

# M2-like macrophage polarization in high lactic acid-producing head and neck cancer

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## Key words

Head and neck squamous cell carcinoma, lactic acid, macrophage polarization, tumor microenvironment, Warburg effect

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Reprogramming of energy metabolism is recognized as a hallmark of cancer.<sup>(1)</sup> Glucose is imported into the cytoplasm by glucose transporters and metabolized to pyruvate. Under aerobic conditions, normal cells convert pyruvate to acetyl-CoA in the mitochondria and metabolize it to CO<sub>2</sub>, H<sub>2</sub>O, and ATP through the tricarboxylic acid cycle. Tumor cells exhibit increased glucose uptake and convert pyruvate to lactate under aerobic conditions, resulting in a state referred to as the Warburg effect, or aerobic glycolysis.<sup>(2,3)</sup> Hence, tumor cells express metabolic enzymes and transporters involved in glycolysis at higher levels than normal cells.<sup>(4)</sup> Glucose transporter 1, which transports glucose into the cytoplasm, is commonly overexpressed in human malignancies,<sup>(4,5)</sup> and tumor cells are thereby able to import large amounts of glucose. Lactate dehydrogenase, a tetramer composed of two subunits, LDHA and LDHB, catalyzes the interconversion of pyruvate and lactate. Lactate dehydrogenase A promotes conversion of pyruvate to lactate and is the predominant isoform in highly glycolytic tissues, especially in tumors.<sup>(6,7)</sup> The enhanced glycolysis in tumor cells produces large amounts of lactic acid, and higher levels of lactic acid are correlated with distant metastasis and poor prognosis in head and neck cancer and cervical cancer.<sup>(8)</sup>

Reprogramming of glucose metabolism in tumor cells is referred to as the Warburg effect and results in increased lactic acid secretion into the tumor microenvironment. We have previously shown that lactic acid has important roles as a pro-inflammatory and immunosuppressive mediator and promotes tumor progression. In this study, we examined the relationship between the lactic acid concentration and expression of *LDHA* and *GLUT1*, which are related to the Warburg effect, in human head and neck squamous cell carcinoma (HNSCC). Tumors expressing lower levels of *LDHA* and *GLUT1* had a higher concentration of lactic acid than those with higher *LDHA* and *GLUT1* expression. Lactic acid also suppressed the expression of *LDHA* and *GLUT1* *in vitro*. We previously reported that lactic acid enhances expression of an M2 macrophage marker, *ARG1*, in murine macrophages. Therefore, we investigated the relationship between the lactic acid concentration and polarization of M2 macrophages in HNSCC by measuring the expression of M2 macrophage markers, *CSF1R* and *CD163*, normalized using a pan-macrophage marker, *CD68*. Tumors with lower levels of *CD68* showed a higher concentration of lactic acid, whereas those with higher levels of *CSF1R* showed a significantly higher concentration of lactic acid. A similar tendency was observed for *CD163*. These results suggest that tumor-secreted lactic acid is linked to the reduction of macrophages in tumors and promotes induction of M2-like macrophage polarization in human HNSCC.

Tumors are infiltrated by many types of innate and adaptive immune cells. These immune cells were classically thought to function to eliminate tumors, but recent studies have indicated that they induce inflammation in tumors and promote tumor progression through induction of angiogenesis and tissue remodeling in the tumor microenvironment, resulting in increased tumor invasion and metastasis.<sup>(9,10)</sup> Macrophages are typically classified into two polarization states: M1 and M2 phenotypes. M1 macrophages are potent effector cells that kill target cells and produce inflammatory cytokines such as IL-12, IL-1 $\beta$ , tumor necrosis factor- $\alpha$ , and IL-6. In contrast, M2 macrophages reduce inflammatory responses and adaptive Th1 immunity, and promote angiogenesis and tissue remodeling.<sup>(11–13)</sup> Tumor-associated macrophages polarize into the M2 phenotype and suppress the host anticancer immune response, leading to tumor progression. M2 macrophages are characterized by secreted cytokines (IL-4, IL-13, IL-10, and CSF1), Toll-like receptor ligands, and glucocorticoids.<sup>(12,14)</sup>

We previously showed that lactic acid secreted by tumor cells enhances expression of *ARG1*, an enzyme that metabolizes L-arginine into L-ornithine and urea and converts macrophages to inhibit T cell proliferation and activation in mice.<sup>(15)</sup>

Depletion of L-arginine from the tumor microenvironment through ARG1-dependent consumption causes suppression of T cell activation and proliferation, and lactic acid secreted by tumor cells plays an important role as an inducer of M2-like macrophages or immunosuppressive macrophage polarization in mice.<sup>(16,17)</sup> However, upregulation of ARG1 occurs in murine M2 macrophages, but not in human M2 macrophages; thus, human M2 macrophages cannot suppress T cells in an ARG1-dependent manner, and it is unknown whether lactic acid secreted by tumors induces M2 macrophage polarization in humans.<sup>(18)</sup> Therefore, to investigate the relationship between lactic acid and M2 macrophage polarization in human tumors, we measured the concentration of lactic acid and the expression of M2 macrophage markers in HNSCC.

