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Troglitazone, a Selective Ligand for PPARγ, Induces Cell-cycle Arrest in Human Oral SCC Cells

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Abstract. *Aim: We attempted to clarify the role of Peroxisome proliferator-activated receptor γ (PPARγ) and its ligand, troglitazone (TRO) on oral squamous cell carcinoma (SCC). Materials and Methods: The expression of PPARγ gene was examined in 47 human oral SCC tissues and two human oral SCC cell lines, CA9-22 and HSC-4. The effects of TRO on the growth and cell-cycle progression of human oral SCC cells were examined. Results: PPARγ mRNA was detected in 20 of 47 oral SCC tissues and two human oral SCC cells. TRO significantly suppressed the growth of the cells, but did not induce apoptosis. CA9-22 cells treated with TRO showed an increased fraction in the G_1 phase and decreased fractions in the S and G_2-M phases. Conclusion: TRO did not induce apoptosis in oral SCC cells, but did inhibit the growth of the cells by arresting the cell cycle at G_1 phase.*

Of all human malignancies reported worldwide, head and neck cancer accounts for 5.4%, with an annual incidence of 550,000 cases (1, 2). Oral squamous cell carcinoma (SCC), laryngeal SCC, pharyngeal SCC, and salivary gland cancer account for most head and neck cancers. Head and neck cancer has a relatively poor prognosis, with most patients having severe dysfunction and a poor appearance following curative treatment.

There are several treatment methods for oral SCC including surgery, irradiation, and chemotherapy, based on the clinical stage and/or the patient's overall general health. The effectiveness of conventional chemotherapy for head and neck cancer is dependent on the patient. In some patients, the tumor response to chemotherapy is very good, whereas in others, the tumors show complete resistance to treatment. Thus, a new approach that can improve the prognosis of oral SCC patients is needed.

Peroxisome proliferator-activated receptor γ (PPARγ) is a member of the nuclear hormone receptor superfamily of ligand-activated transcription factors; it functions as a regulator of adipocyte differentiation and lipid metabolism (3, 4). PPARγ forms heterodimers with the retinoid X receptor (RXR) (5), and can be activated by various ligands, such as the thiazolidinediones, including troglitazone (TRO) (6-8). Synthetic PPARγ ligands are used as orally active antidiabetic agents (8) or as non-steroidal anti-inflammatory drugs (9). PPARγ ligands have been shown in many studies to induce the differentiation of human liposarcoma (10) and breast cancer tissues (11) and inhibit cell growth of various carcinomas, including breast (12), prostate (13), lung (14), colon (15), stomach (16), bladder (17), esophagus(18)and pancreas (19). In a case of human colon cancer, mutations of the *PPARγ* gene were found to be associated with loss of its function (20).

In the present study, *PPARγ* gene expression was evaluated in 47 human oral SCC tissues and two human oral SCC cancer cell lines. In addition, the effects on growth and cell-cycle progression of human oral SCC cells of TRO, a synthetic ligand for PPARγ, were also examined.

Materials and Methods

Ethics statement. The Ethical Committee of the Dokkyo Medical University Hospital approved the study protocol (approval number: R-24-18J). The study was performed in accordance with the ethical standards of the Declaration of Helsinki. All patients provided written informed consent for participation in this study.

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR). RNA was isolated from tumor tissue and cultured cells. Human oral SCC tissues were obtained at the Department of Oral and Maxillofacial Surgery, Dokkyo Medical University School of

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Key Words: Peroxisome proliferator-activated receptor γ, troglitazone, oral SCC, apoptosis, G_1 arrest.

Medicine. Total RNA was extracted from tissue and cultured cancer cells using ISOGEN (Nippon Gene, Tokyo, Japan), according to the manufacturer's instructions. cDNA synthesis was performed using random oligonucleotide primers (5 μM; Thermo Fisher Scientific, Waltham, MA, USA) and Molony murine leukemia virus reverse transcriptase (Thermo Fisher Scientific) with incubation at 42˚C for 60 min. The cDNA of interest was amplified from total cDNA (1 μl) by PCR in a reaction mixture containing deoxynucleotide triphosphates (200 μM), specific oligonucleotide primers (1 μM each), and Ex Taq DNA polymerase (0.05 U/μl) (Takara Biomedicals, Kusatsu, Japan). Amplification was performed in a Takara Thermal Cycler MP (Takara Biomedicals) with denaturation at 94˚C for 5 min, followed by 30 cycles of 94˚C for 1 min, 60˚C for 1 min, 72˚C for 1 min, and final extension at 72°C for 4 min.

