



Original Article

Resveratrol antagonizes thyroid hormone-induced expression of checkpoint and proliferative genes in oral cancer cells



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Abstract *Background/purpose:* Dysregulation of cell cycle checkpoint control may lead to the independence of growth regulating signals. Checkpoint protein such as the PD-1/PD-L1 immune checkpoint involving tumor cells and host immune defense lymphocytes is a well-studied therapeutic target in oncology. Acting at a cell surface receptor on plasma membrane integrin $\alpha v \beta 3$, thyroxine stimulates intracellular accumulation of PD-L1 in cancer cells. Although resveratrol also binds to integrin $\alpha v \beta 3$, it reduces PD-L1 expression.

Materials and methods: In current studies, we investigated the roles of resveratrol and thyroxine in regulating expression of proliferation-related genes and checkpoint genes, *PD-L1*, *BTLA* in two oral cancer cell lines.

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Results: Thyroxine suppressed the expression of pro-apoptotic *BAD* but induced proliferative *CCND1* expression in SSC-25 cells and OEC-M1 cells. It activated expression of *PD-L1* and *BTLA* in both cell lines. On the other hand, resveratrol suppressed the expression of all. Alternatively, it activated *BAD* expression. Thus thyroxine induces checkpoint gene expression which may promote proliferation in cancer cells. Alternatively, resveratrol reverses the stimulatory effects of thyroid hormone to induce anti-proliferation.

Conclusion: These findings provide new insights into the antagonizing effect of resveratrol on the thyroxine-induced expression of checkpoint genes and proliferative genes in oral cancers. © 2019 Association for Dental Sciences of the Republic of China. Publishing services by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Introduction

Negative checkpoint regulators down-regulate immune responses to prevent out-of-proportion immune activation, minimize collateral damage and maintain peripheral self-tolerance.¹ The most actively studied two negative checkpoint regulators are cytotoxic T lymphocyte (CTL)-associated antigen 4 (CTLA-4, CD152) and programmed cell death protein 1 (PD-1, CD279).² They regulate immune responses at completely different levels via different mechanisms.³ CTLA-4 primarily regulates the amplitude of the early stages of T cell activation. On the other hand, PD-1 predominantly regulates effector T cell activity within tissue and tumors where the immune response is ongoing. Receptor-mediated signaling pathways are involved in the induction of PD-L1.⁴ B- and T-lymphocyte attenuator (BTLA) is a checkpoint co-inhibitory receptor classified to CD28 superfamily (also known as the Immunoglobulin [Ig] superfamily). It presents in a wide range of immune cells, including T cells, B cells, and NK cells.⁵ BTLA is structurally and functionally related to CTLA-4 and PD-1.⁶ The increased BTLA level correlates with the development and poor prognosis of gastric cancer.⁷ Therefore, over-expression of checkpoint genes affects the cell cycle, cell proliferation, carcinogenesis, and apoptosis.

Resveratrol induces anti-cancer growth in different cancer cells. By binding to its receptor on integrin $\alpha v \beta 3$, resveratrol inhibits cell proliferation in several types of human cancer cells. Extracellular signal-regulated kinase-1 and -2 (ERK1/2) activated by resveratrol is vital for resveratrol-induced nuclear accumulation of inducible cyclooxygenase (COX)-2. Nuclear phosphorylated ERK1/2 (pERK1/2) in conjunction with the phosphorylation of p53 at Ser-15 promotes anti-proliferation in cancer cells. Thyroid hormones, L-thyroxine (T_4) and 3, 5, 3'-triiodo-L-thyronine (T_3) are able to enhance cancer cell proliferation. Recent studies also indicate that thyroid hormone promotes growth of human lung and ovarian cancer cell through cross-talk between estrogen receptor α (ER α) and cell surface $\alpha v \beta 3$ integrin receptors.^{8,9} Evidence indicates that thyroxine activates ERK1/2 induces PD-L1 gene expression and PD-L1 protein abundance consequently in various cancers.^{10–12} In addition, thyroxine interferes with resveratrol-induced anti-proliferative effect in cancer cells by disrupting resveratrol-induced pERK1/2-dependent nuclear COX-2 complex.