## Materials and Methods

**Patients.** Tumor tissue samples were obtained from 20 patients who underwent primary surgical resection of HNSCC between 2013 and 2015 at Gifu University Hospital (Gifu, Japan). The backgrounds of the patients are shown in Table S1. Normal pharyngeal tissues were obtained from four patients who underwent tonsillectomy for recurrent tonsillitis or IgA nephropathy and from one patient who underwent total laryngectomy for dysphagia at our hospital. Normal pharyngeal tissues were isolated from surrounding tonsillar or laryngeal tissue. Surgical tissue specimens were immediately put into a vial containing RNAlater Stabilization Reagent (Qiagen, Valencia, CA, USA) and then stored at  $-80^{\circ}\text{C}$  for isolation of total RNA or homogenized in Dulbecco's PBS for measurement of the lactic acid concentration. All patients who provided HNSCC tissue or normal pharyngeal tissue gave informed consent in accordance with the institutional ethics committee requirements and the Declaration of Helsinki. This study was approved by the Institutional Review Board of Gifu University Graduate School of Medicine (approval no. 26-181).

**Cell lines and cell culture.** Human HNSCC cell lines, HSC-2 and HSC-4, were shown to be derived from the JCRB0622 and JCRB0624 cell lines, respectively, in the JCRB cell bank using short tandem repeat analysis (Fig. S1). HSC-2 and HSC-4 cells were cultured in RPMI-1640 supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin, and 100  $\mu\text{g}/\text{mL}$  streptomycin at  $37^{\circ}\text{C}$  under a 5%  $\text{CO}_2$  atmosphere. L-lactic acid was purchased from Sigma-Aldrich (St. Louis, MO, USA). For treatment with L-lactic acid,  $2 \times 10^5$  cells were cultured in 0.5 mL culture medium/well (24-well plate) or  $1 \times 10^5$  cells were cultured in 5 mL culture medium/well (6-well plate). In deacidification experiments,  $1 \times 10^5$  cells were cultured in 5 mL culture medium/well (6-well plate) in the presence of 0, 20, or 30 mM lactic acid for 24 h and

further cultured in 5 mL fresh culture medium without lactic acid for 24 h.

**Real-time RT-PCR.** Total RNA was isolated from tissues or cells using a RNeasy Mini kit (Qiagen). Complementary DNA was synthesized at  $37^{\circ}\text{C}$  for 15 min using a PrimeScript RT reagent kit (Takara Bio, Otsu, Japan). Real-time RT-PCR was carried out using a TaqMan gene expression assay system (Applied Biosystems, Foster City, CA, USA), Premix Ex Taq and a Thermal Cycler Dice Real Time System TP800 (Takara Bio). The following TaqMan probes and primer sets were used: *CD68*, Hs02836816\_g1; *CD163*, Hs00174705\_m1; *ARG1*, Hs00968979\_m1; *CSF1R*, Hs00911250\_m1; *LDHA*, Hs00855332\_g1; *SLC2A1* (*GLUT1* is named *SLC2A1* in the HUGO gene nomenclature), Hs00892681\_m1, and *18S rRNA*, 4352930E. The relative expression of each gene was normalized to that of *18S rRNA* or *CD68* and calculated using the  $\Delta\Delta\text{Ct}$  method.<sup>(19)</sup>

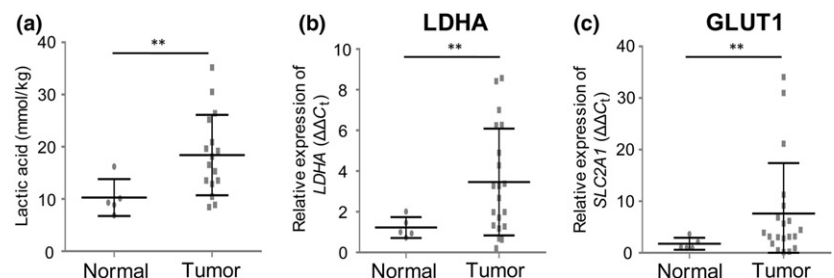
**Measurement of lactic acid concentration in tumors.** Tissue specimens were homogenized in Dulbecco's PBS (1 mL per 100 mg tumor) and centrifuged for 5 min at  $440 \times g$ . The supernatant was mixed with an equal volume of 0.8 N perchloric acid and centrifuged for 5 min at 3000 rpm. The concentrations of lactate in the obtained supernatants were measured using a Determiner LA Kit (Kyowa Medical Corp., Shizuoka, Japan).