The expression of *PPARγ* mRNA in human oral SCC tissues was examined using the oligonucleotide primer pair 5'-TCT CTC CGT AAT GGA AGA CC-3' (upstream) and 5'-GCA TTA TGA GAC ATC CCC AC-3' (downstream) (21). The primers used to amplify glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA were 5'-TTC ATT GAC CTC AAC TAC AT-3' (upstream) and 5'-GAG GGG CCA TCC ACA GTC TT-3' (downstream) (22).

Cell lines and cultures. Two human oral SCC cell lines, CA9-22 and HSC-4, were used in this experiment. These cell lines were kindly provided by the Department of Oral and Maxillofacial Surgery, Kanazawa University, School of Medicine, and grown in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific), 100 U/ml penicillin, and 100 μg/ml streptomycin (Thermo Fisher Scientific), hereafter referred to as complete medium, and maintained at 37˚C in a humidified atmosphere containing 95% air and 5% CO_2 (23).

Cell growth assay. Cells were seeded in complete growth medium in a flat-bottomed, 6-well plate (Falcon, Becton Dickinson, Franklin Lakes, NJ, USA) at a density of 5×10^4 cells per well. After 24 h, incubation of cells with TRO (0-100 μM) was started. The cells were treated for 3 days in medium containing several concentrations of TRO, and the cell number was counted using a hemocytometer.

Terminal deoxynucleotidyl transferase mediated biotin-dUTP nick end labeling (TUNEL) method. Approximately 1×10^4 cells were cultured in Lab-Tek Chamber Slide (Nalgen Nunc International, Naperville, IL, USA). The cells were treated with 10, 50, and 100 μM TRO for 2 days. Induction of apoptosis in the cells was analyzed by the TUNEL method (24) using the Apoptosis *in situ* detection kit (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). The cells were fixed with 10% neutralized formalin solution for 10 min at room temperature. The cells were then washed with phosphate buffered saline (pH 7.4) twice and incubated in TdT reaction mixture (terminal deoxynucleotidyl transferase and biotinylated dUTP) in a moist chamber for 30 min at 37˚C. The cells were then treated with 3% H_2O_2 methanol solution for 5 min at room temperature to inactivate intrinsic peroxidase activity. The cells were finally treated with peroxidase-conjugated avidin for 10 min at 37˚C. Positive reactions were visualized with a 0.3% diaminobenzidine solution and counterstained with 2% methylgreen. As a positive control, the cells were treated with DNAase I before TdT treatment. As a negative control, a similar process without TdT treatment was conducted.

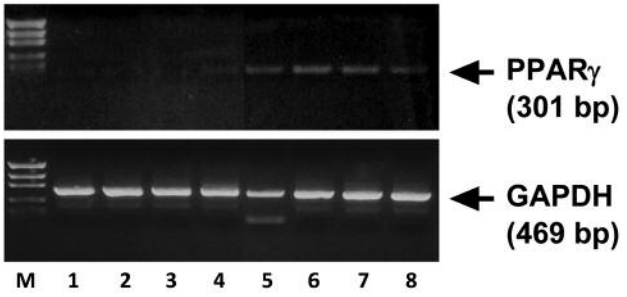

Figure 1. *Expression of PPARγ in human oral SCC tissues. The expression of PPARγ mRNA in human oral SCC was examined by RT-PCR. PPARγ mRNA was detected in 20 of 47 ORAL SCC tissues. Representative cases of negative expression (lanes 1 to 4) and positive expression (lanes 5 to 8) of PPARγ mRNA are shown.*

Analysis of DNA fragmentation. The cells were cultured with TRO (0, 50, or 100 μM) for 2 days. They were lysed in 100 μM of cell lysis buffer (10 mM Tris-HCI pH 7.4, 10 mM EDTA, and 0.5% Triton X-100) for 10 min on ice and centrifuged at 12,000 rpm for 20 min. The resulting supernatant containing the fragmented DNA was treated with RNaseA (0.4 mg/ml) for 1 h at 37˚C. After precipitation with 5 M NaCl and isopropanol, DNA was collected and resuspended in 20 μl of TE buffer (10 mM Tris-HCl; 1 mM EDTA, pH 8.0). The ApoAlert LM-PCR Ladder Assay Kit (CLONTECH, Palo Alto, CA, USA) was used to detect DNA fragmentation according to the manufacturer's protocol.