In the current report, we studied the action of T_4 on the expression of checkpoint genes, *PD-L1* and *BTLA*, in addition to proliferative gene, *CCND1* in oral cancer cells. The promotive effects were inhibited by resveratrol. On the other hand, thyroxine suppressed pro-apoptotic gene, *BAD* expression was reversed by resveratrol co-treatment. In addition, resveratrol suppressed thyroxine-induced *PD-L1* accumulation in nuclei. These results suggest that In addition to PD-L1, thyroxine activates other checkpoint gene expression which protects cancer to escape immune-surveillance. On the other, resveratrol is able to reduce the promotive effect of thyroid hormone.

Materials and methods

Cell cultures

Human oral epidermoid carcinoma cell line, OEC-M1 cells, was a gift from Dr. Hsien-Chung Chiu (Department of Periodontology, School of Dentistry, National Defense Medical Center and Tri-Service General Hospital, Taipei, Taiwan). Human squamous carcinoma of the tongue, SCC-25 cells (ATCC[®] CRL-1628[™]) were obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA). SCC-25 cells had been tested and authenticated by Bioresource Collection and Research Center (BCRC, Hsinchu, Taiwan) (isoenzyme analysis, Mycoplasma, cytogenetics, tumorigenesis, receptor expression testing). Cells were maintained in RPMI-1640 supplemented with 10% FBS in the incubator with 5% CO₂ at 37 °C, and then used for experiments until passage 15. Before the study, cells were placed in 0.25% hormone-depleted serum-supplemented medium for 2 days.

Quantitative real-time PCR

As previous description,^{13–16} total RNA was extracted by Illustra RNeasy Mini RNA Isolation Kit (GE Healthcare Life Sciences, Buckinghamshire, UK) with eliminating genomic DNA. cDNA was prepared using one μ g of DNase I-treated total RNA by RevertAid H Minus First Strand cDNA Synthesis Kit (Life Technologies Corp.). The cDNA was used as the template for real-time PCR reactions. The real-time PCR reactions were conducted using QuantiNova[™] SYBR[®] Green PCR Kit (QIAGEN, Hilden, Germany) on CFX Connect[™]

Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, U.S.A.). This involved an initial denaturation at 95 °C for 5 min, followed by 40 cycles of denaturing at 95 °C for 5 s and combined annealing/extension at 60 °C for 10 s, as detailed in the manufacturer's instructions. The primer sequences were shown as follows: *Homo sapiens* programmed death ligand 1 (*PD-L1*) (*CD274*), forward 5'-GTTGAAGGACCAGCTCTCCC-3' and reverse 5'-ACCCCTGCATCCTGCAATTT-3' (Accession No. AY254342.1); *H. sapiens* B and T lymphocyte associated (*BTLA*), forward 5'-GAGGAGAGTAGGAAGAGCCTG-3' and reverse 5'-GCAAAAACGTGGTAGAGCGG-3' (Accession No. NM_181780.3); *H. sapiens* cyclin D1 (*CCND1*), forward 5'-CAAGGCCTGAACCTGAGGAG-3' and reverse 5'-GATCACTCTGGAGAGGAAGCG-3' (Accession No. NM_053056); *H. sapiens* Bcl-2-associated death promoter (*BAD*), forward 5'-CTTTAAGAAGGGACTTCTCGCC-3' and reverse 5'-AAGTCCGATCCCACCAGGA-3' (accession no.: NM_004322), forward 5'-; *H. sapiens* 18S ribosomal RNA (*18S*), forward 5'-GTAACCCGTTGAACCCATT-3' and reverse 5'-CCATCAATCGGTAGTAGCG-3' (Accession No. NR_003286). Calculations of relative gene expression (normalized to 18S as reference gene) were performed according to the $2^{-\Delta\Delta CT}$ method. Fidelity of the PCR reaction was determined with melting temperature analysis.