**Statistical analysis.** Gene expression levels and lactate concentrations are shown as mean values  $\pm$  SD. Differences between groups were analyzed by unpaired Student's *t*-test. Relationships between two continuous variables were evaluated by Spearman's rank correlation coefficient.  $P < 0.05$  was considered significant in all analyses.

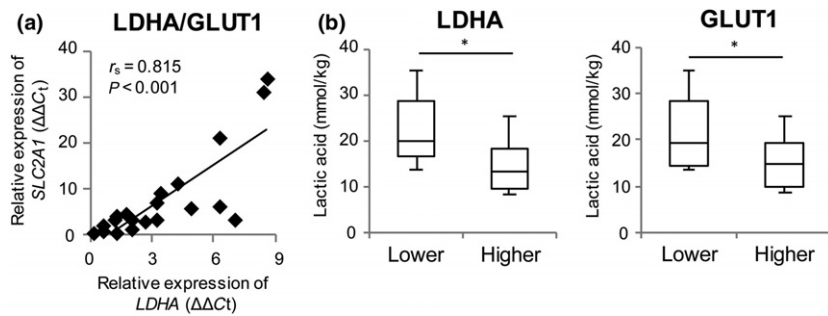
## Results

**Increases in lactic acid and expression of *LDHA* and *GLUT1* in HNSCC.** Aerobic glycolysis is enhanced in tumor cells, and therefore these cells are likely to secrete a large amount of lactic acid. Consistent with this expectation, the lactic acid concentration in HNSCC tissues was significantly higher than that in normal pharyngeal tissues (Fig. 1a;  $P = 0.002$ ). Expression of *LDHA* and *GLUT1* determined by real-time RT-PCR was also significantly higher in HNSCC tissue (Fig. 1b; both  $P < 0.001$ ). These results suggest that tumor cells secrete a large amount of lactic acid due to upregulation of glycolysis, compared to normal cells.

**Lactic acid is decreased in HNSCC expressing higher levels of *LDHA* or *GLUT1*.** Expression levels of *LDHA* and *GLUT1* in each HNSCC were strongly positively correlated (Fig. 2a;  $r_s = 0.815$ ,  $P < 0.001$ ). To investigate the relationship between the concentration of lactic acid and expression of *LDHA* or *GLUT1* in HNSCC, the samples were divided into groups



**Fig. 1.** Increased concentration of lactic acid and expression of *LDHA* and *GLUT1* in head and neck squamous cell carcinoma (HNSCC). (a) Concentrations of lactic acid in normal pharyngeal tissue ( $n = 5$ ) and HNSCC tissue ( $n = 16$ ). (b, c) Relative expression levels of *LDHA* (b) and *GLUT1* (c) in normal pharyngeal tissue ( $n = 5$ ) and HNSCC tissue ( $n = 20$ ) measured by real-time RT-PCR. *GLUT1* is named *SLC2A1* in the HUGO gene nomenclature.  $**P < 0.01$  by Student's *t*-test.



**Fig. 2.** Expression of *LDHA* and *GLUT1* in head and neck squamous cell carcinoma. (a) Correlation between expression of *LDHA* and *GLUT1* mRNA in head and neck squamous cell carcinoma ( $n = 20$ ).  $r_s$  is the Spearman rank correlation coefficient. (b) Concentrations of lactic acid in tissues expressing lower and higher levels of *LDHA* (low,  $<3$ ,  $n = 8$ ; high,  $\geq 3$ ,  $n = 8$ ) or *GLUT1* (low,  $<4.2$ ,  $n = 8$ ; high,  $\geq 4.2$ ,  $n = 8$ ). mRNA of these genes was measured by real-time RT-PCR. \* $P < 0.05$  by Student's *t*-test.

( $n = 8$  in each) expressing lower and higher levels, using median values of 3.0 for *LDHA* and 4.2 for *GLUT1*. The lower expression *LDHA* and *GLUT1* groups had significantly higher levels of lactic acid (Fig. 2b;  $P = 0.017$  for *LDHA*,  $P = 0.045$  for *GLUT1*). However, there was no significant correlation between the lactic acid level and expression of *LDHA* ( $r_s = -0.364$ ,  $P = 0.166$ ) or *GLUT1* ( $r_s = -0.396$ ,  $P = 0.129$ ). These results suggest that a higher concentration of tumor-secreted lactic acid might suppress the expression of *LDHA* and *GLUT1*.