Cell-cycle assay by flow cytometry. HSC-4 and CA9-22 cells treated with TRO or DMSO for 0 or 48 h were collected by centrifugation, permeabilized with ice-cold 70% ethanol for at least 1 h, and then washed with PBS containing 100 mg/ml RNase A (DNase free) at 37˚C for 30 min. After centrifugation, the cells were re-suspended in PBS containing 50 mg/ml propidium iodide and stained at 37˚C for 30 min. DNA contents were analyzed by FACSCalibur (Becton Dickinson) (25).

Results

Expression of PPARγ in human oral SCC tissues. The expression of PPARγ mRNA in human oral SCC was examined using RT-PCR. *PPARγ* mRNA was detected in 20 of 47 ORAL SCC tissues. Representative cases of negative expression (lanes 1 to 4) and positive expression (lanes 5 to 8) of PPARγ mRNA are shown in Figure 1. The functional role of the PPARγ that was expressed in human ORAL SCC was further investigated in two human ORAL SCC cell lines, CA9-22 and HSC-4.

Effect of synthetic PPARγ ligands on the growth of human ORAL SCC cells in vitro. The synthetic ligand, TRO, was applied to cultured cancer cells, and the effect on cell growth was assessed. TRO significantly suppressed the growth of CA9-22 (Figure 2A) and HSC-4 (Figure 2B) cells in a dose-

