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Mild hyperthermia upregulates PD-L1 in the tumor microenvironment and enhances antitumor efficacy of PD-L1 blockade in murine squamous cell carcinoma

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ABSTRACT

Head and neck squamous cell carcinoma (HNSCC) has a low five-year survival rate because of its high rate of recurrence and metastasis. After surgical resection or radiation, the main treatments for HNSCC, patients sometimes experience functional or aesthetic disorders. Therefore, there is a great demand for the development of non-surgical treatment strategies to improve clinical outcomes and patients' quality of life. One such non-surgical treatment is mild hyperthermia (mHT). Many studies have investigated combination treatments with mHT and immune checkpoint inhibitors in preclinical settings. However, there have been no detailed reports on the effects of mHT on immune checkpoint molecules. Here, we investigated the effects of mHT on the tumor microenvironment (TME), particularly on programmed cell death receptor-1 (PD-1)/programmed cell death ligand-1 (PD-L1), in SCCVII cells and a squamous cell carcinoma mouse model. First, we found that *PD-L1* mRNA levels and surface PD-L1 expression significantly increased after mHT. Second, a single tumor model was used to determine the effect of HT on the TME. mHT enhanced the accumulation of $CD4^+$ and $CD8^+$ T cells, elevated PD-L1 expression in the TME, and decreased the PD-1 positive rate of $CD4^+$ T cells. Finally, using a bilateral tumor model, we found that anti-PD-L1 monotherapy and combination therapy resulted in longer survival than the isotype control or mHT monotherapy. Moreover, the combination therapy resulted in a significantly higher survival rate than anti-PD-L1 monotherapy. In conclusion, our findings elucidate changes in PD-L1 expression in the TME and strengthen the rationale for mHT and PD-L1 blockade combination therapy.

Keywords: head and neck squamous cell carcinoma, mild hyperthermia, programmed cell death ligand-1, tumor microenvironment, abscopal effect

Abbreviations:
HNSCC: head and neck squamous cell carcinoma
PD-1: programmed cell death receptor-1
PD-L1: programmed cell death ligand-1

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TME: tumor microenvironment
HT: hyperthermia
mHT: mild hyperthermia

INTRODUCTION

Worldwide, over 890,000 individuals are diagnosed with head and neck squamous cell carcinoma (HNSCC) each year, with approximately 450,000 fatalities.[1] Despite advances in treatment, the five-year survival rates are still low, mainly in advanced HNSCC due to recurrence and metastasis.[2] Currently, surgical resection is the main treatment for HNSCC, which causes severe functional and aesthetic disorders.[3,4] There is great demand for developing non-surgical treatment strategies to improve clinical outcomes and patients' quality of life.

The use of hyperthermia (HT), a non-surgical method for cancer treatment, has been steadily increasing since 1980. HT is classified according to the target temperature. A temperature above 50 °C is used for thermo-ablation, and the use of 41–45 °C is referred to as mild HT (mHT).[5] mHT activates local and systemic immunity with few side effects and can be repeated without limiting the number of cycles, making it suitable for combination with other therapies.[6-10] Among several heating methods, Yoshikawa et al developed a laser hyperthermia system for cervical cancer.[11] This system is suitable and safe for treating superficial lesions, such as HNSCC.

Programmed cell death receptor-1 (PD-1) and programmed cell death ligand-1 (PD-L1) are often activated in cancer and play important roles in tumor escape from host immunity by inhibiting cytotoxic T cell function.[12] Inhibition of the PD-1/PD-L1 pathway has shown a remarkable clinical response in patients with advanced HNSCC and has become a powerful therapeutic strategy.[13] However, most patients with HNSCC are resistant to PD-1/PD-L1 blockade monotherapy. Therefore, combination therapy with PD-1/PD-L1 and other therapeutic modalities has been attempted for many tumors in clinical and preclinical settings to achieve higher therapeutic efficacy.[14,15] Previous reports have shown that the combination of mHT and PD-1/PD-L1 inhibition is effective in mouse models of malignant melanoma, breast cancer, colon cancer, and pancreatic cancer.[16,17] Nevertheless, no reports have examined changes in PD-1/PD-L1 induced by mHT in detail.

Here, we investigated the effects of mHT on the tumor microenvironment (TME), especially on PD-1/PD-L1, and the efficacy of a combination strategy using mHT and an anti-PD-L1 antibody in a squamous cell carcinoma mouse model.