**High lactic acid suppresses expression of *LDHA* and *GLUT1* *in vitro*.** The results in Figure 2(b) are the opposite of our original view that higher expression of *LDHA* and *GLUT1* would lead to an increase in lactic acid in tumors. Therefore, we examined whether a higher concentration of lactic acid affects the expression of *LDHA* and *GLUT1* in human HNSCC cell lines, HSC-2 and HSC-4, *in vitro*. HSC-2 and HSC-4 cells were cultured for 24 h, after which expression of *LDHA* and *GLUT1* and the lactic acid level in culture supernatants were measured. The concentration of lactic acid was significantly higher in the supernatant of HSC-2 cells compared with that of HSC-4 cells (Fig. 3a;  $P < 0.001$ ). Compared with HSC-4 cells, *LDHA* expression was significantly elevated and *GLUT1* expression was significantly reduced in HSC-2 cells (Fig. 3b;  $P = 0.015$  for *LDHA*,  $P = 0.033$  for *GLUT1*).

To explore the effect of lactic acid on expression of these genes, HSC-2 and HSC-4 cells ( $2 \times 10^5$  cells in 0.5 mL/well) were cultured in the presence of 15 mM lactic acid. Lactic acid suppressed expression of *LDHA* in HSC-2 ( $P < 0.001$ ) and HSC-4 cells ( $P = 0.037$ ) and expression of *GLUT1* in HSC-4 cells (Fig. 3c;  $P = 0.024$ ). The concentration of lactic acid in the HSC-2 culture supernatant was  $13.84 \pm 0.17$  mM, which might have been high enough for HSC-2 cells to suppress *GLUT1* expression without additional lactic acid (Fig. 3a). To remove the influence of lactic acid secreted by tumor cells, HSC-2 cells ( $1 \times 10^5$  cells in 5 mL/well) were cultured in a larger amount of medium. The concentration of lactic acid in the supernatant was not increased after 24 h of incubation ( $6.48 \pm 0.16$  mM) and additional lactic acid significantly suppressed expression of *LDHA* and *GLUT1* (Fig. 3d,e;  $P = 0.009$  for *LDHA*,  $P = 0.013$  for *GLUT1*). Moreover, results similar to those with lactic acid were observed after treatment with 10 mM HCl (Fig. 3c).

Next, we investigated whether tumor cells treated with lactic acid can secrete a large amount of lactic acid. HSC-2 and HSC-4 cells were pretreated with lactic acid for 24 h, and then the medium was changed to fresh medium without lactic acid. After 24 h, lactic acid levels in the culture supernatants were measured. The concentration of lactic acid was significantly suppressed in a dose-dependent manner in HSC-2 and HSC-4 cells pretreated with lactic acid (Fig. 3f). These results suggest