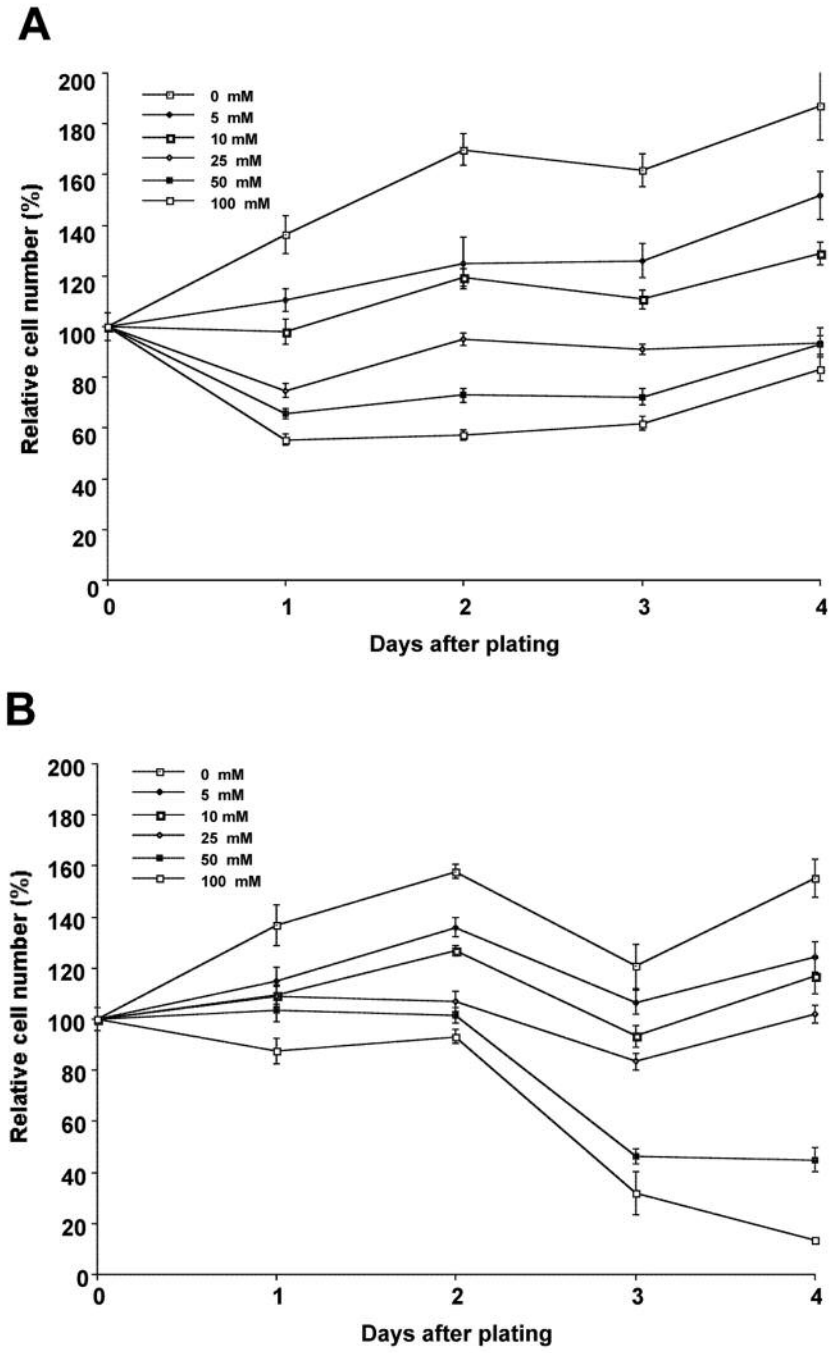

Figure 2. *Effect of synthetic PPARγ ligands on the growth of human ORAL SCC cells in vitro. The synthetic ligand, TRO, was applied to cultured cancer cells, and the effect on cell growth was assessed. TRO significantly suppressed the growth of CA9-22 (A) and HSC-4 (B) in a dose-dependent manner.*

dependent manner. During the experiment, no morphological changes, such as accumulation of lipid droplets, were observed in cancer cells treated with TRO.

TUNEL assay. Apoptosis is characterized by a series of typical morphological features, such as cell shrinkage, fragmentation into membrane-bound apoptotic bodies, and rapid phagocytosis. However, unexpectedly, TRO did not induce apoptosis in both HSC-4 and CA9-22 cells morphologically. Moreover, TUNEL-positive cells were not detectable in TRO-treated CA9-22 (Figure 3A) and TRO-treated HSC-4 cells (Figure 3B).

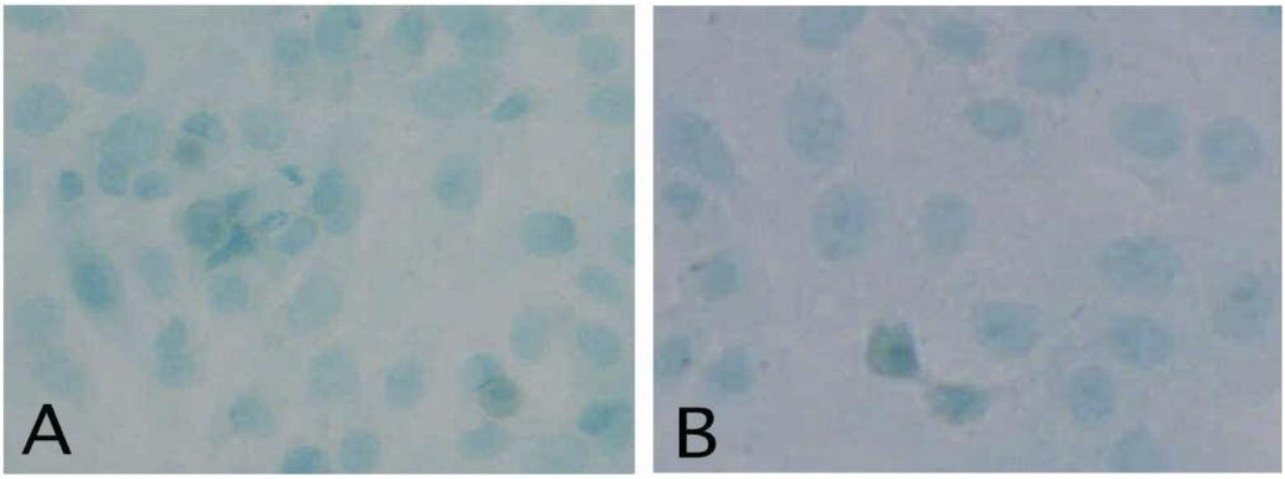

Figure 3. *TUNEL assay. TRO does not induce apoptosis in both HSC-4 and CA9-22 cells morphologically. Moreover, TUNEL-positive cells are not detectable in TRO-treated CA9-22 (A) and TRO-treated HSC-4 cells (B).*

