cases showed complete remission.

The main systemic side effects of photodynamic therapy are photosensitivity that persists for 4-6 days. The patients are asked to avoid direct sunlight and direct light when inside during these periods because skin burns, and blisters can occur. In addition, some pain, slight fever, an increase in urination frequency can occur. This treatment is not recommended for patients showing photosensitivity, poor kidney or poor liver function. The photosensitizer is injected slowly at 15-20 drops per minute and is mixed with 40 cc of normal saline. There is a 15-20 min break after injecting the first 10 cc to determine if there an abnormal response.

In this study, the size of the tumor in the animal experiment was reduced more with the combination therapy (EGFR interception and photofrin-PDT) than with the individual treatments. This shows that the treatments act synergistically through two different molecular pathways, as suggested elsewhere (41,42).

Under normal conditions, the manifestation of EGFR is important for the physiology of a cell using receptor tyrosine kinase. One is the RTK-Grb2-Sos-Ras cascade, which is activated by mitogen-activated protein kinase and phosphatidylinositol3-kinase. The manifestation of these two pathway conversions affects the proteins that induce cell division. The overexpression by cancer cells is increased by Cyclin-D and Myc, phosphorylation of the retinoblastoma protein, activation of factor 4E and a decrease in p27. It is believed that these adjustment proteins disregard the check point, and exhibit the malignancy of an organization by causing the unfit conversion of G1 to S. However, there is the possibility of impeding the progress by suppressing the activity of EGFR. Cells are dependent more on this proliferative pathway after being damaged by photodynamic therapy.

Overexpressed EGFR is common to cancers with considerable resistance to existing treatments or is progressed. On the other hand, PDT is used experimentally in non-reactive chemotherapy and radiotherapy. The union of PDT and an EGFR immune treatment can solve the small issues of each individual treatment. For example, Cetuximab is mainly a cytostatic treatment. Therefore, a long treatment period is needed. However, there are frequent relapses, and drug intoxication. Photodynamic therapy is also a cytotoxic treatment but there is the possibility of residual cancer remaining in cases of single treatments and there are no criteria for use. Therefore, a combined treatment can have an excellent better anticancer effect than the independent treatments despite reduced capacity of Cetuximab or the photosensitizer.

The cell toxicity of photofrin in this study was confirmed by the decrease in cell survival at high concentrations.

Hence, the effective distribution of the photosensitizer in a cell is more important than the concentration (43). Cytotoxicity increases in direct proportion to the energy supplied. Therefore, it is believed that there is an increase in the photophysical phenomenon, which can react with the photosensitizer. This is an important element to be considered in photodynamic therapy. An adequate response time of the photosensitizer in a cell will result in effective treatment.

In conclusion, this study compared the treatment effectiveness of a combined treatment with Cetuximab and PDT with the effects of the individual treatments using *in vivo* and i*n vitro* experiments. A combination of a suitable ratio of biological and photodynamic therapy can be effective in treating malignant tumors and should be considered as a treatment.

However, more cases and a standardized study will be needed to confirm these results. Photodynamic therapy is not confined to an adjuvant therapy and palliative therapy of an existing treatment, and it is believed that photodynamic therapy should be the initial treatment for head and neck cancers and recurrences.

Acknowledgements

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