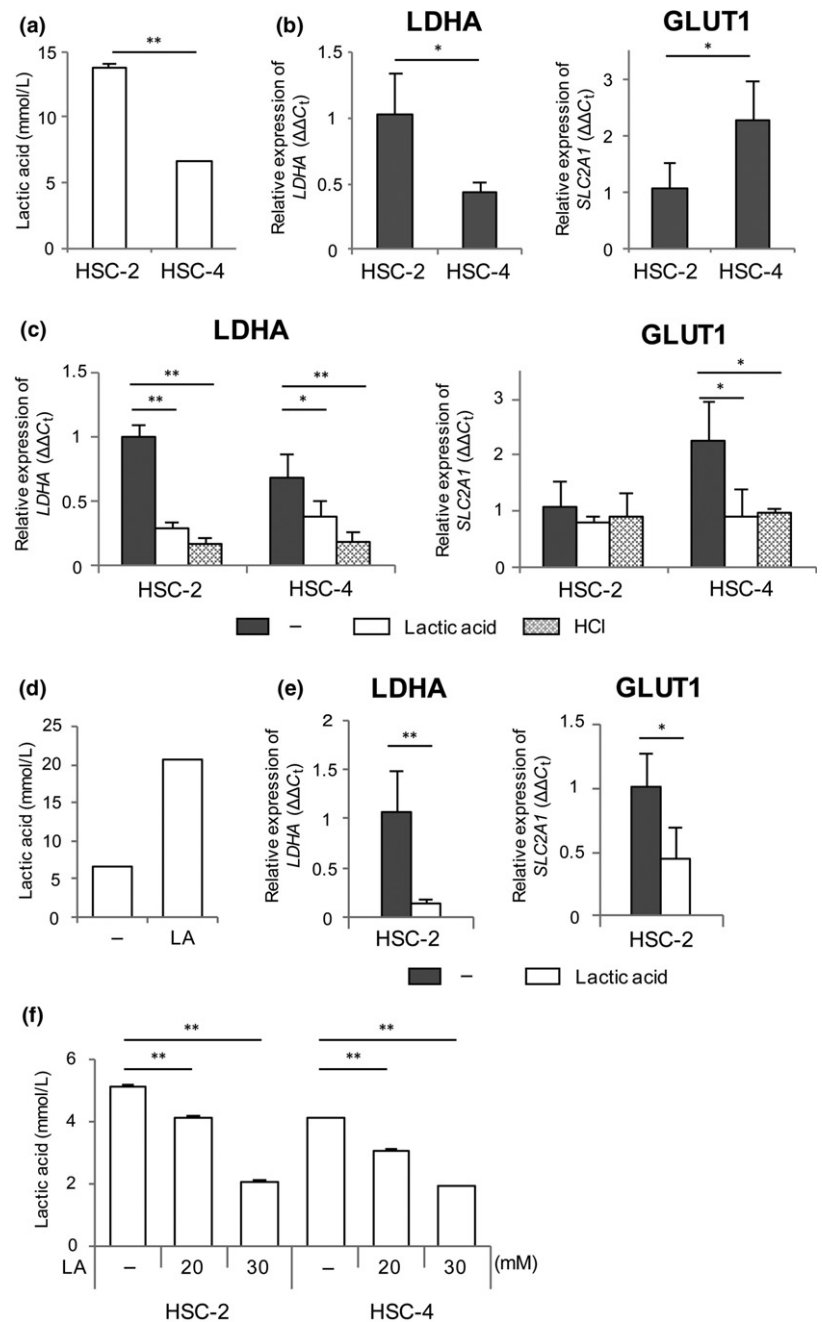
that extracellular acidification with lactic acid suppresses expression of *LDHA* and *GLUT1* in tumor cells, resulting in reduced production of lactic acid from tumor cells.

**Increased expression of *CD68*, *CD163*, and *CSF1R* in HNSCC.** To examine accumulation and polarization of macrophage in tumors, expression of *CD68*, *CD163*, and *CSF1R* was measured by real-time RT-PCR. Expression of *CD68*, a pan-macrophage marker, was normalized using that of *18S rRNA*, and expression of *CD163* and *CSF1R*, which are human M2 macrophage markers, was normalized against that of *CD68*. Compared with normal pharyngeal tissue, HNSCC tissue had significantly higher expression of *CD68*, *CD163*, and *CSF1R* (Fig. 4;  $P = 0.031$ ,  $P = 0.014$ ,  $P = 0.023$ , respectively). These results suggest that macrophages accumulated in the tumor and are polarized to the M2-like phenotype in the tumor. We also measured the expression of *ARG1* mRNA, a murine M2 macrophage marker, because we have previously shown that its expression in murine macrophages is increased by treatment with lactic acid.<sup>(15)</sup> However, *ARG1* showed the opposite result in human tumors: HNSCC tissue had a significantly lower expression of *ARG1* than that in normal pharyngeal tissue (Fig. S2). This result suggests that *ARG1* is not an M2 macrophage marker in human tumor tissue.<sup>(18)</sup>

**M2 like-macrophage polarization is promoted in HNSCC containing higher concentrations of lactic acid.** The HNSCC cases were divided into groups expressing lower and higher levels of *CD68*, *CD163*, or *CSF1R*, and lactic acid levels were compared between each pair of groups (Fig. 5a). There were significantly higher levels of lactic acid in the groups with lower *CD68* expression ( $P = 0.018$ ) and higher *CSF1R* expression ( $P = 0.017$ ), and a tendency for a higher level of lactic acid in the group with higher *CD163* expression ( $P = 0.053$ ). The concentration of lactic acid showed a significant negative correlation with *CD68* expression (Fig. 5b;  $r_s = -0.500$ ,  $P = 0.048$ ) and a significant positive correlation with *CSF1R* expression (Fig. 5b;  $r_s = 0.534$ ,  $P = 0.037$ ). These results suggest that lactic acid negatively regulates the accumulation of macrophages at a tumor site, but promotes M2-like macrophage polarization, as observed in our mouse experiments.<sup>(15)</sup> Finally, we examined the relationship between the lactic acid level and *ARG1* expression, but we did not find a correlation between these parameters in HNSCC tumors (Fig. S3;  $r_s = 0.128$ ,  $P = 0.633$ ).