MATERIALS AND METHODS

Cell line

SCCVII, a murine squamous cell carcinoma cell line, was gifted by Dr. Masunaga (Kyoto University). Cells were cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St. Louis, MO, USA) containing 10% fetal bovine serum (GE Healthcare Bioscience, Little Chalfont, UK) and 1% penicillin-streptomycin (FUJIFILM Wako Chemicals, Osaka, Japan) at 37 °C in 5% CO_2.

In vitro hyperthermia treatment

Approximately 80% confluent SCCVII cells were incubated at 41, 43, or 45 °C in 5% CO_2 humidity for 1 h. Cells were harvested with trypsin-ethylenediaminetetraacetic acid and used for

subsequent experiments.

Cell proliferation assay

Cells were plated at a density of 5.0×10^3 cells/well in 96-well plates and treated with HT as described above. Twenty-four hours after HT, cell viability was measured using a Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions. The absorbance was measured at 450 nm using an Infinite 200 PRO microplate reader (Tecan, Männedorf, Switzerland).

Apoptosis assay

Cells cultured in 6-well plates were treated at 43 °C HT as described above and detached after 24 h of incubation. Detached cells were washed with staining buffer (Becton Dickinson, Franklin Lakes, NJ, USA) and stained with the Annexin V Apoptosis Detection Kit FITC (Thermo Fisher Scientific) according to the manufacturer's protocol. A total of 1×10^5 stained cells were analyzed with a BD FACS Canto II (Becton Dickinson), and data analysis was performed using the FlowJo software (version 10.9.0; Becton Dickinson).

Real-time quantitative reverse transcription polymerase chain reaction

Cells were plated in a 10-cm dish and treated with HT as previously described. Seventy-two hours after HT, total RNA was isolated from SCCVII cells using TRIzol LS Reagent (Thermo Fisher Scientific, Waltham, MA, USA), and cDNA was synthesized using ReverTra Ace qPCR RT Master Mix and gDNA Remover (TOYOBO, Osaka, Japan) according to the manufacturer's protocols. Real-time quantitative polymerase chain reaction was performed using the THUNDERBIRD SYBR qPCR Mix (TOYOBO). The expression of *PD-L1* was normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and relative changes in gene expression were determined using the $2^{-\Delta\Delta Ct}$ method. The primers used in this study are listed in Table 1.

Flow cytometry of the in vitro model

Seventy-two hours after HT, the collected cells were stained with a PE-conjugated Anti-mouse CD274 (PD-L1) antibody (Becton Dickinson) and 7-amino-actinomycin D (Immunostep, Salamanca, Spain). Flow cytometry was performed using the BD FACS Canto II.

Tumor-bearing mice

Six-week-old male C3H/HeJ mice were purchased from CLEA Japan (Tokyo, Japan). All animal experiments were conducted in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals and approved by the Nagoya University School of Medicine Animal Care and Use Committee (permit number M220167-001). To establish a single tumor model, SCCVII cells (5×10^5) were suspended in 50 μL of phosphate-buffered saline (PBS) and subcutaneously injected at the left flank of the mice on day 0. To create a bilateral

Table 1 Sequences of primers used in RT-qPCR

Name of gene	Forward sequence	Reverse sequence
GAPDH	cctggagaaacctgccaagt	tgaagtcgcaggagacaacc
PD-L1 (*CD274*)	acgcctcacttgctcattac	taaggagaggcagtagctgtc

RT-qPCR: real-time quantitative reverse transcription polymerase chain reaction
GAPDH: glyceraldehyde-3-phosphate dehydrogenase

tumor model, SCCVII was implanted on the left side on day 0 and on the right side on day 3. The tumor diameter was measured using a caliper and calculated using the following formula: tumor volume = $L \times W^2 \times 0.5$, where L is the longest dimension and W is the perpendicular dimension. A death event was defined as a tumor that reached 1,500 mm^3 in volume, or 15 mm in any dimension, or the development of ulceration to evaluate survival.

mHT and antibody administration

mHT was performed by exposure to external laser hyperthermal therapy using the diode laser system ADL-20 (Asuka Medical, Kyoto, Japan). Tumor temperature was measured and kept around 43 °C. For the PD-L1 blockade, 200 μg of anti-PD-L1 antibody (BioXcell, Lebanon, NH, USA) was administered intraperitoneally. Equal amounts of rat IgG2b isotype control antibody (BioXcell) were injected into the control group.

