INTRODUCTION

Oral cancer is the 11th most common cancer worldwide, and the majority is oral squamous cell carcinoma (OSCC). Oral and oropharyngeal cancer has a poor survival rate of equal or less than 50% after 5 years regardless of the multidisciplinary treatment. Recently, cancer immunotherapy blocking ligands and receptors in the immune system (immune checkpoints; ICs) and regulating T-cell activation has attracted attention as an advanced treatment. Immune checkpoint inhibitors (ICIs) suppress early and late effects of cancer. However, it is currently unclear what is the immune mechanism for the entry of tumor into dormancy and how stemness is coupled to the tumor cell arrest.

In this study, we revealed that IDO1 induced by IFN-\(\gamma\) activated Trp-KYN-AhR signaling and induced stemness in OSCC cells contributing to the entry of tumor into dormancy. In this mechanism, it was suggested that STAT1 was a key regulator between tumor differentiation and tumor dormancy. The regulation of IDO1-mediated Trp-KYN-AhR signal activation could provide a new strategy for the cancer treatment.

KEYWORDS
IDO1, LGR6, oral squamous cell carcinoma, stemness, tryptophan-kynurenine-AhR signaling

Abstract

The non-proliferative residual tumor cells in cancer therapeutics are considered tumor-repopulating cells (TRCs) and a major issue for tumor recurrences and distant metastases. TRCs are stem cell-like cancer cells repopulating tumors and associating with immune-mediated tumor dormancy. Cell cycle arrest is also coupled to a tumor-initiating or pluripotent capacity. INF-\(\gamma\), a cytokine with an anti-tumor effect, also acts as an inhibitory effect on the cancer immune response via the induction of PD-L1 on the surface of cancer cells. The anti-tumor immune response may shift TRCs from proliferation to dormancy by a specific mechanism. Indoleamine 2, 3-dioxygenase 1 (IDO1) is a metabolic enzyme to produce kynurenine (KYN) from tryptophan (Trp) and is expressed in cancer cells and dendritic cells in the immune system. IDO1 plays an important role for cancer cells to evade the attack from the activated CTLs. Recently, the signaling from the interaction between KYN and aryl hydrocarbon receptor (AhR), a dioxin receptor, has been reported to induce cancer cell dormancy. However, it is currently unclear what is the immune mechanism for the entry of tumor into dormancy and how stemness is coupled to the tumor cell arrest. In this study, we revealed that IDO1 induced by IFN-\(\gamma\) activated Trp-KYN-AhR signaling and induced stemness in OSCC cells contributing to the entry of tumor into dormancy. In this mechanism, it was suggested that STAT1 was a key regulator between tumor differentiation and tumor dormancy. The regulation of IDO1-mediated Trp-KYN-AhR signal activation could provide a new strategy for the cancer treatment.
late co-suppressive signaling via the receptors of cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and programmed cell death protein 1 (PD-1) expressed on the surface of activated anti-tumor T cells. ICIs are currently being used in several solid tumors, including malignant melanoma, lung cancer, and head and neck cancer to evade the immune surveillance of cancer cells. However, the response rate by the ICI treatment only is approximately 10%-40%, and this is why it is necessary to elucidate new mechanisms of cancer immune tolerance and develop more effective cancer immunotherapies.

In cancer therapeutics, non-proliferative residual tumor cells are considered one of the major problems for the tumor recurrence and distant metastasis. These residual tumor cells are considered tumor-repopulating cells (TRCs) which are stem cell-like cancer cells repopulating tumors and associating with immune-mediated tumor dormancy, and tumor dormancy and TRC are thought to be intricably linked. TRCs are a subpopulation of self-renewing and highly neoplastic cancer cells that play an important role in the initiation, promotion, and progression of tumorigenesis. In TRCs, cell cycle arrest is also coupled to a tumor-initiating or pluripotent capacity, namely stemness features. Tumor dormancy was originally defined by Willis and then redefined by Hadfield as a temporary mitotic arrest and a growth arrest. Dormancy was now divided into three categories: cellular dormancy, where tumor cells enter a quiescent state; angiogenic dormancy, where dormancy is kept by a balance between sufficient and insufficient vascularization for tumor cells; and immune-mediated dormancy. The “immune-mediated dormancy” is conceptually attributed to the interaction between the immune system and the tumor, and is considered a major reason why further success of immunotherapies cannot be achieved by such advanced methods of ICIs therapy and oncolytic virus or adoptive T-cell transfer. TRCs have the same potential for self-renewal and differentiation as normal tissues stem cells, but they are characterized by an immune escape mechanism. Even if TRCs escape killing by cytotoxic immune cells, it is possible that the anti-tumor immune response may generate a specific mechanism to inhibit TRC proliferation and shift to a dormant state. However, it is currently unclear how the major immune response is induced when the tumor is going dormant and stemness is coupled to the tumor cell arrest.

In cancer immune-surveillance according to the concept of “cancer immune-editing” developed as 3E theory, dormancy of the tumor mass indicates equilibrium between tumor growth and immune-mediated tumor death, which determines the outcome of tumor escape or elimination. Recently, it is known that metabolizing system of tryptophan (Trp), one of the essential amino acids, is playing an important role in tumor pathophysiology. Indoleamine 2, 3-dioxygenase 1 (IDO1) is a metabolic enzyme to produce kynurenine (KYN) from Trp and is expressed in cancer cells and dendritic cells in cancer immune system. IDO1 plays an important role in the mechanism for cancer cells to evade the attack from the cancer antigen-primed and activated cytotoxic T lymphocytes (CTLs) in the late phase of the cancer immune cycle. In addition, the signaling from the interaction between KYN and aryl hydrocarbon receptor (AhR), a dioxin receptor, has been reported to induce cancer cell dormancy. INF-γ, a cytokine with an anti-tumor effect, also acts as an inhibitory effect on the cancer immune response via the induction of PD-L1 on the surface of cancer cells and macrophages. It is also apparent that INF-γ is expressed in CTLs and natural killer (NK) cells and promotes the induction of IDO1, then activates Trp-KYN-AhR signaling.