## Discussion

The Warburg effect is caused by tumor cells adapting their metabolism to the demand for and limited supply of oxygen<sup>(20)</sup> and by the required production of large amounts of nucleotides, amino acids, and lipids for tumor cell proliferation.<sup>(21)</sup> The Warburg effect results in increased production of lactic

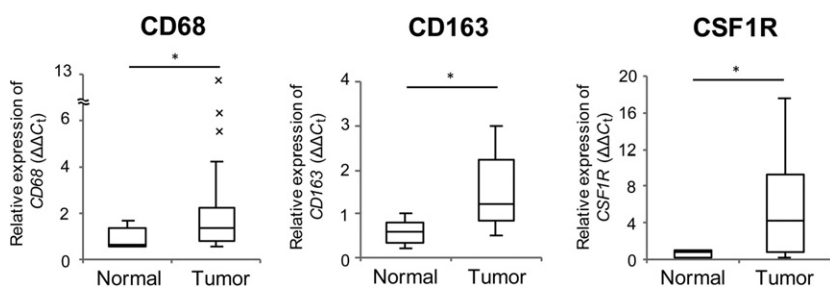


**Fig. 3.** Expression of *LDHA* and *GLUT1* in head and neck squamous cell carcinoma cells. (a, b) HSC-2 and HSC-4 cells ( $2 \times 10^5$  cells in 0.5 mL medium) were cultured for 24 h and the concentration of lactic acid was measured in the culture supernatant (a). *LDHA* and *GLUT1* expression was measured in HSC-2 and HSC-4 cells by real-time RT-PCR (b). (c) HSC-2 and HSC-4 cells were cultured with 15 mM lactic acid or 10 mM HCl for 24 h, and *LDHA* and *GLUT1* expression was measured by real-time RT-PCR. (d, e) HSC-2 cells ( $1 \times 10^5$  cells in 5 mL medium) were cultured in the presence of 20 mM lactic acid (LA) for 48 h, and the concentration of lactic acid was measured in the culture supernatant (d). *LDHA* and *GLUT1* expression was measured in HSC-2 cells by real-time RT-PCR (e). (f) HSC-2 and HSC-4 cells ( $1 \times 10^5$  cells in 5 mL medium) were cultured with lactic acid (LA) for 24 h and then without lactic acid for 24 h. The concentration of lactic acid was measured in the culture supernatant after deacidification. Data are shown as the mean  $\pm$  SD ( $n = 3$ ) for at least two independent experiments. \* $P < 0.05$ ; \*\* $P < 0.01$ . -, water.

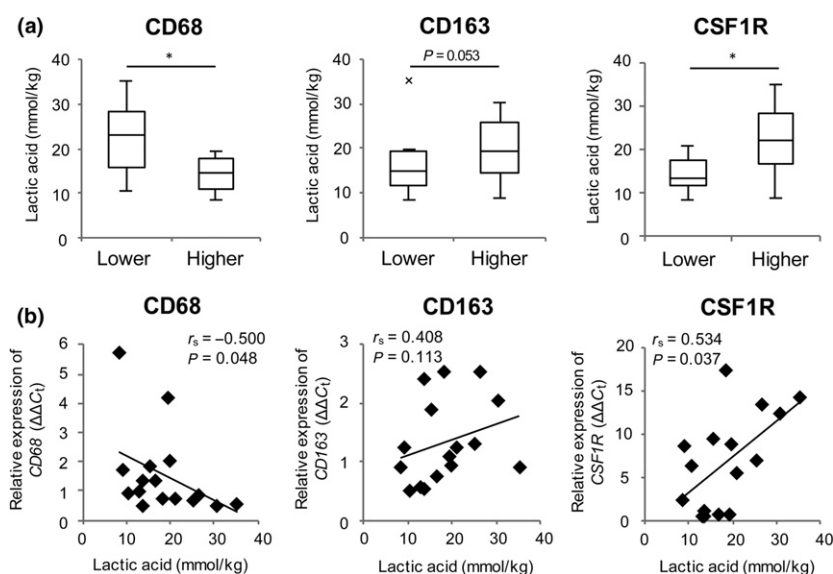
acid, as the final product of glycolysis. In this study, we showed that the concentration of lactic acid in tumors is higher than that in normal tissue. We have shown that lactic acid secreted by tumor cells functions as a pro-inflammatory and immunosuppressive mediator, promotes tumor progression,<sup>(22–24)</sup> and converts macrophages into immunosuppressive macrophages and induces *ARG1* expression in mice.<sup>(15)</sup> Lactic acid secreted by tumor cells also inhibits the functions of natural killer cells, cytotoxic T cells, and dendritic cells, and induces myeloid-derived suppressor cells to form an immunosuppressive environment in tumors.<sup>(25–27)</sup>

In this study, we found that the intratumoral concentration of lactic acid was linked to M2-like macrophage polarization in the tumor microenvironment. However, unlike murine macrophages, *ARG1* expression in human macrophages was not related to the intratumoral concentration of lactic acid.