Confocal microscopy

Exponentially growing oral cancer OEC-M1 cells and SCC-25 cells were seeded on sterilized cover glasses (Paul Mariefeld, Lauda-Königshofen, Germany). After exposure to 0.25% stripped FBS-containing medium for 2 days, oral cancer cells were treated with 40 μ M resveratrol, 10^{-7} M T₄, or their combination for 24 h. As previous description,^{17,18} cells were fixed with 4% paraformaldehyde in phosphate-

buffered saline (PBS) for 30 min and then permeabilized in 0.06% Triton X-100 for 30 min. Cells were incubated with a monoclonal rabbit anti-PD-L1 antibody (1:100, GeneTex International Corporation, Hsinchu City, Taiwan), followed by an Alexa-647-labeled goat anti-rabbit antibody (1:300, GeneTex) and mounted in EverBrite Hardset mounting medium with DAPI (Biotium, Fremont, CA). The fluorescent signals from PD-L1 were recorded and analyzed with the TCS SP5 Confocal Spectral Microscope Imaging System (Leica Microsystems, Wetzlar, Germany). The figures shown are representative of at least four fields for each experimental condition.

Statistical analysis

All of the collected data of immunoblot and nucleotide densities were analyzed by IBM®SPSS® Statistics software version 19.0 (SPSS Inc., Chicago, IL, USA). Two tails *Student's t-test* was conducted and considered significant at *p*-values < 0.05 (*, or #), 0.005 (** or ##) and 0.001 (***) or ###).

Results

Effect of thyroid hormone and resveratrol on expression of proliferative gene and pro-apoptotic gene in human oral cancer cells. Human oral cancer SCC-25 cells were treated with 10^{-7} M T₄, 40 μ M resveratrol and their combination for 24 h. Cells were harvested and total RNA was extracted. qPCR was conducted for proliferative gene, *CCND1* and pro-apoptotic gene, *BAD*. Thyroxine induced *CCND1* expression but inhibited *BAD* expression (Fig. 1). On the other hand, resveratrol reversed thyroxine-induced effect in SCC-25 cells (Fig. 1).

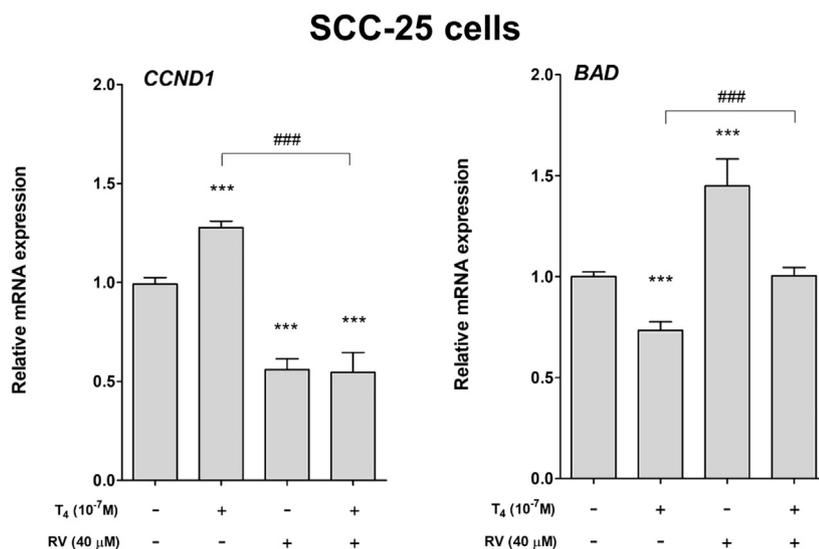


Figure 1 Effect of thyroid hormone and resveratrol on expression of proliferative gene and pro-apoptotic gene in human oral cancer SCC-25 cells. Human oral cancer SCC-25 cells were treated with 10^{-7} M T₄, 40 μ M resveratrol and their combination for 24 h. Cells were harvested and total RNA was extracted. qPCR was conducted for proliferative gene, *CCND1* and pro-apoptotic gene, *BAD*. Number of independent experiments (N) = 3. (Data are expressed as mean \pm SD; ****p* < 0.001, compared with untreated control; ###*p* < 0.001, compared with T₄ treatment.)