In this study, we revealed that IDO1 induced by IFN-γ activated Trp-KYN-AhR signaling and induced stemness in OSCC cells contributing to the entry of tumor into immunological dormancy. In addition, the induction of cancer stemness was inversely correlated with STAT1 phosphorylation suggesting that STAT1 was a key regulator between tumor differentiation and tumor dormancy.

## 2 MATERIALS AND METHODS

### 2.1 Patients and clinicopathological profiles

This clinical study using the patients’ information was done under the permission of the ethics committee in Fukuoka Dental College (ID: 418). The 22 cases (male/female: 13/10, mean age: 63.6; range: 35-86) examined consisted of 22 oral tongue squamous cell carcinomas (OTSCCs). These Japanese patients underwent surgery at Fukuoka Dental College Hospital between 2014 and 2019. The patients were not prescribed chemotherapy or irradiation before surgery. The histological classification was performed according to the criteria of the “2017 WHO Classification of Head and Neck Tumours.” The pathological classifications of histological differentiation, TNM factors, and lymphatic and/or vascular permeation (Ly/V) were adopted as the clinicopathological indices. The clinicopathological profiles of the patients are summarized in Table 1.

### 2.2 Antibodies

Details of antibodies are described in supporting information.

### 2.3 Immunostaining for tissues and cells

Formalin-fixed and paraffin-embedded tissue blocks were cut into 4-μm thick sections for HE and immunohistochemical staining. For immunocytochemistry, the same immunostaining procedure described above was applied for cells after fixation with 4% paraformaldehyde. Details are described in supporting information.

### 2.4 Immunohistochemical assessment

The degree of positivity of immunoreaction in each lesion was determined according to the modified method of the one originally described by Allred et al. Briefly, we randomly chose three areas at the lesion of OTSCC section and counted the number of
immunoreactive atypical cells for IDO1 and leucine-rich repeat-containing G-protein-coupled receptor 6 (LGR6) in their cytoplasm or nuclei at least 300 atypical cells. The percentage of immunoreactive atypical cells was described as proportion score (PS); IDO1 (scored on a scale of 0-3; 0:0%, 1: less than 10%, 2: less than 30%, 3: equal to or more than 50%) and LGR6 (scored on a scale of 0-3: 0:0%, 1: less than 30%, 2:30%-50%, 3: equal to or more than 50%). Staining intensity was also described as intensity score (IS) (scored on a scale of 0-3; 0:negative, 1: weak positive, 2: intermediate positive, 3: strong positive). The proportion and intensity scores were summed to produce total score (TS = PS + IS). Then, the mean score of TS was statistically compared for analyzing the correlation between IDO1 or LGR6 expression and clinicopathological indices.

2.5 | Cell culture

Two human squamous cell carcinoma cell lines established from OTSCC patients, HSC-4 and SAS, were purchased from JCRB cell bank (Osaka, Japan). Cells were grown in Dulbecco’s Modified Eagle’s Medium (DMEM, MilliporeSigma/Sigma-Aldrich), supplemented with 10% fetal bovine serum (PAA Laboratories, Pasching, Austria) at 37°C in 5% CO2/95% air. Cells were reseeded for the next passage after trypsin (Thermo Fisher Scientific) dispersion when they reached ~80% confluency. For the IFN-γ stimulation analyses, cells were incubated in DMEM with 10 ng/mL, 50 ng/mL, or 200 ng/mL IFN-γ (Pepro Tech, Rocky Hill, NJ, USA) for 48 or 72 hours.

2.6 | Kynurenine measurement

To confirm whether the Trp metabolite KYN was actually produced by the activation of IDO1 after the IFN-γ stimulation on HSC-4 cells, we measured the concentration of KYN in the medium. After the treatment with IFN-γ (0, 10, and 50 ng/mL) for 72 hours, the supernatant was collected and the kynurenine concentration (ng/mL) was measured with an enzyme-linked immunosorbent assay (ELISA) kit (ImmuSmol, Bordeaux, France).

2.7 | Proteasome inhibition assay

HSC-4 cells were cultured in DMEM with 10 μM MG132 (MilliporeSigma/Sigma-Aldrich) for 1 hour; then, subsequently, the culture was continued under the IFN-γ stimulation (10 ng/mL) in DMEM without MG132.

2.8 | Western blotting analysis

Western blotting analysis was performed as previously described. Details are described in supporting information.

2.9 | RNA interference

IDO1 and AhR knockdown was carried out using small interfering RNA (siRNA) oligonucleotides (siIDO1, siAhR) synthesized by OriGene Technologies (Rockville, MD, USA). For negative control, siScramble (siScrbl) was also purchased from OriGene Technologies.