Flow cytometry of the in vivo mouse model

Tumors were harvested on day 16, cut into 2- to 3-mm pieces, and dissociated using gentleMACS dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany) in digestion buffer with 200 U/mL collagenase IV (Worthington Biochemical, Lakewood, NJ, USA) and 10 U/mL DNAse (Sigma-Aldrich) in Hank's Balanced Salt Solution for 30 min at 37 °C. After digestion, the cell suspensions were passed through a 100-μm cell strainer, and red blood cells were lysed with a Red Blood Cell Lysis Buffer (BioLegend, San Diego, CA, USA). Cell labeling was performed at 4 °C in the presence of TrueStain FcX PLUS (BioLegend) for CD16/32 blockade. The antibodies and viability dye are listed in Table 2. Flow cytometry was performed on a BD FACS Canto II (Becton Dickinson, Franklin Lakes, NJ, USA) and analyzed using the FlowJo software (version 10.9.0; Becton Dickinson).

Statistical analyses

Unless otherwise indicated, data are expressed as the mean ± SEM. The results were evaluated using the Student's t-test between two groups, one-way analysis of variance for multi-group analysis, and the Kaplan-Meier method with the log-rank test to compare survival between groups. All data were analyzed using GraphPad Prism 8 (GraphPad Software, Boston, MA, USA).

Table 2 The antibodies and viability dye used in flow cytometry of the in vivo mouse model

Antibody or viability dye	Clone	Manufacture
FITC anti-mouse CD45	30-F11	BioLegend
PE Rat Anti-Mouse CD274 (PD-L1)	MIH5	BD
PerCP/Cyanine5.5 anti-mouse/human CD11b	M1/70	BioLegend
PE/Cyanine7 anti-mouse CD279 (PD-1)	29F.1A12	BioLegend
APC anti-mouse CD4	RM4-4	BioLegend
APC/Cyanine7 anti-mouse CD8α	53-6.7	BioLegend
BV421 Rat Anti-mouse CD3	17A2	BD
Zombie Aqua Fixable Viability kit	–	BioLegend

BD: BD Biosciences

RESULTS

Effect of mHT on SCCVII cells

SCCVII cells were treated with HT, and cell proliferation, cell death, and PD-L1 expression were examined. First, the proliferative ability of SCCVII cells decreased at 43 °C or above (Fig. 1A). Based on this result, subsequent experiments were performed at 43 °C. Next, cell death caused by mHT was analyzed using flow cytometry. Both apoptosis and necrosis significantly increased (Fig. 1B). PD-L1 expression was compared with that in untreated SCCVII cells using real-time quantitative reverse transcription polymerase chain reaction and flow cytometry. *PD-L1* mRNA levels and surface PD-L1 expression significantly increased after mHT (Fig. 1C, D).

mHT inhibits SCCVII tumor growth

A single-tumor model was used to determine the effects of mHT on the TME. The experimental design is shown in Fig. 2A. First, we confirmed that the semiconductor laser system rapidly increased the tumor surface temperature and maintained it around 43 °C (Fig. 2B). After three rounds of HT, the tumor volume was significantly smaller in the HT group than that in the control group (Fig. 2C).

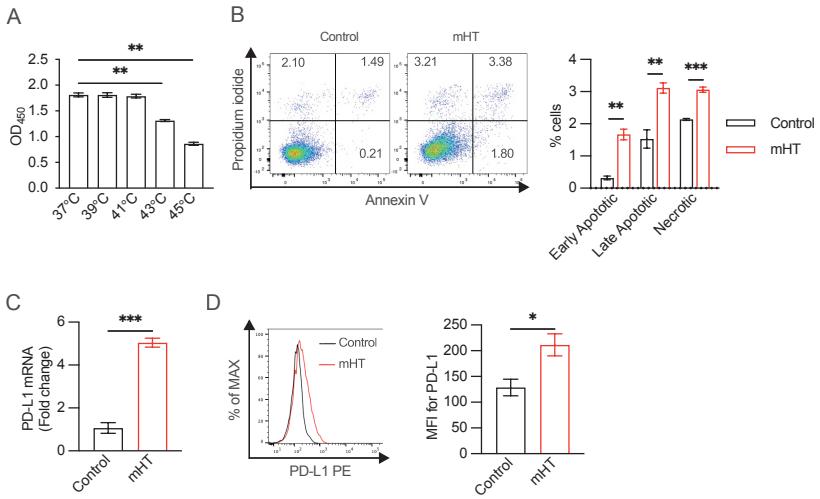

Fig. 1 HT upregulates PD-L1 expression in SCCVII cells in vitro

Fig. 1A: Proliferation of SCCVII cells after HT at various temperatures as determined using Cell Counting Kit-9 (n = 6 independent samples).