Parallel studies were conducted by using another human oral cancer OEC-M1 cell line. Cells were treated with 10^{-7} M T_4 , 40 μ M resveratrol and their combination for 24 h. Cells were harvested and total RNA was extracted. qPCR was conducted for proliferative gene, *CCND1* and pro-apoptotic gene, *BAD*. Thyroxine induced *CCND1* expression but inhibited *BAD* expression (Fig. 2). On the other hand, resveratrol reversed thyroxine-induced effect in OEC-M1 cells (Fig. 2).

Effect of thyroid hormone and resveratrol on expression of checkpoint genes in human oral cancer cells. Human oral cancer SCC-25 cells were treated with thyroid hormone (10^{-7} M), resveratrol or their combination for 24 h. Cells were harvested and total RNA was extracted. qPCR was conducted for two checkpoint genes, *PD-L1* and *BLTA*. Expression of *PD-L1* and *BLTA* was significantly induced by thyroid hormone (T_4) (Fig. 3).

Parallel studies were conducted by using another human oral cancer OEC-M1 cell line. Cells were treated with 10^{-7} M T_4 , 40 μ M resveratrol and their combination for 24 h. Cells were harvested and total RNA was extracted. qPCR was conducted for *PD-L1* and *BLTA*. Thyroxine induced *PD-L1* and *BLTA* expression (Fig. 4). On the other hand, resveratrol reversed thyroxine-induced effect in OEC-M1 cells (Fig. 4).

Resveratrol inhibits thyroxine-induced PD-L1 gene expression and nuclear accumulation in oral cancer cells. Thyroxine induces *PD-L1* gene expression and PD-L1 protein accumulation. Thyroxine-induced PD-L1 can be blocked by co-treatment of resveratrol.¹⁹ To examine the effects of thyroxine and resveratrol on PD-L1 expression in oral cancer, OEC-M1 cells were treated with 10^{-7} M T_4 , 40 μ M resveratrol or their combination for 24 h. Thyroxine increased PD-L1 nuclear accumulation. Resveratrol reduced constitutive expression of PD-L1 (Fig. 5). Interestingly, the

upregulated PD-L1's nuclear accumulation by thyroxine was diminished in the presence of resveratrol (Fig. 5). These results indicated that resveratrol can not only inhibit thyroxine-induced PD-L1 expression but also block its nuclear accumulation when cells were co-incubated with thyroxine and resveratrol.

Discussion

Recently, we have shown that thyroxine induces *PD-L1* expression and its protein accumulation in colorectal cancer, breast cancer, and ovarian cancer cells,^{8,12} Thyroid hormone has been shown to induce cancer cell growth in various types of cancers. From clinical view, the thyroid hormone axis is usually normal and thus activity of the PD-1/PD-L1 tumor cell self-defense system is in part due to endogenous thyroid hormone. In addition, thyroxine also induced the expression of PD-L1 and BTLA in oral cancer cells (Figs. 2 and 4).

BTLA contains an immune-receptor tyrosine-based inhibitory motif (ITIM) and an immune-receptor tyrosine-based switch motif (ITSM), structurally similar to PD-1 and CTLA-4.²⁰ Its ligand HVEM (also known as TNFRSF14) belongs to the tumor necrosis factor receptor (TNFR) superfamily. HVEM presents commonly on hematopoietic cells and on a variety of parenchymal cells such as breast, melanoma, esophageal, colorectal, and ovarian cancer cells.^{21–25} The combination of BTLA to cysteine-rich domains 1 (CRD1) of HVEM makes this pathway an important cross-talk between Ig and TNF superfamily.^{26,27} Furthermore, the BTLA/HVEM pathway appears to be a new possible approach of immune escape and is considered to be a critical factor in the physiological process of inflammation and tumorigenesis.