Expression levels of *CD163*, a member of the scavenger receptor cysteine-rich family, and *CSF1R*, a receptor for macrophage colony stimulating factor, normalized by the pan-macrophage marker *CD68*, were examined as M2 macrophage markers that reflect the rate of M2-like macrophage polarization in tumors. We note that *CD163* and *CSF1R* are expressed in breast and ovarian cancer cells, as well as in normal monocytes and macrophages.<sup>(19,28–32)</sup> Thus, it cannot be excluded that some HNSCC cells might express *CD163* and *CSF1R*, despite there being no reports of expression of these genes in these cells. Indeed, *CD163* was not detected, but *CSF1R* was slightly detected in small fractions of HSC-2 and HSC-4 cells (Fig. S4). Analysis of the expression of these genes in isolated tumor-infiltrating macrophages may more clearly show that lactic acid is linked to M2-like macrophage polarization.



**Fig. 4.** Upregulation of M2 macrophage markers in head and neck squamous cell carcinoma. Expression levels of *CD68* (normalized to expression of *18S rRNA*), *CD163*, and *CSF1R* (normalized to expression of *CD68*) in normal pharyngeal tissue ( $n = 5$ ) and head and neck squamous cell carcinoma tissue ( $n = 20$ ) were measured by real-time RT-PCR. \* $P < 0.05$ ; \*\* $P < 0.01$ , by Student's  $t$ -test.



**Fig. 5.** Accumulation of M2-like macrophages is correlated with a high concentration of lactic acid in head and neck squamous cell carcinoma. (a) Concentrations of lactic acid in cells with different levels of *CD68* (low,  $<0.95$ ,  $n = 8$ ; high,  $\geq 0.95$ ,  $n = 8$ ), *CD163* (low,  $<1.2$ ,  $n = 8$ ; high,  $\geq 1.2$ ,  $n = 8$ ), or *CSF1R* (low,  $<6.6$ ,  $n = 8$ ; high,  $\geq 6.6$ ,  $n = 8$ ). (b) Correlations between lactic acid concentration and expression levels of *CD68*, *CD163*, and *CSF1R* in head and neck squamous cell carcinoma ( $n = 20$ ). Expression of these genes was measured by real-time RT-PCR.  $r_s$  is Spearman's rank correlation coefficient. \* $P < 0.05$  by Student's  $t$ -test.

Our results indicate that macrophages infiltrate tumors more effectively than normal tissues. Chronic inflammation in the tumor microenvironment may cause macrophages to accumulate within the tumor. However, interestingly, more macrophages are likely to accumulate at a tumor site when the intratumoral concentration of lactic acid is lower. Consistent with this finding, a high concentration of lactic acid inhibits monocyte migration *in vitro*.<sup>(33)</sup> These results show that macrophages do not infiltrate well into tumors that produce a large amount of lactic acid, but can polarize toward the M2-like phenotype in tumors that produce a large amount of lactic acid.

Increased expression of *LDHA* and *GLUT1*, which are mainly activated by oncogenes, is important in the Warburg effect.<sup>(34)</sup> It is also well known that expression of *LDHA* and *GLUT1* is increased in response to hypoxia and activation of hypoxia inducible factor-1 $\alpha$ .<sup>(20)</sup> To evaluate induction of the Warburg effect, we measured the concentration of lactic acid and expression of these genes in tumors and normal tissues. In tumor tissues, the concentration of lactic acid was higher, and *LDHA* and *GLUT1* expression was significantly increased, compared with normal tissues. *GLUT1* expression was strongly correlated with *LDHA* expression. However, a lower intratumoral concentration of lactic acid was linked to the higher expression of *LDHA* and *GLUT1*. As upregulation of these genes directly causes the Warburg effect, whereas the accumulation of lactic acid is the result of the Warburg effect, it is not unexpected that the concentration of lactic acid and the expression levels of genes involved in glycolysis do not change synchronously. We suggest that aerobic glycolysis may

be mainly promoted by oncogenes and hypoxia in tumors, and may be suppressed by negative feedback under higher lactic acid conditions. In fact, we showed that a high concentration of lactic acid suppressed the expression of *LDHA* and *GLUT1* in HNSCC cells. Acidification of the culture medium gave the same results. Because lactate anions are co-transported with protons into cells by monocarboxylate transporters, extracellular acidification would suppress efflux of both protons and lactate anions from cells and promote accumulation of lactic acid, resulting in suppression of aerobic glycolysis by negative feedback. Protons also activate G-protein coupled receptors and upregulate cAMP, and this signaling pathway may also suppress the expression of genes involved in glycolysis.<sup>(35,36)</sup> Thus, our results suggest that expression of *LDHA* and *GLUT1* is not a predictive marker for the intratumoral concentration of lactic acid.

