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IDO1-mediated Trp-kynurenine-AhR signal activation induces stemness and tumor dormancy in oral squamous cell carcinomas

Running Title

KYN-AhR signaling promotes OSCC stemness

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Conflict of interest

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Author Contribution

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

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-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. 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-γ 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.

Introduction

Oral cancer is the eleventh most common cancer worldwide, and the majority is oral squamous cell carcinoma (OSCC).[1,2] Oral and oropharyngeal cancer has a poor survival rate of equal or less than 50% after 5 years regardless of the multidisciplinary treatment[3–5] 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.[6] Immune checkpoint inhibitors (ICIs) suppress early and 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.[7] ICIs are currently being used in several solid tumors, including malignant melanoma[8,9], lung cancer[10], and head and neck cancer[11,12] to evade the immune surveillance of cancer cells. However, the response rate by the ICI treatment only is approximately 10 to 40%[13], 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.[14,15] 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[16–18], and tumor dormancy and TRC are thought to be inextricably linked.[19] 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[20] and a growth arrest.[6] Dormancy was now divided into three categories, 1) cellular

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dormancy, where tumor cells enter quiescence, 2) angiogenic dormancy, where dormancy is kept by a balance between sufficient and insufficient vascularization for tumor cells, and 3) immune-mediated dormancy.[21] 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.[22,23] TRCs have the same potential for self-renewal and differentiation as normal tissues stem cells, but they are characterized by an immune escape mechanism.[24,25] 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.[26,27] 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[28,29], 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.[30] 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.[31] 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.

Materials and Methods

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" .[32] 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.

Antibodies

Details of antibodies were described in supporting information.

Immunostaining for tissues and cells

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

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.[33] 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.

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.

Kynurenine measurement

To confirm if 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, 50ng/ml) for 72 hrs, the supernatant was collected and the kynurenine concentration (ng/ml) was measured with an enzyme-linked immunosorbent assay (ELISA) kit (ImmuSmol, Bordeaux, France).

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 (10ng/ml) in DMEM without MG132.

Western blotting analysis

Western blotting analysis was performed as previously described.[34] Details were described in supporting information.

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. 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.

RT-qPCR analysis

Details were described in supporting information.

Wound healing assay

Wounds were prepared by 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 = (w-rw)/w x 100 (%), w: wounded area at the start point, rw: remaining wounded area. Then, the mean value of CR in the IFN-γ (10ng/ml) treated condition was statistically compared with that in the IFN-γ untreated condition (control).

BrdU assay

Cells (5×10^4) were cultured under the IFN-γ untreated (control) and treated (10ng/ml and 200ng/ml) conditions for 72 hrs, 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 1.5M 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

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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-γ untreated (control)

and treated (10ng/ml, 200ng/ml) conditions.

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<0.05, **p<0.01

and ***p<0.001.

Results

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=0.002) (Figure 1C).

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 2; A). LGR6 TS (mean) revealed higher score as 5.13 (M) and 5.25 (MP) compared to 3.50 (W) with statistical significances (**p=0.004 (W vs M), *p=0.015 (W vs MP) (Figure 2; B).

IFN-γ upregulated IDO1, AhR and Lgr6 expressions but downregulated p27 expression

IFN-γ 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-γ 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-γ stimulation of both a low (10ng/ml) and a high (50ng/ml) concentration with statistical significances in comparison with that of IFN-γ unstimulated state (control) (Figure 3; A). Western blotting analysis revealed that IDO1 expression was not apparent in the IFN-γ unstimulated state but was remarkably induced by the IFN-γ (10ng/ml) stimulation. Conversely, p27 was apparently expressed in the IFN-γ unstimulated state but remarkable repressed by the IFN-γ (10ng/ml) stimulation (Figure 3; B). TNF-α (100ng/ml) stimulation did not affect any significant changes on the IDO1 and p27 expressions (Figure 3; B).