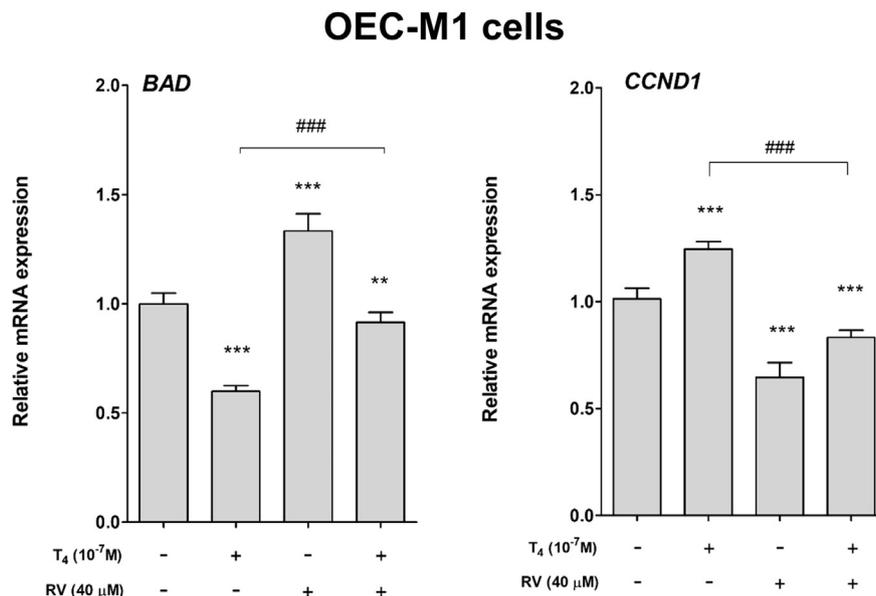


Figure 2 Effect of thyroid hormone and resveratrol on expression of proliferative gene and pro-apoptotic gene in human oral cancer OEC-M1 cells. Human oral cancer OEC-M1 cells were treated with 10^{-7} M T_4 , 40 μ M resveratrol and their combination for 24 h. Cells were harvested and total RNA was extracted. qPCR was conducted for proliferative gene, *CCND1* and pro-apoptotic gene, *BAD*. N = 3. (Data are expressed as mean \pm SD; ** p < 0.01, *** p < 0.001, compared with untreated control; ### p < 0.001, compared with T_4 treatment.)