Fig. 1B: Cell death analyzed using flow cytometry 24 h after 43 °C mHT (n = 3 independent samples).

Fig. 1C–D: PD-L1 expression of SCCVII cells measured using RT-qPCR (C) and flow cytometry (D) (n = 3 independent samples).

HT: hyperthermia
mHT: mild hyperthermia
MFI: mean fluorescent intensity
OD: optical density
PD-L1: programmed cell death ligand-1
RT-qPCR: real-time quantitative reverse transcription polymerase chain reaction
Data are expressed as mean ± SEM.
*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

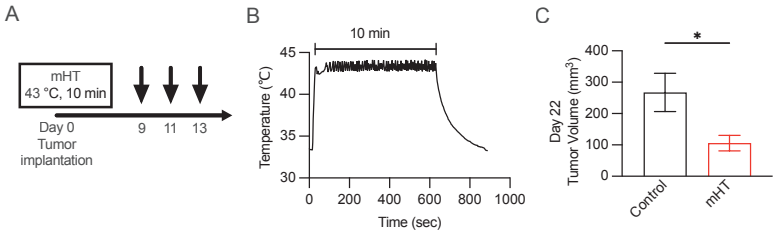

Fig. 2 mHT inhibited SCCVII tumor growth in vivo

Fig. 2A: Schematics of the experimental design.

Fig. 2B: Temperature of tumor surface during mHT.

Fig. 2C: Tumor volume in control and mHT groups on day 22 (n = 4).

mHT: mild hyperthermia

Data are expressed as mean ± SEM.

*p < 0.05, **p < 0.01, ***p < 0.001.

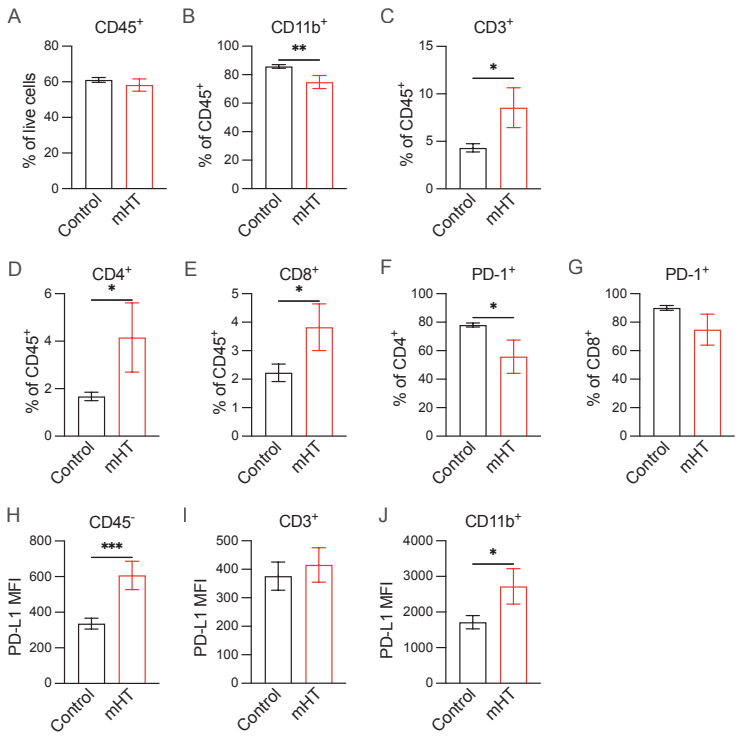

Fig. 3 mHT altered the tumor microenvironment in the SCCVII tumor

Fig. 3A–J: Flow cytometric data of CD45$^+$ cells (A), CD11b$^+$ cells (B), CD3$^+$ cells (C), CD4$^+$ cells (D), CD8$^+$ cells (E), PD-1 positive rate of CD4$^+$ cells (F) and CD8$^+$ cells (G), PD-L1 mean fluorescent intensity of CD45$^-$ cells (H), CD3$^+$ cells (I), and CD11b$^+$ cells (J) (n = 15 in control group; n = 8 in mHT group).

mHT: mild hyperthermia

MFI: mean fluorescent intensity

PD-L1: programmed cell death ligand-1

Data are expressed as mean ± SEM.