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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.[31] However, the expression of p27 in SAS cells were 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 (10ng/ml) stimulation, induction of IDO1, AhR and LGR6 was apparent at 6h, 6h and 48h after IFN-γ stimulation, respectively. On the other hand, p27 was remarkable repressed at 6h after IFN-γ stimulation (Figure 3; C). Immunohistochemically AhR expression was recognized in the nuclei and apparently increased at 72h after IFN-γ (10ng/ml) stimulation (Figure 3; D, green). These results suggested that IFN-γ, even in the low concentration as 10ng/ml, upregulated IDO1 and activates Trp-KYN-AhR signaling, and then, consequently a stem cell marker LGR6 was upregulated in HSC-4 cells.

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 (10ng/ml and 200ng/ml) stimuli but both markers expression patterns by the IFN-γ (200ng/ml) stimulus showed a lower expression tendency compared to those by the IFN-γ (10ng/ml) stimulus (Figure 4; A). By the RT-qPCR analyses, IDO1 mRNA expression was gradually upregulated in accordance with the higher concentration of INF-γ (Figure 4; B). AhR, LGR6 and p27 mRNAs were also upregulated by the IFN-γ stimulus with a low concentration of 10ng/ml. However, these upregulations were downregulated by the IFN-γ stimulus with a high concentration of 200ng/ml (Figure 4; C, D and E).

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 3; C). However, p27 mRNA expression was upregulated by the 48h IFN-γ (10ng/ml) stimulation in HSC-4 cells to compare that of the IFN-γ unstimulated state (control) although there was no statistical significance (Figure 4; E). To clarify if 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 4; F). From these findings, we confirmed that p27 was post-translationally regulated by its degradation through the ubiquitin proteasome system.

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 (Figure5; A). 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

5, C). On the other hand, upregulated expressions of LGR6 and CD133 by the IFN-γ (10ng/ml) stimulus were downregulated by both DMF and siAhR accompanied with the inhibition of the IDO1 upregulation by the IFN-γ (10ng/ml) stimulus (Figure 5; B, D, Fig. 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 5, B, 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.

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 (10ng/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=0.118) (Figure 6; A, B). WST-8 assays revealed a significant decrease in absorbance in the 72hs stimulation with IFN-γ (10ng/ml) compared to that of IFN-γ unstimulated state (control) (***p<0.001) (Figure 6; C). These results revealed that IFN-γ inhibited HSC-4 cell proliferation.

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 200ng/ml) stimulation were significantly decreased compared to that of IFN-γ unstimulated state (control) (Figure 6; D and E). Labeling indices of cleaved caspase 3 in the IFN-γ unstimulated (control) and stimulated (10ng/ml) HSC-4 cells revealed lower values

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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 (200ng/ml) as 11.0% (Figure 6; F and G). From these findings, inhibition of HSC-4

cell proliferation due to slow cell cycling was induced by the IFN-γ low concentrate (10ng/ml) stimulus rather than

by its high concentrate (200ng/ml) stimulus, and apoptosis was more induced by the IFN-γ higher concentrate

(200ng/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 (10ng/ml) stimulated HSC-4 cells (Figure 7; A and C). The ratio of these expressing cells in the IFN-γ

(200ng/ml) stimulated HSC-4 cells was lower than that of the IFN-γ (10ng/ml) stimulated HSC-4 cells (Figure 7; B

and D). From these findings, HSC-4 cells with stem cell-like characteristics were induced by the lower concentration

(10ng/ml) stimulus rather than the higher concentration (200ng/ml) stimulus of IFN-γ.

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. From these findings, we concluded that IDO1-mediated Trp-KYN-AhR signal

activation induced stemness in OSCC cells by inhibiting their proliferation and was considered to contribute to the

entry of tumor into immunological dormancy.

IDO1 and Tryptophan 2,3-dioxygenase (TDO) are known as enzymes that metabolize Trp to KYN in the same

pathway. TDO is mainly 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.[39] 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.[40,41] Then, increased IDO1 expression is associated with tumor survival and poor prognosis in

many solid tumors and hematopoietic tumors.[36–38] 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.[14,15] TRCs are stem cell-like cancer cells with a highly

neoplastic potential such repopulation of tumors and association with immune-mediated tumor dormancy.[16–18,42,43] 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.[31] In our

study, AhR protein and mRNA expressions were upregulated by the IFN-γ stimulation in HSC-4 cells. In addition,

HSC-4 cells expressing AhR were increased by the IFN-γ stimulation. These data suggested that IDO1 induced by

IFN-γ stimulation activated Trp-KYN-AhR signaling and contributed to the entry of tumor into dormancy in HSC-4

cells.