### TABLE 1 Summary of the clinicopathological characteristics of oral squamous cell carcinoma patients examined

| Characteristics                      | Number of patients |
|--------------------------------------|--------------------|
| Age                                  | Mean: 63.6 (range: 35-86) |
| Sex                                  | Male: 12, Female: 10 |
| Pathological diagnosis               | Oral tongue squamous cell carcinoma (OTSCC) |
|                                       | Histological differentiation |
|                                       | Well (W): 10, Moderate (M): 8, Moderate to poor (MP): 4 |
| pT                                    | T1: 5, T2: 11, T3: 3, T4a: 3, T4b: 0 |
| pN                                    | N0: 15, N1: 1, N2a: 0, N2b: 5, N2c: 1, N3a: 0, N3b: 0 |
| Ly/V                                  | +: 7, -: 15 |

Note: The 22 cases (male/female: 12/10, mean age: 63.6; range: 35-86) examined in this study consist of 22 oral tongue squamous cell carcinomas (OTSCCs). The pathological tumor classification reveals as follows: well differentiated (W): 10, moderately differentiated (M): 8, moderately to poorly differentiated (MP): 4. The pathological classifications of TNM factors (pTNM) and lymphatic and/or vascular permeation (Ly/V) are also shown. Lymphatic (Ly) permeation was recognized in 7 of 22 patients.
sirNA was transfected into HSC-4 cells at a final concentration of 25pmol/125μL with Screen Fect A plus (Wako), according to the manufacture's protocol.

2.10 | RT-qPCR analysis

Details are described in supporting information.

2.11 | Wound healing assay

Wounds were prepared using the Culture-Insert 2 well (ibidi, Madison, WS, USA). Cells were cultured for 72 hours in each objective condition and removed the Culture-Insert. After 12 hours sustained culture, the area of remaining wounds was determined using Image J (National Institutes of Health, Bethesda, MD, USA). The closing ratio of wounded area (CR) was calculated according to the following formula; \( CR = \frac{(w-rw)}{w} \times 100 \quad (\%) \), \( w \): wounded area at the start point, \( rw \): remaining wounded area. Then, the mean value of CR in the IFN-\( \gamma \) (10 ng/mL) treated condition was statistically compared with that in the IFN-\( \gamma \) untreated condition (control).

2.12 | BrdU assay

Cells (5x10\(^4\)) were cultured under the IFN-\( \gamma \) untreated (control) and treated (10 ng/mL and 200 ng/mL) conditions for 72 hours; then, cells were treated with 0.03 mg/mL BrdU (5-Bromo-2′-deoxyuridine: Tokyo Kasei, Tokyo, Japan) for 1 hour. After the incubation, cells were fixed with cold 70% ethanol for 5 minutes and incubated for more 30 minutes under the 1.5 M HCl treatment. After the HCl treatment, cells were rinsed two times in PBS for 5 minutes each and were applied for the immunocytochemistry. At least 300 cells were counted in the three different areas and the mean positive % value was adopted as the Labeling Index (LI) representative of each marker. In the dual immunocytochemical analyses, cells with LGR6(+/+)BrdU() or CD133(+/+)/BrdU() were suggested as cells revealing stem cell markers under the slow cell cycling, one of the stem cell characteristics. By this concept, the relative intensity ratio (RIR) of LGR6(+/+)/BrdU() or CD133(+/+)/BrdU() was calculated as the % intensity value against the intensity of cells with LGR6(+/)+BrdU(+) and (-) or CD133(+/)+BrdU(+) and (-). BrdU LI, Cleaved caspase-3 LI, LGR6(+/)+BrdU() RIR and CD133(+/)+BrdU() RIR were statistically compared among the IFN-\( \gamma \) untreated (control) and treated (10 ng/mL, 200 ng/mL) conditions.

2.13 | Statistical analysis

All data were expressed as the mean ± standard error of the mean (SEM). Student’s t test and Mann-Whitney U test were applied for the comparison between two groups. Statistical significance was set as *\( P < .05 \), **\( P < .01 \) and ***\( P < .001 \).

3 | RESULTS

3.1 | IDO1 was expressed in human OSCC cells and infiltrating immune cells

Immunohistochemically, IDO1 expression was recognized in the OSCC cells and infiltrating immune cells, especially dendritic cells. IDO1 expression in cancer cells was apparent in the poorly differentiated OSCCs and at the front of tumor invasion (Figure 1A). To discriminate the expression of IDO1 in between parenchymal tumor and stromal immune cells, we performed the dual immunostaining of IDO1 and ΔNp63, a representative marker of OSCC, against moderately differentiated OSCCs. We confirmed that IDO1 was expressed in cancer cells as well as infiltrating immune cells in and around the cancer cell nests (Figure 1B). In the correlation of Total Score (TS) (mean) between IDO1 expression in cancer cells and histopathological tumor differentiation, IDO1 TS was gradually increased in accordance with the poor tumor differentiation with a statistical significance between W and MP (*\( P = .002 \)) (Figure 1C).

3.2 | LGR6 was expressed in human OSCC cells and became distinct in accordance with the poor tumor differentiation

Leucine-rich repeat-containing G-protein-coupled receptor 6 (LGR6) was reported as a putative stem cell marker in the human lung.\(^{35}\) However, it has not been well known how LGR6 is expressed in human OSCCs. We revealed that LGR6 was immunohistochemically expressed in the cytoplasm of human OSCC cells and a strong expression was seen in the cancer cells of the moderately (M) and moderate-to-poorly (MP) differentiated OSCCs, especially at the front of invasion (Figure 2A). LGR6 TS (mean) revealed higher score as 5.13 (M) and 5.25 (MP) compared to 3.50 (W) with statistical significances (\( **P = .004 \) (W vs M), *\( P = .015 \) (W vs MP)) (Figure 2B).