*p < 0.05, **p < 0.01, ***p < 0.001.

Two independent experiments were conducted, and all data obtained from the experiments were included in the analysis.

mHT modulates the tumor microenvironment

We performed flow cytometry to elucidate the differences in the TME between control and HT-treated tumors. HT enhanced the accumulation of $CD3^+$ and decreased the number of $CD11b^+$ cells while it did not affect the percentage of $CD45^+$ cells (Fig. 3A–C). Among $CD3^+$ cells, the number of both $CD4^+$ and $CD8^+$ T cells increased (Fig. 3D, E). We further investigated changes in the PD-1/PD-L1 pathway. In the control group, SCCVII tumors showed high PD-1 positivity in both $CD4^+$ and $CD8^+$ T cells (Fig. 3F, G). Notably, the PD-1 positivity rate in $CD4^+$ T cells decreased after HT (Fig. 3F). PD-L1 levels were low in $CD45^-$ cells and moderate in $CD11b^+$ cells at baseline, and HT increased PD-L1 expression in these cell populations (Fig. 3H–J).

HT and anti-PD-L1 blockade combination therapy leads to a stronger anti-tumor effect in a bilateral SCCVII tumor model

A bilateral tumor model was used to investigate the combined effects of HT and anti-PD-L1 antibodies. The treatment schematic is shown in Fig. 4A. SCCVII cells were implanted in the left (primary, HT-treated) and right (secondary, not HT-treated) flanks of the mice. On the right side, implantation of cells was performed 3 days after that on the left side to mimic a primary tumor and a smaller metastatic tumor. Nine days after primary tumor implantation, tumors on the left flank were treated with a 43 °C HT for 10 min. Anti-PD-L1 treatment was also initiated on day 9, thrice every third day. In the left tumor, HT, PD-L1 inhibition, or their combination inhibited tumor growth (Fig. 4B). In distant right tumors, combination therapy tended to suppress tumor growth, but the effect was not significant (Fig. 4C). Anti-PD-L1 monotherapy and combination therapy resulted in longer survival than isotype control and HT. Moreover, combination therapy resulted in a significantly higher survival rate than anti-PD-L1 monotherapy (Fig. 4D).

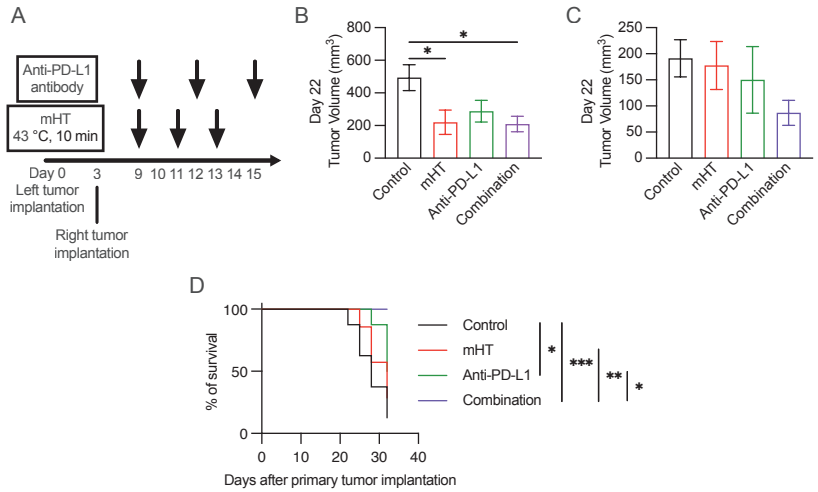

Fig. 4 Anti-tumor effects of mHT and PD-L1 blockade combination therapy

Fig. 4A: Schematics of the experimental design.

Fig. 4B–C: Tumor volume of left (B, primary) tumors and right (C, secondary) tumors on day 22.

Fig. 4D: Survival rate of the tumor-bearing mice.

mHT: mild hyperthermia

Data are expressed as mean ± SEM.