DNA fragmentation. The biochemical hallmark of apoptosis is degradation of DNA by endogenous DNAses that cut the internucleosomal regions into double-stranded DNA fragments of 180-200 base pairs. To study whether TRO could induce apoptosis in HSC-4 and CA9-22 cells, the induction of DNA fragmentation was further analyzed in TRO-treated cancer cells. As shown in Figure 4, TRO clearly induced DNA fragmentation in promyelocytic leukemia cells, HL-60, as a positive control. However, DNA fragmentation was not observed in TRO-treated HSC-4 and CA9-22 cells, although several stacked bands, probably from ribosomal RNA, were seen in both untreated HSC-4 cells and TRO-treated HSC-4 and CA9-22 cells (Figure 4).

Cell-cycle analysis of CA9-22 cells with TRO treatment. Cell-cycle analysis was performed after treatment with TRO for 48 h by a flow cytometer. As shown in Figure 5, untreated CA9-22 cells showed aggressive growth potential, in which cell-fractions in G_1 phase, S phase, and G_2/M phase were 36.6%, 38.1%, and 25.2%, respectively. However, the untreated CA9-22 cells showed a somewhat high population of sub-G_1 fraction (10.8%). On the other hand, CA9-22 cells treated with 50 μM of TRO for 48 h showed an increased fraction in the G_1 phase (63.3%), and decreased fractions in the S and G_2-M phases when compared to the untreated cells. However, CA9-22 cells treated with TRO showed only a small sub-G_1 fraction. These results indicated that TRO did not induce apoptosis, but induced G_1 arrest in CA9-22 cells under this experimental condition.

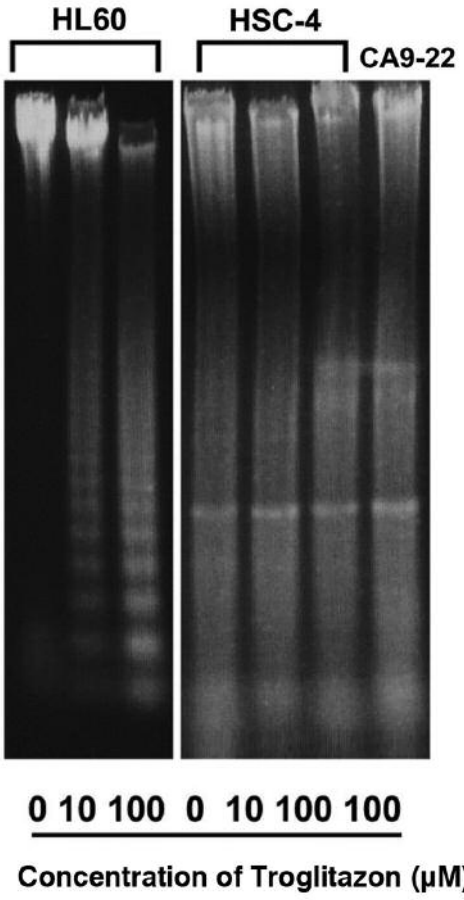

Figure 4. *DNA fragmentation. TRO clearly induced DNA fragmentation in promyelocytic leukemia cells, HL-60, as a positive control. However, DNA fragmentation was not observed in TRO-treated HSC-4 and CA9-22 cells, although several stacked bands, probably from ribosomal RNA, can be seen in both untreated HSC-4 cells and TRO-treated HSC-4 and CA9-22 cells.*

Discussion

This study demonstrated that a PPARγ ligand (TRO) did not induce apoptosis of oral SCC cells (CA9-22, HSC-4), but did inhibit the growth of cells by arresting the cell cycle at the