3.3 | IFN-\( \gamma \) upregulated IDO1, AhR and Lgr6 expressions but downregulated p27 expression

IFN-\( \gamma \) is known to upregulate IDO-1 expression in the tumor microenvironment.\(^{31,36,37,38}\) However, it has not been well known whether IDO1 upregulated by IFN-\( \gamma \) activates Trp-KYN-AhR signaling and consequently stemness is induced in cancer cells or not. In our in vitro study using OSCC cell lines HSC-4 and SAS, we first confirmed that the concentration of KYN, a Trp metabolite, was actually increased by the IFN-\( \gamma \) stimulation of both a low (10 ng/mL) and a high (50 ng/mL) concentration with statistical significances in comparison with that of IFN-\( \gamma \) unstimulated state (control) (Figure 3A). Western blotting analysis revealed that IDO1 expression was not apparent in the IFN-\( \gamma \) unstimulated state but was remarkably induced by the IFN-\( \gamma \) (10 ng/mL) stimulation. Conversely, p27 was
apparently expressed in the IFN-γ unstimulated state but remarkably repressed by the IFN-γ (10 ng/mL) stimulation (Figure 3B). TNF-α (100 ng/mL) stimulation did not affect any significant changes on the IDO1 and p27 expressions (Figure 3B). At the beginning of this study, we considered that p27 was one of the critical factors in the regulation of cell cycle in OSCC cells undergoing tumor dormancy according to the previous literature. However, the expression of p27 in SAS cells was unexpectedly much lower than that in HSC-4 cells. By these points of view and consequences, we investigated expressions of several factors including p27 related to the IDO1-mediated Trp-kynurenine-AhR signal activation after the IFN-γ stimuli using HSC-4 cells in the following experiments. By the time course expression analysis under the IFN-γ (10 ng/mL) stimulation, induction of IDO1, AhR, and LGR6 was apparent at 6 hours, 6 hours, and 48 hours after IFN-γ stimulation, respectively. On the other hand, p27 was remarkably repressed at 6h after IFN-γ stimulation (Figure 3C). Immunohistochemically, AhR expression was recognized in the nuclei and apparently increased at 72h after IFN-γ (10 ng/mL) stimulation (Figure 3D, green). These results suggested that IFN-γ, even in the low concentration as 10 ng/mL, upregulated IDO1 and activates Trp-KYN-AhR signaling, and then, consequently a stem cell marker LGR6 was upregulated in HSC-4 cells.

### 3.4 Stem cell marker expression was upregulated by the IFN-γ stimulation in HSC-4 cells

In our experiment, expressions of stem cell makers LGR6 and CD133 were both upregulated by the IFN-γ (10 ng/mL and 200 ng/mL) stimuli but both markers expression patterns by the IFN-γ (200 ng/mL) stimulus showed a lower expression tendency compared to those by the IFN-γ (10 ng/mL) stimulus (Figure 4A). By the RT-qPCR analyses, IDO1 mRNA expression was gradually upregulated in accordance with the higher concentration of INF-γ (Figure 4B). AhR, LGR6, and p27 mRNAs were also upregulated by the IFN-γ stimulus with a low concentration of 10 ng/mL. However, these upregulations were downregulated by the IFN-γ stimulus with a high concentration of 200 ng/mL (Figure 4C-E).
3.5 | p27 protein expression was post-translationally regulated through the proteasome activity in HSC-4 cells

p27 protein expression was distinctively decreased after 6h in the IFN-γ stimulated state (Figure 3C). However, p27 mRNA expression was upregulated by the 48h IFN-γ (10 ng/mL) stimulation in HSC-4 cells to compare that of the IFN-γ unstimulated state (control), although there was no statistical significance (Figure 4E). To clarify whether this discrepancy in between p27 protein and mRNA expressions came due to a post-translational protein regulation through the ubiquitin proteasome system, we performed the inhibition analysis of proteasome activity by the treatment of MG132, a well-known proteasome inhibitor. In the IFN-γ-stimulated condition, p27 protein expression was gradually downregulated without MG132 treatment but was gradually increased with MG132 treatment in the time-course-dependent manner (Figure 4F). From these findings, we confirmed that p27 was post-translationally regulated by its degradation through the ubiquitin proteasome system.

3.6 | Upregulation of stem cell marker expression by IFN-γ stimulation was dependent on the Trp-KYN-AhR signaling cascade in HSC-4 cells

To clarify whether the upregulation of stem cell marker LGR6 and CD133 expressions was dependent or independent on the Trp-KYN-AhR signaling cascade, the effect of this signaling cascade inhibition on the stem cell marker LGR6 and CD133 expressions was determined with the inhibitor agents 1-MT (IDO1 inhibitor) and DMF (AhR inhibitor) or using siRNAs for IDO1 (siIDO1) and AhR (siAhR). 1-MT did not inhibit IDO1 and did not have any impacts on p27, p-STAT1, STST1, LGR6, and CD133 expressions (Figure 5A). These data might be reflecting the poor effect of IDO1 inhibitors in recently reported clinical trials. However, siIDO1 clearly inhibited IDO1 and LGR6 expressions with the recovery of p27 repression and upregulation of phosphorylated STAT1 (p-STAT1) (Figure 5C). On the other hand, upregulated expressions of LGR6 and CD133 by the IFN-γ (10 ng/mL) stimulus were downregulated by both DMF and siAhR accompanied with the inhibition of the IDO1 upregulation by the IFN-γ (10 ng/mL) stimulus (Figure 5B,D, Figure S1). Upregulation of phosphorylated STAT1 (p-STAT1) and recovery of downregulated p27 expression by the IFN-γ stimulus were also seen by the AhR inhibition with DMF and siAhR (Figure 5B,D). From these findings, the expressions of stem cell markers LGR6 and CD133 were both considered to be dependent on the Trp-KYN-AhR signaling cascade.