* $p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Two independent experiments were conducted, and all data obtained from the experiments were included in the analysis.

DISCUSSION

Several studies have investigated the combination of mHT and immune checkpoint inhibitors, which have shown favorable anti-tumor effects. However, studies on the mHT-induced changes in PD-1/PD-L1 expression are lacking. In this study, we demonstrated that mHT increased PD-L1 expression and decreased PD-1 positivity rate in $CD4^+$ T cells in tumor tissues of a murine squamous cell carcinoma model, indicating that combination therapy with anti-PD-L1 antibodies may be an effective therapeutic strategy. To the best of our knowledge, this is the first report on combination therapy with mHT and PD-L1 blockade in a squamous cell carcinoma model.

PD-L1 expression in the TME can be either constitutive or induced.[18] Previous studies have shown that SCCVII cells exhibit low PD-L1 expression, which can be induced both in vitro and in vivo.[19,20] Our in vitro data indicated that 43 °C HT can induce PD-L1 expression in SCCVII cells. In the TME, it is also known that the infiltration of $CD8^+$ T cells can induce PD-L1 expression.[18] In this study, the infiltration of $CD8^+$ T cells and PD-L1 upregulation occurred concurrently, suggesting that PD-L1 may have been elevated by the direct and indirect effects of mHT. Recent studies have shown that PD-1/PD-L1 inhibition is effective in the presence of $CD8^+$ T cells, which are negatively regulated by the PD-1/PD-L1 pathway.[12] In the present study, mHT promoted $CD8^+$ T cell infiltration into the TME and increased PD-L1 expression in the SCCVII model, which originally showed low PD-L1 expression and a high PD-1 positivity rate in $CD8^+$ T cells, indicating that anti-tumor immunity was negatively regulated. This supports its suitability for use in combination with PD-L1 inhibition.

The abscopal effect, which is the regression of distant lesions outside the radiation area, has been reported in radiation therapy. A similar phenomenon has been reported for combination therapy with mHT,[14] and the effect of combination therapy on distant tumors was investigated in this study. Combination therapy did not show significant anti-tumor effects on primary and secondary tumors in terms of tumor volume. However, combination therapy tended to control the tumors on both sides, indicating the presence of the abscopal effect and improved survival. On the other hand, mHT monotherapy only affected the heated primary tumor, and anti-PD-L1 monotherapy showed insufficient anti-tumor effects to improve survival.

Unlike radiotherapy, HT is considered to have no limit on the number of treatments, but in preclinical studies, HT has been limited to five or fewer sessions. Clinically, there have been reports of hyperthermia combined with radiation in patients with head and neck cancer who received 5–12 sessions of hyperthermia, with no increase in adverse events after 12 sessions compared with radiation alone.[21] Atezolizumab, an anti-PD-L1 antibody used in clinical practice, can be administered at 3-week intervals for up to 12 months (16 doses). Moreover, anti-PD-1 inhibitors (nivolumab and pembrolizumab) have no upper limit to their use. The combination of mHT and immune checkpoint inhibitors may have a strong anti-tumor effect when administered over a long period. Therefore, more effective treatment schedules are required.

We found that the PD-1 positivity rate of $CD4^+$ T cells was decreased by HT. Among the $CD4^+$ T cells, regulatory T cells expressing PD-1 are reactivated by PD-1/PD-L1 inhibition, creating an immune environment resistant to therapy.[22] In the present study, we did not investigate helper and regulatory T cells separately. Further studies are needed to elucidate the effects of mHT on the activation or suppression of $CD4^+$ T cell subpopulations.

Our study had another limitation. We implanted SCCVII cells into flanks, not into head and neck regions. A previous study indicated that the orthotopic SCCVII tumor model better reflects the TME of patients suffering from HNSCC compared with the subcutaneous model.[23] We further used the diode laser system, which cannot heat orthotopic tumors in the oral cavity, pharynx, or larynx accurately in mouse models due to technical issues. The laser system needs to be

improved, and further studies should be conducted in orthotopic tumor models.

In conclusion, our findings elucidate the changes in PD-L1 expression in the TME and strengthen the rationale for combination therapy using mHT and PD-L1 blockade.

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DISCLOSURE STATEMENTS

S. N. and K. Y. are employees of Asuka Medical Inc. The authors declare no conflict of interest regarding the publication of this paper.

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