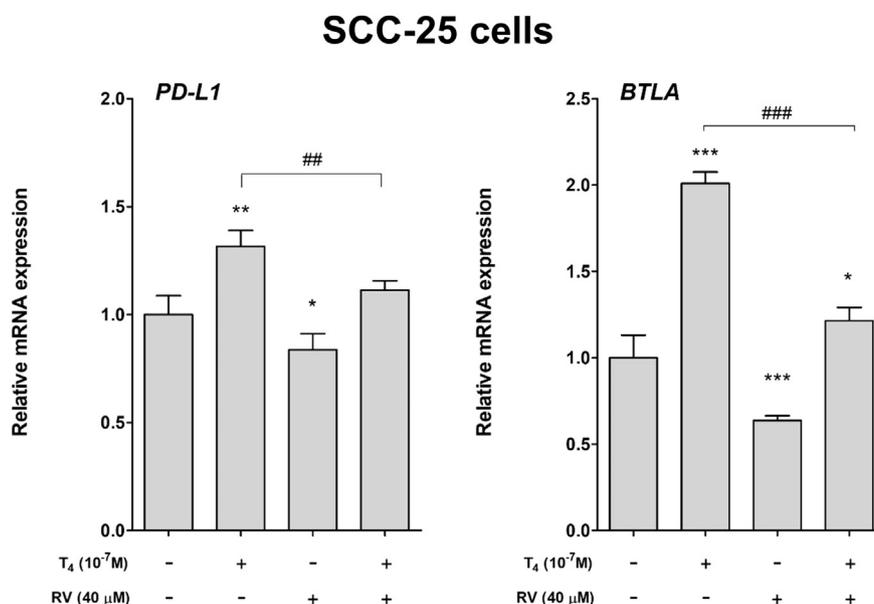


Figure 3 Effect of thyroid hormone and resveratrol on expression of checkpoint genes in human oral cancer SCC-25 cells. Human oral cancer SCC-25 cells were treated with thyroid hormone (10^{-7} M), resveratrol or their combination for 24 h. Cells were harvested and total RNA was extracted. qPCR was conducted for two checkpoint genes, *PD-L1* and *BTLA*. N = 3. (Data are expressed as mean \pm SD; * p < 0.05, ** p < 0.01, *** p < 0.001, compared with untreated control; ## p < 0.01, ### p < 0.001, compared with T₄ treatment.)

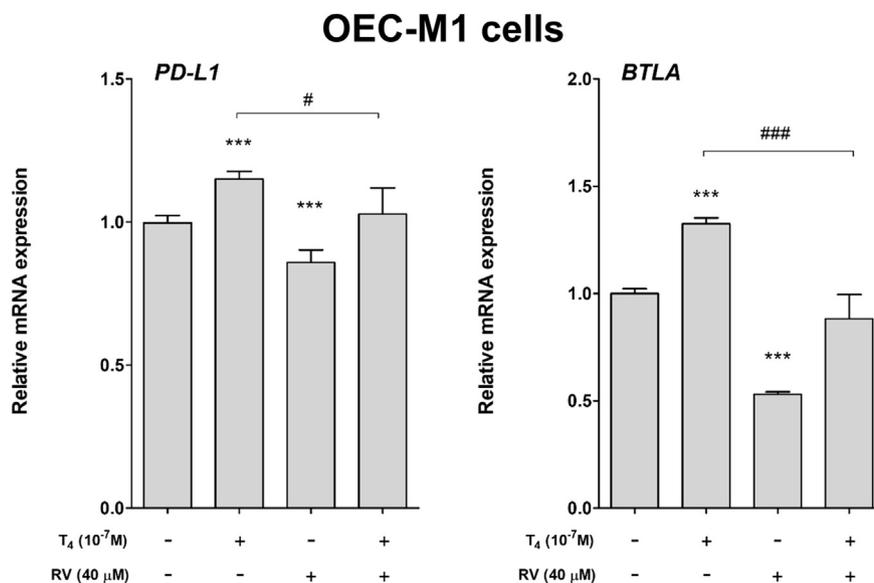


Figure 4 Effect of thyroid hormone and resveratrol on expression of checkpoint genes in human oral cancer OEC-M1 cells. OEC-M1 cells were treated with thyroid hormone (10^{-7} M), resveratrol or their combination for 24 h. Cells were harvested and total RNA was extracted. qPCR was conducted for two checkpoint genes, *PD-L1* and *BTLA*. N = 3. (Data are expressed as mean \pm SD; *** p < 0.001, compared with untreated control; # p < 0.05, ### p < 0.001, compared with T₄ treatment.)

Thyroxine-induced expression of *PD-L1* and *BTLA* was coincidental with increased expression of proliferative gene *CCND1* (Figs. 3 and 4) and down-regulated pro-apoptotic gene, *BAD* expression (Figs. 3 and 4) in two oral cancer cell lines examined.