LGR6 has been reported as a putative stem cell marker in the human lung.[35] LGR6 is an epithelial stem cell

marker and its expression increases with tumor progression and metastasis.[35,44,45] 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.[45] CD133 has been reported as a cancer stem cell marker in several tumors including

oral cancer.[46,47] 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.

Recently, it was reported that p27/CDKN1B (Cyclin-dependent kinase inhibitor 1B), a well-known inhibitor

at Gap 1 (G1) of the cell cycle[48], was involved in slow cell cycling and in the Trp-KYN-AhR signaling pathway,

leading to tumor dormancy.[31] Unexpectedly, in our study, p27 protein expression was decreased, which was an

opposite result compared to that of the previous report.[31] However, p27 mRNA expression was upregulated even by the low concentration of IFN-γ (10ng/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. In other reports, p27 was associated with tumor grade and prognostic factor in OSCCs.[49,50] From this report, 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 was in the state of slow cell cycling rather than apoptotic state by the IFN-γ stimulation even in the low concentration (10ng/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(10ng/ml) rather than higher (200ng/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 cell 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, 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 7; E). 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.

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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
pM	
M0	22
M1	0
Ly/V	
+	7
-	15

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.

Figure legends

Figure 1. IDO1 expression analyses in human OSCCs. A. Histology by H.E. staining (a,c,e) and immunostaining

of IDO1 (b,d,f) in each tumor differentiation of OSCCs (W: Well differentiated (a,b), M; Moderately differentiated

(c,d), MP: Moderately to poorly differentiated (e,f)). The expression of IDO1 in cancer cells becomes distinct in

accordance with the poor tumor differentiation (b,d,f). B. IDO1 and ΔNp63 dual immunofluorescent staining on

OSCCs (IDO1: green, ΔNp63: red). The figure is a representative example of IDO1 (green) and ΔNp63 (red) dual

expression in the moderately differentiated OSCC tumor cells. IDO1 is expressed not only in invading immune

cells (green, arrows), but also in tumor cells (red and green, surrounded by yellow dotted line). Scale bars: 100 μm.

C. Comparison of the mean TS values of IDO1 expression in tumor cells among each degree of tumor

differentiation. IDO1 TS increases in accordance with poor tumor differentiation and there is a significant

difference between W and MP. The statistical significance assessed by Mann-Whitney U test is indicated by **p

<0.01.

Figure 2. LGR6 expression analyses in human OSCCs. A. H.E. staining (a,c,e) and LGR6

immunohistochemistry (b,d,f) in each tumor differentiation of OSCCs (W: Well differentiated (a,b), M: Moderately

differentiated (c,d), MP: Moderately to poorly differentiated (e,f)). The expression of LGR6 in cancer cells becomes distinct in accordance with the poor tumor differentiation or the tip of invasion (b,d,f). B. Comparison of LGR6 TS (mean) in cancer cells among each tumor differentiation. LGR6 TS is increased in accordance with the poor tumor differentiation. There is a statistical significance between W and M, and, W and MP. The statistical significance assessed by Mann-Whitney U test is indicated by *p <0.05.

Figure 3. Effects of IFN-γ stimulation on Trp-KYN-AhR signal cascade and the expression of related factors in HSC-4 cells. A. Kynurenine (KYN) concentration is measured by the ELISA assay system. The concentration of KYN is increased by the IFN-γ stimulation of both a low (10ng/ml) and a high (50ng/ml) concentration with statistical significances in comparison with that of IFN-γ unstimulated state (control). B. Western blot analysis of IDO1 and p27 expressions in HSC-4 and SAS cells after 72 hours IFN-γ (10 ng/mL) or TNF-α (100 ng/mL) stimulation. IDO1 expression is remarkably upregulated but, conversely, p27 expression is remarkably repressed by the IFN-γ stimulation in HSC-4 and SAS cells although the difference of p27 expression is not so obvious in SAS cells. TNF-α stimulation revealed no obvious changes in IDO1 and p27 expressions in both cell lines. C. Time course analysis of several protein expressions in HSC-4 cells under the IFN-γ (10ng/ml) stimulation. The expression of IDO1 is induced and becomes obvious after the 6hrs IFN-γ stimulation. AhR reveals the same time course tendency of the IDO1 expression. In addition, the expression of a stem cell marker LGR6 is apparently upregulated after 48hrs IFN-γ stimulation. Contrary, the obvious decrease of p27 expression is recognized after 3hrs or 6hrs IFN-γ stimulation. D. The immunocytochemical analysis of the AhR expression in HSC-4 cells treated with or without IFN-γ. The