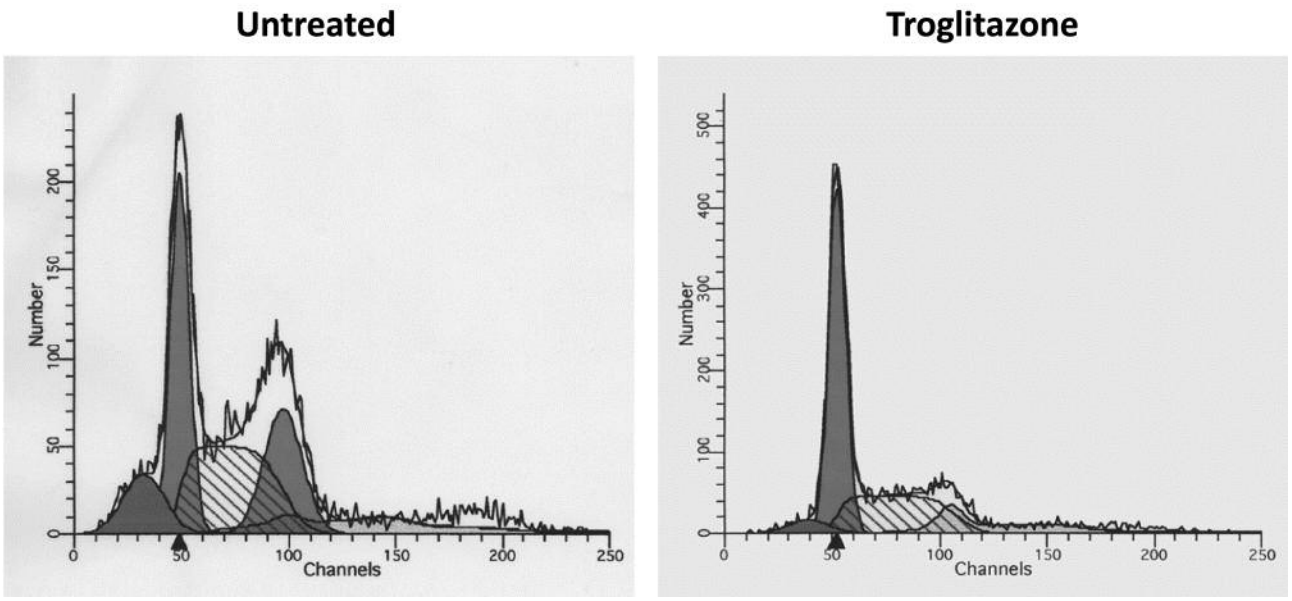

Figure 5. *Cell-cycle analysis of CA9-22 cells under treatment with TRO. Cell cycle analysis was performed after treatment with TRO for 48 h by a flow cytometer. Untreated CA9-22 cells show aggressive growth potentials, in which cell-fractions in G_1 phase, S phase, and G_2/M phase are 36.6%, 38.1%, and 25.2%, respectively. However, the untreated CA9-22 cells show a somewhat high population of sub-G_1 fraction (10.8%). TRO-treated CA9-22 cells show an increased fraction of G_1 phase (63.3%), and decreased fractions of S and G_2-M phases when compared to the untreated cells. However, CA9-22 cells treated with TRO show only a small sub-G_1 fraction.*

G_1 phase. FACS analysis of untreated CA9-22 cells showed that the sub-G_1 population was 35%. However, TRO did not increase, but rather decreased the sub G_1 population. The reasons why untreated CA9-22 cells showed sub G_1 population were as follows: because CA9-22 cells, which almost reached confluency, were used for this analysis, the cells were induced to terminal differentiation by piling-up on the dish or induced to cell death by contact inhibition. When the cells were treated by TRO, the cell cycle was arrested at G_1 phase, and terminal differentiation and cell death were blocked by contact inhibition. Thus, TRO decreased the cell population at sub G_1 phase in CA9-22 cells.