3.7 | Analyses of the behavioral effect of the IFN-γ stimulation on the HSC-4 cell proliferation

We evaluated how IFN-γ stimulation affected on the HSC-4 cell behavior by the methods of wound healing assay and WST-8 live cell assay. Wound healing assays were performed to evaluate the migration ability of HSC-4 cells under the unstimulated and stimulated state with IFN-γ (10 ng/mL) treatment. IFN-γ stimulation resulted in a lower percentage of wound area closing ratio (CR) (45.6%) compared to that of control (60.2%) (P = .118) (Figure 6A,B). WST-8 assays revealed a significant decrease in absorbance in the 72hs stimulation with IFN-γ (10 ng/mL) compared to that of IFN-γ unstimulated state (control) (**P < .001) (Figure 6C). These results revealed that IFN-γ inhibited HSC-4 cell proliferation.
3.8 | IFN-γ induced slow cell cycling and LGR6(+)/BrdU(-) or CD133(+)/BrdU(-) expression in HSC-4 cells

We evaluated the effect of IFN-γ stimulation on HSC-4 cell cycling, apoptosis, and stem cell marker expressions by BrdU assays. The labeling indices of BrdU-positive cells under the IFN-γ (10 and 200 ng/mL) stimulation were significantly decreased compared to that of IFN-γ unstimulated state (control) (Figure 6D,E). Labeling indices of cleaved caspase 3 in the IFN-γ unstimulated (control) and stimulated (10 ng/mL) HSC-4 cells revealed lower values as 1.6% and 5.1%, respectively. These values were two times or much lower than that of the HSC-4 cells stimulated with a higher IFN-γ concentration (200 ng/mL) as 11.0% (Figure 6F,G). From these findings, inhibition of HSC-4 cell proliferation due to slow cell cycling was induced by the IFN-γ low concentrate (10 ng/mL) stimulus rather than by its high concentration (200 ng/mL) stimulus, and apoptosis was more induced by the IFN-γ higher concentrate (200 ng/mL) stimulus in HSC-4 cells. The expression of stem cell markers in the HSC-4 cells under the slow cell cycling, namely, the LGR6(+)/BrdU(-) or CD133(+)/BrdU(-) expression pattern was significantly increased in the IFN-γ (10 ng/mL) stimulated HSC-4 cells (Figure 7A,C). The ratio of these expressing cells in the IFN-γ (200 ng/mL) stimulated HSC-4 cells was lower than that of the IFN-γ (10 ng/mL)-stimulated HSC-4 cells (Figure 7B,D). From these findings, HSC-4 cells with stem cell-like characteristics were induced by the lower concentration (10 ng/mL) stimulus rather than the higher concentration (200 ng/mL) stimulus of IFN-γ.

4 | DISCUSSION

In our clinicopathological studies, IDO1 was expressed in OSCC cells and the expression was significantly increased in accordance with
the poor tumor differentiation or strong in cancer cells at the invasive front. In addition, LGR6 was co-expressed with IDO1 in OSCC cells. In the in vitro studies using OSCC cell lines HSC-4 and SAS cells, we clarified that IDO1 expression was distinctively upregulated by the IFN-γ stimulation; then, stem cell markers LGR6 and CD133 expressions were subsequently induced. The IDO1-mediated activation of Trp-KYN-AhR signaling by the IFN-γ stimulation was confirmed by the significant production of Trp metabolite KYN and upregulation of AhR. In addition, HSC-4 cells expressing AhR in their nuclei were increased by the IFN-γ stimulation. The dependent and specific induction of these stem cell markers expressions on the Trp-KYN-AhR cascade was confirmed by the inhibition assays using IDO1 and AhR specific inhibitors and siRNAs. Furthermore, we found that the induction of stem cell markers LGR6 and CD133 was inversely correlated with STAT1 phosphorylation but not with p27 expression suggesting that STAT1 was a key regulator between tumor differentiation and tumor dormancy. Finally, in the BrdU assays for analyzing cell cycling activity, we revealed that the number of HSC-4 cells expressing stem cell markers LGR6 or CD133 but not BrdU, suggesting cells in the state of slow cell cycling, was significantly increased by the IFN-γ stimulation. These findings suggested that p27 protein expression is post-translationally regulated after the IFN-γ stimulus. Statistical significance was set as *P < .05, **P < .01 and ***P < .001.
found in the liver and is thought to be involved in physiological tryptophan metabolism. On the other hand, IDO1 is enzymatically induced by pro-inflammatory cytokines such as IFN-γ and is strongly expressed in epithelial cells and tumor cells as well as in the antigen-presenting cells. The elevated expression of IDO1 results in Trp deficiency and IDO1 metabolite KYN and other metabolites exert immunosuppressive effects by inhibiting T-cell proliferation and inducing regulatory T-cell (Treg) differentiation in the tumor microenvironment. Then, increased IDO1 expression is associated with tumor survival and poor prognosis in many solid tumors and hematopoietic tumors. Our clinicopathological data also showed that IDO1 expression was significantly correlated with poor tumor differentiation and tumor front invasion, that was one of the poor prognostic factors.