expression of AhR is obviously increased in the nuclei of tumor cells after the IFN-γ stimulation (f, h) compared to that of the control (b, d). Statistical significance was set as ***$p < 0.001$.

Figure 4. Analyses of IDO1, AhR, LGR6 and p27 mRNA expressions by the stimuli with IFN-γ low (10ng/ml) and high (200ng/ml) concentrations. A. Western blot analysis reveals that IDO1 expression is remarkably upregulated but, conversely, p27 expression is remarkably repressed by the IFN-γ stimuli (10 and 200ng/ml) for 72hrs. Expressions of stem cell makers LGR6 and CD133 are both upregulated by the IFN-γ: 10ng/ml and 200ng/ml stimuli although both bands by the IFN-γ (200ng/ml) stimulus show a lower expression tendency compared to those by the IFN-γ (10ng/ml) stimulus. B. IDO1 mRNA expression is gradually upregulated in accordance with the higher concentration of INF-γ with statistical significances. AhR, LGR6 and p27 mRNAs are also upregulated by the IFN-γ stimulus with a lower concentration of 10ng/ml. However, these upregulations are downregulated by the IFN-γ stimulus with a high concentration of 200ng/ml (C, D and E). F. The decreased expression of p27 by the IFN-γ stimulation under the MG132(-) condition is recovered and reveals obviously increased p27 protein expression under the MG132(+) condition. These results suggested that p27 protein expression is post-translationally regulated after the IFN-γ stimulus. Statistical significance was set as *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

Figure 5. Analyses of the IDO1 and AhR inhibition effects on stem cell marker expressions. Analysis of the effect of 1-MT (IDO1 inhibitor) (A) and DMF (AhR inhibitor) (B). Analysis of the effect of siRNAs for IDO1

(siIDO1) (C) and AhR (siAhR) (D). 1-MT did not inhibit IDO1 and did not have any impacts on p27, p-STAT1, STST1, LGR6 and CD133 expressions (A). However, siIDO1 clearly inhibited IDO1 and LGR6 expressions with the recovery of p27 repression and upregulation of phosphorylated STAT1 (p-STAT1) (C). On the other hand, the upregulation of LGR6 and CD133 expressions by the IFN-γ (10ng/ml) stimulus are apparently inhibited by both DMF and siAhR treatments accompanied with the inhibition of the IDO1 upregulation (B, D). The expression of pSTAT-1 is also upregulated by both DMF and siAhR treatments (B, D).

Figure 6. Analyses of the effect of IFN-γ stimulation on the HSC-4 cell proliferation. A. The wound areas are compared by using the 2-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 (10ng/ml) treated condition is lower than that of IFN-γ untreated condition (control) although there is no statistical significance (n=6, Student t-test, p=0.118). 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=0.091 x 10^{-9}). The labeling indices of BrdU positive cells in the IFN-γ (10 and 200ng/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 (10ng/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 (200ng/ml) as 11.0% (F, G). Statistical significance was set as **p<0.01 and ***p<0.001.

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 are significantly increased in the IFN-γ (10ng/ml) stimulated HSC-4 cells. The ratio of these expressing cells in the IFN-γ (200ng/ml) stimulated HSC-4 cells are lower than that of the IFN-γ (10ng/ml) stimulated HSC-4 cells (B, D). Statistical significance was set as *p<0.05, **p<0.01 and ***p<0.001. A schematic illustrating the theoretical mechanism of the induction of LGR6/CD133 and tumor dormancy through IDO1-mediated Trp-KYN-AhR sigunal activation (E).