PD-1 and PD-L1 may have additional functions within tumor cells that are independent of the checkpoint are

indicated by actions of a thyroid hormone analogue, L-thyroxine, on these checkpoint components.¹² Estrogen has been shown to upregulate PD-L1 protein expression in ER α -positive endometrial and breast cancers cells to suppress immune functions of T cells in the tumor microenvironment.²⁸ Another example of hormone-driven cancer progression via PD-L1 pathway is that 1,25-dihydroxyvitamin D

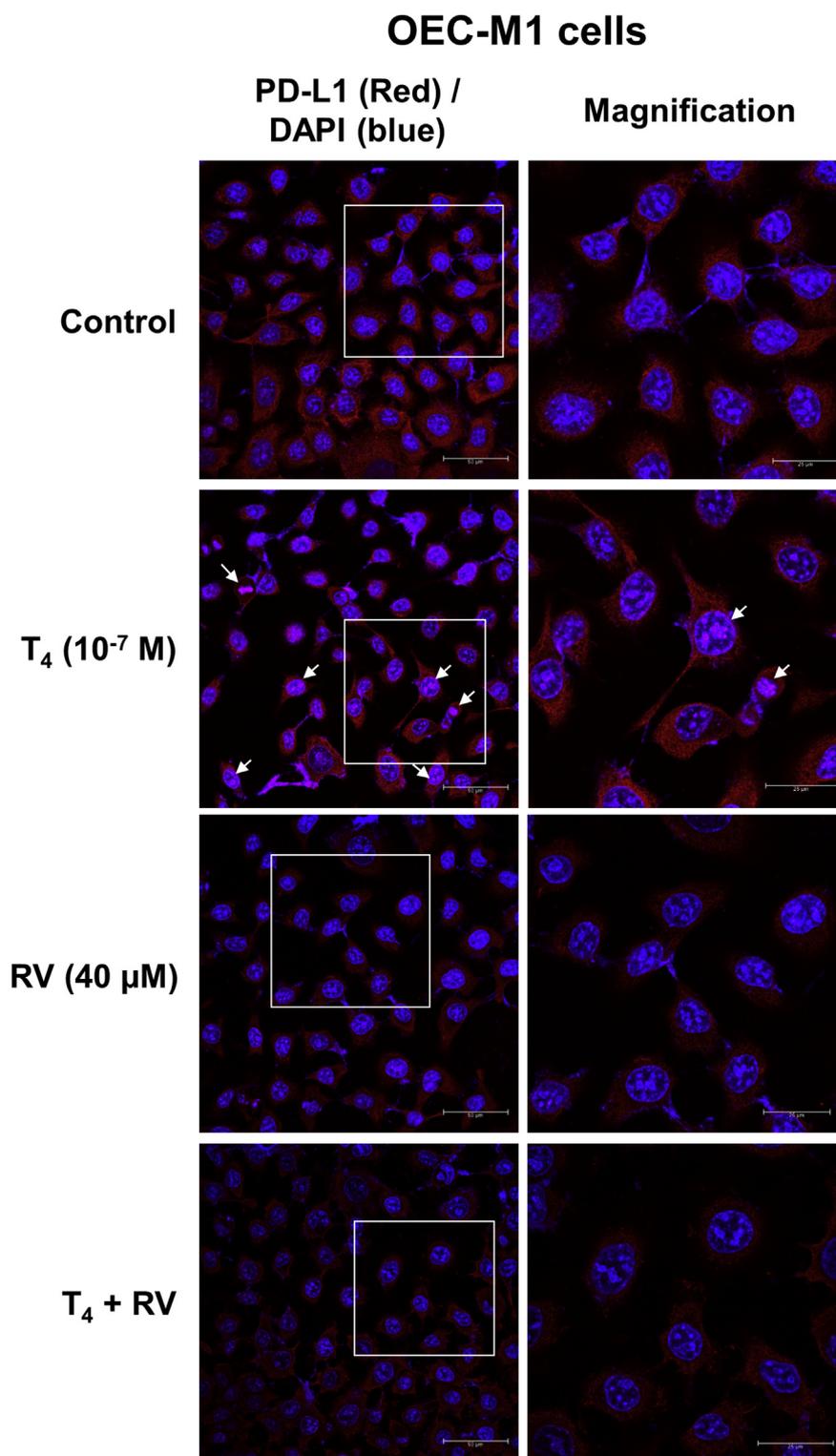


Figure 5 Resveratrol inhibits thyroxine-induced PD-L1 nuclear accumulation in oral cancer cells. OEC-M1 cells were seeded in glass cover slide and were treated with 10^{-7} M T₄, 40 μM resveratrol or their combination for 24 h. Cells were fixed for confocal microscopic analysis of PD-L1 expression (red color) and its nuclear accumulation (purple color, indicated by arrow marker). The nuclei were stained by DAPI as counter staining (blue color).

(1,25D) has shown to be a direct transcriptional inducer of the human genes encoding PD-L1 and PD-L2 through the vitamin D receptor and suggests elevated vitamin D signaling in humans could suppress anti-tumor immunity.²⁹

Interestingly, 17β-Estradiol does not increase PD-L1 mRNA transcription, but stabilized PD-L1 mRNA.²⁸ On the other hand, vitamin D²⁹ and thyroxine^{8,12,16} are able to induce *PD-L1* expression. In addition, 17β-Estradiol's

effects were only observed in estrogen receptor α (ER α)-positive Ishikawa and MCF-7 cells, but not in ER α -negative MDA-MB-231 cells.²⁸ 1,25-dihydroxyvitamin D (1,25D) has shown to induce PD-L1 via the vitamin D receptor.²⁹ On the other hand, thyroxine-induced PD-L1 expression may be integrin α v β 3-dependent.^{12,16}