In cancer therapeutics, non-proliferative residual tumor cells are considered TRCs and one of the major problems for the tumor recurrence and distant metastasis. TRCs are stem cell-like cancer cells with a highly neoplastic potentialities such as repopulation of tumors and association with immune-mediated tumor dormancy. In TRCs, cell cycle arrest is also coupled to a tumor-initiating or pluripotent capacity. It was recently reported that the signaling from the interaction between KYN and AhR, a dioxin receptor, induced cancer cell dormancy. Recently, it was reported that p27/CDKN1B (Cyclin-dependent kinase inhibitor 1B), a well-known inhibitor at Gap 1 (G1) of the cell cycle, was involved in slow cell cycling and in the Trp-KYN-AhR signaling pathway, leading to tumor dormancy. Unexpectedly, in our study, p27 protein expression was decreased, which was an opposite result compared to that of the previous report. However, p27 mRNA expression was upregulated even by the low concentration of IFN-γ (10 ng/mL) stimulation revealing a discrepancy between p27 protein and mRNA expression. We clarified that p27 protein was post-translationally regulated through the ubiquitin proteasome system after the IFN-γ stimulation by the inhibition analysis of proteasome activity under the treatment of MG132 on HSC-4 cells. These data suggested that IDO1-mediated Trp-KYN-AhR signal activation induced stem cell-like properties of tumors.

LGR6 has been reported as a putative stem cell marker in the human lung. LGR6 is an epithelial stem cell marker and its expression increases with tumor progression and metastasis. In addition, LGR6 is enriched in advanced tumor cells in non-small-cell lung cancer and LGR6-positive cancer cells have self-renewal, differentiation, and high oncogenic potential. CD133 has been reported as a cancer stem cell marker in several tumors including oral cancer. However, LGR6 has so far not been analyzed in the head and neck cancers as a stem cell marker. In our study, LGR6 was co-expressed with IDO1 in OSCC cells and LGR6 and CD133 expressions were both upregulated by the IFN-γ stimulation in HSC-4 cells. These data suggested that IDO1-mediated Trp-KYN-AhR signal activation induced stem cell-like properties of tumors.
FIGURE 7  Analyses of the effect of IFN-γ on LGR6 and CD133 expressions by BrdU assays. Dual immunostaining of LGR6 and BrdU (A; LGR6 = Green, BrdU = Red) or CD133 and BrdU (B; CD133 = Green, BrdU = Red) are presented. The expression of stem cell markers in HSC-4 cells under the slow cell cycling, namely, HSC-4 cells with LGR6(+)BrdU(-) (B) or CD133(+)BrdU(-) (D) expression patterns is significantly increased in the IFN-γ (10 ng/mL) stimulated HSC-4 cells. The ratio of these expressing cells in the IFN-γ (200 ng/mL) stimulated HSC-4 cells is lower than that of the IFN-γ (10 ng/mL) stimulated HSC-4 cells (B, D). Statistical significance was set as *P < .05, **P < .01 and ***P < .001. A schematic illustrating the theoretical mechanism of the induction of LGR6/CD133 and tumor dormancy through IDO1-mediated Trp-KYN-AhR signal activation (E)
FIGURE 6 Analyses of the effect of IFN-γ stimulation on the HSC-4 cell proliferation. A, The wound areas are compared using the two-well culture inserts at 12h after IFN-γ stimulation. The wound areas at 0h and 12hrs are revealed inner areas between dashed lines in each figure. B, The closing ratio (CR) in IFN-γ (10 ng/mL) treated condition is lower than that of IFN-γ untreated condition (control), although there is no statistical significance (n = 6, Student’s t test, \( P = .118 \)). C, WST-8 assay is performed on HSC-4 cells. The living cell number is significantly decreased in the IFN-γ (10 ng/mL) stimulated condition compared to that in IFN-γ unstimulated (control) conditions (\( P = .091 \times 10^{-9} \)). The labeling indices of BrdU-positive cells in the IFN-γ (10 and 200 ng/mL) stimulated states were significantly decreased compared to that of IFN-γ unstimulated state (control) (D, E). Labeling indices of cleaved caspase 3 in the IFN-γ unstimulated (control) and stimulated (10 ng/mL) HSC-4 cells revealed lower values as 1.6% and 5.1%, respectively. These values were two times or much lower than that of the HSC-4 cells stimulated with a higher IFN-γ concentrate (200 ng/mL) as 11.0% (F, G). Statistical significance was set as **\( P < .01 \) and ***\( P < .001 \)
the reduction of the p27 expression in the Trp-KYN-AhR signal activation in our result might be related to tumor grade rather than inhibition of cell cycling. In addition, we found that upregulation of LGR6 and CD133 expressions was inversely correlated with STAT1 phosphorylation but not correlated with p27 expression. These data suggested that STAT1 was a key regulator between tumor differentiation and tumor dormancy.

In BrdU and cleaved caspase 3 assays, we confirmed that HSC-4 cells were in the state of slow cell cycling rather than apoptotic state by the IFN-γ stimulation even in the low concentration (10 ng/mL) stimulus. Furthermore, expression of stem cell markers in the HSC-4 cells under the slow cell cycling revealing LGR6(+/−)/BrdU(-) or CD133(+)/BrdU(-) was significantly increased in HSC-4 cells stimulated with the IFN-γ lower (10 ng/mL) rather than higher (200 ng/mL) concentration. These cancer cells in the state of slow cell cycling might be involved in carcinogenesis, invasion, and metastasis.