According to the previous report concerning the effect of PPARγ ligand on several cancers, Takashima *et al*. reported that PPARγ was expressed in esophageal adenocarcinoma cells, and TRO inhibited the growth of these cells (26). They also noted that PPARγ was expressed in esophageal SCC, but the expression of PPARγ was lower in the esophageal SCC than in the esophageal adenocarcinoma cells. Although a synthetic ligand, TRO, significantly suppressed the growth of esophageal adenocarcinoma cells, it did not inhibit the growth of the esophageal SCC cells. On FACS analysis, TRO arrested the cell cycle at G_1 phase in the esophageal adenocarcinoma cells, but it did not affect the cell cycle in the esophageal SCC cells. These results are in part consistent with the result obtained from the current study.

Concerning the effect of PPARγ ligands on head and neck cancer, Nikitakis *et al*. (27) reported that PGJ2, which is a natural ligand of PPARγ inhibited the growth of oral SCC cells, but the synthetic ligands rosiglitazone and ciglitazone did not affect the growth of oral SCC cells. They also reported that the inhibitory action of PGJ2 was related to down-modulation of STAT3 and mediated, at least in part, through a PPARγ-independent mechanism. Mila *et al*. (28) demonstrated that PPARγ was expressed in salivary gland tumor tissues, and they detected the co-expression of PPARγ and RXRα, which formed a heterodimer with PPARγ and an active transcriptional factor. They also showed that the PPARγ ligands PGJ2, TRO, and pioglitazone inhibited the growth of salivary gland cancer cells in a transcription-dependent manner (28). Nakashiro *et al*. examined the expression of PPARγ in oral SCC tissues, and they found that PPARγ was expressed in 17 of 28 SCC tissues. Nakashiro *et al*. (29) also noted that synthetic PPARγ ligands including TRO caused significant dose-dependent inhibition of the growth of oral SCC cells, irrespective of the expression of PPARγ and the function of the PPARγ. At present, the reasons for the inconsistency of the present results with their results are unclear, but it may be caused by the different experimental conditions using different cells.

Yoshida *et al*. (30) reported the molecular mechanism of growth suppression and induction of apoptosis by PPARγ

ligands in stomach cancer cells. They demonstrated that TRO inhibited the activity of a transcription factor, NF-κB (30). Motomura *et al*. (31) reported that TRO induced G_1 arrest and inhibited the growth of pancreatic cancer cells through the increase of $p27^{kip1}$. Chung *et al*. (32) reported that TRO and 15dPDJ2 induced growth suppression in anaplastic thyroid cancer cells *via* a p53-independent, but $p21^{waf1}$- and $p27^{kip1}$-dependent cytostatic pathway. Both HSC4 and CA9-22 cells have been reported to harbor mutated p53 gene (33). Therefore, cell-cycle arrest and growth inhibition of HSC4 and CA9-22 cells by treatment with TRO was induced by a p53-independent pathway.

The goal in cancer treatment is to either completely kill or remove cancer cells. However, a new concept for cancer treatment has recently been proposed "tumor dormancy therapy". The concept of this treatment is to extend cancer patients' survival and maintain quality of life. Examples of tumor dormancy therapy include differentiation-inducing therapy, cell cycle-arresting therapy, apoptosis-inducing therapy, anti-metastasis therapy, and anti-angiogenic therapy (34, 35). Several associations of PPAR expression in head and neck cancer, pre-malignant lesions, and adjacent normal mucosa and clinicopathological characteristics of head and neck cancer have been reported (36, 37). The anti-tumorigenic property of PPAR activation was examined in an animal model of head and neck carcinogenesis (38). Furthermore, two clinical trials of PPAR ligands for head and neck pre-malignant lesions have been performed to date, although the results have not yet been reported in the literature (39, 40). Thus, a PPARγ-ligand, TRO, induced cell-cycle arrest in oral SCC cells. Therefore, PPARγ ligands appear useful as chemopreventive agents for HNSCC, or PPARγ ligand differentiation-inducing activity and conventional chemotherapy or radiation therapy might be used as a second or third-line therapy in patients with advanced oral SCC.

Conflicts of Interest

The Authors declare that they have no competing interests.

Authors' Contributions

Conceived and designed experiments: MS, YF, and HK. Performed the experiments: YF. Analyzed the data: MS, YF, and HK. Contributed reagents/materials/analysis tools: MS, YF, NK, DU, YK, CF, TH, and HK. Wrote the paper: MS, YF, and HK.

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Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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