Tumor cell-induced PD-L1 expression involves several intracellular signaling pathways linked to include nuclear factor (NF)- κ B, ERK1/2, phosphoinositide 3-kinase (PI3K), mammalian target of rapamycin (mTOR), and Janus kinase/signal transducers and activators of transcription (JAK/STAT). Estrogen increases expression of PD-L1 protein via activation of phosphoinositide 3-kinase (PI3K)/Akt pathway in Ishikawa and Michigan Cancer Foundation-7 (MCF-7) cells.²⁸ Phosphoinositide 3-kinase and Akt inhibitors could block estrogen's effects. Thyroxine-induced PD-L1 expression is ERK1/2-dependent.¹²

Resveratrol has been shown to antagonize thyroid hormone-induced proliferation.¹⁶ Both thyroid hormone and resveratrol activate ERK1/2 by binding to cell surface integrin α v β 3.^{9,19} Via ERK1/2 activation resveratrol induces nuclear COX-2 accumulation, p53 phosphorylation leading to COX-2-phosphorylated p53-dependent apoptosis.¹¹ On the other hand, thyroxine induces ERK1/2 activation to activate β -catenin-HMGA2-dependent proliferation.¹⁰ In addition, thyroid hormone induces *PD-L1* expression and its protein PD-L1 traps inducible COX-2 in the cytosol in resveratrol-treated cells.¹⁹ Under physiological condition, resveratrol was able to retain PD-L1 in the cytosol (Fig. 5) or reduce thyroxine-induced PD-L1 nuclear accumulation (Fig. 5).

In summary, thyroxine induces expression of proliferative genes such as *CCND1* and checkpoint genes such as *PD-L1* and *BTLA* in oral cancer cells. On the other hand, resveratrol reduces expression of *PD-L1*, *BTLA*, and *CCND1* but increase expression of pro-apoptotic genes such as *BAD*. How to manage the resveratrol concentration to overcome the stimulatory effect of thyroid hormone concentration in physiological micro-environment will be a big task for future therapeutic concern by using resveratrol in oral cancers.

Conflicts of interest

All co-authors declare no competing financial interests.

Acknowledgments

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