The feature of cancer stemness is different from the embryonic stem cell features such as totipotential differentiation, self-renewal, slow cell cycling and asymmetric division, and difficult to be defined. The term of tumor repopulating (TRP) cells or tumor initiating cells may be used as a similar meaning of cancer stem cells and having the feature of cell quiescence or cell senescence.45 We revealed that HSC-4 cells stimulated by IFN-γ were in the state of quiescence rather than apoptotic state, namely in the state of slow cell cycling by the assay of co-expression analysis of BrdU and cleaved-caspase 3. In addition, tumor cells expressing stem cell markers Lgr6 or CD133 were largely coordinated with these quiescent tumor cells, and then we evaluated HSC-4 cells stimulated by IFN-γ and in the state of cell quiescence as cancer cells with one of the stemness features. However, further investigation should be needed to clarify that tumor cells in the state of cell quiescence, namely tumor dormancy, by the IFN-γ stimuli would functionally reveal anti-tumor drug resistance or radiation treatment resistance and have the tumor repopulating activity.

In summary, we revealed that IDO1 induced by IFN-γ activated Trp-KYN-AhR signal pathway and induced stemness in OSCC cells contributing to the entry of tumor into immunological dormancy (Figure 7E). In addition, the induction of cancer stemness was inversely correlated with STAT1 phosphorylation suggesting that STAT1 was a key regulator between tumor differentiation and tumor dormancy. From these findings, the regulation of IDO1-mediated Trp-KYN-AhR signal activation could be expected to provide a new strategy for the cancer treatment especially by inhibiting recurrences and distant metastases.

ACKNOWLEDGMENTS

This work was supported in part by Grants-in-Aid for Scientific Research (C) (18K07033 to SH), Grant-in-Aid for Young Scientists (B) (17K18301 and 20K18490 to SY) from Japan Society for the Promotion of Science (JSPS)/KAKENHI.

CONFLICT OF INTEREST

All authors have no financial disclosures and no conflict of interest.

REFERENCES

1. Maxwell Parkin D, Bray F, Ferlay J, Pisani P. Estimating the world cancer burden: Globocan 2000. Int J Cancer. 2001;94:153–6.
2. Parkin DM, Bray F, Ferlay J, Pisani P. Global Cancer Statistics, 2002. CA Cancer J Clin. 2005;55:74–108.
3. Jemal A, Bray F, Ferlay J, et al. Global cancer statistics. CA Cancer J Clin. 2011;61:69–90.
4. Sosa MS, Maia AG, Estrada Y, Bosch A, Bragado P, et al. NR2F1 controls tumour cell dormancy via SOX9- and RARβ-driven quiescence programmes. Nat Commun. 2015;6.

AUTHORS' CONTRIBUTIONS

H. Anzai and S. Yoshimoto: Contributed to the conception, design, data acquisition, and interpretation, performed all the statistical analyses, drafted and critically revised the manuscript. K. Okamura and A. Hiraki: Contributed to the conception, design, and critically revised the manuscript. S. Hashimoto: Contributed to the conception, design, data acquisition, and interpretation, drafted and critically revised the manuscript.