We have previously shown that lactic acid secreted by tumors induces immunosuppressive M2-like macrophages in a murine system.<sup>(15)</sup> In addition, blocking of the lactic acid signal pathway directly improves the immunosuppressive function of murine macrophages, even if the intratumoral concentration of lactic acid is not decreased.<sup>(15)</sup> In this study, we showed that a higher concentration of lactic acid was also linked to M2-like macrophage polarization in human HNSCCs. Furthermore, lactic acid-primed tumor cells did not necessarily recover the suppressed production of lactic acid after removal of lactic acid. Therefore, we predict that suppression of lactic acid production and the lactic acid signal pathway that induces M2-like macrophages should improve the immune status of patients with cancer. However, the molecular mechanism

underlying this effect in human tumors is unknown. Lactic acid secreted by tumor cells suppresses antitumor immunity and increases tumor angiogenesis through endothelial cells,<sup>(24,29,37)</sup> and increasing evidence suggests that lactic acid is a tumor-derived mediator that modulates the tumor microenvironment to promote tumor progression.<sup>(5,24,38–40)</sup> Thus, anti-tumor therapies targeting lactic acid production and the lactic acid signaling pathway are of increasing interest. It will be important to determine the details of this signaling pathway.

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## Disclosure Statement

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## Abbreviations

|       |                                       |
|-------|---------------------------------------|
| ARG1  | arginase I                            |
| CSF1  | colony stimulating factor 1           |
| CSF1R | colony stimulating factor 1 receptor  |
| GLUT  | glucose transporter                   |
| HNSCC | head and neck squamous cell carcinoma |
| IL    | interleukin                           |
| LDH   | lactate dehydrogenase                 |

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## Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

**Fig. S1.** Short tandem repeat analyses were carried out by Takara Bio (Otsu, Japan) using the GenePrint 10 system (Promega, Fitchburg, WI, USA). Nine microsatellite loci and the sex-typing marker *Amelogenin* were analyzed in HSC-2 (b) and HSC-4 (d) cell lines. The short tandem repeat profiles of eight microsatellite loci completely matched between our cells and cells registered in the JCRB cell bank (a, c; [http://cellbank.nibioh5n.go.jp/english/cellsearch\\_e/](http://cellbank.nibioh5n.go.jp/english/cellsearch_e/)). Although Y chromosome was detected in our HSC-2 cell but not JACR cell, the HSC-2 cell is originally derived from a man and should include both X and Y chromosomes.

**Fig. S2.** Expression of *ARG1* in head and neck squamous cell carcinoma. *ARG1* expression was measured in normal pharyngeal tissue ( $n = 5$ ) and head and neck squamous cell carcinoma tissue ( $n = 20$ ) by real-time RT-PCR. Expression of *ARG1* was normalized using expression of *CD68* and calculated using the  $\Delta\Delta C_t$  method.  $**P < 0.01$  by Mann–Whitney *U*-test.

**Fig. S3.** Expression of *ARG1* was not correlated with a high concentration of lactic acid in head and neck squamous cell carcinoma. (a) Concentrations of lactic acid in cells expressing higher and lower levels of *ARG1* (low,  $<0.18$ ,  $n = 8$ ; high,  $\geq 0.18$ ,  $n = 8$ ).  $**P < 0.01$  by Student's *t*-test. (b) Correlation between the lactic acid concentration and *ARG1* expression in head and neck squamous cell carcinoma ( $n = 20$ ).  $r_s$  is Spearman's rank correlation coefficient.

**Fig. S4.** Expression of *CD163* and *CSF1R* in head and neck squamous cell carcinoma cell lines. HSC-2 and HSC-4 cells were stained with APC-conjugated anti-human CD163 antibody (GHI/61; BioLegend, San Diego, CA, USA) (a) or APC-conjugated anti-human CD115 (*CSF1R*) antibody (9-4D2-1E4; BioLegend) (b), and examined using a FACSCalibur system (BD Biosciences, San Jose, CA). APC-conjugated mouse IgG1 $\kappa$  (MOPC-21; BioLegend) and APC-conjugated rat IgG1 $\kappa$  (RTK2071; BioLegend) isotype controls were used as anti-human CD163 and anti-human CD115 antibody controls, respectively. The human peripheral monocyte fraction, gated based on forward scatter and side scatter, was used as a positive control. Small fractions of both HSC cell lines expressed CD115 (HSC-2, 1.14%; HSC4, 2.34%), but not CD163.

**Table S1.** Background of patients with head and neck squamous cell carcinoma.