ORCID

Shuichi Hashimoto https://orcid.org/0000-0002-0637-0630

https://oncologia.com
16. Sosa MS, Bragado P, Aguirre-Ghiso JA. Mechanisms of disseminated cancer cell dormancy: an awakening field. Nat Rev Cancer. 2014;14:611–22.
17. Ma J, Zhang Y, Tang K, Zhang H, Yin X, Li Y, et al. Reversing drug resistance of bone tumor-repopulating cells by tumor cell-derived chemotherapeutic microparticles. Cell Res. 2016;26:713–27.
18. Kusumbe AP, Bapat SA. Cancer stem cells and aneuploid populations within developing tumors are the major determinants of tumor dormancy. Cancer Res. 2009;69:9245–53.
19. Sonja Kleflef TS. Tumor dormancy and cancer stem cells: two sides of the same coin? [Internet]. Adv Exp Med Biol. 2013. Available from: https://doi.org/10.1007/978-1-4614-1445-2_8
20. Kennaawy EL. The dormant cancer cell. Br Med J. 1954;2:1225–30.
21. Aguirre-Ghiso JA. Models, mechanisms and clinical evidence for cancer dormancy. Nat Rev Cancer. 2007;7:834–46.
22. Hamid O, Robert C, Daud A, Hodi FS, Hwu W-J, Keeford R, et al. Safety and tumor responses with lambrolizumab (Anti-PD-1) in Melanoma. N Engl J Med. 2013;369:134–44.
23. Senzer NN, Kaufman HL, Amatruda T, Nemunaitis M, Reid T, Daniels G, et al. Phase II clinical trial of a granulocyte-macrophage colony-stimulating factor-encoding, second-generation oncolytic herpesvirus in patients with unresectable metastatic melanoma. J Clin Oncol. 2009;27:5763–71.
24. Noh KH, Kim BW, Song KH, Cho H, Lee YH, Kim JH, et al. Nanog signaling in cancer promotes stem-like phenotype and immune evasion. J Clin Invest. 2012;122:4077–93.
25. Bruttel VS, Wischhusen J. Cancer stem cell immunology: key to understanding tumorigenesis and tumor immune escape? Front Immunol. 2014;5:1–13.
26. Koebel CM, Vermi W, Swann JB, Zerafa N, Rodig SJ, Old LJ, et al. Adaptive immunity maintains occult cancer in an equilibrium state. Nature. 2007;450:903–7.
27. Müller-Hermelink N, Braumüller H, Pichler B, Wieder T, Mailhammer R, Schaak K, et al. TNFR1 signaling and IFN-γ signaling determine whether T cells induce tumor dormancy or promote multistage carcinogenesis. Cancer Cell. 2008;13:507–18.
28. Dunn GP, Old LJ, Schreiber RD. The three Es of cancer immunoediting. Annu Rev Immunol. 2004;22:329–60.
29. Schreiber RD, Old LJ, Smyth MJ. Cancer immunoediting: integrating immunity’s roles in cancer suppression and promotion. Science. 2011;331:1565–70.
30. Chen DS, Mellman I. Elements of cancer immunity and the cancer-immune set point. Nature. 2017;541:321–30.
31. Liu Y, Liang X, Yin X, Lv J, Tang K, Ma J, et al. Blockade of IDO-kynurenine-AhR metabolic circuitry abrogates IFN-γ-induced immunologic dormancy of tumor-repopulating cells. Nat Commun. 2017;8:1–15.
32. El-Naggar AK, Chan JK, Grandis JR, Takata TSP. WHO classification of head and neck tumours. Lyon, France: International Agency for Research on Cancer; 2017.
33. Allred DC, Harvey JM, Berardo M, Clark GM. Prognostic and predictive factors in breast cancer by immunohistochemical analysis. Mod Pathol. 1998;11:155–68.
34. Yoshimoto S, Tanaka F, Morita H, Hiraki A, Hashimoto S. Hypoxia-induced HIF-1α and ZEB1 are critical for the malignant transformation of ameloblastoma via TGF-β-dependent EMT. Cancer Med. 2019;8:7822–32.
35. Oeztuerk-Winder F, Guinot A, Ochalek A, Ventura JJ. Regulation of human lung alveolar multipotent cells by a novel p38α MAPK/miR-17-92 axis. EMBOJ. 2012;31:3431–41.
36. Folgiero V, Goffredo BM, Filippini P, Masetti R, Bonanno G, Caruso R, et al. Indoleamine 2,3-dioxygenase 1 (IDO1) activity in leukemia blasts correlates with poor outcome in childhood acute myeloid leukemia. Oncotarget. 2014;5:2052–64.
37. Godin-Etheri J, Hanafi LA, Piccirillo CA, Lapointe R. Indoleamine 2,3-dioxygenase expression in human cancers: clinical and immunologic perspectives. Clin Cancer Res. 2011;17:6985–91.
38. Moon YW, Hajjar J, Hwu P, Naing A. Targeting the indoleamine 2,3-dioxygenase pathway in cancer. J Immunother Cancer. 2015;3:1–10.
39. Mellor AL, Munn DH. IDO expression by dendritic cells: tolerance and tryptophan catabolism. Nat Rev Immunol. 2004;4:762–74.
40. Mellor AL, Munn DH. Tryptophan catabolism and T-cell tolerance: immunosuppression by starvation? Immunol Today. 1999;20:469–73.
41. Platten M, Wick W, Van Den Eynde BJ. Tryptophan catabolism in cancer: beyond IDO and tryptophan depletion. Cancer Res. 2012;72:5435–40.
42. Li Y, Luo S, Ma R, Liu J, Xu P, Zhang H, et al. Upregulation of cytotoxic phosphoenolpyruvate carboxykinase is a critical metabolic event in melanoma cells that repopulate tumors. Cancer Res. 2015;75:1191–6.
43. Liu J, Tan Y, Zhang H, Zhang Y, Xu P, Chen J, et al. Soft fibrin gels promote selection and growth of tumorigenic cells. Nat Mater. 2012;11:734–41.
44. Huang PY, Kandyba E, Jabouille A, Sjolund J, Kumar A, Halliwill K, et al. Lgr6 is a stem cell marker in mouse skin squamous cell carcinoma. Nat Genet. 2017;49:1624–32.
45. Cortesi E, Ventura J. Lgr6: from stemness to cancer progression. J Lung Health Dis. 2019;3:12–5.
46. Eramo A, Lotti F, Sette G, Pilozzi E, Biffoni M, Di Virgilio A, et al. Identification and expansion of the tumorigenic lung cancer stem cell population. Cell Death Differ. 2008;15(3):504–14.
47. Brien CAO, Pollett A, Gallinger S, Dick JE. A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. Nature. 2007;445:106–10.
48. Polyak K, Lee M, Erdjument-bromage H, Koff A, Roberts JM, Tempst P, et al. Cloning of p27Kip1, a cyclin-dependent kinase inhibitor and a potential mediator of extracellular antimitogenic signals. Cell. 1994;76:59–66.
49. Gao L, Gu W, Zheng J, Ren W, Chang S, Wang X, et al. Clinicopathological and prognostic significance of p27 expression in oral squamous cell carcinoma: a meta-analysis. Int J Biol Markers. 2013;28:329–35.
50. Guan G, Bakr MM, Firth N. Expression of cyclin D1 correlates with p27 KIP1 and regulates the degree of oral dysplasia and squamous cell carcinoma. Oral Surg Oral Med Oral Pathol Oral Radiol. 2018;126:174–83.

SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Anzai H, Yoshimoto S, Okamura K, Hiraki A, Hashimoto S. IDO1-mediated Trp-kynurenine-AhR signal activation induces stemness and tumor dormancy in oral squamous cell carcinomas. Oral Sci Int. 2021;00:1–13. https://doi.org/10.1002/osil.1109